Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 620–624, January 1984
Neurobiology

Free cytoplasmic Ca^{2+} and neurotransmitter release: Studies on PC12 cells and synaptosomes exposed to α-latrotoxin

(cytoplasmic Ca^{2+} concentration/black widow spider venom/quin2/synapsin I)

JACOPO MELDOLESI*, WIELAND B. HUTTNER†, ROGER Y. TSIEN‡, AND TULLIO POZZAN§

*Department of Medical Pharmacology, Consiglio Nazionale delle Ricerche Center of Cytopharmacology, University of Milan, 20129 Milan, Italy; †Max Planck Institute for Psychiatry, 8033 Martinsried, Federal Republic of Germany; ‡Department of Physiology-Anatomy, University of California, Berkeley, CA 94720; and §Institute of General Pathology, Consiglio Nazionale delle Ricerche Center for Physiology of Mitochondria, University of Padova, 35100 Padova, Italy

Communicated by Frank Brink, October 5, 1983

ABSTRACT **The relationship between the free cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_i$, and neurotransmitter release was investigated in guinea pig brain synaptosomes and the neurosecretory cell line PC12. Release was induced by α-latrotoxin, which acts in both Ca^{2+}-containing and Ca^{2+}-free incubation media, or by the classical depolarizing agents high K^+ and veratridine, which require extracellular Ca^{2+}. Two complementary approaches were used to reveal changes of $[Ca^{2+}]_i$: (*i*) direct measurement by a fluorescent Ca^{2+} indicator (quin2) and (*ii*) study of the Ca^{2+}-dependent phosphorylation of a protein, synapsin I, located at the cytoplasmic surface of synaptic vesicles. Depolarizing agents, when applied in Ca^{2+}-containing medium, induced the $[Ca^{2+}]_i$ to increase promptly 3- to 6-fold, drastically increased synapsin I phosphorylation, and caused stimulation of transmitter release. With α-latrotoxin, the $[Ca^{2+}]_i$ increase was delayed and occurred at a slower rate, the increase of synapsin I phosphorylation was less drastic, and the release response was much more pronounced. In Ca^{2+}-free medium, depolarizing agents released no transmitter and had no effect on $[Ca^{2+}]_i$ or synapsin I phosphorylation, whereas with α-latrotoxin these processes were dissociated: considerable stimulation of the release without apparent change of $[Ca^{2+}]_i$ and synapsin I phosphorylation. We conclude that the relationship between average $[Ca^{2+}]_i$ and transmitter release is not straightforward and, in particular, that the release evoked by α-latrotoxin in Ca^{2+}-free medium is mediated by a factor(s) other than bulk redistribution of Ca^{2+} from intracellular stores.**

Evidence indicates that evoked neurotransmitter release occurs by exocytosis, which can be triggered by an increase in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) within axon terminals (1–5). When terminals are depolarized, this increase is due to activation of the late Ca^{2+} channels of the presynaptic membrane and therefore requires Ca^{2+} in the extracellular fluid. However, many treatments [e.g., cardiac glycosides (6), presynaptic toxins (7), alcohols and membrane stabilizers (8), oxidizing and reducing agents (9, 10), and food dyes (11)] evoke transmitter release even in Ca^{2+}-free media. These results have often been explained by assuming calcium redistributions (see, however, ref. 8). Terminals contain large amounts of calcium segregated within organelles. Leakage from these stores could increase the $[Ca^{2+}]_i$ from the resting ≈0.1 μM to the activated level and thus account for release responses in Ca^{2+}-free media. Until recently, no procedures were available for measuring the $[Ca^{2+}]_i$ of vertebrate synaptic terminals. Therefore, questions as to whether the increase in $[Ca^{2+}]_i$ is the only trigger of transmitter release and whether a direct correlation exists between the magnitude of the $[Ca^{2+}]_i$ increase and the release response have remained unanswered.

In the present work, two complementary approaches were used. The first relies on the use of quin2, a fluorescent Ca^{2+} indicator. The hydrophobic tetracetoxymethyl ester of quin2 penetrates the plasma membrane and is then hydrolyzed by cytoplasmic esterases to yield the hydrophilic high-affinity Ca^{2+} ligand. Average cytoplasmic $[Ca^{2+}]$ can thus be monitored directly in intact cells (12, 13). The second approach is based on the consideration that an increase in the $[Ca^{2+}]_i$ should bring about the activation not only of the release but also of other cytoplasmic Ca^{2+}-dependent processes. To obtain information on free $[Ca^{2+}]_i$, we studied a well-known Ca^{2+}-dependent reaction, the phosphorylation of synapsin I (14–16). Quin2 yielded clear results, particularly in PC12 cells (not treated with nerve growth factor), a line of neurosecretory cells that resemble undifferentiated sympathoblasts (17); phosphorylation of synapsin I [which is a protein specific for nerve terminals, where it is bound to the surface of synaptic vesicles (18–20)] was investigated in guinea pig brain synaptosomes.

To stimulate neurotransmitter release, we used depolarizing agents (K^+, veratridine) as well as α-latrotoxin (the major toxin of the black widow spider venom), a high M_r protein devoid of any enzyme or membrane perturbing activity that affects vertebrate synapses (for review, see ref. 7) and PC12 cells (21–23). α-Latrotoxin was an ideal tool for our purpose because (*i*) it activates transmitter release by stimulating exocytosis (3, 7, 23, 24) and (*ii*) it works in both Ca^{2+}-containing and Ca^{2+}-free media (7, 22–25).

EXPERIMENTAL PROCEDURES

Materials. Sources of the materials are specified in refs. 12, 13, 15, 16, 22, and 25.

Incubation Media. Two media were used for PC12 cells. Ca-KR included (in mmol/liter) NaCl, 125; KCl, 5; KH_2PO_4 and $MgSO_4$, 1.2; $NaHCO_3$, 5; $CaCl_2$, 1; Hepes-NaOH buffer (pH 7.4), 25; glucose, 6; bovine serum albumin, 0.08. EGTA-KR differed from Ca-KR by having no Ca^{2+} added, 2.4 mM $MgSO_4$, and 1 mM EGTA. In EGTA-KR medium used for synaptosomes, the EGTA concentration was 0.3 mM. The media used with quin2 lacked bovine serum albumin; those for the phosphorylations lacked added KH_2PO_4 and contained bovine serum albumin at 3 μM.

Cells. PC12 cells (17) were detached, washed, and dissociated (22). Crude and purified synaptosomes were isolated by centrifugation from guinea pig brain cortices (25). Loading of PC12 cells and synaptosomes with [^{3}H]dopamine

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Abbreviations: quin2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline; bis(oxonol), bis(1,3-diethylthiobarbiturate)trimethineoxonol.

and [^{3}H]norepinephrine, respectively, and measurement of transmitter release were made as described (22, 25).

Membrane Potential. Plasma membrane potential changes were qualitatively indicated by bis(oxonol) (12), which responds to depolarization with an increase in fluorescence (excitation, 540 ± 2 nm; emission, 580 ± 5 nm). No attempt was made to convert the dye signal into absolute readings.

Quin2 Measurement of $[Ca^{2+}]_i$. For quin2 loading (13), PC12 cells (1–2 × 10^6/ml) or synaptosomes (2 mg of protein/ml) in RPMI 1640 medium/20 mM Hepes/5 mM $NaHCO_3$, pH 7.4, or Ca-KR, respectively, were mixed with 0.5–1% by volume of a 10 mM solution of quin2 acetoxymethyl ester in dimethylsulfoxide and incubated for 1 hr at 37°C. Immediately before use, the cells were pelleted (10,000 × *g*, 3 or 60 sec for PC12 and synaptosomes, respectively) and then suspended in either Ca-KR or EGTA-KR. Quin2 results were calibrated (13). After each assay (excitation, 339 ± 2 nm; emission, 492 ± 10 nm), 5 mM EGTA and Tris base were added in sequence to increase the pH_o to >8.2 and reduce the extracellular calcium concentration, $[Ca^{2+}]_o$, to ≈1 nM. The decrease in fluorescence that sometimes occurred was attributed to external quin2, because the cytoplasmic dye responds slowly to changes of $[Ca^{2+}]_o$. Triton X-100 was then added (final concentration, 0.1%) to release quin2 from all cells and expose it to 1 mM $[Ca^{2+}]_o$, followed by $CaCl_2$ (final $[Ca^{2+}]_o$, 1 mM). The fluorescence intensities at $[Ca^{2+}]_o$ 1 nM and 1 mM, respectively, represent F_{min} and F_{max} in equation 1 of ref. 13. Intermediate values of $[Ca^{2+}]_i$ were calculated, assuming the cytoplasmic pH and Mg^{2+} concentration of PC12 cells and synaptosomes to be as in lymphocytes [7.05 and 1 mM, respectively (26); K_d for Ca^{2+} binding to quin2 = 115 nM]. Whenever necessary, F_{min} and F_{max} were corrected for changes in cell autofluorescence and for external quin2.

Phosphorylation of Synapsin I. Crude guinea pig brain synaptosomes (≈1.5 mg of protein) in 1 ml of either Ca-KR or EGTA-KR supplemented with carrier-free $^{32}P_i$ (1.2 mCi, 1 Ci = 37 GBq) were incubated at 30°C. Blocker drugs (tetrodotoxin, D600) were applied after 39 min and stimulatory drugs (α-latrotoxin, veratridine), after 45 min. Before this last addition and at various times thereafter, 100-μl aliquots were mixed with a stop solution and boiled (15). ^{32}P incorporation into synapsin I at its two principal domains was analyzed (15) by $NaDodSO_4$/PAGE to isolate synapsin I and then subjecting it to limited proteolysis with *Staphylococcus aureus* V8 protease during a second electrophoresis (27). Gels were dried and autoradiographed. The bands corresponding to the upper and lower phosphorylated fragments of synapsin I, identified in separate experiments in guinea pig synaptosomal lysates with [^{32}P]ATP and either Ca^{2+} or cAMP (16), were cut from the gel and their radioactivity was determined. Values were corrected for background determined by radioactivity of the free area between the upper and lower fragments.

RESULTS

Experiments with Quin2. Quin2 was used to monitor the $[Ca^{2+}]_i$ in cells switched from quiescence to a state of stimulated release. To be adequate for this purpose, the probe should (*i*) be localized primarily in the cytosol (rather than within cytoplasmic organelles) and (*ii*) have no effect on evoked transmitter release responses.

(*i*) PC12 cells were loaded with both quin2 and [^{3}H]dopamine (which is stored within granules) and then exposed to digitonin, which disrupts the permeability of the plasmalemma. We found (data not shown) that quin2 was released at the digitonin concentration (ED_{50} = 12 μM) that caused the cells to become stained by the vital dye trypan blue. This is typical of free cytosolic components (13, 28). [^{3}H]Dopamine was solubilized only at higher concentrations (ED_{50} = 40 μM). Accumulation of quin2 in mitochondria and lysosomes can be excluded based on previous work (13, 29).

(*ii*) Fig. 1 illustrates evoked catecholamine release responses. In Ca-KR buffer, considerable responses were induced by K^+ (PC12 cells) and veratridine (synaptosomes). In EGTA-KR medium, release from the preparations exposed to depolarizing agents did not differ from that of controls (22, 25). The responses induced by α-latrotoxin were larger than those of K^+ and veratridine and were maintained in part also in the EGTA-KR medium. No differences were observed between quin2-loaded and control PC12 cells and synaptosomes with any of the treatments used, with and without Ca^{2+} in the incubation medium.

$[Ca^{2+}]_i$ Measurement in PC12 Cells. Fig. 2*C* shows that high K^+ induces plasma membrane depolarization of PC12 cells as revealed by the potential indicator bis(oxonol) (12). The $[Ca^{2+}]_i$ was measured in resting and K^+-depolarized cells that had been loaded with quin2 at various concentrations. As shown in Fig. 2 *Inset*, both the resting and stimulated $[Ca^{2+}]_i$ were independent of the concentration of quin2. These observations indicate that, although accumulation of the probe increases the Ca^{2+} buffering capacity of the cytoplasm (13), the cellular control mechanisms are able to compensate and thus keep the $[Ca^{2+}]_i$ essentially unchanged. Conclusions along the same line were obtained by the use of Ca^{2+} channel blocker drugs in K^+-depolarized cells. Increasing the extracellular K^+ concentration induced a prompt (Fig. 2*A*), verapamil-inhibitable (Fig. 2*B*) increase in quin2 fluorescence, up to a plateau that then declined slowly (data not shown). The measured $[Ca^{2+}]_i$ was 109 ± 3 nM (mean ± SEM, 29 measurements) and 440 ± 48 nM (21 measurements) in resting and depolarized cells, respectively. Application of verapamil after the K^+-induced $[Ca^{2+}]_i$ increase had leveled off resulted in a rapid fluorescence decrease

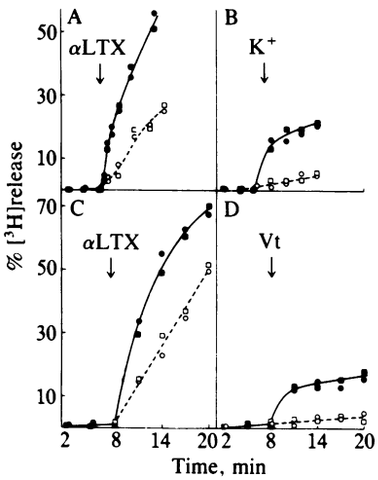

FIG. 1. Effect of previous loading with quin2 on release of [^{3}H]dopamine from PC12 cells (*A* and *B*) or [^{3}H]norepinephrine from purified guinea pig brain synaptosomes (*C* and *D*). PC12 cells and synaptosomes were previously loaded with [^{3}H]catecholamine alone (○, ●) or together with quin2 (□, ■). Final quin2 concentration was 0.8 nmol per 10^6 cells (≈1.3 mM) in PC12 cells and 2.5 nmol/mg of protein (≈0.75 mM) in synaptosomes. After dilution, centrifugation, and washing, PC12 cells and synaptosomes were resuspended (3 × 10^6 cells per ml and 0.5 mg of protein/ml, respectively) in either Ca-KR (●, ■) or EGTA-KR (□, ○) medium containing desmethylimipramine (2 μM) and incubated at 37°C (PC12 cells) or 30°C (synaptosomes). Aliquots (0.15 ml) of these suspensions were centrifuged through oil layers. Additions of stimulatory drugs are marked by arrows: 3 nM α-latrotoxin (dLTX) (*A* and *C*), 50 mM KCl (*B*), 0.1 mM veratridine (Vt) (*D*). Data are expressed as % release. 100% release = ^{3}H radioactivity at the earliest time point investigated (2 min).

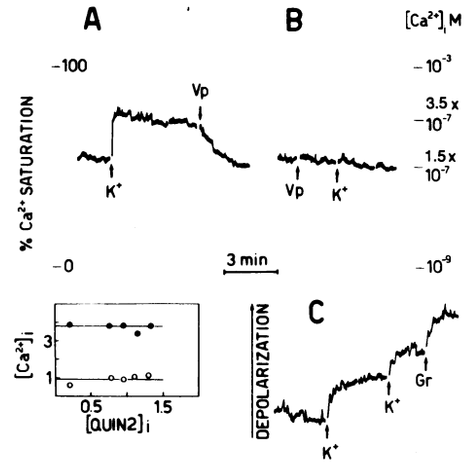

FIG. 2. Effect of K^+ on $[Ca^{2+}]_i$ and plasma membrane potential in PC12 cells. (*A* and *B*) Cells (6×10^5/ml) loaded with quin2 (1 nmol per 10^6 cells) in Ca-KR medium were exposed to KCl (50 mM) (K^+). Where indicated verapamil (Vp) (25 μM) was added. (*C*) Cells (10^5/ml) in Ca-KR medium were incubated at 37°C with 0.1 μM bis-(oxonol) until fluorescence leveled off. K^+ marks additions of KCl, each increasing $[K^+]_o$ by 20 mM. Gr, 0.1 μM gramicidin was added to induce complete depolarization. Spikes in the traces here and in Fig. 4 might be due to cell clumps (23). (*Inset*) Aliquots of PC12 cells, incubated with various concentrations of quin2 acetoxymethyl ester and thus loaded with various concentrations of quin2, were stimulated with 50 mM KCl. Values of $[Ca^{2+}]_i$ in units of 0.1 μM measured in the cells at rest (○) and 15 sec after stimulation (●) are plotted as a function of quin2 concentration within the cells (nmol per 10^6 cells).

(Fig. 2*A*), suggesting that the plateau was due to a persistent increase in the mean activation of the calcium channels. Thus, a sustained alteration in pump–leak balance at the plasma membrane is able to reset the $[Ca^{2+}]_i$ to the same level regardless of the buffering contributed by the dye (13, 28), which fits with the inability of dye loading to affect release (Fig. 1). No changes of quin2 fluorescence were observed when K^+ was applied in EGTA-KR medium.

The results reported so far indicate that quin2 is an adequate probe to monitor average $[Ca^{2+}]_i$ in PC12 cells. Further experiments were therefore carried out with α-latrotoxin (Fig. 3). In Ca-KR medium, the toxin (1 or 3 nM) induced increases in the $[Ca^{2+}]_i$ to levels between 0.3 and 1 μM (five experiments). These increases were preceded by a lag phase, which contrasted with the almost immediate effect of K^+ (compare Fig. 3 *A* and *B* with Fig. 2*A*). A lag phase was also seen to precede the α-latrotoxin-induced membrane depolarization (Fig. 3*D*). Moreover, the rate of $[Ca^{2+}]_i$ increase was much slower with α-latrotoxin than with K^+ (compare Fig. 3 *A* and *B* with Fig. 2*A*). Addition of EGTA (5 mM) when the $[Ca^{2+}]_i$ increase had leveled off caused a return of the $[Ca^{2+}]_i$ to and below the resting level (Fig. 3*A*). The Na^+ channel blocker tetrodotoxin (0.3 μM) inhibited neither the depolarization nor the $[Ca^{2+}]_i$ increase induced by α-latrotoxin (data not shown), while verapamil, which abolished the K^+-induced $[Ca^{2+}]_i$ increase, was without effect on α-latrotoxin (compare Fig. 3*B* with Fig. 2*B*).

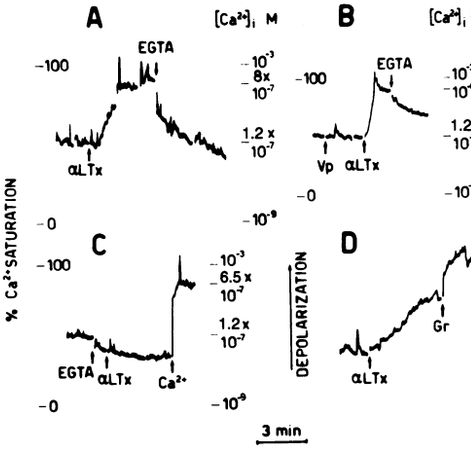

FIG. 3. Effect of α-latrotoxin (αLTx) on $[Ca^{2+}]_i$ and plasma membrane potential in PC12 cells. (*A* and *B*) Cells (6.6×10^5/ml) loaded with quin2 (0.8 nmol per 10^6 cells) and suspended in Ca-KR medium were exposed to α-latrotoxin (*A*, 1 nM; *B*, 3 nM), verapamil (Vp, 25 μM) and EGTA (5 mM) where indicated. (*C*) Cells as in *A* and *B* but without added Ca^{2+} were exposed sequentially to EGTA (1 mM), α-latrotoxin (1 nM), and $CaCl_2$ (Ca^{2+}, 2 mM). (*D*) Conditions as in Fig. 2*C*, but α-latrotoxin (1 nM) was added where indicated.

$[Ca^{2+}]_i$ Measurement in Synaptosomes. Two problems were encountered when using quin2 in synaptosomes (Fig. 4): (*i*) The hydrolysis of quin2 AM was inefficient, possibly because esterases were depleted during synaptosome formation. Thus, appreciable amounts of the uncleaved compound disturbed $[Ca^{2+}]_i$ measurements. (*ii*) Exposure to α-latrotoxin in Ca-KR medium resulted in partial (≈10%) release of the accumulated probe (possibly a sign of α-latrotoxin toxicity), giving rise to a large spurious signal (Fig. 4*B*). Measurements of the $[Ca^{2+}]_i$ could be made only by subtracting this from the total signal and were therefore inaccurate. Despite these limitations, the following conclusions were reached, which agreed with the results in PC12 cells: (*i*) synaptosomes have a resting $[Ca^{2+}]_i$ of about 0.1 μM; treatment with α-latrotoxin in Ca-KR medium resulted in an increase in this level, which was delayed compared with that induced by high K^+ (compare Fig. 4 *A* and *B*), and (*ii*) in EGTA-KR medium, in which measurements are much more accurate than in Ca-KR medium, the resting $[Ca^{2+}]_i$ decreased after application of α-latrotoxin (Fig. 4*C*).

Phosphorylation of Synapsin I. Crude guinea pig brain synaptosomes previously loaded with $^{32}P_i$ were exposed to stimulatory drugs, α-latrotoxin and veratridine, in Ca-KR and EGTA-KR media (Fig. 1 *C* and *D*). Phosphorylation of synapsin I was analyzed after limited proteolysis, which generates two major phosphorylated fragments, an upper fragment (≈30 kilodaltons) corresponding to the tail region of the

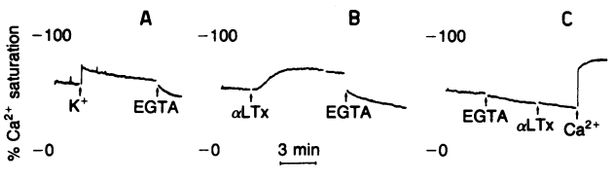

FIG. 4. Effects of K^+ and α-latrotoxin (αLTx) on $[Ca^{2+}]_i$ in guinea pig brain synaptosomes. (*A* and *B*) Synaptosomes (0.8 mg of protein/ml) loaded with quin2 (2.2 nmol/mg of protein) were exposed to KCl (50 mM) or α-latrotoxin; 3 nM, respectively, in Ca-KR medium. (*C*) Conditions as in *A* and *B* but without Ca^{2+} added. Where indicated, EGTA and $CaCl_2$ (Ca^{2+}) were added.

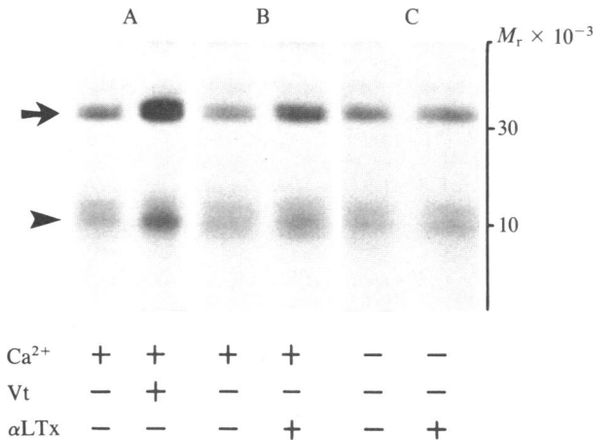

FIG. 5. Effect of veratridine and α-latrotoxin (αLTx) on phosphorylation of synapsin I upper and lower fragments in crude guinea pig brain cortex synaptosomes. Pellets were resuspended in Ca-KR (*A* and *B*) or EGTA-KR (*C*) medium (1.5 mg of protein/ml) containing $^{32}P_i$ (0.8 mCi/ml) and incubated at 30°C for 45 min, after which stimulatory drugs were added. The phosphorylated fragments of synapsin I were revealed by autoradiography of the gels after limited proteolysis. In each pair, the left lane is the control, the right, the experimental sample. (*A*) Veratridine (Vt, 0.1 mM; 20 sec). (*B* and *C*) α-Latrotoxin (3 nM; 60 sec). The arrow marks the upper and the arrowhead marks the lower fragment of synapsin I.

protein and a lower fragment (≈10 kilodaltons) (15, 16). Phosphorylation of the upper fragment is stimulated by Ca^{2+}, whereas that of the lower fragment is less specific, being stimulated by both Ca^{2+} and cAMP (15, 16). Examples of the peptide maps obtained from the guinea pig synapsin I are given in Fig. 5, and the time course of the upper fragment phosphorylation is given in Fig. 6. Application of veratridine in Ca-KR medium resulted in a prompt large increase of both upper and lower fragment phosphorylation (Fig. 5*A*), which was maximal in 20 sec and disappeared at 85 sec (Fig. 6). Omission of Ca^{2+} from the medium (Fig. 6) or pretreatment with blockers of the Na^+ (tetrodotoxin, 0.5 μM) or Ca^{2+} (D600, 0.1 mM) channels (data not shown) abolished this effect.

α-Latrotoxin applied in the Ca^{2+}-containing medium also increased the phosphorylation of synapsin I (Figs. 5*B* and 6). The effect appeared at 0.3 nM and was maximal at 3 nM, which is optimal for the release response. Compared with veratridine, phosphorylation of the Ca^{2+}-specific upper fragment was delayed (peak at 60 sec) and was less (Fig. 6). Basically the same results were obtained with the lower fragment. Phosphorylation of both fragments was unaffected by tetrodotoxin and blocked only in small part by D600 (data not shown).

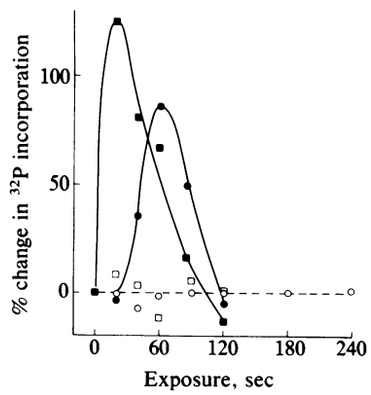

FIG. 6. Time course of radioactive phosphate incorporation in synapsin I upper fragment from crude guinea pig brain cortex synaptosomes exposed to veratridine (0.1 mM; □, ■) or α-latrotoxin (3 nM; ○, ●) in either Ca-KR (■, ●) or EGTA-KR (□, ○) medium. Experimental conditions were as in Fig. 5. Values shown are means of two experiments.

Phosphorylation of synapsin I was also investigated in synaptosomes exposed to α-latrotoxin in EGTA-KR medium. Toxin concentrations ranged from 0.6 to 20 nM and incubation times were 20–480 sec. Under these conditions neurotransmitter release was markedly stimulated (Fig. 1*C*). However, phosphorylation of synapsin I was not stimulated in either the upper or the lower fragment (Figs. 5*C* and 6).

DISCUSSION

The present study on the relationship between the $[Ca^{2+}]_i$ and transmitter release relies primarily on the use of α-latrotoxin. The mechanisms by which this toxin evokes the release are incompletely understood. It is clear, however, that they are not identical to those triggered by action potentials and simple depolarizing agents (7, 21–25, 30). Thus, at the moment our results with α-latrotoxin should be considered as pertaining to the experimental conditions in which they were obtained. Their relevance at the physiological level remains to be investigated.

Two strategies were used in two cellular systems. Quin2 allowed a continuous, sensitive measure of average $[Ca^{2+}]_i$ in PC12 cells, which, being a cloned cell line, were expected to be homogeneous in terms of receptors, Ca^{2+} channels, and response. However, rapid $[Ca^{2+}]_i$ transients or differences in $[Ca^{2+}]_i$ among regions of the cytoplasm would not be revealed. Because quin2 had no effect on the release responses and variations in its initial concentration did not modify either the resting or the stimulated $[Ca^{2+}]_i$ appreciably, its buffering power seems not to have perturbed the essential ionic events. The study of the phosphorylation of synapsin I in brain synaptosomes was interesting (*i*) because of the detailed knowledge of its specific regulation by Ca^{2+} (14–16, 20), (*ii*) because of the strategic localization of this protein around synaptic vesicles (18–20), and (*iii*) because it involved no perturbations of the intraterminal homeostasis. However, the phosphorylation of synapsin I can be regarded only as an indirect indicator of $[Ca^{2+}]_i$. Thus, factors other than $[Ca^{2+}]_i$ fluctuations might also have affected the state of phosphorylation of the protein.

In view of these considerations, the congruency of the data obtained by the two approaches is impressive. The following findings on $[Ca^{2+}]_i$ were revealed by both quin2 and synapsin I phosphorylation. (*i*) $[Ca^{2+}]_i$ increases induced by α-latrotoxin applied in Ca-KR medium were delayed and more gradual than those of the classical depolarizing agents. This delay, which resembles the latency of the α-latrotoxin-induced membrane depolarization (Fig. 3*D* and ref. 25), was not seen when Ca^{2+} was applied to PC12 cells and synaptosomes preexposed to α-latrotoxin in EGTA-KR medium. Thus it might be due to the time needed for α-latrotoxin-receptor binding or for the ensuing activation of ion permeability (21, 25, 30, 31). (*ii*) The Ca^{2+} channel blockers abolished both the quin2 and the phosphorylation responses to classical depolarizing agents but affected α-latrotoxin only slightly, confirming that only a minor part of the Ca^{2+} influx induced by α-latrotoxin goes through the voltage-dependent Ca^{2+} channel (21, 22, 25). (*iii*) There is no single correlation between $[Ca^{2+}]_i$ increases and evoked release responses. The effects on the $[Ca^{2+}]_i$ of depolarizing agents applied in Ca-KR medium were unexpectedly similar to (or, in the case of synapsin I phosphorylation, even higher than) those of α-latrotoxin, which, however, evoked much larger release. (*iv*) α-Latrotoxin in EGTA-KR medium induced no $[Ca^{2+}]_i$ increases, yet evoked considerable release responses. Even

under these conditions, transmitter release is known (24) to occur by exocytosis rather than nonspecific lysis.

Points *iii* and *iv* raise questions about the causal relationship between $[Ca^{2+}]_i$ increases and neurosecretion. When the $[Ca^{2+}]_i$ is increased within nerve terminals and neurosecretory cells, release is certainly stimulated (1, 2, 4, 32). However, the assumption that increased $[Ca^{2+}]_i$ is the only trigger for secretion from intact terminals and cells could not be tested until the $[Ca^{2+}]_i$ could be measured as in this study and ref. 33 (for studies in nonneuronal systems, see refs. 29 and 34).

Our finding that the average $[Ca^{2+}]_i$ measured by quin2 was <1 μM in discharging PC12 cells and synaptosomes agrees with results in adrenal medullary cells (33). However, exocytosis occurs at discrete sites of the plasmalemma, where the $[Ca^{2+}]$ is possibly higher than the average seen by the dye. We doubt that this potential discrepancy is sufficient to explain the poor correlation between secretion and the dye readout of $[Ca^{2+}]_i$, especially the absence of $[Ca^{2+}]_i$ increases during the responses by α-latrotoxin in EGTA-KR medium. PC12 cells and synaptosomes are small, $\approx$10 and $\approx$1 μm in diameter, respectively (23, 25), and both release and $[Ca^{2+}]_i$ are measured on time scales of minutes. Ca^{2+} should approach equilibrium over such space and time dimensions. There is no evidence that the Ca^{2+} influxes induced by depolarization and α-latrotoxin occur in separate domains of the plasmalemma. To the extent that these domains overlap, any diffusional gradients should be similar under the two modes of stimulation. In addition, the synapsin I phosphorylation data, which agree with the dye responses, are expected to reflect $[Ca^{2+}]$ in the immediate environment of synaptic vesicles. Finally, the component of the α-latrotoxin-induced response not explainable by $[Ca^{2+}]_i$ resembles phenomena recently described in neutrophils (29) and platelets (34). In the latter system, there is evidence that release without $[Ca^{2+}]_i$ increase is mediated by a distinct biochemical pathway involving the activation of protein kinase C (34).

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