Use-Dependent Inhibition of Na^+ Currents by Benzocaine Homologs

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ABSTRACT Most local anesthetics (LAs) elicit use-dependent inhibition of Na^+ currents when excitable membranes are stimulated repetitively. One exception to this rule is benzocaine, a neutral LA that fails to produce appreciable use-dependent inhibition. In this study, we have examined the use-dependent phenomenon of three benzocaine homologs: ethyl 4-diethylaminobenzoate, ethyl 4-ethoxybenzoate, and ethyl 4-hydroxybenzoate. Ethyl 4-hydroxybenzoate at 1 mM, like benzocaine, elicited little use-dependent inhibition of Na^+ currents, whereas ethyl 4-diethylaminobenzoate at 0.15 mM and ethyl 4-ethoxybenzoate at 0.5 mM elicited substantial use-dependent inhibition—up to 55% of peak Na^+ currents were inhibited by repetitive depolarizations at 5 Hz. Each of these compounds produced significant tonic block of Na^+ currents at rest and shifted the steady-state inactivation curve (h_∞) toward the hyperpolarizing direction. Kinetic analyses showed that the decaying phase of Na^+ currents during a depolarizing pulse was significantly accelerated by all drugs, thus suggesting that these drugs also block the activated channel. The recovery time course for the use-dependent inhibition of Na^+ currents was relatively slow, with time constants of 6.8 and 4.4 s for ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate, respectively. We conclude that benzocaine and 4-hydroxybenzoate interact with the open and inactivated channels during repetitive pulses, but during the interpulse the complex dissociates too fast to accumulate sufficient use-dependent block of Na^+ currents. In contrast, ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate dissociate slowly from their binding site and consequently elicit significant use-dependent block. A common LA binding site suffices to explain the presence and absence of use-dependent block by benzocaine homologs during repetitive pulses.

INTRODUCTION

Benzocaine is a neutral local anesthetic (LA) that blocks the propagation of action potentials in excitable membranes. The pK_a value of benzocaine is 2.54, an indication that benzocaine is not protonated at physiological conditions (less than 0.01%). The interactions between benzocaine and the voltage-gated Na^+ channel have been extensively studied over the last two decades (for a review see Butterworth and Strichartz, 1990). Benzocaine at moderate concentrations (0.1 to 1 mM) inhibits both Na^+ and gating currents tonically. During repetitive depolarizations benzocaine elicits little use-dependent block of Na^+ or gating currents (Hille, 1977; Neumcke et al., 1981; Schneider and Dubois, 1986). In contrast, tertiary amine LAs and their quaternary derivatives elicit tonic and use-dependent block when the membrane is depolarized infrequently (<0.1 Hz) and repetitively (≥1 Hz), respectively (Strichartz, 1973; Courtney, 1975; Hille, 1977; Cahalan and Almers, 1979). Because quaternary LAs elicit use-dependent block of Na^+ and gating currents, it is suggested that the charged form of tertiary amine LAs is necessary for such a block (e.g., see Schwarz et al., 1977; but see Chernoff and Strichartz, 1989).

There are two hypotheses that can account for the use-dependent block. 1) According to Hille's modulated receptor hypothesis (Hille, 1977), different states of the Na^+ channel may exhibit different affinities for LAs. LAs are proposed to have higher affinities to open and inactivated states than to the resting state. As a result, during repetitive depolarizations Na^+ channels frequently enter their open and inactivated states, which in turn interact more strongly with LAs than the resting state. 2) According to the guarded receptor hypothesis (Starmer et al., 1984), the binding affinity is constant for LAs in the Na^+ channel, but the activation and/or inactivation gate may modulate the access of LAs to their binding site. Upon depolarization, conducting open channels with both gates open leave the LA binding site unguarded and thus accessible to LAs, whereas nonconducting closed channels with gate(s) in the closed conformation act to restrict drug access and possibly to trap charged LAs in LA-bound channels. Both of these hypotheses suggest that there is a common binding site for tertiary amine LAs.

The purpose of this study is to examine why benzocaine fails to elicit significant use-dependent block. Because the structure of benzocaine is relatively simple, it may also be possible to determine the structural basis that prevents it from producing use-dependent block. What are the explanations for the lack of use-dependent block of Na^+ currents by benzocaine? First, benzocaine may occupy a binding site that is distinct from the binding site for tertiary amine LAs (e.g., Huang and Ehrenstein, 1981). Second, the recovery time course of benzocaine is fast (too fast to accumulate the use-dependent block) and is comparable to the recovery time course of fast Na^+ inactivation (Hille, 1977; Yeh and Tanguy, 1985). As a result, upon dissociation neutral benzocaine simply diffuses away through a hydrophobic pathway. One approach to discerning these two alternatives is to use various benzo-

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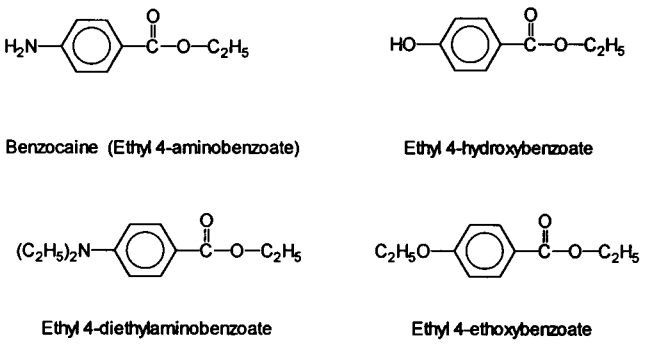
caine homologs to probe the binding site of benzocaine. If the lack of use-dependent block of benzocaine is due to its distinct binding site from other tertiary amine LA binding sites, all benzocaine homologs may exhibit little use-dependent block of Na^+ currents. In this study, we have taken this approach and determined the use-dependent characteristics of benzocaine and its homologs, ethyl 4-diethylaminobenzoate, ethyl 4-ethoxybenzoate, and ethyl 4-hydroxybenzoate.

MATERIALS AND METHODS

Chemicals

Benzocaine was purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl 4-diethylaminobenzoate, ethyl 4-ethoxybenzoate, and ethyl 4-hydroxybenzoate were obtained from Pfaltz and Bauer, Inc. (Waterbury, CT). The chemical structures of these homologs are shown as follows:



Each compound was dissolved in dimethyl sulfoxide (DMSO) at 100–200 mM stock concentration. Stock solutions were stored at 4°C. The final concentration of DMSO was ≤1%. DMSO at 0.15, 0.25, 0.5, and 1% has little effect on Na^+ currents in GH_3 cells ($n = 3$ at each concentration). All other chemicals were reagent grade from commercial sources.

Cell culture and whole-cell voltage clamp

GH_3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1× glutamine (Gibco Laboratories, Grand Island, NY). Cells were plated on 35-mm tissue culture dishes (Falcon Plastics, Cockeysville, MD). Experiments were carried out 1–4 days after plating, with cell density ~30–80% confluent.

The whole-cell variant of the patch clamp method (Hamill et al., 1981) was used to measure Na^+ currents in GH_3 cells. The 35-mm culture dish was rinsed and used as a recording chamber (containing ~0.5 ml solution), which was continuously perfused with the external solution containing (mM): 150 choline·Cl, 0.2 $CdCl_2$, 2 $CaCl_2$, and 10 HEPES adjusted to pH 7.4 with tetramethylammonium hydroxide. Micropipettes were fabricated from borosilicate capillary tubing and had a tip resistance of ~1 MΩ when filled with an internal high-Na^+ solution containing (mM): 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. Under this reversed Na^+ gradient condition, outward I_{Na} is easily visible at all voltages and remains stable up to 45- to 60-min recording time. At the whole-cell configuration, cells were generally allowed to equilibrate with the pipette solution for 5–15 min before experiments.

Data acquisition and analysis

The recording system consisted of a List EPC-7 amplifier (Medical Systems Corp., Greenvale, NY), a homemade leak subtractor, and an

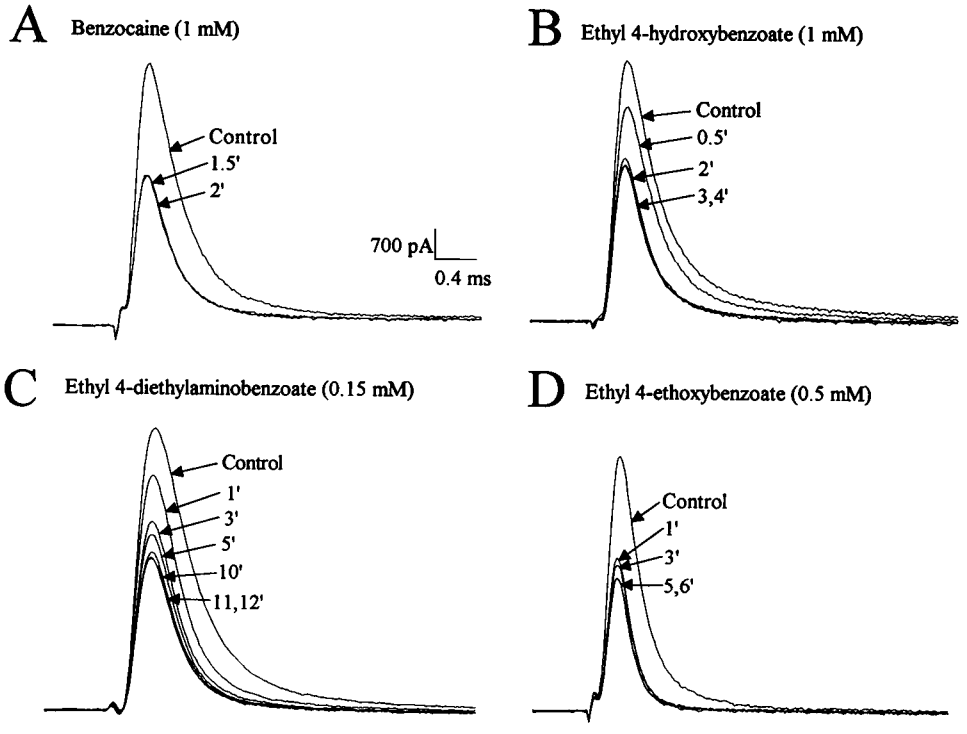


FIGURE 1 Tonic inhibition of Na^+ currents by benzocaine and its homologs. The tonic effects of benzocaine (1 mM), ethyl 4-hydroxybenzoate (1 mM), ethyl 4-diethylaminobenzoate (0.15 mM), and ethyl 4-ethoxybenzoate (0.5 mM) on Na^+ currents were examined in GH_3 cells under reversed Na^+ gradient. Tonic block of Na^+ currents with use of benzocaine (*A*) and ethyl 4-hydroxybenzoate (*B*) was achieved very quickly, after approximately 2 and 6 min, respectively. (*C*) A duration of 11–25 min (mean = 17.6 ± 1.1 min, $n = 23$) is needed to achieve tonic block with ethyl 4-diethylaminobenzoate. (*D*) Tonic block of Na^+ current with ethyl 4-ethoxybenzoate occurred 10.7 ± 2.1 min ($n = 14$) after drug administration. The holding potential was set at −100 mV. Prepulse was set at −130 mV for 100 ms, followed by an interpulse of −100 mV for 0.2 ms to reset the fast activation gate, then a test pulse of +30 mV for 4 ms. Notice that at the drug concentrations used, approximately 40–50% of the maximum current was inhibited by the time tonic block was achieved; tonic block for experiments in *A–D* and other cells was 36.7 ± 4.4% ($n = 3$), 45.6 ± 4.6% ($n = 5$), 54.3 ± 4.2% ($n = 20$), and 51.9 ± 4.6% ($n = 13$), respectively.

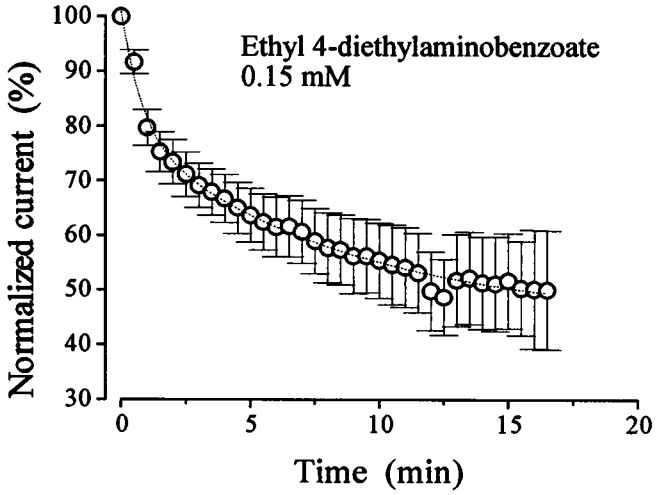


FIGURE 2 The time course of ethyl 4-diethylaminobenzoate block. The currents before and after ethyl 4-diethylaminobenzoate at 0.15 mM were measured at 30-s intervals by the pulse protocol as described in Fig. 1. The peak currents were normalized and plotted against time. The curve was best fitted with a 2-exponential equation. There is a fast phase ($\tau 1$ = 0.79 ± 0.17 min, n = 7, with a component of 21.8 ± 2.8%) and a slow phase ($\tau 2$ = 8.5 ± 1.9 min, with a component of 34.7 ± 1.5%).

IBM-AT computer interfaced by a 125-kHz Labmaster board (Scientific Solutions, Solon, OH). Creation of voltage clamp pulses, data acquisition, and analysis were performed with pClamp software (Axon Instruments, Foster City, CA). On some occasions, leak and capacitance currents were further subtracted by P/-4 protocol (e.g., Figs. 1 and 5). This procedure was omitted whenever possible because of its potential effect on binding interactions and because of its limitation on the maximal frequency one could apply. In particular, when repetitive pulses were applied to the cell no P/-4 protocol was used. Peak Na^+ current was measured at 10-s intervals in steady-state Na^+ channel inactivation (h_∞) protocol. No significant differences were found with a 20-s interval protocol. Series resistance and capacitance current were compensated for by the List EPC-7 device; ~50–80% of series resistance was compensated for. Curve fitting was performed with the Levenberg-Marquardt algorithm (Marquardt, 1963). Results are expressed as mean ± SE.

RESULTS

Tonic inhibition of Na^+ current by benzocaine and its homologs

Benzocaine and its three homologs, without exception, inhibited the peak amplitude of Na^+ currents at moderate concentrations. Benzocaine and ethyl 4-hydroxybenzoate at 1 mM acted rather rapidly: within 2–6 min after application, current reduction reached its steady-state level (Fig. 1, *A* and *B*). In comparison, ethyl 4-diethylaminobenzoate at 0.15 mM was a slow-acting drug (Fig. 1 *C*). The reduction of the peak current appeared to be biphasic over a 20-min period: the fast phase, completed within 3–5 min, followed by a slower phase. It required about 20–30 min of drug treatment to reach the steady-state inhibition (Fig. 2). Even at 0.5 mM, the block by ethyl 4-diethylaminobezoate did not reach completion within 10 min. Ethyl 4-ethoxybenzoate was an intermediate-acting drug, requiring about 5–10 min to reach steady-state (Fig. 1 *D*). The potency of these drugs varied significantly in the following order: ethyl 4-diethylaminobenzoate > ethyl 4-ethoxybenzoate > ethyl 4-hydroxybenzoate > benzocaine. Unfortunately, because of the slow-acting nature of some of these drugs and the low solubility of benzocaine homologs, meaningful dose-response curves for accurate assessment of their potency through current measurements could not be obtained. At

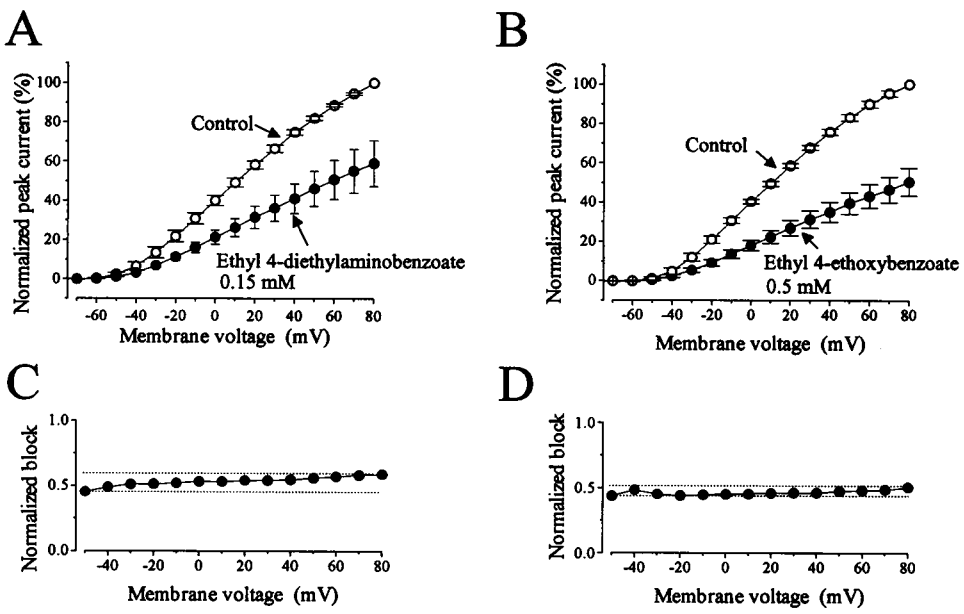


FIGURE 3 Voltage dependence of peak Na^+ current inhibition by benzocaine homologs. A family of peak Na^+ currents was recorded at 10-s intervals with test pulses ranging from −70 to +80 mV for 8 ms. Holding potentials and prepulse potential were set as described in Fig. 2. A complete set of control traces was taken first, then a second set of traces was taken after tonic inhibition by one of the drugs was achieved (*A* and *B* for ethyl 4-diethylaminobenzoate (0.15 mM, n = 5) and ethyl 4-ethoxybenzoate (0.5 mM; n = 6), respectively). The peak current amplitude at each voltage was normalized with respect to the value at +80 mV and plotted against voltages. The degree of reduction of peak current with ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate was normalized by the control value and plotted against membrane voltage (*C* and *D*, respectively). There is little voltage-dependent inhibition of peak current; the slopes for *C* and *D* are 0.0008 and 0.0003, respectively.

minimum, cells will require more than 80-min survival time when four different dosages of ethyl 4-diethylaminobenzoate are applied.

Like benzocaine (Neumcke et al., 1981), none of its homologs produced a voltage-dependent inhibition of the peak current. Fig. 3, *A* and *B*, shows the current families before and after 10- to 20-min treatment with ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate, respectively. The degree of reduction of peak currents at various voltages was relatively constant for these drugs (Fig. 3, *C* and *D*).

Shifts in steady-state inactivation of Na^+ channels by benzocaine homologs

Benzocaine significantly shifted the steady-state inactivation (h_∞) of Na^+ channels to the hyperpolarizing direction by more than 20 mV in GH_3 cells (Wang and Wang, 1994a). A similar degree of shift has been found in other preparations (e.g., Hille, 1977). Like benzocaine, all benzocaine homologs also shifted the h_∞ curve in the hyperpolarizing direction. Fig. 4, *A* and *B*, shows the steady-state inactivation curve measured by a conventional two-pulse protocol (Hodgkin and Huxley, 1952; Meeder and Ulbricht, 1987) for ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate, respectively. Inactivation curves were fitted with the empirical Boltzmann equation, $h_\infty = 1/[1 + \exp(E - E_{0.5})/k]$, where E is the prepulse potential, $E_{0.5}$ is the potential for which $h_\infty = 0.5$, and k is a slope factor. These parameters for benzocaine and its homologs are listed in Table 1. To use this equation, we assumed that inactivation reached steady state during the 50-ms prepulse duration. This assumption is difficult to verify because a longer prepulse duration will elicit slow inactivation of Na^+ channels in GH_3 cells.

Acceleration of the Na^+ current decaying phase by benzocaine homologs

Although there is no voltage-dependent inhibition of peak Na^+ current amplitude as in Na^+ current family measurement (Fig. 3), we noticed that the apparent rate of the Na^+ current decaying phase was consistently accelerated by benzocaine homologs at all voltages. The decay of the Na^+ current at each voltage could be well fitted by a single exponential term (τ in milliseconds) as described earlier (Cota and Armstrong, 1989). Fig. 5, *A* and *B*, shows the current traces before and after ethyl 4-diethylaminobenzoate and before and after ethyl 4-ethoxybenzoate treatment, respectively. The $1/\tau$ values were plotted against each voltage (Fig. 5, *C* and *D*). Significant acceleration of Na^+ current inactivation was found for both drugs. Similar results were found for benzocaine and ethyl 4-hydroxybenzoate (data not shown). Thus, benzocaine and its homologs accelerate the apparent inactivation kinetics of Na^+ currents in GH_3 cells. This finding is consistent with previous reports that benzocaine speeds up the decaying phase of Na^+ currents in frog node of Ranvier (Neumcke et al., 1981; Schneider and DuBois, 1986). Because of their relatively fast kinetics, it is not clear whether the rising phases of Na^+ currents are significantly altered by these drugs.

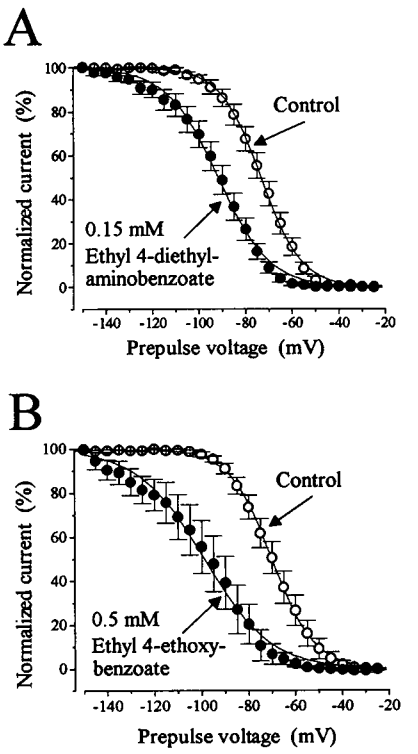


FIGURE 4 Steady-state Na^+ channel inactivation (h_∞) of ethyl 4-diethylaminobenzoate (0.15 mM) and ethyl 4-ethoxybenzoate (0.5 mM). Peak Na^+ current was measured at 10-s intervals with 50-ms prepulse voltages (Epp) ranging from −150 to −20 mV. Holding potential was set at −100 mV, an interpulse was set at −100 mV for 0.08 ms to reset the activation gate, and the test pulse was set at +50 mV for 3.6 ms. First, a complete set of control traces was recorded, then a second set was taken after tonic inhibition was achieved with one of the benzocaine homologs. Each family of peak amplitudes was normalized with respect to the value at −150 mV prepulse voltage. Each curve was then fitted using the Boltzmann equation, $y = 1/\{1 + \exp[(E_{pp} - E_{0.5})/k]\}$, where k is the slope factor and $E_{0.5}$ is the voltage where 50% of Na^+ channels were inactivated. In *A*, the $h_{0.5}$ value shifted from −73.3 ± 0.21 to −91.7 ± 0.42 mV, and k increased from 8.6 ± 0.18 to 10.9 ± 0.37 mV using ethyl 4-diethylaminobenzocate (n = 7). In *B*, $h_{0.5}$ shifted from −70.0 ± 0.16 to −98.9 ± 0.68 mV and k increased from 9.0 ± 0.14 to 14.1 ± 0.60 mV using ethyl 4-ethoxybenzoate (n = 8). The shifts of the fitted $h_{0.5}$ and the ratio of k values are listed in Table 1.

Use-dependent inhibition of Na^+ currents by benzocaine homologs

It is well established that benzocaine elicits little use-dependent block when the membrane is repetitively depolarized at frequencies up to 5 Hz. In GH_3 cells a similar result was found, as shown in Fig. 6, *B* and *C*, for benzocaine and ethyl 4-hydroxybenzoate, respectively. In the absence of these drugs, ~20% of Na^+ currents were inhibited after 60 repetitive pulses of +30 mV for 24 ms at 5 Hz (Fig. 6 *A*). This reduction is probably due to a small fraction

TABLE 1 Shifts in Na^+ channel steady-state inactivation by benzocaine homologs in GH_3 cells

Compounds	Concentration (mM)	$\Delta h_{0.5}$ (mV)	$k_{drug}/k_{control}$
Benzocaine*	1.0	−19.8 (n = 4)	1.10
Ethyl 4-diethylaminobenzoate	0.15	−18.4 (n = 7)	1.27
Ethyl 4-hydroxybenzoate	1.0	−25.9 (n = 4)	1.52
Ethyl 4-ethoxybenzoate	0.5	−28.9 (n = 8)	1.56

Parameters of $h_{0.5}$ and k were first determined as described in Fig. 4. The shift in steady-state inactivation ($\Delta h_{0.5}$) is the difference in $h_{0.5}$ before and after drug treatment. $h_{0.5}$ is the midpoint of the inactivation curve.
*The data for benzocaine are taken from Wang and Wang (1994a).

of inactivated channels unable to recover during the 176-ms interpulse duration. Lesser use-dependent block was found at frequencies of 1 and 2 Hz (Fig. 6 *A*).

In contrast, both ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate elicited significant use-dependent block during repetitive pulses at all three frequencies. The peak current amplitude at each pulse was measured, normalized with respect to the value of the first pulse, and plotted against the pulse number (Fig. 7, *A* and *C*). The current traces in Fig. 7, *B* and *D*, show that up to 50–60% of Na^+ currents are blocked after 60 pulses at 5 Hz by these two compounds. This result demonstrates that alkylation of the 4-amino or 4-hydroxy functional group in benzocaine homologs alters their use-dependent characteristics.

Besides the frequency of the pulse, the duration of the depolarization may also affect the degree of the use-dependent block. Fig. 8, *A* and *B*, shows that this is the case for ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate. Reducing the duration from 24 ms to 10, 5, and 1 ms progressively decreased the level of the use-dependent block elicited during repetitive pulses. Because fast inactivation appears to complete within 5 ms (Fig. 1), this result indicates that binding of these compounds continues long after the Na^+ channels have been inactivated. Furthermore, the steady-state level of the use-dependent block could be reached only after 30–60 pulses, which suggests that during each pulse, the additional binding of these benzocaine homologs is small.

Recovery from the use-dependent inhibition of Na^+ currents

The recovery of Na^+ currents from the use-dependent block was measured by a test pulse with variable durations of rest at the holding potential of −100 mV after 30 repetitive pulses. Na^+ currents recovered from the use-dependent block with a single exponential time course of about 6.8 and 4.1 s for ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate, respectively (Fig. 9). This slow recovery from block is consistent with the observed use-dependent block during repetitive pulses (Fig. 8), because the interpulse is too short (less than 0.5 s) to allow a significant amount of current to recover at the holding potential.

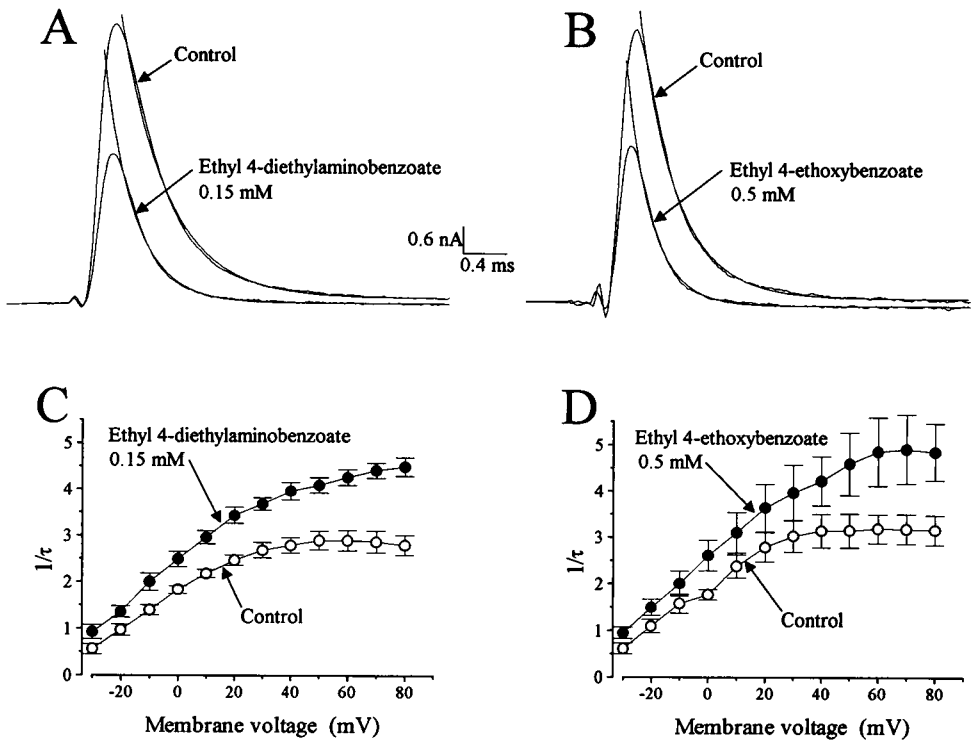


FIGURE 5 The effect of benzocaine homologs on the inactivation kinetics of Na^+ currents in GH_3 cells. *A* and *B* show typical peak Na^+ current traces taken before drug application and after achievement of tonic inhibition with ethyl 4-diethylaminobenzoate (0.15 mM) and ethyl 4-ethoxybenzoate (0.5 mM), respectively. The decay of each current was fitted with a single exponential equation. The τ value decreased from 0.43 to 0.28 ms after treatment with ethyl 4-diethylaminobenzoate. Similarly, τ decreased from 0.35 to 0.24 ms after treatment with ethyl 4-ethoxybenzoate. The R values for the τ fittings were better than 0.998 (thin fitting lines in *A* and *B*). The single-exponential equation was used to obtain τ for each membrane potential of each family as shown in Fig. 3, *A* and *B*. The mean inverse of τ values was then plotted against voltage. At all voltages, both ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate significantly increase the inactivation kinetics of Na^+ current (*C* and *D*, respectively).

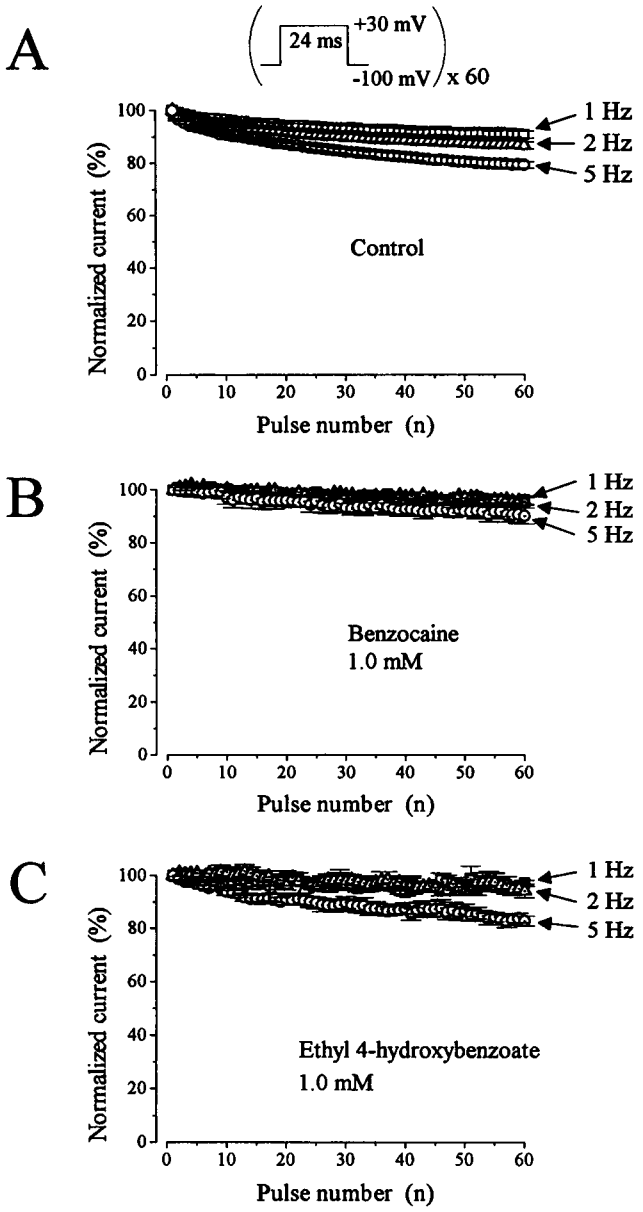


FIGURE 6 Lack of use-dependent block of Na^+ currents before and after benzocaine (1.0 mM) or ethyl 4-hydroxybenzoate (1 mM). Holding potential was set at −100 mV and repetitive pulses were set at +30 mV for 24 ms at 1 to 5 Hz (see inset for pulse protocol). After the control sets of peak amplitudes were recorded at 1, 2, and 5 Hz, the second sets were taken after tonic inhibition was achieved with drug treatment. The peak amplitude of each data set was normalized with respect to the amplitude of the first pulse of its set. The peak amplitudes were then plotted against pulse number. Under the control conditions in *A* there is 20.6 ± 1.3% use-dependent inhibition of Na^+ current at 5 Hz (n = 4) (○). The degree of use-dependent inhibition, which was related to the applied frequency, was 13.3 ± 1.3% at 2 Hz (n = 15) (△) and 9.3 ± 1.3% at 1 Hz (n = 14) (□). Treatment with benzocaine (*B*) elicited even less use-dependent inhibition in Na^+ current than the control. There were 3.8 ± 1.6% and 4.4 ± 1.4% use-dependent block, respectively, at 1 Hz (n = 3) (⊡) and 2 Hz (n = 3) (◬) and 9.8 ± 3% use dependence at 5 Hz (n = 3) (⊙). Treatment with ethyl 4-hydroxybenzoate (*C*) similarly produced little use-dependent inhibition: about 5% at both 1 (n = 6) and at 2 Hz (n = 6). At 5 Hz (n = 7) there was a 17% reduction in peak amplitude; this value is comparable, however, to that of the control at 5 Hz.

DISCUSSION

Use-dependent inhibition of Na^+ currents by benzocaine homologs

The main purpose of this study is to address whether benzocaine homologs can elicit use-dependent block of Na^+ currents. Although numerous other benzocaine homologs have been examined, none of them appears to produce use-dependent block (e.g., Elliott et al., 1987). Our results clearly show that ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate produce use-dependent block of Na^+ currents, whereas benzocaine and ethyl 4-hydroxybenzoate do not. In a previous report (Wang and Wang, 1994b), it was briefly stated that 1 mM ethyl 4-dimethylaminobenzoate can also produce significant use-dependent block (up to 50% at 5 Hz). Together, these data demonstrate that there are two types of benzocaine homologs; only one type can elicit use-dependent block. Thus, the lack of use-dependent block of Na^+ currents by benzocaine and ethyl 4-hydroxybenzoate cannot be used as evidence for the existence of a separate neutral LA binding site apart from the tertiary amine LA binding site (for a review of this possibility see Butterworth and Strichartz, 1990). Our results are consistent with the notion that there is a common LA receptor responsible for eliciting the use-dependent block upon binding, as suggested by Hille (1977). Both ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate probably interact more strongly with the open and inactivated Na^+ channels than with the resting channels for the following reasons. First, these compounds shift the steady-state inactivation curve in the hyperpolarizing direction (Table 1). Such a shift is generally taken as evidence that LAs bind preferentially with the inactivated channel (Hille, 1977; Bean et al., 1983; Meeder and Ulbricht, 1987). Second, they accelerate the decaying phase of the Na^+ current (Fig. 5). Third, they require a long duration, up to 30 ms, of repetitive pulse to elicit maximal use-dependent block (Fig. 8).

Structural determinant of benzocaine homologs for use-dependent block

By examining the chemical structures of benzocaine homologs it becomes apparent that alkylation of the 4-amino or 4-hydroxy position of the phenyl ring will enable the homologs to elicit use-dependent block as well as to increase tonic block. Alkylation of these functional groups on the phenyl ring increases the hydrophobicity and the molecular weight of the compound. We have demonstrated previously in Na^+ channels that the binding site of tertiary amine LAs and quaternary ammonium compounds consists of at least two large hydrophobic binding domains (Wang et al., 1993), each of which may accommodate up to 12 carbons of an alkyl chain. Assuming that there is a common receptor for all LAs, these alkylated compounds, therefore, can easily be accepted by such hydrophobic binding domains.

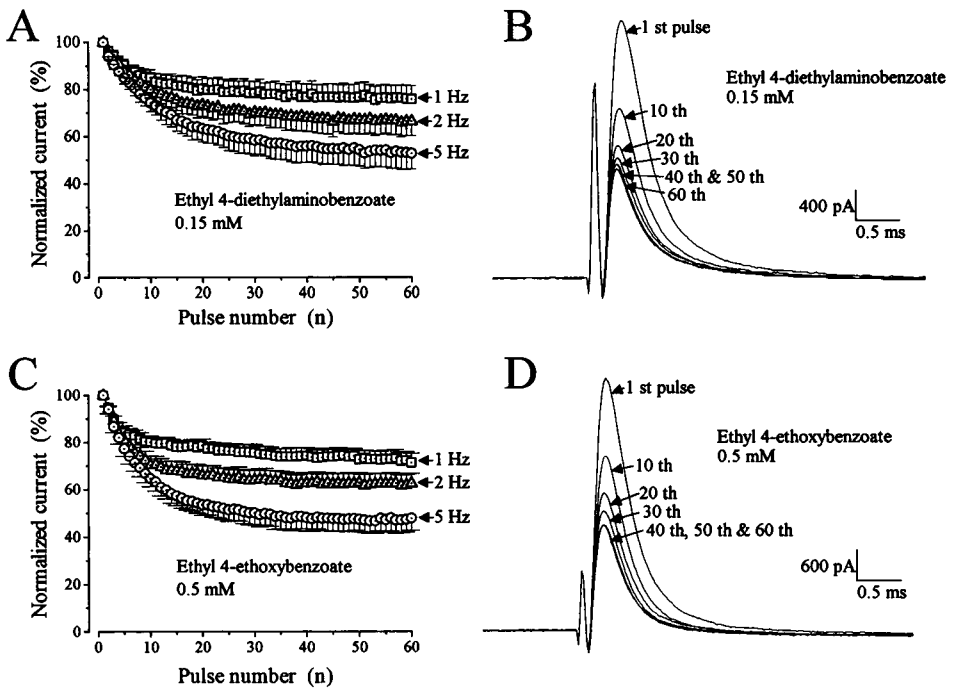


FIGURE 7 Use-dependent inhibition of Na^+ current elicited by ethyl 4-diethylaminobenzoate (0.15 mM) and ethyl 4-ethoxybenzoate (0.5 mM). Using the same pulse protocols, method of measuring, and normalizing peak Na^+ currents as described in Fig. 6, we found that ethyl 4-diethylaminobenzoate (*A*) at 1 Hz produced a 24.4 ± 5.9% (□) reduction in peak amplitude, whereas at 2 Hz (△) and 5 Hz (○) peak amplitudes were reduced by 33.8 ± 6.1% and 47.4% ± 6.6 (n = 5), respectively. Ethyl 4-ethoxybenzoate (*C*) evoked about 5% greater use-dependent inhibition than ethyl 4-diethylaminobenzoate. At 1, 2, and 5 Hz, there was a 28.8 ± 4.1%, 37.6 ± 4.3%, and 52.2 ± 5.3% (n = 5) reduction, respectively. *B* and *D* show the representative Na^+ current traces after repetitive pulsing at 5 Hz in the presence of ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate, respectively.

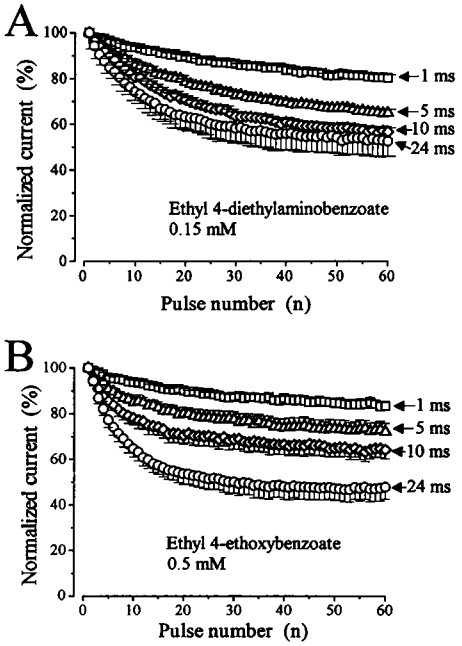


FIGURE 8 The effect of pulse duration on the use-dependent block of peak Na^+ current. Repetitive test pulses were applied at +30 mV, between 1 and 24 ms, at 5 Hz. Holding potential was set at −100 mV. Measurements were recorded when the cells had reached either steady state (as in the case of the controls) or tonic inhibition (as in the case when a drug was administered). Normalized peak current amplitude was plotted against pulse number. It is evident that increasing the duration of the test pulse increases the amount of inhibition of peak amplitude. (*A*) At a pulse duration of 1 (□), 5 (△), 10 (◇), and 24 ms (○), ethyl 4-diethylaminoenzoate (0.15 mM) evoked 19.6 ± 1.3% (n = 6), 35.1 ± 1.6% (n = 6), 43.3 ± 1.8% (n = 6), and 47.4 ± 6.6% (n = 6) inhibition of peak amplitude, respectively. (*B*) Similarly, ethyl 4-ethoxybenzoate (0.5 mM) produced 16.7 ± 1.9% (n = 6), 27.9 ± 3.6% (n = 6), 35.7 ± 4.0% (n = 6), and 52.2 ± 5.3% (n = 5) inhibition at those durations, respectively. In the absence of drugs, repetitive pulses of 1-, 5-, 10-, and 24-ms duration elicited blocks of 4.8 ± 0.8%, 7.7 ± 0.9%, 9.6 ± 1.0%, and 19.7 ± 1.7%, respectively; the degree of block is far less than that with drugs.

How does alkylation of benzocaine and ethyl 4-hydroxybenzoate enable these compounds to elicit use-dependent block of Na^+ currents? Apparently, alkylation slows the recovery time course of benzocaine homologs (Fig. 9). According to Hille (1977) the lack of use-dependent block of Na^+ currents by benzocaine is caused by relatively fast dissociation of benzocaine from its receptor during interpulses and by the quick diffusion of benzocaine in its neutral form away from the pore region through the hydrophobic pathway. According to this same argument, there are two conceivable ways to produce use-dependent block with benzocaine homologs: first, slower dissociation of the alkylated benzocaine homologs from their receptor, and second, slower diffusion of the alkylated homologs away from the pore region through the hydrophobic pathway when the hydrophilic pathway is closed by activation and/or inactivation gate. The first possibility is likely because of the potential increased hydrophobic interactions for the alkylated homologs. For the second possibility, it is not clear how fast the benzocaine homologs can diffuse away from the pore when the channel is closed. The increase in the cylindric dimension of alkylated homologs is minimal, although they do increase the length of the compounds. This steric factor could reduce the diffusion rate of alkylated benzocaine homologs from the pore when the channel is closed. All of these compounds remain neutral and do not carry charges under physiological conditions. Although *N,N*-diethylaniline has a pK_a 2 units higher than that of aniline, the equivalent increase of ethyl 4-diethylaminobenzoate will still have a pK_a of 4.5, or less than 1% in its charged form at our experimental conditions. Our results are in agreement, however, with the conclusion of Chernoff and Strichartz (1989) that neither the presence of a tertiary

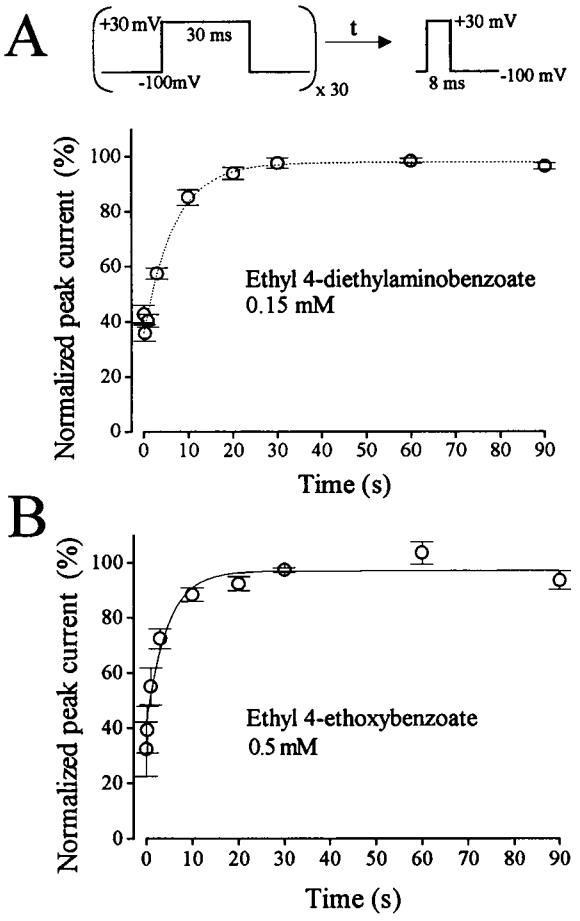


FIGURE 9 Recovery of Na^+ current from use-dependent block. A test pulse of +30 mV for 8 ms was given at various intervals after each train of 20 conditioning pulses of +30 mV for 30 ms at 5 Hz (see inset). Each set of peak Na^+ current amplitudes was measured after cells had achieved tonic inhibition. The mean peak Na^+ current amplitudes were normalized with the amplitude at 90 s and then plotted against time. *A* and *B* show the recovery of Na^+ current from use-dependent inhibition with treatment of ethyl 4-diethylaminobenzoate and ethyl 4-ethoxybenzoate, $\tau = 6.8 \pm 0.7$ s ($n = 6$) and 4.1 ± 0.6 s ($n = 4$), respectively.

amine nor a net charge on the LA is required for use-dependent block of Na^+ channels.

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