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RELATÓRIO TÉCNICO CIENTIFICO DE MARCUS VINICIUS GOMEZ REFERENTE AO PROCESSO CBS1033-92 - EFEITO DOS IONS LANTANIO NA LIBERAÇÃO DE ACETILCOLINA INDUZIDA POR TITYUSTOXINA, OUABAINA E K+ EM FATIAS DO CORTEX CEREBRAL E NO PLEXO MIOENTÉRICO.

Com o auxílio ao projeto acima foram publicados os seguintes trabalhos:

1) Gomez, R.S., Moraes-Santos, T.M. & Gomez, M.V. Effect of lanthanum ions on the release of acetylcholine induced by tityustoxin, K+ and ouabain from myentheric plexus and brain cortical slices. **Toxicon** 31-411-415, 1993

2) M.V. Gomez, T.Moraes-Santos, M.A.M. Prado, A.H.I. Salgado, R.S. Gomez,T.A.A. Casali e C.R. Diniz. Effects of Scorpion Venom Tityustoxin on Central Nervous System. ***Toxicon*** 32: 240, 1993

3)Queiroz, I.B., Turchetti-Maia, R.M.M., Moraes-Santos, T., & Gomez, M.V. Aging and acetylcholine release by tityustoxin from brain cortical slices. **Toxicon** 32: 232-234, 1994

4)Casali, T.A.A., Gomez, R.S., Moraes-Santos, T. & Gomez, M.V. Differential effects of calcium channel antagonist on tityustoxin and ouabain induced release of ^{3}H-acetylcholine from brain cortical slices. **Neuropharmacol.** 34: 599-603, 1995

5)Gomez, M.V., Romano-Silva, M.A., & Prado, M.A.M. Effects of tityustoxin on central nervous system. Journal of Toxicology - **Toxin Reviews** (no prelo), 1995.

6) M.V. Gomez, T. Moraes-Santos, M.A.M. Prado, A.I. Salgado, T.A.A. Casali, R.S. Gomez, A.M. Ribeiro, Z.E.G. Oliveira e C.R. Diniz. Studies on the actions of Tityustoxin on nervous system. **Anais da Academia Brasileira de Ciências Vol 66, nº 3, 1994.**

Durante o período as seguintes teses foram defendidas utilizando o auxílio acima:
Marco Antonio Maximo Prado- Doutoramento-1993
"Estoques de acetilcolina sensíveis e insensíveis ao vesamicol no gânglio cervical superior"

Marco Aurélio Romano Silva- Doutoramento-1994
"Liberação de glutamato de sinaptosomas cerebrocorticais: relações com $[Ca^{+2}]i$ e evidências de um papel regulatório para os íons sódio" - 1994

Ricardo A. Leão - Mestrado-1994
Efeito dos isômeros do vesamicol na liberação de acetilcolina de fatias do cortex cerebral de ratos.

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EFFECT OF LANTHANUM IONS ON THE RELEASE OF ACETYLCHOLINE INDUCED BY TITYUSTOXIN, K^+ AND OUABAIN FROM MYENTERIC PLEXUS AND BRAIN CORTICAL SLICES

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R. S. GOMEZ, T. MORAES-SANTOS and M. V. GOMEZ. Effect of lanthanum ions on the release of acetylcholine induced by tityustoxin, K^+ and ouabain from myenteric plexus and brain cortical slices. *Toxicon* **31**, 411–415, 1993.—Exposure of myenteric plexus longitudinal muscles to 2 mM $LaCl_3$ increased the rate of spontaneous [^{3}H]-ACh release. In brain cortical slices La^{3+} did not affect the spontaneous release of ACh. The release of ACh induced by tityustoxin, ouabain or K^+ 50 mM in brain cortex and myenteric longtudinal muscles slices was inhibited by La^{3+} treatment. It is suggested that tityustoxin, ouabain and K^+ release ACh from the same pool that is inhibited by La^{3+} pretreatment.

INTRODUCTION

LANTHANUM ions (La^{3+}) are known to produce a pronounced increase in the release of acetylcholine (ACh) from cholinergic motor nerve terminals (BLIOCH *et al.*, 1968; HEUSER and MILEDI, 1971; GUNDERSEN and JENDEN, 1981; KRIEBEL and FLOREY, 1983). This release is quantal and associated with a loss of synaptic vesicles from the nerve terminals (MILEDI *et al.*, 1982).

The ACh release mechanism has been intensively studied and two hypotheses, the vesicular (for review see CECCARELLI and HURLBUT, 1980) and the cytoplasmic (for review see ISRAEL and MANARANCHE, 1985) have been proposed to explain this mechanism. Electrical field stimulation of the guinea-pig myenteric plexus has failed to cause a further increase in the release of ACh from La^{3+} pretreated preparations, whereas ouabain released considerable more ACh when compared with controls, suggesting that electrical stimulation and ouabain release ACh from different pools (DOLEZAL *et al.*, 1987).

Tityustoxin, a neurotoxin purified from the Brazilian scorpion *Tityus serrulatus*, mimics many effects of electrical stimulation in rat brain cortical slices and synaptosomes,

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increasing the release of ACh as well as the uptake of sodium and calcium ions, causing a susbtantial depolarization in a tetrodotoxin-sensitive manner (GOMEZ *et al.*, 1973, 1975). Studies on ACh release in brain cortical slices induced by tityustoxin and ouabain suggest that they may mobilize different intracellular pools (GOMEZ, 1980; RIBEIRO and GOMEZ, 1986).

To our knowledge, all published investigations regarding La^{3+} effects on the release of ACh have been performed on frog neuromuscular junction and guinea-pig myenteric plexus. In this paper we have studied the effects of La^{3+} on the release of ACh induced by tityustoxin, ouabain and K^+ 50 mM in slices of guinea-pig myenteric plexus longitudinal muscles. In addition, we have further extended studies on the effect of La^{3+} in the release of ACh from brain cortical slices.

MATERIALS AND METHODS

Tityustoxin was purified as previously described (GOMEZ and DINIZ, 1966). Tityustoxin is an α-scorpiotoxin that activates voltage-sensitive sodium channels of neuronal cells and the release of neurotransmitter from nerve endings. Ouabain and lanthanum chloride were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Holtzman rats (200–250 g) of either sex were killed by decapitation and their cerebral cortices removed and sliced in a McIlwain Tissue Slicer (Brinckman Instruments Inc., U.K.). Pieces of longitudinal muscle with its accompanying plexus, weighing about 40 mg, were obtained from guinea-pigs of either sex, as described by PATON and ZAR (1968).

The release of ACh into the incubating fluid was studied after labelling tissue ACh with methyl [^{3}H]-choline chloride (Amersham Searle, 78 Ci/mmole), following the method described by SUSKIN and TOTH (1986). Tissue ACh stores are first depleted by an incubation in Krebs medium, for 15 min in the presence of 50 Mm K^+. After this the slices were incubated in Krebs medium for 30 min with 0.35 μCi of methyl [3H]-choline to label the endogenous pools of ACh. The slices were then separated from the incubating fluid by centrifugation (5000 *g* 10 min) and then washed three times with 1.0 μM of cold choline. Subsequently the slices were preincubated for 15 min in the presence or absence of 2.0 mM La^{3+} and then stimulated with tityustoxin 2.5 μM, ouabain 10 μM or K^+ 50 mM for 5, 15 and 30 min. To avoid La^{3+} precipitation, the medium used in this incubation contained (mM): NaCl 136; KCl 2.7; $CaCl_2$ 1.8; glucose 5.5; trizma base 10 and diethyl *p*-nitrophenyl phosphate (Paraoxon, Sigma Chem. Co.) 20 μM was added to prevent hydrolysis of ACh. The final pH was adjusted to 7.4.

TABLE 1. EFFECT OF La^{3+} 2.0 mM ON THE RELEASE OF ACh INDUCED BY TITYUSTOXIN, OUABAIN AND K^+ 50mM FROM RAT BRAIN CORTICAL SLICES

		^{3}H-ACh (DPM/g tissue × 10^3)		
Time (min)	Control	Tityustoxin	Ouabain	K^+(50 mM)
A. Without La^{3+}				
5	52.4 ± 3.1	74.2 ± 4.7*	98.6 ± 2.4*	132.0 ± 20.0*
15	69.3 ± 7.3	127.2 ± 11.0*	126.6 ± 10.4*	124.9 ± 12.2*
30	77.2 ± 3.1	113.9 ± 8.6*	120.8 ± 13.2*	154.0 ± 5.4*
B. With La^{3+} (2.0 mM)				
5	53.3 ± 3.7	43.4 ± 5.3	45.1 ± 1.8	48.8 ± 3.0
15	65.3 ± 5.5	71.7 ± 10.7	58.7 ± 2.6	58.7 ± 5.2
30	74.0 ± 7.8	75.6 ± 3.8	75.8 ± 2.7	70.5 ± 4.9

Rat brain cortical slices (±80 mg) were preincubated for 15 min in the absence of (A) or in the presence of 2.0 mM La^{3+} (B). They were then stimulated for 5, 15, and 30 min with tityustoxin 2.0 μM, ouabain 10 μM or K^+ 50 mM. In order to maintain the isotonicity, in the case of high K^+, an equal amount of Na^+ was removed from the incubation medium. For other details see Material and Methods. The values express the means ± S.E. for duplicates of three different experiments. *Statistically different from the control, $P < 0.02$.

TABLE 2. EFFECT OF La^{3+} ON THE RELEASE OF ACh INDUCED BY TITYUSTOXIN, OUABAIN AND K^+ 50mM IN GUINEA-PIG MYENTERIC PLEXUS

	^{3}H-ACh (DPM/g tissue $\times 10^3$)			
Time (min)	Control	Tityustoxin	Ouabain	K^+(50 mM)
A. Without La^{3+}				
5	32.8±6.5	39.4±1.1	29.2± 7.8	80.4±10.1*
15	30.9±2.1	89.8±5.6*	81.9± 9.1*	81.1± 3.3*
30	55.1±9.9	133.5±8.0*	156.2±13.2*	214.5±14.4*
B. With La^{3+} (2.0 mM)				
5	29.1±6.3	33.0±2.1	37.9±7.2	48.1± 9.2
15	62.9±5.0*	44.6±3.9	56.2± 5.3	46.2± 9.9
30	92.9±4.6*	102.2±5.6	109.7±12.3	92.1±17.2

Slices from guinea-pig longitudinal muscles myenteric plexus (±40 mg) were preincubated for 15 min without La^{3+} (A) or with 2.0 mM La^{3+} (B). They were them stimulated for 5, 15 and 30 min with tityustoxin 2.0 μM, ouabain 10 μM or K^+ 50 mM. In order to maintain the isotonicity, in the case of high K^+, an equal amount of Na^+ was removed from the incubation medium. The values express the means±S.E. for duplicates of three different experiments. For other details see Material and Methods. *Statistically different from the control, $P < 0.02$.

In order to characterize the radioactivity released, the supernatants were submitted to high-voltage electrophoresis to separate [^{3}H]-choline and [^{3}H]-acetylcholine. The [^{3}H]-acetylcholine represented 60–72% of the total radioactivity.

The statistical analyses were performed by analysis of variance. Treatments and incubation times formed a factorial with the number of observations in each tissue. Variance homogeneity and normal distribution was assumed. Analysis of variance was then performed to estimate error variance which was used to compare the means using the Student Newman–Kuels test (STEEL and TORRIE, 1960).

RESULTS

Table 1 shows the time-dependent action of La^{3+}, tityustoxin, ouabain and K^+ (50 mM) on the release of ACh from incubated rat brain cortical slices. There was no significant effect of La^{3+} on the spontaneous release of ACh ($P > 0.05$), whereas tityustoxin, ouabain and K^+, as a function of incubation time, cause a pronounced stimulation of the release of ACh ($P < 0.02$). When cortical slices were pretreated with La^{3+} the release of ACh stimulated by tityustoxin, ouabain or K^+ was completely blocked, $P < 0.02$ (Table 1B).

Table 2 shows the time-course effect of La^{3+}, tityustoxin, ouabain and K^+ in the release of ACh from incubated slices of myenteric plexus longitudinal muscles of guinea-pig ileum. As observed with brain cortical slices, tityustoxin, ouabain and K^+, as a function of time, caused stimulation of the release of ACh from incubated myenteric plexus longitudinal muscles, $P < 0.02$. The effect of K^+ on the release of ACh was higher than that of tityustoxin and ouabain. Even at the shortest period of incubation (5 min), K^+ 50 mM was able to increase the release of ACh, $P < 0.02$. La^{3+} pretreatment for 15 and 30 min promoted a significant stimulation of the release of ACh. At 30 min of incubation, the ion almost doubled the release of ACh. However, La^{3+} blocked the release of ACh induced by tityustoxin, ouabain or K^+.

DISCUSSION

As far as we know, there is no report on the effects of La^{3+} ions on the release of ACh from central cholinergic neurons. In this study, we have observed a difference between the effect of La^{3+} on the release of ACh in slices of guinea-pig myenteric plexus longitudinal muscles and rat brain cortex. Indeed, La^{3+} had not effect on the spontaneous release of ACh from incubated cortical slices, while it increased the release of ACh in incubated slices of myenteric plexus. Literature data show that La^{3+} promotes a large increase in the release of ACh in the myenteric plexus (DOLEZAL *et al.*, 1987) and neuromuscular junction (HEUSER and MILEDI, 1971; GUNDERSEN and JENDEN, 1981; MILEDI *et al.*, 1982). We have no explanation for the absence of an effect of La^{3+} on the release of ACh from central cholinergic synapses.

La^{3+} causes depletion of vesicular neurotransmitters (MILEDI *et al.*, 1982); however, the inhibitory effect of La^{3+} ions on the release of ACh induced by tityustoxin, ouabain or K^+ 50 mM is not likely explained by a general depletion of ACh stores. Even in cortical slices, where La^{3+} ions have no effect in the release of ACh, this cation was capable of inhibiting the release of ACh induced by tityustoxin, ouabain or K^+.

Prolonged exposure to La^{3+} was reported to produce miniature end plate potentials of higher amplitudes, suggesting that either a vesicle is re-used several times or a new vesicle population is formed during the process (HEUSER and MILEDI, 1971). The activation of ACh release by La^{3+} is not understood, but the depletion of synaptic vesicles from the cholinergic terminal is a conspicuous consequence of La^{3+}-activated release (DOLEZAL *et al.*, 1987).

Electron micrographs of neuromuscular junctions of muscles treated with La^{3+} showed precipitation of La^{3+} salts (HEUSER and MILEDI, 1971; MILEDI *et al.*, 1980).

Thus, La^{3+} accumulation in the synaptic region might have a blocking action on Ca^{2+} channels. However, washout of La^{3+} for long periods from La^{3+}-pretreated myenteric plexus (DOLEZAL *et al.*, 1987) failed to restore the ACh release on depolarization of La^{3+}-pretreated tissues. The inhibition on the release of ACh by La^{3+} does not seem to depend on extracellular Ca^{2+}, since it was also observed in the presence of ouabain, that causes a calcium-independent release of ACh (GOMEZ *et al.*, 1975; VIZI and LIGETI, 1984).

DOLEZAL *et al.* (1987) reported a result different from our data, but this may be due to methodological differences. Thus, in the myenteric plexus as well in brain cortical slices, La^{3+} was capable of inhibiting the ACh release induced by tityustoxin, ouabain or K^+ 50 mM. Although we cannot distinguish the ACh pools, our data suggest that, in the conditions of our experiments, the release of ACh occurs from only one pool. Thus, La^{3+} may block the vesicular release of ACh induced by tityustoxin, ouabain or K^+.

It is amazing that La^{3+} by itself stimulated release of ACh from myenteric plexus while it blocked the release of ACh induced by tityustoxin, ouabain or K^+. La^{3+} blocks calcium entry into the cells of intestinal smooth muscle (WEISS and GOODMAN, 1969), and it seems to act from the outside of the cells (FLAT *et al.*, 1980). Thus, it is possible to speculate that La^{3+} binds to membrane sites responsible for ACh release. In the first condition, La^{3+} binding may stimulate the ACh release, while in the second condition the sites would be occupied by La^{3+} and unavailable to tityustoxin, ouabain and K^+ to the membrane receptor sites responsible for the ACh release.

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enzyme does not stain well with Coomassie blue. No attempt was made to stain this gel with silver stain.

Since it is known that venom alkaline phosphatase has a mol. wt greater than 90,000 (IWANAGA and SUZUKI, 1979), we selected electrophoresis conditions for detection of reduced proteins between 20,000 and 200,000 mol. wt. However, blots from SDS-PAGE gels boiled for 3 min in buffer containing 2-mercaptoethanol did not develop in the phosphatase substrate (not shown). Therefore, selected venoms were not treated with 2-mercaptoethanol or boiled prior to electrophoresis. Under these conditions, most of the venom proteins (developed with silver stain) were at a mol. wt less than 90,000, and very few bands were discernible in this gel (Fig. 1C). The developed blot from an equivalent SDS-PAGE gel revealed phosphatase activity between 90,000 and 110,000 mol. wts. Corresponding proteins were not seen in the stained gel, even in the venoms having high phosphatase activity, again indicating that the alkaline phosphatase in these venoms is in very low concentrations. The time for development of the enzyme also varied in this blot (Fig. 1D), and reflected the activity determined by the test tube method. Samples that had been frozen and thawed lost activity, as did samples that had been incubated in EDTA (10^{-4} M) or heated for 30 min at 55°C.

Endogenous phosphatases can interfere with alkaline phosphatase-based detection methods when studying snake venom. Preliminary testing to determine whether the venom (or body fluid) possesses endogenous alkaline phosphatase is recommended prior to using alkaline phosphatase-based detection methods to study snake venom. A convenient and simple assay procedure is to test the whole venom or fraction by a dot- or slot-blot method placing the blot in BCIP/NBT shortly after blotting. An alternative is to inactivate endogenous alkaline phosphatase, by using EDTA or heating. Another alternative may be to use horseradish peroxidase or gold-based immunological methods for detecting snake venom proteins.

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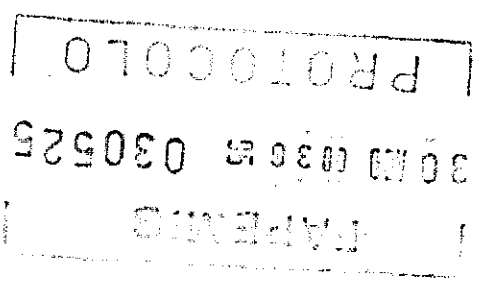

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AGEING AND ACETYLCHOLINE RELEASE BY TITYUSTOXIN FROM BRAIN CORTICAL SLICES

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I. B. QUEIROZ, R. M. M. TURCHETTI-MAIA, T. MORAES-SANTOS and M. V. GOMEZ. Ageing and acetylcholine release by tityustoxin from brain cortical slices. *Toxicon* **32**, 232–234, 1994.—The ability of tityustoxin to trigger acetylcholine release was studied in cerebral cortical slices from 2, 12 and 23-month-old rats. The effect of tityustoxin to induce the release of acetylcholine was reduced by 60% in senescent animals. This result suggests that ageing reduces the sensitivity of cerebral cortical nerve terminals to tityustoxin.

CHOLINERGIC function is reduced with normal ageing. It is firmly established that cholinergic cells degenerate with age. However, the specific functional change caused by this degeneration remains unknown (FIBIGER, 1991). Cells in young rats are more sensitive to acetylcholine (ACh) than those in old rats (HAIGLER *et al.*, 1985). Furthermore, cells in old rats have a lower baseline firing rate (LIPPA *et al.*, 1981). The effect of ageing on cholinergic neurons does not appear to involve a decrease in synthesis or levels of ACh available for the release, since the reduction in ACh release can occur without changes in these presynaptic parameters (PEDATA *et al.*, 1983; MEYER *et al.*, 1984). Ageing reduces calcium ionophore A-23187 and K^+-induced release of [^{3}H-ACh] (MEYER *et al.*, 1986), while release of ACh from the extensor digitorum longus muscle of 27-month-old rats was higher than that released from muscles of mature rats after 1 Hz stimulation (SMITH, 1990).

Tityustoxin from the Brazilian scorpion *Tityus serrulatus* repeats many of the effects of electrical stimulation, causing a substantial increase of the release of ACh from cortical slices of rat brain (GOMEZ *et al.*, 1973). The half maximal concentration (EC_{50}) for the effect of tityustoxin in the release of ACh is 1.1 μM. Therefore, we decided to investigate the stimulation of release of ACh by TsTX in cortical slices of mature and old rats.

Tityustoxin was purified as previously described (GOMEZ and DINIZ, 1966). Slices of cerebral cortex (0.5 mm) were obtained from male Holtzman rats of 2, 12 and 23 months old using a McIlwain Tissue Slicer. After incubation in Tyrode medium (30 min), with or without TsTX (2.3 μM), the slices were removed from the flasks and rinsed with 0.5 ml of incubation medium. The assay of free ACh was performed on the incubating fluids

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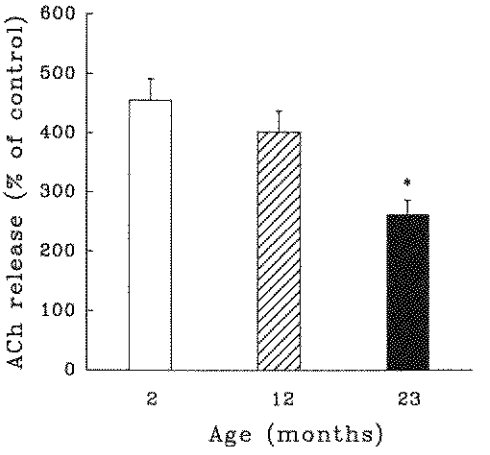

FIG. 1. EFFECT OF TITYUSTOXIN ON THE RELEASE OF ACh FROM BRAIN CORTICAL SLICES OF AGED RATS. Incubation in Tyrode medium at 37°C for 30 min, pH 7.4. □ 2 months old, ▨ 12 months old, and ■ 23 months old. Results are expressed as ACh released (% of control) ± SEM for 14, 11 and 9 animals in each group, respectively. * $P < 0.005$ compared with 2 and 12-month-old groups.

which were each collected individually (by filtration) in 0.1 ml 1 N HCl and boiled for 10 min to inactivate the tityustoxin. Samples were kept frozen at −15°C until assay. Acetylcholine was assayed on a strip of guinea-pig ileum in the presence of morphine, neostigmine and phenergan by the method of PATON (1957) with the precautions of Feldberg (1954) described in detail previously (GOMEZ *et al.*, 1973).

The results in Fig. 1 show the effect of tityustoxin stimulation on the release of ACh from brain cortical slices of 2, 12 and 23-month-old rats. The release of ACh induced by TsTX was 60% lower in 23-month-old animals than in 2 and 12-month-old animals ($P < 0.005$). The value for the release of ACh by tityustoxin in 2-month-old rats is 28.4 ± 1.2 nmol/g. There was no difference in the levels of basal and tissue ACh in the control animals, i.e. without tityustoxin stimulation (data not shown). Thus, the reduced effect of tityustoxin in old animals might not be explained in terms of ACh synthesis available for the release. This is in agreement with the changes in release of ACh induced by K^+ and Ca^{2+} ionophore A-23187 in old animals (PETERSON and GIBSON, 1983; MEYER *et al.*, 1986; PEDATA *et al.*, 1983).

Tityustoxin acts on Na^+ channels, increasing the release of acetylcholine from incubated brain cortical slices (GOMEZ *et al.*, 1973). Thus, we could speculate that in old animals the effect of tityustoxin could be due to a reduction of Na^+ channel tityustoxin binding sites. However, K^+-induced release of ACh does not involve Na^+ channels (SPOKES and DOLLY, 1980) and is also reduced in old animals (MEYER *et al.*, 1986).

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Differential Effects of Calcium Channel Antagonists on Tityustoxin and Ouabain-induced Release of [^{3}H]acetylcholine from Brain Cortical Slices

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Summary—In this paper, the effect of calcium channel blockers on acetylcholine release induced by tityustoxin and ouabain in rat brain cortical slices is described. Cadmium, a non-specific blocker of calcium channels, inhibited the release of ACh induced by tityustoxin. L-type calcium channel blockers (verapamil, nifedipine and diltiazen) had no effect on the release of ACh induced by tityustoxin. The release of ACh was also unaffected by nickel, a T-type calcium channel blocker, and the conotoxins GVIA and MVIIC, blockers of N and Q-type calcium channels. Agatoxin IVA, a specific blocker of the P-type calcium channel, inhibited the release of ACh induced by tityustoxin by 50%. The spontaneous release of ACh as well as ouabain-induced release of ACh was unaffected by any of the calcium channel blockers studied. It is concluded that ACh release induced by tityustoxin is mediated by Ca^{2+} influx via P-type calcium channels.

Keywords—Tityustoxin, ouabain, calcium channel antagonists, acetylcholine ω-conotoxins, ω-conotoxin MVIIC, ω-AgaIVA.

Calcium entry into neurons through voltage-dependent calcium channels (VDCC) is associated with a variety of intracellular processes including neurotransmitter release (Katz and Miledi, 1965). In neurons and other tissues, four main subtypes of VDCC termed L, N, T and P have been defined on the basis of differences in their electrophysiological and pharmacological profiles. Mammalian neurons exhibit a Q-type Ca^{2+} current that co-exists with L, N and P-type channels (Zhang *et al.*, 1993), and is blocked by ω-CgTX MVIIC, a specific antagonist (Wheeler *et al.*, 1994). A transient low-threshold T current is blocked by nickel, whereas calcium channel antagonists with dihydropyridine (nifedipine) or phenylalkylamine (verapamil) structures affect only the L-type channel (Miller, 1987; Tsien *et al.*, 1988). In addition to the organic calcium channel antagonists, the peptide ω-conotoxin GVIA has been found to block N-type calcium channels (Fox *et al.*, 1987; McCleskey *et al.*, 1987; Tsien *et al.*, 1988). 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 (Llinas *et al.*, 1989). P-type calcium channels were subsequently found in many other regions and are specifically blocked by ω-agatoxin IVA, a peptide purified from the venom of *Agelenopsis aperta* (Mintz *et al.*, 1992).

Tityustoxin, a neurotoxin from the venom of the Brazilian scorpion *Tityus serrulatus* mimics many effects of electrical stimulation by increasing the release of acetylcholine (ACh) as well as the uptake of Na^+ and Ca^{2+} in rat brain cortical slices and synaptosomes (Gomez *et al.*, 1975; Henriques and Gomez, 1981). These effects of tityustoxin result in a calcium and sodium-dependent release and synthesis of ACh that can be inhibited by tetrodotoxin (Gomez *et al.*, 1973, 1975). Thus, tityustoxin is useful as a tool for studies on synaptic transmission and neurotransmitter release.

Ouabain treatment of brain cortical slices results in a release of ACh that is independent of extracellular calcium (Gomez *et al.*, 1975, Adam-Vizi and Ligetti, 1984) with almost negligible cell depolarization (Brosemer, 1985; Adam-Vizi and Ligetti, 1986) and that is insensitive to tetrodotoxin inhibition (Gomez *et al.*, 1975). However, there are studies indicating that ouabain can increase levels of intrasynaptosomal free Ca^{2+} in the presence of extracellular calcium (Nachshen, 1985a, b), and that the ouabain induced increase in [^{3}H]ACh release from synaptosomes is inhibited by verapamil, a L-type calcium channel blocker (Satoh

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and Nakasato, 1992). These results suggest that the mechanism of action for ouabain might be different in the presence or absence of Ca^{2+}. Therefore, we tested whether the influx of Ca^{2+} through calcium channels might be involved in the ouabain induced release of ACh.

Although ACh release induced by tityustoxin is a calcium dependent process (Gomez *et al.*, 1975), the mechanisms by which tityustoxin affects Ca^{2+} entry into the cells is presently unknown. In this study we examined whether Ca^{2+} entry through L-, N-, T-, P- and Q-type Ca^{2+} channels is involved in the release of ACh by tityustoxin and ouabain.

METHODS

Tityustoxin was purified as previously described (Gomez and Diniz, 1966). Ouabain, verapamil, nifedipine, diltiazem and ω-conotoxin GVIA (ω-CgTX GVIA) were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). ω-Agatoxin IVA (AgaIVA) from Latoxan (Rosans, France) and ω-conotoxin MVIIC (ω-CgTX-MVIIC) from Peptide (Osaka, Japan). All other chemicals were analytical grade.

Slices of cerebral cortex (0.5 mm) were obtained from Holtzman rats (200–250 g) of either sex using a McIlwain Tissue Slicer. The release of ACh into the incubating fluid was studied after labeling tissue ACh with methyl [^{3}H]choline chloride (Amersham Searle, 78 Ci/mmol) following the method described by Suzskiw and Toth (1986). Tissue ACh stores were first depleted by an incubation in Krebs/Trizma medium, for 15 min in the presence of 50 mM K^+. To label the endogenous pools of ACh, the slices were incubated in Krebs Trizma medium for 30 min with 0.35 μCi of methyl [^{3}H]choline. The slices were then separated from the incubating fluid by centrifugation (5000 *g* for 10 min) followed by three washes with 1.0 μM of cold choline. Subsequently, the slices were preincubated for 15 min in the presence or absence of Cd^{2+}, verapamil, nifedipine, diltiazem, ω-conotoxin-GVIA (ω-CgTX), ω-conotoxin-MVIIC (ω-CgTX-MVIIC) or ω-AgaIVA at the indicated concentrations, followed by stimulation with tityustoxin (2.5 μM) or ouabain (100 μM) for 30 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, the supernatants were submitted to high-voltage electrophoresis to separate [^{3}H]choline and [^{3}H]acetylcholine. The [^{3}H]ACh represented 60–72% of the total radioactivity and tityustoxin did not increase the release of labeled choline.

Statistical analysis was performed by analysis of variance using the Student Newman Kuels test (Steel and Torrie, 1960) with a significance of $P < 0.05$.

RESULTS

The effect of Cd^{2+}, a non-specific blocker of calcium channels (Fox *et al.*, 1987), on the release of ACh induced by tityustoxin and ouabain was studied. Figure 1 shows that Cd^{2+} (100 μM) inhibited the release of ACh induced by tityustoxin (2.5 μM), having no effect on the release of ACh evoked by ouabain (100 μM) in brain cortical slices. Thereafter we examined the effect of L-type calcium channel blockers (verapamil, nifedipine and diltiazen, 1 μM) on tityustoxin and ouabain induced release of ACh. Table 1 shows that tityustoxin and ouabain stimulated ACh release from brain control slices by 127 and 122%, respectively. Verapamil, nifedipine and diltiazem (1 μM) had no effect on the release of ACh evoked by tityustoxin or ouabain ($P < 0.05$). This result suggests that L-type calcium channel blockers are not involved in the release of ACh by tityustoxin and ouabain. Ni^{2+} (100 μM), a T-type calcium channel blocker, had no effect on the release of ACh induced by tityustoxin or ouabain in rat brain cortical slices (Fig. 2). Thus, we decided to test the effect of ω-CgTX-GVIA a blocker of N-type calcium channels (Fox *et al.*, 1987; McCleskey *et al.*, 1987; Tsien *et al.*, 1988), on ACh release evoked by tityustoxin and ouabain. The experiment shown in Fig. 3 shows that ω-CgTX (0.1 μM) has no effect on the release of ACh induced by tityustoxin and ouabain ($P < 0.05$).

Table 2 shows the effect of ω-CgTX-MVIIC and ω-AgaIVA on the release of ACh induced by tityustoxin. The toxin ω-CgTX-MVIIC, a blocker

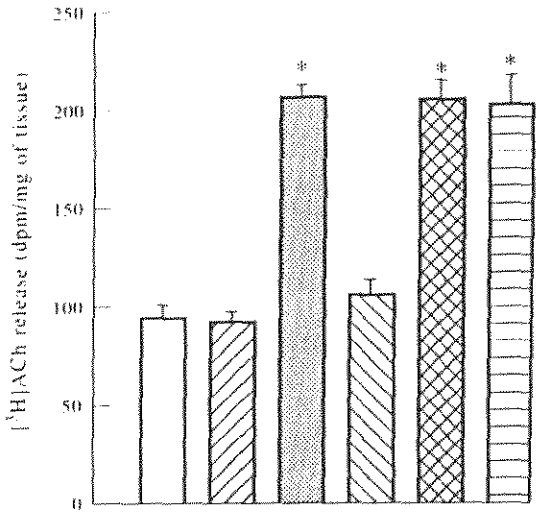

Fig. 1. Effect of cadmium on the release of ACh by tityustoxin and ouabain. Brain cortical slices (±40 mg) were incubated in a Krebs Trizma medium with Cd^{2+} (100 μM) 15 min. They were then stimulated for 30 min with tityustoxin (2.5 μM) or ouabain (100 μM). For other details see Methods. □ Control; ▨ Cd^{2+} 100 μM; ▩ tityustoxin (2.5 μM); ▧ tityustoxin 2.5 μM plus Cd^{2+} 100 μM; ■ ouabain 100 μM; ▤ ouabain 100 μM plus Cd^{2+}, 100 μM. The values represent means ± SEM for duplicates of 3 experiments. *Statistically different from control, $P < 0.05$.

Table 1. Effect of L-type calcium channel blockers on the release of acetylcholine induced by tityustoxin and ouabain

Calcium channel blocker	[^{3}H]-ACh (DPM mg of tissue) Control	Tityustoxin 2.5 μM	Ouabain 100 μM
None	86.3 ± 3.3 (12)	196.6 ± 3.4* (12)	192.1 ± 3.2* (12)
Verapamil 1 μM	93.6 ± 2.0 (9)	190.7 ± 2.4* (3)	183.2 ± 3.1* (3)
Nifedipine 1 μM	86.4 ± 3.3 (6)	185.9 ± 3.9* (3)	191.0 ± 4.0* (3)
Diltiazem 1 μM	92.1 ± 3.4 (6)	197.2 ± 2.8* (3)	188.3 ± 2.1* (3)

*Statistically different from the control value, $P < 0.05$.

Brain cortical slices (±40 mg) were pre-incubated for 15 min in Krebs Trizma medium in the absence (control) or in the presence of verapamil, nifedipine and diltiazem (1.0 μM). They were then stimulated for 30 min with tityustoxin (2.5 μM) or ouabain (100 μM). The values express the means ± SEM for duplicates of the number of experiments in parentheses. For other details see Methods.

Q-type calcium channels, had no effect on the release of ACh induced by tityustoxin. ω-CgTX AgaIVA, a specific blocker of P-type calcium channels, inhibited the release of ACh evoked by tityustoxin by 50% ($P < 0.01$).

DISCUSSION

The effect of tityustoxin on ACh release is Na^+ dependent and thus is inhibited by tetrodotoxin (Gomez *et al.*, 1973, 1975). In the nerve terminals, tityustoxin acts on Na^+ channels, (Gomez *et al.*, 1973, 1975) increasing Na^+ concentrations, causing cell depolarization that

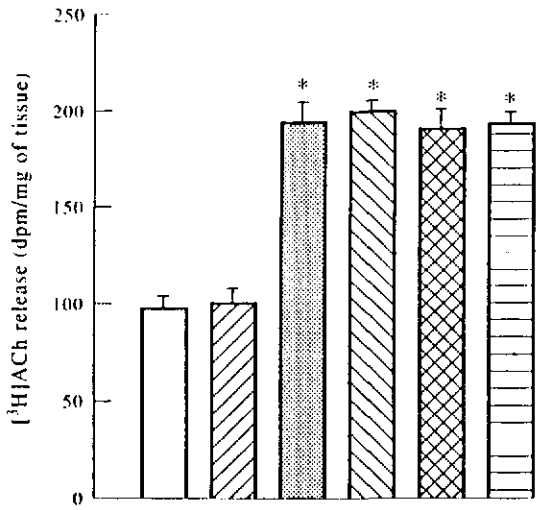

Fig. 2. Effect of nickel on the release of ACh by tityustoxin and ouabain. Brain cortical slices (±40mg) were pre-incubated in a Krebs Trizma medium with Ni^{++} (100 μM) for 15 min. They were then stimulated for 30 min with tityustoxin or ouabain. [ ] Control; ▨ Ni^{++} 100 μM; □ tityustoxin 2.5 μM; ▧ tityustoxin 2.5 μM plus Ni^{++} 100 μM; ■ ouabain 100 μM; ▩ ouabain 100 μM plus Ni^{++} 100 μM. The values represent the means ± SEM for duplicates of 3 experiments. For other details see Methods. *Statistically different from control, $P < 0.05$.

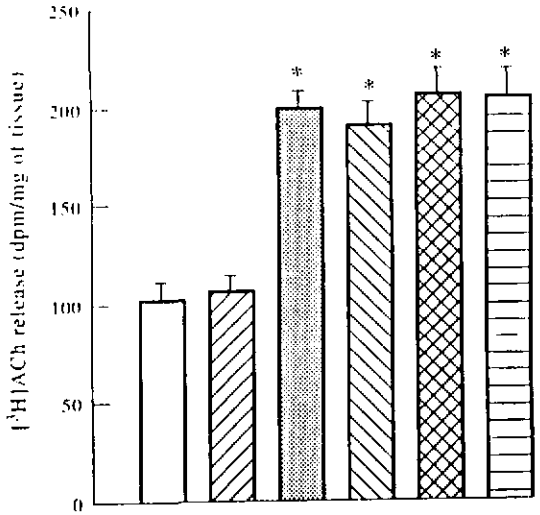

Fig. 3. Effect of ω-conotoxin GVIA on the release of acetylcholine by tityustoxin and ouabain. Brain cortical slices (±40mg) were pre-incubated in a Krebs Trizma medium with ω-CgTX 0.1 μM for 15 min. They were then stimulated for 30 min with tityustoxin or ouabain. □ Control; ▨ ω-CgTX 0.1 μM; □ tityustoxin 2.5 μM; ▧ tityustoxin 2.5 μM plus ω-CgTX 0.1 μM; ■ ouabain 100 μM; ▩ ouabain 100 μM plus ω-CgTX 0.1 μM. The values represent the means ± SEM for duplicates of 3 experiments. For other details see Methods. *Statistically different from control, $P < 0.05$.

opens calcium channels allowing the influx of Ca^{2+}, triggering ACh release.

ACh release from different neurons appears to be regulated by different types of calcium channels. It is known that Cd^{2+} binds strongly to voltage-gated Ca^{2+} channels (Nachshen, 1985a, b; Lansman *et al.*, 1986). Thus, Cd^{2+}, an non-specific blocker of calcium channels (Fox *et al.*, 1987), inhibited the release of ACh induced by tityustoxin, having no effect on the release of ACh evoked by ouabain (Fig. 1). This suggests the involvement of calcium channels in the tityustoxin induced release of ACh. Dihydropyridine-sensitive calcium channels are known to exist in the central nervous system (Miller, 1987) but the functions of L- and T-type calcium channels are largely unknown. There is a consensus that little or no transmitter release in the brain is mediated by L- or T-type calcium channels (Olivera *et al.*, 1994). We

Table 2. Effect of ω-CgTX-NVHC and ω-AgaIVA on the release of acetylcholine induced by tityustoxin in rat brain cortical slices

Calcium channel blocker	[^{3}H]Ach (dpm mg of tissue) Control	Tityustoxin (2.5 μM)
None	97.9 ± 2.2	197.5 ± 1.8*
ω-AgaIVA (0.1 μM)	96.4 ± 3.0	145.6 ± 2.4*†
ω-CgTX-MVIIC (1.0 μM)	97.7 ± 3.6	196.0 ± 3.5*

*Statistically different from the control value, $P < 0.01$.

†Statistically different from the tityustoxin value, $P < 0.01$.

Brain cortical slices (±40 mg) were pre-incubated for 15 min in Krebs Trizma medium in the absence (control) or in the presence of ω-AgaIVA (0.1 μM) or ω-CgTX-MVIIC (1.0 μM). They were then stimulated for 30 min with tityustoxin (2.5 μM). The values express the means ± SEM for duplicates of 3 experiments. For other details see Methods.

observed that L- (Table 1) and T-type (Fig. 2) calcium channels are not involved in the release of ACh induced by tityustoxin and ouabain in rat brain cortical slices.

It was suggested that the N-type calcium channel plays a dominant role in regulating ACh release from the *neocortex and myenteric plexus* (Wessler *et al.*, 1990). However, the literature describes a poor sensitivity of calcium channels to ω-CgTX-GVIA in rat brain cortical slices and synaptosomes (Lundy *et al.*, 1991, Suszkiw *et al.*, 1986, 1989). We have shown that a specific blocker of N-type calcium channels, ω-CgTX-GVIA, had no effect on the release of ACh induced by tityustoxin and ouabain (Fig. 3). This indicates that the N-type channel is not involved in the release of ACh induced by the toxin or ouabain.

The ω-CgTX MVIIC that blocks the Q-type calcium channel (Wheeler *et al.*, 1994) had no effect on the release of ACh induced by tityustoxin (Table 2). Thus the Q-type calcium channel is not involved in tityustoxin-induced release of ACh in rat brain cortical slices.

ω-AgaIVA, a specific blocker of the P-type calcium channel, (Mintz *et al.*, 1992) inhibited the release of ACh evoked by tityustoxin in brain cortical slices by 50% (Table 2). Thus, the P-type calcium channel is involved in the tityustoxin-induced influx of $^{45}Ca^{2+}$ into brain cortical synaptosomes (Henriques and Gomez, 1981). Since the inhibition caused by ω-AgaIVA was only partial it is possible to assume that other calcium channels could be involved in the tityustoxin-induced release of ACh. It has been shown that cooperation can occur among multiple Ca^{2+} channel types in the control of ACh release (Wheeler *et al.*, 1994).

Ouabain stimulates the release of ACh independently of extracellular Ca^{2+} (Gomez *et al.*, 1975; Adam-Vizi and Ligetti, 1984) and causes a release of ACh that is not inhibited by any of the calcium channel blockers studied. Contrary to the results described in this paper, the literature shows that, at a concentration of 500 μM, verapamil inhibited the release of ACh induced by ouabain in synaptosomes (Satoh and Nakazato, 1992). This discrepancy is probably due to the higher concentration of verapamil. At this concentration verapamil is not selective for Ca^{2+} channel blockade and also depresses Na^+ channels (Triggle, 1981; Hille, 1992). It is interesting to note that the spontaneous release of ACh is not calcium dependent and was not affected by any of the calcium channel blockers. In conclusion, our results show that tityustoxin induced influx of calcium and release of ACh are blocked by ω-AgaIVA.

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