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REGULATION OF HEN GRANULOSA CELL FATE DURING OVARIAN FOLLICULAR DEVELOPMENT

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A thesis submitted to the School of Graduate Studies and Research, University of Ottawa
in partial fulfilment of the requirement for the degree of Doctor of Philosophy,
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ABSTRACT

To determine if tumour necrosis factor alpha (TNFα) plays a role in the regulation of follicular growth and selection, studies were performed on F1 and F5,6 granulosa cells in the presence or absence of maximally stimulatory concentrations of luteinizing hormone (LH), transforming growth factor alpha (TGFα) or carbamylcholine chloride (Cch). Assessments of follicular growth were performed primarily using [^{3}H]-thymidine incorporation assays, while survival was assessed using 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide (MTT), DNA degradation and vital staining assays. Basal [^{3}H]-thymidine incorporation was significantly higher in F5,6 (smallest follicles in follicular hierarchy versus F1 (largest follicle in follicular hierarchy) granulosa cells while the opposite was observed for MTT metabolism. LH stimulated [^{3}H]-thymidine incorporation in F5,6 cells, while inhibiting [^{3}H]-thymidine incorporation in F1 cells. In contrast, TGFα stimulated [^{3}H]-thymidine incorporation in F1 cells and to a lesser extent, F5,6 cells. TNFα had no apparent effect on basal, LH- or TGFα-induced [^{3}H]-thymidine incorporation in either F1 or F5,6 granulosa cells. In addition, while neither TNFα nor TGFα alone affected the viability of cultured granulosa cells from either developmental stage, in the presence of both factors an increase in MTT metabolism occurred in F5,6 but not F1 granulosa cells. In contrast to the TNFα-TGFα studies, interactions between TNFα and Cch were not observed irrespective of the stage of follicular development and the rate of MTT metabolism was not affected by

either factor. F5,6 cell viability *in vitro* decreased with time in the presence of TNFα or TGFα, while the combination of these intraovarian regulators significantly delayed this decrease. In contrast, only TGFα increased MTT metabolism in sparsely cultured F5,6 granulosa cells during the first 24 hours of culture, after which time no significant changes were observed. TGFα also significantly decreased the amount of DNA fragmentation, while TNFα had no significant effect in the presence or absence of the growth factor. In contrast, vital staining techniques revealed that TGFα significantly increased the percentage of viable cells following 48 and 72 hrs of culture, while TNFα significantly potentiated TGFα-induced cell survival. Moreover, the primary type of cell death which occurred was necrosis rather than apoptosis. Cch had no significant effect on granulosa cell necrosis irrespective of the presence of TNFα. In conclusion, these studies demonstrate the existence of a stage-dependent interaction between TNFα and TGFα in the regulation of cell survival. Moreover, a role for cholinergic innervation in the regulation of granulosa cell growth and survival was not detected.

Studies of Cch-induced $[Ca^{2+}]_i$ and inositol 1,4,5-trisphosphate (IP_3) production in granulosa cells during follicular development were performed to characterize differentiative changes in cholinergic regulation of granulosa cell function. Intracellular free calcium ($[Ca^{2+}]_i$) was measured in fura-2-loaded cells about 20-36 h after their isolation. The percentage of cells responding to a maximal stimulatory concentration of Cch was higher in the F1 granulosa cells than in cells from the F3 and the F5,6 follicles. Most of the Ca^{2+} transients that were elicited in F1 granulosa cells were characterized by large, fast increases

in $[Ca^{2+}]_i$ followed by a slow, uneven decrease in $[Ca^{2+}]_i$ to the resting concentration. In contrast, Cch-induced changes in $[Ca^{2+}]_i$ in F3 and F5,6 granulosa cells were generally both smaller and slower than those observed in cells from the largest follicle. Removal of external Ca^{2+} did not alter the large, fast increases in $[Ca^{2+}]_i$, although it did inhibit slow Ca^{2+} transients. IP_3 production was elevated in F1 granulosa cells after 1 min of either Cch or Ach treatment, whereas inositol bisphosphate (IP_2) production and inositol monophosphate (IP) production were elevated only after longer incubations. In conclusion, the present studies demonstrate that the response of the IP_3-Ca^{2+} signalling system to muscarinic input is dependent on the stage of follicular development.

As slow Cch-induced changes in $[Ca^{2+}]_i$ were dependent on external $[Ca^{2+}]$, Cch-induced changes in Ca^{2+} current were studied using F1 granulosa cells. The major Ca^{2+} current observed using the perforated patch technique exhibited characteristics typical of T-type Ca^{2+} current. In all cells studied, carbachol caused an inhibition of this current (elicited by depolarizing pulses from -70 to -20 mV) to an average maximal decrease of $90 \pm 2\%$ of basal values. In some 50% of the cells, the Ca^{2+} current also partially recovered during exposure to the muscarinic agonist. These effects were prevented by the muscarinic antagonist atropine. This inhibition was due to increases of intracellular free-Ca^{2+} concentrations. Under these conditions, carbachol failed to produce the expected $[Ca^{2+}]_i$ transients but rather caused a small decrease of basal $[Ca^{2+}]_i$ attributable to its diminution of Ca^{2+} current. Thus, the results demonstrated an important muscarinic inhibition of the T-type Ca^{2+} current not related to $[Ca^{2+}]_i$ fluctuations. They indicate, on the other hand, that $[Ca^{2+}]_i$

can strongly modulate carbachol-induced mobilization of Ca^{2+} from the intracellular stores.

Regulation of Ca^{2+} signalling by TNFα was studied on both basal and Cch-induced $[Ca^{2+}]_i$ in F1 and F5,6 granulosa cells. TNFα induced a small and delayed transient increase in $[Ca^{2+}]_i$. The percentage of cells that responded to the cytokine was greater in F5,6 than F1 granulosa cells. These responses were completely abolished in Ca^{2+}-free media. In addition, pretreatment with TNFα increased the magnitude of Cch-induced Ca^{2+} transients more effectively in F1 than F5,6 granulosa cells which had been shown to only respond to Cch with small Ca^{2+} transients. These studies demonstrate a complex, follicular stage-dependent interaction between cytokine and cholinergic input in the control of Ca^{2+} signalling for the regulation of granulosa cell function.

The interactions between TNFα andTGFα resulting in increased survival in culture were cell density-dependent, suggesting a possible mediatory role for cell attachment factors. The production of fibronectin and integrins β1 and β3 were studied by Western blot using monoclonal mouse anti-human antibodies known to cross-react with their respective hen homologues. Two distinct cellular associated forms of fibronectin (200 kD and 140 kD) and a secreted form (210 kD). All three forms of fibronectin bands were expressed more strongly in F1 than F5,6 cells, which were not influenced by TNFα, irrespective of the presence of Cch or TGFα. Cch decreased fibronectin secretion but had no influence on its cellular content. Moreover, TGFα significantly increased the production of both cellular and secreted fibronectin in F5,6 but not in F1 cells. Studies of integrin β1, revealed poor antibody specificity, despite a clear band of approximately 140 kD in the positive control. In contrast,

Western blots against integrin β3 revealed a band of the expected size (90 kD) and two other bands (35 and 40 kD), presumably representing degraded products. Although neither TNFα nor Cch, alone or in combination, affected the expression of integrin β3, TGFα significantly increased integrin β3 expression in F5,6 cells which also exhibited significant interactions between TNFα and TGFα. In conclusion, although TNFα failed to influence fibronectin secretion in hen granulosa cells throughout follicular development, it induced integrin β3 production in the presence of TGFα in F5,6 cells. This supports a role for integrins as potential mediators of TNFα-TGFα-dependent cell survival.

Although TNFα has long been demonstrated to be a potent inhibitor of gonadotropin-induced ovarian cytodifferentiation in many mammalian species, its early signal transduction events are poorly understood. The focus of this study was to determine the role of ceramide in TNFα-induced Ca^{2+} regulation. Exogenous SMase failed to influence basal $[Ca^{2+}]_i$, but increased the magnitude of Cch-induced Ca^{2+} transients in a manner similar to TNFα. While C8-ceramide, but not C2-ceramide mimicked this effect of SMase, sphingosine induced TNFα-like increases in basal $[Ca^{2+}]_i$. In addition, both exogenous SMase and C2-ceramide inhibited LH-induced progesterone production in F1 and F5,6 cells, although exposure to C8-ceramide resulted in increases in both basal and LH-stimulated progesterone synthesis. In contrast, TNFα had no effect on either basal or LH-induced steroidogenesis. Lastly, measurements of sphingomyelin and ceramide content in both F1 and F5,6 cells revealed that TNFα did not activate an endogenous SMase. Consequently, although ceramide regulates $[Ca^{2+}]_i$ and progesterone secretion, the sphingolipid does not appear to play a role in the

action of TNFα in avian granulosa cells. Furthermore, ceramide mediated responses were highly dependent on acyl chain length, potentially reflecting differences in the abilities of these ceramides to access, bind to and/or activate ceramide-dependent signal transduction mechanisms. Nonetheless, since TNFα did not increase the production of ceramide, the physiological regulator(s) of these responses remains unknown.

In conclusion, the objective of this research was to study TNFα and its interactions with Cch, TGFα and LH in the regulation of granulosa cell fate, ie proliferation, differentiation and apoptosis, as well as the possible signalling mechanisms involved during follicular development. Exposure of granulosa cells to TNFα alone resulted in changes in $[Ca^{2+}]_i$, but did not affect granulosa cell fate based upon any of the endpoints studied. Nonetheless, follicular stage-dependent interactions between the cytokine and both Cch and TGFα were observed. This suggests that TNFα actions are modulated by intraovarian factors in a manner dependent on cytodifferentiation, resulting in granulosa cell responses to the cytokine which are appropriate to their stage of follicular development.

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II. LIST OF ABBREVIATIONS

Ach	acetylcholine
BSA	bovine serum albumin
Ca^{2+}	ionic calcium
$[Ca^{2+}]_i$	cytosolic ionic calcium concentration
Cch	carbamylcholine chloride
cGMP	cyclic guanine monophosphate
c-IAP1	cytosolic inhibitor of apoptosis 1
c-IAP2	cytosolic inhibitor of apoptosis 2
CAM kinase II	calmodulin kinase II
CCK	cholecystekinin
CL	corpus luteum
DETAPAC	diethylenetriaminepentaacetic acid
DMSO	dimethylsulphoxide
dPBS	Dulbecco's modified phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N, N, N' N'-tetraacetic acid
ERK	extracellular signal-regulated kinase
F1	largest follicle in hen ovary
F5,6	smallest two follicles in follicular hierarchy in hen ovary
FAN	factor associated with N-sphingomyelinase activation
FBS	fetal bovine serum
Fura-2AM	fura-2-acetoxymethyl ester
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GnRH	Gonadotropin Releasing Hormone
GRF	Growth Hormone Releasing Factor
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IL-1β	Interleukin-1β
IP	inositol phosphate
IP_2	inositol bisphosphate
IP_3	inositol trisphosphate
IP_4	inositol tetraphosphate
I-TRAF	TRAF-interacting protein
ITS	insulin (5 mg/l)-transferrin (5 mg/l)-selenite (5 μg/l)
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LWF	large white follicle
M199	medium 199

MEM	minimum essential medium
Mn^{2+}	manganese
NBS	normal buffer solution
NF-κB	nuclear factor-κB
NMDG	N-methyl-D-glutamine
P_4	progesterone
PA	plasminogen activator
PBS	phosphate buffered saline
PBSG	phosphate buffered saline-gelatin
PCA	perchloric acid
PGF	prostaglandin F
PLC	phospholipase C
POF	post-ovulatory follicle
RAIDD	RIP associated ICH-1/CED3 homologous protein with a death domain
Rb	Retinoblastoma Protein
RIA	radioimmunoassay
RIP	Receptor Interacting Protein
SAPK	stress-activated protein kinase
SH2	Src homology 2
SM	sphingomyelin
Smase	sphingomyelinase
SOS	son of sevenless
SPP	sphingosine-1-phosphate
SWF	small white follicle
TANK	TRAF Family Member Associated NF-κB Activator
TBS	tris-buffer saline
TBST	tris-buffered saline-tween20
TLC	thin layer chromatography
TCA	trichloroacetic acid
TNFα	tumour necrosis factor alpha
TNFR	tumour necrosis factor alpha receptor
TNFR1	tumour necrosis factor alpha receptor 1
TNFR2	tumour necrosis factor alpha receptor 2
TRAF1	tumour necrosis factor alpha receptor activated factor 1
TRAF2	tumour necrosis factor alpha receptor activated factor 2
TRAK-60	TNFα receptor-associated protein kinase-60
TRAK-80	TNFα receptor-associated protein kinase-80
TRIP	TNFα receptor activated factor 2 inhibitory protein
VIP	vasoactive intestinal peptide

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I. INTRODUCTION

The Oxford definition of a living system is an animate object capable of the intake of organic matter, metabolism, excretion of waste, the capacity for growth & development and the ability to reproduce. As such, reproduction is one of the most fundamental elements of all living species. Although the objectives of reproduction are common to all forms of life, different species have developed their own strategies to accomplish this function. The primary interest of reproductive research today is to further our understanding of human reproduction for the development of new contraceptive approaches and the treatment of infertility and reproductive pathologies. Nonetheless, indepth research on human reproduction is highly difficult due to ethical considerations, limited access to tissues and an inability to design complete experiments. Furthermore, reproduction in mammais is highly complex and its regulation has considerable species specificity. Consequently, many researchers have chosen to study areas of reproduction in simpler systems which can provide unique advantages.

The focus of the current study was to examine differentiative changes in granulosa cells as follicles prepare themselves for ovulation, using egg-laying hens as a model. Unlike in mammals, the developmental stages of ovarian follicles in avian species can be precisely identified in the final 6 days prior to ovulation. Furthermore, since hens ovulate daily, granulosa cell responses from follicles separated in their development by only one day can be collected from a single animal and compared. In addition, the relatively large size of avian

granulosa cells and their high cytoplasm-nuclear ratio makes these cells ideal for fluorescence imaging and electrophysiological studies. Although changes that take place in the differentiation of avian cells differ in many ways from events occurring in the human, these studies can provide important fundamental information on the biology of the ovary and clues for the focus of subsequent research. Moreover, despite cross-species differences in hormonal activity and post-ovulatory function, the process of oocyte expulsion itself is accomplished in a very similar manner between birds and mammals. As such, species from these two classes share many commonalities in their preparation for this process.

II. LITERATURE REVIEW

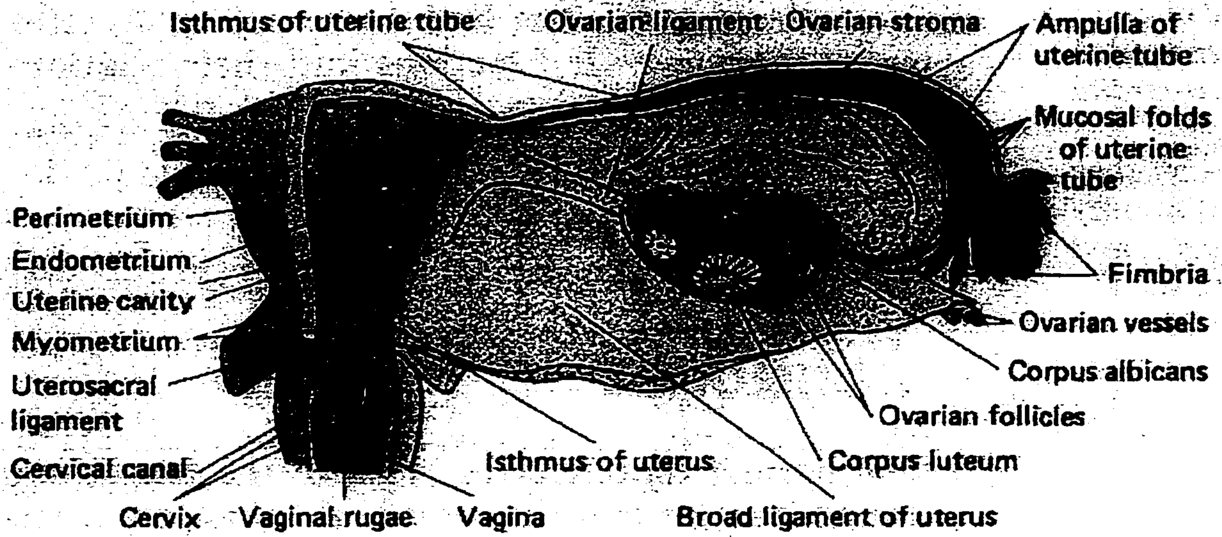
A. The Female Reproductive System

1. Avian versus Mammalian Reproduction

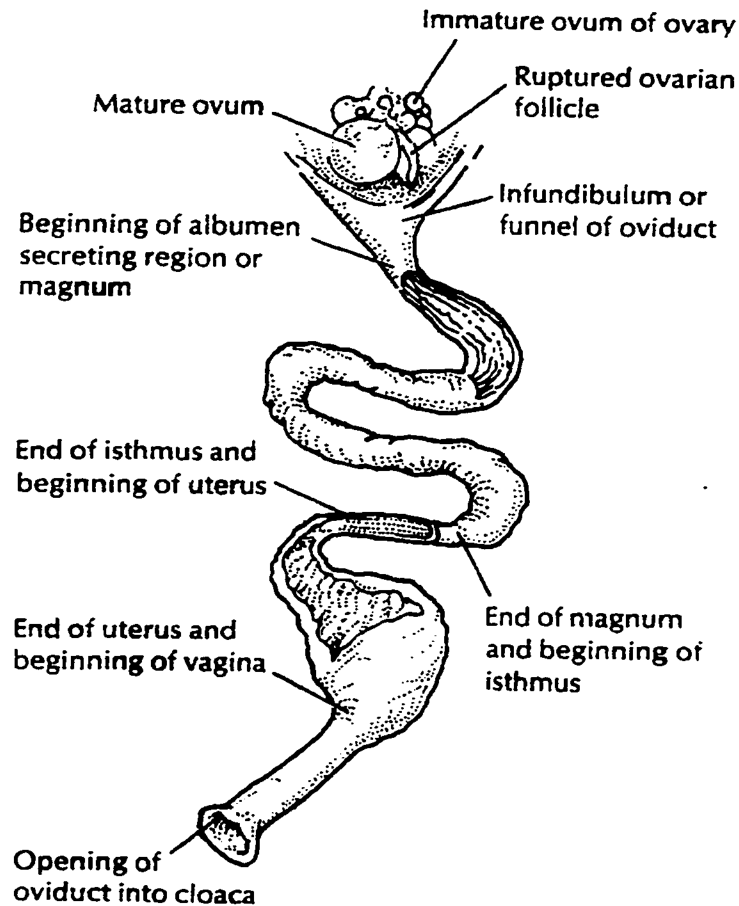
The reproductive strategies of mammals and birds have many similarities and differences. The anatomies of the female reproductive systems in birds and mammals are structurally quite similar. Females from both classes have ovaries, oviducts, a uterus and a birth canal (Fig 1). As such, the primary differences between animals from these two classes are the functions of these organs. In mammals, after ovulation, the ovum moves through the oviduct to the uterus where, if fertilized, it implants into the endometrium and gestates for

Figure 1: The reproductive system. **Panel A:** Mammalian (Guyton and Hall, 1996). **Panel B:** Avian (Johnson, 1986).

A



B



a substantial period of time. Consequently, mammals must be prepared to cease reproductive activity during the process of gestation. This break in the reproductive cycle is accomplished as a result of signals from extra-embryonic tissue resulting in the maintained function of post-ovulatory follicular cells (corpus luteum). In contrast, avian gestation occurs entirely outside of the mother, in an environment she creates in a ~25 hour post-ovulatory period termed oviposition. This provides hens with an important functional advantage: the ability to continue to ovulate after fertilization. Since a break in the reproductive cycle is not required, post-ovulatory follicular cells do not coordinate their activities with the embryo and degenerate soon after oviposition. Although it is not immediately clear how the differences between the post-ovulatory functions of avian and mammalian follicular cells affect their pre-ovulatory nature, this must be considered when making cross-species comparisons.

2. The Hypothalamic-Pituitary-Ovarian Axis

Reproductive function is influenced by a number of different hormonal, paracrine, autocrine and neurocrine factors. Nonetheless, in both mammalian and avian systems, the processes of selecting, preparing and releasing a mature ovum are primarily controlled by the inter-regulation of hormones produced in the hypothalamus, pituitary gland and the ovary. Reproductive maturation is initiated by an increase in the production of Gonadotropin Releasing Hormone (GnRH) by the hypothalamus. This increase in GnRH output stimulates

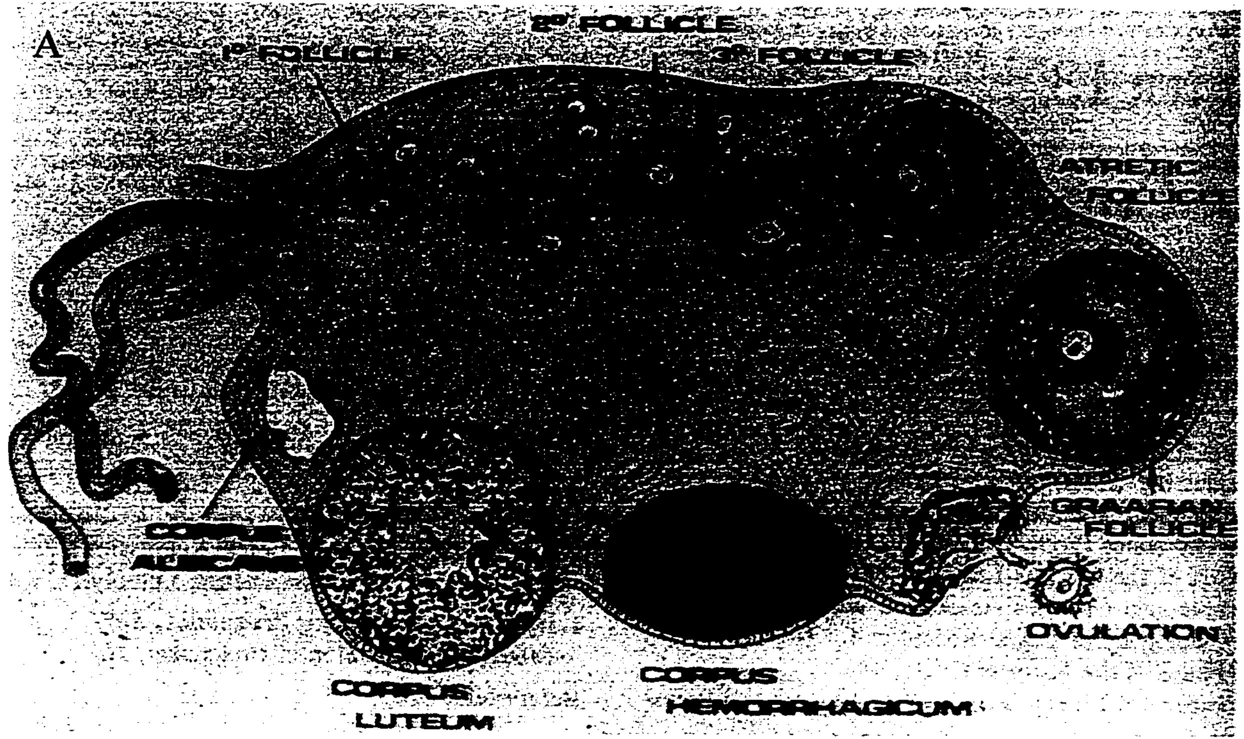
pituitary secretions of the gonadotropins Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Although gonadotropins influence the function of the ovary in a number of different ways, their primary roles are to stimulate follicle growth, steroidogenesis and ovulation. In mammals, the major steroid secreted by the ovary prior to ovulation are estrogens, while progesterone is secreted primarily in the peri- and post-ovulatory periods. In the hen, estrogens are the major steroid products early in follicular development; during the preovulatory period, their production decreases while progesterone production increases. Despite this difference, one of the primary functions of these steroids, along with the ovarian peptide inhibin, is to inhibit GnRH and gonadotropin production. Consequently, since maturing follicles secrete increasing amounts of steroids into circulation, serum gonadotropin and GnRH levels decrease until the day of ovulation in mammals and ~12 hours prior to ovulation in hens (which ovulate daily). At this point, for reasons not yet clear, this negative feedback system suddenly switches to a positive feedback system, resulting in a sudden surge in both LH and FSH production (although primarily LH). This surge in LH production initiates the processes of oocyte maturation and expulsion in both birds and mammals, after which LH concentrations return to normal levels and the ovulatory cycle repeats itself.

B. The Ovary

1. Overview

The mammalian and avian ovaries appear to be fundamentally different in structure (Fig 2), although functionally they are quite similar. Prior to follicular selection, ovarian follicles of both mammalian and avian origin are small and undifferentiated. While most of these follicles die via the process of atresia, those few which survive must grow and develop in preparation for the processes of ovulation, fertilization and pregnancy (mammals) or oviposition (birds). Consequently, after the expulsion of the oocyte, the ruptured follicles are converted into the corpus luteum in mammals and the Post-Ovulatory Follicle (POF) in birds. This results in changes in both the nature and quantity of steroids produced, followed by tissue degradation via the process of apoptosis. These changes in follicular cell fate from proliferation to differentiation (steroidogenesis) to apoptosis are regulated by the actions and interactions of gonadotropins, steroids, growth factors, cytokines and neurotransmitters. Characterization of the roles of these factors in the regulation of follicular development has been the primary focus of research in the ovary for many years. As such, studies of the primary actions of most intraovarian factors are reasonably well understood. Nonetheless, since cells are exposed to many of these factors simultaneously *in vivo*, clarification of their interactions is required and represents the primary interest of these studies.

Figure 2: The ovary. **Panel A:** Mammalian (Niswender and Nett, 1988). **Panel B:** Avian. F1 - F6: Follicles within the follicular hierarchy. The F1 follicle is ~12 hours from ovulation, while each of the smaller follicles is 24 hours less developed. SYF: small yellow follicles. LWF: large white follicle. SWF: small white follicle. Scale is ~1:1.



F4
F6
B
F3
SWF
SYF
LWF
F2
F1
POF
F5

2. Follicular Development

The ovarian follicle consists of three distinct cell types, ie the theca, the granulosa and the oocyte. Since the current research project focused upon granulosa cells, the subsequent review will primarily discuss aspects of follicular development related to changes in granulosa cell function.

In most species, both oocyte and follicle formation occur almost entirely during or immediately after embryonic development (for review, see Tokarz, 1978). Briefly, primordial germ cells migrate into the developing ovary where differentiation into oogonia takes place. At this point, these cells have committed themselves to oocyte formation but still contain the full genetic material of a somatic cell. Furthermore, before the initiation of meiosis and oocyte differentiation, oogonia enter a phase of rapid proliferation in order to create sufficient numbers for normal reproductive capacity. As this proliferative phase ends, the process of meiosis begins. In both mammals and birds, meiosis stops in prophase I, at which point the cell is termed a primary oocyte.

In mammals, follicle formation occurs in the fetus during midgestation shortly after the formation of primary oocytes. In contrast, in the hen, folliculogenesis does not begin until 4 to 5 days after hatching. Despite this difference in timing, the process of folliculogenesis is actually quite similar in both species. In essence, what occurs is that prefollicular epithelial cells surround and flatten around the oocyte while forming overlapping cell processes;

thereby creating a distinct structure termed the primordial (mammals) or Small White Follicle (SWF; birds). Although the factors which regulate this process are not entirely understood, the oocyte itself is believed to serve a primary role in early follicle formation. This is based primarily on the observation that follicle formation does not occur if germ cells are destroyed experimentally (Merchant-Larios, 1976) or by genetic deficiency (eg. Turner's syndrome; Singh and Carr, 1966). In addition, hypophysectomy does not block follicle formation, suggesting that gonadotropins are not involved (Tokarz, 1978).

During the process of follicular development, there are several points at which follicles exhibit distinct and major changes. The primordial (mammal) or small white (birds) follicle stages represent the largest pool of follicles within the ovary. For reasons which have not been clearly identified, certain follicles from this pool begin to grow and differentiate. The follicle increases in size, the granulosa cells expand and become cuboidal, while they begin to express FSH (Tisdall *et al*, 1995) and c-kit receptors (Yoshida *et al*, 1997) as demonstrated in ovine and murine ovaries, respectively. The factors which mediate this transformation are not known, however, the implications of these changes are clear. Due to the expression of c-kit, and perhaps other growth factor receptors, granulosa cells begin to divide. Furthermore, once they become FSH-responsive, granulosa cells begin to produce steroids [Franchimont *et al*, 1993 (mammals); Li and Johnson, 1993a (hens)]. These are the first key steps in follicular development. Once a follicle begins to exhibit these characteristics, it is considered a primary (mammal) or Large White (LWF; birds) follicle.

From this point, there are only two possible outcomes: follicular development and ovulation or follicular atresia.

The transition from the primary to the secondary or preantral stage in mammals, which is analogous to the growth phase of the LWF in birds, is considered the next major change in the development of the follicle. The primary changes which occur during this transition are increases in the expression of Follicle Stimulating Hormone Receptor (FSHR) (Kaipai and Hsueh, 1997), the layering of the granulosa cells, the recruitment of the theca and for birds, increases in yolk transfer to the oocyte (Johnson, 1986). Furthermore, those follicles which do not succeed in making this transition die via the process of follicular atresia. Since FSH can drive the transition between the primary and secondary stages *in vitro* (Cain *et al*, 1995), it seems logical that differences in FSHR expression determine whether or not a follicle will survive or die. Nonetheless, development of primary follicles to the secondary and tertiary stages can occur in the absence of gonadotropin support, suggesting that this is not the case (Hirschfield, 1991). Furthermore, a protective role for Cyclic Guanine Mono-Phosphate (cGMP), an effector of Interleukin-1β (IL-1β)-induced nitric oxide production has been reported (Chun *et al*, 1995; McGee *et al*, 1997). Nonetheless, the factors determining which follicles will be exposed to IL-1β and/or other potential protective factors are, unfortunately, unclear. Additional work focussing on the actions and interactions of intraovarian factors which, presumably, regulate early follicle selection is required for the comprehension of this process.

Perhaps the most important step in follicular development is the transition between the secondary (LWF in the hen) and tertiary [Small Yellow Follicles (SYF) in the hen] stages. It is at this point where the final decision is made regarding which follicles will ovulate and which will become atretic and degenerate (Tilly *et al*, 1991a). As such, follicles which survive this transition are committed to the process of ovulation. Perhaps due to the fact that this stage represents commitment to ovulate, this is the point in development which is the most competitive; most follicles which attempt to reach the tertiary stage die by the process of follicular atresia (Kaipai and Hsueh, 1997). The changes in the characteristics of the follicle which must be accomplished during this transition are increased steroidogenic capacity (Tilly *et al*, 1991b), LH receptor expression within the granulosa cell layer (Zeleznik *et al*, 1974), growth of the antrum (mammals; McNatty, 1978), further increases in the rate of yolk deposition (hen; Marza and Marza, 1935), follicular cell proliferation (Johnson, 1986), the separation of the granulosa cell layer into cumulus (adjacent to the oocyte) and mural cells (mammals; Cain *et al*, 1995) and the theca into the interna and externa layers (Brambell, 1928). Although the primary driving force for this process is FSH, the identification of intermediary factors for FSH in the regulation of each of these events has been a major issue for study and will be further addressed in later sections of this thesis. Moreover, the features that differentiate the one or few early antral follicles destined for ovulation from those which will become atretic remains one of the most important questions in current reproductive research.

Once committed to ovulation, follicles enter the rapid growth phase of follicular maturation. For mammalian follicles, this involves increases in the number and size of follicular cells and the growth of the antrum. Once ready for ovulation, these follicles have reached the Graafian stage. The time frame of this aspect of follicular development can vary from species to species and is based largely upon their ovulatory cycle, however, in the hen, this process takes approximately 11 days. Furthermore, due primarily to the large quantity of yolk required for gestation, the diameter of these follicles increases dramatically, from approximately 9 mm to 40 mm (Marza and Marza, 1935). Since a single follicle is recruited each day in preparation for the hen's daily ovulatory cycle, follicles in the final 6 days prior to ovulation (termed F6 through F1) are easily distinguishable by size and are said to have entered the follicular hierarchy (Johnson, 1986). In contrast, mammalian ovaries can, at any one time, contain follicles at varying points in follicular development. Furthermore, since the degree of follicular growth is less drastic, small differences between the developmental stages of follicles cannot be precisely identified. Lastly, in the hen, the granulosa cell layer itself exists as a monolayer of cells strongly connected to each other by an extracellular matrix (Asem *et al*, 1984). In contrast, the mammalian granulosa cell layer is a multi-layered tissue which cannot be easily separated from oocytes and blood cells. Consequently, cultures of granulosa cells from well defined stages can be compared within the same ovary and between hens. As such, the hen model offers distinct advantages for the study of late changes in follicular development.

3. Follicular Atresia

The process of follicular development is a complex and intricate process, which few follicles can successfully complete. As such, follicle(s) which do ovulate are the ones that survive, while the remainder (>99%) die by the process of follicular atresia. Consequently, most follicles in the ovary are destined for atresia rather than ovulation. In theory, the reason for this complexity would be to select the most suitable follicles for fertilization. Nonetheless, this does not appear to be the case. In the presence of high concentrations of LH or LH analogues (ie hCG), multiple ovulations can be induced (Tan, 1994). Furthermore, many of these artificially rescued follicles can be fertilized, resulting in healthy offspring (Tan, 1994). Lastly, oocytes which are not normal are also selected for ovulation *in vivo*, resulting in miscarriage, stillbirth or any of a number of genetically transmitted diseases. Consequently, since follicular atresia does not always result in the selection of the most viable oocytes, its function may be only to limit the number of ovulations per cycle, rather than selection based upon viability. Nonetheless, additional study of the process of atresia may provide new strategies for the selection of viable oocytes, potentially offering new approaches for these types of problems.

When follicles exit the process of follicular development, they degenerate via follicular atresia. In primordial follicles, this process is initiated by the death of the oocyte, followed by apoptosis of the granulosa cells and reabsorption of the follicle (Odor, 1960).

In contrast, later in follicular development, the first signs that a follicle will become atretic can be observed in the mural granulosa cell layer (Jolly *et al*, 1997). Once these first cells begin to die, signals of an unknown nature transmit this death signal to their neighbouring cells, resulting in the destruction of the entire granulosa cell layer. Furthermore, as the granulosa cell layer dies, steroid production decreases. In the absence of this required support, the oocyte itself ultimately dies. The cycle completes itself when phagocytes digest the cellular debris and newly recruited follicles grow into the space formerly taken by the now completely reabsorbed follicles.

4. Ovulation

As described above (section B2; Pg 8), follicular selection is primarily under the control of FSH. As the time of ovulation approaches, follicular steroidogenesis comes primarily under the control of LH in both mammalian and avian species. In the hen, increases in LH receptor expression on granulosa cells induces a large increase in progesterone production in preovulatory follicles (Kato *et al*, 1995; Bahr *et al*, 1983; Marrone and Hertelendy, 1983; Etches and Duke, 1984). In mammalian systems, however, the LH-induced preovulatory increase in steroidogenesis results primarily in estrogen production in conjunction with a much smaller increase in progesterone secretion. As described above (section A2; Pg 4), ovarian steroids inhibit gonadotropin production throughout most of the

ovulatory cycle. Nonetheless, immediately prior to ovulation, these steroids temporarily begin to stimulate LH release (Johnson and van Tienhoven, 1980). This switch from negative to positive feedback results in rapid increases in LH concentration. Since LH stimulates ovarian steroid output, the result is a rapid incease in the plasma concentrations of LH, progesterone and/or estrogen. Ultimately, this increase in LH signals the initiation of ovulation, completing the process of follicular development.

Ovulation is the process by which mature oocytes are ejected from the follicle through the ovarian epithelium. In both mammals and birds, oocyte ejection is initiated at a discrete spot termed the stigma, although our understanding of these events remains somewhat speculative. LH, the initiator of ovulation, induces prostaglandin production (Beers and Strickland, 1978; Koos and Clark, 1982) which increases the activity of plasminogen activator (PA) in both birds (Tilly *et al*, 1992a) and mammals (Beers and Strickland, 1978). The role of PA in the degradation of extracellular matrix is well established, occurring via the activation of plasminogen located in the extracellular space. Plasmin itself can degrade extracellular matrix, however, it is also capable of activating other latent proteases such as fibrin and collagenase. Since PA is up-regulated by LH and LH induces ovulation, it is reasonable to suggest that the purpose of this activation is to weaken the follicular wall. This, in combination with the high hydrostatic pressure within the preovulatory follicle occurring because of the increased follicular cell number, antral fluid content (mammals) and yolk content (birds), results in follicular rupture at the stigma. The

oocyte is then released into the abdominal cavity where it enters the oviduct, ready for fertilization.

5. Corpora Lutea versus Postovulatory Follicles

Following ovulation, the ruptured follicles form corpora lutea (CL) in mammals (see Niswender and Nett, 1988) or POFs in hens (Chalana and Guraya, 1978). In both mammalian and avian ovaries, the collapse of the follicle during ovulation results in the breakdown of the basement membrane, allowing theca and granulosa cells to come in direct contact and blood vessels to invade the follicular cavity. In mammals, this results in distinct increases in cell size and progesterone production along with a decrease in estrogen production. Since progesterone is the primary follicular steroid in the hen, no significant steroidogenic changes occur between the F1 and POF stage (Nitta *et al*, 1993). Nonetheless, it is interesting to note that a switch in the primary follicular steroid product from estrogen to progesterone does occur in the hen prior to recruitment into the follicular hierarchy (Armstrong, 1985). In any case, the function of the CL/POF is to prepare for the gestation of the embryo. Specifically, progesterone from the CL prepares the uterus for implantation and maintains its function for the first two months of pregnancy, while the POF is believed to determine the time of oviposition (~25 hours after ovulation; Rothchild and Fraps, 1944; Gilbert *et al*, 1978) and to influence nesting behaviour (Wood-Gush and Gilbert, 1975) possibly via the production

of prostaglandin F (PGF) and/or an unidentified oxytocin-like factor (Tanaka and Goto, 1976). After the CL/POF have fulfilled their function, they rapidly degenerate via the process of apoptosis (CL and POF, respectively; Yoshinaga, 1978 and Lofts and Murton, 1973), thereby completing a successful ovulatory cycle.

C. Granulosa cells

During follicular development, there are considerable differentiative changes which occur in granulosa cells. In primordial follicles, granulosa cells are essentially quiescent; they do not divide and are not steroidogenically competent. As follicles are recruited into the developmental pool, they begin to produce steroids and rapidly proliferate. Furthermore, as the time of ovulation approaches, apoptotic granulosa cells are apparent in atretic follicles, while cells in follicles destined to ovulate become more steroidogenically active and stop proliferating. Furthermore, at each developmental stage, there are distinct subpopulations of granulosa cells representing varying stages of differentiation in both hen (Marrone *et al*, 1990) and mammalian (Eppig *et al*, 1997) follicles. Cumulus (mammalian) or germinal disk region (hen) granulosa cells interact directly with the oocyte via gap junctions (Yoshimura *et al*, 1993; Tischkau *et al*, 1997). As a result, relative to more distally located granulosa cells, these cells are more proliferative than steroidogenic in nature. Furthermore, early atresia is initially characterized by apoptosis of granulosa cells distal from the oocyte. The

implication of these observations is that regional changes in granulosa cell function can precede overall changes in follicular function. As such, the study of granulosa cell proliferation, differentiation and apoptosis and their regulation provides important insights into the processes of follicular development, atresia and ovulation.

1. Proliferation

a. The Process

As described previously (Section B2; Pg 8), recruitment for ovulation is characterized by a rapid growth phase, during which granulosa cells rapidly proliferate. Within the ovary, cell proliferation is a tightly regulated process. Granulosa cells in primordial follicles are quiescent, however, between the primordial and preovulatory stages, granulosa cells undergo rapid cell division. Furthermore, since granulosa cells are embedded in a basement membrane and attached to each other by extracellular matrix, tissue remodelling is required to incorporate these new cells. The initiation and coordination of cell division and tissue remodelling are under the control of growth factors, cytokines and gonadotropins. As such, in order to fully understand follicular growth, studies focusing on these aspects of granulosa cell function have primary importance.

The process of cell cycle progression has long been a subject for study in several different tissues. Progression through the cell cycle involves several protein

phosphorylation/dephosphorylation events (Nigg *et al*, 1996; Cox and Lane, 1995; Lee, 1995), cytoskeletal remodelling (Billger *et al*, 1988; Nigg *et al*, 1996) and Ca^{2+}/calmodulin activation (Whitfield *et al*, 1995). Nonetheless, there are certain key events of primary importance to this process, which will be summarized here. Briefly, there are four distinct stages to the cell cycle: G_0/G_1, S, G_2 and M. In the absence of a mitogenic signal, granulosa cells will generally remain in the G_0 phase. This state is characterized as cells which have left the cell cycle and contain one copy of their genome. As such, the G_1 phase only differs from G_0 in that cells in the G_1 phase are actively preparing themselves for replication. As this preparation is completed, the production of cyclins C, D and E occurs, resulting in the activation of cell cycle protein kinases and the beginning of S-phase or chromosome replication (Santella, 1998). Once a completed second copy of the genome has formed, the cell enters the G_2-phase to prepare itself for mitosis. During this preparation, cyclins A and B are produced which activate a different set of cell cycle protein kinases, signalling the beginning of M-phase (Santella, 1998). After the completion of mitosis, each of the two daughter cells re-enter the G_1 phase, finishing one complete cycle of cell division.

In order to physically accommodate the presence of new cells, considerable tissue remodelling is required. Tissue remodelling in the ovary is believed to be primarily regulated by activation of the Plasminogen Activator system (PA; Tilly *et al*, 1992a). PA activation results in the conversion of the zymogen plasminogen to plasmin which is the active form of the enzyme. Plasmin is a non-specific protease which not only degrades extracellular

matrix but also activates tissue metalloproteinases which assist in this process (Lipner, 1988). Furthermore, as granulosa cells proliferate, they produce fibronectin (Asem *et al*, 1992), laminin (Leardkamolkarn and Abrahamson, 1992) and collagen (Huet *et al*, 1997), presumeably to replace extracellular matrix degraded to compensate for new cells and to accomodate the growing follicle. Moreover, PA activity is highest in late follicular development in both mammals (Karakji and Tsang, 1995a,b,c) and birds (Tilly *et al*, 1992a), with the exception of the periovulatory period, at which point both mitosis and PA activity drastically decrease. Whatever role extracellular matrix may play in peri-ovulatory granulosa cells, its production and degradation are primarily coordinated with periods in follicular development associated with granulosa cell proliferation. This supports the concept that tissue remodelling and granulosa cell division are related and perhaps co-dependent events during follicular development.

b. Regulation of Proliferation

Although the importance of FSH in follicular growth is well established, several intraovarian factors are believed to have key roles as regulators of granulosa cell proliferation. The proliferation of granulosa cells in response to a number of different growth factors, including Insulin-like Growth Factor-1 (IGF-1), Epidermal Growth Factor (EGF), Transforming Growth Factor Alpha (TGFα), Fibroblast Growth Factor (FGF), Transforming

Growth Factor Beta (TGFβ) and Platelet-derived Growth Factor (PDGF) has long been demonstrated (see van Nassauw *et al*, 1997, Kol and Adashi, 1995). As such, these growth factors may have a role as stimulators of granulosa cell proliferation during follicular growth. Nonetheless, more complete information regarding the localization of these intraovarian regulators and their receptors as well as the timing and regulation of their expression is required to fully understand the role of these growth factors in follicular growth.

Considerable species specificity has been observed regarding the ability of FSH to stimulate granulosa cell proliferation during follicular growth. In both ovine (Monniaux and Pisselet, 1992) and avian (Onagbesan and Peddie, 1995) species, FSH did not induce granulosa cell proliferation *in vitro*. In contrast, FSH stimulated the proliferation of both bovine (Armstrong *et al*, 1996) and rat (Kanzaki *et al*, 1996) granulosa cells *in vitro*. Interestingly, IGF-1, which induces granulosa cell proliferation in both the avian (Lafrance *et al*, 1993a) and mammalian (Savion *et al*, 1981; Baranao and Hammond, 1984) models, is produced by granulosa cells in response to FSH and increases FSH-induced DNA synthesis in both rat (Kanzaki *et al*, 1996; Chun *et al*, 1994) and bovine granulosa cells (Armstrong *et al*, 1996). This suggests that, aside from the direct effects of the gonadotropins, FSH can regulate granulosa cell proliferative activity indirectly, via the production of growth factors. In the hen, however, IGF-1 is produced exclusively in the theca (Armstrong and Hogg, 1996), suggesting that IGF-1 may not mediate FSH action in the hen. Moreover, studies by Onagbesan and Peddie (1995) demonstrated that LH induces the

proliferation of hen granulosa cells in F3, F2 and F1 follicles in the presence of IGF-1, suggesting LH-IGF-1 interactions may play a key role in the late growth phase of hen follicles. Whether or not FSH-IGF-1 interactions have similar importance to early follicular growth remains to be determined.

TGFα and EGF induce the proliferation of both avian (Tilly and Johnson, 1990; Lafrance *et al*, 1993a; Peddie *et al*, 1994; Li and Tsang, 1995) and mammalian (Gospodarowicz *et al*, 1977; Gospodarowicz and Bialecki, 1979; Skinner *et al*, 1987a) granulosa cells via the EGF receptor. Interestingly, EGF production was not detected in rat (Yeh *et al*, 1993) or human ovaries (Tamura *et al*, 1995) and only weakly detected in the porcine system (Singh *et al*, 1995). In contrast, TGFα mRNA has been detected in both rat (Yeh *et al*, 1993) and porcine (Singh and Armstrong, 1995) granulosa cells, suggesting that TGFα is the primary activator of the EGFR in the ovary. In human ovaries, however, the production of TGFα was primarily localized to the theca (Tamura *et al*, 1995), supporting a paracrine rather than autocrine role for the growth factor. Interestingly, similar observations were made in the hen, in that a TGFα-like substance was found in theca-conditioned medium (Peddie *et al*, 1994), although this finding has not been confirmed.

The induction of EGFR but not TGFα by FSH has been observed in both mammalian (Feng *et al*, 1987; Fujinaga *et al*, 1992; Fujinaga *et al*, 1994) and avian (Onagbeson *et al*, 1996) granulosa cells. This demonstrates a mode of action for the stimulation of follicular growth (alteration of receptor density) by FSH which is distinct from its direct actions or via

growth factor production (ie IGF-1). Consequently, although the secretion of TGFα is paracrine in nature with respect to the granulosa cell layer and is independent of FSH, the characteristics of the granulosa cell response are under the gonadotropin's control. As such, both TGFα and IGF-1 have roles as mediators of FSH-induced granulosa cell proliferation.

FGF has been shown to induce granulosa cell proliferation in both mammalian (Gospodarowicz *et al*, 1977; Gospodarowicz and Bialecki, 1979) and avian (Lafrance *et al*, 1993a) models, although no studies have demonstrated gonadotropic regulation of its production. Basic FGF has been detected in bovine (Massoglia *et al*, 1987; Grothe and Unsicker, 1989; Grothe *et al*, 1990), porcine (Makris *et al*, 1989) and rat (Shimasaki *et al*, 1988; Koos and Olsen, 1989) ovaries, while the acidic FGF has been reported in the porcine (Makris *et al*, 1989) and rat (Koos and Siedel, 1989) systems. In addition, the production of basic FGF by bovine granulosa (Neufeld *et al*, 1987) and luteal (Stirling *et al*, 1990) cells *in vitro* has been demonstrated.

PDGF has also been shown to induce granulosa cell proliferation in both porcine (Hammond and English, 1987) and avian (Lafrance *et al*, 1993a) systems. Nonetheless, there is no evidence for the presence of PDGF within the granulosa cell layer. Interestingly, PDGF was not detected in the follicular fluid of patients undergoing a natural cycle (Svalander *et al*, 1991), but was detected in 70% of those undergoing ovarian stimulation (McWilliam *et al*, 1995). This suggests that the production of PDGF may be stimulated by gonadotropin; however, this contention remains to be demonstrated.

TGFβ, an inhibitor of rat granulosa cell mitosis (Skinner *et al*, 1987b), was found to stimulate granulosa cell DNA synthesis in the hen (Lafrance *et al*, 1993a). TGFβ is an intraovarian factor produced by both granulosa and theca cells throughout follicular development in both avian (Law *et al*, 1995; Van Nassauw *et al*, 1996) and mammalian (McWilliam *et al*, 1995; Roy and Hughes, 1994; Ghiglieri *et al*, 1995; May *et al*, 1996) systems. This apparent difference in its function between the avian and mammalian models is interesting, although the importance of TGFβ is tissue specific and varies considerably from proliferation to differentiation, apoptosis and gene expression (see Alevizopoulos and Mermod, 1997). TGFβ remains an important autocrine/paracrine regulator of granulosa cell function and additional study of its interactions with other factors *in vivo* may clarify the precise role of this factor.

In summary, considerable increases in the number of granulosa cells occur as the follicle grows in preparation for ovulation. In order to accommodate this increase in cell number, increases in extracellular matrix production and degradation must also occur. Although this increase is believed to be stimulated primarily by FSH, the intraovarian factors IGF-1 and TGFα have key mediatory roles in this process. Furthermore, the growth factors FGF, PDGF and TGFβ have been shown to stimulate granulosa cell proliferation, although their physiological roles within the ovary remain to be established.

2. Differentiation

During follicular development, granulosa cells differentiate from prefollicular epithelial cells in immature ovaries to tightly regulated secretory cells in preovulatory follicles. Specifically, as granulosa cells differentiate, progesterone [avian (Bahr *et al*, 1983) and mammalian (Gore-Langton, 1988)], estrogen (mammalian; Gore-Langton, 1988), inhibin (Steinberger and Ward, 1988) and fibronectin (Novero and Asem, 1993; Asem *et al*, 1992) secretion increase several fold. As established in section B2 (Pg 8), follicular development, and hence, granulosa cell differentiation, are primarily under the control of FSH early in follicular development and then LH as the time of ovulation approaches due to the patterns of expression of their respective receptors on granulosa cells (see Richards *et al*, 1995, Tisdall *et al*, 1995). Nonetheless, intraovarian factors such as vasoactive intestinal peptide (VIP; Tornell *et al*, 1988; Moretti *et al*, 1990, Karakji and Tsang, 1995a) and growth hormone-releasing factor (GRF; Spicer and Enright, 1991, Karakji and Tsang, 1995a) also induce granulosa cell differentiation via a shared receptor (Moretti *et al*, 1990). Furthermore, members of the growth factor (Armstrong and Webb, 1997; van Nassauw *et al*, 1997; Kol and Adashi, 1995) and cytokine (van Nassauw *et al*, 1997; Kol and Adashi, 1995; Vinatier *et al*, 1995) families have inhibitory roles in this process. Considering the high degree of complexity in the regulation of differentiation, it is not surprising that the granulosa cells of most follicles do not fully differentiate. Moreover, since follicles which are not selected die

via follicular atresia, it seems reasonable that the timing of granulosa cell differentiation is one of the key factors determining which follicle(s) will ovulate.

a. Steroidogenesis

The primary differentiated function of granulosa cells is the production of reproductive steroids. As steroidogenic synthetic pathways have been well described previously (see Gore-Langton, 1988) and are not a focus of this research, they will only be briefly mentioned here. All steroids (ovarian, testicular and adrenal origin) are produced via a series of chemical modifications of cholesterol. Since the granulosa cell layer is avascular, this cholesterol is primarily supplied by hormone-regulated *de novo* synthesis (Chang *et al*, 1976; Simpson *et al*, 1980). Furthermore, the conversion of cholesterol to pregnenolones, progesterones, androgens and estrogens is accomplished via the upregulation of several steroidogenic enzymes during the process of differentiation.

Steroid biosynthesis is initiated by the conversion of cholesterol via the mitochondrial enzyme cytochrome P450 side chain cleavage enzyme (P450 scc) resulting in pregnenolone (P5) production. P5 can be transformed into 17α-hydroxypregnenolone (17α-P5) via 17α-hydroxylase, while C17,20-lyase converts 17α-P5 to DHEA (dehydroepiandrosterone). Conversion between the P5 and progesterone (P4) pathway is mediated by 3β-hydroxysteroid dehydrogenase (3β-HSD) which can convert each of P5, 17α-

P5 and DHEA into P4, 17α-progesterone (17α-P4) and androstenedione, respectively. Furthermore, P4 and 17α-P4 are also substrates for 17α-hydroxylase and C17,20-lyase, respectively, indicating that 3β-HSD activity does not alter substrate recognition for these enzymes. Androstenedione serves as substrate for two key enzymes: aromatase P450 and 17β-hydroxysteroid dehydrogenase (17β-HSD). Similar to the above described enzymes, the order of activation does not alter the function of aromatase P450 or 17β-HSD. Consequently, while 17β-HSD converts androstenenedione to testosterone and estrone to estradiol-17β, aromatase P450 converts androstenedione to estrone and testosterone to estradiol-17β.

The changes in the steroidogenic capacities of granulosa cells during follicular development has been extensively studied in several mammalian species. Briefly, the primary class of steroid produced by granulosa cells is estrogen, although these cells are also capable of progesterone production (see Richards *et al*, 1995). The first stage at which granulosa cells exhibit steroidogenic competence is at the primary stage (Tisdall *et al*, 1995). Increases in granulosa cell steroid production occur throughout follicular development, with the largest increase occurring between the tertiary and Graafian stages as the dominant follicle(s) prepare for ovulation.

In contast to mammalian granulosa cells, hen granulosa cells do not produce estrogen (Bahr *et al*, 1983; Etches and Duke, 1984; Kato *et al*, 1995). These cells do not express any steroidogenic enzymes until the LWF stage (Nitta *et al*, 1993; Tilly *et al*, 1991a; Tilly *et al*, 1991b), at which time FSH-induced expression and activation of P450 side chain cleavage

enzyme (Li and Johnson 1993a; Tilly *et al*, 1991b), 3β-HSD (Nitta *et al*, 1993; Tilly *et al*, 1991a) and 17α-hydroxylase (Li and Johnson, 1993b) results in the production of progesterone (Li and Johnson, 1993a) and androstenedione (Li and Johnson, 1993b). The expression of P450 scc (Tilly *et al*, 1991b) increases throughout follicular development, with the largest change occurring between the F3 and F1 stages. Moreover, 3β-HSD expression and activation in granulosa cells increases between the LWF and SYF stages (Nitta *et al*, 1993), after which its expresion remains relatively constant (Asem and Hertelendy, 1985; Tilly *et al*, 1991a; Tilly *et al*, 1991b). In addition, 17α-hydroxylase production and activity in granulosa cells decreases to mimimal levels between the LWF and F3 stages (Li and Johnson, 1993b). Due to the described changes in the expression of steroidogenic enzymes during follicular development, granulosa cell progesterone production increases, while androgen production decreases. Moreover, granulosa cells at the F1 stage are the primary steroid source of the avian ovary, producing massive quantities of progesterone (Kato *et al*, 1995; Bahr *et al*, 1983; Marrone and Hertelendy, 1983; Etches and Duke, 1984) without significant androgen production (Li and Johnson, 1993b).

b. Regulation

Granulosa cell differentiation is regulated by the actions and interactions of hormonal and intraovarian factors. Consequently, the decision to differentiate is the result of a balance

between pro- and anti-differentiative signals. The primary signal for early differentiation of granulosa cells is FSH, while pre-, peri- and post-ovulatory changes in the characteristics of granulosa cell are driven primarily by LH. Nonetheless, the responsiveness of granulosa cells to gonadotropins is modulated by differences in receptor expression, the nature and quantity of steroidogenic enzymes and intraovarian factors.

Differences in both the type and density of receptor expression on granulosa cells of different developmental stages of the follicle represent the first level of regulation of gonadotropin responses. FSHR is the first gonadotropin receptor to be expressed during follicular development, which begins at the primary (mammalian; Tisdall *et al*, 1995) or LWF (avian; Li and Johnson, 1993a; Tilly *et al*, 1991b) stages. Limited information is available regarding the *in vivo* regulation of FSHR expression, however, roles for VIP and/or GRF have been postulated. VIP and GRF are believed to bind to a shared receptor which stimulates cAMP production (Moretti *et al*, 1990). Moreover, GRF was shown to stimulate folliculogenesis when administered together with FSH in infertile women resistant to gonadotropin therapy.

During follicular development, FSHR expression on granulosa cells increases through the secondary (mammalian) or LWF (avian) stages, after which, its expression begins to decrease in both mammalian (Kaipai and Hsueh, 1997) and avian (Zhang *et al*, 1997a) granulosa cells. In contrast, Luteinizing Hormone Receptor (LHR) is not expressed until the F6 (avian; Zhang *et al*, 1997a) or tertiary (mammalian; Kaipai and Hsueh, 1997)

stages, after which its expression increases until luteolysis (mammalian) or POF degeneration (avian). In mammalian granulosa cells, estradiol stimulates FSH-induced cAMP production and the number of cytosolic cAMP binding sites, without an increase in FSHR (Richards, 1979). This results initially in increased granulosa cell responsiveness to FSH, followed by the expression of the LHR (Segaloff *et al*, 1990). In the hen, however, progesterone rather than estradiol is the predominant FSH-induced steroid. Moreover, the progesterone receptor is expressed by hen granulosa cells as early as the LWF stage (Isola *et al*, 1987), whereas this receptor is not expressed until after the LH surge in rat granulosa cells (Park and Mayo, 1991; Natraj and Richards, 1993). The predominance of progesterone and the early expression of its receptor in hen granulosa cells would seem to suggest that progesterone rather than estradiol synergizes with FSH to induce LH receptor expression in this species, although this remains to be demonstrated.

Although gonadotropins are highly effective inducers of steroidogenesis, IGF-1 is believed to play a key role as a mediator of these responses. In mammals, IGF-1 amplifies FSH-induced progesterone and estrogen production (Adashi *et al*, 1985, 1988; Hutchinson *et al*, 1988; Schams *et al*, 1988). Moreover, IGF-1 inhibits the expression of 17α-hydroxylase in hen LWF granulosa cells (Li and Johnson, 1993b), but later potentiates LH-induced progesterone production (F1 to F3 stages; Onagbesan and Peddie, 1995). Consequently, in addition to its role as an inducer of proliferation, IGF-1 is a key differentiative factor for granulosa cells. The fact that IGF-1 induces both proliferation and differentiation

(progesterone production) in granulosa cells from the same developmental stage may suggest that differentiation and proliferation are not mutually exclusive. Nonetheless, since follicles contain granulosa cells of varying degrees of differentiation (Marrone *et al*, 1990; Tischkau and Bahr, 1996; Tischkau *et al*, 1997), it is also possible that subpopulations of cells respond to IGF-1 in a distinct and cytodifferentiation-dependent fashion.

Gonadotropin-induced steroidogenesis is regulated by growth factors, estrogens and androgens. TGFα and EGF inhibit the initiation of steroidogenic competence in LWF granulosa cells by inhibiting the expression of P450 scc (Li and Johnson, 1993a) and 17α-hydroxylase (Li and Johnson, 1993b). Moreover, the presence of these growth factors results in inhibition of both mammalian (Adashi *et al*, 1987; May *et al*, 1990, Karakji and Tsang, 1995b) and avian (Lafrance *et al*, 1993b; Onagbesan and Peddie, 1995) granulosa cell steroidogenesis throughout follicular development, although the extent of this inhibition decreases as the time of ovulation approaches. In addition, androgens and estrogens are also effective inhibitors of granulosa cell steroidogenesis in hen F1 granulosa cells (Lee and Bahr, 1990; Johnson *et al*, 1988). Moreoever, this inhibition was more effective in less mature F1 granulosa cells than cells from follicles nearer the time of ovulation (Johnson *et al*, 1988). The fact that growth factor, estrogen and androgen-induced suppression of steroidogenesis becomes less effective during differentiation, suggests that their roles are to control the rate of follicular development, thereby decreasing the chances of multiple ovulations.

In summary, granulosa cell differentiation is the process whereby the pre-epithelial

cells surrounding newly formed oocytes develop into the highly influential secretory cell of the mature preovulatory follicle. As the timing of follicular development is a key determinant to its function, this process is carefully regulated by the interactions of endocrine and intraovarian factors. Although our understanding of the nature and regulation of these events has been greatly improved, very little information is available regarding the process of follicular selection, which remains a central aspect of current reproductive research.

3. Extracellular Matrix

In both mammalian and avian species, granulosa cells grow on a basement membrane consisting primarily of the extracellular matrix proteins fibronectin (Asem *et al*, 1992), laminin (Leardkamolkarn and Abrahamson, 1992) and collagen (Huet *et al*, 1997). Moreover, follicular stage-dependent changes in granulosa cell production of fibronectin has been reported in both avian (Asem and Novero, 1993; Novero and Asem, 1993; Asem and Novero, 1994; Peddie *et al*, 1994; Asem and Conkright, 1995; Conkright and Asem, 1995) and mammalian (Skinner and Dorrington, 1984; Skinner *et al*, 1985; Carnegie, 1990) follicles. In addition to the structural importance of the basement membrane, matrix-cellular interactions play an important role in the regulation of granulosa cell differentiation. These interactions are mediated by the integrin family of receptors which bind to extracellular matrix proteins, resulting in intracellular signals which modify the actions of autocrine,

Table 1: Integrin receptors and their ligands (Tibdall and Albrecht, 1998).

Integrins	Ligands
$\alpha 1\beta 1$	collagen, laminin
$\alpha 2\beta 1$	collagen, laminin, tenascin, integrin $\alpha 3\beta 1$, fibronectin
$\alpha 3\beta 1$	collagen, fibronectin, laminin, integrin $\alpha 2\beta 1$, integrin $\alpha 3\beta 1$, epiligrin, entactin
$\alpha 4\beta 1$	fibronectin, invasin, VCAM-1, ICAM-2
$\alpha 5\beta 1$	fibronectin, collagen
$\alpha 6\beta 1$	laminin
$\alpha 7\beta 1$	laminin
$\alpha 8\beta 1$	tenascin, fibronectin, vitronectin
$\alpha 9\beta 1$	tenascin
$\alpha v\beta 1$	fibronectin, RGD, vitronectin, collagen, osteopontin
$\alpha L\beta 2$	ICAMs
$\alpha m\beta 2$	ICAMs
$\alpha x\beta 2$	fibrinogen
$\alpha II\beta 3$	collagen, disintegrin, fibronectin, RGD, vitronectin
$\alpha v\beta 3$	tenascin, disintegrin, collagen, fibronectin, RGD, vitronectin, osteopontin
$\alpha 6\beta 4$	laminin
$\alpha v\beta 5$	vitronectin, RGD
$\alpha v\beta 6$	fibronectin, tenascin
$\alpha 4\beta 7$	fibronectin, VCAMs, mucosal ACAM-1
$\alpha E\beta 7$	E-cadherin
$\alpha v\beta 8$	vitronectin

Table 2: The influence of integrin receptors on apoptosis (Tibdall and Albrecht, 1998).

Integrin Type	Influence on Apoptosis	Cell Type
α5β1	-	Chinese hamster ovary cells (CHO)
α?β1	-	Mammary epithelial cells
α?β1	-	Human umbilical vein endothelial cells (HUVECs)
αvβ3	-	Endothelial cells (angiogenesis)
α6β4	+	Epithelial cells (rectal carcinoma, RKO)
αvβ?	-	Colon carcinoma (LIM 1863)

paracrine and endocrine factors. Integrin receptors consist of two subunits (α and β), of which there are several different isoforms (see Table 1). Moroever, both apoptotic and protective roles for these receptors have been demonstrated in several different cell types (see Table 2). Consequently, differentiative changes in both the types and abundance of extracellular matrix proteins and integrin receptors may play a key role in the regulation of granulosa cell function.

During follicular development, the structure of the basement membrane is altered by increased production and deposition of fibronectin. In the hen, fibronectin secretion by granulosa cells is under the control of FSH (Novero and Asem, 1993), LH (Asem and Conkright, 1995) and TGFα/EGF (Asem and Novero, 1994; Peddie *et al*, 1994). Furthermore, intracrine roles for cAMP (Asem and Novero, 1993), progesterone (Conkright and Asem, 1995) and Ca^{2+} (Conkright and Asem, 1995) have been demonstrated in the mediation of these responses. Since the production of all three of these signal transduction mediators increases during follicular development, it is not surprising that the secretion of fibronectin follows a similar pattern. Although differences in the components of the basement membrane between follicles from different stages have not been demonstrated, increases in the tensile strength and sensitivity to enzymatic digestion have been observed during follicular development (Soboloff and Tsang; unpublished observations). Since fibronectin production is the only matrix component known to increase during follicular development, it is possible that these observed differences are due to a proportional increase

of fibronectin in the extracellular matrix, occurring as the follicle prepares for ovulation.

In contrast to the hen model, production of fibronectin by rat granulosa cells decreased significantly with follicular development (Skinner and Dorrington, 1984; Carnegie, 1990). Furthermore, FSH decreased granulosa cell fibronectin secretion (Skinner and Dorrington, 1984; Skinner *et al*, 1985; Carnegie, 1990), while GnRH stimulated its secretion (Dorrington and Skinner, 1986). In contrast, laminin and procollagen III production increased during follicular development in human follicles (Christiane *et al*, 1988). Although the reasons for this difference are not known, unlike in the hen, granulosa cells in the rat are organized in a multi-layer fashion, such that most granulosa cells are not in direct contact with the basement membrane. This fact makes comparisons of the secretion of basement membrane components between granulosa cells of these two species difficult to interpret.

The role of integrin receptors as regulators of cell function has been well established, however, very little information is available regarding their presence in ovarian cells. Giebel *et al* (1996) studied the expression of integrin subunits in the marmoset ovary. While the α1, α4 and α5 subunits were not expressed in the ovary, immunoreactivity of α3 was associated with follicular atresia, α2 with primary and luteinizing follicles and α6 was present throughout follicular development. In addition, with the exception of atretic follicles, integrin β1 was expressed throughout follicular development. This study demonstrates the potential importance of differential expression of integrin receptors, although additional information regarding the expression of other integrin β subunits and the role of integrins as modulators

of granulosa cell function would be helpful in establishing if cell-matrix interaction is an important cellular determinant in the control of the fate of the developing follicle.

D. Ovarian Innervation

In addition to autocrine, paracrine and endocrine influences, the ovary is infused by sympathetic, cholinergic and peptidergic nerves (Gilbert, 1965; Gilbert, 1969; Dahl, 1970; Ahmad *et al*, 1986). In the hen, the adrenal, renal and aortic plexuses supply branches that enter via the ovarian stalk and innervate the ovary (Johnson, 1925; Biswal 1954; Bradley and Grahame, 1961; Gilbert, 1967), along with branches directly from the sympathetic chain (Gilbert, 1968). Furthermore, Gilbert (1965, 1969) has demonstrated that bundles of nerve fibers directly innervate ovarian follicles, with specific fibers contacting thecal-interstitial cells, smooth muscle cells and blood vessels. Ferrando and Nalbandov (1969) found that intravenous injection of dibenzyline (α-adrenergic blocker) inhibited ovulation. In addition, Soliman and Walker (1976) demonstrated that isolated follicle strips taken from preovulatory ovaries showed spontaneous contractions, while those from less developed follicles showed no contractions without the addition of acetylcholine, norepinephrine or epinephrine. As such, these studies support the concept that, in addition to autocrine, paracrine and endocrine influences, the nervous system has a role in the regulation of ovarian function.

1. Cholinergic innervation

The precise role of cholinergic innervation in the control of ovarian function is not fully understood. Several studies have demonstrated the involvement of autonomic input in the control of follicular development (Brink and Grob, 1972), compensatory ovarian hypertrophy (Burden and Lawrence, 1977), ovulation (Bahr *et al*, 1974), ovarian blood flow (Gibson and Roche, 1986) and steroidogenesis (Kawakami *et al*, 1981). Furthermore, it has been demonstrated that Ach increases oxytocin and progesterone production via muscarinic receptors *in vivo* in sheep ovaries (Heap *et al*, 1989) and *in vitro* in bovine granulosa cells (Luck *et al*, 1990) and human corpora luteal cells (Casper and Cotterell, 1984). In the hen granulosa cell (F1), neither Ach nor the cholinergic agonist carbamyl choline chloride (carbachol; Cch) induced any change in basal or LH-induced cAMP or progesterone production (Morley *et al*, 1992a). However, it has been shown by Morley *et al* (1997) that exposure to Cch induces an increase in protein secretion in F1 granulosa cells. This was demonstrated by pre-loading the granulosa cells with [^{35}S]-methionine and measuring an increase in radioactivity in the medium following Cch challenge. Consequently, while the precise role for cholinergic innervation is unclear, it may be that the secretion of growth and/or inhibitory factors is under neural control.

Muscarinic responses are mediated by 5 different subtypes of receptor termed M1-M5 (see Felder, 1995). Although these receptors have considerable homology (> 90%) and

are all G-protein-coupled, they can be divided into two distinct functional groups. The M1, M3 and M5 receptors are associated with increases in $[Ca^{2+}]_i$ via both intra- and extracellular sources, as well as the activation of phospholipases A_2, C and D, along with protein kinase C and adenylate cyclase. In contrast, M2 and M4 are associated primarily with inhibition of adenylate cyclase, although weak activation of PLC (Ashkenazi *et al*, 1987) and augmentation of Ca^{2+}-induced phospholipase A_2 activity (Felder *et al*, 1991) have been reported. In hen F1 granulosa cells (Morley *et al*, 1992a) and human granulosa-lutein cells (Mayerhofer *et al*, 1992), challenge with Ach resulted in Ca^{2+} transients from both intra- and extracellular sources, suggesting that granulosa cells express M1, M3 and/or M5 receptors. In addition, Cch induced a Ca^{2+}-dependent, Na^+-independent increase in pH_i in hen granulosa cells (Li *et al*, 1992), suggesting the presence of a novel Ca^{2+}/H^+ exchanger.

In conclusion, although cholinergic innervation induces granulosa cell progesterone secretion in sheep, bovine and human ovaries, this was not observed in the hen. Nonetheless, muscarinic-induced increases in $[Ca^{2+}]_i$ were detected in both avian and human granulosa cells. Although not related to steroidogenesis, these changes in $[Ca^{2+}]_i$ appeared to play a key role in protein secretion and perhaps other aspects of granulosa cell function. Moreover, innervation played a role in follicular development, suggesting that muscarinic-induced changes in granulosa cell function may be follicular stage-dependent.

E. Cytokines

A number of intraovarian secretory products, including cytokines, growth factors, prostaglandins and neurotransmitters are believed to regulate the growth and differentiation of follicular cells in a paracrine and/or autocrine fashion (see Ackland *et al*, 1992; Leung and Steele, 1992). Cytokines are proteins which have generally been thought of as autocrine or paracrine regulators of the immune system, but are now known to have a significant role in the regulation of follicular function, ovulation and luteal regression (see Pate, 1995). Specifically, IL-1β, IL-2, IL-6, TNFα, IFN-γ and IFN-α have been shown to inhibit FSH-stimulated estrogen production (Spicer and Alpizar, 1994). Furthermore, the ovary has been identified as a source of TNFα (see Chen *et al*, 1993) as well as IL-1, IL-6 and granulocyte-macrophage colony stimulating factor (Ziltener *et al*, 1993). Nonetheless, since the current studies focussed on the role of TNFα as an intraovarian regulator, the subsequent review will be limited to this cytokine.

1. Tumour Necrosis Factor Alpha (TNFα)

Interest in the action of TNFα was initiated some 20 years ago when it was observed that the cytokine could cause tumor regression. Although subsequent research failed to identify a major role for TNFα as an anti-cancer agent, the multifunctional nature of the

cytokine has since been under intense study. TNFα has been shown to induce apoptosis, proliferation or growth arrest along with numerous cell type-specific differentiative functions. Since these types of responses are, by definition mutually exclusive, the cell type, cell state and the nature of other co-stimulatory factors must be considered in order to determine the *in vivo* role of this cytokine.

a. Regulation of ovarian function

Immunoreactive TNFα has been identified in the ovaries of several mammalian species (Chen *et al*, 1993; Roby *et al*, 1990; Sancho-Tello *et al*, 1992). Moreover, macrophages, leukocytes, oocytes, granulosa cells and theca cells have all been identified as sources of TNFα within the ovary (see Chen *et al*, 1993). The role of TNFα in the ovary is primarily thought of as anti-differentiative; TNFα inhibits gonadotropin-induced follicular production of progesterone (Roby and Terranova, 1990), estrogen (Adashi *et al*, 1989) and androgen (Andreani *et al*, 1991). In addition, a role for TNFα as an inducer of follicular atresia has been suggested (Kaipai *et al*, 1996; Witty *et al*, 1996).

Since both ovarian follicular estrogen and androgen production were inhibited by TNFα, it is not surprising that both granulosa and theca cells have been identified as TNFα targets. In both murine and swine granulosa cell cultures, TNFα inhibited gonadotropin-induced progesterone production (Adashi *et al*, 1990; Veldhuis *et al*, 1991). Furthermore,

the cytokine inhibited gonadotropin-induced plasminogen activator activity and progesterone production in rat granulosa cells while reversing gonadotropin-induced inhibition of DNA synthesis throughout follicular development (Karakji and Tsang, 1995c). In addition, in the absence of FSH or LH, TNFα stimulated prostaglandin production in granulosa cells from preovulatory follicles (Veldhuis *et al*, 1991; Zolti *et al*, 1990). Similar to granulosa cells, TNFα inhibited LH-induced progesterone production in the theca, although the cytokine stimulated progesterone production in the absence of LH (Roby and Terranova, 1990). This inhibition of LH-induced steroidogenesis was attributed primarily to downregulation of the LH receptor (Zachow *et al*, 1993).

TNFα is a highly pleiotropic factor, with numerous physiological responses attributed to its action, including apoptosis. Furthermore, its influence on prostaglandin production, steroidogenesis and DNA synthesis are consistent with its role as an atretogenic factor. TNFα has been demonstrated to induce apoptosis in hen LWF, but not F1 granulosa cells (Witty *et al*, 1996) and rat follicle cultures (Kaipai *et al*, 1996). Nonetheless,TNFα is a known pleitropic factor. Considering the number of autocrine, paracrine and endocrine factors present within the ovary, additional study of the interactions of the cytokine with other ovarian regulators are required to draw firm conclusions as to its *in vivo* role.

b. *Signal Transduction*

TNFα signals are mediated by two distinct receptors TNFR1 (55 kD) and TNFR2 (75 kD; see Darnay and Aggarwal, 1997). TNFR1 is generally considered the predominant receptor and can mediate both apoptotic and survival signals. TNFR2, however, is not believed to induce apoptosis and is more specific for the survival response. The nature of the TNFα receptors(s) present within the ovary has yet to be determined. Nonetheless, since the cytokine induces apoptosis in preantral (Kaipai *et al*, 1996; rat) and LWF (Witty *et al*, 1996; hen) granulosa cells, TNFR1 is likely to be present in granulosa cells at that stage of follicular development. Moreover, TNFα-induced apoptosis decreases during follicular development (Witty *et al*, 1996). This suggests either a developmentally regulated switch in the predominant receptor from TNFR1 to TNFR2, or a change in TNFR1-induced signals from cell death to cell survival during granulosa cell differentiation.

TNFα-induced death signals are believed to occur via interactions between TNFR1 cytosolic proteins containing *death domain homology regions* (see Darnay and Aggarwal, 1997; Fig 3). TNFα Receptor Activated Death Domain-containing Protein (TRADD) interacts directly with TNFR1 and has a role in both the apoptotic and survival pathways. Similarily, Receptor-Interacting Protein (RIP), which can bind both TRADD and TNFR, mediates both apoptosis and survival signals (see Baker and Reddy, 1996). The first proteins within the TNFR1 signal transduction complex which are specific for apoptosis are Fas-

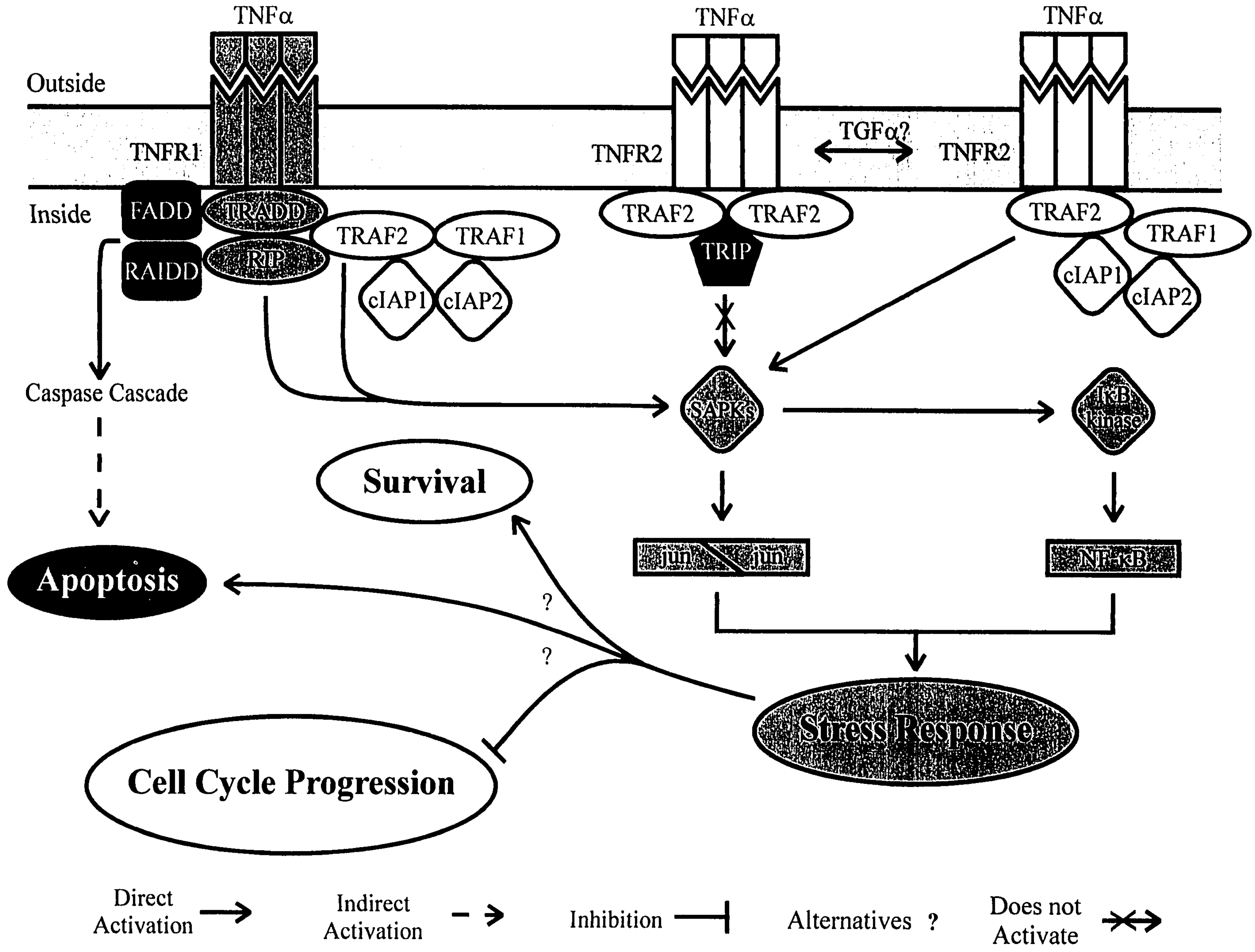
Associated Death Domain Protein (FADD; Hsu *et al*, 1996) and RIP Associated ICH-1/CED-3-Homologous Protein with a Death Domain (RAIDD; Duan and Dixit, 1997), which bind to TRADD and RIP, respectively. FADD and RAIDD activate the caspase network of proteases which initiates the process of apoptosis (see Villa *et al*, 1997).

The TNFα survival response occurs primarily via activation of the transcription factor nuclear factor κb (NF-κB; see Fig 3). Moreover, the primary mediators of TNFα-induced NF-κB activation have been identified (see Darnay and Aggarwal, 1997). Similar to the apoptotic pathway, TNFR1-induced activation of NF-κB results from the activation of the TNFR1 binding protein TRADD (Hsu *et al*, 1995). Interactions between TRADD and TNFR Activated Factor 2 (TRAF2) then results in the activation of NF-κB via stimulation of MEKK1 (Mitogen Activated Protein Kinase Kinase Kinase; Lee *et al*, 1997a; Natoli *et al*, 1997), a member of the Stress Activated Protein Kinase (SAPK) family. Although the mechanism for this has not been fully characterized, the serine-threonine protein kinases RIP (Hsu *et al*, 1996) and TNFα Receptor-Associated Protein Kinase-60 (TRAK-60; Darnay *et al*, 1995) have both been shown to contribute to TNFR1-mediated activation of NF-κB. Activation of SAPKs (mediators of NF-κB activation) occurs primarily by phosphorylation and may, therefore, be targets of RIP and TRAK-60; however, this remains to be demonstrated.

Similar to TNFR1, TNFR2-induced activation of NF-κB results from activation of TRAF2, although in this case, TRAF2 binds directly to the receptor (Rothe *et al*, 1994;

Rothe *et al*, 1995a; see Fig 3). Moreoever, a number of proteins have been identified which regulate the TNFR2-TRAF2-induced activation of NF-κB (see Fig 3). TNFα Receptor-Associated Protein Kinase-80 (TRAK-80) is a serine/threonine kinase which binds to TNFR2 and promotes the activation of NF-κB via serine-threonine phosphorylation (Darnay *et al*, 1994). TNF Receptor-associated Factor 1 (TRAF1) is known to bind to TRAF2 and has been shown to increase TRAF2-mediated NF-κB activation in a tissue-type specific manner (Speiser *et al*, 1997). While TRAF2 is ubiquitously expressed, the expression of TRAF1 has been demonstrated in the testis, but not studied in the ovary (Rothe *et al*, 1994). In addition, TRAF1 expression is tissue-type specific, suggesting that TRAF1 may be responsible for the tissue specificity of TNFα action. Cytosolic Inhibitor of Apoptosis Proteins 1 and 2 (cIAP-1 and cIAP-2) have been shown to bind to the TRAF1-TRAF2 heterodimer; however, the role of this interaction is unclear since cIAP-1 and cIAP-2 expression does not appear to influence the activation of NF-κB (Rothe *et al*, 1995b). In addition, the expression of c-IAP-1 has been demonstrated during follicular development within the ovary (rat; Li *et al*, 1998) along with its hen homologue (Inhibitor of T Cell Apoptosis or ITA; Digby *et al*, 1996; Johnson *et al*, 1998). TRAF-Interacting Protein (I-TRAF) or TRAF Family Member Associated NF-κB Activator (TANK) has been shown to bind to TRAF2-TRAF1

Figure 3: A hypothetical model depicting TNFα receptor-effector interactions. cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; FADD, Fas associated death domain; I-κB kinase, Inhibitor subunit of NF-κB; NF-κB, nuclear factor κB; RAIDD, RIP associated ICH-1/CED-3-homologous protein with a death domain; RIP, receptor interacting protein; SAPKs, stress-activated protein kinase cascade; TNFα, tumour necrosis factor alpha; TNFR1, TNFα receptor 1; TNFR2, TNFα receptor 2; TRADD, TNFα receptor activated death domain-containing protein; TRAF1, TNFα receptor activated factor 1; TRAF2, TNFα receptor activated factor 2; TRIP, TRAF interacting protein



TNFα
TNFα
TNFα
Outside
Inside
TNFR1
TNFR2
TGFα?
TNFR2
FADD
TRADD
RAIDD
RIP
TRAF2
TRAF1
cIAP1
cIAP2
TRAF2
TRAF2
TRIP
TRAF2
TRAF1
cIAP1
cIAP2
Caspase Cascade
SAPKs
IκB kinase
Survival
Apoptosis
jun
jun
NF-κB
?
?
Stress Response
Cell Cycle Progression
Direct Activation
Indirect Activation
Inhibition
Alternatives ?
Does not Activate

heterodimers as well as TRAF1 and TRAF2 homodimers and to either inhibit or facilitate TRAF2-mediated NF-κB activation at high or low concentrations, respectively (Cheng and Baltimore, 1996; Rothe *et al*, 1996). Interestingly, TANK-TRAF2 interactions are known to block TRAF2-TNFR2 binding, suggesting a likely mechanism for inhibition by TANK.

Lastly, TRIP can inhibit TRAF2-mediated activation of NF-κB without interfering with TNFR2-TRAF2 binding (Lee *et al*, 1997b), but is able to bind to TRAF2 homodimers, not TRAF1 (alone or dimerized) or TRAF1-TRAF2 heterodimers. A possible role for TRAF1, cIAP-1 and cIAP-2 is therefore suggested as TRAF2-binding molecules which passively promote TRAF2-mediated NF-κB activation by competitively inhibiting the binding of TRIP and TANK and promoting TRAF2-TNFR2 interaction (as TANK inhibits this interaction). This hypothesis is supported by the fact that lymphocyte proliferation signalled by the presentation of antigens resulted in the downregulation of TRIP and the upregulation of TRAF1, cIAP-1 and cIAP-2 (Lee *et al*, 1997b). This suggests that TNFα may promote proliferation via NF-κB activation only in cells which are already proliferatively active.

TNFα has been shown to increase $[Ca^{2+}]_i$ in 30A5 preadipocytes (Lee *et al*, 1990), human and murine fibroblasts (Bouchelouche *et al*, 1990; Corkey *et al*, 1991), neutrophils (Richter *et al*, 1990; Schumann *et al*, 1993) anterior pituitary cells (Koike *et al*, 1991) and sympathetic neurons (Soliven and Albert, 1992). The degradation of sphingomyelin into ceramide and phosphocholine is considered a key early event in TNFα action (see

Pushkareva *et al*, 1995). Interestingly, the SM metabolites sphingosine, sphingosine-1-phosphate (Spp) and sphingosylphosphorylcholine have been shown to induce changes in $[Ca^{2+}]_i$ (Ghosh *et al*, 1990; Zhang *et al*, 1991). In several non-ovarian cell lines, roles for ceramide have been demonstrated in TNFα-induced apoptosis, growth inhibition and proliferation (see Literature Review G2a; Pg 62). There have, however, been several studies on the mediatory role of ceramide in TNFα action within the ovary. In rat granulosa cells, ceramide has been shown to inhibit the steroidogenic enzymes aromatase (Santana *et al*, 1995), P450 side chain cleavage enzyme (Santana *et al*, 1996) and 3β-hydroxysteroid dehydrogenase isomerase (Santana *et al*, 1996). In addition, ceramide analogues can induce apoptosis in hen granulosa cells (Witty *et al*, 1996) and both exogenous sphingomyelinase (SMase) and ceramide have been shown to mimic TNFα-induced prostaglandin synthesis as well as the inhibition of gonadotropin-induced progesterone production (Santana *et al*, 1996). As such, identification of the mechanisms of TNFα action in the ovary requires additional study.

Although activation of NF-κB is well established as a key event in TNFα signal transduction, there are no published studies on this transcription factor in the ovary. It has been demonstrated that exposure of granulosa cells to TNFα for more than 6 hours is required to inhibit gonadotropin-induced steroidogenesis (Sancho-Tello *et al*, 1991). These findings suggest that gene transcription might be required for these TNFα responses, although the transcription factor mediating TNFα responses in the ovary is unknown.

Whether or not NF-κB is this elusive transcription factor remains to be determined.

F. Ca^{2+} Signalling

As described previously, granulosa cell functions are closely regulated by several different autocrine, paracrine, neurocrine and endocrine factors. Although the mechanisms of action of these factors can differ markedly, one event common to most major changes in granulosa cell function is an increase in $[Ca^{2+}]_i$. Although the role of Ca^{2+} in hen granulosa cell function is poorly understood, studies in other systems demonstrate a mediatory role for Ca^{2+} as a promoter of cell cycle progression, apoptosis and protein secretion along with several cell type specific differentiative changes (eg. steroidogenesis; see section F1b; Pg 55). Since Ca^{2+} transients can drive such a variety of different changes in cellular behaviour, the source and intracellular location of the Ca^{2+} transient along with the nature of other signal transduction intermediates must be considered to determine the physiological implications of these observations.

Increases in $[Ca^{2+}]_i$ occur due to the entry of Ca^{2+} into the cytosol from both extracellular and intracellular Ca^{2+} sources. Entry of Ca^{2+} ions from outside of the cell can occur via several different plasma membrane spanning Ca^{2+} channels, however, only T- and L-type Ca^{2+} channel activity has been observed in hen granulosa cells (Schwartz *et al*, 1989). L-type Ca^{2+} channel activity is characterized by a slow rate of inactivation and sensitivity to

nifedipine, while T-type Ca^{2+} channels are rapidly inactivated and blocked by Ni^{2+}. Direct determinations of the regulation of this Ca^{2+} channel activity have not previously been performed. Nonetheless, transmembrane influx of Ca^{2+} has been implicated in both ATP- (Morley *et al*, 1994) and Ach- (Morley *et al*, 1992a) induced Ca^{2+} transients in hen granulosa cells. This suggests that the regulation of these Ca^{2+} channels may be key to the signal transduction of these intraovarian factors. Nonetheless, full understanding of the nature of the roles for these Ca^{2+} channels in the action of these factors will require clarification of their regulation and studies of their relationships to functional changes in granulosa cell activity.

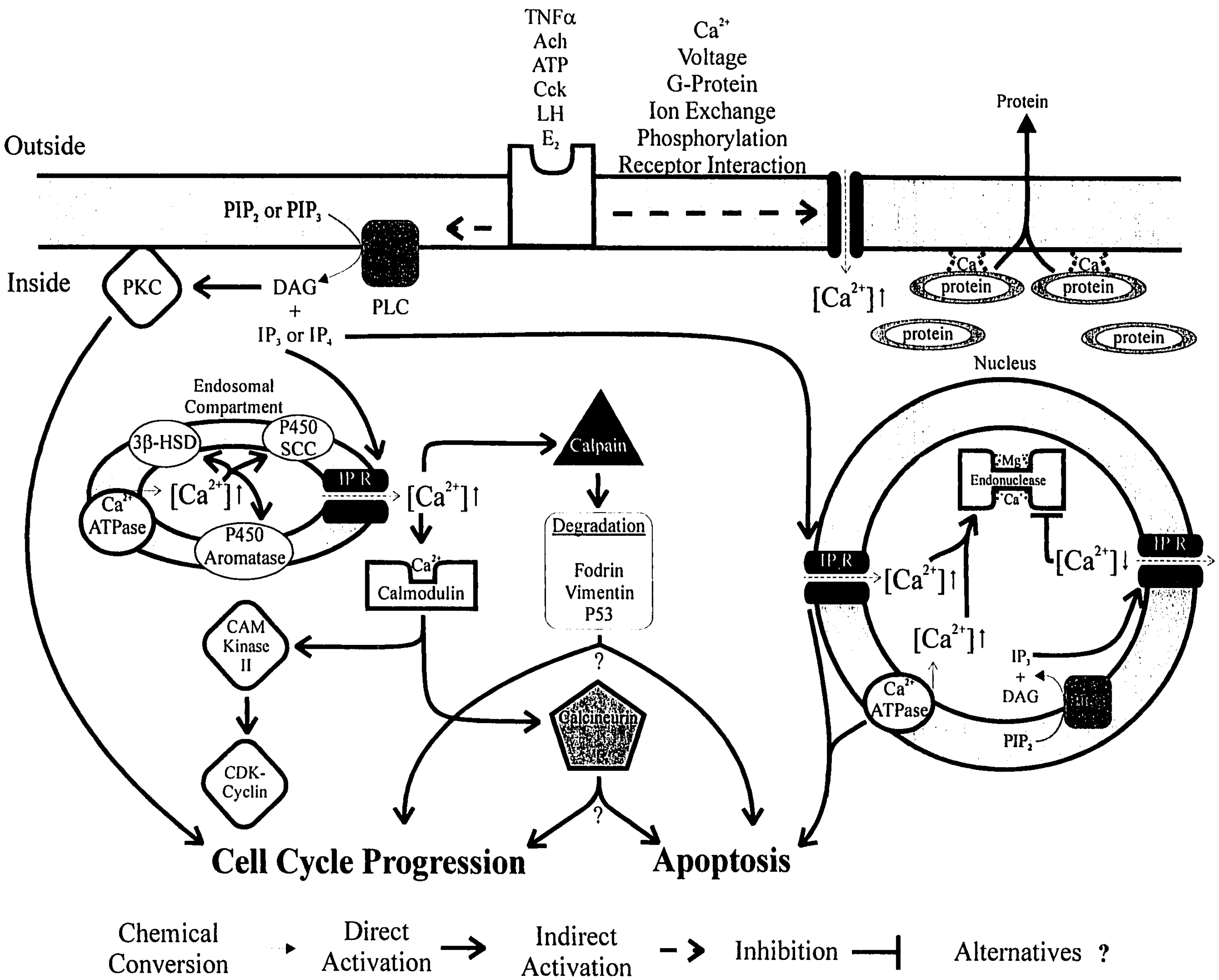
Under physiologic conditions, cytosolic $[Ca^{2+}]$ is approximately 1 million times lower than that outside of the cell and that inside cytoplasmic organelles. As such, Ca^{2+} located in these intracellular stores provides an additional source for ligand-induced changes in $[Ca^{2+}]_i$. Mobilization of Ca^{2+} from intracellular stores is a process known to be mediated by IP_3, Ca^{2+} or cADP ribose in several different systems. In addition, arachidonic acid has been shown to directly stimulate Ca^{2+} release from IP_3-sensitive stores in the hen (Hertelendy *et al*, 1992). Although well established in other systems, activation of the IP_3-Ca^{2+} system in the hen has only been demonstrated in response to luteinizing hormone (LH; Hertelendy *et al*, 1989), while neither Ca^{2+}- nor cADP ribose-induced Ca^{2+} release has been reported. Nonetheless, estrogen (Morley *et al*, 1992b), Ach (Morley *et al*, 1992a), cholecystokinin (Morley *et al*, 1993) and ATP (Morley *et al*, 1994) all increase $[Ca^{2+}]_i$ from intracellular

sources in hen granulosa cells. Consequently, hen granulosa cells exhibit Ca^{2+} signalling mechanisms which are distinct, but highly comparable to those observed in other systems. Still poorly understood, however, are the mechanisms regulating intracellular Ca^{2+} mobilization and their relationships to functional changes in hen granulosa cells.

1. Ca^{2+}-induced Physiological Changes

Increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) result in a number of different physiological responses (see Fig 4). Hormone-induced steroid production in hen, rat and swine granulosa cells has been shown to require the presence of Ca^{2+} (Higuchi *et al*, 1976; Veldhuis and Klase, 1982a; Tsang and Carnegie, 1983; Veldhuis *et al*, 1983; Asem and Hertelendy, 1986a; Davis *et al*, 1986). Cell cycle progression has also been demonstrated to require the activation of calmodulin by Ca^{2+} when $[Ca^{2+}]_i$ attains micromolar levels in several different systems (see Lu and Means, 1993). Protein secretion, which occurs primarily by exocytosis, is triggered by increases in $[Ca^{2+}]_i$ (see Livett *et al*, 1993; Martin, 1994). Lastly, apoptosis or programmed cell death can be induced by increases in $[Ca^{2+}]_i$ (Fesus *et al*, 1987; Fesus *et al*, 1989; Shi et al; 1989; Makrigiannis *et al*, 1994; Amendola *et al*, 1994; Bonnefoy-Berard *et al*, 1994; Boone *et al*, 1995). While it is difficult to understand how it is possible for cells to respond with such apparent lack of specificity, the duration, intracellular location and the magnitude of change in $[Ca^{2+}]_i$ are likely determinants of the

Figure 4: A hypothetical model depicting the role of Ca^{2+} in cell function. 3β-HSD, 3β -hydoxysteroid dehydrogenase; Ca^{2+}, ionic calcium; $[Ca^{2+}]_i$, ionic Ca^{2+} concentration; DAG, diacylglycerol; E_2, estradiol; Mg^{2+}, ionic magnesium; P450-SCC, P450 side chain cleavage enzyme; PA, phosphatidic acid; PC, phosphatidylcholine; PIP_2, triphosphatidylinositol; PIP_3, tetraphosphatidylinositol; PLC, phospholipase C; PKC, protein kinase C.



Outside
Inside
TNFα
Ach
ATP
Cck
LH
E2
Ca2+
Voltage
G-Protein
Ion Exchange
Phosphorylation
Receptor Interaction
Protein
PIP2 or PIP3
PLC
PKC
DAG
+
IP3 or IP4
[Ca2+]↑
Ca
protein
Nucleus
Endosomal
Compartment
3β-HSD
P450
SCC
Ca2+
ATPase
P450
Aromatase
IP R
Calpain
Degradation
Fodrin
Vimentin
P53
Ca2+
Calmodulin
CAM
Kinase
II
CDK-
Cyclin
Calcineurin
?
Mg
Endonuclease
Ca
[Ca2+]↓
IP3
+
DAG
PIP2
Cell Cycle Progression
Apoptosis
Chemical
Conversion
Direct
Activation
Indirect
Activation
Inhibition
Alternatives ?

response to an increase in $[Ca^{2+}]_i$. In addition, the physiological state of the cell before the reception of the Ca^{2+} signal, along with the nature of other signal transduction systems activated concomitantly must further influence the nature of cell responses to Ca^{2+} transients.

a. Proliferation

Transient increases in $[Ca^{2+}]_i$ have been observed at several points in the cell cycle and are believed to be a requirement for the $G_0 \rightarrow G_1$, $G_1 \rightarrow S$, $G_2 \rightarrow M$ and metaphase→anaphase transitions (Santella *et al*, 1998; Whitfield *et al*, 1995). Despite these observations, the mediators of Ca^{2+}-induced cell cycle progression have been only partially identified. The primary mediator of Ca^{2+}-induced cell cycle progression is calmodulin. At the $G_1 \rightarrow S$ transition, Ca^{2+}/calmodulin activates DNA polymerase α (Rixon *et al*, 1989; Youdale *et al*, 1985; Agell *et al*, 1991; López-Girona *et al*, 1992) as well as increasing the amount of proliferating cell nuclear antigen (a cofactor of DNA polymerase δ; Wang, 1991) and DNA polymerase δ at the level of gene transcription (Colomer *et al*, 1994; Bachs *et al*, 1994). Furthermore, Ca^{2+}/calmodulin initiates mitosis via activation of Cdc2•cyclin protein phosphatases which, in turn, activate Cdc2•cyclin protein kinases believed to be involved in the initiation of chromosome condensation and nuclear envelope breakdown in prophase (Whitfield *et al*, 1995).

Although calmodulin is the primary mediator of Ca^{2+}-induced proliferation, several

calmodulin activated proteins play key mediatory roles in this process. Calmodulin kinase II (CAM kinase II) is a serine-threonine kinase activated by Ca^{2+}/calmodulin and believed to play a role in the G_2→M transition (Baitinger *et al*, 1990; Waldmann *et al*, 1990). Moreover, one of the substrates for CAM kinase II is cdc25 (Whitaker, 1995), a phosphatase which activates p34cdc2/cyclin B, suggesting a likely mechanism for CAM kinase II-induced cell cycle progression. Moreover, the calmodulin-dependent phosphatase calcineurin was recently shown to be essential for the G_1→S transition (Santella, 1998), suggesting that this factor may also play a role in Ca^{2+}/calmodulin-dependent cell cycle progression.

The Ca^{2+}-dependent proteases calpain I and II have been demonstrated to contribute to several key steps within the cell cycle. P53, a key negative regulator of the G_1-S transition, has been identified as a target for proteolysis by calpain I (Zhang *et al*, 1997c). Moreover, the entry of extracellular Ca^{2+} was identified as a requirement for calpain-induced P53 degradation. In addition, calpain distribution within the cell is altered during mitosis. During metaphase and anaphase, calpain I accumulated in the spindle fiber region, while calpain II remained diffuse until telophase where it accumulated at the midbody (Lane *et al*, 1992). This suggests that calpains may play a role in cytoskeletal remodelling during mitosis, a concept supported by the observed degradation of the cytoskeletal proteins fodrin (Martin *et al*, 1995) and vimentin (McConkey and Orrenius, 1996) by calpain.

b. Steroidogenesis

While it is clear that the presence of Ca^{2+} is a requirement for steroidogenesis, the extent to which $[Ca^{2+}]_i$ and steroid production are related has not been without controversy. There have been numerous studies showing that either the removal of extracellular Ca^{2+} with the Ca^{2+} chelator EGTA or the addition of Ca^{2+} channel blockers (verapamil, Mn^{2+} or Co^{2+}) can markedly inhibit FSH-, LH-, GnRH- or cAMP-induced steroidogenesis in hen, rat and swine granulosa cells (Higuchi *et al*, 1976; Veldhuis and Klase, 1982a; Veldhuis and Klase, 1982b; Carnegie and Tsang, 1983; Tsang and Carnegie, 1983; Veldhuis *et al*, 1983; Carnegie and Tsang, 1984; Tsang and Carnegie, 1984; Asem and Hertelendy, 1986a). Furthermore, increases in $[Ca^{2+}]_i$ following exposure to the Ca^{2+} ionophore A23187, inositol 1,4,5-trisphosphate (IP_3) or membrane depolarization stimulated basal progesterone production *in vitro* in both rat and swine granulosa cells (Higuchi *et al*, 1976; Veldhuis and Klase, 1982a; Veldhuis and Klase, 1982b; Carnegie and Tsang, 1984; Tsang and Carnegie, 1984; Carnegie and Tsang, 1987; Sadighian *et al*, 1989). In hen granulosa cells (F1), however, increases in $[Ca^{2+}]_i$ following the use of the Ca^{2+} channel activators BAY-K8644 and CGP-28392 (Asem and Tsang, 1987), the Ca^{2+}-ATPase inhibitor thapsigargin (Morley P *et al*, 1992c), cholecystokinin (Morley *et al*, 1993) or acetylcholine (Ach; Morley *et al*, 1992a) did not stimulate progesterone production. Consequently, while Ca^{2+} is a consistent requirement for steroidogenesis in granulosa cells for most species, increases in $[Ca^{2+}]_i$ in the hen do not

result in increased steroid production.

c. Apoptosis

Apoptosis or programmed cell death is a highly regulated form of cell suicide which can be initiated by a variety of different signals. The role of Ca^{2+} in apoptosis has been studied extensively for over 20 years by a number of different laboratories. This concept was first demonstrated by Kaiser and Edelman (1977) based upon their demonstration that glucocorticoid-induced Ca^{2+} influx in immature thymocytes was associated with apoptosis. The role of Ca^{2+} in programmed cell death has since been confirmed in several different systems, including avian granulosa cells (quail; D'Herde and Leybaert, 1997). Although the mechanism of Ca^{2+}-induced apoptosis is not fully understood, the Ca^{2+}-dependent protease calpain has been suggested as one of the key components based on findings in lymphocytes that apoptosis induced by glucocorticoids, γ-irradiation and T-cell receptor can be blocked by calpain antagonists (Squier *et al*, 1994; Sarin *et al*, 1993; Martin *et al*, 1995). Furthermore, the cytoskeletal proteins fodrin (Martin *et al*, 1995) and vimentin (McConkey and Orrenius, 1996) have been demonstrated as targets for calpain hydrolysis and the degradation of cytoskeletal proteins is one of the hallmark characteristics of apoptosis.

Perhaps the best defined event in the process of apoptosis is the degradation of DNA into oligonucleosome fragments via the activation of a Ca^{2+}/Mg^{2+} dependent endonuclease.

In granulosa cells, this endonuclease has been identified as DNase I (Boone and Tsang, 1997), however, the nature of its regulation remains somewhat undetermined. Since this enzyme is Ca^{2+} activated, one distinct possibility is that increases in nuclear [Ca^{2+}] ([Ca^{2+}]$_n$) may signal its activation. Much like cytosolic [Ca^{2+}], [Ca^{2+}]$_n$ is regulated via the interaction of various Ca^{2+} channels. Increases in [Ca^{2+}]$_n$ are mediated by Ca^{2+}-ATPase activity (Nicotera and Rossi, 1994) and IP_4 receptors (Malviya, 1994; Koppler *et al*, 1993), while nuclear IP_3 receptors mediate the release of Ca^{2+} into the cytosol (Malviya, 1994; Nicotera *et al*, 1990). Although studies identifying the involvement of specific nuclear Ca^{2+} channels in apoptosis have not been identified, Bellomo *et al* (1992) demonstrated that TNFα induces a specific increase in [Ca^{2+}]$_n$ preceding programmed cell death in mammary adenocarcinoma cells. This supports the concept that one of the signals for apoptosis is an increase in [Ca^{2+}]$_n$ resulting in endonuclease activation and DNA degradation.

Transglutaminases are a group of Ca^{2+}-activated enzymes which catalyse the post-translational coupling of amines into proteins and the cross-linking of proteins via gamma glutamyl lysine bridges. The expression of this enzyme markedly increases during apoptosis (Fesus *et al*, 1987) and its transfection results in apoptosis (Melino *et al*, 1994). While further work would be required to identify the nature of transglutaminase activation and action in apoptosis, these observations support the concept that these enzymes may have a role in the induction of apoptosis.

Calcineurin, a Ca^{2+}/calmodulin dependent serine/threonine phosphatase, is involved

in the G_1→S transition as well as apoptosis. The immunosuppressant cyclosporin A inhibits Ca^{2+}-dependent apoptosis via activation of cyclophilins (Amendola *et al*, 1994; Bonnefoy-Berard *et al*, 1994; Makrigiannis *et al*, 1994; Shi *et al*; 1989), known inhibitors of calcineurin (Liu *et al*, 1991). Two reported actions of calcineurin are induction of the promoters of apoptosis: Nur77 (Yazbanbakhsh *et al*, 1995) and Fas ligand (Ancel *et al*, 1995). Consequently, although further study may identify additional targets for calcineurin, the above studies suggest a role for the phosphatase as a mediator of Ca^{2+}-stimulated apoptosis unrelated to its involvement in cell cycle progression.

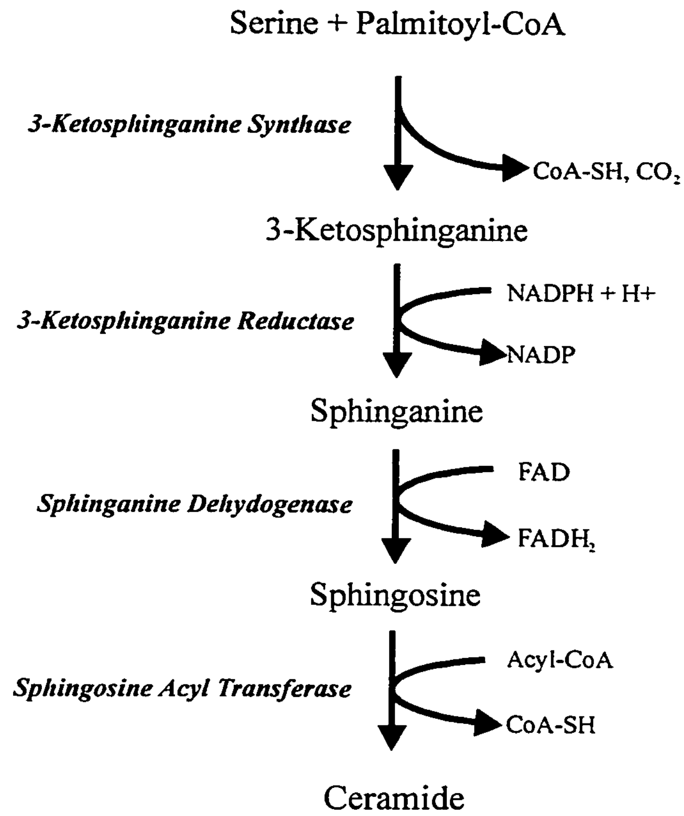
G. SM Degradation System

1. Sphingolipid Synthesis

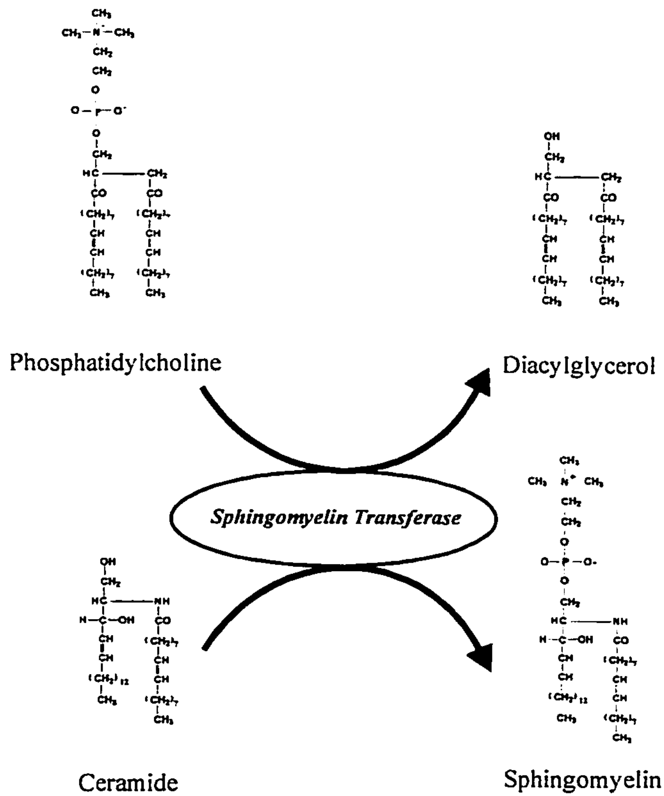
Sphingolipids can be synthesized via a series of reactions in most cell types. The base unit of all sphingolipids is sphingosine (Fig 5A; Lehninger, 1976). The first step in sphingosine production is the fusion of palmitoyl CoA with serine to form 3-ketosphinganine via decarboxylation. 3-Ketosphinganine is reduced to sphinganine by the conversion of the carbonyl group to a hydroxy group via an NADPH-redox reaction. Sphinganine can then converted to sphingosine by an $FADH_2$-redox reaction and the formation of a double bond. The conversion from sphingosine to ceramide can then be accomplished by acylation of sphingosine with acyl-CoA. Finally, the production of the membrane phospholipid

Figure 5: Sphingolipid synthetic pathways. **Panel A:** The ceramide synthesis pathway. **Panel B:** Sphingomyelin synthesis.

A



B

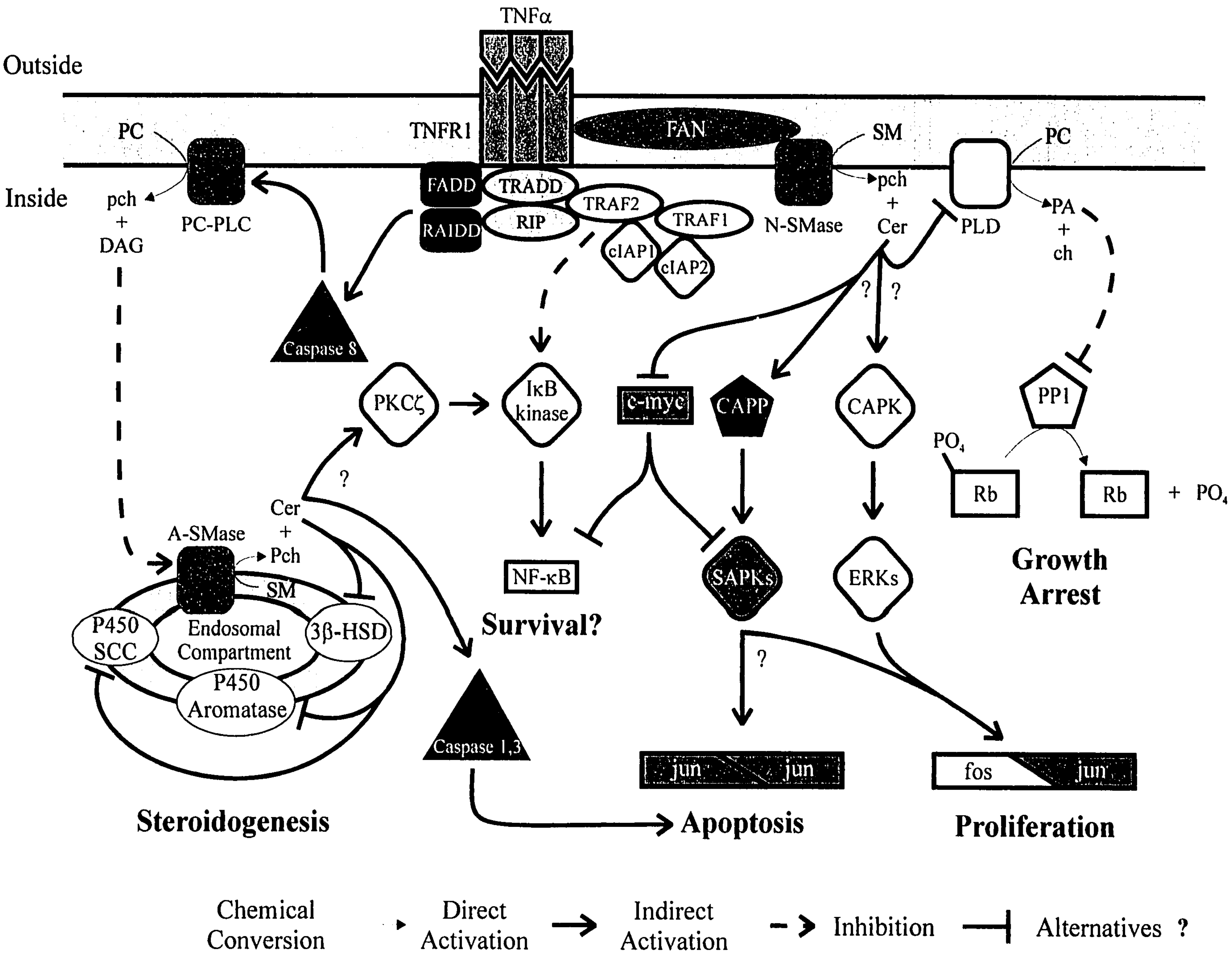


sphingomyelin occurs by the transfer of a phosphocholine from phosphatidylcholine to ceramide, leaving diacylglycerol (DAG) as a biologically active waste product (Fig 5B; Lehninger, 1976).

2. Ceramide Generation

In addition to the above mentioned synthetic pathways, ceramide can be produced by the activation of endogenous sphingomyelinase (SMase) as a second messenger. SMase has been identified as a mediator of TNFα, although it is also activated by Fas (Cifone *et al*, 1994), interferon-γ (Kim *et al*, 1991), interleukin-1β (Mathias *et al*, 1993), nerve growth factor (Dobrowsky *et al*, 1994) and vitamin D_3 (Okazaki *et al*, 1990). Furthermore, two different SMases have been identified which are activated by divergent signals following ligand binding and appear to have different functions: neutral SMase and acidic SMase (Wiegmann *et al*, 1994; Figure 6). Although their are no apparent differences in the nature of the ceramide produced by the two different SMases, the intracellular location of the enzymes, and hence the ceramide product, has been shown to strongly influence the nature of the cellular response (Wiegmann *et al*, 1994; Zhang *et al*, 1997b). The neutral SMase is a membrane-bound enzyme activated by TNFR1 via the mediation of a novel tryptophan-aspartic acid (WD)-repeat protein termed Factor Associated with N-sphingomyelinase Activation (FAN; Adam-Klages *et al*, 1996). Membrane production of ceramide results in

Figure 6: A hypothetical model depicting the regulation of cell function by ceramide. A-SMase, acidic sphingomyelinase; 3β-HSD, 3β -hydoxysteroid dehydrogenase; CAPK, ceramide activated protein kinase; CAPP, ceramide activated protein phosphatase; Cer, ceramide; ch, choline; DAG, diacylglycerol; FAN, factor associated with N-sphingomyelinase activation; ERKs, extracellular signal-regulated kinase cascade; NF-κB, nuclear factor κB; N-SMase, neutral sphingomyelinase; P450-SCC, P450 side chain cleavage enzyme; PA, phosphatidic acid; PC, phosphatidylcholine; pch, phosphocholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PKCς, protein kinase Cς; PLD, phospholipase D; PP1, protein phosphatase type 1; Rb, retinoblastoma protein; SAPKs, stress activated protein kinase cascade; TNFα, tumour necrosis factor α; TNFR1, TNFα receptor 1



TNFα
Outside
Inside
PC
pch
+
DAG
PC-PLC
TNFR1
FADD
RAIDD
TRADD
RIP
TRAF2
TRAF1
cIAP1
cIAP2
FAN
SM
N-SMase
pch
+
Cer
PC
PLD
PA
+
ch
Caspase 8
PKCζ
IκB kinase
c-myc
CAPP
CAPK
PP1
PO4
Rb
Rb
+ PO4
NF-κB
Survival?
SAPKs
ERKs
Growth Arrest
A-SMase
Cer
+
Pch
SM
P450 SCC
Endosomal Compartment
3β-HSD
P450 Aromatase
Caspase 1,3
jun
jun
fos
jun
Steroidogenesis
Apoptosis
Proliferation
Chemical Conversion
Direct Activation
Indirect Activation
Inhibition
Alternatives ?

the activation of ceramide-activated protein kinase (CAPK; Liu *et al*, 1994), Ceramide-Activated Protein Phosphatase (CAPP; Dobrowsky and Hannun, 1992), the MAP kinase cascade (Raines *et al*, 1993), SAPK (Westwick *et al*, 1995a), RAF-1 (Yao *et al*, 1995), protein phosphatase 2A (Law and Rossie, 1995) and phospholipase A_2 (Wiegmann *et al*, 1994). In contrast, the acidic SMase acts on lysosomal sphingomyelin and is activated by phosphatidylcholine-specific phospholipase C (PC-PLC)-induced diacylglycerol (Schütze *et al*, 1992). The acidic SMase is associated with the activation of both NF-κB (Wiegmann *et al*, 1994) and PKCς (Lozano *et al*, 1994).

a. Apoptosis versus Growth Inhibition vs Proliferation

As has been demonstrated in the action of TNFα, activation of SMase has been associated with apoptosis, growth inhibition and proliferative signals in a variety of different cell types. In U937 cells, ceramide is produced following exposure to TNFα and both C_2-ceramide (Obeid *et al*, 1993) and natural ceramide (Ji *et al*, 1995) induce apoptosis in a manner similar to the cytokine, suggesting a mediatory role for ceramide in TNFα-induced apoptosis. Furthermore, ceramide analogues induce granulosa cell apoptosis in F1 and LWF hen follicles (Witty *et al*, 1996) and early antral rat follicles (Kaipai *et al*, 1996). The mechanisms of ceramide-induced apoptosis are now, at least partially, understood. Activation of CAPP was shown to closely match the specificity of ceramide-analogue-

induced apoptosis, while okadaic acid, an inhibitor of serine/threonine protein phosphatases, can inhibit ceramide-induced apoptosis (Hannun, 1994). More recently, Verheij *et al* (1996) demonstrated that ceramide initiates apoptosis via activation of the SAPK cascade following TNFα-receptor interaction, while Reyes *et al* (1996) showed that c-Jun is a downstream target for CAPP. In addition, ceramide decreases the expression of the proto-oncogene c-myc (Kim *et al*, 1991). Recent studies in fibroblasts demonstrated that c-myc inhibits SAPK activation (Klefstrom *et al*, 1997). These studies provide strong support for the concept that ceramide-induced apoptosis occurs due to the activation of SAPK via CAPP and/or c-myc. In addition, the fact that CAPP activation is associated with the neutral-SMase suggests that ceramide produced at the plasma membrane is responsible for apoptosis. Nonetheless, the involvement of the acidic SMase in apoptosis has also been demonstrated. Mutations of TNFR1 which abolish acidic SMase activation have been shown to block TNFα-induced apoptosis (Skowronski *et al*, 1996). Moreover, Genestier *et al* (1998) showed that activation of Fas-induced acidic SMase activation occurs via the activation of a zVAD (non-specific caspase inhibitor)-sensitive and a YVAD/DEVD (inhibitors of caspases 1 and 3)-insensitive protease (most likely Caspase 8). Moreover, the activation of caspases 1 and 3 were required for apoptosis, but dependent on acidic SMase-induced ceramide production. Considered together, these studies seem to suggest there is little difference between ceramide produced from different sources. Nonetheless, an important distinction between these observations is that ceramide produced from the neutral SMase was capable of intitiating apoptosis, while

the role of acidic SMase produced ceramide in the apoptotic process appears to be more secondary in nature.

Ceramide production is well known to induce apoptosis, however, in some cell types, the production of ceramide can be better described as anti-proliferative. Ceramide analogues cause cell cycle arrest in the G0/G1 phase in HL-60 cells (Okazaki *et al*, 1990), Molt-4 leukemia cells (Jayadev *et al*, 1995) and fibroblasts (Venable *et al*, 1995; Gomez-Muñoz *et al*, 1994). The mechanism(s) regulating cell cycle progression are not fully understood, however, the retinoblastoma gene product (Rb) has been implicated as an important regulator of cell-cycle progression in response to growth suppressing agents and serum deprivation (Weinberg, 1991). Several lines of evidence suggest that Rb mediates ceramide-induced anti-proliferative activity. First, it has been demonstrated that Rb-deficient cell lines are resistant to ceramide-induced growth suppression (Pushkareva *et al*, 1995). Moreover, C6-ceramide induces cell-cycle arrest along with the dephosphorylation of Rb in Molt-4 leukemia cells (Dbaibo *et al*, 1995). Second, the decline in the proliferative capacity of fibroblasts which occurs as cells approach senescence is accompanied by increases in ceramide concentration, ceramide/DAG ratio, neutral SMase activity and the dephosphorylation of Rb (Venable *et al*, 1995). In addition, the anti-proliferative effects of ceramide in fibroblasts are accompanied by inhibition of phospholipase D activity (Gomez-Muñoz *et al*, 1994). Phosphatidic acid, the primary product of phospholipase D activity, has been shown to inhibit phosphatase activity in smooth muscle (Ito *et al*, 1997). This suggests the possibility

that ceramide-induced growth arrest occurs at least partially because decreases in phosphatidic acid concentration result in increased phosphatase activity and, therefore, Rb dephosphorylation, although direct studies are required to more clearly elucidate this aspect of ceramide signalling.

It is well established that TNFα can initiate proliferative signals in several different cell systems. Although the role of ceramide in TNFα-induced proliferative signals is not entirely clear, two distinct pathways have been suggested. Binding of ceramide to CAPK has been shown to phosphorylate Raf1 at the plasma membrane, resulting in the activation of the extracellular signal-regulated kinase (ERK) cascade (Yao *et al*, 1995). Since this event occurs at the plasma membrane, this suggests that ceramide produced from the membrane-bound neutral SMase is involved in proliferative signals. This concept is supported by the demonstration that both TNFα- and IL-1β-induced proliferative signals in fibroblasts did not involve the acidic SMase, although ceramide production was observed (Andrieu *et al*, 1994). In contrast, several studies in different cell types have suggested that acidic SMase does mediate ceramide-induced proliferation. Ceramide produced from the acidic SMase is known to induce PKCς activity, resulting in NF-κB activation and cell proliferation (Lozano *et al*, 1994; Diaz-Meco *et al*, 1994; Berra *et al*, 1993). In addition, the proto-oncogene c-myc, the expression of which is inhibited by ceramide (Kim *et al*, 1991), has been shown to inhibit NF-κB (Klefstrom *et al*, 1997), suggesting an additional indirect mechanism for ceramide-induced NF-κB activation. The role of ceramide in NF-κB activation has been somewhat

controversial, in that studies in some cell types have failed to demonstrate ceramide-dependent NF-κB activation (Reddy *et al*, 1994; Dbaibo *et al*, 1993). Nonetheless, a study by Wiegmann *et al* (1994) suggested that the acidic SMase, but not the neutral SMase signals NF-κB activation providing a potential explanation for this controversy.

In summary, ceramide has been demonstrated to have a role as a regulator of both proliferative activity and apoptosis. Ceramide induces cell death via the neutral SMase, resulting in stimulation of CAPP-induced and/or c-myc-induced SAPK activity. Moreover, ceramide promotes apoptosis via the acidic SMase via caspase activation. Ceramide is also able to signal G0/G1 cell-cycle arrest via inhibition of phospholipase D activity and stimulating the dephosphorylation of Rb. Furthermore, ceramide from the neutral SMase can signal proliferative activity via stimulation of CAPK-Raf-ERK, while acidic SMase can induce proliferation via activation of PKCς and/or decreased c-myc-inhibition, either of which result in NF-κB activation. The reasons that ceramide signals result in these mutually exclusive cell responses is not entirely clear, although the cellular location of ceramide produced, the presence and nature of costimulatory signals and the physiological state of the cell in question are likely important factors.

b. *Regulation of Steroidogenesis*

Although a considerable amount of information is available regarding the effects of

ceramide on immune cells and fibroblasts, there have been relatively few studies on granulosa cell function. Ceramide production by rat granulosa cells has been demonstrated following exposure to TNFα (Santana *et al*, 1995; Kaipai *et al*, 1996) and Il-1β (Santana *et al*, 1996), although there have been no demonstrations of ceramide production in the hen reproductive system. Furthermore, like TNFα and IL-1β, both C6-ceramide and exogenous SMase inhibit P450 side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase isomerase (Santana *et al*, 1996) and P450-aromatase activity (Santana *et al*, 1995) in a manner similar to TNFα or IL-1β in cultured rat granulosa cells induced by FSH or cAMP. This suggests that ceramide production may be responsible for the inhibition of steroidogenesis by TNFα and IL-1β, although precisely how ceramide and steroidogenesis are related remains to be determined.

3. Sphingosine

The ceramide byproduct sphingosine and its metabolite Spp are sphingolipids which have also been identified as signal transduction intermediates. Although these products can be synthesized from palmitoyl CoA and serine (as described above in section G1; Pg 58), sphingosine can also be produced from ceramide via ceramidase activation. Increases in both SMase and ceramidase activity as a result of both PDGF (Coroneos *et al*, 1995) and TNFα (Oral *et al*, 1997) have been demonstrated in vascular smooth muscle and cardiac myocytes,

respectively, establishing that sphingosine can be produced as a result of SMase activation. Furthermore, Spp can be produced from sphingosine as a result of sphingosine kinase activity induced by PDGF (Olivera and Spiegel, 1993; Choi *et al*, 1996). Exposure to either sphingosine or Spp can have multiple biological effects on cellular function, resulting in additional elements of SMase signalling.

The production of sphingosine has been associated with proliferative signals in several cell types. Increases in sphingosine concentration can result in increases in $[Ca^{2+}]_i$ (Ghosh *et al*, 1990), activation of phospholipase D (Zhang *et al*, 1990) and activation of the mitogenic protein kinase ERK-2 (Coroneos *et al*, 1996). Nonetheless, there is considerable controversy regarding the role of sphingosine in cellular function. Activation of protein kinase C is often associated with proliferative signals (Clemens *et al*, 1992), however, sphingosine is well recognized to inhibit protein kinase C (Hannun *et al*, 1986). In addition, a study by Orlati *et al* (1996) in a pancreatic duct adenocarcinoma cell line showed that sphingosine-induced Ca^{2+} transients are followed by inhibition of capacitive Ca^{2+} entry, resulting in a depletion of Ca^{2+} stores and a subsequent decrease in the ability of the cell to mobilize intracellular Ca^{2+}. Furthermore, studies by Chao *et al* (1992) and Pyne *et al* (1996) demonstrated that sphingosine production is associated with growth arrest via dephosphorylation of Rb and activation of the SAPK pathway, respectively. Lastly, sphingosine was demonstrated to induce apoptosis in a human prostatic cancer line (Shirahama *et al*, 1997), cardiac myocytes (Krown *et al*, 1996), human neutrophils (Ohta *et*

al, 1997; Yatomi *et al*, 1994) and HL-60 cells (Ohta *et al*, 1994; Sakakura *et al*, 1996). As such, determination of the role of sphingosine as a regulator of cell function will require future study.

One possible explanation for the diversity of cell responses to sphingosine is that its proliferative effects are mediated by sphingosine-1-phosphate (Spp). Zhang *et al*, (1991) showed that while both sphingosine and Spp were able to induce Ca^{2+} transients, sphingosine-induced Ca^{2+} transients were mediated by the production of Spp. In addition, Spp, but not sphingosine was shown to activate ERK-2 (Pyne *et al* 1996; contrary to Coroneos *et al*, 1996 in the same cell type). Since Spp can also activate phospholipase D in a manner similar to sphingosine (Desai *et al*, 1992), it may be the mediator of a wide variety of sphingosine-induced responses. In addition, Spp activation has been associated with activation of the Raf/ERK cascade (Wu *et al*, 1995), protein kinase C (Kleuser *et al*, 1996), Na^{+}/H^{+} exchange (Tornquist, 1997) and phospholipase C (Im *et al*, 1997; Okajima *et al*, 1996), as well as inhibition of adenylate cyclase (Im *et al*, 1997), all of which are consistent with a mitogenic role for this factor. Furthermore, Spp has been shown to suppress apoptosis induced by ceramide (Kleuser *et al*, 1996) and to activate the apoptosis-suppressing transcription factor NF-κB (Shatrov *et al*, 1997). Consequently, the role of Spp as a proliferative agent is considerably clearer than that of sphingosine. The role of Spp as the mediator of sphingosine-induced proliferative signals is strongly suggested, but remains to be proven.

III. OBJECTIVES

The overall objective of this research project is to study the actions and interactions of TNFα with intraovarian factors in the regulation of granulosa cell survival and differentiation during follicular maturation. The regulation of granulosa cell survival and differentiation requires the interactions of gonadotropins, growth factors, cytokines and neurotransmitters. TNFα is a cytokine, the actions of which within the ovary and in other systems are both controversial and contradictory. Since cellular responses to TNFα are highly variable, it seems likely that this action is dependent upon the presence or absence of other intra-ovarian factors and the stage of cytodifferentiation. The studies will focus upon the identification of factors which modulate TNFα-induced changes in granulosa cell function during follicular development. In addition, attempts will be made to identify the signal transduction mediators responsible for crosstalk between the signal transduction pathways of TNFα and other intra-ovarian factors. The following specific objectives will be addressed:

1. To study the actions and interactions of TNFα in the regulation of granulosa cell proliferation and survival during follicular development.

TNFα has been shown to either induce apoptosis or promote cell survival in a

number of different systems. Initial studies will be performed in the presence and absence of TNFα and gonadotropins, growth factors and/or the muscarinic agonist Cch using cell number quantitation, [^{3}H]-thymidine incorporation and 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide (MTT) assays. Apoptosis and necrosis will be assessed by DNA labelling and vital staining techniques.

2. Regulation of Ca^{2+} signalling by muscarinic input in hen granulosa cells during follicular maturation.

To assess muscarinic regulation of $[Ca^{2+}]_i$, single cell measurements of fura-2 loaded F1, F3 and F5,6 granulosa cells will be performed using the cholinergic agonist Cch. Developmentally regulated changes will be identified by comparison of the magnitude, rate of $\Delta[Ca^{2+}]_i$, frequency and source of Ca^{2+} for Cch-induced Ca^{2+} transients at each stage. Furthermore, measurements of IP_3 and Ca^{2+} current will be performed to further characterize the differences between Cch-induced Ca^{2+} transients during follicular development.

3. To study TNFα actions and its interactions with muscarinic input in the regulation of $[Ca^{2+}]_i$ in hen granulosa cells during follicular maturation.

The effect of TNFα on granulosa cell $[Ca^{2+}]_i$ will be assessed in fura-2 loaded

granulosa cells removed from F5,6 and F1 follicles. Developmentally regulated TNFα-induced changes in $[Ca^{2+}]_i$ will be characterized by assessments of the magnitude, frequency, rate of $\Delta[Ca^{2+}]_i$, and possible Ca^{2+} source. Furthermore, since the muscarinic agonist Cch is known to induce Ca^{2+} transients in F1 granulosa cells, the effect of Cch on $[Ca^{2+}]_i$ will be similarily assessed in the presence or absence of TNFα.

4. To study the modulatory role of TNFα on hen granulosa cell differentiation.

In several mammalian systems, TNFα has been shown to inhibit gonadotropin-induced differentiation. As such, TNFα-induced progesterone secretion will be assessed in hen granulosa cells in the presence or absence of LH during follicular development. In addition, due to the relationship between Ca^{2+} and steroidogenesis, TNFα-induced progesterone secretion in the presence of the muscarinic agonist Cch will be determined. Progesterone secretion will be determined by RIA.

The production and secretion of fibronectin in hen granulosa cells has been shown to be Ca^{2+}-dependent. In addition, TNFα-induced integrin production has been established in several non-ovarian systems. Consequently, interactions between TNFα and Cch in the production of these proteins will be determined by Western blot of hen granulosa cell lysates collected from F5,6 and F1 follicles. In addition, interactions between fibronectin and integrin receptors play an important role in cell proliferation and resistance to apoptosis.

Consequently, interactions between TNFα and TGFα in the production of fibronectin and integrin β1 and β3 (subunits of fibronectin receptors) will be assessed.

5. To determine if sphingomyelinase mediates TNFα action in hen granulosa cells during follicular development.

The sphingomyelin cycle has been established as a signalling pathway for TNFα in several different systems. Changes in F1 and F5,6 granulosa cell $[Ca^{2+}]_i$ and progesterone secretion due to activation of the sphingomyelin cycle will be studied and compared to TNFα-induced responses. Activation of the cycle will be accomplished via exogenous SMase while sphingomyelin metabolites will be used to identify the metabolite(s) within the pathway regulating the response. To determine if SMase mediates TNFα action, TNFα-induced changes in SM and Cer will be measured in F1 and F5,6 granulosa cells. Lipid concentrations will be assessed via densitometric analysis of radiolabelled lipid extracts separated by thin layer chromatography.

IV. MATERIALS AND METHODS

A. Reagents

[^{32}P]-γ-ATP, [^{32}P]-α-CTP, [^{14}C]-choline, Diacylglycerol Reagents Assay System and [^{3}H]-thymidine were obtained from Amersham Life Science (Oakville, On). Lysophosphatidic acid standard was obtained from Avanti Polar Lipids Inc. (Alabama, USA). Acetone, ammonium formate, bromphenol blue, chloroform, formic acid, iodine and methanol were obtained from BDH Chemicals (Toronto, On). D-sphingosine, N-acetylsphingosine (C2-ceramide) and N-myristoylsphingosine (C8-ceramide) were obtained from BioMol Research Laboratories (Plymouth Meeting, PA). DC protein assay kits, goat anti-mouse IgG (horseradish peroxidase conjugated), nitrocellulose paper, poly-prep chromatography columns, AG 1-X8 Resin and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Hercules, CA). Acrylamide, agarose, 2-amino-2-(hydroxy-methyl)-1,3-propanediol (Tris) and Coomassie Blue were obtained from Boehringer Mannheim (Laval, Quebec). Ionomycin was obtained from Calbiochem-Behring Corp. (La Jolla, CA). [^{3}H]-Inositol polyphosphate mix, [^{3}H]-progesterone and [^{3}H]-*myo*-inositol were obtained from Dupont/NEN Research Products (Mississauga, On). Acetic acid, hydrochloric acid (HCl), lanthanum chloride, potassium hydroxide (KOH), scintillation fluid (Scintiverse BD) and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Ottawa, ON). Disposable cell scrapers, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine

serum, Medium 199, minimum essential medium and trypsin-EDTA were obtained from Gibco Laboratories (Grand Island, NY). TNFα and TGFα were obtained from Immunocorp (Montreal, PQ). Fura-2-acetoxymethyl ester (fura-2AM) and pluronic F-127 were obtained from Molecular Probes Inc. (Eugene, OR). DNA polymerase I, large (Klenow) fragment was obtained from New England BioLabs (Mississauga, ON). Buffer AL and the Nucleotide Removal Kit were obtained from Qiagen Inc. (Santa Clarita, Ca). Ach, acridine orange, aprotinin, Cch, ceramide, collagenase (type 1A), deoxycholate, diethylenetriamine-pentaacetic acid (DETAPAC), dithiothreitol, dimethylsulphoxide (DMSO), ethylene glycol-bis (β-aminoethyl ether)-N, N, N' N'-tetraacetic acid (EGTA), ethidium bromide, fungizone, glycerol, goat anti-mouse IgM (horseradish peroxidase conjugated), isopropanol, ITS supplement [insulin (5 mg/l)-transferrin (5 mg/l)-selenite (5 μg/l)], imidazole/HCl, lithium chloride (LiCl), lysophosphatidylcholine standard, manganese chloride, 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide (MTT), *myo*-inositol, N-hexanoylsphingosine (C6-cer), N-methyl D-glucamine chloride (NMDG), nonidet P-40, nystatin, penicillin-streptomycin, phenol, phenylmethylsulfonly flouride (PMSF), phosphatidic acid, SMase (Bacillus cereus), SM-phosphatidylcholine standard, trypsin inhibitor (Type II-s) and tween-20 were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse anti-human integrin β3, mouse anti-human integrin β1 and mouse anti-human fibronectin were obtained from Transduction Laboratories (Mississauga, ON). N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) was obtained from VWR

Canada Ltd. (Ottawa, ONT). Ethanol was obtained from Ottawa Civic Hospital Stores (Ottawa, ON). Progesterone anti-serum was a gift from Dr. D.T. Armstrong (University of Western Ontario, London, On).

B. Methods

1. Granulosa Cell Isolation

White leghorn hens caged individually in a windowless, air-conditioned room with a 14L:10D cycle were killed by cervical dislocation 10 - 14 h before the expected time of ovulation. Granulosa cell layers from the 1st (F1), 3rd (F3) and 5th and 6th (F5,6) largest developing follicles were removed and individually dispersed by incubation at 37°C in medium 199 containing 270 U/ml collagenase and 0.01% (w/v) trypsin inhibitor for 10, 15 and 20 min, respectively as described by Asem *et al* (1984). The M199 was supplemented with HEPES (25 mM), penicillin (50 U/ml), streptomycin (50 µg/ml) and fungizone (0.625 µg/ml) and is hereafter referred to as M199.

2. [^{3}H]Thymidine incorporation assay

In order to assess DNA synthetic capacity of granulosa cells during follicular development, the incorporation of [^{3}H]-thymidine was determined as previously described (Lafrance *et al*, 1993a). Granulosa cells (2.5 x 10^5) were cultured 18 hours in 24 well plates (Falcon Plastics, Los Angeles, CA) in the presence or absence of TNFα, TGFα or LH followed by an additional 6 hours in the presence of 0.125 μCi/well of [^{3}H]-thymidine. Cells were then washed at room temperature in the presence of unlabelled thymidine (100 μg/ml) in Dulbecco's modified phosphate buffered saline [dPBS; $CaCl_2$ (0.9 mM), $MgCl_2{\cdot}6\,H_2O$ (4.9 mM), KCl (2.7 mM), NaCl (137 mM), K_2HPO_4 (1.15 mM), KH_2PO_4 (8.45 mM; pH 7.4], followed by two additional washed in dPBS alone. Cells were then fixed with trichloroacetic acid (TCA; 5% wt/vol in dPBS; 4°C, maintained for all subsequent washes) and then washed with TCA once more followed by two additional washes in 100% methanol. Cells were then dissolved in KOH (250 μl; 0.5 M, room temperature). Following an incubation period (aprox. 15 min) to ensure complete resuspension of all remaining cell contents, the pH of the cell lysates was adjusted to 7.6 with H_3PO_4 (250 μl; concentration determined by titration). DNA content was assessed in 50 μl samples using the saran wrap method (Sambrook, 1989), while the remaining 450 μl were transferred to scintillation vials containing 5 ml scintillation cocktail. [^{3}H]-thymidine incorporation was expressed as counts per μg DNA.

3. MTT Assay

The MTT assay was used as an estimate of cell viability in culture as described by Mosmann (1983). Briefly, F5,6 or F1 granulosa cells (2.5×10^5 cells/well) were cultured for 0, 24, 48 or 72 hrs in 24 well culture plates (Falcon Plastics, Los Angeles, CA) in the presence or absence of TNFα, TGFα and/or Cch. At the end of the culture period, MTT (5 mg/ml; one tenth total volume) was added to each well and incubated at 37°C (5% CO_2) for 3 to 4 hrs. At the end of this incubation period, an equal volume of acidic isopropanol (0.04 N HCl) was added to each well and the suspensions were thoroughly mixed and incubated overnight for adequate solubilization. In addition, a mixture of MTT (50 μl), MEM (500 μl) and acidic isopropanol (550 μl) was used as a blank. Dye conversion was assessed using a spectrophotometer (570 nm) following background subtraction (630 nm).

4. Cell Death Assessment

a. DNA labelling

Apoptosis in cultured cells was assessed by determining the pattern of DNA fragmentation by an established DNA labelling approach (Rosl, 1992). F5,6 granulosa cells (7×10^5 cells/well) were cultured in 12 well culture plates (Falcon Plastics, Los Angeles, Ca) for 0, 24, 48 or 72 hrs in the presence or absence of TNFα and/or TGFα. At the end of the

culture period, media were centrifuged (700 *g*; 10 min) to collect floating cells, during which time plated cells were harvested by incubation in 1 ml of trypsin (0.05%)-EDTA (530 μM) in PBS. Media was discarded and replaced with the trypsinized cell suspension supplemented with 50 μl of 100 mg/ml trypsin inhibitor. Following cell counting on a hemocytometer, cell suspensions were centrifuged (18 000 *g*; 7 min) and the supernatant was discarded while the pellets were resuspended in PBS (200 μl). The cells were lysed by the addition of Buffer AL (200 μl) and Qiagen Protease (25 μl) followed by 10 min incubation at 70°C.

DNA was extracted from cell lysates with phenol/chloroform extraction and subjected to ethanol precipitation as previously described (Sambrook *et al*, 1989). Briefly, samples (425 μl) were mixed with an equal volume of phenol/chloroform (1:1; pH 8.0), vortexed and centrifuged (18 000 *g*; 30 s). The upper phase (400 μl) was transferred to another tube, mixed with chloroform (400 μl) and incubated overnight at -20°C in the presence of NaCl (5 M; 15 μl) and ethanol (100 %; 780 μl) to precipitate DNA. The suspensions were centrifuged (18 000 g; 4°C; 10 min) and the pellets were washed in ethanol (70%; 750 μl) and centrifuged (18 000 g; 4°C; 5 min). The DNA pellets were then dried and solubilized in tris (10 mM)-EDTA (1 mM; pH 6.7) by pipetting, vortexing and incubation at 50°C (15 min).

DNA was quantified using the saran wrap method (Sambrook *et al*, 1989). Briefly, drops (5 μl) of DNA samples and standards (0, 1, 2, 5, 10 and 20 μg/ml) were placed on saran wrap over a UV box and mixed with ethidium bromide (2 μg/ml; 5 μl). DNA content

was determined by 2-D densitometric analysis using Molecular Analyst software (Bio-Rad; California).

To assess DNA fragmentation, extracted DNA was labelled by the Klenow reaction as described by Rosl (1992). Briefly, DNA was radiolabelled with α-[^{32}P]-dCTP (5 μCi/500 ng/sample) via 30 min incubation with Klenow enzyme (large fragment; 2.5 U). At the end of the incubation period, unbound α-[^{32}P]-dCTP was separated from the DNA using the Qiagen Nucleotide Removal Kit. Radiolabelled DNA was resolved by agarose (2 %) gel electrophoresis in tris-acetate EDTA buffer (tris, 40 mM; glacial acetic acid, 20 mM; EDTA, 1mM) for ~4.5 hours at 60 volts. The gel was dried and exposed to X-ray film or a phosphorimager (Bio-Rad, California) for 0.5-24 hours. The intensity of labelled low molecular weight DNA (< 23 Kb) in each lane was determined by 2-D densitometry using Molecular Analyst software (Bio-Rad; California).

b. Vital Staining

Vital stains were used for mophological assessments of cellular viability and to differentiate between apoptotic and necrotic cell death in culture as previously described (McGahon *et al*, 1995). F5,6 granulosa cells (2.5 x 10^5 cells/well) were cultured in 24 well culture plates (Falcon Plastics, Los Angeles, Ca) for 0, 24, 48 or 72 hrs in the presence or absence of TNFα, TGFα and/or Cch. At the end of the culture period, media containing

floating cells were collected, while plated cells were harvested by incubation in 1 ml of trypsin (0.05%)-EDTA (530 μM) in PBS. The harvested cells were then mixed with the floating cells and centrifuged (18 000 *g*; 7 min). The supernatant was then discarded. The pellets were resuspended in PBS (25 μl) and supplemented with dye mix [1 μl; acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml)]. Aliquots (10 μl), were placed on a microscope slide and viewed with a fluoresecent microscope (Zeiss IM-35; 150 X). Fields of cells were photographed using Kodak film ASA 400. Live cells had normal nuclei (bright green chromatin with organized structure), apoptotic cells had condensed and/or fragmented nuclei (bright green chromatin was defined as early apoptotic, while bright orange chromatin was defined as late apoptotic) while necrotic cells exhibited a bright orange stain and did not have condensed nuclei (either a normal chromatin structure or no nuclear staining). A minimum of 200 cells per treatment were counted (in duplicate) and the number of cells in each group were expressed as a percentage of the total cell number.

5. Progesterone Assay

In order to measure progesterone (P_4) production, granulosa cells (2.5 x 10^5 cells) were cultured in 24 well plates (Falcon Plastics Ltd., Los Angeles, Ca) for 24 hours in 500 μl MEM with and without BSA (0.1%; see results section A1a; Pg 95) ± Cch, TNFα, TGFα, LH, C2-ceramide, C6-ceramide, C8-ceramide or sphingosine. At the end of the culture

period, spent medium was collected, diluted in 100 % ethanol (10:1, ethanol:medium) and stored at -20 °C for subsequent analysis by radioimmunoassay (RIA), as previously described (Yallow, 1985). On the day the assays were performed, samples [duplicate aliquots of spent medium containing between 5 and 160 pg of P_4 (linear range)] and P_4 standards (5-640 pg/tube in triplicate) were dried under a stream of N_2 and resuspended in phosphate buffered saline-gelatin [PBSG; NaH_2PO_4 (76 mM), Na_2HPO_4 (20mM), NaCl (154 mM), EDTA (1.27 mM), NaN_3 (2.92 mM) and gelatin (0.1 g/l); pH 6.9]. [^{3}H]-P_4 (15 000 CPM) and anti-P_4 antibody (at a concentration which achieved 20-30% binding) were dissolved in PBSG and added to each tube to attain a final volume of 300 µl. In addition, each experiment included triplicate assay blanks (1.6 µg/ml unlabelled P_4, 15 000 counts [^{3}H]-P_4 in triplicate and anti-P_4 antibody; represents non-specific binding), triplicate tubes representing "total counts" (15 000 cpm [^{3}H]-P_4) and triplicate tubes termed "B_0" (15 000 cpm [^{3}H]-P_4 and anti-P_4 antibody only). The assay mixtures were then gently mixed and incubated overnight at 4 °C.

On the following day, 750 µl charcoal-dextran suspension [charcoal (3.57 g/l) and dextran (0.357 g/l) in PBS; to adsorb free P_4] was added to all samples and standards which were previously chilled. Assay tubes were then pre-incubated (15 min; 4 °C) and centrifuged (550 x g at 4°C; 15 min) to separate bound from free P_4. Supernatants containing the antibody-P_4 complex were decanted into scintillation vials containing 4 ml scintillation cocktail. The amount of radioactivity in each tube was determined using a scintillation counter. The intra- and inter-assay coefficients of variation were 6.7 and 11.2 %,

respectively, as previously established in this laboratory (Karakji and Tsang, 1995b).

6. Western Blot

F5,6 and F1 granulosa cells (1.2 x 10^6 to 1.5 x 10^6 cells/well) were cultured for 24 hrs in 6 well culture plates (Falcon Plastics, Los Angeles, Ca) in the presence or absence of TNFα, TGFα and/or Cch. At the end of the culture period, media were collected, supplemented with PMSF (10 μg/ml) and aprotinin (57 μg/ml) and stored at 4°C, while plated cells were lifted by scraping with disposable cell scrapers in cold PBS (1 ml). Spent culture media was then centrifuged (4 000 *g*; 15 minutes; 4°C) to pellet any floating cells. Media was then transferred to a microcentrifuge tube and stored for subsequent analysis. Pellets of floating cells were added to the attached cells and centrifuged (18 000 *g*; 10 min; 4°C). Supernatants were discarded and replaced with chilled cell lysis buffer [NP-40 (1% v/v), deoxycholate (0.05% w/v), SDS (0.1% w/v)] containing PMSF (10 μg/ml) and aprotinin (57 μg/ml) followed by gentle mixing with a pipette and incubation on ice (30 min). The samples were then centrifuged (18 000 *g*; 20 minutes; 4°C) and the supernatants were transferred to new microcentrifuge tubes and stored (-20°C) for subsequent analysis. Protein content was quantified using Bio-Rad DC protein assay kits. Proteins (20 μg/ml) were resolved on 8% SDS-PAGE (Laemmli, 1970) using the MiniProtean II minigel apparatus (Bio-Rad Laboratories) and electroblotted to nitrocellulose paper (Bio-Rad

Laboratories, CA; Towbin and Gordon, 1984). After transfer, polyacrylamide gels were stained with Coomassie Blue to verify proper loading.

After transfer nitrocellulose blots were blocked in 5% powdered skim milk (Carnation) in tris buffered saline-Tween 20 (TBST; 10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween-20) for 1 hour at room temperature and subsequently incubated with primary antibodies [mouse anti-human fibronectin IgG (1:2500), mouse anti-human integrin β1 IgG and mouse anti-human integrin β3 IgM (1:250)] in 5% blocking solution for 1 hour at room temperature. Blots were then washed twice (7 minutes) in TBST and incubated with secondary antibody [30 min; goat anti-mouse IgG (for fibronectin and integrin β1) or goat anti-mouse IgM (for integrin β3) conjugated to horse radish peroxidase at a 1:2500 dilution in 5% blocking solution]. Blots were washed for 5 min three times in TBST and once in tris buffered saline (TBS; 10mM Tris pH 8.0, 150mM NaCl). Peroxidase activity was visualized using the ECL kit as per manufacturer's instructions.

7. Microspectrofluorimetry

In order to determine $[Ca^{2+}]_i$, granulosa cells (1×10^5) were plated on glass coverslips (Fisher Scientific, Ottawa, On) using 1 ml of minimum essential medium containing $NaHCO_3$ (26 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), fungizone (0.625 μg/ml), hereafter called MEM and 10% fetal bovine serum (FBS) in 32 mm culture plates (Falcon

Plastics, Los Angeles, Ca) at 39°C under humidified air containing 5% CO_2. After 4 - 8 hours, cells were incubated in MEM without FBS for an additional 17 - 25 hours and loaded with the membrane permeant fluorophore fura-2AM as described by Morley *et al* (1992a). Briefly, cells were loaded with fura-2AM by incubation for 30 min at 37°C in NBS [NaCl (140 mM), KCl (5 mM), $CaCl_2$ (2.5 mM), $MgCl_2$ (1.1 mM), D-glucose (2.6 mM) and HEPES (10 mM); pH 7.2] containing pluronic F-127 (0.00125%; wt/vol) and fura-2AM (2.5 μM). Following three rinses with NBS to wash off unhydrolyzed fura-2AM, cells were further incubated for a period of 10 to 60 min in NBS at room temperature to ensure full hydrolysis of fura-2AM to fura-2. To verify proper loading and to ensure the complete removal of unhydolyzed fura-2AM, excitation spectra from 320 nM to 400 nM were run prior to usage. They were monophasic and peaked around 355 nm, indicating proper fura-2 loading.

Experiments were conducted on single cells or on groups of 6 cells or less at room temperature. Cover slips were placed in a water bath custom-fitted to the stage of an inverted epifluorescence microscope (Nikon Diaphot) equipped with a 40X objective. The cells were superfused at 0.5 ml/min through a pipette tip held in place immediately proximal to the cell being examined, a system which allowed for a rapid exchange of solution with less than 0.1 ml dead space. Measurements were performed using 350 nm and 380 nm excitation wavelengths at a sampling rate of 10 Hz. Fura-2 fluorescence was monitored through a photomultiplier tube with emissions centered at 505 nm. Background fluorescence, determined from unloaded cells, was about one tenth that of fura-2 loaded cells and was

subtracted prior to $[Ca^{2+}]_i$ determination.

The concentration of intracellular free Ca^{2+} was calculated according to the following formula (Grynkiewcz *et al*, 1985):

$$[Ca^{2+}]_i = K_d \times (F_{min}/F_{max}) \times (R - R_{min})/(R_{max} - R),$$

where R is the ratio of the fluorescence intensities measured at 350 nm and 380 nm during the experiments and F is the fluorescence intensity measured at 380 nm. R_{min},R_{max}, F_{min} and F_{max} were determined from *in situ* calibration of unlysed cells using ionomycin (4 μM) in the absence [R_{min} and F_{min}; EGTA (10 mM) in Ca^{2+}-free NBS] and presence (R_{max} and F_{max}) of Ca^{2+}. K_d, the dissociation constant for fura-2 at room temperature, is 135 nM. R_{min} and R_{max} were found to be 0.98 ± 0.047 and 11.03 ± 0.50, respectively, while F_{min}/F_{max} was found to be 7.46 ± 0.27. In addition to *in situ* calibration, cells were lysed with digitonin (20μM) to determine the extent of fura-2 sequestration. A decrease of 82.9 ± 1.6% was observed within 10 min of exposure to the detergent, consistent with that observed in other systems (Roe *et al*, 1990).

8. Electrophysiology

Electrophysiological studies were performed on F1 granulosa cells in order to assess

Ca^{2+} channel activity in granulosa cells using the perforated-patch clamp technique (Korn and Horn, 1992). Cells were plated as previously descibed for measurements of $[Ca^{2+}]_i$. Nystatin perforated-patch recording experiments were performed at room temperature (23 °C) on single granulosa cells using an Axopatch-1D amplifier (Axon Instruments, Foster city, CA). Patch pipettes of N51A glass (od, 1.65 mm; Garner Glass company) had resistances between 2-5 megaohms when filled with the pipette solution. The seal resistance was >2 gigaohms. Transmembrane currents were recorded in a 386 Akran computer at a sampling rate of 2 KHz and were filtered at 2 KHz. The traces were electronically compensated for series resistance and cell capacitance. Data acquisition and analysis were performed with the pCLAMP 5.5.1 software program (Axon Instrument).

The ionic composition of bath solution for all experiments excluding those in which $[Ca^{2+}]_i$ was monitored was: $CaCl_2$ (10 mM), Choline Cl (130 mM), CsCl (5.4 mM), $MgCl_2$ (0.4 mM), glucose (5.6 mM), HEPES (5 mM), pH 7.4. $NiCl_2$ (0.1 mM), nifedipine (10 uM), Cch (0.5 mM) and atropine (1 uM) were added to the bath solution as needed. The composition of pipette solution in all experiments was: CsCl (30 mM), Cs_2SO_4 (50 mM), NaCl (5 mM), $MgCl_2$ (2 mM), HEPES (5 mM), pH 7.3. Nystatin, freshly dissolved in DMSO by sonication, was added in the pipette solution at final concentration of 100-150 ug/ml. For simultaneous measurements of Ca^{2+} current and concentration, the initial bath solution used was NBS, while the final bath solution composition was: $CaCl_2$ (10 mM), NMDG (130 mM), CsCl (5.4 mM), $MgCl_2$ (0.4 mM), glucose (5.6 mM), HEPES (5 mM), pH 7.2.

9. Inositol Phosphate measurements

Production of IP_3 and metabolites was determined using previously described methods with minor modifications (Berridge *et al*, 1983). Briefly, granulosa cells (2 x 10^5 /well) were plated in quadruplicate in 24-well culture plates (Falcon Plastics, Los Angeles, Ca) using 500 μl of MEM containing 10% FBS for 4 - 5 h at 39°C under humidified air containing 5% CO_2. The plating medium was then replaced with 200 μl of inositol-free dMEM containing $NaHCO_3$ (26 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), fungizone (0.625 μg/ml) and 20 μCi/ml ^{3}*H-myo*-inositol (80 mCi/mmol) and the cells were cultured for an additional 16 - 18 h. The labelling medium was then removed and the cells were washed twice (1 ml; 30 min each) with MEM containing 5 mM *myo*-inositol and then once (0.32 ml; 45 min) in MEM without added inositol followed by a final incubation in LiCl (10 mM; 15 min) at 39°C under 5% CO_2 in air. Cells were treated as described in the results (section C2B; Pg 128) with Cch or Ach dissolved in 100 μl of MEM containing 10 mM LiCl and incubated as described above for 1 to 20 min. Medium was then discarded and the cells were incubated for 10 min at 0°C in 1 ml of methanol:chloroform:concentrated HCl (200:100:1). Cellular debris was scraped from the culture plates with a rubber policeman and transferred to borosillicate glass tubes containing 400 μl water and 300 μl chloroform, vortexed and centrifuged (500 X *g* at 4°C). The aqueous (upper) phase was stored at -20°C

for further analysis.

Aqueous extracts of ^{3}H-*myo*-inositol-labelled granulosa cells were applied to columns of AG1-X8 resin (Bio-Rad Labs., Toronto, ONT; approximately 0.8 ml packed volume prewashed with 5 mM *myo*-inositol) and inositol phosphates were eluted in a stepwise fashion with sequential washings with 12 ml of formic acid (0.1 M) solutions containing ammonium formate (0.1 - 1.0 M). Preliminary experiments with radiolabelled standards demonstrated that inositol-1-phosphate (1-IP), 1,4-IP_2, 1,4,5-IP_3 were eluted with 0.2, 0.4 and 1.0 M ammonium formate, respectively. Because this technique separated inositol phosphates on the basis of charge and did not discriminate between all inositol phosphates, the IP_3 fraction contained 1,4,5-IP_3, 1,3,4-IP_3 and 1,3,4,5-IP_4, while the IP_2 and IP fractions likely contained several different bisphosphate and monophosphate isomers, respectively. Since approximately 90% of radiolabel was eluted in the first 3 ml of each wash, only the first 3 ml fractions were retained for analysis. [^{3}H]Inositol phosphates were quantified by liquid scintillation counting of a 0.5 ml aliquot and data were expressed as percentage of control values.

10. SM Assay

Granulosa cell sphingomyelin content was assessed using a modification of the sphingomyelin assay (Jayadev *et al*, 1994). Granulosa cells (3 x 10^{5}) were plated in 24 well

plates (Falcon Plastics, Los Angeles, Ca) in MEM with 10% FBS for 4-6 h and subsequently maintained in serum- and choline-free MEM supplemented with insulin-transferrin-selenite (ITS) and 2 μCi/ml [^{14}C]-choline chloride for 66 hrs. Cells were then treated with TNFα (0.1 to 50 ng/ml) or exogenous SMase (50 mU/ml) for 1 to 60 min, although medium was not changed (changing medium induces sphingomyelin hydrolysis; Smith and Merrill, 1995). At the end of the culture period, media was removed, the cells were scraped in 0.5 ml HCl (2.4 N) with a rubber policeman and then transferred to polypropylene tubes. The wells were then rinsed with 125 μl NaCl (1.0 M) which was then added to the HCl extracts. The samples were then separated using a modified Bligh-Dyer extraction procedure (Bligh and Dyer, 1959). Briefly, 0.75 ml chloroform/methanol (1:2) followed by 0.5 ml chloroform were added to the samples, which were subsequently vortexed (~5 sec) and centrifuged at (207 x *g*; 5 min). The chloroform layer was then transferred to new polypropylene tubes, replaced with 0.75 ml chloroform and vortexed (~5 sec). Following centrifugation (207 x *g*; 5 min), the lower phase was collected and added to the original chloroform extract. The extracts were then dried under N_2, resuspended in 25 μl chloroform/ methanol (2:1), spotted onto thin layer chromatography plates (Fisher Scientific Ltd., Ottawa, ON) and developed in chloroform/methanol/acetic acid/water (50:30:8:5). The sphingomyelin and phosphatidylcholine spots were located using a phosphorimager (Bio-Rad Laboratories Ltd., Mississauga, On) and by comparison to unlabelled lipid standards (located by iodine). The amount of radioactivity in the spots was quantified with 2-D densitometry (Molecular

Analyst Software, Bio-Rad Laboratories Ltd., Mississauga, On). To correct for possible variation in loading between samples, sphingomyelin concentrations were normalized by total phosphatidylcholine.

11. Ceramide Assay

a. Cell culture and lipid extraction

For ceramide quantitation, granulosa cells (7×10^5) were plated for 4-6 hours in 12 well plates (Falcon Plastics, Los Angeles, Ca) in MEM containing 10% FBS (to allow attachment) and subsequently maintained in serum-free MEM supplemented with ITS for 66 hrs. Cells were then treated with TNFα (0.1 to 50 ng/ml) or exogenous SMase (50 mU/ml) for 1 to 60 min, although medium was not changed (changing medium induces sphingomyelin hydrolysis; Smith and Merrill, 1995). At the end of the culture period, media was removed and cells were scraped in 0.6 ml NaCl (1.0 M) with a rubber policeman and transferred to polypropylene tubes. The wells were then rinsed with 200 µl NaCl (1.0 M) which was added to the original sample. The lipids were then separated using a standard Bligh-Dyer extraction (Bligh and Dyer, 1959). Briefly, 3 ml chloroform/methanol (1:2) were added to the samples, which were subsequently vortexed (~5 sec). Chloroform (1 ml) and NaCl (1 ml; 1.0 M) were then added to each sample which were then vortexed (~5 sec) again and centrifuged (207 x *g*; 5 min). The chloroform layer was then transferred to new

polypropylene tubes, replaced with 2 ml chloroform and vortexed (~5 sec). Following centrifugation (207 x *g*; 5 min) the lower phase was again removed and added to the original chloroform extracts.

b. DAG Kinase Assay

The Diacylglycerol Assay Reagents System (Amersham Canada Ltd., Oakville, On) was used to radiolabel lipid extracts in order to detect and quantify cellular ceramide. This kit included the diacylglycerol kinase enzyme which phosphorylates diacylglycerol, ceramide and monoacylglycerol to produce phosphatidic acid, ceramide-phosphate and lysophosphatidic acid, respectively. Samples collected as described in the previous section were dried under a stream of N_2 and resuspended in 20 μl of a detergent solution [n-octyl-β-glucopyranoside (7.5%; wt/vol), cardiolipin (5 mM) in diethylenetriaminepenta-acetic acid (DETAPAC; 1 mM); vortexed (~10 sec) and sonicated (2 min prior to use)]. The assay tubes were then vortexed (~5 sec) and individually sonicated in a sonicating water bath for 2 min. The resulting suspension was then supplemented with 70 μl of "reagent mix" [10 μl of diacylglycerol kinase enzyme (a suspension of *E. coli* membranes enriched in diacylglycerol kinase of undefined activity) dissolved in a potassium phosphate buffer (5 mM) containing glycerol (10%), imidazole/HCl (5 mM), DETAPAC (0.5 mM) and mercaptoethanol (1 mM), pH 6,8; 50 μl of Diacylglycerol Reagents Assay System Assay Buffer (Amersham Canada

Ltd.) containing imidazole/HCl (0.1 M), NaCl (0.1 M), $MgCl_2$ (25 mM) and EGTA (2 mM), pH 6.6 and 10 μl of dithiothreitol (0.02 M)] followed by 10 μl of a tracer solution [ATP (5 mM) in imidazole/HCl (100 mM) containing DETAPAC (1 mM) and [^{32}P]-γ-ATP (1.0 μCi)] and vortexed (~ 5 sec). Following a 30 min incubation period at room temperature, 20 μl of perchloric acid (1% vol/vol; PCA) and 450 μl of chloroform/methanol (1:2; vol/vol) were added and the tubes were vortexed (~5 sec) and incubated for 10 min at room temperature. The assay tubes were then centrifuged (2000 g; 2 min), mixed with 150 μl chloroform and 150 μl PCA and vortexed (3 x 5 second bursts). Following centrifugation (2000 g; 2 min), the aqueous (upper) phase was removed, replaced with 1 ml PCA and vortexed (3 x 5 second bursts). This washing step was again repeated. The chloroform extracts were then dried under N_2, resuspended in 25 μl chloroform/ methanol (95:5), spotted onto TLC plates (Fisher Scientific Ltd., Ottawa, On) and developed in chloroform/methanol/acetone/acetic acid/water (10:4:3:2:1; Jayadev *et al*, 1994). The ceramide-phosphate, phosphatidic acid and lysophosphatidic acid spots were located using a phosphorimager (Bio-Rad Laboratories Ltd., Mississauga, ON) and by comparison to unlabelled lipid standards (located by iodine). The radioactivity in the spots was quantified with 2-D densitometry (Molecular Analyst Software, Bio-Rad Laboratories Ltd., Mississauga, ON).

12. Statistical Analysis

Results are presented as means ± SEM. Data predetermined to be homogeneous was analyzed by one or two way analysis of variance where appropriate. When data was determined to be heterogenous, data was analyzed using Kruskal-Wallis's analysis of variance by ranks. Differences between groups were determined by Bonferonni's or Tukey's tests for multiple comparisons. For $[Ca^{2+}]_i$ studies, relationships between percentage of response were analyzed by the χ_2 test.

V. RESULTS

A. Optimization of conditions for experimental analysis.

1. Establishment of culture conditions.

a. Progesterone production

In the mammal, studies of TNFα-induced granulosa cell proliferation were performed in the presence or absence of FSH (Karakji and Tsang, 1995c). TNFα-induced inhibition of gonadotropin action has been reported in mammalian cells for both LH (Zachow *et al*, 1993) and FSH (Andreani *et al*, 1991). However, in hen granulosa cells from the follicular hierarchy, progesterone production is primarily regulated by LH rather than FSH (Wells *et al*, 1985). As such, LH was considered the more appropriate choice in the hen and was used for all studies related to gonadotropin action.

Initial studies of LH-induced progesterone production demonstrated a lower sensitivity to LH than expected. The maximum concentration for LH-induced progesterone production was 100 ng/ml (F1) and >500 ng/ml (F5,6), while Asem and Hertelendy (1985) reported maximum LH-induced progesterone concentrations of 25 ng/ml (F1) and 100 ng/ml (F5,6). LH is stored in a 0.1 % BSA solution to limit the loss of the hormone by protease degradation and adherence to the walls of the container. As such, subsequent cultures were performed in the presence of 0.1% BSA. Under these conditions, maximum LH-induced progesterone content was observed at 10 ng/ml (F1) and 100 ng/ml (F5,6). As this is

consistent with other studies and BSA had no significant effect on basal progesterone production [9869 ± 2634 (n = 5; F1) and 133 ± 25 (n = 5; F5,6) pg/1 x10^5 cells with BSA and 3250 ±510 (n = 2; F1) and 214 ± 52 (n = 2; F5,6) pg/1 x10^5 in the absence of BSA], this culture system was used for all related studies.

b. Ca^{2+} measurement.

Granulosa cells (1 x 10^5) were cultured on glass coverslips in 1 ml of MEM containing 10% FBS based on previous studies from our laboratory (Morley *et al*, 1992a). Under these conditions, basal $[Ca^{2+}]_i$ was 88 ± 15 nM (n = 34) in F5,6 cells and 73 ± 6.9 nM (n = 32) in F1 cells. In addition, approximately 45 % (n = 31) of F5,6 cells and 61 % (n = 31) of F1 cells responded to challenge with Cch (0.2 mM) with a Ca^{2+} transient. In order to determine if the presence of serum affected subsequent responses, studies comparing cells cultured under these conditions with cells plated 4 - 6 hours in the presence of FBS (to allow cell attachment) and subsequently maintained overnight under serum-free conditions were performed. Basal $[Ca^{2+}]_i$ decreased to 34 ± 2.7 nM (n = 84; $p < 0.05$) in F5,6 cells and 50 ± 5.0 nM (n = 58; $p < 0.05$) in F1 cells under serum-free conditions as determined by ANOVA. Moreover, % of Cch responsive cells increased to 70 % (n = 64; $p < 0.025$) and 89 % (n = 53; $p < 0.005$) in F5,6 and F1 cells, respectively under serum-free conditions as determined using the χ_2 test. As such, all studies were performed on cells incubated overnight in serum-

free medium.

c. Sphingomyelin and ceramide assays.

In order to detect SMase activation, assays for sphingomyelin and ceramide were developed. Granulosa cells were plated for 4-6 hours in the presence of 10 % FBS and subsequently maintained for 66 hours in serum-free medium containing ITS-supplemented choline-free (sphingomyelin only) MEM in the presence (sphingomyelin assay) or absence (ceramide assay) of [^{14}C]-choline (Jayadev *et al*, 1994). Initially, at the end of this incubation period, medium was replaced with ITS-MEM in the presence or absence of SMase or TNFα. As considerable variability of the control group was observed and medium change induces sphingomyelin hydrolysis (Smith and Merrill, 1995), media for subsequent studies were not changed at the end of the incubation period, but merely supplemented with a 1/10 total volume of MEM containing a 10 times concentration of TNFα or SMase.

2. Development of the perfusion system.

Studies measuring $[Ca^{2+}]_i$ were performed on fura-2 loaded granulosa cells perfused at room temperature via a perfusion system developed in our laboratory. Due to the high cost of reagents, we developed a slow perfusion system (0.5 ml/min) which released test agents

(Cch, TNFα, C2-ceramide, C8-ceramide, sphingosine, SMase or ionomycin) through a pipette placed immediately above and behind the cell (Fig 7). As Cch-induced Ca^{2+} transients were observed within 10 seconds of switching solutions, this system was used for all subsequent studies.

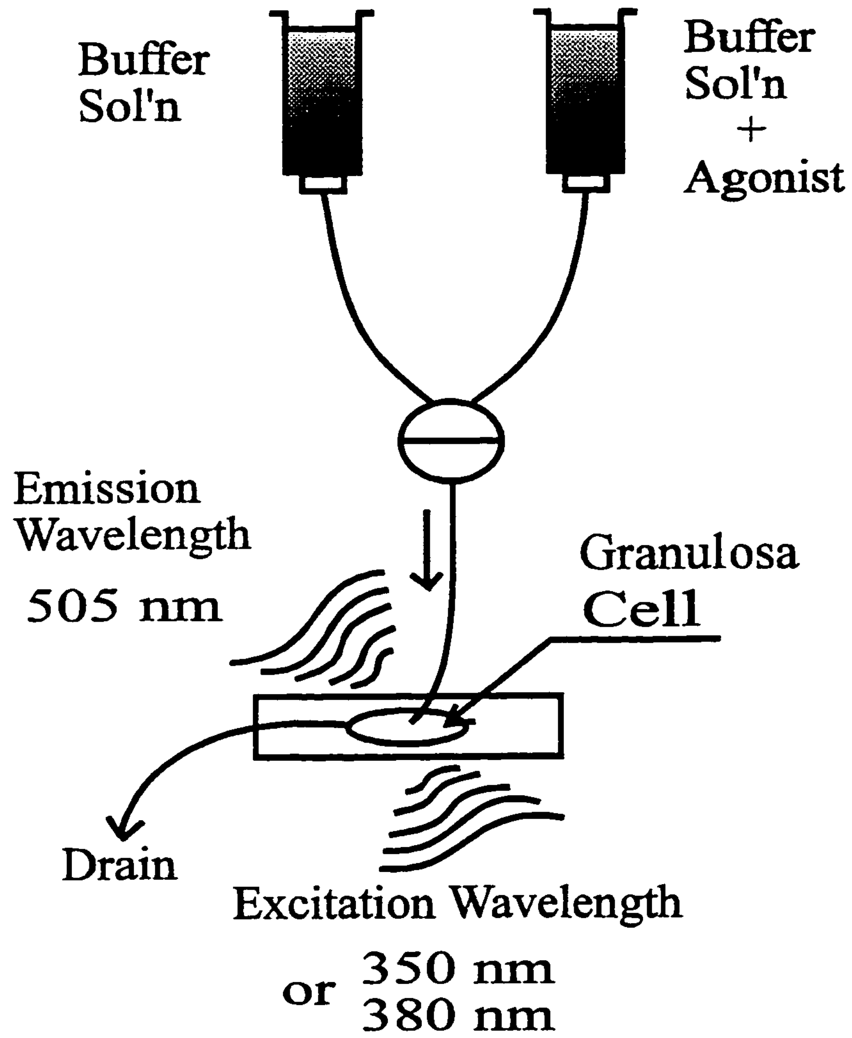
3. Development of conditions for Ca^{2+} current measurements.

a. The nystatin perforated patch whole cell clamp procedure.

Initial studies of Ca^{2+} current were performed using the conventional whole cell clamp configuration. Using this method, suction is applied to form a gigaohm seal and then increased to remove a patch of membrane, but "rundown" of inward current was evident after a few minutes. Rundown of Ca^{2+} current is typical of whole cell recording in a variety of preparations (Xiong *et al*, 1993; Wang *et al*, 1989; Toselli and Lux, 1989; Clapp and Gurnery, 1991) and occurs as a result of the loss of cytosolic material through the excised portion of the membrane. The rapidity of rundown (evident within ~5 min) in the granulosa cells made it difficult to assess and quantify the effect of Cch.

In order to avoid this problem, the perforated patch technique was used to record inward Ca^{2+} current. Using this procedure, the membrane is permeabilized by the addition of the cation permeable pore-forming agent nystatin. After the formation of a gigaohm seal, the series resistance (monitored with a 10-mV hyperpolarization pulse) gradually decreased

Figure 7: Development of the perfusion system. Cells were plated on cover slips and placed in a custom-made cylindrical water bath. A pipette tip was placed immediately above and behind the microscope objective at one end of the water bath, while fluid was removed by suction at the opposite end (drain). The pipette tip was connected through tubing to fluid reservoirs containing treatments. Due to the small volume of dead space (~70 μl), changes in the solution bathing cells located above the objective were accomplished at a flow rate of only 0.5 ml/min within 10 sec. Sol'n - solution

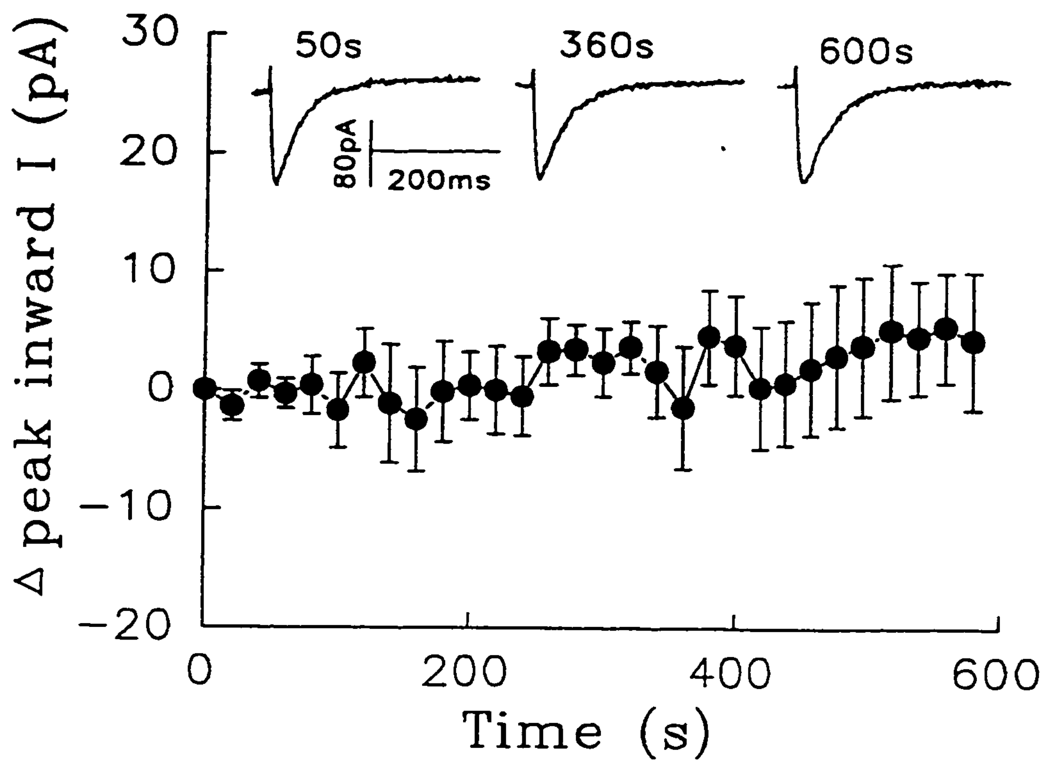

Buffer
Sol'n
Buffer
Sol'n
+
Agonist
Emission
Wavelength
505 nm
Granulosa
Cell
Drain
Excitation Wavelength
350 nm
or
380 nm

as the membrane became permeabilized by the nystatin in the pipette and showed no further decrease after about 15 min. During the 15 min required to permeabilize the patch, the inward current elicited by 50 mV depolarizing square voltage pulses from a holding potential of -70 mV increased gradually, then showed no further change for more than 10 min (Fig. 8). In two cells which were tested for a longer period, there was no change for 30 min. In all subsequent experiments, data were obtained only from recordings made within 10 min after the baseline inward current amplitude had stabilized.

b. *Effect of NMDG solution on $[Ca^{2+}]_i$.*

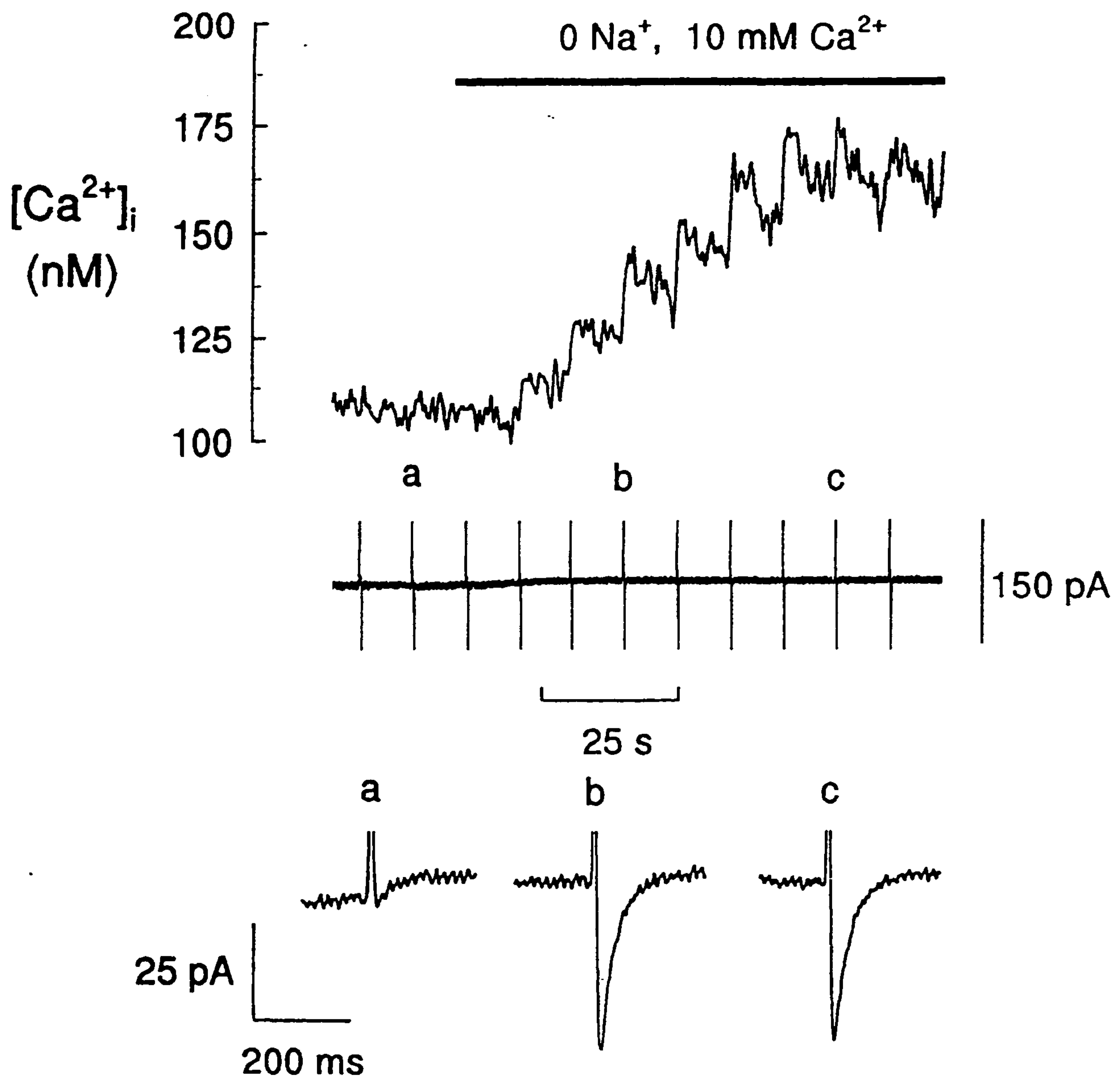
To test whether the observed inhibitory effect of Cch on Ca^{2+} current could be related to changes in intracellular Ca^{2+} levels, simultaneous recordings of $[Ca^{2+}]_i$ in fura-2 loaded cells were also performed. The first series of experiments evaluated the effect of replacement of Na^+ with N-methyl-D-glucamine (NMDG; instead of choline as choline is a weak muscarinic agonist) on granulosa cell Ca^{2+} current and $[Ca^{2+}]_i$. As shown in Figure 9, this manoeuvre promptly induced the expected large augmentation of Ca^{2+} current, with the average amplitude increasing from 19 ± 7 pA in NBS to 86 ± 21 pA after 1 min exposure to the NMDG solution (n = 6). This was significantly less ($p < 0.05$) than the inward current observed when choline was present (185 ± 11 pA), however, Cch (0.5 mM) induced an average maximal suppression of 90 ± 2% (n = 8) in the presence of choline and 92.2 ± 2%

Figure 8: Use of the nystatin perforated-patch configuration prevents run-down of inward current in chicken granulosa cells. "Delta Peak inward I", the ordinate, shows the peak current at time=t minus the peak current at time=0. Measurements were begun at a point when both series resistance and the magnitude of inward current obtained using a standard test pulse became stable. The inset shows, for the times indicated, inward currents recorded using a holding potential of -70 mV and step to -20 mV for 500 ms. n=8.



Δ peak inward I (pA)
30
20
10
0
−10
−20
0
200
400
600
Time (s)
50s
360s
600s
80pA
200ms

Figure 9: Effect of 10 mM Ca^{2+} and 0 Na^{+} on intracellular free-Ca^{2+} concentration and Ca^{2+} current. The granulosa cell was initially superfused with normal buffer solution which was thereafter replaced with the solution used for electrophysiologic recordings (with Na^{+} replaced by N-methyl-D-glucamine), as indicated by the horizontal bar. The upper tracing shows the measured $[Ca^{2+}]_i$ while the middle one represents the membrane current simultaneously recorded. Membrane potential was held at -70 mV and 300-ms depolarizing pulses to -20 mV were applied every 10 s, as can be noticed from the truncated capacitive spikes. Expanded current recordings, taken at the times indicated by the small letters, are given in the lower graph. Superfusion with the NMDG solution induced an increase in Ca^{2+} currents together with the development of small $[Ca^{2+}]_i$ transients that were elicited by these currents but outlasted them for several seconds.



0 Na⁺, 10 mM Ca²⁺
200
175
150
125
100
[Ca²⁺]i
(nM)
a
b
c
150 pA
25 s
a
b
c
25 pA
200 ms

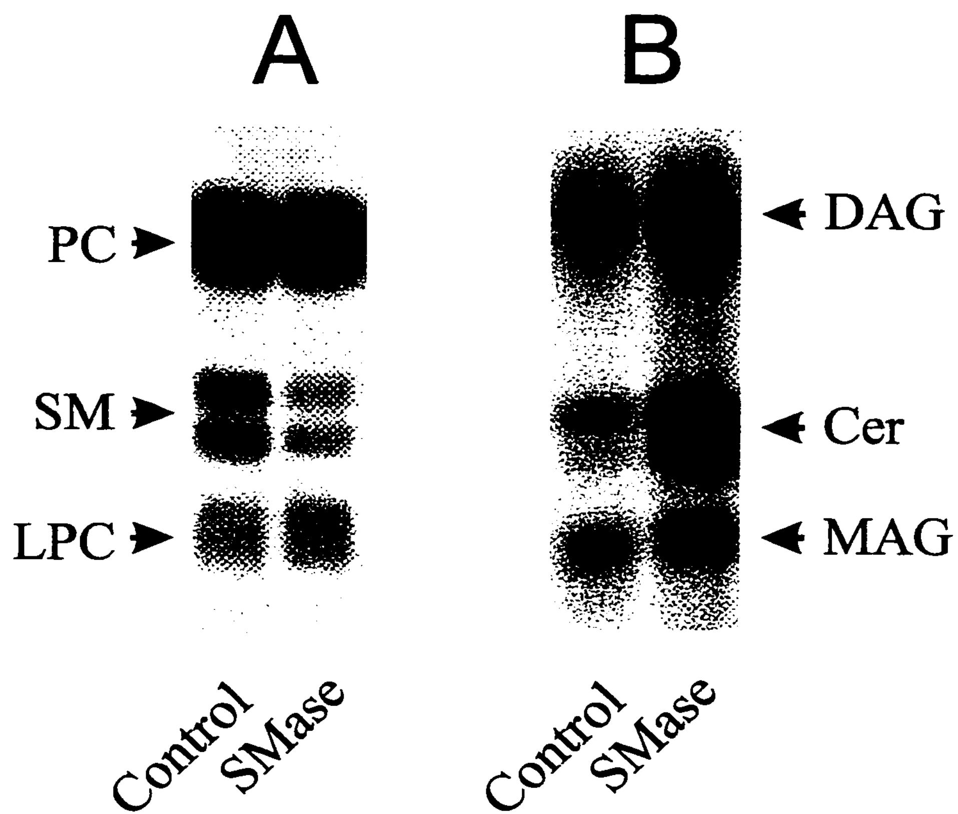
(n = 6) in the presence of NMDG. This confirmed that the Cch response was independent of the presence of the cationic species used to replace extracellular Na^+ (choline or NMDG). As such, the differences between peak currents were attributed to non-specificity and not considered further.

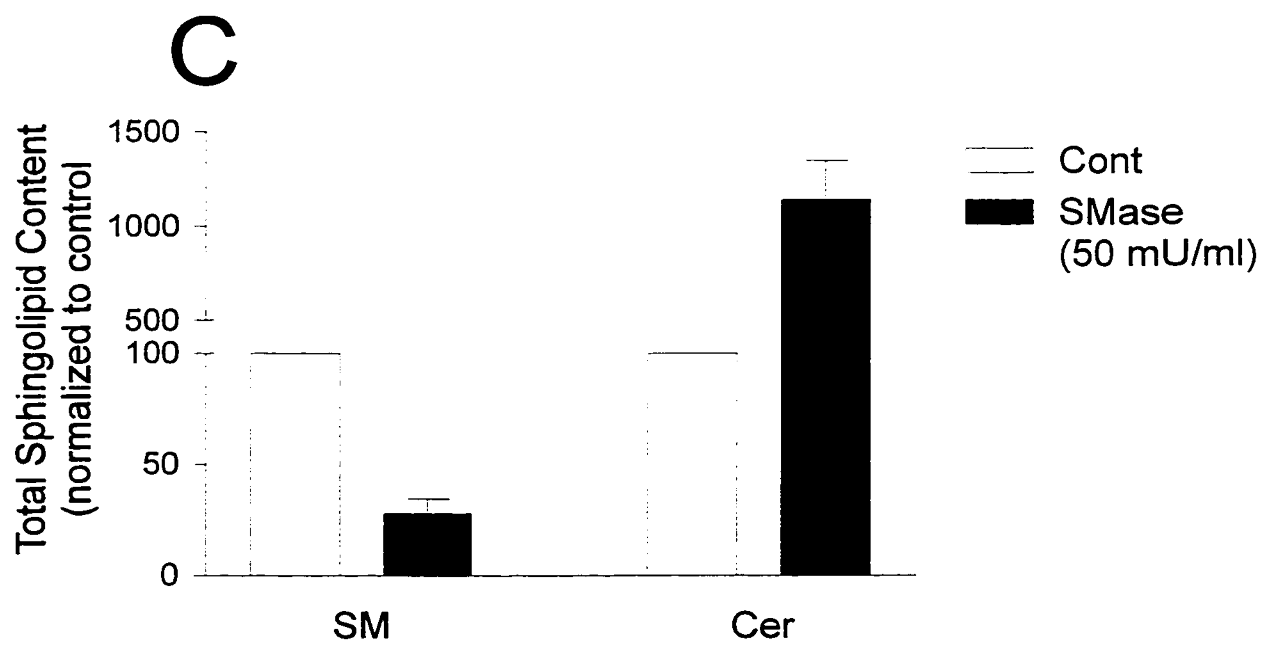
4. Development of the SMase assays.

a. Sphingomyelin assays.

In order to assess the accuracy of the sphingomyelin assay, the spots on the TLC plates were compared to lipid standards which were located by iodine staining. Radioactivity was localized to four spots on the TLC plate (Fig 10A). The upper spot contained the most radioactivity and was identified as phosphatidylcholine. This was based on its colocalization with a phosphatidylcholine standard and observations in other cell systems identifying phosphatidylcholine as the most abundant choline-containing lipid. The two middle spots both roughly corresponded to the sphingomyelin standard. Treatment with exogenous SMase caused the degradation of both spots (Fig 10A) to 28 ± 6.6% of control levels (Fig 10C), indicating that these two spots were actually a doublet band, presumably containing sphingomyelin moities with different length acyl chains. The bottom spot was identified as lysophosphatidylcholine, based on its colocalization with a lysophosphatidylcholine standard.

Figure 10: The effect of exogenous SMase on granulosa cell sphingomyelin and ceramide concentration. **Panel A:** An autoradiograph of [^{14}C]-choline prelabelled lipid extracts. PC, phosphatidylcholine; SM, sphingomyelin; LPC; lysophosphatidylcholine **Panel B:** An autoradiograph of[^{32}P]-γ-ATP labelled lipid extracts. DAG, diacylglycerol; Cer, ceramide; MAG, monoacylglycerol. **Panel C:** Densitometric analysis of the radioactivity of bands corresponding to sphingomyelin and ceramide. Granulosa cells cultured for 66 hours in the presence (sphingomyelin assay) or absence (ceramide assay) of [^{14}C]-choline were treated with 50 mU/ml of exogenous SMase for 1 hour. Lipids were extracted in chloroform using a modification of the Bligh-Dyer procedure and labelled with [^{32}P]-γ-ATP via the DAG kinase reaction (ceramide). Labelled lipids were separated by TLC, detected with a phosphorimager (Bio-Rad Laboratories) and analyzed by 2-D densitometry (Molecular Analyst; Bio-Rad Laboratories).


A
B
PC
SM
LPC
DAG
Cer
MAG
Control
SMase
Control
SMase


C
Total Sphingolipid Content
(normalized to control)
1500
1000
500
100
50
0
SM
Cer
Cont
SMase
(50 mU/ml)

b. Ceramide assays.

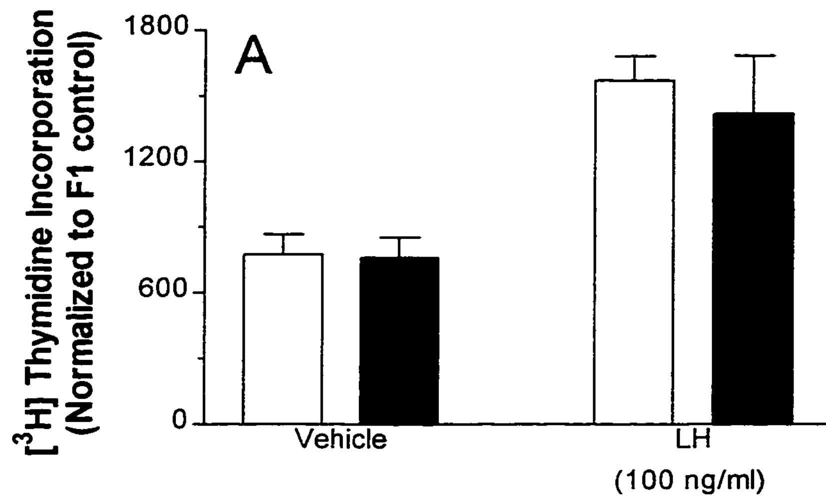
In order to assess the accuracy of the ceramide assay, the spots on the TLC plates were compared to lipid standards which were located by iodine staining. Note that as the spots contained lipids phosphorylated by the DAG kinase assay, phosphorylated lipid standards were used. Radioactivity was localized to four spots on the TLC plate (Fig 10B). The upper spot was identified as representing diacylglycerol, based on its colocalization with a phosphatidic acid standard and its relatively high radioactivity content, which is consistent with most cell types. The bottom spot was identified as monoacylglycerol, based on its colocalization with a lysophosphatidic acid standard. The two middle spots were suspected to represent ceramide, however, ceramide-phosphate standards could not be obtained. Nonetheless, as DAG kinase is known to phosphorylate ceramide, treatment with exogenous SMase caused a large increase in the radioactive content of both spots (Fig 10B) to 1138 ± 206% of control levels (Fig 10C) and sphingomyelin was also represented by a doublet band. The two spots were inferred to represent ceramide moities with different length acyl chains.

B. Regulation of follicular growth by TNFα action and its interactions with intraovarian factors.

1. Does TNFα regulate granulosa cell proliferation during follicular development? Is the action of TNFα influenced by the presence of TGFα or LH?

To determine if TNFα plays a role in the regulation of follicular growth, studies were performed on F1 and F5,6 granulosa cells in the presence or absence of maximally stimulatory concentrations of LH (100 ng/ml; Fig 11) or TGFα (10 ng/ml; Fig 12). Basal [^{3}H]-thymidine incorporation was significantly higher in F5,6 versus F1 granulosa cells ($p < 0.001$) following a 24 hr incubation. Moreover, [^{3}H]-thymidine incorporation was found to be follicular stage dependent ($p < 0.001$), with the gonadotropin stimulating DNA synthesis in F5,6 cells and inhibiting it in F1 cells. The effect of TGFα (10 ng/ml) on [^{3}H]-thymidine incorporation was also found to be follicular stage dependent, although DNA synthesis in the presence of the growth factor increased in both F1 (5 fold) and F5,6 (2 fold) granulosa cells during a similar 24 hr incubation period (Fig 12). TNFα (10 ng/ml) had no significant ($p > 0.05$) effect on basal, LH- or TGFα-induced [^{3}H]-thymidine incorporation in either F1 or F5,6 granulosa cells (2.5×10^5 cells/well) following 24 hr incubation as determined by three way ANOVA (TNFα interactions with TGFα and LH were analyzed separately). These findings suggest that TNFα plays a minimal role in the regulation of granulosa cell proliferation during follicular development.

Figure 11: The effect of TNFα on basal or LH-induced [^{3}H]-thymidine incorporation in hen granulosa cells during follicular development (n = 3). F5,6 (A) or F1 (B) granulosa cells (2.5 x 10^5) were treated with BSA (0.1 %; vehicle) or TNFα (10 ng/ml) in the presence or absence of LH (100 ng/ml) for 24 hrs. [^{3}H]-thymidine was added 6 hours prior to completion of the culture. Cells were washed in dPBS containing cold thymidine, followed by two additional washes in dPBS. DNA precipitation was accomplished by incubation with TCA (5 %). Cells were washed twice in methanol, dissolved in KOH (0.5 M) and quantified in a scintillation counter. Statistical comparisons were performed by three way ANOVA. Although no effect of TNFα was observed on either F1 or F5,6 granulosa cells, the effect of LH was found to be dependent on follicular stage ($p < 0.001$). In addition, F5,6 cell [^{3}H]-thymidine incorporation was significantly higher ($P < 0.001$) than observed in F1 cells.



Cont
TNFα (10 ng/ml)

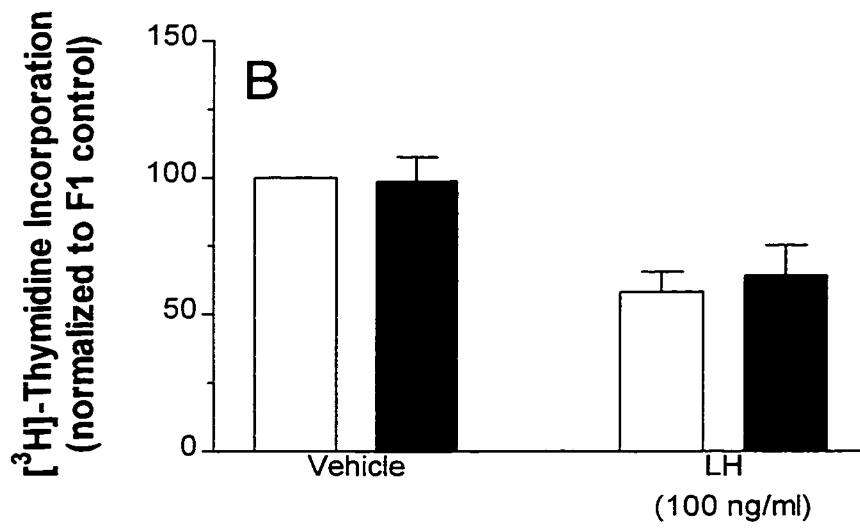
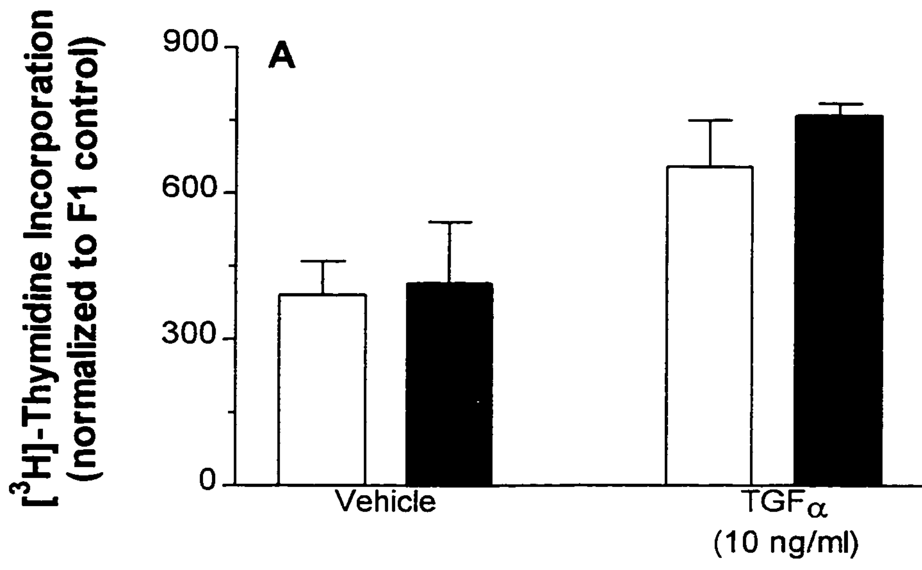


Figure 12: The effect of TNFα on basal or TGFα-induced [^{3}H]-thymidine incorporation in hen granulosa cells during follicular development (n = 3). F5,6 (A) or F1 (B) granulosa cells (2.5×10^5) were incubated with BSA (0.1 %; vehicle) or TNFα (10 ng/ml) in the presence or absence of TGFα (10 ng/ml) for 24 hrs. [^{3}H]-thymidine was added 6 hours prior to completion of the culture. Cells were washed in dPBS containing cold thymidine, followed by two additional washes in dPBS. DNA precipitation was accomplished by incubation with TCA (5 %). Cells were washed twice in methanol and then dissolved in KOH (0.5 M) and quantified in a scintillation counter. Statistical comparisons were performed by three way ANOVA. Although no significant effect of TNFα ($p > 0.05$) was observed on either F1 or F5,6 granulosa cells, the effect of TGFα was found to be follicular stage dependent ($p < 0.002$).



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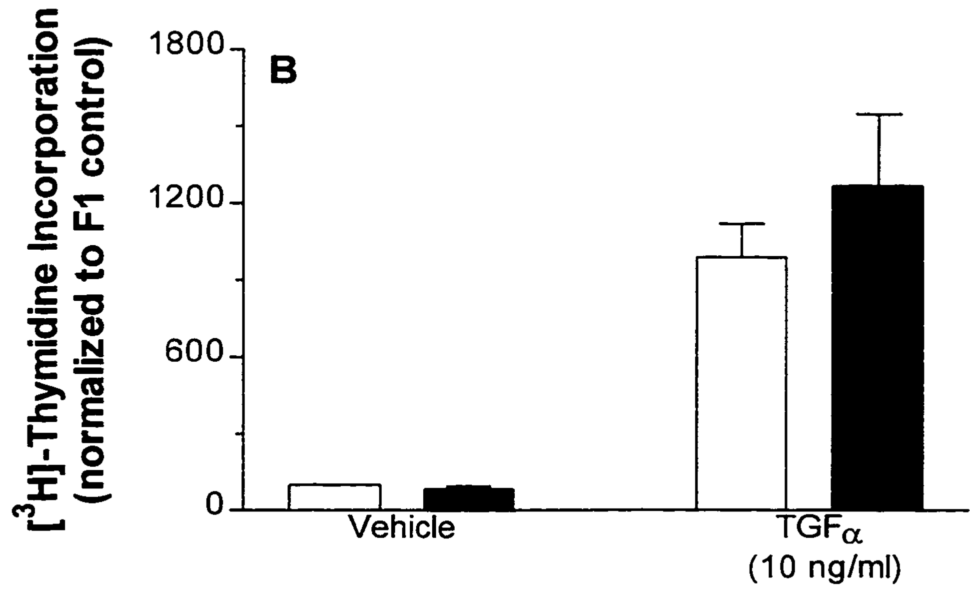
TNFα (10 ng/ml)

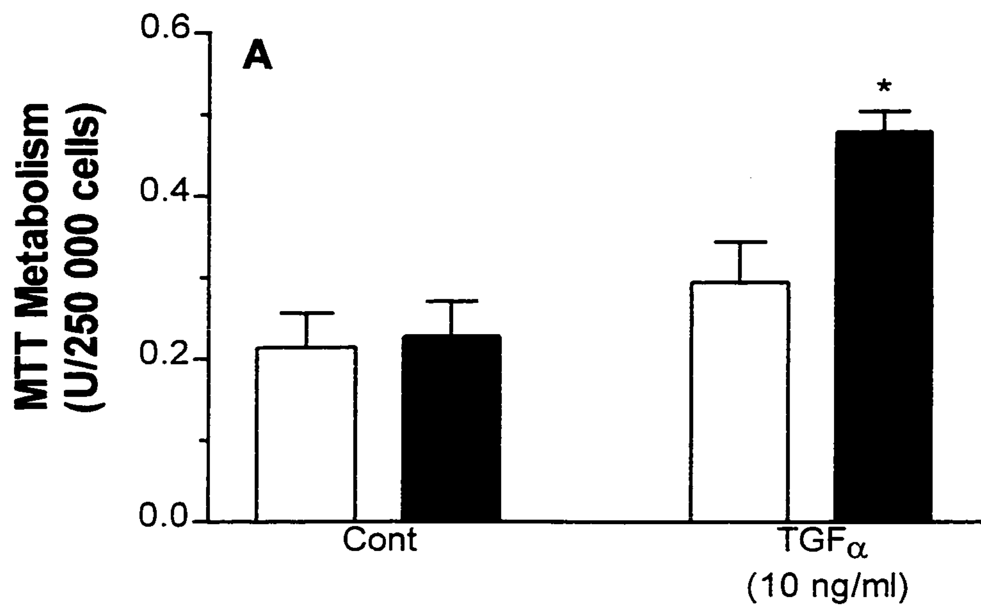


2. Do TNFα and TGFα co-regulate granulosa cell survival during follicular development?

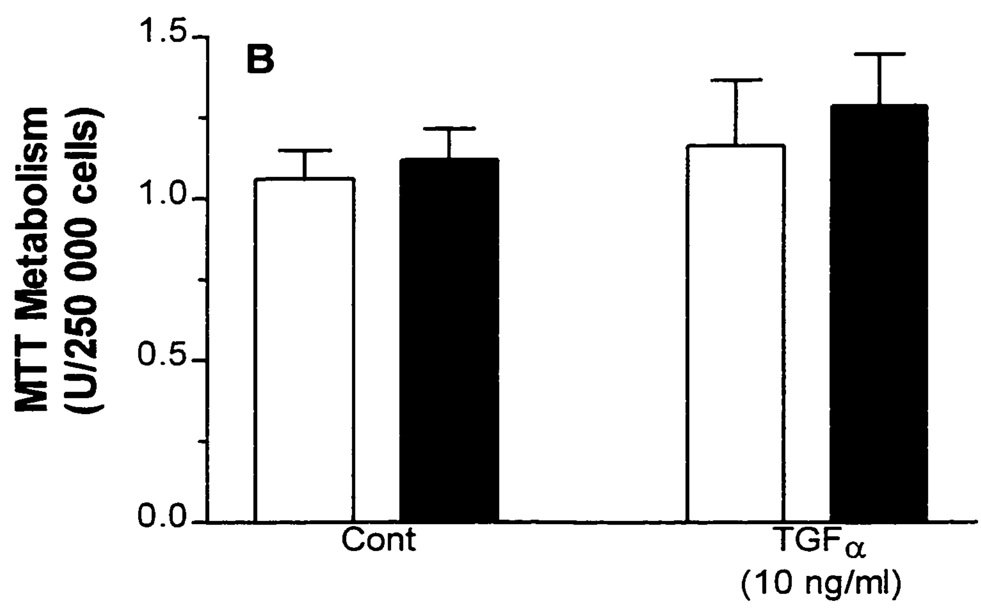
Initial assessments of cell survival were performed using the MTT assay on F1 and F5,6 granulosa cells cultured under confluent conditions (2.5 x 10^5 cells/well) for 24 hrs in serum-free medium containing TNFα (10 ng/ml) and/or TGFα (10 ng/ml; Fig 13). Basal MTT metabolism in F1 cells was significantly higher than F5,6 cells (5 fold; $p < 0.001$), presumably reflecting distinct increases in the metabolic activity of granulosa cells during follicular development. In addition, while neither TNFα nor TGFα affected the cell viability of cultured granulosa cells from either developmental stage, 24 hr incubation in the presence of both factors resulted in an increase in MTT metabolism in F5,6 ($p < 0.05$) but not F1 ($p > 0.05$) granulosa cells.

The increase in MTT metabolism observed in response to TNFα and TGFα is in apparent contrast with results from studies of [^{3}H]-thymidine incorporation (Fig 12). In order to determine if this difference was due to cell proliferation or viability in culture, assessments of the MTT metabolism of F5,6 cells (2.5 x 10^5 cells/well) following 0, 24, 48 and 72 hrs of incubation were determined (Fig 14A). Cell viability *in vitro* decreased with time irrespective of either TNFα or TGFα ($p < 0.001$). Moreover, analysis by three-way ANOVA revealed that only TGFα significantly affected MTT metabolism during the culture period ($p < 0.001$). Surprisingly, significant interactions betweenTNFα and TGFα were not detected

Figure 13: The effect of TNFα on basal or TGFα-induced MTT metabolism in hen granulosa cells during follicular development ($n = 3$). F5,6 (A) or F1 (B) granulosa cells incubated under confluent conditions (2.5×10^5) with BSA (0.1 %; vehicle) or TNFα (10 ng/ml) in the presence or absence of TGFα (10 ng/ml) for 24 hrs. Cells were then incubated in 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide MTT (0.5 mg/ml) for 3 to 4 hours, solubilized in an equal volume of acidic isopropanol (0.04 N HCl) and incubated overnight. Dye conversion was assessed spectrophotometrically (570 nm) with background subtraction (630 nm). Statistical comparisons were performed by two way ANOVA. Significant interactions between TNFα and TGFα were observed in F5,6 ($p < 0.05$), but not in F1 ($p > 0.05$) granulosa cell cultures. In addition, post-hoc analysis of F5,6 cells by Tukey's test revealed that only cells treated with the combination of TNFα and TGFα were significantly different from control (* represents $p < 0.01$). In addition, the MTT metabolism of F5,6 and F1 granulosa cells was statistically different ($p < 0.001$).

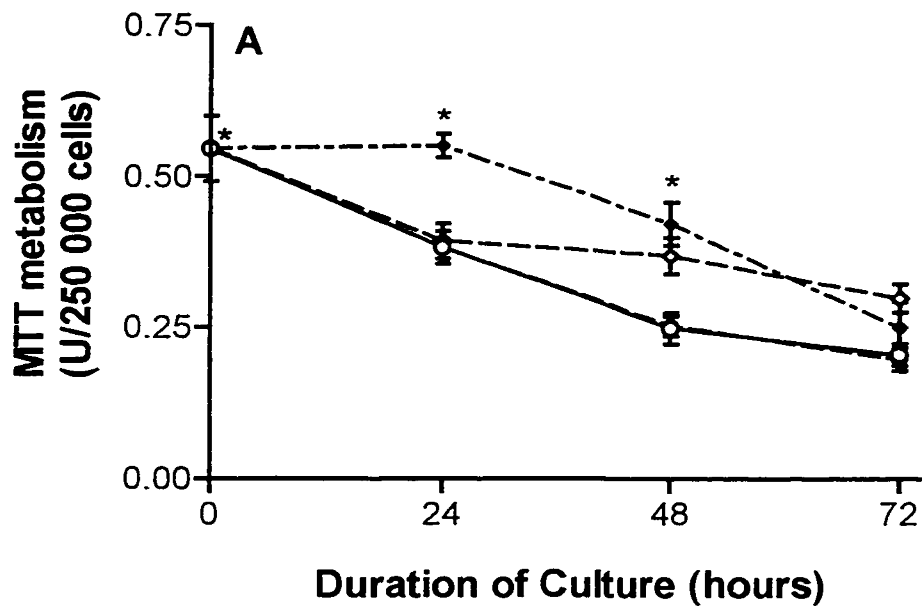


A
*
0.6
0.4
0.2
0.0
MTT Metabolism
(U/250 000 cells)
Cont
TGFα
(10 ng/ml)
Vehicle
TNFα (10 ng/ml)

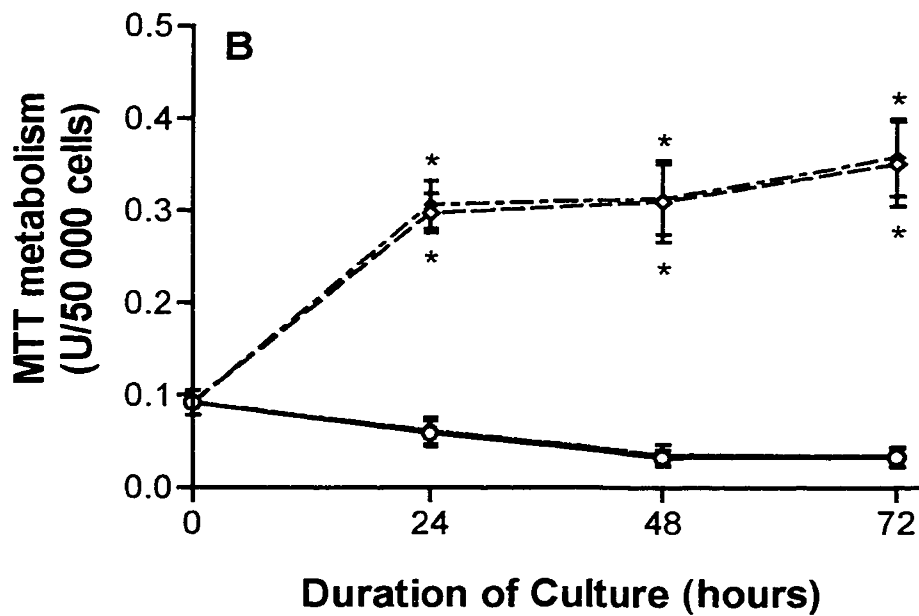


B
1.5
1.0
0.5
0.0
MTT Metabolism
(U/250 000 cells)
Cont
TGFα
(10 ng/ml)

Figure 14: The effect of duration of culture and cell density on the TNFα-TGFα interaction on F5,6 granulosa cell MTT metabolism (n = 3). Sparse (25 000 cells/mm^2; A) or confluent (125 000 cells/mm^2; B) F5,6 granulosa cells were incubated with BSA (0.1 %; vehicle) or TNFα (10 ng/ml) in the presence or absence of TGFα (10 ng/ml) for 0, 24, 48 or 72 hrs. Cells were then incubated in 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide MTT (0.5 mg/ml) for 3 to 4 hours, solubilized in an equal volume of acidic isopropanol (0.04 N HCl) and incubated overnight. Dye conversion was assessed spectrophotometrically (570 nm) with background subtraction (630 nm). Statistical comparisons were performed by three way ANOVA. Significant interactions between TNFα, TGFα and incubation time were not observed for either confluent or sparsely cultured F5,6 granulosa cells ($p > 0.05$). Moreover, TGFα, but not TNFα affected dye conversion in a time dependent manner in both sparse ($p < 0.001$) and confluent ($p < 0.001$) culture. Nonetheless, post-hoc analysis of confluent cells by Tukey's test revealed that cells treated with the combination of TNFα and TGFα for 24 and 48 hours were not significantly different from the time 0 control ($p > 0.05$).* represents significantly different treatment groups ($p < 0.05$).



Cont
TNFα (10 ng/ml)
TGFα (10 ng/ml)
TNFα (10 ng/ml) + TGFα (10 ng/ml)



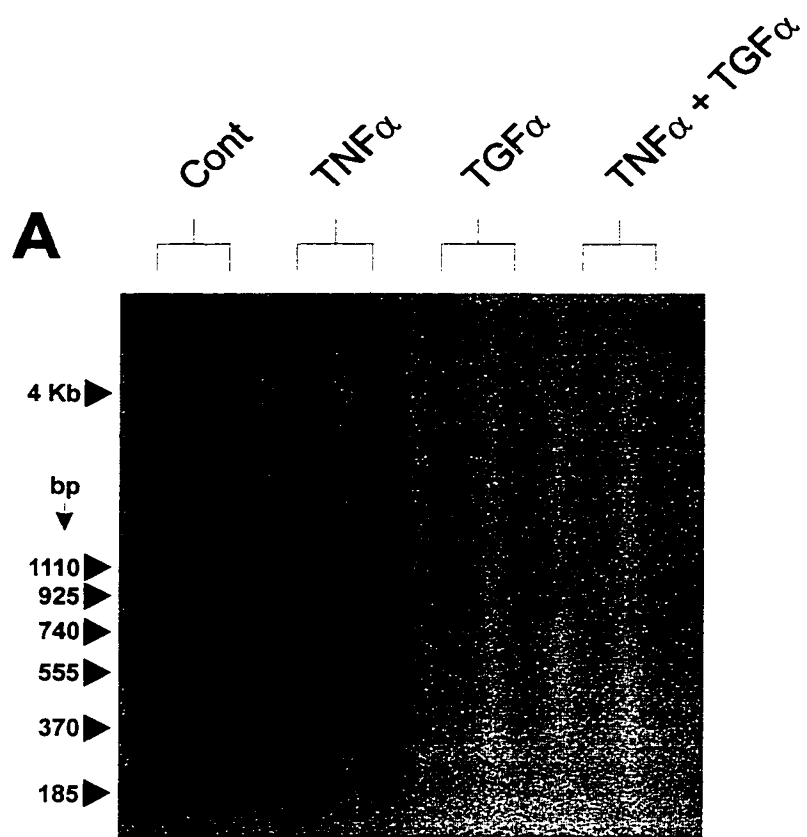
($p > 0.05$), despite the fact that they were detected when only a 24 hour culture was performed (Fig 13). Nonetheless, post-hoc analysis by Tukey's test revealed that the only groups not significantly different from control (time 0) were cells treated with TNFα and TGFα for 24 and 48 hrs ($p > 0.05$). Consequently, the absence of an overall interaction between TNFα and TGFα on MTT metabolism during culture probably reflects the fact that the effect of TNFα decreased with culture duration. In addition, the fact that MTT metabolism decreased with duration of culture suggests that the TNFα-TGFα interaction observed in Figure 13 occurred because of apoptosis in the absence of TNFα and TGFα *in vitro*. Lastly, since these studies were performed at a cell density near confluence (125 000 cells/mm^2) where cell proliferation was inhibited by cell contact, the ability of sparsely (25 000 cells/mm^2) cultured F5,6 granulosa cells to metabolize MTT was assessed (Fig 14B). In contrast to confluent cells, TGFα treatment significantly increased MTT metabolism during the first 24 hours of culture ($p < 0.001$), after which time no significant changes were observed.

In order to assess the relationship between MTT metabolism and apoptosis, DNA degradation and nuclear morphology studies were performed (Fig 15, 16). F5,6 granulosa cells (7×10^5 cells/weli) were cultured in the absence of serum for 0, 24, 48 and 72 hrs. Following extraction, the DNA was labelled with [^{32}P]-dCTP and the amount of low molecular weight DNA fragments was quantified as described (see methods 4A; Pg 78) and depicted in figure 15A. The amount of DNA degradation significantly increased during the

culture period ($p < 0.002$). Moreover, TGFα significantly inhibited apoptosis between 24 and 72 hrs of culture ($p < 0.02$) while the presence of TNFα failed to significantly affect this TGFα response ($p > 0.05$; Fig 15B).

Since the DNA degradation assay cannot differentiate between different stages of apoptosis, the fact that TNFα did not stimulate TGFα-induced cell survival may reflect differences in the time-course of apoptosis. Consequently, the early apoptosis, late apoptosis and necrotic cell populations were individually quantified using acridine orange and ethidium bromide (Fig 16). Three way interactions for cell viability were not detected ($p > 0.05$), although interactions between TNFα and TGFα were observed ($p < 0.02$) that were not dependent on the duration of culture ($p > 0.05$). In contrast, increases in cell viability in the presence of TGFα alone were culture duration-dependent ($p < 0.001$). Consequently, TGFα significantly increased viability which was potentiated by the presence of TNFα. Moreover, the fact that the interaction was not time-dependent probably reflects the fact that no changes in cell viability during the culture period were observed in the presence of both factors (Fig 16A). Distinct differences were also observed regarding the type of cell death which occurred in the presence and absence of these factors. A three way interaction was detected for the incidence of early apoptosis ($p < 0.05$) due to the fact that an increase in the number of early apoptotic cells in the presence of TGFα without TNFα was detected ($p < 0.01$). Interactions for late apoptosis were not detected ($p > 0.05$), although TGFα significantly decreased the number of cells in this group ($p < 0.05$). Lastly, a three way interaction was observed

Figure 15: Apoptosis in cultured F5,6 granulosa cells is inhibited by TGFα irrespective of the presence of TNFα (n = 3). F5,6 granulosa cells were cultured in the presence or absence of TNFα (10 ng/ml) and/or TGFα (10 ng/ml) for 0, 24, 48 or 72 hrs. DNA was extracted from floating and plated cells (combined) by phenol/chloroform extraction and ethanol precipitation. Following labelling with [^{32}P]-CTP, DNA was resolved on an agarose gel and exposed to a phosphorimager (Bio-Rad Laboratories, CAlifornia) for densitometric analysis of low molecular weight (mw) DNA (< 4 Kb; Molecular Analyst Software; Bio-Rad Laboratories, CAlifornia).(A) Representative autoradiogram of labelled DNA resolved by electrophoresis. Molecular weights are indicated on the left. (B) Densitometric quantification of low molecular weight DNA content (< 4 Kb). Statistical comparisons were performed by three way ANOVA. Although no effect of TNFα was observed, low mw DNA content significantly increased during the culture period ($p < 0.002$), while TGFα inhibited this effect ($p < 0.02$).


A
Cont
TNFα
TGFα
TNFα + TGFα
4 Kb
bp
1110
925
740
555
370
185

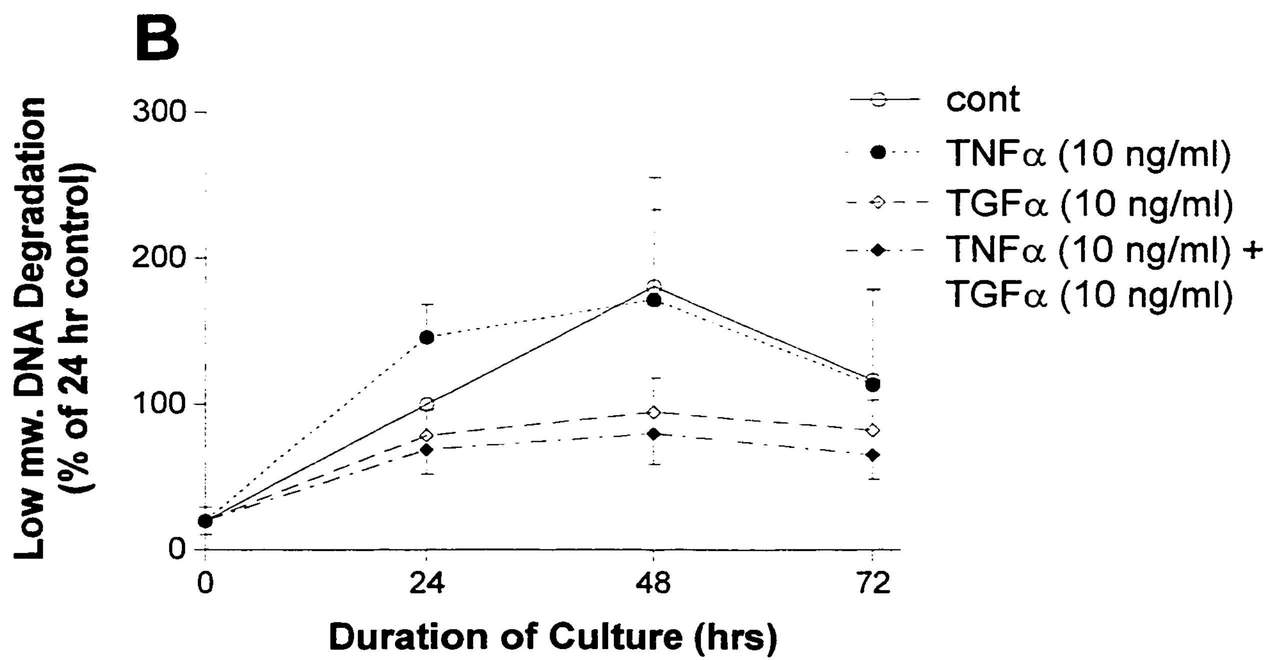
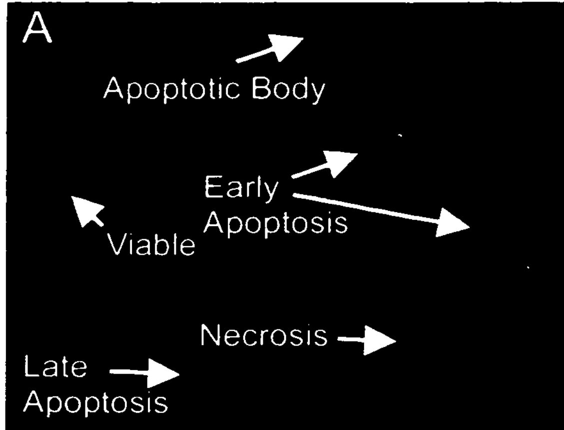
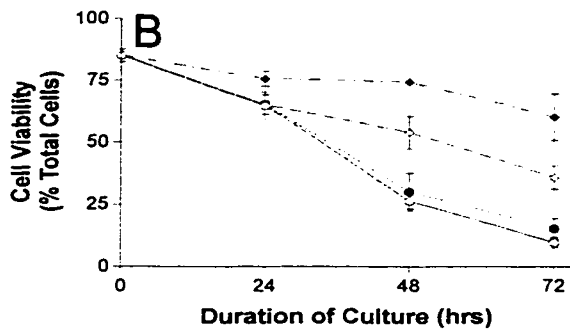
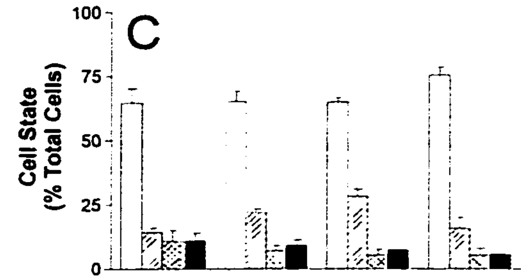

B
Low mw. DNA Degradation (% of 24 hr control)
300
200
100
0
0
24
48
72
Duration of Culture (hrs)
cont
TNFα (10 ng/ml)
TGFα (10 ng/ml)
TNFα (10 ng/ml) + TGFα (10 ng/ml)

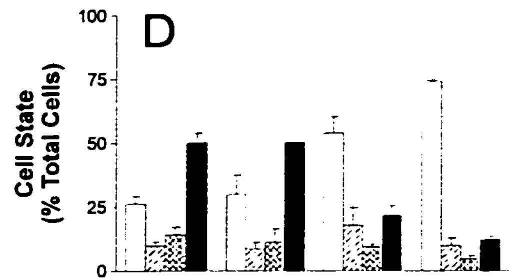
Figure 16: Necrosis in cultured F5,6 granulosa cells is inhibited by TNFα in the presence of TGFα (n = 3). (A) Representive photos of viable, early apoptotic, late apoptotic and necrotic cells following vital staining. (B) Time-course study on the effects of TNFα (10 ng/ml) and/or TGFα (10 ng/ml) on the viability of F5,6 granulosa cells in culture. The percentage of cells at different stages of apoptosis or necrosis following 24, 48 or 72 hrs of culture are depicted in panels C, D, and E, respectively. Floating and plated cells were combined and stained with 1 μl of dye mix [acridine orange (100 μg/ml)-ethidium bromide (100 μg/ml). Live cells had normal nuclei (bright green chromatin with organized structure), apoptotic cells had condensed and/or fragmented nuclei (bright green chromatin was defined as early apoptotic, while bright orange chromatin was defined as late apoptotic) while necrotic cells exhibited a bright orange stain and did not have condensed nuclei (either a normal chromatin structure or no nuclear staining). A minimum of 200 cells per treatment was counted (in duplicate) and the number of cells in each group were expressed as a percentage of the total cell number. Statistical comparisons were performed by three way ANOVA. Three way interactions for cell viability were not detected ($p > 0.05$), however, interactions between TNFα and TGFα were observed ($p < 0.02$) which were not dependent on the duration of the culture period ($p > 0.05$), although increases in cell viability in the presence of TGFα alone were time dependent ($p < 0.001$). A three way interaction was also detected for early apoptosis ($p > 0.05$) due to the fact that an increase in the number of early apoptotic cells in the presence of TGFα without TNFα was detected ($p < 0.01$). Interactions for late apoptosis were not detected ($p > 0.05$), however, TGFα significantly decreased the number of cells in this group ($p < 0.05$). A three way interaction was observed between TNFα, TGFα and the duration of the culture period on the percentage of necrotic cells ($p < 0.02$), indicated that these factors inhibited necrosis in a time-dependent manner.

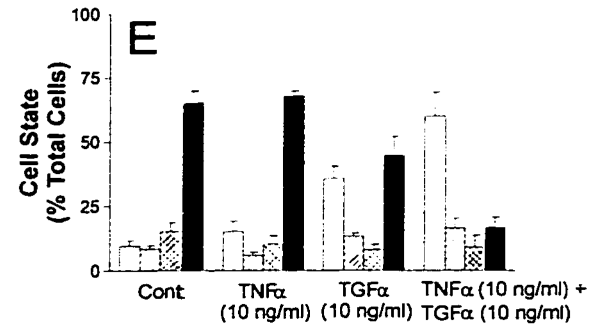




—○— Cont
● TNFα (10 ng/ml)
-◇- TGFα (10 ng/ml)
-◆ -TNFα (10 ng/ml) +
TGFα (10 ng/ml)







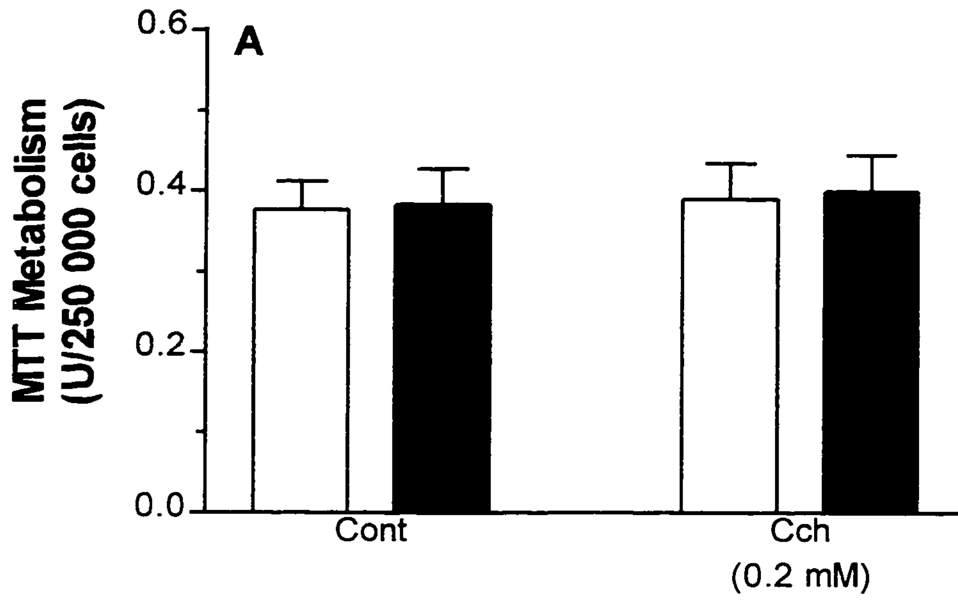
Viable
Early Apoptosis
Late Apoptosis
Necrosis

between TNFα, TGFα and the duration of incubation on the percentage of necrotic cells ($p < 0.02$), indicating that these factors inhibited necrosis in a manner dependent on the culture period. Consequently, the increase in the percentage of early apoptotic cells in the presence of TGFα probably reflects a TGFα-induced delay in cell death, since the growth factor inhibited late apoptosis and necrosis. Moreover, since the presence of TNFα blocked the TGFα-induced increase in early apoptosis and potentiated the TGFα-induced decrease in necrosis, it is suggested that TNFα inhibits both types of cell death *in vitro* in a TGFα-dependent manner.

3. Does Cch regulate granulosa cell fate in the presence or absence of TNFα?

Interactions between TNFα and the muscarinic agonist Cch on cell viability in culture were studied using the MTT and vital staining assays. Unlike TGFα, interactions between TNFα and Cch were not observed for either F1 or F5,6 cells and the rate of MTT metabolism was not affected by the muscarinic agonist (Fig 17). Time-dependent changes in the percentage of viable, early apoptotic, late apoptotic and necrotic cells were detected as described previously (section B2; Fig 18). TNFα significantly inhibited the percentage of late apoptotic cells ($p < 0.05$) without affecting the percentage of viable, early apoptotic or necrotic cells ($p > 0.05$) while no effect of Cch was detected ($p > 0.05$). These findings suggest that Cch plays either a minimal or no role in the regulation of cell fate and that

Figure 17: The effect of TNFα on basal or Cch-induced MTT metabolism in hen granulosa cells during follicular development ($n = 3$). F5,6 (A) or F1 (B) granulosa cells (2.5 x 10^5) were incubated with BSA (0.1 %; vehicle) or TNFα (10 ng/ml) in the presence or absence of Cch (0.2 mM) for 24 hrs. Cells were then incubated in 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide MTT (0.5 mg/ml) for 3 to 4 hours, solubilized in an equal volume of acidic isopropanol (0.04 N HCl) and incubated overnight. Dye conversion was assessed spectrophotometrically (570 nm) with background subtraction (630 nm). Statistical comparisons were performed by two way ANOVA. MTT metabolism of F5,6 and F1 granulosa cells was statistically different ($p < 0.0001$), however, neither Cch nor TNFα affected MTT metabolism alone or in combination in cells from either developmental stage ($p > 0.05$).



Vehicle
TNFα (10 ng/ml)

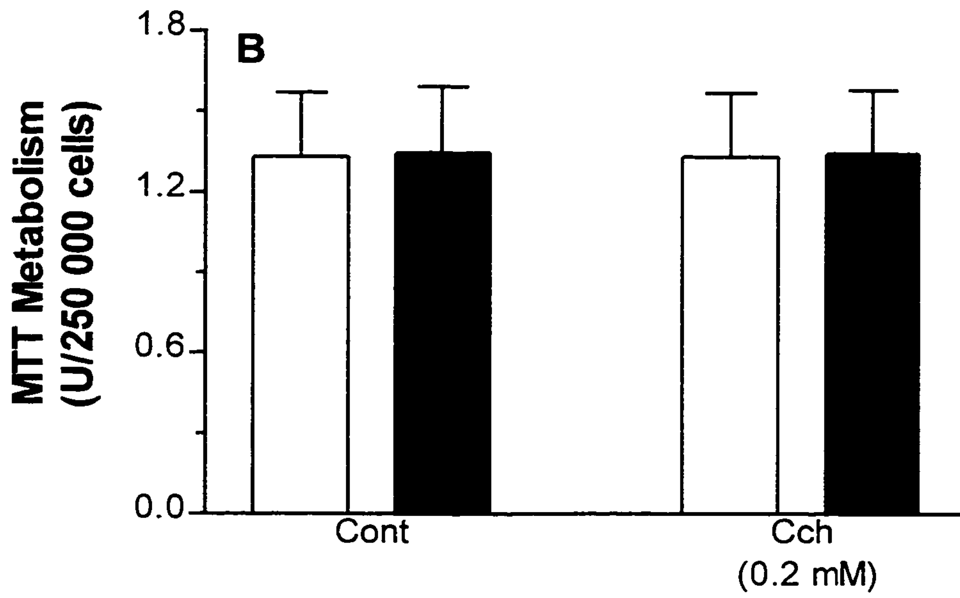
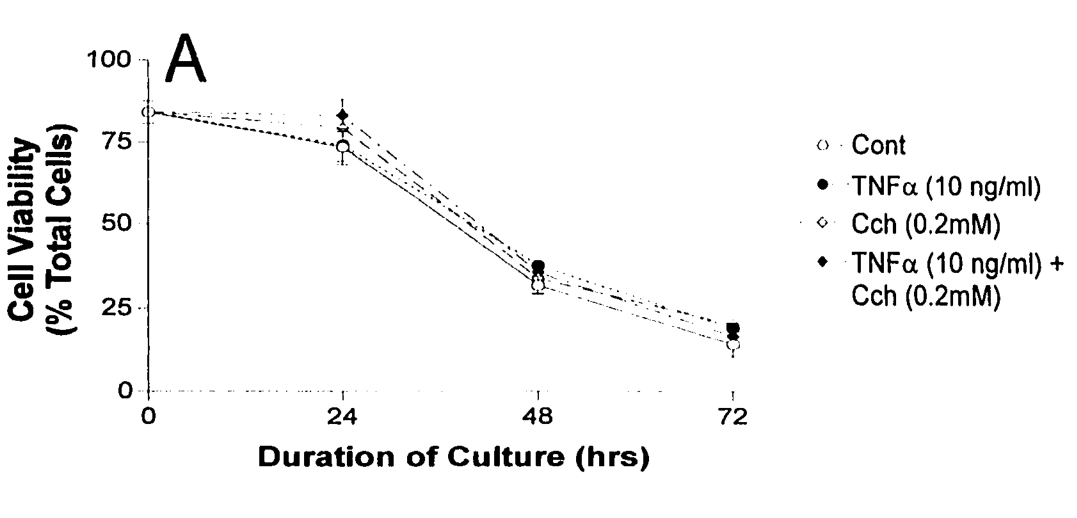
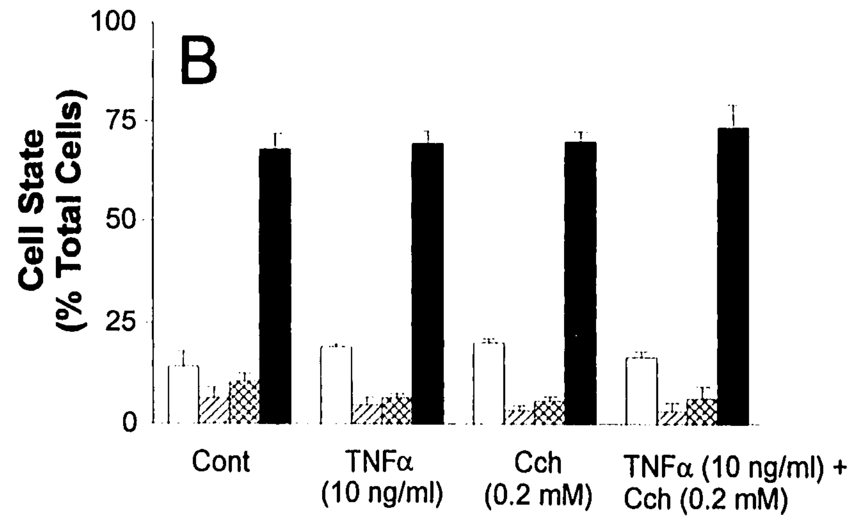
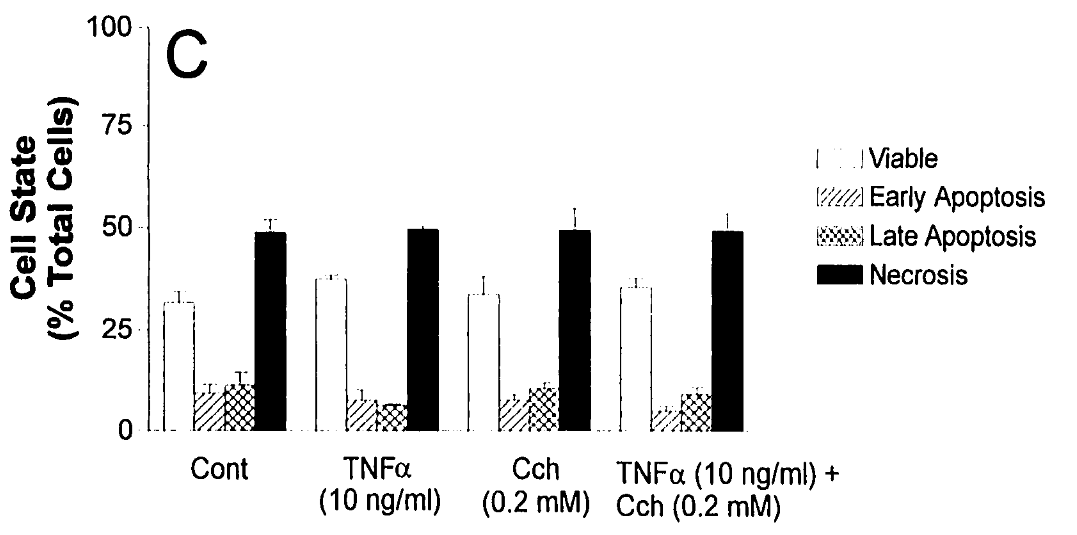
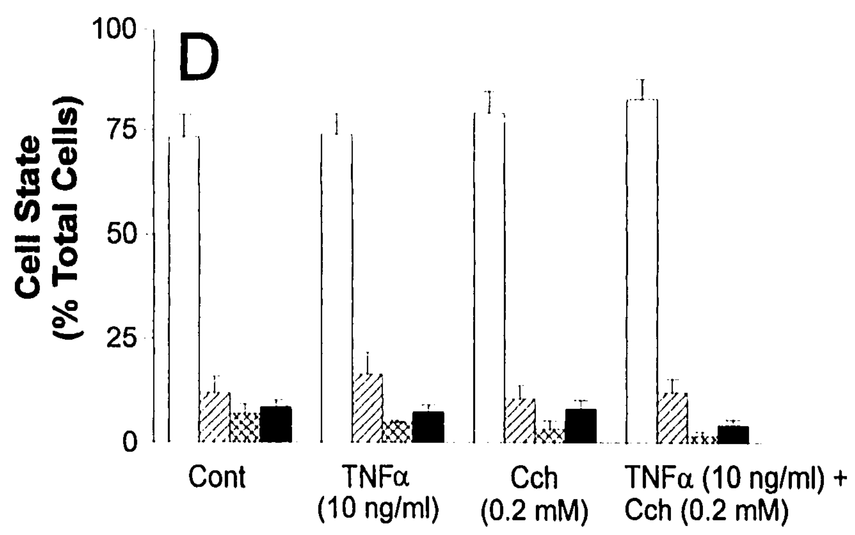


Figure 18: Necrosis in cultured F5,6 granulosa cells is not affected by TNFα in the presence or absence of Cch (n = 3). (A) Time-course study on the effects of TNFα (10 ng/ml) and/or Cch (0.2 mM) on the viability of F5,6 granulosa cells in culture. The percentage of cells at different stages of apoptosis or necrosis following 24, 48 or 72 hrs of culture are depicted in panels B, C and D, respectively. Floating and plated cells were combined and stained with 1 μl of dye mix [acridine orange (100 μg/ml)-ethidium bromide (100 μg/ml)]. Live cells had normal nuclei (bright green chromatin with organized structure), apoptotic cells had condensed and/or fragmented nuclei (bright green chromatin was defined as early apoptotic, while bright orange chromatin was defined as late apoptotic) while necrotic cells exhibited a bright orange stain and did not have condensed nuclei (either a normal chromatin structure or no nuclear staining). A minimum of 200 cells per treatment was counted (in duplicate) and the number of cells in each group was expressed as a percentage of the total cell number. Statistical comparisons were performed by three way ANOVA. The incidence of viable, early apoptotic, late apoptotic and necrotic cells were all time-dependent ($p < 0.01$), however, interactions were not detected. TNFα was found to decrease the incidence of late apoptosis ($p < 0.05$), without affecting the percentage of viable, late apoptotic or necrotic cells ($p > 0.05$). No significant effect of Cch was detected on any of these endpoints ($p > 0.05$).


A
Cell Viability
(% Total Cells)
100
75
50
25
0
0
24
48
72
Duration of Culture (hrs)
Cont
TNFα (10 ng/ml)
Cch (0.2mM)
TNFα (10 ng/ml) +
Cch (0.2mM)


B
Cell State
(% Total Cells)
100
75
50
25
0
Cont
TNFα
(10 ng/ml)
Cch
(0.2 mM)
TNFα (10 ng/ml) +
Cch (0.2 mM)


C
Cell State
(% Total Cells)
100
75
50
25
0
Cont
TNFα
(10 ng/ml)
Cch
(0.2 mM)
TNFα (10 ng/ml) +
Cch (0.2 mM)
Viable
Early Apoptosis
Late Apoptosis
Necrosis


D
Cell State
(% Total Cells)
100
75
50
25
0
Cont
TNFα
(10 ng/ml)
Cch
(0.2 mM)
TNFα (10 ng/ml) +
Cch (0.2 mM)

changes in MTT metabolism reflect differences in the survival of F5,6 cells in culture. Moreover, the percentage of late apoptotic cells was less than 10% irrespective of treatment or duration of incubation, and TNFα did not affect cell viability *in vitro*.

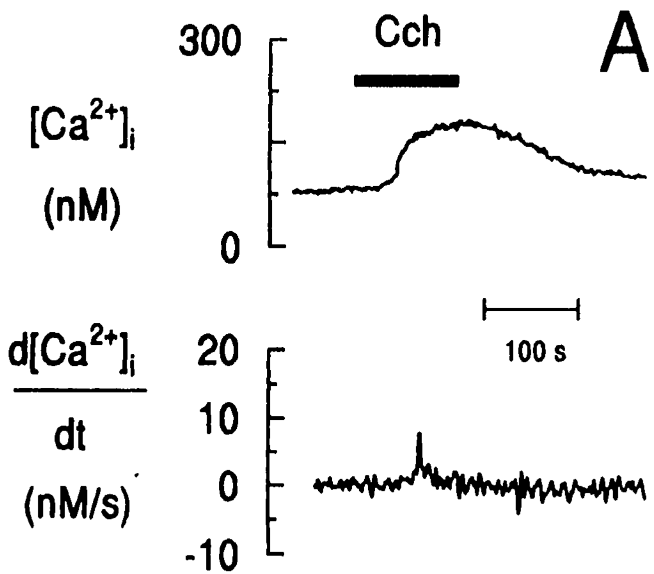
C. Regulation of Ca^{2+} signalling by muscarinic input in hen granulosa cells during follicular maturation.

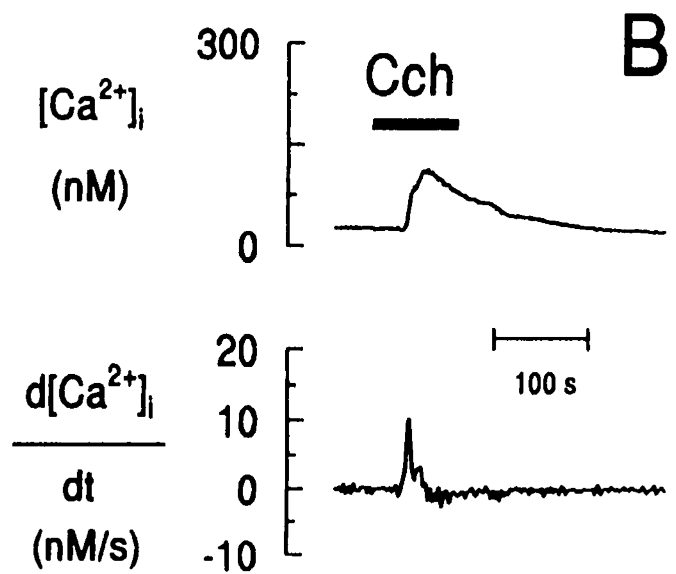
1. Is the regulation of $[Ca^{2+}]_i$ by muscarinic input dependent on follicular maturation?

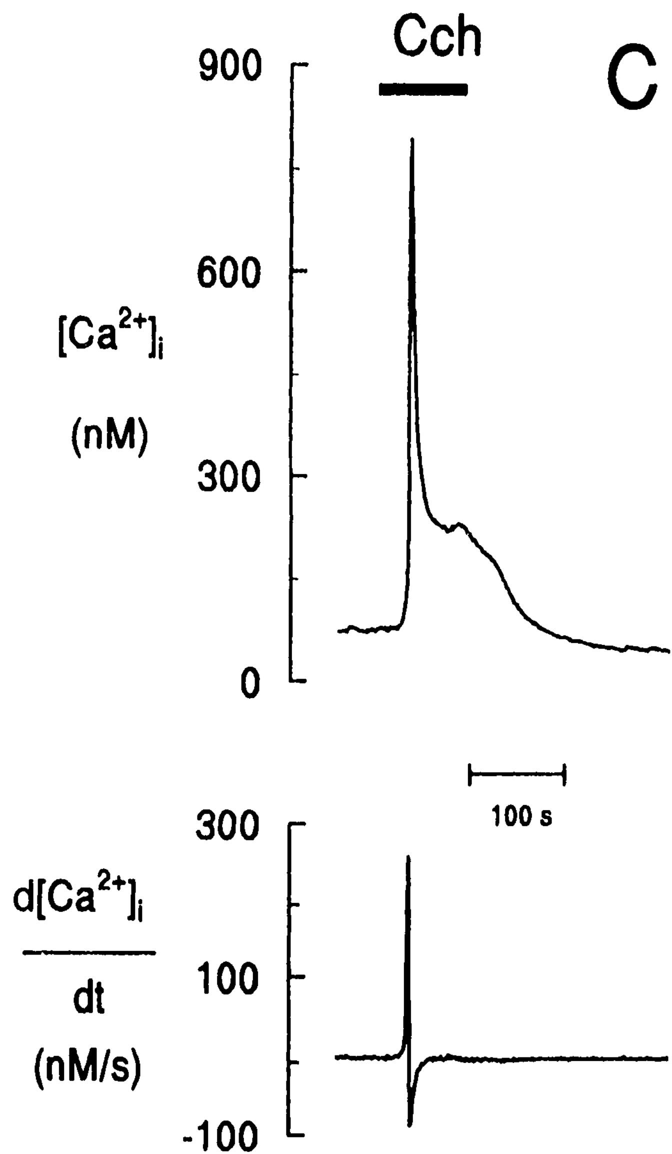
Basal granulosa cell $[Ca^{2+}]_i$ increased with follicular development from 34 ± 2.7 nM ($n = 84$) in F5,6 cells to 54 ± 4.2 nM ($n = 31$) in F3 cells and 50 ± 5.0 nM ($n = 58$) in F1 cells ($p < 0.005$). The typical effects of 0.2 mM Cch on $[Ca^{2+}]_i$ are depicted in Figure 19. The predominant type of Cch-induced Ca^{2+} transient observed was different in the F3 and F5,6 granulosa cells compared to the F1 granulosa cells. In F1 cells (panel C), the Ca^{2+} transients were usually characterized by an initial peak of high amplitude (upper tracing) and a rapid rate of rise (lower tracing). After an initial decrease in $[Ca^{2+}]_i$ that was related to the initial rate of rise, a much slower and occasionally oscillatory decrease was observed. On the other hand, the typical Cch-induced Ca^{2+} transients recorded in F3 and F5,6 cells (panels A and B) were characterized by relatively small and slow changes of basal $[Ca^{2+}]_i$.

Because both basal $[Ca^{2+}]_i$ and Cch-induced $[Ca^{2+}]_i$ changed during follicular

Figure 19: Typical changes in $[Ca^{2+}]_i$ (upper tracings) and $d[Ca^{2+}]_i/dt$ (lower tracings) in response to Cch as predominantly seen in granulosa cells from the F5,6 (panel A; n = 46), F3 (panel B; n = 17) and F1 (panel C; n =47) follicles. The horizontal bars represent the period of exposure to 0.2 mM Cch. Note that the scale of the lower tracing of panel C is different from that of panels A and B.


A
Cch
$[Ca^{2+}]_i$ (nM)
300
0
100 s
$\frac{d[Ca^{2+}]_i}{dt}$ (nM/s)
20
10
0
-10


B
Cch
$[Ca^{2+}]_i$ (nM)
300
0
100 s
$\frac{d[Ca^{2+}]_i}{dt}$ (nM/s)
20
10
0
-10


C
Cch
$[Ca^{2+}]_i$ (nM)
900
600
300
0
100 s
$\frac{d[Ca^{2+}]_i}{dt}$ (nM/s)
300
100
-100

maturation, we attempted to determine whether a relationship existed between these two parameters by plotting the average maximum amplitude of Cch-induced Ca^{2+} transients against their basal $[Ca^{2+}]_i$ in F1, F3 and F5,6 cells. However, all R^2 values were less than 0.08, suggesting that developmental changes in basal and Cch-induced $[Ca^{2+}]_i$ are separate phenomena.

a. *Is the Ca^{2+} response to Cch in hen granulosa cells heterogeneous during follicular development?*

The overall effects of Cch on granulosa cells from three stages of follicular development are summarized in Table 3. While the amplitude as well as the rate of rise of the Ca^{2+} transients was similar in F3 and F5,6 cells, these two values were markedly increased in F1 cells. There was a proportion of the cells that failed to respond to Cch at all stages of development, but this fraction significantly decreased from approximately 30 % in F5,6 and F3 cells to 11 % in F1 cells. Thus, not only do F1 granulosa cells elicit, on average, much more pronounced Ca^{2+} transients than cells obtained from earlier stages, the percentage of responding cells also appears to be increased. Close examination of the distribution of the responses according to amplitude and rate of $[Ca^{2+}]_i$ rise demonstrates that the two distinct types of responses shown in Figure 19 were present at all stages of maturation, albeit in different proportions (Fig 20). While more than 50 % of the F1 cells had Ca^{2+} transients with

Table 3: Effects of follicular stage on the ability of granulosa cells cells to respond to carbachol.[a]

	State of Follicular Development[b]		
	F5,6 (n = 64)	F3 (n = 25)	F1[c] (n = 53)
% Response	72%	68%	89%
$\Delta[Ca^{2+}]_i$[d] (nM)	165 ± 37	154 ± 57	696 ± 119
$d[Ca^{2+}]_i/dt$ (nM/s)	46 ± 16	36 ± 25	260 ± 55

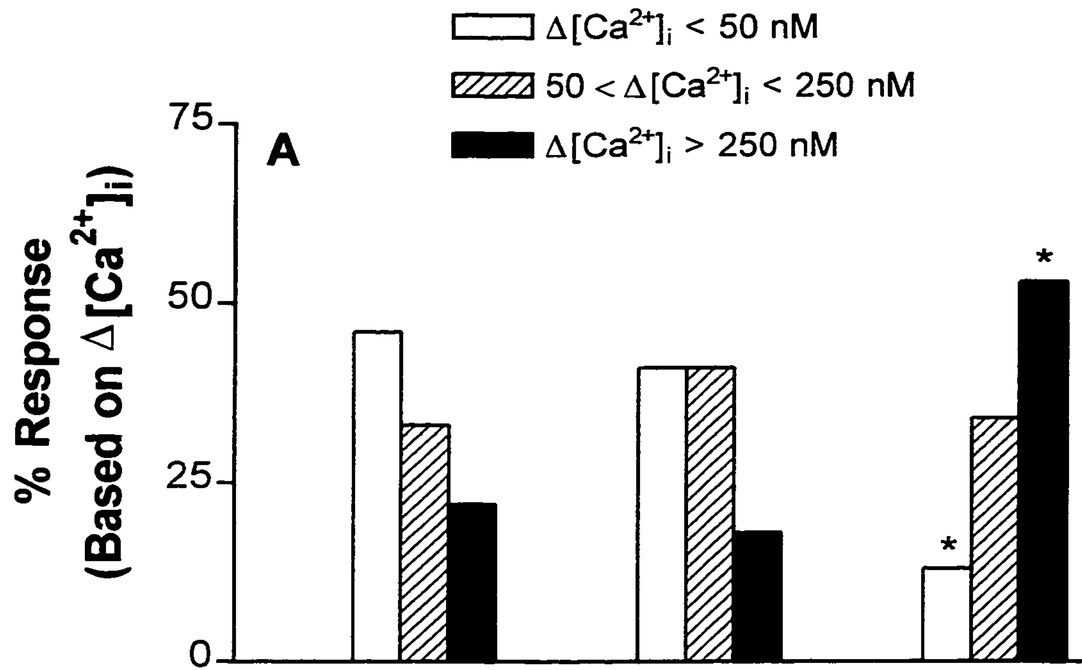
[a] 0.2 mM Cch was added to granulosa cells incubated in serum-free medium overnight and loaded with fura-2.

[b] n represents the number of independent determinations.

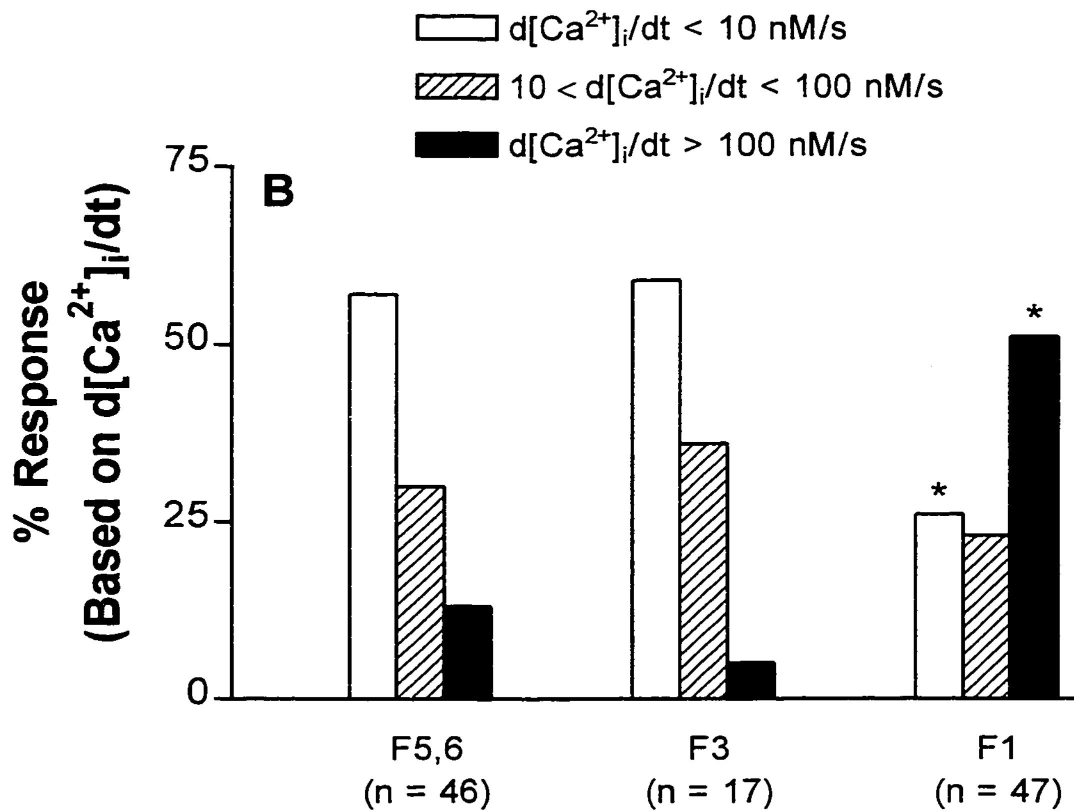
[c] Significantly different ($p < 0.05$) from the corresponding values measured in the F3 and F5,6 follicles.

[d] $\Delta[Ca^{2+}]_i$ is the average maximum amplitude of all cells tested that responded to Cch. $d[Ca^{2+}]_i/dt$ is the average maximum rate of $\Delta[Ca^{2+}]_i$ of all cells tested that responded to Cch.

Figure 20: Distribution of responses to Cch in granulosa cells during follicular maturation. **Panel A:** amplitude of Ca^{2+} transients ($\Delta[Ca^{2+}]_i$). **Panel B:** maximal rate of $[Ca^{2+}]_i$ rise ($d[Ca^{2+}]_i/dt$). % Response is the percentage of cells that responded in the ranges indicated. The distributions within each panel proved to be significantly influenced ($p < 0.005$) by follicular stage, with the asterisk representing the largest differences from the statistically derived expected values that would result if the distribution was not affected by follicular maturation as determined using the chi-square test. Number in parentheses indicates the number of independent determinations (n).



Δ[Ca2+]i < 50 nM
50 < Δ[Ca2+]i < 250 nM
Δ[Ca2+]i > 250 nM
75
50
25
0
A
% Response
(Based on Δ[Ca2+]i)
*
*



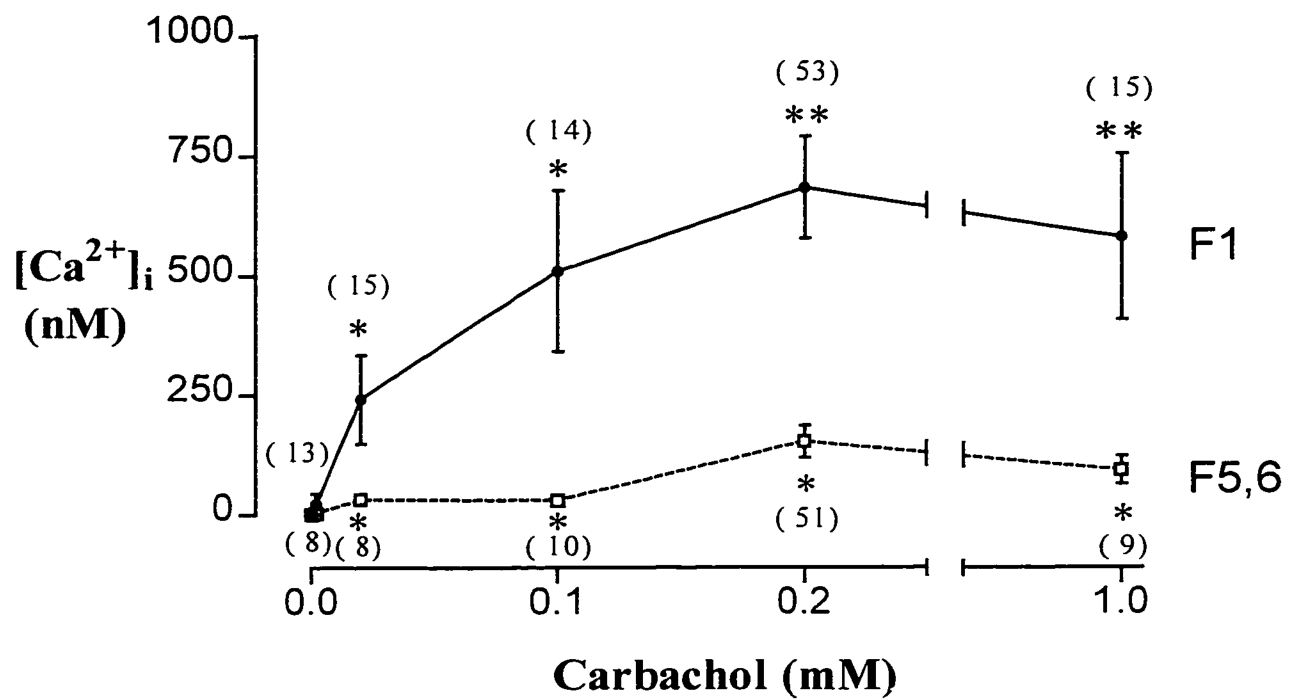
d[Ca2+]i/dt < 10 nM/s
10 < d[Ca2+]i/dt < 100 nM/s
d[Ca2+]i/dt > 100 nM/s
75
50
25
0
B
% Response
(Based on d[Ca2+]i/dt)
*
*
F5,6
(n = 46)
F3
(n = 17)
F1
(n = 47)

amplitudes larger than 250 nM and showed rates of rise greater than 100 nM/sec, about 15 % of the responding cells had amplitude of less than 50 nM. Conversely, the largest proportion of F5,6 and F3 cells showed small and slow responses, although some 10 % of the cells could clearly elicit typical fast responses with amplitude larger than 250 nM and rate of rise greater than 100 nM/sec. Thus, this figure points to the presence of a heterogeneous cell population at all three stages of follicular development. Furthermore, these data clearly support the notion that a major differentiative change occurs in granulosa cells as the follicle matures from F3 to F1, resulting in the presence of a larger proportion of cells capable of producing rapid and large Ca^{2+} transients in response to Cch.

b. What is the concentration-response relationship between Cch and the Ca^{2+} response?

In order to determine whether the Cch concentration tested was maximally stimulatory in granulosa cells from follicles at different stages of development, we examined the influence of graded concentrations of Cch on $[Ca^{2+}]_i$ in F1 and F5,6 granulosa cells (Fig 21). Analysis of variance indicated that there were significant concentration ($p < 0.05$) and follicle ($p < 0.0005$) effects with no significant interactions ($p > 0.05$) between the two factors. Furthermore, because 0.2 mM Cch elicited maximal increases in the Ca^{2+} response in cells from both stages of follicular development, the observed differences between

Figure 21: Concentration-response relationship to carbachol in granulosa cells from the F5,6 and F1 developmental stages. Each point represents the mean maximal $[Ca^{2+}]_i$ ± SEM after challenge of granulosa cells with 0.002 mM, 0.02 mM, 0.1 mM, 0.2 mM or 1 mM Cch. * Indicates significant difference ($p < 0.05$) from the value after challenge with 0.002 mM Cch within the same cell type. ** Indicates significant difference ($p < 0.05$) from values after challenge with both 0.002 mM and 0.02 mM Cch within the same cell type. Number in parentheses indicates the number of independent determinations.



1000
750
500
250
0
$[Ca^{2+}]_i$ (nM)
(53)
(15)
(14)
(15)
(13)
(8)
(8)
(10)
(51)
(9)
F1
F5,6
0.0
0.1
0.2
1.0
Carbachol (mM)

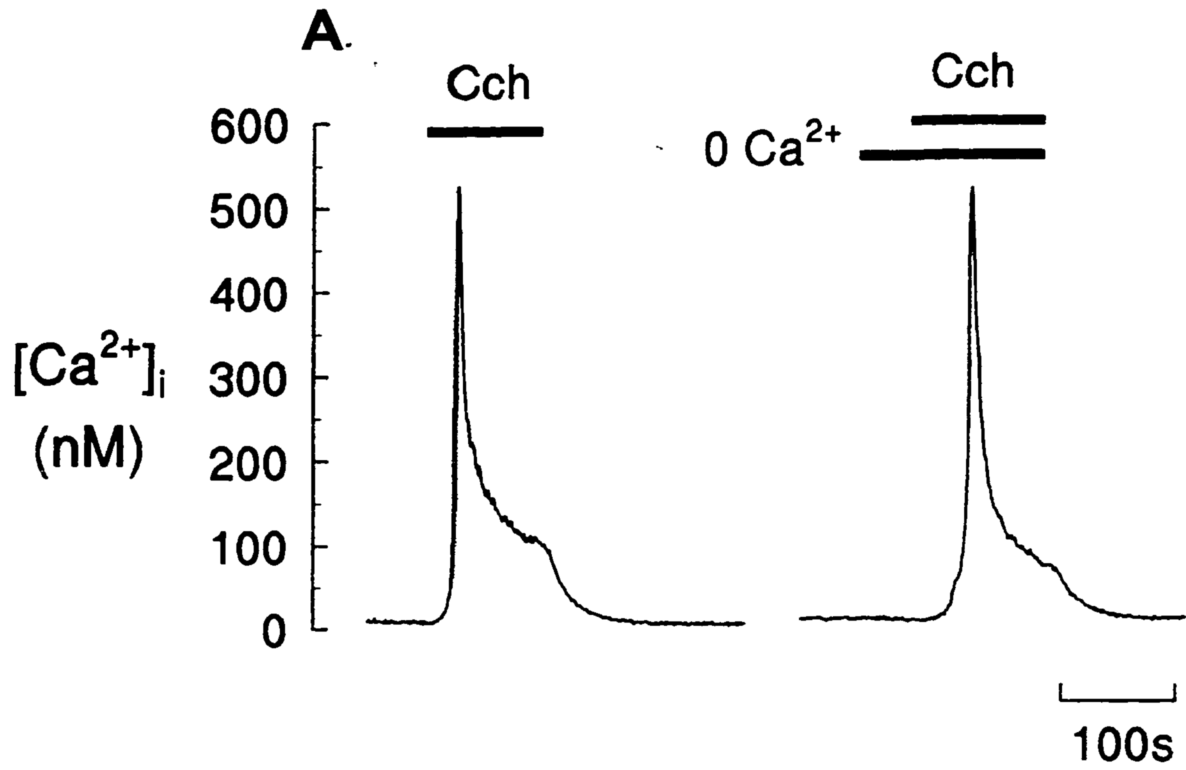
cells removed from different developmental stages are likely not attributable to a difference in their concentration-response relationship.

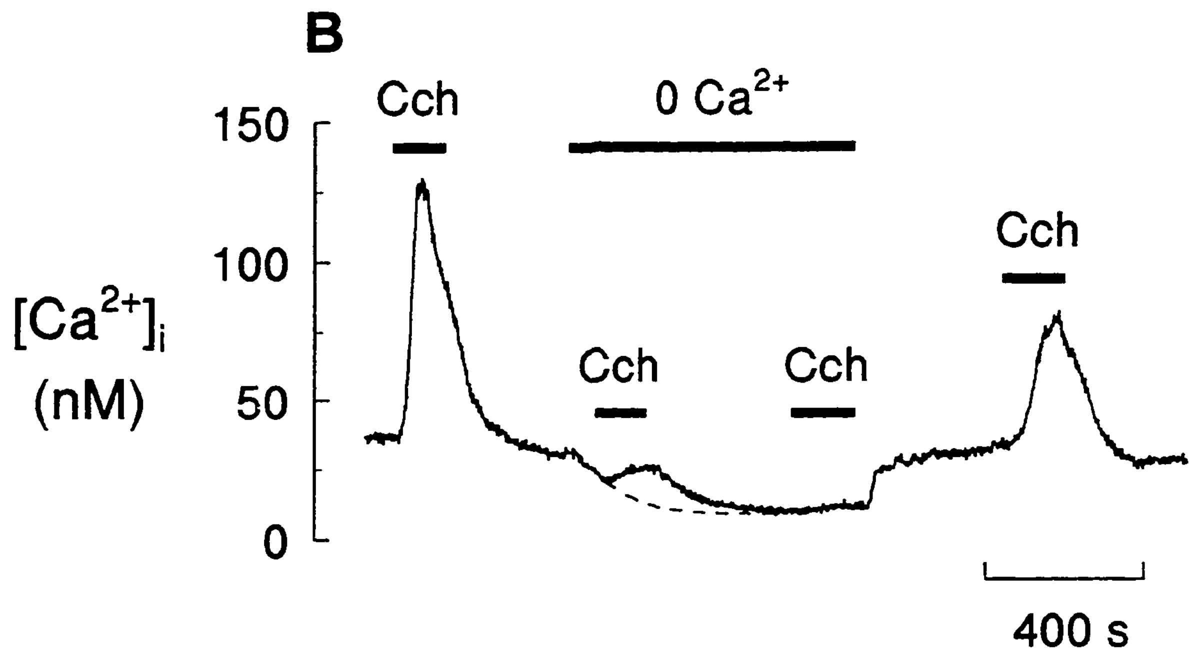
2. What are the cellular mechanisms involved in Cch-induced $[Ca^{2+}]_i$ changes?

a. Are Cch-induced Ca^{2+} transients dependent on extracellular $[Ca^{2+}]$?

To determine the Ca^{2+} sources responsible for the two types of Ca^{2+} transients observed throughout follicular development, experiments were performed with F5,6, F3 and F1 cells exposed to Cch in NBS with 1 mM Mn^{2+} (to replace Ca^{2+}). This concentration of Mn^{2+} was chosen in order to ensure complete blocking of Ca^{2+} channel activity while avoiding nonspecific Mn^{2+} effects such as fura-2 quenching. In addition, measurements of fluorescence at the isosbestic point (359 nm) indicated that the decrease in fluorescence was less than 5 % during Mn^{2+} exposures of 200 sec. This approach failed to inhibit fast Ca^{2+} transients (average rate greater than 100 nM/sec), although it led to a partial block of the sustained or oscillatory phase that occurs after the spike. This was observed in 5 such cells from different follicular stages and is represented by the experiment illustrated in Figure 22A. Slow responses from F5,6, F3 and F1 cells were greatly attenuated by removal of Ca^{2+} from the extracellular milieu. An example of a recording obtained under these conditions is presented in Figure 22B. Note that the first Cch challenge in Ca^{2+}-free medium caused a small, but noticeable increase in $[Ca^{2+}]_i$, whereas during the second Cch challenge in Ca^{2+}-

Figure 22: The influence of removal of external Ca^{2+} on Cch-induced increases in $[Ca^{2+}]_i$. **Panel A:** Representative tracing depicting the effect of removal of extracellular Ca^{2+} on fast Cch-induced Ca^{2+} transients (n = 5). The two Ca^{2+} transients illustrated were recorded from the same cell, separated by a minimum of 4-min washout of Cch. Exposure to 0.2 mM Cch and removal of external Ca^{2+} (Ca^{2+}-free medium containing 1 mM Mn^{2+}) are indicated by their respective horizontal bars. **Panel B:** Representative tracing portraying the effect of removal of external Ca^{2+} on slow Cch-induced Ca^{2+} transients. Note the small effect of the first Cch challenge (emphasized by the dotted line) in Ca^{2+}-free medium and the absence of effect of the second challenge. Note: 12 cells were challenged with Cch in the presence of Mg^{2+} and EGTA, although only 9 were challenged twice in Ca^{2+}-free medium. In addition, 17 cells were challenged with Cch in medium containing Mn^{2+} instead of Ca^{2+}.


A
Cch
Cch
0 Ca^{2+}
600
500
400
300
200
100
0
$[Ca^{2+}]_i$
(nM)
100s

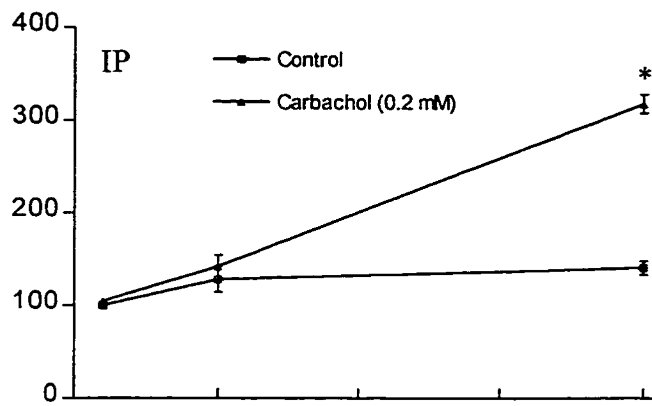

B
Cch
0 Ca^{2+}
150
100
50
0
$[Ca^{2+}]_i$
(nM)
Cch
Cch
Cch
400 s

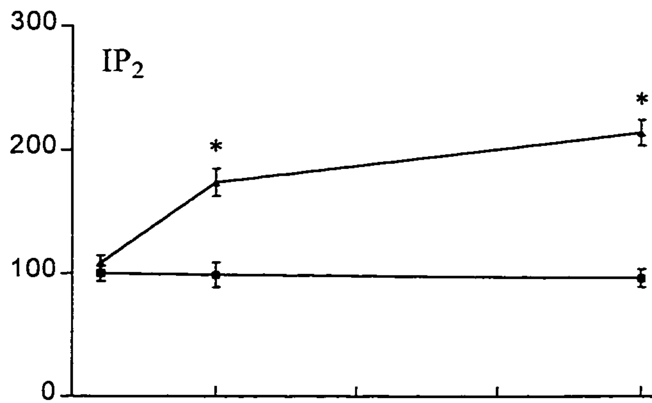
free medium, the Cch response was completely blocked in all cells tested in this way (n = 9). After re-addition of Ca^{2+} to the medium, we again challenged the cells with Cch and observed another Ca^{2+} transient; this indicated that the cells were still capable of responding to Cch challenge. From 17 such cells, with rates under 10 nM/sec, the average amplitude of Cch-induced Ca^{2+} transients decreased from 41 ± 9 nM to 12 ± 4 nM after Cch challenge in Ca^{2+}-free medium containing 1 mM Mn^{2+}. Similar results were observed after replacement of external Ca^{2+} with 2.5 mM Mg^{2+} and 5 mM EGTA (59 ± 13 nM to 20 ± 8 nM; n = 12), supporting the concept that the changes observed in the presence of Mn^{2+} were attributable to the dependence of the Cch response on extracellular Ca^{2+} and not to nonspecific Mn^{2+} effects. In addition, the reduction of the magnitude of Cch-induced Ca^{2+} transients in Ca^{2+}-free medium was performed first or second, suggesting that this is not related to cellular fatigue.

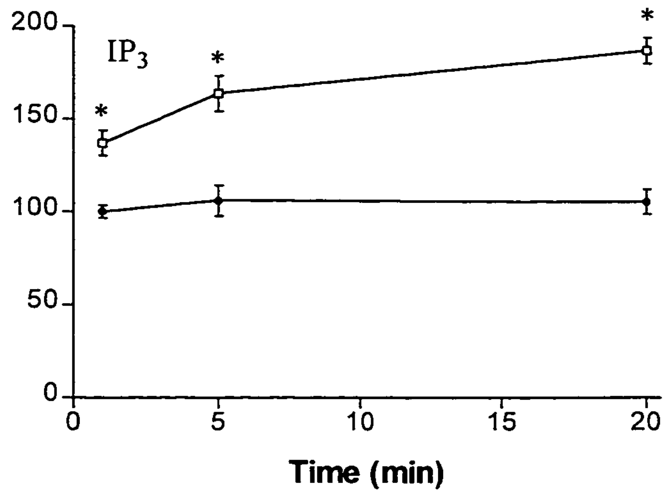
b. Are Cch-induced Ca^{2+} transients associated with changes in IP_3?

Because the large and fast Ca^{2+} transients were shown to be independent of extracellular Ca^{2+}, studies were conducted to examine the influence of Cch on the production of IP_3. To examine the time-course of action of Cch on the phosphatidyl inositol signalling system, inositol phosphate levels were measured in F1 granulosa cells incubated in the presence or absence of 0.2 mM Cch (Fig 23). IP_3 levels were significantly elevated by Cch

Figure 23: Time-course of carbachol (0.2 mM)-induced inositol phosphate production by F1 granulosa cells *in vitro*. Cells were prelabelled with [^{3}H]-*myo*-inositol for 16-18 hrs followed by a 45 min incubation with 10 mM LiCl and then challenged with vehicle (MEM) or carbachol. The subsequent liberation of inositol phosphates was assessed by ion-exchange chromatography of the water soluble fraction of the cells. Data are normalized to control (1 min; 100%) and represent the mean ± SEM of data from three independent experiments (each with four replicates per treatment group). * Indicates significant difference ($p < 0.05$) from control at the same time point.


Inositol Phosphate Production
(% of 1 min control)
400
300
200
100
0
IP
Control
Carbachol (0.2 mM)
*

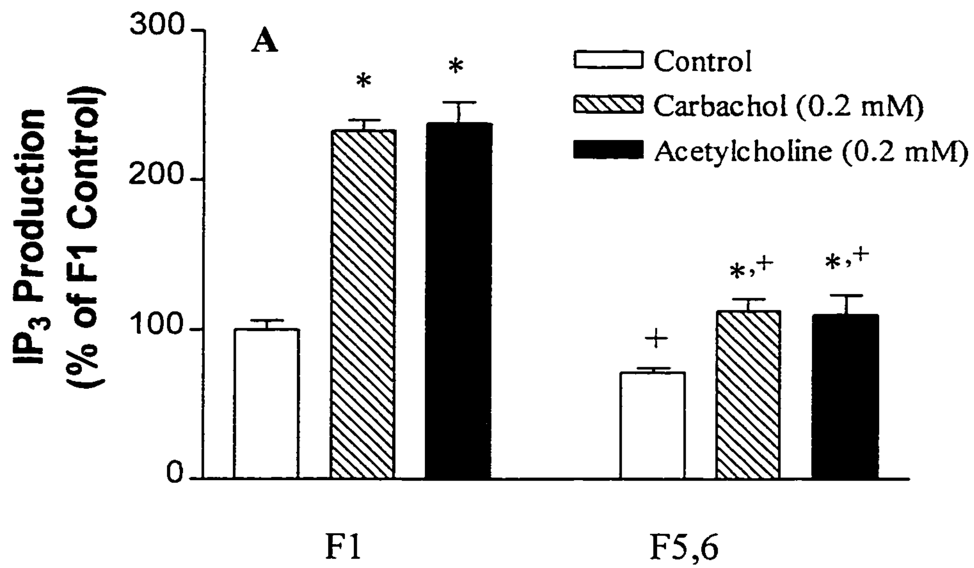

300
200
100
0
IP_2
*
*

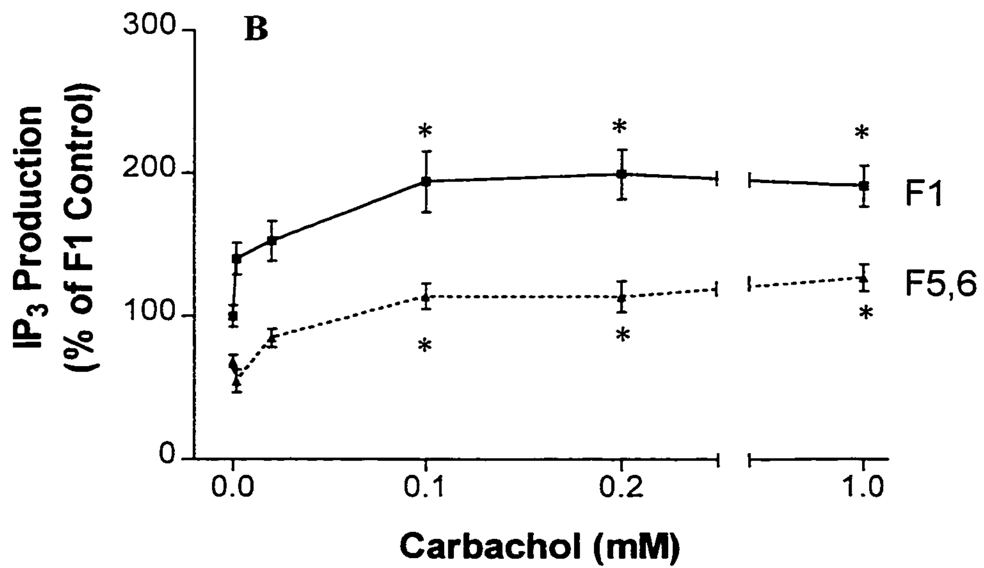

200
150
100
50
0
IP_3
*
*
*
0
5
10
15
20
Time (min)

after a 1-min incubation and these levels continued to rise slowly thereafter. Cch significantly increased the production of IP_2 after 5 min as well as 20 min incubations, whereas it increased IP at the longer incubation period only. In all subsequent experiments, cells were challenged with agonists for 5 min.

To test the influence of developmental stage on Cch- and Ach-induced changes in inositol phosphates, responses of F1 and F5,6 cells were compared (Fig 24A). Basal levels of IP_3 were significantly lower in F5,6 cells compared to F1 cells. Whereas incubation with either Cch or Ach (0.2 mM) stimulated the production of IP_3 in granulosa cells from both stages of follicular development, the relative levels and the proportion of increase over control values were significantly lower in F5,6 cells than in F1 cells (IP_3 levels increased to 143.1 ± 22.7 vs. 205.0 ± 24.8 % by Cch and 143.8 ± 30.4 vs. 214.3 ± 27.2 % by Ach; F5,6 vs. F1). In addition, we determined the concentration-response relationship between Cch and IP_3 production in F1 and F5,6 cells (Fig 24B) in order to determine if 0.2 mM Cch is maximal (as for $[Ca^{2+}]_i$; Fig 22). Analysis of variance of this study showed significant concentration ($p < 0.0001$) and follicle ($p < 0.0001$) effects with no significant interactions ($p > 0.05$) between these factors, indicating that Cch increased IP_3 levels in F1 and F5,6 cells with a similar concentration dependency and that this effect was more pronounced in F1 cells. Consistent with findings from studies on $[Ca^{2+}]_i$, 0.2 mM Cch was maximally stimulatory in F1 and F5,6 cells. These data suggest that these agonists are more potent stimulators of IP_3 production in F1 than in F5,6 granulosa cells.

Figure 24: Effect of follicular stage (Panel A) on carbachol or acetylcholine action or concentration dependency of carbachol action (Panel B) on IP_3 generation. Cells were prelabelled with [^{3}H]-*myo*-inositol for 16-18 hrs followed by a 45 min incubation with 10 mM LiCl and then challenged with vehicle (MEM) or carbachol. The subsequent liberation of inositol phosphates was assessed by ion-exchange chromatography of the water soluble fraction of the cells. Data are corrected for F1 control (100%) and are mean ± SEM of data from three independent experiments (each with four replicates per treatment). * Indicates significant difference ($p < 0.05$) from control of the same cell type.


A
IP3 Production (% of F1 Control)
300
200
100
0
Control
Carbachol (0.2 mM)
Acetylcholine (0.2 mM)
*
*
+
*,+
*,+
F1
F5,6


B
IP3 Production (% of F1 Control)
300
200
100
0
0.0
0.1
0.2
1.0
Carbachol (mM)
F1
F5,6
*
*
*
*
*
*

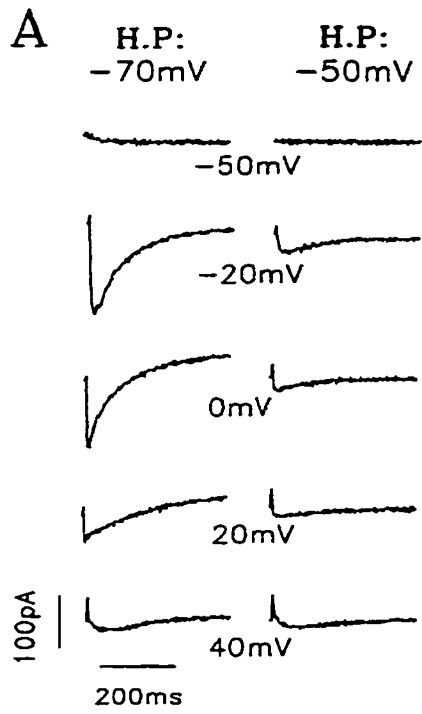
3. Cch increases transmembrane influx of Ca^{2+} throughout follicular development. What is the effect of Cch on Ca^{2+} current in F1 granulosa cells?

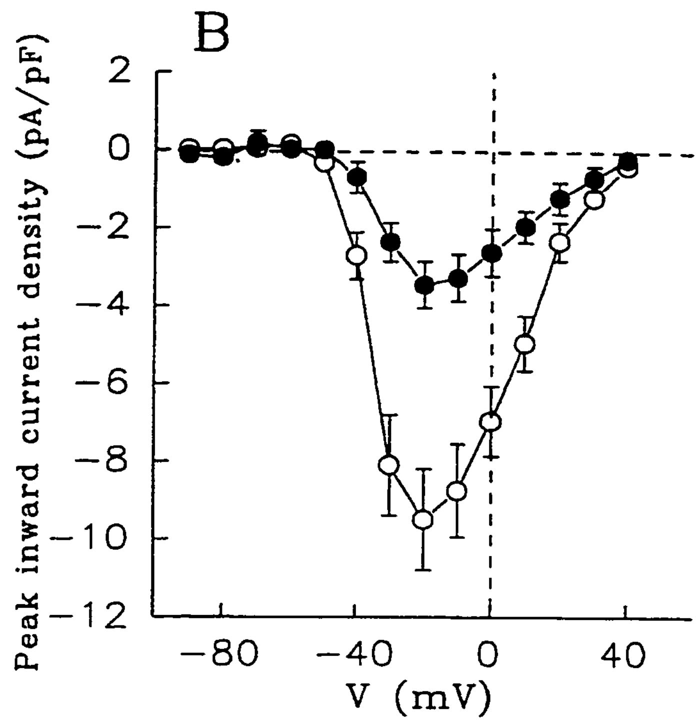
a. What is the nature of the Ca^{2+} current detected in F1 granulosa cells?

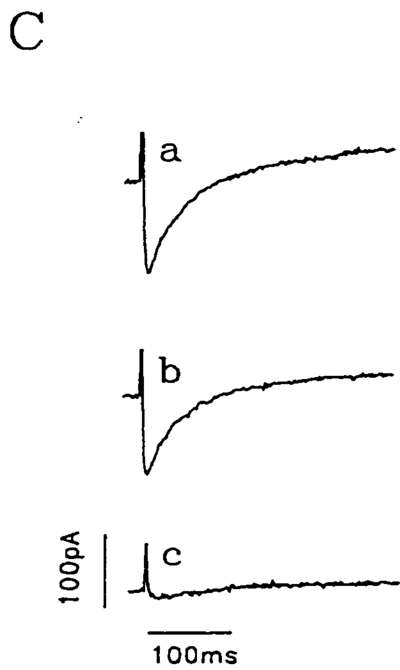
As the perforated patch was established, a single granulosa cell was held at -70 mV. Then current/voltage relationships were generated using 500-msec square pulses at 10 mV increments from -90 to 50 mV. Between pulses, cells were held at -70 mV for 10 sec. As shown in Fig 25A, transient inward currents were obtained between -50 and 40 mV (n = 8). The absence of Na^+ and K^+ to carry these inward currents and the negative resistance I/V relation that asymptotically approached the zero current in the depolarized direction (Fig 25B) are consistent with these Ca^{2+} currents being voltage dependent. In a group of 8 cells, the transient Ca^{2+} current had a threshold between -60 and -50 mV, a maximum amplitude around -20 mV, and a zero current potential of about 42 ± 2 mV. This inward current exhibited fast activation and relatively rapid inactivation. The time for this maximum current (*i.e.* at -20 mV) to reach a peak was 19 ± 2.5 msec. The time-course of inactivation did not follow a simple exponential, but its decay can be roughly characterized by the half-time for the inactivating current to reach steady state, which was 42 ± 3.4 msec at -20 mV.

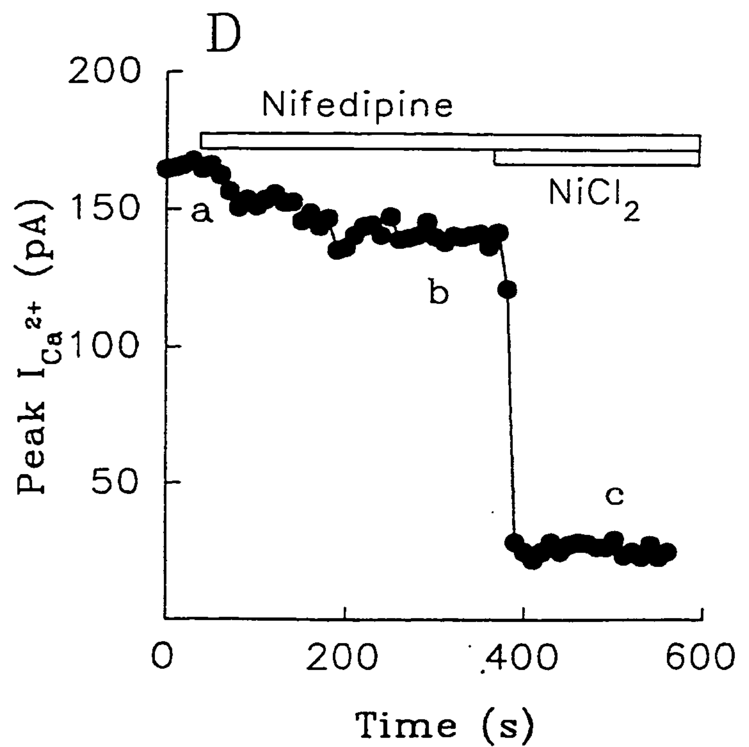
The inward currents obtained with this protocol do not represent a single type of Ca^{2+} conductance, as can be shown by using a depolarized holding potential. In the cells just described, the inward currents were significantly decreased by changing the holding potential

Figure 25: Characterization of the inward Ca^{2+} current in F1 cells. **Panel A:** Representative recordings showing that inward current was reduced by changing the holding potential from -70 mV to -50 mV. Two families of inward current were obtained in same cell. **Panel B:** I/V relation of peak inward current density from 7 cells measured using holding potentials -70 mV (open circles) and -50 mV (closed circles). In both cases, the maximum current density was obtained at about -20 mV. **Panel C:** Sample recordings from a typical experiment demonstrating the pharmacological identity of the inward current. Traces show the effects of nifedipine and $NiCl_2$ on the peak inward currents elicited by a standard test pulse. Traces a,b and c are taken at times indicated in the adjacent graph (D). **Panel D:** Graph for same cell as in C, showing the whole time-course. The concentrations of nifedipine and $NiCl_2$ were 10 μM and 0.1 mM, respectively.


A
H.P:
−70mV
H.P:
−50mV
−50mV
−20mV
0mV
20mV
40mV
100pA
200ms


B
Peak inward current density (pA/pF)
2
0
−2
−4
−6
−8
−10
−12
−80
−40
0
40
V (mV)


C
a
b
c
100pA
100ms


D
Nifedipine
NiCl2
Peak I Ca 2+ (pA)
200
150
100
50
a
b
c
0
200
400
600
Time (s)

from -70 mV to -50 mV (Fig 25A and 25B). Inactivation of these reduced currents still occurred, but was less rapid than with the total currents (half inactivation time of 72 ± 4.9 ms).

These results suggest that the inward current recorded from a holding potential of -70 mV consisted of two Ca^{2+} channel pathways, the L- and T-type channels (long-lasting and transient). In order to determine the relative importance of L- versus T-type Ca^{2+} channel activity in granulosa cells, we sequentially perfused cells with standard solution, then with the classical L-type Ca^{2+} channel blocker, nifedipine, to eliminate the L-type Ca^{2+} current, then with nifedipine and $NiCl_2$, which mainly blocks the T-type Ca^{2+} channel. In 6 cells, 10 μM nifedipine inhibited only 12 ± 5 % of total peak inward current whereas 0.1 mM $NiCl_2$ plus 10 μM nifedipine almost abolished inward current (reduction by 94 ± 3 %; Fig 25C and 25D). Thus, with the cells held at -70mV between pulses, the inward Ca^{2+} current consisted of two types of Ca^{2+} channels, L and T, but the T type dominated.

b. What is the effect of Cch on Ca^{2+} current? Is this Cch response mediated by muscarinic receptors?

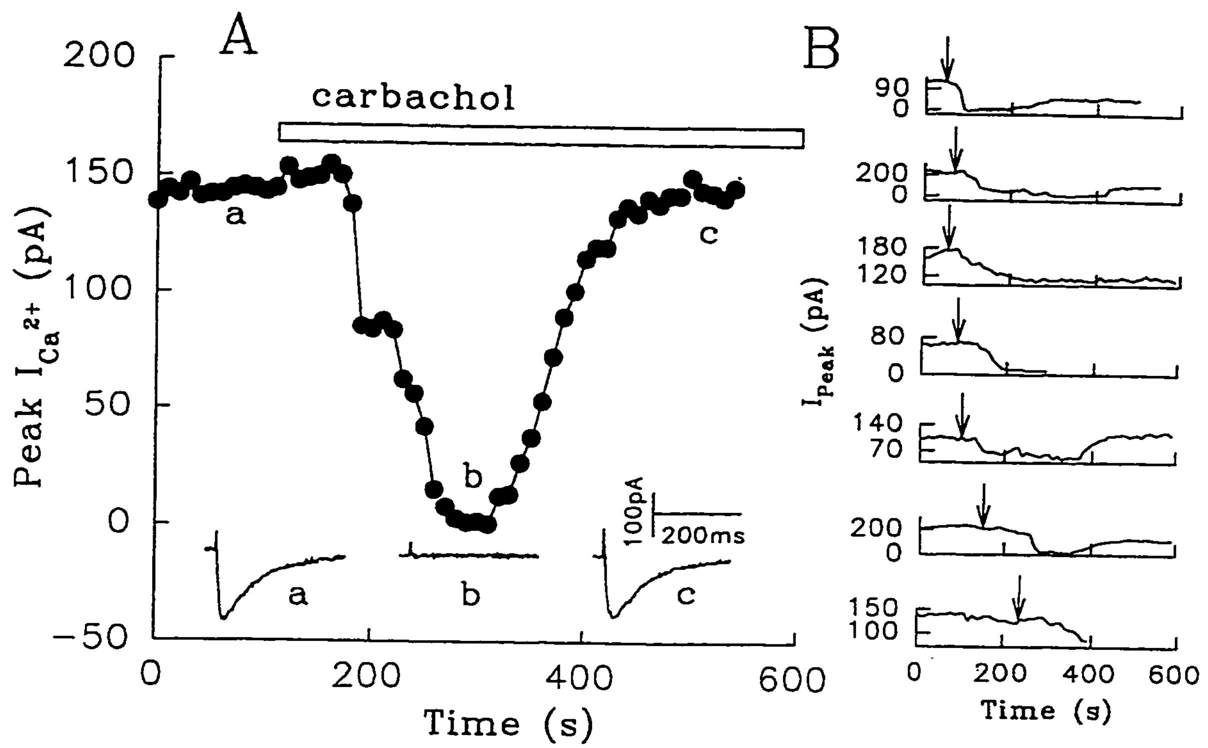
To test whether the Ca^{2+} currents are subject to muscarinic modulation, cells were exposed to 0.5 mM Cch, a concentration about 2.5 times higher than that previously shown to induce maximal changes of $[Ca^{2+}]_i$ in fura-2-loaded hen granulosa cells (Fig 19). The

protocol described above was used in order to obtain the maximum total Ca^{2+} current (stepping from -70 mV to -20 mV for 500 ms). After establishing a baseline over about 1 min, the cells were perfused with 0.5 mM Cch in the bath. Approximately 1 min following Cch challenge, a steep decrease in inward Ca^{2+} current was observed in all cells studied with an average maximal suppression of $90 \pm 2\%$ (Figure 26; n = 8). Figure 26A also shows an apparent desensitization of the response to Cch with the Ca^{2+} current recovering during the prolonged exposure to Cch, an effect clearly observed in 5 out of 8 cells.

To verify that the Cch-induced changes of inward Ca^{2+} current were specifically mediated by a muscarinic pathway, cells were preincubated in the presence of the muscarinic antagonist atropine (1 μM) for 10 min prior to the addition of 0.5 mM Cch. Under these conditions, Cch failed to induce changes of the peak inward current elicited at -20 mV (Fig. 27A). Fig.27B summarizes the result from 6 cells. This result indicates that activation of a muscarinic receptor initiated the Cch responses. Given the complexity of these responses, the stability of the peak inward current magnitude in the presence of Cch plus atropine is reassuring; evidently, Cch did not induce non-specific effects.

Since most of the inward Ca^{2+} current in granulosa cells is associated with T-type channel, it was of interest to determine if the Cch-induced response is associated with these channels. Preincubation for at least 10 min in 10 μM nifedipine to block the L-type Ca^{2+} current did not influence basal or Cch-induced Ca^{2+} current (Fig 28). As observed in the absence of nifedipine, exposure to Cch decreased Ca^{2+} current in all cells studied. Apparent

Figure 26: **Panel A:** The time-course of the effect of carbachol (0.5 mM) on the peak inward current. The inset shows the currents elicited by a standard test pulse at the indicated times. **Panel B:** The time-courses for 7 other cells tested. Carbachol was applied at a time indicated by the arrowhead.



A
B
carbachol
Peak $I_{Ca^{2+}}$ (pA)
200
150
100
50
0
−50
0
200
400
600
Time (s)
a
b
c
100pA
200ms
I_{Peak} (pA)
90
0
200
0
180
120
80
0
140
70
200
0
150
100
0
200
400
600
Time (s)

Figure 27: The effect of atropine on Cch-induced inhibition of inward Ca^{2+} current. **Panel A:** Sample traces from a typical experiment demonstrating that atropine blocks the effects of carbachol. Current traces, elicited by voltage pulses from -70 to -20 mV, were taken at the indicated times, which corresponded to the presence of 1 μM atropine alone (first trace) and that of 1 μM atropine plus 0.5 mM carbachol (last two traces). **Panel B:** Summary of results for 6 cells showing that atropine blocked the effect of carbachol on the inward current. "Delta Peak inward I", the ordinate, shows the peak current at time=t minus the peak current at time=0.

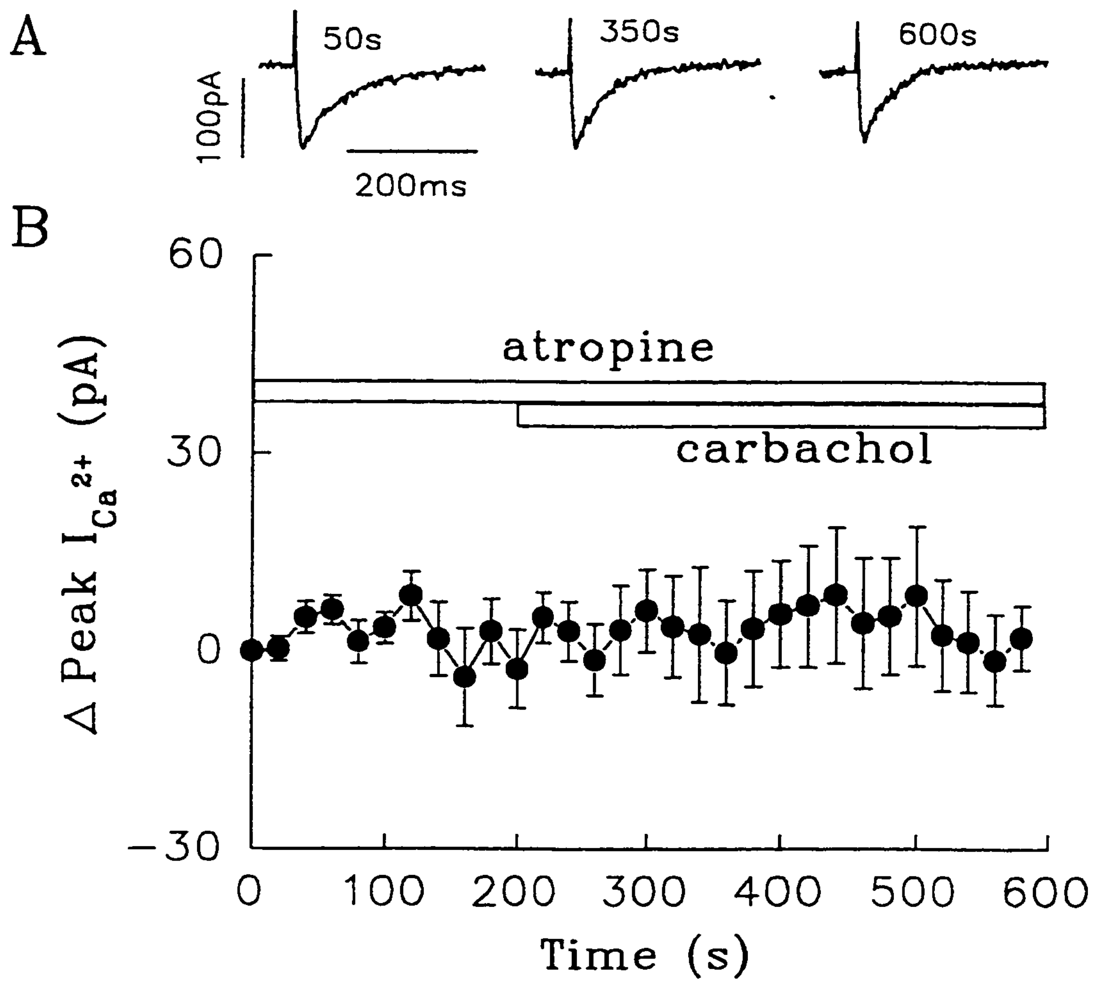
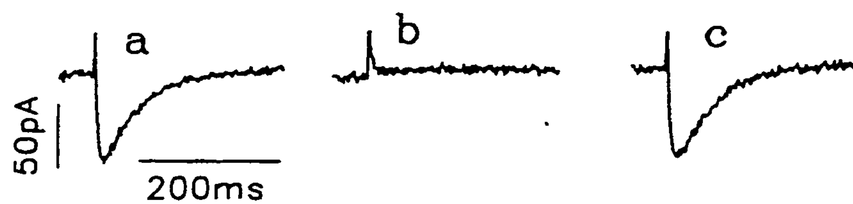
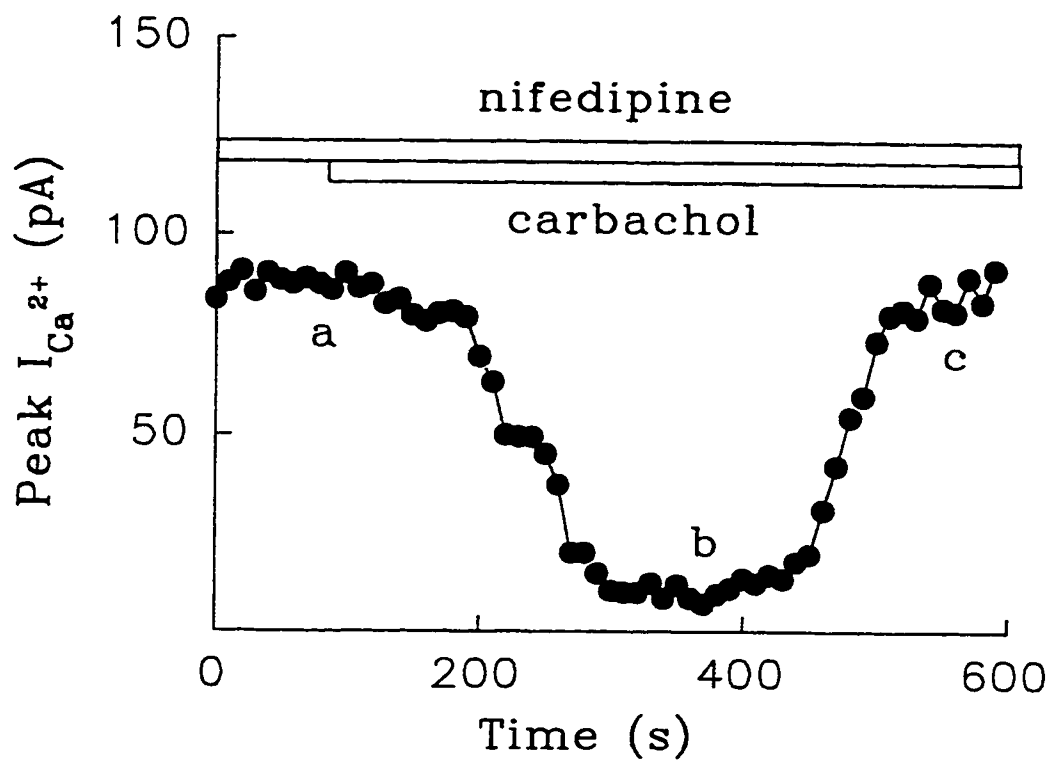

A
50s
350s
600s
100pA
200ms
B
atropine
carbachol
Δ Peak $I_{Ca^{2+}}$ (pA)
60
30
0
−30
0
100
200
300
400
500
600
Time (s)

Figure 28: Muscarinic inhibition of Ca^{2+} current occurs via T-type Ca^{2+} channels. **Panel A:** Sample traces from an experiment demonstrating that carbachol blocks inward current that is still present when cells are exposed to nifedipine. Tracings "a", "b" and "c" represent the Ca^{2+} current at the times indicated in panel B with the corresponding letter. **Panel B:** Graph for same cell as in panel A, showing the whole time-course. The concentrations of nifedipine and carbachol were 10 μM and 0.5 mM, respectively.


A
a
b
c
50pA
200ms


B
150
100
50
nifedipine
carbachol
a
b
c
Peak I_{Ca}^{2+} (pA)
0
200
400
600
Time (s)

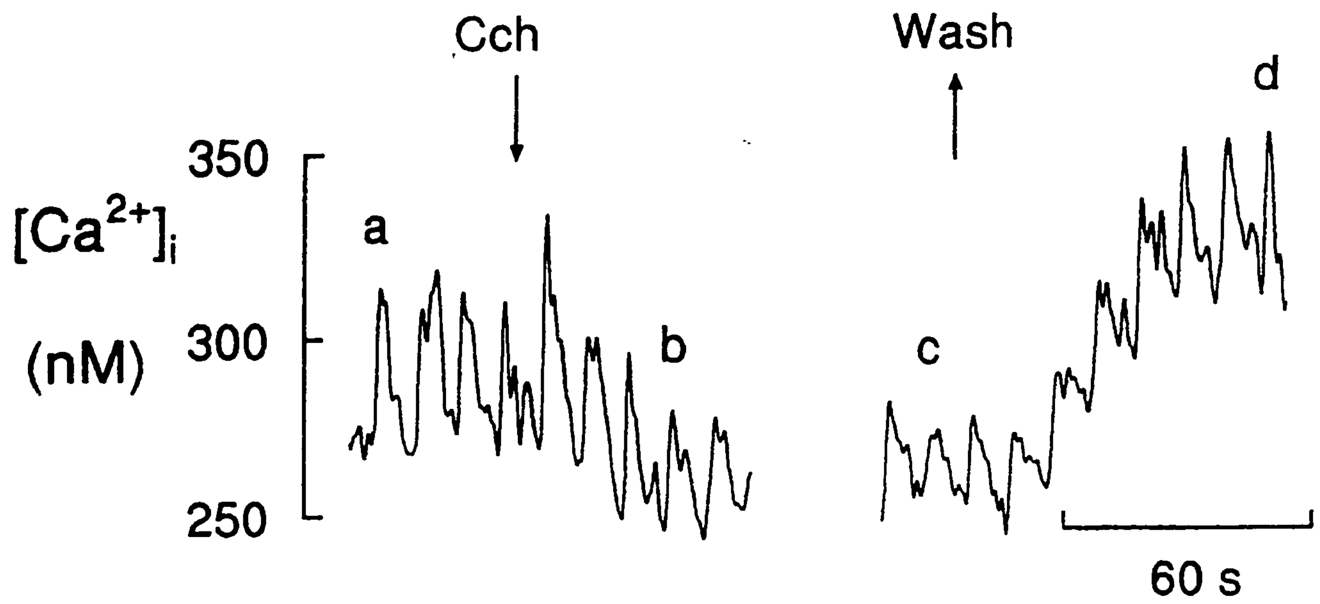
desensitization during prolonged exposure could also be observed under these conditions.

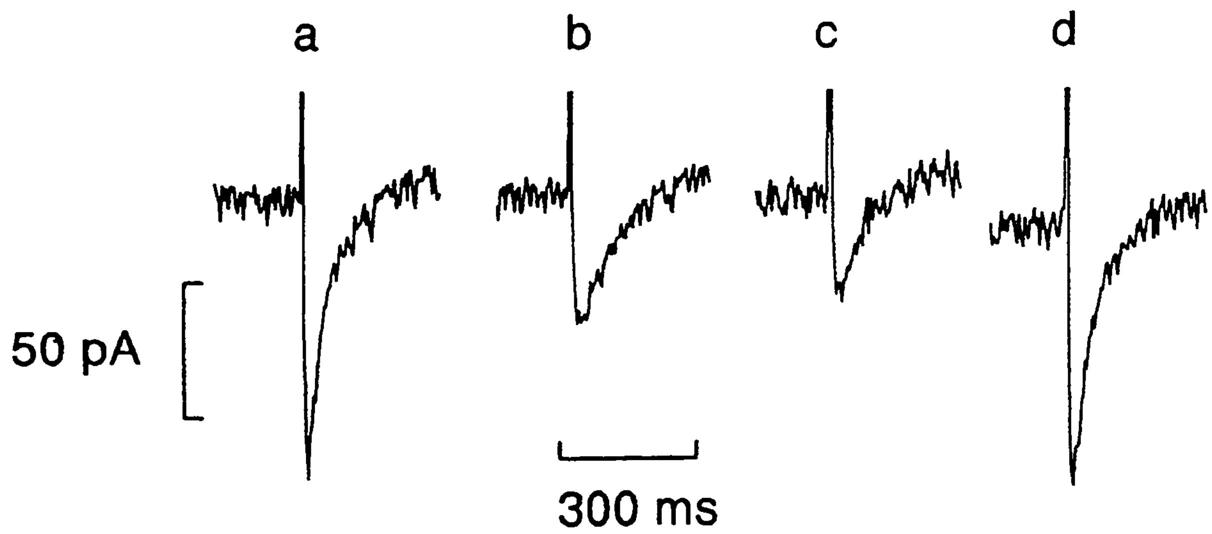
c. *How does $[Ca^{2+}]_i$ change following muscarinic input under electrically stimulated conditions? Is the Cch response dependent on basal $[Ca^{2+}]_i$?*

To test whether the observed inhibitory effect of Cch on Ca^{2+} current could be related to changes in intracellular Ca^{2+} levels, simultaneous recordings of $[Ca^{2+}]_i$ in fura-2 loaded cells were also performed. The first series of experiments evaluated the effect of replacement of Na^+ with NMDG (instead of choline) on granulosa cell Ca^{2+} current and $[Ca^{2+}]_i$. As shown in Figure 9, this manoeuvre promptly induced the expected large augmentation of Ca^{2+} current, with the average amplitude increasing from 19 ± 7 pA in NBS to 86 ± 21 pA after 1 min exposure to the NMDG solution (n = 6). This extracellular environment also caused an elevation of basal $[Ca^{2+}]_i$ which, on average, increased from 0.15 ± 0.02 to 0.25 ± 0.06 μM (n = 6) after 1 min in the NMDG solution. Part of this increase could be attributed to the rise in inward Ca^{2+} current which coincided with appearance of small but clearly distinguishable Ca^{2+} transients elicited by the depolarizing pulses.

Under identical conditions, Cch markedly decreased both basal $[Ca^{2+}]_i$ and the amplitude of the depolarization-induced Ca^{2+} transients (Fig 29). This effect was observed in 5 of the 6 cells studied, such that $[Ca^{2+}]_i$ and the average amplitude of the Ca^{2+} current were significantly decreased to 92.2 ± 2% (0.22 ± 0.06 μM) and 69 ± 7% of control values,

Figure 29: Effect of carbachol on intracellular free-Ca^{2+} concentration and Ca^{2+} current. After 10 min superfusion with the NMDG solution, 0.5 mM carbachol (Cch) was added at the time indicated by the arrow and removed 3 min later (Wash). The upper traces show the measured $[Ca^{2+}]_i$, with a 2 min gap between the 2 recordings. The lower traces represent expanded Ca^{2+} current recordings taken at the times indicated by the small letters. Membrane potential was held at -80 mV and 300-ms depolarizing pulses to -30 mV were applied every 10 s, as can be noticed from the small but noticeable Ca^{2+} transients on the upper trace. Carbachol induced a reversible decrease of both $[Ca^{2+}]_i$ and Ca^{2+} current. The letters "a", "b", "c" and "d" in the upper tracing represent the times at which the Ca^{2+} currents depicted in the lower graph labelled with the corresponding letters were measured.

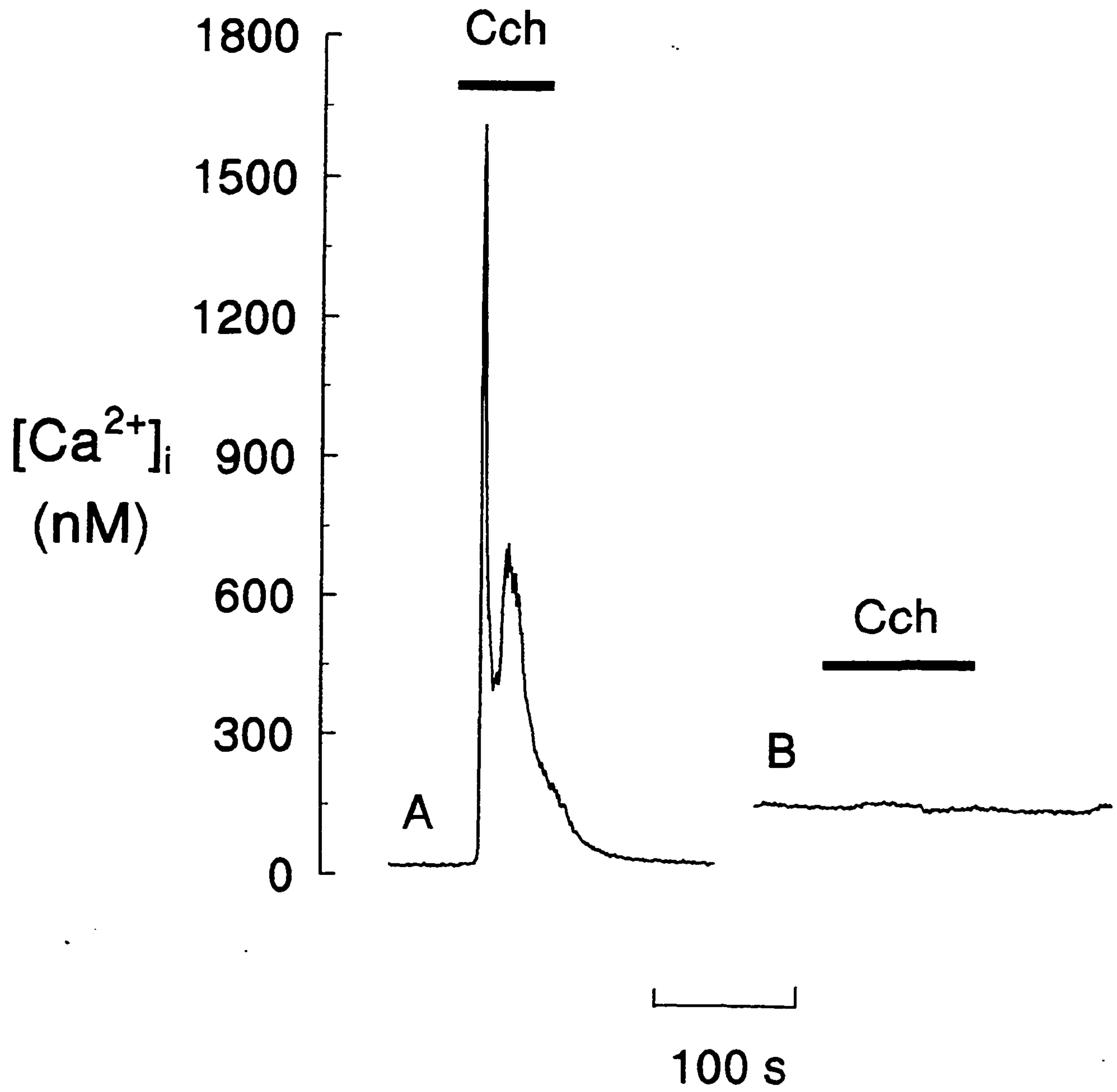

Cch
Wash
d
350
[Ca2+]i
(nM)
a
300
b
c
250
60 s


a
b
c
d
50 pA
300 ms

respectively, following a 1 min exposure to 0.5 mM Cch, similar to those obtained from patch-clamp measurements alone (Fig 26). In addition, Cch washout after 2 to 3 min exposure resulted in a rapid recovery of both Ca^{2+} current and $[Ca^{2+}]_i$ (Fig 29).

The observation that Cch actually induced a decrease in $[Ca^{2+}]_i$ appears to be at odds with previous studies showing a marked and transient increase of $[Ca^{2+}]_i$ upon application of the muscarinic agonist (Morley *et al*, 1992a; Fig 19). One possibility that could account for this apparent discrepancy may be related to the relatively high intracellular Ca^{2+} levels following superfusion in the absence of Na^+ but presence of 10 mM Ca^{2+}. This possible dependency of Cch response on basal $[Ca^{2+}]_i$ was tested by experiments in which fura-2 loaded cells were preincubated in NBS containing 25 mM Ca^{2+}, and $[Ca^{2+}]_i$ was measured in the absence of patch-clamp pipettes to avoid possible artifactual effects from this invasive approach (Fig 30). Exposure of the cells to the high Ca^{2+} containing solution resulted in a population of cells with a wide range of basal $[Ca^{2+}]_i$ values. Of the 55 cells studied, 13 had $[Ca^{2+}]_i$ levels $\leq$ 60 nM while 25 exhibited $[Ca^{2+}]_i \geq$ 120 nM. The majority of the cells with low $[Ca^{2+}]_i$ (77%) responded to Cch with a typical Ca^{2+} transient, as depicted in Figure 30A. In contrast, 80% of the cells with $[Ca^{2+}]_i \geq$ 120 nM failed to elicit any Ca^{2+} transient following Cch challenge (Fig 30B). Furthermore, the amplitude of the Ca^{2+} transients for the remaining 5 responding cells (0.4 ± 0.2 μM) was significantly smaller than those measured from cells with low basal $[Ca^{2+}]_i$ (2.3 ± 0.8 μM; n = 10), demonstrating a clear correlation between the capability of the cells to respond to Cch and their basal $[Ca^{2+}]_i$. Contrary to the

Figure 30: Typical effects of carbachol on intracellular free-Ca^{2+} concentration in cells exposed to 25 mM Ca^{2+}. Carbachol (0.2 mM) was added at the times indicated by the horizontal bars. Tracings are from two different cells, showing the presence of a large Ca^{2+} spike for the cell with an initially low basal $[Ca^{2+}]_i$ value (A; 40 nM) and the absence of carbachol effect for the cell with a relatively high $[Ca^{2+}]_i$ (B; 155 nM).



Cch
1800
1500
1200
$[Ca^{2+}]_i$
(nM)
900
600
300
0
A
B
Cch
100 s

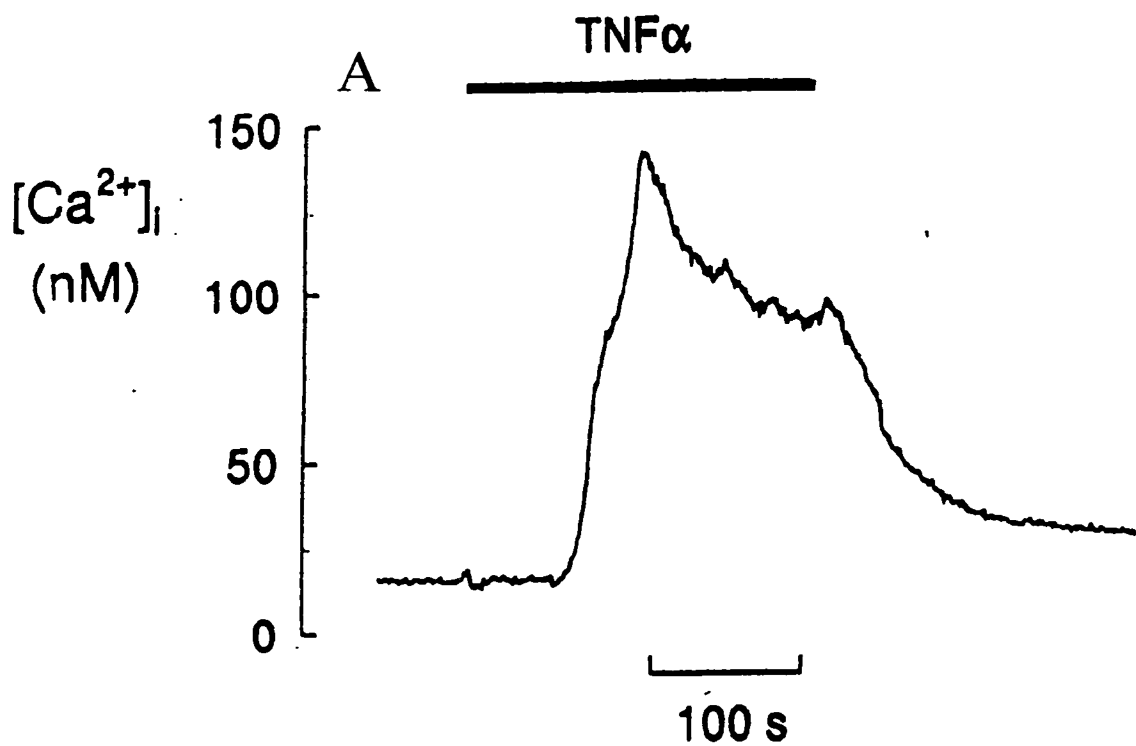
patch-clamp experiments, however, no decrease in $[Ca^{2+}]_i$ could be detected for the non-responding cells. This is likely due to the fact that those cells were not electrically stimulated.

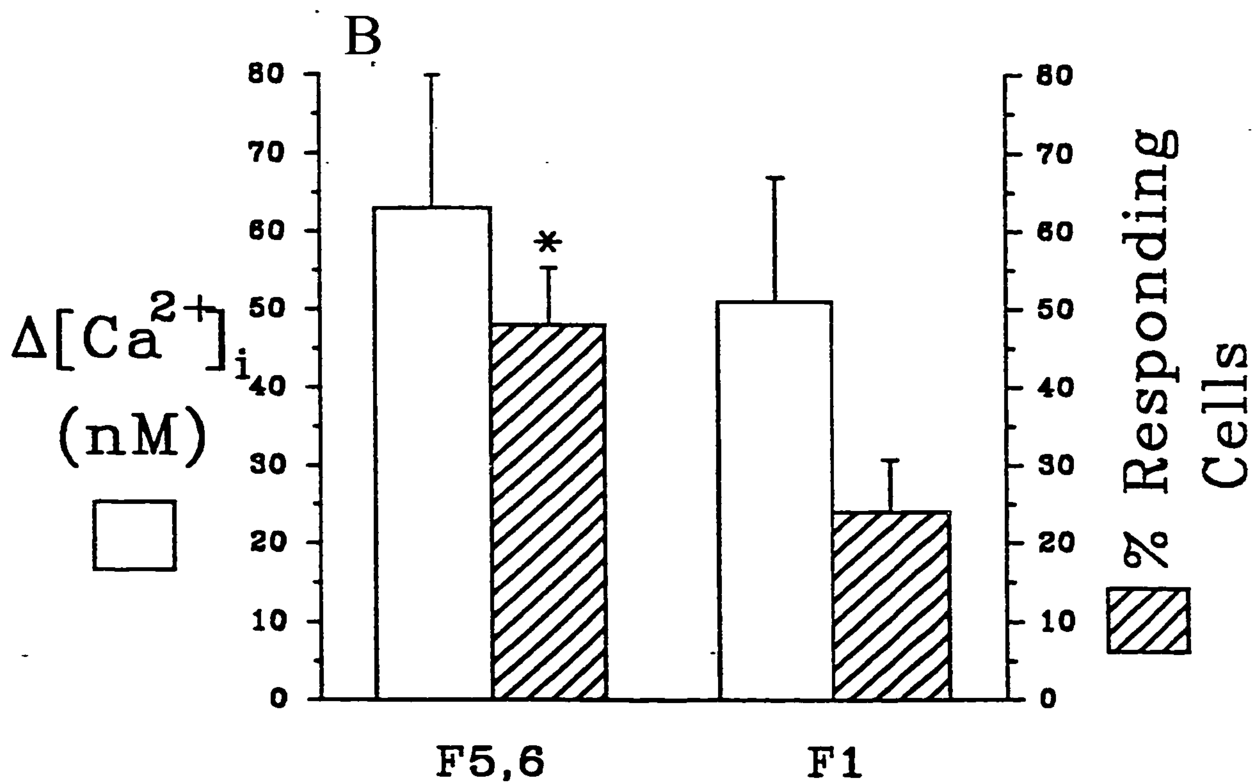
D. Regulation of $[Ca^{2+}]_i$ by TNFα in hen granulosa cells during follicular maturation.

1. What is the effect of TNFα on granulosa cell $[Ca^{2+}]_i$? Are TNFα-induced Ca^{2+} transients dependent on the stage of follicular development?

The typical effects of 10 ng/ml TNFα on $[Ca^{2+}]_i$ in granulosa cells are depicted in Figure 31A. TNFα-induced Ca^{2+} transients observed in hen granulosa cells were characterized by slow (6.37 ± 0.5 nM/sec for F5,6 and 6.28 ± 0.92 nM/sec for F1), small increases in $[Ca^{2+}]_i$ following a delay of approximately 1 min. Higher concentrations of TNFα (100 ng/ml) failed to elicit a quantitatively or qualitatively different response (data not shown). The ability of granulosa cells to respond to TNFα decreased during follicular maturation. Although no significant differences ($p > 0.05$) were found between the magnitude of TNFα-induced Ca^{2+} transients during follicular maturation, the proportion of cells able to respond to TNFα was significantly higher in F5,6 cells than F1 cells ($p < 0.025$; Fig 31B).

Figure 31: Changes in $[Ca^{2+}]_i$ in hen granulosa cells in response to TNFα. **Panel A:** Representative tracing of TNFα-induced changes in F5,6 granulosa cell $[Ca^{2+}]_i$. Horizontal bar represents period of exposure to 10 ng/ml TNFα. **Panel B:** Percentage (hatched bars) and average increases in magnitude (open bars) of TNFα-responsive cells during follicular development. Values reported from F5,6 and F1 cells are based upon 48 and 41 independent determinations, respectively. * Represents significant difference from corresponding value from F1 Cells ($p < 0.025$).


TNFα
A
150
100
50
0
$[Ca^{2+}]_i$
(nM)
100 s


B
80
70
60
50
40
30
20
10
0
$\Delta[Ca^{2+}]_i$
(nM)
*
F5,6
F1
% Responding
Cells

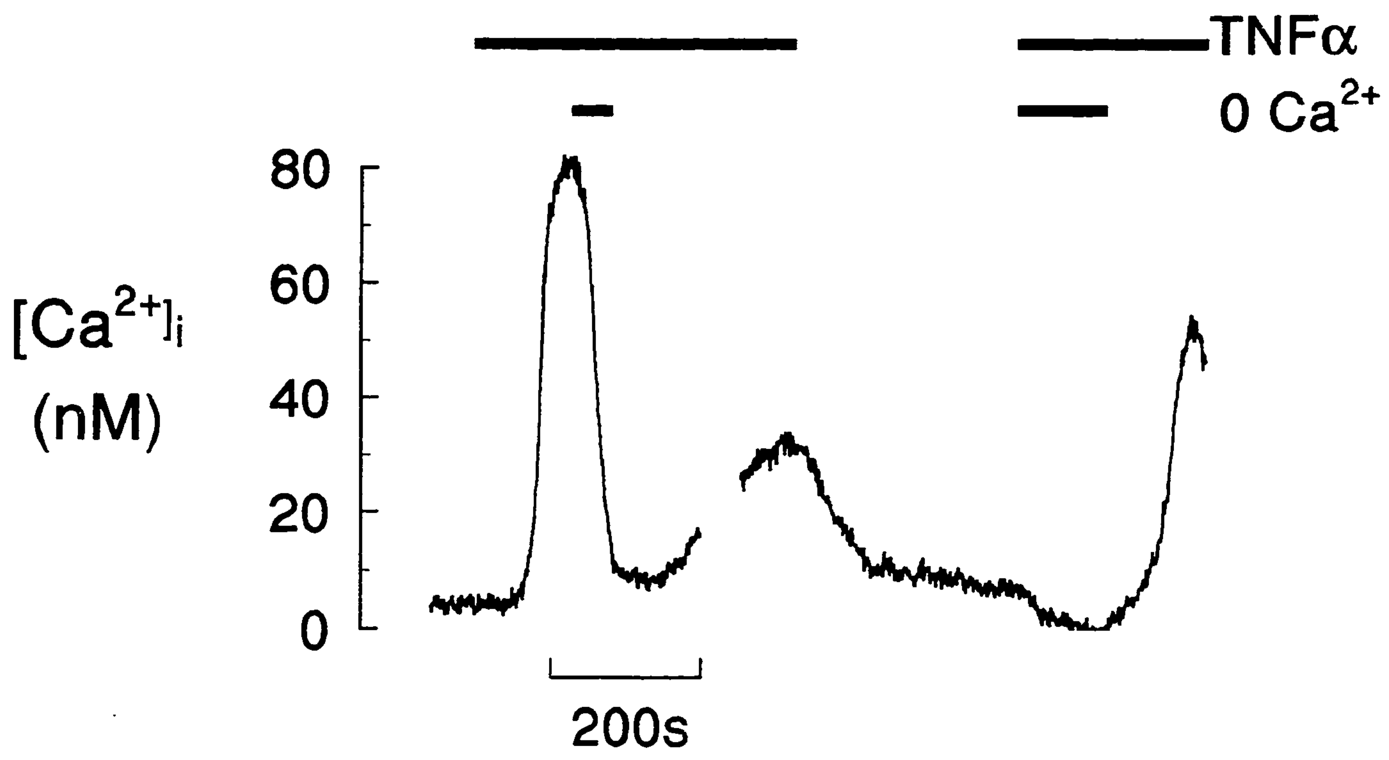
2. Are TNFα-induced Ca^{2+} transients dependent on extracellular $[Ca^{2+}]_i$?

In order to determine the Ca^{2+} sources responsible for TNFα-induced changes in $[Ca^{2+}]_i$, experiments were performed with cells challenged with TNFα in Ca^{2+}-free medium containing 1 mM Mn^{2+} (n = 7). As shown in Fig 32, the TNFα response was completely blocked in the presence of Mn^{2+}, indicating that Ca^{2+} channels must be involved in the Ca^{2+} response of the cell to the cytokine. In addition, approximately 1.5 min following the replacement of Mn^{2+} with Ca^{2+} (still in the presence of TNFα), an increase in $[Ca^{2+}]_i$ above the resting level was observed. These findings were confirmed by replacement of Ca^{2+} with 2.5 mM Mg^{2+} and 5 mM EGTA (n = 5), clearly demonstrating that TNFα-induced Ca^{2+} transients were dependent on extracellular $[Ca^{2+}]$.

3. Does pretreatment with TNFα influence Cch-induced Ca^{2+} transients? Is the TNFα-Cch interaction follicular stage dependent?

The response of granulosa cells to Cch in the presence of TNFα was significantly different from that in the absence of the cytokine. In the absence of TNFα, Cch-induced large (> 250 nM), rapid (> 100 nM/sec) increases in $[Ca^{2+}]_i$ via mobilization of Ca^{2+} from intracellular stores in approximately 50 % of Cch-responsive F1 granulosa cells, but only about 15 % of Cch-responsive F5,6 cells. Pretreatment with TNFα (4 - 5 min) increased the

Figure 32: Influence of extracellular [Ca^{2+}] on TNFα-induced Ca^{2+} transients in F5,6 granulosa cells. Top horizontal bars represent periods of exposure to 10 ng/ml TNFα. Lower horizontal bars represent time periods during which extracellular Ca^{2+} was removed and replaced with 2.5 mM Mg^{2+} and 5 mM EGTA (n = 5) or with 1 mM Mn^{2+} (n = 7).

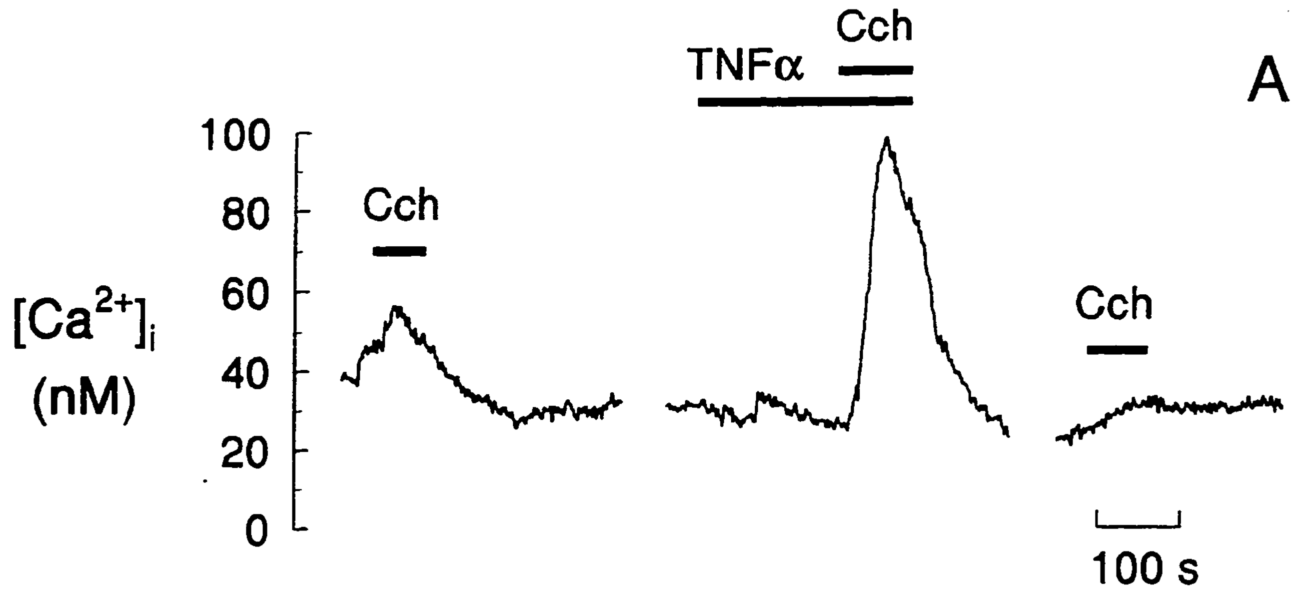


TNFα
0 Ca2+
80
60
40
20
0
[Ca2+]i
(nM)
200s

magnitude of response in F5,6 granulosa cells incapable of producing large Cch-induced changes in $[Ca^{2+}]_i$ (Fig 33A). In contrast, the cytokine had no effect on Cch-induced Ca^{2+} transients in F5,6 granulosa cells already capable of producing large Cch-induced Ca^{2+} transients (Fig 33B). In F1 cells, TNFα produced the same type of effect, only to a greater extent (Fig 34A), such that the Cch response in the presence of TNFα was indistinguishable from fast Cch-induced Ca^{2+} transients as depicted in Figure 34B. In addition, the response of the cells to TNFα was evident irrespective of the number of previous exposures to Cch (one to five) or the order in which the cells were challenged (data not shown). These observations, together with the finding that subsequent challenge with Cch in the absence of TNFα failed to produce large Ca^{2+} transients observed earlier in the presence of the cytokine (Fig 33A and 34A), suggest that the TNFα effect on the Cch response is reversible and not affected by desensitization following multiple Cch challenge.

In order to demonstrate that the change in the response of the F1 cell to Cch was in fact due to pretreatment with the cytokine, we compared the relative change between consecutive Cch-induced Ca^{2+} transients with or without TNFα pretreatment prior to the second Cch challenge. As Figure 35 illustrates, pretreatment with TNFα did induce significant increases in Cch-induced changes in $[Ca^{2+}]_i$ compared to control in both F1 and F5,6 cells that were previously incapable of producing large Ca^{2+} transients. Furthermore, following a prior Cch exposure, the second large Cch-induced Ca^{2+} transients in the presence of TNFα were significantly larger than the second Cch-induced Ca^{2+} transient in the absence

Figure 33: Influence of TNFα on Cch-induced Ca^{2+} transients in F5,6 granulosa cells. **Panel A:** Influence of TNFα on slow Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ less than 250 nM; n = 39). **Panel B:** Influence of TNFα on fast Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ greater than 250 nM; n = 3). Ca^{2+} transients illustrated within each panel were recorded from the same cell separated by a minimum of 4-min washout. Exposure of cells to 10 ng/ml TNFα and 0.2 mM Cch are indicated by their respective horizontal bars.


A
TNFα
Cch
Cch
Cch
[Ca2+]i
(nM)
100
80
60
40
20
0
100 s

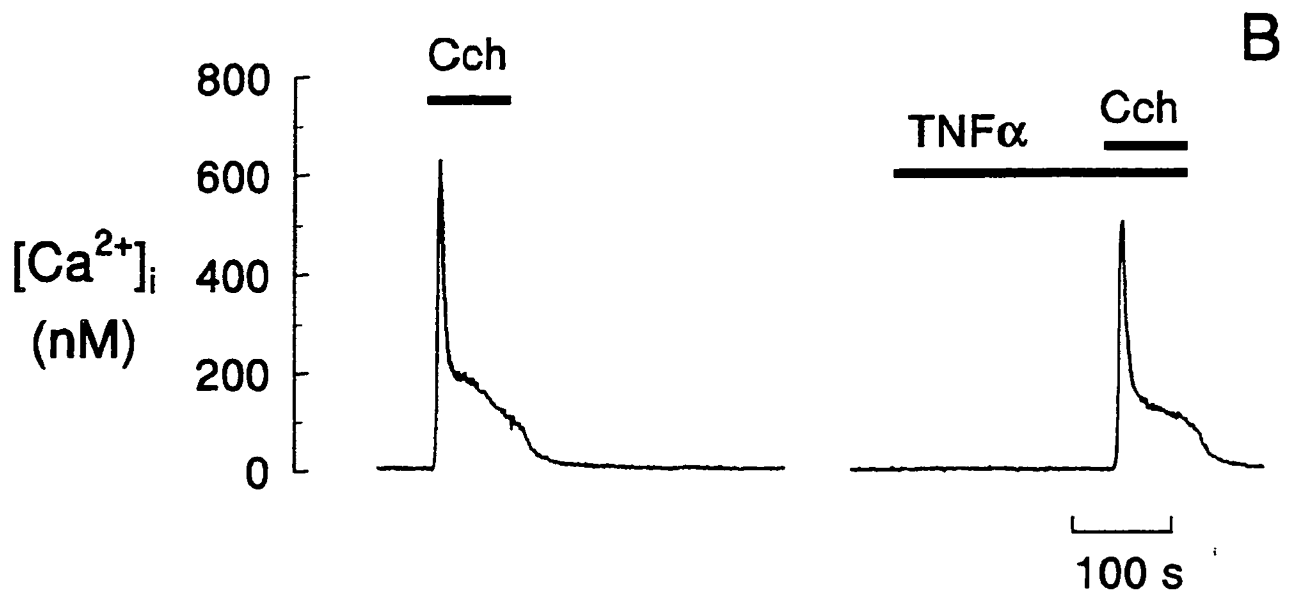
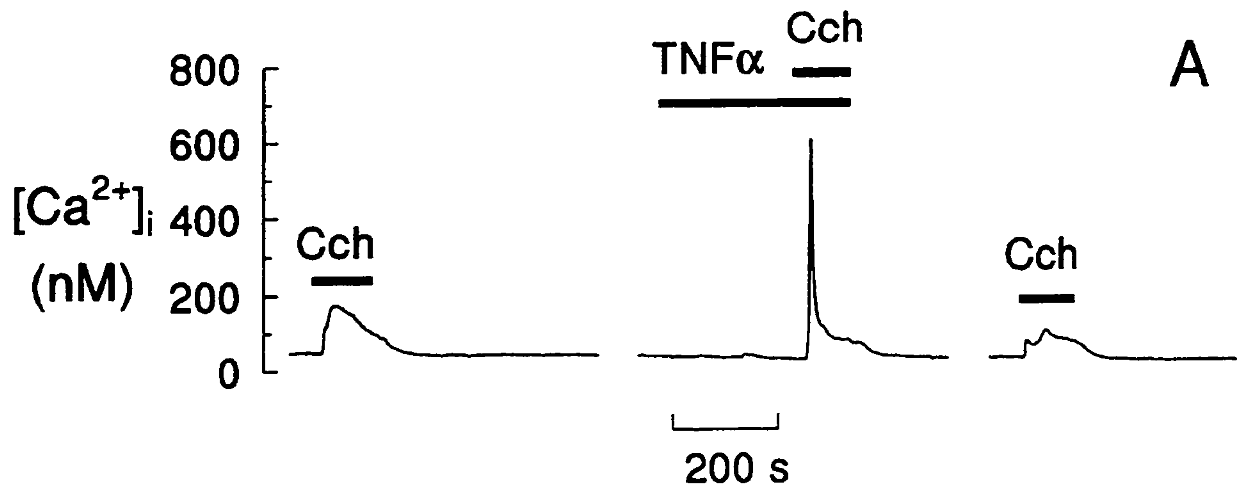

B
Cch
TNFα
Cch
[Ca2+]i
(nM)
800
600
400
200
0
100 s

Figure 34: Influence of TNFα on Cch-induced Ca^{2+} transients in F1 granulosa cells. **Panel A:** Influence of TNFα on slow Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ less than 250 nM; n = 22). **Panel B:** Influence of TNFα on fast Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ greater than 250 nM; n = 11). Ca^{2+} transients illustrated within each panel were recorded from the same cell separated by a minimum of 4-min washout. Exposure of cells to 10 ng/ml TNFα and 0.2 mM Cch are indicated by their respective horizontal bars.


A
Cch
TNFα
Cch
Cch
800
600
400
200
0
[Ca2+]i
(nM)
200 s

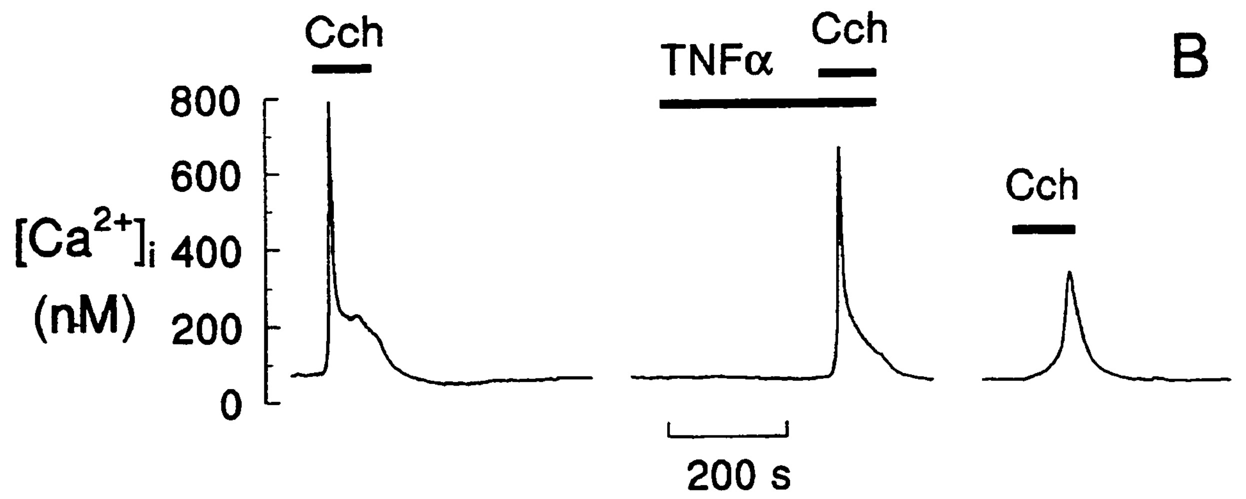
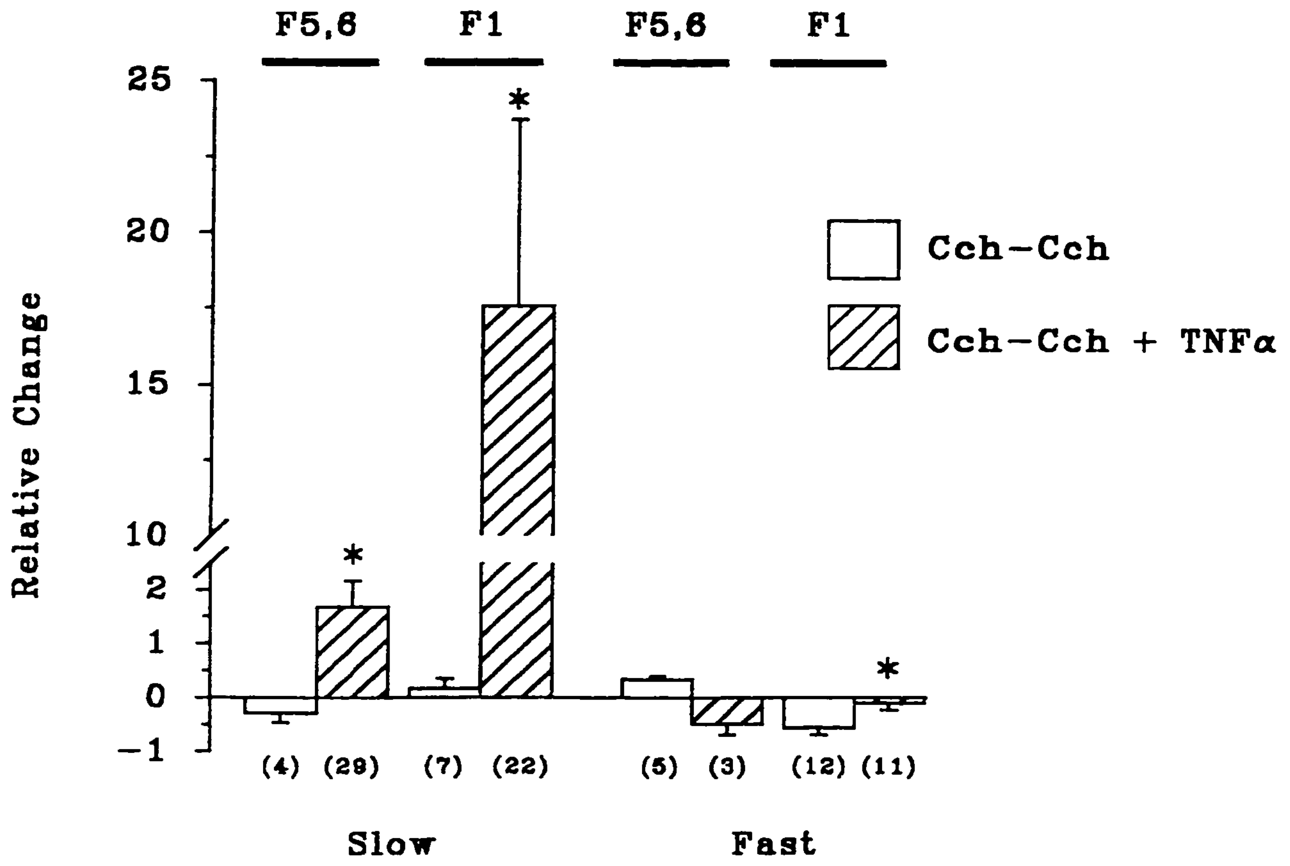

B
Cch
TNFα
Cch
Cch
800
600
400
200
0
[Ca2+]i
(nM)
200 s

Figure 35: Relative change between consecutive Cch-induced Ca^{2+} transients in the presence and absence of TNFα in both F1 and F5,6 granulosa cells. Relative change was calculated by dividing the difference in the magnitude of consecutive Cch-induced increases in $[Ca^{2+}]_i$ by the magnitude of the first Cch-induced Ca^{2+} transient. Slow Ca^{2+} transients were defined as changes in $[Ca^{2+}]_i$ less than 250 nM, while fast Ca^{2+} transients were defined as changes in $[Ca^{2+}]_i$ greater than 250 nM. Bars located underneath the F1 labels represent the relative changes calculated in cells from the F1 follicle, while bars located underneath the F5,6 labels represent the relative changes calculated in cells from the F5,6 follicle. * Represents significant difference with corresponding control values ($p < 0.05$). The number in parentheses represents the number of independent determinations.


F5,6
F1
F5,6
F1
25
20
15
10
2
1
0
−1
Relative Change
Cch−Cch
Cch−Cch + TNFα
*
*
*
(4)
(29)
(7)
(22)
(5)
(3)
(12)
(11)
Slow
Fast

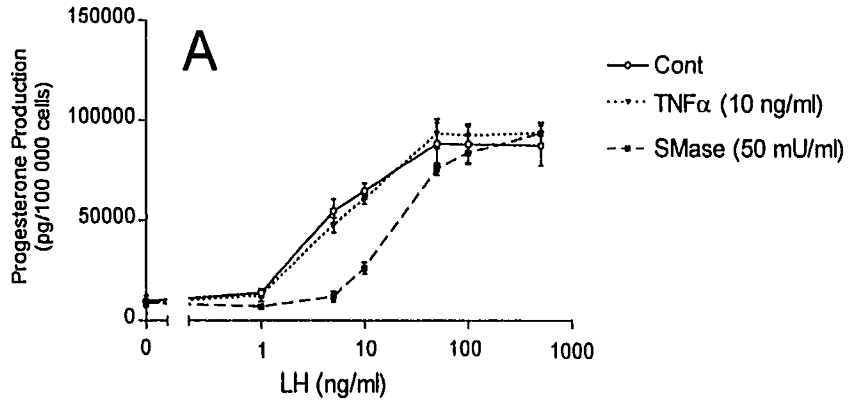
of TNFα. Furthermore, the fact that Cch-induced Ca^{2+} transients in the presence of TNFα were generally smaller than the first Cch-induced Ca^{2+} transient, suggests that the effect of TNFα was to attenuate fatigue in these cells.

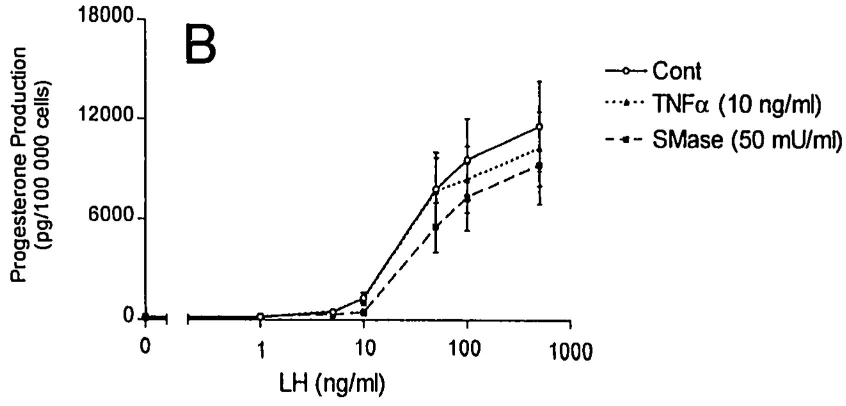
E. Modulatory role of TNFα on granulosa cell differentiation.

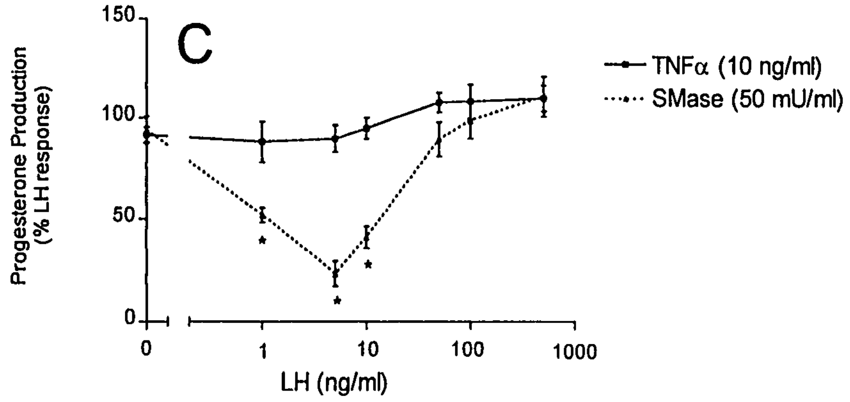
1. Does TNFα regulate basal progesterone production in hen granulosa cells? Does the presence of TNFα alter progesterone production in the presence of LH or Cch?

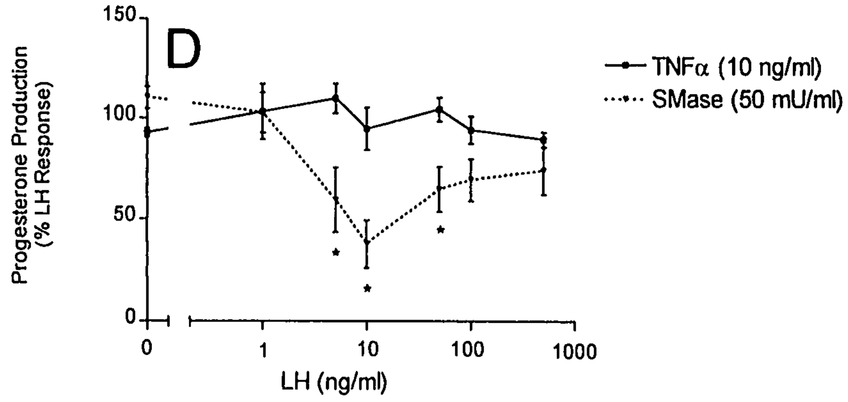
While TNFα is believed to inhibit LH-induced steroidogenesis in rat granulosa cells via ceramide production (Santana *et al*, 1996), the role of TNFα and the possible involvement of the SMase pathway in the regulation of hen progesterone production has not been studied. In the present studies, F1 and F5,6 granulosa cells (2.5×10^5 cells/well) were incubated for 24 hours with TNFα (10 ng/ml) or SMase (50 mU/ml) in the absence or presence of LH (1, 5, 10, 50, 100 or 500 ng/ml) and progesterone secretion was assessed (Fig 36). Although TNFα (10 ng/ml) had no influence on basal or LH-induced granulosa cell progesterone production, exogenous SMase (50 mU/ml) inhibited this response within a limited range of LH concentrations (1-10 ng/ml or 5-50 ng/ml; F1 or F5,6 cells, respectively). The lack of effect of TNFα on basal and LH-stimulated granulosa cell steroid production was independent of the concentration of the cytokine and the stage of follicular

Figure 36: Sphingomyelinase, but not TNFα inhibits LH-induced progesterone production in hen granulosa cells during follicular development. F1 (Panels A and C) and F5,6 (Panels B and D) granulosa cells (2.5 x 10^5) were cultured with BSA (0.1 %; vehicle), SMase (50 mU/ml) or TNFα (10 ng/ml) in the absence or presence of LH (1 to 500 ng/ml) for 24 hours (n = 5). Progesterone secretion was determined by RIA of spent medium. % inhibition (Panels C and D) was calculated by dividing the TNFα- and SMase-induced progesterone content in the presence of LH (1 to 500 ng/ml) by the quantity of progesterone secreted in response to each respective concentration of LH.


A
Progesterone Production
(pg/100 000 cells)
150000
100000
50000
0
0
1
10
100
1000
LH (ng/ml)
Cont
TNFα (10 ng/ml)
SMase (50 mU/ml)


B
Progesterone Production
(pg/100 000 cells)
18000
12000
6000
0
0
1
10
100
1000
LH (ng/ml)
Cont
TNFα (10 ng/ml)
SMase (50 mU/ml)


C
Progesterone Production
(% LH response)
150
100
50
0
0
1
10
100
1000
LH (ng/ml)
TNFα (10 ng/ml)
SMase (50 mU/ml)


D
Progesterone Production
(% LH Response)
150
100
50
0
0
1
10
100
1000
LH (ng/ml)
TNFα (10 ng/ml)
SMase (50 mU/ml)

maturation (Table 4). In addition, due to the established relationship between $[Ca^{2+}]_i$ and steroidogenesis, the muscarinic agonist Cch (0.2 mM) was used in the presence of LH, TNFα or SMase. Progesterone secretions in the presence and absence of Cch were not significantly different in either F1 or F5,6 cells, irrespective of the presence of LH, TNFα or SMase (Figure 37).

2. Does TNFα interact with Cch in the regulation of fibronectin and/or integrin production?

The production and secretion of fibronectin by hen granulosa cells was found to be Ca^{2+}-dependent (Conkright and Asem, 1995). Moreover, the regulation of integrin production in the ovary has not been studied, although TNFα increases integrin production in other cell types (Ahmad *et al*, 1998; Chabot *et al*, 1997; Gailit *et al*, 1996; Lowell *et al*, 1996). Since TNFα interacts with Cch in the regulation of $[Ca^{2+}]_i$ (Results section D3; Pg 145), the role of Cch in the secretion of fibronectin and production of integrins was assessed in the presence or absence of TNFα.

Western blots of cell extracts revealed the presence of two distinct bands: a major band(s) of approximately 200 kDa and a minor 140 kDa band (Fig 38). A secreted form of the protein (approximately 210 kDa) was also detected in spent medium (Fig 39). Both the 200 and 140 kDa ($p < 0.001$ for both bands) cellular fibronectin bands as well as the secreted

Table 4: Granulosa cell progesterone production in the presence of varying concentrations of TNFα and 10 ng/ml LH.[a]

TNFα (ng/ml)	Progesterone Secretion (pg/100 000 cells)[b]			
	F5,6 Granulosa Cells[c]		F1 Granulosa Cells	
	BSA (0.1%)	LH (10 ng/ml)[d]	BSA (0.1%)	LH (10 ng/ml)[d]
0	141 ± 38	1629 ± 301	7807 ± 1357	64386 ± 10717
0.1	152 ± 39	1766 ± 219	6125 ± 988	66248 ± 12240
1	152 ± 49	1662 ± 173	6288 ± 916	66248 ± 10178
10	170 ± 52	1617 ± 255	6011 ± 1120	60858 ± 12314
100	157 ± 41	1456 ± 213	5602 ± 1051	71050 ± 10440

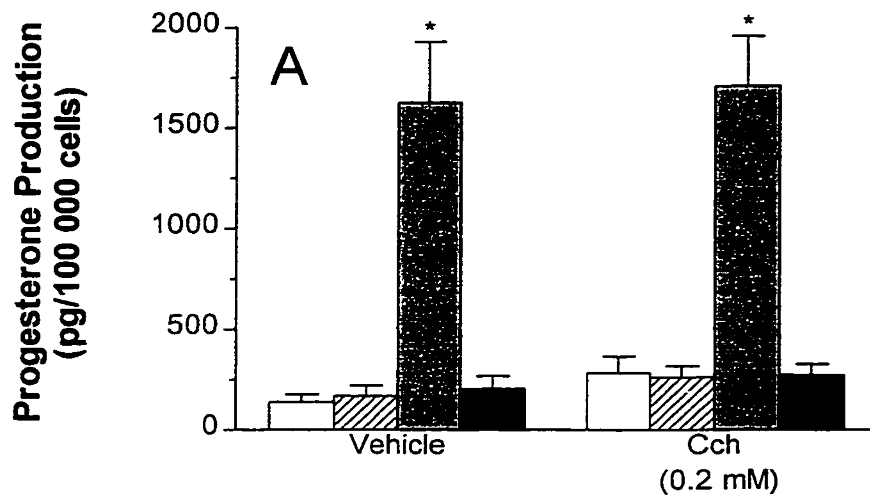
[a] Progesterone content was assessed by RIA of spent medium from 2.5 x 10^5 granulosa cells cultured in 500 ul of MEM for 24 hours.

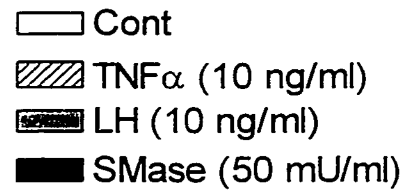
[b] Values presented represent the mean ± SEM of three different cultures with three independent determinations per experiment.

[c] represents significant difference ($p < 0.001$) from F1 as determined by three way ANOVA

[d] represents significant difference ($p < 0.001$) from BSA. TNFα had no effect on BSA or LH-induced progesterone production in F1 or F5,6 cells as determined by three-way ANOVA.

Figure 37: The effect of Cch on progesterone production in the presence and absence of LH, TNFα or SMase. Progesterone production was assessed by RIA of spent medium from F5,6 (Panel A) or F1 (Panel B) cells (2.5 x 10^5) cultured 24 hours in the presence of vehicle, LH (10 ng/ml), TNFα (10 ng/ml) or SMase (50 mU/ml) with or without Cch (0.2 mM; n = 3). Data was analyzed by two way ANOVA. Progesterone secretion in the presence of LH was significantly higher ($p < 0.001$) than all other treatments, although the presence of Cch had no effect on basal, TNFα-, SMase- or LH-induced progesterone secretion. * represents significant difference from control, as determined by Tukey's test.


A
Progesterone Production
(pg/100 000 cells)
2000
1500
1000
500
0
*
*
Vehicle
Cch
(0.2 mM)


Cont
TNFα (10 ng/ml)
LH (10 ng/ml)
SMase (50 mU/ml)

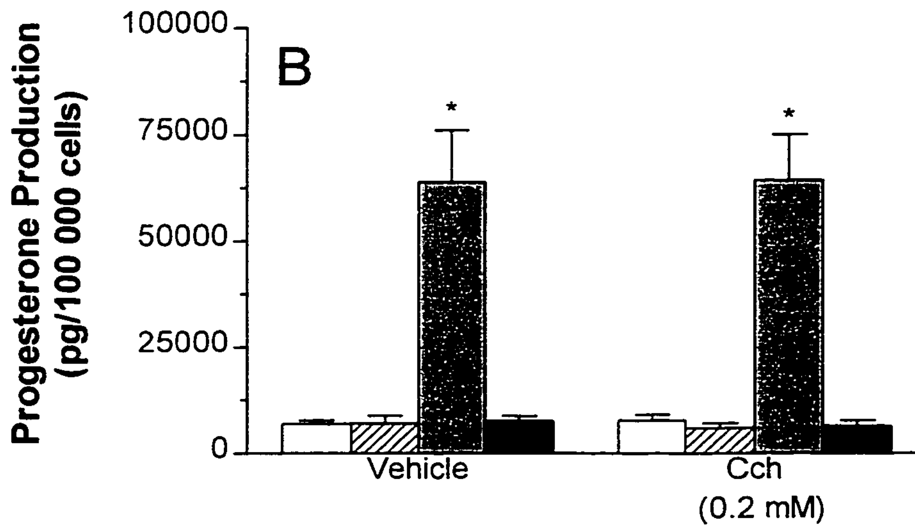
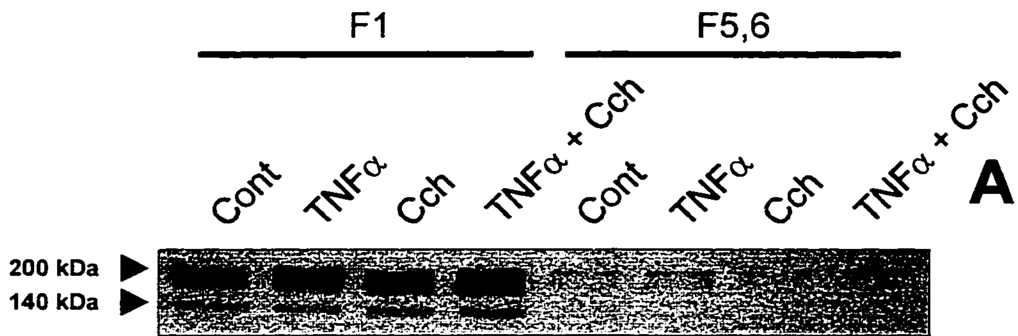
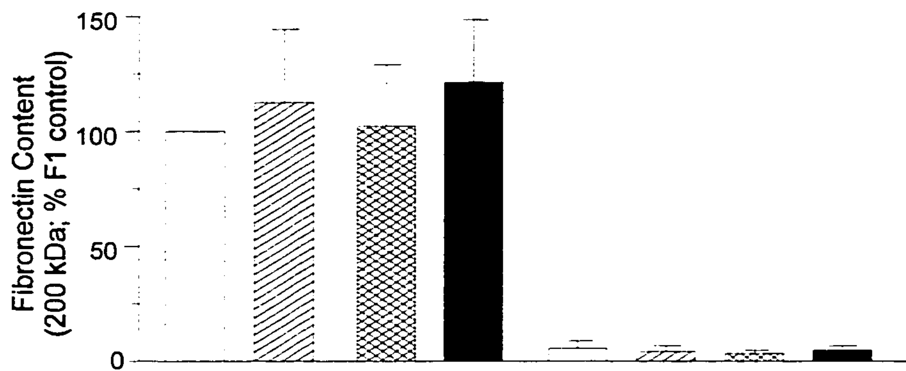

B
Progesterone Production
(pg/100 000 cells)
100000
75000
50000
25000
0
*
*
Vehicle
Cch
(0.2 mM)

Figure 38: Cellular fibronectin content in hen granulosa cells during follicular development in the presence or absence of TNFα and/or Cch (n = 3). F5,6 and F1 granulosa cells were cultured for 24 hrs in the presence or absence of TNFα (10 ng/ml) and/or Cch (0.2 mM). Floating and attached cells were combined and lysed. After protein quantitation, samples (10 μg protein/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human fibronectin IgG, washed, incubated (30 min) in goat anti-mouse IgG and visualized by ECL. A representative blot is depicted in Panel A. Densitometric analysis of the upper (B) and lower (C) bands was performed using Molecular Analyst software (Bio-Rad Laboratories, CA). Three way ANOVA revealed significant differences for both the 200 ($p < 0.001$) and 140 ($p < 0.001$) kDa bands between F5,6 and F1 granulosa cells, although there were no significant differences between the treatment groups within a stage of follicular development ($p > 0.05$).


F1
F5,6
Cont
TNFα
Cch
TNFα + Cch
Cont
TNFα
Cch
TNFα + Cch
A
200 kDa
140 kDa


Fibronectin Content
(200 kDa; % F1 control)
150
100
50
0
B
Cont
TNFα (10 ng/ml)
Cch (0.2 mM)
Cch (0.2 mM) +
TNFα (10 ng/ml)

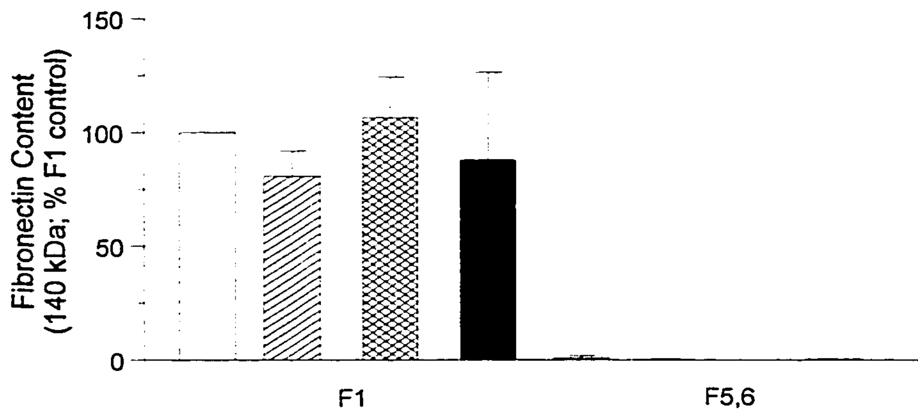
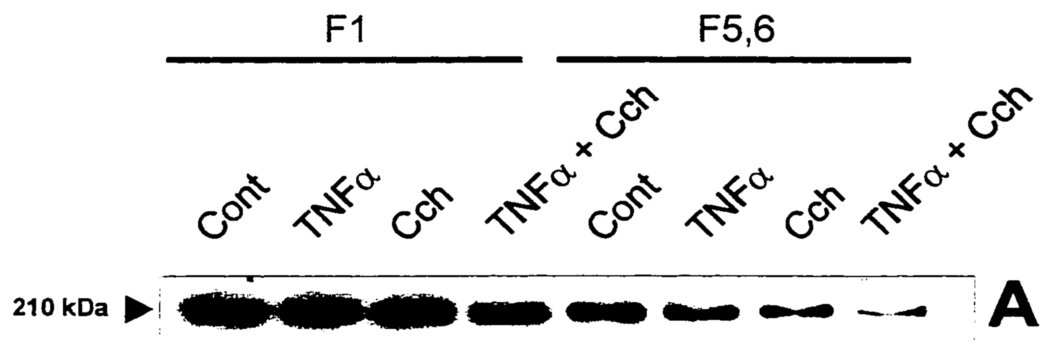
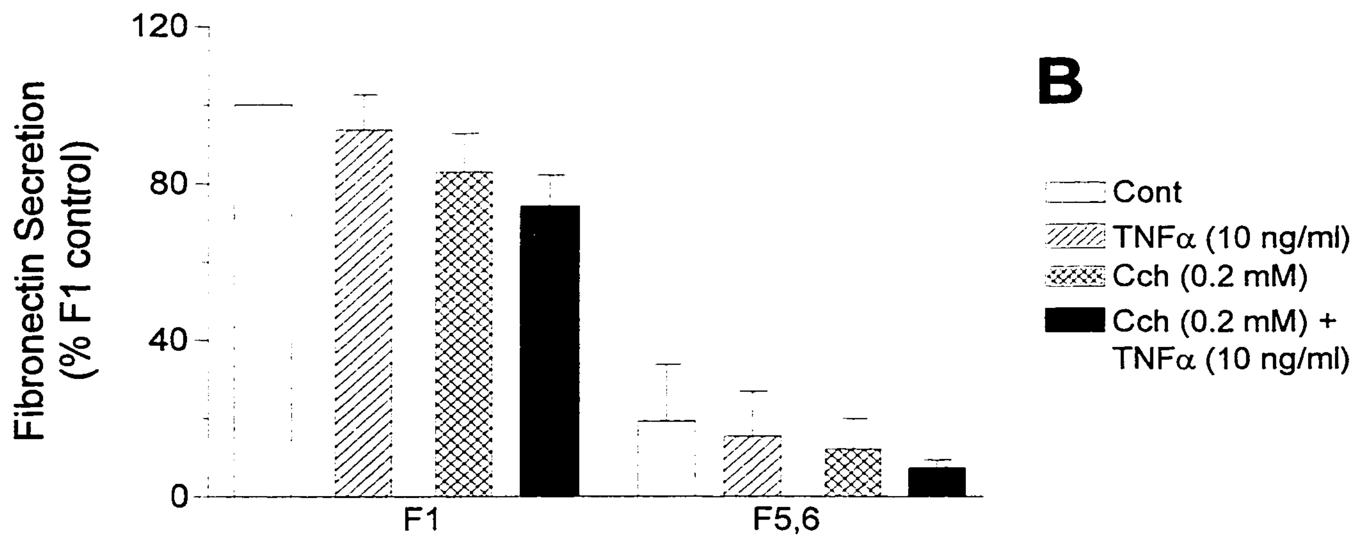

Fibronectin Content
(140 kDa; % F1 control)
150
100
50
0
C
F1
F5,6

Figure 39: Fibronectin secretion by hen granulosa cells during follicular development in the presence or absence of TNFα and/or Cch (n = 3). F5,6 and F1 granulosa cells were cultured for 24 hrs in the presence or absence of TNFα (10 ng/ml) and/or Cch (0.2 mM). Medium was collected and centrifuged to remove floating cells. Samples (30 μl/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human fibronectin IgG, washed, incubated (30 min) in goat anti-mouse IgG and visualized by ECL. A representative blot is depicted in Panel A. Densitometric analysis of the fibronectin (B) band was performed using Molecular Analyst software (Bio-Rad Laboratories, CA). Three way ANOVA revealed significant differences ($p < 0.001$) between F5,6 and F1 granulosa cell fibronectin secretion. In addition, fibronectin secretion was decreased by the presence of Cch ($p < 0.05$), although changes due to TNFα were not observed ($p > 0.05$).



F1
F5,6
Cont
TNFα
Cch
TNFα + Cch
Cont
TNFα
Cch
TNFα + Cch
210 kDa
A

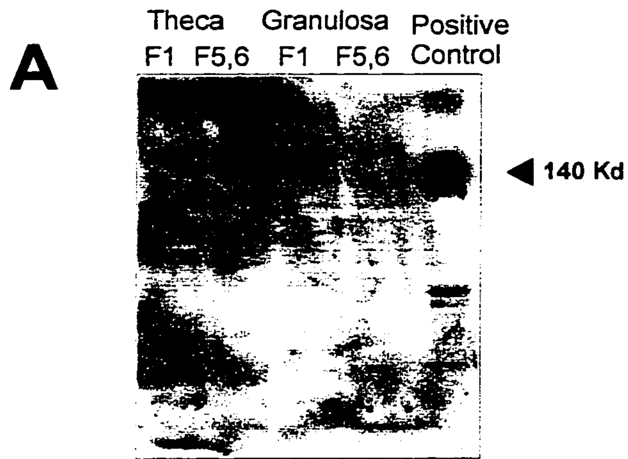


Fibronectin Secretion (% F1 control)
120
80
40
0
F1
F5,6
B
Cont
TNFα (10 ng/ml)
Cch (0.2 mM)
Cch (0.2 mM) + TNFα (10 ng/ml)

band ($p < 0.001$) were present in higher abundance in F1 than F5,6 cells (Fig 38, 39). Although TNFα had no apparent influence on either cellular or secreted fibronectin (irrespective of the presence or absence of Cch), incubation with Cch decreased fibronectin secretion ($p < 0.05$) without affecting total cellular fibronectin content ($p > 0.05$).

Studies of integrin β1, demonstrated poor antibody specificity, as numerous bands were observed in hen ovarian extracts, despite a clear band of approximately 140 kDa in human A431 cells (positive control; Fig 40A). This lack of specificity was confirmed by a Western blot against chicken proteins from several non-ovarian tissues (Fig 40B). In contrast, Western blots against integrin β3 revealed a band of the expected size (90 kDa) along with two bands of 35 and 40 kDa, presumably representing degraded products (Fig 41). Considerable variability was observed in the relative intensities of the 90 kDa band between F5,6 and F1 cells, although a trend of higher integrin β3 levels in F5,6 cells than F1 cells was observed although this did not reach significance ($p = 0.16$; Fig 41). Neither TNFα nor Cch, alone or in combination, affected the cellular content of integrin β3 ($p > 0.05$; Fig 41).

Figure 40: Integrin β1 expression in hen tissues. Tissues were removed from the hen and homogenized using a tissue grinder, filtered and lysed. After protein quantitation, samples (50 μg protein/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human integrin β1 IgG, washed, incubated (30 min) in goat anti-mouse IgG and visualized by ECL. Panel A depicts theca and granulosa tissue extracts from F1 and F5,6 follicles, while Panel B depicts extracts from non-ovarian tissue.


A
Theca
Granulosa
Positive
F1
F5,6
F1
F5,6
Control
140 Kd

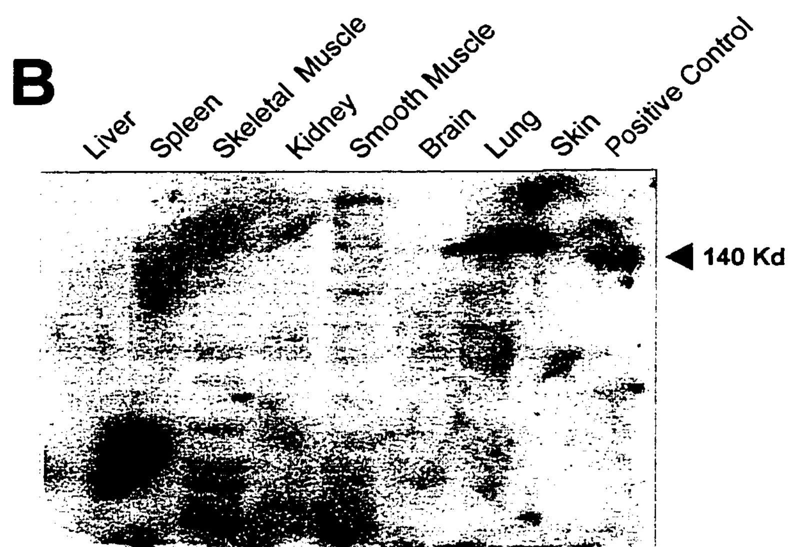
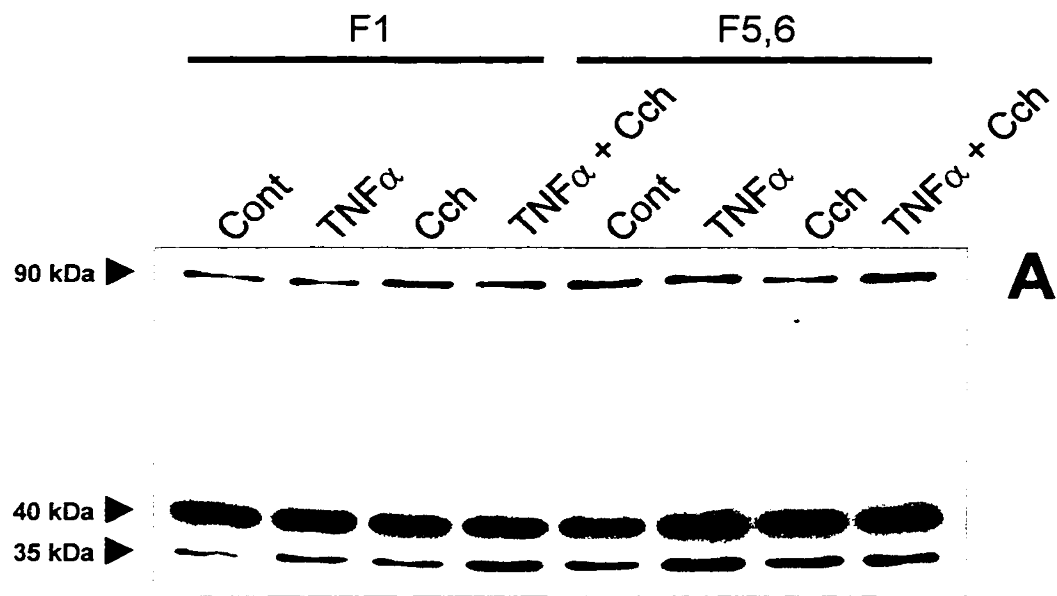
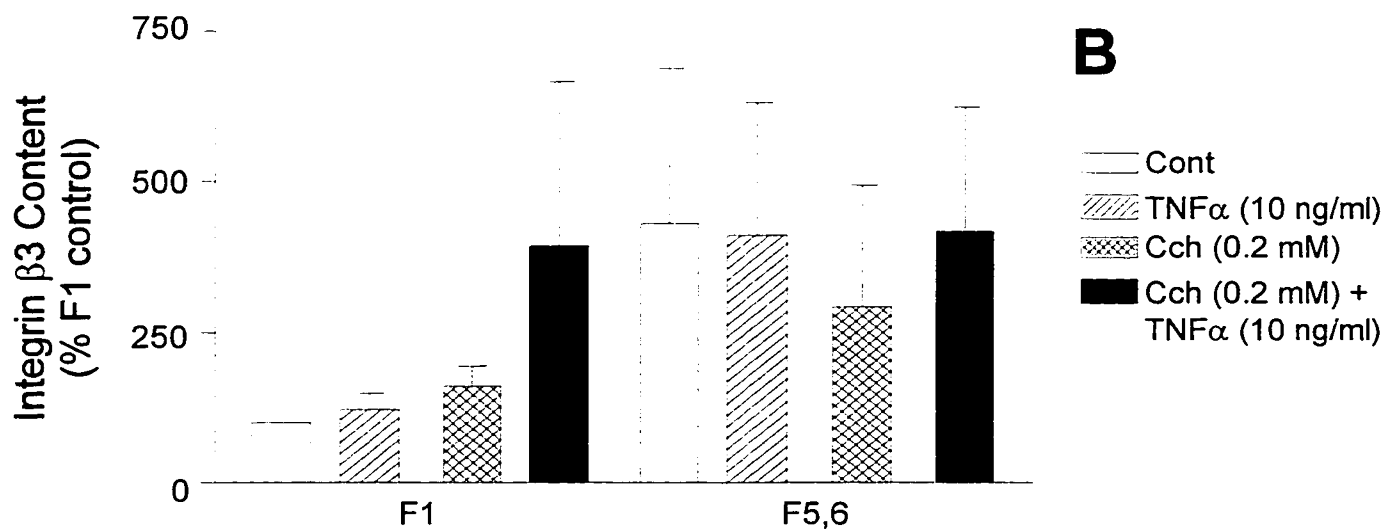

B
Liver
Spleen
Skeletal Muscle
Kidney
Smooth Muscle
Brain
Lung
Skin
Positive Control
140 Kd

Figure 41: Integrin β3 content in hen granulosa cells during follicular development in the presence or absence of TNFα and/or Cch (n = 3). F5,6 and F1 granulosa cells were cultured for 24 hrs in the presence or absence of TNFα (10 ng/ml) and/or Cch (0.2 mM). Floating and attached cells were combined and lysed. After protein quantitation, samples (10 μg protein/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human integrin β3 IgM, washed, incubated (30 min) in goat anti-mouse IgM and visualized by ECL. A representative blot is depicted in Panel A. Densitometric analysis of the 90 kDa band (B) was performed using Molecular Analyst software (Bio-Rad Laboratories, CA). Three way ANOVA demonstrated no significant differences between F5,6 and F1 granulosa cells, irrespective of the presence or absence of TNFα and/or Cch.



F1
F5,6
Cont
TNFα
Cch
TNFα + Cch
Cont
TNFα
Cch
TNFα + Cch
90 kDa
40 kDa
35 kDa
A



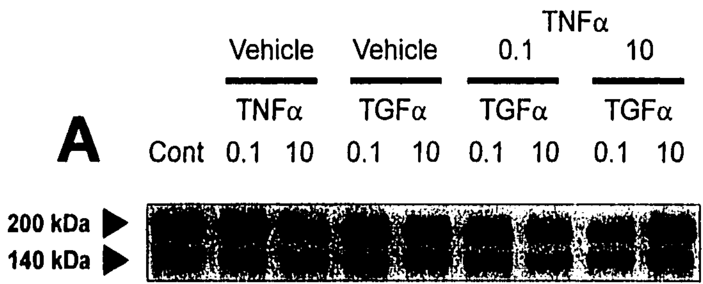
B
Integrin β3 Content
(% F1 control)
750
500
250
0
F1
F5,6
Cont
TNFα (10 ng/ml)
Cch (0.2 mM)
Cch (0.2 mM) +
TNFα (10 ng/ml)

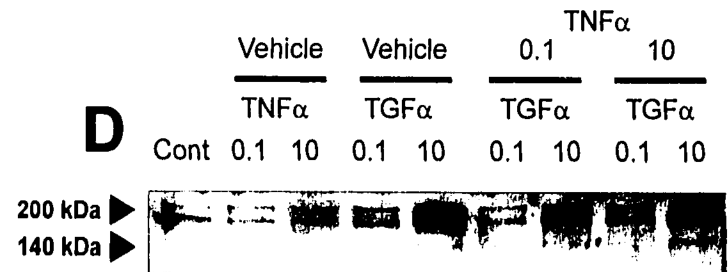
3. Do TNFα and TGFα interact in the regulation of integrin production? Is fibronectin production regulated by TNFα-TGFα interactions?

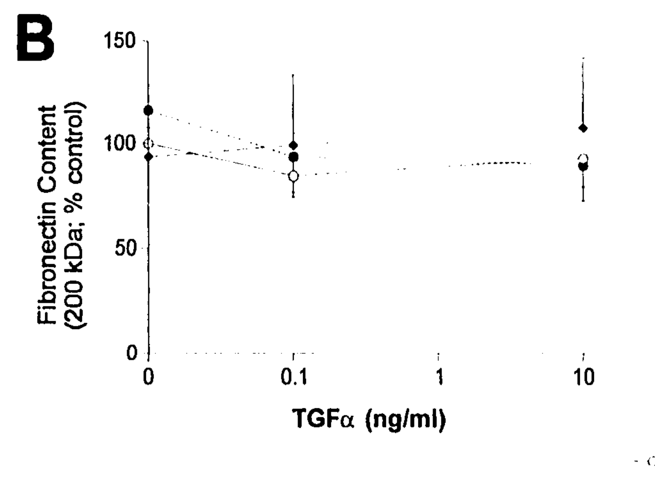
The interactions between TNFα andTGFα resulting in increased survival in culture were cell density-dependent (see Fig 14). TGFα has been previously shown to increase fibronectin production in hen granulosa cells (Asem and Novero, 1994). Moreover, it has been shown that integrin production is cell density-dependent (Stanley *et al*, 1995). Since a relationship between extracellular matrix-integrin signals and cell survival has been well established in several systems (see Howe *et al*, 1998; Giancotti, 1997), TNFα-induced integrin production may have a role as a mediator of the observed survival response.

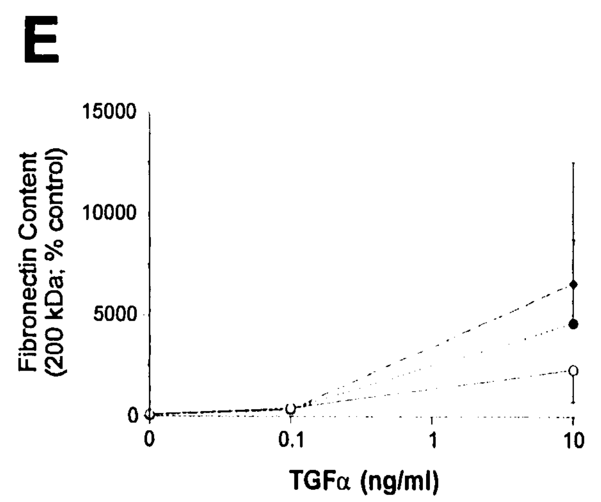
The production and secretion of fibronectin was studied in the presence of TNFα (0.1 or 10 ng/ml) and TGFα (0.1 or 10 ng/ml) in cells cultured under confluent conditions to determine if concentration-dependent interactions between these factors could be detected. Incubation with TGFα significantly increased the production of both the 200 and 140 kDa cellular bands [Fig 42, $p < 0.001$ (after log transformation) and $p < 0.01$ (with or without log transformation), respectively] as well as the secreted band (Fig 43; $p < 0.05$) in F5,6 but not F1 cells, with significant differences observed in the presence of either 0.1 (200 kDa band only) or 10 ng/ml (either band) concentrations of the growth factor ($p < 0.05$). Significant interactions between TNFα and TGFα were not detected, although a notable trend of higher fibronectin content for the 140 kDa band due to TNFα in the presence of TGFα was

Figure 42: Cellular fibronectin content in hen granulosa cells during follicular development in the presence or absence of TNFα and/or TGFα (n = 3). F5,6 and F1 granulosa cells were cultured for 24 hrs in the presence or absence of TNFα (0.1 or 10 ng/ml) and/or TGFα (0.1 or 10 ng/ml). Floating and attached cells were combined and lysed. After protein quantitation, samples (10 μg protein/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human fibronectin IgG, washed, incubated (30 min) in goat anti-mouse IgG and visualized by ECL. Representative blots of F1 and F5,6 extracts are depicted in panels A and D, respectively. Densitometric analysis of the upper (F1 and F5,6; Panels B and E, respectively) and lower (F1 and F5,6; Panels C and F, respectively) bands were performed using Molecular Analyst software (Bio-Rad Laboratories, CA). Two way ANOVA revealed significant dose-dependent differences due to TGFα in F5,6, but not F1 granulosa cells for both the 200 ($p < 0.001$; after log transformation) and 140 kDa ($p < 0.01$; with or without log transformation) fibronectin bands with significant differences observed in the presence of either 0.1 (200 kDa band only) or 10 ng/ml (either band) concentrations of the growth factor ($p < 0.05$). Significant differences due to TNFα were not detected.

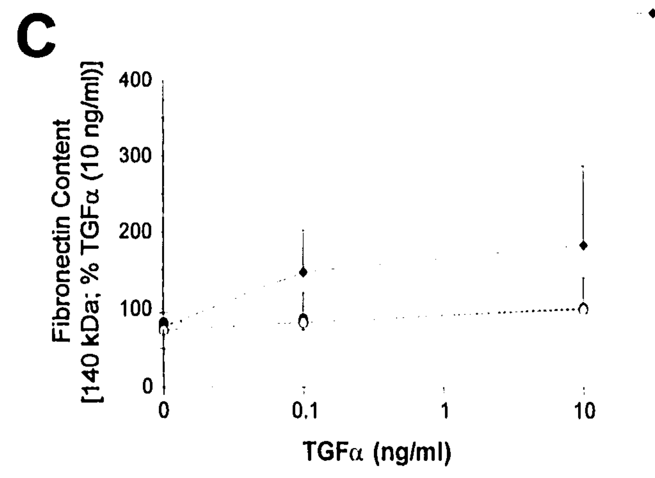








Cont
TNFα (0.1 ng/ml)
TNFα (10 ng/ml)



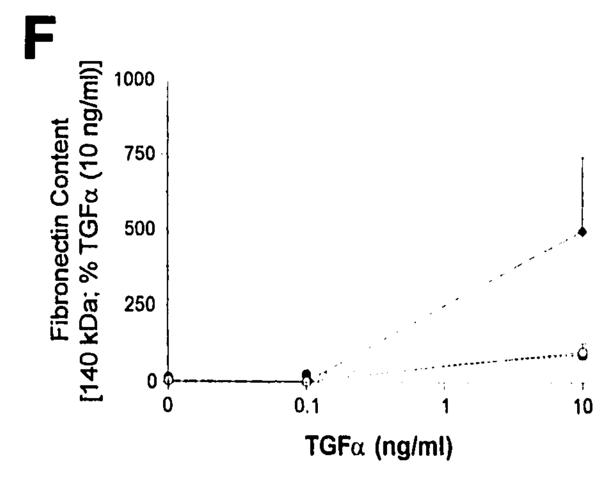
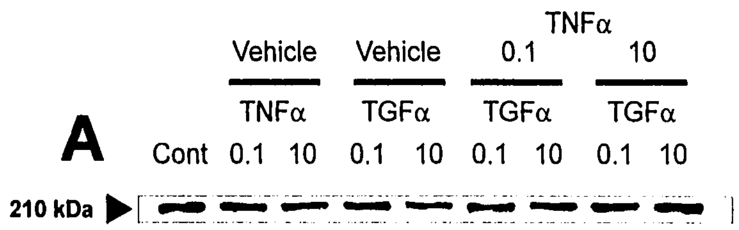
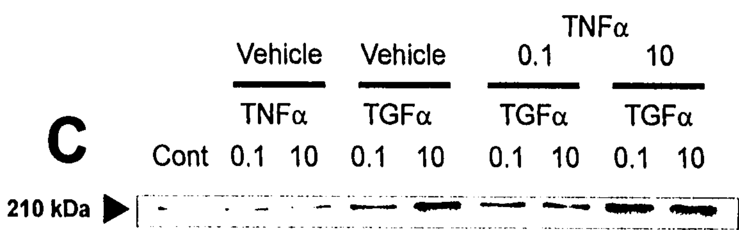
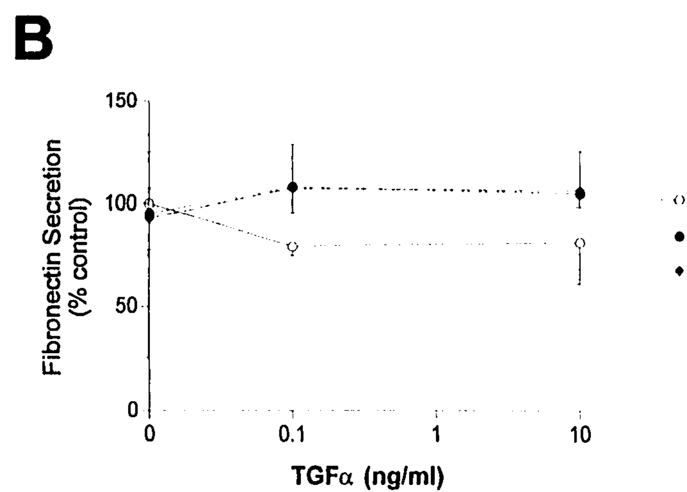
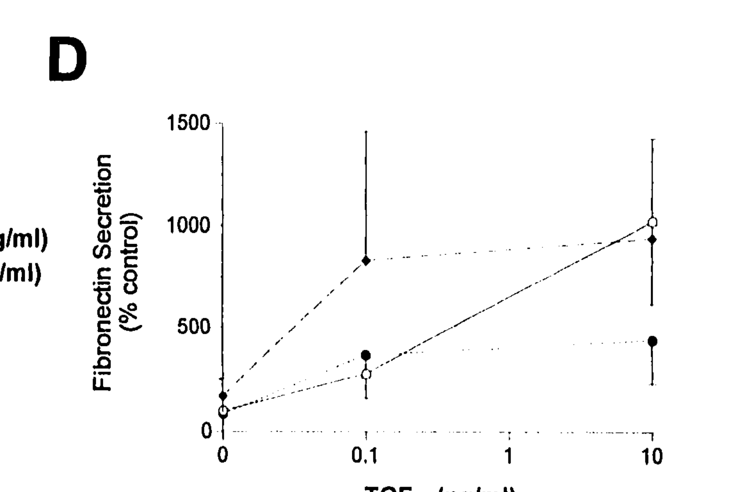


Figure 43: Fibronectin secretion by hen granulosa cells during follicular development in the presence or absence of TNFα and/or TGFα (n = 3). F5,6 and F1 granulosa cells were cultured for 24 hrs in the presence or absence of TNFα (0.1 or 10 ng/ml) and/or TGFα (0.1 or 10 ng/ml). Medium was collected and centrifuged to remove floating cells. Samples (30 μl/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human fibronectin IgG, washed, incubated (30 min) in goat anti-mouse IgG and visualized by ECL. Representative blots of F1 and F5,6 extracts are depicted in panels A and C, respectively. Densitometric analysis of the fibronectin band (F1 and F5,6; Panels B and D, respectively) was performed using Molecular Analyst software (Bio-Rad Laboratories, CA). Two way ANOVA demonstrated that TGFα induced a dose-dependent increase ($p < 0.05$) in fibronectin secretion by F5,6 but not F1 granulosa cells,with significant differences observed in the presence of 10 ng/ml concentrations of the growth factor ($p < 0.05$). Significant differences due to TNFα were not detected.


A
Vehicle
Vehicle
TNFα
0.1
10
TNFα
TGFα
TGFα
TGFα
Cont 0.1 10 0.1 10 0.1 10 0.1 10
210 kDa


C
Vehicle
Vehicle
TNFα
0.1
10
TNFα
TGFα
TGFα
TGFα
Cont 0.1 10 0.1 10 0.1 10 0.1 10
210 kDa


B
Fibronectin Secretion (% control)
150
100
50
0
0
0.1
1
10
TGFα (ng/ml)
Cont
TNFα (0.1 ng/ml)
TNFα (10 ng/ml)


D
Fibronectin Secretion (% control)
1500
1000
500
0
0
0.1
1
10
TGFα (ng/ml)

observed, although this did not reach significance ($p = 0.07$). Significant differences in the secretion of fibronectin due to TNFα were not detected ($p > 0.05$).

The production of integrin β3 was studied in the presence of TNFα (0.1 or 10 ng/ml) and TGFα (0.1 or 10 ng/ml) in cells cultured under confluent conditions to determine if concentration-dependent interactions between these factors could be detected. Integrin β3 expression was significantly increased by TGFα in F5,6 cells ($p < 0.02$), but not F1 cells ($p > 0.05$; Fig 44) with significant differences observed in response to 10 ng/ml concentrations of the growth factor ($p < 0.05$). Moreover, significant interactions between TNFα (10 ng/ml)and TGFα (10 ng/ml) were detected in F5,6 cells only ($p < 0.01$), but only in the presence of 10 ng/ml concentrations of either factor ($p < 0.05$). Consequently, interaction between TNFα and TGFα for integrin β3 production was found to require maximally stimulatory concentrations of both factors within the scope of the current study.

Figure 44: Integrin β3 content in hen granulosa cells during follicular development in the presence or absence of TNFα and/or TGFα ($n = 3$). F5,6 and F1 granulosa cells were cultured for 24 hrs in the presence or absence of TNFα (0.1 or 10 ng/ml) and/or TGFα (0.1 or 10 ng/ml). Floating and attached cells were combined and lysed. After quantitation, samples (10 μg/lane) were resolved by 8% SDS-PAGE and electroblotted on to nitrocellulose paper. Blots were blocked (1 hr) in 5% skim milk, incubated (1 hr) in mouse anti-human integrin β3 IgM, washed, incubated (30 min) in goat anti-mouse IgM and visualized by ECL. Representative blots of F1 and F5,6 extracts are depicted in panels A and C, respectively. Densitometric analysis of the 90 kDa band in F1 (B) and F5,6 (D) extracts were performed using Molecular Analyst software (Bio-Rad Laboratories, CA). Analysis by two way ANOVA revealed no differences due to TNFα or TGFα, alone or in combination in F1 cells. In F5,6 cells, however, TGFα significantly increased integrin β3 production ($p < 0.02$) with significant differences due to 10 ng/ml concentrations of the growth factor. Moreover, significant interactions between TNFα and TGFα were observed ($p < 0.01$) with significant differences due to 10 ng/ml concentrations for either factor ($p < 0.05$).

A

TNFα

Vehicle | Vehicle | 0.1 | 10

TNFα | TGFα | TGFα | TGFα

Cont 0.1 10 0.1 10 0.1 10 0.1 10

90 kDa

40 kDa

35 kDa

C

TNFα

Vehicle | Vehicle | 0.1 | 10

TNFα | TGFα | TGFα | TGFα

Cont 0.1 10 0.1 10 0.1 10 0.1 10

90 kDa

40 kDa

35 kDa

B

Integrin β3 Content (% control)

1500
1000
500
0

0 0.1 1 10

TGFα (ng/ml)

Cont

TNFα (0.1 ng/ml)

TNFα (10 ng/ml)

D

Integrin β3 Content (% control)

1500
1000
500
0

0 0.1 1 10

TGFα (ng/ml)

*

F. Role of SMase in the regulation of granulosa cell function.

1. Do SM metabolites increase basal $[Ca^{2+}]_i$? Can they enhance Cch-induced Ca^{2+} transients?

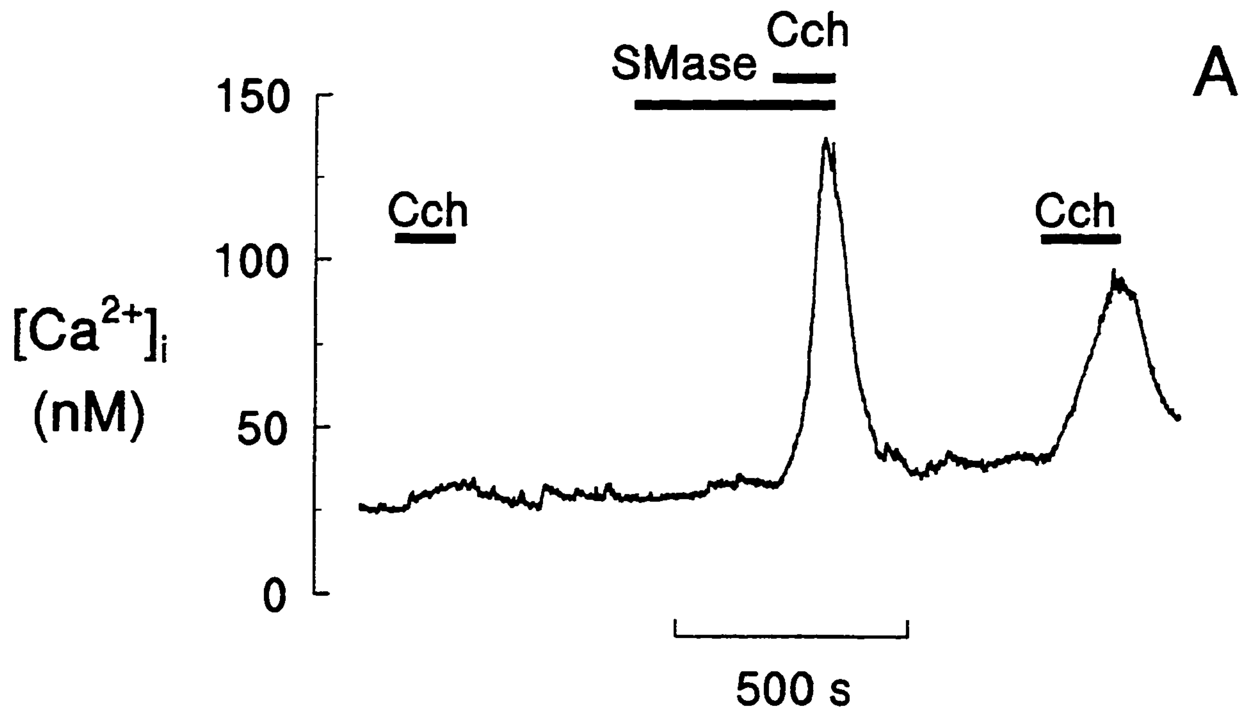
a. Does exogenous SMase mimic TNFα-induced changes in basal or Cch-induced $[Ca^{2+}]_i$?

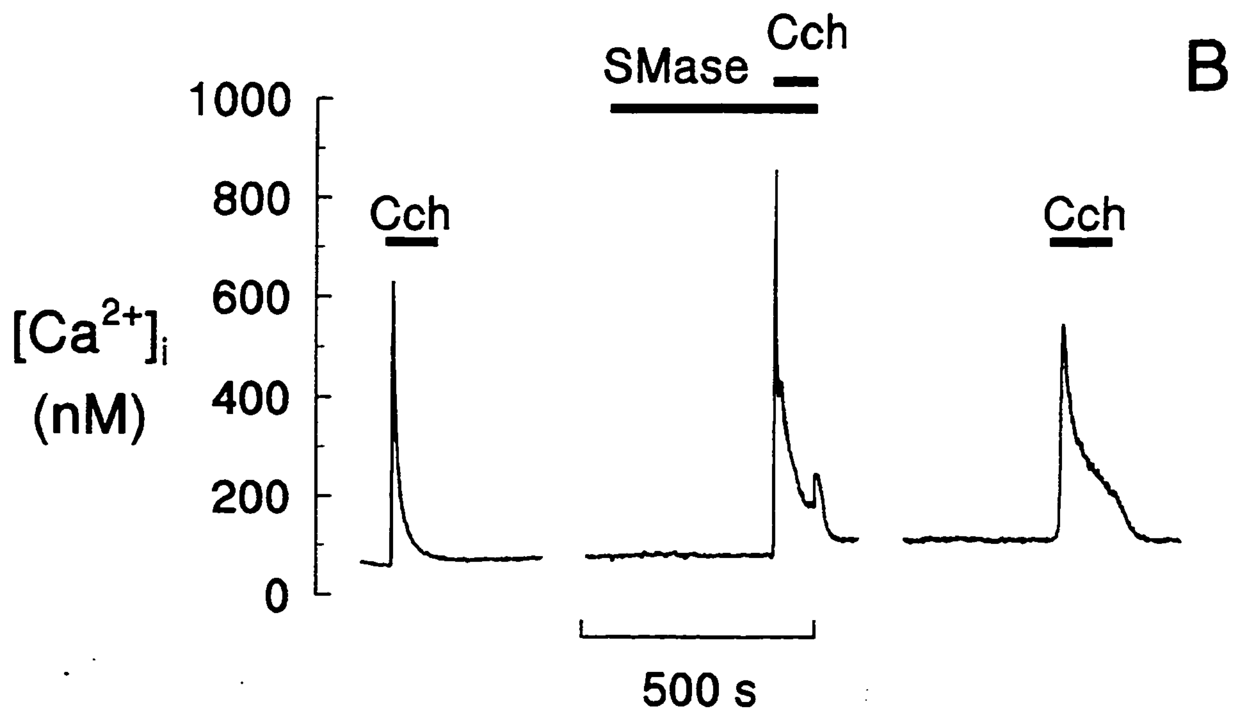
The primary action of SMase is to degrade sphingomyelin to produce ceramide.The typical effects of exogenous SMase treatment on $[Ca^{2+}]_i$ in granulosa cells are depicted in Figure 45. While exogenous SMase had no significant effect on basal $[Ca^{2+}]_i$ (SMase-induced Ca^{2+} transients were observed in only 1 of 24 cells), Cch-induced Ca^{2+} transients in the presence of SMase were larger than those observed in the absence of this enzyme in both F1 and F5,6 cells (11 of 24 cells studied). Unlike TNFα, which only increased the magnitude of small Cch-induced Ca^{2+} transients, SMase appeared able to increase the magnitude of both large (>250 nM) and small (<250 nM) Cch-induced Ca^{2+} transients.

b. Can ceramide analogues increase basal and Cch-induced $[Ca^{2+}]_i$? Does sphingosine regulate $[Ca^{2+}]_i$?

Since ceramide itself is too hydrophobic to be delivered to cells in aqueous solution, the short chain ceramide analogues C2-ceramide (3 μM) and C8-ceramide (3 μM) were used

Figure 45: The effect of exogenous SMase on basal and Cch-induced Ca^{2+} transients in hen granulosa cells. **Panel A:** Influence of SMase on slow Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ less than 250 nM; n = 22). **Panel B:** Influence of SMase on fast Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ greater than 250 nM; n = 11). Ca^{2+} transients illustrated within each panel were recorded from the same cell separated by a minimum of 4-min washout. Exposure of cells to 50 mU/ml SMase and 0.2 mM Cch are indicated by their respective horizontal bars.


SMase
Cch
A
150
100
50
0
Cch
Cch
[Ca2+]i
(nM)
500 s


SMase
Cch
B
1000
800
600
400
200
0
Cch
Cch
[Ca2+]i
(nM)
500 s

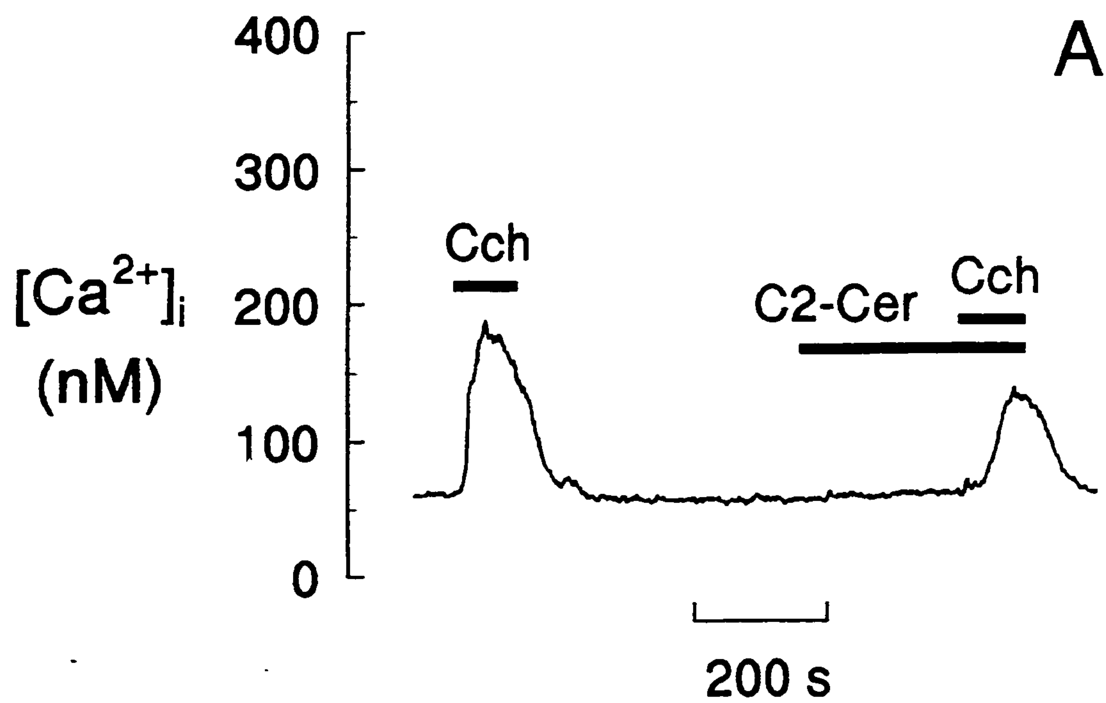
to determine if ceramide could mimic the action of TNFα on $[Ca^{2+}]_i$. While C2-ceramide had no apparent effect on basal or Cch-induced $[Ca^{2+}]_i$ (Fig 46A; n = 12), pretreatment with C8-ceramide enhanced Cch-induced Ca^{2+} transients in a manner similar to exogenous SMase, although changes in basal $[Ca^{2+}]_i$ were not observed (Fig 46B; n = 7). The inability of C2-ceramide to mimic TNFα notwithstanding, the C8-ceramide-Cch interaction implies that Cer production via SMase activation could mediate the TNFα-Cch interaction.

Sphingosine, a product of ceramide degradation, has been shown to increase $[Ca^{2+}]_i$ in several different systems (Ghosh *et al*, 1990; Zhang *et al*, 1991). In F1 and F5,6 granulosa cells, sphingosine (3 μM) induced large increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i = 343 \pm 83$ nM; n = 9) with a relatively slow rate of rise ($d[Ca^{2+}]_i/dt = 7.83 \pm 2.58$ nM/s; n = 9) and a characteristic delay (Fig 47). Although the magnitude of sphingosine-induced Ca^{2+} transients was much greater than observed in response to TNFα (Fig 31), $[Ca^{2+}]_i$ increased at a similar rate in response to both factors. This supports the concept that sphingosine, if produced locally at appropriate concentrations, could mediate TNFα-induced Ca^{2+} transients.

2. Do sphingomyelin metabolites regulate progesterone secretion?

Since exogenous SMase inhibited LH-induced progesterone production (Fig 36), the abilities of ceramides of different acyl chain length (C2-, C6- and C8-ceramide) and sphingosine to inhibit LH (10 ng/ml)-induced progesterone production were also assessed

Figure 46: The effect of ceramide analogues on basal and Cch-induced Ca^{2+} transients in hen granulosa cells. **Panel A:** Influence of C2-ceramide on slow Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ less than 250 nM; n = 12). **Panel B:** Influence of C8-ceramide on slow Cch-induced Ca^{2+} transients (changes in $[Ca^{2+}]_i$ less than 250 nM; n = 7). Ca^{2+} transients illustrated within each panel were recorded from the same cell separated by a minimum of 4-min washout. Exposure of cells to 3 μM ceramide and 0.2 mM Cch are indicated by their respective horizontal bars.


A
400
300
200
100
0
[Ca2+]i
(nM)
Cch
C2-Cer
Cch
200 s

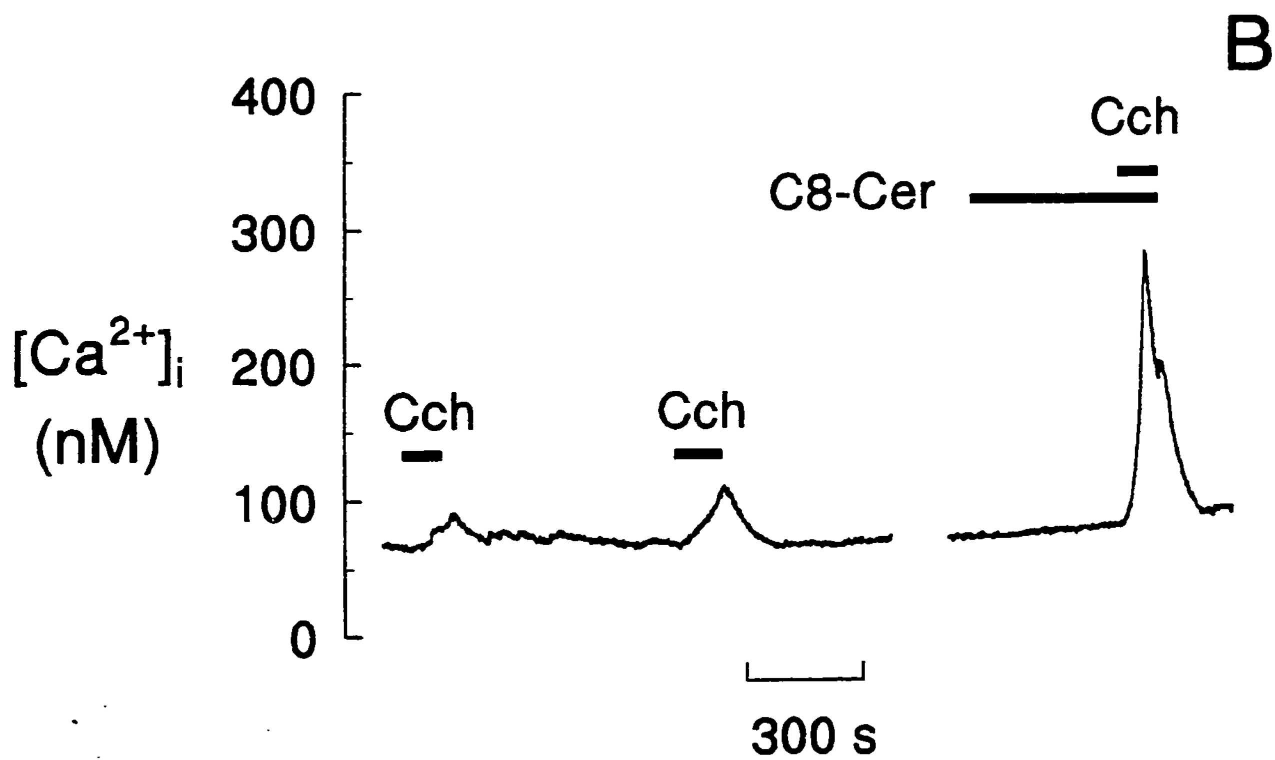
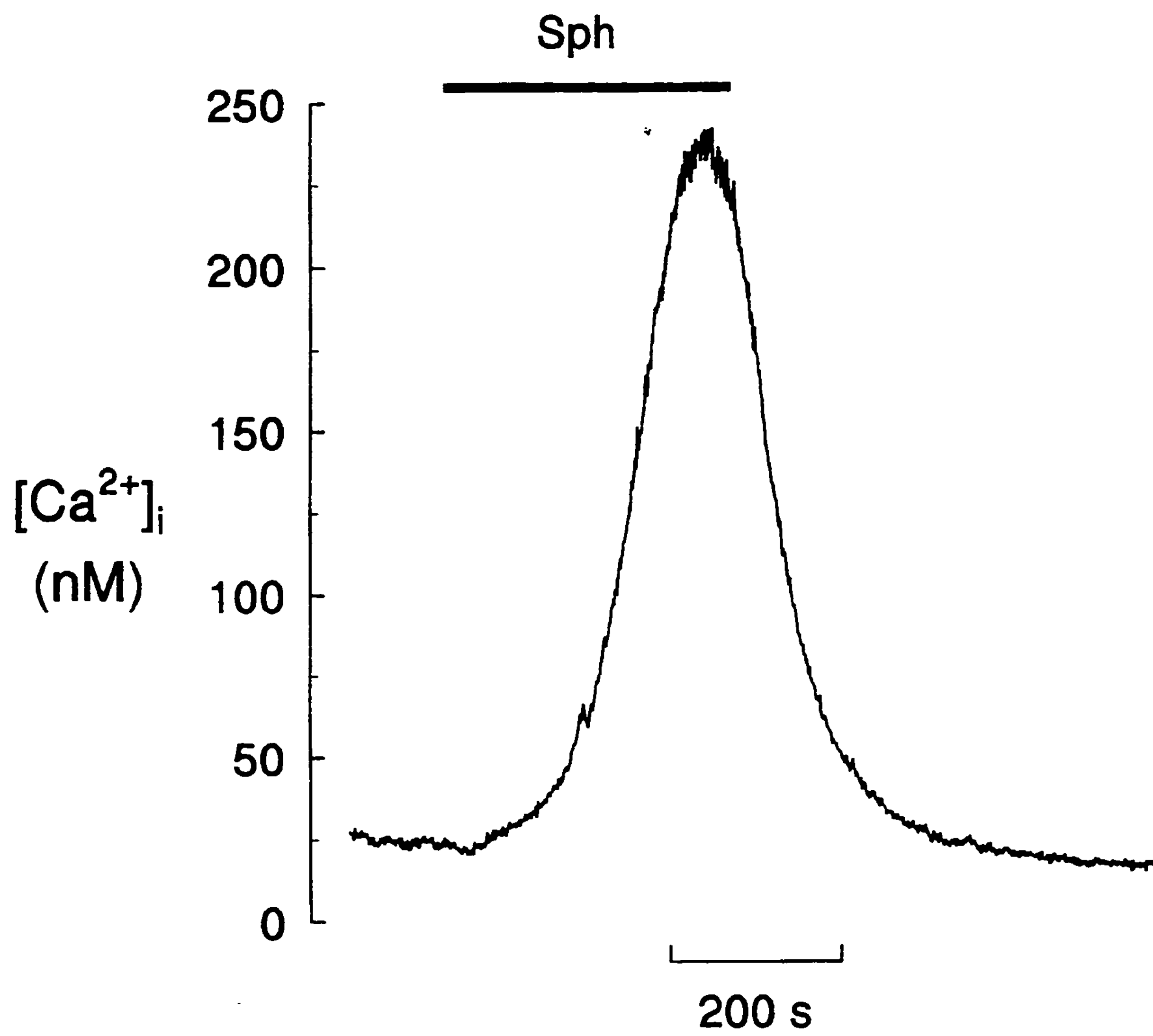

B
400
300
200
100
0
[Ca2+]i
(nM)
Cch
Cch
C8-Cer
Cch
300 s

Figure 47: A representative tracing depicting the effect of sphingosine on $[Ca^{2+}]_i$ in hen granulosa cells. Sphingosine (Sph; 3 μM) induced an approximately 343 ± 83 nM increase in $[Ca^{2+}]_i$ at a rate of 7.83 ± 2.58 nM/s in both F1 and F5,6 granulosa cells (n = 9). Exposure of cells to 3 μM sphingosine is indicated by the horizontal bar.

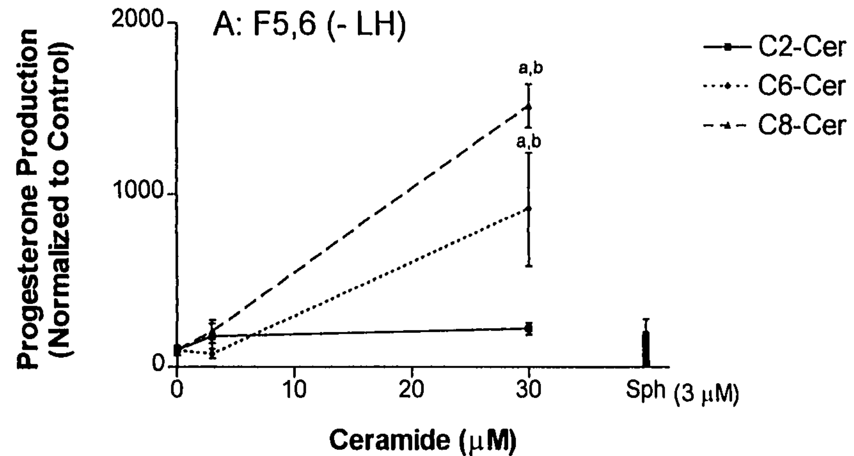


Sph
250
200
150
100
50
0
[Ca2+]i
(nM)
200 s

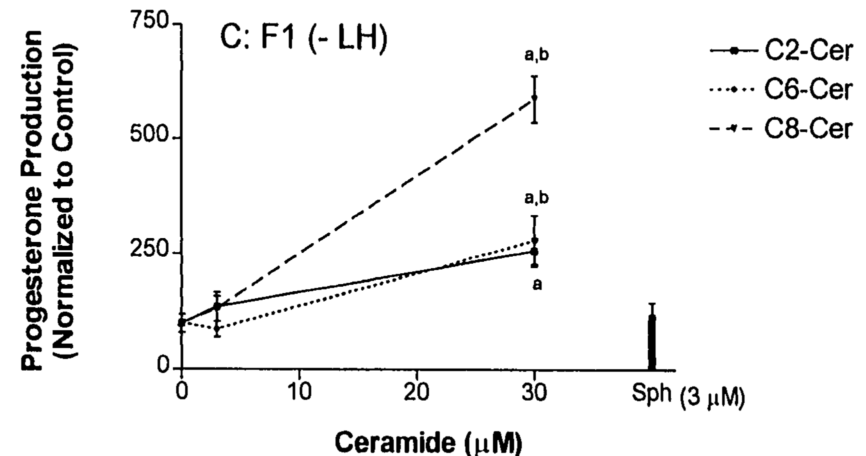
to confirm that the effects of SMase were mediated via ceramide and not through non-specific actions of the enzyme (Fig 48). At concentrations of 3 μM, neither ceramide nor sphingosine influenced granulosa cell progesterone secretion irrespective of the presence of LH (10 ng/ml) or the stage of follicular development. At a higher concentration (30 μM), however, C2-ceramide inhibited LH (10 ng/ml)-induced progesterone production in F5,6, but not F1 cells. Moreover, exposure of either F1 or F5,6 granulosa cells to 30 μM of C6- or C8-ceramide resulted in increases in basal progesterone production in a manner similar to LH (Fig 48). Furthermore, C8-ceramide enhanced LH-induced progesterone production in F5,6, but not F1 cells, while C6-ceramide had no effect on LH-induced steoidogenesis. Sphingosine at 30 μM was not studied as this concentration was cytotoxic, resulting in immediate cell death (data not shown).

Since C8-ceramide interacted with Cch in the regulation of $[Ca^{2+}]_i$, Cch-induced progesterone secretion was also assessed in the presence of these sphingomyelin metabolites (sphingosine, C2-ceramide, C6-ceramide and C8-ceramide; Fig 49). Progesterone secretion was not affected by Cch, indicating that the Cch-ceramide interactions were not related to steroidogenesis.

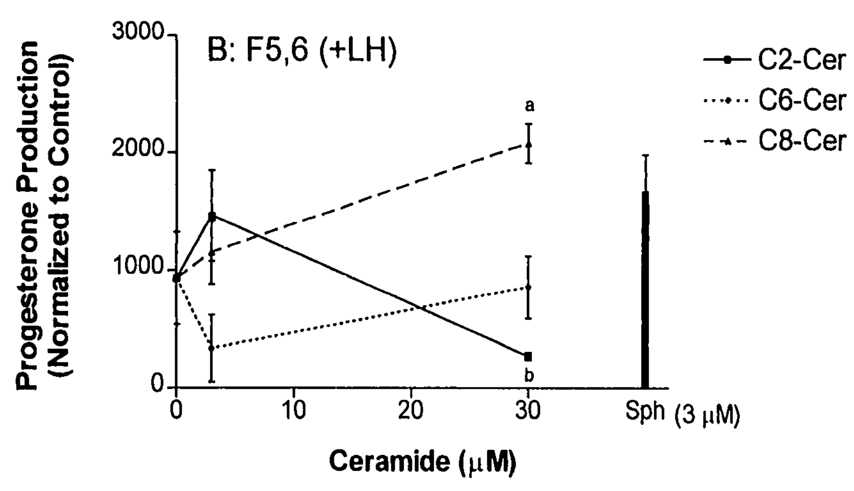
Figure 48: The effect of ceramide analogues and sphingosine on basal and LH-induced progesterone production in hen granulosa cells during follicular development. Progesterone levels in spent medium from F1 or F5,6 cells (2.5 x 10^5) cultured 24 hours in the presence of vehicle, C2-ceramide (C2-Cer; 3 or 30 μM), C6-ceramide (C6-Cer; 3 or 30 μM), C8-ceramide (C8-Cer; 3 or 30 μM) or sphingosine (Sph; 3 μM) were assessed by RIA. Basal (Panels A and C) and LH-induced (10 ng/ml; Panels B and D) progesterone production in F5,6 (Panels A and B) and F1 (Panels C and D) granulosa cells ($n = 3$). Data was analyzed by two way ANOVA. Significant interactions for the ceramide analogues in the absence of LH were observed in both F1 and F5,6 granulosa cells, indicating that ceramide-induced progesterone secretion was concentration-dependent. Significant interactions between LH and C2-ceramide ($p < 0.05$) and C8-ceramide ($p < 0.05$) were observed in F5,6, but not F1 granulosa cell secretions. "a" represents significant difference ($p < 0.05$) from control, while "b" represents significant difference ($p < 0.05$) from the corresponding 3 μM ceramide concentration, as determined by Tukey's test.



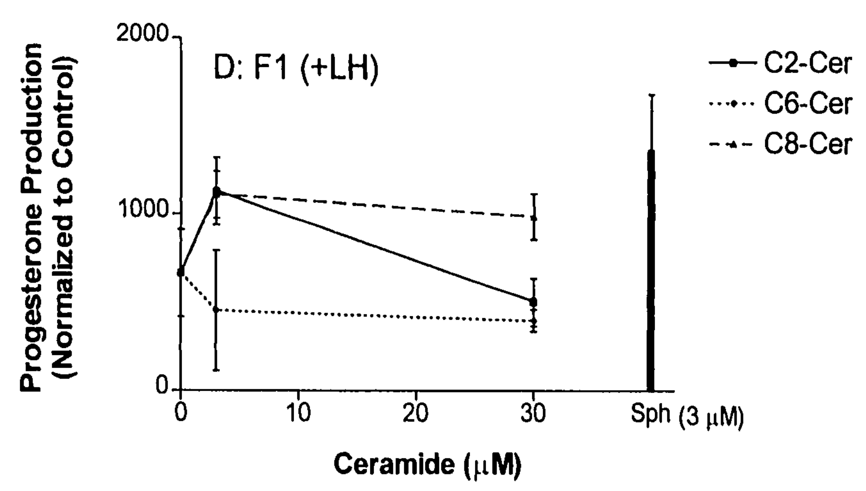
A: F5,6 (- LH)
Progesterone Production (Normalized to Control)
2000
1000
0
0
10
20
30
Sph (3 μM)
Ceramide (μM)
a,b
a,b
C2-Cer
C6-Cer
C8-Cer



C: F1 (- LH)
Progesterone Production (Normalized to Control)
750
500
250
0
0
10
20
30
Sph (3 μM)
Ceramide (μM)
a,b
a,b
a
C2-Cer
C6-Cer
C8-Cer

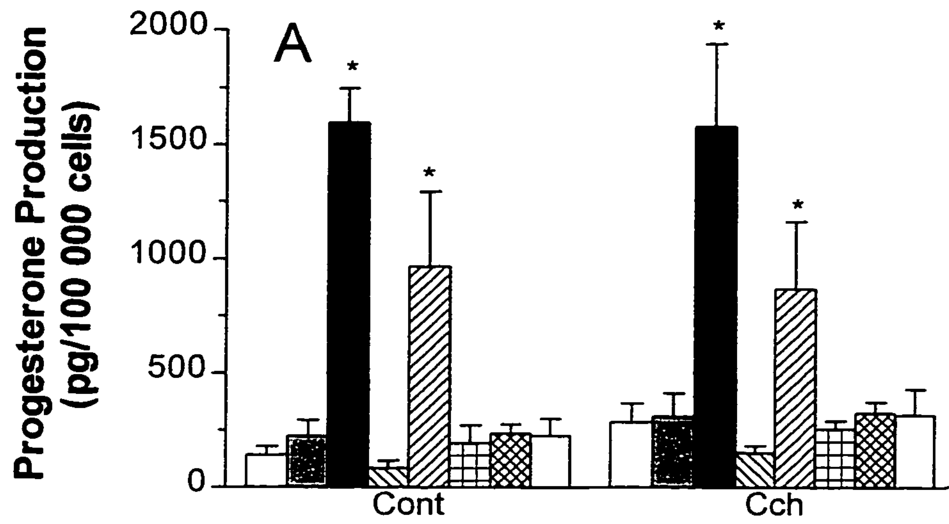


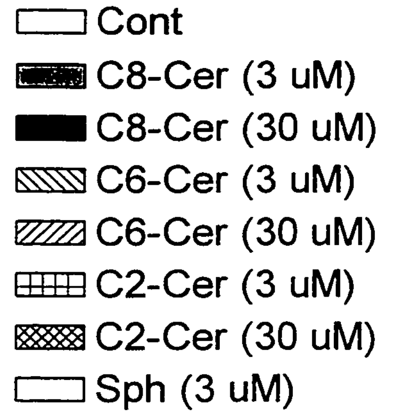
B: F5,6 (+LH)
Progesterone Production (Normalized to Control)
3000
2000
1000
0
0
10
20
30
Sph (3 μM)
Ceramide (μM)
a
b
C2-Cer
C6-Cer
C8-Cer

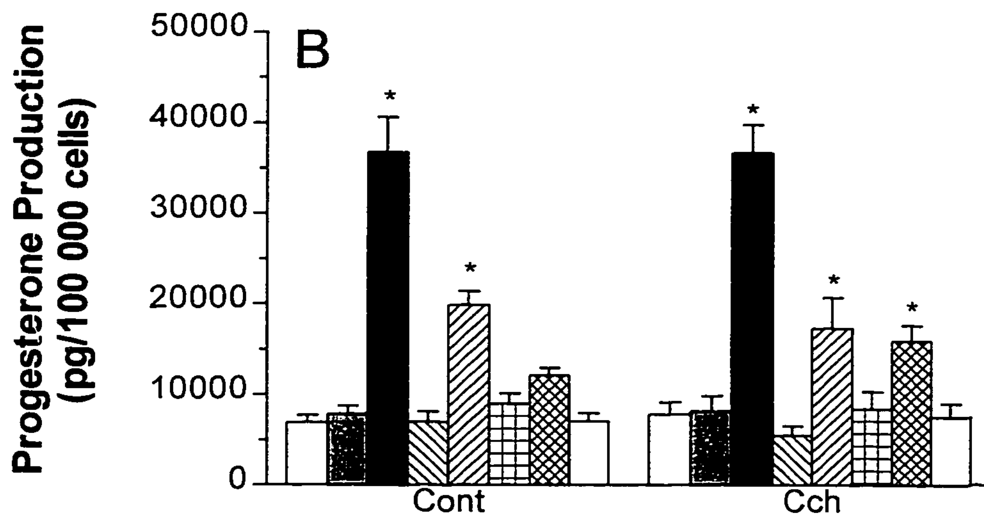


D: F1 (+LH)
Progesterone Production (Normalized to Control)
2000
1000
0
0
10
20
30
Sph (3 μM)
Ceramide (μM)
C2-Cer
C6-Cer
C8-Cer

Figure 49: The effect of Cch on progesterone production in the presence and absence of ceramide analogues or sphingosine (n = 3). Progesterone levels in spent medium from F1 or F5,6 cells (2.5 x 10^5) cultured 24 hours in the presence of vehicle, C2-ceramide (C2-Cer; 3 or 30 μM), C6-ceramide (C6-Cer; 3 or 30 μM), C8-ceramide (C8-Cer; 3 or 30 μM), sphingosine (Sph; 3 μM) and or Cch (0.2 mM) were assessed by RIA. Data was analyzed by two way ANOVA. Progesterone secretion in the presence of either C8-ceramide (30 μM; $p < 0.01$) or C6-ceramide (30 μM; $p < 0.01$) was significantly higher than all other treatments in both F1 and F5,6 follicles. C2-Cer (30 μM; $p < 0.05$) increased progesterone secretion in the presence of Cch in F1 granulosa cells only although no overall effect of Cch was observed. * represents significant difference from control, as determined by Tukey's test.


A
Progesterone Production
(pg/100 000 cells)
2000
1500
1000
500
0
Cont
Cch
*

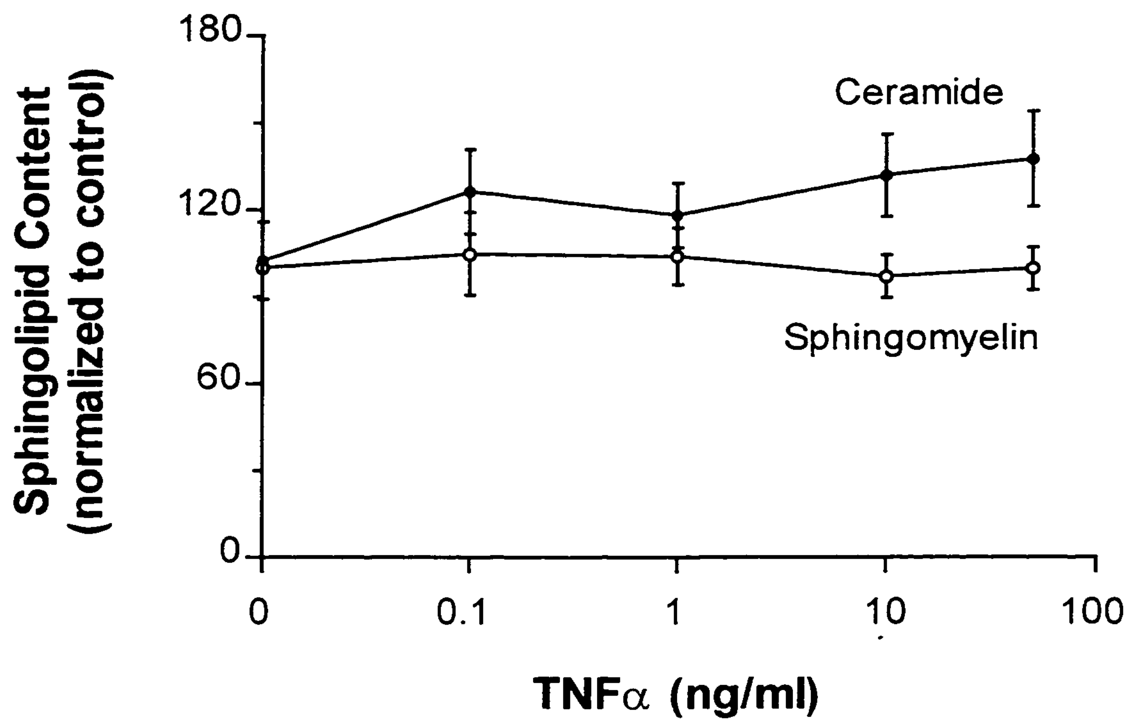

Cont
C8-Cer (3 uM)
C8-Cer (30 uM)
C6-Cer (3 uM)
C6-Cer (30 uM)
C2-Cer (3 uM)
C2-Cer (30 uM)
Sph (3 uM)


B
Progesterone Production
(pg/100 000 cells)
50000
40000
30000
20000
10000
0
Cont
Cch
*

3. Does TNFα activate SMase in hen granulosa cells?

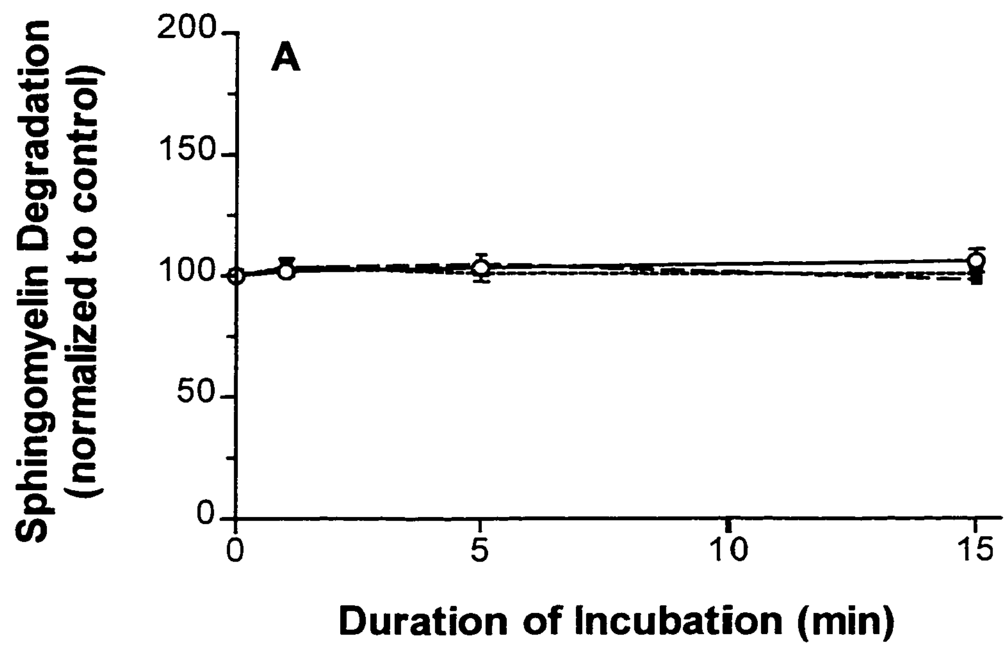
Following a pre-incubation period of 66 hours in the presence or absence of [^{14}C]-choline, F1 granulosa cells were challenged with vehicle or TNFα (0.1, 1, 10 or 50 ng/ml) to determine if the cytokine activates SMase. No significant changes in sphingomyelin or ceramide concentration were observed either at 60 min of exposure to TNFα (Fig 50) or during shorter incubation periods (1, 5 and 15 min; Fig 51). In contrast, these cells responded to exogenous SMase with a significant decrease in sphingomyelin content (28 ± 6.6%; n = 3) and a concomitant rise in ceramide concentration (1138 ± 206%; n = 7; Fig 10). Since TNFα-induced Ca^{2+} transients were developmentally regulated, the influence of TNFα on sphingomyelin and ceramide contents were also determined in F5,6 cells. Neither sphingomyelin (TNFα = 10 ng/ml; 105 ± 28% over control, n = 3) nor ceramide content (TNFα = 1 and 50 ng/ml; 90 ± 16% and 83 ±29% compared to control, respectively; n = 3) was significantly affected by the presence of the cytokine. Finally, since the sphingomyelin protocol required a lengthy preincubation, the possibility that TNFα (10 ng/ml) could induce ceramide production was assessed in cells preincubated only 24 hours. Following a 60 min incubation, TNFα-induced ceramide production was 119 ± 22% (n = 2) in F1 cells and 90 ± 14% (n = 2) in F5,6 cells. As TNFα and Cch interact in the regulation of $[Ca^{2+}]_i$, the possibility that Cch potentiates the TNFα-induced SMase activation was investigated with F1 granulosa cells following Cch (0.2 mM) challenge. No significant changes in sphingomyelin or ceramide

Figure 50: TNFα does not activate a sphingomyelinase in F1 granulosa cells following 60 min incubation. F1 granulosa cells (2.5 x 10^5 cells or 7 x 10^5 cells, sphingomyelin or ceramide assay, respectively; n = 4) were plated in FBS (10%) for 4 to 6 hours and then incubated in the presence (sphingomyelin assay) or absence (ceramide assay) of [^{14}C]-choline for 66 hours. Cells were then challenged with TNFα (0.1-50 ng/ml) or vehicle for 60 min. Lipids were extracted using modifications of the Bligh-Dyer procedure (Methods section 6a) and labelled with [^{32}P]-γ-ATP using the DAG kinase assay (ceramide measurement; Methods section 6b). Labelled lipids were separated by TLC and quantified with 2-D densitometry using a phosphorimager with Molecular Analyst Software (Bio-Rad Laboratories, CA).

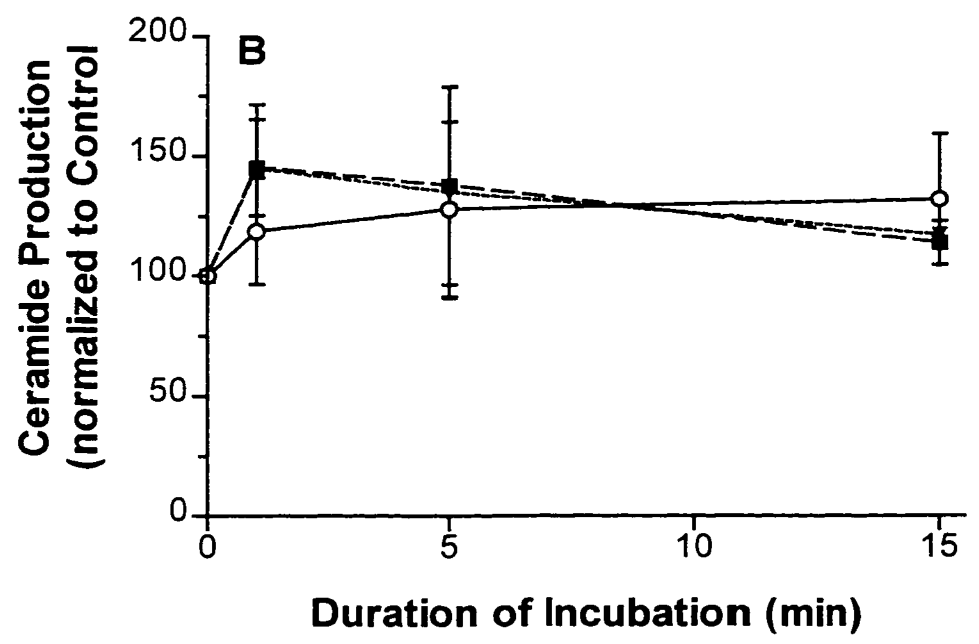


180
120
60
0
Ceramide
Sphingomyelin
Sphingolipid Content
(normalized to control)
0
0.1
1
10
100
TNFα (ng/ml)

Figure 51: TNFα does not regulate sphingomyelinase activity during short incubations in F1 granulosa cells. **Panel A:** Sphingomyelin (2.5 x 10^5 cells; n = 4). **Panel B:** Ceramide (7 x 10^5 cells; n = 4). F1 granulosa cells were plated in FBS (10%) for 4 to 6 hours and then incubated in the presence (sphingomyelin assay) or absence (ceramide assay) of [^{14}C]-choline for 66 hours. Cells were then challenged with TNFα (1 or 50 ng/ml) or vehicle for 0, 1, 5 or 15 min. Lipids were extracted using modifications of the Bligh-Dyer procedure (Methods section 6a) and labelled with [^{32}P]-γ-ATP using the DAG kinase assay (ceramide measurement; Methods section 6b). Labelled lipids were separated by TLC and quantified with 2-D densitometry using a phosphorimager with Molecular Analyst Software (Bio-Rad Laboratories, CA).

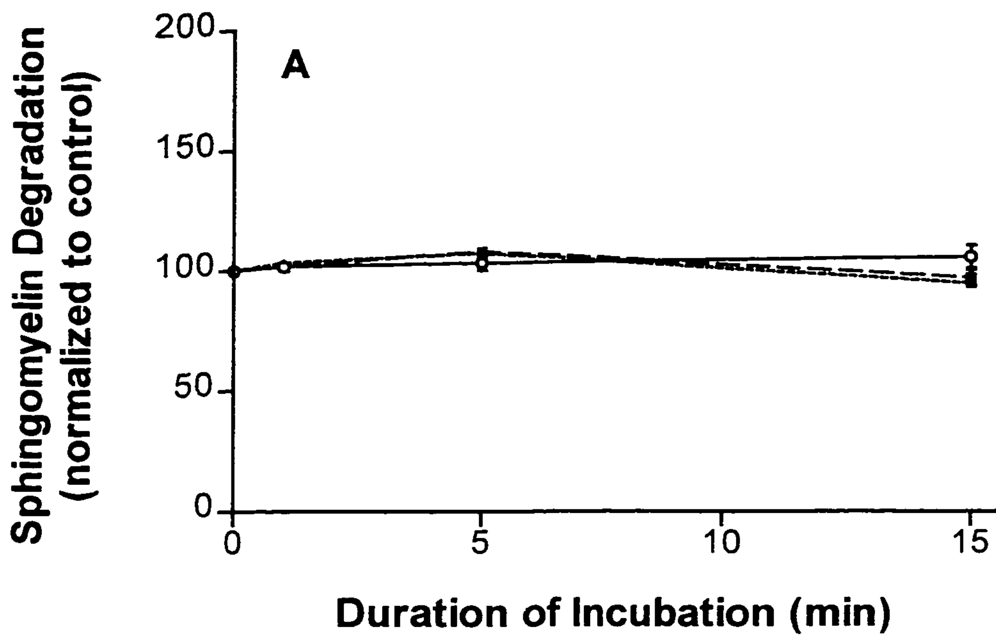


—○— Cont
--▼-- TNFα (1 ng/ml)
–■– TNFα (50 ng/ml)



content were observed irrespective of the duration of incubation (1, 5 or 15 min; Fig 52).

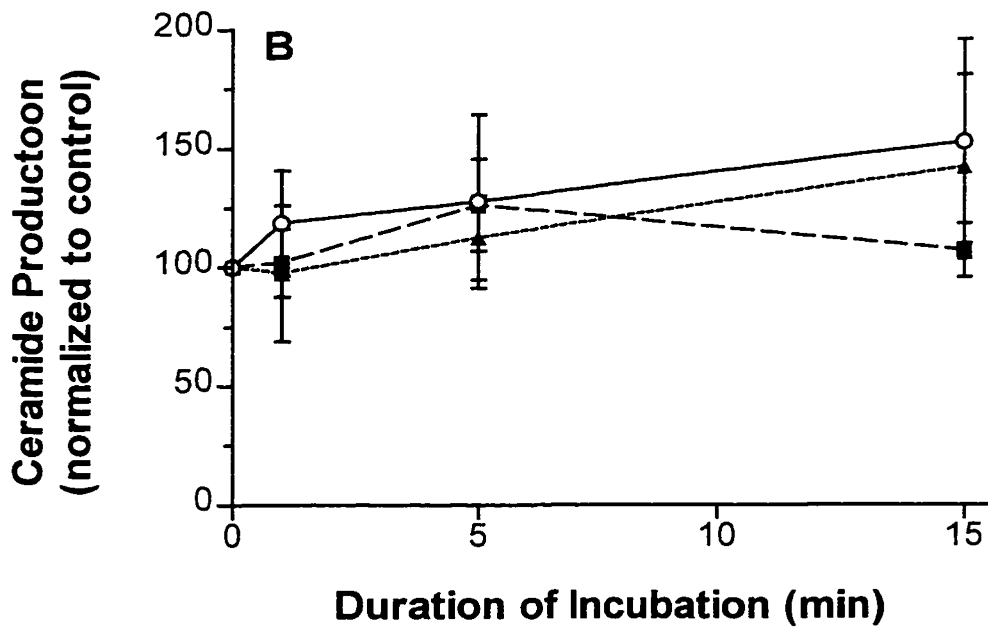
Figure 52: The muscarinic agonist Cch has no influence on TNFα-induced sphingomyelinase activity. **Panel A:** Sphingomyelin (2.5 x 10^5 cells; n = 4). **Panel B:** Ceramide (7 x 10^5 cells; n = 3). F1 granulosa cells were plated in FBS (10%) for 4 to 6 hours and then incubated in the presence (sphingomyelin assay) or absence (ceramide assay) of [^{14}C]-choline for 66 hours. Cells were then challenged with vehicle or Cch (0.2 mM) in the presence or absence of TNFα (10 ng/ml) for 0, 1, 5 or 15 min. Lipids were extracted using modifications of the Bligh-Dyer procedure (Methods section 6a) and labelled with [^{32}P]-γ-ATP using the DAG kinase assay (ceramide measurement; Methods section 6b). Labelled lipids were separated by TLC and quantified with 2-D densitometry using a phosphorimager with Molecular Analyst Software (Bio-Rad Laboratories, CA).



—o— Cont

---▲--- Cch (0.2 mM)

—■— Cch (0.2 mM) +
TNFα (10 ng/ml)



VI. DISCUSSION

A. The role of TNFα in hen granulosa cell fate.

In mammalian systems, the role of TNFα as a regulator of ovarian function is primarily anti-gonadotropic; TNFα inhibits gonadotropin-induced follicular production of progesterone (Roby and Terranova, 1990), estrogen (Adashi *et al*, 1989) and androgen (Andreani *et al*, 1991). Furthermore, this cytokine attenuates FSH-induced inhibition of granulosa cell DNA synthesis and stimulation of plasminogen activator activity (Karakji and Tsang, 1995c). Inconsistent with its apparent role as an anti-differentiative factor, however, TNFα stimulates progesterone (Roby and Terranova, 1990) production in theca cells from preovulatory follicles in the absence of gonadotropin support. In the hen, TNFα did not influence progesterone production at any point during follicular development with or without gonadotropin stimulation. Furthermore, the observed potentiation of TGFα-induced increases in cell viability *in vitro* by TNFα represents the first evidence supporting a role for TNFα as a survival factor in granulosa cells. These findings reveal aspects of the TNFα's role in the regulation of ovarian function not previously considered.

The decrease in viability of F5,6 granulosa cells in culture appeared to be associated with necrosis rather than apoptosis. The fact that necrosis, rather than apoptosis occurred is somewhat surprising, in that the *in vitro* death of both avian and mammalian granulosa cells under serum-free conditions is well established to be apoptotic in nature (Tilly *et al*, 1992b;

Johnson *et al*, 1996). Moreover, unlike apoptosis, necrosis generally occurs as a result of trauma rather than spontaneously (ie due to the absence of survival signals). One possibility, however, is that the necrosis observed in the current study was actually "secondary necrosis" which has been found to occur in apoptotic cells which cannot be phagocytosed, both *in vivo* (apoptotic cells extruded into a lumen; see Schwartzman and Cidlowski, 1993) and *in vitro* (Lieberthal *et al*, 1996). This suggests that the increase in necrosis occurring in F5,6 granulosa cells *in vitro* may actually be due to programmed cell death, however, additional study would be required to confirm this possibility.

The decreased viability of F5,6 granulosa cells *in vitro* was independent of cell density. This contradicts prior studies demonstrating that apoptosis is inhibited by cell contact in rat granulosa cells (Peluso, 1997; Peluso *et al*, 1996; Peluso and Pappalardo, 1994), a phenomenon also observed in neutrophils (Hannah *et al*, 1998) and ovarian cancer cells (Mathieu *et al*, 1995). Nonetheless, programmed cell death induced by confluent culture has been reported for primary hepatocytes (Maeda *et al*, 1995), HL-60 (Saeki *et al*, 1997; Nishizawa *et al*, 1998) and NB-40 cells (Taimi and Breitman, 1997). As in the current findings, evidence for both apoptosis and necrosis was provided in these studies. Specifically, extensive DNA fragmentation was observed in hepatocytes, while only 2 to 3% of the cells exhibited condensed nuclei (Maeda *et al*, 1995). Moreover, density-induced cell death in hepatocytes was blocked by inhibitors of protein synthesis, calmodulin and mitochondrial function, suggesting that a form of programmed cell death is occurring despite

differences from classical apoptotic morphology. In addition, dimethyl-sulfoxide, an inducer of leukocyte differentiation, inhibited density-dependent cell death in HL-60 cells (Nishizawa *et al*, 1998). Hence, the results of the studies on MTT metabolism indicating that neither TNFα nor TGFα increased cell viability in F1 cells may be explained by the suggestion that the highly differentiated nature of F1 cells may have rendered them insusceptible to this form of programmed cell death.

The observed cell density-dependent differences in the response of granulosa cells to TNFα and TGFα revealed distinct differences in the types of responses regulated by these two factors. TGFα-induced proliferation of hen granulosa cells has been previously demonstrated (Tilly and Johnson, 1990; Lafrance *et al*, 1993a; Peddie *et al*, 1994; Li and Tsang, 1995), suggesting that the increase in MTT metabolism observed in sparse culture may be due to an increase in cell number. Moreover, the fact that TGFα did not increase MTT metabolism in confluent cultures is likely due to contact inhibition which normally occurs in non-transformed cells *in vivo* and *in vitro* (Wieser *et al*, 1990). In contrast, the effect of TNFα appeared to asssociated with inhibition of cell death rather than induction of proliferation, since MTT metabolism decreased in confluent cells cultured in its absence (even in the presence of TGFα) while freshly isolated granulosa cells (time 0) had the highest MTT metabolism, irrespective of treatment. Since this response was observed only in F5,6 cells cultured only at one cell density *in vitro*, it is important to consider which situation is represented *in vivo*. In F5,6 follicles, granulosa cells exist as a monolayer, densely packed

within an extracellular matrix. Moreover, considerable cell contact has been demonstrated *in vivo* in the form of gap junctions (Tischkau *et al*, 1997, Yoshimura *et al*, 1993). Consequently, although considerable differences between the *in vivo* and *in vitro* conditions exist, confluent rather than sparse culture may be more representative of the physiological conditions for granulosa cells *in vivo*.

TNFα-induced cell survival/proliferation has been observed in fibroblasts (Guy *et al*, 1991), lymphocytes (Lee *et al*, 1997b) and cancer cells (Wu *et al*, 1992; Wu *et al*, 1993), although earlier studies in granulosa cells had suggested the cytokine was apoptogenic (Witty *et al*, 1996; Kaipai *et al*, 1996). Nonetheless, both studies demonstrating TNFα-induced apoptosis in granulosa cells were performed using granulosa cells from follicles not yet committed to ovulation (LWF and preantral follicles in the hen and rat, respectively). It is generally accepted that once a follicle is destined to ovulate, follicular atresia (and hence apoptosis) does not occur *in vivo* (see Kaipai and Hsueh, 1997). Consequently, the current studies suggest that once committed to ovulation, a developmentally regulated change in the nature of TNFα action on granulosa cells occurs. Whether this occurs because of differences in receptor expression or post-receptor signal transduction mechanisms remains to be determined.

The nature of tumour necrosis factor alpha receptor (TNFR) expression in the ovary is a subject of speculation, since studies of TNFR expression in the ovary have not been performed in any species. Nonetheless, based upon findings in other cell types, TNFR1

activation is associated with both apoptosis and cell survival, while TNFR2 is specific for the survival response. TNFR1 activation results in the activation of neutral SMase via the adaptor protein FAN (Adam-Klages *et al*, 1996). Consequently, the fact that TNFα-induced increases in ceramide production were not observed in F1 or F5,6 granulosa cells suggests that TNFR1 was not present. This appears to support the concept that the observed developmental change from TNFα-induced apoptosis to TNFα-induced survival occurs as a result of differences in receptor expression from TNFR1 to TNFR2. Nonetheless, since TNFR1 can also mediate survival responses and downregulation of either FAN or neutral sphingomyelinase would result in the absence of ceramide production, this hypothesis requires confirmation.

In the current study, TNFα-induced cell survival was follicular stage-dependent and occurred only in the presence of TGFα. These findings suggest the presence of crosstalk mechanism(s) between the signal transduction pathways of TGFα and TNFα. Furthermore, since this response was observed in F5,6 but not F1 granulosa cells, this crosstalk mechanism must be blocked during the process of cytodifferentiation as follicles mature. Although we have not studied the mechanism(s) of this interaction, the results of several analogous studies on TNFα-TGFα interactions in other cell systems provide possible explanations for our findings.

Schmiegel *et al* (1993) demonstrated that TNFα induced the expression of both TGFα and EGFR in several human pancreatic cancer cell lines. Furthermore, a subsequent

study from the same laboratory demonstrated that TNFR2 mediated the increase in TGFα expression, while TNFR1 mediated the increase in EGFR expression (Kalthoff *et al*, 1993). Since TNFα-TGFα interaction in the current study was observed in the presence of maximally stimulatory concentrations of TGFα, it is unlikely that an increased production of the ligand could have elicited the observed increase in granulosa cell survival without TNFα-induced increases in EGFR expression. Consequently, if the TNFα-TGFα interaction in the granulosa cell is mediated by changes in EGFR and TGFα expression similar to pancreatic cells, this would support the hypothesis that granulosa cells express TNFR1 rather than TNFR2. Nonetheless, since this interaction may be regulated downstream of receptor expression, the concept that TNFR2 is the mediator of the TNFα-TGFα interaction cannot be excluded.

It is well established that TGFα-induced protection from apoptosis is mediated via stimulation of tyrosine phosphorylation of the EGFR. Phosphorylation of the receptor causes it to dimerize, resulting in the binding and activation of several src homology-domain containing proteins including PI-3 kinase, phospholipase Cγ, GTPase-activating proteins and Src family protein kinases (Birge and Hanafusa, 1993). Activation of these proteins results in cell survival via several distinct signals including mobilization of $[Ca^{2+}]_i$, activation of protein kinase C and activation of the ERK cascade. Nonetheless, it is the activation of the ERK cascade, which is believed to be the primary driving force for EGFR-induced survival signals (Crews and Erickson, 1993; Cobb and Goldsmith, 1995; Hall, 1994). TNFα is also

recognized as a weak activator of the ERK cascade (Boylan *et al*, 1996; Guy *et al*, 1991; Van Lint *et al*, 1992; Guesdon *et al*, 1993), although this occurs via ceramide production rather than tyrosine phosphorylation (Raines *et al*, 1993). Since we did not observe TNFα-induced ceramide production, it is unlikely that the cytokine was able to activate ERK. In addition, since the ability of TNFα to induce ERK activation is far weaker than that of members of the tyrosine kinase receptor family (Boylan *et al*, 1996), ERK activation itself may not be adequate to account for the TNFα-TGFα interaction.

The SAPK phosphorylation cascade is also activated by both TNFα (Natoli *et al*, 1997; Boylan *et al*, 1996; Hibi *et al*, 1993; Derijard *et al*, 1994; Kyriakis *et al*, 1994) and TGFα (Boylan *et al*, 1996; Minden *et al*, 1994). In contrast to the ERK cascade, however, TNFα stimulates this cascade far more effectively than TGFα (Boylan *et al*, 1996). Consequently, exposure of granulosa cells to both TNFα and TGFα results in maximal stimulation of the ERK and SAPK cascades. The role of the SAPK cascade is highly controversial. Although several studies have shown a relationship between SAPK activation and apoptosis (Zanke *et al*, 1996; Verheij *et al*, 1996; Luo *et al*, 1998; Toyoshima *et al*, 1997; Seimiya *et al*, 1997; Ichijo *et al*, 1997; Graves *et al*, 1996), several studies have suggested a protective role for SAPK (Natoli *et al*, 1997; Nishina *et al*, 1997; Smith *et al*, 1997; Boylan and Grupposo, 1996; Westwick *et al*, 1995b). Consequently, the effect of SAPK activation must be dependent on the cell type, the presence or absence of co-stimulatory factors and/or the extent of its activation. Although additional experiments would

clearly be necessary to clarify the role of SAPK in granulosa cell function, it is reasonable to suggest that its action may be similarly pleiotropic in nature and dependent on cytodifferentiation.

NF-κB is a well established TNFα-induced regulator of inflammation and immune responses (Finco *et al*, 1995). Of more interest to the current study, however, is the fact that activation of NF-κB suppresses apoptosis (Van Antwerp *et al*, 1996; Beg and Baltimore, 1996; Wang *et al*, 1996). Consequently, the activation of NF-κB may be a key factor in determining whether TNFα is apoptogenic or protective in different cell types. In lymphocytes, antigen presentation is known to stimulate TNFα-induced NF-κB activation via inhibition of TRIP expression (inhibitor of TNFR2-induced NF-κB activation), increased TRAF1, cIAP1 and cIAP2 expression (facilitators of TNFR2-induced NF-κB activation), proliferation and resistance to apoptosis (Lee *et al*, 1997b; see Fig 3). It is, therefore, possible that TGFα exerts its influence on TNFα action via a similar mechanism, ie TGFα-induced facilitation of TNFR2-induced NF-κB activation.

In conclusion, these studies demonstrate the existence of a stage-dependent interaction between TNFα and TGFα in the regulation of cell survival. Although the mechanism(s) of the interaction remains unidentified, changes in EGFR density and/or crosstalk between the signal transduction pathways of TNFα and TGFα are likely to account for this phenomenon. Identification of the regulatory factor(s) involved will further our understanding of the roles of both TNFα and TGFα along with their mediators in granulosa

cell survival and follicular selection.

B. TGFα-dependent TNFα-induced fibronectin-integrin interactions.

The purpose of this study was to determine the role of cell attachment in TNFα responses in granulosa cells. The involvement of extracellular matrix production and remodelling in the ovary and the regulatory mechanisms involved in these processes has been under study for some time. Nonetheless, only one prior study had demonstrated the presence of integrin receptors (receptors for extracellular matrix) on ovarian cells (marmoset; Giebel *et al*, 1996) and no information regarding the regulators of their production within the ovary has been published. Nonetheless, the involvement of integrins as modulators of proliferative, differentiative and apoptotic signals has been demonstrated in many different cell types (see Giancotti, 1997; Howe *et al*, 1998) and TNFα has been identified as one of the regulators of integrin production. Moreover, the fact that the TNFα response was limited to confluent cultures suggests a potential role for cell-cell and/or cell-matrix interactions in this response. Consequently, integrin receptors may play a pivotal role as mediators of the TNFα-TGFα-induced increase in granulosa cell survival *in vitro* in F5,6 cells.

Western blots of granulosa cell extracts for fibronectin revealed two distinct bands of approximately 200 and 140 kDa in size. Moreover, a slightly larger band estimated at 210 kDa was detected in granulosa cell spent medium. Analysis of the differences between these

three different bands was not performed, although prior studies on the different isoforms of fibronectin may provide potential interpretations for these findings. Fibronectin is a relatively well conserved protein containing two binding domains for fibrin and one for each of heparin, collagen and integrin as well as a region containing a series of type III repeats of unknown function (see Kornblihtt *et al*, 1996). In addition, there is a 120 amino acid V region present in secreted rather than cytosolic fibronectin (Schwarzbauer *et al*, 1989), suggesting a function for this region either in signalling the secretion of the molecule or as a modulator of extracellular interactions. This suggests that the observed decrease in mobility of secreted fibronectin may reflect the presence of this V region. In addition, two 20 amino acid regions termed EIIIA and EIIIB have been identified as alternatively spliced regions of chicken fibronectin (Norton and Hynes, 1987). Nonetheless, alternative splicing of these regions could not have accounted for the observed difference of approximately 60 kDa between the two cellular bands, although the notable width of the upper band may have been due to this phenomenon. Moreover, although mixed reports of the size of fibronectin are available, it is estimated to be in the 200 to 250 kDa range (see Kornblihtt *et al*, 1996; Huet *et al*, 1997). Consequently, the 140 kDa band may simply reflect proteolytic product of fibronectin, although subsequent studies are needed to clarify this question.

Although direct studies of fibronectin and integrin as mediators of TNFα-TGFα-dependent F5,6 granulosa cell survival were not performed, several interesting correlations were observed. TGFα effectively increased both the production and secretion of fibronectin

during a 24 hr culture period independently of TNFα. In addition, the growth factor stimulated integrin β3 production within the initial 24 hr culture and protected cells from death *in vitro* following 48 and 72 hr culture periods. Although TNFα-induced regulation of fibronectin secretion was not detected in these studies, the cytokine stimulated the production of integrin β3 during the initial 24 hr culture period and improved cell viability *in vitro* following subsequent incubation when TGFα was present. Since differences in integrin β3 expression were observed prior to changes in cell viability, this receptor may have served a protective role in granulosa cell function.

TNFα-induced integrin β3 production was dependent on the presence of TGFα. Although the mechanism whereby TNFα and TGFα interact has not been identified, there are several reasonable possibilities. TNFα-induced expression of vascular cell adhesion molecule-1 (Ahmad *et al*, 1998) and integrin α2 (Xu *et al*, 1998) are under the control of the transcription factors NF-κB and AP-1. As summarized previously (see Discussion section A; Pg 179) NF-κB and AP-1 are potential mediators of TNFα-TGFα interactions. Although their role in integrin β3 production has not been demonstrated, their involvement in the production of related adhesion molecules suggests that activation of these transcription factors could result in integrin β3 production. Alternatively, the increase in fibronectin itself could have resulted in integrin upregulation, since a TGFα-induced increase in fibronectin production was observed in the absence of TNFα. Nonetheless, TNFα did not stimulate MTT metabolism when plated on fibronectin (data not shown), suggesting that the presence of

fibronectin itself is not sufficient to mimic the growth factor.

The importance of cell attachment for cell survival has long been recognized. Moreover, integrins have been identified as the effectors of this phenomenon. In fact, the fibronectin receptors, integrins $\alpha_5\beta_3$ and $\alpha_v\beta_3$, were shown to activate the Ras-ERK pathway via the tyrosine kinase Shc (Wary *et al*, 1996), resulting in inhibition of apoptosis. In addition, the tyrosine kinase FAK is well known to play a role in the control of adhesion-dependent cell survival, possibly via activation of PI-3 kinase and c-Abl tyrosine kinase (Lewis *et al*, 1996; Gertler *et al*, 1996). Although the identity of the α subunits expressed on granulosa cells has not been determined, the increase in β3 expression probably reflects increased expression of the entire molecule. Moreover, the presence of fibronectin in the extracellular matrix would seem to support the concept that its receptors would be present. Consequently, TNFα-induced upregulation of integrin receptors in the presence of TGFα may play a significant role in the mediation of granulosa cell survival.

The regulation of fibronectin production by TGFα and/or TNFα was limited to F5,6 rather than F1 cells. Although this was the first study in granulosa cells of the role of TNFα on fibronectin production, prior studies of TGFα-induced fibronectin during follicular development have been performed (Asem *et al*, 1994). Interestingly, TGFα-induced fibronectin production in F1 cells was detected in that study, albeit at much lower levels than earlier in follicular development. Nonetheless, one of the key differences between these two experiments was that the current study quantified fibronectin levels by densitometric analysis

of Western blots, while the previous study used ELISA. Although ELISA is a more quantitative assay, non-specific binding of the antibody cannot be eliminated. In any case, both studies do agree that while TGFα-induced fibronectin production and secretion decrease during follicular development, basal fibronectin production and secretion increase. Downregulation of TGFα receptors between the F5,6 and F1 developmental stages in hen granulosa cells has been documented (Onagbesan *et al*, 1996) and represents a likely mechanism for this phenomenon. In addition, the extremely high basal expression of fibronectin production by F1 cells may represent a maximal rate of production.

Integrin β3 was expressed at fairly similar levels in F1 and F5,6 cells, although TGFα and/or TNFα were only effective in stimulating its expression in F5,6 cells. The lack of effect of TGFα may be attributed to the downregulation of TGFα receptors (Onagbesan *et al*, 1996), although no information is available regarding TNFR expression in granulosa cells. Nonetheless, since the effect of TNFα in F5,6 cells was only observed in the presence of maximally stimulatory concentrations of TGFα, the TGFα signal in F1 cells may not have been sufficient to modulate the TNFα response even in the absence of changes to the TNFR. Alternatively, follicular stage-dependent changes in the post-receptor signalling mechanisms of TNFα and TGFα could be responsible for the differences in their responses to these two agonists. Additional study is required to identify the intracellular regulators of integrin production and the nature of TNFR expression in granulosa cells in order to determine the nature of the differentiative change between F5,6 and F1 granulosa cells.

In conclusion, TNFα induced integrin β3 production was dependent on maximally stimulatory concentrations of TGFα and inhibited by cytodifferentiation. Moreover, integrin β3 may have a key role as a protective factor for F5,6 granulosa cells under the control of TNFα. These observations provide important clues regarding the nature of crosstalk between TNFα and TGFα in the regulation of the fate of granulosa cells during the process of follicular selection. Further study towards the identification of integrin α subunits, the mediators of integrin production and the actions of integrins as regulators of granulosa cell function may further our understanding of the roles of TNFα, TGFα and extracellular matrix in the regulation of follicular selection and granulosa cell fate.

C. Regulation of Ca^{2+} signalling via muscarinic input during follicular development.

1. The follicular stage dependence of IP_3-mediated Ca^{2+} mobilization following muscarinic input.

This study demonstrated for the first time that the muscarinic agonist Cch modulated IP_3 production and intracellular Ca^{2+} levels in hen granulosa cells during follicular development. Moreover, the nature and magnitude of these responses to Cch were dependent on the extent of follicular maturation and thus the degree of cellular differentiation; the $[Ca^{2+}]_i$ responses were generally small at the early stages, in contrast to the large Ca^{2+} spikes observed almost exclusively in F1 cells. As the Cch-stimulated increase in IP_3 production was higher in F1 vs. F5,6 cells, these data suggest that there is an increased capacity for muscarinic activation of PIP_2 cleavage with development.

The Cch-stimulated increases in inositol phosphate synthesis in hen granulosa cells occurred with a time dependency similar to that seen in previous studies on granulosa cells (Flores *et al*, 1992; Dimino *et al*, 1987) and other cell types (Downes and Wusteman, 1983). They are also consistent with recent studies in a variety of cell types demonstrating that some, but not all, muscarinic receptor subtypes are coupled to inositol phospholipid-specific phospholipase C (PLC; see Lambert, 1993). Activation of this pathway leads to the release of 1,4,5-IP_3 into the cytosol, where it mobilizes intracellular stores of Ca^{2+} until it is

phosphorylated to form 1,3,4,5-IP_4 and/or is dephosphorylated to form various inactive metabolites (see Berridge, 1993). The observation that IP_3 is produced prior to IP_2 and IP is consistent with this concept. Although shorter time points could not be examined with this model because of technical complications, it is likely that IP_3 levels reach effective concentrations between 5 and 10 sec after Cch challenge, given the dynamics of inositol phosphate production in other cell types (see Berridge, 1993).

Dependency on intracellular Ca^{2+} was demonstrated by experiments in which large and fast Ca^{2+} transients were not blocked when Cch challenge occurred in Ca^{2+}-free medium containing EGTA and Mg^{2+} or Mn^{2+}. As evidenced by these findings and the demonstration of Cch-stimulated IP_3 production, the rapid transients were a likely consequence of IP_3 mobilization of Ca^{2+} from intracellular stores. In contrast, the slow responses could be primarily attributed to a transmembrane influx of Ca^{2+}, as the magnitude of these Ca^{2+} transients was greatly reduced when these cells were challenged in Ca^{2+}-free medium. However, the inability of Ca^{2+} removal to completely block the slow response during the first Cch challenge suggests that an intracellular component may also be operational. In support of this concept, our finding that F5,6 granulosa cells produce IP_3 in response to Cch indicates that this effect is probably partially mediated by IP_3 in these cells. Furthermore, the fact that this small increase in $[Ca^{2+}]_i$ was never observed during subsequent Cch challenge prior to re-exposure of the cells to Ca^{2+} is consistent with these findings, as complete refilling of Ca^{2+} stores would not occur in Ca^{2+}-free medium. Thus it can be concluded that the Cch-induced

Ca^{2+} transients changed from being primarily dependent on extracellular Ca^{2+} at early developmental stages to being primarily dependent on intracellular Ca^{2+} in cells from mature follicles.

The intracellular component of Cch-induced changes in $[Ca^{2+}]_i$ was minor in all but approximately 10% of the cells from the F3 and F5,6 follicles. This could be attributed to poorly developed intracellular Ca^{2+} pools in these cells, low IP_3 receptor density on existing Ca^{2+} stores, low production of IP_3 in response to receptor-ligand, low muscarinic receptor density and/or a change in the subtype of muscarinic receptor. This study demonstrated that muscarinic stimulation of IP_3 production was greater in F1 than in F5,6 granulosa cells, suggesting that the increased ability of F1 cells to mobilize intracellular Ca^{2+} stores is due, at least in part, to an increased capacity to generate this second messenger. Similarly, Hertelendy *et al* (1989) showed that while there were no differences between Ca^{2+} transients induced by IP_3 in permeabilized hen granulosa cells from different stages of maturation, LH-induced IP_3 production increased during late follicular development. This suggests that the increased ability of Cch to cause mobilization of Ca^{2+} from intracellular stores in F1 (as compared to F5,6) granulosa cells is due either to an increased coupling of muscarinic receptors to PLC, changes in muscarinic receptor number and/or subtype during follicular maturation. Similarly, increased Cch-induced IP_3 production during cytodifferentiation could also account for the differences observed in the percentage of cells capable of producing measurable Ca^{2+} transients between the F5,6 and F1 stages.

The average basal $[Ca^{2+}]_i$ was significantly lower in granulosa cells from F5,6 than from F3 and F1, showing that intracellular Ca^{2+} levels rise at some point late in follicular maturation. This conclusion is in agreement with the study reported by Hertelendy *et al* (1989). However, those authors indicated that both the F5,6 and the F3 granulosa cells had lower basal $[Ca^{2+}]_i$ than the F1 granulosa cells. Furthermore, the average basal $[Ca^{2+}]_i$ values they reported were about 6 to 7 times higher than our measured values. This discrepancy can be accounted for by differences in experimental conditions, as experiments in the present study were performed on single, cultured cells whereas Hertelendy *et al* performed experiments on a suspension of fresh cells. Tucker *et al* (1990) demonstrated differences in both basal and stimulated $[Ca^{2+}]_i$ between suspended and attached cells; this suggests that experimental conditions influence $[Ca^{2+}]_i$ responses. Conversely, it is also conceivable that our $[Ca^{2+}]_i$ values have been somewhat underestimated because of the possible presence of unhydrolyzed fura-2AM in the cells (see Morgan, 1993).

The precise role of cholinergic innervation in the control of ovarian function is poorly understood. Several studies have demonstrated the involvement of autonomic input in the control of follicular development (Brink and Grob, 1972), compensatory ovarian hypertrophy (Burden and Lawrence, 1977), ovulation (Bahr *et al*, 1974), ovarian blood flow (Gibson and Roche, 1986) and steroidogenesis (Kawakami *et al*, 1981). Furthermore, it has been demonstrated that Ach increases ovarian oxytocin and progesterone secretion *in vivo* in sheep (Heap *et al*, 1989) and bovine granulosa cell oxytocin and progesterone production

in vitro (Luck, 1990). Previous studies from our laboratory have demonstrated that muscarinic input has at least two mechanisms of action in hen granulosa cells: increasing $[Ca^{2+}]_i$ (Morley *et al*, 1992a) and intracellular pH (Li *et al*, 1992). Nonetheless, Cch did not increase progesterone production in F1 (Morley *et al*, 1992a; the present study) or F5,6 granulosa cells. This confirms previous reports performed only in F1 granulosa cells (Asem and Tsang, 1987; Morley *et al*, 1992a; Morley *et al*, 1992c; Morley *et al*, 1994) that increases in $[Ca^{2+}]_i$ do not, by themselves, cause steroidogenesis in hen granulosa cells. Moreover, the observation that Cch did not affect MTT metabolism in F1 or F5,6 granulosa cells suggests that the muscarinic activator is not involved in cell proliferation or cell fate.

Although muscarinic regulators are not involved in steroid production, cell proliferation or cell survival, another interesting possibility is that protein secretion via exocytosis is under neural control. The role of Ca^{2+} in exocytosis has been well established in several cell types (Livett, 1993; Martin, 1994) and Cch induced protein secretion in F1 granulosa cells was demonstrated, although the nature of the proteins secreted was not determined (Morley *et al*, 1997). In the current study, Cch inhibited fibronectin secretion in F1, but not F5,6 cells. This suggests that Cch-induced protein secretion is highly specific in nature. Nonetheless, $[Ca^{2+}]_i$ following long-term exposure has not been determined and fibronectin secretion was measured following a 24 hr incubation period in order to accumulate sufficient protein for analysis. Although Cch cannot be degraded by cholinesterase, receptor downregulation and/or depletion of Ca^{2+} stores may occur during a

24 hr incubation, which could have altered Cch-induced changes in $[Ca^{2+}]_i$. Moreover, under conditions of high $[Ca^{2+}]_i$, we observed Cch-induced inhibition of Ca^{2+} current. Although we were not able to determine if this decrease in Ca^{2+} current can occur under normal physiological conditions, if so, it would provide an alternative explanation for the observed decrease in fibronectin secretion.

In conclusion, our results demonstrate that the ability of granulosa cells to mobilize intracellular Ca^{2+} via the production of IP_3 in response to muscarinic stimulation is dependent on the stage of follicular development. While the precise role of cholinergic innervation remains unclear, the results of the present study, in combination with those of previous investigations, are consistent with the concept that Ca^{2+}-mediated muscarinic activation during follicular development plays an important role in the maturation of the ovarian follicle.

2. The role of muscarinic input in Ca^{2+} homeostasis.

This study demonstrated for the first time that activation of muscarinic receptors inhibits T-type Ca^{2+} current. Using the perforated-patch technique, which resulted in a complete prevention of current rundown during the time period of our experiments, the major inward current we observed presented characteristics typical of T-type Ca^{2+} current (Caffrey *et al*, 1986; Ohya *et al*, 1987; Marchetti and Brown, 1988; Tsien *et al*, 1988; Rane *et al*,

1989; Schwartz *et al*, 1989; Toselli and Lux, 1989; Wang *et al*, 1989; Schroeder *et al*, 1990; Clapp and Gurnery, 1991; Jones, 1993; Xiong *et al*, 1993): maximal amplitude at about -20 mv, rapid inactivation at depolarizing potentials, inhibition by 100 μM Ni^{2+} and insensitivity to the dihydropyridine Ca^{2+} channel antagonist, nifedipine. A previous single-channel study showed that both T- and L-type Ca^{2+} channels occur in chicken granulosa cells (Schwartz *et al*, 1989). Our study also revealed both types of current at the macroscopic level but the T-type current largely predominated under our conditions, as was also recently demonstrated in porcine granulosa cells (Kusaka *et al*, 1993). Thus, the observation that Cch could almost completely inhibit the Ca^{2+} current in some cells readily demonstrated its action on the T-type Ca^{2+} channels. This was also confirmed by the observed Cch-induced inhibition of the Ca^{2+} current in the presence of nifedipine, a condition that should have singled out the T-type Ca^{2+} current. That this inhibition was specifically mediated by a muscarinic pathway was further demonstrated by using the complete blockage of the Cch response by the muscarinic inhibitor atropine.

Prolonged exposure to Cch caused an apparent desensitization, although the extent of the time-course greatly varied between cells. The cellular mechanisms accounting for this desensitization and its variability are likely to be multiple and may include down regulation of receptors or intracellular signalling systems, alterations in the state of T-type Ca^{2+} channels or variation of $[Ca^{2+}]_i$. Due to the possible complexity of this phenomenon, the observed variability of the desensitization was not surprising. It is interesting to note that

Cch-induced $[Ca^{2+}]_i$ responses also varied greatly and were dependent on the state of cytodifferentiation. Furthermore, the extent of this variability can be reversibly reduced by *in vitro* exposure of the cells to tumour necrosis factor alpha. As such, the variability in desensitization of T-type Ca^{2+} current to muscarinic activation may also reflect a genuine heterogeneity in the F1 granulosa cell population which is comprised of cells in functionally different states.

Large transient increases in $[Ca^{2+}]_i$ are typically elicited upon activation of muscarinic receptors in F1 granulosa cells (Morley *et al*, 1992a; the present study). The mechanisms underlying these changes are believed to involve a fast Ca^{2+} release from intracellular stores triggered by inositol trisphophate (IP_3) and, to a much smaller extent and following a slower time-course, increased Ca^{2+} entry through yet to be defined Ca^{2+} channels. Such Cch-induced $[Ca^{2+}]_i$ increases could have conceivably contributed to the inhibition of the T-type Ca^{2+} current following the consequent dissipation of Ca^{2+} electrochemical gradient and/or some direct effect of intracellular Ca^{2+} ions on the channel gating properties. However, our experiments involving simultaneous measurements of $[Ca^{2+}]_i$ excluded these possibilities since Cch never induced the expected large transient increases in $[Ca^{2+}]_i$ under these experimental conditions. On the contrary, intracellular $[Ca^{2+}]_i$ slightly decreased during Cch exposure, an effect that could be attributed to the diminished Ca^{2+} influx through the T-type Ca^{2+} channels which we have observed.

One possible explanation for the observed absence of Cch-induced Ca^{2+} transients in

our patch-clamp experiments would be that these measurements were done under conditions leading to high basal $[Ca^{2+}]_i$. It has indeed been well recognized in several other cell types that high cytosolic Ca^{2+} levels can inhibit IP_3-induced Ca^{2+} release (Pietri *et al*, 1990; Ino, 1990; Bezprozvanny *et al*, 1991). In our patch-clamp experiments, superfusion in high extracellular $[Ca^{2+}]$ in the absence of Na^+ induced an increase in basal $[Ca^{2+}]_i$ to approximately 0.25 μM, due primarily to the enhancement of the repeatedly evoked Ca^{2+} currents by the increased extracellular $[Ca^{2+}]$ although reduction of Ca^{2+} extrusion mechanisms such as the Na^+-Ca^{2+} exchanger following removal of extracellular Na^+ could also have contributed to increased basal $[Ca^{2+}]_i$. In addition, the fact that inhibition of Cch-induced Ca^{2+} transients was observed in cells with high cytosolic $[Ca^{2+}]_i$ ($\geq$ 0.12 μM) but not in cells with low cytosolic $[Ca^{2+}]_i$ ($\leq$ 0.06 μM) following incubation in 25 mM extracellular Ca^{2+} supports the concept that the absence of Cch-induced Ca^{2+} transients under our patch clamp experiments was due to the increased cytosolic $[Ca^{2+}]_i$ and not the experimental conditions.

Muscarinic agonists have been shown to modulate Ca^{2+} current in a variety of cellular preparations (Marchetti and Brown, 1988; Tsien *et al*, 1988; Rane *et al*, 1989; Toselli and Lux, 1989). Specifically, activation of the muscarinic receptor can result in the inhibition of adenylate cyclase via G_i proteins, leading to an inhibition of L- and N-type Ca^{2+} channels (Marchetti and Brown, 1988; Tsien *et al*, 1988; Rane *et al*, 1989). However, a previous study from our laboratory has demonstrated that a challenge with either acetylcholine or Cch failed

to significantly influence basal or LH-stimulated cAMP levels in hen granulosa cells (Morley *et al*, 1992a). Furthermore, Kusaka *et al* (1993) showed that neither LH nor cAMP had any effects on T-type Ca^{2+} current. In fact, actetylcholine actually increased T-type Ca^{2+} current in hippocampal neurons, an effect antagonized by atropine (Toselli and Lux, 1989). On the other hand, activation of protein kinase C by phorbol esters and diacylglycerol analogues has been shown to inhibit T-type Ca^{2+} currents in chick (Marchetti and Brown, 1988; Rane *et al*, 1989) and rat (Schroeder *et al*, 1990) sensory neurons. Although such an effect remains to be demonstrated in granulosa cells, activation of protein kinase C may well constitute the biochemical pathway leading to our observed muscarinic inhibition of T-type Ca^{2+} current. The fact that Cch induced the production of IP_3 suggests that diacylglycerol was generated. As such, Cch-induced stimulation of protein kinase C must also occur in granulosa cells. Activation of this kinase is also known to inhibit muscarinic responses and has been considered to play an important role as a negative feedback mechanism (El-Fakahany *et al*, 1988). In this regard, it is tempting to propose that inhibition of T-type Ca^{2+} channels is a constituent of this feedback response in granulosa cells. T-type Ca^{2+} current has been invoked as a generator of electrical activity which then leads to further Ca^{2+} entry through slowly inactivating channels (Tsien *et al*, 1988), and granulosa cells have been shown to have Ca^{2+}-dependent action potentials (Mealing *et al*, 1994). Delayed muscarinic inhibition of this current would then decrease cell excitability and prevent deleterious Ca^{2+} overload that otherwise could occur during prolonged stimulation.

In conclusion, activation of muscarinic receptors results in the inhibition of T-type Ca^{2+} current with a concomitant decrease in $[Ca^{2+}]_i$ in hen granulosa cells when faced with an elevated $[Ca^{2+}]_i$. These findings therefore suggest that Cch may serve a protective role in granulosa cells by modulating Ca^{2+} current to keep $[Ca^{2+}]_i$ within an as yet unidentified range. As such, the purpose of muscarinic input in granulosa cells may be in the long-term maintenance of granulosa cell health in terms of excitability and electrochemical balance. If so, muscarinic input may also regulate the concentrations of other divalent or monovalent cations, however, additional study is required to confirm or deny this hypothesis.

D. Regulation of $[Ca^{2+}]_i$ by TNFα during follicular development.

This study demonstrates for the first time that TNFα influences $[Ca^{2+}]_i$ in ovarian cells. Several studies have indicated that the action of TNFα is mediated by sphingomyelin breakdown (Schutze *et al*, 1992; Jayadev *et al*, 1994; Santana *et al*, 1995). Ceramide, the immediate product of sphingomyelin breakdown, has been demonstrated to activate a ceramide-activated protein kinase (Mathias *et al*, 1991), PKCξ (Lozano *et al*, 1994), as well as a cytosolic ceramide-activated protein phosphatase (Dobrowsky and Hannun, 1992). Nonetheless, it seems clear that sphingolipids alone cannot account for all of the pleiotropic effects of this cytokine. Furthermore, while Ca^{2+} is known to be involved in TNFα action in 30A5 preadipocytes (Lee *et al*, 1990), human and murine fibroblasts (Bouchelouche *et al*, 1990; Corkey *et al*, 1991), neutrophils (Richter *et al*, 1990; Schumann *et al*, 1993), anterior pituitary cells (Koike *et al*, 1991) and sympathetic neurons (Soliven and Albert, 1992), its role in ovarian cells has not previously been demonstrated.

A challenge of granulosa cells with TNFα in Ca^{2+}-free medium containing EGTA and Mg^{2+} or Mn^{2+} blocked the small, delayed increases in $[Ca^{2+}]_i$ observed in response to the cytokine, suggesting the involvement of a transmembrane influx of Ca^{2+} in the action of the cytokine in these ovarian cells. These observations, together with the 1 - 10 min delay in response time, are consistent with the concept that TNFα may be acting through a second messenger-operated Ca^{2+} channel rather than direct receptor-channel interaction. This is in

contrast to the quick response observed with Cch-induced Ca^{2+} transients that occurs within the first 10 sec of exposure to the agonist at a much greater rate of rise. In contrast to the mode of action of TNFα, regulation of Ca^{2+} signalling by muscarinic agonists appears to involve both mobilization of Ca^{2+} from intracellular stores and transmembrane influx of Ca^{2+}, presumably via G-protein mediated Ca^{2+} channel activation (Morley *et al*, 1992a).

The reported interaction between TNFα and Cch reveals the presence of complex, stage-dependent changes in the control of Ca^{2+} signalling and/or in the cross-talk between TNFα and muscarinic-signalling pathways. Although the precise mode of this interaction in the induction of Ca^{2+} mobilization from intracellular stores is unclear, the present findings suggest participation of a complex regulatory mechanism(s) upstream of the Ca^{2+} surge. The fact that pretreatment with TNFα only increased the magnitude of slow Cch-induced Ca^{2+} transients is interesting, as it implies that this portion of the granulosa cell population failed to maximally respond to Cch challenge despite the fact that higher concentrations of Cch will not induce larger Ca^{2+} transients in these slow responding cells. Consequently, the inability of this portion of the granulosa cell population to mobilize intracellular Ca^{2+} is attributed to their failure to access and/or fill Ca^{2+} stores. It is proposed that TNFα facilitates the filling of these stores by either 1) activation of Ca^{2+}-ATPase on Ca^{2+} stores with a concomitant influx of extracellular Ca^{2+} directly into these Ca^{2+} stores as suggested by the model of capacitive Ca^{2+} entry (Berridge, 1993), or, 2) Cch-induced IP_3 production, as we have previously demonstrated that fast Ca^{2+} transients in F1 cells are mediated by IP_3. Determining

which (if either) of the mechanisms proposed above accurately describe the mechanisms behind the TNFα-Cch interaction will require further experimentation.

Although the TNFα response was dependent on follicular maturation, a large portion of granulosa cells failed to respond to TNFα challenge with an increase in $[Ca^{2+}]_i$ irrespective of developmental stage or responsiveness to Cch. This could be explained by either changes in the density or nature of TNFα receptors in these cells or by uncoupling of receptor-ligand binding with Ca^{2+} mobilization. However, since cells that were incapable of TNFα-induced Ca^{2+} transients were capable of promoting Cch-induced intracellular Ca^{2+} mobilization, the hypothesis that changes in receptor density are responsible for this developmental inhibition is unlikely. Instead, we suggest that while receptor-ligand binding does occur, developmental changes in the predomimant receptor subtype or postreceptor effectors must account for these differentiative changes in the nature of the Ca^{2+} response to the cytokine.

Although no direct evidence exists regarding the mechanism(s) regulating the effects of TNFα on $[Ca^{2+}]_i$, the many recent studies indicating an involvement of sphingolipids cannot be ignored. Prior studies in other cell systems have demonstrated that sphingosine augments EGF-stimulated IP_3 levels in A-431 cells (Wahl and Carpenter, 1988) and Ach-stimulated IP_3 levels in C62B glioma cells (Brook *et al*, 1987). Clearly, our findings that both SMase and C8-ceramide mimicked the TNFα-Cch interaction as well as our demonstration that TNFα-induced increases in basal $[Ca^{2+}]_i$ could be mimicked by sphingosine suggest that the actions of TNFα in hen granulosa cells may be mediated by sphingolipids. Nonetheless,

since we were not able to demonstrate TNFα-induced ceramide production the roles of sphingolipids as mediators of TNFα action in hen granulosa cells remains highly suspect and will be discussed in greater detail below (Discussion Section E; Pg 209).

Data from the present study has demonstrated that the nature and magnitude of Cch-induced Ca^{2+} transients are modified by pretreatment with TNFα and that this TNFα effect increases with follicular development. This developmental increase in TNFα responsiveness coincides with large increases in granulosa cell progesterone production (Johnson, 1990) and basal fibronectin production (Asem *et al*, 1992) as well as a precipitous decline in [^{3}H]-thymidine uptake (Lafrance *et al*, 1993a). Furthermore, Cch-induced increases in progesterone production have been observed previously in both ovine (Heap *et al*, 1989) and bovine (Luck, 1990) ovaries. Nonetheless, Cch-induced increases in hen granulosa cell progesterone production were not observed irrespective of the presence or absence of cytokine or gonadotropin support. Consequently, the reported increase in the responsiveness of granulosa cells to both TNFα and Cch must be unrelated to the regulation of steroidogenesis. Moreover, unlike TGFα, the presence of Cch did not stimulate TNFα-induced cell survival or proliferative activity based upon MTT and vital staining studies. Although Cch inhibited fibronectin secretion in F1 granulosa cells, TNFα had no significant effect on this phenomenon. Consequently, the physiological implications of this interaction remain unknown and additional studies *in vivo* may be required to identify its role in the regulation of granulosa cell function.

In conclusion, our results demonstrate that 1) TNFα regulates granulosa cell $[Ca^{2+}]_i$, presumably via transmembrane influx of Ca^{2+}, 2) these changes are suppressed during follicular maturation, 3) TNFα enhances Cch-induced changes in $[Ca^{2+}]_i$, possibly by promoting mobilization of Ca^{2+} from intracellular stores and 4) the TNFα-Cch interaction is enhanced with follicular maturation. Irrespective of the mechanism(s) involved, the observed changes in $[Ca^{2+}]_i$ in response to TNFα and muscarinic input represent a novel and important finding and further studies should help to delineate the complex regulatory mechanisms involved in the control of Ca^{2+} signalling in ovarian cells during follicular development.

E. The regulation of granulosa cell function by sphingomyelinase during follicular development is not regulated by TNFα.

The primary purpose of this study was to determine if SMase mediates TNFα-induced changes in $[Ca^{2+}]_i$ and progesterone production in hen granulosa cells. Exogenous SMase, C8-ceramide and sphingosine mimicked TNFα-induced changes in $[Ca^{2+}]_i$, while C2-ceramide was ineffective. Nonetheless, TNFα-induced SMase activity was not detected either early or late in follicular development. This concept that TNFα action is independent of SMase activity in hen granulosa cells was further supported by the fact that while exogenous SMase and C2-ceramide inhibited LH-induced progesterone production, C6- and C8-ceramide stimulated progesterone production and TNFα challenge failed to influence granulosa cell steroidogenesis. Consequently, it can be concluded that although sphingolipids mimic TNFα-induced changes in $[Ca^{2+}]_i$, TNFα does not activate a SMase in this cell type.

Ceramide is known to result in mutually contradictory changes in cell survival, cell cycle progression and differentiative character in several cell types and experimental conditions. Ceramide is produced following exposure of U937 cells to TNFα. Furthermore, the addition of both C2-ceramide (Obeid *et al*, 1993) and natural ceramide (Ji *et al*, 1995) induced apoptosis in this cell line in a manner similar to the cytokine, suggesting a mediatory role for ceramide in TNFα-induced apoptosis. C8-ceramide and exogenous SMase induced apoptosis in granulosa cells of F1 and LWF hen follicles (Witty *et al*, 1996), while C2-

ceramide induced apoptosis in early antral rat follicles (Kaipai *et al*, 1996). In contrast, ceramide induced cell cycle arrest in HL-60 cells (Okazaki *et al*, 1990), Molt-4 leukemia cells (Jayadev *et al*, 1995) and fibroblasts (Gomez-Munoz *et al*, 1994) where the sphingolipid was also associated with cellular senescence (Venable *et al*, 1995). Finally, ceramide induced proliferation via ERK activation in HL-60 cells (Yao *et al*, 1995) and fibroblasts (Andrieu *et al*, 1994) or via activation of NF-κB and PKCς in Jurkat T cells (Lozano *et al*, 1994). These findings indicate that ceramide-induced responses vary depending upon both the cell type and the conditions of each independent study, perhaps reflecting different elements of ceramide signal transduction.

TNFα-induced ceramide production has been studied previously in both mammalian and avian ovaries. In rat preantral granulosa cells, the cytokine induced ceramide production (Santana *et al*, 1995), while Fas, a member of the TNFα family, induced ceramide production in theca cells (Foghi *et al*, 1998). Although TNFα-induced ceramide production has never been reported in the hen, TNFα and C8-ceramide induced apoptosis in large white follicle (LWF) granulosa cells, while only C8-ceramide induced apoptosis in F1 granulosa cells (Witty *et al*, 1996). Moreover, preliminary data from our laboratory demonstrated that pharmacological concentrations of TNFα (50 ng/ml) induced a small increase in ceramide production in LWF granulosa cells (138 ± 8.7 %, n = 2; data not shown). Since TNFα-induced ceramide production in F5,6 and F1 cells was not observed even at 50 ng/ml, this suggests the presence of a developentally regulated switch in either TNFα receptor subtype

or the expression of SMase. Although additional study would be required to confirm this hypothesis, this concept is consistent with current developmental models, since a large proportion of LWFs become atretic, while follicles within the follicular hierarchy are committed to ovulation *in vivo*.

In rats, TNFα-induced ceramide production has been suggested to mediate TNFα-induced inhibition of gonadotropin-induced steroidogenesis by inhibition of P450 side-chain cleavage enzyme (Santana *et al*, 1996), 3β-hydroxysteroid dehydrogenase isomerase (Santana *et al*, 1996) and P450-aromatase activity (Santana *et al*, 1995). Since TNFα did not induce the production of ceramide in hen granulosa cells, it is not surprising that TNFα did not inhibit gonadotropin-induced steroidogenesis. In addition, TNFα did not increase basal progesterone production in the hen. Since a key difference between TNFα action in avian and mammalian granulosa cells is their ability to increase the production of ceramide, it is possible that ceramide also mediates this TNFα effect. Furthermore, this suggests that there may be a difference in the type of TNFR expressed in avian and mammalian granulosa cells.

Our finding that both C6- and C8-ceramide stimulated basal progesterone production was the first demonstration of ceramide-induced steroidogenesis in any system. In contrast, both C2- and C6-ceramide inhibited LH-induced progesterone production in rat granulosa cells without ceramide-induced increases in basal progesterone production. Unlike in mammalian systems, granulosa cells in the hen are the primary producers of progesterone and androstenedione, while the theca produces estrogens (Nitta *et al*, 1993). Consequently,

ceramide-induced changes in hen granulosa cell steroidogenesis may better reflect the theca of mammalian follicles. Irrespective of the implications of ceramide-induced ovarian progesterone production in other species, the current study suggests that ceramide is a key signal transduction mediator regulating granulosa cell progesterone production. Although several different activators of the SMase pathway have been identified in other systems, the activators of this signal transduction pathway in hen granulosa cells have not been identified. Nonetheless, the fact that ceramide regulates both granulosa cell progesterone secretion and $[Ca^{2+}]_i$ reveals the presence of ceramide-regulated signal transduction mechanisms, suggesting that activators of this pathway are likely to exist.

Although ceramide-induced changes in Cch-induced Ca^{2+} transients occurred throughout follicular development, differences in progesterone production by F5,6 and F1 granulosa cells were observed. Specifically, C8-ceramide stimulated LH-induced progesterone production by F5,6, but not F1 cells. Consistent with prior studies (Asem and Hertelendy, 1985), progesterone production by F1 cells was approximately 10X higher than that observed in F5,6 cells. Furthermore, 10 ng/ml LH was nearly maximally stimulatory for F1 cells, but near the threshold for stimulation of F5,6 cells. Consequently, this follicular stage-dependent difference in C8-ceramide-induced progesterone production in the presence of LH may reflect the fact that F1 granulosa cells reached their maximum for progesterone secretion, while F5,6 cells did not. This concept is supported by observations that LH-induced increases in cAMP occur in granulosa cells removed from follicles from all of the

stages within the follicular hierarchy (with the largest increase observed at the F3-F4 developmental stages), yet LH-induced steroidogenesis continues to increase throughout these stages (Asem and Hertelendy, 1986b).

Activation of the SMase pathway resulted in changes in both basal and Cch-induced $[Ca^{2+}]_i$. Since TNFα did not activate SMase, the similarities between these observations and our previous findings of TNFα-induced Ca^{2+} transients may merely be coincidental. Nonetheless, although sphingosine-induced Ca^{2+} transients have been previously reported to occur via mobilization of Ca^{2+} from intracellular stores (Ghosh *et al*, 1990, Zhang *et al*, 1991), this study represents the first demonstration that ceramide regulates $[Ca^{2+}]_i$. Cch-induced Ca^{2+} transients occur via IP_3-mediated mobilization of Ca^{2+} from intracellular stores and/or transmembrane influx of Ca^{2+} (Morley *et al*, 1992a; Results section C; Pg 193). Similar to TNFα, pretreatment with either C8-ceramide or SMase stimulated slow Cch-induced Ca^{2+} transients, raising the interesting possibility that ceramide may facilitate either the filling of Ca^{2+} stores and/or Cch-induced IP_3 production. Unlike the cytokine, however, SMase treatment resulted in increases in fast Cch-induced Ca^{2+} transients. IP_3-induced Ca^{2+} efflux occurs in an all or none fashion (Berridge *et al*, 1993). These studies are consistent with the concept that ceramide-induced stimulation of Cch-induced Ca^{2+} transients occurs as a result of increased loading of Ca^{2+} stores, however, additional studies are required to confirm this conclusion.

The role of Ca^{2+} in steroidogenesis has been extensively studied. There have been

numerous studies showing that either the removal of extracellular Ca^{2+} with the Ca^{2+} chelator EGTA or the addition of Ca^{2+} channel blockers (verapamil, Mn^{2+} or Co^{2+}) can markedly inhibit FSH-, LH-, GnRH- or cAMP-induced steroidogenesis in hen, rat and swine granulosa cells (Higuchi *et al*, 1976; Velduis *et al*, 1982; Velduis *et al*, 1982a; Velduis *et al*, 1983; Carnegie and Tsang, 1983; Tsang and Carnegie, 1983; Carnegie and Tsang, 1984; Tsang and Carnegie, 1984; Asem and Hertelendy, 1986a). Nonetheless, since Cch (Morley *et al*, 1992a), ATP (Morley *et al*, 1994) and cholesystokinin (Morley *et al*, 1993) induce Ca^{2+} transients but not progesterone production, the role of Ca^{2+} in steroidogenesis is facilatory rather than direct. Similarly, since ceramide and Cch interacted to induce Ca^{2+} transients but not progesterone production, it can be concluded that Ca^{2+} has no role in ceramide-induced changes in steroidogenesis.

Although several studies have demonstrated that ceramide responses are acyl chain length dependent, the present study is the first to show that ceramides of different acyl chain length can elicit different responses within the same cell type. C8-ceramide induces apoptosis in hen granulosa cells while C2-ceramide is ineffective (Witty *et al*, 1996). In addition, activation of heterotrimeric protein phosphatase 2A (PP2A) from either bovine brain or heart by ceramide was dependent on acyl chain length in a cell-free system, with C10 > C6 > C2-ceramide while C18-ceramide had no effect (Dobrowsky *et al*, 1993). The acyl chain of sphingomyelin, the substrate for SMase, is normally 22 or 26 carbons in length. Consequently, the fact that C18-ceramide did not activate PP2A is surprising, since it is the

ceramide analogue most similar to membrane ceramide. Nonetheless, the presence of C2-ceramide in HL-60 cells was recently reported (Lee *et al*, 1996), suggesting that short chain ceramides may also be produced *in vivo*. If true, the differences in granulosa cell responses to different ceramide analogues noted in the present study may be physiologically relevent, irrespective of their relationship to the SMase pathway.

Although there is no evidence to date showing the locations within cells where ceramides of different acyl chain length accumulate, different cellular sites of ceramide production have been demonstrated. Linardic and Hannun (1994) have shown that only sphingomyelin in the inner leaflet of the plasma membrane is degraded by the neutral SMase. Although exogenous bacterial SMase was unable to induce apoptosis, it is apoptogenic when cloned into a mammalian expression vector and induced in the same cell line (Zhang *et al*, 1997b). In addition, TNFα-induced acidic SMase activity is present in endosomal/lysosomal compartments (Wiegmann *et al*, 1994) and ceramide produced from the acidic, but not neutral SMase can activate NF-κB (Wiegmann *et al*, 1994, Reddy *et al*, 1994, Schütze *et al*, 1992). This suggests the possibility that the acyl chain length dependence of ceramide responses may be due to differences in their intracellular location, resulting in the activation of different ceramide-induced signal transduction pathways.

In conclusion, although TNFα does not induce ceramide production, ceramide analogues regulate hen granulosa cell progesterone production and $[Ca^{2+}]_i$ during follicular development in an acyl chain length dependent manner. Considering the established

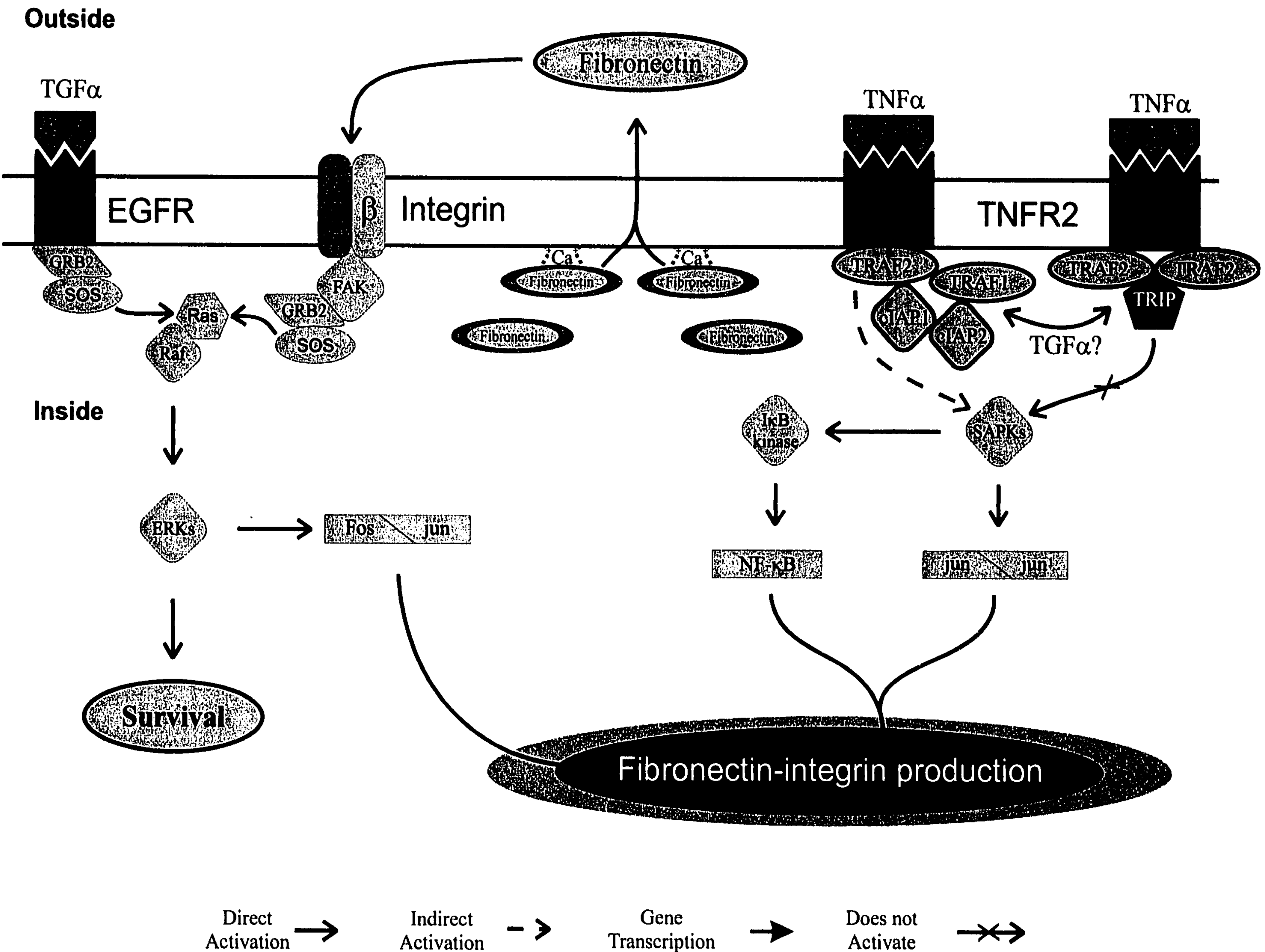
differences between ceramide responses under different conditions and cell types, the acyl chain length dependence of ceramide responses may reflect distinct differences in their abilities to access, bind to and/or activate ceramide-dependent signal transduction mechanisms. Prior studies have shown a role for ceramide as an inducer of cell death in hen granulosa cells, however, the current studies demonstrate the ability of ceramide to modulate granulosa cell $[Ca^{2+}]_i$ and progesterone production. As such, these studies provide novel insight into a role for ceramide as a regulator of granulosa cell differentiation.

VII. CONCLUSIONS

The primary objective of these studies was to study the actions and interactions of TNFα in hen granulosa cells in the regulation of granulosa cell fate during follicular development. Follicular stage-dependent interactions between TNFα and TGFα in the regulation of integrin β3 production and cell survival have been identified. Although the involvement of integrins in TNFα-TGFα-induced cell survival has not been established, integrin β3 has been shown to protect cells of different origins from apoptosis. Moreover, since TNFα was shown to induce apoptosis in LWF granulosa cells (Witty *et al*, 1996), TNFα may play a pivotal role in the determination of granulosa cell fate.

A hypothetical model depicting cross-talk between the TNFα, TGFα and integrin signal transduction mechanism(s) in the regulation of cell survival is proposed in Fig 53. A mechanism for the facilitation of TNFα-induced integrin production by TGFα has not been determined. Nonetheless, antigen receptors on lymphocytes have been shown to upregulate TRAF1, cIAP1 and cIAP2 and downregulate TRIP, thereby facilitating TNFα-induced NF-κB activation (Lee *et al*, 1997b). A similar mechanism is proposed for granulosa cells, although a mediatory role for SAPKs is included based upon the findings of Natoli *et al*, 1997). Based upon observations that these transcription factors can up-regulate vascular cell adhesion molecule-1 (Ahmad *et al*, 1998) and integrin α2 (Xu *et al*, 1998), it is proposed that the transcription factors NF-κB and AP-1 (jun/jun) mediate TNFα-induced integrin β3

Figure 53: A hypothetical model depicting TNFα-TGFα interactions. cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; EGFR, epidermal growth factor receptor; ERKs, extracellular signal-regulated kinase cascade; FAK; focal adhesion kinase; GRB2, growth factor receptor-bound protein 2; I-κB kinase, inhibitor subunit of NF-κB; NF-κB, nuclear factor κB; SAPKs, stress-activated protein kinase cascade; SOS, son of sevenless; TGFα, transforming growth factor alpha; TNFα, tumour necrosis factor alpha; TNFR2, TNFα receptor 2; TRAF1, TNFα receptor activated factor 1; TRAF2, TNFα receptor activated factor 2; TRIP, TRAF interacting protein



Outside
Inside
TGFα
EGFR
GRB2
SOS
Ras
Raf
ERKs
Survival
Fos
jun
β
Integrin
FAK
GRB2
SOS
Fibronectin
Ca
Fibronectin
Fibronectin
Fibronectin
Fibronectin
TNFα
TNFα
TNFR2
TRAF2
TRAF1
cIAP1
cIAP2
TRAF2
TRAF2
TRIP
TGFα?
SAPKs
IκB kinase
NF-κB
jun
jun
Fibronectin-integrin production
Direct Activation
Indirect Activation
Gene Transcription
Does not Activate

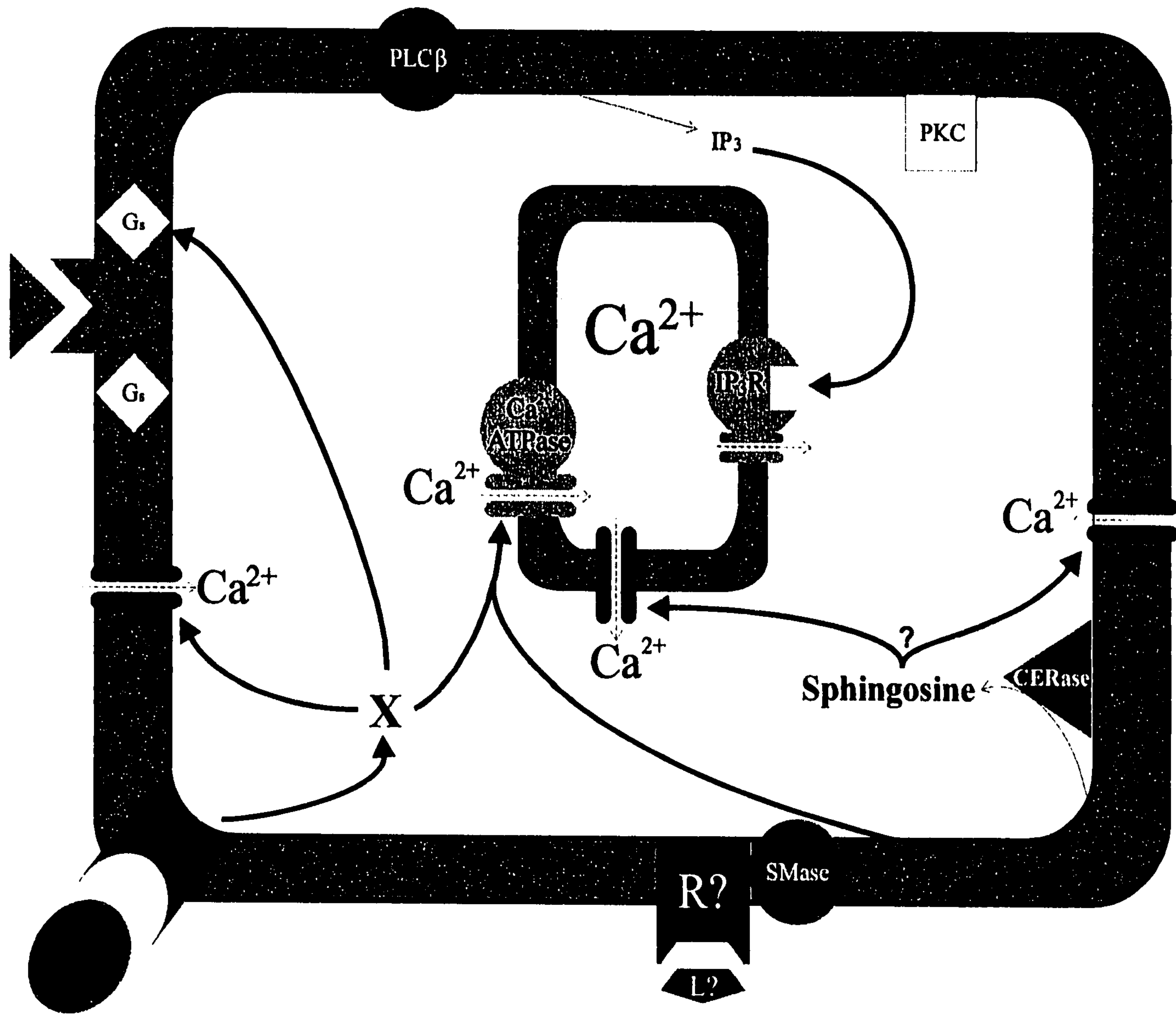
production. Both the fibronectin-integrin (via the tyrosine kinase FAK; Giancotti, 1997) and TGFα-EGFR (Birge and Hanafusa, 1993) interactions result in Ras activation through GRB2 (growth factor receptor-bound protein 2)-mediated stimulation of the guanine-nucleotide exchange factor SOS (son of sevenless), resulting in the activation of the ERK cascade. ERKs activate AP-1 (fos/jun), which may facilitate TGFα-induced integrin production and protection from apoptosis.

A second objective of these studies was to study the mechanism(s) of action and physiological role of the muscarinic agonist Cch. The ability of granulosa cells to mobilize intracellular Ca^{2+} via IP_3 production in response to the muscarinic agonist increased duringfollicular development. Moreover, in the face of elevated $[Ca^{2+}]_i$, muscarinic agonists inhibited T-type Ca^{2+} current with a concomitant decrease in $[Ca^{2+}]_i$ in F1 granulosa cells. Although a challenge with Cch had no effect on cell proliferation, survival, progesterone or integrin production, fibronectin secretion by F1 granulosa cells was inhibited in the presence of the muscarinic agonist irrespective of the presence of TNFα or LH. Since a relationship between $[Ca^{2+}]_i$ and protein secretion has been established, the inhibition of fibronectin secretion may be related to inhibition of Ca^{2+} current, although the role of the IP_3-induced Ca^{2+} transients has not been determined. Nonetheless, the degree of increase in the magnitude of Cch-induced Ca^{2+} transients in granulosa cells between the F3 and F1 developmental stages suggests that muscarinic Ca^{2+} transients may have important differentiative functions in granulosa cells.

Interactions between TNFα and Cch were also observed. TNFα was found to increase $[Ca^{2+}]_i$ in a small percentage of cells that decreased during follicular maturation. In contrast, TNFα enhanced Cch-induced changes in $[Ca^{2+}]_i$, in a manner that was enhanced by cytodifferentiation. Moreover, although the sphingomyelin metabolites ceramide and sphingosine mimicked these responses, TNFα-induced ceramide production was not observed, suggesting that this was only coincidental. Ceramides regulate progesterone production in an acyl chain length-dependent manner, although neither TNFα nor Cch (alone or in combination) affected granulosa cell steroidogenesis. Consequently, although the physiological significance of the TNFα-Cch interaction has not been determined, a role for ceramide as a regulator of granulosa cell steroidogenesis has been identified. Although the inducers of ceramide production for hen granulosa cells are unknown, the extent and location of its production may strongly influence its action since ceramide-induced progesterone production was both acyl-chain length- and concentration-dependent.

A hypothetical model depicting the regulation of hen granulosa cell $[Ca^{2+}]_i$ by TNFα, Ach and sphingolipids is proposed in Fig 54. Cholinergic activation of muscarinic receptors results in G-protein mediated transmembrane influx of Ca^{2+} and IP_3-mediated mobilization of Ca^{2+} from intracellular stores. Although the mediators of TNFα-induced $[Ca^{2+}]_i$ have not been identified, TNFα-induced Ca^{2+} transients also involve transmembrane influx of Ca^{2+}. It is also proposed that the cytokine increased the magnitude of Cch-induced Ca^{2+} transients via either increased IP_3 production or increased loading of Ca^{2+} in IP_3-sensitive Ca^{2+} stores.

Figure 54: A hypothetical model illustrating the regulation of $[Ca^{2+}]_i$ by TNFα and Ach in hen granulosa cells. "X" represents the unidentified mediators of TNFα-induced actions and interactions in the regulation of $[Ca^{2+}]_i$. Ach, acetylcholine; CERase; ceramidase; DAG, diacylglycerol, G_s, stimulatory G-protein subunit, IP_3, inositol (1,4,5) trisphosphate, IP_3R, inositol (1,4,5) trisphosphate receptor; L?, unidentified ligand; MR, muscarinic receptor, PKC, protein kinase C, PLCβ, phospholipase Cβ, R? unidentified receptor TNFα, tumour necrosis factor alpha; TNFR, TNFα receptor


PLCβ
IP3
PKC
Gs
Gs
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
Ca
ATPase
IP3R
X
?
Sphingosine
CERase
R?
SMase
L?
Regulation
Production
Movement

Although the activators of SMase in hen granulosa cells have not been identified, ceramide was found to similarily increase Cch-induced Ca^{2+} transients, presumably via increased loading of Ca^{2+} in IP_3-sensitive Ca^{2+} stores. Moreover, sphingosine increases $[Ca^{2+}]_i$ with a time-course similar to TNFα, although the source of sphingosine-induced Ca^{2+} transients is not known. Whereas TNFα and SMase regulate $[Ca^{2+}]_i$ in a similar fashion, this similarity is likely only coincidental, since TNFα did not activate a SMase in hen granulosa cells.

This research focused on TNFα and its interactions with Cch, TGFα and LH in the regulation of progesterone production, cell attachment, survival, proliferation, $[Ca^{2+}]_i$ and ceramide production during follicular development. Although changes in $[Ca^{2+}]_i$ in response to TNFα were detected in 25 to 50% of the cells studied, exposure of granulosa cells to the cytokine alone did not affect progesterone, cell attachment, cell survival or cell proliferation. Nonetheless, in the presence of TGFα, TNFα upregulated the attachment factor integrin β3 and protected granulosa cells from apoptosis. Moreover, the cytokine potentiated Cch-induced Ca^{2+} transients. In several different cell types, mutually contradictory responses to TNFα have been observed, including cell survival vs. apoptosis, differentiation vs. anti-differentiation and cell-cycle progression vs. cell-cycle arrest. The current studies demonstrate that both differentiative and proliferative factors modulate TNFα responses in a manner dependent on cytodifferentiation, resulting in granulosa cell responses to the cytokine which are appropriate to their stage of follicular development.

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IX. BIBLIOGRAPHY

AWARDS AND SCHOLARSHIPS

External Awards:

- Ontario Graduate Scholarship 1997-1998
  - Value: $11 859
- Natural Science and Engineering Research Council Studentship (Ph.D.) 1995-1997
  - Value: $34 800
- Ontario Graduate Scholarship 1994-1995
  - Value: $11 859

University Awards:

- University of Ottawa Excellence Scholarship 1997-1998
  - Value: $2500
- University of Ottawa Excellence Scholarship 1995-1997
  - Value: $14 174
- University of Ottawa Excellence Scholarship 1994-1995
  - Value: $2500
- University of Ottawa Admission Scholarship 1993-1994
  - Value: $3228

Others:

- Society for the Study of Reproduction Travel Award 1996
  - Value: $100
- Taichman Award for M.Sc. Research 1994-1995
  - Value: $150

REFEREED PUBLICATIONS:

1. **Soboloff J**, Tsang BK (1998) Follicular stage dependent TNFα-induced cell survival in the presence of TGFα in hen granulosa cells. *Manuscript in preparation.*

2. **Soboloff J**, Désilets M, Sorisky A, Tsang BK (1998) Acyl-chain length specific ceramide-induced changes in intracellular Ca^{2+} concentration and progesterone production are not regulated by TNFα. Biol Reprod *in press.*

3. Wan X, Désilets M, **Soboloff J**, Morris C, Tsang BK (1996) Muscarinic activation inhibits T-type Ca^{2+} current in hen granulosa cells. Endocrinology 137:2514-2521.

4. **Soboloff J**, Désilets M, Tsang BK (1995) Influence of tumour necrosis factor alpha on intracellular Ca^{2+} in hen granulosa cells *in vitro* during follicular development. Biol Reprod 53:546-552.

5. **Soboloff J**, Wade MG, Wells G, Désilets M, Tsang BK (1995) Influence of the muscarinic agonist carbachol on intracellular Ca^{2+} in chicken granulosa cells: I. Dependence on follicular maturation. Biol Reprod 52:721-728.

INVITED REFEREED REVIEWS

1. **Soboloff J**, Désilets M, Tsang BK (1997) Ca^{2+} signalling in avian granulosa cells during ovarian follicular development. In: Etches R and Harvey S (eds.) Perspectives in Avian Endocrinology. London, UK: Journal of Endocrinology Ltd., pp 225-240.

ABSTRACTS AND PRESENTATIONS

1. **Soboloff J**, Tsang BK (1998) Follicular stage-dependent TNFα-TGFα interactions in hen granulosa cells. Presented at the 17th Annual Ottawa Reproductive Biology Workshop. Ottawa, Ontario.

2. **Soboloff J**, Tsang BK (1997) The influence of TNFα and the sphingomyelinase pathway on granulosa cell progesterone production and DNA synthesis during follicular development. Presented at the 16th Annual Ottawa Reproductive Biology Workshop. Ottawa, Ontario.

3. **Soboloff J**, Sorisky A, Désilets M, Tsang BK (1996) TNFα-induced sphingomyelin degradation in avian granulosa cells. Presented at the 29th Annual Meeting of the Society for the Study of Reproduction, London, Ontario. Biol Reprod 55; supplement 1.

4. **Soboloff J**, Boone D, Sorisky A, Désilets M, Tsang BK (1996) Sphingomyelin Metabolites and Apoptosis. Presented at the 15th Annual Ottawa Reproductive Biology Workshop. Ottawa, Ontario.

5. **Soboloff J**, Sorisky A, Désilets M, Tsang BK (1995) Sphingomyelin degradation in TNFα signal transduction in hen granulosa cells. Presented at the 14th Annual Reproductive Biology Workshop. Ottawa, Ontario.

6. **Soboloff J**, Désilets M, Tsang BK (1994) The influence of the muscarinic agonist

carbachol on intracellular Ca^{2+} in chicken granulosa cells: interaction with tumour necrosis factor alpha. Presented at the 27th Annual Meeting of the Society for the Study of Reproduction, Ann Arbor, Michigan. Biol Reprod 53; supplement 1.

7. **Soboloff J**, Désilets M, Tsang BK (1994) The influence of the muscarinic agonist carbachol and tumour necrosis factor α on Ca^{2+} signalling in chicken granulosa cells. Presented at the 13th Annual Ottawa Reproductive Biology Workshop. Ottawa, Ontario.

8. **Soboloff J**, Désilets M, Tsang BK (1994) The influence of the muscarinic agonist carbachol on intracellular Ca^{2+} in chicken granulosa cells: I. Dependence on follicular maturation. Presented at the 41st Annual Meeting of the Society for Gynecologic Investigation. Chicago, Illinois.

9. **Soboloff J**, Désilets M, Tsang BK (1993) Modulation of intracellular Ca^{2+} concentration in chicken granulosa cells by tumour necrosis factor and carbachol. Presented at the 12th Annual Reproductive Biology Workshop. Ottawa, Ontario.