A Novel α-Conotoxin Identified by Gene Sequencing Is Active in Suppressing the Vascular Response to Selective Stimulation of Sensory Nerves in Vivo †

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A Novel α-Conotoxin Identified by Gene Sequencing Is Active in Suppressing the Vascular Response to Selective Stimulation of Sensory Nerves in Vivo†

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ABSTRACT: We describe the identification of a conopeptide sequence in venom duct mRNA from *Conus victoriae* that suppresses a vascular response to pain in the rat. PCR-RACE was used to screen venom duct cDNAs for those transcripts that encode specific antagonists of vertebrate neuronal nicotinic acetylcholine receptors (nAChRs). One of these peptides, Vc1.1, was active as an antagonist of neuronal nAChRs in receptor binding and functional studies in bovine chromaffin cells. It also suppressed the vascular responses to unmyelinated sensory nerve C-fiber activation in rats. Such vascular responses are involved in pain transmission. Furthermore, its ability to suppress C-fiber function was greater than that of MVIIA, an ω-conotoxin with known analgesic activity in rats and humans. Vc1.1 has a high degree of sequence similarity to the α-conotoxin family of peptides and has the 4,7 loop structure characteristic of the subfamily of peptides that act on neuronal-type nAChRs. The results suggest that neuronal α-conotoxins should be further investigated with respect to their potential to suppress pain.

Marine snails from the *Conus* genus produce venom with an unusually wide array of neurotoxic peptides that are the active components of the venom. One subset of biologically active peptides is the disulfide-rich conotoxins that have been studied extensively in milked or harvested venom in a range of species of *Conus.* These have been shown to be highly specific agonists or antagonists of different types of ion channels involved in neuronal and neuromuscular signaling (*1*−*3*). The diversity of functions of conotoxins in specifically targeting a wide array of ion channels is apparently a reflection of the biodiversity demonstrated by the genus *Conus*. Not only are there 500 different *Conus* species colonizing widely divergent habitats and feeding on a wide array of prey, but also the conotoxin genes appear to be among the most rapidly evolving genes studied (*4*). As a consequence it is estimated that there are up to 100000 different toxic peptides in the combined venoms from this remarkable genus.

Despite their large numbers and their sequence diversity, individual conotoxins show a high degree of selectivity in their ability to agonize or antagonize particular ion channels. This is evident in the α-conotoxins where modification of a single amino acid, Ala_{10} to Leu_{10}, in the conotoxin PnIA completely changed its specificity for nicotinic acetylcholine receptors (nAChRs)[1] from α3β2-preferring to α7 preferring (*5*). ω-Conotoxins MVIIA (Ziconotide) and CVID that target N-type calcium channels without significantly affecting P/Q type calcium channels have shown analgesic activity in rats and humans (*6*). Such blocking of N-type calcium channels results in substantial pain relief (*2*).

We have investigated the extent to which an alterative type of conotoxin, an α-conotoxin, that targets neuronal-type nicotinic acetylcholine receptors (nAChRs) can interfere with the pathways of pain perception.

In this paper we describe the identification of a previously uncharacterized conopeptide sequence in venom duct mRNAs from an Australian muscivorous cone snail, *Conus victoriae.* PCR-RACE was used to screen venom duct cDNAs for those transcripts that encode specific antagonists of the vertebrate neuronal nAChRs. We show that one of these peptides, Vc1.1, active as an antagonist of neuronal nAChRs in bovine chromaffin cells, also suppressed the vascular responses to C-fiber activation (involved in pain transmission) in rats. The results are indicative of the ability of this α-conotoxin to suppress pain. Furthermore, its ability to suppress C-fiber function was compared to that of ω-conotoxin MVIIA with known analgesic activity in rats and humans.

In a subsequent paper (*31*), we report on the ability of this novel α-conotoxin to inhibit sensory function in vivo

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; PCR-RACE, polymerase chain reaction−rapid amplification cDNA ends; SNP, sodium nitroprusside; PCA, perchloric acid; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; SEM, standard error of the mean; ANOVA, analysis of variance; ESI-MS, electrospray ionization−mass spectrometry.

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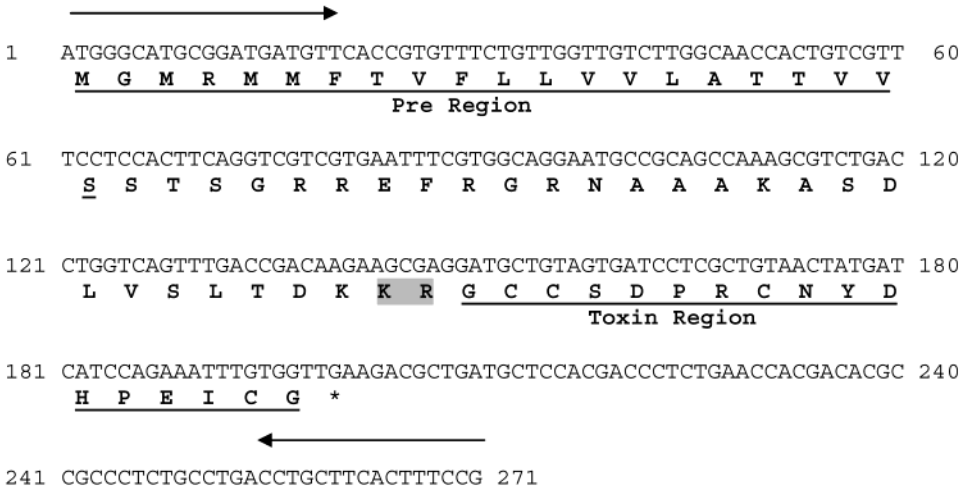

FIGURE 1: Nucleotide sequence and deduced amino acid sequence for Vc1.1. The coding sequence is 201 base pairs which translates to 66 amino acids. The predicted pre-region is 21 amino acids in length (underlined), the mediated pro-region is 29 amino acids in length, and the predicted mature peptide is 17 amino acids in length (underlined) and is reduced to 16 amino acids following removal of the C-terminal glycine during C-terminal amidation. The expected proteolytic site KR is shaded. The mature peptide has four cysteine residues in a CC-$(X)_4$-C-$(X)_7$-C arrangement. Arrows indicate the positions of primers α1 and α2, respectively.

as shown by its effectiveness in alleviating chronic pain and accelerating functional recovery in an animal model of peripheral neuropathy.

MATERIALS AND METHODS

Compounds and Solutions. Pentobarbitone sodium (Nembutal) was obtained from Boehringer Ingelheim Pty. Ltd. (Queensland, Australia) and was prepared in 0.9% saline solution (Delta West Pty. Ltd., Western Australia). Sodium nitroprusside (SNP) was purchased from Sigma Chemical Co. (St. Louis, MO). A synthetic form of *ω*-conotoxin MVIIA [SNX-111 (*7*)] was obtained from Auspep Pty. Ltd. (Melbourne, Australia), and α-conotoxin Vc1.1 was custom synthesized by Auspep Pty. Ltd.

All experimental procedures adhered to the National Health and Medical Research Council (NHMRC) and International Association for the Study of Pain (IASP) guidelines and were approved by the Royal Melbourne Hospital Research Foundation Animal Ethics Committee.

Conotoxin cDNA Isolation and Sequencing. Specimens of *C. victoriae* were collected from Broome, Western Australia, and transported live to Melbourne; the venom ducts were removed and snap frozen in liquid nitrogen. mRNA was extracted from individual ducts and purified using a Dynabeads mRNA direct kit (Dynal, Norway), and a cDNA library was created from this mRNA using a Marathon cDNA amplification kit (Clontech) as previously described (*8*). Following purification of the cDNA a partially double-stranded adaptor (Marathon cDNA Adaptor; Clontech) was ligated onto the 5′ and 3′ ends to allow rapid amplification of cDNA ends (RACE).

PCR amplification of conotoxin genes was carried out using the *C. victoriae* cDNA, a forward α_1 primer (ATG CGC ATG CGG ATG ATG TT), a reverse α_2 primer (CGG AAA GTG AAG CAG GTC AG), Taq polymerase (Roche), and deoxynucleotides in a buffer supplied by the manufacturer. Samples were incubated at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final step of 72 °C for 5 min. Primers α_1 and α_2 matched regions highly conserved at the 5′ and 3′ ends, respectively, of α-conotoxin genes (Figure 1). Amplification products were purified after separation on a 1.5% agarose gel using a Qiaquick gel extraction kit (Qiagen). The purified PCR products were TA cloned into the pCR2.1 vector (Invitrogen) and transformed into INVαF′ *Escherichia coli* cells. Plasmids containing inserts of approximately 250 bp in size were sequenced by the dideoxy chain termination method using an ABI PRISM dye terminator cycle sequence ready reaction kit (Perkin-Elmer).

Peptide Synthesis. Conotoxin peptide sequences as deduced from cDNA sequences were synthesized using standard BOC chemistry by Auspep Pty. Ltd. and allowed to form disulfide bridges by stirring in solution (0.3 mg/mL in 25 mM NH_4HCO_3) at room temperature.

Biological Assays: (A) Assay of Neuronal Nicotinic Acetylcholine Receptor Activity in Bovine Chromaffin Cells. Adrenal chromaffin cells were isolated from adult bovine adrenal glands as previously described (*9*). Isolated cells were plated on collagen-coated 24-well plates at a density of 2.8 $\times 10^5$ cells/cm^2. Three- to four-day-old cultured chromaffin cells were allowed to equilibrate to room temperature for 5 min. The incubation medium was removed by two consecutive washes in Locke's buffer [154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 10 mM D-glucose, 1.18 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.5% (w/v) bovine serum albumin, pH 7.4] for 5 min. Cells were then incubated with different concentrations of the peptide Vc1.1 for 5 min, before stimulation with either 1−4 μM nicotine or 56 mM KCl for a further 5 min. The incubation mixture was separated from the cells and acidified to a final concentration of 0.4 M perchloric acid (PCA). The catecholamines remaining in the chromaffin cells were released by lysing the cells with 0.01 M PCA and then further acidified by addition of an equal volume of 0.8 M PCA. Precipitated proteins were removed by centrifugation at 8000*g* for 10 min. Basal release of catecholamines was measured in the presence of Vc1.1 but the absence of nicotine or KCl. Maximal release of catecholamines was determined following stimulation with

1−4 μM nicotine or 56 mM KCl in the absence of Vc1.1. Catecholamines were measured by electrochemical detection (650 mV BAS model LC-3A) following reversed-phase high-performance liquid chromatography as previously described (*10*) and the catecholamines in each release sample expressed as a percentage of the total cell content.

(B) [^{3}H]Epibatidine Binding Assay. The preparation of bovine adrenomedullary membranes was adapted from the method of MacAllan (*11*), who initially described the preparation of membranes from rat brain. Bovine adrenal glands obtained from the local abattoir were dissected, and the medulla was removed. The medulla was placed into ice-cold 0.32 M sucrose supplemented with 1 mM EDTA, 0.1 mM PMSF, and 0.01% (w/v) sodium azide. The medulla was homogenized using a Polytron, until a homogeneous solution was obtained. The homogenate was centrifuged at 100*g* for 10 min at 4 °C. The resultant supernatant was decanted and the pellet resuspended in 0.32 M sucrose supplemented as above (5 mL/g of original medulla weight). The resuspended pellet was again centrifuged at 100*g* for 10 min at 4 °C. The two supernatants were combined and centrifuged at 12000*g* for 30 min at 4 °C. The resultant pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 0.01% (w/v) sodium azide, 0.1 mM PMSF, and 1 mM bovine serum albumin and centrifuged at 12000*g* for 30 min at 4 °C. This step was repeated once to wash the pellet. The washed pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4, and stored at −70 °C.

Adrenal medulla membranes (300−500 μg of protein/tube) were incubated at 37 °C for 2 h in the presence of 1 nM [^{3}H]epibatidine and the test ligand (epibatidine, carbachol, nicotine, or Vc1.1 over a concentration range of 1 nM−1 mM). [^{3}H]Epibatidine was the radioligand of choice as it has broad nAChR subtype affinity. After the incubation period each assay tube was filtered and washed with phosphate-buffered saline (150 mM NaCl, 8 mM K_2HPO_4, 2 mM KH_2PO_4) in a Brandel filtration system. Glass fiber filters used in the filtration step were first soaked overnight in phosphate-buffered saline containing 5% (w/v) polyethylenimine. Membrane filters obtained from this filtration were subjected to liquid scintillation counting. Nonspecific binding was detected using 2 mM nicotine (pH adjusted). Total binding was obtained by substituting the displacing test ligand with milliQ water. Specific binding was determined by subtracting the nonspecific from total binding. Saturation binding experiments ($n = 3$) determined a K_d value of 0.28 nM for [^{3}H]epibatidine binding to adrenomedullary membranes. This value was determined using nonlinear regression analysis (Prism, GraphPad, San Diego, CA). From the displacement experiments, Hill coefficients and K_i values were determined using nonlinear regression analysis. K_i values were determined using the Cheng−Prusoff rule, whereby $K_i = IC_{50}/(1 + [[^3H]epibatidine])/K_d$ of [^{3}H]epibatidine.

(C) Assays of Nicotinic Acetylcholine Receptor Activity at the Neuromuscular Junction Using the Rat Phrenic Nerve Hemidiaphragm Preparation. Electrically stimulated muscle twitch was analyzed in a rat phrenic nerve/diaphragm assay as previously described (*12*).

In Vivo Assays: Blister Induction, Antidromic Electrical Stimulation of the Sciatic Nerve, and Measurement of Peripheral Sensory Vascular Response. Unless otherwise stated, conotoxins were dissolved first in HPLC-grade water (doubly deionized) and then in Ringer's solution (0.15 M NaCl, 6 mM KCl, 3 mM $CaCl_2$, 12 mM $NaHCO_3$, pH 7.4).

Outbred young (2−3 months) male Sprague-Dawley rats, weight 250−350 g, were obtained from Monash University Animal Services. Rats were housed in groups of four in a plastic cage, in a constant temperature room (21 ± 0.5 °C), with a 12 h/12 h light/dark cycle and had free access to food and water. Each experimental group consisted of four to eight rats. Anesthesia was induced with pentobarbitone sodium (60 mg/kg ip) and maintained by supplementary injections (15 mg/kg) to ensure a constant state of anesthesia. Body temperature was maintained at 37 °C. Animals were sacrificed by anesthetic overdose at the end of the experiment.

A blister was induced on the glabrous skin of the right hind paw of the anesthetized rat by applying a vacuum pressure of −40 kPa, for approximately 30 min, using a metal suction cap heated to 40 °C, and the blister was encased in a perfusion chamber as described previously (*13*, *14*). Relative blood flow was monitored over time by a laser Doppler flowmeter (Periflux, PF2B; Perimed, Sweden) via a probe positioned in the chamber directly above the blister base.

To permit antidromic electrical stimulation of a subset of the sensory nerve (unmyelinated C-fibers) to induce vasodilation, the sciatic nerve was separated from the surrounding connective tissue and cut as proximately as possible before being connected to a Grass S48 stimulator (Grass Intruments) as previously described (*15*, *16*). Bipolar platinum electrodes were fixed in such a position that electrical leakage to adjacent nerve and muscle structures was minimized, and the distal portion of the sciatic nerve was stimulated at 20 V, 5 Hz, 2 ms square waves for 1 min duration. These parameters were previously shown to selectively stimulate the unmyelinated C-fibers to induce vasodilation (*15*, *16*).

To determine the effects of conotoxins on sensory nerve mediated vascular response, Ringer's solution was initially perfused over the blister base until a stable baseline blood flow was achieved (approximately 30 min). Sodium nitroprusside (SNP) (100 μM) in Ringer's solution, a direct-acting smooth muscle vasodilator, was then perfused for 10 min over the blister base prior to the stimulation, followed by a 10 min washout with Ringer's solution until blood flow was returned to baseline. The 1 min electrical stimulation was then applied and perfusion continued for a further 20 min. In control groups, Ringer's solution was continuously perfused over the blister base during the 1 min stimulation as well as during a poststimulation period of 20 min. In the experimental groups, Vc1.1 (in Ringer's solution) was perfused for 30 min prestimulation and continuously during the stimulation and poststimulation period of 20 min. A dose−response curve was constructed for Vc1.1 to determine the optimal dose that produces a near 50% inhibition of sensory nerve activity.

Upon the completion of the experiments, SNP was perfused again for 10 min over the blister base to see whether the vascular action of compounds tested was dependent or independent of an action on smooth muscle reactivity.

To compare the efficacy of α-conotoxin Vc1.1 to that of ω-conotoxin MVIIA, each of the peptides was tested at 0.1 μM concentration using the above protocol.

Measurement of the Physiological Responses and Data Analysis. SNP and stimulation-induced vascular responses

Table 1: Alignment of Putative Conotoxin Vc1.1 with Known α-Conotoxins[a]

Name	nAChR	Amino Acid Sequence	
Vc1.1		G C C S D P R C N Y D H P E I C *	
EpI	neuronal	G C C S D P R C N M N N P D Y*C *	(*12*)
MII	neuronal	G C C S N P V C H L E H S N L C *	(*22*)
PnIB	neuronal	G C C S L P P C A L S N P D Y*C *	(*20*)
PnIA	neuronal	G C C S L P P C A A N N P D Y*C *	(*20*)
AulA	neuronal	G C C S Y P P C F A T N S D Y C *	(*27*)
AulC	neuronal	G C C S Y P P C F A T N S G Y C *	(*27*)
AulB	neuronal	G C C S Y P P C F A T N P D - C *	(*27*)
SI	muscle	I C C - N P A C G - P K - Y S C *	(*28*)
GI	muscle	E C C - N P A C G - R H - Y S C *	(*29*)

[a] Vc1.1 is most homologous with α-EpI,α-MII, α-PnIA, and α-PnIB, all of which have 4/7 loop sizes and block the neuronal nAChR. * indicates amidated C-terminus. Y* indicates sulfated tyrosine.

were calculated from the area under the response curve measured in cm^2 for 10 or 20 min, respectively. Stimulation-induced vascular responses were adjusted to take into account individual variability in smooth muscle reactivity, using the equation [individual area under response curve to electrical stimulation × (mean SNP response for group ÷ individual SNP response)]. Since SNP is a direct smooth muscle vasodilator, this correction effectively uses the SNP response as an internal standard for individual variations in vascular responses that are independent of sensory nerve activation. The effect of perfusion of different compounds on the vascular response was analyzed using one-way ANOVA followed by the post hoc Student−Newman−Keuls test. All of the results were expressed as the mean ± SEM. The level of significance was chosen as $p < 0.05$.

RESULTS

Cloning. PCR amplification of *C. victoriae* venom duct cDNA using the α1 and α2 primers produced an amplicon approximately 250 bp in length. Primer α1 was based on the consensus sequence of the 5′ end of pre-pro-α-conotoxins in the NCBI database and primer α2 on a previously unrecognized highly conserved region in the 3′ untranslated region of α-conotoxins. The resultant PCR products were cloned, and multiple clones were isolated and sequenced. An insert 271 bp in length was further characterized. It contained an open reading frame that encoded a protein 66 amino acids in length, with a structure typical of a conotoxin propeptide (Figure 1). The conotoxin precursor contained an N-terminal pre-region 21 amino acids in length and marked by a predominance of hydrophobic residues; this was followed by a predicted pro-region of 29 amino acids in length.

The candidate conotoxin is separated from the pro-region by the proteolytic site KR, which would yield a peptide of 17 amino acids in length, further truncated to 16 amino acids in length by removal of the C-terminal glycine during C-terminal amidation. This putative conotoxin, designated Vc1.1 in accordance with current nomenclature (*17*), contains four cystinyl residues in a CC-C-C arrangement which is characteristic of the α-conotoxins (*18*). The peptide sequence of Vc1.1 has the highest similarity to α-EPI, α-MII, α-PnIA, and PnIB, all of which are known to be antagonists at neuronal nAChRs (*12*, *19*, *20*) (Table 1).

Significantly, Vc1.1 has the potential to form four- and seven-residue loops separating the cystinyl bridges formed by oxidation of the cysteines and characteristic of many of the α-conotoxins that are specific antagonists of neuronal nAChRs (*3*, *5*). Previous studies indicated that the terminal glycine is often removed from α-conotoxins in their native state and the C-terminus amidated. Accordingly, Vc1.1 was synthesized without the final glycine residue and with an amidated C-terminus. The synthetic peptide was allowed to undergo spontaneous oxidation to facilitate the formation of disulfide bonds.

Physical Properties. As synthesized, Vc1.1 consisted of a peptide >70% purity on HPLC and with an average mass of 1806.6 by ESI-MS (Figure 2). Behavior on HPLC suggests a single predominant form, i.e., a species with a single connectivity rather than a mixed connectivity. This remained unchanged in solution for 8 weeks.

Neuronal-Type Nicotinic Receptor Assay. The ability of the synthetic peptide Vc1.1 to inhibit neuronal nAChRs was tested using chromaffin cell cultures. The synthetic peptide Vc1.1 potently inhibited nicotine-induced catecholamine release from the bovine chromaffin cells. The IC_{50} of Vc1.1 for both noradrenaline and adrenaline release was in the range of 1−3 μM (Figure 3A), with an inhibitory effect at concentrations as low as 0.1 μM (data not shown). K^+-stimulated catecholamine release was not inhibited by the synthetic peptide Vc1.1, indicating that the synthetic peptide inhibits nAChRs but does not block voltage-gated ion channels (Figure 3B). Vc1.1 by itself had no effect on catecholamine release in the absence of nicotine or K^+.

Vc1.1 was a competitive inhibitor of nicotine-stimulated release of catecholamines from chromaffin cells. As shown in panels C and D of Figure 3, the ability of Vc1.1 to inhibit release of both noradenaline (Figure 3C) and adrenaline (Figure 3D) was dependent on the concentration of nicotine and was completely reversed at the higher nicotine concentrations. In contrast, Vc1.1 at concentrations up to 50 μM did not inhibit transmission at the neuromuscular junction as assessed by electrical stimulation of the rat phrenic nerve/hemidiaphragm preparation (data not shown).

Binding of Vc1.1 to nAChRs. As shown in Figure 4 and Table 2, Vc1.1 also competed for binding of the nicotinic agonist [^{3}H]epibatidine to membrane preparations of bovine adrenal chromaffin cells. Preliminary [^{3}H]epibatidine saturation binding experiments to chromaffin cell membranes revealed a K_d of 0.28 nM with a Hill coefficient of 1.08 for [^{3}H]epibatidine.

As shown in Figure 4, α-conotoxin Vc1.1 was able to displace [^{3}H]epibatidine from chromaffin cell membranes over a concentration range similar to that of nicotine and carbachol. Other studies aimed at characterizing nAChR subtypes expressed by bovine adrenomedullary membranes (*30*) revealed that 1 nM [^{3}H]epibatidine labeled two distinct populations of nAChRs. Vc1.1 displaced epibatidine from both populations of nAChR subtypes. However, Vc1.1 showed high affinity for only one of these sites with a K_i value of 2.3 nM (Table 2). Vc1.1 showed a lower affinity for the second site, with a K_i value of 3.7 μM. This demonstrates a definite selectivity of Vc1.1 for one of the nAChR populations.

Nicotine also showed two-site displacement of [^{3}H]-epibatidine from bovine adrenomedullary membranes while

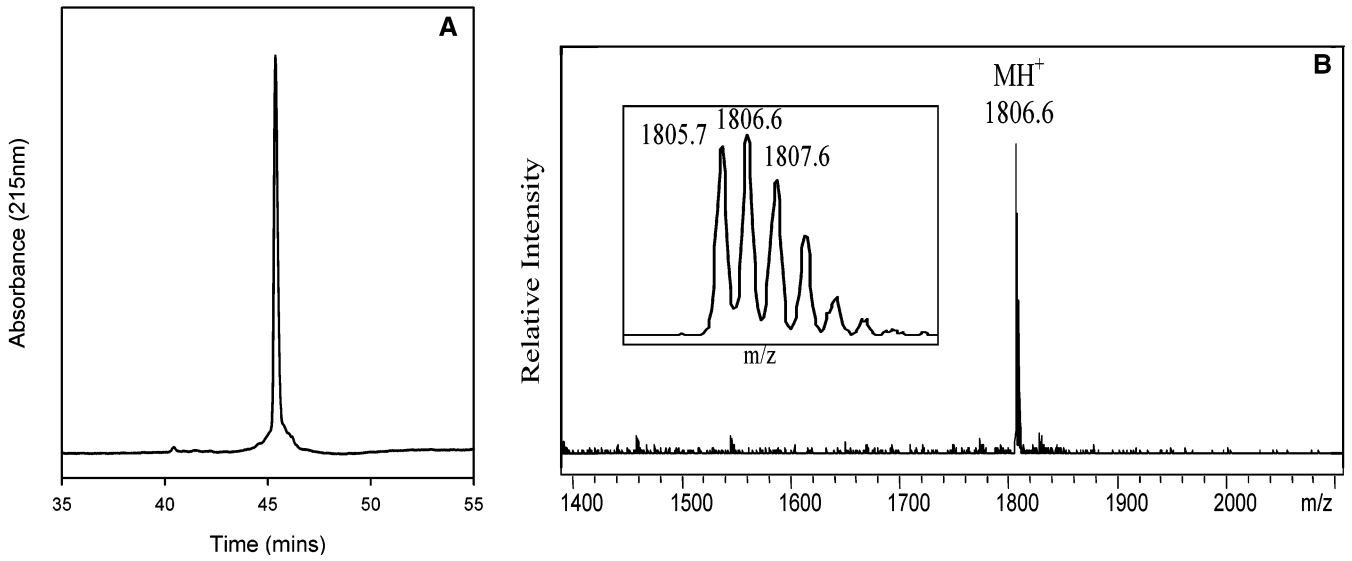

FIGURE 2: (A) HPLC analysis of synthetic Vc1.1. Analysis of the synthetic peptide Vc1.1 by RP-HPLC was performed on a Synergi MAX-RP column (2.0 mm × 150 mm) (Phenomenex). The peptide was loaded onto the column in 95% buffer A and eluted in a gradient of 5−60% buffer B with a flow rate of 0.3 mL/min over 60 min. Buffer A = 0.1% TFA; buffer B = 0.08% TFA and 90% acetonitrile. Absorbance was measured at 215 nm. (B) MS data on Vc1.1. The mass profile of synthetic Vc1.1 was determined by ESI-MS.

Table 2: Ability of α-Conotoxin Vc1.1, Carbachol, and Nicotine To Displace 1 nM [^{3}H]Epibatidine from Bovine Adrenal Medullary Membranes[a]

ligand	subtype selectivity	K_{i1}	K_{i2}	type of displacement
epibatidine	α4β2 > α3β4	0.26 ± 0.05 nM		one site
Vc1.1	unknown	2.3 ± 0.8 nM	3.7 ± 0.51 μM	two site
nicotine	α4β2 > α3β4	6.3 ± 0.3 nM	148 ± 5.1 nM	two site
carbachol	α3β4	2.8 ± 0.37 μM		one site

[a] K_i values were determined from IC_{50} values in accordance with the Cheng−Prusoff rule. Competition binding displacement was determined using nonlinear regression with the GraphPad Prizm program.

carbachol showed one-site displacement of the radioligand. Together, these results suggest that Vc1.1 is a specific competitive inhibitor of nicotine stimulation of the particular nAChR subtypes in bovine chromaffin cells that bind epibatidine, most likely, a heterotrimeric α3β4 receptor also containing α5 and/or α7 subunits.

Effects of Vc1.1 on Sensory Nerve Activity. The ability of the unmyelinated C-fibers to mount a vascular inflammatory response upon selective activation by antidromic electrical stimulation (20 V, 2 ms for 1 min at 5 Hz) is an established method to determine their function (*15*, *16*). The conotoxin peptides were perfused over the base of a blister raised on the footpad of an anesthetized rat that is innervated by peripheral terminals of the sciatic nerve and is accessible to the perfused compounds. Changes in the microvascular blood flow were measured from the base of the blister using laser Doppler flowmetry (*21*). Vc1.1 produced a significant dose-dependent inhibition of the vascular response to low-frequency electrical stimulation of the sensory nerves at concentrations ranging from 0.01 to 10 μM (Figure 5A). Furthermore, the degree of inhibition induced by 0.1 μM Vc1.1 was greater than that induced by the same concentration of ω-conotoxin MVIIA (Figure 5B).

As the vascular response measured in response to electrical stimulation of sensory nerves reflects not only the ability of these nerves to release neurotransmitters to cause vasodilation but also the ability of the smooth muscle cells to dilate, we used sodium nitroprusside as an internal control to indicate any changes in smooth muscle reactivity. SNP is a direct smooth muscle cell dilator that is independent of sensory nerves. Perfusion of Vc1.1 did not alter the response to SNP (Figure 6), indicating that the inhibitory effect of Vc1.1 on the vascular response to sensory nerve stimulation is mediated via an action that is independent of changes in smooth muscle reactivity. The most likely explanation is that Vc1.1 inhibits the release of sensory neurotransmitters from the stimulated sensory nerves.

DISCUSSION

A molecular approach has been taken to identify a novel conopeptide, α-conotoxin Vc1.1, whose amino acid sequence was deduced by sequencing a cDNA library obtained by RT-PCR from the mRNA extracted from the venom ducts of a tropical marine snail, *C. victoriae*, found in waters off the coast near Broome, Western Australia. The coding sequence was 201 base pairs, which translates into 66 amino acids. The predicted pre-region of the protein precursor was 21 amino acids, the pro-region 29 amino acids, and the mature peptide 16 amino acids in length. The peptide Vc1.1, as synthesized by solid-phase techniques, had 16 amino acids with a C-terminal amide and with two disulfide bonds connected in a 4,7 loop structure. Purification by RP-HPLC gave a single major peak (>70% pure) whose mass was confirmed by ESI-MS as 1806.6. The peptide was soluble in water and stable in solution when tested after 8 weeks. Conotoxin Vc1.1 was shown in bovine and rat assay systems in vitro and in vivo, respectively, to inhibit in vitro a neuronal nicotinic acetylcholine receptor response and to inhibit in vivo a vascular response to pain.

The characteristics of Vc1.1 are those of a specific antagonist of neuronal nAChRs. In the in vitro assays used in this study, that means an antagonist specific to nAChRs

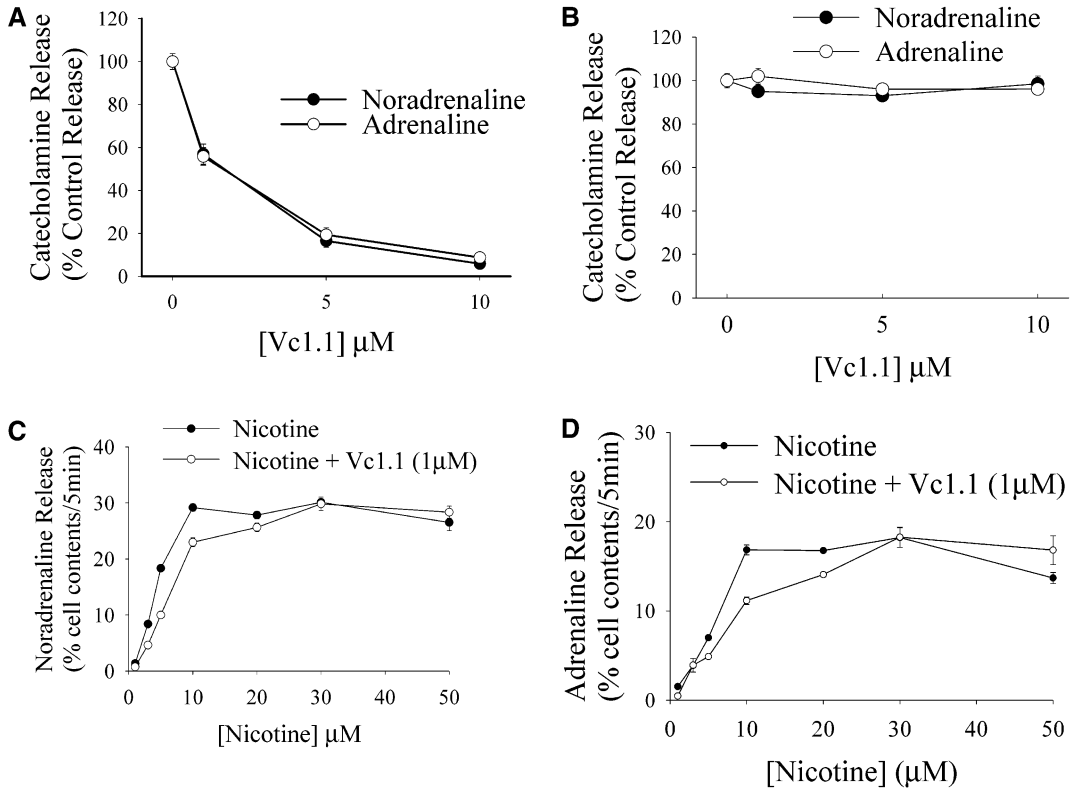

FIGURE 3: (A) Effects of Vc1.1 on nicotine (1 μM) evoked release of adrenaline and noradrenaline from primary monolayer cultures of bovine adrenal chromaffin cells. (B) Effects of Vc1.1 on the release of adrenaline and noradrenaline from primary monolayer cultures of bovine adrenal chromaffin cells evoked by 56 mM K^+ that bypasses the nAChR and causes Ca^{2+} entry and catecholamine secretion via activation of voltage-activated ion channels. (C) Effect of nicotine on Vc1.1 (1 μM) inhibition of the release of noradrenaline from primary monolayer cultures of bovine adrenal chromaffin cells. (D) Effect of nicotine on Vc1.1 (1 μM) inhibition of the release of adrenaline from primary monolayer cultures of bovine adrenal chromaffin cells.

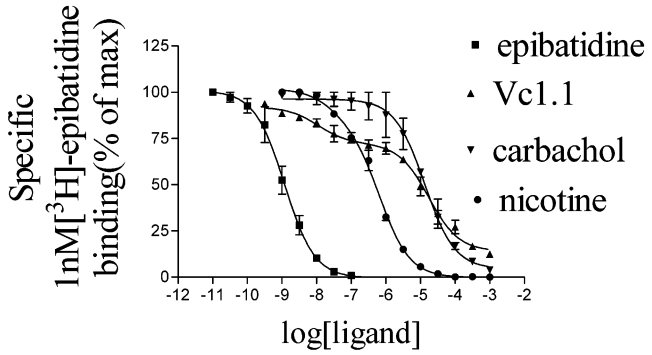

FIGURE 4: Ability of α-conotoxin Vc1.1, carbachol, and nicotine to displace 1 nM [^{3}H]epibatidine binding from bovine chromaffin cell membranes. Data are compared to displacement with epibatidine, and experiments were performed three times in triplicate where data represent the mean ± standard error of mean (SEM) ($n = 3$). Curves represent nonlinear regression, and Vc1.1 and nicotine results are fitted to a two-site model, whereas epibatidine and carbachol are fitted to a single site model.

present in bovine chromaffin cells. Like conotoxins MII and EpI, and other 4,7 loop conopeptides (*12*, *22*), Vc1.1 selectively antagonized the neuronal-type nAChRs but had no effect on nAChRs at the neuromuscular junction. In the case of MII this was demonstrated with α3β2 nAChR's expressed in *Xenopus* oocytes (*22*) and for EpI (*12*) using the same assays (in bovine chromaffin cells and rat phrenic nerve/hemidiaphragm) as in the present study. Bovine chromaffin cells are known to contain mRNA encoding α3, α5, α7, and β4 nAChR subunits. It has been previously assumed that these subunits are assembled in chromaffin cells predominantly into separate α3β4 heteromeric and α7 homomeric nAChRs. However, a recent report (*23*) suggests that the predominant forms of nAChR in bovine chromaffin cells are heteromeric receptors (α3α7β4 or α3α5β4). Such heteromeric neuronal nAChRs are therefore the most likely receptors for the inhibition shown by Vc1.1 in bovine chromaffin cells.

Conotoxins that have previously shown analgesic activity in rats and humans, ω-conotoxins, MVIIA ,and CVID, specifically target N-type calcium channels involved in pain pathways without significantly affecting P/Q-type calcium channels (*6*). N-type calcium channels are electronically linked to acetylcholine receptors at neuronal synapses (*24*); the analgesic action of ω-conotoxins apparently depends on their ability to reduce neurotransmission in sensory pathways. Such blocking of N-type calcium channels results in substantial pain relief (*2*). Inhibition of the acetylcholine receptors electronically linked to these N-type calcium channels in pain pathways could therefore be expected to reduce activation of the N-type calcium channels, as it does elsewhere in the brain (*24*), and bring about analgesia.

The present results obtained in vivo with α-conopeptide Vc1.1 demonstrate its ability to inhibit neurotransmitter release from sensory neurones involved in inflammation and pain transmission. This is demonstrated by its effectiveness in reducing the ability of these nerves to mount a peripheral inflammatory vascular response. This mechanism of action could contribute to the ability of this novel α-conotoxin to alleviate pain in animals with peripheral neuropathy (*31*). Its ability to suppress C-fiber function was greater than that

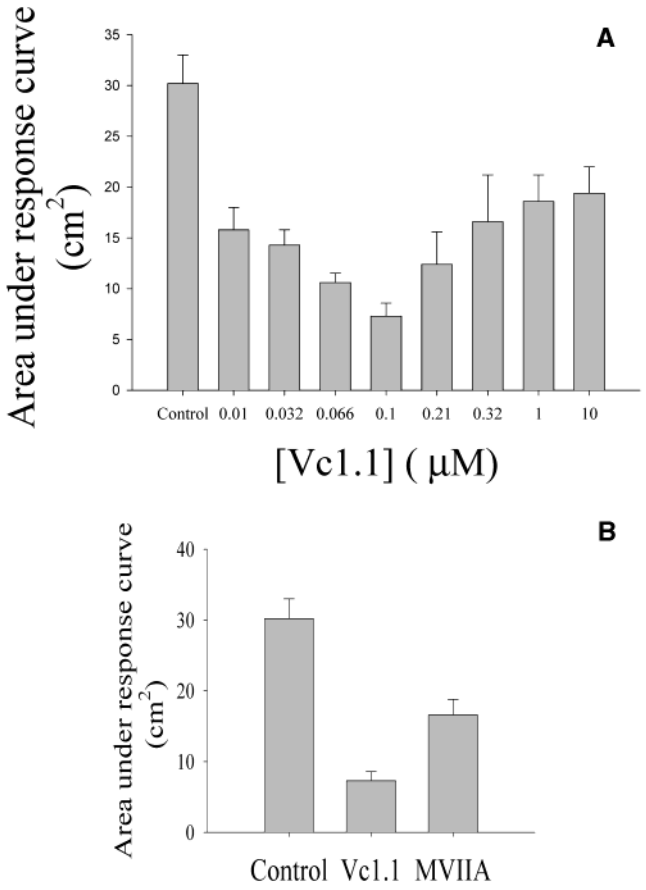

FIGURE 5: (A) Effect of conotoxin Vc1.1 at different concentrations on the vascular response to low-frequency electrical stimulation (20 V, 2 ms for 1 min at 5 Hz) of the sciatic nerve. Results are expressed as the mean ± SEM ($n = 5-8$). Statistical analyses were determined using one-way analysis of variance (ANOVA) followed by Student−Newman−Keuls post hoc tests using the SPSS statistical package. All treatments were significantly different from the control group ($p < 0.05$). (B) Effect of ω-conotoxin MVIIA and α-conotoxin Vc1.1 at concentrations of 0.1 μM on the vascular response to low-frequency electrical stimulation of the sciatic nerve. Results are expressed as the mean ± SEM ($n = 5-8$). Statistical analyses were determined using one-way analysis of variance (ANOVA) followed by Student−Newman−Keuls post hoc tests. Both treatments were significantly different from the control group ($p < 0.05$).

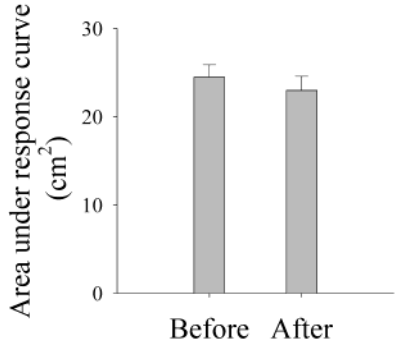

FIGURE 6: Effect of Vc1.1 on the vascular response to SNP. Statistical analysis using one-way analysis of variance (ANOVA) ($n = 41$) showed no significant difference ($p > 0.05$).

of MVIIA, an ω-conotoxin with known analgesic activity in rats and humans. Inhibition of C-fiber-mediated activity by Vc1.1 could be the result of its ability to block neuronal nAChRs as shown from the in vitro work utilizing bovine chromaffin cell assays (Figures 3 and 4). Likely targets are a set of neuronal nAChRs expressed in sensory nerves (*25*) and whose increased expression in damaged sensory nerves could constitute a source of phantom pain. Such nAChRs would be an alternative to N-type Ca^{2+} channels to target by conotoxins to inhibit the influx of Ca^{2+} into damaged nerves. These experiments with Vc1.1 suggest that directly inhibiting the response of neuronal nAChR's using selective neuronal α-conotoxins is a plausible approach to modulation of pain pathways (see ref *26* for review) that does not involve direct action on voltage-operated calcium channels. In another study (*31*), we show that Vc1.1 acts as a potent analgesic in a model of human neuropathic pain in the rat.

The in vitro and in vivo experiments in bovine and rat assay systems show that Vc1.1 successfully discriminated between neuronal and neuromuscular nAChRs. We suggest that there may be sufficient conservation between species in the functional regions of the neuronal and neuromuscular nAChRs for this discrimination to apply also to human nAChRs.

We propose, on the basis of the above results, that blocking the neuronal-type nicotinic acetylcholine receptors on sensory nerves by selective neuronal α-conotoxins is an alternative strategy that can be further developed for the treatment of pain in humans.

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REFERENCES

1. Olivera, B. M. (1997) E. E. Just Lecture, 1996. *Conus* venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology, *Mol. Biol. Cell 8*, 2101−2109.
2. McIntosh, J. M., and Jones, R. M. (2001) Cone venom-from accidental stings to deliberate injection, *Toxicon 39*, 1447−1451.
3. Dutton, J. L., and Craik, D. J. (2001) Alpha-conotoxins: nicotinic acetylcholine receptor antagonists as pharmacological tools and potential drug leads, *Curr. Med. Chem. 8*, 327−344.
4. Duda, T. F., Jr., and Palumbi, S. R. (1999) Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod Conus, *Proc. Natl. Acad. Sci. U.S.A. 96*, 6820−6823.
5. Luo, S., Nguyen, T. A., Cartier, G. E., Olivera, B. M., Yoshikami, D., and McIntosh, J. M. (1999) Single-residue alteration in alpha-conotoxin PnIA switches its nAChR subtype selectivity, *Biochemistry 38*, 14542−14548.
6. Lewis, R. J., Nielsen, K. J., Craik, D. J., Loughnan, M. L., Adams, D. A., Sharpe, I. A., Luchian, T., Adams, D. J., Bond, T., Thomas, L., Jones, A., Matheson, J. L., Drinkwater, R., Andrews, P. R., and Alewood, P. F. (2000) Novel omega-conotoxins from *Conus catus* discriminate among neuronal calcium channel subtypes, *J. Biol. Chem. 275*, 35335−35344.
7. Bowersox, S. S., and Luther, R. (1998) Pharmacotherapeutic potential of omega-conotoxin MVIIA (SNX-111), an N-type neuronal calcium channel blocker found in the venom of *Conus magus*, *Toxicon 36*, 1651−1658.
8. Maksel, D., Guranowski, A., Ilgoutz, S. C., Moir, A., Blackburn, M. G., and Gayler, K. R. (1998) Cloning and expression of diadenosine 5′,5‴-P1,P4-tetraphosphate hydrolase from *Lupinus angustifolius* L, *Biochem. J. 329*, 313−319.

9. Livett, B. G., Mitchelhill, K. I., and Dean, D. M. (1987) Adrenal chromaffin cells—their isolation and culture, in *In-Vitro Methods for Studying Secretion* (Poisner, A. M., and Trifaro, J. M., Eds.) pp 171−175, Elsevier, Amsterdam.
10. Livett, B. G., Marley, P. D., Mitchelhill, K. I., Wan, D, C.-C., and White, T. D. (1987) Assessment of adrenal chromaffin cell secretion: presentation of four techniques, in *In-Vitro Methods for Studying Secretion* (Poisner, A. M., and Trifaro, J. M., Eds.) pp 177−204, Elsevier, Amsterdam.
11. Macallan, D. R., Lunt, G. G., Wonnacott, S., Swanson, K. L., Rapoport, H., and Albuquerque, E. X. (1988) Methyllycaconitine and (+)-anatoxin differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems, *FEBS Lett. 226*, 357−363.
12. Loughnan, M., Bond, T., Atkins, A., Cuevas, J., Adams, D. J., Broxton, N. M., Livett, B. G., Down, J. G., Jones, A., Alewood, P. F., and Lewis, R. J. (1998) Alpha-conotoxin EpI, a novel sulfated peptide from *Conus episcopatus* that selectively targets neuronal nicotinic acetylcholine receptors, *J. Biol. Chem. 273*, 15667−15674.
13. Khalil, Z., and Helme, R. D. (1989) Sequence of events in substance P-mediated plasma extravasation in rat skin, *Brain Res. 500*, 256−262.
14. Helme, R. D., White, D. M., and Andrews, P. V. (1985) Neurogenic inflammation in skin blisters, *Exp. Brain Res. 59*, 382−387.
15. Khalil, Z., Ralivic, V., Bassirat, M., Dusting, G. J., and Helme, R. D. (1994) Effect of ageing on sensory nerve function in rat skin, *Brain Res. 641*, 265−272.
16. Lembeck, F., and Holzer, P. (1979) Substance P as a neurogenic mediator of antidomic vasodilation and neurogenic plasma extravasation, *Naunyn-Schmiedeberg's Arch. Pharmacol. 310*, 175−183.
17. Walker, C. S., Steel, D., Jacobsen, R. B., Lirazan, M. B., Cruz, L. J., Hooper, D., Shetty, R., DelaCruz, R. C., Nielsen, J. S., Zhou, L. M., Bandyopadhyay, P., Craig, A. G., and Olivera, B. M. (1999) The T-superfamily of conotoxins, *J. Biol. Chem. 274*, 30664−30671.
18. McIntosh, J. M., Santos, A. D., and Olivera, B. M. (1999) Conus peptides targeted to specific nicotinic acetylcholine receptor subtypes, *Annu. Rev. Biochem. 68*, 59−88.
19. Luetje, C. W., Wada, K., Rogers, S., Abramson, S. N., Tsuji, K., Heinemann, S., and Patrick, J. (1990) Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations, *J. Neurochem. 55*, 632−640.
20. Fainzilber, M., Hasson, A., Oren, R., Burlingame, A. L., Gordon, D., Spira, M. E., and Zlotkin, E. (1994) New mollusc-specific alpha-conotoxins block aplysia neuronal acetylcholine receptors, *Biochemistry 33*, 9523−9529.
21. Merhi, M., Dusting, G. J., and Khalil, Z. (1998) CGRP and nitric oxide of neuronal origin and their involvement in neurogenic vasodilatation in rat skin microvasculature, *Br. J. Pharmacol. 123*, 863−868.
22. Cartier, G. E., Yoshikami, D., Gray, W. R., Luo, S., Olivera, B. M., and McIntosh, J. M. (1996) A new alpha-conotoxin which targets alpha3beta2 nicotinic acetylcholine receptors, *J. Biol. Chem. 271*, 7522−7528.
23. Maneu, V., Rojo, J., Mulet, J., Valor, L. M., Sala, F., Criado, M., Garcia, A. G., and Gandia, L. (2002) A single neuronal nicotinic receptor a3a7b4* is present in the bovine chromaffin cell, *Ann. N.Y. Acad. Sci. 971*, 165−167.
24. Kulak, J. M., McIntosh, J. M., Yoshikami, D., and Olivera, B. M. (2001) Nicotinic-evoked transmitter release from synaptosomes: functional association of specific presynaptic acetylcholine receptors and voltage-gated calcium channels, *J. Neurochem. 77*, 1581−1589.
25. Genzen, J. R., Cleve, W. V., and McGhehee, D. S. (2001) Dorsal root ganglion neurons express multiple nicotinic acetylcholine receptor subtypes, *J. Neurophysiol. 86*, 1773−1782.
26. Flores, C. M. (2000) The promise and pitfalls of a nicotinic cholinergic approach to pain management, *Pain 88*, 1−6.
27. Luo, S., Kulak, J. M., Cartier, G. E., Jacobsen, R. B., Yoshikami, D., Olivera, B. M., and McIntosh, J. M. (1998) Alpha-conotoxin Au1B selectively blocks alpha3 beta4 nictoinic acetylcholine receptors and nicotine-evoked norepinephrine release, *J. Neurosci. 18*, 8571−8579.
28. Zafaralla, G. C., Ramilo, C., Gray, W. R., Karlstrom, R., Olivera, B. M., and Cruz, L. J. (1988) Phylogenetic specificity of cholinergic ligands: alpha-conotoxin SI, *Biochemistry 27*, 7102−7105.
29. Gray, W. R., Luque, A., Olivera, B. M., Barrett, J., and Cruz, L. J. (1981) Peptide toxins from *Conus geographus* venom, *J. Biol. Chem. 256*, 4734−4740.
30. Polidano, M. A., Marley, P. D., and Livett, B. G. (2003) [^{3}H]-Epibatidine labels at least two α3-containing neuronal nicotinic receptor subtypes in the bovine adrenal medulla, *Neurosci. Lett.* (submitted for publication).
31. Satkunanathan, N., Livett, B., Gayler, K., Sandall, D., Down, J., and Khalil, Z. (2003) A novel α-conotoxin alleviates neuropathic pain and accelerates functional recovery of injured neurons, *Pain* (submitted for publication).

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