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Inhibition of Na^+ Channels by the Novel Blocker PD85,639

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SUMMARY

This study examined the actions of the novel Na^+ channel blocker PD85,639. In whole-cell voltage-clamp recordings from Chinese hamster ovary cells transfected with a cDNA encoding the rat brain type IIA Na^+ channel and from dissociated rat brain neurons, PD85,639 attenuated Na^+ currents when applied either in the external bath or in the internal pipette solution. Block had a tonic component that occurred in the absence of stimulus pulses and an additional use-dependent component that developed during a train of pulses. The EC_{50} for tonic block was 30 μM and was not strongly dependent on the holding potential. Use-dependent block was first detectable at 1 μM and was pronounced at higher concentrations, even at stimulus frequencies as low as 1 pulse/2 min. The marked use-dependent block was due to rapid drug binding during depolarizing pulses and very slow recovery of drug-bound channels between the pulses (τ = 11 min at −85 mV). Use-dependent block was greater at more depolarized potentials, suggesting that the drug binding site was partway across the membrane electric field. The block that developed with strong depolarizations was rapidly reversed by opening channels with trains of unblocking pulses to more negative potentials. These characteristics of block by PD85,639 suggest that it is a local anesthetic drug with novel properties.

The Na^+ channel is a large membrane protein responsible for Na^+ influx during action potential depolarization in nerve and muscle cells (1). The channel has three different voltage-dependent conformational states. At negative membrane potentials, most channels are in a closed resting state. In response to membrane depolarization, the channels open and then inactivate within a few milliseconds.

In mammalian cells, Na^+ channels consist of a 260-kDa α subunit and one (in muscle) or two (in brain) smaller β subunits (2–4). cDNAs encoding several different α subunit subtypes have been cloned from brain, heart, and skeletal muscle cDNA libraries (5–8) and, more recently, a clone for the rat brain β1 subunit has also been isolated (9). The α subunit alone can form functional channels when expressed in *Xenopus* oocytes (6–8) or mammalian cells (10). The β subunits may modulate α subunit function and stabilize the protein in the cell membrane, because Na^+ currents in *Xenopus* oocytes are larger and have more normal inactivation when rat brain α and β1 subunits are coexpressed than when the α subunit is expressed alone (9).

Na^+ channels are the site of action of a number of clinically important drugs, including local anesthetic, antiarrhythmic, and anticonvulsant agents. For example, local anesthetics like benzocaine and lidocaine inhibit pain by blocking Na^+ channels responsible for impulse propagation in peripheral nerves (11). Lidocaine also inhibits cardiac arrhythmias by blocking cardiac Na^+ channels. Lidocaine is effective because it selectively blocks arrhythmic activity but has little effect on normal cardiac activity (12, 13). This selective inhibition stems from two properties of local anesthetic block of Na^+ channels. First, local anesthetics are more potent blockers at the more depolarized membrane potentials characteristic of cardiac cells during arrhythmic activity. Second, drug block accumulates during activity and thus filters out abnormal high frequency depolarizations with only nominal effects on normal activity. Similar selective block of abnormally active brain Na^+ channels also appears to be the main mechanism of action of the anticonvulsants phenytoin and carbamazepine (13, 14).

Because of the important therapeutic applications of local anesthetics and related drugs, characterization of the binding site for these drugs is important. However, binding studies have been limited by the absence of potent ligands. PD85,639 [α-(phenyl)-*N*-[3-(2,6-dimethyl-1-piperizinyl)-α-propyl]benzeneacetamide] was identified as a high affinity inhibitor of veratridine-activated Na^+ channels in an extensive drug screen (15) and may represent a novel class of local anesthetic-like drugs that bind with high affinity to Na^+ channels (see accompanying paper). In the present study, we have characterized rat brain Na^+ channel blockade by PD85,639 in detail and have shown that its blocking properties are similar to those of local anesthetics.

Experimental Procedures

Materials. PD85,639 was generously provided by Dr. Sheryl Hayes, Parke-Davis Research Division, Warner-Lambert Co. (Ann Arbor, MI).

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Cell culture. Most experiments were performed using the cell line CNaIIA-1, a Chinese hamster ovary cell line (CHO-K1; American Type Culture Collection) that has been stably transfected with the α subunit of the type IIA rat brain Na^+ channel and expresses high levels of functional Na^+ channels (10). The cells were grown on glass coverslips in 35-mm plastic Petri dishes in RPMI 1640 medium (GIBCO) with 2.5% fetal calf serum, 200 μg/ml G418, 10 μg/ml streptomycin, and 20 μg/ml penicillin, in the presence of 5% CO_2.

Experiments were also performed on primary cultures of rat brain neurons prepared from 20-day-old embryos as described previously (6). Briefly, embryonic rat brains were minced with scissors and then treated for 40 min with trypsin (2 mg/ml). The cells were then resuspended in medium (Dulbecco's modified Eagle's medium with 10% heat-inactivated horse serum, 5% fetal calf serum, 20 μg/ml penicillin, and 10 μg/ml streptomycin), filtered through Nitex filters, and seeded onto 35-mm plastic Petri dishes.

Electrophysiology. Na^+ currents were recorded using the patch-clamp recording technique in the whole-cell configuration (7). For recording from CNaIIA-1 cells, the bath solution contained (in mM) NaCl, 130; KCl, 5.0; $CaCl_2$, 1.5; $MgCl_2$, 1.0; glucose, 5.0; and HEPES, 5.0; pH 7.4 with NaOH. Patch pipettes were pulled from microhematocrit tubing and were filled with an intracellular solution containing (in mM) KF, 70; EGTA, 8; KCl, 45; NaF, 8; and HEPES, 8; pH 7.4 with KOH. Pipettes had input resistances of about 1 MΩ when filled with intracellular solution.

Brain neurons in primary culture have processes that are difficult to space-clamp effectively. Therefore, to prevent regenerative activity in poorly clamped regions of these processes we recorded from neurons using a reverse Na^+ gradient. The bath solution contained (in mM) choline chloride, 115; tetraethylammonium chloride, 20; NaCl, 10; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1; glucose, 5; and HEPES, 5; pH adjusted to 7.4 with NaOH. The pipette solution contained (in mM) NaCl, 140; CsCl, 20; and HEPES, 5; pH adjusted to 7.4 with CsOH.

The cells were clamped using either a List L/M EPC-7 clamp, an Axopatch-1C, or a locally made voltage clamp based on the original design of Hamill *et al.* (17). Data acquisition and analysis were performed using a personal computer and commercially available software (Basic-Fastlab; Indec Systems). The data were filtered at 7.4 kHz. Capacitive transients were canceled and series resistance was compensated using the internal voltage-clamp circuitry. In some experiments, remaining capacitance and leakage currents were subtracted by the *P*/4 procedure (18). Theoretical curves were fit to the data using the Sigma Plot graphics program (Jandel Scientific). All recordings were at room temperature (about 20–22°).

Drug application. Stock solutions (20 mM) of PD85,639 were prepared in dimethylsulfoxide and then diluted into the bath or pipette solution to the desired concentration. Up to 1% dimethylsulfoxide has no detectable effects on Na^+ currents in CNaIIA-1 cells or brain neurons. For most experiments, the drug was applied extracellularly by superfusion. In a few experiments the drug was applied by addition of the appropriate volume of the stock solution directly to the bath, and in one experiment it was applied intracellularly by addition to the pipette solution.

Results

Inhibition of Na^+ channels by PD85,639. Fig. 1 compares the chemical structure of PD85,639 with those of the local anesthetics lidocaine and tetracaine. Like these anesthetics, PD85,639 has a tertiary amine group that can exist in both neutral and positively charged forms. The drug has a pK_a of 9.3 and an octanol/water partition coefficient of 1.2 (19). Thus, at physiological pH values most of the drug is in the positively charged form, but the neutral form of the drug is hydrophobic and probably can pass freely through cell membranes.

Fig. 2 shows the main effects of PD85,639 on Na^+ currents

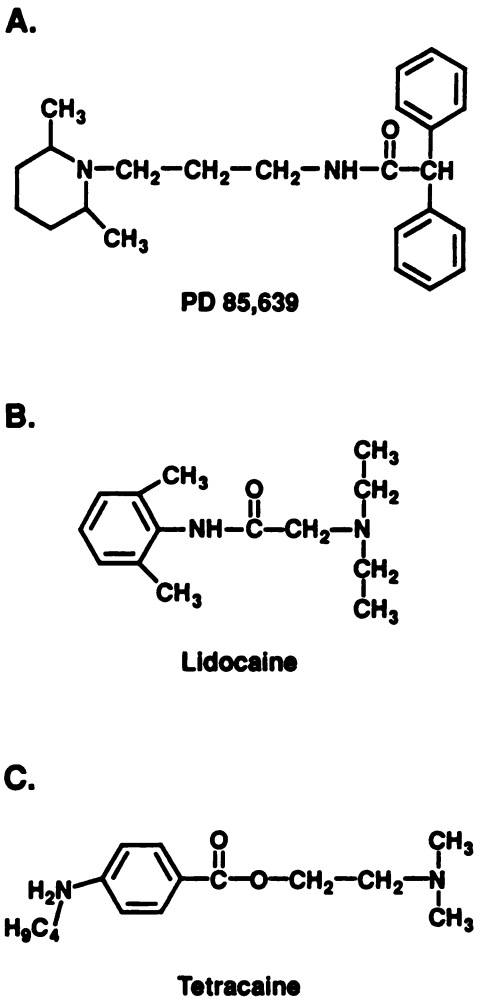

Fig. 1. Structures of PD85,639 and tertiary local anesthetics.

in CNaIIA-1 cells, which express rat brain type IIA Na^+ channel α subunits (Fig. 2, *top*), and the time course of the tonic and use-dependent components of block (Fig. 2, *bottom*). In this experiment, transient inward Na^+ currents were evoked by applying 5-msec stimulus pulses to 0 mV. In control conditions, the amplitudes of the currents elicited by pulses applied at 10-sec intervals (0.1 Hz) were similar and stable over time (Fig. 2, *bottom*). After several minutes, the pulses were stopped, and 40 μM PD85,639 was superfused into the external bath solution. Currents evoked by single pulses applied at 5-min intervals after the start of drug application were attenuated by about 70% (Fig. 2, *bottom*). Additional attenuation of the currents, to <10% of control, developed during a train of pulses applied at 0.1 Hz (Fig. 2, *bottom*). When the stimulus rate was returned to 1 pulse/5 min, the currents relaxed back to approximately 70% block over 15 min (Fig. 2, *bottom*). We refer to the initial attenuation of the currents as tonic block and the additional block that accumulated during more frequent pulse trains and decayed when the pulses were stopped as use-dependent block. Similar use-dependent block was observed when PD85,639 was applied intracellularly from the recording pipette in the whole-cell recording configuration (Fig. 3). Evidently, PD85,639 can reach its receptor site from either side of the membrane.

PD85,639 was also a potent blocker of Na^+ currents in primary cultures of rat brain neurons (Fig. 4). During repetitive

stimulation in the presence of 25 μM PD85,639, the Na^+ current was reduced 75% in 10 min and >90% in 20 min (Fig. 4A). A lower concentration of PD85,639 (2.5 μM) reduced Na^+ current by approximately 50%, and a similar reduction was observed across a broad range of test pulse potentials (Fig. 4B).

The structure of PD85,639 and its properties as a Na^+ channel blocker suggested that it might be a drug with novel local anesthetic properties. To critically compare PD85,639 with other local anesthetics, we characterized the tonic and use-dependent components of Na^+ channel blockade more completely.

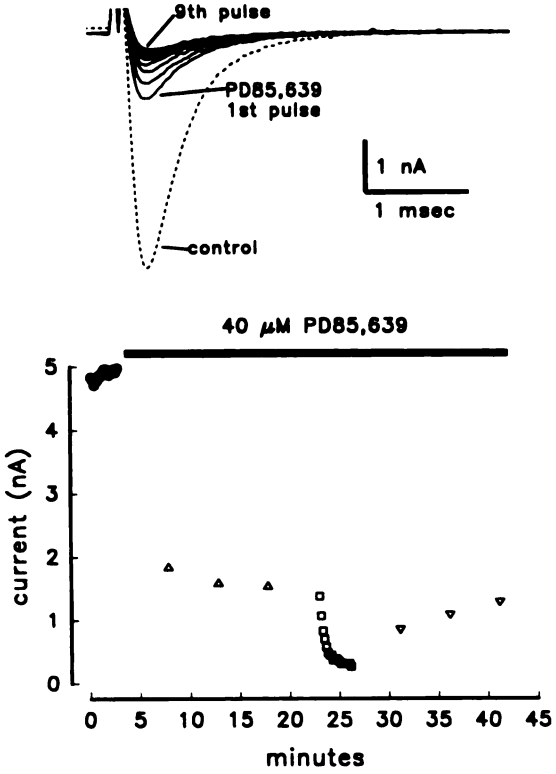

Fig. 2. Tonic and use-dependent block by PD85,639 of rat brain Na^+ channels expressed in CNaIIA-1 cells. Currents were recorded during 5-msec stimulus pulses to 0 mV from a holding potential of −85 mV. Pulses were first applied under control conditions at a rate of 0.1 Hz (○). Pulsing was then stopped and PD85,639 was superfused into the external bath. Single pulses were then given at 5-min intervals (△) to determine the level of tonic block. Twenty minutes after the start of drug application, a 0.1-Hz pulse train was applied to assess use-dependent block (□). After the 0.1-Hz train, the frequency was returned to 1 pulse/5 min (▽) to determine the recovery from use-dependent block. *Top*, currents elicited by test pulses under control conditions (*dashed trace*) and by pulses 1 through 9 applied at 0.1 Hz after application of PD85,639. *Bottom*, magnitude of peak inward current plotted as a function of time. *Bar*, total period of PD85,639 application.

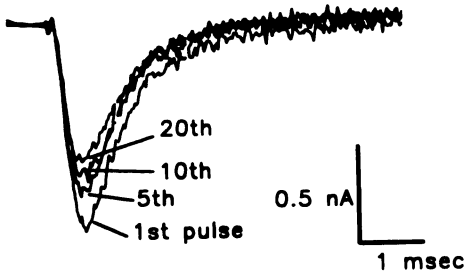

Fig. 3. Intracellular application of PD85,639 blocked Na^+ currents in CNaIIA-1 cells. PD85,639 (100 μM) was dissolved in the pipette solution. Ten minutes after breaking into the cell, the extent of block was determined by giving 5-msec stimulus pulses to 0 mV from a holding potential of −85 mV, at a frequency of 1 pulse/5 sec. The *traces* show currents elicited by pulses 1, 5, 10, and 20 in the train.

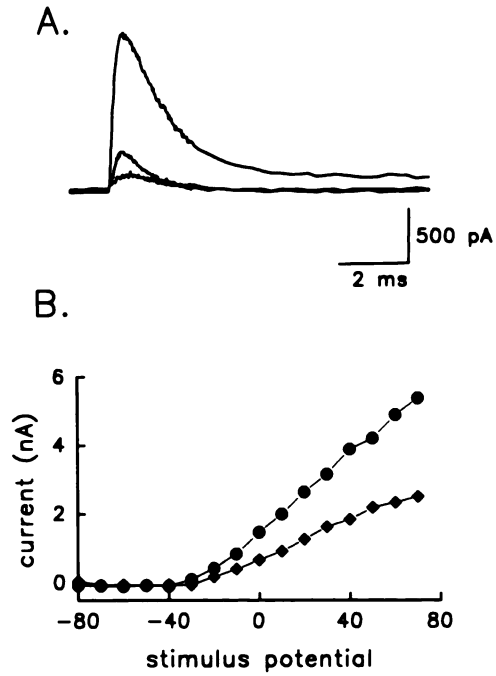

Fig. 4. PD85,639 blocked Na^+ currents in primary cultures of rat brain neurons. A, Na^+ currents were recorded during stimulus pulses to −20 mV after 100-msec prepulses to −120 mV at 2-sec intervals. *Traces* shown were obtained under control conditions (*largest trace*) and then 10 and 20 min after application of 25 μM PD85,639. Each *trace* is an average of three stimulus pulses. Pulses were applied continuously during drug application, so the attenuation of the currents represents both tonic and use-dependent block. Currents were outward due to the reversed Na^+ gradient (see Experimental Procedures). B, Current-voltage relationship from a different neuron before (●) and after (♦) application of 2.5 μM PD85,639. Peak current is plotted versus stimulus potential.

Tonic block with PD85,639. To investigate tonic block in more detail, we determined the relationship between drug concentration and block in CNaIIA-1 cells. Tonic block was just detectable at 5 μM, and the EC_{50} was about 40 μM when the drug was applied extracellularly (Fig. 5). Therefore, the potency of PD85,639 as a tonic blocker of brain Na^+ channels was similar to that of other local anesthetic drugs (20, 21).

Local anesthetics and anticonvulsants are more potent blockers at relatively positive holding potentials than at more negative holding potentials, reflecting an overall negative shift of up to 10–20 mV in the relationship between membrane potential and steady state inactivation (12, 20–22). We investigated the effect of PD85,639 on steady state inactivation by determining the extent of block at various holding potentials. The normalized data from three such experiments are shown in Fig. 6A. The smooth line through the data points was the best fit of the equation

$$h = 1/[1 + \exp(E_m - E_{1/2})/k] \quad (1)$$

where h is the relative channel availability, E_m is the holding potential, $E_{1/2}$ is the potential corresponding to 50% inactivation, and k is a slope factor. The $E_{1/2}$ values obtained for control and 20 μM PD85,639 were −63 and −68 mV, respectively. Non-normalized data from a single cell are replotted in Fig. 6B,

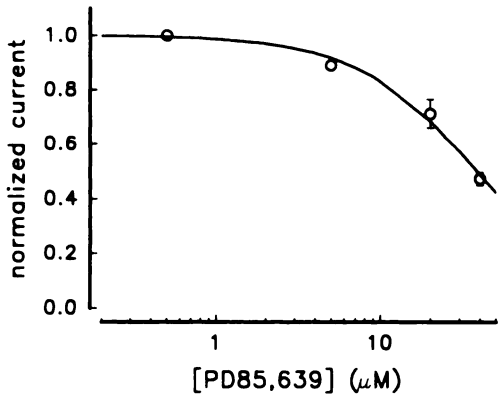

Fig. 5. Dose-response relationship for PD85,639. Peak currents were recorded during stimulus pulses to 0 mV for 5 msec from a holding potential of −85 mV, under control conditions and in the presence of various concentrations of PD85,639. The cell was held at −85 mV without pulsing as each concentration of drug was perfused into the bath. After 5–6 min, a single test depolarization was applied. Peak currents during this test pulse were normalized with respect to the amplitude of the current elicited under control conditions. The *smooth line* represents a least-squares fit of $1-(1/[1 + \exp(EC_{50}/[PD85,639])^n])$ to these data, with EC_{50} = 38.6 μM and n = 1.18.

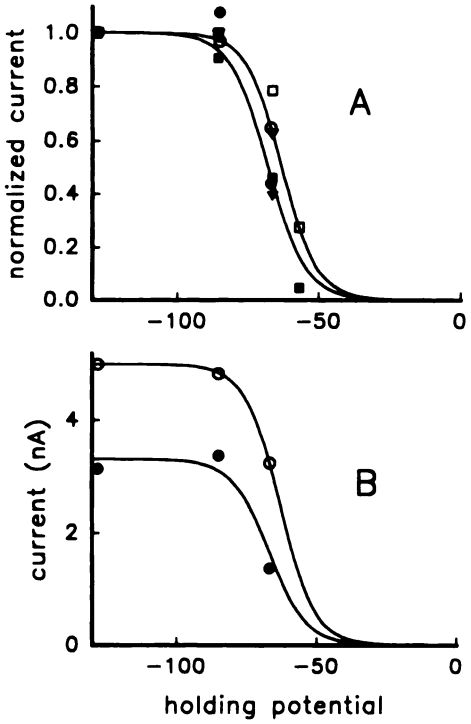

Fig. 6. Effect of PD85,639 on the relationship between holding membrane potential and steady state inactivation. A, The data are from three different cells under control conditions (*open symbols*) and in the presence of 20 μM PD85,639 (*filled symbols*). Currents were recorded during single test depolarizations to 0 mV for 5 msec after the cell was held for 1 min at membrane potentials ranging from −128 mV to −56 mV. Current amplitudes normalized with respect to the current obtained at a holding potential of −128 mV are plotted as a function of holding potential. The *smooth lines* through the data are the best fit of eq. 1, with $E_{½}$ = −63 and −68 mV and k = 6.3 and 6.7, respectively. Mean block of current by 20 μM PD85,639 was 29.7 ± 0.08% at a holding potential of −128 mV. B, Data from one of the cells, plotted without normalization.

showing the approximately 30% reduction in current at all potentials. Thus, PD85,639 caused a relatively small negative shift in steady state inactivation, and most of its tonic effects were due to this holding potential-independent block.

Use-dependent block with PD85,639. The most striking effect of PD85,639 was its pronounced use-dependent block of Na^+ currents. Use-dependent block was detectable with 1 μM PD85,639 and was profound at higher concentrations, even at much lower stimulus frequencies than those required for use-dependent block with tertiary amine local anesthetics. For example, use-dependent block of type IIA Na^+ channels with the local anesthetic lidocaine requires stimulus frequencies greater than about 0.5 Hz (20), whereas substantial use-dependent block was seen with PD85,639 at 0.1 Hz (Fig. 7A) and block was still observed when pulses were applied at a rate of 1 pulse every 2 min (0.008 Hz; Fig. 7B).

The pronounced use-dependent block with PD85,639 was due to rapid drug binding during depolarizing stimulus pulses and very slow repriming of drug-bound channels between the pulses. Under control conditions, Na^+ channels in CNaIIA-1 cells recovered from the inactivated to the resting state within milliseconds when a depolarizing stimulus pulse was terminated and the cell returned to a negative holding potential (e.g., Ref. 20). In contrast, in the presence of PD85,639 the additional use-dependent block that developed during a train of stimulus pulses reversed over 10–30 min when the pulse train was terminated and the cell membrane returned to a negative potential (Fig. 8). This reflects recovery from the use-dependent component of block, as illustrated in Fig. 2 (*bottom*).

Use-dependent block with PD85,639 was strongly voltage dependent, becoming more pronounced at more positive stimulus potentials (Fig. 9). In the presence of 5 μM PD85,639, a train of stimulus pulses to −30 mV produced no block, as assessed by a test pulse to 0 mV after the train, whereas almost 80% block was obtained by a series of depolarizing pulses to +80 mV (Fig. 9A). The increased block that developed with the strong depolarizations was rapidly reversed by a train of "unblocking" pulses to −30 mV (Fig. 9B). The rate of this use-dependent unblocking was much faster than the rate of repriming of blocked channels at hyperpolarized potentials. Apparently drug dissociation from closed channels was greatly accelerated by opening of the channels at relatively negative potentials that favored drug dissociation.

Measurement of Na^+ channel block at a series of membrane potentials (Fig. 10) gave a voltage dependence similar to that which has been observed previously for use-dependent block

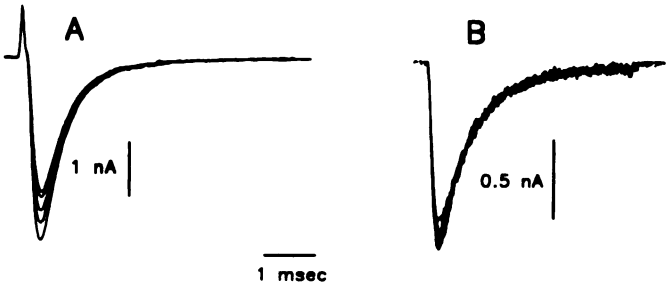

Fig. 7. Use-dependent block in the presence of PD85,639. Currents were evoked by five consecutive stimulus pulses to 0 mV for 5 msec. A, Pulses were applied at 10-sec intervals in the presence of 20 μM PD85,639. B, Pulses were applied at 2-min intervals in the presence of 40 μM PD85,639. In both cases, the drug was applied long enough for tonic block to reach a steady state before the pulses were applied.

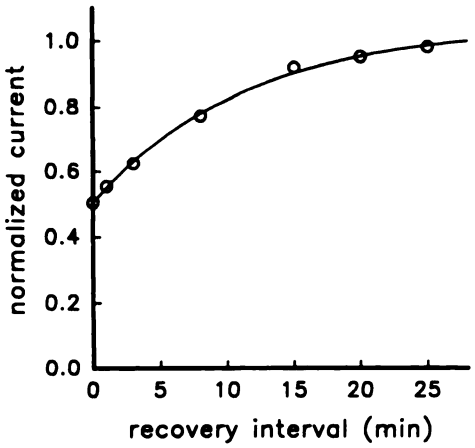

Fig. 8. Slow repriming of channels blocked by PD85,639. Use-dependent block of Na^+ channels was developed by applying a train of conditioning pulses at 1 Hz to 0 mV for 5 msec, from a holding potential of −85 mV, in the presence of 20 μM PD85,639. The time course of recovery from this block was assessed by giving test pulses to 0 mV at 1–5-min intervals. The currents evoked by each pulse were normalized with respect to the current evoked by the first pulse in the conditioning pulse train. The *solid line* represents an exponential fit to the recovery data, with a time constant of 11.2 min.

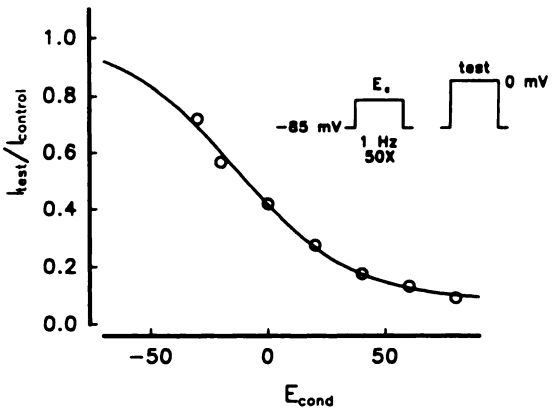

Fig. 10. Voltage dependence of use-dependent block by PD85,639. Trains of 50 conditioning pulses were given to varying potentials, followed by a test pulse to 0 mV for 5 msec in the presence of 20 μM PD85,639. Peak test pulse current (I_{test}) was normalized to test pulse current obtained without conditioning pulses ($I_{control}$) and plotted versus conditioning pulse potential. Control experiments showed that the steady state block at the end of a 50-Hz train was independent of the degree of block before the train and was the same as that obtained after application of the same pulse train to a rested cell. The data shown were obtained from one cell during successive trains using increasingly more positive conditioning depolarizations. The *smooth line* through the data is a least squares fit of eq. 2, with $\delta = 0.98$, $E_{½} = -13.6$ mV, and $b = 0.082$.

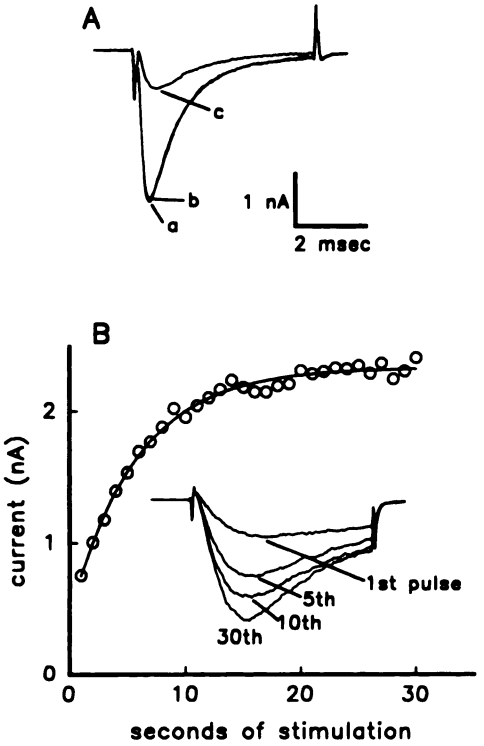

Fig. 9. Voltage-dependent block and unblock by PD85,639. A, Currents evoked by stimulus pulses to 0 mV for 5 msec in the presence of 5 μM PD85,639, after a long rest period (*a*) or immediately after a train of conditioning pulses to −30 mV (1 Hz, 50 pulses) (*b*) or +80 mV (*c*). B, Data from the same cell. The *data points* show the amplitudes of the currents elicited by a 1-Hz train of test pulses to −30 mV immediately after a 1-Hz train of conditioning pulses to +80 mV. The *smooth line* is an exponential fit of the data, with a time constant of 5.8 sec. *Inset*, currents evoked by test pulses 1, 5, 10, and 30. The *scale bars* are for both sets of *current traces*.

with local anesthetic drugs (23–25). Unlike the shifts in steady state inactivation described above, this voltage dependence probably did not reflect different drug affinities for distinct functional states of the channel but rather was due to the intrinsic voltage dependence of drug binding resulting from the positively charged form of the drug moving through part of the membrane electric field to reach its binding site (26). The smooth line through the data points shown in Fig. 10 is the best fit of the equation

$$I_{test}/I_c = (1 - b)/[1 + \exp(\delta(E - E_{½})/(RT/F))] + b \quad (2)$$

where I_{test}/I_c is the relative block after a train of stimulus pulses to a given conditioning potential E, $E_{½}$ is the conditioning potential where the block is 50%, b is the maximum block, R is the gas constant, T is the absolute temperature, F is Faraday's constant, and δ is a slope factor reflecting the proportion of the membrane electric field that the charged drug molecule must move through to reach its binding site. The value for δ obtained with PD85,639 was 0.98, which is similar to values that have been obtained previously with other local anesthetic drugs (23–25). Although this value can be interpreted to mean that the positively charged form of the drug molecule moves almost completely across the membrane field through the channel pore to reach its binding site, it is probably also affected by other factors such as inward ionic current through the channel that reduces drug block at negative potentials (24, 27). Whatever its physical meaning, δ remains a useful descriptive value for comparison of use-dependent block with that produced by other local anesthetics.

Discussion

We have characterized a novel Na^+ channel blocker, PD85,639. PD85,639 blocked Na^+ currents at concentrations of 1–40 μM in both primary cultured rat brain neurons and

CNaIIA-1 cells expressing the rat brain type IIA Na^+ channel α subunit. The lipophilic nature of the drug and its pK_a of 9.3 suggest that it crosses the membrane easily. This is consistent with the finding that the drug was an effective blocker when applied from either side of the membrane. The drug appeared less potent when it was applied intracellularly via the recording pipette. This may reflect the fact that the extracellular space can act as a large sink for a rapidly membrane-permeant drug, preventing the intracellular concentration from ever reaching the pipette concentration.

A large component of tonic block was attainable in the absence of stimuli that opened substantial numbers of channels. This block was only weakly dependent on holding potential, as reflected in a slight negative shift in steady state inactivation. Interestingly, the characteristics of use-dependent block were reminiscent of those of more hydrophilic local anesthetics. Use-dependent block by PD85,639 was pronounced even at extremely low stimulus frequencies. Such block is due to drug binding to open and/or inactivated channels during stimulus pulses and slow drug dissociation from closed channels during the interpulse intervals. Although block by PD85,639 was virtually irreversible at the holding potential when channels were closed, the drug dissociated readily if channels were opened, as in Fig. 9. Block by PD85,639 was strongly voltage dependent, suggesting that the cationic form of the drug had to move across a substantial fraction of the membrane electric field to reach its binding site. In these respects, use-dependent block by PD85,639 was similar to that produced by quarternary local anesthetics such as QX-314 (11–13, 22), suggesting that PD85,639 acts by a similar mechanism and possibly binds to the same site within the sodium channel pore.

Local anesthetic/antiarrhythmic drugs like lidocaine and anticonvulsant drugs like phenytoin are clinically useful because they inhibit abnormal electrical activity in cardiac cells and neurons, respectively, but have little effect on normal activity in these cells. This selective inhibition probably stems from the dependence of block on resting membrane potential and frequency of activity. However, tonic block with PD85,639 was not strongly dependent on holding potential, and use-dependent block was pronounced at stimulus frequencies far below those that are physiologically relevant in cardiac cells or brain neurons. Thus, PD85,639 may not be an effective antiarrhythmic or anticonvulsant drug. The most important use of PD85,639 may be as a high affinity probe for the local anesthetic binding site on the Na^+ channel because, as we show in the accompanying paper (19), it binds with high affinity to a local anesthetic receptor site on brain Na^+ channels.

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