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Optimal Requirements for High Affinity and Use-Dependent Block of Skeletal Muscle Sodium Channel by *N*-Benzyl Analogs of Tocainide-Like Compounds

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ABSTRACT

Newly synthesized tocainide analogs were tested for their state-dependent affinity and use-dependent behavior on sodium currents (I_{Na}) of adult skeletal muscle fibers by means of the Vaseline-gap voltage clamp method. The drugs had the pharmacophore amino group constrained in position α [*N*-(2,6-dimethylphenyl)pyrrolidine-2-carboxamide (To5)] or β [*N*-(2,6-dimethylphenyl)pyrrolidine-3-carboxamide (To9)] in a proline-like cycle and/or linked to a lipophilic benzyl moiety as in *N*-benzyl-tocainide (Benzyl-Toc), 1-benzyl-To5 (Benzyl-To5), and 1-benzyl-To9 (Benzyl-To9). I_{Na} were elicited with pulses to −20 mV from different holding potentials (−140, −100, and −70 mV) and stimulation frequencies (2 and 10 Hz). All compounds were voltage-dependent and use-dependent channel blockers. The presence of a proline-like cycle increased the potency; i.e., To5 was 3- and 10-fold more effective than Toc in blocking I_{Na} at the holding potential of −140 and −70 mV, respectively. The benzyl group on the amine further enhanced drug effectiveness with the following scale: Benzyl-To9 ≥ Benzyl-Toc > Benzyl-To5. At a holding potential of −100 mV and 10-Hz stimulation, Benzyl-To9 blocked I_{Na} with a half-maximal concentration of 0.5 μM, being 60 and 400 times more potent than To9 and Toc, respectively. The similar effectiveness of Benzyl-Toc and Benzyl-To9 was paralleled by a similar spatial arrangement by equilibrium geometry modeling. In addition, the latter had a higher pK_a value that probably contributed to a slow kinetic during its high use-dependent behavior. Benzyl-To5 had its lowest energy level at a more folded conformation that justifies the less favorable profile among the *N*-benzylated analogs. The new compounds are the most potent tocainide-like sodium channel blockers so far described and have high therapeutic potentials.

The block of voltage-gated Na^+ channel by local anesthetic-like (LA) drugs has therapeutic value for numerous disorders characterized by abnormal membrane excitability (Clare et al., 2000). Different channel types show different sensitivities to LAs; however, the structural basis for this difference is still poorly understood (Wang et al., 1996; Li et al., 1999; 2002). The selective activity on tissue displaying abnormal excitability pattern is based on the state-dependence of LA effect (i.e., a stronger potency at depolarized membrane potential or during high frequency trains of channel activity) (Catterall, 2002). This effect is related to drug interaction with a receptor whose affinity for ligands changes with the state-dependent transitions of the channel, the affinity being the highest when the channels open or inactivate. Mutagenesis studies identified various amino acid residues, localized in the S6 segments of each homologous domain of the α subunit, as critical for LA binding and activity on Na^+ channels of various excitable tissues (Ragsdale et al., 1994, 1996; Wright et al., 1998; Li et al., 1999; Nau et al., 1999; Wang et al., 2000; Yarov-Yarovoy et al., 2001, 2002). LAs may interact weakly with the channel at the resting state, presumably through hydrophobic interactions with two aromatic amino acids, Phe and Tyr, on D4-S6 (Ragsdale et al., 1996; Wright et al., 1998; Li et al., 1999; Nau et al., 1999). Voltage-dependent gating movements of the channel protein may allow a stronger interaction of the drug at these and other residues, leading to a high-affinity stabilization of the channel in the

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ABBREVIATIONS: LA, local anesthetic; Toc, tocainide; Benzyl-Toc, *N*-benzyl-tocainide; To5, *N*-(2,6-dimethylphenyl)pyrrolidine-2-carboxamide; Benzyl-To5, 1-benzyl-*N*-(2,6-dimethylphenyl)pyrrolidine-2-carboxamide; To9, *N*-(2,6-dimethylphenyl)pyrrolidine-3-carboxamide; Benzyl-To9, 1-benzyl-*N*-(2,6-dimethylphenyl)-pyrrolidine-3-carboxamide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HP, holding potential; fUDB, fractional use-dependent block (percentage reduction of I_{Na} at the steady-state versus the first pulse of 10-Hz train); TB, tonic block.

inactivated state (Li et al., 1999; Wang et al., 2000; Yarov-Yarovoy et al., 2001). The parts of LA molecule with recognized pharmacophore roles are the ionizable amino group and the aromatic ring at the opposite extremity, which are proposed to establish cation-π and π-π interactions, respectively, with the two hydrophobic domains cited above (Dougherty, 1996; Ragsdale et al., 1996). In addition, a site in D1-S6 segment may account for the stereoselective action of chiral compounds (De Luca et al., 1997a, 2000; Nau et al., 1999), probably as a consequence of a third point interaction driven by the stereogenic center, which is the carbon atom near the pharmacophore amino group. An increase in the lipophilicity and/or hindrance at this level (i.e., with an aromatic ring) enhances the potency of stereoselective compounds for blocking Na^+ currents in skeletal muscle by up to 10-fold (De Luca et al., 1997a, 2000; Desaphy et al., 2001). We proposed that the lipophilic group at this level could establish with the hydrophobic pockets in D4-S6 a π-π interaction stronger than the π-cation interaction made by the amino group (Wright et al., 1998; De Luca et al., 2000, 2003). However, the chiral center is strictly adjacent to the amino group; thus, the above-described changes could lead to a general amelioration of the binding properties of the main pharmacophore. Interesting results were obtained by constraining the stereogenic center and the amino group in a proline-like cycle (Talon et al., 2001). In line with the more strict spatial conformation, the α proline-like analog of tocainide, conventionally named To5, showed an increased stereoselectivity. In parallel, the eutomer (*R*)-To5 was 5- and 20-fold more potent than tocainide in producing a tonic and a 10-Hz use-dependent block of sodium currents, respectively (Talon et al., 2001). These findings suggest a better interaction of the pharmacophoric groups with the binding site. The aim of the present study was to better clarify the role of the pharmacophore amino group in the drug-receptor interaction. This has been accomplished 1) by spacing out the amino group from the stereogenic center in the rigid proline-like cycle (β-proline analog To9) to maintain the spatial structure of the molecule and improve basicity; 2) by increasing the lipophilicity on the nitrogen atom by introducing a benzyl moiety on both tocainide (Benzyl-Toc) and its proline-like analogs (Benzyl-To5 and Benzyl-To9) (Fig. 1). The rationale for testing the effect of the original *N*-benzylation versus the more classic *N*-alkylation was aimed to combine the effect of a second aromatic moiety, able to establish high-affinity interaction with one of the two aromatic residues, with the change in basicity of the pharmacophore. This latter can influence drug potency; in fact, charged amino moiety can

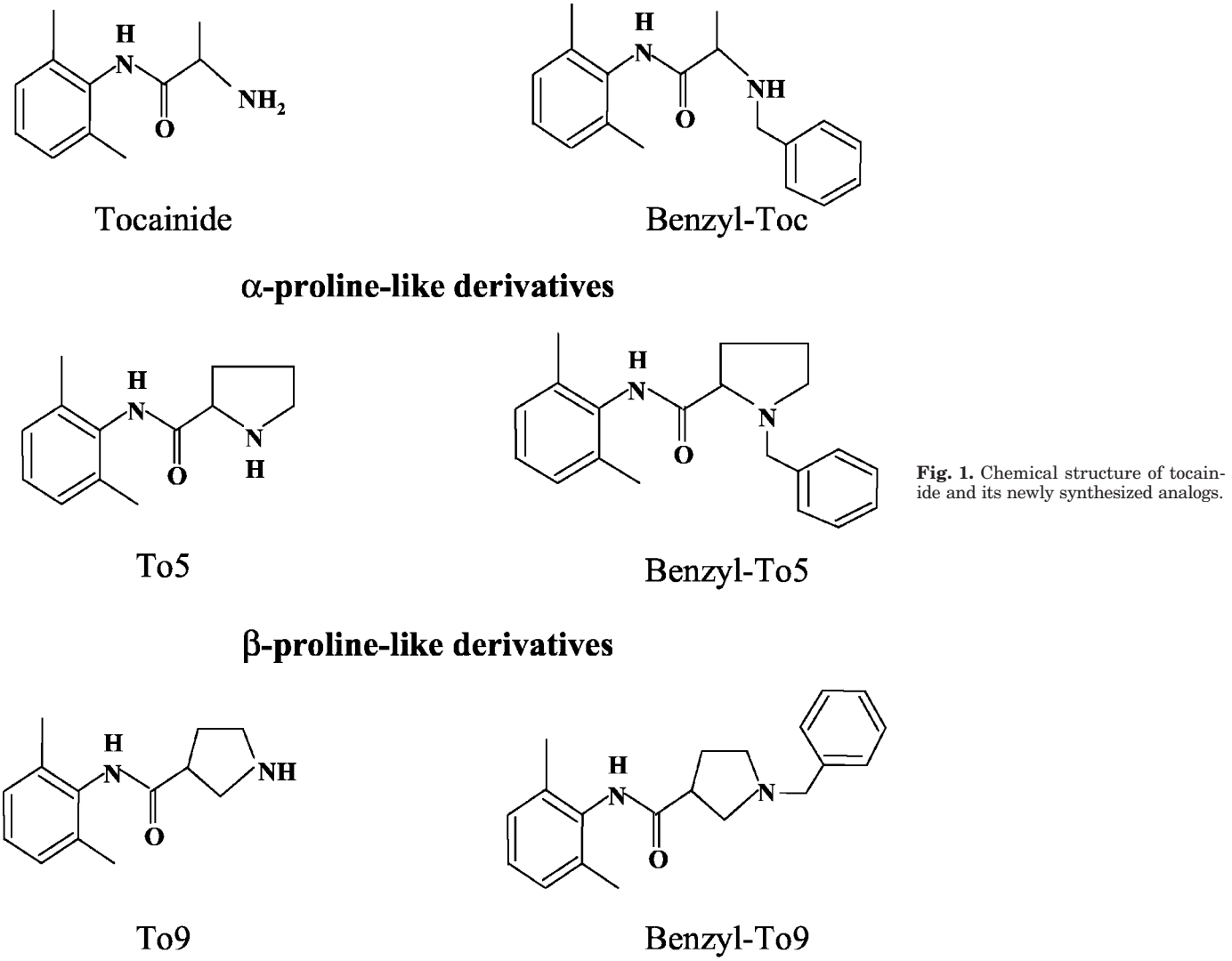

Fig. 1. Chemical structure of tocainide and its newly synthesized analogs.

influence drug diffusion through the membrane and/or establish hydrogen bonds with the third site in D1S6 (Nau et al., 1999; Liu et al., 2003).

We found that the *N*-benzyl analogs showed a remarkable gain of potency; Benzyl-To9 was up to 400 times more potent than tocainide as an inactivated use-dependent channel blocker. A conformational analysis proved that the increase in affinity parallels the spatial disposition of the two aromatic rings, corroborating their direct role in establishing specific interactions with the binding site. An optimal basicity of the amino group, influenced by its substituents, confers a high use-dependent behavior. The results shed further light on the molecular interaction of LAs with their receptor. Also, the new potent agents deserve further investigation for their wide potential therapeutic application, because previous studies indicate a good correlation between the scale of potency determined by this approach and the in vivo and in vitro antimyotonic activity of the drugs in animal models of the human disease (De Luca et al., 1997b; Talon et al., 2001).

Materials and Methods

Fiber Preparation and Voltage Clamp Apparatus. Segments of undamaged single muscle fibers (about 1 cm long) were obtained by microsurgery (plucking procedure) from the ventral branch of the semitendinosus muscle of *Rana esculenta* bathed in normal physiological solution at room temperature. The cut-end fiber was then perfused with an internal solution and mounted across three chamber partitions, which delineated the four pools. Three strips of Vaseline were applied over the fiber and carefully sealed to the fiber to reduce leakage. The width of the gaps of the central pools (A and B) had been previously set to 70 to 100 μm and 200 μm, respectively. Four KCl/agar bridges electrodes connected the recording chamber to the voltage clamp amplifier based on methods described by Hille and Campbell (1976) and detailed elsewhere (Hille and Campbell, 1976; De Luca et al., 1997a, 2000). For recordings, the solution in pool A was replaced with the external solution; after about 10 min of equilibration, the recordings were performed at 10°C. The usual holding potential (HP) was −100 mV. However, as detailed below, different holding potential values have been used in protocols aimed at evaluating voltage-dependence of drug effect.

Sodium currents were recorded using an amplifier connected via an analog-to-digital and digital-to-analog Digidata 1200 Interface (Axon Instruments, Union City, CA) to a 486 DX2/66 personal computer and stored on the hard disk. The stimulation protocols and data acquisition was driven by the Clampex program (pClamp6; Axon Instruments). The currents flowing in response to depolarizing command voltages were low-pass filtered at 10 kHz (Frequency Devices, Haverhill, MA), visualized on an oscilloscope, and sampled at 20 kHz. The acquired traces were analyzed later using Clampfit program (pClamp6 and 8.1; Axon Instruments).

Drugs and Solutions. The following solutions were used: normal physiological solution, 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO_4, and 0.85 mM NaH_2PO_4; external solution, 77 mM NaCl, 38 mM choline-Cl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO_4, and 0.85 mM NaH_2PO_4; internal solution, 105 mM CsF, 5 mM MOPS, 2 mM $MgSO_4$, 5 mM EGTA, and 0.55 mM Na_2ATP. The pH was adjusted to 7.2 with a standard NaOH concentrated (5 N) solution.

The compounds tested and shown in Fig. 1 were tocainide (Toc), *N*-benzyl-tocainide (Benzyl-Toc); the α-proline derivatives *N*-(2,6-dimethylphenyl)pyrrolidine-2-carboxamide (To5) and its benzyl derivative 1-benzyl-*N*-(2,6-dimethylphenyl)pyrrolidine-2-carboxamide (Benzyl-To5); and the β-proline derivatives *N*-(2,6-dimethylphenyl)pyrrolidine-3-carboxamide (To9) and its benzyl derivative 1-benzyl-*N*-(2,6-dimethylphenyl)pyrrolidine-3-carboxamide (Benzyl-To9).

All the compounds were prepared in our laboratories as hydrochloride or hydroiodide salts according to procedures described in details elsewhere (Franchini et al., 2000). All compounds were racemates, with the exception of Toc and To5, synthesized as pure *R*- and *S*-enantiomers (Franchini et al., 2000). Racemates of these latter to be tested on I_{Na} were obtained by exactly mixing equimolar solutions of the single enantiomers.

Stock solutions of Toc, To5, and To9 were prepared by dissolving the compounds in external solution, whereas stock solutions in external solution containing dimethyl sulfoxide (<1%) were used for the benzyl-derivatives. Dimethyl sulfoxide at the highest concentration used for dilution (0.2%) was without effect on the parameters recorded. All other chemicals used were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Pulse Protocols and Statistical Analysis. In agreement with previous studies (Nau et al., 1999; De Luca et al., 2000; Yarov-Yarovoy et al., 2002), the voltage-dependent block exerted by the compounds was evaluated on nearly maximal I_{Na}-elicited single depolarizing 10-ms test pulses to −20 mV from two different HP: −140 and −70 mV. The evaluation of concentration-dependent drug effect, expressed as half-maximal blocking concentration (IC_{50}), at −140 mV allowed calculation of the affinity constant for the resting state (K_r); the amount of closed-state inactivation at this potential was negligible. The IC_{50} value calculated from the HP of −70 mV was instead influenced both by the higher proportion of channels entering a closed-state inactivation at this potential and by the ability of the drug to modify this proportion in favor of more channels being inactivated, based on the ability of the drug to act as inactivated channel blocker. Because the inactivated state was nonconductive, the voltage-dependent block exerted by the drugs was used to estimate the affinity constant for the inactivated channel (K_i) using the above IC_{50} values, the relative distribution of channels in the resting and/or inactivated states at the holding potentials used (from the steady-state inactivation curve) and the equations described below.

The use-dependent block exerted by each compound was evaluated as the cumulative block of I_{Na} upon increase of the stimulation frequency. For this protocol, the membrane potential was held at −100 mV, a HP closer to the physiological values at which almost all the channels are in the resting closed state (De Luca et al., 2000). Trains of depolarizing 10-ms pulses to −20 mV from the HP were applied at the frequency of 2 and 10 Hz for 30 s. At both frequencies, the drug-induced reduction of peak I_{Na} at the first pulse was considered tonic block (block of the channel in the resting state). The use-dependent behavior was estimated by the further reduction of the current observed in the presence, but not the absence, of use-dependent compounds that progressively cumulated over the tonic block in a frequency-dependent manner until a new equilibrium was reached. The value of the current at the equilibrium normalized with respect to the current in the absence of drug was used to calculate the absolute potency of the drug for blocking the channels under conditions of excessive stimulation (e.g., high-frequency firing). The normalization of each current trace composing the 10-Hz train in the presence of the drug with respect to the current at the first pulse after drug, allowed evaluation, for each compound, of the concentration-dependent time course to reach the steady state for use-dependent blockade. For any drug and at each drug concentration, this process was well described with a single exponential decay ($\chi^2 < 0.0005$), which led to estimate the concentration-dependent time constants as well as the fractional use-dependent block (i.e., the block of I_{Na} by the use-dependent compound eliminating the tonic block component).

Steady-state inactivation (h_∞) curves were determined by cyclic protocol of pulse sequences. Each sequence consisted of a conditioning pulse to −140 mV for 500 ms (to have most of the sodium channels in the "activatable" state), a prepulse of variable potential of 1000-ms duration, and the 10-ms test pulse to −20 mV; after a pause of 1 s, the sequence was cyclically repeated 18 to 20 times, with

the prepulse potential value increased each time by 5-mV steps (De Luca et al., 1997a, 2000).

Modeling Studies. All the analogs tested are weak basic amines, and it is likely that in the physiological environment, they are largely present in the protonated charged form. This plays an important role for their pharmacological profile, in that they are able to influence use-dependent behavior and gain access to the receptor through the hydrophilic pathway of the open channel by sensing the electric gradient across the membrane (Bean et al., 1983; Liu et al., 2003). Thus, the modeling studies were conducted on the protonated cationic form. Models were constructed by fragments, assuming the starting geometry to be similar to that found for lidocaine hydrochloride monohydrate crystal structure (Hanson and Röhrl, 1972). Equilibrium geometries were calculated starting with a systematic conformer distribution analysis. Conformers were grouped into families based on relevant torsion angle values. The best (i.e., most stable) representative of each family was submitted to an AM1 semiempirical geometry optimization and a single-point energy calculation by a Hartree-Fock calculation at the 3-21G(*) level. On the best conformer of each analog, a 3-21G(*) Hartree-Fock geometry optimization was performed. Energies were corrected for aqueous solvation using the Cramer/Truhlar SM5.4 model (Chambers et al., 1996). The pK_a values of the newly synthesized compounds were calculated by using the approach proposed by D'Souza et al. (2000). This method is based on the calculation of the proton affinity of a nitrogen base. Proton affinity corresponds to the energy of a reaction in which a nonbonded electron pair is replaced by a bonded pair. A good estimation of the energy for this type of reaction may be derived calculating the energy difference (ΔE) between the lowest energy conformations of the protonated and unprotonated forms. This energy has been shown to be related to the pK_a value of the base for several nitrogen bases (D'Souza et al., 2000). Running the same calculations on a series of nitrogen bases with different experimental pK_a values, a straight-line plot is obtained when plotting ΔE versus the experimental pK_a values. Hence, the unknown experimental pK_a value for a nitrogen base may be derived by calculating the relative energy of protonation (i.e., ΔE) and interpolating the latter in the curve. Thus, the 3-21G(*) estimated free energy difference (ΔE) between the protonated and unprotonated forms of eight pharmacologically relevant nitrogenous bases (experimental pK_a values in the range 6.9–10.1) was calculated, and a straight-line plot correlating experimental pK_a values and ΔE was derived ($\Delta E = 3.7632\ pK_a + 268.22$, $r = 0.97$, $\sigma_{pK_a} = 0.3$). By interpolating the ΔE values calculated for the newly synthesized compounds, the corresponding estimated pK_a values were obtained. All calculations and graphical representations were performed by using the SPARTAN PRO software package (Wavefunction, Inc., Irvine, CA).

Data Analysis and Statistics. The data were expressed as mean ± S.E.M. The estimates of S.E.M. of normalized I_{Na} values have been obtained as described previously (De Luca et al., 1997a, 2000). Molar concentrations of the drugs tested that produce a 50% block of I_{Na} (IC_{50}) in the various experimental conditions were determined by using a nonlinear least-squares fit of the concentration-response curves to the following logistic equation: $\text{Effect} = -100/[1 + (K/[\text{drug}])^n]$, where Effect is the percentage change of I_{Na}, −100 is the maximal percentage block of I_{Na}, K is IC_{50}, n is the logistic slope factor, and [drug] is the molar concentration of the compound. The h_∞ curves have been fitted with a single Boltzmann distribution, and the potential at which 50% of the sodium channels were inactivated ($Vh_{1/2}$) was calculated at the inflection point of the curves (DeLuca et al., 1991). The left shift of the h_∞ curve produced by the drug was a function of the concentration used and of the relative affinity for the channel in the inactivated state. The difference between $Vh_{1/2}$ at each drug concentration and the control $Vh_{1/2}$ ($\Delta Vh_{1/2}$) measured in the same fiber, as a function of the drug concentration, were well fitted by the nonlinear equation $\Delta Vh_{1/2} = k\ \ln\{(1 + [\text{drug}]/K_r)\ (1 + [\text{drug}]/K_i)^{-1}\}$, where k is the mean slope factor of the h_∞ curves at each drug concentration [drug] (taken constant to 5.5), K_r is the affinity constant for resting state obtained from the fit of the concentration-response curve at −140 mV, and K_i is the apparent affinity of drug for the inactivated state (Bean et al., 1983). A more detailed evaluation of absolute K_i was obtained from the voltage-dependent distribution of the channels in the resting (h) and inactivated state ($1 - h$) according to the h_∞ curve, using the equation $1/K_{-70} = h/K_r + (1 - h)/K_i$, where K_{-70} and K_r are the IC_{50} values obtained from dose-response curves at −140 and −70 mV, whereas h and $(1 - h)$ represent the fraction of channel present at resting and inactivated state at −70 mV, respectively (Bean et al., 1983).

Statistical significance of differences between couples mean values has been estimated by unpaired Student's t test and considered significant with $p < 0.05$. The statistical significance between IC_{50} values ± S.E. obtained from the fit was also evaluated by a Student's t distribution using a number of degrees of freedom equal to the total number of preparations determining each point of the curve minus the number of means determining the curve minus two for the free parameters (De Luca et al., 2000).

CLogP is the calculated log value of the octanol/water partition coefficient obtained by using C Log Software v. 3.0 (Biobyte Corp., Clermont, CA), and logD is the calculated log value of distribution coefficient at physiological pH, considering the nature of weak electrolytes and the different lipophilicity of unprotonated and protonated forms. LogD is then calculated at pH 7.4 from the cLogP and pK_a values of each compound according to the equation $\text{logD} = \text{LogP} - \log(1 + 10^{pK_a - pH})$.

Correlation analysis was evaluated by fitting the experimental data points to linear regression analysis. Nonlinear equation fitting and processing for data graphics were done by Fig P Software (Biosoft, Cambridge, UK).

Results

Voltage Dependent Block of Na^+ Channels by Proline-Like and Benzylated Tocainide Derivatives: Evaluation of Apparent Affinity Constants for Channel States. Sodium channels transit between voltage-dependent conformational states showing different affinities for LA-like compounds (Bean et al., 1983). In response to a depolarizing step, the channel passes from a closed resting state to an open state, rapidly entering an inactivated state on a millisecond time scale (open-state inactivation). Direct transitions from closed to inactivated states, so called closed-state inactivations, also occur as a result of membrane depolarization below the threshold for channel opening (Bean et al., 1983; Aldrich et al., 1983; Takahashi and Cannon, 2001). Because of the dynamic equilibrium between the states and the fact that inactivation is a nonconductive state, the evaluation of the state-dependent drug affinity is not an easy task. In our experimental conditions, we evaluated the state-dependent drug effect by testing the block of I_{Na} exerted by the newly synthesized compounds in response to a depolarizing step from two different HP, −140 mV, at which all channels are in the resting activatable state, and −70 mV, a membrane potential in the physiologically excitable range at which a considerable amount of closed-state inactivation is present. As can be seen in Fig. 2, each drug produced a block of sodium currents that was generally more pronounced at the HP of −70 mV than at −140 mV. In accordance with previous results (Talon et al., 2001), the α-proline derivative To5 at 100 μM produced a block of I_{Na} that was comparable with that produced by 500 μM Toc. Interestingly, the β-proline-analog To9 showed almost the same potency as To5, suggesting that the increased distance of the amino group by one methylene does not greatly modify the interaction of this

constrained molecule with the receptor. In contrast, all benzylated derivatives were markedly more potent that the related unbenzylated parent compounds, with higher potency shown by Benzyl-To9, which produced a substantial block at concentrations as low as 1 μM (Fig. 2).

For each compound, we constructed concentration-response curves for the block produced at both HP (Fig. 2). From the fit of the experimental points, we obtained the concentrations for half-maximal block of I_{Na} (IC_{50}) at both -140 and -70 mV. Although the IC_{50} value at the former HP can be considered as the affinity constant for closed-resting (K_r) channels, the IC_{50} at -70 mV (K_{-70}) is strongly influenced by the relative proportion of resting channels in equilibrium with closed-inactivated ones and by the ability of the drug to influence such an equilibrium based on its affinity for the inactivated state (Table 1) (Nau et al., 1999). In fact, as expected from inactivated channel blockers, a great increase of potency was observed when the membrane potential was held at -70 mV: the concentration-response curves of all compounds were clearly shifted to the left with respect to those obtained at -140 mV. The gain of potency for each compound at the more depolarized potential ranged between 4- and 15-fold; the smallest gain was observed with tocainide and the greatest with To5. When looking at the absolute values of IC_{50}, we confirmed that To5 and To9 were almost equipotent, both being about 3- and 10-fold more potent than Toc at -140 and -70 mV, respectively (Table 1). As anticipated, a marked change in the potency of the compounds was observed when a benzyl group was introduced on the amine one (Table 1). Figure 3 shows the potency of each compound in comparison with that of tocainide at both holding potentials. As can be seen, the scale of potency was Benzyl-To9 > Benzyl-Toc > Benzyl-To5 > To5 = To9. Benzyl-To9 was up to 200- and 400-fold more potent than tocainide for block at

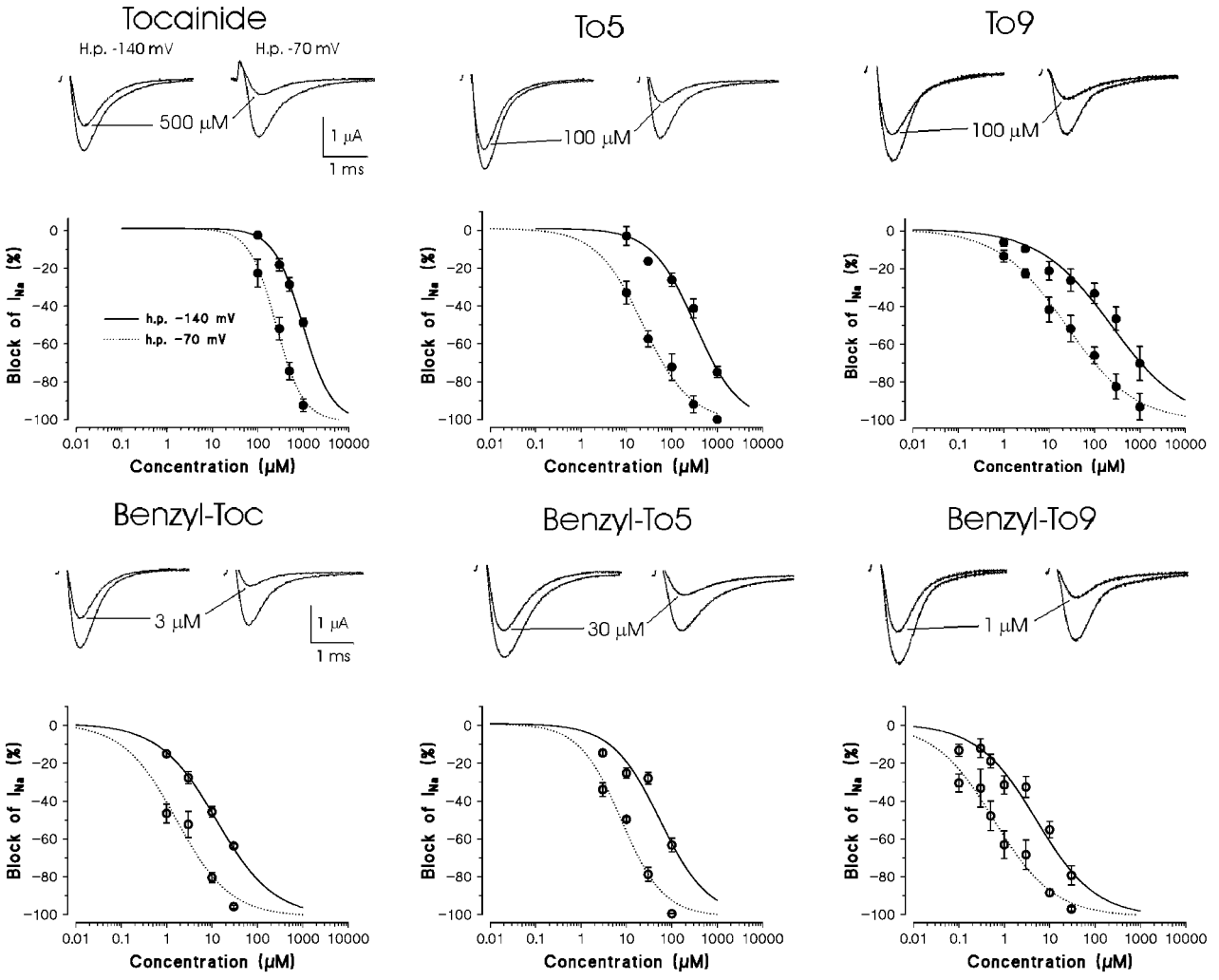

Fig. 2. Evaluation of the voltage-dependence of drug effect. Traces of sodium current transients have been recorded by the three Vaseline-gap voltage-clamp method from single fibers of frog semitendinosus muscle in the absence and presence of tocainide (Toc) and of its analogs. It is shown as the reduction of sodium current elicited with a 20-ms test pulse to -20 mV from two different holding potentials [i.e., -140 mV (current traces on the left) and -70 mV (current traces on the right)]. In each condition and group of traces, the greatest one is the control current obtained in the absence of drug. The current trace elicited at the HP -70 mV has a less amplitude with respect to that elicited at -140 mV, as expected by an increased number of inactivated channels at the more depolarized potential. The test compounds blocked peak sodium currents, I_{Na} with different potency and all produced a more pronounced block at the more depolarized holding potential. The reduction of I_{Na} observed at increasing drug concentrations allowed construction of the dose-response curves, shown below each trace sample, at the two holding potentials of -140 mV (full line) and -70 mV (dotted line). The curves fitting the experimental points were obtained using the logistic function described under *Materials and Methods* and allowed calculation of the half-maximal concentrations in the two recording conditions. Each value is the mean ± S.E.M. from four to seven fibers of the percentage block of I_{Na} observed in the presence of each concentration of drugs versus I_{Na} in the absence of the drug in the same fiber.

−140 and −70 mV, respectively (Table 1). Focusing on the specific effect on potency caused by the simple introduction of the benzyl moiety, we found that the gain of potency observed was different for each benzylated compound although more potent than the relative nonbenzylated ones. The most dramatic increase was observed for Benzyl-Toc, which was 80- and 150-fold more potent than tocainide in determining the block at −140 and −70 mV, respectively. Benzyl-To9 showed at both potentials a similar 40-fold increase in potency compared with its parent compound (Table 1). Taking into account that To9 is already significantly more potent than Toc, its benzylated analog turned out to be the most potent tocainide-like compound described so far, with half-maximal concentration at −70 mV as low as 0.5 μM. The introduction of the benzyl group on the α-proline derivative To5 also markedly increase the potency; however, such an increase was significantly less with respect to that observed with Toc and To9 (Table 1).

The activity of the compounds as inactivated channel blockers was confirmed by evaluating their effects on the voltage dependence of steady-state channel availability by constructing h_∞ curves in the absence and in the presence of the test compounds. All the compounds produced a shift of the h_∞ curve and consequently of the potential for inactivating 50% of the channels ($Vh_{1/2}$) toward more negative potentials, corroborating the view that the test compounds preferentially bind and stabilize the channel in the inactive state (Fig. 3). The shift of the h_∞ curve produced by each drug was clearly concentration-dependent and related to the relative potency in blocking I_{Na}. In fact, the curve describing the concentration-dependent shift of the h_∞ curve (taken as $\Delta Vh_{1/2}$) by Benzyl-To9 was markedly shifted to the left with respect to that of To9 (Fig. 4). The fit of the data points to the equation described under *Materials and Methods*, and previously used to calculate drug affinity for the inactivated state (Bean et al., 1983), led to K_i values of 27 ± 2.2 and 0.38 ± 0.04 μM for To9 and Benzyl-To9, respectively. As can be seen, these values were in the same range but did not exactly overlap the IC_{50} values obtained at −70 mV (Table 1); the K_i value found for Benzyl-To9 was about 1.6-fold lower, and this trend was also observed with some other compounds (data not shown). This is related to the fact that the value obtained at −70 mV is the result of the mixed binding of the drug to both resting and inactivated channel, although the high-affinity binding to the inactivated channel tends to overwhelm (Bean et al., 1983). To validate this view, the values of K_{-70} were used to calculate the absolute K_i using the equation $1/K_{-70} = h/K_r + 1 - h/K_i$, which takes into consideration the relative distribution of channels between the two states according to the membrane potential (Bean et al., 1983). The resulting K_i values, listed in Table 1, were almost halved with respect to K_{-70}, as expected given the contribution of the resting-state binding. However, the relative potency between the compounds was largely maintained. By this approach, the K_i of Benzyl-To9 was as low as 0.25 μM. Although the absolute values of K_i could be better estimated with more sophisticated biophysical approaches, our results indicate that the high potency shown by the compounds at depolarized membrane potentials is related to their high affinity for the inactivated state and to their ability to markedly shift the equilibrium of closed-state inactivation.

Use-Dependent Block of Na+ Channels by Proline-Like and Benzylated Tocainide Derivatives. Use-dependent behavior of tested compounds has been evaluated at HP of −100 mV, closer to the physiological values, with 10-ms depolarizing pulse to −20 mV applied at the frequency of 2 and 10 Hz. At both frequencies, the first trace recorded in the presence of the compound was reduced versus the control because of the tonic block exerted by the drug. When the fiber was repetitively stimulated at high frequency, the block progressively cumulated over the initial tonic block until a new equilibrium was reached. Figure 5 shows, for each compound, a sample of the progressive use-dependent block of sodium currents at 10 Hz; the concentration-response curves comparing the block before and after steady-state, use-dependent block at 10 Hz are shown just below the sample traces. The reduction of the current at the first pulse of the train was influenced only by the membrane potential and by the relative distribution of the channels between resting and inactivated states. In fact, the curves describing the tonic block and the relative calculated IC_{50} values were intermediate between those obtained at −140 and −70 mV (Table 2). The use-dependent block adds cumulatively to the tonic block; thus, to evaluate the absolute drug potency in real conditions

TABLE 1

Voltage-dependent block of sodium currents by tocainide, proline-like derivatives, and related benzylated analogs

The columns from left to right are as follows: drug used; concentrations able to produce the half-maximal block of sodium currents (IC_{50}) with single 10-ms test pulses to −20 mV from a holding potential of either −140 or −70 mV. The IC_{50} values have been obtained during nonlinear least squares fit of the concentration-response data to the logistic equation described under *Materials and Methods*. Inactivated state-block refers to the affinity constants for inactivated sodium channels (K_i) calculated from the IC_{50} values at −140 mV (assumed as affinity for resting state, K_r) and at −70 mV (K_{-70}) and the relative distribution of channels between resting and inactivated state at −70 mV evaluated from steady-state inactivation curves, according to the equation described under *Materials and Methods*. The gain of potency of the new compounds versus Toc on both resting and inactivated channels is shown as ratios between related constants values, $K_{r\ Toc}/K_{r\ drug}$ and $K_{i\ Toc}/K_{i\ drug}$, respectively. The gain of potency of each compound versus inactivated state is shown as the ratio between related K_r and K_i values (K_r/K_i).

Drug	Voltage-Dependent Block			Inactivated-State Block		
	HP −140 mV IC_{50}; K_r	$K_{r\ Toc}/K_{r\ drug}$	HP −70 mV IC_{50}; K_{-70}	Calculated K_i	$K_{i\ Toc}/K_{i\ drug}$	K_r/K_i
	μM		μM	μM		
Toc	985 ± 49.3	1	254.0 ± 15.0	115	1	4
Benzyl-Toc	12.5 ± 0.9[a,b]	79	1.7 ± 0.4[a,b]	1.1	105	11
To5	329 ± 50[c]	3	23.3 ± 2.6[c]	10.9	11	30
Benzyl-To5	54.9 ± 14[a,b]	18	7.7 ± 1.2[a,b]	3.9	29	14
To9	240 ± 51[c]	4	24 ± 2.3[c]	10.1	11	24
Benzyl-To9	5.1 ± 1.0[b]	193	0.6 ± 0.1[b]	0.25	460	20

[a] Significant difference of benzyl analogs with respect to Benzyl-To9 ($P < 0.01$ and less).
[b] Significant difference of benzyl analogs with respect to related non-benzylated parent compounds ($P < 0.001$).
[c] Significant difference of proline-like analogs with respect to tocainide ($P < 0.001$).

of high-frequency depolarizing trains, we constructed the dose-response curves by considering the residual current after the high-frequency stimulation with respect to that in the absence of drug. The IC_{50} values obtained from experimental data fitting at both 2 and 10 Hz, along with the gain of potency of each compound caused by the use-dependent behavior, are listed in Table 2. As previously shown, To5 was markedly more use-dependent with respect to Toc, with gain of potency up to 8-fold at 10 Hz; surprisingly, To9 showed use-dependent behavior similar to that of Toc, producing a similar block at 2 and 10 Hz (Table 2). Each benzylated compound showed a specific pattern of use-dependent behavior. In fact Benzyl-Toc maintained the use-dependent behavior of Toc, although it was more potent in absolute terms (Fig. 6). On the other hand, the presence of the benzyl moiety on the α-proline derivative To5 dramatically reduced use-dependent behavior, the gain of potency ranging around 2-fold at 10 Hz. In contrast, Benzyl-To9, was also remarkably use-dependent, showing gain of potency values approaching those observed with To5 (Fig. 6 and Table 2). Thus Benzyl-To9, which proved to be the most potent analog, was also the one showing almost the highest use-dependence.

A comparison between the IC_{50} values listed in Tables 1 and 2 allows us to appreciate that the high-stimulation frequency lowers the effective concentration of the compounds in a manner similar to, but not overlapping, that produced by the depolarized membrane potential. In fact, use-dependent behavior is a complex dynamic process involving the kinetics of drug binding and unbinding to the channel in relation to both state-dependent drug affinity and physicochemical properties; these properties influence the ability of the drug to access the receptor site. To gain insight into this dynamic process, the time course of reaching steady state after a 10-Hz stimulation was determined for each drug at each

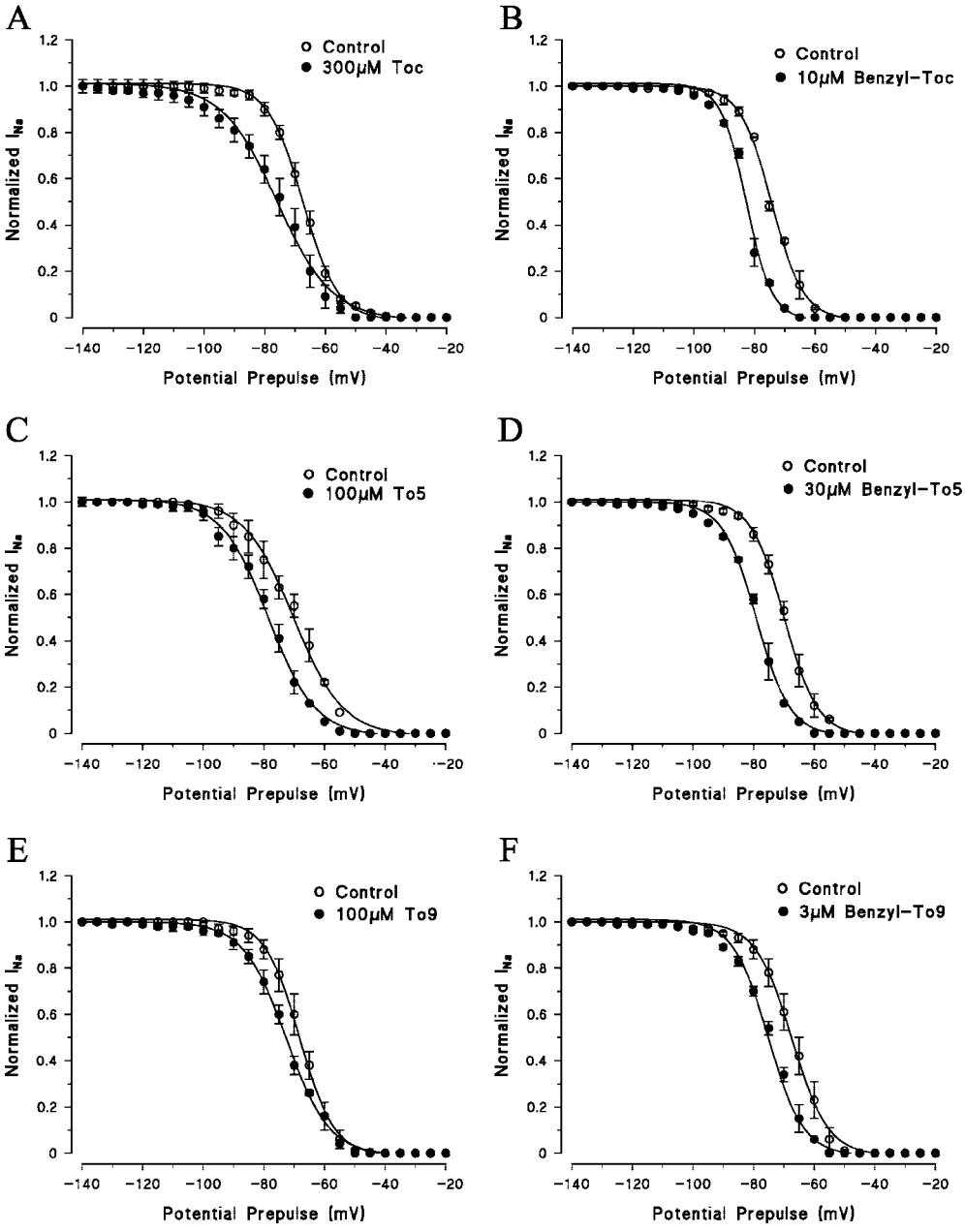

Fig. 3. Steady-state inactivation curves constructed in the absence and in the presence of tocainide and its analogs. For each compound, we used approximately the half-maximal concentration. At each membrane potential, maintained for 1 s, the current amplitude has been normalized to the I_{Namax} value obtained at −140 mV. Each point is the mean ± S.E.M. of four to seven experiments. Curves were fitted by a single Boltzmann distribution that enabled determination of $Vh_{1/2}$, the membrane potential at which 50% of channels are inactivated in the absence and presence of the drug. The values of $Vh_{1/2}$ for each compound is as follows: A, control, −67.4 ± 0.16 mV and 300 μM; Toc, −75.9 ± 0.5 mV; B, control, −74.4 ± 0.23 mV and 10 μM; Benzyl-Toc, −82.7 ± 0.28 mV; C, control, −70.2 ± 0.4 mV and 100 μM; To5, −78.5 ± 0.3 mV; D, control, −69.9 ± 0.2 mV and 30 μM; Benzyl-To5, −79.3 ± 0.3 mV; E, control, −68.2 ± 0.2 mV and 100 μM; To9, −72.8 ± 0.26 mV; F, control, −67.6 ± 0.29 mV and 3 μM; Benzyl-To9, −74.8 ± 0.26 mV. For each compound, the shift observed was significant with $p < 0.001$ by Student's t test.

concentration. The temporal pattern was obtained by normalizing all subsequent current traces versus the first pulse, so as to exclude tonic block, until the steady-state fractional block was reached. Figure 6, A and B, shows the pulse-dependent current reduction at 10 Hz for two extreme compounds in terms of potency of I_{Na} block: tocainide and Benzyl-To9. For both compounds, the steady state was reached within 10 s of stimulation over the 30 s of total train duration, and this time was chosen in Fig. 6 to better show graphically the different time course between the compounds. For each concentration of these two compounds, as well as for all the others, the current reduction was well fitted with a single exponential function that allowed calculation of both the time course to steady state and the fractional use-dependent block from the residual current amplitude. Toc turned out to be the fastest compound, whereas Benzyl-To9, able to produce a notable fractional use-dependent block at much lower concentrations, reaches the steady state with a remarkably slower time course. Figure 6C shows the IC_{50} values of all the compounds for producing the fractional steady-state, use-dependent block. Toc, Benzyl-Toc, and Benzyl-To5 produced a fractional block at 10 Hz with IC_{50} values of about 400, 40, and 25 μM, respectively. These values are similar to those observed for tonic block in Table 2, whereas the gain of potency for absolute use-dependent block for these compounds at 10 Hz, calculated without excluding the contribution of the tonic block (TB), was around 2. For To5, To9, and Benzyl-To9, a significantly lowering of IC_{50} values versus those for tonic block was instead observed, with ratios TB/fractional use-dependent block (fUDB) of 1.8 (To9), 4.6 (To5), and 5.2 (Benzyl-To9). This phenomenon (i.e., the degree of apparent change in drug potency during the high frequency stimulation) seems influenced by the ability of the compounds to equilibrate more or less rapidly with the channel in relation to the interpulse time. This point is addressed in Fig. 6D, which compares the time constants required to reach the steady-state block for the various drugs.

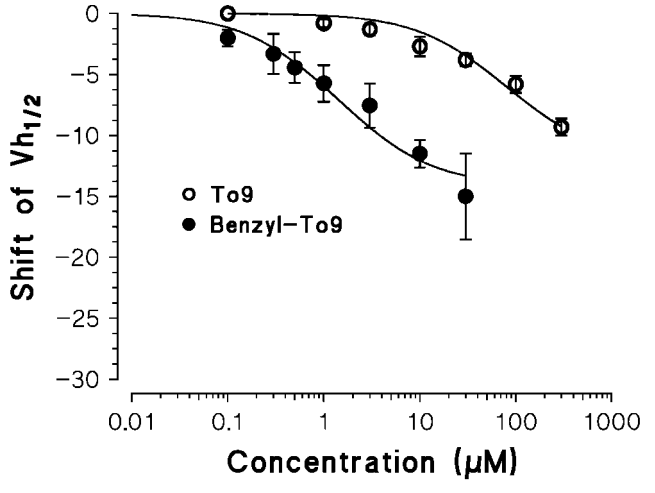

Fig. 4. Concentration-related shift of steady-state inactivation curve for To9 and Benzyl-To9. At each concentration, we evaluated the mean ± S.E.M. (from four to six fibers) of the shift of the h_∞ curve produced by the drug. The experimental points have been fitted to the equation $\Delta Vh_{1/2} = k \ln\{(1 + [drug]/K_r)\ (1 + [drug]/K_i)^{-1}\}$ to calculate the apparent affinity constant for the inactivated state (K_i) (see text). The shifts produced by Benzyl-To9 at 10 and 30 μM are significantly different (by Student's t test) from those produced by the same concentrations of To9 ($p < 0.05$).

Because of the great differences in potency between the compounds, the time constants have been compared at equieffective points (i.e., at concentrations able to produce the same steady-state block). The time constants were inversely proportional with the amount of block and consequently with the drug concentration; thus, the higher the block, the shorter the time constant. Beside this general view, marked differences were observed between the various compounds. Generally, the compounds characterized by a high use-dependent behavior showed long time constants, with values ranging also in the tenths of seconds. However, this phenomenon was not correlated with the absolute potency for either the resting or the inactivated state, because some potent compounds, such as Benzyl-Toc and Benzyl-To5, showed a faster time constant compared with less potent compounds such as To9 and To5. This result suggests the involvement of other determinants in the kinetic of drug block and in use-dependent behavior.

Interpretation of Potency and Use-Dependence in Terms of Physicochemical Properties and Conformational Features. The physicochemical properties of the compounds are shown in Table 3. The ClogP values follow the expected increase in relation to 1) the presence of the proline-like cycle and 2) the presence of the benzyl moiety, whereas the logD undergoes more complex variations, and is highly influenced by pK_a (see below). No linear correlation could be found between these parameters and drug potency, although the strong potency of the benzylated analogs was mirrored by their high lipophilicity. According to our previous hypotheses, this could be related to the presence of the second aromatic ring in a crucial pharmacophore position rather than to lipophilicity by itself (De Luca et al., 2002). In fact, Benzyl-To5, which was the compound with the best "ideal" properties in terms of logP and logD for diffusion through the membrane, was the least potent of the three benzylated analogs. To gain insight in this feature, we performed a molecular modeling study aimed at evaluating the lowest conformation geometry for these compounds. As can be seen in Fig. 8, Benzyl-Toc and Benzyl-To9 assume a similar spatial conformation, with the two benzyl groups in outstretched position. On the contrary, Benzyl-To5 attains stability in a more folded conformation, with the aromatic groups almost facing each other. The calculated distance between the centroid of the aromatic rings turned out to be higher than 8 Å for both Benzyl-Toc and Benzyl To-9, whereas it was >1 Å less for Benzyl-To5. These data strongly corroborate that the higher and similar potency of the two former compounds is related to the similar distance between the two aromatic rings, which allows a similar interaction with the hydrophobic binding pockets.

A large change in estimated pK_a values was also observed. In fact, according to previous data (De Luca et al., 2000; Talon et al., 2001), To5 showed a higher pK_a than Toc, and the homologating of the amino group by one methylene further increases the basicity. As generally observed for tertiary amines compared with secondary amines in an aqueous environment (Aue et al., 1972), Benzyl-To5 and Benzyl-To9 were less basic than the respective nonbenzylated analogs. No linear correlation was found between pK_a and drug affinities for either resting or inactivated channels, so we focused on the relationship between pK_a and use-dependent behavior. In fact, as mentioned in the previous paragraph, this

latter is a dynamic process resulting from state-dependent affinity of the protonated molecule and physicochemical properties that influence drug access to and egress from the binding site. We evaluated the relationship between the use-dependent properties of each compound, expressed as the ratio between TB and fUDB and pK_a. As can be seen in Fig. 8A, no linear correlation could be observed when considering all the compounds ($r^2 = 0.219$). However, the deviation from linearity is mainly caused by the high pK_a value of To9; in fact, when excluding this compound from the fit, the correlation rose to $r^2 = 0.969$. This high correlation needs to be better verified, because the points are relatively few and mostly clustered around two pK_a values. However, the data might suggest that use-dependence is indeed correlated in a gaussian-like manner to the pK_a, with a preferential range of pK_a values leading to the highest use-dependent block. Other than the absolute use-dependent potency, pK_a may influence the kinetic of drug-receptor interaction. To qualitatively test this hypothesis, we evaluated the relation between the time constants to reach the steady-state 20% fractional block of I_{Na} after a train at 10 Hz and the pK_a of the various compounds (Fig. 8A). We found that the cluster of compounds with pK_a of 7.4 to 7.7 were those with shorter time constants (i.e., Toc, Benzyl-Toc, and Benzyl-To5). However, remarkable differences are present between the compounds of this group; the compounds being faster with the following order Toc > Benzyl-Toc > Benzyl-To5. Interestingly, the fastest Toc was also the compound with the lowest affinity for inactivated state, which is believed to contribute to the accumulation of use-dependent blockade. Benzyl-To5, slightly less potent than Benzyl Toc, has a slightly higher p*K*a value (7.7 versus 7.4) which can account for its longer time constant. The increase of pK_a value of one unit was paralleled by a further prolongation of the time constants with respect to the previous group, suggesting that the higher quote of ionized compound required a longer time or a higher number of openings

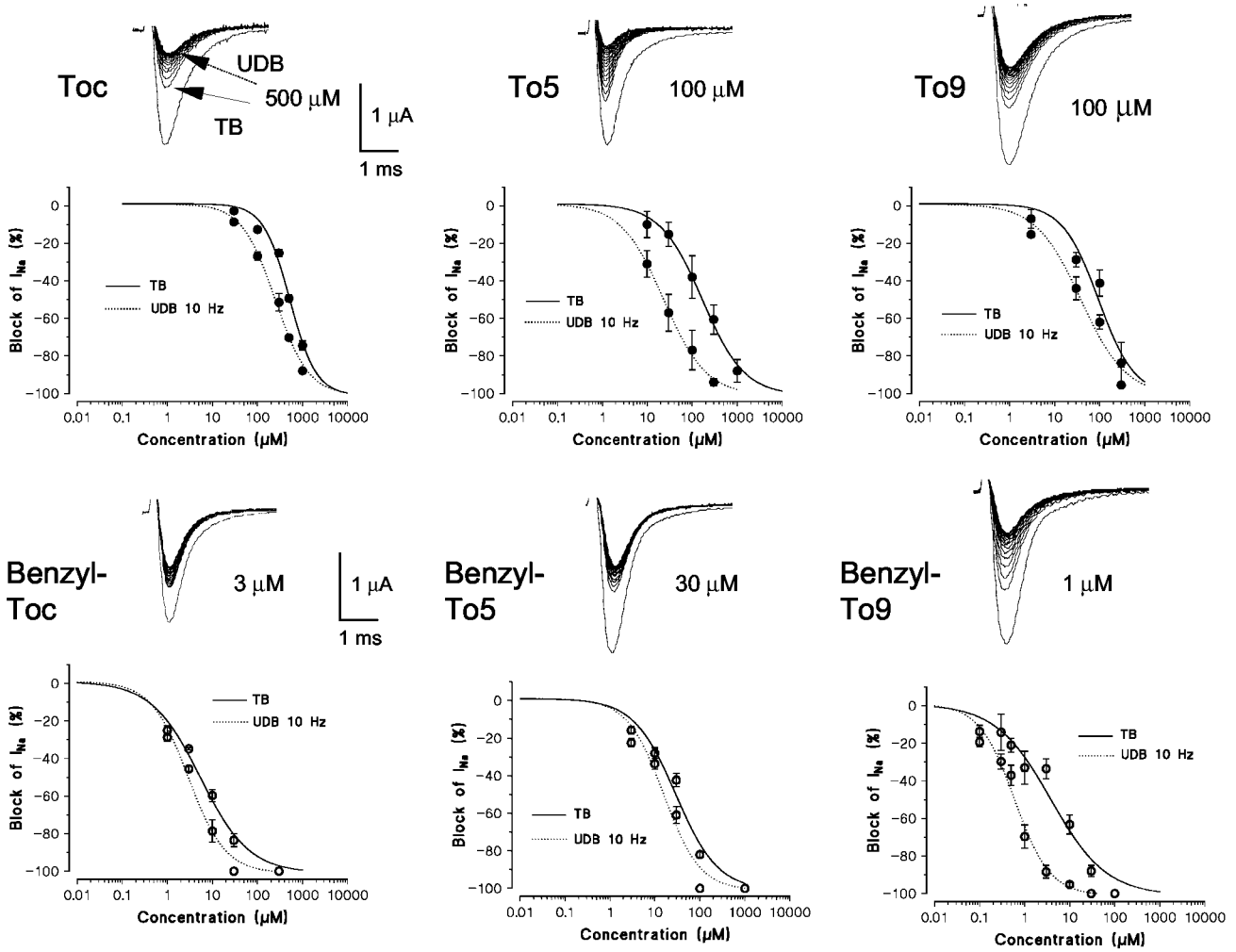

Fig. 5. Traces of sodium current transients recorded by the three Vaseline-gap, voltage-clamp method from single fibers of frog semitendinosus muscle and use-dependent block exerted by tocainide (Toc) and its analogs. In each group of traces, the greatest one has been recorded in the absence of drug, with a depolarizing step from the HP of −100 mV to −20 mV for 10 ms. The same depolarizing stimulus 10 min after the application of the test compound allowed estimation of the tonic block (filled arrow). Afterward, similar depolarizing steps were repetitively applied at a 10-Hz frequency for 20–30 s, which produced an additional cumulative block caused by a use-dependent behavior until a new equilibrium was reached (dashed arrow). The figure shows the use-dependent block exerted by each compound tested near the half-maximal concentration for tonic block. The dose-response curves for tonic (filled lines) and use-dependent block at 10 Hz (dotted lines), calculated as the percentage reduction of current at the steady state versus control, for each compound are shown below the corresponding current trace family. The curves fitting the experimental points were obtained using the logistic function described under *Materials and Methods*. Each value is the mean ± S.E.M. from four to seven fibers of the percentage block of I_{Na} observed in the presence of each concentration of drugs versus I_{Na} in the absence of the drug in the same fiber.

to reach the receptor site (Liu et al., 2003). In this case, it is worth notice that Benzyl-To9 showed a time constant value that was more than 2-fold longer that of To5, probably in relation to the 40-fold higher affinity for the inactivated state of the former. This finding supports the role of a high-affinity drug-receptor interaction in contributing to the kinetic of drug binding. Interestingly, To9, which in the previous analysis was the compound deviating from linear correlation between TB/fUDB ratio and pK_a, had the same K_i values as To5 but a higher pK_a value, which is probably responsible for the

TABLE 2

Use-dependent block of sodium currents by tocainide, proline derivatives, and related benzylated analogs

Potency of drug in producing a use-dependent block of sodium currents. For each drug used, the concentration for determining the 50% block of the current (IC_{50}) is shown, evaluated from the residual current in the presence of drug after 30-s trains of 10-ms test pulses to −20 mV from a holding potential of −100 mV at the frequency of either 2 or 10 Hz. The block at the first pulse was caused by the block of the resting channel before the start of the high-frequency stimulation, and it has been considered tonic block produced at −100 mV of holding potential. The IC_{50} values were obtained during non-linear least squares fits of the concentration-response data to the logistic equation described under *Materials and Methods*. The gain of potency over the tonic block is shown for each compound as the ratio between the IC_{50} values for tonic block and use-dependent block (TB/UDB) at either 2 or 10 Hz. The higher the ratio, the stronger the gain of potency obtained during the high frequency of stimulation.

Compound	Tonic Block (HP −100 mV) IC_{50}	Use-Dependent Block			
		2 Hz		10 Hz	
		IC_{50}	TB/UDB	IC_{50}	TB/UDB
	μM	μM		μM	
Toc	523 ± 32	350 ± 11	1.5	248 ± 14	2.1
Benzyl-Toc	5.4 ± 0.6[a,b]	5.5 ± 0.6[a,b]	0.98	2.9 ± 0.4[a,b]	1.9
To5	168 ± 12[c]	47 ± 4.8[c]	3.6	23.2 ± 1.4[c]	7.2
Benzyl-To5	29.7 ± 4.9[a,b]	29.8 ± 6[a,b]	0.99	15.8 ± 3[a,b]	1.9
To9	94 ± 19[c]	42.1 ± 9.6[c]	2.2	38.2 ± 9[c]	2.5
Benzyl-To9	3.9 ± 0.8[a]	1.2 ± 0.2[a]	3.3	0.50 ± 0.06[a]	7.8

[a] Significant difference of benzyl-analogs with respect to related nonbenzylated parent compounds ($P < 0.05$ for Benzyl-To5; $P < 0.001$ for Benzyl-Toc and Benzyl-To9).
[b] Significant difference of benzyl-analogs with respect to Benzyl-To9 ($P < 0.001$).
[c] Significant difference of proline-like analogs with respect to tocainide ($P < 0.001$).

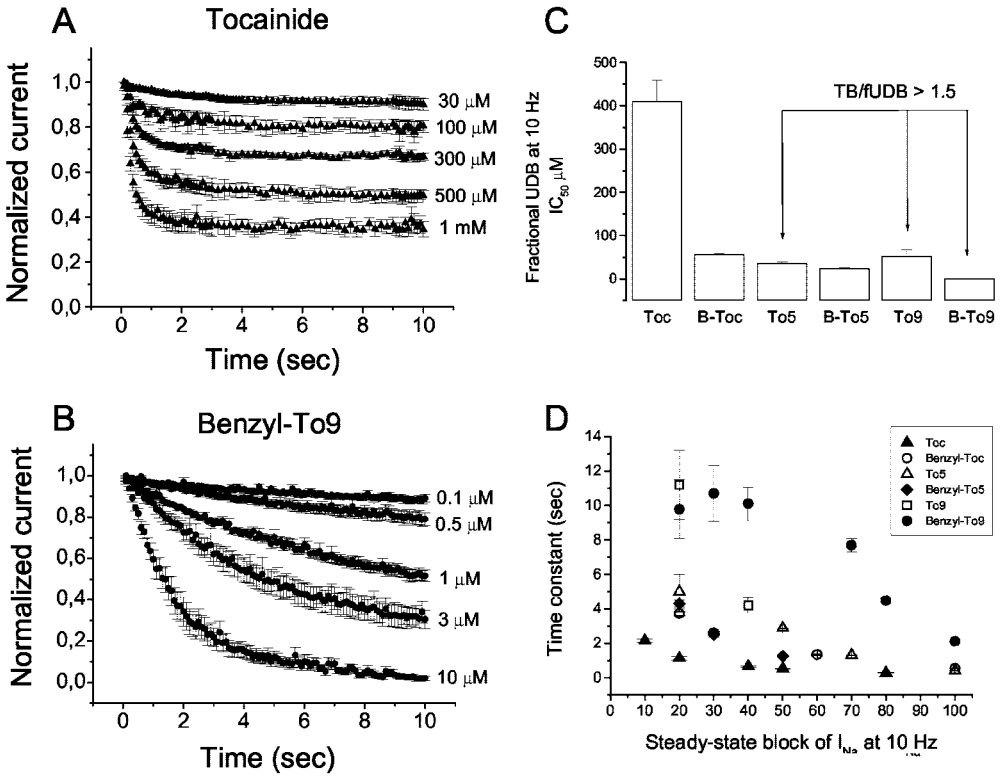

Fig. 6. Time course for development of use-dependent block. A and B, time course of I_{Na} reduction exerted by increasing concentration of Toc and Benzyl-To9 during a 10-Hz stimulation frequency, respectively. Each temporally subsequent current trace has been normalized versus the first one of the train to null the tonic block. Each point is the mean ± S.E.M. from three to six fibers. For graphical resolution, the time course during the first 10-s stimulation time for both compounds is shown; for Toc, which was very quick to reach equilibrium, every other trace shows the interval from 5 to 9 s. For all compounds, the time course followed a first-order kinetic from which we estimated the amount of residual current at the equilibrium and the time constant at each concentration. This analysis allowed estimation of the fractional use-dependent block (current at the steady state versus first pulse) exerted by each compound, excluding the contribution of tonic block. The IC_{50} values ± S.E. calculated from the relative concentration-dependent curves are shown in C. The arrows show the compounds for which the ratio between the IC_{50} value for tonic block (shown in Table 2) and that for fractional use-dependent block (TB/fUDB) are greater that 1.5, thus suggesting a significant gain of potency versus tonic block. D, the time constant values of each compound are compared. Because of the remarkable difference in potency, the time constants have been grouped according to the equieffectiveness of the compounds in producing the fractional block. Each point is the time constant ± S.E. calculated from the exponential fit of the time course of I_{Na} reduction.

remarkable slowing of the time constant. These results again corroborate the idea that lower or higher pK_a values can be detrimental for use-dependence, preventing proper receptor interaction or proper access to the binding site.

Discussion

Our study was aimed at clarifying the molecular determinants of LA-like drugs to find potent and selective blockers of skeletal muscle sodium channels that could be safely used in the treatment of excitability disturbance of patients affected by channelopathies, such as myotonic syndromes and periodic paralyses (Cannon, 1996; Jurkat-Rott and Lehmann-Horn, 2001). Tocainide and mexiletine are in fact among the few drugs clinically used to symptomatically solve myotonic-like hyperexcitability (Rüdel et al., 1994). However, as a general outcome, an amelioration of the therapeutic profile, in terms of potency and use-dependence, may lead to interesting compounds for other excitability disorders (Clare et al., 2000). Site-directed mutagenesis has moved structure-activity relationship studies from drug to protein, and crucial residues for biophysical and pharmacological properties of sodium channels have been identified (Catterall, 2002). To design high-affinity ligands that are more selective, the hypotheses drawn from mutagenesis experiments have to be validated with new compounds synthesized informally. According to this approach, we identified a series of tocainide analogs that are potent voltage- and use-dependent sodium channel blockers with affinity constants for inactivated channels in the submicromolar range. In particular, potency and use-dependent behavior were strongly increased by 1) constraining the amino terminal group in a rigid proline-like cycle and/or 2) introducing a benzyl moiety on the amino terminal group. The advantage in constraining the molecule in a rigid proline-like cycle (Franchini et al., 2000; Talon et al., 2001) was confirmed. Even in racemic form, To5, up to 10-fold more potent than tocainide, was one of the most use-dependent analogs, thus accounting for its highly effective antimyotonic activity in the adr/adr mouse model (De Luca et al., 1997b; Conte Camerino et al., 2000; Talon et al., 2001). The simple change of the α- with a β-proline cycle did not substantially change the drug potency and produced a decrease in the use-dependent behavior, probably in relation to less favorable physicochemical properties (see below). The most interesting observation was that the introduction of a benzyl group on the pharmacophore amino terminal, a structural change that has been exploited to a limited degree in

TABLE 3

Physicochemical parameters of tocainide and its analogs evaluated during conformational modeling

The pK_a values and the distances between the two centroids have been theoretically derived as described in detail under *Materials and Methods*. The pK_a values of the newly synthesized compounds were calculated by interpolating the ΔE of protonation calculated for the newly synthesized compounds to a straight-line plot correlating the experimental pK_a values and ΔE of protonation previously determined for a training set formed by eight nitrogenous bases. All calculations and graphical representations were performed using the SPARTAN PRO software package. Theoretical ClogP values (log value of the octanol/water partition coefficient) were obtained by using C log Software. LogD values for each compound was obtained at physiological pH, from log P and pK_a value, according to the classic equation, as described under *Materials and Methods*.

Compound	pK_a	ClogP	Log D	Distance Aromatic Rings
				Å
Toc	7.7	0.3	−0.2	
Benzyl-Toc	7.4	2.5	2.2	8.4
To5	8.7	1.0	−0.4	
Benzyl-To5	7.7	3.3	2.8	7.0
To9	9.7	0.7	−1.6	
Benzyl-To9	8.7	3.0	1.7	8.8

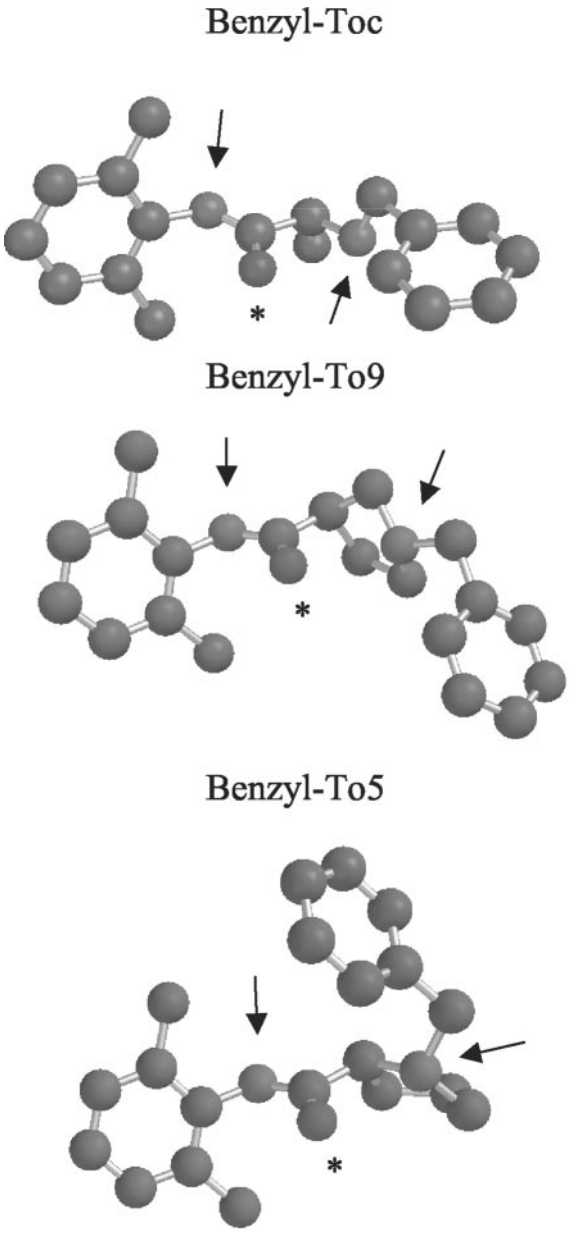

Fig. 7. Lowest energy conformation and spatial geometry of protonated forms of the benzylated analogs Benzyl-Toc, Benzyl-To9, and Benzyl-To5. Models were constructed using the protonated forms, assuming the starting geometries to be similar to that found for lidocaine hydrochloride monohydrate crystal structure. Equilibrium geometries were calculated starting with a systematic conformer distribution analysis. Conformers were grouped into families based on relevant torsion angle values. The most stable representative of each family was submitted to an AM1 semiempirical geometry optimization and the best HF/3-21G(*)//AM1 conformer was optimized at the 3-21G(*) level. As can be seen, Benzyl-Toc and Benzyl-To9 assume a similar spatial geometry, with the two aromatic rings in the outstretched position, although the second, the benzyl moiety linked to the rigid proline-like cycle, was a bit more constrained. In contrast, the Benzyl-To5 assumes a more folded geometry, with the two aromatic rings almost facing each other. The asterisk indicates the O atom; the arrows show the nitrogen atoms. All calculations and graphical representations were performed using the SPARTAN PRO software package.

local anesthetic compounds, increases drug potency up to hundred times. Previous data favor the amelioration of drug affinity by inserting a second aromatic moiety in the structure, probably for the establishment of specific hydrophobic interactions with the binding site (De Luca et al., 2000, 2003; Kuo et al., 2000; Desaphy et al., 2001; Yang and Kuo, 2002). This view is supported by the following results from the present study: 1) the presence of the benzyl group directly on the pharmacophore amino group led to the highest increase in potency observed so far and 2) the three benzylated compounds differ in potency in relation to the spatial conformation and the distance between the two aromatic groups. Both Benzyl-Toc and Benzyl-To9 are hundreds times more potent than tocainide. Their similar potency is mirrored by a similar spatial conformation and a similar distance of about 8.5 Å between the aromatic moieties. Accordingly, Benzyl-To5, the least potent of newly synthesized benzylated compounds, assumes a more folded geometry, with a minor distance between the two aromatic groups. Thus, two aromatic moieties, with a specific spatial orientation, are pivotal for the interaction with the receptor site, probably establishing π-π interactions (Dougherty, 1996) with the two aromatic Phe and Tyr residues on D4-S6 contributing to the binding site (Ragsdale et al., 1996; Wright et al., 1998). The calculated distance between the two aromatic rings is in line with a proper interaction at this level, because the two amino acids are spaced of about two turns of the α helix at an ideal distance of 11 Å (Ragsdale et al., 1994), which is likely to be less in relation to the nature of the amino acids composing the helix, of their side groups, and of the state-dependent conformational changes. Other data with less potent diphenyl compounds support the importance of the spatial disposition between the two aromatic rings for exerting the block of sodium channel (Brown et al., 1999; Kuo et al., 2000). However, we cannot exclude the idea that one of the aromatic rings establishes a π-cation interaction with a positively charged residue. A possible candidate is the Lys1273 in the selectivity filter, which has been found to affect LA binding (Sunami et al., 1997). Alternatively, the higher potency could be related to the doubled likelihood that a hydrophobic interaction would occur in the presence of a second aromatic moiety. The data available do not allow us to draw clear conclusions from this regard. However, following the approach recently used by Yang and Kuo (2002), we estimated that the gain in affinity during either resting or inactivated state block observed with Benzyl-To9 with respect to the nonbenzylated To9 is paralleled by an increase in binding energy of about 2 kcal/mol. This value corresponds to the energy level generally displayed by a π-π interaction (McGaughey et al., 1998), thus supporting the hypothesis that the second aromatic ring independently and specifically reinforces the interaction with the binding site. According to this hypothesis, the structural changes introduced allow a better interaction with residues involved in block of resting channel as well as in the tonic block (i.e., the noncumulative current reduction observed in low-frequency stimulation). Furthermore, the presence of a second aromatic ring linked to the pharmacophore amino group directly influences inactivated and use-dependent channel block through the change in the physicochemical properties and of the steric structure of the molecule. A parallel increase in lipophilicity and molecular dimension can help reinforce hydrophobic interactions during state-dependent conformational change of the channel vestibule and slow the kinetic to reach a new equilibrium between free and drug-bound channels (Yarov-Yarovoy et al., 2001). In addition, a change in the pK_a of the basic amine can in turn contribute to the inactivated and use-dependent behavior, on the basis of the state-dependent affinity of protonated amine and drug ability to access to and egress from the receptor (De Luca et al., 1991, 1997a; Catterall, 2002; Liu et al., 2003). In this study, we confirmed that the high use-dependent behavior of To5 (Talon et al., 2001) is paralleled by a high estimated pK_a value, which slows down

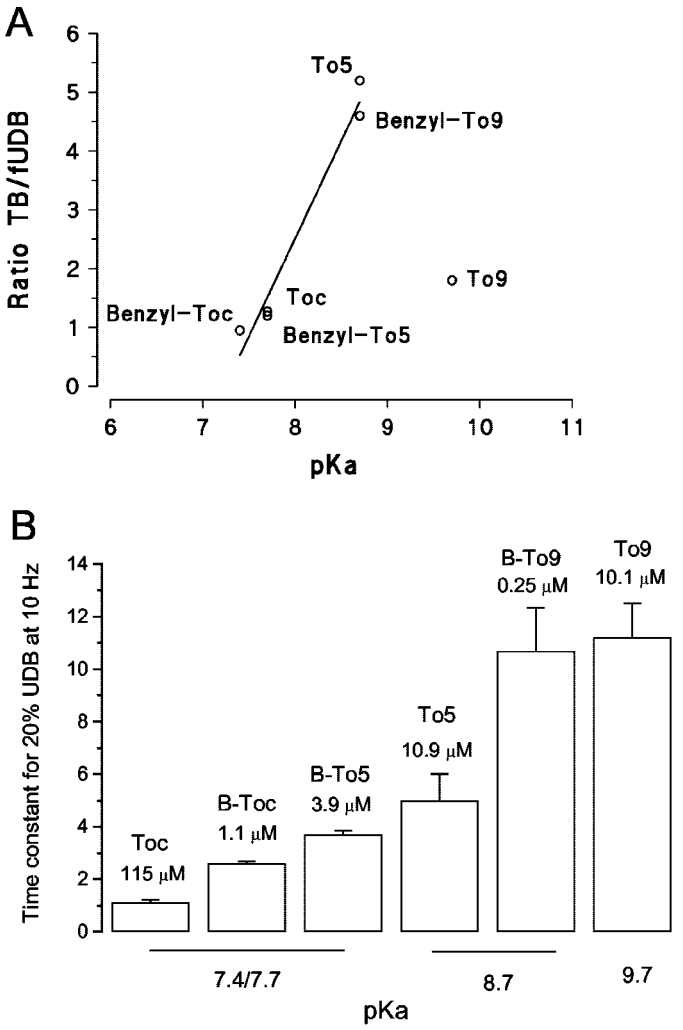

Fig. 8. A, evaluation of the relationship between use-dependent behavior (taken as the ratio between IC_{50} values calculated during tonic and use-dependent block at 10 Hz) and pK_a of the compounds. The pK_a values of the newly synthesized compounds were calculated as described under *Materials and Methods*. When considering all values, no correlation was found between the two parameters. However, when excluding To9 (open circle) which shows less use-dependent behavior and high pK_a values, a linear correlation was found ($r^2 = 0.969$). However, such a correlation has to be taken with caution, because the limited data are almost distributed in two clusters. All points are well correlated by a polynomial function, indicative of a gaussian-like distribution, but they are insufficient to allow a definitive conclusion at this point. B, pK_a and development of use-dependent block. Each bar is the time constant of each drug for producing a 20% fractional use-dependent block at 10 Hz, grouped according to pK_a values. Above the bars (shown as values ± S.E. estimated from the exponential fit described in Fig. 6) are indicated the calculated values for inactivation state affinity (see Table 1), which is the other parameter that influences use-dependent behavior.

the kinetic of use-dependent block with respect to compounds such as Benzyl-Toc and Benzyl-To5, both of which have a higher affinity for both resting and inactivated states but a pK_a value that is one unit lower. The relation between use-dependent properties and basicity of the amine group was also straightforward between the benzylated derivatives. Benzyl-To9 and Benzyl-Toc had an overlapping potency, but the former showed a higher and slower use-dependent behavior, almost comparable with that of To5, whereas Benzyl-Toc resembled its unbenzylated parent compound in this respect. In fact, Benzyl-To9 has an estimated pK_a value almost overlapping that of To5, whereas the pK_a of Benzyl-Toc was comparable with that of tocainide. The pK_a value has to be within a certain range for the optimal use dependence. In fact, To9, which, as expected, was the most basic among the analogs, showed a decrease rather than an increase of the use-dependent behavior. The protonated amino group can interact with other residues described to contribute to LA binding. Most of them have been identified on S6 segments of the other domains and are either apolar aliphatic, such as leucine and isoleucine, or polar, such as asparagine and serine (Nau et al., 1999; Wang et al., 2000; Yarov-Yarovoy et al., 2001; 2002). Although these residues seem rather "secondary" with respect to the two on D4-S6, they contribute toward stabilizing the binding of the drug during conformational transitions (Yarov-Yarovoy et al., 2001). The asparagine at 434 on D1 (in the skeletal muscle isoform), involved in the stereoselective interaction, can directly establish hydrogen-bonds with the positive charged moiety of LAs during channel inactivation (Nau et al., 1999). A further kinetic analysis of the recovery from inactivation will be pivotal in clarifying the contribution of drug affinity to inactivated state in determining the use-dependent behavior of the test compounds (De Luca et al., 1991, 1997a). However, a preliminary evaluation of the time course of recovery from use-dependent block of equieffective concentrations of To5 and Toc showed that the former is only slightly slower in recovering (data not shown), whereas we have shown herein that To5 is surely slower in the development of the use-dependent block, probably in relation to the higher fraction of charged molecule, which hinders a rapid equilibration with the internal receptor site. This observation reinforces the view that use-dependence is a complex melange between parallel changes in affinity and physicochemical properties (Liu et al., 2003). Thus we propose that the two aromatic moieties with a correct distance interact properly with the two hydrophobic pockets in D4-S6, whereas the protonated secondary or tertiary amine, near the chiral center, can establish the third and stereoselective bond. The importance of the amino group can be highly enhanced if one of its substituents is the second aromatic ring.

Based on their high-affinity, state-dependent block, the newly synthesized compounds may have a wide and rational therapeutic use. Specific residues and gating differences account for the higher affinity of cardiac versus skeletal muscle and nerve sodium channel (Wang et al., 1996; Li et al., 2002). Also, others' and our results have shown that this class of compounds can selectively suppress the abnormal opening of myotonia-causing sodium channel mutants, based on a combination of their state-dependent action and mutation-related gating differences (Weckbecker et al., 2000; Desaphy et al., 2001; Takahashi and Cannon, 2001). Consequently, the newly synthesized compounds may offer an improvement of the therapeutic profile and selectivity with respect to tocainide.

In particular, the *N*-benzylated analogs have a combined increase of potency and use-dependence and can selectively address pathological signs at very low dosages. In parallel, the increase in lipophilicity may also offer advantages in pharmacokinetic properties and tissue distribution, whereas the more constrained conformation of the proline-like analogs may help the validation of the presumed map of residues involved in drug binding.

References

Aldrich RW, Corey DP, and Stevens CF (1983) A reinterpretation of mammalian sodium channel gating based on single channel recording. *Nature (Lond)* **306:**436–441.

Aue DH, Webb HM, and Bowers MT (1972) A thermodynamic analysis of solvation effects on the basicities of alkylamines. An electrostatic analysis of substituent effects. *J Am Chem Soc* **98:**318–329.

Bean PB, Cohen CJ, and Tsien RW (1983) Lidocaine block of cardiac sodium channels. *J Gen Physiol* **81:**613–642.

Brown ML, Zha CC, Van Dyke CC, Brown GB, and Brouillette WJ (1999) Comparative molecular field analysis of hydantoin binding to the neuronal voltage-dependent sodium channel. *J Med Chem* **42:**1537–1545.

Cannon SC (1996) Ion-channel defects and aberrant excitability in myotonia and periodic paralysis. *Trends Neurosci* **19:**3–10.

Catterall WA (2002) Molecular mechanisms of gating and drug block of sodium channels. *Novartis Found Symp* **241:**206–218.

Chambers CC, Hawkins GD, Cramer CJ, and Truhlar DG (1996) Model for aqueous solvation based on class IV atomic charges and first solvation shell effects. *J Phys Chem* **100:**16385–16398.

Clare JJ, Tate SN, Nobbs M, and Romanos MA (2000) Voltage-gated sodium channels as therapeutic targets. *Drug Discov Today* **5:**506–520.

Conte Camerino D, Pierno S, De Luca A, and Bryant SH (2000) Antimyotonic effects of tocainide enantiomers on skeletal muscle fibers of congenitally myotonic goats. *Neuromuscul Disord* **10:**160–164.

De Luca A, Natuzzi F, Desaphy JF, Loni G, Lentini G, Franchini C, Tortorella V, and Conte Camerino D (2000) Molecular determinants of mexiletine structure for potent and use-dependent block of skeletal muscle sodium channels. *Mol Pharmacol* **57:**268–277.

De Luca A, Natuzzi F, Falcone G, Duranti A, Lentini G, Franchini C, Tortorella V, and Conte Camerino D (1997a) Inhibition of frog skeletal muscle sodium channels by newly synthesized chiral derivatives of mexiletine and tocainide. *Naunyn-Schmiedeberg's Arch Pharmacol* **356:**777–787.

De Luca A, Pierno S, Natuzzi F, Franchini C, Duranti A, Lentini G, Tortorella V, Jockusch H, and Conte Camerino D (1997b) Evaluation of the antimyotonic activity of mexiletine and some new analogs on sodium currents of single muscle fibers and on the abnormal excitability of the myotonic ADR mouse. *J Pharmacol Exp Ther* **282:**93–100.

De Luca A, Pröbstle T, Brinkmeier H, and Rüdel R (1991) The different use dependences of tocainide and benzocaine are correlated with different effects on sodium channel inactivation. *Naunyn-Schmiedeberg's Arch Pharmacol* **344:**596–601.

De Luca A, Talon S, De Bellis M, Desaphy JF, Franchini C, Lentini G, Catalano A, Corbo F, Tortorella V, and Conte Camerino D (2003) Inhibition of skeletal muscle sodium currents by mexiletine analogues: specific hydrophobic interactions rather than lipophilia per se account for drug therapeutic profile. *Naunyn-Schmiedeberg's Arch Pharmacol* **367:**318–327.

Desaphy JF, De Luca A, Tortorella P, De Vito D, George AL Jr, and Conte Camerino D (2001) Gating of myotonic Na channel mutants defines the response to mexiletine and a potent derivative. *Neurology* **57:**1849–1857.

Dougherty DA (1996) Cation-π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science (Wash DC)* **271:**163–168.

D'Souza F, Zandler ME, Deviprasad GR, and Kutner W (2000) Acid–base properties of fulleropyrrolidines: experimental and theoretical investigations. *J Phys Chem A* **104:**6887–6893.

Franchini C, Corbo F, Lentini G, Bruno G, Scilimati A, Tortorella V, Conte Camerino D, and De Luca A (2000) Synthesis of new 2,6-prolylxylidide analogues of tocainide as stereoselective blockers of voltage-gated Na^+ channels with increased potency and improved use-dependent activity. *J Med Chem* **43:**3792–3798.

Hanson AW and Röhrl M (1972) The crystal structure of lidocaine hydrochloride monohydrate. *Acta Crystallogr Sec B* **28:**3567–3571.

Hille B and Campbell DT (1976) An improved Vaseline gap voltage clamp for skeletal muscle fibers. *J Gen Physiol* **67:**265–293.

Jurkat-Rott K and Lehmann-Horn F (2001) Human muscle voltage-gated ion channels and hereditary disease. *Curr Opin Pharmacol* **1:**280–287.

Kuo CC, Huang RC, and Lou BS (2000) Inhibition of Na^+ current by diphenhydramine and other diphenyl compounds: molecular determinants of selective binding to the inactivated channels. *Mol Pharmacol* **57:**135–143.

Li HL, Galue A, Meadows L, and Ragsdale DS (1999) A molecular basis for the different local anesthetic affinities of resting versus open and inactivated states of the sodium channel. *Mol Pharmacol* **55:**134–141.

Li RA, Ennis IL, Tomaselli GF, and Marban E (2002) Structural basis of differences in isoform-specific gating and lidocaine block between cardiac and skeletal muscle sodium channels. *Mol Pharmacol* **61:**136–141.

Liu H, Atkins J, and Kass RS (2003) Common molecular determinants of flecainide

and lidocaine block of heart Na^+ channels: evidence from experiments with neutral and quaternary flecainide analogues. *J Gen Physiol* **121:**199–214.
McGaughey GB, Gagnés M, and Rappé AK (1998) π-stacking interactions. *J Biol Chem* **273:**15458–15463.
Nau C, Wang SY, Strichartz GR, and Wang GK (1999) Point mutations at N434 in D1–S6 of Mu1 Na^+ channels modulate binding affinity and stereoselectivity of local anesthetic enantiomers. *Mol Pharmacol* **56:**404–413.
Ragsdale DS, McPhee JC, Scheuer T, and Catterall WA (1994) Molecular determinants of state-dependent block of Na^+ channels by local anesthetics. *Science (Wash DC)* **265:**1724–1728.
Ragsdale DS, McPhee JC, Scheuer T, and Catterall WA (1996) Common molecular determinants of local anesthetic, antiarrhythmic and anticonvulsant block of voltage-gated Na^+ channels. *Proc Natl Acad Sci USA* **93:**9270–9275.
Rüdel R, Lehmann-Horn F, and Ricker K (1994) The non-dystrophic myotonias, in *Myology* (Engel AG and Franzini-Armstrong C eds) pp 1291–1302, McGraw-Hill Companies, New York.
Sunami A, Dudley SC Jr, and Fozzard HA (1997) Sodium channel selectivity filter regulates antiarrhythmic drug binding. *Proc Natl Acad Sci USA* **94:**14126–14131.
Takahashi MP and Cannon SC (2001) Mexiletine block of disease-associated mutations in S6 segments of the human skeletal muscle Na^+ channel. *J Physiol (Lond)* **537:**701–714.
Talon S, De Luca A, De Bellis M, Desaphy JF, Lentini G, Scilimati A, Corbo F, Franchini C, Tortorella P, Jockusch H, et al. (2001) Increased rigidity of the chiral centre of tocainide favours stereoselectivity and use-dependent block of skeletal muscle Na^+ channels enhancing the antimyotonic activity in vivo. *Br J Pharmacol* **134:**1523–1531.
Wang DW, Nie L, George AL Jr, and Bennett PB (1996) Distinct local anesthetic affinities in Na^+ channel subtypes. *Biophys J* **70:**1700–1708.
Wang SY, Nau C, and Wang GK (2000) Residues in Na^+ channel D3–S6 segment modulate both batrachotoxin and local anesthetic affinities. *Biophys J* **79:**1379–1387.
Weckbecker K, Wurz A, Mohammadi B, Mansuroglu T, George AL Jr, Lerche H, Dengler R, Lehmann-Horn F, and Mitrovic N (2000) Different effects of mexiletine on two mutant sodium channels causing paramyotonia congenita and hyperkalemic periodic paralysis. *Neuromuscul Disord* **10:**31–39.
Wright SN, Wang SY, and Wang GK (1998) Lysine point mutations in Na^+ channel D4–S6 reduce inactivated channel block by local anesthetics. *Mol Pharmacol* **54:**733–739.
Yang Y-C and Kuo C-C (2002) Inhibition of Na^+ current by imipramine and related compounds: different binding kinetics as an inactivation stabilizer and as open channel blocker. *Mol Pharmacol* **62:**1228–1237.
Yarov-Yarovoy V, Brown J, Sharp EM, Clare JJ, Scheuer T, and Catterall WA (2001) Molecular determinants of voltage-dependent gating and binding of pore-blocking drugs in transmembrane segment IIIS6 of the Na^+ channel α subunit. *J Biol Chem* **276:**20–27.
Yarov-Yarovoy V, McPhee JC, Idsvoog D, Pate C, Scheuer T, and Catterall WA (2002) Role of amino acid residues in transmembrane segments IS6 and IIS6 of the Na^+ channel α subunit in voltage-dependent gating and drug block. *J Biol Chem* **277:**35393–35401.

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