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Block of Single Cardiac Na^+ Channels by Antiarrhythmic Drugs: The Effect of Amiodarone, Propafenone and Diprafenone

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Summary. Cell-attached patch-clamp experiments were performed in cultured cardiocytes of neonatal rats at 19°C to analyze elementary currents through single Na^+ channels under control conditions and in the presence of the class 1 antiarrhythmic drugs amiodarone, propafenone, and diprafenone. As observed in a cell-attached patch with only one functioning Na^+ channel, repetitive stepping of the membrane at 0.4 Hz triggered periodically channel openings except during a silent period of about 1.5 min. The latter began and ceased abruptly and did not fit the monoexponential distribution of the run length of sweeps without activity (blank sweeps). Treating the cardiocytes with amiodarone, propafenone or diprafenone (10 to 20 μmol/liter) led rapidly to a blockage and reduced the likelihood that membrane depolarization triggers the opening of Na^+ channels. The number of blank sweeps increased at the expense of the number of sweeps with activity. The fraction of activity sweeps with superpositions, indicating the simultaneous activation of two or more Na^+ channels, also declined. As tested with amiodarone, the run length of blank sweeps is voltage- and time-dependent, analogous to the intensity of the block of macroscopic Na^+ currents. Open time, open-time distribution, unitary current size and the tendency to reopen did not differ in unblocked cardiac Na^+ channels (i.e. that channel fraction capable of opening in the presence of amiodarone or propafenone) from the respective control values obtained before superfusing the cardiocytes with these drugs. Apart from its blocking action, the propafenone derivative diprafenone exerted additionally a modifying effect and reduced mean open time by up to 45%. In contrast to the block, this reduction in conducting state proved insensitive to changes in holding potential, at least between −130 and −150 mV, the range tested. This means that block was attenuated on hyperpolarization whereas the reduction in open time persisted. It is concluded that, in the presence of these drugs, unblocked cardiac Na^+ channels share a number of properties with normal Na^+ channels in the absence of these drugs. Shortening of channel lifetime by diprafenone may be indicative of a channel modification brought about possibly by a receptor-mediated facilitation of the transition from the open to the inactivated state.

Key Words patch clamp · Na^+ channels · modification · class 1 antiarrhythmic drugs

Introduction

Blockage of Na^+ currents in excitable membranes, including heart muscle, by local anesthetics and related antiarrhythmic drugs represents a biophysically well-documented principle for depressing excitability and impulse conduction. Drug-induced blockage attracted considerable theoretical interest during the last two decades in an attempt to define the properties of voltage-gated Na^+ channels in greater detail and to elucidate the site of drug action. A channel-associated binding site which is part of the channel is assumed to exist (Khodorov et al., 1976; Hille, 1977; Hondeghem & Katzung, 1977; Starmer, Grant & Strauss, 1984) whose interaction with these structurally heterogeneous drugs is proposed to convert the channel finally to a nonconducting configuration. The latter seems to be reached in an all-or-none fashion. State-dependent drug affinity (Hille, 1977; Hondeghem & Katzung, 1977) or, to mention a very recently developed hypothesis, state-dependent drug access (Starmer et al., 1984) to the site of action within the Na^+ channels provide valuable theoretical models for understanding the block phenomenology. Depending on the history, tonic and phasic blockade (Strichartz, 1973; Courtney, 1975) are the two possible manifestations of such a drug interaction. The anesthetic binding site may be considered to be one of several other receptor-like subunits of the Na^+ channel. Among others, this receptor family comprises specific sites for blocking marine toxins (tetrodotoxin, saxitoxin), for the modifying batrachotoxin (BTX) including several likewise lipophilic alkaloides, and for the modifying sea anemona toxin ATX including some scorpion toxins.

Block manifestation on the single-channel level, however, still needs to be elucidated in greater de-

tail. The proposed all-or-none blockade predicts that gating of unblocked Na^+ channels should be essentially normal. The present patch-clamp experiments in neonatal cardiocytes with single Na^+ channels stressed this hypothesis and defined the blocking effect of class 1 antiarrhythmic agents, including its voltage and time dependence, as a reduction in open probability. Blocked cardiac Na^+ channels are trapped in a nonactivatable state. A formally not discernible, likewise long-lasting nonactivatable state may also be attained in the absence of these drugs and prevents a Na^+ channel from opening for a longer time than usual. Blocked, i.e. drug-associated, Na^+ channels are unlikely to influence their unblocked neighbors.

Materials and Methods

Employing the standard patch-clamp technique (Hamill et al., 1981), single Na^+ channel currents were recorded with an L/M-EPC 5 amplifier (List Electronics, Darmstadt) in cell-attached patches from cultured neonatal rat cardiocytes. Details of the cell culture which combines the techniques elaborated by Mark and Strasser (1966) with those by Blondel, Riojeu and Cheneval (1971) have already been described (Kohlhardt, Fröbe & Herzig, 1986). The disaggregated cardiocytes were short-time cultured in CMRL medium supplemented with 10% fetal calf serum (Biochrom, Berlin) so that a major fraction of the cultured cells still showed a more or less spherical shape. Such cells were used for the patch-clamp experiments as this configuration facilitates the contact of the patch pipette with the cell surface and the formation of stable seals. Seal resistances between the pipette lumen and the bath of 100 to 200 GΩ were easily achieved. The culture dishes were washed with modified Tyrode solution (solution A) and equilibrated to this medium for 10 min before the cells were exposed to an isotonic K^+ solution (solution B).

A major disadvantage of cultured neonatal cardiocytes is their tendency to beat spontaneously. Automaticity may also occur after a longer exposure to a physiological saline. This, consequently, introduces a notorious error in relating single-current measurements to a definite membrane potential in cell-attached patch-clamp experiments. On bathing the cells in isotonic K^+ solution, this problem could be overcome since automaticity was abolished and, as found in whole cell clamp measurements, the cells were depolarized to about 0 mV. Under these conditions, very stable patches with a lifetime of up to 2 hr were obtained. However, a varying fraction of cardiocytes did not tolerate the isotonic K^+ solution without morphological changes but, within some 10 sec, reacted with an increase in cytoplasmic granulation mostly accompanied by a loss of the initially spherical configuration. Therefore, another 10-min lasting equilibration period was awaited until a suitable cell with an apparently normal morphological structure was elected for a patch-clamp experiment.

Conventional fire-polished patch-clamp pipettes fabricated from borosilicate glass were used. After filling with pipette solution (solution C), they had a resistance between 4 and 8 MΩ.

Elementary Na^+ currents were elicited by step deplorizations of 120 msec duration delivered from a conventional stimulator. The patches were continuously pulsed at 0.4 to 0.5 Hz. Despite this low rate of stimulation, the development of slow inactivation cannot be ruled out. The patch-clamp recordings were filtered at 1 kHz by an eight-pole Bessel filter, digitized by a microcomputer with a sampling rate of 5 kHz and stored on floppy discs. A residual component of the capacity transients which could not be compensated at the level of headstage was eliminated by averaging records without channel activity and subtracting the average from the records. Furthermore, the records were corrected for leakage currents.

Under these recording conditions, the dead time was 0.18 msec so that channel detection requires a minimal open time of 0.2 msec. To increase the yield of single openings and to minimize the error in determining the open state from overlapping events (Horn & Standen, 1983), the holding potential was adjusted to a level 100 to 130 mV more negative than the resting potential. As the K^+-depolarized cardiocytes exhibit a quite uniform resting potential close to 0 mV, holding and step potentials will be given, for convenience, in absolute terms.

Open time of single Na^+ channels was analyzed by setting a threshold for a transition, i.e. opening or closing, at 50% of the unitary current amplitude (Colquhoun & Sigworth, 1983). Mean open time ($\bar{t}_{open}$) was calculated from $\bar{t}_{open} = \Sigma_i n_i T_i / n$ (Fenwick, Marty & Neher, 1982), where T_i is the duration of n_i open channels and n means a count of events. The frequency distribution of the open time yielded τ_{open} which resulted from the best fit of the open time histograms using the least-squares (X^2) method. The shut time between events resulted also from the 50% analysis and is expressed analogously as mean shut time ($\bar{t}_{shut}$) or as τ_{shut}. The elementary Na^+ current size (i_{unit}) was obtained from a Gaussian analysis of amplitude histograms.

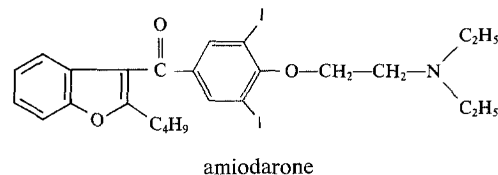
Records showing channel openings are referred to as activity sweeps and those without detectable openings as blank sweeps. A run consists of the sequence of identical sweeps, activity sweeps or blank sweeps.

Solutions (composition in mmol/liter)

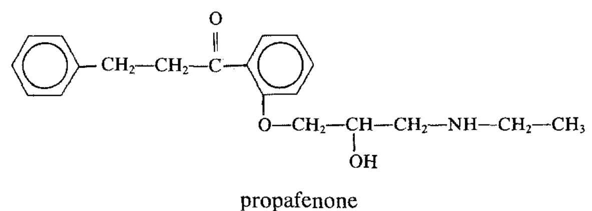
Solution A (modified Tyrode solution): NaCl 137; KCl 5.4; $CaCl_2$ 0.1; $MgCl_2$ 5; Na-pyruvate 5; glucose 20; HEPES 10. pH 7.4. *Solution B:* KCl 140; $CaCl_2$ 0.1; $MgCl_2$ 5; Na-pyruvate 2.5; glucose 20; HEPES 10. pH 7.4. *Solution C* (pipette solution): NaCl 200; $CaCl_2$ 0.2; $MgCl_2$ 5; HEPES 10. pH 7.4. Bath temperature (controlled by a Peltier element device) 19 ± 1°C.

Drugs

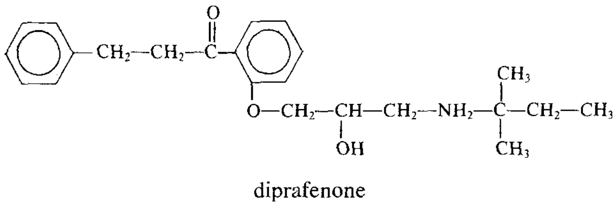
Amiodarone (Sanofi, Montpellier), propafenone and diprafenone (Helopharm KG, Berlin) were used. The structural formulae of these compounds are as follows



amiodarone



propafenone



diprafenone

From a serum-ethanol-amiodaronehydrochloride stock solution, an appropriate amount was diluted in solution B to give a final amiodarone concentration of 1 to 2 × 10^{-5} mol/liter. Propafenonehydrochloride and diprafenonehydrochloride were freshly dissolved in solution B before use.

Results

CARDIAC Na^+ CHANNELS MAY ATTAIN SEVERAL NONACTIVATABLE STATES

Stepping the cell-attached patches from holding potentials between −100 and −130 mV to a suprathreshold membrane potential triggered single Na^+ channel currents which, as judged from overlapping events, were mostly due to the presence of 2 to 3 functioning Na^+ channels. After patch formation, repetitive stimulation at 0.4 Hz revealed a high incidence of activity sweeps while blank sweeps were rare. This pattern remained in most of the cases over the whole patch lifetime as long as blocking agents were absent. In about 30% of the cell-attached patches, a run-down developed similar to that in cell-free patches after excision (Cachelin et al., 1983) so that within 10 min after patch formation, the number of activity sweeps including the frequency of overlapping events declined drastically. Interestingly, an increase in holding potential proved ineffective in restoring the initially high channel activity.

Figure 1 demonstrates the response of the only patch with one functioning Na^+ channel to repetitive stimulation. After an initial equilibration period of 10 min which was allowed in all experiments to exclude a run-down development, an ensemble of 400 samples was collected during an analysis period of 14 min. Consistent with the findings in the literature, a count of the resolved events (Fig. 1*A*) revealed that a considerable fraction (29%) of the activity sweeps showed two or more closely timed openings so that opening sequences occurred. From the exponential frequency distribution of events in the sequences, a mean number of 2.12 openings per sequence was obtained. The true value must be expected to be larger, since missed openings and missed closings are not considered. The shut time distribution between openings fits best a single exponential and yielded a value for τ_{shut} of 0.46 msec. In patches with two or three functioning Na^+ channels, a similar value was obtained at the same membrane potential (−50 mV), but the mean number of openings per sequence was larger and varied between 2.4 and 2.6. The maximal number of sequential openings in a three-channel patch was 18. This indicates reopening (Kunze et al., 1985), i.e. Na^+ channels attain more than once their open configuration in response to membrane depolarization.

Figure 1(*B*) shows still another and unexpected phenomenon which justifies to describe this one-channel patch in greater detail. Although repetitive pulsing of the membrane from −130 to −50 mV had periodically triggered channel openings during a period of about 6.5 min, Na^+ channel activity suddenly and abruptly ceased. An extremely long period occurred in which the same command impulse failed to induce detectable channel openings. This silent period lasted about 1.5 min and seems to indicate that the Na^+ channel became longer than usual nonactivatable. Subsequently, channel activity reappeared, and, again, there occurred periodically activity sweeps with one or more openings. Ensemble averaging before and after the silent period revealed reconstructed macroscopic Na^+ currents (Fig. 1*C*) of identical magnitude (0.28 and 0.29 pA, respectively). This is important since the identical Na^+ currents may exclude some kind of possible hang-over or run-down which might be suspected to remain from or be induced by the silent period and could finally reduce channel activatability. Further experimental support for unaltered channel activatability after the silent period was overcome can be obtained from the exponential frequency distribution of the run length of activity sweeps (Fig. 2*A*). To present the overall mean value for the whole 14-min lasting analysis period, 2.46 activity sweeps could be triggered before the occurrence of a blank sweep. A split analysis before and after the silent period gave values of 2.44 and 2.49, respectively.

A statistical run analysis (Horn & Vandenberg, 1984) revealed that activity sweeps and blank sweeps tended to alternate. Ninety-five runs were obtained prior to and 87 runs after the silent period. These experimental data equal the values (94 and 87, respectively) predicted by $2\,np\,(1-p)$. In this term, n is the total number of the collected samples and p means open probability calculated by dividing the number of activity sweeps by n. Consequently, the standardized random variable Z which is defined as

$$Z = -\frac{R - 2\,np\,(1-p)}{2\sqrt{n}\,p\,(1-p)}$$

and where R denotes the number of runs approached a value of zero (−0.75 and −0.15, respec-

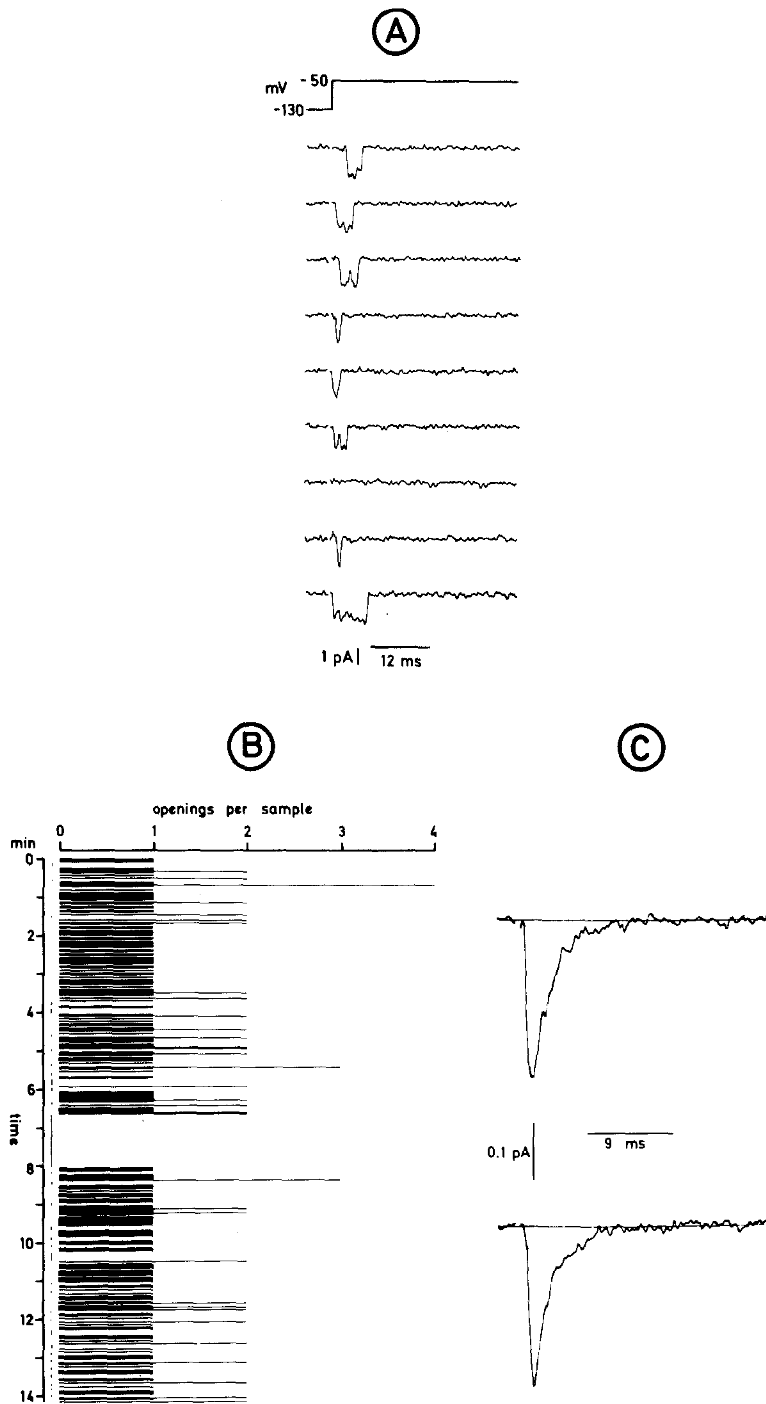


Fig. 1. Silent period in cardiac Na^+ channels. (*A*) Successive samples with and without elementary Na^+ currents from a cell-attached patch stepped with 0.4 Hz as indicated. During 24 min, overlapping openings or superpositions were never observed. Note the closely timed sequential openings in the 3rd and 6th sweep. (*B*) Channel openings in consecutive records during the 14-min lasting analysis period of this patch. Each bar indicates a sample with one or more (sequential) openings. (*C*) Reconstructed macroscopic Na^+ currents obtained by ensemble averaging before (upper I_{Na}) and after (lower I_{Na}) the silent period. The horizontal lines represent zero current. The decay of both I_{Na}'s could be best fitted by a single exponential (τ_{decay} 2.2 msec). Patch 341 CA

tively). Positive values of Z indicate grouped openings, negative values mean that activity sweeps and blank sweeps alternate.

Some insight into the nature of the silent period was obtained from the frequency distribution of the run length of blank sweeps. As found in five other patches with more than one functioning Na^+ channel, where 500 to 700 samples were collected from each patch under the same experimental conditions, the run length was always monoexponentially distributed (correlation coefficients of 0.90 or larger). This may be indicative of a uniform nonactivatable state. Figure 2(*B*) shows that the run with the largest number of blank sweeps (39) must be disregarded if the frequency distribution is to be fitted by a single exponential. It is, therefore, tempting to assume that the silent period reflects another, probably more stable nonactivatable state.

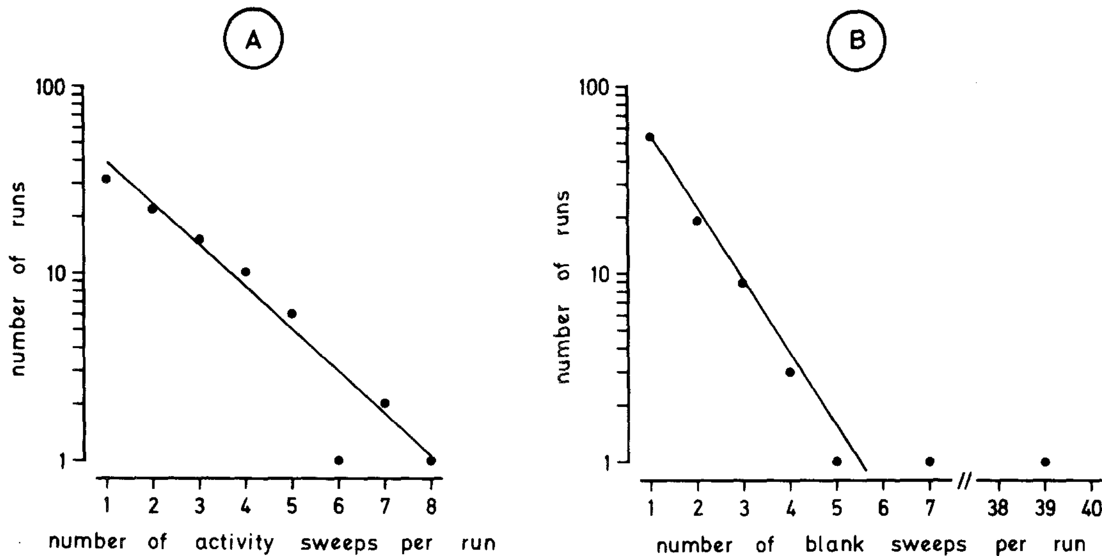


Fig. 2. Frequency distribution of the run length of activity sweeps (*A*) and of the run length of blank sweeps (*B*) in the one-channel patch demonstrated in Fig. 1

Class 1 Antiarrhythmic Agents Prevent Cardiac Na^+ Channels from Opening

Before the cardiocytes were superfused with a drug-containing isotonic K^+ solution, the cell-attached patches had been kept under control conditions for at least 30 min in order to collect a representative ensemble mostly of 400 to 700 samples on stepping them to −50 mV at 0.4 to 0.5 Hz. External application of amiodarone, propafenone, or diprafenone in concentrations of 10 to 20 μmol/liter produced within a few seconds a Na^+-channel blockade, i.e. the number of blank sweeps in the ensemble increased at the expense of the number of the activity sweeps, and, furthermore, the fraction of activity sweeps with superpositions declined. In the patch illustrated in Fig. 3, for example, the mean run length of blank sweeps increased from 1.3 to 1.8 blank sweeps in response to 10 μmol/liter amiodarone. The latter value resulted from a 13-min collecting period where the patch was stepped at the same (0.5 Hz) frequency. Increasing the step frequency to 1 Hz accentuated the block and led to a drastic rise of the number of blank sweeps (*see* Fig. 3*A*) so that the mean run length went further up to 5.4 blank sweeps. Consequently, as obtained from ensemble averaging (Fig. 4*A*), the peak amplitude of the reconstructed macroscopic I_{Na} declined from 0.72 pA under control conditions to 0.64 pA in the presence of the drug at 0.5 Hz and to 0.14 pA at 1 Hz. Moreover, block development was found to be accompanied by an altered frequency distribution of the run length of blank sweeps (Fig. 4*B*). Particularly at 1 Hz, very long runs occurred. Unlike under control conditions, the frequency distribution is more complicated and did not fit a single exponential. Experiments with 20 μmol/liter propafenone and with 20 μmol/liter diprafenone confirmed this latter result and allow to conclude that the frequency distribution of the run length of blank sweeps is no longer monoexponential when Na^+ channel blockage occurs.

The sensitivity of the run length of blank sweeps to changes in stimulation frequency reflects use dependence of the amiodarone action in blocking cardiac Na^+ channels (Mason, Hondeghem & Katzung, 1984). Use dependence of action is a typical feature of class 1 antiarrhythmic agents and related local anesthetics. It depends on the drug-specific dissociation constant. The latter determines the liberation of the channel from the drug during the interstimulus interval. Block modulation can also be achieved by variations in holding potential which is, consequently, also expected to alter the run length of blank sweeps. As tested in another amiodarone-treated cell-attached patch, the mean run length was 5.4 blank sweeps in presence of the drug at a holding potential of −110 mV, but returned on hyperpolarization to −150 mV to the initial control value of 1.1. However, this apparent normalization was not accompanied by a removal of blockade (Fig. 5, *A* and *B*). The reconstructed macroscopic Na^+ current increased in response to this hyperpolarization from 0.36 pA to only 0.88 pA and failed to attain the control current size of 1.4 pA, i.e. a significant block of 38% persisted. Inspecting the activity sweeps showed that the fraction with superpositions or overlapping openings which indi-

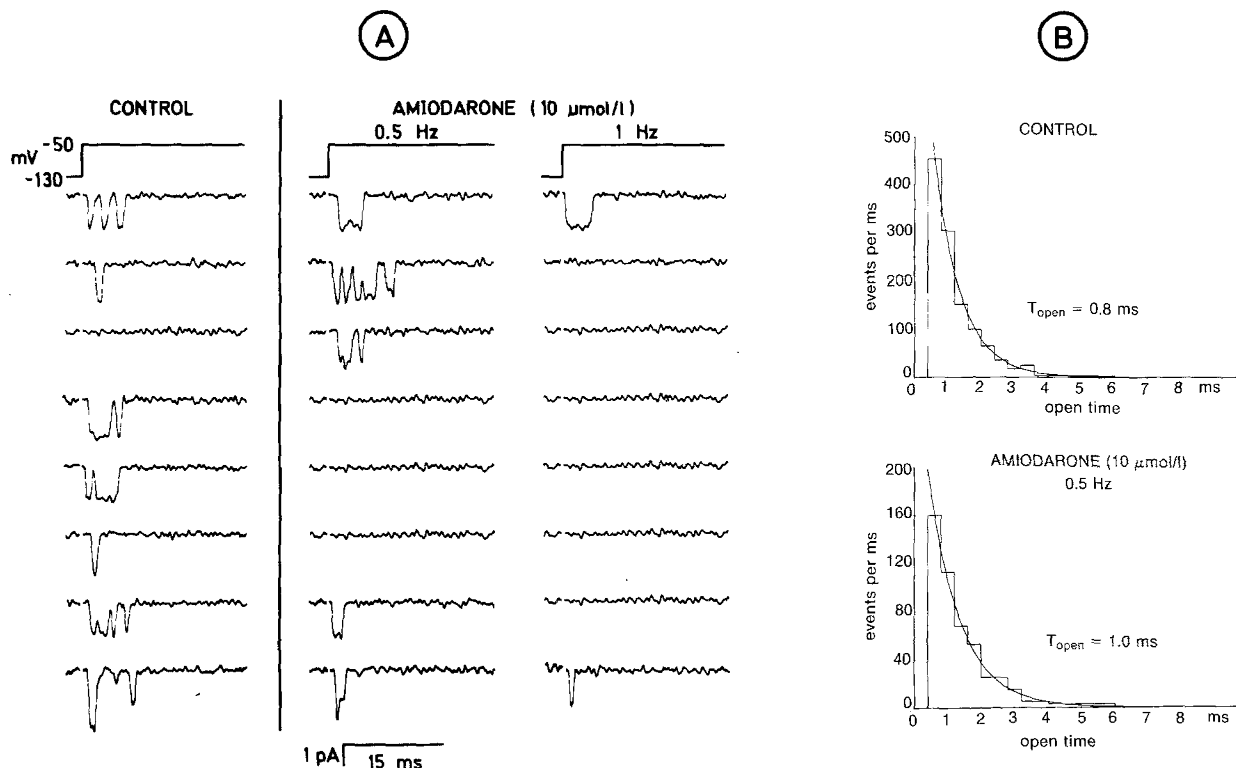


Fig. 3. (*A*) Successive recordings of elementary Na^+ currents before (control) and after treating the cardiocyte with amidarone on repetitive stimulation with 0.5 and 1 Hz, respectively. The patch contained two functioning Na^+ channels. (*B*) Open-time histograms of the same patch before (upper part) and after (lower part) amiodarone treatment. Both histograms are best fitted by a single exponential with time constants as indicated. Patch 336 CA. Membrane potential −50 mV

cate the simultaneous activity of two or three Na^+ channels remained small. This fraction was 35% under drug-free control conditions, declined to 4% after amiodarone treatment, but increased only slightly to 8% on membrane hyperpolarization from −110 to −150 mV. It is clear from this finding that the mean run length of blank sweeps does not necessarily coincide quantitatively with the block intensity, at least in this particular situation where removal of resting inactivation will augment the number of activatable Na^+ channels.

In order to define the pattern of channel opening, alternating or clustering, in the presence of these blocking drugs, the randomness run analysis was applied in two cell-attached patches. The ensemble collected in the one patch consisted of 187 runs under control conditions and was slightly smaller than predicted (210). In this case, the random variable Z was positive (+2.76) indicating that sweeps with and without activity tended to occur in groups. The variable Z declined in presence of amiodarone and took values which became smaller (0.96 at 0.5 Hz and −0.38 at 1 Hz) with stronger I_{Na} blockade. The other experiment yielded a conflicting result. Here, Z was +0.57 prior to but +4.2 after amiodarone treatment. This is noteworthy in that both patches contained the same number (3) of functioning Na^+ channels and, furthermore, the I_{Na} blockade which appeared in response to amiodarone was of identical strength (81% and 75%, respectively).

Properties of Unblocked Na^+ Channels

In the presence of antiarrhythimc drugs, two fractions of Na^+ channels may be assumed to coexist, one of them representing the blocked and the other unblocked, activatable channels. Although some evidence has been presented which might suggest a mutual interaction between neighboring Na^+ channels in mouse neuroblastoma cells (Kiss & Nagy, 1985), the present experiments did not yield evidence that unblocked Na^+ channels could somehow sense their blocked neighbor. The open-time histograms obtained in the presence of amiodarone (Fig. 3) and the other blocking agents employed were monoexponentially distributed as under control condi-

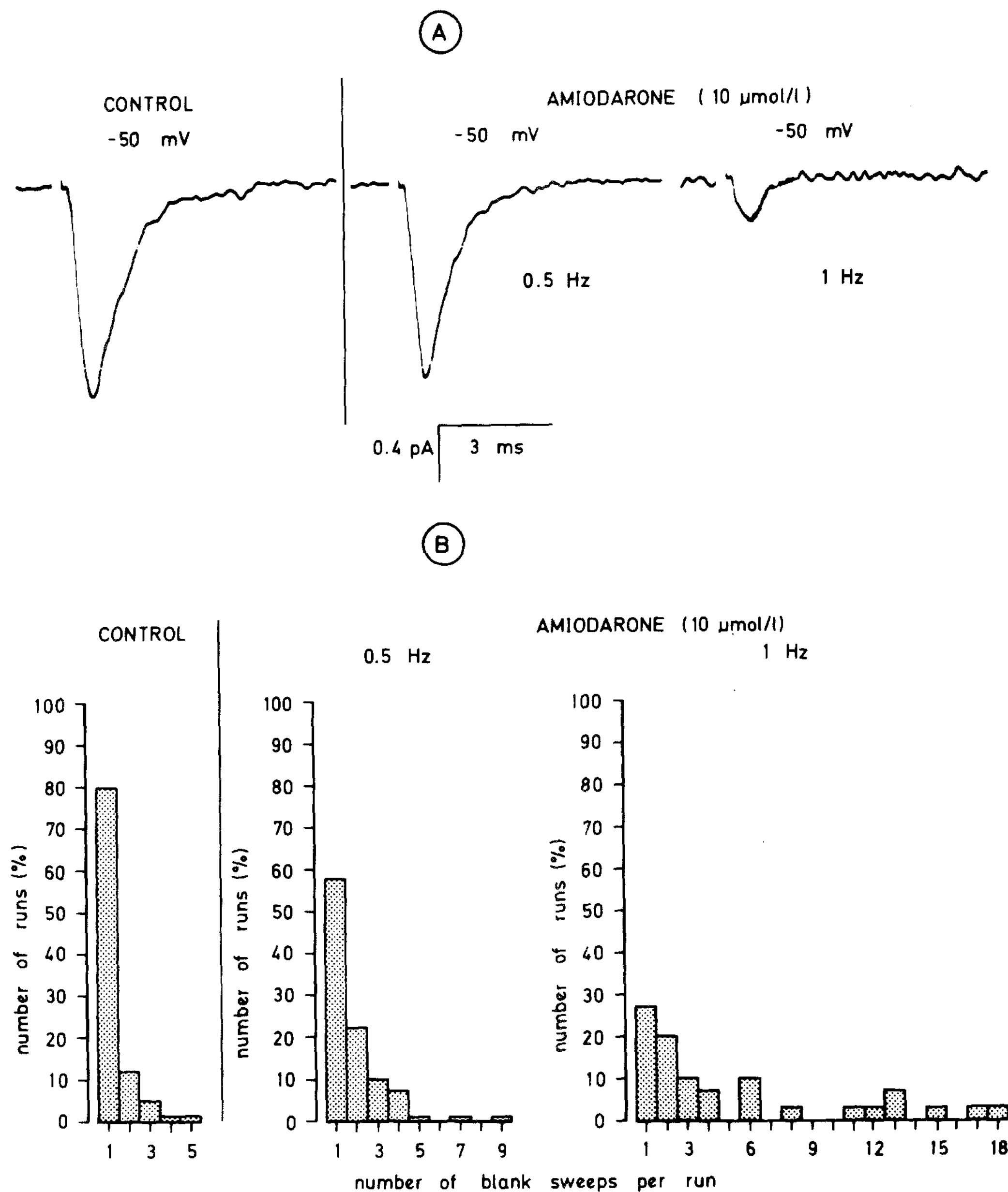


Fig. 4. (*A*) Use-dependent block of reconstructed macroscopic I_{Na} by amiodarone. Ensemble average from 651 samples collected under control conditions (left), 461 samples under amiodarone at 0.5 Hz (middle) and 234 samples under amiodarone at 1 Hz (right). Membrane potential −50 mV. (*B*) Frequency distribution of the run length of blank sweeps in the same patch before (left) and after (middle, at 0.5 Hz; right, at 1 Hz) amiodarone treatment. The histogram was normalized (100% refers to the total number of blank sweep runs observed) in order to demonstrate the change in distribution in greater clarity. Membrane potential −50 mV. Patch 335 CA

tions. Moreover, the dwell time in the open state remained unchanged after amiodarone and propafenone treatment (*see* Table 1). Table 1 also shows that unblocked Na^+ channels allow the same unitary current to flow as under control conditions, at least at −50 mV. Differences were only detected in the shut time between sequential openings. Two patches in which a sufficiently large ensemble could be collected for a histogram analysis after drug application showed a tendency of τ_{shut} to rise. It is, therefore, tempting to conclude that the reopening kinetics are affected. It should be emphasized, however, that even extremely closely timed sequential openings might occasionally be due to successively activating Na^+ channels.

These antiarrhythmic agents proved incapable of reducing the probability that a Na^+ channel reopens. As analyzed in the one-channel patch

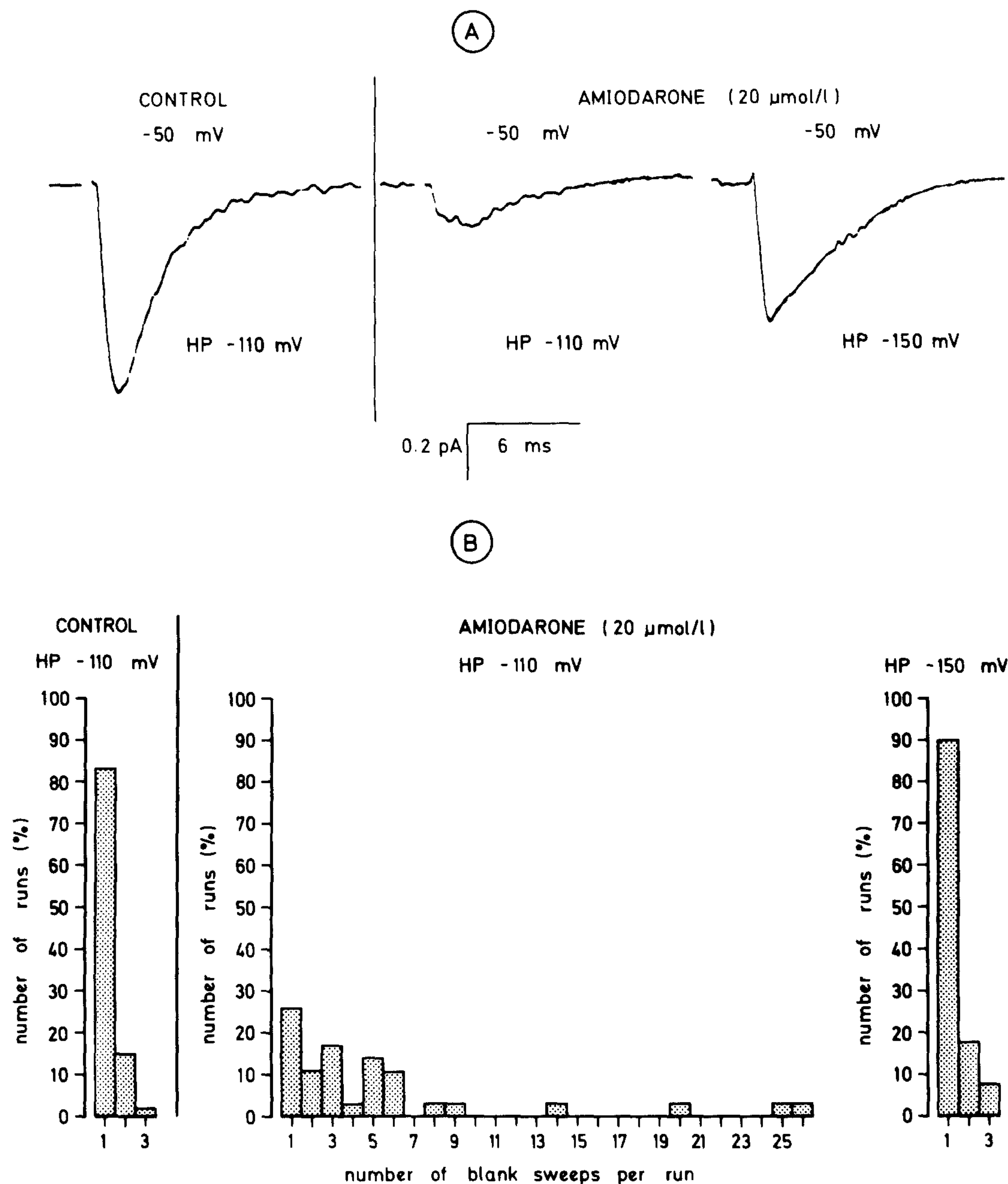


Fig. 5. (*A*) Voltage-dependent block of reconstructed macroscopic I_{Na} by amiodarone. Ensemble average from 484 samples collected under control conditions (left), 266 samples under amiodarone at the same holding potential (−110 mV) (middle), and 117 samples under amiodarone at −150 mV (right). Membrane potential −50 mV. (*B*) Frequency distribution of the run length of blank sweeps before (left), after amiodarone (middle), and, in the continued presence of the drug, after increasing the holding potential to −150 mV (right). The histogram was normalized (100% refers to the total number of blank sweep runs observed). Membrane potential −50 mV. Patch 336 CA

treated with diprafenone (patch 341 CA), the fraction of activity sweeps with multiple openings may even tend to rise, at the expense of the fraction of activity sweeps with a single opening, in the presence of the drug (Fig. 6*A*). Moreover, the number of openings during sequences remained either unchanged or varied insignificantly by 10% or less. It is noteworthy that this number was found to be slightly enhanced in patch 341 CA although the block was most strongly pronounced (80%) in comparison with the other patches (Fig. 6*B*).

This is consistent with the observation that some kind of repetitive activity may occur in presence of blocking drugs. Figure 7 demonstrates a

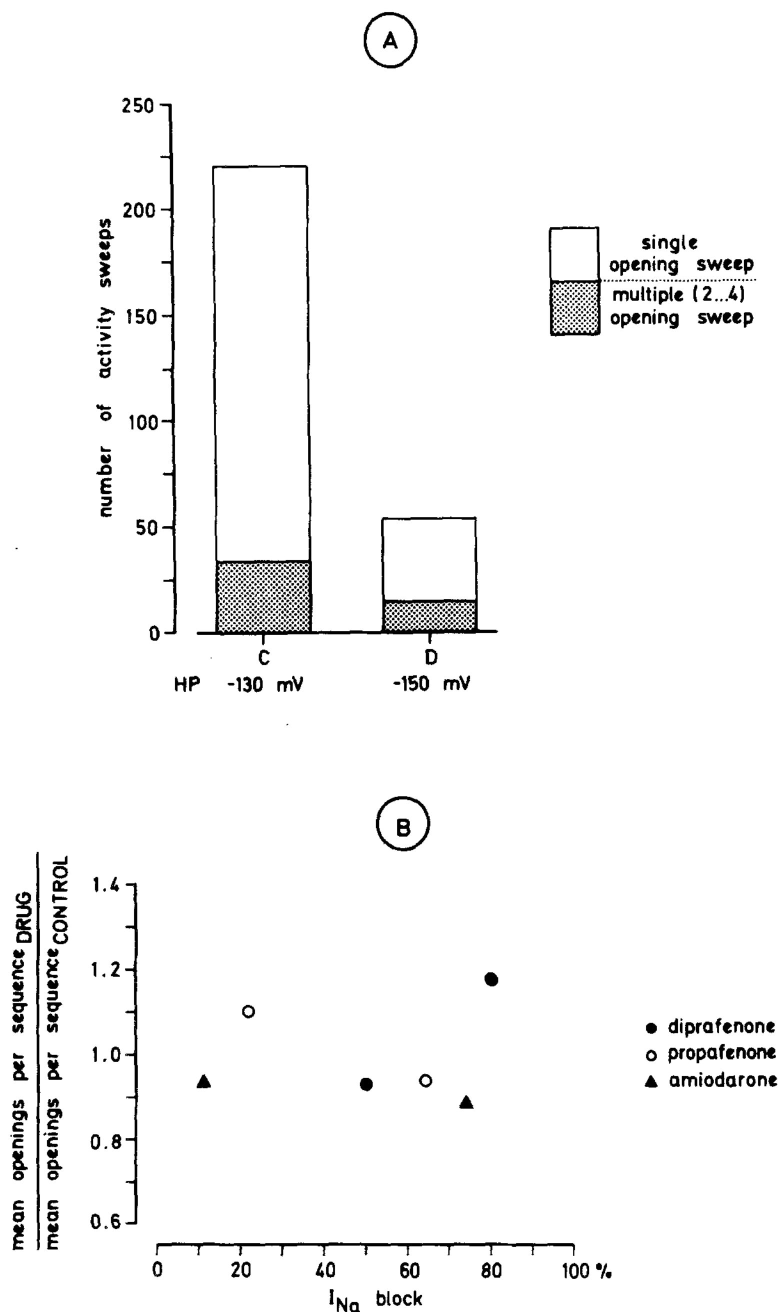


Fig. 6. (*A*) The tendency of reopening before (*C*) and after (*D*) diprafenone treatment (20 μmol/liter) in a one-channel patch (patch 341 CA). The columns represent the total number of activity sweeps observed prior to and after drug treatment, respectively. Their shadowed part indicates the number of sweeps with two or more sequential openings. The increase in holding potential was necessary in order to remove the total channel block evoked by diprafenone at −130 mV. At −150 mV, block strength was still strongly pronounced and amounted to 81%. The ratio sweeps with multiple openings to sweeps with a single opening was 0.15 under control conditions and 0.29 in presence of diprafenone. Membrane potential −50 mV. (*B*) Failing dependence of the number of openings in a sequence on the strength of I_{Na} block. Each symbol represents a cell-attached patch treated with 20 μmol/liter of the drug as indicated. The significantly varying block strength is due to differences in holding potential. Each patch was stepped with 0.4 Hz to −50 mV

cell-attached patch in which two ultralong opening sequences with 11 and 18 events were observed under control conditions. They did not fit the exponential event frequency distribution. The mean open time in these sequences (1.7 and 1.8 msec) was close to $\bar{t}_{open}$ (1.9 msec) of the whole ensemble. A similar sequence appeared during amiodarone treatment after the holding potential was hyperpolarized from −110 to −150 mV. It is important to note that $\bar{t}_{open}$ in the latter sequence (consisting of 14 events) was almost twice as large as in the activity sweeps with the normal, i.e. much lower open probability (3.4 msec instead of 1.8 msec). This strongly resembles a rarely occurring phenomenon, spontaneous failure of inactivation (Kohlhardt et al., 1987) although channel activity did not persist during the whole membrane depolarization (*see* Fig. 7).

Diprafenone Shortens Na⁺ Channel Lifetime

Apart from its blocking action, the propafenone derivative diprafenone exerts an additional effect on

Table 1. Open state, shut time between sequential openings and unitary current (i_{unit}) in cardiac Na^+ channels under control conditions and after application of amiodarone (amiod.) and propafenone (propaf.)[a]

	Open time		i_{unit} (pA)	Shut time	I_{Na} (pA)	HP (mV)	Patch (CA)
	$\bar{t}_{open}$ (msec)	τ_{open} (msec)		τ_{shut} (msec)			
Control	1.1	0.8	1.25	0.5	0.72	−130	336
amiodar. 10 μmol/liter 0.5 Hz	1.2	1.0	1.37	0.9	0.64	−130	
1 Hz	1.1				0.14	−130	
Control	1.8	1.6	1.33	0.4	1.4	−110	334
amiod. 20 μmol/liter	2.1	1.7	1.33		0.36	−110	
	1.8	1.5			0.88	−150	
Control	1.2	0.9	1.37	0.7	1.2	−100	339
propaf. 20 μmol/liter	1.2	0.9	1.33	1.0	0.9	−130	
Control	1.4	1.1	1.33	0.7	1.5	−130	338
propaf. 20 μmol/liter	1.3	1.1	1.33		0.54	−140	

[a] I_{Na} means the size of the macroscopic Na^+ current reconstructed by ensemble averaging. HP is the holding potential. All patches were stepped to −50 mV. In the patches 334 CA and 338 CA, the shut-time kinetics could not be analyzed completely because of the shortage of data. Time constants were obtained from the best fit of event distributions.

cardiac Na^+ channels. As demonstrated in Fig. 8(*A*), the lifetime of unblocked Na^+ channels appeared significantly shorter than expected from the control value. Open-time histograms confirmed this difference (Fig. 8*B*). In the presence of diprafenone and consistent with control conditions, there was evidence of a single open state. However, τ_{open} was 0.7 msec instead of 1.3 msec, the control value in the patch illustrated in Fig. 8 prior to drug treatment. A similar decline was obtained in two other experiments (*see* Table 2). Another peculiarity also contrasting with the normal properties of unblocked Na^+ channels in the presence of amiodarone and propafenone was the decline in unitary current size. A decrease of i_{unit} of up to 20% was found. This result, however, is difficult to interpret because of the shortcomings inherent in amplitude measurements with limited bandwidth. It cannot be excluded that the shift in the amplitude histograms is due to the increased contribution of events shorter than 1 msec, the critical lower limit in detecting the true event amplitude at 1 kHz bandwidth.

In an attempt to define the nature of the drug-induced abbreviation of channel lifetime, the holding potential was systematically varied in order to modulate the blocking diprafenone action (Fig. 9). As judged from the reconstructed Na^+ currents, the channel block evoked by 20 μmol/liter diprafenone declined on hyperpolarizing the holding potential from 90% at −130 mV to 53% at −150 mV. This proves the voltage dependence of the blocking drug action and should mainly reflect the voltage sensitivity of drug-induced tonic I_{Na} blockade (Kohlhardt & Seifert, 1983). Block attenuation was not accompanied by an increase in open time of unblocked Na^+ channels. Rather the smallest value for $\bar{t}_{open}$ occurred at −150 mV where the blocking diprafenone action became less pronounced. This indicates that drug-induced shortening of open time cannot be modulated, in contrast to the drug-induced blockade of Na^+ channels, by changes in holding potential, at least within the range tested. It is, therefore, tempting to conclude that the two drug effects are unrelated to each other.

Discussion

The major results of the present patch-clamp experiments can be briefly summarized as follows: (1) Cardiac Na^+ channels may attain two nonactivatable states, a dominating rather short-living one, and a long-lasting one. (2) Blockade of Na^+ channels by amiodarone, propafenone and diprafenone consistently reduces open probability. Unblocked Na^+ channels are indistinguishable from Na^+ channels in the absence of these antiarrhythmic agents. (3) Na^+ channels respond to drugs like diprafenone

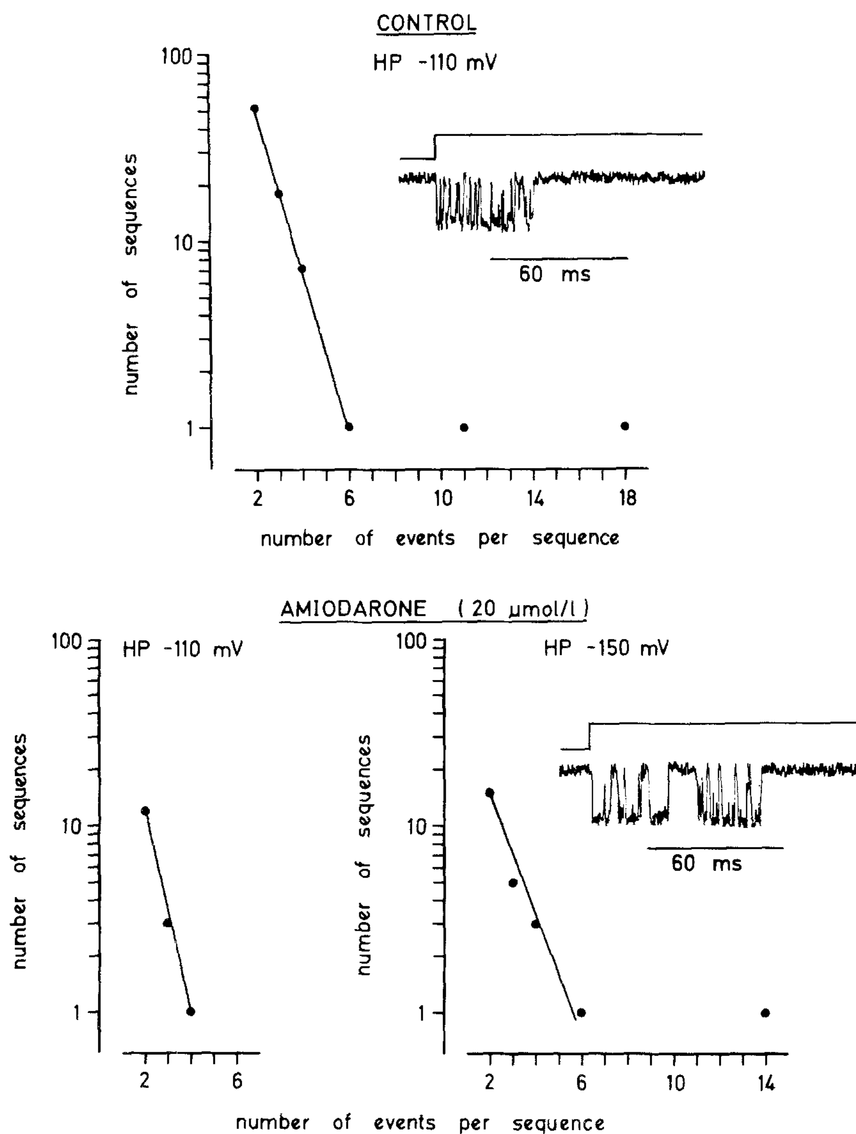


Fig. 7. Frequency distribution of events in a sequential opening before (upper part) and after amiodarone application (lower part). The insets show the samples with the largest number of events observed prior to and after drug application. Holding potentials as indicated. Membrane potential −50 mV. Patch 334 CA

Table 2. Open state, shut time between sequential openings and unitary current (i_{unit}) in cardiac Na^+ channels under control conditions and after application of diprafenone (dipraf.)[a]

	Open time		i_{unit} (pA)	Shut time	I_{Na} (pA)	HP (mV)	Patch (CA)
	$\bar{t}_{open}$ (msec)	τ_{open} (msec)		τ_{shut} (msec)			
Control	1.6	1.1	1.21	0.5	0.48	−130	341
dipraf. 20 µmol/liter	1.1	0.8	1.05	0.6	0.10	−150	
Control	1.7	1.3	1.41	0.6	1.8	−120	337
dipraf. 20 µmol/liter	0.9	0.7	1.13		0.9	−140	
Control	1.8	1.2	1.25		0.95	−130	322
dipraf. 20 µmol/liter	1.0	0.7	1.10		0.31	−140	

[a] I_{Na} means the size of the macroscopic Na^+ current reconstructed by ensemble averaging. HP is the holding potential. Patches 337CA and 341CA were stepped to −50 mV, patch 322CA to −40 mV. Time constants were obtained from the best fit of event distributions.

Fig. 8. Successive recordings of elementary Na^+ currents under control conditions and in the presence of diprafenone (*A*). The patch contained two functioning Na^+ channels. The open-time distributions (*B*) prior to and after drug application could be best fitted by a single exponential with a time constant as indicated. Membrane potential −50 mV. Patch 337 CA

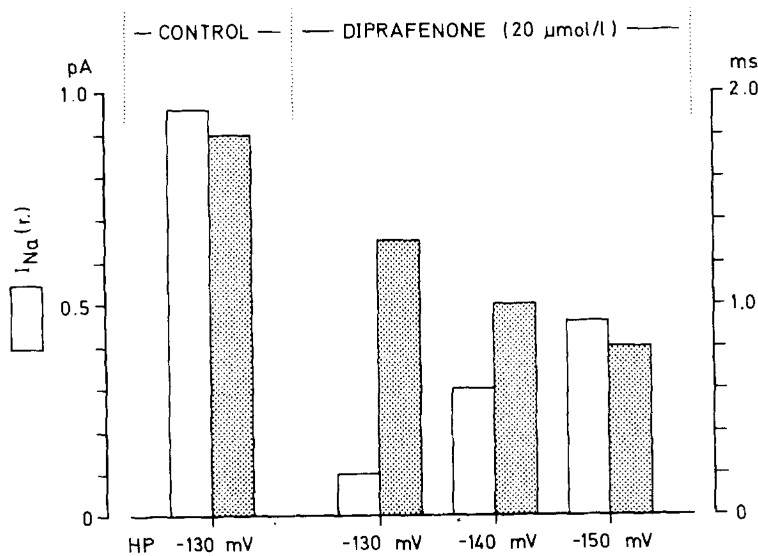


Fig. 9. Different voltage-dependent of the blocking and the open-time reducing diprafenone effect. The light columns indicate the size of the reconstructed macroscopic Na^+ currents obtained from ensemble averaging of 401 control samples, of 450 diprafenone samples at a holding potential of −130 mV, of 287 diprafenone samples at −140 mV and 265 diprafenone samples at −150 mV. The shadowed columns indicate the mean open times found under these conditions. Membrane potential −40 mV. Patch 321 CA

with an abbreviation of their open state and leave the conducting configuration at up to a 1.85-fold increased rate.

Heterogeneous Gating Kinetics of Na^+ Channels

As previously shown in skeletal muscle, an individual Na^+ channel is capable of displaying long-lasting, burst-like activity with significantly varying open times (Patlak, Ortiz & Horn, 1986). Repetitive activity during the whole membrane depolarization was likewise observed in heart muscle but appeared with an extremely low likelihood in that tissue (Patlak & Oritz, 1985; Kohlhardt et al., 1987) and represents, *per se*, a mode of activity not consistent with the usual pattern of channel opening. At least in heart muscle, evidence is provided that the inactivated, absorbing (Aldrich, Corey & Stevens, 1983) state cannot be reached during such an episode (Kohlhardt et al., 1987).

Another example of heterogeneous gating kinetics are the several nonactivatable states which are suggested by the silent period in the one-channel patch of the present experiments. Evidence of a nonactivatable state which is slowly reached and left has been obtained in GH_3 cells (Horn, 1984) from the clustering of records with and without activity. In the one-channel patch showing the silent period, such a clustering was not observed. This might well be due to the large interstimulus interval (2380 msec) which could be too long in relation to the cycling from the nonactivatable to the activatable state (Horn et al., 1984) from which the Na^+ channel can reach the open configuration on membrane depolarization. It is, therefore, impossible to define quantitatively the nonactivatable state in the present experiments. Nevertheless, the run length of blank sweeps will be determined by the nonactivatable state and is, consequently, related to the likelihood with which a voltage jump triggers a channel opening. This likelihood is reduced during the silent period.

The proposed change from one to the other nonactivatable state might be more frequent than expected from the present experiments, since a silent period as pronounced as that observed here will barely happen in patches with several functioning Na^+ channels. This change would further stress the concept of a tautochannel (Benoit, Corbier & Dubois, 1985), which may switch, by definition, between several molecular arrangements. It remains to be tested whether the spontaneously occurring, poorly activatable state is unique for neonatal cardiac Na^+ channels or represents a general feature of Na^+ channels in excitable membranes.

Interaction of Class 1 Antiarrhythmic Drugs with Na^+ Channels

The ineffectiveness of permanently charged lidocaine derivatives to block Na^+ currents on external application (Frazier, Narahashi & Yamada, 1970; Strichartz, 1973; Courtney, 1975) was an important observation for elucidating the mode of action of local anesthetics and led to the concept that these drugs and the related antiarrhythmic compounds find a binding site within the Na^+ channel not accessible to them from the outer channel mouth (Hille, 1977). The extremely narrow aperture of the selectivity filter hinders the larger drug molecules to pass the latter (Hille, 1975) and compels them to utilize a lipophilic route. The drug reaches the binding site either laterally from the surrounding lipid matrix or, after passing the membrane, by entering the channel from its cytoplasmic mouth. Cardiac Na^+ channels possess a binding site of identical localization, namely somewhere between the selectivity filter and cytoplasmic mouth since bath application of amiodarone, propafenone and diprafenone effectively blocked Na^+ channels in the present cell-attached patch-clamp experiments.

The very rapid onset of block which took only a few seconds to appear after the cardiocyte was superfused with drug-containing solutions indicates that the diffusion of the neutral drug molecules through the sarcolemma and their arrival at the site of action is readily accomplished. This clearly contrasts with results in papillary muscles where several minutes are needed to establish the full inhibitory propafenone effect (Kohlhardt & Seifert, 1980). A well-developed glycocalyx which covers the surface membrane in normal cardiac tissue but is lacking in enzymatically disaggregated cardiocytes (Isenberg & Klöckner, 1980) could render drug diffusion and might explain this discrepancy.

Unblocked Na^+ channels share many properties with normal Na^+ channels. The most interesting finding was their unchanged tendency to reopen. This is noteworthy in that the effectiveness of local anesthetics and related antiarrhythmic drugs in evoking a channel blockade is state dependent, and is supposed to be accentuated on transition from the rested to the open and/or inactivated channel configuration (Hondeghem & Katzung, 1977). A reduction in the number of openings during a sequence, nevertheless, did not occur. There are two possible explanations of this surprisingly normal behavior. First, a Na^+ channel once opened remains drug free until the inactivated state will be reached. This hypothesis is intimately related to both, the genesis of reopening, including repetitive activity like that demonstrated in Fig. 7, and the predominant affinity

of an individual drug for a definite channel state. Presupposing the inactivation to be an absorbing state (Aldrich et al., 1983), such activity suggests that the inactivated state could not yet be reached, thus allowing the Na^+ channel to cycle between the open and a closed configuration. At least in the presence of amiodarone, a block preferentially of inactivated Na^+ channels must be expected to develop (Mason, Hondeghem & Katzung, 1983). If propafenone and diprafenone follow the same rule, all these drugs would attack the Na^+ channel only after it has terminated its reopening activity. Second, the rate constant of the drugs employed in the present experiments is not large enough to install channel block within a restricted period of time, say 5 to 15 msec, the usual duration of sequences. Our own unpublished results with propafenone and diprafenone in DPI-modified cardiac Na^+ channels revealed a value for the blocking rate constant of 8 to 10×10^6 mol^{-1} sec^{-1} (at a membrane potential of -45 mV). This might be critically low in relation to the duration of sequences. It should be emphasized that this second explanation tacitly proposes these drugs to be capable of interacting with open Na^+ channels.

Diprafenone—A Novel Type of Channel Modifier?

A great variety of chemicals such as protein reagents (aldeydes, N-bromoacetamide and others), naturally occurring toxins (ATX, BTX, and others), and organic compounds like the diphenylpiperazinylindole derivative DPI are capable of modifying Na^+ channels. As shown in single-channel studies with N-bromoacetamide (Patlak & Horn, 1982), BTX (Quandt & Narahashi, 1982), and DPI (Kohlhardt et al., 1986), these interventions uniformly prolong the conducting state tremendously and cause repetitive channel activity, kinetic modifications which reflect removal of inactivation. Since Na^+ channels evidently possess specific binding sites for each toxin (for review *see* Hille, 1984) and for DPI (Romey et al., 1987), the kinetic modification evoked by them is most likely to result from interactions with their respective receptor. This might directly or allosterically provoke a presently still unknown molecular reaction which finally prevents the Na^+ channel from attaining its absorbing inactivated state during membrane depolarization.

It is tempting to assume that the transition from the open to the inactivated state might also be sensitive to another group of compounds represented by the propafenone derivative diprafenone, but exerting a completely different effect from the channel modifiers mentioned above. Although propafenone and diprafenone are structurally closely related, only diprafenone has, in addition and independent of its channel blocking effect, a modifying effect which is, in a strictly formalistic sense, opposite to the action of DPI and causes Na^+ channels to leave their open state faster than under control conditions. As a hypothetical molecular interpretation whose verification requires binding studies, diprafenone could find still another site of action in utilizing the lipophilic channel access route not identical with that receptor which is proposed to interact with diprafenone in blocking the Na^+ channel. Detecting and binding of diprafenone by this hypothetical site could ultimately facilitate the exit from the open state.

Another, but rather unlikely possibility is that the reduction in open time reflects an open-channel block. Blockade of open Na^+ channels is the mode of action proposed for a certain subgroup of antiarrhythmic drugs including quinidine and lidocaine (Hondeghem, 1987), which develops extremely rapidly but keeps a channel blocked at least for several ten milliseconds. This can be concluded from the relaxation kinetics of the phasic lidocaine I_{Na} blockade, one of the fastest drugs in this respect, having a rate constant in the order of magnitude of 5 sec^{-1} (Kohlhardt & Seifert, 1985). Consequently, the tendency to reopen and the number of openings during sequential activity would be expected to be reduced, a prediction which is in striking contrast to the experimental results. Probably the most convincing piece of evidence against the objection that the observed reduction in open time might be due to an open-channel block arises from our own, still unpublished cell-attached patch-clamp experiments in neonatal cardiocytes with the open-channel blocker lidocaine. Even in the presence of a very high concentration (300 μmol/liter), the open time of cardiac Na^+ channels remained unchanged.

Besides its theoretical significance in elucidating Na^+ channel properties in greater detail, modulation of Na^+ channel lifetime is also of interest from a more functional aspect since it has been recognized as an alternative and important principle to influence cardiac contractile strength (for review *see* Honerjäger, 1982). The resultant change in cellular Na^+ load during each excitation influences, via transmembrane Na^+/Ca^{2+} exchange, the amount of intracellular Ca^{2+} available for excitation-contraction coupling. Drugs like diprafenone which combine an open-time shortening effect with a Na^+ channel blocking effect are predicted to depress cardiac contractility more strongly than other class 1 antiarrhythmic drugs lacking this modifying capability and interfering with contractile force (Honerjäger et al., 1986) only by Na^+ channel blockade.

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