The $\alpha_2\delta$ Auxiliary Subunit Reduces Affinity of $\omega$-Conotoxins for Recombinant N-type (Ca$_{2.2}$) Calcium Channels*

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The $\omega$-conotoxins from fish-hunting cone snails are potent inhibitors of voltage-gated calcium channels. The $\omega$-conotoxins MVIIA and CVID are selective N-type calcium channel inhibitors with potential in the treatment of chronic pain. The $\beta$ and $\alpha_2\delta$-1 auxiliary subunits influence the expression and characteristics of the $\alpha_1B$ subunit of N-type channels and are differentially regulated in disease states, including pain. In this study, we examined the influence of these auxiliary subunits on the ability of the $\omega$-conotoxins GVIA, MVIIA, CVID and analogues to inhibit peripheral and central forms of the rat N-type channels. Although the $\beta_3$ subunit had little influence on the on- and off-rates of $\omega$-conotoxins, co-expression of $\alpha_2\delta$ with $\alpha_1B$ significantly reduced on-rates and equilibrium inhibition at both the central and peripheral isoforms of the N-type channels. The $\alpha_2\delta$ also enhanced the selectivity of MVIIA, but not CVID, for the central isoform. Similar but less pronounced trends were also observed for N-type channels expressed in human embryonic kidney cells. The influence of $\alpha_2\delta$ was not affected by oocyte deglycosylation. The extent of recovery from the $\omega$-conotoxin block was least for GVIA, intermediate for MVIIA, and almost complete for CVID. Application of a hyperpolarizing holding potential (~120 mV) did not significantly enhance the extent of CVID recovery. Interestingly, [R10K]MVIIA and [K10R]CVID had greater recovery from the block, whereas [K10R]CVID had reduced recovery from the block, indicating that position 10 had an important influence on the extent of $\omega$-conotoxin reversibility. Recovery from CVID block was reduced in the presence of $\alpha_2\delta$ in human embryonic kidney cells and in oocytes expressing $\alpha_1B$-Lore. These results may have implications for the antinociceptive properties of $\omega$-conotoxins, given that the $\alpha_2\delta$ subunit is up-regulated in certain pain states.

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The N-type (Ca$_{2.2}$) voltage-gated calcium channels play an important role in the control of neurotransmitter release from nerve terminals (1, 2) and are important drug targets for the treatment of pain (3, 4) and ischemic brain injury (5). Native N-type Ca$_{2+}$ channels are hetero-oligomers that comprise a pore-forming $\alpha_1$ subunit ($\alpha_{1n}$) and at least two auxiliary subunits, $\beta$ and $\alpha_2\delta$, which modulate the $\alpha_1$ subunit function (6, 7). Two splice variants of the $\alpha_{1n}$ subunit have been identified that occur predominantly in the central ($\alpha_{1n,c}$) and peripheral ($\alpha_{1n,p}$) nervous systems (8, 9). Additional splice variants that lack large parts of the domain II-III linker region, including the synaptic protein interaction site, have also been isolated from human brain cDNA libraries (10). Multiple isoforms of the $\beta$ and $\alpha_2\delta$ subunits also exist that can interact with the $\alpha_1$ subunit to produce N-type Ca$_{2+}$ channels with different gating properties, allowing the fine tuning of synaptic transmissions (11).

A distinguishing feature of N-type channels is their high sensitivity to block by $\omega$-conotoxins, which are relatively small (~25 residue) polypeptides isolated from the venom of the marine snail of the genus Conus (12, 13). $\omega$-Conotoxins have been used as research tools to help define the distribution and physiological roles of specific N-type calcium channels (14–17) and have potential therapeutic value as intrathecal treatments for pain. MVIIA from Conus magus is being tested in clinical trials as a treatment for neuropathic pain, but dose-limiting side effects are a concern (18–20). $\omega$-Conotoxin CVID from Conus catus (21), which appears to have a wider therapeutic window (22), is also in clinical trials (23).

The modulatory effects of auxiliary subunits on the biophysical properties of Ca$_{2+}$ channel $\alpha_1$ subunits have been well characterized. When expressed alone, $\alpha_1$ subunits produce functional channels with kinetic properties that differ substantially from those of the native channel (24, 25). The most commonly observed effect of the $\beta$ and $\alpha_2\delta$ subunits when they are co-expressed with the $\alpha_1$ subunit in a heterologous expression system such as Xenopus oocytes or HEK (23) 293 cells is to increase the amplitude of macroscopic Ca$_{2+}$ channel currents (typically carried by Ba$_{2+}$ ions) and influence the rate of channel activation and inactivation (26–29). Recently, a negative regulatory effect of the overexpressed $\beta_3$ subunit on N-type Ca$_{2+}$ channel currents has been demonstrated in Xenopus oocytes (30).

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The atomic coordinates and structure factors (codes 1TTK, 1TT3, 1TTL, and 1TR6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The abbreviations used are: HEK, human embryonic kidney; I-V, current-voltage.
N-type Ca\(^{2+}\) channels are widely distributed in brain and peripheral neurons with a variety of co-localized auxiliary subunits (31–34). This heterogeneity in subunit composition contributes to functional and, potentially, to pharmacological heterogeneity. Although the pathophysiological role of auxiliary subunits is poorly understood, up-regulation of \(\alpha_{2}\delta\) subunits has been reported in association with neuropathic pain (35–38). In a previous study, we showed that CVID was a highly selective N-type Ca\(^{2+}\)-channel inhibitor (21) that induced depolarization-repolarization-related transmitter release at preganglionic parasympathetic nerve terminals (15). Both CVID and MVIIA inhibited currents mediated by the central and peripheral isoforms of the \(\alpha_{1B}\) subunit expressed in Xenopus oocytes in the absence of the \(\alpha_{2}\delta\) auxiliary subunit (21). In the present study, we show that the affinity of the \(\omega\)-conotoxins CVID and MVIIA to block N-type Ca\(^{2+}\) channel current is reduced in the presence of \(\alpha_{2}\delta\), and we identify position 10 in \(\omega\)-conotoxins as having an important influence on the extent of recovery from channel block.

EXPERIMENTAL PROCEDURES

**Oocyte Injection and Recording—** Oocytes (stages V–VI) were surgically removed from mature Xenopus laevis frogs anesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester (MS-222). The follicular cell layer was removed by incubating oocytes in Ca\(^{2+}\)-free solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), and 5 mM HEPES (pH 7.4) plus 2 mg/ml collagenase (Sigma type 1) for 2 h at room temperature. Oocytes were rinsed several times, sorted, and maintained at 18 °C in an ND96 storage solution that contained 96 mM NaCl, 2 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES, and 5 mM pyruvate plus 50 \(\mu\)g/ml gentamycin (pH 7.4).

\(\omega\)-Conotoxin CVID and MVIIA were injected with 46 nl of 50 mM 1,2-bis(\(\omega\)-aminophenoxy)ethane-N\(_2\)N\(_3\)N\(_4\)-tetraacetate solution at least 15 min prior to recording. Data were leak-subtracted and analyzed with Clampfit 8.0 (Axon Instruments Inc.). Series resistance was compensated to 95%. Membrane currents were evoked every 15 s by 150-ms test pulses from −100 mV to +10 or +20 mV. Cells were perfused directly with control or toxin-containing bath solutions by a custom-built perfusion system allowing complete solution changes in <1 s. Data were leak-subtracted and analyzed with Clampfit 8.0 (Axon Instruments Inc.).

**Peptide Synthesis**—

The \(\omega\)-conotoxins CVID, [K10R]CVID, MVIIA, [K10R]MVIIA, GVIA, and [O10K]GVIA (see Fig. 1) were assembled using standard solid-phase peptide synthesis (39), deprotected, cleaved, and purified as described previously (40). The purified reduced peptides were oxidized in a cuvet containing 0.33 mM NH\(_4\)OAc and 0.5 M guanidine-HCl (pH 7.8) for 2–4 days at 4 °C in the presence of reduced and oxidized glutathione (molar ratio 1:100:1). Oxidized peptides were purified by preparative reversed phase high pressure liquid chromatography (40). Mass spectra were measured on a time-of-flight mass spectrometer (PerSeptive Biosystems) equipped with an electrospray atmospheric pressure ionization source.

**Radioligand Binding Assays—** A rat brain membrane was prepared as described previously (41). Data are from at least three experiments, each performed in triplicate.

**NMR Spectroscopy**—

Three-dimensional solution structures were calculated using the torsion angle dynamics/simulated annealing protocol in XPLOR version 3.853, as described previously (41). The structures were deposited in the Protein Data Bank under the following accession numbers: 2NAD, 2NMJ, 2NMF, 2NMG, 2NMS, and 2NMT.

**RESULTS**

Effects of \(\beta\) and \(\alpha_{2}\delta\) Subunits on the Biophysical Properties of N-Type Ca\(^{2+}\) Channels Expressed in Oocytes—

| loops | connectivity |
|-------|--------------|
| 1     | CVID         |
| 2     | [K10R]CVID   |
| 3     | MVIIA        |
| 4     | [R10K]MVIIA  |
|       | [O10K]GVIA   |

Using Prism software (GraphPad). The \(K_d\) for each toxin was then calculated using Equation 1,

\[
K_d = K_{on} + K_m
\]

where \(K_{on} = t_{1/2} \cdot \alpha_{2}\delta\) (s\(^{-1}\)) and \(K_m = (t_{1/2} - K_{off})/\alpha_{2}\delta\) (s\(^{-1}\)).

**Fig. 1.** Amino acid sequence alignment of the \(\omega\)-conotoxins CVID (from C. catus), MVIIA (C. magus), and GVIA (C. geographus) and the analogues [K10R]CVID, [R10K]MVIIA, and [O10K]GVIA. Shown are the positions of the four loops and the disulfide connectivity that characterize \(\omega\)-conotoxins. Position 10 residues are boxed.
 Auxiliary Subunit Influences on \( \omega \)-Conotoxin Pharmacology

3–5 days following the injection of 2.5 ng of cRNA encoding for the \( \alpha_{1B-d} \) (central isoform) of the N-type Ca\(^{2+} \) channel (Fig. 2). Currents evoked by 150-ms voltage steps to 0 mV from a holding potential of \(-80\) mV typically ranged from 150–500 nA in peak current amplitude (Fig. 2A). Co-expression of the \( \beta_3 \) subunit at a 1:1 ratio with \( \alpha_{1B-d} \) increased the peak current of current-voltage (I-V) relationships 2-fold compared with \( \alpha_{1B-d} \) expressed without the addition of recombinant \( \beta_3 \). In agreement with a previous report (8), \( \beta_3 \) had only minor effects on the activation and inactivation kinetics of Ca\(^{2+} \) channel current (Fig. 2A). Co-expression of the \( \beta_3 \) subunit caused a significant (\( p < 0.001 \)) hyperpolarizing shift in the half-maximal activation voltage \( (V_{1/2}) \) from \(-2.6 \pm 0.8 \) (\( n = 11 \)) for \( \alpha_{1B-d} \) alone to \(-8.3 \pm 0.8 \) (\( n = 16 \)) in the presence of \( \beta_3 \) (Fig. 2, B and C).

Co-expression of the \( \alpha_{1B-d} \) and \( \beta_3 \) subunits together with the \( \alpha_{\delta} \) subunit resulted in an additional enhancement of the peak current amplitude (Fig. 2, A and B). On average, the peak current amplitude from the I-V curve obtained in the presence of the \( \alpha_{\delta} \) subunit was 2-fold larger than that obtained with the \( \alpha_{1B-d} + \beta_3 \) subunit combination alone. Ca\(^{2+} \) channel currents recorded from oocytes expressing \( \alpha_{\delta} \beta_3 \) also exhibited faster activation and inactivation kinetics during the 150-ms test pulse (Fig. 2A). Co-expression of \( \alpha_{\delta} \beta_3 + \beta_3 \) caused a small but significant (\( p < 0.01 \)) depolarizing shift of \( V_{1/2} \) for \( \alpha_{1B-d} \) activation to \(-5.2 \pm 0.6 \) mV (\( n = 16 \)) compared with \( \beta_3 \) alone (\(-8.3 \pm 0.8 \) mV) (Fig. 2, B and C). A similar \( \alpha_{\delta} \beta_3 \)-induced depolarizing shift of I-V curves has been reported for N- and R-type Ca\(^{2+} \) channels (29, 42). The \( \alpha_{1B-d} \) (peripheral isoform) channel current amplitude and channel properties were also modulated by \( \beta_3 \) and \( \alpha_{\delta} \) subunits in a manner similar to their influence on \( \alpha_{1B-d} \).

Effect of \( \beta_3 \) and \( \alpha_{\delta} \) Subunits on the Inhibition by \( \omega \)-Conotoxins of N-type Ca\(^{2+} \) Channel Current Expressed in Oocytes—To assess the influence of different auxiliary subunits on the pharmacology of \( \omega \)-conotoxins at N-type Ca\(^{2+} \) channels, we compared the ability of the \( \omega \)-conotoxins CVID and MVIIIA and
the analogs [K10R]CVID and [R10K]MVIIA (see Fig. 1) to inhibit N-type currents mediated by either α1B alone, α1B + β3, or α1B + β3 + αδ. On- and off-rates of channel block and estimates of potency (Kᵢ) for the different Ca²⁺ channel subunit combinations were obtained following application and washout of each toxin (Fig. 3, A and B). In all cases, the current decrease attributed to toxin block reached a steady state and was fitted by a single exponential function. A single exponential function also fitted recovery from block during toxin washout (Fig. 3C). The kinetics, extent, and recovery from block were determined for each toxin from curve fits. Data obtained for the peripheral (α1B-B) and central (α1d-d) isoforms of the N-type Ca²⁺ channel are presented in Tables I and II, respectively.

For the α1δ subunit expressed alone or in combination with the β3 subunit, the addition of 1 nM CVID or MVIIA inhibited a substantial portion of the Ba²⁺ current mediated by either the peripheral or the central isoform of the N-type Ca²⁺ channel (Tables I and II). CVID was less potent than MVIIA, especially at the α1B,δ subunit (Tables I and II; Fig. 4A). The analogues [K10R]CVID and [R10K]MVIIA had similar profiles of inhibition of α1δ to CVID and MVIIA, respectively.

Co-expression of the αδ subunit with α1B + β3 subunit dramatically reduced the apparent affinity of the N-type Ca²⁺ channel for the ω-conotoxins (Figs. 4 and 5), ranging from 150–680-fold at α1B-B, to 80–220-fold at α1B,δ (Tables I and II). At both splice variants, the effect of αδ was most prominent for [K10R]CVID and MVIIA compared with MVIIA and [R10K]MVIIA. The reduction in potency induced by αδ arose from a markedly reduced on-rate for these ω-conotoxins without any significant effect on off-rate kinetics (Tables I and II; Fig. 5). The same influence of αδ on the kinetics and potency of ω-conotoxin block at α1B were also observed in the absence of the added β3 (Table III). Similar estimates of potency (log IC₅₀) were obtained using equilibrium concentration-response data obtained for ω-conotoxins CVID (−9.93 ± 0.03, −7.66 ± 0.03) and MVIIA (−10.19 ± 0.07, −8.30 ± 0.03) inhibition of α1d-d + β3 in the absence or presence of αδ, respectively (Fig. 4B).

Two batches of oocytes expressing α1B,δ + β3 were found to be particularly sensitive to MVIIA block (Fig. 4B, diamonds); this sensitivity was pronounced when the oocytes were maintained longer and was similar to results obtained previously for CVID at α1B,δ alone (21), indicating that the picomolar inhibition occasionally observed is an oocyte batch-dependent phenomenon. Interestingly, a plot of τᵢ on as a function of ω-conotoxin concentration gives a linear relationship with a slope >−1. This difference was most pronounced for τᵢ on obtained in the absence of αδ (−0.48 ± 0.03) but still significantly different from unity in the presence of αδ (−0.73 ± 0.01). Assuming constant off-rate kinetics, this change in the on-rate gives rise to different potency estimates, depending on the concentration of ω-conotoxin tested. In the absence of αδ, at 0.1 nM CVID, the estimated Kᵢ was 0.06 nM, whereas at 10 nM CVID the estimated Kᵢ was 20-fold greater (1.3 nM), suggestive of channel heterogeneity.

Reversibility of ω-Conotoxin Inhibition of N-type Ca²⁺ Channel Current Expressed in Oocytes—As observed previously for MVIIA (43, 44), recovery from block (reversibility) for MVIIA and CVID was found to be incomplete (Fig. 6; Tables I and II). For α1B-B expressed alone, recovery from block was less (−45%) for toxins with Arg at position 10 compared with those with a Lys at position 10 (−85%). For α1d-d, channels expressed alone (Table II), CVID and [K10R]CVID had similar levels of recovery (−45%), whereas [R10K]MVIIA (83%) had enhanced recovery compared with MVIIA (25%). For α1B,δ + β3 channels, recovery from block was also greater for peptides containing Arg at position 10, and for α1B,δ + β3 channels it was greater for [R10K]MVIIA. Co-expression of αδ with α1B + β3 further reduced recovery from block for CVID and [K10R]CVID but not for MVIIA and [R10K]MVIIA (Fig. 6, Tables I and II). In contrast, for α1d-d + β3 + αδ channels the recovery from block was significantly reduced for MVIIA and [R10K]MVIIA but not for CVID and [K10R]CVID. Overall, recovery was least for MVIIA (13%) at α1B,δ and greatest for [R10K]MVIIA at α1B,β (85%). Previous studies have suggested that the extent of recovery from ω-conotoxin block is enhanced at hyperpolarizing holding potentials (45). In the present study, applying a hyperpolarizing holding potential was found to have no significant influence on the extent of recovery from block, at least for CVID (Table III).

GVIA has a sequence that is distinct from those of other ω-conotoxins (see Fig. 1) and shows pseudo-irreversible binding to the N-type Ca²⁺ channel (45). On the basis that position 10 was found to influence the reversibility of MVIIA and CVID inhibition of N-type calcium channels, we synthesized the analogue [O10K]GVIA to investigate the importance of position 10 for GVIA reversibility. Interestingly, this analogue also showed enhanced reversibility compared with GVIA (Fig. 6, B and C), suggesting that an interaction between the toxin residue at position 10 and the N-type calcium channel is a key determinant in stabilizing the toxin-bound state.

ω-Conotoxin CVID Inhibition of N-type Ca²⁺ Channel Current Expressed in tsA201 Cells—In contrast to the results ob-
were also observed for MVIIA (data not shown). Consistent with the results obtained in oocytes, coexpression of the αδ subunit reduced CVID affinity, but to a lesser extent (Fig. 7, A and B). Washout of CVID was again incomplete, especially in the presence of the αδ subunit (Fig. 7C).

Radioligand Binding Results—Potencies (pIC<sub>50</sub>) to displace <sup>125</sup>I-GVIA from rat brain membrane were also determined to establish whether position 10 changes the affinity for the native N-type channels found centrally (Table IV). These results showed that the mutated α-conotoxins had a potency similar to that of their native counterparts at brain N-type calcium channels.

1H NMR and Three-dimensional Structure Calculations—To further probe the role of residue 10 in affinity to oocyte-expressed α<sub>1β</sub>. 1H NMR was used to identify any structural changes that may be associated with this residue replacement. Comparing Hα chemical shifts with random coil values for a series of related peptides is a sensitive method for identifying changes introduced in the peptide backbone. Replacing residue 10 in MVIIA and GVIA from CVID introduced minor changes in the secondary Hα chemical shift in [R1O6K]MVIIA across residues 10–14, whereas the changes in the secondary Hα chemical shift in [O1O6K]GVIA were larger in magnitude and spread across residues 8–14. Replacing Lys-10 in CVID with an Arg residue did not result in alterations of the secondary Hα chemical shift (15). Three-dimensional solution structures were calculated for [R1O6K]MVIIA and [O1O6K]GVIA, which showed significant secondary Hα chemical shift changes from their native counterparts. Analysis of these structures revealed that replacing Arg-10 in MVIIA with the similar Lys residue did not alter the overall three-dimensional structure of the peptide (Fig. 8B), which is consistent with the relatively small changes in the secondary Hα chemical shift observed (Fig. 8A). For [O1O6K]GVIA, which showed larger Hα chemical shift changes, the Hyp to Lys replacement resulted in destabilization of loop 2 residues 9–14 as compared with GVIA. The remainder of the [O1O6K]GVIA peptide backbone, however, was not perturbed by the Hyp to Lys residue substitution and retained a similar overall structure to GVIA (Fig. 8B).

**DISCUSSION**

The biophysical effects of auxiliary β and αδ subunits on the pore-forming α subunit of calcium channels have been well documented (26, 28, 29, 42). The aim of the present study was to determine how these auxiliary subunits influence the po-
tency of ω-conotoxins to inhibit currents mediated by N-type (Ca_{2.2}) calcium channels. Expression of the peripheral (α_{1B-b}) and central (α_{1B-d}) isoforms in *Xenopus* oocytes yielded Ca^{2+} channel currents that were enhanced 2-fold upon co-expression with the α_{1B-d} + β3 subunit combination. However, the α_{1B-d} + β3 subunit had no significant effect on the ability of the ω-conotoxins CVID, MVIIA, and analogues to inhibit currents mediated by the α_{1B-d} subunit. In general, the central α_{1B-d} isoform was more sensitive to block than the peripheral α_{1B-b} isoform for all ω-conotoxins studied, although the maximum difference observed was <5-fold.

In addition to further enhancing the peak current amplitudes of both the central and peripheral isoforms of the N-type Ca^{2+} channel and shifting the V_{1/2} for activation as observed previously (29), the α_{2δ} subunit dramatically reduced ω-conotoxin affinity for N-type channels expressed in oocytes. Co-expression of the α_{2δ} subunit together with α_{1B} + β3 subunits caused a ~100-fold decrease in the sensitivity of the channel to block by CVID, MVIIA, and analogues. K_{d} values obtained for the inhibition of N-type currents by ω-conotoxin MVIIA in the presence of the α_{2δ} subunit were similar to values reported previously (44, 46, 47). The α_{2δ} subunit caused a decrease in toxin sensitivity at both the peripheral and central isoforms of the N-type Ca^{2+} channel. In the presence of the α_{2δ}, selectivity for the central over the peripheral isoform was MVIIA [K10R]CVID > [R10K]MVIIA ~ CVID (Fig. 4A; Tables I and II), indicating that Arg at position 10 contributes to central selectivity. Consistent with the results obtained in *Xenopus*...
Although the effect was not as dramatic. This result agrees with an earlier observation in HEK cells in which \( \alpha_{2}\delta \) decreased the affinity to GVIA, a 3-fold (48).

Our results demonstrate that the decrease in affinity caused by \( \alpha_{2}\delta \) was achieved through a slowing of the \( \omega \)-conotoxin association rate \( (k_{\text{on}}) \). It is possible that this effect may arise from electrostatic shielding or repulsion, perhaps through the proximity of the bulky and heavily glycosylated extracellular \( \alpha_{2} \) domain to the \( \omega \)-conotoxin binding site, which presumably resides in the outer vestibule of the channel pore (47). Indeed, oocytes may uniquely glycosylate membrane proteins, thus accounting for the differences observed between the two expression systems used here. However, the effect of the \( \alpha_{2}\delta \) on \( \omega \)-conotoxin pharmacology was not influenced by oocyte glycosylation (Table III). If the effects were indeed due to the increased charge-screening capability of 20 mM Ba\( \text{II} \), unfortunately, we were unable to directly test this possibility, because currents from \( \alpha_{1B} \) expressed in HEK cells in the absence of \( \alpha_{2}\delta \) were too small to measure by using 5 mM Ba\( \text{II} \) as the charge carrier.

A direct physical restriction of \( \omega \)-conotoxin's access to its binding site on the N-type Ca\( \text{II} \) channel protein seems unlikely, as this would be expected to slow both the association and dissociation rates. In contrast, Felix et al. (49) reported that the binding of \([\text{H}]\text{PN200–110}\) to the cardiac L-type \( (\alpha_{1C}\) channel was increased by the \( \alpha_{2}\delta \) moieties of \( \alpha_{2}\delta \), presumably by affecting the conformation of the \( \alpha_{1C} \) subunit. It is therefore possible that the opposite effect occurs at \( \alpha_{1B} \). In addition to decreasing conotoxin sensitivity, coexpression of the \( \alpha_{2}\delta \) subunit reduced the percentage of current recovery from conotoxin block following washout. Surprisingly, this effect appeared to be specific to both the \( \alpha_{1B} \) isofrom expressed and to the type of toxin used, with the effect being greatest for CVID analogues in cells expressing the peripheral subunit. The reason for this difference in the \( \alpha_{2}\delta \) effect on recovery between \( \alpha_{1B} \) isofrom is not clear. The effects of \( \alpha_{2}\delta \) on \( \omega \)-conotoxin affinity appears to be unrelated to gabapentin action, because application of gabapentin (100 \( \mu \)M) failed to produce any immediate inhibition of \( \alpha_{1B}\delta + \alpha_{2}\delta + \beta \) current expressed in oocytes.²

It was previously reported that the sensitivity of \( \alpha_{1B}\delta \) to CVID block was two orders of magnitude greater when expressed in the absence of the \( \beta \) subunit (21). In the present study, however, only occasional batches of oocytes expressing \( \alpha_{1B}\delta \) alone had high \( \omega \)-conotoxin affinity (20-fold enhanced; see Fig. 4B); in this instance, the affinity was for MVIIA. Apparently, low picomolar affinity is not restricted to CVID and presumably arises from heterogeneity in \( \alpha_{1B} \) pharmacology in oocytes, perhaps as a result of post-translational processing

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² T. Yasuda, D. J Adams, and R. J. Lewis, unpublished observations.
or auxiliary protein heterogeneity. Similar potency differences were also obtained when $K_d$ estimates at $\alpha_1B$ expressed alone were calculated using on-rates of block determined at different concentrations of CVID, which is again suggestive of heterogeneity in $\alpha_1B$ pharmacology in oocytes.

In addition to the effect of auxiliary subunits on toxin potency, an overall difference in the extent of recovery from block by different $\omega$-conotoxins was also observed. Recovery from block by CVID was greater than that for MVIIA, whereas GVIA block was virtually irreversible. Interestingly, CVID reversibility was reduced for the peripheral form of the N-type channel in the presence of $\alpha_2B$, whereas MVIIA reversibility was reduced at the central form in the presence of $\alpha_2B$. In the spinal cord, this difference may allow CVID to preferentially inhibit the ascending presynaptic transmission presumably driven by peripheral subtypes and permit MVIIA to preferentially inhibit the postsynaptic and descending presynaptic transmission presumably driven by central subtypes. A 3-fold preference for the central isoform displayed by MVIIA (but not CVID) is expected to further increase such central selectivity. Central versus pe-

![Fig. 7. $\omega$-Conotoxin block of N-type ($\alpha_1B + \beta_2$) channel currents transiently expressed in HEK tsA201 cells in the absence or presence of $\alpha_2B$. A and B, $\omega$-conotoxin CVID inhibition of N-type channels is reduced by the calcium channel $\alpha_2B$ subunit. Representative current traces recorded in 20 mM Ba$^{2+}$ external solution depict N-type current block by 100 nM CVID in the presence or absence of $\alpha_2B$. Scale bar labels correspond to both sets of traces; note different scale bar sizes. Data are plotted as mean ± S.E.; numbers in parentheses indicate number of experiments. Asterisks indicate $p < 0.05$ in unpaired t-tests. C, time courses illustrate current reduction by 3 mM CVID (b) and recovery during washout (gray triangles, + $\alpha_2B$; black squares, − $\alpha_2B$) with 20 mM Ba$^{2+}$ external solution (a). Inset shows that the percentage of current recovery from CVID block after 2 min of washing is greater in cells lacking the $\alpha_2B$ subunit (black) than in $\alpha_2B$-expressing cells (gray).

![Fig. 8. $^1$H NMR studies. A, Ha chemical shift for the three residue 10-substituted $\omega$-conotoxins and their native counterparts. B, three-dimensional solution structures of MVIIA (accession number RCSB022889; Protein Data Bank (PDB) code 1TTK) (15), [R10K]MVIIA (accession number RCSB02280; PDB code 1TT3) (15), GVIA (accession number RCSB0228890; PDB code 1TTL), and [O10K]GVIA (accession number RCSB022862; PDB code 1TR6). Superimposition of the 20 lowest energy conformations across the entire backbone are shown with the exception of [O10K]GVIA, which was superimposed over residues 1–9 and 15–27.

Table IV

| $\omega$-Conotoxin Potency (pIC$_{50}$) | 95% CI       |
|---------------------------------------|--------------|
| CVID                                 | 10.42        | 10.32–10.52 |
| [K10K]CVID                            | 10.24        | 10.14–10.33 |
| MVIIA                                | 10.71        | 10.66–10.77 |
| [R10K]MVIIA                           | 10.60        | 10.50–10.70 |
| GVIA                                 | 10.55        | 10.45–10.63 |
| [O10K]GVIA                            | 10.73        | 10.64–10.82 |

*a* Confidence interval.
rpheral selectivity may have relevance to pain management, given that a dorsal root ganglion-specific splice variant of the N-type calcium channel has recently been identified in peripheral pain pathways (50). It is possible that the selectivity of MVIIA for central isoforms of the N-type calcium channel may explain why it has a narrower therapeutic window than CVID in rat models of inflammatory (22) and neuropathic pain (51).

In an attempt to identify which residues on ω-conotoxins influence reversibility, we investigated a number of position 10 mutants that had previously been identified as influencing calcium channel selectivity (15). [R10K]MVIIA had enhanced reversibility as compared with MVIIA for both the central and peripheral isoforms of the N-type Ca++ channel. Relative to CVID, [K10R]CVID had diminished reversibility to the peripheral isoform of the N-type Ca++ channel or little influence at the central isoform. The influence of position 10 on toxin reversibility was extended to GVIA, with [O10K]GVIA showing enhanced recovery. Taken together, these results suggest that position 10 is a key determinant of ω-conotoxin reversibility. This effect appears to take place through a specific interaction between residue 10 of the toxin and another residue on the N-type Ca++ channel protein, because the shape of the toxin is little altered but apparently more flexible in the mutants. Regions of the δ1 subunit crucial for the binding of GVIA in the external vestibule of the channel (domain III; SS-S6 region) have been identified (52). In a recent study by Feng et al. (47), residue Gly-1326 was found to be a major determinant of GVIA/H9251 position 10 is a key determinant of enhanced recovery. Taken together, these results suggest that the αδ subunit is up-regulated in animal models of neuropathic pain (37), differences in potency and recovery from block may influence how effectively ω-conotoxins reverse different painful conditions in vivo.

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