Local Anesthetics Noncompetitively Inhibit Function of Four Distinct Nicotinic Acetylcholine Receptor Subtypes

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ABSTRACT

Local anesthetics (LAs) are considered to act primarily by inhibiting voltage-gated Na⁺ channels. However, LAs also are pharmacologically active at other ion channels including nicotinic acetylcholine receptors (nAChR). nAChR exist as a family of diverse subtypes, each of which has a unique pharmacological profile. The current studies established effects of LAs on function of four human nAChR subtypes: naturally expressed muscle-type (α1*-nAChR) or autonomic (α3β4*-nAChR) nAChR, or heterologously expressed nAChR containing α4 with either β2- or β4-subunits (α4β2- or α4β4-nAChR). Of the LAs tested, those with structures containing two separated aromatic rings (e.g., proadifen and adiphenine) had the greatest inhibition potency (IC₅₀ values between 0.34 and 6.3 μM) but lowest selectivity (~4-fold) across the four nAChR subtypes examined. From the fused, two-ring (isoquinoline backbone) class of LAs, dimethisoquin had comparatively moderate inhibition potency (IC₅₀ values between 2.4 and 61 μM) and ~30-fold selectivity across nAChR subtypes. Lidocaine, a commonly used LA from the single ring category of LAs, blocked nAChR function with IC₅₀ values of between 52 and 250 μM and had only ~5-fold selectivity across nAChR subtypes. Its quaternary triethyl ammonium analog, QX-314, had greater inhibition potency, but the trimethyl ammonium derivative, QX-222, was the least potent LA at all but the α4β2-nAChR subtype. With only a few exceptions, LA effects were consistent with noncompetitive inhibition of nAChR function and occurred at therapeutic doses. These studies suggest structural determinants for LA action at diverse nAChR subtypes and that nAChR likely are clinically relevant targets of LAs.

LAs block nerve conduction in the peripheral nervous system (Arias, 1999). They also have a wide range of behavioral effects implicating actions in the central nervous system (CNS). Among these effects are restlessness, euphoria, muscle twitching, and tremor, which have been attributed to selective depression of inhibitory neurons by LAs. Other effects of LAs suggesting CNS actions include drowsiness, disorientation, slurred speech, respiratory depression, tinnitus, and sedation. At high concentrations, LAs may cause loss of consciousness or even death (Naguib et al., 1998; Hodgson et al., 2000).

LAs also differ from one another in several ways, and different bases have been used to classify LAs. LAs have been classified into three categories by Arias (1999). Agents from one category of LAs (group I; see Fig. 1), typified by tetra- caine, procaine and lidocaine, possess only one aromatic ring. An amide (lidocaine) or an ester (tetracaine and procaine) linkage couples the ring to one aliphatic chain that typically ends in a ternary amino group (tetracaine, procaine, and lidocaine) or a quaternary ammonium ion (the charged lidocaine analog QX-314 or the dimethylammonio variant QX-222). The group I esters also have a second amino (procaine) or alkylamino (butylamino for tetracaine) chain para to the ester-linked alkylamine chain. The group I amides include compounds (lidocaine, QX-314, and QX-222) having two methyl groups ortho to the alkylamine or alkylammonio chain. Thus, group I compounds may be subdivided into two subgroups representing para-(alkyl)amino-phenyl-alkylesters and ortho-dimethyl-phenyl-alkylamides. A second category of LAs (group II; see Fig. 1) includes molecules with two aromatic rings separated and linked by a single α-carbon chain and is typified by proadifen and adiphenine. Group II LAs also have an ester linking the ring region through the α-carbon to an aliphatic chain that ends in a ternary amino group (proadifen and adiphenine) or in a quaternary ammonium ion (charged proadifen derivative meproadifen; not shown). In some cases, group II LAs have an additional alkyl...
Molecular Structures of LA tested

II. proadifen

II. adiphenine

III. dimethisoquin

I. tetracaine

I. QX-314

I. lidocaine

I. procaine

I. QX-222

Fig. 1. Molecular structures of LAs used in this study. Names for each LA and their position according to the classification scheme of Arias (1999) in groups I, II, or III are shown for each structure [note however that dimethisoquin, proadifen, and adiphenine are ternary amino ligands rather than quaternary ammonium ions as Fig. 2 in Arias (1999) may implicate]. Structures are listed left top-to-bottom and then right top-to-bottom in the rank order, with some minor exceptions, observed for actions at nAChR. See the text, Figs. 2 through 9, and Table 1 for experimental details.

chain (propyl for proadifen) linked to the α-carbon bridging the two aromatic rings. A third category (group III; see Fig. 1), represented by dimethisoquin, contains drugs having a fused, two-ring structure (i.e., an isoquinoline backbone). An aliphatic chain ending again in either a ternary amino group (dimethisoquin) or in a quaternary ammonium ion (charged dimethisoquin analog trimethisoquin; not shown) is coupled via an ester linkage to the isoquinoline ring, which is further
derivatized with an alkyl chain (butyl for dimethisoquin) meta to the ester link.

Whereas peripheral effects of LAs are attributed to actions on voltage-sensitive Na⁺ channels, mechanisms of LA action on presumably CNS-mediated behaviors are not well understood. Ionotropic neurotransmitter receptors such as cholinergic and serotoninergic receptors have been investigated as potential targets of LA action in the brain (Katz and Mileti, 1975; Neher and Steinbach, 1978; Lukas and Bennett, 1979; Horn et al., 1980; Forman and Miller, 1989; Charnet et al., 1990; Revah et al., 1991; Barann et al., 1993; Dilger and Vidal, 1994; Pan and Weight, 1994).

Nicotinic acetylcholine receptors (nAChR) are neurotransmitter-gated ion channels. At least 16 distinct genes encode nAChR subunits that combine in a variety of ways to form diverse, pentameric nAChR ion channels (for review, see Lukas, 1998). Some physiological roles of diverse nAChR subtypes are known whereas others remain incompletely defined (Albuquerque et al., 1996, 2000). However, each nAChR subtype has a unique pharmacological profile, and this may also translate into differences in sensitivity to LAs (Dani, 1993; Eterovic et al., 1993; Cuevas and Adams, 1994). Conversely, nAChR may prove to contribute to clinically relevant therapeutic actions and/or side effects of LAs.

This study assessed effects of several members from the three categories of LAs on function of four different, human nAChR subtypes. A preliminary report of these findings has appeared (Gentry et al., 2000).

### Materials and Methods

**Drug Dilutions.** All drugs were prepared fresh the day of the assay as stock solution in as much as 50% ethanol depending on the drug and then diluted so that final concentrations of ethanol were no higher than 5% in assay buffer (130 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂·2H₂O, 5 mM glucose, and 50 mM HEPES) at the highest concentrations of LA used in each experiment. Ethanol alone up to 50% in assay buffer had no effects on function of any of the examined nAChR subtypes during a 3-min exposure (data not shown). Ethanol, α-phenylbenzeneacetic acid-2-(diethylamino)ethyl ester HCl (adiphenine), carbamylcholine chloride (carb), 2-diethylamino-(2,6-dimethylphenyl)acetamide HCl (lidocaine), α-phenyl-α-propylbenzeneacetic acid-2-(diethylamino)ethyl ester HCl (proadifen; SKF-525A), 4-aminobenzoic acid-2-(diethylamino)ethyl ester HCl (procarbazine), and 4-(butylamino)benzoic acid 2-(dimethylamino)ethyl ester HCl (tetracaine) were purchased from Sigma (St. Louis, MO). 3-Butyl-1-[2-(dimethylamino)ethoxy] isoquinoline HCl (3-butyl-1-(2-diethylamino)ethoxy) isouquinoline HCl (dimethisoquin) was purchased from ICN Biomedicals, Inc. (Plainview, NY) (discontinued; now available from Research Diagnostics, Inc., Flanders, NJ).

**Model Cell Lines and Cell Culture.** The present study used low passage (less than 50) human cell lines naturally or heterologously expressing different nAChR subtypes to examine inhibition potencies of LAs. The TE671/RD human cell line naturally expresses muscle-type nAChR (α1*-nAChR according to suggested nomenclature) (Lukas et al., 1999) as an assembly of two α1-subunits and one each of β1-, δ-, and γ-subunits (Lukas, 1988, 1989; Schoepfer et al., 1988; Luther et al., 1989). Human neuroblastoma-derived SH-SY5Y cells naturally express two nAChR subtypes found in autonomic neurons. However, the subtype that contains δ- and β4-subunits (α3β4*-nAChR) dominates ion flux assay measures of nAChR function in these cells (Lukas, 1993; Lukas et al., 1993; Conroy and Berg, 1995). nAChR containing α4- and β2-subunits (α4β2-nAChR) are a major form of high affinity nicotine binding site in mammalian brain, and nAChR containing α4- and β4-subunits (α4β4-nAChR) may also be expressed substantially in the CNS. Human α4β2- and α4β4-nAChR were expressed stably and heterologously in native nAChR-null SH-EP1 human epithelial cells to create SH-EP1-ho4β2 and SH-EP1-ho4β4 cell lines, respectively, using techniques that have been reported previously (Peng et al., 1999; Eaton et al., 2000; Ferchmin et al., 2001). Cells were maintained at 37°C, under 95% O₂/5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 10% horse serum (Invitrogen, Carlsbad, CA), 1% sodium pyruvate (Cellgro; AK by Mediatech Inc., Herndon, VA), 2% glutamine penicillin-streptomycin (Invirogene, Santa Ana, CA) and 0.02% amphotericin B (Sigma).

**Assays of nAChR Function.** Rubidium-86 (⁸⁶Rb⁺) efflux assays were performed using TE671/RD, SH-SY5Y, SH-EP1-ho4β2, or SH-EP1-ho4β4 cells according to the procedure of Lukas and Cullen (1988). Cells were cultured (~2 × 10⁵ cells per 15.5-mm-diameter well; ~150 µg of total cell protein per well) on Falcon 24-well culture plates (BD Biosciences, Bedford, MA) precoated with poly-γ-lysine (M₇₀, 70,000 to 150,000 (Sigma) according to Benchref et al. (1995)). Cells were allowed to grow (overnight) until a confluent monolayer
was formed and cells adhered to culture plate. Confluence was monitored using light microscopy. $^{86}$Rb$^+$ was obtained from PerkinElmer Life Sciences (Boston, MA). In all cases, cells were loaded with $^{86}$Rb$^+$ for no less than 4 h and subsequently rinsed twice with 2 ml per well of drug-free assay buffer. Cells expressing each nAChR subtype of interest were exposed to efflux buffer alone or experimental concentrations. Data points are from three or more independent experiments (mean ± S.E.M.). Smooth curves drawn through the data points are from iterative fits to the logistic function ($b = -0.26$ to 5.1%; see Materials and Methods). Values for the parameter $a$ (as a percentage of specific $^{86}$Rb$^+$ efflux) and $r^2$ values (in parentheses) are $103.7 ± 4.0\%$ (0.90) for untreated control, $76.3 ± 2.9\%$ (0.97) for 1 $\mu$M adipine, $63.2 ± 3.3\%$ (0.95) for 2.5 $\mu$M adipine, $41.6 ± 3.3\%$ (0.90) for 5 $\mu$M adipine, $72.4 ± 0.9\%$ (0.99) for 1 $\mu$M dimethisioquin, $48.7 ± 1.5\%$ (0.99) for 3 $\mu$M dimethisioquin, $29.5 ± 1.2\%$ (0.97) for 10 $\mu$M dimethisioquin, $78.4 ± 2.2\%$ (0.99) for 3 $\mu$M tetracaine, $47.5 ± 2.2\%$ (0.98) for 10 $\mu$M tetracaine, $21.8 ± 1.8\%$ (0.94) for 32 $\mu$M tetracaine, $84.6 ± 0.8\%$ (0.99) for 32 $\mu$M procaine, $64.5 ± 1.4\%$ (0.99) for 100 $\mu$M procaine, and $36.1 ± 1.4\%$ (0.98) for 316 $\mu$M procaine. None of the carb dose-response profiles displayed any significant shift from EC$_{50}$ value of control (39 $\mu$M ×/− 0.05).

**Fig. 3.** Dose-response profiles for carbamylcholine-stimulated $^{86}$Rb$^+$ efflux through $\alpha_1$-nAChR at different concentrations of LA. Measurements of specific $^{86}$Rb$^+$ efflux (ordinate, percentage of 1 mM carbamylcholine control) were made with TE671/RD cells assayed in the presence of carb alone (○) at the indicated doses (absissa, molar log scale) or in the presence of carb with adipine, dimethisioquin, tetracaine, or procaine at indicated concentrations. Data points are from three or more independent experiments (mean ± S.E.M.). Smooth curves drawn through the data points are from iterative fits to the logistic function ($b = -0.26$ to 5.1%; see Materials and Methods). Values for the parameter $a$ (as a percentage of specific $^{86}$Rb$^+$ efflux) and $r^2$ values (in parentheses) are $103.7 ± 4.0\%$ (0.90) for untreated control, $76.3 ± 2.9\%$ (0.97) for 1 $\mu$M adipine, $63.2 ± 3.3\%$ (0.95) for 2.5 $\mu$M adipine, $41.6 ± 3.3\%$ (0.90) for 5 $\mu$M adipine, $72.4 ± 0.9\%$ (0.99) for 1 $\mu$M dimethisioquin, $48.7 ± 1.5\%$ (0.99) for 3 $\mu$M dimethisioquin, $29.5 ± 1.2\%$ (0.97) for 10 $\mu$M dimethisioquin, $78.4 ± 2.2\%$ (0.99) for 3 $\mu$M tetracaine, $47.5 ± 2.2\%$ (0.98) for 10 $\mu$M tetracaine, $21.8 ± 1.8\%$ (0.94) for 32 $\mu$M tetracaine, $84.6 ± 0.8\%$ (0.99) for 32 $\mu$M procaine, $64.5 ± 1.4\%$ (0.99) for 100 $\mu$M procaine, and $36.1 ± 1.4\%$ (0.98) for 316 $\mu$M procaine. None of the carb dose-response profiles displayed any significant shift from EC$_{50}$ value of control (39 $\mu$M ×/− 0.05).

**TABLE 1**

Parameters for functional nAChR block by LA

<table>
<thead>
<tr>
<th>Protein</th>
<th>$IC_{50}$ μM (×/− S.E.M.)</th>
<th>$a$</th>
<th>$a_{34}$</th>
<th>$a_{48}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipine</td>
<td>1.9 (1.1)</td>
<td>1.8 (1.3)</td>
<td>3.7 (1.3)</td>
<td>6.3 (1.2)</td>
</tr>
<tr>
<td>Dimethylisoquin</td>
<td>2.4 (1.5)</td>
<td>4.7 (1.1)</td>
<td>61 (1.2)</td>
<td>7.2 (1.1)</td>
</tr>
<tr>
<td>Tetracaine</td>
<td>13 (1.1)</td>
<td>8.3 (1.2)</td>
<td>27 (1.1)</td>
<td>30 (1.1)</td>
</tr>
<tr>
<td>QX-314</td>
<td>19 (1.5)</td>
<td>9.2 (1.2)</td>
<td>68 (1.3)</td>
<td>64 (1.1)</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>52 (1.1)</td>
<td>63 (1.4)</td>
<td>190 (1.5)</td>
<td>250 (1.2)</td>
</tr>
<tr>
<td>Procaine</td>
<td>230 (1.4)</td>
<td>87 (1.2)</td>
<td>2100 (5.2)</td>
<td>1030 (4.6)</td>
</tr>
<tr>
<td>QX-222</td>
<td>&gt;3400</td>
<td>150 (1.4)</td>
<td>390 (1.4)</td>
<td>&gt;2500</td>
</tr>
</tbody>
</table>

* Rank order inhibition potency not in agreement with muscle-type nAChR results.
trations of LA and/or carbamylcholine for 3 min. Radioactivity released into the extracellular medium was quantified by Cerenkov counting using a Wallac TriLux system (40% efficiency). Levels of nonspecific ion flux from each cell line were comparable whether defined with samples containing agonist plus antagonist 100 μM d-tubocurarine or with control samples containing buffer alone without agonist. Specific nAChR function was defined as total, experimentally determined ion flux evoked by agonist carb in the absence or presence of LA minus nonspecific ion flux. Typical values for specific and nonspecific 86Rb+ efflux for cell samples are 9000 and 1200 cpm for TE671/RD cells, 2900 and 900 cpm for SH-SY5Y cells, 3500 and 600 cpm for SH-EP1-h4/32 cells, and 9000 and 800 cpm for SH-EP1-h4/34 cells.

Data Analysis. Dose-response curves were fit to data points by the general equation \( Y = b + [(a - b) / 1 + 10^{(n \cdot X)}] \) where \( Y \) is the observed specific 86Rb+ efflux response (% of control), \( X \) is the experimental concentration of LA, \( b \) is nonspecific flux, \( a \) is total ion flux (1 mM carb control), \( c \) is the log IC50 value for antagonist dose-response profiles at fixed agonist concentration or the log EC50 value for agonist dose-response profiles at fixed agonist concentration, and \( n \) is the Hill coefficient (<0 for antagonist dose-response profiles; >0 for agonist dose-response profiles). Best fit, nonlinear regression variable slope curves were determined by an iterative process using GraphPad Prism (San Diego, CA) software, from which values of \( a \), \( b \), \( c \), and \( n \) were derived for each experiment. Values of \( a \) and \( b \) parameters were normalized to percentage of control flux. Because LA acted as noncompetitive inhibitors in nearly all cases, there was no dependence on concentration of agonist used to stimulate nAChR function for LA IC50 values and there was no need to apply modifications, such as the Cheng-Prusoff correction, to the data. Results from three to 16 independent measurements were fit to the logistic equation and plotted as mean ± S.E.M.

Results

Acute Effects of LAs on α1*-nAChR Function. 86Rb+ efflux assays using the TE671/RD cell line expressing muscle-type α1*-nAChR were used to evaluate receptor function during acute exposure to selected LAs. Simultaneous exposure of cells to increasing concentrations of LA and 1 mM carb indicated that all LAs inhibited α1*-nAChR function in a concentration-dependent manner (Fig. 2). Concentrations at which half-maximal block of TE671/RD cell α1*-nAChR function were achieved ranked ordered from high to low inhibition potency were (IC50 value precedes drug’s name): 0.34 μM proadifen > 1.9 μM adiphenine > 2.4 μM dimethisoquin > 13 μM tetracaine > 19 μM QX-314 > 52 μM lidocaine > 230 μM procaine ≈ 3.4 mM QX-222 (Table 1). Thus, representative group II LAs exhibited greatest inhibitory potency at α1*-nAChR followed by group III LAs, with group I LAs exhibiting the lowest inhibitory potency (Fig. 1). Hill coefficients for antagonist concentration-response profiles ranged between −0.74 proadifen to −1.6 for lidocaine (Table 1).

Subsequent experiments were completed in which carbamylcholine concentration-response profiles were obtained at fixed concentrations of LAs near to and encompassing their IC50 values for inhibition of 1 mM carb response. Representative LAs from each of the structural categories were used for these studies. Group I was represented by tetracaine and procaine; group II was represented by adiphenine; and group III was represented by dimethisoquin. For each of the LAs tested, functional block produced by near IC50 concentrations was insurmountable by increasing the concentration of carb, suggesting a noncompetitive mechanism for functional inhibition of α1*-nAChR (Fig. 3).

Acute Effects of LAs on α3β4*-nAChR Function. 86Rb+ efflux assays using SH-SY5Y cells expressing α3β4*-nAChR showed concentration-dependent functional blockade by all LAs tested (Fig. 4). Concentrations at which half-maximal block (IC50) of SH-SY5Y cell α3β4*-nAChR function was achieved were (by rank order: high to low inhibition potency): 0.6 μM proadifen > 1.8 μM adiphenine > 4.7 μM dimethisoquin > 8.3 μM tetracaine ≈ 9.2 μM QX-314 > 63 μM lidocaine > 87 μM procaine > 150 μM QX-222 (Table 1). Thus, the general rank order inhibition potency of LAs observed in the TE671/RD cell line (group II > group III > group I) held for α3β4*-nAChR expressed by the SH-SY5Y cell line (Figs. 2 and 4; Table 1). Hill slopes ranged between −0.78 for proadifen and −1.23 for lidocaine.

Carb dose-response profiles at increasing concentrations of LAs revealed that functional block of α3β4*-nAChR produced by each of the representative LAs was consistent with a noncompetitive mechanism of functional inhibition (Fig. 5).
Acute Effects of Drugs on α4β2- or α4β4- nAChR Function. Acute effects of selected LAs on function of nAChR containing α4-subunits were also evaluated by 86Rb⁺ efflux assay. SH-EP1 cells in which α4β2- or α4β4- nAChR were expressed heterologously were exposed simultaneously to test concentrations of LA and 1 mM carb. All LAs inhibited α4*-nAChR function in a concentration-dependent manner with one exception (Figs. 6 and 7). The quarternary LA, QX-222, at a concentration of 2.5 mM only weakly inhibited 1 mM carb-evoked response mediated by α4β4-nAChR (Fig. 7). The rank order inhibition potency for LAs tested at α4β4-nAChR was identical with that for α1*- or α3β4*-nAChR and was (IC₅₀ value precedes drug name): 1.5 μM proadifen > 6.3 μM adipine = 7.2 μM dimethisoquin > 30 μM tetracaine > 64 μM QX-314 > 250 μM lidocaine > 1.0 mM procaine > 2.5 mM QX-222 (Table 1). Interestingly, the rank order inhibition potency for LAs at α4β2-nAChR was somewhat unique. Rank order and IC₅₀ values for LA inhibition of α4β2-nAChR function were: 2.0 μM proadifen > 3.7 μM adipine > 27 μM tetracaine > 61 μM dimethisoquin = 68 μM QX-314 > 190 μM lidocaine > 390 μM QX-222 > 2.1 mM procaine (Fig. 6; Table 1). Thus, in general, group II LAs (adipine and proadifen) exhibited the greatest ability to inhibit carb-evoked α4*-nAChR function, whereas group I compounds exhibited weaker ability to inhibit. However, the group III compound has an inhibition potency for α4β2-nAChR within the range of potencies tested for group I compounds. Moreover, the diethylamino group I ligand, lidocaine, its trimethylammonium derivative, QX-314, and the trimethylammonium analog, QX-222, had the most similar inhibitory capability for α4β2-nAChR compared with other nAChR subtypes (Figs. 1 and 6; Table 1). Hill slopes were shallowest for proadifen (−0.73 to −0.94) and steepest for tetracaine (−1.24 to −1.61) at α4*-nAChR (Table 1).

Concentration-response profiles for carb-evoked efflux exhibited both right- and downward shifts with increasing LA concentration suggesting that LAs tested inhibit α4β2-nAChR-mediated responses via a combination of noncompetitive and competitive mechanisms (Fig. 8). Carb concentration-response profiles at antagonist concentrations fixed near their respective IC₅₀ value suggested that most of the representative LAs produce functional block of α4β4-nAChR via a noncompetitive mechanism (Fig. 9). The exception is for procaine, for which a rightward shift in the agonist dose-re-
three separate experiments) are from iterative fits to the logistic function

\[ \text{Smooth curves drawn through data points (means} \pm \text{S.E.M. from at least three separate experiments) are from iterative fits to the logistic function } \]

\[ (a = 92 \text{ to } 101\% ; \text{ see Materials and Methods). Results yield } IC_{50} \text{ values, Hill coefficients, and } r^2 \text{ values indicated in the text and/or in Table 1. Values for the parameter } b \text{ as a percentage of control } ^{86}\text{Rb}^+ \text{ efflux are } 5.3 \pm 6.8\% \text{ for proadifen, } 16.5 \pm 5.1\% \text{ for dimethisoquin, } -4.1 \pm 7.7\% \text{ for QX-314, } n/a \text{ for procaine, } -12.2 \pm 4.9\% \text{ for adiphene}, 2.3 \pm 2.5\% \text{ for tetracaine, } -3.1 \pm 20\% \text{ for lidocaine, and } n/a \text{ for QX-222.} \]

Discussion

The primary findings of this study are: 1) that LAs have reasonably strong ability to inhibit diverse, human nAChR subtypes (\(\alpha_1\)-, \(\alpha_3\beta_4\)-, \(\alpha_4\beta_2\)-, and \(\alpha_4\beta_4\)-nAChRs), and 2) that, with a few exceptions, LAs act at these nAChR subtypes as noncompetitive functional inhibitors. Two exceptions to the noncompetitive inhibition rule are for LAs acting at \(\alpha_4\beta_2\)-nAChR and for procaine acting at \(\alpha_4\beta_4\)-nAChR. Although LAs can be classified in several ways, our data regarding LA function at nAChR are well segregated based on the structural classification for LAs as put forth by Arias (1999) (represented in Fig. 1). Although the sample size of LAs representing each structural category is limited, the data serve as a foundation for studies of LA-nAChR structure-activity relationships.

Group II LAs proadifen and adiphene were the most potent functional inhibitors at all nAChR tested, with proadifen having 2- to 6-fold greater inhibition potency across nAChR subtypes than adiphene (see Table 1). The only structural difference that may account for the greater inhibition potency of nAChR for proadifen is its additional propyl moiety linked to the \(\alpha\)-carbon (see Fig. 1).

In general, the group III ligand built on the butyl-isouquinoline backbone, dimethisoquin, had the next greatest ability to inhibit each nAChR subtype tested (see Table 1). Dimethisoquin, like the group II LAs, has a two carbon chain separating amino from ester moieties on one aliphatic chain (see Fig. 1). However, dimethisoquin is a dimethylamino rather than a diethylamino compound, suggesting that presence of the more compact amino group may contribute to lower inhibition potency for nAChR.

Group I drugs generally have the lowest inhibition potencies of the four nAChR subtypes examined. Tetracaine, which has a dimethylamino residue ending its ester-linked aliphatic chain but also has a markedly longer \(\alpha\)-amino substituent, consistently has \(\geq\)10-fold greater potency for the nAChR subtypes tested than the diethylamino, \(\alpha\)-amino ligand, procaine (see Table 1 and Fig. 1). The ability of
SH-EP $\alpha_4\beta_2$ cells

Fig. 8. Dose-response profiles for carb-stimulated $^{86}$Rb$^+$ efflux through $\alpha$4$\beta$2-$\alpha$ChR at different concentrations of LA. Measurements of specific $^{86}$Rb$^+$ efflux (ordinate, percentage of 1 mM carb control) were made with SH-EP1-h4$\alpha$2$\beta$2 cells assayed in the presence of carb alone (C) at the indicated doses (abscissa, molar log scale) or in the presence of carb with adphenine, dimethisoquin, tetracaine, or procaine at indicated concentrations. Data points are from three or more independent experiments (mean $\pm$ S.E.M.). Smooth curves drawn through the data points are from iterative fits to the logistic function ($b = -3.7$ to 1.2%; see Materials and Methods). Values for the parameter $a$ (as a percentage of specific $^{86}$Rb$^+$ efflux), and $r^2$ values (in parentheses) are 100.8 $\pm$ 1.4% (0.98) for untreated control, 47.2 $\pm$ 3.0% (0.81) for 1 $\mu$M adphenine, 36.9 $\pm$ 2.5% (0.83) for 3 $\mu$M adphenine, 22.1 $\pm$ 2.6% (0.70) for 6 $\mu$M adphenine, 54.1 $\pm$ 0.7% (0.99) for 32 $\mu$M dimethisoquin, 40.0 $\pm$ 3.2% (0.82) for 100 $\mu$M dimethisoquin, 13.2 $\pm$ 1.6% (0.95) for 320 $\mu$M dimethisoquin, 65.4 $\pm$ 1.5% (0.97) for 10 $\mu$M tetracaine, 44.0 $\pm$ 1.1% (0.98) for 32 $\mu$M tetracaine, 6.9 $\pm$ 0.3% (0.92) for 100 $\mu$M tetracaine, 85.4 $\pm$ 1.9% (0.98) for 100 $\mu$M procaine, 71.6 $\pm$ 2.9% (0.95) for 320 $\mu$M procaine, and 27.4 $\pm$ 1.3% (0.94) for 1000 $\mu$M procaine. All of the carb dose-response profiles produced a shift in EC$_{50}$ value from (19 $\mu$M $\pm$ 0.03) for control to higher values (up to $\sim$400 $\mu$M $\pm$ 0.08) in the presence of LAs.

tetracaine to inhibit all $\alpha$ChR subtypes tested is only 4- to 8-fold greater than that of the diethylamino amide lidocaine. The quaternary, trimethylammonium amide QX-314 generally has greater (up to 7-fold) inhibition potency for $\alpha$ChR than does the ternary, diethylamino analog lidocaine, but QX-314 has much greater inhibition potency than does the more compact quaternary, trimethylammonium amide QX-222. Thus for group I esters, presence of a diethylamino (procaine) moiety rather than a more compact dimethylamino (tetracaine) group and perhaps the shortened length of the para-(alkyl)amino aliphatic chain correlates with diminished potency for $\alpha$ChR (see Fig. 1). However, for group I amides, the diethylammonium (QX-314) has greater inhibition potency than does the more compact dimethylammonium (QX-222). The diethylammonium (QX-314) also has greater inhibition potency than does the diethylamino (lidocaine) compound. These findings indicate that structural determinants of LA ability to inhibit $\alpha$ChR include bulk at the amino/ammonio moiety. However, these findings also indicate that these structural determinants differ between group I esters and amides, suggesting that ligands in group I should be further subclassified.

Of the $\alpha$ChR subtypes tested, muscle-type $\alpha_1^*$- and ganglionic $\alpha_3\beta_4^*$-$\alpha$ChR naturally expressed in the periphery each were generally more sensitive to block by LAs than were CNS-type $\alpha_4\beta_2$- or $\alpha_4\beta_4$-$\alpha$ChR. $\alpha_1^*$- and $\alpha_3\beta_4^*$-$\alpha$ChR had similar sensitivity to block by most of the LA tested. As an exception, $\alpha_3\beta_4^*$-$\alpha$ChR showed nearly 20-fold higher sensitivity than $\alpha_1^*$-$\alpha$ChR to QX-222. Another notable exception was for $\alpha_4\beta_2$-$\alpha$ChR, which was $\sim$9-fold more sensitive than $\alpha_1^*$-$\alpha$ChR to QX-222 block. Thus, across $\alpha$ChR subtypes, the only rank order inhibition potency profile that deviated from proadifen $>$ adphenine $>$ dimethisoquin $>$ tetracaine $>$ QX-314 $>$ lidocaine $>$ procaine $\approx$ QX-222 was for $\alpha_4\beta_2$-$\alpha$ChR, which had the profile tetracaine $>$ dimethisoquin $\sim$ QX-314 $>$ lidocaine $\simeq$ QX-222 $\approx$ procaine. Comparisons between $\alpha_4\beta_2$- and $\alpha_4\beta_4$-$\alpha$ChR indicated comparable inhibition potency or slightly higher potency at $\alpha_4\beta_2$-$\alpha$ChR for most of the LAs tested except for dimethisoquin ($\sim$9-fold greater inhibition potency at $\alpha_4\beta_4$-$\alpha$ChR) and QX-222 ($\sim$6-fold greater inhibition potency at $\alpha_4\beta_2$-$\alpha$ChR). Therefore, the small differences between $\beta_2$- and $\beta_4$-subunits influence potency for only a select few LAs. Curiously, $\alpha_4\beta_2$-$\alpha$ChR showed the highest level of discrimination between
Fig. 9. Dose-response profiles for carb-stimulated 86Rb efflux through α4β4-nAChR at different concentrations of LA. Measurements of specific 86Rb efflux (ordinate, percentage of 1 nM carb control) were made with SH-EP1-hα4β4 cells assayed in the presence of carbamylcholine alone (C) at the indicated doses (abscissa, molar log scale) or in the presence of carb with adiphenine, dimethisoquin, tetracaine, or procaine at indicated concentrations. Data points are from three or more independent experiments (mean ± S.E.M.). Smooth curves drawn through the data points are from iterative fits to the logistic function \( b = -1.5 \) to 1.6% (see Materials and Methods). Values for the parameter \( a \) (as a percentage of specific 86Rb efflux) and \( r^2 \) values (in parentheses) are 97.9 ± 2.6% (0.99) for untreated control, 70.0 ± 3.1% (0.93) for 2 μM adiphenine, 48.7 ± 2.4% (0.91) for 4 μM adiphenine, 25.8 ± 3.0% (0.66) for 8 μM adiphenine, 68.9 ± 3.5% (0.97) for 4 μM dimethisoquin, 46.2 ± 2.9% (0.96) for 8 μM dimethisoquin, 52.0 ± 51.6% (0.89) for 16 μM dimethisoquin, 81.2 ± 1.2% (0.99) for 8 μM tetracaine, 72.2 ± 1.8% (0.97) for 16 μM tetracaine, 51.9 ± 1.6% (0.98) for 32 μM tetracaine, 85.0 ± 4.2% (0.95) for 250 μM procaine, 73.5 ± 8.6 (0.86) for 500 μM procaine, and 39.3 ± 7.4% (0.89) for 1000 μM procaine. The dose response profile for procaine produced a rightward shift in EC50 value (from 50 μM X±0.03 control to 316 μM X+/2.25 in the presence of 1000 μM procaine) indicating that procaine may partially compete with carb for a binding site on the α4β4-nAChR. Adiphenine, dimethisoquin, tetracaine, and procaine dose-response profiles all produced functional blockade of the α4β2-nAChR insurmountable with increasing concentrations of carb suggesting noncompetitive binding properties.
physiological or ion flux assays), or perhaps degrees of nAChR desensitization (Ryan and Baenziger, 1999) could account for differences in IC_{50} values observed in different studies.

Rank order inhibition potency for LA action at *Torpedo marmorata* electric organ α*-nAChR measured using electron spin resonance and fluorescence techniques was procaine > tetracaine > QX-222 (Arias et al., 1990) and contrasts to our findings. This probably is because the biophysical measures taken may not relate to functional effects of LA at nAChR.

With regard to clinical relevance of LA inhibition of nAChR function, pharmacokinetic findings and ease of LA access to nAChR are of primary importance. Actions of charged ammonium LAs are likely restricted to the periphery. Also, peripheral routes of administration are less likely to yield LA concentrations high enough to induce neurotoxicity (Ritchie and Greengard, 1966). Roles played by blood-brain barrier-permeable LAs in neurological effects depend on perineural concentrations achieved rather than the dose of administered LA (Johnson, 2000), and plasma, cerebrospinal fluid (CSF), and brain concentrations of LAs are influenced by rates and routes of LA administration (Robinson et al., 1994; Xuecheng et al., 1997; Youngs, 1999).

For example, lidocaine is widely used for clinical procedures that include topical ointment application, epidural anesthesia, spinal administration, and systemic bolus administration. Peak plasma concentrations of lidocaine after topical application of 5% ointment to intact skin of healthy volunteers was 9 ng/ml (33.2 nM) and occurred 24 h after the treatment. Thus, absorption of lidocaine from intact skin is poor, and even if applied to damaged skin, effects of topical lidocaine at nAChR (IC_{50} values of 52–250 μM) would be unlikely to occur. However, plasma and CSF concentrations of lidocaine after systemic bolus administration in humans peak at ~1.7 μg/ml (~6 μM) within 5 to 15 min rapidly declining thereafter (Tsai et al., 1998). Intravenous (i.v.) infusion of rabbits with lidocaine at a rate of 4 mg kg\(^{-1}\) min\(^{-1}\) produces whole brain ≈ cortical brain > plasma > CSF concentrations of 373, 295, 166, and 77.5 μM, respectively, at the time of lidocaine-induced seizure onset (718 s) (Momota et al., 2000). These concentrations, especially those in cortical or whole brain, in which lidocaine accumulates, are higher than IC_{50} values for blockade of nAChR. An independent investigation of adiphene metabolism showed that concentration of ethyl-[\(^{14}\)C]adiphene 5 min after i.v. administration is 106.7 ± 17.2 ng/g brain tissue versus 7.1 ± 0.4 ng/g in blood, a difference of nearly 15 times more concentrated in brain tissue (Michelot et al., 1981). Spinal administration, despite dilution of drug in the subarachnoid space, of anesthetics provides perhaps the greatest opportunity for LA to access CNS receptors (Rigler et al., 1991).

Spinally administered LAs are distributed nonhomogeneously and can briefly reach very high local concentrations (Drausner et al., 1994; Robinson et al., 1994). For example, 5 min after routine spinal administration of a 5% solution of lidocaine, the mean CSF concentration can reach 14 mM (Van Zundert et al., 1996), which clearly would have effects at nAChR.

Collectively, these considerations and our results suggest therapeutic application of LA may produce peak plasma and/or brain concentrations capable of inducing significant functional inhibition of nAChR in the periphery or centrally. Enhanced deposition of LAs in the brain may actually make their concentrations there higher than in the periphery, meaning that inhibition of nAChR in the brain (e.g., α*-nAChR and perhaps central α3*-nAChR) would occur even if their acute sensitivity to LAs is lower than LA sensitivity of nAChR in the periphery. Thus, nAChR need to be considered as potential targets for therapeutic actions of LAs and in mediation of side effects of LA action. Moreover, LAs present a broad and interesting family of pharmacophores, action of which at nAChR can be used to help define structural determinants on drugs and receptors that could be exploited to develop novel nAChR antagonists and safer and more effective LAs.

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