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Omega *Conus geographus* toxin: a peptide that blocks calcium channels

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We previously reported that omega *Conus geographus* toxin (ωCgTX), blocks evoked-release of transmitter at synapses in frog and attenuates the Ca^{2+} component of the action potential of chick dorsal root ganglion neurons. We report here voltage-clamp experiments on cultured chick dorsal root ganglion neurons which demonstrate that ωCgTX produces a persistent block of voltage-gated Ca^{2+} currents. Thus, we conclude that ωCgTX inhibits synaptic transmission by blocking Ca^{2+} channels in the presynaptic nerve terminal. The toxin had no effect on K^+ currents; however, in some but not all neurons, ωCgTX reduced Na^+ currents by 10–25%. These findings suggest that ωCgTX should be useful as a probe to examine synaptic Ca^{2+} channels.

Voltage-clamp; Neurotoxin; Ca^{2+} channel; *(Conus geographus)*

1. INTRODUCTION

An obligatory step in chemical synaptic transmission is the influx of Ca^{2+} into the presynaptic terminal through voltage-gated Ca^{2+} channels [1,2]. Our previous experiments [3,4] suggested that these Ca^{2+} channels may be blocked by ωCgTX, a 27 amino acid neurotoxic peptide from the venom of the marine snail *Conus geographus* [5], apparently by blocking the influx of Ca^{2+} into the presynaptic nerve terminal. This effect of ωCgTX could result directly from a reduction of Ca^{2+} conductance (e.g. [6]), or indirectly from an increase in K^+ conductance (e.g. [7]). To distinguish between these possibilities we examined membrane currents of dissociated neurons from chick dorsal root ganglia (DRG) directly with the whole cell voltage-clamp technique [8]. Some of these findings have been reported in preliminary form [9].

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Abbreviations: DRG, dorsal root ganglion; ωCgTX, omega *Conus geographus* toxin; TEA, tetraethylammonium; TTX, tetrodotoxin

2. MATERIALS AND METHODS

2.1. *Cell culture*

Dissociated cell cultures were prepared from DRG of 9–11-day-old chick embryos by a modification of the procedure of Okun [10]. Cells were grown on collagen-coated glass cover slips in Leibowitz's L-15 medium supplemented with 10% fetal calf serum and 10–25 ng/ml nerve growth factor, and incubated at 36°C in air. Cells were treated with 5 μM cytosine arabinoside to kill dividing non-neuronal cells [11].

2.2. *Physiological recording*

Cells (2–14 days in culture) were placed in a 0.2 ml bath perfused (1 ml/min) at room temperature with either 'Ca-Ringer's', or 'Co-Ringer's', as specified below. Whole-cell voltage-clamp recordings [8] were tape-recorded (low-pass

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filtered at 2500 Hz, −3 dB) for subsequent digitization and computer analysis. Currents illustrated in the figures are raw traces (leakage and holding currents not subtracted), unless otherwise noted, with capacitative transients blanked out.

Ca^{2+} currents were recorded with pipets containing (in mM): 120 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 11 Na-EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 2 Mg-ATP, 10 Na-Hepes (pH 7.2), while the cells were bathed in Ca-Ringer's consisting of (in mM): 135 NaCl, 10 $CaCl_2$, 3 KCl, 1 $MgCl_2$, 10 Na-Hepes (pH 7.3), 2 TEA-Cl, 1 μg/ml tetrodotoxin (TTX, Sigma) and 1.1 g/l glucose. Potassium currents were recorded with pipets containing: 140 KCl, 11 K-EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 K-Hepes (pH 7.2) and a bathing solution of Co-Ringer's, which was similar to Ca-Ringer's except that 10 mM $CoCl_2$ replaced $CaCl_2$, and TEA-Cl was absent. Na^+ currents were recorded with pipets containing (in mM): 95 CsCl, 5 NaCl, 10 TEA-EGTA, 3 $MgCl_2$, 2 Mg-ATP, 40 TEA-Hepes (pH 7.3) and a bathing solution like Co-Ringer's, except that 2 mM TEA was present, and TTX was absent. Current-clamp recordings of action potentials were made with conventional intracellular electrodes filled with 3 M KCl and having resistances of ~50 MΩ.

2.3. *Puffer application of toxin and specific ions*

The ωCgTX used throughout these experiments was of the subtype GVIA [5,12]. Solutions containing ωCgTX or specific ions were applied by pressure ejection from extracellular 'puffer' pipets [13] with 3–5 μm tip diameters. These pipets were filled with ωCgTX dissolved in a solution identical with that in the bath but with 0.1% cytochrome *c* added to minimize non-specific binding of the toxin, particularly to the pipet glass. Air in the pipet above the toxin solution was displaced with oil to minimize capillary flow of bath solution into the pipet tip. Toxin was administered by application of constant pressure (1–2 lb/inch^2, maintained for tens of seconds to several minutes) to the pipet, whose tip was positioned ~20 μm from the cell body of the target neuron just before puffing. Control experiments showed that puffer application of carrier solution containing cytochrome *c* alone, without toxin, had no effect on holding, leakage, or any of the ionic currents examined in these experiments.

The ionic environment immediately surrounding the clamped cell could be momentarily changed by ejection of an alternate Ringer's solution from a second puffer pipet placed close to the cell. Thus, a cell could be 'puffer-perfused' with Co-Ringer's to reversibly block its Ca^{2+} channels (e.g. fig.1). Likewise, when K^+ or Na^+ currents were being recorded in Co-Ringer's, the neuron could be puffer-perfused with Ca-Ringer's (lacking TEA or TTX, as appropriate) to reveal Ca^{2+} currents, whose disappearance upon ωCgTX treatment confirmed that ωCgTX had been properly delivered when the Na^+ or K^+ current was unaffected (e.g. figs 3,4A).

3. RESULTS

3.1. *Ca^{2+} currents*

Fig.1 shows the effects of ωCgTX on Ca currents. Depolarization to 10 mV from a holding potential of −90 mV elicited inward Ca^{2+} currents (trace a) similar to those described by others for chick DRG neurons [14,15]. As shown in trace b, these currents were completely blocked by Co^{2+} applied from a puffer pipet (see section 2). The block by Co^{2+} was reversible (not shown). Puffer application of 5 μM ωCgTX rapidly blocked >90% of the inward Ca^{2+} currents (trace c). The kinetics of toxin block is illustrated in fig.2A, where the peak Ca^{2+} current is plotted on a

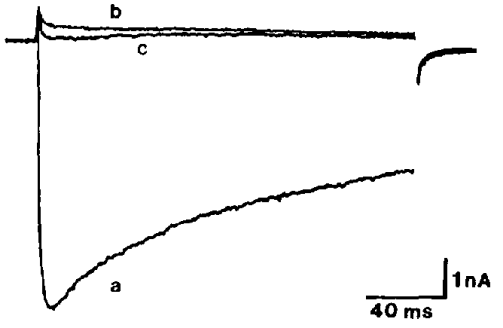

Fig.1. ωCgTX blocks Ca^{2+} currents. Three superimposed current traces are shown in response to a potential step to 10 mV from a holding potential of −90 mV. (a) Ca^{2+} current recorded in the presence of Ca-Ringer's. (b) Response while cell was exposed to Co^{2+} (as described in section 2, with 2 mM TEA added). (c) Response in Ca-Ringer's after exposure to ωCgTX.

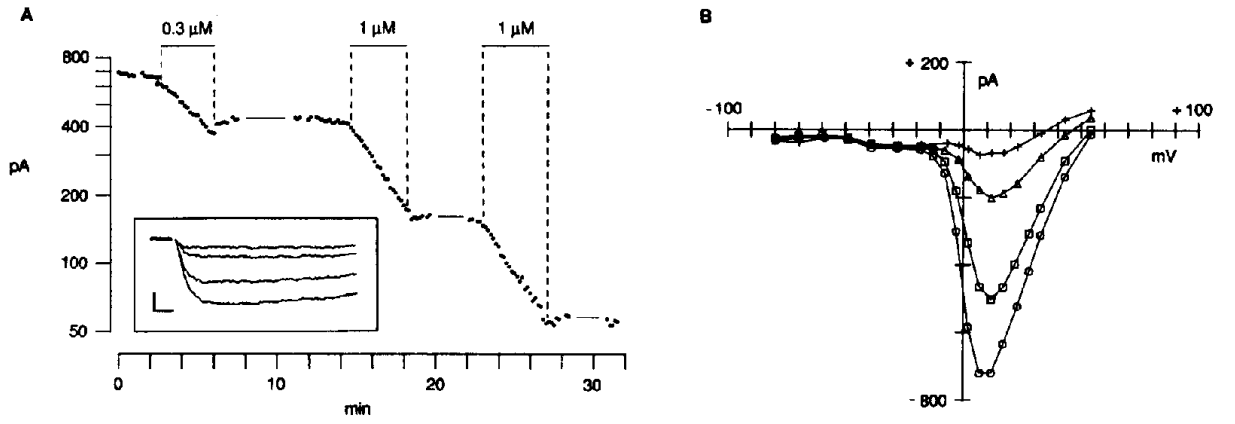

Fig.2. (A) Kinetics of Ca current inhibition by ωCgTX. A cell was voltage-clamped as in fig.1, and the membrane potential was stepped to 7 mV from a holding potential of −80 mV every 10 s. The toxin-sensitive component of each response was obtained by subtracting out toxin-insensitive currents. The peaks of the toxin-sensitive Ca^{2+} currents are plotted on a log scale as a function of time. Three puffs of ωCgTX were applied during the times indicated by the bars. The first puff was from a pipet containing 0.3 μM toxin, and the succeeding two were from a pipet with 1 μM toxin. The small (40 pA) reversible displacement in current that occurred during the first puff is presumably a puffer artifact (cf. [6]), and was ignored. Data points are absent over time intervals during which current vs voltage (*I-V*) curves (see fig.2B) were obtained. Inset: Ca^{2+} current traces (with leakage currents subtracted), before toxin and after each puff of toxin. Calibration bars: 200 pA, 5 ms. (B) *I-V* curves obtained during the experiment illustrated in A, before (circles) and after, three successive toxin applications (squares, triangles, +, respectively).

logarithmic scale as a function of time. Three steady puffs (3–4 min each) of toxin were applied to the cell. The first puff, from a pipet containing 0.3 μM toxin, was followed by two puffs from another pipet containing 1 μM toxin. Although Ca^{2+} current decreased during each puff of toxin, the time course of the toxin-attenuated responses appear unaltered (see inset), and when the traces are normalized (not shown), they are essentially superimposable. In addition, Ca^{2+} currents remained essentially constant between puffs, suggesting the irreversible reaction:

$$\omega\text{CgTX} + \text{channel(active)} \xrightarrow{k} \text{channel(blocked)},$$

where k is the rate constant of block. During a steady puff of toxin, the target cell should be subjected to a constant [ωCgTX] approximately that contained in the pipet (cf. [6,13]). Under these conditions, the block by toxin should obey first-order kinetics, i.e. the log of the response decays linearly as a function of time with the magnitude of the slope given by the pseudo first-order rate constant, k'. The results presented in fig.2A are consistent with this expectation. With 0.3 and 1 μM ωCgTX, k' was 0.0026 and 0.004 s^{-1}, respectively. In an experiment on another cell with 5 μM ωCgTX (not shown), k' was 0.0299 s^{-1}. The second-order rate constants, k, obtained upon dividing the aforementioned k' values by the [ωCgTX] used are (in $M^{-1} \cdot s^{-1}$) 8700, 4000 and 6000, respectively. Some variability in the k obtained with different pipets is anticipated since the actual concentration of toxin reaching the cell will vary depending on the pipet's tip diameter and puff pressure, as well as the distance between the pipet and target. Taken together, these results indicate that the rate of block is proportional to the applied [ωCgTX] with k likely to be within a factor of two of 6000 $M^{-1} \cdot s^{-1}$.

In some cells, such as that in fig.1, a small fraction of cobalt-sensitive inward current remained even after prolonged application of toxin. This residual current is presumably due in part to a minor, transient component of Ca^{2+} current that activates near −50 mV [14,15], which has been shown in recent experiments [16] to be relatively insensitive to ωCgTX. Fig.2B shows *I-V* curves during the progressive block of Ca^{2+} current by

ωCgTX in the experiment of fig.2A. It is evident that the Ca^{2+} current which activates near -50 mV was insensitive to ωCgTX, whereas that with a higher activation threshold (>20 mV) was progressively blocked by the toxin without obvious alteration in its voltage sensitivity.

After the toxin was washed out, the block of Ca^{2+} currents by ωCgTX persisted for at least as long as the currents were monitored (many minutes). To examine the duration of block of Ca^{2+} currents in DRG neurons over a longer period, we used conventional intracellular current-clamp recording to sample the action potentials of a number of neurons which were bathed in Ca-Ringer's (without TTX, but with 2 mM TEA). Under these conditions, depolarizing current pulses elicited action potentials with pronounced Ca^{2+} plateaus (cf. Fig.4 in [4]) in nearly all neurons examined (13 out of 15 cells tested). In contrast, such plateaus were not detectable in any neurons (10 out of 10 cells) within 30 min after the cultures were exposed to 100 nM ωCgTX. Furthermore, no recovery of the Ca^{2+} plateaus was evident after washing at room temperature with toxin-free solution for 2 h. However, after overnight incubation at 36°C in fresh culture medium, most neurons once again had detectable Ca^{2+} plateaus (4 out of 5 cells). It remains to be established whether this recovery results, for example, from the dissociation of ωCgTX from its receptor or from insertion of new Ca channels into the neuronal membrane.

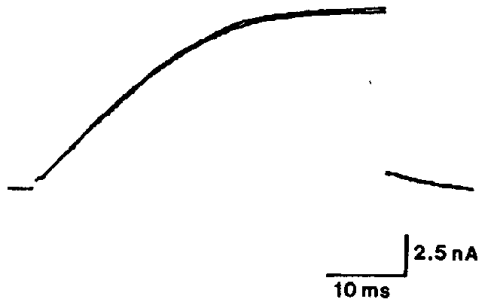

Fig.3. ωCgTX has no effects on K^+ currents. Potassium currents before and after ~30 s application of 50 μM ωCgTX are superimposed. Membrane potential was stepped to 0 mV from a holding potential of -80 mV.

3.2. *K^+ currents*

Voltage-gated K^+ currents were recorded under conditions where Ca^{2+} and Na^+ channels were blocked. Outward K^+ currents were not altered by 50 μM ωCgTX (fig.3), indicating that ωCgTX does not affect voltage-gated K^+ channels in these neurons. Effective delivery of ωCgTX onto this

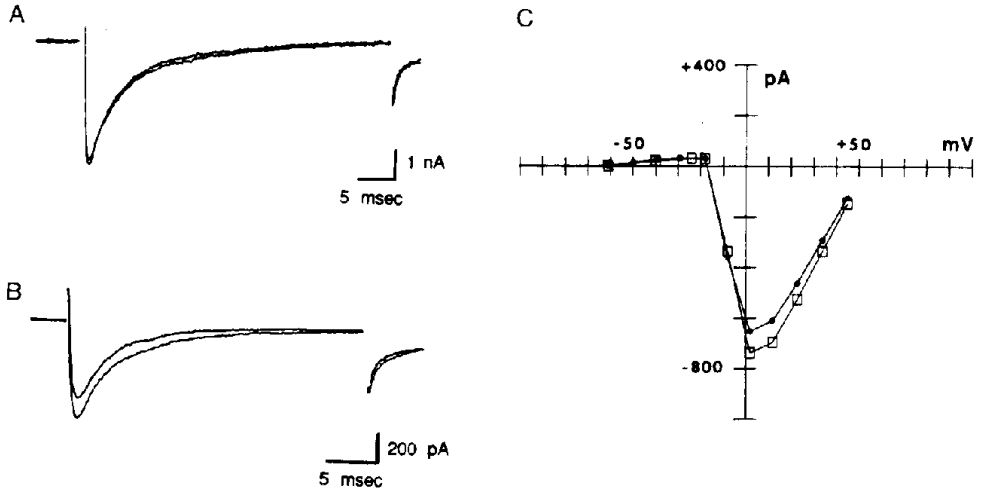

Fig.4. ωCgTX has variable effects on Na^+ currents. (A) Example of Na^+ currents of a cell in which a ~75 s application of 10 μM ωCgTX produced no effects (traces before and after toxin are superimposed). Potential stepped to -9 mV from holding potential of -60 mV. (B) Example from a cell where Na^+ currents were reduced about 20% by a ~90 s application of 5 μM toxin. Potential stepped to 8 mV from a holding potential of -60 mV. (C) *I-V* curves before (open squares) and after (filled circles) exposure to ωCgTX, from another cell whose Na^+ current was reduced by toxin.

cell was confirmed by examination of Ca^{2+} currents during puffer perfusion of Ca-Ringer's, as described in section 2.

In addition to voltage-gated K^+ currents, chick DRG neurons also have Ca^{2+}-activated K^+ currents (see [17]). In our experiments in Co-Ringer's, these currents, which could be revealed during puffer perfusion of Ca^{2+}, were blocked by ωCgTX (not shown). The toxin's effect was presumably mediated by its block of Ca^{2+} channels, and hence Ca^{2+} entry; however, our recording methods precluded direct examination of Ca^{2+}-activated K^+ currents.

3.3. *Na^+ currents*

Previous studies [4] indicated that ωCgTX has little, if any, effect on sodium action potentials. Nevertheless, to scrutinize the specificity of ωCgTX more closely, we examined its possible action on Na^+ currents in DRG neurons, and found that it partially inhibited this current in six out of eight cells. In the remaining two cells, ωCgTX produced no obvious change in the Na^+ current (see fig.4A) even though it was demonstrated (by puffer perfusion of Ca-Ringer's) that ωCgTX had blocked the Ca^{2+} current in the same cells. In the neurons that were affected, toxin produced a 10–25% reduction of Na^+ current, while leakage and holding currents remained unchanged (fig.4B,C). Fig.4C also shows that ωCgTX reduced the magnitude of the Na^+ current without appreciably altering its voltage sensitivity. The attenuated Na^+ current remained unchanged for several minutes after the toxin was applied; this suggests that the effect of ωCgTX on Na^+ currents is not readily reversible.

4. DISCUSSION

Previous experiments on synapses in frogs [3,4] showed that ωCgTX produced a long-lasting block of evoked-release of transmitter at a variety of synapses in the frog by reducing the quantal content of transmitter release; this suggested that ωCgTX interferes with Ca^{2+} influx into the presynaptic terminal. The present results with chick DRG neurons provide a direct demonstration that ωCgTX blocks Ca^{2+} channels, and that its effect, like that at synapses, is long-lasting. Hence, we conclude that the toxin blocks synaptic transmission by blocking presynaptic Ca^{2+} channels that are essential for the evoked-release of transmitter. ωCgTX thus differs from the well-known organic Ca^{2+} channel blockers verapamil and the dihydropyridines, which do not appear to affect synaptic transmission ([18–20, but see [21]).

In contrast to its dramatic inhibition of Ca^{2+} currents, ωCgTX had no effect on voltage-sensitive K^+ currents and little or no effects on Na^+ currents. These results are consistent with previous observations that the toxin causes no apparent changes in Na^+ action potentials recorded from chick DRG neurons in intact ganglia, and from various nerves in the frog [3,4]. However, modest effects of ωCgTX on Na^+ channels, such as those observed in the present study, could have been overlooked in studies examining Na^+ action potentials instead of Na^+ currents.

ωCgTX could affect Ca^{2+} channels by binding directly to them, or by binding to a receptor and initiating events that inactivate these channels (cf. [6,22]). Patch-clamping experiments indicate that ωCgTX's effect on Ca^{2+} channels is probably not mediated by a soluble second messenger or cofactor other than the ATP supplied by the recording pipet [16]. Thus, it is likely that toxin-binding sites and Ca^{2+} channels are, if not the same molecular entity, physically close to each other. The block of synaptic transmission and inhibition of Ca^{2+} channels by ωCgTX are long-lasting, and a recent study [23] has shown that the specific binding of radioiodinated ωCgTX to membrane fragments isolated from chick brain is not readily reversible. All of these observations are consistent with the idea that ωCgTX binds tightly and directly to Ca^{2+} channels. Thus, ωCgTX should be useful in the biochemical purification of synaptic Ca^{2+} channels and as a probe for studies of the distribution, function, and metabolism of these channels.

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