Block of Wild-Type and Inactivation-Deficient Cardiac Sodium Channels IFM/QQQ Stably Expressed in Mammalian Cells

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ABSTRACT The role of inactivation as a central mechanism in blockade of the cardiac Na^+ channel by antiarrhythmic drugs remains uncertain. We have used whole-cell and single channel recordings to examine the block of wild-type and inactivation-deficient mutant cardiac Na^+ channels, IFM/QQQ, stably expressed in HEK-293 cells. We studied the open-channel blockers disopyramide and flecainide, and the lidocaine derivative RAD-243. All three drugs blocked the wild-type Na^+ channel in a use-dependent manner. There was no use-dependent block of IFM/QQQ mutant channels with trains of 20 40-ms pulses at 150-ms interpulse intervals during disopyramide exposure. Flecainide and RAD-243 retained their use-dependent blocking action and accelerated macroscopic current relaxation. All three drugs reduced the mean open time of single channels and increased the probability of their failure to open. From the abbreviation of the mean open times, we estimated association rates of $\sim 10^6$/M/s for the three drugs. Reducing the burst duration contributed to the acceleration of macroscopic current relaxation during exposure to flecainide and RAD-243. The qualitative differences in use-dependent block appear to be the result of differences in drug dissociation rate. The inactivation gate may play a trapping role during exposure to some sodium channel blocking drugs.

INTRODUCTION

Frequency-dependent block of the cardiac Na^+ channel is central to antiarrhythmic drug action. Rapid beats during tachycardia are strongly suppressed, whereas the normal beats are minimally affected. The greater block during repetitive excitation indicates the Na^+ channel conformations occupied during depolarization have greater drug affinity or more accessible binding sites (Hille, 1977; Hondeghem and Katzung, 1977; Starmer and Grant, 1985). It is the channel conformation, not the membrane potential, that enhances drug binding to the Na^+ channel (Grant et al., 1993). Drugs bind to open and inactivated channels during membrane depolarization. Evidence from a wide range of studies supports the role of the inactivation process in channel blockade. Many antiarrhythmic drugs, e.g., the class IB drugs lidocaine, mexiletine, and amiodarone, produce progressive block as the duration of depolarization is increased beyond the initial transient inward current to times when channels became inactivated (Sanchez-Chapula et al., 1983; Bean et al., 1983; Clarkson et al., 1988).

Attempts to further define the role of the inactivation process in block by slowing or disabling inactivation have produced conflicting results. Superfusion of the cytoplasmic surface of the sarcolemma with endopeptidases, such as pronase and chymotrypsin, amino acid-modifying agents, such as chloramine-T and the pyrethyroid toxins, slow inactivation (Armstrong et al., 1973; Cohen and Barchi, 1993; Wang et al., 1987; Wasserstrom et al., 1993). The Na^+ current assumes a compound waveform, showing an initial transient component followed by a persistent component, after treatment with inactivation modifiers. Frequency-dependent block of the persistent component of neuronal Na^+ current by lidocaine and tetracaine was abolished after chloramine-T modification, whereas block by the open channel blockers N-propyl ajmaline and KC 3791 remained (Zaborovskaya and Khodorov, 1984). Use-dependent block of neuronal Na^+ channels by ethidocaine and QX314 persisted after chloramine-T treatment, whereas block is abolished after pronase treatment (Wang et al., 1987). Both modifying agents exerted similar effects on inactivation. For the cardiac sodium channel, enzymatic removal of inactivation shifts activation to more negative voltages (Benitah et al., 1996). This shift in activation could influence drug action (Lawrence et al., 1996). The nonspecific nature of chemical modification of inactivation may account for the inconclusive results.

The mutation IFM/QQQ in the linker between the third and fourth domains ($ID_{III/IV}$) of the Na^+ channel disables inactivation (West et al., 1992; Hartmann et al., 1994). This provides an attractive alternative model for examining the role in inactivation in channel blockade. Bennett et al. (1995) showed ~80% blockade of the wild-type channel by 25 μM lidocaine at a stimulus frequency of 5 Hz, but <18% block of the IFM/QQQ mutant with 100 μM lidocaine. Balser et al. (1996) further showed that lidocaine strongly suppresses the persistent component of current in this mutant channel, restoring the wild-type phenotype. Based on their modeling of the change in the current time course, they indicated that the action of lidocaine could not be explained by the modulated receptor model of Hille (1977) and Hondeghem and Katzung (1977). Basler et al. suggested that antiarrhythmic drugs functioned as allosteric modula-

Received for publication 10 April 2000 and in final form 30 August 2000.
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0006-3495/00/12/3019/17 $2.00

tors of channel inactivation, enhancing inactivation at late times during depolarization. However, current reduction at late times cannot be uniquely attributed to inactivation or to block.

The role of inactivation in channel block was also examined by measurement of gating currents in squid giant axon and cardiac sodium channels. Block of the Na^+ current in squid giant axon by QX314 immobilizes the same component of gating change subjected to physiologic immobilization during inactivation (Armstrong & Bezanilla, 1977). However, in cardiac muscle, the gating current data suggest drug action on channel activation (Hanck et al., 2000).

There are a number of important unanswered questions on the role of inactivation in channel blockade using the IFM/QQQ mutant model. It is not clear whether other antiarrhythmic drugs, e.g., typical open channel blockers like disopyramide, also require intact inactivation to effect use-dependent block. Courtney (1975) has suggested that the inactivation gate may trap open channel blockers in the channel pore. If the enhancement of current relaxation in the IFM/QQQ mutant results from the enhancement of inactivation, it should not be evident with "open-state blockers." Alternatively, if a mechanism of local anesthetic action is to reduce burst duration by enhancing transition into slow inactivated state(s), the enhanced current relaxation should be evident with open channel blockers. The need to expand the class of sodium channel blocking drugs examined in these mutant channels is made more pressing by the recent studies by Vedantham and Cannon (1999) suggesting that at least for the lidocaine-class local anesthetics, it is activation rather than inactivation that is crucial for block.

We have expressed the wild-type and IFM/QQQ mutant of the human cardiac Na^+ channel hH1 stably in HEK-293 cells. Using whole-cell and single-channel recordings, we have examined channel blockade by the open channel blockers, disopyramide and flecainide, and the lipid-soluble lidocaine derivative RAD-243. All three drugs block Na^+ channels with intact inactivation, and disopyramide is also trapped in the channel. We show that disopyramide blocks open IFM/QQQ Na^+ channels but produces no use-dependent block. Flecainide blocks open Na^+ channels from a site accessed from the extracellular space, produces significant use-dependent block, and accelerates current relaxation like "inactivated-state blockers"; RAD-243 produces use-dependent block, abbreviates mean channel open time, and enhances current relaxation by shortening burst duration. The role of inactivation in the block of the Na^+ channel by antiarrhythmic drugs is dependent on kinetics of drug binding to the channel; it may influence the action of open- and inactivated-state blockers. Our preliminary results have been published in abstract form (Grant et al., 1998).

METHODS

Construction of stable cell lines expressing IFM/QQQ

The human cardiac Na^+-channel gene, hH1, was cloned into the mammalian expression plasmid pcDNA3. We mutated the triplet IFM in the III–IV interdomain linker to QQQ using recombinant PCR (Higuchi, 1990). HEK-293 cells were transfected with the plasmid pcDNA3/hH1QQQ using lipofectamine. Cells were cultured in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). G-418 (500 μg/ml) was used as the dominant selective marker. Clones were screened for Na^+-channel expression using whole-cell recordings. All three positive clones isolated showed Na^+-channel currents with markedly slowed current relaxation, but peak current amplitudes were usually $<$100 pA. The resting membrane potential of the cells was ~ -60 to -50 mV, i.e., close to the threshold for Na^+-channel activation.

We suspected that Ca^{2+} overload secondary to high intracellular $[Na^+]$ was suppressing channel expression. In native cells, elevation of $[Ca^{2+}]_i$ decreases peak Na^+ current (Chiamvimonvat et al., 1995). Culture in Na^+- or Ca^{2+}-free medium, high concentrations of TTX, or the membrane-permeable Ca^{2+}-chelator BAPTA-AM resulted in poor cell survival or no increase in channel expression. In developing neurons, sodium channel expression is down-regulated by the sodium channel activators veratridine and α-scorpion toxin (Giraud et al., 1998). The process is calcium-independent, and mimicked by membrane depolarization. We examined whether an intervention that could increase membrane potential would affect current amplitude. We co-transfected HEK-293 cells with the inward rectifier K^+ channel IRK1 and IFM/QQQ using G-418 and hygromycin as dominant selective markers. Positive clones expressed Na^+ currents of $\sim$0.5 to several nA.

Experimental setup

Whole-cell and single Na^+-channel currents were measured in HEK-293 cells expressing the wild-type and mutant Na^+ channels. Cells were superfused with a Na^+ external solution for whole-cell recordings. The micropipettes were filled with a Cs^{2+} internal solution. Na^+ currents were the only ionic current recorded under these conditions. Cells were superfused with a high-K^+ external solution for the cell-attached single channel recordings. The high $[K^+]$ external solution reduced the membrane potential to 0 mV. Membrane potentials are reported as absolute values. The major cations in micropipette solution are Na^+ and K^+. Occasionally we observed outward single channel currents positive to -30 mV. These currents were kinetically distinct from the Na^+ channel and we presumed that these were single K^+-channel currents. Trials showing such current were excluded from analysis.

Solutions

The solutions used in these experiments had the following compositions (mM): Na^+ external solution: NaCl 130, KCl 4, $CaCl_2$ 1, $MgCl_2$ 5, HEPES 5, and glucose 5 (pH adjusted to 7.4 with NaOH); Cs^+ micropipette solution: CsCl 130, $MgCl_2$ 1, MgATP 5, BAPTA 10, HEPES 10 (pH adjusted to 7.2 with CsOH); high K^+ external solution: potassium-aspartate 140, KCl 10, $MgCl_2$ 2, $CaCl_2$ 1, glucose 5, HEPES 5 (pH adjusted to 7.4 with KOH); Na^+-micropipette solution for single-channel recordings: NaCl 140, KCl 5, $MgCl_2$ 2.5, $CaCl_2$ 0.5, HEPES 5 (pH adjusted to 7.4 with NaOH). All experiments were performed at room temperature (20–22°C).

Antiarrhythmic drugs

We examined the use-dependent blocking action of three sodium channel blockers. We selected disopyramide as an example of an antiarrhythmic drug that produces discrete block of open sodium channels, has distinct structural differences from lidocaine, and has been well studied in native cells (Sunami et al., 1991; Koumi et al., 1992; Zilberter et al., 1994; Grant et al., 1996). Flecainide has a pK_a of 9.3 and is 99% protonated at pH 7.4. It blocks sodium channels from an extracellular site; no block is observed with intracellular application (Nitta et al., 1992). The extent of flecainide

block does not depend on the duration of depolarization. It is presumed to be an open channel blocker. However, we are not aware of any studies at the single channel level demonstrating such block. The third drug we studied was the lidocaine derivative RAD-243. A butyl group replaces one of the ethyl substituents on the tertiary amine, increasing lipid solubility 50-fold. It blocks inactivated channels. However, unlike lidocaine, it also produces discrete open-channel block with intermediate kinetics that can be readily resolved (Liu et al., 1994). Disopyramide and flecainide were obtained from Sigma Chemical Co. (St. Louis, MO). RAD-243 was generously provided by Dr. Rune Sandberg (Astra A Lab, AB, Sodertalije, Sweden). Disopyramide was prepared as a 10^{-2} M stock solution in dilute HCl. Flecainide was prepared as a 10^{-2} stock solution in water. RAD-243 was prepared as a 10^{-2} stock solution in DMSO.

Recording techniques

Whole-cell and single-channel recordings were performed with an Axopatch-200 or EPC-7 patch clamp amplifier (Axon Instruments, Inc., Foster City, CA, and Adams and List, Great Neck, NY, respectively). Whole-cell currents were recorded with 0.5–1.5 MΩ microelectrodes coated with a hydrophobic elastomer (Sylgard 184) to their tips. Series resistance and capacitive transients were compensated using standard techniques. At the beginning of each experiment, adequacy of voltage control was assessed as previously described (Gilliam et al., 1989). As the peak currents in cells expressing the IFM/QQQ mutant were usually <2 nA, adequate control was obtained in all but the rare cell. Whole-cell currents were filtered at 10 kHz and digitized at 20–40 kHz.

Single Na^+-channel currents were recorded with high-resistance (10–15 MΩ) microelectrodes. The holding potential was set at −90 mV, and 200-ms test pulses were applied to −20, −30, −40, and −60 mV. Currents were filtered at 2.5 kHz and digitized at 20 kHz.

Data analysis

Analysis of the whole-cell and single-channel currents are described in prior publications from this laboratory (Grant and Starmer, 1987; Grant et al., 1989). Peak whole-cell currents were determined from digitized currents using custom software written in C programming language. We subtracted leakage current and capacity transients before analysis. Currents during each depolarizing trial were scanned, and the null sweeps collected and averaged. The averaged null sweep was subtracted from each trial. Single-channel current amplitude was determined from an all-points histogram of the leakage-subtracted currents. Single-channel openings were identified using an automatic detection algorithm with the threshold set at 0.5 times the single-channel amplitude. Closed times were determined from trials that had no overlapping openings.

Histograms of open and closed times were fit using a least-square procedure. The bin width was set at an integer multiple of the sampling interval (Hurwitz et al., 1991). Open times were usually fit by single-exponential functions. The distribution of closed times usually required two exponentials for fitting. Values are quoted as means ± SE unless otherwise stated. Comparisons were made using unpaired or paired *t*-tests as appropriate; $p < 0.05$ was considered significant.

RESULTS

The properties of the wild-type sodium channels stably expressed in HEK-293 cells have been described in prior studies from this laboratory (Chandra et al., 1998). We shall first describe the properties of the IFM/QQQ mutant sodium channel expressed in the same cells. Although the antibiotic selection should have produced clones of cells, we noted some variability between cells in the effect of the mutation on the time course of inactivation. Whole-cell currents from representative cells are illustrated in Fig. 1, *A* and *C*. From a holding potential of −100 mV, 500-ms conditioning steps were performed from −100 to −20 mV in 5-mV increments, each followed by a test depolarization to −20 mV. *Panels A* and *C* show currents at the end of the conditioning pulse to −100, −80, −60, −40, −20, and the test pulses. The whole-cell currents in *panel A* show essentially no relaxation. The normalized maximum current as a function of conditioning potential is plotted in *panel B*; 500-ms conditioning depolarizations to potentials as low as −20 mV had no effect on maximum current. Clearly, the fast inactivation process has been removed by the IFM/QQQ mutation. Currents elicited in another cell from the same line using the above protocols are illustrated in *panel C*. Currents elicited from conditioning potentials of −100 to −60 mV show some inactivation, with a steady-state current 63% of the peak current for the most negative conditioning potentials. Conditioning voltages that fail to open sodium channels elicit very little steady-state inactivation, suggesting that closed-state inactivation remains disabled. It is only at potentials to −60 mV, i.e., positive to the current threshold, that significant inactivation of the current occurs. As shown in Fig. 1 *D*, steady-state inactivation is incomplete. The potential for half-inactivation of the current was −56 mV. This is ~30 mV positive to that of the wild-type channel expressed in the same cells (Chandra et al., 1998). The shift is in part a reflection of impaired closed-state inactivation of the IFM/QQQ mutant channel. We are unsure of the basis for the difference in the degree of inactivation between cells. A significant endogenous current could be contributing to the total current in Fig. 1 *C*. However, in nontransfected cells or during the screening process to identify the positive clones, a majority of cells showed no endogenous sodium current, or peak currents in the 10–20 pA range. Another possibility is that the cells may produce a milieu for varying susceptibility to fast and slow modes of gating (Bennett, 1999). The variable expression of a β_1-subunit could account for contribution of fast and slow gating modes to the current time course (Lawrence et al., 1996). However, we are not aware of the expression of an endogenous β_1-subunit in HEK-293 cells. For the remainder of the reported studies, we tried to select cells expressing currents of the waveform shown in Fig. 1 *A* for the analysis of blockade by antiarrhythmic drugs.

Antiarrhythmic drug blockade of wild-type sodium channels

Fig. 2 illustrates block of wild-type sodium channels by disopyramide during pulse train stimulation. Forty pulses of 40-ms duration were applied from −100 to −20 mV at a cycle length of 150 ms. During control, there was little difference between the peak current for pulses 1 and 40.

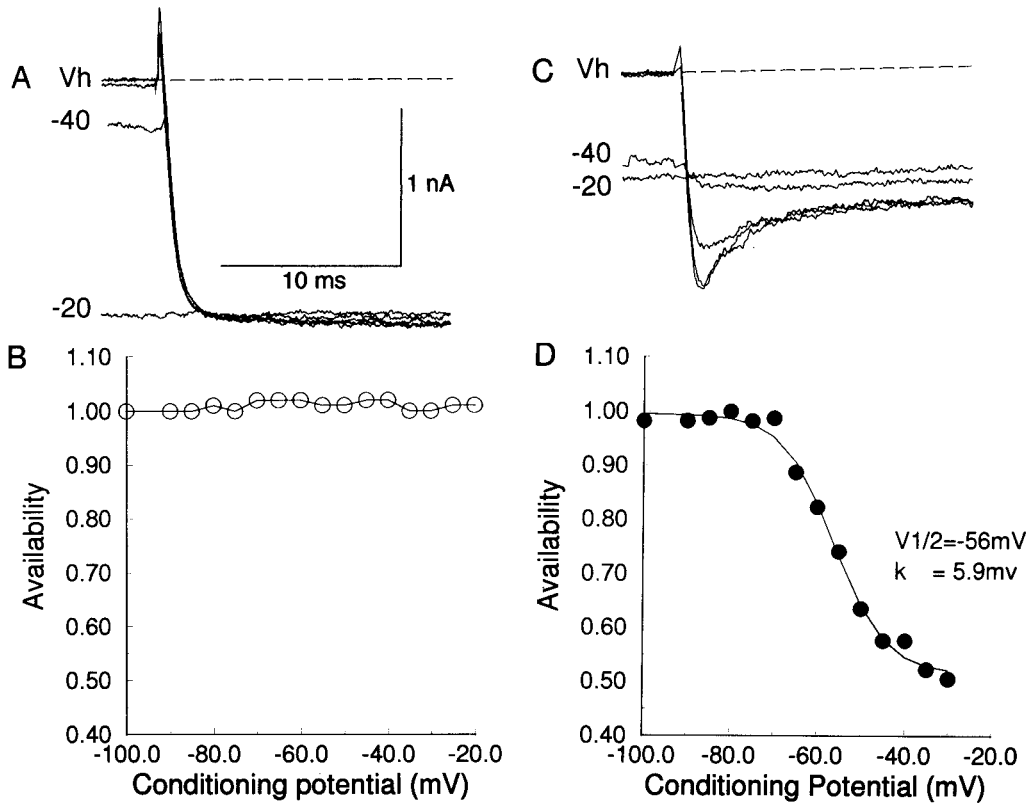

FIGURE 1 Variability of macroscopic inactivation in HEK-293 cells expressing the IFM/QQQ mutant cardiac sodium channel. Steady-state inactivation was determined by applying 500-ms conditioning pulses from a holding potential of −100 mV to various potentials followed by a test pulse to −20 mV. (*A*) and (*C*) show currents at the end of the conditioning and test pulse recorded from separate cells. V_h indicates the overlapping currents during conditioning potentials of −100, −80, and −60 mV. The maximum (peak) current has been normalized and plotted against the conditioning potential. The currents in (*A*) show no inactivation. The currents in (*C*) show incomplete inactivation. The potential for half-inactivation, V_2, was −56 mV; the slope was factor 5.9 mV.

Exposure to 200 μM disopyramide resulted in 21% use-dependent block. The drug was washed out for 5 min, with no activating voltage pulses applied during washout. At the end of the washout period, 1-mV pulses to check the capacity compensation were applied, followed by the pulse trains from −100 to −20 mV. A progressive increase in current was observed between pulses 1 and 40. This represents use-dependent unblocking. It is not likely to be a technical artifact of the methods, as the pulse sequence was immediately repeated and the current during pulses 1 and 40 were virtually superimposable. Data for all pulses during each of the trains are presented in Fig. 2 *D*. Block and recovery were both monoexponential, with rates of 0.098 and 0.1 per pulse, respectively. Fig. 2 *E* summarizes data from five experiments during control and disopyramide exposure and three experiments in which washout of drug was also obtained.

Disopyramide can also use an intracellular access route to its blocking site. To contrast this access site with that of flecainide to be reported below, we performed four experiments in which 200 μM disopyramide was included in the micropipette. After waiting at least 5 min after intracellular access, pulse train stimulation was applied. Block with intracellular disopyramide averaged 22 ± 0.4%. In six separate control cells with no drug in the micropipette, no use-dependent block was observed. These data are also summarized in Fig. 2 *E*.

The same protocols were used to examine block during exposure to flecainide (Fig. 3). Ten micromolar flecainide produced 55% use-dependent block during pulse stimulation. The onset of block was relatively slow, with an onset rate of 0.027/pulse. The drug was then washed out without interval-depolarizing pulses. The pulse-train applied 5 min after washout showed similar current during pulses 1 and 40. In contrast to disopyramide, flecainide displayed no use-dependent *unblocking*. Summary data are presented in Fig. 3 *E*.

We examined a potential intracellular access to its binding site by including 10 μM flecainide in the micropipette solution. We saw no use-dependent block during pulse-train stimulation. The tissue bath could act as a large sink for any drug applied intracellularly. However, the high pK_a of 9.3 would force 99% drug into the charged form that would be retained in the cell. We increased the drug concentration in

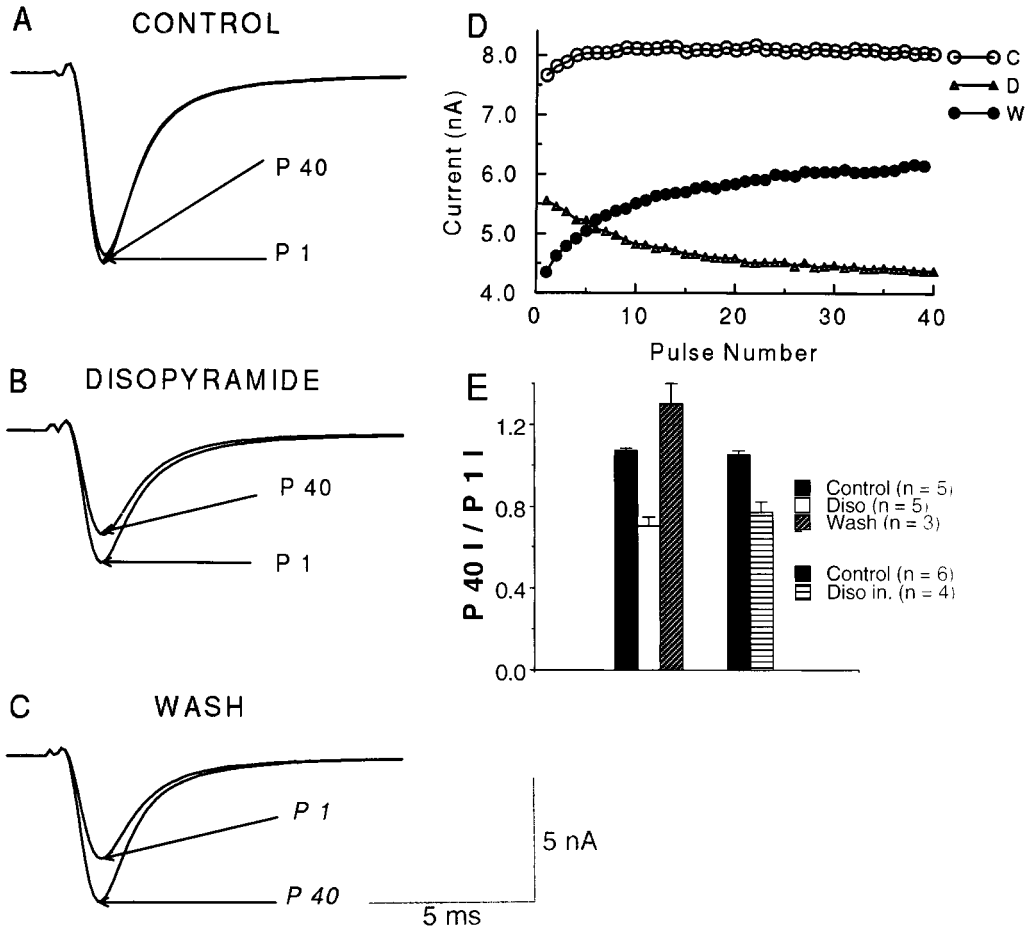

FIGURE 2 Block of the wild-type cardiac Na^+ channel by disopyramide during pulse-train stimulation. Membrane currents were elicited by 40-ms pulses from a holding potential of −100 mV to a test potential of −20 mV. The cycle length was 150 ms. Currents during the first and 40th pulses of the train during control, exposure to disopyramide, and washout of drugs are shown in *A–C*, respectively. Peak currents during each pulse of the trains are presented in (*D*). Block developed at a rate of 0.098 per pulse during drug exposure and unblocking occurred at a rate of 0.1 per pulse after drug washout. Summary data during both external and internal application of disopyramide are presented in (*E*). The summary data are presented in a normalized form by dividing the current during the 40th pulse, P40, by that during the pulse, P1.

the micropipette to 50 μM and even after delaying pulse-train stimulation for 10 min after intracellular access, saw no use-dependent block with subsequent pulse-train stimulation. When 50 μM flecainide was then applied externally to each cell (i.e., 50 μM flecainide inside and outside), 71% use-dependent block was observed. After washout of the external flecainide for 5 min, a residual of 11% use-dependent block was evident. Summary data are present in Fig. 3 *E*. They support the results of Nitta et al. (1992) that flecainide accesses its binding site through an extracellular route.

We examined the use-dependent blocking action of RAD-243 (extracellular application only) in five experiments. We observed 41 ± 6% ($n = 6$) use-dependent block. The major difference from disopyramide and flecainide was that block reached a steady state after few pulses. The rapid onset of block is similar to that which we reported for native cells (Barber et al., 1992). No use-dependent unblocking was observed during washout.

Antiarrhythmic drug blockade of inactivation-deficient sodium channels: whole-cell studies

Fig. 4 shows whole-cell currents elicited by pulse-train stimulation in a cell during control, exposure to 200 μM disopyramide, and after washout of the disopyramide. The holding potential was −100 mV and 20 (P1–P20) 40-ms test pulses to −20 mV were applied at an interpulse interval of 150 ms. During control (*A*), there was no significant relaxation of the current during the pulses and the maximum current was the same during the first (P1, 0.81 nA) and the last (P20, 0.8 nA) pulse of the train. During exposure to 200 μM disopyramide, peak and plateau current were reduced to 0.56 nA and 0.54 nA for the first and the last pulses of the train. The reduction in first-pulse current can be attributed to tonic block, implying block occurring at the resting potential or block that equilibrated rapidly during the activation process (duration <1 ms). After washout, the maximum and

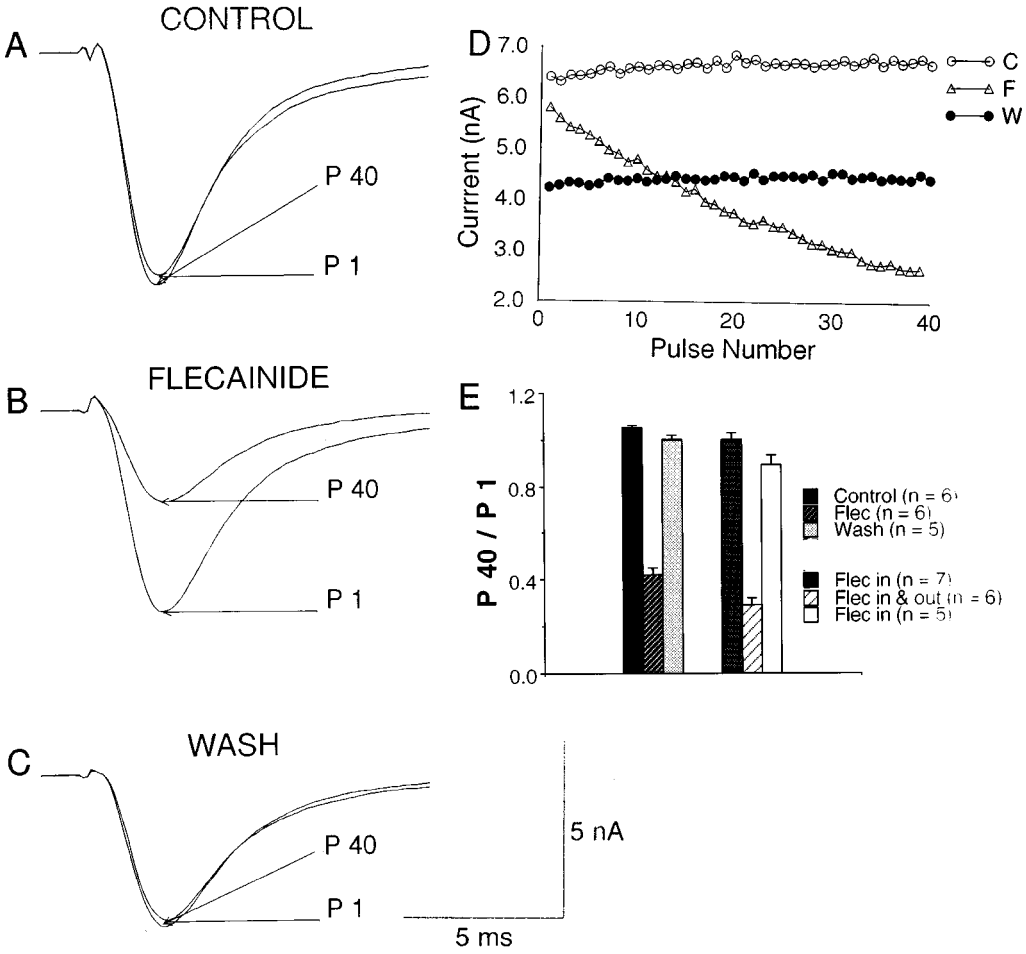

FIGURE 3 Block of wild-type cardiac Na^+ channels by flecainide during pulse-train. Membrane currents elicited at −20 mV from a holding potential of −100 mV are illustrated for the first and 40th pulses of the train during control (*A*), exposure to flecainide (*B*), and after drug washout (*C*). Peak current for all 40 pulses of each train are presented in (*D*). Summary data for experiments with external, internal (flec in), and combined (flec in and flec out) exposure to flecainide are presented in (*E*). For the summary data, peak currents during the 40th pulse, P40, are normalized against those in the first pulse, P1.

plateau currents recovered (*C*). The maximum and plateau current for all 20 pulses in the train are presented in *panels D* and *E*. Disopyramide produces no use-dependent block of the IFM/QQQ channel. Clearly, the occurrence first-pulse block is not dependent on the presence of inactivation at the holding potential (−100 mV). The concentration of disopyramide used produces 20–30% use-dependent block of wild-type cardiac sodium channels (Zilberter et al., 1994). Summary results are presented in Fig. 5.

Results of an experiment using the same protocol but with 50 μM flecainide are illustrated in Fig. 6. In the absence of drug, there is a small amount of relaxation of the current: peak current 0.76 nA, plateau current 0.68 nA for P1. Again, there is no change in peak and plateau current during the pulse train in the absence of drugs (Fig. 6, *D* and *E*). This is in striking contrast to the effect observed during exposure to flecainide. Peak current was reduced to 0.57 nA and there was a monoexponential relaxation of the current to a plateau of 0.24 nA. There was little recovery in the peak current amplitude in the second and subsequent pulses in the train. The plateau level of P1 was similar to the peak and plateau levels of P20. As shown in Fig. 6 *C*, the effect is partially reversible on washout of flecainide.

From the average block at steady state, b_{ss}, the time constant for the exponential relaxation of the current, τ_b, and the drug concentration, the association and dissociation rate constant can be calculated: $\tau_b = 1/(k_{on}D + k_{off})$; $b_{ss} = k_{on}D/(k_{on}D + k_{off})$, where k_{on} and k_{off} are the association and dissociation rate constants, respectively ($K_D = k_{off}/k_{on}$). From the data in Fig. 5, $k_{on}[D] = 161.7$/s, so $k_{on} = 3.2 \times 10^6$/M/s and $k_{off} = 88.3$/s. This dissociation rate is much faster than that observed at normal resting potentials in native cells (Campbell and Vaughan Williams, 1983). Deactivation of the inactivation deficient channel evidently markedly slows the dissociation of flecainide. Flecainide retained its use-dependent blocking action in the IFM/QQQ mutant and resulted in substantial relaxation of the current despite its lack of inactivated state block.

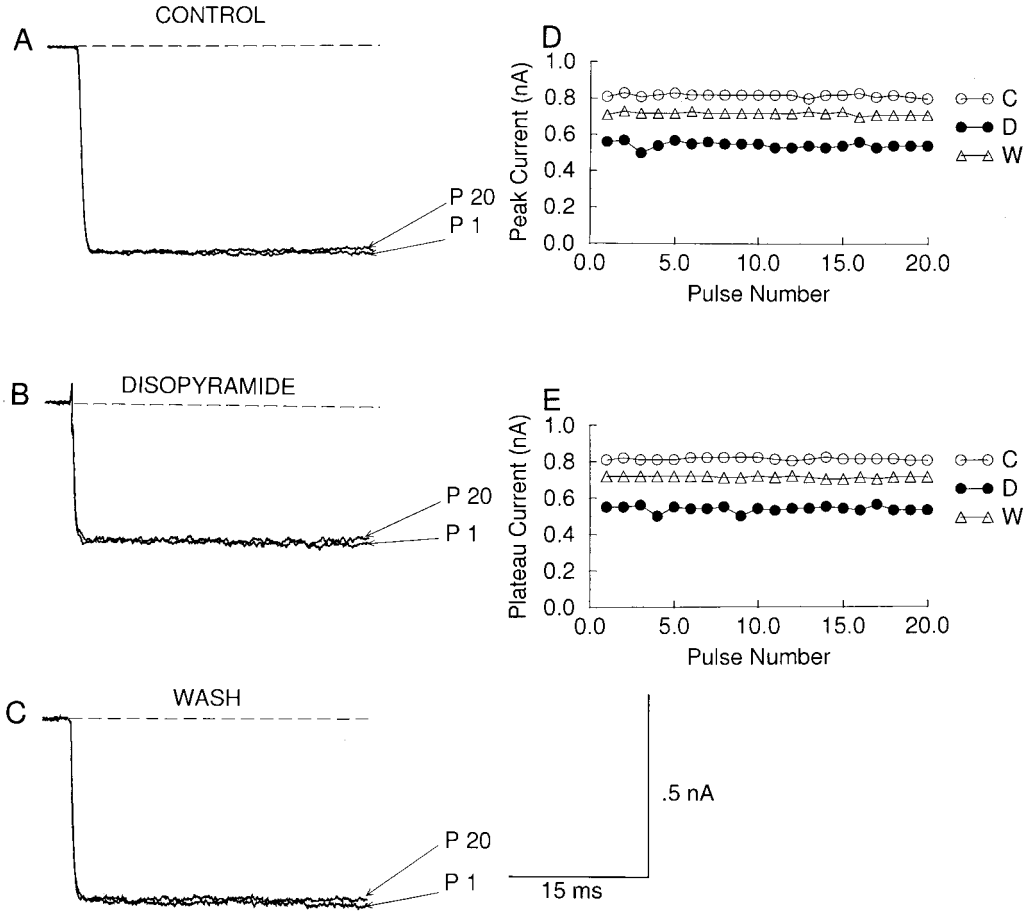

FIGURE 4 Block of the whole-cell sodium current by disopyramide during pulse-train stimulation. Trains of 20 40-ms pulses from a holding potential of −100 mV to −20 mV at intervals of 150 ms were applied during control (*A*), exposure to 200 μM disopyramide (*B*), and after washout of the drug (*C*). The current during the first (P1) and the 20th (P20) pulse are shown in *A–C*. The peak and plateau current for each pulse of the train have been plotted in (*D*) and (*E*). The open circles and C denote data obtained during control; the filled circles and D denote data obtained during disopyramide exposure; the open triangles and W denote data obtained after washout of disopyramide. Time and current calibrations are shown at the bottom.

Results with 100 μM RAD-243 are shown in Fig. 7, using protocols as described in Fig. 4. During control, there was little change of the peak and the plateau currents during pulse-train stimulation. Exposure to RAD-243 reduced the peak current from 0.3 nA to 0.14 nA, with progressive decline of the current during the first pulse. Peak current during the 20 pulses declined to 0.05 nA. RAD-243 retained its use-dependent blocking action in the IFM/QQQ mutant channel. From the rate of relaxation of the current and level of block at the end of the pulse, we estimated $k_{on}D = 83$/s and $k_{off} = 17$/s. Later, we show that these parameters cannot account for the substantial first pulse block, and we must assume that RAD-243 produces ~50% tonic block. At the holding potential of −100 mV, the dissociation of RAD-243 was sufficiently fast and complete that sodium channel availability could be determined in a time frame when currents are stable. Fig. 7 *C* shows sodium channel availability at a test potential of −20 mV following 500-ms conditioning pulses to various potentials. Without drug, channel availability decreased by 20% at conditioning potentials that open the channel (positive to −60 mV). With RAD-243, availability declined at a conditioning potential of −85 mV and was zero at −55 mV. This experiment does not distinguish between a shift in availability and voltage-dependent block during the conditioning pulse.

Antiarrhythmic drug blockade of inactivation-deficient sodium channels: single-channel analysis

We performed single-channel current recordings in cell-attached patches in cells expressing the IFM/QQQ mutation. To record from patches with single active channels, we used high-resistance electrodes for these recordings. Patches with a single channel usually were observed in one of 10 seals. The holding potential was −90 mV, and 200-ms test pulses were applied to −20, −30, −40, and −60 mV.

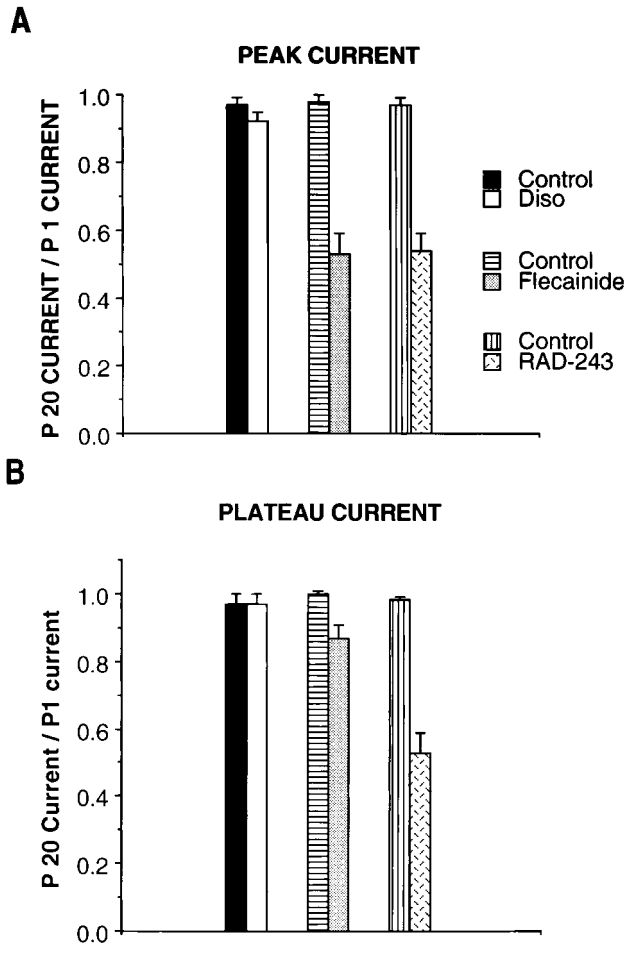

FIGURE 5 Block of whole-cell sodium current by disopyramide, flecainide, and RAD-243 during pulse-train stimulation. In (*A*) the peak currents of the last pulse (P20) of pulse trains have been expressed as a fraction of the current for the first pulse of the train (P1). The ratio is a measure of the use-dependent block of the sodium current during the trains. There is little use-dependent reduction of current during control. Significant use-dependent block is observed during exposure to flecainide and RAD-243 only. The ratio for the plateau to steady-state current is plotted in (*B*). In both panels, the means were obtained from eight experiments with disopyramide, four with flecainide, and six with RAD-243; the error bars are standard errors of the means.

At each test potential, 100–400 trials were performed, followed by drug, washout, and a repeat of the protocol. The long-term stability of cell-attached patches dictated that we limit the number of potentials examined with each drug. The majority of the single-channel data reported was obtained at −20 and −40 mV.

Single-channel recording during control and exposure to disopyramide were obtained during continuous recordings in five cell-attached membrane patches. Currents for five consecutive trials together with the average current from 100–200 trials are shown in Fig. 8, *A–C*. The test potential was −20 mV. The single-channel amplitude was 1.45 pA during control. For most trials, the single channel present in this patch showed repetitive openings and closures throughout the 200-ms pulse. Some of the channel closures during the bursts were incompletely resolved at the recording bandwidth of 2.5 kHz. The average current shows no significant relaxation during the 200-ms pulse. The mean open time was 3.8 ± 4.9 ms. During exposure to disopyramide, most of the bursts still lasted the entire duration of the 200-ms pulse, and average current showed no relaxation. The openings were interrupted by many rapid closures due to channel blockade. Mean open time was reduced to 1 ± 1 ms, and single-channel current amplitude was unchanged at 1.45 pA. The probability that a channel would fail to open during a depolarizing trial increased from 0.21 during control to 0.28 during exposure to disopyramide. Since the recording micropipette in this cell-attached recording does not contain disopyramide, the drug is accessing its binding site through some intramembrane or cytoplasmic route. As illustrated in Fig. 8 *C*, the fast block and reduction of the average current are reversed on washout of disopyramide.

The distribution of open and closed times is analyzed in more detail in the histograms shown in Fig. 9. During control, the distribution of open times was fit by two exponentials, with the slower component (λ_1 = 0.21/ms) accounting for 30% of the distribution. During exposure to disopyramide, the distribution was fit with a single exponential with a rate constant λ of 1.1/ms, intermediate between the fast and slow rate constants during control. Comparing the two open-time distributions, open times greater than 5 ms were largely eliminated by disopyramide. The closed-time distributions during control and disopyramide exposure were best fit by two exponentials. However, both distributions were dominated by brief closures that accounted for 98 and 92% of the distribution during control and drug exposure, respectively. Disopyramide increased the slow-opening rate constant. Summary data are presented in Table 1. In two of five experiments, the distribution of closed times was fit by a single exponential during disopyramide exposure. In the remaining experiments, there was a trend toward an increase in the slower opening rate (0.71 ± 0.3/ms and 1 ± 0.16/ms, n = 3 during control and disopyramide exposure). The single-channel experiments show that disopyramide retains its open-channel blocking action in the IFM/QQQ mutant channels. The absence of use-dependent block during the whole-cell recordings indicates that the interstimulus interval of 150 ms is long enough to permit drug dissociation from its blocking site(s) at the holding potential of −100 mV.

To our knowledge, single-channel analysis of the blocking action of flecainide has not been reported. Since the drug blocks from the external side of the sarcolemma, the options for analysis were outside-out patch recordings or cell-attached recordings with control and drug data obtained in separate patches (Carmeliet et al., 1989). Outside-out patches invariably contained multiple channels and were not suitable for analysis. We elected to compare single-channel

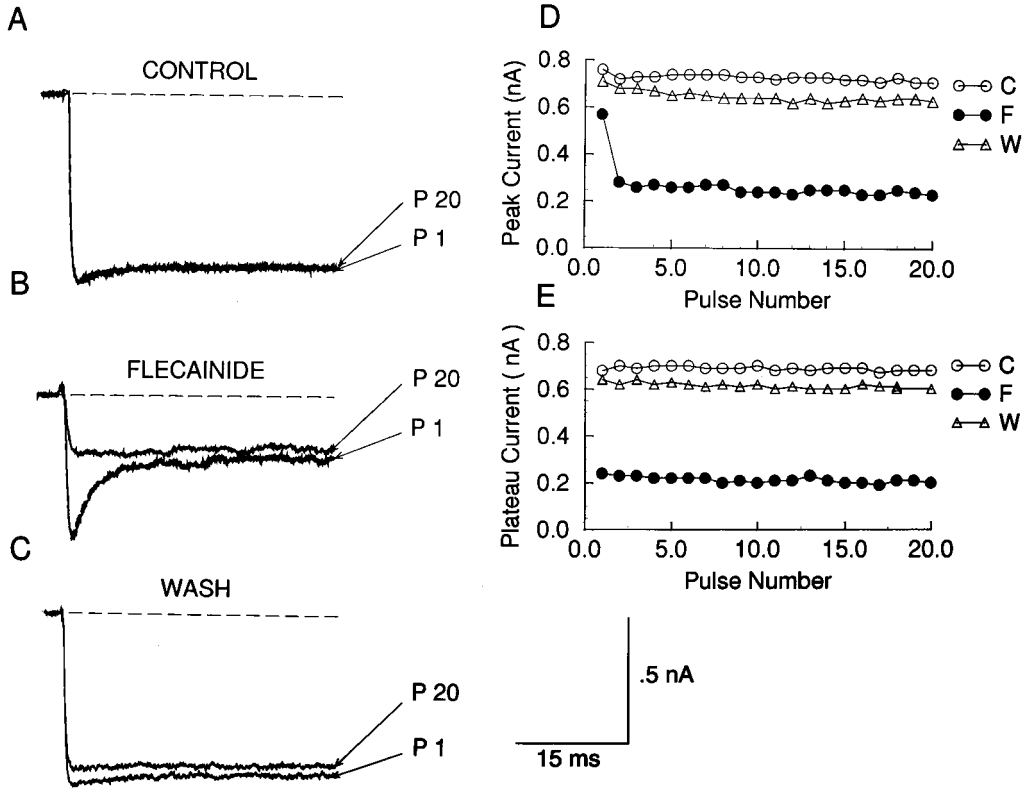

FIGURE 6 Block of the whole-cell sodium current by flecainide during pulse-train stimulation. P1 and P20 are the whole-cell currents during the first and last pulses of a train of 40-ms pulses from the holding potential of −100 to a test potential of −20 mV. The interpulse interval was 150 ms. Recordings were obtained during control (*A*), exposure to flecainide (*B*), and after washout of flecainide (*C*). The single exponential fit (time constant 3.57 ms) to the relaxation of the current during flecainide is superimposed as a dashed line in (*B*). Peak and plateau currents are plotted in (*D*) and (*E*). The open circles and C denote data obtained during flecainide exposure, and the open triangles and W denote data obtained after washout of the drug. For the first pulse during flecainide exposure, the current relaxed to a plateau level. There was little decline in the peak and plateau current amplitude during the second and subsequent pulses.

current in separate control and drug-exposed patches. A similar strategy was used by Carmeliet et al. (1989) to examine the blocking action of penticainide in isolated myocytes. Single-channel currents in separate control and test patches are shown in Fig. 10. The holding potential was −90 mV and the test potential −20 mV. Each panel shows current during five consecutive trials and the average current from 200 trials. The records are representative of five experiments in drug-free patches and four test patches in which the micropipette solution contained 50 μM flecainide. The average current in the control patch showed little change in amplitude during the 200-ms depolarization. In contrast, the average current in the flecainide-treated patch declined progressively. Mean open time was 4.1 ± 4.7 ms in the control patch and 2.3 ± 2.2 ms in the flecainide-treated patch. Under both recording conditions the distribution of closed times was biexponential. The fast and slow rate constants were 0.36 and 5.9/ms for the control patch and 0.11 and 1.5/ms in the test patch. Probability of failure to open was 0.07 and 0.56 for the control and test patch, respectively. Summary data are presented in Table 1. Control and test data were compared with an unpaired *t*-test. Open times were significantly less during flecainide exposure at test potentials of −20 and −30 mV. The briefer open times at −40 and −60 mV were not significantly affected by flecainide. The drug also increased the probability of failure of the channel to open at −20 and −30 mV. The distribution of closed times was fit by two exponentials at test potentials of −20, −30, and −40 mV. One exponential was required in one of four experiments at a test potential of −60 mV. The slow opening rate λ was significantly prolonged at −20, and −30 mV in flecainide-exposed patches.

From a direct inspection of the current recording in Fig. 10 *B*, it is evident that single-channel current amplitude was not decreasing as the average current relaxed. The current relaxation can be explained by a shortening of the total burst duration. Because of hardware limitations, the pulse duration was limited to 200 ms. Therefore, we can only compare relative total burst duration, as at least during control most bursts would have had longer durations if the duration of the depolarizing pulse were increased. We define the relative burst duration as the time interval between the first opening

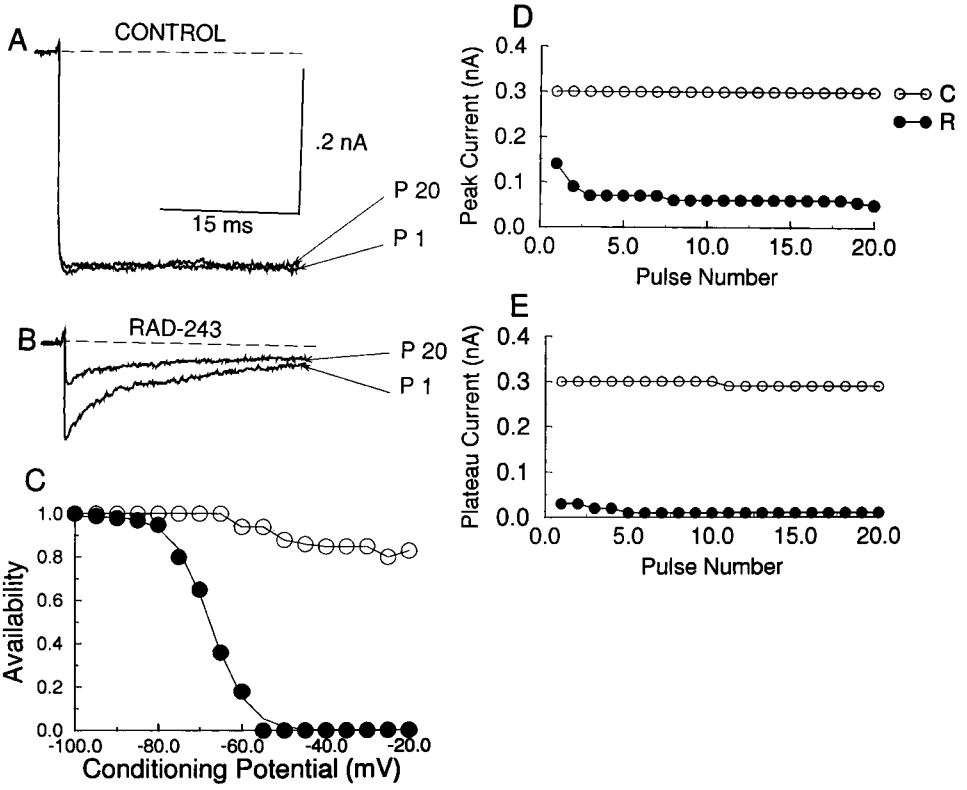

FIGURE 7 Block of the whole-cell sodium current by RAD-243 during pulse-train stimulation. P1 and P20 are currents during the first and last pulses of a train of 40-ms pulses elicited from a holding potential of −100 mV to a test potential of −20 mV. Recordings were obtained during control (*A*) and exposure to RAD-243 (*B*). The peak and plateau currents for each pulse in the train are plotted in (*C*) and (*D*). The open symbols and C denote control; the filled symbols and R denote data obtained during exposure to RAD-243. The current during RAD-243 exposure declined throughout the 40-ms pulse. The current at the end of the pulse is plotted as the plateau current.

and the last closure in a trial. The channel may be open at the end of the 200-ms pulse, but the last event without channel closure would be ignored. Using this operational definition, mean burst duration was 170 ± 4 ms during control, and 144 ± 8 ms during exposure to flecainide. Mean burst duration was decreased significantly for the group of experiments, 170 ± 4 ms during control ($n = 5$) and 112 ± 14 ms during exposure to flecainide ($n = 4$).

Single-channel conductance for control and test patches are compared in Fig. 11. Both single-channel *I-V* relationships were linear with chord conductances of 15.3 and 16 pS for control and test patches, respectively. The representative single channel records in *panels A* and *B* are consistent with the data in Fig. 10. Open times are reduced and closed times increased in the drug-exposed test patches. A qualitative comparison of the data in Figs. 8, 10, and 11 indicates that

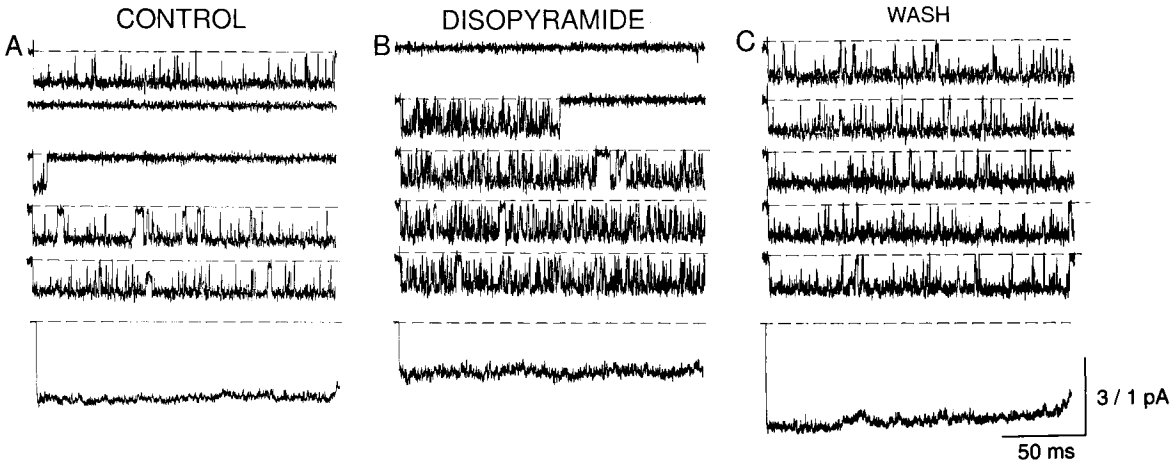

FIGURE 8 Effects of disopyramide on single sodium channel currents. Single sodium channel currents were recorded from a cell-attached membrane patch during control (*A*), exposure to disopyramide (*B*), and after washout of the drug (*C*). The holding potential was −90 mV and the test potential −20 mV. Each panel shows membrane current during five consecutive trials and the average current of 100 trials. There was evidence for a single functioning channel in this patch.

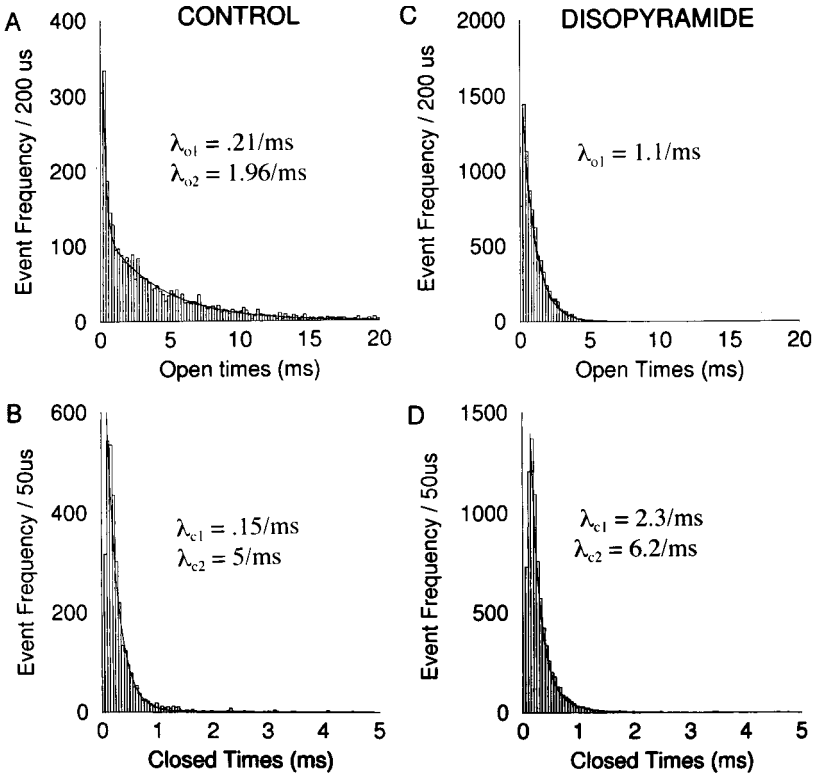

FIGURE 9 Disopyramide action on the open and closed time distribution. Histograms of open time during control (*A*) and exposure to disopyramide (*B*) were plotted with a bin width of four sampling intervals (200 μs); histograms of closed times were plotted with a bin width of one sampling interval (50 μs). The distribution of open times during control was fit with two exponentials, and with one exponential during disopyramide exposure. Both closed time distributions were fit with two exponentials. The closing and opening rate constants are shown in the insets.

at −20 mV, the dissociation rate of flecainide is less than that of disopyramide.

Single-channel recordings during control and exposure to RAD-243 were obtained in five cell-attached membrane patches. Stimulus protocol and recording conditions were the same as those used for the disopyramide recording. Fig. 12 shows records obtained from a patch during control, exposure to RAD-243, and after drug washout. The average current in the lowest row show a clear drug-induced reduction of the peak current, and progressive decline in current amplitude for the duration of the 200-ms pulse. Open times during control and drug exposure were fit with single exponentials. The mean open times were 3.3 ± 3.6, 1.9 ± 1.9, and 3.8 ± 3.5 during control, exposure to RAD-243, and

TABLE 1 Effects of disopyramide, flecainide, and RAD-243 on single sodium channel properties

	V_m (mV)	i (pA)	⟨to⟩ (ms)	λ_1 (/ms)	λ_2 (/ms)	p_f
Data obtained from the same patch						
Control (n = 5)	−20	1.5 ± 0.03	4.4 ± 0.4		—†	0.2 ± 0.03
Disopyramide		1.5 ± 0.04	0.96 ± 0.05*		—†	0.4 ± 0.15
Control (n = 5)	−20	1.6 ± 0.02	3.4 ± 0.6	0.61 ± 0.16	4.76 ± 0.19	0.13 ± 0.06
RAD-243		1.6 ± 0.02	1.6 ± 0.3*	0.55 ± 0.14	3.9 ± 0.65	0.6 ± 0.08*
Control (n = 5)	−40	2.0 ± 0.02	1.4 ± 0.22			0.09 ± 0.05
RAD-243		1.9 ± 0.04	0.8 ± 0.13			0.52 ± 0.14*
Data obtained from separate patches						
Control (n = 5)	−20	1.6 ± 0.08	4.3 ± 0.3	0.53 ± 0.1	5.5 ± 0.14	0.07 ± 0.015
Flecainide (n = 4)		1.4 ± 0.1	1.9 ± 0.54*	0.15 ± 0.05*	3.7 ± 1.0	0.5 ± 0.06*
Control (n = 5)	−30	1.82 ± 0.1	3.7 ± 0.2	0.62 ± 0.1	4.6 ± 0.25	0.04 ± 0.02
Flecainide (n = 4)		1.75 ± 0.18	1.7 ± 0.5*	0.21 ± 0.1*	3.5 ± 1.5	0.47 ± 0.05*
Control (n = 5)	−40	1.97 ± 0.04	2.1 ± 0.2	0.63 ± 0.12	3.9 ± 0.16	0.14 ± 0.05
Flecainide (n = 3)		1.75 ± 0.18	1.7 ± 0.4	0.4 ± 0.3	3.1 ± 0.14	0.46 ± 0.18
Control (n = 4)	−60	2.26 ± 0.04	0.72 ± 0.1		—†	0.07 ± 0.02
Flecainide (n = 3)		2.06 ± 0.04	0.77 ± 0.17		—†	0.26 ± 0.1

V_m, test potential; i, single channel current amplitude; ⟨to⟩, mean open time; λ_1 and λ_2, slow and fast rate constants of closed time distribution; p_f, probability that a channel failed to open.

*$p < 0.05$.

†One or two exponentials required to describe the distribution of closed times.

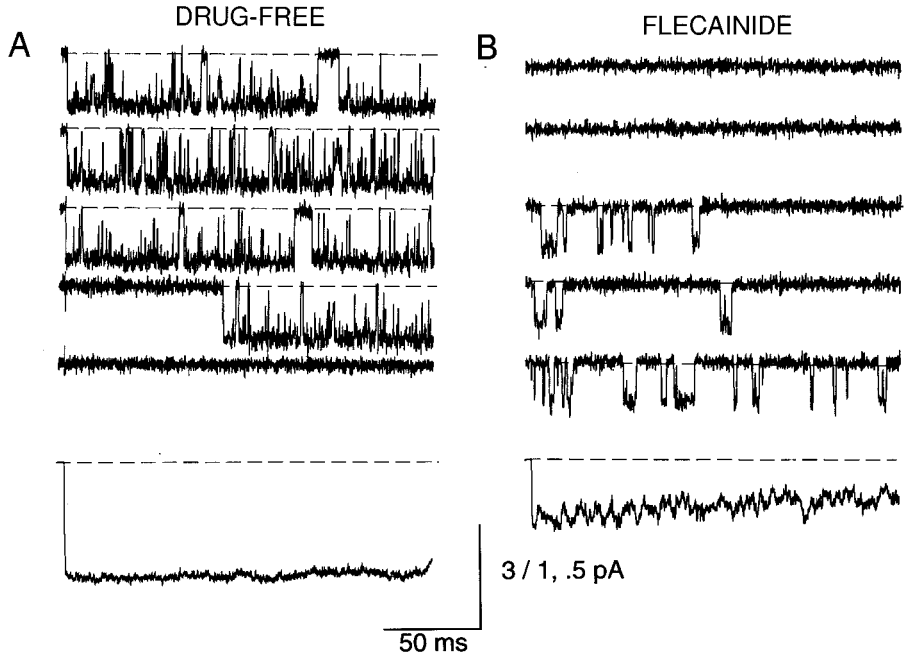

FIGURE 10 Flecainide action of single cardiac sodium channel currents. Membrane current recorded from separate patches during the drug-free state and exposure to flecainide. The holding potential was set at −90 mV and the test potential −20 mV. Each panel shows current during five consecutive trials and the average current of 200 trials.

drug washout, respectively. Two exponentials were required to fit the distribution of closed times, but the distributions were dominated by the faster rate constant; λ_1 was 0.23, 0.66, and 0.4; and λ_2 was 5, 4.4, and 4.5. The probability of failure of the channel to open increased from 0.04 during control to 0.59 during drug exposure. This was partially reversed on drug washout (pf = 0.17). Summary data from all five patches are presented in Table 1.

Mean burst duration was 180 ± 4 ms during control and 94 ± 7 ms during exposure to RAD-243. For the five experiments, apparent burst duration was significantly shortened, 177 ± 2.7 ms during control and 62 ± 12 ms

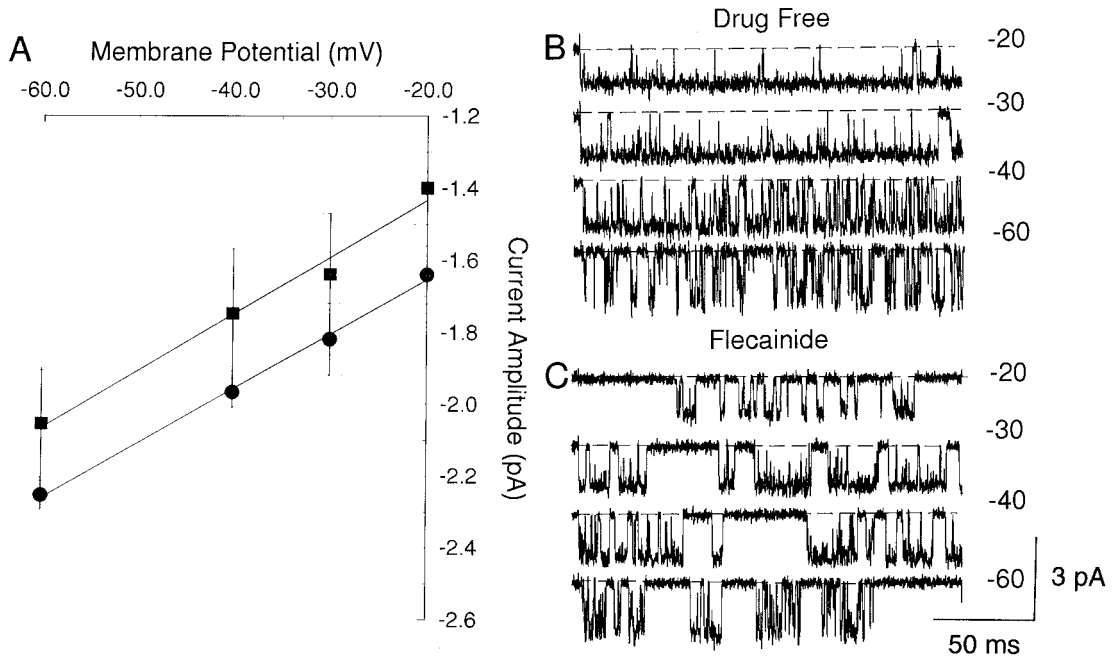

FIGURE 11 Flecainide action of single sodium channel conductance. The mean single channel current from three to five experiments are plotted against test potential during control (*filled circles*) and exposure to flecainide (*filled squares*) in (*A*). Exemplar records from drug-free- and flecainide-exposed patches are shown in (*B*) and (*C*), respectively. Single channel chord conductance was 15.3 pS in the drug-free patches, and 16 pS in the flecainide exposed patches.

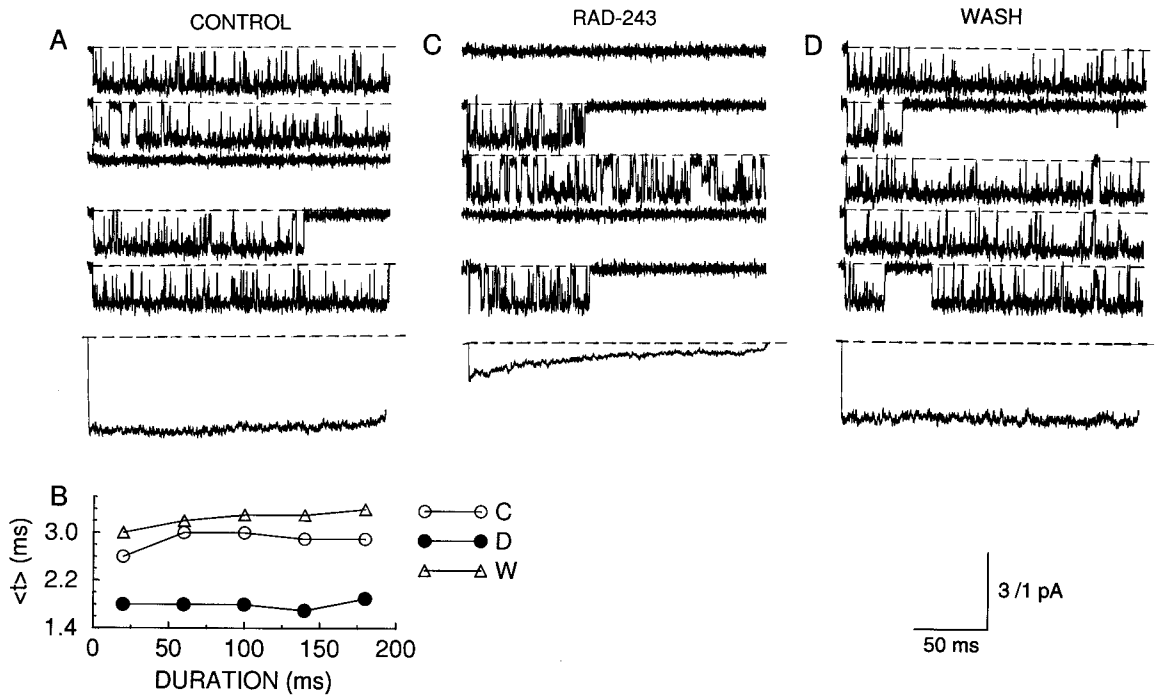

FIGURE 12 RAD-243 action on single sodium channel currents. Single sodium channel currents were recorded form a cell-attached membrane patch during control (*A*), exposure to RAD-243 (*C*), and after washout of drug (*D*). The holding potential was −90 and the test potential −20 mV. Panels *A*, *C*, and *D* show currents during five consecutive trials and the average current from 200 trials. The average current shows little time variation during control and washout, but declined progressively during exposure to RAD-243. The 200-ms pulse was divided into five 40-ms segments, and the mean open time for events falling in each segment determined. These have been plotted for control (*open circle*), exposure to RAD-243 (*filled circle*), and after drug washout (*open triangle*). There is no tendency for open times to shorten during the 200 ms, suggesting that the closure mechanism is stationary.

during exposure to RAD-243. The shortening of the apparent burst during drug exposure can account for the marked relaxation of the macroscopic current. It also indicates that the channel can close without dissociation of the drug from the channel. If drug dissociation were required for channel closure, the apparent duration would have increased (Neher, 1983). An increase in the rate of channel closure during the 200-ms pulse could also contribute to the faster relaxation of the current during drug exposure. We divided the 200-ms pulse into five 40-ms segments and compared the mean open time during each segment. As shown in Fig. 12 *B*, mean open time did not decrease during the 200-ms pulse. This indicates that the mechanism(s) of channel closure is stationary throughout the pulse.

DISCUSSION

The aim of these studies was the comparison of the block of cardiac sodium channels with disabled inactivation by a group of drugs with different blocking mechanisms. Our studies were performed with the wild-type and IFM/QQQ mutant human cardiac sodium channel stably expressed in HEK-293 cells. In preliminary studies, we found the frog oocyte expression unsuitable for these studies. The wild-type and IFM/QQQ mutant channels expressed readily. However, drug response varied markedly and voltage control was very slow. Cell lines offer the convenience of ready availability and cells of suitable size and geometry for whole-cell voltage clamping. Establishing the stable cell lines of the IFM/QQQ mutant with robust currents proved formidable. Our initial transfection yielded cells with small currents of <100 pA. We assumed that sodium overload from spontaneous channel openings inhibited channel expression. Drug-induced persistent activation of the sodium channel or membrane depolarization of developing neurons also down-regulated sodium channel expression (Giraud et al., 1998). It was only by co-expression with the inward rectifier K^+ channel that robust currents were obtained. The gating of the mutant channel was similar to that previously reported in frog oocytes (West et al., 1992; Hartmann et al., 1994; Bennett et al., 1995). There was a small and variable component of inactivation that persisted in these cells. This component of inactivation appeared to be dependent on channel opening. The preparation was adequate to address the major role of inactivation in channel blockade.

Although block of the sodium channel by disopyramide, flecainide, and RAD-243 has been examined in native sodium channels in prior studies, we have reexamined block using the wild-type sodium channel in the same heterologous system as the IFM/QQQ mutant. Disopyramide block

of wild-type channels developed slowly with either intra or extracellular application. Reversal of the block following extracellular drug application required repetitive depolarization. This is an example of use-dependent unblocking. This phenomenon of use-dependent unblocking was first described by Strichartz in nerve (Strichartz, 1973). It has also been described for potassium and sodium channel blockers in nerve and cardiac muscle and heterologously expressed ion channels (Strichartz, 1973; Carmeliet, 1988; Holmgren et al., 1997). The interpretation is that drug is trapped behind the closed activation- or inactivation-gate and requires cycling of the channel through various states to relieve block. The present experiments do not identify the actual site of trapping. Onset of block by flecainide was also very slow, approaching a steady state only after trains of 40 pulses. Our data support extracellular access of flecainide to its binding site. Both upstroke velocity and whole-cell sodium currents have shown that unlike nerve, cardiac sodium channels are blocked by external application of quaternary ammonium derivatives of lidocaine. Disopyramide and flecainide have similar pK_a values. Therefore, differences in the state of dissociation are not likely to account for the difference of access. The absence of use-dependent unblocking with flecainide further supports the idea of difference in pathways for dissociation of the two drugs. RAD-243 had very rapid onset of block and showed no use-dependent unblocking.

Disopyramide produced block during the initial depolarizing pulse, but no frequency-dependent block in cells that clearly did not demonstrate any fast inactivation. With the wild-type channel, there is always the uncertainty that this initial block reflects block of a small fraction of channels with residual fast inactivation, tonic block, or rapid block that attains equilibrium during activation. The experiments with the IFM/QQQ mutant channel indicate that this initial block can occur independent of channel inactivation. If we assume that this initial block reaches equilibrium in the ~1 ms (4τ) required for activation, we can also estimate association and dissociation rates of 5.10^6/M/s and 2950/s for the first pulse block, as was implemented for flecainide and RAD-243. The lack of use-dependent block of the IFM/QQQ mutant channel by disopyramide differs from the results of Yeh and Ten Eick (1987) who observed enhanced use-dependent block with a disopyramide analog in pronase-treated squid giant axons. The most likely explanation for the differences in results is that pronase produced other effects on the channel in addition to disabling inactivation.

The single-channel studies confirm the removal of fast inactivation in the IFM/QQQ mutant. However, there was some complexity to the gating; open times were not always well-fit by single exponentials. Closed times were always fit by two exponentials, indicating that the kinetic scheme contains at least two closed states. A more appropriate description of the functional consequence of the IFM/QQQ mutant is that inactivation becomes freely reversible (Hartmann et al., 1994); the channel may then open by the normal activation pathway, and by a reversal of inactivation.

$$C_N \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O \underset{\delta}{\overset{\gamma}{\rightleftharpoons}} I$$

where C_N is the terminal resting state, O the open state, I the inactivated state, and α, β, γ, and δ the rate constants for the indicated transitions. In the scheme, δ is significant compared with the gating of the wild-type channel.

The single-channel studies provide direct evidence of block of the IFM/QQQ sodium channel by disopyramide, despite the absence of use-dependent block. The reduction of the mean open time during drug exposure provides an estimate of the association rate constant of 4.10^6/M/s. The rate is similar to that which we previously observed in native cardiac sodium channels (Grant et al., 1993). The closures and blocking events occurred at similar rates. Therefore, we could not identify a specific closed-time rate constant with the blocking events (no prolonged shut periods between bursts were observed).

The lack of use-dependent block during disopyramide exposure is consistent with the establishment of equilibrium of the drug-channel reaction during the initial moment of the channel activation process. Whether the drug is able to dissociate from the channel during the repolarization or the rest potential interval is uncertain. In a prior study of the block of the whole-cell sodium current in cardiac myocytes we showed that there were components of disopyramide block that recovered with fast and slow rates at the resting potential (Zilberter et al., 1994). In wild-type channels in which both inactivation and deactivation were slowed by exposure to the pyrethyroid toxin deltamethrin, we showed that disopyramide dissociated rapidly (time frame of milliseconds) from open sodium channels at the normal resting potentials (Grant et al., 1993). Kuo and Bean (1994) have shown that sodium channels are open very briefly on repolarization to high membrane potentials. The transient opening on repolarization may provide a pathway for rapid recovery. The IFM/QQQ mutant may have eliminated the slow pathway for recovery, but facilitated complete recovery from block in the 150 ms between pulses by an open channel pathway.

Flecainide retained its use-dependent blocking action in the IFM/QQQ mutant channel. About 50% block occurs during the first pulse, and little additional block occurs during the subsequent pulses of the train. A difference between the reduction of the peak and the plateau current may be taken as evidence for differing sensitivities of the peak and plateau current to be blocked by flecainide (Balser et al., 1996). An alternative interpretation is a simpler activated-state blocking model. The monoexponential decline of the current during P1 of Fig. 6 is consistent with this interpretation. The single-channel data presented in Fig. 10 and Table 1 also support this interpretation. At −20 and

−30 mV, flecainide reduced the mean single-channel open time significantly by >50%. Estimated drug association rates at −20 and −30 mV were 5.6×10^6 and 6×10^6/M/s, respectively. The drug also increased the probability that the channel would not open during depolarization. The latter action would scale the average current, but would not alter the current time course. The slow rate constant for the distribution of closed times is reduced by flecainide at −20 and −30 mV. The return of channels from the blocked state is slowed. This contrasts with the action of disopyramide, which produced no apparent change in the distribution of closed times.

RAD-243 shared some of the blocking actions of flecainide: rapid relaxation of the current during the first pulse of the train and use-dependent block during the subsequent pulses. The rapid relaxation of the current during the pulses was a result of a shortening of the apparent burst duration. The reduction of mean open times at −20 and −40 mV are consistent with association rates of 3.3 and 5.3×10^6/M/s. The similar association rates estimated from the reduction in mean open time for all three drugs depends on the assumption that they share the same relationship between drug bulk and receptor-site concentration. In view of the marked differences in drug lipid solubility and pK_a, such an assumption may not be warranted. Drug dissociation should not depend on this assumption; the difference in the extent of use-dependent blockade may more directly reflect differences in dissociation rate of the drug from the channel.

We have explored mechanisms of blockade using the Lou-Rudy model, which includes the Ebihara-Johnson Na^+ channel model. We disabled inactivation by setting the fast (*h*) variables to one. Using *m* as the activation variable, we defined the drug alterations of the sodium current with $I(\mathrm{Na}) = g\mathrm{Na}^*m^*(1 - b)^*(V - V\mathrm{Na})$, where *b* is the fraction of blocked channels. We explored a model of blockade based on dynamic control of the channel-binding site. Because inactivation was absent, we limited our exploration to that where activation controlled binding site access and is described by the differential equation:

$$db/dt = k^*_{on}D^*m^*(1 - b) - k^*_{off}b$$

where k_{on} is the association rate constant, *D* is the drug concentration, *m* is the channel activation variable, k_{off} is the dissociation rate constant, and *b* is the fraction of blocked channels.

From the whole-cell recordings, we estimated the rate constants for block at the test potential of −20 mV by fitting an exponential to the decreasing phase of the sodium current in order to evaluate the time constant. The results were:

Disopyramide:

$$k_{on}D = 1.05/\mathrm{ms} \quad k_{off} = 2.95/\mathrm{ms} \quad k_{on} = 5 \cdot 10^6/\mathrm{M/s}$$

Flecainide:

$$k_{on}D = 0.16/\mathrm{ms} \quad k_{off} = 0.088/\mathrm{ms} \quad k_{on} = 3.2 \cdot 10^6/\mathrm{M/s}$$

RAD-243:

$$k_{on}D = 0.083/\mathrm{ms} \quad k_{off} = 0.017/\mathrm{ms} \quad k_{on} = 8.3 \cdot 10^5/\mathrm{M/s}$$

In Fig. 13 we show the results of the numerical studies, showing good agreement with the observed currents.

The simulations suggest association rates that are of similar order of magnitude to those observed with the single-channel recordings. However, the routes of access to the receptor may differ between the whole-cell and single-

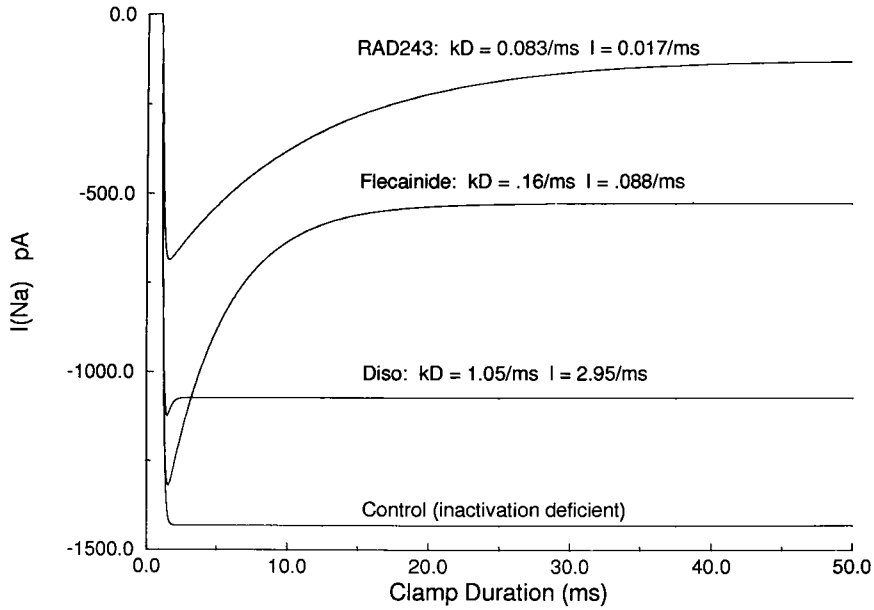

FIGURE 13 Simulations of block of the IFM/QQQ mutant sodium channel by disopyramide (diso), flecainide, and the lidocaine derivative RAD-243. The simulations were implemented using the Ebihara-Johnson sodium current model as contained in the Lou-Rudy model. The fast and slow inactivation rates have been set to zero and the association rates optimized from a fit of the macroscopic currents.

channel recordings. The greatest uncertainties are the rates for disopyramide, as the reduction of first-pulse current amplitude with this drug may truly reflect tonic block. Dissociation rates should be concentration-independent, and the simulations suggest almost a 200-fold variation between them. The fastest dissociation rate was for disopyramide, which showed considerable flicker in the single-channel recordings. The dissociation rate for flecainide was ~30-fold less, which would result in a decreased number of channel openings (Fig. 9) during the depolarizing trials. Little rest-recovery occurred between pulses, suggesting that the drug may be trapped by closure of the activation gate. RAD-243, however, dissociates roughly four times slower, but appears not to be trapped in the channel. The results with RAD-243 indicate that there are probably two binding processes for lidocaine and lidocaine derivatives: one that is associated with a tonic component of block and a second component of slow block that accounts for relaxation of the current during the depolarizing pulses. That both types of binding exist has also been suggested by experiments in sodium channels with intact inactivation or chemically modified channels (Gilliam et al., 1989; Gingrich et al., 1993). Confirming these results, Starmer et al. (1991) demonstrated that both the channel availability and current-voltage curves were shifted in a manner consistent with binding site access controlled by the inactivation voltage sensor and the activation voltage sensor (Starmer et al., 1991).

Our results need to be reconciled with the earlier studies of Bennett et al. (1995), Balser et al. (1996), and Vedantham and Cannon (1999) on the block of inactivation-deficient sodium channel (IFM/QQQ or IFM/IQM) by lidocaine. Bennett et al. observed no use-dependent block with 100 μM lidocaine. We believed that the differences in our observations and those of Bennett et al. are largely quantitative. As shown in their Fig. 8, they did observe block with higher lidocaine concentrations. They showed a similar increase in the current relaxation rate that could be fit to an activated-state blocking model. The additional requirement for tonic block in our model is consistent with our prior studies in native cells showing that RAD-243 produces a substantial amount of tonic block when compared with lidocaine (Liu et al., 1994). Our interpretation of the increase in relaxation rate with different reductions of the peak and plateau current is different from that of Balser et al. (1996). Their model assumed different K_D values for the reduction of the peak and plateau current. However, since they did not show that an equilibrium was achieved at the peak of the current, a K_D value cannot be estimated for the reduction of the peak current. Some tonic block and a single fixed K_D value are enough to capture the change in current time course. Furthermore, the single-channel studies suggest that the blocking mechanism is stable during the 200-ms pulse (Fig. 12).

Vedantham and Cannon (1999) report no effect of lidocaine on the apparent rate of recovery of lidocaine-blocked sodium channel from inactivation. This was inferred from the reactivity of the ICM mutant to modification by MTS-ET. However, their estimate of the reaction time constant was 0.027/s. We are concerned that the measurement may not have had the requisite precision to follow the inactivation process, which has a time constant of <5 ms. Furthermore, the kinetics of the modifying reaction was of the same order of our blocking rate of 10^6/M/s. We agree with their conclusion that the ICM mutation may have changed the relationship between drug blockade and inactivation. Our results with disopyramide are consistent with such a conclusion (Grant et al., 1996). We can clearly demonstrate open block of wild-type and mutant sodium channels, but use-dependence is lost with the IFM/QQQ mutant. Prior studies of inactivation-deficient mutant sodium channels suggest no change in channel activation (Hartmann et al., 1994). Therefore, we cannot make a compelling case for a change in activation as a basis for the change in use-dependent block. Our studies suggest that the local anesthetic-class drugs cannot be regarded as a uniform class. Although they may share common receptors, there may be important differences in their access and mode of block at their receptor site(s).

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