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THE EFFECT OF THE SECOND MESSENGER CYCLIC AMP ON SCINDERIN REDISTRIBUTION, F-ACTIN DISASSEMBLY AND CATECHOLAMINE SECRETION IN BOVINE ADRENAL CHROMAFFIN CELLS

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Thesis submitted to the Department of Pharmacology in partial

fulfillment of the requirements for the degree of

Master of Science

University of Ottawa

Ottawa, Ontario, Canada

August 1993

ISBN 0-315-95971-1

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Work in our laboratory has demonstrated that stimulation of bovine chromaffin cells with nicotine elicits as a prelude to exocytosis, a) redistribution of scinderin (Sc), a novel 80 kD Ca^{2+}-dependent actin filament severing protein and b) cortical F-actin disassembly. The work presented in this thesis shows that the second messenger, cyclic AMP (cAMP) modulates Sc redistribution, F-actin disassembly and catecholamine (CA) secretion. Here we show that forskolin (F) produces a concentration-related (10 μM - 50 μM) inhibition of Sc redistribution, F-actin disassembly and CA release in response to 10 μM nicotine. F also induces a concentration-dependent (10 μM -50 μM) increase in cAMP levels. Increasing intracellular cAMP with 50 μM F or incubation with the F analogs 6-acetyl-7-deacetylforskolin or deacetylforskolin (100 μM) as well as the cAMP membrane permeant analog, 8-Bromo cAMP (2.5mM) for 40 s also inhibits Sc redistribution, F-actin disassembly and CA secretion. The inhibitory effect of F on nicotine-induced Sc redistribution and F-actin disassembly is observed even upon 5 s of incubation while inhibition of CA secretion cannot be detected until 20 s of incubation with F. These effects are accompanied by an increase in cAMP. The discrepancy in timing between inhibition of both Sc redistribution and F-actin disassembly in relation to CA secretion may be explained by the fact that Sc redistribution and F-actin disassembly seem to occur simultaneously and to precede secretion. Although work on cAMP by others has yielded conflicting results, our findings suggest

that cAMP may play a role in modulating cytoskeleton dynamics during secretion. This also implies that this second messenger can attenuate the secretory response either by preventing disassembly of F-actin or activation of Sc thus, denying the secretory vesicles access to exocytotic sites. Alternatively, the observed effects may represent a modulation of nicotinic receptor activity by cAMP.

STATEMENT OF THE PROBLEM

It has been proposed that adrenal medullary chromaffin cells possess a network of actin filaments localized underneath the plasma membrane (Lee and Trifaró, 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986; Nakata and Hirokawa, 1992). This actin network is thought to serve as a physical obstruction (Trifaró et al., 1982; Trifaró et al., 1984; Cheek and Burgoyne, 1986; Burgoyne and Cheek, 1987; Trifaró et al., 1989b) which prevents the interaction between the specialized organelles which store the secretory products of chromaffin cells, the chromaffin granules (Smith, 1968; Trifaró, 1977) and the plasma membrane at sites of exocytosis by precluding granule movement to these regions (Trifaró et al., 1982, 1984, 1989b; Cheek and Burgoyne, 1986; Burgoyne and Cheek, 1987). Release of the granule contents to the exterior milieu via exocytosis requires that the granules be able to interact with the plasma membrane and this interaction cannot occur unless the cortical actin barrier is removed. Nicotinic stimulation has been observed to elicit a transient disassembly of the cortical F-actin network (Cheek and Burgoyne, 1986; Trifaró et al., 1989b; Marxen and Bigalke, 1990; Vitale et al., 1991) allowing movement of chromaffin granules to sites of low viscosity where exocytosis has been observed to occur preferentially (Vitale et al., 1991). Several lines of evidence exist which indicate that catecholamine secretion is accompanied by a temporary disruption of the cortical cytoskeleton which causes facilitation of exocytosis. Treatment of chromaffin cells with

either tetanus or botulinum A toxins inhibits both actin network disassembly (Marxen and Bigalke, 1990) and catecholamine secretion (Knight, 1986; Penner et al., 1986; Marxen and Bigalke, 1989). Moreover, experimental findings obtained in studies using intact (Friedman et al., 1980) and digitonin- or streptolysin-O-permeabilized chromaffin cells (Lelkes et al., 1986; Sontag et al., 1988) exposed to agents known to destabilize actin microfilaments such as cytochalasin D or DNase I have shown that these agents facilitate stimulus-induced secretion. The existence of endogenous actin-binding proteins (ie. gelsolin and scinderin) which regulate actin network dynamics (Yin and Stossel, 1979; Craig and Pollard, 1982; Stossel et al., 1985; Maekawa et al., 1989; Rodriguez Del Castillo et al., 1990) strongly suggests a role for these proteins in the process of stimulus-evoked disassembly of actin filaments. Furthermore, the Ca^{2+} signal which has been proposed to trigger exocytosis seems to modulate cytoskeletal actin dynamics by modulating the activity of endogenous actin-regulatory proteins (Weeds, 1982; Forscher, 1989; Rodriguez Del Castillo et al., 1990). Therefore, it could be proposed that the cortical cytoskeleton may represent a target for other second messengers and a strategic site for the control of catecholamine secretion. Second messengers such as Ca^{2+} and PKC have been associated with scinderin redistribution (Vitale et al., 1992a), regulation of actin network dynamics (Grant and Aunis, 1990) and enhancement of secretion in bovine adrenal chromaffin cells (Bittner and Holz, 1990; Vitale et al., 1992a). The second messenger cAMP, like Ca^{2+}, has been

found to modulate cytoskeletal dynamics (Kreisberg et al., 1985; Mills and Lubin, 1986; Cheek and Burgoyne, 1987; Fox et al., 1987; Wessels et al., 1989; Goldman and Abramson, 1990; Downey et al., 1991; Egan et al., 1991; Perrin et al., 1992) and secretion (Baker et al., 1985; Morita et al., 1987a,b; for a review see Harper, 1988) in a variety of cells. Consequently, it was of interest to investigate the role of cAMP in modulation of actin-binding regulatory proteins such as scinderin, an actin-filament severing protein (Rodriguez Del Castillo et al., 1990) as well as in the regulation of actin microfilament disassembly and secretory dynamics in bovine adrenal chromaffin cells. Thus, the project described in this thesis was undertaken in fulfilment of the following objectives.

1. To examine the effects of the membrane permeant cAMP analog, 8-Bromo cAMP (8Br-cAMP) and forskolin, a compound known to augment intracellular cAMP by direct activation of the adenylate cyclase catalytic subunit (Seamon and Daly, 1986) and analogs of forskolin on nicotine-evoked scinderin redistribution.

2. To observe if modulation of nicotine-evoked scinderin redistribution by the 8Br-cAMP, forskolin and forskolin analogs results in modulation of the cortical actin network.

3. To correlate modulation of scinderin redistribution and F-actin disassembly by 8Br-cAMP, forskolin and forskolin analogs with regulation of nicotine-evoked catecholamine secretion in bovine adrenal chromaffin cells.

ACKNOWLEDGEMENTS

My deepest gratitude is extended to Dr. Jose-Maria Trifaró, my supervisor to whom I am indebted for not only his kindness, support and friendship but also, for the advice and guidance he provided which led to edification of my research and analytical skills.

I would also like to express my appreciation to Pierre Drouin, Sara Dunn, Simon Nsonwah, Carole Laionde and Manohar Parulekar for all of their assistance and kindness throughout the duration of my training.

A special thank you is extended to Drs. Mary E. Morris, Maria Laura Novas Schimdt, Gabriela Obrocea, Antonio Rodríguez Del Castillo, Andrei Rosen, Liouben Tchakarov and Maria Leiza Vitale as well as Rainy Tang for being wonderful friends, providing me with guidance and being a source of moral support. To the faculty members of the Department of Pharmacology "Thank you all for the knowledge and inspiration each of you has imparted and for the opportunity to have worked with you."

Lastly, I would like to thank those to whom this thesis is dedicated; my parents, family and my husband Mark J. Lavergne. I cannot express in words the gratitude I feel for the affection and motivation with which you have provided mme but suffice it to say, that you have been the beacon which will continue to guide and comfort me throughout the years.

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ABBREVIATIONS USED IN THESIS

cAMP:	3′,5′-cyclic adenosine monophosphate
DβH:	Dopamine-β-hydroxylase
DNase I:	deoxyribonuclease I
EBSS:	Eagle's Balanced Salt Solution
EDTA:	Ethylenediamine tetraacetic acid
EGTA:	Ethylene glycol-bis (β-Aminoethyl ether) N,N,N′,N′-tetraacetic acid
F-actin:	Filamentous actin
FITC-IgG:	Fluorescein isothiocyanate conjugate IgG
G-Actin:	Globular actin (monomeric actin)
GDP:	Guanosine diphosphate
GTP:	Guanosine triphosphate
[^{3}H]NA:	Tritiated noradrenaline
NGF:	Nerve growth factor
PBS:	Phosphate buffered saline
TCA:	Trichloroacetic acid
Tris:	Hydroxymethyl methylamine

CHAPTER I

INTRODUCTION

A. THE ADRENAL MEDULLA: ANATOMY AND MORPHOLOGICAL ULTRASTRUCTURE

In 1563, Bartholomeus Eustachius became the first anatomist to provide a comprehensive and accurate description of the adrenal glands (Eustachi, 1563). He referred to the pair of glands as, "glandulae renibus incumbentes" to imply that the glands functioned as accessories to the kidneys (Lenard, 1951). The adrenal glands are paired structures weighing approximately between 3-5 g each in humans and are localized anterior or medial to the kidney in quadrupeds and on the superior poles of each kidney in human specimens at the level of the eleventh or twelfth vertebra (Yeasting, 1986). They are embedded in adipose tissue and encapsulated by extraperitoneal connective tissue termed, renal fascia, which although providing a means of attachment of the adrenal to the kidney also separates it from the kidney rendering it an autonomous organ (Soffer et al., 1961). Each gland is highly vascularized and possesses a vast network of arterial branches. This network of arterial branches which include the superior, middle and inferior suprarenal arteries originate from the inferior diaphragmatic artery, aorta and renal artery respectively and then divide into smaller branches which sheath the surface of the adrenal capsule, penetrating it, and continuing on to sinusoids in the interior of the gland (Yeasting, 1986; Carmichael, 1987). These sinusoids intercalate between proximate rows of cortical cells and on into the adrenocorticomedullary junction and medulla (Yeasting, 1986). The venous

drainage system of each adrenal gland is such that the medullary chromaffin tissue at the core of the gland is nourished by a sparse network of venules to a central vein, which appears as a groove on the anterior surface of each adrenal gland (Yeasting, 1986). From this point the blood flow in the case right adrenal gland, continues into the inferior vena cava and in the case of left adrenal gland, to the renal vein (Yeasting, 1986). Histologically, the adrenal gland may be dichotomized into two distinct types of endocrine tissue of diverse primordial origin, secretory products and function. As previously implied, the adrenal medulla is found at the central core of the adrenal gland. The medulla is brownish-red and constitutes approximately one tenth of the total adrenal gland cross-sectional width (Soffer et al., 1961). Encasing the medullary core is the adrenal cortex which upon dissection can be visually distinguished from the medulla due to its characteristic yellowish colouring and lipid-like appearance (Soffer et al., 1961; Yeasting, 1986). Nominal and functional distinction between these two facets of the adrenal gland was established as early 1836 by Nagel, who first coined the terms "cortex" and "medulla" and by von Kölliker who would provide the first complete description of the ultrastructural anatomy of the glands (Kölliker, 1852, 1854). The functional disparity which exists between the two facets of the adrenal gland arises as a result of their divergent lineages. The cortical area of the gland is steroidogenic and develops from the coelomic mesoderm of the genital ridge of the embryo in the early weeks of gestation and is then permeated by tissue of

sympathetic ganglionic lineage which evolves into the adrenal medulla. Initial insight as to the embryological origin of the adrenal medulla came from studies conducted by Remak (1847-1855) and was confirmed by the experimental findings of Le Douarin and Teillet on quail-chick chimeras (Le Douarin and Teillet, 1971; Teillet and Le Douarin, 1974). The work of these investigators was the first to demonstrate that the medulla is related embryologically to sympathetic ganglia and other neural crest derivatives in the cervico-thoracic region. In the last decade the common ancestral origin of post-ganglionic sympathetic neurons and the neuron-like cells of the adrenal medulla has become unquestionable. Recent findings have demonstrated not only that the cells of the adrenal medulla can be induced to differentiate into cells possessing the characteristics of sympathetic neurons under the influence of agents such as nerve growth factor (NGF; Levi-Montalcini and Aloe, 1980), but also that embryonic neural crest cells must be in close propinquity to the notochord and somite tissues for the development of adrenergic attributes (Le Douarin, 1980).

A.1. Innervation of the adrenal glands and the adrenal medulla Electron microscopic studies conducted on rat specimens have demonstrated that innervation of the adrenal gland ensues either directly via non-myelinated fibres to the gland or indirectly by way of the coeliac ganglia (Coupland, 1965b,c) with the appearance of nerve endings exhibiting physical characteristics typical

to cholinergic synaptic endings (Tomlinson and Coupland, 1990). Recently, studies using Fast Blue injected into the medulla to label retrogradely the neurons have demonstrated that in adult rat, the nerve tracts which influence the adrenal medulla arise from the perikarya of preganglionic sympathetic neurons which emanate from the intermediolateral cell column of the spinal cord at segments T1 to L1 (Keese et al., 1988). These nerve tracts traverse the thoracic splanchnic nerves and have been implicated in the control of catecholamine secretion (Dreyer, 1899; Elliott, 1912, 1913; Feldberg et al., 1934). Within the medulla nerves are arranged in a three dimensional lattice comprised of axons, Schwann cells and connective tissue (Hillarp, 1947, 1954). Individual groups ofnerve fibres in this network then progress between chromaffin cells and synapse as "bouton en passage" or synaptic endings located within indentations of the chromaffin cells (Coupland, 1965b,c; Tomlinson and Coupland, 1990).

A.2. Adrenal chromaffin cells and their ultrastructural characteristics

In 1902, Kohn created the term "chromaffin cell" to describe the neuroendocrine cells of the adrenal medulla which Alfred Vulpian (1856) and Henle (1865) found could be stained with chromium salts. Isolated chromaffin cells are spherical entities, 20μm in diameter with a nucleus 5μm in width and in the medulla are arranged in groups which are separated by connective tissue and situated around blood vessels (Carmichael, 1987) such as the medullary

arteries, capillaries and venous sinuses (Tomlinson and Coupland, 1990). Elucidation of the sub-cellular features of these cells was initially conducted during the advent of electron microscopic studies. It is known that the distribution of chromaffin cells within the medulla and organelles within the chromaffin cell itself are not random (Carmichael, 1987). Polarization has been found to occur with respect to nerve endings, which are located on one side of the cells within the medulla and blood vessels, which are associated with the opposing pole (Carmichael, 1987). The Golgi apparatus and secretory organelles are usually found near the pole of the nucleus closest to blood vessels. This polarization is consistent with nervous input being received at one pole of the chromaffin cell and secretion occurring at sites closest to blood vessels at the diametric pole (Carmichael, 1987). Analysis of the chromaffin cell on a microscopic level demonstrates that the internal portion of the chromaffin cell, which is the site at which neurochemical input is received, houses a nucleus possessing 1-2 nucleoli, the rough endoplasmic reticulum and the Golgi apparatus. The last two structures of which are involved in the biogenesis of chromaffin granules and cellular proteins (Benedeczky, 1983; Spagnoli et al., 1987). Mitochondria, which are relatively numerous are distributed throughout the cytoplasm along with lysosomes, multivesicular bodies, centrioles, cilia and microtubules (Carmichael, 1987). One of the most arresting morphological attributes of the chromaffin cell was found to be the granular appearance of the cytoplasm. Studies examining centrifuged

samples of homogenized medullary tissue containing catecholamines led to the initial isolation of the adrenal medullary secretory organelle (Blaschko and Welch, 1953; Hillarp et al., 1953). In 1955, Lever would provide the first electron micrographs which would clearly establish the identity of the agent causing this granular appearance as being, " the chromaffin granule", a term introduced by Sjöstrand and Wetzstein (1956). At present, chromaffin granules are among the most characterized secretory organelles in existence as a result of the relative ease which with they are isolated. Biochemical findings in correlation with morphological observations have demonstrated the presence of three diverse types of secretory organelles in chromaffin cells. The first type of granule present in chromaffin cells is represented by the large dense-core vesicles (LDCV 750-1000 Å) related to the LDCV found in the neurons of the sympathetic nervous system (Trifaró et al., 1992) and second type is comprised of the small electrontransluscent vesicles (400-500 Å) found in sympathetic nerves (Navone et al., 1986, 1989; Trifaró et al., 1992). These findings are in agreement with early light microscopic studies utilizing the iodate method (Hillarp and Hökfelt, 1953) and analysis of vesicular ultrastructure (Unsicker, 1976) which have demonstrated that three types of chromaffin cells exist in the adrenal medulla. The two LDCV types are adrenaline-containing (A) and noradrenaline-containing (NA) cells and the third is the small granule chromaffin cell (SGC). The dense-core vesicles of the different populations of chromaffin cells (NA and A) originally classified by Erankö (1955) possess

unique characteristics which enable them to be distinguished from each other. "NA" for example, can be distinguished from "A" as a consequence of the fact, that "NA" granules are characteristically more dense than "A" granules when fixation with gluteraldehyde-osmium tetroxide is employed. The reason for the increased density of "NA" as opposed to "A" -containing vesicles when the afore-mentioned procedure is utilized is that a tight complex is formed between norepinephrine and gluteraldehyde rendering it more osmiophilic and electron dense (Coupland and Hopwood, 1966). Additionally, morphological examination shows that "NA" and "A" vesicles have been found to differ in that "NA" vesicles tend to exhibit an irregular appearance while "A" are spherical. Moreover, the distribution of these two types of cells in the medulla is such that in most mammals, "A" comprise 85-95% of the chromaffin cell population, but it should be noted that extensive species variation does exist (Benchimol and Cantin, 1977). The other subtype of chromaffin cell cited previously is the "SGC" cell, which was initially isolated in the mouse adrenal gland (Kobayashi and Coupland, 1977). The function of the "SGC" cell has not been conclusively established but some investigators have proposed that cells containing this type of vesicle may act as interneurons or cells with both neuronal and adrenal chromaffin cell character (Unsicker et al., 1978). Chromaffin cells with this vesicle type possess a dense-core vesicle and variant small electrontransluscent vesicle which is thought to differ from the other two traditional vesicle types primarily in that it may store diverse secretory

products. In chromaffin cells, as in cholinergic and other neurons housing both LDCV and small electrontransluscent vesicles, the latter are thought to store classical neurotransmitters (acetylcholine, norepinephrine, glutamate, serotonin and GABA) whereas, the former are thought to contain peptides and amines (Fillenz, 1971). The SGC type of chromaffin cell is very rarely observed in the adrenal medulla of mammals other than the species where it was initially identified, the mouse where it comprises 4-5 % of the chromaffin cell population and is primarily dopamine-containing (Coupland, 1984). At the electron microscopic level, the appearance of the vesicles of SGC cells seem to suggest that these cells possess both "A" and "NA" (Coupland, 1989). It has been implied that the SGC cell might represent a variant attenuated form of ganglionic origin, the small intensely fluorescing cell (SIF) type I. The presence of the small intensely fluorescent (SIF) cell, a chromaffin-like cell encountered in sympathetic ganglia was first described years ago (see Coupland 1965a, 1978) and was first noted by formaldehyde-induced fluorescent technique for catecholamines in rat sympathetic neurons in the superior cervical ganglia (Eränkö and Harkonen, 1963). SIF cells are divided into two groups, type I and type II (Chiba and Williams, 1975). SIF type II cells are virtually indistinguishable from chromaffin cells whereas, SIF type I cells superficially resemble cultured chromaffin cells which have been exposed to NGF or glucocorticoids and in some respects, resemble SGC cells of the mouse adrenal medulla (Kobayashi and Coupland, 1977; Kobayashi et al., 1978).

A.3. Integral membrane proteins of the chromaffin granule and membrane-associated proteins

As previously mentioned, chromaffin granules are well characterized and the composition of these granules has been described in several reviews (Winkler et al., 1986; Phillips, 1987). Although the most abundant components present in the chromaffin granule membrane have been found to be dopamine-β-hydroxylase (DβH) and cytochrome b_{561}, other proteins are present in the granule membrane which may be involved in exocytosis. Three integral membrane proteins have been isolated and characterized from brain synaptic vesicles, p65 (Matthew et al., 1981), synaptophysin or p38 (Jahn et al., 1985; Weidenmann and Franke, 1985) and SV2 (Buckley and Kelly, 1985).

A.3.1. Synaptophysin (p38)

This protein is a major component of the membrane of synaptic vesicles (Jahn and Maycox, 1980; Jahn et al., 1985; Wiedenmann and Franke, 1985; Navone et al., 1986, 1988; Rehm et al., 1986; Decamilli and Navone, 1987). Characterization of synaptophysin by different laboratories has revealed that it is a glycoprotein with an apparent molecular weight of 38,000 daltons as determined by electrophoresis. However, electrophoresis under non-reducing conditions shows an apparent molecular weight of 76,000 daltons (Jahn and Maycox, 1980). The protein is an integral component of the granule membrane, requiring Triton X-100 buffer for extraction. Synaptophysin is able

to bind Ca^{2+} (Rehm et al., 1986) and in addition, it has been shown that the native protein is a hexamer with topology similar to channel proteins and upon introduction into lipid bilayers exhibits voltage-dependent channel activity (Thomas et al., 1988). Recently, three independent groups have published the cloning and sequences of cDNAs encoding synaptophysin (Buckley et al., 1987; Leube et al., 1987; Südhof et al., 1987). These studies have shown that the protein is composed of 307 amino acids with membrane topology showing four hydrophobic transmembrane regions with both amino and carboxyl terminals oriented toward the cytoplasm. The Ca^{2+} binding domain of the protein has been found to be in the cytoplasmic domain. Recent phosphorylation studies seem to indicate that synaptophysin is a substrate for tyrosine kinase (Pang et al., 1988) and tyrosine kinase present in two forms of $p60^{c\text{-}src}$ has been found to be associated with chromaffin granules (Parsons and Crentz, 1986). Immunocytochemical studies have revealed that synaptophysin is present in all synapses of the mammalian nervous system (Decamilli and Navone, 1987; Navone et al., 1988) and has been found to be present in chromaffin cells (Fournier and Trifaró, 1988a; Fournier et al., 1989; Trifaró, 1990) and pheochromocytoma (PC_{12}) cells as well as in other neoplasms (Wiedenmann and Franke, 1985; Lowe et al.,1988). In chromaffin cells however, in contrast to p65, an integral granule protein which will be discussed below, synaptophysin is not detected in the plasma membrane of chromaffin cells (Fournier and Trifaró, 1988b; Trifaró et al., 1989a).

A.3.2. Glycoprotein SV2

The transmembrane glycoprotein (SV2) is also an integral secretory vesicle component and has a molecular weight of 100,000 daltons (Buckley and Kelly, 1985). This protein, which was originally described in synaptic vesicles, has subsequently been found to be present in tissues such as the adrenal medulla, endocrine pancreas and pituitary (Buckley and Kelly, 1985), as well as in cells lines such as PC_{12} (derived from rat pheochromocytoma), GH_3 (derived from mouse anterior pituitary) and H1T (derived from insulin secreting line). These different cell types have been found to express SV2 proteins of differing molecular weights as a result of variations in the composition of their carbohydrate moieties (Buckley and Kelly, 1985).

A.3.3. 65 kDA calmodulin-binding protein (65-CMBP, p65)

The Ca^{2+} regulatory protein, calmodulin has been shown to bind to chromaffin granules (Geisow et al., 1982; Hikita et al., 1984; Bader et al., 1985) as well as other secretory vesicle types including synaptic vesicle (Moskowitz et al., 1983), neurohypophyseal granules (Olsen et al., 1983), platelet α-granules (Grinstein and Furuya, 1982) and pancreatic islet cell secretory granules (Watkins and White, 1985). Recent work has demonstrated the presence of a 65,000 dalton protein in chromaffin granule membranes which exhibits high affinity binding to calmodulin (Hikita et al., 1988; Bader et al., 1985). Many secretory tissues has been found to show cross-reactivity to

a monoclonal antibody prepared against a vesicle antigen of 65,000 molecular weight found in rat brain synapses (p65). The development of radioimmunoassay techniques using antibodies against p65 has led to the demonstration of the presence of p65 in anterior and posterior pituitaries as well as in the cell lines, GH_3, PC_{12}, and AtT_{20} (Matthew et al., 1981; Lowe et al., 1988). Subsequently, the presence of the 65-CMBP has been demonstrated in chromaffin, neurohypophyseal and synaptic secretory granules (Fournier and Trifaró, 1988a). The protein was extracted from each vesicle membrane types with Triton X-100 buffer and then consequently purified by calmodulin affinity chromatography. Immunoblot analysis of the secretory vesicle 65-CMBP using monoclonal antibodies against p65 demonstrated the immunological identity of the calmodulin-binding proteins isolated from each of the three types of vesicle membranes (Fournier and Trifaró, 1988a).

A.3.4. Membrane-associated proteins

There are several proteins which, although not existing as integral components of the granule membrane, are associated with the chromaffin granule membrane and may also be involved in the secretory process. These proteins may modulate interactions between granules and the cytoskeleton or fusion of the granules and the plasma membrane, and include fodrin (Aunis and Perrin, 1984) and α-actinin (Bader and Aunis, 1983) which will be discussed in a D.2.2 of this thesis, as well as caldesmon (Burgoyne et al., 1986),

calmodulin (discussed in section on 65-CMBP/p65) (Geisow et al., 1982; Hikita et al., 1984; Bader et al., 1985), calpactin (Burgoyne and Gelsow, 1989; Burgoyne, 1990) and GTP-binding proteins (discussed in section A.3 of the discussion in this thesis) (Burgoyne and Morgan, 1989; Doucet et al., 1989; Ngsee et al., 1990).

A.3.4.i. Caldesmon

Caldesmon is a calmodulin-dependent actin-binding protein which at low Ca^{2+} concentrations (10^{-7}M), binds and cross links actin filaments (Burgoyne et al., 1986). The binding of caldesmon to actin microfilaments is inhibited by the presence of micromolar Ca^{2+} concentrations, conditions under which caldesmon interacts reversibly with chromaffin granule membrane (Burgoyne et al., 1986). This indicates that caldesmon may be important in granule function during changes in intracellular Ca^{2+} which occur during stimulation.

A.3.4.ii. Calpactin

Calpactin is another Ca^{2+}-dependent-binding protein which belongs to a family called the annexins (Burgoyne and Gelsow, 1989; Burgoyne, 1990). The protein exhibits the ability to bind reversibly to chromaffin granules in the presence of micromolar Ca^{2+} concentrations (Burgoyne and Cheek, 1987; Burgoyne, 1988; Drust and Creutz, 1988a,b). In vitro experiments indicate that calpactin induces secretory vesicle aggregation in the presence of Ca^{2+}

(Drust and Creutz, 1988a) and that aggregation of vesicles is followed by membrane fusion if arachidonic acid is present (Drust and Creutz, 1988a).

B. CHROMAFFIN CELLS IN CULTURE : A MODEL SYSTEM FOR THE STUDY OF NEUROSECRETORY MECHANISMS

The bovine adrenal chromaffin cell has provided an outstanding model for the study of neuronal function and development, endocrine mechanisms and basic biological, pharmacological, biochemical and mechanistic aspects of the secretory process. The success of the chromaffin cell as model has come as a result of the relative ease with which large homogeneous populations of these cells can be isolated and the fact that a number of biochemical, morphological and electrophysiological analyses can be performed utilizing them. As a system in which to study neuronal and endocrine function, the chromaffin cell affords the advantage of a shared primordial origin with sympathetic neurons and the possession of common ultrastructural features of endocrine cells. Chromaffin cells possess many of the same characteristics as neurons and are classified as "paraneurons", a term used to describe relatives of neurons who store their secretory products in membrane-bound secretory vesicles and exhibit regulated mechanisms of secretion which was introduced by Fujita and Kobayashi (1975). Figure 1 demonstrates the similarity in the way which members of the paraneuron family which include, gastroenteric endocrine cells, adrenal chromaffin cells, mast cells, melanocytes, pancreatic islet cells, pinealocytes,

adenohypophysial cells, parafollicular cells and Merkel cells (Fujita, 1980) respond to stimuli. Isolated chromaffin cells can be maintained in culture for long periods of time (Trifaró and Lee, 1980; Livett et al., 1983) and begin to develop processes which increase in length in proportion to the population's duration in culture and which have been observed to display growth cones (Trifaró and Lee, 1980). These processes are neurite-like in nature, appear to synapse on the cell bodies and varicosities of other neurosecretory cells and contain high levels of catecholamines and neuropeptides (Trifaró and Lee, 1980). Several trophic factors affect the growth, differentiation and ontogeny of chromaffin cells in culture. Some investigators have speculated that NGF and glucocorticoids may affect the morphogenesis, biochemical properties and outgrowth of varicosities in chromaffin cells in vitro (Unsicker, 1982; Doupe et al., 1985; Grothe et al., 1985). This finding varies from what is encountered in culture and is seen more in the case of dissociated sympathetic neuron and pheochromocytoma cells (PC_{12}) cells obtained from rats. Cultured chromaffin cells have been found to spontaneously grow varicosities in the absence of NGF or presence of antibodies against NGF (Livett et al., 1978; Trifaró and Lee, 1980; Unsicker et al., 1980; Unsicker and Hofmann, 1981, 1983). This spontaneous outgrowth of processes in these cell has been suggested to result as a consequences of factors produced by non-chromaffin cells present in the culture, which are liberated into the culture medium (Trifaró and Lee, 1980; Unsicker and Hofmann, 1981, 1983). Like their neuronal counterparts cultured

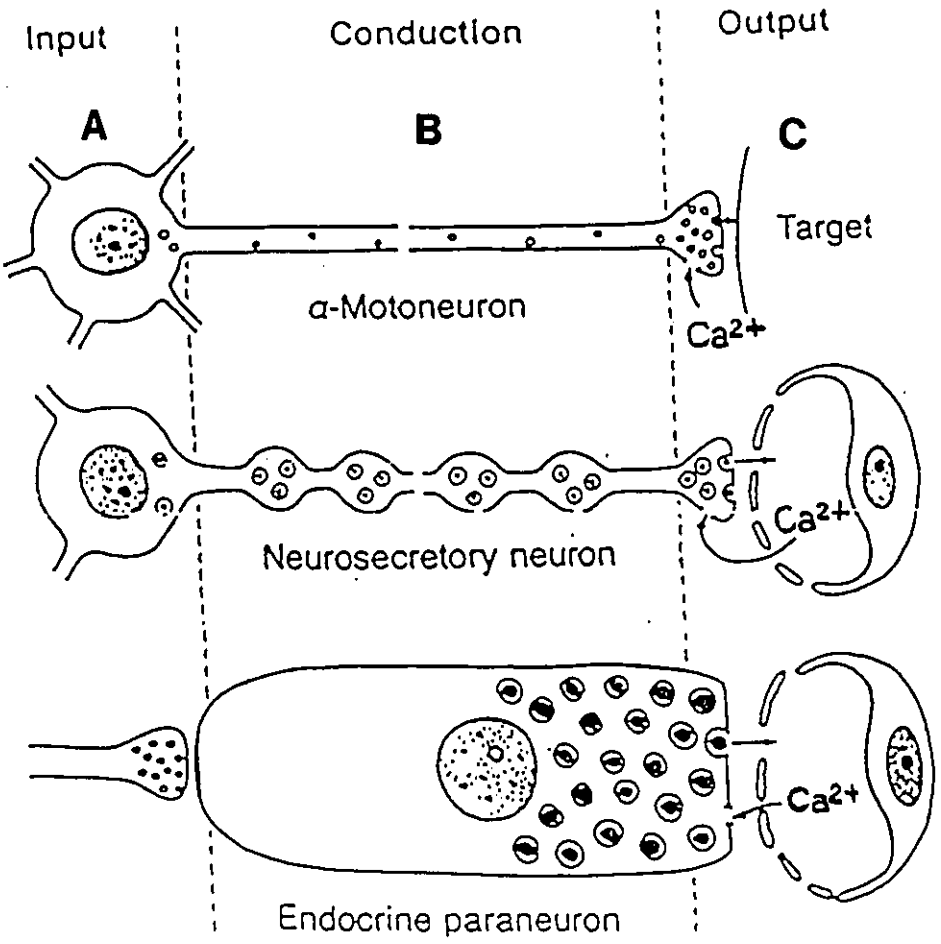


Fig.1
Illustration of the existing similarities between an *α*-motoneuron, neurosecretory cell and paraneuron of endocrine origin. Also depicted are the input portion of the cell which is the site of action of various stimuli (A), the conducting region and hence, the site of nerve impulse propagation (B) and the area where the secretory response is engendered (C). (Taken from "The Paraneuron", Fujita T., Kanno T. and Kobayashi S., 1988).

chromaffin cells possess secretory vesicles, Na^+, K^+ and Ca^{2+} channels as well as neuronal-specific proteins such as neurofilaments (Burgoyne, 1991; Bader et al., 1984). In the tradition of neurons, these cells in culture display the presence of an amine-uptake mechanism which has high affinity for noradrenaline, but no stereochemical specificity for (-) noradrenaline, is saturable and complies with Michaelis-Menten kinetics, exhibits absolute Na^+ dependence and can be blocked by 10^{-7} -10^{-8} M desipramine (Kenigsberg and Trifaró, 1980; Trifaró, 1982). Nicotinic receptor activation in chromaffin cells as in sympathetic neurons precipitates a chain of events which will be discussed in more detail and can be summarized into the following phases: 1) Influx of Na^+ and Ca^{2+} ions via receptor-linked ion channels and initial depolarization; 2) Activation and opening of voltage-dependent Ca^{2+} and Na^+ channels eliciting further increases in intracellular Ca^{2+} and Na^+ concentration; 3) Granule translocation to the plasma membrane; 4) Fusion of granule and plasma membranes and exocytosis; 5) Endocytosis and membrane retrieval. Depolarization leads not only to activation of voltage-dependent ion channels, but may also elicit activation of phospholipase C, IP_3 and Ca^{2+}-induced calcium release via the Ca^{2+}- mobilizing signal triggered by IP_3 (Berridge and Irvine, 1989). Though several types of receptors have been identified on the chromaffin cell plasma membrane which stimulate secretion, it is the nicotinic receptor which has been observed to engender the greatest secretory response (for review see Burgoyne, 1991) and which leads to catecholamine secretion

(Trifaró, 1982). The outcome of stimulation of the alternate cholinergic receptor, the muscarinic receptor is IP_3 generation and hence, augmentation of intracellular Ca^{2+} (Cheek and Burgoyne, 1985; Forsberg et al., 1986; O'Sullivan and Burgoyne, 1989). In some species, such as bovine, the muscarinic receptor produces inhibition of nicotinic-receptor elicited secretion (Burgoyne, 1984) while in contrast in others such as the rat, it has been found to enhance nicotine-evoked secretion (Wakade, 1987). The previously described characteristics have established the fact that cultured chromaffin cells emulate neurons in several aspects. It is for this reason that they as a system, have been crucial to the development of a model in which neurochemical transmission ensues via regulated liberation of the soluble vesicular contents of neurosecretory cells through the process of exocytosis (Douglas, 1974; Viveros, 1974; Trifaró, 1977). As comprehension of the intricacies of this model becomes more feasible due to the advent of advanced technological strategies the cultured chromaffin cell will continue to be a tool which science can employ to probe the complexities which underlie the process of neurochemical transmission.

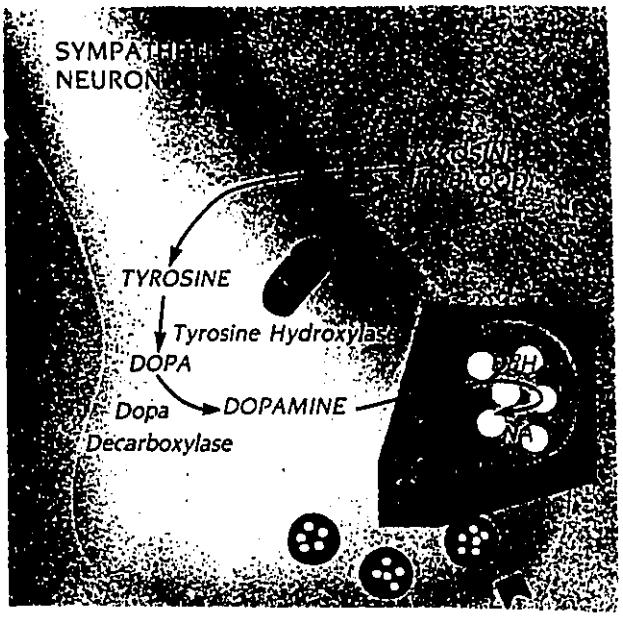
C. NEUROCHEMICAL TRANSMISSION IN CHROMAFFIN CELLS

C.1. Biosynthesis and storage of secretory products

The chromaffin secretory vesicle consists of a membrane and a soluble

content destined for regulated secretion via exocytosis (Smith, 1968; Trifaró, 1977; Trifaró and Poisner, 1982). The soluble proteins of the vesicle are released in fixed amounts as opposed to the constitutive or unregulated mode of secretion in which secretion is continuous and independent of extracellular signals (Kelly, 1985). The soluble contents are protected from degradation by storage in the vesicles and were initially collectively named chromogranins (Blaschko et al., 1967). Subsequently, the soluble proteins are known to include the primary protein constituents, dopamine β-hydroxylase, enkephalin precursors, glycoprotein III, opioid peptides, endorphins, and acidic proteins rich in glutamic acid known as, chromogranin A, B and C (Winkler, 1976; Viveros et al., 1979, 1980; Winkler et al., 1986; Eiden et al., 1987). Moreover, the granules contain nucleotides, ascorbic acid, Ca^{2+} and catecholamines, the chemical signals responsible for neurotransmission (Winkler, 1976). The pathway of synthesis of catecholamines was originally proposed by Blaschko in 1939 and was confirmed nearly thirty years later when tyrosine hydroxylase, the enzyme responsible for catalyzing the rate-determining step in the pathway was isolated (Nagatsu et al., 1964). Further insights as to the elaborate individual steps involved in the process came to fruition when the cellular location and distribution of the enzymes catalyzing each phase of catecholamine biosynthesis were elucidated (Kirschner, 1957; Kirschner and Goodall, 1957; Sabban and Goldstein, 1984). Although the detailed steps are illustrated (Fig.2) some of the important highlights will be noted in the

discussion at hand. The biogenesis of catecholamines is initiated by the amino acid L-tyrosine, which is taken up into the cell via a Na^+-dependent uptake mechanism where a mixed function oxidase present in the cytosol of the secretory cell, tyrosine hydroxylase (TH), in association with its required tetrahydropteridine cofactor catalyses its conversion to L-dopa. This initial step in the pathway is a major site of regulation of catecholamine synthesis because it represents the rate-limiting step in biosynthesis as was previously mentioned and TH is subject to feedback inhibition by catecholamines and analogs of itself as well as induction by prolonged exposure to stimuli of cholinergic origin and may be subject to modulation by second messengers (Nagatsu et al., 1964; Thoenen, 1975; Axelrod, 1977; Melegeni et al., 1982). L-dopa is converted to dopamine by dopa decarboxylase, an enzyme which as its name intimates decarboxylates L-dopa yielding dopamine. Following its formation dopamine is actively taken up into chromaffin granules. In the granule membranes and cytosol of chromaffin cells there exists another mixed function oxidase, dopamine-β-hydoxylase which requires O_2 as an electron donor and Cu^{2+} for activation of its catalytic potential. This enzyme catalyses the transformation of dopamine into noradrenaline by inducing aerobic hydroxylation of the dopamine precursor. In adrenal medullary chromaffin cells, L-noradrenaline can be methylated in the cytoplasm of chromaffin cells and converted to adrenaline via the enzyme, Phenylethanolamine N-methyltransferase (PMNT) in the presence of a methyl donor, S-adenosyl methionine. Since PMNT is a cytosolic



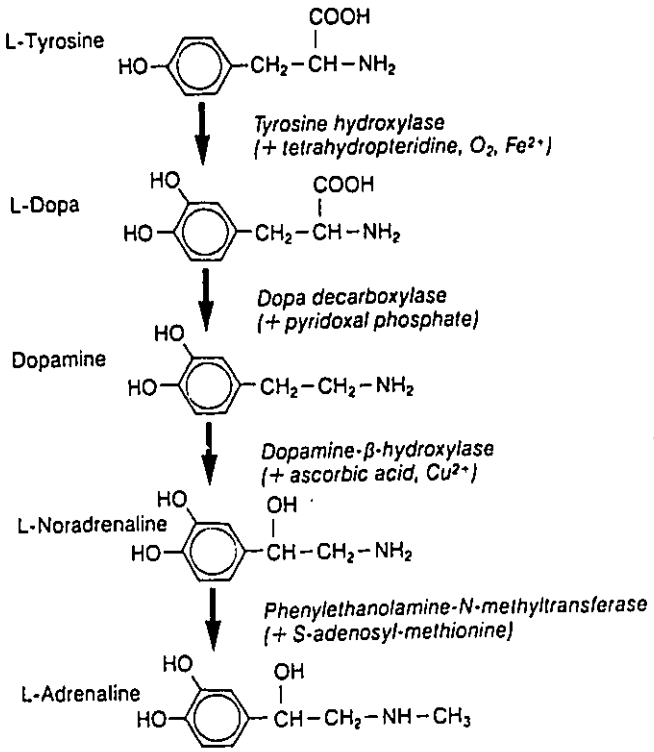


Fig.2
Schematic depiction of the pathway of catecholamine synthesis and the enzymes and cofactors required in the catalysis of the individual stages. (Taken from Principles of Medical Pharmacology, Kalant H. and Roschlau W.H.E., 1989).

enzyme, in order for adrenaline synthesis to occur noradrenaline must exit the granule and be present in the cytosol. This final step is of interest from a stoichiometric standpoint because it represents another site of regulation. In the adrenal medulla, PMNT levels can be induced to increase in the presence of steroid hormones such as the glucocorticoids, which travel to the medulla from the cortex by route of a portal system within the adrenal gland (Weiner, 1975). Following synthesis, catecholamines are taken up into the granules by way of an amine transporter driven by an Mg^{2+}-dependent, ATP-induced electrochemical proton gradient across the chromaffin granule membrane which has been purified in its functional state (Stern-Bach et al., 1990) and are stored bound in a complex with ATP in a ratio of 4:1 until stimulation initiates the secretory process prompting them to be co-secreted with other neuropeptides, ATP,dopamine β-hydroxylase and chromogranin A (Banks and Helle, 1965; Douglas et al., 1965; Viveros et al., 1968; Lastoweka and Trifaró, 1974).

C.2. The secretory process in chromaffin cells

Activation of the nicotinic cholinergic receptor by agonists such as nicotine or acetylcholine, with the latter being released from the splanchnic nerve terminals or stimulation with a depolarizing concentration of potassium (K^+) leads to opening of voltage-dependent channels. These channels are L-type or dihydropyridine-sensitive and dihydropyridine-insensitive, ω-conotoxin sensitive and insensitive (possibly P-channels; Rosario et al., 1989) Ca^{2+}

channels as well as receptor-linked Na^+ channels (Rosario et al., 1989; Bansal et al., 1990; Jan et al., 1990; Burgoyne, 1990). This elicits depolarization, Ca^{2+} influx, and exocytosis. Exocytosis was first described by De Robertis and Vaz Ferreira in 1957 and involves the complex interaction of second messenger systems, nucleotides, ions, cellular proteins and the cell cytoskeleton. The process is initiated by the translocation of secretory granules to sites of exocytosis and fusion of the secretory vesicle membrane with the inner surface of the plasma membrane and is terminated by the extrusion of the soluble vesicular contents to the cell exterior. Once exocytosis has occurred, the empty granules are selectively retrieved and recycled via endocytosis (Meldolesi and Ceccarelli, 1981; Trifaró and Poisner, 1982).

C.2.1. Requirements for the exocytotic process

Several intracellular requisites must be fulfilled in order for exocytosis to occur. Houssay and Molinelli (1928) were the first investigators to propose that Ca^{2+} played a pivotal role in hormonal secretion and evidence of the micromolar Ca^{2+} requirement of the secretory process was confirmed by studies conducted by Douglas and co-workers (1961) who illustrated that if this second messenger was absent from the extracellular milieu catecholamine secretion did not occur. Subsequently, advances in the examination of the role which Ca^{2+} plays in neurosecretion have been aided by studies with Ca^{2+} indicators such as Fura-2, $^{45}Ca^{2+}$ and Ca^{2+}-selective ionophores as well as

streptolysin-O- (Sontag et al.,1988) electrically, saponin and digitonin-permeabilized cells in which Ca^{2+} alone in the absence of other secretagogues has been sufficient to elicit secretion (Burgoyne, 1991). Furthermore, exclusion of Ca^{2+} from the extracellular environment has been found to inhibit secretion and may be corrected by the addition of the divalent cation, Ba^{2+} (Douglas, 1968; Trifaró, 1977; Trifaró and Bourne, 1981). In addition to the acute Ca^{2+} requirement, other cellular proteins such as, GTP-binding proteins (Knight et al., 1989; Gomperts, 1990) and second messengers such as phosphoinositides and the nucleotide ATP coupled with Mg^{2+}, which has a direct effect on ATP intracellular levels are also required. Phosphoinositides have been implicated to act as substrates for protein binding to membranes (Burgoyne, 1991; Rodriguez Del Castillo et al., 1992).

C.3. Evidence of release of soluble vesicular contents via exocytosis

Although the complex mechanisms by which exocytosis occurs are currently still under investigation, valuable insights have been obtained from biochemical, electrophysiological and in vitro analyses. Quantitative isolation of vesicular contents from the fluid surrounding stimulated medullary cells, as well as sub-cellular fractionation analyses have provided evidence which supports a mechanism of secretion in which the soluble contents of the granule are discharged to the exterior of the cell in an "all or none" fashion (Poisner et al., 1967; Sage et al., 1967; Trifaró et al., 1967; Viveros et al., 1969; Smith et al., 1970; Serck-Hanssen, 1972; Trifaró, 1977). Moreover,

electrophysiological studies conducted in chromaffin cells substantiate that the probable means by which this "all or none" discharge ensues is via the route of exocytosis. Findings from these analyses demonstrate that membrane capacitance in chromaffin cells increases where substantial exocytosis is expected to occur (Neher, 1992) and that this substantial increase in membrane capacitance reflects the incorporation of the chromaffin granule membrane into the plasmalemma of the cell (Neher and Marty, 1982). Exocytosis via fusion of the granule and plasma membranes is also supported by biochemical data which illustrates that integral constituents of the secretory vesicle membrane persist within the cell after the secretory event (Trifaró et al., 1967; Poisner et al., 1967; Viveros et al., 1969) and by quick freeze electron and video-enhanced microscopic studies which have allowed the stages in processes of fusion of the secretory vesicle membrane and extrusion of vesicular contents into the extracellular milieu to be visualized (Ornberg and Reese, 1981; Heuser and Reese, 1981). Furthermore, it has been proposed that there is an actin filament network localized beneath the plasma membrane of adrenal chromaffin cells (Lee and Trifaró, 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986) which acts as a barrier opposing the movement of chromaffin granules to sites of exocytosis at the plasma membrane (Trifaró et al., 1982, 1984, 1989b; Cheek and Burgoyne, 1986, 1987; Burgoyne and Cheek, 1987; Burgoyne et al., 1989; Burgoyne, 1991). Removal of the cortical actin barrier allows translocation of granules to exocytotic sites (Vitale et al., 1992a). Nicotinic

stimulation of chromaffin cells induces a transient disassembly of the cortical filamentous actin lattice (Cheek and Burgoyne, 1986; Trifaró et al., 1989b; Vitale et al., 1991; Marxen and Bigalke, 1991). Recent work conducted in our laboratory has led to the isolation and characterization of scinderin, a novel Ca^{2+}-dependent actin-filament severing protein which modulates actin network dynamics in bovine adrenal chromaffin cells (Rodriguez Del Castillo et al., 1990). Scinderin is found in other tissues with high secretory activity (Tchakarov et al., 1990; Rodriguez Del Castillo et al., 1992). Immunofluorescence microscopy and biochemical studies have revealed that scinderin under resting conditions exists in the two regions of the chromaffin cell, the cytoplasm and the subplasmalemmal region, where the protein is bound to membrane phospholipids (Rodriguez Del Castillo et al, 1992). Nicotinic stimulation elicits a rise in intracellular Ca^{2+} which is followed by activation of protein kinase C (PKC). PKC once activated in turn activates the Na^+/H^+ antiport which elicits a rise in intracellular pH. These events together with a Ca^{2+} increase have been proposed to be associated with release of scinderin from membrane phospholipids (Rodriguez Del Castillo et al., 1992). Once released from the membrane, scinderin is able to interact with its target, filamentous actin. Scinderin actin-filament severing capacity is activated at an intracellular Ca^{2+} concentration of 10^{-6}M, a concentration easily attained upon depolarization (Rodriguez Del Castillo et al., 1990).

D. THE ROLE OF THE CYTOSKELETON IN SECRETORY DYNAMICS

Secretion (stimulus-secretion coupling), the primary function of the adrenal chromaffin cell, is a dynamic process which shares many common features with excitation-contraction coupling in muscle (for reviews see Douglas, 1975; Trifaró, 1977). It has therefore, been proposed that release of catecholamines in chromaffin cells in response to stimulation is mediated by the force of contractile proteins associated with the chromaffin granules (Poisner and Trifaró, 1967; Trifaró, 1978) and the cytoskeletal matrix of these cells (Trifaró et al., 1982; Trifaró et al., 1984). These assertions have served as the impetus which led to the creation of the term "stimulus-secretion coupling" (Douglas, 1968) to forge the link between the secretory process and excitation-contraction coupling in muscle cells. Biochemical and immunological studies support the proposal that there are similarities between secretion and contraction, which are both Ca^{2+}-dependent processes and have revealed that contractile proteins exist in adrenal chromaffin cells. Actino-myosin (Poisner, 1970; Trifaró and Ulpian, 1975), myosin (Trifaró and Ulpian, 1976; Creutz, 1977; Trifaró et al., 1978), heavy meromyosin (Trifaró and Lee, 1978) and actin (Lee and Trifaró, 1979) similar to that found in muscle cells have been isolated in chromaffin cells. In addition, an interaction between rabbit actin, chicken myosin and bovine chromaffin granules has been reported (Burridge and Phillips, 1975). Immunohistochemical and electron microscopic studies utilizing quick freeze, deep-etching technique have revealed that the skeletal matrix of

chromaffin cells is comprised of an intricate three-dimensional lattice of filament systems which form cross-connections with each other and serve to link adjacent membrane-bound organelles in continuum with each other and the plasma membrane (Trifaró et al. 1985a, 1985b, 1988a; Aunis et al., 1987). The network of fibres houses three filament systems and a diverse population of cytoskeleton-associated proteins which have been classified on the basis of function and whose characteristics have been studied extensively (Trifaró and Ulpian, 1976; Lee et al., 1979; Aunis et al., 1980; Trifaró et al. 1982, 1984, 1985a, 1985b, 1988a, 1989b). Examination of the interaction between the filament systems of chromaffin cells has illustrated that although cytoplasmic movement in these secretory cells might be analogous to contraction in striated muscle in many aspects, that there are basic differences in the properties of the proteins common to both which exist due to the specialized demands of the secretory process. In non-muscle systems such as the chromaffin cell, the cytoskeletal macrostructure is designed to be more dynamic in order to comply with rapidly changing cell requirements and represents a strategic site for modulation of the secretory process by virtue of the Ca^{2+}-dependent endogenous proteins with which it is intimately associated. These endogenous proteins serve the functions of 1) favouring actin microfilament assembly or gelation (actinogelins; Trifaró, 1990), 2) favouring actin microfilament disassembly (gelsolin; Bader et al., 1986 and scinderin; Rodriguez Del Castillo et al., 1990), 3) anchoring cytoskeletal elements to membranes (fodrin; Aunis

and Perrin, 1984, α-actinin; Bader and Aunis, 1983;) and 4) regulating phosphorylation of cytoskeletal components (calmodulin; Burke and De Lorenzo, 1981; Walsh, 1981; Kakiuchi and Sobue, 1983). Ca^{2+}-ions in this system serve to promote the activation of cytoskeleton-associated regulatory proteins which mediate the assembly or disassembly of microfilaments thus, enabling the cortical cytoplasm to undergo transitions in viscosity and stiffness and effectuate stasis or movement of organelles such as the secretory vesicles to sites of exocytosis (Trifaró, 1978; Weeds, 1982; Trifaró, 1990; Hartwig and Kwiatkowski, 1991).

D.1. The cytoskeleton: Foundations for its role in secretion

A plethora of cells including those of muscle and non-muscle origin possess a prominent cytoskeleton. This structure which is involved in a vast number of cellular functions such as cytokinesis, cell motility, secretion and phagocytosis most eminently serves to preserve cellular structural integrity (Pollard and Weihing 1974; Clarke and Spudich, 1977; Korn, 1978; Stossel, 1978; Goldman, Milsted, Schloss, Starger and Yerna, 1979; Weeds, 1982). Initial insight as to the possible role which the cytoskeleton could play in the process of secretion in various cells types came from work conducted in pancreatic islet cells which led to the finding that in these cells a microfilament web localized beneath the plasma membrane could function by limiting secretory granule access to the plasma membrane (Orci et al., 1972). The first

findings providing support for this hypothesis were however, obtained much later in studies using adrenal chromaffin cells. It was found that in these cells stimulated secretion was accompanied by reorganization of the actin microfilament network and a reduction in the amount of actin associated with the cytoskeleton (Burgoyne et al., 1989) as well as redistribution of cytoskeletal proteins such as fodrin (Perrin and Aunis, 1985; Fujimoto and Ogana, 1989), which is responsible for self-assembly and binding of actin filaments (for review see Glenney et al., 1982; Goodmann et al., 1988). Moreover, the introduction of anti-alpha-fodrin antibodies into permeabilized chromaffin cells was reported to produce a 50% inhibition of catecholamine release (Perrin et al., 1987). Further evidence of an active role for the cytoskeleton in the secretory process came from electron microscopic studies of ultra rapidly frozen actively secreting cells in which the cytoskeleton is shown to possibly modulate exocytosis. In studies conducted examining the process of exocytosis from Limulus amoebocytes (Ornberg and Reese, 1981) and chromaffin cells (Pollard et al., 1985) secretion seems to occur accompanied by plasma membrane invaginations toward the secretory vesicles. These alterations in the shape of the plasma membrane have been proposed to be related to changes in microfilament organization (Harper, 1988). The phenomenon of stimulus-evoked cytoskeletal rearrangement is observed in numerous cell types in which secretion occurs in a regulated manner. In platelets for example, disruption of the actin network-membrane interaction and

hydrolysis of actin-binding proteins is necessary for the shedding of microvesicles containing pro-coagulants such as fibrinogen, β-thromboglobulin and platelet activating factor 4 from the plasma membrane produced by exposure to the ionophore, A23187 or dibucaine (Fox et al., 1990). Secretion of amylase from cultured guinea pig parotid acinar cells is also associated with a transient reorganization of the cytoskeleton which is accompanied by redistribution of the actin-binding protein, fodrin (Perrin et al., 1992). Furthermore, in unstimulated neutrophils, the actin network is associated with the plasma membrane and aggregates during secretion, leaving areas of plasma membrane devoid of actin microfilaments (Boyles and Bainton, 1981). Moreover, in permeabilized rat mast cells Ca^{2+}, the signal thought to trigger exocytosis has been found to cause a progressive decrease in F-actin levels as measured by flow cytometry and incubation of these cells with microfilament destabilizing agents such as cytochalasin E was found to lower the intracellular Ca^{2+} requisite for exocytosis (Koffer et al., 1990). Additional pharmacological evidence also exists supporting the view of cytoskeletal involvement in secretory dynamics. Studies conducted using compounds known to destabilize actin microfilaments and destroy the actin network such as cytochalasin B, cytochalasin D or DNase I have been found to enhance catecholamine secretion in intact (Friedman et al., 1980) and digitonin or streptolysin-O-permeabilized chromaffin cells (Lelkes et al., 1986; Sontag et al., 1988) as well as glucose-induced insulin secretion in islet cells (Orci et al., 1972).

D.2. The chromaffin cell cytoskeletal architecture: Actin and actin-binding proteins

Immunofluorescence microscopy has demonstrated that the endogenous scaffolding of the chromaffin cell is pervaded by actin microfilaments, microtubules and intermediate filaments.

Intermediate filaments and microtubules although responsible for providing a great deal of mechanical support and being involved in other functions in the chromaffin cell (Trifaró et al., 1972; Bader et al., 1981, 1984) unlike microfilaments, do not seem to be consequential in the process of exocytosis. Intermediate filaments are present in isolated chromaffin cells in culture as well as, in the intact adrenal gland (Bader et al., 1984; Trifaró and Kenigsberg, 1987). In lieu of the fact that the primary progenitor of chromaffin cells, the neural crest is of neuronal origin these cells express neurofilament proteins and hence, intermediate filaments of the neurofilament subtype (Bader et al ., 1984; Trifaró et al., 1985a). Often associated with microtubules, which in cultured chromaffin cells are disbursed throughout the cell body and other structures such as growth cones and neurites in a system of thin varicosities (Bader et al., 1981, 1984; Trifaró et al.,1985a, b) intermediate filaments in the chromaffin cell, as in other cell types, tend to be localized throughout the cytoplasm encompassing the nucleus and providing the cell with structural support. In addition to this role, intermediate filaments have also been implicated in the processes of saltatory particle movement and transport and positioning of

cellular structures such as the nucleus (for review see Traub, 1985; Trifaró et al., 1985b). The function of microtubules within the confines of the cytoskeletal matrix is primarily that of transport of cell components the most notable of which are, the chromaffin secretory vesicles. Despite the fact, that microtubules partake in the movement of vesicles to the periphery of the chromaffin cell architecture, the extensively ramified microtubular system of chromaffin cells does not seem to be involved in stimulus-evoked exocytosis (Trifaró et al., 1972; Bader et al., 1981; Schneider et al., 1981; Burgoyne, 1984; Trifaró et al., 1985b; Trifaró et al., 1989b; Vale, 1987; Hammerschlag and Brady, 1988; Hirokawa et al., 1989).

D.2.1. Actin

Actin, the building block of actin microfilaments is the principal molecular component of the adrenal chromaffin cell cytoskeleton and is a major constituent of a wealth of eukaryotic cell types, comprising up to 10% of a typical eukaryotic cell's total protein content (Forscher, 1989). The presence of actin in chromaffin cells was elucidated upon isolation and characterization of the protein from the cytosolic fraction of cultured chromaffin cells utilizing DNase I affinity chromatography (Lee et al., 1979). Two distinct isoforms (β and γ) of the protein possessing equivalent molecular weights but differing isoelectric points from muscle actin (α) were found to exist in these neuroendocrine cells (Lee et al., 1979).

Actin microfilaments are comprised of subunit monomers of molecular

weight 42,000 (G-actin) which in the presence of ATP and at physiological salt concentrations self-associate to yield helical polymers of actin (F-actin) with each individual polymer consisting of two staggered, parallel rows of monomers non-covalently bound and twisted into a helix (Weeds, 1982; Forscher, 1989; Stossel, 1989). Recent electron microscopic analysis has determined these helical actin microfilaments to be approximately 9 nm in diameter in chromaffin cells (Nakawa and Hirokawa, 1992). Work conducted in our laboratory as well as in others has illustrated that in chromaffin cells this network of microfilaments runs parallel to the plasma membrane (Nakata and Hirokawa, 1992), is localized predominantly in the subplasmalemma and is less dense in the cytosol (Lee and Trifaró, 1981; Trifaró et al., 1984; 1989b; Cheek and Burgoyne, 1986, 1987). The actin-rich cortex of chromaffin cells can be visualized in addition to antibodies (Lee and Trifaró, 1981), with rhodamine-labelled phalloidin, a bicyclic heptapeptide (Wulf et al., 1979) obtained from the green death cap (Faulstich, 1988) which is coupled with a fluorescent dye, rhodamine and specifically binds to filamentous actin (Faulstich, 1983). In unstimulated adrenal chromaffin cells, this actin-replete region constitutes a 200-400 nm deep zone from which chromaffin secretory vesicles are excluded (Burgoyne et al., 1982, 1988a). Moreover, it has been proposed that chromaffin secretory vesicles are entrapped within the actin lattice (Kondo et al., 1982; Nakata and Hirokawa, 1992) as a result of interactions which exist between the secretory vesicle membrane and actin microfilaments which are

mediated by anchorage such as, α-actinin, (Trifaró et al., 1982; Bader and Aunis, 1983) and fodrin (Perrin and Aunis, 1985).

D.2.2 Alpha actinin and fodrin

Actin anchorage proteins mediate the interaction of actin microfilaments and secretory vesicle membranes. Alpha actinin (α-actinin), a protein which is abundant in the Z-line of skeletal muscle and fodrin are two proteins thought to anchor actin filaments (Trifaró et al., 1992) which have been found to exist in adrenal chromaffin cells (Aunis and Perrin, 1984; Trifaró et al., 1984, 1985a,b). The actin-binding proteins α-actinin (Jockusch et al., 1977; Bader and Aunis, 1983) and fodrin (Aunis and Perrin, 1984) are extrinsic components of the secretory vesicle membrane with fodrin also being localized on the inner surface of the plasma membrane of secretory vesicles (Aunis and Perrin, 1984). Co-existing with the two actin-binding proteins on the surface of vesicle membranes is the contractile protein, actin (Jockusch et al., 1977; Bader and Aunis, 1983). Actin nuclei on the secretory vesicle surface bound to and stabilized by the anchorage proteins promote actin polymerization and membrane-bound actin polymers (Trifaró et al., 1992). Biochemical evidence obtained from studies utilizing isolated secretory vesicle membranes has demonstrated that in the absence of Ca^{2+} the vesicles bind to and become cross-linked by filamentous actin (F-actin). As increasing amounts of F-actin become bound to the vesicle membrane surface cross-linkages are formed

between individual polymers of actin evoking the formation of a highly viscous actin gel and thickening of the cortical cytoplasm of chromaffin cells (Fowler and Pollard, 1982). The presence of micromolar Ca^{2+}, a concentration attainable during depolarization of chromaffin cells, reduces both cross-linking and binding of F-actin to anchorage proteins on the vesicle surface (Fowler and Pollard, 1982). As a consequence of these findings and the fact that cross-linking of actin polymers and actin gel formation are Ca^{2+}-dependent it has been suggested that in unstimulated cells, where intracellular Ca^{2+} concentration is low, that the actin matrix forms a corporeal barrier which prevents the movement of secretory vesicles within adrenal chromaffin cells to sites of exocytosis thus, modulating the release of vesicular contents to the cell exterior (Trifaró et al., 1982; Aunis and Bader, 1988).

D.2.3. Stimulation-induced disassembly of cortical F-actin in adrenal chromaffin cells

Stimulation of chromaffin cells with secretagogues as mentioned previously in this work, effectuates Ca^{2+}-influx, the signal which triggers the occurrence of the secretory event and is known to regulate facets of actin-filament organization (Bennet and Weeds,1986). Reorganization of the actin lattice in chromaffin cells as a result of treatment of the cells with a secretagogue or destabilization of actin microfilaments with cytochalasin D or DNase I (Friedman et al., 1980) disrupts the granule-cytoskeleton interaction

and disintegrates the actin macrostructure thus, allowing the granules to translocate to exocytotic sites (Lelkes et al., 1986; Cheek and Burgoyne, 1986, 1987; Lelkes et al., 1986; Trifaró and Fournier, 1987; Burgoyne et al., 1989; Sontag et al., 1988; Trifaró et al., 1989b) and enabling catecholamine secretion to ensue. The groundwork for the above proposal extends from the following evidence. Immunocytochemical experiments carried out utilizing rhodamine-labelled phalloidin and anti-actin antibodies demonstrate that in unstimulated chromaffin cells a continuous actin network exists in the cortical region of chromaffin cells as evidenced by strong and continuous fluorescence in this region (Lee and Trifaró, 1981; Cheek and Burgoyne , 1986, 1987; Trifaró et al., 1989b). Stimulation of cholinergic receptors on the chromaffin cell surface evokes a transient disassembly of cortical actin filaments as indicated in chromaffin cells treated with rhodamine-labelled phalloidin by fragmentation of cortical fluorescence in areas devoid of F-actin (Cheek and Burgoyne, 1986, 1987; Trifaró et al., 1989b; Vitale et al., 1991). This disassembly of the cortical F-actin network in stimulated cells parallels a decrease in the amount of F-actin which can be recuperated from the Triton X-100 insoluble cytoskeletons of stimulated chromaffin cells (Cheek and Burgoyne, 1986; Burgoyne et al., 1989; Trifaró et al., 1990) and is accompanied by an increase in monomeric (G-actin) content as evaluated by DNase I inhibition assay (Cheek and Burgoyne, 1986; Trifaró et al., 1989b). Moreover, previous findings obtained in our laboratory utilizing anti-DβH and employing DβH as a marker for

exocytosis indicate that exocytosis occurs preferentially at sites of the plasma membrane devoid of filamentous actin (Vitale et al., 1991). Thus suggesting, that transient disassembly of the cortical cytoskeleton in chromaffin cells may be necessary for catecholamine secretion to occur.

D.3. Adrenal chromaffin cell actin-filament severing proteins

The existence of endogenous factors such as actin-binding proteins known to regulate the organization of the actin networks (Yin and Stossel, 1979; Craig and Pollard, 1982) and remodel cellular architecture intimates strongly that these proteins may play an eminent role in actin assembly and disassembly and hence, in chromaffin cell secretory dynamics (Trifaró et al., 1985b; Bader et al., 1986; Maekawa et al., 1989; Rodriguez Del Castillo et al.,1990). Two such actin-binding proteins which effectuate the disassembly of actin microfilaments have been observed to exist in chromaffin cells. These two actin-filament severing proteins are gelsolin (Trifaró et al., 1985a; Bader et al., 1986) and scinderin (Rodriguez Del Castillo et al., 1990).

D.3.1. Gelsolin

Chromaffin cell gelsolin is a 91 kDA (Trifaró et al., 1985b; Bader et al., 1986) actin filament-severing and capping protein found in chromaffin cells and other tissues which was initially isolated in rabbit lung macrophages (Yin and Stossel, 1979; Yin et al., 1981; Stossel et al., 1985). Gelsolin undergoes a

conformational change when bound to Ca^{2+} that triggers its capacity to effectuate the disassembly of actin-containing structures (Rouayrenc et al., 1986). The protein binds to actin reversibly and inhibits actin polymerization in a Ca^{2+}-dependent manner, decreases the average length of actin filaments (Trifaró et al., 1992) and prevents gelation of actin microfilaments by actin-binding proteins (Yin and Stossel, 1979). In the presence of micromolar Ca^{2+}, gelsolin binds to the site of actin microfilaments to which actin monomers add preventing elongation of filaments (Trifaró et al., 1992) and decreases the length of existing microfilaments eliciting fragmentation of the actin network (Bader et al.,1986; Trifaró et al., 1988a).

D.3.2. Scinderin: A novel Ca^{2+}-dependent actin filament-severing protein

Previous work conducted in our laboratory which led to isolation and characterization of chromaffin cell actin additionally resulted in the observation that chromaffin cell gelsolin of molecular weight 91 kDA and pI 6.0-6.2 could be eluted by a guanidine hydrochloride -containing buffer concomitantly with actin from DNase I affinity columns (Lee et al., 1979; Trifaró et al., 1985a; Bader et al., 1986). The principle underlying the use of the DNase I affinity column is that monomeric actin (G-actin) has been assessed to possess a great affinity for DNase I, an enzyme which was initially isolated and crystallized from bovine pancreas by Kunitz (1948) and whose sole enzymatic activity to date is degradation of DNA (Lazarides and Lindberg, 1974). Monomeric actin, which

is abundant in all eucaryotic cells when in contact with DNase I forms a tight complex with the enzyme, modifies its structure and induces inhibition of the enzyme's catalytic activity. Once coupled to sepharose beads DNase I in the presence of Ca^{2+} provides a powerful tool which permits the isolation of Ca^{2+}-dependent actin-binding proteins. Subsequent re-analysis of initial experiments leading to the isolation of chromaffin cell actin was conducted utilizing a DNase I affinity chromatography column washed extensively with a 0.5M K^{+} solution to reduce non-specific binding and eluted with an EGTA containing buffer followed by elution with guanidine hydrochloride (Trifaró et al., 1985b, 1988a; Bader et al., 1986). Under these conditions, the electrophoretic pattern obtained demonstrated that a second actin-binding protein of molecular weight 80-85 kDA (Bader et al.,1986) could be isolated from the adrenal medullary cytosol in addition to the originally isolated 91 kDA polypeptide (gelsolin). This second polypeptide was established to be a unique Ca^{2+}-dependent actin binding protein. As a consequence of the peptide's actin-filament severing capacity, the peptide was named scinderin, a named derived from the latin word, scindere, which means "to cut" (Rodriguez Del Castillo et al., 1990). The protein, which was subsequently found to exhibit the characteristics summarized in Table.1 was consequently purified in our laboratory utilizing a four step chromatographic process in which the cytosolic fraction of chromaffin cells obtained from homogenized medullary tissue was subjected to ion exchange chromatography, gel filtration, DNase I affinity chromatography, and

HLPC using an ion exchange column (Rodriguez Del Castillo et al., 1990). Once freed from impurities, antiserum to the purified peptide was raised in rabbits and employed in immunohistochemical and biochemical analyses. Sodium dodecyl sulfate and double dimension gel electrophoresis (SDS-PAGE) and immunoblotting analyses showed an apparent molecular weight for scinderin of 79,500 ± 450 Daltons and three isoforms of isoelectric points 6.0, 6.1, and 6.2 (Rodriguez Del Castillo et al., 1990). Equilibrium dialysis experiments indicated that scinderin possessed two Ca^{2+}-binding sites (K_d 5.85 x 10^{-7}M, B_{max} 0.81 mol Ca^{2+}/ mol protein and K_d 2.85 x 10^{-6}M, B_{max} 1.87 mol Ca^{2+}/ mol protein). Once some of the biochemical parameters of the protein had been ascertained, the function of scinderin was investigated. Functional experiments employing actin solutions demonstrated that although scinderin was unable to modify the viscosity of actin solutions in the absence of Ca^{2+}, that in the presence of 10^{-8} -10^{-6}M Ca^{2+} scinderin was capable of decreasing the viscosity of actin solutions (Rodriguez Del Castillo et al., 1990; Trifaró et al., 1992). Maximum decreases in the viscosity of actin solutions in the presence of scinderin were observed at a Ca^{2+} concentration of 10^{-6}M (Rodriguez Del Castillo et al., 1990; Trifaró et al., 1992). Electron microscopic analysis of the interaction of scinderin and actin demonstrated that the Ca^{2+}-dependent ability of scinderin to modify actin solutions was due to the protein's ability to shorten actin filaments (Rodriguez Del Castillo et al., 1990). Additional experiments indicated that the interaction between actin and scinderin was such that two

SCINDERIN

* apparent MW of 79000 ± 450 D
* three isoforms (pI: 6.0, 6.1 and 6.2)
* two Ca^{2+} binding sites
* two Ca^{2+} -dependent actin binding sites
* a Ca^{2+} -dependent actin filament severing activity
* binds to PS and PIP_2 liposomes in a Ca^{2+}- and pH -dependent manner
* 90% of scinderin is cytosolic and 10% associates with the microsomal fraction
* leaks out from digitonin-permeabilized chromaffin cells and is retained by Ca^{2+}
* is mainly present in tissues with high secretory activity

Table 1
Summary of the characteristics of the Ca^{2+}-dependent actin-filament severing protein, scinderin.

molecules of actin formed a complex with one molecule of scinderin and that this interaction was Ca^{2+}-dependent (Trifaró et al., 1992). These findings led to the suggestion that scinderin may possess two actin-binding sites. Limited proteolytic digestion of scinderin with either Staphylococcus V8 protease or chymotrypsin yielded two peptide fragments of molecular weight 38 and 40kDA and differing isoelectric points (Rodriguez Del Castillo et al., 1990). These fragments were separated by ion exchange chromatography and the actin-binding properties of each fragment examined. Each of the fragments was found to exhibit the capacity to bind one G-actin monomer in a Ca^{2+}-dependent manner suggesting that each fragment contains an actin and a Ca^{2+}-binding site (Trifaró et al., 1992). Immunofluorescence studies utilizing scinderin antiserum and examining the localization of scinderin in cultured chromaffin cells showed that scinderin exhibits weak and diffuse cytoplasmic staining accompanied by a strong continuous fluorescence in the cortical region of cells indicating that the peptide is predominantly localized in the subplasmalemmal region of cultured chromaffin cells (Rodriguez Del Castillo et al., 1990; Vitale et al., 1991). Immunoblotting analysis revealed that the actin-filament severing peptide, scinderin is predominantly in tissues with high secretory activity such as bovine brain, anterior and posterior pituitary, kidney, salivary glands and testis and is absent from the liver, plasma and heart and skeletal muscle (Tchakarov et al., 1990) thus, intimating the peptide may play a crucial role in secretion. As discussed previously in this work, cytochemical

findings from experiments conducted in resting chromaffin cells using rhodamine-labelled phalloidin and anti-actin antibodies have allowed visualization of a continuous network comprised of actin microfilaments localized in the cortical region of chromaffin cells (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986, 1987; Trifaró et al., 1989b). Cholinergic receptor stimulation or depolarization with high K^+ causes fragmentation of the cortical network, as indicated by fragmentation of the continuous cortical fluorescent pattern exhibited in resting cells exposed to rhodamine-labelled phalloidin. This fragmentation is indicative of areas in the subplasmalemma which are devoid of F-actin and represents removal of the actin lattice barrier which has been proposed to impede contact of the chromaffin granules with exocytotic sites (Cheek and Burgoyne, 1986, 1987; Trifaró et al., 1989b). In chromaffin cells which were incubated with Locke's in the absence or presence of 10μM nicotine for 5, 20 and 40 s and processed for immunofluorescence with scinderin antiserum #6, FITC-IgG and rhodamine-phalloidin, as described in the materials and methods section, it was found that nicotine elicited fragmentation of the scinderin cortical fluorescence pattern, which in control cells exposed only to Locke's, is continuous in the subplasmalemmal region of these cells and diffuse in the cytoplasm (Vitale et al., 1991). Disruption of scinderin cortical fluorescence, leaving "patches" of scinderin in the equatorial plane of the cells was evident even at 5 s of stimulation of chromaffin cells with nicotine and was found to occur concomitantly with fragmentation of F-actin cortical

fluorescence (Vitale et al., 1991). In fact, in 88% of nicotine-stimulated cells exhibiting scinderin redistribution, concomitant redistribution of scinderin and F-actin disassembly occurred (Vitale et al., 1991; Trifaró et al., 1992). Although similar results were obtained when high K^+ (56mM) was used to depolarize the cells, maximum values in the percentage of cells displaying redistribution of scinderin cortical fluorescence were attained after 20 s of stimulation with K^+ and 40 s of stimulation with nicotine (Vitale et al., 1991; Trifaró et al., 1992). After removal of the stimulus, the number of cells displaying a discontinuous cortical fluorescence pattern for scinderin and F-actin returned to control values, with scinderin recovery occurring before F-actin (Vitale et al., 1991). Comparison of the time-courses of scinderin redistribution and F-actin disassembly in cells stimulated with either nicotine or high K^+ with the time-course of catecholamine output in these cells demonstrated that after stimulation with nicotine or high K^+ there was a sharp rise in catecholamine output. However, the sharp increase in catecholamine output lagged 10-15 s behind scinderin redistribution and F-actin disassembly (Vitale et al., 1991; Trifaró et al., 1992). Therefore, these results seem to suggest that scinderin redistribution and F-actin filament disassembly in response to stimulation with nicotine or high K^+ precede exocytosis (Vitale et al., 1991; Trifaró et al., 1992). A mechanism for the modulation of scinderin has been proposed (Rodriguez Del Castillo et al., 1992) and is presented in Figure 3. Briefly, under resting conditions (pCa = 8; pH = 6.8) scinderin is divided into two pools, a

soluble one and a pool bound to membrane phospholipids. These two pools can be observed using immunofluorescence microscopy and correspond to the diffuse cytoplasmic staining in the cytoplasm and continuous cortical fluorescence observed in chromaffin cells respectively. Under these conditions, the binding of scinderin to phospholipids is possible because half maximal binding to phospholipids is observed at 10^{-8}M Ca^{2+} and scinderin is not bound to actin because scinderin actin-filament severing activity is evident at 10^{-7}M Ca^{2+} (Rodriguez Del Castillo et al., 1990). Nicotinic stimulation elicits Ca^{2+} entry (pCa = 5-6) and this increase in intracellular Ca^{2+} induces release of scinderin from the membrane-associated pool (redistribution of scinderin as indicated by discontinuity of scinderin cortical fluorescence) and binding of scinderin to actin (co-localization of scinderin and F-actin cortical discontinuous areas) (Vitale et al., 1991) as well as activation of scinderin actin-filament severing activity. Nicotine-induced scinderin redistribution has been associated with a rise in intracellular pH (Rodriguez Del Castillo et al., 1992). The increase in pH ($pH_i = 7.10$) within the cell results because intracellular Ca^{2+} elevation leads to activation of PKC, and this enzyme activates the Na^+/H^+ antiport (Rodriguez Del Castillo et al., 1992). Under these conditions, the low affinity binding site of scinderin might be activated. During recovery, intracellular Ca^{2+} decreases due to extrusion or sequestration, and the scinderin-actin complex begins to dissociate (low affinity Ca^{2+} binding site) (Rodriguez Del Castillo et al., 1992). When Ca^{2+} concentration is approximately 10^{-7}M and the pH is still

high ($pH_i = 7.1$), conditions in which scinderin expresses maximum affinity for phospholipids, the remaining scinderin-actin complex is dissociated by competing phospholipids thus, explaining why scinderin continuous cortical fluorescence is recuperated upon removal of the stimulus more rapidly then that of F-actin (Vitale et al.,1991). Upon return of the intracellular Ca^{2+} concentration to resting levels (pCa = 8), PKC activity decreases, chromaffin cell intracellular pH attains resting values and scinderin is in equilibrium between the soluble and membrane-bound pools (Rodriguez Del Castillo et al., 1992).

D.3.3. Two distinct actin-filament severing proteins: Comparison between scinderin and gelsolin

Scinderin was consequently established not to be a breakdown product of gelsolin and antiserum against the purified scinderin protein was found not to cross-react immunologically with gelsolin or other cytoskeleton proteins (Rodriguez Del Castillo et al., 1990; Vitale et al., 1991). Scinderin is structurally, immunologically and biochemically distinct from gelsolin. The two peptides possess distinct molecular weights, isoelectric points, amino acid composition and peptide maps (Rodriguez Del Castillo et al., 1990). As mentioned previously, two dimensional gel electrophoresis showed three isoforms of scinderin exist (pI = 6.0, 6.1, and 6.2). The same type of electrophoretic analysis demonstrated the presence of three isoforms for gelsolin which were distinct from those of scinderin (pI = 5.8, 5.9 and 6.0)

(Trifaró et al., 1992). The molecular weights of the fragments obtained after limited proteolytic digestion of adrenal medullary gelsolin with Staphylococcus V8 protease or chymotrypsin also differed from the fragments obtained from proteolytic digestion of scinderin. As cited in a prior section of this thesis, limited proteolysis of scinderin yielded two peptide fragments 38kDA (pI = two isoforms, 5.7, 5.9) and 40kDA (two isoforms pI = 6.0, 6.1) (Trifaró et al., 1992). Limited proteolysis of gelsolin in contrast, yielded five peptide fragment (Kwiatkowski et al., 1985). The molecular weights of these fragments and their respective isoelectric points were 80kDA (pI = 6.0), 50kDA two isoforms, (pI = 5.5 and 5.7), 46.5kDA (pI = 5.5) and 16kDA (pI = 6.1) (Trifaró et al., 1992). The amino acid composition of the two actin-filament severing peptides is also different. Moreover, in chromaffin cells, cytochemical studies conducted with anti-gelsolin and a mouse-monoclonal antibody against a 47kDA fragment of gelsolin, the cortical cytoplasmic pattern of gelsolin was weaker than that observed in cells stained with anti-scinderin and stimulation of chromaffin cells with nicotine or high K^+ although inducing fragmentation of F-actin cortical fluorescence, did not cause modification in gelsolin cortical fluorescence (Vitale et al., 1991; Trifaró et al., 1992). An additional difference between the two proteins is that in studies using digitonin-permeabilized chromaffin cells scinderin as well as other cytosolic proteins leak out of the cell, but gelsolin does not (Vitale et al., 1992b). Addition of micromolar Ca^{2+} produced retention of scinderin and other cytosolic protein in permeabilized cells and was

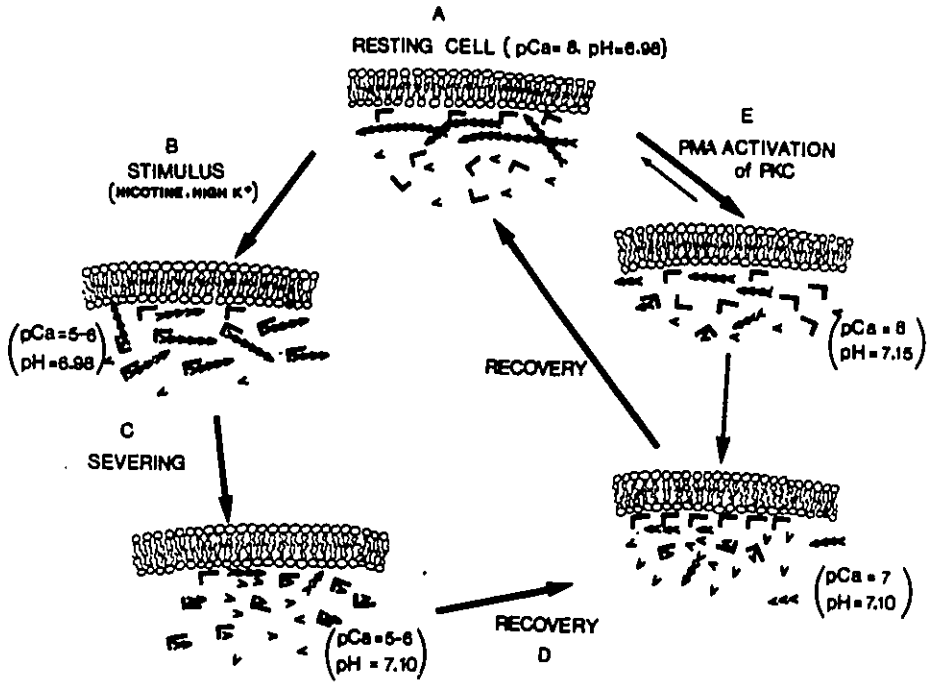


Fig.3
Schematic model of the proposed interactions of scinderin with actin and membrane phospholipids (PS and PIP_2) and the effects of intracellular pH and free Ca^{2+} on these interactions. (Taken from J. Cell Biology, Rodríguez Del Castillo A., Vitale M.L. and Trifaró J-M., 1992).

accompanied by enhancement of Ca^{2+}-dependent catecholamine secretion (Vitale et al., 1992). Scinderin therefore, seems to be the actin-binding protein in bovine adrenal chromaffin cells which is the best candidate as a modulator of actin microfilament dynamics and catecholamine secretion.

E. THE SECOND MESSENGER CYCLIC AMP

The cortical cytoskeleton has been proposed to be a possible target for second messengers and has been proposed to be involved in the regulation of secretion in chromaffin cells. Several lines of evidence suggest the involvement of the second messenger Ca2+ (Houssay and Molinelli, 1928; Douglas and Rubin, 1961; Douglas, 1968; Trifaró, 1977) and Ca^{2+} and phospholipid-dependent protein kinase C in catecholamine secretion in intact (Burgoyne and Norman, 1984; Brocklehurst et al., 1985; Morita et al., 1985; Pocotte et al., 1985; Terbush et al., 1988; Bittner and Holz, 1990) and permeabilized chromaffin cells (Knight and Baker, 1983; Pocotte et al., 1985; Burgoyne et al., 1988b; Knight et al., 1988; Bader et al., 1989; Bittner and Holz, 1990; Tachikawa et al., 1990; Isosaki et al., 1991). In adrenal medullary chromaffin cells, PKC activation has been associated with reorganization of actin filaments (Grant and Aunis, 1990; Vitale et al., 1992a), scinderin redistribution and an increase in catecholamine release (Vitale et al., 1992a). These two second messengers however, may not be the only intracellular signals which modulate cytoskeletal proteins such as scinderin, actin network dynamics and

catecholamine secretion. A labyrinth of intracellular message relay systems may in fact be acting to regulate enhancement and inhibition of catecholamine secretion. The plasma membrane of adrenal chromaffin cells as well as that of other cell types, represents a physical demarcation which separates the intracellular environment of cells from the surrounding extracellular milieu and which is thus, responsible for maintaining homeostasis in the internal environment of each cell. In order for cells to perceive signals from the immediate exterior vicinity and effectuate adaptive responses to changes in the external medium, cells have evolved complex mechanisms which allow signals generated in the external surroundings to transcend the plasma membrane barrier and be propagated throughout the intracellular domain. At the molecular level, this message relay system encompasses ions and a series of proteins native to the plasma membrane each of which serves as a point source for the transfer of information by inducing conformational changes in adjacent proteins (Berridge, 1985). The components of this hierarchical system constitute a group collectively called the "second messengers". The identification and characterization of a heat-stable factor which could be generated by treatment of the particulate sub-cellular fraction obtained from rat liver with adrenaline and which came to be called, 3',5'-cyclic adenosine monophosphate (cAMP) (Sutherland and Rall, 1958) was the driving force which led to the creation of the concept of second messenger systems by Dr. Earl Sutherland, (1958), making cAMP the first compound to be established as a second messenger

(Morgan, 1989). Sutherland and Rall in the late 1950s proposed that transmembrane signalling was accomplished by hormones binding to specific receptors on the cell surface which mediated an increase in intracellular second messengers such as cAMP (Rall et al., 1957; Rall and Sutherland, 1961).

E.1. Synthesis of cAMP by adenylate cyclase and degradation of cAMP by phosphodiesterase

Binding of external signals to receptors on the cell surface elicits a conformational change in guanyl nucleotide-binding proteins (G-proteins) (Berridge, 1985), peripheral plasma membrane proteins comprised of three subunits, α (45kD), β (35kD) and γ (7kD) (Fig. 4) that couple the activation of hormone receptors to stimulation of effector enzymes (Gilman, 1987). The α subunit of these proteins contains the guanine nucleotide-binding domain which in the non-activated state of the G-protein, is occupied by guanosine diphosphate (GDP). Activation of G-proteins results in an exchange of GDP for GTP and a subsequent dissociation of the β and γ from the α-GTP complex (Weiss et al., 1988). The α-GTP complex is responsible for the activation of adenylate cyclase, a plasma-membrane associated enzyme present in most eukaryotic cells which catalyzes the biosynthesis of cAMP from ATP in the presence of Mg^{2+} ions (Stryer, 1988). Since many α-GTP complexes are formed the original signal is greatly amplified (Stryer, 1988). Termination of activation of G-proteins results due to the inherent ability of G-proteins to

function as GTPases and effectuate the hydrolysis of GTP in the α-GTP complex to GDP (Stryer, 1988).

Various types of G-proteins exist, one stimulating adenylate cyclase (G_s) and the other inhibiting activation of the enzyme and hence, cAMP generation (G_i) (Weiss et al., 1988). The mechanism by which G_i inhibits adenylate cyclase is not yet well understood (Harper, 1988).

The primary mechanism known to exist for reduction of intracellular cAMP is the degradation of cAMP by cyclic nucleotide phosphodiesterases. At least 20 members of the phosphodiesterase family have been characterized and the sequence information is available on 3 gene products (McKnight, 1991). Although there are multiple isoforms of phosphodiesterases which could contribute to removal of cAMP, diverse phosphodiesterases possess varying affinities for the nucleotide. Most cells contain at least four forms of phosphodiesterase which are generally classified as, low K_m, cAMP-preferring, Ca^{2+}/calmodulin-regulated (preferring cGMP, K_m $\sim 3\mu M$ but with significant activity against cAMP), and cGMP-stimulated and cGMP-inhibited forms preferring cAMP (for review see, Thompson et al., 1979; Beavo et al.,1982). In general, phosphodiesterases degrade cAMP by hydrolyzing the second messenger to 5'AMP (Stryer, 1988). There have also been reports that in some more primitive cells decreases in cellular cAMP levels result due to exportation of cAMP out of the cell in an active, regulated manner. (for review see, Barber and Butcher, 1983).

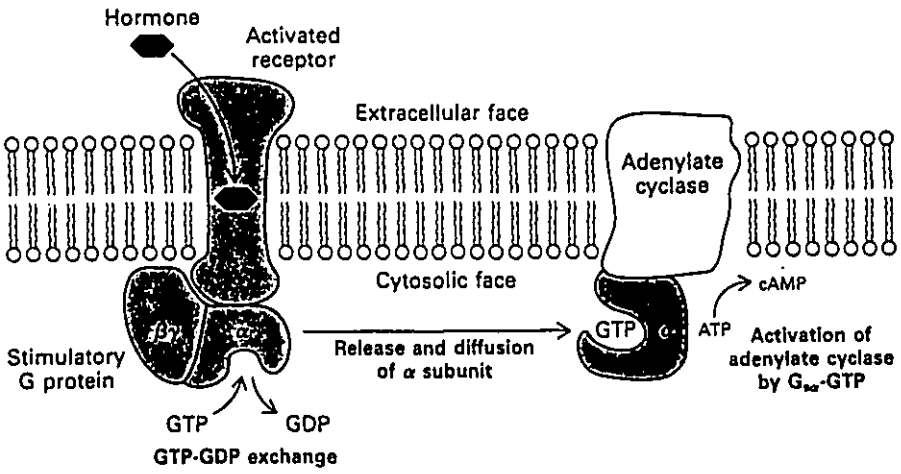


Fig. 4
Mechanism for the activation of adenylate cyclase by the binding of a hormone to its specific receptor is mediated by G_s, the stimulatory G-protein. (Taken from Biochemistry, Stryer L., 1988).

E.2. Cyclic AMP-dependent protein kinases

Cyclic AMP protein kinases (protein kinase A; PKA) allow cAMP to manifest its physiological effects in eukaryotic cells (Johnson and Wong, 1989). The protein kinases which respond to cAMP are formed as inactive tetramers containing two regulatory subunits and two catalytic subunits (holoenzyme). Binding of two molecules of cAMP to each regulatory subunit monomer results in release of the catalytic subunits which in turn, can phosphorylate intracellular targets (McKnight, 1991) such as regulatory proteins at serine and in some cases, threonine residues eliciting conformational changes which alter their biological properties (Cohen and Hardie, 1991).

E.3. Compounds known to stimulate cAMP formation

Several agents exist which are known to increase levels of cAMP by activation of adenylate cyclase. These agents include sodium fluoride (NaF), non-hydrolyzable analogs of GTP, cholera toxin and forskolin and its biologically active derivatives (Seamon and Daly, 1986). The agent which was utilized to conduct the majority of the work in this thesis was, forskolin. As a result, brief mention of this compound will be made at this time in this thesis. A more lengthy discussion on the agent in a later section of this work.

E.3.1. Forskolin

Forskolin, is a naturally occurring diterpene which has been shown to

directly activate adenylate cyclase and augment cAMP thus, eliciting cAMP-dependent physiological responses (Laurenza et al., 1989). This ability of forskolin to activate adenylate cyclase directly, without the need for a G-protein intermediary distinguishes the compound and it biologically active derivatives from other compounds known to increase cAMP by adenylate cyclase activation such as cholera toxin, sodium fluoride, and non-hydrolyzable analogs of GTP (Seamon and Daly, 1983). Moreover, this characteristic of forskolin has provided new method for assessing the role of cAMP is physiological responses.

CHAPTER II

MATERIALS AND METHODS

A. CHROMAFFIN CELL CULTURE

A.1. Adrenal gland preparation and dissection

A near homogeneous population of bovine adrenal chromaffin cells was obtained utilizing a method previously described in our laboratory (Trifaró and Lee, 1980). Eight glands obtained from a local slaughterhouse were rinsed in Ca^{2+} and Mg^{2+}-free Locke's solution (in millimolar: NaCl,154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.50; glucose, 10; pH 7.0) containing phenol red, penicillin (200mg/l), streptomycin (50mg/l) and gentamycin (50mg/l). On arrival to the laboratory the glands were rinsed again and perfused in a retrograde fashion with the same buffer via a polyethylene tube inserted in the central adrenal vein. The glands were dissected and the individual cortices were removed. The decorticated medullae were then perfused with the above buffer for 5-10 min.

A.2. Chromaffin cell isolation and purification

Each decorticated gland was then perfused utilizing a closed circuit perfusion chamber for 60 min at 37°C with 25-30 ml of enzyme mixture previously sterilized through a millepore filter. The enzyme mixture consisted of 5.3 mg DNase and collagenase (548.13 units/gland) per 400 ml of Ca^{2+} and Mg^{2+}-free Locke's buffer containing phenol red, penicillin (100mg/l), streptomycin (100mg/l), mycostatin (25,000 U/l), gentamycin (50mg/l). Once

flaccid, traces of the remaining cortex of each gland were removed and the medullae were minced. The medullae were then transferred to a "Trypsinizing flask" containing enzyme mixture pre-warmed to 37°C and stirred for 30 min in a water bath at the same temperature. Undigested tissue was then filtered and rinsed through a 44μm sterile cloth mesh into a graduated cylinder containing Locke's solution (in millimolar: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.5; $MgCl_2$, 1.2; $CaCl_2$, 2.2; glucose, 10; pH 7.2). with phenol red, penicillin, streptomycin, mycostatin and gentamycin in the afore mentioned concentrations. The cell number was determined utilizing a hemocytometer (Neubauer, Levy chamber Cat. No. 500). The above mixture was centrifuged for 10 min at room temperature at 50 x g and the pellet obtained was washed in the same buffer used for rinsing which was cited above and re-centrifuged for 15 min under the same conditions. Following re-centrifugation the pellet obtained was suspended in Eagle's Balanced Salt Solution (EBSS) and this mixture was added to a solution of Percoll (colloidal silica coated with polyvinylpyrrolidone at pH 7.2) in EBSS such that each gradient contained EBSS (1X), Percoll and EBSS (10X) in an 8:9:1 ratio. The tubes were then centrifuged in a Sorvall centrifuge at 20°C and 45,000 x g for a period of 20 min. A diagram of a self-generating Percoll gradient is depicted in Fig.5. To obtain the chromaffin cell population desired, 4.5 ml of the top of the gradient was discarded and the chromaffin cell band was collected (Fig.5). The combined gradient fractions were then diluted with 5 vol of EBSS containing

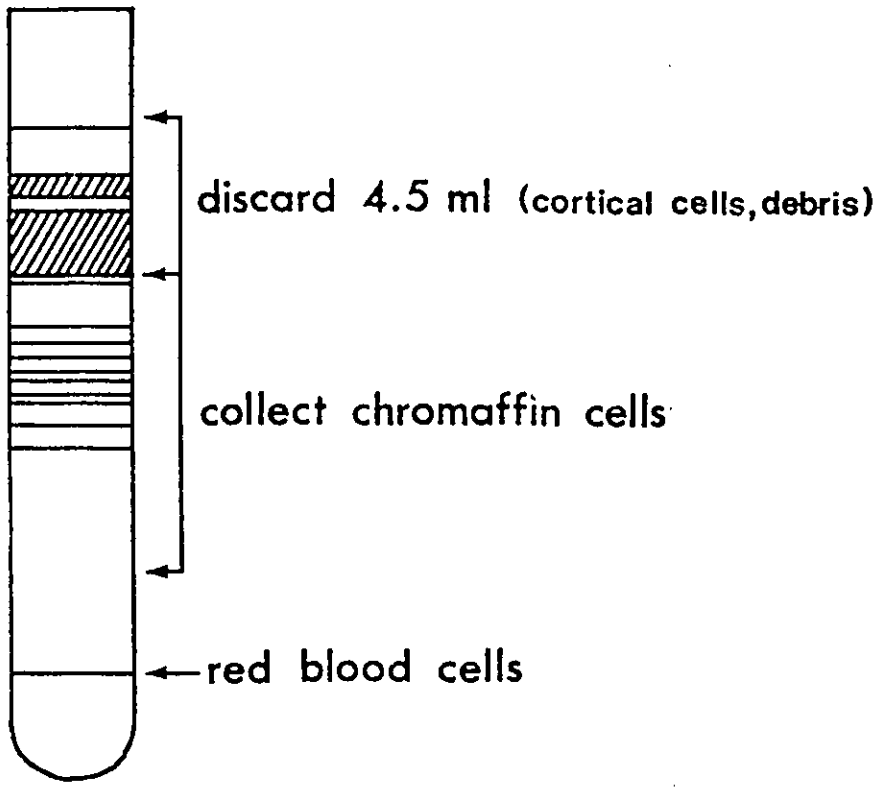


Fig.5
!llustration depicting the layers formed by the self-generating Percoll density gradient employed in the isolation of chromaffin cells. The top layer is discarded and contains cortical cells, cellular debris and fibroblasts allowing isolation of the second layer which contains the chromaffin cell population.

antibiotics and centrifuged at 50 x g at room temperature for 15 min. The supernatant was then discarded and the pellet was washed once with EBSS containing antibiotics (penicillin, 100mg/l; streptomycin, 100mg/l; nystatin, 25,000 U/l; gentamycin, 50mg/l). An aliquot was removed and the cell number was determined to calculate the cell yield prior to plating.

A.3. Preparation of chromaffin cell primary cultures

The final cell pellet was then suspended in Dulbecco's modified Eagle's medium fortified with 10% fetal calf serum, 0.1mM ascorbic acid, 0.1% glucose and 15mM Hepes, pH 7.2. Also included in the culture medium were penicillin (100μg/ml), streptomycin (100μg/ml), gentamycin (50μg/ml), nystatin (25,000 U/l) and 5-fluorodeoxyuridine (10^{-5}M) as well as, fresh cytosine arabinose (cytosar, 10^{-5}M) to arrest cell division and thus,the proliferation of fibroblasts.

Cells utilized for immunohistochemistry purposes were plated on collagen-coated glass coverslips at a density of $0.3x10^6$ cells/35-mm diameter dish while those employed in cAMP assays and catecholamine output studies were plated on collagen-coated petri dishes at a density of $1x10^6$/35-mm diameter dish. Cells were cultured at 37°C in a NAPCO 6300 humidified incubator under a CO_2 + air atmosphere (5% : 95%) for an initial period of 24h. This initial period allowed for attachment of the cells. Once the cells had adhered to the collagen-coated surfaces they were fed every 2-3 days with a solution

comprised of sterile complete feeding medium comprised of non-sterile Dulbecco's modified Eagle's medium containing cytosine arabinose (10^{-5}M) and 5-fluorodeoxyuridine (10^{-5}M). The age in culture of chromaffin cells utilized in all of the studies described in this thesis was between 48-72h. Fig.6 depicts two-day old chromaffin cells in culture.

B. IMMUNOHISTOCHEMISTRY

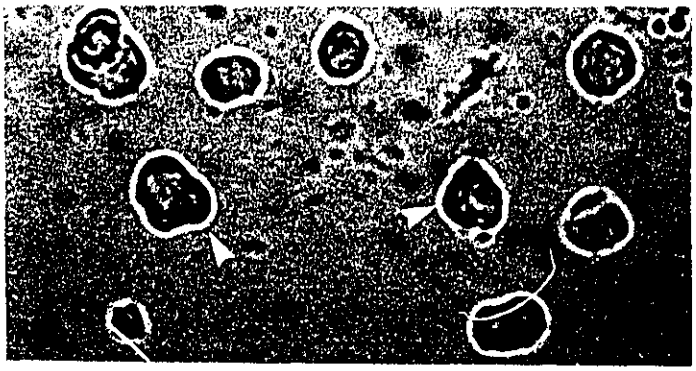
B.1. Source of scinderin antiserum

Scinderin antiserum was produced by raising polyclonal antibodies to purified bovine scinderin in rabbits. The antiserum employed throughout all of the work described in this thesis was derived from a bleeding obtained from rabbit number 6 and was hence, termed scinderin antiserum #6. Scinderin antiserum #6 has been determined to possess a high degree of specificity toward scinderin and does not cross-react with the other cytoskeletal proteins (Vitale et al., 1991) and actin-binding proteins such as gelsolin (Rodriguez and Castillo et al., 1990; Tchakarov et al., 1990).

B.2. Indirect immunofluorescence of adrenal chromaffin cells

Chromaffin cells utilized in immunohistochemisty studies were processed for immunofluorescence as described by Lee and Trifaró (1981) and Vitale et al. (1991). As shown in Fig.7 at the onset of experiments the cells were

A.



B.

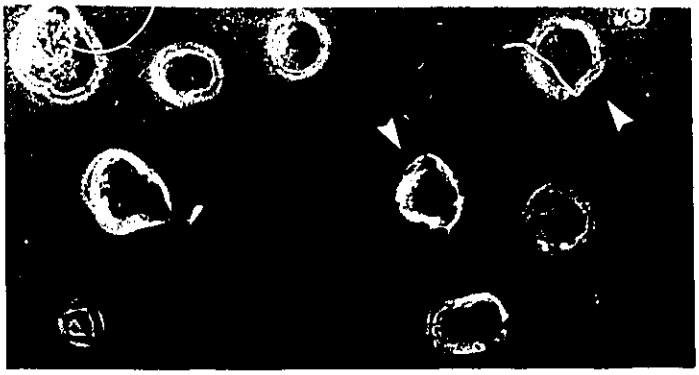


Fig.6
Two day old bovine adrenal chromaffin cells in culture as seen using phase contrast (A) or bright field (B) microscopy at a magnification of 20x.

bathed three times with 1ml of Locke's solution. Following the initial rinsing period the cells were incubated with Locke's in the absence (control) or presence (stimulated) of the diverse compounds examined for the incubation times outlined in each experimental protocol. The cells were then fixed in 3.7% formaldehyde in Locke's solution for 20 min. Fixed chromaffin cells were then subjected to 6 successive washes with phosphate buffered saline (PBS) (in millimolar: NaCl, 130; 76, Na_2HPO_4; 24, NaH_2PO_4; pH 7.2) and were permeabilized by three successive exposures of 5 min each to 50%, 100%, 50% acetone (50% acetone was made by dilution of 100% acetone with distilled water) and then rinsed again several times with PBS.

B.2.1 Single fluorescence-labelling of chromaffin cells

After rinsing with PBS, preparations were incubated at 37°C for 60 min with scinderin antiserum #6 (dilution, 1:80), washed 6 times with PBS and incubated with goat-anti rabbit immunoglobulin G-fluorescein isothiocyanate conjugate (FITC-IgG) (dilution, 1:160) for 60 min at 37°C. The coverslips were rinsed again several times in PBS and were mounted in glycerol-PBS (1:1; vol:vol) as outlined in Fig.7. Control preparations were then incubated either with scinderin antiserum #6 or with the second antibody alone.

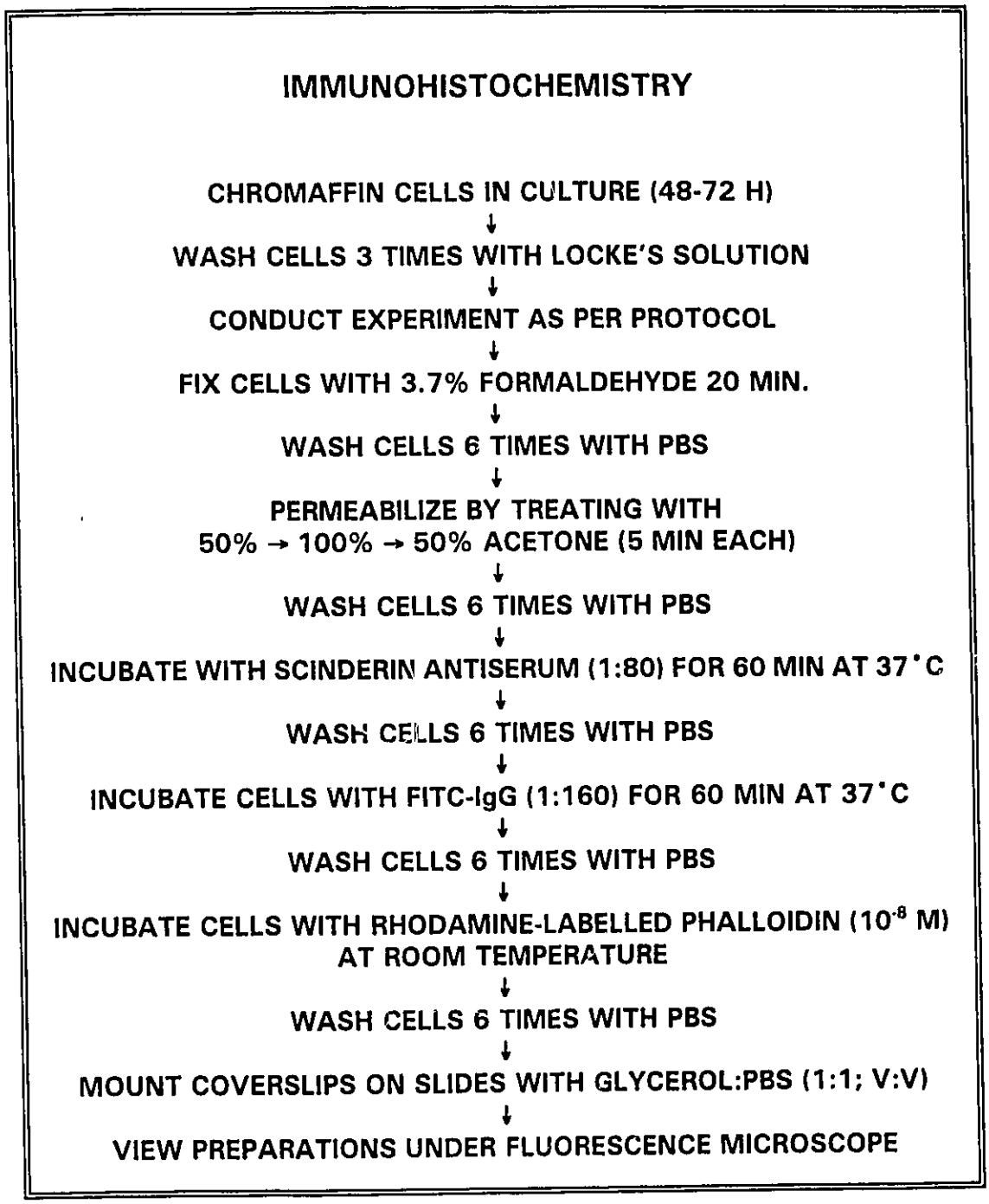


Fig.7
Flow chart describing the protocol followed in immunohistochemistry of chromaffin cell scinderin and F-actin fluorescence.

B.2.2 Double fluorescence-labelling of chromaffin cells

The above protocol was also utilized for double staining with the only variation being that following incubation with (FITC-IgG) the cells were washed again 6 times with PBS and incubated with 10^{-8}M rhodamine-labelled phalloidin, a probe for filamentous actin (Faulstish et al., 1988), for an additional 20 min this time at room temperature. All other steps of the protocol remained unchanged and are illustrated in Fig.7.

B.3. Data analysis

Preparations were observed with a Leitz-Ortholux II fluorescent microscope equipped with a 200-W high pressure lamp and Ploemopack II incident light illuminator possessing an I-filter block (KP 490 plus 1mm GG 455 exciting filter, TK dichroic beam splitting mirror, K 515 suppression filter) for fluorescein and an M-filter block (2mm BG plus S 546 exciting filter , TK 580 dichroic beam splitting mirror , K 580 suppression filter) for rhodamine as previously reported (Vitale et al.,1991, 1992a). One-hundred individual spherical cells per coverslip were examined. Cell containing regions were initially pinpointed under low magnification; then, under high magnification, each single rounded cell in the area was then classified as exhibiting either a discontinuous patched staining pattern in the cortical region of the cell (see Fig.11, 40s in results section) or a continuous cortical fluorescence (see Fig.11, 0s in results section). In an effort to avert bias in the classification of cells,

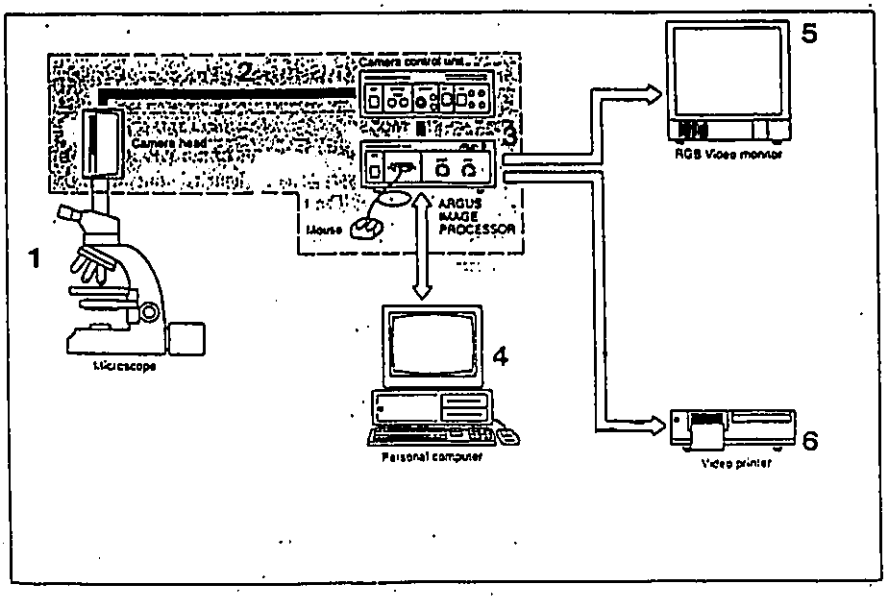
code numbers were assigned to each of the coverslips to be examined and the procedure was conducted without knowing if the cells being observed were from control or treated populations (single-blind); only after all coverslips were examined and the results obtained recorded were the codes revealed and the experimental treatments employed elucidated (Vitale et al., 1992a). Once data was procured utilizing the afore described single-blind protocol the percentage of chromaffin cells for each condition examined displaying "discontinuous" cortical fluorescence was graphically represented. In most cases 400-800 cells per condition were examined and in the case of F-actin only, scinderin positive cells were analyzed.

C. VIDEO-ENHANCED MICROSCOPY OF CHROMAFFIN CELLS

C.1. Description of equipment

Once viewed under the fluorescence microscope, cells were again classified as exhibiting either "continuous" or "discontinuous" cortical scinderin and F-actin fluorescence and quantitative analysis of fluorescent intensity was conducted employing a Hamamatsu Photonics K.K. Argus-50/CL image processor. The components of the system were arranged as depicted in Fig.8. The image observed under the fluorescence microscope was fed into a Zeiss Venus III camera head which was in turn connected to a Hamamatsu Argus-50/CL Image processor. The image processor was linked to a Compaq Desk

ARGUS-50 C/L SYSTEM CONFIGURATION



1. LEITZ ORTHOLUX II FLUORESCENCE MICROSCOPE
2. ZEISS VENUS III CAMERA HEAD/CAMERA CONTROL UNIT
3. ARGUS-50/CL IMAGE PROCESSOR
4. COMPAQ DESK PRO 386s/20
5. SONY MARVIGRAPH UP-3000 COLOUR VIDEO PRINTER
6. SONY TRINITRON COLOUR MONITOR

Fig.8
Diagram of the video-enhanced microscopy system configuration employed to visualize variations in fluorescence intensity in resting and stimulated chromaffin cells.

Pro 386s/20 personal computer equipped with Argus-50 version 3.0 software for Windows.

C.2. Computer analysis of data and graphic representation

Analysis of fluorescent intensity of preparations utilizing video-enhanced microscopy was conducted employing a three-dimensional display option furnished by the Argus program. This function transforms a two-dimensional distribution of the intensities of a chromaffin cell displayed on the monitor and designated by a window into a three-dimensional graphic representation. The three-dimensional representation may be viewed in four distinct directions and at four levels of resolution with respect to the intensity of the sample. The coordinates of the surface of a cell of interest are represented on the X and Y axes and the intensity of fluorescence of the chromaffin cell on the Z axis. The output from the image processor was displayed on a Sony 3300 colour monitor and subsequently printed with a Sony Marvigraph UP-3000 Colour Video Printer. Images of cells printed in pseudocolour were then analyzed according to fluorescence intensity.

D. CATECHOLAMINE RELEASE STUDIES

D.1. Catecholamine output assay

Catecholamine output was determined utilizing the method described

previously by Trifaró and Lee (1980). As described in the afore cited reference chromaffin cells which are loaded with [^{3}H]noradrenaline ([^{3}H]NA) under carefully controlled conditions (Kenigsberg and Trifaró, 1980) exhibit concomitant and parallel release of endogenous catecholamines and [^{3}H]NA labelled amines upon stimulation by secretagogues (Trifaró and Lee, 1980; Trifaró and Bourne, 1981). Briefly, chromaffin cells were washed three times in special medium (in millimolar: NaCl, 110; $NaHCO_3$, 40; KCl, 5; $MgSO_4$,1; NaH_2PO_4,1; Na-pyruvate, 1; $CaCl_2$, 2; $Fe(NO_3)_3$, $2.5x10^{-4}$; ascorbic acid, 0.1; pH 7.2 adjusted with Hepes) and their catecholamine stores labelled by incubating each culture dish with 1ml of special medium containing 10^{-7}M [^{3}H]NA (sp. act, 56.9 Ci/mmol, New England Nuclear, Boston, MA) for 5 min at room temperature. Following the labelling of catecholamine stores each dish was rinsed with 1ml of regular Locke's solution six times with each wash having a duration of 10 min for a total of 60 min prior to initiation of the experimental phase. The cells were then incubated with Locke's solution alone (control) or exposed to Locke's buffer containing the treatments prescribed for the individual experiments. The incubation medium of each dish was collected at different time intervals and radioactivity of the sample determined. [^{3}H]NA cell content was determined by treating each culture dish with 1ml of 10% Trichloroacetic acid (TCA) for 10 min followed by two 0.5ml volumes of 6% TCA. The three aliquots were then combined and 5 ml of Cytoscinct scintillation fluid obtained from ICN Biochemicals Inc., Irvine, CA was added.

Radioactivity was determined using a liquid scintillation spectrometer (Beckman Instruments Fullerton, CA).

D.2. Data analysis

In order to ascertain total [^{3}H]NA cell content, the amount released into the medium was added to the amount of [^{3}H]NA extracted with TCA. Percentage of catecholamine output was obtained by dividing the amount released into the medium by the total [^{3}H]NA content. The sensitivity of this protocol was such that it allowed for the determination of an amount of catecholamine released corresponding to 0.35% of total catecholamine content (Vitale et al., 1991). A minimum of 4 culture dishes were used per condition tested and the mean ± S.E.M of values obtained was plotted.

E. CYCLIC AMP DETERMINATION

E.1. Measurement of intracellular cAMP

Cultured chromaffin cells were rinsed three times with regular Locke's solution and exposed to the diverse drug treatments described by each experiment. Once the experiment was completed, the cells were treated with acid ethanol (1ml 1M HCl: 100ml ethanol) at room temperature for 5 min. After acid ethanol treatment the cells were scraped off of the Petri dish surface and each culture dish was rinsed to ensure removal of cells. The samples were

then placed on ice and sonicated for three successive periods of 5-10 s, each at 4°C. Subsequent to sonication, samples were centrifuged at the same temperature in an Eppendorf microcentrifuge for 2 min. The supernatant was then collected and the pellet was washed with 1ml of ethanol/water (2:1; vol:vol). The wash was then added to the supernatant previously collected and each sample was evaporated under a nitrogen stream at 55°C utilizing a Reacti-Vap III evaporator (Pierce; Rockford, IL.). Once evaporated, each sample was re-suspended in 200μl of assay buffer containing 4mM EDTA and 0.05M Tris at pH 7.5 and vortexed until dissolved. A [^{3}H] cAMP assay kit (Amersham Canada Limited) was then employed to determine cellular cAMP content as follows. Fifty microliters were removed from each sample containing an unknown amount of cAMP and from prepared standard solutions containing 1, 2, 4, 8 and 16 picomoles of unlabelled cAMP for assay purposes and to this, the following solutions were added in sequential order:

1) 50μl of [8-^{3}H] Adenosine 3', 5'-cyclic phosphate.

2) 100μl of binding protein, purified from bovine muscle.

Two distinct tubes containing 150μl of assay buffer and 50μl of [8-^{3}H] Adenosine 3', 5'-cyclic phosphate were employed to ascertain blank radioactive counts for the assay followed by an additional two tubes containing 150μl of assay buffer and the amount of the compounds specified above to determine binding in the absence of unlabelled cAMP. The total counts for the assay were determined by including two assay tubes containing 50μl [8-^{3}H]

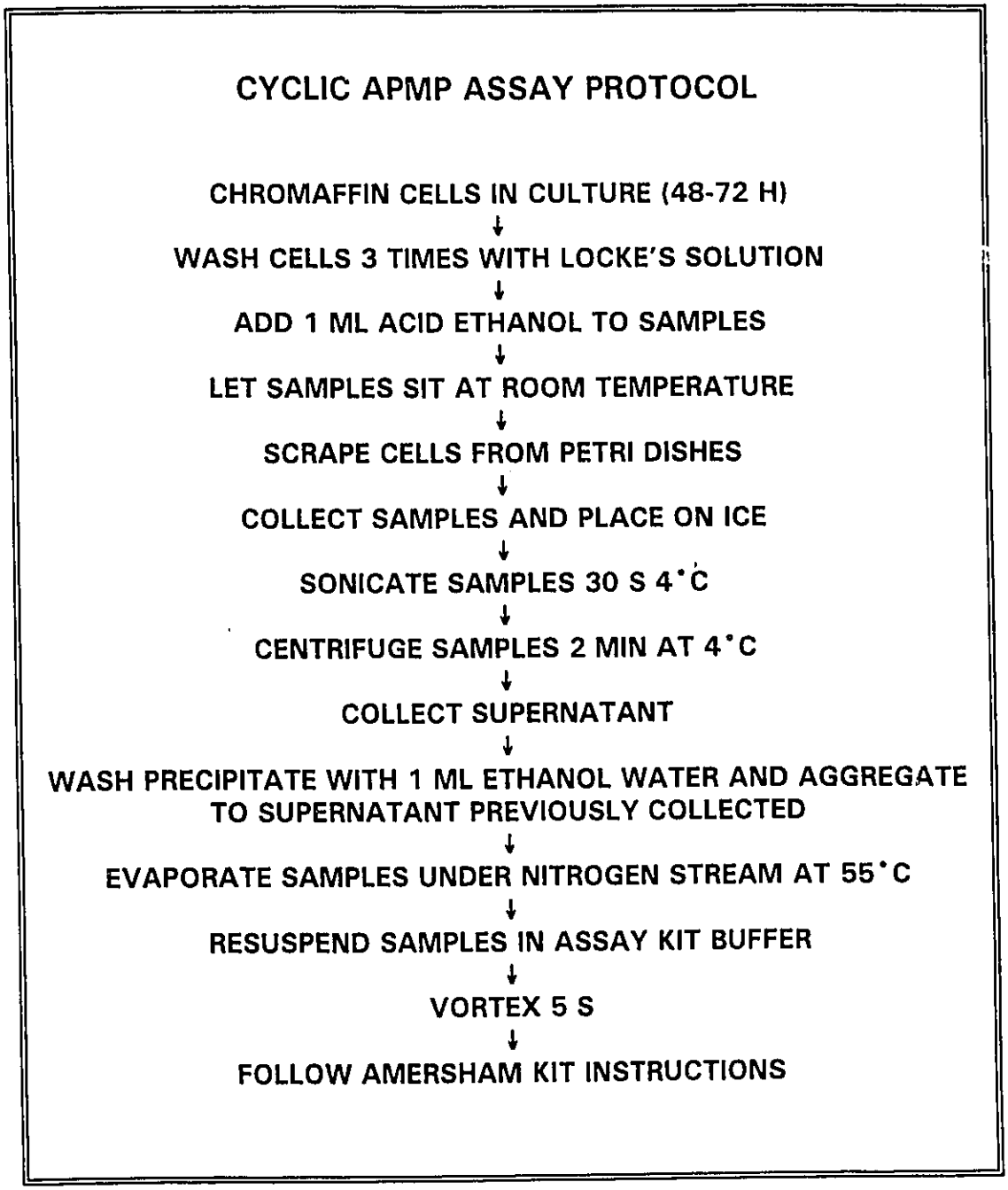


Fig.9
Schematic representation of the methodology utilized in the preparation of samples for determination of intracellular cAMP content by radioimmuno assay.

Adenosine 3', 5'-cyclic phosphate and 250μl of the assay buffer. The samples were vortexed 5 s and then allowed to incubate at 4°C for 2h. At the end of the incubation period 100μl of cold absorbent charcoal suspension was added to each sample in order separate protein bound cAMP from unbound nucleotides. Individual samples were then vortexed 10 s and centrifuged twice at 4°C to ensure removal of charcoal containing free nucleotide (Brown et al.,1971). Without disturbing the charcoal sediment, 200μl of clear liquid was then removed from each sample and the radioactivity determined in a liquid scintillation spectrometer. Samples containing standard solutions of unlabelled cAMP were analyzed in duplicate and samples containing unknown amounts of cAMP were in most cases examined in triplicates and quadruplets.

E.2. Data analysis

To obtain the cpm bound in the presence of unknown (sample) or standard unlabelled cAMP (C_x), the radioactive blank counts/min (cpm) were subtracted from the individual cpm obtained. The average of the two tubes representing binding in the absence of unlabelled nucleotide was obtained, and the blank subtracted from this value to yield the cpm bound in the absence of unlabelled cAMP (C_o). This value (C_o) was the divided by each C_x in order to obtain the C_o/C_x. The value of C_o/C_x obtained for each sample was then compared to a standard curve and the corresponding picomole value for each

C_o/C_x was obtained. A typical standard curve is depicted in Fig.10. The mean picomoles ± S.E.M of a minimum of three and in most cases four determinations per condition was represented graphically. The Amersham cAMP assay kit provides a specific method for the determination of cAMP in the range of 0.2-16 picomoles per sample with a maximum precision in the range of 0.5-8 picomoles. All samples examined were within the range of maximum sensitivity for the assay with intracellular cAMP levels in control chromaffin cells being 1.808 ± 0.122 picomoles/10^6 cells (n=39, 9 cell culture preparations). Although this average value of intracellular cAMP content in control chromaffin cells is within the range of values reported by others (Cheek and Burgoyne, 1987; Wilson, 1988), comparison to all values cited in the literature is difficult because of discrepancies which exist in experimental protocols and measurement techniques.

F. MATERIALS

Products employed for the culture of bovine adrenal chromaffin cells and to conduct experiments were obtained from the following sources: Earl's Balanced Salt Solution, Dulbecco's modified Eagle's medium, penicillin stock and streptomycin stock were purchased from GIBCO (Burlington, ON, Canada). Nystatin stock, gentamycin, 5-fluorodeoxyuridine, cytosar, collagenase, 6-acetyl-7-deacetylforskolin, deacetylforskolin, 8-Bromo cAMP (sodium salt), Forskolin, FITC-IgG and Nicotine (Hydrogen tartrate salt) were obtained from

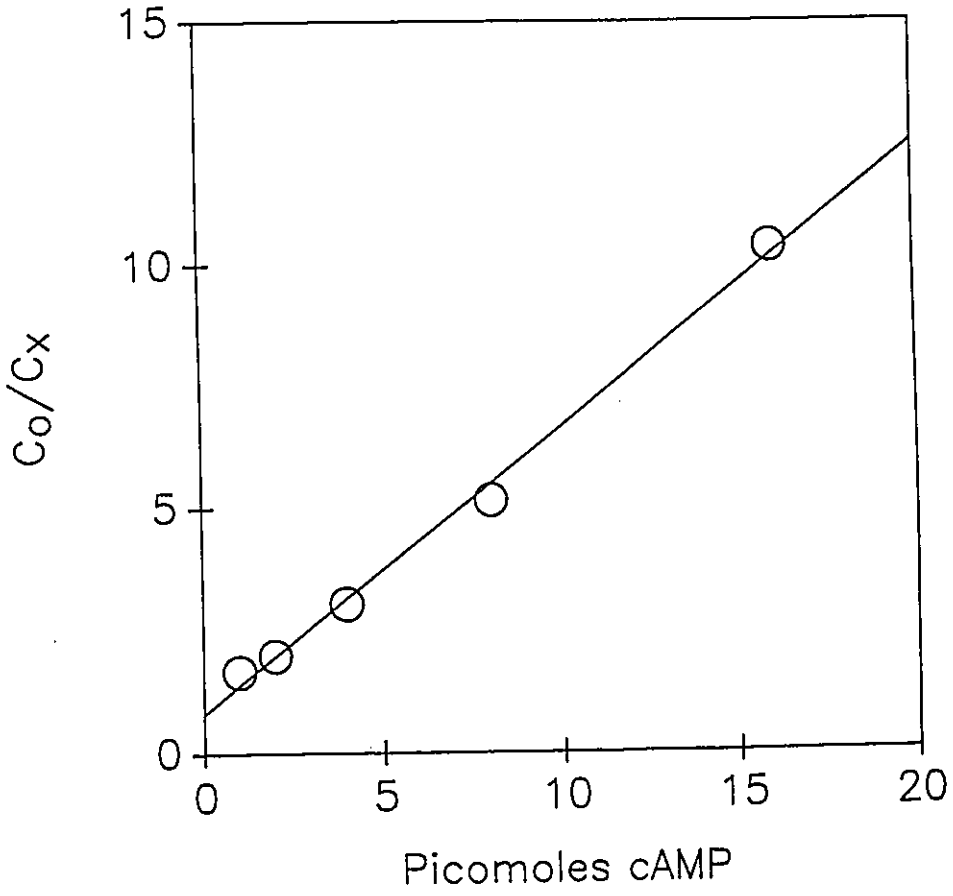


Fig.10
Typical standard curve obtained and used in the analysis of cAMP levels employing radioimmuno assay. C_o represents cpm bound in the absence of unlabelled cAMP. C_x represents cpm bound in the presence of an unknown (sample) or standard amount of unlabelled cAMP.

Sigma Chemical company (St.Louis, MO, U.S.A.). DNAse I Type I was obtained from Boehringer (Laval, Quebec, Canada). Culture dishes were purchased from Corning (Kirkland, Quebec, Canada). Rhodamine-labelled phalloidin was purchased from Molecular probes, Inc. (Eugene, OR, U.S.A.).

CHAPTER III

RESULTS

A. IMMUNOHISTOCHEMICAL ANALYSIS AND VIDEO-ENHANCED MICROSCOPY OF SCINDERIN AND F-ACTIN CORTICAL FLUORESCENCE IN RESTING AND NICOTINE STIMULATED CELLS

The existence of an actin network located beneath the plasma membrane of the chromaffin cell which opposes the movement of secretory vesicles to exocytotic sites has been described (Burgoyne et al., 1989; Trifaró et al., 1989b). Immunohistochemical and video-enhanced microscopy studies in chromaffin cells cultured for 48 h and stained with scinderin antiserum #6 followed by FITC-IgG and rhodamine-labelled phalloidin, a heptapeptide which binds preferentially to filamentous actin (F-actin) and not monomeric actin (G-actin) permit the scinderin and the actin network to be visualized. In cells incubated only in Locke's solution (resting cells) F-actin (a) and scinderin (a') (Fig.11, 0s) display an analogous pattern in individual cells characterized by diffuse cytoplasmic staining and a bright cortical fluorescent ring. Measurement of fluorescent intensity utilizing video-enhanced microscopy in these cells demonstrates that both F-actin and scinderin under resting conditions (Fig.12a, a') display a high level of continuous fluorescence in the cortical region of the cell in agreement with previous findings obtained from immunohistochemical studies conducted in this laboratory (Vitale et al., 1991). Upon stimulation of chromaffin cell cholinergic receptors for 40 s with 10μM nicotine, scinderin and F-actin cortical fluorescent rings become fragmented and the cell exhibits "patchy" F-actin (b) and scinderin (b') (Fig.11, 40s)

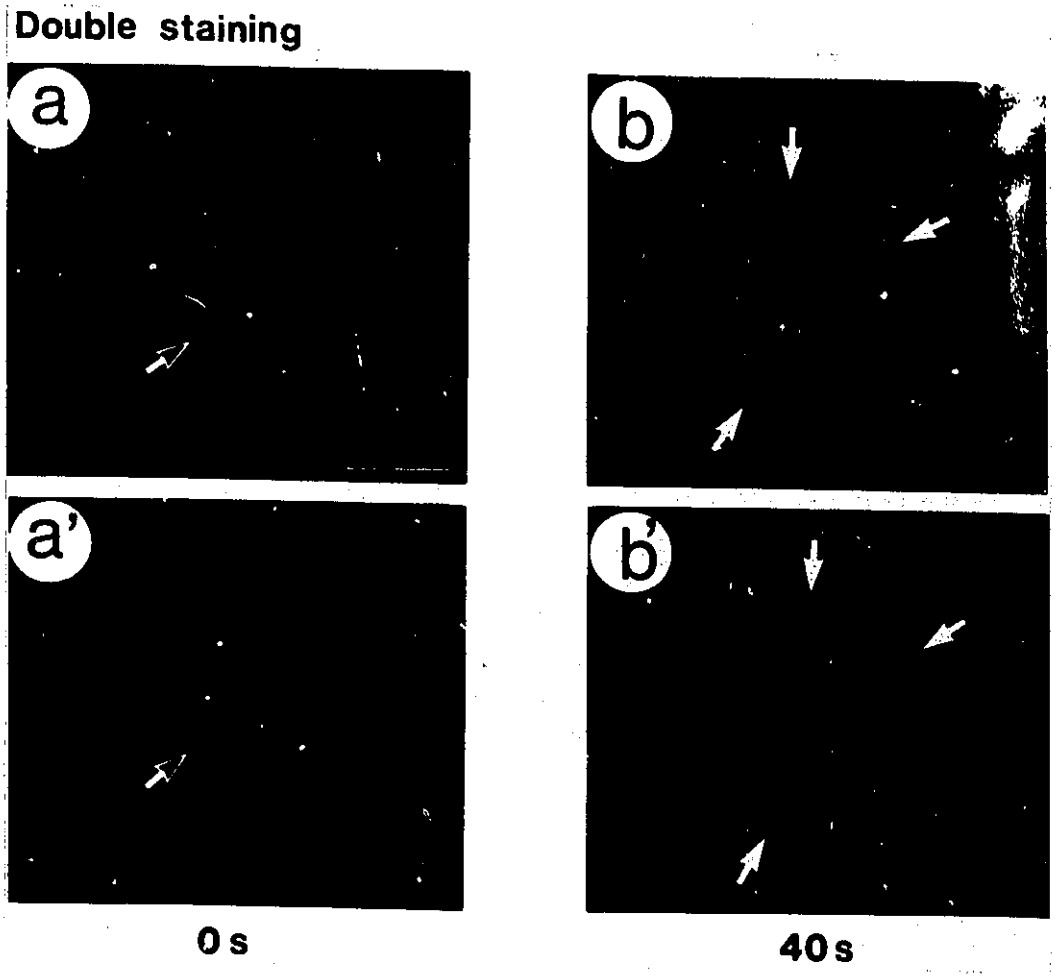


Fig.11
Colour photographs of double-staining for cortical scinderin (a',b') and actin (a,b) fluorescence in control (left, 0s incubation with nicotine) and nicotine-stimulated (right, 40s incubation with 10μM nicotine) chromaffin cells.

fluorescence. This loss of fluorescence which denotes cortical scinderin redistribution and partial disassembly of the cortical F-actin network, coincides with a decrease in fluorescent intensity such that areas devoid of F-actin (Fig.12b, 40s) and scinderin (Fig.12b', 40s) are depicted as valleys and only remaining cortical fluorescent segments of the cells show fluorescent intensity which is similar to cortical areas in control cells.

B. INHIBITION BY 8-BROMO CYCLIC AMP OF NICOTINE-EVOKED SCINDERIN REDISTRIBUTION, F-ACTIN DISASSEMBLY AND CATECHOLAMINE OUTPUT

It is well known that the second messenger, cAMP plays an important role in the regulation of hormonal activity, enzymes and other cellular processes in physiological systems (Sutherland et al., 1968; Jost and Rickenburg, 1971; Robinson et al., 1971) and elicits its biological effects as a result, of its ability to activate protein kinases (Stryer, 1988).

In order to study the modulatory effects of the second messenger, cAMP on nicotine-induced scinderin redistribution, F-actin disassembly and catecholamine output, 8Br-cAMP, an analog of cAMP which can permeate the plasma membrane was tested. First, fluorescence microscopy was utilized in order to examine the effect of the cAMP analog on scinderin and F-actin staining. Chromaffin cells were incubated with scinderin antiserum #6 followed by FITC anti-rabbit IgG and rhodamine-labelled phalloidin as described in the methods.

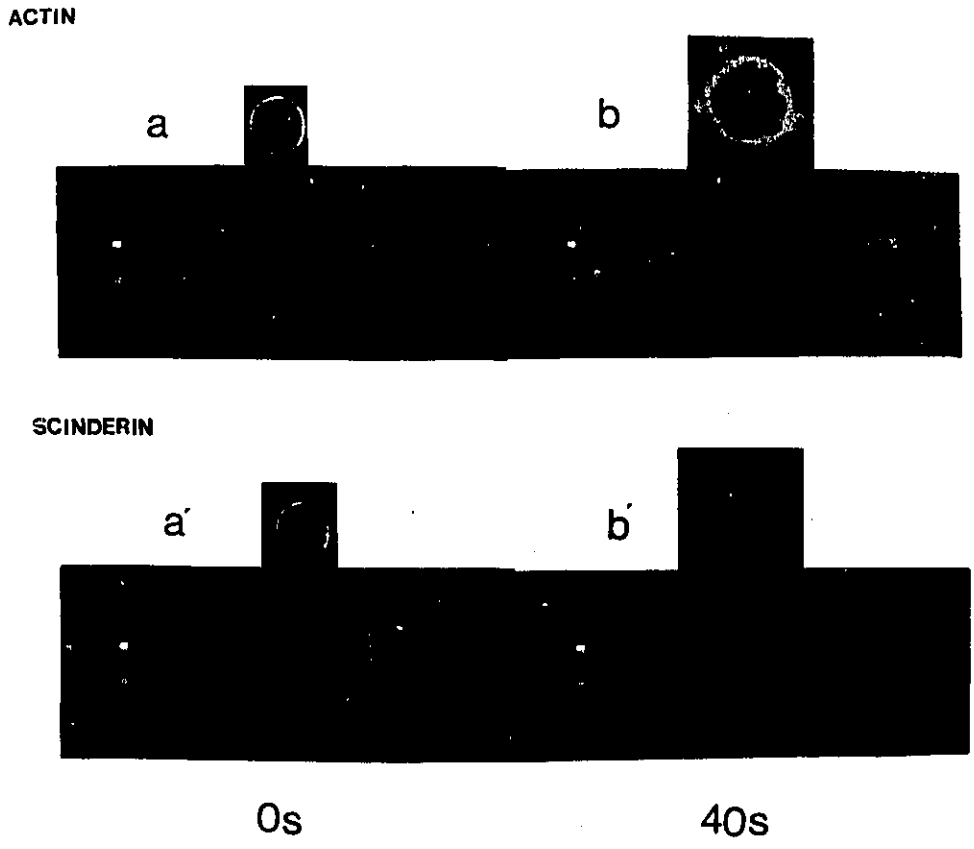


Fig.12
Analysis of variations in fluorescence intensity of cortical actin (a,b) and scinderin (a',b') in the control (0s) and nicotine-stimulated (40s) chromaffin cells depicted in Fig.11.

In accordance with previous results obtained in our laboratory (Vitale et al., 1991), control cells (Fig.13B) displayed a minimal percentage of cells with cortical scinderin redistribution and F-actin disassembly (13.25 ± 3.9 and 14.4 ± 3.15 respectively, n = 400-800 cells). Incubation of the cells with Locke's solution containing 8Br-cAMP for 40 s also did not elicit redistribution of scinderin or disassembly of F-actin (11.0 ± 2.63 and 11.2 ± 1.98, n = 400-800 cells). As expected, stimulation of the cells with nicotine elicited a dramatic increase in both the percentage of cells showing scinderin redistribution and F-actin disassembly (83.8 ± 3.74 and 81 ± 4.15, n = 400-800 cells). When 2.5mM 8Br-cAMP was present in the medium during nicotinic stimulation there was a significant inhibition of nicotine-mediated effects (Fig.13B, 42 ± 3.83 and 41.2 ± 3.03, n = 400-800 cells).

Earlier work conducted in our laboratory has demonstrated that scinderin redistribution and F-actin disassembly precede chromaffin cell secretion (Vitale et al., 1991) thus, one would expect that inhibition of nicotine-induced scinderin redistribution and F-actin disassembly would accompany suppression of nicotine-induced catecholamine secretion. In order to investigate this possibility catecholamine release studies were conducted following the same protocol. The findings depicted in Figure 13A indicate that in resting cells exposed to 8Br-cAMP only limited amounts of secretion occur and this release was not different from controls. This result correlates with the fact that in these cells the percentage of scinderin redistribution and F-actin disassembly

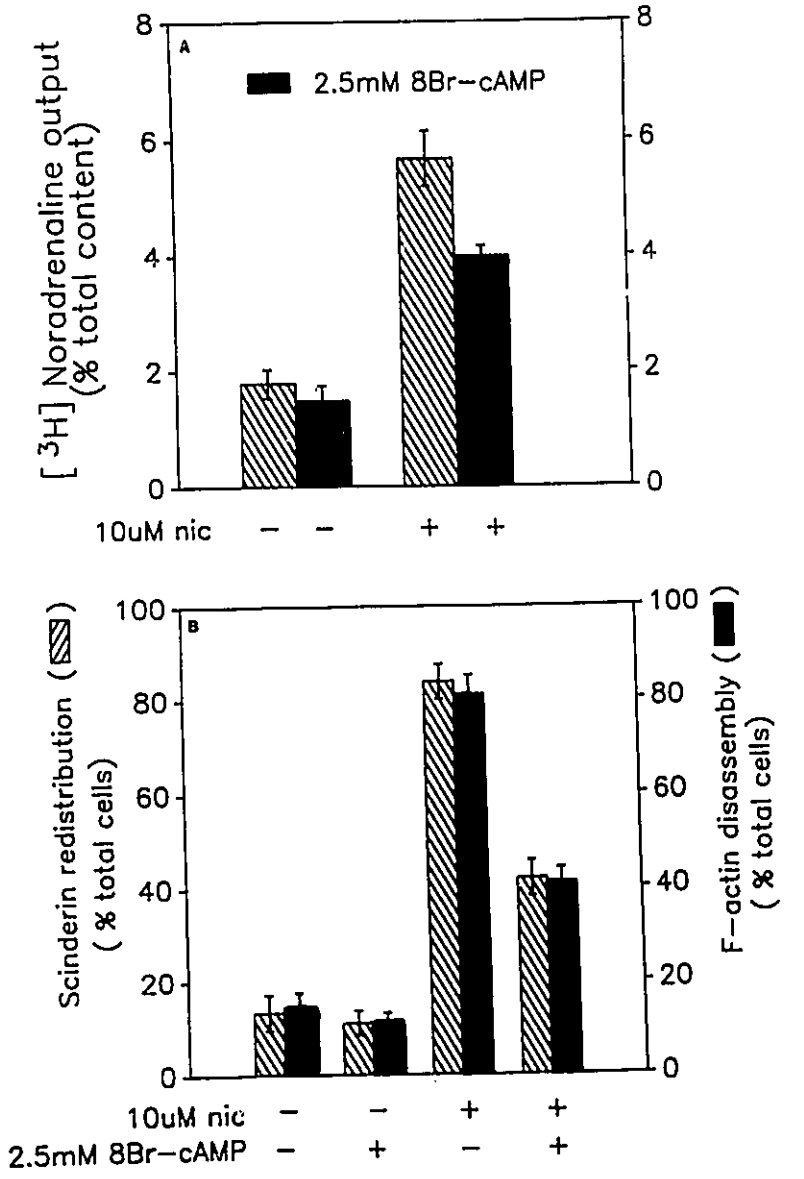


Fig.13
[^{3}H] Noradrenaline output (A), scinderin redistribution and F-actin disassembly (B) in resting and nicotine-stimulated cells exposed to 2.5mM 8Br-cAMP for 40 s. Each value represents the mean ± S.E.M. of results from 4-8 coverslips per condition (n = 400-800 cells examined) and 2 cell preparations.

are similar to that observed in cells incubated with Locke's solution alone Fig.11,Os. Stimulation of the cells with nicotine as expected, induced an increase in catecholamine secretion which was directly consistent with the observed augmentation in the percentage of cells showing scinderin redistribution and fragmentation of the cortical F-actin network. Moreover, as in the case of nicotine-evoked scinderin redistribution and cortical F-actin disassembly, catecholamine secretion in response to nicotine was also partially inhibited (52.3%) by exposure of to 2.5mM 8Br-cAMP during the stimulation period (Fig.13A).

C. INTRACELLULAR CYCLIC AMP CONTENT IN CHROMAFFIN CELLS EXPOSED TO INCREASING CONCENTRATIONS OF FORSKOLIN

On the basis the above results which provided evidence indicating that cAMP could possibly be modulating scinderin redistribution, F-actin disassembly and catecholamine secretion in response to nicotinic stimulation other compounds known to increase endogenous cAMP levels were employed and their impact on these parameters examined. The first such compound to be tested in these studies was forskolin, a cardioactive diterpene obtained from the methanolic extracts from the roots of the plant Coleus forskohlii which increases cAMP by activating adenylate cyclase (Seamon and Daly, 1986). In order to establish the concentration of forskolin which would effectively increase intracellular cAMP levels, chromaffin cells were first exposed to 10,

20, 35 and 50μM forskolin for 40 s and intracellular cAMP content measured by radioimmunoassay as indicated in the methods section. Cyclic AMP in chromaffin cells increased in a dcse-dependent manner upon incubation of cells with increasing concentrations of forskolin (Fig.14). Furthermore, although low doses of the compound were found to produce small but, albeit significant increases in intracellular cAMP levels, 50μM forskolin was selected as the concentration to be utilized in our studies to ensure that nucleotide content was sufficiently elevated in the cells.

D. EFFECT OF FORSKOLIN ON NICOTINE-EVOKED SCINDERIN REDISTRIBUTION, F-ACTIN DISASSEMBLY AND CATECHOLAMINE OUTPUT

Cultured chromaffin cells were incubated with regular Locke's solution alone or containing 10μM nicotine in the presence or absence of 50μM forskolin for 40 s. The cells were then fixed in formaldehyde and processed for double staining fluorescence microscopy as described in the methods section. Examination of both F-actin and scinderin fluorescence (Fig.15) demonstrated that resting cells either in the absence (a, a') or presence of forskolin (b, b') exhibited a continuous cortical fluorescent ring.

On the other hand, nicotine-induced F-actin disassembly and scinderin redistribution (discontinuous cortical fluorescent ring, d, d') were inhibited in the presence of 50μM forskolin (c, c'). These findings were reinforced by results obtained with analysis of fluorescence intensity utilizing video-enhanced

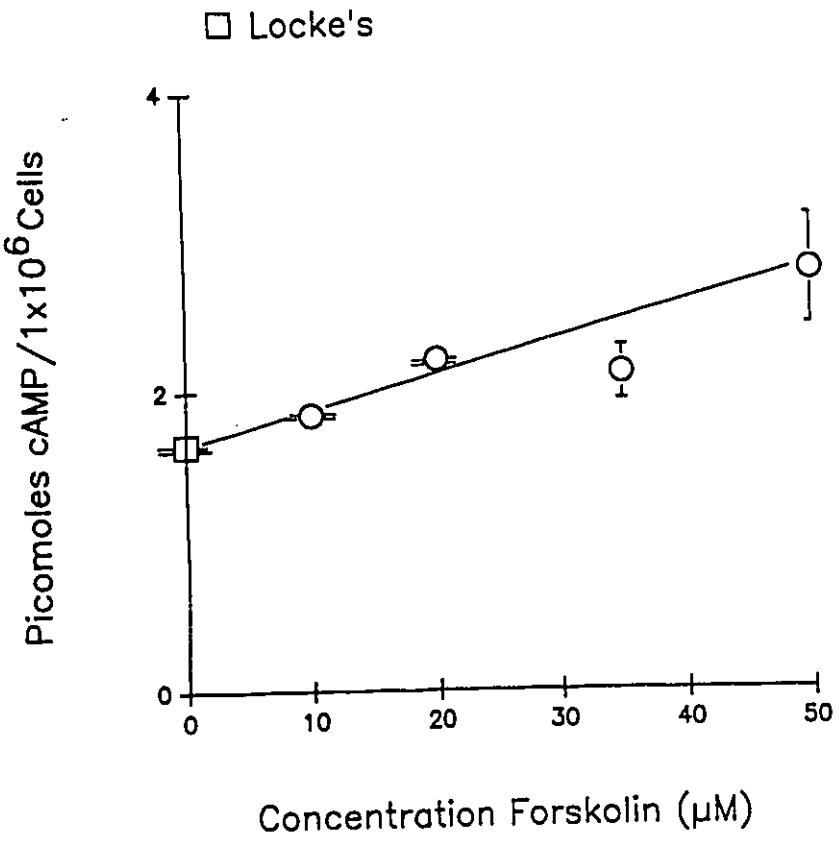


Fig.14
Intracellular levels of cAMP in unstimulated (resting) cultured (48-72 h) chromaffin cells incubated with Locke's solution in the absence (control, □) or presence (○) of increasing concentrations of forskolin. Cells were incubated with 10,20,35 and 50μM forskolin for 40 s and intracellular cAMP content measured by a radioimmuno assay (see methods). Each point represents the mean ± S.E.M. of at least n = 4 culture dishes and a minimum of 2 cell culture preparations.

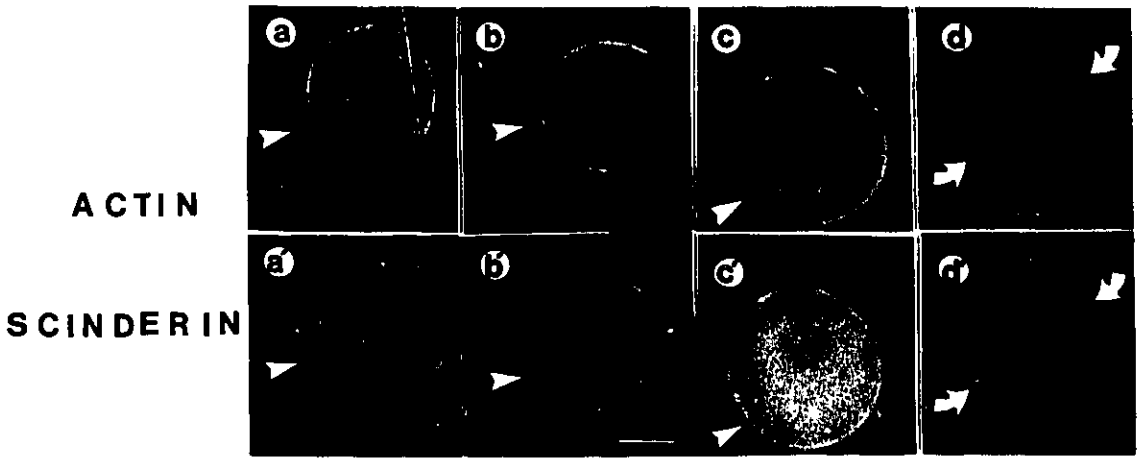


Fig.15
Actin and scinderin cortical fluorescence in chromaffin cells subjected to treatment with Locke's (a, a'), 50μM forskolin (b, b') and 10μM nicotine in the presence of 50μM forskolin (c, c') or 10μM nicotine (d,d') for 40s.

microscopy (Fig.16). Control cells in the absence (Fig.16a, a') and presence (Fig.16b, b') of forskolin exhibited continuous and usual resting levels of fluorescence intensity in the cortical region of the cell indicating that subplasmalemmal F-actin disassembly and scinderin redistribution had not been induced by forskolin. Stimulation of the cell with nicotine (Fig.16d, d') in contrast, produced a decrease in fluorescence in the cortical region of the cell. This was consistent with both cortical F-actin disassembly and scinderin redistribution. Both F-actin disassembly and scinderin redistribution in response to nicotine were inhibited by the presence of 50μM forskolin (Fig.16c, c'). Fluorescence intensity was analogous for actin and scinderin in resting cells (Fig.16a, a') and in cells in which nicotine-induced scinderin redistribution and F-actin disassembly had been inhibited by forskolin (Fig.16c, c').

E. TIME COURSE OF CYCLIC AMP LEVELS, SCINDERIN REDISTRIBUTION, F-ACTIN DISASSEMBLY AND CATECHOLAMINE OUTPUT IN RESTING AND NICOTINE STIMULATED CELLS EXPOSED TO FORSKOLIN

Intracellular cAMP was initially measured in cells subjected to the same experimental conditions (40 s incubation period) described in the previous section. The results obtained indicate that while no significant increase in intracellular cAMP are observed in cells stimulated for 40 s with 10μM nicotine

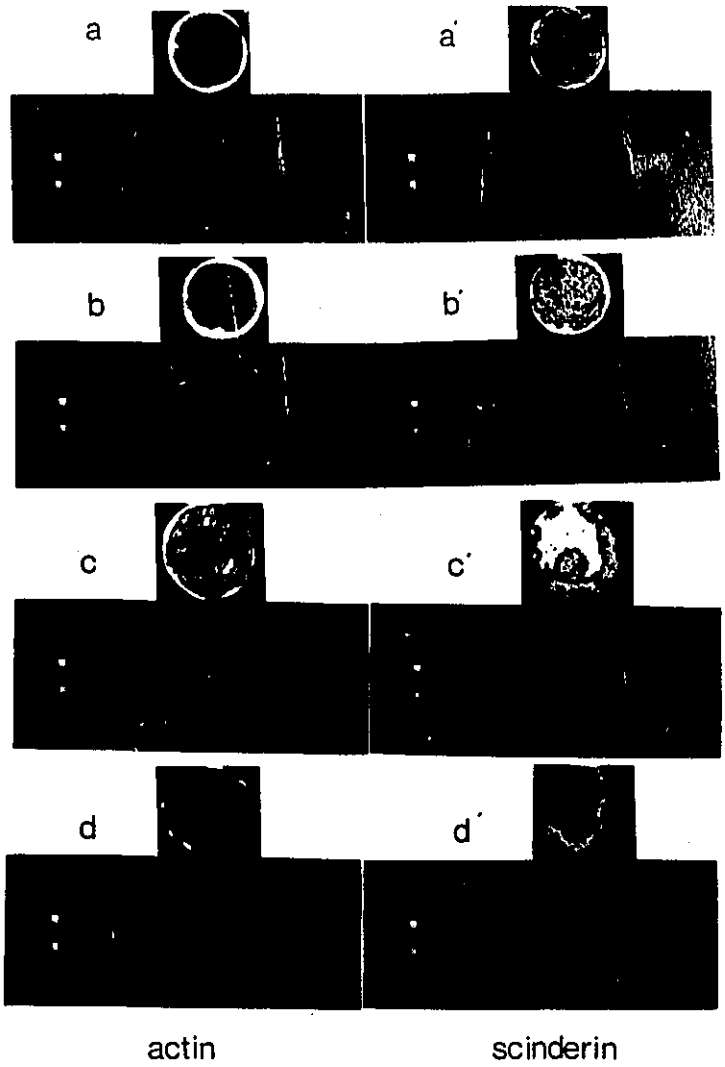


Fig.16
Analysis of variations in cortical actin and scinderin fluorescence intensity by video-enhanced microscopy in cells treated with Locke's (a, a'), 50μM forskolin (b, b') and 10μM nicotine in the presence of 50μM forskolin (c, c') and 10μM nicotine (d, d') for 40 s.

(△), cAMP levels in resting and stimulated cells exposed to 50μM forskolin for 40 sec showed comparable and significant increases in cellular cAMP levels (Fig.17, ♦, ○).

Because of forskolin-induced cAMP elevation in unstimulated and stimulated cells during the 40 s incubation period it became necessary to perform a time course study of cAMP levels in chromaffin cells exposed to forskolin under the same experimental conditions. Resting and stimulated cells were incubated with forskolin for 5, 10,20, 30 and 40 s. Furthermore, some cell populations were subjected to a 40 s incubation which was followed by a 50 s wash with Locke's solution in order to determine if removal of forskolin from the incubation medium terminated cAMP augmentation. The effect of these treatments was assessed (Fig.17) and the findings suggest that intracellular cAMP in these cells begins to increase in parallel in both resting and nicotine stimulated cells upon incubation with forskolin for 5-10 s. The observed elevation of cAMP levels continues with increasing time of incubation with forskolin and continues to augment despite removal of forskolin from the incubation medium. In order to link suppression of catecholamine output observed in time in stimulated cells incubated with forskolin with the previously observed inhibition of scinderin redistribution and F-actin disassembly, two-day old chromaffin cells were subjected to comparable conditions and fluorescence microscopy was performed. After exposure of the cells to the experimental conditions of interest, the cells were fixed with formaldehyde and chromaffin

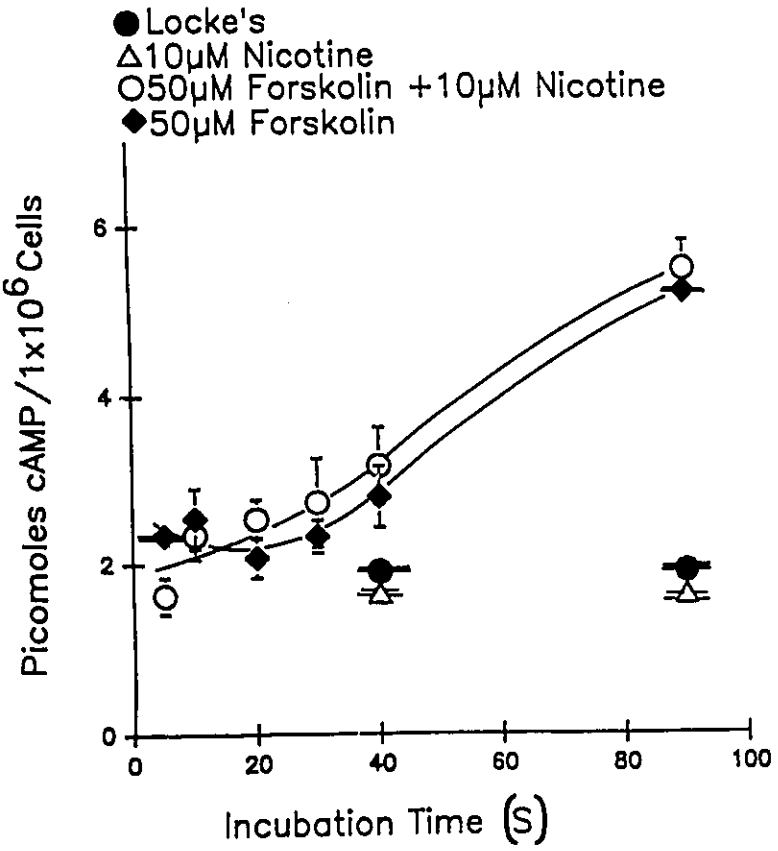


Fig.17
Time course of intracellular cAMP concentration in resting and stimulated chromaffin cells incubated with 50μM forskolin for increasing periods of time. Bovine adrenal chromaffin cells cultured for 48-72 h were incubated with Locke's solution alone (●) or containing either 50μM forskolin (◆) or 10μM nicotine in the absence (△) or presence (○) 50μM forskolin for 5,10,20,30 and 40 s as well as, for 40 s followed by a 50 s incubation with Locke's solution. Following the treatments, intracellular cAMP was determined by radioimmuno assay. Individual points represent mean ± S.E.M. of results obtained from a minimum of n=6 from 2 different cell cultures.

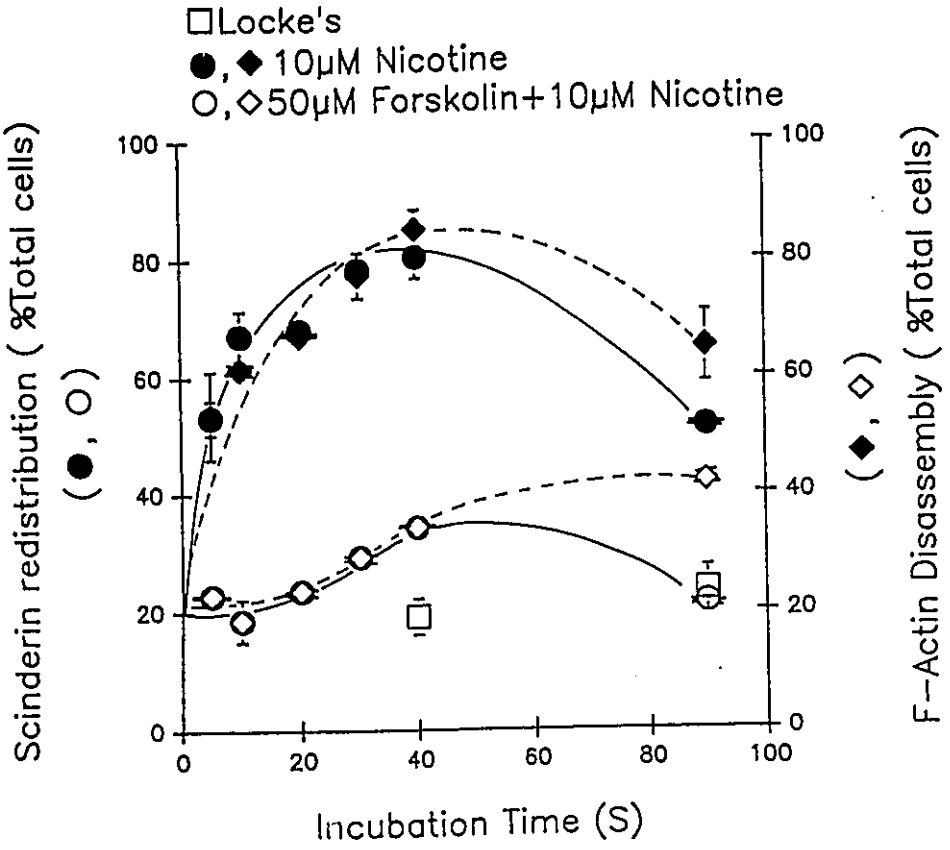


Fig.18
Time course of nicotine-evoked scinderin redistribution and F-actin disassembly in stimulated cells in the presence or absence of forskolin. Two-day old cultured chromaffin cells were incubated with Locke's solution containing 10μM nicotine alone (○, ◆) or 10μM nicotine and 50μM forskolin (○, ◇) for 5,10,20,30 and 40 s as well as, followed by incubation with Locke's solution until the end of stimulation. Furthermore, cells were also incubated with Locke's solution in the absence of all other compounds (□, control). The cells were fixed with formaldehyde, permeabilized and incubated with scinderin antiserum #6 followed by FITC anti-rabbit IgG and rhodamine-labelled phalloidin, a probe for F-actin. Chromaffin cells were then classified as exhibiting continuous or discontinuous scinderin and F-actin cortical fluorescence as per the methods section. Data points depicted represent the mean ± S.E.M. of results obtained from 400 cells examined for each condition.

cells prepared for double-staining fluorescence microscopy. Examination of the data obtained (Fig. 18, each point represents 400-800 cells) disclosed that inhibition of nicotine-evoked scinderin redistribution and F-actin disassembly occurs even as early as 5-10 s of incubation of stimulated cells with forskolin and that the time courses of inhibition of scinderin redistribution and F-actin disassembly in stimulated cells also exhibit a temporal pattern of inhibition analogous to the time course of cAMP elevation observed. Catecholamine output in cells subjected to the same experimental parameters was also examined and the findings of these studies indicate that inhibition of nicotine-evoked secretion can be observed at 20 s of incubation with forskolin and is comparable to the time course of intracellular cAMP augmentation (Fig.19). Synthesis of all the results illustrates that the temporal sequence of inhibition of both nicotine-evoked scinderin redistribution, F-actin disassembly and catecholamine release (Figs. 18, 19 and 20) follows the time course of intracellular cAMP augmentation (Fig.17) and that inhibition of scinderin redistribution and F-actin disassembly precedes suppression of catecholamine secretion. The results would comply with a model in which cAMP increases induce suppression of catecholamine output in response to nicotinic stimulation by modulation of scinderin redistribution and hence, F-actin disassembly. Prior results obtained in our laboratory have demonstrated that rate recovery of scinderin cortical fluorescence upon removal of 10μM nicotine from the incubation medium after 40 s is much quicker than that of cortical F-actin

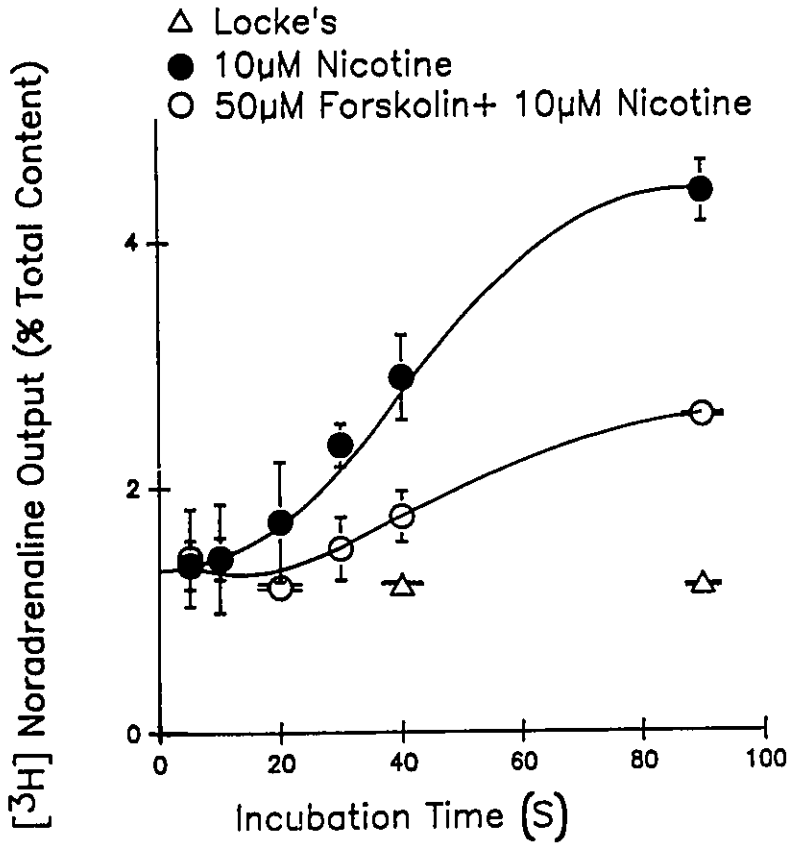


Fig.19
Time course of nicotine-evoked [^{3}H] noradrenaline output from 3 day old cultured chromaffin cells subjected to treatment with forskolin. Cells were exposed to 10μM nicotine (●) or 10μM nicotine in the presence of 50μM forskolin (○) for 5,10,20,30 and 40 s followed by 50 s incubation with Locke's solution. Basal catecholamine output was determined by incubation of the cells with Locke's solution. Catecholamine output was then determined as indicated in the methods section. Each individual point represents the mean ± S.E.M of results obtained from at least n = 8 culture dishes from 4 different cell cultures.

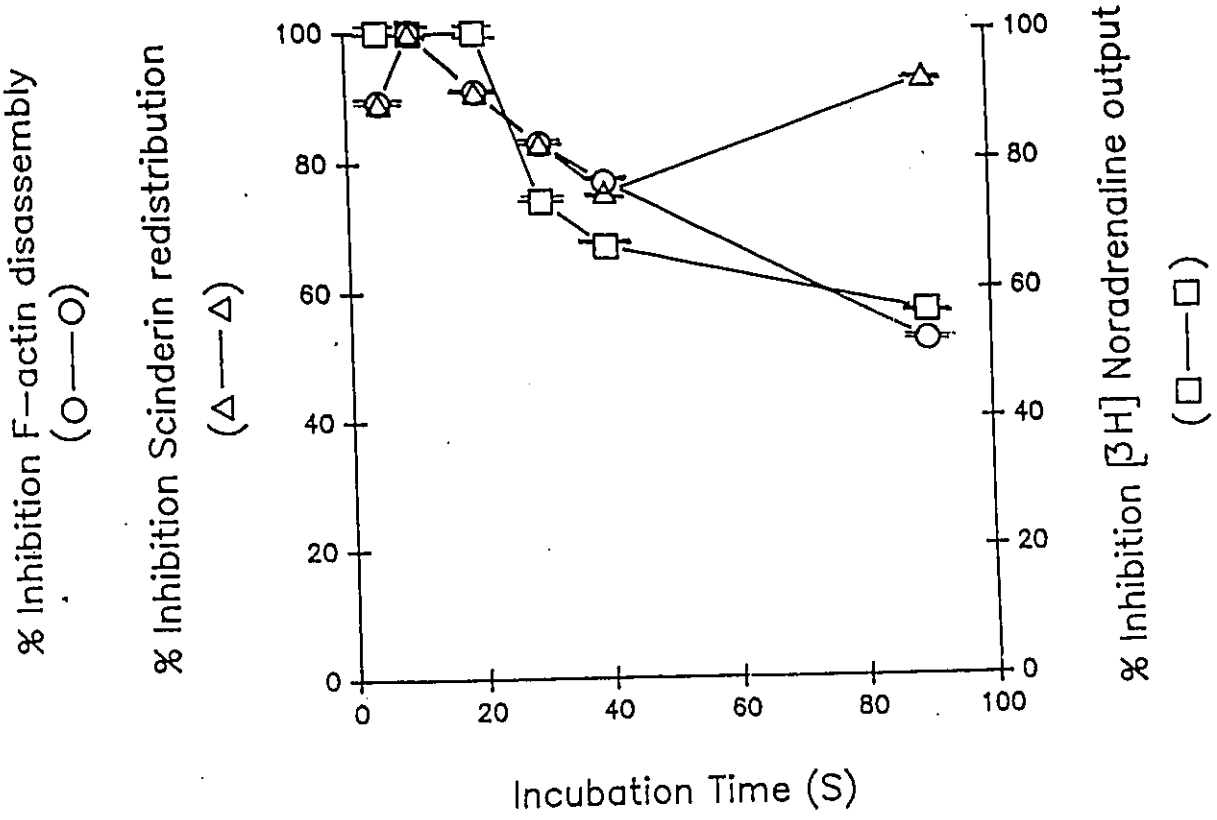


Fig.20
Time course of forskolin-induced inhibition of scinderin redistribution, F-actin disassembly and catecholamine output in chromaffin cells subjected to the same experimental conditions delineated in the legends to Figs.18,19. Briefly, to obtain this plot basal values (Locke's solution) were subtracted from maximum values obtained upon stimulation with 10 μM nicotine at each time and the differences were considered as 100%. The values obtained in cells treated with 10 μM nicotine and 50 μM forskolin corrected for basal values were then expressed as a percentage of the corresponding maximum value.

(Vitale et al., 1991). Recent work conducted in our laboratory suggests that the observed difference in the rates of recovery of scinderin and F-actin upon removal of the stimulus from the incubation milieu is due to binding of cortical scinderin to plasma membrane phospholipids such as, phosphatidylserine and phosphatidylinositol 4,5,-bisphosphate (Rodriguez Del Castillo et al., 1992). This phenomenon was not attenuated by presence of forskolin in the incubation medium (Fig.18) and may serve to explain why forskolin induced inhibition of scinderin redistribution is terminated faster than forskolin-induced inhibition of F-actin disassembly and catecholamine output (Fig.20).

F. DOSE-DEPENDENT INHIBITION BY FORSKOLIN OF SCINDERIN REDISTRIBUTION, F-ACTIN DISASSEMBLY AND CATECHOLAMINE RELEASE IN NICOTINE-STIMULATED CELLS

Cells were incubated with Locke's in the absence (control) or presence of 10 μM nicotine containing either 10,20,35, or 50 μM forskolin for 40 s and were then processed for scinderin redistribution and F-actin disassembly and catecholamine output assays. The data illustrate (Fig.21) that there is a parallel inhibition of nicotine-evoked scinderin redistribution, F-actin disassembly and catecholamine secretion in the presence of increasing concentrations of forskolin. It should be noted that the inhibitory effect was produced even at doses of forskolin (10 μM) which induced only small but yet, significant increase in intracellular cAMP levels (see Fig.14).

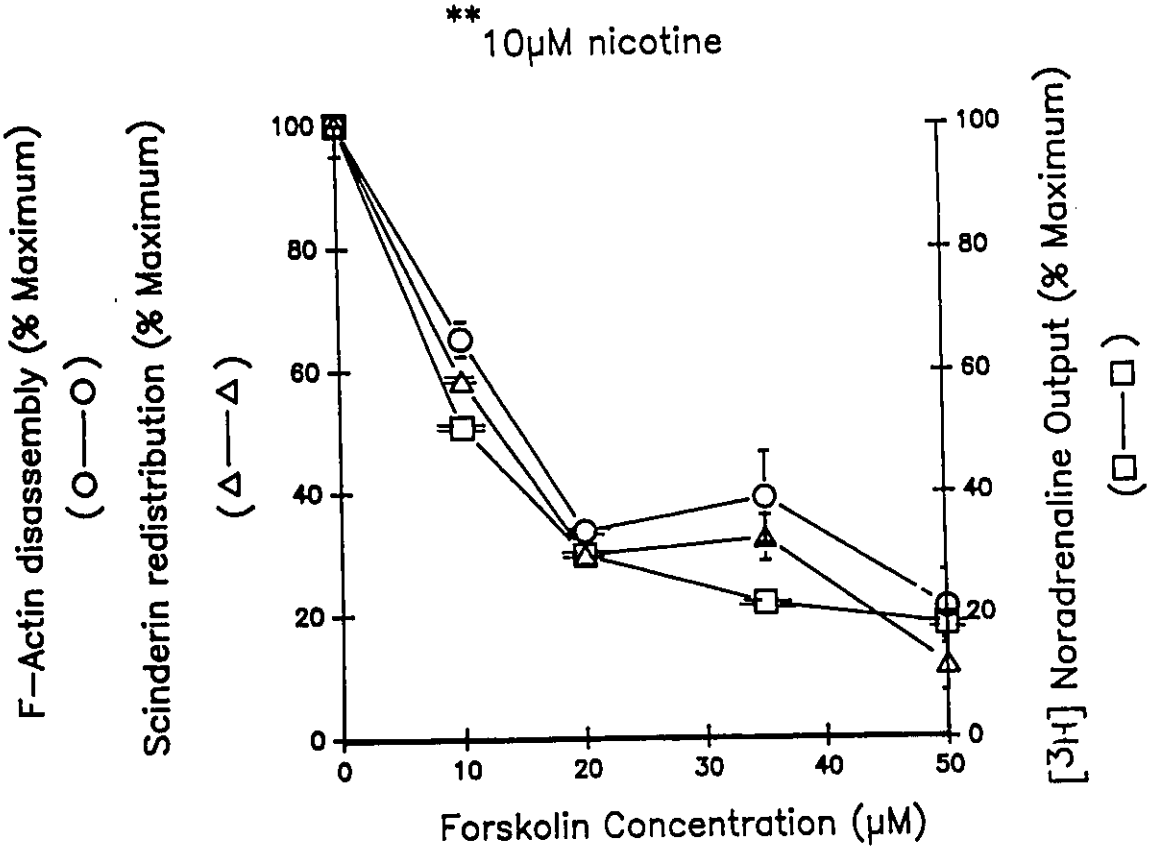


Fig.21
Dose-dependent inhibition of scinderin redistribution, F-actin disassembly and [^{3}H]-NA output in nicotine stimulated cells in the presence of increasing concentrations of forskolin (10,20,35, or 50μM) for 40 s. Each data point depicted represents the mean ± S.E.M. of results obtained from n = 300-700 cells for fluorescence microscopy studies and the mean ± S.E.M. results obtain in n = 4-8 culture dishes from 2 different cell cultures for [^{3}H]-NA output studies.

G. EFFECTS OF FORSKOLIN ANALOGS ON cAMP LEVELS, SCINDERIN REDISTRIBUTION, F-ACTIN DISASSEMBLY AND CATECHOLAMINE SECRETION IN RESPONSE TO NICOTINE

The forskolin analogs, 6-acetyl-7-deacetylforskolin and deacetylforskolin also increase cAMP levels. However, they are much less potent with respect to their ability to stimulate adenylate cyclase than forskolin (Seamon and Daly, 1986). The decreased potency of the analogs is also shown in the present results which demonstrate that a 100μM concentration of the afore mentioned analogs elevates cAMP levels to levels comparable to those observed in cells treated with 50μM forskolin . In order to investigate if these analogs, like forskolin could inhibit scinderin redistribution, F-actin disassembly and catecholamine output, cells were incubated with Locke's solution in the absence (control) or presence of 10 μM nicotine alone or containing either 100 μM 6-acetyl-7-deacetylforskolin or 100 μM deacetylforskolin for 40 s. The findings suggest that both of these analogs induced inhibition of nicotine-evoked scinderin redistribution, cortical F-actin fragmentation (Fig.22) and catecholamine secretion (Fig.23). As in the case of forskolin, this inhibition seems to be the result of increases in cellular cAMP levels (Fig.24).

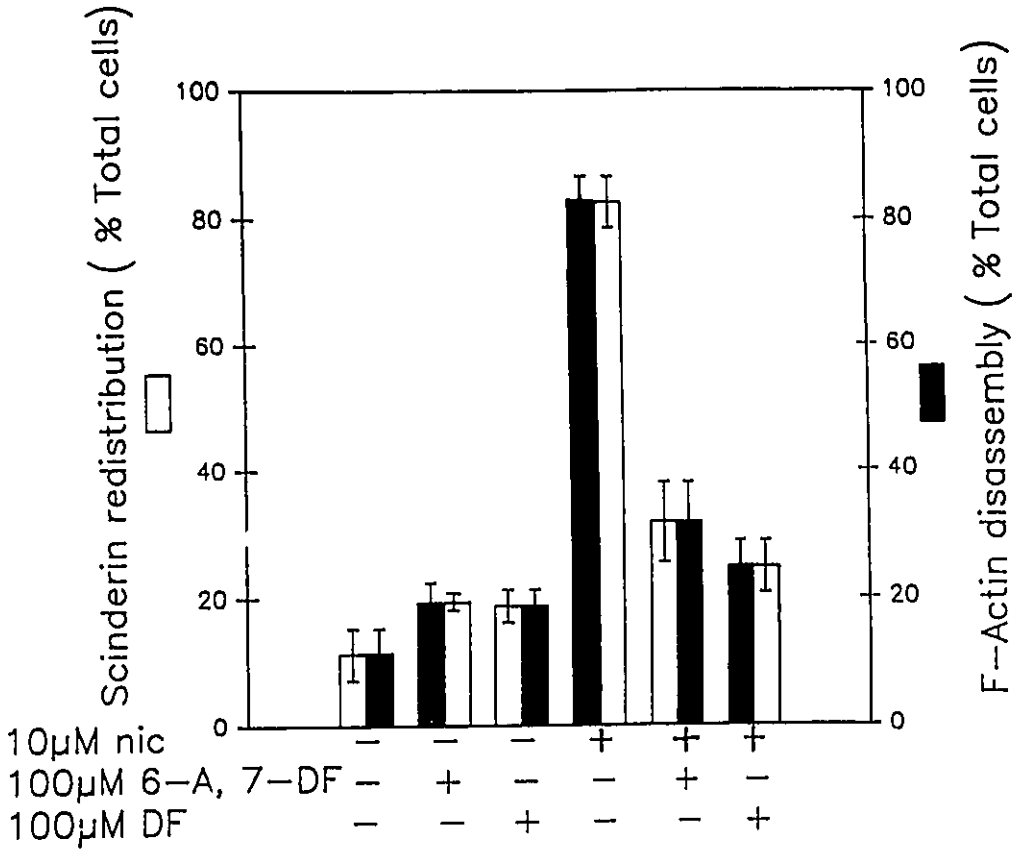


Fig.22
Scinderin redistribution and F-actin disassembly in chromaffin cells incubated with Locke's solution alone, 100μM 6-acetyl-7-deacetylforskolin (6-A, 7-DF) in the absence and presence of 10μM nicotine, 100μM deacetylforskolin (DF) in the absence and presence of 10μM nicotine, and nicotine alone for 40 s. Data illustrated represents the mean ± S.E.M. of n = 400 cells.

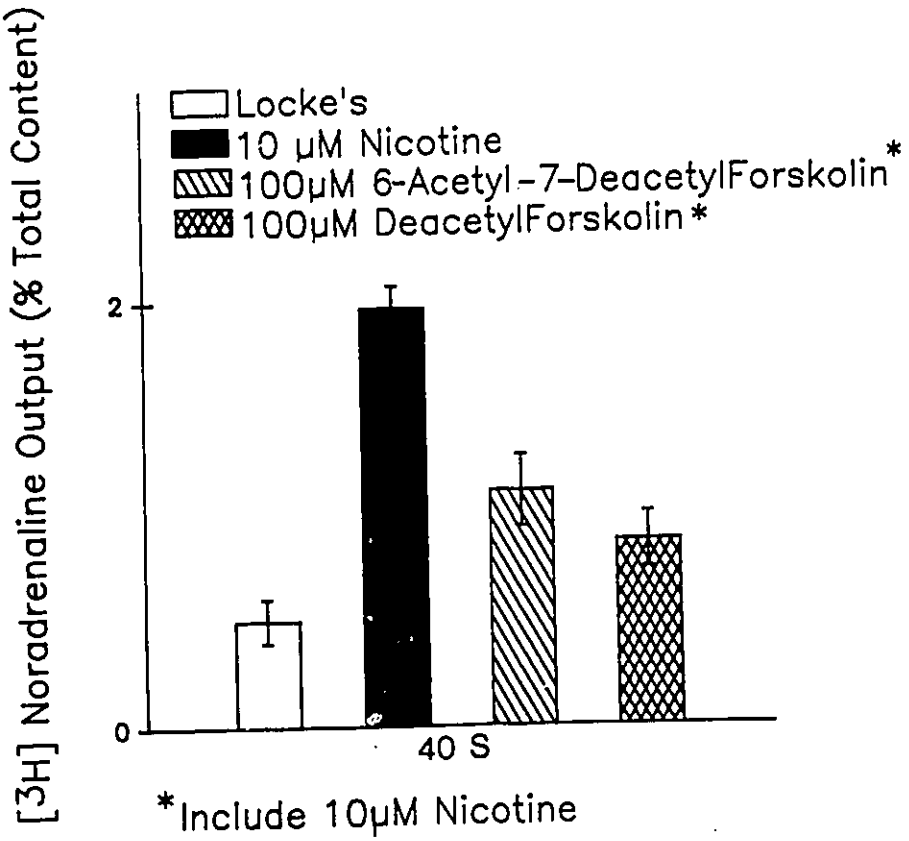


Fig.23
Effects of 100μM 6-acetyl-7-deacetylforskolin and 100μM deacetylforskolin on [^{3}H] noradrenaline output from stimulated chromaffin cells. Chromaffin cells cultured for 48-72 h were incubated with 10μM nicotine in the absence (■) and presence of either 100μM 6-acetyl-7-deacetylforskolin (▨) or 100μM deacetylforskolin (▩) for 40 s. The values represent the mean ± S.E.M. of results obtained in a minimum of n = 4 culture dishes.

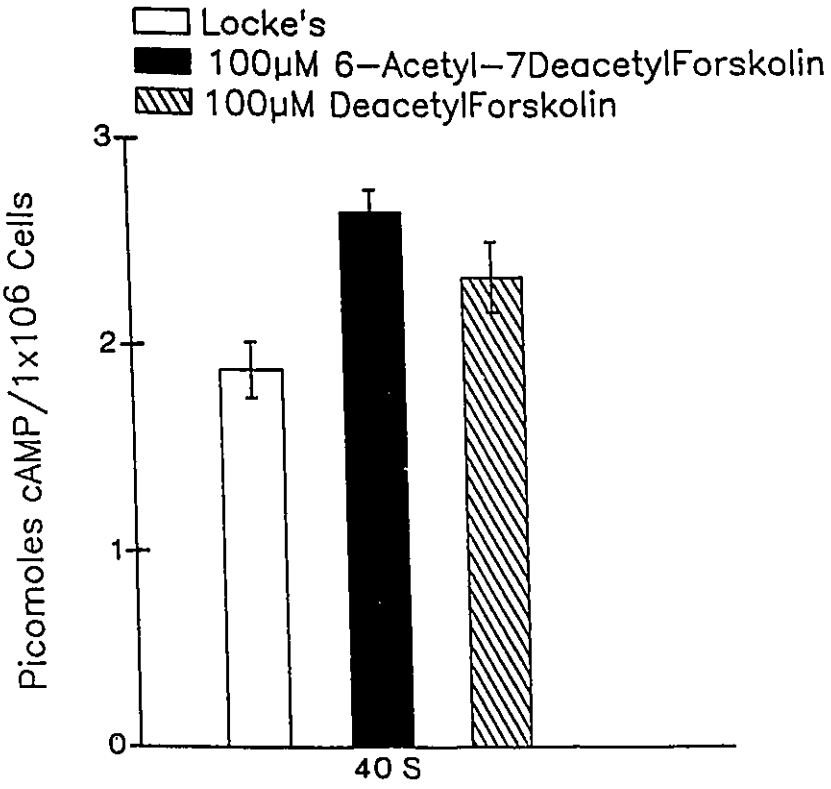


Fig.24
Determination of intracellular cAMP content in two-day old cultured chromaffin cells incubated with Locke's solution alone (□), 100μM 6-acetyl-7-deacetylforskolin (■) or 100μM deacetylforskolin (▨) for 40 s. Cellular cAMP levels were determined by radioimmuno assay. Each value represents the mean ± S.E.M. of results obtained from cells present in at least 4 different culture dishes.

CHAPTER IV

DISCUSSION

A. MODULATION OF SECRETION IN BOVINE ADRENAL CHROMAFFIN CELLS

Adrenal chromaffin cells, the parenchymal cells of the adrenal medulla are cells of neuroendocrine origin which store their secretory products in specialized membrane-bound organelles, the chromaffin granules (Smith, 1968; Trifaró, 1977) and exhibit regulated mechanisms of secretion. As a result, they have become an exemplary prototype for the study of the process exocytosis. Exocytosis in chromaffin cells occurs as a result of an increase in the activity of presynaptic splanchnic nerves originating in the spinal cord which innervate the adrenal medulla or in synaptic contacts made by splanchnic nerve endings on individual chromaffin cells (Coupland, 1965b,c) which elicits the release of the neurotransmitter principally contained and released by splanchnic neurons, acetylcholine (Feldberg et al., 1934). Alternatively, direct exposure of chromaffin cells to depolarizing concentrations of K^+, nicotinic agonists or Ca^{2+} ionophores also elicits exocytosis. Acetylcholine or nicotinic agonists interact with postsynaptic cholinergic receptors on the surface of chromaffin cells and as with depolarizing concentrations of K^+, this results in depolarization, Ca^{2+} influx and extrusion of the soluble granule contents to the cell exterior via exocytosis. In bovine adrenal chromaffin cells, the cholinergic receptor associated with evoking exocytosis upon interaction with an agonist is the nicotinic receptor (Burgoyne, 1991) and exocytosis evoked by nicotinic agonists has been found to be blocked by the nicotinic antagonist,

hexamethonium (Douglas and Poisner, 1965; Lee and Trendelenburg, 1967). Stimulation of the nicotinic receptor in bovine adrenal chromaffin cells engenders the production or activation of an assortment of second messengers including, Ca^{2+}, diacylglycerol, IP_3, Ca^{2+} and phospholipid-dependent protein kinase C (PKC) (Burgoyne, 1991) and cAMP (Guidotti and Costa, 1974; Marriott et al., 1988; Wilson, 1988). These second messengers carefully orchestrate the secretory response and may be involved in the modulation of secretion. A brief discussion of some of the ways in which second messengers may modulate secretion in chromaffin cells is presented below.

A.1. Calcium

The importance of the second messenger, Ca^{2+} in secretion has been established (Douglas, 1968). The Ca^{2+} signal in addition to initiating secretion, is responsible for the control of a multiplicity of regulatory proteins. These proteins modulate actin polymerization and disassembly as well as network organization and actin microfilament-membrane interactions in non-neuronal and neuronal cells (Forscher, 1989). In chromaffin cells, Ca^{2+} is necessary for the activity of actin-binding proteins such as scinderin and gelsolin and regulates actin microfilament-membrane and -vesicle interactions. The second messenger regulates these interactions by attenuating the ability of actin filaments to form cross-linkages or to become bound to anchorage proteins such as α-actinin and fodrin. Moreover, Ca^{2+} may control the activity of voltage-dependent Ca^{2+}

channels since the inactivation of these channels seems to be a Ca^{2+}-dependent process (Artalejo et al., 1987) and interacts with calmodulin, a Ca^{2+} regulatory protein found in chromaffin cells (Trifaró and Fournier, 1987). Calmodulin seems to involved in fusion of the secretory granule and plasma membranes during exocytosis (Burgoyne et al., 1982) and in the regulation of cytoskeletal proteins such as α-actinin (Trifaró et al., 1982, 1984; Bader and Aunis, 1983) and fodrin (Perrin and Aunis, 1985). The regulation of these proteins by calmodulin has been reported to be carried out by a "flip-flop mechanism" (Sobue et al., 1983) in which under resting conditions (low Ca^{2+}), the anchorage proteins interact with the granule, whereas during stimulation (high Ca^{2+}), they bind to calmodulin (Trifaró et al., 1992).

A.2. Protein kinase C

The second messenger, PKC is an enzyme which was discovered by Nishizuka and co-workers (Takai et al., 1979) and which plays an eminent role in a wealth of physiological processes (Nishizuka, 1984; Huang, 1989; Rana and Hokin, 1990). Activation of receptor-linked phospholipase C (PLC) or Ca^{2+}-dependent activation of PLC upon depolarization initiates phosphoinositide hydrolysis and elicits the generation of diacylglycerol (DAG) which activates PKC (Burgoyne, 1991). Under resting conditions, PKC is located in the cytoplasm and upon activation of the enzyme in response to synaptic, hormonal (Nishizuka, 1986) or phorbol ester (Castagna et al., 1982) stimulation it is translocated to the membrane where it associates with phosphatidylserine and

DAG. PKC activity depends on Ca^{2+} and the presence of lipids within the enzyme's immediate environment such as, phosphatidylserine. Since DAG increases PKC affinity for Ca^{2+} it is essential to the activation of PKC. In many cell types, activation of PKC has been found to produce a stimulatory effect on secretion (Rink et al., 1983). In contrast, some reports show that activation of PKC by phorbol esters in adrenal chromaffin cells has been found not to evoke neurotransmitter release (Brocklehurst and Pollard, 1985; Pocotte et al., 1985; Bader et al., 1989; Bittner and Holz, 1990; Vitale et al., 1992a). Neurotransmitter release in response to a physiological stimulus in chromaffin cells is however, enhanced by activation of PKC (Brocklehurst and Pollard, 1985; Pocotte et al., 1985; Terbush et al., 1988; Bader et al., 1989; Bittner and Holz, 1990). The enzyme possesses a variety of substrates which may account for the ability of PKC to modulate stimulus induced secretion in secretory cells. These substrates include cytoskeletal and cytoskeletal-associated proteins such as actin, myosin light chain kinase, troponin, caldesmon, talin, filamin and neurofilament subunits (Katoh et al., 1983; Naka et al., 1983; Nishizuka et al., 1983; Howard and Meyer, 1984; Kawamoto and Hidaka, 1984; Werth and Pastan, 1984; Lichtfield and Ball, 1986; Ohta et al.,1987; Phatak et al., 1988; Sihag et al., 1988; Georges et al., 1989; Pappadopoulos and Hall, 1989; Zalewski et al., 1990; Isosaki et al., 1991). PKC activation elicits reorganization of actin microfilaments in chromaffin cells (Grant and Aunis, 1990, Vitale et al., 1992a) and neutrophils (Downey et al.,

1992) and causes redistribution of actin anchorage proteins such as fodrin in 3T3 cells (Sobue et al., 1988). Work conducted in our laboratory has shown that PKC causes scinderin redistribution and F-actin disassembly independent of extra or intracellular Ca^{2+} as indicated by measurement of Ca^{2+} transients (Rodriguez Del Castillo et al., 1992; Vitale et al., 1992a) as well as an increase in the initial rate of nicotine-evoked exocytosis (Vitale et al., 1992a). The effect of PKC on scinderin was not found to be due to phosphorylation of the protein by PKC but was instead, found to be mediated by pH. Experiments conducted with Na^+/H^+ antiport inhibitors using intracellular pH determinations demonstrated that PKC-mediated scinderin redistribution was a consequence of an increase in intracellular pH, a phenomenon which occurs during nicotinic receptor stimulation and which may be due to PKC translocation and activation upon Ca^{2+} entry (Vitale et al., 1992a). Furthermore, two pools of scinderin have been proposed to exist in chromaffin cells, one cytosolic and the second, a pool which under resting conditions was found to be bound to plasma membrane phospholipids (Rodriguez Del Castillo et al., 1992). Scinderin binding to membrane phospholipids was noted to be both Ca^{2+}- and pH-dependent (Rodriguez Del Castillo et al., 1992). The physiological significance of scinderin binding to membrane phospholipids under resting conditions was suggested to be that this allowed scinderin to be close to it's target, filamentous actin (Rodriguez Del Castillo et al., 1992). Additionally, scinderin redistribution induced by nicotine was suggested to be caused by Ca^{2+}- and pH

induced release of scinderin from membrane phospholipids and denotes the activation of scinderin actin-filament severing activity. An increase in pH occurs only after Ca^{2+} influx takes place eliciting the activation of PKC which in turn stimulates the Na^+/H^+ antiport (Rodriguez Del Castillo et al., 1992). Since nicotine-induced scinderin redistribution was found to be partially blocked either by inhibitors of PKC or the Na^+/H^+ antiport, it was proposed that the mechanism by which PKC modulates secretion in bovine adrenal chromaffin cells is by activating the Na^+/H^+ antiport and eliciting a rise in pH. A rise in pH in conjunction with Ca^{2+} influx, seem to be necessary for the release of scinderin from membrane phospholipids and for activation of the actin-filament severing capacity (Rodriguez Del Castillo et al., 1992).

A.3. GTP-binding proteins

GTP-binding proteins (G-proteins) transduce hormonal, neurotransmitter and sensory signals across the plasma membranes of cells (Gilman, 1987). Although recently, G-proteins have been implicated in ion channel regulation (Brown and Birnbaumer, 1990) and in the vectorial transport of membrane and membrane-associated components in the secretory pathway (Bourne, 1988) the exact function that these proteins play in the process of secretion is unknown. Low molecular weight G-proteins have been implicated to be involved with constitutive secretion in yeast (Bourne, 1988) and there is some evidence of

their role in exocytosis (Gomperts, 1990) but in chromaffin cells, findings are contradictory. In electro-permeabilized bovine chromaffin cells and PC_{12} cells permeabilized with staphylococcal α-toxin, initial reports have indicated that GTPγS inhibits secretion (Knight and Baker, 1985; Knight et al., 1985; Ahnert-Hilger et al., 1987). In contrast, some reports indicate that in digitonin-permeabilized chromaffin cells non-hydrolyzable GTP analogs have no effect on Ca^{2+}-induced secretion but enhance Ca^{2+}-independent secretion (Bittner et al., 1986; Morgan and Burgoyne, 1990). More recent findings indicate that non-hydrolyzable analogs of GTP may have a dual effect on secretion in chromaffin cells, enhancing Ca^{2+}-dependent secretion indirectly through a mechanism involving the activation of PKC (Bader et al., 1989; Sontag et al., 1992) and inhibiting secretion by a PKC-independent pathway (Sontag et al., 1992). Moreover, it has been proposed that the enhancing and inhibitory effects observed on secretion in chromaffin cells are modulated by two diverse G-proteins, one acting at the early stages of secretion to enhance release and the other at the late stages of stimulus-secretion coupling to inhibit release (Sontag et al., 1992). The identity of the second G-protein thought to be inhibiting stimulated release has recently, been proposed to possibly be an G_o inhibitory protein located on the secretory granule membrane (Bader et al., 1993).

B. THE CHROMAFFIN CELL CYTOSKELETON IN THE MODULATION OF EXOCYTOSIS

As discussed previously in this work, in most cell types electron

microscopic and immunocytochemical techniques have demonstrated the presence of a dense network of actin microfilaments intimately associated with a variety of cytoskeletal proteins and localized beneath the plasma membrane (Heuser and Kirschner, 1980; Lee and Trifaró, 1981; Schliwa and Vanblerkom, 1981). Several lines of evidence indicate that the actin lattice in secretory cells functions as a barrier opposing the movement of secretory organelles to sites of exocytosis thus, modulating regulated secretion (Trifaró et al., 1982; Howard and Meyer, 1984; Bernstein and Bamburg, 1989; Koffer et al., 1990; Trifaró, 1990; Burgoyne, 1991; Perrin et al., 1992). This has led to the proposal that in chromaffin cells, in order for interaction between the secretory granules and exocytotic sites on the plasma membrane surface to occur the cortical actin barrier must be removed (Trifaró et al., 1982, 1989b; Cheek and Burgoyne 1986; Burgoyne et al., 1989; Burgoyne, 1991; Vitale et al., 1991). Stimulation of bovine adrenal chromaffin cells produces disassembly of cortical F-actin networks (Cheek and Burgoyne, 1986; Trifaró et al., 1989b; Vitale et al., 1991) and redistribution of actin regulatory proteins such as fodrin (Perrin and Aunis, 1985) and scinderin (Vitale et al., 1991), a novel actin-filament severing protein isolated in our laboratory (Rodriguez Del Castillo et al., 1990). Scinderin is a protein found in tissues with high secretory activity (Tchakarov et al., 1990; Rodriguez Del Castillo et al., 1992) which possesses two Ca^{2+} binding sites and two Ca^{2+}-dependent actin binding sites (Rodriguez Del Castillo et al., 1990). Under resting conditions, one pool of the scinderin exists in the

cytoplasm while another pool, is bound to plasma membrane phospholipids in close proximity to the cortical F-actin network (Rodriguez Del Castillo et al., 1992). Upon stimulation of chromaffin cells and augmentation of intracellular Ca^{2+} to a concentration of approximately 10^{-6}M scinderin is released from membrane phospholipids (as indicated by redistribution of cortical scinderin in immunofluorescence microscopy studies; Fig. 11) and each molecule of scinderin interacts with two molecules of actin to elicit a decrease in the viscosity of the cortical actin network by severing actin filaments (Rodriguez Del Castillo et al., 1990). A decrease in viscosity of actin gelatinous solutions elicited by scinderin in the presence of Ca^{2+} has been observed in vitro using electron microscopic analysis (Rodriguez Del Castillo et al., 1990). This effect of scinderin has also been suggested by immunofluorescence microscopy studies and image analysis (Fig.12, this thesis) which have demonstrated that regions of scinderin redistribution coincide with areas of F-actin disassembly (Vitale et al., 1991; Fig.11 of this thesis). In contrast, gelsolin, the other Ca^{2+}-dependent actin-filament severing protein which has been isolated from bovine adrenal chromaffin cells (Trifaró et al., 1985; Bader et al., 1986) does not exhibit redistribution in response to stimulation (Vitale et al., 1991) thus, suggesting that scinderin seems to be the actin-filament severing protein responsible for reorganization of the actin lattice upon stimulation of bovine adrenal chromaffin cells.

C. MODULATION OF CYTOSKELETAL DYNAMICS AND SECRETION BY CYCLIC AMP IN BOVINE ADRENAL CHROMAFFIN CELLS

As discussed in the previous section of the thesis, second messengers including Ca^{2+} and PKC have been reported to regulate scinderin redistribution (Rodriguez Del Castillo et al., 1992; Vitale et al., 1992a), cytoskeletal dynamics and secretion. The second messenger, cyclic AMP has also been reported to modulate cytoskeletal dynamics and secretion (Rivken et al., 1975; Sedgwick et al., 1985; Marone et al., 1986; Park et al., 1986; Burde et al., 1989; Mueller and Sklar, 1989; Sikdar et al., 1990; Zawalich and Rasmussen, 1990; Ervens et al., 1991; Shapiro et al., 1991; Wenzel-Seifert et al., 1991; Perrin et al., 1992) and cytoskeletal organization in an assortment of cell types (Kreisberg et al., 1985; Varnum et al., 1985; Mills and Lubin, 1986; Fox et al., 1987; Hall et al., 1988; Soll et al., 1989; Wessels et al., 1989; Goldman and Abramson, 1990; Lomri and Marie, 1990; Downey et al., 1991; Egan et al., 1991; Shapiro et al., 1991; Perrin et al., 1992). In some cell types, such as mouse osteoblastic cells (Lomri and Marie, 1990), neutrophils (Downey et al., 1991) and parotid acinar cells (Perrin et al., 1992), cAMP has been found to evoke secretion by causing reorganization of the microfilament component of the cell cytoskeleton. Cyclic AMP-evoked secretion of chloride from T84 human epithelial cells has also been proposed to be caused by cAMP-elicited cytoskeletal redistribution (Shapiro et al., 1991). Cytoskeletal reorganization in these cells was reported to be inhibited by nitrobenzoxadiazole-phallacidin,

an agent which binds to F-actin with high affinity and stabilizes it preventing depolymerization and reorganization of actin microfilaments (Shapiro et al., 1991). In contrast, in other cells such as dictyostelium, cAMP has been reported to redistribute actin microfilaments from other regions such as the pseudopodia to the cytoplasmic cortex beneath the plasma membrane, doubling F-actin content in this area of the cell (Wessel et al., 1989) and inducing increased cell rounding (Varnum et al., 1985; Hall et al., 1988; Soll, 1989). The effects produced by cAMP in dictyostelium are accompanied by prevention of cytoplasmic particle movement (Soll, 1989; Wessels et al., 1989). In this work we show that cAMP also appears to modulate scinderin redistribution, F-actin disassembly and catecholamine secretion in response to stimulation of chromaffin cells with nicotine. In order to examine the effect that cAMP has on these parameters, chromaffin cells were exposed to forskolin, 6-acetyl, 7-deacetylforskolin, deacetylforskolin and the 8Br-cAMP, an analog of cAMP capable of permeating the plasma membrane. Nicotinic stimulation of bovine adrenal chromaffin cells for 5, 10, 20 or 40 s with 10μM nicotine followed by an additional 50 or 80 s period with regular Locke's solution has demonstrated that the percentage of cells exhibiting discontinuous cortical fluorescence for scinderin (scinderin redistribution) and F-actin (disassembly) reaches a maximum at 40 s of stimulation with nicotine and is accompanied by maximum catecholamine output which increases slightly after 40 s of stimulation and reaches a plateau after removal of the stimulus (Vitale et al., 1991 and Fig.19

this thesis). This time period of 40 s was thus, chosen to conduct initial experiments analyzing the effect of 8Br-cAMP on scinderin redistribution, F-actin disassembly and catecholamine output. Our findings indicate that 8Br-cAMP alone was not found to evoke scinderin redistribution, F-actin disassembly or catecholamine release after 40 s of incubation at the concentration tested (2.5mM). The analog of cAMP, however, at the same concentration, was found to partially inhibit nicotine-evoked scinderin redistribution, F-actin disassembly and catecholamine secretion. These findings are in agreement with other reports which have shown that incubation with 2.3 mM 8Br-cAMP for 3 h is able to inhibit F-actin disassembly and secretion evoked by nicotine in bovine adrenal chromaffin cells (Cheek and Burgoyne, 1987) and that the cAMP analog, dibutyryl cAMP is also able to reduce catecholamine secretion in these cells in response to nicotine (Negishi et al., 1989). The validity of the method employed in this work as well as in other work conducted in our laboratory (Vitale et al., 1991, 1992a) to assess cortical F-actin disassembly is based on the following evidence. Staining of chromaffin cells with actin antibodies or rhodamine-labelled phalloidin, a probe which binds with high affinity and specificity to F-actin (Faulstish et al., 1988) demonstrates that in unstimulated chromaffin cells, an actin network can be visualized beneath the plasma membrane as a continuous cortical fluorescent ring (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986; Trifaró et al., 1989; Vitale et al., 1991, 1992a). Stimulation of chromaffin cells with secretagogues such as

nicotine or depolarization with high K^+ results in fragmentation of the actin lattice which is exhibited by disappearance of rhodamine fluorescence in areas devoid of F-actin (discontinuous cortical fluorescence) (Cheek and Burgoyne, 1986; Trifaró et al., 1989b) and by a decrease in the amount of F-actin recovered from the Triton X-100 insoluble (cytoskeleton) proteins (Burgoyne et al., 1989; Trifaró, 1990). In contrast to other methods used in the evaluation of F-actin content such as DNase I inhibition assay, the method used in our studies allows for analysis of changes in the state of cortical F-actin independently of the changes that occur in actin networks in other regions of the cell (Vitale et al., 1992a) and permits assessment of changes in actin dynamics in a specific cell population. Moreover, the cells used in the experiments described here were double-stained for actin and scinderin, a protein not expressed in other cell populations such as fibroblasts and adrenal cortical cells commonly found to co-exist in primary cultures of chromaffin cells (Tchakarov et al., 1990; Vitale et al., 1991). Following the observation that 8Br-cAMP did appear to regulate cytoskeletal dynamics and secretion in our system, studies were initiated to explore the effect of forskolin and its analogs on these parameters.

C.1. Forskolin and analogs of forskolin

Forskolin was discovered (Bhat et al., 1982; De Souza et al., 1983) as a result of programs directed by Dr. N.J. De Souza at Hoechst Pharmaceutical

Research in Bombay, India devised to screen plant extracts for cardiovascular and other pharmacological activities. These studies led to the isolation of the methanolic extracts from the root of a plant named Coleus forskolii (Seamon and Daly, 1986) and to the isolation of a compound which could be sequestered with other derivatives thereof from the extracts. The compound came to be named forskolin by virtue of the plant source from which it was obtained and to honour the Swedish naturalist, Pehr Foskål (Seamon and Daly, 1983) and like its derivatives is a diterpene, a chemical family which resembles the hexoses and steroids in chemical structure (Laurenza et al.,1989). Subsequent work demonstrated that forskolin exhibited hypotensive and anti-spasmolytic activities (Linder et al., 1978) which could be attributed to its ability to increase the activity of cAMP-dependent protein kinases in rabbit heart slices by increasing membrane adenylate cyclase activity (Metzger and Linder, 1981). Others also noted that forskolin and its derivatives possessed the ability to stimulate adenylate cyclase in isolated membranes as well as intact cells (Seamon and Daly, 1981). The ability of forskolin to stimulate adenylate cyclase was found to be unique because forskolin was able to stimulate a rapid and reversible increase in adenylate cyclase in the absence of hormonal agonists and in the absence of a functional guanine nucleotide regulatory protein (Seamon and Daly, 1986). This indicates that forskolin exerts its actions by directly activating the catalytic subunit of adenylate cyclase or indirectly by interacting with an unidentified protein closely

associated with the catalytic subunit, distinguishing forskolin from hormones and other compounds known to activate adenylate cyclase such as sodium fluoride, guanine nucleotides and cholera toxin (Seamon and Daly, 1986). Activation of adenylate cyclase by forskolin occurs with an EC_{50} of 5-20μM and 10-40μM in membrane preparations and intact cells respectively (Seamon and Daly, 1983). Less potent analogs of forskolin such as, 6-acetyl,7-deacetylforskolin and deacetylforskolin (Seamon and Daly, 1986) with an EC_{50} of > 50μM (Laurenza et al.,1989) have also been used. More recently, forskolin has been found to exhibit several cAMP-independent actions such as inhibition of glucose transport, enhancement of nicotinic receptor desensitization, decay of acetylcholine-elicited currents through the nicotinic receptor (White, 1988), inhibition of carbachol-mediated ion fluxes through the nicotinic receptor and modulation of voltage-dependent K^+ channels at higher concentrations (McHugh and McGee, 1986; Laurenza et al., 1989). Analysis of the potential effect of different concentrations of forskolin (10-50μM) to increase intracellular cAMP in unstimulated chromaffin cells during 40 s of incubation revealed that there was a concentration-dependent increase in cAMP in these cells. Even the lowest concentration tested (10μM) was found to elicit small but, significant increase in cAMP (Fig.14). The concentrations of forskolin tested were selected in accordance with the fact that forskolin has been found to stimulate adenylate cyclase in intact cells at a concentration ranging from 10-40μM (Seamon and Daly, 1983). These concentrations were

found to elicit a dose-dependent inhibition of scinderin redistribution, F-actin disassembly and catecholamine output in response to stimulation of cultured chromaffin cells with 10μM nicotine (Fig.21). Since inhibition of these parameters could be observed at low doses of forskolin (10μM-20μM) which produced small but albeit, significant increases in cAMP, the observed inhibitory effects could most likely be due to the cAMP-related effects of forskolin rather that to the cAMP-independent effects of the diterpene such as inhibition of ion influxes through the nicotinic receptor and modulation of voltage-dependent ion channels which occur at higher concentrations of forskolin ($>$ 20μM) (McHugh and McGee, 1986; Laurenza et al., 1989). Although basal levels of cAMP (1.808 $\pm$ 0.122, n=39) obtained were similar to those reported by others (Morita et al., 1987a; Marriott et al., 1988; Wilson, 1988), levels of cAMP obtained upon stimulation with forskolin over all the concentrations tested were lower than those reported in other studies in response to forskolin in the concentration range of 5-30μM. This may be explained by the fact that the incubation periods were longer than those used in the work presented here (40 s) and ranged between 6-12 min (Baker et al., 1985; Cheek and Burgoyne, 1987; Marriott et al., 1988). Studies were then conducted with the concentration which elicited the greatest increase in cAMP upon 40 s of incubation of resting chromaffin cells with forskolin (50μM). Analysis of the effect of forskolin on cAMP levels in our system was carried out using a [^{3}H] cAMP radioimmunoassay. The assay utilized for measurement of cAMP is

based on the competition between unlabelled cAMP and a fixed quantity of tritium labelled cAMP for binding to a protein with high specificity for cAMP, presumably a cAMP-dependent protein kinase (Gilman, 1970). The amount of labelled cAMP-protein complex formed is inversely related to the amount of unlabelled cAMP present in the assay. Separation of the protein bound cAMP from the unbound nucleotide is achieved by absorption of the free nucleotide on charcoal followed by centrifugation, as described by Brown et. al. (1971). The advantages of using this assay system are that the sensitivity of the assay is high and the assay is simple to perform on a large number of samples (130 tests can be run using 1 assay kit) in a short time frame (2h). Moreover, assay conditions are such that destruction of cAMP is not an important factor and interference by other substances is minimal. Time course studies in resting and nicotine-stimulated cultured adrenal chromaffin cells exposed to 50μM forskolin for 5 s or longer show that cAMP levels in these cells begin to increase even at 5-10 s of incubation with forskolin (which was the shortest time period we were capable of measuring in all of the studies presented here) and continue to increase after 40 s (Fig.17).

Moreover, no differences in cAMP levels were found between resting and nicotine-stimulated cells exposed to forskolin over the time periods examined. Additionally, 10μM nicotine alone was not found to increase intracellular cAMP at 40 s of stimulation. Our finding that nicotine or cholinergic stimulation alone does not increase levels of cAMP in chromaffin cells is in agreement with other

reports which have illustrated that there are no differences in the levels of cAMP between control cells and chromaffin cells incubated with nicotine (Marriott et al., 1988) or carbamylcholine (Baker et al., 1985). Other results in contrast, have demonstrated that cAMP levels in chromaffin cells are increased by several minutes of nicotinic cholinergic stimulation (Boonyaviroj and Gutman, 1977; Tsujimoto et al., 1980; Morita et al., 1987a,b; Eiden et al., 1984; Pocotte et al., 1986). For instance, some have found that stimulation of chromaffin cells with nicotine for 3-8 min (Jaanus and Rubin, 1974; Wilson, 1988; Anderson et al., 1992) and acetylcholine (3-8 min) (Jaanus and Rubin, 1974) or carbamylcholine for 24 min (Guidotti and Costa, 1974) does evoke an increase in levels of cAMP in chromaffin cells. Moreover, some studies have shown that exposure of chromaffin cells to nicotine and forskolin concomitantly, potentiates the increases in cAMP levels observed in response to forskolin alone (Marriott et al., 1988). Once again, the time frame of incubation with nicotine and forskolin in this study (Marriott et al., 1988), as in the studies discussed above in which nicotinic receptor stimulation by agonists was shown to increase cAMP, was long (12 min). In this time period, which is much longer than the time frame of 40 s employed in the studies presented in this thesis many other factors could be the eliciting an increase in cAMP besides nicotine. Calcium for instance, has been reported to be necessary for cAMP augmentation (Boonyaviroj and Gutman, 1977; Pocotte et al., 1986; Anderson et al., 1992) and to modulate cAMP levels in adrenal

chromaffin cells (Keogh and Marley, 1991). Additionally, some of these studies employed higher concentrations of nicotine (Marriott et al., 1988; Anderson et al., 1992) than the concentration of nicotine used here. The time course of inhibition of the parameters of scinderin redistribution and F-actin disassembly was found to closely parallel the time course of cAMP augmentation (Fig.17) and inhibition of nicotine-evoked scinderin redistribution and F-actin disassembly in response to 50μM forskolin was found to occur at 5-10 s of exposure of nicotine-stimulated cells to forskolin and to coincide with the time period in which an initial rise in cAMP levels could be detected. Incubation of the cells with Locke's solution following the 40 s incubation period elicited an decrease in the percentage of cells exhibiting discontinuous scinderin and F-actin cortical fluorescence. As indicated in previous studies conducted in our laboratory (Vitale et al., 1991) and in the findings in this thesis (Fig.18), the percentage of cells exhibiting scinderin discontinuous cortical fluorescence was lower than the percentage of chromaffin cells displaying discontinuous F-actin cortical fluorescence. This was due to the fact that during recovery of chromaffin cells after stimulation, Ca^{2+} concentrations within the cell are low ($\sim 10^{-7}$M) and pH is still high (7.1). Under these conditions scinderin has been proposed to exhibit a maximum affinity for plasma membrane phospholipids and phospholipids are competing with actin for scinderin and causing dissociation of scinderin from actin (Rodriguez Del Castillo et al., 1992). Scinderin (normal) distribution being almost totally recovered can be explained by the fact that

scinderin is probably bound to phospholipids at this time. Scinderin redistribution and F-actin disassembly in response to nicotine in cultured chromaffin cells has been found to precede catecholamine output by 10-15 s (Vitale et al., 1991). The fact that no elevation in catecholamine output was observed during the first 10-15 s of stimulation was not due to low sensitivity in the catecholamine assay employed. The sensitivity of the method used in determination of catecholamine output in studies conducted in our laboratory, is high and is such that it permits determination of an amount of catecholamine released corresponding to 0.35% of total catecholamine content (Vitale et al., 1991). Examination of the time course of nicotine-evoked catecholamine output (Fig.19) in bovine adrenal chromaffin cells in response to 50μM forskolin indicates that inhibition of catecholamine output in response to forskolin lags 10-15 s behind augmentation of cAMP levels. Moreover, the percentage of inhibition of both scinderin redistribution and F-actin disassembly over time (Fig.20) coincides with the time course of cAMP elevation (Fig.17).

These results are consistent with a model in which inhibition of catecholamine output by cAMP is due to attenuation of nicotine-evoked scinderin redistribution and actin filament disassembly and is compatible with other results in bovine adrenal chromaffin cells which demonstrate that forskolin is able to reduce secretion in response to nicotinic receptor stimulation (Baker et al., 1985; Marriott et al., 1988). The inhibitory effects engendered by forskolin and cAMP analogs on agonist-induced secretion have also been observed in other

secretory cell types. Incubation of sensitized human basophils and mast cells with forskolin in a concentration range (~ 30μM) similar to that employed in the studies in this thesis for instance, caused inhibition of IgE-mediated histamine release from these cells (Marone et al., 1986). Similar effects have been observed on superoxide formation in response to neutrophil activation (Sedgwick et al., 1985; Mueller and Sklar, 1989; Burde et al., 1989; Ervens et al., 1991), neutrophil functions such as chemotaxis, oxygen radical production and enzyme secretion (Rivken et al., 1975) as well as on exocytosis, aggregation and Ca^{2+} influx in response to chemoattractants in human neutrophils exposed to cAMP analogs (Wenzel-Seifert et al., 1991).

Analogs of forskolin known to be less potent than forskolin in stimulating adenylate cyclase and at increasing cAMP were also analyzed during the course of our studies and the effect they engendered on the parameters of scinderin redistribution, actin microfilament disassembly and catecholamine output studied. We found that neither 100μM 6-acetyl,7-deacetylforskolin nor deacetylforskolin were able to elicit significant scinderin redistribution or F-actin disassembly at 40 s. These compounds however, like forskolin, partially inhibited these parameters in response to stimulation with nicotine. Furthermore, as in the case of forskolin, these inhibitory effects could be correlated with an increase in cAMP (Fig.24) and a reduction in nicotine-evoked secretion (Fig.23).

Our findings are in sharp contrast with other studies which have shown

that exposure of chromaffin cells to agents which increase cAMP levels (forskolin, 8Br-cAMP, dibutyryl cAMP) facilitates secretion (Adams and Boarder, 1987; Morita et al., 1987a,b). Some of the discrepancies which exist between these studies and our catecholamine release work as stated previously are due to differences in times of incubation and drug concentrations used. Aams and Boarder (1987) incubated chromaffin cells with the cAMP analog, (1mM) dibutyryl cAMP and forskolin (10μM) for 72 h. Morita et al. (1987a,b) for example, has proposed that cAMP analogs and forskolin enhance catecholamine secretion in bovine adrenal chromaffin cells in response to nicotinic stimulation and reported that the ability of cAMP to enhance secretion is due to inhibition of the Na^+-K^+-ATPase in the plasma membrane of chromaffin cells. This it has been proposed leads to accumulation of Na^+, thereby increasing intracellular Ca^{2+} through a mechanism involving Na^+/Ca^{2+} exchange (Morita et al., 1991a,b). In these experiments however, the initial concentrations of forskolin used ranged from 0.3-1μM and the incubation period was 3 min. These low concentrations of forskolin have been associated with enhancement of secretion in response to stimulation (Seamon and Daly, 1986). Enhancement of catecholamine secretion in nicotine-stimulated cells exposed only to low concentrations of forskolin ($\sim$0.1-5μM) has been reported by investigators who at the same time have found that higher doses of forskolin ($\geq$ 10μM) reduced nicotine-evoked secretion (Marriott et al., 1988). Moreover, Morita et al. (1987b) also observed that at higher doses of forskolin ($>$ 10μM) an inhibitory

effect is found on ^{45}Ca uptake. This could be a mechanism by which forskolin could reduce Ca^{2+}-dependent secretion.

The afore-cited contradictions serve to underscore the fact that some of the observed discrepancies in the literature on this subject seem to be due to differences in the times of incubation and forskolin concentrations employed.

D. POSSIBLE SITES AT WHICH CYCLIC AMP MAY MODULATE CHROMAFFIN CELL CYTOSKELETAL DYNAMICS AND SECRETION

D.1. The cytoskeleton: Phosphorylation and cAMP-dependent kinase

Many proteins involved in synaptic function which play a role in neurotransmitter release, voltage-dependent ion channel and receptor function have been shown to be phosphorylated by cAMP-dependent mechanisms (Hemmings et al., 1989; Huganir and Greengard, 1987; Greengard et al., 1987; Decamilli et al., 1990). Work conducted by others has illustrated other ways in which cAMP can regulate both the membrane and cytoskeleton of cells. It has been shown that in non-muscle cells, cAMP-dependent protein kinase can phosphorylate myosin light chain kinase and reduce affinity of the kinase for Ca^{2+}-calmodulin hence, eliciting a decrease in enzyme's function and

attenuation of cytoskeletal dynamics (Conti and Adelstein, 1980; Egan et al., 1991). Additionally, cAMP-dependent phosphorylation of the membrane-cytoskeleton has been found to increase the activity of Ca^{2+}-calmodulin dependent protein kinase by releasing it from the cytoskeleton (Saltoh and Schwartz, 1985) thus, possibly enhancing the phosphorylation of cellular components. At present it is not known if phosphorylation could account for modulation of actin dynamics in the chromaffin cell but involvement of cAMP and the cytoskeletal network in modulation of secretion is indicated by our findings. Cyclic AMP has been cited to modulate cytoskeletal dynamics in mesanglial cells (Kreisberg et al., 1985), MDKC cells (Mills and Lubin, 1986), astrocytes (Goldman and Abramson, 1990), neutrophils (Downey et al., 1991), platelets (Fox et al., 1987), dictyostelium (Wessels et al.,1989), osteoblasts (Egan et al., 1991) and parotid acinar cells (Takuma, 1990; Perrin et al., 1992) and some of cAMP-related actions on these systems may be mediated by phosphorylation. In some cases, phosphorylation of key cytoskeletal proteins has been proposed to be responsible for modulation of cytoskeletal organization by cAMP (Kreisberg et al., 1985; Mills and Lubin, 1988; Fox et al., 1987; Goldman and Abramson, 1990). This is also the case in cultured mouse osteoblastic cells. In these cells, a rapid and transient rise in cAMP elicited by parathyroid hormone promotes disassembly of cytoskeletal actin and myosin in a mechanism involving phosphorylation of myosin light chain kinase by cAMP (Egan et al., 1991). Phosphorylation of myosin light chain kinase reduces the

enzyme's affinity for calcium-calmodulin and decreases the ability of myosin light chain kinase to phosphorylate myosin light $chain_{20}$ (Egan et al., 1991). The ability of cAMP-dependent protein kinase (PKA) to phosphorylate proteins involved in the modulation of actin dynamics as well as the fact that PKA has been directly implicated in the desensitization of acetylcholinergic receptors in (Albuquerque et al., 1986; Middleton et al., 1986) (specifically nicotinic receptors; Huganir et al., 1986) may provide evidence that desensitization of the receptor by PKA could account for attenuation of nicotine-induced scinderin redistribution, F-actin disassembly and catecholamine secretion. Cyclic AMP-dependent protein kinase has also been found to phosphorylate the sodium channels of electrically excitable cells such as cardiac myocytes inhibiting sodium currents and depolarization (Sorbera and Morad, 1991). Moreover, there is evidence that cAMP-dependent protein kinase regulates the electrical properties of neurons and phosphorylates neuronal voltage-dependent ion channels (Catterall, 1988; Kaczmarek, 1988) thus, affecting secretion. If modulation of ion channels such as Ca^{2+} channels by cAMP is occurring in chromaffin cells, this could account for inhibition of both scinderin redistribution and F-actin disassembly since these processes are Ca^{2+}-dependent.

D.2. The nicotinic receptor

Some investigators have reported that the inhibitory effect produced on F-actin disassembly (Cheek and Burgoyne, 1987) and secretion (Baker et al.,

1985) by cAMP seems to be engendered specifically in response to nicotinic stimulation. Similar results were obtained by other investigators employing forskolin and dibutyryl cAMP (Negeshi et al., 1989). These findings are consistent with the results shown in this thesis. since scinderin redistribution is primarily an event associated with the nicotinic receptor activation (Vitale et al., 1991). This would suggest that the nicotinic receptor might be a site of modulation. Evidence does exist which demonstrates that cAMP can modulate nicotinic receptor function. It has been reported, that old and new nicotinic cholinergic receptors on the chromaffin cell surface respond differently to analogs of cAMP (Higgins and Berg, 1988). The nicotinic response to agonists is greater in receptors which have been newly inserted into the plasma membrane and cannot be affected by cAMP analogs (Higgins and Berg, 1988). In contrast, older receptorS engender a response to agonists which can be affected by cAMP analogs (Higgins and Berg, 1988).

D.3. Possible cAMP interaction with other second messengers in the modulation of chromaffin cell secretion

In studies in chromaffin cells which have been exposed to veratridine (40μM) and high K+ (50μM) for 5 min, levels of cAMP have been found to increase in a Ca^{2+}-dependent manner (Keogh and Marley, 1991). Calcium entry, the signal which triggers not only, secretion but also scinderin activation, has been proposed to regulate cAMP levels in chromaffin cells by activating the

Ca^{2+}-sensitive isoenzyme of adenylate cyclase (Keogh and Marley, 1991). Since our findings indicate that nicotine does not increase cAMP at 40 s of stimulation one could speculate that this could be another route through which cAMP could accumulate intracellularly. Moreover, it has been proposed that the secretory vesicles of chromaffin cells contain a large pool of Ca^{2+} which is released with other vesicle contents during exocytosis (Von Grafenstein and Powis, 1989). It could thus, be possible that cAMP may involved in a mechanism which terminates secretion triggered by Ca^{2+} influx perhaps by preventing reorganization of actin microfilaments and redistribution of scinderin. Data has been obtained which shows that cAMP presence and metabolism in biological systems often accompanies the Ca^{2+} signal (Rasmussen and Barrett, 1984) and can modulate the Ca^{2+} signal and sensitize or desensitize the secretory apparatus to the effects of Ca^{2+} (Plascik et al.,1980). Cyclic AMP has been found to possess the capacity to extrude intracellular calcium (Feinstein et al., 1983; Egan et al., 1991). This decrease in cytosolic Ca^{2+} could prevent scinderin redistribution and F-actin disassembly and hence, secretion. Several lines of evidence indicate that activation of PKC leads to sensitization of the adenyl cyclase system (Nishizuka, 1986) and has been found to mediate activation of adenyl cyclase in PC_{12} cells (Hollingsworth et al., 1986) and a number of other cell types (Yoshimasa et al., 1987; Choi and Toscano, 1988). Recent work conducted in our laboratory has shown that PKC activation by phorbol esters induces Ca^{2+}-independent scinderin redistribution

(Rodriguez Del Castillo et al., 1992) and F-actin disassembly and initially enhances the exocytotic response to nicotine (Vitale et al., 1992a; for explanation see introduction section of this thesis). The mechanism does not involve scinderin phosphorylation by PKC (Rodriguez Del Castillo et al., 1992). Instead calcium entry upon depolarization may induce PKC translocation from the cytoplasm to membranes (Terbush et al., 1988; Vitale et al., 1992a), a process known to be associated with activation of the kinase (Nishizuka, 1986). In cells systems such as neutrophils, platelets and lymphocytes the signals that elicit the inositol phospholipid cascade, which activates release reactions are usually antagonized by cAMP (Nishizuka, 1986). The mechanism by which cAMP antagonizes activation in the cells cited above, where signals induce breakdown of phospholipids, is by way of a feedback mechanism in which cAMP activates PKA which inhibits diacylglycerol and in turn, PKC (Nishizuka, 1986). Moreover, in chromaffin cells exposed to phorbol esters, compounds that activate PKC, cAMP is elevated indicating that the Ca^{2+}-dependent nicotinic stimulation of adenyl cyclase cited in studies employing stimulation with nicotine in the time frame of minutes could be mediated by PKC (Anderson et al.,1992). This mode of activation of adenyl cyclase is contradicted by our findings since nicotine does not augment cAMP after 40 s of stimulation even though PKC is activated (Vitale et al., 1992). Opposing effects of PKC and cAMP-dependent kinase on actin polymerization have been observed (Ohta et al.,1987). It is thus, possible to speculate that in chromaffin

cells, PKC and cAMP-dependent protein kinase may be exerting opposing effects on actin dynamics and hence, on secretion in response to nicotinic stimulation.

E. CONCLUDING REMARKS

In conclusion, it appears that cAMP might regulate nicotine-induced secretion in chromaffin cells by modulation of scinderin redistribution and cortical F-actin disassembly. These events precede exocytosis and appear to be crucial for secretion in bovine adrenal chromaffin cells to occur. The findings shown here underscore the fact that a plethora of second messenger systems act at different levels to modulate the secretory response utilizing a labyrinth of diverse mechanisms. More conclusive elucidation of the role that cAMP may play in the modulation of the afore discussed nicotine-induced parameters will require that the possibility of cAMP-dependent protein kinase activity in our system be explored. Analysis of the possibility that cAMP might be exerting its effects via PKA or by release of the RI and RII regulatory subunits of the kinase, the two primary routes through which cAMP regulates processes (Rogers et al., 1988; Perrin et al., 1992) might represent a possible area to be explored. As well, insight as to the site at which cAMP might act to modulate secretory dynamics may be gained by the use of receptor-independent methods of depolarization. Additionally, since cAMP-dependent kinase phosphorylates several intracellular proteins in nerve cells (Tsujimoto et al., 1991) it might be

of interest to explore whether phosphorylation of actin regulatory proteins such as scinderin, might account for the observed results. The involvement of G-proteins in inhibition of nicotine-induced scinderin redistribution, F-actin disassembly and catecholamine secretion by cAMP could also add new insight as to the role of cAMP in the secretory process. It is also noteworthy, that cAMP might play a different role in different tissues and may exert different modulatory actions at various phases of the secretory process. For instance, depending on the duration and strength of stimulation neuromodulators which act through cAMP may produce effects at the level of gene expression and catecholamine biosynthesis or may act at the level of the exocytotic machinery to facilitate or terminate catecholamine release. A number of neuroreactive peptides that were originally characterized in non-neuronal tissues have been found to co-exist with biogenic amines in the storage vesicles of neuronal tissues (Dohi et al., 1991; for review see Hökfelt et al., 1986). These biologically active peptides which act as neuromodulators and elicit second messenger generation are present in the central and peripheral nervous system (Dohi et al., 1991). Chromaffin cells for example, produce the neuroactive peptide, vasoactive intestinal polypeptide (VIP) (Dohi et al., 1991). Primary cultures of bovine adrenal chromaffin cells have been found not only to synthesize, but also to release VIP in response to stimulation with agents which evoke release of catecholamines such as nicotine, veratridine and high potassium (Eiden et al., 1983). Moreover, VIP has been reported to increase

cAMP levels in cultured chromaffin cells and to modulate secretion in these cells evoked by nicotine (Wilson, 1988). The possibility has been proposed that bioactive peptides such as VIP in chromaffin cells, may be contained in a diverse sub-population of chromaffin cells containing not only biogenic amines, but also peptides. Different types, strengths and durations of stress may cause different chromaffin cell populations to respond. For instance, one type of cell may respond to exogenous stress by releasing catecholamines and peptides while another type may respond to a metabolic stress by releasing only catecholamines (Vaupel et al., 1988). If this is the case, it might be of interest to conduct studies to investigate the role that VIP, which has been reported to increase cAMP and modulate secretion in bovine adrenal chromaffin cells (Wilson, 1988) might play in regulation of nicotine-evoked scinderin redistribution, actin network disassembly and catecholamine secretion in these cells.

Finally, there is no doubt that use of a multi-disciplinary approach to clarify the intricacies of the process of exocytosis will continue to aide in the construction of a clear picture of the molecular mechanisms governing secretion.

CHAPTER V

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REFERENCES

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