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Characterization of Hormone and Protein Release from α-Toxin-permeabilized Chromaffin Cells in Primary Culture*

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Addition of *Staphylococcus aureus* α-toxin to adult bovine chromaffin cells maintained in primary culture causes permeabilization of cell membrane as shown by the release of intracellular $^{86}Rb^+$. The α-toxin does not provoke a spontaneous release of either catecholamines or chromogranin A, a protein marker of the secretory granule, showing the integrity of the secretory vesicle membrane. However the addition of micromolar free Ca^{2+} concentration induced the co-release of noradrenaline and chromogranin A. In α-toxin-treated cells, the released chromogranin A could not be sedimented and lactate dehydrogenase was still associated within cells, which provides direct evidence that secretory product is liberated by exocytosis. By contrast, permeabilization of cells with digitonin caused a Ca^{2+}-dependent but also a Ca^{2+}-independent release of secretory product, a dramatic loss of lactate dehydrogenase, as well as release of secretory product in a sedimentable form. Ca^{2+}-dependent exocytosis from α-toxin-permeabilized cells required Mg^{2+}-ATP and did not occur in the presence of other nucleotides. Thus α-toxin is a convenient tool to permeabilize chromaffin cells, and has the advantage of keeping intracellular structures, specifically the exocytotic machinery, intact.

The final step in exocytosis, the fusion of the secretory vesicle membrane with the cell membrane, is not well understood. Studies using intact cells are limited because the plasma membrane acts as a barrier to the cell interior. Intervesicular fusion of isolated secretory vesicles has been successfully used to determine the ionic requirements of membrane fusion (1). Therefore, the development of an *in vitro* system using isolated secretory vesicles and cell membranes (2) would be advantageous. However, cell membrane fractions with suitable orientations for the analysis of exocytosis are difficult to obtain and attempts to use these experimental setups have been not fully satisfactory. A major step has recently been accomplished using permeabilized cells; this system represents a compromise between intact cells and *in vitro* systems.

Chromaffin cells and other secretory cells have been successfully permeabilized by high voltage discharge (3). Although this method certainly is selective for the plasma membrane, it can only be used for cells in suspension. Chromaffin cells can also be made permeable using detergents. Digitonin and saponin have been applied to chromaffin cells maintained in primary culture (4–6). Although these molecules provoke formation of pores, their use is limited because it is impossible to control the number and size of pores, and the overall impact on cell cytoplasm. In addition, some results obtained using electrically permeabilized cells contradict those obtained using detergent-permeabilized cells.

Recently α-toxin from *Staphylococcus aureus* has been developed as a tool to selectively permeabilize cell membranes. This exotoxin secreted by *S. aureus* causes lysis of erythrocytes (7). The native form of the toxin assembles into characteristic ring structures in the target membrane creating stable transmembrane pores (8). In the erythrocyte membrane, the α-toxin pore is a ring-structured hexamer with a diameter of 2–3 nm (8). Similar rings have been observed in plasma membrane of hepatocytes (9) and fibroblasts (10) treated with α-toxin. This property has been used to study contractility in smooth muscle cells (11), enzymatic activities of internal hepatocyte organelles (12), and secretion in PC12 cells (13, 14). α-Toxin channels do not permit the native α-toxin molecule to cross the plasma membrane and therefore the toxin does not affect intracellular membranes (8). Thus, the plasma membrane is specifically and easily permeabilized with α-toxin.

In the present study, α-toxin has been used to permeabilize adult bovine adrenal medullary chromaffin cells maintained in primary culture. The minimal requirements necessary to induce the optimal release of the low molecular weight secretory product, noradrenaline, and the high molecular weight granule protein, chromogranin A, were studied.

EXPERIMENTAL PROCEDURES

Culture of Chromaffin Cells—The procedure for isolating chromaffin cells from bovine adrenal medullae has been described previously (15). Briefly, bovine adrenal glands were perfused retrogradely with calcium-free Krebs solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5 mM HEPES,[1] pH 7.4) containing 0.1% *clostridium histolyticum* collagenase (Boehringer, GmBH, Mannheim, Federal Republic of Germany) and 0.5% bovine serum albumin. Cells were then dissociated by filtration through a nylon sieve, and chromaffin cells were further purified on self-generating Percoll (Pharmacia, Bois d'Arcy, France) gradients. Subsequently, chromaffin cells

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[1] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PBS-T, phosphate-buffered saline containing 0.05% Tween 20; ELISA, enzyme-linked immunoabsorbent assay.

were washed by low-speed centrifugation in Dulbecco's modified Eagle's medium and finally suspended in that medium supplemented with 10% fetal calf serum and containing 10^{-5} M cytosine arabinoside (Aldrich Chimie, Strasbourg, France) and 10^{-5} M 5-fluorodeoxyuridine (Aldrich). Cells were grown on 24 multiple 16-mm well Costar plates (Costar, Data Packaging Corp., Cambridge, MA) in a humidified 5% CO_2/95% air atmosphere. The cell density was 5.10^5 cells/well for experiments with digitonin and 2.5×10^5 cells/well for experiments with α-toxin.

[^{3}H]Noradrenaline Release from Permeabilized Cells—For release experiments, 3–6 day-old cultured chromaffin cells were loaded with [7-^{3}H]noradrenaline (Amersham France, Les Ulis, France; 16 Ci/mmol). The culture medium was removed and cells were incubated for 60 min with the same medium containing 125 nM noradrenaline but no amino acids. The amount of radioactive noradrenaline taken up by the cells was in the range of 10–15%. Cells were then washed four times with Locke's solution containing calcium (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM HEPES, pH 7.5) and two times with calcium-free Locke's solution (containing 1 mM EGTA). Washing intervals were set constant at 10 min. Cells were then incubated 10 min in calcium-free permeabilizing medium (KG medium: 150 mM glutamate, potassium salt, 10 mM PIPES, 5 mM nitrilotriacetic acid, 0.5 mM EGTA, 0.2% bovine serum albumin, pH 7.2, adjusted with 1 M KOH) and subsequently permeabilized with digitonin (Merck, Darmstadt, FRG) or α-toxin in the KG medium containing calcium to give the indicated free-calcium concentration. Changes in this standard procedure are indicated in the text. Catecholamine release was monitored by determining the radioactivity present in the incubation medium after centrifugation for 2 min in an Eppendorf centrifuge. Cells were precipitated with 0.4 N perchloric acid and scraped off the plates. Radioactivity was measured with scintillation vials containing 10 ml of BIOFLUOR (New England Nuclear, Dreieich, Federal Republic of Germany) in an SL-4000 Intertechnique scintillation counter. [^{3}H]Noradrenaline release was expressed as per cent of total radioactivity present in the cells before stimulation.

Calcium Concentration—The exact free calcium concentration in KG medium was calculated as described (16) using the stability constants given by Sillen and Martell (17). The free calcium concentration was also measured using a selective calcium minielectrode (kindly provided by Dr. W. Simon, Swiss Federal Institute of Technology, Zurich, Switzerland), operating with a neutral carrier incorporated into a polyvinyl chloride membrane (18).

S. aureus α-Toxin—α-Toxin was a generous gift from Dr. S. Bhakdi (Institut fur Medizinische Mikrobiologie, Justus Liebig Universitat, Giessen, Federal Republic of Germany). Preparations of purified toxin were assayed for hemolytic activity against 2.5% rabbit erythrocytes in 25 mM phosphate-buffered saline. The dilution of toxin hemolyzing 50% of the red cells (EC_{50}) was estimated and the reciprocal of this value was taken as the number of hemolytic units/ml of the undiluted toxin solution (19). Usually chromaffin cells (2.5×10^5 cells) were permeabilized for 30 min at 37 °C in 200 μl of KG medium containing 40–80 units/ml of α-toxin.

Permeability of the Cells—Permeability of the cell plasma membrane was assessed by the release of $^{86}Rb^+$ (13). Cultured cells were washed twice with Locke's solution where K_2HPO_4 was replaced by Na_2HPO_4 and then incubated for 2 h at 37 °C in the same saline solution containing 40 μCi of $^{86}Rb^+$ (1–8 mCi/mg; Amersham, France)/multiwell plate. During this period, $^{86}Rb^+$ enters the cells through the Na,K-ATPase (20). Then extracellular $^{86}Rb^+$ was removed by washing with the saline solution. Permeabilization was performed as usual with α-toxin for 30 min or digitonin for 15 min in KG solution. $^{86}Rb^+$ was estimated in the extracellular fluid and in sodium dodecyl sulfate lysates of the cells. $^{86}Rb^+$ release was expressed as per cent of the total radioactivity present in the cells before permeabilization.

Chromogranin A—Chromogranin A was assayed by the ELISA (enzyme-linked immunoabsorbent assay) technique. Cells were permeabilized with digitonin or α-toxin and stimulated with calcium. Chromogranin A was measured in extracellular fluids and in distilled water lysates of the cells. The assay was performed in disposable flat-bottom polystyrene microtest plates (Dynatech, Rungis, France). Wells were coated overnight at 4 °C with 100 μl of samples diluted in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6. All samples were assayed in triplicate at dilutions calculated to have protein concentration in the linear range of the assay (usually 0.5–20 ng of chromogranin A, partially purified from bovine chromaffin granules). After removing unabsorbed material by washing each well three times with phosphate (25 mM)-buffered saline containing 0.05% Tween 20 (PBS-T), 100 μl of anti-chromogranin A antiserum diluted to 1:10,000 in PBS-T containing 1% bovine serum albumin was added to the coated wells, and plates were left at room temperature for 2 h. The antiserum against chromogranin A (from rabbit europium) is specific to bovine chromogranin A as shown by biochemical and immunocytochemical techniques.[2] Subsequently the plate was rinsed three times with PBS-T and 100 μl of peroxidase-coupled goat anti-rabbit immunoglobulins (1:100 dilution in PBS-T (Nordic Immunological Laboratories, Tilburg, The Netherlands)) was added to each well. After a 2-h incubation at room temperature in the dark, the wells were extensively washed with PBS-T, and bound antibody was detected by adding 50 μl of phosphate-buffered saline containing 0.012% H_2O_2 and 0.25 mg/ml *p*-phenylenediamine (Sigma). The color was developed for 30 min in the dark. The absorbance at 492 nm was directly read in a micro-ELISA minireader (Titertek Multiskan MCC).

Lactate Dehydrogenase—Release of L-lactate dehydrogenase (EC 1.1.1.27) has been estimated from chromaffin cells permeabilized with either digitonin or α-toxin in KG media containing calcium. Incubation times and free calcium concentrations are given in the figure legends. Enzyme activity was determined in the supernatant and in the cells scraped off the plates in 10 mM phosphate buffer, pH 7.4. Enzyme activity was determined in the presence of 50 mM sodium phosphate, pH 7.4, 180 μM NADH, and 600 μM pyruvate (21). The rate of NADH disappearance was monitored at 340 nm.

Proteins—Proteins were estimated according to Lowry *et al.* (22) with bovine serum albumin (Sigma) as standard.

Presentation of Data—All experiments described have been carried out on three different cell preparations. In the figures, data are given as the mean of triplicate determinations on the same cell preparation.

RESULTS

Comparison of Digitonin and α-Toxin Effects on Chromaffin Cells—Chromaffin cells maintained in primary cultures were loaded with $^{86}Rb^+$ and treated with various concentrations of digitonin or α-toxin. The release of intracellular $^{86}Rb^+$ was used as a measure of the permeabilization of the cell membrane. α-Toxin as well as digitonin increased the release of $^{86}Rb^+$ from preloaded chromaffin cells in a dose-dependent manner. Net $^{86}Rb^+$ release is expressed as the difference in radioactivity released into the medium by permeabilized cells and by intact cells (Fig. 1). Chromaffin cells were exposed to digitonin concentrations from 1 to 100 μM for 15 min at 37 °C. Maximal net $^{86}Rb^+$ release, approximately 50%, was observed with 50 μM digitonin when calcium was present in the incubation medium. The absence of calcium did not modify $^{86}Rb^+$ release. A calcium-dependent $^{86}Rb^+$ release was also observed when chromaffin cells were treated with α-toxin concentration ranging from 1 to 300 units/ml. Maximal net $^{86}Rb^+$ release was seen with 80 units/ml α-toxin following a 30-min incubation at 37 °C (Fig. 1). Thus, the efficiency of α-toxin in permeabilizing the cell membrane to small molecules is comparable to that of digitonin.

In Fig. 2, catecholamine release evoked by various concentrations of digitonin and α-toxin is shown. Chromaffin cells were first loaded with [^{3}H]noradrenaline and then titrated with digitonin (15 min) or α-toxin (30 min) in the presence or absence of 100 μM free calcium. Both digitonin (Fig. 2*A*) and α-toxin (Fig. 2*B*) elicited a dose-dependent noradrenaline release when calcium was in the external medium. Cells released approximately 40% of the total [^{3}H]noradrenaline uptake after exposure to 100 μM digitonin or 100 units/ml α-toxin in presence of 100 μM free calcium. Catecholamine release from intact cells is not induced by this calcium concentration; the high potassium concentration in the KG media would depolarize cells, but a free calcium concentration of

[2] Ehrhart, M., Grube, D., Aunis, D., Bader, M. F., and Gratzl, M., (1986) *J. Histochem. Cytochem.*, submitted for publication.

100 μM is too low to induce significant secretion from intact cells (14, 23).

Although in both types of permeabilized cells catecholamine release was similar, there was a marked difference in calcium dependence of the process. The release of [^{3}H]noradrenaline from α-toxin-treated cells was absolutely calcium-dependent (Fig. 2*B*) even in high concentrations of α-toxin (up to 300 units/ml). In 5 and 20 μM digitonin, a similar calcium dependence of [^{3}H]noradrenaline release was observed (Fig. 2*A*); but at higher concentrations the cells displayed a dramatic calcium-independent release (Fig. 2*A*). The net release, calculated as the difference in the release of [^{3}H]noradrenaline in the presence and absence of calcium, clearly indicates that digitonin induces a calcium-independent release of catecholamine in chromaffin cells (Fig. 2*C*). This result demonstrates that *S. aureus* α-toxin, in contrast to digitonin, does not affect intracellular storage structures and thereby the regulatory mechanism controlling release.

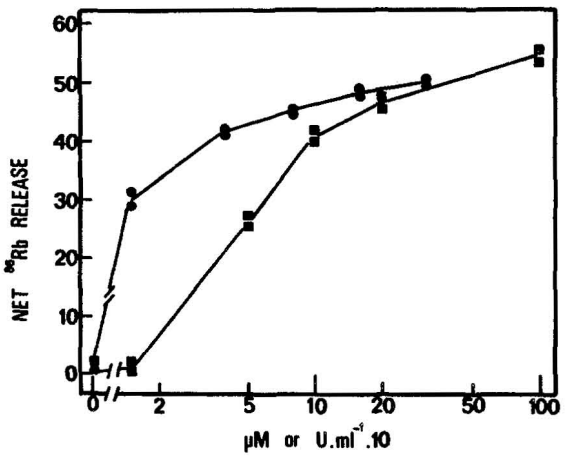

FIG. 1. **^{86}Rb$^+$ release from α-toxin- and digitonin-permeabilized chromaffin cells.** Chromaffin cells were loaded with ^{86}Rb$^+$ as described under "Experimental Procedures" and subsequently permeabilized with α-toxin (*circles*) or with digitonin (*squares*) at the indicated concentrations. The radioactivity present in the supernatant was estimated after incubations of 15 min with digitonin or 30 min with α-toxin. ^{86}Rb$^+$ release was calculated as a percentage of the total radioactivity present in the cells at the beginning of the experiment. In the absence of permeabilizing agents, cells released 20% of the total radioactivity within 15 min of incubation. The *ordinate* is the net ^{86}Rb$^+$ release, *i.e.* the difference in release of ^{86}Rb$^+$ in the presence and absence of permeabilizing agents. The *abscissa* is the final concentration of α-toxin in units/100 μl or digitonin in μM.

Evidence That Calcium-dependent Catecholamine Release from α-Toxin-treated Cells Occurs by Exocytosis—In order to show that the calcium-dependent release of catecholamine from α-toxin-treated chromaffin cells occurs from the intragranular compartment, extracellular fluids were assayed for chromogranin A, the major soluble granule protein. The activity of lactate dehydrogenase, a marker of the cytosol, was also measured. The time dependence of release of [^{3}H]noradrenaline, chromogranin A, and lactate dehydrogenase from α-toxin-permeabilized cells is shown in Fig. 3. Chromaffin cells were exposed to KG media containing 40 units/ml α-toxin in the presence or absence of 100 μM free calcium. Less than 3% of the total [^{3}H]noradrenaline and chromogranin A was released when chromaffin cells were permeabilized for 40 min in calcium-free media (Fig. 3*B*). However, by increasing the external free calcium concentration to 100 μM, 30% of the [^{3}H]noradrenaline and chromogranin A was released in 40 min.

The release of chromogranin A paralleled that of [^{3}H] noradrenaline (Fig. 3*B*, *inset*), an observation which suggests that the two molecules come from the same intracellular compartment and provides evidence for a release by exocytosis. The latter was confirmed by the observation that the chromogranin A released into the extracellular fluids could not be sedimented by high-speed centrifugation (30 min,

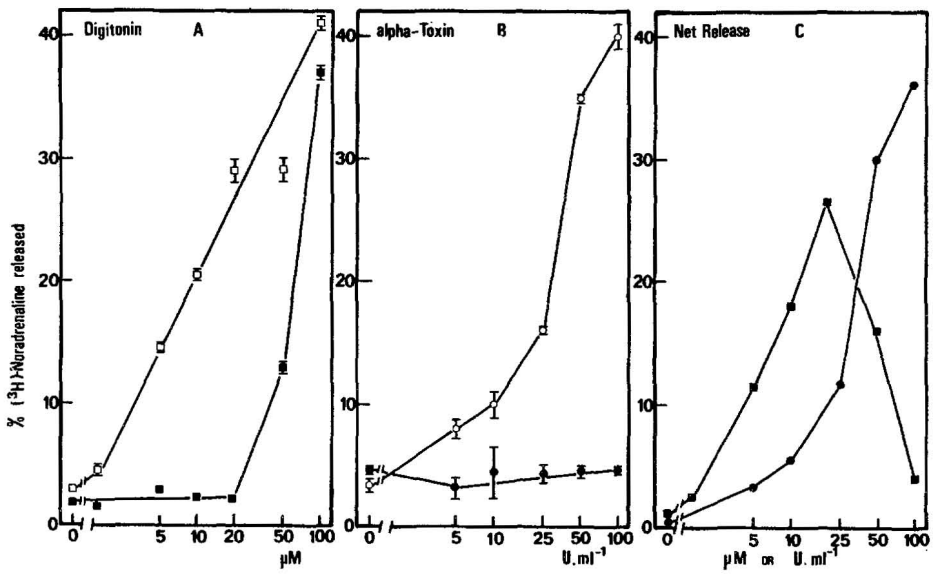

FIG. 2. **Effect of various concentrations of digitonin or α-toxin on catecholamine release from chromaffin cells.** Cells loaded with [^{3}H]noradrenaline were incubated in KG media containing various concentrations of digitonin (*A*) or α-toxin (*B*) in the presence (*open symbols*) or absence (*closed symbols*) of 100 μM free calcium. [^{3}H]Noradrenaline release was measured after a 15-min incubation period with digitonin or 30 min with α-toxin (n = 3). Digitonin as well as α-toxin induced a dose-dependent [^{3}H]noradrenaline release in the presence of calcium. However, the release from α-toxin-treated cells was absolutely calcium-dependent (*B*), whereas a calcium-independent component of [^{3}H]noradrenaline release occurred between 20 and 100 μM digitonin (*A*). *C* represents the difference between [^{3}H]noradrenaline release in the presence and absence of calcium from digitonin- (*square*) and α-toxin (*circle*)-permeabilized cells.

100,000 × *g*), as shown in Table I.

Interestingly, as little as 3% of total cell lactate dehydrogenase was found in the extracellular fluids of α-toxin-treated cells during the 40-min incubation period. In contrast, lactate dehydrogenase was gradually released from chromaffin cells permeabilized with 10 μM digitonin despite the use of a low detergent concentration which induced virtually no calcium-independent catecholamine release (Fig. 2*A*). After a 15-min incubation, 50% of the total cell lactate dehydrogenase activity was released from cells permeabilized with digitonin in the absence of calcium. This value dropped to 20% when calcium was present at 100 μM in the external medium (data not shown). Since lactate dehydrogenase is a cytoplasmic protein of 142,000 daltons, such a massive lactate dehydrogenase leakage suggested the presence of large lesions in the plasma membrane of digitonin-treated cells. Therefore, the possibility that digitonin-treated cells release intact chromaffin granules was also examined. For this purpose, chromaffin cells were permeabilized with 10 μM digitonin in the presence or absence of 100 μM free calcium. After a 15-min incubation, external media were centrifuged first at 800 × *g* for 10 min to remove detached cells and then at 100,000 × *g* for 30 min in the Airfuge Ultracentrifuge. Chromogranin A was assayed in the supernatants. As shown in Table I, 23% of the chromogranin A released in Ca^{2+} medium could be sedimented by high-speed centrifugation, suggesting the diffusion of intact secretory granules through digitonin-induced holes in the plasma membrane.

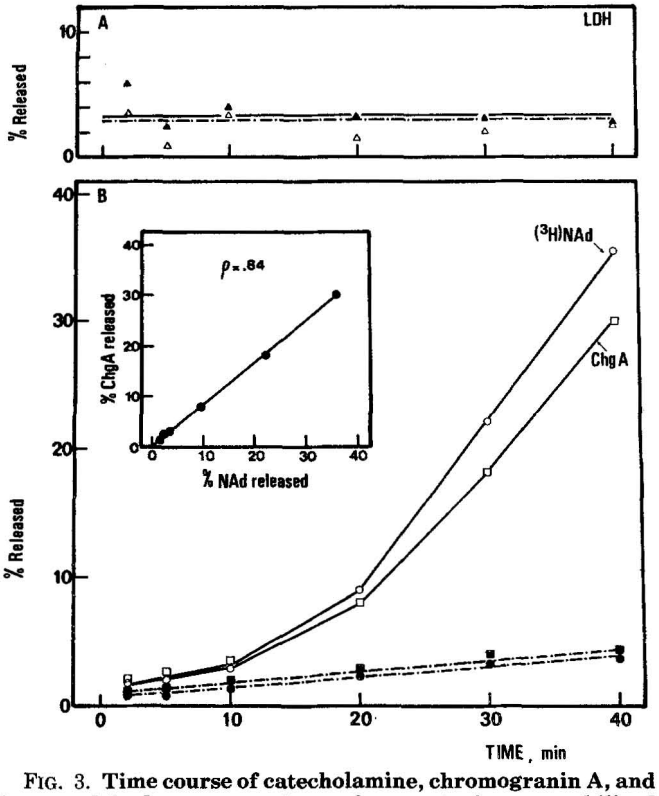

FIG. 3. **Time course of catecholamine, chromogranin A, and lactate dehydrogenase release from α-toxin-permeabilized chromaffin cells.** Chromaffin cells were incubated in KG medium containing 40 units/ml α-toxin in the presence of 100 μM free calcium (*open symbols*), or in the absence (*closed symbols*) of free calcium. At the indicated times, extracellular fluid was removed and [^{3}H]noradrenaline ([^{3}*H*]*NAd, circle*), chromogranin A (*ChgA, square*), and lactate dehydrogenase (*LDH, triangle*) activity were determined in the medium and also cells. Data are the mean of triplicate determinations. α-Toxin caused no lactate dehydrogenase release from the cells during the 40-min incubation period (*A*). As shown in the *inset*, [^{3}H]noradrenaline and chromogranin A were released in parallel (*B*).

Calcium Dependence of Secretion of α-Toxin-permeabilized Chromaffin Cells—Catecholamine secretion from α-toxin-permeabilized chromaffin cells was triggered by the addition of calcium to the incubation medium. The release of [^{3}H]noradrenaline, chromogranin A, and lactate dehydrogenase was examined at different free calcium concentrations. Chromaffin cells were permeabilized for 30 min with 40 units/ml α-toxin in KG medium containing calcium ranging from 1 to 100 μM. As shown in Fig. 4, calcium triggered a secretion of both [^{3}H]noradrenaline and chromogranin A (Fig. 4, *inset*) but had no effect on lactate dehydrogenase release. Maximal catecholamine and chromogranin A secretion (approximately 25% of the total cell content) occurred in 100 μM calcium. Under these conditions, only 4% of the lactate dehydrogenase activity was found in the external medium. The minimum free calcium concentration required to induce significant noradrenaline release was 4 μM. It appears that calcium concentration rather than the number of toxin pores in the plasma membrane was the limiting factor, since at calcium concentrations less than 20 μM, the amount of noradrenaline released was independent of α-toxin concentration (40 to 300 units/ml, data not shown).

Effect of ATP—The energy requirement of the secretory process in permeabilized chromaffin cells is a major point of interest. As mentioned previously, it is possible to induce secretion from α-toxin- and digitonin-permeabilized cells by placing cells in the permeabilizing agents in media containing calcium but no Mg-ATP. However, chromaffin cells may retain some endogenous ATP which obscures the actual ATP requirement of secretion. Therefore, two types of experiments were designed. In the first experiment, cells were permeabilized with 40 units/ml α-toxin in KG media containing increasing free calcium concentration, in the presence or absence of Mg-ATP. As shown in Fig. 5*A*, catecholamine release from α-toxin-treated cells was stimulated when 5 mM Mg-ATP (final free Mg^{2+} concentration adjusted to 1 mM with Mg acetate) was in the incubation medium. Mg-ATP produced a 25%

TABLE I

Measurements of 10^5 g sedimented chromogranin A released from α-toxin- and digitonin-treated cells in the absence or in the presence of 100 μM free calcium

Chromogranin A content in 3–7-day-old cultured chromaffin cells was 28.6 ± 1.6 pmol/10^5 cells. Chromogranin A was measured by the ELISA technique in duplicate in four different wells. Values are given as the mean ± S.D.

	Digitonin-treated cells		α-Toxin-treated cells	
	Chromogranin A in 800 × *g* supernatants	Chromogranin A in 100,000 × *g* supernatants	Chromogranin A in 800 × *g* supernatants	Chromogranin A in 100,000 × *g* supernatants
	pmol/5 × 10^5 cells		*pmol/2.5 × 10^5 cells*	
0 Ca^{2+}	1.3 ± 0.04	1.1 ± 0.03	1.1 ± 0.02	1.2 ± 0.04
100 μM Ca^{2+}	44.3 ± 2.2	34.3 ± 1.1	25.2 ± 1.3	25.6 ± 0.6

increase in the [^{3}H]noradrenaline release evoked by 100 μM free calcium but did not change the minimal calcium requirement (4 μM). When 1 mM free Mg^{2+} (adjusted with Mg acetate) alone was added, a significant increase (13%) of the calcium-dependent catecholamine release was observed (data not shown). However, magnesium could not be substituted for calcium; no release occurred with Mg^{2+} in the absence of free Ca^{2+}. In order to determine the actual ATP requirement in the secretory process, cells were first permeabilized for 30 min with 40 units/ml α-toxin in calcium- and Mg-ATP-free KG medium and subsequently stimulated for 10 min with KG media containing the free calcium concentrations indicated in Fig. 5*B*. [^{3}H]Noradrenaline release did not occur when ATP was absent during stimulation even in the presence of 1 mM free magnesium, but was observed when Mg-ATP was added to the external medium. This provides strong evidence of a role for Mg-ATP in the calcium-dependent release from α-toxin-permeabilized cells. This requirement for ATP is only seen when permeabilized cells are depleted of their cytoplasmic ATP by permeabilization in ATP-free KG media. When the permeabilization was performed in the presence of Mg-ATP, calcium-induced release could be obtained in Mg-ATP-free media. The specificity was restricted to Mg-ATP. As shown in Fig. 6, GTP, UTP, and adenosine 5-*O*-(3-thiotriphosphate) (the ATPγS-analogue of ATP) in presence of 1 mM free Mg^{2+}, induced relatively little [^{3}H]noradrenaline release from α-toxin-treated cells depleted of endogenous ATP prior to stimulation.

DISCUSSION

In order to gain access to the intracellular exocytotic sites and to determine the minimal requirements of the exocytotic reaction, several techniques have been developed to increase cell membrane permeability. Knight and Baker (3) reported that brief pulses of high voltage electrical currents make the plasma membrane freely permeable to small molecules but not to proteins such as lactate dehydrogenase. By a suitable choice of voltage, the cell membrane remains permeable for at least 1 h after exposure to the electrical field and intracellular membrane-bound organelles are not affected. Although the technique is attractive because of the small pores of 1–2 nm formed in the plasma membrane, it is somewhat difficult to use because of the specialized experimental setup required. Moreover, the technique involves the use of freshly isolated chromaffin cells dispersed by collagenase treatment and cannot be easily applied to cultured chromaffin cells.

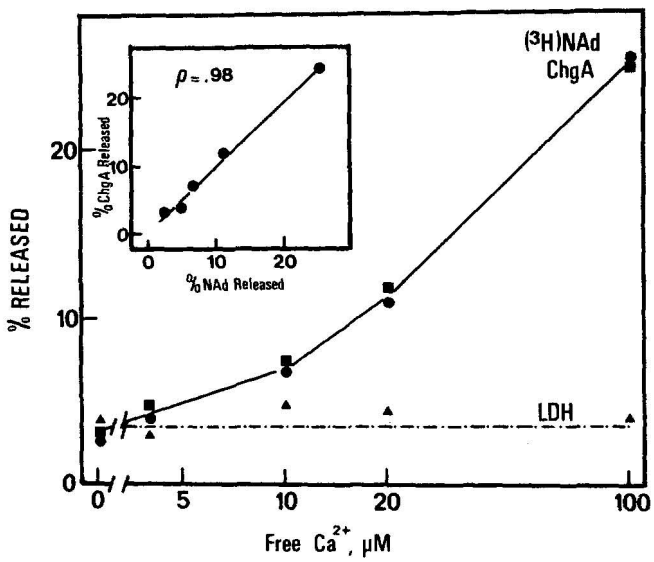

FIG. 4. **Effect of calcium concentration on secretion from α-toxin permeabilized chromaffin cells.** Chromaffin cells were exposed to 40 units/ml α-toxin in KG medium containing free calcium at the indicated concentration. After 30-min incubation, the solution was removed and [^{3}H]noradrenaline ([^{3}*H*]*NAd, circle*), chromogranin A (*Chg A, square*), and lactate dehydrogenase (*LDH, triangle*) were assayed in the solution and remaining cells. Calcium triggered a parallel secretion of [^{3}H]noradrenaline and chromogranin A (*inset*) but had little effect on lactate dehydrogenase release from α-toxin-permeabilized chromaffin cells.

Another approach to make the plasma membrane permeable involves the use of detergents. Digitonin (4, 5) and saponin (6) have been used to permeabilize chromaffin cells in culture. However, for several experimental reasons, detergents are difficult to handle and give unsatisfactory results. The ideal permeabilizing agent is one that specifically reacts with the cell membrane, and neither induces deterioration of the cellular morphology nor causes release of cytosolic components. Digitonin and saponin complex membrane-bound cholesterol. Since the chromaffin granule membrane and the plasma membrane contain cholesterol, detergents affect both membranes. Indeed, a calcium-independent release of catecholamines was observed with digitonin, clearly indicating intracellular damages to secretory granules. Detergents must be applied in a narrow range of time and concentrations which makes their use difficult. Moreover, treatment of chromaffin cells with digitonin or saponin induces an important leakage of lactate dehydrogenase (4, 5; this paper) and phenylethanolamine *N*-methyltransferase (5) from the cytosol indicating a major disruption of intracellular organization. Brooks and Carmichael (24) examined the cell surface of saponin-treated chromaffin cells by scanning electron microscopy and described the presence of holes ranging in size from 100 nm to 1 μm. Since the diameter of a secretory vesicle is approximately 250 nm, intact granules would be able to escape through some of these large holes. In the present study, measurement of calcium-dependent chromogranin A release, a marker of the soluble content of the chromaffin granule, showed that about 23% of the released chromogranin A was sedimented by high-speed centrifugation. These data suggested that release from digitonin-treated cells does not occur solely by the exocytotic reaction. Surprisingly, Holz and Senter (25) reported in a recent paper different results. They measured the catecholamine and dopamine β-hydroxylase (EC 1.14.17.1) activity released from digitonin-treated cells and found that neither catecholamines nor dopamine β-hydroxylase could be sedimented by centrifugation. Dopamine β-hydroxylase is a membrane-bound enzyme and should be sedimented if intact granules are released. However, low dopamine β-hydroxylase activity is difficult to detect particularly when metal chelators are present in the media (26). Moreover, residual digitonin may induce vesicle instability and cause catecholamine leakage from intact granules. In these respects, detergents are not fully satisfactory agents for selectively permeabilizing the plasma membrane. In fact, digitonin concentrations used to permeabilize chromaffin cells inhibit exocytosis in cells permeabilized by high voltage discharge (27).

The present study shows that α-toxin generates functional transmembrane channels in the plasma membrane of cultured chromaffin cells, as seen by the rapid and dose-dependent release of radioactive Rb^+. Interestingly, and in marked contrast to detergents, no lactate dehydrogenase output was detected from α-toxin-treated cells, indicating that intracellular structures are not affected by the toxin. Catecholamines are released from α-toxin-permeabilized chromaffin cells in a

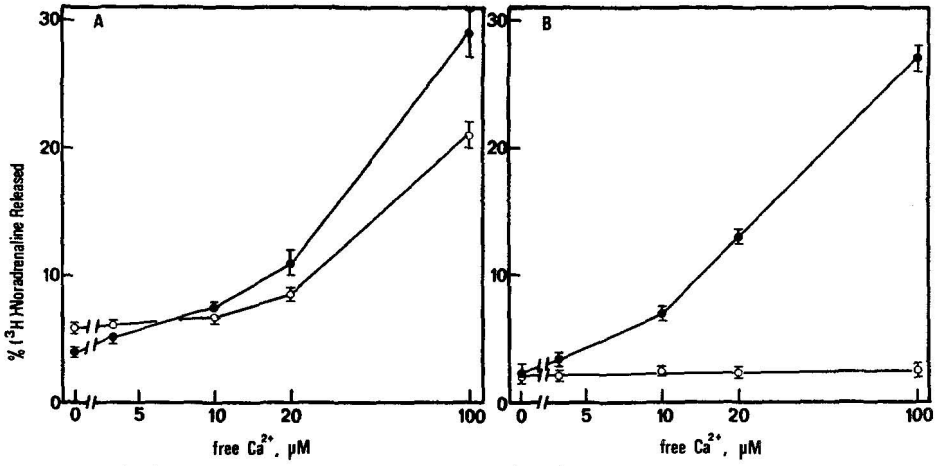

FIG. 5. **Effect of Mg-ATP on calcium-evoked catecholamine release from α-toxin-permeabilized chromaffin cells.** Chromaffin cells were permeabilized for 30 min with 40 units/ml α-toxin in KG media containing indicated free calcium concentration, in the presence (*filled circle*) or absence (*open circle*) of Mg-ATP (*A*). Experiments with Mg-ATP were performed in KG media containing added 5 mM Mg-ATP, $CaCl_2$, and Mg acetate to give a final free Mg^{2+} concentration of 1 mM and final free Ca^{2+} concentration indicated on *abscissa*. A 25% increase in catecholamine secretion from α-toxin-treated cells evoked by 100 μM free calcium was observed when 5 mM Mg-ATP was included in the incubation medium. For *B*, chromaffin cells were first permeabilized with 40 units/ml α-toxin in calcium- and ATP-free KG medium. Cells were then stimulated with calcium-containing KG media in the presence (*filled circle*) or absence (*open circle*) of 5 mM Mg-ATP and 1 mM free magnesium. Under these conditions, [^{3}H]noradrenaline release was observed only when ATP was present in the incubation medium. Neither calcium, nor calcium supplemented with 1 mM Mg^{2+} was able to induce catecholamine secretion in the absence of exogenous ATP.

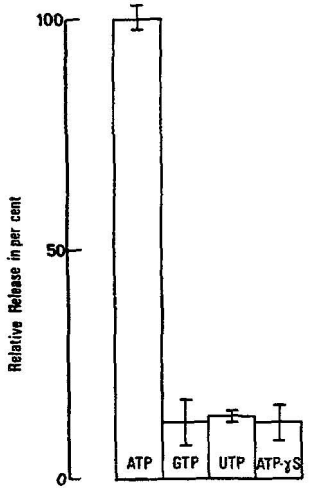

FIG. 6. **Nucleotide dependence of calcium-evoked catecholamine release from α-toxin-permeabilized cells.** Chromaffin cells were permeabilized with α-toxin (40 units/ml) in calcium- and ATP-free KG medium. Cells were then stimulated with KG medium containing 100 μM free calcium, 1 mM free magnesium, and 5 mM of the Mg-nucleotide complex indicated. [^{3}H]Noradrenaline release was measured after 10-min stimulation and release in the absence of calcium (basal release) was substracted. There was no significant difference in the calcium-independent basal release over the range of nucleotide tested (3% of total [^{3}H]noradrenaline cell content). Results are expressed relative to the response seen with 5 mM Mg-ATP (22% of total [^{3}H]noradrenaline cell content). Of the nucleotides tested, ATP is the most effective in supporting calcium-dependent release in α-toxin-treated chromaffin cells.

calcium-dependent manner. We were unable to detect any calcium-independent loss of catecholamines even in very high concentrations of α-toxin. Moreover, the calcium-dependent co-release of catecholamines and of chromogranin A suggests that release occurs by exocytosis.

It is interesting to compare the calcium concentration required for secretion in toxin-treated cells with those found for detergent- or electrically permeabilized chromaffin cells. In α-toxin-permeabilized cells 20 μM free calcium stimulates a release similar to that obtained from intact cells in response to nicotine or acetylcholine in the presence of external 2.2 mM calcium (23, 25, 28, 29). Our data are in agreement with results on saponin-skinned cells (6) and digitonin-treated cells (5). On the other hand, electrically permeabilized chromaffin cells show a higher sensitivity to calcium (2). However, the freshly isolated cells used in that study may not be directly comparable to cells maintained in primary culture. Structural and functional differences between the two preparations have been reported (30). Digitonin has been used by two laboratories to permeabilize chromaffin cells. There are conflicting results concerning the free calcium concentration (0.05 *versus* 2 mM) required to produce the same quantity of catecholamine release (4, 5). We compared the calcium requirement for release in digitonin- and α-toxin-permeabilized cells. Chromaffin cells permeabilized with 10 μM digitonin released 15% of their total catecholamines in 20 μM free calcium in the external medium. Under the same conditions, α-toxin-treated cells released fewer catecholamines. In other words, α-toxin-treated cells require more calcium than digitonin-treated cells to produce a similar secretory response. This result may be explained by intracellular effects of digitonin. For instance, recent studies have shown that calcium is required to induce solation of the subplasmalemmal cytoskeletal network in stimulated chromaffin cells (31). Such a network is completely perturbed by digitonin,[3] which would explain the lower calcium concentration required to induce a given secretory response from digitonin-treated cells. Furthermore, since about 23% of the released soluble granule proteins from digitonin-permeabilized cells could be sedimented by high-speed centrifugation, it is quite possible that the additional catecholamine release observed in digitonin-treated cells corresponds

[3] D. Perrin and D. Aunis, unpublished observation.

to granules that crossed the plasma membrane through large holes created by digitonin. In toxin-permeabilized chromaffin cells, this mechanism of release is unlikely because the toxin-induced channels have a functional diameter of only 2 nm.

α-Toxin has been used successfully to study secretion in PC12 cells (13, 14), a pheochromocytoma cell line from rat. In this system, micromolar free calcium induced dopamine release without added ATP. In contrast, electrically permeabilized chromaffin cells show an absolute requirement for Mg-ATP (3). A partial requirement for Mg-ATP has also been described in digitonin- and saponin-permeabilized chromaffin cells. We found that Mg-ATP is essential to stimulate release from α-toxin-permeabilized chromaffin cells, and that other nucleotides cannot serve as substitutes. It is surprising that α-toxin-permeabilized PC12 cells, which are tumoral chromaffin cells, do not require Mg-ATP to secrete dopamine, whereas chromaffin cells are absolutely ATP-dependent. Although chromaffin cells and PC12 cells are closely related, some morphological and functional differences have been described for cells maintained in culture (32–35). For instance, the distribution of secretory granules is quite different: granules are concentrated in the subplasmalemmal region in cultured PC12 cells (36), whereas granules are more randomly distributed in chromaffin cells (32, 33). Thus, the translocation of granules toward the cell membrane is probably a pre-exocytotic step in chromaffin cells but may not be essential in PC12 cells. Recently, Allen *et al.* (37) and Vale *et al.* (38) reported that organelles move along microtubules, and this movement requires ATP. Further studies using factors which affect organelle movement in α-toxin-permeabilized chromaffin cells may determine whether granule movement is the ATP-dependent step of exocytosis.

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