MEMBRANE FUSION

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Permeabilized Cells
An Approach to the Study of Exocytosis

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I. INTRODUCTION

Biological membranes are highly asymmetrical structures that separate cells or subcellular compartments differing greatly in their composition. Well-known cellular activities, such as secretion by exocytosis, uptake of extracellular material by phagocytosis, formation of the multinucleated myotubes during development of striated skeletal muscle, and fertilization, require direct (open) communication between cells or subcellular compartments. This is achieved by the process of membrane fusion. At least two types of membrane fusion can be distinguished: in one type, extracellular membrane surfaces interact with each other (e.g., cell-cell fusion), while in the other, interactions occur between membrane surfaces facing the cytoplasm (e.g., exocytosis).

Exocytotic membrane fusion is difficult to analyze because the interacting membrane surfaces are not accessible from the outside of the cell. Moreover, the complexity of the processes in the chain of events between stimulation of a cell and release of secretory product makes it difficult to study or manipulate adequately membrane fusion in intact cells.

Within the past few years, procedures have been developed that allow exocytotic membrane fusion to be investigated by permeabilization of the plasma membrane of secretory cells. While they leave the exocytotic machinery intact, they allow for modifying the cytoplasmic composition as desired and determining exocytotic output as a function of various manipulations. In this way, the properties of exocytotic membrane fusion can be recorded and compared with the fusion properties of more simple model systems or with the secretory process as exhibited by intact cells.

II. PERMEABILIZATION TECHNIQUES

Three different techniques have been used to permeabilize cells: application of physical force (high-voltage discharges), of detergents, and of pore-forming proteins.

When cells in suspension are briefly exposed to electric fields, their membranes become permeable to solutes. The size of the membrane-bound structure permeabilized depends on the strength of the electric field applied. Hence, one can permeabilize the cell membrane, permitting access to the cytosol, without risking damage of intracellular organelles. This technique has been initially applied to bovine adrenal medullary cells (1-3) and subsequently also to other secretory cells. After this treatment, the adrenal medullary cells behave as if their cell membrane contains pores allowing passage for at least 1 h of substances of up to a molecular weight of about 1000.

Detergents such as digitonin and saponin have also been applied to chromaffin cells (4-9). Within a narrow range of concentration and time, these substances are suitable for permeabilizing the cell membrane without causing leakage of substances from secretory vesicles. Since the size of the pores created is not uniform, i.e., the holes obtained with saponin vary between 0.1 μm and 1 μm (10), intact chromaffin secretory vesicles, which have a diameter of about 0.25 μm, can escape. A careful examination of digitonin-permeabilized chromaffin cells shows that compared to alpha toxin poration (see below) significant changes in the ultrastructure and in the secretory behavior occur with digitonin (10a). The cells also lose proteins essential for exocytosis (10b). During measurement of Ca^{2+}-induced release of chromogranin A and noradrenalin from digitonin-permeabilized chromaffin cells in primary cultures, it has been shown that part of the secretory product can be sedimented by centrifugation (9), which indicates that the secretory product remains membrane bound. Thus, the release observed from digitonin-treated cells does not occur solely by exocytosis. A further drawback of the use of detergents as permeabilizing agents is their possible influence on the exocytotic process itself, a conclusion based on the observed inhibition by detergents of catecholamine release from electrically permeabilized cells (2).

Natural proteins produced from T lymphobytes (11) or bacteria (12,13) or derived from the complement complexes (14) insert in target membranes and lead to the formation of "stabilized" pores because every hole is surrounded by a protein ring (14a).

Two of these proteins, the staphylococcal α-toxin and streptolysin O from streptococci, were valuable for probing the exocytotic process. They attack both the cell membrane of PC 12 cells (a pheochromocytoma cell line from rat) as well as of bovine adrenal chromaffin cells in culture (9,15,16,16a-f).

Unlike the pores created by electrical discharges or detergents, all α-toxin pores are the same size (see Fig. 1). In target membranes, the water-soluble toxin monomers (molecular weight 34 kD) form ring-structured hexamers surrounding a pore with a diameter of 2-3 nm (12). These structures do not permit the passage of myoglobin (17 kD) or Dextran 4 (4 kD) (12,13). Consequently, the toxin monomers cannot enter the cells. Also, the cytoplasmic enzyme lactate dehydrogenase is not released from chromaffin cells in primary cultures or from PC 12 cells under this treatment. However, the rapid efflux of $^{86}Rb^{+}$ from the cells demonstrates complete accessibility of the cytosol for small molecules (9,15,16). Also, the rapid equilibration of cellular ATP or Ca^{2+} or externally added substances like inositol-1,4,5-trisphosphate, different vanadate species, and so forth, indicates an effective poration of the plasma membrane for molecules up to a molecular mass of about 1 kD (16a,16g). Since catecholamines or the protein chromogranin A was not released from the secretory vesicles, α-toxin's attack is strictly confined to the plasma membrane. Therefore, the technically simple permea-

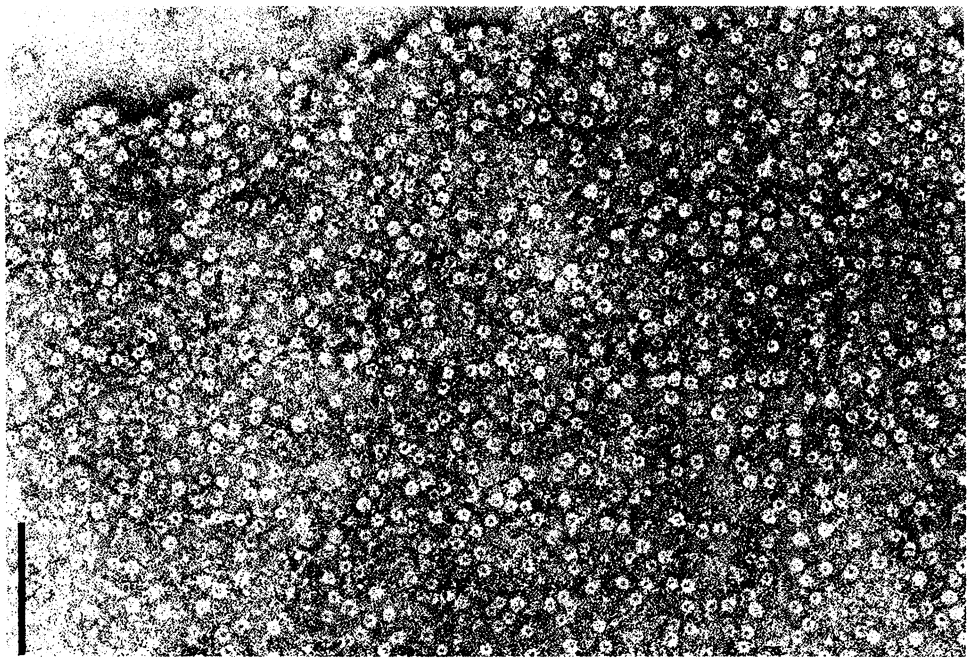

FIGURE 1 Electron micrograph of a negatively stained fragment of rabbit erythrocyte membrane, lysed with *Staphylococcus aureus* α-toxin (20 μg toxin/10^8 cells). Numerous ring-shaped toxin polymers are seen over the membrane. The hexameric form of the toxin has an outer diameter of 10 nm and exhibits a central stain deposit that indicates an inner diameter of about 2.5 nm. Sodium silicotungstate staining. Scale bar indicates 100 nm. (Courtesy of J. Tranum-Jensen, Institute of Anatomy, Department C, University of Copenhagen, Denmark.)

bilization by α-toxin represents an ideal technique for studying exocytotic membrane fusion.

Streptolysin O (SLO) from beta-hemolytic streptococci produces large pores in target membranes (14a). Catecholamine-secreting cells permeabilized with SLO retain an intact exocytotic machinery and antibodies to intracellular constituents, as well as tetanus toxin and botulinum A toxin, can be introduced directly into these cells (16d-f).

In addition to the three procedures described here, another closely related technique is the use of hemolytic Sendai virus as a permeabilizing agent (17). However, the size of the holes created with this procedure cannot be precisely defined. Therefore, this technique must be considered inferior to permeabilization with α-toxin or streptolysin O.

III. RELEASE STUDIES WITH PERMEABILIZED CHROMAFFIN CELLS

A. Ca^{2+} Requirement

The concept of stimulus-secretion coupling was developed around 25 years ago (18). This coupling concept describes calcium as having a crucial role in the regulation of secretion by exocytosis. Injection of Ca^{2+} into mast

cells (19) and nerve (20) provided direct evidence for the role of intracellular Ca^{2+} as a trigger substance.

The Ca^{2+} concentration is precisely controlled within secretory cells by systems present in the cell membrane and in subcellular structures. In resting cells, the free Ca^{2+} concentration is close to 10^{-7} M but increases upon receptor activation and/or depolarization owing to an influx of Ca^{2+} from the extracellular space which contains high (mM) concentrations of Ca^{2+} (cf. Refs. 21-23). Receptor activation and linked processes can be circumvented by ionophores which have often been used to facilitate the Ca^{2+} influx. A complete exchange of the intracellular fluid as well as its exact control (e.g., by buffering substances) can be achieved if pores sufficiently large in size to permit exchange of small or even high-molecular weight substances can be created in the cell membrane.

Compared to intact cells, permeabilized cells require much less Ca^{2+} for the release of secretory product. An example is given for pheochromocytoma cells (PC 12) (Fig. 2). Intact cells require mM concentrations of Ca^{2+} but α-toxin permeabilized cells respond already to μM Ca^{2+}.

Exocytosis in intact cells is the complete transfer of small as well as large molecules from a intracellular vesicular compartment to the extracellular space, a process morphologically characterized by fusion of secretory vesicles with the cell membrane. Therefore, a parallel release of small and large secretory products (catecholamines as well as dopamine-*B*-hydroxylase and chromogranins) but not of cytoplasmic lactate dehydrogenase was used as a biochemical parameter for secretion by exocytosis from chromaffin cells (cf. Refs. 24-26). Cell preparations permeabilized by high-voltage discharges or by α-toxin or streptolysin O meet these criteria, suggesting that release occurs by exocytosis (1-3,9,15,16). Proof for exocytotic release from permeabilized catecholamine-secreting cells was also obtained in a study of the catecholamine metabolism in these cells (16c). It was shown that the enzymes oxidizing catecholamines, which are present in the cytoplasm, cannot come into contact with the membrane-bound catecholamines indicating that the cytoplasm is avoided during the release of catecholamines in α-toxin permeabilized cells (16c). As an example, the Ca^{2+}-dependent release of the soluble contents of the storage vesicles, but not of the cytosolic marker enzyme, from α-toxin permeabilized adrenal medullary chromaffin cells in tissue culture is shown in Figure 3.

The range of Ca^{2+} concentrations required for exocytosis in permeabilized cells certainly is in accordance with the measurements of Ca^{2+} within intact cells. Adrenal medullary chromaffin cells or pheochromocytoma cells (PC 12) contain about 0.1 μM free Ca^{2+} within the cytoplasm (27,28). Stimulation increases this value by a factor of roughly 3 or more. The measurement of free Ca^{2+} concentrations within cells certainly is not an easy procedure. One can assume that the true Ca^{2+} concentration in these cells is slightly higher than estimated for the following two reasons. First, Ca^{2+} determination using indicator substances only gives the average concentration within a given cell and not the concentration close to the plasma membrane where, owing to its influx from outside, the largest increase could be envisaged. Second, these substances, in order to provide a good signal, must be present within the secretory cells in fairly high amounts, and the indicator, being a Ca^{2+} chelator itself, would thus report a smaller Ca^{2+} concentration than actually exists. In addition to the difficulty of adjusting free Ca^{2+} concentrations to the low values used in studies with permeabilized cells, the possible influence on subcellular Ca^{2+} pools by the permeabilization procedure or by the media used may also have con-

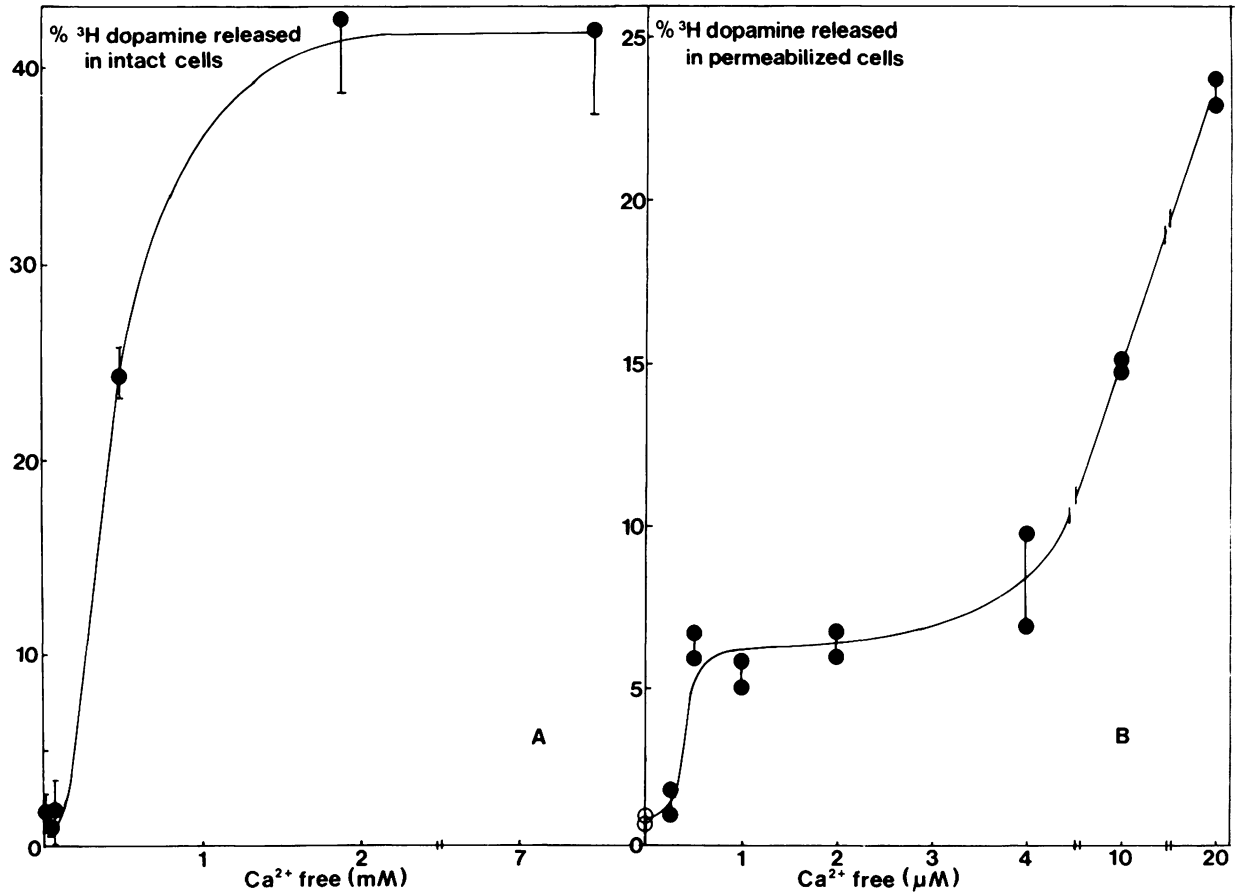

FIGURE 2 Ca^{2+} requirement of dopamine release by intact (A) and by α-toxin permeabilized PC12 cells. (From Ref. 15, by permission).

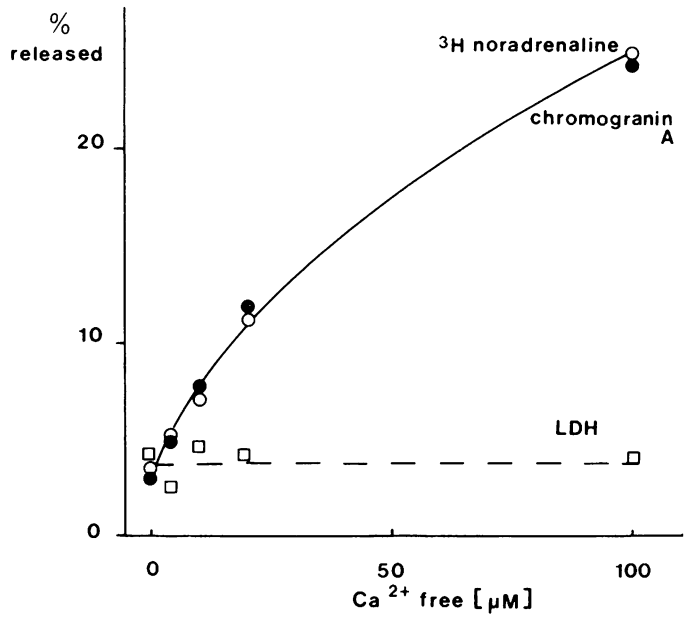

FIGURE 3 Ca^{2+} dependence of noradrenalin and chromogranin release from α-toxin-permeabilized chromaffin cells. Ca^{2+} triggers parallel secretion of noradrenalin and chromogranin A but has no effect on lactate dehydrogenase from the cells. (From Ref. 9, by permission.)

tributed to the reported differences in the Ca^{2+} sensitivity of catecholamine release by permeabilized chromaffin cells. Nonetheless, there is no doubt that about 1000 times less Ca^{2+} needs to be added to elicit hormone release from permeabilized chromaffin cells than from intact cells.

B. Role of ATP

Freshly isolated adrenal medullary cells permeabilized electrically require ATP to be stimulated successfully with μM concentrations of Ca^{2+} (1-3). If the same cells are kept in tissue culture and permeabilized with detergents, the effect of ATP is inconsistent (4-6). PC 12 cells permeabilized with α-toxin do not require ATP at all (16). These differences in ATP requirement may reflect cell-type-specific properties of the release process or the different permeabilization procedures used.

It is likely that the lack of ATP sensitivity of permeabilized PC 12 cells is inherent to this pheochromocytoma cell line. While chromaffin cells in primary culture exhibit decreased secretion when ATP production by glycolysis and oxidative phosphorylation is blocked (29,30), PC 12 cells do not respond to such a treatment (31). Thus, the properties of secretion by intact cells are in accordance with findings obtained with permeabilized cells. The reason for the different behavior of PC 12 and chromaffin cells is not clear. However, a fact worth considering is that, in PC 12 cells, the preferential arrangement of secretory vesicles is near the cell membrane (32), whereas in chromaffin cells the secretory vesicles are distributed throughout the cytoplasm (cf. Ref. 33). In other words, movement of

secretory vesicles toward the cell membrane seems to be essential in chromaffin cells but not in PC 12 cells. In conjunction with this, the sole requirement of organelle movement for ATP is of considerable interest (34,35). Another possibility could be that a phosphorylation step is essential to prime for exocytosis. Along this line, studies have been carried out using intact chromaffin cells, permeabilized cells, or subcellular fractions (36-39). Finally, an effect of ATP-dependent proton translocation across the secretory vesicle membrane has been considered (40).

Despite the efforts carried out, the mode of ATP action in exocytosis is still unknown. It is noteworthy that chromaffin cells kept in tissue culture, similar to PC 12 cells after permeabilization with α-toxin, were not dependent on ATP. However, after a washout period the ATP requirement of catecholamine release by the chromaffin cells could be clearly demonstrated (Fig. 4). Under these precisely controlled conditions ATP could not be substituted by any of the nucleotides tested (9). A parallel finding has been reported with freshly isolated chromaffin cells (3) This clear-cut difference in the ATP requirement between chromaffin cells and PC 12 cells may help to find the ATP-dependent step in exocytotic secretion.

C. Effect of Neurotoxins

The clostidial neurotoxins tetanus toxin and botulinum A toxin belong to the most poisonous substances known (20a). By means of the large pores generated by SLO, neurotoxins can be introduced into chromaffin cells (16d-f). It has been observed that tetanus toxin and botulinum A toxin following separation of their disulfide linked-chains are able to block Ca^{2+}-induced catecholamine release. The heavy chain of the toxins has no effect, but the light chains block exocytosis (16d-f). Thus, a new tool, the light chains of the clostridial neurotoxins for the analysis of the components involved in exocytosis, has been detected (16d-f). Furthermore, appropriate permeabilized cells should permit identification of the target of tetanus toxin within the cells, as well as detection of its reactive domain within the light chain, and consequently provide an approach to elucidate the mechanism of the action of these neurotoxins at the molecular level.

D. Modulation of Catecholamine Release

All observations reported agree that the amount or nature of monovalent cations does not modify the characteristics of secretion by permeabilized cells. From the divalent cations tested Mg^{2+} could not be substituted for Ca^{2+} and high concentrations (>mM) and Mg^{2+} inhibit the release elicited by μM Ca^{2+} (1-3,6,16). The reported ability of Sr^{2+}, Cd^{2+}, Mg^{2+}, and Ba^{2+} to replace or block the effect of Ca^{2+} (6) must be interpreted with caution because the experiments were carried out with very high Ca^{2+} concentrations (mM). Moreover, no chelators were present during these experiments, i.e., it is not known how much Ca^{2+} was still present or whether a redistribution of ions or an actual influence on the Ca^{2+}-sensitive target was the cause. Thus, further experiments are required to characterize the effect of divalent cations in order to obtain a solid basis for the comparison of secretion by intact secretory cells with such permeabilized cell preparations.

Freshly isolated chromaffin cells permeabilized electrically demonstrate a large inhibition of Ca^{2+}-induced catecholamine release by chloride and

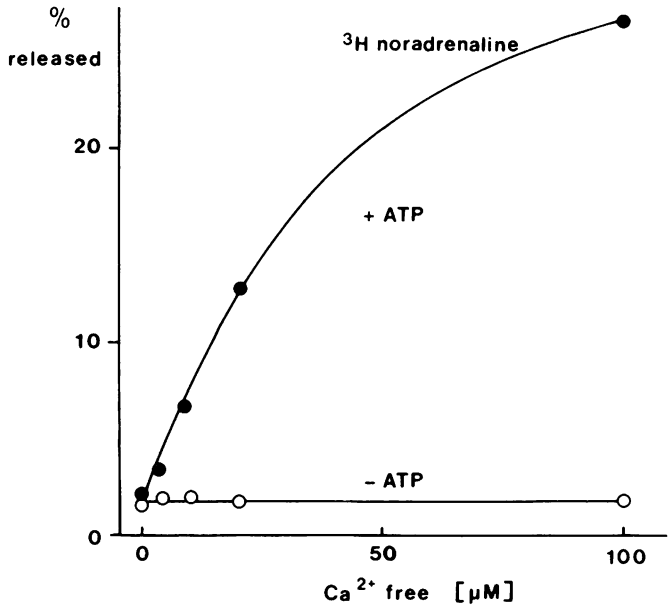

FIGURE 4 Effect of ATP on Ca^{2+}-evoked catecholamine release from α-toxin-permeabilized chromaffin cells. (From Ref. 9, by permission.)

other anions (3), but chromaffin cells kept in primary cultures and permeabilized with digitonin or saponin (4,6) are not affected by the nature of the anions present. This difference is not yet understood. Recently, it has been observed that changes in the medium composition with respect to anions likewise do not modify dopamine release from α-toxin-permeabilized PC 12 cells in culture (16).

Permeabilized cells appear to be an almost ideal tool to elucidate the role of intracellular messengers. That is why the participation of the protein kinase C system in secretion by exocytosis also has been analyzed by using such preparations. Phorbolesters, which can substitute the natural diacylglycerol as activator of protein kinase C, apparently decrease the requirement for Ca^{2+} of catecholamine release in freshly isolated, electrically permeabilized cells (41), as well as in digitonin-permeabilized chromaffin cells in primary culture (42). The latter, however, is less sensitive to this drug. Surprisingly 1-oleyl-2-acetyl-glycerol (OAG), an activator of purified kinase C, does not show a detectable effect in electrically permeabilized cells (43). In permeabilized rat pheochromocytoma cells (PC 12) activation of protein kinase C by the diacylglycerol analog OAG or the phorbolester TPA ameliorates Ca^{2+} induced exocytosis (16c,43a). Using α-toxin-permeabilized PC 12 cells, it could also be shown that the activation by TPA is dependent on the presence of Mg^{2+}/ATP (16c), which clearly indicates the involvement of an active protein kinase C system. Exposure of these cells to GTP-γ-S inhibits Ca^{2+}-dependent release in bovine cells but stimulates it in chicken cells. As the inhibitory action on bovine cells persisted in the presence of phorbolester, it was concluded that a GTP binding protein (G protein) may inhibit the action of protein kinase C (43). A Ca^{2+}-independent stimulatory effect of GTP analogs was reported for bovine chromaffin cells in primary culture (43b), whereas

Ca^{2+}-triggered exocytosis in α-toxin-permeabilized pheochromocytoma cells (PC 12) was inhibited by $GTP_{\gamma}S$ (16c). This effect could be abolished by pertussis toxin but not by cholera toxin, indicating that exocytosis by PC 12 cells can be modulated by a pertussis toxin-sensitive G protein (16c). Further analysis of the G proteins present in chromaffin cells seems to be necessary to fully understand the role of these proteins in intracellular signal transduction. In this context it is noteworthy that low- and high-molecular-mass GTP binding proteins have been detected at a strategic location, namely the secretory vesicle membrane in chromaffin cells (43c,d).

Application of drugs interacting with calmodulin in intact chromaffin cells (44,45) and injection of anticalmodulin antibodies into chromaffin cells using erythrocytes as a vehicle (46) suggested the possibility of calmodulin controlling the Ca^{2+} sensitivity of the release process. The reported data using permeabilized cells do not support this view, at least as far as trifluoperazine is concerned. Neither electrically nor detergent-permeabilized cells responded to low concentrations of this drug; 10 μM trifluoperazine caused the Ca^{2+}-independent catecholamine release to increase, suggesting a damage of secretory vesicles (3,16d,47). Since introduction of an antibody against calmodulin into SLO-permeabilized PC 12 cells did not affect the Ca^{2+}-dependent secretory response, an involvement of calmodulin in the final steps of exocytosis is less likely (16d).

The different techniques to permeabilize the cell membrane of chromaffin cells allow for introducing small and large molecular substances into the intracellular environment which would not penetrate otherwise. The examples given above demonstrate the usefulness of permeabilized cell preparations to determine the molecular requirements for exocytosis.

IV. COMPARISON WITH OTHER PERMEABILIZED SECRETORY CELLS

A. Endocrine and Exocrine Pancreas

Much of our knowledge on the process of secretion by exocytosis stems from numerous investigations of the chromaffin cell. It was therefore feasible to use mainly chromaffin cells as an object for permeabilization studies.

However, such studies have also been conducted on other endocrine cells: The pancreatic islet cells produce several key hormones in carbohydrate metabolism, including insulin, glucagon, and somatostatin. Knowledge of the release process of these hormones is of particular importance in understanding diabetes and other endocrine disorders. The high-voltage technique has been used to determine the molecular requirements for exocytosis in islet cells (48-51).

It is not surprising that also in these cells μM Ca^{2+} was sufficient to release insulin after permeabilization. The ATP requirement has been studied in more detail (48). Cells that had received an ATP washout treatment were found to be dependent on this nucleotide. On the other hand, cells that were not washed out did not require the addition of ATP. This is similar to the situation seen with α-toxin-permeabilized chromaffin cells in primary culture, which also exhibit ATP dependence only after a washout period (9).

Elevation of the glucose level in the extracellular fluid in the presence of Ca^{2+} is the natural stimulus for insulin secretion from intact pancreatic

B cells. Neither glucose nor glucose-6-phosphate modified insulin release from permeabilized cells, but phosphoenolpyruvate stimulated the release. This suggests that the latter substance may act as an intracellular modulator of insulin release. Further experiments along this line may be helpful to settle a long-lasting controversy on whether glucose itself or some of its metabolites may act as the principal stimulus of insulin release (cf. Ref. 52).

Inhibition of insulin release from pancreatic islets has been reported for phalloidin (50), which stabilizes F-actin, cytochalasin B (49), which alters microfilament function, and vinblastin (49), which impairs microtubule function. By contrast, none of these substances affected catecholamine release from electrically permeabilized chromaffin cells (3).

Insulin secretion has also been investigated using digitonin-permeabilized islets of Langerhans (53,54). Both forskolin and phorbolesters, in addition to Ca^{2+}, have been observed to stimulate insulin release in this preparation (53). Concerning phorbolesters, a parallel finding was recently noted using electrically permeabilized islets; thus a physiological role for protein kinase C cascade in insulin secretion can be envisioned (51).

Also, exocrine pancreatic acinar cells which produce digestive enzymes were rendered permeable by intense electric fields (55). Without Ca^{2+} present, less than 0.5% of the cellular content of amylase was released but about 4% was released upon addition of 10 μM free Ca^{2+}. Cyclic nucleotides did not affect the exocytotic machinery within these cells. However, similar to the behavior of pancreatic B cells and chromaffin cells, amylase release was increased by phorbolesters.

B. Platelets and Mast Cells

Secretion from and aggregation of platelets are important processes cooperating in hemostasis. Both can be elicited by thrombin, a protein with a central role in the regulation of blood clotting. This protein induces serotonin release from intact platelets even in the absence of extracellular Ca^{2+}. It also causes phosphatidyl inositol breakdown leading to diacylglycerol formation, activation of protein kinase C, and phosphorylation of proteins with molecular masses of 40 kD and 20 kD, the latter being light-chain platelet myosin (cf. Ref. 56). When permeabilized by high-voltage discharges, platelets in the presence of ATP and μM concentrations of Ca^{2+} release serotonin from the storage vesicles and acid hydrolases from the lysosomes. This release is increased by further addition of thrombin (57-61). In permeabilized platelets with low (0.1 μM) concentration of Ca^{2+}, thrombin also increases diacylglycerol formation, presumably by hydrolysis of phosphatidylinositols (61) and phosphorylation of proteins with a molecular weight of about 40 kD and 20 kD. Diacylglycerol itself causes enhanced phosphorylation of these two proteins (59). The results obtained so far are consistent with the view that secretion from platelets as studied with permeabilized cells is dependent on μM Ca^{2+} and members of the phosphatidylinositol/protein kinase C cascade are probably potent modulators. The relation of protein phosphorylation to the secretory response is not yet known (cf. Chapter 25). However, further studies may help to provide some insight into the nature of mechanisms involved in this ATP-dependent step of exocytosis.

Mast cells permeabilized with Sendai virus release histamin upon addition of μM Ca^{2+}. This requires no ATP and a nonhydrolyzable ATP derivative had no effect (17). However, when permeabilized with streptolysin O, rat mast cells that have been pretreated with metabolic inhibitors secrete histamine provided that Ca^{2+} and a nucleoside triphosphate are present (59a).

C. Sea Urchin Eggs

Within seconds after fusion of sperm and egg, the membranes of cortical vesicles fuse with the cell membrane. This example of massive exocytosis has been studied with intact eggs, with a preparation containing cortical vesicles plus cell membrane ("isolated cortices"), as well as with permeabilized eggs, with the aim of obtaining information on the mechanism of exocytosis in these cells.

The properties of the co-called cortical reaction in intact eggs are very similar to the secretion by exocytosis observed in other cells. It is associated with a rise in intracellular free Ca^{2+} (62,63), can also be activated by the ionophor A23187 (64), as well as by injection of Ca^{2+} (65). As a measure of secretion, enzymes present in the cortical vesicles are determined (66,67).

A preparation of cortical vesicles attached to the inner surface of the plasma membrane (isolated cortices) can be obtained from sea urchin eggs adhering to a coated surface, after shearing away the upper part of the cell (68). Addition of Ca^{2+}, Ba^{2+}, and Sr^{2+}, but not of Mg^{2+}, elicits discharge. Treatment with drugs affecting microtubules or microfilaments, with cyclic nucleotides, or with ATP neither enhanced nor inhibited this process (69). Experiments carried out with isolated cortices as well as with eggs permeabilized by high-voltage discharges have shown that a supply of ATP is not necessary in these preparations (70). However, the sensitivity of exocytosis to Ca^{2+} is increased by ATP and the fraction of vesicles reacting falls in the absence of this nucleotide. Finally, in a reconstituted system, consisting of purified cortical vesicles and plasma membrane, it was shown that ATP was not necessary for exocytosis (71). In vitro exocytosis in isolated egg cortices is inhibited by elevated Mg^{2+} concentrations. This inhibition can be overcome by increasing the free Ca^{2+} concentration (72). Many other conceivable modulators of the Ca^{2+}-dependent cortical reaction did not show any effect. Trifluoperazine inhibited Ca^{2+}-dependent exocytosis, but because of the increased Ca^{2+}-independent release observed at slightly higher concentrations of this drug, the authors were not confident in attributing its inhibitory effects to an action on calmodulin rather than to its detergent-like activity (72). Another group using an antibody to calmodulin, which inhibits exocytosis in isolated cortices, concluded that calmodulin or other similar Ca^{2+}-binding modulatory proteins may be involved in exocytosis (73).

V. CONCLUSION

Investigation of exocytotic membrane fusion in permeabilized cell preparations has shown that there is an absolute requirement for Ca^{+} in the μM range. The target (receptor) of this cation, i.e., the protein and/or the lipid it interacts with, remains to be identified.

The specificity of Ca^{2+} binding to its receptor is to be compared with the ionic requirements of the secretory vesicle/plasma membrane interaction. While there is agreement on the fact that Mg^{2+} in high (mM) concentrations inhibits the effect elicited by μM Ca^{2+} and that Mg^{2+} cannot replace Ca^{2+}, it has not yet been completely worked out whether other divalent or trivalent cations can act like Ca^{2+} does.

Another unsolved problem is the question of exactly how ATP acts in exocytotic secretion. It must be borne in mind that some endocrine cells can do without ATP. Also, in the sea urchin egg there is no direct influence

of ATP. Furthermore, isolated secretory vesicle fusion requires no ATP added (74-76). Thus, it may be concluded that the exocytotic membrane fusion catalyzed by Ca^{2+} has to be differentiated from other ATP-requiring processes which may precede the fusion step. In particular, ATP may assure the movement of the secretory vesicles toward the cell membrane or the docking of the secretory vesicle to the cell membrane prior to membrane fusion.

Modulation of exocytosis via the protein kinase C system is certainly an ATP-dependent step. Within the intact cell this pathway is affected by receptor-coupled, GTP-binding proteins at the level of the plasma membrane. Modulation of exocytosis via intracellular GTP binding proteins is indicated by the effects of GTP analogs on Ca^{2+}-triggered exocytosis in permeabilized cells and first reports on the intracellular distribution of these proteins within secretory cells. However, the nature and the role of these proteins in exocytotic membrane fusion remain to be elucidated.

Recent reports on the inhibitory effect of the clostridial neurotoxins on exocytotic release of catecholamines from permeabilized chromaffin cells indicate that a novel tool has been detected to define intracellular molecules participating in exocytotic membrane fusion. Further work concerning the intracellular action of tetanus toxin and botulinum A toxin will certainly allow us to define the mechanism of action of these highly poisonous neurotoxins.

The processes of exocytotic membrane fusion in different secretory cells probably have the same principal basic mechanisms. Permeabilizing cell preparations appears to be an ideal method for following these mechanisms.

ACKNOWLEDGMENTS

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