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N- and P-type Ca^{2+} channels are involved in acetylcholine release at a neuroneuronal synapse: Only the N-type channel is the target of neuromodulators

(ω-conotoxin/funnel-web-spider toxin/*Aplysia californica*)

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ABSTRACT **Cholinergic transmission in an identified neuro-neuronal synapse of the *Aplysia* buccal ganglion was depressed by application of a partially purified extract of the funnel-web-spider venom (FTx) or of its synthetic analog (sFTx). This specific blocker of voltage-dependent P-type Ca^{2+} channels did not interfere with the effect of the N-type Ca^{2+} channel blocker ω-conotoxin, which could further decrease synaptic transmission after a previous application of FTx. Similar results were obtained when the reversal order of application of these two Ca^{2+} channel blockers was used. Both P- and N-type Ca^{2+} currents trigger acetylcholine release in the presynaptic neuron. The neuromodulatory effects of FMRF-amide, histamine, and buccalin on transmitter release disappeared after the blockade of the N-type Ca^{2+} channels but remained still effective in the presence of FTx. These results indicate that only N-type Ca^{2+} channels appear to be sensitive to the neuromodulators we have identified.**

P-type Ca^{2+} channels were first identified in Purkinje cells from mammalian cerebellum (1) because of their sensitivity to FTx, a polyamine molecule present in a low molecular weight fraction extracted from the venom of the funnel-web spider *Agenelopsis aperta*. A synthetic derivative of FTx, sFTx, also blocks P-type channels with high specificity (2). With the use of these new toxins, it was possible to show that P-type Ca^{2+} channels were involved in the triggering of transmitter release at the squid presynaptic terminal (1) and at the mammalian neuromuscular junction (3).

Other types of high voltage-activated Ca^{2+} channels have also been associated with the release of neurotransmitters. Thus, for example, Ca^{2+} influx through dihydropyridine-sensitive L-type channels triggers the release of substance P from dorsal root ganglion neurons (4) and also the release of cathecholamine from chromaffin cells (5). N-type channels, blocked by ω-conotoxin, are involved in the release of neurotransmitters such as norepinephrine (6–10), acetylcholine (Ach) (11–15), and adenosine (16). These N-type Ca^{2+} channels are also present in sensory nerves (17–19) and central neurons (20–24) and function in autonomic neurotransmission (25).

However, in most cases, the use of any single drug (dihydropyridine, ω-conotoxin, or FTx) does not produce complete blockade of transmitter release. Brain synaptosomes are only partially sensitive to either ω-conotoxin, dihydropyridine (26, 27), or FTx (3). It has been suggested that both P- and N-type channels participate in Ach release in *Torpedo* synaptosomes (28). However, the possible involvement of more than one type of high voltage-activated Ca^{2+} channels in triggering neurotransmitter release has not been investigated for synapses *in vivo*.

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The aim of this work was to identify the presynaptic Ca^{2+} channels responsible for triggering Ach release at a well-known neuroneuronal synapse of *Aplysia californica* (29). We have previously shown that nifedipine, a dihydropyridine derivative, does not affect Ach release (30), whereas ω-conotoxin is only partially effective (31). Based on these results, we concluded that N-type, but not L-type, Ca^{2+} channels were associated with transmitter release in our preparation. We have now extended our analysis to include the P-type channel by taking advantage of the specificity of FTx. Thus the effects of FTx on both Ach release and presynaptic Ca^{2+} influx were studied. Moreover, since we have shown that activation of presynaptic heteroreceptors by Phe-Met-Arg-Phe-NH_2 (FMRFamide), histamine, and buccalin controls Ach release by modulating a nifedipine-resistant Ca^{2+} current (29, 32), this preparation offers the opportunity for testing which type of Ca^{2+} channel is the target for modulation.

MATERIALS AND METHODS

Experiments were performed at 22°C on neurons of the buccal ganglion of *A. californica*. The ganglia were pinned to the bottom of a 1-ml Sylgard-coated perfusion chamber and carefully freed of connective tissue to expose the cells. Identified presynaptic (B4 or B5) and postsynaptic (B3 or B6) neurons were each penetrated with two microelectrodes with resistance varying between 0.5 and 1.5 MΩ when the electrodes were filled with 3 M KCl. B4 and B5 cells are cholinergic interneurons containing the small peptide FMRF-amide (33).

The experimental chamber contained 1 ml of artificial seawater of the following composition (mM): NaCl, 460; KCl, 10; $CaCl_2$, 11; $MgCl_2$, 25; $MgSO_4$, 28; Tris·HCl buffer, 10 (pH 7.8). FTx was purified from the crude venom of American funnel-web spiders as described (1). The concentration of the stock solution was considered to be 10^{-1} (vol/vol). Because our stock of toxin was very limited, FTx was added directly to the experimental chamber to obtain a final concentration of 10^{-3} (vol/vol). The absence of perfusion did not modify the amplitude of postsynaptic responses. sFTx was used in identical conditions as native FTx. ω-Conotoxin (Calbiochem) was prepared as a 100 μM stock solution in distilled water. It was applied to the preparation at a concentration of 3–10 μM in artificial seawater with reduced Ca^{2+} (1 mM $CaCl_2$) and Mg^{2+} (1 mM $MgCl_2$) (complemented with equimolar concentrations of Tris·HCl). After 30 min of application of the toxin, normal bathing solutions were reintroduced and allowed to equilibrate for 10 min. Nifedipine (Sigma) was

Abbreviations: Ach, acetylcholine; FTx, funnel-web-spider toxin; sFTx, synthetic FTx; IPSC, inhibitory postsynaptic current; LDIPSC, long-duration induced postsynaptic current; MPSC, miniature postsynaptic current.

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solubilized in dimethyl sulfoxide and was used at a final concentration of 10 μM in 0.1% dimethyl sulfoxide. This concentration of dimethyl sulfoxide was previously shown to have no effect on transmitter release or ionic currents (30). The peptides FLRFamide and buccalin were obtained from Bachem.

Electrical Recordings. Cells were voltage-clamped with a conventional two-electrode system. Postsynaptic responses were expressed as conductances (nS) by dividing their amplitude (measured in nA) by the driving force (29). The presynaptic neuron was maintained at −50 mV by using an independent current electrode. The amplitude of the inhibitory postsynaptic current (IPSC) recorded in the voltage-clamped postsynaptic neuron (at −80 mV) is directly related to the quantal release of Ach. Alternatively, to study transmitter release independently of spike conduction or modifications of presynaptic ionic fluxes other than Ca^{2+}, we evoked long-duration induced postsynaptic currents (LDIPSCs) by applying 3-s depolarizing commands (from −50 to +10 mV) to the presynaptic neuron in the presence of 0.1 mM tetrodotoxin (Sigma) to block the Na^+ channels. The statistical analysis of this current response, which has been described in detail (32), allows us to determine LDIPSC amplitude (A), evoked miniature postsynaptic current (MPSC) mean amplitude (g_{min}), MPSC decay time constant (τ), and the number of quanta released (Q), with $Q = (3A)/(g_{min}\tau)$. This calculation of Q is independent of modifications at the postsynaptic level because such modifications affect A and g_{min} to equal extents.

Measurement of Ca^{2+} Currents. To isolate the inward Ca^{2+} current, contaminating Na^+ and K^+ currents were blocked with an extracellular solution containing 100 μM tetrodotoxin, 50 mM tetraethylammonium chloride (Sigma), and 4 mM 4-aminopyridine (Sigma). Under these conditions, K^+ currents are greatly reduced (34) and do not significantly contaminate the inward Ca^{2+} current (35). To increase the magnitude of the Ca^{2+} current recorded in the presynaptic neuron, the extracellular $CaCl_2$ was raised to 55 mM. All Ca^{2+} currents were corrected for leakage. In this preparation, it is possible to evoke transmitter release through a voltage step in the clamped presynaptic neuron (32, 36). No Ca^{2+} influx was detected focally in the presynaptic soma by the aequorin method (unpublished results). Therefore, we think that the Ca^{2+} current recorded in the soma largely reflects Ca^{2+} influx at the terminals.

RESULTS

Effects of FTx on Synaptic Transmission. The application of partially purified FTx resulted in depression of synaptic transmission: as shown in Fig. 1, the amplitude of the IPSC in response to an action potential was decreased by 7%, 20%, and 35% in the presence of 3×10^{-4}, 5×10^{-4}, and 10^{-3} (vol/vol) FTx, respectively. FTx at a concentration of 10^{-3} depressed the response by an average 35% ± 0.7% (four experiments) without having any apparent effect on the presynaptic action potential (Fig. 1). Similarly, sFTx, reported to have FTx-like activity (37), decreased the amplitude of postsynaptic responses by 43% when applied at 1 mM (Fig. 2*A*).

The observed depressive effect could be attributed to a pre- and/or postsynaptic action of the toxin. Indeed FTx might partially block the postsynaptic receptor channel complex as already reported at the neuromuscular junction (3). We tested this possibility by recording the current response to ionophoretic application of Ach onto the postsynaptic soma. Somatic receptors activated by this procedure exhibit properties identical to those of the synaptic area (38). A representative example is shown in Fig. 2*B*. The current response (655 ± 12 nS, $n = 3$) was indeed decreased by about 20% in

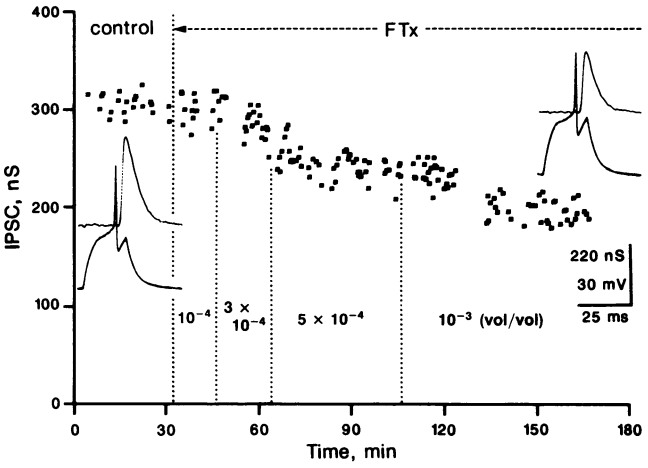

FIG. 1. Effect of increasing concentrations of partially purified FTx on the amplitude of IPSCs evoked by an action potential. Insets represent the IPSC (upper trace) and the presynaptic action potential (lower trace) in the control situation (left recording) and after application of FTx (right recording).

the presence of 1 mM sFTx (528 ± 8 nS, $n = 7$). However, the percentage of blockade of this postsynaptic response was lower than the decrease in IPSC amplitude. Consequently, it seems likely that a presynaptic mechanism also contributes to the synaptic depression induced by FTx.

Action of FTx on the Ca^{2+} Current Recorded in the Presynaptic Neuron. The peak amplitude of the Ca^{2+} current was decreased by 11% and 31% in the presence of 1 mM and 3 mM sFTx, respectively (Fig. 3*A*).

The action of partially purified FTx was assayed after the L-type component of the Ca^{2+} current was blocked with 10 μM nifedipine. This concentration of nifedipine, which decreased the Ca^{2+} current by about 33% (Fig. 3*B*), had no effect on the amplitude of the postsynaptic response, clearly indicating that L-type Ca^{2+} channels were not involved in triggering Ach release (30). Under these conditions, FTx (10^{-3}, vol/vol) decreased the peak Ca^{2+} current by 8.6 ± 0.4% (three experiments) (Fig. 3*B*) and the postsynaptic response by 35% (Fig. 3*C*). As expected, the magnitude of the synaptic depression was the same regardless of the presence of nifedipine (see Fig. 1). Subsequent application of ω-cono-

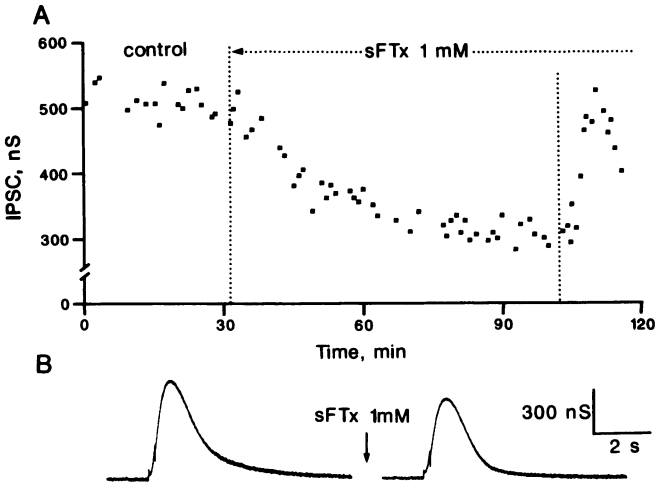

FIG. 2. (*A*) Action of 1 mM sFTx on IPSC amplitude. After the IPSC amplitude was reduced by sFTx, bath application of 0.1 mM FLRFamide (indicated by the second vertical dotted line) transiently potentiated the postsynaptic response. (*B*) sFTx decreased the postsynaptic current response evoked by ionophoretic application of Ach on the postsynaptic neuron.

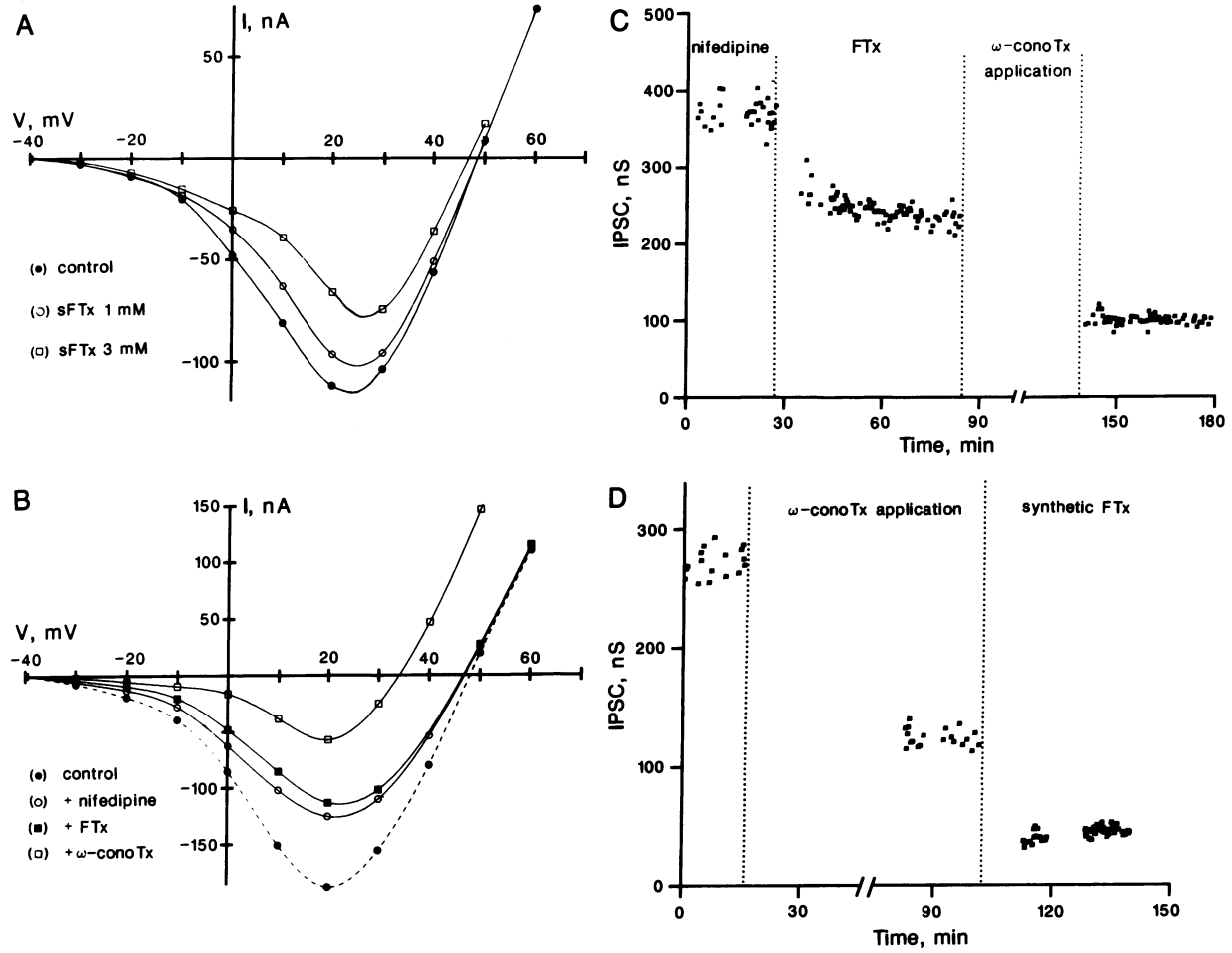

FIG. 3. (*A*) Inhibitory effect of sFTx on the presynaptic Ca^{2+} current. Current–voltage (*I–V*) curves have been leakage-subtracted. (*B*) After the blockade of L-type Ca^{2+} channels by nifedipine (10 μM), FTx (10^{-3}, vol/vol), and ω-conotoxin (ω-conoTx, 5 μM) have additive decreasing effects on the remaining presynaptic Ca^{2+} current. (*C* and *D*) Whatever the order of application, FTx and ω-conotoxin exhibit additive depressing effects on the amplitude of IPSCs. In *C*, the IPSC amplitude is not decreased by nifedipine (10 μM) and is used as a control.

toxin (5 μM), which blocks N-type Ca^{2+} channels, further decreased the remaining peak Ca^{2+} current by 50% (Fig. 3*B*) and finally depressed the IPSC amplitude by 58% (Fig. 3*C*) with respect to the situation under FTx. We have previously shown that application of ω-conotoxin alone depresses the IPSC amplitude by 54 ± 7% (31). These experiments indicate that FTx and ω-conotoxin have cumulative effects on the amplitude of IPSCs. To confirm a conjoined involvement of P- and N-type Ca^{2+} channels in the initiation of Ach release, the cumulative effect of ω-conotoxin and FTx on the amplitude of IPSCs was checked by blocking first N-type channels and then P-type channels. The blockade of N-type Ca^{2+} channels with ω-conotoxin induced a decrease in the postsynaptic response by 56% (Fig. 3*D*). In this situation, the application of sFTx (4 mM) produced a further decrease of 63% in IPSC amplitude. This additional decrease was partly the consequence of the postsynaptic blocking effect of FTx (Fig. 2*B*). This decrease is in good agreement with the result reported in Fig. 3*C* and is in favor of the involvement of both P-type and N-type Ca^{2+} channels in triggering Ach release.

Modulation of Ach Release After Blockade of P-Type Ca^{2+} Channels. The presynaptic Ca^{2+} current is modulated by a number of neuroactive substances, including FMRFamide, buccalin, and histamine (29, 32). The FMRFamide-induced increase in presynaptic Ca^{2+} current is linked to phosphorylation of the Ca^{2+} channels by protein kinase C (39) and results in facilitation of Ach release. Conversely, histamine and buccalin depress the presynaptic Ca^{2+} current, leading to a decrease of Ach release. Tail-current analysis of the Ca^{2+} current shows that histamine and FMRFamide shift the voltage dependence of the nifedipine-resistant Ca^{2+} channels toward higher voltages and lower voltages, respectively (29). Buccalin also depresses the amplitude of the nifedipine-resistant Ca^{2+} current, but without affecting the voltage dependence of the channels (29). Given our present results that Ach release was sensitive to both ω-conotoxin and FTx, it became of interest to determine whether both N- and P-type Ca^{2+} channels were targeted by these neuromodulators.

As shown in Fig. 4*A*, histamine (0.1 mM) reduced the postsynaptic current (by about 40%), even after L-type and P-type channels were blocked respectively by nifedipine (10 μM) and FTx (10^{-3}, vol/vol). Subsequent addition of buccalin (10 μM) to the bath produced a further decrease in the postsynaptic response of about 47% with respect to the level obtained after histamine application. In the representative experiment of Fig. 4*A*, the effects of histamine and buccalin were quite similar in magnitude to those that have been observed in the absence of FTx (29, 32). FLRFamide was also still able to increase the amplitude of the postsynaptic response when P-type Ca^{2+} channels were blocked (Figs. 2*A* and 4*B*). Statistical analysis of LDIPSCs revealed that the number of evoked quanta released was decreased by 45% (Fig. 4*B*) after application of sFTx (1 mM) and by 33% after the application of partially purified FTx (10^{-3}, vol/vol). As this calculation is independent of any postsynaptic effect (40), the decrease in Ach release confirms the presynaptic action of FTx. After blockade of P-type Ca^{2+} channels (Fig. 4*B*), FLRFamide still increased the number of released quanta by

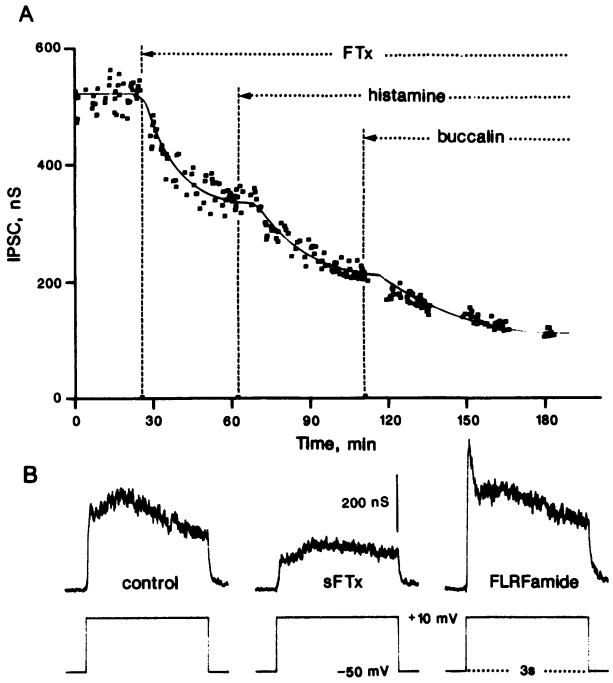

FIG. 4. (*A*) In the presence of FTx (10^{-3}, vol/vol) the amplitude of IPSCs is reduced. Histamine (0.1 mM) and buccalin (10 μM) further decrease IPSC amplitude by the same extent as in control conditions (29). (*B*) Amplitude of the postsynaptic response (LDIPSC, upper trace) evoked by a constant depolarization of the voltage-clamped presynaptic neuron (lower trace) is lowered by sFTx. In this condition, FLRFamide (0.1 mM) increases the amplitude of the response. The mean number of quanta released was 38,860 in the control, 21,470 after FTx, and 43,235 after FLRFamide (which represents an increase of >100% with respect to the situation under FTx). The mean amplitude of MPSCs (1.7 nS) was reduced by 12% after FTx with respect to control (1.93 nS). The mean decay time of the MPSC remained constant (12 ms).

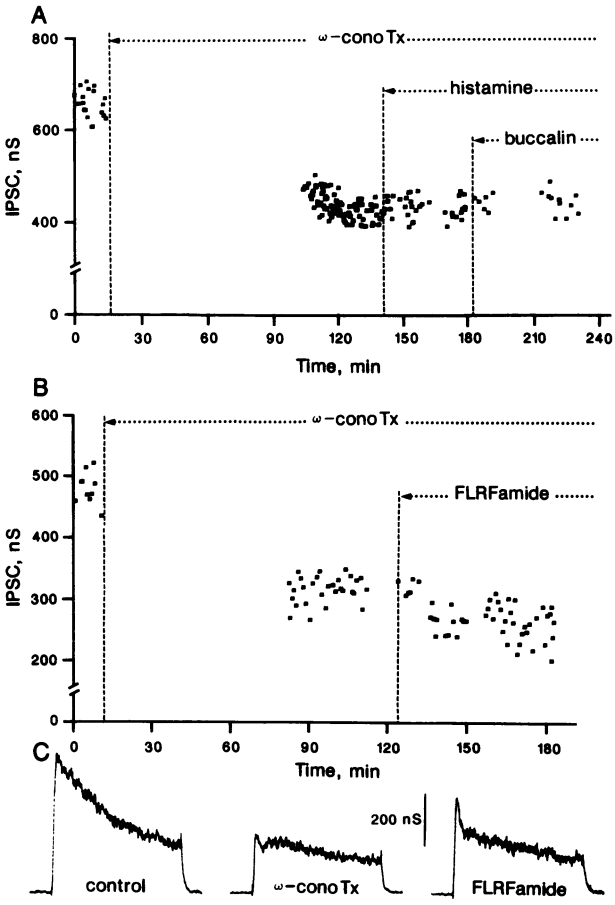

FIG. 5. (*A*) Histamine (0.1 mM) and buccalin (10 μM) are no longer able to decrease the amplitude of the IPSCs after the effect of ω-conotoxin (ω-conoTx, 5 μM). (*B*) Potentiating effect of FLRFamide (0.1 mM) on IPSC amplitude is prevented by pretreatment with ω-conotoxin (5 μM). (*C*) Amplitude of the postsynaptic response (LDIPSC) evoked by a constant depolarization of the voltage-clamped presynaptic neuron is lowered by ω-conotoxin. Subsequent application of FLRFamide produces only a slight increase of the amplitude of the LDIPSC. Under ω-conotoxin, the mean number of released quanta (25,192) was reduced with respect to the control (46,526). In the presence of FLRFamide, the number of released quanta was 29,508, which represents a mean increase of only 17% with respect to the situation under ω-conotoxin. The mean amplitude and decay time of MPSCs, 1.22 nS and 13 ms, respectively, were unaffected by any of the treatments.

>100%. No significant difference in the potentiation of Ach release was observed whether FTx was present (Fig. 4*B*) or not (32, 39).

Absence of Modulation of Ach Release After Blockade of N-Type Ca^{2+} Channels. Blockade of N-type Ca^{2+} channels by ω-conotoxin, which reduced the IPSC amplitude by 44%, rendered ineffective the modulation of synaptic transmission by histamine and buccalin (Fig. 5*A*). FLRFamide induced a slight decrease of the IPSCs (Fig. 5*B*) in contrast to its classical transient potentiating effect (see Fig. 2*A* and ref. 32). Similarly, FLRFamide produced only a small increase in the LDIPSCs (Fig. 5*C*) when N-type Ca^{2+} channels had been blocked by ω-conotoxin. This potentiation was much smaller than that observed when FLRFamide was applied in the absence of any Ca^{2+} channel blocker (32) or after FTx (Fig. 4*B*). In the experiment depicted in Fig. 5*C*, the increase in the number of released quanta was <20%.

Effect of FTx on the Mean Amplitude of Evoked MPSCs. The calculation of the number of released quanta and of the amplitude of evoked MPSCs by a statistical analysis of LDIPSCs allows one to differentiate between pre- and postsynaptic effects of a drug (40). The mean amplitude of the MPSCs was reduced by 12% and 19% (two experiments) in the presence of sFTx (1 mM) and by 17% in the presence of partially purified FTx (10^{-3}, vol/vol) with respect to the controls. These results are in good agreement with the decrease in the postsynaptic response evoked by iontophoretic application of Ach in the presence of sFTx (Fig. 2*B*).

DISCUSSION

Our electrophysiological results favor the notion that two types of Ca^{2+} channels, N and P types, trigger Ach release in an identified cholinergic neuroneuronal synapse of *Aplysia* (29, 32). The presence of P-type Ca^{2+} channels coexisting with another nifedipine-insensitive channel on the same terminal was suggested by Uchitel *et al.* (3) for cerebral cortex synaptosomes on the basis that FTx did not completely block the K^+-induced uptake of $^{45}Ca^{2+}$. In *Torpedo* synaptosomes, P-type Ca^{2+} channels mediate 50% of the KCl-evoked Ach release which is moderately inhibited by ω-conotoxin (28). We have shown in this report that ω-conotoxin and FTx have cumulative depressive effects not only on the Ca^{2+} influx recorded in the presynaptic neuron but also on Ach release.

In our experiments FTx and ω-conotoxin were applied successively at 10^{-3} (vol/vol) and 5 μM, respectively. At this concentration of FTx, Uchitel *et al.* (3) have blocked completely the P-type Ca^{2+} channels at the mammalian neuromuscular junction. Because the only channels at this synapse are of the P type, the removal of the Ca^{2+} current was total. We could expect that the situation would be similar in our preparation after the blockade of L-, N-, and P-type channels. However, we always recorded a residual Ca^{2+} influx

and Ach release. It is possible that yet another channel type, insensitive to ω-conotoxin and FTx and also involved in transmitter release, is present in *Aplysia* terminals. A high voltage-activated R-type Ca^{2+} channel insensitive to the classical blockers of L-, N-, and P-type channels was described recently (41). Alternatively, our experimental protocol for the application of ω-conotoxin and/or FTx may not allow optimal effects of the toxins.

Also, the fact that the conjoined use of ω-conotoxin and FTx did not completely block either the nifedipine-resistant Ca^{2+} current or Ach release may have been due to nonselective effects of these toxins. In a mammalian neuroblastoma–glioma cell line (42), ω-conotoxin can bind to various types of Ca^{2+} channels. This action may partially prevent the specific action of FTx on its target.

A preferential modulation of N-type channels with respect to L-type channels by various neuromodulators has been reported in frog sympathetic neurons (43), although these actions were not specifically linked to a modulation of transmitter release. In synapses between *Aplysia* sensory and motor neurons in culture (44), Ca^{2+} current through nifedipine-resistant channels appears to be the trigger of transmitter release and the target of neuromodulators. In our preparation, where both N- and P-type channels are involved in Ach release, only N-type channels are targeted by all presynaptic neuromodulators that we have identified, whereas P-type channels are insensitive to them.

It is generally accepted that Ca^{2+} channels triggering transmitter release are situated very close to the release sites in the terminal (45). In this respect, in our preparation, both N- and P-type channels would be localized at these strategic sites. Because P-type channels appear to be unrelated to modulation of transmitter release, they might support an influx of Ca^{2+} triggering a basal release of Ach whatever the physiological state of the synapse. Further experiments to localize the N- and P-type channels in the terminal will be of interest.

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