Hydrophobic and Ionic Factors in the Binding of Local Anesthetics to the Major Variant of Human $\alpha_1$-Acid Glycoprotein

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ABSTRACT

Understanding the interaction of local anesthetics (LAs) with plasma proteins is essential to understanding their systemic pharmacology and toxicology. The molecular determinants of LA binding to the major variant (F1*S) of human $\alpha_1$-acid glycoprotein (AGP) were therefore investigated spectrofluorometrically using whole AGP and a novel, F1*S variant-selective probe previously developed in our laboratory. Equilibrium-competitive displacement of this probe by LAs, observed by the recovery of AGP’s fluorescence as the quenching probe was displaced from its high-affinity site, was characterized by inhibitory dissociation constants for the various LAs. The importance of electrostatic factors was assessed by examining the pH dependent binding of an ionizable LA, lidocaine, using the quaternary lidocaine derivative QX-314 [N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride] to control for pH dependent ionization of AGP. Uncharged lidocaine bound with at least 8 times the affinity of protonated lidocaine ($K_D = 4.0 \pm 0.6 \mu M$ and $>32 \mu M$, respectively). This result is inconsistent with the current model of the AGP-binding site, which depicts a buried pocket having a negatively charged region that interacts with the amino termini of basic drugs. Consistent with the model, however, two sets of structurally homologous LAs (mepivacaine, ropivacaine, bupivacaine, and lidocaine, RAD-240, RAD-241, RAD-242, L-30, W-6603) demonstrated a strong positive correlation between hydrophobicity (measured as the octanol:buffer partition coefficient of the neutral species) and their free energies of dissociation. Given that the tertiary structure of AGP has proven refractory to resolution, these structure-activity studies should contribute to understanding the nature of the binding site on this important protein.

For example, the local anesthetic bupivacaine binds tightly to AGP (with $K_D = 1 \mu M$) (Essassi et al., 1989), and this binding may mitigate the systemic toxicity of bupivacaine (Wulf et al., 1991; Mazoit et al., 1996).

Indeed, altered pharmacokinetics have been observed following the binding of AGP to the LAs lidocaine and cocaine, and the same holds true with other clinically relevant amphipathic amines: amitriptyline, chlorpromazine, nicardipine, and verapamil (Benet and Hoener, 2002). For example, AGP concentration has been shown to correlate inversely with the free fraction of the LA ropivacaine 60 min after epidural injection (Porter et al., 2001). Also, one of the AGP variants has been shown to limit the blood-to-brain transfer of the amphipathic amines imipramine, disopyramide, and methadone (Jolliet-Riant et al., 1998).

Understanding the interaction of local anesthetics with plasma proteins is essential to predicting their systemic pharmacology and toxicology. Many basic drugs, including local anesthetics (LAs) and class I antiarrythmics, are bound in blood principally or in part by AGP (Routledge, 1986; Stanski and Watkins, 1986; Wood, 1986; Kremer et al., 1988). The binding of a drug to a plasma protein reduces its free fraction in serum, thus reducing its availability for active uptake or diffusion into surrounding tissue and significantly influencing its pharmacokinetics, toxicity, etc. (Wood, 1986).

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1 These authors contributed equally to this work.

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ABBREVIATIONS: LA, local anesthetic; AGP, $\alpha_1$-acid glycoprotein, human; RAD-240, N-(2,6-dimethylphenylcarbamoylmethyl) ethylmethylamine (HCl salt); RAD-241, N-(2,6-dimethylphenylcarbamoylmethyl)ethylpropylamine (HCl salt); RAD-242, N-(2,6-dimethylphenylcarbamoylmethyl)ethylpentyamine (HCl salt); L-30, N-(2,6-dimethylphenylcarbamoyl)ethylidihyamine; W-6603, N-(2,6-dimethylphenylcarbamoyl)propylidihyamine; QX-314, N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride; DEDIC, 2-hydroxy-3,5-diido-N-[2-(diethylamino)ethyl]benzamide; F1*S, refers collectively to the closely related F1 and S variants of AGP which together comprise roughly 70% of pooled source AGP; SAM, standard aqueous medium, containing 0.15 M NaCl and 5 mM MOPS buffer adjusted to pH 7.40; $F(\lambda_{exc}/\lambda_{em})$, corrected, normalized fluorescence with excitation and emission wavelengths of $\lambda_{exc}$ and $\lambda_{em}$. 
Therefore, there has been much interest in determining the affinities of drugs (or potential drugs) for $\alpha_1$-acid glycoprotein (Belaiba et al., 1989; Belpaire and Bogaert, 1989). Indeed, the ability to predict both effective doses for drugs and their potential for toxicity hinges on our knowledge of these drugs’ affinities for AGP. The ability to predict affinities in turn depends on an understanding of the molecular determinants of their binding.

Here we report the affinities of three clinically used local anesthetics for the major ($F1^*S$) variant of AGP (bupivacaine, lidocaine, and mepivacaine), along with the affinity of a racemic mixture of ropivacaine (LEA-103) and its enantiomer, LEA-104. We also report the affinities of several lidocaine homologs, including the ionizable compounds RAD-240, RAD-241, RAD-242, L-30, and W-6603, and the quaternized lidocaine derivative QX-314. These molecules were chosen to assess the contributions of both hydrophobicity and electrostatics to the binding of LAs to AGP. Hydrophobic contributions were examined at physiologic pH (7.40) by correlating the binding affinity of the LAs with their partition coefficients between octanol solvents and aqueous buffer. Ionic contributions were examined both by repeating the binding analyses with lidocaine at a higher pH (8.40), where the majority of the local anesthetic is unprotonated ($pK_a = 8.19$ at 25°C; Strichartz et al., 1990), and by using the permanently positively charged lidocaine analog QX-314. We contrast our results for the representative LAs examined here with the prevailing pharmacophoric model developed using a series of structurally related antihistamines (Kaliszan et al., 1996).

Affinities were determined in competition experiments with the probe molecule 2-hydroxy-3,5-diodo-N-[2(diethylamino)ethyl]benzamide (DEDIC), recently developed in our laboratory (Cogswell et al., 2001). Addition of DEDIC, an iodinated local anesthetic analog, to AGP results in quenching of the protein’s native tryptophan fluorescence by binding with a known $K_D$. Subsequent addition of a LA, with [DEDIC] kept constant, results in fluorescence recovery from which the concentration dependence permits determination of its affinity. The local anesthetics used in this study were themselves not efficient quenchers, and therefore, it was not possible to determine their affinities directly.

A major advantage to using DEDIC, as opposed to other probes of drug binding, is that it binds selectively to a high-affinity site on the major ($F1^*S$) variant of AGP. This selectivity permits the determination of drug affinities to the $F1^*S$ variant in naturally occurring heterogeneous AGP (as is available commercially) and therefore obviates the need to first separate the variants chromatographically (Cogswell et al., 2001). The importance of determining affinities to the variants individually is underscored by observations that the major and minor variants bind to many drugs with differing affinities (Hervé et al., 1998). The failure to account for this heterogeneity would preclude the determination of individual binding constants and, therefore, the development of more accurate pharmacokinetic models, and greatly complicates the consideration of structure-activity relationships for protein binding.

Materials and Methods

**Materials.** Human $\alpha_1$-acid glycoprotein, lot 128H7606 (AGP), bupivacaine, and lidocaine, all $\geq 99\%$ pure, were obtained from Sigma-Aldrich (St. Louis, MO). Ethanol, 100%, anhydrous, in glass bottles was obtained from Aldrich Chemical Co. (Milwaukee, WI). Mepivacaine, LEA-103, LEA-104, RAD-240, RAD-241, RAD-242, L-30, W-6603, and QX-314 were obtained from Dr. Rune Sandberg, Astra Pharmaceuticals (Sodertalje, Sweden); and bupivacaine enantiomers were obtained from CellTech Group PLC (London, UK). The probe molecule DEDIC was previously developed and synthesized in our laboratory (Cogswell et al., 2001). Water was doubly deionized to $>18.2$ MΩ-1 with a Milli-Q ultra pure water system (Millipore Corp., Bedford, MA) and was free of fluorescent contaminants. The structures of DEDIC and the drugs used in this study are given in Fig. 1.

**Preparation of biochemical solutions.** All solutions were prepared in standard aqueous medium (SAM), containing 0.15 M NaCl and 5 mM MOPS buffer adjusted to pH 7.40. Standard practices for the preparation and storage of solutions have been described previously (Cogswell et al., 2001).

**Fluorescence Assays.** Emission measurements were taken at 22°C with an SLM-Aminco SPF-500 spectrophotofluorometer operating in ratio mode, using rhodamine B (3 g/l in ethylene glycol) as a standard for longitudinal stability. Repeated measures of the fluorescence spectrum of a stable solution of AGP demonstrated a precision better than $\pm 2\%$ for recordings collected over 3 to 4 h. The excitation and emission bandpasses were set at 2.5 nm and 20 nm, respectively. As previously reported (Cogswell et al., 2001), the shape of the emission spectrum of AGP was essentially independent of excitation wavelength over the range 265 to 295 nm, indicating that tyrosine residues did not contribute significantly to the observed fluorescence. Therefore, to yield the maximal fluorescence signal, the protein was excited at either 278 nm or 285 nm, near its wavelength of maximum absorbance (278 nm). However, in experiments with QX-314, the protein was excited at 295 nm to avoid large, inaccurate (i.e., greater than a factor of 2) inner filter corrections due to absorbance by drug at shorter wavelengths.

**Fluorescence Quenching Experiments with DEDIC.** The use of DEDIC as a variant $F1^*S$-selective antagonist of drug binding to whole AGP has already been described in detail (Cogswell et al., 2001), and only essential details are repeated here. Although DEDIC is structurally similar to local anesthetics, it contains two iodine atoms, which are believed to be responsible for its efficient quenching of proteins’ intrinsic fluorescence. One of the noteworthy physical properties of DEDIC is that it is zwitterionic at physiologic pH, with its phenolic and amino hydrogens having $pK_a$ values of 5.0 and 9.8, respectively (Fig. 1). Therefore, DEDIC does not undergo major

![Fig. 1. Structures of the ligands used in this study. All are tertiary amines, except for quaternary QX-314.](image-url)
changes in protonation at pH levels near the pKₐ values of most local anesthetics (ca. 7.5–8.5). In the present work, this property of DEDIC greatly facilitated its use as a probe of drug binding at more than one pH.

For all experiments a titration method was used, as described in Cogswell et al. (2001). At both pH 7.4 and 8.4, 250 µl of 5 µM AGP was added to 2.25 ml of SAM in a quartz cuvette and allowed to stand for 5 min with stirring. Stock solutions of DEDIC (2, 20, or 200 µM) were then added to the cuvette in successive aliquots, with continued stirring, and the fluorescence was measured at an analytical emission wavelength of 345 nm. The volume of each aliquot ranged from 5 to 200 µl, and DEDIC concentrations were distributed evenly along a logarithmic concentration axis. Data were first corrected for inner filter effects (Lakowicz, 1999) and then for volume dilution. To minimize the theoretical possibility of degradation associated with repeated interrogation of the sample, single-wavelength measurements (λ_em = 345 nm) were collected instead of complete emission scans, and the excitation shutter was closed between brief periods of illumination. In control experiments, no evidence of photodegradation was observed when AGP or AGP/DEDIC solutions were permitted to stand in the light path for a period of time that exceeded several fold the cumulative exposure of completely titrated solutions.

Resultant quench curves were generated by plotting the normalized corrected fluorescence, \( F \), versus the total (nominal) drug concentration, \( D_T \). At the concentrations of drug and protein used in this study the free drug concentration was not well approximated by that of total drug, and so the data were fitted to isotherms where the independent variable was total drug. The data were best fitted by a two-site model, as given in eq. 1, where \( D \) is given in eq. 2 below (derived in Cogswell et al., 2001).

\[
F = 1 - A_1 \left( \frac{[D]}{K_{D1} + [D]} \right) - A_2 \left( \frac{[D]}{K_{D2} + [D]} \right)
\]

where \( A_1 \) and \( A_2 \) are defined as the amplitudes of the quench associated with DEDIC binding to the apparent higher- and lower-affinity binding site, respectively. Equation 2 defines the concentration of free DEDIC, \( [D] \), in terms of the nominal drug concentration, \( D_T \), the nominal concentration of each class of binding site, \( P_1^m \) and \( P_2^m \), and the equilibrium dissociation constants from the higher- and lower-affinity sites (\( K_{D1} \) and \( K_{D2} \)).

\[
[D](D_T, P_1^m, P_2^m, K_{D1}, K_{D2}) = \frac{a}{3} - \frac{b}{3} \sqrt{\frac{a^2 - 3b \cdot \cos \theta}{3}}
\]

where

\[
\begin{align*}
a & = K_{D1}^2 + K_{D2}^2 + P_1^m + P_2^m + D_T, \\
b & = K_{D1}^2 K_{D2}^2 + P_1^m K_D^2 (K_D^2 - K_D^2) - D_T (K_D^2 + K_D^2), \\
c & = -K_{D1}^2 K_{D2}^2 D_T, \\
\theta & = \arccos \left( \frac{-2a^3 + 9ab - 27c}{2 \sqrt{a^2 - 3b \cdot \cos \theta}} \right)
\end{align*}
\]

We previously demonstrated that the apparent high-affinity binding site observed for DEDIC binding to whole AGP is the high-affinity site on the FI'TS variant (Cogswell et al., 2001). Therefore, the affinity of DEDIC for FI'TS may be readily identified from the quench curve. For each of the replicate quench curves, eq. 1 was fitted to the data yielding the parameters \( P_1^m, P_2^m, K_{D1}, K_{D2}, A_1 \), and \( A_2 \). Replicate determinations of each parameter were averaged.

**Fluorescence Recovery Experiments with Las.** The affinities of the test compounds were determined in displacement experiments with DEDIC at either pH 7.4 or 8.4, as noted. Again, a titration method was used, taking the same precautions to prevent photodegradation. Two hundred fifty microliters of a 5 µM AGP solution were added to 2.19 ml of SAM in a cuvette and equilibrated for 5 min before an initial fluorescence reading was taken. Then, 62.5 µl of either 20 µM, 40 µM, or 80 µM DEDIC solution was pipetted into the cuvette to make a nominally 0.5 µM, 1 µM, or 2 µM solution of DEDIC, respectively. After stirring for 4 min to allow for equilibration, a second fluorescence reading was taken. Subsequent readings were taken 15 s after addition of successive aliquots of local anesthetic directly to the cuvette. Again, the volume of each aliquot was variable, ranging from 5 to 200 µl.

Fluorescence data were first corrected for inner-filter effects (Lakowicz, 1999) and then corrected for dilution by multiplying each datum by the appropriate dilution factor. From the resulting fluorescence recovery curves, the affinities of the local anesthetics were determined by fitting the data by a model of competitive antagonism at a single site (eq. 3 derived in Cogswell et al., 2001). The single-site assumption is appropriate since, at the DEDIC concentrations used in the displacement experiments, it binds almost exclusively to the high affinity site on the FI'TS variant. Given knowledge of \( K_{D1} \) (DEDIC) and the concentration of high affinity sites, eq. 3 was used to determine an inhibition constant (\( K_i \)). Data from replicate experiments were fitted individually by eq. 3, and the fit parameters were averaged. Best-fit lines for each set of replicate recovery curves were then generated from these averaged parameters.

\[
F = 1 - A_1 \left( \frac{[P]}{K_{D1} + [P]} \right) - A_2 \left( \frac{[P]}{K_{D2} + [P]} \right)
\]

where \( A_1 \) and \( A_2 \) are the amplitudes of fluorescence quenching that would result from the binding of infinite nominal concentrations of DEDIC (\( D^{\infty} \)) or LA (\( D^{\infty} \)) to protein with dissociation constants of \( K_{D1} \) and \( K_{D2} \), respectively. The concentration of free protein \( [P] \) is given in terms of nominal protein and ligand concentrations by eq. 4.

\[
[P](P_T, D_{1}^{\infty}, D_{2}^{\infty}, K_{b1}, K_{b2}) = \frac{a}{3} + \frac{2}{3} \sqrt{\frac{a^2 - 3b \cdot \cos \theta}{3}}
\]

Fluorescence recovery experiments were performed using 0.5 µM AGP, which had been allowed to pre-equilibrate with each of three DEDIC concentrations (0.5, 1, and 2 µM). Experiments at each DEDIC were performed at least in duplicate.

**Physicochemical Properties of LA Homologs.** Octanol:buffer partition coefficients were determined at 25°C by an optical method, as described in Sanchez et al. (1987) and Strichartz et al. (1990), using well buffered aqueous solutions of 0.15 M NaCl. These data, obtained by G. Strichartz and V. Sanchez, have not been previously reported. pKₐ values of the LAS and homologs are taken from Strichartz et al. (1990) and from Bokesch et al. (1986).

Using lidocaine, the competitive nature of the interaction was established by determining the average inhibition constant overall. The independence of \( K_i \) on DEDIC would be consistent with competitive antagonism. Additionally, for lidocaine, at each [DEDIC], IC₅₀ values were calculated graphically from the recovery curves, and a Schild plot was generated. If the lidocaine-DEDIC interaction was competitive, a linear dependence of IC₅₀ on [DEDIC] would be expected.

**Calculation of LA Surface Areas and Volumes.** To probe the relation between the affinity of the LA homologs (Fig. 1) and several indices of hydrophobicity, the van der Waals surface areas and the volumes of the LAS' amino termini were calculated using Alchemy
Control Experiments. To investigate the combined effects of LA fluorescence and LA quenching of AGP, each LA was added to 0.5 μM AGP without DEDIC over the range of the concentrations used in this study. In cases where the magnitude of apparent quenching was greater than the signal variance (ca. 2% of the protein’s unquenched fluorescence), the data were corrected for this effect. Although no correction was necessary for bupivacaine, L-30, or RAD-242, corrections were made for mepivacaine, LEA-103/104 (“racemic ropivacaine”), QX-314, (R)-bupivacaine, (S)-bupivacaine, RAD-241, RAD-240, and lidocaine, which at the highest concentrations encountered in these experiments quenched approximately 15, 10, 8, 8, 8, 7, 6, and 5% of the original AGP signal, respectively. These curves served as DEDIC-independent baselines for the titration experiments with LAs and were subtracted from the corresponding recovery curves prior to their analysis.

Calculating the Affinity of Neutral and Protonated Lidocaine to AGP at pH 7.4. Lidocaine, an ionizable LA with a pKₐ of 8.19 ± 0.03 (mean ± S.D.) at 25°C (Strichartz et al., 1990), exists as a mixture of positive and neutral species, each of which may bind to AGP with different affinities. By determining the affinity of LA to neutral and protonated species, respectively. However, in this analysis, pH dependent changes of the binding site must be properly accounted for. The protonizable positively charged QX-314 was therefore used to assess the influence on LA binding of possible pH dependent changes in the protein-binding site. The effect of protein ionization on LA affinity, if present, could then be factored into the calculation of affinities of neutral and protonated lidocaine for AGP at pH 7.4. The derivation of equations that describe these behaviors is given in the Appendix.

In this paper, eqs. 12 and 13 (Appendix) are used to determine the affinity of AGP at pH 7.4 for the neutral and protonated forms of lidocaine, respectively. To do this, however, it is essential to know the factor $\chi$ for lidocaine (see eq. 8, Appendix), the ratio expressing the relative affinities of protein for the charged form of the drug between the higher and lower pH. This was achieved in control experiments with the permanently positively charged lidocaine homolog QX-314, wherein $\chi^{\text{QX-314}}$ was readily determined. On the basis of the near structural identity of the two molecules, $\chi^{\text{lidocaine}}$ was then assumed to equal $\chi^{\text{QX-314}}$.

Results

Determination of $K_D$ ($F1^S/S$) for DEDIC at pH 7.4 and 8.4. The addition of DEDIC to 0.5 μM AGP results in the quenching of up to 75 to 80% of the protein’s intrinsic fluorescence. These data for pH 7.4, shown in Fig. 2, are well fitted by a two-site model (eq. 1 to yield a $K_D$ ($F1^S/S$)/DEDIC) of 0.057 ± 0.013 μM (mean ± S.D.) with $P^1_T = 0.21 ± 0.02$ μM, and a $K_D$ of 9.82 ± 2.60 μM, with $P^2_T$ indeterminate, for the high-affinity and lower-affinity site, respectively (Here $P^1_T$ and $P^2_T$ refer to the apparent molar concentrations of binding sites.) The normalized amplitudes of the fluorescence changes for the high- and the low-affinity processes are unchanged by this elevation of pH: 0.49 ± 0.02 and 0.27 ± 0.01, respectively (mean ± S.D.).

Fluorescence Recovery on Addition of LAs (pH 7.4). Addition of increasing concentrations of lidocaine, from 0.5 μM to 10 mM, to pre-equilibrated mixtures of DEDIC and AGP restores the original protein fluorescence in a concentration-dependent manner (Fig. 3). At each of the three DEDIC concentrations used (0.5, 1, and 2 μM), primarily the major ($F1^S$) variant of AGP is bound by the quencher. Also note that the independent variable in the plot is the nominal concentration of AGP following addition of the probe molecule, DEDIC, to the nominal concentrations shown on the abscissa. The lines are the best fits of eq. 3 for all fitted curves in Fig. 2 was 0.999.

Fig. 2. Quenching of the intrinsic (tryptophan) fluorescence of 0.5 μM AGP following addition of the probe molecule, DEDIC, to the nominal concentrations shown on the abscissa. The lines are the best fits of eq. 3 for all fitted curves in Fig. 2 was 0.999.

Fig. 3. Fluorescence recovery on addition of lidocaine to premixed solutions of 0.5 μM AGP and DEDIC at pH 7.4. Three sets of recovery curves (each with two replicates) are shown, corresponding to a different [DEDIC]: 0.5 μM, open symbols; 1.0 μM, cross symbols; and 2.0 μM, closed symbols. Best-fit lines for eq. 3 are shown for each set of replicates. Inset, IC₅₀ versus each [DEDIC].
concentration of LA and not the free LA concentration. The increase of IC50 for lidocaine (Fig. 3, inset) with increasing nominal DEDIC concentration confirms the competitive nature of the interaction between DEDIC and lidocaine, a behavior that held for all the compounds studied here. From these data we calculated the average Kf for lidocaine binding to the F1\*S variant at each DEDIC concentration using eq. 3. The averaged Kf values at 0.5, 1, and 2 μM (DEDIC) were 30.2 ± 4.9, 20.1 ± 2.9, and 26.0 ± 2.3 μM, and the overall average of all these determinations was Kf = 25.4 ± 5.3 μM (mean ± S.D.), n = 6. These calculated Kf values agree well with values in the literature, further validating this method (see Table 1). The average coefficient of determination (R²) for all fitted curves in Fig. 3 was 0.991.

Similar recovery curves at pH 7.4 were obtained for the remaining compounds: mepivacaine, LEA-103/104 (racemic ropivacaine), bupivacaine, (R)-bupivacaine, (S)-bupivacaine, RAD-240, RAD-241, RAD-242, L-30, W-6603, and QX-314. A summary of Kf values from these fluorescence recovery experiments is given in Table 1.

**Local Anesthetics: Ionic Factors in Binding to AGP.** To examine the effects of charge on the drug-protein interaction, we determined the affinity of lidocaine for the major (F1\*S) and charged (F1\*S) drug species, and pKᵦ. Graphs of several of these molecular properties versus the apparent free energy of binding reveal the features of LAs that systematically correlate with AGP affinity, and those features that do not.

The strongest correlation was between ΔG and P⁰ (r² = 0.82; Fig. 4), and a much weaker correlation (r² = 0.40) was observed for ΔG versus P⁺ (not shown). Curiously, the latter correlation is significantly strengthened (r² = 0.86) if L-30 and W-6603 are not included in the regression. Unlike the remaining compounds in the study, which have only one carbon between the carbonyl group and the amine, L-30 and W-6603 have two and three carbon linkers, respectively.

Similarly, despite weak correlations between ΔG and the number of amino group-linked carbons (r² = 0.22; Fig. 5A), amino surface area (r² = 0.12, not shown), and amino volume (r² = 0.18, not shown), the omission of L-30 and W-6603 from the regression gave strong correlations, with r² equal to 0.90, 0.85, and 0.90, respectively. Comparison of the free energy of binding of neutral lidocaine to AGP to the corresponding values in the literature (R₁ and whole AGP, respectively). With the exception of DEDIC, previous determinations were made in buffers of varying compositions, and different from our own. The number of independent determinations is given in parentheses.

**Table 1**

Local anesthetic binding to the major variant of AGP (F1\*S) at pH 7.4 and pH 8.4.
The first two columns give the Kf values determined for ligand binding to the major (F1\*S) variant of human α₁-acid glycoprotein at pH 7.4 and pH 8.4, respectively. For comparison with our data at pH 7.4, columns three and four give previously reported Kf values (where available) for drug binding to F1\*S and whole AGP, respectively. With the exception of DEDIC, previous determinations were made in buffers of varying compositions, and different from our own. The number of independent determinations is given in parentheses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apparent Kf F1*S (mean ± S.D.)</th>
<th>Apparent Kf F1*S Lit./pH 7.4 (mean ± S.D.)</th>
<th>Apparent Kf AGP Lit./pH 7.4 (mean ± S.D.)</th>
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<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 8.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>DEDIC</td>
<td>0.057 ± 0.013 (3)</td>
<td>0.11 ± 0.034 (3)</td>
<td>0.041 ± 0.013 (3)</td>
</tr>
<tr>
<td>Mepivacaine</td>
<td>10.3 ± 0.11 (3)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>LEA-103/104</td>
<td>2.75 ± 0.14 (2)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>(R)-Bupivacaine</td>
<td>2.97 ± 0.23 (10)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>(S)-Bupivacaine</td>
<td>2.36 ± 0.19 (2)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>RAD-240</td>
<td>53.6 ± 2.12 (2)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>Lidoceine</td>
<td>25.4 ± 5.36 (3)</td>
<td>6.75 ± 0.65 (3)</td>
<td>37 (37)</td>
</tr>
<tr>
<td>RAD-241</td>
<td>16.2 ± 0.92 (2)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>RAD-242</td>
<td>4.19 ± 0.15 (2)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>W-6603</td>
<td>385 ± 29 (2)</td>
<td>N.D.</td>
<td>N.R.</td>
</tr>
<tr>
<td>QX-314</td>
<td>90.3 ± 7.66 (3)</td>
<td>52.2 ± 14.8 (3)</td>
<td>N.R.</td>
</tr>
</tbody>
</table>

N.D., not determined; N.R., not reported.
* P < 0.01 versus Kf at pH 7.4; ** P < 0.001 versus Kf at pH 7.4; *** P < 0.001 versus Kf at pH 7.4.
* Cogswell et al., 2001.
* Hartrick et al., 1984.
* Compound is a racemic mixture of ropivacaine (LEA-103) and its enantiomer.
* Densen et al., 1984.
* Maziot et al., 1996.
* Hervé et al., 1996.
TABLE 2

Gibbs free energy of dissociation and several indices of compound hydrophobicity

<table>
<thead>
<tr>
<th>Compound a</th>
<th>DG (mean ± S.D.) kcal/mol</th>
<th>Number of Amino Carbons</th>
<th>Surface Area of Amino Terminus</th>
<th>Q^14</th>
<th>p^0</th>
<th>p^+</th>
<th>pK_w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mepivacaine</td>
<td>6.74 ± 0.01</td>
<td>6</td>
<td>152</td>
<td>21</td>
<td>304</td>
<td>0.09</td>
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<td>7.48 ± 0.03</td>
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<td>190</td>
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<td>775</td>
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<td>Bupivacaine</td>
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<tr>
<td>LID= Bupivacaine</td>
<td>7.58 ± 0.02</td>
<td>9</td>
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<td>1.5</td>
<td>8.21</td>
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<tr>
<td>RAD-240</td>
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<td>214</td>
<td>346</td>
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<td>1.5</td>
<td>8.21</td>
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<tr>
<td>RAD-241</td>
<td>7.44 ± 0.02</td>
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<td>106</td>
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<td>W-6603</td>
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<td>145</td>
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<tr>
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<tr>
<td>W-6603</td>
<td>5.46 ± 0.05</td>
<td>7</td>
<td>137</td>
<td>341</td>
<td>49</td>
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<td>QX-314</td>
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<td>7</td>
<td>148</td>
<td>0.306</td>
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a Distribution:partition coefficients and pK_w for lidocaine, mepivacaine, LEA-103/104, and bupivacaine are from Strichartz et al. (1990). Remaining data are from G. R. Strichartz and V. Sanchez: Comparison of experimentally determined and theoretically calculated octanol:buffer partition coefficients for a homologous series of local anesthetics (unpublished observation).

b Compound is a racemic mixture of ropivacaine (LEA-103) and its enantiomer (LEA-104).

AGP binding versus carbon number with the free energy of octanol:buffer partitioning of the neutral species versus carbon number (r^2 = 0.34; Fig. 5B) shows a striking similarity. Not only are the slopes similar, 0.29 and 0.35 kcal/mol/methylene group, respectively, but both L-30 and W-6603 are outliers from the best fit with anomalously low free energies for protein dissociation or solvent partitioning.

In contrast, very weak overall correlations existed between ΔG and either pK_w (r^2 = 0.26; Fig. 6) or the octanol:buffer distribution coefficient (r^2 = 0.08; Fig. 7), which accounts for the partitioning of both protonated and neutral species of LAs. The omission of L-30 and W-6603 from these regressions did not improve these correlations.

Discussion

The affinities of the major AGP variant for the clinically important drug mepivacaine, racemic ropivacaine, the quaternary lidocaine derivative QX-314, and the tertiary amine lidocaine homologs RAD-240, RAD-241, RAD-242, W-6603, and L-30 are reported here for the first time. Although values for bupivacaine and lidocaine were previously reported (Denson et al., 1984; Kaliszan et al., 1996; Cogswell et al., 2001), we repeated these determinations using our current lot of AGP and buffer system to allow valid comparison with the present determinations of the other compounds. Not only can the buffer affect the K_D (Ravis et al., 1988), complicating comparisons of drug affinities under different conditions, but the composition and quality of commercial AGP are also known to vary significantly, even from lot to lot from the same supplier (Lunde et al., 1986; Morin et al., 1986; Hervé et al., 1997). Nevertheless, our results for bupivacaine and lidocaine binding to the major AGP variant are consistent with those reported in the literature (Table 1).

Hydrophobic Effects on LA Binding. For the two series of local anesthetics, the linear alkyl amino homologs of lidocaine and the piperidine ring-containing homologs of mepivacaine, we found a linear relationship (r^2 = 0.82) between ΔG (dissociation) for AGP binding and the octanol:buffer partition coefficient of the neutral drug species. The strong correlation between hydrophobicity and the affinity for the LAs is in accord with studies showing that generalized hydrophobicity is a major determinant of binding for other drug classes, including basic drugs such as antihistamines and antihypertensives (Kaliszan et al., 1996), diazepines (Maruyama et al., 1992), and phenothiazines (Miyoshi et al., 1992), as well as acidic drugs such as coumarin antiagulants (Maruyama et al., 1990). That the same linear correlation holds for both homologous series, each with a different conformation of carbons around the amino nitrogen, implies that this hydrophobic interaction is relatively forgiving, suggesting that the binding site is a broad hydrophobic surface or a flexible pocket that can accommodate ligands with a range of sizes.

The correlation of AGP affinity with the number of amino-linked carbons (Fig. 5A) was not as strong as that for the (neutral) partition coefficient (Fig. 4), because the molecules that have increasing carbon numbers between the amide bond and the amine nitrogen, namely W-6603 and L-30 (Fig. 1), fall well off the line. Interestingly, this same deviation occurs when the ΔG of octanol:buffer partitioning is graphed...
against carbon number (Fig. 5B), suggesting that the properties of the binding pocket have fortuitous similarity to octanol, including hydrophobicity and, possibly, hydrogen bonding activity.

Although the two bupivacaine enantiomers have been assigned different affinities for whole (unfractionated) AGP (Mazoit et al., 1996), with a stereoselectivity ($R:S$) of ca. 2, we resolved no significant stereoselectivity for bupivacaine binding to the high affinity site on $F1^*S$. Therefore, for the purpose of examining trends in drug binding with changes in drug hydrophobicity, the use of apparent affinities for the piperidine ring-containing racemates seems acceptable.

**Electrostatic Effects on LA Binding.** The effects of pH on local anesthetic pharmacokinetics are complex and poorly understood, yet clearly important (Porter et al., 2000). Given that LAs, particularly the notoriously cardiotoxic bupivacaine, are reported to bind tightly to AGP (Essassi et al., 1989), it seemed plausible that pH dependent changes in LA pharmacology/toxicology could be mediated through changes in their affinity for the protein.

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**Fig. 5.** A, relationship between $\Delta G$ (dissociation) of structurally homologous LAs from the $F1^*S$ variant of AGP and the number of alkyl-amino carbons ($r^2 = 0.22$). B, analogous relationship between $\Delta G$ (partitioning) of neutral species between octanol and buffer (G. R. Strichartz and V. Sanchez, unpublished observation) ($r^2 = 0.34$).
changes on the drug could not be separated from those on the protein. Moreover, it is essential that the ligand used as a control (here QX-314) be structurally homologous to the drug under study, ensuring that the drug and the probe bind in an identical position. Likeriness of charge is equally important. Indeed, an uncharged probe would probably be less sensitive than a cationic one to changes in protein ionization, given the relative importance of ionic interactions in ligand binding.

In control experiments, we found that F1*S bound QX-314 only about twice as tightly at pH 8.4 as it did at pH 7.4. A likely interpretation is that the neutralization of basic amino acid side chains at the higher pH decreases the net positive charge near the binding site, reducing electrostatic repulsion of cationic QX-314. The affinity of the F1*S variant for neutral lidocaine was calculated to be at least 8 times that of protonated lidocaine, supporting the model of a region of overall positive charge in the binding pocket.

Curiously, in the case of zwitterionic DEDIC, the trend was the opposite to lidocaine and QX-314: DEDIC bound only about half as tightly at the higher pH. Because the $pK_a$ values of DEDIC's phenolic and amino moieties are 5.5 and 9.8, respectively, increasing the pH from 7.4 to 8.4 does little to alter its ionization. Ionization of the protein, therefore, must predominate in the change in affinity, and since the binding energy is reduced, the attraction of the putative basic residue for DEDIC's negatively charged phenolic group must be greater than its repulsion of the positively charged amine. The protonatable charges of AGP near the LA binding site appear to be positive and located closer to the LA's aromatic group rather than its amino terminus.

This finding is significant in that it contradicts the predictions of the current model of the AGP binding site for amphipathic amines, namely, a region of negative charge near the bottom of a hydrophobic pocket (Kaliszan et al., 1995). Kaliszan et al. (1995) noted a strong correlation between the affinities of basic drugs (antihistamines and antihypertensives) for unfractionated AGP and the calculated excess electron density on their amino nitrogens. Since amino electron density correlates with $pK_a$ values of the drugs, they postulated that a negatively charged region on the protein was stabilizing the binding of basic drugs by interacting with their protonated amino termini. Their model was likewise consistent with previous observations that the $pK_a$ values of phenothiazines correlated with their apparent affinities for unfractionated AGP (Miyoshi et al., 1992). However, for the LAs reported here, there was no correlation between $pK_a$ and affinity (Fig. 6).

One possible explanation for the discrepancy between our results and those of Kaliszan et al. (1995) might arise from their use of whole AGP, whereas our method selectively probed the F1*S variant. Perhaps the compounds used by Kaliszan et al. (1995) bound preferentially to the A variant, which might differ from F1*S in the effects of drug protonation on affinity. However, Hervé et al. (1998) found that several antihistamines and antihypertensives had no consistent preference for either variant.

A second possibility is that whereas we measured the affinity of free drug to free protein, Kaliszan et al. (1995) actually measured the retention of drug on an AGP column; it is conceivable that the AGP binding site is altered during chemical crosslinking to silica. A third explanation lies in the potentially different binding modalities of LAs and those
drug classes examined by Kaliszan et al. (1995), principally antihistamines and class II antiarrhythmics.

The model of Kaliszan et al. (1995) is also inconsistent with a report on the binding of unfractoned AGP to six amphi-
pathic amines: two antihypertensives, two antidepressants, an antipsychotic, and a coronary vasodilator (Urien et al., 1991). The neutral forms of these drugs bound to AGP more tightly than did their protonated counterparts by factors of 5 to 20. Although unfractoned AGP was used, dipyriramole and imipramine (two of the drugs studied) are now known to possess greater than 10-fold selectivity for the F18S and A variants, respectively (Hervé et al., 1998). These data therefore appear to contradict the model of Kaliszan et al. (1995), yet are consistent with the results reported here.

As a result of the current work, we propose a binding site for local anesthetics on F18S of AGP that is largely composed of a structurally accommodating hydrophobic pocket, perhaps with the ability to hydrogen bond to H-donor and -ac-
tceptor groups on the ligand (by analogy with the hydroxyl moiety of octanol), and with a basic residue, mostly charged at neutral pH, that is located close to the aromatic group on bound drug molecules.

Appendix: Derivation of Equations to Calculate the Affinity of Neutral and Protonated LAs at a Given pH

The apparent (observed) dissociation constant of an ionizable drug for a protein is given by

$$K_D^{app} = \frac{([D] + [DH^+]) \cdot [P]}{([PD] + [PDH^+])} \tag{A1}$$

where [P] is the concentration of free protein, [D] and [DH+] are the concentrations of neutral and protonated drug, re-
spectively, and [PD] and [PDH+] represent the drug-protein complexes. In addition, the dissociation constants for each drug species, individually, are given by

$$K_D^0 = \frac{[DH^+] \cdot [P]}{[PDH^+]} \tag{A2}$$

$$K_D^- = \frac{[D] \cdot [P]}{[PD]} \tag{A3}$$

Finally, the protonation equilibrium is expressed by

$$K_a = \frac{[D] \cdot [H^+]}{[DH^+]} \tag{A4}$$

Rearranging and substituting eqs. 2 to 4 into eq. 1 gives

$$K_D^{app} = K_D^0 \cdot K_D^- \cdot (K_a + [H^+] + K_D^-) \tag{A5}$$

If there are no pH dependent changes in the binding site, then $K_D^0$ and $K_D^-$ are pH independent. Consequently, if $K_D^{app}$ is determined at two pHs, eq. 5 may be used to determine the two dissociation constants:

$$K_D^0 = \frac{K_D^{app}(1)[H^+]^1 + [H^+]^2)}{2}$$

$$K_D^- = \frac{K_D^{app}(1)[H^+]^2 + K_D^{app}(2)[K_a + [H^+]^1] + [H^+]^2)}{K_D^{app}(2)[K_a + [H^+]^2]}$$

Here the superscripts 1 and 2 distinguish the variables for the two different pHs.

In the more general case, however, changes in pH will cause a change in the binding site, $K_D^0$ and $K_D^-$ are not pH independent, and eqs. 6 and 7 are not appropriate. For the two pHs, let the factor $\chi$ define the ratio of the dissociation constants $K_D^+$ at the two pHs.

$$\chi = \frac{K_D^+(1)}{K_D^+(2)} \tag{A8}$$

Further, assume that whereas binding of the positively charged drug species $DH^+$ is affected electrostatically by ionization of the protein, binding of the neutral species $D$ is not affected. Then

$$K_D^+(1) = K_D^+(2) \tag{A9}$$

At each of the two pHs, then eq. 5 gives the relations

$$K_D^{app(1)} = \frac{K_D^0(1), [K_a + [H^+]^1)}{K_D^0(1) \cdot [K_a + [H^+]^1) \cdot K_D^0} \tag{A10}$$

$$K_D^{app(2)} = \frac{K_D^0(2), [K_a + [H^+]^2)}{K_D^0(2) \cdot [K_a + [H^+]^2) \cdot K_D^0} \tag{A11}$$

After using eq. 8 to express $K_D^0(1)$ in terms of $K_D^0(2)$ and using eq. 9 to replace $K_D^{app}(2)$ with $K_D^{app}(1)$, eqs. 10 and 11 may be solved simultaneously for $K_D^0$.

$$K_D^0 = \frac{K_D^{app(1)}(1)[K_a + [H^+]^1)}{K_D^{app(1)}(2)[K_a + [H^+]^2)}$$

Equation 10 may then be solved for $K_D^+(1)$.

$$K_D^+(1) = \frac{1}{\chi} \left[ -K_D^{app(1)}(K_a + [H^+]^2) - K_D^{app(2)}(K_a + [H^+]^2) \right] \tag{A13}$$

Finally, $K_D^+(2)$ may be readily determined, if desired, by substitution of eq. 13 into eq. 8.

References


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