**β Subunit Coexpression and the α1 Subunit Domain I–II Linker Affect Piperidine Block of Neuronal Calcium Channels**

Gerald W. Zamponi, Tuck W. Soong, Emmanuel Bourinet, and Terry P. Snutch

Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

The effects of local anesthetics were examined on a family of transiently expressed neuronal calcium channels. Fomocaine, a local anesthetic containing a morpholine ring, preferentially blocked α1E channels ($K_i = 100 \mu M$), and had a lower affinity (3- to 15-fold) for α1A, α1B, and α1C channels. Block was completely reversible, followed 1:1 kinetics, and did not affect steady-state inactivation properties. Fomocaine block was sensitive to the concentration of permeant ion and enhanced in the presence of external pore blockers, suggesting a site of action in the conducting pathway. Flecainide, which carries a piperidine ring, and the diphenylbutylpiperidine antipsychotic, penfluridol, caused qualitatively similar block, suggesting that morpholine rings are compatible with the piperidine receptor site. In contrast, procaine, which contains an alkyl chain, caused reversible low affinity block of the different calcium channels ($K_i$ values between 2 and 5 mM) and was least effective on α1E and did not compete with fomocaine, suggesting that local anesthetics interact with at least two distinct receptor sites. Compared to coexpression with the Ca channel β1 subunit, block at the piperidine receptor site was significantly weakened with the β2a subunit suggesting that the nature of the β subunit contributes to drug binding. Amino acid changes in the cytoplasmic linker between domains I and II resulted in decreased fomocaine and penfluridol blocking affinity. Furthermore, the blocking affinity observed with α1B was conferred on α1A by substitution of the domain I–II linker of α1B into α1A. Taken together, the data suggest that β subunit binding and the domain I–II linker contribute to the piperidine receptor site on neuronal calcium channels.

**Key words:** local anesthetics; Xenopus oocytes; functional expression; barium current; fomocaine; penfluridol; procaine.
Figure 1. Chemical structures of the compounds fomocaine, flecainide, procaine, diethylcarbamazine, piperazine, and penfluridol. Note that both fomocaine and flecainide carry their tertiary nitrogens as part of a saturated ring (dotted box), whereas the N terminus in procaine is part of an alkyl chain. Diethylcarbamazine is a hybrid between the N-terminal portions of procaine and fomocaine. The methylated nitrogen in the piperazine ring of diethylamine has a pKa of 7.3. Piperazine itself is essentially permanently uncharged (Ka = 6.5 × 10⁻⁵) at physiological pH.
nels, we compared the blocking action of procaine with that of fomocaine (a morpholine-based local anesthetic) on four types of cloned neuronal calcium channels transiently expressed in Xenopus oocytes. The results suggest the existence of two distinct receptor sites for local anesthetics, one that weakly interacts with procaine, and a second higher-affinity receptor that binds piperidine- and morpholine-based compounds. The data are consistent with the notion that the procaine-binding site is directly accessible from the extracellular side, whereas block by compounds such as fomocaine likely occurs at the piperidine receptor site on the intracellular side of the channel. We also present evidence indicating that piperidine binding is affected both by β subtype coexpression and by amino acid substitutions in the α₂ subunit domain I–II linker.

**MATERIALS AND METHODS**

Functional expression of calcium channels in Xenopus oocytes. Ovaries were surgically removed from mature, anesthetized (0.17% 3-aminobenzoic acid ethyl ester; MS 222) Xenopus laevis (Xenopus One, Ann Arbor, MI) and agitated in 2 mg/ml collagenase (type IA; Sigma, St. Louis, MO) for 2–3 hr. The collagenase was dissolved in calcium-free OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5). Before nuclear injection, oocytes were allowed to recover for 2–24 hr in SOS medium (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) containing 2.5 mM sodium pyruvate and 10 µg/ml gentamicin sulfate. Each nucleus was injected (−10 nl final volume) with −1–2 ng of each calcium channel subunit cDNA subcloned into the pCI expression vector, pMT2 (Stea et al., 1995). Oocytes were incubated in SOS medium for 2–5 d before recording.

**Voltage-clamp and data analysis.** Two-electrode voltage-clamp experiments were carried out using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) linked to an IBM-compatible PC equipped with pClamp version 5.5 software (Axon Instruments). Microelectrodes were filled with 3 M KCl and showed typical resistances from 0.5 to 2.5 MΩ. BAPTA (10–30 mM) from a 100 mM stock) was injected into each oocyte during recording to suppress leak currents carried by endogenous calcium-activated chloride channels (Chae et al., 1994). The oocytes were bathed in 2 mM BaCl₂, 36 mM tetraethylammonium chloride (TEA), 2 mM CaCl₂, 5 mM niflumic acid, 30 mM sucrose, 20 mM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 5 mM HEPES, pH 7.6, or in 10 mM CaCl₂, 36 mM TEA, 5 mM niflamic acid, 30 mM sucrose, 20 mM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 5 mM HEPES, pH 7.6. Drugs were dissolved in the appropriate recording solution before application and perfused into the bath. Fomocaine (Aldrich, Milwaukee, WI) was dissolved in ethanol to make a stock solution of 500 mM fomocaine; procaine (a kind gift from Dr. Robert French), piperazine (Sigma), and diethylcarbamazine (Sigma) were dissolved directly in the recording solution to give a stock of 500 mM; flecainide (a gift from Dr. Robert French) was dissolved in ethanol as a 250 mM stock, penfluoridol (a kind gift from Janssen Pharmaceuticals, Berse, Belgium) was dissolved in ethanol at a stock concentration of 200 mM. The chemical structures of the compounds used in this study are depicted in Figure 1. ω-Conotoxin GVIA was dissolved in water to make a stock of 200 µM. The final ethanol concentration in the bath during drug application did not cause significant block of the currents as verified by application of ethanol alone (n = 5). Unless otherwise stated, currents were elicited by a 0.033 Hz train of 400 msec pulses from a holding potential of −100 to +10 mV. In most cases block fully developed within 60 sec of drug application. Unless otherwise stated, fomocaine and flecainide were allowed to equilibrate for 90 sec, whereas procaine, piperazine, and diethylcarbamazine were applied for 30–60 sec before determining their blocking actions. Currents were allowed to stabilize before drug application to minimize contributions from run-up and run-down. Data were filtered at 1 kHz and recorded directly on the hard drive of a personal computer. In most cases, last subtraction was carried out on line using a pClamp protocol. Peak currents were analyzed using Clamp version 5.5 and 6.0. Macroscopic current–voltage relations were fit and described as a single Boltzmann function with the equation: I = 1/(1 + exp(-(V_m - V_1/2)/G)), where V_m is the peak potential, V_1/2 is the half-maximal activation potential, G is the maximal slope conductance, and 5 reflects the steepness of the activation curve and is an indication of the apparent gating charge movement. Inactivation curves were constructed by normalizing the currents after a prepulse to those in the absence of a prepulse and then fit to the equation: I/II = 1/(1 + exp(-(V_m - V_1/2)/G)), where V_m is the holding potential, V_1/2 is the zero-inactivation potential, and I is the normalized peak current. Data were fit using a least squares algorithm (Winplotr, Jandel Scientific). Data are reported as means ± SEM with n = 3–4 for all experiments.

**RESULTS**

**Fomocaine preferentially blocks the α₁E channel**

Figure 2 shows the effects of fomocaine on whole cell barium currents resulting from α₁A₄α₁Bα₂Cα₃ or α₁E calcium channels (with α₂ and β₁b or β₃ subunits) transiently expressed in Xenopus oocytes. In the absence of blockers, the different channel subtypes show varying electrophysiological profiles; α₁A₄α₁Bα₂Cα₃ and α₁E activate rapidly and inactivate with distinct time courses whereas α₁C activates more slowly and exhibits little inactivation. Fomocaine reduced peak currents of each channel subtype to varying degrees. Block of the barium currents developed within 15 sec and reached a steady state within 1 ½ min of drug application (Fig. 2A). The effects of fomocaine were partially reversible and complete recovery did not occur during the time course of the experiments (Fig. 24,B,D,E). The relative order of potency for fomocaine block was α₁E > α₁C > α₁A₄α₁Bα₂Cα₃ (Fig. 2B–E) and was maintained over a range of fomocaine concentrations (Fig. 3). The dose–response curves were well fit by simple hyperbolas suggesting a 1:1 drug–channel interaction. The IC₅₀ values determined from the fits were 95 µM, 334 µM, and 777 µM for α₁E, α₁C, and α₁A₄α₁Bα₂Cα₃ channels, respectively. Fomocaine application (up to 600 µM) did not result in any significant changes to the steady-state activation curves of the four calcium channel types examined (not shown).

The piperidine-based drug flecainide also blocked the neuronal Ca channels in a qualitatively similar manner to that for fomocaine (e.g., subtype specificity and degree of reversibility) but with a somewhat lower affinity for α₁E. (IC₅₀: α₁E + β₁b + α₂, 320 µM, n = 7; α₁C + β₁b + α₂, 380 µM, n = 4; α₁A₄α₁Bα₂Cα₃ + β₁b + α₂, 630 µM, n = 4; not shown). Also similar to that for fomocaine, the effect of flecainide was partially reversible. Overall, the relative order of potencies is consistent with previous reports indicating that piperidine-based antipsychotics preferentially block lower threshold calcium channels (Galizzi et al., 1989; Tynear et al., 1992).

**Fomocaine block is sensitive to the concentration of permeant ion**

As evidenced from Figure 3, raising the external barium concentration from 2 to 10 mM resulted in a substantial (~6- to 10-fold) decrease in the fomocaine blocking affinity for α₁A₄α₁Bα₂Cα₃ and α₁E.
channels. The IC50 values obtained from the fits were $\alpha_{\text{a}, \text{b}}$: 2 mM Ba = 777 μM, 10 mM Ba = 4953 μM; $\alpha_{\text{E}}$: 2 mM Ba = 95 μM, 10 mM Ba = 916 μM. These data suggest that fomocaine may act at a region of the channel accessible to external barium ions. Barium ions might act by competitively inhibiting fomocaine binding at the drug site. Alternatively, if fomocaine physically occludes the pore from the intracellular side then permeating barium ions might repel fomocaine accessing its binding site and result in an
increase in the IC₅₀ of fomocaine block as has been proposed for local anesthetic action on sodium channels (Cahalan and Almers, 1979; Wang, 1988; Zamponi and French, 1993; Zamponi et al., 1993a).

**Blocking effects of procaine**

In contrast to that for the piperidine-based fomocaine and flecainide, the local anesthetic procaine only weakly blocked the transiently expressed neuronal calcium currents. For example, Figure 4 shows that procaine at concentrations as high as 2 mM only resulted in ~25% block of the α₁Aa current. A further distinction was that unlike block by fomocaine, the block by procaine was readily reversible after washout. In addition, the subtype specificity of procaine action was distinct from that of fomocaine and flecainide with α₁C being the most sensitive channel subtype followed by α₁B > α₁Aa > α₁E (Fig. 4). Application of 6 mM diethylamine (pKa = 10.5; Zamponi and French, 1993) or 6 mM TEA did not result in any significant effect on the macroscopic currents suggesting that procaine does not act by screening diffuse surface charges (not shown). This result also suggests that the diethylamine tail of procaine is not the only structural component that determines blocking affinity and is consistent with the more potent action of the procaine analog tetracaine on neuronal calcium channels (Sugiyama and Muteki, 1994).

As with fomocaine, increasing the concentration of external barium ions from 2 mM to 10 mM resulted in a decreased blocking affinity. In five experiments the effect of 2 mM procaine was studied under 2 mM and 10 mM barium on the same oocyte and the degree of procaine block of α₁Aa decreased from 24 ± 6 to 13 ± 1% at the higher barium concentration (data not shown). Although these values are significantly different (p < 0.0013, paired t test) and reflect an approximately twofold increase in IC₅₀, the degree of change is much smaller than that observed with fomocaine (Fig. 3). Overall, the qualitative and quantitative differences between block by fomocaine and procaine are suggestive of distinct mechanisms of block on neuronal calcium channels.

**Structural requirements for drug action and interactions between fomocaine and procaine**

A more detailed investigation into the mechanisms of action of fomocaine and procaine was carried out on α₁Aa because this channel type gives the most robust expression in oocytes (Stea et al., 1994). To define the drug structural requirements for block, we examined the effects of piperazine and diethylcarbamazine, antihelmintic agents used in the treatment of conditions such as filariasis (Gelband, 1994). Structurally, piperazine closely resembles the amine-containing portions of fomocaine and flecainide (Fig. 1), and is essentially permanently uncharged at a pH of 7.6 (pKa = 4.2). Application of piperazine at concentrations between 2 and 6 mM had little effect on α₁C + (Ye + β₄, + α₂ (Fig. 3). The data were fitted with the equation $I/I_{drug\ free} = 1/(1 + [F]/IC₅₀)$, where $I$ and $I_{drug\ free}$ are, respectively, the peak currents measured in the presence and the absence of fomocaine, [F] is the fomocaine concentration, and IC₅₀ is the concentration at 50% current inhibition. The data are well described by a Hill coefficient of 1, suggesting a 1:1 interaction between the channel and the drugs.
**Figure 4.** Current traces obtained from the major types of neuronal calcium channels in the absence and presence of 2 mM procaine. Procaine blocks $\alpha_{1Aa}$ and $\alpha_{1C}$ more effectively than $\alpha_{1B}$ channels. Note the complete reversibility of procaine block (A, C, D). The experimental conditions are as described in Figure 2.

$\alpha_2 + \beta_{1b} (n = 3)$, or $\alpha_{1B} + \alpha_2 + \beta_{1b} (n = 4$; data not shown). Although it is possible that other portions of local anesthetics are also required for a high-affinity interaction, these results are consistent with the notion that the protonated species is required for block.

Diethylcarbamazine is essentially a hybrid between the amine-containing portions of procaine and fomocaine. Diethylcarbamazine contains three amino groups: one is methylated and has a pKa of 7.3 and the other two have permanently protonated groups (pKa > 12; see Fig. 1). This compound would not be expected to permeate cell membranes easily. Application of 2 mM diethylcarbamazine to oocytes expressing $\alpha_{1Aa} + \alpha_2 + \beta_{1b}$ resulted in a rapidly developing, fully reversible block to $-60\%$ of the control level (Fig. 5A). Decreasing the pH to 7.1 from 7.6 (which increases the concentration of drug with a protonated methyl-amino group) did not significantly affect block ($n = 3$; data not shown). This result suggested that the structure crucial for diethylcarbamazine block is more likely to be the diethylated amino group that resembles the head group of procaine, rather than the portion of diethylcarbamazine that resembles the amine-containing portion of fomocaine. If this assumption is correct, diethylcarbamazine and procaine would be expected to compete for binding whereas diethylcarbamazine and fomocaine would not compete. To test this hypothesis, a series of coapplication experiments were performed. Consistent with a direct competition model, Figure 5A shows that coapplication of 2 mM procaine and 2 mM diethylcarbamazine resulted in only a 10% increase in block compared with that for diethylcarbamazine alone. Because diethylcarbamazine is essentially membrane impermeant this result implies that procaine is also likely to act on the extracellular side. In contrast, coapplication of 600 $\mu$M fomocaine and 2 mM diethylcarbamazine resulted in a potentiated block that exceeded the predictions for both direct competition and independence (see Materials and Methods, Fig. 5A) and supports the hypothesized existence of separate receptors for procaine and fomocaine. Similarly, coapplication of procaine and fomocaine produced block that exceeded predictions for direct competition between the two compounds (Fig. 5B). The results indicate that procaine and fomocaine do not compete at a common blocking site.

A possible mechanism of enhanced fomocaine block in the presence of diethylcarbamazine was suggested by the coapplication of nickel and fomocaine. Figure 5C shows that application of
Figure 5. Effects of diethylcarbamazine, procaine, and nickel on fomocaine block of α1A-β4 in 2 mM barium saline. The magnitudes of the bars reflect the fraction of the channels blocked on a scale from 0 to 1. Note that coapplication of diethylcarbamazine and fomocaine (A) or procaine and fomocaine (B) result in block that exceeds the degree of block expected from a simple competition model (see Materials and Methods). The open bars indicate S.E.s. In A, the degree of block by diethylcarbamazine + procaine and diethylcarbamazine + fomocaine was determined in paired experiments on the same oocyte. C and D show current-voltage relationships obtained for α1A-β4 in 2 mM Ba. Addition of 600 μM fomocaine reduces the current by 35%, whereas coapplication of 600 μM fomocaine and 1 mM nickel eliminates virtually all of the current (C). Wash with 600 μM fomocaine reveals an enhanced degree of fomocaine block (C). The bar graphs indicate the fractional block by 600 μM fomocaine before and after application of nickel from five different paired experiments on a scale from 0 to 1. The open bars indicate standard errors. D illustrates the reversibility of block by 1 mM nickel alone. The current-voltage relationships were fitted as described in Materials and Methods.

600 μM fomocaine resulted in an ~35% reduction in the peak α1A-β4 current and that coapplication of 1 mM nickel and 600 μM fomocaine eliminated all of the current. Of particular note, wash-out of the nickel with a solution containing 600 μM fomocaine revealed an ~100% increase in the degree of fomocaine block (n = 5; Fig. 5C). Because the level of fomocaine block stabilizes rapidly (Fig. 2A), the enhanced block is unlikely attributable to longer exposure to fomocaine. Furthermore, nickel block is completely reversible (Fig. 5D) and suggests that the enhanced fomocaine block is most likely attributable to an effect of nickel on permeation. Because barium ions appear to inhibit fomocaine binding (Fig. 3), one possible explanation is that external nickel ions physically occlude barium permeation resulting in an increased fomocaine block. In this scenario, because fomocaine block is only weakly reversible, the additional fomocaine block developing in the presence of nickel ions persists after nickel removal. This qualitative behavior might be expected given the high sensitivity of fomocaine block to barium flux through the channel. If our interpretation is correct, these data suggest that fomocaine must act from the cytoplasmic side, consistent with the lack of competition between the membrane impermeant diethylcarbamazine molecule and fomocaine. Furthermore, such a mechanism might account for the potentiation of fomocaine block by diethylcarbamazine.

Overall, the data suggest the presence of at least two separate local anesthetic receptor sites on neuronal calcium channels: a low-affinity site that is accessible directly from the extracellular side and that binds compounds such as procaine, and a second, higher affinity site that interacts with piperidine and morpholine-based compounds on the intracellular side (see also below).
**Figure 6.** The effect of the β₂a subunit on fomocaine block. Whole-cell currents were recorded from α₁A.a (A), α₁C (B), and α₁E (C) channels coexpressed with β₁a, β₂a, β₃a, and β₄. Coexpression of α₁ subunits with β₂a significantly reduces the degree of inactivation. In addition, the degree of block by 600 μM fomocaine is reduced compared with that obtained when the channels are coexpressed with β₁a, β₂a, β₃a, and β₄ subunits (D). Experimental conditions were as described in Figure 2.

**Fomocaine block is sensitive to the type of β subunit present**

To investigate the effects of β subunits on fomocaine block, we coexpressed α₁A.a with four different neuronal β subunits (β₁b, β₂a, β₃, and β₄). There were no discernible differences between fomocaine block of α₁A.a channels in the absence of a β subunit or when coexpressed with either β₁b, β₂a, or β₄ (Fig. 6). In contrast, coexpression with β₂a significantly decreased the blocking affinity of fomocaine. Similar decreases in fomocaine block were also observed when α₁E and α₁C subunits were coexpressed with β₂a (Fig. 6). The β₂a subunit has been shown to significantly reduce the degree of calcium channel inactivation (Stea et al., 1994) (Fig. 6). To investigate the possibility that the effect on fomocaine block may arise from secondary effects on inactivation properties, steady-state inactivation profiles were determined in the presence and absence of fomocaine. Fomocaine application did not significantly affect \( V_{\text{h}}(\text{control}) = -61.1 ± 2.9 \text{ mV}, V_{\text{h}}(\text{fomocaine}) = -60 ± 3.6 \text{ mV}, n = 5 \) steady-state inactivation (Fig. 7), consistent with previous reports of flunarizine action on native calcium channels (Takahashi and Akaike, 1991). The data suggest that the effect of β₂a on fomocaine block does not arise secondarily from removal of voltage-dependent inactivation. This notion is supported by the observation that α₁C shows little voltage-dependent inactivation regardless of the type of β subunit coexpressed; however, the β₂a still reduced the fomocaine blocking affinity (see Fig. 6).

**Amino acid substitutions in the α₁A.a subunit domain I-II linker affect fomocaine block**

Recently, several variants of the α₁A.a subunit that result from alternative splicing have been identified (Fig. 8A; Soong and Snutch, unpublished observations). The α₁A.a isoform differs from α₁A.a in that it contains a valine insertion in the domain I-II linker (position #472), an insertion of asparagine and proline in the domain IV S3-S4 loop and several amino acid substitutions in the EF motif of the C terminus. The α₁A.a isoform differs from α₁A.a in a single glycine deletion in the domain I-II linker (position #471; Fig. 8; T. W. Soong and T. P. Snutch, unpublished observations). Examination of an α₁A.a variant that differed from α₁A.a only by the valine insertion (Soong and Snutch, unpublished observations) showed that 600 μM fomocaine produced significantly less block (1/2 ± 2%, n = 13) compared with α₁A.a (Fig. 8B,E). The α₁A.a variant, when coexpressed with α₂ + β₄, exhibits kinetic...
block observed with \( \alpha_{1-4} \) did not differ significantly from \( \alpha_{1-4} \) in combination with any of the \( \beta \) subunits tested (\( p > 0.15 \), not shown). Together with the competition experiments and the difference in reversibility of fomocaine and procaine block, these data support the idea of separate receptors for procaine and fomocaine.

**Fomocaine acts at the piperidine receptor site**

To determine whether fomocaine acts at the piperidine receptor site we performed a series of experiments using the clinically active antipsychotic, penfluridol, a compound shown to act at the piperidine site (King et al., 1989). Application of penfluridol resulted in block of whole cell barium currents that were qualitatively similar to that for fomocaine but with an \( \sim \)10-fold higher affinity (Fig. 9A–D) and a slower development (equilibration after \( \sim \)3 min). Penfluridol block was not reversible after washout as with fomocaine (not shown). Penfluridol exhibited a similar subtype specificity as fomocaine, blocking \( \alpha_{1E} + \beta + \beta \) with an IC\(_{50}\) of 13 \( \mu \)M (\( n = 6 \)) > \( \alpha_{1C} + \beta + \alpha_{2} \) (47 \( \mu \)M, \( n = 4 \)) > \( \alpha_{1A-a} + \beta + \alpha_{2} \) (127 \( \mu \)M, \( n = 8 \)) > \( \alpha_{1A-a} + \beta + \alpha_{2} \) (117 \( \mu \)M, \( n = 5 \)) > \( \alpha_{1B} + \beta + \alpha_{2} \) (400 \( \mu \)M, \( n = 3 \)). Penfluridol block of \( \alpha_{1A-a} \) was significantly reduced by \( \beta_{2a} \) coexpression (1170 \( \mu \)M, \( n = 9 \)). Amino acid substitutions in the \( \alpha_{1A} \) domain I–II linker also significantly reduced penfluridol block as for fomocaine (Fig. 9E–H; \( \alpha_{1A-b} + \beta + \alpha_{2} \); 488 \( \mu \)M, \( n = 8 \); \( \alpha_{1A-c} + \beta + \alpha_{2} \) 334 \( \mu \)M, \( n = 7 \)). Coapplication of fomocaine and penfluridol to \( \alpha_{1A-a} \) resulted in a degree of block consistent with a simple competition between the two compounds (\( n = 5 \), not shown).

Overall, the data suggest that morpholine/piperidine based local anesthetics block calcium channels at the piperidine receptor.

**DISCUSSION**

Morpholine rings are compatible with the piperidine receptor site

A range of structurally unrelated compounds block calcium channels. For L-type calcium channels, the presence of at least seven distinct drug binding domains has been suggested, including sites for binding of phenylalkylamines (PAAs), DHPs, benzothiazepines (BTZs), and piperidines (for review, see Glossmann and Striessnig, 1990; Striessnig et al., 1994). The locations of the PAA binding site and the region for DHP binding have been mapped on the primary structure of the channel, with PAAs binding at the C terminal region of the S6 segment of the fourth domain, and DHPs binding near the pore-forming region of domain III and near the extracellular end of the S6 region in domain IV (Striessnig et al., 1991, 1994). Furthermore, there is some evidence that BZTs act at least in part at the S5–S6 region of domain IV (Watanabe et al., 1993). However, the location of the piperidine receptor on the calcium channel primary sequence is not known. Piperidines were originally thought of as highly potent antagonists of the D\(_{2}\) receptor, and clinically used to treat various forms of psychosis (Seeman and Lee, 1975; Seeman et al., 1976). However, over the past few years, increasing evidence suggests that piperidine-based antipsychotics are also powerful antagonists of voltage-dependent calcium channels, with affinities ranging from several nanomolar into the micromolar range (Gould et al., 1983; Sah and Bean, 1984; Galizi et al., 1986; King et al., 1989; Enyeart et al., 1992; Grantham et al., 1994; Xu and Lee, 1994). Several members of the piperidine class have been shown to preferentially block T-type calcium channels in neurons (Enyeart et al., 1992; Im et al., 1993). Here, we show that a morpholine-based local anesthetic is also capable of interactions with the...
Figure 8. Effect of 600 μM fomocaine on amino acid substitutions in the domain I–II linker. A, Primary structure of the domain I–II linker of α subunit variants (Soong et al., 1995). Whole-cell currents were recorded in 2 mM barium from αIβγ+β4 (B), αIβγ+α2 (C), and a chimeric channel in which the I–II loop of αII is inserted into αIAβD (D). αIAβ and αIAB channels are identical to αIAβ channels except for a single glycine deletion (αIAβ) or a single valine insertion (αIAB) in position 472. Note that αIAB exhibits activation and inactivation properties similar to that for αIAβ, whereas αIAB channels exhibit little inactivation. Both αIAβ and αIAB exhibit a reduced sensitivity to fomocaine block compared with αIAβ. The chimeric channel exhibits a reduced blocking sensitivity that is comparable with that seen with the intact αIB channel (D, E). Experimental conditions were as described in Figure 2.
Figure 9. Block of the neuronal calcium channels by the piperidine-based antipsychotic penfluridol. As can be seen from the records, α₁E is the most significantly affected subtype (D), followed by α₁C (C), α₁Aa (A), and α₁Bb (B). Coexpression of α₁Aa with β₂ (F) results in a reduced blocking affinity, as do amino acid substitutions in the I–II loop (G, H; compare with E). The experimental conditions were as outlined in Figure 2.
piperidine receptor site. Fomocaine qualitatively mimics the action of penfluridol, a compound known to act at the piperidine receptor site, in subtype specificity, lack of reversibility, dependence on ancillary subunits, and sensitivity to mutations in the I-II loop. Furthermore, copptlication of fomocaine and penfluridol results in blockage consistent with direct competition between the two compounds. Overall, these data indicate that morpholine rings are sufficient for interaction with the piperidine receptor. Given the similar blocking behavior seen with flecainide and its structural similarity to bupivacaine, we suggest that the potent calcium channel block reported for bupivacaine might also arise from interactions with the piperidine receptor, thus providing a molecular basis for the observation that some local anesthetics can substantially affect calcium channels, whereas others such as lidocaine or procaine do not.

Comparison with previous work and clinical significance
Fomocaine preferentially blocks $\alpha_{1E}$ with an $IC_{50}$ of 95 $\mu$M, whereas $\alpha_{1C}$, $\alpha_{1As}$, and $\alpha_{1B}$ are blocked with, respectively, 3.5-fold, 8-fold, and 15-fold lower affinity. A qualitatively similar order of blocking affinities has been observed with penfluridol and flecainide. The $\alpha_{1E}$ channel has been proposed to be a novel type of low to mid threshold calcium channel (Soong et al., 1993), and the subtype-specific action observed in this study is consistent with the subtype-specificity seen with block of native low threshold channels by piperidine antipsychotics and related compounds (Enyeart et al., 1992).

Relatively little is available concerning the blocking action of fomocaine on native calcium channels. Fomocaine has been previously reported to exhibit pronounced antiaarrhythmic properties at concentrations as high as 30 $\mu$M (Braeunig et al., 1989), which is similar to clinically used blood plasma concentrations of a range of antiaarrhythmic agents (Sheldon et al., 1987). At this concentration, ~25% of the calcium current through $\alpha_{1E}$ was blocked by fomocaine, suggesting that calcium channel block by fomocaine occurs at clinically significant concentrations. We know of only two reports describing calcium channel block by flecainide (Scamps et al., 1989; Yamashita et al., 1995). In both of these studies, flecainide weakly blocked cardiac calcium channels (i.e., 15% block at 10 $\mu$M concentrations; Yamashita et al., 1995). The structurally related bupivacaine has been reported to block cardiac calcium channels with $IC_{50}$ between 100 and 300 $\mu$M (Sanchez-Chapula, 1988; Wulf et al., 1994) and N-type calcium channels from bullfrog sensory ganglion cells with an $IC_{50}$ of ~50 $\mu$M (Guo et al., 1992). For comparison, between 1 and 25 $\mu$M bupivacaine is required to block 50% of the currents through cardiac and neuronal sodium channels (Clarkson and Hondegem, 1985; Butterworth and Strichartz, 1990; Chernoff, 1990). Hence, the blocking effects on calcium channels seen with fomocaine, flecainide and bupivacaine could be of direct clinical importance, especially in the light of the CAST study (Cardiac Arrhythmia Suppression Trial Investigators, 1989) and in view of reports that bupivacaine is lethal when administered to the cardiovascular system (Clarkson and Hondegem, 1985).

There are several similarities between the blocking action of fomocaine and what is seen with block of low threshold calcium channels by piperidine/piperazine-based compounds in isolated and cultured neurons. First, the degree of block by flunarizine increases by ~10-fold when the external calcium concentration is dropped from 10 $\mu$M to 2.5 $\mu$M (Takahashi and Akaike, 1991). Similarly, the efficacy of penfluridol increases with decreasing external calcium concentrations (Enyeart et al., 1992). Second, flunarizine has only negligible effects on the position of the steady-state inactivation curve along the voltage axis (Takahashi and Akaike, 1991). Furthermore, inactivation is not required for the development of use-dependent block of T-type calcium channels (Enyeart et al., 1992; but see Im et al., 1993). Third, penfluridol block of calcium channels in neuronal C cells is irreversible (Enyeart et al., 1992). Fourth, binding of radiolabeled fluspirilene is enhanced in the presence of external divalent cation blockers (King et al., 1989) (for review, see Kaczorowski et al., 1994). Finally, block of high voltage-activated currents by penfluridol, flunarizine, and fluspirilene is about one order of magnitude weaker than that of low threshold channels (Akaike et al., 1989; Enyeart et al., 1992) (for review, see Peters et al., 1991). Overall, these data support the hypothesis that fomocaine acts at the piperidine receptor site.

We know of only a few reports describing procaine block of calcium channels (Johansen and Kleinhaus, 1985; Sugiyama and Muteki, 1994). Sugiyama and Muteki reported a $K_i$ for procaine of ~2.5 mm whereas Johansen and Kleinhaus reported a 40% inhibition of calcium channels in leech neurons by 5 mm procaine. Based on a pH experiment (raising the pH from 7.4 to 8.5 resulted in a drastic increase in the degree of procaine block), Johansen and Kleinhaus concluded that procaine had to act from the cytoplasmic side. In three experiments, we could not detect a significant increase in procaine block after a pH increase from 7.6 to 8.65 (not shown), despite an associated 12-fold increase in the concentration of uncharged, and thus membrane-permeant, form of the drug (procaine pKa = 9.0; Ritchie and Greengard, 1961). Furthermore, procaine block was mimicked by, and competitively inhibited by membrane-impermeant diethy carbamazine, again suggesting that the procaine site is directly accessible from the extracellular side. It is possible that the discrepancy between our data and those of Johansen and Kleinhaus might arise from structural differences between leech and mammalian calcium channels.

Is the piperidine receptor site in the domain I-II linker?
We have presented four lines of evidence that are consistent with the I-II loop forming part of the piperidine receptor. First, coexpression of $\alpha_1$ subunits with $\beta_2$ significantly reduces the degree of fomocaine and penfluridol block of $\alpha_{1A\cdot\alpha_0}$, $\alpha_{1C}$, and $\alpha_{1E}$ channels. Second, a single glycine deletion or valine insertion in position 472 in loop I-II also reduced the blocking affinity. Third, inserting the I-II loop of $\alpha_{1B}$ into $\alpha_{1A}$ resulted in block consistent with that seen with $\alpha_1$. Finally, fomocaine block was antagonized by external divalent cations and enhanced in the presence of external pore blockers, suggesting a cytoplasmic action close to the narrow region of the pore. Although both coexpression of $\alpha_1$ subunits with $\beta_2$ and the insertion of a valine residue in position 4/2 have been shown to reduce the degree of inactivation (Soong et al., 1995), the observation that the steady-state inactivation curve was not affected by the presence of fomocaine suggests that inactivated channels are not blocked (Hille, 1977; Bean et al., 1983). Furthermore, there are no resolvable differences in the electrophysiological properties of $\alpha_{1A\cdotA}$ and $\alpha_{1A\cdotC}$ (Soong and Snutch, unpublished observations), arguing against a global conformational change induced by the glycine deletion, and yet the drug affinity is reduced. Although we are unable at this point to demonstrate direct binding to the I-II loop, our data are consistent with a direct involvement of the I-II loop in fomocaine block
and not with a secondary effect arising from altered channel kinetics or a global conformational change.

We can only speculate as to a putative mechanism by which ancillary subunits and amino acid substitutions might affect drug affinity. If there is a similarity to sodium channel block, one would expect the charged (note that the permanently uncharged piperazine is ineffective) N-terminal nitrogen atom of fomocaine to be the predominant blocking structure that would physically occlude the pore, whereas the phenyl rings and the morpholine ring would serve to increase the blocking affinity by binding to additional structures near the pore mouth (Zamponi and French, 1994). Coexpression with $\beta_2$ or small structural changes in the I–II loop might abolish some of these additional interactions, leaving only a weak interaction between the N terminal and the narrow region of the pore. Such a mechanism would be consistent with the observation that 600 μM fomocaine always produces a minimal level of $\sim 15$–20% block for any of the various combinations of $\alpha$, and $\beta$ subunits. Although far-reaching $\beta$ subunit effects on calcium channel pharmacology have been reported (i.e., an increased affinity for the external blockers $\omega$-conotoxin and some DHPs when $\alpha$ subunits were coexpressed with a $\beta$ subunit compared with expression of $\alpha$ alone; Williams et al., 1992a,b; Nishiimura et al., 1993), the observation that removal or addition of a single amino acid residue within the I–II loop results in a comparable effect suggests the possibility of a simple structural rearrangement of the I–II loop that occurs after binding of $\beta_2$, but not after binding of any of the other $\beta$ subunits studied. Although there is a substantial degree of homology between the different $\beta$ subunits especially in the regions that are involved in binding of the $\beta$ subunit to the $\alpha$ subunit, (De Waard et al., 1994; Pragnell et al., 1994), there are significant structural differences between $\beta_2$ and other types of $\beta$ subunits. One could envision a scenario in which a unique region on the $\beta_2$ subunit causes a small displacement of the I–II loop, thereby altering the three-dimensional conformation of the drug receptor. Such a mechanism appears plausible, especially in light of a recent study by Olcese et al. (1994) demonstrating that a short sequence of pza affects of inactivation.

Although we acknowledge the possibility of alternative interpretations, we view the data as consistent with the idea that the I–II linker is critical for block of calcium channels by piperidine compounds and perhaps forms part of the piperidine receptor.

REFERENCES

Akaike N, Kostyuk PG, Osipchuk YV (1989) Dihydropyridine-sensitive low threshold calcium channels in isolated rat hypothalamic neurones. J Physiol (Lond) 412:181–191.

Bean BP, Cohen CJ, Tisien RW (1983) Loidocaine block of cardiac sodium channels. J Gen Physiol 81:613–642.

Birnbaumer L, Campbell KP, Catterall WA, Harpold MM, Hofmann F, Horne WA, Mori Y, Schwartz A, Snutch TP, Tanelue T, Tisien RW (1994) The naming of voltage-gated calcium channels. Neuron 13:505–506.

Birnbaumer L, Flockerzi V, Hofmann F, Lazzara R, Rosen MR, Schwartz PJ, Buttenvorth JF, Strichartz GR (1990) Molecular mechanisms of local anaesthesia. Annu Rev Pharmacol Toxicol 30:549–586.

Birnbaumer L, Brown AM (1991) Normalization of current kinetics by small structural changes in the I-II loop that occurs after binding of $\beta_2$, but not after binding of any of the other $\beta$ subunits studied. Although there is a substantial degree of homology between the different $\beta$ subunits especially in the regions that are involved in binding of the $\beta$ subunit to the $\alpha$ subunit, (De Waard et al., 1994; Pragnell et al., 1994), there are significant structural differences between $\beta_2$ and other types of $\beta$ subunits. One could envision a scenario in which a unique region on the $\beta_2$ subunit causes a small displacement of the I–II loop, thereby altering the three-dimensional conformation of the drug receptor. Such a mechanism appears plausible, especially in light of a recent study by Olcese et al. (1994) demonstrating that a short sequence of pza affects of inactivation.

Although we acknowledge the possibility of alternative interpretations, we view the data as consistent with the idea that the I–II linker is critical for block of calcium channels by piperidine compounds and perhaps forms part of the piperidine receptor.

REFERENCES

Akaike N, Kostyuk PG, Osipchuk YV (1989) Dihydropyridine-sensitive low threshold calcium channels in isolated rat hypothalamic neurones. J Physiol (Lond) 412:181–191.

Bean BP, Cohen CJ, Tisien RW (1983) Loidocaine block of cardiac sodium channels. J Gen Physiol 81:613–642.

Birnbaumer L, Campbell KP, Catterall WA, Harpold MM, Hofmann F, Horne WA, Mori Y, Schwartz A, Snutch TP, Tanelue T, Tisien RW (1994) The naming of voltage-gated calcium channels. Neuron 13:505–506.

Birnbaumer L, Flockerzi V, Hofmann F, Lazzara R, Rosen MR, Schwartz PJ, Buttenvorth JF, Strichartz GR (1990) Molecular mechanisms of local anaesthesia. Annu Rev Pharmacol Toxicol 30:549–586.

Birnbaumer L, Brown AM (1991) Normalization of current kinetics by small structural changes in the I-II loop that occurs after binding of $\beta_2$, but not after binding of any of the other $\beta$ subunits studied. Although there is a substantial degree of homology between the different $\beta$ subunits especially in the regions that are involved in binding of the $\beta$ subunit to the $\alpha$ subunit, (De Waard et al., 1994; Pragnell et al., 1994), there are significant structural differences between $\beta_2$ and other types of $\beta$ subunits. One could envision a scenario in which a unique region on the $\beta_2$ subunit causes a small displacement of the I–II loop, thereby altering the three-dimensional conformation of the drug receptor. Such a mechanism appears plausible, especially in light of a recent study by Olcese et al. (1994) demonstrating that a short sequence of pza affects of inactivation.

Although we acknowledge the possibility of alternative interpretations, we view the data as consistent with the idea that the I–II linker is critical for block of calcium channels by piperidine compounds and perhaps forms part of the piperidine receptor.

REFERENCES

Akaike N, Kostyuk PG, Osipchuk YV (1989) Dihydropyridine-sensitive low threshold calcium channels in isolated rat hypothalamic neurones. J Physiol (Lond) 412:181–191.

Bean BP, Cohen CJ, Tisien RW (1983) Loidocaine block of cardiac sodium channels. J Gen Physiol 81:613–642.

Birnbaumer L, Campbell KP, Catterall WA, Harpold MM, Hofmann F, Horne WA, Mori Y, Schwartz A, Snutch TP, Tanelue T, Tisien RW (1994) The naming of voltage-gated calcium channels. Neuron 13:505–506.

Birnbaumer L, Flockerzi V, Hofmann F, Lazzara R, Rosen MR, Schwartz PJ, Buttenvorth JF, Strichartz GR (1990) Molecular mechanisms of local anaesthesia. Annu Rev Pharmacol Toxicol 30:549–586.

Birnbaumer L, Brown AM (1991) Normalization of current kinetics by small structural changes in the I-II loop that occurs after binding of $\beta_2$, but not after binding of any of the other $\beta$ subunits studied. Although there is a substantial degree of homology between the different $\beta$ subunits especially in the regions that are involved in binding of the $\beta$ subunit to the $\alpha$ subunit, (De Waard et al., 1994; Pragnell et al., 1994), there are significant structural differences between $\beta_2$ and other types of $\beta$ subunits. One could envision a scenario in which a unique region on the $\beta_2$ subunit causes a small displacement of the I–II loop, thereby altering the three-dimensional conformation of the drug receptor. Such a mechanism appears plausible, especially in light of a recent study by Olcese et al. (1994) demonstrating that a short sequence of pza affects of inactivation.

Although we acknowledge the possibility of alternative interpretations, we view the data as consistent with the idea that the I–II linker is critical for block of calcium channels by piperidine compounds and perhaps forms part of the piperidine receptor.

REFERENCES

Akaike N, Kostyuk PG, Osipchuk YV (1989) Dihydropyridine-sensitive low threshold calcium channels in isolated rat hypothalamic neurones. J Physiol (Lond) 412:181–191.

Bean BP, Cohen CJ, Tisien RW (1983) Loidocaine block of cardiac sodium channels. J Gen Physiol 81:613–642.

Birnbaumer L, Campbell KP, Catterall WA, Harpold MM, Hofmann F, Horne WA, Mori Y, Schwartz A, Snutch TP, Tanelue T, Tisien RW (1994) The naming of voltage-gated calcium channels. Neuron 13:505–506.

Birnbaumer L, Flockerzi V, Hofmann F, Lazzara R, Rosen MR, Schwartz PJ, Buttenvorth JF, Strichartz GR (1990) Molecular mechanisms of local anaesthesia. Annu Rev Pharmacol Toxicol 30:549–586.
interaction between the α1 and β subunits of the skeletal muscle dihydropyridine-sensitive Ca²⁺ channel. Nature 352:247-250.

Mori Y, Friedrich T, Kim M-S, Mikami A, Nakai J, Ruth P, Bosse E, Hofmann F, Flockerzi V, Furutichi T, Mikoshiba K, Imoto K, Tanabe T, Numa S (1991) Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. Nature 340:230-233.

Nishimura S, Takehashi H, Hofmann F, Flockerzi V, Imoto K, Tanabe T, Numa S (1991) Primary structure and functional expression from complementary DNA of a brain calcium channel. Nature 350:398-402.

Olcese R, Quin N, Schneider T, Neely A, Wei X, Stefani E, Birnbaumer L (1994) The amino terminus of a calcium channel beta subunit sets rates of channel inactivation independently of the subunits effects on activation. Neuro 13:1421-1438.

Peters T, Willibert B, Vanhoutte PM, van Zwieten PA (1991) Calcium channels in the brain as targets for the calcium-channel modulators used in the treatment of neurological disorders. J Cardiovasc Pharmacol 18(Suppl 8):S1-S5.

Patterson M, De Waard M, Mori Y, Tanabe T, Snutch T, Campbell K (1994) Calcium channel β-subunit binds to a conserved motif of the I-II cytoplasmic linker of the α1-subunit. Nature 368:67-70.

Ritchie JM, Greengard P (1961) On the active structure of local anesthetics. J Pharmacol Exp Ther 133:241-245.

Sanchez-Chapula J (1988) Effects of bupivacaine on membrane currents of guinea pig ventricular myocytes. Eur J Pharmacol 156:303-308.

Scamps F, Undrovinas A, Vassort G (1989) Inhibition of I(to) in single frog cardiac cells by quinidine, flecainide, etmozin, and ethacizin. Am J Physiol 257:H1773-H1781.

Seeman P, Lee T (1975) Antipsychotic drugs: direct correlation between rates of channel inactivation independently of the subunits effects on activation. Proc Natl Acad Sci USA 88:10769-10773.

Stei A, Soong TW, Snutch TP (1995) Voltage-gated calcium channels. In: Handbook of receptors and channels: ligand- and voltage-gated ion channels. Proc Natl Acad Sci USA 32:1117-1126.

Striessnig J, Hering S, Berger W, Vatterall W, Glossmann H (1994) Calcium antagonist binding domains of L-type calcium channels. In: Ion channels in the cardiovascular system: function and dysfunction, (Spooner P, ed), pp 441-458. Armonk, NY: Futura.

Sugiya K, Muteki T (1994) Local anesthetics depress the calcium current of rat sensory neurons in culture. Anesthesiology 80:1369-1378. Takahashi K, Akaike N (1991) Calcium antagonist effects on low-threshold (T-type) calcium current in rat isolated hippocampal CA1-pyramidal neurons. J Pharmacol Exp Ther 256:169-175.

Tomlinson WJ, Stea A, Bourinet E, Charnet P, Nargeot J, Snutch TP (1993) Functional properties of a neuronal class C L-type channel. Neuropharmacology 22:1117-1126.

Wang GK (1988) Cocaine-induced closures of single batrachotoxin-activated Na⁺ channels in planar lipid bilayers. J Gen Physiol 92:747-765.

Watanabe T, Kalam H, Yabana H, Kuniyasu A, Mersen J, Igoaki G, Vaghy PL, Naito K, Nakayama H, Schwartz A (1993) Azidobutyl clenazepam, a new photoactivable diltiazem analog, labels benzothiazepine binding sites in the α1 subunit of the skeletal muscle calcium channel. FEBS Lett 334:261-264.

Weidmann S (1955) The effect of calcium and local anesthetics on the electrical properties of Purkinje fibers. J Physiol (Lond) 129:568-582.

Williams ME, Feldman DH, McCue AF, Brenner R, Velicicli E, Ellis SB, Harpold MM (1992a) Structure and functional expression of α1, α2, and β subunits of a novel human neuronal calcium channel subtype. Neuron 8:71-84.

Williams ME, Brust PF, Feldman DH, Pattni S, Simerson S, Marohti C, McCue AF, Velicicli E, Ellis SB, Harpold M (1992b) Structure and functional expression of an α-conotoxin-sensitive human N-type calcium channel. Science 257:389-395.

Williams ME, Murablio LM, Deal CR, Hans M, Brust PF, Philipson LH, Miller RJ, Johnson EC, Harpold MM, Ellis SB (1994) Structure and functional characterization of neuronal α1H calcium channel subtypes. J Biol Chem 269:22347-22357.

Wulf H, Groedcke J, Herzog S (1994) Functional interaction between local anesthetics and calcium antagonists in guinea pig myocardium: electrophysiological studies with bupivacaine and nifedipine. Br J Anesthesiol 73:364-370.

Xu X, Lee KS (1994) A selective blocker for rested T-type Ca²⁺ channels in guinea pig atrial cells. J Pharmacol Exp Ther 269:1135-1142.

Yamashita T, Nakajima T, Hamada E, Hazam H, Omata M, Kurachi Y (1995) Flecainide inhibits the transient outward current in atrial myocytes isolated from the rabbit heart. J Pharmacol Exp Ther 274:315-321.

Zamponi GW, French RJ (1993) Dissecting lidocaine action: diethylamidine and phenol mimic separate modes of lidocaine block of sodium channels from heart and skeletal muscle. Biophys J 65:2325-2347.

Zamponi GW, French RJ (1994) Amine blockers of the cytoplasmic mouth of sodium channels. A small structural change abolishes voltage dependence. Biophys J 67:1015-1027.

Zamponi GW, Doyle DD, French RJ (1993a) Fast lidocaine block of cardiac and skeletal muscle sodium channels. One site with two routes of access. Biophys J 65:80-90.

Zamponi GW, Doyle DD, French RJ (1993b) State-dependent block underlies the tissue specificity of lidocaine action on BTX-activated cardiac sodium channels. Biophys J 65:91-100.

Zamponi GW, Sui X, Cooding PW, French RJ (1993c) Dual proarrhythmia action on batrachotoxin-activated cardiac sodium channels: open channel block and prevention of inactivation. Biophys J 65:2324-2334.