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The Role of Inactivation in Open-Channel Block of the Sodium Channel: Studies with Inactivation-Deficient Mutant Channels

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SUMMARY

Inactivation has been implicated as an important determinant of the block of Na^+ channel by local anesthetic-class drugs. This proposition has been difficult to examine because agents used to modify inactivation change other channel properties and both inactivated and blocked channels do not conduct. We used site-directed mutagenesis of Phe1304 to glutamine in the linker between the third and fourth domains of the μ-1 Na^+ channel to slow inactivation. Wild-type and mutant channels were expressed in frog oocytes. Macropatch and single-channel currents were recorded in cell-attached membrane patches. The F1304Q mutation increased mean open time (1.7 fold at −20 mV) and reduced the probability that the channel would fail to open. Closed times were best fit by a double-exponential function, suggesting that the inactivated state transitions were no longer absorbing. In wild-type channels, 100 μM disopyramide decreased mean open time from 1.64 ± 0.08 to 0.34 ± 0.04 msec. Total open time per trial was decreased 2-fold. There also was a marked increase in the fraction of null sweeps. In the inactivation-deficient mutant channel, mean and total open times were also reduced. These data indicate that even when inactivation is slowed by a localized specific mutation, open-channel block by disopyramide persists. Inactivation may not be a necessary requirement for open-channel block.

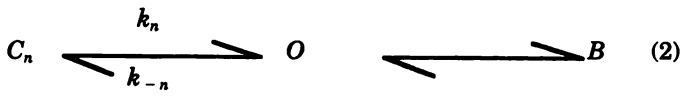
Local anesthetic-class drugs produce tonic and frequency-dependent inhibition of the Na^+ current. Early analyses of block of cardiac Na^+ channels and the neuronal Na^+ channel suggested a central role of channel inactivation in the development of block (1–4). The steady state inactivation relationship is shifted to more negative potentials during exposure to local anesthetic-class drugs. The degree of block is increased as pulse duration is extended beyond the time required for the relaxation of the Na^+ current toward zero (5–9). Because the inactivated state is the principal state occupied at late times during depolarization, such experiments suggest significant binding to that state. Inactivation results in immobilization of gating charge. Block of Na^+ channels by local anesthetic-class drugs produces similar charge immobilization (10, 11). The various models of Na^+ channel blockade suggest that the small tertiary amine local anesthetics such as lidocaine have a high affinity for inactivated Na^+ channels. They bind to and stabilize the inactivated channel state.

The relationship between block and Na^+ channel inactivation has been difficult to establish because neither channel state conducts. A widely used approach is to compare block of Na^+ channels with normal inactivation and those in which inactivation has been slowed or eliminated by enzymes, oxidants, or naturally occurring toxins (12). The action of these agents is not very specific; they may produce widespread effects on the Na^+ channel. The modifying enzymes cleave specific peptide bonds (e.g., trypsin cleaves those associated with lysine and arginine residues). Their action is not likely to be restricted to the susceptible sequences that play a role in inactivation; conceivably, they could modify or eliminate independent inactivation and local anesthetic-binding loci on the channel protein. Similarly, amino acid-modifying agents such as chloramine-T interact with susceptible residues at multiple sites within the protein (13, 14). The inactivation-modifying toxins such as batrachotoxin may also alter channel activation, conductance, and selectivity (15). No consistent relationship between inactivation and block has been demonstrated; some studies reported an abolition of block in inactivation-deficient channels, and other studies reported little change in block (16–21).

The successful cloning and sequencing of the Na^+ channel from brain, heart, skeletal muscle, and eel electroplax have permitted the analysis of the relationship between Na^+ channel structure and function (22). The hydrophobic triplet of isoleucine, phenylalanine, and methionine in the loop between the third and fourth domains of the α subunit plays a

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

critical role in inactivation (23). Mutation of one or more of these residues to glutamine results in the slowing or abolition of channel inactivation. Because the mutation is very localized, the functional consequences are likely to be more specific. Other aspects of channel function, such as conductance and activation, seem to be unaffected (24). These mutant channels provide useful tools with which to examine the relationship between channel inactivation and block (25). In the current study, we focus on specific questions regarding the role of inactivation in open-channel block. The following is the blocking scheme:



(2)

where C_n is one or more closed states, O is the open state, and B is the blocked state. The current integral is unchanged in the presence of a blocker, and single openings are converted to bursts of n openings (26–28). Total open time should be unchanged (28). Therefore, to effect a reduction in current, other mechanisms must participate. One hypothesis is that bursts are terminated prematurely (after m openings; $m < n$) through an absorbing transition to the inactivated state. The inactivation process may therefore play a critical role in open-channel block. We examined this hypothesis by comparing disopyramide blockade of single Na^+ channels in frog oocytes expressing wild-type and inactivation-deficient mutant μ-1 Na^+ channels. Preliminary results have been presented in abstract form (29).

Materials and Methods

Cloning and mutagenesis. Experiments were performed on frog oocytes expressing mutant and wild-type Na^+ channels prepared as outlined below.

Our method for Na channel mutation has been described previously (30). We began with the full-length Na^+ channel cDNA sequence (31) flanked 5′ and 3′ by frog hemoglobin sequence (32). To construct the F1304Q mutation, we used Kunkel-style mutagenesis with a mutagenic 54-mer with four base changes. The nucleotide change resulting in the phenylalanine-to-glutamic acid mutation was TTC4359-4361CAG. To select mutants, we simultaneously engineered a new, unique *Ssp*I site with the nucleotide change C4385T. Mutant cDNAs, when digested with *Ssp*I and *Kpn*I, yielded 8-, 6-, 1.7-, 0.7-, and 0.4-kb fragments compared with wild-type cDNAs, which yielded 9.3-, 1.7-. and 0.4-kb fragments. the mutation was confirmed by the new restriction digest pattern, by sequencing (Lark Sequencing Technologies, Houston, TX), and by the phenotypic change in Na^+ channel gating. Wild-type and mutant cDNAs were linearized with *Sal*I, and capped RNA was transcribed using the SP6 promoter (Megascript; Ambion, Austin, TX). The ratio of cap analog to GTP was 4:1.

Stage V–VI oocytes were injected with 2.5–12 ng of wild-type or mutant Na^+ channel cRNA and incubated in Barth's solution at 19°. Oocytes were used for electrophysiological studies after 48 hr of incubation.

Oocyte preparation, solutions, and electrodes. The follicular cell layer and the vitelline membrane were removed before recording. Batches of 5–10 oocytes were incubated in Ca^{2+}-free modified Barth's medium containing 1 mg/ml collagenase (Worthington, Freehold, NJ) for 1 hr at room temperature. The oocytes were washed repeatedly with enzyme-free medium. The follicular cell layer was then removed mechanically. Oocytes were transferred to a hypertonic stripping solution. After 5–10 min, the transparent vitelline membrane separated from the plasma membrane and was removed with blunt forceps. The oocyte was allowed to settle on the base of the recording chamber for 5–10 min and was perfused with a high-potassium solution. The high-potassium solution depolarized the cell membrane to ~0 mV. Transmembrane potential is expressed in absolute voltage.

The modified Barth's solution contained 88 mM NaCl, 1 mM KCl, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM Na^+ pyruvate, and 15 mM HEPES, pH adjusted to 7.6 with NaOH. Immediately before use, 10 mg/ml penicillin and 0.1 mg/ml streptomycin were added. The hypertonic stripping medium contained 200 mM K^+ aspartate, 20 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES, pH adjusted to 7.4 with KOH. Micropipettes were filled with a Na^+-containing solution of 115 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES, pH adjusted to 7.2 with NaOH. During cell-attached recordings, each oocyte was superfused with a high-potassium solution containing 140 mM K^+ aspartate, 10 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH adjusted to 7.4 with KOH. Disopyramide was obtained from Sigma Chemical (St. Louis, MO). A 0.1 M stock solution of disopyramide was prepared by dissolving the base in 0.1 N HCl and adjusting pH to 7.2 with NaOH. Aliquots of the stock solution were added to the perfusate to give a final disopyramide concentration of 100 μM.

Micropipettes were fabricated from thick-walled Pyrex glass tubing (absorbance, 1.8 mm; i.d., 0.8 mm; Drummond Scientific, Bromall, PA). For single-channel recordings, we used 5–10 MΩ microelectrodes, and for macroscopic recordings, we used 2–5 MΩ microelectrodes. Micropipettes were coated to their tips with Sylgard 184 (Dow Corning, Midland, MI) and fire polished immediately before use.

Recording techniques. Macroscopic and single Na^+ channel currents were recorded with a Dagan 3900 A integrating patch-clamp amplifier (Dagan, Minneapolis, MN). Each microelectrode was coupled to the input of the amplifier with a silver/silver chloride wire coated with Teflon to its tip. A similar silver/silver chloride wire embedded in agar/micropipette solution formed the reference electrode. Offset voltages were typically 1–3 mV and were nulled before membrane seal formation. Currents were analog filtered at a corner frequency of 2–2.5 KHz with an eight-pole Bessel filter (model 902 LPF; Frequency Devices, Haverhill, MA). Filtered currents were digitized at 20 KHz and stored on the fixed drive of a microcomputer (Compaq 386/20). Voltage command pulses were also provided with a microcomputer equipped with a D/A interface (TL1 interface with Labmaster boards; Axon Instruments, Burlingame, CA). Voltage-clamp protocols are given in Results.

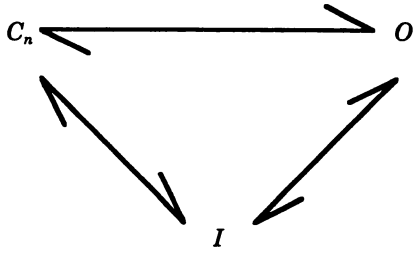
Data analysis. The techniques of data analysis are similar to those previously reported (33). The currents during each depolarizing trial were scanned, and those without events (nulls) were separated. The nulls were averaged, and the averaged current was subtracted from each trial to remove residual leakage and capacitive currents. In most of the experiments with the mutant channel, there were insufficient nulls for leakage and capacitive transient subtraction. In those experiments, 20–40 trials with step depolarizations of 10 or 40 mV were averaged, scaled, and used for leakage and capacitive current subtraction. Single-channel current amplitude was determined from histograms of leakage-subtracted current trials or the mean of clearly resolved events. An automatic detection algorithm with threshold set at 0.5× the single-channel amplitude was used to identify channel openings. The performance of the algorithm was routinely checked by comparing leakage-subtracted current traces with the idealized records generated by the program.

Closed times were determined in experiments in which only one channel was apparent. Histograms of open and closed time distributions were fitted to exponentials using a least-squares procedure. The procedures for exponential fitting of the histogram data are described in detail in a prior single-channel study (33). One or two exponentials were fit to the data. When the exponential rates were nearly equal, the estimation procedure became unstable because of a singular design matrix. The bin width was set at an integer multiple of the sampling interval.

Results

Na^+ channel kinetics in the absence of drug. Like in naturally occurring preparations, a time-dependent shift in channel gating kinetics was also observed in these studies performed in frog oocytes (34). The shift resulted in a change in channel open time (usually an increase at most test potentials) and a decrease in first latency so that the averaged current relaxed faster. The magnitude of the change was variable between patches. When shifts were apparent within 4–5 min of GΩ seal formation, they tended to be large; such experiments were abandoned. We arbitrarily waited for 10 min, and if large gating shifts were not apparent, we proceeded with the experiments. We elected to obtain a large number of trials at few test potentials rather than a few trials at many potentials.

Fig. 1 shows 10 consecutive trials of a total of 329 recorded from a membrane patch containing the F1304Q mutant channel depolarized to −20 mV from a holding potential of −100 mV. The cycle length was 4 sec. There was evidence for a single active channel in this patch. Channel openings were noted throughout the duration of the trials; there was an average of 44 openings per trial. Only one null was noted in the 329 trials; this suggests that in this patch, direct transition from the closed to the inactivated state in the scheme shown below is rare.



Alternatively, the I-to-C_n transition may be occurring at a significant rate.

In describing the gating transitions of Na^+ channel, we use the nomenclature of modes originally proposed for the Ca^{2+} channel by Hess *et al.* (35) and later used to describe the gating of transiently expressed Na^+ channels by Ukomadu *et al.* (36). Mode *O* describes groups of depolarizing trials during which a channel fails to open. Mode 1 refers to trials in which a channel opens a few times and then remains closed for the remainder of the depolarization. Mode 2 describes groups of depolarizing trials in which a channel opens repetitively for the duration of the trial. Gating of the mutant channel was modal. Gating with openings distributed throughout the trial (i.e., mode 2) was the predominant gating mode. We occasionally observed prolonged shifts into a nonconducting (*O*) mode (Fig. 2). Pulses of 200 msec were applied from a holding potential of −100 mV to a test potential of −20 mV. There was evidence for a single channel in this patch. Openings occurred throughout the duration of the pulse. The averaged current (Fig. 2, *middle*) indicates that there is a sustained component of current at the end of the 200-msec pulse. The history plot (Fig. 2, *bottom*) demonstrates a sustained decrease of channel activity. Except for a single trial with one event, the channel was quiescent from trials 8–50. This mode of gating was observed only in patches containing one or two channels. Presumably, the presence of multiple channels in a patch obscured the identification of such behavior of a constituent channel.

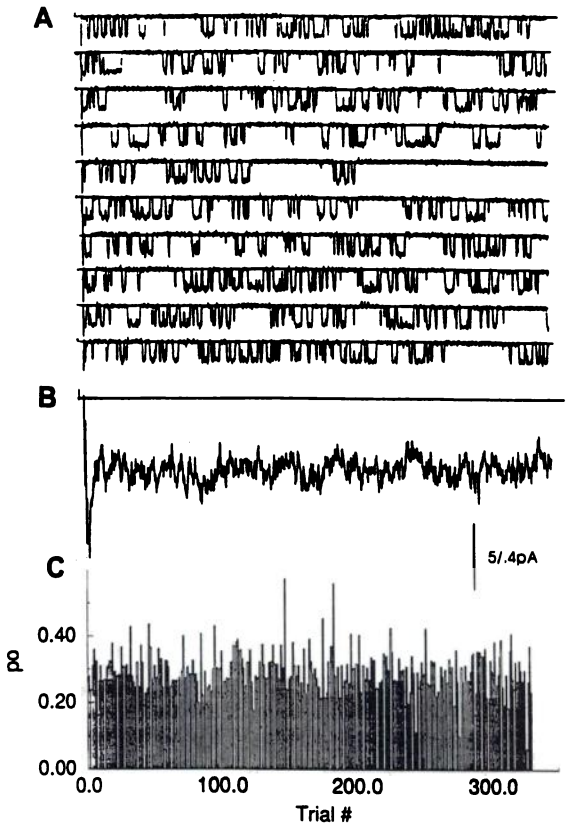


Fig. 1. Gating of inactivation-deficient mutant Na^+ channels. A, Current responses to 10 consecutive 200-msec pulses from −100 to −20 mV. Channel openings are downward. B, Current obtained by averaging 329 trials. C, Trend of events. Channel openness (*po*; $n = 1$) is plotted against pulse number. Currents were filtered at 2.5 kHz.

We did not observe switches from modes with a large number of openings to the mode with few openings (1–2 openings per trial; i.e., from mode 2 to mode 1). Trials with openings distributed throughout the depolarization were almost the exclusive mode of gating of the mutant channel. On the other hand, mode 1-to-mode 2 switches were observed with the wild-type channel (data not shown) and have been well described for Na^+ channels in native membranes (33, 37).

Fig. 3 shows a comparison of the mean open time of wild-type ($n = 3$) and mutant ($n = 5$) Na^+ channels. For both the wild-type and mutant Na^+ channels, the distribution of open times was fit by a single exponential. The mean open times of the channel types were similar at a test potential of −60 mV. However, the open times diverged with increasing depolarization. The mean open time of wild-type channels was weakly voltage dependent; that of the mutant channel increased progressively at depolarized potential. The difference in the voltage dependence of the open time suggests fundamental differences in the mechanisms of channel closure.

For the mutant channel, the large number of events in each trial allows analysis of the closed-time distribution. However, this distribution is most readily interpreted if there is a single functioning channel in the patch. Closed-time distributions from two single-channel patches expressing the F1304Q mutant channel are illustrated in Fig. 4. The histograms were based on 1,600–14,000 events. In each case, the closed-time distribution was best fit by two exponentials. The

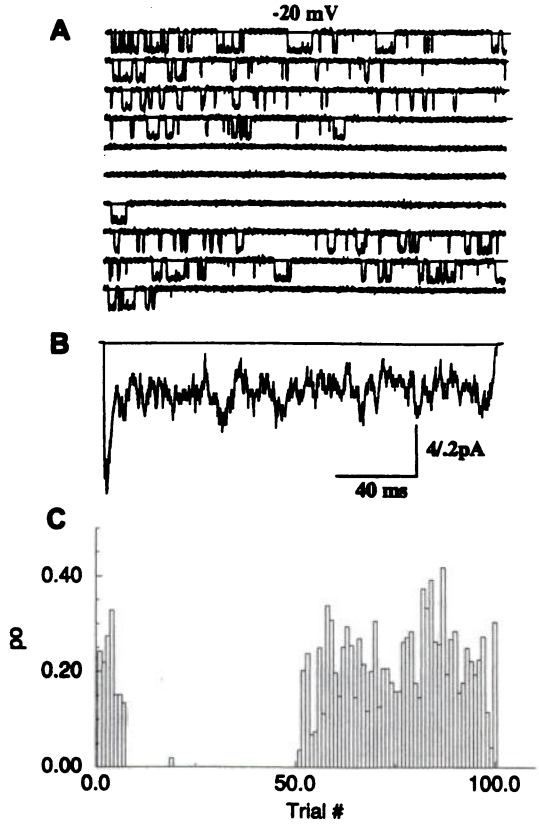


Fig. 2. Modal gating of the inactivation-deficient mutant Na^+ channel. A, Currents in response to 200-msec depolarizing pulses from −100 to −20 mV. Selective trials 5–8 and 49–54 are shown. B, Average current. C, Channel openness (*po*; n = 1) is plotted against pulse number (1–100).

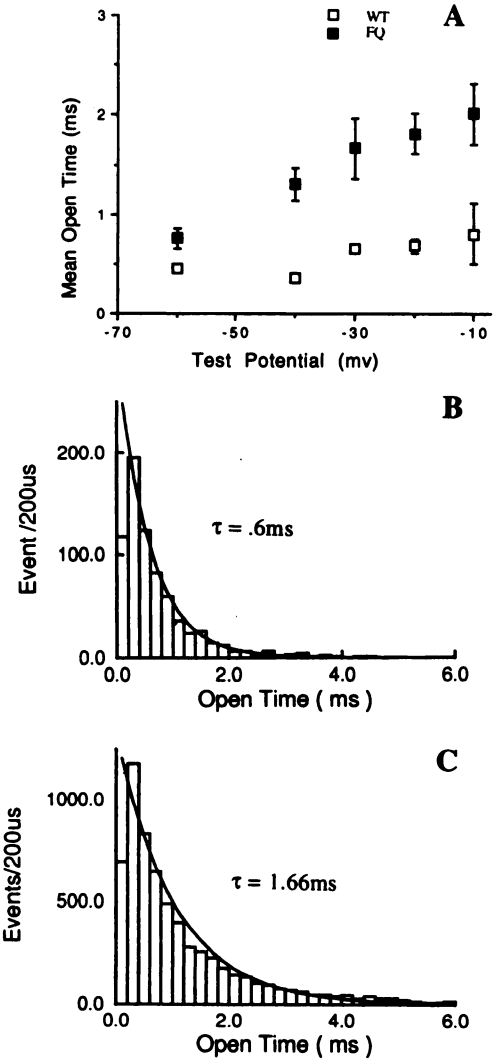


Fig. 3. Comparison of the voltage dependence of the mean open time of wild-type and mutant Na^+ channel openings. A, Mean open time of mutant channels were more strongly voltage dependent than that of wild-type channels. □, Wild-type Na^+ channel. ■, Mutant Na^+ channel. B and C, Distribution of open times of (B) wild-type and (C) mutant Na^+ channels. In each case, the test potential was −20 mV. The distribution at open times was fit by a single exponential with time constant τ. The fitting procedure uses a theoretical correction for the reduced number of entries in the first bin (33).

average value of the fast and slow time constants was 0.19 ± 0.06 and 3.4 ± 1 msec, respectively. The biexponential distribution of closed times suggests that the mutant Na^+ channels are opening from two distinct closed states. One pathway should reflect the passage from the resting closed states. At −20 mV, transition from these states is primarily dependent on the terminal transition (rate k_n in eq. 1). Another measure of this transition is the mean first latency. The mean first latency for the four experiments was 2.6 to 5.9 msec. This is of the order of magnitude of the slow time constant of the closed-time distribution. A strict equivalence would not be anticipated. The fast time constant should reflect return from the inactivated state or states.

Block of wild-type and mutant Na^+ channels by disopyramide. For the wild-type channel, we had five complete experiments in which stable recordings were obtained through a 10-min stabilizing period, acquisition of control data, a minimum of a 15-min exposure to disopyramide, and acquisition of data during drug exposure. Representative recordings are shown in Fig. 5. Fig. 5 (*top*) shows current responses to 10 consecutive depolarizations. The 200-msec pulses to −20 mV were applied from −100 mV at a cycle length of 4 sec. In 199 trials, there were 80 nulls. The probability of transition into an inactivated (absorbing) state was 0.63. Open times were exponentially distributed, with a mean ± standard deviation of 0.65 ± 0.67 msec. Fig. 5 (*bottom*) shows records obtained during exposure to 100 μM disopyramide. The predominant effect was an increase in the fraction of null trials. Of a total of 200 trials, there were 170 nulls. The probability that a channel would fail to open, corrected for the apparent maximal number of functioning channels, increased from 0.63 to 0.85. This is a conservative estimate because action of the drug decreases the likelihood of observing overlapping events; the maximal number of overlaps was two during control and one during exposure to disopyramide.

Single-channel mean open time decreased from 0.65 ± 0.67 to 0.3 ± 0.2 msec. In fact, for the 200 trials during disopyramide exposure, there were only 33 events, which is too few to allow examination of the distribution of open times. The total open time (the product of the total number of openings and the mean open time) decreased from 257.4 to 9.9 msec. Summary data are presented in Table 1. The mean association rate constant, which was estimated from the abbreviation of open time and drug concentration, was 2.7 ± 1.5 ×

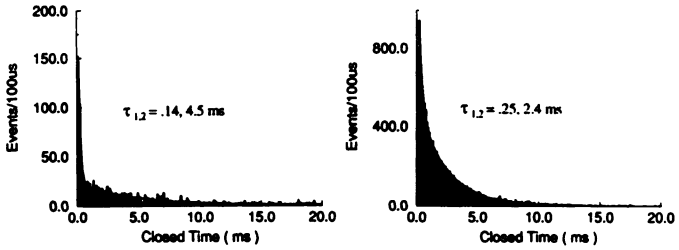


Fig. 4. Closed-time distribution in two (*left* and *right*) membrane patches containing a single active channel. The bin width is 100 μsec. Each distribution is fit with a double exponential function. *Inset*, fast and slow time constants, τ_1 and τ_2. The ratio of the slow and fast time constants averaged 20:1 for all experiments (4).

TABLE 1
Wild-type

Exp.	Exp. no.	Mean open time [1] (ms)	p_f[a]
		msec	
Control	040795	0.65 ± 0.67	0.63
Drug		0.3 ± 0.2	0.85
Control	033095	0.33 ± 0.31	0.71
Drug		0.4 ± 0.2	0.94
Control	031395	0.69 ± 0.76	0.6
Drug		0.25 ± 0.2	0.88
Control	082995	0.5 ± 0.5	0.37
Drug		0.15	0.98
Control	091694	0.8 ± 1.1	0.43
Drug		0.46 ± 0.30	0.58

[a] Probability that the channel will fail to open.

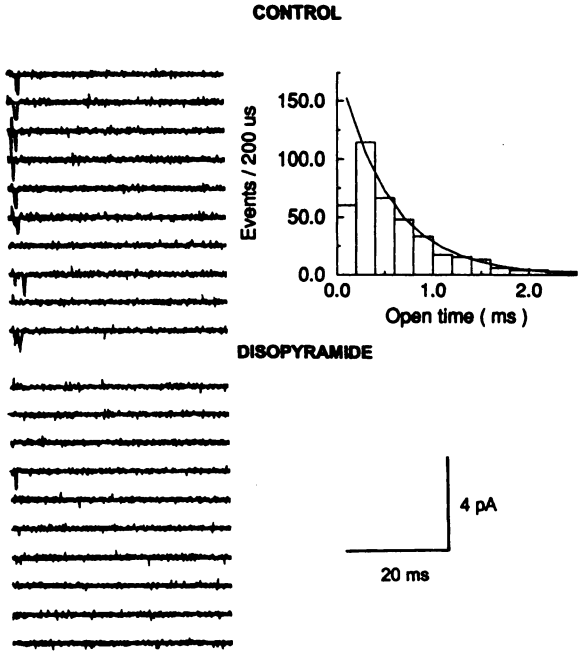


Fig. 5. Block of wild-type Na^+ channels. *Top left*, membrane current during 10 consecutive 200-msec pulses from −100 to −20 mV. Single-channel open time was distributed as a single exponential (*top right*) with a mean closing-time constant of 0.53 msec. Currents were recorded from the same patch during exposure to 100 μM disopyramide. Because of the high frequency of nulls, there are insufficient events in the 200 trials during drug exposure to plot a histogram.

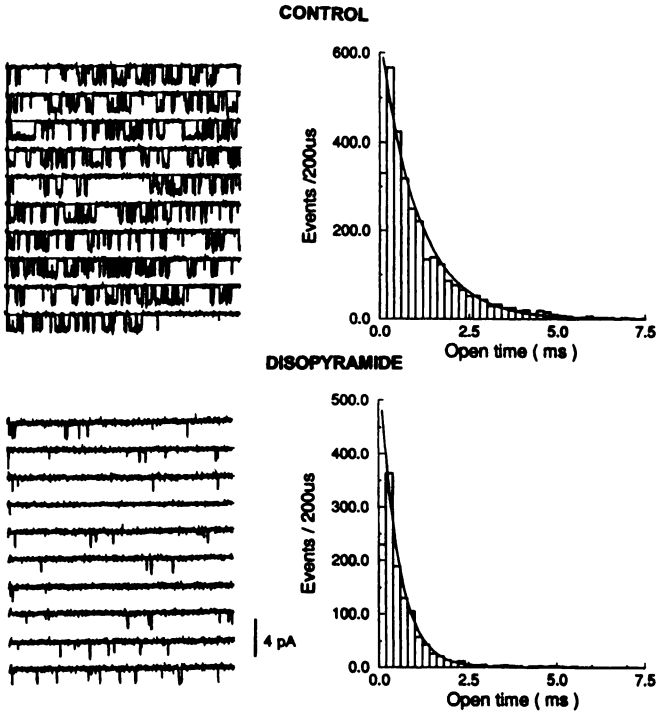


Fig. 6. Block of mutant Na^+ channel by disopyramide. *Top left*, current responses to 10 consecutive 200-msec depolarizing trials from −100 to −20 mV during control. *Right*, histogram of open times. Open times were exponentially distributed with a mean closing-time constant of 1 msec. *Bottom*, current responses and open-time histogram during exposure of the same patch to disopyramide. Mean closing time constant decreased to 0.53 msec.

10^7/M/sec (28). Reduction in the mean open time and an increase in the fraction of null sweeps resulted in a decrease in current during drug exposure. Disopyramide did not change the latency to first opening (data not shown). During drug exposure, the abbreviated openings of the wild-type channel occurred promptly after depolarization.

Complete experiments in which the effects were examined of disopyramide on the inactivation-deficient mutant Na^+ channel were obtained on nine oocytes. In three oocytes, the membrane patch remained stable during the acquisition of the control data, exposure to 100 μM disopyramide, and acquisition of postdrug data. In six experiments, pairs of data sets were obtained during control and drug exposure on separate patches of the same oocytes. Membrane currents during control and exposure to disopyramide are compared in Fig. 6. Fig. 6 (*top*) shows 10 consecutive of a total of 200 depolarizing trials during control. The holding potential was −100 mV, and the depolarizing steps were 200-msec pulses to −20 mV. Channel openings were distributed throughout the duration of the pulse. Open times were exponentially distributed with a mean of 1.2 ± 1.5 msec. There were two nulls in the total of 200 trials. Current responses to consecutive depolarizations during disopyramide exposure are shown in Fig. 6 (*bottom*). The number of events per trial was markedly reduced (control, 31 events/trial; disopyramide exposure, 6.2 events/trial). Mean open time was reduced to 0.6 ± 0.78 msec. The association rate constant was 1.0 ± 0.8 × 10^7/M/sec, a value similar to that observed for the wild-type channel. Summary data are presented in Table 2. Despite these clear indicators of block, the number of nulls remained small: five in a total of 200 trials. Although channels opened promptly during control, the first opening seemed to be considerably delayed during exposure to diso-

TABLE 2

Phenylalanine-to-glutamine mutant

A, control and drug data obtained on the same seal; B, control and drug data obtained on separate seals on the same oocyte.

Exp.	Exp. no.	Mean open time	pf[a]
		msec	
A			
Control	020795	1.2 ± 1.5	0.1
Drug		0.6 ± 0.8	0.16
Control	112294	1.14 ± 1.24	
Drug		0.29 ± 0.28	
Control	021494	1.9 ± 2.2	
Drug		0.8 ± 1	0.32
B			
Control	041594a	1.4 ± 1.6	0.27
Drug	04159aa	0.4 ± 0.39	0.3
Control	013194bd	2.7 ± 3	0.08
Drug	013194eh	1.2 ± 2.1	0.3
Control	020794c	2 ± 2.6	
Drug	020794ac	1.8 ± 2.1	0.22

[a] Probability that the channel will fail to open.

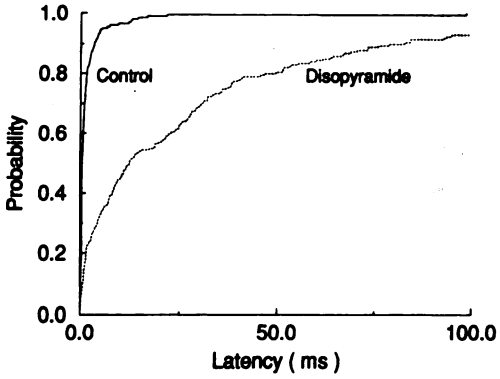


Fig. 7. Latency to first opening in the phenylalanine-to-glutamine mutant during control and exposure to disopyramide. Data were obtained during control (*continuous line*) and exposure (*dotted line*) to disopyramide. Data were obtained from currents in the path illustrated in Fig. 6. Latency to first opening was delayed during exposure to disopyramide.

pyramide. This was formally examined by comparing the cumulative latency to the first opening during control and exposure to disopyramide (Fig. 7). During control, the asymptotic cumulative probability was ~1, reflecting the rarity of nulls. Mean latency to first opening was 5.4 msec. Latency to first opening was substantially delayed to 18.4 msec during exposure to disopyramide. The data suggest that the decrease in the rate of closed to inactivated state transition permits late opening of the channel during drug exposure. The corresponding transition in the wild-type channel is faster, favoring the occurrence of a high proportion of null sweeps.

Discussion

Influence of the F1304Q mutation on the gating of Na^+ channels. The influence of the mutation F1304Q on the α subunit of the skeletal muscle Na^+ channel has not been previously reported. As in the case of the rat brain II and human cardiac muscle Na^+ channel, the predominant effect of the F1304Q mutation was a slowing of inactivation (23, 24). There were several expressions of slowed inactivation: (i) voltage dependence of the mean open time (Fig. 3), (ii) slowed relaxation of the macroscopic current, and (iii) rarity of null sweeps except during modal shifts of gating. The frequency of nulls reflects the rate of inactivation of closed channels.

Except at the threshold potential, mean open time of the mutant channel is markedly prolonged compared with the wild-type channel. We observed no change in latency to first opening. For the wild-type channel, closure occurs by the transition O-to-C_n, a reversal of the initial opening process (38–40). With increasing depolarization, closure occurs primarily by inactivation (38). Because this process is weakly voltage dependent, mean open times show little voltage dependence. In contrast, inactivation plays a smaller role in the closure of the mutant channel. The O-to-C_n transition plays a significant role in closure, even at depolarized potentials. Because this process is inherently more voltage dependent than inactivation, greater voltage dependence of the mean open time is observed (38). These conclusions are based on qualitative arguments. The determination of the fraction of channels closing by inactivation would require estimates of the fraction of null sweeps (rare for the mutant channel) and is based on the theory that the inactivated state is absorbing (39, 40).

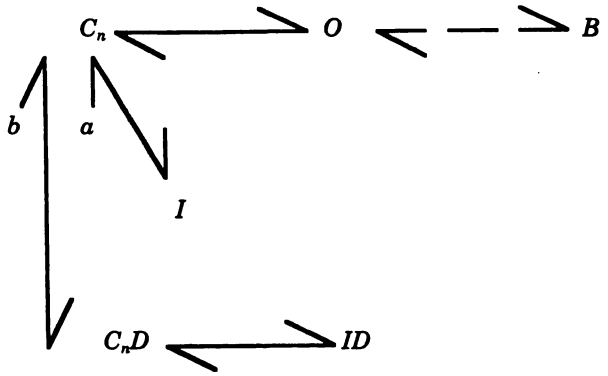
Relaxation of the macroscopic current in the phenylalanine-to-glutamine mutant channel of the μ-1 α subunit is more marked than that observed in the corresponding mutant in the rat brain II channel. It should be noted that much longer pulses (200 msec) were used in the current study compared with prior studies of the inactivation-deficient mutant channels. However, the extent of current relaxation is similar to that observed after exposure to chloramine-T (Ref. 13, Fig. 7) or batrachotoxin (Ref. 21, Fig. 1). The voltage dependence of the residual current at 100 msec in the mutant channel suggests the persistence of a significant component of inactivation in the phenylalanine-to-glutamine mutant (data not shown). Inactivation is slowed but not eliminated by the mutation.

The rarity of nulls except during modal transitions is similar to that reported by Hartman *et al.* (24) in the cardiac Na^+ channel isoform. It is as striking an effect of the mutation as the marked slowing of current relaxation. The rarity of nulls is consistent with marked slowing of the net C_n-to-I transition. It suggests that a single motif, IFM, is critical for both open- and closed-channel inactivation. It is this slowing of the C_n-to-I transition that may be critical to the difference in block of the wild-type and mutant channels by disopyramide.

The distribution of closed times was not examined in the prior studies of the F1304Q mutant brain and cardiac muscle Na^+ channels. The large number of events in each depolarizing trial of patches with the mutant channel permitted an analysis of the distribution of closed times. We limited the analysis to patches containing a single functioning channel. The closed-time distribution was clearly biexponential, with a ratio of the time constants of 18:1. This observation indicates that the mutant channel is opening from two distinct states. One of these states is the terminal closed state, C_n, on the pathway to opening. Openings are also occurring from an "inactivated" state that is no longer absorbing. As pointed out by Hartman *et al.* (24), it is necessary to postulate multiple inactivated states to account for the fast and residual slow components of inactivation.

Block of wild-type and mutant Na^+ channels by disopyramide. In the wild-type Na^+ channel, the predominant effect of disopyramide on the Na^+ channel was an

increase in the fraction of null sweeps. The probability that a channel would fail to open increased from 0.55 ± 0.06 to 0.85 ± 0.05 ($p < 0.05$; Table 1). This is similar to the effect observed in the cardiac Na^+ channel isoform in native cell membrane (41). It is a blocking mechanism shared by open and inactivated-state blocking drugs (42–44). The increase in the fraction of null sweeps has always posed a problem for the analysis of open-channel block. A substantial number of depolarizing trials must be performed to obtain a sufficient number of events for analysis. The proposed model for this block that increases the fraction of nulls with the wild-type channels is shown in the following scheme:



where D is the drug, and a and b are the association and dissociation rate constant, respectively. Even with 200-msec test pulses, we did not observe first opening occurring at late times. This suggests that b is small. Significant reversal of block to the C_n would permit transitions to O or I or a return to C_nD. As late events were not observed, we have to conclude that b is small. An additional possibility is C_nD may undergo further transitions, for example to the blocked inactivated state, ID.

Disopyramide reduced the mean open time of the wild-type μ-1 Na^+ channel. Therefore, as a *minimum* requirement, the model includes an additional element, as follows, to account for open-channel block:

Despite the brief open time and the reduction in the number of events per trial, a reduction in open time was documented in four of five patches expressing the wild-type channel.

There also was a trend for the number of nulls to increase when the mutant channel was exposed to disopyramide. Mean open time was decreased by 50%. Disopyramide reduced the number of openings per trial. We would have predicted that if inactivation were essential for the reduction of the total open time per trial, then the marked slowing of inactivation would be associated with little reduction in the total open time per trial. The results indicate that disopyramide caused a substantial decrease in total open time even when inactivation is markedly slowed. Disopyramide prolonged the latency to first opening of the mutant channel. This may be related to reversibility of the ID-to-C_nD-to-C_n transitions. Return to the C_n state late during the depolarizing trial will result in openings occurring with long first latency but with a smaller proportion of null sweeps compared with the wild-type channel.

Prior studies of block of inactivation-deficient Na channels. The influence of inactivation on the block of Na^+ channels by local anesthetic-class drugs has been examined in both nerve and cardiac muscle. Zaborovskaya and Khodorov (16) examined the block of chloramine-T-treated Na^+ channel in frog node of Ranvier. They identified two classes of response. The use-dependent block produced by the small tertiary-amine local anesthetics tetracaine and lidocaine was markedly attenuated in the inactivation-deficient channels. On the other hand, use-dependent block by the open-channel blockers *N*-propyl-ajamaline and KC 3791 persisted. The concept of two categories of Na^+ channel-blocking agents was developed further by Cahalan *et al.* (18). Certain drugs, such as QX-314 and QX-222, are "inactivation enhancers." Voltage-dependent block by these compounds is diminished or abolished by removal of inactivation. Like the normal inactivation process, they cause partial immobilization of gating charge. Other drugs, such as pancuronium, are "gate immobilizers." Use-dependent block persists after inactivation is removed by pronase, and the drug may cause complete immobilization of gating charge. The two categories of blocking agents raised the possibility of multiple receptor sites for the local anesthetic-class drugs.

In nerve, the influence of inactivation on block depends on the agent used to retard inactivation. Ulbricht and Stoye-Herzog (45) showed that benzocaine produced rapid reduction ($t_{1/2}$ = 60 msec) of chloramine-T-induced sustained Na^+ current in frog node of Ranvier. However, a similar experiment in the veratridine-treated node resulted in a much slower block ($t_{1/2}$ = 3.6 sec).

Koumi *et al.* (19, 46) used chloramine-T treatment to examine the role of inactivation in the block of whole-cell Na^+ current by quinidine and disopyramide in guinea pig ventricular myocytes). Chloramine-T treatment reduced the current amplitude by 40%, indicating the presence of other actions in addition to the slowing of inactivation. Use-dependent block persisted in the inactivation-deficient channels. In the case of disopyramide, fast and slow phases of block onset and recovery could be defined. The fast phase of disopyramide block was abolished in the inactivation-deficient channels. Persistence of slow component or components of inactivation may account for the residual block. A similar persistence of use-dependent block of the cardiac Na^+ current by lidocaine is observed when inactivation is slowed with enzyme treatment or exposure to batrachotoxin (21). Our studies show the persistence of block when inactivation is slowed by a point mutation in the IMF motif controlling inactivation, which is in contrast with the recent studies reported by Bennett *et al.* (25) and Benz and Kohlhardt (47). Benz and Kohlhardt used reduced temperature (9°) to show the inactivation of the Na^+ current recorded from inside-out membrane patches. Propafenone and lidocaine did not block open channels with slowed inactivation. Bennett *et al.* performed the triple mutation IFMQQQ on the human cardiac Na^+ channel. There was no relaxation of the Na^+ current, with pulses of duration as long as ~100 msec (Fig. 6 of Ref. 25). Use-dependent block by lidocaine was almost completely eliminated, even at pulsing frequencies of 10 Hz.

There are a number of potential causes for the difference in results. In addition to slowing inactivation, the reduced temperature may have decreased the blocking potency of the drugs in the study of Benz and Kohlhardt (47). Inactivation is almost completely eliminated in the channels expressing the triple mutant. The residual inactivation in the studies on the

native Na^+ channels and our studies in the mutant μ-1 Na^+ channel may have contributed to the residual component of block. The studies of Bennett *et al.* (25) involved the expression of only the α subunit of hH1 in frog oocyte. The β subunit contributes to the rapid inactivation of the Na^+ current (48). This rapid component of inactivation may play a role in block. Lidocaine has weak, if any, open-channel-blocking properties and predominantly blocks inactivated channels (34, 44). Further studies are required that that use expression of both the QQQ mutant α and β subunits or express in a mammalian cell system in which inactivation of the expressed channel is similar to that in native cells. Block by disopyramide and lidocaine should be contrasted in such channels.

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