Journal of Cellular Biochemistry 33:289–303 (1987)

Detergents Inhibit Exocytosis in PC 12 Cells: Evidence for an Effect on Ion Fluxes

Carlo Tapparelli, Marianne Grob, and Max M. Burger

Department of Biochemistry, Biozentrum, University of Basel, CH-4056 Basel, Switzerland

Membrane events in exocytosis were studied by examining the effect of different detergents on the K^+-stimulated release of noradrenaline in the secretory cell line PC 12. The nonionic detergent Triton X-100 and the cationic detergent cetyltrimethylammonium bromide (CTAB) inhibit the noradrenaline release evoked by 55 mM K^+ by 50% at very low concentrations (30 μM and 10 μM, respectively). These values are tenfold lower than the critical micellar concentrations (CMC). No such effect was seen with the anionic detergent sodium dodecyl sulphate ($NaDodSO_4$).

The inhibitory effect of 30 μM Triton X-100 is reversible, and the recovery from inhibition correlates with the loss of detergent from the cells as demonstrated by binding studies using [^{3}H]Triton X-100. The possible relationship between this inhibition of secretion and the structural properties of the detergent was investigated. The inhibition in the presence of purified Triton X-100 subfractions turned out to be a function of the length of the oligometric ethyleneglycol chain (C_6 to C_{26}). The maximal effect was observed for Triton X-100 molecules having a chain length of 16 carbon atoms, which can penetrate just half of the lipid bilayer of the membrane. Additionally, the phase transition at 13–14°C observed in an Arrhenius plot of noradrenaline release in stimulated cells was abolished.

In the presence of 30 μM Triton X-100, $^{22}Na^+$ uptake, $^{86}Rb^+$ release, and $^{45}Ca^{2+}$ uptake were reduced by 50–60%. These data suggest that the site of action of Triton X-100 is at the level of altering the movement of ions in PC 12 cells during the stimulatory phase of secretion.

Key words: detergents, noradrenaline secretion

The release of physiologically important substances via exocytosis is common to many secretory cell types in which it has been sought [1–5]. Exocytosis involves the fusion of two membrane systems (secretory vesicles and plasma membrane) in response to a given stimulus. To date, little is understood about exocytosis at the molecular level. Owing to the ease of measurement, the role of small molecules and ions in this phenomenon has become well characterized [6]. On the other hand, much

Carlo Tapparelli's present address is Preclinical Research, Sandoz Ltd., CH-4002 Basel, Switzerland.

Received February 1, 1986; revised and accepted November 3, 1986.

© 1987 Alan R. Liss, Inc.

less evidence has been accumulated concerning the role played by the individual membrane systems and their respective components in a physiologically relevant manner. This has been, in part, due to a lack of suitable experimental systems in which exocytotic release could be modulated.

In the past few years secretory cell lines have been isolated that retain their ability to respond to an appropriate stimulus. PC 12 cells, a derivative of a rat pheochromocytoma (tumor of the adrenal medulla), have been shown to be able to release dopamine and noradrenaline in response to elevated K^+ concentrations [7,8] by exocytosis. Furthermore, these cells behave identically to adrenal medulla regarding cholinergic stimulation, ion fluxes, and energy requirements [8–10]. It is thus clear that PC 12 cells fulfill the requirements for being a system in which exocytosis could be studied in vitro.

Amphipathic agents or drugs are used in a classical approach to examining membrane-mediated processes involving either the perturbation of the native phospholipid environment or, at lower concentrations, the specifical modulation of cellular receptors [11]. The use of detergents has become widespread in membrane biology as a good deal of knowledge exists concerning their molecular structure, functions, and interaction with membrane components [12,13]. It therefore seemed appropriate to examine the effect of various detergents on the triggered release of catecholamines in the PC 12 cells.

In this study, the effects of the nonionic detergent Triton X-100, the cationic detergent cetyltrimethylammonium bromide (CTAB), and the anionic detergent sodium dodecyl sulfate ($NaDodSO_4$) on K^+-mediated noradrenaline release were examined. In particular, the effects of TX-100 were singled out for further characterization with respect to known release parameters such as Na^+, K^+, and Ca^{2+} fluxes and membrane fluidity.

MATERIALS AND METHODS

Cell Culture

PC 12 cells, a clonal line derived from the original [7] by culturing on collagen, were grown at 37°C under an atmosphere of 10% CO_2 and 90% air on plastic tissue culture dishes. The culture medium, Dulbecco's modified Eagle's medium (DME) containing horse serum (10%) and fetal calf serum (5%), streptomycin (100 μg/ml), and penicillin (100 μg/ml) was changed every 2 days. The cells were used 4 to 5 days after subculturing.

Uptake and Release of [^{3}H]Noradrenaline

Cell cultures were incubated with [^{3}H]noradrenaline (0.25 μCi/ml medium; 28.3 Ci/nmol, New England Nuclear) for 1 hr at 37°C. After incubation, this medium was removed by aspiration, and the cultures were washed twice with 5 mM K^+-medium (K-RM), a HEPES-buffered modified Krebs Ringer saline consisting of 127 mM NaCl, 5 mM KCl, 2.2 mM $CaCl_2$, 0.33 mM $NaHPO_4$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 6.5 mM glucose, and 10 mM HEPES, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid, pH 7.2. The washed cells were harvested in a stream of 5 mM K-RM delivered gently via a Pasteur pipet, pooled in 5 mM K-RM, and divided into equal aliquots of 10^6 cells containing 30,000–40,000 cpm. After centrifugation for 5 min at 110g the supernatant was removed, and the cells were incubated for 10

min at 37 °C in the desired medium. In all cases in which different KCl-concentrations were used, the NaCl concentration was lowered correspondingly to maintain isoosmolarity. For the measurement of the released [^{3}H]noradrenaline, the supernatant from the 110g centrifugation was transferred to a scintillation vial and, following the addition of 10 ml Instagel (Packard), was assayed for radioactivity.

Distribution of Endogenous Noradrenaline and [^{3}H]Noradrenaline in Subcellular Fractions of PC 12

Secretory granules were isolated from cells incubated with [^{3}H]noradrenaline, washed, and harvested as described. A cell homogenate containing 1–2 mg of protein per ml was produced by 40 up and down strokes in a DUAL 23 homogenizer (Kontes, Vineland, NJ) at 0 °C. The homogenate was centrifuged at 1,000g for 15 min at 4 °C. The supernatant was further centrifuged at 10,000g for 20 min at 4 °C, and the resulting pellet, containing the chromaffin granules, was resuspended in 5 mM K-RM. All the fractions were kept on ice until use.

Endogenous noradrenaline was measured, with some modifications, as described by Westerink and Korf [14]. The samples were acidified at 0 °C to give a final concentration of 0.4 M $HClO_4$. The pH was adjusted to 2.7 by addition of a solution of 43% KOH in 23% HCOOH. After a 1,500g centrifugation for 10 min at 4 °C, 1.0 ml supernatant was passed through a 7 cm × 5 mm ID Sephadex G-10 column and eluted first with 1.0 ml 10 mM HCOOH, second with 1.5 ml 10 mM HCOOH, and third with 1.0 ml 5 mM phosphate buffer, pH 8.5. More than 85% of noradrenaline was recovered in the second and third fraction. Noradrenaline was assayed with the trihydroxyindol (THI) reaction [15]. Fluorescence measurements were made on a Perkin-Elmer Fluorescence Spectrophotometer 204-A. [^{3}H]Noradrenaline was assayed as described above. Protein was assayed using bovine serum albumin as standard [16].

Two separate measurements showed that the chromaffin granule fraction contained 84.1% noradrenaline and 82.8% [^{3}H]noradrenaline, respectively, whereas the cytoplasmic fraction contained 16.3% noradrenaline and 18.5% [^{3}H]noradrenaline. The recovery, determined by measuring total noradrenaline, [^{3}H]noradrenaline, and protein, ranged between 98.0 and 99.0% for noradrenaline and [^{3}H]noradrenaline and 102.0 and 103.5% for the protein.

Cell Viability

The possible cytotoxic effect of the detergents on PC 12 cells was tested by exposing the cells under sterile conditions for 20 min to the various detergent-containing media and examining cell growth by reculturing for a period of 2 days. Cell number was counted with a hematocytometer. In addition, cell viability was also tested after incubation by the Trypan blue exclusion method.

Triton X-100 Binding

Aliquots of 7×10^6 cells were incubated in 1.0 ml 5 mM K-RM containing different concentrations of TX-100, which contained a $1:10^5$ dilution of [^{3}H]Triton X-100 (1.56 mCi/mg; Amersham) for a defined period of time at 37 °C (10^6 cells corresponding to 10,000–11,000 cpm). Cells and supernatants were separated by centrifugation at 110g for 5 min. The radioactivity was then measured in both fractions. The release of bound TX-100 was determined by reincubating the pellet of

treated cells for 10 min at 37°C in 5 mM K-RM. Cells and medium were again separated by centrifugation and assayed for radioactivity.

Isolation and Identification of the Triton X-100 Fractions

Separation of the heterogeneous components of TX-100 by high-pressure liquid chromatography (HPLC) was carried out in a Kontron-HPLC, equipped with a 25 cm × 4 mm-ID (inner diameter) stainless steel column packed with Lichosorb SI 100 (7 μm). Flow rate and pressure were between 1 and 2 ml/min and 64 and 136 bar, respectively. Ethyl acetate or ethyl acetate saturated with water was used as the mobile phase. Fractions were dried under nitrogen. For the gas chromatographic separation, aliquots of the fractions were dissolved in tetrahydrofuran containing 10% N-methyl N-trimethylsilyl-trifluoroacetamid (MSTFA; Macherey-Nagel, Düren, FRG) to a final concentration of 1 mg detergent/ml. Complete derivatization was achieved by heating the sample to 100°C for 10 min. Gas chromatography-mass spectrometry (GC/MS) analyses were performed with a Hewlett-Packard HP 5992 equipped with a 1.6 m × 2 mm-ID column packed with 3% OV-17 on Chromosorb W/aw 80–100 mesh (Applied Science).

Determination of Cellular ATP Level

Cellular ATP level was determined according to the method of Holm-Hansen and Karl [17] by use of the firefly luciferin-luciferase bioluminescence reaction. Washed cells were incubated in either 5- or 55-mM K-RM aliquots in the presence or absence of 30 μM Triton X-100. After various periods of incubation at 37°C, cell suspensions were assayed for ATP by mixing a 0.5-ml aliquot with a 2.0-ml solution of firefly lantern extract (FLE-150, Sigma). Luminescence was measured in a luminator (SKAN, Basel, Switzerland) using ATP as a standard.

Determination of $^{22}NA^+$, $^{86}Rb^+$, and $^{45}Ca^{2+}$ Fluxes in PC 12 Cells

Ion fluxes were determined using the radioactive isotopes $^{22}NaCl$ (100–1,000 mCi/mg Na^+; Amersham), $^{86}RbCl$ (1–8 mCi/mg Rb^+, Amersham), and $^{45}CaCl_2$ (0.96 Ci/mol; New England Nuclear) according to the methods of Kikuchi et al [18] and J.C. Henquin [19]. Washed cells were incubated in either 5- or 55-mM K-RM aliquots containing the radioisotopic tracer in concentration between 2.0 and 10 μM, in the absence or presence of 30 μM TX-100. After various periods of incubation (see "Results") at 37°C, 150 μl of the cell suspension was layered over a gradient composed of 50 μl of 6 M urea overlaid with 200 μl of a mixture composed of 77% dibutyl phtalate (Fluka, Buchs, Switzerland) and 23% bis -3,5-5-trimethylcyclohexylphtalate (Fluka) in a 0.4-ml plastic tube. The cells were separated from the incubation layer by centrifugation at 10,000g for 1 min. The portion of the tube containing the cells was cut off with a razor blade, transferred to a scintillation vial, and, following the addition of 10 ml Instagel, assayed for $^{22}Na^+$ and $^{45}Ca^{2+}$ radioactivity.

$^{86}Rb^+$ release was determined by centrifugation of 300 μl of the cell suspension at 8,000g for 5 sec, and radioactivity was assayed in both the supernatant and cell pellet.

RESULTS

Effect of Detergents on the Release of Noradrenaline

Previous studies [8,21,22] have shown that dopamine and noradrenaline in PC 12 cells are located mainly in the secretory granules, and that the release of these catecholamines can be stimulated by increasing the external K^+-concentration. In our experiments [23], PC 12 cells released 65–70% of the endogeneous noradrenaline as well as of the preloaded [^{3}H]noradrenaline upon stimulation by 55 mM K^+, which was significantly higher than in other cell lines of the same origin in which values of 40–50% were observed [8]. The amount of [^{3}H]noradrenaline detected in the supernatant increased rapidly, reaching a maximal level within 2 min. This maximal level remained constant at least 20 min after stimulation. Both endogeneous noradrenaline and loaded [^{3}H]noradrenaline were located to about 84% in the chromaffin granule fraction (see "Materials and Methods").

Of the three detergents tested, TX-100 and CTAB displayed a strong inhibitory effect on noradrenaline secretion (Fig. 1A,B), while $NaDodSO_4$ failed to have any effect throughout the range of detergent concentrations employed (Fig. 1C). In the case of CTAB this inhibition was observed as low as 3–10 μM, while for Triton X-100 the inhibition persisted up to 30 μM. Only at higher detergent concentrations (64 μM and above) could an apparent stimulation be observed in controls (5 mM K-RM), which was probably due to detergent-mediated agonistic effect and/or membrane leakiness.

It was therefore essential to separate these two phenomena and to focus on the range of detergent concentrations in which inhibition was maximal prior to detergent-induced spontaneous release. It should be mentioned that none of the three detergents had any effect on cell viability, even in concentrations of up to 100 μM (see "Materials and Methods").

As TX-100 is better characterized in terms of its physicochemical and biological action, it was decided that further studies should focus upon the influence of this detergent on the release process.

Reversibility of Triton X-100 Inhibition

An obvious question is whether detergent is exerting its effect at the extracellular or the intracellular level. It was therefore decided to administer the detergent, to wash the cells, and then to reexamine the effect of K^+ stimulation. In this way the reversibility of the detergent effect could be explored. As can be seen in Table I, the inhibition brought about by 30 μM TX-100 was reflected by a drop from 68% release to 35% (Exp. Ac). In TX-100 pretreated cells (exp. B), stimulation levels remained below 40% (exp. Bc). If TX-100 was present during the stimulation of detergent-treated cells, release dropped to a value of 24% (exp. Bc). This result indicates that inhibition persisted in the absence of detergent-containing medium and was even increased in its presence. When a 10-min detergent-free incubation period was introduced (column b), recovery back to 60% was observed (exp. Dc). In this case, either the detergent effect was in some way neutralized by the cell during the 10-min detergent-free incubation period or the Triton X-100 was washed away and was no longer associated with the cells. The use of radiolabeled Triton X-100 allowed the differentiation between these two alternate hypotheses.

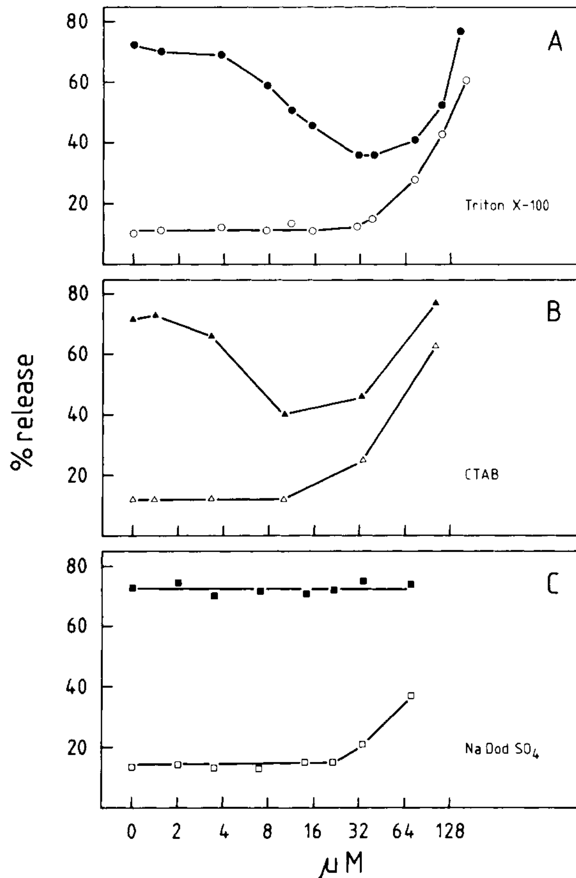

Fig. 1. Effect of detergents on K^+-stimulated [^{3}H]noradrenaline ([^{3}H] NA) release in PC 12 cells. Cells were loaded with [^{3}H] NA and incubated as described in "Materials and Methods." Detergent concentrations are given on the abscissa. Release is expressed as the percent value of [^{3}H]NA released into the medium during the incubation period compared to the cellular [^{3}H]NA content prior to stimulation. Open symbols: unstimulated cells (5 mM K-RM); closed symbols: stimulated cells (55 mM K-RM). Values represent the averages of ten different experiments, with a SD $\leqslant \pm 2.0\%$. The recovery of total activity was $96.0 \pm 7.3\%$. Panels refer to values obtained in the presence of (**A**) Triton X-100, (**B**) CTAB, and (**C**) $NaDodSO_4$.

The concentration of TX-100 that brought maximal inhibition of secretion (30 μM) is very close to that which enabled maximal detergent binding without loss of cell viability (Fig. 2).

In further experiments, loss of labeled Triton X-100 from the cells during detergent-free incubation periods could be directly demonstrated (Table II). The total amount of detergent present following a 10-min recovery period in detergent-free medium dropped from 30 μM to 8.4 μM. A comparison of the data in Table I, exp. Dc, with the results presented in Figure 1A shows that the release level of 60% following a 10-min recovery was very close to the value obtained when cells were stimulated in the presence of 8 μM TX-100 (Fig. 1A). It would appear that the TX-100-induced inhibition was due to a partitioning of a critical amount of detergent from the medium to the cells.

TABLE I. Release of [^{3}H]NA in PC 12 Cells After Different Treatment With 30 μM Triton X-100*

Experiment	a (5 mM K-RM) −TX-100	a (5 mM K-RM) +TX-100	b (5 mM K-RM)	c (55 mM K-RM) −TX-100	c (55 mM K-RM) +TX-100
A	12.7		—	67.9	34.9
B		17.0	—	39.3	24.4
C	12.7		15.1	67.6	35.9
D		17.0	15.9	60.8	33.6

*Cells were loaded with [^{3}H]NA and incubated as described in "Materials and Methods." After the first incubation period (column a), cell pellets, obtained by centrifuging at 110g for 5 min, were split into two aliquots and reincubated in 55 mM K-RM (c). In the experiments C and D a detergent-free incubation period of 10 min was introduced (b). Incubation media are given on top of the columns; release was calculated as mentioned in Figure 1. Values represent the averages of eight different experiments with a SD $\leqslant \pm 1.5\%$.

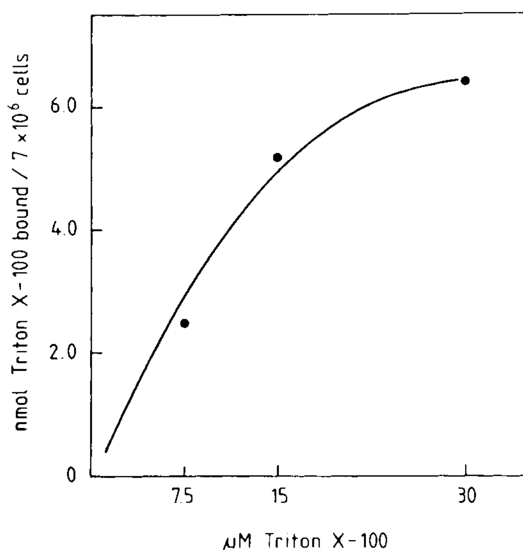

Fig. 2. Triton X-100 binding to PC 12 cells. Binding of TX-100, containing [^{3}H]TX-100, was assayed as described in "Materials and Methods." Total amount of bound detergent was calculated from the radioactivity present in the cells after incubation. Values represent the averages of four different experiments with an SD $\leqslant \pm 4.5\%$.

Commercially available preparations of TX-100 are composed of a mixture of amphipathic molecules differing in the length of their oligomeric ethyleneglycol chains. One could assume therefore that individual species could interact with cells to a different extent depending upon the length of their oligomeric chains. The result of this would be a correlation of inhibitory activity with the chain length most capable of interacting with biological membranes. To investigate this point, HPLC of TX-100 was undertaken to separate it into pure fractions of a defined oligoethyleneglycol chain length. Figure 3 indicates that as many as 11 components could be purified by this procedure. The relative amounts of the different species are also indicated. Mass spectrometry confirmed that fractions were homogenous and of a defined molecular weight.

The ability of each of these purified detergent fractions to inhibit the K^+-stimulated release of noradrenaline from PC 12 cells was tested, and the results are

TABLE II. Loss of Bound Triton X-100 From PC 12 Cells*

Detergent distribution measured	Triton X-100 (cells + medium) nmol/7 × 10^6 cells	Ratio TX-100 in cells/ TX-100 in medium
At 1st incubation	30	
		28:1
Following 1st incubation	8.4	
		65:1
Following 2nd incubation	4.0	
		65:1
Following 3rd incubation	1.9	

*Cells were incubated in 30 μM Triton X-100 containing [^{3}H]Triton X-100 as described in "Materials and Methods." After centrifugation at 110g for 5 min cell pellets were reincubated in detergent-free 5 mM K-RM. This procedure was repeated three times, and distribution of Triton X-100 between cells and medium was determined by measuring the radioactivity in the separated fractions. Ratio of Triton X-100 in cells to Triton X-100 in medium was calculated from a total cell volume of 14 μl for 7 × 10^6 cells (cell volume: 2 × 10^{-9} cm^3, diameter of 16 μm determined microscopically in phase contrast) resuspended in 1.0 ml medium.

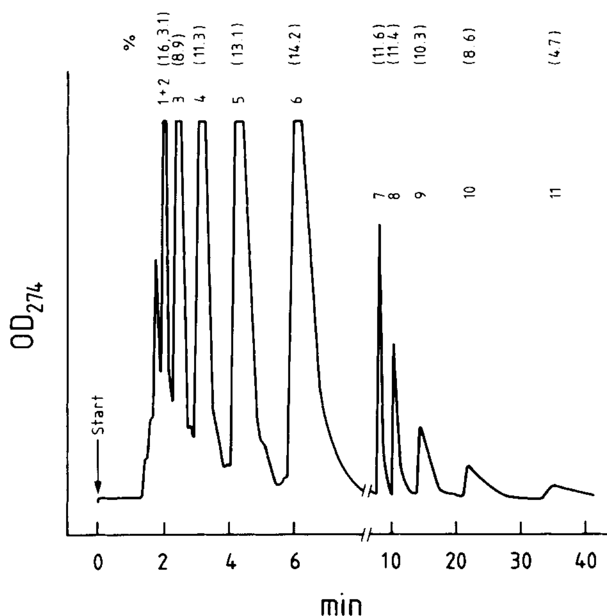

Fig. 3. Chromatographic separation of TX-100 oligomeric components by HPLC. Commercial TX-100 (2.0 mg) dissolved in 20 μl ethylacetate was separated as described in "Materials and Methods." Except for the first two fractions (numbered on top of the peaks), all other fractions could be separated by using ethylacetate saturated with water as mobile phase. The first two fractions were separated in a second run with ethylacetate as mobile phase. Each single fraction was reanalyzed by HPLC, and their degree of purity was 95% or higher (judged by integrated area of the major peak vs total peaks area). Distribution in percentage (given in parentheses on the top of the peaks) was determined by measuring the optical density (OD) at 274 nm of the isolated fractions. At this wavelength only the aromatic moiety of the detergent molecule contributes to absorption. According to GC/MS analysis, number of ethyleneglycol units in the single fraction = fraction number (on top of the peak) + 2.

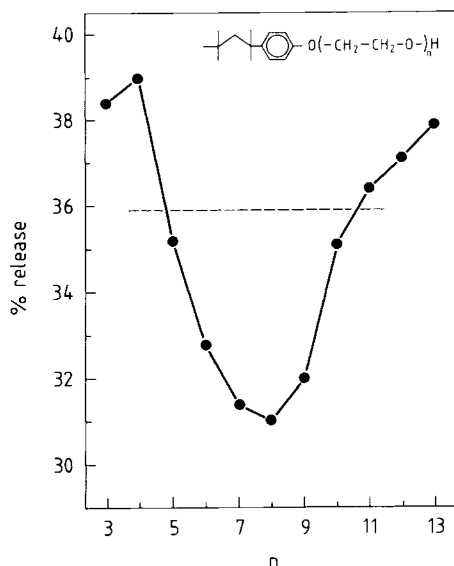

Fig. 4. Inhibition of [^{3}H]noradrenaline ([^{3}H]NA) release in PC 12 cells by purified TX-100 fractions. The isolated fractions were diluted with 55 mM K-RM to the same optical density (274) as 30 μM unfractionated TX-100. [^{3}H]NA-loaded cells were incubated with the detergent fractions, and release was determined as described in Figure 1. The release in absence of TX-100 was 67.2% (not shown). The dotted line represents the value obtained with unfractionated detergent. Values represent the averages of four different experiments with a SD $\leqslant \pm 1.0\%$. The number (n) of ethyleneglycol units of the oligomeric chain given in the abscissa was determined by GC/MS as described in "Materials and Methods."

shown in Figure 4. A clear relationship between chain length and inhibitory effect could be detected. Both short (6 carbons) and long (26 carbons) chains of ethyleneglycol are inhibitory, and maximal inhibition was achieved by detergent molecules having oligometric ethyleneglycol chains of 16 carbon atoms in length, which corresponds to the length of the fatty acid side chains of phospholipids in biological membranes.

Assuming that inhibition of noradrenaline release reflects an incorporation or intercalation of detergent molecules into the lipid bilayer, one can hypothesize that a change in the physical state of membrane lipids may have taken place. Such a change, revealed functionally in an inhibition of noradrenaline release, might be detectable by the application of the Arrhenius relationship between a physiological function and temperature.

Accordingly, K^+-stimulated noradrenaline release was examined in the presence (30 μM) and absence of TX-100 at different temperatures. As can be seen in the Arrhenius plot (Fig. 5), the discontinuity that is supposed to reflect a phase transition in the lipid bilayer was abolished in presence of the detergent. Such results have been interpreted to be indicative of a stabilization of the lipid bilayer with a corresponding decrease in membrane fluidity [24], although firm molecular interpretation is not permitted from Arrhenius plot changes.

Effect of Triton X-100 on Cellular Ion Fluxes

Changes in membrane fluidity have been shown to have a profound influence on a variety of membrane processes [11,25]. It was therefore essential to ascertain

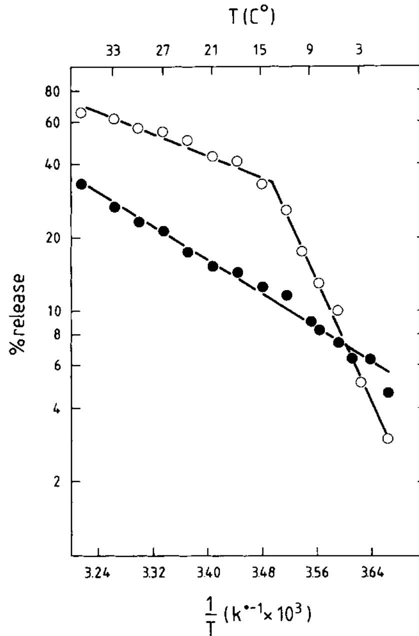

Fig. 5. Arrhenius plot of [^{3}H]noradrenaline ([^{3}H]NA) release in PC 12 cells in the presence of TX-100. [^{3}H]NA release was assayed in 55 mM K-RM, in the presence (●) and absence (○) of 30 μM TX-100. Values represent the averages of ten different experiments with a SD $\leqslant \pm 1.6\%$. The plot in the absence of detergent was clearly biphasic, with r = 0.97 for the slope from 0°C to 12°C and r = 0.98 for the one from 15°C to 37°C. Temperature dependence in presence of TX-100 could only be fitted by a single slope with a high degree of confidence (r = 0.98).

which of the individual events leading to secretion was influenced by this detergent-evoked inhibition.

In our system, K^+ functions as a trigger that depolarizes the plasma membrane, and the inhibition evoked by the detergent could cause an impairment of ion fluxes involved in the processes leading to release. Movements of ions such as Na^+, K^+, and Ca^{2+} can easily be measured by using radioactive isotopes as tracers [10,19,20]. As is shown in Figure 6, 30 μM TX-100 inhibited the inward flux of $^{22}Na^+$ and the efflux of $^{86}Rb^+$ (as a tracer for K^+) by about 50–60% under stimulating conditions. Ion fluxes examined under nonstimulating conditions (5 mM K^+) were not affected by the detergent (data not shown). This reduction of permeability may evoke an inhibition of the Ca^{2+}-influx, one of the crucial steps in the release process [26].

An initial study was undertaken to quantitate the Ca^{2+} dependence of K^+-stimulated release in our system. As is shown in Figure 7, optimal release was achieved in the presence of 2.2 mM Ca^{2+}, while in the absence of Ca^{2+}, K^+ has no effect. Therefore, subsequent experiments were conducted in the presence of 2.2 mM extracellular Ca^{2+} supplemented with 2.0 μM $^{45}Ca^{2+}$. As a control, 2.0 mM extracellular Co^{2+} [27] was used in all cases to correct for nonspecific Ca^{2+} binding. The data in Figure 8 indicate that after a 2-min stimulation with 55 mM K^+, when maximal release has occurred, the initial influx of Ca^{2+} in controls is dramatically reduced in the presence of 30 μM TX-100. The possible impairment of ATP-dependent processes such as the (Ca^{2+}, Mg^{2+})-ATPase Ca^{2+} transporter by TX-100

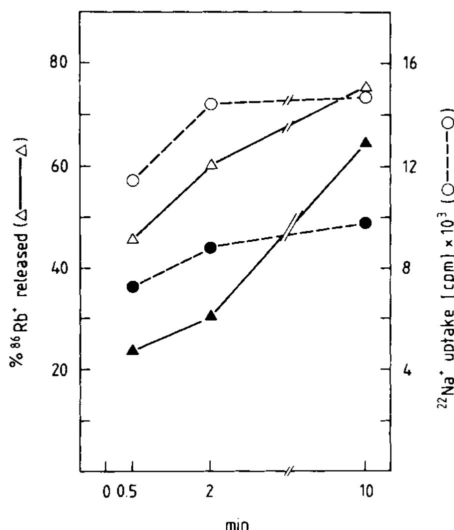

Fig. 6. $^{22}Na^+$ uptake and $^{86}Rb^+$ release in PC 12 cells. Aliquots of 10^6 cells were incubated for different times as described in "Materials and Methods." The incubation media were 55 mM K-RM (○, $^{22}Na^+$; △, $^{86}Rb^+$) and 55 mM K-RM containing 30 μM TX-100 (●,▲). $^{86}Rb^+$ efflux is expressed as the percent value of $^{86}Rb^+$ present in the washed cells prior to stimulation (100% correspond to 16,000–17,000 cpm). Values represent the averages of four different experiments with a SD $\leqslant$ 11.2% (eg, 23.7 ± 2.6% for the ▲ value after 0.5 min).

cannot be completely ruled out, although the total cellular ATP levels (Table III) tend to corroborate the hypothesis that the detergent does not inhibit enzymatic activity. The method used here is not sufficiently sensitive to allow such a subtle discrimination. In order to support the finding that the effect of TX-100 on release is a function of its inhibition of Ca^{2+} influx, it was necessary to demonstrate that the Ca^{2+} uptake followed the same inhibition pattern as a function of various detergent concentrations (Fig. 9), as it was seen in the case of noradrenaline release (see Fig. 1A) and $^{22}Na^+$ uptake (Fig. 6). The Ca^{2+} uptake inhibition curve is virtually superimposable onto the curve shown in Figure 1A, suggesting that the site of action of TX-100 is also the level of altering the movement of Ca^{2+} into the PC 12 cells during stimulation.

DISCUSSION

The results of this study indicate that exocytosis is exquisitely sensitive to very low concentrations of detergent. This effect seems to manifest itself in a stabilizing of the lipid bilayer, which results in an inhibition of Na^+, Rb^+, and Ca^{2+} movements in and out of the cell. In this way a significant inhibition of noradrenaline release is brought about.

The most remarkable feature is the fact that *micromolar* concentrations of TX-100 exerted a profound effect on a physiological process. Up to now such concentrations were only common to drug- or hormone-mediated alterations. It should be pointed out that at concentrations as low as 20 μM (0.001% w/v), a full order of magnitude less than the lowest critical micellar concentration reported [12], significant inhibition was achieved.

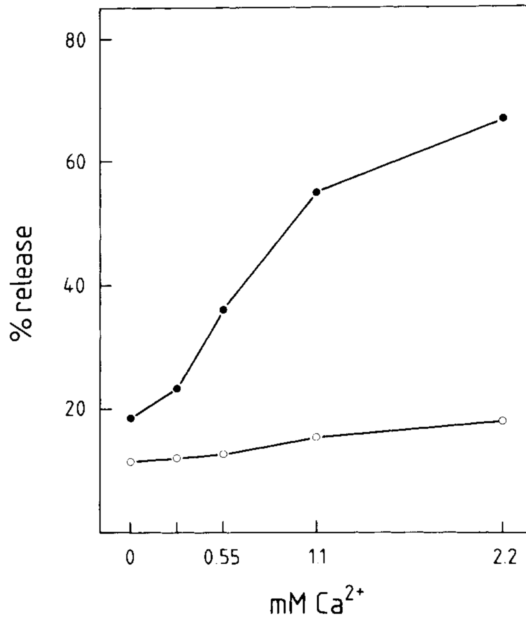

Fig. 7. Ca^{2+} dependence of [^{3}H]noradrenaline ([^{3}H]NA) release in PC 12 cells. [^{3}H]NA-loaded PC 12 cells were incubated as described in "Materials and Methods." Percentage of release was determined as described in Figure 1. Values represent the averages of five different experiments, with a SD $\leqslant \pm 2.1\%$. ○, unstimulated cells (5 mM K-RM); ●, stimulated cells (55 mM K-RM).

A plausible explanation of such an observation could be that the majority of the studies investigating detergent effects have been carried out on isolated membrane or enzyme preparations as well as on model systems [28–30]. In this case intact viable cells were used, whose sensitive regulatory and control mechanisms were undoubtedly functional and more sensitive to perturbation by detergents. Significantly, the effect was reversible and could be completely correlated with the amount of detergent that had been partitioned into the cellular phase.

As we do not have exact information about either the real extension of the plasma membrane or about how many detergent molecules are inserted into it, it is difficult to judge whether the inhibition represents a specific effect on membrane proteins and/or their boundary lipid region, or is just a dilution of the membrane lipid by TX-100. By assuming that at saturation of the plasma membrane by 6×10^8 detergent molecules/cell only 10% of the molecules are inserted into the lipid bilayer (eg, amphotericin B is incorporated at 4–8% into erythrocytes membrane, [31]) and by extrapolating the amount of 2×10^8 lipid molecules in the erythrocyte membrane [31] to the PC_{12} cells with four times the surface area, the resulting ratio of detergent to lipid becomes 1:17. This ratio would rather favor an unspecific incorporation of TX-100 into the membrane. The separation of TX-100 into homogenous subfractions indicated that a certain specificity was a part of the detergent-mediated inhibition. When the oligomeric ethyleneglycol chain was 16 carbons in length, the most profound inhibitory effect was observed. Similar results have been obtained using detergents of defined chain length in studying their effect on systems such as Ca^{2+}-ATPase in sarcoplasmic reticulum [32] and others [33]. The abolition of a phase

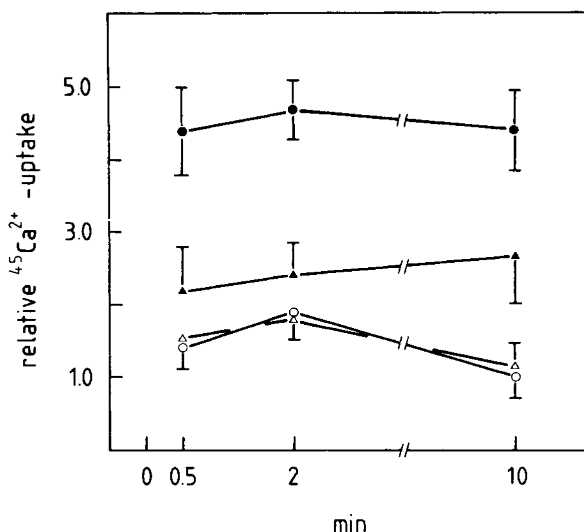

Fig. 8. $^{45}Ca^{2+}$ uptake in PC 12 cells in the presence of 30 μM TX-100. Cells were incubated for different times as described in "Materials and Methods." The incubation media were 5 mM K-RM (○), 55 mM K-RM (●), 5 mM K-RM containing 30 μm TX-100 (△), and 55 mM K-RM containing 30 μM TX-100 (▲). Bars indicate the range of SD of four different experiments. The relative $^{45}Ca^{2+}$-uptake was determined in comparison to the amount of $^{45}Ca^{2+}$ taken up by the cells after 10 min in the presence of 5 mM K-RM in the absence of TX-100, previous correction of the nonspecific uptake in the presence of 2.0 mM Co^{2+}. The relative value of 1.0 corresponds to about 2,000 cpm.

TABLE III. ATP Level in PC 12 After Incubation in 30 μM Triton X-100*

Incubation time (min)	5 mM K^+ −TX	5 mM K^+ +TX	55 mM K^+ −TX	55 mM K^+ +TX
0.5	0.60	0.58	0.65	0.65
2	0.61	0.63	0.67	0.65
5	0.61	0.72	0.72	0.67
10	0.76	0.73	0.76	0.72

*Cells were incubated in the presence or absence of Triton X-100, as shown on top of the columns. After incubation for various periods at 37°C, cells were separated by centrifugation and assayed for ATP as described in "Materials and Methods." Values are expressed in μg/mg protein and represent the averages of three different experiments with a SD $\leqslant \pm 8.5\%$.

transition caused by TX-100 as seen in the Arrhenius plot (Fig. 5) can be interpreted as being the result of a stabilization of the lipid bilayer in PC 12 cells. Data to support this notion have been obtained with detergents in model lipid systems [34].

What is not clear is the exact level at which this membrane stabilization is acting to inhibit release. The data presented here point to altered ion fluxes. Although it is impossible to say at this point whether the changes in cellular permeability of Na^+ (and Rb^+, respectively) could alone account for altered Ca^{2+} fluxes and the subsequent inhibition of noradrenaline release, the fact that Na^+ and Rb^+ fluxes are affected by detergent in a similar fashion to Ca^{2+} suggests that it is an inhibition of Ca^{2+} influx (as opposed to increased Ca^{2+} efflux) [35] which is affecting exocytosis.

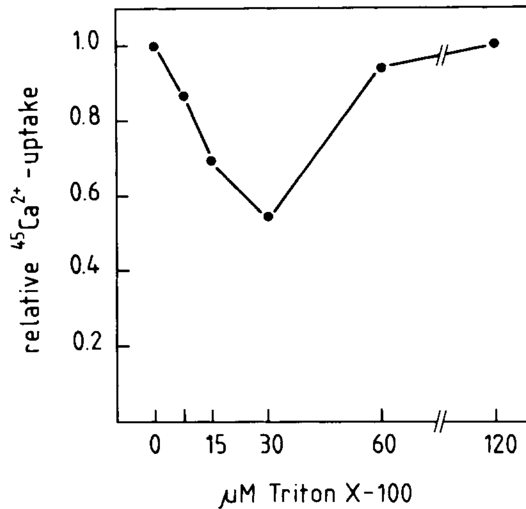

Fig. 9. $^{45}Ca^{2+}$ uptake in PC 12 cells in the presence of different concentrations of Triton X-100. A comparison was made of the relative uptake after 2 min incubation between stimulated cells in the presence and absence of detergent. Values represent the averages of three different experiments with an S.D. $\leqslant \pm 5.6\%$.

ACKNOWLEDGMENTS

This work was supported by grant 3.513.79 from the Swiss National Foundation for Scientific Research, as well as by the Ministry of the City and Canton of Basel.

C.T. was recipient of a predoctoral fellowship, Erziehungsdirektion Canton Baselland and Sandoz Ltd.

We wish to thank Dr. D.I. Meyer for the helpful discussions and a critical reading of the manuscript.

REFERENCES

1. Palade G: Science 189:347, 1975.
2. Llinas RR, Heuser JE: Neurosci Res Prog Bull 15 (4): 563–687, 1977.
3. Pollard HB, Pazoles CJ, Creutz CE, Zinder O: Int Rev Cytol 58:159, 1979.
4. Lawson D: In Gilula NB (ed): "Membrane-Membrane Interactions." New York: Raven Press, 1980, Vol. 34, p 27.
5. Plattner H: Cell Biol Int Rep 5:435, 1981.
6. Hagiwara S: Annu Rev Neurosci 4:69, 1981.
7. Greene LA, Tischler AS: Proc Natl Acad Sci USA 73:2424, 1976.
8. Greene LA, Rein G: Brain Res 129:247, 1977.
9. Schubert D, Klier FG: Proc Natl Acad Sci USA 74:5184, 1977.
10. Lawrence T: J Biol Chem 257:13189, 1982.
11. Poste G, Allison AC: Biochim Biophys Acta 300:421, 1973.
12. Helenius A, Simons K: Biochim Biophys Acta 415:29, 1975.
13. Seeman P: Pharmacol Rev 24:583, 1972.
14. Westerink BHC, Korf J: J Neurochem 29:697, 1977.
15. Smith JE, Lane JD, Aprison MH: Anal Biochem 64:149, 1975.
16. Lowry OH, Rosebrough NS, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
17. Holm-Hansen O, Karl DM: Methods Enzymol 57:73, 1978.
18. Kikuchi M, Wollheim CB, Cuendet GS, Renold AE, Sharp GWG: Endocrinology 102:1339, 1978.

19. Henquin JC: Nature 280:66, 1979.
20. Tatsumi M, Takahashi M, Ohizumi Y: Mol Pharmacol 25:379, 1984.
21. Rebois RV, Reynolds EE, Toll L, Howard BD: Biochemistry 19:1240, 1980.
22. Roda LG, Nolan JA, Kim SU, Hogue-Angeletti RA: Exp Cell Res 128:103, 1980.
23. Tapparelli C: "Secretion of Noradrenaline in Rat Pheochromocytoma PC 12 Cells." Thesis, University of Basel, 1980.
24. Lenaz G, Curatola G, Mazzanti L, Parenti-Castelli G: Mol Cell Biochem 22:3, 1978.
25. Papahadjopoulos D, Jacobson K, Poste G, Shepherd G: Biochim Biophys Acta 394:504, 1975.
26. Stallcup WB: J Physiol (Lond) 286:541, 1979.
27. Ritchie AK: J Physiol (Lond) 286:541, 1979.
28. Sharma RK, Wang JH: Biochem Biophys Res Commun 100:710, 1981.
29. Tanford C: "The Hydrophobic Effect." New York: John Wiley & Sons, 1973.
30. Niggli V, Adunyah ES, Penniston JT, Carafoli E: J Biol Chem 256:395, 1981.
31. Schneider E, Haest CWM, Plasa G, Deuticke B: Biochim Biophys Acta 855:325, 1986.
32. Dean WL, Suarez CP: Biochemistry 20:1743, 1981.
33. Makino S, Reynolds JA, Tanford C: J Biol Chem 248:4926, 1973.
34. Madden TD, Cullis PR: Biochim Biophys Acta 684:149, 1982.
35. Barritt GJ: Trends Biochem Sci 6:322, 1981.