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CHROMAFFIN CELL SCINDERIN REDISTRIBUTION, CORTICAL F-ACTIN DISASSEMBLY AND SECRETION ARE EVOKED BY HISTAMINE THROUGH ACTIVATION OF THE H_1 RECEPTOR-PHOPHATIDYLINOSITOL 4,5-BISPHOSPHATE TRANSDUCTION PATHWAY

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Abstract

Nicotinic stimulation of chromaffin cells causes disassembly of cortical F-actin networks and redistribution of scinderin, a Ca^{2+}-dependent F-actin severing protein. These Ca^{2+}-dependent events precede exocytosis. Activation of scinderin by Ca^{2+} may cause severing of F-actin and disassembly of actin networks leaving cortical areas of low cytoplasmic viscosity which are the sites of exocytosis. Histamine is a known chromaffin cell secretagogue which induces Ca^{+}-dependent release of catecholamines. However, conflicting evidence exists as to the source of Ca^{2+} utilized in histamine-evoked secretion. Here we report that histamine-H_1 receptor activation induces scinderin redistribution, F-actin disassembly and catecholamine release. Histamine evoked similar patterns of distribution of scinderin and filamentous actin. The rapid responses to histamine occurred in the absence of extracellular Ca^{2+} and were triggered by release of Ca^{2+} from intracellular stores. The trigger for the release of Ca^{2+} was inositol 1,4,5-triphosphate (IP_3) since U-73122, a phospholipase C inhibitor, but not its inactive isomer (U-73343), inhibited the increases in IP_3, intracellular Ca^{2+}, scinderin redistribution, cortical F-actin disassembly and catecholamine release in response to histamine. Thapsigargin, an agent known to mobilize intracellular Ca^{2+}, blocked the rise in intracellular Ca^{2+}, scinderin redistribution, F-actin disassembly and catecholamine secretion in response to histamine. However, thapsigargin did not modify the increase in IP_3 induced by histamine. Calphostin C and chelerythrine, two inhibitors of protein kinase C, blocked all responses to histamine with the exception of the release of Ca^{2+} from intracellular stores. This

suggests that protein kinase C is involved in histamine induced responses. The results also show that without F-actin disassembly, raises in intracellular Ca^{2+} are not by themselves capable of triggering catecholamine release.

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ABBREVIATIONS

ATPase	adenosine 5'-triphosphatase
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
DβH	dopamine β hydroxylase
DNase I	deoxyribonuclease I
EBSS	eagle's balanced salt solution
EDTA	ethylenediamine tetraacetic acid
F-actin	filamentous actin
FITC-IgG	fluorescein isothiocyanate conjugate IgG
G-actin	globular actin (monomeric actin)
[^{3}H]NA	tritiated noradrenaline
kDa	kilodalton
PBS	phosphate buffered saline
TCA	trichloroacetic acid
PNMT	phenylethanolamine N-methyltransferase
PMA	phorbol 12-myristate 13-acetate

CHAPTER I

INTRODUCTION

A. The Adrenal Medulla and Adrenal Chromaffin Cells

1. General description

The adrenal glands are paired structures localized anterior or medial to the kidney in quadrupeds. In humans, they lie on top of each kidney at the level of the 11th or 12th vertebra. They are embedded in adipose tissue and encapsulated by the renal fascia which separates them from the kidney. The adrenal gland has two anatomically distinct parts: the medulla, the central core of the gland, and the adrenal cortex, which encases the medullary core. Nominal and functional distinction between these two areas of adrenal gland was established by Nagel (1886), who first used the terms "cortex" and "medulla", and by von Kolliker, who provided the first complete description of the structural anatomy of the gland (Kolliker, 1852, 1854).

The combined weight of the medullae (both glands) is about 1.0g in human. The adrenal medulla is composed almost entirely of chromaffin cells which are irregularly shaped polyhedrons surrounded by nerves, connective tissue, and blood vessels. They contain numerous chromaffin granules, which are electron-dense vesicles of 100 to 300 nm in diameter. These organelles resemble the dense-core vesicles of sympathetic nerve endings. The granules are important for the storage and secretion of catecholamines (Blaschko and Welch, 1953; Hillarp et al., 1953; Sjostrand and Wetzstein, 1956). Chromaffin cells contain large amounts of either noradrenaline or adrenaline and traces of dopamine. In humans, 85% of the adrenomedullary catecholamine store is adrenaline

(Benchimol and Cantin, 1977). As with the sympathetic nerve endings, a variety of potential noncatecholamine mediators have been identified in adrenomedullary chromaffin cells (Benchimol and Cantin, 1977). The adrenal medulla has both arterial and portal venous circulations (Yeasting, 1986). The medullary artery traverses the cortex and supplies the cortex from the subcapsular plexus, which drains centripetally toward the medulla. In the zona reticularis the capillaries coalesce to form venous sinuses that drain into and supply the medullary tissue. The portal system contains high concentrations of steroid hormones derived from the adrenal cortex. Medullary capillaries are fenestrated, which may allow free diffusion of released catecholamines. These capillaries coalesce and eventually form a single adrenal vein that usually drains into the inferior vena cava on the right and into the renal vein on the left. The adrenal medulla is innervated by typical cholinergic preganglionic sympathetic neurons carried in the splanchnic nerves (Tomilinson and Coupland, 1990). The major portion of the innervation is from the ipsilateral greater splanchnic nerve (T5-9).

2. Adrenal medullary chromaffin cells

As the name implies, chromaffin cells have historically been characterized as being reactive with dichromat salts (Kojima, 1917). Isolated chromaffin cells are spherical 10-20 μm in diameter with a nucleus 5 μm in width. In the medulla, chromaffin cells are arranged in groups separated by connective tissue and situated around blood vessels (Carmichael, 1979). It is now known that the distribution of chromaffin cells within the

medulla and that of organelles within the chromaffin cell is not random (Bornstein et al, 1991). Polarization has been found to occur with respect to nerve endings, which are located on one side of the cells whereas blood vessels are associated with the opposite pole (Bornstein et al, 1991). The Golgi apparatus and secretory organelles are usually found near the pole of the nucleus which is close to the blood vessels. This polarization is consistent with nervous input being received at one pole of the chromaffin cell and secretion occurring at the opposite site in contact with blood vessels (Bornstein et al, 1991). Microscopic analysis of the chromaffin cell indicates that the surface close to the nucleus (possessing 1-2 nucleoli), the rough endoplasmic reticulum and the Golgi apparatus, is the side at which neurochemical input is received. The rough endoplasmic reticulum and the Golgi apparatus are involved in the biogenesis of chromaffin granules and other cellular organelles (Hortnagl, 1976; Trifaró, 1978). Elucidation of the sub-cellular features of chromaffin cells was concomitant with the development of electromicroscopic techniques. In 1955, Lever provided the first electron micrographs of "the chromaffin granule", a term introduced by Sjöstrand and Wetzstein (1956). Biochemical findings in correlation with morphological observations have demonstrated the presence of three types of secretory granule in chromaffin cells. These are adrenaline-containing cells(A), noradrenaline-containing cells (NA), and small granule chromaffin cells (SGC). NA and A are represented by the large dense-core vesicles (750-1000 Å) related to the LDCV found in neurons of the sympathetic nervous system, and SGC is comprised of the small dense-core vesicles (400-500 Å) also found in sympathetic nerves (Trifaró et al., 1992). Moreover, electron-translucent vesicles have been described in

chromaffin cells (Navone et al., 1986,1989). However, the function of these vesicles have not been established. Eranko (1955) originally distinguished NA from A organelles that in NA containing granules were more dense than A containing granules after fixation with gluteraldehyde-osmium tetroxide. Additionally, morphological examination shows NA and A containing vesicles have been found that in NA storage vesicles tend to exhibit an irregular appearance while A storage vesicles are spherical. The distribution of the two types of cells in the medulla is such that in most mammals, A containing cells comprise 85-95% of the chromaffin cell population, but it should be noted that extensive species variation does exist (Benchimol and Cantin, 1977). The function of SGC, which were initially isolated in the mouse adrenal gland (Kobayashi and Coupland, 1977), has not been conclusively established but some investigators have proposed that these cells may act as interneurons or being cells with both neuronal and adrenal cell phenotypes. At the electron microscopy level, the appearance of the vesicles in SGC seem to suggest, that, these cells possess both A and NA (Coupland, 1989). Small intense fluorescence (SIF) cells are divided into two types: type I and type II (Chiba and Williams, 1975). SIF type II cells are virtually indistinguishable from chromaffin cells, while SIF type I cells resemble chromaffin cells which have been exposed to NGF or glucocorticoids (Kobayashi and Coupland, 1977; Kobayashi et al, 1978). In cholinergic and other neurons housing both LDCV and small electron-translucent vesicles, the latter are thought to store classical neurotransmitters (acetylcholine, noradrenaline, glutamate, serotonin and GABA), whereas the former are thought to contain peptides (Fillenz, 1971). Whether this is true for chromaffin cells remains to be elucidated.

B. Culture Chromaffin Cell: A Model 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, and biochemical aspects of the secretory process. The reason is mainly based on the relative ease with which large homogeneous populations of these cells can be isolated and different type of experiments can be performed on them. As a system for studying neuronal and endocrine function, the chromaffin cell has the advantage of sharing primordial origin with sympathetic neurons and, on the other hand, possessing common ultrastructural features with the endocrine cell. Fujita and Kobayashi (1975) introduced the term "paraneuron" for this type of cells. The paraneuron family includes gastroenteric endocrine cells, adrenal chromaffin cells, mast cells, melanocytes, pancreatic islet cells, pinealocytes, adenohypopophysial cells, parafollicular cells and Merkel cells (Fujita, 1980). Figure 1 shows the similarities between this family and the neurons. Isolated chromaffin cells maintained in culture for long time begin to develop processes which increase in length in proportion to the time in culture (Trifaró and Lee, 1980; Livett et al., 1983). These processes are neurite-like in nature and contain high level of catecholamines and neuropeptides (Trifaró and Lee, 1980).

C. Secretory Process in the Adrenal Chromaffin Cell

It is clear that the major activity of chromaffin cells is to secret primary amines and neuropeptides in response to appropriate stimuli (Knight and Bader, 1987), and the main

organelle involved in the secretory response is the chromaffin secretory vesicle which consists of a membrane and a soluble content (Winkler, 1976). In addition to adrenaline and noradrenaline, chromaffin granules contain a diverse mixture of proteins, such as chromogranin A, B, and C, enkephalins and neuropeptides, that may have widespread effects on other organs. The content of the chromaffin granule is thought to be released to the exterior by the process of exocytosis. A lot of evidence supports the idea that exocytosis is the mechanism for secretion. Direct evidence for exocytosis from studies of chromaffin cells of cattle (Trifaró et al., 1967; Dowd and Edwards, 1981; Englert et al., 1982) and rat (Edwards et al., 1982) has been published. Biochemical studies suggest that all soluble components of the secretory vesicles are released together (Douglas and Rubin, 1961; Trifaró et al., 1967) and there is a good correlation between incorporation of vesicle membrane antigens into the plasma membrane and secretion (Lingg et al., 1983; Phillips et al., 1983). Electron microscopy and image analysis microscopy gave a clear appearance of vesicles bursting open at the surface of cultured cells (Ornberg and Reese, 1981). These findings are further substantiated by data from studies utilizing false transmitters, whereby chemically modified transmitter substances fail to be secreted if they are not stored within secretory vesicles (Smith and Winkler, 1972). More recently, electrical capacitance measurements of chromaffin cell membranes under conditions favouring secretion have proven that vesicles do fuse with plasma membrane prior to the release of their contents (Fernandez et al., 1984). Extensive research on the secretory mechanisms has established exocytosis as the final phase of stimulus-secretion coupling in secretory cells as well as the primary release mechanism for all neurotransmitters and hormones, except for the steroids secreted by adrenal cortical cells and gonads (Trifaró, 1977; Trifaró and Cubbedu, 1979; Trifaró and Poisner, 1982; Knight and Baker, 1987).

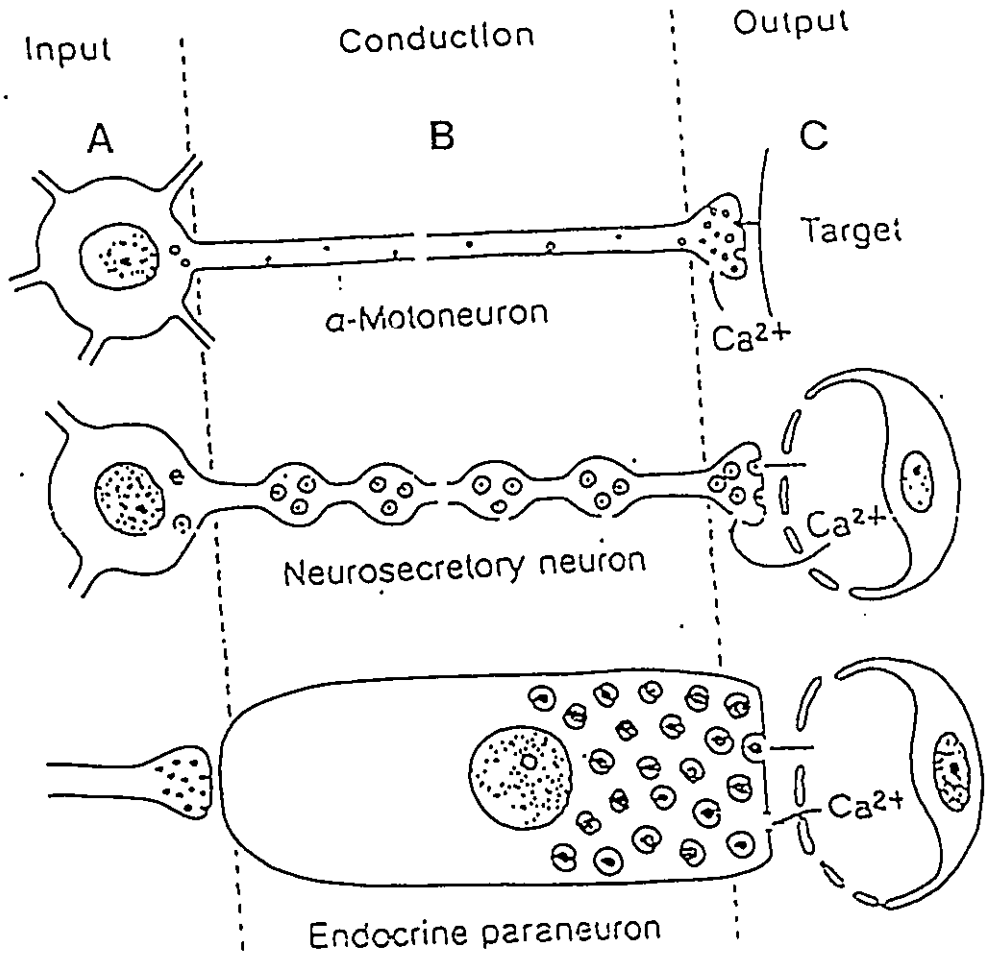


Fig.1 Illustration of the existing similarities between an α-motoneuron, neurosecretory cell and paraneuron of endocrine origin. Also depicted are the input portion of the cell which is the site of action of various stimuli (A), the conducting region and hence, the site of nerve impulse propagation (B)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

It has become clear in recent years that secretion by cells can take two forms: constitutive and regulated (Tartakoff et al., 1978; Gumbiner and Kelly, 1982; Green and Shields, 1984; Kelly, 1985). Constitutive secretion is the form of secretion which is unregulated and closely follows the rate of synthesis of secretory products. The other form of secretion is highly regulated and is triggered by an increase in intracellular calcium.

Several requirements do exist in order for exocytosis to occur which indicate the involvement of second messenger systems. Houssay and Molinelli (1928) first mentioned the pivotal role of Ca^{2+} and this hypothesis was confirmed by studies conducted by Douglas and Rubin (1961) who showed that if Ca^{2+} was absent from the extracellular milieu, catecholamine secretion would not occur. 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). Ca^{2+} alone in the absence of other secretagogues in digitonin-permeabilized cells has been sufficient to elicit secretion (Burgoyne,1991). Other factors, such as phosphoinositides, GTP-binding proteins, and phospholipid-depend protein kinase C (PKC) may also be required for exocytosis for some kinds of cells. Phosphoinositides have been implicated to act as substrates for protein binding to membranes (Burgoyne, 1991; Rodríguez del Castillo et al., 1992a). It is now generally agreed that many agonists, including several neurotransmitters, do interact with receptors that are linked via a guanine nucleotides protein to phospholipase C (Nahorski, 1988; Berridge, 1987). The primary substrate for the initial receptor-mediated effect is phophatidylinositol (4,5)-bisphosphate (PIP_2) and the immediate products D-myo-inositol (1,4,5)-trisphosphate [$(1,4,5)IP_3$] and sn(1,2)-diacylglycerol (DAG) almost certainly play important second

messenger roles in many cells, including neurons and chromaffin cells. The subsequent increase in IP_3 then leads to release of Ca^{2+} from intracellular stores, while increase of DAG may activate the phospholipid-dependent protein kinase C (Berridge and Irvine, 1984; Berridge, 1987). Several lines of evidence demonstrated the involvement of IP_3 (Noble et al., 1986; Boarder and Challiss, 1992) and PKC (Burgoyne and Norman, 1984; Pocott et al, 1985; Bittner and Holz, 1990) in catecholamine release from chromaffin cells. Fig.2 shows a schematic model of second messengers which may be involved in catecholamine secretion in chromaffin cells.

D. Chromaffin Cell Cytoskeleton and Its Changes During Exocytosis

1. Cytoskeleton role on exocytosis

The cytoskeleton is a framework of cytoplasmic filaments which display ultrastructural functions in almost all cell types. Several aspects related to the organization and function of cytoskeletal filament networks and their associated proteins have been extensively reviewed (Stossel et al., 1985; Olive et al., 1985). Initial experiments tested the possible role of cytoskeleton in the process of secretion in pancreatic islet cells. These studies led to the finding that in these cells, there was a microfilament web localized beneath the plasma membrane (Orci et al., 1972). Although the requirement of Ca^{2+} in secretion was observed for couple of decades (Houssay and Molinelli, 1928; Harvey and MacIntoch, 1940; Douglas and Rubin, 1961; Douglas, 1968), the site of action of these divalent cation is still poorly understood. One attractive hypothesis is that the action of Ca^{2+} in secretion is mediated through the control of cellular cytoskeleton networks

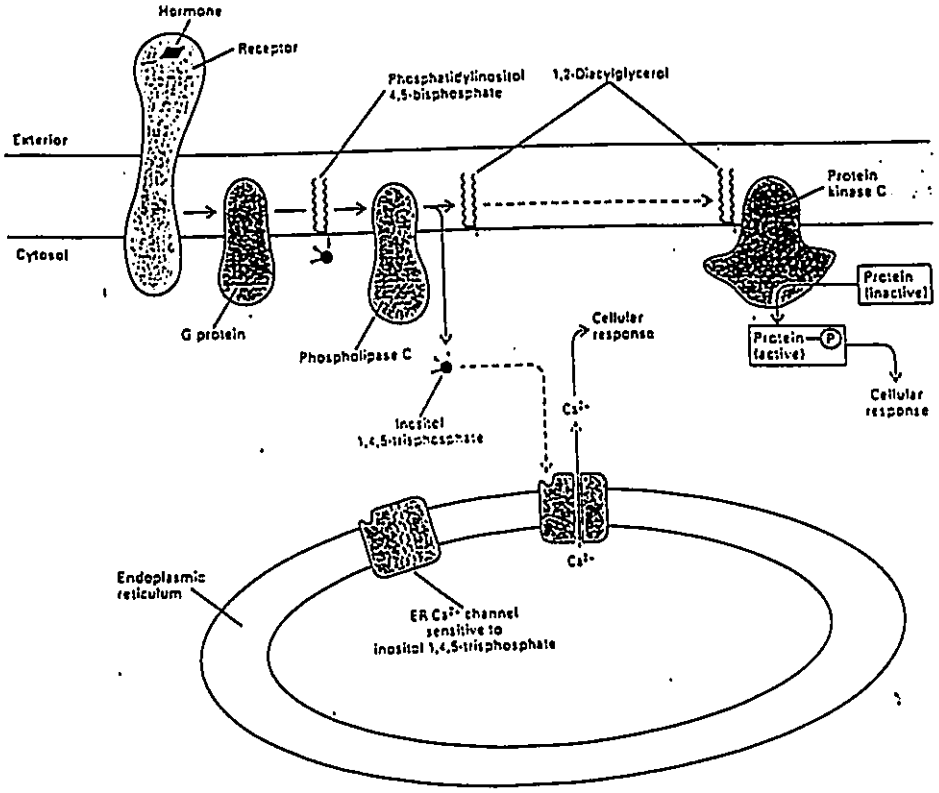


Fig 2. Illustration of a schematic model of second messengers which may be involved in catecholamine release from chromaffin cells (Taken from Berridge, 1985).

(Trifaró, 1990). A group of the specific proteins, some of which are previously known from studies on muscle, form a cytoplasmic network in all non-muscle cells including neurons and neurosecretory cells as chromaffin cells (Trifaró, 1978; Birchmeier, 1984). Immunocytochemical studies have shown at least three types of filament systems: microfilaments, microtubules, and intermediate filaments (Birchmeier, 1984). In addition, a large variety of cytoskeleton-associated proteins has been found and characterized (Trifaró et al., 1984; Trifaró et al., 1985a,b). These cytoskeleton-associated proteins can be classified according to their functions,ie., proteins that favour (actinogelin) or disrupt (gelsolin, scinderin) microfilament formation, proteins that serve as anchorage for cytoskeleton elements to membranes (vinculin, spectrin or fodrin, α-actinin, ankyrin), and proteins that regulate the phosphorylation of cytoskeleton elements (calmodulin).

2. Actin microfilaments and actin binding proteins

2.1 Actin

Actin is one of the main components of the chromaffin cell cytoskeleton and a major protein constituent of eukaryotic cells, comprising up to 10% of the cell's total protein content (Forscher, 1989). Work on the exposure of glycerol-treated chromaffin cells to fluorescent-labelled heavy meromyosin first suggested the presence of actin in chromaffin cells (Trifaró and Lee, 1978). Actin was isolated and characterized from the chromaffin cell cytosol by DNase I affinity chromatograph (Lee et al., 1979). A SDS-PAGE of the fraction eluted from DNase I column by 3M guanidine hydrochloride revealed two bands: a major one (92%) of molecular weight of 42 kDa, which comigrated with muscle actin,

and a minor one (10%) of molecular mass 90-91 Kda, which was probably an actin-binding proteins such as gelsolin (Lee et al., 1979). Actin has two isometric form (α and β) which have the same molecular weight but different isoelectric points (Lee et al., 1979). In the presence of ATP, actin monomers (42 Kda) can self-associate to yield helical polymers of actin (F-actin) that each individual polymer consists of two staggered, parallel rows and twists into a helix (Weeds, 1982; Forscher, 1989; Stossel, 1989). Antibodies against actin were also used to investigate localization of actin in cultured bovine chromaffin cells by indirect immunofluorescence (Trifaró and Lee, 1978; Aunis et al., 1980; Lee and Trifaró, 1981). The staining pattern showed actin widely distributed in the cells and associated with many cellular structures. A strong membrane and a weak cytosol fluorescence were observed when freshly isolated or 1-day-old cultured cells were used (Lee and Trifaró, 1981; Trifaró et al., 1984, 1989; Cheek and Burgoyne, 1986, 1987). More recent studies using rhodamine-labelled phalloidin, a substance with affinity only for filamentous actin, demonstrated the presence of fluorescence in the subplasmalemma area of chromaffin cells, confirming again the presence of an actin network in this region (Burgoyne and Cheek, 1987; Trifaró et al., 1989).

Actin antibodies were further used to localize actin at the electron microscopic level. The protein A-gold technique (Bendayan et al., 1982) used in these studies revealed the contribution of electron opaque gold particles in the proximity of the dense core granules, thus, suggesting that actin-binding sites were closely associated with secretory vesicles (Trifaró et al., 1985a,b). In addition to labelling around the granules, a small number of gold particles were also seen in the cell web underneath the plasma membrane. Furthermore, stereo electromicroscopy of polyethylene glycol-embedded rat adrenal medulla has revealed a three-dimensional lattice of microtrabeculae that is continued with

the surface of the chromaffin granules and also with the inner surface of the plasma membrane (Kondo et al., 1982).

2.2 α-Actinin and Fodrin

The interaction of actin with secretory vesicles needs the presence of actin anchorage proteins like those in the Z-line of the skeletal muscle. α-Actinin is the richest component in this area of muscle to serve as an anchorage for actin filaments. Antibodies against muscle α-actinin were used to investigate and localize the α-actinin in chromaffin cells (Trifaró et al., 1984, 1985a,b). The staining pattern obtained showed α-actinin distributing over a whole chromaffin cell body.Some diffuse fluorescence was detectable in the cytoplasm around the nucleus, but the nucleus itself was not stained. In addition, a punctate staining pattern was observed in the cytoplasm and along the neurites. The staining was very intense in growth cones where α-actinin appeared to accumulate. A similar staining pattern was observed when chromaffin cells were labelled with either dopamine β-hydroxylase (DβH) or chromogranin A antibodies to visualize secretory granule distribution (Trifaró et al, 1984, 1985a,b). This similarity suggested an association of α-actinin with chromaffin granule membranes. Therefore, an actin-binding protein similar to α-actinin should be expected to be present in the secretory granule organelles. An SDS-PAGE of chromaffin granule membranes showed the presence of a protein component that comigrated with purified muscle α-actinin (Trifaró et al, 1982, 1984, 1985a,b). Furthermore, an α-actinin-like protein was extracted from purified chromaffin granules and recognized by α-actinin antibodies in immunoblotting. This protein had molecular weight of 97 Kda and isoelectric point of 6.4. (Bader and Aunis,

1983; Trifaró et al, 1984, 1985a,b). In addition, the results from pronase digestion of intact and broken granules suggested localization on the cytoplasmic surface of the granule for both actin and α-actinin (Bader and Aunis, 1983). Chromaffin granule membranes contain significant number of actin nuclei that are able to promote actin polymerization and formation of membrane-bound actin filaments. Granule membranes preincubated with α-actinin antibodies showed a reduced number of binding sites, suggesting the absence of actin nuclei in those pretreated membranes (Trifaró et al, 1985b). Therefore, α-actinin molecules are either the nuclei themselves or they stabilize the actin nuclei, probably by anchoring these nuclei to the granule membrane (Trifaró et al, 1985b).

Recently, another actin-binding protein, fodrin, was found to be present in the cytoplasmic surface of chromaffin granules and on the inner surface of the plasma membrane (Aunis and Perrin, 1984). The presence of two different actin-binding proteins (α-actinin and fodrin) in granule membranes indicates the existence of two different actin-secretory granule associations, with each protein involving in one type (Trifaró, 1984b). In summary, actin microfilaments not only interact with the inner surface of the plasma membrane but also with the cytoplasmic surface of chromaffin granule, probably through their binding to α-actinin and fodrin. Moreover, chromaffin granule membranes are able to crosslink actin filaments and increases the viscosity of F-actin gels in a Ca^{2+}-dependent manner. Therefore, actin network seems to increase the viscosity of the cytoplasm and then prevents the free movement of secretory granules.

3. Chromaffin cell actin filament-severing proteins

3.1. Gelsolin

First isolated from rabbit lung macrophages, gelsolin is a globular protein that regulates the network structure of actin filaments (Yin and Stossel, 1979). In the presence of micromolar concentrations of Ca^{2+}, gelsolin binds to Ca^{2+} and actin microfilaments and prevents gelation of these filaments by actin-binding proteins (Yin and Stossel, 1979). Moreover, gelsolin is complexed with Ca^{2+} and binds the end of actin microfilaments, to which monomers add during elongation. The final average length of actin microfilaments formed in the presence of gelsolin-Ca^{2+} is shorter than that formed in their absence.

In early experiments on the isolation of chromaffin cell actin by DNase I affinity chromatography, it was noticed that in addition to actin, another polypeptide (91 Kda; Pi=6.0-6.2) was eluted from the column by the guanidine hydrochloride-containing buffer (Lee et al., 1979). In view of this, the early experiments on the isolation of chromaffin cell actin has been repeated. However, in this case, the DNase I affinity column was eluted first with an EGTA-containing buffer prior to elution with 3M guanidine hydrochloride. Under these conditions, the EGTA buffer eluted a 91 polypeptide. The 91 Kda polypeptide was immunologically (immunoblotting) reactive with antibodies against rabbit lung macrophage gelsolin (Trifaró et al., 1985a). Chromaffin cell gelsolin binds reversibly to actin and inhibits active polymerization in a Ca^{2+}-dependent fashion, as measured by the low shear falling ball viscometer technique (Bader et al., 1986; Trifaró et al., 1988). Work by electron microscopy has demonstrated that actin filaments obtained in the presence of Ca^{2+} and gelsolin were much shorter than those obtained in the absence of gelsolin (Bader et al., 1986; Trifaró et al., 1988).

3.2 Scinderin

This protein has been isolated and purified by others in our laboratory (Rodríguez Del Castillo et al., 1990). It is a Ca^{2+}-dependent actin-severing protein which binds to actin and severs actin filaments with a consequent decrease in the viscosity of actin gels. Trifaró and co-workers (1989) have named this protein "scinderin" (a name derived from Latin "scindere", mean to cut) (Rodríguez Del Castillo et al., 1990). The molecular weight of scinderin determined by SDS-PAGE is 79,600 $\pm$ 450 Daltons. Equilibrium dialysis experiments indicated that scinderin has 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).

The addition of scinderin did not modify the viscosity of actin solutions when Ca^{2+} was absent (Rodríguez Del Castillo et al, 1990). However, in the presence of Ca^{2+}, a decrease in the viscosity of actin solutions was observed with molar ratios of scinderin to actin higher than 1:3200. At the scinderin-actin molar ratio of 1:800, a decrease in viscosity from 480 to 10 cp was observed (Rodríguez Del Castillo et al., 1990). The effect of Ca^{2+} concentration on scinderin-induced decreases in actin viscosity was also tested at a fixed molar ratio (1:1600). Changes in the viscosity were observed between Ca^{2+} concentrations of 10^{-8} and 10^{-6}M, with the maximum fall in viscosity observed at 10^{-6}M Ca^{2+}.

The interaction of scinderin with actin has been also investigated by electron microscopy. Under conditions suitable for polymerization of actin, the addition of scinderin in the absence of Ca^{2+} made actin form a network of very long filaments. Short

filaments were never observed under these conditions. In contrast, only short filaments were observed when scinderin was added to actin networks in the presence of Ca^{2+} (Rodríguez Del Castillo et al., 1990). Under these conditions, the filaments observed were shorter than 0.8 μm with more than 50% of the filaments with lengths of 0.2 μm or shorter and the average filament length observed was 0.32 $\pm$ 0.04 μm (n=183). This length corresponds to filaments formed by approximately 58 actin monomers (Rodríguez Del Castillo et al., 1990).

Additional experiments indicated that two molecules of actin formed a complex with one molecule of scinderin and that this interaction was Ca^{2+}-dependent (Trifaró et al, 1992). This would suggest the presence of two actin-binding sites in scinderin. Moreover, two main fragments (40 and 38 Kda) can be obtained from scinderin by limited proteolytic digestion with chymotrypsin. Each fragment interacts with G-actin in a Ca^{2+}-dependent manner (Trifaró et al, 1992). The results suggest that each fragment contains an actin- and a Ca^{2+}-binding site. Furthermore, amino acid sequence experiments have also indicated that both scinderin and the 40 Kda fragment have their N-terminal blocked. Therefore, the 40 Kda fragment might be derived from the N-terminal region of scinderin (Trifaró et al, 1992).

Immunofluorescence studies with antiscinderin on cultured chromaffin cells showed a weak and diffused cytoplasmic staining and a strong fluorescence ring pattern at the cortical cytoplasmic region, thus suggesting a preferential subplasmalemmal localization for this actin-severing protein (Rodríguez Del Castillo et al., 1990; Vitale et al., 1991). A more diffused cortical cytoplasmic fluorescence pattern was obtained when antigelsolin was used (Vitale et al., 1991). Scinderin antibodies were also used to determine if scinderin was expressed in other tissues. Cytosol fractions from bovine brain, anterior

and posterior pituitary, kidney, salivary gland, testis, liver, skeletal muscle, heart muscle, platelet, and plasma were prepared and incubated in the presence of Ca^{2+} with actin DNase I Sepharose 4B beads (Tchakarov et al., 1990; Rodríguez Del Castillo et al., 1990). Immunoblots with antiscinderin antibody demonstrated the presence of scinderin in all of the above mentioned tissues with the exception of liver, plasma, and skeletal and heart muscles, thus suggesting that scinderin might be preferentially expressed in secretory tissues. Gelsolin, on the other hands, was expressed in all of the above tissues (Tchakarov et al., 1990).

3.3 Comparison of scinderin and gelsolin

Polyclonal antibodies raised against chromaffin cell gelsolin did not crossreact with scinderin when tested in immunoblots and vase versa (Rodríguez Del Castillo et al., 1990; Vitale et al., 1991). In addition to the difference in tissue distribution among these two actin filament-severing proteins (Tchakarov et al., 1990), other differences were found. Not only were the molecular weight and pI of gelsolin and scinderin different, but also, the one- and two-dimensional peptide maps obtained after limited proteolytic digestion with either Staphylococcus V8 protease or chymotrypsin showed marked differences in peptide composition and mobility (Rodríguez Del Castillo et al., 1990).

Two-dimensional electrophoresis showed the presence of three isoforms of pI = 5.8, 5.9, and 6.0 for adrenal medullary gelsolin and three distinct isoforms for scinderin (pI= 6.0, 6.1, 6.2). Upon incubation using a protein to enzyme ratio of 400:1, five fragments were obtained for gelsolin. Molecular weights and pI of these fragments were 80 (pI= 6.0), 50 (2 isoforms of pI= 5.5 and 5.7), 46.5 (pI= 5.5), 31 (pI= 5.5), and 16 (pI=

6.1).

Chymotrypsin digestion of scinderin under similar experimental conditions gave a quite different peptide pattern (Rodríguez Del Castillo et al, 1990). The polypeptide pattern showed two main proteolytic fragments of molecular weight 40 (2 isoforms of pI= 6.0 and 6.1) and 38 Kda (2 isoforms of pI= 5.7 and 5.9) and a small 32 Kda (pI= 5.8) fragment (Rodríguez Del Castillo et al, 1990). The amino acid composition of purified scinderin was also determined and compared with gelsolin composition (Rodríguez Del Castillo et al, 1990). The content of lysine is lower in scinderin than in gelsolin; however, scinderin is a more basic protein than gelsolin, since the content of acidic residues (aspartic and glutamic) is much greater in the latter (Rodríguez Del Castillo et al, 1990). Moreover, the content of isoleucine and tyrosine residues is higher in scinderin than in gelsolin, thus indicating that scinderin cannot be a breakdown product of gelsolin (Rodríguez Del Castillo et al, 1990).

Most recent analysis of nucleotide sequences has revealed that scinderin was at least 67% homology with gelsolin (Marcu et al., 1994). As gelsolin, scinderin has internal repeats of shorter sequence motifs which occur six times as approximately equal intervals (Marcu et al., 1994). A sequence of a peptide obtained by Edman degradation of scinderin was also identified. The N-terminal half of the molecule is the functional domain of scinderin and two sequences corresponding to actin-binding domains are present together with two other additional PPI binding domains. One of them shows the consensus sequence R(X)XXXKXRR, typical PPI binding sites of phospholipase C and gelsolin (Marcu et al., 1994).

4. Dynamic changes in cytoskeleton during exocytosis

Previous publications have demonstrated that filamentous actin is mainly localized in the cortical surface of the chromaffin cells (Lee and Trifaró, 1981; Trifaró et al., 1984, 1989; Cheek and Burgoyne, 1986). Trifaró et al (1984, 1989) have also suggested that cortical F-actin acts as a barrier to the secretory vesicles, impeding their contact with the plasma membranes. Chromaffin vesicles contain α-actinin and fodrin, anchorage proteins which mediate filamentous actin association with these vesicles (Aunis et al, 1980; Trifaró et al, 1982; Perrin and Aunis, 1985).

Stimulation of chromaffin cells produces disassembly of actin networks and removal of the barrier (Cheek and Burgoyne, 1986,1987; Burgoyne et al., 1989; Trifaró et al., 1982, 1984, 1989). This interpretation is based on the following evidence. Immunocytochemical experiments with rhodamine labelled phalloidin and actin antibodies indicate that in resting chromaffin cells a filamentous actin network is visualized as a strong cortical fluorescent ring (Lee and Trifaró, 1981; Cheek and Burgoyne, 1986,1987; Trifaró et al., 1989). Nicotinic and high K^+ stimulation showed a fragmentation of the fluorescence ring with areas devoid of fluorescence in the subplasmalemmal area. DNase I inhibition assay also showed the decrease in F-actin with a concomitant increase in G-actin. Furthermore, there was also a decrease in the amount of F-actin recovered with the Triton X-100 insoluble protein. F-actin network disassembly has also been observed in mast cells upon stimulation (Koffer et al, 1990) and in depolarized synaptosomes (Bernstein and Bamburg, 1985). Moreover, the presence of scinderin and gelsolin suggests that disassembly of F-actin network upon stimulation may due to activation either or both of these two actin filament-severing proteins. Immunocytochemistry

experiments utilizing scinderin and gelsolin antibodies shows scinderin distribution in control cells as a bright and continuous cortical fluorescent ring and a less intense and diffused cytoplasmic fluorescence. Nicotinic stimulation causes fragmentation of the bright fluorescence ring and patches of scinderin fluorescence, appear as a fragmented fluorescent ring at the equatorial plane of the cells (Vitale et al., 1991). Stimulation also caused a disruption of the rhodamine phalloidin cortical fluorescent pattern, suggesting depolymerization of F-actin. Under nicotine stimulation, cells showing scinderin redistribution also show a concomitant F-actin disassembly. On the other hand, cells stained with antigelsolin showed a cortical cytoplasmic fluorescent patterns and fluorescent rings under the plasma membrane which were weaker than those observed in antiscinderin stained cells. In this case, nicotine stimulation of chromaffin cells did not cause any reorganization of gelsolin cortical fluorescence. When a mouse monoclonal antibody against the 47-Kda chymotrypsin fragment of gelsolin was used, a similar result was obtained (Vitale et al., 1991). Therefore, the above results suggested that only scinderin was involved upon nicotine stimulation on chromaffin cells.

Scinderin redistribution can be seen as early as 5 sec of stimulation (Vitale et al, 1991). Maximum effects on scinderin redistribution can be seen at 20 sec (high K^+) to 40s (nicotine) stimulation. Catecholamine output can also be seen as a sharp increase upon either nicotine or high K^+ stimulation, but scinderin redistribution and actin disassembly always precede catecholamine release (Vitale et al., 1991).

The similar rates of actin disassembly and scinderin redistribution and concomitant localization of scinderin and actin upon stimulation suggest that scinderin shows a subplamalemmal distribution in resting cells because it is bound to filamentous actin. On the other hand, after removal of the stimulus, the rate of recovery of scinderin cortical

fluorescence was faster than that of rhodamine phalloidin fluorescence (Vitale et al., 1991). This would suggest that scinderin is retained in the cortical region of the resting cells through its binding to a site other than the filamentous actin. It is known that Ca^{2+} increase is absolute requirement for catecholamine release and nicotine and high K^+ induce extracellular Ca^{2+} influx and then the rise in cytosolic Ca^{2+} (Douglas and Rubin, 1961; Trifaró and Bourne, 1981; Cheek et al., 1989; Kim and Westhead, 1989). On the other hand, muscarine treatment does increase Ca^{2+} from intracellular stores (Wilson and Kirshner, 1977; Kim and Westhead, 1989) but fail to release catecholamines (Wilson and Kirshner, 1977; Fisher et al., 1981) or induce scinderin redistribution and F-actin disassembly (Vitale et al., 1991). It is believed that muscarine effect is independent of extracellular Ca^{2+} (Kao and Schneider, 1985) and mediated by inositol 1,4,5-trisphosphate (Hughes and Putney, 1990). Thus, the intracellular release of Ca^{2+} induced by muscarine in bovine chromaffin cells is not enough to trigger either catecholamine release or F-actin disassembly (Vitale et al., 1991).

E. Histamine: Another Secretagogue for Studying the Molecular Events Involved in Exocytosis.

In 1926, Burn and Dale reported that the secondary rise in blood pressure produced by histamine in anaesthetized cats was abolished by adrenalectomy and suggested that this rise in blood pressure was due to histamine-evoked catecholamine release (Burn and Dale, 1926). Histamine is secreted from mast cells and basophils in response to the binding of antigens to IgE molecules on the surface of these cells (Choi et al., 1993).

The release of histamine from mast cells plays a role in immediate hypersensitivity or allergic reactions (Dale, 1920). Prominent pharmacological effects of histamine include vasodilatation, bronchoconstriction, and catecholamine secretion (Choi et al., 1993). Subsequently, Wada et al. (1940) demonstrated that histamine increased adrenaline levels in the adrenal venous blood of dogs. Although histamine may stimulate adrenal catecholamine secretion in part by increasing splanchnic nerve activity, studies with isolated perfused adrenal glands (Schneider, 1969) and chromaffin cells (Livett and Marley, 1986; Noble et al., 1988) revealed that histamine can stimulate catecholamine secretion by acting directly on chromaffin cells. Furthermore, histamine is also found within the adrenal gland itself (Endo & Ogwa, 1974) and has recently been localized by immunohistochemistry to the noradrenaline-containing chromaffin cells in the rat (Happola et al., 1985). It is possible, therefore, that histamine may play a local regulatory role within the adrenal medulla.

In addition to nicotine and high K^+ as previously described in the study of chromaffin cell exocytosis (Vitale et al., 1991, 1992a,b; Rodríguez Del Castillo, et al., 1990, 1992a), histamine is another chromaffin cell secretagogue (Noble et al, 1986, 1988; Marley et al, 1991; Choi et al., 1993). At least two types of histamine receptors (H_1 and H_2) so far have been described in chromaffin cells (Noble et al., 1986, 1988; Choi et al., 1993; Marley et al., 1991). H_1 receptors seem to mediate catecholamine release (Choi et al., 1993) and PIP_2 breakdown (Noble et al., 1986) whereas H_2 receptor activation produces increases in cAMP (Marley et al., 1991). Stimulation of chromaffin cells with histamine may increase intracellular Ca^{2+} by at least two mechanisms: an increase in extracellular Ca^{2+} influx and a release of Ca^{2+} from intracellular stores (Livett & Marley, 1986; Staudermann and Murawsky, 1991; Bunn & Boyd, 1992). However, conflicting

evidence exists as to the source of Ca^{2+} involved in the secretory response to histamine (Bunn & Boyd, 1992; Borges, 1993; Cheek et al., 1993).

F. Statement of the Problem

As discussed above, adrenal medullary chromaffin cells possess a network of actin filaments localized underneath the plasma membrane. This actin network is thought to serve as a physical obstruction which prevents the moving of the secretory vesicle to plasma membrane. Release of the granule contents to the exterior milieu via exocytosis requires that the granules be able to interact with the plasma membrane. This interaction cannot occur unless the cortical actin barrier is removed. Previous studies have proved that nicotinic stimulation of chromaffin cells causes disassembly of cortical F-actin networks and redistribution of scinderin, a Ca^{2+}-dependent filament-severing protein (Trifaró et al., 1989b; 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). The existence of endogenous actin-binding proteins (scinderin) which regulate actin network dynamics (Rodríguez Del Castillo et al., 1990) strongly suggests a role for these proteins in the stimulus-evoked disassembly of actin filaments. Furthermore, the Ca^{2+} signal to trigger exocytosis seems to influence actin dynamics by modulating the activity of endogenous actin-regulatory proteins (Forscher, 1989; Rodríguez Del Castillo et al., 1990). Therefore, it could be proposed that the cortical cytoskeleton may represent a target for other secretagogues or second messengers and a strategic site for the control of catecholamine release.

In view of the existence of at least two types of histamine receptors in chromaffin

cells and of the observation that histamine increases intracellular Ca^{2+} by two distinct mechanisms, it becomes important to determine whether histamine stimulation induces scinderin redistribution, F-actin disassembly and catecholamine release as we described above for nicotine and high K^+ and, if this is the case, which receptor subtype, source of Ca^{2+}, and second messenger systems are involved in the response. Therefore, the present project was undertaken, using histamine as secretagogue, for the following purposes.

1. To examine the effect of histamine on scinderin redistribution and cortical F-actin disassembly and determine the role of extracellular Ca^{2+} in the response to histamine.
2. To determine whether the catecholamine release stimulated by histamine also correlates with scinderin redistribution and F-actin disassembly.
3. To determine the receptor type involved in histamine-evoked effects.
4. To examine whether histamine acts on either adrenaline-containing cells, noradrenaline-containing cells or both.
5. To observe whether PKC is involved in histamine-evoked responses.
6. To study the role of PLC-PIP_2 cascade pathway in histamine-induced responses.

CHAPTER II

METHODS AND MATERIALS

Chromaffin Cell Culture

1. Isolation of chromaffin cells

A homogeneous population of bovine adrenal chromaffin cells was obtained as previously described by Trifaró and Lee (1980). Glands were rinsed at the local slaughterhouse with buffer I (Ca^{2+},Mg^{2+}-free Locke's buffer in millimolar: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.50; glucose, 10; pH 7.0; plus antibiotics: 200mg/l penicillin, 50mg/l streptomycin, 50mg/l gentamycin, 5 mM Hepes and 15 mg/l Phenol red). On arrival at the laboratory, glands were rinsed again and perfused with the same buffer via a polyethylene tube inserted in the central adrenal vein for 10 min. The glands were then dissected and cortexes were removed. Each gland was then perfused utilizing a closed circuit perfusion chamber for 60 min with 25-30 ml enzyme mixture previously sterilized through a millipore filter at 37^0C at a rate of 10 ml/min. The enzyme mixture consisted of 5.3 mg DNase I, 548.13 U/gland collagenase and 25,000 U/L mycostatin in 400 ml above buffer I. Once flaccid, the remaining cortex of each gland was removed and then the medullae were minced. The minced medullae were transferred to a "Trypsinizing flask" containing the same enzyme mixture pre-warmed and stirred for 30 min in a water bath at 37^0C. Tissue was then filtered and rinsed through a 44 μ sterile cloth mesh into a graduated cylinder containing buffer II (buffer I plus 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, pH 7.2). Cell number was determined by using a haemocytometer (Neubauer, Levy chamber Cat. No. 500) in order to determine the number of the Percoll

gradient to be used. The above solution was centrifuged 10 min at room temperature at 50 x g and the sediment was washed again with 100 ml buffer II and centrifuged again at 50 x g for 15 min at room temperature. The sediment was then suspended in Eagle's Balance Salt Solution (EBSS 1x) containing antibiotics (Penicillin 100mg/l, Streptomycin 100mg/l, Mystatin 25,000U/l, Gentamycin 50mg/l). According to number of the Percoll gradients to be used, the suspension was mixed with Percoll (colloidal silica coated with polyvinylpyrrolidone at pH 7.2) and EBSS(10x) in an ratio 8:9:1 respectively. The mixture was centrifuged in a Sorvall centrifuge at 45,000 x g at 20^0C for 20 min. Fig.3 showed a diagram of a self-generating Percoll gradient. To obtain a desired chromaffin cell population, 4.5 ml of the top of the gradient was discarded and the chromaffin cell bands were collected (Fig.3). The gradient fractions were then diluted with 5 vol of EBSS (1x) containing antibiotics and centrifuged again at 50 x g at room temperature for 15 min. The supernatant was then discarded and the sediment was washed once with EBSS (1x) containing antibiotics as previously described. An aliquot was removed and cell number was determined to calculate the cell yield prior to plating.

2. Chromaffin cell culture

The final cell sediment was suspended in Dulbecco's modified Eagle's medium (DMEM: 10% fetal calf serum, 0.1mM ascorbic acid, 0.1% glucose and 15mM Hepes, pH 7.2) containing 100μg/ml penicillin, 100μg/ml streptomycin, 50μg/ml gentamycin, 25,000U/l nystatin, 10mM 5-fluorodeoxyuridine, and 10mM cytosar. Cells utilized for

immunohistochemistry were plated on collagen-coated glass coverslips at a density of $0.3x10^6$ cells/35-mm diameter dish while those employed for catecholamine output studies were plated on collagen-coated plastic Petri dishes at a density of $1x10^6$/35-mm diameter dish. Cells were incubated at 37^0C in a NAPCO 6300 humidified incubator under a CO_2 + air atmosphere (5% : 95%) for an initial period of 24h for cell attachment. Once the cells had adhered to the collagen-coated surfaces they were fed every 2-3 days with the medium described above. The age of the cultured chromaffin cells utilized in all studies described in this thesis was between 48-72h.

Immunocytochemistry

1. Source of antibodies:

Scinderin antiserum was produced by raising polyclonal antibodies against purified adrenal medullary scinderin in rabbits. (Rodríguez del Castillo et al., 1990). The antiserum used in this thesis was derived from a bleeding obtained from rabbit #6. Scinderin antiserum #6 has been purified and determined to possess a high degree of specificity toward scinderin, since it does not cross-react with other cytoskeletal proteins (Vitale et al., 1991) or actin-binding proteins such as gelsolin (Rodríguez del Castillo et al., 1990; Tchakarov et al., 1990). Anti-bovine PNMT antiserum was a generous gift from Dr.Dona Lee Wong (Department of Psychiatry and Behaviour Sciences, Stanford

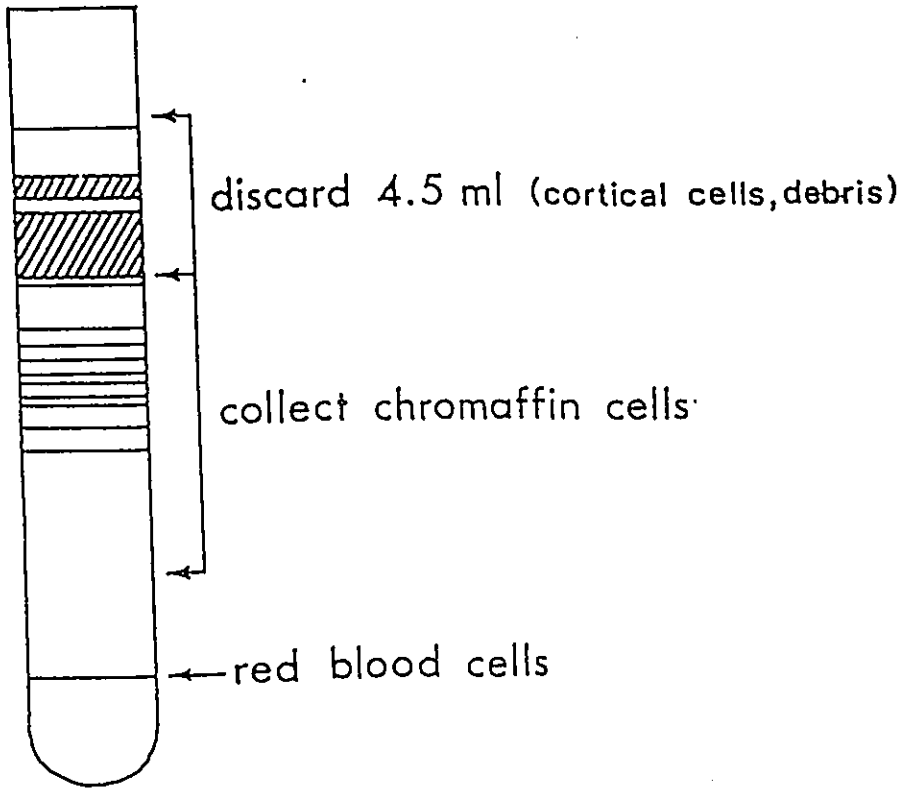


Fig.3 Illustration depicting the layers formed in the self-generating Percoll density gradient employed in the isolation of chromaffin cells. The top layer is discarded and contains cortical cells, cellular debris and fibroblasts allowing isolation of the second layer which contains the chromaffin cell population.

University Medical Center) and mouse monoclonal anti-DβH was a kind present from Dr.B. Tugal (Department of Biochemistry, University of Edinburgh).

2. Immunofluorescence Microscopy Studies

Cultured chromaffin cells were rinsed three times with 1 ml of regular Locke's solution. When Ca^{2+}-free Locke's solution was used, Ca^{2+} was removed and 1 mM EGTA was added. Cells were then incubated with Locke's in the absence (control) or presence (stimulated) of different compounds for the incubation times outlined in each experimental protocol. Cells were immediately fixed in 3.7% formaldehyde in Locke's solution for 20 min at room temperature. Fixed chromaffin cells were subjected to 6 washes with phosphate-buffered saline (PBS in mM : NaCl, 130; Na_2HPO_4, 76; NaH_2PO_4, 24; pH 7.2), permeabilized by three successive exposure of 5 min each to 50%, 100%, and 50% acetone, and then rinsed again 6 times with PBS. The following steps were different for different kinds of labelling.

a. Single fluorescence-labelling of chromaffin cells with scinderin antiserum

After rinsing with PBS, preparations were incubated at 37^0C for 60 min with scinderin antiserum #6 (1:80 dilution). Then, cells were washed with PBS 6 times and incubated with goat-anti rabbit immunoglobulin G-fluorescein isothiocyanate conjugate (FITC-IgG, 1:160 dilution) for another 60 min at 37^0C. Coverslips were rinsed again 6 times in PBS and then mounted in glycerol-PBS (1:1; vol:vol). Control preparations were

incubated with either antiserum #6 scinderin or the second antibody alone.

b. Double labelling of chromaffin cells with scinderin antiserum and rhodamine-labelling phalloin

The first steps in the procedure were similar to those described above and after incubation with FITC-IgG, cells were then washed again 6 times with PBS and incubated for another 20 min at room temperature with rhodamine-labelled phalloin (1:250 dilution), a probe for filamentous actin (Faulstish et al., 1988). All other steps remained unchanged. Fig.4 shows the flow chart of double labelling with scinderin and F-actin fluorescence.

c. Double staining of chromaffin cells with Anti-PNMT antiserum and rhodamine-labelled phalloin

This method utilized the protocol b described above with substitution of scinderin antiserum #6 by anti-phenylethanolamine N-methyltransferase antiserum (PNMT, 1:1000 dilution).

d. Double labelling of chromaffin cells with antibodies against PNMT and DβH

After rinsing with PBS, cells were then incubated at 37^{0}C for 60 min with 1% BSA. Then, cells were washed with PBS for 6 times and incubated with anti-PNMT antiserum (1:1000 dilution) and anti-DβH IgG (1:10,000 dilution) at 37^{0}C for 60 min. The cells were then rinsed with PBS again and incubated with FITC-IgG (1:200 dilution) for PNMT and anti-mouse-IgG-TRITC (1:800 dilution) for DβH at 37^{0}C for 60 min.

Slides were observed with a Leitz-Ortholux II fluorescent microscope equipped with

a 200w high pressure lamp and a Ploemopack II incident light illuminator possessing an I-filter block (KP 490 plus 1 mm GG 455 exciting filter, TK dichroic beam splitting mirror, K 515 suppression filter) for fluorescein and an M-filter block (2 mm BG plus S 546 exciting filter, TK 580 dichroic beam splitting mirror, K 580 suppression filter) for rhodamine as previously reported (Rodríguez del Castillo et al., 1992a). One hundred spherical cells per coverslip were examined and classified as having either a discontinuous patched staining pattern in the cortical region of the cell (see Fig. 6) or a continuous cortical fluorescence "ring" (see Fig.6). To avoid personal bias, code numbers were assigned to each slide to be examined and the procedure was conducted without knowing whether cells being observed were from control or treated groups until all slides were examined (single blind design, Rodríguez del Castillo et al, 1992a). Once data were obtained, the percentage of chromaffin cells for each examined condition displaying "discontinuous" cortical fluorescence was graphically represented. In all cases 500-800 cells per condition were examined.

Video-Enhanced Microscopy of Chromaffin Cells

a. Equipment

Once cells viewed under the fluorescence microscope were again classified as having either "continuous" or " discontinuous" cortical scinderin and F-actin fluorescence, quantitative analysis of fluorescent intensity was conducted by using a Hamamatsu

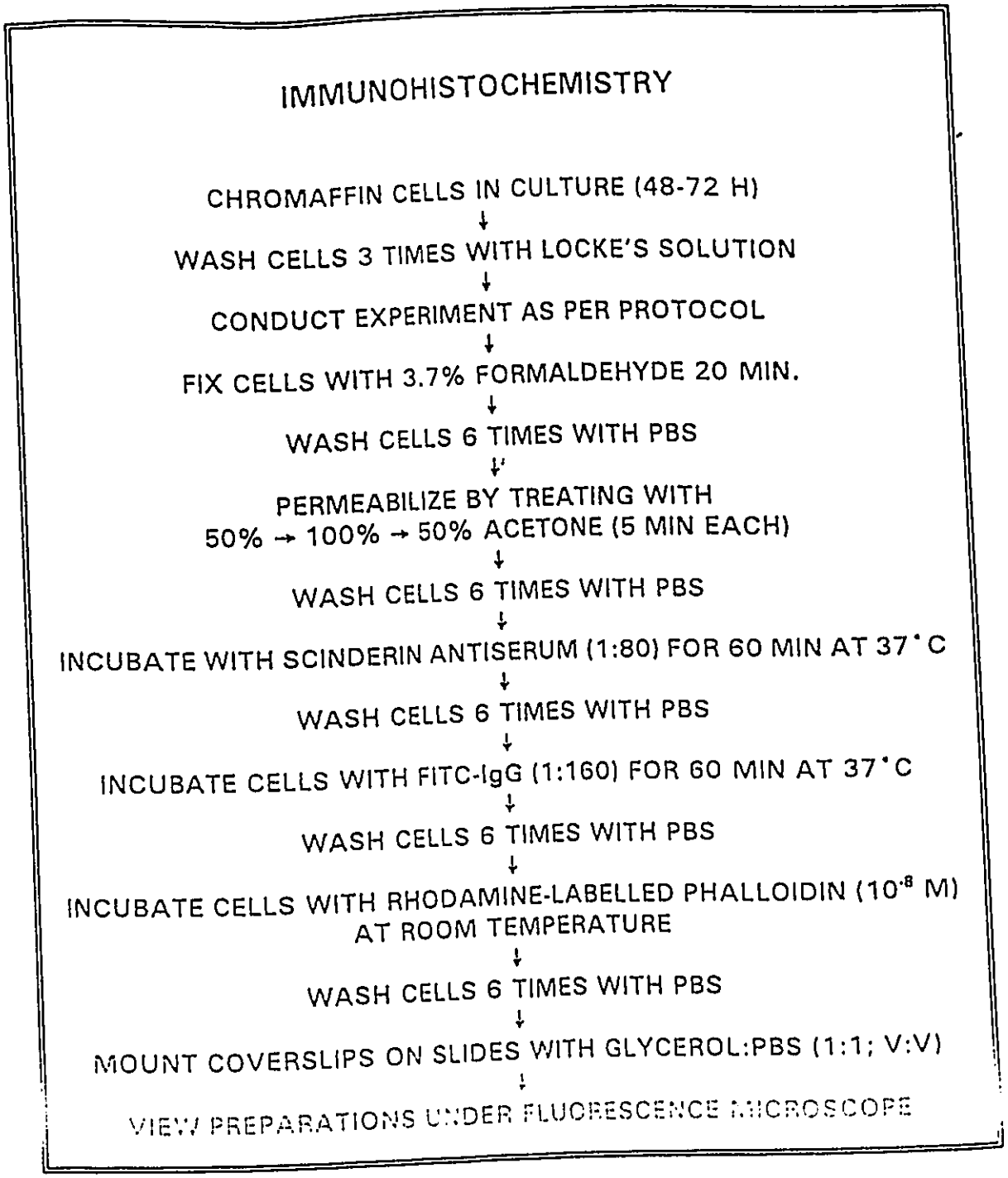
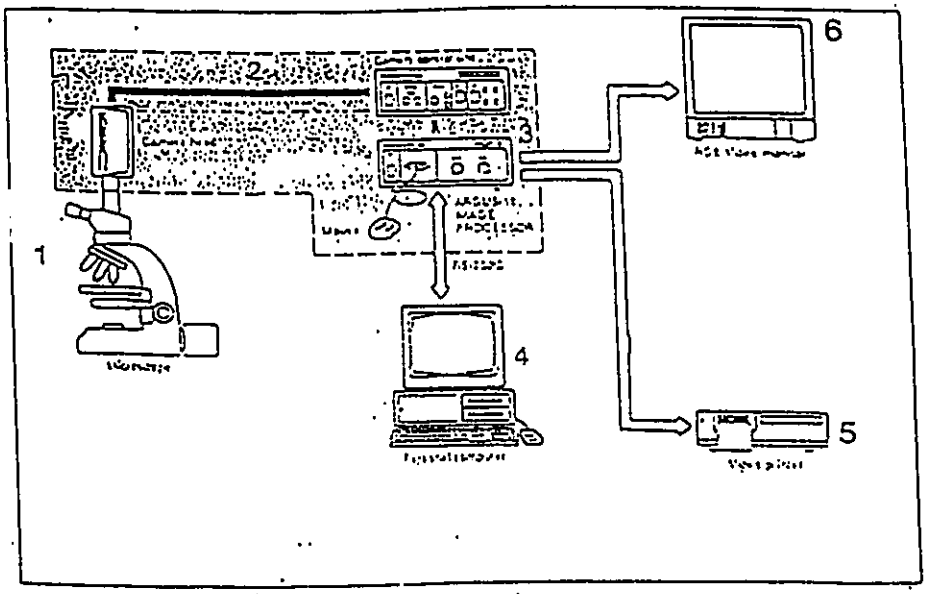


Fig.4 Flow chart describing the protocol followed in immunohistochemistry of chromaffin cell scinderin and F-actin fluorescence.

Photonics K.K. Argus-50/CL image processor. The components of the system were arranged as depicted in Fig.5. 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 Pro 386s/20 personal computer equipped with Argus-50 version 3.0 software for Windows.

b. Computer analysis of data and graphic representation

Analysis of fluorescent intensity of preparations using video-enhanced microscopy was conducted in a three-dimensional display option furnished by the Argus program. This program can transform a two-dimensional distribution of the intensities of a chromaffin cell displayed on the monitor 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 cell on the Z axis. The output from the image processor was displayed on a SONY Marvigraph UP-3000 colour Video Printer. Images of cells printed in pseudocolour were then analyzed according to fluorescence intensity.



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.5 Diagram of the video-enhanced microscopy system configuration employed to visualize variations in fluorescence intensity in resting and stimulated chromaffin cells (Taken from Ramirez-Lavergne, 1993)

Catecholamine Release Studies

Catecholamine output was determined by measuring [^{3}H]noradrenaline ([^{3}H]NA) as described previously by Trifaró and Lee (1980). 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 mM: NaCl, 110; NaHCO3, 40; KCl, 5; MgSO4, 1; NaH_2PO4, 1; Na-pyruvate, 1; $CaCl_2$,2; $Fe(NO_3)_3$, $2.5x10^{-4}$; ascorbic acid, 0.1; pH 7.2 adjusted with 0.6 g/ml Hepes) and then labelled by incubating with 0.75 ml of special medium containing 10^{-7}M [^{3}H]NA (42.7 Ci/mmol sp act; Amersham Canada, Oakville, Ontario) for 5 min at room temperature. Following the labelling of catecholamine stores, each dish was rinsed with 1 ml of regular Locke's solution six times for a total of 60 min prior to initiation of the experimental phase. Cells were then incubated with Locke's solution alone (control) or exposed to Locke's buffer containing the treatments prescribed for the different experiments. After incubations, the entire 1 ml incubation medium of each dish was collected and radioactivity of the sample was measured in a scintillation spectrometer. The total [^{3}H]NA cell content of each dish was determined by treating each dish with 1 ml of 10% Trichloroacetic acid (TCA) for 10 min followed by additional two washes with 0.5 ml of 6% TCA. Three aliquots were collected together into a Scintillation vial.

Ten ml of Cytoscinct scintillation fluid (ICN Biochmicals Inc.,Irvine, CA) was added to each vial and radioactivity was measured using a liquid scintillation spectrometer (Beckman Instruments Fullerton, CA).

Data analysis

In order to obtain total [^{3}H]NA cell content, the amount of [^{3}H]NA 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 of [^{3}H]NA released into the medium by the total [^{3}H]NA content. The sensitivity of this protocol was that it allowed for the determination of an amount of catecholamine released corresponding to 0.35% of total cell catecholamine content (Vitale et al., 1991). A minimum of 6 dishes were used per condition and the mean $\pm$ S.E.M. of each value obtained was plotted.

Measurement of Intracellular Ca^{2+}

Intracellular Ca^{2+} concentrations were measured by using the fluorescent Ca^{2+} indicator Fura-2 (Grynkiewicz et al., 1985).Cells attached to collagen-coated plastic petri dishes were used in this study. Chromaffin cells, grown at a density of 5 X 10^6/100 mm diameter dish, were incubated for 10s with assay buffer (in mM: NaCl, 118; KCl, 4.6; Hepes, 20 [pH 7.2]; $CaCl_2$, 1; D-glucose, 10) in the absence of Ca^{2+} but containing 5

mM EDTA. Then cells were incubated for an additional 2 min period, with Ca^{2+}-free assay buffer containing 2 mM EDTA. The cells were then resuspended with a pipette using assay buffer. Suspension was then centrifuged and the sediment containing the cells was resuspended with same buffer. The suspension was next incubated with Fura-2 (10^{-8}M, final concentration) for 1 hr in dark at room temperature. After the loading step, cells were rinsed three times with assay buffer and final suspension was adjusted to 2ml for each sample. Fluorescence of the cells was monitored using a dual wavelength luminescence spectrophometer (Ratio fluorescence microfluorometer model #RF-M2011, Photon Technology International Inc., London, Ontario, Canada). Drugs were added in 10-μl aliquots to obtain the final concentrations mentioned in the text.

Measurement of Inositol (1,4,5) Trisphosphate

The Inositol (1,4,5) Trisphosphate (IP_3) was measured using high-performance-liquid-chromatograph (HPLC), as previously described by Dean and Moyer (1987).

a) Preparation of [^{3}H]inositol-labelled chromaffin cells

Two million chromaffin cells/dish were used in this study. Cells were washed three times in special medium described before for catecholamine release studies and then labelled for 48 hr at 37^{0}C with 40 μCi/dish of myo-[^{3}H]inositol (105 Ci/mmol sp act; Amersham Canada, Oakville, Ontario). After loading, each dish was rinsed three times with Locke's solution and incubated in Locke's solution containing 10 mM LiCl for 10

min at room temperature. The cells were then exposed to different experimental conditions. 10% TCA was used to stop the reaction and dishes were kept on ice for 15 min. The suspension was centrifuged for 1 min at 11,000 X g and the supernatant was removed and extracted twice with water-saturated diethyl ether. The pH of samples was adjusted to 5 by NaOH before loading into the HPLC system.

b) IP_3 Measurement by HPLC

IP_3 levels were measured on a Waters Partisil SAX Anion Exchange column (8mm X 100mm, Waters Millipore, Mississauga, Ontario, Canada) with ammonium phosphate ($NH_4H_2PO_4$), pH 3.8, at a flow rate of 1ml/min. Briefly, the gradients used to elute $[^3H]IP$, $[^3H]IP_2$ and $[^3H]IP_3$ were of 0.01-0.08 M (20 min), 0.2-0.28 M (30 min) and 0.5-0.58 M (30 min) ammonium phosphate respectively. 1-ml fractions were collected and mixed with 15 ml aqueous scintillant Ecolite (ICN Biomedicals Inc., Irvine, CA). Radioactivity was determined by liquid scintillation spectrometer (Beckman Instrument Fullerton, CA).

MATERIALS

Products used for cell culture and 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, FITC-IgG, Anti-

mouse IgG TRITC, nicotine (Hydrogen tartrate salt), cimetidine, pyrilamine (Maleate Salt), thapsigargin and histamine (Dihydrochloride) were obtained from Sigma Chemical Company (St.Louis, MO, USA). DNAse I Type I was purchased from Boehringer (Laval, Quebec,Canada). Phospholipase C inhibitor U-73122 and its inactive isomer U-73343 were from Biomol Res. Lab. Inc. (Plymouth Meeting, PA, USA). Protein kinase C inhibitor Chlerythrine and Calphostin C were obtained from LC Laboratories (Woburn, MA, USA). [^{3}H]NA and Myo-[^{3}H]Inositol were from Amersham Canada (Oakville, ON, Canada). Rhodamine-labelled phalloidin and Fura-2 were purchased from Molecular probes Inc. (Eugene, OR, USA).

CHAPTER III

RESULTS

A. Immunocytochemical analysis of histamine effects on chromaffin cells

1. Effect of histamine stimulation on scinderin redistribution and F-actin disassembly.

Chromaffin cells cultured for 48 h were incubated in regular Locke's solution alone or in the presence of 100 μM histamine for 5, 20 and 40 s. At the end of these treatments, cells were processed for double-staining fluorescence microscopy using scinderin antibodies from rabbit #6 and rhodamine-labelled phalloidin, a probe for filamentous actin. As demonstrated in previous work (Vitale et al., 1991), scinderin distribution in control cells show a bright and continuous cortical fluorescence ring and a less intense and diffused cytoplasmic fluorescence (see Fig.6). Histamine stimulation causes a fragmentation of the bright fluorescent ring suggesting redistribution of cortical scinderin (Fig.6). Patches of scinderin appear clearly as a fragmented fluorescent ring at the equatorial plane of the cell. The effect of histamine on scinderin reorganization was seen as early as 5 s of stimulation. Furthermore, as shown before in work from this laboratory (Vitale et al., 1991), distribution of F-actin in resting chromaffin cells shows a continuous cortical fluorescent ring (Fig.6). Stimulation of cells with histamine produces a disruption in the rhodamine-phalloidin cortical fluorescence (Fig.6). The areas in which the fluorescent ring is interrupted correspond, as previously demonstrated for nicotinic stimulation (Vitale et al., 1991), to cortical areas of F-actin disassembly. Again, the effect of histamine on F-actin disassembly was also seen as early as 5s of stimulation.

Moreover, histamine stimulated cells show concomitant scinderin redistribution and cortical F-actin disassembly (Fig. 6).

These structural changes induced by histamine were measured for up to 40 s of stimulation and during this time scinderin redistribution and cortical F-actin disassembly were the same in the presence or in the absence of extracellular Ca^{2+} (Fig. 7, 8, 9). These results are in contrast to those obtained in our laboratory with nicotine stimulation. Nicotine-evoked scinderin redistribution and cortical F-actin disassembly have an absolute requirement for extracellular Ca^{2+} (Fig.7A). The above observation suggested the intracellular Ca^{2+} store as the source of Ca^{2+} in histamine responses. The magnitude of the dynamic changes in scinderin redistribution and cortical F-actin disassembly stimulated by histamine can be measured by using image analysis and this is illustrated in Fig.7B.

2. Time course of scinderin redistribution and cortical F-actin disassembly in response to histamine stimulation.

Time courses of scinderin redistribution and cortical F-actin disassembly are illustrated in Fig.8 and Fig.9. Chromaffin cells cultured for 48 h were incubated for 0, 5, 10, 20, 30 or 40s with 100 μM histamine followed by an additional 40, 35, 30, 20 and 10s respectively with regular Locke's solution with 2.2 mM Ca^{2+} or without (1 mM EGTA) the divalent cation. After these periods of incubation, cells were immediately fixed, permeabilized and processed for fluorescence microscopy using scinderin

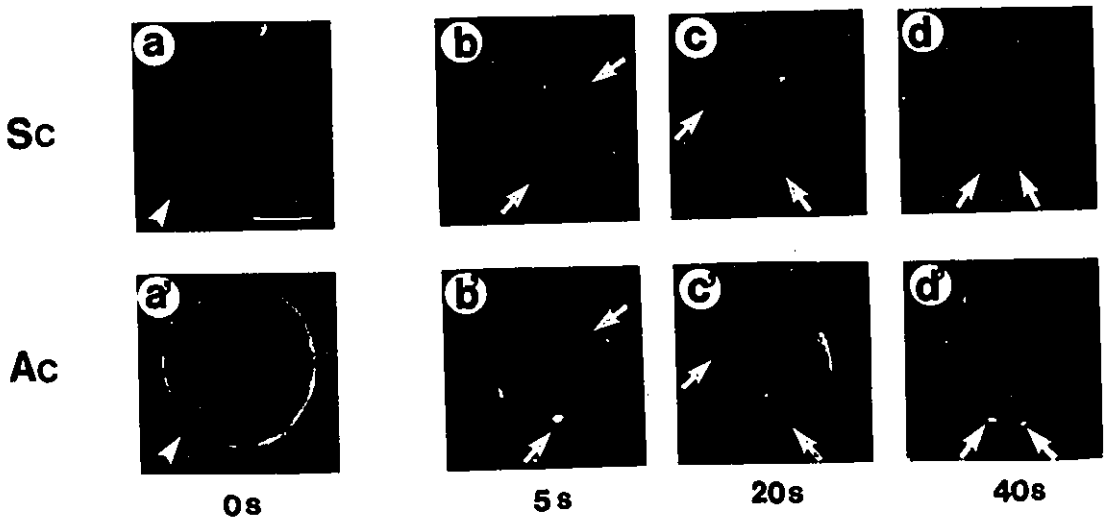


Fig.6 Localization of scinderin and F-actin by fluorescence microscopy. Chromaffin cells were incubated with Locke's solution in the absence (control 0s) or presence of 100 μM histamine for 5 (b,b'), 20 (c,c') or 40s (d,d'). Cells were sequentially stained with rhodamine-labelled phalloidin followed by scinderin antibody and FITC anti-rabbit IgG. A control cell in (a,a') shows continuous and intense rings of fluorescence for scinderin (a) and F-actin (a') colocalized at the subplasmalemmal region (arrowheads). Histamine stimulated cells display a disrupted cortical fluorescent pattern for scinderin (b,c,d) and F-actin (b,c,d'). There is a correspondence between the patched distribution of both scinderin and F-actin in each cell (compare b and b', c and c', and d and d'). Some patches are indicated by arrows. Bar, 10 μm.

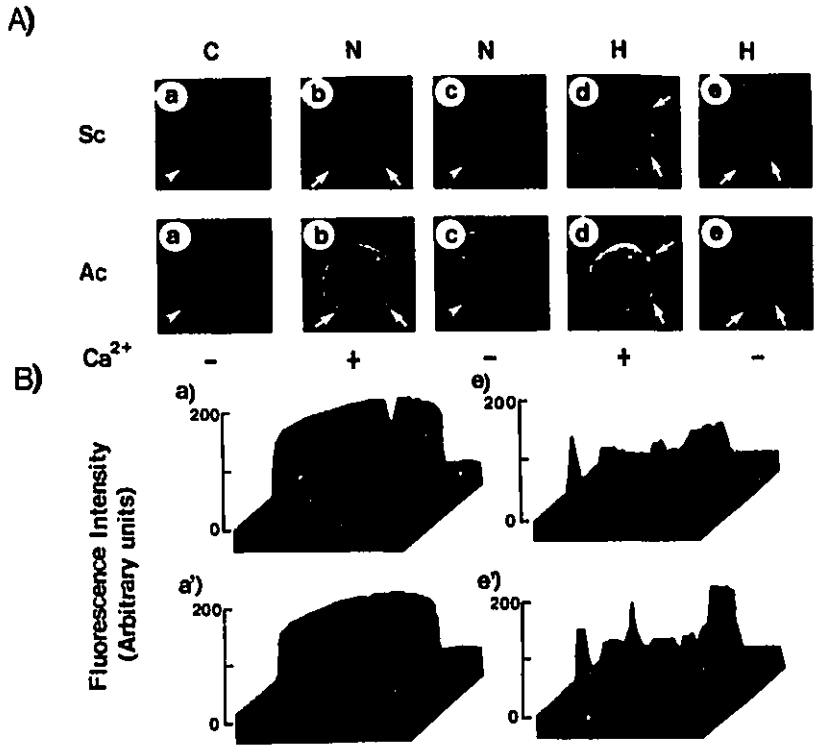


Fig.7 Effects of extracellular Ca^{2+} on the changes in the fluorescent patterns of scinderin and F-actin induced by either histamine or nicotine stimulation.

A) Two-day old chromaffin cells were incubated with Locke's solution in the absence (1 mM EGTA) or present of Ca^{2+} (2.2 mM). Cells were then stimulated with 10 μM nicotine (N) or 100 μM histamine (H) for 40s, both in the present or absence of Ca^{2+} and immediately processed for fluorescence microscopy using anti-scinderin and rhodamine phalloidin. A control cell (C) shows the characteristic rings of fluorescence for scinderin (a) and F-actin (a'). Cells stimulated by nicotine (N) show disrupted fluorescent rings (patched fluorescence) for scinderin (b) and F-actin (b') only in the presence of extracellular Ca^{2+}. On the contrary, cells incubated with histamine (H) show disrupted fluorescence rings for scinderin (d,e) and F-actin (d',e') in the presence as well as in the absence of extracellular Ca^{2+}.

B) Video-enhanced image analysis of scinderin and F-actin distribution in control (C, a and a') and the changes produced by histamine stimulation (H, e and e'). The three-dimensional plots show the distribution of the fluorescence intensity of single chromaffin cells under resting conditions (C) or after incubation with 100 μM histamine in the absence of Ca^{2+} in the medium. Bars, 10 μm.

antiserum #6 (Fig.8) and rhodamine-phalloidin (Fig.9) as described in Methods and Materials. Maximum responses were reached within 10s of stimulation and the effects were quite similar either in the presence or in the absence of extracellular Ca^{2+}. The results obtained with Ca^{2+}-free solutions suggested that histamine might evoke scinderin redistribution and cortical F-actin disassembly by increasing cytoplasmic Ca^{2+} levels through the release of the divalent cation from intracellular stores.

3. Responses to histamine are concentration-dependent.

Concentration-response curves of scinderin redistribution and cortical F-actin disassembly in response to histamine stimulation are illustrated in Fig.10. Chromaffin cells cultured for 48 h on collagen coated coverslips were incubated with Ca^{2+}-free Locke's solution for 40s in the presence of increasing concentrations of histamine. Responses to histamine stimulation are concentration-dependent and maximum responses (scinderin redistribution and F-actin disassembly) are reached at a histamine concentration of 10^{-3}M. The concentration of histamine used in the experiments described in this thesis (100 μM) produced nearly maximum effect as shown in Fig.10.

B. Time course of catecholamine output in response to histamine stimulation.

Fig. 11 shows the time course of catecholamine output in response to histamine stimulation. Chromaffin cells were cultured for 48 h and catecholamine stores were

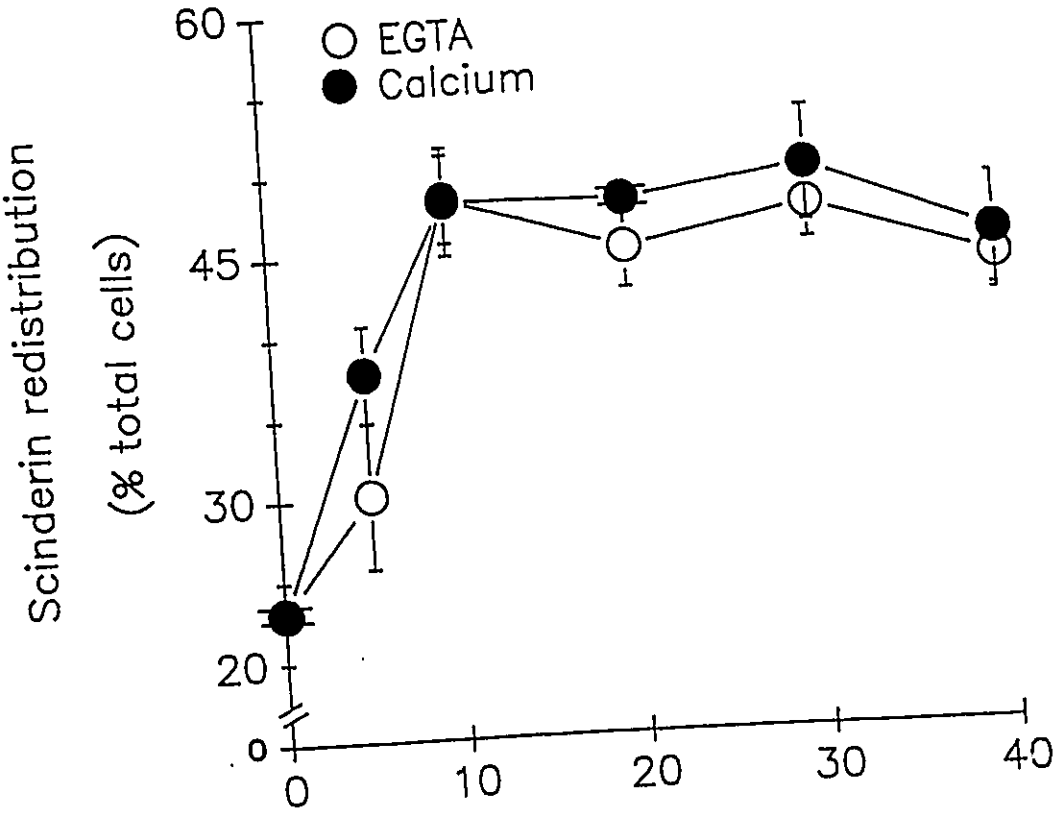


Fig.8 **Time course of scinderin redistribution in response to histamine stimulation in the presence or absence of extracellular Ca^{2+}.**

Chromaffin cells cultured for 48h were incubated for 0, 5, 10, 20, 30 or 40s with 100 μM histamine followed by an additional 40, 35, 30, 20, 10s respectively with regular Locke's solution with 2.2 mM Ca^{2+} or without (1 mM EGTA) the divalent cation. After these periods of incubation, cells were immediately fixed, permeabilized and processed for fluorescence microscopy using scinderin antiserum as described in Methods and Materials. One hundred cells per coverslip were examined and classified as having either a "continuous cortical fluorescent pattern" (see Fig.6, a,a') or a "discontinuous cortical fluorescent pattern" (see Fig.6, b-d,b'-d'). This was done as described in Methods and Materials. Each value shown represents the mean ± SEM of the percentage of discontinuous cortical fluorescent pattern of 6-8 coverslips (600-800 cells for each value) containing cells from 4 different chromaffin cell cultures.

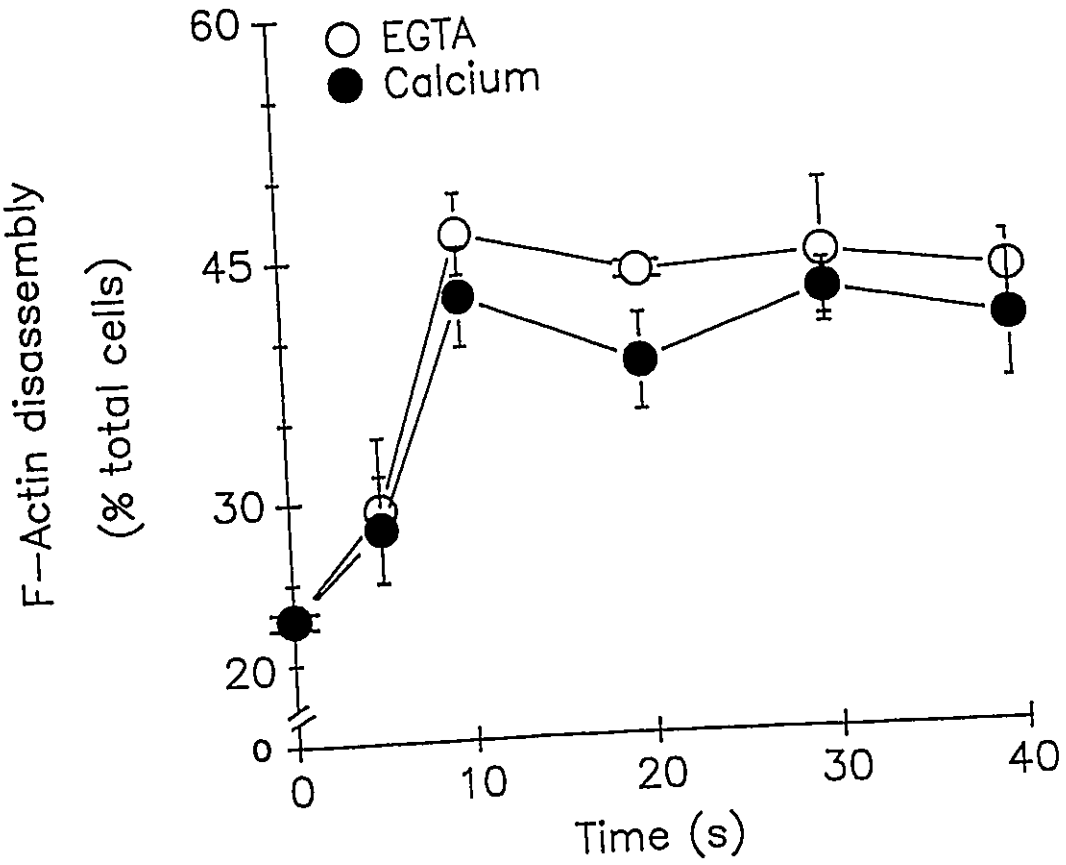


Fig.9 **Time course of F-actin disassembly in response to histamine stimulation in the presence or absence of extracellular Ca^{2+}.**
After the incubations cells were immediately fixed, permeabilized, and processed for fluorescence microscopy using rhodamine-phalloidin. Other conditions as described in legend to Fig.8.

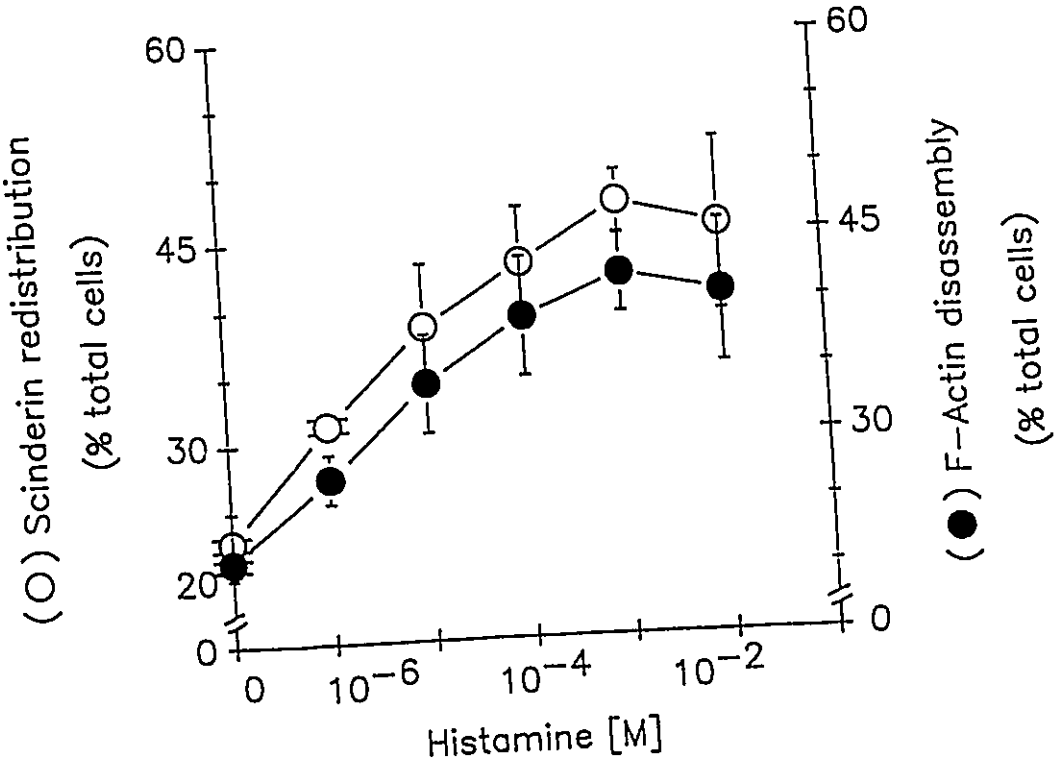


Fig.10 **Concentration-dependent effect of histamine-evoked scinderin redistribution and F-actin disassembly.**

Chromaffin cells cultured for 48h on collagen coated coverslips were incubated with Ca^{2+}-free Locke's solution for 40s in the present of increasing concentrations of histamine. After the incubations, cells were immediately fixed in 3.7% formaldehyde and stained for scinderin and F-actin fluorescence as described in Methods and Materials. Preparations were viewed under the fluorescence microscope and cells were classified as described in legend to Fig.8. Each value represents the mean ± SEM of the percentage of cells with disrupted fluorescent rings in 4-5 coverslips (400-500 cells examined for each value).

labelled with [^{3}H]NA as indicated in Methods and Materials. Cells were then incubated with Locke's solution containing 100 μM histamine in the presence of 2.2 mM Ca^{2+} or in the absence (1 mM EGTA) of the divalent cation. Significant catecholamine output was observed as early as 5s and maximum effect was reached on 20s. Again, catecholamine release was similar in the presence or absence of extracellular Ca^{2+}. These observations together with the results from immunocytochemical analysis on scinderin redistribution and F-actin disassembly suggest that the histamine might act through the release of Ca^{2+} from intracellular stores.

C. Comparison between nicotine and histamine stimulation on bovine chromaffin cells.

The previous work from our laboratory already demonstrated that nicotine induced effect on scinderin redistribution, F-actin disassembly along with catecholamine release. Above results from histamine responses showed that histamine had similar response on scinderin redistribution, F-actin disassembly, and catecholamine release. It is quite reasonable to compare these two secretagogues' effect on bovine chromaffin cells. Table 1 shows the comparison between these two stimulus and it clearly shows that under conditions of my experiments, nicotine was found to be a more effective secretagogue than histamine.

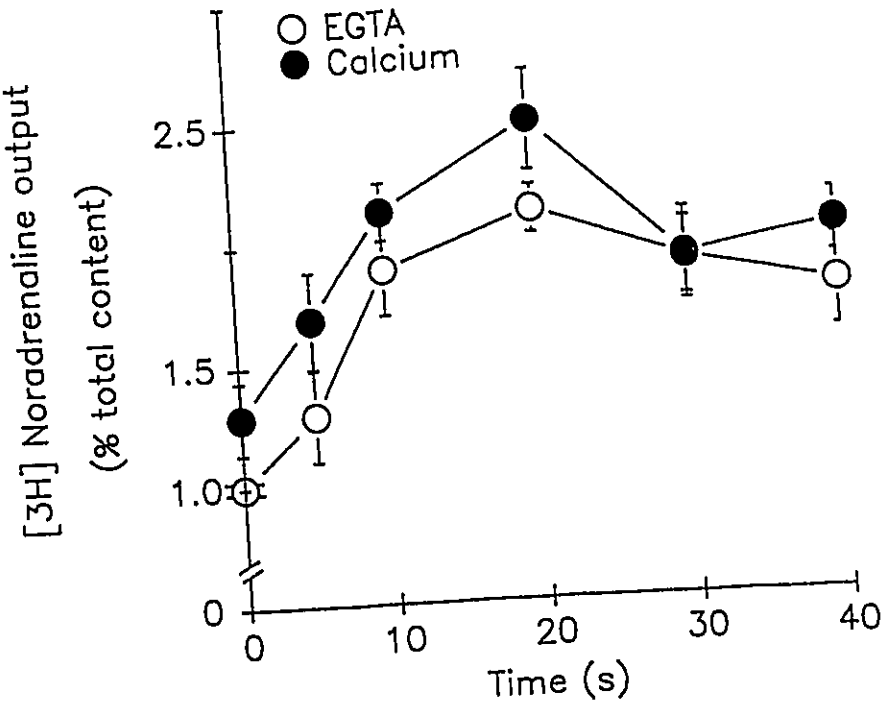


Fig.11 **Time courses of [^{3}H]noradrenaline ([^{3}H]NA) output in response to histamine stimulation in presence or absence of Ca^{2+}.**

Chromaffin cells were cultured for 48h and catecholamine stores were labelled with [^{3}H]NA as indicated in Methods and Materials. Cells were then incubated with Locke's solution containing 100 μM histamine in the present of 2.2 mM Ca^{2+} or in the absence (1 mM EGTA) of the divalent cation. Incubations with histamine were for 0, 5, 10, 20, 30 and 40s. After each of those periods, media were removed and their radioactivity were measured. [^{3}H]NA output is expressed as percentage of total [^{3}H]NA cell content. Each point represents the mean ± SEM of values obtained from eight different culture dishes.

Table 1. Comparison between nicotine and histamine effects on bovine chromaffin cells		
	Histamine*	**Nicotine***
Scinderin redistribution	20-30%	50-60%
Actin disassembly	20-30%	50-60%
Net catecholamine output (% of total contents)	0.5-1.5%	2-2.5%
Extracellular Ca^{2+}	Not required	Required
* 40 sec stimulation.		

Table 1. Comparison between nicotine and histamine effects on bovine chromaffin cells.

Table 1 shows the comparison between nicotine and histamine effects on chromaffin cells. Histamine concentration was 100 μM while nicotine was 10 μM and the stimulation period was 40 s. Response to histamine was in the absence of extracellular Ca^{2+} while response to nicotine was in the presence of extracellular Ca^{2+}.

D. The receptor type involved in histamine-induced effects.

The presence of both H_1 and H_2 receptors in chromaffin cells have been described (Noble et al., 1988; Choi et al., 1993; Marley et al., 1991). H_1 receptors have been associated with histamine-evoked catecholamine release (Noble et al., 1988; Choi et al., 1993) whereas H_2 receptors have been associated with increases in chromaffin cell cAMP levels (Marley et al., 1991). Therefore, it became necessary to determine what receptor type mediates histamine-evoked changes in cytoskeleton dynamics. Cultured chromaffin cells were preincubated with Ca^{2+}-free Locke's solution for 5 min in the presence of either 10 μM pyrilamine (H_1 antagonist) or 10 μM cimetidine (H_2 antagonist). This was followed by 40s incubation with the same solution containing 100 μM histamine. After incubations, cells were fixed in 3.7% formaldehyde and immediately processed for immunofluorescence staining with scinderin antiserum (Fig.12) or rhodamine-phalloidin (Fig.13). The experiments depicted in Fig. 12, 13, and 14 together show that the three responses evoked by histamine (scinderin redistribution, cortical F-actin disassembly and catecholamine release) were completely blocked by 10 μM pyrilamine, an H_1 antagonist, whereas 10 μM of cimetidine, an H_2 antagonist, had no effect on histamine responses. These results clearly demonstrate the H_1 receptor involvement in histamine-induced effects.

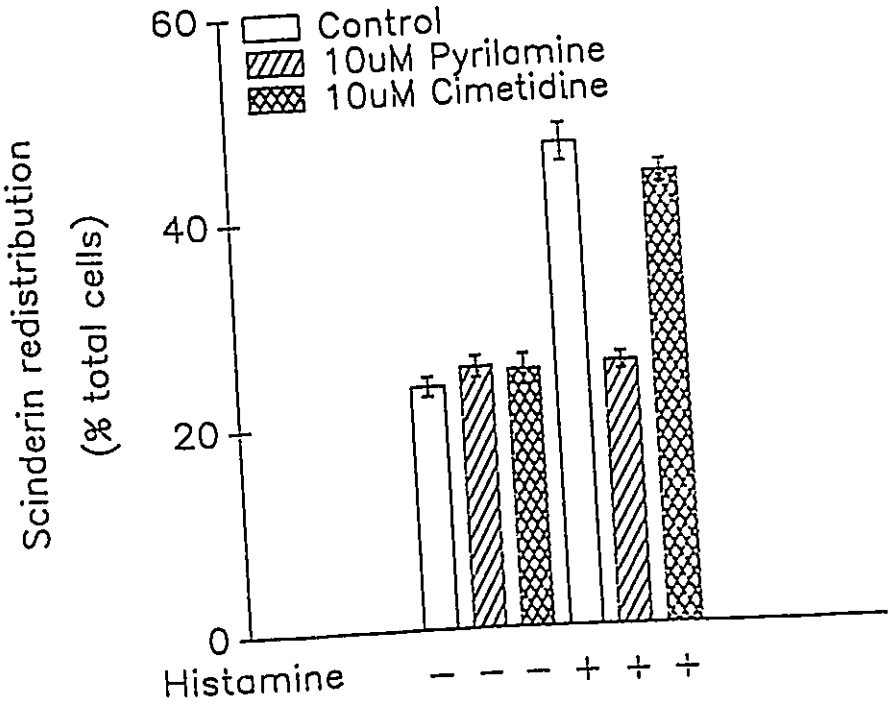


Fig.12 **Effect of H_1 and H_2 receptor antagonists on histamine-induced scinderin redistribution.**

Chromaffin cells cultured for 48h were preincubated with Ca^{2+}-free Locke's solution for 5 min in the presence of either 10 μM pyrilamine (H_1 antagonist) or 10 μM cimetidine (H_2 antagonist). This was followed by incubation for 40s with the same solutions containing 100 μM histamine. After incubation, cells were fixed in 3.7% formaldehyde and immediately processed for immunofluorescence staining with scinderin antiserum. Preparations were viewed under fluorescence microscope and cells were classified as described in legend to Fig.6. Each bar represents the mean ± SEM of the percentage of cells with disrupted scinderin fluorescent rings in 5 coverslips (500 cells were examined for each value).

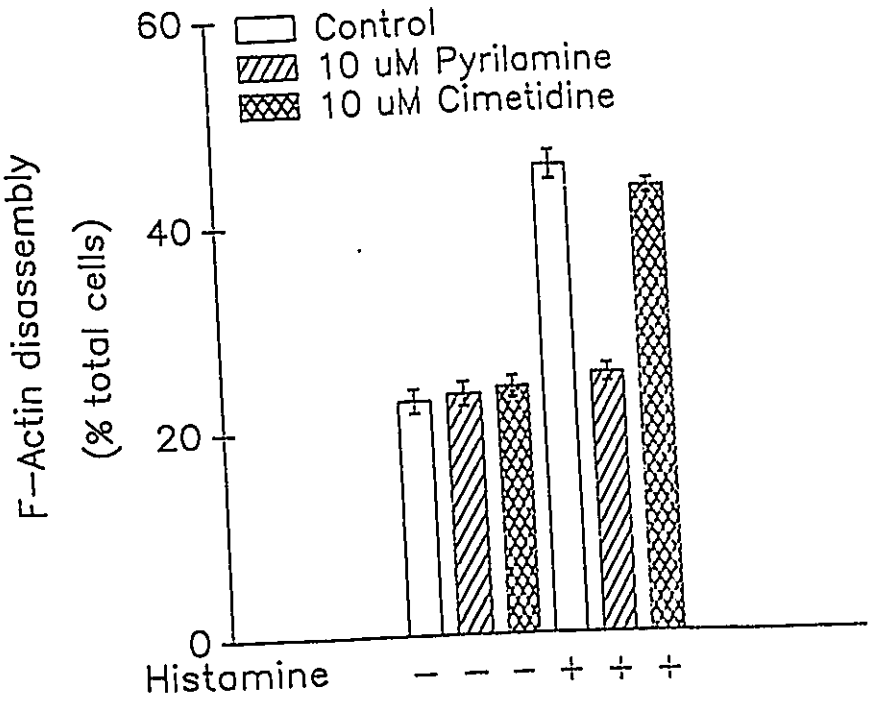


Fig.13 **Effect of H_1 and H_2 receptor antagonists on histamine-induced F-actin disassembly.**

After incubation, cells were fixed in 3.7% formaldehyde and immediately processed for fluorescence staining with rhodamine-phalloidin. Each bar represents the mean ± SEM of the percentage of cells with disrupted F-actin fluorescent rings in 5 coverslips (500 cells were examined for each value). Other conditions as indicated in legend to Fig.12.

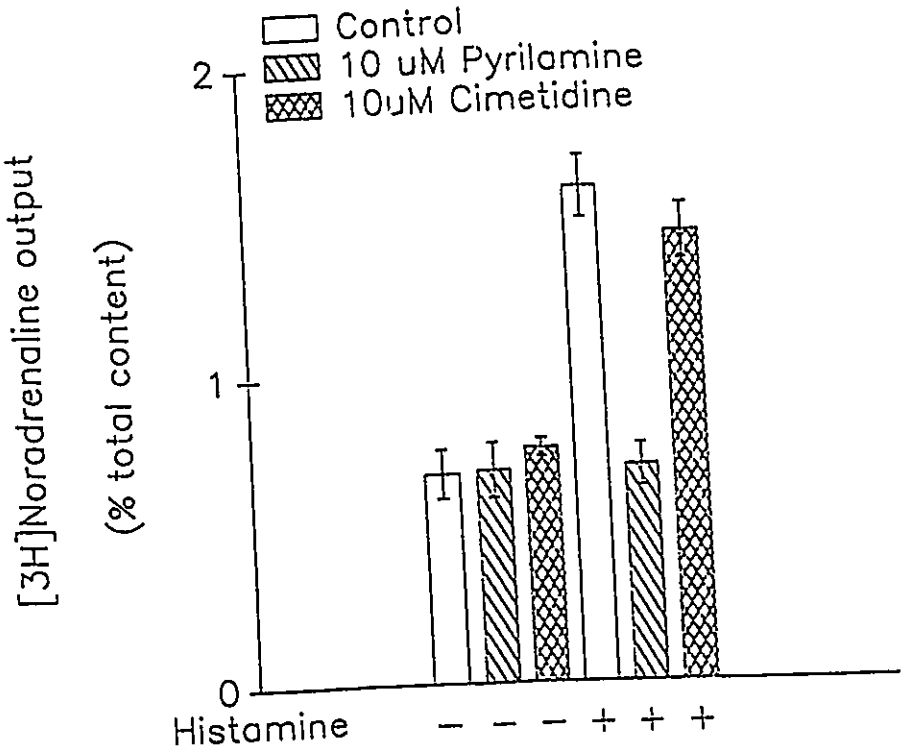


Fig.14 **Effect of H_1 and H_2 receptor antagonists on histamine-induced catecholamine release.**

Chromaffin cells with catecholamine stores labelled with [^{3}H]NA (see Methods and Materials) were incubated with Ca^{2+}-free Locke's solution for 5 min in the presence of 10 μM pyrilamine or 10 μM cimetidine. This was followed by incubation for 40s with the same solutions, this time containing 100 μM histamine. After the incubations, media were removed and their radioactivity measured. [^{3}H]NA output is expressed as percentage of total [^{3}H]NA cell content. Each bar represents the mean ± SEM of values obtained from eight different culture dishes.

E. Effects of histamine on adrenaline and noradrenaline-containing cells.

It has been suggested from experiments on partially purified chromaffin cell populations of adrenaline and noradrenaline-containing cells that histamine is a much better stimulus for the release of adrenaline than noradrenaline (Choi et al., 1993). The apparent reason for this is the presence of a large density of histamine receptors in adrenaline-containing cells (Choi et al., 1993). In order to determine whether scinderin redistribution and cortical F-actin disassembly observed in chromaffin cells in response to histamine were localized in one cell type or both, immunocytochemical studies were carried out on cells simultaneously immunostained with antibodies against dopamine β-hydroxylase (DβH), or phenyl N-methyltransferase (PNMT) and subsequently stained for actin with rhodamine-phalloidin. DβH positive cells represent all adrenaline and noradrenaline-containing cells in the cell cultures. Among the DβH positive cells, those showing PNMT positive fluorescence are adrenaline-containing cells, whereas those PNMT negative are noradrenaline-containing cells. Chromaffin cells cultured for 48 h on collagen coverslips were incubated for 40s in Ca^{2+}-free solution in the presence of 100 μM histamine while a second group of cells were also incubated for 40s with regular Locke's solution in the presence of 10 μM nicotine. Fig. 15 shows that close to 80% of DβH positive cells were also PNMT positive (adrenaline-containing cells) whereas the rest were noradrenaline-containing cells. Among the cells showing cortical F-actin disassembly in response to histamine, there was the same percentage of PNMT positive (adrenaline-containing cells) and PNMT negative cells (noradrenaline-containing cells)

as that found in the total chromaffin cell populations (Fig. 16). Moreover, when cortical F-actin disassembly is induced by cholinergic nicotinic receptor stimulation, the same proportion of PNMT positive and negative cells were affected (Fig. 16). Consequently, the results indicate that when chromaffin cells are exposed for short periods of time with either histamine in the absence of extracellular Ca^{2+} or nicotine in the presence of Ca^{2+}, both cell types are equally stimulated.

F. Effects of inhibition of protein kinase C (PKC) activity on histamine-induced scinderin redistribution, cortical F-actin disassembly and catecholamine release.

In order to determine the second messenger (PKC) involved in histamine stimulation, two PKC inhibitors, calphostin C and chelerythrine, were tested for their effects on histamine-induced responses.

Chromaffin cells cultured for 48 h were preincubated for 6 min with calphostin C in increasing concentrations and then stimulated with 100 μM histamine for 40s in the presence of the corresponding concentrations of calphostin C utilized during the preincubation period. Following the last incubation period (40s) cells were immediately fixed and processed for scinderin and F-actin staining with scinderin antiserum #6/FITC-IgG and rhodamine-phalloidin as described in Methods and Materials. Calphostin C caused a concentration-dependent inhibition of all chromaffin cell responses to histamine:

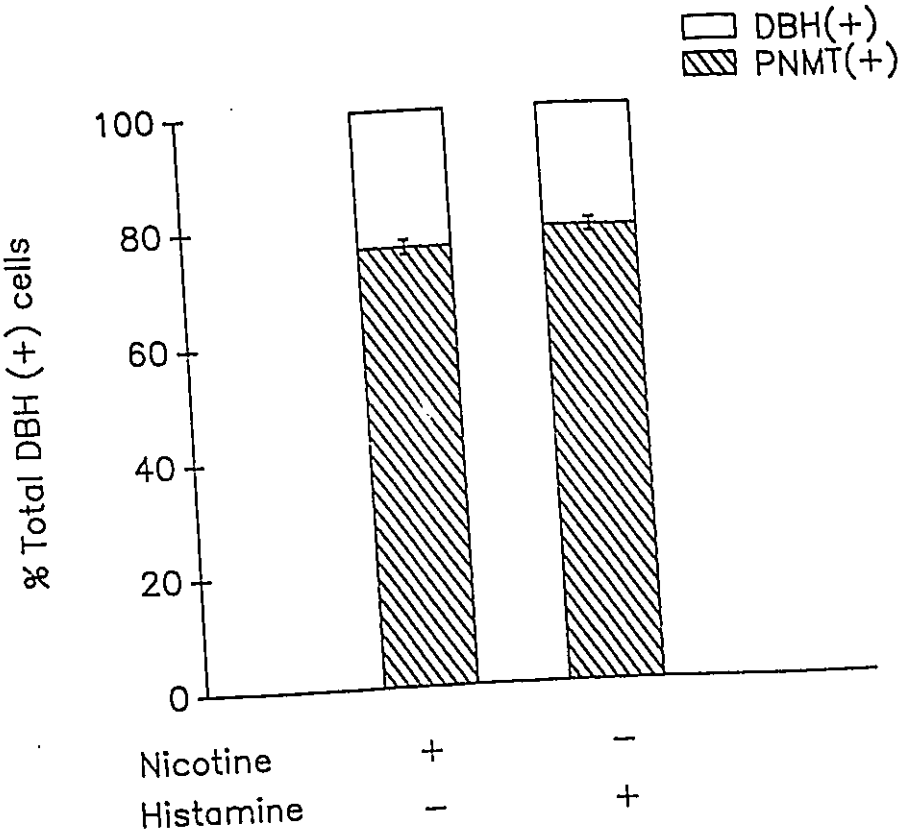


Fig.15 **Histamine stimulate scinderin redistribution on both adrenaline- and noradrenaline-containing cells.**

Chromaffin cells cultured for 48h on collagen coated coverslips were incubated for 40s in Ca^{2+}-free solution in the presence of 100 μM histamine. A second group of cells were also incubated for 40s with regular Locke's solution in the presence of 10 μM nicotine. After these periods of time, cells were fixed in 3.7% formaldehyde and processed for immunofluorescence using antibodies against DβH and for F-actin staining using rhodamine-phalloidin. Preparations were viewed under a fluorescence microscope and cells were classified for DβH positive cells and among these were subclassified as PNMT positive or negative cells. Graph shows the percentage of PNMT positive cells (adrenaline-containing cells) present in the entire chromaffin cell population (DβH positive cells) in the groups treated with nicotine or histamine. The PNMT negative cells [DβH(+) minus PNMT(+)] correspond to noradrenaline-containing cells. Each bar represents the mean ± SEM of cells present in eight different coverslips (800 cells examined for each value).

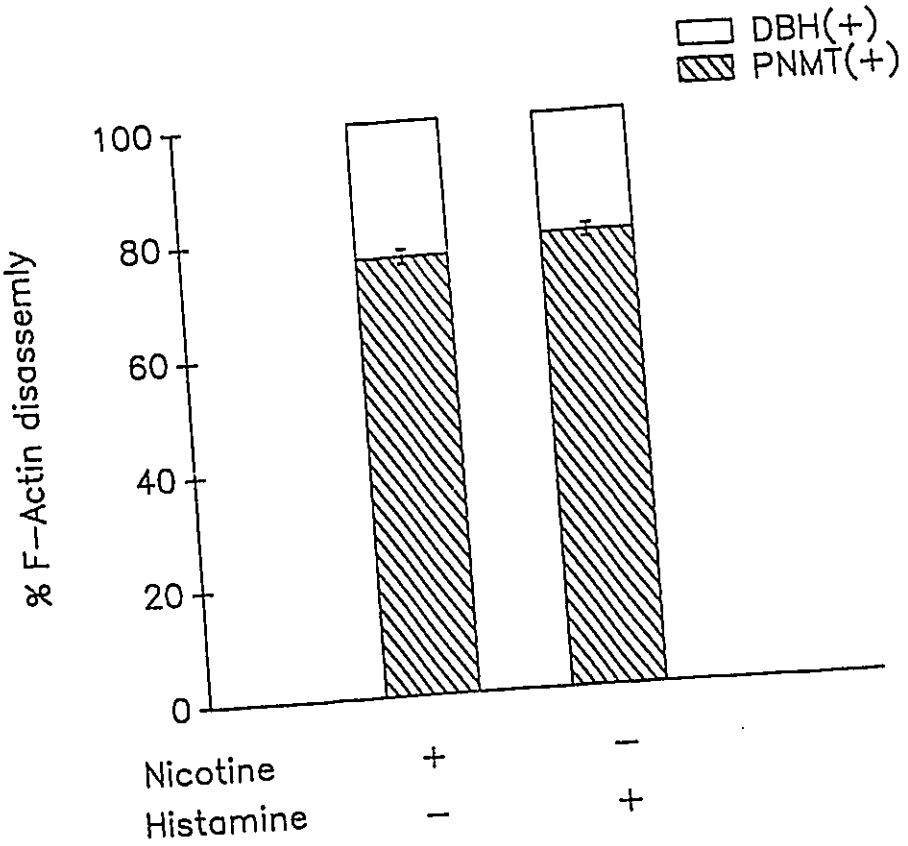


Fig.16 **Histamine stimulates F-actin disassembly on both adrenaline-and noradrenaline-containing cells.**

Graphs shows the percentage of PNMT positive cells (adrenaline containing cells) among all cells (DβH +) showing F-actin disassembly for both nicotine and histamine treated preparations. Each bar represents the mean ± SEM of cells present in eight different coverslips (800 cells examined for each value). Other conditions are as described in legend to Fig.15.

scinderin redistribution, cortical F-actin disassembly and catecholamine release (Fig. 17, 18). Preincubation of chromaffin cells for 3 min with chelerythrine did not modify basal parameters but inhibited in a concentration-dependent manner scinderin redistribution, cortical F-actin disassembly and catecholamine release in response to histamine stimulation (Fig. 19, 20). The highest inhibitory effect for the three histamine-evoked responses among these two inhibitors was obtained with 10^{-4}M chelerythrine. The results also show that calphostin C was 10 times more potent than chelerythrine in inhibiting histamine-induced responses.

G. Phospholipase C (PLC)- phosphatidylinositol 4,5-bisphosphate (PIP_2) cascade transduction pathway is involved in histamine-evoked scinderin redistribution, cortical F-actin disassembly and catecholamine release.

The results described in the previous section suggest that activation of PKC might play a role in the response to histamine measured in chromaffin cells. Because PKC is activated by diacylglycerol and intracellular Ca^{2+} and the responses to histamine are observed in the absence of extracellular Ca^{2+} , it is possible that histamine-induced responses are the result of a G-protein activation of PLC. This would produce phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis with formation of diacylglycerol (activator of PKC) and IP_3 which in turn releases Ca^{2+} from intracellular stores. To test this hypothesis four different approaches were followed: 1) PLC inhibitors; 2)

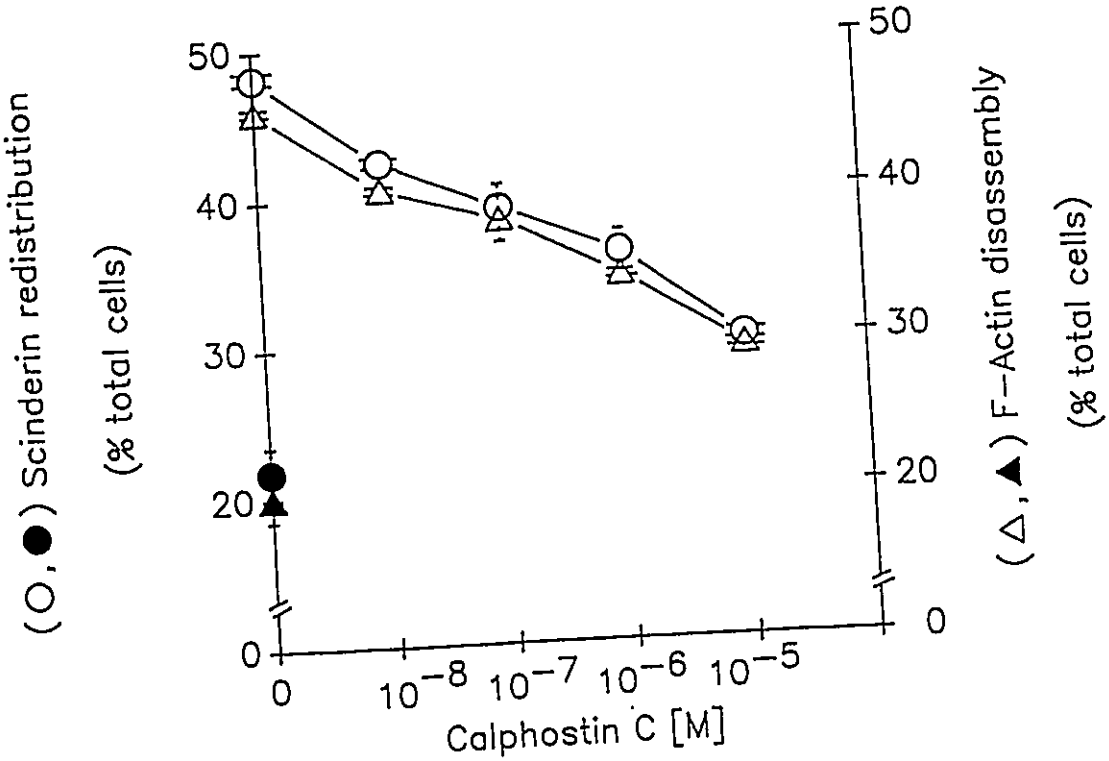


Fig.17 **Concentration-dependent inhibitory effects of calphostin C on histamine-evoked scinderin redistribution and F-actin disassembly.**

Chromaffin cells cultured for 48h on collagen coated coverslips were preincubated with Ca^{2+}-free Locke's solution with increasing concentrations of calphostin C and were then further incubated for 40s with Ca^{2+}-free Locke's solution containing 100 μM histamine and the inhibitor used in the preincubation step. Following the last incubation period (40s) cells were immediately fixed and processed for scinderin and F-actin staining with scinderin antiserum #6/FITC-IgG and rhodamine-phalloidin as described in Methods and Materials. Preparation were observed under the fluorescence microscope. One hundred cells per coverslip were examined and classified as having either a "discontinuous" or "continuous" cortical fluorescence ring as explained in the legend to Fig.8. Each value plotted represents the mean ± SEM of the percentage of cells showing a "discontinuous" cortical staining of 7-8 coverslips (700-800 cells for each value) containing cells from 3 different cultures.

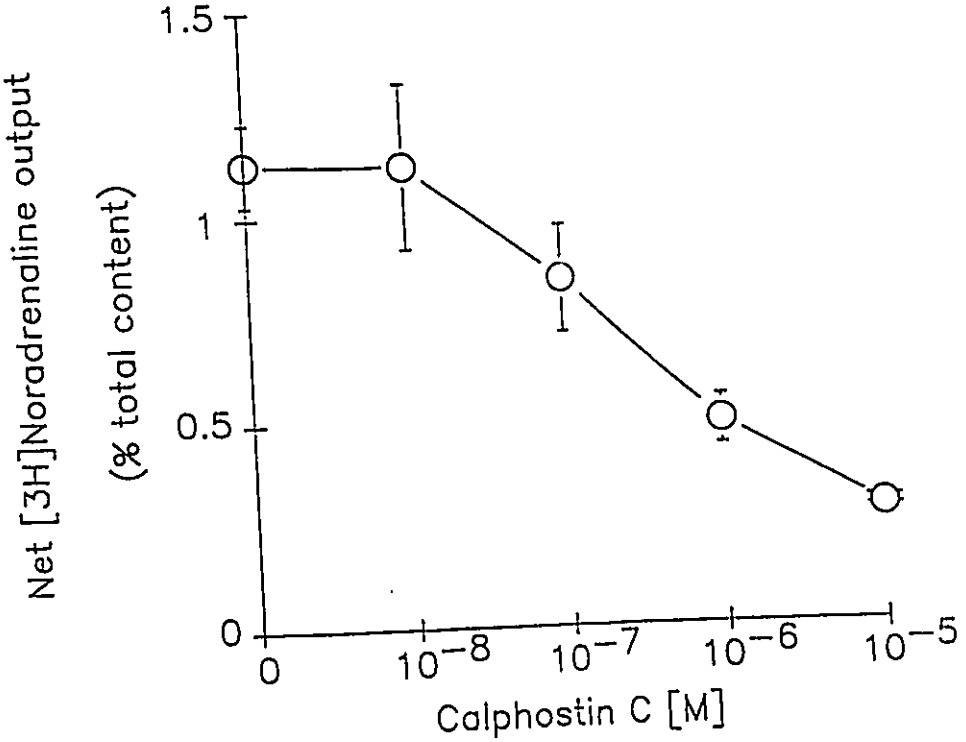


Fig.18 **Concentration-dependent inhibitory effects of calphostin C on histamine-evoked catecholamine release.**

Forty-eight-hour old chromaffin cells were loaded with [^{3}H]NA as described in Methods and Materials. Cells were preincubated with Ca^{2+}-free Locke's solution with increasing concentrations of calphostin C and were then further incubated for 40s with Ca^{2+}-free Locke's solution containing 100 μM histamine and the PKC inhibitor used in the preincubation step. Following the histamine stimulation, radioactivity was measured in both media and cells. Histamine-evoked [^{3}H]NA output is expressed as the percentage of total [^{3}H]NA cell content. Each point represents the mean ± SEM of values obtained from 7-8 different culture dishes (700-800 cells).

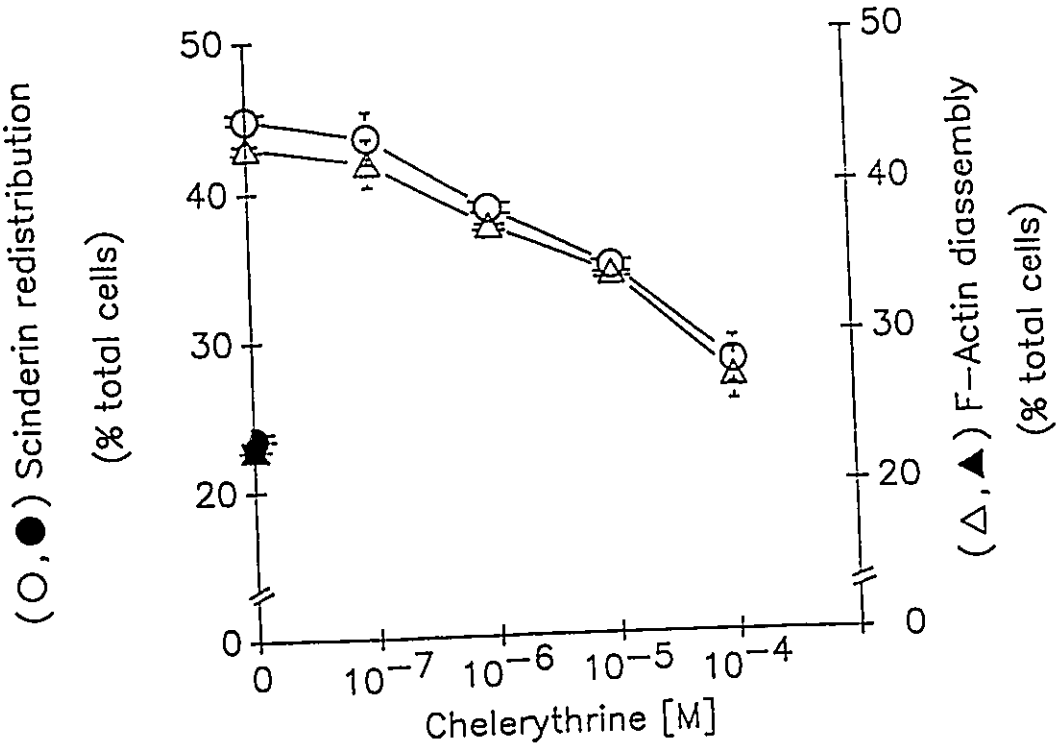


Fig.19 **Concentration-dependent inhibitory effects of chelerythrine on histamine-evoked scinderin redistribution and F-actin disassembly.**
Each value plotted represents the mean ± SEM of the percentage of cells showing a "discontinuous" cortical staining for 7-8 coverslips (700-800 cells for each value) containing cells from 3 different cultures. Other conditions were as described in legend to Fig.17.

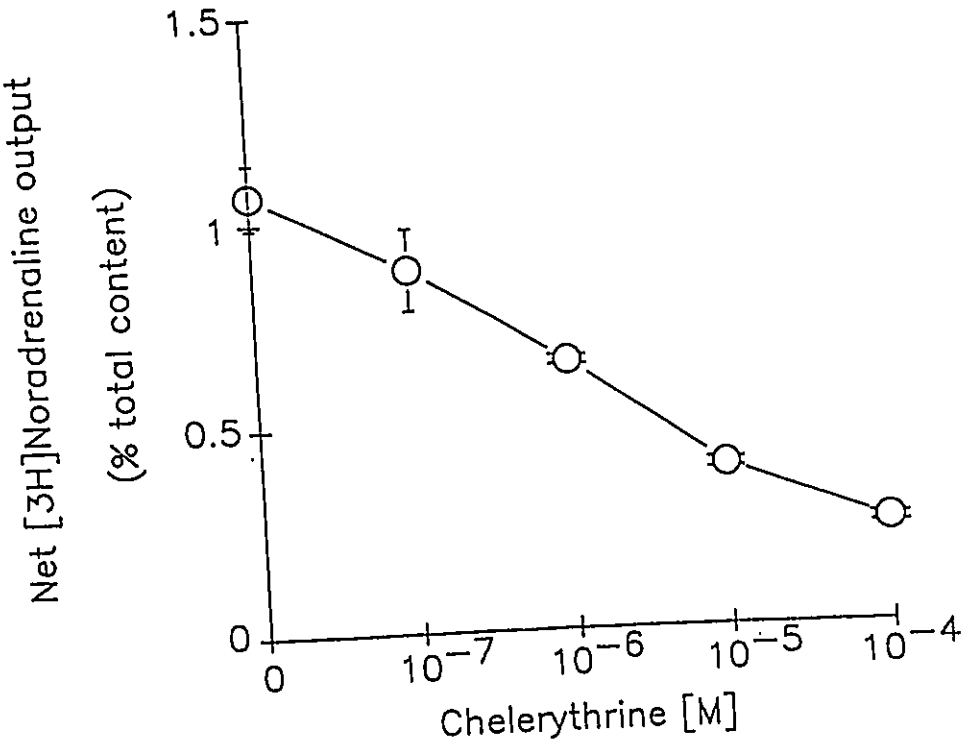


Fig.20 **Concentration-dependent inhibitory effects of chelerythrine on histamine-evoked catecholamine release.**

Each point represents the mean ± SEM of values obtained from 7-8 different culture dishes (700-800 cells for each condition). Other conditions were as described in legend for Fig.18.

histamine on IP_3 synthesis; 3) histamine on Ca^{2+} transients under different experimental conditions; 4) thapsigargin induced depletion of intracellular stores of Ca^{2+} on histamine-evoked responses.

1) Effect of PLC inhibitors.

Chromaffin cells were cultured 48 h at 37°C and preincubated for 3 min with increasing concentrations of either the PLC inhibitor 1-[6-[[17β-3-Methoxyestra-1,3,5 (10)-trien-17-yl]amino]hexyl]-IH-pyrole-2,5-dione (U-73122) (Smith et al., 1990) or its inactive isomer 1-[6-[[17β-3-Methoxyestra-1,3,5 (10)-trien-17-yl]amino]hexyl]-2,5-pyrrodine-dione (U-73343) and then stimulated for 40s with 100 μM histamine in the presence of the corresponding concentration of these compounds utilized during the preincubation period. Cells were immediately processed for scinderin and F-actin fluorescence (Fig.21). Catecholamine release was measured in cells pre-labelled with [^{3}H]NA (Fig.22). U-73122 inhibited scinderin redistribution (Fig.21,23), cortical F-actin disassembly (Fig. 21,23) and catecholamine release (Fig. 22). Inhibition produced by U-73122 on histamine-evoked stimulation was concentration-dependent (Fig.22, 23). On the other hand, U-73343, the inactive isomer, did not modify the responses to histamine stimulation (Fig. 22, 23).

2) Effect of histamine on IP_3 synthesis.

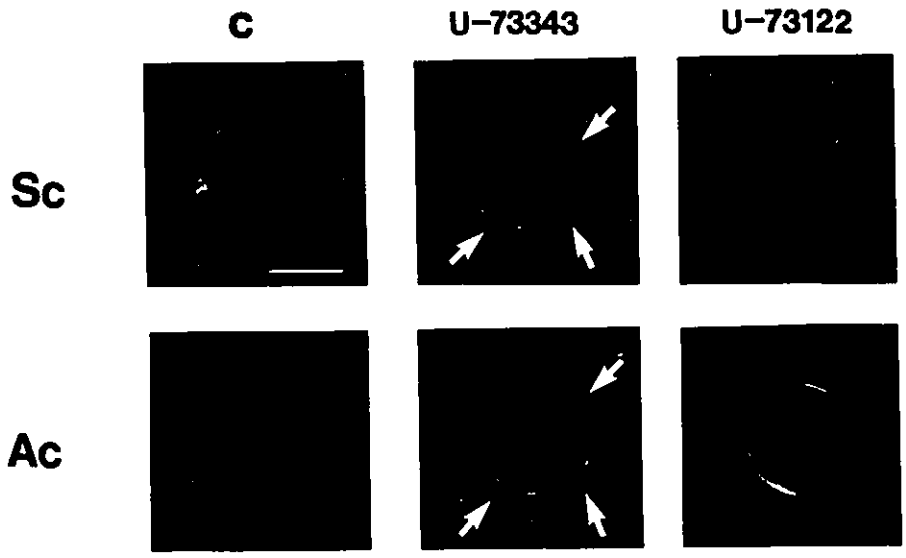


Fig.21 **Effect of the PLC inhibitor, U-73122, and its inactive isomer, U-73343, on histamine-induced scinderin redistribution and F-actin disassembly.**

Chromaffin cells cultured for 48h on collagen coated coverslips were preincubated for 3 min with Ca^{2+}-free Locke's solution either 10 μM U-73122 or 10 μM U-73343 and were then further incubated for 40s with the same solutions containing 100 μM histamine. A control cell shows the typical continuous intense rings of fluorescence for scinderin and F-actin colocalized at the subplasmalemmal region. Histamine stimulated cells display a disrupted cortical fluorescent pattern for scinderin and F-actin only when cells were incubated in the presence of U-73343 (inactive isomer). Some fluorescent patches are indicated by arrows. In the presence of the PLC inhibitor U-73122, histamine failed to show any changes in scinderin and F-actin distribution. Bar, 10 μm.

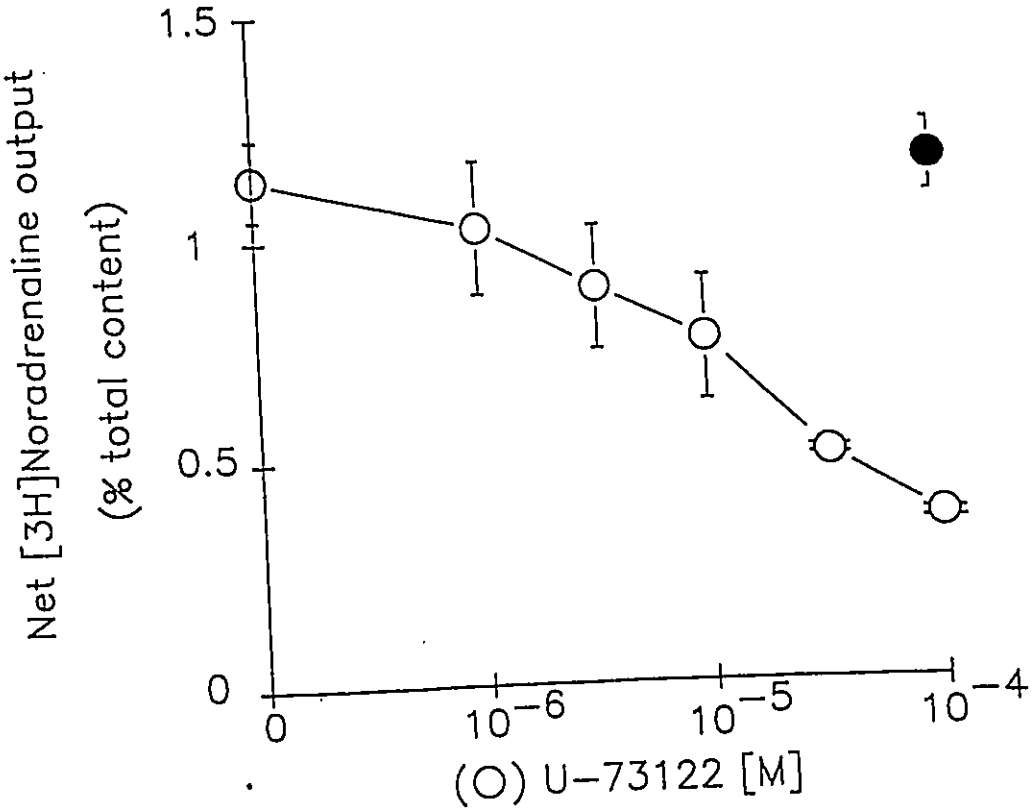


Fig.22 **Concentration-dependent inhibitor effect of U-73122 on histamine-evoked catecholamine release.**

Chromaffin cells with catecholamine stores labelled with [^{3}H] were preincubated for 3 min with Ca^{2+}-free Locke's solution containing increasing concentrations of U-73122 or 100 μM U-73343 and subsequently challenged with histamine (40s) as indicated above in (A). After the incubations, media were removed and their radioactivity measured. [^{3}H]NA output is expressed as percentage of total [^{3}H]NA cell content. Each point represents the mean ± SEM of values obtained from 8 different dishes. The symbol (●) represents cells incubated in the present of U-73343 following stimulated by histamine.

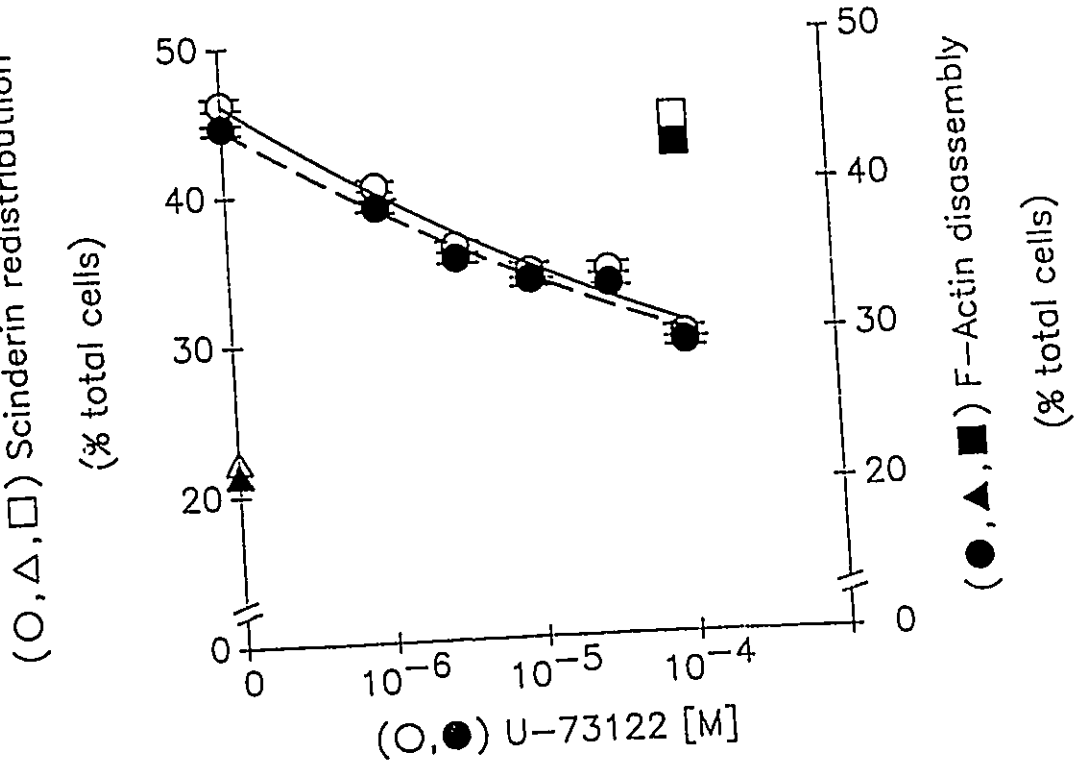


Fig.23 **Concentration-dependent inhibitor effect of U-73122 on histamine-evoked scinderin redistribution and F-actin disassembly.**

Chromaffin cells cultured for 48h on collagen coated coverslips were preincubated for 3 min with Ca^{2+}-free Locke's solution with increasing concentrations of U-73122 or 100 μM U-73343 (inactive isomer) and were then further incubated for 40s with Ca^{2+}-free Locke's solution containing 100 μM histamine and the inhibitor used in the preincubator step. Cells were fixed and immediately processed for scinderin and F-actin staining as described in Methods and Materials. Preparations were viewed under the fluorescence microscope and cells were classified as described in the legend for Fig.6. Each value represents the mean ± SEM of the percentage of cells with disruptive fluorescent rings in 5 coverslips (500 cells examined for each value). The △, ▲ symbols represent control values (absence of histamine) whereas □, ■ represent cells incubated in U-73343 and histamine.

The above results demonstrated the apparent involvement of PLC in the histamine-evoked responses. Chromaffin cells IP_3 levels were then measured by using the HPLC system described in Methods and Materials under resting conditions, 5s of stimulation with histamine, and preincubation with either U-73122 for 3 min or thapsigargin for 5 min followed by 5s histamine stimulation. Fig.24 A,B,C,D show the profiles of the peaks obtained from HPLC fractions under these conditions. Data obtained from the experiments are depicted in Fig.25. Each bar represents the mean ± SEM of values obtained from 3-6 different cultures. The results (Fig.24, 25) indicate that histamine stimulation significantly increased IP_3 levels and that this increase is blocked in the presence of the PLC inhibitor, U-73122. Thapsigargin, on the other hand, is unable to block the IP_3 increase evoked by histamine stimulation.

3) Effect of histamine on Ca^{2+} transients under different experimental conditions.

Ca^{2+} transients were also measured in cultured chromaffin cells under the different experimental conditions. Chromaffin cells cultured for 48 h were loaded with fura-2 for 40 min and the changes in intracellular Ca^{2+} levels were monitored using a double-wavelength luminescence spectrophotometer (see Methods and Materials). Figure 26 shows the traces of representative experiments. Histamine stimulation was able to produce a rapid increase in intracellular Ca^{2+} levels. This was observed in the presence or in the absence of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+}, after the initial rapid phase of increase in intracellular Ca^{2+}, the intracellular Ca^{2+} levels reached

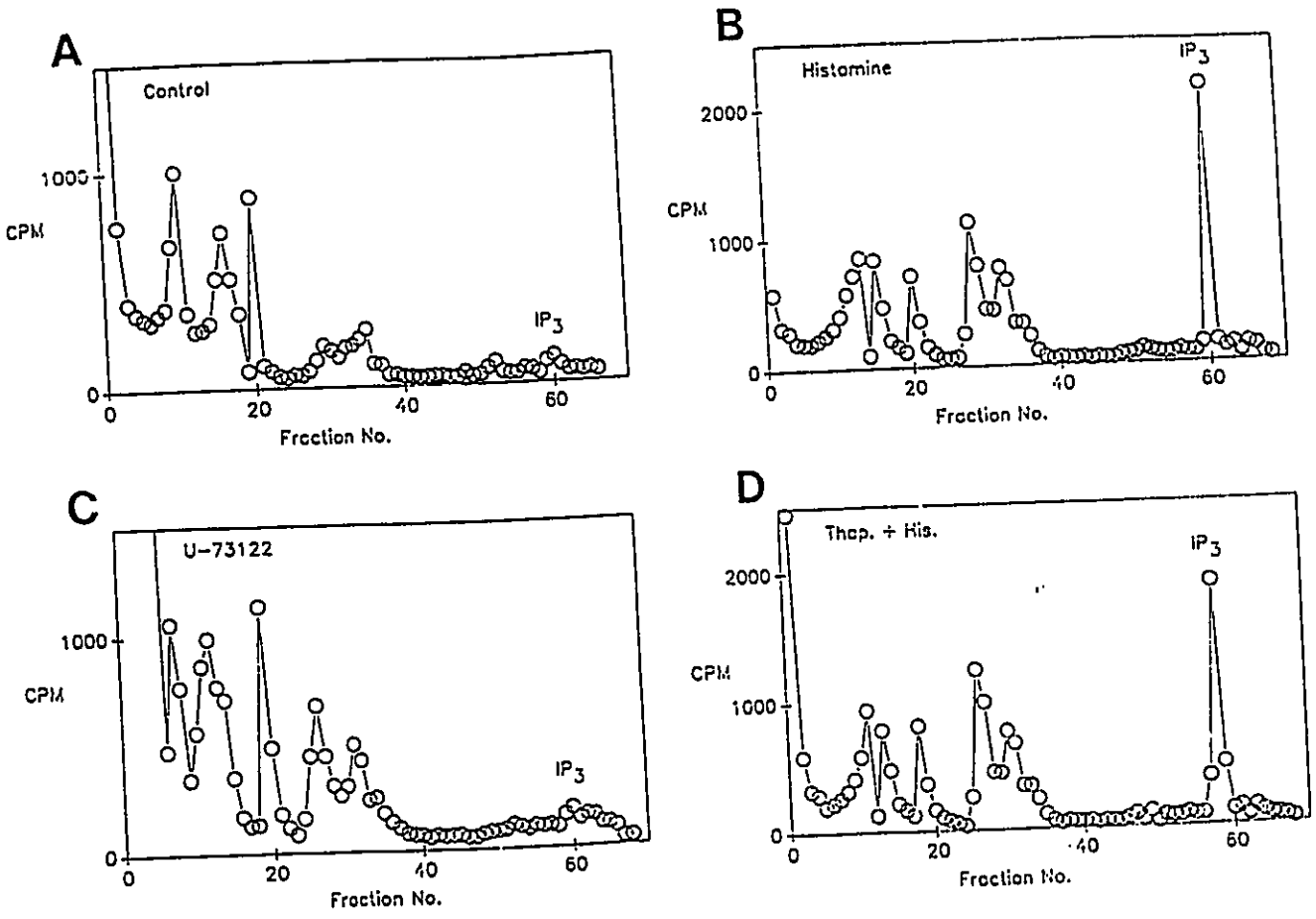


Fig.24 **Effect of U-73122 and thapsigargin on the changes in IP_3 cellular levels evoked by histamine stimulation.**

Peaks show the radioactivity in HPLC fractions. A, control; B, 5s stimulation by histamine; C, 3 min preincubation of U-73122 followed by 5 s histamine stimulation; D, 5 min preincubation of thapsigargin followed by 5s histamine stimulation.

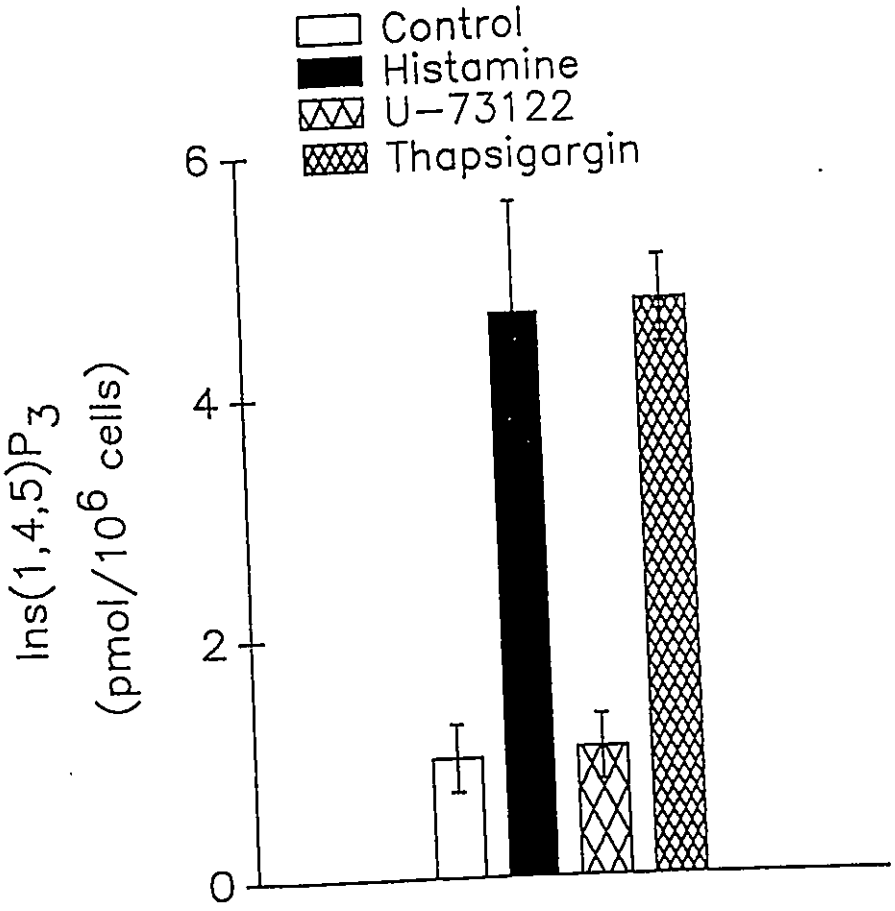


Fig.25 **Effect of U-73122, a PLC inhibitor, and thapsigargin on the changes in inositol 1,4,5-trisphosphate (IP_3) cellular levels evoked by histamine stimulation.**

Chromaffin cells labelled for 48h at 37°C with myo[^{3}H]inositol as indicated in Methods and Materials were incubated for 5s with Ca^{2+}-free Locke's solution alone or the same solution containing 100 μM histamine in presence or of either 10 μM U-73122 or 20 nM thapsigargin or in the absence of the compounds. Cells treated with U-73122 were preincubated with the PLC inhibitor for 3 min prior to the 5s stimulation period. Cells were also preincubated with thapsigargin for 5 min prior to the 5 s stimulation period. IP_3 levels were measured by HPLC as described in Methods and Materials. Each bar represents the mean ± SEM of values obtained from 3-6 different culture dishes.

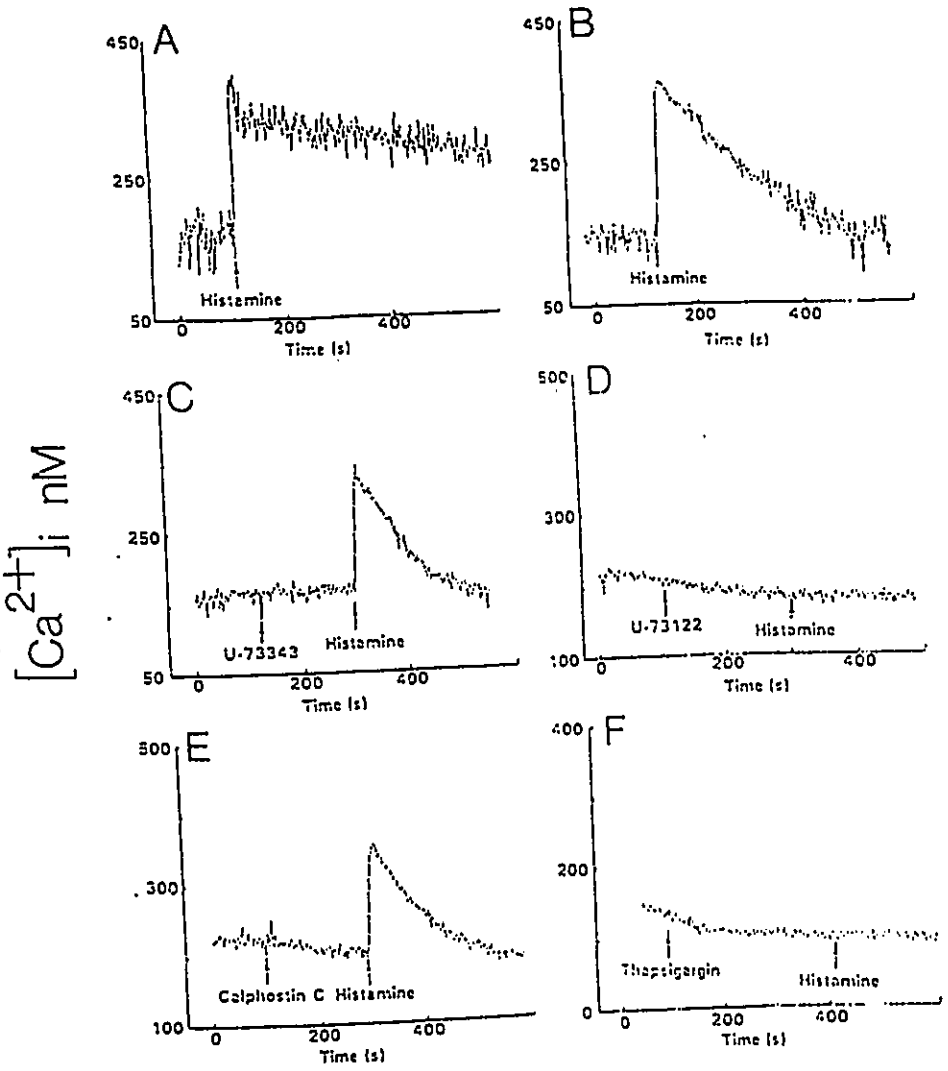


Fig.26 **Effects of calphostin C, U-73122, U-73343 and thapsigargin on histamine-induced changes in intracellular Ca^{2+} levels.**

Chromaffin cells labelled for 48h were loaded with fura-2 for 40 min and the changes in intracellular Ca^{2+} levels were monitored using a double-wavelength luminescence spectrophotometer. Ten microliters of a solution of histamine (final concentration 100 μM) were introduced into the cuvette at the time indicated by the arrow. Cells were incubated in regular Locke's solution in (A) and in Ca^{2+}-free Locke's solution in (B). In (C) to (F) cells were also incubated in Ca^{2+}-free Locke's solution. At the time indicated by arrows 10 μl containing U-73122 (D, , final concentration 10 μM), U-73343 (C, final concentration 10 μM), calphostin C (E, final concentration 1 μM) and thapsigargin (F, final concentration 30 nM). The figure shows traces of representative experiments. Similar results were obtained from 4-5 tests performed for each condition.

a plateau which was then maintained during the entire length of the experiment (Fig.26A). In the absence of extracellular Ca^{2+}, only the initial phase was observed (Fig.26B). The magnitude of initial increase phases of both conditions was in the same level. The rest of the tests were then conducted in the absence of extracellular Ca^{2+} (1 mM EGTA). PLC inhibitor, U-73122, was able to block completely the rise in intracellular Ca^{2+} produced by histamine stimulation (Fig.26D). As suspected, neither U-73343, the inactive isomer of U-73122, nor calphostin C, one of the PKC inhibitors used in the experiments described above, block the Ca^{2+} transients in response to histamine (Fig. 26C,E). Similar results were obtained from 4-5 tests performed for each conditions.

4) Effect of thapsigargin-induced depletion of Ca^{2+} intracellular stores on histamine-induced responses.

The results from Ca^{2+} transients again suggest that the IP_3 sensitive Ca^{2+} store is involved in the responses to histamine. If this was the case, depletion of intracellular Ca^{2+} stores should block Ca^{2+} transients as well as scinderin redistribution, cortical F-actin disassembly and catecholamine release in response to histamine. Therefore, thapsigargin, an agent known to mobilize intracellular Ca^{2+} (Thastrup et al., 1990; Sagara et al., 1991), was used to test the hypothesis. Chromaffin cells were preincubated for 5 min with 30 nM thapsigargin and then stimulated by 100 μM histamine. After thapsigargin treatment, the increase in intracellular Ca^{2+} in response to histamine was not

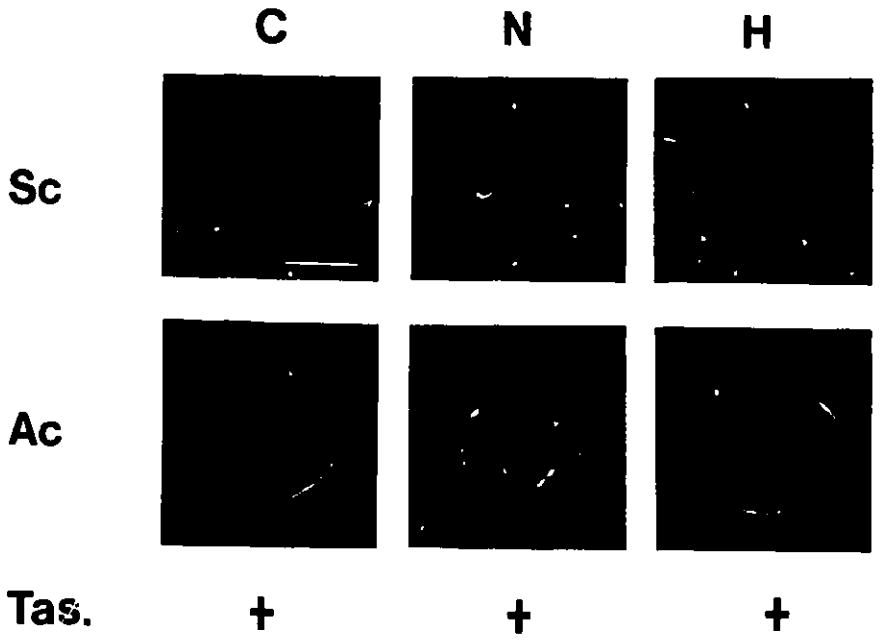


Fig. 27 Effect of thapsigargin on histamine-induced scinderin redistribution and F-actin disassembly.

Chromaffin cells cultured for 48h on collagen coated coverslips were preincubated for 3 min with 30 nM thapsigargin in either regular Locke's solution or in Ca^{2+}-free Locke's solution. Cells were then further incubated for 40s with either 10 μM nicotine (regular Locke's solution) or 100 μM histamine (Ca^{2+}-free Locke's solution). Cells were immediately fixed and processed for cytochemical staining for scinderin and F-actin. A control cell shows the typical continuous intense ring of fluorescence for scinderin and F-actin colocalized to the cortical region. When cells were incubated with thapsigargin and stimulated with nicotine, they displayed a disrupted cortical fluorescent pattern for scinderin and F-actin indicating the lack of effect of thapsigargin on nicotine-induced response. On the other hand, treatment with thapsigargin blocked the response to histamine stimulation. Bar, 10 μm.

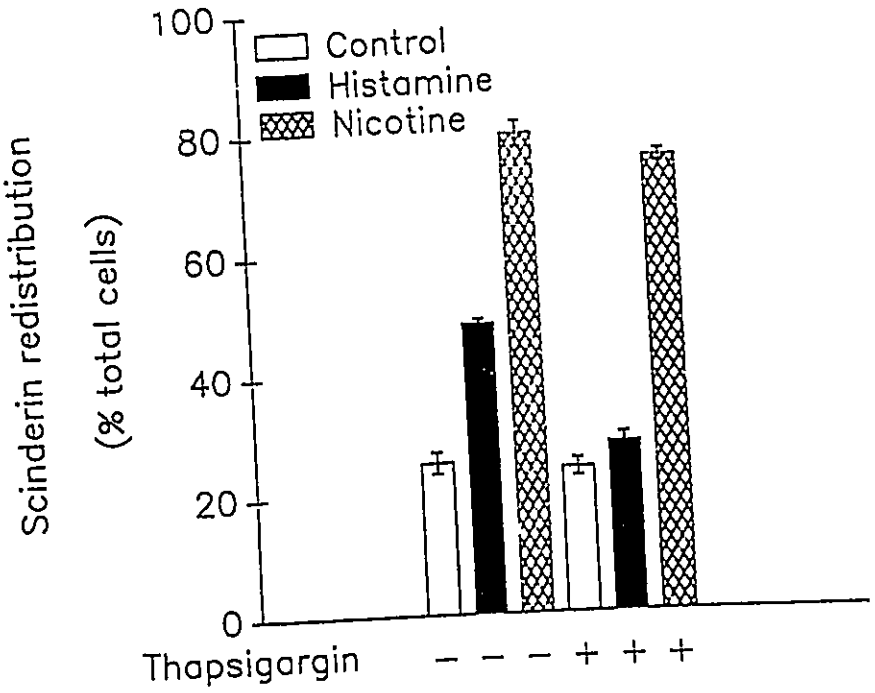


Fig.28 **Depletion of intracellular Ca^{2+} stores by thapsigargin blocks histamine-induced scinderin redistribution.**

Chromaffin cells cultured for 48h on collagen coated coverslips were preincubated for 3 min with 30 nM thapsigargin in either regular Locke's solution or Ca^{2+}-free Locke's solution. Cells were further incubated for 40s with either 10 μM nicotine (regular Locke's solution) or 100 μM histamine (Ca^{2+}-free Locke's solution). Cells were immediately processed for scinderin and F-actin staining. Preparations were viewed under the fluorescence microscope and cells were classified as described in the legend to Fig.8. Each bar represents the mean ± SEM of the percentage of cells with disrupted fluorescent rings in 5 coverslips (500 cells for each value) from 2 different cell cultures.

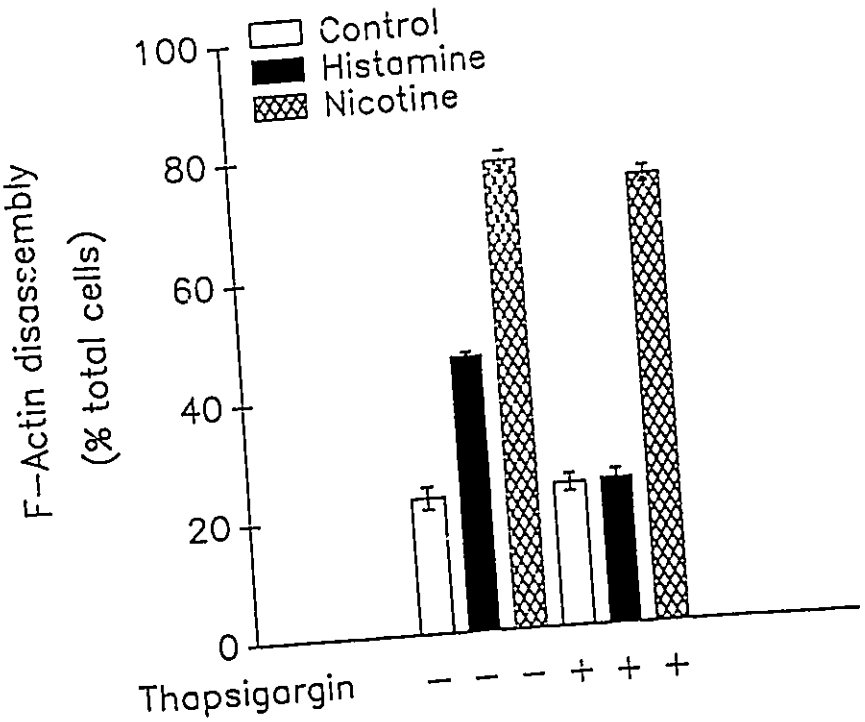


Fig.29 **Depletion of intracellular Ca^{2+} stores by thapsigargin blocks histamine-induced F-actin disassembly.**

Each bar represents the mean ± SEM of the percentage of cells with disrupted fluorescent rings in 5 coverslips (500 cells for each value) from 2 different cell cultures. Other conditions are described in the legend to Fig.28.

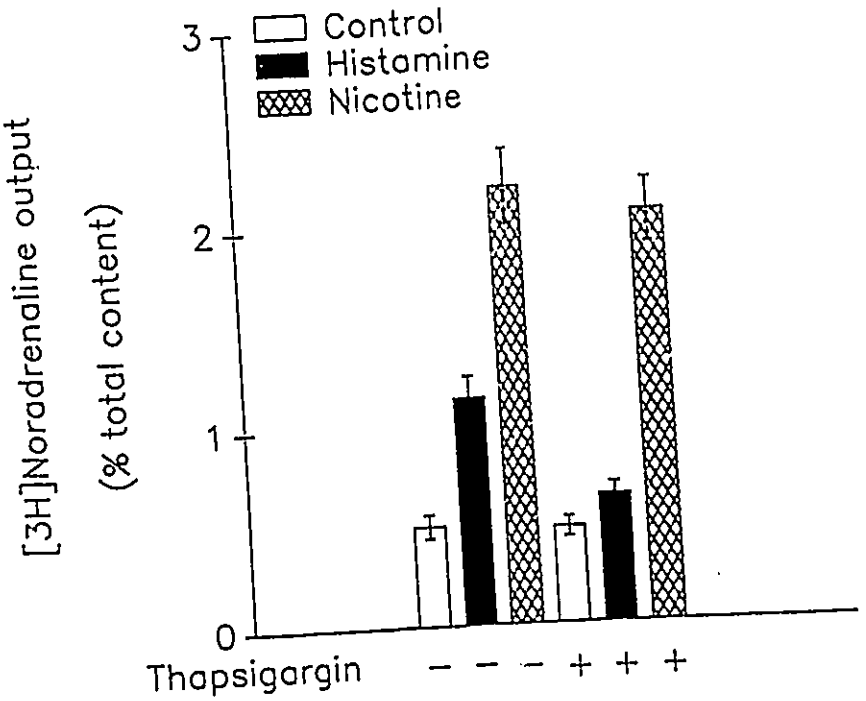


Fig.30 **Depletion of intracellular Ca^{2+} stores by thapsigargin blocks histamine-induced catecholamine release.**

Chromaffin cells were cultured for 48h and catecholamine stores were labelled with [^{3}H]NA as described in Methods and Materials. Cells were then incubated for 3 min with 30 nM thapsigargin in either regular or Ca^{2+}-free Locke's solutions and subsequently stimulated for 40s with either nicotine or histamine as indicated above in legend for Fig.28. [^{3}H]NA output is expressed as percentage of total [^{3}H]NA cell content. Each bar represents the mean ± SEM of values obtained from seven different culture dishes. Here again thapsigargin blocked the responses to histamine without affecting those to nicotine.

observed (Fig. 26F). Furthermore, the same thapsigargin treatment blocked scinderin redistribution (Fig.27,28), cortical F-actin disassembly (Fig.27,29) and catecholamine release (Fig.30) in response to histamine. Chromaffin cells were also stimulated by 40s 10 μM nicotine in the presence or absence of thapsigargin. As expected, thapsigargin was ineffective in blocking the nicotine-evoked responses (Fig.27,28,29,30). Furthermore, thapsigargin was ineffective in blocking chromaffin cell IP_3 level stimulated by histamine (Fig.24,25).

CHAPTER IV

DISCUSSION

A. Dynamic changes in cortical actin microfilament network during exocytosis

Previous work has already proved that filamentous actin is mainly localized in the cortical surface of the chromaffin cell (Lee and Trifaró, 1981; Trifaró et al, 1984,1989; Cheek and Burgoyne, 1986). Immunocytochemical experiments with rhodamine phalloidin and actin antibodies indicate that the actin filamentous network can be seen as a strong fluorescent ring in control chromaffin cells. Upon cholinergic stimulation, the fluorescent ring show areas devoid of fluorescence, suggesting disassembly of F-actin network (Cheek and Burgoyne, 1986,1987; Trifaró et al, 1989; also seen Fig.7 in this thesis). Biochemical approaches under same conditions also showed: a) F-actin decrease with concomitant G-actin increase when the DNase I inhibition assay was used (Cheek and Burgoyne, 1986; Trifaró et al, 1989) and b) a decrease in the amount of F-actin recovered with the Triton-X-100 insoluble proteins (Burgoyne et al, 1989; Trifaró, 1990). F-actin network disassembly has also been seen in stimulated mast cells (Koffer et al, 1990) and in depolarized (high K^+) synaptosomes (Bernstein and Bamburg, 1985; Trifaró and Vitale, 1993). Based on above evidence, it was suggested that cortical F-actin acted as a barrier to prevent the secretory vesicle freely contact with the plasma membranes and that upon stimulation, the actin network shows disassembly, thus removal of the barrier (Cheek and Burgoyne, 1986,1987; Burgoyne et al, 1989; Trifaró et al, 1982,1984,1989). The two actin binding proteins in chromaffin vesicles (α-actinin and fodrin) have the effect to mediate filamentous actin association with these vesicles (Aunis et al, 1980; Trifaró et al, 1982; Perrin and Aunis, 1985).

It has been shown that rates of actin disassembly and scinderin redistribution upon stimulation are similar and that areas devoid of filamentous actin are also devoid of cortical scinderin (Vitale et al, 1991). It seems that, in resting cells, scinderin shows a subplamalemmal distribution because its binding to filamentous actin. However, this suggestion can not explain why after removal of the stimulus, the rate of recovery of scinderin fluorescence was always faster than that of rhodamine phalloidin (Vitale et al, 1991). The faster recovery rate of scinderin indicates that there is another side for scinderin binding in the cortical region of resting cells. Further experiments revealed that scinderin might be bound to plasma membrane phospholipids under resting conditions (Rodríguez Del Castillo et al, 1992a). It is known that a Ca^{2+} rise is absolutely required for catecholamine release (Douglas and Rubin, 1961; Trifaró and Bourne, 1980). Nicotinic or high K^{+} stimulation induces the rise in cytosolic Ca^{2+} as a result of influx of Ca^{2+} from the extracellular milieu (Trifaró and Bourne, 1980; Cheek et al, 1989; Kim and Westhead, 1989). On the contrary, muscarine increases intracellular Ca^{2+} release from intracellular stores (Wilson and Kirshner, 1977; Kim and Westhead, 1989). This is independent of extracellular Ca^{2+} (Kao and Schneider, 1985) and mediated by inositol 1,4,5-trisphosphate (Huges and Putney, 1990). The most important fact is that muscarine stimulation can not release catecholamines (Wilson and Kirshner, 1977; Fisher et al, 1981), or produce F-actin disassembly and scinderin redistribution (Vitale et al, 1991). This seems to suggest that Ca^{2+} release from intracellular store under muscarine stimulation is not enough to trigger catecholamine release.

B. Histamine effect on actin network changes during exocytosis

The present experiments clearly demonstrate that stimulation with histamine also induces scinderin redistribution and cortical F-actin disassembly together with catecholamine release (Fig.6,7,8,11). These effects were concentration-dependent (Fig.10) and with EC_{50}s of 3 and 5 μM for scinderin redistribution and cortical F-actin disassembly, respectively. These values are of the same order of magnitude as those EC_{50} values (1 and 3 μM) published for histamine-evoked catecholamine release (Noble et al., 1988; Choi et al., 1993). The effect of histamine on scinderin redistribution and cortical F-actin disassembly were mediated through H_1 receptors (Fig.12,13,14).

It has been suggested that adrenaline-containing cells show three times more H_1 receptors per cell when compared to noradrenaline-containing cells (Choi et al., 1993). The preferential release of adrenaline from chromaffin cell preparations in response to histamine has been attributed to the high density of H_1 receptors in adrenaline-containing cells (Choi et al., 1993). The studies presented here demonstrate that, under our experimental conditions (short incubation time with histamine in the absence of extracellular Ca^{2+}), both adrenaline-containing (PNMT positive) and noradrenaline-containing (PNMT negative) cells were similarly stimulated (Fig.15,16). Moreover, short incubation periods with nicotine, but this time, in the presence of extracellular Ca^{2+}, yielded similar results (Fig.15,16). The reason for the discrepancy of our findings with previous studies (Choi et al., 1993) is not clear. However, in the latter experiments, cells or tissues were exposed to histamine for longer periods of time (30-120 min) always in

the presence of extracellular Ca^{2+} (Choi et al., 1993; Cheek et al., 1993). In our experiments we were only concerned with the initial events by which histamine triggers secretion.

The results presented in this thesis also show that scinderin redistribution, cortical F-actin disassembly and catecholamine release were all of similar magnitude either in the presence or in the absence of extracellular Ca^{2+} (Fig.8,9,11). In contrast to our observations, previous studies have concluded that histamine-induced catecholamine release from chromaffin cells was largely or totally dependent on extracellular Ca^{2+} (Livett and Marley, 1986; Noble et al., 1988; Cheek et al.,1993). A difference between our and these experiments was that the periods of stimulation in the other studies were very long (20-30 min) and, consequently, it is quite possible that during these long, and perhaps, unphysiological periods of stimulation, the cells were Ca^{2+} starved and, therefore, they required extracellular Ca^{2+} for the operation of the exocytotic machinery. The fact that the initial phase of histamine stimulation does not require extracellular Ca^{2+}, does not rule out the importance of extracellular Ca^{2+} in exocytosis. On the contrary, the initial phase of catecholamine release and, for this matter of scinderin redistribution and cortical F-actin disassembly, in response to either nicotine or a depolarizing concentration of K^+, requires the presence of extracellular Ca^{2+} (Vitale et al., 1991). Therefore, to initiate the operation of the exocytotic machinery, a rise in intracellular Ca^{2+} at the specific site is a necessary and absolute requirement.

However, the origin of this Ca^{2+} could be either from external or internal sources. The experiments presented in this thesis clearly indicate that initial phase of scinderin

redistribution, F-actin disassembly and catecholamine release in response to histamine are triggered by an increase in intracellular Ca^{2+} from internal stores. It has been previously demonstrated that histamine induces PIP_2 breakdown with a subsequent increase in IP_3 and diacylglycerol (Noble et al., 1986; Plevin and Boarder, 1988; Staudermann and Pruss, 1990). The results presented here agree with these earlier observations and in addition showed that U-73122, a phospholipase C inhibitor, not only blocked the increase in IP_3 (Fig.24,25) and the subsequent increase in intracellular Ca^{2+} (Fig.26D), but also blocked the three histamine-evoked responses-scinderin redistribution, cortical F-actin disassembly and catecholamine release (Fig.21,22,23). These blocking effects were not observed with U-73343, an inactive isomer of U-73122. These results were strengthened by the observation on the effects of thapsigargin. Thapsigargin, a plant-derived sesquiterpene lactone, is a potent cell permeable substance which interferes with the control of intracellular Ca^{2+} via direct discharge from intracellular stores without hydrolysis of inositol phospholipids (Thastrup et al., 1990; Sagara et al., 1991). The $[Ca^{2+}]_i$ mobilization of thapsigargin is known to be supported by a specific mechanism in which the agent selectively inhibits the endo-sarcoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990; Sagara et al., 1991) and has no direct effect on PKC or protein phosphatase activities (Thastrup et al., 1990; Sagara et al., 1991). In our experiments, this agent blocked the rise in intracellular Ca^{2+} in response to histamine, in spite of the fact that a rise in IP_3 levels was observed during histamine stimulation (Fig.24,25,26E). Similarly, a brief exposure to thapsigargin was enough to block scinderin redistribution, cortical F-actin disassembly and catecholamine release in response to histamine

(Fig.28,29,30).

It should also be noticed that increases in Ca^{2+} from intracellular stores do not always trigger catecholamine release. Muscarine stimulation of chromaffin cells induces PIP_2 breakdown and release of Ca^{2+} from intracellular stores (Kim and Westhead, 1989) but fails to redistribute scinderin or produce F-actin disassembly and catecholamine release (Vitale et al., 1991). Comparison of muscarine and histamine effects on chromaffin cells suggests that for exocytosis to occur, either the increase in intracellular Ca^{2+} should be at a very specific site or that other events (i.e. scinderin activation, F-actin disassembly) not activated by muscarine, are essential.

C. Regulation of actin filament network by second messengers

Diacylglycerol (DAG) is produced as a result of PIP_2 breakdown and this substance is the physiological activator of protein kinase C (Nishizuka, 1986). The two inhibitors of PKC tested (calphostin C and chelerythrine) produced a concentration-dependent inhibition of the three responses to histamine (Fig.17,18,19,20), suggesting the involvement of PKC in histamine-evoked responses. Inhibitions of scinderin redistribution, cortical F-actin disassembly and catecholamine release were of the same magnitude. These results together with previous observations (Rodríguez Del Castillo et al., 1992a; Vitale et al., 1992a) suggest that if scinderin redistribution and cortical F-actin disassembly are inhibited, catecholamine release is also proportionally inhibited. Indeed, 30-40% of the total nicotine-evoked scinderin redistribution and F-actin

disassembly was blocked in a dose-dependent manner by sphingosine, staurosporine and calphostine C, all inhibitors of PKC (Rodríguez Del Castillo et al., 1992a; Vitale et al., 1992a). These inhibitors produced similar blockades in nicotine-induced catecholamine release (Vitale et al., 1992a). These latter observations suggest that the PKC role, at least in nicotine-induced responses, is modulatory but not essential. In the case of histamine-induced responses described here, complete inhibition of the responses were observed and these occur in the absence of any changes in histamine-induced increases in intracellular Ca^{2+}. This indicates that histamine-induced PLC activation, IP_3 production and the subsequent release of intracellular Ca^{2+} are not affected by PKC inhibition. Moreover, the results suggest that a PKC-dependent process distal to the increase in Ca^{2+} is involved in scinderin redistribution and F-actin disassembly. The results also show that increases in intracellular Ca^{2+} of a magnitude which by themselves would trigger catecholamine release under normal circumstances, fail to do so when scinderin redistribution and cortical F-actin disassembly are inhibited. The findings discussed above also strongly support Trifaró's hypothesis on cortical F-actin networks as barrier to the movement of secretory vesicles to exocytotic sites and as important components of exocytotic machinery (Fig.31) (Trifaró and Vitale, 1993).

Previous work used a phorbol ester, PMA, to determine the involvement of PKC in regulation of actin filament network (Rodríguez Del Castillo et al, 1992a; Vitale et al, 1992a). The results showed that PMA caused scinderin redistribution (Rodríguez Del Castillo et al, 1992a; Vitale et al, 1992a) and cortical F-actin disassembly (Vitale et al, 1992a) in the absence of extracellular Ca^{2+} and without release of Ca^{2+} from intracellular

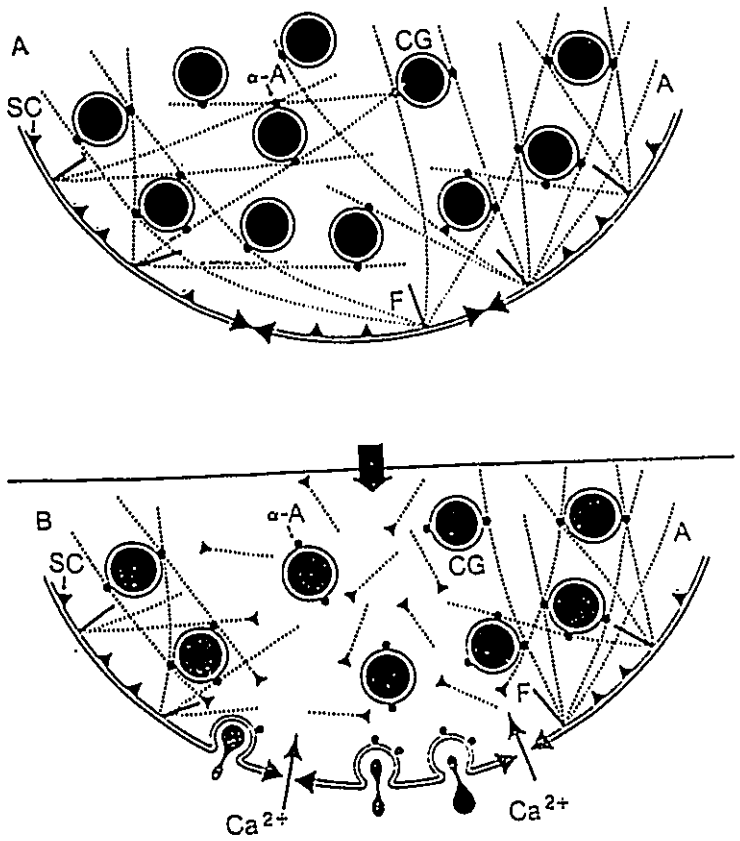


Fig.31 **The possible involvement of the cortical actin filament network in secretory granule exocytosis from the chromaffin cell.**

A) In resting cells, cortical actin filaments (A) are anchored to the plasma membrane and to chromaffin granules (CG) through fodrin (F) and α-actinin (α-A). The cortical actin filament network restrains chromaffin granules from interacting with exocytotic sites on the plasma membrane. B) Cell stimulation induces the influx of Ca^{2+}, which in turn detaches chromaffin granules bound to actin filaments and simulateneously activates proteins that sever actin filaments [such as scinderin (SC)]. This creates subplasmalemmal areas of low viscosity in which chromaffin granules are highly mobile; these areas are the exocytotic sites. (Taken from Trifaró and Vitale, 1993).

stores. There are several lines to suggest that disassembly of F-actin by phorbol ester is mediated by activation of PKC: 1) PMA can produce translocation of PKC from the cytoplasm to membranes (Vitale et al, 1992b); 2) 4α-PMA, an inactive isomer of PMA, cannot produce any redistribution of cortical staining (Vitale et al, 1992b); 3) PKC inhibitors (sphingosine, staurosporine and calphostine C) can inhibit PMA induced cortical F-actin disassembly (Vitale et al, 1992b); and 4) PMA cannot modify intracellular Ca^{2+} (Vitale et al, 1992b; Rodríguez Del Castillo et al, 1992a). Although PMA can induce scinderin redistribution and F-actin disassembly, no catecholamine release had been observed (Vitale et al, 1992a), suggesting that activation of PKC and F-actin disassembly are not enough to trigger catecholamine release. This confirmed again the necessary requirement for an increase in intracellular Ca^{2+}. On the other hand, as discussed above, an intracellular Ca^{2+} rise without cortical F-actin disassembly can not produce catecholamine release. These observations suggest that both, F-actin disassembly and increase in intracellular Ca^{2+} are necessary requirements for catecholamine release. PKC activation might be involved to destabilize the cortical actin network (Vitale et al, 1992a). In view of this, the site of action of PKC in the control of cytoskeleton dynamics should be determined. Two possibilities exist. Firstly, PKC induces an increase in intracellular pH with a corresponding scinderin activation. Although experiments to determine pH have not been carried out in this thesis, previous work has showed a rise in intracellular pH upon PKC activation (Rodríguez Del Castillo et al, 1992a). These experiments have also shown that alkalinization decreases the binding of scinderin to phospholipids in vitro and that activation of PKC, redistributes

scinderin from the cortical region to the interior of the cell (Rodríguez Del Castillo et al, 1992a). Moreover, isometyl amiloride, a blocker of the Na^+/H^+ antiport, blocked the scinderin redistribution induced by PKC activation (Rodríguez Del Castillo et al, 1992a). In summary, cytosolic alkalinization induced by PKC activation will activate scinderin. The activated scinderin will be able to sever cortical F-actin filaments. Another possibility is that PKC might induce phosphorylation of MARCKS. MARCKS is that a protein known to crosslink actin filaments in its unphosphorylated state (Hartwig et al, 1992; Aderem, 1992). MARCKS is also a substrate for calmodulin-dependent kinases (Hartwig et al, 1992; Aderem, 1992). It might be possible that phosphorylation of MARCKS by PKC would reorganize the cortical actin cytoskeleton and remove the barrier to secretory vesicle movement. This possibility needs further study.

D. SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The exact mechanisms underlying the secretory process is not yet clearly known. However, the biological and biochemical data available from the study of chromaffin cells suggest that actin based cytoskeleton may undergo dynamic assembly and disassembly as the physiological state of the cell changes. The present studies have described that histamine, another chromaffin cell secretagogue, have the following effects on chromaffin cells.

1. Histamine induces scinderin redistribution, F-actin disassembly and catecholamine release via H_1 receptor. Histamine stimulation evoked similar patterns of distribution of scinderin and filamentous actin and the magnitude of these responses were concentration-dependent.

2. The rapid responses to histamine occurred in absence of extracellular Ca^{2+} and were triggered by release of Ca^{2+} from intracellular stores. Thapsigargin, an agent known to mobilize intracellular Ca^{2+}, blocked the rise in intracellular Ca^{2+}, scinderin redistribution, F-actin disassembly and catecholamine release in response to histamine.

3. The trigger for the release of Ca^{2+} was inositol 1,4,5-triphosphate (IP_3). U-73122, a PLC inhibitor, inhibited the increase in IP_3, intracellular Ca^{2+}, scinderin redistribution, cortical F-actin disassembly and catecholamine release in response to histamine whereas U-73343, an inactive isomer of U-73122, was without effect.

4. PKC may also be involved in the response to histamine since calphostin C and chelerythrine, two inhibitors of PKC, blocked all responses to histamine without affecting the release of Ca^{2+} from intracellular stores.

5. One of the most important aspects of the results presented here is that in the absence of F-actin disassembly, rises in intracellular Ca^{2+} are not by themselves capable of triggering substantial catecholamine release. This is an important observation since it has

been stated over and over that increases in intracellular Ca^{2+} are always accompanied by neurotransmitter release.

The above results clearly demonstrate that cortical F-actin network disassembly is a necessary event in the activation of the exocytotic machinery and this is a new concept in neurosecretion.

CHAPTER V

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