Determinants of Inhibition of Transiently Expressed Voltage-gated Calcium Channels by ω-Conotoxins GVIA and MVIIA*

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The Conus magus peptide toxin ω-conotoxin MVIIA is considered an irreversible, specific blocker of N-type calcium channels, and is now in clinical trials as an intrathecal analgesic. Here, we have examined the action of MVIIA on mutant and wild type calcium channels transiently expressed in tsA-201 cells. Although we have shown previously that mutations in a putative external EF-hand motif in the domain IIISS–H5 region alters block by both ω-conotoxin GVIA and MVIIA (Feng, Z. P., Hamid, J., Doering, C., Bosey, G. M., Snutch, T. P., and Zamponi, G. W. (2001) J. Biol. Chem. 276, 15728–15735), the introduction of five point mutations known to affect GVIA blocking (and located downstream of the EF-hand) affected MVIIA block to a smaller degree compared with GVIA. These data suggest that despite some overlap, MVIIA and GVIA block does not share identical channel structural determinants. At higher concentrations (3–μM), MVIIA reversibly blocked L-, P/Q-, and R-type, but not T-type channels, indicating that the overall architecture of the MVIIA site is conserved in all types of high voltage-activated calcium channels. A kinetic analysis of the MVIIA effects on the N-type channel showed that MVIIA blocked resting, open, and inactivated channels. Although the development of MVIIA block did not appear to be voltage-, nor frequency-dependent, the degree of recovery from block strongly depended on the potential applied during washout. Interestingly, the degree of washout was highly variable and appeared to weakly depend on the holding potential applied during toxin application. We propose a model in which N-type calcium channels can form both reversible and irreversible complexes with MVIIA.

A number of predatory species such as spiders, scorpions, and fish hunting mollusks have developed venomous to rapidly stun and kill their prey. Their venoms typically contain a mixture of potent peptide toxins that have evolved to specifically bind to voltage- and ligand-gated ion channels (1). Among channel calcium blocking peptides, ω-conotoxin GVIA (isolated from Conus geographus) and ω-conotoxin MVIIA (isolated from Conus magus) are perhaps the most widely known. Both of these toxins are thought to specifically block the pore of N-type calcium channels from a variety of species. Block by both toxins is considered to be virtually irreversible at normal membrane potentials (i.e. Refs. 2 and 3), but complete reversibility can be obtained upon strong hyperpolarization (4), or by removal of extracellular divalent carrier ions (5). Our current understanding of the molecular basis of N-type channel block came initially from a study by Ellinor et al. (6) who showed that block of transiently expressed N-type channels by ω-conotoxin GVIA was dramatically attenuated by sequence substitution in the domain III S5–S6 region of the Ca2.2 α1 subunit. More recently, Feng et al. (7) showed that additional point mutations in an EF-hand consensus motif in this region affected the development of, and recovery from, block by both GVIA and MVIIA, consistent with binding studies showing that both toxins compete for the same site on the α1 subunit of the channel (1, 8).

MVIIA has received particular notoriety because a synthetic version of this toxin blocks pain transmission when injected intrathecally (9). Indeed, under the names “ziconotide” and “SNX-111,” MVIIA is currently in phase III clinical trials, however, in some cases, severe side effects have been reported (10–12). Although complete knockout of Ca2.2 in mice does not result in serious adverse effects (13, 14), it is possible that the acute shutdown of N-type channel activity by toxin administration may well result in a different physiological response compared with gene knockout. It therefore remains unclear as to whether MVIIA side effects are related to the N-type channel blocking action of this toxin. As a consequence, it is important to elucidate in detail the intricacies of MVIIA action on N-type, and other types of voltage-gated calcium channels.

Here, we have examined some of the molecular and kinetic details of MVIIA action on calcium channels transiently expressed in tsA-201 cells. Our data show that manipulations of the domain III S5–S6 region in the rat Ca2.2 calcium channels differentially affect GVIA and MVIIA block. Whereas MVIIA completely inhibited Ca2.2 channel action at concentrations as low as 100 nM, other types of high voltage-activated calcium channels including Ca1.2, Ca2.1, and Ca2.3 were reversibly blocked in the micromolar range, indicating that an ω-conotoxin docking site may be generally conserved in many members of the calcium channel family. Ca2.2 channels were blocked in the resting, open, and inactivated conformations.
but block was neither frequency, nor voltage-dependent. As reported by Stocker et al. (4), MVIIA block of Ca,2.2 channels could be reversed during strong membrane hyperpolarization, however, in our hands, reversibility remained incomplete. The degree of recovery varied greatly from cell to cell, and appeared however, in our hands, reversibility remained incomplete. The degree of recovery varied greatly from cell to cell, and appeared

**MATERIALS AND METHODS**

**Molecular Biology and cDNA Constructs**—The wild-type rat calcium channel CMV-α1C (Ca,2.2), pMT2α1A (Ca,2.1), pMT2α1C (Ca,2.3), pMT2α1B (Ca,1.2) pMT2β1α, pMT2β2α, and pMT2β2α,β subunits cDNAs were kindly donated by Dr. Terry Snutch. The human pMT2α1G (Ca,3.1) cDNA used here has been described by us recently (15). The pEGFP cDNA construct used for cell selection was purchased from Clontech (Palo Alto, CA).

Mutagenesis of selected amino acid residues was performed as follows. The entire α1D coding region was first subcloned as a Sali/NeoI fragment into pBluescript (Stratagene). A unique KpnI restriction fragment of that clone (spanning 3520 to 4849 nucleotides) was subcloned into a new pUC19 vector. The pUC subclone, which contained all six residues to be mutated, was generated with amino acid substitutions within the “upstream” primer containing the PstI restriction site. High fidelity PCR amplification (Proofstart, Qiagen) was performed using the wild type clone as template and the specific upstream primer with a primer downstream of the NcoI site. Clone 5M had the following amino acid substitutions: Q1327K, D1330L, E1334N, E1337K, and Q1339R inserted by PCR. Clone 6M1 had an additional substitution, E1332R, in combination with the five aforementioned mutations. Sequences of the four PstI/NcoI fragments were confirmed by nucleotide sequencing before assembling sequentially back into the pUC subclone, then the pBluescript full-length clone, and finally inserts of all full-length mutants were cloned into the vector pCDNAS1(-) (Invitrogen).

The activation and inactivation behaviors of the mutant N-type channel constructs were indistinguishable from those of the wild type channels. Although we did not examine this here, we note that our previous single channel analysis indicates that a mutation of residue Glu-1332 to arginine alters the relative permeabilities of barium and calcium (16), hence, we may expect a similar effect in clone 6M1.

**Tissue Culture and Transient Transfection**—Unless stated otherwise, the calcium channel β2 and αδ-δ subunits and the pEGFP construct were coexpressed with either wild type or mutant calcium channel α1 subunit cDNAs. For experiments involving α1C, β2δ, and αδ-δ, cDNA constructs of calcium channel cDNAs have been reported by us on several occasions (for example, see Ref. 7). In brief, tsA-201 cells were grown at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented by fetal calf serum and penicillin/streptomycin, and split 12 h prior to transfection with 6 μg of each calcium channel cDNA and 1 μg of enhanced green fluorescent protein (EGFP) cDNA, using the calcium phosphate method. Twelve hours after transfection, cells were washed with fresh Dulbecco’s modified Eagle’s medium, and after a recovery period of 12 h, the cells were moved to a 28 °C, 5% CO2 incubator for maintenance. Recordings were then carried out between 2 and 5 days subsequently.

**Electrophysiology**—Whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with pClamp v9.0. Patch pipettes (Sutter borosilicate glass, BF 150-86-15) were pulled with a Sutter P-87 microelectrode puller, and subsequently fire polished using a Narishige microforge. Pipettes (in the range of 2–4 MΩ) were filled with the standard solution containing 118 mM Cs-methanesulfonate, 4 mM MgCl2, 9 mM EGTA, 9 mM HEPES (pH 7.2 adjusted with tetraethylammonium-OH). The cells were transferred to a 3-cm culture dish containing recording solution comprised of 5 mM BaCl2, 1 mM MgCl2, 10 mM HEPES, 40 mM tetraethylammonium-Cl, 10 mM glucose, 97.5 mM CsCl (pH 7.2 adjusted with tetraethylammonium-OH). Currents were elicited by stepping from the various holding potentials to various test potentials using Clampex software. ω-Conotoxin GVIA (Sigma) and MVIIA (Sigma) were dissolved first, respectively, in water and 1% acetic acid, and then diluted in the external recording solution. Solutions containing various concentrations of toxin were delivered directly to the cells by means of a gravity-driven microperfusion system that allows complete solution exchange in less than 1 s. Micropipettes with acetic acid containing carrier solution corresponding to 3 μM MVIIA did not affect current amplitudes of the various Ca, channels examined in this study. In one set of experiments (Fig. 3C), it was necessary to apply 30 μM MVIIA. In this case, the pH of the toxin containing recording solution needed to be re-adjusted with tetraethylammonium-OH. Perfusion with toxin-free mock solution did not affect N-type-channel activity. Data were filtered at 1 kHz using a 4-pole Bessel filter, and digitized at a sampling frequency of 2 kHz. Series resistance was compensated, but online leak subtraction protocol was not used. Data were analyzed using Clampfit (Axon Instruments). The time constant for development of block was fitted monoexponentially, and plotted as an inverse of the time constant for development and unblocking rate constants. All current fittings and preparation of initial figures were carried out using Sigmaplot 4.0 (Jandel Scientific). All error bars are standard errors, numbers in parentheses displayed in the figures reflect numbers of experiments. Statistical analysis was carried out using SigmaStat 2.0 (Jandel Scientific). Differences between mean values from each experimental group were tested using a Student’s t test for two groups and one-way analysis of variance for multiple comparisons. Differences were considered significant if p < 0.05.

**RESULTS**

**Distinct Ca,2.2 Channel Structural Determinants Underlie GVIA and MVIIA Block**—We have recently shown that point mutations in a putative EF-hand region contained in the domain III S5–H5 region of the Ca,2.2 α1 subunit (see Fig. 1A) regulate the blocking and unblocking rate constants for both ω-conotoxins MVIIA and GVIA (7). Based on these results, and on binding experiments showing that both toxins compete for the same macrosite on the N-type calcium channels, we concluded that there is significant overlap in the channel structural determinants that govern block by these toxins. Ellinor et al. (6) reported that a set of five point mutations in the domain III S5–S6 region (see Fig. 1A) dramatically attenuated GVIA blocking of N-type calcium channels expressed in Xenopus oocytes. To determine whether the MVIIA block was also affected by these mutations, we recreated these amino acid substitutions, and compared their effects on block by GVIA and MVIIA by coexpressing the channels with β2δ and αδ-δ, in tsA-201 cells and carrying out whole cell patch clamp recordings. As seen in Fig. 1B, the time course for development of the GVIA block was significantly slowed in the quintuple mutant. Fig. 1C examines the dependence of the inverse of the time constant for development of block on toxin concentration. The data are nicely described by a linear regression line, as expected from a 1:1 interaction between the channel and the toxin. The blocking rate constant determined from the slope of the fit was decreased 21-fold compared with that observed for the wild type channel. Whereas this is qualitatively consistent with the work of Ellinor et al. (6) in Xenopus oocytes, these authors reported a somewhat larger reduction in the blocking rate constant (44-fold decrease), which could be because of differences in the expression system, or ion composition in the external recording solution. Fig. 1, D and E, shows an analogous experiment for ω-conotoxin MVIIA. Similar to GVIA, blocking by MVIIA was slowed in the mutant N-type channel, however, the degree of slowing was much smaller than that seen with GVIA (3.75-fold decrease in the blocking rate constant between mutant and wild type channel).

We next wanted to determine whether the effects of these five mutations were additive to a separate point mutation, E1332R, which was found previously to slow the time constant of development of the block (7). As shown in Fig. 1, B–E, the additional substitution of residue 1332 for arginine mediated a substantial further slowing of the time constants for development of block by each toxin, causing an overall 30-fold slowing of the kinetics of the GVIA block, and a 10-fold slowing of MVIIA block. We also added the G1326P mutation (which by
itself dramatically speeds the recovery from toxin block (7) to the quintuple mutant; however, the mutant channels were expressed too poorly to allow a meaningful characterization (not shown). Overall, our data indicate that although both toxins act at the same macrosite within the N-type channel, MVIIA and GVIA do not share identical channel structural determinants for block. This suggests that some of the individual contact points between the channel and the toxin differ

**FIG. 1.** Mutagenesis of the \( \omega \)-conotoxin binding site on the \( \text{Ca}_{2.2} \) calcium channel \( \alpha_{1B} \) subunit differentially affects GVIA and MVIIA block. A, schematic representation of the transmembrane topology of the \( \alpha_{1B} \) subunit, and amino acid sequence of the putative \( \omega \)-conotoxin binding region in the domain IIIS6 region. Residues shown with the triangle indicate an external EF-hand motif that we have shown previously to affect MVIIA and GVIA block (7). Residues shown with the circle were shown previously to be involved in GVIA block (6). B, time course of GVIA blocking seen with wild type (WT), quintuple (5 mut), or sextuple (6 mut) mutant N-type calcium channels, in which the residues comprising the putative GVIA binding site (i.e., those shown in A) were substituted with corresponding residues found in \( \text{Ca}_{2.1} \) channels (i.e., Q1327K, D1330L, E1334N, E1337K, and Q1339R for the quintuple mutant plus an additional substitution of E1332R in the sextuple mutant). Cells were held at \(-100 \) mV and depolarized to \(+10 \) mV every \(10 \) s, and \(1 \) \( \mu \)M GVIA was applied until complete block of the current was observed. Note that block of the mutant channels occurs more slowly than that of the wild type channels. C, effects of the quintuple and sextuple mutation on the time constant for development of GVIA block. The time constants were determined from monoexponential fits to time courses such as that shown in panel B, and their inverses were plotted as a function of toxin concentration. Note that the blocking rate constant determined from the slope of the regression line was reduced 21- and 30-fold, respectively, in the quintuple and sextuple mutant channels, whereas the unblocking rate determined from the intercept was not markedly affected. D and E, effect of the quintuple and sextuple mutations on MVIIA blocking. The experimental conditions were the same as those described for panels B and C with the exception of the toxin concentrations used. Note that the quintuple and sextuple mutations slowed MVIIA action only about 3.75- and 10-fold, respectively.
between GVIA and MVIIA. Alternatively, the toxin-specific changes in the rate of development of block may be because of a steric effect on the access pathway, which may differentially affect the abilities of GVIA and MVIIA to reach the blocking site.

At the holding potential (i.e. $-100 \text{ mV}$) used in the experiments shown in Fig. 1, the mutations did not measurably increase reversibility of MVIIA and GVIA block, indicating that the mutations did not reduce the stability of the toxin-channel complex. This is supported, in principle, by examination of the unblocking rate constant (i.e. $y$ intercept) obtained from the linear regressions shown in Fig. 1, C and E, which, if anything, revealed a slight decrease in the extrapolated unblocking rate constants in the presence of the mutations. It is, however, important to note that for poorly reversible blockers, unblocking rate constants obtained from analysis such as that shown in Fig. 1, C and E, are easily skewed by even small changes in the slope of the regression line and must thus be viewed cautiously. As shown below, a more rigorous analysis of the effects of mutations on toxin unblock is precluded by a large variability in the degree of recovery from the MVIIA block (see Fig. 4).

**Development of MVIIA Block Is Independent of Stimulus Frequency and Channel State**—Many blockers of voltage-gated calcium channels display state and use-dependent block. To determine whether MVIIA action on N-type calcium channels is frequency-dependent, we examined the time course of development of MVIIA inhibition at stimulus frequencies of 0.1 and 0.5 Hz. As shown in Fig. 2, A and B, application of 300 nM MVIIA resulted in complete block of N-type channel activity with a time constant of about 15 s, irrespective of pulse frequency. We then examined the rate of development of MVIIA block at three different holding potentials. In each case, 300 nM MVIIA completely blocked N-type channel activity, and the time constant for development of block was virtually identical at all holding potentials tested (Fig. 2C). Hence, the development of MVIIA block of transiently expressed N-type channels neither appears to be use-dependent, nor holding potential-dependent. The latter data are in contrast with those reported for another pore-blocking $\omega$-conotoxin molecule, SNX-331, whose blocking action is strongly holding potential-dependent (4) and suggest that MVIIA block is not correlated with channel availability.

Next, we examined whether the development of MVIIA block required channel opening. To address this issue, cells were held at a holding potential of $-100 \text{ mV}$, and current amplitude stability and size were assessed with a series of test depolarizations. Subsequently, MVIIA was applied without evoking further depolarizations for 1 min, before application of a single test pulse to assess current size. In each of the five cells examined, the application of 300 nM MVIIA resulted in the complete block (100%) of current activity (see Fig. 3A for a typical example), indicating that MVIIA is capable of blocking the channel in its resting state, and thus explaining the lack of use dependence shown in Fig. 2, A and B.

We then wanted to determine whether MVIIA also blocked inactivated channels. Fig. 3B shows a typical example of a set of eight experiments designed to address this issue. As illustrated in Fig. 3B, the cells were held at $-100 \text{ mV}$ and currents were elicited by stepping to $+10 \text{ mV}$. The holding potential was then switched to 0 mV until currents were completely inactivated, and the cell was repolarized to $-100 \text{ mV}$ to monitor the time course for recovery from inactivation. Subsequently, the channels were again inactivated by holding the cells at 0 mV, and 300 nM MVIIA was applied. One minute after MVIIA application, the toxin was washed and the cell was repolarized to a holding potential of $-100 \text{ mV}$, revealing that currents could no longer be recovered (see Fig. 3B). In the eight cells examined, only $6.6 \pm 1.8\%$ of the current amplitude observed prior to toxin application remained at the end of the test paradigm, indicating that MVIIA could effectively interact with inactivated channels.

Finally, we wanted to assess whether MVIIA also blocks open channels. To determine this, we coexpressed the $\text{Cav}_2.2\alpha_1$ and $\alpha_2$-$\delta_1$ with rat $\beta_2\alpha_1$ to slow voltage-dependent inactivation, allowing us to maintain some channel activity for up to 5 s. Immediately after channel opening, we perfused a high concentration of MVIIA onto the cells and monitored current activity. As shown in Fig. 3C, application of 3 $\mu\text{M}$ MVIIA ($n = 4$) increased the rate of current decay, consistent with open channel block developing during the test depolarization. To more clearly isolate this effect, we increased the MVIIA concentration by 1 order of magnitude, and repeated the experiment. As seen in Fig. 3C, after a short ($\sim 500 \text{ ms}$) perfusion lag, increasing the MVIIA concentration to 30 $\mu\text{M}$ ($n = 5$) dramatically

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**Fig. 2.** MVIIA block is neither frequency- nor voltage-dependent. A, time course of MVIIA block of wild type Ca$_2.2$ calcium channels at stimulus frequencies of 0.1 and 0.5 Hz. The holding potential was $-100 \text{ mV}$, the test potential was $+10 \text{ mV}$. Note that the time required for complete block is similar at both frequencies. B, time constant for development of MVIIA block for two different stimulus frequencies. The time constants were determined from monoeponential fits to data such as those shown in panel A. The two data sets were not statistically different ($p > 0.05$). C, time constant for development of MVIIA block at three different holding potentials. Note that the time constant for development of block is not correlated with channel availability.
accelerated the development of current inhibition, as expected from the increase in the blocking rate and consistent with open channel block. Taken together, our data thus suggest that the MVIIA binding site on the N-type channel molecule is accessi-
bility might depend on the duration for which the toxin was applied. We therefore analyzed the degree of recovery as a function of duration of application at a holding potential of −80 mV. As shown in Fig. 4C, no correlation between duration of application and degree of recovery was evident under these circumstances. This suggests that the relative proportion of reversibly and irreversibly blocked channels is perhaps deter-
mined before formation of the toxin-channel complex. Taken together, it appears as if N-type calcium channels can undergo two MVIIA blocked conformations that differ in their stabili-
ties, with the relative proportions of reversibly and irreversibly blocked channels varying from cell to cell, and with holding potential.

**MVIIA Can Interact with Other Members of the Calcium Channel Family**—The observation that the clinical use of MVIIA has been associated with a number of side effects (10–12) may indicate that MVIIA interacts with other targets, such as other types of calcium channels. To test this, we examined MVIIA block for several types of transiently expressed voltage-
gated calcium channels under identical experimental condi-
tions. As shown in Fig. 5, all high voltage-activated calcium channels examined (i.e. L-type Ca,1.2, P/Q-type Ca,2.1, and R-type Ca,2.3) underwent a small (10–20%), but statistically significant inhibition in the presence of 3 μM MVIIA, with the L-type channel being perhaps the most sensitive channel iso-
form. Unlike with the N-type channel, block of any of the other calcium channels examined could be reversed upon washout (not shown). In contrast, Ca,3.1 (T-type) calcium channels were not inhibited at this toxin concentration. It is important to note that the toxin affinity for these channels is at least 2 orders of magnitude lower than that seen with the N-type. Hence, while it seems unlikely that our findings might account for the side effects of MVIIA during clinical use, our data nonetheless in-
dicate that the geometries of the outer vestibules of all high voltage-activated calcium channel families are sufficiently sim-
ilar to allow them to accommodate ω-conotoxin molecules.

**DISCUSSION**

**State Dependence of MVIIA Action**—In this paper, we pres-
ten several novel insights into the state dependence of ω-cono-
toxin MVIIA action, with regard to both the development of block and its recovery. We presented three key experiments
that allow us to determine with which kinetic state of the channel MVIIA is able to interact. To fully appreciate our findings, we need to recall the basic biophysical profile of the N-type channel when bathed in 5 mM external barium. As we have shown previously (17, 18), N-type calcium channels activate at around −20 mV, peak at about +5 mV, and display a half-inactivation potential of about −65 mV under these recording conditions. Thus, at a holding potential of −100 mV, all of the channels are expected to be in the resting state, whereas the channels will be completely inactivated at a holding potential of 0 mV. We also note that under control conditions, recovery from inactivation occurs on the order of several seconds (see Fig. 2B). When MVIIA was perfused at a holding potential of −100 mV without evoking any step depolarization during toxin application, current activity assessed by a single test pulse at the end of toxin application was completely abolished. Hence, we can conclude that MVIIA potently inhibits channels in the resting state. This finding is consistent with the lack of use dependence of MVIIA block illustrated in Fig. 2. When MVIIA was applied to cells held at a prolonged holding potential of 0 mV, channel activity could no longer be recovered after washout by applying a hyperpolarization to −100 mV, a membrane potential that would normally be sufficient to quickly recover channels from the inactivated state. Thus, our data suggest that MVIIA is capable of blocking inactivated N-type calcium channels.

The most appropriate means of assessing the open channel block is an examination of mean open times in single channel recordings. However, in the case of MVIIA action on calcium channels, this is a virtually insurmountable challenge, because the poor reversibility of MVIIA action (leading to likely no more than one long blocked event per recording), and the inability to control application and washout in cell-attached patch recordings would preclude measurement of MVIIA effects on mean open times. Instead, we attempted to use whole cell recordings to study open channel block by slowing channel inactivation through coexpression of the rat β2 subunit, and applying the toxin in the middle of a sustained inward barium current. Consistent with open channel block, MVIIA depressed this inward current with a time course that depended on toxin concentration. We must, however, acknowledge the following caveat in interpreting these experiments. Even during a sustained membrane depolarization, it is possible that channels cycle between open and resting states, and given that MVIIA was an effective resting channel blocker, this could potentially
contribute to the observed inhibition during sustained depolarizations. We attempted to circumvent this complication by stepping to a test potential past the peak of the current-voltage relation where the open state is clearly favored (+10 mV). It is also worth noting that the structurally related \( \mu \)-conotoxin GIIIA blocks the open state of batrachotoxin-activated sodium channels (19). Taken together, our data are therefore consistent with an ability of MVIIA to block N-type calcium channels irrespective of the kinetic state of the channel. From a channel structural point of view, this may therefore indicate that the outer vestibule of the pore does not undergo conformational changes during channel gating that prevents the docking of MVIIA.

Recovery from Inhibition—Our data showing that recovery from block was favored by strong membrane hyperpolarization are qualitatively consistent with those of Stocker et al. (4). However, unlike Stocker et al. (4) we could not completely reverse the effects of MVIIA under any experimental condition. This apparent discrepancy could be because of the particular N-type calcium channel isoform used, or may be linked to the use of different transient expression systems. Stocker and colleagues (4) also suggested that pore-blocking \( \omega \)-conotoxins could stabilize the activated state of the channel, and that the enhanced reversibility of blocking seen at hyperpolarizing voltages might reflect an enhanced recovery from a toxin-stabilized activated state. Our data showing that the degree of recovery from block appeared to increase when the toxin was applied at more depolarized holding potentials is qualitatively consistent with this idea. However, in our hands, currents could never be completely recovered, even when the toxin was applied at holding potentials at which all channels would be inactivated. The fact that block could be partially, but never completely, recovered suggests that two populations of MVIIA-blocked channels may exist, one population that tightly interacts with MVIIA, and a second population that appears to bind MVIIA less strongly, and can be recovered from block with membrane hyperpolarization. Within the confines of such a model, the relative proportion of reversibly and irreversibly bound channels appears to be independent of the amount of time the toxin is applied, but to increase slightly when the toxin is applied at depolarized voltages. Yet, the notion that for a particular experimental condition (for example, Fig. 4B at a holding potential of 0 mV: identical pulse paradigm, identical duration of MVIIA application (60 s), and identical batch of toxin), recovery from block could range from 10 to about 85% suggests that there is an overriding, but unknown mechanism that determines the relative proportion of reversibly and irreversible blocked channels.

We can only speculate about the molecular basis for the occurrence of two distinct putative blocked states. The MVIIA molecule could conceivably adopt two orientations in the outer vestibule of the N-type channel pore. Alternatively, it has been shown by NMR analysis that MVIIA can dynamically swap between two conformations by rearranging the disulfide backbone (20, 21). Future work examining MVIIA mutants with a single defined structure, or other related peptides such as \( \omega \)-conotoxin CVID (22) might serve to test such a hypothesis. It is also interesting to note that in the absence of divalent carrier ions, block of the N-type calcium channels by \( \omega \)-conotoxin GVIA becomes fully reversible (5, 23). It has been suggested that divalent cations might mediate this effect by binding to a specific site on the extracellular side of the channel, thereby regulating toxin affinity (5). In the context of our observations, any variability in barium occupancy of this site would then secondarily result in a variable degree of recovery from the block. Such a mechanism would potentially fit with our previous work identifying an EF-hand motif located adjacent to the putative conotoxin binding site in the Ca_2.2 III S5–S6 region (7). Disruption of the EF-hand motif via insertion of a proline residue renders GVIA and MVIIA block fully reversible, and accelerated the rate of development of block similar to what is seen upon removal of external divalent cations, hence hinting at a common molecular mechanism.

Channel Structural Determinants of Toxin Block and Channel Subtype Specificity—Although the N-type calcium channels are by far the most effectively MVIIA-blocked calcium channel species, the observation that MVIIA appears to have some ability to block other types of high voltage-activated calcium channels raises two important considerations. First, \( \omega \)-conotoxins are frequently used in the micromolar range during physiological studies on isolated neurons and brain slices. Such studies may no longer be appropriate given that 3 \( \mu \)M MVIIA mediated significant block of several types of non-N-type calcium channels. Second, the fact that all types of high voltage-activated calcium channels examined were susceptible to MVIIA block suggests that the overall architecture of the outer vestibule of the pore (the likely site of action of MVIIA) may be generally conserved across multiple calcium channel families. By contrast, the more distantly related T-type calcium channels did not appear to be affected in the concentration range examined here. The nonselective action of what are considered highly selective peptide blockers does not appear to apply only to pore blockers, but also to gating inhibitors: \( \omega \)-agatoxin IVA, once considered a specific inhibitor of P/VQ-type channels, appears to block several other types of voltage-gated calcium channels, albeit only in the micromolar range (24). SNX-314, a supposedly specific blocker of R-type channels, also significantly inhibits Ca_1.2 L-type channels in the same concentration range (25).

The approximate location of the conotoxin binding region on the N-type calcium channel was originally identified through chimeric Ca_2.1–Ca_2.2 channels (6). Therefore, the notion that Ca_2.1 channels appear to have a basic ability to interact with \( \omega \)-conotoxins raises the possibility that the domain III S5–S6 region identified via these chimeras may simply be responsible for the differences in conotoxin affinity for the parent channels, rather than identifying all of the regions capable of interacting with these toxins. Indeed, both our previously characterized mutations in the domain III S5–S6 EF-hand region (7) and the five downstream mutations examined here seem to only modulate conotoxin action, rather than preventing it from occurring. Moreover, in a chimeric N-type channel in which 50 amino acid residues in the domain III S5–S6 region were substituted with a sequence from a conotoxin-insensitive invertebrate Ca_2.2 channel homolog (26), both GVIA and MVIIA blocking persisted. Together with evidence from the sodium channel field that implicates multiple transmembrane domains in \( \mu \)-conotoxin block (27), it seems likely that regions outside the domain III S5–S6 regions may contribute to toxin action. Further experiments will be required to identify putative conotoxin interaction sites outside of the domain III S5–S6 region. In addition, further mutagenesis of the III S5–S6 region and/or the toxins will be needed to discern whether the differential effects of our mutations on GVIA and MVIIA blocking rates reflect differences in the contact points with the channel, or a differential steric effect on the toxin access pathway (i.e. because of differences in shape/volume of the two toxins). Overall, we present a number of novel insights into the action of \( \omega \)-conotoxin MVIIA on voltage-gated calcium chan-

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1 C. J. Doering, Z. P. Feng, J. D. Spafford, and G. W. Zamponi, unpublished observations.
nels, which may require us to re-examine our current thinking about the site of action on the conotoxin molecule, and to take particular care in selecting conotoxin concentrations that ensure selectivity for N-type channels in physiological experiments and possibly clinical applications.

Acknowledgment—We thank Dr. Terry Snutch for providing wild type rat calcium channel subunits.

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Zhong-Ping Feng, Clinton J. Doering, Robert J. Winkfein, Aaron M. Beedle, J. David Spafford and Gerald W. Zamponi

doi: 10.1074/jbc.M300581200 originally published online March 24, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300581200

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