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Minimal Requirements for Exocytosis

A STUDY USING PC 12 CELLS PERMEABILIZED WITH STAPHYLOCOCCAL α-TOXIN*

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Gudrun Ahnert-Hilger, Sucharit Bhakdi‡, and Manfred Gratzl

From the Abteilung Klinische Morphologie der Universität Ulm, Oberer Eselsberg, D-7900 Ulm, Federal Republic of Germany and the ‡Institut für Medizinische Mikrobiologie der Universität Giessen, Frankfurter Strasse 107, D-6300 Giessen, Federal Republic of Germany

The membrane-permeabilizing effects of streptolysin O, staphylococcal α-toxin, and digitonin on cultured rat pheochromocytoma cells were studied. All three agents perturbed the plasma membrane, causing release of intracellular $^{86}Rb^+$ and uptake of trypan blue. In addition, streptolysin O and digitonin also damaged the membranes of secretory vesicles, including a parallel release of dopamine. In contrast, the effects of α-toxin appeared to be strictly confined to the plasma membrane, and no dopamine release was observed with this agent. The exocytotic machinery, however, remained intact and could be triggered by subsequent introduction of micromolar concentrations of Ca^{2+} into the medium. Dopamine release was entirely Ca^{2+} specific and occurred independent of the presence or absence of other cations or anions including K^+ glutamate, K^+ acetate, or Na^+ chloride. Ca^{2+}-induced exocytosis did not require the presence of Mg^{2+}-ATP in the medium. The process was insensitive to pH alterations in the range pH 6.6–7.2, and appeared optimal at an osmolarity of 300 mosm/kg. Toxin permeabilization seems to be an excellent method for studying the minimal requirements for exocytosis.

The concept that Ca^{2+} plays a key role in exocytotic secretion processes has received direct support from microinjection studies (1–3) as well as from experiments using ionophores in conjunction with measurements of free intracellular Ca^{2+} concentrations (*cf.* 4). In order to elucidate further the requirements for secretion, a novel approach has been introduced that involves permeabilization of cell plasma membranes. For this purpose, secretory cells have been treated in various fashions with the ultimate objective of selectively permeabilizing their plasma membranes while retaining an intact exocytotic machinery. Methods previously used to achieve such selective permeabilization include application of high voltage discharge to chromaffin cells (5, 6), platelets (7), and pancreatic islet cells (8); use of detergents such as digitonin and saponin on chromaffin cells (9–12), mechanical disruption of the plasma membrane of sea urchin egg cells (13–15); and permeabilization of mast cells with Sendai virus (16).

Secretion by chromaffin cells has been our specific area of interest. It is clear that Ca^{2+} is essential for triggering the release of catecholamines, but requirement of other factors such as Mg^{2+}-ATP, and the effects of anions such as chloride appear controversial. Thus, an absolute requirement of Mg^{2+}-ATP was reported initially (5, 6), whereas only partial dependence was subsequently found by others (9–11). Clarification of this issue is important, since Mg^{2+}-ATP dependence could implicate the involvement of some phosphorylation steps in the control of exocytosis. Chloride was reported to be inhibitory towards the secretory process (6), but this contention was subsequently challenged (10). A further controversial issue relates to the role of calmodulin: a role for this component was proposed (6) and debated (10, 11).

Some of the discrepancies possibly derive from the different methods used to permeabilize the cells. Ideally, a permeabilizing agent should create permanent leaks across the cell membrane; "non-stabilized" pores such as are probably generated by high voltage discharge (5–8) or detergents (9–12) would probably be subject to some resealing and thus not fulfill this requirement. Moreover, detergents can only be applied in a narrow range of time and concentrations (9–12), and their use harbors the hazards of incurring direct damage to the secretory vesicles and/or of compromising other cellular functions. Indeed, the data obtained with detergents *versus* high voltage discharge appear to stand in fundamental conflict with each other, since digitonin and saponin at concentrations used for permeabilization (9–12) *inhibited* exocytosis in cells exposed to high voltage discharges (5).

On this background, we sought for an alternative procedure for inserting stable pores into the plasma membrane of secretory cells. Transmembrane protein channels as formed by certain bacterial exotoxins such as staphylococcal α-toxin and streptolysin O (17) appeared to be promising candidates in this respect. The native forms of the toxins assemble into supramolecular amphiphilic polymers in the target lipid bilayers to generate stable transmembrane pores. α-Toxin channels comprise a homogeneous population of ring-structured hexamers with an effective functional pore diameter of 2–3 nm (18). Streptolysin O channels are heterogeneous, with an approximately 10-fold larger diameter (19). In this study, we compare the membrane permeabilizing effects of digitonin, streptolysin O, and α-toxin on the pheochromocytoma cell line PC 12 (20). We demonstrate that α-toxin represents an attractive permeabilizing agent for the study of exocytosis in these cells.

EXPERIMENTAL PROCEDURES

Materials—Carbachol, human placenta collagen, and digitonin were obtained from Sigma, München, and verapamil, bovine serum albumin (BSA[1]), NADH, and sodium pyruvate from Serva, Heidel-

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[1] The abbreviations used are: BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BPS, buffered physiological saline; SLO, streptolysin O; DMEM, Dulbecco's modified Eagle's medium.

berg. DMEM dried medium, horse serum, and fetal calf serum were from Seromed Biochrom KG, Berlin. Culture dishes were purchased from Falcon, Becton Dickinson, Heidelberg. [^{3}H]Dopamine, 30 Ci/mmol was from New England Nuclear, and $^{86}Rb^+$, (1–8 mCi/mg) from Amersham Buchler, Braunschweig.

PC 12 Cultures—The rat pheochromocytoma cell line PC 12 was obtained through the kindness of H. Thoenen, Max Planck Institute for Psychiatry, Martinsried, Federal Republic of Germany. Cells were grown on plastic dishes in DMEM supplemented with 10% horse serum and 5% fetal calf serum in a humidified atmosphere of 10% CO_2. They were routinely subcultured every week. Experiments were performed with cultures from the 15th to the 40th passage. For experiments, cells were grown on multiwells coated with 25 μg/ml human placenta collagen (21) in order to optimize cell adherence.

[^{3}H]Dopamine Release—Cells cultured for 5–8 days were loaded with 0.25 μM [^{3}H]dopamine (11.5 Ci/mmol) corresponding to 0.3 μCi/well in serum-free DMEM stabilized with 1 mM ascorbic acid for 2 h at 37 °C. Cells were washed twice with prewarmed Ca^{2+}-free buffered physiological saline (BPS), containing (millimole/liter): NaCl, 130; K_2HPO_4, 1.2; $MgSO_4$, 2.5; glucose, 11; Pipes, 10; EGTA, 1; pH 7.2, or in buffered KG medium containing (millimole/liter): K^+ glutamate, 150; Pipes, 10; EGTA, 5; pH 7.2, adjusted with KOH. Overall [^{3}H] dopamine uptake was in the range of 10 and 20% corresponding to 25–50 pmol/mg cell protein.

In the release studies 200 μl of the indicated buffers were added and supernatant radioactivity measured after 20 min. Subsequently the cells attached to the plates were lysed in 300 μl of 0.2% sodium dodecyl sulfate and cell-associated protein and radioactivity determined. [^{3}H]Dopamine release was expressed as per cent of total radioactivity in a given sample.

Toxins—*Staphylococcus aureus* α-toxin was obtained by Sephacryl chromatography of a lyophilized α-toxin preparation from Behringwerke (Marburg) as described (18). The hemolytic activity (EC_{50}) against 2.5% rat erythrocytes in phosphate (50 mM)-buffered saline, pH 7.3, was in the range of 0.9–1.7 μg/ml as also reported in literature (22). For permeabilization of PC 12 cells, between 20 and 50 μg/ml α-toxin, usually dialyzed against buffered KG medium was used corresponding to a toxin total cell protein ratio of 0.1–0.3 (w/w). Streptolysin O (SLO) from group A β-hemolytic streptococci was purified as described (19). This toxin lysed PC 12 cells at concentrations between 0.1 and 1 μg/ml after activation with 4 mM dithiothreitol.

Ca^{2+} Determination—The exact free Ca^{2+} concentration in buffered KG medium was determined either with the aid of a Ca^{2+}-selective electrode (23), the necessary membranes were kindly provided by W. Simon, ETH Zürich, Switzerland, or calculated by means of a computer program as described (24), using the stability constants given (25).

Permeability of Plasma Membranes—Permeability of the cell membranes was assessed by trypan blue dye exclusion and by the release of either $^{86}Rb^+$ or lactate dehydrogenase. For determination of dye uptake cells were first incubated with the permeabilizing agents, and 0.2% trypan blue in BPS subsequently added. About 100 cells from each culture were counted. Experiments with $^{86}Rb^+$ were performed with BPS plus 0.2% BSA where K_2HPO_4 was replaced by Na_2HPO_4. Cells were loaded with 1.7 μCi of $^{86}Rb^+$ for 2 hours at 37 °C on plates. After suspension by gentle pipetting the cells were washed twice and the release was initiated by addition of the substances to be tested. 20 min later the incubation was stopped by centrifugation (10,000 × *g* for 2 min) and $^{86}Rb^+$ was estimated in the supernatant and in sodium dodecyl sulfate-lysed cell pellets. About 6% of the $^{86}Rb^+$ offered was present at the beginning of the release experiments. For assaying lactate dehydrogenase release, cells were treated and incubated as described for the dopamine release experiments (see above). Enzyme activity was determined in the supernatants and in distilled water lysates of the cells after 20 min as described (26). Protein was measured in 0.2% sodium dodecyl sulfate with BSA as standard (27).

RESULTS

Effects of Digitonin, SLO, and α-Toxin on Cell Membrane Permeability—The low molecular weight, charged trypan blue stains nuclei and intracellular proteins when it gains access to the cytoplasm. This dye was excluded by 95% of untreated PC 12 cells. Following 5-min exposure to α-toxin (10 μg/ml) or digitonin (12 μM), 24% and 41% of the cells were stained with the dye, respectively. After a further 40 min incubation, both agents caused dye uptake in 60–70% of the cells.

Release of intracellular $^{86}Rb^+$ was measured as a second parameter for plasma membrane permeabilization. Digitonin, SLO, and α-toxin all augmented release of this marker from pre-loaded cells in a dose-dependent manner that was entirely Ca^{2+} independent. Maximal release of approximately 90% after 20 min incubation was obtained with 1 μg/ml SLO, 10 μg/ml α-toxin, or 500 μM digitonin (Fig. 1).

In contrast to these uniform effects of the three agents on cell membrane permeability towards small molecules, a marked difference was discerned when lactate dehydrogenase release was measured. Digitonin (12 μM) and SLO (1 μg/ml) again both caused Ca^{2+}-independent release of this intracellular macromolecule (Fig. 2). However, no lactate dehydrogenase release was observed with α-toxin following a 20-min incubation despite the use of a high toxin concentration (40 μg/ml) (Fig. 2). Prolonged incubation resulted in gradual lactate dehydrogenase release, possibly due to cell swelling (not shown). These results collectively support the contention that SLO generates very large holes in the target membrane that permit egress of macromolecules from the cells (17, 19, 28, 29), whereas α-toxin channels are smaller in size and thus permit passage of ions and small molecules, but not of macromolecules across the plasma membrane (17, 18, 22, 30).

Effects of Toxins and Digitonin on Dopamine Release: Dependence on Anions, Cations, Glucose, and ATP—As reported by earlier investigators, nicotinic stimulation or depolarization induces dopamine release from the PC 12 cells in the presence of millimolar Ca^{2+} concentrations, whereas the sodium ionophore monensin causes a Ca^{2+}-independent release (Refs. 20, 31, 32, and Table I). In the presence of 2.5 mM Ca^{2+}, all three permeabilizing agents tested in this study elicited a dose-dependent dopamine release, with EC_{50} values of approximately 16 μg/ml for α-toxin, 12 μM for digitonin, and 0.1 μg/ml for SLO (Fig. 3). When the Ca^{2+} dependence of the processes was investigated, however, striking divergencies became apparent. Thus, virtually no Ca^{2+} dependence

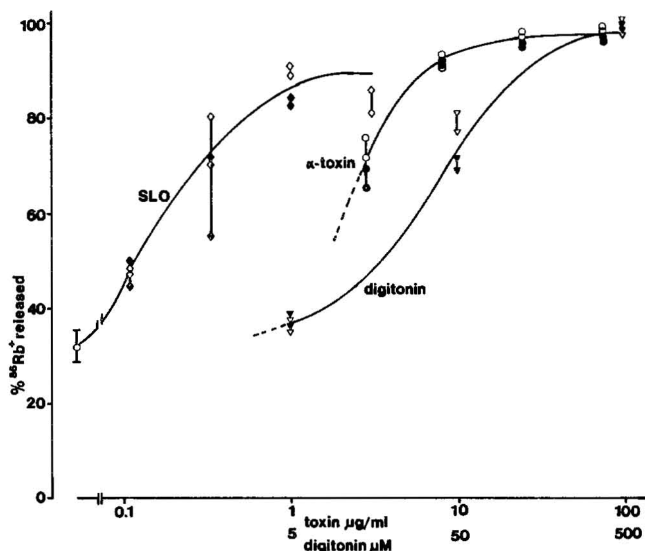

FIG. 1. **$^{86}Rb^+$-release by PC 12 cells induced by SLO, α-toxin, and digitonin.** Cells were treated with the given substances as detailed under "Experimental Procedures" in the presence (*filled symbols*) or absence (*open symbols*) of 2.5 mM Ca^{2+}. All three agents caused a dose-dependent and Ca^{2+}-independent release of $^{86}Rb^+$ from the cells. The amount of $^{86}Rb^+$ present at the beginning of the release experiment was set as 100%. The *abscissa* gives the final concentration of toxin in micrograms/milliliter or digitonin in micromolar.

was observed with SLO and digitonin, *i.e.* dopamine release appeared to occur as a nonspecific result of primary damage to the vesicle membranes induced by these substances (Fig. 4). In marked contrast, α-toxin-permeabilized cells released little or no dopamine in the absence of Ca^{2+}, but secretion could be triggered by the addition of micromolar amounts of Ca^{2+} to the medium (Fig. 4). Thus, no severe primary damage to the secretory vesicles was observed with this agent. The Ca^{2+}-dependent dopamine release in α-toxin-treated cells occurred to the same extent at pH 6.6 and 7.2.

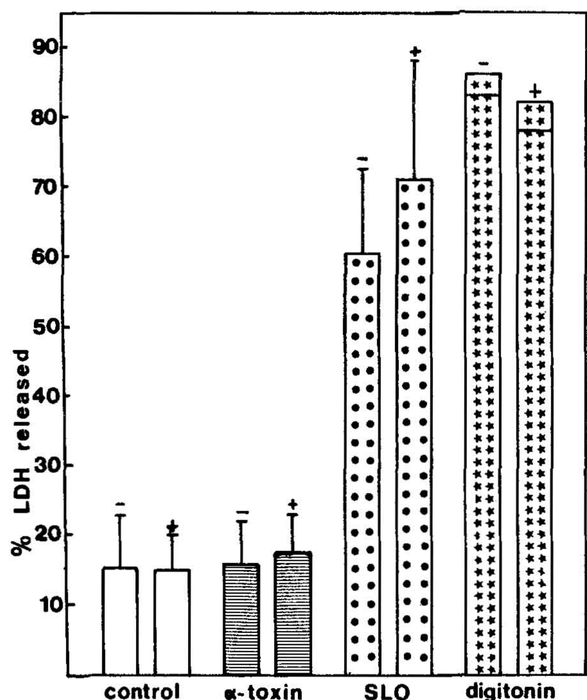

FIG. 2. **Lactate dehydrogenase (*LDH*) release from PC 12 cells.** Cells suspended for 60 min in serum-free DMEM were washed once with BPS and preincubated for 10 min in this buffer supplemented with 0.2% BSA. Lactate dehydrogenase release elicited in the presence (+) or absence (−) of 50 μM free Ca^{2+} by α-toxin (40 μg/ml), SLO (1 μg/ml), or digitonin (12 μM) was measured following a 20-min incubation period. SLO and digitonin, but not α-toxin caused release of lactate dehydrogenase from the cells. Values derive from duplicate or triplicate determinations.

Ions other than Ca^{2+} appeared to play no significant role. Thus, extracellular K^+ could be replaced by Na^+, and dopamine release also occurred to the same extent when glutamate was replaced by acetate or chloride (not shown). Ca^{2+}-dependent secretion was even observed when ions were totally replaced by sucrose, whereby release appeared maximal at 300 mM of this substance (not shown). The presence or absence of neither glucose (11 mM) nor Mg^{2+}-ATP (5 mM) influenced the Ca^{2+}-dependent dopamine release from α-toxin-permeabilized cells (not shown). In the presence of 5 mM Mg^{2+}-ATP the medium contained approximately 150 μM free Mg^{2+}. Since dopamine release was not observed under these conditions, we concluded that Mg^{2+} could not substitute for Ca^{2+}.

Concentrations of Free Ca^{2+} Required to Stimulate Dopamine Release in Toxin-permeabilized Cells—When dopamine release was determined as a function of the free Ca^{2+} concentration, two phases could be distinguished (Fig. 5). A first increase plateauing at 1–4 μM Ca^{2+} was followed by a second rise occurring at approximately 10 μM. The latter plateaued at approximately 20 μM Ca^{2+}. The first rise was half-maximal at the extremely low Ca^{2+} concentration of 0.5 ± 0.2 μM (n = 5, S.D.). Verapamil, 10 μM, which is considered a Ca-channel blocker, did not prevent the Ca^{2+}-induced catecholamine release in permeabilized cells, whereas the release elicited through K^+ depolarization of intact cells was effectively blocked (not shown). Thus, extracellular Ca^{2+} appeared to

TABLE I

Release of [³H]dopamine from PC 12 cells in response to various types of stimulation

Preloaded cells were washed with BPS and preincubated for 15 min in the same buffer supplemented with 0.2% BSA. The buffer was discarded and replaced by BPS or BPS minus EGTA plus 2.5 mM Ca^{2+} containing the substances indicated. After 10 min, [³H]dopamine release was determined in the supernatant. 10 mM Hepes buffer was used instead of Pipes.

Medium	% [³H]dopamine released
BPS	5.2; 5.8
BPS plus Ca^{2+}	4.5; 5.4
BPS, 54 mM K^+	5.7; 5.7
BPS, 54 mM K^+ plus Ca^{2+}	30.0; 29.0
BPS, 100 μM carbachol	5.0; 5.8
BPS plus Ca^{2+}, 100 μM carbachol	8.0; 7.8
BPS, 2 μM monensin	71.0; 65.7
BPS plus Ca^{2+}, 2 μM monensin	67.2; 67.9

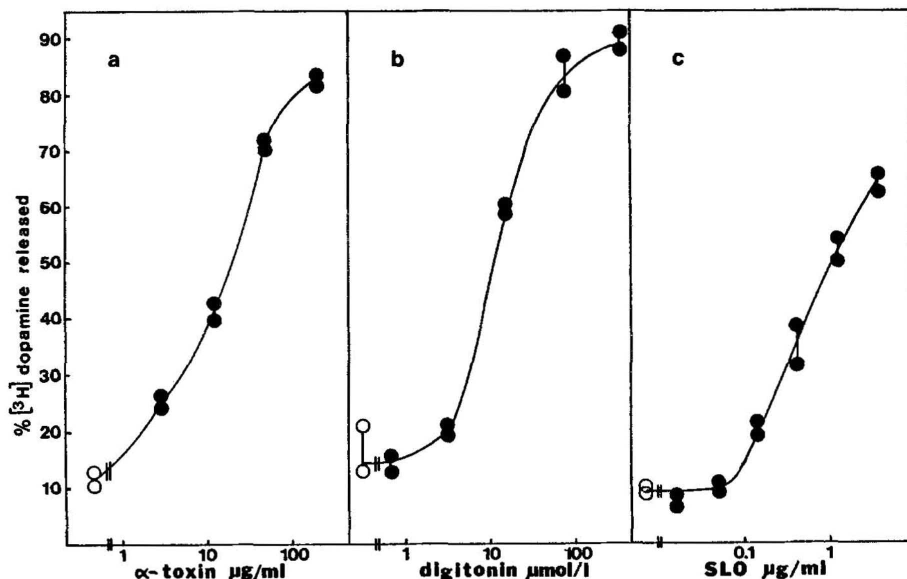

FIG. 3. **Effect of α-toxin (*a*), digitonin (*b*), and SLO (*c*) on [³H]dopamine release by PC 12 cells.** Cells were preloaded with [³H]dopamine as described under "Experimental Procedures." After washing with BPS they were preincubated 15 min at 37 °C in the same buffer supplemented with 0.2% BSA. The preincubation medium was discarded and replaced by BPS/2.5 mM Ca^{2+} with or without the permeabilizing substances at the given concentrations. [³H]Dopamine release was measured after a 20-min incubation period (n = 3). All three premeabilizing agents induced [³H]dopamine release in the presence of Ca^{2+}.

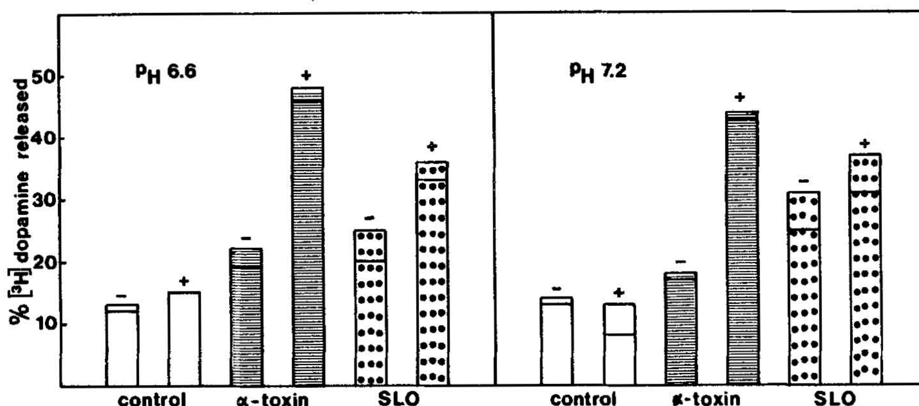

FIG. 4. **Effect of pH on Ca^{2+}-stimulated release of [^{3}H]dopamine by permeabilized cells.** [^{3}H]Dopamine preloaded cells were treated with toxins as described under "Experimental Procedures" and incubated for 20 min in KG medium titrated to pH 6.6 or 7.2 with KOH. The free Ca^{2+} concentrations, measured by the Ca^{2+}-sensitive electrode in pH 6.6 buffer were 22 μM and in pH 7.2 buffer 80 μM. Each *bar* represents two determinations indicated by the short lines: *open*, controls (no toxin added); *hatched*, α-toxin (40 μg/ml); *dotted*, SLO (1 μg/ml). No dependence of [^{3}H]dopamine release in permeabilized PC 12 cells on pH was observed.

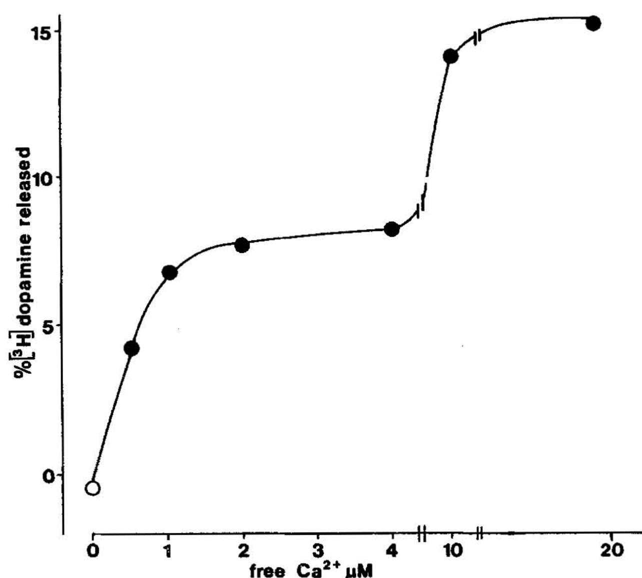

FIG. 5. **[^{3}H]Dopamine release as a function of free Ca^{2+} concentration in α-toxin permeabilized PC 12 cells.** Preloaded cells were washed twice in KG medium with 5 mM nitrilotriacetic acid, 0.5 mM EGTA instead of 5 mM EGTA. Incubation (20 min) was started by adding the same buffer supplemented with 0.2% BSA with or without α-toxin (37 μg/ml) and the free Ca^{2+} concentrations given at the *abscissa*. Release in the absence of toxin was substracted from release by toxin-treated cells.

penetrate to the cytoplasm via a nonphysiological route, probably represented by the toxin channels themselves.

DISCUSSION

Selective membrane permeabilization has recently been introduced for the study of the minimal requirements for exocytosis. High voltage discharges have been applied to cells in suspension, and the lifetime of the "holes" thus generated was reported to be sufficient to allow such investigations to be performed in chromaffin cells (5, 6). Another approach involves the use of detergents (9–12), which are, however, difficult to handle since no biochemical basis exists for a confinement of their effect to the plasma membrane. Indeed, we have found that digitonin although possibly useful in other cell systems (9–12) is not an appropriate agent for permeabilizing PC 12 pheochromocytoma cells. This substance induced lactate dehydrogenase leakage and a Ca^{2+}-independent release of dopamine; thus, perturbation of the plasma membrane appeared to be inseparably linked to parallel damage of the secretory vesicle membrane. The principle difficulties inherent in either of the above mentioned approaches are underlined by the controversial finding that exocytosis in electrically permeabilized chromaffin cells was actually inhibited by detergents (5).

Membrane permeabilization by channel-forming toxins represents a novel approach in this area. Conceptually, the method is related to Sendai virus permeabilization (16). Toxin permeabilization is, however, technically simpler and permeabilization is more stable, permitting investigations to be performed over longer time periods. Unsurprisingly, not all channel formers appear suited for these experiments. For example, plasma membrane damage by SLO was invariably coupled to unspecific catecholamine release, *i.e.* secretory vesicle membrane damage. In this respect, SLO-induced permeabilization showed similarities to that caused by detergents. This could be due to primary generation of very large holes in the plasma membrane that permitted passage of native toxin molecules to the cell cytoplasm. At the same time, these holes cause egress of the large intracellular marker enzyme lactate dehydrogenase. In striking contrast, the channels formed by α-toxin are apparently much smaller. In the erythrocyte model, α-toxin channels have been sized to 2–3 nm (18). They do not permit passage of myoglobin (M_r = 17,000) or Dextran 4 (mean M_r = 4,000) (18, 33). It is highly unlikely that native α-toxin molecules (M_r = 34,000) should be able to cross such channels and gain access to the vesicle membranes in PC 12 cells. That functional transmembrane channels are generated by α-toxin in these cells is apparent from the rapid and dose-dependent release of Rb^+; this release is not paralleled by lactate dehydrogenase liberation. Similar findings of selective markers release made in other model systems (23, 34, 35) support the notion that small channels are indeed formed by α-toxin in nucleated cells.

Thus α-toxin could represent an ideal membrane permeabilizer: its attack is strictly directed and confined to the plasma membrane, where it generates stable transmembrane channels of dimensions that would permit free passage of small molecules, but that totally restrict diffusion of macromolecules. Studies on the minimal requirements for exocytosis in this system have already brought forth some clear and partly surprising results. We found that Ca^{2+} was the only ion required to trigger secretion, and the Ca^{2+} concentrations eliciting catecholamine release were remarkably low. Thus, secretion was induced by 0.5 μM and reached maximal values at 20 μM Ca^{2+}. These values are to be compared with the Ca^{2+} levels required in previous permeabilization systems (5–12). The observed initiation of dopamine release at 0.5 μM Ca correlates remarkably well with the increase of intracellular Ca^{2+} from 0.1 to 0.5 μM measured in intact chromaffin and PC 12 cells during exocytosis, with quin 2 (4, 36). The reasons for augmentation of release at higher Ca^{2+} concentrations are

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speculative at present. It is worth mentioning, however, that quin 2 measurements yield average intracellular Ca^{2+} concentrations, and that the true concentrations just beneath the plasma membrane may be higher as a consequence of the Ca^{2+} influx during stimulation.

We observed dependence of exocytosis solely on extracellular Ca^{2+}, and no inhibitory effect of chloride ions in the present system. These findings thus support the data of Wilson and Kirshner (10). We also found no influence of K^+ as opposed to Na^+, or of variations in pH (from pH 6.6 to 7.2) on the Ca^{2+}-dependent secretion process. The latter results contrast to those of Knight and Baker (5, 6), who reported a marked pH dependence of catecholamine release in electrically permeabilized chromaffin cells. A slight dependence of secretion on the osmolarity in the medium was found in this study, and these results appear in accord with those of Knight and Baker (5, 6).

A final issue relates to the requirement for Mg^{2+}-ATP, which has been reported as absolute (5–9, 13, 15) or partial (10, 11) by previous investigators. Surprisingly, we were unable to demonstrate any requirement whatsoever in the present system. This finding obviously harbors important implications and is currently being investigated further. Until now we cannot exclude that permeabilized cells still retain enough ATP to sustain the energy requirements of the exocytotic machinery.

In sum, the use of α-toxin as a membrane permeabilizing agent appears to represent a novel and useful tool for studying the minimal requirements for exocytosis. The apparently sole requirements for low Ca^{2+} levels for secretion in PC 12 cells resembles the minimal requirements observed previously for fusion processes as studied with secretory vesicle membranes or with liposomes in the presence of adrenal medullary proteins (37, 38).

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