Catecholamine Secretion from Digitonin-treated PC12 Cells

EFFECTS OF Ca\(^{2+}\), ATP, AND PROTEIN KINASE C ACTIVATORS*

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PC12 cells, a cloned rat pheochromocytoma cell line, were treated with digitonin to render the plasma membrane permeable to ions and proteins. At a cell density of 2-6 x 10\(^6\) cells/cm\(^2\), incubation with 7.5 \(\mu\)M digitonin permitted a Ca\(^{2+}\)-dependent release of 25-40\% of the catecholamine within 18 min in the presence of 10 \(\mu\)M Ca\(^{2+}\). Half-maximal secretion occurred at 0.5-1 \(\mu\)M Ca\(^{2+}\). PC12 cultures at lower cell densities were more sensitive to digitonin and gave more variable results. Secretion in the presence of digitonin and Ca\(^{2+}\) began after a 2-min lag and continued for up to 30 min. When cells were treated for 3 min in digitonin and then stimulated with Ca\(^{2+}\) in the absence of digitonin, secretion occurred in the same manner but without the initial lag. Optimal secretion from PC12 cells was also dependent upon the presence of Mg\(^{2+}\) and ATP. Permeabilized PC12 cells exhibited a slow time-dependent loss of secretory responsiveness which was correlated with the release of a cytosolic marker, lactate dehydrogenase (134 kDa). This suggests that digitonin permeabilization allows soluble constituents necessary for secretion to leave the cell in addition to allowing Ca\(^{2+}\) and ATP access into the cell interior. Ca\(^{2+}\)-dependent secretion was completely inhibited by exposure of digitonin-permeabilized cells to 100 \(\mu\)g/ml trypsin (27 kDa), whereas secretion was only slightly inhibited by trypsin exposure prior to digitonin treatment. Thus, an intracellular, trypsin-sensitive protein is probably involved in secretion. The data also indicate that the same population of digitonin-treated cells which responded to Ca\(^{2+}\) was permeable to a 27-kDa protein, 1,2-Dioctanoylgllycerol and phorbol esters which activate protein kinase C enhanced the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent secretion in digitonin-permeabilized PC12 cells. Thus, protein kinase C appears to be involved in the regulation of catecholamine secretion from permeabilized PC12 cells.

Secretion of prepackaged neurotransmitters and hormones occurs by exocytosis and is usually triggered by an increase of cytosolic Ca\(^{2+}\). Although the process of exocytosis has been well-described in a number of systems, its underlying molecular mechanisms are not understood.

A cloned rat pheochromocytoma cell line, PC12, established by Greene and Tishler (1) expresses many of the characteristics of adrenal chromaffin cells, including catecholamine synthetic enzymes, dense-core granules, nicotinic cholinergic receptors, and a high affinity, saturable norepinephrine uptake mechanism. Exposure to nerve growth factor (NGF) causes differentiation into cells similar to sympathetic neurons with extensive neurite development, loss of cell replication, enhanced responsiveness to acetylcholine, and induction of several adrenergic and cholinergic synthetic enzymes (for review, see Ref. 2). PC12 cells release catecholamine (3) and acetylcholine (4) in a Ca\(^{2+}\)-dependent manner in response to K\(^+-\)induced depolarization. The ability to have homogeneous and reproducible cultures of neurons makes PC12 cells an attractive system for the study of the biochemical mechanisms underlying neurotransmitter release.

Protein kinase C is Ca\(^{2+}\)-and phospholipid-dependent (5, 6). Diacylglycerol in the presence of phosphatidylycerine increases maximal activity and increases the Ca\(^{2+}\) sensitivity of the enzyme to micromolar or perhaps submicromolar concentrations (7). TPA can substitute for diacylglycerol in vitro (8) and similarly increases the Ca\(^{2+}\) sensitivity of the enzyme. Most important, TPA activates protein kinase C and protein phosphorylation in intact cells and enhances secretion (8, 9). Thus, protein kinase C may play an important role in exocytosis. Indeed, the phorbol ester TPA and a relatively water-soluble diacylglycerol, 1-oleoyl-2-acetylglycerol, which are activators of protein kinase C (10), induce a slow and sustained catecholamine release from PC12 cells and potentiate the response to ionomycin, a Ca\(^{2+}\) ionophore (11).

One of the problems in studying the molecular mechanisms underlying exocytosis has been the inability to manipulate the internal milieu of the cell. To overcome this, a number of techniques have recently been developed. Baker and Knight (12), by exposing suspended adrenal medulla chromaffin cells to high voltage discharge, rendered the plasma membrane permeable to ions and small molecules. Secretion of catecholamine was stimulated in the absence of secretagogue by micromolar concentrations of Ca\(^{2+}\) and required the presence of Mg\(^{2+}\) and ATP. More recently, Dunn and Holz (13) and Wilson and Kirshner (14), using digitonin, and Brooks and Treml (15), using saponin, increased the plasma membrane permeability of monolayer cultures of chromaffin cells. Micromolar Ca\(^{2+}\) caused an ATP-dependent release of catecholamine from digitonin-treated chromaffin cells which occurred by exocytosis.

In the present study, we have adapted the digitonin permeabilization technique for application with nondifferentiated and NGF-treated PC12 cells. We demonstrate that cells permeabilized by digitonin release catecholamine into the medium in the absence of secretagogue in a Ca\(^{2+}\)- and ATP-dependent manner. This suggests that digitonin permeabilization allows soluble constituents necessary for secretion to leave the cell in addition to allowing Ca\(^{2+}\) and ATP access into the cell interior. Ca\(^{2+}\)-dependent secretion was completely inhibited by exposure of digitonin-permeabilized cells to 100 \(\mu\)g/ml trypsin (27 kDa), whereas secretion was only slightly inhibited by trypsin exposure prior to digitonin treatment. Thus, an intracellular, trypsin-sensitive protein is probably involved in secretion. The data also indicate that the same population of digitonin-treated cells which responded to Ca\(^{2+}\) was permeable to a 27-kDa protein, 1,2-Dioctanoylgllycerol and phorbol esters which activate protein kinase C enhanced the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent secretion in digitonin-permeabilized PC12 cells. Thus, protein kinase C appears to be involved in the regulation of catecholamine secretion from permeabilized PC12 cells.

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1 The abbreviations used are: NGF, nerve growth factor; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KGEF, potassium/glutamate/EGTA/PIPES solution; TPA, 12-O-tetradecanoylphorbol-13-acetate.
MgATP-dependent manner consistent with exocytosis. In addition, we show that exogenous diacylglycerol and phorbol esters that activate protein kinase C enhance secretion from permeabilized PC12 cells.

MATERIALS AND METHODS AND RESULTS

DISCUSSION

Optimal Conditions for Ca\(^{2+}\)- and MgATP-dependent Secretion from Digitonin-permeabilized PC12 Cells—Digitonin treatment rendered the plasma membrane of PC12 cells permeable to ions and to molecules as large as lactate dehydrogenase (134 kDa). Submicromolar and micromolar concentrations of Ca\(^{2+}\) within the reported cytosolic range for PC12 cells (11, 20) elicited Mg\(^{2+}\)- and ATP-dependent catecholamine secretion from the permeabilized cells (Figs. 3 and 4). Cell density and digitonin concentration were critical factors in obtaining optimal permeabilization and secretion. In PC12 cultures untreated with NGF at low cell density (less than 2 \(\times 10^5\) cells/cm\(^2\)), it was difficult to define a digitonin concentration that allowed Ca\(^{2+}\)-dependent release of catecholamine and did not cause significant release of catecholamine in the absence of Ca\(^{2+}\) (Fig. 1). Excellent Ca\(^{2+}\)-dependent secretion with minimal catecholamine release in the absence of Ca\(^{2+}\) was routinely obtained when cells were subcultured at 2 \(\times 10^5\) cells/cm\(^2\) and permeabilized with 7.5 \(\mu\)M digitonin 4–6 days later (when densities were 4–6 \(\times 10^5\) cells/cm\(^2\)). Cell density has also been shown to greatly affect the specific activities of choline acetyltransferase and tyrosine hydroxylase in PC12 cultures (4, 21). Therefore, changes associated with cell density may alter the membrane composition (e.g. cholesterol content) and influence the sensitivity of the plasma membrane and secretory granule membranes to digitonin.

Permeability Characteristics of Digitonin-treated Cells—The plasma membranes of PC12 cells permeabilized by digitonin did not readily reseal after removal of digitonin, but remained permeable to Ca\(^{2+}\) for a prolonged time (Figs. 2 and 5) and continued to release lactate dehydrogenase (Figs. 6 and 7). The ability of the cells to respond to Ca\(^{2+}\) in the medium slowly declined with time after permeabilization. The greater decline in Ca\(^{2+}\) responsiveness which occurred with longer exposure to digitonin was associated with a greater release of lactate dehydrogenase. The decline in Ca\(^{2+}\)-dependent secretion after permeabilization may have resulted, therefore, from the escape of cytosolic factors required for exocytosis.

Ca\(^{2+}\)-dependent secretion from permeabilized cells was completely inhibited when a low concentration of trypsin was applied to cells after, but not before, permeabilization (Fig. 8). Thus, the same population of cells which underwent Ca\(^{2+}\)-dependent secretion after digitonin treatment was permeable to trypsin, a 27-kDa protein. In addition, the data indicate that an intracellular, trypsin-sensitive protein is involved in exocytosis. Trypsin also inhibits Ca\(^{2+}\)-dependent secretion from digitonin-treated bovine chromaffin cells.\(^5\)

Effects of Diacylglycerol and Phorbol Esters on Secretion from Permeabilized Cells—Ca\(^{2+}\)-dependent secretion from

\(^5\) Portions of this paper (including "Materials and Methods," "Results," Figs. 1, 2, and 5–9, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-519, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

\(^6\) R. W. Holz and R. A. Senter, unpublished results.
PC12 cells was enhanced by exogenously added diacylglycerol and phorbol esters that activate protein kinase C, but not by phorbol esters that do not activate protein kinase C (Table I). Therefore, protein kinase C activation by diacylglycerol, an endogenous product of receptor-mediated phospholipase C activity (22), could regulate Ca**+-dependent secretion. The ability of diacylglycerol and phorbol esters to increase secretion from permeabilized PC12 cells is consistent with many investigations suggesting a role for protein kinase C in exocytosis (23).

The observation that phorbol esters, and to a lesser extent diacylglycerol, stimulated Ca**+-independent secretion from permeabilized PC12 cells was unexpected since it had not been observed in intact or permeabilized bovine adrenal chromaffin cells (18, 19). However, it is consistent with the small degree of secretion induced by TPA in intact PC12 cells which had been depleted of intracellular Ca**+ by preincubation with EGTA and ionomycin (11). Ca**+-independent stimulation of secretion by TPA has also been observed in intact neutrophils (24). Perhaps, in PC12 cells, protein kinase C in the absence of Ca**+ stimulates secretion via the constitutive pathway present in the cells (25). In bovine adrenal chromaffin cells, the constitutive pathway may be less apparent.

Comparison to Previous Work—It has been suggested that secretion from intact PC12 cells is ATP-independent because metabolic inhibitors which depleted total cellular ATP by 90% had little effect on Ca**+-dependent secretion (26). The present study, however, indicates that ATP and Mg**+ are required for secretion (Fig. 4), as has been previously shown for bovine adrenal chromaffin cells (12-14) and platelets (27). A period of time (approximately 6 min) was required after permeabilization of PC12 cells prior to stimulation with Ca**+ in order to demonstrate Mg**+ and ATP dependence. This interval was probably necessary to deplete cytosol of ATP and Mg**+.

In a recent study in which PC12 cells were permeabilized by staphylococcal α-toxin (28), a requirement of Ca**+-dependent secretion for MgATP was not observed. The conditions of the experiment were not specified, and it was not determined whether Mg**+ and ATP were depleted from the cells prior to the introduction of Ca**+. In the same study, Ca**+-dependent secretion of catecholamine could not be demonstrated in cells permeabilized with digitonin. Instead, catecholamine release was entirely Ca**+-independent, consistent with disruption by digitonin of the intracellular storage granules. Cell density, digitonin concentration, and Ca**+ concentrations used in the investigation of Ca**+-dependence were not specified. As discussed above, we obtained similar results with low density cultures and with high digitonin concentrations. A proper choice of cell density and digitonin concentration is critical for obtaining optimal Ca**+-dependent secretion without disruption of the secretory vesicles in the absence of Ca**+.

In summary, these experiments with digitonin demonstrate the feasibility of increasing plasma membrane permeability of PC12 cells to proteins as large as 134 kDa while retaining the Ca**+-responsive reactions of exocytosis. The introduction of trypsin into the permeabilized cells and experiments with diacylglycerol and phorbol esters demonstrate the potential value of this system to study the biochemical mechanisms of secretion. The ability to study Ca**+-dependent secretion from NGF-differentiated PC12 cells with long neurites (Fig. 9) will allow the direct study of the intracellular mechanisms responsible for exocytosis in neuron-like cells.

Acknowledgment—We are grateful to Dr. Lloyd A. Greene for giving us the PC12 cells and his advice in culturing them.

REFERENCES
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Figure 1. Effects of diacerein concentration and r-mglutamyl as gliadin on the concentration of diacerein.

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Secretion from Digitonin-treated PC12 Cells

The effects of various concentrations of Ca²⁺ on secretion induced by PC12 were further investigated. Briefly, treatment with 50 μM digitonin for 5 minutes caused a significant increase in Ca²⁺ influx in the absence of extracellular Ca²⁺ (Fig. 1). Inhibition of Ca²⁺ influx by EGTA (1 mM) significantly reduced the secretory response. These results suggest that Ca²⁺ influx is a crucial factor in the mechanism of secretion in PC12 cells.

Figure 1. Effect of Ca²⁺ on secretion. Cells were treated with 50 μM digitonin in the presence or absence of extracellular Ca²⁺. A: The percentage of secretory response to Ca²⁺ was determined by measuring the release of total Ca²⁺ from the cells. B: The time course of total Ca²⁺ release from the cells following digitonin treatment. The Ca²⁺ influx was measured using a radioactive dye, and the release was quantified by monitoring the fluorescence of the dye.

Table 1. Effects of various compounds on secretion from Digitonin-treated PC12 Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect on Secretion</th>
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<tr>
<td>10 μM nigericin</td>
<td>Enhanced secretion</td>
</tr>
<tr>
<td>10 μM FCCP</td>
<td>Enhanced secretion</td>
</tr>
<tr>
<td>10 μM ruthenium tartrate</td>
<td>Enhanced secretion</td>
</tr>
<tr>
<td>10 μM amiloride</td>
<td>Enhanced secretion</td>
</tr>
<tr>
<td>10 μM apamin</td>
<td>Enhanced secretion</td>
</tr>
<tr>
<td>10 μM hexamethonium</td>
<td>Enhanced secretion</td>
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Cells treated with nigericin were assayed for Ca²⁺ influx by measuring the fluorescence of a dye, and the results were compared with those obtained from control cells. The results showed that nigericin enhanced Ca²⁺ influx, which may be responsible for the enhanced secretion observed. Similar results were obtained with FCCP, ruthenium tartrate, apamin, and hexamethonium, which are known to enhance Ca²⁺ influx.