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Chapter 4

Poration by α-Toxin and Streptolysin O: An Approach to Analyze Intracellular Processes

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I. Introduction

Permeabilized Cells—Preparations between Intact Cells and Isolated Organelles

Stimulation of a cell from the outside leads to a chain of intracellular events that finally result in a physiological response. In intact cells the

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single steps of such a cascade, also termed signal transduction, are difficult to resolve.

Exocytosis is a process common to various cells including neurons, endocrine and exocrine cells, mast cells, leukocytes, and lymphocytes. During the final steps of exocytosis the secretory-vesicle membrane fuses with the plasma membrane, which allows the vesicular content to leave the cell. The investigation of the intracellular processes involved in the regulation of exocytosis is hampered by the bordering plasma membrane. However, selective permeabilization of this barrier has allowed complete control of the cell interior because the extracellular and intracellular spaces thereby become continuous.

Three different techniques—high-voltage discharge (Baker and Knight, 1978; Knight and Baker, 1982), the use of detergents such as saponin and digitonin (Brooks and Treml, 1983; Dunn and Holz, 1983; Wilson and Kirshner, 1983), and the application of pore-forming toxins such as α-toxin (Ahnert-Hilger *et al.*, 1985a,b; Bader *et al.*, 1986) and streptolysin O (Ahnert-Hilger *et al.*, 1985a, 1989a,b; Howell and Gomperts, 1987; Howell *et al.*, 1987)—have been inaugurated as valuable instruments for this purpose. Here we describe the purification and handling of α-toxin and streptolysin O (SLO) for the poration of cells. This contribution also deals with the subsequent use of the permeabilized secretory cells in the analysis of intracellular Ca^{2+} sequestration and release, as well as of regulation of exocytosis.

II. Purification and Analysis of Pore-Forming Toxins

Stable transmembrane pores can be formed by specialized proteins in the plasma membrane of target cells. Examples of pore-forming proteins are the C5b–9 complex of the complement (Bhakdi and Tranum-Jensen, 1984, 1987), the cytolysin of cytotoxic T lymphocytes (Podak and Konigsberg, 1984; Henkart *et al.*, 1984; Masson and Tschopp, 1985), or various exotoxins produced by several strains of *Staphylococcus* and *Streptococcus* (for review, see Bhakdi and Tranum-Jensen, 1987; Thelestam and Blomquist, 1988). Two of the latter group, α-toxin from *Staphylococcus aureus* and SLO from β-hemolytic streptococci, have hitherto been used to permeabilize the plasma membrane selectively in order to control the composition of the cytosol.

A. α-Toxin

α-Toxin from *Staphylococcus aureus* (strain Wood 46, ATCC 10832, DSM 20491, kindly provided by S. Bhakdi, Giessen, Federal Republic of Germany) can be purified from the culture supernatant as described by Lind *et al.* (1987). After 18 hours of bacterial growth the soluble proteins of the culture medium are precipitated by the addition of 75% ammonium sulfate. The collected precipitate can be stored at −20°C for months without loss of activity. After dialysis against sodium acetate (10 mmol/liter, pH 5, containing 20 mmol/liter NaCl), the soluble material is subjected to cation exchange chromatography (Mono S column HR 5/5, Pharmacia, Freiburg, FRG). α-Toxin elutes with 170 m*M* NaCl in the column buffer. Then, it is further purified by gel filtration (Superose 12-HR, Pharmacia). Cation exchange chromatography can also be carried out using less expensive S-Sepharose. Figure 1 shows SDS–PAGE of the culture supernatant and the toxin preparations obtained after cation exchange chromatography and gel filtration. Besides the rapid procedure using the FPLC technique described by Lind *et al.* (1987), α-toxin produced in various other ways (for review, see Möllby, 1983; Füssle *et al.*, 1981) yields preparations of similar specific activity [20,000–40,000 hemolytic units (HU)/mg of protein].

The purified toxin, dialyzed against the permeabilization buffer (see Sections IV, A and B), can be lyophilized and stored at −20°C for months. Toxin solutions can also be stored at 4°C for 2–3 weeks without loss of toxicity. Commercially available α-toxin preparations can be obtained from Calbiochem (Frankfurt) and from the Institut Pasteur (Paris). They appear to be less active and contain additional high and low molecular weight protein bands. Because staphylococci produce several different toxins, it is not clear which of them contributes to the lytic activity of these preparations (Wadström, 1983; Möllby, 1983). However, these toxin preparations of relatively low titer can still be used for cell permeabilizations provided they are concentrated and further purified by two successive ammonium sulfate precipitations (55% first and 65% second), followed by dialysis against permeabilization buffer (Schrezenmeier *et al.*, 1988a,b).

B. Streptolysin O

Highly purified SLO was isolated from culture supernatants of group A β-hemolytic streptococci by ammonium sulfate and polyethylene glycol precipitation, DEAE–ion exchange chromatography, preparative isoelec-

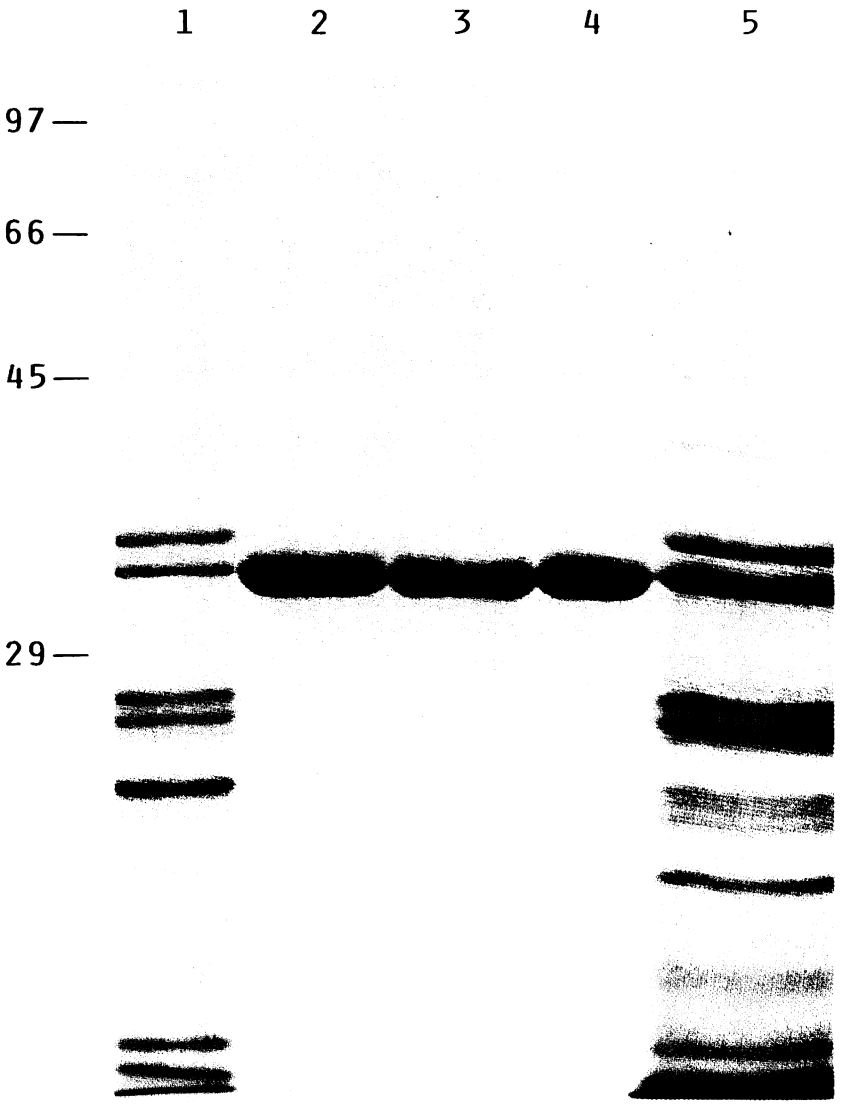

FIG. 1. Control of α-toxin purification by SDS–PAGE. The samples were separated in 12% acrylamide gel and stained with Coomassie blue. The indicated molecular mass values (kDa) correspond to the following standards run in parallel: carbonic anhydrase, 29; ovalbumin, 45; albumin, 66; and phosphorylase *b*, 97. Lanes 1 (1.5 μg) and 5 (15 μg) represent culture supernatant. Lanes 2 and 3 are peak fractions (1.5 μg) eluted by cation exchange chromatography (Mono S). Lane 4 is peak fraction (1.5 μg) after gel chromatography (Superose), which removes low molecular weight compounds. From Lind *et al.* (1987), by permission.

tric focusing, and chromatography on Sephacryl S-300 (Bhakdi *et al.*, 1984b). The excellent final product (kindly provided for the investigations in the authors' laboratory by S. Bhakdi, Giessen), using the hemolytic assay described in the following, exhibited ~250,000 HU/mg of protein. Also commercially available SLO preparations have been used for permeabilization of secretory cells. Streptolysin O from the Institute Pasteur is mainly prepared for diagnostic purposes. This product (1 "titrage") contains ~40 HU in the rabbit erythrocyte assay described in Section II,C. The low activity of this preparation can be increased by precipitation with ammonium sulfate (75%). Then it can be stored at 4°C for months and used, for example, for the permeabilization of adrenal medullary chromaffin cells in primary culture (Sontag *et al.*, 1988; Ahnert-Hilger *et al.*, 1989a,b). The toxin must be activated with 4 mmol/liter dithiothreitol (DTT) (Bhakdi *et al.*, 1984b). Another SLO preparation obtained from Sigma (St. Louis, MO), when tested as described in the following, contained ~800 HU per batch. Streptolysin O as a partially purified culture filtrate, obtained from Wellcome Diagnostics (Dartford, United Kingdom), has been used for permeabilization of rat mast cells (Howell and Gomperts, 1987; Howell *et al.*, 1987).

C. Determination of Toxin Activity

During purification and storage the toxicity of α-toxin and SLO is checked by determining the hemolytic titer. Because rabbit erythrocytes compared to that of human or bovine origin are very sensitive against both toxins, they are used routinely (see Füssel *et al.*, 1982; Möllby, 1983; Bhakdi *et al.*, 1984a,b; Bhakdi and Tranum-Jensen, 1987). For SLO, erythrocytes from other species can also be taken (Bhakdi and Tranum-Jensen, 1987).

To avoid clotting, fresh rabbit blood is immediately mixed with 4% sodium citrate. After three washes with 50 mmol/liter phosphate-buffered saline (PBS), pH 7.0, the erythrocytes are diluted 1 : 40 in the same buffer. This erythrocyte suspension (2.5%) can be used for 3–4 days when stored at 4°C. Dilutions of toxins are performed in PBS containing 0.1 bovine serum albumin (BSA) and in tests for SLO with an additional 2 mmol/liter DTT present. The latter is necessary to reduce the SH groups of SLO thereby activating the toxin (Bhakdi *et al.*, 1984b; Bhakdi and Tranum-Jensen, 1987). Then, 5μl of the appropriate toxin dilution are mixed with 50 μl of the erythrocyte suspension. Hemolysis is monitored after 40 minutes of incubation at 37°C. The samples are briefly mixed followed by centrifugation (2 minutes at 12,000 *g*). Released hemoglobin is determined spectrophotometrically at 412 nm after addition of 1 ml distilled water to

30 μl of the supernatant. Total hemolysis is determined after addition of SDS (0.2. w/v final), which gives an extinction of ~1.2. The dilution of toxin hemolyzing 50% of the erythrocytes is determined, and the reciprocal of the value obtained is taken as the number of HU per milliliter of the undiluted toxin solution (Lind *et al.*, 1987). The procedure described earlier results generally in lower values for the hemolytic activity compared to an assay in which the onset of hemolysis is determined with 2% erythrocytes (Füssle *et al.*, 1981).

III. Application for Cell Poration

α-Toxin permeabilizes cells for low molecular weight substances, whereas SLO permeabilizes cells for both high and low molecular weight substances (Table I). The properties of the SLO-permeabilized preparations, in this respect, are very similar to the cells treated with detergents such as digitonin (Table I). Besides the procedures listed in Table I, which are frequently used in the authors' laboratory, other methods including the measurement of release of amino acids from cells (Thelestam and Möllby, 1979) are also of great value.

A. Determination of Permeability for Low Molecular Weight Substances

Increased permeability of a cell can be rapidly checked by the use of membrane-impermeable dyes, which stain either various components of the cell body or the nucleus (Wilson and Kirshner, 1983; Ahnert-Hilger *et*

TABLE I

PERMEABILITY OF CELLS AFTER TREATMENT WITH α-TOXIN, STREPTOLYSIN O, AND DIGITONIN

	α-Toxin	Streptolysin O	Digitonin
Ions ($^{86}Rb^+$, ATP^{4-}, Ca^{2+}) and $^{51}CrO_4^{2-}$-labeled material	+	+	+
Dyes (azur A, trypan blue, eosin)	+	+	+
Proteins (lactate dehydrogenase, Immunoglobulins, tetanus toxin)	−	+	+

al., 1985a). Intact cells exclude the dye and are therefore not stained. The cationic phenothiazine dye azur A stains mainly the nucleus of cells provided the plasma membrane has been previously permeabilized. The cell interior of permeabilized cells can also be stained by trypan blue or eosin. Permeabilized cells and the dye solution, both in an isotonic medium containing potassium or sodium as a main cation (see media described in Sections IV, A and B), are mixed to yield a final dye concentration of ~0.2%. Because prolonged incubation inevitably leads to staining of intact cells, the percentage of stained cells must be immediately determined in a Neubauer cell-counting chamber.

Release of enzymatically loaded $^{86}Rb^+$ is a sensitive indicator for increased permeability of the plasma membrane after treatment with cytolysins. For this assay the cells ($\sim 5 \times 10^5$) are first loaded with $4–8 \times 10^{-4}$ Bq $^{86}Rb^+$ for 2 hours at 37°C in 2 ml of a physiological salt solution containing (in millimoles per liter): 150 NaCl, 1.2 Na_2HPO_4, 1 $CaCl_2$, 2.5 $MgSO_4$, 11 glucose, 10 PIPES, pH 7.2, and 0.2% BSA on culture plates of 60 mm diameter. After suspending the cells by gentle pipetting and washing, release is initiated by the addition of the permeabilizing agent to be tested. After 20 minutes the cells are centrifuged (10,000 *g* for 2 minutes) and $^{86}Rb^+$ is estimated in the supernatant as well as in the cell pellets lysed with SDS. About 6% of the $^{86}Rb^+$ provided is present within the cells at the beginning of the release experiment. This protocol was used to analyze the effects of α-toxin on PC12 and RINA2 cells (Ahnert-Hilger *et al.*, 1985a; Lind *et al.*, 1987). In a similar experimental design, $^{86}Rb^+$ release can be performed directly on the culture plates as done with adrenal medullary chromaffin cells in primary culture (Bader *et al.*, 1986). An example of $^{86}Rb^+$ release from SLO-treated PC12 cells is shown in Fig. 2.

The release of intracellular ATP from the cell is also a sensitive indicator for the permeability of the plasma membrane. The ATP released can be easily measured using the firefly assay obtained from Boehringer, Mannheim (FRG). Permeabilization with cytolysins is performed in an "intracellular" buffer [KG buffer, containing (in millimoles per liter): 150 K^+-glutamate, 0.5 EGTA, 5 NTA, 10 PIPES, pH 7.2]. The released ATP was determined in the supernatant (after centrifugation for 2 minutes at 12,000 *g*) as well as in the cell extract [performed in assay medium (in millimoles per liter): 10 Mg^{2+}-acetate, 1.5 EGTA, 50 Tris, pH 7.8] after heating to 95°C for 5 minutes (Lind *et al.*, 1987). Luciferase and its substrate luciferin were dissolved in the assay buffer as outlined in the manual of the firefly assay kit. α-Toxin (like digitonin) causes release of the ATP dose dependently from PC12 (Fig. 3) and RINA2 cells (Lind *et al.*, 1987). Similar effects have been observed with SLO (Fig. 4; the effect

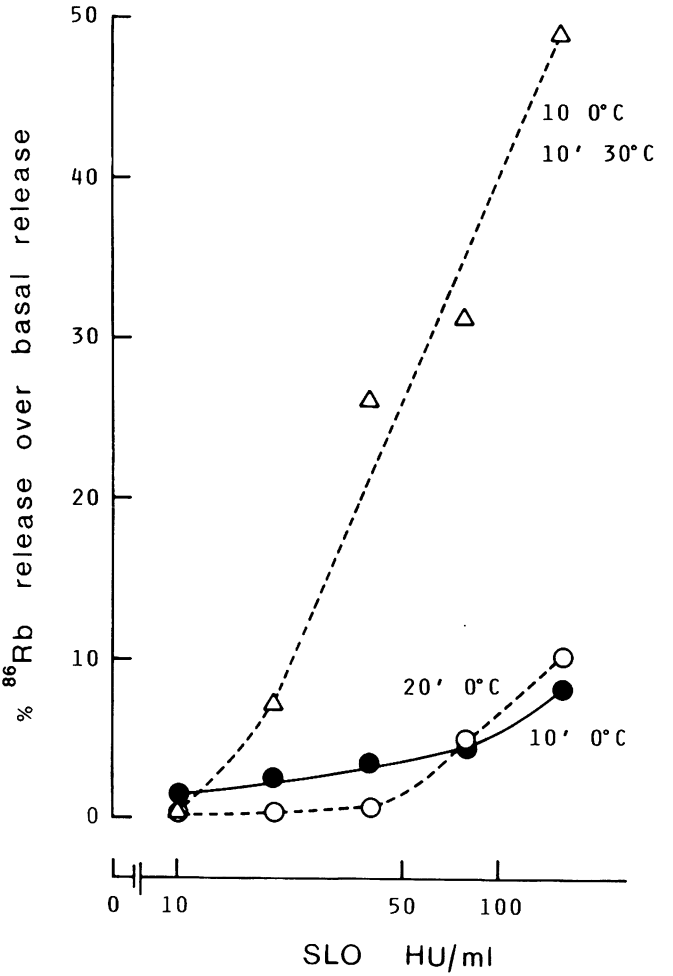

FIG. 2. $^{86}Rb^+$ release from RINA2 cells by SLO. RINA2 cells grown on polylysin (10 μg/ml)-coated plates were loaded with $^{86}Rb^+$ at 37°C for 1 hour. After three washes in the loading buffer, the cells were first incubated for 10 minutes at 0°C with different SLO concentrations and the released radioactivity determined (●). Further incubation for 10 minutes at 0°C did not increase $^{86}Rb^+$ release (○). By contrast, incubation at 37°C (△) resulted in an increased release of $^{86}Rb^+$ as a function of the SLO concentration. Basal release (2%/minute) was subtracted. The experiment clearly shows that, after binding at 0°C, pore formation with SLO can be induced by elevation of the temperature to 37°C. Similar results were obtained with PC12 cells (Ahnert-Hilger *et al.*, 1989b).

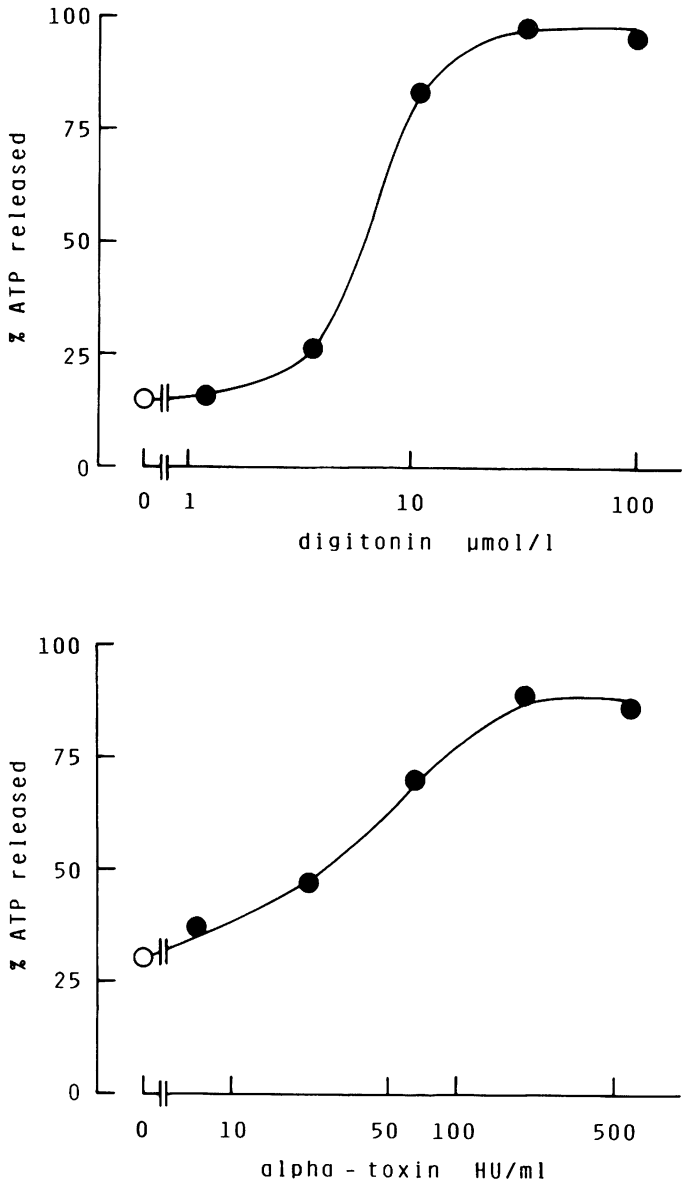

FIG. 3. Release of ATP from PC12 cells treated with α-toxin or digitonin. PC12 cells were washed and suspended in a buffer containing (in millimoles per liter): 150 NaCl, 5 KCl, 10 glucose, 10 HEPES, pH 7.4, supplemented with 0.1% BSA. After 30 minutes at 30°C the buffer was exchanged for KG buffer (see Fig. 8) containing BSA and glucose. About 2×10^6 cells were incubated at 30°C for 20 minutes (digitonin) or 30 minutes (α-toxin) with the given concentrations of the permeabilizing agent. ATP was determined in the supernatant and the lysate of the cells (see Section III, A) using the firefly assay (Lind *et al.*, 1987). Values are the mean of duplicates expressed as percentage of total (~0.5 nmol ATP per sample) present at the beginning of the experiment.

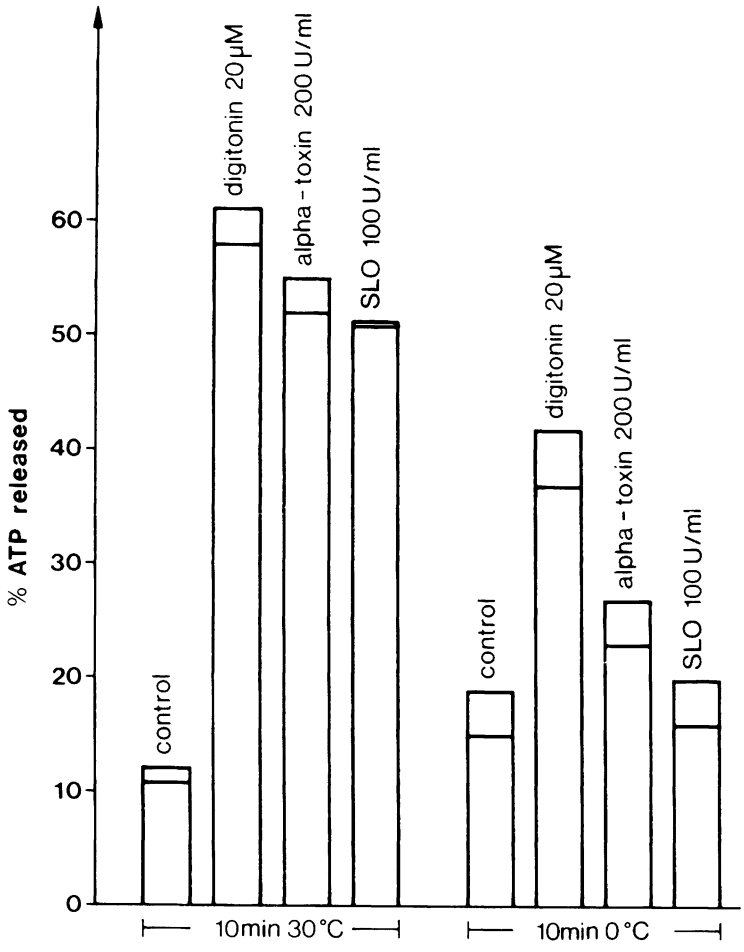

FIG. 4. Effect of temperature on ATP release from SLO-, α-toxin-, and digitonin-treated PC12 cells. PC12 cells were incubated as described in the legend to Fig. 3. After suspending them in KG buffer, the cells were incubated with the indicated compounds either at 0°C or at 30°C for 10 minutes. After centrifugation, ATP was measured in the supernatant and in the extract of the cells (see also Fig. 3).

of temperature on the toxins' attack is described in Section III, C). Thus the observations described here indicate that even the small pores of α-toxin are large enough for the free passage of nucleotides (Ahnert-Hilger and Gratzl, 1987; Lind *et al.*, 1987).

Living cells can be easily labeled with $^{51}CrO_4^{2-}$, which binds to cytoplasmic material, of which 90% have a molecular weight <4000

(Martz, 1976). Release of $^{51}CrO_4^{2-}$-labeled material indicates an increased permeability of cells such as cytotoxic T lymphocytes when treated with α-toxin or other cytolysins (Martz, 1976; Schrezenmeier *et al.*, 1988a,b). Because the ^{51}Cr-labeled material is not uniform, the exact data on the size of the pore generated by a particular cytolysin cannot be determined. Nevertheless, labeling with $^{51}CrO_4^{2-}$ is suitable to determine rapidly the effects of temperature, toxin concentration, and other parameters for optimal poration.

B. Determination of Permeability for High Molecular Weight Substances

α-Toxin, SLO, and detergents allow the passage of small molecules through the plasma membrane. The release of cytoplasmic enzymes or the introduction of antibodies directed against intracellular proteins after application of a permeabilizing agent would indicate that the pores were large enough for the free passage of proteins.

The release of cytoplasmic lactate dehydrogenase (LDH) (MW 135,000) has been demonstrated for digitonin-permeabilized adrenal medullary chromaffin cells (Dunn and Holz, 1983; Wilson and Kirshner, 1983), for SLO-permeabilized mast cells (Howell and Gomperts, 1987; Howell *et al.*, 1987), and for SLO-permeabilized PC12 cells (Ahnert-Hilger *et al.*, 1985a). This observation is consistent with data using erythrocytes, which indicate that SLO produces large transmembrane pores in the target cell (Bhakdi *et al.*, 1985; Hugo *et al.*, 1986; Bhakdi and Tranum-Jensen, 1987). On the other hand, the absence of an increased LDH release and other cytosolic proteins by α-toxin-permeabilized cells indicates the small size of the pores generated (Ahnert-Hilger *et al.*, 1985a; Bader *et al.*, 1986; Grant *et al.*, 1987; Bhakdi and Tranum-Jensen, 1987; Thelestam and Blomquist, 1988; Schrezenmeier *et al.*, 1988a). Lactate dehydrogenase was determined in the supernatant of SLO- or α-toxin-treated cells in permeabilization buffer (see Section IV, B) and in the hypotonic lysate of the cells (Ahnert-Hilger *et al.*, 1985a), using a modification (Gratzl *et al.*, 1981) of the procedure described by Kornberg (1955). Whereas α-toxin-treated cells exhibited no increased LDH release during 40 minutes of incubation (Ahnert-Hilger *et al.*, 1985a; Schrezenmeier *et al.*, 1988a), SLO induces immediate release of this cytoplasmic marker (Ahnert-Hilger *et al.*, 1985; Howell and Gomperts, 1987).

Because LDH is able to leave SLO-permeabilized cells, immunoglobulins (with roughly the same molecular weight) should be able to enter them. Indeed, SLO-treated PC12 cells accumulate antibodies directed against calmodulin as well as synaptophysin/p38 (Ahnert-Hilger *et al.*,

1989b), an integral membrane protein of small clear vesicles and secretory vesicles in neuroendocrine cells (cf. Rehm *et al.*, 1986; Navone *et al.*, 1987; Schilling and Gratzl, 1988). As shown in Fig. 5, cells permeabilized with SLO can also be stained with anticalmodulin. Digitonin also provides access for the antibody directed against calmodulin (Fig. 5), actin, or chromaffin vesicle constituents (Schäfer *et al.*, 1987). Thus the large pores generated by SLO (or digitonin) are useful to investigate the intracellular action of high molecular weight neurotoxins such as tetanus toxin or botulinum A toxin with yet-unidentified intracellular targets (Habermann and Dreyer, 1986; Ahnert-Hilger *et al.*, 1989a,b; Stecher *et al*, 1989).

C. Selective Permeabilization of the Plasma Membrane

Permeabilization with α-toxin is limited to the plasma membrane because the pores formed (diameter ~1–2 nm) are too small for the free passage of the α-toxin monomer (34 kKa) into the cell (Bhakdi *et al.*, 1981; Füssle *et al.*, 1981; Bhakdi and Tranum-Jensen, 1987). In contrast, the lesions generated by digitonin, saponin, or SLO are certainly large enough to allow access of unbound detergent molecules or of SLO monomers to the cytoplasmic space. These pores would also be highly desirable for the introduction of antibodies or other big molecules into secretory cells.

In order to limit the effects of SLO to the plasma membrane without affecting intracellular structures, an improved protocol was developed for the permeabilization of endocrine cells, which is based on previous experience obtained in the erythrocyte model (Hugo *et al.*, 1986). Incubation of cells with SLO at 0°C results only in toxin binding to the cell surface but not in pore formation. However, permeabilization occurs rapidly upon additional incubation at 30°C, as indicated by the $^{86}Rb^{+}$ release with RINA2 cells (Fig. 2) or the ATP release by PC12 cells (Fig. 4). α-Toxin can also be bound to the cells in the cold and poration of the cells triggered by warming (Fig. 4). By contrast, membrane permeabilization by digitonin is insensitive to the incubation and thus cannot be

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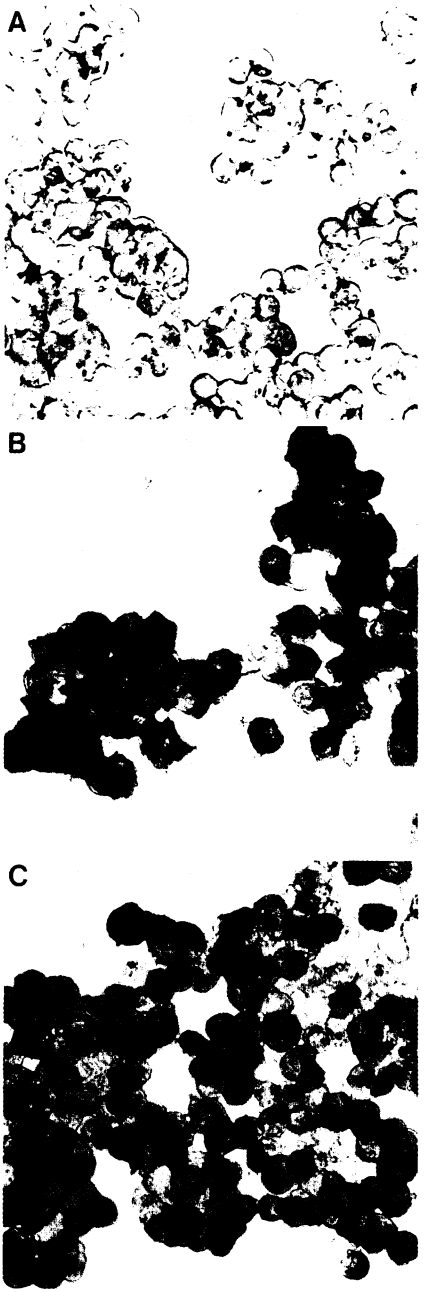
FIG. 5. Access of immunoglobulins to the interior of PC12 cells permeabilized by either digitonin or SLO. PC12 cells were washed twice with KG buffer and then incubated for 10 minutes at 30°C in the same buffer without (A) or with 60 HU/ml SLO (B) or 20 μmol/liter digitonin (C). The incubation was stopped by fixation of the cells with a 4% paraformaldehyde. Immunocytochemistry was performed with a calmodulin antibody (final dilution 1 : 200) using the PAP technique. The intracellular antigen is only accessible to the antibody in the permeabilized cells (Ahnert-Hilger *et al.*, 1989b).


A
B
C

controlled by using different temperatures for binding and permeabilization (Ahnert-Hilger *et al.*, 1989b).

IV. Pore-Forming Toxins as Tools in the Study of Intracellular Processes

α-Toxin and SLO are powerful instruments for the poration of various cells and have been applied to the analysis of intracellular Ca^{2+} movements and the regulation of exocytosis by endocrine cells. Depending on whether low or high molecular weight compounds are to be introduced into the cells, the type of toxin required can be selected.

A. Intracellular Ca^{2+} Sequestration in Permeabilized Endocrine Cells

The intracellular free-Ca^{2+} concentration is regulated by a number of Ca^{2+}-transport systems present in the plasma membrane, mitochondria, secretory vesicles, and the endoplasmic reticulum. Permeabilized cells are suitable for investigation of intracellular systems participating in the regulation of intracellular free-Ca^{2+} concentrations. Indeed, permeabilized cells have been instrumental in the analysis of the "IP_3-sensitive pool" (Streb *et al.*, 1983; for review, see Berridge, 1987). Using a Ca^{2+}-selective electrode, dynamic changes in intracellular Ca^{2+} sequestration can be registered in a suspension of α-toxin-permeabilized cells. Highly reliable measurements of the free-Ca^{2+} concentration in the micromolar range have been made possible since the development of a suitable carrier (Simon *et al.*, 1978). A further improvement of this technique is due to a new carrier (ETH 129, kindly provided by W. Simon, ETH Zürich), which is characterized by a high specifity of Ca^{2+} over Mg^{2+} and H^+, and allows one to measure free-Ca^{2+} concentrations as low as 10^{-9} *M* (Ammann *et al.*, 1987). A Ca^{2+} electrode can be obtained from Glasmanufaktur Möller, Zürich.

In order to measure the Ca^{2+} fluxes, cells are first permeabilized with α-toxin in a medium containing (in millimoles per liter): 150 KCl, 5 NaN_3, 1 EGTA, 20 MOPS, pH 7.2. After several washings with the same buffer without EGTA, the ATP-dependent Ca^{2+} uptake into the endoplasmic reticulum can be followed with the electrode. Azide in these experiments blocks mitochondrial Ca^{2+} uptake. Increasing amounts of IP_3 can release the stored Ca^{2+} (Streb *et al.*, 1983, cited for Berridge, 1987). Figure 6 gives an example of an ATP-driven Ca^{2+} uptake followed by an IP_3-induced Ca^{2+} release by both α-toxin-permeabilized PC12 and RINA2

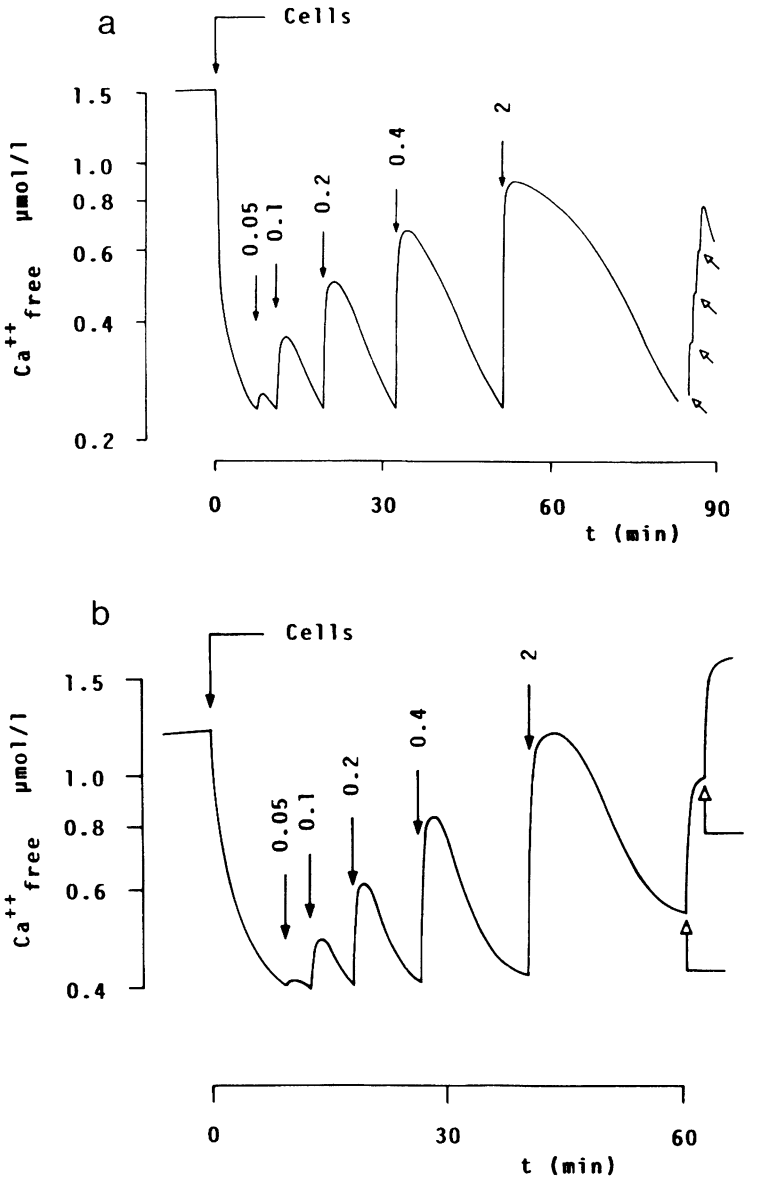


FIG. 6. Analysis of IP_3-induced Ca^{2+} release from α-toxin-permeabilized RINA2 (a) and PC12 (b) cells. Permeabilization was carried out with 300 HU α-toxin per 10^7 cells (10 minutes on ice, 30 minutes at 30°C) in a medium consisting of (in millimoles per liter) 150 KCl, 1 EGTA, 20 MOPS, pH 7.2. For measurement of Ca^{2+} fluxes, cells were incubated in the same medium containing no EGTA but 2 mmol/liter Mg^{2+}/ATP. The arrows indicate the addition of increasing amounts of IP_3 (between 0.05 and 2 nmol) corresponding to final concentrations of 0.125 μmol/liter and 5 μmol/liter, respectively. The calibration of the Ca^{2+} release was carried out by the sequential addition of 1 nmol Ca^{2+} (open arrows) at the end of the experiment.

cells. In these cell preparations Ca^{2+} flux measurements can be conducted over hours, indicating the high stability of this preparation. The rapid onset of the effects of ATP and IP_3 on the ambient free-Ca^{2+} concentration is a further indicator for the effective permeabilization of the cells by α-toxin.

In a similar experimental design, intracellular Ca^{2+} stores can be labeled with $^{45}Ca^{2+}$. These experiments were carried out with cells attached to a culture plate. Figure 7 shows the time course of ATP-dependent $^{45}Ca^{2+}$ uptake by SLO-permeabilized PC12 cells. Following loading of the ATP-dependent subcellular compartment, release experiments can be carried out under a variety of conditions.

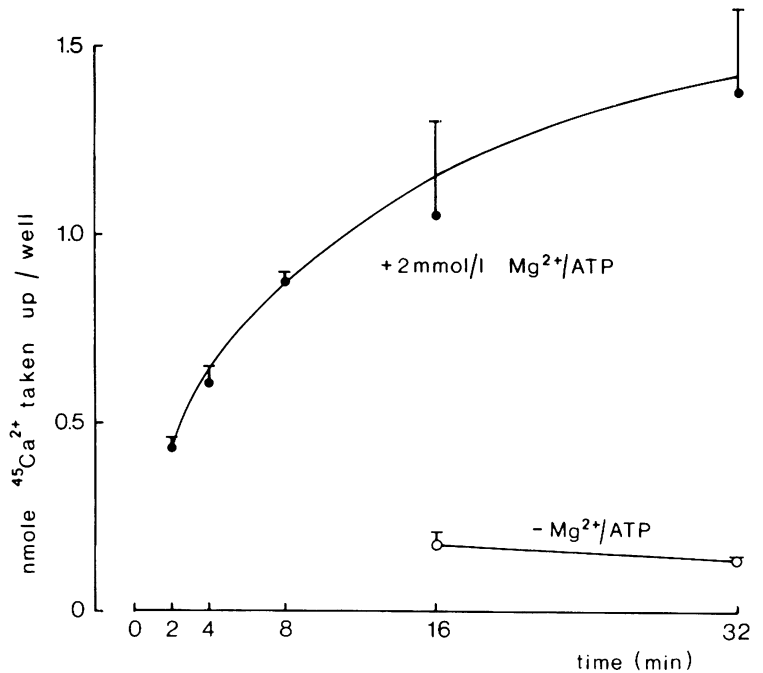


FIG. 7. ATP-dependent $^{45}Ca^{2+}$ uptake by SLO-permeabilized PC12 cells. PC12 cells were cultivated on polylysin (10 μg/ml)-coated multiwell plates ($\sim 2 \times 10^5$ per well). The cells were washed twice in a medium containing (in millimoles per liter): 150 NaCl, 1 EGTA, 10 PIPES, pH 7.2 and then in a medium containing (in millimoles per liter 150 KCl, 20 PIPES, pH 7.2. Incubation with SLO (60 HU/ml) was performed for 10 minutes at 0°C in the same buffer supplemented with 0.1% BSA and 1 mmol/liter DTT. This medium was removed and replaced by 200 μl fresh medium containing 14 nmol $^{45}Ca^{2+}$ ± 2 mmol/liter Mg^{2+}/ATP. The amount of radioactivity taken up by the cells was determined in the SDS lysate of the cells as well as in the supernatant.

B. Exocytosis by Permeabilized Secretory Cells

Toxin-permeabilized cells have been used to study the molecular requirements for exocytosis (Ahnert-Hilger *et al.*, 1985a,b, 1989a,b; Bader *et al.*, 1986; Grant *et al.*, 1987; Ahnert-Hilger and Gratzl, 1987, 1988; Howell and Gomperts, 1987; Howell *et al.*, 1987; Schrezenmeier *et al.*, 1988a,b; Sontag *et al.*, 1988). During this type of secretion the fusion of the secretory-vesicle membrane with the plasma membrane is a crucial event. Because poration of the latter with α-toxin or SLO does not affect this process, an extensive investigation of exocytosis by various secretory cells has been made possible.

In most of the studies dealing with exocytosis by permeabilized cells an "intracellular buffer" system was used, containing potassium as a main cation and glutamate as an anion. Glutamate was chosen (Baker and Knight, 1978) because of its impermeability to the chromaffin secretory-vesicle membranes (Phillips, 1977). Because the free-Ca^{2+} concentrations under resting conditions as well as during stimulation within the cells are in the micromolar range, this ion must be carefully controlled in the media used. A combination of EGTA and NTA is suitable to buffer the free-Ca^{2+} concentration between 0.1 and 100 μmol/liter. Thus a typical buffer for permeabilization contains (in millimoles per liter): 150 potassium glutamate, 0.5 EGTA, 5 NTA, 10 PIPES, pH 7.2. Added Mg^{2+} and ATP as well as the pH of the medium must be also considered, because they influence the equilibrium between Ca^{2+} and the chelators present. The free Ca^{2+} and Mg^{2+} concentrations are calculated by means of a computer program (Flodgaard and Fleron, 1974) kindly provided by T. Saermark, University of Copenhagen, using the stability constants given by Sillen and Martell (1971). Each Ca^{2+} buffer is prepared separately from stock solutions with a final check of pH and pCa by the Ca^{2+}-selective electrode (see Section IV,A). Solutions free of ATP can be stored at −20°C, whereas solutions containing ATP must be prepared freshly prior to the experiment.

Adrenal medullary chromaffin cells or pheochromocytoma cells from rat (PC12; Greene and Tischler, 1982) take up labeled catecholamines, store them in vesicles, and release them upon stimulation. Thus the assay for exocytosis in permeabilized chromaffin cells includes the loading of the cells with [^{3}H]dopamine (PC12) or [^{3}H]norepinephrine (adrenal medullary chromaffin cells), washing, and permeabilization. Stimulation is carried out with micromolar concentrations of Ca^{2+}, which then triggers the release of the stored labeled catecholamines. Table II summarizes the procedure for either PC12 cells or adrenal medullary chromaffin cells

TABLE II

ASSAY FOR EXOCYTOSIS

Release of catecholamines by permeabilized PC12 cells (chromaffin cells in primary culture)

1. Loading of cells with tritium labeled dopamine (norepinephrine) for 1–2 hours
2. Washing with Ca^{2+} free balanced salt solutions several times
3. Suspension of the cells in permeabilization buffer = KG-buffer containing (in millimoles per liter): 150 K^+-glutamate, 10 PIPES, 5 NTA, 0.5 EGTA, pH 7.2, and 0.1% BSA (+2 mmol/l Mg/ATP and 1 mmol/l free Mg^{2+}).
4. Treatment with pore-forming toxins: α-toxin: 30 min at 30°C or 37°C SLO: 5 min at 0°C (1 or 2 min at 30°C or 37°C)
5. Centrifugation at low speed and removal of supernatant (if done on plates, removal of supernatant)
6. Stimulation with micromolar amounts of free Ca^{2+} (+ATP) for 10 min at 30°C or 37°C
7. Centrifugation and counting of released catecholamines in the supernatant
8. Solubilization of cells with SDS and counting of the remaining catecholamines

using α-toxin or SLO. α-Toxin-permeabilized cells respond to micromolar concentrations of Ca^{2+} for >1 hour (Ahnert-Hilger and Gratzl, 1987), whereas SLO-treated cells respond for ~40 minutes (Ahnert-Hilger *et al.*, 1989a,b). Later they become insensitive to stimulation with Ca^{2+} (Sarafian *et al.*, 1987). In a comparable experimental design, exocytosis can be measured by α-toxin-permeabilized cytotoxic T lymphocytes. Here the release of a vesicular serine esterase (Pasternack *et al.*, 1986; Henkart *et al.*, 1984) is taken as a measure for exocytosis. This release is triggered with increasing Ca^{2+} concentrations, provided ATP and GTPγS are present (Schrezenmeier *et al.*, 1988a,b). Furthermore, mast cells previously permeabilized with small amounts of SLO release histamine upon addition of Ca^{2+} and a nucleotide (Howell and Gomperts, 1987; Howell *et al.*, 1987).

Besides exocytosis, the observed release of secretory product by permeabilized cells may be due to leakiness of secretory vesicles or even loss of intact secretory vesicles from the cells. Detergents like saponin or digitonin may destroy the secretory-vesicle membrane or support the escape of intact secretory vesicles through the large membrane lesions generated (Brooks and Carmichael, 1983; Bader *et al.*, 1986). The parallel release of low and high molecular weight secretory products is a convincing indication for exocytosis, provided that under the same conditions large cytoplasmic constituents do not leak out. Such a situation has been found with α-toxin-permeabilized adrenal medullary chromaffin cells, which release labeled catecholamines and the vesicular

protein chromogranin A but not cytoplasmic LDH in the presence of micromolar concentrations of Ca^{2+} (Bader *et al.*, 1986). Similarly, exocytosis is indicated by the parallel release of catecholamines and dopamine β-hydroxylase from electrically permeabilized adrenal medullary chromaffin cells (Knight and Baker, 1982) and the release of vesicular serine esterase by α-toxin-permeabilized cytotoxic T lymphocytes (Schrezenmeier *et al.*, 1988a,b), but not of cytoplasmic proteins. In all these preparations the pores in the plasma membrane are too small to allow a direct escape of the vesicular proteins.

Another approach takes the metabolism of catecholamines in the cytoplasm of α-toxin-permeabilized PC12 cells as an indicator of whether or not the secretory products leave the cell by exocytosis. Since cytoplasmic enzymes such as LDH remain entrapped in these cells after permeabilization, the enzymes involved in the metabolism of catecholamines are also retained. Thus, the discharge of vesicular dopamine into the cytoplasm (e.g., by nigericin) results in its enzymatic oxidation to 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylethanol (DOPET). In contrast, if catecyholamines are released by exocytosis, the cytoplasm is avoided and no metabolism can occur. The pattern of catecholamines and their metabolites released by α-toxin-permeabilized PC12 cells is in accordance with these predictions. Ca^{2+} only results in a release of dopamine and norepinephrine, whereas the release of the metabolies resembles that seen with either unstimulated or intact control cells (Ahnert-Hilger *et al.*, 1987; Fig. 8). Therefore, it can be concluded that permeabilized PC12 cells release catecholamines by exocytosis with micromolar amounts of free Ca^{2+}.

The three types of secretory cells permeabilized by pore-forming toxins differ in their molecular requirements for exocytosis. Whereas in PC12 cells permeabilized with α-toxin or SLO, Ca^{2+} alone is sufficient to release the stored catecholamines (Figs. 9, 10; Ahnert-Hilger and Gratzl, 1987; Ahnert-Hilger *et al.*, 1985a, 1989a,b), adrenal medullary chromaffin cells require additional Mg^{2+}/ATP (Bader *et al.*, 1986; Grant *et al.*, 1987). The situation is even more complicated in the permeabilized cytotoxic T lymphocyte, where Ca^{2+} and ATP alone result only in a small release of the serine esterase. Only when a G protein is activated by GTPγS, can a full exocytotic response be obtained (Schrezenmeier *et al.*, 1988a,b). In SLO-permeabilized mast cells, Ca^{2+} plus a nucleotide must be present (Howell *et al.*, 1987; Howell and Gomperts, 1987).

In permeabilized PC12 cells the Ca^{2+}-stimulated dopamine release is not affected by the composition of the medium. Potassium can be exchanged for sodium as well as glutamate for chloride (Ahnert-Hilger *et al.*, 1985a, 1987). This holds also for cytotoxic T lymphocytes (Schrezen-

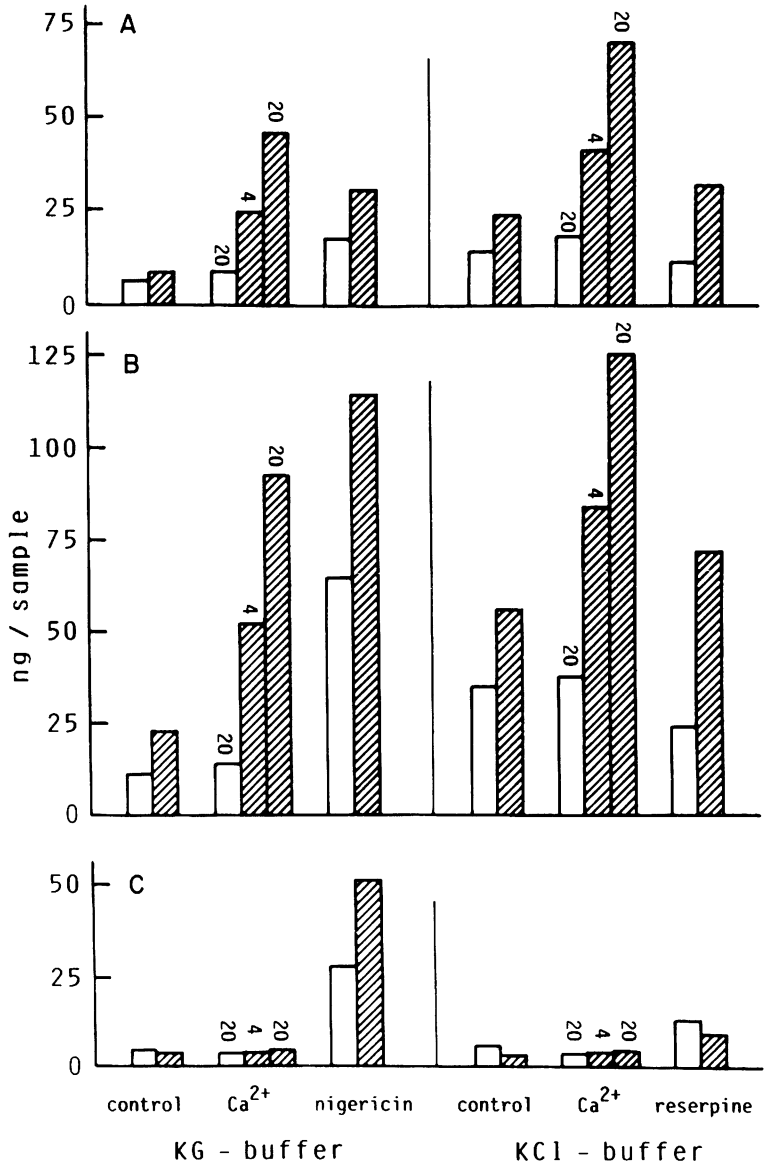


FIG. 8. Effects of Ca^{2+}, nigericin, and reserpine on the release of (A) norepinephrine, (B) dopamine, and (C) its metabolite DOPAC by permeabilized and intact PC12 cells. PC12 cells were first permeabilized with α-toxin in KG buffer or KCl buffer (K^+ glutamate was exchanged for KCl), and then incubated with buffer (controls), with 4 or 20 μmol/liter free Ca^{2+}, with nigericin (1 μmol/liter), or with reserpine (0.2 μmol/liter) for 10 minutes at 30°C. Catecholamines were determined in the supernatant and the lysate of cells by the HPLC technique. The hatched bars represent permeabilized cells, the open ones intact cells. The samples contained 560±32 μg of protein. From Ahnert-Hilger *et al.* (1987), by permission.

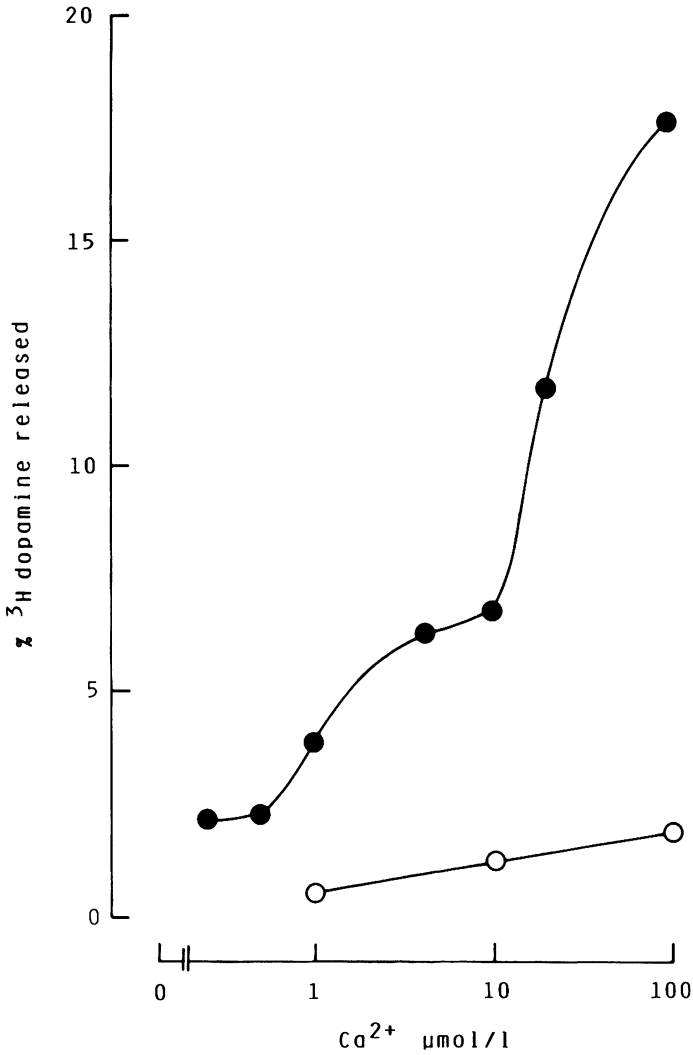


FIG. 9. Ca^{2+} dependency of [^{3} H]dopamine release by α-toxin permeabilized PC12 cells. Cells were loaded with [^{3} H]dopamine, washed, and treated with α-toxin (●) or KG buffer alone (○), as described in Fig. 8. The permeabilization medium was exchanged for a fresh one containing the amount of the free-Ca^{2+} concentration given in the abscissa. Each point represents the mean of two samples. The release in the absence of Ca^{2+} (7.5% for α-toxin-treated cells and 8% for intact cells) was subtracted. Micromolar amounts of Ca^{2+} release dopamine only from α-toxin-permeabilized cells. From Ahnert-Hilger and Gratzl (1987), by permission.

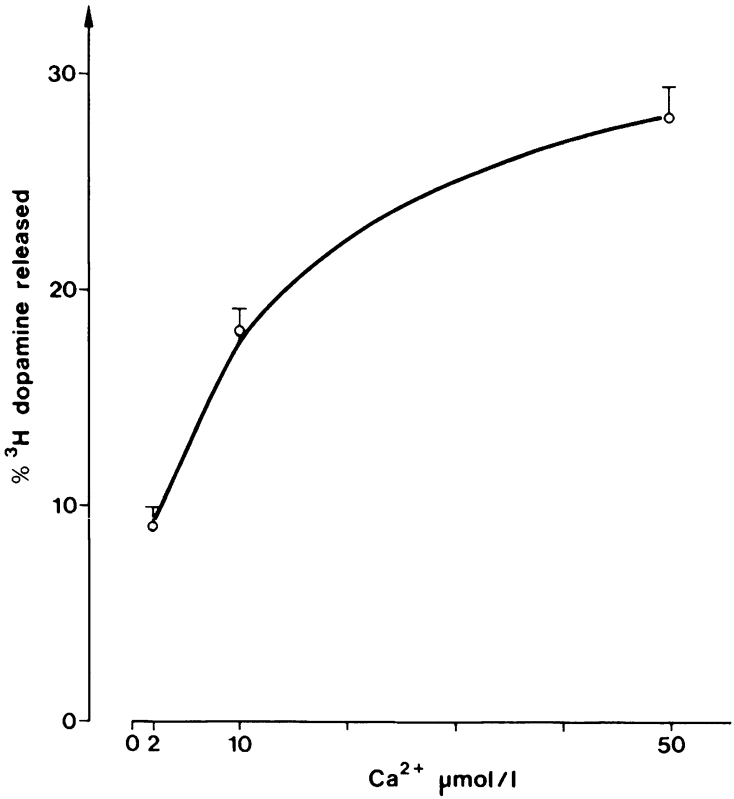


FIG. 10. Ca^{2+} dependency of [^{3}H]dopamine release by SLO-permeabilized PC12 cells. Preloaded PC12 cells were treated and incubated with SLO as given in Fig. 8, prior to stimulation with the given free-Ca^{2+} concentrations (abscissa). Values represent the Ca^{2+}-stimulated release of three samples (±SD).

meier *et al.*, 1988a,b). By contrast, in electrically permeabilized adrenal medullary chromaffin cells exocytosis has been reported to be inhibited by chloride (Knight and Baker, 1982). Even when all ions are replaced by sucrose or the pH is varied between 6.6 and 7.2, the Ca^{2+}-stimulated release remains unchanged (Ahnert-Hilger *et al.*, 1985a; Ahnert-Hilger and Gratzl, 1987).

Permeabilized PC12 cells are a very useful preparation to study the modulation of exocytosis by intracellular regulator systems such as protein kinase C and G proteins, because exocytosis by these cells can be triggered by Ca^{2+} alone. In these cells Mg^{2+} in the millimolar range increases the Ca^{2+}-induced exocytosis (Ahnert-Hilger and Gratzl, 1987),

whereas in the adrenal medullary chromaffin cells Mg^{2+} alone has no effect but must be complexed with ATP to sustain exocytosis (Bader *et al.*, 1986; Knight and Baker, 1982). Very high amounts of Mg^{2+} have even been reported to be inhibitory in these cells (Knight and Baker, 1982). Activation of protein kinase C by the diacylglycerol analog 1-oleyl-2-acetylglycerol (OAG) or the phorbol ester 1-*O*-tetradecanoylphorbol-13-acetate (TPA) ameliorates Ca^{2+}-induced exocytosis by both permeabilized PC12 cells (Peppers and Holz, 1986; Ahnert-Hilger *et al.*, 1987) and adrenal medullary chromaffin cells (Knight and Baker, 1983). In PC12 cells this effect was shown to be dependent on the presence of Mg^{2+}/ATP (Ahnert-Hilger and Gratzl, 1987). In adrenal medullary chromaffin cells the modulatory role of protein kinase C is difficult to analyze because exocytosis by these cells also depends on ATP.

G proteins are specialized membrane proteins involved in the transduction of various signals (Gilman, 1987). In permeabilized PC12 cells, activation of G proteins by GTPγS results in an incomplete inhibition of exocytosis in the presence of Mg^{2+}. The amounts of GTPγS (between 2 and 100 μM) did not interfere with the free-Ca^{2+} concentration in the buffer system as measured by the Ca^{2+}-sensitive electrode. When cells were pretreated during the loading period (see Fig. 8) with pertussis toxin (11 μg/ml, obtained from List Biological Laboratories, Campbell, CA), the inhibitory effect of GTPγS could be overcome Fig. 11). In a similar experimental design, cells were pretreated with cholera toxin (100 μg/ml; obtained from Sigma, Munich) which did not alter the GTPγS-induced inhibition of exocytosis. Thus, it can be concluded that exocytosis by PC12 cells can be modulated by a pertussis toxin-sensitive G protein (Ahnert-Hilger *et al.*, 1987). Similar results have been reported for freshly isolated bovine adrenal medullary chromaffin cells (Knight and Baker, 1985), whereas a stimulatory effect of GTPγS has been obtained in adrenal medullary chromaffin cells from chicken (Knight and Baker, 1985), from bovine tissue (Bittner *et al.*, 1986), and in mast cells (Howell and Gomperts, 1987; Howell *et al.*, 1987; Neher, 1988). Besides the fact that different cell preparations were used, these contradictory results may reflect the modulation of exocytosis by different G proteins. Figure 12 summarizes our current data concerning the regulation and modulation of exocytosis in PC12 cells.

Experimental data from our laboratory show that the large pores generated by SLO are also well suited to study the intracellular effects of tetanus toxin, a neurotoxin of 150 kDa from clostridium tetani. This toxin when applied extracellulary inhibits exocytosis from neurons but not from endocrine cells (cf. Habermann and Dreyer, 1986; Knight, 1986). Upon intracellular injection, however, tetanus toxin becomes capable of inhibit-

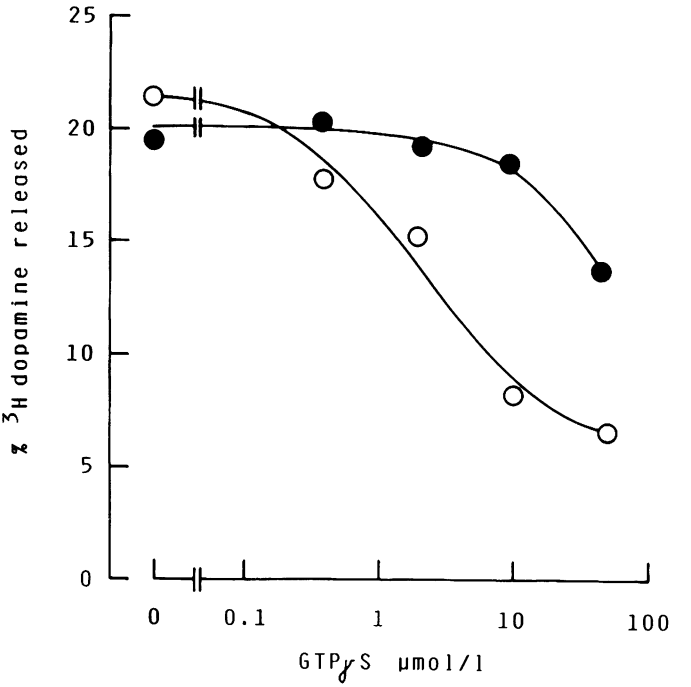


FIG. 11. Pretreatment (●) of PC12 cells with pertussis toxin (11.5 µg/ml) overcomes the inhibitory effects of GTPγS on exocytosis. PC12 cells were treated with or without (○) pertussis toxin for 4 hours during the loading period. Then the cells were washed and permeabilized with α-toxin described in Fig. 8. Free Mg^{2+} (1 mmol/liter) and the indicated amounts of GTPγS were also present. The medium was exchanged for a fresh one containing the same constituents and 10 µmol/liter free Ca^{2+}. From Ahnert-Hilger *et al.* (1987), by permission.

ing exocytosis from adrenal medullary chromaffin cells (Penner *et al.*, 1986), indicating that it attacks a step during exocytosis which is common to neurons and endocrine cells. In contrast to the study using single injected cells, SLO-permeabilized cells allow the application of defined dosis of toxin to a great number of cells and further biochemical analysis of the mechanism of tetanus toxin action. Tetanus toxin consists of a heavy and a light chain covalently linked by a disulfide bond (Habermann and Dreyer, 1986). Chain separation by reduction of this bond initiates the inhibitory action on exocytosis of the toxin in SLO-permeabilized chromaffin and PC12 cells (Ahnert-Hilger *et al.*, 1989a,b). Also cleavage of the disulfide bond linking the heavy and light chain of botulinum A toxin is necessary for its inhibitory action on exocytosis (Stecher *et al.*, 1989). Even the light chain of tetanus toxin alone is fully active whereas the heavy chain has no effect on exocytosis (Ahnert-Hilger *et al.*, 1989b).

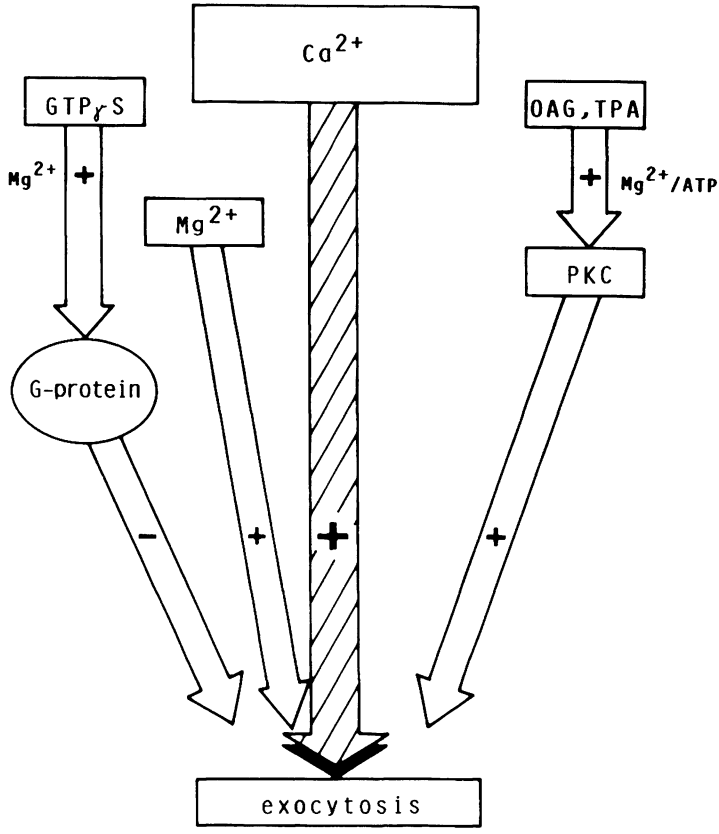


FIG. 12. Regulation and modulation of Ca^{2+}-dependent exocytosis by permeabilized PC12 cells. In permeabilized PC12 cells exocytosis can be stimulated by Ca^{2+}. Ca^{2+}-stimulated exocytosis can be augmented by Mg^{2+}, and activators of the protein kinase C provided Mg^{2+}/ATP is present. Ca^{2+}-induced exocytosis is inhibited by GTPγS in the presence of Mg^{2+} because of activation of a pertussis-sensitive G protein.

Thus tetanus toxin has to be reduced before the light chain can exert its biological effect on exocytosis.

V. Concluding Remarks

In contrast to other permeabilizing procedures, the pores inserted into the plasma membrane with the aid of bacterial toxins are stabilized by a proteinaceous ringlike structure. Depending on the aim of an experiment, the cells can be made permeable either for large or for small molecules by

selection of a suitable bacterial pore-forming protein. This allows permanent access to the cells' interior in order to investigate intracellular processes as diverse as fusion of secretory vesicles with the inner surface of the cell membrane, contraction, metabolism of hormones, fluxes of ions, or glucose metabolism. Besides the different secretory cells already referred to in this article, poration by α-toxin or SLO was carried out with hepatocytes (McEwen and Arion, 1985), rat basophilic leukemia cells (Hohman, 1988), fibroblasts (Thelestam and Möllby, 1979), and smooth muscle cells (Cassidy *et al.*, 1978). This indicates that the novel approach of permeabilization of cells by channel-forming toxins has already become a widely used tool for the investigation of a variety of intracellular processes in situ.

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