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α-TOXIN PERMEABILIZED RAT PHEOCHROMOCYTOMA CELLS: A NEW APPROACH TO INVESTIGATE STIMULUS–SECRETION COUPLING

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The channel forming α-toxin of *Staphylococcus aureus* (about 50 μg/ml) markedly reduces the Ca^{2+} requirement for dopamine release by the rat pheochromocytoma cell line (PC 12). Maximal secretion by intact cells requires approximately 1 mM Ca^{2+}, whereas release by α-toxin-permeabilized cells can already be triggered with μM concentrations of Ca^{2+}. The latter process reaches a plateau at about 1 μM free Ca^{2+} and increases again with 10–20 μM free Ca^{2+}. The sensitivity to low concentrations of Ca^{2+} indicates that the toxin, as a selective cell membrane permeabilizing agent, can be used as a powerful instrument to study stimulus–secretion coupling.

In order to gain access to the intracellular sites involved in the regulation of exocytosis, secretory cells have been permeabilized using different techniques [3, 4, 10, 13]. However, the results obtained with cells subjected to high-voltage discharges or treated with membrane perturbants such as digitonin and saponin are contradictory. For example, the release of secretory products observed with electrically permeabilized chromaffin cells [1] was inhibited by digitonin, a substance used by other groups for permeabilization [4, 13]. The conflicting data obtained so far may be mainly due to the unspecificity of the procedures used. In particular, substances such as digitonin may affect cell components other than the cell membrane, thereby compromising functions other than membrane permeability in the cells.

Here, we report an alternative approach to circumvent these problems. A rat pheochromocytoma cell line (PC 12) has been used as a secretory system, and α-toxin from *Staphylococcus aureus* as a cell membrane permeabilizing agent. This toxin forms stable, transmembrane channels with an effective diameter of 2–3 nm [2, 6], which are too small to permit passage of toxin monomers into the cell. The effects of this toxin would therefore be expected to be confined strictly to the cell plasma membrane. α-Toxin as a selective membrane permeabilizing agent indeed appears to be an excellent tool for studying stimulus–secretion coupling. In this contribution, we demonstrate that μM concentrations of free Ca^{2+} are sufficient to trigger dopa-

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mine release in α-toxin-treated cells, whereas millimolar extracellular concentrations Ca^{2+} are required for secretion elicited by depolarization.

Exocytosis is paralleled by an increase in the cytoplasmic concentration of free Ca^{2+} in chromaffin and other secretory cells (cf. ref. 11). Primary cultures from adrenal medulla or the rat pheochromocytoma cell line PC 12 [9] are often used to study secretion. The release of dopamine from PC 12 cells due to nicotinic stimulation or

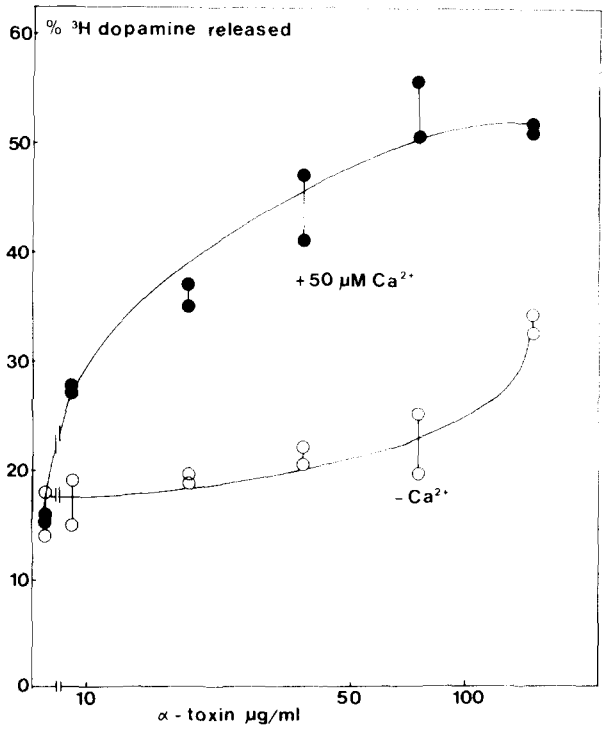

Fig. 1. Effect of different concentrations of α-toxin on the [^{3}H]dopamine release of PC 12 cells in the presence (●) or absence (○) of Ca^{2+}. α-Toxin from *Staphylococcus aureus* was purified as described [6]. The PC 12 cells were kindly supplied by H. Thoenen (Max Planck Institut für Psychiatrie, Martinsried, F.R.G.). Cells were grown on plastic dishes in Dulbecco's modified Eagle's medium supplemented with 10% horse serum–5% fetal calf serum in a humidified atmosphere of 10% CO_2 as described [8]. For loading, the cells were first incubated with 0.25 µM [^{3}H]dopamine (spec.act. 11.5 Ci/mmol) for 2 h in culture medium without serum. Subsequently they were washed with Ca^{2+}-free incubation buffer (K^+ glutamate 150, PIPES 10, EGTA 5 mM, pH 7.2) and resuspended in the same buffer supplemented with 0.2% bovine serum albumin. Incubation buffer with defined amounts of Ca^{2+} in the presence of absence of α-toxin was added, and incubations were performed for 20 min at 37°C. The exact concentration of free Ca^{2+} was calculated by means of a computer program as described [5] using the stability constants listed [12] and/or determined with the aid of a Ca^{2+}-specific electrode (the necessary membranes were kindly provided by W. Simon, ETH Zürich, Switzerland). Radioactivity was determined by liquid-scintillation counting in the supernatant as well as in the cells. [^{3}H]Dopamine released was given as percent of total present before incubation with toxin. Samples contained 50–80 µg cell protein. The average uptake of [^{3}H]dopamine was approximately 55 pmol/mg cell protein.

depolarization requires mM concentrations of Ca^{2+} (0.5-1 mM) in the extracellular fluid (cf. ref. 9, compare Fig. 2A).

By contrast, μM concentrations of free Ca^{2+} were found to be sufficient to elicit release of dopamine in α-toxin-permeabilized cells. In these experiments, α-toxin was used at concentrations ranging from 10 to 100 μg/ml with negligible changes in basal release (Fig. 1). Using these concentrations, Ca^{2+}-dependent [^{3}H]dopamine release was increased in a dose-dependent manner. Above 100 μg/ml, α-toxin induced a Ca^{2+}-independent release of [^{3}H]dopamine, probably due to general leakiness of the cells (Fig. 1).

Fig. 2 compares the amount of free Ca^{2+} necessary in the medium for secretion by depolarized cells as opposed to α-toxin-permeabilized cells. Depolarization by potassium was half-maximal with a concentration of 0.5 mM free Ca^{2+}. Below a concentration of 0.2 mM free Ca^{2+}, release of [^{3}H]dopamine was approximately the same as in the absence of Ca^{2+} (Fig. 2A). By contrast, very low concentrations of free Ca^{2+} sufficed to initiate dopamine release from α-toxin-permeabilized cells (37 μg/ml toxin). The release induced by Ca^{2+} reached a first plateau between 1 and 4 μM, and further release was induced at 5–20 μM Ca^{2+} to reach maximal levels at the latter Ca^{2+} concentrations (Fig. 2B, compare value found with the same amount of toxin

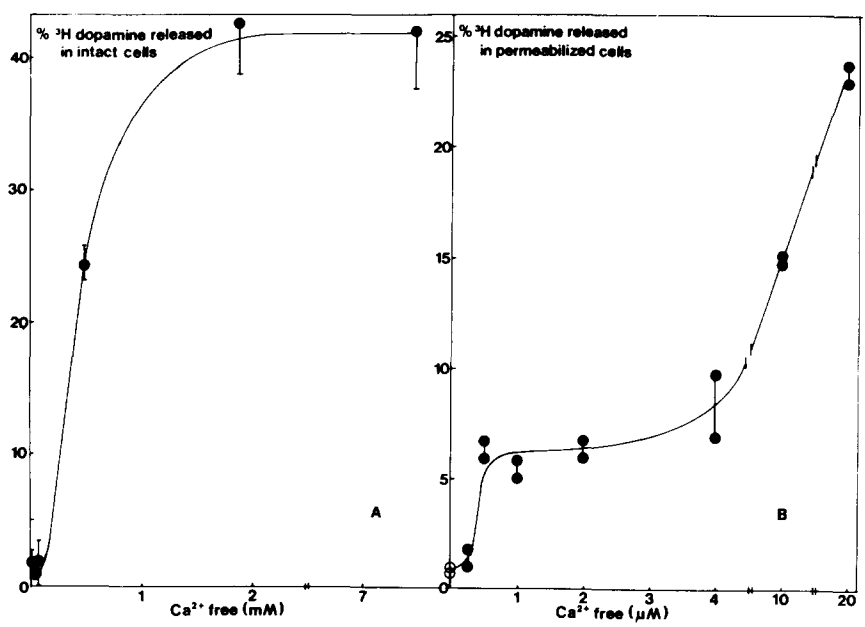

Fig. 2. Ca^{2+} requirement of [^{3}H]dopamine release by PC 12 cells after depolarization (A) and α-toxin permeabilization (B) as a function of the free Ca^{2+} concentration. In B, permeabilization buffer was slightly modified containing 0.5 mM EGTA plus 5 mM NTA instead of 5 mM EGTA (see legend to Fig. 1). The basal release of [^{3}H]dopamine in the absence of Ca^{2+} (A) or α-toxin (B) were 15.6 $\pm$ 1.7 (n = 3) or 18 $\pm$ 1.7% (n = 16), respectively. These values were substracted from the release observed in the presence of Ca^{2+} or α-toxin (37 μg/ml).

at a free Ca^{2+} concentration of 50 μM in Fig. 1). The first increase was observed with a half-maximal free Ca^{2+} concentration of 0.5 $\pm$ 0.2 μM (n = 5). It is of interest that intracellular free concentrations of Ca^{2+} in chromaffin cells indeed have been found to rise from 0.1 to approximately 0.5 μM under physiological stimulation as determined with the sensitive intracellular Ca^{2+} indicator Quin 2 [11].

The elucidation of the chain of events taking place between recognition of the stimulus and release of the secretory product deserves different experimental approaches. Intact cells are certainly too complex to permit delineation of these events. On the other hand, experiments with isolated membranes involved in the physiological fusion reaction only allow the investigation of certain aspects of the secretory process [7]. We therefore feel that the introduction of selective membrane-permeabilizing reagents as described for α-toxin in this paper provides a generally useful approach to the study of stimulus-secretion coupling.

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