Drastic facilitation by α-latrotoxin of bovine chromaffin cell exocytosis without measurable enhancement of Ca^{2+} entry or $[Ca^{2+}]_i$

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1. Latrotoxin (LTX, 1–3 nM) caused a gradual increase of the spontaneous catecholamine release rate in bovine adrenal chromaffin cells superfused with normal Krebs–Hepes solution containing 2·5 mM Ca^{2+}. Ca^{2+} removal abolished this effect. LTX enhanced also the secretory responses to high K^+ (35 or 70 mM) and to acetylcholine (ACh, 30 μM).

2. The application of Ca^{2+} pulses to cells previously superfused with a 0 Ca^{2+} solution (Krebs–Hepes deprived of $CaCl_2$) induced secretory responses that gradually reached 400–800 nA of catecholamines, provided that LTX was present. The responses to ACh or 35 mM K^+ pulses (in the presence of Ca^{2+}) were also enhanced by LTX, from around 100–200 nA to over 1000 nA. Though such enhancement remained in the presence of Ca^{2+} channel blockers, it disappeared upon the lowering of $[Na^+]_o$ or in electroporated cells.

3. Using protocols similar to those of secretion, LTX did not enhance basal $^{45}Ca^{2+}$ uptake, whole-cell Ca^{2+} currents or basal $[Ca^{2+}]_i$. In fact, LTX attenuated the K^+- or ACh-evoked increases in $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$.

4. It is proposed that the secretory response to brief periods of Ca^{2+} reintroductions is triggered by local subplasmalemmal Ca_i^{2+} transients, produced by the Na^+–Ca^{2+} exchanger of the plasma membrane working in the reverse mode. This situation might be physiologically reproduced during ACh stimulation of chromaffin cells, which is followed by the firing of Na^+-dependent action potentials.

Ionophoric compounds have been important tools in clarifying the role of ions in controlling various physiological processes (Pressman, 1973). At the time when patch-clamp techniques were unavailable, Na^+ and Ca^{2+} ionophores were widely used to modify ion gradients in secretory cells and study the functional consequences for the Ca^{2+}-dependent exocytotic process. Pioneering works showed that the Ca^{2+}-selective ionophore A23187 triggered the secretion of histamine from mast cells in a Ca^{2+}-dependent manner (Foreman, Mongar & Gomperts, 1973; Cochrane & Douglas, 1974). Subsequently García, Kirpekar & Prat (1975) showed that A23187 evoked the secretion of catecholamines from the perfused cat adrenal gland in a Ca^{2+}-, concentration- and time-dependent manner. Then, it was demonstrated that other non-selective ionophores such as X537A, or the highly selective Ca^{2+} ionophore ionomycin also triggered catecholamine release from sympathetic neurones (Pascual, Horga, Sánchez-García & García, 1977) and adrenal medullary chromaffin cells (Carvalho, Prat, García & Kirpekar, 1982). Those earlier ionophores behave as carriers that transport ions across membranes along favourable gradients. The appearance of pore-forming ionophores such as alamethicin stimulated further research trying to mimic the physiological behaviour of native ion channel pores (Eisenberg, Hall & Mead, 1973; Latorre & Alvarez, 1981). Alamethicin was shown to activate the entry of Ca^{2+} and Mn^{2+} into bovine chromaffin cells and to trigger a Ca^{2+}-dependent secretory signal (Fonteríz, López, García-Sancho & García, 1991).

A toxin that stimulates a massive release of neurotransmitters from presynaptic nerve terminals is α-latrotoxin (LTX), a component of the venom from the black widow spider. The mechanisms underlying those drastic effects are still puzzling. One hypothesis is that LTX binds to synaptic neurexins whose activation causes the recruitment of

transmitter-storing vesicles to active exocytotic sites at the presynaptic plasma membrane (Petrenko, 1993). Another hypothesis attributes pore-forming properties to LTX, thus causing Na^+ and Ca^{2+} entry into the axoplasm and the activation of the secretory machinery (Rosenthal, Zacchetti, Madeddu & Meldolesi, 1990; Filippov, Tertishnikova, Alekseev, Tsurupa, Pashkov & Grishin, 1994). A still more recent hypothesis attributes to LTX direct action on the protein secretory machinery (Surkova, 1994).

In vivo and *in vitro* studies concluded that LTX has no effects on [^{3}H]noradrenaline release from suspensions of cultured rat chromaffin cells. Picotti, Bondiolotti & Meldolesi (1982) concluded that 'LTX binding and its amine releasing effect seems, therefore, to be specific for neurons and absent from other cells, even those, like adrenomedullary cells and blood platelets, which share with neurons their origin and/or other important characteristics'. In contrast to this earlier observation, the study presented here shows profound effects of LTX on spontaneous as well as on the evoked catecholamine release in bovine chromaffin cells. These facilitatory effects are completely dependent on extracellular Ca^{2+} (Ca_o^{2+}) though, paradoxically, they are not accompanied by a concomitant enhancement of Ca_o^{2+} entry or the cytosolic concentration of Ca^{2+}, $[Ca^{2+}]_i$. While this study complements others in the literature seeking to understand the actions of LTX on various secretory systems, it has also a clear-cut physiological implication: that Na^+ ion movements and the Na^+–Ca^{2+} exchanger might be regulating the catecholamine secretory signal triggered by acetylcholine, the neurotransmitter at the splanchnic nerve–chromaffin cell synapse (Douglas & Rubin, 1961).

METHODS

Preparation and culture of bovine chromaffin cells

Bovine adrenal medulla chromaffin cells were isolated following standard methods (Livett, 1984) with some modifications (Moro, López, Gandía, Michelena & García, 1990). Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 i.u. ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. For secretion experiments, cells were plated in 5 cm diameter Petri dishes (3×10^6 cells per 5 ml DMEM). For $^{45}Ca^{2+}$ uptake studies, cells were plated in twenty-four-well plates at a density of 5×10^5 cells per well. For measurements of cytosolic Ca_i^{2+} transients and ionic currents cells were plated on 1 cm diameter glass coverslips at a density of 5×10^4 cells per coverslip.

Catecholamine release from intact cells

Cells (3×10^6) were placed in a microchamber and superfused at room temperature (23 ± 2 °C) with Krebs–Hepes solution of the following composition (mM): NaCl, 144; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; Hepes, 10; and glucose, 10; pH 7·4.

The rate of superfusion was 1 ml min^{-1}; the liquid flowing from the perfusion chamber reached an amperometric detector through a thin polyethylene tube. Electrochemical detection of standard and released catecholamines was performed according to Borges, Sala & García (1986), with a Methrom amperometric detector equipped with a glassy carbon working electrode, a Ag–AgCl reference electrode and a gold auxiliary electrode. Catecholamines were oxidized at a potential of +0·65 V and the oxidation current signal was digitized and recorded with a computer.

Known concentrations of adrenaline were administered directly to the detector to obtain a standard curve. Under these conditions, 1 μg ml^{-1} adrenaline produced an amperometric signal of 74 nA. Absolute values of catecholamine release from the cells could be quantified by measuring peak heights and comparing them with those obtained with adrenaline standards.

Before starting the experiments, the cells were superfused for 10–15 min with Krebs–Hepes solution containing Ca^{2+} (2·5 mM) or deprived of Ca^{2+} (nominal 0 Ca^{2+} solution), and allowed to reach a steady-state rate of basal secretion. After this, LTX (1–3 nM) was introduced under the different conditions described in Results. Changes of superfusion solution were performed by using miniature solenoid valves connected to a computer. Catecholamine release was studied under resting conditions (spontaneous output) or in response to brief pulses of acetylcholine (ACh) or high K^+ solutions. The stimulation solutions are referred to as ACh (30 μM ACh dissolved in normal Krebs–Hepes solution containing 2·5 mM Ca^{2+}), 30 mM K^+ or 70 mM K^+ (Krebs–Hepes solutions with 35 mM or 70 mM K^+, with equimolar reduction of NaCl to keep isotonicity), or Ca^{2+} (the normal Krebs–Hepes solution containing 2·5 mM Ca^{2+}, which was reintroduced after a period of cell superfusion with 0 Ca^{2+}).

Catecholamine release from electroporated cells

Cells were washed in Ca^{2+}-free Krebs–Hepes solution and resuspended (4×10^6 cells ml^{-1}) in an electroporation buffer of the following composition (mM): potassium glutamate, 140; $MgCl_2$, 3; EGTA, 1; Hepes, 20; K_2-ATP, 2; and 0·5% bovine serum albumin; pH 7. Cells were rendered permeable by ten discharges of 1500 V at a capacitance of 3 μF, using a Gene Pulser apparatus (Bio-Rad) with 10 s in between the discharges, in a cuvette (0·8 ml) with two electrodes 0·4 cm apart. Electroporated cells were then trapped in a microchamber and superfused with electroporating solution for 10 min to reach a steady-state basal rate of secretion. Secretion was then activated by application of given Ca^{2+} concentrations.

Electrophysiological recordings

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature (23 ± 2 °C) with a control Tyrode solution containing (mM): NaCl, 137; $MgCl_2$, 1; $CaCl_2$, 2; Hepes, 10; glucose, 10; pH 7·4, titrated with NaOH. For current recording, 10 mM Ca^{2+} was used as the charge carrier and 5 μM tetrodotoxin (TTX) was added to the perfusion solution. Cells were dialysed with an intracellular solution containing (mM): NaCl, 10; CsCl, 110; TEACl, 20; EGTA, 14; Hepes, 20; MgATP, 5; GTP, 0·3; pH 7·2, titrated with CsOH.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 8900 patch-clamp amplifier, allowing cancellation of capacitative transients and compensation of series resistance. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pCLAMP software (Axon Instruments) were used to acquire

and analyse the data. Control and test solutions were changed using a multi-barrelled concentration-clamp device (Gandía, García & Morad, 1993*b*), the common outlet of which was placed within 100 μm of the cell to be patched. The flow rate (0·2–0·5 ml min^{-1}) was regulated by gravity to achieve a complete replacement of the cell surroundings within less than 1 s.

Measurement of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake studies were carried out in cells after 2–3 days in culture. Before the experiment, cells were washed twice with 0·5 ml Krebs–Hepes solution of the following composition (mM): NaCl, 140; KCl, 5·9; $MgCl_2$, 1·2; $CaCl_2$, 0·5; glucose, 11; Hepes, 10; pH 7·2 and 37 °C. $^{45}Ca^{2+}$ uptake into chromaffin cells was studied by incubating the cells at 37 °C with $^{45}Ca^{2+}$ at a final concentration of 3 μCi ml^{-1} in the presence of Krebs–Hepes solution (basal uptake), high K^+ solution (Krebs–Hepes solution containing 70 mM KCl or 35 mM KCl with isosmotic reduction of NaCl), or acetylcholine (30 μM). This incubation was carried out for 1 min or 10 s; at the end of this period, the test medium was rapidly aspirated and the uptake reaction was ended by adding 0·5 ml of a cold Ca^{2+}-free Krebs–Hepes solution containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals.

To measure the radioactivity retained by chromaffin cells, the cells were scraped with a plastic pipette tip while adding 0·5 ml 10% trichloroacetic acid; 3·5 ml of scintillation fluid (Ready Micro, Beckman) was added and the samples counted in a Packard beta counter. Results are expressed as a percentage of $^{45}Ca^{2+}$ taken up by control cells.

Intracellular Ca^{2+} measurements

Cells attached to glass coverslips were loaded with the acetoxymethyl ester form of the fluorescent dye fura-2 (fura-2 AM) (2·5 μM for 40 min at 25 °C, in the dark). Then, the cells were washed with Krebs–Hepes solution and kept for 10 min at 37 °C in an incubator before being placed on the stage of an inverted microscope, in a chamber allowing their continuous superfusion with Krebs–Hepes solution. Solutions were applied to the cell under investigation using the fast superfusion device employed in the electrophysiological studies. Only one experimental protocol was run on each single coverslip. Single cell fluorescence measurements were performed by exciting the fura-2-loaded cells with alternating 360 and 390 nm filtered light. The apparent $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence signals according to Grynkiewicz, Poenie & Tsien (1985):

$$[Ca^{2+}]_i = K_{eff}(R - R_0)/(R_1 - R),$$

where K_{eff} is an 'effective binding constant', R_0 is the fluorescence ratio at zero Ca^{2+} and R_1 is the limiting ratio at high Ca^{2+}. These calibration constants were experimentally determined as described by Almers & Neher (1985).

Materials and solutions

α-Latrotoxin was obtained from Alomone Labs (Jerusalem, Israel), or Latoxan (Rosans, France) and reconstituted in distilled water to obtain a final concentration of 1 μM. Toxin was kept in aliquots at −20 °C until use. Furnidipine was a gift from Drs Carlos Sunkel and Miguel Fau (Laboratorios Alter, Madrid); it was dissolved in ethanol (10 mM) and diluted in the 0 Ca^{2+} solution. ω-Conotoxin MVIIC was purchased from Bachem Ltd (Bubendorf, Switzerland), dissolved in distilled water and kept frozen at −20 °C until use. Fura-2 AM was obtained from Molecular Probes. DMEM, fetal calf serum and antibiotics were purchased from Gibco (Madrid). Collagenase type B from *Clostridium histolyticum* was obtained from Boehringer Mannheim (Madrid). Other chemicals were obtained either from Sigma or Merck (Madrid). Stimulation solutions are referred to as the ACh solution (a Krebs–Hepes solution containing 30 μM ACh and 2·5 mM Ca^{2+}), the 35 or 70 mM K^+ solutions (Krebs–Hepes solutions containing 35 or 70 mM K^+, with 2·5 mM Ca^{2+}, with concomitant isosmotic reduction of Na^+).

Statistical analysis

Results are expressed as means ± S.E.M. The statistical differences between means of two experimental results were assessed by Student's t test. A value of P equal to or smaller than 0·05 was taken as the limit of significance.

RESULTS

The effects of LTX on spontaneous and K^+-evoked secretion under conditions of continuous superfusion of cells with a Ca^{2+}-containing Krebs–Hepes solution

These initial experiments were performed to test the effects of LTX on spontaneous and evoked catecholamine release from chromaffin cells superfused continuously with a normal Krebs–Hepes solution containing 2·5 mM Ca^{2+} (Fig. 1). In Fig. 1*A*, cells were challenged with three 10 s pulses of a 70 mM K^+ solution given at 10 min intervals. Secretory peaks of about 100 nA were produced. Switching to 0 Ca^{2+} caused an immediate drop of release to basal levels. Ca^{2+} reintroduction caused a sharp secretory peak of 200 nA, that declined gradually as LTX washed off. In Fig. 1*B*, after an initial 10 min equilibration period, the resting release of catecholamines stabilized at about 20 nA. A 20 s pulse of 70 mM K^+ produced a sharp secretion peak of 350 nA. Subsequently, LTX (1 nM for 5 min) produced a delayed increase of the basal rate of secretion that reached a maximum of 80 nA and reversed slowly after the toxin washout. In spite of this reversion, two final 70 mM K^+ pulses produced secretion peaks as high as 800 and 700 nA. Figure 1*C* shows the original traces obtained in a different batch of cells subjected to a similar protocol, but using 3 nM LTX during a 5 min period. Here the K^+ peaks reached 200–250 nA height. LTX enhanced the basal catecholamine output at a rate much higher than that of 1 nM and reached a maximum at around 200 nA (compare with Fig. 1*B*). Again, the first 70 mM K^+ response (given during the decline of the secretory response produced by LTX) was slightly potentiated (about 420 nA), but the subsequent responses were similar to those obtained before giving the toxin.

In the experiments of Fig. 2, the effects of LTX (1 nM in Fig. 2*A*, 3 nM in Fig. 2*B*) on basal secretion and on the secretion evoked by alternating 10 s pulses of ACh or 35 mM K^+ were studied. Moderate concentrations of ACh (30 μM) and K^+ (35 mM) were chosen to avoid saturation of secretory responses, so that the possible LTX-enhancing effects on evoked secretion could be unmasked. Pulses of ACh and 35 mM K^+ were alternated at 2 min intervals; this

frequent pattern of stimulation required the increase of the superfusion flow from 1 to 2 ml min^{-1} so that secretion could reach basal levels between two stimuli. A strong depolarizing 10 s pulse of 70 mM K^+ was given at the beginning and at the end of each experiment, to assess the viability of the cells' secretory machinery. A maximum initial secretory response of near 800 nA was generated by the 70 mM K^+ pulse. The initial 35 mM K^+ response was 300 nA and that of ACh amounted to around 200 nA. On repeated stimulation, the ACh but not the K^+ response underwent a progressive decline, probably reflecting a gradual desensitization of the nicotinic acetylcholine receptor (nAChR; Schiavone & Kirpekar, 1982). At 1 nM (Fig. 2*A*), LTX enhanced slightly and gradually the baseline secretion. The 35 mM K^+ response remained unchanged and that of ACh initially increased and then declined. At 3 nM (Fig. 2*B*), the basal secretion increased initially to over 200 nA and then declined even before the washout of LTX. Both the ACh and the 35 mM K^+ responses were enhanced first, and then declined even in the continued presence of the toxin.

Effects of LTX on catecholamine release stimulated by pulses of Ca^{2+}, ACh or 35 mM K^+ under conditions of continuous superfusion of cells with a nominal 0 Ca^{2+} solution

In the experiments of Fig. 3, cells were superfused continuously at 2 ml min^{-1} with a 0 Ca^{2+} solution (nominal 0 Ca^{2+}). After a 10 min initial equilibration period to stabilize the basal secretory rate (usually at around 50–100 nA), catecholamine release was activated by alternating 10 s pulses of Ca^{2+} and either ACh (Fig. 3*A*) or 35 mM K^+ solution (Fig. 3*B*). A strong depolarizing 10 s 70 mM K^+

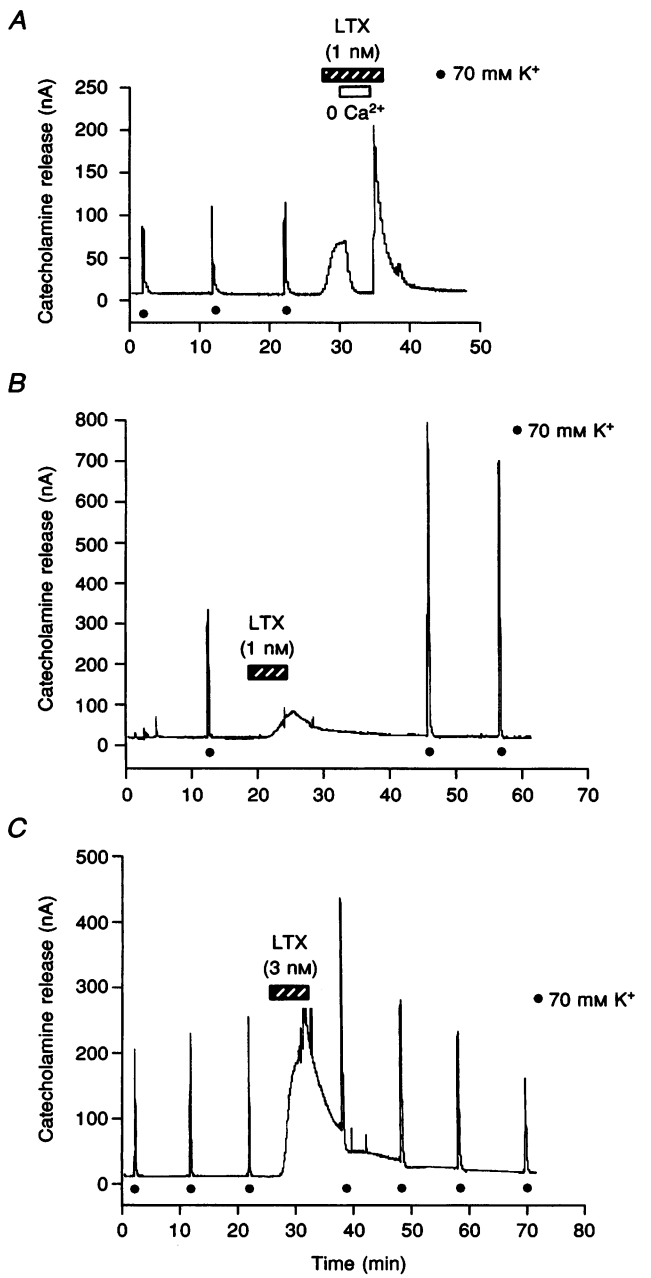

Figure 1. Latrotoxin (LTX) enhanced the spontaneous catecholamine output, and potentiated the secretory response to 70 mM K^+, in cells superfused continuously with a solution containing 2·5 mM Ca^{2+}

Bovine chromaffin cells were superfused continuously with a Krebs–Hepes solution containing 2·5 mM Ca^{2+} at a rate of 1 ml min^{-1}. Depolarization-evoked secretion was triggered by applying 20 s pulses of a 70 mM K^+ solution (circles at the bottom). LTX (1 nM in *A* and *B*, 3 nM in *C*) was applied as shown by the horizontal bars. Secretion was monitored on-line using an electrochemical detector. In *A*, the Ca^{2+}-containing Krebs–Hepes solution was changed to a nominal 0 Ca^{2+} solution during the time period shown by the open bar. Catecholamine release is expressed as nanoamps of oxidation current.

pulse was given at the beginning and at the end of each individual experiment to test the viability of the secretory machinery. When the secretory test signal induced by 70 mM K^+ was smaller than 200 nA, cells were discarded.

The Ca^{2+} pulses produced initially almost undetectable secretory responses, usually about 20 nA above basal. The initial ACh pulses enhanced the basal secretion from 50 to 200 nA. The response to ACh gradually desensitized to reach 80 nA at the fourth pulse. After a delay of 4–6 min, LTX (1 nM) caused a drastic and gradual increase of the responses to Ca^{2+} and ACh. Thus, the Ca^{2+} responses which were undetectable in the absence of LTX, reached 640 nA after 20 min superfusion with the toxin, and those of ACh reached 1000 nA (Fig. 3*A*). Secretion was maintained at a high level during the 20 min period of superfusion with LTX, and then gradually declined. After a 20 min washout period, ACh responses remained elevated (around 400 nA), the Ca^{2+} responses being at about 150 nA. The final 70 mM K^+ response was even higher than that evoked by the initial 70 mM K^+ test pulse, thus indicating that the secretory machinery was still fully functional at the end of the experiment, and that cells were not unspecifically damaged by the toxin. It is worth noting that LTX did not by itself modify the basal secretion, as expected from the fact that cells were being continuously superfused with 0 Ca^{2+} solution, and LTX required Ca_o^{2+} in order to enhance the rate of basal secretion (Figs 1 and 2).

Figure 3*B* shows a similar experiment, but here cells were stimulated with alternating 10 s pulses of Ca^{2+} and 35 mM K^+. The initial Ca^{2+} pulses gave increments of secretion of only 20 nA, while the K^+ pulses increased it to around 200 nA. Contrary to ACh, the responses to 35 mM K^+ scarcely declined. Again, after a 4–6 min delay LTX (1 nM) enhanced the secretory responses to Ca^{2+} (near 200 nA) and

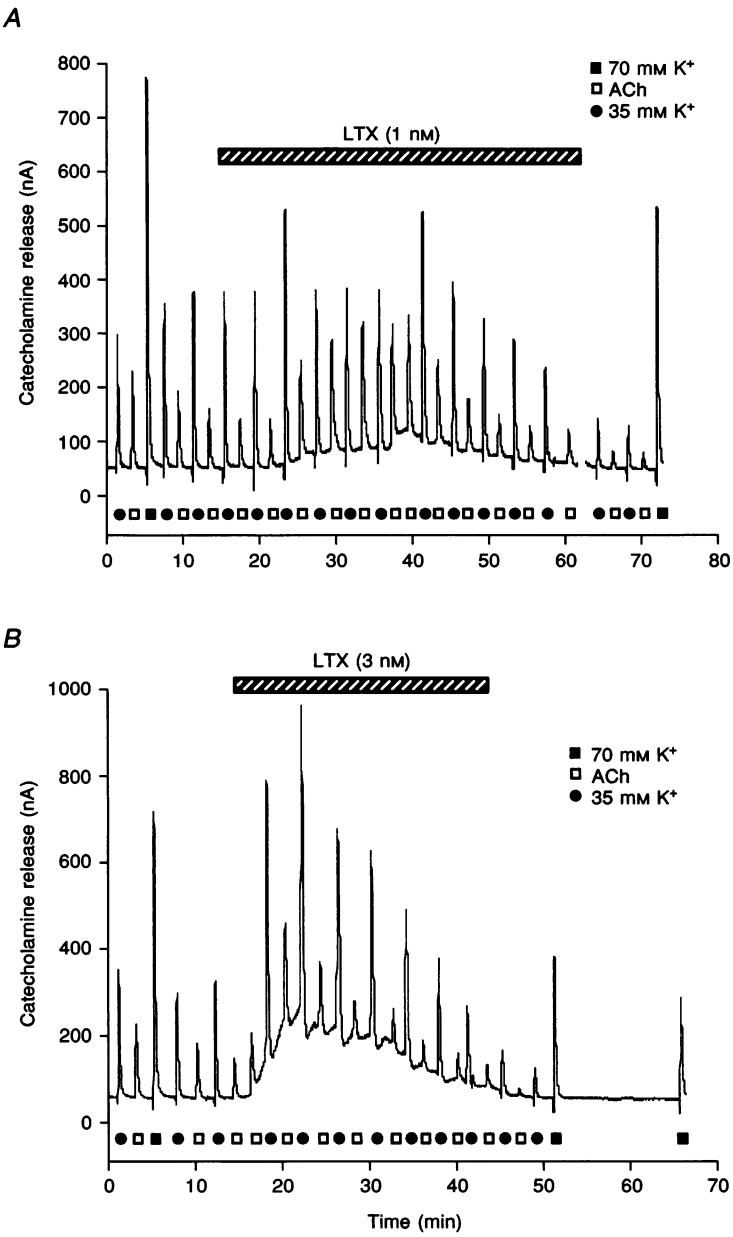

Figure 2. Latrotoxin (LTX) augments the basal as well as the evoked catecholamine output, induced by ACh or 30 mM K^+ pulses, in cells superfused continuously with Krebs–Hepes solution containing 2·5 mM Ca^{2+}

Chromaffin cells were superfused continuously with a Krebs–Hepes solution containing 2·5 mM Ca^{2+} at a rate of 2 ml min^{-1}. Once the basal secretion stabilized, evoked secretion was triggered by alternating every 2 min ACh pulses (30 μM ACh for 10 s) and K^+ pulses (35 mM K^+ for 10 s). Peaks labelled 70 mM K^+ correspond to 70 mM K^+ pulses given at the beginning and at the end of each experiment, to test the viability of the secretory machinery. LTX was applied at 1 nM (*A*) or 3 nM (*B*) during the period shown by the horizontal bars.

to 35 mM K^+ (near 1000 nA). Once more, the enhancement of the response was partially reversible after washout of LTX. The final 70 mM K^+ test pulse gave a secretory response (near 800 nA) similar to that obtained at the beginning.

Figure 3*C* shows a similar experiment but using 3 nM instead of 1 nM LTX. After LTX introduction, the secretory responses to 35 mM K^+ pulses quickly jumped from around 200 nA to near 800 nA; the responses to Ca^{2+} pulses were increased also to near 800 nA after LTX. Note that this potentiation developed much faster than with 1 nM LTX and in the case of Ca^{2+} pulses, the potentiation of secretion was greater.

In summary, LTX transforms a mild Ca_o^{2+}-dependent secretory response induced by moderate concentrations of ACh or K^+, or an almost undetectable secretory response to Ca^{2+} reintroduction, into supramaximal secretory responses. This drastic potentiation amounted to 621 ± 61% for ACh ($n = 6$), 341 ± 16% for 35 mM K^+ ($n = 6$) and 1592 ± 239% for Ca^{2+} pulses ($n = 12$).

Blockade of Ca^{2+} entry through voltage-dependent Ca^{2+} channels did not prevent the potentiation of K^+-evoked secretion induced by LTX

Interruption of Ca^{2+} entry through different voltage-dependent Ca^{2+} channel subtypes by using specific Ca^{2+} channel blockers did not substantially decrease the potentiating effects of LTX on secretion induced by 35 mM K^+ pulses. Thus, 1 μM furnidipine, a selective blocker of L-type Ca^{2+} channels in bovine chromaffin cells (Gandía, Albillos & García, 1993*a*), inhibited substantially the initial

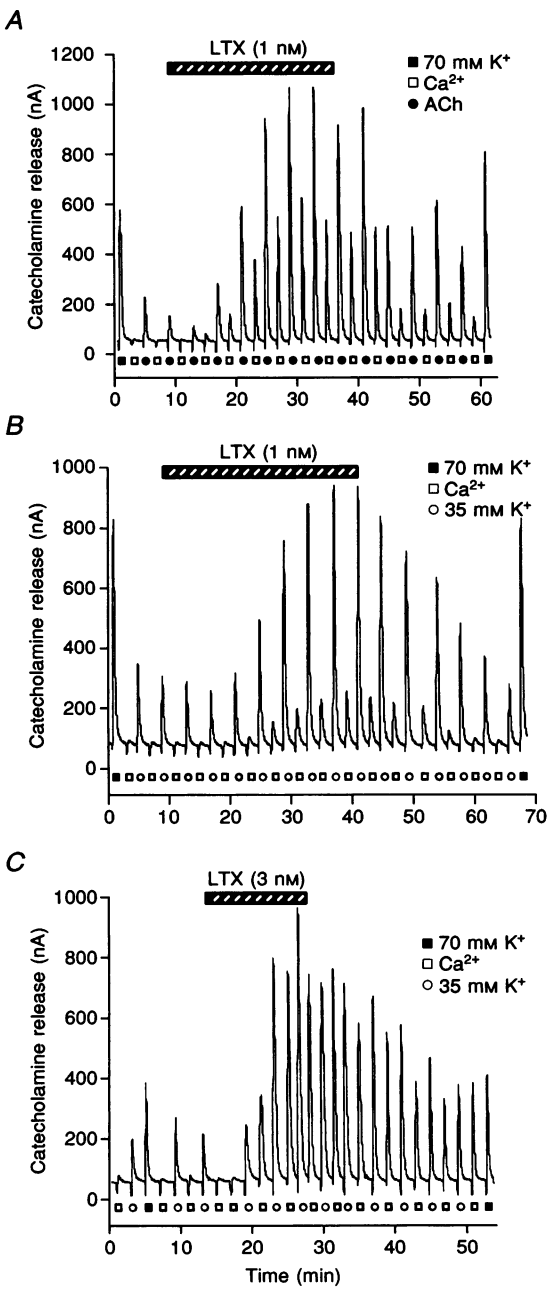

Figure 3. Latrotoxin (LTX) potentiates the secretion of catecholamines evoked by pulses of Ca^{2+}, ACh or K^+, in cells superfused continuously with a nominal 0 Ca^{2+} solution

Cells were continuously superfused with a nominal 0 Ca^{2+} solution (Krebs–Hepes solution without added $CaCl_2$). In *A*, cells were challenged alternately with 10 s pulses of Ca^{2+} (2·5 mM Ca^{2+} in Krebs–Hepes solution) and with 30 μM ACh plus 2·5 mM Ca^{2+}. In *B* and *C*, cells were stimulated at 2 min intervals, alternately with the Ca^{2+} and with the 35 mM K^+ solution; LTX was given at 1 nM (*A* and *B*) or at 3 nM (*C*), during the time shown by the horizontal bars. At the beginning and at the end of the experiment, cells were challenged for 10 s with a solution containing 70 mM K^+ and 2·5 mM Ca^{2+} (■).

secretory responses to 35 mM K^+ pulses (Fig. 4*A*). However, in subsequent 35 mM K^+ pulses in the presence of 1 nM LTX, the secretory signal rose from about 50–75 nA to 1300 nA. In the presence of ω-conotoxin MVIIC, a blocker of N, P and Q subtypes of Ca^{2+} channels in bovine chromaffin cells (López *et al.* 1994), the potentiating effects of LTX on secretion were also very marked. So, in the experiment shown in Fig. 4*B*, the secretory response rose from 50 nA to about 800 nA. Finally, the combined use of ω-conotoxin MVIIC plus furnidipine (1 μM of each) did not prevent the potentiation of the secretion signal (Fig. 4*C*). In one experiment, the secretory response was decreased in cells treated with furnidipine plus ω-conotoxin MVIIC.

Transient elevations of $[Ca^{2+}]_i$ are not required for development of the LTX-potentiating effects on secretion

In the experiments of Figs 3 and 4, cells were superfused with a 0 Ca^{2+} solution and exposed intermittently to Ca^{2+} during the 10 s depolarizing pulses applied every 2 min. Therefore, it was not clear whether LTX required transient elevations of cytosolic Ca^{2+} to facilitate the subsequent evoked secretory responses, i.e. by increasing the transport of catecholamine-storing vesicles from a reserve pool to the ready-release vesicle pool (Von Rüden & Neher, 1993). In fact, the gradual development of the potentiation by 1 nM LTX suggests that such intermittent cytosolic Ca^{2+} transients could be required. To test this hypothesis, an experiment

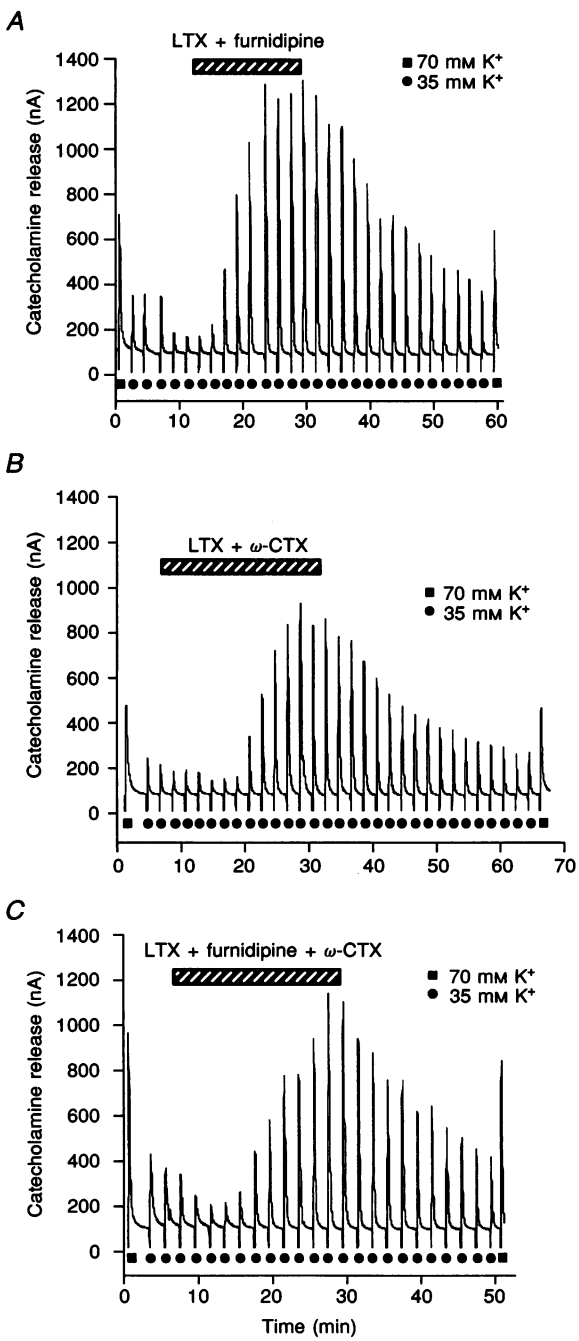

Figure 4. Selective blockade of Ca^{2+} entry through voltage-dependent Ca^{2+} channels did not prevent the potentiation of K^+-evoked secretion induced by LTX

Cells were continuously superfused with a 0 Ca^{2+} solution and challenged at 2 min intervals with 10 s pulses of 35 mM K^+ (circles at the bottom of each spike). The first and the last pulses were given with a 70 mM K^+ test solution. LTX (1 nM) was given together with 1 μM furnidipine (*A*), 1 μM ω-conotoxin MVIIC (*B*) or furnidipine plus ω-conotoxin MVIIC (*C*) for the time period shown by the top horizontal bars in each panel.

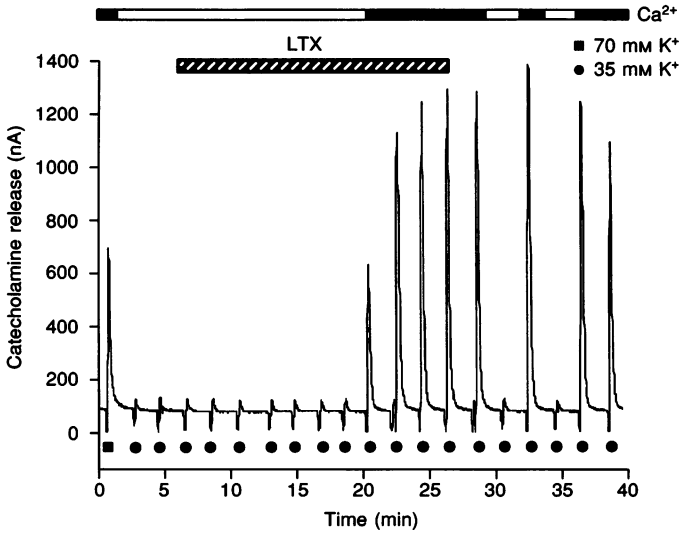

Figure 5. Transient elevations of $[Ca^{2+}]_i$ are not required for development of the LTX-potentiating effects on secretion

Cells were superfused with a nominal 0 Ca^{2+} solution (□) or with a normal Krebs–Hepes solution containing 2·5 mM Ca^{2+} (■). LTX (1 nM) was given during the period shown by the top horizontal bar. The first secretory peak was obtained with a 10 s pulse of 70 mM K^+. The subsequent depolarizing pulses were given with a 35 mM K^+ solution with or without Ca^{2+}, as shown by the horizontal bar at the top and the circles.

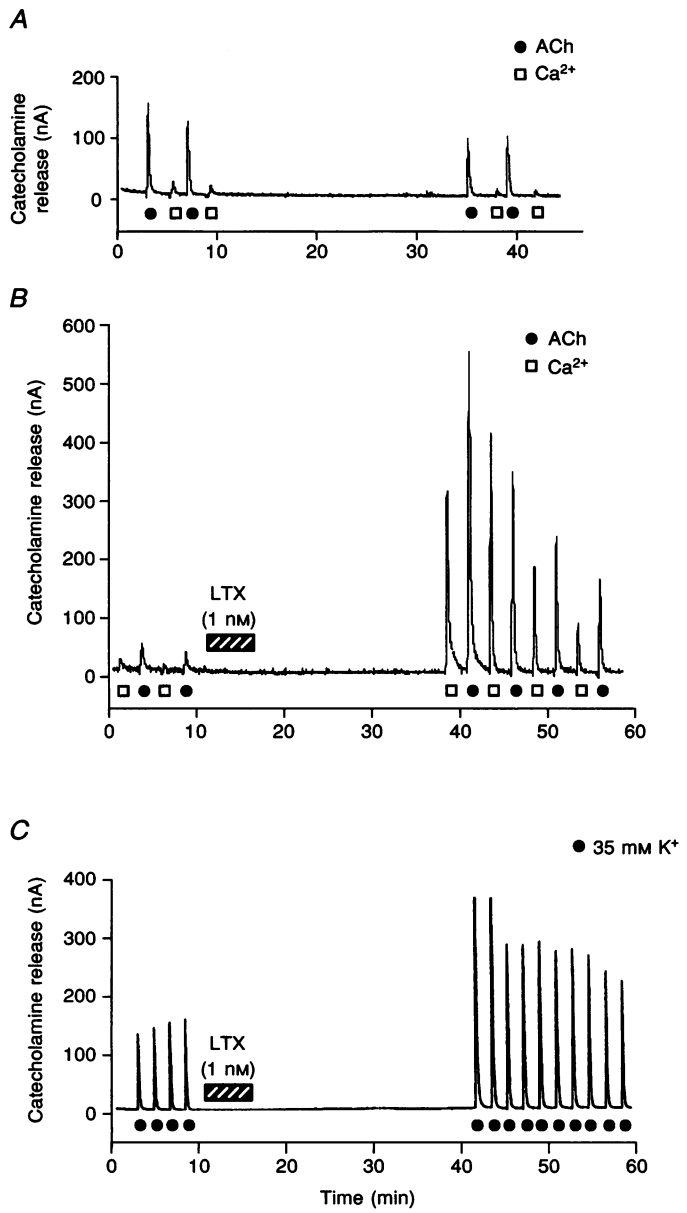

Figure 6. A short treatment with LTX followed by a 'silent' 20 min washout period (without stimulation) in 0 Ca^{2+} secures the potentiation of secretion

Cells were continuously superfused with 0 Ca^{2+}. The protocol consisted of four parts: (i) stimulation with pulses of ACh (30 μM for 10 s), 35 mM K^+ or Ca^{2+}; (ii) treatment with LTX in 0 Ca^{2+} (1 nM) for 5 min as shown by the horizontal bar; (iii) a 20 min period washout with 0 Ca^{2+}; and (iv) stimulation again with ACh, 35 mM K^+ or Ca^{2+}. In *A* (control cells) LTX was not given. In *B*, LTX was given as shown, and secretion was triggered by alternating pulses (2 min intervals) of ACh or Ca^{2+}. In *C*, LTX was given as shown, and cells were stimulated by 35 mM K^+ pulses applied at 2 min intervals (circles at bottom of the secretory peaks).

was planned in which cells were challenged at 2 min intervals with a solution containing 35 mM K^+ (as in Fig. 3) but in the nominal absence of Ca^{2+} (35 mM K^+–0 Ca^{2+}), while they were being treated with LTX. As expected, the 35 mM K^+–0 Ca^{2+} pulses produced almost undetectable secretory responses, which remained unmodified by 1 nM LTX (Fig. 5). After 14 min superfusion with LTX in 0 Ca^{2+} (always with intermittent application of 10 s pulses of 35 mM K^+–0 Ca^{2+}), 10 s K^+ challenges were applied in 2·5 mM Ca^{2+} (35 mM K^+). Alternating K^+ challenges in 0 Ca^{2+} or in 2·5 mM Ca^{2+} produced responses of 50 and 1200–1400 nA, respectively.

The potentiation of secretion by LTX develops in the absence of depolarization and of Ca^{2+} entry, and after a long period of washout

The experiments shown in Fig. 6 demonstrate that neither depolarization nor brief cytosolic Ca^{2+} transients were required for the LTX-potentiating effects to be established. Cells (continuously superfused with 0 Ca^{2+}) were initially stimulated to determine the size of secretory responses to alternating pulses of ACh and Ca^{2+} (*A* and *B*), or 35 mM K^+ (*C*). In the control experiment shown in *A*, these secretory responses declined by 30% after a 25 min 'silent period' of superfusion with 0 Ca^{2+}. In *B*, the silent period consisted of a 5 min superfusion with 1 nM LTX in 0 Ca^{2+}, followed by a 20 min superfusion with 0 Ca^{2+}; after this, the secretory responses to ACh or Ca^{2+} were potentiated more than 10-fold. *C*, a similar experiment using K^+ as secretagogue; 5 min treatment with LTX followed by 20 min washout with 0 Ca^{2+} produced a clear enhancement of the responses to 35 mM K^+ pulses. These experiments also indicate that the time required for development of the potentiating effects of LTX was short (5 min), and that those effects remained even after a long washout period of the toxin (20 min).

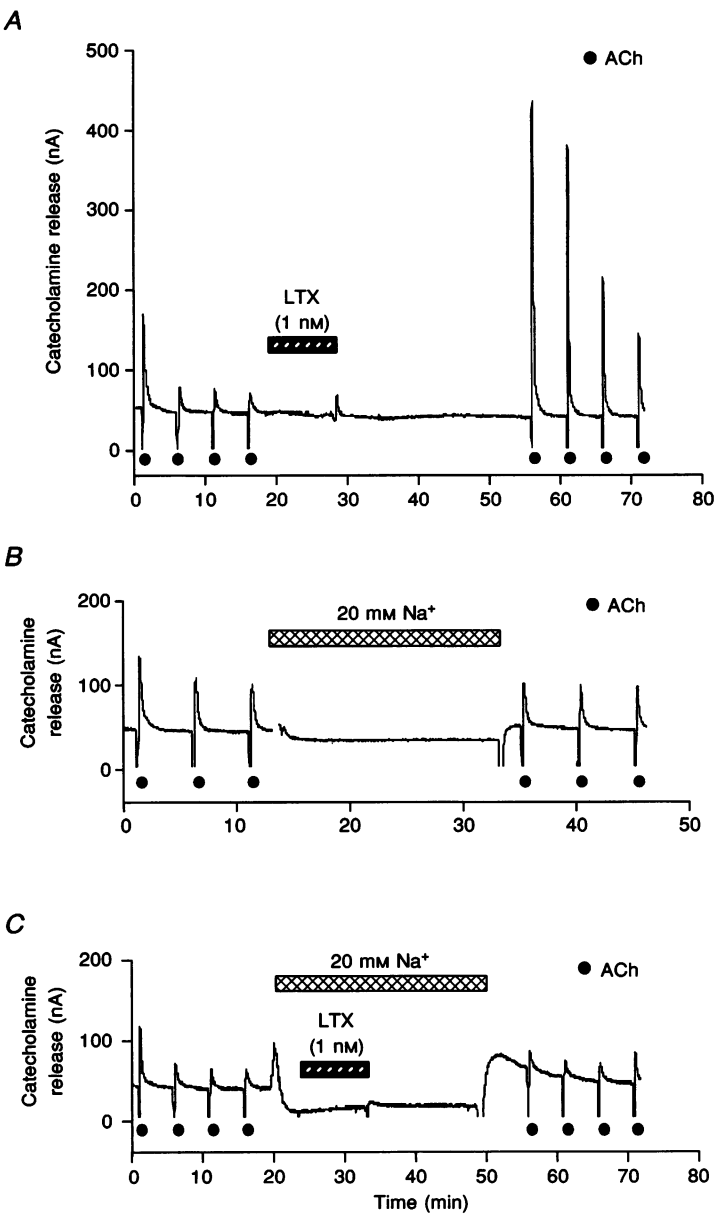

Figure 7. Augmentation by LTX of ACh-induced secretion is Na^+ dependent

These experiments were performed using a protocol essentially similar to that described in Fig. 6. The only difference was that the superfusion solutions containing LTX and that used for the washout 'silent' period lacked Ca^{2+} and had only 20 mM Na^+ (124 mM NaCl was replaced by *N*-methylglucamine chloride, NMG). *A*, an experiment starting with control secretory responses to ACh, followed by a 'silent' period of superfusion with LTX (1 nM), and then new pulses of ACh were applied (30 μM for 10 s, given at 5 min intervals). In *B*, a similar experiment is shown, in which the cells were superfused for 25 min with a 20 mM Na^+ solution, but without LTX treatment. In *C*, LTX was given for 5 min in 20 mM Na^+, followed by an additional 20 min 'silent' period with 20 mM Na^+ and then 0 Ca^{2+}, and back to the ACh pulses.

The potentiation by LTX of ACh- and K^+-induced secretion is Na^+ dependent

Progressive gaining of Na^+ by LTX-treated cells could be the cause for the potentiation of ACh-evoked secretion. This hypothesis was tested through the following experiments (Fig. 7). Figure 7*A* shows a control experiment to demonstrate the repeated observation that LTX (given for 5 min in 0 Ca^{2+}, followed by a 20 min washout period) caused a sharp potentiation of secretion signals evoked by ACh pulses (30 μM for 10 s). In the experiment of Fig. 7*B*, cells were superfused with 0 Ca^{2+} and challenged first with ACh (10 s pulses at 5 min intervals) followed by a 'silent' 20 min period with a 20 mM Na^+ solution (nominal 0 Ca^{2+}, with 20 mM Na^+ and 124 mM *N*-methylglucamine chloride, NMG). Cells were then superfused again with 0 Ca^{2+} and rechallenged with ACh pulses. The low-Na^+ 'silent' period had little effect on the ACh secretory response. Thus, ACh evoked tiny secretory responses similar to those produced at the beginning of the experiment. If LTX (1 nM) was given during the period of superfusion with 20 mM Na^+, the potentiation of the subsequent ACh response did not develop (Fig. 7*C*). It seems, therefore, that LTX requires the presence of Na^+ ions, in order to cause a potentiation of the subsequent secretory responses to ACh.

Effects of LTX on Ca^{2+}-evoked secretion from electroporated cells

It was of interest to study whether the potentiation of secretion by LTX was a plasma membrane-restricted mechanism. Thus, experiments were performed with the idea of bypassing the plasmalemma, and looking for a possible direct intracellular mechanism in secretion. Catecholamine release induced by intermittent applications of Ca^{2+} pulses (100 μM Ca^{2+} in the potassium glutamate electroporating solution described in Methods, for 10 s at 2 min intervals) was explored in electroporated chromaffin cells. Upon repeated Ca^{2+} challenging, the secretory peaks tended to decline rapidly (Fig. 8*A*). The presence of LTX (3 nM) did not alter this pattern of relaxation of the secretory response (Fig. 8*B*).

In another series of experiments, electroporated cells were challenged to secrete with a prolonged exposure to Ca^{2+} (100 μM for 5 min). This produced a secretory curve consisting of a rapid peak of catecholamine release followed by a slowly declining plateau (Michelena, Vega, Montiel, López, García-Pérez, Gandía & García, 1995). LTX did not modify significantly any of the parameters of the secretory profiles (basal secretion, peak secretion, peak area, or total secretion; data not shown). In other experiments, secretion

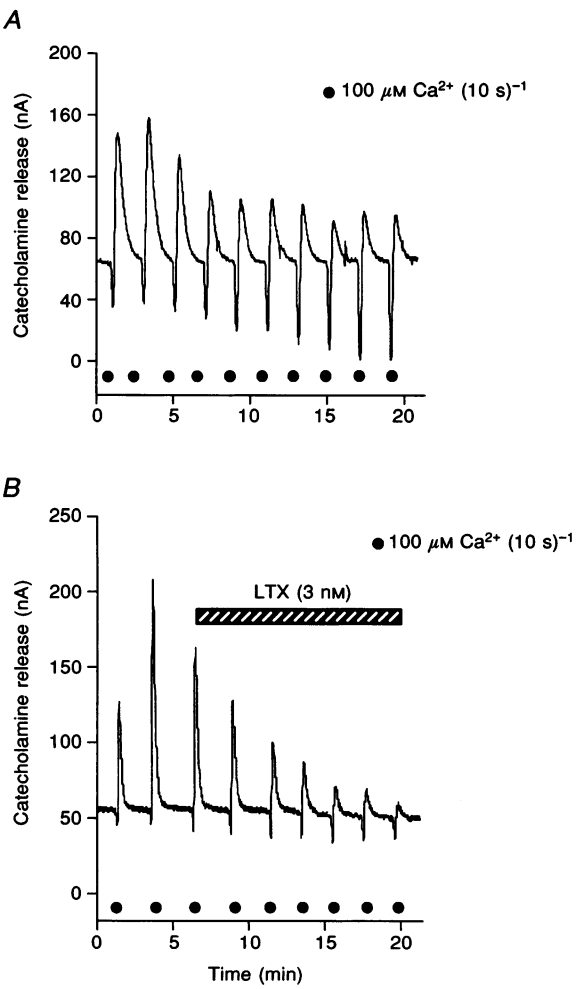

Figure 8. LTX did not modify the secretory responses induced by brief Ca^{2+} pulses in electroporated cells

Electroporated chromaffin cells were superfused with the potassium glutamate-based electroporating solution containing EGTA (free Ca^{2+} concentration smaller than 100 nM). Secretion was triggered by 10 s pulses of 100 μM free Ca^{2+} applied at 2 min intervals. *A* shows the secretory responses in the absence of LTX, and *B* in its presence, given at 3 nM during the period shown by the horizontal bar.

was triggered by exposing the electroporated cells to 1 μM Ca^{2+} for 10 s or 5 min. The secretory responses were similar in control and LTX (3 nM)-treated cells (not shown).

Effects of LTX on basal and stimulated $^{45}Ca^{2+}$ uptake

Upon its insertion into the plasmalemma, LTX has been found to form ion-permeable pores (Rosenthal *et al.* 1990; Filippov, Tertishnikova, Alekseev, Tsurupa, Pashov & Grishin, 1994). Thus, the potentiation of secretion could be due to extra Ca^{2+} entry through those pores upon Ca^{2+} reintroduction, in addition to Ca^{2+} entering through Ca^{2+} channels recruited by ACh or K^+ during the 10 s pulses. Experiments to study the effects of LTX on basal $^{45}Ca^{2+}$ entry and on $^{45}Ca^{2+}$ uptake induced by ACh or K^+ were, therefore, indicated.

Basal $^{45}Ca^{2+}$ uptake (in the presence of 2·5 mM $^{40}Ca^{2+}$) into chromaffin cells amounted to 522 ± 45 c.p.m. ($n = 11$ wells). Net evoked $^{45}Ca^{2+}$ uptake (evoked minus basal) amounted to 820 ± 96 c.p.m. for ACh and to 430 ± 53 c.p.m. for K^+ (35 mM for 10 s). Incubation of resting cells with 1 or 3 nM LTX for 20 min did not modify the basal $^{45}Ca^{2+}$ uptake (Fig. 9*A*). At 1 nM, LTX inhibited the ACh-evoked $^{45}Ca^{2+}$ uptake by 33 ± 6%; at 3 nM, the inhibition further increased to 69 ± 7%. K^+-evoked $^{45}Ca^{2+}$ uptake was unaffected by 1 nM LTX, but was halved by 3 nM (Fig. 9*B*).

Effects of LTX on whole-cell currents through Ca^{2+} channels

The possible blocking effects by LTX of Ca^{2+} entry through voltage-dependent Ca^{2+} channels was explored more directly by measuring the Ca^{2+} entry through those channels in voltage-clamped chromaffin cells. Figure 10*A* shows the time course of I_{Ca} generated by 50 ms depolarizing pulses from a holding potential of −80 mV to 0 mV, applied at 10 s intervals, with 14 mM EGTA in the intracellular pipette solution. In the example shown, LTX (3 nM) reduced by 10% the initial I_{Ca} (850 pA). Insets show original traces and averaged results from eleven cells (means ± S.E.M.). Figure 10*B* shows the results in a cell with a recording pipette without EGTA in the intracellular solution and 10 mM Ca^{2+} in the extracellular solution. I_{Ca} was inhibited around 40% by 3 nM LTX. This greater inhibition in the absence of EGTA might be related to a greater accumulation of $[Ca^{2+}]_i$ and with Ca_i^{2+}-inactivation of Ca^{2+} channels.

Effects of LTX on $[Ca^{2+}]_i$

The effects of LTX on Ca^{2+} entry was further studied by measuring the changes of $[Ca^{2+}]_i$ in single fura-2-loaded chromaffin cells. In the four experiments shown in Fig. 11, single fura-2-loaded cells were continuously superfused with a nominal 0 Ca^{2+} Krebs–Hepes solution and after 10 min they were alternately challenged for 10 s at 2 min intervals,

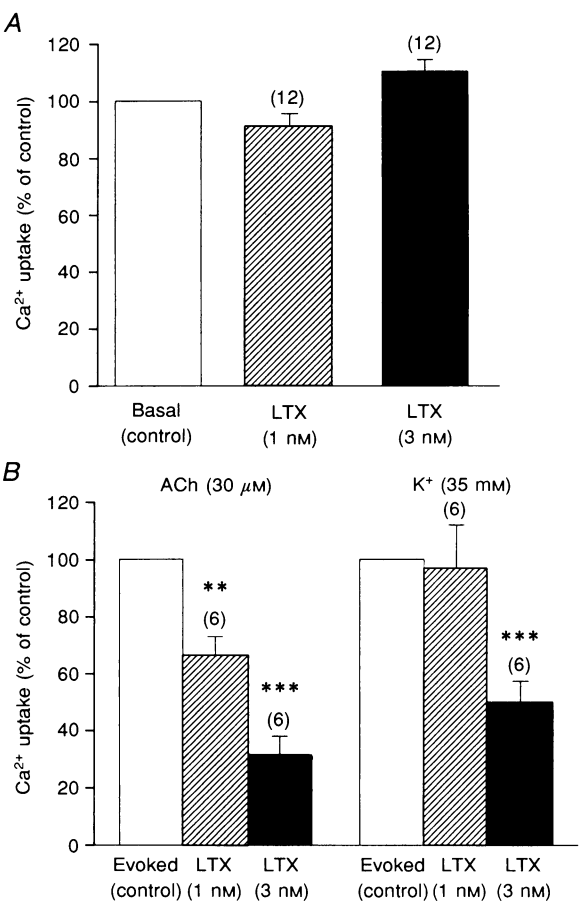

Figure 9. Effects of LTX on basal (*A*) and evoked $^{45}Ca^{2+}$ uptake (*B*)

In *A*, cells were pre-incubated in a 0 Ca^{2+} Krebs–Hepes solution without (Basal) or in the presence of 1 or 3 nM LTX for a 10 min period. Then $^{45}Ca^{2+}$ plus $^{40}Ca^{2+}$ were added. In *B*, cells were treated the same way, but the 10 s period of $^{45}Ca^{2+}$ plus $^{40}Ca^{2+}$ exposure was performed in the presence of ACh (30 μM) or high K^+ (35 mM). Data are means ± S.E.M. of the number of wells shown in parentheses, from at least two different cell batches. ** $P < 0.005$, *** $P < 0.001$ compared with control uptake.

with the 2·5 mM Ca^{2+} solution and either the ACh solution or the 35 mM K^+ solution. Figure 11*A* shows an experiment in a cell whose initial basal $[Ca^{2+}]_i$ was around 0·08 μM. A 10 s challenge with Ca^{2+} did not increase the $[Ca^{2+}]_i$ above basal levels; the ACh pulse increased the $[Ca^{2+}]_i$ to 0·45 μM. LTX (1 nM) completely blunted the ACh responses. The blocking effect developed in 4 min and was reversed 4 min after washing out the toxin. In the experiment shown in Fig. 11*B*, an initial 10 s test pulse with 70 mM K^+ gave an increase of $[Ca^{2+}]_i$ to near 1·5 μM. The Ca^{2+} pulse produced an initial $[Ca^{2+}]_i$ increase to 0·5 μM; in subsequent pulses, however, no increase of $[Ca^{2+}]_i$ was produced upon the Ca^{2+} challenge. This was the usual pattern seen in several cells superfused with 0 Ca^{2+}. The Ca^{2+} pulse did not produce a Ca_i^{2+} transient even in the presence of 1 nM LTX (Fig. 11*A* and *B*). The 35 mM K^+ pulses increased the $[Ca^{2+}]_i$ to around 0·5–0·6 μM. This signal was maintained at a similar level through the 24 min of superfusion with LTX, with values oscillating between 0·3 and 0·7 μM. Washout of LTX produced a rebound effect, with an initial increase of $[Ca^{2+}]_i$ to near 1 μM, followed by a progressive decline in subsequent 35 mM K^+ challenges. These experiments suggest that the drastic changes induced by LTX on secretion occur in the absence of any measurable changes in Ca_o^{2+} entry into the cells, or in averaged changes of $[Ca^{2+}]_i$, both in basal or in stimulating conditions.

Figure 11*C* and *D* shows two experiments performed with a protocol similar to that used for secretion experiments in Fig. 6. Cells were superfused continuously with a nominal 0 Ca^{2+} solution. The three initial ACh pulses produced $[Ca^{2+}]_i$ peaks of 0·3–0·5 μM, with a tendency to decline. LTX (1 nM) was then given, followed by a 15 min washout period. Rechallenging of the cell with ACh did not produce an enhanced $[Ca^{2+}]_i$ response, as was the case for secretion (Fig. 6). A similar result was observed with the 35 mM K^+ pulses, which were drastically reduced after the period of LTX treatment and washout (Fig. 11*D*).

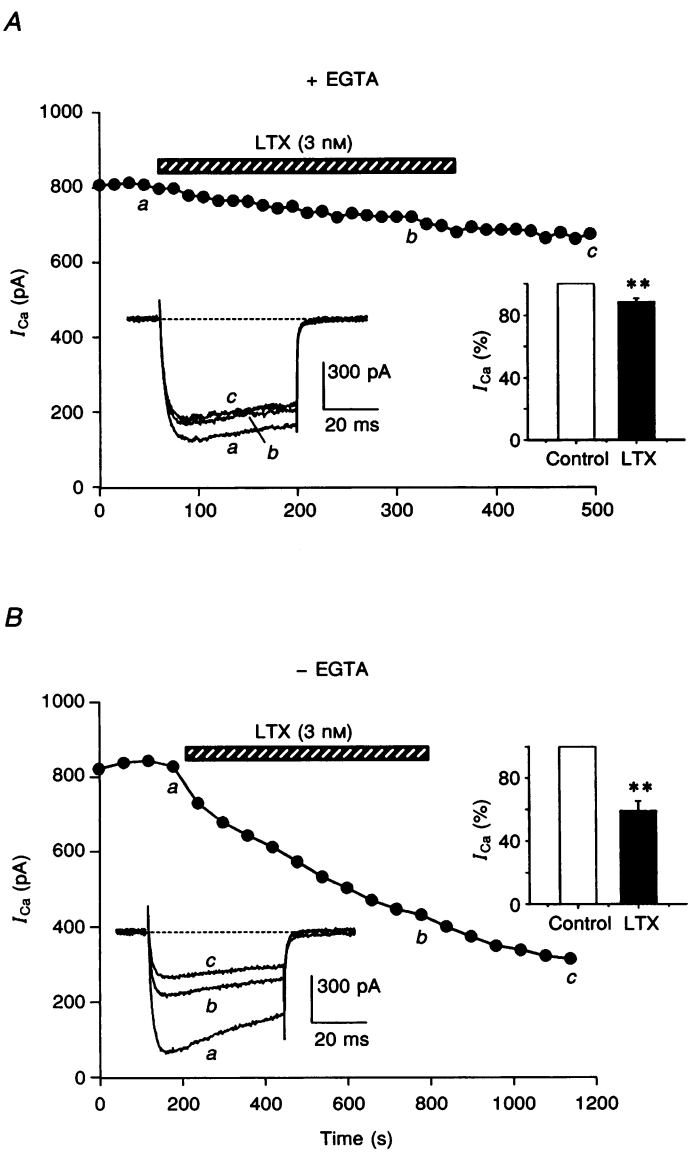

Figure 10. LTX partially inhibits the whole-cell Ca^{2+} currents (I_{Ca}) in voltage-clamped chromaffin cells

The extracellular solution contained 10 mM Ca^{2+}, used as charge carrier. The holding potential was maintained at −80 mV and currents were elicited by means of 50 ms depolarizing pulses at 0 mV, given at 5 (*A*) or 60 s (*B*) intervals. In *A* the intracellular solution contained 14 mM EGTA; in *B*, no EGTA was added. Each panel shows the time course of I_{Ca}, the original traces taken at the points indicated by the letters *a*, *b* and *c*, and the averaged values of I_{Ca} in the absence and the presence of 3 nM LTX (inset histograms). Data are means ± S.E.M. of 11 cells in each case. ** $P < 0·001$, compared with control I_{Ca}.

DISCUSSION

In nerve terminals, LTX elicits a Ca_o^{2+}-independent high rate of spontaneous transmitter release (Longenecker, Hurbult, Mauro & Clark, 1970). This effect of LTX has been extensively studied, and two potential mechanisms have been proposed (Rosenthal *et al.* 1990; Petrenko, 1993). First, LTX may initiate a chain of biochemical events by binding to neurexin receptors in the axon terminal membrane, thus favouring the transport of synaptic vesicles to exocytotic sites, and culminating in an enhancement of spontaneous release. Second, LTX may form non-specific, cation-permeable pores in the axon terminal.

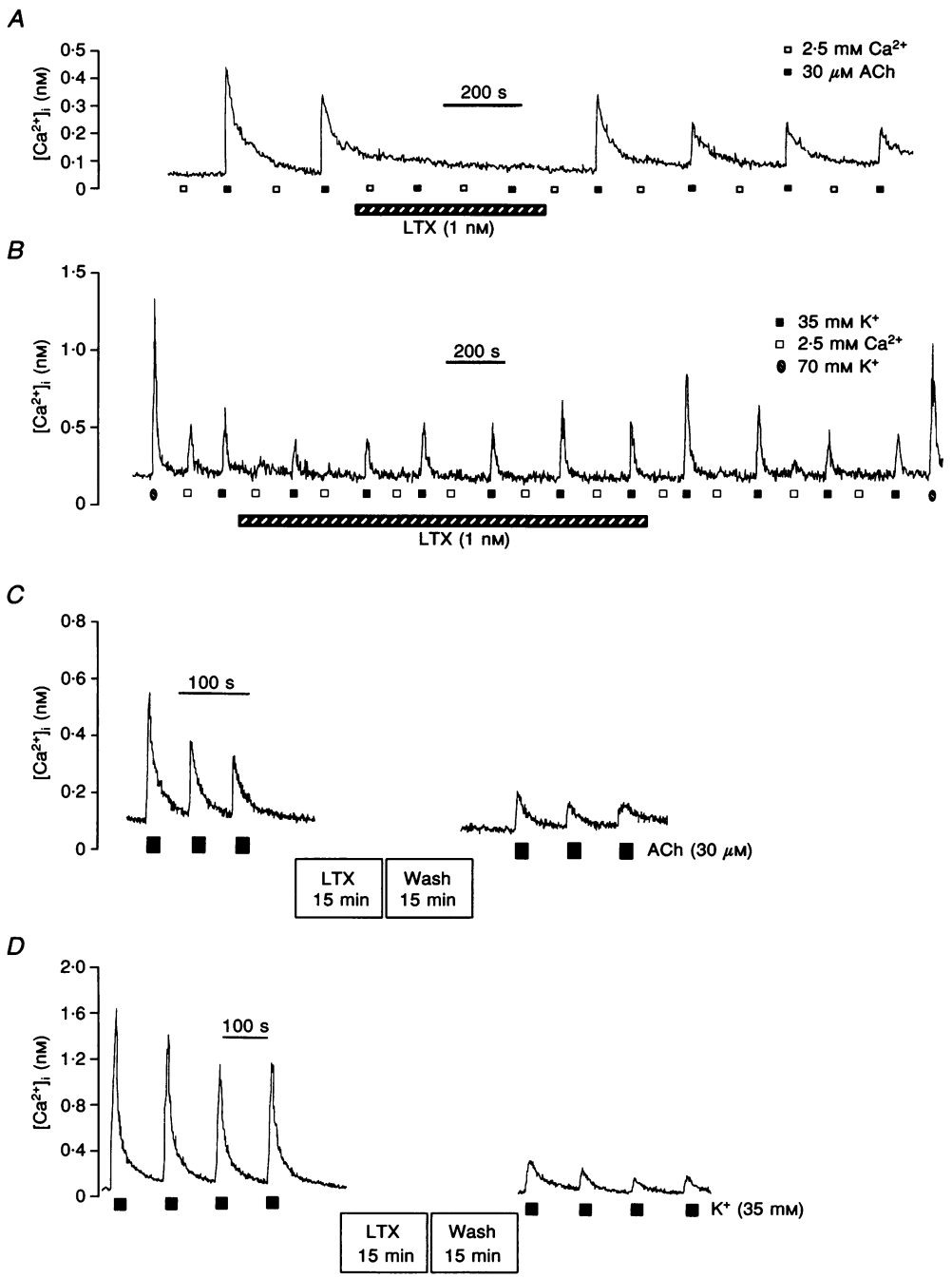

Figure 11. Effects of LTX on $[Ca^{2+}]_i$ transients in fura-2-loaded single chromaffin cells

Four cells are shown (*A–D*); they were continuously superfused with a 0 Ca^{2+} solution. After an initial 10 min superfusion with this solution, the cell in *A* was alternately challenged with 10 s pulses of Ca^{2+} and ACh solutions, at 2 min intervals, and that in *B* with Ca^{2+} and 35 mM K^+ pulses. LTX (1 nM) was applied during the time period shown by the horizontal bars. In *C* and *D*, the cells were initially challenged with ACh or 35 mM K^+. Then, in a resting condition over a 30 min period, they were exposed for 15 min to LTX (1 nM) followed by a 15 min washout period, as shown by the boxes at the bottom.

Contrary to nerve terminals, LTX enhanced the spontaneous catecholamine output from superfused chromaffin cells in a strictly Ca_o^{2+}-dependent manner (Figs 1–3). In addition, LTX enhanced drastically the catecholamine release responses to brief pulses of Ca^{2+}, ACh or 35 mM K^+ solutions, through a mechanism also dependent on Ca_o^{2+} (Figs 3 and 4). Since the adrenal gland does not express either LTX binding sites or neurexins (Ushkarjov, Petrenko, Geppert & Südhof, 1992), the effects of LTX on catecholamine release cannot be explained through the first of the mechanisms proposed, i.e. an action on the exocytotic machinery. This agrees with the lack of effects of LXT on Ca^{2+}-induced secretion from electroporated chromaffin cells, though in digitonin-permeabilized bovine chromaffin cells, LTX (12 nM) doubled the secretion induced by 10 μM Ca^{2+} along 1 h incubation period (Surkova, 1994). These conditions, however, are very different from ours, i.e. 10 s exposure to 1 or 100 μM Ca^{2+}, in electroporated cells, and using a lower concentration (3 nM) of the toxin (Fig. 8). We therefore favour the formation of cation-permeable pores in the plasmalemma of chromaffin cells, to explain the secretory actions of LTX.

A priori, the simplest mechanism could be an enhanced Ca_o^{2+} entry upon Ca_o^{2+} reintroduction through LTX-formed ion pores in the plasmalemma. If so, LTX should have increased $^{45}Ca^{2+}$ entry as well as $[Ca^{2+}]_i$ in resting cells, but it did not. Another possibility is enhanced Ca_o^{2+} entry through voltage-dependent Ca^{2+} channels during cell depolarization with ACh or 35 mM K^+. But blockade by LTX of $^{45}Ca^{2+}$ entry or $[Ca^{2+}]_i$ rises induced by these two stimuli make this explanation also unlikely. This conclusion is reinforced by the inhibition by LTX of whole-cell Ca^{2+} currents, as well as by the fact that its potentiating effects on K^+-induced secretion remained in the presence of various blockers of the Ca^{2+} channel subtypes expressed by bovine chromaffin cells. In spite of this, it is clear that the mechanism responsible for triggering the enhanced secretory responses in LTX-treated cells during applications of pulses of ACh plus Ca^{2+}, K^+ plus Ca^{2+}, or Ca^{2+} alone must be the entry of Ca_o^{2+} into the cell, to gain access to its target receptor in the secretory machinery. The strong dependency upon Na_o^+ ions of the LTX-potentiating effects led us to involve the chromaffin cell Na^+–Ca^{2+} exchanger (Török & Powis, 1990; Liu & Kao, 1990; Chern, Chueh, Lin, Ho & Kao, 1992; Fuente, Maroto, Esquerro, Sánchez-García & García, 1996) in an attempt to explain the ion movements responsible for the enhanced secretory responses to Ca^{2+}, ACh or K^+ challenging of chromaffin cells.

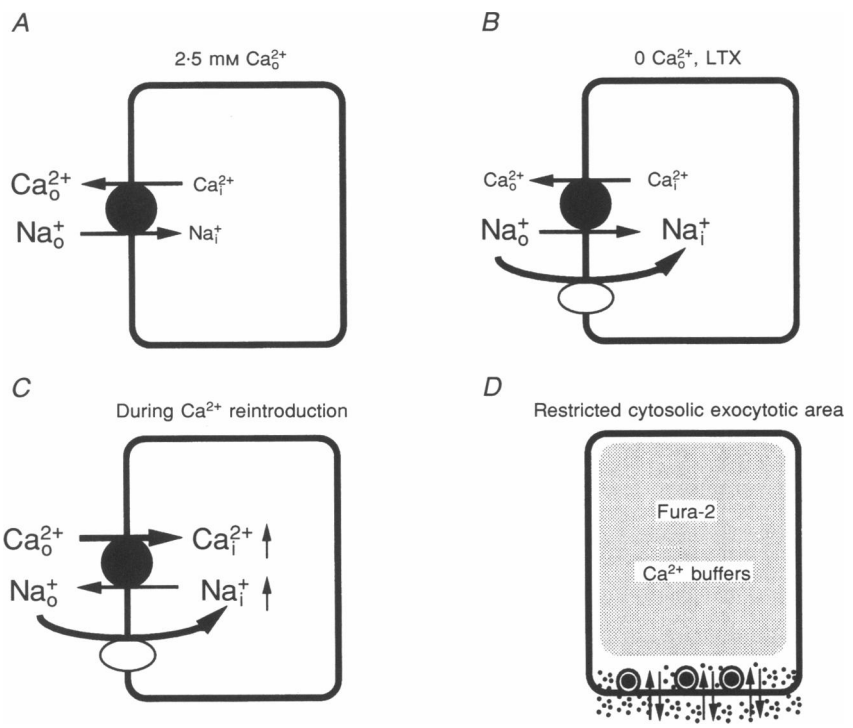

Figure 12. Scheme showing the Na^+ and Ca^{2+} movements likely to be responsible for the LTX-potentiating effects of secretion in bovine chromaffin cells

A, in normal conditions the Na_o^+–Ca_i^{2+} transport system works forwardly by exchanging Na_o^+ for Ca_i^{2+}, thus extracting Ca^{2+} from the cytosol. *B*, superfusion with a nominal 0 Ca^{2+} solution, in the presence of LTX causes a gradual loading of the cell. *C*, the reintroduction of Ca_o^{2+} to Na_i^+-loaded cells reactivates the exchanger that now works in reverse, extracting Na_i^+ and pushing Ca_o^{2+} entry into the cell. *D*, these ionic changes must take place at a restricted subplasmalemma secretory surface, since fura-2 did not detect a measurable global $[Ca^{2+}]_i$ increase. The endogenous Ca^{2+} buffers might restrict the diffusion of Ca_o^{2+} entering through the Na^+–Ca^{2+} exchanger, from the subplasmalemma to inner parts of the cytosol.

Figure 12 shows the proposed sequence of ion movements taking place before and during LTX treatment of cells. In normal cells bathed in 2·5 mM Ca_o^{2+}, the Na^+–Ca^{2+} exchanger promotes the efflux of Ca_i^{2+} in exchange for Na_o^+. In cells bathed in nominal 0 Ca_o^{2+}, the $[Ca^{2+}]_i$ is low and the exchanger works at low rate. It is well documented that LTX favours the accumulation of Na^+ inside cells by forming Na^+-permeable pores in their plasmalemma (Deri & Adam-Vizi, 1993; Filippov *et al.* 1994; Hurbult, Chieregatti, Valtorta & Haimann, 1994). The insertion in the plasmalemma of LTX Na^+-permeable pores in our chromaffin cells will allow the entry of Na_o^+, which tends to accumulate gradually in a time-dependent manner (Fig. 12*B*). As time elapses, the Na_o^+/Na_i^+ gradient dissipates. Thus, when Ca^{2+} is reintroduced (either alone, or with ACh or 35 mM K^+), the Na^+–Ca^{2+} exchanger operates in the reverse mode, and Ca_o^{2+} goes into the cell as Na_i^+ moves out of the cell during the 10 s of duration of the pulse (Fig. 12*C*).

It is puzzling, however, that in fura-2-loaded cells treated with LTX in 0 Ca^{2+}, the reintroduction of Ca_o^{2+} alone for 10 s periods did not evoke an increase in the global $[Ca^{2+}]_i$ (Fig. 12). Yet this Ca_o^{2+} reintroduction caused a drastic secretory response (Figs 3 and 6) suggesting that Ca_o^{2+} must indeed gain access to the cytosol. Why then is this Ca_i^{2+} signal not detected by fura-2? A plausible explanation rests on the limited Ca_o^{2+} entry through a finite number of Na^+–Ca^{2+} exchanger molecules. The bovine chromaffin cell has powerful endogenous buffers that limit the diffusion of Ca^{2+} (Neher & Augustine, 1992). Therefore, Ca^{2+} entering through the exchanger will be sequestered by those buffers, thus impeding a measurable build-up of the global $[Ca^{2+}]_i$ (Fig. 12*D*). In spite of this, at subplasmalemmal exocytotic sites enough Ca_o^{2+} must be delivered by the Na^+–Ca^{2+} exchanger to trigger a healthy secretory response. This response was comparable to that produced by Ca^{2+} given with ACh or 35 mM K^+, strongly suggesting that under depolarizing conditions the Na^+–Ca^{2+} exchanger might contribute to controlling the local $[Ca^{2+}]_i$ and, therefore, the extent and duration of exocytosis.

Contrary to Ca_o^{2+} entering through the Na^+–Ca^{2+} exchanger, Ca^{2+} entering via voltage-dependent Ca^{2+} channels during ACh or 35 mM K^+ stimulation did generate $[Ca^{2+}]_i$ transient elevations. This pathway does not saturate and intracellular Ca^{2+} buffers cannot obviously fully mitigate the diffusion of Ca^{2+} entering the cell through an enormous favourable gradient. LTX substantially reduced the ACh- and 35 mM K^+-induced $[Ca^{2+}]_i$ elevations, probably through direct blockade of the nAChR and/or Ca^{2+} channels, as the $^{45}Ca^{2+}$ uptake (Fig. 9) and whole-cell Ca^{2+} currents experiments suggest (Fig. 10). This inhibition of Ca_i^{2+} transients, however, was accompanied again by a drastic augmentation of secretion, indicating that the Ca^{2+} required for such a response gained access to the secretory machinery through the reverse Na^+–Ca^{2+} exchanger, rather than through Ca^{2+} channels. The fact that Ca^{2+} channel blockers did not attenuate the responses to 35 mM K^+ (Fig. 5) supports this conclusion.

LTX created an interesting experimental situation in which a Ca^{2+}-dependent drastic increase of catecholamine release occurred, without any measurable change in the global $[Ca^{2+}]_i$. In trying to find a physiological explanation for this situation, we considered how the Na^+–Ca^{2+} exchanger might be working in a physiological situation. ACh stimulation of chromaffin cells triggers bursts of action potentials that are dependent on Na^+ and Ca^{2+}. A clear role for Na^+ ions and voltage-dependent Na^+ channels of chromaffin cells (Fenwick, Marty & Neher, 1982) has not yet been discovered. This is in part due to the fact that tetrodotoxin, which efficiently blocks Na^+ channels, has little effect on the nAChR-mediated secretory response (Ceña, Nicolás, Sánchez-García, Kirpekar & García, 1983). It is plausible that during action potential firing, Na_o^+ entering the chromaffin cells through Na^+ channels might modulate the duration of the secretory signal by changing the $[Na^+]_i$ at subplasmalemmal sites; in this manner, these local changes of Na^+ might regulate the activity of the Na^+–Ca^{2+} exchanger and, therefore, the local $[Ca^{2+}]_i$ and the duration and extent of the secretory response.

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