Effects of Toxins on Ca^{2+} Currents and Peptide Release from Nerve Terminals[a]

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INTRODUCTION

Voltage-activated calcium channels in nerve terminals of the neurohypophysis are instrumental for triggering the exocytotic release of the neuropeptides, oxytocin (OT) and vasopressin (AVP). In depolarization-secretion coupling, Ca^{2+}, acting as a second messenger, flows into the terminals through these channels and consequently evokes release.[1,2] In neurons, the major voltage-gated Ca^{2+} channels were originally named and distinguished as the T- (low-voltage-threshold), N-, and L- (high-voltage-threshold) types.[3,4] Both of the high-voltage Ca^{2+} channel types may be involved in the process of neurotransmitter or neuropeptide release.[5-8]

A number of pharmacological agents, including toxins, have been used to further

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distinguish these different Ca^{2+} channel types. ω-Conotoxin GVIA (ω-CgTx), a 27 amino-acid peptide from the venom of the marine snail *Conus geographus*, has been shown to block the activity of voltage-sensitive Ca^{2+} channels and subsequent neurotransmitter release in neuronal tissues.[9,10] Most previous electrophysiological, binding, or release studies have demonstrated that ω-CgTx can block the "N"-type Ca^{2+} channels.[11-13] In contrast, the results obtained with ω-CgTx on the long-lasting-type Ca^{2+} channels have been rather inconclusive. The long-lasting-type Ca^{2+} channels have been shown to be insensitive to ω-CgTx in many preparations.[13,14] On the other hand, it has been shown that long-lasting-type, along with "N"-type, Ca^{2+} channels were persistently blocked by higher concentrations of ω-CgTx.[4,5,11,12,15] Therefore, one cannot exclude possible effects of ω-CgTx on the long-lasting-type Ca^{2+} channels and one should question the original claim that ω-CgTx binds only to "N"-type Ca^{2+} channels.[10,14]

Another useful agent, (+)-tetrandrine (tetrandrine), a bis-benzylisoquinoline alkaloid (6,6',7,12-tetramethoxy-2,2'-dimethylberbaman), is now employed in China for the treatment of hypertension and cardiac arrhythmia. Pharmacological experiments *in vivo* and *in vitro* have explored the mechanisms by which tetrandrine exerts its clinical hypotensive and antiarrhythmic effects. Tetrandrine exerted a negative inotropic effect on myocardium and blocked high K^+-evoked contraction of artery strips.[16] It decreased the amplitude of the Ca^{2+}-mediated action potential and inhibited the inward Ca^{2+} current in cardiac Purkinje fibers.[16,17] Recent patch-clamp studies showed that tetrandrine blocked long-lasting Ca^{2+} currents in GH_3 anterior pituitary cells[18] and posterior pituitary nerve terminals,[19] as well as a low-voltage-activated transient channel in adrenal cortical cells.[20] Tetrandrine is thereby considered to be a new Ca^{2+} channel blocker, of herbal origin, specific to T and L types.

Recently, another type of high-voltage-threshold Ca^{2+} channel, the P-type channel, has been characterized in neurons.[21] Pharmacologically, the P-type channel currents are blocked by either a funnel-web spider toxin polyamine (FTX) or peptide (ω-Agatoxin IVA),[22] but are insensitive to other Ca^{2+} channel blockers such as ω-conotoxin GVIA or the dihydropyridines. The two funnel-web spider toxins therefore provide us with effective probes to distinguish P-type channel current from other Ca^{2+} channel currents. Kinetically, almost all P-type Ca^{2+} channel currents investigated thus far are slowly inactivating.[21,23-25]

It has been reported that FTX eliminated the undershoot of the compound action potential, recorded using optical measurement techniques, and reduced the magnitude of the action potential in frog neurohypophyseal nerve terminals. After maximal inhibition by ω-conotoxin GVIA, the active calcium response could be further inhibited by FTX.[26] In addition, a "secretory wave" thought to possibly reflect release of peptidergic hormones from the terminal was found to be partially inhibited by either FTX or ω-conotoxin GVIA. These results imply that at least two populations of calcium channels, one being sensitive to ω-conotoxin GVIA and another to FTX, are present and are required for neuropeptide release in the frog neurohypophyseal nerve terminals.[27]

The isolated neurohypophyseal nerve terminals of the rat are an accessible model system for investigating ionic channels in presynaptic nerve terminals using patch-clamp techniques.[7,28] Although both a transient, dihydropyridine-resistant N_t-type and a long-lasting, dihydropyridine-sensitive L-type Ca^{2+} channel have been pre-

viously characterized in the rat neurohypophyseal terminals,[7,19,29] there is still the possibility that other types of Ca^{2+} channel populations exist and function in this preparation. In the present study, using the "whole-cell" patch-clamp recording method, we examined the effects of ω-CgTx, tetrandrine, FTX, and ω-AgaIVA on both the transient and long-lasting components of macroscopic calcium currents (I_{Ca}) in these nerve terminals. We examined evidence for a voltage-activated P-type Ca^{2+} channel in order to better understand any contributions made by the different types of Ca^{2+} channels to depolarization-secretion coupling in presynaptic terminals.

MATERIALS AND METHODS

Terminal Isolation

Experiments were conducted on the freshly isolated neurohypophyseal nerve terminals of male CD rats (Charles River Laboratory, Boston, Massachusetts). Briefly, as previously described,[28,30,31] the rat, after being anesthetized by CO_2, was decapitated. The brain was removed and then the pituitary gland was excised. After careful removal of the anterior pituitary and the pars intermedia, the posterior pituitary was homogenized in the following solution (mM): sucrose, 270; HEPES, 10; EGTA, 0.01; pH 6.8. The isolated terminals were then placed into a 35-mm petri dish coated with poly-*l*-lysine and perfused with 2.2 mM Ca^{2+} Locke's solution (mM): NaCl, 145; KCl, 5; $CaCl_2$, 2.2; $MgCl_2$, 1; HEPES, 10; glucose, 15; pH 7.3. Typically, the peptidergic nerve terminals could be identified using an inverted microscope equipped with phase and Hoffman-modulated contrast optics (Nikon, Tokyo, Japan). The fact that they are terminals can be confirmed by immunocytochemistry of vasopressin, oxytocin, or neurophysins,[32] and the isolated terminals attached to the bottom of the dish have been identified by immunofluorescence staining for vasopressin or oxytocin (Thorn and 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.[33] Only terminals 6–9 μm in diameter were chosen for patch-clamping. All experiments were done at room temperature (22–24°C).

"Whole-Cell" Patch-Clamp Recording

After approximately one-hour perfusion with 2.2 mM Ca^{2+} Locke's solution, the terminals were perfused with 10 mM Ca^{2+} modified Locke's solution (mM): TEA-Cl, 100; NaCl, 30; $CaCl_2$, 10 (or $BaCl_2$, 10); KCl, 5; $MgCl_2$, 1; HEPES, 10; glucose, 15; pH 7.3. Pipette solution contained the following (mM): NMG-Cl, 130; EGTA-Cs, 10; HEPES, 20; MgATP, 2; cyclic AMP, 0.2; pH 7.2. The resistances of soft glass pipettes (Drummond Sci. Company, Broomall, Pennsylvania), which had been double-pulled (David Kopf 700C, Tujunga, California), Sylgard-coated, and fire-polished (Narashige, Kyoto, Japan), were 5–7 MΩ. The pipette was pressed against the surface of the terminal membrane and mild suction was applied until the giga-seal was formed. Additional mild suction could lead to the rupture of the membrane and the "whole-

cell" configuration.[34] The currents were recorded by using an EPC-7 amplifier (List Electronic, Darmstadt, Germany); were filtered at 1-kHz corner frequency, −3 dB, with an 8-pole Bessel filter (902LPF, Frequency Devices Incorporated, Haverhill, Massachusetts); and were saved on high-density disks for later analysis. PClamp computer software (Axon Corporation, Burlingame, California) and an A-D converter (homemade) were used to generate command voltage potentials.

Drug Application

Most of the terminals were picked up from the bottom of the petri dish after giga-seal formation so that the entire area of their membrane would have direct contact with the perfused medium. The "rundown" of I_{Ca} in the neurohypophyseal nerve terminal was so fast that, in most cases, I_{Ca} values for the control and the drug-treated were recorded from separate terminals. FTX, purified chromatographically from the crude venom of American funnel-web spiders (*Agelenopsis aperta*), was a kind gift from R. Llinas and B. Cherksey (Department of Physiology and Biophysics, New York University Medical Center). ω-AgaIVA purified from the same venom was a kind gift of Michael E. Adams (Department of Entomology, University of California at Riverside). ω-Conotoxin GVIA (Peninsula Laboratories) was prepared as 40 mM stock solution in 2% BSA solution. Tetrandrine, an alkaloid extracted from the root of *Stephania tetrandra* S. Moore, was a kind gift from X. G. Zong (Institute of Pharmacology and Toxicology, Technical University of Munich).

Release Assays

For release studies, the isolated terminals are resuspended in normal saline and loaded on a 0.22-μm Millipore filter. After experimental manipulations, perfusate samples are then collected. Hormone release is determined by radioimmunoassay, as described previously.[30,31] AVP and OT antibodies are used at a final concentration of 1/80,000 and 1/40,000, respectively. The cross-reactivity of OT in the AVP assay is less than 1:1000. Similarly, the cross-reactivity of AVP in the OT assay is less than 1:200.

Data Analysis

PClamp software was also used for analysis of the data. The inward I_{Ca} was expressed as downward. In most cases, the leakage currents were subtracted from the total currents in order to analyze the pure inward I_{Ca}. The amplitude of I_{Ca} at the end of the 200-ms steps was considered to be that of the long-lasting component of the I_{Ca}, and the difference in amplitude between this component of I_{Ca} and the peak I_{Ca} is considered to be that of the transient I_{Ca} (see FIGURE 1B). Data are given as mean ± SEM. Student's t test was used to examine statistical significance of paired or unpaired data.

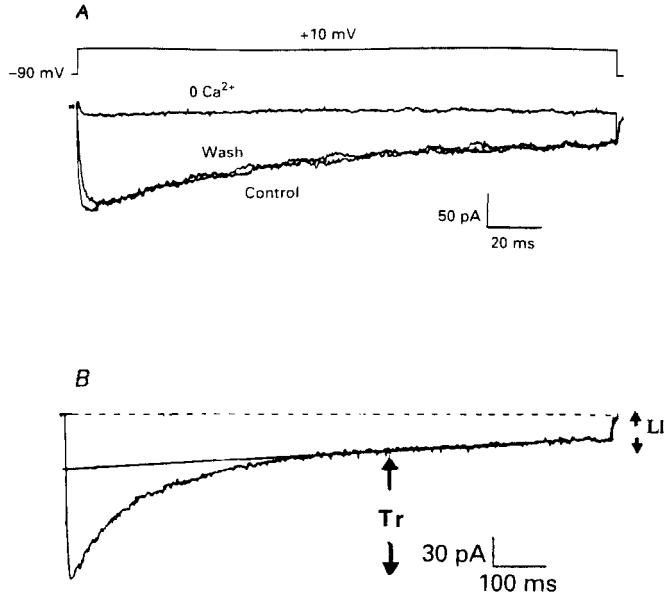

FIGURE 1. The transient- and long-lasting-type components of neurohypophyseal terminal I_{Ca}. (A) An example of current traces recorded from a nerve terminal in 10 mM Ca^{2+} bath solution. Current obtained from a holding potential (HP) of -90 mV, stepping to $+10$ mV. I_{Ca} disappears in 0 Ca^{2+} (replaced by 10 mM TEA-Cl) bath solution. The complete I_{Ca} can be washed back after returning to 10 mM Ca^{2+} bath solution. (B) The inactivating phase of I_{Ca} exhibits two exponential functions: a fast-inactivating, transient (Tr) component with a τ_i of ~ 30 ms and a slow-inactivating, long-lasting (Ll) component with a τ_i of ~ 1000 ms. Normally, the last 100 (minimum) points in the trace were averaged in order to measure the long-lasting (Ll) component. This value was subtracted from the peak (total) current to provide a measurement of the transient (Tr) component. The dashed line represents baseline current and the solid line is the fit to the Ll component.

RESULTS

Long-lasting and Transient Components of I_{Ca} in Peptidergic Nerve Terminals

We recorded, by stepping to $+10$ mV from -90 mV, a macroscopic calcium current (I_{Ca}) from individual, isolated nerve terminals (as shown in FIGURE 1A), whose peak followed a TTX-sensitive I_{Na} and which consisted of fast-inactivating (transient) and slowly inactivating (long-lasting) components.[7] Furthermore, both components of the inward current can be abolished by either removing all Ca^{2+} (FIGURE 1A) or adding 50 μM Cd^{2+} to the bathing solution.[29] Thus, the inward currents are indeed I_{Ca}. Under control conditions, because the slow activation (~ 10 ms) of the long-lasting Ca^{2+} current contaminates the faster activation (~ 2 ms) and

inactivation (23 ms) of the transient-type Ca^{2+} current as elicited by a depolarization from -90 mV to $+10$ mV,[7,29] the distinction between the two Ca^{2+} current components during a 250-ms pulse is not completely obvious; however, it is nevertheless possible to differentiate the two phases of the total current (see FIGURE 1B). The inactivating phase of the current evoked from a holding potential (HP) of -90 mV can be fitted with the sum of two exponentials (see FIGURE 1B): one is a rapidly decaying transient component with a time-constant of inactivation (τ_i) of 30–40 ms, corresponding to the transient type; the other is a long-lasting component with a τ_i of 700–1000 ms, corresponding to the long-lasting type.[7,29] Thus, only the long-lasting component is evident at the end of these 150-ms-long traces. This observation has been used in protocols to separate the two components using "whole-cell" recordings.[29] The long-lasting-type component of I_{Ca} can be more selectively activated by holding at a potential of -50 mV (FIGURE 2B), where the rapidly decaying transient component is inactivated.[7,29] This protocol might be the most effective in isolating the long-lasting-type component.

ω-CgTx 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 "rundown" or "washout" of I_{Ca}.[35] I_{Ca} "rundown" can occur very rapidly, within 3–5 minutes, in these nerve terminals. On the other hand, because ω-CgTx is known to irreversibly block Ca^{2+} channels,[11] no recovery of currents can be obtained. Our strategy for this toxin, in particular, was the following: Currents from a number of individual terminals were recorded immediately after breakthrough 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, we found that the untreated terminals did not show a change in baseline I_{Ca} during time periods (3–4 h) longer than those that separated the first control group and the last drug-treatment group.[29]

FIGURE 2A 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 transient-type I_{Ca} was significantly reduced at a concentration of 30 nM ω-CgTx (FIGURE 2C) and the inhibition was dose-dependent. The half-maximal inhibition (IC_{50}) of transient-type I_{Ca} by ω-CgTx was calculated to be 50 nM. At higher concentrations, we found that ω-CgTx also reduced the long-lasting component of the I_{Ca}. FIGURE 2B 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 the peak long-lasting I_{Ca} was significantly reduced at a concentration of 300 nM ω-CgTx (FIGURE 2D) and this reduction was dose-dependent. The half-maximal inhibition (IC_{50}) of long-lasting-type current by ω-CgTx was calculated to be 513 nM. Even at the highest concentration (2 μM) tested, however, an appreciable amount ($\sim 20\%$) of transient I_{Ca} remained (FIGURE 2C).

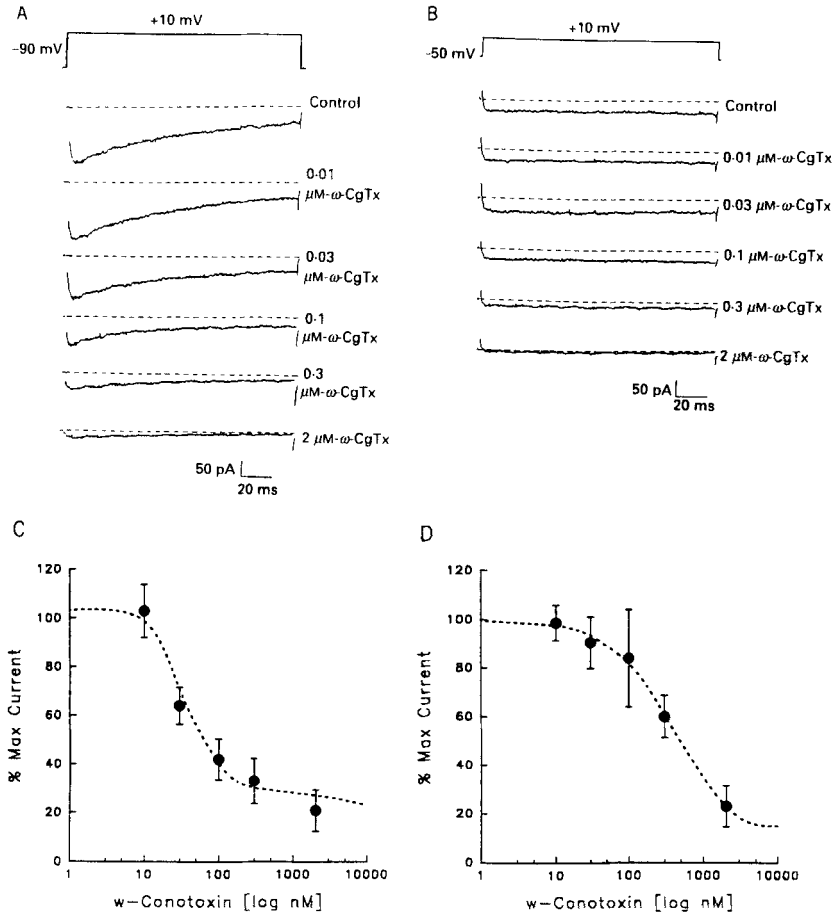

FIGURE 2. Effects of ω-CgTx on the terminal I_{Ca}. (A) Representative traces of the transient-type component of I_{Ca}, elicited as in FIGURE 1A, inhibited by ω-CgTx at the concentrations as noted at right. (B) Effects of ω-CgTx on the long-lasting-type component elicited as indicated above. Representative traces of I_{Ca} inhibited by ω-CgTx at the concentrations as noted at right. (C) Dose-response relationship shows the mean ± SEM (n = 5-9) amplitudes of Tr peak currents, as measured in FIGURE 1B, elicited by a voltage step as indicated above in the absence or presence of various ω-CgTx concentrations. These data were fit (dashed line) using the Langmuir adsorption isotherm, $y = B_{max} \cdot \{1/[x/(IC_{50} + x)]\}$, where x is the CgTX concentration. (D) Dose-response relationship shows the mean ± SEM (n = 5-10) amplitudes of the peak currents, elicited as in part B, in the absence or presence of various ω-CgTx concentrations. These data were also fit (dashed line) using the Langmuir adsorption isotherm. Modified from reference 29.

Inhibition of AVP Release by ω-CgTx

The above findings corroborate peptide release studies from the same preparation. Release of the peptide hormones, arginine vasopressin (AVP) and oxytocin, from these nerve terminals can be measured by radioimmunoassay (for review, see reference 32). The binding of ω-CgTx to specific receptors on these nerve terminals and its effects on high potassium and electrically induced release of AVP have been examined.[31] There is a high affinity for ω-CgTx in these terminals and, at nM concentrations, the toxin can inhibit both high K^+ and electrically stimulated AVP release from the nerve terminals of the neurohypophysis (FIGURE 3). The maximum inhibition of release, however, was only 78%, even at the highest (300 nM) concentration tested (FIGURE 3, inset). This was true even when maximal concentrations of DHP antagonist and ω-CgTx were added together.[31] In order to determine what other, if any, Ca^{2+} channels could underlie the resistant Ca^{2+}-dependent release, other toxins were tested.

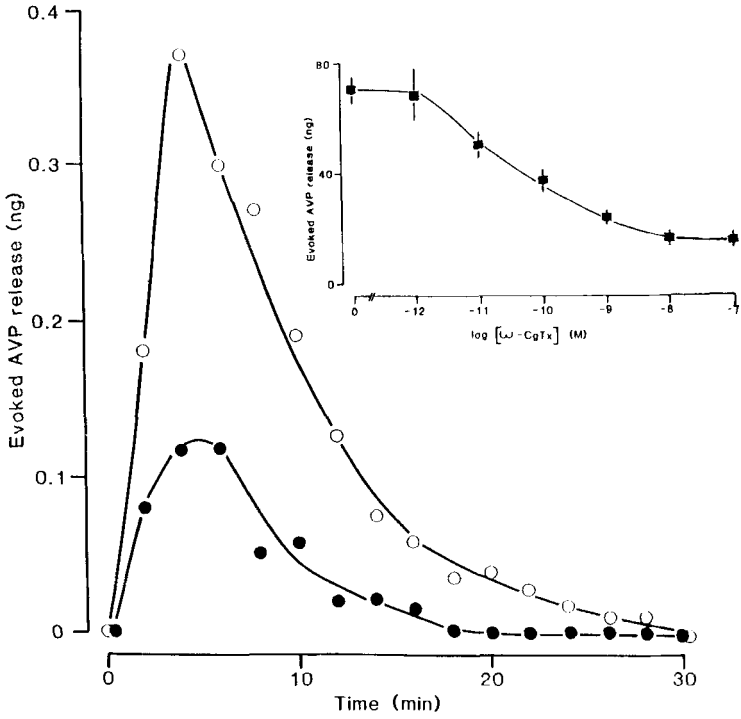

FIGURE 3. Effects of ω-CgTx on high K^+ and electrically stimulated AVP release. The neural lobe was stimulated with a pattern of firing mimicking the electrical activity of magnocellular neurons of the hypothalamus. Ten nM ω-CgTx (closed circles) inhibited the evoked AVP release down to 28% of control (open circles). Insert: Dose-response curve of ω-CgTx effects on high (100 mM) K^+-evoked AVP release. The results are given as mean ± SEM ($4 < n < 7$). Modified from reference 31.

Dose-dependent Inhibition of the Transient I_{Ca} by External FTX

The effects of funnel-web spider toxin (FTX), a polyamine that reportedly specifically blocks P-type Ca^{2+} channels,[36] on the Ca^{2+} currents of the neurohypophyseal nerve terminals were investigated. The long-lasting I_{Ca} component elicited by depolarizations from HP = −50 mV was insensitive to the external application of high doses of FTX (FIGURE 4), except for the residual transient component normally seen at this HP.[29] FTX had little effect on the long-lasting I_{Ca} component at any voltage (FIGURE 4a), but there is an approximately 10-mV depolarizing shift in the *I*-V relationship of the long-lasting I_{Ca} component.[37] The transient component of I_{Ca} elicited from −90 mV, however, was significantly inhibited by externally applied FTX in a dose-dependent manner (FIGURE 4b). This inhibition by FTX could be reversed by washing.

Inhibition of Long-lasting Component of I_{Ca} by Tetrandrine

In FIGURE 5A, the external application of 33 μM tetrandrine, a bis-benzylisoquinoline alkaloid, strongly inhibited the long-lasting I_{Ca}, whereas the I_{Na} and the transient-type I_{Ca} remained unaffected.[19] The IC_{50} for tetrandrine to inhibit the noninactivating Ca^{2+} current is 10.1 μM.[19,38] The transient-type I_{Ca} could only be partially inhibited if the concentration of tetrandrine was increased to 50 μM. Any

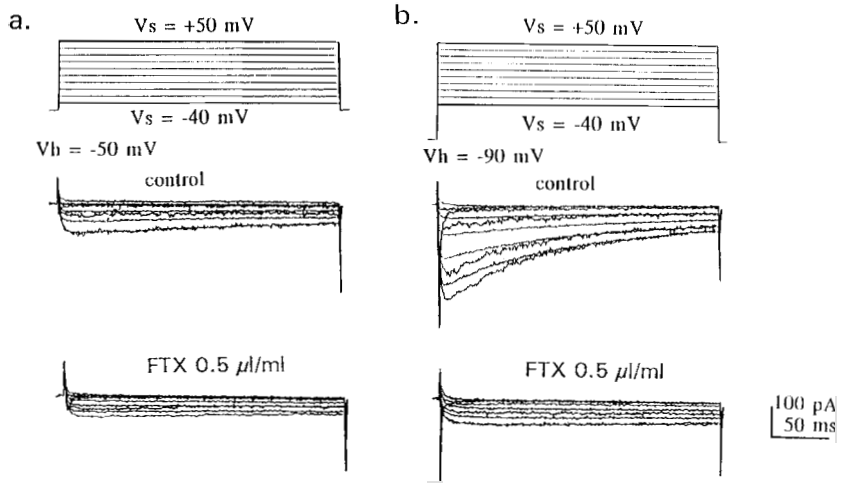

FIGURE 4. Effects of external application of FTX on high-voltage-activated I_{Ca} recorded from isolated rat neurohypophyseal nerve terminals using the "whole-cell" configuration. (a) Representative traces of the long-lasting I_{Ca} elicited by steps from HP = −50 mV to the voltages indicated. As much as 0.5 μL/mL FTX failed to affect this current. (b) Representative traces of both transient and long-lasting I_{Ca} elicited by stepping from HP = −90 mV to the voltages indicated. There is a reduction by FTX of the amplitude of only the transient component of I_{Ca}.

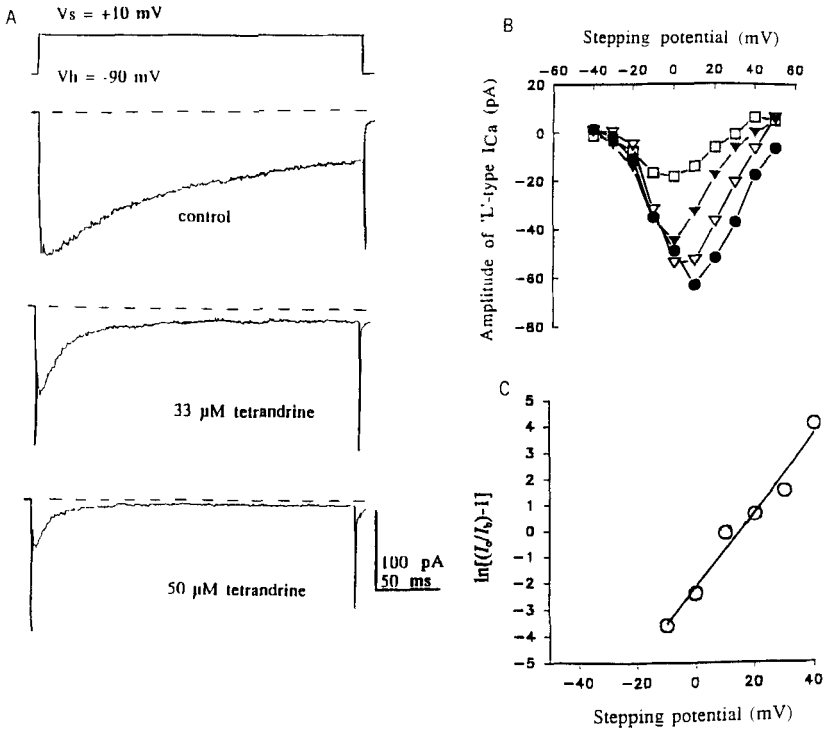

FIGURE 5. Effects of external application of tetrandrine on I_{Ca} recorded from rat neurohypophyseal nerve terminals using the "whole-cell" configuration. (A) The I_{Ca} values in control conditions and in the presence of tetrandrine were recorded from different terminals. The current was maximally elicited by a depolarization from −90 mV to +10 mV. Note that the initial, faster I_{Na} has been cut off in this figure. The dashed lines indicate the baselines of the currents. (B) The *I*-V curves of the L-type I_{Ca} in control conditions (●) and in the presence of 3 μM (▽), 10 μM (▼), and 30 μM (□) tetrandrine. (C) Voltage-dependence of inhibition of the L-type I_{Ca} by tetrandrine. The ratios of currents ($\ln[(I_o/I_b) - 1]$, where I_o and I_b are the control and the blocked current, respectively) of the macroscopic L-type I_{Ca} (○) were plotted against the step potentials using the results of control versus 10 μM tetrandrine from part B. Modified from reference 19.

effect of tetrandrine on the inactivated time-constant of transient-type I_{Ca} was evaluated as well. The mean time-constant of the total I_{Ca} treated with 33 μM tetrandrine appeared to be decreased to 16 ± 3.5 ms (n = 4) from 72.7 ± 16 ms of the control (n = 3), but this is similar to the time-constant for inactivation (23 ms) of the isolated transient component.[7] Moreover, treatment with a higher concentration (50 μM) of tetrandrine did not further reduce the inactivation time-constant (17.8 ± 8.2 ms, n = 3), indicating that tetrandrine has no direct effect on the inactivating rate of the transient-type I_{Ca}. Inhibition of both I_{Ca} components could be reversed by washing.

In order to evaluate any voltage-dependence of inhibition of the L-type I_{Ca} by tetrandrine, we examined its effects at different step potentials. The *I*-V curves of

this current, in the absence and presence of tetrandrine, reveal that the inhibition by tetrandrine of the L-type I_{Ca} is not proportionally the same at each potential (FIGURE 5B). Examination of the plot of $\ln[(I_o/I_b) - 1]$ against membrane potential (FIGURE 5C) demonstrates that the slope of the inhibition for the L-type I_{Ca} is 14, about 11 times steeper than that for $I_{K(Ca)}$, indicating that tetrandrine's inhibition of the noninactivating I_{Ca} is strongly voltage-dependent.[19]

FTX-sensitive I_{Ca} versus Tetrandrine-insensitive I_{Ca}

In order to avoid any dihydropyridine-dependent artifacts, we chose tetrandrine, a nondihydropyridine L-type Ca^{2+} channel blocker (see references 19 and 38, as well as above), to block the long-lasting Ca^{2+} current. Thus, we could more clearly examine the interaction of FTX with the remaining transient I_{Ca}. FTX could fully inhibit the remaining transient I_{Ca} in a dose-dependent manner (FIGURE 6A). Fitting the dose-response curve using the Langmuir adsorption isotherm to the data gave an IC_{50} of 0.04 μL/mL (FIGURE 6B). A pipette-perfusion system was utilized to apply FTX to a terminal so rapidly that the I_{Ba} could be recorded under both control conditions and in the presence of FTX before obvious "rundown" of the current could take place. As illustrated in FIGURE 7, 0.1 μL/mL FTX eliminated only the inactivating I_{Ba}, whereas the long-lasting I_{Ba} remained unaffected. The FTX-sensitive I_{Ba} (upper trace) is the difference between control I_{Ba} and FTX-treated I_{Ba}.[37]

ω-AgaIVA Effects on Neurohypophyseal Terminal I_{Ca}

The funnel-web spider polypeptide toxin, ω-AgaIVA, potently and specifically blocks P-type Ca^{2+} channels in neuronal cell bodies.[22,24] FIGURE 7 shows that the inhibition of the inactivating Ca^{2+} current by ω-AgaIVA-toxin appears similar to that observed using the polyamine, FTX. The inactivating Ca^{2+} current was strongly inhibited by externally applied ω-AgaIVA with an IC_{50} of approximately 15 nM.[39] In contrast, the noninactivating Ca^{2+} current was not significantly affected by the same concentration of ω-AgaIVA. Thus, ω-AgaIVA is a potent blocker of the high-threshold, fast-inactivating Ca^{2+} current of neurohypophyseal terminals.[39]

The neurohypophyseal transient Ca^{2+} current shows voltage-dependent recovery from block by ω-AgaIVA (FIGURE 8). Trains of strong depolarizations (to +80 mV) were able to apparently "knock off" the peptide toxin from the terminal Ca^{2+} channel, as has been reported for neuronal cell bodies.[24]

Block of the Transient I_{Ca} by a Combination of ω-Conotoxin GVIA and FTX

We have shown that extracellular ω-conotoxin GVIA dose-dependently inhibited the transient I_{Ca} in the nerve terminals, but with a maximal reduction of about 80%.[29] Because the present results demonstrate that the funnel-web spider toxins are also able to inhibit the transient I_{Ca}, it seemed necessary to test if the ω-conotoxin GVIA-resistant I_{Ca} could be further affected by the spider toxins. As illustrated in FIGURE

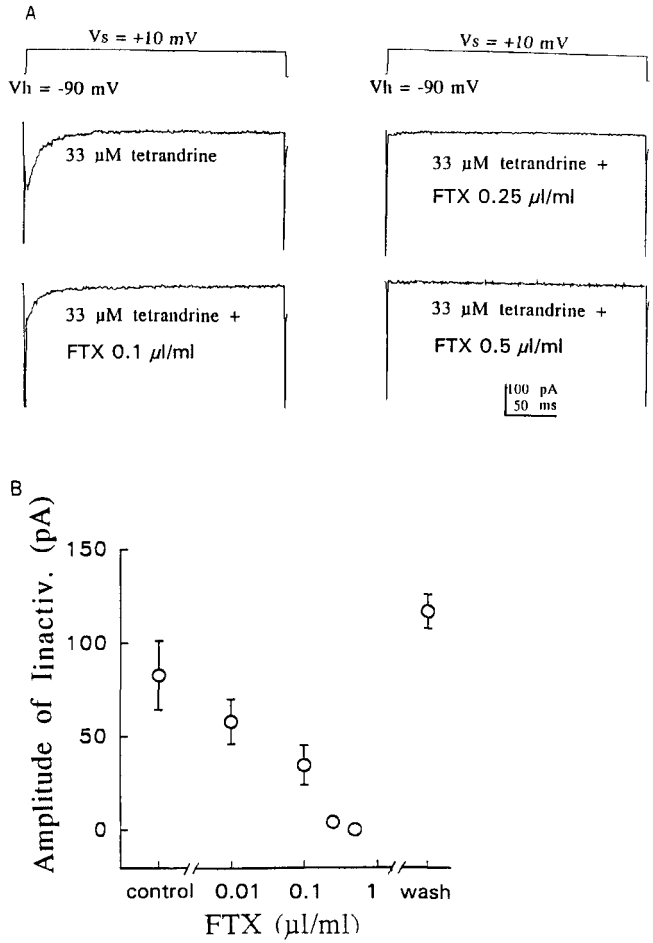

FIGURE 6. FTX blocks the tetrandrine-resistant component of I_{Ca} of the nerve terminals. (A) I_{Ca} was elicited by stepping from −90 mV to +10 mV as indicated. The external application of tetrandrine produced profound inhibition on the long-lasting I_{Ca} (see FIGURE 5A). The remaining transient I_{Ca} could be blocked by FTX in a dose-dependent manner. These effects could be reversed by washing. (B) The dose-response inhibition by FTX of the tetrandrine-resistant transient I_{Ca} could be fit using the Langmuir adsorption isotherm, giving an $IC_{50} \approx 0.1$ μL/mL. This is from a total of 16 terminals (5 controls, 2 at 0.01, 4 at 0.1, 3 at 0.25, and 2 at 0.5 μL/mL FTX).

9, after pretreatment with 700 nM ω-conotoxin GVIA, the amplitudes of transient and long-lasting calcium currents, when the terminals were depolarized by stepping from −90 mV to 0 mV, were 88.4 ± 6.7 pA and 75.2 ± 2 pA, respectively (n = 3). If 700 nM ω-conotoxin GVIA and 0.25 μL/mL FTX were simultaneously present in the bath solution, the amplitudes of transient I_{Ca} were reduced to 9.8 ± 10.9 pA

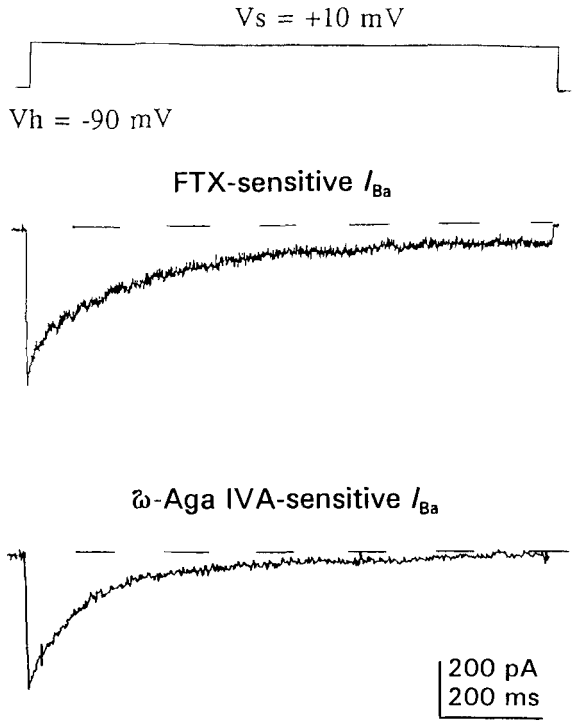

FIGURE 7. Similar inhibition by FTX and ω-AgaIVA of the transient I_{Ba} (using 10 mM Ba^{2+} solution) of the neurohypophyseal terminals. Using the perfusion pipette, FTX (0.1 μL/mL) was externally applied to the same terminal following a control recording by stepping as indicated above. The FTX-treated current was subtracted from the control to obtain the FTX-sensitive I_{Ba} (upper). Similarly, treatment with 15 nM ω-AgaIVA resulted in an ω-AgaIVA-sensitive I_{Ba} (lower). The dashed lines are baseline currents.

($n = 4$, $P < 0.01$), whereas the amplitudes of long-lasting I_{Ca} were not significantly affected (83.6 ± 5 pA, $n = 4$, $P > 0.05$). Thus, FTX can effectively block the ω-CgTx-resistant transient Ca^{2+} current of neurohypophyseal terminals.

Effects of FTX on AVP Release

FIGURE 10 shows preliminary evidence that high K^+-induced release of AVP was partially (22%) inhibited by a high dose (0.5 μL/mL) of FTX. Whether this partial reduction can be attributable to a P-type channel component and/or if this corresponds to the Ca^{2+}-dependent release that has been shown to be resistant to organic blockers requires more experiments. Taken together, these data strongly suggest that, in these nerve terminals of the neurohypophysis, multiple types of I_{Ca} are important for peptide release.

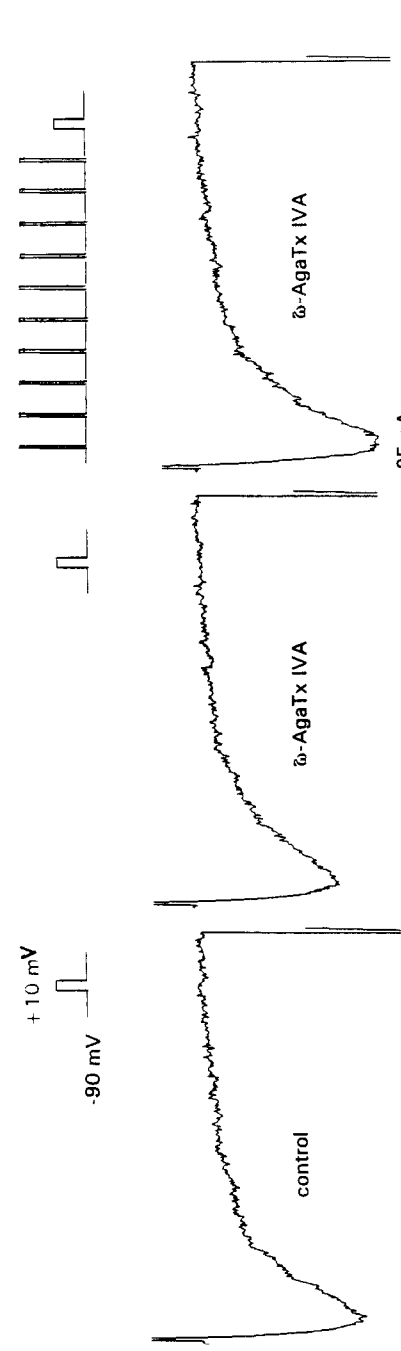

FIGURE 8. Voltage-dependent "knockoff" of ω-AgaIVA inhibition. Relief of block by ω-AgaIVA on Ca^{2+} currents was recorded using the perforated patch method. The I_{Ca} was elicited by stepping from −90 to +10 mV (left panel). The external application of 13 nM ω-AgaIVA partially inhibited the transient I_{Ca} (middle panel). Using a train of 10 strong depolarizing pulses to +80 mV (as indicated), the blocked Ca^{2+} current was fully recovered in the continuous presence of ω-AgaIVA (right panel).

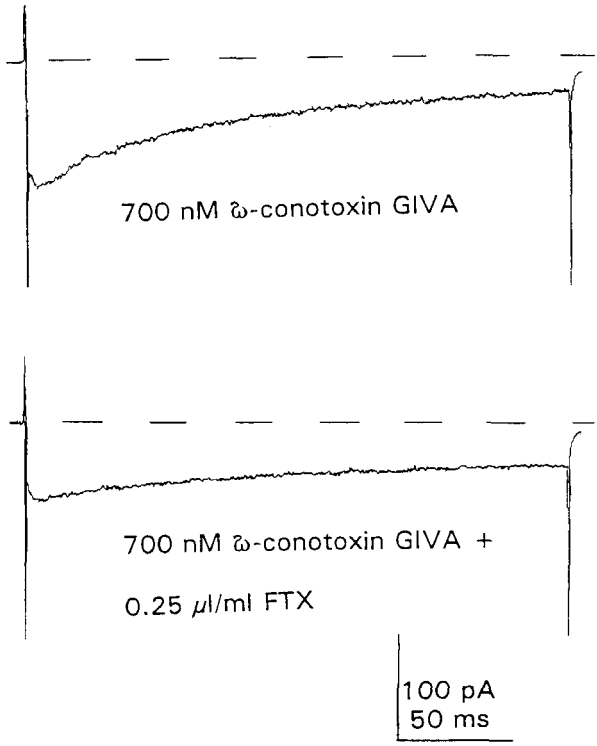

FIGURE 9. FTX blocks the ω-conotoxin GVIA–resistant component of the transient I_{Ca}. The transient I_{Ca}, elicited by stepping to +10 mV from −90 mV, could be partially inhibited by external application of 700 nM ω-conotoxin GVIA (top). Addition of 0.25 μL/mL FTX to the 700 nM ω-conotoxin GVIA in the bath could significantly reduce the amplitude of the transient I_{Ca} that is resistant to ω-conotoxin GVIA (bottom). The dashed lines are baseline currents.

DISCUSSION

Two Kinetically Distinct Components of I_{Ca}

There are at least two different (transient and long-lasting) types of high-threshold, voltage-gated Ca^{2+} channels in the isolated neurohypophyseal peptidergic nerve terminals.[7,29] The properties of the two types of Ca^{2+} channels in these nerve terminals have been previously studied using single-channel patch-clamp techniques.[7] The transient channels had a smaller conductance, high threshold for activation, were inactivated rapidly, and also were not sensitive to dihydropyridines. The long-lasting-type channels, which inactivated slowly, had a larger conductance, high threshold for activation and were dihydropyridine-sensitive to both agonists and antagonists.

ω-CgTx can reduce both the transient and long-lasting components of I_{Ca} in these

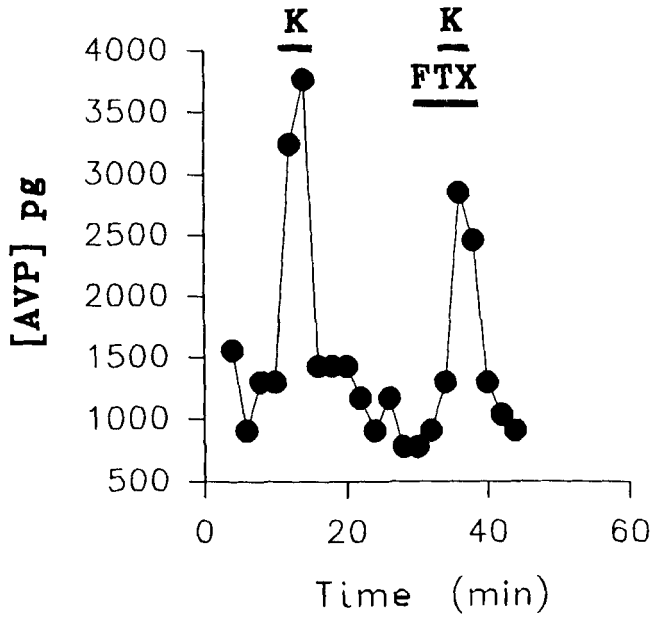

FIGURE 10. FTX effects on high K^+-stimulated AVP release. AVP release was stimulated by high (50 mM) potassium as indicated by the solid bars with the K above. FTX (0.5 μL/mL) was applied (solid bar) only during the second stimulation. All experiments were performed in 40 mM Na^+ saline so as not to reduce Na^+ and increase K^+ at the same time.

nerve terminals. The effects on the transient type are in agreement with many previous findings showing that ω-CgTx can block "N"-type Ca^{2+} channels. However, our studies have demonstrated that the long-lasting-type component of I_{Ca} can also be reduced by higher concentrations of ω-CgTx, suggesting that long-lasting-type Ca^{2+} channels might also be sensitive to ω-CgTx, although 10 times less so than the transient type. Nevertheless, ω-CgTx can affect the long-lasting-type Ca^{2+} channels in these nerve terminals. These findings corroborate AVP release studies from the same preparation. The influx of Ca^{2+} via membrane voltage-gated channels is usually associated with transmitter release from nerve terminals. The activation of the long-lasting-type Ca^{2+} channels is the main factor for inducing an increase in cytoplasmic Ca^{2+} (see references 40 and 41) and in AVP release.[8] AVP release from the nerve endings, which is also highly sensitive to dihydropyridines,[30] can be strongly inhibited by ω-CgTx (FIGURE 3).[31]

Previous electrophysiological studies,[11,13] together with our present data, have shown that I_{Ca} is not completely blocked by this toxin. Even though the L-type channels are selectively blocked by nifedipine and the "N"-type channels by ω-CgTx, recent studies by Schroeder and colleagues[15] on rat dorsal root ganglion neurons have shown that the two together fail to block all of I_{Ca}. Biochemical studies[31] also showed that ω-CgTx could not totally abolish the rise in nerve terminal free internal

Ca^{2+} concentrations induced by high potassium depolarization and that, subsequently, a small, but substantial amount (~20%) of AVP release was insensitive to both this toxin and the dihydropyridines.

Other Types of Ca^{2+} Channels in Neurohypophyseal Terminals?

In order to determine what other, if any, Ca^{2+} channels could underlie the above-mentioned resistant Ca^{2+}-dependent release, other toxins were tested. Tetrandrine, a Chinese plant toxin, has a strong hypotensive action due not only to vasodilation, but also possibly due to its inhibiting the release of vasopressin from the neurohypophyseal terminals. Tetrandrine has been reported to have verapamil-like actions on the cardiovascular system.[16] Binding studies showed that tetrandrine blocked diltiazem and D-600 binding, but stimulated nitrendipine binding.[18] It has been shown to be a more effective blocker of "T"-type versus "L"-type channels.[20,38] Our results indicate that the inhibition by the alkaloid of the long-lasting I_{Ca} differs from that of the transient-type I_{Ca} in that lower concentrations (3–33 μM) block only the long-lasting Ca^{2+} channel; however, at higher concentrations ($\geq$50 μM), it could also block the transient-type Ca^{2+} channel. This is another bit of evidence showing that the transient I_{Ca} in the terminals is not a T-type channel. Our results show that the IC_{50} for tetrandrine's effect on the long-lasting Ca^{2+} current of nerve terminals is 10.1 μM, almost identical to that observed for vessel smooth muscles *in vitro*.[16] The fact that the ability of tetrandrine to inhibit the long-lasting Ca^{2+} channel current was strongly enhanced by depolarization (FIGURE 5C) could account for its voltage-dependent negative inotropism on the myocardium.[16]

Venom of the funnel-web spider, *Agelenopsis aperta*, contains the polyamine FTX and the peptide ω-AgaIVA. The present findings showing that FTX potently inhibits the transient component of I_{Ca} in these nerve terminals, with little effect on the long-lasting I_{Ca}, indicate that there are structural differences in the Ca^{2+} channels underlying these I_{Ca}. Because this toxin has been shown to be a specific P-type Ca^{2+} channel blocker,[21,23,25,36] it should nominally be a P-type Ca^{2+} channel that underlies the FTX-sensitive transient I_{Ca} in the neurohypophyseal nerve terminals. However, the high-threshold Ca^{2+} conductances sensitive to FTX or ω-Agatoxin IVA in neuronal cells and expressed in *Xenopus* oocytes from rat brain mRNA show little inactivation.[21,23–25] It is not clear why the kinetics of the funnel-web spider toxin–sensitive Ca^{2+} conductance in these nerve terminals are quite different, but they are similar to each other in terms of sensitivity to the spider toxins and in single channel conductance.

Conclusions

There are at least two (transient and long-lasting) types of high-threshold, voltage-activated Ca^{2+} channels that coexist in the peptidergic nerve terminals of the rat neurohypophysis. ω-CgTx can block both types of Ca^{2+} channels, but with the transient type being 10 times more susceptible. This toxin also strongly inhibited the potassium and electrically induced release of vasopressin from the neurohypophyseal

nerve terminals. The results from these two observations indicate that a Ca^{2+} channel with a high affinity for ω-conotoxin GVIA binding in the terminals underlies the transient I_{Ca} that plays an important role in depolarization-secretion coupling of neurohormones. However, a small, but significant depolarization-induced vasopressin release is insensitive to both ω-conotoxin GVIA and the dihydropyridine antagonist nicardipine,[31] indicating that there could be another Ca^{2+} channel involved in this process. Preliminary data (see FIGURE 10) indicate that FTX can also inhibit Ca^{2+}-dependent peptide release.[26,27] Taken together, these data strongly suggest that, in these nerve terminals of the neurohypophysis, more than two types of I_{Ca} are important for peptide release.

Our findings show that FTX and ω-AgaIVA, like ω-conotoxin GVIA, can inhibit the transient I_{Ca} in the nerve terminal. Possible mechanisms that could account for these overlapping inhibitions of the transient I_{Ca} could be as follows: First, whereas the ω-conotoxin GVIA-sensitive Ca^{2+} channel underlies only a portion of the transient I_{Ca}, the funnel-web spider toxin-sensitive Ca^{2+} channel underlies the remainder of the transient I_{Ca}. Therefore, the high-threshold, inactivating Ca^{2+} current in these isolated nerve terminals has a "P"-type Ca^{2+} channel component that is, however, different in its inactivation from previously reported "P"-type Ca^{2+} currents. Second, there may be binding sites for both ω-conotoxin and the funnel-web spider toxins on the same "novel" channel underlying the transient I_{Ca}. These differences in structure and function and in the interactions of the blockers with the inactivating Ca^{2+} conductance need to be probed by more direct methods, such as single channel recording.

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