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Responsiveness of Cardiac Na^+ Channels to Antiarrhythmic Drugs: The Role of Inactivation

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Summary. Elementary Na^+ currents were recorded at 9°C in inside-out patches from cultured neonatal rat heart myocytes. In characterizing the sensitivity of cooled, slowly inactivating cardiac Na^+ channels to several antiarrhythmic drugs including propafenone, lidocaine and quinidine, the study aimed to define the role of Na^+ inactivation for open channel blockade.

In concentrations (1–10 μmol/liter) effective to depress NP_o significantly, propafenone completely failed to influence the open state of slowly inactivating Na^+ channels. With 1 μmol/liter, τ_{open} changed insignificantly to 96 ± 7% of the control. Even a small number of ultralong openings of 6 msec or longer exceeding τ_{open} of the whole ensemble several-fold and attaining τ_{open} (at −45 mV) in cooled, (−)-DPI-modified, noninactivating Na^+ channels proved to be drug resistant and could not be flicker-blocked by 10 μmol/liter propafenone. The same drug concentration induced in (−)-DPI-modified Na^+ channels a discrete block with association and dissociation rate constants of 16.1 ± 5.3 × 10^6 mol^{-1} sec^{-1} and 675 ± 25 sec^{-1}, respectively. Quinidine, known to have a considerable affinity for activated Na^+ channels, in lower concentrations (5 μmol/liter) left τ_{open} unchanged or reduced, in higher concentrations (10 μmol/liter) τ_{open} only slightly to 81% of the predrug value whereas NP_o declined to 30%, but repetitive blocking events during the conducting state could never be observed. Basically the same drug resistance of the open state was seen in cardiac Na^+ channels whose open-state kinetics had been modulated by the cytoplasmic presence of F^- ions. But in this case, propafenone reduced reopening and selectively abolished a long-lasting open state. This drug action is unlikely related to the inhibitory effect on NP_o since hyperpolarization and the accompanying block attenuation did not restore the channel kinetics. It is concluded that cardiac Na^+ channels cannot be flicker-blocked by antiarrhythmic drugs unless Na^+ inactivation is removed.

Key Words single cardiac Na^+ channels · open-state kinetics · drug-induced blockade · (−)-DPI

Introduction

Drug-induced blockade of voltage-dependent Na^+ channels constitutes an important principle in local anesthesia and offers in heart muscle an opportunity to suppress cardiac arrhythmias. I_{Na} block phenomenology has been extensively analyzed during the last years in neuronal tissues, skeletal and cardiac muscle, but attracted growing interest in membrane biology not primarily for pharmacological reasons. The greater fascination relies on the fact that the complicated, puzzling block manifestation reflects some basic properties of Na^+ channel organization. The effectiveness of local anesthetics and related antiarrhythmic drugs to depress I_{Na} in excitable membranes is voltage and time dependent and is additionally determined by their physicochemical properties. Circumstantial evidence has been presented in favor of a channel-associated binding site (for review *see* ref. [9]) which can be modeled as modulated [11, 12] or as guarded [35] receptor. The former type possesses a state-dependent drug affinity that increases with a transition from the rested to the activated/inactivated channel configuration. The guarded receptor explains the use-dependent I_{Na} blockade with a facilitated drug access during the activated state, but has been recently somehow invalidated by the demonstration that the antiarrhythmic agent quinidine may occupy guarded Hodgkin-Huxley Na^+ channels in heart muscle [34].

Single-channel experiments in cardiac Na^+ channels confirmed the theoretical postulate that blocking by antiarrhythmic drugs occurs in an all-or-none fashion [15]. All-or-none blockade represents a common principle regardless of the nature and the site of action of a particular blocking molecule and is likewise evoked by naturally occurring toxins such as tetrodotoxin [30]. Drug-occupied Na^+ channels basically fail to conduct in response to membrane depolarization as if they could not undergo a transition in the open configuration. Alternatively, normal gating properties might be maintained, i.e., local anesthetics and related agents are not capable of preventing the Na^+ channel protein from undergoing conformation changes as underlying the formation of a Na^+ pore but exert their inhibitory action by

blocking the latter, thus hindering Na^+ permeation. Charge immobilization reported from gating current measurements to occur after drug treatment provide no support for the latter hypothesis. Another, second type of block having a quite different biophysical phenomenology and not being involved in the conventional I_{Na} blockade operates additionally in kinetically modified Na^+ channels. After pharmacological or chemical removal of Na^+ inactivation, they become blocked repetitively during their open state [17, 37, 38]. This microscopic or flicker blockade visualizes the arrival and departure of a blocking molecule and may be considered to reflect the reaction kinetics of the drug-receptor interaction that underlies the channel blockade.

The nature of the flicker blockade remains to be defined. It is not yet clear whether this type of block is causally related to removal of Na^+ inactivation. Because noninactivating Na^+ channels dwell for a longer fraction of time in the conducting state than normal Na^+ channels, this kinetic peculiarity itself could be suspected to allow flicker blockade by increasing the likelihood that a blocking molecule interfers with the Na^+ channels during the open state. The present inside-out patch-clamp experiments with several antiarrhythmic drugs tried to answer this question. By cooling to 9°C, the open state of normal cardiac Na^+ channels was several-fold prolonged and the drug response of these slowly inactivating Na^+ channels was analyzed which may dwell as long as (−)-DPI-modified Na^+ channels in the conducting state. It will be shown that a prolonged open state remains protected against flicker blockage by antiarrhythmic drugs if Na^+ inactivation is operative.

Materials and Methods

Elementary Na^+ currents were recorded in inside-out patches from cultured neonatal rat myocytes with an L-M/EPC 5 amplifier by employing the standard patch-clamp technique [8]. Cell culturing and the handling of the short-time (18–24 hr) cultured myocytes, which were incubated in a depolarizing isotonic K^+ saline (solution A) during the patch-clamp experiments, were essentially the same as already described in detail [18]. Before excision, the patches were kept for 10 min at 19°C in the cell-attached configuration in order to stabilize the recording conditions and to achieve stable Na^+ channel activity. Immediately after excision, the inside-out patches were cooled by lowering the bath temperature to 9°C and, subsequently, equilibrated for 10 min in order to avoid transitory temperature effects on Na^+ channel activity until elementary Na^+ currents were recorded. During the control period of 15–20 min, a sufficiently large ensemble of sweeps was collected to yield, as a lower limit, 400 single-channel events, thereby carefully monitoring the Na^+ channel activity mode. An experiment was discarded when, as observed in a few cases, a run-down developed and Na^+ channel activity gradually declined with time.

The inside-out patches were kept at holding potentials between −110 and −130 mV and repetitively depolarized by rectangular command impulses of 70-msec duration at a rate of 0.67 Hz to a suprathreshold test potential. With normal Na^+ channels, a near-threshold potential between −65 and −55 mV was intentionally chosen in order to minimize overlapping events and to facilitate channel reopening which increases the yield of single-channel events and improves the analysis of burst-like activity. Each patch contained at least two functioning Na^+ channels but no attempt was made to determine their true number.

The patch-clamp recordings were filtered at 1 kHz with an 8-pole Bessel filter, digitized with a sampling rate of 5 kHz and stored on floppy discs. The dead time was 0.2 msec.

Idealized patch-clamp recordings resulted from the subtraction of leakage and residual capacity currents. Open time and gap times between nonoverlapping single events were analyzed by setting a threshold at 50% of the unitary current amplitude [4]. Probability density functions in individual patches before and after drug treatment yielded τ_{open} and τ_{closed}, respectively, and were based on an unweighted fit meaning that each bin was considered to be of identical significance. Late and, therefore, rare events were fitted by lumping several bins with a certain minimum of events arbitrarily chosen to be four. The best fit of the probability density functions resulted from the least squares (X^2) method. Mean open time was calculated from $\bar{t}_{open} = \Sigma_i n_i T_i / n$ [6]. The presence of more than one Na^+ channel seriously complicated the analysis of sequential, burst-like activity. Although activity sweeps without overlapping events were exclusively taken for analysis and short gaps in the submillisecond range dominated by far between repetitive openings, they do not indicate unambiguously the repetitive activity of one and the same individual Na^+ channel. Counting sequential openings will, consequently, not yield a statistically valid measure of the reopening tendency but may only provide a certain estimate.

Burst analysis in DPI-modified Na^+ channels was essentially based on the bimodal closed-time distribution as individually determined in each patch. Gaps within bursts can be discriminated from gaps between bursts by introducing a certain critical gap time depending on the ratio between $\tau_{closed(1)}$ and $\tau_{closed(2)}$ which was calculated from the geometric mean of both time constants.

Ensemble averaging of 120–180 sweeps yielded the macroscopic I_{Na}. Since $I = iNP_o$, peak I_{Na} refers to the moment during membrane depolarization where NP_o attains its maximum and was taken as an index of the latter. From the decline of NP_o in the presence of antiarrhythmic drugs, their potency to block Na^+ channels can be calculated according to

$$K_D = \text{drug concentration} \cdot \frac{I_{Na(drug)}}{I_{Na(control)} - I_{Na(drug)}}.$$

Whenever possible, the data are expressed as mean ± SEM.

Solutions (Composition in mmol/liter)

A. Solutions facing the cytoplasmic membrane surface in inside-out patches: (*i*) Isotonic K^+ solution: K^+ aspartate 120; KCl 20; $MgCl_2$ 5; Na-pyruvate 2.5; glucose 20; EGTA 1; HEPES 10; pH 7.4. (*ii*) KF solution: KF 140; Na-pyruvate 2.5; glucose 20; EGTA 1; HEPES 10; pH 7.4. B. Pipette solution: NaCl 150; $MgCl_2$ 1;

$CaCl_2$ 0.035; HEPES 10; pH 7.4. Temperature (controlled by a Peltier element): 9°C.

Drugs

Propafenonehydrochloride (Helopharm KG, Berlin), lidocainehyhdrochloride (Sigma Chemie, München), and quinidinesulfate (Sigma Chemie, München) were freshly dissolved in solution (*i*) or (*ii*) before use. (−)-DPI (Sandoz, Basle), dissolved in dimethylsulfoxid (Sigma Chemie, München), was added to the pipette solution to give a final concentration of 5–10 μmol/liter.

Results

Flicker Blockade in Cooled DPI-Modified Na^+ Channels

In the first series of experiments, the inhibitory effect of class 1 antiarrhythmic drugs on DPI-modified Na^+ channels was analyzed in order to establish that they also can be flicker-blocked after lowering the temperature to 9°C. The experimental protocol basically included an initial equilibration to the modifying (−)-DPI in order to install stable modified channel activity which is characterized by the occurrence of two distinct types of activity sweeps, one with a low ($P_o < 0.1$) and the other type with a high ($P_o > 0.1$) open probability. The latter shows repetitive, burst-like activity which persists during membrane depolarization and a several-fold prolonged, strongly voltage-dependent open time and, thus, strikingly differs from the activity sweeps with a low P_o. Activity sweeps with $P_o > 0.1$ were considered to reflect (−)-DPI-bound, modified Na^+ channels (*see* Fig. 1; left part). Analogous to their response at 19°C [17], treating the cytoplasmic membrane surface with propafenone (5–10 μmol/liter) increased, by chopping long-lasting openings, the number of transitions between the conducting and a nonconducting state. In the experiment depicted in Fig. 1, for example, 5 μmol/liter propafenone led to a rise of the mean number of openings per $P_o > 0.1$ activity sweep by a factor of 1.41 and concomitantly decreased τ_{open} from 9.43 to 5.30 msec (at −45 mV). The interpretation of this effect is intimately related to the molecular mode of the (−)-DPI action. If (−)-DPI interacts with a channel-associated binding site [31] in order to remove Na^+ inactivation, these kinetic changes are indicative for a flicker blockade. Although several lines of evidence strongly support the hypothesis that (−)-DPI bound Na^+ channels cannot inactivate, an alternative mechanism might be that (−)-DPI acts as channel opener, i.e., repetitive openings and closings

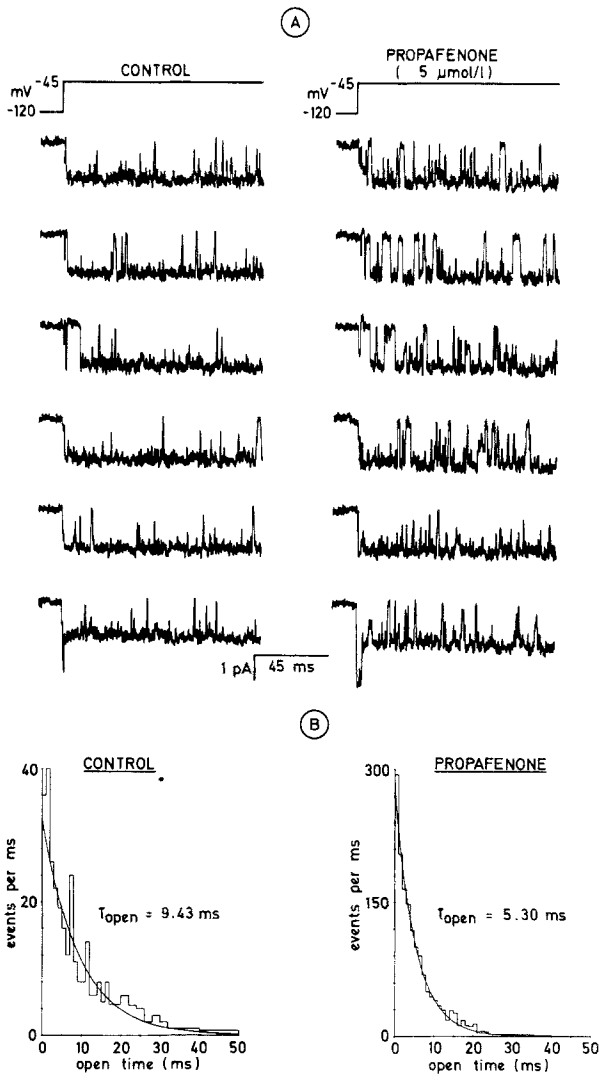


Fig. 1. (*A*) Selected recordings of elementary Na^+ currents from (−)-DPI-modified Na^+ channels before (left row) and after (right row) the cytoplasmic presence of 5 μmol/liter propafenone. (*B*) Open-time probability density functions of the same experiment based on 350 events in the absence and of 1581 events in the presence of the drug. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 32 \exp(-t/0.00943)$ and by $N(t) = 282 \exp(-t/0.0053)$, respectively. Patch 603I0; test potential −45 mV; 9°C

emerge from repetitive binding and unbinding of (−)-DPI during membrane depolarization. If this holds true, the decrease in τ_{open} seen after the treatment with propafenone and related antiarrhythmic agents would result from a displacement of (−)-DPI by those drugs. Testing the temperature coefficient of τ_{open} offers an opportunity to clarify this point and a Q_{10} of 1.3 must be postulated for the case that (−)-DPI acts as channel opener. Experimentally, this

postulate is not fulfilled. When cooling from 19 to 9°C, τ_{open} increased (at −45 mV) with a Q_{10} value of 2.44 which is in the order of magnitude that is typical for a gating process.

The flicker blockade can be described, in the simplest case, by the reaction scheme

$$\text{closed} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{open} \underset{d}{\overset{aD}{\rightleftharpoons}} \text{blocked}$$

[25, 38]. Accordingly, DPI-modified Na^+ channels may attain, on leaving their open configuration, two different nonconducting states, namely a closed or, after interaction with propafenone, a blocked state. The rate constants a and d correspond to the association and dissociation rate constants of the drug; a was calculated from $(1/\tau_{open(drug)} - 1/\tau_{open(control)})$; and d resulted from the mean shut time within bursts. At −45 mV and 9°C, values for a and d of 16.1 ± 5.3 × 10^6 mol^{-1} sec^{-1} (n = 3) and of 675 ± 25 sec^{-1} (n = 3), respectively, were obtained. Since K_D = d/a, the mean K_D amounted to 4.9 ± 1.4 × 10^{-5} mol.

The Effects of Class 1 Antiarrhythmic Drugs on Cooled Na^+ Channels with Intact Inactivation

Gating of Na^+ channels and the underlying conformation changes of the α-subunit of the channel protein exhibit almost the same temperature sensitivity as many enzyme reactions and become decelerated with temperature coefficients of 2 or more on lowering the temperature from 19 to 9°C. The resultant prolongation of the conducting state is paralleled by an increase of the fraction of long openings. Classifying events with a life time of 4 msec or longer in a potential range between −65 and −55 mV arbitrarily as long openings, their contribution to the whole ensemble rose from 1.7 ± 0.2% at 19°C to 12.1 ± 2.8% at 9°C (n = 3). Bearing in mind the open-state kinetics of DPI-modified Na^+ channels (mean τ_{open} at 9°C and −45 mV: 6.6 ± 0.5 msec, n = 5), cooled Na^+ channels with operating inactivation may leave with a likelihood of about 10% their conducting state with a very similar exit rate.

Nevertheless, as observed in cooled Na^+ channels exposed cytoplasmatically to a quasi-physiological anionic milieu, their open-state kinetics proved to be insensitive to class 1 antiarrhythmic drugs. Figure 2 demonstrates an inside-out patch treated cytoplasmatically with 1 μmol/liter propafenone. Within a few seconds, the drug evoked a channel blockade. At the expense of the fraction of activity sweeps the fraction of blank sweeps increased (Fig. 2*A*), leading to a reduction of the reconstructed peak I_{Na} from 2.11 to 1.55 pA, i.e., NP_o decreased to 73% of the predrug level. The fraction of conducting Na^+ channels retained normal elementary properties including unaffected open-state kinetics (Fig. 2*B*) when compared with the control data in the same patch before drug administration. In a total of seven experiments with 1 μmol/liter propafenone, τ_{open} varied insignificantly to 96 ± 7% of the control. A biased analysis concentrated on activity sweeps with events having a mean open time which is at least threefold larger than τ_{open} of the whole ensemble and confirmed that even extremely long-lasting openings cannot be attacked by propafenone and are not chopped into multiple transitions between the conducting and a nonconducting state (Fig. 3). This is remarkable because $\bar{t}_{open}$ may attain values during an individual activity sweep (between 6.0 and 8.4 msec in the experiment illustrated in Fig. 3) which are close to τ_{open} in DPI-modified Na^+ channels. It becomes also evident from Fig. 3 that the events with the longest dwell time in the open state can occur in the presence of the drug. Another important argument against a flicker blockade arises from the observation that the mean number of openings during the 70-msec lasting membrane depolarization remained mostly unchanged or, as shown in Fig. 3*B*, declined in a few experiments.

To ensure that the absence of flicker blockade is not related to the comparatively low drug concentration, the effect of 10 μmol/liter propafenone was additionally studied in two inside-out patches at −60 mV. Since NP_o responded with an extreme decline to 10% of the control value, block attenuation as achieved by a 20-mV hyperpolarization of the holding potential was necessary in order to collect a sufficiently large ensemble of single-channel events. The reliability of this experimental protocol resides in the experimentally established fact that class 1 antiarrhythmic agents interfere with open DPI-modified Na^+ channels with a differential voltage dependence: flicker blockade has been shown to be highly insensitive to changes in holding potential [17]. τ_{open} amounted to 1.86 ± 0.01 before and 1.77 ± 0.21 msec after propafenone treatment. Moreover, the mean number of openings per activity sweeps failed to increase.

This persistence of open-state kinetics confirms earlier results with propafenone and amiodarone at 19°C [15] and was also observed with lidocaine. A treatment of the cytoplasmic membrane surface with 5 μmol/liter lidocaine led to a reduction of the reconstructed peak I_{Na} to 72 ± 5% (n = 2) of the control value but left τ_{open} unchanged: it was, at −60 mV,

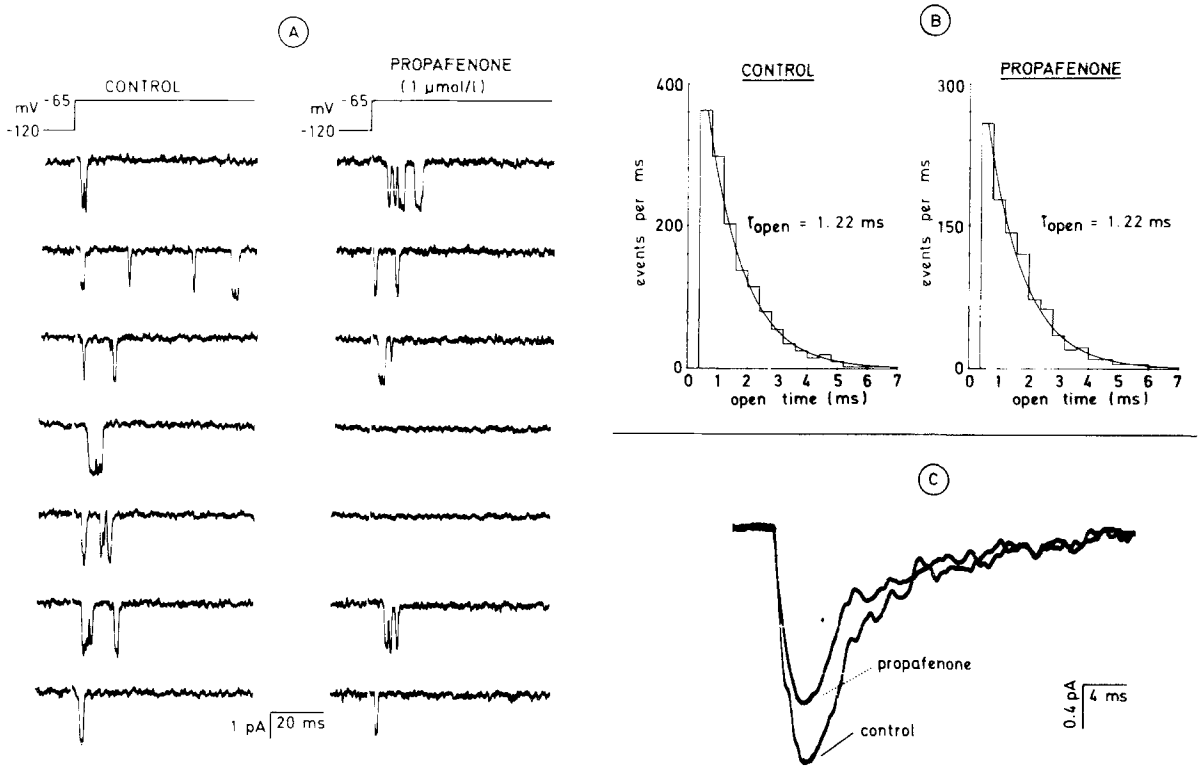


Fig. 2. The response of cooled Na^+ channels exposed cytoplasmatically to a quasi-physiological anionic milieu to propafenone. (*A*) Consecutive recordings of elementary Na^+ currents before (left row) and after (right row) drug treatment. (*B*) Open-time probability density functions based on 644 events under control conditions (left) and on 426 events with propafenone (right). By disregarding the first bin of 0.4 msec, they were best fitted by $N(t) = 624 \exp(-t/0.00122)$ and $N(t) = 429 \exp(-t/0.00122)$, respectively. (*C*) Superimposed, reconstructed macroscopic I_{Na} before and after drug treatment. Each I_{Na} is the ensemble average of 160 consecutive sweeps at the same holding potential (−120 mV). Patch 588I0; test potential −65 mV; 9°C

1.45 ± 0.05 before and 1.50 ± 0.07 msec after lidocaine application. The apparent lidocaine resistance of the open-state kinetics disagrees with the reported response of adult cardiac Na^+ channels in the guinea pig heart at 20°C [27] but is consistent with observations of Grant et al. [7] likewise in adult cardiac Na^+channels at 18°C, but from rabbit myocardium, and largely excludes that lidocaine interferes with the channel during its open configuration. In fact, circumstantial evidence has been presented [7, 13] that lidocaine blocks preferentially inactivated Na^+ channels.

The antiarrhythmic agent quinidine has a considerable affinity for activated Na^+ channels [13] and was, therefore, employed for testing the idea as to whether this drug type would influence the open-state kinetics. The open-time probability density functions shown in Fig. 4 strongly suggest that 5 μmol/liter quinidine is ineffective in this respect although the cytoplasmic drug application exerted an inhibitory effect of NP_o and reduced peak I_{Na} to 71% of the control. Higher quinidine concentrations may shorten the conducting state (Fig. 4*B*) as observed in another inside-out patch with 10 μmol/liter, and likewise at −60 mV. τ_{open} decreased from 2.11 to 1.70 msec, i.e., to 81% of the predrug value. When compared with the response of peak I_{Na}, which declined to 30% of the control, it becomes evident that quinidine exerts a much stronger pronounced action on NP_o.

Other properties such as reopening, including the capability of cardiac Na^+ channels to switch occasionally in a particular activity mode characterized by ultralong bursting episodes [16, 28], have been found to be preserved in the presence of propafenone and lidocaine. Accordingly, drug treatment failed to influence the decay kinetics of reconstructed macroscopic I_{Na}. Even quinidine left the mean number of sequential openings unchanged: at −60 mV, this estimate of reopening slightly increased to 109% of the control with 5 μmol/liter and to 106% of the control with 10 μmol/liter.

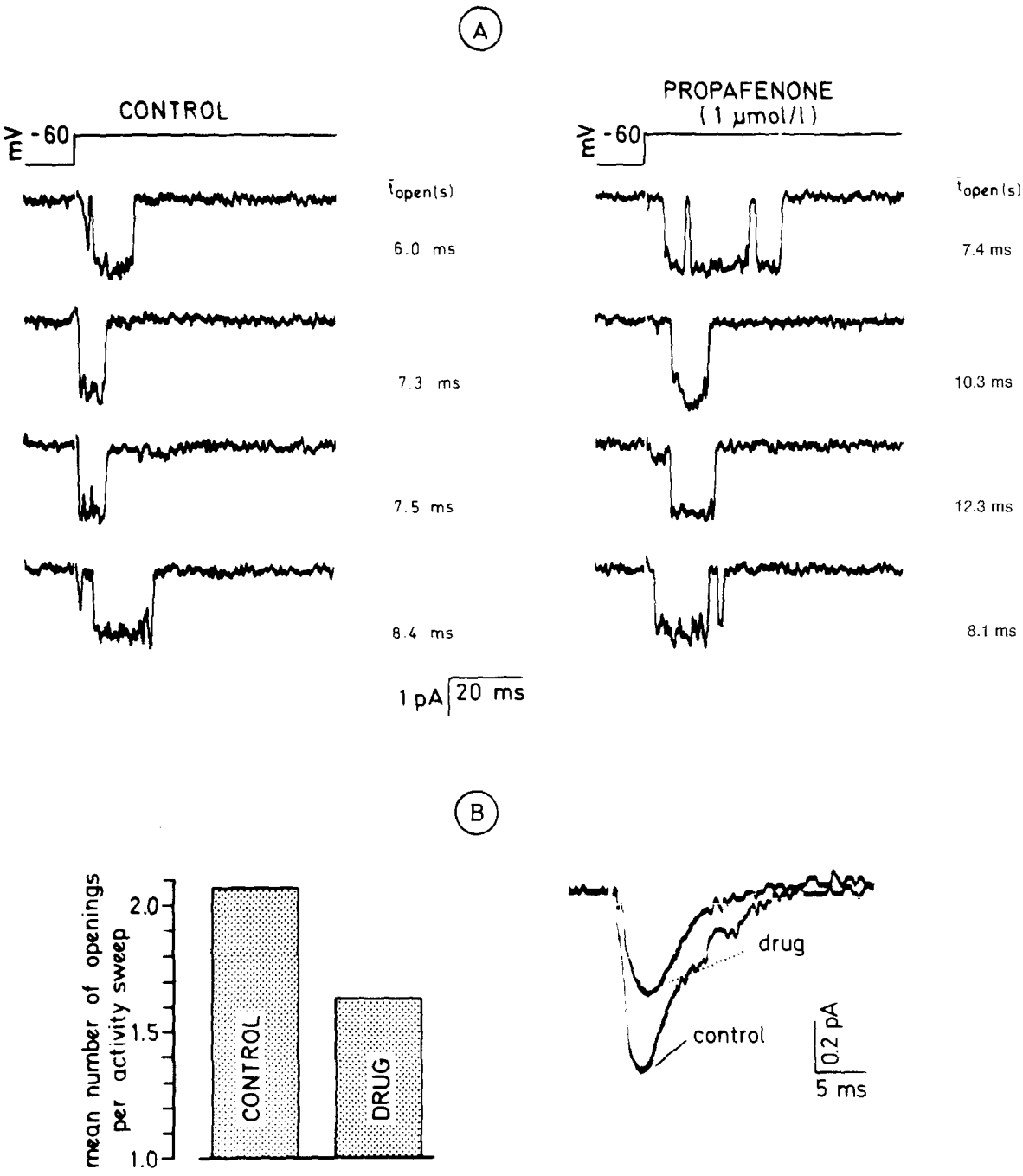


Fig. 3. Propafenone resistance of ultralong Na^+ channel openings. (*A*) Selected activity sweeps showing long-lasting openings; $\bar{t}_{open(s)}$ indicates the mean open time in each sweep. (*B*) Left: Mean number of openings per activity sweep before and after drug treatment. Right: Superimposed reconstructed macroscopic I_{Na} before and after drug treatment. Each I_{Na} is the ensemble average from 160 consecutive sweeps at the same holding potential (120 mV). Patch 59110; test potential −60 mV; 9°C

Exceptional Drug Response of F^--Treated, Cooled Na^+ Channels

It is well established from internally perfused axons that Na^+ inactivation reacts sensitively to the cytoplasmic presence of the artificial anion F^- [23]. Retardation in inactivation as it develops after F^- treatment seems to be accompanied by enhanced burst activity and is particularly pronounced below 19°C. At −60 mV and 9°C, the mean number of sequential openings amounted to 3.50 ± 0.11 (n = 4) and significantly exceeded the value (3.00 ± 0.15; n = 6) in

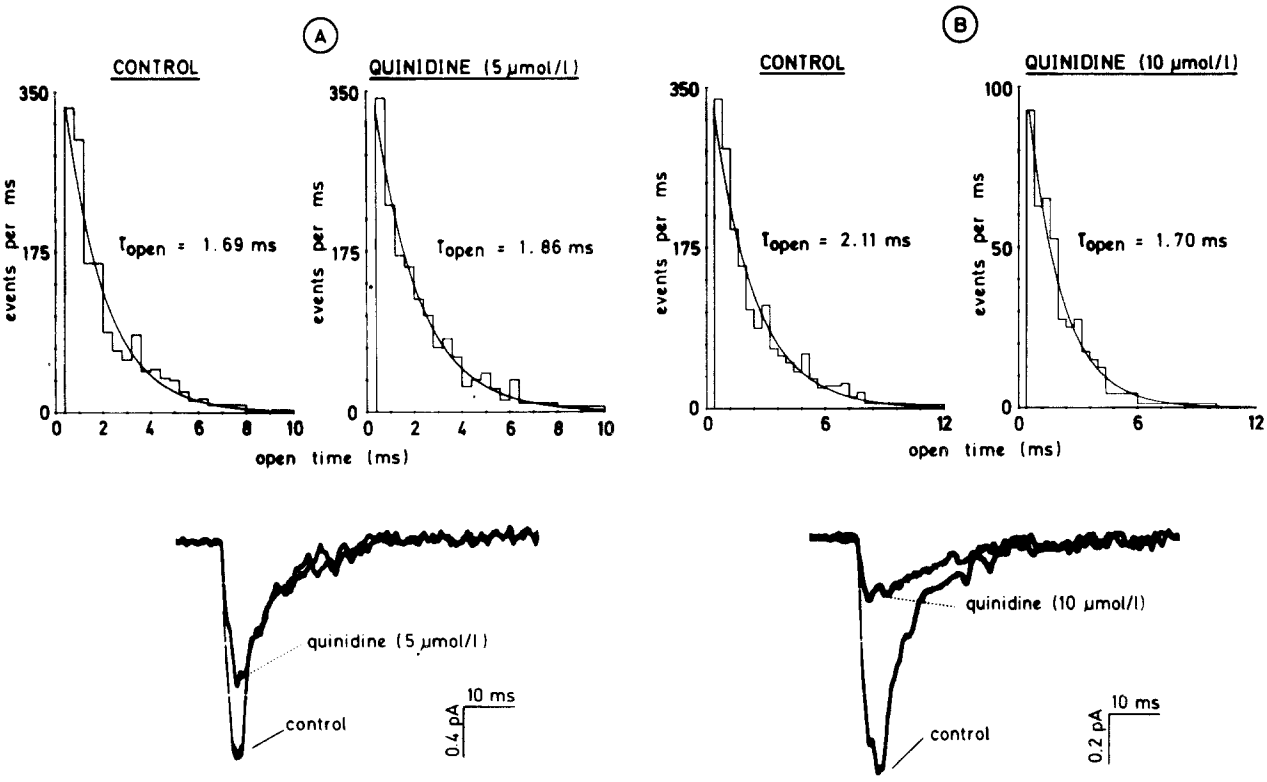


Fig. 4. I_{Na} blocking potency and the effect of quinidine on open-state kinetics. (*A*) Upper part: Open-time probability density functions before and after drug treatment with 5 µmol/liter as based on 689 and 718 events, respectively. By disregarding the first bin of 0.4 msec, they could be best fitted by $N(t) = 435 \exp(-t/0.00169)$ and $N(t) = 408 \exp(-t/0.00186)$, respectively. Lower part: Superimposed, reconstructed macroscopic I_{Na} before and after drug treatment at the same holding potential (−120 mV). (*B*) Open-time probability density functions before and after drug treatment with 10 µmol/liter from 792 and 187 events, respectively. By disregarding the first bin of 0.4 msec, they could be best fitted by $N(t) = 389 \exp(-t/0.00211)$ and $N(t) = 127 \exp(-t/0.00170)$, respectively. Lower part: Superimposed, reconstructed macroscopic I_{Na} before and after drug treatment at the same holding potential (−120 mV). Patches 61110 (*A*) and 61210 (*B*); test potential −60 mV; 9°C

Na^+ channels whose internal surface faced a quasi-physiological anion milieu instead of an isotonic KF solution.

Some gating properties of F^--treated Na^+ channels proved to be sensitive to the presence of antiarrhythmic drugs. As demonstrated in Fig. 5 with 1 µmol/liter propafenone, the drug not only reduced NP_o but also diminished burst activity. At −65 mV, the mean number of sequential openings declined from 3.97 to 3.30 which mirrors in a decrease of τ_{decay} of the reconstructed macroscopic I_{Na} (Fig. 5*C*) from 21 to 9 msec. In a total of seven experiments of this type, the mean number of sequential openings decreased to 83 ± 2% of the control. This fundamentally contrasts with the largely preserved burst activity of Na^+ channels not exposed to F^-: with 1 µmol/liter propafenone, the mean number of sequential openings was 101 ± 7% ($n = 7$) of the control.

The other exceptional response was a loss of the second open state (Fig. 5*B*). In three out of six F^- experiments, the open-time histogram analysis revealed a bimodal event distribution under predrug conditions. As calculated from the area under both exponentials, the ratio of second open-state events to first open-state events varied, at −65 mV, between 1.72 (in the experiment illustrated in Fig. 5) and 2.63 which indicates in a purely formalistic sense the dominance of events governed by $\tau_{open(2)}$. Because these probability density functions were constructed from events collected from multichannel patches, they cannot be interpreted unambiguously as being indicative for a Na^+ channel which has two open states and attains its long-lasting conducting configuration with greater likelihood. $\tau_{open(2)}$ was found to vary between 1.83 and 3.33 msec, thus exceeding $\tau_{open(1)}$ by a factor of 1.58–3.28. To avoid analytical problems, the open-time density functions were based in the presence of propafenone on an event number which was identical with or not smaller than 90% of the control ensemble. With propafenone, a single exponential function best fitted the histograms with values for τ_{open} being close to

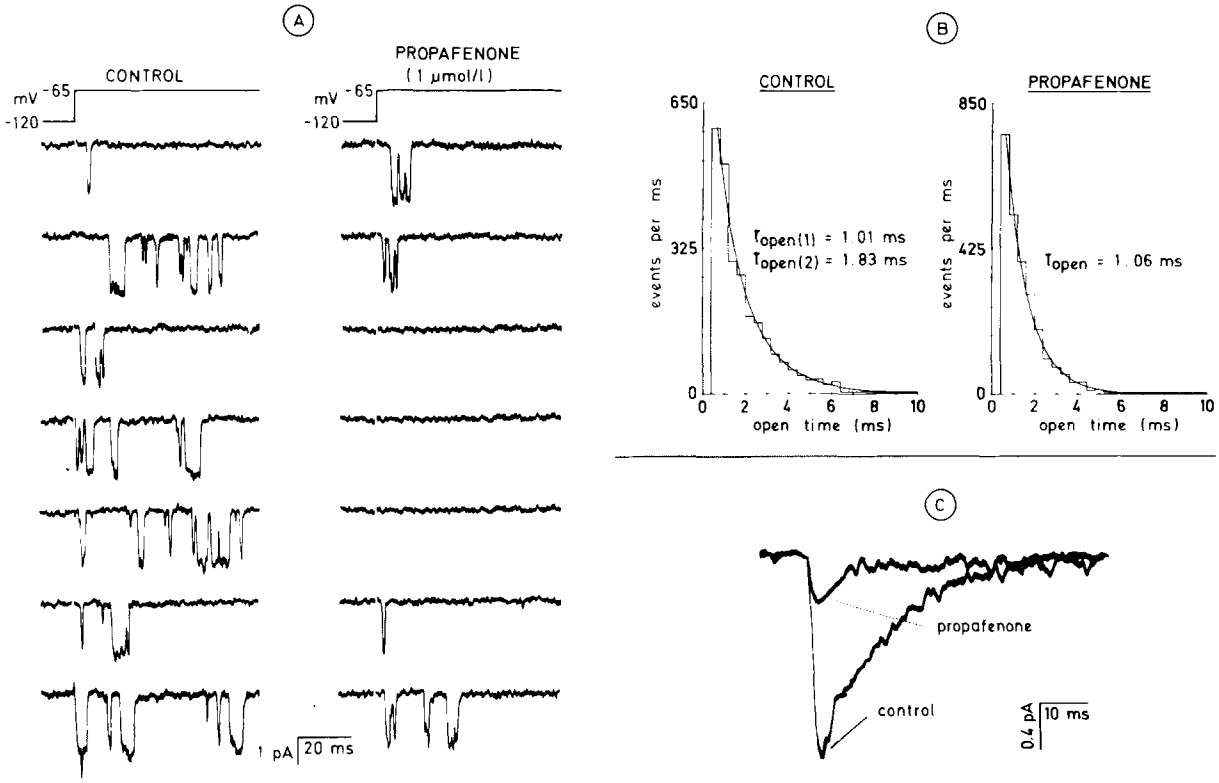


Fig. 5. The response of cooled Na^+ channels exposed cytoplasmatically to F^- ions to propafenone. (*A*) Consecutive recordings of elementary Na^+ currents before (left row) and after drug treatment (right row). (*B*) Open-time probability density functions from 1135 events under control conditions (left) and from 1182 events with propafenone (right). By disregarding the first bin of 0.4 msec, they were best fitted by $N(t) = 530 \exp(-t/0.00101) + 462 \exp(-t/0.00183)$ and $N(t) = 1240 \exp(-t/0.00106)$, respectively. (*C*) Superimposed, reconstructed macroscopic I_{Na} before and after drug treatment. Each I_{Na} is the ensemble average of 160 consecutive sweeps at the same holding potential (−120 mV). Patch 583I0; test potential −65 mV; 9°C

$\tau_{open(1)}$ under control conditions. It was interesting to see that block attenuation would not restore the bimodal event distribution. In the experiment illustrated in Fig. 5, for example, a hyperpolarizing shift of the holding potential from −120 to −140 mV augmented peak I_{Na} from 0.42 to 0.75 pA but the best fit remained monoexponential (*not shown*). τ_{open} corresponded to the value obtained at −120 mV.

Another property of F^--treated Na^+ channels is an enhanced likelihood to open late during membrane depolarization and can be likewise readily explained by assuming a retarded development of inactivation. At −60 mV, for example, the fraction of events occurring 15 msec after the onset of the command impulse and later was found to be 31 ± 4% (n = 6), in contrast to 14 ± 3% (n = 7) in cooled Na^+ channels not exposed to F^-. In reducing the fraction of late events from 31 ± 4% to 21 ± 5%, propafenone virtually abolished this specific property of F^--treated Na^+ channels since a similar value (18 ± 3%; n = 7) was obtained in Na^+ channels not exposed to F^-. This comparison also indicates that drug sensitivity of late openings is specific for F^--treated Na^+ channels. It seems very unlikely that the capability of propafenone to block late openings increases with time during membrane depolarization, since the drug induced an almost parallel shift of the curve relating the fraction of late events to the time after the onset of the command impulse (Fig. 6). Moreover, as analyzed in events occurring later than 15 msec, propafenone reduced $\bar{t}_{open}$ to the same extent as in events within the first 15 msec of step depolarization.

No evidence could be obtained that F^--treated Na^+ channels possess their own sensitivity to the blocking action of antiarrhythmic drugs. As tested with propafenone at an uniform holding potential of −120 mV and the same stimulation rate of 0.67 Hz, peak I_{Na} was reduced with an apparent K_D of 0.9 ± 0.21 × 10^{-6} mol (n = 3) in the presence and of 1.00 ± 0.38 × 10^{-6} mol (n = 6) in the absence of F^- ions. It should be mentioned that the inhibitory propafenone action is strongly voltage and time dependent [14], making this apparent K_D value specifically

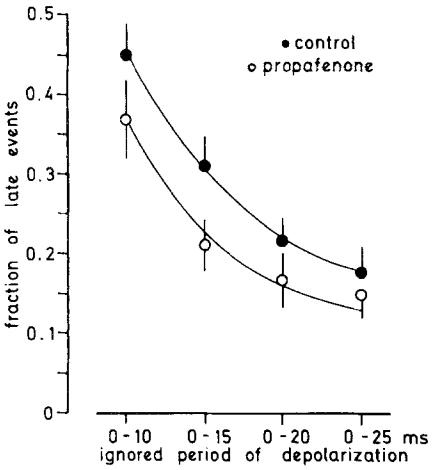


Fig. 6. The influence of propafenone on late Na^+ channel openings. The ignored period refers to the time of the 70-msec lasting membrane depolarization which was neglected in counting opening events. Each symbol indicates the mean of six inside-out patches exposed cytoplasmatically to F^- ions; the curves are drawn by eye. Vertical bars mean SEM. The fraction of late events resulted from the ratio of the number of late openings to the total number of openings during the 70-msec lasting depolarization

related to the actual holding potential and driving rate.

Discussion

The results obtained in the present patch-clamp experiments with isolated neonatal cardiac Na^+ channels can be briefly summarized as follows: (*i*) antiarrhythmic drugs such as propafenone, lidocaine and quinidine, in concentrations effective to reduce NP_o by inducing a conventional time- and voltage-dependent I_{Na} blockade, cannot interfere with open Na^+ channels unless their inactivation is eliminated; (*ii*) F^--treated cardiac Na^+ channels may have sensitive open-state kinetics, despite operating inactivation but respond, nevertheless, not with a flicker blockade as DPI-modified Na^+ channels.

Removal of Inactivation Enables Antiarrhythmic Drugs to Block Open Na^+ Channels Repetitively

Blockade in most ionic channels resides typically in a temporary occlusion of the open pore and is brought about by an interference of a blocking molecule with a site within the aqueous pore which is involved in ionic permeation. Repetitive transitions between the conducting and a nonconducting state, for example, occur in many K^+ channels when internally exposed to Ba^{2+} or Mg^{2+} or in Ca^{2+} channels as characteristic response to the external presence of the inhibitory cations Cd^{2+} or Mg^{2+} [21]. Despite a substantial amino acid sequence similarity between Ca^{2+} channels and voltage-dependent Na^+ channels (for review *see* ref. [3]), which had caused Tanabe et al. [36] to propose an analogous transmembrane structure, external inhibitory influences including protons, Ca^{2+} [32, 39] or Mg^{2+} [5] evoke in voltage-dependent Na^+ channels of excitable membranes a flicker blockade with reaction kinetics being several-fold faster than in other ionic channels. This discrete blockade is too fast to be resolved and becomes, therefore, manifest as decline in unitary current size. It seems important to note that even tetramethrin- [39] and batrachotoxin- [5] modified, noninactivating Na^+ channels react in the same way to external Ca^{2+} and Mg^{2+}. These particular flicker-block kinetics in Na^+ channels may be regarded as being structurally related to the selectivity filter [10] modeled to act as a barrier near the external entrance, which largely facilitates Na^+ permeation, thereby excluding cations other than Na^+ from the passage.

Well-resolved flicker blockade with reaction kinetics similar to those seen with inhibitory cations in K^+ and Ca^{2+} channels may be also evoked by a number of organic compounds having a much larger molecular size when compared with inhibitory cations. Examples are local anesthetics first demonstrated by Neher and Steinbach [26] in acetylcholine receptor channels to be effective in this respect or the class of the Ca^{2+} inhibitory phenylalkylamines. Apart from a depression in NP_o, verapamil interferes also with cardiac L-type Ca^{2+} channels during the open state in order to block them repetitively [22]. Again, voltage-dependent Na^+ channels behave exceptionally since flicker blockage by local anesthetics and the related antiarrhythmic agents essentially requires removal of inactivation. As shown in the present experiments with ultralong openings in channels with operating inactivation exceeding the mean open time several-fold, no flicker blockade occurred after propafenone application. Openings of comparable duration in DPI-modified Na^+ channels, however, show multiple transitions between a conducting and a nonconducting state in presence of the same drug concentration. It is, therefore, tempting to conclude that, in a purely formalistic sense, the inactivation process prevents a drug interference with the open-channel configuration. In fact, noninactivating Na^+ channels consistently become flicker-blocked by local anesthetics and related compounds regardless of the tool experimentally applied to modify them, (−)-DPI, batrachotoxin [24] or, as shown in neuroblastoma cells after N-bromoacetamide treatment [38], covalently binding protein re-

agents. Evidence in support of the hypothesis that local anesthetics penetrate in the Na^+ pore comes from the voltage dependence of the flicker-block reaction kinetics proven for propafenone [17] and several local anesthetics including cocaine, bupivacaine and the lidocaine derivative QX-314 [37]. The proposed existence of a binding site in the interior of the Na^+ pore is also stressed by the modulating influence of the external Na^+ concentration on the flicker-block reaction kinetics [17, 37].

According to the model of Hille [11], the local anesthetic binding site is located distal to the selectivity filter within the Na^+ pore. Assuming a filter aperture of a few Angström, the larger drugs must use a lipophilic lateral route or a hydrophilic pathway from the cytoplasmic channel surface in order to bypass this narrow part and to gain access to the receptor. Alpert et al. [1] showed in voltage-clamped cardiac Purkinje cells that, in contrast to noncardiac tissues, the permanently charged QX-314 effectively inhibits I_{Na} upon external application. A second drug binding site must be, therefore, assumed in cardiac Na^+ channels. The absolute drug resistance of the open state in cooled, slowly inactivating Na^+ channels seen with propafenone, lidocaine and quinidine in the present study further stresses the concept of an all-or-none blockade [15] and also fulfills the postulate that drug dissociation from the binding site restores instantly normal conducting properties. Propafenone and quinidine, for example, dissociate, at 35°C, with a rate close to 0.2 sec^{-1} [20] as determined from the relaxation of use-dependent I_{Na} blockade in ventricular myocardium. This is important since several-fold faster dissociation kinetics can be obtained by calculating the rate from flicker-blocked DPI-modified Na^+ channels: it was close to 700 sec^{-1} at 9°C. Because the latter rate constant is in the same order to magnitude than the dissociation rate constant in 8-bromoacetamide-modified Na^+ channels flicker-blocked by 9-aminoacridine [38], this discrepancy cannot be related to the (−)—DPI treatment. To assume that modifying agents, in interfering with the Na^+ channels, may secondarily influence drug dissociation kinetics would imply that recovery from use-dependent I_{Na} blockade simultaneously proceeds faster, a postulate not fulfilled in our own unpublished DPI experiments.

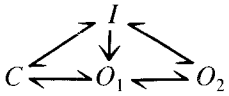
Even a fast block recovery process whose existence was recently identified with quinidine in normal cardiac Na^+ channels [34] proceeds, at 17°C and −160 mV, with a rate not larger than 10 sec^{-1} [34], far below the drug dissociation in DPI-modified Na^+ channels at 9°C and −45 mV. The true difference is still larger since the drugs dissociate in both normal and modified Na^+ channels in a voltage-dependent fashion in that the rate constant decreases upon shifting the membrane potential in the positive direction. Another peculiarity of DPI-modified Na^+ channels is that they become inevitably flicker-blocked once the open configuration has been attained [17]. In fact, this strongly resembles the response of other ionic channels to inhibitory cations and may be analogously explained by a competition with Na^+ for a site needed for Na^+ permeation.

In an attempt to interpret both the conventional I_{Na} blockade and the flicker blockade in noninactivating Na^+ channels as being caused by a drug interaction with one and the same binding site, the most important difficulty arises from the heterogeneous drug dissociation kinetics. Although the potency of propafenone to reduce NP_o was found to be approximately 10 times larger than its potency to flicker-block DPI-modified Na^+ channels, this virtual difference in K_D can, for methodological reasons, not *a priori* be taken as an additional argument in favor of a separate binding mechanism specifically involved in flicker blockade. Radioligand assays with 3H-batrachotoxinin A 20α-benzoate in myocytes [33] confirmed results in neuronal tissues [29] by demonstrating the capability of antiarrhythmic drugs to inhibit BTX binding, but provide no relevant information concerning the number and nature of binding sites for those drugs. The question how the hypothetically proposed second binding site may be protected against drug occupancy in normal Na^+ channels with intact inactivation is crucial indeed, but remains open until its existence has been clearly established.

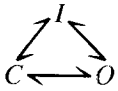
Sensitivity of Long-Lasting Na^+ Channel Openings—An Exceptional Propafenone Action

The present propafenone experiments demonstrate for the first time that, under special environmental conditions, antiarrhythmic drugs can selectively abolish long-lasting openings in Na^+ channels with operating inactivation. Although cardiac Na^+ channels were consistently reported to possess a single open state, a bimodal open-time distribution may be occasionally observed under particular experimental conditions as, for example, after lowering the temperature from 19 to 9°C [14] or in the cytoplasmic presence of F^- ions. In the latter case, long-lasting openings even dominate. In view of their well-established ability to react with a variety of cellular polypeptides and proteins including phosphatases and several G-proteins [2], F^- ions might directly attack a certain domain of the channel protein which could be followed by altered kinetic properties. Because multichannel patches had to be used for analysis,

the resultant biexponentially distributed open-time histograms are ambiguous in nature. They are either indicative for a two-open-state channel according to the Markovian reaction scheme



or reflect alternatively two channel populations each of them modeled by the scheme



but characterized by their own, differential exit rate from *O*. *C*, *O* and *I* mean closed, open and inactivated configuration in the conventional sense where *C* combines, for convenience, multiple rested states.

To explain the distribution change in the open-time histograms from a biexponential to a monoexponential mode seen in the F^--treated inside-out patches after drug application, propafenone either could prevent a two-open-state Na^+ channel to attain O_2 or might block the population with a prolonged *O* state. Since hyperpolarization and the accompanying block attenuation could not restore the initial bimodal distribution mode, this latter possibility seems less likely.

The O_1–O_2 scheme and, consequently, the former interpretation gains support from earlier observations with F^- showing that two open states could only be discriminated in an individual patch with multiple Na^+ channels when the temperature had been lowered from 19 to 9°C (M. Kohlhardt, *unpublished*). It seems, therefore, more attractive to propose that propafenone abolishes O_2. This hypothesis has two interesting implications. First, Na^+ channels pharmacologically hindered to attain O_2 remain basically unblocked and normally conduct during O_1. Second, they are suspected to possess still another "vulnerable site" not identical with the drug receptor that mediates the conventional blocking effect on I_{Na}.

It should be emphasized that this novel propafenone action is rather specifically related to F^--treated, cooled Na^+ channels with modulated open-state kinetics, but remains undetectable under more physiological conditions. This will barely lessen the theoretical importance of this kinetic response since the novel propafenone action clearly establishes that, in the presence of antiarrhythmic drugs, the functional correlate not only consists in a blockade of Na^+ permeation.

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