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Responsiveness of Cardiac Na^+ Channels to a Site-Directed Antiserum against the Cytosolic Linker between Domains III and IV and Their Sensitivity to Other Modifying Agents

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Abstract. Elementary Na^+ currents were recorded in inside-out patches from neonatal rat heart cardiocytes to analyze the influence of a site-directed polyclonal anti-serum against the linker region between the domains III and IV (amino acids 1489–1507 of the cardiac Na^+ channel protein) on Na^+ channel gating and to test whether this part of the α-subunit may be considered as a target for modifying agents such as the (-)-enantiomer of DPI 201–106.

Anti-SLP 1 serum (directed against amino acids 1490–1507) evoked, usually within 10–15 min after cytosolic administration, modified Na^+ channel activity. Antiserum-modified Na^+ channels retain a single open state but leave, at −60 mV for example, their conducting configuration consistently with an about threefold lower rate than normal Na^+ channels. Another outstanding property of noninactivating Na^+ channels, enhanced burst activity, may be quite individually pronounced, a surprising result which is difficult to interpret in terms of structure-function relations. Removal of inactivation led to an increase of reconstructed peak I_{Na} (indicating a rise in NP_o) and changed I_{Na} decay to obey second-order kinetics, i.e., open probability declined slowly but progressively during membrane depolarization. The underlying deactivation process is voltage dependent and responds to a positive voltage shift with a deceleration but may operate even at the same membrane potential with different rates. Iodate-modified Na^+ channels exhibit very similar properties including a conserved conductance. They are likewise controlled by an efficient, voltage-dependent deactivation process. Modification by (-)-DPI 201–106 fundamentally contrasts to the influence of anti-SLP 1 serum and the protein reagent iodate since (-)-DPI-modified Na^+ channels maintain their open probability for at least 120 msec, i.e., a deactivation process seems lacking. This functional difference suggests that the linker region between the domains III and IV of the α-subunit may not be the only target for (-)-DPI 201-106 and related compounds, if at all.

Key words: Noninactivating cardiac Na^+ channels — Removal of inactivation — Cardiac Na^+ channel protein — α-subunit

Introduction

Voltage-gated Na^+ channels represent the molecular substrate of action potential generation in excitable tissues. In skeletal and heart muscle, they are a heterodimeric composed of the 260 kD α-subunit which consists of four homologous domains each containing six membrane-spanning segments (for review *see* Catterall, 1988) and β_1-like subunits (Gordon et al., 1988). The principal functional properties including Na^+ permeation, selectivity and gating are structurally related to the α-subunit. The α-subunit also provides the binding site for tetrodotoxin (Terlau et al., 1991) and, very probably, for other toxins and drugs capable of interfering with Na^+ channels. Two highly conserved regions in the multigene Na^+ channel family (Rogart et al., 1989) are the S4-segment in the domains I–IV identified as the voltage sensor of the channel (Stühmer et al., 1989) and the

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cytosolic linker region between the domains III and IV shown in site-directed mutagenesis experiments (Stühmer et al., 1989) and with site-directed antibodies (Vassilev, Scheuer & Catterall, 1988, 1989) to be involved in Na^+ inactivation. Na^+ inactivation is of particular functional significance since it restricts the Na^+ conductance of excitable membranes to a very few milliseconds and less, an important principle involved in maintaining excitability and keeping the cellular Na^+ load minimal during excitation of nerves, skeletal muscle and myocardium.

Na^+ inactivation reacts sensitively to a great variety of influences. Naturally occurring but structurally unrelated toxins including batrachotoxin (BTX), scorpion toxin (ScTX) and some plant alkaloids or a novel group of drugs as represented by the (-)-enantiomer of DPI 201–106, a diphenylpiperazinylindol derivative, are established to interfere with Na^+ channels by interacting with distinct channel-associated binding sites. Channel occupancy by these agents has a unique functional consequence, namely conversion of the normal channel kinetics to a noninactivating gating mode. Proteolysis or chemical modification of the cytosolic channel region eliminates Na^+ inactivation irreversibly. This also alters fundamentally the responsiveness to local anesthetics since these inhibitory drugs are now allowed to repetitively block the open Na^+ pore. Particularly the nature of the (-)-DPI binding site remains to be elucidated. It could be argued that the conserved cytosolic linker region between the domains III and IV formed in the cardiac α-subunit by the amino acids 1489–1507 could be involved as a target for those compounds.

By analyzing the influence of a site-directed, specific antiserum against the amino acids 1490–1507 of the cardiac Na^+ channel α-subunit, the present inside-out patch-clamp study aimed to further substantiate the significance of the linker region between the domains III and IV for cardiac Na^+ channel gating. Another goal was to compare antiserum-modified with other noninactivating cardiac Na^+ channels, particularly after modification with the protein reagent iodate and with (-)-DPI 201–106, in order to define the degree of consensus with regard to their kinetic properties. Functional evidence will be presented in favor of the hypothesis that this linker region is not specifically involved in the interference of (-)-DPI 201–106 with cardiac Na^+ channels.

Materials and Methods

Elementary Na^+ currents were recorded at 19°C in inside-out patches from cultured neonatal rat cardiocytes with an L-M/EPC 5 amplifier by using the standard patch-clamp technique (Hamill et al., 1981). Cultivating and handling of the short-time (19–24 hr) cultured cardiocytes were essentially the same as already described in detail earlier (Kohlhardt, Fröbe & Herzig, 1986). K^+-depolarized, quiescent cardiocytes with a resting potential close to 0 mV were used for patch formation. Patch excision occurred after an equilibration of the patch for 20–25 min in the cell-attached configuration and was followed by a second equilibration period of about 10 min to verify that Na^+ channel activity attained a steady state.

Na^+ channel openings were triggered by 120 msec lasting rectangular membrane depolarizations from a holding potential (between −120 and −130 mV) to a test potential (between −60 and −30 mV) at a rate of 0.5 Hz. The patch-clamp recordings were filtered at 1 kHz, on-line digitized with a sampling rate of 5 kHz (dead time 0.2 msec under these recording conditions), and stored on floppy disks.

Idealized records obtained after subtracting leakage and residual capacity currents were taken for analysis. Open times of and gap times between nonoverlapping events were analyzed by setting a threshold at 50% unitary current amplitude (Colquhoun & Sigworth, 1983). Probability density functions yielded τ_{open} and τ_{closed}, respectively, and, by neglecting the first bin of 0.4 msec, were based on an unweighted fit. 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-square method. Reopening analysis was based on a count of sequential openings in activity sweeps without superpositions, an analytical method which will yield only a rough estimate of reopening properties because no patch could be obtained with only one Na^+ channel. Therefore, repetitive activity particularly under control conditions cannot be considered a priori to reflect the reopening of one and the same Na^+ channel. Burst analysis, in modified Na^+ channels, was based on the bimodal closed time distribution. Gaps within bursts can be discriminated from gaps between bursts by introducing a certain critical gap time. The latter depends on the ratio between $\tau_{closed(1)}$ and $\tau_{closed(2)}$ and was calculated from the geometric mean of both time constants.

The macroscopic I_{Na} was reconstructed by ensemble averaging. Since $I = i \times N \times P_o$, where i means unitary current size, N is the number of channels and P_o their open probability, I_{Na} decay will give the time-dependent change of open probability, $P_o(t)$.

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

Solutions (composition in mmol/liter)

A. Isotonic K^+ solution: K^+ aspartate 120; KCl 20; $MgCl_2$ 5; Na-pyruvate 2; 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 device): 19 ± 0.5°C.

Drugs

(-)-DPI (Sandoz, Basle) dissolved in dimethylsulfoxid (Sigma, Munich) was added before use to solution A.

Peptide Synthesis

The synthetic linker peptide SLP 1 consisting of amino acids 1490–1507 of the cardiac Na^+ channel protein (TEEQKKYY-NAMKKLGSKK) was synthesized on the peptide synthesizer

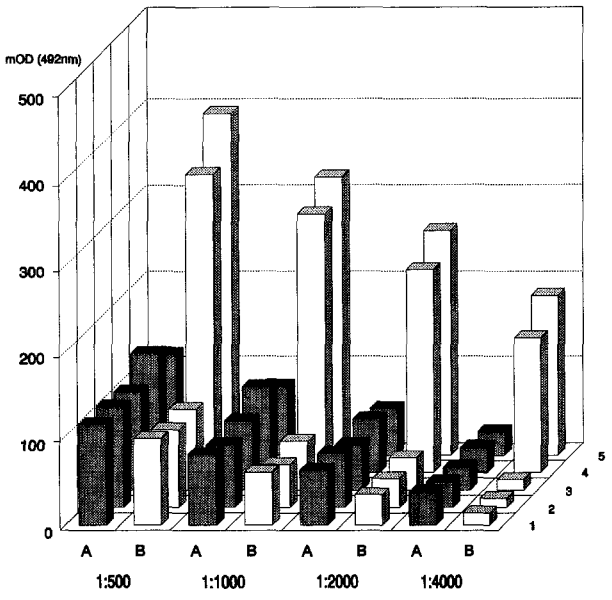


Fig. 1. Specific binding of polyclonal rabbit antiserum to cardiac Na^+ channel sequence SLP 1 in ELISA. *A*: preimmune serum; *B*: serum after immunization. Columns 1–5 refer to Pam_3Cys-Ser, Pam_3Cys-Ser-Ser-FMDV, Pam_3Cys-Ser-T_h, Pam_3Cys-Ser-Ser-SLP 1, and Pam_3Cys-Ser-Ser-T_h-SLP 1, respectively.

430A (Applied Biosystems, Weiterstadt) using 9-fluorenylmethoxy-carbon/tert.-butyl-strategy. After elongation of the resin-bound octadecapeptide SLP 1 by two serine residues, the lipoamino acid N-palmitoyl-S-[2,3-bis(palmitoyloxy)propyl]-cysteine (Pam_3Cys) was introduced (Metzger et al., 1991). The lipotripeptide Pam_3Cys-Ser-Ser acts as a built-in adjuvant and allows immunization without any further adjuvant (Jung et al., 1985; Wiesmüller, Jung & Hess, 1989). The lipopeptide conjugate Pam_3Cys-Ser-Ser-SLP 1 was analyzed by electrospray mass spectrometry (Metzger et al., 1992) on an API III triple quadrupole mass spectrometer equipped with an IonSpry™ source (Sciex, Toronto): 3240.2 ± 0.4 amu found, 3241.2 amu calculated.

Immunization

The lipopeptide conjugate Pam_3Cys-Ser-Ser-SLP 1 was injected in multiple sites on rabbits at three-week intervals. Antisera were collected after the third injection and tested by ELISA procedures.

ELISA Procedures

The specificity of the antiserum was analyzed by ELISA as described (Böltz et al., 1988; Fig. 1). Preimmune serum (*A*) and serum after immunization with Pam_3Cys-Ser-Ser-SLP 1 (*B*) were tested at dilutions of 1 : 500, 1 : 1000, 1 : 2000, and 1 : 4000. Five lipopeptides were used for coating: (1) Pam_3Cys-Ser (Prass et al., 1987), (2) Pam_3Cys-Ser-Ser-FMDV (containing a 20-mer sequence (Wiesmüller et al., 1989) comparable in basicity to SLP 1), (3) Pam_3Cys-Ser-T_h (containing a mainly hydrophobic 16-mer T-helper epitope sequence (Wiesmüller et al., 1992), (4) Pam_3Cys-Ser-Ser-SLP 1, and (5) Pam_3Cys-Ser-T_h-SLP 1 (sequence of SLP 1 elongated by T_h). The lipopeptides containing the Na^+ channel sequence SLP 1 (4 and 5) are obviously recognized by the antiserum (*B*), whereas the control sequences (1–3) are not. The preimmune serum (*A*) shows no specific binding to any of the sequences. The antibody concentrations (IgG + IgM) of the sera were determined in a titration assay. Affinity purified rabbit-gamma-globulin and goat-anti-rabbit (Dianova, Hamburg) were used for calibration. Concentrations determined by comparison of the midpoints of the titration curves were as follows: (*A*) 7 mg/ml whole IgG + IgM; anti-SLP 1 serum (*B*): 8 mg/ml whole IgG + IgM and 1 mg/ml specific IgG + IgM.

Results

1. The Effect of Anti-SLP 1 Serum

First of all, a series of five inside-out experiments examined the cytosolic influence of rabbit preimmune serum in order to verify that Na^+ channel properties remained unchanged in this particular environment. Switching from isotonic K^+ solution to rabbit preimmune serum had no effect on I_{Na} decay kinetics, open state and reopening properties even after an extended time of exposure of up to 50 min. Moreover, NP_o did not change with time.

Anti-SLP 1 serum evoked channel modification (Fig. 2): after cytosolic administration, Na^+ channel openings no longer clustered during the first few milliseconds of membrane depolarization. Instead, repetitive, burst-like activity occurred which may persist for 100 msec or longer and was sometimes only terminated by back-clamping to the holding potential. Two types of activity sweeps could be discriminated, a low activity type with a P_o of 10% or less (*see* rows 3 and 4 in the right part of Fig. 2) which corresponds to the activity mode under control conditions prior to anti-SLP 1 treatment, and a high activity type with a several-fold higher P_o. Reconstructed macroscopic peak I_{Na} increased (to 132% in the experiment illustrated in Fig. 2) accompanied by a deceleration of I_{Na} decay. I_{Na} decay may proceed with second-order kinetics. In this case, $\tau_{decay(1)}$ was close to the value found under control conditions (2.5–4.5 ms, at −60 mV) while $\tau_{decay(2)}$ was several-fold larger, 48 msec, in the experiment shown in Fig. 2. Consistent with observations in type II rat brain Na^+ channels (Vassilev et al., 1988), the anti-SLP 1 serum needed 10–15 min to exert this modifying effect. In only one out of 20 inside-out experiments, an immediate response occurred. Since the effectiveness of N-bromoacetamide and proteolytic enzymes to destroy Na^+ inactivation is voltage dependent (Salgado, Yeh & Narahashi, 1985), it is important to note that also the fast response was obtained by applying the same experimental protocol, i.e., repetitive depolarization from

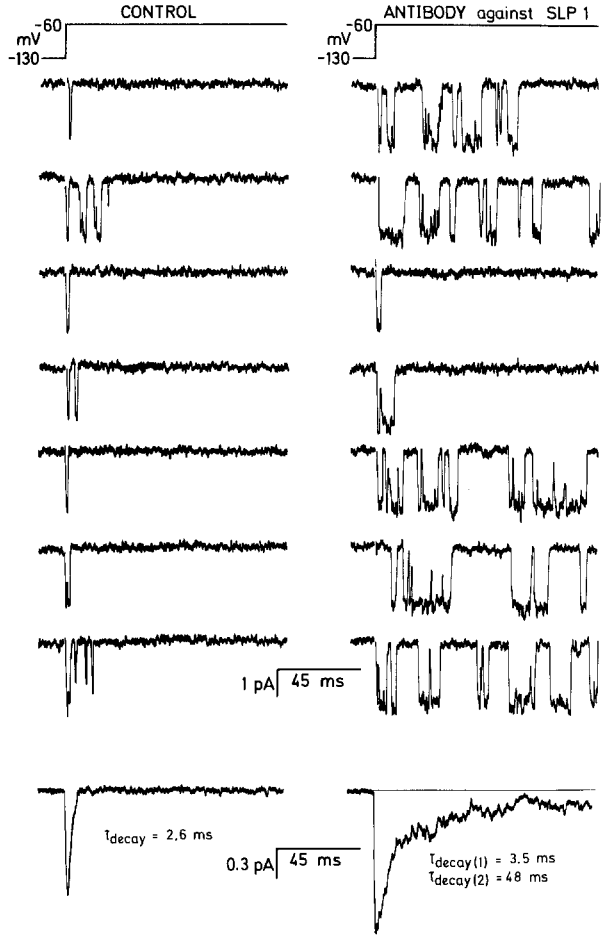


Fig. 2. The effect of anti-SLP 1 serum on cardiac Na^+ channels. Upper part: Selected records of elementary Na^+ currents before (left) and 15 min after cytosolic application of the antiserum (right). Lower part: Reconstructed macroscopic I_{Na} obtained from ensemble averaging of 120 sweeps before (left) and 15 after switching to antiserum (right). The line in the right I_{Na} indicates zero current. Patch 700I0; holding potential −130 mV, test potential −60 mV.

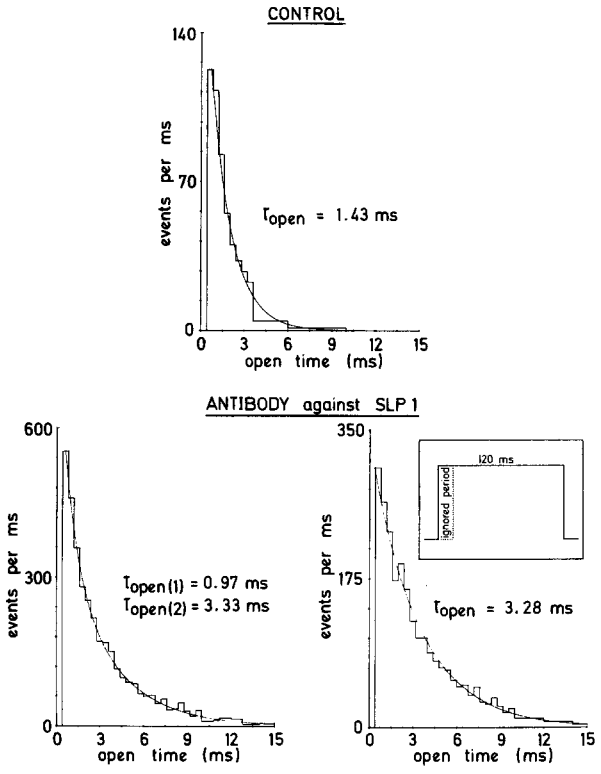


Fig. 3. The effect of anti-SLP 1 serum on Na^+ channel open state kinetics. Open time histograms before (*control*) and after antiserum treatment (15–25 min). By disregarding the first bin of 0.4 msec, the best fits were as follows: control: $N(t)$ = 202exp (-t/0.00132); antibody against SLP 1 (left): $N(t)$ = 416exp (-t/0.00097) + 398exp (-t/0.00333); antibody against SLP 1 (right, biased analysis by disregarding the first 15 msec; *see* ignored period in the inset): $N(t)$ = 340exp (-t/0.00328).

a holding potential of −120 mV. Steady-state conditions were difficult to achieve since, as evidenced from a progressive deceleration of I_{Na} decay, the modifying effect of anti-SLP 1 serum may continue to develop during 5–10 min. After switching to an antibody-free solution, channel modification may persist for periods of 20 min or longer. This poor reversibility could reflect both restricted diffusion and the particular dissociation kinetics of antibodies which are governed by rates in the order of several minutes.

Anti-SLP 1 serum-modified Na^+ channels preserve normal permeation properties since no changes of i_{unit} occurred. In 1 out of 15 experiments, a careful inspection of several hundreds of openings detected a single subconductance event with a size of 0.65 pA, in contrast to 1.95 pA (at −60 mV) for fully sized openings. To consider this substate as being specifically related to the antiserum treatment would, however, ignore that the low conductance permeation mode has been established as a property of normal cardiac Na^+ channels (Scanley & Fozzard, 1987).

Anti-SLP 1 serum-modified Na^+ channels attain at least two closed states, a short-lasting one in the submillisecond range and a several-fold longer nonconducting configuration. Open time histogram analysis revealed consistently a bimodal event distribution (Fig. 3). At −60 mV, $\tau_{open(1)}$ amounted to 0.93 ± 0.12 msec (n = 9) being, thus, close to the value for τ_{open} (0.69 ± 0.06 msec; n = 9) obtained under control conditions while $\tau_{open(2)}$ was 2.90 ± 0.49 msec (n = 9). This indicates either two open states or, because multichannel patches had to be used for analysis, two classes of Na^+ channels. The latter possibility implies the coexistence of antibody-bound and antibody-free Na^+

channels in an individual patch. This idea was tested in a biased open time analysis by neglecting all events during the first 15 msec of membrane depolarization (*see* Fig. 3) so that potentially non-modified channel openings become eliminated. In fact, such biased open time histograms could be best fit by a single exponential. This strongly suggests that anti-SLP 1 serum-modified Na^+ channels attain, like normal Na^+ channels, a single open state but leave their conducting configuration with an about threefold lower rate (at −60 mV). In fact, expression of mutants with deletions or cuts in the linker region between the domains III and IV were reported to yield noninactivating Na^+ channels with a single open state (Stühmer et al., 1989).

The voltage dependence of I_{Na} decay kinetics could be examined in two experiments. A depolarizing shift of membrane potential from −60 to −30 mV caused $\tau_{decay(1)}$ to decline from 3.0 ± 0.8 to 1.5 ± 0.7 msec while $\tau_{decay(2)}$ showed the opposite response and increased from 12.7 ± 2.5 to 24.8 ± 3.0 msec. This voltage dependence of $\tau_{decay(2)}$ fundamentally differs from the behavior in Na^+ channels with operating inactivation where $\tau_{decay(2)}$ declines in responding to a positive potential shift (Brown, Less & Powell, 1981) and, therefore, excludes that the slowly decaying I_{Na} component seen after anti-SLP 1 serum treatment reflects slow Na^+ inactivation.

From a quantitative point of view, cardiac Na^+ channels may respond distinctly different to the cytosolic presence of anti-SLP 1 serum. Figure 4 demonstrates both extreme reactions: in exp 700I0 (Fig. 4*A*), high P_o activity sweeps occurred and contributed to a considerable extent to the whole ensemble but were lacking in exp 706I0 (Fig. 4*B*) although likewise analyzed at −60 mV. Moreover, the mean number of openings rose, in exp 700I0 from 2.7 to 6.8 but, in exp 706I0, from 2.7 to only 3.7. This mirrors different I_{Na} decay kinetics (*see* lower part in Fig. 4*A* and *B*) since a second I_{Na} decay component could only be detected in exp 706I0. Consequently, at −60 mV, $\tau_{decay(2)}$ was found to vary between 9.2 and 48 msec. Interestingly, anti-SLP 1 serum may even fail to enhance burst activity and, thus, in modifying cardiac Na^+ channels, prolong their open state selectively (Fig. 5). In the experiment illustrated in Fig. 5, even extremely long-lasting openings were virtually not chopped by closings, suggesting that a rather stabilized conducting configuration can be attained. The bimodal I_{Na} decay with a slowly decaying current component found after ensemble averaging is, therefore, only related to the prolonged dwell-time in the open state.

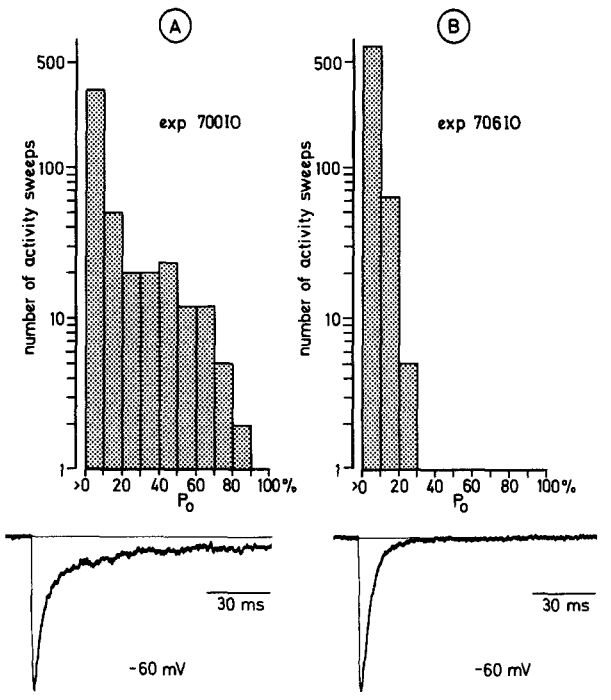


Fig. 4. Differential response of cardiac Na^+ channels to anti-SLP 1 serum: high P_o (*A*) and low P_o (*B*) activity. Upper part in *A* and *B*: Histograms of P_o for activity sweeps of two individual ensembles recorded in exp 700I0 (*A*) and exp 706I0 (*B*). Steady-state P_o was calculated for each activity sweep from $P_o = I/i$, where I is the current averaged over the whole 120 msec lasting depolarization and i is the unitary current. Lower part in *A* and *B*: Normalized reconstructed I_{Na} (horizontal lines indicate zero current) from a total ensemble average in exp 700I0 and exp 706I0, respectively. Test potential in *A* and *B*: −60 mV.

The Effects of Iodate and (-)-DPI 201–106

Two other series of inside-out experiments dealt with the influence of iodate (5 mmol/liter) and (-)-DPI (5×10^{-6} mol/liter) in order to compare their modifying action with the modifying effect of anti-SLP 1 serum. Iodate and (-)-DPI caused modified Na^+ channel activity after cytosolic administration characterized by enhanced burst activity and a prolonged open state. Although substates occasionally appeared, both modifiers left i_{unit} unchanged when compared with the value under control conditions.

Iodate-modified Na^+ channels resemble antiserum-modified Na^+ channels with respect to their burst kinetics: at −55 mV, for example, $\bar{t}_{burst}$ was 4.35 ± 0.05 msec (n = 3) and 5.60 ± 1.01 msec (n = 3), respectively. DPI-modified Na^+ channels, however, attain an about twofold longer bursting state. A still more striking difference becomes apparent by comparing $P_o(t)$ (*see* Fig. 6*A–C*). With (-)-DPI, the reconstructed macroscopic I_{Na} typically shows a sustained current component which fails to

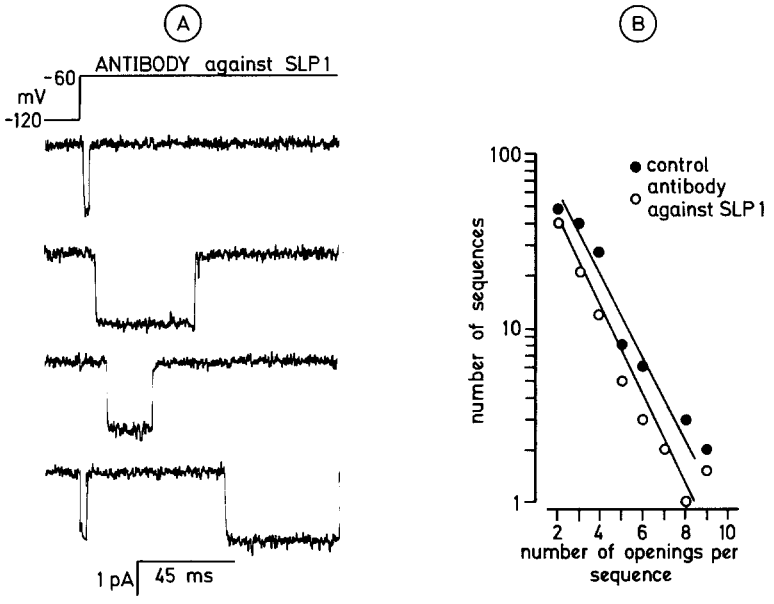


Fig. 5. Failing response of reopening of cardiac Na^+ channels to anti-SLP 1 serum. (*A*) Selected recordings of elementary Na^+ currents with the prevailing type of channel opening. (*B*) Frequency distribution of the number of openings per sequence before (filled circles) and after antiserum treatment (open circles). Patch 718I0; holding potential −120 mV, test potential −60 mV.

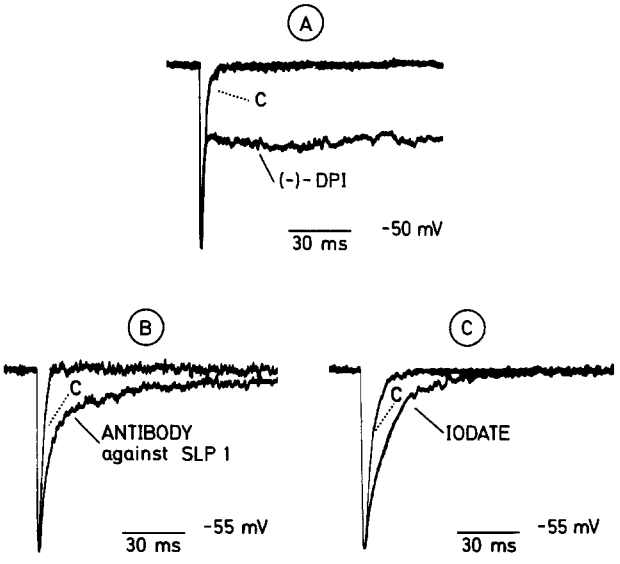


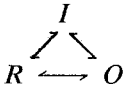
Fig. 6. I_{Na} decay changes after (-)-DPI treatment (5×10^{-6} mol/liter; *A*), with anti-SLP 1 serum (*B*),and in the cytosolic presence of iodate (5 mmol/liter; *C*). Test potential −50, −55, and −55 mV, respectively. *C* indicates the I_{Na} reconstructed under control conditions. All ensemble averages were uniformly based on an ensemble of 120 sweeps.

relax and is virtually time independent, at least for 120 msec lasting membrane depolarizations (Fig. 5*A*), i.e., open probability is maintained for a long period of time regardless of the membrane potential, −60 or −30 mV. When modified with anti-SLP 1 serum or iodate, however, P_o remains a function of time and declines progressively during membrane depolarization as it becomes evident from the I_{Na} decay (Fig. 5*B* and *C*). It seems important to note that also a prolonged exposure to anti-SLP 1 serum or iodate up to 50 min fails to eliminate I_{Na} decay since elimination of Na^+ inactivation by the cytoplasmic presence of proteolytic enzymes was reported to proceed in two distinct stages. In an early stage, I_{Na} decay becomes decelerated but remains time dependent while, in a late stage, I_{Na} decay is abolished (Zilberter & Motin, 1991).

Discussion

The present patch-clamp experiments can be briefly summarized as follows: (i) cardiac Na^+ channels can be kinetically modified by a site-directed polyclonal antibody against the conserved region between the domains III and IV of their α-subunit to attain a several-fold prolonged open state but burst activity may vary significantly; (ii) a voltage-dependent deactivation operates to terminate channel activity during membrane depolarization, a common property of anti-SLP 1-modified and iodate-modified Na^+ channels, while (-)-DPI-modified Na^+ channels maintain their open probability for a long period of time.

Voltage-gated Na^+ channels can be modeled, in the simplest case, by a Markovian reaction scheme according to



where R combines several nonconducting, closed states (C_1,C_2, C_3 in the model of Horn & Vandenberg, 1984), O means the open and I the inactivated, absorbing (Aldrich et al., 1983) configuration. Antibody binding to the linker region between the domains III and IV of the α-subunit may be supposed to critically reduce or to hinder the transition from O to I. This would stabilize the O state and could also favor a repetitive cycling between O and R, thus enhancing burst activity. A C_3-C_2-C_1-O reaction can account for anti-SLP 1 serum-modified Na^+ channels when C_3 represents a stable, nonconducting configuration, or, alternatively, when the forward reaction rate governing the transition from C_3 to C_2 would be rate-limiting: once trapped in the C_3 state, the channel is deactivated. This can also explain the deactivation in iodate-modified Na^+ channels. BTX-modified (Keller et al., 1986), deltametrin-modified (Chinn & Narahashi, 1986) or (-)-DPI-modified (Kohlhardt, Fröbe & Herzig, 1987) Na^+channels attain likewise a single open state, in contrast to noninactivating Na^+ channels after treatment with chloramin T (Nagy, 1988), proteolytic enzymes (Quandt, 1987) or iodate (Kohlhardt, Fichtner & Fröbe). Their more complicated O_1-O_2 kinetics might be related to the less specific action of these chemical modifiers. Most of these modifiers react with more than one species of amino acids and, furthermore, the preferential reactants have different positions in the Na^+ channel protein.

Compared with anti-SLP 1 serum, the sensitivity of cardiac Na^+ channels to iodate is noteworthy with respect to the chemical reactants. Iodate preferentially reacts with cysteine to cleave S-S bonds (Gorin & Godwin, 1966) but the sequence 1489–1507 forming the linker region between the domains III and IV of the cardiac α-subunit lacks this particular amino acid. However, it cannot be excluded that the linker might have been allosterically affected by an iodate-induced chemical reaction in a neighboring region.

It was surprising to see that anti-SLP 1 serum-modified Na^+ channels may exhibit quite individual burst properties. The antibody interaction with the linker can be supposed to prevent the linker from controlling channel gating. This interaction is determined by geometric arrangement as well as by the epitope structure. The assumption that epitope binding of the polyclonal antibody might underlie the differentially pronounced burst activity is purely speculative: it implies a nonuniformity of the linker function in that several linker epitopes are not equivalent in the control of burst activity. The major problem in an attempt to relate Na^+ channel gating to the linker function resides on the molecular mechanism of Na^+ inactivation being poorly understood, if at all. The chain-and-ball hypothesis supposes a positively charged cytosolic ball, i.e., the linker between the domains III and IV (Guy & Seetharamulu, 1986; Noda et al., 1986; Stühmer et al., 1989). The ball is electrostatically attracted to a negatively charged site at or in close vicinity to the Na^+ pore, a site which becomes exposed when the Na^+ pathway has formed. Not consistent with the ball function are point mutations of positively charged residues in the linker. Conversion of lysines to asparagines and the resultant reduction of the number of positive charges were reported not to decelerate Na^+ inactivation (Moorman et al., 1990) but to exert the opposite gating change, an acceleration of Na^+ inactivation. Moreover, as recently demonstrated with rat brain Na^+ channels, coexpression of β_1-subunits with the α-subunit accelerates Na^+ inactivation and shifts the voltage dependence of h_∞ in the hyperpolarizing direction (Isom et al., 1992). Consequently, channel gating may be also influenced by the quaternary structure of the Na^+ channel protein. Despite its fundamental importance for Na^+ inactivation, it would be, therefore, an oversimplification to consider any modulation of the inactivation process as being primarily caused by the linker region.

Assuming that antibody-linker interaction eliminates Na^+ inactivation in an all-or-none fashion, the observed differences in burst activity would indicate that cardiac Na^+ channels are heterogeneous with respect to their deactivating properties. Another example for functional heterogeneity are different reopening properties found also in Na^+ channels from neonatal cardiac myocytes (Kohlhardt et al., 1988) but which might be specifically related to Na^+ channels expressed in the prenatal cell stage.

Noninactivating Na^+ channels can be distinguished by their deactivating properties: deactivation is present in anti-SLP 1 serum-modified and iodate-modified Na^+ channels, but virtually absent in (-)-DPI-modified Na^+ channels. (-)-DPI 201–106 represents a highly lipophilic, positively charged molecule. It interacts with a channel-associated binding site whose location and nature is, however, still unknown. The DPI-binding site is distinct from the binding site for ScTX and ATX II (Scholtysik, Quast & Schaad, 1986; Romey et al., 1987) and could be located either at the intramembrane channel portion or at the cytoplasmic channel surface. The conserved region of the linker between the domains III and IV bears positively charged residues. It, therefore, provides no binding site(s) which could electrostatically attract the (-)-DPI molecule. A hydrophobic (-)-DPI interaction, however, could conceivably occur. It seems attractive to assume that such a drug

interference may be followed by the same or by very similar gating changes as the interference of anti-SLP 1 serum with the linker region between domains III and IV. However, anti-SLP 1 serum-modified Na^+channels deactivate, in striking contrast to (-)-DPI-modified Na^+ channels. This discrepancy can be explained by the hypothesis that (-)-DPI interacts with still another site which is distinct from the linker region between domains III and IV but also involved in controlling Na^+ inactivation. The S6 segment of domain IV has been recently identified in skeletal muscle as such a region (Rojas et al., 1991) but, for functional reasons, seems less likely to be (-)-DPI sensitive. A methionine to valine mutation in this segment alters Na^+ channel properties in a fairly complex fashion (Rojas et al., 1991). So mutated Na^+ channels are not only devoid of the inactivation process but also lose the normal conductance (Lehmann-Horn et al., 1991). Thus, they resemble to some extent veratridine-modified Na^+ channels (Barnes & Hille, 1988). By contrast, (-)-DPI-modified Na^+ channels preserve normal permeation properties, i.e., (-)-DPI removes selectively the inactivation process.

Further support for the hypothesis that Na^+ inactivation can be influenced by an alteration of channel portions other than the linker between the domains III and IV arises from the Na^+ channel sensitivity to ATX II and ScTX. These toxins are well established to act at the external channel surface (for review *see* Hille, 1984). It cannot be excluded that such an interaction affects allosterically the linker region between domains III and IV. Alternatively, elimination of Na^+ inactivation by these toxins might be the direct functional consequence of receptor occupation meaning that this part of the α-subunit is also involved in gating control.

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