British Journal of Pharmacology (1997) 120, 88–92 © 1997 Stockton Press All rights reserved 0007–1188/97 $12.00

Different effects of reducing agents on ω-conotoxin GVIA inhibition of [^{3}H]-acetylcholine release from rat cortical slices and guinea-pig myenteric plexus

T.A.A. Casali, *R.S. Gomez, **T. Moraes-Santos, M.A. Romano-Silva, M.A.M. Prado & [1]M.V. Gomez

Departamento de Farmacologia, ICB-UFMG; *Departamento de Cirurgia, Faculdade de Medicina da UFMG, and **Departamento de Alimentos, Faculdade Farmácia, UFMG, MG, Brasil

1 The effect of reducing reagents on ω-conotoxin GVIA (ω-CgTX) inhibition of the release of [^{3}H]-acetylcholine ([^{3}H]-ACh) induced by tityustoxin, K^+ 50 mM and electrical stimulation was investigated in rat brain cortical slices.

2 In cortical slices the inhibition of tityustoxin or electrically-stimulated [^{3}H]-ACh release by ω-CgTX was dramatically increased by reducing reagents ascorbate or β-mercaptoethanol. Dehydroascorbic acid did not substitute for ascorbate

3 Depolarization induced by K^+ 50 mM caused [^{3}H]-ACh release from cortical slices which was not inhibited by ω-CgTX, even in the presence of ascorbate.

4 In the guinea-pig myenteric plexus, ω-CgTX inhibition of the tityustoxin induced release of [^{3}H]-ACh was independent of ascorbate.

5 It is suggested that N-type-like calcium channels in guinea-pig myenteric plexus may have pharmacological/biochemical diversity from similar channels of rat cerebral cortex.

Keywords: Acetylcholine release; calcium channels; ω-conotoxin GVIA; reducing reagents; ascorbic acid

Introduction

Voltage-dependent Ca^{2+} channels (VDCC) are ubiquitous components of excitable tissues from virtually all types of animals. Electrophysiological studies have revealed several classes of VDCC in neurones. These channels are products of different genes and are classified mainly by their characteristic currents, potentials for activation or inactivation, their α_1 subunit and their sensitivity to ω-toxins (Zhang *et al*., 1993; Olivera *et al*., 1994). Calcium channels have been classified as of the L, N, T, P or Q-type. The first known neuronal-type calcium channel, the N-type, has been implicated in controlling transmitter release in some species (Hirning *et al*., 1988). ω-Conotoxin GVIA (ω-CgTX), a peptide isolated from *Conus geografus* blocks N-type calcium channels selectively and reversibly (Fox *et al*., 1987; Tsien *et al*., 1988). However, ω-CgTX only partially blocks transmitter release from mammalian central nervous system (Reynolds *et al*., 1986) and in some studies has been found to have no effect on evoked neurotransmitter release (Mangano *et al*., 1991; Casali *et al*., 1995).

The ω-CgTX binds irreversibly and with high specificity to VDCC in lower vertebrates (Rivier *et al*., 1987) and in mammals (Feigenbaum *et al*., 1988; Wagner *et al*., 1988), but it inhibits only slightly the depolarization-induced Ca^{2+} influx in rat brain synaptosomes (Lundy *et al*., 1991). K^+ induced Ca^{2+} uptake by chicken synaptosomes was blocked by ω-CgTX but this toxin had no effect on the depolarization-induced Ca^{2+} entry into rat synaptosomes from frontal lobe (Maubecin *et al*., 1995). A low sensitivity to ω-CgTX of calcium channels coupled to transmitter release was observed in rat cortical slices (Lundy *et al*., 1991; Perrier *et al*., 1992) and synaptosomes (Suszkiw *et al*., 1986). These data argue against a major contribution of ω-CgTX-sensitive, N-type calcium channels to the induced Ca^{2+} entry and release of neurotransmitter in mammalian brain cortical slices.

In the course of an investigation of the release of acetylcholine ([^{3}H]-ACh) from brain cortical slices, we observed that the inhibitory effect of ω-CgTX was enhanced by ascorbate. The aim of this work was to explore this point further by studying the effect of the reducing reagents ascorbate and β-mercaptoethanol on the effects of ω-CgTX. Since variations in stimulus conditions may alter the effectiveness of ω-CgTX in inhibiting neurotransmitter release (Wessler *et al*., 1990), we experimented with different depolarizing conditions, such as electrical field stimulation, high K^+, or tityustoxin, a scorpion toxin that increases Na^+ entry through tetrodotoxin-sensitive Na^+ channels (Gomez *et al*., 1973).

Methods

Materials were as follows: ACh chloride, ATP, choline chloride, dithiothreitol (DTT), 3-heptanone, diethyl-*p*-nitrophenyl phosphate (paraoxon), physostigmime free base, 1-4-bis [5-phenyl 2 oxazolyl] benzene-2, 2′-*p*-phenylene bis [5-phenyl oxazole] (POPOP), 2,5 diphenyloxazol (PPO), sodium tetraphenylborate (TPB), ascorbic acid, β-mercaptoethanol and ω-conotoxin GVIA were from Sigma Chemical Co. (St. Louis, Missouri). Tityustoxin was purified as previously described (Gomez & Diniz, 1966). All the other chemicals were analytical grade. Holtzman rats (200–250 g) were killed by decapitation. The cerebral cortex was removed and sliced in a McIlwain Tissue Slicer (Brinkman Instruments Inc., UK). Pieces of guinea-pig myenteric plexus with its accompanying plexus were obtained from guinea-pigs of either sex, as described by Rang (1964). The release of [^{3}H]-ACh into the incubating medium was studied after labelling tissue ACh with [methyl-^{3}H]-choline (78 Ci mM^{-1}; Amershan Searle) as previously described by Casali *et al*. (1995) and Gomez *et al*. (1995). Briefly tissue ACh stores were first depleted by incubation in Krebs/Trizma medium for 15 min in the presence of 50 mM K^+. To label the endogenous pools of ACh, the slices or the strips of myenteric plexus were incubated in Krebs-Trizma medium 30 min with 0.12 μCi ml^{-1} of methyl[^{3}H]-choline.

[1]Author for correspondence at: Departamento de Farmacologia-ICB-UFMG, Caixa Postal 2486, 31160-970-Belo Horizonte-MG-Brasil.

Then the brain cortical slices or the strips of myenteric plexus were separated from the incubating fluid by centrifugation followed by three washes with 1.0 μM cold choline. Subsequently, the slices or the strips were preincubated for 15 min in the presence or absence of ω-CgTX 0.1 μM, ascorbate 0.57 mM, dehydroascorbic 0.57 mM or β-mercaptoethanol 0.1 mM followed by 30 min stimulation with tityustoxin 2.5 μM, K^+ 50 mM or electrical field stimulation at 10 Hz (10 V, 2 ms for 3 min). The Krebs-Trizma medium contained (in mM): NaCl 136, KCl 2.7, $CaCl_2$, 1.8, Trizma base 10, glucose 5.5 and diethyl *p*-nitrophenylphosphate (Paraoxon, Sigma Chemical Co.) 20 μM was added to prevent hydrolysis of ACh. The final pH was adjusted to 7.4. In order to characterize the radioactivity released, [^{3}H]-choline and [^{3}H]-acetylcholine were separated from the supernatants by the choline kinase method (Goldberg & McCamman, 1973) or by high voltage electrophoresis. [^{3}H]-acetylcholine represented 60–72% of the total radioactivity. Statisticals analyses were performed by analysis of variance (ANOVA).

Results

Reducing reagents allow ω-CgTX to inhibit the evoked release of [^{3}H]-ACh in rat cortical slices

In agreement with the consensus that ω-CgTX has little or no effect on transmitter release in the mammalian central nervous system, it failed to inhibit tityustoxin-evoked release of [^{3}H]-ACh from rat cortical slices in the absence of ascorbate (Figure 1a), confirming our previous observations (Casali *et al*., 1995). However, in the presence of ascorbate 0.57 mM, ω-CgTX 0.1 μM inhibited by 90% the release of [^{3}H]-ACh induced by tityustoxin 2.5 μM (Figure 1a). Ascorbate alone or in the presence of tityustoxin did not interfere with the release of [^{3}H]-ACh. To determine whether the ascorbate acts as a reducing agent, we tested the oxidized form of ascorbic acid, dehydroascorbic. Figure 1b shows that in rat brain cortical slices the presence of dehydroascorbate, ω-CgTX has no inhibitory effect on the release of [^{3}H]-ACh evoked by tityustoxin. Thus, the ascorbate enhancement of the inhibition by ω-CgTX on the evoked release [^{3}H]-ACh appears to be related to its reducing properties. Therefore we tested another reducing reagent, β-mercaptoethanol. Figure 1c shows that in the presence of β-mercaptoethanol 0.1 mM, ω-CgTX inhibited by 70% the release of [^{3}H]-ACh induced by tityustoxin from rat brain cortical slices. In the absence of β-mercaptoethanol, ω-CgTX had no effect on the evoked release of [^{3}H]-ACh by tityustoxin from rat brain cortical slices. Like ascorbate, β-mercaptoethanol alone did not interfere with the spontaneous release of [^{3}H]-ACh or the release evoked by tityustoxin depolarization of rat brain cortical slices.

Sensitivity of rat cortical slices to ω-CgTX in the presence of ascorbate and under different conditions of stimulation

The effectiveness of ω-CgTX as an inhibitor of neurotransmitter release depends on the stimulus (Wessler *et al*., 1990; Keith *et al*., 1993; Turner & Dunlap, 1995). To study further the effect of ω-CgTX and ascorbate on the release of [^{3}H]-ACh from rat brain cortical slices we used two other stimuli: electrical field stimulation (Figure 2a) or K^+ depolarization (Figure 2b). In the presence of ascor-

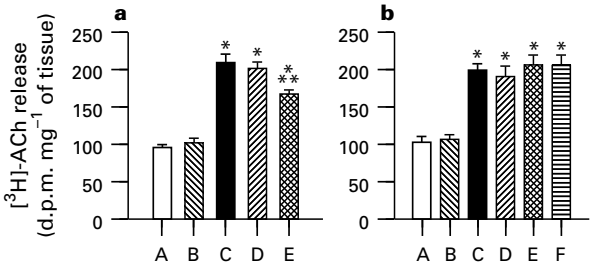

Figure 2 The effect of ascorbate on the ω-CgTX inhibition of [^{3}H]-ACh release induced by electrical and KCl stimulation of rat brain cortical slices. Slices (±40 mg) were pre-incubated in a Krebs-Trizma medium in the presence or absence of ascorbate 0.57 mM and ω-CgTX 0.1 μM for 15 min. They were then stimulated with electrical pulses of 10 Hz (a) or K^+ 50 mM for 30 min (b). (a) Column A, control; B, ascorbate 0.57 mM; C, electrical stimulation 10 Hz; D, electrical stimulation 10 Hz plus ω-CgTX 0.1 μM; E, electrical stimulation 10 Hz ascorbate 0.57 mM plus ω-CgTX 0.1 μM. (b) Column A, control; B, ascorbate; C, KCl 50 M; D, KCl 50 mM plus ω-CgTX 0.1 μM; E, KCl 50 mM plus ascorbate 0.57 mM; F, KCl 50 mM, ascorbate 0.57 mM plus ω-CgTX 0.1 μM. The values represent the means±s.e.mean for duplicates of 3 experiments. For other details see Methods. *Statistically different from the control value, $P<0.01$. **Statistically different from the tityustoxin value, $P<0.02$.

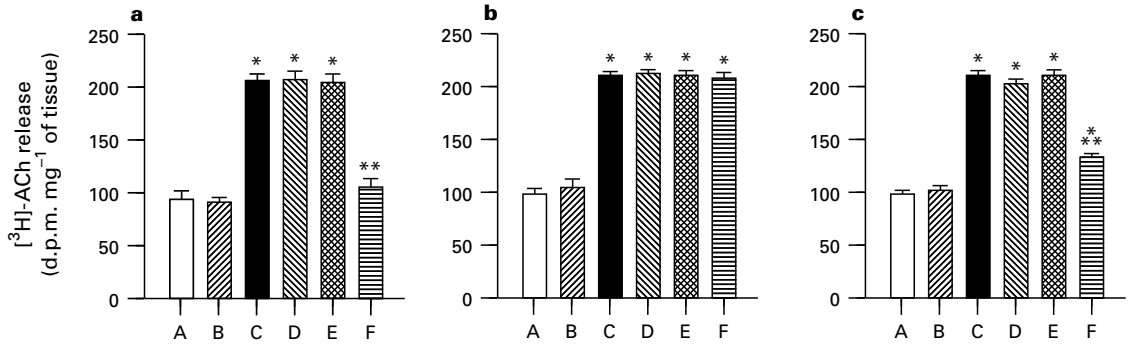

Figure 1 The effect of ascorbate, dehydroascorbic or β-mercaptoethanol on the ω-CgTX inhibition of [^{3}H]-ACh release induced by tityustoxin in rat brain cortical slices. Slices (±40 mg) were pre-incubated in a Krebs-Trizma medium in the presence or absence of ascorbate 0.57 mM (a), dehydroascorbic acid 0.57 mM (b), β-mercaptoethanol, 0.1 μM (c) and ω-CgTX 0.1 μM for 15 min. They were then stimulated for 30 min with tityustoxin 2.5 μM. (a) Column A, control; B, ascorbate 0.57 mM; C, tityustoxin 2.5 μM; D, tityustoxin 2.5 μM plus ω-CgTX 0.1 μM; E, tityustoxin 2.5 μM plus ascorbate 0.57 mM; F, tityustoxin 2.5 μM; ascorbate 0.57 mM plus ω-CgTX 0.1 μM. (b) Column A, control; B, dehydroascorbic acid 0.57 mM C, tityustoxin 2.5 μM; D, tityustoxin 2.5 μM plus ω-CgTX 0.1 μM; E, tityustoxin 2.5 μM plus dehydroascorbic acid 0.57 mM; F, tityustoxin 2.5 μM, dehydroascorbic acid 0.57 mM plus ω-CgTX 0.1 μM. (c) Column A, control; B, β-mercaptoethanol 0.1 μM; C, tityustoxin 2.5 μM; D, tityustoxin plus ω-CgTX, 0.1 μM, E, tityustoxin 2.5 μM plus β-mercaptoethanol 0.1 μM; F, tityustoxin, 2.5μM, β-mercaptoethanol 0.1 μM plus ω-CgTX 0.1 μM. The values represent the means±s.e.mean for duplicates of 3 experiments. For other details see Methods. *Statistically different from the control value, $P<0.01$. **Statistically different from the tityustoxin value, $P<0.01$.

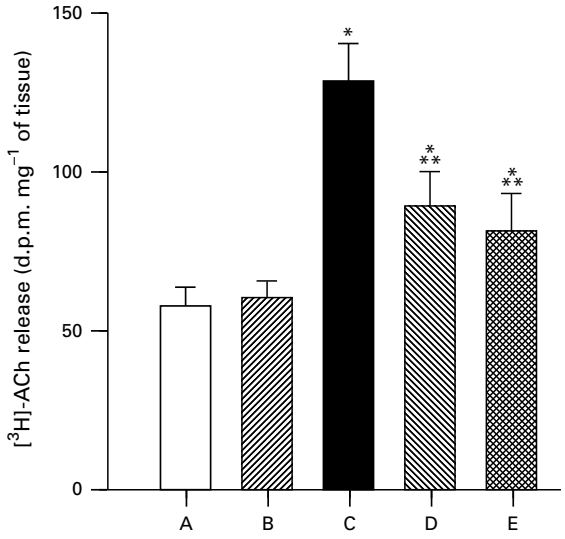

Figure 3 The effect of ascorbate on the ω-CgTX inhibition of [^{3}H]-ACh release induced by tityustoxin in guinea-pig myenteric plexus. Pieces of longitudinal muscles with its accompanying plexus (±40 mg) were pre-incubated for 15 min in Krebs-Trizma medium in the presence or absence of ascorbate 0.57 mM or ω-CgTX 0.1 μM for 15 min. They were then stimulated with tityustoxin 2.5 μM for 30 min. Column A, control; B, ascorbate 0.57 mM; D, tityustoxin 2.5 μM; E, tityustoxin 2.5 μM plus ω-CgTX 0.1 μM; F, tityustoxin 2.5 μM, ascorbate 0.57 mM plus ω-CgTX 0.1 μM. The values represent the means±s.e.mean for duplicates of 3 experiments. For other details see Methods. *Statistically different from the control value, $P<0.01$. **Statistically different from the tityustoxin value, $P<0.02$.

bate, ω-CgTX inhibited by 36% the release of [^{3}H]-ACh induced by electrical stimulation with pulses of 10 Hz for 3 min. Without ascorbate, ω-CgTX had no effect on the release of neurotransmitter induced by electrical stimulation. The effect of ascorbate and ω-CgTX on the evoked release of [^{3}H]-ACh by 50 mM K^+ depolarization is shown in Figure 2b. K^+ 50 mM increased the release of neurotransmitter to levels comparable to those produced by tityustoxin or electrical stimulation of rat brain cortical slices. This increased release of [^{3}H]-ACh was not inhibited by ω-CgTX, even in the presence of ascorbate.

Sensitivity of guinea-pig myenteric plexus to ω-CgTX in the presence of ascorbate

Figure 3 shows the effect of ascorbate and ω-CgTX on the evoked release of [^{3}H]-ACh by tityustoxin in guinea-pig myenteric plexus. Tityustoxin 2.5 μM doubled the release of neurotransmitter and ω-CgTX inhibited this release by 79% with ascorbate and 69% without ascorbate. These conditions were not statistically significant from each step ($P>0.05$). Thus, the inhibition of tityustoxin-induced release of [^{3}H]-ACh by ω-CgTX was independent of the presence of ascorbate.

Discussion

It has been shown in rat brain slices (Lundy *et al*., 1991; Perrier *et al*., 1992), synaptosomes (Suszkiw *et al*., 1989) and neuromuscular junctions (Uchitel *et al*., 1992) that the calcium channels are insensitive to ω-CgTX. However, ω-CgTX abolishes neurotransmitter release in the chicken or frog (Olivera *et al*., 1994). We have also found that ω-CgTX has no effect on the release of [^{3}H]-ACh induced by tityustoxin in incubated brain cortical slices of the rat (Casali *et al*., 1995). However, the present results show that ω-CgTX inhibits the release of [^{3}H]-ACh induced by tityustoxin when reducing reagents, ascorbate (Figure 1a) or β-mercaptoethanol (Figure 1c) are present. In the absence of paraoxon, an inhibition of 70% by ω-CgTx of the evoked release of [^{3}H-]ACh was also dependent of ascorbate (data not shown). Thus the result is not a consequence of the conditions used to recover [^{3}H]-ACh from the medium (inhibition of cholinesterase by paraoxon), because the same observation was made when measuring ^{3}H efflux in hemicholinium-3 treated slices.

In rat brain cortical slices, ω-CgTX did not inhibit [^{3}H]-ACh release when ascorbate was replaced by its oxidized form, dehydroascorbic acid (Figure 1b). Ascorbate also allowed ω-CgTX to inhibit the release of [^{3}H]-ACh from rat cortical slices stimulated by electrical field stimulation (Figure 2a). The weak inhibitory effect may be related to the high frequency stimulation used in our experiments. However, ascorbate had no effect on the blocking action of ω-CgTX on the release of [^{3}H]-ACh induced by K^+ depolarization (Figure 2b). This apparent discrepancy is explained by known differences in the effects of the different stimuli used. For example, ω-CgTX, in the presence of ascorbate and EDTA, is more effective in blocking release of ACh in response to electrical field stimulation than K^+ stimulation in the myenteric plexus indicating different types of N-type calcium channels in these tissues (Wessler *et al*., 1990). Data from the literature show the similarity between the effects of electrical stimulation and tityustoxin (Gomez *et al*., 1973; Warnick *et al*., 1976; Prado *et al*., 1992) as well as the different effects of electrical stimulation and K^+ depolarization on the release of neurotransmitters (Momyama & Takahashi, 1994).

Thus, mammalian myenteric plexus may have a Ca^{2+} channel subtype with a mixed pharmacology. The ascorbate-independence of the ω-CgTX inhibition of the evoked [^{3}H]-ACh release in guinea-pig myenteric plexus suggests a pharmacological difference between the calcium channels in this tissue and those in rat brain cortex.

At present, we do not know how reducing agents enhance ω-CgTX blockade in the central but not in the peripheral nervous systems of these two species studied. The site of action of the reducing reagents that induce ω-CgTX inhibition of the release of [^{3}H]-ACh from rat brain cortical slices may not be on the functional active -S-S groups of ω-CgTX. This hypothesis is supported by the finding that ω-CgTX inhibition of the evoked release of [^{3}H]-ACh from guinea-pig myenteric plexus did not require ascorbate (Figure 3).

Molecular biological investigations have demonstrated that the main determinant of Ca^{2+} channel phenotype is the α1 subunit, of which 5 classes have been cloned from vertebrates (Zhang *et al*., 1993; Dunlap *et al*., 1995). In addition to the α1 subunit that is the pore for calcium, a number of clones have been isolated for accessory subunits (e.g. β, α2δ) that modify Ca^{2+} channel properties. The α2δ subunits are usually considered to be a single molecular entity, since they are encoded by a single gene that generates two invariably disulphide linked protein moieties (De Jongh *et al*., 1990; Jay *et al*., 1991; Witcher *et al*., 1995). The disulphide-linked tertiary structure is necessary for a proper α2δ transmembrane interaction with the α1 subunit (Gurnett *et al*., 1996). Ascorbate or β-mercaptoethanol may act on S-S bridge(s) of α2δ-subunits allowing, in rat brain cortical slices, inhibition of the release of [^{3}H]-ACh by ω-CgTX-GVIA. Under reducing conditions, in the presence of β-mercaptoethanol, the complex α2δ is separated into two components, the 143,000 Da form of the α2-subunit and the smaller, 27,000 Da for the δ-subunit (De Jongh *et al*., 1990; Jay *et al*., 1991).

Ascorbic acid inhibits Na^+ dependent calcium uptake in rat cultured astrocytes (Takuma *et al*., 1995). The release of ACh stimulated by tityustoxin is Na^+ and Ca^{2+}- dependent (Gomez *et al*., 1973; 1975) and ascorbate had no effect on the tityustoxin induced release of [^{3}H]-ACh (Figure 1a). Therefore, any ascorbate involvement with the Na^+-dependent Ca^{2+} uptake does not explain the ω-CgTX induced inhibition of the release of [^{3}H]-ACh.

Finally the data suggest a pharmacological/biochemical diversity among N-like calcium channels of guinea-pig myenteric plexus and rat brain cortical slices. However, differences between rat brain and guinea-pig myenteric plexus could reflect species differences in the cellular environment, VDCC regulation or VDCC composition/structure. Further experiments are necessary to clarify these differences.

We thank Adriane A. Pereira, Andreia G. Alves and Antonio C.S Gomes for technical assistance and Dr Willian Van der Kloot for reading and suggestions in this manuscript. This work was supported by grants of Fapemig, CNPq, PADCT and Finep.

References

CASALI, T.A.A., GOMEZ, R.S., MORAES-SANTOS, T. & GOMEZ, M.V. (1995). Differential effects of calcium channels antagonists on tityustoxin and ouabain-induced release of [^{3}H]acetylcholine from brain cortical slices. *Neuropharmacology*, **34,** 599 – 603.

DE JONGH, K.S., WARNER, C. & CATTERALL, W.A. (1990). Subunits of purified calcium channels: $\alpha 2\delta$ are encoded by the same gene *J. Biol. Chem.*, **265,** 14738 – 14741.

DUNLAP, K., LUEBKE, J.I. & TURNER, T.J. (1995). Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci.*, **18,** 89 – 98.

FEIGENBAUM, P., GARCIA, M. & KACZOROWSKI, G.J. (1988). Evidence for distinct site receptor in rat brain synaptic plasma membrane vesicles. *Biochem. Biophys. Res. Commun.*, **154,** 299 – 305.

FOX, A.P., NOWYCKY, M.C. & TSIEN, R.W. (1987). Kinetics and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. *J. Physiol.*, **394**, 149 – 172.

GOLDBERG, A.M. & McCAMAN, R.E. (1973). The determination of picomoles amounts of acetylcholine in mammalian brain. *J. Neurochem.*, **20,** 1 – 8.

GOMEZ, M.V., DAI, M.E.M. & DINIZ, C.R. (1973). Effects of scorpion venom tityustoxin on the release of acetylcholine from incubated slices of rat brain. *J. Neurochem.*, **20,** 1054 – 1061.

GOMEZ, M.V. & DINIZ, C.R. (1966). Separation of toxic components from the Brazilian scorpion Tityus serrulatus venom. *Mem. Inst. Butantan. Symp. Int.*, **33,** 899 – 902.

GOMEZ, M.V., DINIZ, C.R. & BARBOSA, T.S. (1975). A comparison of the effect of scorpion venom tityustoxin and ouabain on the release of acetylcholine from incubated slices of rat brain slices of rat brain. *J. Neurochem.*, **24,** 331 – 336.

GOMEZ, R.S., CASALI, T.A.A., ROMANO-SILVA, M.A., CORDEIRO, M.N., DINIZ, C.R., MORAES-SANTOS, T., PRADO, M.A.M. & GOMEZ, M.V. (1995). The release of ^{3}H-acetylcholine induced by tityustoxin and potassium in brain cortical slices and myenteric plexus. *Neurosci. Lett.*, **196,** 131 – 133.

GURNETT, C.A., DE WAARD, M. & CAMPBELL, K.P. (1996). Dual function of the voltage-dependent Ca^{2+} $\alpha 2\delta$ subunit in current stimulation and subunit interaction. *Neuron*, **16,** 431 – 440.

HIRNING, FOX, A.P., MCCLESKEY, E.W., OLIVERA, B., THAYER, S.A., MILLER, R.J. & TSIEN, R.W. (1988). Dominant role of N-type calcium channels in evoked release of norepinephrine from sympathetic neurons. *Science*, **239,** 57 – 61.

JAY, S.D., SHARP, A.H., KAHL, S.D., VEDVICK, T.S., HARPOLD, M.M., & CAMPBELL, K.P. (1991). Structural characterization of the dihydropyridine-sensitive calcium channel $\alpha 2$-subunit and the associated delta peptides. *J. Biol. Chem.*, **266,** 3287 – 3293.

KEITH, R.A., HORN, M.B., PISER, T.M. & MANGANO, T.J. (1993). Effects of stimulus intensity on the inhibition by ω-conotoxin GVIA and neomycin of K^+-evoked [^{3}H]norepinephrine release from hippocampal brain slices and synaptosomal calcium influx. *Biochem. Pharmacol.*, **45,** 165 – 171.

LUNDY, P.M., FREW, R., FULLER, T.W. & HAMILTON, M.G. (1991). Pharmacological evidence for an ω-conotoxin dihydropyridine-insensitive neuronal Ca^{2+} channels. *Eur. J. Pharmacol.*, **206,** 61 – 68.

MANGANO, T.J., PATEL, J., SALAMA, A.I. & KEITH, R.A. (1991). Inhibition of K^+ evoked [^{3}H] D-aspartate release and neuronal calcium calcium influx by verapamil, diltiazen and dextromethorphan: evidence for non-L/non-N voltage sensitive calcium channels. *Eur. J. Pharmacol.*, **192,** 9 – 17.

MAUBECIN, V.A., SANCHEZ, V.N., ROSATO SIRI, M.D., CHERKSEY, B.D., SUGIMORI, M., LLINÁS. R. & UCHITEL, O.D. (1995). Pharmacological characerization of the voltage dependent Ca^{2+} channels present in synaptosomes from rat and chicken central nervous system. *J. Neurochem.*, **64,** 2544 – 2550.

MOMYAMA, A. & TAKAHASHI, T. (1994). Calcium channels responsible for potassium-induced transmitter release at rat cerebellar synapses. *J. Physiol.*, **476,** 197 – 202.

OLIVERA, B.M., MILJANICH, G.P., RAMACHANDRAN, J. & ADAMS, M.E. (1994). Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins. *Ann. Rev. Biochem.*, **63,** 823 – 867.

PERRIER, M.L., SCATTON, B. & BENAVIDES, J. (1992). Dihydropyridine- and ω-conotoxin-resistant, neomycin-sensitive calcium channels mediate the depolarization-induced increase in internal calcium levels in cortical slices from immature rat brain. *J. Pharmacol. Exp. Ther.*, **261,** 324 – 330.

PRADO, M.A.M., GOMEZ, M.V. & COLLIER, B. (1992). The mobilization of readily releasable pool of acetylcholine from a sympathetic ganglion by tityustoxin in the presence of vesamicol. *J. Neurochem.*, **59,** 544 – 552.

RANG, H.P. (1964). Stimulant action of volatile anesthetics on smooth muscle. *Br. J. Pharmacol.*, **22,** 356 – 365.

REYNOLDS, I.J., WAGNER, J.A., SNYDER, S.H., THAYER, S.A., OLIVERA, B.M. & MILLER, R.J. (1986). Brain voltage sensitive calcium channel subtypes differentiated by ω-conotoxin fraction GVIA. *Proc. Natl. Acad. Sci. U.S.A.*, **83,** 8804 – 8807.

RIVIER, J., GALYNEAN, R., GRAY, W.R., AZIMI-ZONOOZ, A., MCINTOSH, J.M., CRUZ, L.J. & OLIVERIA, B.M. (1987). Neuronal calcium channel inhibitors. Synthesis of ω-conotoxin GVIA and effects on ^{45}Ca uptake by synaptosomes. *J. Biol. Chem.*, **262,** 1194 – 1198.

SUSZKIW, J.B., O'LEARY, M.E., MURAWISKY, M.M. & WANG, T. (1986). Presynaptic calcium channels in rat cortical synaptosomes: fast kinetics of phasic calcium influx channel inactiviation, and relationship to nitrendipine receptors. *J. Neurosci.*, **6,** 1349 – 1357.

SUSZKIW, J.B., MURAWSKY, M.M. & SHI, M. (1989). Futher characterization of phasic calcium influx rat cerebral cortical synaptosomes: interference regarding calcium channel type(s) in nerve endings. *J. Neurochem.* **52,** 1260 – 1269.

TAKUMA, K., MATSUDA, T., ASANO, S. & BABA, A. (1995). Intracellular ascorbic acid inhibits Na^+-Ca^{2+} exchange in cultured rat astrocytes. *J. Neurochem.,* **64,** 1536 – 1540.

TSIEN, R.W., LIPSCOMBE, C., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channels and their selective modulation *Trends Neurosci.* **11,** 431 – 438.

TURNER, T.J. & DUNLAP, K. (1995). Pharmacological characterization of presynaptic calcium channels using subsecond biochemical measurements of synaptosomal neurosecretion. *Neuropharmacology* **34,** 1469 – 1478.

UCHITEL, O., PROTTI, D., SANCHEZ, V., CHERKSEY, B., SUGIMORI, M. & LLINÁS, R. (1992). P-Type voltage dependent calcium channel mediates presynaptic calcium influx and transmitter and transmitter release in mammalian synapses. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 3330 – 3333.

WAGNER, J., SNOWMAN, A., BISWAS, A., OLIVERA, B. & SNYDER, S. (1988). ω-CgTX GVIA binding to high-affinity receptor in brain: characterization, calcium sensitivity and solubilization. *J. Neurosci.*, **8,** 3354 – 3359.

WARNICK, J.E., ALBUQUERQUE, E.X. & DINIZ, C.R. (1976). Electrophysiological observation on the action of the purified scorpion venom, tityustoxin, on nerve and muscle preparation of the rat. *J. Pharmacol. Exp. Ther.*, **198,** 155 – 167.

WESSLER, I., DOOLEY, D.J., WERHAND, J. & SCHLEMMER, F. (1990). Differential effects of calcium channel antagonists (ω-conotoxin GVIA, nifedipine, verapamil) on the electrically evoked-evoked release of [^{3}H]acetylcholine from the myenteric plexus, phrenic nerve and neocortex of rats. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **341,** 288 – 294.

WITCHER, D.R., DE WAARD, M., LIU, H., PRAGNELL, M. & CAMPBELL, K.P. (1995). Association of native Ca^{2+} channel beta subunits with alpha 1 subunit interaction domain. *J. Biol. Chem.*, **270,** 18088 – 18093.

ZHANG, J.F., RANDALL, A.D., ELLINOR, P.T., HORNE, W.A., SATHER, W.A., TANABE, T., SCHWARZ, T.L. & TSIEN, R.W. (1993). Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology* **32,** 1075 – 1088.

(Received July 1, 1996
Revised September 18, 1996
Accepted September 25, 1996)