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THE CALCIUM CHANNEL ANTAGONIST ω-CONOTOXIN INHIBITS SECRETION FROM PEPTIDERGIC NERVE TERMINALS

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SUMMARY - The binding of ω-conotoxin to isolated rat neurohypophysial nerve terminals, its effect on the depolarization-induced increase of cytoplasmic Ca^{2+} and on the potassium and electrically-induced release of vasopressin (AVP) have been studied. The results show that isolated neurosecretory nerve endings have calcium channels with a high affinity for ω-CgTx and that this toxin inhibits neurohormone release at very low concentration (IC_{50}= 0. 1nM). Although secretion of vasopressin is inhibited to a great extent by the toxin it is shown that a small but significant amount of the depolarization-induced AVP release is insensitive to ω-CgTx and to the dihydropyridine molecule nicardipine.

A new class of toxins isolated from the venom of the fish-hunting snail *Conus geographus* has recently been described (1). One of these toxins, ω-conotoxin GVIA (hereafter abbreviated ω-CgTx) has been shown to block voltage-sensitive calcium channels at the frog neuromuscular junction (2) as well as two distinct calcium currents (termed N and L) in chick sensory neurons (3). In brain synaptosomes, the peptide ω-conotoxin fraction GVIA inhibits only a small percentage of the depolarization-induced $^{45}Ca^{2+}$ uptake and neurotransmitter release (4). In the present study we have analyzed the binding of ω-conotoxin to isolated rat neurohypophysial nerve terminals, its effects on the depolarization-induced increase of cytoplasmic Ca^{2+} and on the potassium and electrically-induced release of vasopressin (AVP).

MATERIALS AND METHODS

AVP release from neural lobes and isolated neurohypophysial nerve terminals. The neural lobes were prepared as described earlier (5, 6). They were perfused with normal saline containing (mM):NaCl, 140; $KHCO_3$, 5; $CaCl_2$, 2.2; $MgCl_2$, 1; glucose, 10; HEPES, 10; pH 7.2 and to which bovine serum albumine at a final concentration of 0.01 % was

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added. The saline was continuously gassed with 5 % CO_2 in O_2. After 60 min of perfusion, at a flow rate of 100 µl x min^{-1}, the neurohypophyses were perfused for 20 min with Na-free saline containing 140 mM choline chloride. Depolarization, for a period of 10 min, was induced with 100 mM potassium, choline being decreased accordingly in order to maintain the osmolarity constant. The evoked AVP release was calculated by subtracting the mean basal release determined in the fractions preceding the onset of the stimulus from the amount of hormone found in each fraction.

Electrical stimulation of the neural lobe. The neural lobes were obtained from male rats (250-300 g body weight) and the pars intermedia carefully dissected out. The neural lobe was impaled on one of a pair of electrodes which were inserted in a small perspex chamber which had an internal volume of about 50 µl. The gland was continuously perfused with normal saline (See Fig. 2 legend) in the presence or absence of ω-CgTx (1 nM). Fractions (100 µl x min^{-1}) were collected every 2 min. and AVP in the perfusate was determined. The neurohypophyses were stimulated electrically with biphasic electrical pulses which had a pattern of discharge similar to the bursting activity of AVP cells (see ref. 6). Briefly the electrical activity of vasopressin cell recorded *in vivo* was used as a command pulse to trigger the stimulator and thus generate a series of pulses (2 ms duration, 2 mA) similar to the bursting pattern observed *in vivo*. The neural lobes were stimulated with four"AVP-like" bursts separated by intervals of 21 s.

Binding of ω-CgTx. ω-CgTx (Peptide Institute, Osaka, Japan) was iodinated by the lactoperoxydase method (7,8) and the monoiodinated derivative was obtained by HPLC 7,8). Membranes (8 µg protein per assay) were prepared by lysing the isolated nerve endings and by further centrifugation of the disrupted membranes. They were incubated with ω-CgTx for 60 mn at 37° in a final volume of 150 µl of a medium containing (mM) : NaCl, 140; KCl, 5; $MgSO_4$, 0.8; glucose, 10; HEPES, 20 and 0,5% bovine serum albumin adjusted to pH 7.2 with Tris-base. Bound toxin was separated by rapid filtration over polyethyleneimine pretreated glass fiber filters (Whatman GF/C).

Binding of (3H) PN 200-110. The binding of (^{3}H)PN 200-110 (85 Ci/mmole, Amersham Corp.) was measured in 170 mM Tris, 1 mM $CaCl_2$ (pH 7.4) in a final volume of 150 µl containing membranes equivalent to three neural lobes. (^{3}H) PN200-110 (2 nM) was incubated with membranes for 30 min at 37°C under red light. Non specific binding was measured in the presence of 100 nM nifedipine (Sigma). Binding was terminated by rapid filtration over polyethyleneimine pretreated glass fiber filter (Whatman GF/C).

RESULTS

Binding of ω-CgTx and PN 200-110 to nerve endings.

Figure 1a shows the results of typical experiments demonstrating a saturable binding of ω-CgTx to nerve terminal membranes. As already demonstrated (7,8), the binding of the toxin with its receptor is irreversible and thus the ligand-receptor interaction can be described by the following equation : $R + L \xrightarrow{k} RL$ where k is the association kinetic constant. After linearization of the results (Fig. 1b; see ref. 8) the association kinetic constant had a value of 5.6 x 10^6 $M^{-1}s^{-1}$ at 37°C and the total number of binding sites was 85 fmoles x mg^{-1} of protein. In an other series of experiments we looked if the calcium channel antagonists diltiazem (0.1 µM) and nifedipine (0.1 µM) could compete with the toxin for its binding site. Both on membranes isolated from

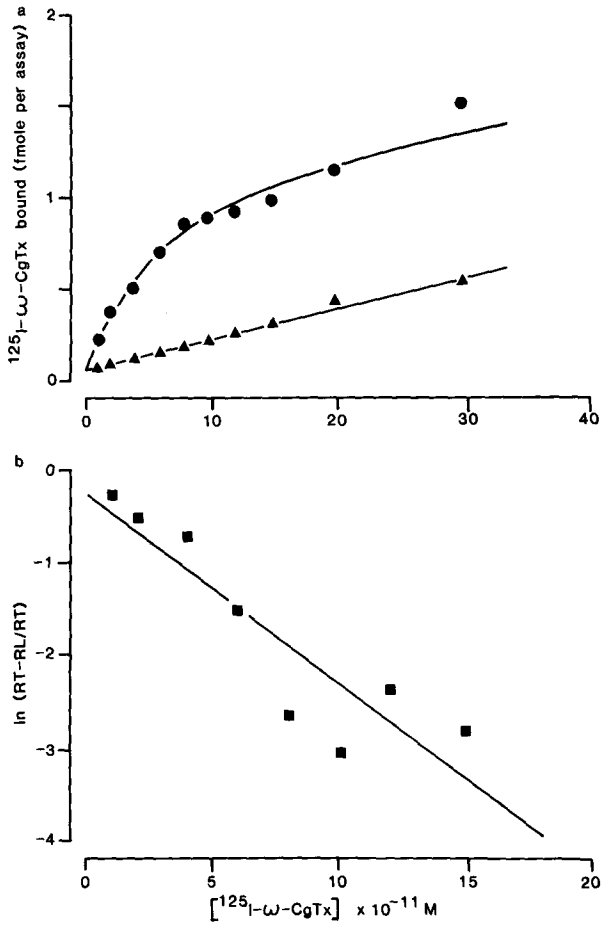

Figure 1. Binding of ω-CgTx to isolated rat neurohypophysial nerve terminal membrane. **a**, the membranes were incubated with increasing concentrations of (^{125}I)-ω-CgTx in the presence (▲, non specific binding) or in the absence of 0.1 μM native toxin (●, total binding). A saturable binding is observed. **b**,the specific binding (i.e. the difference between total and non specific binding illustrated in a) was linearized according to Ref. 8.

the neurohypophysial nerve endings and on synaptosomes from the cortex the antagonists did not affect significantly (less than 10%) the binding of ω-CgTx to its receptor (not illustrated). We also found that ω-CgTx up to a concentration of 0.1 μM did not compete with (^{3}H)-labeled dihydropyridine PN 200-110. In two sets of experiments the number of sites (fmol/neural lobe) was determined for both ω-CgTx and PN 200-110. The mean values were 4.4 (4.8-4.0) and 0.6 (0.6-0.6) respectively. This suggests that the number of binding sites for ω-CgTx is 6.7 to 8.0 larger than that of the dihydropyridines binding sites. Similarly, in lysed brain synaptosomes isolated from the rat, the number of binding sites for the toxin and PN 200-110 were 500 and 88 fmol x mg^{-1} respectively; thus the ratio of 5.7 between the two categories of sites is very similar to that measured in the neurosecretory nerve endings.

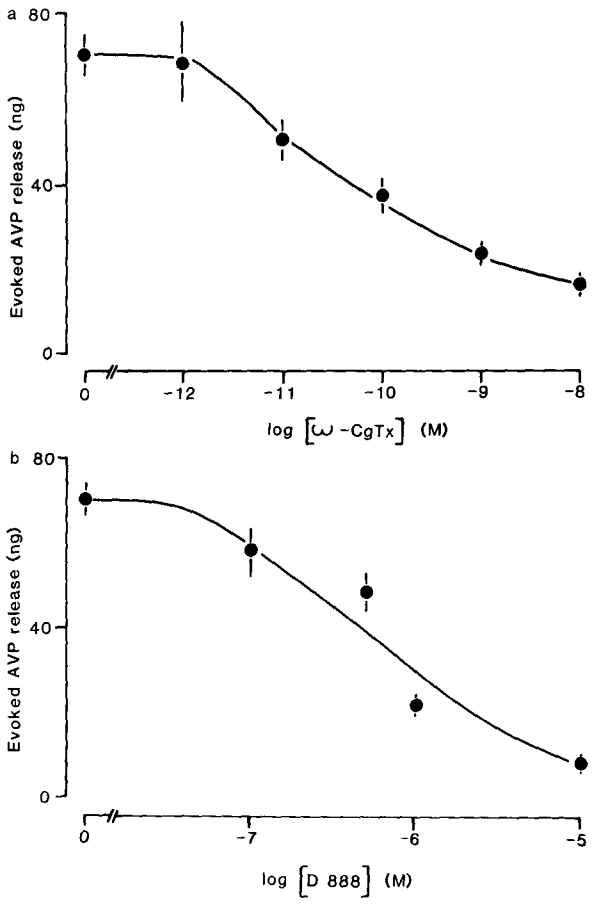

Figure 2. The effects of increasing concentrations of ω-CgTx (**a**) and D888 (**b**) on high potassium evoked AVP release from isolated neural lobes. ω-CgTx and D888, at concentrations indicated on the abcissa were present 10 min before and during the K+ stimulation. The results are given as mean ± S.E.M. (4<n<7).

K^+-induced AVP release.

Figure 2 shows the dose-response curves of high K+-evoked AVP release from isolated neural lobes in the presence of ω-CgTx (Fig. 2a) or in the presence of another calcium channel antagonist, D 888 (desmethoxyverapamil; Fig. 2b); half-maximal hormone release was observed at concentrations of 0.1 nM and 1 μM respectively. In another series of experiments we studied the effect of the toxin on AVP release from isolated nerve endings. These were obtained from rat neural lobes by methods already described (5). Depolarization of the nerve terminals with 100 mM potassium evoked an AVP release of 3.8 ± 0.3 ng (n=4) whereas in the presence of 0.1 μM ω-CgTx there was a 80% inhibition of the evoked AVP release. Thus, although verapamil derivatives (Fig. 2b) and some dihydropyridines can inhibit depolarization-induced release from the neural lobe, ω-CgTx is the most potent inhibitor of secretion so far described. From the data presented in Figs. 1 and 2 it is possible to correlate the degree

of occupancy of the toxin receptors and the extend of hormone release inhibition. One can calculate that, during a 10 min incubation period, the ω-CgTx concentration necessary for half of its membrane receptors to be occupied is 0.2 nM. On the other hand half maximal K^+-evoked AVP release is observed following 10 min exposure of the neural lobes to 0.1 nM ω-CgTx (Fig. 2a). The fact that these two values are very similar suggest strongly that ω-CgTx interacts with the voltage-sensitive calcium channels involved in the mechanism leading to neuropeptide release. However K^+-induced AVP release is never completely abolished by the toxin. Similarly, although we have already presented data on the potent inhibitory effect of nitrendipine and nicardipine on AVP release (5), depolarization-induced secretion could not be totally abolished in the presence of 5 μM nicardipine. Whereas in the presence of 0.3 μM ω-CgTx the K^+ induced AVP release was inhibited by 78±6 % (n=4), in the presence of 5 μM nicardipine the degree of inhibition was 58 ± 6 % (n=9). In order to analyze further the inhibition of AVP release by ω-CgTx and the dihydropyridine, neurosecretory nerve terminals were incubated in the presence of 30 μM nicardipine and hormone release was triggered with 100 mM K^+ for a period of 10 min. The neurohypophyses were then allowed to rest for a period of 30 min in the presence of dihydropyridine molecule. Hormone release was induced a second time with 100 mM potassium but in the presence of nicardipine and 30 nM ω-CgTx. Thus the effect of the toxin in the presence of nicardipine on the evoked hormone release could be calculated by comparing the amount (S_2) released during the second stimulation with that (S_1) observed during the first exposure to elevated potassium concentration. Neural lobes stimulated twice with K^+ in the presence of nicardipine were used as controls. We found that ω-CgTx inhibited by 22.0 + 2.4 % (n=6) the release of AVP induced in the presence of nicardipine.

Electrically-induced AVP release.

Figure 3 shows that ω-CgTx also reduced electrically induced AVP release from the neurohypophysis. The neural lobe was stimulated with a pattern of firing mimicking the electrical activity of magnocellular neurons of the hypothalamus(9). ω-CgTx (1 nM) considerably decreased the electrically-evoked release. Four AVP-like bursts induced the release of 1.74 ± 0.34 ng (n=3) of AVP whereas in the presence of the toxin the amount of hormone secreted was 0.50 ± 0.07 ng (n=3). In another series of experiments we used the S_2/S_1 paradigm (see above) to analyze further the effects of ω-CgTx and nicardipine on the electrically induced AVP release. The neural lobes were first stimulated with four AVP like bursts in the absence of the drug. The neurohypophyses were then let to rest for a period of 30 min. The neural lobes were then stimulated with four AVP like bursts in the presence of nicardipine (5 μM) or ω-CgTx (30 nM) which were added 10 min before the onset of stimulation. In control experiments the neural lobes were stimulated twice in the absence of the calcium channel blockers. Figure 4 summarizes the data obtained for both the electrically and

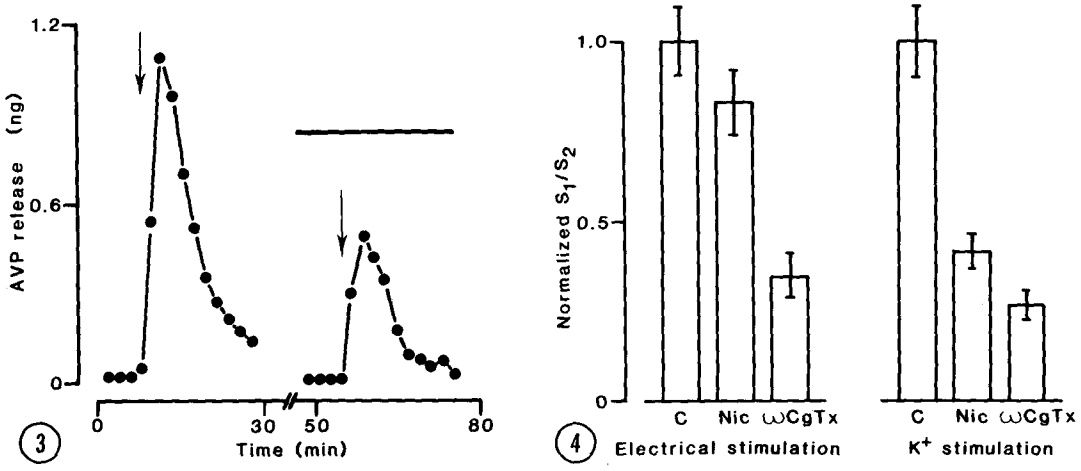

Figure 3. Effects of ω-CgTx on vasopressin release from electrically stimulated rat neural lobe. The neural lobe was stimulated (arrows) with four bursts of pulses mimicking the electrical activity of vasopressin-containing cells during haemorrhage. AVP in the perfusate was measured by radioimmunoassay. ω-CgTx (10 nM) was present, as indicated by the bar, 10 min before, during and after the second stimulus.

Figure 4. Effects of ω-CgTx and nicardipine on the electrically and potassium-induced AVP release. The neural lobes were stimulated twice with four AVP-like bursts (see Materials ands Methods). A period of 30 min was given between the two stimuli and the toxin (30 nM) or nicardipine (5 μM) were added 10 min before the onset of the second stimulation. Similarly isolated neurosecretory nerve terminals were stimulated with 100 mM potassium for two periods of 10 min separated by 30 min and the calcium channel blockers were added 10 min before the onset of the depolarization. The results are given as the ratio between the amount of AVP released during the second stimulation (S_2) and during the first (S_1). They have been normalized in such a way that for control experiments this ratio was 1.0. The results are given as mean ± S.E.M.($16<n<4$).

potassium-induced AVP release. Whereas ω-CgTx greatly inhibited AVP release in both cases, nicardipine was much more potent when hormone release was triggered with an increase of external K^+ concentration then when stimulated with electrical pulses. This may be due to the well-known voltage dependency of dihydropyridine binding, the affinity being higher on depolarized membranes.

Depolarization-induced $(Ca^{2+})_i$ increase.

The effect of the toxin on the depolarization-induced increase in internal $(Ca^{2+})_i$ was measured using the Ca^{2+} indicator fura-2 (10) as described in ref. 11. Under basal conditions the resting Ca^{2+} concentration in control and toxin-treated (1 nM) terminals was 422 ± 90 nM (n=7) and 504 ± 117 nM (n=7), respectively. Depolarization of the nerve terminals with 50 mM potassium gave rise to an increase of 341 ± 4 nM (n=7) whereas in the presence of the toxin the change of internal Ca^{2+} was 209 ± 28 nM (n=7). Using a higher toxin concentration (10 nM) the K^+- induced $(Ca^{2+})_i$ was inhibited by 50 ± 8% (n=7).Thus ω-CgTx inhibits but does not abolish totally the rise in free internal Ca^{2+}concentration induced by the activation of the voltage dependent calcium channels.

To see if the toxin could also act at a step following the increase in ionized cytoplasmic Ca^{2+} concentration induced by the activation of the voltage-dependent calcium channels, it was added before and during a period when internal calcium was artificially increased without depolarizing the membrane. This involves making the nerve terminals permeable, with digitonin, in a medium containing micromolar concentrations of calcium (12). ω-CgTx (1 nM) did not modify the total evoked AVP release. In controls, AVP levels were 1.5 $\pm$ 0.3 ng (n=4) whereas in the presence of the toxin the amount secreted was 1.3 $\pm$ 0.1 ng (n=4). These data show that the toxin does not act at a step following the opening of calcium channels.

DISCUSSION

The present study shows that ω-CgTx is an extremely potent inhibitor of depolarization-induced secretion from the neurohypophysis. Electrophysiological studies on other excitable tissues have shown that the toxin acts on the N- and L-types of calcium channels as defined in ref. 13. Furthermore it has been found in chick sensory neurons that the L-type calcium channel is sensitive to dihydropyridines whereas the N and T types are not (3,13). Because AVP release from the nerve endings is highly sensitive to dihydropyridines (5) and can be greatly inhibited by ω-CgTx, we can reasonably assume that in the neural lobe activation of the L-type calcium channels is mainly responsible for inducing an increase of $(Ca^{2+})_i$ and subsequent secretion. This conclusion is supported by the recent findings showing that in the isolated neurohypophysial nerve endings a predominant Ca-inward current corresponds to L-type channels and is sensitive to dihydropyridines (14). In contrast with our findings, the $^{45}Ca^{2+}$ uptake (15) and the release of the noradrenalin and serotonin from brain synaptosomes are only slightly inhibited by ω-CgTx at concentrations higher than those used in the present study (4). Furthermore, in contrast to what has been observed in the neural lobe, calcium influx and transmitter release from synaptosomes are insensitive to dihydropyridines (4, 16, 17) (but see ref. 18). However it is interesting to note that in a paper published very recently it was shown that the release of (^{3}H)-labeled noradrenalin from superior cervical ganglion neurons in culture was inhibited by concentrations of ω-CgTx relatively similar to those used in the present study (19). Thus, the sensitivity to ω-CgTx varies among different preparations used. Our results suggest that neuropeptide release from the neurohypophysis can be modulated by the activation of both N and L-type channels whereas the results obtained from brain synaptosomes suggest that neurotransmitter release is mostly triggered by the opening of the N-type calcium channels.

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