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ARTICLE

I. Benz · W. Beck · W. Kraas · D. Stoll · G. Jung
M. Kohlhardt

Two types of modified cardiac Na^+ channels after cytosolic interventions at the α-subunit capable of removing Na^+ inactivation

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Abstract Failure of inactivation is the typical response of voltage-gated Na^+ channels to the cytosolic presence of proteolytic enzymes, protein reagents such as N-bromoacetamide (NBA) or iodate, and antibodies directed against the linker between domains III and IV of the α-subunit. The present patch clamp experiments with cardiac Na^+ channels aimed to test the hypothesis that these interventions may provoke the occurrence of non-inactivating Na^+ channels with distinct kinetic properties. A site-directed polyclonal antibody (anti-SLP2, target sequence 1481–1496 of the cardiac Na^+ channel α-subunit) eliminated fast Na^+ inactivation to induce burst activity which was accompanied by the occurrence of two open states. A deactivation process terminated channel activity during membrane depolarization proceeding with time constants of close to 40 ms (at –40 mV). NBA-modified and iodate-modified Na^+ channels were kinetically indistinguishable from the anti-SLP2-modified type since they likewise deactivate and, thus, attain an only moderate P_o of close to 20%. This is fundamentally different from the behaviour of enzymatically-modified Na^+ channels: after cytosolic proteolysis with α-chymotrypsin, trypsin or pronase, mean P_o during membrane depolarization amounted to approximately 40% because deactivation operated extremely slowly and less efficiently (time constants 100–200 ms at –40 mV, as a minimum) or was virtually non-operating. In-vitro cleavage of the synthetic linker sequence 1481–1496 confirmed that this part of the α-subunit provides a substrate for these peptidases or reactants for NBA but cannot be chemically modified by iodate. This iodate resistance indicates that iodate-modified Na^+ channels are based on a structural alteration of still another region which is also involved in Na^+ inactivation, besides the linker between domains III and IV of the α-subunit. Endogenous peptidases such as calpain did not affect Na^+ inactivation. This stresses the stochastic nature of a kinetic peculiarity of cardiac Na^+ channels, mode-switching to a non-inactivating mode.

Key words Single cardiac Na^+ channels · Site-directed antipeptide antibody · Proteolysis · Protein reagents · Calpain · Stochastic mode switching

I. Benz · M. Kohlhardt (✉)
Physiological Institute University of Freiburg,
Hermann-Herder-Strasse 7, D-79104 Freiburg/Br., Germany

W. Beck · W. Kraas · D. Stoll · G. Jung
Institute for Organic Chemistry, University of Tübingen,
D-72076 Tübingen, Germany

Introduction

Voltage-gated Na^+ channels are encoded by a multigene family (Rogart et al. 1989) and represent the molecular substrate for excitability in many excitable tissues, including heart muscle. In myocardium, I_{Na} generation triggers excitation-contraction coupling. The principal part of the Na^+ channel protein, the ~260 kDa α-subunit exhibits a characteristic architecture and is formed by an assembly of four intramembrane domains each consisting of six helical segments with the outstanding positively charged S4-segment (for review see Catterall 1988, 1995). Elementary Na^+ channel properties including permeation and selectivity, activation and inactivation have their structural equivalent in the α-subunit. The conformational change underlying activation is postulated to be related to an outward movement of the S4-segment along its helical axis (Stühmer et al. 1989; Yang and Horn 1995). A major structural part involved in inactivation has been identified in a similarly conserved region of the cytosolic linker between domains III and IV (Stühmer et al. 1989; Vassilev et al. 1988). More recently, a residue in S5 of domain II and another amino acid in S6 of domain IV with a location at the very cytosolic end of either segment turned out to be also critical for Na^+ inactivation (Cannon and Strittmatter 1993).

The active part of this cytosolic linker region is obviously formed by three adjacent amino acids, the IFM-cluster corresponding to position 1487–1489 in the cardiac Na^+ channel α-subunit which is supposed to interact hydrophobically with the open Na^+ pore (West et al. 1992) by a

hinged-lid mechanism, thereby terminating the open state. The mechanism initially assumed to mediate Na^+ inactivation, the chain-and-ball mechanism (Armstrong and Bezanilla 1977), has, however, received no support from recent mutagenesis experiments (Moorman et al. 1990).

Na^+ inactivation effectively switches off Na^+ conductance within a few milliseconds or less. The importance of this process for the normal function of excitable cells becomes clearly apparent from some inherited disorders. Autosomal dominant hyperkalemic periodic paralysis and congenital long QT-syndrome have been recognized as being causally related to non-inactivating Na^+ channels (Lehmann-Horn et al. 1987; Bennett et al. 1995). The partial depolarization caused by the sustained I_{Na} is associated with severe skeletal weakness or, in heart muscle, predisposes to ventricular arrhythmias with sudden death. The molecular origins, at least in periodic paralysis, are point mutations in S5 of domain II and in S6 of domain IV of the α-subunit (Ptacek et al. 1991; Rojas et al. 1991; Cannon and Strittmatter 1993). Interestingly, the linker sequence between domains III and IV corresponds to the wild type.

Na^+ inactivation reacts sensitively to a great number of influences including plant alkaloides, some naturally occurring toxins or certain drugs and can also be eliminated by cytosolic proteolysis or, more specifically, by site-directed antipeptide antibodies against the cytosolic linker between domains III and IV of the α-subunit (Vassilev et al. 1988). Another critical region seems to be accomodated in the external channel surface where α-ScTX or ATX II act to remove Na^+ inactivation (for review see Hille 1992), surprisingly with regard to the currently established structure-function relationship and a cytoplasmically localized inactivation particle. Functional differences between antipeptide antibody-modified and (–)-DPI-modified cardiac Na^+ channel (Beck et al. 1993) suggest a (–)-DPI target not localized in the cytoplasmic linker between domains III and IV.

To test the hypothesis that structural alterations of the cytoplasmic channel surface may have distinct functional consequences, the influence of peptidases and protein reagents such as NBA and iodate on single cardiac Na^+ channels were comparatively analyzed in the present patch clamp experiments. The channel response to an antipeptide antibody directed against the cytosolic linker between domains III and IV of the α-subunit and including the IFM-cluster as target was considered as the prototype of a non-inactivating Na^+ channel. In fact, fast and ultra-slow deactivating modified Na^+ channels can be distinguished. Also of interest is the modifying iodate effect since this protein reagent cannot chemically react with the cytosolic linker sequence.

Methods

1. Patch clamp experiments

Elementary Na^+ currents were recorded at 19 °C in the cell-attached and in the inside-out patch configuration with an L-M/EPC5 amplifier by using the standard patch clamp technique (Hamill et al. 1981). The patches were formed from short-time (18–24 h) cultivated neonatal rat cardiocytes. Cultivating and handling of the cells were essentially the same as described in detail earlier (Kohlhardt et al. 1989). To improve patch formation, K^+-depolarized, quiescent cardiocytes with a resting potential of close to 0 mV were used. Patch excision occurred after a 10 min equilibration in the cell-attached configuration. This was necessary to judge membrane stability and to verify stable Na^+ channel activity.

Na^+ channels openings were triggered by 120 ms rectangular membrane depolarizations from a holding potential (between –90 mV and –120 mV) to a test potential of –40 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, and stored on floppy discs.

Idealized records resulted from subtraction of leakage and residual capacity currents and were taken for analysis. Open times of and gap times between non-overlapping events were analyzed by the 50% threshold method (Colquhoun and Sigworth 1983). Probability density functions yielded τ_{open} and τ_{closed}, respectively. The fit procedure ignored the first bin of 0.4 ms and was based on the least χ^2 method. Modified Na^+ channel activity was considered to happen when P_o during the 120 ms membrane depolarization exceeded a level of 10%; a rationale for this bias was given earlier (Kohlhardt et al. 1989). $P_o \geq 0.1$ activity sweeps (and showing no superpositions) were selected for reopening analysis. Reopening was estimated from a count of events but the value obtained will be an underestimation of the true value because of missed events. The ratio reopening/P_o yielded a normalized value of reopening in order to take into account that the reopening frequency will be primarily a function of P_o. Burst analysis was likewise restricted to $P_o \geq 0.1$ activity sweeps without superpositions. To discriminate gaps within and gaps between bursts, a critical time was introduced as calculated from the geometric mean of $\tau_{closed(1)}$ and $\tau_{closed(2)}$.

The macroscopic I_{Na} was reconstructed by ensemble averaging. Since $I = i \times P_o \times N$ where i means unitary current size, P_o open probability and N the number of channels, I_{Na} decay provides a measure for $P_o(t)$ during membrane depolarization. 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; $MgCl_2$ 1; $CaCl_2$ 0.2; Hepes 10; pH 7.4.

Temperature (19 ± 0.5 °C) was controlled by means of a Peltier element. Proteases (α-chymotrypsin, trypsin, pro-

nase, thermolysin), protein reagents (NBA, iodate) and calpain were purchased from Sigma-Chemie, München, and freshly dissolved in solution A just before use. Except for calpain, the proteases were applied in concentrations between 80 μg/ml and 150 μg/ml. To modify Na^+ channels with NBA and iodate, a concentration of 5 mmol/l of either chemical was chosen.

2. Peptide synthesis

Two peptides were synthesized from N_α-Fmoc-protected amino acids with a peptide synthesizer 433A (Applied Biosystems, Weiterstadt) following conventional strategies. The 53-mer peptide has a sequence corresponding to amino acids 1473–1525 of the cardiac Na^+ channel α-subunit and, thus, represents the primary structure of the cytosolic linker between domains III and IV. It was used as substrate for in-vitro cleavage experiments. The 16-mer peptide (called SLP2) corresponds to the amino acid sequence 1481–1496 of the Na^+ channel α-subunit and served as immunogen. Peptide identity was verified by electrospray mass spectrometry and peptide purification was achieved by means of semi-preparative HPLC using a Nucleosil 300 C18 column (5 μm particle size).

After elongation of SLP2 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_3-Cys-Ser-Ser acts as a built-in adjuvant (Wiesmüller et al. 1989) and allows immunization without any further adjuvant.

3. Immunization

The lipopeptide conjugate Pam_3-Cys-Ser-Ser-SLP2 was injected in multiple sites on rabbits at three weeks intervals. Antisera were collected after the third injection and tested by ELISA procedures to verify the specificity of the polyclonal antiserum against SLP2. The antibody concentration (IgG + IgM) was determined in a titration assay using affinity-purified rabbit gamma globulin and goat-anti-rabbit (Dianova, Hamburg) for calibration.

Results

1. Functional properties of modified cardiac Na^+ channels

Cardiac Na^+ channels responded to the cytoplasmic presence of anti-SLP2 serum with modified activity. Usually within 5–10 min, burst-like activity occurred (Fig. 1) lasting for several tens of milliseconds during membrane depolarization so that openings no longer clustered during the very initial stage of membrane depolarization. Repolarization to the holding potential (not shown) promptly terminated channel activity. Furthermore, untriggered and spontaneous channel openings at the holding potential appeared in none of the 8 anti-SLP2 serum experiments suggesting that antibody intervention would not abolish the principal voltage dependence of cardiac Na^+ channels: activation essentially requires a step depolarization as in the absence of anti-SLP2 serum. Ensemble averaging revealed that, in contrast to the control conditions, the decay of macroscopic I_{Na} followed second-order kinetics (see Fig. 1, lower part): $\tau_{decay(1)}$ was 2.4 ± 1.1 ms ($n = 6$) and, thus, close to τ_{decay} prior to anti-SLP2 treatment at –40 mV but $\tau_{decay(2)}$ had a several-fold larger value (39.6 ± 4.8 ms; $n = 6$). This can be explained with two channel populations, an antibody-free fraction and an antibody-occupied fraction and is consistent with the notion that the presence of antibody cannot stabilize burst activity over an extended period of time. Accompanied were changes of open state kinetics with a several-fold prolongation of the conductive state to be described in detail later.

The protein reagents NBA and iodate, well established as Na^+ channel modifiers (Patlak and Horn 1982, Kohlhardt et al. 1989), exert the same effect which, more importantly, cannot be distinguished quantitatively from the action of anti-SLP2 serum. Removal of Na^+ inactivation occurred within 5–7 min in an apparently all-or-none fashion since the likelihood to observe $P_o \geq 0.1$ activity sweeps did not increase with time. As in the presence of anti-SLP2 serum, the permeation properties remained preserved. Occasional substate openings with a frequency of 0.1% or less cannot be considered as an effect of these chemicals since normal Na^+ channels can attain, with a similar likelihood, this particular permeation mode (Scanley et al. 1987). The P_o of NBA-modified and iodate-modified Na^+ channels declined progressively during membrane depolarization. This became evident from I_{Na} decay kinetics: $\tau_{decay(2)}$ amounted to 20.8 ± 5.2 ms ($n = 9$) with NBA and to 22.0 ± 6.4 ms ($n = 7$) with iodate. If reopening and P_o during membrane depolarization can adequately define the bursting mode of modified Na^+ channels, NBA-modified and iodate-modified channels share with anti-SLP2 serum modified channels common functional properties (Table 1a).

Table 1 a Comparable reopening and burst properties of cardiac Na^+ channels modified by anti-SLP2 serum, NBA and iodate. As in Table 1b, reopening is given as dimensionless number and refers to the count of resolved openings. The numbers in brackets indicate the number of inside-out experiments. Test potential –40 mV

	Anti-SLP2 serum ($n = 8$)	NBA ($n = 9$)	Iodate ($n = 7$)
Reopening (in activity sweeps with $P_o \geq 0.1$)	4.9 ± 0.7	5.4 ± 0.4	5.9 ± 0.9
Mean P_o (in activity sweeps with $P_o \geq 0.1$)	$20.7 \pm 2.5\%$	$20.1 \pm 0.8\%$	$21.9 \pm 2.3\%$
Reopening/mean P_o (in activity sweeps with $P_o \geq 0.1$)	0.24 ± 0.03	0.27 ± 0.02	0.27 ± 0.02
t_{burst}	8.9 ± 0.9 ms	8.4 ± 0.8 ms	8.9 ± 0.9 ms

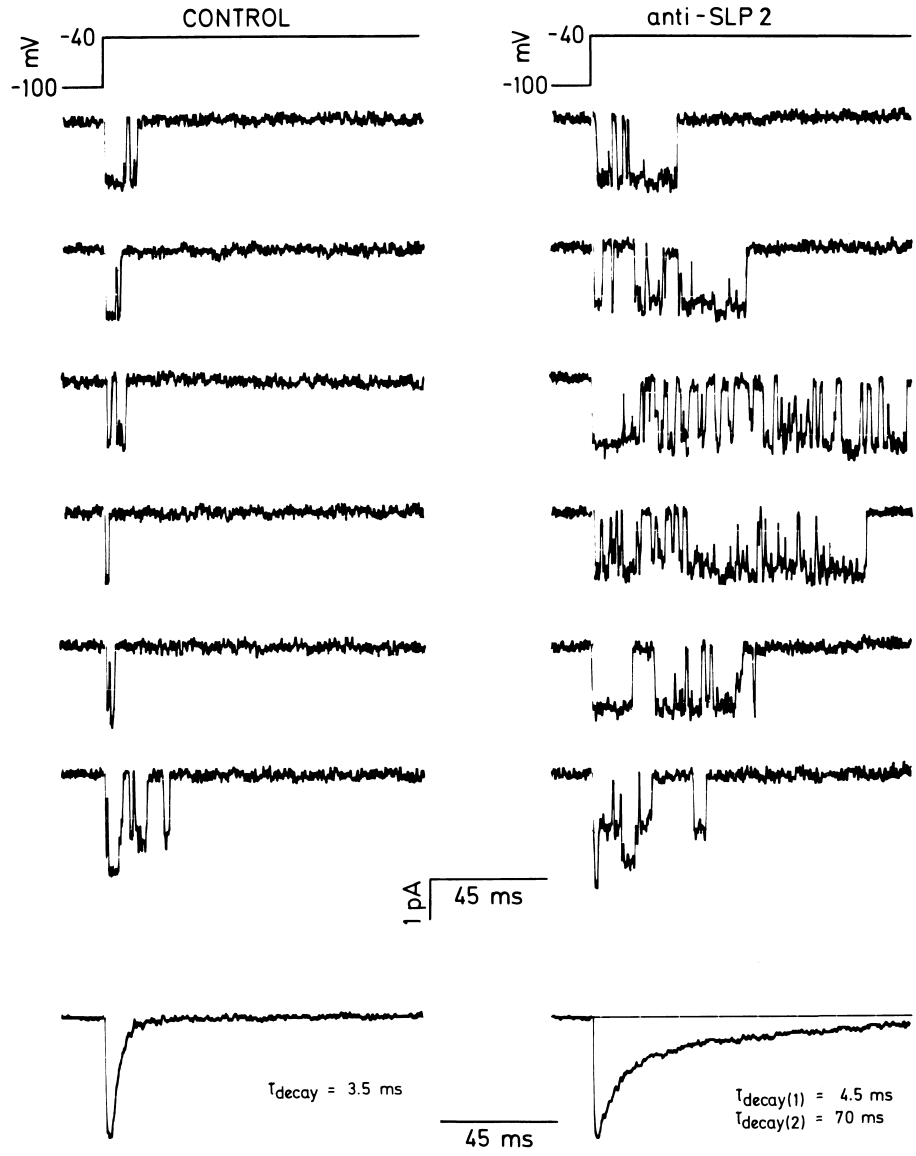


Fig. 1 The effects of anti-SLP2 serum on cardiac Na^+ channels. *Upper part:* Continuous recordings of elementary Na^+ currents in cell-free conditions before (*left*) and 7 min after the cytoplasmic administration of the anti-peptide antibody (*right*). *Lower part:* reconstructed macroscopic Na^+ currents obtained from ensemble averaging of 150 sweeps under control conditions (*left*) and of 300 sweeps in the presence of antibody (*right*). The I_{Na} traces are presented in a normalized form and the straight line in the right I_{Na} indicates zero current. Patch 803IO; holding potential –100 mV, test potential –40 mV

Cytosolic proteolysis seems the most rigorous intervention to alter channel structure and represents the classical approach to destroy Na^+ inactivation (Armstrong et al. 1973). A series of inside-out experiments with peptidases should answer the question as to whether cytosolic channel proteolysis is accompanied by a specific functional correlate when compared with the influence of anti-SLP2 serum. With α-chymotrypsin (Fig. 2), trypsin or pronase, channel modification developed within 5–10 min after cytosolic administration. Remarkably, thermolysin failed to exert a modifying effect in another series of 9 experiments even after a prolonged exposure of up to 45 min. Once modified by α-chymotrypsin or the other effective peptidases, the non-inactivating Na^+ channels attain a high activity burst mode (see Table 1b) with a mean P_o of about 40%, a level wich is twice as high as in anti-SLP2 serum

Table 1b Comparable reopening and burst properties of cardiac Na^+ channels modified by peptidases. Test potential –40 mV

	α-chymotrypsin (n = 10)	Trypsin (n = 7)	Pronase (n = 10)
Reopening (in activity sweeps with $P_o \geq 0.1$)	16.5 ± 1.00	16.2 ± 0.85	15.3 ± 0.62
Mean P_o (in activity sweeps with $P_o \geq 0.1$)	41.1 ± 4.1%	34.5 ± 3.3%	43.1 ± 4.0%
Reopening/mean P_o (in activity sweeps with $P_o \geq 0.1$)	0.44 ± 0.07	0.47 ± 0.07	0.35 ± 0.03
t_{burst}	11.3 ± 0.9 ms	9.3 ± 1.3 ms	9.5 ± 0.8 ms

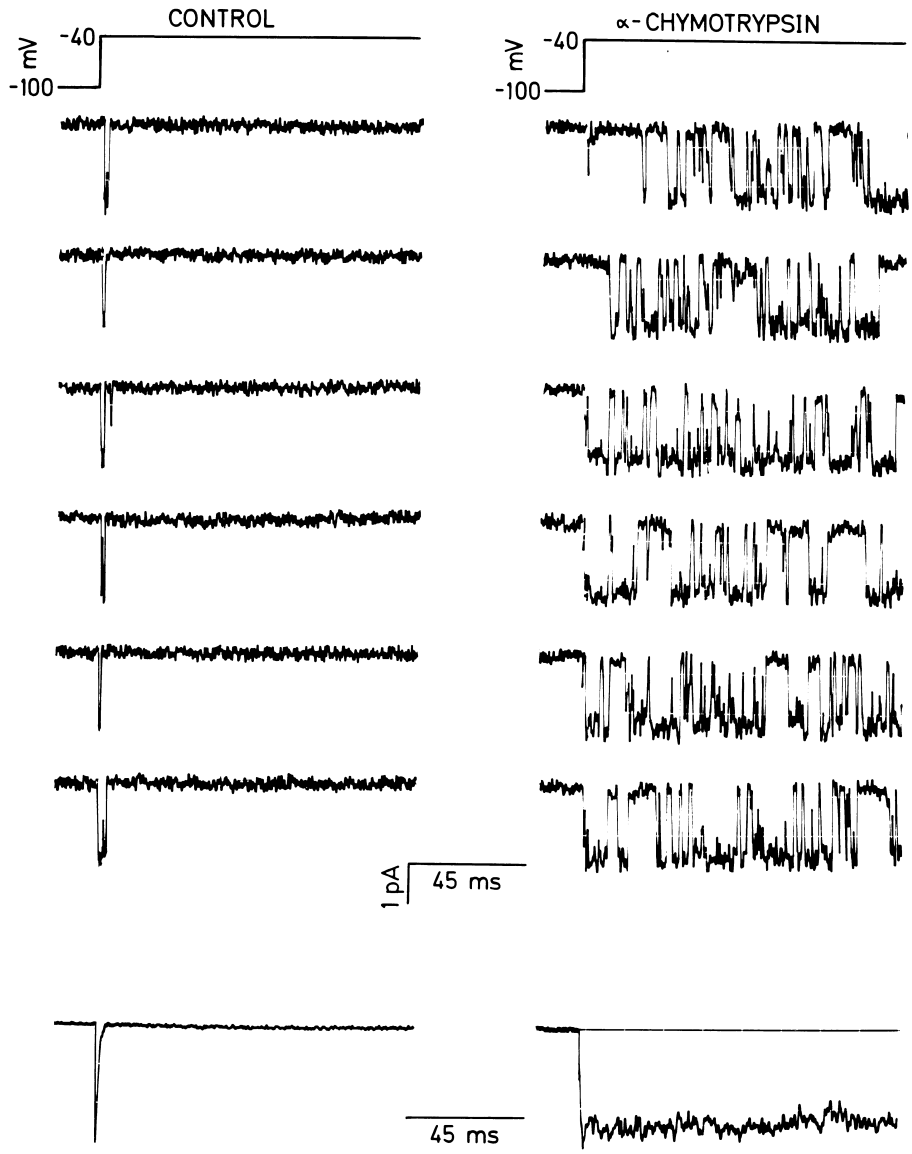


Fig. 2 The effect of α-chymotrypsin on cardiac Na^+ channels. *Upper part:* Continuous recordings of elementary Na^+ currents before (*left*) and after the cytosolic administration of α-chymotrypsin (*right*). *Lower part:* Reconstructed macroscopic Na^+ currents obtained from ensemble averaging of 150 sweeps before (*left*) and of 300 sweeps during peptidase treatment (*right*). The I_{Na} traces are normalized, the straight line in the right I_{Na} indicates zero current. Note that the I_{Na} after proteolysis apparently lacks a decay. Patch 789IO; holding potential –100 mV, test potential –40 mV

modified Na^+ channels or Na^+ channels modified by protein reagents. Since cytosolic proteolysis may proceed slowly with time so that the final functional result will only appear in a later stage (Zilberter and Motin 1991), this possibility was addressed by a separate P_o analysis during the early (7–9 min) and a later (14–16 min) time of exposure to α-chymotrypsin: in 3 experiments, mean P_o was consistently close to 40%. Interestingly, enzymatically modified Na^+ channels can gate more frequently per unit time. They can preserve their burst activity over an extended period of time during membrane depolarization and this is reflected in the drastically reduced decay of I_{Na} (see Fig. 2, lower part). τ_{decay} amounted to 103 ± 13 ms ($n = 5$), thus exceeding the control value by a factor of about 50. This, however, ignores the observation in 5 other α-chymotrypsin experiments where I_{Na} essentially failed to decay, at least during the 120 ms membrane depolarization.

Figure 3 illustrates these distinct single channel properties on the macroscopic I_{Na} level. With effective peptidases, the ratio of sustained I_{Na} to peak I_{Na} took values between 0.36 and 0.47 to exceed the ratios obtained in presence of anti-SLP2 serum, NBA or iodate by a factor of at least 5.1.

Low P_o bursting and high P_o bursting Na^+ channels share common open state kinetics. With any modifying agent, probability density functions were in most of the experiments biexponentially distributed (see Fig. 4). With anti-SLP2 serum, however, the bimodal event distribution is ambigious and may indicate two channel fractions, one with a short open time and the other population with a several-fold longer open time (which were consistent with the idea of an antibody-free and an antibody-occupied fraction mentioned above). This possibility could be excluded by a biased analysis (Kohlhardt et al. 1989) thereby ignoring events during the first 15 ms of membrane depolarization

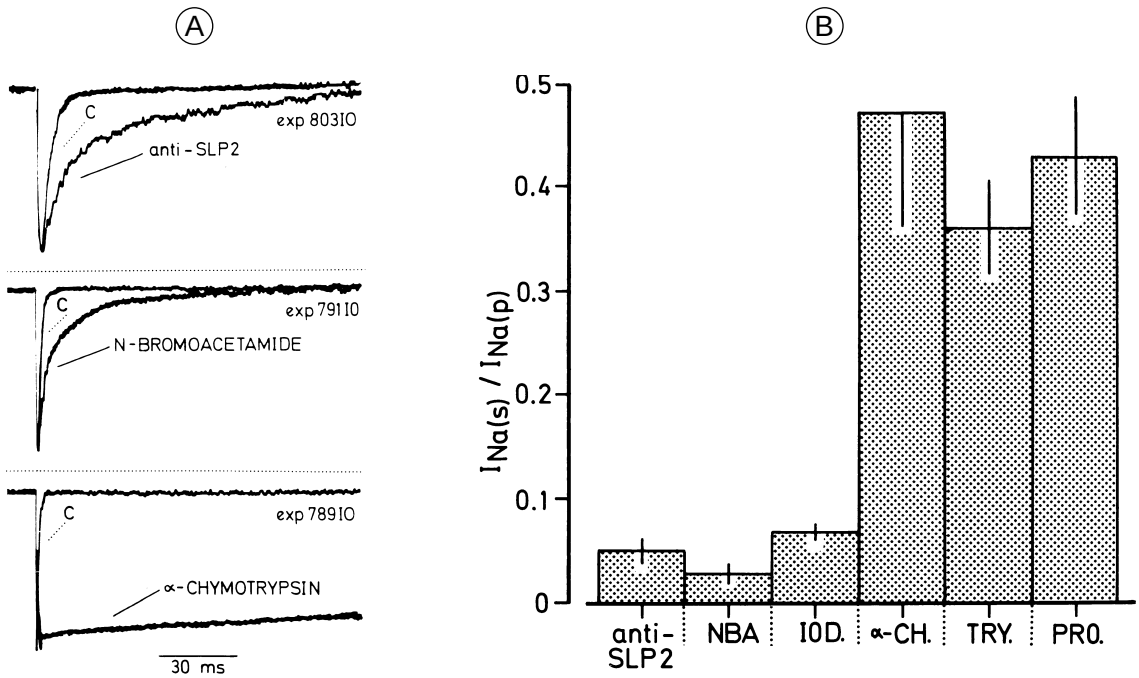


Fig. 3A, B Distinct influences of channel modifiers on I_{Na} decay kinetics. **A** Superimposed reconstructed macroscopic (*normalized*) Na^+ currents before (*C*) and after anti-SLP2 serum (*upper row*), before (*C*) and after N-bromoacetamide (*middle row*), before (*C*) and after α-chymotrypsin (*lower row*). I_{Na} reconstruction was based on ensemble averaging of 150 sweeps under control conditions and of 300 sweeps in the presence of the modifiers. Test potential –40 mV. **B** The ratio of sustained I_{Na} ($I_{Na(s)}$) to peak I_{Na} ($I_{Na(p)}$) as a measure of the persistent Na^+ conductance at the end of the 120 ms test depolarization. Any column represents the mean (*vertical bars* are SEM) of n experiments: n = 8 with anti-SLP2 serum, n = 9 with NBA, n = 7 with iodate (IOD), n = 10 with α-chymotrypsin (α-CH), n = 7 with trypsin (TRY), n = 10 with pronase (PRO). Differences between the mean values for anti-SLP2 serum, NBA and iodate and between the mean values for α-chymotrypsin, trypsin and pronase are statistically insignificant. The macroscopic I_{Na} was reconstructed from ensemble averaging of uniformly 300 sweeps. Test potential –40 mV

Table 2 Open state kinetics in modified cardiac Na^+ channels at –40 mV. A bimodal open time distribution was confirmed in a biased analysis where early occurring (i.e. within the first 15 ms) and potentially regular openings are largely excluded. Not included in Table 2 are five trypsin experiments with a monoexponential open time distribution: τ_{open} was 3.0 ± 0.5 ms, in contrast to the value of 1.4 ± 0.1 ms (n = 45) in normal cardiac Na^+ channels

	$\tau_{open(1)}$	$\tau_{open(2)}$
Anti-SLP2 serum (n = 8)	1.22 ± 0.2 ms	5.43 ± 0.61 ms
NBA (n = 9)	1.04 ± 0.2 ms	3.35 ± 0.56 ms
Iodate (n = 7)	1.41 ± 0.3 ms	5.40 ± 1.10 ms
α-chymotrypsin (n = 10)	1.01 ± 0.2 ms	3.50 ± 0.40 ms
Trypsin (n = 7)	1.10 ± 0.2 ms	3.03 ± 0.50 ms
Pronase (n = 10)	1.29 ± 0.2 ms	3.46 ± 0.21 ms

where normal Na^+ channels cluster: again, the best histogram fit was biexponential. Obviously, antibody-modified Na^+ channels can also attain two 0-states, in contrast to the single 0-state found in more than 50 control experiments at –40 mV. A single 0-state in normal Na^+ channels agrees with recent observations under significantly improved recording conditions at high bandwidth (Böhle and Benndorf 1995). The apparent O_2-kinetics are rather uniform in modified Na^+ channels since each type dwells for almost the same time in this particular configuration (see Table 2) whilst the dwell time in the O_1-state resembles the open time in normal Na^+ channels (τ_{open} between 1.1 and 1.8 ms, at –40 mV). As shown in Fig. 4, O_2-events can significantly contribute to the whole event ensemble with a percentage of about 50%. This results from analyzing the open time histogram area but has to be regarded with reservation because of the limited recording conditions. It still remains to be stressed that, after removal of Na^+ inactivation, Na^+ channels can reach the O_1-state with almost the same likelihood as the O_2-state.

Enzymatically modified Na^+ channels retain a predictable temperature dependence. As evidenced with pronase, open state kinetics ($\tau_{open(1)}$, $\tau_{open(2)}$) and the normalized reopening responded to cooling from 19 °C to 9 °C with Q_{10}'s of close to 1.75. Figure 5 shows that I_{Na} decay may react particularly sensitively since cooling decelerated I_{Na} decay to such an extent that the ratio of sustained I_{Na} to peak I_{Na} approached unity. Thus, this ratio had the highest Q_{10} (2.30). Likewise consistent with normal cardiac Na^+ channels (Kohlhardt 1990), the Q_{10} of i_{unit} was 1.25.

2. In-vitro cleavage of the cytosolic linker

In-vitro cleavage experiments should verify that the cytosolic linker between domains III and IV of the α-subunit

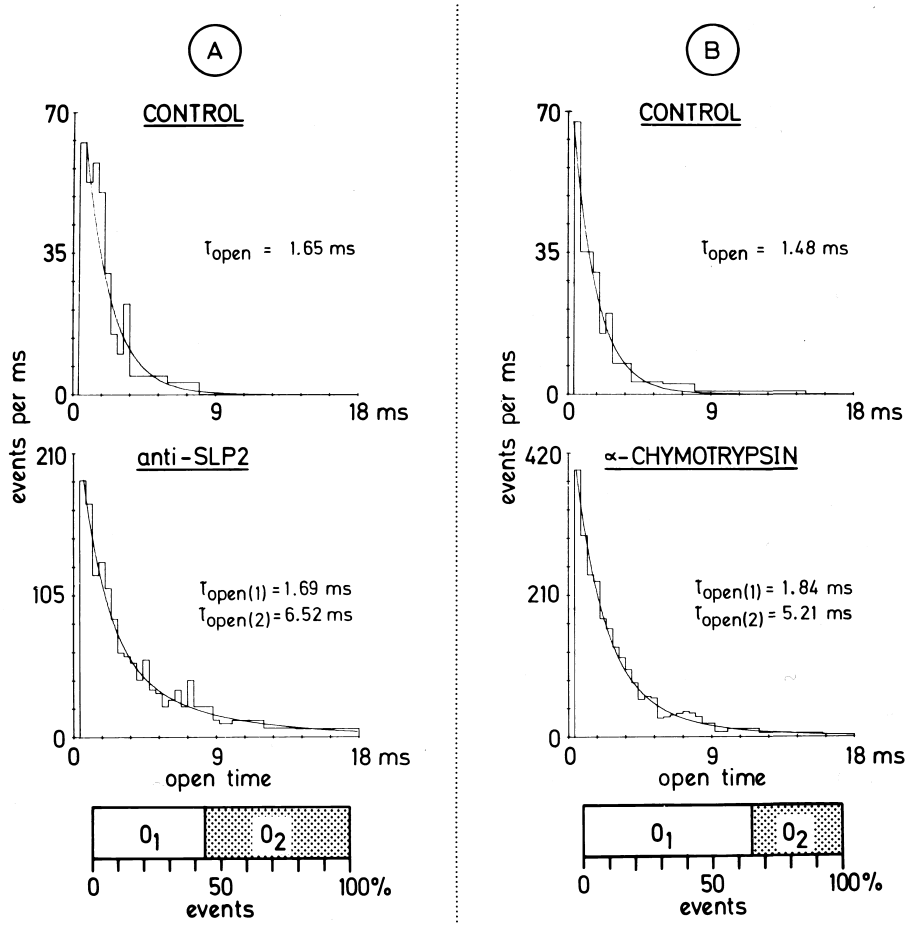


Fig. 4A, B The influence of anti-SLP2 serum (Part A) and of α-chymotrypsin (Part B) on open state kinetics of cardiac Na^+ channels. By disregarding the first bin of 0.4 ms, the best fits of the open time probability density functions were as follows: **A**: control $N(t) = 101 \exp(-t/0.00165)$; anti-SLP2 serum $N(t) = 200 \exp(-t/0.00169) + 60 \exp(-t/0.00652)$ Patch803IO, holding potential –100 mV, test potential –40 mV; **B**: control $N(t) = 130 \exp(-t/0.00148)$ α-chymotrypsin $N(t) = 430 \exp(-t/0.00184) + 76 \exp(-t/0.00521)$ Patch 789IO, holding potential –100 mV, test potential –40 mV. The relative contribution of O_1- and O_2-events to the whole open event ensemble (see lowest graph in Part A and Part B) refers to and was obtained from the bimodal distributed probability density functions by calculating the area under the curve according to

$$\int_{0.2}^{\infty} a\,\tau_{open(1)} \exp(-0.2/\tau_{open(1)}) + \int_{0.2}^{\infty} a\,\tau_{open(2)} \exp(-0.2/\tau_{open(2)}).$$

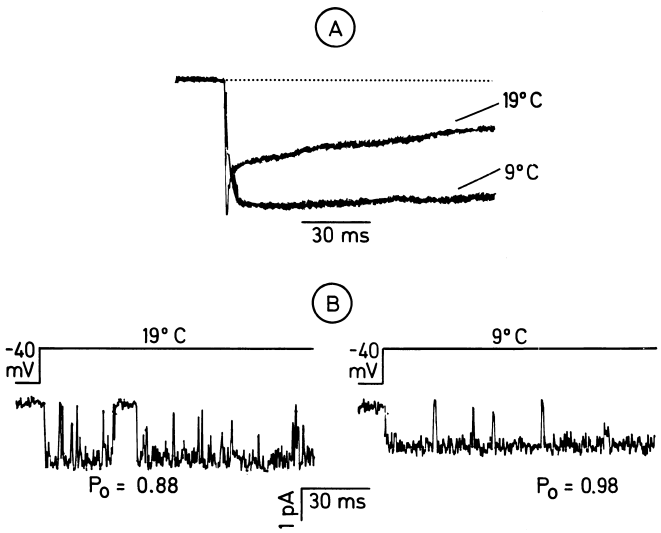


Fig. 5A, B Temperature sensitivity of pronase-modified cardiac Na^+ channels. **A** Reconstructed macroscopic Na^+ currents (superimposed and *normalized*) at 19 °C and at 9 °C. **B** Selected recordings of elementary Na^+ currents at 19 °C and at 9 °C. Shown are the two outstanding sweeps with the highest P_o of a channel during membrane depolarization of an ensemble of 350 sweeps (19 °C) and of 160 sweeps 8 (9 °C). Patch 824IO. holding potential –100 mV, test potential –40 mV

actually provides the substrate for peptidases and protein reagents employed in the patch clamp experiments as channel modifiers. To simulate in-situ conditions where the functional correlate of cytosolic proteolysis was analyzed during ~20 min protease treatment, the 53-mer linker peptide was dissolved in a physiological saline (pH 7.4; temperature 19 °C) and exposed to α-chymotrypsin (5 µg, i.e. 1/20 of the peptide quantity by weight) for 10 min. The fragment analysis (Fig. 6) was based on HPLC-mass spectrometry techniques and predominantly focused on the question of whether fragments can be identified under the influence of α-chymotrypsin and the other peptidases of interest which are consistent with theoretically predicted cleavage sites at the peptide. In fact, this relatively short time of exposure was sufficient to obtain the predicted cleavage products with α-chymotrypsin, trypsin and thermolysin. The cleavage products also contained the functionally important I1487, F1488, M1489 cluster. The sensitivity of the 53-mer peptide towards thermolysin was not surprising but this result is worth mentioning with respect to the ineffectiveness of this peptidase to remove Na^+ inactivation.

Both protein reagents, iodate and NBA, had opposite effects on the 53-mer peptide. Mass spectrometry excluded the possibility that iodate (5 mmol/l) influenced this pep-

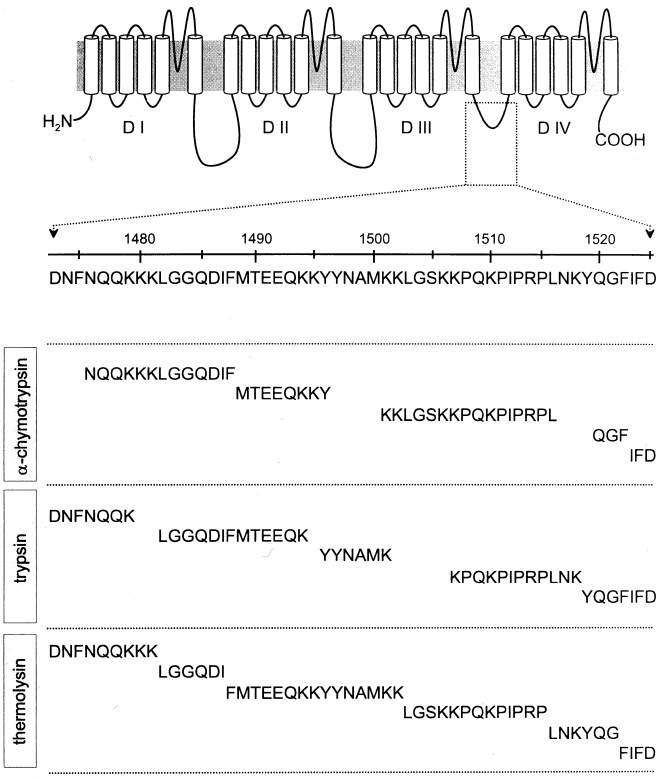


Fig. 6 Dissection of the 53-mer linker peptide by α-chymotrypsin, trypsin and thermolysin. Shown are the principal fragments identified in the presence of these peptidases by means of electrospray mass spectrometry. The uppermost graph demonstrates the predicted transmembrane topology of the Na^+ channel α-subunit with the four (D I–D IV) domains. The primary structure of the cytosolic linker between D III and D IV (marked by the dotted window) corresponds to the 53-mer linker peptide

tide, in contrast to NBA (5 mmol/l). With NBA, the 53-mer peptide became structurally altered as evidenced from a change of molecular weight from 6.345 kDa to one triplet of products of 6.616 kDa, 6.695 kDa, 6.775 kDa and another triplet of products of 6.710 kDa, 6.790 kDa, 6.870 kDa. Either triplet product constantly differed by 80 kDa. This is consistent with the assumption that NBA binds, at least partially, to the 53-mer peptide and thereby adds bromine to the aminoacid residues. Tyrosine and phenylalanine, which are widely distributed over this linker peptide (including the sequence 1473–1525) represent the candidates that provide potential bromination sites on their aromatic rings.

3. Calpain insensitivity of cardiac Na^+ channels

Modal gating determines the kinetic behaviour of voltage-dependent Na^+ channels (Patlak and Ortiz 1985, 1989, Kohlhardt et al. 1987, Nilius 1988, Böhle and Benndorf 1995) since the rate constants governing the transitions between the closed, open and inactivated state can independently and spontaneously change (Patlak and Ortiz 1989). The most dramatic mode switch keeps the channel non-inactivating with persisting burst activity (Patlak and Ortiz 1985, Kohlhardt et al. 1987). Figure 7 demonstrates a multi-channel experiment in the cell-attached configuration with an abruptly occurring episode where high NP_o samples clustered (Fig. 7A), obviously due to mode switching of one or more Na^+ channels to a slowly or non-inactivating mode (Fig. 7B). In this situation, a bimodal open time distribution (Fig. 7C) was found: $\tau_{open(1)}$ corresponded to τ_{open} during the observation period with the normal NP_o level but $\tau_{open(2)}$ was 4-fold larger, the other peculiarity which agrees with the properties of artificially modified Na^+ channels described in the previous section.

Endogenous proteases such as calpain find substrates within the cytoplasmic space which are closely associated with the membrane (for review see Saido et al. 1994) and might be expected to play a role as a Na^+ channel modifier. The present experiments exclude this possibility. The two types of calpain experiments performed in the inside-out patch configuration differed in the concentration of activator Ca^{++}, 4 µmol/l or 50 µmol/l. Calpain (800 µg/ml) proved ineffective and failed to evoke any sign of modified channel activity even on prolonged cytosolic presence for up to 50 min. Ensemble averaging revealed that the τ_{decay} of macroscopic I_{Na} rose during a 30 min calpain treatment to 141 ± 5% (n = 4; 50 µmol/l Ca^{++}) of the control, an unspecific increase since τ_{decay} of I_{Na} tends to rise in prolonged cell-free control conditions to approximately the same extent (Kohlhardt 1991). The calpain resistance stresses the genuine character of mode switching in the non-inactivating mode even under in-situ conditions.

Discussion

The results of the present patch clamp experiments demonstrate, first of all, that non-inactivating cardiac Na^+ channels differ in some functional properties. Anti-SLP2-modified, NBA-modified and also iodate-modified Na^+ channels underlie an effective deactivation process which terminates burst activity almost completely during a 120 ms test membrane depolarization. The phenotype after cytosolic proteolysis exhibits extremely slow deactivation kinetics or can even maintain burst activity over this test depolarization. Proteolytic destruction is also followed by increased reopening as if this intervention would facilitate gating. The insensitivity to calpain excludes the possibility that this peptidase plays a role as channel modifier.

Site-directed antipeptide antibodies against segments of the cytoplasmic linker between domains III and IV of the Na^+ channel α-subunit have a typical functional effect, inhibition of Na^+ inactivation (Vassilev et al. 1988). To consider the possible mode of action of anti-SLP2 serum, antibody interference with the hinged-lid mechanism (West et al. 1992) seems a plausible hypothesis. Accordingly, the IFM-cluster as the active part of the linker region (West et al. 1992) could be prevented from interacting with its

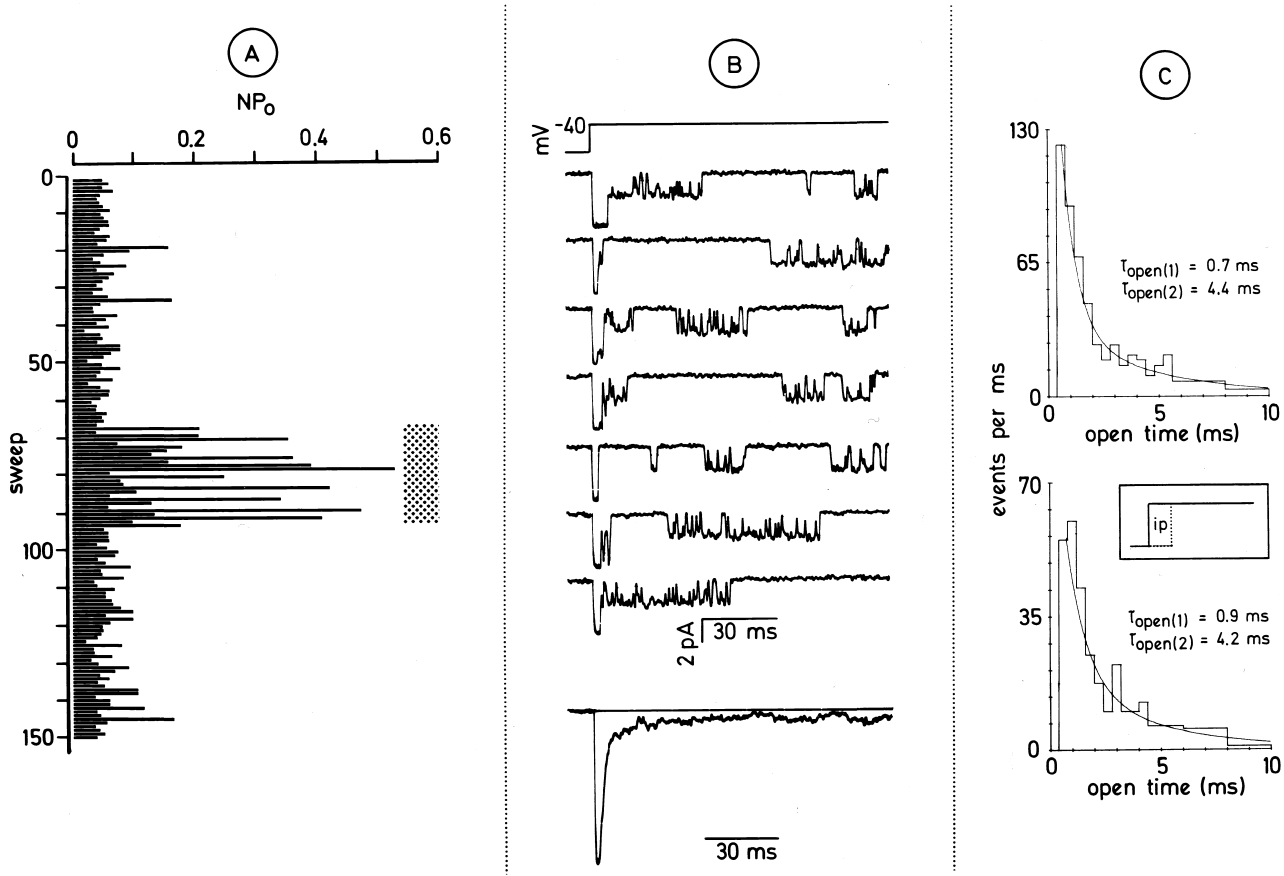


Fig. 7A–C Mode switching of normal cardiac Na^+ channels under in-situ conditions. **A** NP_o profile over an observation period of 5 min. **B** Continuous records of elementary Na^+ currents during the period of the NP_o profile labeled by the *dotted column* with ensemble average (*lower part*). The *continuous line* in the I_{Na} trace indicates zero current which is not approached during the test depolarization of 120 ms. **C** Open time probability density functions constructed from events out of the hatched part of the NP_o profile shown in **A**. The lower of both histograms presents the open time probability density function in a biased form: as symbolized in the *inset*, events during the early 25 ms of test depolarization were ignored (*ip* means ignored period) to exclude the overwhelming majority of regular openings which cluster (at –40 mV) during the first 5 ms but occur 20 ms later with a likelihood of less than 2%. Nevertheless, the best fit remained biexponential according to $N(t) = 86 \exp(-t/0.00094) + 20 \exp(-t/0.00422)$. Patch 829CA, holding potential –90 mV, test potential –40 mV

receptor at the cytoplasmic pore mouth, either by the formation of an IFM-antibody complex or, indirectly, by antibody binding at a neighbouring epitope with concomitant "shielding" of the IFM-cluster. Alternatively, antibody binding could also have an influence on the α-helical secondary structure (Beck et al. 1994) thereby reducing linker mobility to somehow immobilize the linker in such a way that its approach to the receptor becomes altered. The functional correlate of this latter possibility is conceivably retardation instead of removal of inactivation. This question seems crucial indeed since anti-SLP2 serum-modified Na^+ channels cannot maintain their open probability during membrane depolarization. Recent observations with a anti-peptide antibody recognized by the sequence 1490–1506 of the cardiac Na^+ channel α-subunit provide evidence for a deactivation process (Beck et al. 1993) which operates after removal of Na^+ inactivation and can be distinguished by a unique voltage dependence. I_{Na} decay becomes slowed on shifting the membrane potential in the positive direction, opposite to the behaviour seen in the presence of intact Na^+ inactivation. In fact, P_o of anti-SLP2 serum-modified Na^+ channels declines with similar kinetics during membrane depolarization.

The conformity between the effects of anti-SLP2 serum and iodate is interesting with respect to structure-function relationships. Iodate reacts preferentially with cysteine to cleave S-S bonds (Gorin and Godwin 1966) but the linker between domains III and IV is devoid of this amino acid. Furthermore, in-vitro experiments with the 53-mer linker peptide exclude the possibility that iodate finds a non-cysteine reactant in this region. Consequently, in-situ, the chemical must have reacted with a part of the α-subunit distinct from the cytosolic linker. This so-far unidentified target is predicted to be rather superficially located because it cannot otherwise be reached by the membrane-impermeant iodate. The β-subunit shown in coexpression experiments with the α-subunit in rat brain Na^+ channels to hasten Na^+ inactivation (Isom et al. 1991) is not a candidate since, in heart muscle, the α-subunit seems not to be associated with auxiliary subunits (Satin et al. 1992; Cohen and Levitt 1993). Other regions critical for fast Na^+ inactiva-

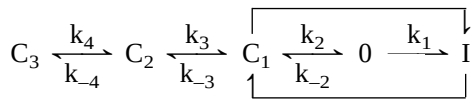
tion comprise, in skeletal muscle Na^+ channels, T698 and M1585 near the cytosolic end of domains II and IV, respectively (Cannon and Strittmatter 1993) and, in the rat brain Na^+ channel, three recently identified adjacent hydrophobic residues at the cytosolic end of S6 in domain IV of the α-subunit (McPhee et al. 1994). The latter region is suspected to form the receptor for the IFM-cluster of the cytosolic linker (McPhee et al. 1994) but likewise does not provide the preferential iodate reactants.

Assuming that cytosolic proteolysis destroys the linker between domains III and IV of the α-subunit, antibody binding is comparatively the much less rigorous influence since the linker structure remains undissected. The functional result of both interventions is, nevertheless, difficult to predict, i.e. enzymatically-modified Na^+ channels may be distinct from the antibody-modified type or not. In-vitro cleavage confirmed the predicted cleavage sites for α-chymotrypsin, trypsin, pronase and thermolysin, indicating that in-situ proteolysis may also be expected to dissect the linker region in several fragments. Linker dissection seems comparable with segment deletion in channel mutagenesis (Stühmer et al. 1989). Although τ_h in mutant Na^+ channels with linker deletions is about 30-fold larger than in the wild-type (Stühmer et al. 1989), I_{Na} can principally decay. After proteolysis, however, I_{Na} decay was virtually eliminated in a considerable percentage of the present experiments. Nevertheless, this discrepancy is difficult to interpret with respect to structure-function relationships and is not necessarily indicative for still another cytosolic proteolysis target in addition to the linker between domains III and IV. Mutant human heart Na^+ channels where the IFM-cluster had been replaced by three glutamines fail to inactivate and generate a I_{Na} with a 6600-fold smaller decay rate when compared with the wild-type (Hartmann et al. 1994), i.e. they maintain, just like the enzymatically modified Na^+ channels in the present experiments, a high P_o during membrane depolarization and deactivate very much less effectively, if at all.

Proteolytic cleavage mapping (Zwerling et al. 1991) of skeletal muscle Na^+ channels has identified accessible protease-sensitive sites. These sites are localized in the cytosolic linker region between domains II and III (excluded in site-directed antibody experiments by Vassilev et al. (1988) as playing a role in Na^+ inactivation) and in the C-terminus of the α-subunit with a particular sensitivity of the latter. Most interestingly, the linker between domains III and IV of the α-subunit can be less easily cleaved. This has been explained by Zwerling et al. (1991) as due to poor in-situ enzyme access, possibly owing to a more compactly folded structure of this functionally important region. This may cast some doubt on the seemingly unequivocal assumption that peptidases remove Na^+ inactivation by proteolysis of the linker between domains III and IV. Furthermore, thermolysin left the inactivation process unchanged although in-vitro cleavage confirmed that the linker region provides a good substrate for this peptidase, a discrepancy which may well result from this access problem.

A recently introduced octagonal structure model which considers Na^+ channel gating alternatively as sliding movement of the P-region along a guiding pore where S4-segments act as a rail, proposes that the C-terminus of the α-subunit has a role in Na^+ inactivation (Sato and Matsumoto 1995). However, it is purely speculative to assume that proteolysis of the C-terminus could have functional consequences unless the significance of this channel part for gating is elucidated in mutagenesis experiments.

Kinetically "normal" voltage-dependent Na^+ channels can be modelled by a Markovian reaction scheme according to



(Horn and Vandenberg 1984). C_3–C_1 are closed states, 0 means the open and I the absorbing (Aldrich et al. 1983) inactivated state. O can be left to I or C_1, the latter exit requires that $k_{-2} \gg k_1$ and allows reopening or burst activity. To follow this formalism, a large k_{-2}/k_1 ratio characterizes Na^+ channels in the non-inactivating mode which can be attained either stochastically by mode switching or under artificial circumstances when lesions of the α-subunit exist. Apart from the interventions analyzed in the present experiments, any other Na^+ channel modifier, including batrachotoxin (Keller et al. 1986), deltametrin (Chin and Narahashi 1986), α-ScTX, ATX II or the piperazinylindole derivative (–)-DPI 201–106 (Kohlhardt et al. 1986) prolong the O-state. It is, therefore, tempting to assume that the non-inactivating mode and the prolongation of the O-state have a common structural equivalent. As evidenced in the present observations, even removal of Na^+ inactivation by stochastic mode switching is accompanied by a prolongation of the O-state. An impaired hinged-lid mechanism seems unlikely to be involved in this change of open state kinetics because normal Na^+ channels with intact inactivation do not dwell longer in the O-state during reopening episodes (Kunze et al. 1985, Kohlhardt et al. 1988). It should be mentioned in this context that the external presence of α-ScTX and ATX II eliminates powerful Na^+ inactivation, although these peptides cannot reach the functionally critical regions at the cytosolic channel surface and, hence, will not directly interfere with the hinged-lid mechanism. Interestingly, recent mutagenesis experiments suggest that, in skeletal muscle Na^+ channels, gating may have a structural equivalent in the P-loop. Point mutations in this region reduced open time and caused an abbreviation of the latency to the first event, apart from a decline in single channel conductance (Tomaselli et al. 1995).

More complicated open state kinetics represent a common kinetic feature in chemically-modified (Nagy 1988; Kohlhardt et al. 1989) and enzymatically-modified (Quandt 1987) Na^+ channels. Even anti-SLP2 serum-modified Na^+ channels and Na^+ channels with stochastic removal of inactivation follow this rule: a second O-state with a much smaller exit rate determines the prolonged dwell time in the conductive configuration. This clearly invalidates the above mentioned Markovian reaction scheme but the valid scheme with the two O-states incorporated

(the normal O_1-state and the several-fold O_2-state) remains to be defined.

It might be inferred from coexpression experiments with skeletal muscle Na^+ channels that stochastic removal of inactivation in cardiac Na^+ channels might be facilitated by their apparent lack of a modulating β-subunit (Satin et al. 1992, Cohen and Levitt 1993). Non-cardiac Na^+ channels can gate in the fast and in the slow inactivating mode when the α-subunit is expressed alone but prefer the conventional fast inactivating mode on coexpression with the β_1-subunit (Patton et al. 1994; Bennett et al. 1993). Nevertheless, mode switching in cardiac Na^+ channels is rare enough to be functionally irrelevant for cardiac excitation. The calpain resistance observed in the present experiments has the implication that Na^+ inactivation is protected against this kind of proteases which can be found as physiological constituents of the cytosol in a variety of cells.

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