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TWO TYPES OF HIGH-THRESHOLD CALCIUM CURRENTS INHIBITED BY ω-CONOTOXIN IN NERVE TERMINALS OF RAT NEUROHYPOPHYSIS

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SUMMARY

1. The neurohypophysis comprises the nerve terminals of hypothalamic neurosecretory cells, which contain arginine vasopressin (AVP) and oxytocin. The secretory terminals of rat neurohypophyses were acutely dissociated. The macroscopic calcium currents (I_{Ca}) of these isolated peptidergic terminals were studied using 'whole-cell' patch-clamp recording techniques.

2. There are two types ('N_t' (where the subscript 't' denotes terminal) and 'L') of high-threshold voltage-activated I_{Ca} in the terminals, which can be distinguished by holding at different potentials i.e. −90 and −50 mV. Replacement of Ca^{2+} in the bathing solution by Ba^{2+} increased the amplitude of I_{Ca}, primarily due to an increase in the L-type component. Both inward currents were eliminated by adding 50 μM-Cd^{2+} or when in a Ca^{2+}-free bathing solution.

3. ω-Conotoxin GVIA (ω-CgTx) has been widely used as a Ca^{2+} channel blocker. However, whether this toxin can discriminate between different types of Ca^{2+} channels is still a subject of controversy. We applied ω-CgTx over a wide range of concentrations (0·01–2 μM) to examine its effects on both N_t- and L-type I_{Ca} in these terminals. At a concentration of 30 nM, ω-CgTx selectively reduced, by 48%, the amplitude of N_t-type I_{Ca}. In contrast, a higher concentration (300 nM) of ω-CgTx was necessary to inhibit the L-type I_{Ca}.

4. ω-CgTx inhibited both N_t- and L-type I_{Ca} in a dose-dependent manner, and the half-maximum inhibition (IC_{50}) of the I_{Ca} by the toxin was 50 and 513 nM, respectively, which was approximately a tenfold difference. The reduction in both types of currents did not result from any shift in their current–voltage or steady-state inactivation relationships.

5. In contrast, ω-CgTx, at a concentration of 300 nM, had no effect on the tetrodotoxin-sensitive sodium current (I_{Na}) of the isolated peptidergic nerve terminals. Furthermore, ω-CgTx did not reduce the long-lasting, non-inactivating I_{Ca} in the isolated non-neuronal secretory cells of the pars intermedia (PI) (intermediate lobe of the pituitary).

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6. Our studies suggest that ω-CgTx might exert specific blocking effects on both N_t- and L-type Ca^{2+} channels, but that in the isolated peptidergic nerve terminals, the N_t-type component is more susceptible to this toxin.

INTRODUCTION

It is known that there are at least two types of high-threshold voltage-activated Ca^{2+} channels in many neuronal tissues. A dihydropyridine-insensitive Ca^{2+} channel characterized by short-duration openings has been named the 'N'-type, while a dihydropyridine-sensitive Ca^{2+} channel with long-duration openings has been named the 'L'-type (Nowycky, Fox & Tsien, 1985; Tsien, Lipscombe, Madison, Bley & Fox, 1988). Either or both types of Ca^{2+} channels may be involved in the process of neurotransmitter or neuropeptide release (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988; Holz, Dunlap & Kream, 1988; Lemos & Nowycky, 1989). ω-Conotoxin GVIA (ω-CgTx), a twenty-seven amino acid-long peptide from the venom of the marine snail *Conus geographus*, has been shown to block the activity of voltage-activated Ca^{2+} channels and subsequent neurotransmitter release in neuronal tissues (Kerr & Yoshikami, 1984; Olivera, McIntosh, Cruz, Luque & Gray, 1984; Reynolds, Wagner, Snyder, Thayer, Olivera & Miller, 1986; Olivera, Rivier, Clark, Ramilo, Corpuz, Abogadie, Mena, Woodward, Hillyard & Cruz, 1990). Most previous electrophysiological, binding, or release studies have demonstrated that ω-CgTx can block the N-type Ca^{2+} channels. Experiments have been performed on a number of preparations, such as chick dorsal root ganglion neurons (Kasai, Aosaki & Fukuda, 1987; McCleskey, Fox, Feldman, Cruz, Olivera, Tsien & Yoshikami, 1987), presynaptic calyces of ciliary ganglia of chick embryo (Yawo, 1990), rat phaeochromocytoma (PC12) cells (Plummer, Logothetis & Hess, 1989), and slices of rat hippocampus (Rijnhout, Hill & Middlemiss, 1990). In contrast, the results produced by ω-CgTx on the L-type Ca^{2+} channels have been rather inconclusive. The L-type Ca^{2+} channels have been shown to be insensitive to ω-CgTx in many preparations, such as fetal chick brain synaptosomes (Cruz & Olivera, 1986), mouse motor nerve terminals (Anderson & Harvey, 1987), PC12 cells (Sher, Pandiella & Clementi, 1988; Plummer *et al.* 1989), human neuroblastoma cell lines (Sher *et al.* 1988), rat hippocampal neurons (Toselli & Taglietti, 1990) or slices (Rijnhout *et al.* 1990) and cultured fetal rat brain neurons (Martin-Moutot, Seager & Couraud, 1990). On the other hand, it has been shown that both L- and N-type Ca^{2+} channels were persistently blocked by 10 μM-ω-CgTx in chick dorsal root ganglion neurons (McCleskey *et al.* 1987; Tsien *et al.* 1988; Yawo, 1990), frog dorsal root ganglion neurons (Oyama, Tsuda, Sakakibara & Akaike, 1987) and rat sympathetic neurons (Hirning *et al.* 1988). Both 'sustained' and 'transient' components of the Ca^{2+} current were also partially blocked by 1 μM-ω-CgTx in rat dorsal root ganglion neurons (Schroeder, Fischbach, Mamo & McCleskey, 1990), or by 10 μM-ω-CgTx in a rat dorsal root ganglion cell line (Boland & Dingledine, 1990). The L-type was only weakly or transiently blocked in chick sensory neurons (Kasai *et al.* 1987; Aosaki & Kasai, 1989). The discrepancy in the results with ω-CgTx on Ca^{2+} channels, particularly the L-type, may depend on many important factors, including doses used, heterogeneity of cell types, and means of toxin delivery. Therefore, one cannot exclude possible effects of ω-CgTx on the L-type Ca^{2+} channels and should question

the original claim that ω-CgTx binds only to a subset of neuronal Ca^{2+} channels, i.e. N-type (Cruz & Olivera, 1986; Reynolds *et al.* 1986; Olivera *et al.* 1990). In fact, most electrophysiological studies have tested only a single dose of ω-CgTx. Thus, more detailed studies, applying a range of doses of ω-CgTx, seem necessary in order to better define the role of this toxin in blocking these two populations of Ca^{2+} channels in neuronal tissue, and subsequently inhibiting neurotransmitter or neurohormone release. In particular, it is important to understand the effects of this toxin at the sites of such release, the nerve terminals. Moreover, successful electrophysiological isolation of different channel populations will allow the determination of the target channel for drugs such as ethanol, which exerts powerful effects on I_{Ca} in isolated nerve terminals (Wang, Lemos, Dayanithi, Nordmann & Treistman, 1991*a*).

A physiologically competent preparation of isolated rat peptidergic nerve terminals, dissociated from the neurohypophysis and free from any contamination by postsynaptic or other neuronal structures, has been developed. Release of the peptide hormones arginine vasopressin (AVP) and oxytocin from these secretory terminals can be measured by radiommunoassay (for review see Nordmann, Dayanithi & Lemos, 1987). Recently, the binding of ω-CgTx to these nerve terminals and its effects on high potassium and electrically induced release of AVP have been examined (Dayanithi, Martin-Moutot, Barlier, Colin, Kretz-Zaepfel, Couraud & Nordmann, 1988). There is a high affinity for binding of ω-CgTx in these terminals, and AVP release can be greatly inhibited, up to 80%, by this toxin.

Single-ion channel activity from the terminals of rat neurohypophyses has been studied using patch-clamp techniques (Lemos & Nordmann, 1986; Mason & Dyball, 1986). Recently, two types of high-threshold voltage-activated Ca^{2+} channels have been identified in the isolated terminals dissociated from the rat neurohypophysis (Lemos & Nowycky, 1989). One corresponds to the L-type and the other more close to the N-type, but the terminal N-type Ca^{2+} channel differs quantitatively (see Lemos & Nowycky, 1989) from that found in cell bodies of other nerve tissues, such as chick dorsal root ganglion neurons (Nowycky *et al.* 1985; Tsien *et al.* 1988). Thus, we have called it the 'N_t'-type ('t' for terminals). In the present study, using the 'whole-cell' patch-clamp recording method, we have examined the effects of ω-CgTx at different concentrations on both N_t- and L-type macroscopic calcium currents (I_{Ca}) in these nerve terminals. In addition, the effects of this toxin on sodium currents (I_{Na}) in the nerve terminals and on I_{Ca} in the non-neuronal secretory cells of the pars intermedia (PI) (intermediate lobe of the pituitary) were examined.

METHODS

Tissue preparation

Male CD rats weighing 155–195 g (Charles River, Boston, MA, USA) were anaesthetized with CO_2 and then decapitated. The brain and pituitary were quickly separated, and the pituitary was placed in a dish filled with 4–5 ml of low-Ca^{2+} Locke solution (in mM: NaCl, 145; HEPES, 10; glucose, 10; KCl, 5; EGTA, 2; $CaCl_2$, 1·9; $MgCl_2$, 1; pH 7·2). The intact posterior lobe was dissected free from the intermediate and anterior lobes of the pituitary. The nerve terminals, which are the axon terminals of hypothalamic neurosecretory cells, were dissociated by homogenizing the lobe in 200 μl of 270 mM-sucrose, 10 μM-EGTA and 10 mM-HEPES (pH 6·8), as previously described (Nordmann *et al.* 1987; Lemos & Nowycky, 1989; Thorn, Wang & Lemos, 1991). The terminals were attached to the bottom of a dish coated with poly-L-lysine hydrobromide (molecular weight 15000–30000; Sigma, St Louis, MO, USA) for 10–15 min, then perfused with low-Ca^{2+}

Locke solution (see above). The dish was mounted on the movable stage of a Nikon Diaphot inverted microscope. The poly-L-lysine-coated (1 mg ml^{-1} distilled water) dish was prepared 24 h before experiments.

Dot immunobinding assay

Recently, we have developed a novel technique (Wang, Treistman & Lemos, 1990, 1991*b*, *c*), which allows identification of the peptide contents of individual isolated nerve terminals after 'whole-cell' recordings. Briefly, the whole terminal was aspirated into the recording electrode after

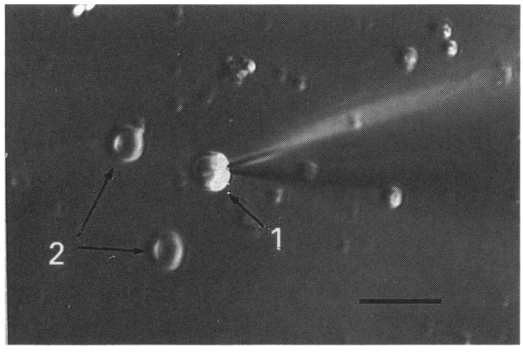

Fig. 1. The appearance of the neurohypophysial preparation during an experiment. An isolated nerve terminal viewed under light microscopy with Hoffman modulation contrast. Arrow 1, a terminal sealed to a recording electrode. Arrow 2, red blood cells. Scale bar = 10 μm.

collecting electrophysiological data. Later, the contents were spotted onto a nitrocellulose membrane (pore size = 0·1 μm, CMS, Houston, TX, USA) for the dot immunobinding (also called immunoblotting) assay (Hawkes, Niday & Gordon, 1982; Scopsi & Larsson, 1986). Vectastain Elite-ABC kits (Vector Laboratories, Burlingame, CA, USA) were used for the assay. The spotted membrane was immersed in Tween Tris-buffered saline (TTBS) (0·1 % (v/v) Tween, 100 mM-Tris, 0·9 % NaCl, pH adjusted to 7·5 with HCl) for 30 min, and then in diluted normal blocking serum (150 μl (10 ml)$^{-1}$ TTBS) for 20 min. The membrane was incubated in a solution of the primary antibodies, i.e. either AVP antibody (at final dilution of 1 : 10000) or oxytocin antibody (at 1 : 1000) for 18–24 h. Then the membrane was transferred to solutions of biotinylated secondary antibodies and of the ABC reagent for 30 min each. Finally, the membrane was placed in the peroxidase substrate (3,3′-diaminobenzidine plus nickel chloride) solution, which produced a black reaction product (black dot). The staining sensitivity of the assay can be 5–10 pg spots of target antigen (Scopsi & Larsson, 1986; Wang *et al.* 1991*b*).

'Whole-cell' recordings

Standard 'whole-cell' patch-clamp recording techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were used. Recordings were made with an EPC-7 patch-clamp amplifier (List-Electronic, Germany) at room temperature (20–25 °C). 'Whole-cell' current records were filtered at 3 kHz, sampled every 0·3 ms, and analysed using pCLAMP software (Axon Instruments Inc., Burlingame, CA, USA). The interpulse interval was 4 s. Glass capillaries (Drummond, Scientific Co., Broomall, PA, USA) were double pulled (Kopf 700C, Tujunga, CA, USA). Pipette tips were coated with Sylgard (Dow Corning Co., Midland, MI, USA) and fire-polished on a microforge (Narashige, Kyoto, Japan). The pipette resistances ranged from 8 to 10 MΩ. The pipette was filled with internal medium containing (in mM): *n*-methyl-D-glucamine chloride, 130; HEPES, 40; EGTA, 10; Mg-ATP, 2; cyclic AMP, 0·2; pH 7·2. 'Whole-cell' configurations with seal resistances > 5 GΩ were produced by gentle mouth suction from the back of the electrode, and then, after

compensating the electrode capacitance, the sealed membrane patch was ruptured by additional gentle suction. After an initial 20 min perfusion (50 ml) with low-Ca^{2+} Locke solution (see above), the dissociated terminals were incubated in a 10 mM-Ca^{2+} bath solution (in mM: tetraethylammonium chloride (TEA-Cl), 95; NaCl, 25; glucose, 19; $CaCl_2$, 10; HEPES, 10; $MgCl_2$, 1; pH 7·3). In some experiments, $CaCl_2$ was replaced by an equal concentration of $BaCl_2$. Current traces were leak current and capacity transient subtracted. All the experiments were performed on isolated nerve terminals which were 6–8 μm in diameter (see Fig. 1).

Drugs

ω-Conotoxin GVIA, a twenty-seven amino acid long peptide, was purchased from Peptide Institute of Protein Research Foundation, Osaka, Japan. The toxin was dissolved in the 10 mM-Ca^{2+} bathing solution (see above) together with bovine serum albumin (BSA, 1 mg ml^{-1}). Without BSA, ω-CgTx was far less effective at all concentrations tested. Concentrations of 0·01, 0·03, 0·1, 0·3 and 2 μm-ω-CgTx were locally perfused through a pipette (1 mm inner diameter, World Precision Instruments, Inc., New Haven, CT, USA). Firstly, a seal between the electrode and a terminal was formed. Secondly, the sealed terminal was lifted from the bottom of the dish and moved close to the perfusion pipette containing ω-CgTx. Thirdly, the terminal was positioned just inside the opening of the perfusion pipette for 2 min, and then the terminal membrane was ruptured and recording commenced. Sucrose, $BaCl_2$ and $CdCl_2$ were obtained from Mallinckrodt, and TEA chloride, EGTA, ATP and cyclic AMP from Sigma.

Statistics

Most data are expressed as the means and standard error of the means (S.E.M.) (n = number of experiments). The significance of data changes was determined using Student's t test or a two-tailed t test. Curve fits to data were accomplished using Sigma Plot Scientific Graphing Software (Jandel Scientific, Corte Madera, CA, USA). The half-maximum inhibition (IC_{50}) of I_{Ca} by ω-CgTx was calculated by the following equation,

$$\frac{I}{I_{max}} = 1 - \frac{x}{IC_{50} + x},$$

where I is the current amplitude in the presence of toxin, I_{max} is the maximum current in the absence of toxin, x is the test concentration of toxin and IC_{50} is the concentration of toxin that blocks 50% of the control current. The IC_{50} for N_t-type current was obtained by subtracting the current at the end (140 ms) of the pulse from the peak current.

RESULTS

Terminal identification

Individual dissociated nerve terminals can be identified as such using a combination of phase and interference (Hoffman modulation) optics (Modulation Optics Inc., Greenvale, NY, USA). The terminals showed no visible nuclei while other cells contained nuclei (such as the PI cells and pituicytes) or other distinctive characteristics, such as the red blood cell's 'biconcave' feature (Fig. 1). That they are terminals can be confirmed by immunocytochemistry of vasopressin, oxytocin, or neurophysins (Nordmann *et al.* 1987), and the isolated terminals attached to the bottom of the dish have been identified by immunofluorescence staining for vasopressin or oxytocin (P. J. Thorn and J. R. Lemos, unpublished results). Furthermore, after each 'whole-cell' patch-clamp recording, the peptide contents (AVP or oxytocin) of individual terminals can be identified using the dot immunobinding assay (Wang *et al.* 1990, 1991*b*, *c*).

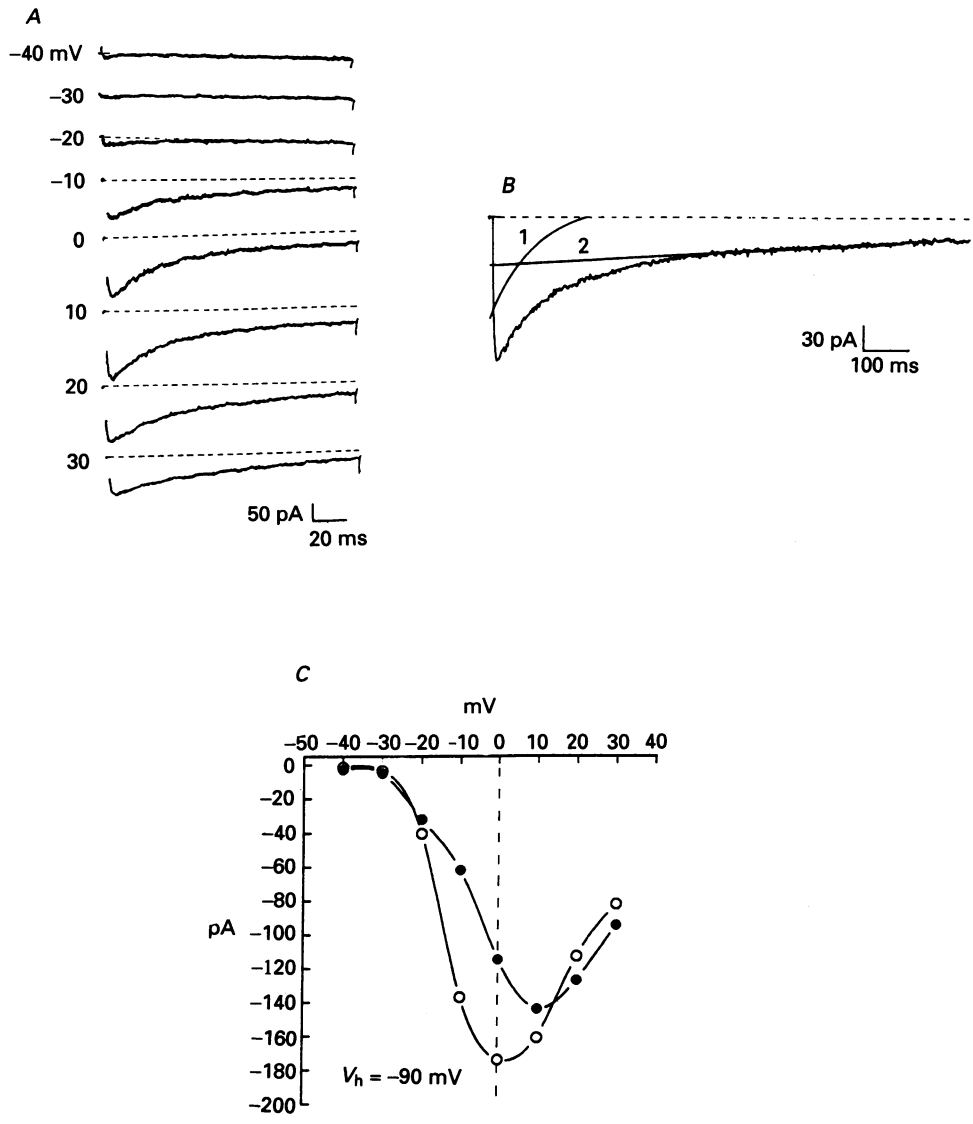

Fig. 2. The mixed N_t- and L-type components of I_{Ca}. *A*, an example of transient and long-lasting current traces recorded from a nerve terminal in the 10 mM-Ca^{2+} bathing solution. The inward currents were elicited from a holding potential (V_h) of −90 mV to different test potentials (−40 to +30 mV). *B*, the inactivating phase of I_{Ca} exhibits two exponential functions: a fast-inactivating component with a τ of 95·8 ms (1) and a slow-inactivating component with a τ of 1295·4 ms (2). Current obtained from a holding potential of −90 mV, stepping to +10 mV. *C*, current–voltage relationships of the mean peak current amplitudes of the isolated nerve terminals in 10 mM-Ca^{2+} (●, $n = 8$) or in 10 mM-Ba^{2+} (○, $n = 5$) bathing solutions.

Two types of I_{Ca}

Two components of I_{Ca}, i.e. N_t- and L-type, can be distinguished by stepping to the same depolarizing potentials from different holding potentials. Figure 2*A* shows a

series of inward current traces obtained from a holding potential of −90 mV to different depolarizing potentials, which evoked both the N_t- and L-type components of I_{Ca}. A sustained inward current starts to be activated at about −20 mV. The inactivating phase of the current evoked from a holding potential of −90 mV can be

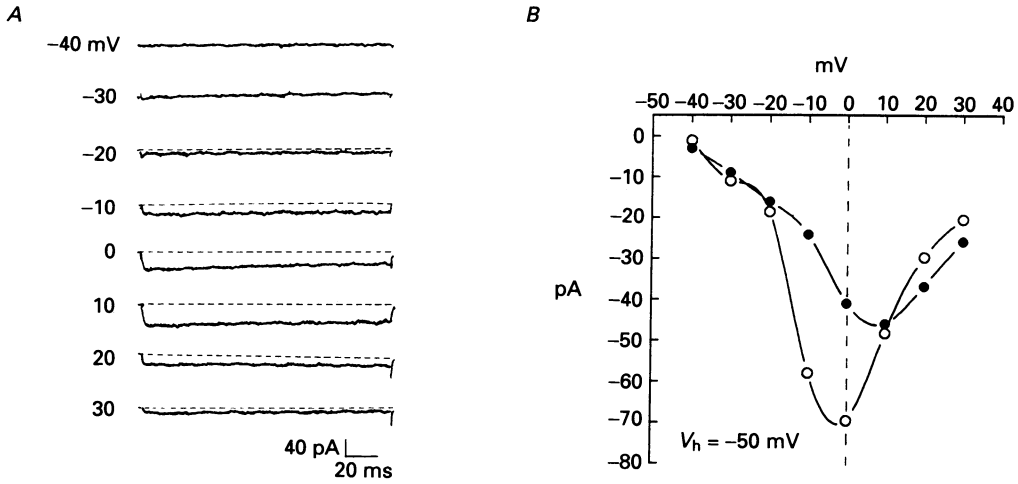

Fig. 3. The isolated L-type component of I_{Ca}. *A*, an example of long-lasting current traces recorded from a nerve terminal in a 10 mM-Ca^{2+} bathing solution. The inward currents were elicited from a holding potential (V_h) of −50 mV to different test potentials (−40 to +30 mV). *B*, current–voltage relationships of mean peak current amplitudes of the isolated nerve terminals in 10 mM-Ca^{2+} (●, $n = 10$), or in 10 mM-Ba^{2+} (○, $n = 5$) bathing solutions.

fitted with the sum of two exponentials (for an example see Fig. 2*B*): one is a rapidly decaying transient component with a time constant (τ) of 94·7 ± 4·2 ms ($n = 5$), corresponding to the N_t-type; the other is a long-lasting component with a τ of 1251·7 ± 121·4 ms ($n = 5$), corresponding to the L-type (Lemos & Nowycky, 1989; Wang *et al.* 1991*a*). Thus, only the long-lasting L-type component is evident at the end of these 150 ms long traces. This observation has been used in protocols to distinguish the two components in 'whole-cell' recordings (Wang *et al.* 1991*a*). Previous studies in nerve terminals of the chick ciliary ganglion (Yawo, 1990) have shown that two components, similar to the N_t- and L-types, evoked at one holding potential represent two distinct channel populations, rather than two different states of a single type of Ca^{2+} channel. The maximum current was reached at a test potential of +10 mV in 10 mM-Ca^{2+}, but shifted to 0 mV in a 10 mM-Ba^{2+} bathing solution (Fig. 2*C*).

The long-lasting, L-type component of I_{Ca} can be more selectively activated by holding at a potential of −50 mV (Fig. 3*A*), where the rapidly decaying, transient component (N_t-type) is inactivated (Lemos & Nowycky, 1989; Wang *et al.* 1991*a*). This protocol might be the most effective in isolating the L-type component. This inward current elicited from a holding potential of −50 mV starts to be activated at about −30 mV, and is voltage dependent. The peak current was also maximal

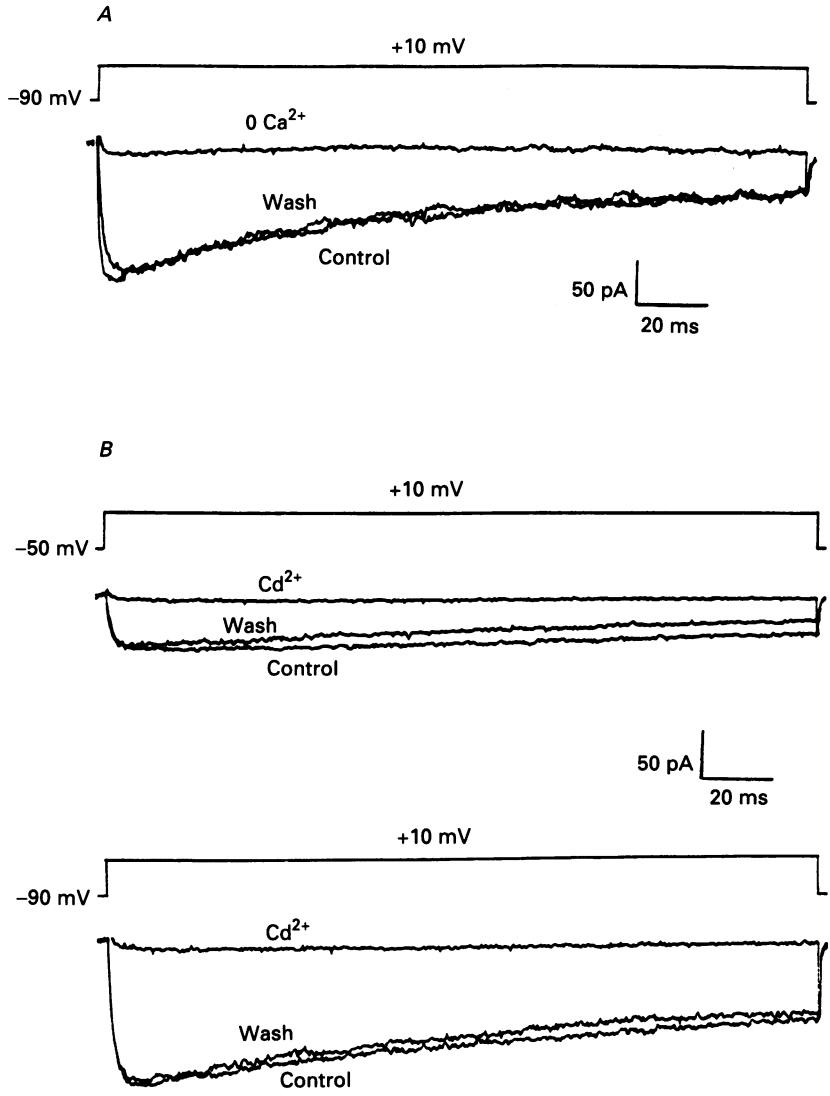

Fig. 4. Effects of ion substitution and the Ca^{2+} channel antagonist Cd^{2+} on I_{Ca}. *A*, I_{Ca} disappears in 0 Ca^{2+} (replaced by 10 mM-TEA chloride) bathing solution. The complete I_{Ca} can be recovered almost immediately after returning to the 10 mM-Ca^{2+} bathing solution. *B*, the L- and N_t-type currents were elicited by stepping to +10 mV from a holding potential of either −50 or −90 mV in the 10 mM-Ca^{2+} bathing solution. Both types of current were blocked by adding 50 μM-Cd^{2+} to the 10 mM-Ca^{2+} bathing solution, and the blocking effects by Cd^{2+} were reversible immediately after returning to the original solution.

at a test potential of +10 mV in 10 mM-Ca^{2+}, but shifted to 0 mV in 10 mM-Ba^{2+} (Fig. 3*B*).

Equimolar replacement of Ca^{2+} by Ba^{2+} affects the inward currents (Figs 2*C* and 3*B*). The amplitude of current evoked at either holding potential (−90 or −50 mV) is larger in Ba^{2+} than in Ca^{2+}. This effect is primarily due to an increase in the L-type

component (compare Figs 2*C* and 3*B*), as seen previously (Tsien *et al.* 1988). The activation threshold is similar in both solutions, but the peak of the current–voltage relation is shifted by 10 mV in the hyperpolarizing direction in 10 mM-Ba^{2+}.

Furthermore, both components of the inward current can be abolished by either removing all the Ca^{2+} from the bathing solution (Fig. 4*A*), or adding 50 μM-Cd^{2+} to the bathing solution (Fig. 4*B*). Thus, these macroscopic inward currents are indeed I_{Ca}.

ω-Conotoxin inhibits both components of I_{Ca}

In these studies, control and experimental measurements were carried out on different terminals (unless otherwise mentioned in the text) to avoid the complications associated with 'run-down' or 'wash-out' of I_{Ca} (for review see Eckert & Chad, 1984). I_{Ca} 'run-down' can occur very rapidly, within 2–4 min, in these nerve terminals. On the other hand, since ω-CgTx is known to irreversibly block Ca^{2+} channels (Olivera *et al.* 1990), no recovery of currents can be obtained. Our strategy was the following: currents from a number of individual terminals were recorded immediately after break-through while in the control perfusion pipette. Currents from another group of terminals in the same dish were recorded while in the same perfusion pipette but containing ω-CgTx. In control studies where only 6–8 μm diameter terminals were used, we found that the undisturbed terminals did not show a change in baseline I_{Ca} during time periods (3–4 h) longer than those which separated the first control group and the last drug treatment group. Peak currents holding at −90 mV, with mixed N_t- and L-type components, were 139·9 ± 6·6 pA ($n = 29$) (early group) *vs.* 140·7 ± 6·5 pA ($n = 19$) (late group) and the peak L-type component currents holding at −50 mV were 53·5 ± 3·3 pA ($n = 29$) (early group) *vs.* 53·6 ± 5·7 pA ($n = 12$) (late group).

Figure 5*A* shows representative inward current records elicited by stepping to +10 mV (peak response) from a holding potential of −90 mV in the absence and presence of different concentrations of ω-CgTx. The amplitude of the peak N_t-type I_{Ca} was significantly reduced, by 48%, at a concentration of 30 nM-ω-CgTx. The inhibition was dose dependent (Fig. 5*B*). The half-maximum inhibition (IC_{50}) of N_t-type I_{Ca} by ω-CgTx was calculated to be 50 nM. Figure 5*C* shows that the reduction in current amplitude by ω-CgTx did not result from any shift in the current–voltage relationship of I_{Ca}.

We found that at higher concentrations, ω-CgTx also reduced the L-type component of the I_{Ca}. Figure 6*A* shows representative inward current records elicited by stepping to +10 mV from a holding potential of −50 mV in the absence and presence of different concentrations of ω-CgTx. The amplitude of peak L-type I_{Ca} was significantly reduced, by 38%, at a concentration of 300 nM-ω-CgTx (Fig. 6*B*). This reduction was dose dependent. The half-maximum inhibition (IC_{50}) of L-type current by ω-CgTx was calculated to be 513 nM. Again, like the N_t-type, the reduction in the L-type current amplitude by ω-CgTx did not result from any shift in the current–voltage relationship (Fig. 6*C*). In addition, if we measured the late portion of I_{Ca} at a holding potential of −90 mV, which also represents the L-type component (see above or Wang *et al.* 1991*a*), this long-lasting component was reduced significantly (Fig. 6*D*), at the same concentrations of ω-CgTx as those that were

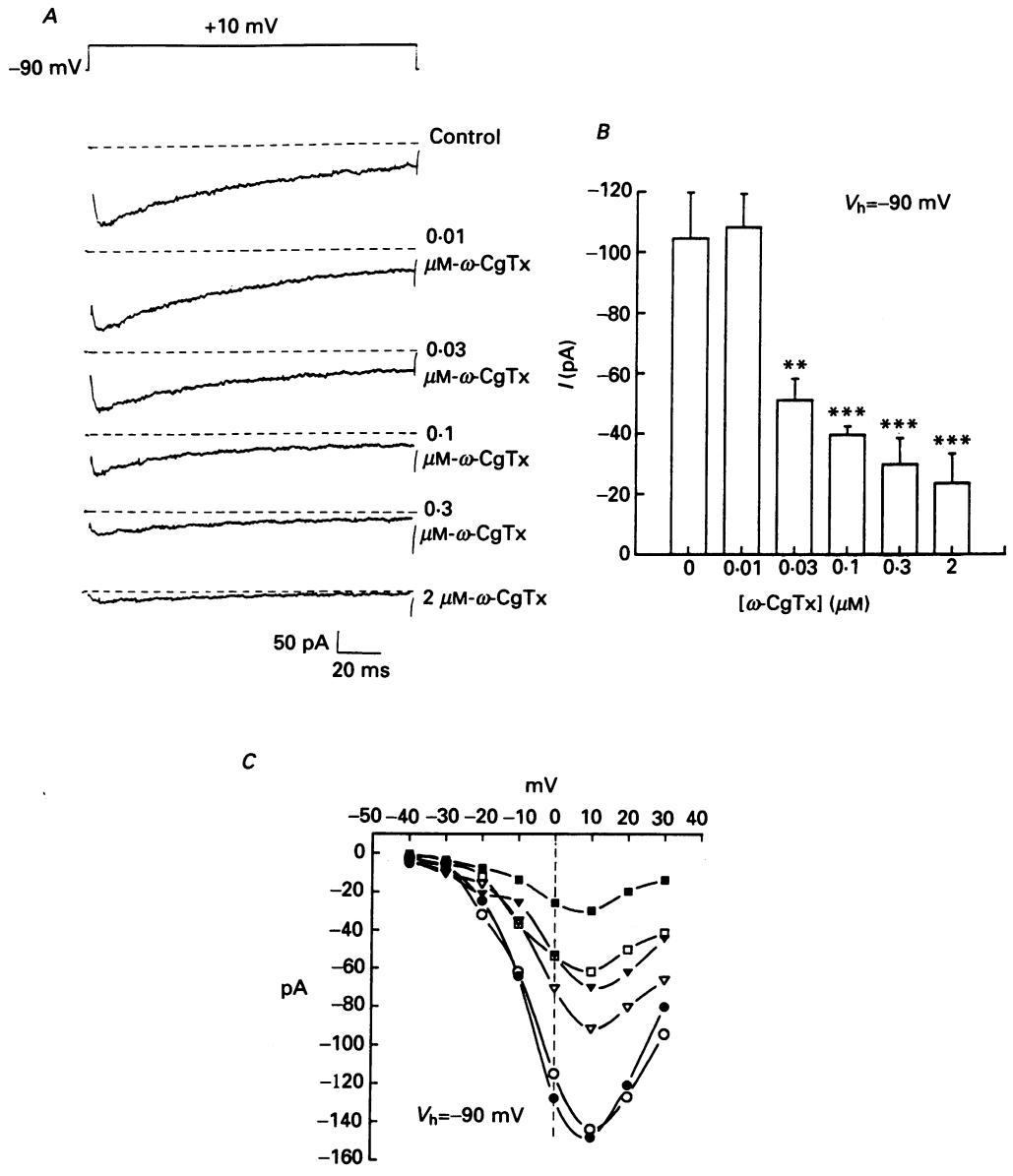

Fig. 5. Effects of ω-CgTx on the N_t-type component of I_{Ca}. *A*, representative traces of I_{Ca} inhibited by ω-CgTx at the concentrations as noted at right. *B*, dose–response relationship. Histogram shows the means ± S.E.M. ($n = 4$–8) amplitudes of peak currents (after subtracting the late portion (140 ms) of the current) elicited by a voltage step to +10 mV from a holding potential (V_h) of −90 mV in the absence or presence of various ω-CgTx concentrations. Statistical significance: $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ (student's *t* test). *C*, current–voltage relationships of the mean peak current amplitudes at holding potential (V_h) of −90 mV in the absence (○, $n = 8$) or presence of ω-CgTx (●, 0·01 μM, $n = 7$; ▽, 0·03 μM, $n = 8$; ▼, 0·1 μM, $n = 4$; □, 0·3 μM, $n = 6$; ■, 2 μM, $n = 6$).

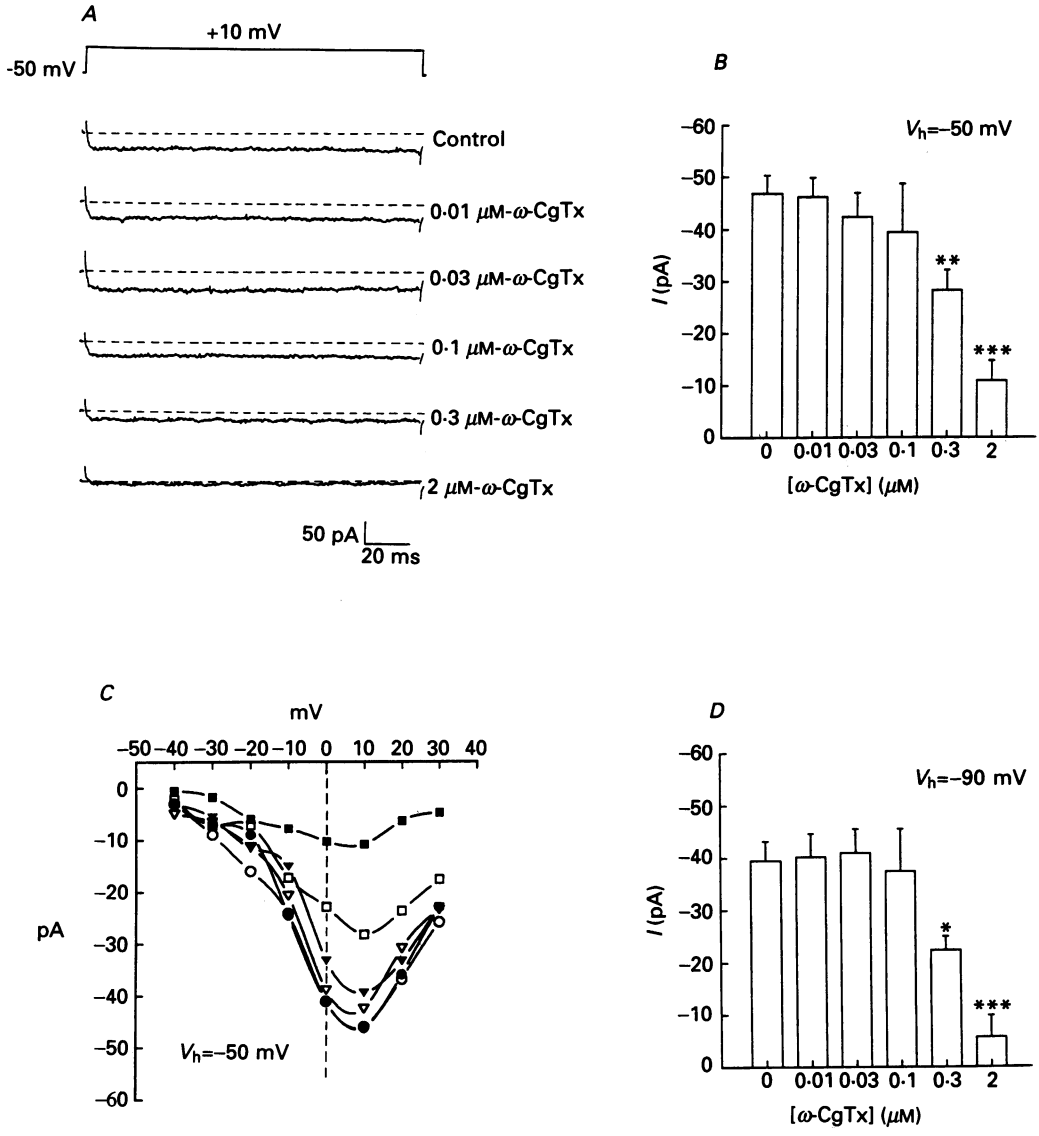

Fig. 6. Effects of ω-CgTx on the L-type component of I_{Ca}. *A*, representative traces of I_{Ca} inhibited by ω-CgTx at the concentrations as noted at right. *B*, dose–response relationship. Histogram shows the means ± S.E.M. (n = 5–10) amplitudes of peak currents elicited by a voltage step to +10 mV from a holding potential (V_h) of −50 mV in the absence or presence of indicated ω-CgTx concentrations. Statistical significance: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ (Student's t test). *C*, current–voltage relationships of the mean peak current amplitudes at holding potential of −50 mV in the absence (○, n = 10) or presence of ω-CgTx (●, 0·01 μM, n = 7; ▽, 0·03 μM, n = 8; ▼, 0·1 μM, n = 5; □, 0·3 μM, n = 6; ■, 2 μM, n = 8). *D*, dose–response relationship as in *B*, but measuring the late portion (at 140 ms) of the currents elicited by a holding potential (V_h) of −90 mV in the absence and presence of indicated concentrations of ω-CgTx. Statistical significance: $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$ (Student's t test, n = 5–9).

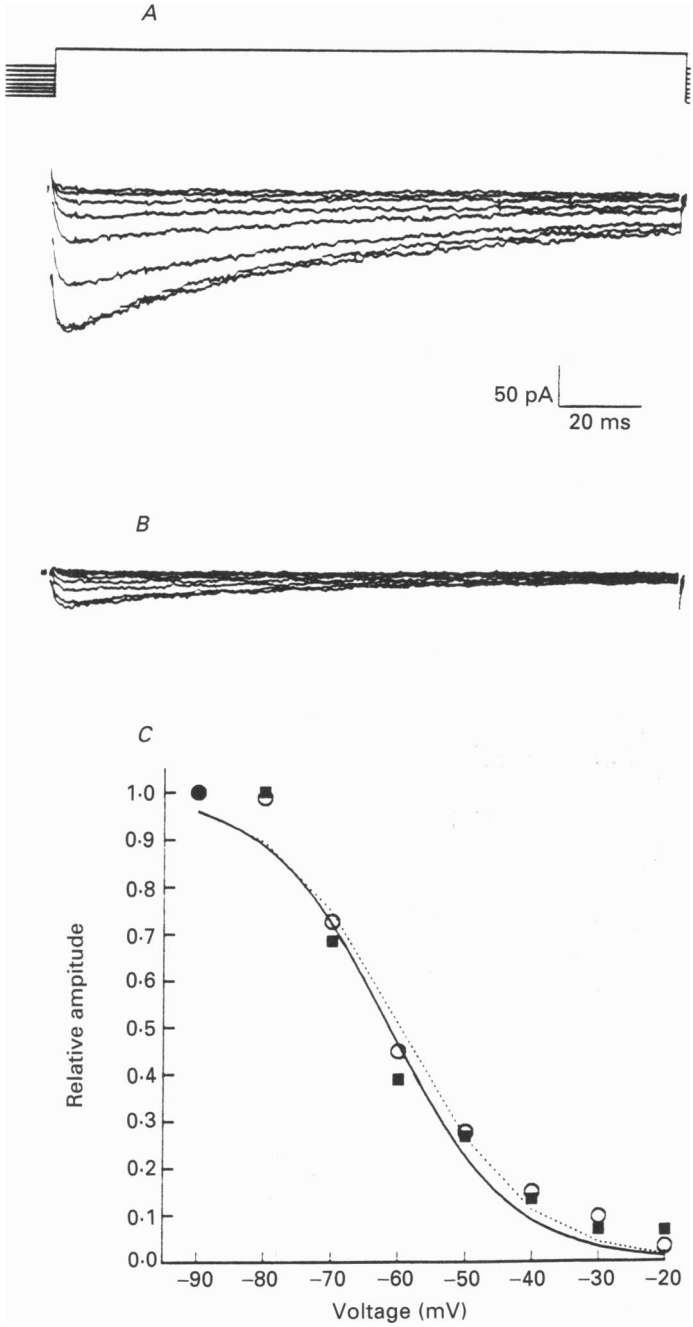

Fig. 7. Effects of ω-CgTx on steady-state inactivation of I_{Ca}. *A*, as a control, the nerve terminal current traces for steady-state inactivation were elicited from a range of holding potentials (−90, −80, −70, −60, −50, −40, −30 and −20 mV, respectively, with the holding potential applied for 8 s before making each step), and stepped to the same membrane potential (0 mV). *B*, using the same voltage protocols as in *A* in the presence of 300 nM-ω-CgTx. *C*, steady-state inactivation curves of mean I_{Ca} in the absence (○,

effective on the current elicited from a holding potential of −50 mV (see Fig. 6*B*). Thus, the difference in the effectiveness of the toxin is not due to the difference in holding potentials.

The effects of ω-CgTx on the steady-state inactivation of mixed N_t- and L-type I_{Ca} were also examined. Steady-state inactivation was elicited by prepulse potentials varying between −90 and −20 mV, and was assessed by measuring the current during steps to 0 mV. The voltage dependence of steady-state inactivation of I_{Ca} in the absence (Fig. 7*A*) or presence (Fig. 7*B*) of 300 nM-ω-CgTx was fitted by the Boltzmann distribution, and both showed nearly identical half-maximal inactivation voltages, −59·7 and −60·7 mV, respectively (Fig. 7*C*). There was no significant difference in this parameter between controls and ω-CgTx-treated terminals (two-tailed *t* test).

ω-Conotoxin does not affect the terminal I_{Na}

Previous studies have mentioned that ω-CgTx did not influence I_{Na} in neuronal cells, but actual data were not shown (McCleskey *et al.* 1987; Oyama *et al.* 1987). In this group of experiments, control and experimental data were both collected from the same terminals since I_{Na} did not exhibit 'run-down'. The I_{Na} was activated by depolarization steps from a holding potential of −90 mV and the peak of the current–voltage relationship occurred at −20 mV. The current rapidly activated and then inactivated within a few milliseconds during a steady depolarization (Thorn *et al.* 1991). This fast, transient inward current can be blocked completely by 1 μM-tetrodotoxin (Lemos & Nowycky, 1989). Figure 8 shows that the terminal I_{Na} was not affected by the addition of 300 nM-ω-CgTx, a dose which significantly blocked both types of I_{Ca}.

ω-Conotoxin has no effect on non-neuronal cells

In general, mammalian PI cells are involved in the secretion of melanophore-stimulating hormone (MSH) and opioid peptides (Reichlin, 1985). A small number of PI cells were contained in most of our preparations. These PI cells can be distinguished easily from the nerve terminals by their nuclei and larger size (10–20 μm in diameter). The properties of their I_{Ca} are strikingly different from that of the nerve terminals. In particular, the I_{Ca} of the PI cells showed only a long-lasting, non-inactivating component. Furthermore, these non-neuronal secretory cells do not exhibit fast 'run-down' of I_{Ca}. At the same concentration that blocked both types of I_{Ca} in the nerve terminals, ω-CgTx did not affect the long-lasting, non-inactivating component of I_{Ca} in the PI cells (Fig. 9).

$n = 8$), and in the presence (■, $n = 6$) of 300 nM-ω-CgTx. The lines were fitted according to the Boltzmann equation:

$$\frac{I}{I_{max}} = \frac{1}{1+\exp\frac{(V-V_{\frac{1}{2}})}{k}}.$$

For the control, the half-maximal inactivation potential, $V_{\frac{1}{2}}$, = −59·7 mV and the steepness factor, k, = 9·47; after ω-CgTx treatment, $V_{\frac{1}{2}} = -60{\cdot}7$ mV and $k = 9{\cdot}23$.

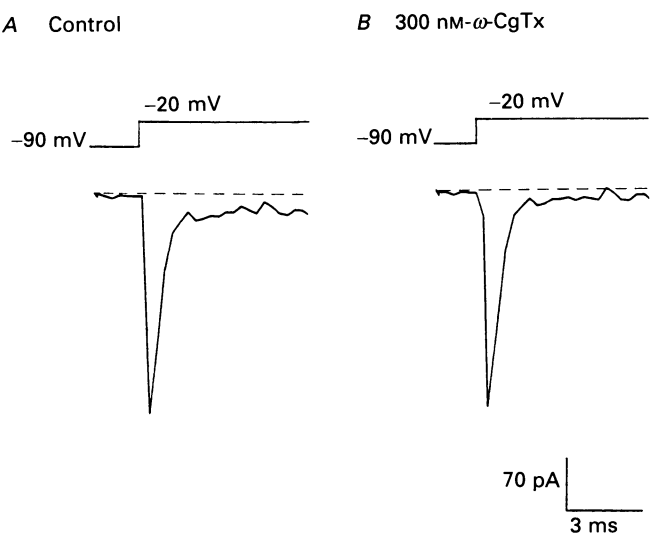

Fig. 8. Effects of ω-CgTx on the I_{Na} of the nerve terminals. *A*, before addition of ω-CgTx, an inward, fast, transient current was elicited by a depolarizing voltage pulse (as shown above). *B*, using the same protocol in the same cell, but in the presence of 300 nM-ω-CgTx. Both were superfused with the 10 mM-Ca^{2+} bathing solution, but 25 mM-NaCl was substituted for an equal amount of TEA chloride.

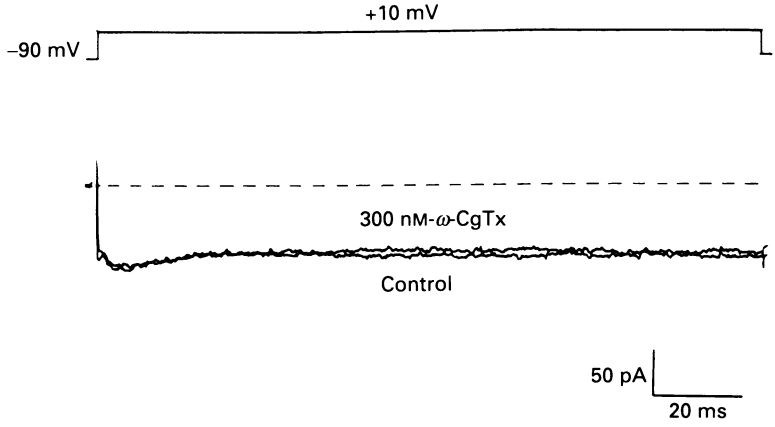

Fig. 9. Effects of ω-CgTx on the I_{Ca} of a non-neuronal secretory cell of the PI. The I_{Ca} was elicited by applying depolarizing voltage pulses (as shown above) to the same cell in the absence and presence of 300 nM-ω-CgTx in the 10 mM-Ca^{2+} bathing solution.

DISCUSSION

There are two different types (N_t and L) of high-threshold voltage-actived Ca^{2+} channels in the neurohypophysial peptidergic nerve terminal membrane (Lemos & Nowycky, 1989; Wang, *et al.* 1991*a*). These two types of macroscopic terminal I_{Ca} can be distinguished from each other by manipulation of the holding potentials. The thresholds for activation of the two inward current components were about −20 and −30 mV, respectively. The N_t-type component inactivated rapidly (τ = 94·7 ms) while the L-type component inactivated much more slowly

(τ = 1251·7 ms). The properties of the two types of Ca^{2+} channels in these nerve terminals have been studied previously using single-channel patch-clamp techniques (Lemos & Nowycky, 1989). The N_t-type channels had a small conductance, high threshold for activation, and inactivated rapidly. The L-type channels, which inactivated slowly, had a large-conductance, high threshold for activation and were dihydropyridine sensitive. In terms of their permeability to Ca^{2+} and Ba^{2+}, and their sensitivity to Cd^{2+}, the peptidergic nerve terminal Ca^{2+} channels are similar to those found in the terminals of the chick ciliary ganglion (Yawo, 1990). The influx of Ca^{2+} via membrane voltage-activated channels is usually associated with transmitter release from nerve terminals. A functional significance for these two types of Ca^{2+} channels has been proposed: release from small, clear vesicles is mediated via the N-type, while release from large, dense-core vesicles is mediated via L-type (Hirning *et al.* 1988).

ω-Conotoxin can affect both the N_t- and L-type I_{Ca} in these nerve terminals. These findings confirm a previous study (Obaid, Flores, Salzberg, 1989) which showed ω-CgTx blockade of Ca^{2+} spikes in the intact neurohypophysis, and indicate that both components could be involved in action potential generation. The inhibiting effects on the N_t-type of I_{Ca} are in agreement with many previous findings that ω-CgTx can block N-type Ca^{2+} channels, and help to confirm that the transient I_{Ca} in these secretory nerve terminals is a member of this high-threshold I_{Ca} 'family' (Tsien *et al.* 1988). Furthermore, our studies have demonstrated that the L-type component of I_{Ca} can also be reduced, but only by higher concentrations of ω-CgTx. However, such an interpretation needs to be viewed with caution since it has been recently shown that N-type Ca^{2+} channels can sometimes remain open (i.e. do not inactivate) for as long as L-type Ca^{2+} channels (Plummer *et al.* 1989; Regan, Sah & Bean, 1991). Nevertheless, we fell that ω-CgTx can affect the L-type Ca^{2+} channels in these secretory nerve terminals. These findings corroborate AVP release studies from the same preparation. The activation of the L-type Ca^{2+} channels is a major factor in inducing an increase in cytoplasmic Ca^{2+} (Brethes, Dayanithi, Letellier & Nordmann, 1987; Stuenkel, 1990) and in AVP release. AVP release from the nerve endings, which are highly sensitive to dihydropyridines (Cazalis, Dayanithi, & Nordmann, 1987), can also be strongly inhibited by ω-CgTx (Dayanithi *et al.* 1988). Taken together these data strongly suggest that, in these nerve terminals of the neurohypophysis, both types of I_{Ca} are important for peptide release.

The reduction of I_{Ca} by ω-CgTx does not result from a shift in the I_{Ca} current–voltage relationship. Steady-state inactivation of the terminal I_{Ca} is also unaffected by ω-CgTx. Thus, it appears that the block by this toxin is not voltage dependent and does not change the inactivation voltage dependence. However, the degree of inhibition of either type of current is dose dependent. The half-maximum inhibition (IC_{50}) of I_{Ca} is 50 nM for the N_t-type Ca^{2+} channel and 513 nM for the L-type Ca^{2+} channel, which is approximately a tenfold difference.

The mechanism by which ω-CgTx blocks the Ca^{2+} channels is not well understood. The blocking effects may be due to a direct binding to Ca^{2+} channels, or a binding to specific receptors which then inactivate these channels (Cruz & Olivera, 1986; Cruz, Johnson & Olivera, 1987). It has been suggested that the ω-CgTx blocking action does not involve a second messenger system or a GTP-binding protein, but rather

that direct binding to Ca^{2+} channels is likely (McCleskey *et al.* 1987). ω-Conotoxin depresses ion permeation through the channels primarily when Ca^{2+} is the permeating ion (Carbone & Lux, 1988). Previous electrophysiological studies (McCleskey *et al.* 1987; Boland & Dingledine, 1990), together with our present data, have shown that I_{Ca} is not completely blocked by this toxin. Recent studies by Schroeder and colleagues (Schroeder *et al.* 1990) on rat dorsal root ganglion neurons have shown that even though the L-type Ca^{2+} channels are selectively blocked by nifedipine and the N-type Ca^{2+} channels by ω-CgTx, the two together fail to block all of I_{Ca}. Also, it was found that both transient and sustained Ba^{2+} currents have ω-CgTx-sensitive and -insensitive components in a rat dorsal root ganglion cell line (Boland & Dingledine, 1990). Furthermore, some studies, using Ba^{2+} as the charge carrier, suggested that some other population of high-threshold Ca^{2+} channels which are resistant to both ω-CgTx and dihydropyridine blockers might exist in several different peripheral and central neurons (Mogul & Fox, 1991; Regan *et al.* 1991). Biochemical studies (Dayanithi *et al.* 1988) also showed that ω-CgTx cannot totally abolish the rise in nerve terminal internal free Ca^{2+} concentrations induced by high-potassium depolarization, and that subsequently, a small amount of AVP release was insensitive to both this toxin and the dihydropyridine nicardipine. Possible interactions between ω-CgTx and other Ca^{2+} channel blockers is unclear, and the Ca^{2+} channel diversity and complex channel behaviour remain to be elucidated.

Voltage-activated Na^+ channels have been studied in many excitable nerve cells and they play an essential role in the process of transmitter release via the generation of action potentials in nerve terminals (Lemos, Nordmann, Cooke & Stuenkel, 1986). In this study, ω-CgTx at a concentration (300 nM) which significantly inhibits both N_t- and L-type I_{Ca} in the nerve terminals does not affect the fast, transient, tetrodotoxin-sensitive voltage-gated I_{Na} in the same tissue. This observation confirms that ω-CgTx can discriminate between Ca^{2+} and Na^+ channels in the same membrane (McCleskey *et al.* 1987; Oyama *et al.* 1987; Olivera *et al.* 1990).

No binding sites for ω-CgTx have been found in heart and skeletal muscle (Cruz *et al.* 1987; Barhanin, Scmid & Lazdunski, 1988), and patch-clamping studies have shown that L-type I_{Ca} was not influenced by ω-CgTx in those tissues (McCleskey *et al.* 1987). Here, we have shown that this toxin, using present protocols, did not block Ca^{2+} channels in the non-neuronal, secretory PI cells. This finding supports the general view that ω-CgTx might be highly tissue specific and perhaps only works on neuronal tissue (for review see Olivera *et al.* 1990). There is an exception since only the L-type Ca^{2+} channel was blocked in clonal rat anterior pituitary GH_3 cells (Suzuki & Yoshioka, 1987). All results should, therefore, be interpreted with caution and more carefully designed future experiments are needed. Here, however, it is unlikely that the lack of effect seen on the PI cells (or terminal I_{Na}) is a result of the different protocols being used, i.e. voltage-clamping cells that have already been exposed to toxin, or that were exposed to toxin after recording control currents. Actually, using the same protocol as in the studies of the PI I_{Ca} and terminal I_{Na}, we have found that the toxin was still effective at blocking I_{Ca} in the nerve terminals (data not shown).

In summary, there are at least two different types of high-threshold voltage-activated Ca^{2+} currents which co-exist in the peptidergic nerve terminals of the rat

neurohypophysis. ω-Conotoxin can block both of these types of Ca^{2+} currents, but the N_t-type is much more susceptible. These observations help establish the distinction between these two channel types, and provide evidence that the transient component is 'N'-like (Tsien *et al.* 1988).

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