A New ω-Conotoxin That Targets N-Type Voltage-Sensitive Calcium Channels with Unusual Specificity†

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ABSTRACT: A new specific voltage-sensitive calcium channel (VSCC) blocker has been isolated from the venom of the fish-hunting cone snail *Conus consors*. This peptide, named ω-Ctx CNVIIA, consists of 27 amino acid residues folded by 3 disulfide bridges. Interestingly, loop 4, which is supposed to be crucial for selectivity, shows an unusual sequence (SSSKGR). The synthesis of the linear peptide was performed using the Fmoc strategy, and the correct folding was achieved in the presence of guanidinium chloride, potassium buffer, and reduced/oxidized glutathione at 4 °C for 3 days. Both synthetic and native toxin caused an intense shaking activity, characteristic of ω-conotoxins targeting N-type VSCC when injected intracerebroventricularly to mice. Binding studies on rat brain synaptosomes revealed that the radioiodinated ω-Ctx CNVIIA specifically and reversibly binds to high-affinity sites with a K_d of 36.3 pM. Its binding is competitive with ω-Ctx MVIIA at low concentration (K_i = 2 pM). Moreover, ω-Ctx CNVIIA exhibits a clear selectivity for N-type VSCCs versus P/Q-type VSCCs targeted respectively by radioiodinated ω-Ctx GVIA and ω-Ctx MVIIC. Although ω-Ctx CNVIIA clearly blocked N-type Ca^{2+} current in chromaffin cells, this toxin did not inhibit acetylcholine release evoked by nerve stimuli at the frog neuromuscular junction, in marked contrast to ω-Ctx GVIA. ω-Ctx CNVIIA thus represents a new selective tool for blocking N-type VSCC that displays a unique pharmacological profile and highlights the diversity of voltage-sensitive Ca^{2+} channels in the animal kingdom.

Peptide toxins from *Conus* venoms represent useful tools for studying the structure and functional properties of ionic channels and receptors. For the past 2 decades, a number of conotoxins have been shown to target voltage-gated Na^+, K^+, and Ca^{2+} channels, as well as ligand-gated nicotinic acetylcholine and 5-HT receptors (*1*). Voltage-sensitive Ca^{2+} channels are heteromultimeric proteins consisting of an α1 main subunit and auxiliary α2δ and β subunits. The α1 subunit forms the ion-conducting pore and contains receptor sites for ligands that modify channel activity (*3*). ω-Conotoxins selectively inhibit different subtypes of voltage-sensitive calcium channels (VSCCs)[1] (*2*). Thus, ω-Ctx GVIA and ω-Ctx MVIIA, isolated respectively from the venom of the fish-hunting *C. geographus* and *C. magus*, are widely used as pharmacological agents to block the N-type calcium channel (*4*, *5*). Recently, ω-Ctx CVID isolated from *Conus catus* was shown to exhibit the highest selectivity for N-type versus P/Q-type calcium channels, as revealed by radioligand binding assays (*42*). The primary structures of two other ω-conotoxins were identified from a cDNA library constructed from the venom duct of *C. magus* (*6*, *7*). The pharmacological profile of these two conotoxins, ω-Ctx MVIIC and ω-Ctx MVIID, exhibits broader specificity than ω-Ctx GVIA or ω-Ctx MVIIA for non-L-type VSCCs. Both conotoxins inhibit P/Q-type VSCCs at low concentrations and block ω-Ctx GVIA-sensitive channels at higher concentrations. Few other ω-conotoxins have been identified from the venom of *C. striatus*, *C. tulipa*, and *C. radiatus* that provided guidelines for structure−activity studies (*8*). The unique properties of ω-conotoxins make them valuable tools for discrimination, characterization, and localization of calcium channel subtypes in vertebrate preparations such as frog and mouse neuromuscular junctions (*9*), rat brain (*10*, *11*), and

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[1] Abbreviations: ω-Ctx CNVIIA, ω-conotoxin-CNVIIA from *Conus consors*; ω-Ctx GVIA, ω-conotoxin-GVIA from *Conus geographus*; ω-Ctx MVIIA and ω-Ctx MVIIC, ω-conotoxin-MVIIA and ω-conotoxin-MVIIC from *Conus magus*; ω-Ctx CVID, ω-conotoxin-CVID from *Conus catus*; VSCC, voltage-sensitive calcium channel; TFA, trifluoroacetic acid; EPP, endplate potential; HPLC, high-performance liquid chromatography.

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chromaffin cells (*12*). Indeed, at the frog neuromuscular junction, nerve-evoked quantal acetylcholine (ACh) release is mediated by N-type VSCCs based on the strong inhibitory effect of ω-Ctx GVIA (*13*, *14*) and the lack of effect of dihydropyridines (L-type VSCC blockers) and ω-Ctx MVIIC (P/Q-type VSCC blockers). In contrast, evoked quantal ACh release is mediated by P/Q-type VSCCs at mouse and rat mature neuromuscular junctions (*15*). In addition to their use as neurobiological tools, clinical studies have shown that ω-conotoxins which target N-type VSCCs have therapeutic potential in pain and ischemic brain injury (*16*).

In the search of new VSCC peptide inhibitors, we have investigated the venom of *C. consors*, an Indo-Pacific species known to contain α-conotoxins and a sodium channel activator (*17*, *18*). Present results describe the isolation, the chemical synthesis, and the pharmacological characterization of a new specific N-type calcium channel-targeted peptide.

EXPERIMENTAL PROCEDURES

Materials. Specimens of *C. consors* were collected in Chesterfield Island (New Caledonia) and immediately frozen at −80 °C. *C. consors* venom was obtained as previously described for *C. geographus* (*19*). Briefly, the venom was squeezed out from freshly dissected venom ducts, extracted with H_2O/0.8% TFA, and then lyophilized. All Fmoc-protected amino acids, 4-(2′,4′-dimethoxyphenyl-hydroxy)-methylphenoxy polystyrene resin (Rink amide resin, 0.55 mequiv/g), were obtained from Novabiochem (Laüfelfingen, Switzerland). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate was obtained from Novabiochem. *N*-Methylpyrrolidone, trifluoroacetic acid, and acetonitrile were from SDS (Peypin, France). Other solvents and chemicals were purchased from commercial sources and were of the highest purity commercially available. All HPLC purifications and analyses were performed at room temperature on a Thermo Separation Products (TSP) high-pressure liquid chromatography, equipped with a TSP UV-150 wavelength detector. The elution buffers used for all HPLC were the following: buffer A, H_2O/0.8% TFA; buffer B, H_2O/CH_3CN 40/60, unless otherwise stated.

Purification of the Toxin. Lyophilized *C. consors* venom (50 mg) was dissolved in H_2O/0.8% TFA, and the extract was loaded in batches of 10 mg on a semipreparative C18 Vydac column. Further purification steps were carried out using an analytical C18 Vydac column. Detection of peptides was carried out by monitoring the absorbance at 220 nm.

Amino Acid Analysis, Sequencing, and Mass Spectrometry. The purified peptides were sequenced using an Applied Biosystems 477A automatic microsequencer, and phenylthiohydantoin derivatives were identified using an Applied Biosystems 120A on-line analyzer. Electrospray ionization mass spectra (ESI-MS) were obtained on an API III+ mass spectrometer (Perkin-Elmer Sciex) equipped with a nebulizer-assisted electrospray source.

Chemical Synthesis. The peptidic toxin was synthesized by solid-phase methods on an Applied Biosystems−Perkin-Elmer (ABI−PE) 430A peptide synthesizer (Foster City, CA), using the Fmoc methodology. Chain elongation was performed step-by-step using 0.1 mmol of Rink amide resin (NovaBiochem) (0.55 mequiv/g) and 1 mmol of Fmoc amino acid derivatives. Amino acids were activated by 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate according to Fastmoc chemistry methodology (ABI−PE). Side chain protecting groups were trityl for Cys and His, butyloxycarbonyl for Lys, *tert*-butyl for Tyr, Asp, Ser, and Hyp, and Ng-2,2,5,7,8-pentamethylchroman-6-sulfonyl for Arg. After full assembly was completed, the linear peptide was cleaved from the resin by treatment with 10 mL of TFA/*m*-cresol/thioanisole/triisopropylsilane (92.5/2.5/2.5/2.5) for 2 h. After filtration, the reaction medium was precipitated in cold diethyl ether and centrifuged for 5 min at 500*g*, and the pellet (crude peptide) was washed 3 times. The linear peptide was then purified by preparative reverse-phase HPLC with a linear gradient of 10−80% B in 70 min at 10 mL/min flow rate (wavelength detection at 275 nm). The lyophilized linear peptide was diluted in water (0.02 mM) in the presence of guanidinium chloride (0.5 M), potassium dihydrogen phosphate (0.05 M), reduced glutathione (5 mM), and oxidized glutathione (0.5 mM). The pH of the solution was adjusted to 7.8 with KOH and stirred for 3 days at 4 °C. After acidification with 80% AcOH, the solution was directly loaded on a semipreparative C18 Vydac column eluted with a gradient of 0−60% B in 60 min (detection at 275 nm). An additional purification step was carried out on a semipreparative C18 Vydac column eluted with a linear gradient of 5−25% acetonitrile in 0.6% triethylammonium phosphate buffer (pH 2.25) followed by a final purification on a reverse-d column for desalting.

In Vivo Bioassays. The biological effects of the native and the synthetic toxin were studied after intramuscular (im) injections to fish (*Gambusia affinis*). Fish were injected with 5 μL of a saline solution (150 mM NaCl) containing different toxin concentrations. The toxin was also tested at different concentrations by intracerebroventricular (icv) injections (5 μL) in Swiss Webster mice (20−22 g) using a stereotaxic micromanipulator (Harvard/ASI Apparatus, Kent, England).

Electrophysiological Recordings on Isolated Neuromuscular Preparations. Cutaneous pectoris nerve−muscle preparations were removed from double-pithed male frogs (*Rana esculenta*) weighing 20−25 g. The isolated nerve−muscle preparation was bathed in standard frog physiological solution composed of (mM) 115 NaCl, 2.1 KCl, 1.8 $CaCl_2$, and 5 HEPES. In some experiments, excitation−contraction was uncoupled by treating the preparations with formamide (2 M) for 20 min (*20*). Left and right hemidiaphragm muscles with their associated phrenic nerves were isolated from Swiss Webster mice (20−25 g) killed by dislocation of the cervical vertebrae followed by immediate exsanguination. The two hemidiaphragms were separated, and each was mounted in a Rhodorsil-lined organ bath (2 mL volume) superfused with a physiological solution of the following composition (mM): 154 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES buffer, 11 glucose. For quantal analysis of transmitter release, experiments were performed in low Ca^{2+} (1 mM), high Mg^{2+} (6 mM) solution. The solution gassed with pure O_2 had a pH = 7.4. Membrane potentials and synaptic potentials were recorded with intracellular microelectrodes filled with 3 M KCl solution (resistance 8−12 MΩ) at room temperature (22−24 °C) as previously described (*17*). The motor nerve of the isolated neuromuscular preparation was stimulated via a suction microelectrode, and recordings were made continuously from the same endplate before and throughout application of the ω-conotoxin tested.

Patch-Clamp Recordings on Primary Cultures of Rat Embryonic Chromaffin Cells. Primary cultures of rat embryonic chromaffin cells were performed according to methods previously detailed (*21*). The whole-cell configuration of the patch-clamp technique was used (*40*). Patch electrodes were pulled from glass capillary tubes (Blu-Tip, Oxford, MA) using a programmable micropipet puller (model P-87, Sutter Instruments, Novato, CA) and coated with sticky wax (S. S. White, England) to reduce capacitance. The tip of the patch pipet had a resistance of 2−4 MΩ when filled with the pipet solution (for composition see below).

Membrane calcium channel currents were recorded with Ba^{2+} as a charge carrier using a RK 400 patch-clamp amplifier (Biologic, France). Pulse generation and acquisition of signals were performed through a TL-l Labmaster DMA board (Scientific Solutions, USA). The pClamp software (V.5.5 I., Axon Instruments, Foster City, CA) was used for data acquisition and analysis. The barium channel currents were evoked by depolarizing pulses (100 ms duration) from a holding potential (V_h) of −80 mV. It was usually possible to compensate for up to 80% of the series resistance without introducing oscillations into the recorded current. Although leak and capacitive currents were compensated electronically at the beginning of each experiment, subtraction of residual capacitive and leakage currents was performed with an online P/N protocol provided by the pClamp software.

The recording chamber was filled with a solution containing the following (mM): 137 NaCl, 1 $MgCl_2$, 10 $BaCl_2$, 10 HEPES−NaOH, 0.005 tetrodotoxin (pH 7.4). The patch pipet solution contained the following (in mM): 10 NaCl, 100 CsCl, 20 tetraethylammonium chloride, 5 MgATP, 14 ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 20 HEPES, pH adjusted to 7.2 with CsOH. ω-Conotoxins were dissolved in the external solution; nifedipine was dissolved in poly(ethylene glycol) in aliquots of 100 mM and diluted at 1 mM in external solution. The channel blockers were applied externally through a pipet placed near the target cell after the current amplitude became stable.

Radioiodination. The synthetic toxins ω-Ctx CNVIIA (this report), ω-Ctx MVIIC (Latoxan, France), and ω-Ctx GVIA (Latoxan, France) were radioiodinated using 1 nmol of toxin and 0.5 mCi of carrier-free $Na^{125}I$ in a potassium phosphate buffer (pH 7.25) containing H_2O_2 (10 μL of a 1/50 000 solution) and lactoperoxidase (0.7 unit, EC 1.11.1.7 from bovine milk). The reaction was terminated after 2 min by 1/10 dilution in buffer A, and direct injection on a Vydac C18 column. The iodinated toxins were eluted with a gradient of acetonitrile from 10 to 50% B in 50 min at a flow rate of 1 mL/min. The concentration of the radiolabeled toxin was determined according to the specific activity of the ^{125}I corresponding to 2500−3000 dpm/fmol of monoiodotoxin, depending on the age of the radiotoxin and by estimation of its biological activity (60−65% for ^{125}I-ω-Ctx CNVIIA).

Binding to Rat Brain Synaptosomes. Brain synaptosomes were prepared from adult Sprague−Dawley rats (300 g) according to the method described by Kanner (*22*). Equilibrium competitions were performed using increasing concentrations of unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. Hot competitions were realized using increasing concentrations of iodinated toxins. To obtain saturation curves, the specific radioactivity and the amount of bound toxin were calculated and determined for each toxin concentration. Equilibrium saturation experiments were analyzed by the iterative computer program LIGAND (Elsevier Biosoft, U.K.), and competition binding experiments were analyzed by the program Kaleidagraph (Synergy Software, USA) using a nonlinear Hill equation (for IC_{50} determination). The K_i values were calculated by the Cheng and Prusoff equation (*23*): $K_i = IC_{50}/(1 + L^*/K_d)$, where L^* is the concentration of the hot ligand and K_d is its dissociation constant. The standard binding medium was composed as follows (mM): 20 HEPES (pH 7.25), 75 NaCl, 0.1 EDTA, 0.1 EGTA, and 0.1% BSA. The washing medium consisted of (mM) 20 HEPES (pH 7.25), 125 NaCl, and 0.1% BSA. Rat brain synaptosomes (5−15 μg) were suspended in 2 mL of binding buffer containing the radioligand. After incubation for 30 min, the reaction mixture was filtered through GF/C filters presoaked in 0.3% polyethylenimine. Filters were rapidly washed with 2 × 2 mL of washing buffer. Nonspecific toxin binding was determined in the presence of a 100 nM sample of the cold toxin and consisted of 20−40% of the total binding for both ω-Ctx CNVIIA and ω-Ctx MVIIC. The kinetic data for ligand association and dissociation rates were subjected to analysis according to the method described by Weiland and Molinoff (*44*).

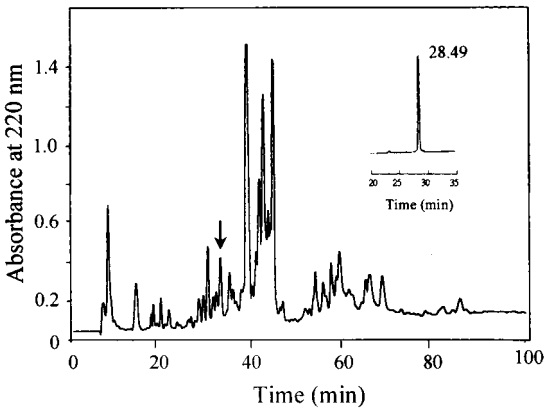

FIGURE 1: Purification of ω-Ctx CNVIIA from the venom of *Conus consors*. Main panel: Crude venom was applied to a semipreparative C18 Vydac column. The gradient program was 0−8% B/5 min, 8−80% B/70 min, 80−100% B/10 min, followed by 100% B/10 min (flow rate, 2 mL/min; buffer A = 0.1% TFA; buffer B = 0.1% TFA, 80% acetonitrile). Inset: Material eluting at 34.5 min (arrow in main panel) was run several times on an analytical C18 Vydac column with the gradient program 10−40% B/60 min (flow rate, 1 mL/min; buffer A = 0.1% TFA; buffer B = 0.1% TFA, 60% acetonitrile) to obtain the purified product.

RESULTS

Isolation and Biochemical Characterization of ω-Ctx CNVIIA. The crude venom of the cone snail *C. consors* was fractionated by liquid chromatography on a reverse-phase column. Each fraction was tested for biological activity by im injections to fish, the natural prey of *C. consors*. The fractions were also tested by icv injections to mice. The bioactive fraction eluting at 22% ACN (Figure 1) produced paralytic symptoms when injected im to fish and tremors when injected icv to mice. The purification step led to a sharp peak in the analytical HPLC, and the isolated compound was submitted to amino acid sequence analysis (Table 1) and mass spectrometry. The peptide is composed of 27 amino acids and possesses the cysteine framework of previously

Table 1: Edman Degradation of ω-Ctx CNVIIA

cycle	assigned residue[a]	yield (pmol)	cycle	assigned residue[a]	yield (pmol)
1	Cys	−	15	Cys	−
2	Lys	141	16	Cys	−
3	Gly	169	17	His	17
4	Lys	125	18	Gly	44
5	Gly	162	19	Ser	21
6	Ala	147	20	Cys	−
7	Hyp	147	21	Ser	16
8	Cys	−	22	Ser	20
9	Thr	78	23	Ser	14
10	Arg	42	24	Lys	8
11	Leu	76	25	Gly	14
12	Met	68	26	Arg	11
13	Tyr	38	27	Cys	−
14	Asp	33			

[a] Hyp is *trans*-4-hydroxyproline.

characterized conotoxins (Table 2). Further confirmation of the purity and the sequence assignment of the peptide was provided by electrospray mass spectrometry. The mass determination yielded a single product with a molecular mass of 2847.74 Da, which is in accordance with the calculated mass of the amidated form of the peptide with three disulfide bridges (2848.34 Da). The peptide is highly basic with a positive net charge of +4. Based on the chemical and electrophysiological results (see below), the peptide is designated as ω-Ctx CNVIIA.

Chemical Synthesis. To make ω-Ctx CNVIIA available for its pharmacological characterization, the chemical synthesis with an amidated C-terminus was carried out on the basis of the mass spectrometry analysis and sequence assignment. Starting with a Rink amide resin, the polypeptide chain assembly was performed using the Fmoc strategy with uronium activation (see Experimental Procedures). Deprotection and cleavage from the resin led to a major product with the desired mass. The purified linear peptide was subjected to different folding conditions in order to maximize the folding process. Temperature had to be maintained between 0 and 5 °C during the whole folding process for reasonable results. Low peptide concentrations (0.01−0.04 mM) and the use of guanidinium chloride significantly improved the yield of the cyclization by comparison with higher substrate concentrations. In our case, the redox couple GSH/GSSG proved to be more efficient than ambient air and cysteine/DTT to perform the folding step (Figure 2A,B). Most remarkably, the potassium phosphate buffer contributed to much better results than ammonium acetate (Figure 2C). Finally, the folding of the peptide (0.03 mM) was carried out at 4 °C for 3 days in the presence of guanidinium chloride (0.5 M), reduced/oxidized gluthatione (5 mM/0.5 mM) in a potassium hydrogen phosphate buffer (0.05 M, pH 7.8). Under these conditions, the linear precursor led to a major product with the nativelike disulfide bonding. A subsequent purification step by the TEAP/ACN (pH 2.25) chromatographic system allowed correct purification of the desired peptide. The folding yield of the synthetic peptide corresponded to approximately 10% of the linear peptide. The identity of the synthetic ω-Ctx CNVIIA with the natural toxin was confirmed by peptide sequencing; mass spectrometry analysis yielded a single product with a molecular mass of 2847.78 Da and coelution experiments by reverse-phase HPLC (Figure 2D). Correct synthesis of the desired product was also confirmed by the similar toxicological effects of the synthetic and native toxin in fish and mice. All experiments were thereafter carried out with the synthetic ω-Ctx CNVIIA.

Bioassays. The icv injection of native and synthetic ω-Ctx CNVIIA to mice caused shaking activity characteristic of ω-conotoxins targeting N-type VSCC (*24*). ω-Ctx CNVIIA, at 1.5 pmol/g, produced mild tremors in mice that became more intense as the amount injected was increased. The purified native toxin proved also to be very toxic by im injection to fish. Indeed, fish were unable to resist a weak water current and became paralyzed. ω-Ctx CNVIIA at 15 pmol/g of fish produced lethal effects. This lethal dose can be compared to that of ω-conotoxins SVIA and SVIB from *C. striatus* that paralyzed fish at doses of 20 pmol/g by im injection.

ω-Ctx CNVIIA at Vertebrate Neuromuscular Junctions. It was of interest to study the effect of ω-Ctx CnVIIA as compared to other ω-conotoxins on isolated frog and mouse mature neuromuscular preparations in which the N- and P/Q-type Ca^{2+} channels are respectively expressed in motor nerve terminals. Thus, the effects of the new ω-Ctx CNVIIA were investigated on quantal evoked transmitter release from nerve terminals. When applied to frog cutaneous pectoris nerve−muscle preparations, ω-Ctx CNVIIA (10 μM) affected neither the amplitude of endplate potentials (EPPs) nor that of miniature endplate potentials recorded on the same junctions as compared to controls (Figure 3A). These results indicate that ω-Ctx CNVIIA had no effect on the quantal content of EPPs ($n = 4$) and do not block N-type VSCCs of motor nerve terminals. In contrast, when using the same experimental approach, the N-type ω-Ctx GVIA (1 μM) markedly reduced the quantal content of EPPs by reducing EPP amplitudes, as shown in Figure 3A. Furthermore, when experiments were performed in mature mouse neuromuscular preparations that express P/Q-type Ca^{2+} channels in motor nerve terminals, ω-Ctx CNVIIA had no action on the quantal content of EPPs whereas 1 μM ω-Ctx MVIIC produced a strong decrease in the quantal content of EPPs (Figure 3B). It was therefore of interest to determine whether ω-Ctx MVIIA, which previously was shown to act on mammalian N-type VSCCs, was active on frog motor nerve terminals. However, when ω-Ctx MVIIA (0.5−7 μM) was tested in the same conditions as ω-Ctx CnVIIA, no effect was detected on the quantal content of EPPs ($n = 4$; data not shown).

Binding of ^{125}I-ω-Ctx CNVIIA to Rat Brain Synaptosomes. In view that ω-Ctx CNVIIA proved to be very effective in inducing shaking symptoms in mice, we therefore tested its ability to bind to rat brain synaptosomal preparations. Monoiodotoxin labeled on tyrosine 13 was obtained following iodination and HPLC purification. Hot saturation analysis indicates that ^{125}I-ω-Ctx CNVIIA binds with high affinity ($K_d = 36.3 \pm 1.7$ pM, $n = 3$) to rat brain synaptosomes with a receptor site capacity (B_{max}) of 320 ± 60 fmol/mg of protein ($n = 3$), as shown in Figure 4A (inset). The binding of ^{125}I-ω-Ctx CNVIIA was fully competed by ω-Ctx CNVIIA, ω-Ctx MVIIA, and ω-Ctx MVIIC (Figure 4A) with a K_i of 2.02 ± 0.73 pM ($n = 4$), 1.13 ± 0.3 pM ($n = 3$), and 137 ± 5 pM ($n = 3$), respectively. To quantify the reversibility of ω-Ctx CNVIIA, association and dissociation kinetic studies were performed. The association rate constant was 260×10^6 M^{-1} s^{-1}, and the dissociation, induced by

Table 2: Sequence Comparison of ω-Conotoxins Isolated from *Conus* Venoms[a]

Peptide	Sequence	*Conus Sp.*	Reference
CnVIIA	C K G K G A O C T R L M Y D C C H G S C S S S K G R C *	*C.consors*	this work
MVIIA	C K G K G A K C S R L M Y D C C T G S C - R S G K - C *	*C. magus*	[27]
MVIIC	C K G K G A P C R K T M Y D C C S G S C G R R G K - C *	"	[6]
CVID	C K S K G A K C S K L M Y D C C S G S C S G T V G R C *	*C. catus*	[42]
GVIA	C K S O G S S C S O T S Y N C C R - S C N O Y T K R C Y *	*C. geographus*	[4]
GVIB	C K S O G S S C S O T S Y N C C R - S C N O Y T K R C Y G *	"	"
GVIC	C K S O G S S C S O T S Y N C C R - S C N O Y T K R C *	"	"
SVIA	C R S S G S P C G V T S I - C C - G R C - Y R G K - C T *	*C.striatus*	[28]
SVIB	C K L K G Q S C R K T S Y D C C S G S C G R S G K - C *	"	"
TVIA	C L S O G S S C S O T S Y N C C R - S C N O Y S R K C	*C. tulipa*	[16]
RVIA	C K P O G S O C R V S S Y N C C S - S C K S Y N K K C G	*C. radiatus*	"

[a] Asterisks denote an amidated C-terminal; O is for hydroxyproline.

addition of cold toxin (Figure 4B, arrow), was fast and complete with an off-rate of 8×10^{-3} s^{-1}. The half-life of ω-Ctx CNVIIA was 1.44 min.

Binding Competition with ^{125}I-ω-Ctx GVIA. The properties of ω-Ctx CNVIIA binding on rat brain synaptosomes were studied using the N-type calcium channel blocking peptide ^{125}I-ω-Ctx GVIA. Hot saturation analysis (Figure 4C, inset) indicates that ^{125}I-ω-Ctx GVIA binds with high affinity (K_d between 15.6 and 19.3 pM) to rat brain synaptosomes with a receptor site capacity (B_{max}) between 490 and 560 fmol/mg of protein. As shown in Figure 4C, both ω-Ctx GVIA and ω-Ctx CNVIIA displace completely ^{125}I-ω-Ctx GVIA with a K_i from 3.7 to 4.3 pM and from 2.7 to 3.7 pM, respectively.

Binding Competition with ^{125}I-ω-Ctx MVIIC. To study the selectivity of ω-Ctx CNVIIA between N- and P/Q-type VSCCs, several competition experiments were carried out using the ^{125}I-ω-Ctx MVIIC on rat brain synaptosomes (Figure 4D). ω-Ctx MVIIC possess an affinity of 11 and 86 pM for the P/Q- and N-type VSCC, respectively (*25*). Its binding was then performed with a low concentration of hot ω-Ctx MVIIC and corresponded to a single P/Q site displacement. Thus, ω-Ctx MVIIC was able to displace ^{125}I-ω-Ctx MVIIC at picomolar concentrations ($K_i = 30.3 \pm 4.7$ pM) whereas both ω-Ctx CNVIIA and ω-Ctx MVIIA inhibited the binding with K_i of 179 ± 34 and 156 ± 29 nM, respectively (Figure 4D).We compared the B_{max} values of ^{125}I-ω-Ctx GVIA, CNVIIA, and MVIIC on the same batch of rat brain synaptosome preparation. Hot saturation experiments were realized according to the Figure 4E. Results show that both ^{125}I-ω-Ctx GVIA and ^{125}I-ω-Ctx CNVIIA exhibit very close VSCC capacity (0.4−0.5 pmol of VSCC/mg of protein) whereas that of ^{125}I-ω-Ctx MVIIC is 3 times higher.

ω-Ctx CNVIIA Effect on Calcium Channel Current. To confirm the selectivity of ω-Ctx CNVIIA for N-type VSCC, its effects were also tested in rat chromaffin cells that have been described to contain L-, N-, P/Q-, and T-type Ca^{2+} channels (*26*). Application of 2.5 μM ω-Ctx CNVIIA clearly induced a fast blockade of barium current (I_{Ba}) that amounted to 49.2% ± 3.2 (SEM, $n = 5$) of the currents recorded (Figure 5). Once the blockade-induced ω-Ctx CNVIIA was reached, application of ω-Ctx GVIA (1 μM) did not induce additional blockade, suggesting that full blockade of N-type current had been achieved by ω-Ctx CNVIIA. Furthermore, the remaining current was reduced by sequential addition of ω-Ctx MVIIC (2 μM) and nifedipine (2.5 μM), indicating the presence of P/Q-, L-, and R-type VSCCs in these cells.

DISCUSSION

Using bioassay on fish and mice (*45*), we have isolated a new ω-conotoxin from the venom of *Conus concors*, named ω-Ctx CNVIIA. As will be detailed below, this toxin provides a new selective tool, which may enable new insights into structure−function relationships of calcium channels.

Chemical Synthesis of ω-Ctx CNVIIA. An optimized peptide synthesis led to nativelike product in sufficient quantity, allowing pharmacological characterization of the new ω-conotoxin. To obtain synthetic ω-Ctx CNVIIA, the folding procedure proved to be the key step for a reasonable yield. Difficulties in the synthesis of ω-Ctx MVIIA were previously reported by Olivera et al. (*27*) with the obtention of very low yield of toxin (<0.1%). Alternative syntheses of ω-conotoxins were later devised using Boc or Fmoc chemistry and using air oxidation, DMSO, DTT/cysteine, or gluthatione as oxidating agents (*28*−*31*). For the synthesis of MVIID, a sequential deprotection of cysteines was used to lead to the biologically active product (*7*). Difficulties in folding linear ω-conotoxins into the three-disulfide bridge conformation can arise from one particular residue. Single substitutions on ω-Ctx GVIA, (G5A, N20A, or T23A) have been reported to fail in providing the wanted product in good yield (*31*). Our aim to produce the synthetic ω-Ctx CNVIIA in large amounts led us to carefully monitor all the parameters involved in the folding process. The low temperature and substrate concentrations were revealed to be crucial to reduce aggregation, as was previously noted (*32*). Moreover, combination of oxidized/reduced gluthatione in potassium phosphate buffer allowed proper conversion of the linear peptide into the desired isomer. The use of this buffer solution proved to be much better than the widely used ammonium acetate buffer. The different solvatation effects on the positive charges of the linear peptide may be responsible for this observation.

ω-Ctx CNVIIA Selectively Blocks N-Type VSCCs. Binding results show that the B_{max} of ^{125}I-ω-Ctx CNVIIA on synaptosomes corresponds to the capacity of the N-type ω-Ctx

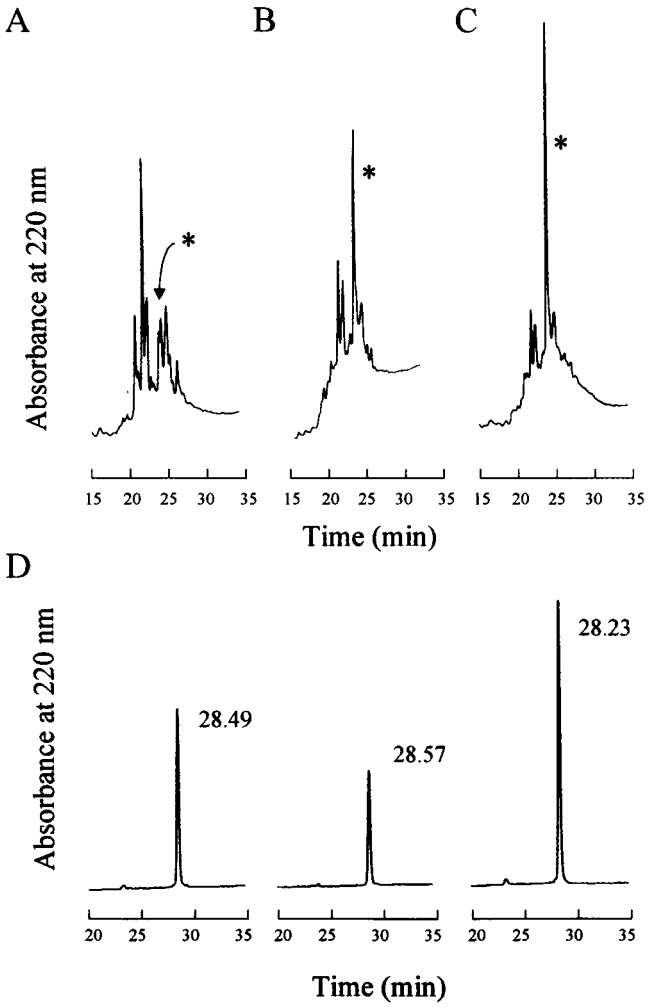

FIGURE 2: Folding and coelution experiment of the native and synthetic ω-Ctx CNVIIA. (A) HPLC of the oxidation mixture in the presence of ammonium acetate buffer (0.3 M, pH 7.8) and DTT/Cys (0.15 mM/0.3 mM). (B) HPLC of the oxidation mixture in the presence of ammonium acetate buffer (0.3 M, pH 7.8) and reduced/oxidized glutathione (3 mM/0.3 mM). (C) HPLC of the oxidation mixture in the presence of potassium phosphate buffer (0.05 M, pH 7.8) and reduced/oxidized glutathione (3 mM/0.3 mM). All oxidation reactions were carried out in the presence of 0.03 mM purified linear peptide, 0.5 M guanidinium chloride for 3 days at 4 °C. (D) From left to right, HPLC of the native toxin ω-Ctx CNVIIA, the synthetic toxin, and the mixture of native and synthetic toxins. Note the small product eluting at 23.5 min in all chromatograms corresponding to ω-Ctx CNVIIA with an oxidized methionine.

GVIA and that ω-Ctx CNVIIA displaces ^{125}I-ω-Ctx CNVIIA and ^{125}I-ω-Ctx GVIA with the same affinity (see Table 3). These results suggested that the two toxins possess the same channel receptor site. In addition, ω-Ctx CNVIIA as well as ω-Ctx MVIIA displace the binding of ^{125}I-ω-Ctx MVIIC with inhibition constants of 156 and 179 nM, respectively. The new ω-Ctx CNVIIA presents the same subtype selectivity (5 log) as ω-Ctx MVIIA (3.9 log) and ω-Ctx GVIA (5 log, Table 3). As a comparison, the recently isolated ω-Ctx CVID was found to be 10−100-fold more selective for the N-type VSCC than ω-Ctx MVIIA and ω-Ctx CNVIIA (*42*). In addition, one can remark that a 20-fold difference in affinity exists between ^{125}I-ω-Ctx CNVIIA (iodinated on position 13, K_d = 36.3 pM) and ω-Ctx CNVIIA (K_d = 2.0 pM). Moreover, Sato et al. (*46*) have shown that Tyr 13 of ω-Ctx MVIIC is essential for the binding with both N- and

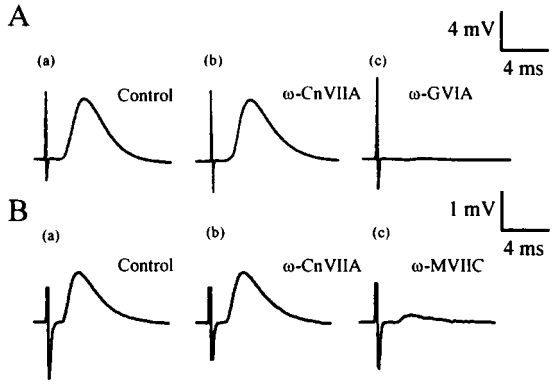

FIGURE 3: Differential effects of ω-Ctx GVIA, ω-Ctx MVIIC, and ω-Ctx CNVIIA on frog and mouse neuromuscular junctions. (A) EPPs recorded on a frog cutaneous pectoris nerve−muscle preparation in control conditions (a) and after 1 h treatment with 10 μM ω-Ctx CNVIIA (b) and 1 μM ω-Ctx GVIA (c). Each trace corresponds to the average of 10 recorded endplate potentials (resting membrane potential preset at −50 mV). (B) Endplate potentials recorded on a mouse hemidiaphragm preparation under control conditions (a) and after 1 h treatment with 5 μM ω-Ctx CNVIIA (b) and 1 μM ω-Ctx MVIIC (c). Each superimposed tracing is the average of 10 endplate potentials. Resting membrane potential during recordings, −60 mV. Notice that ω-Ctx CNVIIA did not affect EPPs recorded on frog and mouse neuromuscular junctions while ω-Ctx GVIA and ω-Ctx MVIIC completely inhibited EPPs recorded on frog and mouse neuromuscular junctions, respectively.

P/Q-type calcium channels. It seems likely that Tyr 13 of ω-Ctx CNVIIA is directly involved in the binding with its target.

It was previously shown that the whole-cell barium current (I_{Ba}) in rat chromaffin cells exhibits at least four components and that sequential addition of ω-Ctx GVIA and ω-Ctx MVIIC produced partial and additive inhibition of I_{Ba} (*26*). Thus, chromaffin cells can be used to discriminate between N- and P/Q-type VSCC blockers. By using the whole-cell configuration of the patch-clamp technique, we clearly showed that ω-Ctx CNVIIA (2.5 μM) induced a fast blockade of I_{Ba} which corresponded to the N-type VSCC. In conclusion, both the electrophysiological and binding experiments demonstrate that this new conotoxin inhibits selectively the N-type calcium channels.

ω-Ctx CNVIIA, a New Tool To Study Structure−Activity Relationships of VSCC Subtypes. A striking sequence homology between the toxin isolated in this work and the previously identified ω-Ctx MVIIA, ω-Ctx MVIIC, and ω-Ctx CVID can be noticed when comparing their primary sequences (see Table 2). As previously shown, ω-conotoxins are all relatively rigid peptides presenting a common structural scaffold (*33*). It seems that ω-Ctx CNVIIA would adopt a similar structure given its cysteine pattern. By comparing the amino acid sequences of ω-Ctx CNVIIA, ω-Ctx MVIIA, ω-Ctx MVIIC, and ω-Ctx CVID, we can notice a change in the amino acid composition in the first loop and more significantly in the fourth loop (Table 2). According to the pharmacological results obtained in this study, the natural mutations in the first loop do not lead to selectivity between N- and P/Q-type VSCCs. This is in agreement with the work of Nielsen et al. (*34*), that defined loops 2 and 4 as the prominent factors contributing to VSCC subtype selectivity. To that effect, loop 4 of ω-conotoxins is the most diverse in all ω-conotoxins so far studied (see

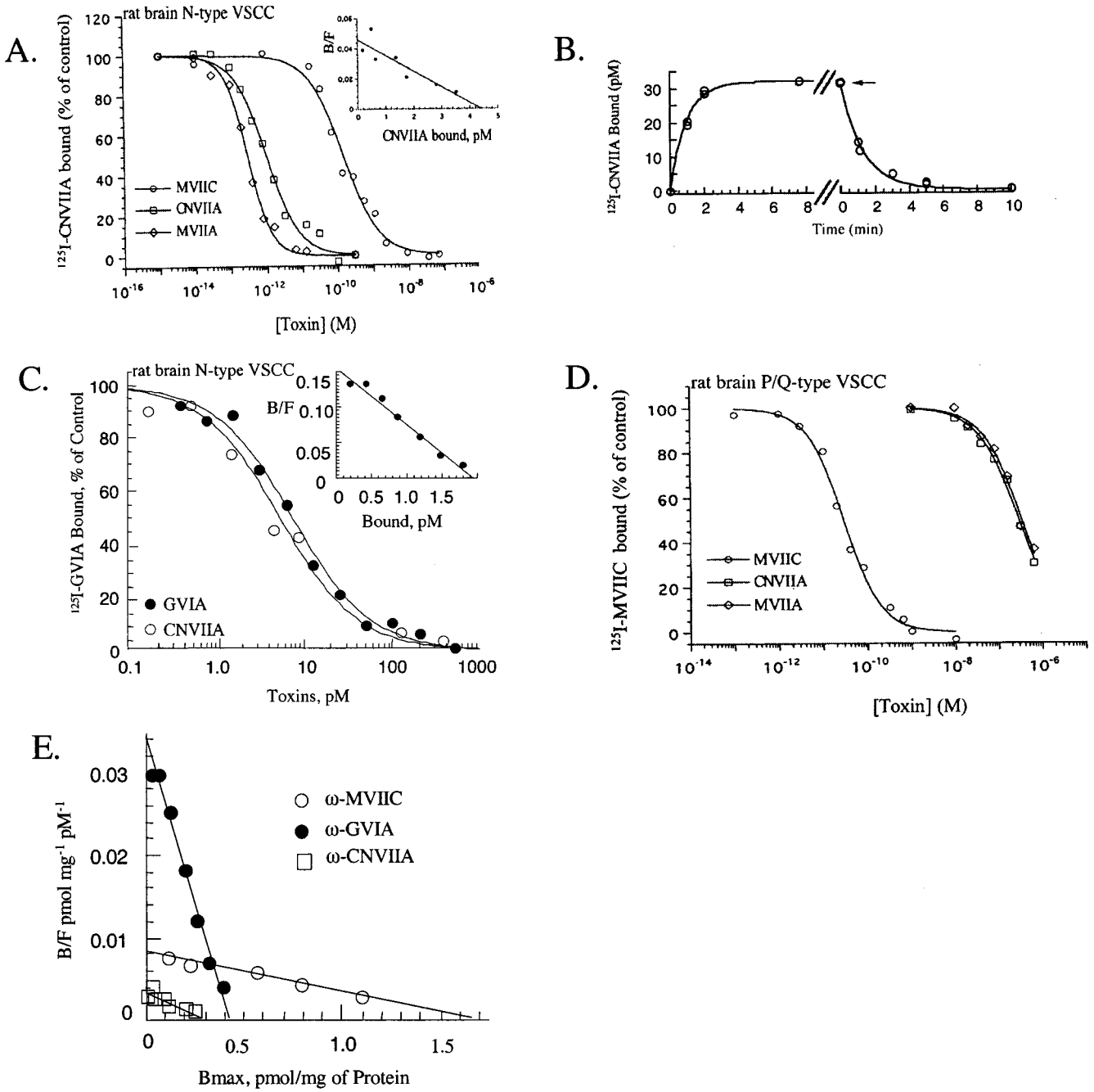

FIGURE 4: Binding interaction of ω-conotoxins with rat brain synaptosomes. (A) Competition for ^{125}I-ω-Ctx CNVIIA binding by increasing the concentration of ω-Ctx CNVIIA, ω-Ctx MVIIA, and ω-Ctx MVIIC. Nonspecific binding was determined in the presence of 10 nM ω-Ctx CNVIIA and subtracted from the data. Inset: Scatchard plot of the specific binding of ^{125}I-ω-Ctx CNVIIA with rat brain synaptosomes. (B) Kinetic analysis of the binding of ^{125}I-ω-Ctx CNVIIA. Nonspecific binding determined in the presence of cold toxin (10 nM) is time-invariant and was subtracted from the experimental points. After 10 min of association, complex was dissociated by 10 nM cold ω-Ctx CNVIIA (arrow) for different periods of time. (C) Competition for ^{125}I-ω-Ctx GVIA binding by increasing the concentration of ω-Ctx CNVIIA and ω-GVIA. Nonspecific binding was determined in the presence of 100 nM ω-Ctx GVIA and subtracted from the data. Inset: Scatchard plot of the specific binding of ^{125}I-ω-Ctx GVIA with rat brain synaptosomes. (D) Competition curves for ^{125}I-ω-Ctx MVIIC binding inhibition by ω-Ctx CNVIIA, ω-Ctx MVIIA, and ω-Ctx MVIIC. Nonspecific binding was determined in the presence of 100 nM ω-Ctx MVIIC and subtracted from all data points. (E) Scatchard plot of the specific binding of ^{125}I-ω-Ctx CNVIIA, ^{125}I-ω-Ctx GVIA, and ^{125}I-ω- Ctx MVIIC with the same membrane preparation.

Table 2). This loop, very close to the second loop in space, is also of importance for selectivity (*34*), and it acts by direct interaction with the receptor site and/or by influencing the position of some residues in the second loop. Interestingly, it was proposed by Lewis et al. (*41*) that ω-Ctx CVID lacks the secondary binding residues present in loop 4 of ω-Ctx MVIIA (Arg 21) (*30*) and ω-Ctx GVIA (Lys 24, Tyr 22) and utilizes the relatively exposed Val 24 for interaction with the VSCC. ω-Ctx CNVIIA and ω-Ctx CVID possess the same number of residues in each loop, and the identity in loops 1−3 and 4 is 75% and 50%, respectively (Table 2). As the presence of a basic residue in ω-Ctx CNVIIA at position 24 is the main difference between this toxin and ω-Ctx CVID, it seems likely that this position influences both the *selectivity* and *affinity* for N-type VSCC: (i) ω-Ctx CVID is 10−100-fold more selective for the N-type than

Table 3: Affinities and Kinetic Parameters of N-Type ω-Conotoxins on Rat Brain Synaptosomes[a]

	affinity parameters		inhibition constants (pM) against VSCC		log ($K_{i,P/Q-type}$ vs $K_{i,N-type}$)	kinetic parameters		
	K_d (pM) hot sat.	B_{max} (fmol/mg)	N-type	P/Q-type		K_{on} ($\times 10^6$ M^{-1} s^{-1})	K_{off} ($\times 10^{-3}$ s^{-1})	K_d (pM) K_{off}/K_{on}
GVIA	15.6−19.3	490−560	3.7−4.3[&] 2.02[@]	1.050.000[(42)]	5.4	6[(41)]	−[(25)]	
CNVIIA	36.3	330	2.7−3.7[&] 1.13[@]	179.000	5.0	260	8.0	40
MVIIA	37[(42)]	100[(42)]	1.0[#(25)]	156.000	3.9[(42)]	1333	8.8[(42)]	7[(42)]
CVID	67[(42)]	70[(42)]	70[&(42)]	55.000.000[(42)]	5.9[(42)]	400[(42)]	9.3[(42)]	23[(42)]

[a] Abbreviations: @, against ^{125}I-ω-Ctx CNVIIA; #, against ^{125}I-ω-Ctx MVIIA; &, against ^{125}I-ω-Ctx GVIA.

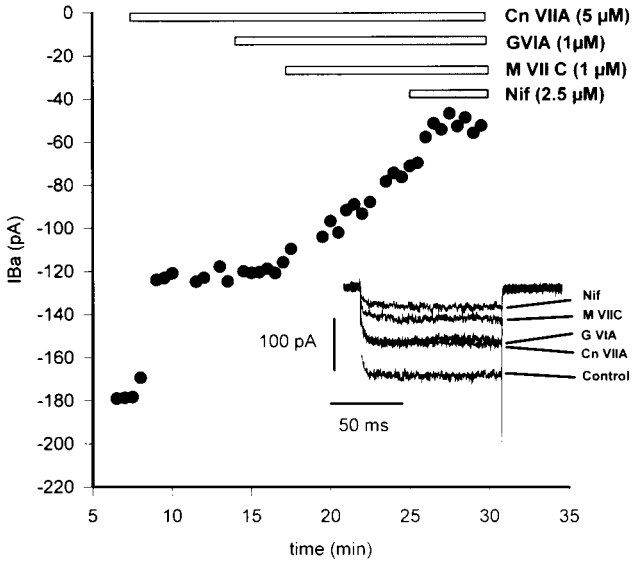

FIGURE 5: Effects of the sequential addition of ω-Ctx CNVIIA (2.5 μM), ω-Ctx GVIA (1 μM), ω-conotoxin MVIIC (2 μM), and nifedipine (2.5 μM) on I_{Ba} recorded in a single rat embryonic chromaffin cell. The cell was held at −80 mV and stimulated with depolarizing pulses of 100 ms duration to 0 mV. The inset shows representative current traces obtained during the sequential application of toxins.

ω-Ctx CNVIIA and (ii) ω-Ctx CNVIIA has a 35-fold greater affinity for its target than ω-Ctx CVID (2.02 versus 70 pM).

Differential Interaction with N-Type VSCCs. One of the major findings in the present study is the original pharmacological profile of ω-Ctx CNVIIA in vertebrate preparations. Binding experiments demonstrated VSCC N-type selectivity in rat brain, and this was corroborated by current recordings in rat chromaffin cell preparations. Unexpectedly, ω-Ctx CNVIIA did not show any blocking activity at amphibian neuromuscular junctions, in marked contrast to the well-known N-type VSCC blocker ω-Ctx.

To the best of our knowledge, this represent the first evidence of differential activity of N-type VSCC blockers at the vertebrate neuromuscular junction. Such pharmacological diversity was also presented by Lewis et al. (*46*) concerning N-type VSCC antagonists in rat and human brain. Considering these properties and because of their high subtype selectivity between VSCC subtypes, N-type blockers could be useful for many potential therapeutic applications (*16*). To that effect, ω-Ctx MVIIA has been demonstrated to protect against global ischemia (*35*) and to suppress neuropathic pain (*36*). Because an adequate level of selectivity for the N-type channel is an essential requirement for selection of such analgesic candidates, combinatorial mutagenesis of ω-conotoxin loop 4 provides a strategic approach for the design of peptidomimetic inhibitors of N-type VSCCs. As a general rule, an interesting feature of the venomous snails of the Conidae family is their ability to produce mutated toxins possessing a similar cysteine motif (*37*−*39*). In other words, *Conus* venoms provide highly specific toxins, each possessing original sequences with common three-dimensional structure. These venoms can thus be considered as an unprecedented natural source of toxins with potential therapeutic applications. Characterization of ω-Ctx CNVIIA from the venom of *Conus consors* presented in this work emphasizes the diversity of VSCCs in the animal kingdom and highlights the potential in providing specific and diverse ligands for ion channels.

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