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Chemically Modified Cardiac Na^+ Channels and Their Sensitivity to Antiarrhythmics: Is There a Hidden Drug Receptor?

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Abstract. Elementary Na^+ currents were recorded at 19°C in inside-out patches from cultured neonatal rat cardiocytes. In analyzing the sensitivity of chemically modified Na^+ channels to several class 1 antiarrhythmic drugs, the hypothesis was tested that removal of Na^+ inactivation may be accompanied by a distinct responsiveness to these drugs, open channel blockade.

Iodate-modified and trypsin-modified cardiac Na^+ channels are noninactivating but strikingly differ from each other by their open state kinetics, a O_1–O_2 reaction ($\tau_{open(1)}$ 1.4 ± 0.3 msec; $\tau_{open(2)}$ 5.4 ± 1.1 msec; at −40 mV) in the former and a single open state (τ_{open} 3.0 ± 0.5 msec; at −40 mV) in the latter. Lidocaine (150 μmol/liter) like propafenone (10 μmol/liter), diprafenone (10 μmol/liter) and quinidine (20 μmol/liter) in cytoplasmic concentrations effective to depress NP_o significantly can interact with both types of noninactivating Na^+ channels to reduce the dwell time in the conducting configuration. Iodate-modified Na^+ channels became drug sensitive during the O_2 state. At −40 mV, for example, lidocaine reduced $\tau_{open(2)}$ to 62 ± 5% of the control without detectable changes in $\tau_{open(1)}$. No evidence could be obtained that these inhibitory molecules would flicker-block the open Na^+ pore. Drug-induced shortening of the open state, thus, is indicative for a distinct mode of drug action, namely interference with the gating process. Lidocaine proved less effective to reduce $\tau_{open(2)}$ when compared with the action of diprafenone. Both drugs apparently interacted with individual association rate constants, $a_{lidocaine}$ was 0.64×10^6 mol^{-1} sec^{-1} and $a_{diprafenone}$ 13.6×10^6 mol^{-1} sec^{-1}. Trypsin-modified Na^+ channels also appear capable of discriminating among these antiarrhythmics, the ratio $a_{diprafenone}/a_{lidocaine}$ even exceeded the value in iodate-modified Na^+ channels. Obviously, this antiarrhythmic drug interaction with chemically modified Na^+ channels is receptor mediated: drug occupation of such a hypothetical hidden receptor that is not available in normal Na^+ channels may facilitate the exit from the open state.

Key words: Single noninactivating Na^+ channels — Iodate — Trypsin — (−)-DPI 201-106 — Drug-sensitive open state — Channel-associated binding sites

Introduction

Blockade of voltage-gated Na^+ channels by local anesthetics and related drugs represents an important principle to depress action potential generation in excitable tissues including myocardium. I_{Na} blockade by these drugs attracted considerable interest during the last two decades mainly for two reasons: to elucidate Na^+ channel properties in greater detail and to characterize the nature of the channel-associated binding sites. The puzzling I_{Na} block phenomenology, as arising mainly from the voltage- and use-dependent drug action, can be modeled by a modulated channel-associated receptor with a state-dependent drug affinity (Hille, 1977; Hondeghem & Katzung, 1977) or, alternatively, by a guarded receptor proposed to be accessible for local anesthetics only during the open channel configuration (Starmer, Grant & Strauss, 1984). Both models are based on a single local anesthetic receptor but, in several neuronal tissues, there is functional evidence in favor of multiple targets for those drugs (Khodorov et al., 1976; Mrose & Ritchie, 1978; Huang & Ehrenstein, 1981). Recent expression studies with rat brain Na^+

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channels identified the Na^+ channel α-subunit to accommodate such a binding site (Ragsdale, Schever & Catterall, 1991) but its location at this principal 260 kD subunit, the structural equivalent for Na^+ permeation, selectivity and gating, remains to be established. Drug occupation may hinder Na^+ channels from gating by trapping the channel in a nonconducting configuration, but the underlying molecular event responsible for the P_o depression is unknown.

Apart from this conventional Na^+ channel blockade, still another type of blockade with a distinct biophysical phenomenology can occur in chemically or pharmacologically modified Na^+ channels (Yamamoto & Yeh, 1984; Wang, 1988; Kohlhardt, Fichtner & Fröbe, 1989). Noninactivating Na^+ channels possess, in striking contrast to normal Na^+ channels (Kohlhardt & Fichtner, 1988; Grant et al., 1989; Benz & Kohlhardt, 1991), a highly drug-sensitive open state in that they may respond to local anesthetics and some other but structurally unrelated organic compounds with a well-resolved flicker blockade. Obviously, after removal of Na^+ inactivation, these compounds may compete with the permeant Na^+ for a site involved in Na^+ permeation within the pore. DPI-modified cardiac Na^+ channels were recently shown to discriminate among several antiarrhythmics since lidocaine fails to flicker-block them, in contrast to propafenone and prajmalium (Benz & Kohlhardt, 1992), another supportive argument for a drug-binding site within the Na^+ pore clearly not involved in the common pharmacological action of local anesthetics and antiarrhythmics but of considerable theoretical interest.

Although flicker blockade by inhibitory organic molecules represents a widespread phenomenon in ionic channels, its nature in noninactivating Na^+ channels is only poorly understood. The question seems crucial indeed as to whether Na^+ channel modification demasks or creates this particular drug response, i.e., flicker blockade may be well due to this artificial channel state and not be a genuine event. The present inside-out patch clamp experiments concentrated on this problem by analyzing the response of three types of modified cardiac Na^+ channels to antiarrhythmic drugs including lidocaine, propafenone, diprafenone, quinidine and prajmalium. The modifying agents essentially differed in their mode and sidedness of action: iodate belongs to a group of protein reagents and cleaves like the proteolytic enzyme trypsin chemical bonds while (−)-DPI 201-106 finds a specific, channel-associated binding site (Romey et al., 1987) to modify reversibly cardiac Na^+ channels (Kohlhardt, Fröbe & Herzig, 1986). It will be shown that, despite individual kinetic properties, iodate-modified and trypsin-modified cardiac Na^+ channels share the common feature of a drug-sensitive open state but cannot be flicker-blocked, in contrast to DPI-modified Na^+ channels.

Materials and Methods

Elementary Na^+ currents were recorded in inside-out patches excised from short-time (18–24 hr) cultured neonatal rat heart cardiocytes with an L-M/EPC5 amplifier using the standard patch clamp technique (Hamill et al., 1981). Cultivating and handling of the cardiocytes were essentially the same as already described in detail (Kohlhardt et al., 1989). Briefly, patches were formed in quiescent, K^+-depolarized mostly rod-shaped cardiocytes, equilibrated in the cell-attached mode for 15–20 min, and excised in isotonic K^+ solution containing appropriate amounts of iodate or trypsin for channel modification. Isotonic K^+ solution lacking these compounds faced the internal surface of the inside-out patches in the experiments with (−)-DPI 201-106.

Na^+ channel openings were triggered by 120-msec-lasting rectangular membrane depolarizations from a holding potential (between −110 and −140 mV and depending on the number of active Na^+ channels in an individual patch) to a test potential (−40 or −30 mV, respectively) at a rate of 0.5 Hz. The patch clamp recordings were filtered at 1 kHz, digitized with a sampling rate of 5 kHz, and stored on line on floppy disks. The dead time was 0.2 msec under these recording conditions.

By subtracting leakage and residual capacity currents, idealized records were obtained for analysis. The 50% threshold method (Colquhoun & Sigworth, 1983) was used to analyze open times of and gap times between nonoverlapping events. Probability density functions yielded τ_{open} and, by neglecting the first bin of 0.4 msec, were based on an unweighted fit. The best fit of the probability density functions resulted from the least-squares method. Burst analysis was based on a critical gap time as calculated from the geometric mean of $\tau_{closed(1)}$ and $\tau_{closed(2)}$. Both time constants were obtained from individual closed time density functions.

Iodate (cytoplasmic application of 5 mmol/liter), trypsin (cytoplasmic application of 75–150 μg/ml) and (−)-DPI 201-106 (6 μmol/liter, added to the pipette solution thus applied externally) were used as Na^+ channel modifiers. Modified Na^+ channel activity was considered to happen during membrane depolarization when open probability (P_o) exceeded a level of 10%; a rationale for this bias was given earlier (Kohlhardt et al., 1989). $P_o \geqslant 0.1$ activity sweeps were chosen for the determination of the number of transitions accomplished simply by counting sequential events in sweeps without superpositions before and after drug administration; an increased number of transitions between the conducting and a nonconducting state is well established to indicate flicker blockade.

Ensemble averaging of 60–120 sweeps yielded the reconstructed macroscopic I_{Na}. Since $I = iNP_o$, I refers to the moment during membrane depolarization where NP_o attains its maximum and was taken as an index of the latter to analyze the conventional blocking effect of antiarrhythmics. Whenever possible, the data are expressed as mean ± SEM.

Solutions (Composition in mmol/liter)

(A) Isotonic K^+ solution (used to equilibrate the cardiocytes in a saline environment; facing the cytoplasmic membrane surface after patch excision): K^+ aspartate 120; KCl 20; $MgCl_2$ 5; Na^+ pyruvate 2.5; glucose 20; EGTA 1; HEPES 10; pH 7.4. (B) Pipette solution (facing the external membrane surface): NaCl 200; $MgCl_2$ 5; $CaCl_2$ 1; HEPES 10; pH 7.4.

The temperature (19 ± 0.5°C) was controlled by means of a Peltier element device.

Chemicals and Drugs

Iodate and trypsin were purchased from Sigma Chemical, Munich and dissolved just before use in solution A. Iodate-containing solutions

were protected from light. Propafenone · HCl and diprafenone · HCl were provided by Helopharm KG, Berlin, prajmaliumbitartrate was a gift of Kali-Chemie, Hannover, and lidocaine · HCl and quinidine · SO_4 were purchased from Sigma, Munich. The (−)-enantiomer of DPI 201–106 was kindly provided by Sandoz, Basle. All these drugs were freshly dissolved just before use. DPI was dissolved in dimethylsulfoxid (Sigma, Munich) and added to the pipette solution (solution B) to give a final concentration of 6 μmol/liter.

Results

Kinetic Differences between Trypsin- and Iodate-modified Na^+ Channels

The cytoplasmic presence of trypsin caused modified channel activity (Fig. 1*A*) within 4–7 min: membrane depolarization to −40 mV triggered long-lasting burst-like activity which may persist for several ten milliseconds, sometimes only terminated by back-clamping the membrane to the holding potential. The open time was several-fold prolonged when compared with normal Na^+ channels from control inside-out patches, but trypsin-modified Na^+ channels retain a single open state since open time histograms could be consistently fitted by a single exponential. A careful inspection of more than 4,000 activity sweeps collected in 19 individual patches failed to detect subconductance openings as they were reported to occur in the presence of other modifying agents including DPI (Nilius, Vereecke & Carmeliet, 1989; Schreibmayer et al., 1989) or iodate (Kohlhardt et al., 1989), i_{unit} did not differ from the value in normal Na^+ channels suggesting that their permeation properties remain preserved. However, after trypsin modification, Na^+ channels cannot maintain their initial open probability. A run analysis revealed that $P_o \geqslant 0.1$ activity sweeps clustered predominantly (Fig. 1*A*) in the early stage of trypsination but, later, their incidence gradually declined or abruptly disappeared. Concomitantly, the number of blank sweeps rose.

Cytosolic treatment with proteolytic enzymes may be followed in several excitable tissues including neuroblastoma cells (Gonoi & Hille, 1987) and adult myocardium (Clarkson, 1990) by a significant shift of the G_{Na}–E_m relationship in the hyperpolarizing direction. The final loss of Na^+ channel activity in the present trypsin experiments proved irreversible since an increase in holding potential by 20–40 mV failed to reduce the number of blank sweeps.

Since Na^+ channel activity was restricted to a period of maximal 15 min after trypsin exposure, complete removal of Na^+ inactivation, reported to need 40–60 min in isolated cardiac Na^+ channels (Zilberter & Motin, 1991) and more than 25 min in neuronal Na^+ channels *in situ* (Gonoi & Hille, 1987), could not be

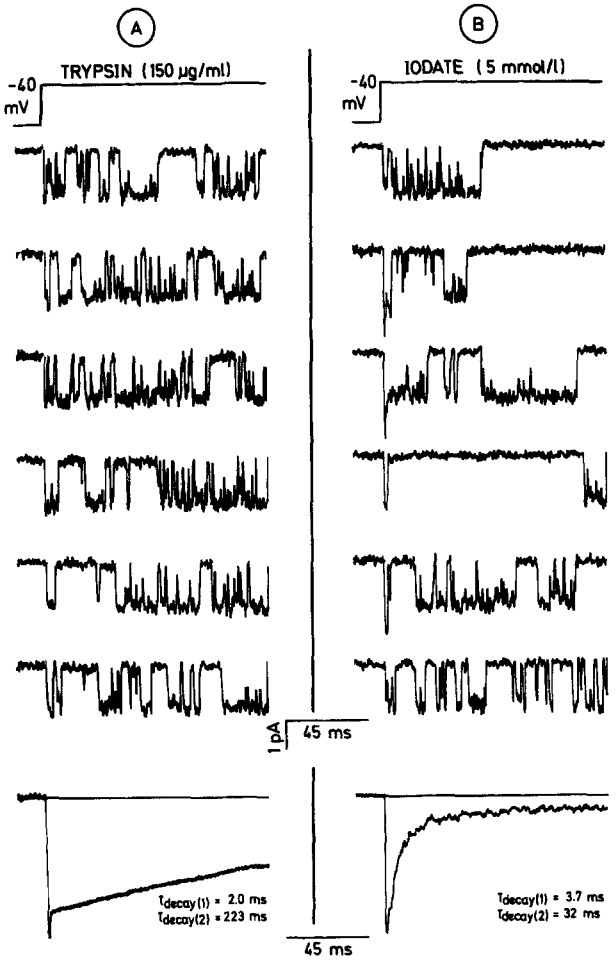

Fig. 1. Upper Panel: Selected recordings of elementary Na^+ currents through trypsin-modified (*A*) and through iodate-modified (*B*) cardiac Na^+ channels. Note the identical size of i_{unit} in both types of noninactivating Na^+ channels. Lower panel: Reconstructed macroscopic Na^+ currents (shown in normalized form), each obtained from an ensemble average of 120 consecutive sweeps; the unbroken line indicates zero current. Patches 753I0 (trypsin) and 735I0 (iodate); holding potential −120 mV, test potential −40 mV.

achieved. This becomes evident from the I_{Na} decay (Fig. 1*A*, lower panel) showing second-order kinetics: an initial fast decay component proceeded with time constants close to 2 msec, the value of τ_{decay} in I_{Na} at −40 mV obtained in control inside-out patches not treated with trypsin. The subsequent slow I_{Na} decay component was governed by a time constant of 176 ± 16 msec (at −40 mV; $n = 5$). Basically, the same results were obtained at a reduced (75 instead of 150 μM/ml) enzyme concentration.

In six other inside-out patches, no signs of modified Na^+ channel activity could be detected after cytosolic trypsin treatment. Instead, the number of blank sweeps increased dramatically within the first 60–90 sec, a loss in activity which could not be neutralized by hyperpo-

Table. Comparison of open state kinetics, open state probability during membrane depolarization and the sustained I_{Na} fraction (at the end of the 120-msec lasting membrane depolarization) in iodate-modified and trypsin-modified Na^+ channels

	Iodate-modified Na^+ channels	Trypsin-modified Na^+ channels
$\tau_{open(1)}$	1.4 ± 0.3 msec (n = 7)	
$\tau_{open(2)}$	5.4 ± 1.1 msec (n = 7)	
τ_{open}		3.0 ± 0.5 msec (n = 5)
Mean number of openings in $P_o \geqslant 0.1$ activity sweeps	5.9 ± 0.9 (n = 13)	16.2 ± 0.9 (n = 5)
Mean P_o per $P_o \geqslant 0.1$ activity sweep	21.9 ± 2.3% (n = 11)	34.5 ± 3.3% (n = 5)
$\bar{t}_{burst}$	8.9 ± 0.9 msec (n = 9)	9.3 ± 1.3 msec (n = 5)
$I_{Na\,(sust)}/I_{Na\,(peak)}$	0.07 ± 0.01 (n = 7)	0.36 ± 0.05 (n = 5)

The number in parentheses refers to the number of inside-out patches. Membrane potential −40 mV.

larizing the holding potential, thus suggesting that the Na^+ channels lost the capability to attain a conducting configuration.

The modifying effect of iodate (Fig. 1*B*) took usually 5–10 min to occur but was not followed by any signs of channel deactivation. Figure 1 demonstrates another outstanding difference between both types of modified Na^+ channels which becomes apparent by comparing the reconstructed macroscopic I_{Na}'s: the slowly decaying I_{Na} component proceeds much faster in iodate-modified Na^+ channels with a mean τ_{decay} of 22.0 ± 6.4 msec (at −40 mV; n = 7). Obviously, their deactivation process (Kohlhardt et al., 1989) operates with an about eightfold larger rate during membrane depolarization than in trypsin-modified Na^+ channels under comparable conditions. This enables the latter to switch more frequently between the conducting and a nonconducting state and to attain a higher P_o (*see* Table).

Another distinguishing criterion is the open state kinetics. Consistent with earlier results (Kohlhardt et al., 1989), open time analysis in iodate-modified Na^+ channels consistently revealed a bimodal event distribution, in contrast to trypsin-modified Na^+ channels.

The Interaction of Antiarrhythmics with Modified Na^+ Channels

A first series of experiments dealt with the effectiveness of antiarrhythmics to depress NP_o in iodate-modified Na^+ channels. The cytoplasmic treatment with lidocaine (150 μmol/liter) led within a few seconds to a rise in the number of blanks, at the expense of the number of activity sweeps if the inside-out patches were repetitively depolarized at an appropriate rate (0.5 Hz). Thus, the reconstructed macroscopic I_{Na} declined, in the experiment shown in Fig. 2 from 2.4 to 1.2 pA. A similar reduction of I_{Na} to 50% of the control was obtained in three other lidocaine experiments at the same concentration accompanied by changes in I_{Na} decay. The slow I_{Na} decay component sometimes proved highly drug sensitive so that $\tau_{decay(2)}$ may decrease significantly, from 8.4 to 4.3 msec (at −40 mV) in the experiment illustrated in Fig. 2. Propafenone (10 μmol/liter), its derivative diprafenone (10 μmol/liter) or quinidine (20 μmol/liter), likewise applied cytoplasmically, also reduced NP_o reversibly. No attempt was undertaken to distinguish between the tonic and the phasic component of the drug-induced I_{Na} depression amounting (at 0.5 Hz) to 49 ± 4% (n = 4) with propafenone and to 40 ± 5% (n = 4) with quinidine. Bearing in mind the inhibitory potency of these antiarrhythmics (Kohlhardt & Seifert, 1985) under more physiological conditions, most of the NP_o depression obtained in the present experiments should be mainly due to a phasic channel blockade. Recent whole-cell I_{Na} measurements in adult myocytes have demonstrated that intact Na^+ inactivation is actually no requirement for the use-dependent drug action (Koumi et al., 1991), although the question remains as to whether removal of Na^+ inactivation leaves the use-dependent blockade completely unaffected.

Since proteolysis was followed by a loss in Na^+ channel availability, the effect of these antiarrhythmics on NP_o in trypsin-modified Na^+ channels could not be studied with reliable accuracy. Another insurmountable difficulty arose from the drastically reduced lifetime of inside-out patches when exposed to trypsin. Therefore, it cannot be decided whether proteolysis might partially destroy the drug sensitivity as found with several local anesthetics in squid axons (Cahalan, 1978).

A microscopic block analysis revealed that both types of modified Na^+ channels are characterized by a drug-sensitive open state. A first series of experiments dealt with the influence of the above-mentioned antiarrhythmics on iodate-modified Na^+ channels in concentrations effective to depress NP_o. Open time histogram analysis clearly identified the prolonged, second open state to be drug sensitive. In the lidocaine experiment depicted in Fig. 2, for example, $\tau_{open(2)}$ declined from 6.70 to 4.08 msec (at −40 mV), without detectable changes of $\tau_{open(1)}$. Moreover, as calculated from the area under both exponentials of the open time distribution, the ratio second-open state events to first-open

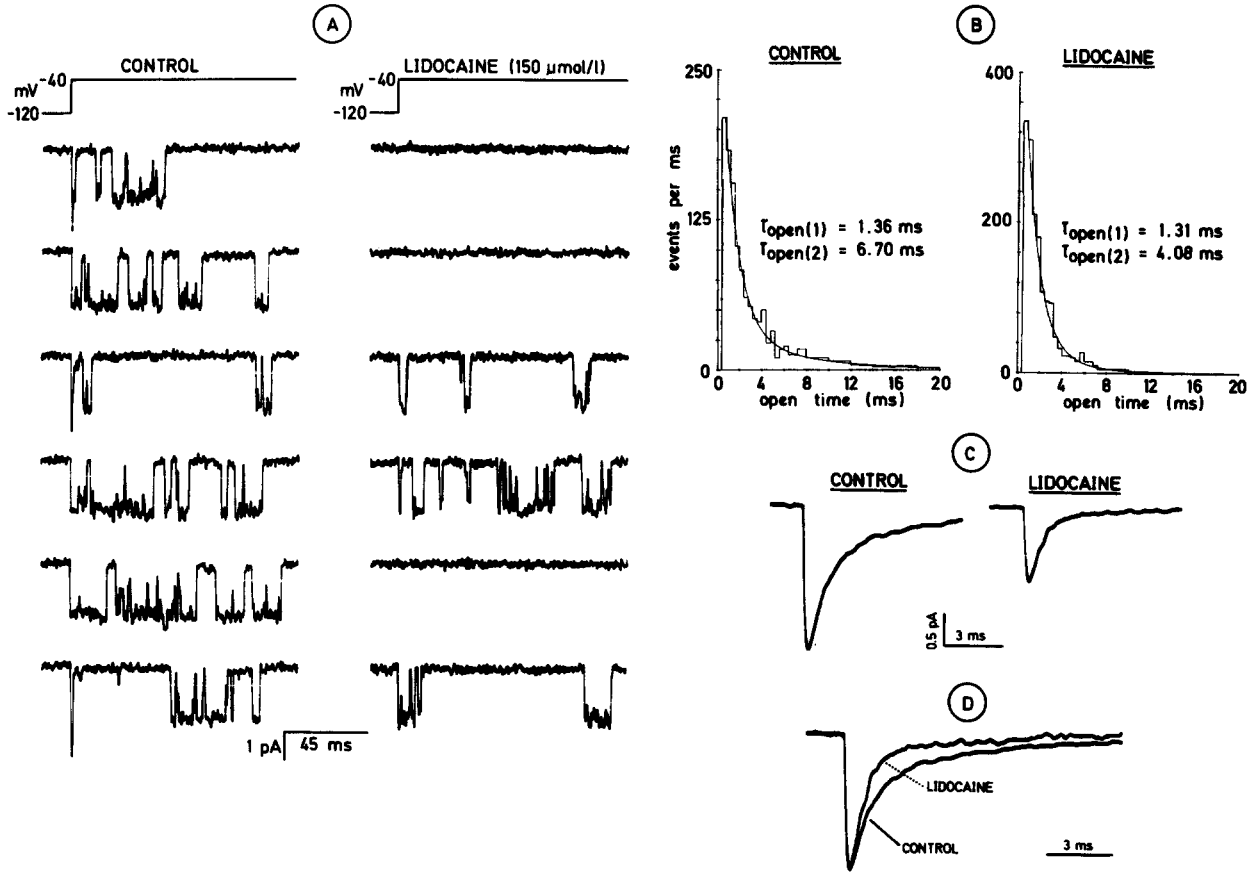

Fig. 2. The influence of lidocaine on iodate-modified cardiac Na^+ channels. (*A*) Consecutive records of elementary Na^+ currents before (left) and after (right) cytosolic drug application. (*B*) Open time probability density functions before (left) and after (right) lidocaine application. By disregarding the first bin of 0.4 msec, the best fits were as follows: control $N(t) = 304\exp(-t/0.00136) + 32\exp(-t/0.0067)$; drug $N(t) = 542\exp(-t/0.00131) + 29\exp(-t/0.0048)$. (*C*) Reconstructed macroscopic Na^+ currents before (left; taken under control conditions during the last 2 min before lidocaine application) and after (right) lidocaine treatment. (*D*) Superimposed, normalized macroscopic I_{Na}'s. Note the accelerated I_{Na} decay under the influence of lidocaine. Patch 728I0; holding potential −120 mV, test potential −40 mV, stimulation rate 0.5 Hz.

state events decreased, in this case from 0.59 to 0.19. Thus, the dominance of the short-lasting open state became accentuated as if lidocaine-associated channels are hindered to attain their second open state. In fact, a few other lidocaine experiments yielded open time histograms which could be best fitted by a single exponential with values for τ_{open} close to the value for $\tau_{open(1)}$ (2.0 ± 0.4 msec; at −40 mV; $n = 4$) under predrug conditions. Experiments with propafenone (10 µmol/liter), diprafenone (10 µmol/liter) and quinidine (20 µmol/liter) confirmed this apparently selective sensitivity of $\tau_{open(2)}$. In a quantitative aspect, however, $\tau_{open(2)}$ responded differentially to these drugs (Fig. 2*A*): a reduction to about 60% of the control value needed (µmol/liter)10 diprafenone, 20 quinidine and as much as 150 lidocaine.

Drug-induced open channel blockade can be modeled, in the simplest case, by the reaction scheme

$$\text{closed} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{open} \xrightarrow[d]{a[D]} \text{blocked}$$

where closed, open and blocked mean channel configurations, a and d correspond to the association and dissociation rate constant, respectively. Considering the reduction of $\tau_{open(2)}$ as a block manifestation, this scheme must be adopted to the more complicated open state kinetics of iodate-modified Na^+ channels by introducing two open states

$$\text{closed} \underset{\delta}{\overset{\gamma}{\rightleftharpoons}} \text{open}_{(1)} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{open}_{(2)} \xrightarrow[d]{a[D]} \text{blocked.}$$

Then, the association rate constant can be calculated from the term $(1/\tau_{open(2)(drug)} - 1/\tau_{open(2)(control)})$ to estimate the affinity of lidocaine, quinidine and diprafenone for open iodate-modified Na^+ channels. The following

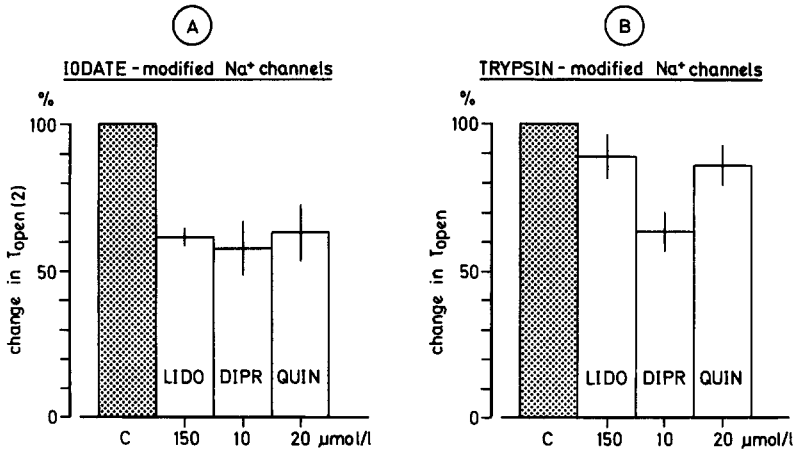

Fig. 3. The drug sensitivity of the open state in iodate-modified (*A*) and trypsin-modified (*B*) cardiac Na^+ channels. The dotted columns refer to the control value (100%) in predrug conditions. The open columns represent the mean changes in $\tau_{open(2)}$ (*A*) and τ_{open} (*B*) obtained with lidocaine (*LIDO; n* = 3 in *A*, *n* = 4 in *B*), diprafenone (*DIPR; n* = 4 in *A* and *B*), and quinidine (*QUIN; n* = 4 in *A* and *B*) in individual inside-out patches. Vertical bars indicate SEM. Step potential was uniformly −40 mV.

association rate constants were obtained: $a_{lidocaine}$ 0.64 ± 0.07 × 10^6 mol^{-1} sec^{-1} (*n* = 3); $a_{quinidine}$ 5.1 ± 0.09 × 10^6 mol^{-1} sec^{-1} (*n* = 4); $a_{diprafenone}$ 13.6 ± 1.9 × 10^6 mol^{-1} sec^{-1} (*n* = 4). It should be mentioned that the validity of the calculated values for "a" essentially resides on an identical drug concentration in both compartments, the bulk phase (in this case at the cytoplasmic membrane surface and corresponding to the drug concentration in the isotonic K^+ solution) and the vicinity of the channel-associated drug binding site. Another critical point is related to the limitations of the recording conditions and possible errors in determining the channel open time, but the difference between $a_{lidocaine}$ and both other association rate constants seems large enough to be reliable.

Open trypsin-modified Na^+ channels, in another series of experiments with these antiarrhythmics, were found to be basically drug sensitive, too. The experimental protocol was complicated by the reduced lifetime of trypsin-treated patches as mentioned above and compared, therefore, control patches with inside-out patches exposed a priori cytoplasmically to lidocaine (150 µmol/liter), diprafenone (10 µmol/liter) and quinidine (20 µmol/liter). Reliable results can be expected from such an interindividual comparison since trypsin-modified Na^+ channels maintain open state kinetics and, as evidenced from the analysis of $P_o \geqslant 0.1$ activity sweeps, open probability during membrane depolarization, although NP_o declines with time. Interestingly, a significant reduction of τ_{open} to 63 ± 7% (*n* = 4) was only obtained with diprafenone. Figure 3 also shows that quinidine and, particularly, lidocaine was less effective and reduced τ_{open} only slightly. The following association rate constants were calculated: $a_{lidocaine}$ 0.26 ± 0.06 × 10^6 mol^{-1} sec^{-1} (*n* = 4); $a_{quinidine}$ 2.7 ± 0.9 × 10^6 mol^{-1} sec^{-1} (*n* = 4); $a_{diprafenone}$ 19.3 ± 1.5 × 10^6 mol^{-1} sec^{-1} (*n* = 4).

Both types of modified Na^+ channels could not be flicker-blocked by these antiarrhythmics. As demonstrated in Fig. 4 with iodate-modified Na^+ channels, long-lasting openings persisted and were not chopped into multiple transitions between the conducting and a nonconducting state, despite the application of drug concentrations effective to depress NP_o and also to reduce $\tau_{open(2)}$. Iodate-modified and trypsin-modified Na^+ channels can dwell for 20 msec or longer without going to be blocked. Failing flicker blockade was observed with any drug tested, lidocaine (150 µmol/liter), propafenone (10 µmol/liter), diprafenone (10 µmol/liter) and quinidine (20 µmol/liter). Evidence was provided by counting transitions between the conducting and a nonconducting state in $P_o \geqslant 0.1$ activity sweeps since this number varied in the presence of drugs either insignificantly or even declined (Fig. 5*A* and *B*). A validating analysis related the number of transitions in an individual $P_o \geqslant 0.1$ activity sweep to its P_o in order to take into account that these antiarrhythmic drugs can reduce the open state probability during membrane depolarization. For example, diprafenone diminished in trypsin-modified Na^+ channels the mean P_o in $P_o \geqslant 0.1$ activity sweeps from 34 ± 3.3% (*n* = 5) in control patches to 20.3 ± 4.6% (*n* = 4). But the ratio number of transitions to P_o did not increase as it would be expected to occur if a blocking molecule blocks repetitively the open pore.

This contrasts fundamentally to the responsiveness of (−)-DPI-modified Na^+ channels (Fig. 5*C*) to propafenone and the antiarrhythmic prajmalium. A last set of inside-out experiments dealt with the influence of these drugs, likewise after cytosolic administration in individual inside-out patches exposed externally to 6 µmol/liter (−)-DPI 201-106, in order to demonstrate how drastically the number of transitions between the conducting and a nonconducting state may rise, with 10 µmol/liter propafenone, for example, by a factor of 3.5. As tested noncumulatively, the number of transitions is concentration dependent, the predicted result when this parameter reflects the number of blocking events.

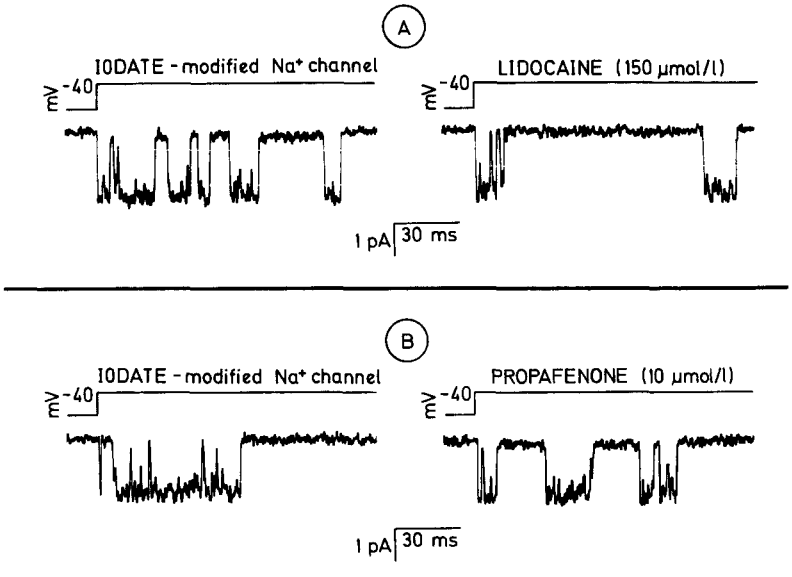

Fig. 4. Selected activity sweeps with long-lasting openings of iodate-modified cardiac Na^+ channels in the presence of lidocaine (*A*) and propafenone (*B*). Holding potential in the experiment depicted in *A*: −130 mV; in *B*: −120 mV; step potential −40 mV in both experiments.

Another observation with iodate-modified and trypsin-modified Na^+ channels is worth mentioning, namely their persisting permeation properties in the presence of these drugs (*see* Fig. 4). Amplitude histogram analysis in $P_o \geqslant 0.1$ activity sweeps confirmed that the unitary current size changed only insignificantly, in a ±5% range after drug treatment and, thus excluded that, in the concentration range tested, lidocaine, propafenone, diprafenone or quinidine might evoke a flicker blockade with kinetics too fast to be resolvable at the present recording conditions with limited bandwidth (dead time 0.2 msec).

Discussion

The present inside-out experiments with single cardiac iodate-modified and trypsin-modified Na^+ channels can be briefly summarized as follows: (i) although sharing the common feature to be noninactivating, chemically modified Na^+ channels may be distinguished by individual kinetic properties, i.e., different open state kinetics and a distinctly effective deactivation process; (ii) after chemical channel modification, antiarrhythmics such as lidocaine, propafenone, diprafenone or quinidine are allowed to exert a dualistic effect, besides the conventional NP_o depression, a shortening of the open state arising from drug interference with Na^+ channel gating.

Removal of Na^+ inactivation by proteolytic enzymes may result primarily from a structural alteration of the cytosolic linker between domains III and IV of the Na^+ channel α-subunit identified in mutagenesis experiments (Stühmer et al., 1989) and with site-directed antibodies (Vassilev, Scheuer & Catterall, 1988) as a molecular substrate of Na^+ inactivation. This conserved region bears a number of positively charged residues known as a good substrate for trypsin. Iodate, the other modifier used in the present experiments, should also find a cytoplasmically located target. Since this chemical reactant reacts preferentially with cysteine to cleave S-S bonds (Gorin & Godwin, 1966), iodate will not find a target in the functionally important linker sequence 1489–1507. However, as recently recognized by point mutations in position 698 and 1585 in skeletal muscle Na^+ channel α-subunits (Cannon & Strittmatter, 1993), other regions than the linker may play likewise a role in gating control and, if accessible for iodate, might be directly or allosterically affected.

Several types of noninactivating Na^+ channels cannot maintain their initially high open probability during membrane depolarization (Quandt, 1987; Kohlhardt et al., 1988, 1989; Nagy, 1988). A surviving slow Na^+ inactivation process is unlikely to be the reason for this time-dependent P_o decline. Slow Na^+ inactivation operates also in heart muscle with kinetics having the same voltage dependence as fast Na^+ inactivation, i.e., $\tau_{h(2)}$, like $\tau_{h(1)}$ declines with membrane depolarization (Brown, Lee & Powell, 1981). $P_o(t)$ in iodate-modified cardiac Na^+ channels (Kohlhardt et al., 1989) or in neuronal Na^+ channels with enzymatically destroyed inactivation (Quandt, 1987), however, shows the opposite voltage dependence, meaning that a depolarizing voltage shift causes a retardation of I_{Na} decay. Iodate-modified Na^+ channels differ from trypsin-modified Na^+ channels by the kinetics of this deactivation process, extremely slow in the latter channels when compared with the former. A C_3–C_2–C_1–O scheme was recently proposed to model deactivation in modified Na^+ channels devoid of inactivation (Beck et al., 1993): once trapped in the stable C_3 state, the channel is deactivated. Con-

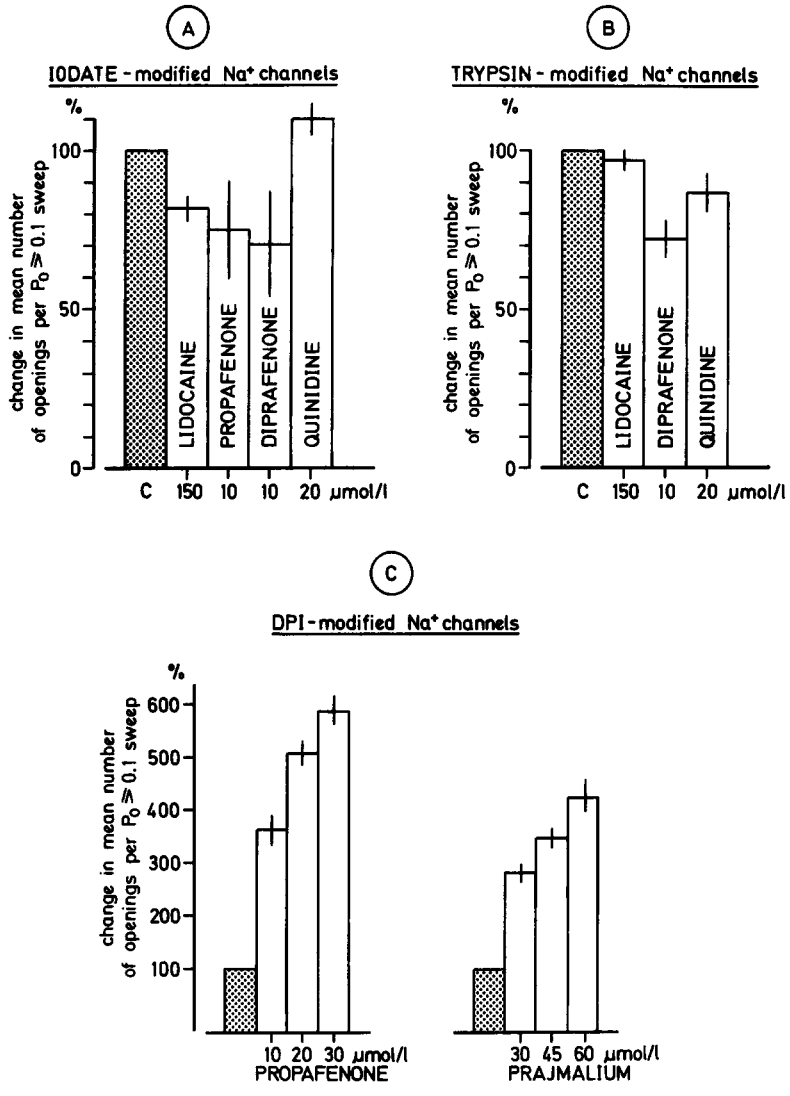

Fig. 5. The influence of antiarrhythmic drugs on the number of openings in iodate-modified (*A*), trypsin-modified (*B*) and DPI-modified (*C*) cardiac Na^+ channels during membrane depolarization. The dotted columns symbolized the control values (100%) in predrug conditions. Each open column represents the mean of 3–4 experiments, vertical bars indicate SEM. The holding potential varied individually between −110 and −130 mV, step potential was −40 mV except in the experiments with DPI-modified Na^+ channels (−30 mV).

sequently, the modifying agents iodate and trypsin might influence the transition from C_2 to C_3 or, alternatively, the exit rate from C_3 individually, a kinetic peculiarity which may be related to the different chemical mode of action of both modifiers. It is important to note that deactivation likewise operates in Na^+ channels modified by site-directed antibodies against the linker between domains III and IV of the α-subunit (Beck et al., 1993). Obviously, deactivation is a genuine property of noninactivating Na^+ channels.

Trypsin as well as iodate may well be supposed to find also a substrate in β-subunits of the Na^+ channel protein. Coexpression of β-subunits with the α-subunit was shown in rat brain Na^+ channels to accelerate Na^+ inactivation and to shift h_∞ in the hyperpolarizing direction (Isom et al., 1991). That a putative structural alteration of the β-subunit can barely explain some of the present observations in trypsin-modified Na^+ channels suggests recent expression experiments with the cardiac Na^+ channels α-subunit (Satin et al., 1992). I_{Na} generated from α-subunits remains stable over a long period of time without any signs of rundown (Satin et al., 1992). The loss of activity in trypsin-modified Na^+ channels, therefore, is most likely the final consequence of an enzymatic destruction of important regions of the α-subunit which are essential for channel function.

According to the model of Hille (1977), local anesthetics and related antiarrhythmics find a channel-associated binding site located distal of the selectivity filter. This proposed receptor location is essentially based on the fact that, in noncardiac excitable membranes, hydrophilic lidocaine analogues fail to depress I_{Na} when applied externally. In isolated cardiac Purkinje cells, however, the permanently charged QX-314 was recently shown to exert a significant inhibitory effect on I_{Na} upon external application (Alpert et al., 1989; Baumgarten, Makielski & Fozzard, 1991). Obviously, cardiac Na^+ channels, unlike their relatives in

other excitable tissues, dispose of a second local anesthetic binding site located with respect to the Hille model proximal to the selectivity filter thus accommodated in the outer channel mouth. Drug occupation of these receptors causes monotonically an NP_o reduction, i.e., open state kinetics remains essentially unaffected unless Na^+ inactivation is eliminated (Benz & Kohlhardt, 1991). Iodate-modified and trypsin-modified Na^+ channels follow basically this rule. Apart from an NP_o depression, they responded to the cytoplasmic presence of lidocaine and the other antiarrhythmics tested with a shortening of the open state. Importantly, as evidenced with lidocaine and diprafenone in micromolar concentrations, these drugs may interfere with individual association reaction kinetics to reduce the dwell time in the conducting configuration. For example, the ratio $a_{diprafenone}/a_{lidocaine}$ amounted in iodate-modified Na^+ channels to 22 and in trypsin-modified Na^+ channels even to 73. This strongly suggests that the channels can discriminate among those inhibitory molecules. Consequently, drug-induced shortening of the open state in these chemically modified Na^+ channels seems to be receptor mediated.

This putative drug target may be called, simply for clarity, the hidden receptor, to take into account that normal open Na^+ channels with operating inactivation are resistant to propafenone, lidocaine, quinidine and related antiarrhythmics (Kohlhardt & Fichtner, 1988; Grant et al., 1989; Benz & Kohlhardt, 1991). Then, chemical channel modification and the resultant removal of the Na^+ inactivation may be the event making the hidden receptor available for drugs, but the underlying molecular mechanisms are unknown. The question would be crucial indeed whether the native α-subunit bears such a hypothetical hidden receptor and, if so, how antiarrhythmics are normally hindered to interact with it. Drug occupancy of the hidden receptor might be suspected to facilitate the exit rate from the open state but a serious problem arises from this hypothesis in iodate-modified Na^+ channels since antiarrhythmic drugs were not only found to reduce $\tau_{open(2)}$ but also acted to reduce the likelihood that the channel attains the O_2 state, i.e., the entrance rate into the O_2 state appears also drug sensitive. Interestingly, in F^--treated cardiac Na^+ channels not devoid of inactivation but likewise following an O_1-O_2 scheme, micromolecular propafenone concentrations had a similar effect and selectively abolished the O_2 state (Benz & Kohlhardt, 1991). To interpret this complicated response in terms of drug-receptor interactions, still another drug target must be postulated to exist.

Noninactivating Na^+ channels from neuronal tissues, skeletal muscle and myocardium can be flicker-blocked by a number of structurally quite unrelated organic molecules including 9-aminoacridine (Yamamoto & Yeh, 1984), cocaine and derivatives (Wang, 1988) or antiarrhythmics (Carmeliet, Nilius & Vereecke, 1989; Kohlhardt et al., 1989; Gruber, Vereecke & Carmeliet, 1991). As inferred from the voltage dependence of the flicker blockade (Yamamoto & Yeh, 1984; Wang, 1988; Kohlhardt et al., 1989) and its modulation by external Na^+ variations (Wang, 1988; Kohlhardt et al., 1989), these flicker-blocking agents find a target in some distance from the inner channel entrance to compete with the permeant Na^+ for a site involved in forming the permeation pathway. Interestingly, association rate constants in the order of magnitude of 10^7 mol^{-1} sec^{-1} have been found with propafenone and prajmalium in DPI-modified cardiac Na^+ channels (Kohlhardt et al., 1989) which correspond to the value calculated by Starmer and Grant (1985) from experimental I_{Na} block data obtained with propafenone in papillary muscles of guinea pigs (Kohlhardt & Seifert, 1983). This coincidence is remarkable with respect to the putative drug receptors, one involved in flicker blockade and the other in the conventional NP_o depression, and suggests their uniform drug affinity, at least for propafenone, although both drug targets most likely represent distinct sites at the α-subunit.

Assuming that flicker blockade and NP_o depression may actually emerge from drug occupancy of distinct receptor sites with similar affinity, it is surprising that chemically modified Na^+ channels remain resistant against flicker blockade when exposed to antiarrhythmic drugs in pharmacologically relevant, i.e., micromolar concentrations effective to reduce their open probability. Most important seems the ineffectiveness of diprafenone to repetitively block the Na^+ pore for reasons related to its reaction kinetics. As mentioned above, diprafenone shortened the open state in iodate-modified and trypsin-modified Na^+ channels with an estimated association rate constant close to 10^7 mol^{-1} sec^{-1}, i.e., with a much higher association rate constant than the other drugs tested, particularly lidocaine. Association rate constants in the range of 10^7 mol^{-1} sec^{-1} determine the interaction of many molecules in blocking repetitively conducting ionic channels including propafenone and prajmalium with DPI-modified Na^+ channels (Kohlhardt et al., 1989). The objection, therefore, finds no rationale that failing flicker blockade seen in the present experiments basically resides in the choice of the inappropriate drug.

Obviously, removal of Na^+ inactivation per se will not basically enable antiarrhythmics to interfere with the Na^+ permeation pathway. To envisage the possible reason for this observed flicker block resistance, it seems tempting to speculate that iodate and trypsin, in destroying several vulnerable regions of the α-subunit, might also structurally alter the inner Na^+ pore mouth where blocking molecules are thought to interact with DPI-modified Na^+ channels, in analogy to other ionic channels. Unfortunately, recent observations with

pronase-modified cardiac Na^+ channels (Gruber et al., 1991) which react sensitively to micromolar concentrations of the local anesthetic penticainide with a flicker blockade are not consistent with this explanation.

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