National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

Modulation Of Cyclic AMP Levels by EGF and Effect of cAMP on the Mitogenicity of Serum and EGF

by

Sanjay Kumar Pandey

Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Sciences.

Department of Biochemistry
Faculty Of Medicine
University of Ottawa
Ottawa, CANADA

ISBN 0-315-82542-1

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Epidermal Growth Factor (EGF) modulates cyclic AMP (cAMP) levels in non-neoplastic T51B rat liver epithelial cells induced by the β-adrenergic agonist isoproterenol (IPR) and by forskolin (FSK). In general the effect obtained depends upon whether pretreatment has taken place in the presence of serum (stimulation) or hepes (inhibition) on the duration of the pretreatment and on whether EGF was added before or after IPR or FSK. Activation of protein kinase C (PKC) by the tumor promoting phorbol ester, TPA, potentiated cAMP synthesis stimulated by FSK in both EGF pretreated cells and untreated cells although EGF pretreatment partially attenuated the effect of TPA.

Cyclic AMP secretion is one method by which cells regulate the changes in intracellular cAMP. It was observed that cAMP secretion gradually increased with time when untreated and EGF pretreated cells were stimulated with FSK or IPR. The increase in cAMP secretion did not account for all the changes in intracellular cAMP. In fact, although all treatments resulted in some accumulation of external cAMP, there was no correlation between the level of internal cAMP obtained or the decrease in internal cAMP and the amount secreted.

DNA synthesis was induced in serum deprived (0.2% BCS) cells by EGF or serum (10% BCS). This induction was inhibited by most cAMP elevating agents except isoproterenol and pertussis toxin. A combination of forskolin and the phosphodiesterase inhibitor, RO 201724 was most effective in inhibiting the DNA

synthesis stimulated by EGF. Forskolin and RO 201724 inhibited DNA synthesis in a time dependent manner whereas maximum isoproterenol inhibition (approximately 22%) was observed regardless of time of isoproterenol addition.

Genistein, a tyrosine kinase inhibitor, also inhibited EGF induced DNA synthesis. The inhibitory effect of genistein and cAMP elevating agents were not additive.

These results suggest that EGF modulates cAMP levels and the mitogenic effect of serum and EGF is inhibited by cAMP elevating agents.

DEDICATION

This thesis is dedicated to my grandparents Shri Kapil Deo Pande and Smt. Gangajali Devi Pande who always support me with their special love and kindness

ACKNOWLEDGEMENT

It is my great pleasure to acknowledge my supervisor Dr. L.P. Kleine, who has spent his valuable time reading, rewording and bringing this scattered project to a readable form. I am indebted to him for his valuable suggestions during the whole period of my work. I am also grateful to Dr.D.J.Franks for his good suggestions in various areas of the project. I would like to thank Dr.V.K.Shukla, my colleague for his time and patience in preparing the figures. The love and courage given to me by my parents (Dr.S.N.Pandeya and Mrs.Shanti Pandeya) and my in-laws (Dr.L.S.Dube and Mrs.U.Dube) helped maintain my morale high and kept me going. Special thanks to my wife Mrs. Archana Pandey and my sons Vineet and Rahul; without them these acknowledgements would be incomplete. I accept the affection shown by my brothers (Rajeev and Rajesh) and sisters (Sunita and Alka) which helped me during my work. I thank my sister in-law Ms.Ratna Dube for her technical help with computers. My youngest sister in-law, Meena Dube and brother in-law Abhay, made life easier for me.

I acknowledge Rakesh Sharma, who is working on a related project, with whom I spent many hours discussing his and my project. I would like to thank Caroline Brabyn, a graduate student in my laboratory and David Pyke, who worked in Dr.Kleine's laboratory when I started this project for their co-operation in the early days when this project started. Lastly, I thank those who directly or indirectly helped me during the course of my work in the department.

TABLE OF CONTENTS

List of Figures:

List of Abbreviations:

AC	Adenylate Cyclase.
ATP	Adenosine tri phosphate
BCS	Bovine Calf Serum.
BME	Eagle's Basal Medium
cAMP	cyclic Adenosine 3',5',-monophosphate.
cDNA	complimentary deoxyribonucleic acid.
CT	Cholera toxin
DAG	Diacylglycerol.
DNA	Deoxyribonucleic acid.
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethylsulfoxide.
db cAMP	Dibutyryl cyclic adenosine 3',5'-monophosphate.
EDTA	Ethylene diamine tetraacetic acid.
EGF	Epidermal Growth Factor.
EGF-R	Epidermal Growth Factor-Receptor.
FSK	Forskolin.
G	Guanine nucleotide binding protein.
G_i	Inhibitory G-protein of adenylate cyclase.
Gs	Stimulatory G-protein of adenylate cyclase.
GTP	Guanosine Triphosphate.
Hepes	4-(2-Hydroxyethyl-1-piperazine ethane sulfonic acid)
IP_3	Inositol 1,4,5-trisphosphate.
IBMX	3-Isobutyl-1-methyl-xanthine.
IPR	isoproterenol.
NCB-20	Neuroblastoma-Chinese-Hamster embryo Brain Explant.
NIH 3T3	a mouse fibroblast cell line (whole embryo/fetus)
PBS	phosphate buffered saline.
PC	phosphatidyl choline.
PDE	cyclic nucleotide phosphodiesterase.
PDEI	cyclic nucleotide phosphodiesterase inhibitor.
PE	phosphatidyl ethanolamine.
PGE_{1or2}	prostaglandin $E_{1 \text{ or } 2}$.
PGA_{1or2}	prostaglandin $A_{1 \text{ or } 2}$.
PI	phosphatidylinositol.
PIP	phosphatidylinositol-4-phosphate.
PIP2	phosphatidylinositol-4,5-bisphosphate.

PKA	protein kinase A.
PKC	protein kinase C.
PLC	phospholipase C.
PLD	phopholipase D.
PT	pertussis toxin.
PTK	protein tyrosine kinase.
RO	RO-201724
Tdr	deoxythymidine.
TGF-α	tumor growth factor-α.
T51B	a rat liver epithelial cell line.
TPA	12-O-tetradecanoyl-phorbol-13-acetate.

Chapter 1

INTRODUCTION

Transduction of signals from hormone or growth factor occupied receptors at the cell surface and by second messenger producing effector enzymes, e.g. phospholipase C (PLC), adenylate cyclase (AC), on the cytoplasmic face of the plasma membrane plays an important role in cell proliferation and differentiation. Guanine nucleotide binding proteins (G- proteins) function as transmembrane couplers which transmit the signals from the outside of the plasma membrane to the inside of the cell.

There are basically three classes of pathways which are involved in signal transduction:

(1) The Phospholipid Hydrolysis pathway: Ligands, when bound to receptors on the cell surface, stimulate the enzyme PLC. This activated enzyme in turn hydrolyses phosphatidyl inositol-4,5-bisphosphate (PIP_2) into inositol 1,4,5-tris-phosphate (IP_3) and 1,2 diacylglycerol (DAG) on the plasma membrane. IP_3 mobilizes Ca^{++} from intracellular stores while DAG activates protein kinase C (PKC). Example of such ligands are: vasopressin, oxytocin, α1-adrenergic agents, angiotensin and histamines which binds to H_1 receptors

(Watson,S. and Abbot, A; 1991). However, many reports show that PKC is activated by DAG which is also produced by hydrolysis of other phospholipids besides PIP_2. These include phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) (Exton, 1990; Nishizuka,Y; 1992) .

(2) Adenylate Cyclase and Cyclic AMP pathway: Stimulation of receptors by another group of agonists results in generation of cyclic AMP (cAMP) as a consequence of the action of a specific G-protein, Gs, which activates adenylate cyclase. Examples of this group of ligands are: glucagon, prostaglandin E_1, β-adrenergic agents, dopamine, adenosine and histamines that bind to H_2 receptors (Watson,S. and Abbot, A; 1991).

(3) Tyrosine Kinase Pathways: The receptors for several hormones and growth factors possess an intrinsic tyrosine kinase activity. These receptors have similar structures. They possess an extracellular ligand binding domain which is glycosylated, a single hydrophobic transmembrane domain and a cytoplasmic domain which has tyrosine kinase catalytic activity. A variety of oncogene products (e.g. Src and ros) also have an intrinsic tyrosine kinase activity. Examples include: epidermal growth factor receptor (EGF-R), insulin like growth factor receptor and platelet derived growth factor receptor.

Besides the above mentioned three major signal transduction systems, there

are other less characterized but nevertheless important systems such as the guanylate cyclase activating receptors and receptors which are ion channels. The ion channels span the plasma membrane and regulate the influx and efflux of cations and anions and therefore the physiological state of the cell. Examples of a guanylate cyclase activating ligand is atrial natriuretic factor and of ion channel regulating ligands are: excitatory amino acid γ-amino-butyric acid and glycine in the synapse, acetylcholine in the synapse and nicotinic acid.

1.1 EPIDERMAL GROWTH FACTOR

Epidermal growth factor (EGF) is a polypeptide with 53 amino acids and a molecular weight of 6045. EGF has a globular tertiary structure and is a stable polypeptide because there are three disulphide bridges (Holladay, Savage, Cohen and Puette; 1976). It was first isolated from male mouse submaxillary gland by Stanley Cohen and found to enhance the growth and maturation of epidermis in newborn mice (Cohen; 1962 and Cohen and Elliot; 1963) and of an organ culture of epithelial tissues (Cohen, 1965; Turkington, 1969; Bertsch and Marks, 1974; Starkey, Cohen and Orth, 1975). Its amino acid sequence is known (Savage, Inagami and Cohen, 1972). It has been shown that EGF is an extremely potent mitogen for fibroblasts (Armelin, 1973; Hollenberg and Cuatrecasas, 1973). EGF has been shown to stimulate DNA synthesis in hepatocytes both in vivo (Buchner, Patel and Cohen; 1978) and in vitro (Richman, Claus, Pilkis and Friedman; 1976).

1.2 EPIDERMAL GROWTH FACTOR RECEPTOR

Epidermal growth factor receptor (EGF-R) is a transmembrane glycoprotein (Das, Miyakawa, Fox, Pruss, Aharonov and Herschman; 1977 and Wrann and Fox; 1979) and its complete primary structure from cDNA is known (Ullrich, Coussens, Hayflick, Dull, Gray, Tam, Lee, Yarden, Liberman, Schlessinger, Downward, Mayer, Whittle, Waterfield and Seeburg; 1984). The receptor has two cysteine-rich extracellular domains connected by a single transmembrane domain which has 23 hydrophobic amino acids and a cytoplasmic tyrosine kinase domain of 542 amino acids. The receptor closely resembles the avian erythroblastosis virus erb-B oncogene product which has lost the EGF binding domain (Downward, Yarden, Mayes, Scrace, Totty, Stockwell, Ullrich, Schlessinger and Waterfield; 1984). In addition to the autophosphorylation of tyrosine residues by the receptor's tyrosine kinase, the intracellular domain of the receptor contains many phosphorylation sites for other kinases including PKC (Hunter, Ling and Cooper; 1984). In particular the phosphorylation of residue Thr-654 by PKC may play an important role in receptor functioning since phosphorylation of this residue is associated with a decrease in ligand affinity as well as in tyrosine kinase activity (Cochet, Gill, Meisenhelder, Cooper and Hunter, 1984; Freidmann, Frackelton, Ross, Connors, Fujiki, Sugimura and Rosner, 1984). It has been shown that a point mutation in the EGF-R at Lys-721, which is the ATP binding site, abolishes EGF induced tyrosine kinase activity as well as inositol lipid hydrolysis, ionic signalling and DNA synthesis. Chen et al. provide evidence that activation of tyrosine kinase activity

is essential but not sufficient for the multiple biological effects of EGF (Chen, Lazar, Poenie, Tsien, Gill and Rosenfeld, 1987).

1.3 MECHANISM OF ACTION OF EGF-R

The signal is transferred from the EGF-binding domain by an intermolecular mechanism (Yarden and Schlessinger, 1987). It has been shown that the allosteric aggregation model is suitable to explain the activation of the receptor kinase function by EGF. Briefly, this model depicts the existence of EGF receptors in equilibrium between oligomeric and monomeric states. It is assumed that the oligomeric state has high affinity towards ligand and therefore upon binding of EGF, equilibrium shifts towards active receptor oligomers. It has been observed that the EGF receptor exist in high affinity (10%) and low affinity (90%) states (Massague,1983b). After performing its biological function both EGF and its receptor are internalized via a clathrin-coated endosome system, (Bergeron, Cruz, Khan and Posner, 1985; Goldstein, Anderson and Brown, 1979; Maxfield and Yamashiro, 1987) and delivered to lysosomes where the receptor and its ligands are degraded (Carol and Hubbard, 1991a; Carol and Hubbard, 1991b).

1.4 INTERACTION OF EGF AND PKC

An increase in DAG, a physiological activator of PKC, has been reported in response to EGF in fibroblasts (Wright, Ragan, Shin and Raben, 1988) and in a muscle cell line (Farese, Nair, Sierra, Standaert, Pollet and Cooper, 1989).

However, there is no correlation between the increase of DAG and phosphoinositide turnover in both of the above cases. Recently it has been shown that PC may be a source of agonist induced DAG production (Exton, 1990). It is already known that tumor promoting phorbol esters such as, 12-O-tetradecanoyl phorbol-13-acetate (TPA), can substitute for DAG and directly activate PKC (Ashendel, 1985; Bjorge and Kudlow, 1987; El-Fakahary, Alger, Lai, Pitler, Worley and Barabam, 1988; Takada, Amino, Tetsumoto and Miyai, 1988).

It is possible that EGF activates PKC by other mechanisms such as that suggested recently for platelet activating factor (Pelech, Charest, Howard, Paddon and Salari, 1990). It has been shown that EGF exerts its effect, at least in part, through activation of PKC in freshly isolated rat hepatocytes (Conricode and Ochs, 1990). These effects include stimulation of lactate production and of the pentose phosphate pathway. There is also a report of PKC being activated by EGF in A431 cells (King and Cooper, 1986; Whitley and Glasner, 1986). It has been shown that PKC phosphorylates the EGF receptor at Thr-654 (Hunter et. al. 1984; Davis and Czech, 1985). Phosphorylation of EGF-R at Thr-654 in vitro causes an inhibition of the protein tyrosine kinase activity of the receptor (Cochet et al. 1984; Davis, 1988; Downward, Waterfield and Parker, 1985). It has been shown that phosphorylation of Thr-654 in phorbol ester treated NIH 3T3 cells causes an inhibition of the EGF induced mitogenic signals (Livneh, Dull, Berent, Ullrich and Schlessinger, 1988) but it is not clear whether growth inhibition is solely due to phosphorylation of Thr-654 on the EGF-R or if it is a result of some other effect of

phorbol ester on cultured cells. Recently it has been shown that phosphorylation of Thr-654 is regulated largely by the activity of PKC (Brown, Stanley, Selva and Davis, 1991). Therefore the physiological role of Thr-654 phosphorylation of EGF-R is not clear but it is clear that activation of EGF-R has significant effects on PKC and that activation of PKC in turn affects the activity of the EGF-R.

1.5 INTERACTION OF EGF AND cAMP/ADENYLATE CYCLASE SYSTEM

There are reports suggesting a role for cross-talk between the cAMP and EGF systems. It has been shown that under some circumstances EGF, like insulin, can antagonize cAMP-elevating hormones in isolated rat hepatocytes (Bosch, Bouscarel, Slaton, Blackmore and Exton, 1986). Another group suggested that cAMP is required for EGF stimulation of C-fos and C-myc mRNA production in A431 human epidermoid carcinoma cell line (Ran, Dean, Levine, Henkle and Campisi, 1986). Cholera toxin,(CT), which activates adenylate cyclase via G-proteins, was used to demonstrate the involvement of G- proteins in EGF stimulation of an ATPase activity of human hepatoma cells (Knowles, Salas-Prato and Villela, 1985). EGF has also been shown to act like glucagon, a cAMP inducer (Moule and McGivan, 1987). It has been suggested that protein kinase A phosphorylates the EGF-R in isolated plasma membranes of A431 cells or of rat liver in the presence of EGF (Ghosh-Dastidar and Fox, 1984; Rackoff, Rubin and Earp, 1984). In contrast, it was recently observed that pre-incubation of A431 cells with agents that elevate intracellular cAMP level, results in depression of EGF-R

phosphorylation and modulation of EGF-mediated inhibition of cell growth. For A431 cells, it was suggested that this effect is partly due to cAMP-mediated inhibition of DAG production induced by EGF via protein kinase A (Iwashita-Mitsui, Shoji-Kasai and Senshu-Miyaike, 1990). In the same cell line it was shown that EGF may modulate the activity of a component of the adenylate cyclase system resulting in stimulation of cAMP synthesis (Ball, Tanner and Carpenter, 1990). This increase might be due to modulation of cAMP metabolism by EGF which acts synergistically with cAMP elevating agents in A431 cells. The mechanism by which EGF and cAMP co-operate to stimulate epithelial cell proliferation is unknown. There is another report which shows that EGF increases cellular cAMP in perfused rat heart by stimulating the stimulatory guanine nucleotide binding protein Gs (Nair, Rashed and Patel, 1989). Furthermore it was observed that antiserum CS1, which is generated against the carboxy terminus of Gsα, is involved in mediating the effects of EGF on cardiac adenylate cyclase and the antiserum specifically inhibited Gsα function (Nair, Parikh, Milligan and Patel, 1990).

1.6 INTERACTION BETWEEN EGF AND PHOSPHOLIPASES

It is known that EGF upon binding to its cell surface receptors stimulates tyrosine phosphorylation of its substrates including EGF-R autophosphorylation (Carpenter and Cohen, 1979; Hunter and Cooper, 1985; Schlessinger, 1986; Schlessinger, 1988; Yarden and Ullrich, 1988) which leads to activation of PLC, (or phosphoinositidase). Activated PLC hydrolyses PIP_2 into IP_3 and DAG which

then mobilizes Ca^{++} from intracellular stores and activates PKC respectively (Majerus, Wilson, Connoly,Bross and Neufeld, 1985). Sawyer and Cohen (1981) first reported an increase in phosphatidyl inositol turnover in response to EGF in A431 cells. Gilligan et al. (Gilligan, Prentki, Gelennon and Knowles, 1988) found that EGF increases inositol phosphates and Ca^{++} in a liver tumor cell line and Johnson et al. (Johnson, Connelly, Sisk, Pobiner, Hewlett and Garrison, 1986) found an increase in cytosolic Ca^{++} in response to EGF with isolated hepatocytes. A small increase in inositol phosphates in response to EGF in hepatocytes was reported by Johnson and Garrison (Johnson and Garrison 1987).

Mammalian PLC is a family of at least four isozymes namely PLC-α (m.w.=62-68 kd), PLC-β (m.w.=150-154 kd), PLC-γ (m.w.=145-148 kd) and PLC-δ (m.w.=85-88 kd) (Rhee, Suh, Ryu and Lee, 1989). These isozymes have been differentiated mainly by immunological methods. All of them are single polypeptides and products of discrete genes (Rhee et al, 1989; Ryu, Pann, Cho, Lee and Lee; 1984). They share similar amino acid sequences in two separate regions which are important for their catalytic function. All isozymes show dependence on Ca^{++} and hydrolyse three phosphoinositides i.e. phosphatidyl inositol (PI), phosphatidyl inositol-4-phosphate (PIP) and PIP_2. They do not hydrolyse PC or PE. Their preference for substrate changes as the Ca^{++} concen-tration and pH are varied. It was proposed that there is a fifth isoform of PLC called PLC-ε (Rhee et al. 1989) but it is now believed to be very similar to PLC-δ. It has been observed that PLC-γ (and not PLC-β or PLC-δ) has sequence similarity with the Src or non-receptor

protein-tyrosine kinase family in the Src homologous domains of SH_2 and SH_3 regions. These regions are thought to be involved in PLC-γ activity and may provide substrate specificity (Pawson, 1988; Stahl, Ferenz, Kelleper, Kriz and Knopf, 1988). It has been observed that tyrosine phosphorylation of PLC-γ correlates well with the increased turnover of PIP_2 (Huckle, Hepler, Rhee, Harden and Earp, 1990). Thus EGF upon binding to its receptor elicits phosphorylation of several tyrosine residues at its carboxy terminus (Carpenter and Cohen, 1990). These phosphotyrosine residues are recognised by SH_2-domains of PLC-γ1 and phosphorylation of PLC-γ1 tyrosine residue 771,783 and 1254 by the receptor kinase domain occurs. These phosphorylated tyrosine residues in turn elicit many biological responses via hydrolysis of phospho-inositides. Although it has been shown that the EGF receptor upon binding of EGF activates PLC-γ through tyrosine phosphorylation, another means of regulating this interaction is via G-protein. In some membrane systems PLC activity can be stimulated by GTP-γ-s (Cockroft and Gomperts, 1985). Pertussis toxin,(PT), which inhibits Gi, inhibits EGF-mediated inositol phosphate accumulation in hepatocytes (Johnson and Garrison, 1987) and renal epithelial cells (Teitelbaum, Strasheim and Berl, 1990). However, PT does not attenuate the effect of EGF in some other cell types e.g. murine epithelial A431 cells, 3T3 fibroblast, vascular smooth muscle and liver epithelial WB cells. Thus different cell types respond differently to EGF in terms of coupling to PLC via G-proteins. A G-protein augments the tyrosine phosphorylation dependent activation of PLC-γ1 and the G-proteins may be

involved in activation of other isoforms of PLC.

The enzymatic hydrolysis of PC which constitutes up to 50% of phospholipids in mammalian tissues, is emerging as a wide-spread signal transduction pathway (Billah, Anthes,1990). Activation of PLC, phospholipase D (PLD) or both results in PC breakdown (Asaoka, Nakamura, Yoshida and Nishizuka, 1992). The hydrolytic products vary among cell types and even within the same cell depending on the agonist (Billah and Anthes, 1990). Available evidence suggest that PLD, the principal enzyme which hydrolyses PC, is regulated by multiple factors including G-proteins, Ca^{++} and PKC (Asaoka et al. 1992). The forms of PLD that are involved in stimulated cells are rather poorly defined in molecular terms because they have not been purified, cloned or sequenced (Asaoka et al. 1992) .

1.7 INTERACTION BETWEEN EGF AND TGF-α

The effect of EGF may either be mimicked or modulated by transforming growth factor-α (TGF-α). TGF-α is structurally similar to EGF (Massague, 1983a) and can bind and activate the EGF receptor (Massague, 1983b; Carpenter, Stoscheck, Preston and DeLarco, 1983). Therefore, it appears that all the biological effects of TGF-α are mediated through the EGF receptor. Initially in mammalian cells EGF and TGF-α were generally believed to be similar (Massague, 1983a). However, it has now been shown that there are marked differences in biological activities of EGF and TGF-α (Korc, Chandrasekar and Shah, 1991). The naturally active TGF-α and EGF show about 35-40% amino acid

homology with conservation of all six cysteine residues. The binding of TGF-α and EGF to the same receptor appears due to the similarity in conformation (Massague, 1983b; Tam, Marquardt, Roseberger, Wong and Todaro,1984). TGF-α is synthesized in embryos during early fetal development (Lee, Rochford, Todaro and Villareal, 1985), in virally transformed cells (Todaro, Lee, Webb, Rose, Brown, 1985) and in a variety of human tumors (Derynak, Goeddel, Ullrich, Gutterman, Williams, Bringmann and Berger, 1987). Although both peptides trigger many biological effects in a similar manner, but there are differences. Thus TGF-α exerts a greater stimulatory effect than EGF with respect to calcium mobilization in fetal rat long bones (Ibbotson, Harrod, Gowen, D'Souza, Smith, Winkler, Derynak and Mundy, 1986) angiogenesis in the hamster cheek pouch model (Schultz, White, Mitchell, Brown, Lynch, Twardzik and Todaro, 1987) and keratinocyte megacolony formation (Barrandon and Green, 1987). Thus from the above information it seems that TGF-α and EGF, although they utilize the same receptors, may differently regulate signal transduction pathways in different cell types.

1.8 CROSS-TALK BETWEEN PKC AND CYCLIC AMP/AC SYSTEMS

It has been demonstrated that PKC and the adenylate cyclase signalling systems interact. It is currently believed that PKC can phosphorylate and modify the activity of one or more of the components of the adenylate cyclase system. It was shown that, TPA, causes phosphorylation and activation of the catalytic subunit of adenylate cyclase in frog erythrocytes (Yoshimasa, Sibley, Bouvier,

Lefkowitz and Caron, 1987). However direct phosphorylation of the catalytic component of adenylate cyclase by PKC was shown in frog erythrocytes both in vivo and in vitro (Sibley, Benovic, Caron and Lefkowitz, 1988).

There is also evidence showing that PKC can modulate the adenylate cyclase system via G-proteins. In one report it was observed that activation of PKC by phorbol esters or diacylglycerol sensitizes adenylate cyclase to agents that act through Gs (Sugden, Vanecek, Klein, Thomas and Anderson, 1985; Aasheim, Kleine and Franks, 1989). Treatment of intact human platelets and S49 Cyc$^-$ lymphoma cells with phorbol esters caused phosphorylation and inactivation of Gi (Bauer and Jacobs, 1988; Watanabe, Horn, Bauer and Jacobs, 1985). It has also been demonstrated that the inhibition of adenylate cyclase is switched off by PKC phosphorylation of Gi in studies done with platelets in vitro (Katada, Gilman, Watanabe, Bauer and Jacobs; 1985).

There are also reports of PKC phosphorylating β-adrenergic receptors. Phorbol esters have been shown to result in phosphorylation of β-adrenergic receptors in duck erythrocyte (Sibley, Nambi, Peters and Lefkowitz, 1984) and rat reticulocytes (Yamashita, Kurokawa, Daneura, Higashi and Ishibashi; 1986) which results in desensitization of the receptor. Evidence is also accumulating which shows that increase in cAMP affects PKC. For example it has been shown that FSK treatment of NCB-20 cells, a mouse neuroblastoma-chinese hamster embryo brain explant hybrid cell line, increases cAMP levels and results in down regulation of membrane PKC (McAtee and Dawson; 1989).

Whether a cell proliferates, differentiates or enters a quiescent state is probably the result of a combination of different extracellular signals which act by different signalling pathways. The cross-talk between signalling pathways, either synergistic or antagonistic interactions, could provide a mechanism to fine tune proliferation and differentiation.

1.9 EGRESS OF CYCLIC AMP

Davoren and Sutherland were the first to report the efflux of cAMP from intact animal cells when they observed cyclic nucleotide in the medium of pigeon erythrocytes stimulated by L-epinephrine (Davoren and Sutherland; 1963). Since the first report, there have been reports of egress of cAMP from different cultured cell types and lower organisms e.g. bacteria (Makman and Sutherland; 1965), Dictyostelium discoideum (Konijin, van de Meene, Bonner and Barkley, 1967), and other metazoan cells (Barber and Butcher; 1983).

It has been shown that the process of efflux is unidirectional (King and Mayer; 1974) and it requires energy (Rindler, Bashor, Spitzer and Saier, 1978; Brunton and Mayer, 1979). In some cell lines there are reports which show that there is a cAMP binding site on the surface of these cells, e.g. the T51B rat liver epithelial cells (Kleine and Whitfield; 1987), and the Y-1 adrenal tumor cells (Wen, Chang, Reitherman and Harding; 1985). In some cells e.g. S49, HeLa and Chinese hamster ovary cells, cAMP-dependent ecto protein kinase activity was demonstrated (Kubler, Pyerin, Bill, Hotz, Sonka and Kinzel; 1989).

Efflux of cAMP can occur after binding of ligands to their receptors and activation of adenylate cyclase, for example, occupation of the adenosine receptor in pig aortic smooth muscle (Fehr, Dickinson, Goldman and Slakey; 1990). Another report demonstrated extrusion of cAMP by pigeon erythrocytes stimulated by the β-adrenergic agonist, isoproterenol (Brunton and Mayer, 1979). Some reports show that agents which activate adenylate cyclase directly and bypass receptor mediated adenylate cyclase activation lead to cAMP efflux e.g. CT (Brunton and Mayer; 1979) and forskolin (Zeytin, Mougin, Brazeau and Ling; 1984).

Some prostaglandins e.g. PGE_1, PGE_2, PGA_1 and PGA_2 activate adenylate cyclase by occupying receptors at the cell surface. This G-protein mediated activation leads to an increase in intracellular cAMP and inhibition of efflux (Rindler, Bashor, Spitzer and Saier; 1978). Prostaglandin PGA_1 and PGA_2 specifically inhibit cAMP efflux (Brunton and Mayer, 1979; Heasley and Brunton, 1985). It has been shown that inhibition by prostaglandins of cAMP efflux occurs by an intracellular effect at the site (undetermined as yet) of cAMP efflux (Heasley and Brunton, 1985; Hagey, Kanter and Brunton, 1988).

The amino acid sequence of adenylate cyclase from bovine brain shows similarity to many transporters and channels (Krupinski, Coussen, Bakalyar, Tang, Feinstein, Orth, Slaughter, Reed and Gilman; 1989). Most importantly, it has striking similarity to P-glycoprotein which is a multidrug transporter. Therefore it may be possible that cAMP is transported by adenylate cyclase itself.

Efflux of cAMP, seems not to be regulated significantly by cAMP-dependent

kinases in smooth muscle (Fehr et.al.; 1990). It has also been demonstrated that the S49 lymphoma cell line, which is deficient of catalytic subunit of cAMP-dependent kinase shows efflux of cAMP (Brunton, 1981).

The efflux of cyclic guanosine 3,'5,'-monophosphate has now been reported for cultured vascular smooth muscle and endothelial cells after induction by atrial natriuretic factor (Hamet, Pang and Tremblay, 1989).

1.10 CELL CYCLE

All eukaryotes from yeast to human have a similar cell cycle. The cell cycle is divided into four different stages: the G1, S, G2 and M phase. However there is another phase which is called G0 or quiescent phase during which cells do not divide or DNA does not replicate. The Go phase arises due to deprivation of growth factor(s) (Whitfield, 1992).

Transition from G0 to G1 requires changes in the level of many agents. During this phase of entry into the cell cycle from quiescence, observed changes include increase in Ca^{++} with corresponding cAMP, adenylate cyclase, PLC isozyme activities and a transient surge of membrane associated PKC (Whitfield, 1990; Martin, 1991;). During the S phase, DNA replication takes place and in the G2 phase cells prepare for mitosis. Finally in the M phase cell division takes place.

The G1 phase is the most interesting phase because it is during this phase cells prepare for DNA synthesis and it is in this phase where the controls of proliferation as well as differentiation are expected to occur (Whitfield,1990,1992).

In a variety of cell types cAMP and cAMP dependent kinases (Boynton and Whitfield, 1983), Ca^{++} calmodulin (Boynton, Whitfield and MacManus; 1980), protein kinase C (Boynton, Kleine, Whitfield and Bossi, 1985) and EGF (St.Hilaire and Jones, 1982) are involved in G1 transit or G1-S transition.

Cyclic AMP levels change transiently during the cell cycle and if one maintains a high level of cAMP using its analogue, dibutyryl cAMP (db cAMP), progression through the cell cycle is blocked. One of the reasons for this could be due to the fact that db cAMP is slowly metabolized. Thus it remains high for a prolonged period including S phase, where it blocks progression. It has been demonstrated that cAMP promotes proliferation (Boynton and Whitfield, 1983). A requirement for cAMP can be demonstrated in mutant yeast cells which possess defective adenylate cyclase. They can not normally transit the G1 phase and thus require exogenously added cAMP in order to proliferate (Kataoka, Powers, Cameron, Fasano, Goldfarb and Wigler, 1985).

The role of PKC in G1 or G1-S transit was also demonstrated when activators of PKC, (e.g. TPA and Oleyl-acetyl-glycerol)) stimulated cell cycle transition of cells that were blocked in late G1 (Boynton et al. 1985).

The mitogenic response of growth factors requires many enzymes among which are membrane bound PKC and cAMP dependent kinases. It has been observed that phosphorylation of the EGF receptor by PKC results in modulation of the receptor. However, PKC also stimulates adenylate cyclase which in turn increases cAMP and cAMP dependent kinases (Boney, Fink, Schlicter, Carr and

Wicks, 1983). This activation of cAMP dependent kinase in turn can affect the actions of growth factors. Thus it appears that both cAMP-dependent kinases and PKC together mediate mitogenic actions of various growth factors. Some other factors inhibit cell growth e.g. tumor necrosis factor (Broxmeyer, Williams, Lu, Cooper, Anderson, Beyer, Hoffman and Rubin, 1986).

Thus, growth factors whether they inhibit or stimulate cell growth, are convenient tools to elucidate the mechanism of cell cycle regulation. Finally, calmodulin also affects cells during the G1 phase as has been demonstrated for lymphocytes (Gorbachevskaya, Borokova, Rubin and Fedorov, 1983) and B-16 melanoma cells (MacNeil, Walker, Senior, Bleehen, Tomlinson; 1984) as well as for T51B rat liver cells (Boynton et al. 1980).

1.11 T51B Rat Liver Cell Line

T51B cells are non-neoplastic epitheloid derivatives of cells obtained from the liver of a normal adult Fischer rat (Swierenga, 1984). I chose this cell line because it was well characterized in terms of the roles of cAMP and PKC in proliferation. Proliferation of these cells has been shown to be regulated by both Ca^{++} and cyclic AMP (Swierenga, Whitfield, Boynton, MacManus, Rixon, Sikorska, Tsang and Walker, 1980). However Kleine et al. (1986) have shown that phosphorylation of cell surface proteins may be involved in the initiation of DNA synthesis. These proteins are phosphorylated in a Ca^{++} dependent manner.

Cross-talk between PKC and adenylate cyclase has been demonstrated in

these cells (Aasheim et al. 1989). It was demonstrated that the PKC activator,TPA, sensitizes the adenylate cyclase system to stimulation by IPR and FSK.

Epidermal growth factor is a good mitogen for most cell types. Hill et al.(1988) have shown that EGF stimulates DNA synthesis in T51B cells. They also showed that EGF stimulated DNA synthesis requires an influx of extracellular Ca^{++} and does not involve IP_3, since EGF does not lead to hydrolysis of PIP_2 in these cells and therefore no IP_3 is produced. EGF activates PKC via production of DAG which is produced by the hydrolysis of PC. The PIP_2 hydrolysis pathway operates in these cells but seemingly not in response to EGF.

The available evidence suggests that there are complex interactions between the EGF-R, adenylate cyclase and PKC. The interplay between these signalling pathways is not clear. However a technician in Dr.Kleine's laboratory in a few preliminary experiments has shown that EGF potentiates the cAMP levels stimulated by FSK or IPR.

Therefore I propose that EGF may affect the synthesis of cAMP and cAMP may in turn affect some of the mitogenic response of EGF. Activation of PKC can modulate the effect of EGF on the cAMP/adenylate cyclase signalling system.

The studies in this thesis are aimed at determining:

- the effect of EGF (potentiation or inhibition) on IPR and FSK stimulated cAMP synthesis and secretion.
- that PKC can modulate these effects of EGF.
- that cAMP elevating agents can inhibit the mitogenic action of EGF.

Chapter 2

MATERIALS AND METHODS

2.1 Materials:

Genistein and RO 201724 (RO) were purchased from BIOMOL (Philadelphia, PA). Epidermal growth factor was ordered from Upstate Biotechnology Incorporated (Lake Placid, N.Y.). Bovine Calf Serum (BCS) was obtained from Colorado Serum Co.(Denver, CO). Eagle's Basal Medium (BME) was ordered from GIBCO (Montreal, Canada). Cholera toxin, pertussis toxin, cyclic AMP, ethylene diamine tetraacetic acid (EDTA), 4-(2-hydroxyethyl-1-piperazine ethane sulfonic acid (Hepes), 12-O-tetradecanoyl phorbol-13-acetate (TPA), isobutyl methyl xanthine (IBMX), isoproterenol, forskolin and gentamicin sulfate were purchased from Sigma Chemical Co. Ltd. (St-Louis, MO). 2-8 ^{3}H-adenine and ^{14}C cAMP were obtained from NEN Research Products (Du Pont, Montreal, Canada). All other chemicals, of reagent grade, were purchased from BDH Chemicals or Fisher Scientific (Ottawa, Ontario).

2.2 Cell Cultures:

T51B rat liver epithelial cells were cultured as described by Jones et al. (1982). Cells were detached by brief exposure to 0.05% (w/v) trypsin in phosphate buffered saline (PBS). They were then plated in 100 mm dishes or 6-well multidishes in a medium consisting of 10% BCS and 90% BME containing 100

mg/l gentamicin sulfate. The cultures were then grown to confluence at 37 °C in an atmosphere consisting of 95% air and 5% CO_2. Cells were used for experiments 1 or 2 days after they reached confluence.

2.3 Cyclic AMP Synthesis:

The intracellular ATP pool was labelled by addition of 0.125 µCi of 2-8 ^3H adenine to the medium for 16-17 hours. The culture medium was changed two hours before the start of each experiment into fluid containing 10mM Hepes pH 7.2 in BME. All incubations were performed at 37 °C in an atmosphere containing 95% air and 5% CO_2 on a shaking platform.

In all experiments, RO was added to the cultures to prevent the degradation of newly synthesized cyclic AMP by cyclic nucleotide phosphodiesterase (PDE). Agents to be tested for their ability to stimulate cAMP synthesis were added in small volumes of BME or BME:DMSO (1:1) so that the final concentration of DMSO (dimethyl sulfoxide) in the medium never exceed 0.025%. Controls received only carrier. Incubations continued until appropriate times. Experiments were terminated by rapidly removing and saving the medium, washing the cells with cold PBS and then lysing them in 0.5 ml of lysis buffer containing 150 mM NaCl,10 mM Na_2HPO_4,1mM EDTA,0.1 mM cyclic AMP,0.1mM IBMX, 1% Nonidet P-40 and 0.001 µCi(^{14}C)-cyclic AMP to monitor [^3H]-cyclic AMP recovery. Labelled nucleotides were separated by ion exchange chromatography as described by Franks et al. (1985).

2.4 Cyclic AMP Secretion:

After terminating the reactions, the medium was removed and loaded onto ion exchange columns and processed as above.

2.5 DNA Synthesis:

T51B rat liver epithelial cells were plated in six-well dishes. After reaching confluence, cells were serum deprived by fluid changing into BME containing 0.2% BCS for 48 hours prior to addition of the agents to be tested. DNA synthesis was monitored by the addition of 0.5 μCi/ml [^{3}H]-thymidine for 24 hours. After the end of this time period the medium was removed and the cells were washed with ice cold PBS and fixed with PBS/formalin. DNA was extracted from acid insoluble material by heating at 80-90 °C in 0.5 N perchloric acid. Radioactivity incorporated into DNA was then determined by liquid scintillation counting.

2.6 Statistical Analysis:

All the statistical calculations were performed by the computer assisted INSTAT programme. The level of significance (P-values) was obtained using the paired two-tailed t-test comparing the values from untreated (control) to EGF pretreated values. The results are plotted as mean ± SE. The P-values <0.05 were considered as significant.

Chapter 3

RESULTS

3.1 Dose Response: Effect of EGF On Cyclic AMP Synthesis:

EGF has been shown to affect second messenger systems in a variety of cell types including effects on adenylate cyclase (Ball et al., 1990; Wright et al.,1988; Farese et al.,1989). However EGF alone does not affect the level of cAMP except in cardiac myocytes (Yu et al,.1992). Work in our laboratory has also shown that EGF alone does not affect cAMP levels in T51B cells (unpublished observation). EGF though has been shown to potentiate cAMP synthesis stimulated by cAMP elevating agents (Ball et al.,1990). Therefore I planned experiments to determine if EGF could potentiate cAMP synthesis stimulated by cAMP elevating agents and to determine the optimal concentration of EGF required.

Forskolin (FSK), one of the cAMP elevating agents, stimulates cAMP synthesis by directly activating adenylate cyclase. The optimal concentration of FSK in T51B cells had been shown to be 33μM. For the following experiments RO,(30μM), a phosphodiesterase inhibitor, was always present to stop the degradation of newly synthesized cAMP. Cells were stimulated with FSK (33μM) and two and half minutes after addition of FSK, different doses of EGF (0.015, 0.15, 1.5, 4.5, 15 and 150 ng/ml) were added to the cultures and the reaction was

terminated 30 minutes after the initial addition of FSK. Figure 1 shows that the optimal concentration of EGF lies between 15 and 150 ng/ml. Therefore I used the initial optimal dose of EGF (16.7 ng/ml) for all the following experiments.

3.2 Effect Of Adding EGF Before And After Forskolin Addition:

It had been previously shown that cAMP synthesis peaks 2.5 minutes after adding FSK to T51B cells (Aasheim et al,.1989) and remains high for up to 15 minutes. However work in our laboratory had shown that, in the presence of EGF, cAMP synthesis peaks at 30 minutes (i.e. 30 minute after FSK addition).

Cells were treated with EGF (16.7 ng/ml) 2.5 minutes after adding FSK (33 μM) and the reaction was terminated at various times thereafter. Figure 2 demonstrates that FSK by itself gives a peak at 2.5 minutes and when EGF is present the signal is prolonged culminating in a peak at 30 minutes. In each case, with or without EGF, cAMP remains elevated for at least up to 1 hour. To determine the optimal length of time that EGF should be present, I planned a series of experiments where EGF was added at different times before and after addition of FSK. FSK was always present for 30 minutes prior to termination of reaction. The results in Figure 3 are plotted as percentage of control (FSK alone). The results show that EGF is effective in potentiating the effect of FSK when present for a minimum of 20 minutes and a maximum of 50 minutes before lysis. This experiment demonstrates that EGF potentiates cAMP synthesis and that there is a window of 20 minutes before and 10 minutes after FSK addition where the

Figure 1: Effect of EGF dose response on cAMP synthesis in response to forskolin:

T51B cultures were fluid changed from serum/medium to Hepes/medium two hours before the start of the experiment. After this time cells were treated with FSK(33 μM) for 2.5 minutes before adding EGF. EGF was added at varying concentrations (0.015, 0.15, 1.5, 4.5, 15 and 150 ng/ml). The reaction was terminated 30 minutes after EGF addition. The results on the Y-axis are plotted as cyclic AMP (cpm x 10^{-3}) and on the X-axis EGF dose is expressed as ng/ml. The results are expressed as the mean ± SE of four different experiments. Significant differences, obtained when controls (no EGF,4.9 ± 0.28) are compared to the values obtained with different EGF doses by the two-tailed paired t-test, are indicated by stars as; * $P<0.007$, ** $P<0.005$, *** $P<0.0001$ and **** $P<0.0006$.

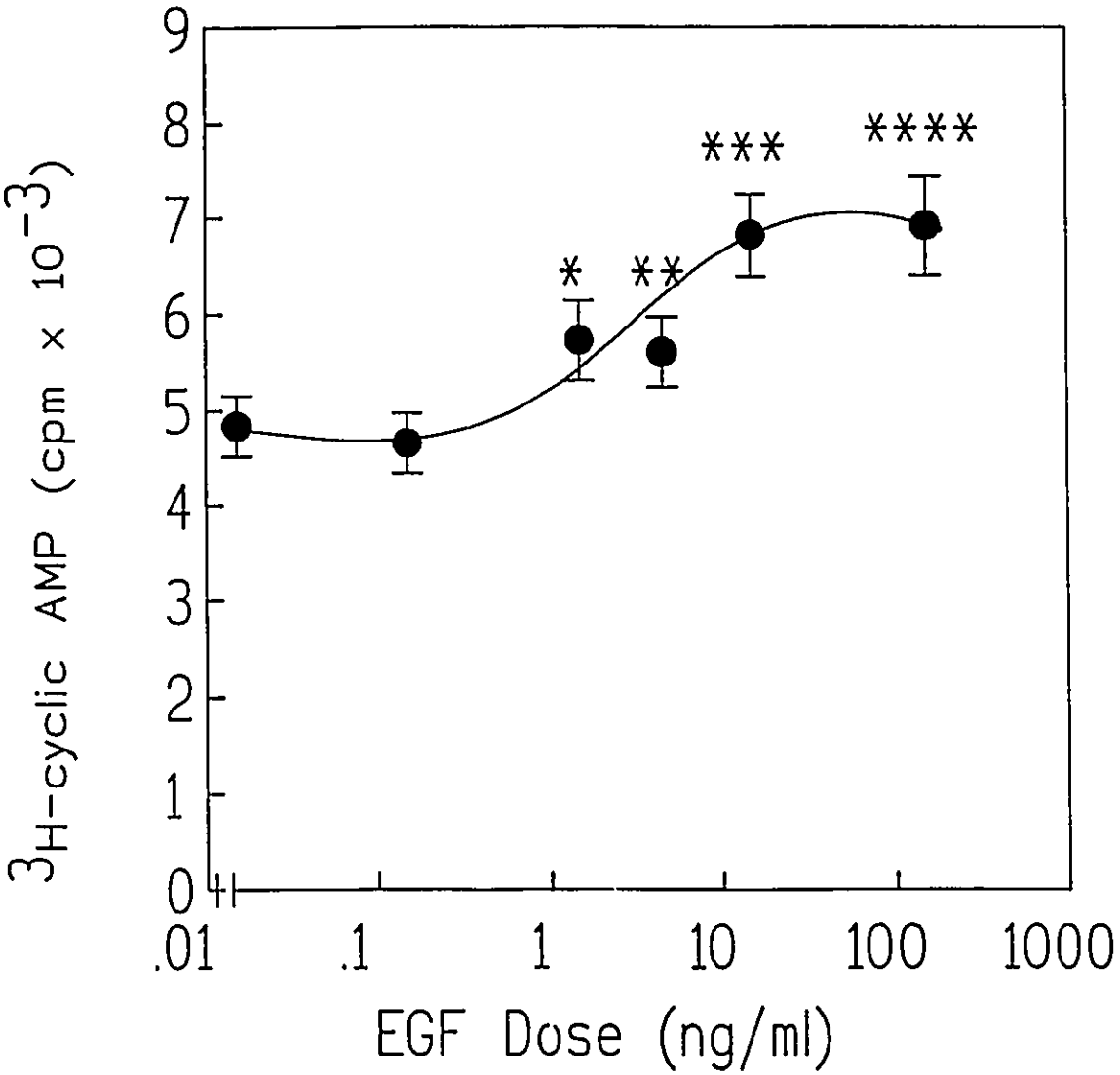

^{3}H-cyclic AMP (cpm x 10^{-3})
9
8
7
6
5
4
3
2
1
0
*
**
***
****
.01
.1
1
10
100
1000
EGF Dose (ng/ml)

Figure 2: Effect of EGF on the time course of forskolin stimulated cAMP synthesis:

T51B cells were incubated with Hepes/medium for two hours and then treated with FSK (33 μM) for different times. EGF (16.7 ng/ml) was added 2.5 minutes after FSK addition and the reaction was terminated at various times after FSK addition. Statistical analysis performed was the two-tailed paired t-test, comparing the values from three different experiments of control (no EGF) and treated (with EGF) cultures at 15, 30 and 60 minutes. The level of significance is indicated as : *$P<0.0001$, **$P<0.0043$ and ***$P<0.005$.

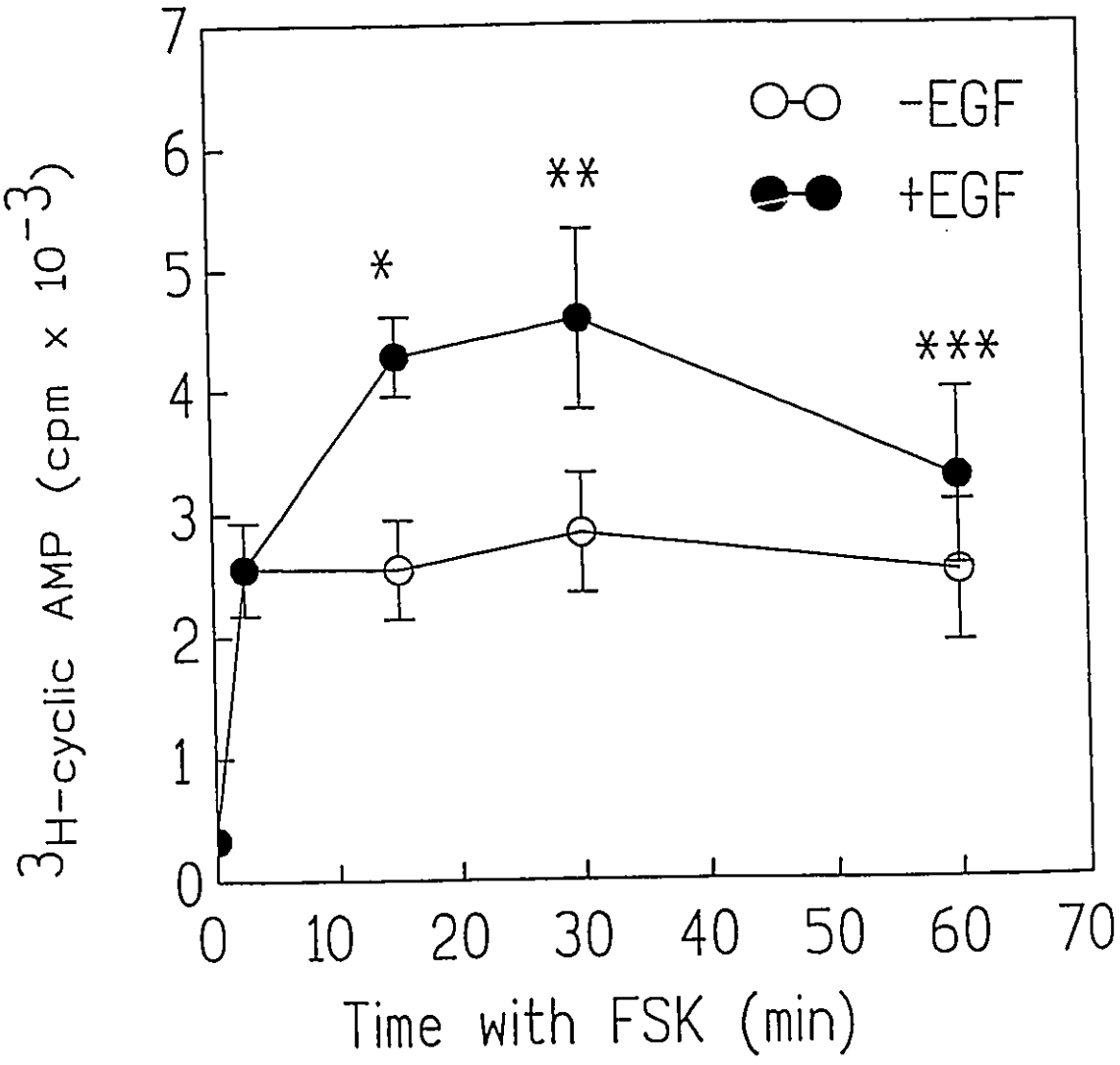
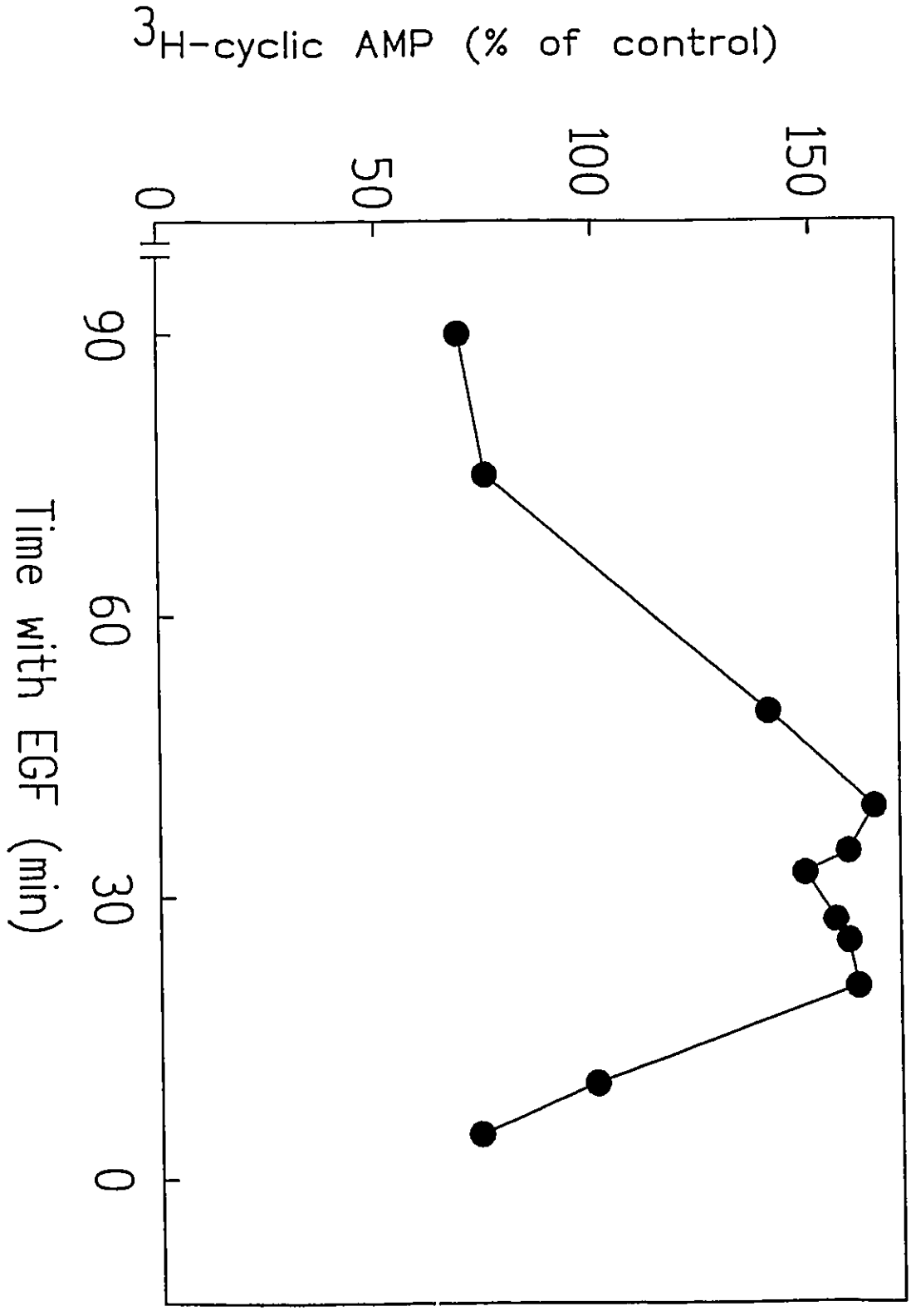

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
*
**
***
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

Figure 3: Time course of the effect of EGF on forskolin stimulated cyclic AMP synthesis:

For all culture dishes, FSK (33 μM) was present for 30 minutes. EGF (16.7 ng/ml) was added at various times, as indicated, either before or after addition of FSK. The results are expressed as percentage of control (without EGF) and are presented as mean ± S.E. of three different experiments.


^{3}H-cyclic AMP (% of control)
0
50
100
150
Time with EGF (min)
0
30
60
90

EGF effect can be demonstrated. It was also observed that adding EGF 5 minutes before lysis or 45 minutes or longer before FSK causes an inhibition of FSK stimulated cAMP synthesis.

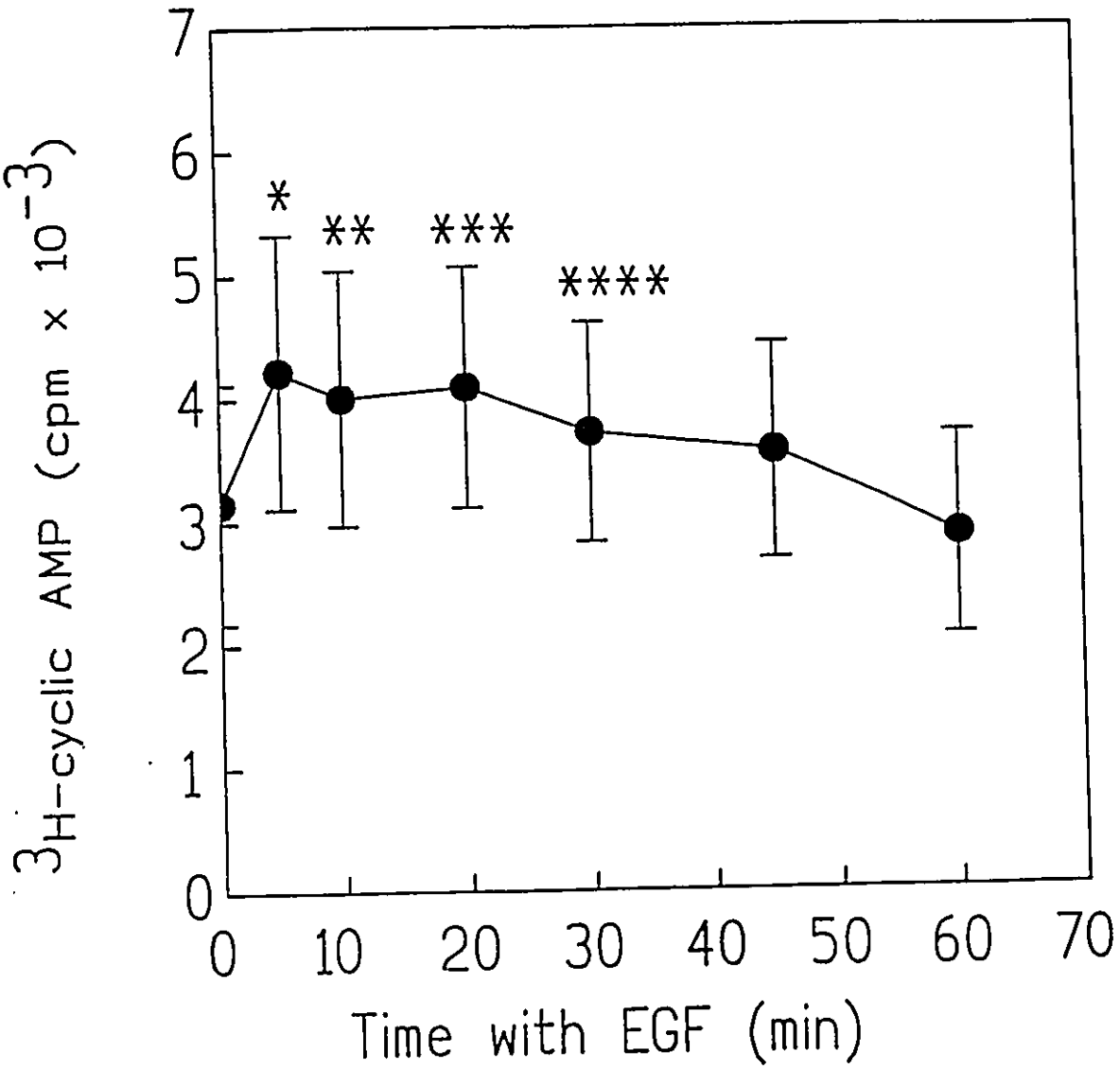
3.3 Temporal Effect of EGF on cAMP synthesis in response to Isoproterenol:

The effect of EGF on the cellular response to the β-adrenergic agonist IPR was studied. IPR (1μM) had previously been shown in our laboratory to give a peak of cAMP synthesis at 2.5 minutes. Therefore in the following experiment IPR was present for 2.5 minutes for each time point and EGF was present for various lengths of time. Figure 4 demonstrates that addition of EGF before adding IPR enhanced the response to IPR. However when cells are pretreated with EGF for longer times (1 hour or more) the potentiation of the IPR response was decreased.

The results from Figure 4 demonstrate that minimal potentiation of IPR-stimulated cAMP synthesis is obtained by EGF pretreatment. Nevertheless, preliminary experiments by myself and by a technician before I started the project indicated that EGF could potentiate the effect of IPR to a greater extent than was observed in Figure 4. It should be mentioned that preliminary results were not always consistent. I thus investigated this phenomenon further by examining the effect of the presence of serum during the EGF pretreatment (the best preliminary experiment seems to indicate that serum influences the EGF effect). The next two experiments report on the effect of a one hour EGF pretreatment in serum or

Figure 4: Time course of EGF effect on cAMP synthesis in response to isoproterenol (IPR) :

T51B cells were fluid replaced from serum/medium to Hepes/medium two hours before the start of the experiment. EGF (16.7 ng/ml) was added to cultures at different times before adding IPR. For each time point IPR (1 μM) was present for 2.5 minutes. The results are expressed as [^{3}H] cAMP (cpm x 10^{-3}) and represent the mean ± S.E. of four different experiments. The statistical significance (P-values) are indicated as: *P<0.0020, **P<0.0024, ***P<0.0002 and ****P<0.02.


^{3}H-cyclic AMP (cpm x 10^{-3})
7
6
5
4
3
2
1
0
*
**
***
****
0
10
20
30
40
50
60
70
Time with EGF (min)

Hepes. For Figure 5, cultures were pretreated for 1 hour in Hepes/medium and then IPR was added and cAMP levels were determined at various times after adding IPR.

Previously it had been shown that after IPR addition the maximum stimulation occurred at 2.5 minutes (Aasheim et al.1989) which is consistent with the result in Figure 5. The pretreatment with EGF did not alter the time at which cAMP synthesis peaked. The decrease in cAMP synthesis in the EGF pretreated cells may not actually represent a decrease in IPR stimulation of adenylate cyclase, since cAMP synthesis has been decreased by an equal amount in the cells to which IPR was not added (zero time). The EGF-pretreatment seems to have inhibited background adenylate cyclase and inhibition of this pool of adenylate cyclase is reflected in the cultures treated with IPR. The statistical analysis, by the two-tailed paired t-test, at times 0, 15, 30 and 60 minutes, showed that the P-values (level of significance) are: $^{*}P<0.0001$, $^{**}P<0.003$, $^{***}P<0.02$ and $^{***}P<0.02$ respectively.

Opposite results were observed when cells were pretreated with EGF (16.7 ng/ml) for one hour in the presence of serum/medium instead of Hepes/media (Fig.6). After incubation, the serum/medium was replaced with fresh EGF-free Hepes/medium and the cells were incubated for two hours in the Hepes/medium before they were stimulated with IPR. Cyclic AMP synthesis was found to be significantly elevated in EGF-pretreated cells as compared to untreated cells. The effect of EGF pretreatment gives opposite results depending on whether the

Figure 5: Effect of EGF pretreatment (1 hour in Hepes/medium) on the time course of IPR stimulation of cAMP synthesis:

T51B cells were incubated with EGF (16.7 ng/ml) for 1 hour in Hepes/medium. Control cultures did not receive EGF. The cells were then stimulated with IPR (1 μM) for different times. The results are expressed as [^{3}H] cAMP (cpm x 10^{-3}) and represent the mean ± S.E. of four different experiments. The statistical analysis performed was the two-tailed paired t-test. A significant difference between EGF-pretreated and untreated cultures was observed. The level of significance (P-value) is indicated as: * $P<0.0001$, ** $P<0.003$, ***$P<0.02$.

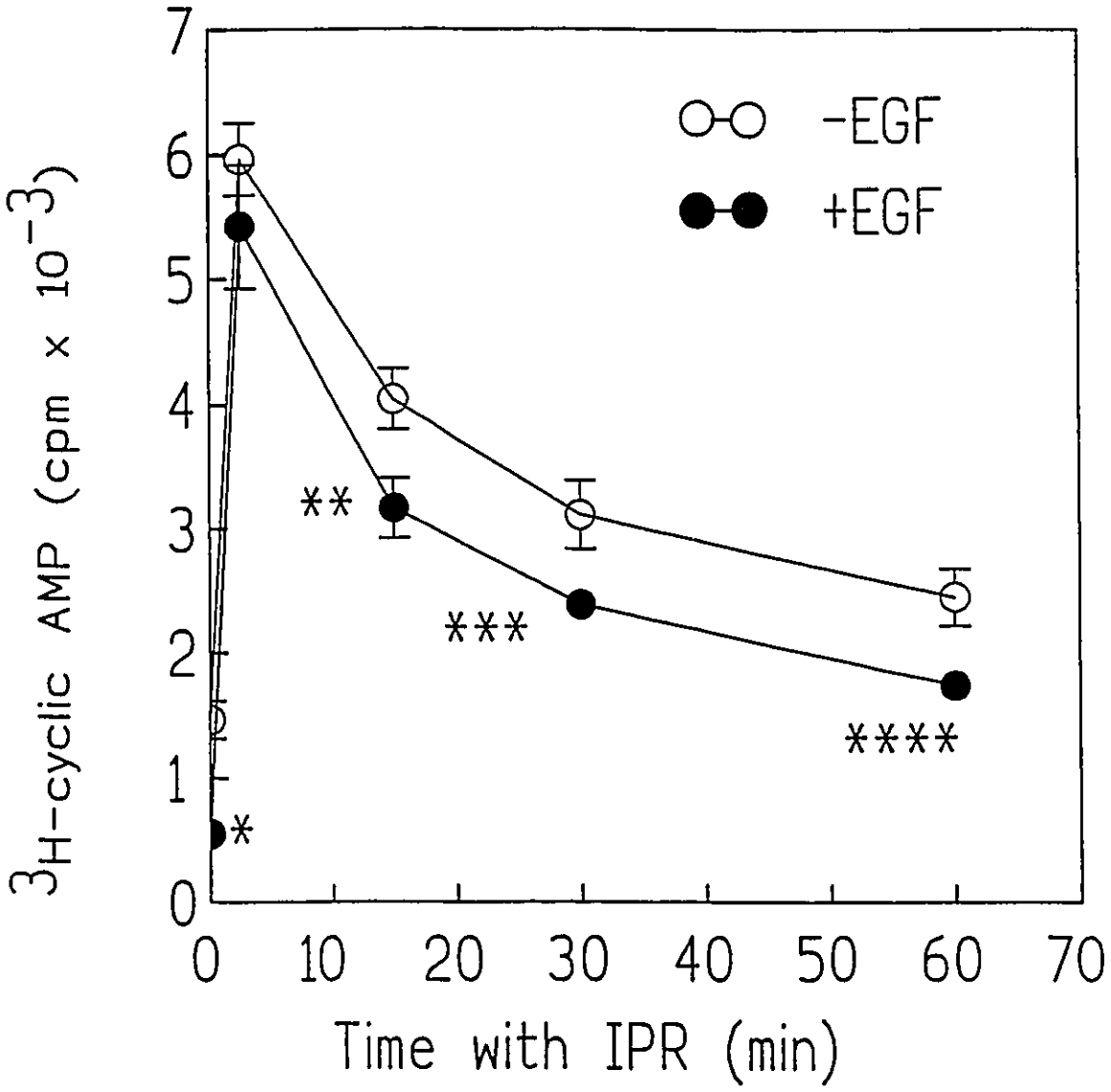
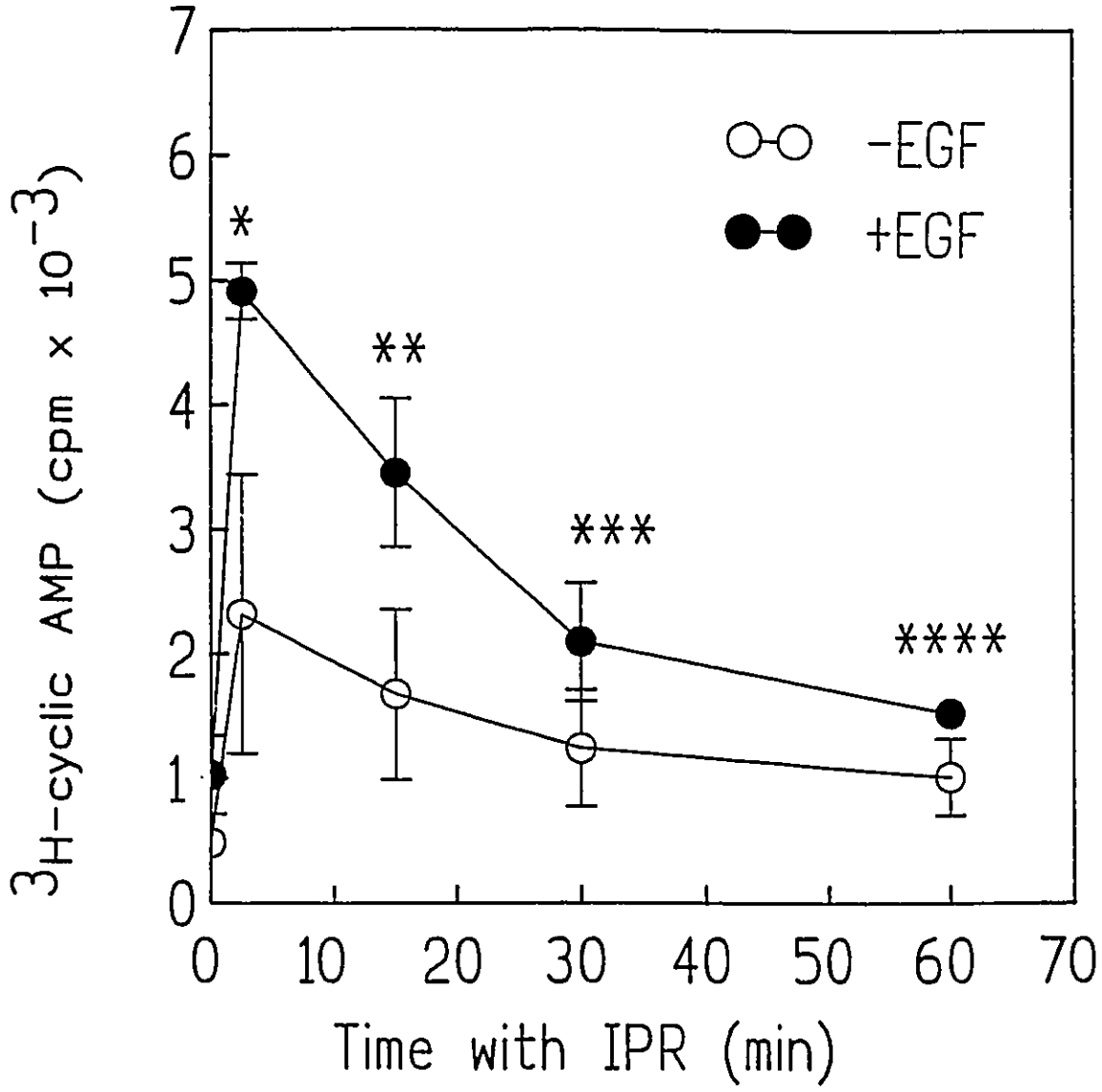

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
*
**
***
****
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with IPR (min)

Figure 6: Effect of EGF pretreatment (1 hour in serum/medium) on the time course of IPR stimulation of cAMP synthesis:

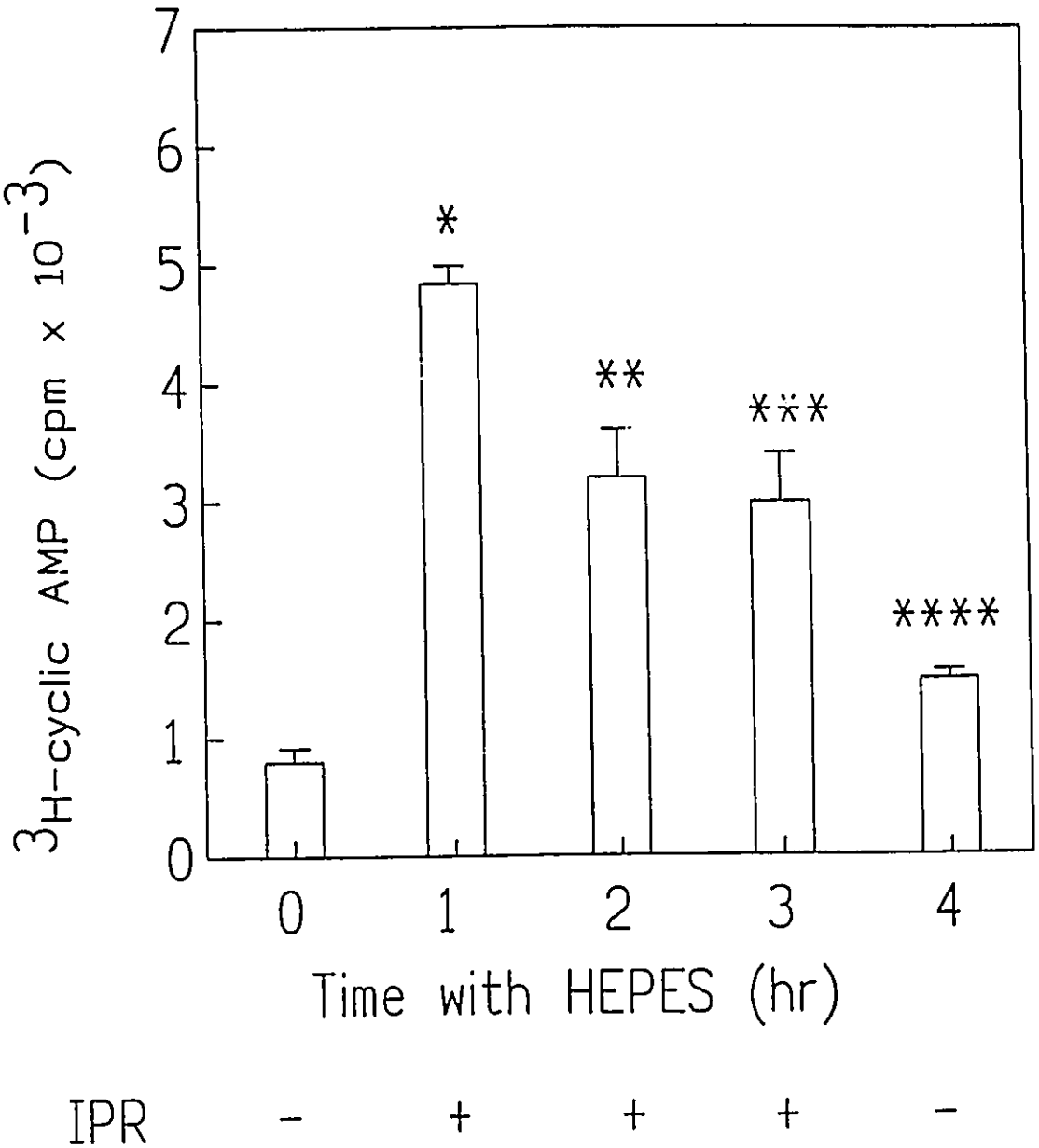
EGF (16.7 ng/ml) was added to T51B cultures for 1 hour in the presence of serum/medium. After one hour of incubation the serum was removed and cells were incubated with Hepes/medium for the next two hours. Control cultures did not receive EGF. The cells were then stimulated with IPR (1 μM) for different times. The results are expressed as [^{3}H] cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. The statistical analysis performed was the two-tailed paired t-test. A significant difference between EGF-pretreated and untreated cultures was found. The level of significance is indicated as: *P<0.0001, **P<0.025, and ***P<0.024.


7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
*
**
***
****
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with IPR (min)

pretreatment has taken place in the presence of serum/medium (stimulation, Fig.6) or Hepes/medium (inhibition, Fig.5). The level of cAMP is about two-fold higher at 2.5 and 15 minutes in EGF-pretreated cells as compared to untreated cells. The level of significance (P-value) at times 2.5, 15 and 60 minutes are marked as: $^{*}P<0.0001$, $^{**}P<0.025$, and $^{***}P<0.024$ respectively. These opposite effects could be due to the presence of serum during the EGF pretreatment or they could be due to the length of time that the cells were incubated in Hepes/medium (without serum). For Figure 5, the cells were exposed to Hepes for 1 hour in the presence of EGF and for Figure 6, the cells were exposed to Hepes for 2 hours (post-EGF/serum pretreatment). Thus for untreated cells, cAMP synthesis in response to IPR was much lower when cells had been exposed to Hepes/medium for two hours (Fig.6, controls) as compared to cells treated with Hepes/medium for one hour (Fig.5, controls). It seems that longer exposure to Hepes/medium (2 hours) has rendered the cells less responsive to IPR. This observation suggests that a three hour exposure to Hepes/medium would make the cells even less responsive to IPR. This is observed in Figure 7, where cells show different responsiveness to IPR depending upon the length of incubation in Hepes/medium. Figure 7 also demonstrates that not only is there a decrease in IPR responsiveness, but an increased time of exposure to Hepes/medium aiso results in an increase in background cAMP synthesis. Also, for Figure 4, cultures had been pre-incubated with Hepes/medium before starting EGF treatment. Thus at zero time, cells had been exposed to Hepes for only two hours whereas at the last time point (60

Figure 7: Temporal effect of incubation in Hepes/medium on cAMP synthesis stimulated with IPR:

T51B cells were stimulated with IPR (1 μM) after cells had been exposed for different times to Hepes/medium. IPR was added after 1, 2, and 3 hours of incubation in Hepes/medium and cells were lysed 2.5 minutes later. Control cells did not receive IPR (1 and 3 hours). Statistical analysis performed was two-tailed paired t-test comparing the controls (1 hour with no IPR) to all other treatments. The P-values are indicated as *P<0.0004, ** P<0.017 *** P<0.0225 and **** P<0.0087.


7
6
5
4
3
2
1
0
^{3}H-cyclic AMP (cpm x 10^{-3})
*
**
***
****
0
1
2
3
4
Time with HEPES (hr)
IPR
-
+
+
+
-

minutes EGF pretreatment) the cells had been exposed to Hepes for a total of 3 hours. The increase in time of exposure to Hepes could account for the loss of EGF effect at 1 hour (Fig.4).

Thus longer incubation in Hepes/medium, or probably more to the point, incubation in the absence of serum, results in the desensitization of the cells to IPR. Under conditions where cells are still highly responsive to IPR (Fig.5) EGF has a minimal effect, possibly due to a decrease in the activity of an adenylate cyclase pool that is not responsive to IPR. In contrast, when cells are no longer very responsive to IPR (Fig.6) EGF seems to resensitize the cells to IPR. Note that the curves for EGF-pretreated cells are identical in Figures 5 and 6. In other words, the maximal potentiation by EGF is observed under conditions where the β-adrenergic receptors would be down regulated or inactivated (i.e. with longer incubation in Hepes/medium). Although the results on the effect of Hepes/medium pretreatment are very interesting and require further work, I did not pursue these studies since my project involved mainly the study of effects of EGF on the adenylate cyclase/cAMP signalling pathway.

3.4 Effect of EGF Pretreatment on forskolin stimulated cAMP Synthesis:

It was of interest to see if the inhibition of FSK induced cyclic AMP synthesis by one hour pre-incubation with EGF in Hepes/medium (Fig 3) could be inhibited further by a more prolonged pretreatment with EGF. T51B cultures were incubated for two hours with EGF (16.7 ng/ml) in the presence of Hepes/medium.

After this time cells were stimulated with FSK (33 μM) and the reaction was stopped at different times after addition of FSK. It can be seen that there is no significant difference in the level of cAMP between EGF-pretreated and untreated cultures (Fig 8). Thus the inhibition observed with one hour pretreatment can no longer be seen if the incubation with EGF is increased to two hours in Hepes/medium. Figure 9 shows the effect of EGF-pretreatment (16.7 ng/ml) for one hour in serum/medium followed by incubation for two hours in EGF-free Hepes/medium. The cultures were then stimulated with FSK for different times. The results show that cAMP synthesis is enhanced in EGF-pretreated cells as compared to untreated cells. The increase in cAMP synthesis was more prominent between 2.5 and 30 minutes. The statistical analysis for Figure 9 was performed by the two-tailed paired t-test comparing the values at each time point between EGF-pretreated and untreated cultures. The P-values (level of significance) at 2.5, 15, and 30 minutes are marked as: $^{*}P<0.022$, $^{**}P<0.005$,$^{***}P<0.001$ respectively. The effect of length of time of incubation in Hepes/medium was not examined. For Figures 1, 2, 3 and 8, cultures had in all cases been exposed to Hepes/medium for two hours. Although prolonged treatment with EGF in Hepes/medium leads to a disappearance of the potentiation of FSK stimulated cAMP synthesis (Figures 3 and 8) the potentiation of cAMP synthesis can be observed if the EGF pretreatment has taken place in serum (Fig.9). It should be noted that the control curves for FSK alone are almost identical for Figures 8 and 9.

Figure 8: Effect of EGF pretreatment (2 hours in Hepes/medium) on the time course of forskolin stimulation of cAMP synthesis:

EGF (16.7 ng/ml) was added to T51B cultures for 2 hours in the presence of Hepes/medium. Control cultures did not receive EGF. After 2 hours of incubation cells were stimulated with FSK (33 μM) for different times. The results are expressed as ^{3}H-cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. No significant difference was observed between EGF pretreated and untreated cultures when values were compared by two-tailed paired t-test.

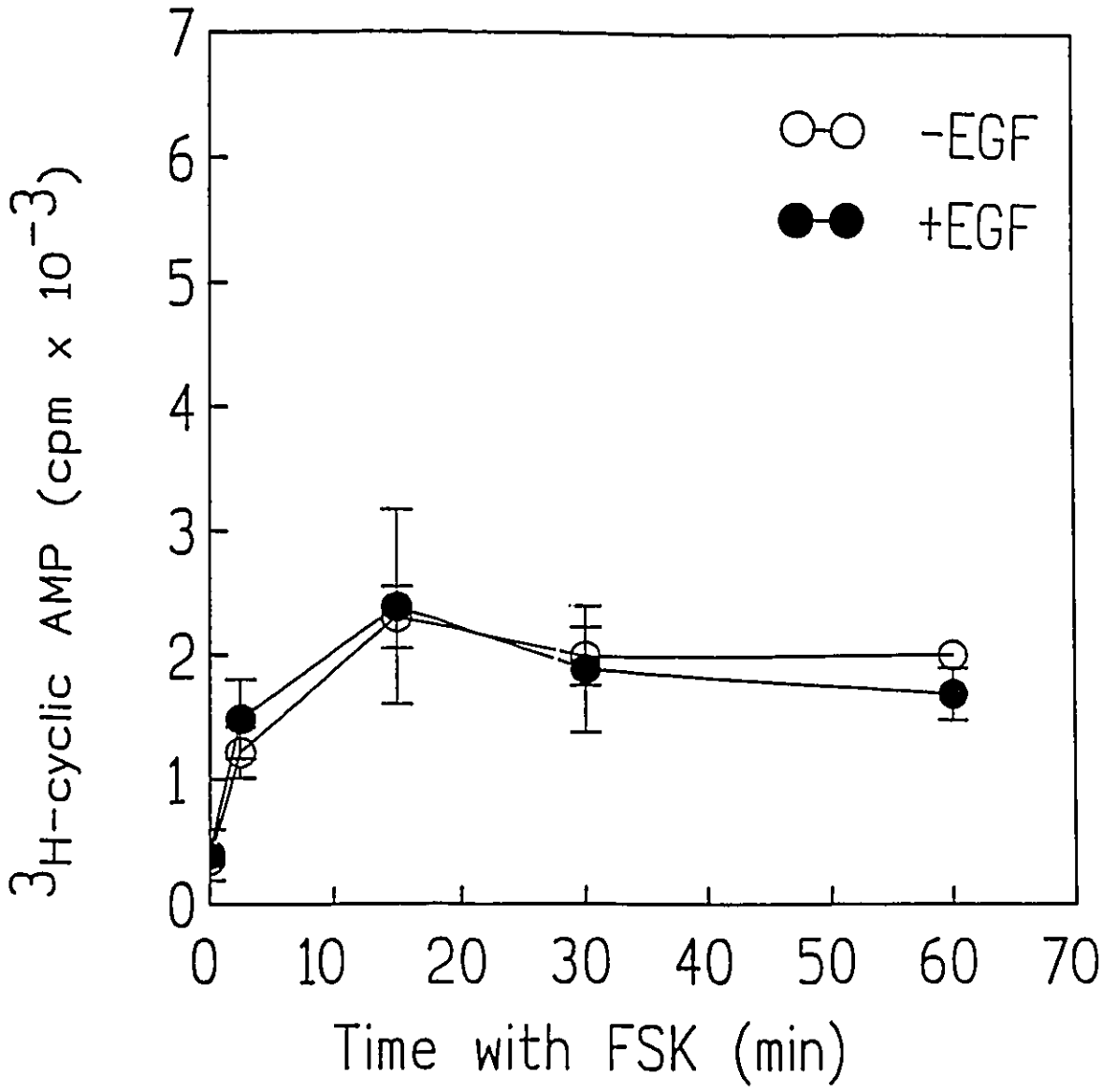
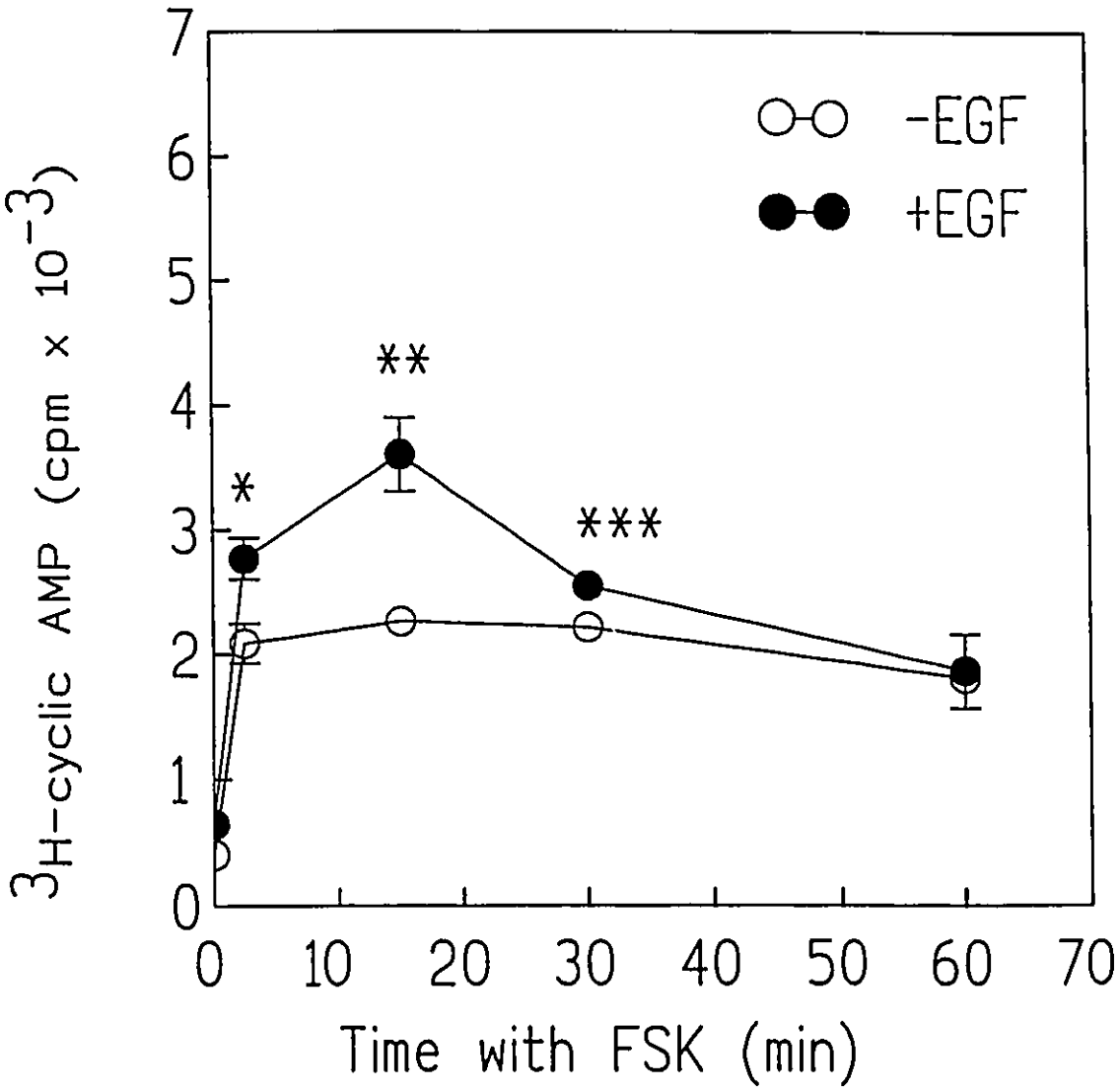

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

Figure 9: Effect of EGF pretreatment (1 hour in serum/medium) on the time course of forskolin stimulation of cAMP synthesis:

EGF (16.7 ng/ml) was added to T51B cultures for 1 hour in the presence of serum/medium. After 1 hour serum/medium was removed and cells were incubated with Hepes/medium for the next 2 hours. Control cultures did not receive EGF. The cells were then stimulated with FSK (33 μM) for different times. The results are expressed as ^{3}H-cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. The statistical analysis performed was the two-tailed paired t-test. A significant difference between EGF pretreated and untreated cultures was obtained when corresponding values are compared at various times. The level of significance is indicated as: * $P<0.022$, ** $P<0.005$, *** $P<0.001$.


7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
*
**
***
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

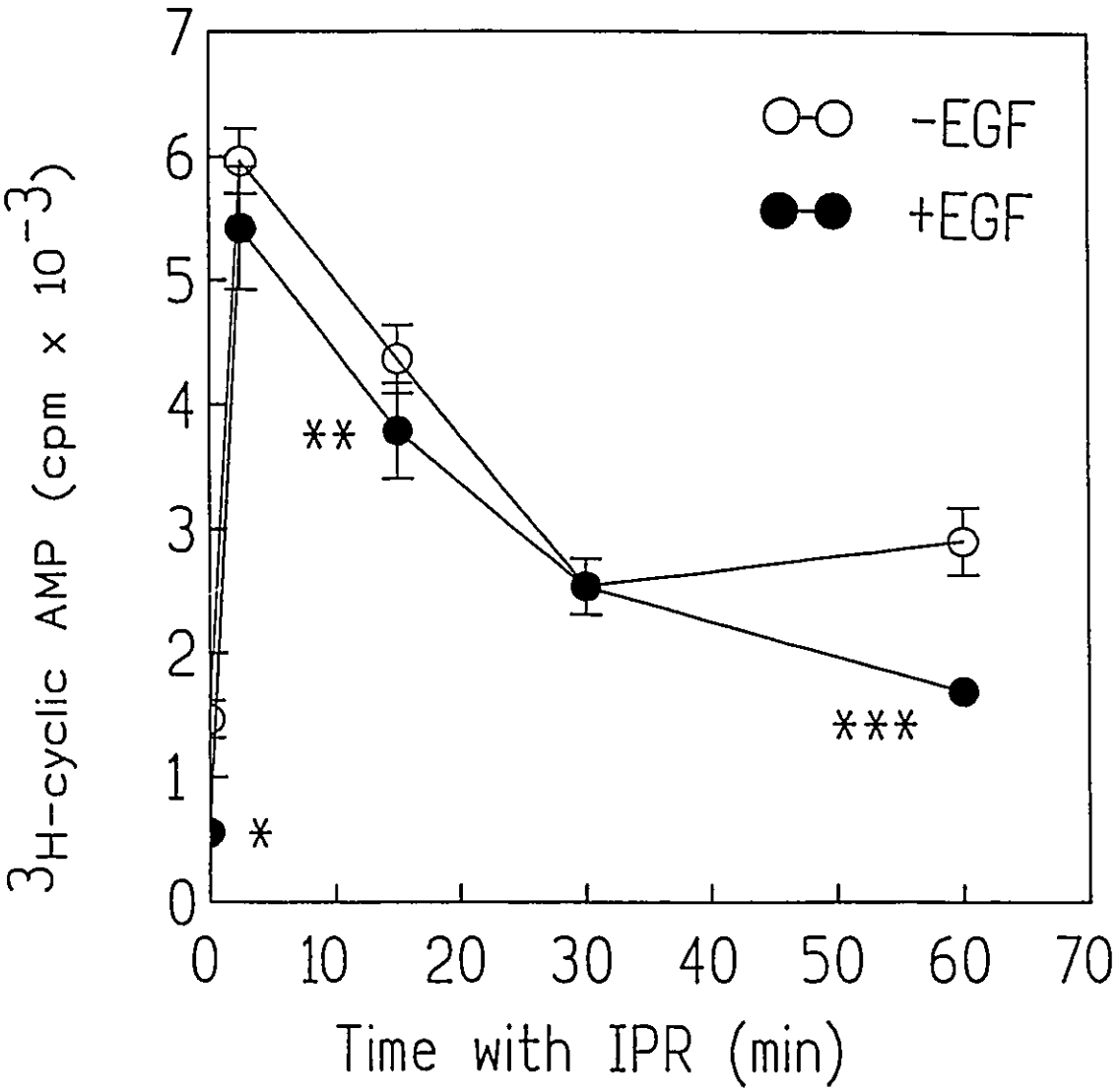
3.5 Effect of TPA on cAMP Synthesis:

Since TPA had been shown to potentiate the effect of FSK or IPR in T51B cells (Aasheim et al. 1989), it was of interest to know if there is any role of PKC in modulating the effect of EGF-pretreatment on the potentiation of IPR or FSK stimulated adenylate cyclase activity. The next experiments were therefore conducted with the PKC activator TPA. TPA (100 nM) was added 2.5 minutes after either FSK or IPR addition. Assays were terminated at different times after the IPR or FSK addition. The experiments reported in the following four Figures were done at the same time as those for Figures 5, 6, 8 and 9. Thus the results from Figures 5, 6, 8 and 9 serve as the controls (no TPA) for the results reported in Figures 10, 11, 12 and 13 respectively (with TPA).

T51B cultures were incubated (Fig 10) as for Figure 5 (1 hour EGF pretreatment in Hepes/medium). TPA (100 nM) was added 2.5 minutes after IPR (1 μM). A decrease in cAMP synthesis in EGF-pretreated cells was observed as compared to untreated cells. However, if the results of Figures 5 and 10 are compared, one observes almost no difference between the two figures. The slight inhibition due to EGF observed in Figure 10 seems to be due to an inhibition of background adenylate cyclase activity as was observed in Figure 5. In either case TPA did not have any effect. This could be due to two possibilities: (a) either the TPA used was not active (not effective) or (b) activation of PKC by TPA does not modulate the IPR-stimulated adenylate cyclase signalling system under the conditions used. These possibilities will be discussed later.

Figure 10: Effect of EGF pretreatment (1 hour in Hepes/medium) on the time course of IPR stimulation and TPA activation of cAMP synthesis:

T51B cultures were incubated with EGF (16.7 ng/ml) for 1 hour in the presence of Hepes/medium. Control cultures did not receive EGF. After 1 hour of incubation cells were stimulated with IPR (1 μM) for different times. TPA (100 nM) was added 2.5 minutes after IPR addition. Reaction was terminated at different times after IPR addition. The results are expressed as ^{3}H-cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of four different experiments. A significant difference between EGF pretreated and untreated cultures was obtained upon statistical analysis by the two-tailed paired t-test. The level of significance is indicated as: * $P<0.0035$, ** $P<0.03$ and *** $P<0.0012$.


7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
**
*
***
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with IPR (min)

In the next set of experiments (Fig.11) cells were EGF-pretreated in the presence of serum/medium for one hour as in Figure 6. The serum/medium was replaced with EGF-free Hepes/medium and the cells were incubated for two hours in this medium before they received TPA and IPR. TPA (100 nM) was added 2.5 minutes after IPR (1 μM) addition. The results in Figure 11 show that cAMP synthesis is significantly higher in EGF-pretreated cells as compared to untreated cells. However if one compares the result from Figures 6 and 11 it can be seen that the two Figures are almost identical indicating that TPA has minimal effect on IPR stimulation of adenylate cyclase and on the EGF potentiation. The only difference between the two figures is that TPA seems to result in a sharper peak in the EGF-pretreated cultures (i.e. at 15 and 30 minutes, there is less potentiation due to EGF when TPA was present).

Since the prolonged pretreatment (2 hours) with EGF in the presence of Hepes/medium did not affect cAMP synthesis (Fig.8) stimulated by FSK, an experiment was designed to determine if PKC activation affects FSK stimulated adenylate cyclase in EGF-pretreated and untreated cells. When the results of Figure 12 (+TPA) are compared to the results of Figure 8 (-TPA) it is clearly seen that TPA potentiates the cAMP synthesis stimulated by FSK. For the control cultures (no EGF pretreatment), the peak of cAMP (cpm x 10^{-3}) rises from 2.3 to 6.8 in the presence of TPA. The peak of cAMP synthesis shifts from 15 to 30 minutes and the cAMP levels remain elevated for at least one hour. Similarly for the EGF-pretreated cells, the peak of cAMP rises from 2.3 to 5 in the presence of

Figure 11: Effect of EGF pretreatment (1 hour in serum/medium) on the time course of IPR stimulation and TPA activation of cAMP synthesis:

T51B cultures were incubated with EGF (16.7 ng/ml) for 1 hour in the presence of serum/medium. After 1 hour of incubation serum/medium was replaced with Hepes/medium and the cultures were incubated in this medium for the next 2 hours. Control cells did not receive EGF. The cells were then stimulated with IPR (1 μM) for different times. TPA (100 nM) was added 2.5 minutes after IPR stimulation. Reaction was terminated at different times after IPR addition. The results are expressed as ^{3}H-cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. The statistical analysis, by two-tailed paired t-test, showed a significant difference between EGF pretreated and untreated cultures. The level of significance (P-values) is indicated as: * $P<0.0006$, ** $P<0.016$, *** $P<0.018$.

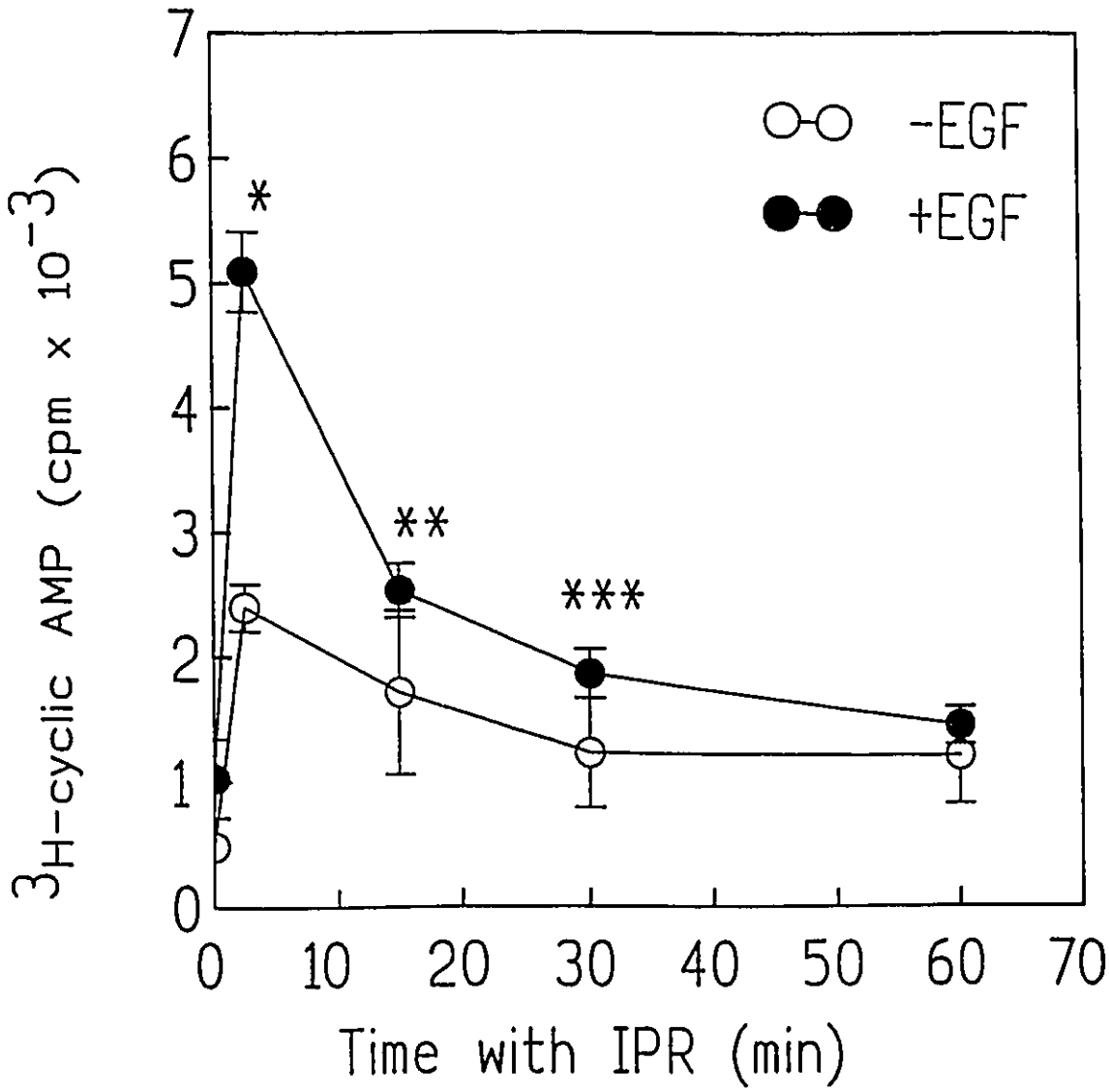
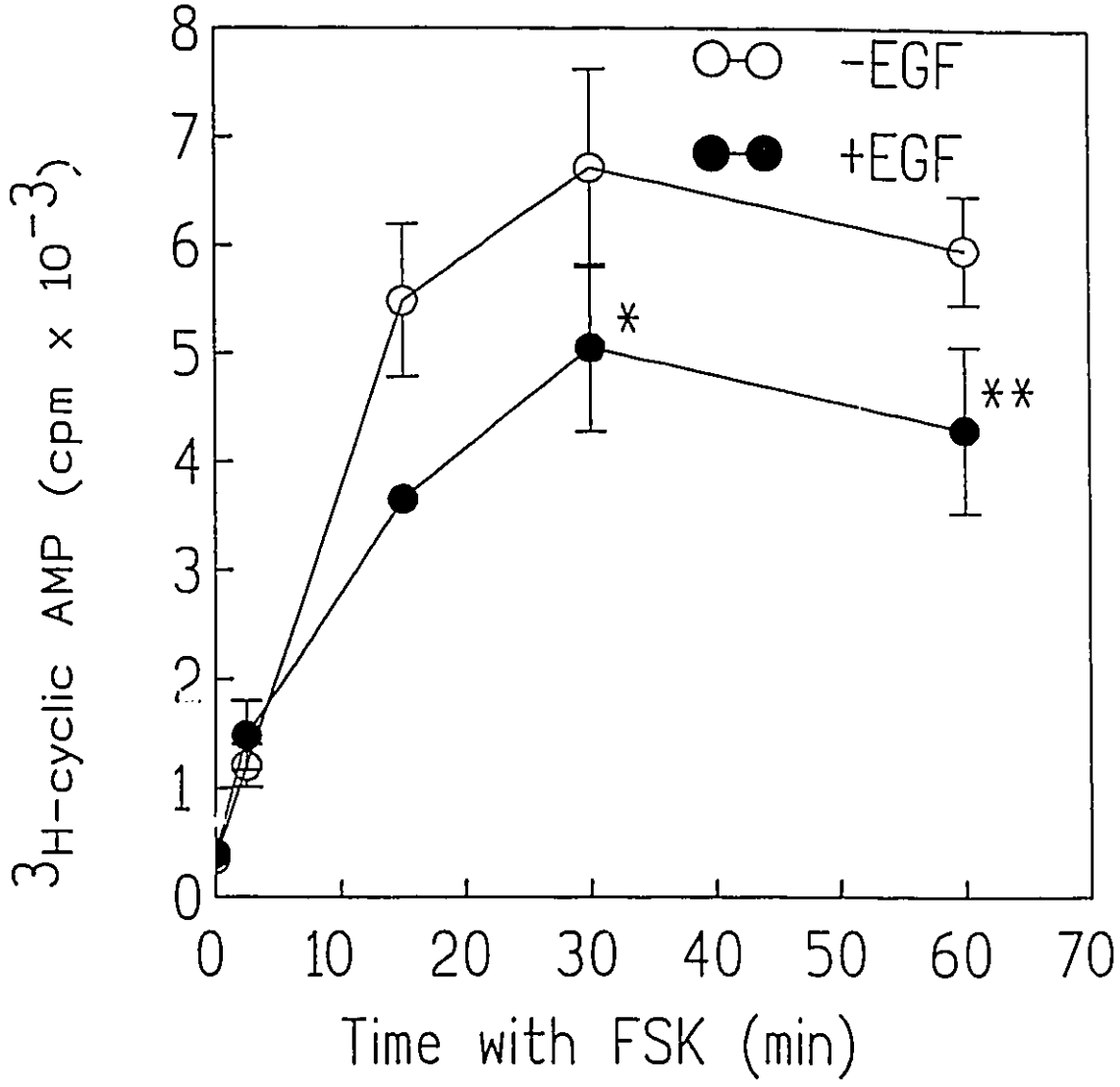

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
*
**
***
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with IPR (min)

Figure 12: Effect of EGF pretreatment (2 hours in Hepes/medium) on the time course of forskolin stimulation and TPA activation of cAMP synthesis:

EGF (16.7 ng/ml) was added to T51B cultures in the presence of Hepes/medium for 2 hours. Control cultures did not receive EGF. After 2 hours cells were stimulated with FSK (33 μM) for different times. TPA (100 nM) was added 2.5 minutes after FSK. Reaction was terminated at different times after FSK addition. The results are expressed as ^{3}H-cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. Statistical analysis performed was the two-tailed paired t-test. A significant difference was obtained between EGF pretreated and untreated cultures. The P-values (level of significance) is indicated as: *$P<0.009$, and **$P<0.006$ at times 30 and 60 minutes respectively.

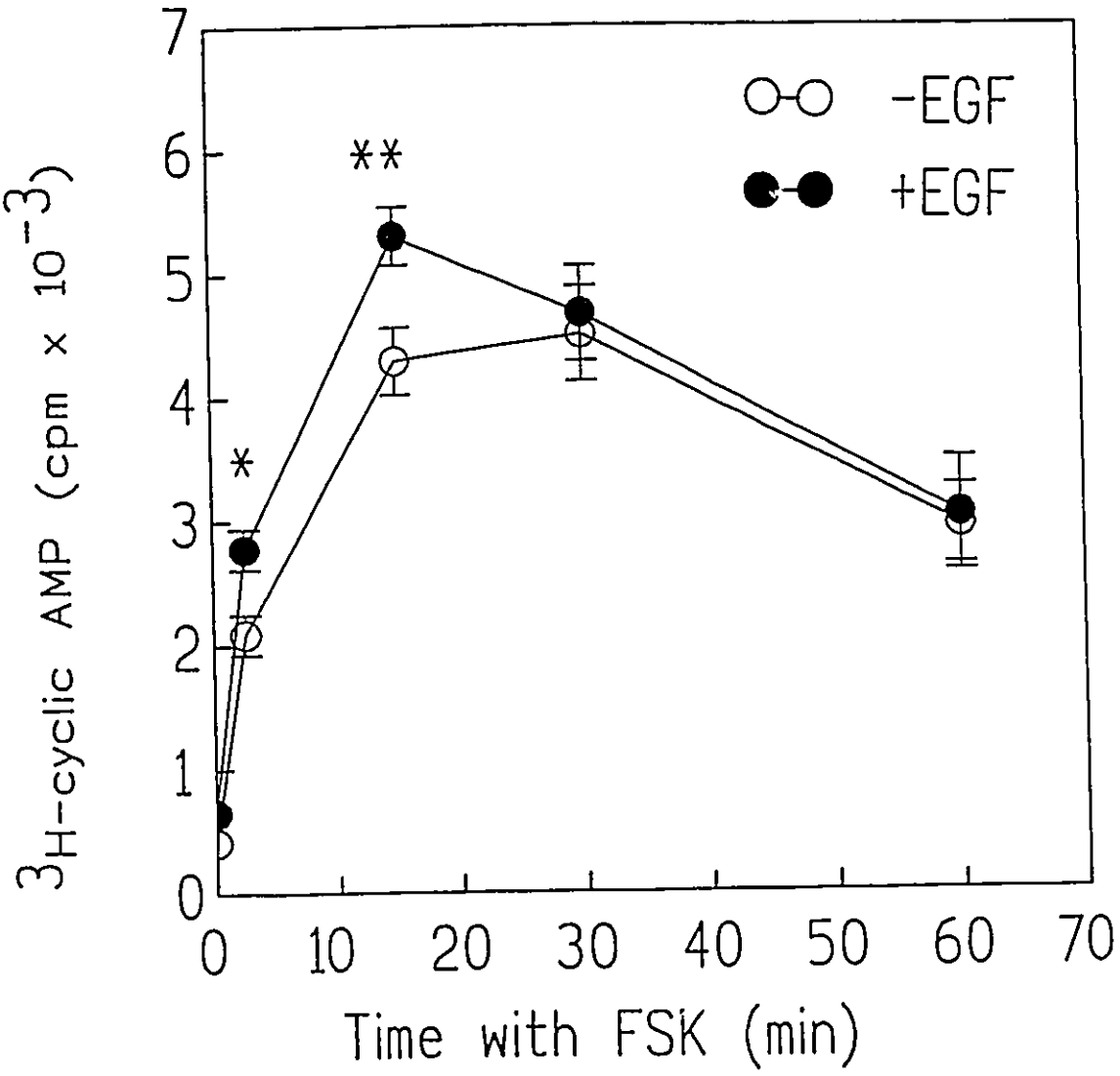

-EGF
+EGF
*
**
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70

TPA and the peak shifts from 15 to 30 minutes. Furthermore it can be observed that without TPA, EGF had no stimulatory or inhibitory effect on the FSK stimulated cAMP synthesis. In contrast, in the presence of TPA, EGF inhibited FSK stimulated cAMP synthesis. Therefore cyclic AMP synthesis potentiated by TPA in FSK stimulated cells, is attenuated by EGF-pretreatment.

I then examined the effect of TPA on cells that had been EGF exposed for one hour in serum/medium and then incubated for two hours in EGF-free Hepes/medium (Fig.13). T51B cells were incubated with EGF for one hour as in Figure 9 but activated with TPA (100 nM) (Fig.13). The results in Figure 13 show that cAMP synthesis was significantly higher (at 2.5 and 15 minutes) in EGF-pretreated cells as compared to untreated cells. When Figures 9 and 13 are compared, it can be seen that TPA has a marked effect. Thus the result from Figures 9 and 13 show that TPA potentiates the FSK stimulated adenylate cyclase signalling pathway in T51B cells as was observed previously (Aasheim et al.1989). This was observed in the EGF-pretreated and untreated cells. TPA seems to have a minimal effect on EGF potentiation of FSK stimulated cAMP synthesis. Although the fold stimulation (EGF-treated versus untreated cells) is less in the presence of TPA, however the actual increase in cAMP synthesis (cpm x 10^{-3}) is approximately the same. Therefore in contrast to its lack of effect on IPR stimulation of cAMP synthesis, activation of PKC potentiates cyclic AMP synthesis by FSK in both EGF-pretreated and untreated cultures.

Figure 13: Effect of EGF pretreatment (1 hour in serum/medium) on the time course of forskolin stimulation and TPA activation of cAMP synthesis:

EGF (16.7 ng/ml) was added to T51B cultures in the presence of serum/medium. After 1 hour of incubation, serum/medium was replaced by Hepes/medium and the cells were incubated in this medium for the next 2 hours. Control cultures did not receive EGF. The cells were then stimulated with FSK (33 μM) for different times. TPA (100 nM) was added 2.5 minutes after FSK. Reaction was terminated at different times after FSK addition. The results are expressed as ^{3}H-cyclic AMP (cpm x 10^{-3}) and represent mean ± S.E. of three different experiments. The statistical analysis performed was the two-tailed paired t-test. A significant difference between EGF pretreated and untreated cells was found. The level of significance (P-values) is indicated as: * P,0.022, ** P<0.005.


7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
**
*
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

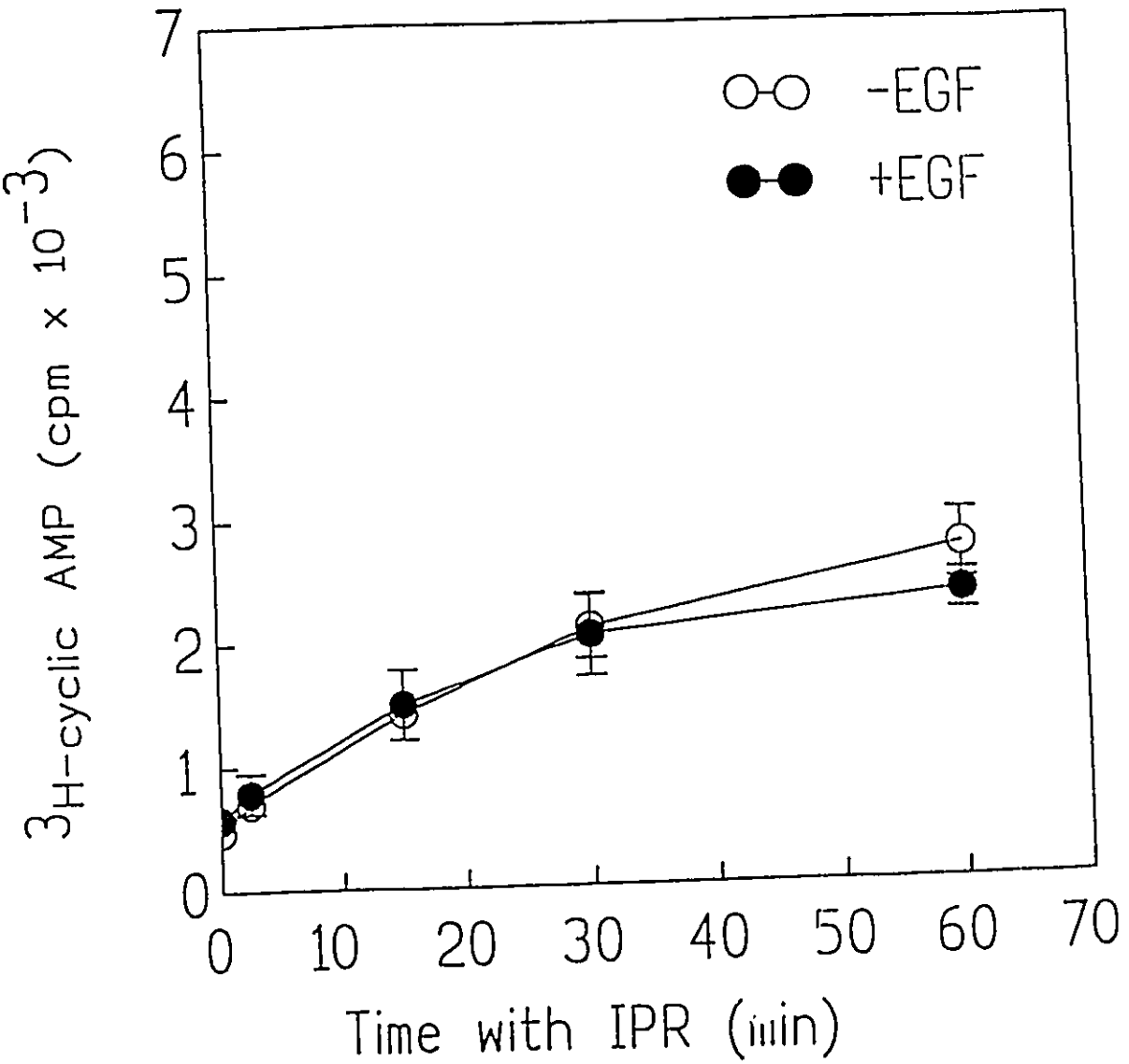
3.6 Cyclic AMP Secretion:

In this section, I will present results on the secretion of cAMP from the cells during FSK or IPR stimulation in EGF-pretreated and untreated cultures. I will also present the results of the effect of TPA on the secretion.

Figure 14 shows cyclic AMP secretion in EGF-pretreated (1 hour in Hepes/medium) and untreated cells. The secreted cAMP was measured from the same culture dishes that were used in Figure 5, by pipetting out the assay medium and loading it onto the columns (as done with the cell lysates for cyclic AMP synthesis in Fig.5). Therefore cAMP synthesis and secretion were measured from the same culture dishes (i.e. Fig.14 reports the results on cAMP secretion into the medium and Figure 5 reports on the synthesis of cAMP within the cell). It can be seen that cAMP secreted by EGF-pretreated and untreated cells is not significantly different. However, for both treatments, there is a gradual increase in secretion with time. It can be seen from Figure 5 that cAMP inside the cell decreases from a peak of 6 at 2.5 minutes to 2.8 at 60 minutes. Most of this decrease could be accounted for by the rise in extracellular cAMP which rises from 0.5 to a level of 2.5 (Fig. 14). However, not all of the decrease in the internal cAMP can be accounted for by secretion into the medium. This discrepancy could be due to the possibility that not all of the cAMP (intracellular or extracellular) was recovered. It could also be due to degradation of cAMP by phosphodiesterase (PDE) although sufficient RO 201724 (inhibitor of PDE) was present to presumably inhibit most of the PDE activity. Although cAMP synthesis in EGF-pretreated cells is

Figure 14: Effect of EGF-pretreatment (one hour in Hepes/medium) on the time course of IPR stimulation of cAMP secretion:

T51B cultures were incubated with EGF (16.7 ng/ml) for one hour in Hepes/medium. Control cultures did not receive EGF. After one hour of incubation cells were stimulated with IPR (1 μM) for different times. The secretion was measured by measuring labelled cAMP in the medium. The results are expressed as ^{3}H cyclic AMP ($cpmx10^{-3}$) and represent the mean ± S.E. of four different experiments. Statistical analysis by two-tailed paired t-test did not detect any significant difference between treated and untreated cultures.


-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time with IPR (min)

significantly lower than for untreated cells (Fig.5), the secretion of cAMP in EGF-pretreated cells is not significantly lower than untreated cells.

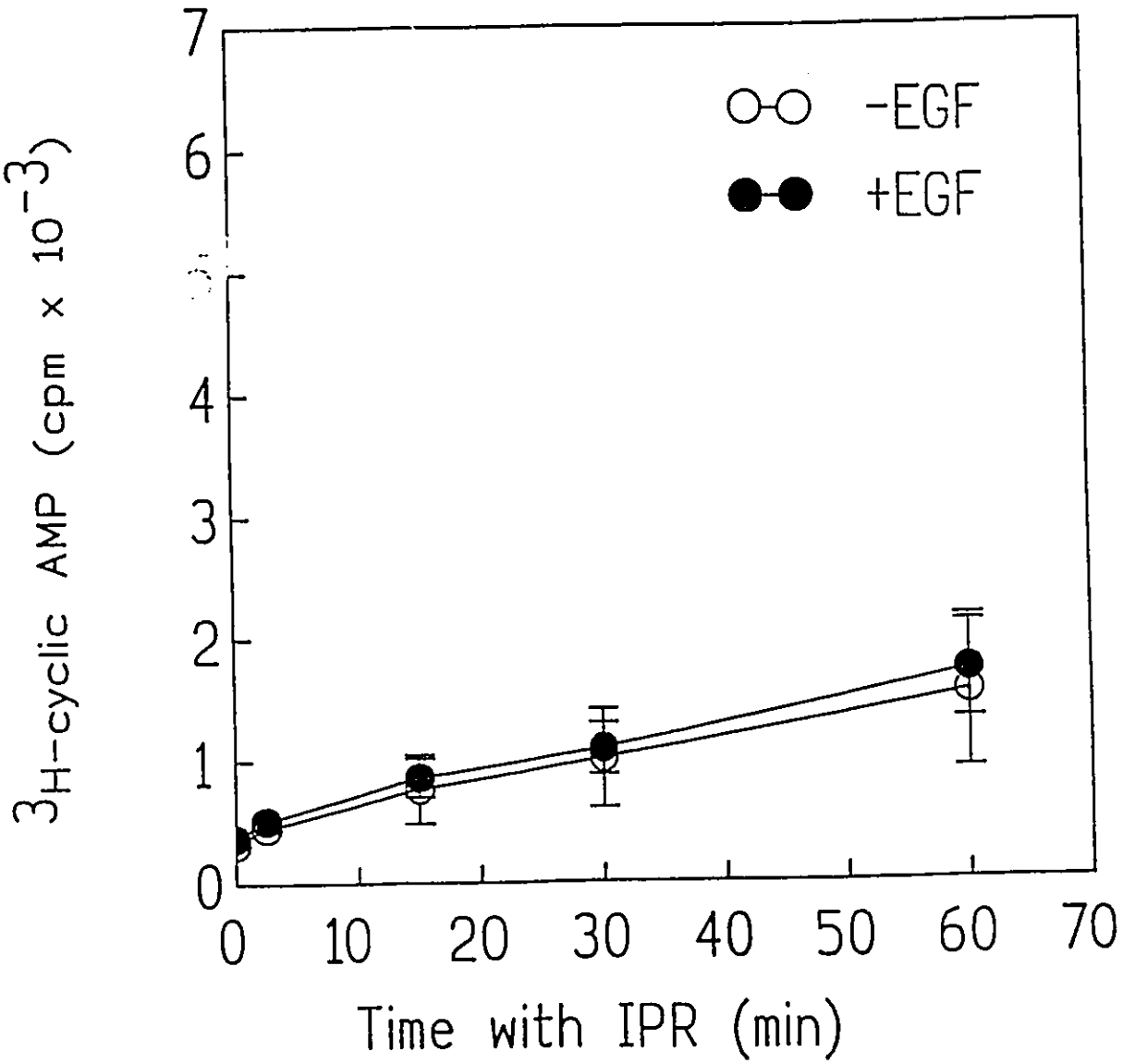
When cultures were EGF-pretreated for one hour in serum/medium as in Figure 6 followed by a 2 hour incubation in Hepes/medium, secreted cAMP was not different from untreated cultures (Fig 15). It can be seen from Figure 6 that intracellular cAMP in untreated cells decrease over time from 2.3 to 1 whereas it decreases from 5 to 1.8 in the EGF-pretreated cells. This difference in intracellular cAMP decrease is not reflected by a difference in extracellular cAMP (Fig.15). If cAMP secretion were simply a passive uncontrolled event, one would expect any decrease in intracellular cAMP to be reflected by a corresponding increase in cAMP in the medium. Obviously, the results do not substantiate this idea. There must be other factors which control the amount of cAMP that is secreted.

Similarly, since for the EGF-pretreated cultures, cAMP has decreased from 5.5 to 2 in Figure 5 and from 5 to 1.8 in Figure 6, one would expect equivalent levels of cAMP to be secreted in the medium. This is not the case, in fact there is less cAMP secretion in Figure 15 (corresponding to Fig.6) compared to that found in Figure 14 (corresponding to Fig.5).

The results also indicate that cAMP observed in the medium is not due to the presence of dead ruptured cells since cultures with high intracellular cAMP should yield correspondingly higher levels of extracellular cyclic AMP. It could be suggested that different treatments could specifically increase the death of cells. This is not the case since few floating dead cells were observed in any cultures.

Figure 15: Effect of EGF pretreatment (one hour in serum/medium) on the time course of IPR stimulation of cAMP secretion:

EGF (16.7 ng/ml) was added to cultures for one hour in the presence of serum/medium. After this time serum/medium was replaced with EGF-free Hepes/medium and cells were incubated for two hours. Control cells did not receive EGF. The cells were stimulated with IPR (1 μM) for different times.The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. Statistical analysis performed was the two-tailed paired t-test which did not give significant differences between EGF pretreated and untreated cells.


^{3}H-cyclic AMP (cpm x 10^{-3})
7
6
4
3
2
1
0
-EGF
+EGF
0
10
20
30
40
50
60
70
Time with IPR (min)

When cultures were preincubated with EGF for two hours in Hepes/medium and then stimulated with FSK, the amount of cAMP secreted is not different in these cells compared to untreated cells (Fig.16). Similarly, when the cultures were pre-incubated with EGF for one hour in the presence of serum/medium followed by 2 hour in EGF-free Hepes/medium and then stimulated with FSK for different times (Fig.17), it can be seen that extacellular cAMP levels are not different (very marginally significant at 60 min.) as compared to the cultures not treated with EGF (Fig.17). Yet a significant decrease in intracellular cAMP (Fig.9) had been observed for the EGF pretreated cells. Also the cAMP secretion observed after stimulation with IPR (Fig.15) or FSK (Fig.16) seems to give identical results, yet the decrease in intracellular cAMP under these conditions are very different (Figures 6 and 8). Therefore, it appears that modulation (increase or decrease) of cAMP levels in EGF-pretreated and untreated cultures is not solely due to the changes (increase or decrease) in cAMP export outside the cells.

In a previous section it was demonstrated that TPA has minimal effects on IPR stimulated cAMP synthesis in either untreated or EGF pretreated cultures (1 hour in Hepes/medium, Fig.10). This is reflected in cAMP secretion where TPA again has minimal effect (compare Fig. 18 to Fig.14). The secretion increases from 0.5 at 0 minutes to 3.0 at 60 minutes but this is not significantly different from Figure 14 where TPA was not used. Similarly TPA has no effect on cAMP secretion in cells that had been preincubated with EGF for 1 hour in serum (compare Fig.19 to Fig.15).

Figure 16: Effect of EGF pretreatment (one hour in serum/medium) on the time course of Forskolin stimulation of cAMP secretion:

Cultures were pretreated with EGF (16.7 ng/ml) in the presence of serum/medium for one hour and after this time serum/medium was replaced with EGF-free Hepes/medium. Cells were incubated for two hours under this condition. The cells were then stimulated with FSK (33 μM) for different times. The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. The statistical analysis by two-tailed paired t-test showed a significant difference between EGF pretreated and untreated cells at time 60 minutes. The P-value (level of significance) is $^{*}P<0.02$.

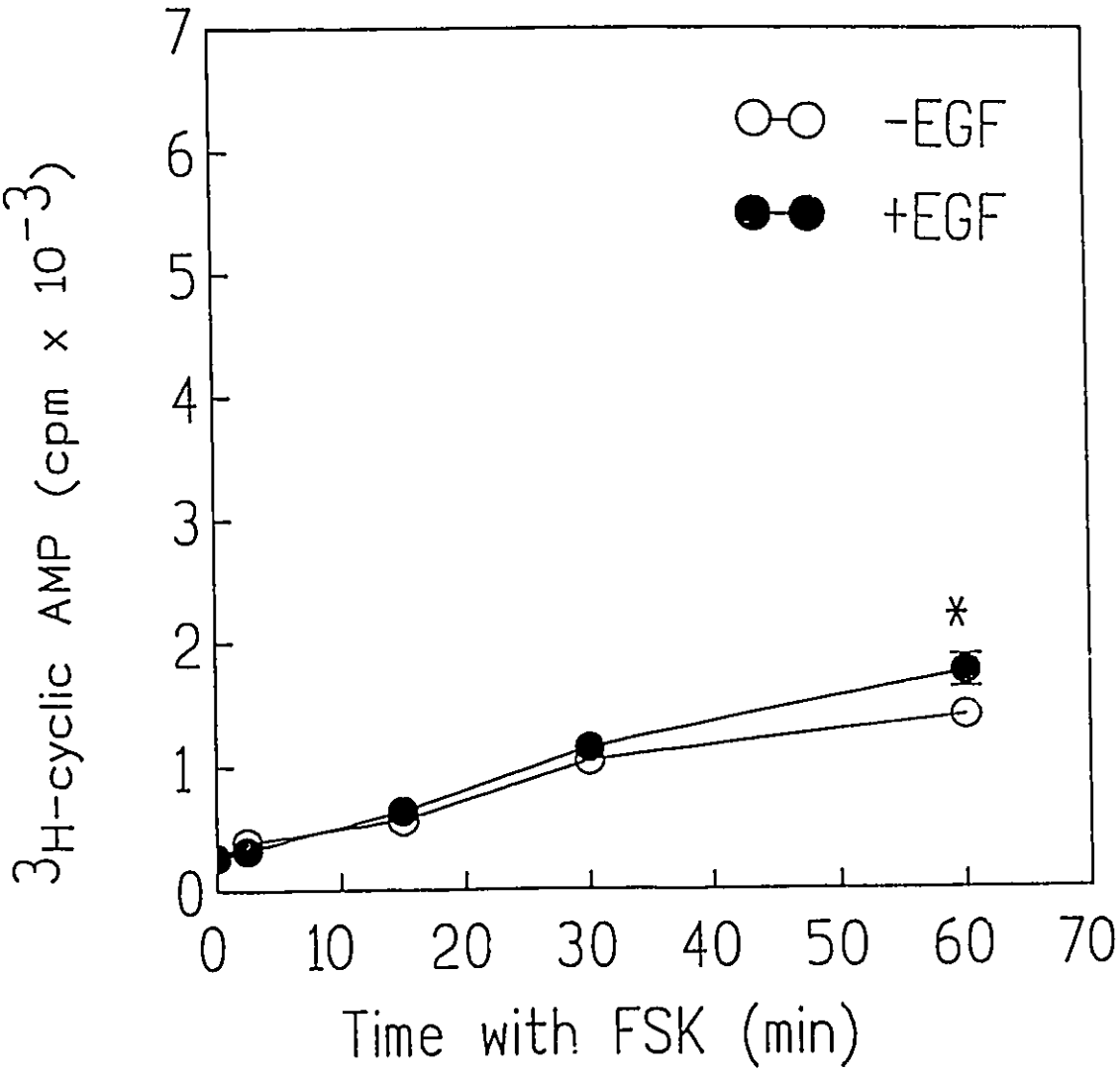

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
*
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

Figure 17: Effect of EGF pretreatment (two hours in Hepes/medium) on the time course of FSK stimulation of cAMP secretion:

EGF (16.7 ng/ml) was added to T51B cells for two hours in the presence Hepes/medium. After two hours, cells were stimulated with FSK (33 μM) for different times. The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent mean ± S.E. of three different experiments. The statistical analysis by two-tailed paired t-test showed no significant difference between EGF pretreated and untreated cells.

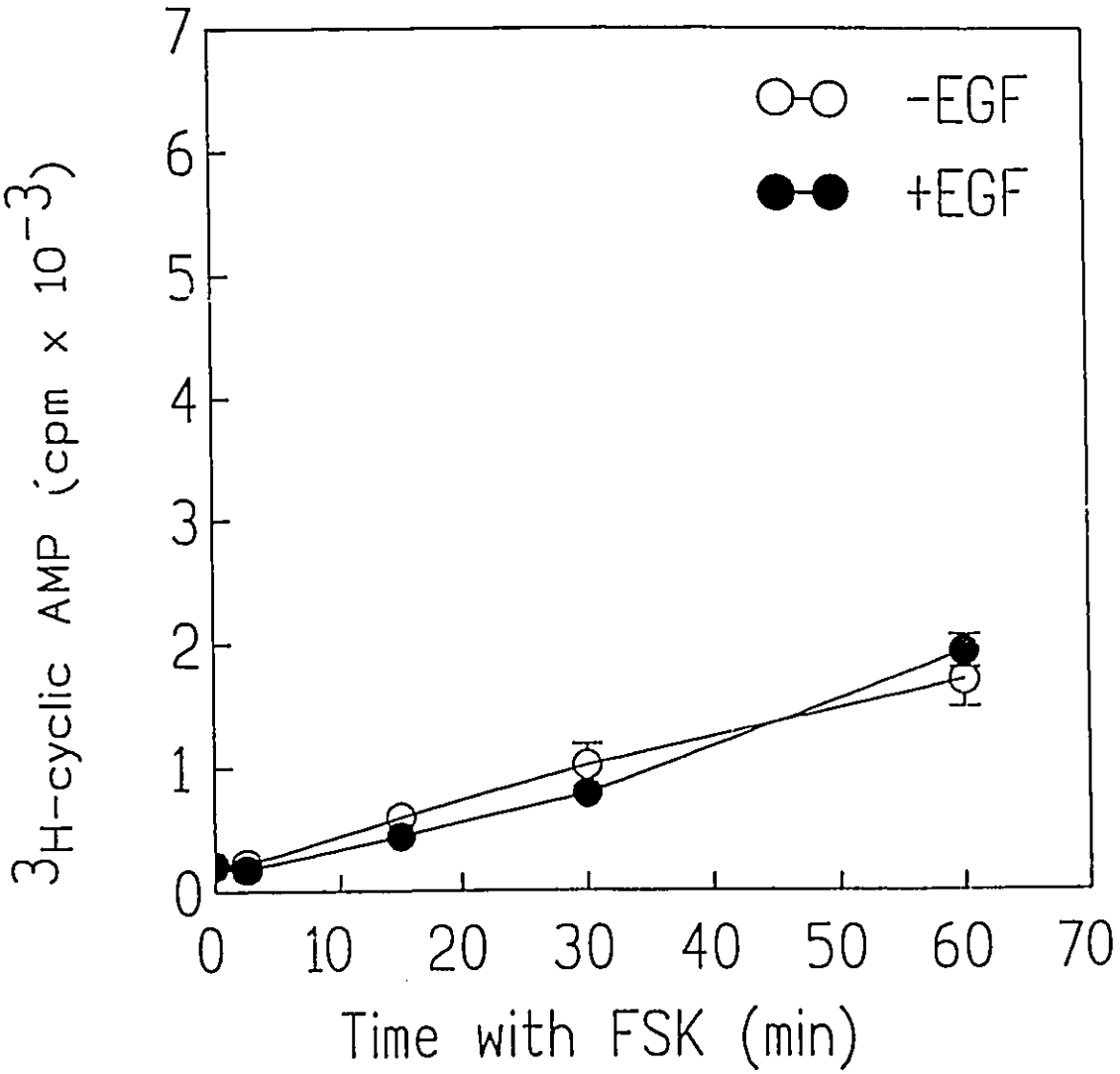

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

Figure 18: Effect of EGF pretreatment (one hour in Hepes/medium) on the time course of IPR stimulation and TPA activation of cAMP secretion:

T51B cells were incubated with EGF (16.7 ng/ml) for one hour in the presence of Hepes/medium. Control cultures did not receive EGF. After one hour of incubation cells were stimulated with IPR (1 μM) for different times. TPA (100 nM) was added 2.5 minutes after IPR addition. Reaction was terminated at different times after IPR addition. The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent mean ± S.E. of four different experiments. The statistical analysis performed by two-tailed paired t-test did not show any significant difference between EGF pretreated and untreated cultures.

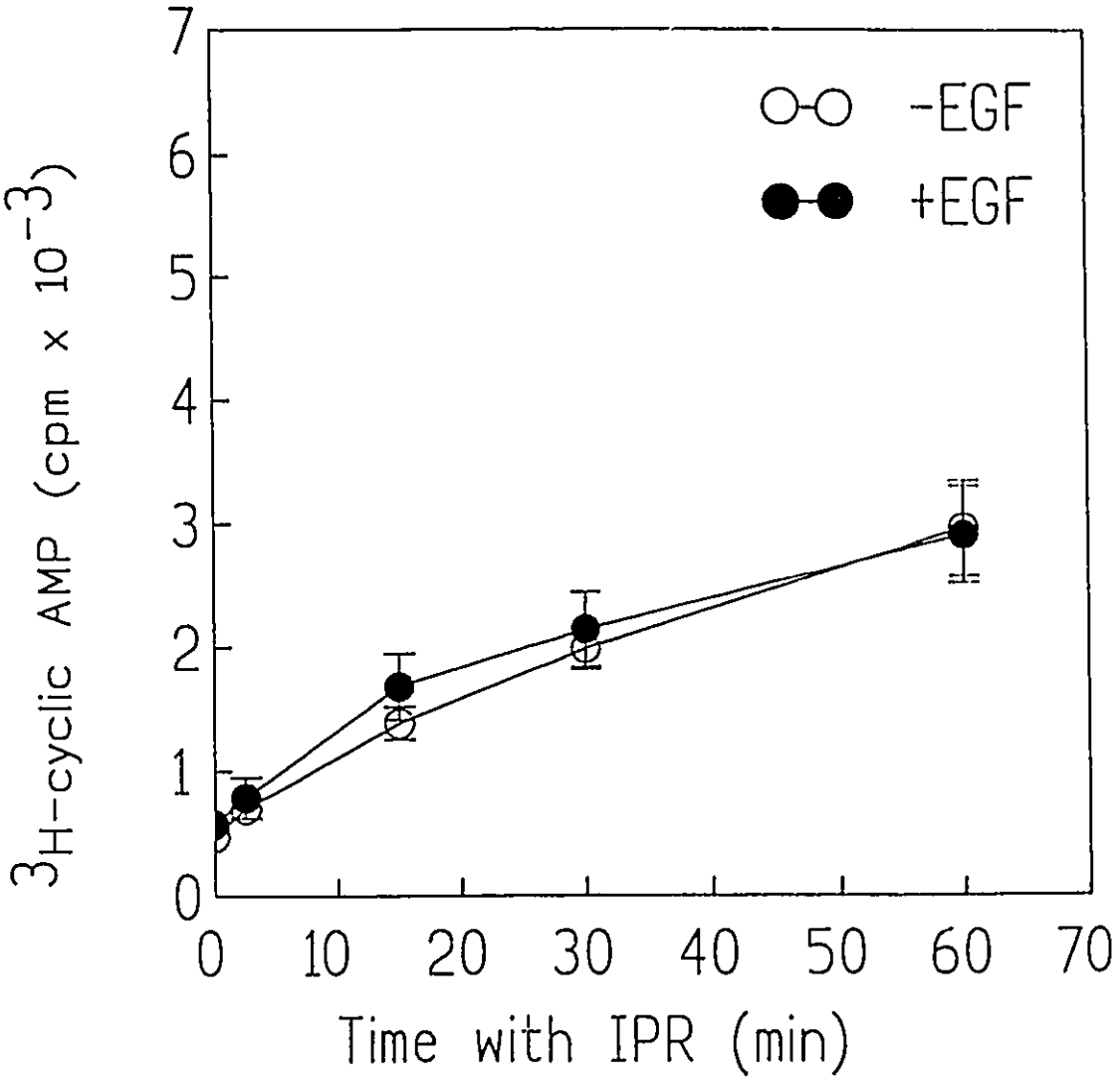
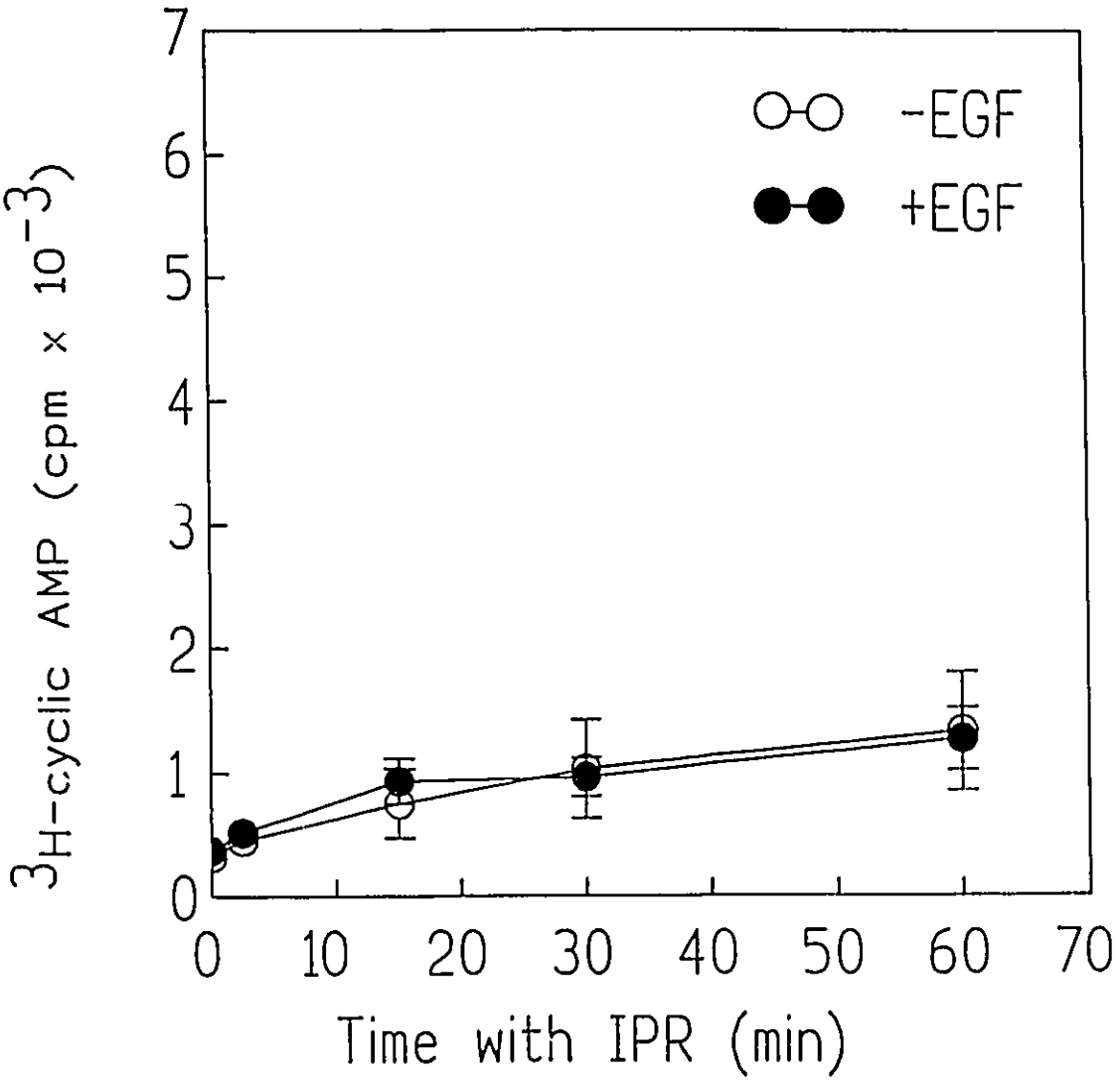

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with IPR (min)

Figure 19: Effect of EGF pretreatment (one hour in serum/medium) on the time course of IPR stimulation and TPA activation of cAMP secretion:

T51B cells were incubated with EGF (16.7 ng/ml) for one hour in the presence of serum/medium. After this time serum/medium was replaced with EGF-free Hepes/medium for two hours. The cultures were then stimulated with IPR (1 μM) for different times. TPA (100 nM) was added after 2.5 minute of IPR addition. The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent the mean ± S.E. of three different experiments. The statistical analysis by two-tailed paired t-test showed no significant difference between treated and untreated cultures.


7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with IPR (min)

Prolonged pretreatment (2 hours in Hepes/medium, Fig. 20) followed by activation with TPA (100 nM) and FSK results in the increase in cAMP secretion gradually from 0.1 to 2.5 in EGF-pretreated cells and from 0.1 to 3.1 in untreated cells. Results from Figures 16 and 20, in which cultures received similar treatments except that TPA was used in Figure 20, suggest that activation of PKC by TPA causes enhancement of cAMP secretion from untreated cells as compared to EGF-pretreated cells (at 60 minutes). Similar to that reported in section 3.5 (Fig.12) where EGF-pretreatment attenuated the TPA potentiation of FSK-stimulated cAMP synthesis, the presence of EGF also attenuated TPA-induced cAMP secretion. In both cases, for untreated and EGF pretreated cells, the presence of TPA caused almost a doubling of cAMP synthesis (e.g. from 2 to 4.3, Figures 8 and 12), yet the presence of TPA has only increased cAMP secretion from 1.5 to 2.5 (Figures 16 and 20).

Figure 21 demonstrates the cAMP secreted in untreated and EGF-pretreated cells (1 hour in serum/medium followed by 2 hours incubation in Hepes/medium) and stimulated with FSK. The secretion gradually increases from 0.2 at 2.5 minutes to 2.4 at 60 minutes in EGF pretreated cultures. It seems that activation of PKC by TPA gave a slight increase in cAMP secretion at 60 min. in EGF-pretreated cells as compared to untreated cells. However when the results from Figure 17 are compared to the results from Figure 21, it appears that TPA affects cAMP secretion in both EGF-pretreated as well as untreated cultures to a similar degree. If one compares Figures 19 and 21, where cultures were treated

Figure 20: Effect of EGF pretreatment (one hour in serum/medium) on the time course of FSK stimulation and TPA activation of cAMP secretion:

EGF (16.7 ng/ml) was added in the presence of serum/medium. After one hour of incubation serum/medium was replaced with EGF-free Hepes/medium and cultures were incubated for two hours in this condition. Control cultures did not receive EGF. The cells were then stimulated with FSK (33 μM) for different times. TPA (100 nM) was added 2.5 minute after FSK addition. The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent mean ± S.E. of three different experiments. The statistical analysis by two-tailed paired t-test did not show any significant difference between EGF pretreated and untreated cultures.

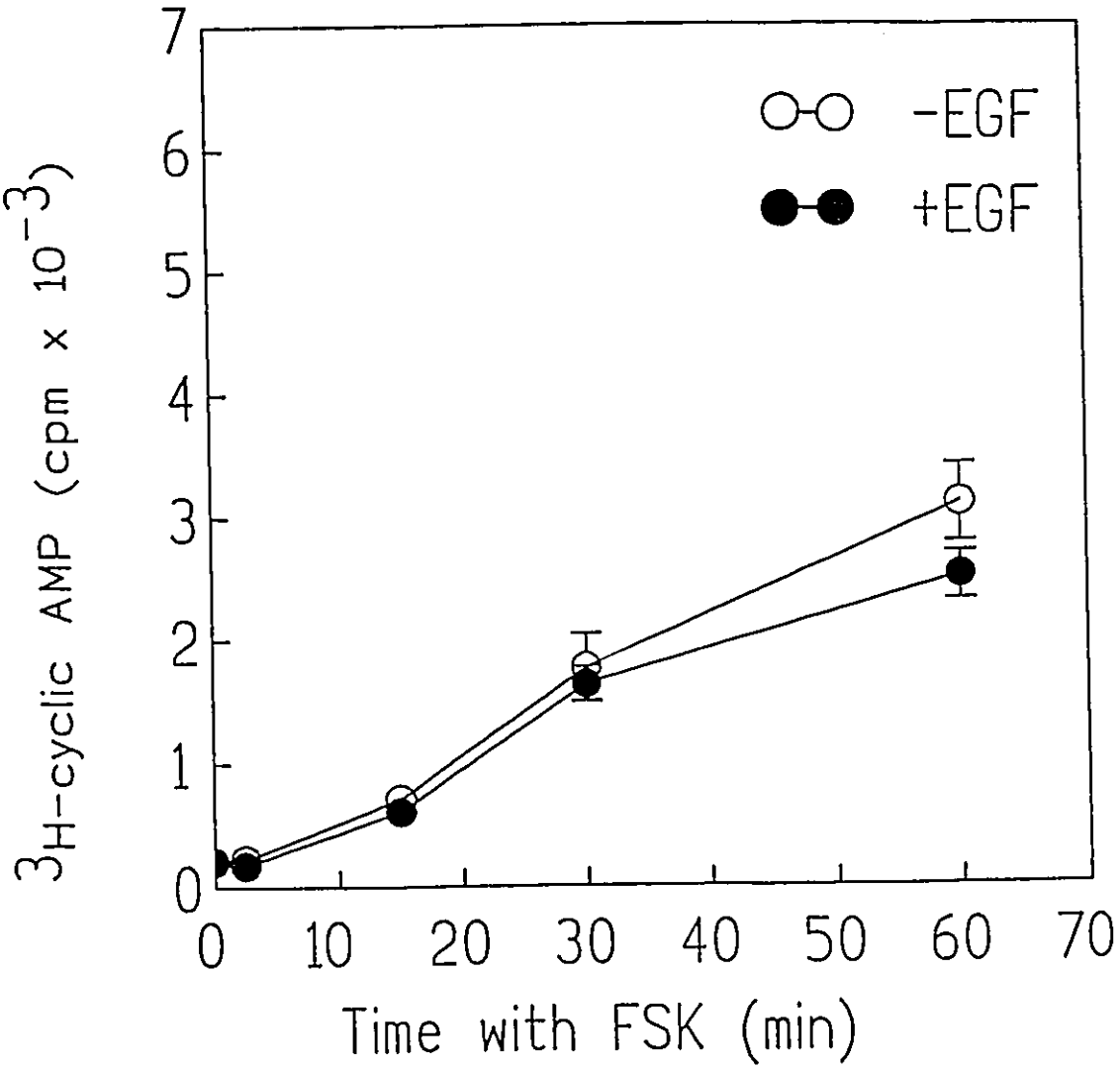
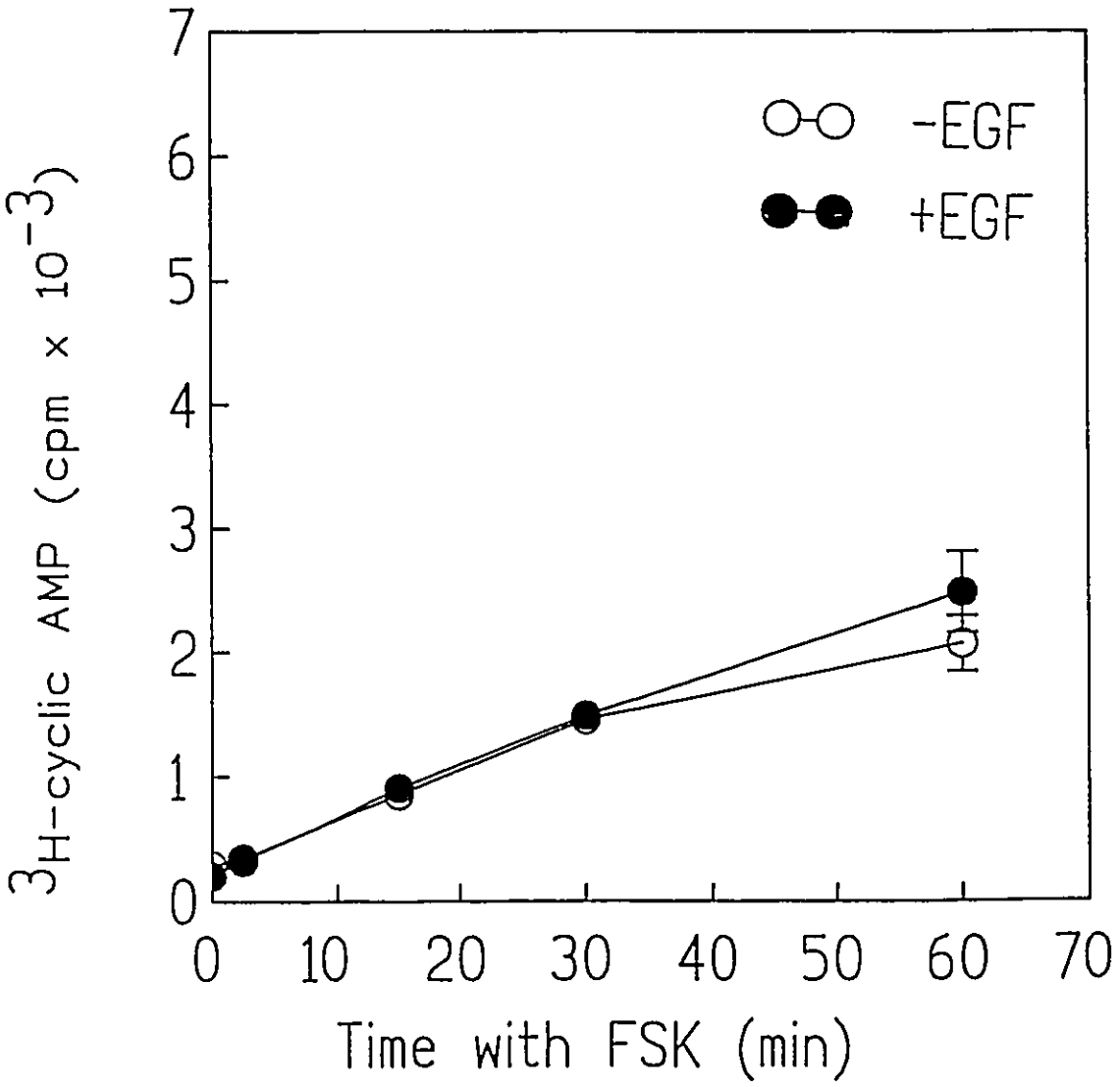

7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

Figure 21: Effect of EGF pretreatment (two hours in Hepes/medium) on the time course of FSK stimulation and TPA activation of cAMP secretion:

EGF (16.7 ng/ml) was added to T51B cultures for two hours in the presence of Hepes/medium. Control cultures did not receive EGF. After two hours of incubation cells were stimulated with FSK (33 μM) for different times. TPA (100 nM) was added 2.5 minute after FSK addition. The results are expressed as ^{3}H cyclic AMP (cpm x 10^{-3}) and represent mean ± S.E. of three different experiments. Statistical analysis by two-tailed paired t-test did not show any significant difference between treated and untreated cultures.


7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
-EGF
+EGF
^{3}H-cyclic AMP (cpm x 10^{-3})
Time with FSK (min)

similarly except that cells were stimulated with either IPR (Fig.19) or FSK (Fig.21) it can be seen that cAMP secretion increases gradually from 0.2 to 2.4 in Figure 21 as compared to Figure 19 which had a very slight increase from 0.2 to 1.1. This is interesting since, for the EGF-pretreated cells there is a large decrease in internal cAMP from 5 to 1.8 (Fig.11, IPR stimulated) whereas there is less of a decrease in internal cAMP (from 5.2 to 3) when cells were stimulated with FSK (Fig.13). Thus one would expect more secretion in Figure 19 (IPR) as compared to Figure 21 (FSK) if the decrease in internal cAMP is caused by an increased efflux. This is obviously not the case; in fact more secretion is seen in Figure 21.

3.7 DNA Synthesis:

3.7:1 Effect Of cAMP Elevating Agents On EGF Mitogenicity:

EGF has been shown to be a potent mitogen in a variety of cell types (McGowan et al. 1981; Hill et al. 1989). Hill et al. have demonstrated that EGF is also a mitogen for T51B cells. They observed that DNA synthesis induced by EGF requires extracellular Ca^{++} and not IP_3 mobilized intracellular Ca^{++}. Initially I was interested in demonstrating the effect of cAMP elevating agents on the mitogenicity of EGF in serum starved (0.2 % BCS) T51B cells. This model system was chosen so as to rule out the possible effects of mitogens, including EGF, present in serum. Others in our laboratory and Dr. Franks' laboratory have determined that the optimal mitogenic dose of EGF for serum starved T51B cells is between 5 and 10 ng/ml. This is half of the optimal dose used for the cAMP synthesis experiments.

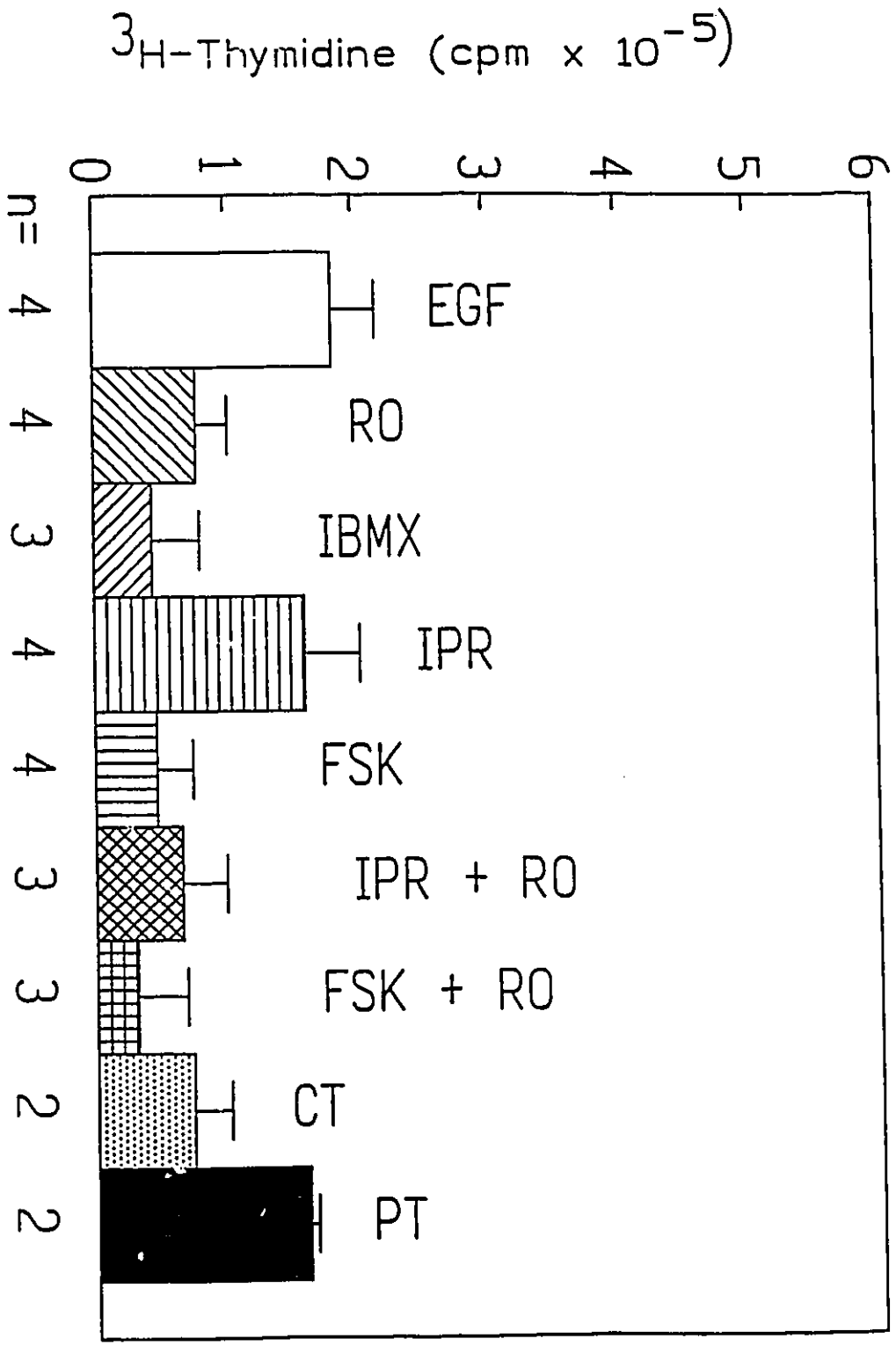
T51B cultures were serum starved for 48 hour at which time tritiated thymidine (^{3}H-Tdr) was added with EGF either alone or in combination with various cAMP elevating agents. The assay was stopped 24 hour later. When cAMP elevating agents were added to control cultures (0.2 % serum), DNA synthesis was unaffected or slightly decreased as compared to the basal level (data not shown). However when the cAMP elevating agents are present with EGF, they inhibited DNA synthesis induced by EGF to various degrees. FSK (33 μM) and RO (30 μM) when present together, were able to inhibit the EGF mitogenic response down to approximately basal levels (0.2 % serum alone, Fig. 22). RO (approximately 50 % inhibition) was less effective than IBMX (approximately 65-70 % inhibition) in inhibiting the EGF mitogenic response. CT (0.1 μg/ml) inhibited DNA synthesis by 55 % and a combination of IPR and RO inhibited DNA synthesis by 60 percent. However IPR (1 μM) alone or PT, 0.16 μg/ml) alone were the least effective in inhibiting EGF induced DNA synthesis (10 % to 15 % inhibition).

3.7:2 Effect Of Genistein on DNA synthesis:

EGF acts by binding to its receptor (EGF-R) and activating the tyrosine kinase activity of the receptor (Hunter and Cooper, 1985). It is presumed that EGF mitogenic activity is due to activation of this protein tyrosine kinase (PTK) activity. Genistein is known to be an effective PTK inhibitor (Dean et al. 1989) including EGF-R PTK in T51B cells. Therefore I became interested in demonstrating that DNA synthesis induced by EGF could be blocked by inhibiting the EGF receptor

Figure 22: Effect of cAMP elevating agents on EGF mitogenicity in serum starved (0.2% BCS) T51B cultures:

T51B cells were serum starved for 48 hours after they become confluent. At that time EGF (8 ng/ml) and ^{3}H-thymidine (0.5 µCi/ml) along with various cAMP elevating agents either alone or in combination were added in the same conditioned (i.e. serum starved) medium. The concentrations of the agents are; RO- (30 µM), FSK- (33 µM), IPR- (1 µM), IBMX- (33 µM), CT- (0.1µg/ml) and PT- (1.6µg/ml). The results are expressed as ^{3}H-thymidine incorporation (cpm x 10^{-5}) after subtracting the control values (0.2% BCS alone). The number of experiments for each treatment is indicated as n.


^{3}H-Thymidine (cpm x 10^{-5})
0
1
2
3
4
5
6
n=
4
4
3
4
4
3
3
2
2
EGF
RO
IBMX
IPR
FSK
IPR + RO
FSK + RO
CT
PT

PTK activity. This would show that tyrosine kinase activity is possibly involved in the mitogenic response of EGF. Genistein (15 μM) was able to inhibit DNA synthesis by more than 50 % (Fig. 23). The presence of genistein with cAMP elevating agents did not give results significantly different from genistein alone. All the above observations were made in the serum starved (0.2 % BCS) condition. These results suggest that the tyrosine kinase activity of EGF receptor may be involved in induction of DNA synthesis, and that cAMP elevating agents may also be involved in DNA synthesis and that their effects are not additive.

3.7:3 Effect Of cAMP elevating Agents on Serum Mitogenicity in Serum Replenished (10 % BCS) Condition:

The inhibitory effect of cAMP elevating agents on EGF mitogenicity was presented in section 3.7:1. This approach was taken to eliminate the possibility that other factors present in serum would stimulate DNA synthesis or could modulate the EGF mitogenic response. However, I was interested in determining if cAMP elevating agents could also inhibit the serum induced mitogenic response. Therefore, cultures were serum starved (0.2 % BCS) for 48 hours and then stimulated with serum (10 % BCS) and various cAMP elevating agents (Fig.24). DNA synthesis induced by serum (Fig.24) was higher than that obtained with EGF alone (Fig.22). The cAMP elevating agents, when present in 10 % serum, inhibited serum induced DNA synthesis to various degrees (Fig. 24). FSK and RO together inhibited the mitogenic response of serum by 74% and a similar degree of

Figure 23: Effect of the tyrosine kinase inhibitor, genistein, when added with cAMP elevating agents on the EGF mitogenicity in serum starved (0.2% BCS) cultures:

T51B cells were serum starved (0.2% BCS) for 48 hours after they reached confluence. After this time EGF (8 ng/ml) and ^{3}H-thymidine (0.5 µCi/ml) were added. Genistein (15 µM) was added alone or in combination with various cAMP elevating agents. The experiment was stopped 24 hour after adding EGF. Each bar represents the mean ± S.E. of three different experiments. The results are expressed as ^{3}H thymidine (cpm x 10^{-5}).

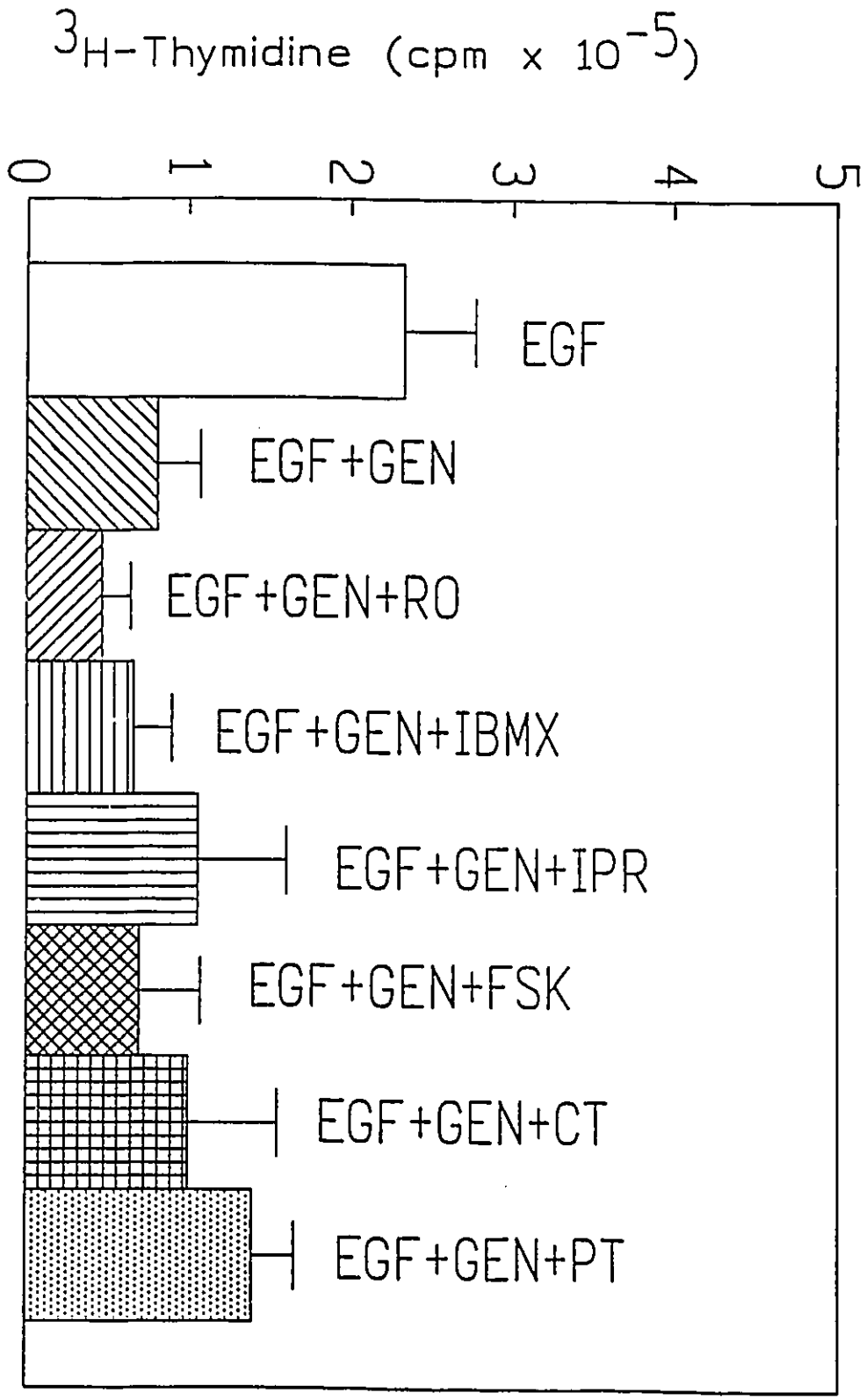
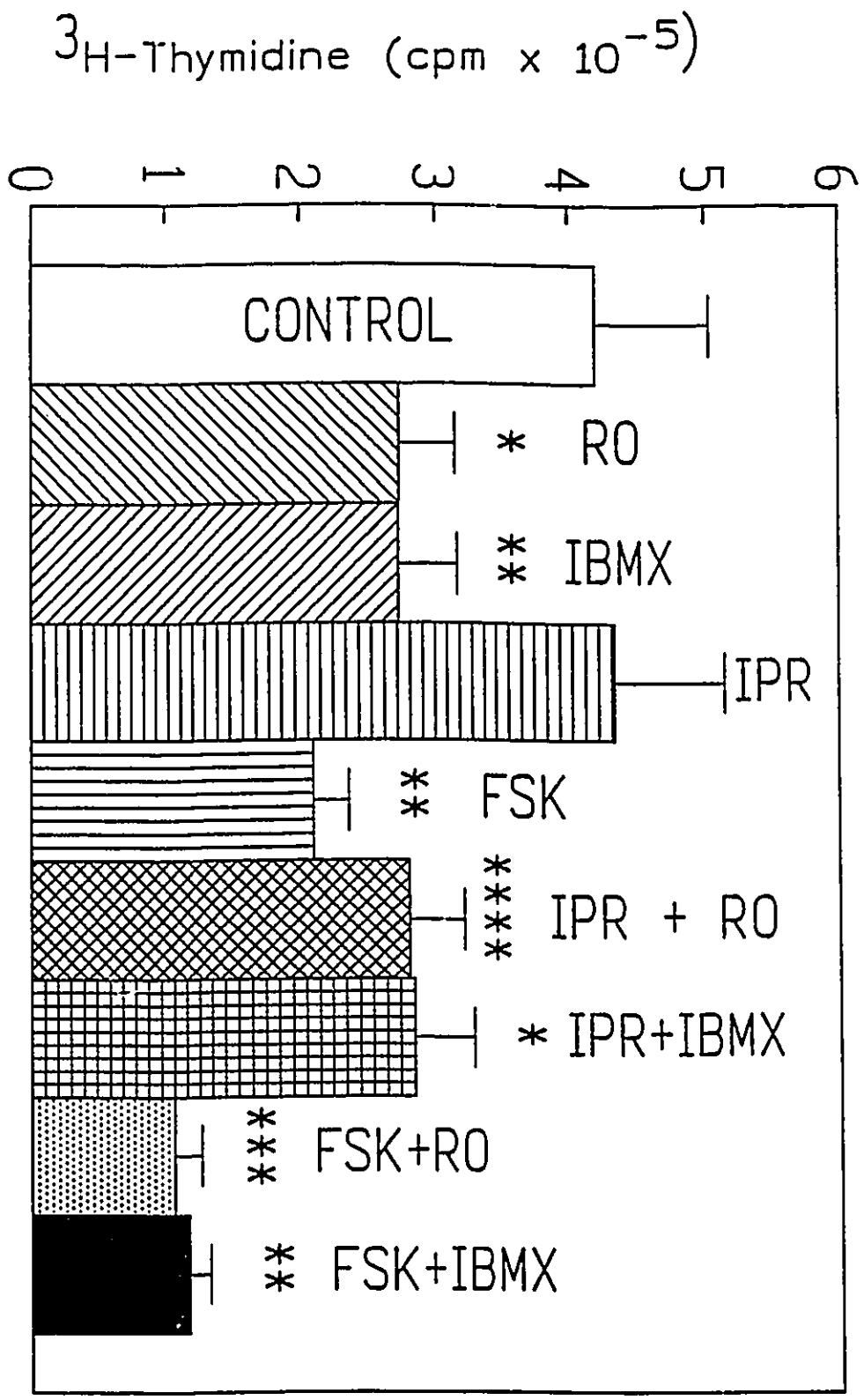

^{3}H-Thymidine (cpm x 10^{-5})
0
1
2
3
4
5
EGF
EGF+GEN
EGF+GEN+RO
EGF+GEN+IBMX
EGF+GEN+IPR
EGF+GEN+FSK
EGF+GEN+CT
EGF+GEN+PT

Figure 24: Effect of cyclic AMP elevating agents on serum mitogenicity (10% BCS):

T51B cells were serum starved (0.2% BCS) for 48 hours after they reached confluence. After this time ^{3}H-thymidine (0.5 μCi/well) was added and cultures were serum replenished (10% BCS) for 24 hours. Various cAMP elevating agents were added either alone or in combination to the cultures. The figure is the presentation of mean ± S.E. of five different experiments. The statistical analysis performed was two-tailed paired t-test comparing the control (10% serum alone) to other additions. The level of significance is indicated as : * $P<0.03$, ** $P<0.02$, *** $P<0.01$ and **** $P<0.05$.


^{3}H-Thymidine (cpm x 10^{-5})
0
1
2
3
4
5
6
CONTROL
* RO
** IBMX
IPR
** FSK
*** IPR + RO
* IPR+IBMX
*** FSK+RO
** FSK+IBMX

inhibition was observed when FSK was present with IBMX. IPR (1 μM) did not inhibit the mitogenic response of serum. FSK (33 μM), RO (30 μM) and IBMX (33 μM) when present separately were able to significantly inhibit DNA synthesis induced by serum by 50%, 40% and 40% respectively. A combination of RO and IPR or IBMX and IPR inhibited DNA synthesis to a similar extent (approximately 35 %) as did RO or IBMX alone.

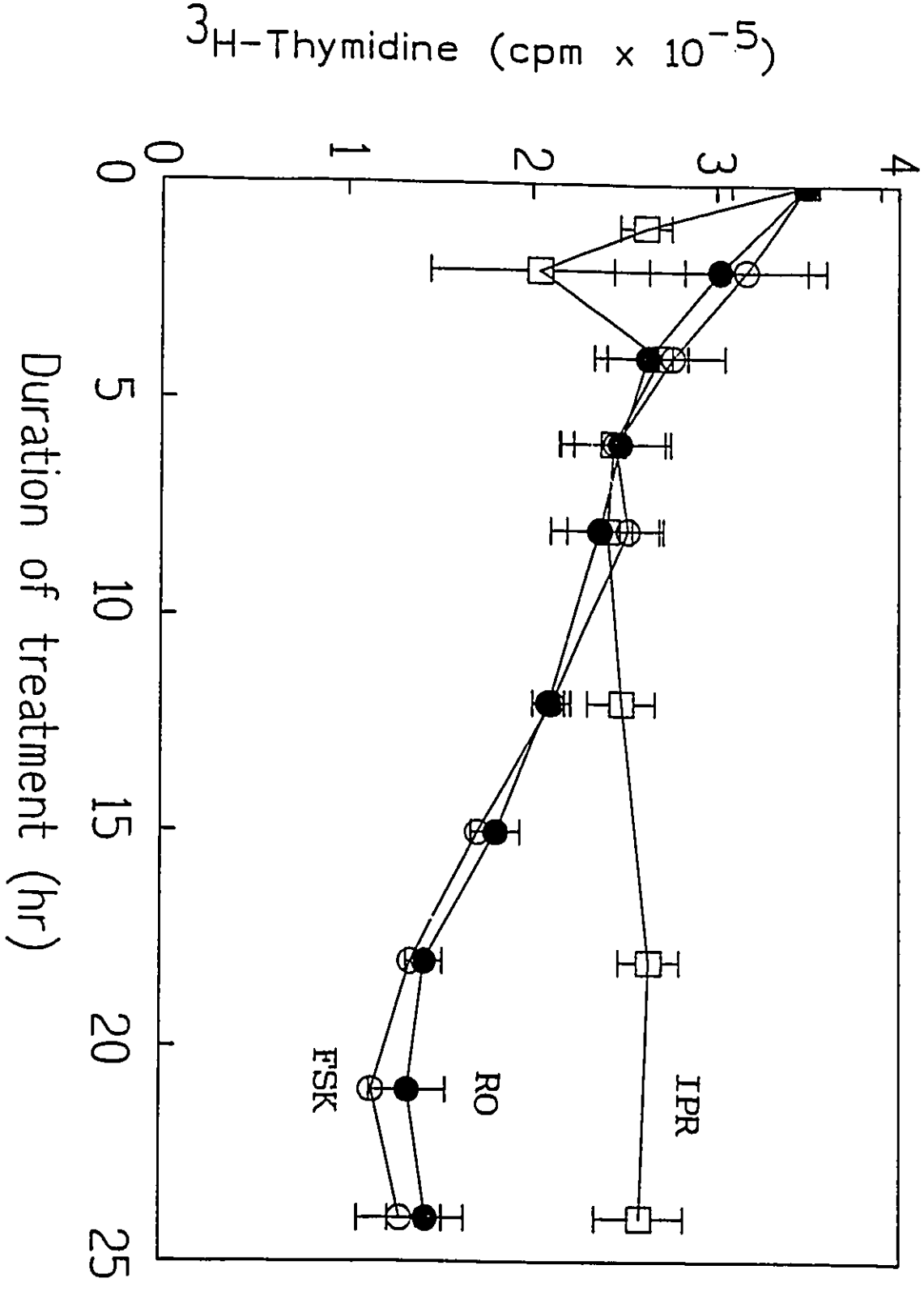
The results suggest that all cAMP elevating agents except IPR inhibited DNA synthesis to various degrees possibly by raising cAMP levels.

3.7:4 Time Course Of The Effect Of cAMP Elevating Agents On The EGF Induced DNA Synthesis:

It was of interest to determine the temporal relationship between elevation of cAMP and inhibition of EGF-stimulated DNA synthesis. In other words, how long after stimulation with EGF could cAMP elevating agents be added and still be effective in inhibiting the DNA synthetic response of EGF. Cells were serum starved for 48 hours at which time EGF (8 ng/ml) and ^{3}H-Tdr were added. One of the following cAMP elevating agents RO (30 μM), FSK (33 μM) or IPR (1 μM) was added at the same time as EGF or at different times after EGF addition. The experiments were always terminated 24 hours after EGF had been added. FSK and RO inhibited DNA synthesis in a time dependent manner (Fig.25). Maximal inhibition (65%) was obtained if RO or FSK were added at the same time as EGF or added as long as 6 hours later. Even adding RO or FSK 2 hours before the

Figure 25: Temporal effect of cAMP elevating agents on the mitogenic response of EGF in serum starved (0.2% BCS) T51B cells:

Cells were serum deprived for 48 hours. At this time EGF (8 ng/ml) and ^{3}H-thymidine (0.5 μCi/ml) were added to each 6-well culture dish. RO (30 μM), FSK (33 μM) or IPR (1 μM) were added with EGF or at different times after EGF addition. Reaction was terminated 24 hours after EGF addition. The results represent mean ± S.E. of three different experiments. The values on the Y-axis show ^{3}H-Tdr incorporated (cpm x 10^{-5}) and on the X-axis represent the duration of treatment with various agents before terminating the reaction. The statistical analysis performed was the two-tailed paired t-test which compares values at zero time (EGF alone) to values at different times for which agents were present. The level of significance (P-values) shows that most of the values are significant (using $p<0.05$) except FSK and RO at 2 and 4 hours; FSK at 12 hours; RO at 6 hours and IPR at 1,2 and 24 hours.


^{3}H-Thymidine (cpm x 10^{-5})
0
1
2
3
4
0
5
10
15
20
25
Duration of treatment (hr)
FSK
RO
IPR

experiment was terminated resulted in some inhibition of DNA synthesis. IPR (1μM) did not have as dramatic an effect on DNA synthesis as did RO or FSK. Maximal inhibition (approximately 20-25%) was observed regardless of when IPR was added.

Chapter 4

DISCUSSION

EGF has been shown to affect cell proliferation and differentiation by modulating second messenger systems (Wright et al. 1988; Farese et al. 1989; Carpenter et al. 1979 and Yarden et al. 1988.). I was interested in examining the effect of EGF on cAMP levels, DNA synthesis as well as modulation of these activities by PKC. Most previous studies have used medium containing serum. This complicates the analysis of cAMP synthesis and secretion as well as DNA synthesis, since serum contains an ill-defined mixture of hormones and growth factors. Therefore serum free or serum starved model systems were utilized to detect EGF related alterations in cAMP levels and DNA synthesis. This discussion is divided into three sections: Section 4.1 deals with interactions among EGF, PKC, and the cAMP signalling pathway. Section 4.2 describes cAMP secretion which may contribute to changes in intracellular cAMP. Section 4.3 describes the effect of cAMP elevating agents on the mitogenic actions of EGF and serum.

Section 4.1: Cyclic AMP Synthesis.

Section 4.1.1: Effect of EGF on forskolin and isoproterenol stimulated cAMP synthesis:

The optimal concentration range of EGF used to potentiate the FSK stimulated cAMP accumulation in T51B cells was between 10 to 150 ng/ml

(Fig. 1). For all the experiments the EGF dose used was 16.7 ng/ml. This concentration was used because the initial optimal dose was economical. A similar concentration range for potentiation of cAMP accumulation by EGF has been reported by Ball et al.(1990) and Yu et al. (1992). In general, EGF by itself does not measurably alter the basal level of cAMP (Ball et al; 1990). However, there are exceptions; Yu et al. demonstrated that EGF stimulates cAMP accumulation by directly altering adenylate cyclase activity in cardiac myocyte cultures. Therefore it seems that EGF by itself can affect cAMP synthesis in some systems while in other models EGF acts by potentiating the cAMP response to agents which stimulate adenylate cyclase. Since EGF is mitogenic for T51B cells (Hill et al; 1989) and a surge of cAMP is required for DNA synthesis in these cells (Boynton and Whitfield, 1979), it was of interest to determine if EGF acts by modulating adenylate cyclase and if so, whether EGF by itself was sufficient to raise cAMP levels or if EGF potentiates the action of cAMP elevating agents. Therefore a series of experiment was conducted to monitor cAMP synthesis in cultures pretreated with EGF under a variety of conditions; for example EGF-pretreatment in Hepes/medium or in serum/medium for one or two hours followed by stimulation with either FSK (33 μM) or IPR (1 μM). Control cultures did not receive EGF. All the assays were performed in the presence of Hepes/medium whether cultures were pretreated with EGF in serum/medium or in Hepes/medium. Firstly, for all the experiments that were performed, regardless of the experimental conditions, EGF did not by itself stimulate adenylate cyclase. Under some conditions EGF slightly

inhibited background cAMP synthesis (Fig.5). Sometimes a slight increase (not statistically significant) in background was observed in the presence of EGF (Fig.6). In preliminary experiments, I observed that cells pretreated with EGF in Hepes/medium responded differently to IPR and FSK. It was observed that cAMP synthesis peaked at 2.5 minutes when cells were stimulated with IPR, supporting the previous observation of Aasheim et al.(1989). This was observed in both EGF pretreated and untreated cells (Fig 5). However cAMP synthesis was significantly lower in EGF pretreated cells as compared to untreated cells (Fig.5). As already mentioned in the results, EGF pretreatment inhibited background adenylate cyclase and inhibition of this pool of adenylate cyclase is reflected in the cultures treated with IPR. Thus under these conditions (1 hour EGF pretreatment in Hepes) EGF does not seem to affect the IPR stimulation of adenylate cyclase. However, when cells are stimulated with IPR after one hour EGF pretreatment in serum/medium (Fig 6), the results were opposite to that observed with a one hour pretreatment in Hepes/medium. EGF under these conditions potentiates cAMP synthesis (Fig.6) It can be suggested that pretreatment in serum/medium may provide an essential coupling environment for the β-adrenergic receptor/adenylate cyclase signalling system to interact with the EGF-receptor. An interaction that is maintained untill the β-adrenergic receptor is activated. Thus, I observed higher cAMP synthesis in the cultures which were EGF pretreated in serum/medium compared to pretreatment in Hepes/medium followed by stimulation with IPR.

When cells were pretreated with EGF in Hepes/medium longer than one hour, the response of cAMP synthesis to IPR was decreased (Figs.4 and 7).

Therefore, it seems that length of time of exposure to Hepes/medium affects cAMP synthesis in response to IPR. The cAMP level was higher for cultures incubated one hour in Hepes/medium and significantly lower in cultures exposed to Hepes/medium for two or three hours (Fig. 7). These differences may be due to changes in responsiveness of β-adrenergic receptors to IPR. As cells spend more time in Hepes/medium away from the normal serum environment, their β-adrenergic receptors may become desensitized to IPR (Reviewed by Lefkowitz,Stadel and Caron, 1983).

In comparing the results for the one hour and three hour Hepes/medium exposed cultures, it can be seen that the decrease in fold stimulation in the three hour exposure is due to both an increase in background (no IPR) cAMP synthesis and to a decrease in IPR stimulation.

Similarly prolonged pretreatment with EGF (two hours in Hepes/medium) did not result in any change in cAMP synthesis compared to untreated cells (Fig.8) upon FSK stimulation unless the pretreatment occurred in the presence of serum (Fig.9). Cyclic AMP synthesis in EGF pretreated cells is higher in the IPR stimulated (Fig.6) as compared to FSK stimulated (Fig.9) cultures.

Section 4.1.2: Role of Protein Kinase C:

Protein kinase C is believed to play an important role in signal transduction (Wright et al. 1988; Farese et al. 1989) and is activated by its physiological

activator DAG. However it has been reported that the EGF induced activation of PKC in T51B cells does not involve DAG produced by hydrolysis of inositol lipids but by DAG, that is produced by the hydrolysis of PC (Hill et al., 1989).

The tumor promoting phorbol ester, TPA, which activates PKC directly (El-Fakahary et al. 1989; Takada et al. 1988), was used to explore the possibility that activation of PKC might affect the adenylate cyclase signalling system. Activation of PKC by TPA (100 nM) results in neither stimulation nor inhibition of cAMP synthesis stimulated with IPR (1 μM) for either EGF pretreated or untreated cultures either in Hepes/medium (Fig. 10) or serum/medium (Fig. 11). This observation is in contrast to that reported by Aasheim et al.(1989) in T51B cells. However, cAMP synthesis stimulated by FSK is potentiated by TPA (Fig. 12) in both EGF pretreated and untreated cells. It is possible that the TPA used for the IPR experiments was inactive. This is unlikely since the same TPA stocks were used for the experiments reported in Figures 12 and 13 where TPA potentiated cAMP synthesis stimulated with FSK. The discrepancy between the report by Aasheim et al.(1989) and my results may be due to the culture medium and passage number as well as the state of the cells.

There are a number of reports in the literature which demonstrate that cAMP synthesis varies with passage number and with the culture medium that is used. For example, it has been reported that basal level of cAMP in cultured bovine tracheal gland cells vary according to the culture medium and passage number (Benali, Dupuit, Chevilard, Jacquot, Haye and Puchelle; 1991). I am not

aware of any studies that specifically demonstrate the effect of passage number and culture medium on TPA or IPR induced cAMP synthesis.

There are a number of factors which could explain the discrepancy between my results and those of Aasheim et al. The T51B cells used in the report by Aasheim et al. were grown in 85% DMEM and 15% BCS. The reactions were performed in 49.9% Hams F12, 49.9% DMEM, 10 mM Hepes and 0.2% BCS. In contrast, for my experiments, cells were grown in 90% BME, 10% BCS and the experiments were performed in BME, 10 mM Hepes. Furthermore and most importantly, for the experiments reported by Aasheim et al. (1989) the TPA was added at the same time as IPR. For my experiments TPA was added 2.5 minutes after IPR, at the time when cAMP synthesis had already reached its maximum. Since Aasheim et al. (1989) and a technician in Dr. Kleine's laboratory had already demonstrated that TPA potentiates the cAMP stimulatory action of IPR, I was not interested in repeating these experiments. I was interested in longer term effects of TPA such as whether TPA (or activation of PKC) could prolong the effect of IPR in the untreated and EGF pretreated cells. If PKC acts downstream of the IPR receptor, for example a direct effect on adenylate cyclase, then the cyclase may be sensitized or modulated in such a way that the IPR response could be prolonged. The results indicate that TPA was added too late to have an effect on the IPR response. How and where PKC acts on these cells remain to be determined.

The experiments with FSK activated cAMP synthesis has uncovered a

number of interesting complex interactions between adenylate cyclase, EGF, PKC and serum. For example, EGF potentiates FSK activation of adenylate cyclase if EGF is added any time between 20 minutes before or 10 minutes after FSK (Figs.2 & 3). If EGF is added one or two hours before FSK no potentiation of the cAMP synthesis is obtained even though EGF is still present at the time of FSK addition (Figures 3 and 8). In fact, an inhibition of cAMP synthesis is often observed (Fig. 3). Whether this is due to downregulation of the EGF receptors (internalization) is not known. Nevertheless, there seems to be a narrow window within which EGF must be added in order to potentiate the effect of FSK. However, EGF potentiation of FSK stimulated adenylate cyclase is observed if the cultures were pretreated for one hour in the presence of serum/medium followed by a two hour incubation in EGF-free Hepes/medium (Fig.9). Thus, either the presence of serum during the EGF pretreatment or the two hours incubation in EGF-free Hepes/medium has allowed for the EGF effect to be expressed three hours after EGF had been added.

In contrast to the result with IPR, FSK stimulated adenylate cyclase is increased at least two fold by activation of PKC by TPA (Figures 12 and 13). This occurs whether or not the cells had been pretreated with EGF; however the presence of EGF seems to attenuate the potentiation due to TPA (Fig.12). Interestingly, the presence of serum during the EGF pretreatment (or the 2 hours incubation in EGF-free Hepes) has abolished this attenuation due to EGF (Fig.13).

Thus EGF can under certain conditions potentiate the action of FSK and this

potentiation can be modulated by serum. Activation of PKC is involved in potentiation of FSK stimulated adenylate cyclase and this effect of PKC can be modulated by EGF and possibly by serum.

Section 4.2: Cyclic AMP Secretion:

Cyclic AMP secretion occurs in many different cell types (reviewed by Barber and Butcher, 1984). In avian erythrocytes the release of cAMP was found to be a significant mechanism for the control of intracellular cAMP levels (Barber and Butcher, 1984; Brunton and Mayer, 1979). However, egress of cAMP in cultured fibroblasts contributed little to the overall control of intracellular cAMP levels (Barber and Butcher, 1981; Barber, Ray and Butcher, 1980). T51B cells have been shown to display cAMP binding sites (cAMP-dependent protein kinase) on their surfaces (Kleine and Whitfield, 1987) which suggests that secretion of cAMP may occur in order to activate the extracellular protein kinases. Therefore, it was of interest to determine whether or not secretion of cAMP occurred in order to regulate the level of intracellular cAMP. If cAMP secretion is involved in controlling the levels of internal cAMP then differences in the extracellular accumulation may reflect changes in the intracellular cAMP. Therefore experiments were planned to observe the time course of changes in both intracellular as well as extracellular cAMP.

Cyclic AMP secretion by cells pretreated with EGF had not been studied before. Egress of cAMP in EGF pretreated cells (1 hour Hepes, IPR stimulated) and untreated cells is not significantly different (Fig. 14) but there is a gradual increase in secretion with time. Although cAMP accumulation outside the cell does not parallel the intracellular decrease (Fig. 5), the decrease of intracellular cAMP can be accounted for, in part, by secretion. Barber and Butcher, 1988, have demonstrated that in the presence of phospho-diesterase inhibitors (PDEI) not only the amount of cAMP is increased but also the process of efflux may be inhibited.

Similar results were obtained regardless of the conditions for EGF pretreatment and of the activators used (IPR or FSK). There was always a gradual increase in extracellular cAMP. The amount of cAMP egress did not parallel the total amount of internal cAMP. For example, a two fold difference in internal cAMP (Fig.6) did not result in any difference in secreted cAMP (Fig.15). Also, experiments where there was no decrease in internal cAMP (Fig.8) still resulted in an increase in cAMP secretion (Fig.16). Due to these differences, it appears that cAMP secretion probably does not occur in order to regulate levels of internal cAMP. It is also relevant to mention that labelled ATP inside and outside the cell remained constant throughout the experimental period. In contrast to cAMP, external ATP levels did not increase with time.

The PTK inhibitor genistein, blocks cAMP secretion but does not seem to affect cAMP synthesis (unpublished observation from Dr. Kleine's laboratory). Therefore it is possible that the EGF receptor tyrosine kinase may have a role in

cAMP secretion although it should be pointed out that genistein is not a specific inhibitor of tyrosine kinase. Other kinases e.g. cAMP-dependent kinase, do not significantly regulate secretion in smooth muscle cells (Fehr et al. 1985). Significant differences exist between different cell types; in some, secretion predominates over intracellular accumulation (WI-38 cells) in contrast to what is observed in other cell lines such as VA-1 cells (Chalpowask, Kelly and Butcher, 1988).

These results suggest that cAMP egress takes place not simply to regulate internal cAMP but it may be one of the mechanisms that helps cells to maintain their physiological state. The results also suggest that it is not simply a preset percentage of cAMP that is secreted. Egress is not by simple passive diffusion but this process seems to be tightly regulated. This still leaves the possibility that cAMP is secreted for the purpose of activating external PKA (Kleine and Whitfield, 1987) although it was not the purpose of this thesis to study this phenomenon. There may be many other factors that are controlling and thereby harmonizing these two processes i.e. cAMP synthesis and secretion.

Section 4.3: DNA Synthesis

Proliferation of T51B cells had been shown to be regulated by cAMP and Ca^{++} (Swierenga et al. 1980). Recently it had been demonstrated that EGF stimulates DNA synthesis in T51B cells (Hill et al. 1989) a process that requires an influx of extracellular calcium ions. It has also been shown that the continuous

presence of EGF stimulates DNA synthesis and mitosis of hepatocytes in culture (McGowan et al. 1981; Skouteris et al. 1980). Serum, which is a rich source of many hormones and growth factors also induces DNA synthesis (Richman et al. 1987). Therefore, it was of interest to plan experiments so that interference due to serum could be reduced to a minimal level when EGF or cAMP elevating agents are added to the cultures.

Cells were serum starved (0.2 % BCS) for 48 hours followed by addition of EGF either alone or in combination with various cAMP elevating agents (Figures 22 to 25). Serum starvation would deprive cultures of growth factors and hormones which otherwise might interfere with the effects of exogenously added EGF and cAMP elevating agents.

The results from these experiments demonstrate that, presumably by elevated cAMP levels, most of the cAMP elevating agents tested (RO, FSK, CT and IBMX) inhibited the mitogenic response of EGF. However PT has no effect. This observation was made previously by Lise Aasheim (M.Sc. thesis 1990). The reason for this is unknown. IPR has very little effect. Since the IPR gives only a brief surge of cAMP (within minutes) it appears that this brief cAMP surge does not last long enough to give prolonged inhibition of DNA synthesis. Therefore, hormones or agents that act via cAMP may stimulate or inhibit cell proliferation depending upon the duration of the cAMP increase (Boynton and Whitfield, 1983; Christoffersen and Brosted, 1980; Domont, Jauniaux and Roger, 1989).

The DNA synthesis induced by EGF was inhibited by genistein, a tyrosine

kinase inhibitor. However, the ^{3}H-Tdr incorporation was not further inhibited when genistein was added in combination with cAMP elevating agents. It appears that genistein blocks EGF dependent DNA synthesis in T51B cells but the results do not rule out the possibility that genistein may affect DNA synthesis which is independent of EGF. This idea seems reasonable due to the fact that genistein is not a specific inhibitor of tyrosine kinase activity which is associated with EGF-R. Dean et al. (1989) have also shown that genistein inhibits DNA synthesis in T51B cells as well as in mouse 10T1/2 fibroblasts.

Temporal effects of cAMP elevating agents on EGF mitogenicity in serum deprived cultures show that inhibition due to FSK as well as RO occurred in a time dependent manner whereas IPR did not have as dramatic an effect as the others two agents (Fig. 25). FSK and RO produce a sustained elevation of cAMP which may have inhibited the EGF mitogenic response as opposed to IPR which gives a brief surge of cAMP. The small inhibition observed with IPR may be due to the fact that there is always a small population of cells that are in the S phase. IPR stimulated cAMP levels might have inhibited only these cells. (Whitfield, 1992).

The serum stimulated mitogenicity was also not inhibited by IPR in serum replenished cultures (Fig.24). However, when RO or IBMX are present along with IPR, 35 % inhibition was observed. This suggests that inhibition of ^{3}H-Tdr incorporation may be due to prolonged increases in cAMP levels stimulated by RO or IBMX. These agents prevent cAMP degradation by PDE and therefore keep the cAMP concentration elevated. FSK, which activates adenylate cyclase directly and

bypasses receptor occupancy, inhibited DNA synthesis by 50 % and when the PDEI's RO or IBMX are present along with FSK, ^{3}H-Tdr incorporation was inhibited by 70%. This suggest that maximal cAMP elevation such as is obtained by RO and FSK or RO and IBMX together results in maximal inhibition of the serum and EGF mitogenic response.

Epidermal growth factor when present with 10 % serum, enhanced DNA synthesis as compared to serum alone (data not shown). This also suggests that other factors in serum apart from EGF are mitogenic. This is probably a valid conclusion since many other factors have been shown to be mitogenic for T51B cells and most of these agents would be expected to be present in serum (Whitfield, 1990).

To summarize, it can be suggested that cAMP elevating agents inhibited the mitogenic response of EGF as well as serum. The tyrosine kinase inhibitor, genistein, blocked EGF induced DNA synthesis but did not further decrease the inhibition of ^{3}H-Tdr incorporation induced by cAMP elevating agents.

Chapter 5

CONCLUSION

The evidence based on the results presented in this thesis suggests that EGF by itself did not stimulate cAMP synthesis but pretreatment with EGF results in differential modulation of IPR and FSK stimulated cAMP synthesis depending on the pretreatment conditions. The interaction between the EGF signalling pathway and receptor mediated (IPR) activation of adenylate cyclase seems to be minimal since at best only slight inhibition of cAMP synthesis was observed following short term (up to one hour) pretreatment with EGF. Although very significant potentiation of IPR stimulated cAMP synthesis was observed following long term (up to three hours) pretreatment with EGF, this potentiation was not real since all of the apparent effect could be accounted for by a sharp decline in IPR stimulated cAMP synthesis (untreated cells) as cultures were incubated with increasing time in Hepes. The presence of EGF in fact is protecting against desensitization or downregulation of the β-adrenergic receptor that is induced by the presence of Hepes (or the lack of serum).

In contrast, the presence of EGF, for up to 40 minutes before adding FSK, leads to a significant potentiation of FSK stimulated cAMP synthesis. Longer term preincubation with EGF in Hepes does not alter the FSK effect but if the EGF preincubation has taken place in serum (up to three hours before adding FSK) the potentiation of FSK induced cAMP synthesis can once again be observed.

The presence of TPA potentiated FSK stimulated cAMP synthesis in untreated and EGF pretreated cells. An interesting interaction was observed in that EGF pretreatment in Hepes partially attenuated the TPA effect.

In some experiments, the large decrease in intracellular cAMP that follows immediately after the initial peak can be accounted for by accumulation of cAMP in the medium. There was however no correlation between internal decrease and external accumulation. In fact, for some experiments no intracellular decrease was observed, yet a large increase in external cAMP was obtained. These results suggest that cAMP secretion is not a passive event which occurs simply to terminate the signalling process but that it is a tightly regulated event. Further work is required to characterize the controls involved.

Various cAMP elevating agents, except IPR and pertussis toxin inhibited EGF and serum induced DNA synthesis. The inhibition is probably due to the fact that these agents have induced a sustained elevated level of cAMP.

Taken together, these results provide answers to the questions that were initially asked. EGF can under the appropriate conditions modulate IPR and FSK stimulated cAMP synthesis although caution must be taken in interpreting the results since pretreatment conditions (Hepes, serum) can by themselves have marked effects. Interesting interactions between the EGF, PKC and adenylate cyclase signalling pathways were observed including the novel observation that EGF can protect against β-adrenergic desensitization.

REFERENCES

Aasheim, L.H., Kleine, L.P. and Franks, D.J. (1989) Activation of protein kinase C sensitizes the cyclic AMP signalling system of T51B rat liver cells. Cellular Signalling; 6, 617-625.

Armelin, H.A. (1973) Pituitary extract and steroid hormones in the control of 3T3 cell growth. Proc. Natl. Acad. USA 70; 2702-2706.

Asaoka, Y., Nakamura, S., Yoshida, K. and Nishizuka, Y. (1992) Protein Kinase C, Calcium and phospholipid degradation. Trends. Bioch. Sci. 17; 414-417.

Ashendel, C.L. (1985) The phorbol ester receptor, a phospholipid regulated protein kinase, Biochem. Biophys. Acta. 822;219-242.

Ball, R.L., Tanner, D.K. and Carpenter, G. (1990) Epidermal growth factor potentiates the cyclic AMP accumulation in A431 cells. J. Biol. Chem. 265(22); 12836-12845.

Barber, R. and Butcher, R.W. (1981) The quantitative relationship between intracellular concentration and egress of cyclic AMP from cultured cells. Mol. Pharmacol. 19; 38-43.

Barber, R. and Butcher, R.W. (1983) The Egress of cyclic AMP from metazoan cells. Adv. Cyclic. Nucl. Res. 15; 119-135.

Barber, R. and Butcher, R.W. (1988) cAMP turnover in intact cell. Methods in Enzymology. 159;50-74.

Barber, R., Ray, K.P. and Butcher, R.W. (1980) Turnover of adenosine 3',5',-monophosphate in WI-38 cultured fibroblasts. Biochemistry. 19; 2560-2567.

Barrandon, Y. and Green, H. (1987) Cell migration is essential for sustained growth of keratinocyte colonies: The role of transforming growth-α and epidermal growth factor. Cell. 50;1131-1137.

Bauer, S. and Jacobs, K.H. (1988) Phorbol ester treatment impairs hormone but not stable GTP analog induced inhibition of adenylate cyclase. FEBS. Lett. 198; 43-46.

Benali, R., Dupuit, F., Chevillard, M., Jacquot, J., Haye, B. and Puchelle, E. (1991) Modulation of epithelial phenotype and functional activity of cultured bovine tracheal gland cells: dependence on the culture medium and passage number. Biology of the Cell. 73(1); 49-56.

Bergeron, J.J.M., Cruz, J., Khan, M.N. and Posner, B.I. (1985) Uptake of insulin and other ligands into receptor-rich endocytic compartments of target cells: The endosomal apparatus. Ann. Rev. Physiol. 47; 383-403.

Bertsch, S. and Marks, F. (1974) Effect of fetal calf serum and epidermal growth factor on DNA synthesis in explant of chick embryo epidermis. Nature. 251; 517-519.

Beyer, G.S., Hoffman, R. and Rubin, B.Y. (1980) The suppressive influence of tumor necrosis factor on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: Synergism of tumor necrosis factor and interferon γ. J. Immunol. 136; 4487.

Billah, M.M. and Anthes, J.C. (1990) The regulation and cellular functions of phosphatidyl choline hydrolysis. Biochem. J. 269; 281-291.

Boney, C., Fink, D., Schlichter, D., Carr, K and Wicks, W. (1983) Direct evidence that protein kinase catalytic subunit mediates the effect of cAMP on tyrosine aminotransferase synthesis. J. Biol. Chem. 258; 4911-4918.

Bjorge, J.D. and Kudlow, J.E. (1987) Epidermal growth factor receptor synthesis is stimulated by phorbol ester and epidermal growth factor. J. Biol. Chem. 262; 6615-6622.

Bosch, F., Bouscarel, B., Slaton, J., Blackmore, P.F. and Exton, J.H. (1986) Epidermal growth factor mimics insulin Effect in rat hepatocytes. Biochem. J. 239; 523-530.

Boynton, A.L. and Whitfield, J.F. (1979) The cyclic AMP dependent initiation of DNA synthesis by T51B rat liver epithelial cells. J. Cell. Physiol. 101; 139-148.

Boynton, A.L. and Whitfield, J.F. (1983) The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. Adv. Cyclic. Nucl. Res. 15;193-294.

Boynton, A.L., Kleine, L.P., Whitfield, J.F., Bossi, D. (1985) Involvement of the Ca^{2+}/phospholipid-dependent protein kinase in G1 transit of T51B rat liver epithelial cells. Exp. Cell. Res. 160; 197-205.

Boynton, A.L., Whitfield, J.F. and MacManus, J.P. (1980) Calmodulin stimulates DNA synthesis by rat liver cell. Biochem. Biophys. Res. Commun. 95; 745-749.

Brown, S., Stanley, C., Selva, E. and Davis, R.J.(1991) Constitutive phosphorylation of the epidermal growth factor receptor blocks mitogenic signal transduction. J. Biol. Chem 266; 1162-1169.

Broxmeyer, H.E., Williams, D.E., Lu, L., Cooper, S., Anderson., S.L., Beyer , S., Hoffman, R. and Rubin, B.Y. (1986) The suppressive influence of human tumor necrosis factor on bone marrow hematopoietic progenitor cells from normal donors and patient with leukemia: synergism of tumor necrosis factor and interferon γ. J. Immunol. 136; 4487-4494.

Brunton,L.L. (1981) Mechanisms mediating the extrusion of cyclic AMP from S49 lymphoma cells. Adv. Cycl. Nucl. Res. 14;672.

Brunton, L.L. and Mayer, S.E. (1979) Extrusion of cyclic AMP from pigeon erythrocytes. J. Biol. Chem. 254; 9714-9720.

Buchner, N.L.R., Patel, U. and Cohen, S. (1978) Hormonal factors and liver growth. Adv. Enz. Reg 16; 205-213.

Carol, A.R. and Hubbard, A.L.(1991a) Sequential processing of epidermal growth factor in early and late endosomes of rat liver. J. Biol. Chem. 266;4348-4356.

Carol, A.R. and Hubbard, A.L.(1991b) Degradation of epidermal growth factor receptor in rat liver. J. Biol. Chem. 266; 21265- 21273.

Carpenter, G., Stoscheck, C.M., Preston, Y.A. and Delarco, J. E. (1983) Antibodies to the epidermal growth factor receptor block the biological activities of sarcoma growth factor. Proc. Natl. Acad. Sci. U.S.A. 80;5627-5630.

Carpenter, G. and Cohen, S.(1990) Epidermal growth factor. J. Biol. Chem. 265;7709-7712.

Carpenter, G. and Cohen, S.(1979) Epidermal growth factor. Ann. Rev. Biochem. 48;193-216.

Chalpowski, F.J., Kelly, L., A. and Butcher, R.W. (1975) Cyclic nucleotides in cultured cells. Adv. Cyclic. Nucleotide. Res. 6; 245-338.

Chen, W.S., Lazar, C.S., Poenie, M., Tsien, Y., Gill, G, N. and Rosenfeld, M.G. (1987) Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature. 328; 820-823.

Christoffersen, T. and Bronstad, GO.(1980) A commentary on the role of cyclic nucleotide in cell growth and malignancy. Prog. Pharmacol. 4;117-135.

Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A. and Hunter, T.(1984). C kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259; 2553-2558.

Cockcroft, S. and Gomperts, B.D.(1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature. 314;534-536.

Cohen, S.(1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animals. J. Biol. Chem. 237;1555-1562.

Cohen, S. and Elliot, G.A. (1963) The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse. J. Invest. Dermatol. 40; 1-5.

Cohen, S. (1965) The stimulation of epidermal proliferation by a specific protein (EGF). Dev. Biol. 12; 394-407.

Conricode, K.M. and Ochs, R.S.(1990) Epidermal growth factor and 12-O-tetradecanoyl phorbol 13-acetate stimulate lactate production and pentose phosphate pathway in freshly isolated rat hepatocytes. J. Biol. Chem. 265; 20931-20937.

Das, M., Miyakawa, T., Fox, C.F., Pruss, R.M., Aharonov, A. and Herschman, H.K.(1977) Specific radiolabelling of a cell surface receptor for epidermal growth factor. Proc. Natl. Acad. Sci. USA 74; 2790-2794.

Davis, R.J.(1988) Independent mechanisms account for the regulation by protein kinase C of the epidermal growth factor receptor affinity and tyrosine-protein kinase activity. J. Biol. Chem. 263; 9462-9469.

Davis, R.J. and Czech, M.P.(1985) Tumor-promoting phorbol esters causes the phosphorylation of epidermal growth factor receptor in normal human fibroblasts at threonine-654. Proc. Natl. Acad. Sci. USA 82; 1974-1978.

Davoren, P.R. and Sutherland, E.W.(1963) The effect of L-epinephrine and other agents on the synthesis and release of Adenosine 3', 5',-Phosphate by whole pigeon erythrocytes. J. Biol. Chem. 238; 3009-3015.

Dean, N.M., Kanemitsu, M. and Boynton, A.L. (1989) Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. Biochem. Biophys. Res. Commun. 165; 795-801.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H.(1987) Synthesis of messenger RNA for transforming growth factor-α and β and epidermal growth factor receptor by human tumors. Cancer. Res. 47;707-712.

Domont, J.E., Jauniaux, J.C. and Roger, P.P.(1989) The cAMP-mediated stimulation of cell proliferation. Trends. Biochem. Sci. 14; 67-71.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich., Schlessinger, J. and Waterfield, M.D.(1984) Close similarity of epidermal growth factor receptor and V-erbB oncogene protein sequence. Nature. 307; 521-527.

Downward, J., Waterfield, M.P. and Parker, P.J.(1985) Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. J. Biol. Chem. 260; 14538-14546.

El-Fakahary, E.E., Algar, B.E., Lai, W.S., Pitter, T.A., Worley, P.F. and Baraban, J.M. (1988) Neuronal muscarinic responses:role of protein kinase C. FASEB. J. 2; 2575-2583.

Exton, J.H.(1990) Signalling through phosphatidylcholine breakdown. J. Biol. Chem. 265; 1-4.

Farese, R.V., Nair, G.P., Sierra, C.G., Standaert, M.L., Pollet, R.J. and Cooper, D.R.(1989) Insulin-like effects of epidermal growth factor and Insulin-like growth factor-I on [^{3}H]2-deoxyglucose uptake, diacylglycerol generation and protein kinase C activation in BC3H-1 myocytes. Biochem. J. 261; 927-934.

Farrel, P.H.O., Edger, B.A., Lakich, D. and Lehner, C.F.(1989) Directing cell division during development. Science. 246;635-640.

Fehr, T.F., Ellen, S.D., Goldman, S.J and Slakey, L.L. (1990) Cyclic AMP efflux is regulated by occupancy of the adenosine receptor in pig aortic smooth muscle cells. J. Biol. Chem. 265; 10974-10980.

Franks, D.J., Whitfield, J.F. and Durkin, J.P. (1985) The mitogenic/oncogenic P^{21} RAS protein stimulates adenylate cyclase activity early in G1 phase of NRK rat kidney cells. Biochem. Biophys. Res. Commun. 127; 318-325.

Friedmann, B., Frackelton, A.R.Jr., Ross.A.H., Connors, J.M., Fujuki, H., Sugimura, T. and Rosner, M.R.(1984) Tumor promoter block tyrosine-specific phosphorylation of the epidermal growth factor receptor. Proc. Natl. Acad. Sci. USA. 81; 3034-3038.

Ghosh-Dastidar, P. and Fox, C.F.(1984) Cyclic AMP dependent protein kinase stimulates epidermal growth factor-dependent phosphorylation of epidermal growth factor receptor. J. Biol. Chem. 259; 3864-3869.

Gilligan, A., Prentiki,M., Gelennon, M.C. and Knowles, B.B.(1988) Epidermal growth factor-induced increase in inositol tris-phosphate, inositol tetrakisphophate and cytosolic Ca^{2+} in a human hepatocellular carcinoma-derived cell line. FEBS. Lett. 233; 41-46.

Giuffre, L., Schreyer, M., Mach, J.P. and Carrel, S.(1988) Cyclic AMP induces differentiation in vitro of human melanoma cells. Cancer. 61; 1132-1141.

Goldstein, J.L., Anderson.R.G.W. and Brown, M.S.(1979) Coated pits, Coated vesicles and receptor mediated endocytosis. Nature. 279; 679-685.

Gorbachevskaya, L.V., Borovkova, T.V., Rybin, V.O. and Fedorov, N.A. (1983) Calmodulin stimulates lymphocyte proliferation. Bull. Exp. Med. Biol. 95; 361-363.

Hagey, L.R., Kanter, J.R. and Brunton, L.L.(1988) Inhibition of cyclic AMP efflux by insect pheromones and fatty acids. FASEB. J. 2;1065.

Hamet, P., Pang, S.C. and Tremblay, J.(1989) Atrial natriuretic factor-induced egression of cyclic Guanosine 3':5'-monophosphate in cultured vascular smooth muscle and endothelial cells. J. Biol. Chem. 264;12364-12369.

Heasley, L.E. and Brunton, L.L.(1985) Prostaglandin A1 metabolism and inhibition of cyclic AMP extrusion by avian erythrocytes. J. Biol. Chem. 260;11514-11519.

Hill, T.D., Kindmark, K.H. and Boynton, A.L. (1988) Epidermal growth factor-stimulated DNA synthesis requires an influx of extracellular calcium. J. Cellular. Biochem. 38; 137-144.

Holladay, L.A., Savage, C.R.Jr., Cohen, S. and Puette, D.(1976) Conformation and unfolding thermodynamics of Epidermal growth factor and derivatives. Biochemistry 15; 2624-2633.

Hollenberg, M.D. and Cuatrecasas, P.(1973) Epidermal growth factor: Receptor in human fibroblasts and modulation of action by cholera toxin. Proc. Natl. Acad. Sci. USA. 70;2964-2968.

Huckle, W.R., Hepler, J.R., Rhee, S.G., Harden, T.K. and Earp, H.S.(1990) Protein kinase C inhibits epidermal growth factor dependent tyrosine phosphorylation of phospholipase C-γ and activation of Phosphoinositide hydrolysis. Endocrinology. 127; 1697-1705.

Hunter, T., Ling, N. and Cooper, J.A.(1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature. 311; 480-483.

Hunter, T. and Cooper, J.A.(1981) Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell. 24; 741-752.

Hunter, T. and Cooper, J.A.(1985) Protein tyrosine kinase. Ann. Rev. Biochem. 54; 897-930.

Ibbotson, K.J., Harrod, J., Gowen, M., D, Souza, S., Smith, D.D., Winkler, M.E., Derynck, R. and Mundy, G.R.(1986) Human recombinant transforming growth factor-α stimulates bone resorption and inhibits its formation in vitro. Proc. Natl. Acad. Sci. USA. 83; 2228-2232.

Iwashita, S., Mitsui, K., Shoji-Kasai, Y. and Senshu-Miyaike, M. (1990) cAMP-mediated modulation of signal transduction of epidermal growth factor (EGF) receptor systems in Human epidermoid carcinoma A431 cells. J. Biol. Chem. 265; 10702-10708.

Johnson, R.M., Connelly, P.A., Sisk, R.B., Pobiner, B.F., Hewlett, E.L. and Garrison, J.C.(1986) Pertussis toxin or phorbol, 12-myristate β-acetate can distinguish between epidermal growth factor and angiotensin stimulated signals in hepatocytes. Proc. Natl. Acad. Sci. USA. 83;2032-2036.

Johnson, R.M. and Garrison, J.C.(1987) Epidermal growth factor and Angiotensin II stimulate formation of inositol 1, 4, 5-and inositol 1,3,4,-trisphosphate in hepatocytes. J. Biol. Chem. 262; 17285-17293.

Jones, A., Boynton, A.L., MacManus, J.P. and Whitfield, J.F (1982) Ca-calmodulin mediates the DNA-synthetic response of calcium deprived liver cells to the tumor promoter TPA. Exp. Cell. Res. 138; 87-93.

Katada, T., Gillman, A.G., Watanabe, Y., Bauer, S. and Jacobs, K.H.(1985) Protein kinase C phosphorylates the inhibitory guanine nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. Eur. J. Biochem. 151; 431-437.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J. and Wigler, M. (1985) Functional homology of mammalian and yeast RAS genes. Cell. 40; 19-26.

King, C.D and Mayer, S.E.(1974) Inhibition of egress of 3', 5'-monophosphate from pigeon erythrocytes. Mol. Pharmacol. 10; 941-953.

King, C.S. and Cooper, J.A.(1986) Effects of protein kinase C activation after epidermal growth factor binding on epidermal growth factor receptor phosphorylation. J. Biol. Chem. 261; 10073-10078.

Kleine, L.P. and Whitfield, J.F. (1987) Serum-activated T51B rat liver cells transiently accumulate cyclic AMP-dependent protein kinases on their surfaces during the G1 phase. J. Cell. Physiol. 132; 354-358.

Kleine, L.P., Whitfield, J.F. and Boynton, A.L. (1986) Ca^{2+}-dependent cell surface protein phosphorylation may be involved in the initiation of DNA synthesis. J. Cell. Physiol. 129; 303-309.

Knowles, A.F., Salas-Prato, N. and Villela, J.(1985) Epidermal growth factor inhibits growth while increasing the expression of an ecto-Ca^{2+}-ATPase of a human hepatoma cell line. Biochem. Biophys. Res. Commun. 126; 8-14.

Konijin, T.M., van de Meene, J.G.C., Bonner, J.T. and Barkley, D.S. (1967) The acrasin activity of adenosine-3', 5'-cyclic phosphate. Proc. Natl. Acad. Sci. USA. 58; 1152-1154.

Korc, M., Chandrasekar, B. and Shaw, G.N.(1991) Differential binding and biological activities of epidermal growth factor and Transforming growth factor-α in a human pancreatic cell line. Cancer. Res. 51; 6243-6249.

Krupinski, J., Coussen, F., Bakalyar, H.A., Tang, W.-J., Feinstein, P.G., Orth, K., Slaughter, C., Reed, R.R and Gilman, A.G. (1989) Adenylyl cyclase Amino Acid sequence:Possible channel or transporter-like structure. Science. 244; 1558-1564.

Kubler, D., Pyerin, W., Bill, O., Hotz, A., Sonka, J. and Kinzel, V. (1989) Evidence for Ecto-protein kinase activity that phosphorylates kemptide in a cyclic AMP-dependent mode. J. Biol. Chem. 264; 14549-14555.

Lee,D.C.,Rochford,R.M.,Todaro,G.J. and Villareal,L.P. (1985) Developmental expression of rat transforming growth factor-alpha mRNA. Mol. Cell. Biol. 5; 3644-3646.

Lefkowitz, R.J., Stadel, J.M. and Caron, M.G.(1983) Adenylate cyclase-coupled beta-adrenergic receptors: Structure and mechanisms of activation and desensitization. Ann. Rev. Biochem. 52; 159-186.

Livneh, E., Dull, T.J., Berent, E., Prywesi, R., Ullrich, A. and Schlessinger, J.(1988) Release of a phorbol ester induced mitogenic block by mutation at Thr-654 of the epidermal growth factor receptor. Mol. Cell. Biol. 8; 2302-2308.

MacNeil, S., Walker, S.W., Senior, H.J., Bleehen, S.S. and Tomkinson, S. (1984) Effects of extracellular calmodulin and calmodulin antagonist on B16 melanoma cell growth. J. Invest. Derm. 83; 15-19.

Majerus, P.W., Wilson, D.B., Connoly, T.M., Bross, T.E. and Neufeld, E.J.(1985) Phosphoinositide turnover provides a link in stimulus response coupling. Trends. Biochem. Sci.10; 168-171.

Makman, R.S. and Sutherland, E.W.(1965) Adenosine 3, '5'-cyclic monophosphate in E.coli. J. Biol. Chem. 240; 1309-1314.

Martin, T.J.F. (1991) Receptor regulation of phosphoinositidase C. Pharmacol. Ther. 49; 329.

Massague, J.(1983.a) Epidermal growth factor like - transforming growth factor : I isolation, chemical characterization and potentiation by other transforming factors from feline sarcoma virus-transformed rat cells. J. Biol. Chem. 258; 13606-13613.

Massague, J.(1983.b) Epidermal growth factor-like transforming growth factor: II interaction with epidermal growth factor receptors in human placenta membrane and A431 cells. J. Biol. Chem. 258;13614-13620.

Maxfield, F.R. and Yamashiro, D.J.(1987) Endosome acidification and the pathways of receptor-mediated endocytosis. Adv. Exp. Med. Biol. 225; 189-198.

McAtee, P. and Dawson, G.(1989) Rapid dephosphorylation of PKC substrates by PKA activators results from inhibition of diacylglycerol release. J. Biol. Chem. 264; 11193-11199.

McGowan, J.A., Starain, A.J. and Bucher, N.L.R.(1981) DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: Effects of epidermal growth factor, insulin, glucagon and cyclic AMP. J. Cell. Physiol. 108; 353-363.

Moule, S.K. and McGiven, J.D.(1987) Epidermal growth factor, like glucagon, exerts a short-term stimulation of alanine transport in rat hepatocytes. Biochem. J. 247; 233-235.

Nair, B.G., Parikh, B., Milligan, G. and Patel, T.B.(1990) Gsα mediates epidermal growth factor elicited stimulation of rat cardiac adenylate cyclase. J. Biol. Chem. 265; 21317-21322.

Nair, B.G., Rashed, N.M. and Patel, T.B.(1989) Epidermal growth factor stimulation of cardiac adenylate cyclase through a GTP-binding regulatory protein. Biochem. J. 264; 563-571.

Nemecek, G.M., Wells, J.N. and Butcher R.W.(1980) Inhibition of Fibroblast cyclic AMP escape and cyclic nucleotide phosphodiesterase activities by xanthines. Mol. Pharmacol. 18; 57-64.

Nishizuka, Y. (1992) Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. Science. 258; 607-614.

Pawson, T.(1988) Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene. 3; 491-495.

Pelech, S.L., Charest, D.L., Howard, S.L., Paddor, H.B. and Salari, H.(1990) Protein kinase C activation by platelet- activating factor is independent of enzyme translocation. Biochem. Biophys. Acta. 1051; 100-107.

Pines, M., Ashkenazi, A., Cohen-Chapnikn., Binder, L. and Gertler, A. (1988) Inhibition of the proliferation of Nb2 cells by femtomolar concentration of cholera toxin and partial reversal of the effect of 12-O-tetradecanoyl-phorbol-13-acetate. J. Cell. Biochem. 37; 119-129.

Rackoff, W.R., Rubin, R.A. and Earp, H.S.(1984) Phosphorylation of the hepatic EGF receptor with cAMP-dependent protein kinase. Mol. Cell. Endocr. 34; 113-119.

Ran, W., Dean, M., Levine, R.A., Hanckle, C. and Campisi, J.(1986) Induction of C-fos and C-myc mRNA by epidermal growth factor or calcium ionophore is cAMP dependent. Proc. Natl. Acad. Sci. USA. 83; 8216-8220.

Rhee, S.G., Suh, P.G., Ryu, S.H. and Lee, S, Y.(1989) Studies of inositol phospholipid specific phospholipase C. Science. 244; 546-550.

Richman, R.A., Claus, T.H., Pilkis, S.J. and Friedman, D.L. (1976) Hormonal stimulation of DNA synthesis in primary cultures of rat hepatocytes. Proc. Natl. Acad. Sci.USA. 73; 3589-3593.

Rindler, M.J., Bashor, M.M., Spitzer, N and Saier, Jr.M.H. (1978) Regulation of adenosine 3', 5'-monophosphate efflux from animal cells. J. Biol. Chem. 253; 5431-5436.

Ryu, S.H., Pann-G, S., Cho, K.S., Lee, K.Y. and Rhee, S.G. (1984) Bovine brain cytosol contains three immunologically distinct forms of inositol-specific phospholipase C. Proc. Natl. Acad. Sci. 84; 6649-6653.

Savage, C.R., Inagami, T. and Cohen, S. (1972) The primary structure of epidermal growth factor. J. Biol. Chem. 247; 7612-7621.

Sawyer, S.T. and Cohen, S.(1981) Enhancement of calcium uptake and phosphatidyl inositol turnover by epidermal growth factor in A-431 cells. Biochemistry. 20; 6280-6286.

Schlessinger, J.(1988) Signal transduction by allosteric receptor oligomerization. Trends. Biochem. Sci. 13; 443-447.

Schlessinger, J.(1986a) Allosteric regulation of the epidermal growth factor receptor kinase. J. Cell. Biol 103; 2067-2072.

Schultz, G.S., White, M., Mitchelle, R., Brown, G., Lynch, J., Twardzik, D.R. and Todaro, G.J. (1987) Epithelial wound healing enhanced by transforming growth factor-α and vaccinia growth factor. Science. 235; 350-352.

Sibley, D.R., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J.(1988) Phosphorylation of cell surface receptors: a mechanism for regulating signal transduction pathways. Endocr. Rev. 9; 38-56.

Sibley, D.R., Nambi., Peters, J.R. and Lefkowitz, R.J.(1984) Phorbol diesters promotes β-adrenergic receptor phosphorylation and adenylate cyclase desensitization in duck erythrocytes. Biochem. Biophys. Res. Commun. 121; 973-979.

Skouteris, G.G., Ord, M.G. and Stocken, L.A. (1988) Regulation of the proliferation of primary rat hepatocytes by eicosanoids. J. Cell. Physiol. 135; 516-520.

St.Hilaire, R.J. and Jones, A.L. (1982) Epidermal growth factor: Its biological and metabolic effects with emphasis on the hepatocyte. Hepatology. 2; 601.

Stahl, M.L., Ferenz, C.R., Kelleher, K.L., Kriz, R.W. and Knopf, J.L.(1988) Sequence similarity of phospholipase C with the non-catalytic regions of Src. Nature. 332; 269-272.

Starkey, R.H., Cohen, S. and Orth, D.N.(1975) Epidermal growth factor: Identification of a new hormone in human urine. Science. 189; 800-802.

Sugden, D., Vanecek, J., Klein, D.C., Thompson, T.P. and Anderson, W.B.(1985) Activation of protein kinase C potentiates isoprenaline induced cAMP accumulation in rat pinealocytes. Nature. 314; 359-361.

Swierenga, S.H.H. (1984) In: In vitro models for cancer Research, (ed.M.M.Webber and L.I. Skelely), CRC Press, Boca Raton, FL; vol 2; 61-89.

Swierenga, S.H.H., Whitfield, J.F., Boynton, A.L., Macmanus, J.P., Rixon, R.H., Sikorska, M., Tsang, B.K and Walker, P.R (1980) Regulation of proliferation of normal and neoplastic rat liver cells by calcium and cyclic AMP. Ann. N.Y. Acad. Sci. 349; 294-311.

Takada, K., Amino, N., Testsumoto, T. and Miyaik, K.(1988) Phorbol esters have dual actions through protein kinase C in regulation of proliferation of FRTL-5 cells. FEBS. Lett. 234; 13-16.

Tam, J.P., Marquardt, M., Roseberger, D.F., Wong, T.W. and Todaro, G.J.(1984) Synthesis of biologically active rat transforming growth factor α. Nature. 309; 376-378.

Teitelbaum, I., Strasheim, A. and Berl, T.(1990) Epidermal growth factor-stimulated phosphoinositide hydrolysis in cultured rat inner medullary collecting tubule cells. J. Clin. Invest. 85; 1044-1055.

Todaro, G.J., Lee, D.C., Webb, N.R., Rose, T.H. and Brown, J.P.(1985) In: Cancer cells 3.Eds; J.Feramisco, B.Ozanne and C.Stilles. (Cold Spring Harbour Laboratory, N.Y.) Vol 3;pp 51-58.

Turkington, R.W.(1969) The role of epithelial growth factor in mammary gland and development in vitro. Exp. Cell. Res. 57; 79-85.

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayer, E.L.V., Whittle, N., Waterfield, M.D. and Seeburg, P.H.(1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature. 309; 418-425.

Watanabe, Y., Horn, F., Bauer, S. and Jacobs, K.H.(1985) Protein kinase C interferes with Ni-mediated inhibition of human platelet adenylate cyclase. FEBS. Lett. 192; 23-37.

Watson, S. and Abbott, A.(1991) Tips receptor nomenclature. Supplement. Trend. Pharm. Sci.1-35

Wen, S.C., Chang, C.-M., Reitherman, R.W and Harding, B.W. (1985) Characterization of cell surface adenosine 3', 5'-monophosphate-Binding proteins in Y-1 mouse adrenal tumor cells. Endocrinology. 116; 935-944.

Whitfield, J, F (1990) Calcium, Cell cycle and Cancer. CRC Press, Boca Raton FL.

Whitfield, J.F. (1992) Calcium signals and cancer. Critical reviews in Oncogenesis. 3(1,2); 55-90.

Whitley, B. and Glasner, L.(1986) Epidermal growth factor promotes phosphorylation at threonine-654 of the EGF-receptor: possible role of protein kinase C in Homologous regulation of the EGF- receptor. J. Cell. Biol. 103; 1355-1362.

Wrann, M. and Fox, F.(1979) Identification of Epidermal growth factor receptors in a Hyperproducing Human epidermoid carcinoma cell line. J. Biol. Chem. 254; 8083-8086.

Wright, T.M., Rangan, L.A., Shin, H.S. and Raben, D.M.(1988) Kinetic analysis of 1, 2-Diacylglycerol mass levels in cultured fibroblasts. J. Biol. Chem. 263; 9374-9380.

Yamashita, A., Kurokawa, T., Dan, ura, T., Higashi, K. and Ishibashi, S.(1986) Induction of desensitization by phorbol-ester to β-adrenergic agonist stimulation in adenylate cyclase system of rat reticulocytes. Biochem. Biophys. Res. Commun. 138; 125-130.

Yarden, Y. and Schlessinger, J.(1987) Self-phosphorylation of epidermal growth factor receptor : Evidence for a model of intracellular allosteric activation. Biochemistry. 26; 1434-1442.

Yarden, Y. and Ullrich, A.(1988) Growth factor receptor tyrosine kinase. Ann. Rev. Biochem. 57; 443-478.

Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J. and Caron, M.G.(1987) Cross-talk between cellular signalling pathways suggested by phorbol-ester induced adenylate cyclase. Nature. 327; 67-70.

Yu, Y., Nair, B.G. and Patel, T.B. (1992) Epidermal Growth Factor stimulates cAMP accumulation in cultured rat cardiac myocytes. J. Cellular. Physiol. 150;559-567.

Zeytin, F., Mougin, C., Brazeau, P. and Ling, N. (1984) Effects of Forskolin and Cholera toxin on cyclic AMP release in a neurotensin secreting rat C-cell line. Biochem. Biophys. Res. Commun. 120; 540-547.

CURRICULUM VITAE

NAME	SANJAY K.PANDEY
PLACE OF BIRTH	Varanasi, India
CITIZENSHIP	Indian (Permanent resident)
EDUCATION	High School: Central Hindu School,Varanasi, India, (Grades 6-10)
	College: Central Hindu School,Varanasi, India, (Grades 11-12)
	University: Banaras Hindu University
	India, B.Sc.(Hons) Chemistry, 1985
	M.Sc. in Biochemistry, 1987
AWARDS	University Grant Commission (UGC)
	Graduate Scholarship,New Delhi, India, 1987-89
	CSIR-UGC Joint Scholarship,India, 1989-90
	University of Ottawa Entrance Award, 1990.
MEETING	Attended 34th Annual CFBS meeting in Kingston, Ontario,1990.
EXPERIENCE	Lab Demostrator-BCH 3946, 2936
	University of Ottawa, 1990-1992
EXTRA CURRICULAR ACTIVITIES	Member of N.A.C.O.I. (National Association Of Canadians with origins in India),
	Sports: Soccer, Swimming, Badminton, Chess