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Block of Voltage-dependent Calcium Channel by the Green Mamba Toxin Calcicludine

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Abstract. A number of peptide toxins derived from marine snails and various spiders have been shown to potently inhibit voltage-dependent calcium channels. Here, we describe the effect of calcicludine, a 60 amino-acid peptide isolated from the venom of the green mamba (*Dendroaspis angusticeps*), on transiently expressed high voltage-activated calcium channels. Upon application of calcicludine, L-type (α_{1C}) calcium channels underwent a rapid, irreversible decrease in peak current amplitude with no change in current kinetics, or any apparent voltage-dependence. However, even at saturating toxin concentrations, block was always incomplete with a maximum inhibition of 58%, indicating either partial pore block, or an effect on channel gating. Block nonetheless was of high affinity with an IC_{50} value of 88 nM. Three other types of high voltage activated channels tested (α_{1A}, α_{1B}, and α_{1E}) exhibited a diametrically different response to calcicludine. First, the maximal inhibition observed was around 10%, furthermore, the voltage-dependence of channel activation was shifted slightly towards more negative potentials. Thus, at relatively hyperpolarized test potentials, calcicludine actually upregulated current activity of (N-type) α_{1B} channels by as much as 50%. Finally, the use of several chimeric channels combining the major transmembrane domains of α_{1C} and α_{1E} revealed that calcicludine block of L-type calcium channels involves interactions with multiple structural domains. Overall, calcicludine is a potent and selective inhibitor of neuronal L-type channels with a unique mode of action.

Key words: Calcium channel blockers — Peptide toxins — Pharmacology — Partial pore block — Calcium channel chimeras — Channel gating

Introduction

Calcium influx through voltage-gated calcium channels triggers a range of intracellular events, including gene expression, neurotransmitter release, calcium-dependent second messenger cascades, and cell proliferation. Most neurons express multiple subtypes of voltage-dependent calcium channels, which have been classified on the basis of their biophysical and pharmacological characteristics (Bean, 1989; McCleskey & Schroder, 1991). Low-voltage-activated (LVA), or T-type channels activate within the −70 to −50 mV range and are very sensitive to changes in the resting membrane potential (Akaike, Kostyuk & Osipchuck, 1989; Takahashi, Ueno & Akaike, 1991). High-voltage-activated (HVA) channels, subclassified into L-, N-, P-, Q- and R-types, activate at more positive potentials (generally higher than −30 mV) and can be distinguished by their pharmacological profiles (Nowicky, Fox & Tsien, 1985; Fox, Nowycky & Tsien, 1987; Tsien et al., 1988, 1991; Hess, 1990; Zhang et al., 1993; McCleskey, 1994). Calcium channels are typically formed through association of multiple subunits, α_1, α_2-δ, and β (i.e., Catterall, 1991; McCleskey, 1994; Stea, Soong & Snutch, 1995). While the α_1 subunit is sufficient to form a functional channel, the ancillary α_2-δ and β subunits are critical modulators of the function of the α_1 subunit. To date, nine different neuronal α_1 subunit genes have been identified which correspond to native calcium channels. Functional expression and immunoprecipitation studies demonstrate that α_{1C}, α_{1D}, and α_{1F} encode dihydropyridine-sensitive L-type channels (Williams et al., 1992a; Hell et al., 1993; Tomlinson et al., 1993; Bech-Hansen et al., 1998), α_{1B} defines an N-type channel (Dubel et al., 1992; Williams et al., 1992*b;* Fujita et al., 1993; Stea et al., 1993), P- and Q-type channels are likely encoded by splice variants of α_{1A} (Mori et al., 1991; Sather et al., 1993; Stea et al., 1994; Bourinet et al., 1999), and T-type currents are encoded by α_{1G}, α_{1H}, and α_{1I} (Perez-Reyes et al., 1998; Cribbs et al., 1998; Lee et al., 1999). The α_{1E} channel may be one of the channels underlying the resistant current (Soong et al., 1993; Ellinor et al., 1993; Zhang et al., 1993; Williams et al., 1994; Tottene, Moretti & Pietrobon, 1996).

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A number of peptide toxins from marine snails (conotoxins) and spiders (agatoxins, grammotoxin, DW13.3) are known to potently inhibit the activities of voltage-dependent calcium channels (*see* Adams et al., 1993; Zamponi, 1997). Whereas the conotoxins are usually small (~22–30 AA) peptides, the spider toxins are typically larger (~60–90 AA) molecules (see Adams et al., 1993; Olivera et al., 1985, 1994; Sutton et al., 1998). The individual calcium channel blocking toxins exhibit a great variation in calcium channel subtype specificity. For example, ω-aga IVA (*Agenelopsis aperta*) selectively inhibits P/Q type channels, ω-aga IIIA (*Agenelopsis aperta*) and DW13.3 (*Filistata hibernalis*) block all types of high voltage-activated channels, ω-conotoxin GVIA (*Conus greographus*) selectively blocks N-type channels, and ω-conotoxin MVIIC (*Conus magus*) blocks both P/Q- and N-type channels (for review, *see* Zamponi, 1997).

A structurally distinct class of calcium channel blockers, calcicludine (CaC) is a 60 amino acid polypeptide isolated from the venom of the green mamba *Dendroaspis angusticeps* (Schweitz et al., 1994; Gilquin et al., 1999). Previous reports suggested that CaC irreversibly blocks L-, N-, and P-type channels from rat and chicken cerebellar granule cells, cardiac cells, and peripheral dorsal root ganglion cells with high affinity, while leaving the R-type current unaffected. Furthermore, CaC sensitivity appears to be both tissue and species dependent, with rat cerebellar granule L-type channels being most potently blocked (EC_{50} = 0.2 nM) (Schweitz et al., 1994). Based on evidence from binding experiments (Schweitz et al., 1994) and peptide structure determination (Gilquin et al., 1999), CaC has been predicted to interact with calcium channels at sites distinct from those targeted by classical L- or N-type blockers. Here, we examine the effect of CaC on four types of neuronal calcium channels transiently expressed in TSA-201 cells. CaC was found to have the greatest effect on L-type α_{1C} channel conductance ($\alpha_{1C} > \alpha_{1B} \approx \alpha_{1A} \approx \alpha_{1E}$). However, while L-type channel block occurred with high affinity (K_d = 88 nM), it did not surpass a maximal inhibition of 58%. For non L-type channels, CaC shifted the current-voltage relation towards slightly (~5 mV) more hyperpolarized potentials while having only a minor (10%) effect on conductance. Chimeric channels combining the major structural domains of the wild type α_{1C} and α_{1E} channels revealed that each of the four internal homologous repeats comprising the α_1 subunit are essential for block of L-type channels. Overall, our data indicate that CaC may interact with multiple transmembrane domains to mediate either a partial occlusion of the conductance pathway or a reduction in the steady-state open probability of the L-type channel. In contrast, CaC may interact with domains II and III to elicit effects on activation of non L-type channels.

Materials and Methods

Tissue Culture

Human embryonic kidney (HEK) TSA-201 cells were grown in standard DMEM medium, supplemented with 10% fetal bovine serum, 0.5 mg/ml penicillin streptomycin and 0.4 mg/ml neomycin at 37°C with 5% CO_2. The cells were grown to 85% confluence, split with trypsin-EDTA and plated on glass coverslips at 10% confluence 12 hr prior to transfection. Immediately prior to transfection, the medium was replaced with fresh DMEM. Using a standard calcium phosphate protocol, the cells were transiently transfected with cDNA subunits encoding for wild type or chimeric calcium channels (α_1, β_{1b}, and α_2-δ), as well as green fluorescent protein (pEGFP-C1) at a 1:1:1:0.5 molar ratio. After 12 hr incubation, the cells were washed with fresh DMEM and allowed to recover for 12 hr. Subsequently, the cells were incubated at 28°C in 5% CO_2 for 1–3 days prior to recording. Wild type calcium channel cDNAs were kindly donated by Dr. Snutch, chimeric α_{1C}-α_{1E} subunits were created as described in detail in Spaetgens & Zamponi (1999).

Electrophysiology

Immediately prior to recording, individual coverslips were transferred to a 3 cm culture dish containing external recording solution comprised of (in mM): 5 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 TEA-Cl, 10 Glucose, and 90 CsCl (pH 7.2 with TEA-OH). Whole-cell patch clamp recordings were performed on pEGFP-Cl labeled cells using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with pClamp v 6.0. Patch pipettes (Sutter borosilicate glass, BF150-86-15), with a typical resistance of 3–5 Mohms, were pulled using a Sutter P-87 microelectrode puller, and fire polished using a Narashige microforge. The internal pipette solution contained (in mM): 108 Cesium Methanesulfonate (CsMs), 4 $MgCl_2$, 9 EGTA, and 9 HEPES (pH 7.2 with TEA-OH).

After giga-seal formation and rupture, cells were allowed to dialyze for 5 min before recordings were performed. Current-voltage (*I–V*) relations were typically obtained by holding the cell membrane potential at −100 mV and applying a step protocol in 10 mV increments between −50 and +30 mV for 100 msec using Clampex software (Axon Instruments). After a control current had been recorded, CaC (Alomone Labs, Israel), diluted in external solution to its appropriate final concentration from a 10 mM stock (in H_2O), was directly prefused onto the cell for up to 3 min using a gravity driven microperfusion system. The time course of drug effect was monitored by stepping from a holding potential of −100 to 10 mV every 10 sec during application. After drug effect had stabilized, another *I–V* relation was obtained in the presence of CaC. After drug application, the cells were washed with control external solution for up to 10 min to determine if any effects were reversible.

Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Data were analyzed using Clampfit (Axon Instruments). Curve fitting was carried out in Sigmaplot 4.0 (Jandel Scientific) and Clampfit. *IV* relations were fit with the Boltzmann equation,

$$I = (1/(1 + exp(-(V - V_h)/S)))(V - E_{rev})G,$$

where V and V_h are the test and half-activation potential respectively, E_{rev} is the reversal potential, S is the slope factor, and G is the maximum slope conductance. The dose-response curve was fit with a modified Michaelis-Menten equation to accommodate the partial pore blocking effect of CaC, where

$$F_{unblock} = X + (1 - X) * (1/(1 + ([C]/K_d)\wedge n)),$$

X is the current fraction remaining at saturating concentrations of CaC, $[C]$ is the concentration of blocker, K_d is the blocker dissociation constant, and n is the Hill coefficient. Time constants of activation and inactivation were fitted monoexponentially. Steady-state inactivation curves were fitted with the Boltzmann equation (*see* Spaetgens & Zamponi, 1999). In all figures shown error bars represent standard errors, and numbers in parentheses reflect the number of experiments per group. Differences between mean values of experimental groups were tested using a two-tailed Student's *t*-test. Differences were considered significant if P values were less than 0.05.

Results

Calcicludine is a High Affinity Partial Blocker of L-type Calcium Channels

As CaC has been reported to be a potent blocker of native L-type calcium channels, we first assessed its ability to inhibit transiently expressed α_{1C} channels (coexpressed with β_{1b}, and α_2-δ subunits). Figure 1*A* shows that bath application of 250 nM CaC rapidly and irreversibly blocked whole cell α_{1C} currents carried by 5 mM Ba. As seen in the figure, block developed rapidly, plateaued at about 60% inhibition, and could not be reversed upon washout. The current remaining after CaC application could be completely blocked by nifedipine (Fig. 1*B*). Block did not significantly affect the extrapolated reversal potential (*see* inset in Fig. 1*A*), nor the time course of activation (−CaC: $\tau_a = 1.7 \pm 0.23$ msec; +CaC: $\tau_a = 2.2 \pm 0.3$ msec at +10 mV; $P > 0.05$; $n = 8$). In some cells, the inactivation kinetics appeared slightly faster, but overall, this effect was not statistically significant (−CaC: $\tau_h = 158 \pm 37$ msec; +CaC: $\tau_h = 179 \pm 59$ msec at +10 mV; $P > 0.05$). To assess the dose dependence of block, the effects of CaC on α_{1C} channels were examined at five different CaC concentrations spanning a range between 30 and 750 nM. As seen in Fig. 2, the dose-dependence could be nicely described via a Michaelis-Menton isotherm; however, two unusual properties became apparent. First, block saturated at 58% inhibition. In addition, a Hill coefficient of 1.44 was required to describe the raw data. The IC_{50} value obtained from the fit was 88.2 nM, indicating a relatively high affinity of CaC for L-type channels. Overall, these data suggest that CaC may either mediate a partial occlusion of the L-type calcium channel pore, or perhaps reduce the steady state open probability in a voltage-independent manner.

Calcicludine Interacts with Non L-type Channels

To assess whether the effects of CaC were specific to L-type calcium channels, we examined the action of CaC on three additional types of HVA channels under identical experimental conditions. Figure 3 illustrates the response of α_{1A}, α_{1B}, and α_{1E} channels (coexpressed with β_{1b} and α_2-δ subunits) to application of 100 nM CaC.

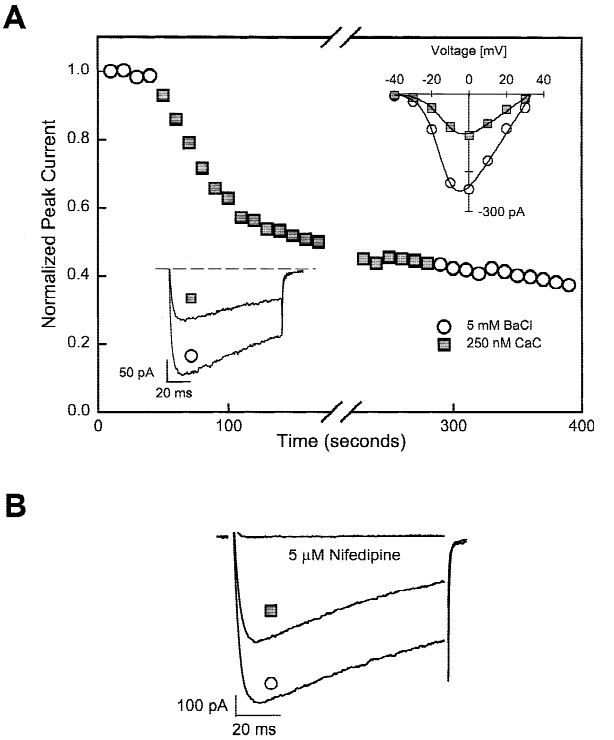

Fig. 1. (*A*) Time course of the effects of 250 nM CaC on the α_{1C} L-type channel (coexpressed with β_{1b} and α_2-δ) at a test potential of +10 mV. Block develops rapidly and is irreversible. The break in the time axis reflects an interruption in the step protocol for the purpose of acquisition of a current voltage relation. Inset (top right): Current-voltage relations were measured at the beginning and during CaC application of the experiment shown in the main panel. Circles correspond to currents obtained under control conditions and filled squares correspond to currents obtained after the addition of 250 nM CaC. Data were fit with the Boltzmann equation described in Materials and Methods, resulting in the following parameters: control: $V_h = -14.2$ mV, $E_{rev} = 33.5$ mV, $S = 4.8$, and $G = 7.4$, and CaC: $V_h = -12.0$ mV, $E_{rev} = 32.7$ mV, $S = 5.6$, and $G = 3.4$. Inset (bottom left): Current traces corresponding to times $t = 40$ sec (control) and $t = 170$ sec (250 nM CaC) of the experiment shown in the main panel. Time constants for activation and inactivation were as follows: −CaC: $\tau_a = 1.8$ msec, $\tau_h = 181$ msec; +CaC: $\tau_a = 1.9$ msec, $\tau_h = 152$ msec. The test potential was +10 mV. (*B*) Raw current traces illustrating the effect of 5 μM nifedipine on the CaC resistant current. Nifedipine completely blocks the CaC resistant current component.

The data shown in Fig. 3 reveal that application of 100 nM CaC mediates a small reduction in current amplitude at a test potential of +10 mV (current traces in Fig. 3), and a decrease in the peak of the macroscopic current-voltage relations by, respectively, $9.0 \pm 6.0\%$ ($n = 12$), $9.9 \pm 3.0\%$ ($n = 8$); $6.0 \pm 4.0\%$ ($n = 12$) for α_{1A}, α_{1B} and α_{1E} (compared with $34.0 \pm 6.0\%$ for α_{1C}). However, the *I–V* relations displayed in Fig. 3 also show that there is a slight (~5 mV) shift in the predicted reversal potential. This effect was small, but it was consistently observed in every single case for non-L-type channels, whereas it never occurred with L-type channels (*see* Fig.

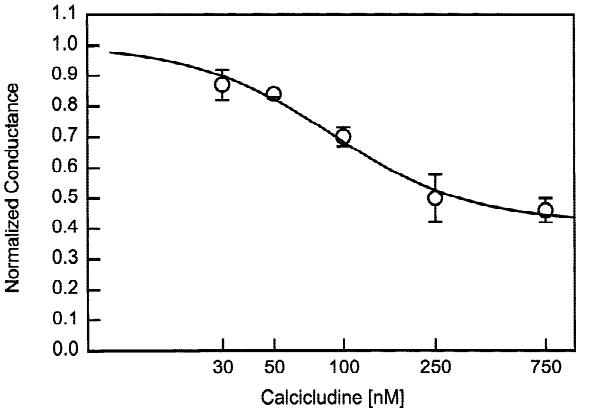

Fig. 2. Dose dependence of CaC block of α_{1C} channels in the form of slope conductance ratios obtained from current-voltage relations. The experimental conditions were as described in Fig. 1. Data were fitted with the Michaelis-Menten equation assuming partial block (*see* Materials and Methods). The fitting parameters were as follows: $X = 0.42$, $K_d = 88.2$ nM and $n = 1.44$.

1*A*). A detailed quantitative analysis of the effect was not carried out, because our whole cell recordings are not sufficiently sensitive to provide a clean access to the true reversal potential (i.e., near the reversal potential there is likely contamination from outward cesium currents). Nonetheless, the reduced driving force associated with any putative reversal potential shifts would result in a reduction of peak current amplitude at our typical test potentials (+10 mV), thereby leading to an overestimation of physical block of the channel. Hence, in order to compare the effects of CaC among the four types of channels tested independently of such effects, we determined the maximum slope conductance from Boltzman fits of the whole cell current voltage relation in the absence and the presence of CaC and used it as a measure for the CaC effects on the four types of channels tested. As shown in Fig. 4, application of 100 nM CaC reduced the whole-cell conductance of α_{1C} channels by 33%, whereas less than 10% reduction was observed for non L-type channels. Taken together, these data indicate that CaC likely interacts to some extent with all types of channels tested, but that the inhibition is fairly selective for L-type calcium channels.

Calcicludine Binding to Non L-type Channels Promotes Channel Activation

Upon examination of the current-voltage curves in Fig. 3 a third effect becomes apparent. For the three non-L-type channels, CaC mediated a small but statistically significant hyperpolarizing shift in the position of the *I–V* curve, which was not observed with L-type channels (*see* Fig. 1*A*). This is illustrated further in Fig. 5*A* in form of half-activation potentials estimated from Boltzman fits to the raw data. As seen in Fig. 5*A*, while L-type channels were unaffected, α_{1B} channels underwent a robust 5 mV negative shift in half activation potential and α_{1A} and α_{1E} channels exhibited a slightly smaller (3–4 mV) shift. Although these shifts were in the direction opposite to that expected from voltage errors due to series resistance, it is conceivable that they could be an artifact of our fitting procedure. To minimize this possibility, we examined the development of CaC block of α_{1B} channels at a more hyperpolarized test potential (−10 mV) where the shift in the half activation potential would be predicted to result in an increase in current activity rather than block. As seen in Fig. 3*B*, this was exactly what we observed. Upon application of 250 nM CaC, current activity became progressively increased before reaching a plateau; thus, supporting our contention that

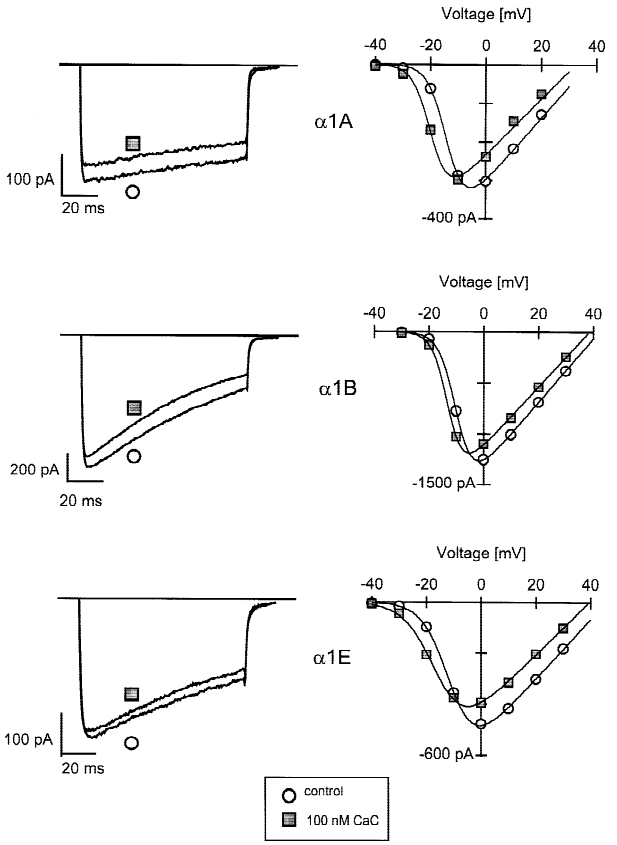

Fig. 3. Effects of 100 nM CaC on transiently expressed non-L-type HVA calcium channels. Raw currents were measured at a test potential +10 mV. Current-voltage relations were fit with a Boltzman equation. In both cases, currents recorded in 5 mM Ba are represented by circles and currents recorded in the presence of 100 nM CaC are represented by filled squares. The parameters obtained from fits of the *I–V* curves were as follows: α_{1A} (control/CaC): $V_h = -13.7$ mV/−19.5 mV, $E_{rev} = 36.8$ mV/32.3 mV, $S = 3.4/3.3$, $G = 8.2/7.3$; α_{1B} (control/CaC): $V_h = -9.7$ mV/−12.8 mV, $E_{rev} = 42.1$ mV/38.5 mV, $S = 3.2/2.9$, $G = 31.3/29.0$; α_{1E} (control/CaC): $V_h = -10.8$ mV/−15.6 mV, $E_{rev} = 45.7$ mV/39.7, $S = 4.8/5.6$, $G = 11.7/10.6$.

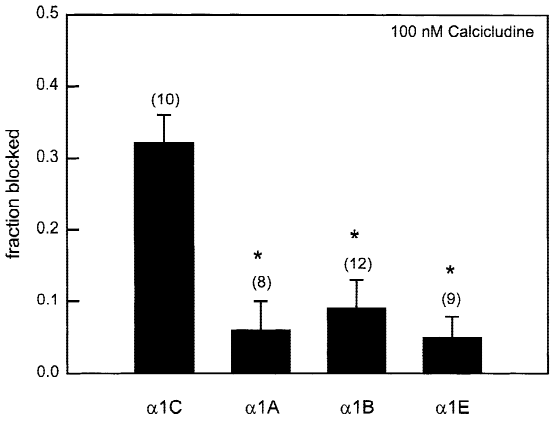

Fig. 4. Summary of the effects of 100 nM CaC on the slope conductance of HVA calcium channels. The error bars reflect standard errors and the numbers in parentheses reflect the number of experiments. Asterisks above subtype groups indicate significant differences compared to the wild-type α_{1C} channels.

CaC did indeed promote channel activation. While the CaC induced shifts in half activation potential were relatively minor, similar toxin induced shifts in half activation potential have also been observed with ω-aga IIIA (~7 mV shift, Mintz et al., 1991) and with sodium channel block by μ-CTX GIIIA (6 mV shift, French et al., 1996). CaC had only a small effect on half inactivation potential of α_{1C}, α_{1B}, or α_{1E} (< 5 mV negative shift), whereas α_{1A} channels underwent a significant leftward shift in the position of the steady-state inactivation curve by 11.8 ± 1.3 mV ($n = 3$). Taken together, the above data indicate that CaC may physically interact with non L-type channels despite its apparent inability to substantially block channel activity. Preincubation of α_{1B} channels with 250 nM CaC did not affect the time course of development of ω-CTX GVIA block (*not shown*), indicating that CaC and ω-CTX GVIA do not compete for a common site of action.

CaC Block Requires an Interaction with Multiple Transmembrane Domains

Based on the clearly different effects of CaC on α_{1C} *vs.* the other HVA channels, several chimeric channels combining the structural features of α_{1C} and α_{1E} were used to discern the molecular structures participating in conductance block. The chimeras examined involved substitution of one of the four transmembrane domains of α_{1C} with the corresponding domain of α_{1E} (*see* Spaetgens & Zamponi, 1999). Figure 6 compares the response of the chimeras and wild-type channels to 250 nM CaC. As seen in Fig. 6*A*, substitution of either domains I, II or III of the wild type α_{1C} channels with the corresponding regions of α_{1E} reduced the ability of CaC to reduce the maximum slope conductance to those levels observed

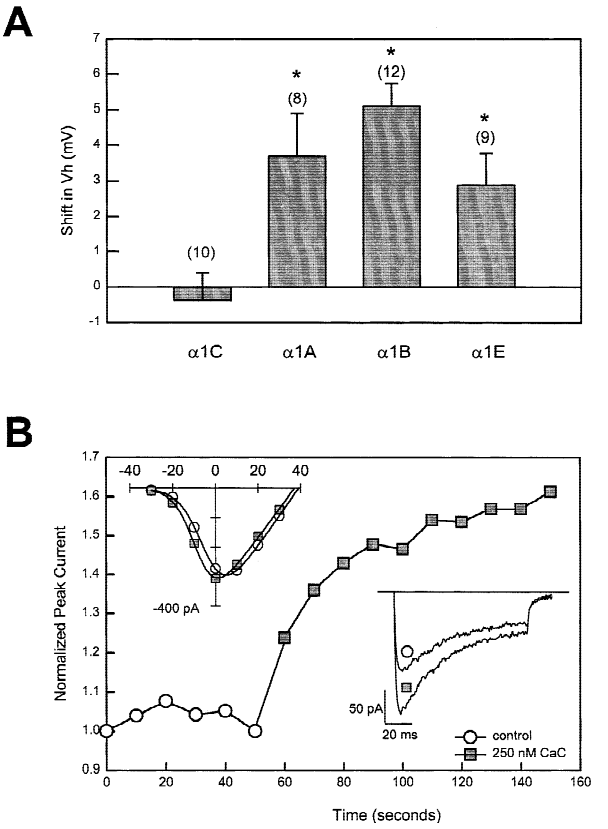

Fig. 5. (*A*) Summary of the effects of 100 nM CaC on the half activation potentials of HVA calcium channels. The error bars reflect standard error and the numbers in parentheses reflect the number of experiments. Asterisks (*) above subtype groups indicate significant differences compared to the wild-type α_{1C} channel. (*B*) Upper inset: Current-voltage relations corresponding to $\alpha_{1B} + \beta_{1b} + \alpha_2$-δ currents recorded under control conditions and in the presence of 250 nM CaC. Data were fitted with a Boltzmann relation: control $V_h = -4.2$ mV, $E_{rev} = 39.0$ mV, $S = 5.5$, $G = 10.3$; CaC $V_h = -8.1$ mV, $E_{rev} = 37.0$ mV, $S = 5.0$, and $G = 9.8$. Main panel: Time course of the effects of 250 nM CaC on α_{1B} currents at a test potential of −10 mV. Note that CaC increases current activity. Bottom panel: Raw currents corresponding to measurements taken at time $t = 0$ sec and $t = 160$ sec during the experiment shown in the main panel.

with wild-type α_{1C} channels, indicating that CaC block of L-type channels involves interactions with multiple structural domains. Interestingly, substitution of domains II or III conferred the ability of α_{1E} channels to undergo leftward shifts in half activation potential onto α_{1C} channels indicating that the structural determinants which underlie this effect may be different from those governing conductance block.

Discussion

Comparison with Previous Work

Under our experimental conditions, CaC fairly selectively, yet incompletely blocked L-type calcium channels

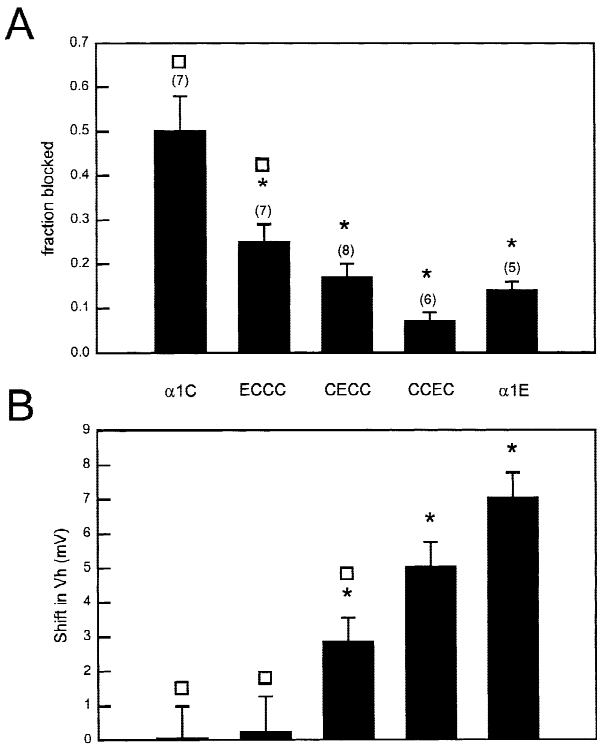

Fig. 6. Summary of the effects of 250 nM CaC on the slope conductance (*A*) and half activation potential (*B*) of wild-type and chimeric calcium channels (coexpressed with ancillary α_2-δ and β_{1b} subunits). The error bars reflect standard errors and the numbers in parentheses reflect the number of experiments (for both panels). Asterisks above subtype groups indicate significant differences compared to the wild-type α_{1C} channels, while squares represent significant differences compared to the wild-type α_{1E} channels.

with an IC_{50} of 88 nM. This contrasts dramatically with previous work on native channels. For example, block of L-type channels in cerebellar granule cells occurs with subnanomolar affinity (IC_{50} = 0.2 nM, Schweitz et al., 1994), and L-type channels of cardiac cells are inhibited with an IC_{50} of 5 nM (Schweitz et al., 1994). In contrast, L-type channels in peripheral dorsal root ganglion cells were blocked with affinities comparable to those seen in our experiments (60–80 nM, Schweitz et al., 1994). In each case, block was irreversible and, unlike in our experiments, complete (Schweitz et al., 1994). A similar disparity exists with N-type channel blockade. Whereas application of 250 nM mediated only a minor depression in the whole cell conductance of α_{1B} in our experiments, N-type channels in rat DRG and cerebellar granule cells were reportedly inhibited, respectively, with IC_{50} values of 60 nM and ~100 nM (Schweitz et al., 1994). Finally, IC_{50} values of 1–5 nM have been reported for block of P-type calcium channels of rat cerebellar granule cells (Schweitz et al., 1994), which again contrasts with our results of less than 10% block of α_{1A} at 100 nM CaC concentrations.

Differences between toxin sensitivities for native and cloned channels do not appear to be unique to CaC, as qualitatively similar observations have been noted for ω-Aga IVA block of native and transiently expressed P/Q-type channels (Bourinet et al., 1999), as well as for block of native and transiently expressed N-type channels by the novel peptide spider toxin DW13.3 (Sutton et al., 1998). There does not appear to be an obvious explanation for these differences. Although these toxins act from the extracellular side of the channel, differential modulation by cytoplasmic messenger molecules could perhaps account for these differences, particularly if the major effect of the toxin was linked to channel gating. Factors such as alternative splicing have been shown to affect toxin sensitivity of P/Q-type calcium channels (Bourinet et al., 1999), however, it is unlikely that this would occur for each of the channels tested here. Another possible mechanism which could contribute to differences between wild-type and transiently expressed calcium channels could be putative effects of calcium channel β subunit coexpression on blocking affinity. The β_{1b} subunit used in our experiments is abundant in rat brain (i.e., Stea et al., 1995), and thus, at least a fraction of the channels in native neurons would be expected to be coassembled with β_{1b} (McEnery et al., 1998), thus arguing against such an effect. Another possibility could be tissue-dependent glycosylation or other tissue specific post-translational modification mechanisms, which might interfere with toxin binding. Regardless, however, of the exact mechanisms underlying the different toxin affinities for native and transiently expressed calcium channels, the affinity of CaC for native L-type calcium channels has been observed to vary by as much as 400-fold with tissue subtype (Schweitz et al., 1994). This suggests that differences between our experiments and those of Schweitz et al. are unlikely to be a simple artifact of our expression system, but rather due to some form of tissue specific modulation.

Molecular Mechanism of L-type Channel Block by Calcicludine

Structurally, CaC is not related to calcium channel blocking peptides isolated from marine snails, spiders, or other types of snakes (Olivera et al., 1985, 1994; Davis et al., 1993; Pallaghy et al., 1993; Sevilla et al., 1993; Skalicky et al., 1993; Adams et al., 1993; Albrand et al., 1995; Sutton et al., 1998). Instead, CaC exhibits structural homology to a number of protease inhibitor molecules (Schweitz et al., 1994; Gilquin et al., 1999), and it has been suggested that the three-dimensional structure of CaC may resemble that of basic pancreatic trypsin inhibitors (Schweitz et al., 1994; Gilquin et al., 1999). Thus, given these structural considerations, a somewhat unique blocking profile may not be unexpected.

For L-type channels examined here, CaC block was always incomplete, with a maximal level of block close to 60%. This could indicate that CaC might perhaps partially occlude the pore, similar to what has been described for ω-agatoxin IIIA (Mintz et al., 1991) and the spider toxin DW13.3 (Sutton et al., 1999). However, we were unable to describe the dose dependence of this partial block by using a simple 1:1 binding scheme (i.e., a Hill coefficient greater than 1 was required). Furthermore, assuming that CaC binds to overlapping regions on both α_{1C} and α_{1B} channels, the observation that the presence of CaC did not affect the time course of development of N-type channel block by ω-conotoxin GVIA — a known pore blocker — might argue against partial pore occlusion. Alternative possibilities include a toxin-induced conformational change, which directly affects the permeation pathway or a voltage-independent reduction in steady-state open probability of the channel. The concept of a gating modifying peptide toxin is not without precedent. For example, ω-aga IVA (Bourinet et al., 1999), grammotoxin (Li-Smerin & Swartz, 1998), α and β scorpion toxins (Na^+ channels, Rogers et al., 1996), and hanatoxin (K^+ channels, Swartz & MacKinnon, 1997) all modulate channel gating. Thus, an effect on open probability is an attractive possibility, however, in lieu of detailed single channel recordings, it is difficult to conclusively discern between a gating and a pore blocking effect.

For non-L-type channels, CaC exerted little effect on the maximum slope conductance. Instead, the position of the *I–V* curve was consistently shifted towards more hyperpolarized potentials. Although we did not attempt to precisely characterize these shifts using tail current protocols, the data shown in Fig. 5 for α_{1B} currents strongly suggest that channel activation is indeed facilitated by the toxin. Furthermore, at least for α_{1A} channels, CaC significantly shifted the midpoint of the steady-state inactivation curve. Hence, we conclude that CaC interacts with non L-type channels without being able to effectively reduce the whole cell conductance. A possible mechanism underlying the effects on half activation and inactivation potentials could be electrostatic interactions between negatively charged residues on the toxin molecule and the gating machinery of the calcium channel α_1 subunit. Such a mechanism would be conceptually similar, but in the opposite direction, to what has been described in detail for the actions of μ-conotoxins on voltage-dependent sodium channels (French et al., 1996). Alternatively, it is conceivable that binding of such a large toxin affects channel conformation, thereby perhaps mediating effects on activation and permeation.

Our experiments with the chimeric α_1 subunits indicate that CaC likely interacts with multiple structural domains of α_{1C} to reduce the whole cell conductance. This is perhaps not surprising, since a 60 amino acid toxin would be predicted to share multiple contact points with the target protein. Nonetheless, these observations indicate that the larger effect of the toxin on L-type channels is due to interactions between the toxin and L-type channel specific residues distributed over at least the first three transmembrane domains. Interestingly, domains II and III, but not domain I of α_{1E} conferred aspects of the toxin's effect on activation of α_{1E} onto α_{1C}. This supports the notion that specific regions of the α_{1E} subunit are able to detect and respond to the presence of the toxin, and further underlines the idea that CaC affects L-type and non L-type channels via separate mechanisms.

In summary, CaC is a potent, high affinity inhibitor of transiently expressed L-type calcium channels. The dramatic differences from previous observations on native calcium channels may suggest that the action of CaC is dependent on the cellular environment to an extent not previously reported for any type of calcium channel blocking toxin.

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