0026-895X/90/060917-07$03.00/0

MOLECULAR PHARMACOLOGY, 38:917-923

Mode of Action of α-Latrotoxin: Role of Divalent Cations in Ca^{2+}-Dependent and Ca^{2+}-Independent Effects Mediated by the Toxin.

LUANN ROSENTHAL, DANIELE ZACCHETTI, LUISA MADEDDU, and JACOPO MELDOLESI

Department of Pharmacology, University of Milan, C.N.R. Center for Cytopharmacology, and Scientific Institute San Raffaele, 20132 Milan, Italy

Received March 9, 1990; Accepted September 19, 1990

SUMMARY

The potent neurotoxin α-latrotoxin (αLTx), from black widow spider venom, induces neurotransmitter release in both Ca^{2+}-containing and Ca^{2+}-free medium, following interaction with a specific cell surface receptor. Binding studies revealed two populations of αLTx binding sites in bovine synaptosomal membranes, showing the same high affinity (K_d, 0.3×10^{-10} M) for αLTx, with approximately 50% of the sites being Ca^{2+} sensitive and the rest being Ca^{2+} insensitive. In contrast, in PC12 cells αLTx binding was completely unaffected by the removal of extracellular Ca^{2+} (K_d, 5×10^{-10} M). The use of La^{3+} as an inhibitor of αLTx action, previously shown in synaptosomes, was extended to PC12 cells. In this system, La^{3+} (100 μM) was shown to inhibit Ca^{2+} influx, both Ca^{2+}-dependent and -independent dopamine release, and polyphosphoinositide (PPI) hydrolysis induced by αLTx. At the same time, La^{3+} did not block αLTx binding or dopamine release evoked by either the ionophore ionomycin (0.5 μM) or the phorbol ester tetradecanoylphorbol acetate (100 nM). La^{3+} also blocked the influx of Mn^{2+} ions through the αLTx-induced cation channel, as measured by quenching of fura-2 fluorescence. In this PC12 cell line, PPI hydrolysis could also be induced by ionomycin, but only when it was present at concentrations that caused an elevation of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) that was not transient but was as persistent as that evoked by αLTx. Our conclusions with regard to the mode of action of αLTx are as follows. (i) All the effects of αLTx in PC12 cells (dopamine release, PPI hydrolysis, and Ca^{2+} influx) can be mediated via a single, Ca^{2+}-insensitive αLTx receptor. (ii) αLTx-induced PPI hydrolysis is most likely due to the activation of a Ca^{2+}-sensitive phospholipase C following the persistent rise in $[Ca^{2+}]_i$ elicited by the toxin in Ca^{2+}-containing medium, and not via direct coupling of the αLTx receptor to the enzyme. (iii) Toxin-evoked Ca^{2+}-independent dopamine release can be blocked by La^{3+} at the extracellular level, most likely by prevention of the entry of divalent cations.

αLTx from black widow spider venom is a high molecular weight protein neurotoxin (M_r 130,000) that induces extensive neurotransmitter release from a variety of preparations including the neuromuscular junction, brain synaptosomes, and neurosecretory cells such as PC12 cells (for review see Refs. 1 and 2). This presynaptic neurotoxin has often been used to investigate specific aspects of neuronal secretion, such as the quantal release of acetylcholine (3, 4) or the differential release of dense core vesicles at the frog neuromuscular junction (5). Understanding of its mode of action is essential for its effective use as an experimental tool and is also expected to provide insight into the overall mechanism(s) of neurotransmitter release.

αLTx is thought to act by binding to a specific plasma membrane receptor protein, which leads to the opening of a nonspecific cation channel and, hence, depolarization and massive calcium entry (6–9). αLTx has also been shown to induce extensive PPI hydrolysis by a process that is entirely dependent on the presence of extracellular Ca^{2+} (10). Previous studies had concluded that this was due to direct coupling of the toxin receptor to a phospholipase C via a transmembrane signaling complex. These events can easily account for the stimulation of neurotransmitter release observed in Ca^{2+}-containing medium.

In addition, αLTx possesses the unusual ability to induce neurotransmitter release in Ca^{2+}-free medium (11–14). Under these conditions, no variations in the intracellular free calcium concentration, $[Ca^{2+}]_i$, can be detected and PPI hydrolysis does not occur. Ca^{2+}-independent secretion has also been observed in various systems with other agents, including phorbol esters (15–19). Up to the present time, the mechanisms responsible for this effect have remained undefined.

In the present study, we have reinvestigated the series of

This work was supported by grants from the C.N.R. (Strategic Project on Transmembrane Signalling and Target Project on Biotechnology and Bioinstrumentation). L. Rosenthal was the recipient of a Muscula Dystrophy Association postdoctoral fellowship.

ABBREVIATIONS: αLTx, α-latrotoxin; PPI, polyphosphoinositide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; KRH, Krebs-Ringer-HEPES; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TPA, tetradecanoylphorbol acetate; Fura-2/AM, Fura-2 penta acetoxy methyl ester.

events induced by αLTx in its targets, from binding up to the release responses. The possible involvement of multiple receptors, previously suggested by the observation that in synaptosomal membranes toxin binding is in part dependent and in part independent of Ca^{2+} (20), appeared inconsistent with results in PC12 cells, where binding was found to be the same with and without Ca^{2+} in the medium. Thus, activation of a single receptor is capable of mediating all the effects of the toxin, including PPI hydrolysis, which is shown here to be not an independent effect but the consequence of the persistent increase of $[Ca^{2+}]_i$ induced by αLTx when it is applied in Ca^{2+}-containing medium. Finally, by the use of La^{3+} as a blocker of the αLTx-activated channel, we provide evidence that divalent cation entry is an essential step in the release effect of the toxin even when it is applied without Ca^{2+} in the medium.

Experimental Procedures

Materials

αLTx was purified to homogeneity, as previously described (21), from the venom glands of the female of the european black widow spider *Latrodectus mactans tredecimguttatus*. Ionomycin and fura-2/AM were from Calbiochem (La Jolla, CA); *myo*-[^{3}H]inositol (80 Ci/mmol), [2,5,6^{3}H]dopamine (20 Ci/mmol), and ^{125}I-Bolton Hunter reagent were from Amersham (Arlington Heights, IL). Lanthanum chloride was from Sigma (St. Louis, MO), Dowex 1X8 resin from Fluka Chemie AG (Buchs, Switzerland), and other reagents from Merck (Darmstadt, FRG).

αLTx binding. αLTx was labeled, using ^{125}I-Bolton Hunter reagent, to a specific activity of 2 μCi/pmol. This procedure has been shown to yield biologically active toxin (20). Approximately 55–70% of the labeled preparation was able to bind competitively with unlabeled toxin in the presence of an excess of synaptosomal membranes. This was taken into account in determining the toxin concentrations.

PC12 cells were cultured in RPMI 1640 medium in the presence of 10% horse serum and 5% fetal calf serum. Synaptosomal membranes were prepared from bovine cerebral cortex, as previously described, using a discontinuous sucrose gradient (7).

For the binding experiments, PC12 cells (1–1.5 × 10^6 cells) or synaptosomal membranes (36 μg) were suspended in 200 μl of KRH buffer containing 125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES-NaOH, pH 7.4, and 2 mM $CaCl_2$, supplemented with 0.1% bovine serum albumin. After addition of ^{125}I-αLTx (2 × 10^6 cpm/pmol) in the presence of varying amounts of unlabeled toxin, the incubation was allowed to proceed at 37° for 20 min and the tubes were then transferred to 0°. The preparations were then centrifuged for 5 min at 10,000 × *g* in an Eppendorf centrifuge, the supernatant was aspirated, and the labeled pellet was rapidly washed once in 500 μl of buffer, spun down, and counted in a γ-counter. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled toxin and was subtracted from total binding. Incubations in nominally Ca^{2+}-free medium were performed using a modified KRH buffer containing no added Ca^{2+}, 2.4 mM $MgSO_4$, and 1 mM EGTA (KRH-EGTA).

For the αLTx release experiments, PC12 cells (45 × 10^6 cells in 1 ml) or synaptosomal membranes (2.4 mg/ml) were preincubated with 5 × 10^{-10} M (750,000 cpm) labeled αLTx at 37° for 20 min and then diluted 100-fold in the same buffer. Aliquots were removed at different times and the amount of bound toxin was quantified as described above. Immediately before dilution, an aliquot was taken to determine the 100% bound value.

Dopamine release assay. Release of dopamine from PC12 cells was performed as previously described (13), in KRH or KRH-EGTA supplemented with 6 mM glucose. αLTx was added to cells at a final concentration of 1 nM and at different times aliquots were removed and centrifuged through a layer of oil. The amount of [^{3}H]dopamine remaining in the cells was determined following solubilization of the samples in Atomlight scintillant (NEN Dupont). When necessary, $LaCl_3$ was added to a final concentration of 100 μM a few minutes before stimulation.

Intracellular calcium measurements. PC12 cells were gently detached from the culture dish, washed once in KRH, suspended in KRH medium (10–15 × 10^6 cell/ml) containing glucose and 5 μM fura-2/AM, and then incubated at 37° with occasional agitation for 30 min. The cell suspension was then diluted 10-fold and left at 37° for 15 min. For each assay, an aliquot of cells (10^6) was pelleted by a brief centrifugation at 10,000 × *g*, washed once, and suspended in 1.5 ml of KRH prewarmed at 37° and containing 250 μM sulfinpyrazone. Fluorescence measurements were performed on a Perkin-Elmer L6-5B fluorimeter connected to an IBM personal computer. In some experiments, the fluorescence at 340 nm was followed directly and $[Ca^{2+}]_i$ was calculated following determination of F_{min} and F_{max} (Fig. 3). For the experiments in Figs. 5 and 6, averaged light intensities over excitation periods of 4 sec at the two wavelengths were used by the computer to calculate 340/380 ratios. The $[Ca^{2+}]_i$ concentrations were calculated following calibration as described by Grynkyewicz *et al.* (22), assuming that the intracellular K_d of fura-2 for Ca^{2+} is 225 nM. R_{min} (1.03), R_{max} (7.17), and S_{fb} (3.97) were determined using fura-2 standards, with low (10^{-8}M) and high (10^{-3}M) Ca^{2+} in the intracellular buffer.

Mn^{2+} influx measurements. PC12 cells were preloaded with fura-2 as described above, except that the final fura-2/AM concentration was decreased to 3 μM and the cells were washed twice in order to minimize the presence of extracellular fura-2. The fluorescence was followed at an excitation wavelength of 362 nm (emission at 495 nm), which was found to be the least sensitive to fluctuations in $[Ca^{2+}]_i$. Mn^{2+} influx was determined as the rate of decrease in 362-nm fluorescence units/sec, and all results were normalized to the total amount of arbitrary fluorescence units present in the cell sample. All measurements were performed within 6 min of cell suspension in the cuvette, to keep the contribution of leaked fura-2 to a minimum.

PPI hydrolysis. PC12 cells were labeled for 24 hr in inositol-free basal medium eagle medium containing 1 μCi/ml *myo*-[^{3}H]inositol, 0.2% horse serum, and 0.1% fetal bovine serum. The PPI hydrolysis experiments and the determination of total inositol phosphates were as previously described (10), in the presence or absence of 10 mM LiCl. The individual inositol phosphates were separated on Dowex AG 1 × 8 resin, as described by Berridge *et al.* (23); following an initial wash with 5 mM unlabeled inositol, they were sequentially eluted first in 1.5 mM sodium tetraborate/60 mM sodium formate and then 0.1 M formic acid/0.2 M ammonium formate, followed by 0.1 M formic acid/0.4 M ammonium formate and 0.1 M formic acid/1.0 M ammonium formate. Aliquots of the various inositol fractions were counted in Ready-gel scintillant (Beckman).

All of the experiments where La^{3+} was present were performed in a modified buffer devoid of sulfates and phosphates (to avoid La^{3+} precipitation), containing 125 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES-Tris, pH 7.4, 6 mM glucose, and 1 mg/ml gelatin.

Results

Effect of Ca^{2+} on αLTx binding. Bovine brain synaptosomes and PC12 cells were employed for these studies, and a Scatchard analysis of the binding data is shown in Fig. 1. In the presence of extracellular Ca^{2+}, αLTx was found to possess a higher affinity for the synaptosomal membrane (Fig. 1A) than for the PC12 cell receptor (Fig. 1B; K_D, 0.07 and 0.5 nM, respectively; see also Refs. 13 and 20). The number of binding sites found on PC12 cells was somewhat variable (5,000–15,000 sites/cell) but comparable to that found in previous studies [6,400 sites/cell (13)], whereas in bovine brain synaptosomal membranes the number was very reproducible from one prep-

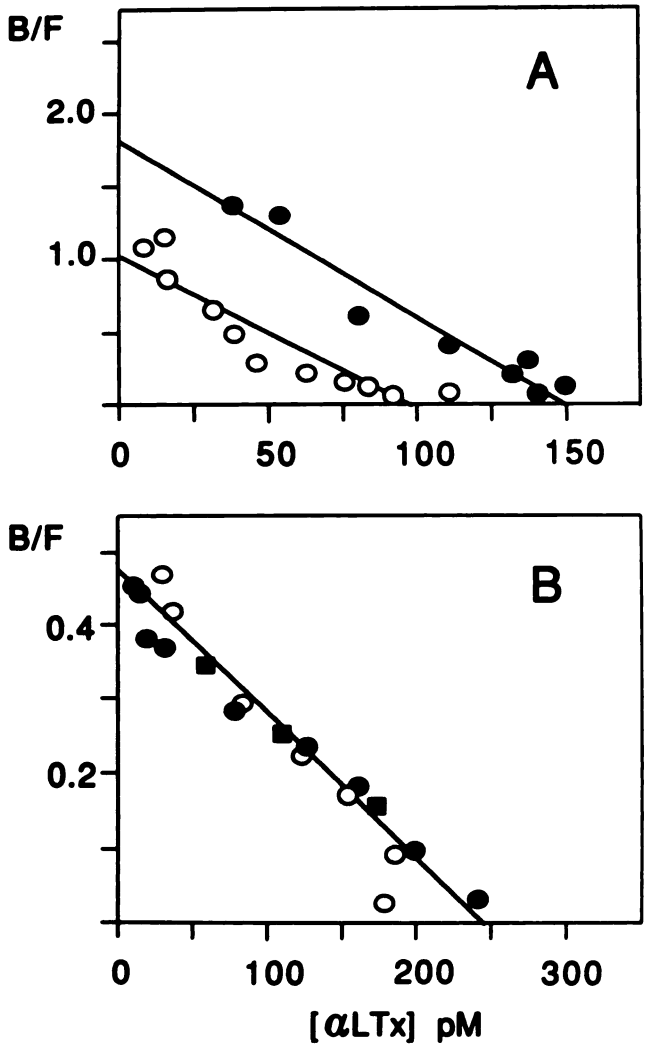

Fig. 1. Scatchard analysis of αLTx binding to synaptosomal membranes (A) and PC12 cells (B). Incubations with ^{125}I-αLTx were performed for 20 min at 37° in complete KRH (●), Ca^{2+}-free KRH-EGTA (○), or phosphate- and sulfate-free buffer containing 100 μM $LaCl_3$ (■). *B/F*, bound/free.

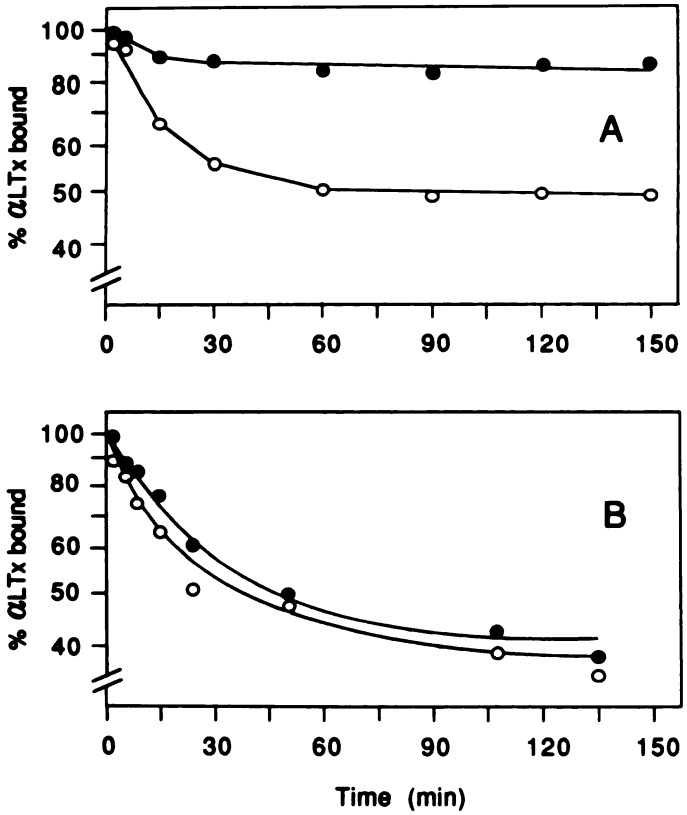

Fig. 2. Time course of the release of ^{125}I-αLTx bound to synaptosomal membranes (A) or PC12 cells (B). Preincubations were performed for 20 min at 37° in KRH buffer, to allow binding of ^{125}I-αLTx to its receptor. At $t = 0$, the preparation was diluted in a 100-fold excess of either KRH (●) or KRH-EGTA (○). The amount of αLTx bound immediately before dilution was taken as 100%.

aration to the next (0.26 ± 0.02 pmol/mg of protein). Removal of Ca^{2+} from the incubation medium caused a 50% decrease in B_{max} with no change of K_D in synaptosomal membranes (Fig. 1A). In contrast, in PC12 cells the αLTx binding constants were virtually identical in Ca^{2+}-containing and Ca^{2+}-free medium (Fig. 1B).

The differential sensitivity to Ca^{2+} of binding in synaptosomes and PC12 cells was further investigated by following the kinetics of αLTx release from its receptor. αLTx was allowed to bind in the presence of Ca^{2+} and then diluted in an excess of either Ca^{2+}-containing or Ca^{2+}-free medium. Release from PC12 cells was slow but progressive (≈50% in the first and 15% more in the second hour), with no significant effect of extracellular Ca^{2+} (Fig. 2B). On the other hand, as previously observed (20), release of αLTx from synaptosomal membranes was found to be extremely slow in the presence of Ca^{2+}; whereas 15% of the toxin was released within the first 60 min, the remaining 85% appeared almost irreversibly bound, with no detectable release during the following 120 min (Fig. 2A). In this case, however, when Ca^{2+} was removed the pattern of release changed considerably, with 50% of the binding sites freed in 60 min (Fig. 2A). Thus, whereas two different types of αLTx binding sites, which differ in their Ca^{2+} sensitivity, exist in synaptosomes, there is no evidence that extracellular Ca^{2+} plays any role in toxin binding to PC12 cells.

Effect of La^{3+} on dopamine release, divalent cation influx, and PPI hydrolysis in PC12 cells. It has recently been shown in synaptosomes that trivalent cations such as La^{3+} and Gd^{3+} are able to block both Ca^{2+} entry and neurotransmitter release caused by αLTx, without interfering with toxin binding (24). The use of lanthanides might, therefore, enable us to evaluate the role of Ca^{2+} at the extracellular level by creating a situation where Ca^{2+} is present in the extracellular medium but cannot enter the cell. As seen in Fig. 3A, the addition of 100 μM La^{3+} immediately before 1 nM αLTx caused the complete inhibition of [^{3}H]dopamine release from PC12 cells. At the same time, the rise in $[Ca^{2+}]_i$ induced by αLTx, as detected by fura-2 fluorescence, was blocked following addition of the lanthanide (Fig. 3B), whereas αLTx binding was completely unaffected (see Fig. 1B). La^{3+}, therefore, inhibits αLTx action in PC12 cells in a manner similar to that in synaptosomes.

The complete inhibition by La^{3+} of the αLTx release effect demonstrates that the trivalent cation blocks not only the Ca^{2+}-dependent but also the Ca^{2+}-independent mechanism of toxin stimulation. Inhibition of the second mechanism could be due either to blockade of influx (divalent cations such as Mg^{2+} are needed for the Ca^{2+}-independent release effect of the toxin to occur) and/or to an independent effect of La^{3+}, for example at

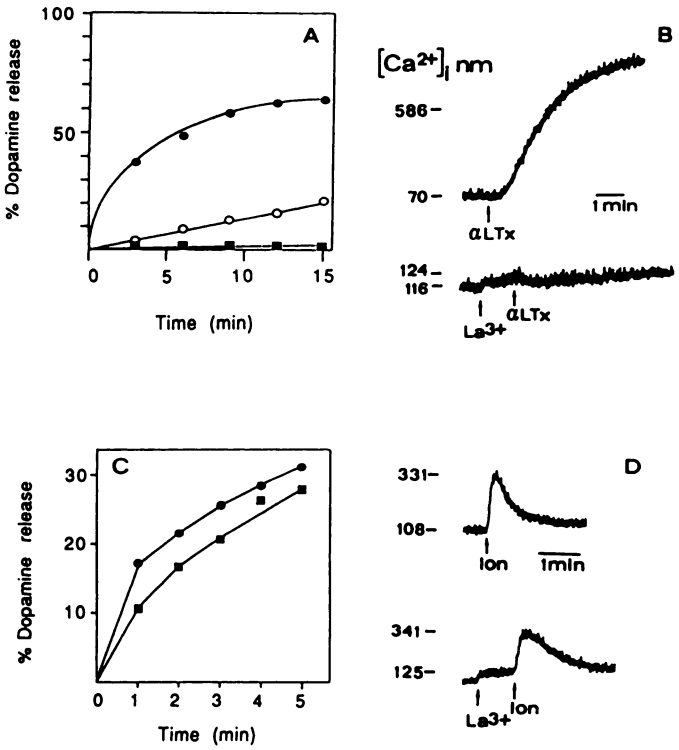

Fig. 3. Effect of La^{3+} on dopamine release (A and C) and Ca^{2+} influx (B and D) in PC12 cells. A and C, Dopamine release was stimulated with 3 nM αLTx (A) or 0.5 μM ionomycin (C) in phosphate- and sulfate-free buffer, following a 5-min preincubation in the absence (●) or presence (■) of 100 μM $LaCl_3$. αLTx-induced Ca^{2+}-independent release (A) was performed in KRH-EGTA (○). B and D, For Ca^{2+} measurements, PC12 cells were preloaded with fura-2 for 30 min, washed, and resuspended in phosphate- and sulfate-free buffer at a concentration of 10^6 cells/ml. Where indicated, $LaCl_3$ was added to a final concentration of 100 μM. The fluorescence was followed at an excitation wavelength of 340 nm. Indicated $[Ca^{2+}]_i$ values correspond to basal and stimulated levels after 2 min of exposure to 1 nM αLTx (B) or to peak stimulated levels after 0.5 μM ionomycin (*Ion*) (D).

the level of exocytosis. In order to investigate this latter possibility, experiments were carried out with ionomycin, a ionophore drug known to increase Ca^{2+} influx not via the activation (or generation) of channels but via electroneutral Ca^{2+}/H^+ exchange. As can be seen in Fig. 3D, the $[Ca^{2+}]_i$ increase peak stimulated by ionomycin (0.5 μM) in PC12 cells was decreased by only 20% in the presence of La^{3+}, and a similar small decrease was observed with the release effect of the ionophore (Fig. 3C). Similar results were obtained when a higher (2 μM) concentration of ionomycin was used to yield an extent of release (50% in 5 min) closer to that induced by αLTx. Likewise, the release response to the phorbol ester TPA (100 nM) was not inhibited by La^{3+} (data not shown). We conclude that exocytosis operates unaffected in La^{3+}-incubated PC12 cells.

In order to determine whether La^{3+} had an effect on the entry of divalent cations other than Ca^{2+}, we monitored the influx of Mn^{2+} ions by following the changes in fluorescence at 362 nm of fura-2-loaded PC12 cells. At this wavelength, intracellular fura-2 fluorescence is insensitive to changes in $[Ca^{2+}]_i$ but can be quenched upon interaction with Mn^{2+} ions (22, 25). When $MnCl_2$ was added to control cells (2 mM final concentration), a basal decrease in 362-nm fluorescence was detected, most likely due in part to interaction of Mn^{2+} with extracellular fura-2 and in part to a slow influx of Mn^{2+} into the cells (Table 1). However, in cells preexposed to 2 nM αLTx, the rate of quench in fura-2 fluorescence was increased 3-fold, as would be expected from Mn^{2+} influx via the toxin-induced cation channel. Prior addition of 100 μM La^{3+} reduced basal Mn^{2+} influx by approximately 35%, but, most importantly, it also completely inhibited any Mn^{2+} entry stimulated by αLTx (Table 1).

TABLE 1

Influx of Mn^{2+} into PC12 cells

PC12 cells were preloaded with fura-2. Fluorescence was followed at an excitation wavelength of 362 nm, with an emission wavelength at 495 nm. Mn^{2+} influx was measured as the initial rate of decrease in fluorescence expressed in arbitrary fluorescence units/sec, following the addition of $MnCl_2$ to a final concentration of 2 mM. La^{3+} (100 μM) was added 1 min before subsequent additions. αLTx (2 nM) was incubated with cells for 3 min before addition of $MnCl_2$.

Preincubation	Rate of quench
	fluorescence units/sec
Control	0.86
αLTx	2.5
La^{3+}	0.56
La^{3+} + αLTx	0.48

As described previously (10), 1 nM αLTx induces extensive PPI hydrolysis to generate large amounts of inositol phosphates (350% of basal inositol 1,4,5-trisphosphate released in 1.5 min; Fig. 4A) and this effect is completely absent when Ca^{2+} is removed from the extracellular medium (Fig. 4B). When 100 μM La^{3+} was added to PC12 cells in Ca^{2+}-containing medium, αLTx-induced PPI hydrolysis was almost completely blocked (Fig. 4C). Such a blockade was not due to a nonspecific inhibition of the reaction, because in parallel experiments the stimulation by 50 nM bradykinin [a peptide known to trigger PPI hydrolysis in PC12 cells via the activation of specific B_2 receptors (26)] was unaffected (data not shown). These results demonstrate that the presence of extracellular Ca^{2+} is not in itself sufficient to allow αLTx-induced PPI hydrolysis.

Relationship between $[Ca^{2+}]_i$ and PPI hydrolysis. We, therefore, chose to reinvestigate the mechanisms of αLTx-induced PPI hydrolysis, in particular the possibility that this effect is a consequence of the toxin-induced $[Ca^{2+}]_i$ rise. As shown in Fig. 5A, after a short lag αLTx induces a dose-dependent rise in $[Ca^{2+}]_i$, which is known to be caused by the opening of both voltage-dependent and voltage-independent cation channels. The pattern of $[Ca^{2+}]_i$ elevation is quite different with respect to other $[Ca^{2+}]_i$-elevating agonists, in that the rise is slow but reaches high levels that are maintained indefinitely.

The dose response for αLTx-induced PPI hydrolysis is shown in Fig. 5B. It is shifted towards slightly higher concentrations, compared with the dopamine release curve (half-maximal effect at 1.0 nM as opposed to 0.5 nM), with a maximal response that can reach 450% of basal levels. Interestingly, the αLTx dose-response curve for PPI closely corresponds to that for $[Ca^{2+}]_i$ increase (not shown).

The calcium ionophore ionomycin, on the other hand, causes an immediate increase in $[Ca^{2+}]_i$, which, at drug concentrations up to 0.1 μM, tends to return to near basal levels within 50 sec (Fig. 6A). These transients greatly resemble those provoked by depolarization with high K^+ (Fig. 6A). PPI hydrolysis in PC12 cells is barely detectable at these concentrations of ionomycin (Fig. 6B) or in the presence of 35 mM K^+ (data not shown). However, at micromolar ionomycin concentrations, the initial $[Ca^{2+}]_i$ rise peak is followed by sustained elevated $[Ca^{2+}]_i$ levels

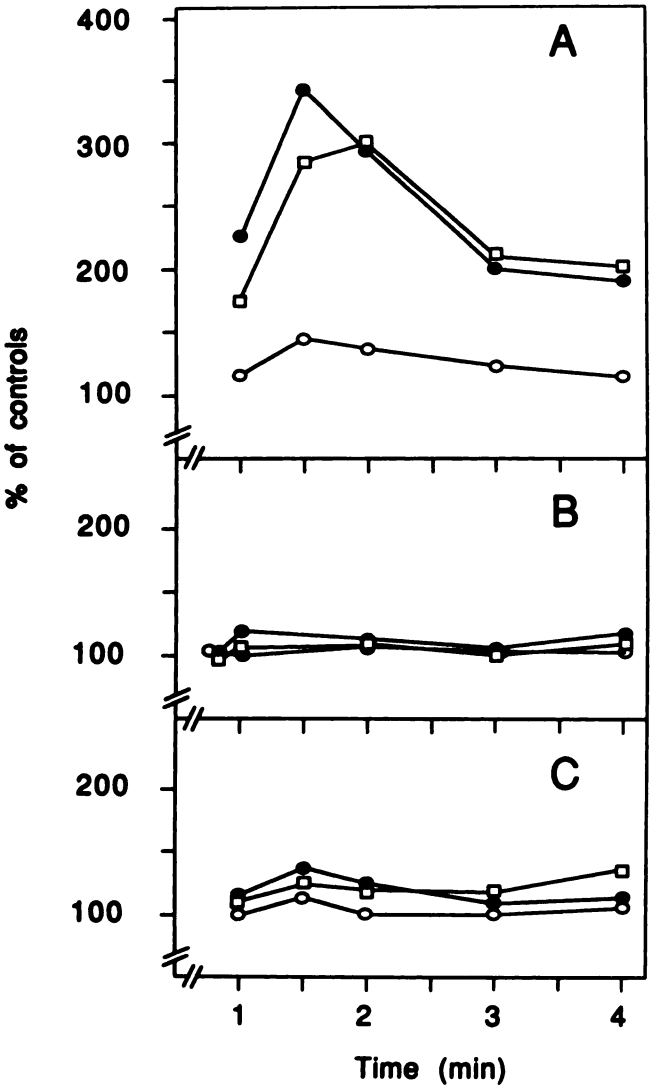

Fig. 4. Effect of La^{3+} on αLTx-induced PPI hydrolysis in PC12 cells. PC12 cells were exposed to 1 nM αLTx in either KRH (A), Ca^{2+}-free KRH-EGTA (B), or phosphate- and sulfate-free buffer containing 100 μM $LaCl_3$ (C). The experiment was performed in the absence of added lithium on cells prelabeled for 24 hr with *myo*-[^{3}H]inositol. The inositol monophosphates (○), inositol bisphosphates (□), and inositol trishosphates (●) were separated by anion exchange chromatography. αLTx-induced PPI hydrolysis in phosphate- and sulfate-free buffer in the absence of added La^{3+} was identical to that observed in A.

(Fig. 6A). Concomitantly, high levels of PPI hydrolysis can be measured, with maxima (420%) close to those obtained with αLTx (Fig. 6B).

Discussion

In this work we have attempted to clarify what role Ca^{2+} plays in toxin binding and in mediating various toxin-induced responses. The present studies provide evidence for two types of αLTx binding sites in bovine brain synaptosomal membranes, one Ca^{2+}-dependent and the other Ca^{2+}-independent. The existence of different subsets of binding sites in synaptosomes had previously been suggested on the basis of salt (20) and temperature sensitivity (27), although there is no direct evidence that binding sites sharing one of those characteristics share them all. Ushkaryov *et al.* (27) have described a model where an αLTx dimer could cross-link two receptor molecules, in which case the two types of binding sites would represent monomeric and dimeric forms of the receptor, a possibility that appears consistent with the ratio of receptors observed in our experiments. However, in PC12 cells only Ca^{2+}-insensitive αLTx binding was observed. These results differ from previous observations in the same cell type, where the absence of calcium appeared to decrease binding after 10 min by approximately 50% (13). It is conceivable that in the present work we have analyzed a subclone of PC12 cells in which only one of the two receptor forms is expressed or the receptor has lost the ability to dimerize. The present situation is, however, fortunate, because it allows us to conclude that both Ca^{2+}-dependent and -independent neurosecretion, as well as PPI hydrolysis, can be mediated via one single type of receptor (Ca^{2+}-insensitive) and that the different toxin-induced responses must, therefore be due to postreceptorial events. The additional Ca^{2+}-sensitive sites present in synaptosomes may be involved in other αLTx effects observed in this system, such as the recently described nonvesicular release of glutamate from guinea pig synaptosomes (28).

An important tool we have employed to reveal the mechanisms of αLTx action has been La^{3+}, whose blocking effects were also investigated in PC12 cells treated with other stimulatory agents, such as ionomycin, TPA, and bradykinin. In brain synaptosomes La^{3+} has recently been shown to markedly inhibit both Ca^{2+} influx and neurotransmitter release induced by αLTx (24), and these same effects have now been documented in PC12 cells. Moreover, our present results demonstrate that the effects of the trivalent cation are highly specific. Indeed, La^{3+} failed to affect αLTx binding, induced no marked inhibition of the exocytotic process *per se* (as revealed by the experiments with ionomycin and TPA), and appeared not to enter PC12 cells (it caused no change of fura-2 signals). Its action appears, therefore, to be restricted to the cell surface, in particular to the processes of ion channel transport, including that across the voltage-independent cation channel activated (or generated) by αLTx. Of particular interest is the fact that La^{3+} inhibition of transmitter release was complete, i.e., it concerned not only the Ca^{2+}-dependent but also the Ca^{2+}-independent stimulation elicited by αLTx. The former process is believed to depend on the large Ca^{2+} influx, whereas the latter is still obscure but requires divalent cations (12, 29–30) in the incubation medium in order to take place. The blockade of Mn^{2+} influx now observed with La^{3+} strongly suggests that these cations operate not outside the cell but after transport across the plasma membrane, a possibility already considered previously, although without any experimental support.

More definite conclusions were obtained by the use of La^{3+} and ionomycin with respect to another effect of αLTx, the stimulation of PPI hydrolysis. La^{3+}, added to the Ca^{2+}-containing medium, was found to block the effect of αLTx and to leave unaffected that induced by an unrelated agent, bradykinin (see also Ref. 31). Such a dissociation excludes the possibility of a nonspecific receptor-effector uncoupling induced by the trivalent cation. Rather, the inhibition of PPI hydrolysis appears to correlate with, and most probably to depend on, the inhibition of Ca^{2+} influx. We conclude, therefore, that Ca^{2+} is not required outside the cell to permit the coupling of the activated toxin receptor to a phospholipase C, as previously suggested (10), but is required inside the cytoplasm, most probably to directly

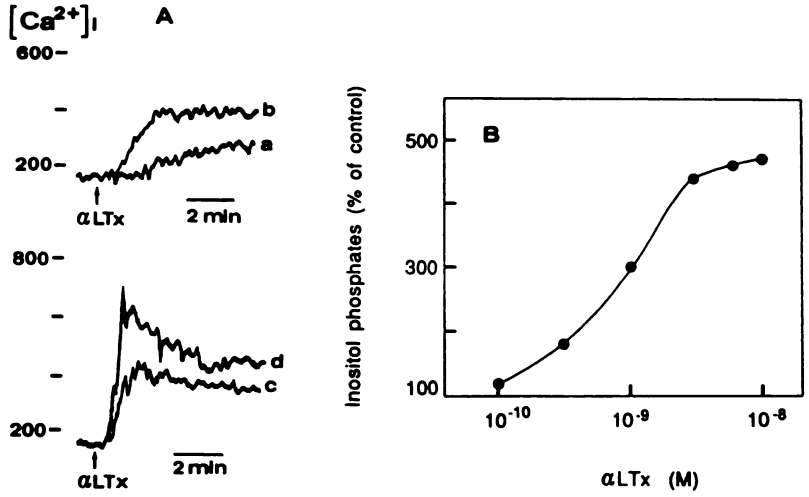

Fig. 5. Dose dependence of $[Ca^{2+}]_i$ levels (A) and PPI hydrolysis (B) induced by αLTx in PC12 cells. A, The results are plotted directly in terms of $[Ca^{2+}]_i$, following computer processing of 340/380 nm ratios and calibration values. Where indicated, αLTx was added to a final concentration of 0.6 nM (*a*), 1 nM (*b*), 3 nM (*c*), or 6 nM (*d*). B, Total [^{3}H] inositol phosphates were quantified after 10 min of toxin stimulation in the presence of 10 mM Li^+.

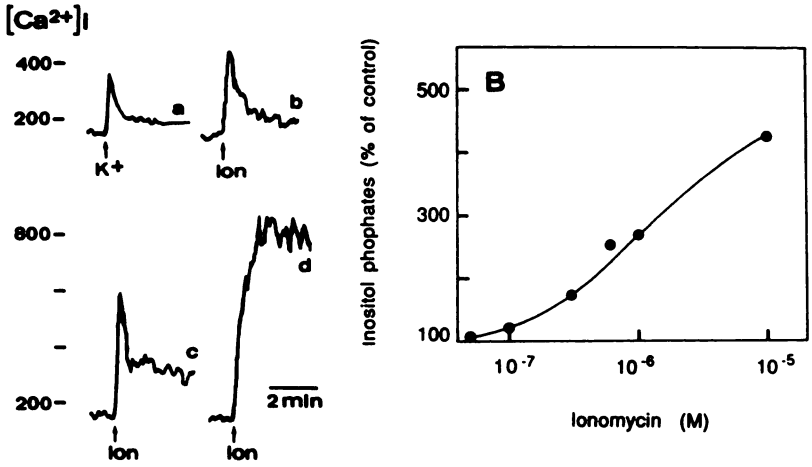

Fig. 6. Dose dependence of $[Ca^{2+}]_i$ levels (A) and PPI hydrolysis (B) induced by ionomycin (*Ion*) in PC12 cells. Both $[Ca^{2+}]_i$ and PPI measurements were performed as in Fig. 5. Where indicated, potassium was added to a final concentration of 35 mM (*a*) and ionomycin to a final concentration of 0.1 μM (*b*), 0.5 μM (*c*), or 5 μM (*d*).

activate a Ca^{2+}-dependent enzyme. This last conclusion might appear at variance with previous results in PC12 cells showing only marginal PPI hydrolysis effects after treatment with other agents known to increase $[Ca^{2+}]_i$, i.e., high $[K^+]$ and ionomycin (10, 31). In the present work, however, we demonstrate that, when ionomycin was used at concentrations large enough to induce an increase in $[Ca^{2+}]_i$ that was not transient but persisted for many minutes, a PPI response very similar to that elicited by αLTx was observed. The observation that PPI hydrolysis can result from the activation of a Ca^{2+}-sensitive phospholipase C following a rise in $[Ca^{2+}]_i$ has been described in numerous cell systems (32–36). In particular, in chick heart cells PPI hydrolysis is stimulated by sodium channel activators that provoke a rise in $[Ca^{2+}]_i$ that is persistent (35). A potential physiological role for this type of phospholipase C activation has been proposed (for review see Ref. 37). Although in some cell types this might well be the case (38), in PC12 cells this seems unlikely, given the levels and duration of $[Ca^{2+}]_i$ increase required. In any case, the Ca^{2+}-induced PPI hydrolysis probably plays a role in αLTx action in Ca^{2+}-containing medium, because it must contribute at the level of both $[Ca^{2+}]_i$ and the activation of protein kinase C, via the generation of inositol 1,4,5-trisphosphate and diacylglycerol, respectively.

In conclusion, the results that we have obtained characterize multiple steps of the action of αLTx and its target process, neurotransmitter release, in PC12 cells, a nerve cell model of widespread use in the field. The importance of divalent cations in the mode of action of αLTx has been reinforced. But, whereas the role of Ca^{2+} in neurotransmitter release is now fairly well understood, the intracellular mechanisms whereby ions such as Mg^{2+} could activate such a process in the absence of a rise in $[Ca^{2+}]_i$ are as yet undefined. In addition, whether these divalent cations are in and of themselves sufficient to cause Ca^{2+}-independent neurotransmitter release or whether some other second messenger is generated at the level of the αLTx receptor are interesting questions for the future. The availability of an inhibitor such as La^{3+} should prove useful in resolving these questions.

References

1. Meldolesi, J., H. Scheer, L. Madeddu, and E. Wanke. Mechanism of action of α-latrotoxin: the presynaptic stimulatory toxin of the black widow spider venom. *Trends Pharmacol. Sci.* **7**:151–155 (1986).
2. Rosenthal, L., and J. Meldolesi. α-Latrotoxin and related toxins. *Pharmacol. Ther.* **42**:115–134 (1989).
3. Ceccarelli, B., and W. P. Hurlbut. Vesicle hypothesis of the release of quanta of acetylcholine. *Physiol. Rev.* **60**:396–441 (1980).
4. Fesce, L. C., J. R. Segal, B. Ceccarelli, and W. P. Hurlbut. Effects of black widow spider venom and Ca^{2+} on quantal secretion at the frog neuromuscular junction. *J. Gen. Physiol.* **88**:737–752 (1986).
5. Matteoli, M., C. Haimann, F. Torri-Tarelli, J. M. Polak, B. Ceccarelli, and

P. De Camilli. Differential effect of α-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **85**:7366–7370 (1988).
6. Wanke, E., A. Ferroni, P. Gattanini, and J. Meldolesi. α-Latrotoxin of the black widow spider venom opens a small, non-closing cation channel. *Biochem. Biophys. Res. Commun.* **134**:320–325 (1986).
7. Scheer, H., and J. Meldolesi. Purification of the putative α-latrotoxin receptor from bovine synaptosomal membranes in an active binding form. *EMBO J.* **4**:323–327 (1985).
8. Scheer, H., G. Prestipino, and J. Meldolesi. Reconstitution of the purified α-latrotoxin receptor in liposomes and planar lipid membranes: clues to the mechanism of toxin action. *EMBO J.* **5**:2643–2648 (1986).
9. Petrenko, A. G., V. A. Kovalenko, O. G. Shamotienko, I. N. Surkova, T. A. Tarasyuk, Y. A. Ushkaryov, and E. V. Grishin. Isolation and properties of the α-latrotoxin receptor. *EMBO J.* **6**:2023–2027 (1990).
10. Vicentini, L., and J. Meldolesi. α-Latrotoxin of black widow spider venom binds to a specific receptor coupled to phosphoinositide breakdown in PC12 cells. *Biochem. Biophys. Res. Commun.* **121**:538–544 (1984).
11. Ceccarelli, B. F., F. Grohovaz, and W. P. Hurlbut. Freeze-fracture studies of frog neuromuscular junctions during intense release of transmitter. I. Effects of black widow spider venom and Ca^{2+}-free solutions on the structure of the active zone. *J. Cell. Biol.* **81**:163–177 (1979).
12. Misler, S., and W. P. Hurlbut. Action of black widow spider venom on quantized release of acetylcholine at the frog neuromuscular junction: dependence upon external Mg^{2+}. *Proc. Natl. Acad. Sci. USA* **76**:991–995 (1979).
13. Meldolesi, J., L. Madeddu, M. Torda, G. Gatti, and E. Niutta. The effect of α-latrotoxin on the neurosecretory PC12 cell line: studies on toxin binding and stimulation of neurotransmitter release. *Neuroscience* **10**:997–1009 (1983).
14. Meldolesi, J., W. B. Huttner, R. Y. Tsien, and T. Pozzan. Free cytoplasmic Ca^{2+} and neurotransmitter release: studies on PC12 cells and synaptosomes exposed to α-latrotoxin. *Proc. Natl. Acad. Sci. USA* **81**:620–624 (1984).
15. Pozzan, T., G. Gatti, N. Dozio, L. M. Vicentini, and J. Meldolesi. Ca^{2+}-dependent and -independent release of neurotransmitter from PC12 cells: a role for protein kinase C activation? *J. Cell. Biol.* **99**:628–638 (1984).
16. Rink, T. J., A. Sanchez, and T. J. Hallam. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature (Lond.)* **305**:317–319 (1983).
17. Pozzan, T., D. P. Lew, C. B. Wollheim, and R. Y. Tsien. Is cytosolic ionized calcium regulating neutrophil activation? *Science (Washington D. C.)* **221**:1413–1415 (1983).
18. Vallar, L., T. J. Biden, and C. B. Wollheim. Guanine nucleotides induce Ca^{2+}-independent insulin secretion from permeabilized RINm5F cells. *J. Biol. Chem.* **262**:5049–5056 (1987).
19. Knight, D. E., H. Von Grafenstein, and C. M. Athayde. Calcium-dependent and calcium-independent exocytosis. *Trends Neurosci.* **12**:451–458 (1989).
20. Meldolesi, J. Studies on α-latrotoxin receptors in rat brain synaptosomes: correlation between toxin binding and stimulation of transmitter release. *J. Neurochem.* **38**:1559–1569 (1982).
21. Frontali, N., B. Ceccarelli, A. Gorio, A. Mauro, P. Siekevitz, M.-C. Tzeng, and W. P. Hurlbut. Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions. *J. Cell Biol.* **68**:462–479 (1976).
22. Grynkyewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
23. Berridge, M., R. M. Dawson, C. P. Downes, T. P. Heslop, and R. F. Irvine. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**:473–482 (1983).
24. Scheer, H. Interactions between α-latrotoxin and trivalent cations in rat striatal synaptosomal preparations. *J. Neurochem.* **52**:1590–1597 (1989).
25. Sage, S. O., J. E. Merritt, T. J. Hallam, and T. J. Rink. Receptor mediated calcium entry in fura-2 loaded human platelets stimulated with ADP and thrombin: dual wavelength studies with Mn^{2+}. *Biochem. J.* **258**:923–926 (1989).
26. Fasolato, C., A. Pandiella, J. Meldolesi, and T. Pozzan. Generation of inositol phosphates, cytosolic Ca^{2+}, and ionic fluxes in PC12 cells treated with bradykinin. *J. Biol. Chem.* **263**:17350–17359 (1988).
27. Ushkaryov, Y. A., and E. V. Grishin. Black widow spider neurotoxin and its interaction with rat brain receptors. *Bioorg. Khim.* **12**:71–80 (1986).
28. McMahon, H. T., L. Rosenthal, J. Meldolesi, and D. G. Nicholls. α-Latrotoxin releases both vesicular and cytoplasmic glutamate from isolated nerve terminals. *J. Neurochem.*, in press.
29. Misler, S., and L. C. Falke. Dependence on multivalent cations of quantal release of transmitter induced by black widow spider venom. *Am. J. Physiol.* **253**:C469–C476 (1987).
30. Ceccarelli, B., W. P. Hurlbut, and N. Iezzi. Effect of α-latrotoxin on the frog neuromuscular junction at low temperature. *J. Physiol. (Lond.)* **402**:195–217 (1988).
31. Gusovsky, F., T. Yasumoto, and J. W. Daly. Calcium-dependent effects of maitotoxin on phophoinositide breakdown and on cyclic AMP accumulation in PC12 and NCB-20 cells. *Mol. Pharmacol.* **36**:44–53 (1989).
32. Griffin, H. D., and J. N. Hawthorne. Calcium-activated hydrolysis of phosphatidyl-*myo*-inositol 4-phosphate and phosphatidyl-*myo*-inositol 4,5-bisphosphate in guinea-pig synaptosomes. *Biochem. J.* **176**:541–552 (1978).
33. Akhtar, R. A., and A. Abdel-Latif. Calcium ion requirement for acetylcholine stimulated breakdown of triphosphoinositide in rabbit iris smooth muscle. *J. Pharmacol. Exp. Ther.* **204**:655–668 (1978).
34. Eberhard, D. A., and R. W. Holz. Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. *J. Neurochem.* **49**:1634–1643 (1978).
35. McDonough, P. M., D. Goldstein, and J. H. Brown. Elevation of cytoplasmic calcium concentration stimulates hydrolysis of phosphatidyl inositol bisphosphate in chick heart cells: effect of sodium channel activators. *Mol. Pharmacol.* **33**:310–315 (1988).
36. Fisher, S. K., L. M. Domask, and R. M. Roland. Muscarinic receptor regulation of cytoplasmic Ca^{2+} concentrations in human SK-N-SH neuroblastoma cells: Ca^{2+} requirements for phospholipase C activation. *Mol. Pharmacol.* **35**:195–204 (1989).
37. Eberhard, D. A., and R. W. Holz. Intracellular Ca^{2+} activates phospholipase C. Trends Neurosci. **11**:517–520 (1988).
38. Vallar, L., L. M. Vicentini, and J. Meldolesi. Inhibition of inositol phosphate production is a late, Ca^{2+}-dependent effect of D2 dopaminergic receptor activation in rat lactotroph cells. *J. Biol. Chem.* **263**:10127–10134 (1988).

Send reprint requests to: Jacopo Meldolesi, Department of Pharmacology, S. Reffaele Institute, 60 via Olgettina, 20132 Milan, Italy.