β Subunit Coexpression and the α_1 Subunit Domain I–II Linker Affect Piperidine Block of Neuronal Calcium Channels

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The effects of local anesthetics were examined on a family of transiently expressed neuronal calcium channels. Fmocaine, a local anesthetic containing a morpholine ring, preferentially blocked α_{1E} channels ($K_i = 100 \mu\text{M}$), and had a lower affinity (3- to 15-fold) for α_{1A} , α_{1B} , and α_{1C} channels. Block was incompletely reversible, followed 1:1 kinetics, and did not affect steady-state inactivation properties. Fmocaine 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_d values between 2 and 5 mM) and was least effective on α_{1E} and

did not compete with fmocaine, suggesting that local anesthetics interact with at least two distinct receptor sites. Compared to coexpression with the Ca channel β_{1b} subunit, block at the piperidine receptor site was significantly weakened with the β_{2a} 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 fmocaine and penfluridol blocking affinity. Furthermore, the blocking affinity observed with α_{1B} was conferred on α_{1A} by substitution of the domain I–II linker of α_{1B} into α_{1A} . 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; fmocaine; penfluridol; procaine

It is well established that local anesthetics are effective blockers of neuronal, cardiac, and skeletal muscle sodium channels (Weidmann, 1956; Strichartz, 1973; Courtney, 1975; Hondeghem and Katzung, 1977; Bean et al., 1983; Butterworth and Strichartz, 1990; Zamponi et al., 1993a–c). Local anesthetics also affect several members of the potassium channel family (Camm et al., 1992; Yamashita et al., 1995), nicotinic ACh receptors, as well as calcium release channels from the sarcoplasmic reticulum. However, the effects of local anesthetics on voltage-gated calcium channels have been poorly described, and neither the structural requirements for calcium channel block nor the specific site(s) of action of these compounds has been identified. Whereas the archetypal local anesthetics lidocaine and procaine appear to be poor inhibitors of voltage-gated calcium channels ($K_i \sim 2$ –5 mM; Johansen and Kleinhaus, 1985; Sugiyama and Muteki, 1994), there have been several reports of potent block (K_i between 50 and 300 μM) of both cardiac and neuronal calcium channels by the local anesthetic bupivacaine (Sanchez-Chapula, 1988; Guo et al., 1992; Sugiyama and Muteki, 1994; Wulf et al., 1994). Bupivacaine exhibits an obvious structural difference compared to procaine and lidocaine in that the tertiary amine-containing portion—a

critical structure for block of both sodium (Zamponi and French, 1994) and potassium channels—forms part of a piperidine ring rather than an alkyl chain (see Fig. 1). Similar ring structures that include tertiary amines also form the core of a range of calcium channel-blocking antipsychotic agents, including penfluridol (see Fig. 1) (Galizzi et al., 1986; Czuczwar et al., 1992; Enyeart et al., 1992; Sah and Bean, 1994), and the existence of a specific binding site for piperidine-based compounds has been proposed (King et al., 1989) (for review, see Glossmann and Striessnig, 1990; Kaczorowski et al., 1994; Striessnig et al., 1994).

Molecular cloning studies have identified gene families encoding Ca channel α_1 and β subunits expressed in the mammalian nervous system (for review, see Birnbaumer et al., 1994; Stea et al., 1995). The various α_1 subunits each encode a Ca channel with distinct physiological and pharmacological characteristics: α_{1A-b} , a high-voltage threshold channel exhibiting characteristics similar to both P- and Q-type channels (Mori et al., 1991; Sather et al., 1993; Stea et al., 1994); α_{1B} , an ω -conotoxin GVIA-sensitive N-type channel (Dubel et al., 1992; Williams et al., 1992b; Fujita et al., 1993; Stea et al., 1993); α_{1C} and α_{1D} , dihydropyridine (DHP)-sensitive L-type channels (Mikami et al., 1989; Snutch et al., 1991; Williams et al., 1992a; Tomlinson et al., 1993); and α_{1E} , a widely expressed channel for which no specific antagonists have yet been described (Soong et al., 1993; Williams et al., 1994). Coexpression of Ca channel β subunits has been shown to influence a number of α_1 subunit functional properties, including increased whole-cell currents as well as altered kinetic and voltage-dependent properties (for examples, see Lacerda et al., 1991; Castellano et al., 1993a,b; Stea et al., 1994).

To test whether local anesthetics containing piperidine-based or similar structural features interact with neuronal calcium chan-

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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 pK_a of 7.3. Piperazine itself is essentially permanently uncharged ($K_a = 6.5 \times 10^{-5}$) 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 subunit coexpression and by amino acid substitutions in the α_1 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 MgCl_2 , 5 mM HEPES, pH 7.5). Before nuclear injection, oocytes were allowed to recover for 3–24 hr in SOS medium (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, pH 7.5) containing 2.5 mM sodium pyruvate and 10 $\mu\text{g}/\text{ml}$ gentamycin sulfate. Each nucleus was injected (~ 10 nl final volume) with ~ 1 –2 ng of each calcium channel subunit cDNA subcloned into the vertebrate expression vector, pMT2 (Stein 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 CsCl and showed typical resistances from 0.5 to 2.5 M Ω . BAPTA (10–30 nl from a 100 mM stock) was injected into each oocyte during recording to suppress leak currents carried by endogenous calcium-activated chloride channels (Charnet et al., 1994). The oocytes were bathed in 2 mM BaCl_2 , 36 mM tetraethylammonium chloride (TEA), 2 mM CsCl, 5 mM niflumic acid, 38 mM sucrose, 20 μM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 5 mM HEPES, pH 7.6, or in 10 mM BaCl_2 , 36 mM TEA, 2 mM CsCl, 5 mM niflumic acid, 30 mM sucrose, 20 μM 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, penfluridol (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, leak subtraction was carried out on line using a p/5 protocol. Peak currents were analyzed using pClamp versions 5.5 and 6.0. Macroscopic current–voltage relations were fitted with the equation: $I = \{1/(1 + \exp(-(V_m - V_h)/S))\} \{(V_m - E_{\text{rev}})G\}$, where V_m is the test potential, V_h is the voltage at which half of the channels are activated, E_{rev} is the reversal potential, G is the maximum slope conductance, and S 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: $I = 1/(1 + \exp((V_m - V_h)/S))$, where V_m is the holding potential, V_h is the half-inactivation potential, and I is the normalized peak current. Competition experiments were analyzed as follows. Direct competition between two compounds is represented by the scheme:

and mathematically described by the equation:

$$I_{\text{norm}} = 1/(1 + [F]/K_{[F]} + [D]/K_{[D]}),$$

where I_{norm} is the normalized peak current, $[F]$ and $[D]$ are the respective concentrations of the two coapplied compounds, and $K_{[F]}$ and $K_{[D]}$ are their respective IC_{50} values. Independent drug binding to two individual blocking sites is represented by the scheme:

and is mathematically described by the relation:

$$I_{\text{norm}} = \{1/(1 + [F]/K_{[F]})\} \{1/(1 + [D]/K_{[D]})\}.$$

The respective IC_{50} values were determined from experiments involving application of a single compound.

RESULTS

Fomocaine preferentially blocks the α_{1E} channel

Figure 2 shows the effects of fomocaine on whole cell barium currents resulting from α_{1A-a} , α_{1B} , α_{1C} , and α_{1E} calcium channels (with α_2 and β_{1b} or β_4 subunits) transiently expressed in *Xenopus* oocytes. In the absence of blockers, the different channel subtypes show varying electrophysiological profiles; α_{1A-a} , α_{1B} , and α_{1E} activate rapidly and inactivate with distinct time courses whereas α_{1C} 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 1/2 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. 2A,B,D,E). The relative order of potency for fomocaine block was $\alpha_{1E} > \alpha_{1C} > \alpha_{1A-a} > \alpha_{1B}$ (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 IC_{50} values determined from the fits were 95 μM , 334 μM , and 777 μM for α_{1E} , α_{1C} , and α_{1A-a} 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 α_{1E} (IC_{50} : $\alpha_{1E} + \beta_{1b} + \alpha_2$, 320 μM , $n = 7$; $\alpha_{1C} + \beta_{1b} + \alpha_2$, 380 μM , $n = 4$; $\alpha_{1A-a} + \beta_4 + \alpha_2$, 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 are consistent with previous reports indicating that piperidine-based antipsychotics preferentially block lower threshold calcium channels (Galizzi et al., 1989; Enyeart 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 α_{1A-a} and α_{1E}

Figure 2. Block of whole-cell currents by fomocaine application. **A**, Time course of development of fomocaine block. Currents recorded from α_{1A-a} coexpressed with β_4 and α_2 (in 2 mM barium saline) were elicited by steps to +10 mV from a holding potential of -100 mV every 15 sec. Application of 600 μM fomocaine results in rapid development of $\sim 35\%$ block and is incompletely reversible after washout. **B–E**, Whole-cell current traces recorded from $\alpha_{1A-a} + \beta_{1b} + \alpha_2$, $\alpha_{1B} + \beta_{1b} + \alpha_2$, $\alpha_{1C} + \beta_{1b} + \alpha_2$, and $\alpha_{1E} + \beta_{1b} + \alpha_2$, in the absence and presence of fomocaine (in 2 mM barium saline). The records were filtered at 1 kHz and were elicited by stepping from -100 to +10 mV.

channels. The IC_{50} values obtained from the fits were α_{1A-a} : 2 mM Ba = 777 μM , 10 mM Ba = 4953 μM ; α_{1E} : 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 IC_{50} 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 $\sim 25\%$ block of the α_{1A-a} 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 α_{1C} being the most sensitive channel subtype followed by $\alpha_{1B} > \alpha_{1A-a} > \alpha_{1E}$ (Fig. 4). Application of 6 mM diethylamine ($\text{pK}_a = 10.5$; 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 α_{1A-a} decreased from 24 ± 6 to $13 \pm 1\%$ 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 IC_{50} , 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 α_{1A-a} 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 ($\text{pK}_a = 4.2$). Application of piperazine at concentrations between 2 and 6 mM had little effect on $\alpha_{1A-a} + \alpha_2 + \beta_{1b}$ ($n = 6$), $\alpha_{1C} +$

Figure 3. Dose-response curves for fomocaine block. $\alpha_{1A-a} + \beta_4 + \alpha_2$ (A), $\alpha_{1E} + \beta_{1b} + \alpha_2$ (B), and $\alpha_{1C} + \beta_{1b} + \alpha_2$ (C) in 2 mM (solid symbols) or 10 mM barium (open symbols). The results indicate that α_{1E} channels are more potently blocked than both α_{1C} and α_{1A-a} and that the external barium concentration significantly reduces the blocking affinity. The data were fitted with the equation $I/I_{(\text{drug free})} = 1/(1 + [F]/\text{IC}_{50})$, where I and $I_{(\text{drug free})}$ are, respectively, the peak currents measured in the presence and the absence of fomocaine, $[F]$ is the fomocaine concentration, and IC_{50} 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 α_{1A-a} and α_{1C} more effectively than α_{1E} 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 $\alpha_{1E} + \alpha_2 + \beta_{1b}$ ($n = 4$; 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 fomicaine. Diethylcarbamazine contains three amino groups: one is methylated and has a pK_a of 7.3 and the other two have permanently protonated groups ($\text{pK}_a > 12$; see Fig. 1). This compound would not be expected to permeate cell membranes easily. Application of 2 mM diethylcarbamazine to oocytes expressing $\alpha_{1A-a} + \alpha_2 + \beta_4$ resulted in a rapidly developing, fully reversible block to $\sim 60\%$ of the control level (Fig. 5*A*). 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 fomicaine. If this assumption is correct, diethylcarbamazine

and procaine would be expected to compete for binding whereas diethylcarbamazine and fomicaine would not compete. To test this hypothesis, a series of coapplication experiments were performed. Consistent with a direct competition model, Figure 5*A* 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 μM fomicaine and 2 mM diethylcarbamazine resulted in a potentiated block that exceeded the predictions for both direct competition and independence (see Materials and Methods; Fig. 5*A*) and supports the hypothesized existence of separate receptors for procaine and fomicaine. Similarly, coapplication of procaine and fomicaine produced block that exceeded predictions for direct competition between the two compounds (Fig. 5*B*). The results indicate that procaine and fomicaine do not compete at a common blocking site.

A possible mechanism of enhanced fomicaine block in the presence of diethylcarbamazine was suggested by the coapplication of nickel and fomicaine. Figure 5*C* shows that application of

Figure 5. Effects of diethylcarbamazine, procaine, and nickel on fomocaine block of $\alpha_{1A-a} + \beta_4 + \alpha_2$ 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 SEs. 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 $\alpha_{1A-a} + \beta_4 + \alpha_2$ 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 $\sim 35\%$ reduction in the peak α_{1A-a} 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 $\sim 100\%$ increase in the degree of fomocaine block ($n = 5$; Fig. 5*C*). Because the level of fomocaine block stabilizes rapidly (Fig. 2*A*), the enhanced block is unlikely attributable to longer exposure to fomocaine. Furthermore, nickel block is completely reversible (Fig. 5*D*) 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 β_{2a} subunit on fomocaine block. Whole-cell currents were recorded from α_{1Aa} (A), α_{1E} (B), and α_{1C} (C) channels coexpressed with α_2 and β_{2a} . Coexpression of α_1 subunits with β_{2a} 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 β_{1b} , β_3 , and β_4 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 α_{1Aa} with four different neuronal β subunits (β_{1b} , β_{2a} , β_3 , and β_4). There were no discernible differences between fomocaine block of α_{1Aa} channels in the absence of a β subunit or when coexpressed with either β_{1b} , β_3 , or β_4 (Fig. 6). In contrast, coexpression with β_{2a} significantly decreased the blocking affinity of fomocaine. Similar decreases in fomocaine block were also observed when α_{1E} and α_{1C} subunits were coexpressed with β_{2a} (Fig. 6). The β_{2a} 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 affects on inactivation properties, steady-state inactivation profiles were determined in the presence and absence of fomocaine. Fomocaine application did not significantly affect ($V_{h(\text{control})} = -61.1 \pm 2.9$ mV, $V_{h(\text{fomocaine})} = -60 \pm 3.6$ 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 β_{2a} on fomocaine block does not arise secondarily from removal

of voltage-dependent inactivation. This notion is supported by the observation that α_{1C} shows little voltage-dependent inactivation regardless of the type of β subunit coexpressed; however, the β_{2a} still reduced the fomocaine blocking affinity (see Fig. 6).

Amino acid substitutions in the α_1 subunit domain I–II linker affect fomocaine block

Recently, several variants of the α_{1Aa} subunit that result from alternative splicing have been identified (Fig. 8A; Soong and Snutch, unpublished observations). The α_{1Ab} isoform differs from α_{1Aa} 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 α_{1Ac} isoform differs from α_{1Aa} 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 α_{1Ab} variant that differed from α_{1Aa} only by the valine insertion (Soong and Snutch, unpublished observations) showed that 600 μM fomocaine produced significantly less block ($17 \pm 2\%$, $n = 13$) compared with α_{1Aa} (Fig. 8B,E). The α_{1Ac} , when coexpressed with $\alpha_2 + \beta_4$, exhibits kinetic

Figure 7. Steady-state inactivation profile recorded from $\alpha_{1A-a} + \beta_4 + \alpha_2$ in the absence and the presence of 600 μM fomocaine in 2 mM barium ($n = 5$). Fomocaine does not significantly affect the steady-state inactivation properties of the channel. The curves were fitted as outlined in Materials and Methods. The voltage at which half of the channels were inactivated was -60.1 mV in the absence and -59.6 mV in the presence of fomocaine.

and voltage-dependent properties essentially identical to those observed for $\alpha_{1A-a} + \alpha_2 + \beta_4$ (Soong and Snutch, unpublished observations). However, as shown in Figure 8, fomocaine block of α_{1A-c} was significantly less pronounced than block of α_{1A-a} ($20 \pm 2\%$, $n = 7$). These data, together with the fact that the region for β subunit binding has been localized to the I–II linker (Pragnell et al., 1994), suggest that a common mechanism may underlie the antagonistic effects of β_{2a} and amino acid substitutions in position 471/472 on fomocaine block.

To further investigate this possibility, we tested a chimeric channel in which the I–II loop of α_{1B} replaced that of α_{1A-a} (Stea et al., 1995). Figure 8*D* illustrates that the chimeric channel exhibits only $\sim 15\%$ block by 600 μM fomocaine, a concentration that produces almost 40% block of α_{1A-a} . The degree of block observed with the chimera did not significantly differ from that for the α_{1B} complex (Fig. 8*E*), suggesting that insertion of the I–II loop of α_{1B} into α_{1A-a} is sufficient to confer the reduced fomocaine sensitivity of α_{1B} onto α_{1A} .

The observation that a single glycine deletion or valine insertion is sufficient to affect fomocaine block, together with the data obtained from the α_{1A} – α_{1B} chimeric channel, the β subunit dependence of block, and the suggestion for a cytoplasmic location of the fomocaine blocking site, is consistent with the notion of a direct involvement of the I–II loop in fomocaine action. The data are summarized in Figures 6*D* and 8*E*. Coexpression with β_{2a} shows significant effects on fomocaine block of each α_{1A-a} , α_{1E} , and α_{1C} , but the complete absence of a β subunit does not significantly affect fomocaine block. Furthermore, an amino acid change at position 472 reduces the drug affinity by $\sim 50\%$, with little additional effect of β_{2a} . In contrast, block by procaine shows a different profile regarding subtype specificity, amino acid substitutions and effects of β subunits. For example, the degree of

block observed with α_{1A-b} did not differ significantly from α_{1A-a} in combination with any of the β 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 ~ 10 -fold higher affinity (Fig. 9*A–D*) and a slower development (equilibration after ~ 3 min). Penfluridol block was not reversible after washout as with fomocaine (not shown). Penfluridol exhibited a similar subtype specificity as fomocaine, blocking $\alpha_{1E} + \beta_{1b} + \alpha_2$ with an IC_{50} of 13 μM ($n = 6$) $> \alpha_{1C} + \beta_{1b} + \alpha_2$ (47 μM , $n = 4$) $> \alpha_{1A-a} + \beta_4 + \alpha_2$ (127 μM , $n = 8$) $> \alpha_{1A-a} + \beta_{1b} + \alpha_2$ (117 μM , $n = 3$) $> \alpha_{1B} + \beta_{1b} + \alpha_2$ (400 μM , $n = 3$). Penfluridol block of α_{1A-a} was significantly reduced by β_{2a} coexpression (1170 μM , $n = 9$). Amino acid substitutions in the α_{1A} domain I–II linker also significantly reduced penfluridol block as for fomocaine (Fig. 9*E–H*; $\alpha_{1A-b} + \beta_4 + \alpha_2$: 488 μM , $n = 8$; $\alpha_{1A-c} + \beta_4 + \alpha_2$: 334 μM , $n = 7$). Coapplication of fomocaine and penfluridol to α_{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 BTZs 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; Galizzi 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 fmocaine on amino acid substitutions in the domain I–II linker. *A*, Primary structure of the domain I–II linker of α_1 subunit variants (Soong et al., 1995). Whole-cell currents were recorded in 2 mM barium from α_{1Aa} - β_4 + α_2 (*B*), α_{1Ac} - β_4 + α_2 (*C*), and a chimeric channel in which the I–II loop of α_{1B} is inserted into α_{1Aa} (*D*). α_{1Ac} and α_{1Ab} channels are identical to α_{1Aa} channels except for a single glycine deletion (α_{1Ac}) or a single valine insertion (α_{1Ab}) in position 472. Note that α_{1Ac} exhibits activation and inactivation properties similar to that for α_{1Aa} , whereas α_{1Ab} channels exhibit little inactivation. Both α_{1Ac} and α_{1Ab} exhibit a reduced sensitivity to fmocaine block compared with α_{1Aa} . The chimeric channel exhibits a reduced blocking sensitivity that is comparable with that seen with the intact α_{1B} 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, $\alpha 1E$ is the most significantly affected subtype (D), followed by $\alpha 1C$ (C), $\alpha 1Aa$ (A), and $\alpha 1B$ (B). Coexpression of $\alpha 1Aa$ with $\beta 2a$ (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. Fmocaine 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, coapplication 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

Fmocaine preferentially blocks α_{1E} with an IC_{50} of 95 μM , whereas α_{1C} , α_{1A-a} , and α_{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 α_{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 information is available concerning the blocking action of fomocaine on native calcium channels. Fmocaine has been previously reported to exhibit pronounced antiarrhythmic properties at concentrations as high as 30 μM (Braeunig et al., 1989), which is similar to clinically used blood plasma concentrations of a range of antiarrhythmic agents (Sheldon et al., 1987). At this concentration, ~25% of the calcium current through α_{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 μM concentrations; Yamashita et al., 1995). The structurally related bupivacaine has been reported to block cardiac calcium channels with IC_{50} s between 100 and 300 μM (Sanchez-Chapula, 1988; Wulf et al., 1994) and N-type calcium channels from bullfrog sensory ganglion cells with an IC_{50} of ~50 μM (Guo et al., 1992). For comparison, between 1 and 25 μM bupivacaine is required to block 50% of the currents through cardiac and neuronal sodium channels (Clarkson and Hondeghem, 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 Hondeghem, 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 mM to 2.5 mM (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 block 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 $\text{pK}_a = 9.0$; Ritchie and Greengard, 1961). Furthermore, procaine block was mimicked by, and competitively inhibited by membrane-impermeant diethylcarbazine, 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 α_1 subunits with β_{2a} significantly reduces the degree of fomocaine and penfluridol block of α_{1A-a} , α_{1C} , and α_{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 α_{1B} into α_{1A-a} resulted in block consistent with that seen with α_{1B} . 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 α_1 subunits with β_{2a} and the insertion of a valine residue in position 472 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 α_{1A-a} and α_{1A-c} (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 β_{2a} 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 ~15–20% block for any of the various combinations of α_1 and β subunits. Although far-reaching β subunit effects on calcium channel pharmacology have been reported (i.e., an increased affinity for the external blockers ω -conotoxin and some DHPs when α_1 subunits were coexpressed with a β subunit compared with expression of α_1 alone; Williams et al., 1992a,b; Nishimura 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 β_{2a} but not after binding of any of the other β subunits studied. Although there is a substantial degree of homology between the different β subunits especially in the regions that are involved in binding of the β subunit to the α_1 subunit, (De Waard et al., 1994; Pragnell et al., 1994), there are significant structural differences between β_{2a} and other types of β subunits. One could envision a scenario in which a unique region on the β_{2a} 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 stretch of amino acids at the β subunit N terminus region (not directly involved in binding to the α subunit) was critical for the effects of β_{2a} on 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.

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