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Multiple Ca^{2+} channel types coexist to regulate synaptosomal neurotransmitter release

(ω-Aga-IVA/ω-conotoxin/glutamate/dopamine/synaptic transmission)

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ABSTRACT **The regulation of excitation–secretion coupling by Ca^{2+} channels is a fundamental property of the nerve terminal. Peptide toxins that block specific Ca^{2+} channel types have been used to identify which channels participate in neurotransmitter release. Subsecond measurements of [^{3}H]-glutamate and [^{3}H]dopamine release from rat striatal synaptosomes showed that P-type channels, which are sensitive to the *Agelenopsis aperta* venom peptide ω-Aga-IVA, trigger the release of both transmitters. Dopamine (but not glutamate) release was also controlled by N-type, ω-conotoxin-sensitive channels. With strong depolarizations, where neither toxin was very effective alone, a combination of ω-Aga-IVA and ω-conotoxin produced a synergistic inhibition of 60–80% of Ca^{2+}-dependent dopamine release. The results suggest that multiple Ca^{2+} channel types coexist to regulate neurosecretion under normal physiological conditions in the majority of nerve terminals. P- and N-type channels coexist in dopaminergic terminals, while P-type and a ω-conotoxin- and ω-Aga-IVA-resistant channel coexist in glutamatergic terminals. Such an arrangement could lend a high degree of flexibility in the regulation of transmitter release under diverse conditions of stimulation and modulation.**

Ca^{2+} channels in the nerve terminal regulate excitation–secretion coupling by controlling the entry of Ca^{2+} necessary for exocytosis (1). Multiple Ca^{2+} channel types in mammalian central neuron somata have been described (2–4), and several types can be defined based on their sensitivity to specific antagonists. One such antagonist, ω-conotoxin GVIA (ω-CgTx), was originally identified as an irreversible blocker of presynaptic release at the frog neuromuscular junction (5) and has been shown to specifically block N-type Ca^{2+} channels (6, 7). Subsequent work showed that neurotransmitter release from peripheral neurons (8, 9) and nerve terminal preparations (synaptosomes) from rat brain (10–14) was partially blocked by ω-CgTx but not by 1,4-dihydropyridine antagonists that are specific for L channels. These results led to the widely accepted notion that neurosecretion is regulated primarily if not exclusively by N channels (15). ω-CgTx can also block synaptic transmission in brain slice preparations (16–18), but the block is incomplete and in one case (18) was overcome by increased stimulus intensity. The partial block of neurotransmitter release as well as synaptic transmission suggested that ω-CgTx-resistant channels may mediate excitation–secretion coupling at central synapses in some cases.

More recently, ω-CgTx-resistant Ca^{2+} channels have been described in mammalian brain. One such channel, the P type, was first characterized in Purkinje neurons, where it is the predominant Ca^{2+} channel (19). P channels, which have subsequently been found in many other regions, are specifically blocked by ω-Aga-IVA, a peptide purified from the venom of *Agelenopsis aperta* (20). We demonstrated previously (21) that synaptosomal [^{3}H]glutamate release was resistant to ω-CgTx and partially but potently blocked by ω-Aga-IVA, providing evidence for a role for P channels at glutamatergic synapses. We now report that [^{3}H]dopamine release evoked by low levels of depolarization is partially blocked by ω-CgTx as well as by ω-Aga-IVA. However, under conditions of strong depolarization where neither toxin is very effective alone, a combination of nanomolar concentrations of both toxins is synergistic, producing substantial block of dopamine release. This observation suggests that in striatum, P-type and N-type Ca^{2+} channels coexist and regulate secretion from dopaminergic nerve terminals, while P-type and a toxin-resistant Ca^{2+} channel type coexist to regulate glutamate release. This arrangement has significant implications for the regulation of excitation–secretion coupling in mammalian central nerve terminals.

MATERIALS AND METHODS

Materials. Peptide toxins from *Agelenopsis aperta* were purified from crude venom (20, 22). Synthetic ω-Aga-IVA was obtained from Peptides International, and synthetic ω-CgTx was from Bachem. Stock solutions (10 μM) of the peptides were made in water and stored at −20°C. L-[G-^{3}H]glutamic acid and [7,8-^{3}H]dopamine were obtained from Amersham Corp. All other reagents were from Fluka or Sigma.

Neurosecretion Assay. Striatal tissue was dissected from rat brain (≈100 mg per brain), and the 10,000 × *g* pellet (P_2 fraction) was suspended in basal saline (145 mM NaCl/5 mM KCl/10 mM D-glucose/1.2 mM $MgCl_2$/0.1 mM EGTA·Tris/10 mM Hepes·Tris, pH 7.43) at a protein concentration of 1 mg/ml and kept on ice. The P_2 fraction was used instead of a more enriched synaptosomal fraction because of low yields from small amounts of tissues. In addition, control experiments indicated that glutamate release rates were indistinguishable when striatal P_2 or cortical synaptosomes were used.

A portion of the suspension was combined with a small volume of a concentrated toxin solution and incubated on ice for at least 30 min prior to release experiments. The terminals were metabolically labeled by combining 50 μl of the suspension with 5 μl of [^{3}H]glutamate (2 μCi/μl; 1 μCi = 37 kBq) or [^{3}H]dopamine (0.4 μCi/μl) and incubating the mixture at room temperature for 12 min. At this time, the suspension was diluted with buffer and applied to a filter sandwich to immobilize the synaptosomes. Because of the limited availability of the toxins, the synaptosomes were exposed to them only during the preincubation and uptake periods. We made the assumption that the toxin binding was essentially irreversible under these experimental conditions, where the time

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Abbreviation: ω-CgTx, ω-conotoxin GVIA.

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between toxin removal by filtration and stimulation of release was 20–30 sec. Recovery of Ca^{2+} currents in Purkinje neurons after removal of toxin has been shown to take many minutes (4). The release was measured on a 70-msec time scale by superfusion as described (21, 23). Results are expressed as the ratio of radioactivity in each fraction to the total radioactivity retained by the synaptosomes (% of total), plotted as the average of values from three to four experiments performed on fresh synaptosomal preparations. To minimize time-dependent changes in transmitter release rate, the order in which the individual conditions were tested was randomized from day to day. The standard deviation (error bars omitted for clarity) for each point was generally <10%, and did not exceed 20%.

RESULTS

The superfusion method used (23) measures synaptosomal transmitter release on a subsecond time scale, providing a rapid biochemical assay for presynaptic Ca^{2+} channel activity (Fig. 1). Depolarizing the synaptosomes with elevated K^+ concentration caused an increase in the rate of transmitter release that was strongly dependent on the presence of external Ca^{2+} (1.2 mM). The pharmacological properties of the voltage-activated Ca^{2+} channels coupled to transmitter release were examined by observing the effects of ω-CgTx and ω-Aga-IVA on both [^{3}H]dopamine and [^{3}H]glutamate release from striatal tissue, a region containing both dopaminergic and glutamatergic terminals (24). As reported previously (21), [^{3}H]glutamate release (Fig. 1 *C* and *D*) was

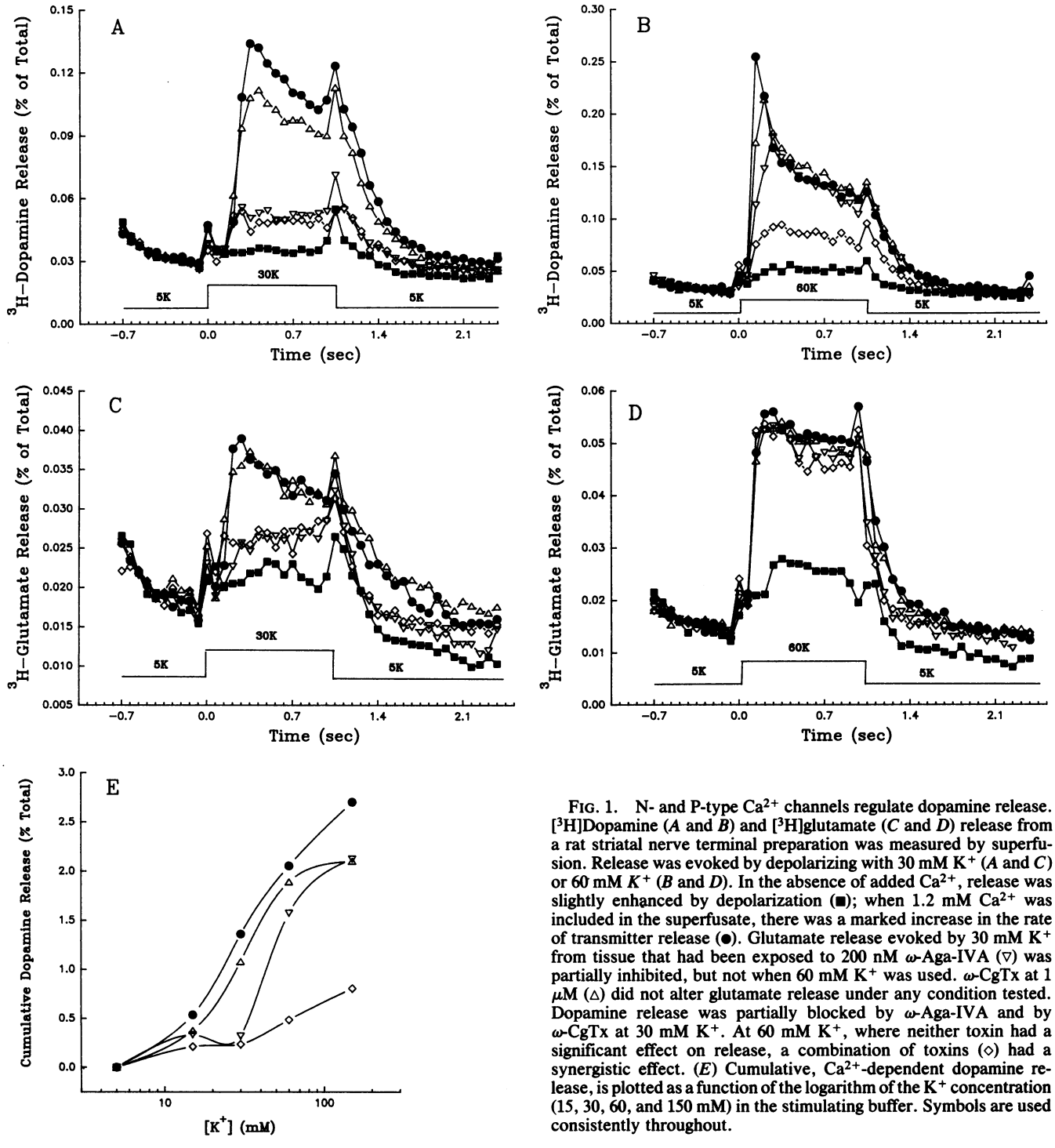

FIG. 1. N- and P-type Ca^{2+} channels regulate dopamine release. [^{3}H]Dopamine (*A* and *B*) and [^{3}H]glutamate (*C* and *D*) release from a rat striatal nerve terminal preparation was measured by superfusion. Release was evoked by depolarizing with 30 mM K^+ (*A* and *C*) or 60 mM K^+ (*B* and *D*). In the absence of added Ca^{2+}, release was slightly enhanced by depolarization (■); when 1.2 mM Ca^{2+} was included in the superfusate, there was a marked increase in the rate of transmitter release (●). Glutamate release evoked by 30 mM K^+ from tissue that had been exposed to 200 nM ω-Aga-IVA (▽) was partially inhibited, but not when 60 mM K^+ was used. ω-CgTx at 1 μM (△) did not alter glutamate release under any condition tested. Dopamine release was partially blocked by ω-Aga-IVA and by ω-CgTx at 30 mM K^+. At 60 mM K^+, where neither toxin had a significant effect on release, a combination of toxins (◇) had a synergistic effect. (*E*) Cumulative, Ca^{2+}-dependent dopamine release, is plotted as a function of the logarithm of the K^+ concentration (15, 30, 60, and 150 mM) in the stimulating buffer. Symbols are used consistently throughout.

partially blocked by nanomolar concentrations of ω-Aga-IVA, with the efficacy being greatest at low levels of depolarization; ω-Aga-IVA had very little effect on glutamate release evoked by extracellular KCl concentrations of 60 mM or more. [^{3}H]Dopamine release (Fig. 1 *A* and *B*) was blocked by ω-Aga-IVA in similar voltage-dependent fashion, although the fractional inhibition was greater than that for [^{3}H]glutamate release at all KCl concentrations. In addition, there was a small effect of ω-CgTx at all levels of depolarization, indicating that some portion of dopamine release was mediated by N channels, as well as by P channels.

The observation that ω-CgTx and ω-Aga-IVA partially block [^{3}H]dopamine release led us to test the additivity of the two toxins. At low levels of depolarization (15 and 30 mM K^+), their effects were slightly less than additive. However, at KCl concentrations of 60 mM or more, where either agent alone produced less than 25% block, the combination of the two toxins was synergistic, blocking 60–80% of Ca^{2+}-dependent dopamine release (Fig. 1 *B* and *E*). ω-CgTx had no effect on glutamate release from the same preparation, either alone or in combination with ω-Aga-IVA, at any KCl concentration tested (data not shown), as reported previously for cortical synaptosomes (21).

Although the combination of ω-CgTx and ω-Aga-IVA was able to block a majority of dopamine release, there remained a significant portion of release that was resistant to the toxins. To assess whether this component was mediated by L channels, we measured release after treatment with ω-CgTx, ω-Aga-IVA, and 100 nM ω-Aga-IIIA, an antagonist of N, P, and L channels (25–27). Adding ω-Aga-IIIA to the toxin combination had no additional effects on dopamine release (Fig. 2*A*), arguing that L channels were not involved. However, the component of release that was resistant to the peptide toxins was completely blocked by 200 μM Cd^{2+} (Fig. 2). The block of dopamine release by Cd^{2+} was potent (IC_{50} = 17 μM; Fig. 2*B Inset*) and suggested that the portion of dopamine release resistant to the peptide antagonists was mediated by a Ca^{2+} channel that has not been pharmacologically defined.

We examined the concentration–response relationship for each toxin by measuring release at a saturating concentration of the other toxin. Thus, we varied ω-CgTx between 0.1 and 1000 nM in the presence of 300 nM ω-Aga-IVA, and varied ω-Aga-IVA between 1 and 300 nM in the presence of 100 nM ω-CgTx (Fig. 3). Both toxins were quite potent, with IC_{50} values of 30 nM for ω-Aga-IVA and 1 nM for ω-CgTx. The potency of ω-CgTx is probably underestimated, since the concentration of ω-CgTx receptor under these conditions was estimated to be about 1 nM (28, 29) which approaches the IC_{50} value seen here. The high potency of the toxins supports the idea that they interact with specific, identified Ca^{2+} channel types.

The blockade of P channels by ω-Aga-IVA has been shown to be reversed by strong depolarizations (4). The release measurements were made within 30 sec after pretreatment of the terminals with ω-Aga-IVA, but not in its continuous presence, and we made the assumption that the toxin effects were long-lasting under these conditions. We were concerned that the apparent loss of efficacy of the toxin at ≥60 mM KCl was due to dissociation of the toxin from its receptor, due to the sustained depolarization produced by elevated K^+ concentration. To test this possibility, we delivered a 980-msec prepulse with 5 mM K^+ (control) or 60 mM K^+, in the absence of Ca^{2+}. We followed the prepulse 500 msec later with a 980-msec test pulse with 30 mM K^+, with or without Ca^{2+}. While the 60 mM K^+ prepulse produced a 20% decrease in dopamine release compared with control samples, the blockade of release from samples pretreated with ω-Aga-IVA was not altered by the prepulse (Fig. 4). This observation, taken together with the synergy of the two toxins, discounts the possibility that the loss of efficacy of ω-Aga-IVA at high K^+ concentrations is due to toxin dissociation from its receptor.

DISCUSSION

We have shown that release of dopamine and glutamate from striatal nerve terminals is partially sensitive to ω-Aga-IVA. Unlike glutamate release, dopamine release was also partially blocked by ω-CgTx. The efficacy of the toxins in blocking release of either transmitter was diminished when strong depolarizations were used to evoke release. However, under conditions of strong depolarization, a combination of the two toxins blocked dopamine release (but not glutamate release) in a synergistic manner. Strong depolarizations are likely to activate sufficient numbers of both P- and N-type Ca^{2+} channels so that Ca^{2+} entry through either type alone can sustain maximal dopamine release. When both channel types

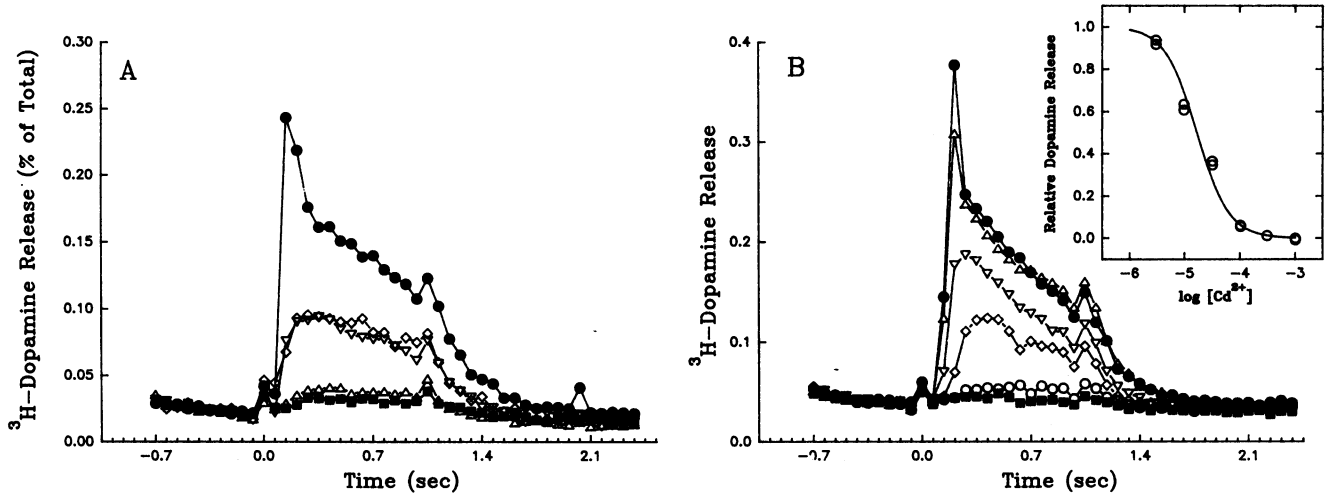

FIG. 2. Unblocked component of dopamine release is mediated by a resistant Ca^{2+} channel type. (*A*) Dopamine release was evoked by superfusion with 60 mM KCl with (●) or without (■) 1.2 mM Ca^{2+}. Release was measured after treatment with ω-CgTx plus ω-Aga-IVA (◇), with ω-CgTx, ω-Aga-IVA, and ω-Aga-IIIA (▽), or with 200 μM Cd^{2+} (△). Each peptide was applied at 100 nM. (*B*) Dopamine release was evoked by superfusion with 60 mM KCl with (●) or without (■) 1.2 mM Ca^{2+} and 3 μM (△), 10 μM (▽), 30 μM (◇), or 100 μM (○) Cd^{2+}. (*Inset*) Concentration–response relationship for Cd^{2+} block of cumulative Ca^{2+}-dependent dopamine release. Smooth curve is described by $y = \{1 + (K_i/[Cd^{2+}])^{n_H}\}^{-1}$, using values for K_i of 16.7 μM and n_H of 1.50 obtained by a Hill analysis of the data (n = 2).

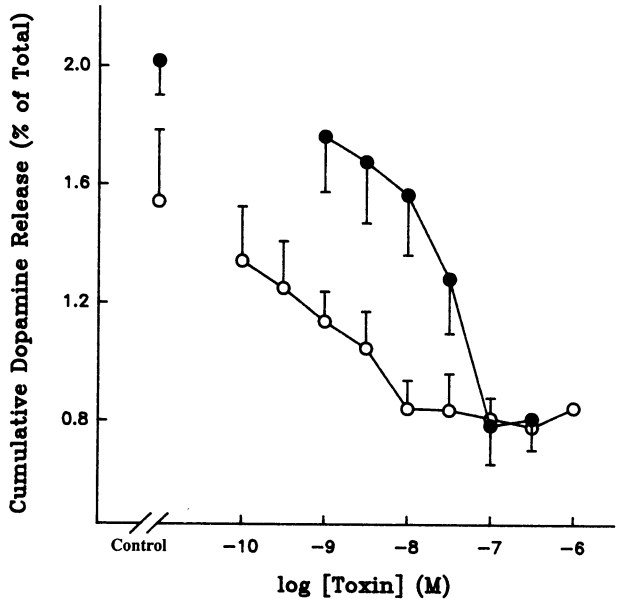

FIG. 3. Concentration–response relationship for block of dopamine release by ω-CgTx and ω-Aga-IVA. Tissue was incubated in a saturating concentration of either ω-CgTx (●, 100 nM) or ω-Aga-IVA (○, 300 nM), and defined concentrations of the other toxin ranging between 0.1 and 1000 nM. Release was evoked by stimulation for 1.05 sec with a buffer containing 60 mM K^+, 1.2 mM Ca^{2+}. Average values for cumulative, Ca^{2+}-dependent release from four experiments are plotted against the logarithm of the toxin concentration. Cumulative, Ca^{2+}-dependent dopamine release from untreated terminals was 1.94 ± 0.28% of total. Error bars denote the standard deviation.

are blocked, however, a profound inhibition of secretion results.

These observations lead us to the following conclusions: (*i*) dopamine release is regulated by both P- and N-type channels, (*ii*) these channels must coexist in the same population of nerve terminals in order to account for the observed synergy, and (*iii*) different transmitter types are regulated by different complements of Ca^{2+} channel, even within a given brain region. There appears to be a component of dopamine and glutamate release that is resistant to ω-CgTx, ω-Aga-IIIA, and ω-Aga-IVA, suggesting that at least some dopaminergic terminals contain a pharmacologically uncharacterized Ca^{2+} entry pathway. Low-voltage activated T channels are not likely candidates for this role, since they would probably be largely inactivated at resting potentials of about −60 mV (3), a typical average value for rat synaptosomes (30). The resistant Ca^{2+} entry pathway may correspond to the ω-CgTx- and ω-Aga-IVA-resistant Ca^{2+} current described in a number of central and peripheral neurons (4).

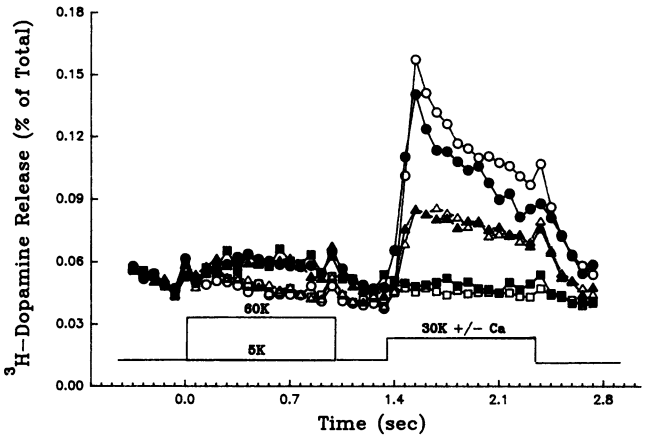

FIG. 4. Depolarization does not reverse ω-Aga-IVA block of dopamine release. A 980-msec prepulse with either basal buffer (5 mM K^+, open symbols) or a depolarizing buffer (60 mM K^+, filled symbols) was delivered 500 msec prior to a 980-msec test pulse with either 30 mM K^+ (□, ■), or 30 mM K^+ plus 1.2 mM Ca^{2+} with (△, ▲) or without (○, ●) treatment with 200 nM ω-Aga-IVA. Results are expressed as an average of three experiments for each condition.

These results are significant in that they suggest that multiple Ca^{2+} channel types can regulate neurosecretion under typical physiological conditions. Previous studies of neurosecretion have suggested that under some circumstances where L channel activity is enhanced by dihydropyridine agonists, both L- and N-type channels can participate in the release process (8, 12, 31). Release of various transmitters was enhanced as much as 50% by Bay K8644. However, dihydropyridine antagonists were only able to block the enhancement and had no effect on the remaining release even at high concentrations. These results were interpreted to suggest that L-type channels do not normally participate in the release process but can serve as "overflow" channels when activated by dihydropyridine agonists, as if they were not optimally oriented with the release apparatus (15). In instances where presynaptic Ca^{2+} currents can be measured (32–34), multiple types of Ca^{2+} current have been observed. In growth cones of sympathetic neurons (32), as well as in large synaptosomes from rat neurohypophysis (33, 34), both N and L channels were described. However, neurosecretion from the neurohypophysis was blocked by ω-CgTx but not by dihydropyridines (35). By contrast, we find that both N and P channels are capable of triggering dopamine release, especially when evoked with strong depolarizations where both channel types must be blocked in order to largely block release. The observation that the efficacy of these toxins is independent of the external Ca^{2+} concentration (data not shown) further suggests that the diffusional distance for Ca^{2+} is very small in these terminals. If Ca^{2+} diffusion in the cytoplasm were significant enough to allow overflow of Ca^{2+} into remote active zones, one would expect an inverse relationship between Ca^{2+} concentration and toxin efficacy. This provides additional evidence that the distinct channel types are intimately associated with the release apparatus at dopaminergic terminals.

We consistently observe that release rates remain elevated for several seconds after the end of the depolarizing pulse. This observation seems to be at odds with the concept of an intimate association between Ca^{2+} channels and the release apparatus, where relatively high Ca^{2+} concentrations are thought to be necessary to drive secretion (36, 37). Accordingly, one might predict that release rates should relax toward baseline on the same rapid time scale as closure of the Ca^{2+} channels, and that slow relaxation would implicate a long-lasting Ca^{2+} transient spread throughout the nerve terminal and not confined to the active zone. However, in our recent work using "synthetic action potentials", even brief (<50 msec) depolarizations that decay with a rate constant of at least 60 sec^{-1} evoke glutamate release that proceeds for several seconds (38). This result, in conjunction with the synergistic blockade of dopamine release by ω-CgTx and ω-Aga-IVA, leads us to conclude that elevation of Ca^{2+} in restricted regions is required to drive secretion and that Ca^{2+} acts as a trigger in a process that lasts several seconds.

Regulation of transmitter release at individual synapses by different Ca^{2+} channel types adds complexity to the ways in which synaptic transmission can be modulated. Subtle changes in synaptic strength could be achieved by preferential modulation of one type of exocytotic Ca^{2+} channel. Furthermore, in order to effectively block transmission at central synapses, a combination of Ca^{2+} channel blockers specific for a given transmitter type must be used. For

example, we predict that in striatum, a combination of ω-CgTx and ω-Aga-IVA would largely block dopaminergic transmission while preserving glutamatergic transmission, since glutamate release is insensitive to ω-CgTx and only partially blocked by ω-Aga-IVA. Multiple types of presynaptic Ca^{2+} channels involved in excitation–secretion coupling may in part account for the partial block of synaptic transmission at mammalian synapses by ω-CgTx (16–18). Discovery of toxins that block the "resistant" Ca^{2+} entry pathways will most likely yield an assortment of pharmacological tools to selectively target diverse transmitter systems on the basis of which Ca^{2+} channel types regulate release at a given class of synapse. Such a pharmacological strategy presents a means of targeting specific transmitter systems for therapeutic purposes and may furnish additional insight into the regulation of neurosecretion in the brain.

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