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STIMULUS-SECRETION COUPLING *in* CHROMAFFIN CELLS

Volume II

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Stimulus-Secretion Coupling in Chromaffin Cells

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INTRODUCTION

The adrenal chromaffin cell occupies a unique place in modern cell and neurobiology. Originating from the neural crest, chromaffin cells are believed to be modified postganglionic sympathetic neurons. Thus, over the past two decades, they have served as one of the most readily available model systems in studying the mechanism(s) of neurotransmitter release. The pioneering experiments of W. W. Douglas and his colleagues in adrenal chromaffin cells have firmly established the now widely accepted concept of calcium-dependent stimulus-secretion coupling as a universal mechanism for the exocytotic secretion of a variety of neurotransmitters and hormones, packaged in intracellular storage organelles of many different cell types.

Over the years, a number of excellent reviews on chromaffin cells have been published, most of them dealing with general aspects of chromaffin cell biology. However, during our own involvement in the study of the mechanism of catecholamine secretion, we felt the need for a more specialized treatise on what we believe the be the central *raison de etre* of chromaffin cells: catecholamines and neuropeptides, synthesized and stored in the cells, are released in a strictly controlled fashion and upon special physiological demand only. So what then are those mechanisms that translate the extracellular signals into the cellular responses, culminating in the secretion of storage products? What do we really know about the intracellular mechanism linking cell activation to secretion?

In order to address these questions in as broad a fashion as possible, we have gathered for these volumes authoritative contributions of leading experts on the adrenal chromaffin cell, and in particular, on the mechanism(s) of stimulus-secretion coupling. As it is unavoidable in multiauthored monographs like this, some of the contributions contain several seemingly redundant treatises of similar issues; however, we deliberately invited such complementary chapters, since every author is presenting his individual concept of a very complex issue.

As is evident from studying the various chapters, we are still far from being able to draw a unified picture of what might be going on during the processes of stimulus-secretion coupling in the adrenal chromaffin cell. On the contrary, the more detailed our information becomes about the intracellular events following stimulation of the cells and leading up to the exocytotic secretion, the more emerges our obvious lack in understanding of the finer tunings of the cell biology of the chromaffin cells. At present, exciting new developments are rapidly changing some of our basic understanding about, for example, membrane biophysics of ion channels, intracellular second and third messengers, the temporal and spatial role of calcium homeostasis, or the role of cytosolic proteins in mediating exocytotic membrane fusion, to name but a few of the unresolved issues. Furthermore, with more details emerging on the biological, biophysical, and molecular biological patterns of stimulus-secretion coupling in chromaffin cells, the more the individuality of this particular cell will become evident.

In editing this book, we therefore attempted to provide, for the first time, an in-depth resume of several aspects of our current (as of 1986) understanding of stimulus-secretion coupling. The authors were asked not only to provide a state-of-the-art review in their respective fields of interest, but also to look ahead and try to address unresolved issues and those relevant questions to be tackled in the near future. Thus, this monograph, centered around a key function in the cellular biology of adrenal chromaffin cells, combines solid evidence on what is presently known as well as more speculative individual assessments as to future developments. Concomitantly, however, this book is published with the cautionary notion on extrapolating our current knowledge about chromaffin cells to apparently similar mechanisms governing stimulus-secretion coupling in other secretory cells or to different endocrine organs.

For technical reasons the 15 chapters have been arranged in two volumes. Nevertheless, these two volumes are intended as a contiguous, single monograph on the multifaceted issue termed ''stimulus-secretion coupling''.

In the first chapter, S. W. Carmichael presents a detailed description of the anatomical morphology of the adrenal medulla. J. H. Phillips deals in two chapters with an extensive discussion of chromaffin granules: beyond the biogenesis of these storage organelles, their structure, and their dynamics, the author discusses the fate of chromaffin granules during the perpetual cycles of exocytosis and endocytosis.

More recently, a number of bioactive peptides, in particular enkephalins, were found to be localized in chromaffin granules and co-released together with the catecholamines; these exciting new findings are summarized by C. D. Unsworth and O. H. Viveros in Chapter 4.

Calcium is generally believed to be of central importance for a number of biochemical processes linking cell stimulation to exocytotic secretion. One of the unresolved issues relates to the question of calcium buffering and calcium homeostasis in chromaffin cells and the possible role of the granules in these processes. In Chapter 5, Gratzl discusses possible pathways for the uptake of calcium into chromaffin granules and the relevance of Na^+/Ca^{2+} exchange across the granules for intracellular calcium homeostasis.

Specific calcium-regulating and -regulated cytosolic proteins, which are believed to be involved in intracellular signal transduction, are discussed in the next group of chapters. As described in Chapter 6, Trifaró and Kenigsberg used classical pharmacological approaches as well the fusion of antibody loaded erythrocyte ghosts with cultured chromaffin cells to probe the central role for calmodulin in stimulus-secretion coupling. The relevance of cytoskeletal proteins in intracellular signal transduction, especially the structural and functional role of a spectrin-related, actin-associated protein α-fodrin is reviewed by Aunis, Perrin, and Langley in Chapter 7. In recent years, a number of cytosolic proteins, such as the phospholipases, proteinkinases, etc., have been proposed to mediate calcium action during exocytosis. In Chapter 8, Pollard and his colleagues primarily discuss the family of synexins and immunologically related proteins, which seem to regulate membrane contact and fusion in a calcium-dependent fashion. P. I. Lelkes describes (Chapter 9) how liposomal vectors can be employed to introduce bioactive (macro) molecules, such as cytoskeletal proteins, etc., into intact chromaffin cells to study their involvement in the cascades of stimulus-secretion coupling.

Recognition of the stimulus at the plasma membrane is the primary event in activating a cellular response. Yet, as summarized by Rosenheck in Chapter 10, our present knowledge of the functional biochemistry at the plasma membrane level is quite limited, presumably due to too scarce a usage of isolated chromaffin cell plasma membrane preparations.

Adrenal chromaffin cells are activated via nicotinic and/or muscarinic receptors, depending on the species and probably also on physiological idiosyncrasies. The importance of muscarinic stimulation has recently been emphasized due to the clear linkage of muscarinic activation to the phosphoinositide metabolism in a number of eukaryotic cells. In Chapter 11, Allan Schneider discusses muscarinic receptor mechanisms in adrenal chromaffin cells.

Cell activation comprises transmembranal ion fluxes. In intact cells, the various ion channels of the plasma membrane have been characterized and the ion fluxes pertaining to the intracellular signal transduction have been widely studied. In Chapter 12, Kirshner presents an overview of the more classical biochemistry and electrophysiology of calcium and sodium channels in intact chromaffin cells. However, over the past few years, electrophysiology has been revolutionized by analyzing single channels using the patch-clamp technique. Thus, Kidokoro summarizes in Chapter 13 these more recent developments in membrane biophysics of chromaffin cells.

Inhibitory modulations of the secretory response, often termed desensitization, are discussed in the final two chapters. In Chapter 14, Garcia and his co-workers review the

evidence for the pivotal role of calcium channel activation for the onset of stimulus-secretion coupling and the inactivation of the secretory response by sustained elevated intracellular calcium concentrations. Finally, in Chapter 15, Bruce Livett summarizes our current understanding of various parameters, in particular the role of neuropeptides, which in vitro and in vivo are involved in the modulation of the secretory response in adrenal chromaffin cells.

During the entire process of compiling and editing this monograph, we were encouraged by Marsha Baker, the senior editor in charge of the Uniscience Series at CRC Press, and guided by the helpful advice and patience of our coordinating editor, Anita Hetzler. We especially appreciate the enduring understanding and support from our families, who for the many years of our close collaboration have tolerated our time-consuming fascination with the chromaffin cells more or less patiently. We therefore dedicate this book to our families, especially to our wives, Alma Rosenheck and Iris Lelkes.

Rehovoth and Bethesda, December 1986

Kurt Rosenheck
Peter I. Lelkes

THE EDITORS

Kurt Rosenheck received his Ph.D. in Physical Chemistry from the Hebrew University of Jerusalem in 1959 for work on polyelectrolytes, under the direction of Aharon Katzir-Katchalsky. He then was a research assistant in the Polymer Department of the Weizmann Institute of Science, Rehovot, Israel, and subsequently worked as a research fellow with Paul Doty in the Chemistry Department of Harvard University.

He spent 1969 as a resident scientist in the Neuroscience Research Program of the Massachusetts Institute of Technology, Cambridge, directed by Francis O. Schmitt. In 1975 he was a visiting investigator in the Endocrinology Section, directed by Martin Sonenberg, of the New York Sloan-Kettering Institute for Cancer Research. In 1977 he was appointed Associate Professor at the Weizmann Institute's Department of Membrane Research. During the years 1984 and 1986 he held Visiting Professorships at the Universities of Constance and Bielefeld, in West Germany, as well as at the University of Berne in Switzerland.

Starting out as a polymer spectroscopist, he later applied ultraviolet light spectroscopic techniques to the study of protein conformation and lipid-protein interactions in biological membranes. This led to his interest in the membrane-linked events occurring during the stimulus-secretion response, a field he has been active in for the last 10 years.

Peter I. Lelkes, born in 1949 in Budapest, Hungary, received his training in physics, biochemistry, cell biology, and membrane biophysics at the Technical University in Aachen, West Germany. In 1977 he joined the Department of Membrane Research at the Weizmann Institute of Science in Rehovoth, Israel, as a postdoctoral fellow to work on physicochemical aspects of protein-lipid interactions of biological membranes. Subsequently, as a staff scientist in the same department, he focused his interest on the mechanism of membrane fusion of biological membranes, and in particular, on the involvement of cytosolic proteins in the stimulus-secretion coupling in chromaffin cells and other secretory systems.

In 1983, he went to the National Institutes of Health, Bethesda, Md., to further study the pathways of intracellular signal transduction of secretory cells. Working in the National Institute of Diabetes, Digestive, and Kidney Diseases, he currently holds the position of Visiting Scientist in the Laboratory of Cell Biology and Genetics, and continues to study cell biological aspects of the activation of adrenal medullary chromaffin and other endocrine cells.

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Chapter 8

CYTOSOLIC PROTEINS AS INTRACELLULAR MEDIATORS OF CALCIUM ACTION DURING EXOCYTOSIS

Harvey B. Pollard, Alexander L. Burns, Andres Stutzin, Eduardo Rojas, Peter I. Lelkes, and Kyoji Morita

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I. INTRODUCTION

It is now beyond argument that calcium plays a critical role in the regulation of secretion from chromaffin cells. However, exactly what this role entails, or whether there are a multiplicity of such roles remains a mystery. Calcium enters the cytosol upon stimulation and then provokes a cascade of events leading to secretion of chromaffin granule contents, but not the granule membrane. This is "exocytosis". However, even such questions as how calcium enters, from which sources, how much is free, and over what time course calcium remains free are, as yet, unanswered with certainty.

Going beyond the quantitative problems of calcium concentration, the consequences of calcium presence seem to be movement of granules to the plasma membrane, contact with the membrane, and eventual fusion. It was once thought that calcium acted directly on the plasma membrane and granule membrane to induce fusion. Presently there is really no compelling experimental reason to support such a contention. On the other hand, there is a number of specific proteins which could mediate calcium action in these various events. Recent candidates include calmodulin, actin, cytoskeletal proteins, synexin, protein kinase C, phospholipase C, metabolic products of phosphatidylinositol and inositol phosphates, metalloendoproteases, and other as yet unspecified fusion factors.

It is our intention in this chapter to focus attention on cytosolic activities possibly involved in calcium signaling and on two specific proteins that interact with chromaffin granule membranes in a calcium-dependent manner, actin and synexin. It is possible that calcium-dependent interactions with actin model events occurring during granule movement through the cytosol, while interactions with synexin may model membrane contact and fusion events occurring during exocytosic secretion.

II. CALCIUM SIGNAL PROCESSES

A. Phospholipase C

Phospholipase C is becoming increasingly appreciated as an important intermediary in a growing number of secretory cell systems.[2] Phospholipase C cleaves phosphatidyl inositol to liberate inositolphosphate (IP) or more phosphorylated derivatives such as inositolbisphosphate (IP_2) or inositoltrisphosphate (IP_3). In the case of skeletal muscle[3] and other systems the IP_3 is believed to mediate release of calcium from the sarcoplasmic reticulum, or, in the case of nonmuscle cells, the endoplasmic reticulum.

In the case of the chromaffin cell, the possible involvement of IP_3 with secretion has been somewhat mysterious. By analogy with other secretory systems one might expect that physiologic secretagogues might evoke IP_3 synthesis and coincident increases in cytosolic Ca^{2+} concentration from internal stores. Perversely, however, nicotine, the specific cholinergic secretagogue, apparently has no effect on phosphatidylinositol metabolism, whereas muscarine, having no secretagogue activity, does indeed have this effect.[4] Until recently, this fact has lurked uncompromisingly in the back of many otherwise optimistic reviews about the likely generality of phospholipase-C activation being coupled to calcium mobilization and secretion.

The solution to this problem has come from studies on the real-time kinetics of ATP release from chromaffin cells[5] using a newly available, highly purified luciferase to detect quantitatively released ATP over lengthy time periods. We noted that muscarine was actually able to potentiate nicotine-induced ATP release from chromaffin cells if the two agonists were added within at least 1 min of one another. Interestingly, the potentiation was barely observed if the two compounds were added simultaneously. In their subsequent work, Forsberg et al.[6] observed the same phenomena for catecholamine release and noted that IP_3 rose quickly during the first 15 sec after muscarinic stimulation. Others[7] had shown that the

calcium concentrations measured by quin-2 rises and falls slightly over a 2-min period when chromaffin cells are exposed to muscarine. Indeed, muscarine is known to cause calcium efflux from chromaffin cells.[8] Thus, the window of opportunity for muscarine to potentiate nicotine-induced release would seem to be an IP_3-mediated rise and fall in intracellular calcium concentration.

To further substantiate this concept, Forsberg et al.[6] noted that a slight depolarization with 10 m*M* KCl could likewise potentiate nicotine-induced release of both ATP and catecholamines. The only common consequence of muscarine and KCl could, on the basis of present knowledge, only be an elevation in cytosolic calcium concentration.

B. Protein Kinase C

As a further consequence of phospholipase-C activity diacylglyceride is also produced. Diacylglyceride and calcium, together with phosphatidyl serine, activate protein kinase C, and in a variety of systems activation of protein kinase C has also been implicated with activation of secretory processes.[9]

The evidence for protein kinase C being involved in regulation of secretion from chromaffin cells is only indirect, but nonetheless remains encouraging. The tumor-promoting phorbol ester TPA is an effective analog of diacylglycerol for activation of protein kinase C, and this same compound has been found to decrease the calcium concentration dependence of catecholamine release from high voltage permeabilized chromaffin cells.[10] However, phorbol esters had no effect on secretions from intact cells. Morita et al.[11] did find that phorbol ester could potentiate A23187-induced catecholamine secretion, but the ester had no effect on secretion induced by carbachol or high potassium. Recent results from Brocklehurst and Pollard[12] also showed that the phorbol ester TPA could enhance calcium-induced catecholamine release from digitonin-permeabilized cells, and also raise the apparent calcium sensitivity of the process. In this case, high voltage-permeabilized cells and digitonin-permeabilized cells behaved similarly.

These results might indicate that protein kinase C could be involved in modulating secretion induced by elevation in bulk cytosolic calcium concentration, but not secretion induced by secretagogues acting on plasma membrane receptors. The concept of mechanistic significance being accorded these two types of calcium compartments is also found in other chapters of this book. It is becoming increasingly apparent that the mere presence of elevated calcium concentration in the cytosol is actually necessary, but not sufficient, to induce release from chromaffin cells.

Protein kinase C has been extensively studied, but seldom purified from biological tissue. Brocklehurst et al.[13] characterized protein kinase C in crude extracts from bovine adrenal medulla with respect to calcium, diolein, and phorbol ester concentration dependencies. More recent studies by Brocklehurst et al.[73] have confirmed that the purified enzyme has quite similar properties. The most troublesome result of these studies has been the fact that the calcium dependence of protein kinase C seems to be substantially greater than the calcium dependence of release from the permeabilized cell systems. However, it remains possible that protein kinase C *in situ* may be under cooperative controls missing in the more purified states. Pocotte and Holz[14] have additionally suggested that phorbol ester could evoke phosphorylation of tyrosine hydroxylase, possibly via protein kinase C. The evidence for this "housekeeping chore" as an aspect of protein kinase C action, however, also remains indirect.

C. Metalloendoprotease Inhibitors and Secretion

Based on certain analogies to viral fusion, Strittmatter and co-workers[15] have recently proposed that metalloendoproteases might be generally involved in fusion of biological membranes. Evidence for this hypothesis is based on experiments in which inhibition of

fusion in rat myoblast cultures was observed in the presence of certain oligopeptides, believed to be specific substrates and/or inhibitors of metalloendoproteases.

Indeed, the same oligopeptide inhibitors that inhibited fusion of myoblast cells,[15] neurotransmitter release at the mouse neuromuscular junction,[16] and histamine release from mast cells[17] were also found to interfere with catecholamine secretion from bovine adrenal chromaffin cells.[18] In mast cells and in chromaffin cells inhibition of secretion was dependent on both the type of stimulus employed and on the concentration of the respective agonists. Most effective inhibition of catecholamine release was observed when stimulating the chromaffin cells via the nicotinic receptor, while secretion evoked by other secretagogues, e.g., elevated extracellular K^+, Ba^{2+}, or veratridine, was much less susceptible to inhibition by the antagonist to metalloendoprotease activity.

This preferential inhibition of receptor-mediated stimulation by the oligopeptides casts doubt about their site of action, since it raises the possibility that these hydrophobic compounds might also exert nonspecific anticholinergic effects. Therefore, conclusions as to the involvement of metalloprotease activity in (exocytotic) membrane fusion, which are based on such inhibition studies, should be taken with great caution.

In the search for alternate cellular effects, we found that metalloprotease inhibitors which interfered with catecholamine secretion also modulated transmembranal calcium fluxes and the concentration of free intracellular Ca^{2+}.[74] Efflux of $^{45}Ca^{2+}$ was accelerated in the presence of the inhibitors, while at the same time the cytoplasmic-free Ca^{2+} was raised to a level, which by itself was not sufficient to cause catecholamine release. This elevated Ca^{2+} level, however, prevented a further increase in the cytoplasmic-free Ca^{2+} concentration upon addition of the various secretagogues.

Thus, with the above-mentioned cautionary note about possible nonspecific effects of the metalloprotease inhibitors in mind, we might speculate that metalloendoprotease activity is involved in regulating intracellular calcium homeostasis and/or calcium-dependent receptor inactivation. Rather than modifying a putative fusion protein, a metalloprotease activity might be required for the proteolytic cleavage of a protein, which itself might be involved in regulating the function of receptor-associated calcium channels.

III. CYTOSKELETAL PROTEINS

A. Interaction of Granules with Actin

The cytoplasm of chromaffin cells is composed of cytoskeletal elements including microfilaments, microtubules, and intermediate filaments,[19-21] and direct interactions between chromaffin granules and cytoskeletal elements have been observed by stereoelectron microscopy of cells embedded in water-soluble media.[22] We presumed there must be quite specific mechanisms regulating granule-cytoskeletal interaction, since the granules clearly move from one region of the cell to another.

Actin and myosin have been widely described in chromaffin cells,[23-30] and actin may actually be associated with the granule membrane. However, in highly purified chromaffin granule membranes, Zinder et al.[31] showed that the band comigrating with authentic actin was not actin. They used a highly specific fingerprint analysis of peptides eluted from the protein in the band. By contrast, a band comigrating with actin on gels of purified plasma membranes from chromaffin cells was, by the same criterion, indeed, actin. The basis of this difference of opinion about actin being on granules may rest with the fact that granule membranes analyzed by the different groups may have differed in purity. However, this does not mean that no association occurs in vivo and, as will be evident, such studies with less pure granule membrane preparations may prove to be more relevant to the in vivo situation.

Indeed, actin interacts with granule membranes when mixed together, as originally described by Burridge and Phillips.[24] Wilkins and Lin[32] also reported that they could detect stable oligomers of actin on granule membranes, using binding of radiolabeled cytochalasin B as an assay. Wilkins and Lin also suggested that these oligomers might be nuclei for the subsequent assembly of actin filaments.

However, Fowler and Pollard[33,34] found that F-actin could interact with highly purified granule membranes depleted of endogenous actin. The technique they used was low shear, falling-ball viscometry, in which the chromaffin granule membranes cross-linked F-actin and thus raised the viscosity of the solution. This interaction was inhibited by calcium, with 50% inhibition occurring at 0.2 μM free Ca^{2+}. Anticalmodulin and antisynexin drugs such as trifluoperazine and promethazine had no influence on this activity. On the other hand, trypsin treatment of membranes blocked the cross-linking activity, indicating that the actin binding site might be protein in nature.

α-Actinin has historically been detected in preparations of granule membranes.[35,36] α-Actinin is a component of Z bands of muscle, implicated by some investigators in the interaction of actin with the organelle and was thus considered a reasonable candidate for the F-actin binding site on granule membranes. However, the purified membranes used by Fowler and Pollard[33,34] were prepared under magnesium-free, low ionic strength conditions designed to elute out α-actinin.[27] Aunis and Perrin[37] have verified that α-actinin is indeed removed and have proposed instead that a spectrin-like protein (fodrin) might be the true granule membrane component responsible for binding F-actin (see Chapter 7 for more details).

The sensitivity to calcium may be the most important property of the actin binding site on granule membranes in terms of exocytosis regulation. It is possible that under resting conditions of cell calcium (≤ 0.1 μM free calcium) granules might be relatively immobilized, attached to F-actin in the cytoplasm. However, upon elevation of cell calcium after stimulation, the interaction between F-actin and granules would break. Granules would then become free to interact with the cell membrane and undergo subsequent fusion processes.[38] Indeed, direct visual evidence for this notion may have been provided in recent experiments by Aunis and co-workers,[39] who found that fodrin was specifically localized around the plasma membrane, and that upon stimulation of cells the distribution of cytoimmunospecific fodrin appeared modified near the plasma membrane.

B. Influence of Cytoskeletal Drugs and Proteins

However, in spite of interesting chemical data linking cytoskeletal proteins to secretory vesicles and plasma membranes, evidence for relevance to actual secretion processes has been difficult to obtain. Early experiments, using anticytoskeletal drugs such as colchicine, vinblastine, or cytocholasin B, were compromised by the findings that inhibition of catecholamine release by these drugs may be predominantly due to their strong anticholinergic effects, rather than to specific action on microfilaments or microtubules.[40,41] Furthermore, the data gathered from the use of anticytoskeletal drugs in electrically permeabilized "leaky" cells have seemed to clearly contradict the hypothesis for a role of cytoskeletal proteins in Ca^{2+}-induced exocytosis.[4] Indeed, these drugs had little, if any, effect on release from permeabilized cells. However, the permeabilized cell as a model for secretion has a major deficiency recognized by most who have studied the system. By virtue of the permeabilization step the natural pathways of signal transduction across the plasma membrane are necessarily short circuited. This means that one cannot tell whether the processes now observed are truly of importance in the intact cell.

Recently, liposome-cell fusion has been employed to overcome the membrane permeability barrier in intact chromaffin cells.[42] Using this technique (see Chapter 9), microfilament-specific macromolecules, such as heavy meromyosin and DNAse I, have been introduced

efficiently into viable isolated chromaffin cells. The outcome of these experiments indicate a modulatory role of the cytoskeleton exactly at the level of transmembranous signaling, a site hitherto inaccessible using permeabilized cells. Following liposome-mediated injection of DNAse I, which is known to shift the G-F equilibrium of actin, Friedman and co-workers[43] observed an increase in catecholamine release concomitant with a significant sustained depolarization of the plasma membrane.

More recent experiments have shown similar effects for heavy meromyosin and its S_1 subfragment, but not if the same compounds were rendered mechanochemically inactive by poisoning them with *N*-ethyl maleimide (NEM).[42] DNAse I and heavy meromyosin, but not NEM-poisoned heavy meromyosin, also induced sustained membrane depolarization concomitant with a significant increase in the influx of sodium and calcium. In addition, Ca^{2+} (20 μM)-induced catecholamine release from digitonin permeabilized cells) was augmented in the presence of DNAse I and heavy meromyosin, but not upon addition of NEM heavy meromyosin. In contrast, addition of F-actin resulted in a marked decrease of Ca^{2+}-stimulated release from digitonin-permeabilized cells.[75]

These data thus indicate that these macromolecular reagents introduced by liposome fusion methods may have actions on membrane sites in addition to cytoskeletal sites, either directly or indirectly. Nonetheless, it is highly likely that specific location and dislocation of granules in the cytoplasm of chromaffin cells, after introduction of calcium, will prove to be a sensitive function of the easily detected interaction between granule membranes and F-actin. Indeed, Morita and Pollard[44] have recently shown that actin also stabilizes a granule ATPase activity against thermal denaturation, thus indicating more extensive effects of actin than mere binding. Actin and myosin may also prove to have concomitant actions on separate plasma membrane functions related to secretion.

IV. SYNEXIN

A. Synexin and Granule Aggregation

For exocytosis to occur the granule membrane must contact and fuse with the plasma membrane. Therefore, to understand the process one must explain how calcium can induce these essentially mechanochemical events. While calcium can act directly in a number of model systems, many biochemical processes depend on a calcium binding protein for specificity and sensitivity. In the case of exocytosis, we have proposed that synexin, or a synexin-like protein, might be a likely mediator of membrane contact and membrane fusion. Indeed, in the presence of calcium only this protein and its functional relatives promote these events when added to isolated chromaffin granules.

Synexin from adrenal medulla is a 47,000-dalton calcium binding protein that causes isolated chromaffin granules to aggregate to one another by pentalaminar membrane contact.[45,46] The activity can be measured by simply following the turbidity of a granule suspension at A 540 nm. Since secretion from chromaffin cells proceeds by both simple and compound exocytosis the formation of such contacts may indeed be of physiological relevance. In simple exocytosis the granule fuses with the plasma membrane, but in compound exocytosis chromaffin granules make contact and fuse with granule membrane remnants of previous exocytotic events remaining on the plasma membrane. This process may be of great advantage in chromaffin and other cells, since upon stimulation only limited movement of granules is necessary, and the resulting tunnels of fused granules may provide a pathway for controlled penetration of calcium-rich extracellular medium into the depths of the cell.

The exact mechanism of synexin-dependent granule aggregation is a source of some controversy. Creutz et al.[46] found that the calcium titration curve for granule aggregation coincided with that for calcium-dependent polymerization of synexin to form 50 × 100-Å rods. They suggested that calcium acted on synexin to form active polymers, which them-

selves caused granules to aggregate. Only calcium promoted both processes, and calcium-dependent polymerization of synexin has indeed also been used as part of a synexin purification scheme by Morris et al.[47] However, Morris and co-workers preferred the interpretation that calcium acted only on the granule membranes and that synexin promoted this calcium effect on a membrane site.

The site to which synexin binds on the granule membrane is similarly a source of controversy. Dabrow et al.[48] found that treatment of granules with proteolytic enzymes could block synexin action. They concluded that the synexin receptor was a protein. However, Hong et al.[49] reported that synexin caused aggregation and fusion of phosphatidyl serine vesicles in a calcium-dependent manner. The latter authors questioned the existence of a protein receptor and suggested instead that the receptor could be a lipid. In circumstantial support of this conclusion were findings, summarized by Creutz et al.,[50] that synexin was among a series of proteins, termed "chromobindins",[51] that had affinity for the lipid fraction of chromaffin granules when these lipids were bound to a sepharose affinity column.

Pursuing the concept that the synexin receptor might be a lipid, Hong et al.[52] investigated the ability of synexin to aggregate and fuse liposomes of defined composition. The specificity of synexin was manifest by the observation that calmodulin slightly inhibited phospholipid vesicle fusion induced by calcium, while other calcium binding proteins such as bovine prothrombin and its proteolipid fragment 1 had a strong inhibitory action. Synexin alone was able to lower the threshold for calcium-induced fusion of phosphatidic acid (PA): phosphatidylethanolamine (PE)::(1:3) liposomes from 1 m*M* Ca^{2+} to 100 μM Ca^{2+} and subsequently to approximately 10 μM Ca^{2+} in the additional presence of 1 m*M* Mg^{2+}. Hong et al.[52] believed that the mechanism of the fusion process depended upon formation of anhydrous Ca^{2+} complexes with acidic phospholipid head groups, and that synexin somehow promoted this effect.

However, one can question whether these liposome studies accurately model the actual interaction of synexin and chromaffin granules. For example, the specific dependence of this liposome interaction on calcium and its potentiation by magnesium are somewhat different from the observed action of synexin on intact chromaffin granules. Calcium and synexin only induce aggregation of granules, not aggregation and fusion as the combination does with PA/PE::(1:3) liposomes. Furthermore, synexin action does not need magnesium to achieve its threshold for granule aggregation at approximately 6 μM Ca^{2+}.[53] Finally, added magnesium neither potentiates nor inhibits calcium-activated synexin activity. Indeed, the critical variable affecting binding of synexin to chromaffin granule membranes, aside from calcium, is pH. The $K_{1/2}$ for calcium dependence of synexin binding to granule membranes is approximately 5 μM at neutral pH and rises as the pH declines.[53]

However, one element in the study by Hong et al.[52] was more reminiscent of the effects of synexin on native chromaffin granules. In a survey of a variety of phospholipids, the authors discovered that phosphatidylinositol (PI) profoundly inhibited fusion, while still allowing liposome aggregation, when it replaced phosphatidic acid in the PA/PE (1:3) liposomes. This was seen as a unique property of PI, possibly due to the inositol head group blocking access of synexin to the phosphate group of the phospholipid. However, when viewed from the perspective of the synexin reaction with granules, it is an intriguing possibility that phosphatidyl inositol may be the bona fide lipid receptor for synexin, rather than the other phospholipids.

B. Synexin and Granule Fusion

While calcium and synexin can only aggregate chromaffin granules, it is indeed possible to cause the granule aggregates to fuse. This is achieved by the addition of a small amount (5 μM) of arachidonic acid. Other fatty acids will also work so long as the fatty acid has a *cis* unsaturated bond(s).[54,55] Fusion can be followed by phase microscopy or by a reduction