

Control of Exocytosis from Adrenal Chromaffin Cells

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SUMMARY

1. Calcium-dependent exocytosis of catecholamines from intact and digitonin-permeabilized bovine adrenal chromaffin cells was investigated.

2. $^{45}\text{Ca}^{2+}$ uptake and secretion induced by nicotinic stimulation or depolarization in intact cells were closely correlated. The results provide strong support for Ca^{2+} entry being the trigger for exocytosis.

3. Experiments in which the H^+ electrochemical gradient across the intracellular secretory granule (chromaffin granule) membrane was altered indicated that the gradient does not play an important role in exocytosis.

4. Ca^{2+} entry into the cells is associated with activation of phospholipase C and a rapid translocation of protein kinase C to membranes.

5. The plasma membrane of chromaffin cells was rendered permeable to Ca^{2+} , ATP, and proteins by the detergent digitonin without disruption of the intracellular secretory granules. In this system in which the intracellular milieu can be controlled, micromolar Ca^{2+} directly stimulated catecholamine secretion.

6. Treatment of the cells with phorbol esters and diglyceride, which activate protein kinase C, enhanced phosphorylation and subsequent Ca^{2+} -dependent secretion in digitonin-treated cells.

7. Phorbol ester-induced secretion could be specifically inhibited by trypsin. The experiments indicate that protein kinase C modulates but is not necessary for Ca^{2+} -dependent secretion.

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INTRODUCTION

Secretion of prepackaged hormones and neurotransmitters generally occurs by exocytosis. Although exocytosis has been extensively studied, little is known about the physiological and biochemical mechanisms underlying it. Studies on the perfused adrenal medulla and adrenal medullary slices helped define the process and demonstrated that in this tissue Ca^{2+} influx initiates secretion (Douglas, 1975; Viveros, 1975). Acetylcholine released from cholinergic nerves interacts with receptors on chromaffin cells to cause Ca^{2+} entry. In the bovine adrenal medulla nicotinic but not muscarinic receptor activation is responsible for these events. Although studies with the perfused gland and adrenal medullary slices led to a greatly increased understanding of secretion and served as a model for studies in other secretory systems, they were often limited in time resolution and in the ability to investigate biochemical events associated with secretion. These limitations have been overcome by the development of techniques to dissociate large numbers of bovine adrenal chromaffin cells and to study secretion either from suspended cells or from monolayer cultures. The following is a brief description of some of the studies we have undertaken in monolayer cultures and with suspended chromaffin cells to elucidate further those factors which control and underlie exocytosis.

METHODS

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures or as suspended cells as previously described (Holz *et al.*, 1982, 1983). Experiments with intact cells were generally performed at 25°C in a physiological salt solution (PSS) containing 142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl_2 , 0.5 mM MgCl_2 , 15 mM Hepes (pH 7.4), 5.6 mM glucose, and 0.5 mM ascorbate. Catecholamine secretion and $^{45}\text{Ca}^{2+}$ uptake were measured as previously described (Holz *et al.*, 1982). Experiments with digitonin-treated cells were usually performed in a solution containing 145 mM potassium glutamate, 5 mM, various concentrations of calcium, 1–5 mM MgATP, and 20 mM Pipes (pH 6.60) (Dunn and Holz, 1983). The free Ca^{2+} concentration was calculated according to Portzehl *et al.* (1964). Catecholamine secretion was measured by determining the fractional release from cells of endogenous catecholamine or of [^3H]norepinephrine in cells which had been preincubated with [^3H]norepinephrine to label intracellular catecholamine stores.

RESULTS AND DISCUSSION

Relationship Between $^{45}\text{Ca}^{2+}$ Uptake and Catecholamine Secretion. Douglas and co-workers demonstrated that extracellular Ca^{2+} is required for secretion from perfused adrenal medulla and that $^{45}\text{Ca}^{2+}$ uptake into the gland was associated with secretion (Douglas, 1975). In monolayer cultures the relationship between calcium uptake and secretion could be determined more precisely

(Kilpartick *et al.*, 1982; Holz *et al.*, 1982). The rate of catecholamine secretion induced by the mixed nicotinic–muscarinic agonist carbachol was maximal during the initial 2 min and slowed thereafter (Fig. 1). Significant carbachol-stimulated Ca^{2+} uptake could be detected at 15 sec before the occurrence of significant catecholamine secretion. The rate of carbachol-induced Ca^{2+} uptake was maximal between 0 and 1 min and significantly slowed after 5 min. Both catecholamine secretion and $^{45}\text{Ca}^{2+}$ uptake were stimulated by selective nicotinic agonists (nicotine, 1,1-dimethyl-4-phenylpiperazinium) and by mixed nicotinic–muscarinic agonists (acetylcholine and carbachol) but not by selective muscarinic agonists (muscarine, methacholine) (Holz *et al.*, 1982). Similarly, nicotinic but not muscarinic antagonists blocked carbachol-induced secretion and $^{45}\text{Ca}^{2+}$ uptake (Holz *et al.*, 1982). Thus, nicotinic receptor activation is responsible for both increased calcium permeability and increased catecholamine secretion.

Secretion and calcium uptake were also stimulated by depolarization induced by elevated K^+ . Calcium probably entered the cell through voltage-sensitive calcium channels. Again, there was a close temporal relationship between calcium uptake and catecholamine secretion.

The studies strongly support the conclusion from the now-classical work of Douglas and co-workers (Douglas, 1975) that Ca^{2+} entry initiates and maintains secretion from adrenal medullary cells.

Absence of a Role in Exocytosis for the H^+ Electrochemical Gradient Across the Secretory Granule Membrane. A common feature of secretory granules is their low internal pH and a membrane-bound electrogenic H^+ translocating ATPase (Njus *et al.* 1986). The most extensively studied secretory granule, the chromaffin granule from adrenal chromaffin cells, has a pH of approximately 5.6 *in vitro* and an electrogenic H^+ translocating ATPase that generates a H^+ electrochemical gradient across the granule membrane of over 120 mV (Johnson and Scarpa, 1976; Casey *et al.*, 1977; Holz, 1978; Njus *et al.*, 1986). Catecholamine uptake into chromaffin granules is coupled to the H^+ electrochemical gradient. The common occurrence of H^+ electrochemical gradients across membranes of a wide variety of secretory granules raised the possibility that the H^+ electrochemical potential of granules may, in addition, be important for exocytosis. However, studies using a variety of different manipulations indicated that the H^+ electrochemical gradient across the granule membrane is probably not involved in exocytosis (Holz *et al.*, 1983). An important aspect of these studies was the use of techniques to assess the effects of various manipulations on the H^+ electrochemical gradient of chromaffin granules within cells. Weak bases such as methylamine (Johnson and Scarpa, 1979) or ammonium or the ionophore nigericin (Johnson and Scarpa, 1976), which exchanges protons and alkali metal ions across membranes, partially collapsed the pH gradient across chromaffin granule membranes *in vitro*. We determined, using ^{31}P nmr of ATP within intracellular chromaffin granules and radiometric techniques based upon the uptake of radioactive methylamine, that 20–30 mM methylamine or ammonium or 1 μM nigericin shifted the pH within intracellular chromaffin granules from 5.3 to 6.3 (Holz *et al.*, 1983). Under these conditions catecholamine secretion was not inhibited.

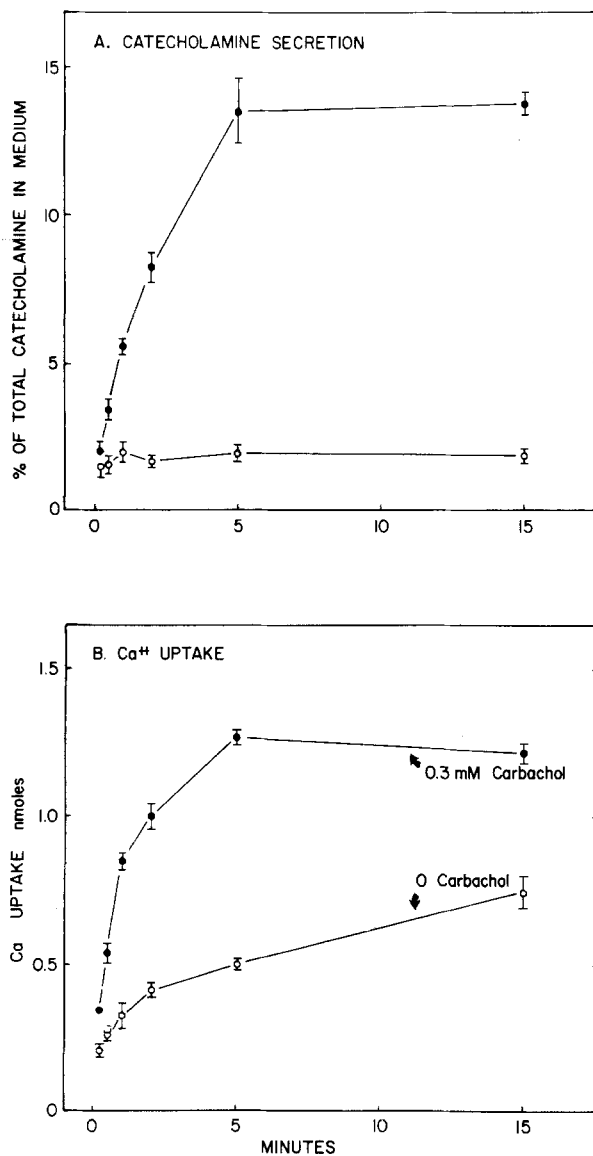


Fig. 1. Time course of carbachol-induced catecholamine secretion (A) and Ca^{2+} uptake (B). Cells were incubated in a solution containing $^{45}\text{Ca}^{2+}$ in the presence (●) or absence (○) of 0.3 mM carbachol. The solution was aspirated at various times and saved for measurement of catecholamine release from the cells. There were four wells per group. (From Holz *et al.*, 1982.)

Another approach involved the use of dicyclohexylcarbodiimide (DCCD), an irreversible inhibitor of H^+ translocating ATPases. Under conditions in which the H^+ translocating ATPase of chromaffin granules within cells was inhibited, there was no effect on elevated K^+ -induced secretion. Similarly, FCCP, a H^+ ionophore, which reduces the granule membrane potential to the H^+ equilibrium potential in isolated chromaffin granules (and, thereby, reduces to zero the H^+ electrochemical gradient) had no effect or caused a small enhancement of secretion induced by nicotinic stimulation at concentrations which maximally uncoupled mitochondria within chromaffin cells (Holz *et al.*, 1983).

These experiments indicate that secretion does not require (1) the complete H^+ concentration gradient across the granule membrane, (2) a normal H^+ electrochemical gradient across the granule membrane, (3) an inside positive granule electrical potential, or (4) an active granule membrane H^+ pump ATPase.

Secretion from Chromaffin Cells with Plasma Membranes Rendered Leaky by Digitonin. One of the major limitations in the study of a complex cellular process such as exocytosis is the inability to control the intracellular milieu. Knight and Baker (1982) permeabilized the plasma membrane of freshly suspended adrenal medullary cells by exposing cell suspensions to high-voltage discharges which caused localized dielectric breakdown of the plasma membrane. They found that micromolar Ca^{2+} without secretagogue induced exocytosis. We have permeabilized monolayer chromaffin cells with the detergent digitonin, which interacts with cholesterol in membranes (Dunn and Holz, 1983). Although digitonin at high concentrations can completely disrupt cells, we found that exposure of cells to low concentrations of digitonin (10 to 20 μM) for short times (less than 15 min) caused the cells to become permeable to low molecular weight ions such as Ca^{2+} and ATP, to carbohydrates as large as the tetrasaccharide stachyose, and to lactate dehydrogenase, a cytosolic protein marker, which exited from the cells with a half-time of 10–15 min. Intracellular chromaffin granules, however, remained intact, did not leak catecholamines, and were able to maintain a H^+ electrochemical gradient (Holz and Senter, 1985). Most importantly, at these low concentrations of digitonin, micromolar Ca^{2+} stimulated catecholamine release (Fig. 2). Similar results were obtained by Wilson and Kirshner (1983). Figure 3 demonstrates that the cells were responsive to calcium from below 1 to 10 μM . The response to Ca^{2+} was specific since the 1 mM free Mg^{2+} which was presented in the solutions did not support catecholamine release. The Ca^{2+} concentrations are within the physiological range which stimulates intracellular events in intact cells and are approximately 1/500th of that necessary to support secretagogue-induced secretion from intact cells (Fig. 3).

If cells are permeabilized for 10 min in the absence of ATP to deplete the cytosol of ATP, subsequent addition of ATP in the presence and absence of Ca^{2+} indicates that at least 75% of the Ca^{2+} -dependent secretion is also ATP dependent. The results suggest that ATP plays a direct role in the secretory process. Baker and Knight (1982), using cells permeabilized by intense electric fields, arrived at the same conclusion.

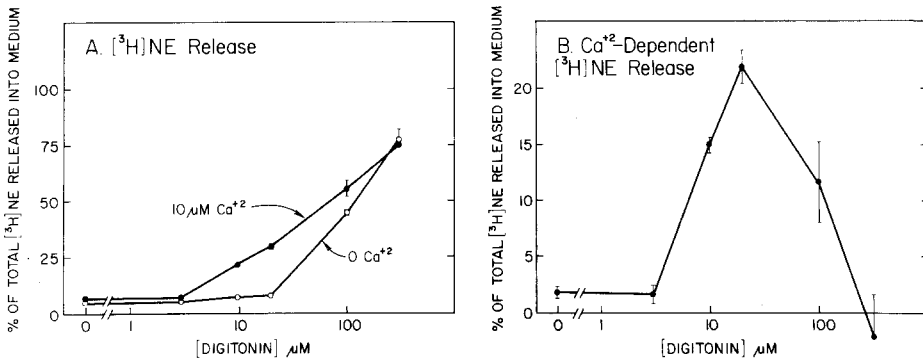


Fig. 2. Effect of various concentrations of digitonin on norepinephrine release from chromaffin cells. Cells containing $[^3\text{H}]$ norepinephrine (NE) were incubated in potassium glutamate solutions containing 5 mM MgATP and various concentrations of digitonin in the presence or absence of 10 μM Ca^{2+} . After 15 min, the solution was removed, and $[^3\text{H}]$ norepinephrine released in the $[^3\text{H}]$ norepinephrine solution and that remaining in the cells were measured. (A) $[^3\text{H}]$ Norepinephrine released into the medium in the presence and absence of 10 μM Ca^{2+} ; (B) difference between release of $[^3\text{H}]$ norepinephrine in the presence and that in the absence of Ca^{2+} . There were three wells per group. (From Dunn and Holz, 1983.)

Phorbol Esters Stimulate Protein Phosphorylation and Secretion. Protein kinase C is Ca^{2+} dependent and requires acidic phospholipids for maximal activity (Wise *et al.*, 1982; Kikkawa *et al.*, 1982). Diglyceride in the presence of phosphatidylserine increases the calcium sensitivity of the enzyme to micromolar concentrations (Kishimoto *et al.*, 1980). Tumor promoting phorbol esters such as tetradecanoylphorbol 13-acetate (TPA) substitute for diglyceride *in vitro* to

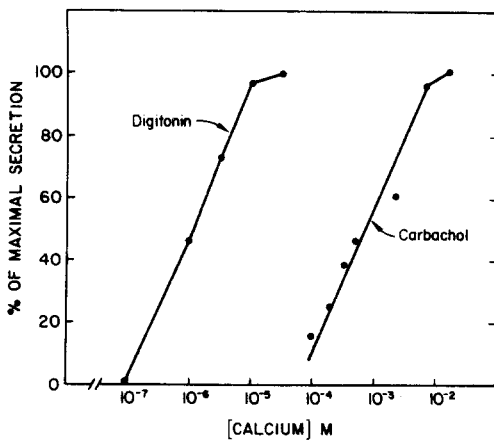


Fig. 3. Effect of various calcium concentrations on secretion in the presence of digitonin or carbachol. Ca^{2+} -dependent $[^3\text{H}]$ norepinephrine release into the medium by digitonin-treated cells was measured in 139 mM potassium glutamate, 20 mM Pipes (pH 6.6), 5 mM MgATP, 5 mM glucose, 0.5 mM ascorbic acid, and 20 μM digitonin after 15 min. Calcium concentrations were buffered with 5 mM EGTA. Carbachol-dependent secretion of endogenous catecholamine was measured in 142 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO_3 , 0.5 mM MgCl_2 , 15 mM Na-Hepes (pH 7.4), 5.6 mM glucose, and 0.5 mM ascorbic acid with various amounts of calcium (in the absence of EGTA) after 2 min. Carbachol concentration was 0.3 mM. Maximal calcium-dependent secretion in the presence of digitonin was 28.5% of the total $[^3\text{H}]$ norepinephrine. Maximal calcium-dependent secretion in the presence of carbachol was 9.6% of the total endogenous catecholamine. There were three samples/groups. (From Dunn and Holz, 1983.)

increase the activity and calcium sensitivity of the enzyme (Castagna *et al.*, 1982). Most importantly, TPA activates protein kinase C and protein phosphorylation in intact platelets and enhances serotonin secretion (Castagna *et al.*, 1982; Yamaniishi *et al.*, 1983).

The effects of various activators of protein kinase C (phorbol esters and dioctanoylglycerol, DiC₈) on secretion and protein phosphorylation were investigated in intact and digitonin-permeabilized cells. Protein phosphorylation was measured using one- and two-dimensional electrophoresis and autoradiography of ³²P-labeled proteins. Because activation of protein kinase C is associated with translocation of the enzyme from cytosol to membranes, changes in the subcellular localization of protein kinase C in response to phorbol esters, DiC₈, nicotinic agonists, and muscarinic agonists were also investigated.

TPA induced a dose-dependent increase in catecholamine release from intact chromaffin cells which occurred gradually over a period of 2 hr (Pocotte *et al.*, 1985; M. A. Bittner and R. W. Holz, unpublished observations). The release at 90 min was approximately 10% of the total catecholamine and contrasts with the rapid release induced by nicotinic agonists or by depolarization of 15–20% of the total catecholamine in 5–10 min. TPA-induced catecholamine release was associated with a proportional release of soluble dopamine- β -hydroxylase (M. A. Bittner and R. W. Holz, unpublished observations), a soluble protein marker for the chromaffin granule interior. Thus, the release was exocytotic.

TPA and other phorbol esters which activate protein kinase C also potentiate secretion induced from intact cells by Ba²⁺ and Ca²⁺ ionophore in the presence of Ca²⁺ (Pocotte *et al.*, 1985). Phorbol esters also enhance catecholamine secretion induced by nicotinic agonists (TerBush, Bittner and Holz, manuscript in preparation). We have recently found that within 2 sec the nicotinic agonist DMPP (1,1-dimethyl-4-phenylpiperazinium) causes a Ca²⁺-dependent translocation of 5–10% of the total cellular protein kinase C to the membrane (TerBush and Holz, 1986; D. R. TerBush, M. A. Bittner, and R. W. Holz, manuscript in preparation). Associated with the translocation of protein kinase C is a DMPP-induced, Ca²⁺-dependent activation of phospholipase C measured by the release of [³H]inositol phosphates from cells prelabeled with [³H]inositol (Eberhard *et al.*, 1987). Thus, it is likely that nicotinic receptor activation results in Ca²⁺ influx and a rise in cytosolic Ca²⁺, which activates phospholipase C. Elevated cytosolic Ca²⁺ together with a possible increase in diglyceride (or basal levels of diglyceride) cause translocation of protein kinase C to the membrane with subsequent effects on secretion.

The effects of activated protein kinase C on Ca²⁺-dependent secretion were directly studied in digitonin-permeabilized chromaffin cells. Cells were incubated with TPA or dioctanoylglycerol (DiC₈) prior to permeabilization to cause translocation of protein kinase C to membranes (TerBush and Holz, 1986) and activation of the enzyme (Pocotte *et al.*, 1985; Lee and Holz, 1986). Upon permeabilization, Ca²⁺-induced secretion was enhanced in cells preincubated with TPA (Fig. 4). A similar effect of TPA on Ca²⁺-dependent secretion had been obtained in suspended chromaffin cells permeabilized by intense electric fields (Knight and Baker, 1983). TPA increased both the sensitivity of the secretory

system to Ca^{2+} and the maximal response to Ca^{2+} . Recent studies indicate that translocation of as little as 2% of the total cellular protein kinase C to membranes resulted in greater than a 25% increase in secretion induced by $1 \mu\text{M}$ Ca^{2+} (D. R. TerBush, M. A. Bittner, and R. W. Holz, manuscript in preparation). Thus, the translocation of 5–10% of the protein kinase C to membranes during nicotinic stimulation of intact cells probably enhances the secretory response to the rise in cytosolic Ca^{2+} .

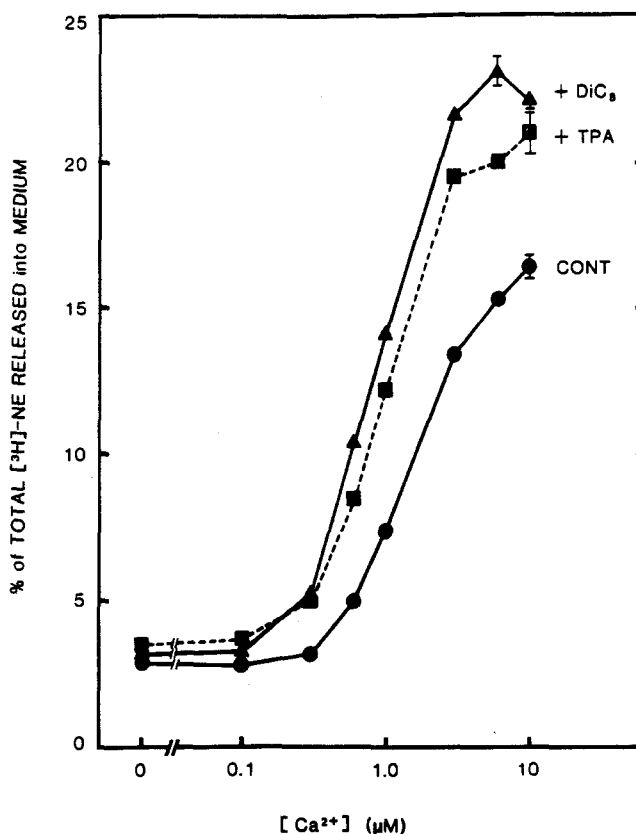


Fig. 4. Effects of dioctanoylglycerol (DiC_8) and TPA on catecholamine secretion from digitonin-treated chromaffin cells. Chromaffin cells prelabeled with [^3H]norepinephrine (^3H]NE) were incubated for 30 min in PSS containing $100 \mu\text{M}$ DiC_8 , 100 nM TPA, or no drug and subsequently stimulated to secrete in KGEP solutions containing $20 \mu\text{M}$ digitonin, $10 \mu\text{M}$ MgATP , 1 mM MgCl_2 , various concentrations of Ca^{2+} , and $100 \mu\text{M}$ DiC_8 , 100 nM TPA, or vehicle. The percentage of [^3H]norepinephrine released into the medium was determined after 6 min. Each group contained three wells. Endogenous intracellular ATP was not allowed to exit before the addition of $10 \mu\text{M}$ ATP. Thus, the intracellular ATP concentration was probably considerably greater than $10 \mu\text{M}$. Similar effects of DiC_8 and TPA were obtained in 1 mM MgATP . CONT, control. (From Lee and Holz, 1986.)

It is unlikely that activation of protein kinase C is obligatory in the secretory pathway in chromaffin cells. Experiments with trypsin in permeabilized cells reveal that the phorbol ester-mediated enhancement of secretion can be inhibited without inhibition of phorbol ester-independent secretion (Holz and Senter, 1988). Thus, it is likely that protein kinase C plays a modulatory, rather than an obligatory, role in secretion induced by nicotinic agonists.

In summary, nicotinic stimulation results in Ca^{2+} influx. The rise in cytosolic Ca^{2+} has at least three effects which are important for secretion.

- (1) Ca^{2+} directly activates the secretory pathway through yet unknown mechanisms.
- (2) Ca^{2+} activates phospholipase C and may generate diglyceride.
- (3) Ca^{2+} , perhaps acting together with diglyceride, causes the translocation and activation of protein kinase C. The activation of protein kinase C enhances Ca^{2+} -dependent secretion.

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