Interaction of SNX482 with Domains III and IV Inhibits Activation Gating of $\alpha_{1E}$ (Ca\(_{2.3}\)) Calcium Channels

Emmanuel Bourinet,* Stephanie C. Stotz,† Renée L. Spaetgens,† Govindan Dayanithi,‡ José Lemos,§ Joël Nargeot,*, and Gerald W. Zamponi†

*Physiopathologie des Canaux Ioniques, Institut de Génétique Humaine, CNRS UPR1142, 34396 Montpellier Cedex 5, France; †Departments of Physiology and Biophysics and Pharmacology and Therapeutics, Neuroscience Research Group, University of Calgary, Alberta T2N 4N1 Canada; ‡INSERM U432, Université Montpellier II, cc089, 34095 Montpellier, France; and §Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655 USA

ABSTRACT We have investigated the action of SNX482, a toxin isolated from the venom of the tarantula 

Hysterocrates gigas,

on voltage-dependent calcium channels expressed in tsa-201 cells. Upon application of 200 nM SNX482, R-type $\alpha_{1E}$ calcium channels underwent rapid and complete inhibition, which was only poorly reversible upon washout. However, upon application of strong membrane depolarizations, rapid and complete recovery from inhibition was obtained. Tail current analysis revealed that SNX482 mediated an $\sim$70 mV depolarizing shift in half-activation potential, suggesting that the toxin inhibits $\alpha_{1E}$ calcium channels by preventing their activation. Experiments involving chimeric channels combining structural features of $\alpha_{1E}$ and $\alpha_{1C}$ subunits indicated that the presence of the domain III and IV of $\alpha_{1E}$ is a prerequisite for a strong gating inhibition. In contrast, L-type $\alpha_{1C}$ channels underwent incomplete inhibition at saturating concentrations of SNX482 that was paralleled by a small shift in half-activation potential and which could be rapidly reversed, suggesting a less pronounced effect of the toxin on L-type calcium channel gating. We conclude that SNX482 does not exhibit unequivocal specificity for R-type channels, but highly effectively antagonizes their activation.

INTRODUCTION

Voltage gated calcium channels (VGCCs), are transmembrane proteins involved in the regulation of cellular excitability and Ca\(^{2+}\) homeostasis. VGCCs, classified as L-, N-, P-, Q-, R-, and T-type based on their functional and pharmacological properties, are critical for many cellular functions including muscular contraction, neurotransmitter release, and excitability. To date, nine neuronal Ca\(^{2+}\) channel genes have been identified and termed $\alpha_{1A}$ through $\alpha_{1I}$ (Perez-Reyes et al., 1998). When transiently expressed in a host system together with their ancillary $\beta$ and $\alpha_{2-\delta}$ subunits, $\alpha_{1A}$ exhibits the characteristics of P- and Q-type channels (Bourinet et al., 1999), $\alpha_{1B}$ encodes an N-type channel (Dubel et al., 1992), $\alpha_{1C}$, $\alpha_{1D}$, and $\alpha_{1E}$ constitute three different L-type channels (Tomlinson et al., 1993; Williams et al., 1992; Bech Hansen et al., 1998), and $\alpha_{1E}$ appears to correlate to what has been described as R-type channels (Soong et al., 1993; Williams et al., 1994). Finally, $\alpha_{1G}$, $\alpha_{1H}$, and $\alpha_{1I}$, the most recent genes to be identified, have been unambiguously characterized as members of the T-type Ca\(^{2+}\) channel family (Perez-Reyes et al., 1998; Cribbs et al., 1998; Lee et al., 1999).

The identification of the native counterparts of the cloned calcium channel isoforms has been made possible in part by their pharmacological properties. For example, L-type channels are characterized via their selective inhibition by dihydropyridines (Bean, 1984; for review, see Zamponi, 1997). Furthermore, the isolation of highly specific toxins from the venoms of predatory animals such as spiders or cone snails has yielded highly selective blockers of non-L-type channels. For example, $\omega$-conotoxin GVIA (Conus geographus marine snail) and $\omega$-agatoxin IVA (American funnel web spider) are specific blockers of, respectively, N-type and P/Q-type calcium channels (Olivera et al., 1984; Mintz et al., 1992; Adams et al., 1993). These two toxins differ fundamentally in their mechanisms of current inhibition, with $\omega$-conotoxin GVIA mediating physical pore block (see Zamponi, 1997), whereas $\omega$-agatoxin IVA functions as an activation gating inhibitor (Mintz et al., 1992; McDonough et al., 1997a). More recently, SNX482, a toxin from the tarantula Hysterocrates gigas has been described as the first potent and selective antagonist of $\alpha_{1E}$ calcium channels (Newcomb et al., 1998) and has started to be used as a tool to unravel the physiological role of these channels (Wang et al., 1999). Structurally, SNX482 shares a similar size and cysteine disulfide bond arrangement with two other spider toxins, hanatoxin (Swartz and McKinnon, 1995) and grammotoxin SIA (McDonough et al., 1997b), that respectively block potassium and calcium channels by altering their gating, suggesting the possibility that SNX482 might perhaps also function as a gating modifier.

Here, we have examined the mechanism by which SNX482 inhibits recombinant rat brain $\alpha_{1E}$ channels in HEK cells. Our results indicate that SNX482 is a member of the group of gating-modifying toxins and exhibits a mechanism of action reminiscent of that observed with $\omega$-agatoxin IVA block of $\alpha_{1A}$ calcium channels. Using a series of chimeric calcium channel $\alpha_1$ subunits, we show that interactions between the toxin and domains III and IV of the $\alpha_{1E}$
subunit are required for the pronounced effects on \( \alpha_{\text{1E}} \) channel gating. Finally, we show for the first time that SNX482 mediates partial and reversible block of transiently expressed L-type channels. Thus, SNX482 is not entirely selective for \( \alpha_{\text{1E}} \) channels, and appears to inhibit R-type and L-type calcium channels.

MATERIALS AND METHODS

Construction of chimeras

The calcium channel chimeras used here are identical to those described in detail in Spaetgens and Zamponi (1999). Briefly, cDNAs encoding rat brain \( \alpha_{\text{1E}} \) and \( \alpha_{\text{1C}} \) (GenBank accession numbers L15453, M67515) were used to introduce silent restriction enzyme sites via site-directed mutagenesis at the carboxyl end of domain I, II, and III of each \( \alpha_{\text{1}} \) subunit, and chimeras were constructed by using these unique sites. Given the positions of the restriction sites, each domain remains linked to the preceding cytoplasmic linker. The nomenclature used herein is in the form of a four-letter code indicating the origins of the individual transmembrane domain (i.e., CEEM contains domain I of \( \alpha_{\text{1C}} \) and domains II, III, and IV of \( \alpha_{\text{1E}} \)).

Transient expression of recombinant calcium channels

cDNAs encoding wild-type and chimeric \( \alpha_{1}, \alpha_{2}, \) and \( \beta \) subunits and a reporter gene (CD8 or GFP) were inserted in vertebrate expression vectors (\( \alpha_{\text{1E}}/\alpha_{\text{1C}} \) chimeras in pMT2, rat brain \( \alpha_{\text{1E}}, \alpha_{\text{1C}}, \alpha_{\text{1B}}, \beta_{2a}, \beta_{2b}, \) and \( \alpha_{2}-\delta \) in pMT2: Stea et al., 1994, 1999; Soong et al., 1993; Tomlinson et al., 1993; CD8 and GFP in a CMV promoter-driven vector). Human embryonic kidney cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (v/v). For optimal transfection, cells were plated at 50–70% confluence. A calcium phosphate transfection procedure was used with an \( \alpha_{1-}\alpha_{2}-\delta-\beta-CD8 \) (or GFP) cDNA mix at a molar ratio of 1:1:1:0.1. Cells were plated at low density 24 h after transfection and used for patch clamp studies 24 h later. Positively transfected cells were identified using anti-CD8 antibody-coated beads (Dynal) or via fluorescence (for GFP).

Electrophysiology

Positively transfected cells were examined via whole-cell patch clamp using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Leak and capacitive currents were subtracted using a P/-5 method. Unless indicated otherwise, currents were evoked with 50-ms-long depolarizing pulses from \(-100 \text{ mV}\) to the potential giving the maximum inward current delivered at 0.1 Hz. The extracellular solution contained (in mM): 5 BaCl\(_2\), 160 TEACl, 10 HEPES (pH to 7.4 with TEAOH). To avoid toxin adsorption to tubing, 1 mg/ml BSA (fraction IV, Sigma, L’Isle d’Abeau Chesnes, France) was added to the recording solution. Pipettes of typical resistance of 0.9–2 MΩ, made of borosilicate glass, were filled with an internal solution containing (in mM): 110 CsCl, 3 MgCl\(_2\), 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.6 GTP (pH to 7.2 with CsOH). Synthetic SNX482 and \( \omega \)-grammotoxin SIA were prepared daily in the external recording solution from a 1 mM stock. The various dilutions were applied to cells by gravity-driven perfusion controlled by solenoid valves. A recording chamber with a small volume (~200 μl) was used to minimize the amount of toxin applied.

Data were acquired and analyzed using pCLAMP V. 6. Fitting of the raw data was carried out with Prism software. Figures were prepared using Freelance Graphics (Lotus). Steady-state activation curves were fitted using a single Boltzmann equation. All error bars indicate standard errors, \( p \) values reflect Student’s \( t \)-tests.

RESULTS

SNX482 irreversibly blocks \( \alpha_{\text{1E}} \) calcium channels

The calcium imaging data of Newcomb et al. (1998) indicate that SNX482 prevents calcium influx via R-type calcium channels. To more directly investigate the detailed action of this toxin, we transiently transfected \( \alpha_{\text{1E}} \) (+\( \beta_{2a} + \alpha_{2-}\delta \)) calcium channels into tsa-201 cells, and studied the effect of SNX482 via whole-cell patch clamp.

Fig. 1 A depicts current records obtained from \( \alpha_{\text{1E}} + \beta_{2a} + \alpha_{2-}\delta \) calcium channels in the absence and presence of 200 nM SNX482 in 5 mM external barium. As evident from the figure, application of 200 nM SNX482 completely abolished barium currents carried by \( \alpha_{\text{1E}} \), consistent with the imaging and electrophysiological data of Newcomb et al. (1998) and Wang et al. (1999). Block was dependent on the membrane potential, such that outward currents at very positive potentials were affected to a much smaller degree (Fig. 1 B). Fig. 1 C depicts the time course of development of and recovery from SNX482 inhibition of \( \alpha_{\text{1E}} \) channels. Application of 200 nM SNX482 mediated rapid (\( t_{\text{on}} = 33 \pm 7 \text{ s}, n = 14 \)) and complete inhibition of the channels within \(-1 \text{ min} \) of application. The inhibition was only poorly reversible upon washout (\( t_{\text{off}} = 496 \pm 73 \text{ s}, n = 6 \)); however, consistent with the strong voltage dependence revealed in Fig. 1 B, currents could be recovered nearly completely within one minute upon application of a train of strong depolarizing prepulses (\( t_{\text{off}} = 22 \pm 5 \text{ s}, n = 10 \)). These data suggest that the dissociation of SNX482 from the channel is highly voltage-dependent and appears reminiscent of the actions of \( \omega \)-agatoxin IVA and \( \omega \)-grammotoxin SIA on \( \alpha_{\text{1A}} \) calcium channels (Bourinet et al., 1999; McDonough et al., 1997a, b).

We also examined the dose dependence of SNX482 action on \( \alpha_{\text{1E}} \) channels. Whereas complete block was obtained at each of the concentrations examined (not shown), the time constant for the development of current inhibition was dependent on the concentration of SNX482. Fig. 1 D illustrates the dependence of the inverse of the blocking time constant on the toxin concentration. A linear relation consistent with a 1:1 interaction between the channel and SNX482 nicely describes the data. The slope (\( k_{\text{on}}: 1.498 \times 10^{-4} \text{ nM}^{-1} \text{ s}^{-1} \)) and the intercept (\( k_{\text{off}}: 2.843 \times 10^{-3} \text{ s}^{-1} \)) of the regression line predict an equilibrium dissociation constant (\( K_d = k_{\text{off}}/k_{\text{on}} \) ) of 19 nM, consistent with the \( IC_{50} \) of 30 nM found by Newcomb et al. (1998). Overall, our data indicate that SNX482 binds to R-type \( \alpha_{\text{1E}} \) calcium channels with high affinity and in a voltage-dependent manner.
SNX482 prevents activation gating

The strong voltage-dependence observed in Fig. 1 together with the prepulse relief of toxin action suggests the possibility that SNX482 might act as an inhibitor of R-type calcium channel activation gating. To examine this possibility, we used tail current protocols to record steady-state activation curves in the presence and absence of the toxin. Fig. 2 A depicts representative current traces illustrating tail currents in the presence and absence of the toxin at three different test potentials. With increasingly positive test potentials, a substantial tail current develops in both the absence and the presence of the toxin. However, whereas the tail current under control conditions is already saturated at /V11001 60 mV, tail current amplitude continues to increase in the presence of the toxin to membrane potentials as high as /V11001 140 mV. This is examined in more detail in the form of steady-state activation curves (Fig. 2 B) for a single representative cell. As seen from the figure, the half-activation potential underwent a dramatic shift toward more positive potentials, which on average amounted to 66 ± 3.9 mV (n = 4). In addition, the slope of the activation curve was reduced ~4-fold in the presence of the toxin (3.79 ± 0.43). The plateau level of the activation curve, however, remained reduced over the whole range of test potentials. To rule out the possibility that the observed effects were due to the type of /H9275 subunit used in our experiments, a set of recordings was conducted using /H9251 1E channels coexpressed with /H9252 1b and /H9254, and identical results to those shown in Figs. 1 and 2 were obtained (data not shown).

As shown in Fig. 2, the magnitude of inhibition of the /H11001 1E outward current at +140 mV induced by SNX482 was clearly less prominent than that of the inward tail current at −80 mV. This asymmetric effect of SNX482 occurred in every single cell and resembles previous observations with /H9251-agatoxin IVA on P-type currents (McDonough et al., 1997a). The reduction in tail current amplitude following a strong membrane depolarization may reflect rapid deactivation of the channel in the presence of the toxin upon repolarization (deactivation time constants at −60 mV: \( \tau_{\text{control}} 0.42 \text{ ms} (n = 5), \tau_{\text{SNX}} 0.27 \text{ ms} (n = 5) \)). These results and the slowing of the activation kinetics at positive potentials

FIGURE 1 SNX482 block of \( \alpha_{1E} \) calcium channels. (A) Current records obtained from \( \alpha_{1E} + \beta_{2a} + \alpha_{2-8} \) calcium channels transiently expressed in tsa-201 cells. Currents were elicited from a holding potential of −100 mV to a test depolarization of 0 mV. Application of 200 nM SNX482 mediates complete block of current activity. (B) Current-voltage relation obtained under the same conditions as in (A). Note that little block of outward currents is observed in the presence of SNX482. (C) Typical time course of development and recovery from block. Currents were elicited from a holding potential of −100 mV to a test potential of 0 mV. SNX482 block develops rapidly and is poorly reversible upon washout unless a train of strong depolarizing prepulses is applied (to +150 mV). (D) Dependence of the blocking kinetics on SNX482 concentration. The time constant for development of block was determined from experiments such as that shown in (C), and its inverse plotted as a function of SNX482 concentration. The slope from the regression line and the intercept were, respectively, \( 1.498 \times 10^{-4} \text{ nM}^{-1} \text{ s}^{-1} \) and \( 2.843 \times 10^{-3} \text{ s}^{-1} \). Means of 5 to 14 experiments are included in the figure, and error bars represent standard errors.
closely parallel previous observations with \( \omega \)-agatoxin IVA block of the P-type, and suggest that SNX482 is a gating modifier of rat brain \( \alpha_{1E} \) channels.

**Comparison of SNX482 and \( \omega \)-grammotoxin SIA action**

SNX482 shares a number of sequence similarities and a similar cysteine disulfide bond arrangement with \( \omega \)-grammotoxin SIA, another spider toxin known to potently inhibit N-type calcium channels (Newcomb et al., 1998). To determine any putative similarities in the modes of action of these two toxins, we compared the properties of SNX482 inhibition of \( \alpha_{1E} \) to those seen with \( \omega \)-grammotoxin SIA block of transiently expressed N-type (\( \alpha_{1B} \)) calcium channels under identical experimental conditions. The action of the two toxins displayed a number of similarities, including a shift of the activation curve to more positive potentials and more rapid dissociation following application of train of positive prepulses (not shown, but see McDonough et al., 1997b). However, in contrast with our observations with SNX482 (Fig. 2), the degree of \( \omega \)-grammotoxin SIA inhibition of outward currents at very positive potentials was similar to that of the inward tail current at negative potentials. This is likely due to the notion that \( \omega \)-grammotoxin SIA exerts only a moderate speeding of the deactivation kinetics of the N-type calcium channels compared with the effects of SNX482 on \( \alpha_{1E} \) deactivation, which is clearly evident upon comparison of the effects of the two toxins on the tail current kinetics at 0 mV (Fig. 3). The relatively small effect of \( \omega \)-grammotoxin SIA on deactivation kinetics is consistent with data obtained previously in intact neurons (McDonough et al., 1997b).

Finally, whereas saturating SNX482 concentrations mediated only little kinetic slowing of currents activated by a strong membrane depolarization, the activation kinetics of N-type calcium channels in the presence of saturating concentrations of \( \omega \)-grammotoxin SIA were dramatically slowed. This may reflect a more stable interaction between N-type calcium channels and \( \omega \)-grammotoxin SIA.

**SNX482 does not affect R-type channel permeation**

One of the characteristics of gating modifier toxins is that whereas they mediate complete inhibition of all the inward...
currents by divalent cations, outward currents carried by monovalent cations can be observed despite the presence of the toxin. To determine whether SNX482 affects the permeation characteristics, we recorded instantaneous current-voltage (*I*-*V*) relationships in the presence and the absence of the toxin. As seen in Fig. 4, instantaneous *I*-*V* curves obtained with α₁₃E channels display a sigmoidal shape, which reflects their higher conductances at negative and positive potentials compared to the region near the reversal potential. Application of SNX482 shows that there is no apparent change in the reversal potentials, showing that the toxin does not affect channel selectivity. However, the shape of the measured instantaneous *I*-*V* relationship changed slightly, with more reduction of the inward current than outward current. This could reflect small effects on permeation, but the most dramatic reduction of current at hyperpolarized potentials is probably at least partially due to the difficulty in resolving the very fast tail currents, which were faster in the presence of the toxin. Similar observations were made for ω-grammotoxin SIA inhibition of N-type calcium channels (Fig. 4, B and D), although the reversal potential obtained with α₁₃B channels was more negative than that seen with α₁₃E channels, which may reflect a greater permeability of N-type channels for cesium ions, and the shape of the *I*-*V* relationship was negligibly affected.

Overall, the characteristics of SNX482 inhibition of R-type α₁₃E channels appear strikingly similar to the actions of other calcium channel gating modifier toxins known to date.

**SNX482 incompletely blocks L-type channels**

To assess whether SNX482 was specific for R-type channels, we applied the toxin to two other types of high voltage-activated calcium channels, α₁₃A and α₁₃C. P/Q-type channels generated by α₁₃A did not exhibit any detectable inhibition in the presence of 200 nM SNX482 (not shown). However, as shown in Fig. 5 A, L-type channels underwent ~25% inhibition in the presence of 200 nM SNX482. Unlike in the case of α₁₃E, block of α₁₃C was rapidly reversible upon washout (Fig. 5 B). To test whether 200 nM produced a maximal effect we applied a higher concentration of the toxin. Whereas 500 nM produced only a smaller increase in block (36 ± 5%, n = 2), application of 1.5 μM SNX substantially increased α₁₃C current inhibition (56 ± 4%, n = 4), but the inhibition was still partial and completely reversible (Fig. 5 B). We tested whether the SNX482 effect on α₁₃C could be reversed by applying trains of positive prepulses in analogy with α₁₃E (Fig. 1 C). Indeed, even in the continued presence of 1.5 μM of the toxin, a substantial portion of the inhibition could be removed by application of depolarizing prepulses such that only 22.4 ± 4% (n = 4) block remained (compared with 56 ± 4% inhibition without...
prepulses, \( n = 4 \), see Fig. 5 B). The effect did not depend on the type of calcium channel \( \beta \) subunit coexpressed, as similar results were obtained in the presence of \( \beta_{1b} \) (data not shown).

In the presence of SNX482, the current-voltage relation was shifted toward slightly more positive potentials; however, this effect was much smaller compared to that observed with \( \alpha_{1E} \). This is illustrated in the form of tail current analysis in Fig. 5 C. As shown in the figure, the presence of SNX482 mediated only a small shift in half-activation potential by \( 9 \pm 0.5 \) mV (\( n = 4 \)) with little change in the slope of the activation curve, which may account for the small inhibition seen at typical test potentials. Application of 1.5 \( \mu \)M SNX482 revealed a slightly more pronounced effect (shift in half-activation potential of 16 mV). Consistent with Fig. 5 B, the shift in half-activation potential was fully reversible upon washout. Thus, the interaction between SNX482 and the L-type calcium channels appears to be less stable than that seen with the \( \alpha_{1E} \) channel.

Overall, these data suggest that the basic mechanism underlying the inhibition is conserved in \( \alpha_{1C} \) and \( \alpha_{1E} \) channels, but that the gating machinery of \( \alpha_{1E} \) is affected to a much greater degree.

**Domains III and IV of the \( \alpha_{1E} \) subunit participate in the inhibition of activation gating**

To investigate the channel structural determinants that participate in the inhibition of activation gating of \( \alpha_{1E} \) channels, we examined several chimeric calcium channels combining the major transmembrane domains of wild-type \( \alpha_{1E} \) and \( \alpha_{1C} \) channels (see Spaetgens and Zamponi, 1999). To maximize the magnitude of the differences in the effects of the toxin on the two channel types, and to conserve the limited supply of the toxin available to us, 200 nM concentrations were chosen as the standard in all chimeric experiments. Fig. 6 compares the time course of development of, and recovery from, SNX482 for the wild-type channels and a series of C-E chimeras. Replacement of the first two transmembrane domains of \( \alpha_{1E} \) with the corresponding regions of \( \alpha_{1C} \) (CCEE) had little effect of SNX482 block.
Block remained rapid, complete, and was not reversible upon washout. In contrast, additional replacement of domain III (CCCE) resulted in block that corresponded closely to the behavior of wild-type /H92511C channels, suggesting that domain III is an important determinant of the inhibition of /H92511E channels by SNX482. Interestingly, however, CCEC failed to exhibit /H92511E-like inhibition, indicating that the presence of domain III of /H92511E is not sufficient for /H92511E-like inhibition, and that domain IV of /H92511E may also participate in the large effects of SNX482 on R-type channel gating. As with wild-type /H92511E channels, the inhibition of CEEE and CCEE could be reversed only following application of strong depolarizing prepulses (Fig. 6 C). Furthermore, any chimeras containing domains III plus IV of /H92511E exhibited a dramatic positive shift in half-activation potential in response to SNX482 application (Fig. 6 D). In contrast, chimeras lacking either domain III or IV of /H92511E did not undergo shifts in half-activation potential, nor were prepulses required for recovery from block. Taken together, our results indicate that the pronounced toxin-mediated effects on /H92511E channel activation gating are primarily due to an interaction between SNX482 and the domain III and IV regions of the channel. The notion that both CECC and CCCE exhibited /H92511C-like partial inhibition by the toxin, and that CCEC exhibited almost no block, indicates that domain III may be involved in the weak inhibition seen with L-type calcium channels, suggesting that there may be some overlap in the structural requirements for /H92511E and /H92511C block.

DISCUSSION

SNX482 belongs to the family of calcium channel gating blockers

A vast number of peptide toxins isolated from the venoms of various predatory animals have been shown to interfere with cellular signaling and electrical activity in mammals. Among the prime targets of many of these toxins are voltage-gated ion channels. For example, agitoxin, charybdotoxin (from scorpion venom), and hanatoxin (tarantula spider) block various types of potassium channels (Park and Miller, 1992; Swartz and McKinnon, 1995, 1997), the /H9262-conotoxins (i.e., Conus geographus marine snail) block voltage-gated sodium channels (French et al., 1996; Becker...
et al., 1992), and \( \omega \)-agatoxins (American funnel web spider) and \( \omega \)-conotoxins ({*Conus geographus*} and {*Conus magus*} marine snails) selectively block voltage-dependent calcium channels (Olivera et al., 1984; Adams et al., 1993). These blockers fall into two principal categories: they either physically occlude the pore of the channel (i.e., conotoxins and charybdotoxin), or they prevent channel opening through either direct or allosteric interactions with the voltage sensor of the channel (i.e., \( \omega \)-agatoxin IVA, hanatoxin, \( \omega \)-grammotoxin SIA). Our present data indicate that SNX482 appears to belong to the latter class of molecules. Qualitatively similar to what has been reported for \( \omega \)-agatoxin IVA (McDonough et al., 1997a) and \( \omega \)-grammotoxin SIA (McDonough et al., 1997b), SNX482 mediating a large (~70 mV) depolarizing shift in the midpoint of the steady-state activation curve without apparent change in reversal potential, and its blocking action could only be recovered upon application of strong depolarizing prepulses. For comparison, the shifts in \( V_{0.5} \) observed with \( \omega \)-agatoxin IVA block of native P-type calcium channels (McDonough et al., 1997a, b) and \( \omega \)-grammotoxin block of native N-type and P-type calcium channels (McDonough et al., 1997b) were respectively ~55 mV, ~85 mV, and ~100 mV. Thus, our data fit well with previous observations obtained with other gating modifiers.

**Block of L-type channels**

The incomplete inhibition of the L-type calcium channels can also be explained by inhibition of channel activation, although this effect was much less pronounced than that seen with the \( \alpha_{1E} \) channel. In the presence of the toxin, the L-type channels underwent a reversible small shift in the position of the steady-state activation curve. In the range of the typical test potentials, this shift could account for the inhibition seen at high toxin concentrations. In contrast with the R-type channels, the inhibition of the L-type channels was fully reversible upon washout. Furthermore, voltage-dependent destabilization of the toxin effect occurs at very positive potentials. This likely reflects a less stable interaction between the L-type channels and the toxin, in line with
what it was recently shown for ω-aga IVA and N-type calcium channels (Sidach and Mintz, 2000).

Although SNX482 was shown to exhibit some effects on N-type channels, the notion that transiently expressed rat brain L-type channels showed some sensitivity to the toxin is surprising in view of previous observations of Newcomb et al. (1998) with the GH3 cell line. Although these cells express α1C, they express α1D channels at higher levels, such that any effects on α1C channels may have been masked. Nonetheless, our observations indicate that SNX482 is not as commonly believed an entirely selective antagonist of R-type calcium channels in the submicromolar range.

**Structural determinants of SNX482 block**

Our experiments with chimeric calcium channel subunits suggest that the pronounced effect on R-type channel gating requires the presence of α1E domains III plus IV. In view of the size of the toxin, the involvement of multiple binding sites may not be surprising. Indeed, the involvement of multiple contact points on the channels has been suggested previously by McDonough et al. (1997b) regarding the action of the closely related ω-grammotoxin SIA molecule. We have shown previously that gating block of α1A calcium channels by ω-agatoxin IVA is strongly reduced upon insertion of an asparagine and proline residue (Asn-Pro) in the extracellular loop connecting the S3 and S4 regions of domain IV (Bourinet et al., 1999), and Winterfield and Swartz (2000) have reported that a single glutamate residue in this region is an essential determinant of ω-agatoxin IVA action. More generally, this linker is thought to be important for the action of all gating modifier toxin known to date (Li-Smerin and Swartz, 1998). It is possible that SNX482 involves a similar interaction with both domain III and IV S3-S4 regions of α1E. The S3-S4 regions of α1E in domain III and IV differ from those of other calcium channel isoforms (see Stea et al., 1995), thereby perhaps accounting for the selectivity of SNX482 for R-type channels. Ultimately, however, a detailed examination of the domain III and IV S3-S4 linkers with point mutations would be required to determine whether these regions are indeed involved in SNX482 block of the channel.

The presence of α1E domain III was sufficient to mediate the weak inhibition and complete reversibility seen with wild-type α1C channels. It is thus possible that the toxin is capable of binding to both α1C and α1E channels, but that unique structural features contained within domains III and IV of α1E result in a larger effect on gating of this channel subtype. In addition, the notion that the presence of domains III and IV was required for an all-or-none effect may perhaps be indicative of some cooperativity between the two domains. The notion that the CCEC construct did not exhibit any detectable block could indicate that the toxin either does not bind to this channel at all, or that binding does not affect gating of this channel. Taken together, while there is partial overlap in the domain requirements for L-type and R-type channel block, it is likely that specific determinants contained within domains III and IV are responsible for the toxin’s distinct actions on R- and L-type channels. These distinct actions appear to be due to a combination of a smaller effect of the toxin on L-type channel activation, and a lower binding affinity.

Recent work of Tottene et al. (2000) suggests the existence of multiple R-type channel isoforms in mammalian brain. By using single-channel patch clamp recordings the authors identified three channel subtypes with distinct sensitivities to SNX482, one of which was blocked with an IC50 of 6 nM, a second blocked with ~10-fold lower affinity (IC50 = 81 nM), and a third, an SNX482 insensitive isoform. Antisense treatment directed against the α1E sequence reduced expression of all three components, suggesting that all three might encode variants of the α1E gene. Given the lack of calcium channel β subunit-dependence of the SNX482 blocking effects reported here, the differences in SNX482 sensitivity observed with native channels is more likely due to alternative splicing of the α1E gene rather than β subunit heterogeneity. In view of the importance of domains III and IV in gating block of α1E channels, it will thus be of interest to examine the possibility of alternatively spliced regions in the α1E channel gene in one or more of the extracellular loops connecting the individual transmembrane segments. Given the parallels with ω-agatoxin IVA block of α1A channels (Bourinet et al., 1999), the domain IV S3-S4 region could be a prime candidate for such an effect.

At this point, it is not clear whether both α1E variants identified by Tottene et al. (2000) exhibit gating block or if, under certain circumstances, pore block of α1E channels by SNX482 could occur. Identification of the nature of the putatively spliced regions and subsequent electrophysiological characterization will be required to further elucidate the nature of the differential interaction of this toxin with various types of α1E calcium channels. Nonetheless, despite the lack of complete selectivity for α1E channels reported here, the unique blocking profile of SNX482 may form a convenient tool for subclassification of native R-type calcium channels, and therefore identification of their physiological role.

This article is dedicated to the memory of Dr. Rob Newcomb for his contribution to ion channel physiology through the discovery of highly selective specific toxins. The synthetic SNX482 used for that study was kindly provided by Dr. Newcomb. We are grateful to Dr. Snutch for providing the wild-type calcium channel subunit cDNAs, to Dr. Perez-Reyes for the β2 subunit, and to Dr. Lamp for the ω-grammotoxin SIA. We thank Steve Dubel for reading of the manuscript.

This work was supported by NATO Grant CRT971546 (to E.B. and G.W.Z.), by Association Française contre les Myopathies (AFM), Fondation pour la Recherche Médicale (FRM), Association de Recherche contre le Cancer (ARC), Institut UPSA de recherche contre la Douleur (IUD), and Région Languedoc-Roussillon (to the J.N. group), and by operating grants.
from the Heart and Stroke Foundation of Alberta and the Northwest Territories and from the Canadian Institutes of Health Research (CIHR) (to G.W.Z.). G.W.Z. holds Scholarships from the Alberta Heritage Foundation for Medical Research (AHFMR), CIHR, and the EJLB Foundation. R.L.S. was supported by a studentship award from the AHFMR, and S.C.S. holds a studentship award from the AHFMR.

REFERENCES


