0026-895X/95/020348-06$03.00/0

MOLECULAR PHARMACOLOGY, 47:348-353, 1995.

Characterization of Presynaptic Calcium Channels with ω-Conotoxin MVIIC and ω-Grammotoxin SIA: Role for a Resistant Calcium Channel Type in Neurosecretion

TIMOTHY J. TURNER, RICHARD A. LAMPE, and KATHLEEN DUNLAP

Departments of Physiology and Neuroscience, Tufts University School of Medicine, Boston, Massachusetts 02111 (T.J.T., K.D.), and Department of CNS Pharmacology, Zeneca, Inc., Wilmington, Delaware 19897 (R.A.L.)

Received September 16, 1994; Accepted November 2, 1994

SUMMARY

The peptide Ca^{2+} channel antagonists ω-conotoxin (ω-CTX) MVIIC and ω-grammotoxin (ω-GTX) SIA were studied by measuring their effects on the release of [^{3}H]glutamate from rat brain synaptosomes. The pseudo-first-order association constant for ω-CTX MVIIC (1.1×10^4 M^{-1} sec^{-1}) was small, relative to that for ω-GTX SIA (3.6×10^5 M^{-1} sec^{-1}). Equilibrium experiments showed that ω-CTX MVIIC blocked ~70% of Ca^{2+}-dependent glutamate release evoked by 30 mM KCl (IC_{50} ~ 200 nM), whereas ω-GTX SIA virtually eliminated release, with lower potency (IC_{50} ~ 700 nM). At stronger depolarizations (60 mM KCl), neither toxin (at 1 μM) showed significant block of release, but when these or other Ca^{2+} channel antagonists (ω-CTX GVIA or ω-agatoxin IVA) were used in combination a substantial fraction of release was blocked. [^{3}H]Glutamate release that was resistant to ω-CTX MVIIC was characterized with respect to its sensitivity to block by ω-GTX SIA and the inorganic blocker Ni^{2+}. Both ω-GTX SIA and Ni^{2+} were relatively weak blockers of the resistant release. These results suggest that a previously uncharacterized Ca^{2+} channel exists in nerve terminals and can be distinguished on the basis of its resistance to ω-CTX MVIIC and its weak sensitivity to ω-GTX SIA and Ni^{2+}. Thus, at least three channel types (P, N, and a "resistant" type) contribute to excitation-secretion coupling in nerve terminals.

Interneuronal communication in the brain takes place largely as a result of synaptic transmission. A critical regulatory step in the process of synaptic transmission is the entry of Ca^{2+} into the presynaptic terminal, triggering a series of events that lead to exocytosis of neurotransmitters (1). A wealth of evidence suggests that Ca^{2+} channels in presynaptic terminals are located in the active zone and are closely associated with a complex of proteins found on both the synaptic vesicle and the plasma membrane. Entry of Ca^{2+} produces a local Ca^{2+} transient, and it has been suggested that one or more synaptic vesicle proteins act as low affinity Ca^{2+} receptors that can promote rapid exocytosis of transmitter (2, 3). Thus, voltage-gated Ca^{2+} channels in the presynaptic terminal play a pivotal role in regulating synaptic transmission.

Progress towards defining the role of Ca^{2+} channels in the neurosecretory process has been slowed by the lack of selective antagonists for the multiple types of Ca^{2+} channels found at nerve terminals. Peptide antagonists purified from the marine snail species *Conus* (4, 5) have been instrumental in the characterization of neuronal Ca^{2+} channels. The first of these peptides, ω-CTX GVIA, was shown to be a potent and selective blocker of neuronal Ca^{2+} channels, particularly at peripheral synapses (6, 7). After some confusion regarding its specificity, it was determined that ω-CTX GVIA is a specific blocker of N-type channels (8–10). Although ω-CTX GVIA decreased synaptic transmission at some central synapses, its effects were weak and variable (11, 12). Recently, another family of peptides from the funnel web spider *Agelenopsis aperta* has been identified (5, 13, 14); ω-Aga IVA has been shown to be a potent and selective blocker of P-type channels in cerebellar Purkinje neurons, without effects on either N-type or L-type channels. ω-Aga IVA-sensitive Ca^{2+} channels are involved in neurosecretion; ω-Aga IVA partially but potently blocks [^{3}H]glutamate (15), [^{3}H]norepinephrine (16), and [^{3}H]dopamine release (17) from rat brain synaptosomes or slices. Likewise, ω-Aga IVA strongly reduces synaptic transmission at a number of central synapses (18–22). We have shown that ω-CTX GVIA- and ω-Aga IVA-sensitive channels are co-localized within individual nerve terminals, by the observed synergy of ω-CTX GVIA and ω-Aga IVA in blocking striatal [^{3}H]dopamine release (17) and hippocampal [^{3}H]glutamate release (18).

Despite these recent advances, the pharmacological characterization of presynaptic Ca^{2+} channels remains incomplete.

This work was supported by Grant NS28815 from the United States Public Health Service to K.D.

ABBREVIATIONS: ω-CTX, ω-conotoxin; ω-GTX, ω-grammotoxin; ω-Aga, ω-agatoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Even when saturating concentrations of ω-CTX GVIA and ω-Aga IVA were applied, some toxin-resistant neurotransmitter release and synaptic transmission remained (18–22), indicating that one or more resistant Ca^{2+} channels can regulate neurosecretion. The aim of this study has been to characterize the resistant Ca^{2+} channels (as defined by resistance to ω-CTX GVIA and ω-Aga IVA) in presynaptic terminals, by measuring [^{3}H]glutamate release from synaptosomes with a superfusion device (23). We have used two peptide toxins, ω-CTX MVIIC (from *Conus magus*) (24) and ω-GTX SIA (from the spider *Grammastola spatulata*) (25), that are relatively nonselective blockers of high-voltage-activated Ca^{2+} channels in neurons, in combination with the relatively selective agents ω-CTX GVIA and ω-Aga IVA. Our results confirm the nonselective nature of ω-CTX MVIIC and ω-GTX SIA and show that glutamate release is blocked partially by ω-CTX MVIIC and completely by ω-GTX SIA.

Materials and Methods

Male Sprague-Dawley rats (50–150 g) were decapitated, and their brains were removed and bisected into hemispheres, which were placed in ice-cold 0.32 M sucrose. The thalamus, hippocampus, and striatum were removed and discarded, and the remaining cortical tissue (~0.5 g) was homogenized in 0.32 M sucrose, 1 mM EDTA, in a 2-ml Wheaton glass/Teflon homogenizer. Synaptosomes were prepared using discontinuous Percoll (Pharmacia) gradients (26). The final pellet (~5 mg of protein) was resuspended in 2.5 ml of basal buffer (145 mM NaCl, 2.7 mM KCl, 10 mM glucose, 10 mM HEPES-Tris, pH 7.4) that contained 1 mg/ml bovine serum albumin and 0.5 mM ascorbic acid. The synaptosomes were divided into a number of 25-μl portions, and each portion was combined with a small defined volume of concentrated toxin solution and incubated on ice for at least 30 min before the beginning of the release experiment, to allow for toxin binding for equilibrium experiments. To initiate the loading reaction, each synaptosomal suspension was combined with 5 μl of [^{3}H]glutamate (50 Ci/mmol, 2 μCi/μl; Amersham International) that had been prepared by evaporation of the aqueous stock solution under N_2 gas and resuspension in basal buffer. The final concentration of exogenous glutamate ranged between 1.8 and 2.8 μM. The loading proceeded for 12 min, at which time the reaction was stopped by addition of 820 μl of basal buffer and application of the suspension to a filtration sandwich composed of cellulose ester and glass fiber filters, as described previously (23). Release was measured using a superfusion device in conjunction with a fraction collector modified from a phonograph turntable (23, 27). The standard protocol was to depolarize cells by superfusion for 1.00 sec with a stimulus buffer containing an elevated concentration of KCl and to collect 70-msec fractions (16 rpm). Some experiments (Fig. 3; Table 1) were conducted by collecting 15-msec fractions (78 rpm). Radioactivity in each fraction and the amount remaining on the filter at the end of the experiment were determined by addition of 1.5 ml of liquid scintillation cocktail (BioSafe II; Research Products, Mt. Prospect, IL) and counting in a Beckman LS7000 scintillation counter. Data were analyzed using Lotus 1-2-3. Ca^{2+}-dependent release was calculated by measuring release in the presence of various Ca^{2+} concentrations (generally between 0.5 and 1.0 mM) and subtracting release evoked with a stimulus buffer that contained no added Ca^{2+} (free Ca^{2+} concentration, ~3 μM). Results were expressed as the ratio of counts in each fraction to the total radioactivity remaining on the filter (× 100%). Data are the average of at least three separate experiments performed on different days with freshly prepared synaptosomes. Standard deviations (error bars were omitted from kinetic plots for clarity) were generally <10% and never exceeded 20%. To account for possible time-dependent changes in release rates, the order of the experimental conditions was randomized for each experiment.

Experiments in which release was characterized with respect to Ni^{2+} sensitivity (Fig. 4) were performed by exposing the synaptosomes to a saturating concentration (3 μM) of ω-CTX MVIIC and subsequently superfusing the preparation with buffers containing the indicated concentrations of Ni^{2+}. The rate of glutamate release (in the absence of Ni^{2+}) was 31.8% of maximum, due to block produced by ω-CTX MVIIC. Release from ω-CTX MVIIC-treated synaptosomes was then normalized and plotted as a function of the Ni^{2+} concentration. The toxin-free release rate at 1 mM Ni^{2+} was 16.8% of control (no additions), whereas the release from ω-CTX MVIIC-treated samples was 20.5%.

The peptide toxins were prepared as 100 μM stock solutions in water, divided into 10-μl portions, and stored at −20°. Working stocks were prepared by diluting the concentrated solutions 10-fold with basal buffer that contained 1 mg/ml bovine serum albumin and 0.5 mM ascorbic acid. These working solutions could be subjected to several freeze-thaw cycles without apparent loss of activity. The peptides were obtained from the following sources: ω-Aga IVA, Peptides International (Frankfort, KY); ω-CTX GVIA, Bachem (Torrence, CA); ω-CTX MVIIC, Dr. J. Michael McIntosh (University of Utah) or Research Biochemicals (Natick, MA); and ω-GTX SIA, Dr. Richard Lampe (Zeneca Pharmaceuticals, Wilmington, DE). All other reagents were purchased from Fluka.

Results

ω-CTX MVIIC and ω-GTX SIA define a resistant component of transmitter release. The release of [^{3}H]glutamate from cortical synaptosomes has been shown to be partially sensitive to ω-Aga IVA and relatively insensitive to ω-CTX GVIA (15, 17, 18). We tested both ω-CTX MVIIC and ω-GTX SIA for effects on [^{3}H]glutamate release, to determine whether they might affect neurosecretion resistant to ω-Aga IVA and ω-CTX GVIA. Because the association rate for ω-CTX MVIIC has been reported to be slow (24, 28, 29), we performed kinetic experiments to estimate the minimum duration of incubation necessary for toxin binding to an approximately steady state level. Synaptosomes were incubated for intervals between 1 and 12 min with ω-CTX MVIIC (500 nM) and for intervals between 5 and 60 sec with ω-GTX SIA (200 nM). The binding of toxin was stopped by addition of a 30-fold excess of basal buffer, and glutamate release evoked by 30 mM KCl and 0.5 mM Ca^{2+} was measured by superfusion. Independent experiments showed that recovery of glutamate release from block by ω-Aga IVA was very slow, with <20% recovery being seen after 30 min (data not shown). Thus, the dissociation rate was assumed to be negligible, relative to the association rate, at these toxin concentrations. The association rate could be estimated by plotting the natural logarithm of the peak release rate versus toxin exposure time (Fig. 1). The values obtained from this analysis were 1.1×10^4 M^{-1} sec^{-1} for ω-CTX MVIIC and 3.5×10^5 M^{-1} sec^{-1} for ω-GTX SIA. Similar experiments (data not shown) were performed to obtain k_1 values for ω-Aga IVA (2.8×10^5 M^{-1} sec^{-1}). Because ω-CTX GVIA has relatively weak effects on cortical glutamate release, we estimated the k_1 (4.1×10^5 M^{-1} sec^{-1}) for this toxin by measuring the rate of block of [^{3}H]dopamine release from striatal synaptosomes (17). Assuming a linear relationship between association rate and toxin concentration, we calculated that the standard protocol would require a minimum of 40 min of incubation for the binding reaction to come within 95% of the equilibrium value at 100 nM ω-CTX MVIIC, whereas the same reaction would require about 2 min for the other three peptides.

The concentration-response relationship for ω-CTX MVIIC and ω-GTX SIA was determined over the range of 10 nM to 10 μM, using 30 mM KCl plus 1.2 mM Ca^{2+} to stimulate [^{3}H]-

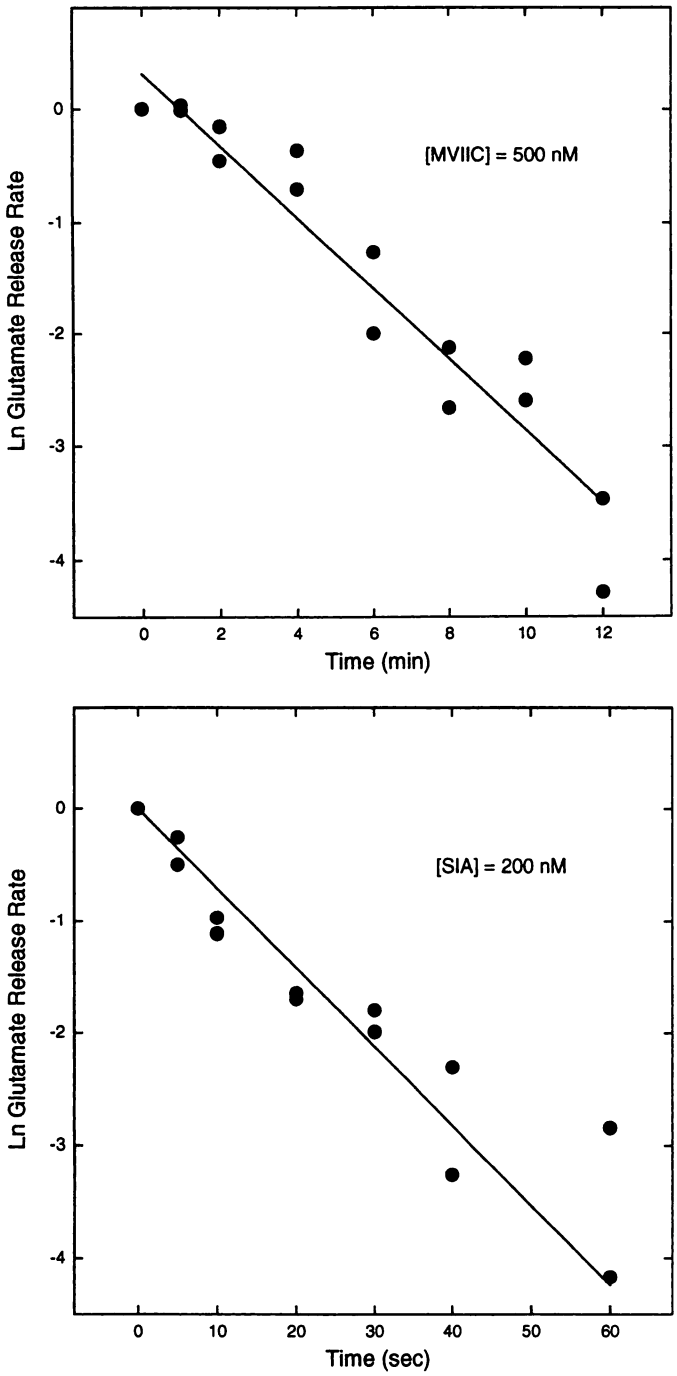

Fig. 1. Kinetics of ω-CTX MVIIC (A) and ω-GTX SIA (B) block of synaptosomal glutamate release. Cortical synaptosomes (50 μl) were incubated in the presence of 500 nM ω-CTX MVIIC or 200 nM ω-GTX SIA for the times indicated. The reaction was quenched with 800 μl of basal buffer, and rates of release (evoked by 30 mM KCl and 1.2 mM Ca^{2+}) were measured within 60 sec. Duplicate values averaged from two experiments are plotted, and the linear regression lines fitted to those data indicate pseudo-first-order rate constants for association of 1.1×10^4 M^{-1} sec^{-1} (ω-CTX MVIIC) and 3.5×10^5 M^{-1} sec^{-1} (ω-GTX SIA).

glutamate release (Fig. 2). ω-CTX MVIIC showed a concentration-dependent and saturable but partial block of release, with a maximal efficacy of ~70% at concentrations of ≥1 μM. In contrast, ω-GTX SIA blocked 93 ± 7.3% of the release at 10 μM. This is most clearly seen in the concentration-response analysis (Fig. 2C), where the effect of ω-CTX MVIIC was

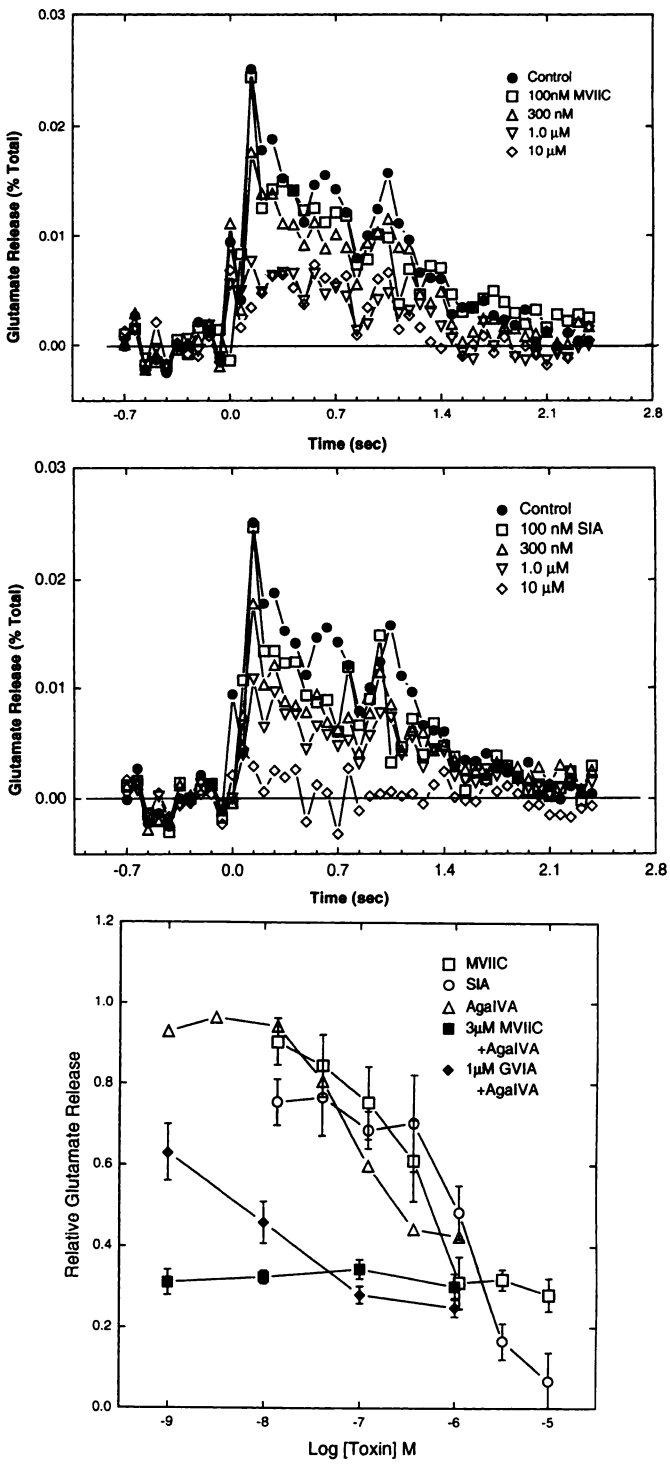

Fig. 2. Concentration-response relationship for ω-CTX MVIIC or ω-GTX SIA inhibition of synaptosomal glutamate release. Synaptosomes were preincubated on ice for at least 30 min in the presence of toxin and were then loaded with [^{3}H]glutamate at room temperature as usual. Release was evoked by 30 mM KCl and 1.2 mM Ca^{2+} in the presence of the indicated concentration of ω-CTX MVIIC (*MVIIC*) (A), ω-GTX SIA (*SIA*) (B), or ω-Aga IVA (C). Net cumulative glutamate release was normalized to control values and plotted versus the logarithm of the concentration of ω-CTX MVIIC, ω-GTX SIA, or ω-Aga IVA. Values represent the mean ± standard error of five experiments.

incomplete but saturating, whereas block by ω-GTX SIA was nearly complete. Based on previous reports (5, 24) that ω-CTX MVIIC blocks both P- and N-type channels, we measured the concentration-response relationship for ω-Aga IVA block of glutamate release in the presence of saturating concentrations of ω-CTX MVIIC (3 μM) or ω-CTX GVIA (1 μM). ω-CTX MVIIC occluded the effect of ω-Aga IVA (Fig. 2C), whereas ω-CTX GVIA plus ω-Aga IVA produced a saturable block of release that was indistinguishable from the level of block produced by ω-CTX MVIIC.

We showed previously that, when synaptosomes were depolarized with 60 mM KCl, neither ω-Aga IVA nor ω-CTX GVIA applied alone was able to alter glutamate release from hippocampal synaptosomes (18) but the two toxins combined were synergistic, blocking >70% of Ca^{2+}-dependent release. Our interpretation of this observation is that strong depolarizations produce more complete activation of Ca^{2+} channels, so that Ca^{2+} entry through a single channel type is sufficient to evoke maximal release. Therefore, we performed a series of experiments aimed at evaluating the effects of the toxins, both alone and in combination, on glutamate release measured during the first 150 msec of depolarization, under conditions designed to favor maximal channel activation (Fig. 3). Control experiments (data not shown) demonstrated that an increase in sampling rate (provided by an increase of the fraction collector speed to 78 rpm) improved our ability to measure toxin effects on the initial fast component of glutamate release. As observed previously, the individual toxins had relatively weak effects on glutamate release rates evoked by 60 mM KCl (Table 1). However, with recording at 78 rpm, the combination of any two toxins showed a significant degree of synergy. The experiments in which ω-CTX GVIA and ω-GTX SIA were combined consistently showed some degree of antagonism between these two peptides. The combination of all four peptides produced >95% block of release.

The effects of ω-CTX MVIIC and ω-GTX SIA were consistent with a nonselective blockade of calcium channels coupled to glutamate release, with considerable overlap between the two peptides but with ω-GTX SIA showing a broader spectrum. We wanted to examine the properties of the release that was

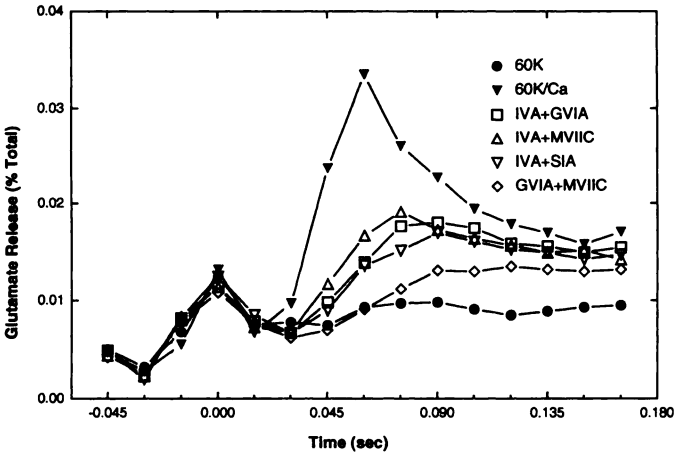

Fig. 3. Interactions of the peptide toxins. Synaptosomes were incubated on ice with 1 μM concentrations of each toxin (except ω-Aga IVA, which was used at 200 nM), and release was evoked by 60 mM KCl (*60K*) and 1.2 mM Ca^{2+}. Fractions were collected at 78 rpm. Data are averages of four experiments. *IVA*, ω-Aga IVA; *GVIA*, ω-CTX GVIA; *MVIIC*, ω-CTX MVIIC; *SIA*, ω-GTX SIA.

TABLE 1

Effects of toxin combinations

The concentrations of toxins used were as follows; ω-Aga IVA (A), 200 nM; ω-CTX GVIA (G), 1 μM; ω-CTX MVIIC (M), 1 μM; ω-GTX SIA (S), 1 μM. Synaptosomes were combined with the toxins for 12 min at room temperature during the loading reaction. Release was evoked with a 1-sec pulse with 60 mM KCl, with or without 1.0 mM Ca^{2+}. Cumulative Ca^{2+}-dependent glutamate release was determined over the first 150 msec and normalized to the control value (0.1106%) determined without added toxins. Results are the average ± standard error of four separate experiments conducted with fresh preparations each day.

Condition	Relative release
	%
Control	100.0 ± 1.5
A	94.4 ± 7.9
G	95.4 ± 16.6
M	91.5 ± 8.3
S	89.9 ± 16.1
A + G	54.2 ± 7.6
A + M	56.1 ± 6.5
A + S	46.2 ± 8.5
G + M	29.4 ± 16.5
G + S	69.9 ± 14.1
M + S	30.3 ± 16.4
A + G + M	23.4 ± 10.7
A + G + S	6.0 ± 6.2
A + M + S	0.0 ± 6.0
G + M + S	31.5 ± 6.2
A + G + M + S	3.0 ± 6.9

resistant to saturating concentrations of ω-Aga IVA, ω-CTX GVIA, and ω-CTX MVIIC, by measuring the concentration-response relationship for ω-GTX SIA block of glutamate release from synaptosomes exposed to a saturating concentration (3 μM) of ω-CTX MVIIC. ω-GTX SIA completely blocked the ω-CTX MVIIC-resistant release but did so at relatively high concentrations (IC_{50} ~ 3 μM) (data not shown). We then examined the concentration-response relationship for the divalent metals Cd^{2+} and Ni^{2+} (Fig. 4), which have been used previously to distinguish between high- and low-threshold calcium channel types (29–32). In the absence of peptide toxins, the concentration-response relationship for these metals showed that Cd^{2+} (EC_{50} = 4.3 μM) was significantly more potent than Ni^{2+} (IC_{50} = 113 μM), as reported previously (30). The release that was resistant to 3 μM ω-CTX MVIIC was less sensitive to Ni^{2+} than controls, with an IC_{50} value of ~1 mM.

Discussion

The discovery of peptides that selectively block neuronal calcium channels has been instrumental in the dissection of the role of pharmacologically diverse calcium channel types in mediating excitation-secretion coupling. The peptides characterized in this study, ω-CTX MVIIC and ω-GTX SIA, were shown to be relatively nonselective calcium channel blockers. We have used relatively specific Ca^{2+} channel blockers in combination with these two peptides to characterize presynaptic Ca^{2+} channels involved in excitation-secretion coupling. We observed the following: 1) ω-CTX MVIIC occluded a larger fraction of cortical glutamate release than did ω-Aga IVA (a relatively selective P channel blocker), 2) saturating concentrations of ω-CTX MVIIC inhibited the action of a saturating concentration of ω-Aga IVA, 3) the combined effect of saturating concentrations of ω-Aga IVA and ω-CTX GVIA (an N channel blocker) was equal to the effect of a saturating concen-

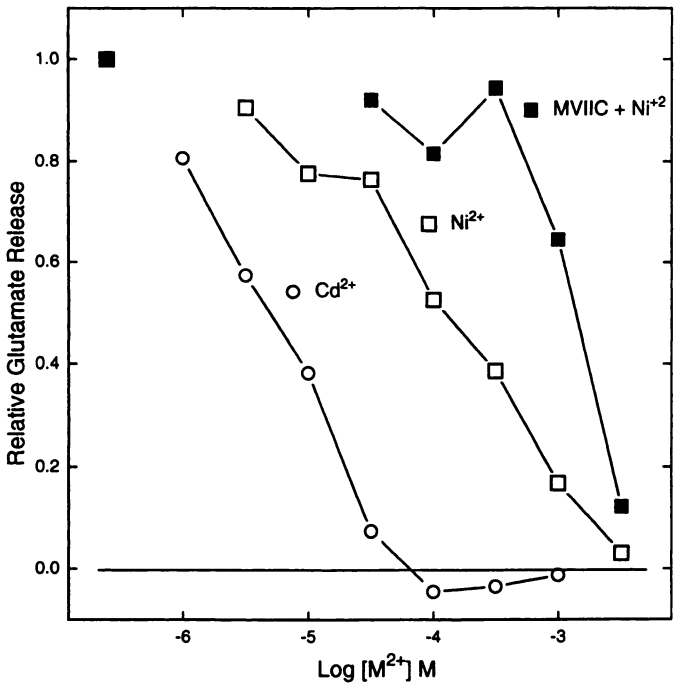

Fig. 4. Block of glutamate release by inorganic antagonists. Synaptosomes were preincubated with (*filled symbols*) or without (*open symbols*) 1 μM ω-CTX MVIIC (*MVIIC*) for at least 30 min. Release was evoked by 30 mM KCl and 1.2 mM Ca^{2+}, with the indicated concentrations of Cd^{2+} (*circles*) or Ni^{2+} (*squares*). Data are the average of four experiments for control and two experiments for ω-CTX MVIIC-treated samples.

tration of ω-CTX MVIIC, and 4) ω-GTX SIA blocked >95% of glutamate release. Taken together, these results suggest that three classes of Ca^{2+} channels participate in glutamate release, i.e., P-type, N-type, and a resistant type. ω-Aga IVA blocks the P-type channel, ω-CTX GVIA blocks the N-type channel, and ω-CTX MVIIC blocks both P- and N-type channels. Based on the observed block of >95% of glutamate release by ω-GTX SIA, it appears that this peptide is nonselective, blocking the P-type channel, the N-type channel, and the resistant channel. Despite its relatively low potency, ω-GTX SIA appears to be a useful reagent to characterize Ca^{2+} channels involved in neurosecretion, on the basis of its broad spectrum of action.

Glutamate release supported by Ca^{2+} entry though the ω-CTX MVIIC-resistant pathway was characterized based on sensitivity to the nonselective peptide ω-GTX SIA and the inorganic blockers Cd^{2+} and Ni^{2+}. This pathway was relatively insensitive to ω-GTX SIA and was blocked by this peptide in the micromolar range after pretreatment of synaptosomes with a saturating concentration of ω-CTX MVIIC. Release from synaptosomes that had been pretreated with 3 μM ω-CTX MVIIC was notably less sensitive to Ni^{2+} than was the control release. This observation suggests that this Ca^{2+} entry is not mediated by T-type channels, because T channels are relatively Ni^{2+} sensitive (30). By the same criterion, this channel does not resemble doe-1 (31) or class E $\alpha 1$ subunits, as expressed in oocytes (32). This pathway may represent a distinct channel type that is similar to those previously characterized (by virtue of sensitivity to nonselective blockers) but uniquely resistant to the peptide that blocks N- and P-type channels, i.e., ω-CTX MVIIC.

To ensure the accuracy of equilibrium measurements with the peptide antagonists, we first determined the time course for onset of blockade of glutamate release. As reported previously (24, 25, 28), the association rate for ω-CTX MVIIC (1.1×10^4 M^{-1} sec^{-1}) was slow, relative to that for the other toxins. Thus, it was important to determine association rates, so that equilibrium measurements were not compromised by insufficient incubation times. The association constants for the other three peptides used in this study were all very similar (approximately 3×10^5 M^{-1} sec^{-1}). The k_1 values for block of calcium currents by ω-CTX GVIA (in rat sympathetic neurons) (33) and by ω-Aga IVA (in rat Purkinje neurons) (34) have been reported to be 2×10^5 M^{-1} sec^{-1} and 2.4×10^5 M^{-1} sec^{-1}, respectively, in reasonable agreement with our measurements. However, because of the apparent competition between the peptide toxins and Ca^{2+} (or Ba^{2+}), the rate of block of glutamate release with 0.5 mM Ca^{2+} would presumably be less than with 2 mM Ba^{2+} in the stimulus buffer.

Previous results have shown that multiple channel types coexist in individual terminals (17–19). In the present study, we observed synergistic interactions between combinations of any two peptides, including combinations with the N-type channel blocker ω-CTX GVIA. We expect that, as more specific calcium channel antagonists are developed, the resistant pathways will be more precisely defined. The eventual goal will be to ascertain the molecular identity of the diverse neuronal calcium channel types that give rise to the rich pharmacology of neurosecretion.

References

1. Augustine, G. J., M. P. Charlton, and S. J. Smith. Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.* **10**:633–693 (1987).
2. Bennett, M. K., and R. H. Scheller. A molecular description of synaptic vesicle membrane trafficking. *Annu. Rev. Biochem.* **63**:63–100 (1994).
3. Jahn, R., and T. C. Sudhoff. Synaptic vesicles and exocytosis. *Annu. Rev. Neurosci.* **17**:219–246 (1994).
4. Olivera, B. M., J. Rivier, C. Clark, C. A. Ramilo, G. P. Corpuz, F. C. Abogadie, E. E. Mena, S. R. Woodward, D. R. Hillyard, and L. J. Cruz. Diversity of *Conus* neuropeptides. *Science (Washington D. C.)* **249**:257–263 (1990).
5. Olivera, B. M., G. Miljanich, J. Ramachandran, and M. E. Adams. Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. *Annu. Rev. Biochem.* **63**:823–867 (1994).
6. McClesky, E. W., A. P. Fox, D. H. Feldman, L. J. Cruz, B. M. Olivera, R. W. Tsein, and D. Yoshikami. ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. USA* **84**:4327–4331 (1987).
7. Hirning, L. D., A. P. Fox, E. W. McClesky, B. M. Olivera, S. A. Thayer, R. J. Miller, and R. W. Tsien. Dominant role of N-type calcium channels in evoked release of norepinephrine from sympathetic neurons. *Science (Washington D. C.)* **239**:57–61 (1988).
8. Plummer, M. R., D. E. Logothetis, and P. Hess. Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron* **2**:1453–1463 (1989).
9. Regan, L. J., D. W. Sah, and B. P. Bean. Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin. *Neuron* **6**:269–280 (1991).
10. Cox, D. H., and K. Dunlap. Pharmacological discrimination of N-type from L-type calcium current and its selective modulation by transmitters. *J. Neurosci.* **12**:906–914 (1992).
11. Kamiya, H., S. Sawada, and C. Yamamoto. Synthetic ω-conotoxin blocks synaptic transmission in the hippocampus *in vitro*. *Neurosci. Lett.* **91**:84–88 (1988).
12. Horne, A. L., and J. A. Kemp. The effect of ω-conotoxin GVIA on synaptic transmission within the nucleus accumbens and hippocampus of the rat *in vitro*. *Br. J. Pharmacol.* **103**:1733–1739 (1991).
13. Mintz, I. M., V. J. Venema, K. Swiderek, T. Lee, B. P. Bean, and M. E. Adams. P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature (Lond.)* **355**:827–829 (1992).
14. Mintz, I. M., M. E. Adams, and B. P. Bean. P-type calcium channels in rat central and peripheral neurons. *Neuron* **9**:85–95 (1992).
15. Turner, T. J., M. E. Adams, and K. Dunlap. Calcium channels coupled to glutamate release identified by ω-Aga-IVA. *Science (Washington D. C.)* **258**:310–313 (1992).
16. DeFeo, P. A., T. J. Mangano, M. E. Adams, and R. A. Keith. Inhibition of ω-conotoxin GVIA insensitive neurotransmitter release by ω-Aga-IVA. *Pharmacol. Commun.* **1**:273–278 (1992).
17. Turner, T. J., M. E. Adams, and K. Dunlap. Multiple Ca^{2+} channel types

coexist to regulate synaptosomal neurotransmitter release. *Proc. Natl. Acad. Sci. USA* **90**:9518–9522 (1993).
18. Luebke, J. I., K. Dunlap, and T. J. Turner. Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron* **11**:895–902 (1993).
19. Takahashi, T., and M. Momiyama. Different types of calcium channels mediate central synaptic transmission. *Nature (Lond.)* **366**:156–158 (1993).
20. Castillo, P. E., M. G. Weisskopf, and R. A. Nicoll. The role of Ca^{2+} channels in hippocampal mossy fiber synaptic transmission and long-term potentiation. *Neuron* **12**:261–269 (1994).
21. Regehr, W. G., and I. M. Mintz. Participation of multiple calcium channel types in transmission at single climbing fiber to Purkinje cell synapses. *Neuron* **12**:605–613 (1994).
22. Wheeler, D. B., A. Randall, and R. W. Tsien. Roles of N-type and Q-type Ca^{2+} channels in supporting synaptic transmission. *Science (Washington D. C.)* **264**:107–111 (1994).
23. Turner, T. J., L. B. Pearce, and S. M. Goldin. A superfusion system designed to measure release of radiolabeled neurotransmitters on a subsecond time scale. *Anal. Biochem.* **178**:8–16 (1989).
24. Hillyard, D. R., V. D. Monje, I. M. Mintz, B. P. Bean, L. Nadasdi, J. Ramachandran, G. Miljanich, A. Azimi-Zoonooz, J. M. McIntosh, L. J. Cruz, J. S. Imperial, and B. M. Olivera. A new *Conus* peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron* **9**:69–77 (1992).
25. Lampe, R. A., P. A. DeFeo, M. D. Davison, J. Young, J. L. Herman, R. C. Spreen, M. B. Horn, T. J. Mangano, and R. A. Keith. Isolation and pharmacological characterization of ω-grammotoxin SIA: a novel peptide inhibitor of neuronal voltage-sensitive calcium channel responses. *Mol. Pharmacol.* **44**:451–460 (1993).
26. Dunkley, P. R., P. E. Jarvie, J. W. Heath, G. J. Kidd, and J. A. P. Rostas. A rapid method for isolation of synaptosomes on Percoll gradients. *Brain Res.* **372**:115–129 (1986).
27. Forbush, B. An apparatus for rapid kinetic analysis of isotope efflux from membrane vesicles and of ligand dissociation from membarne proteins. *Anal. Biochem.* **140**:495–505 (1984).
28. Sather, W. A., T. Tanabe, Z.-F. Zhang, Y. Mori, M. E. Adams, and R. W. Tsien. Distinctive biophysical and pharmacological properties of class A (BI) calcium channel α_1 subunits. *Neuron* **11**:291–303 (1993).
29. Zhang, J.-F., A. D. Randall, P. T. Ellinor, W. A. Horne, W. A. Sather, T. Tanabe, T. L. Schwarz, and R. W. Tsien. Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology* **32**:1075–1088 (1993).
30. Fox, A. P., M. C. Nowycky, and R. W. Tsien. Kinetic and phamacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol. (Lond.)* **394**:149–172 (1987).
31. Ellinor, P. T., J.-F. Zhang, A. D. Randall, M. Zhou, T. L. Schwarz, R. W. Tsien, and W. A. Horne. Functional expression of a rapidly inactivating neuronal calcium channel. *Nature (Lond.)* **363**:455–458 (1993).
32. Soong, T. W., A. Stea, C. D. Hodson, S. J. Dubel, S. R. Vincent, and T. P. Snutch. Structure and functional expression of a member of the low voltage-activated calcium channel family. *Science (Washington D. C.)* **260**:1133–1136 (1993).
33. Boland, L. M., J. A. Morrill, and B. P. Bean. ω-Conotoxin block of N-type calcium channels in frog and rat symapthetic neurons. *J. Neurosci.* **14**:5011–5027 (1993).
34. Mintz, I. M., and B. P. Bean. Block of calcium channels in rat neurons by ω-Aga-IVA. *Neuropharmacology* **32**:1161–1169 (1993).

Send reprint requests to: Timothy J. Turner, Department of Physiology, Room M709, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.