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Modification of Single Cardiac Na^+ Channels by DPI 201-106

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Summary. In inside-out patches from cultured neonatal rat heart cells, single Na^+ channel currents were analyzed under the influence of the cardiotonic compound DPI 201-106 (DPI), a putative novel channel modifier. In absence of DPI, normal cardiac single Na^+ channels studied at −30 mV have one open state which is rapidly left with a rate constant of 826.5 sec^{-1} at 20°C during sustained depolarization. Reconstructed macroscopic currents relax completely with 7 to 10 msec. The current decay fits a single exponential. A considerable percentage of openings may occur during relaxation of the macroscopic current. In patches treated with 3×10^{-6} M DPI in the pipette solution, stepping to −30 mV results in drastically prolonged and usually repetitive openings. This channel activity mostly persists over the whole depolarization (usually 160 msec in duration) but is abruptly terminated on clamping back the patch to the holding potential. Besides these modified events, apparently normal openings occur. The open time distribution of DPI-treated Na^+ channels is the sum of two exponentials characterized by time constants of 0.85 msec (which is close to the time constant found in the control patches, 1.21 msec) and 12 msec. Moreover, DPI-modified Na^+ channels exhibit a sustained high, time-independent open probability. Similar to normal Na^+ channels, the mean number of open DPI-modified Na^+ channels is voltage-dependent and increases on shifting the holding potential in the hyperpolarizing direction. These kinetic changes suggest an elimination of Na^+ channel inactivation as it may follow from an interaction of DPI with Na^+ channels.

Key Words inside-out patch clamp · kinetic behavior of cardiac Na^+ channels · chemically eliminated inactivation · piperazine indole · isolated heart cells

Introduction

Under physiological conditions, excitability of atrial and ventricular myocardium depends essentially on the availability of Na^+ channels whose ability to form a pathway for Na^+ inward movements underlies the upstroke phase of the fast action potential. Despite their importance for heart muscle function, a more precise insight into some fundamental properties of cardiac Na^+ channels was only obtained during the last few years after novel techniques had been developed allowing I_{Na} measurements with sufficient accuracy (Colatsky & Tsien, 1979; Lee et al., 1979; Ebihara et al., 1980). The most promising results for understanding the molecular substrate of Na^+ permeability, however, can be expected from the application of the improved patch-clamp technique (Hamill et al., 1981) in suitable cardiac cells. As verified in single Na^+ channel recordings (Cachelin et al., 1983*a*; Grant et al., 1983), their kinetic behavior makes cardiac Na^+ channels indistinguishable from Na^+ channels in other excitable cells. This largely rules out the idea that cardiac Na^+ channels might be somehow exceptional as might be argued from the comparatively low TTX affinity (Cohen et al., 1981).

Na^+ inactivation conventionally defined as h-process (Hodgkin & Huxley, 1952) becomes dominant during depolarization. It switches off Na^+ permeability very rapidly by terminating the open state thereby preventing the channel to attain other configurations. Removal of inactivation first demonstrated by Rojas and Armstrong (1971) in giant axons internally perfused with the proteolytic enzyme pronase may be, therefore, a valuable tool in elucidating the channel kinetics. The present patch-clamp experiments concentrated on this point. The diphenylmethyl-piperazine-indole derivative DPI is a novel cardiotonic compound which prolongs cardiac action potentials (Scholtysik et al., 1985) and is therefore suspected to be a Na^+ channel modifier. The susceptibility of cardiac single Na^+ channels was analyzed in order to obtain a closer insight into their kinetic properties. Action potential prolongation by DPI is of particular interest in heart muscle since it represents an antiarrhythmic principle.

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Materials and Methods

Cardiac Cell Culture

Cardiac myocytes have been cultured according to methods described by Mark and Strasser (1966), Blondel et al. (1971), and Werdan et al. (1980). The hearts of newborn, 2- to 4-day-old rats were rapidly excised under sterile conditions, transferred into a phosphate-buffered, nominally Ca^{2+}-free saline, and minced with razor blades. For disaggregation, the ventricle pieces were exposed to a trypsin (0.1% Boehringer Mannheim)-salt solution at 37°C for 50 min. The cell suspension was carefully washed by incubating in CMRL medium and centrifugating at $100 \times g$. After reincubating in this medium supplemented with 10% fetal calf serum (Biochrom, Berlin), cardiac myocytes and fibroblasts were separated from each other by the differential attachment technique (Blondel et al., 1971).

Cardiac myocytes were cultivated (seeding density 5×10^4 cells/cm^2) either immediately following 2 hr preincubation to eliminate the fibroblasts from the cell suspension or after storing them in growth medium at 4°C for 1 to 5 days. Cultured cells were kept in an incubator at 35°C in a water-saturated atmosphere. A considerable fraction firmly attached to the bottom of the culture dish within 5 to 10 hr. Initially, they were completely spherical in shape. The subsequent development to a more or less rod-shaped cell needed 1 to 3 days depending on the storage duration prior to seeding and led to the formation of structural connections between individual cells, finally resulting in multicellular clusters. Blebs, as formed in the surface membrane of damaged adult single cells, represent an extremely rare phenomenon in neonatal heart cells while the percentage of myocytes showing granules in their cytoplasm may vary and usually increases on extending the storage at 4°C.

Spherical myocytes proved the most suitable cell type for patch-clamp experiments because this shape greatly favors patch formation by allowing the pipette to be pressed against the cell surface. Additionally, unlike further developed cells, they rarely beat spontaneously.

Solutions (Composition in mmol/liter)

a) Salt solution: NaCl 137; KCl 2.7; KH_2PO_4 1.5; Na_2HPO_4 8.3; glucose 15; pH 7.4.
b) Storage and culture medium: CMRL 1415 ATM, bicarbonate-free (Biochrom, Berlin), supplemented with 10% fetal calf serum (Biochrom, Berlin) and 0.05 mg/ml gentamycin containing an increased (15 mmol/liter) glucose concentration; pH 7.4.

Patch-Clamp Experiments

Single Na^+ channel currents were recorded in the inside-out configuration with an L/M-EPC 5 amplifier (List Electronic, Darmstadt) employing the patch-clamp technique developed by Hamill et al. (1981). Stabile conditions of cell-free patches essentially required high seal resistances of at least 100 GΩ which could be easily achieved in spherical cells with 3 to 10 MΩ (filled with extracellular solution, *see below*) pipettes. Lifetimes of the inside-out patches up to 90 min were obtained.

Culture dishes with 18 to 20 hr cultured cardiocytes were carefully rinsed with Ca^{2+}-poor (0.2 mmol/liter) Tyrode solution in order to remove a smaller fraction of cells not yet attached to the bottom and brought to an inverted microscope whose stage was temperature controlled by a Peltier element device. The bath temperature was kept at 20 ± 1°C in order to improve the time resolution of single Na^+ channel currents. An increased (5 or 10 mmol/liter) Mg^{2+} concentration in the solution facing the external side of the membrane served for saturating negative surface charges. Before dissecting the patch from the cell membrane, the Na^+ ions were replaced by Cs^+ by rinsing the cell culture with an intracellular solution.

Solutions (Composition in mmol/liter) at the Membrane Surfaces of the Inside-Out Patch

a) Bathing solution (intracellular solution): CsCl 130; NaCl 2.5; KCl 5.4; $MgCl_2$ 2.5; glucose 10; EGTA 2; HEPES 10. pH 7.4.
b) Pipette solution (extracellular solution): NaCl 137; $CaCl_2$ 0.2; $MgCl_2$ 10; HEPES 10; pH 7.4.

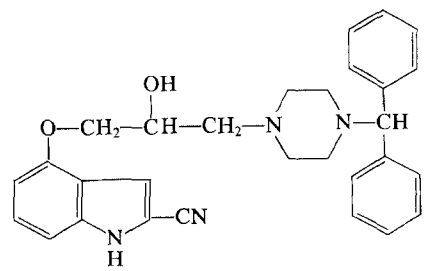
Data Acquisition and Analysis

The single-channel current recordings were filtered at 1 kHz using an eight-pole Bessel filter, digitized by a microcomputer with a sampling rate of 5 kHz and stored on floppy discs. Residual capacity transients not compensated at the level of the headstage were eliminated by averaging records without channel activity and subtracting the average from the records. Moreover, the records were corrected for leakage currents.

Under these recording conditions, channel detection requires a minimal open time of 0.20 msec. Single Na^+ channel currents were analyzed by setting a threshold for a transition, i.e., opening or closing, at 50% of the unitary current amplitude (Colquhoun & Sigworth, 1983). Superpositions of single-channel openings and overlapping events were systematically excluded from kinetic analysis except for determining the waiting time. The latter was defined as latency of first event after the depolarizing step, averaged and given as mean value. The mean open time either refers to the time constant of open time distribution or is given as an average calculated from $\bar{t}_o = \Sigma_i n_i T_i / n$, where T_i is the duration of n_i open channels and n means a count of nonoverlapping openings.

Drug

Freshly dissolved racemic DPI 201-106 was used. This piperazinyl-indole (4[3-(4-diphenyl-methyl-1-piperazinyl)-2-hydroxy-propoxy]-1H-indole-2-carbonitrile) is characterized by a pronounced hydrophobicity. The partition coefficient for octanol/50 mmol/liter phosphate buffer was ≥1800 (U. Quast, *personal communication*). DPI 201-106 was provided by Sandoz Ltd., Basle, Switzerland. The structure formula is as follows:



Results

Normal Single Na^+ Channel Currents

After removal of resting inactivation, voltage-activated single Na^+ channel currents could be observed in 60 to 70% of the inside-out patches. Removal of resting inactivation required a holding potential of at least −105 mV indicating a shift of the voltage dependence of inactivation to more negative potentials. This shift seems to be evoked by the cell-free patch configuration (Cachelin et al., 1983; Nagy et al., 1983) and could result from surface charge changes. Resting inactivation was almost completely abolished at −150 mV. Step depolarizations of 125-msec duration and at a rate of 0.5 Hz were delivered from a conventional stimulator. Depolarizing the inside-out patches beyond a threshold of about −75 mV triggered single Na^+ channel currents (Fig. 1*A*). Their unitary amplitude amounted to -1.4 ± 0.07 pA ($n = 1200$) at −60 mV and gradually decreased with further depolarization thereby exhibiting a linear voltage dependence tested between −60 and −20 mV. A slope conductance (γ) of 13.8 ± 1.1 pS was obtained in six patches. This agrees with the Na^+ channel conductance in neuroblastoma cells (Quandt & Narahashi, 1982; Nagy et al., 1983) likewise determined in excised patches but contrasts with earlier observations in cultured cardiac cells (Cachelin et al., 1983*a*). These authors found a value for γ about twice as large (27.8 pS) when determined in cell-free patches which, however, had been exposed on either side to a symmetrical Na^+ concentration. Whether or not the missing Na^+ gradient across the membrane might be related to this enlarged conductance remains to be elucidated.

As in neuroblastoma cells (Horn et al., 1984), Na^+ channel openings tended to occur in clusters in that records without and with channel activity are grouped. Even in inside-out patches held at −110 mV, more than one functioning channel could be detected. The incidence of simultaneous or overlapping channel openings largely increased with stronger depolarizations while, conversely, the number of sequential openings (illustrated in the right part of Fig. 1*A*, 1st and 3rd record) declined. Sequential openings focus on the important problem as to whether an individual Na^+ channel may open only once during a step depolarization (Aldrich et al., 1983). In this case, up to 15 channels, as a minimum, would be present in our inside-out patches which exceeds the number of channels estimated from simultaneous openings by far. By inspecting a minimum of 150 samples, the latter minimum estimate yielded never more than 3 channels

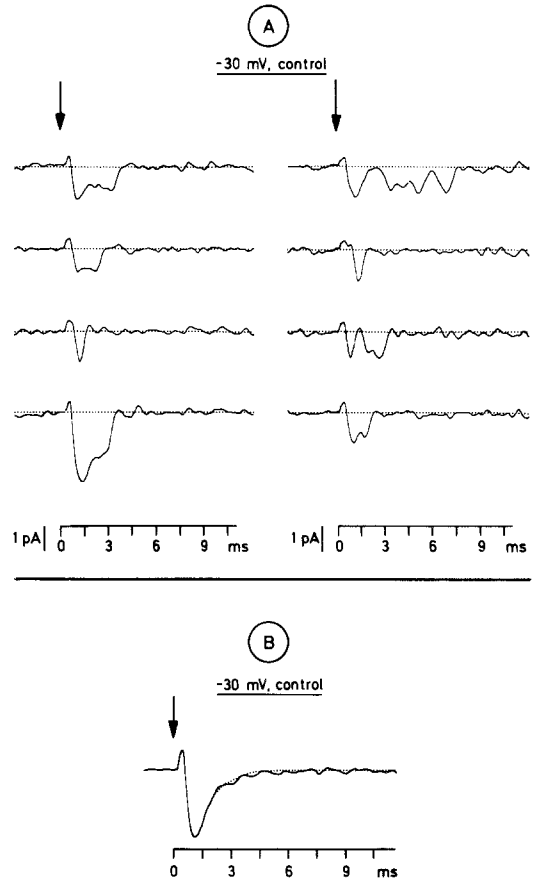


Fig. 1. *A*. Consecutive patch-clamp records of normal single Na^+ channel currents at −30 mV. The arrows indicate the onset of the step depolarization and the dotted lines the zero current. Patch 150, kept at −130 mV and 20°C. Low-pass filtered at 1 kHz. *B*. Average current from 224 runs of the same patch. The arrow indicates the onset of the step depolarization to −30 mV. The dotted line was fitted by eye

even in cases where most of the resting inactivation had been removed by setting the holding potential to −150 mV.

As demonstrated in Fig. 1*B*, a macroscopic current can be reconstructed by averaging single Na^+ channel currents from inside-out patches. It decays almost perfectly with a monoexponential time course, the time constant ranging between 0.75 and 1.45 msec at −30 mV in four experiments (mean: 1.10 ± 0.14 msec). At the same membrane potential and a similar temperature, I_{Na} of adult isolated myocytes was found by Brown et al. (1981) to decay in two distinct phases, an initial fast one with a time constant of 1.4 ± 0.3 msec, followed by a component three times slower. As a tentative explanation for this discrepancy, a second channel population with different kinetics may have remained undetected in the single-channel analysis, possibly be-

Table. Kinetic data of normal cardiac Na^+ channels[a]

	Mean open time	*n*	Mean waiting time	*n*	Membrane potential
Patch 144–155	1.43 msec	1793	1.11 msec	1865	−30 mV

	Reconstructed current τ_{decay} (msec)	*n* (samples)	Mean open time (msec)	*n* (single events)
patch 145	1.45	129	1.88	105
patch 147	0.75	234	1.32	199
patch 149	1.12	88	1.20	65
patch 150	1.08	224	1.33	191

[a] Upper part: Mean open time (average from 1793 single events) and mean waiting time (average from 1865 events) from 12 inside-out patches at −30 mV.
Lower part: Time constants for the decay of the reconstructed current and the corresponding mean open time (average value) in four individual patches at −30 mV.

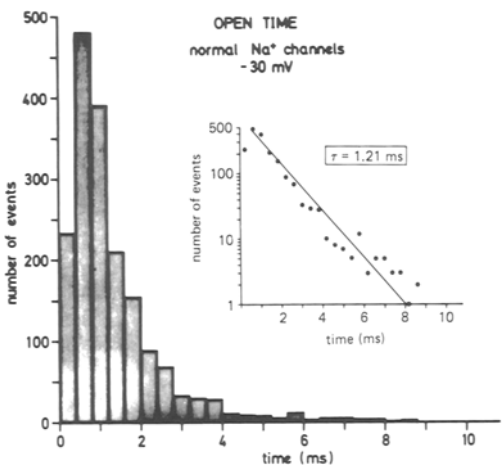


Fig. 2. Open time distribution (1793 events) of normal single Na^+ channels at −30 mV. The inset shows the plot of the histogram in a semilogarithmic scale

cause of a very low density. I_{Na} in chick embryonic heart cell clusters, on the other hand, decays monoexponentially (Ebihara et al., 1980). This gives rise to the speculation that the proposed low density might somehow be related to cultured cardiac cells in an early developmental stage. A considerable percentage of openings, 18.5% in the patch depicted in Fig. 1, happened during the decay phase. Moreover, the events with the longest open time can be detected during the relaxation of the macroscopic current. A close coincidence between mean open time and τ_{decay} of macroscopic currents was obtained (Table).

The reconstructed macroscopic current reflects the time-dependent open probability $P_o(t)$ of single Na^+ channels. At −30 mV, $P_o(t)$ reached the maximum within 1.25 ± 0.08 msec (n = 4). Subsequently, the open probability declined reaching 0.5% of $P_o(t)_{maximal}$ 8.5 ± 0.3 msec after the onset of step depolarization.

The histogram of the open time distribution of 1793 single events grouped in 0.4-msec bins provided evidence that cardiac Na^+ channels have only one open state (Fig. 2). Apart from the first 0.4 msec, the histogram obeys a monoexponential function with a time constant of 1.21 msec, which is consistent with the mean open time found by averaging the open-time durations (Table). This agrees with earlier results in cardiac cells (Grant et al., 1983) and neuroblastoma cells (Horn & Vendenberg, 1984) but a biexponential open-time distribution has also been observed (Nagy et al., 1983). Analytical problems mainly arising from overlapping openings included in the biexponential histogram have been argued to be underlying this discrepancy (Horn & Vandenberg, 1984). By selecting single and not overlapping events, however, the longest openings may be excluded from the analysis since the likelihood of superpositions increases with increasing open time. Therefore, the true mean open time may be slightly underestimated in the present experiments.

Modification of Na^+ Channels by DPI

In a separate set of experiments performed in four inside-out patches, the influence of the piperazinylindole was studied by admixing 3×10^{-6} mol/liter DPI 201-106 to the pipette solution. The experimental protocol included an initial equilibration period of 10 min which proved long enough to establish the full effect. During the subsequent recording phase, the patches were stepped to −30 mV for 150 to 160 msec with a rate of 0.33 Hz and the samples were

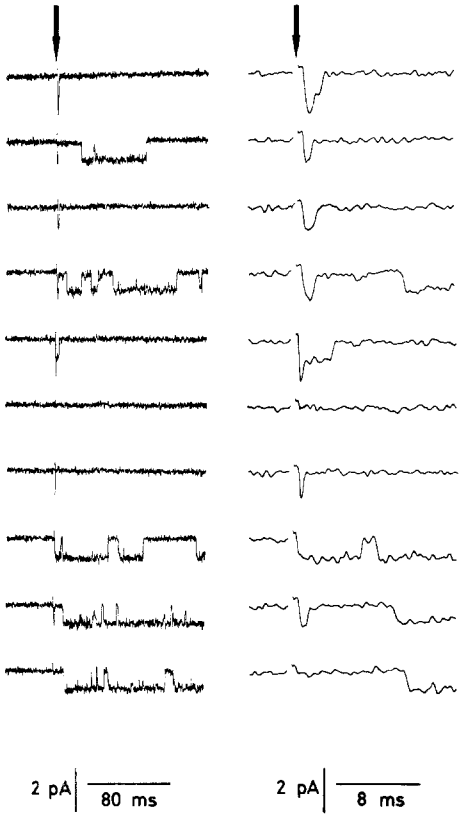


Fig. 3. Consecutive patch-clamp records of single Na^+ channel currents at −30 mV in the presence of DPI. In the left part of this figure, the whole sample is demonstrated. The right part shows in an expanded time scale the first 15 msec of the step depolarization whose onset is indicated by the arrows. Patch 130, kept at −130 mV and 19.5°C. Low-pass filtered at 1 kHz

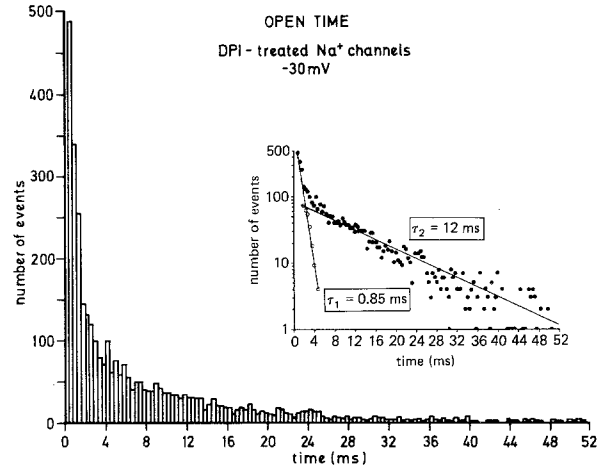


Fig. 4. Open time distribution (3759 events) of DPI-treated single Na^+ channels at −30 mV. In the inset, the histogram is plotted in a semilogarithmic scale

continuously registered. The holding potential was adjusted to the level with the lowest incidence of overlapping channel openings in order to increase the yield of single openings which were exclusively considered in determining open and closed times.

Single Na^+ channel currents were drastically affected by DPI (Fig. 3). A step depolarization may elicit a sequence of repetitive channel openings sometimes with extremely prolonged lifetimes which usually continued to appear during the whole depolarization. As can be seen from the ensemble depicted in Fig. 3, samples with modified events alternated with runs where channel activity was restricted to the first 4 to 5 msec. The latter openings strongly resemble single Na^+ channel currents recorded in the control patches. Moreover, samples could be detected where such an initial, apparently normal single Na^+ channel current was followed after some 10 msec by a train of modified openings covering the whole depolarization.

The proposed increase in lifetime of DPI-modified single Na^+ channels was evidenced by analyzing the open-time distribution (Fig. 4). In contrast to the control patches, the histogram was the sum of two exponentials. The early fast component with a time constant of 0.85 msec was followed by a considerably slower component characterized by a time constant of 12.0 msec. This may indicate either that there are two distinct populations of channels (one with a normal and one with an about 10 times longer lifetime) or that DPI causes certain Na^+ channels to enter a second open state which is left with a 10 times lower rate. Both alternatives permit the conclusion that DPI strongly favors the open state.

In an attempt to find out whether two channel populations might coexist, the samples were classified into two groups depending on the time-dependent open probability. According to the results in the control patches at −30 mV, normal channels were defined as having a $P_o(t)$ of 0.5% of $P_o(t)_{\text{maximal}}$ at 10 msec after step depolarization. Openings beyond this time limit were considered as arising from modified channel activity. After splitting up the samples, a monoexponential open-time distribution with time constants of 0.95 msec for the apparently normal group and of 11.5 msec for the modified records (2750 single events) were obtained.

Moreover, averaging apparently normal and modified single Na^+ channel currents separately yielded in the former case a macroscopic current not distinguishable from the one obtained in untreated patches (Fig. 5*A*). Again, the current reached the peak within 1.25 to 1.30 msec and subsequently relaxed completely in a monoexponential fashion with time constants between 0.8 and 1.2 msec. By contrast, a sustained macroscopic current resulted from the modified events (Fig. 5*B*) which was almost completely lacking a decay phase. This

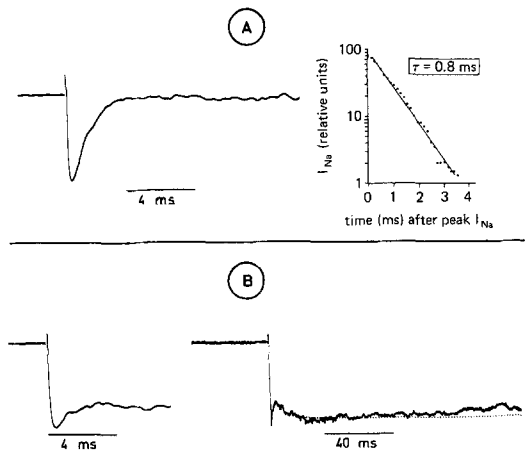


Fig. 5. Reconstructed Na^+ currents from single Na^+ channel currents in a DPI-treated patch (patch 130). *A*. Average current from 285 runs with apparently normal single Na^+ channel currents. The inset shows the semilogarithmic plot of the current decay phase yielding a time constant as indicated. *B*. Average current from 323 runs showing modified single Na^+ channel currents either exclusively or together with apparently normal current events. The whole current deflection in the right part corresponds to the duration of the step depolarization to −30 mV. The dotted line symbolizes time independence

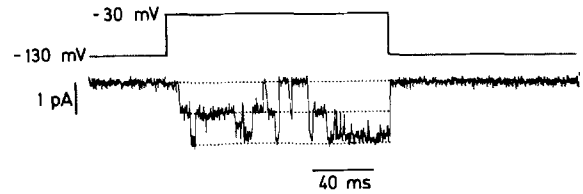


Fig. 6. Absence of spontaneous activity of DPI-modified single Na^+ channels at the holding potential. Each interval between the dotted lines represents the unitary current amplitude, 1 pA at −30 mV. An increased noise level during prolonged openings was occasionally detected and might be pretended by poorly resolved fast transitions (*see* Materials and Methods) between the closed and the open state. Low-pass filtered at 1 kHz. Patch 129, kept at −30 mV and 20°C

indicates a sustained high open probability of DPI-modified Na^+ channels which seems to be time independent during the 160-msec lasting depolarization.

The interpretation of the DPI effect is intimately linked with the number of channels functioning in a patch. As might be judged from the transitions from one state to another in samples with modified events under the premise that Na^+ channels open only once during depolarization (Aldrich et al., 1983), at least five channels were present in the patch demonstrated in Fig. 3. To consider every transition as being indicative for one individual channel is not justified since the number of events depends on the sample duration and strongly increases with a prolongation of the sample from 250 to 350 msec. At −30 mV, the majority of the records was free of superpositions despite the tenfold prolonged dwell time in the open state which strongly increases the likelihood of overlapping events. Simultaneously activated modified Na^+ channels (Fig. 6) were detected in a comparatively small percentage of records. The likelihood (L) that a train of nonoverlapping modified events may arise from sequential openings of at least two channels was statistically judged from

$$L_{(N)} = \left(\frac{1}{1 + \left(\frac{N-1}{N}\right) \cdot \frac{\mathrm{MOT}}{\mathrm{MC}}} \right)^n$$

(Colquhoun & Hawkes, 1983). N means the number of channels (in this case 2), MOT and MC are mean open time and mean closed time, respectively, and n indicates the number of single openings. Taking, for example, the 23 modified openings shown in Fig. 3 (having an average MOT of 10.0 msec and an average MC of 3.0 msec), results in a value for $L_{(N=2)}$ of already as small as 0.38[23]. This permits the conclusion that, with a likelihood of more than 99.99%, the nonoverlapping, sequentially occurring openings are due to repetitive activity of one channel.

DPI-modified Na^+ channels might have lost their ability to inactivate which could enable them to cycle between the open and two closed states (Fig. 7) during depolarization. Nevertheless, their mode of activation remained exclusively voltage-sensitive since openings never occurred without stepping the membrane to potentials above threshold. Even on extending a resting period to 10 min when the patches were continuously kept at the holding potential of −110 mV or more, no spontaneous channel activity could be detected. Moreover, DPI-modified channels promptly responded to clamping back the membrane to the holding potential (Fig. 6). Repolarization abruptly terminated the open state and induced channel closing.

The incidence of samples with modified Na^+ channel activity varied considerably from one patch to another. First of all, this incidence will be related to the ratio of runs to records in an ensemble since a large number of blanks, i.e., records without openings, must be expected to diminish also the number of records with modified channel activity. But even in patches with the same run/record ratio, a quite different contribution of modified samples can be observed (Fig. 8). Unfortunately, the most important postulate in interpreting this difference, a defined DPI concentration near the binding site, is possibly not fulfilled. *In vitro* experiments with tri-

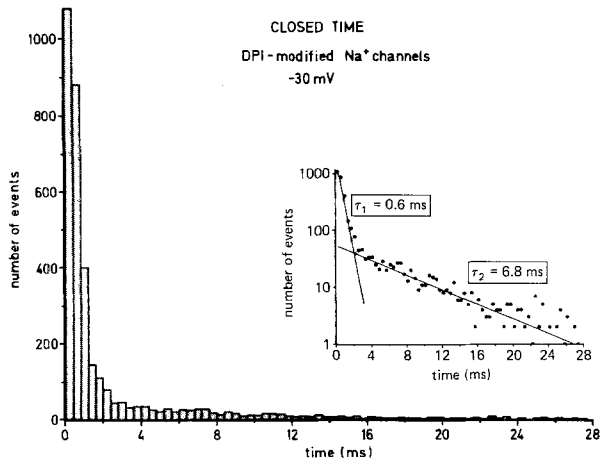


Fig. 7. Histogram of closed time distribution (3429 events) of DPI-modified cardiac Na^+ channels. The inset shows the plot of the histogram in a semilogarithmic scale. The curve is defined by the sum of two exponentials. Patches 129 and 130 at −30 mV

tium-labeled DPI demonstrated a pronounced tendency of the drug to adsorb to glass surfaces (U. Quast, *unpublished*), due to the extremely hydrophobic diphenylmethyl-piperazine group. This may reduce the drug concentration in the bulk phase considerably. Depending on the surface/volume ratio near the mouth as an individual factor, the actual DPI concentration may, therefore, differ from patch to patch. On the other hand, the minimum number of functioning channels estimated from overlapping, apparently normal openings was not identical in both patches depicted in Fig. 8. It amounted to 3 in the patch with the higher incidence of modified samples but to only 2 in the other.

The number of open modified Na^+ channels proved to be voltage-dependent. This has been verified in an inside-out patch where the holding potential was systematically varied during the experiment between −120 and −150 mV. From the peak current I the number of functioning channels in each normal and modified sample was calculated according to $pN = I/i$, where p is the open probability and i means unitary current amplitude. As blanks are included in the analysis, the mean number of samples may be smaller than 1. A certain time-dependent fluctuation can be detected in that the mean number of open modified channels increased more or less periodically up to a maximum subsequently decreasing to a minimum within a few minutes (Fig. 9*A*). This phenomenon became evident on stepping the membrane from holding potentials between −130 and −150 mV. Shifting the holding potential from −120 mV in the hyperpolarizing direction strongly increased the mean number of open modified channels. In the experiment shown in Fig. 8, it

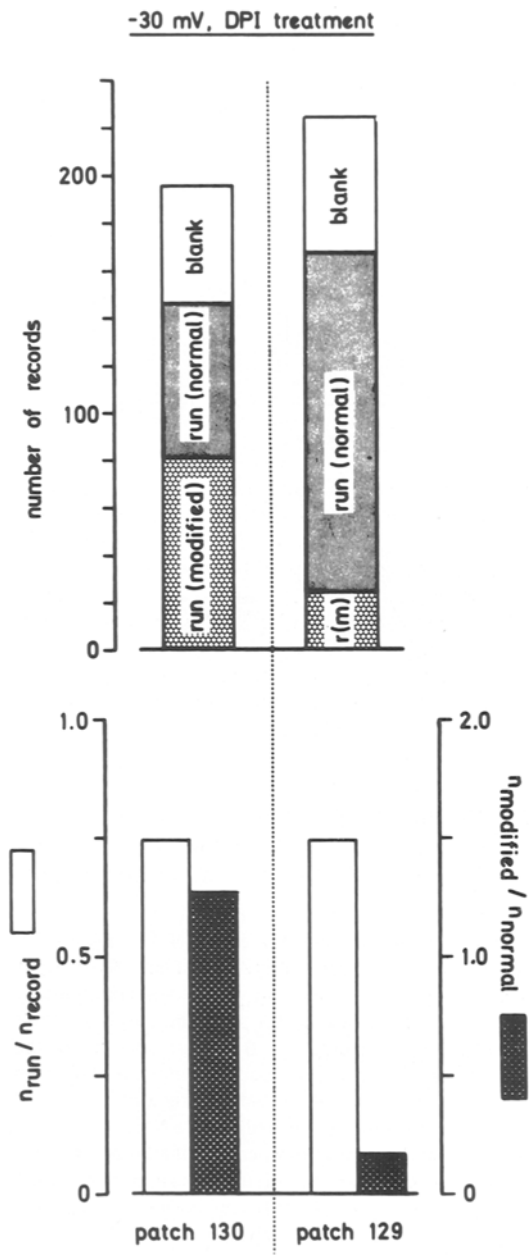


Fig. 8. Different frequency of runs with modified Na^+ channel activity in two inside-out patches. An ensemble of records symbolized by the columns in the upper part of this figure consists of records with (runs) and without (blanks) detectable openings. In DPI-treated patches, a certain number of runs shows modified single Na^+ channel currents. Their relative contribution is indicated in the lower part of this figure by the shadowed columns while the light columns represent the run/record ratio

rose from 0.14 to 0.69 in response to a 30-mV shift to −150 mV. This seems to be related to the simultaneous increase in the number of open normal Na^+ channels (Fig. 9*B*). Two reasons for this voltage dependence could be envisaged: (1) there is a given

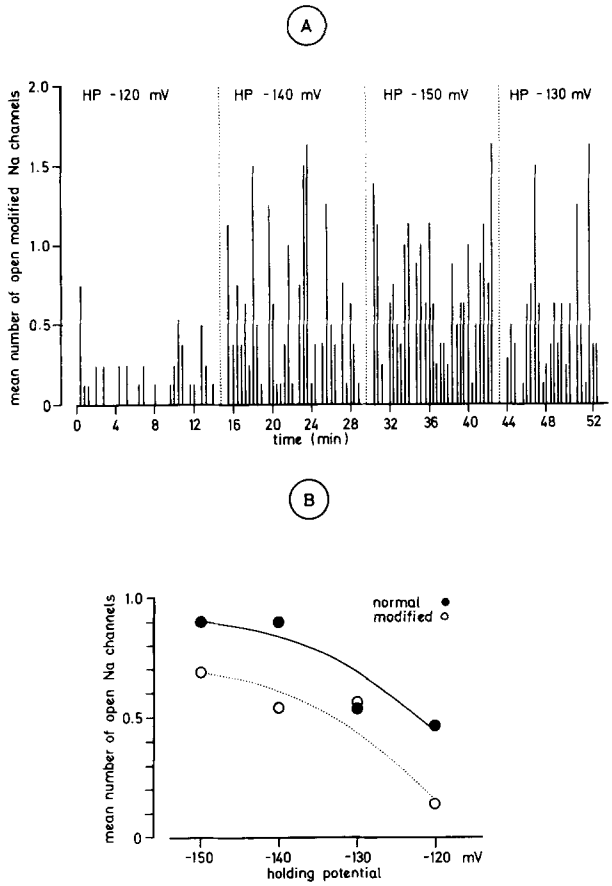


Fig. 9. Voltage dependence of the mean number of open modified Na^+ channels (patch 131; DPI-treated). *A*. The time distribution of the mean number of open modified Na^+ channels at different holding potentials. The inside-out patch was continuously stepped to −30 mV with a rate of 0.33 Hz. The abscissa shows the real time of the experiment, time 0 refers to the end of the initial equilibration period of 10 min. Each bar indicates the mean number of open channels due to step depolarization calculated from eight consecutive samples according to $pN = I/i$ as minimum estimate. *B*. Mean number of open modified (open circles) and open normal (filled circles) Na^+ channels as a function of the holding potential. To match the criterion normal, an opening must occur during the first 10 msec of the sample

fraction of DPI-labeled channels whose resting inactivation declines similar to normal Na^+ channels with hyperpolarization of the membrane; (2) hyperpolarization facilitates the drug-receptor interaction by increasing the number of functioning channels. The latter possibility implies an increased number of available binding sites.

Discussion

Cardiac single Na^+ channels which have been shown in the present inside-out patch-clamp experiments to leave the open state very rapidly (rate constant 826.5 sec^{-1} at −30 mv and 20°C) may be affected by a novel class of organic compounds represented by the piperazine derivative DPI. DPI prolongs the conducting state of single Na^+ channels tremendously, possibly by removing inactivation which might be the final consequence of an interaction with a subunit of the channel. DPI-modified channels left their open state with an about tenfold reduced rate constant (83.3 sec^{-1}).

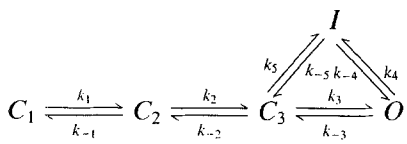
The DPI action strongly resembles the effect of certain other small molecules comprising protein reagents such as N-bromoacetamide (Patlak & Horn, 1982), various aldehydes (Nonner et al., 1980) and glutaraldehyde (*unpublished results*) although these other agents destroy, probably by covalent binding the inactivation process irreversibly. Certain toxins represent another group of Na^+ channel modifiers. Batrachotoxin (BTX), proven in neuronal cells to compete stereoselectivity with a number of local anesthetics for a common channel site (Postma & Catterall, 1984), induces in the node of Ranvier and neuroblastoma cells (Khodorov et al., 1975; Huang et al., 1982) changes like the sea anemone toxin ATX II in isolated cardiac myocytes (Isenberg & Ravens, 1984), namely a very slowly and incompletely inactivating I_{Na} component. BTX seems additionally to interfere with the voltage control of channel gating since repolarization fails to terminate channel activity abruptly (Quandt & Narahashi, 1982) and spontaneous openings may occur (Grant et al., 1983). Still another difference to the DPI action is that BTX reduces the unitary current amplitude (Quandt & Narahashi, 1982) indicating a decrease of conductance or a reduced channel selectivity for Na^+ ions, or both. In any case, and in contrast to DPI, channel modification evoked by BTX seems to be much more complex. It would be, nevertheless, highly speculative to ascribe to DPI a selective effect of the inactivation process before the properties of DPI-modified Na^+ channels are fully elucidated.

Although modification by DPI can be achieved after treating the external side of the membrane, the drug does not necessarily find a target in the outer part of the Na^+ channel freely accessible from the extracellular space. Due to its lipophilic properties, DPI may be able to pass through the lipid phase so that the molecule could gain access, in analogy to local anesthetics (Hille, 1977), to its site of action either laterally during membrane permeation or, after reaching the cytoplasmic side, from the inner channel mouth. To localize the DPI target in the latter channel region rests on the vulnerability of I_{Na} inactivation seen after exposing the *inner* side of the membrane to proteolytic enzymes (Rojas & Arm-

strong, 1971) but neglects the possibility that DPI-induced removal of inactivation might well be a secondary event. In the latter case, a DPI reaction with neighboring channel structures would allosterically attack the inactivation process.

Because of its hydrophobicity, DPI is most likely to interact with hydrophobic pockets, i.e. protein domains formed predominantly by apolar amino acid residues such as alanine, valine, leucine or isoleucine. Despite this variety of possible candidates for binding, they do not necessarily include the constituents of the subunit involved in channel inactivation. It is interesting to note that two other amino acids, tyrosine (Oxford et al., 1978) and arginine (Rojas & Rudy, 1976), have been proposed to play a role for this gating process.

Single Na^+ channels may attain three configurations, closed, open and inactivated. Depolarization initiates the open (O) and the inactivated (I) configuration whereas resting conditions at the holding potential favor the closed (C) configuration. Among 25 different kinetic models, an extensive statistical analysis considered the best reaction scheme to be one which consists of three closed and one open state and where the inactivated configuration can be reached from O and from C_3 (Horn & Vandenberg, 1984) according to



where the k's are the rate constants. The DPI action should primarily induce a decline of the ratios k_4/k_{-4} and k_5/k_{-5} to values far below 1. It cannot be excluded that modifiers like DPI might additionally favor the open state by making the forward reaction from C_3 to O more dominant than in normal channels. As judged from the biexponential closed time histogram of modified channels (*see* Fig. 7), it may be presumed that cardiac Na^+ channels can attain at least two closed states.

It is interesting to note that noninactivating Na^+ channels kinetically resemble another class of inward channels, Ca^{2+} channels. Ca^{2+} channels in heart muscle and other excitable cells can be described by a similar reaction scheme (Cavalie et al., 1983*b*; Cachelin et al., 1983; Lux & Brown, 1984) consisting of two closed and one open state. Particularly in the presence of the agonistic 1,-4-dihydropyridine derivative Bay K 8644, long-lasting openings and a sustained high open probability over the whole depolarization occur giving rise to average currents which are lacking a decay phase (Hess et al., 1984). The DPI-modified single Na^+ channel currents recorded in the present patch-clamp experiments are almost indistinguishable from the single Ca^{2+} channel currents in Bay K 8644-treated heart cells (Hess et al., 1984).

Removal of Na^+ inactivation may be an important antiarrhythmic principle in heart muscle and represents a new mode of action of drugs classified by Vaughan Williams (1975) as class 3 antiarrhythmics. This class is rather formally defined since prolongation of the action potential as the typical feature is ambiguous in nature and can result from both an increase of inward currents or a decrease of outward currents. DPI-modified Na^+ channels must be expected to be exclusively responsible for the prolongation of the action potential (Scholtysik et al., 1985) because TTX abolishes the DPI effect and normalizes the shape of the action potential (Buggisch et al., 1985). Thus, Na^+ channel modification reduces cardiac excitability during diastole by prolonging the absolute refractory period.

Still another consequence of Na^+ channel modification by DPI is worth mentioning. DPI exerts a strong positive inotropic effect which is, together with the prolonged duration of the action potential, abolished by TTX (Buggisch et al., 1985). Obviously, the increased Na^+ load of the cardiac cell as it results from the noninactivating portion of I_{Na} during depolarization facilitates excitation-contraction coupling in heart muscle, presumably via an influence on the transmembrane Na^+/Ca^{2+} exchange thereby increasing the amount of intracellular Ca^{2+} available for contractile activity.

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