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Gating Properties of Cardiac Na^+ Channels in Cell-Free Conditions

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Summary. In patches from neonatal rat heart myocytes, elementary Na^+ currents were recorded at near threshold potentials in order to compare cardiac Na^+ channels kinetics in the cell-attached mode with those in the inside-out mode.

The transition from cell-attached to cell-free recording conditions caused a small prolongation of the conductive state of about 20%. This appeared within 8 min after patch excision regardless of the anionic composition (in mmol/liter) at the cytoplasmic membrane surface: 20 Cl^- plus 120 aspartate, 140 Cl^-, or 140 F^-. Prolonged exposure (up to 50 min) to cell-free conditions evoked no additional changes and, specifically, left the monoexponential open-time distribution unchanged. Increased burst activity only developed in the cytoplasmic presence of F^-, indicating that it is this artificial anion which influences reopening, but not the isolation of the Na^+ channels from their natural environment *per se*. The mean number of openings per sequence (increase by a factor of 1.23 ± 0.04) and τ_{decay} of reconstructed macroscopic I_{Na} (increase by a factor of 1.32 ± 0.06) responded rather weakly to F^-. Cooling from 19 to 9°C accentuated this F^- effect significantly and led, at −65 mV, to pronounced burst activity. Moreover, the combined influence of F^- and cooling induced a second, long-lasting and sometimes dominating open state. It is concluded that isolated cardiac Na^+ channels largely preserve their intrinsic kinetic properties when facing a cytoplasmic environment with a quasi-physiological anionic composition.

Key Words single cardiac Na^+ channels · open-state kinetics · Na^+ inactivation · cytoplasmic anionic environment · rat heart myocytes

Introduction

Tetrodotoxin-sensitive Na^+ channels which are encoded by a multigene family (Rogart et al., 1989) and expressed in numerous excitable cells including heart muscle constitute the prototype of a voltage-regulated ionic channel installed in the surface membrane. Their gating provides a transmembrane pathway for Na^+ ions and is effectively controlled by an inactivation process conventionally termed as h-process which represents an outstanding property making the Na^+ channel different, even kinetically, from many other ionic channels. Na^+ inactivation was recently shown in mutagenesis experiments to be structurally related to the intracellular segment between repeats III and IV of the channel protein (Stühmer et al., 1989) and reacts sensitively to a number of naturally occurring toxins (for review *see* Hille, 1984) or to a novel drug family of the diphenylpiperazinylindole type (Buggisch et al., 1985; Kohlhardt, Fröbe & Herzig, 1986).

The significance of the cytosolic environment for Na^+ channel gating and its sensitivity to intracellular metabolic events is not yet fully understood. Phosphorylation of sites located in the intracellular segment between repeats I and II of the channel protein and brought about by cAMP-dependent protein kinase has been recognized to be a principle which modulates to a certain degree Na^+ channel availability (for review *see* Catterall, 1988) and may be of some functional importance in β_1-adrenergic stimulation of heart muscle. But I_{Na} recordings in whole-cell clamped myocytes from mammalian and amphibian hearts (Brown, Lee & Powell, 1981; Clark and Giles, 1987; Follmer, Ten Eick & Yeh, 1987) largely exclude the possibility that dialyzing the cell interior would significantly alter Na^+ channel activity. Only the isolation of Na^+ channels from their natural environment can be followed by dramatic changes. Apart from a run-down in activity (Cachelin et al., 1983) that is usually evoked by a so far unexplained negative voltage shift of steady-state inactivation, enhanced burst activity was also reported to develop after patch excision (Horn & Vandenberg, 1986; Nilius, 1988; Kirsch & Brown, 1989). This particular gating mode was supposed as being specifically related to the cell-free conditions, i.e., to the loss of the cellular contact (Nilius, 1988), but could alternatively reflect an artificial ionic composition at the cytoplasmic membrane surface. Presumably the presence of fluoride might be crucial since F^- has been proven in internally perfused axons to retard Na^+ inactivation significantly (for review *see* Meves, 1978). Batrachotoxin-modified, noninactivating Na^+ channels, on the other hand, even retain

their intrinsic kinetic properties when reconstituted in artificial lipid planar bilayers (Krueger, Worley & French, 1983).

The present patch-clamp experiments with neonatal rat heart myocytes dealt primarily with the gating properties of isolated cardiac Na^+ channels and should further substantiate their response to isolation from the cellular environment. It will be shown that patch excision and the transition from cell-attached to cell-free recording conditions remains meaningless for major kinetic properties such as reopening unless the artificial anion F^- faces the cytoplasmic channel surface. F^- most likely interferes directly with the Na^+ channel thereby facilitating burst activity.

Materials and Methods

Preparation and handling of short-time (18–24 hr) cultured myocytes which were disaggregated from the hearts of 2–3 day-old rats and used for the patch-clamp experiments were essentially the same as already described in detail (Kohlhardt et al., 1986). By bathing in isotonic K^+ saline, the cardiocytes were depolarized to about 0 mV (0 ± 5 mV). The presence of the Ca^{2+}-buffering EGTA prevented effectively the development of morphological signs of Ca^{2+} intolerance and stabilized the cellular structure for several hours. Elementary Na^+ currents were triggered in cell-attached and inside-out recording conditions by rectangular depolarizations of 70-msec duration at a rate of 0.67 Hz and recorded with an L-M/EPC 5 amplifier by employing the standard patch-clamp technique (Hamill et al., 1981). By the choice of an appropriate holding potential between −100 and −120 mV, both the number of activity sweeps with superpositions and the number of blanks (i.e., sweeps without detectable channel openings) could be minimized. The test potential ranged between −65 and −35 mV. The patch-clamp recordings were filtered at 1 kHz, digitized with a sampling rate of 5 kHz and stored on floppy discs.

Data analysis was based on idealized patch-clamp recordings as obtained from the subtraction of capacity and leakage currents. By employing the 50% unitary current method (Colquhoun & Sigworth, 1983), open time of and gap times between nonoverlapping single-channel events were analyzed. Open-state kinetics were assessed from $\bar{t}_{open}$ and τ_{open}. The latter resulted from probability density functions and, by neglecting the first bin of 0.4 msec, were based on an unweighted fit, i.e., each bin was considered to be of identical significance. Late and, therefore, rare events were fitted by lumping several bins with a certain minimum of events arbitrarily chosen to be four. Reopening analysis was essentially complicated by the fact that all patches contained more than one Na^+ channel. Consequently, sequential openings cannot be regarded *a priori* to reflect reopening of an individual Na^+ channel even in activity sweeps without superpositions, the type of activity sweeps exclusively taken for analysis. Although gap times in the submillisecond range were found to dominate by a factor of 5–6 over longer (i.e., in the range of 3–4 msec) gap times, counting sequential openings will only yield a rough estimate for reopening under the particular conditions of multichannel patches.

Groups each consisting of 40 sweeps and periodically collected every minute from the continuously stepped patches were ensemble averaged to reconstruct the macroscopic I_{Na}. I_{Na} is the product of the number of functioning Na^+ channels (N), the open probability (P_o) and the unitary current size (i_{unit}) and provides a measure of NP_o in cases when i_{unit} is known or remains constant; peak I_{Na} refers to the moment when NP_o reaches its maximum during membrane depolarization and may serve as an indicator of NP_o. The time-dependent I_{Na} analysis yields an NP_o profile and can detect eventual spontaneous changes in Na^+ channel activity on a minute time scale during the lifetime of an individual patch.

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

Solutions (Composition in mmol/liter)

(A) Bathing solutions (facing the cytoplasmic membrane surface in inside-out patches): (*i*) KCl solution: KCl 140, $MgCl_2$ 5, Na-pyruvate 2.5, glucose 20, EGTA 1.0, HEPES 10, pH 7.4; (*ii*) K^+ aspartate solution: K^+ aspartate 120, KCl 20, $MgCl_2$ 5 (or 0), Na-pyruvate 2.5, glucose 20, EGTA 1, HEPES 10, pH 7.4; (*iii*) KF solution: KF 140, Na-pyruvate 2.5, glucose 20, EGTA 1, HEPES 10, pH 7.4.

(B) Pipette solution (facing the external membrane surface): NaCl 200, $MgCl_2$ 1.0, $CaCl_2$ 0.035, HEPES 10, pH 7.4. Temperature (controlled by a Peltier element): 19 (or 9) ± 0.5°C.

Results

NP_o Profiles in Cell-Attached and in Cell-Free Recording Conditions

It is well established that patch formation may be rapidly followed by a significant decline in Na^+ channel activity (Kunze et al., 1985; Kohlhardt & Fichtner, 1988) recently reported to be associated with a negative voltage shift of steady-state inactivation (Kimitsuki, Mitsuiye & Noma, 1990), a process whose nature still remains to be elucidated and which usually takes several minutes to be accomplished. As an experience with more than 250 cell-attached multichannel patches, the initial run-down is unusual to continue or to reappear after the initial 10 min of patch lifetime. Nevertheless, as found in the present experiments by systematically analyzing NP_o in 16 cell-attached patches stepped to a test potential between −65 and −55 mV, the Na^+ channel activity attained an only labile steady state during the later stage since spontaneous and unpredictable fluctuations of I_{Na} may frequently occur. These fluctuations can be quantitatively quite differently pronounced (Fig. 1): peak I_{Na} varied in patch 544CA strongly between 0.79 and 0.16 pA, i.e. by a factor of 5, but in patch 549CA only slightly between 2.65 and 2.00 pA, i.e., by a factor of 1.3. Calculating the mean I_{Na} and its standard deviation for the whole 15-min lasting analysis period revealed that an activity mode with relatively less variance is characteristic

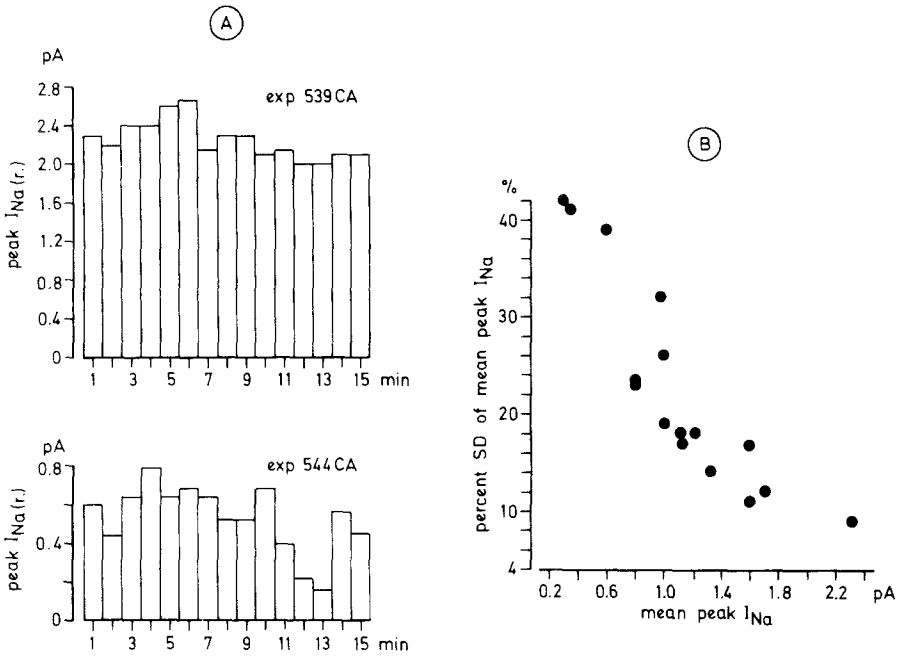

Fig. 1. NP_o profiles of Na^+ channels in cell-attached patches. (*A*) Momentary I_{Na} changes in two individual patches (539CA, upper part, and 544CA, lower part; test potential −55 mV). (*B*) The dependence of percent SD of mean peak I_{Na} on mean peak I_{Na}. Mean peak I_{Na} was calculated from the NP_o profile; each symbol represents an individual cell-attached patch

for patches with a large average I_{Na}, i.e., when the number of functioning Na^+ channels and/or the open probability is comparatively large (*see* Fig. 1*B*). Similar current fluctuations have been reported to occur in voltage-clamped myelinated nerve fibers of the frog and reflect a stochastic channel gating (Sigworth, 1980).

Very similar NP_o profiles were obtained in a series of 17 inside-out patches irrespective of the anionic milieu facing the cytoplasmic channel surface, chloride, chloride plus aspartate, or fluoride. Figure 2*A* also shows that quasi-steady-state conditions of I_{Na} may be preserved for several 10-min periods. Again, the intensity of NP_o fluctuations is inversely related to the size of mean I_{Na} calculated for the whole analysis period. The percent SD of mean I_{Na} amount to 22.2 ± 5% (n = 15) and corresponded to the value (24.3 ± 6%; n = 16) obtained in the cell-attached experiments.

Systematic NP_o changes occurred in two inside-out patches. In one of them (Fig. 2*B*), I_{Na} increased steadily after patch excision, reached a plateau within 10 min but subsequently relaxed. This suggests an initial run-up which is followed by a run-down likewise proceeding with a rate in a minute time scale. In the other inside-out patch, a similar slow run-down developed from the beginning of the cell-free recording mode (*see* Fig. 2*C*). It seems important to note that these systematic I_{Na} changes only appeared after patch excision and were absent during the preceding cell-attached recording. Figure 2*C* also demonstrates that an increase in holding potential (from −105 to −125 mV in the experiment depicted) has no influence on the run-down development. Although I_{Na} initially responded with an increase, the run-down continued to develop with practically the same rate (time constant ~10 min at −105 and −125 mV, respectively). The lifetime of this particular inside-out patch was not long enough in order to see whether Na^+ channel activity would completely disappear. Amplitude histograms excluded an eventual change of i_{unit} with time: i_{unit} (at −60 mV) was 1.95 pA during the first 15 min but 1.92 pA during the subsequent 12 min of patch lifetime, thus corresponding to the value (1.98 pA) as obtained at the same membrane potential in the preceding cell-attached recording mode. A careful inspection of the activity sweeps failed to detect rim currents.

Na^+ Channel Gating in Cell-Free Recording Conditions

Na^+ channel gating was found to be remarkably insensitive to a change of the recording conditions from the cell-attached to the inside-out mode (Fig. 3). A first series of experiments examined the influence of patch excision in a K^+ saline containing 20 mmol/liter Cl^- plus 120 mmol/liter aspartate so that a quasi-physiological anionic milieu was maintained at the cytoplasmic channel surface. As judged from sampling periods prior to and after patch excision each 8 min in duration, τ_{open} increased slightly, in

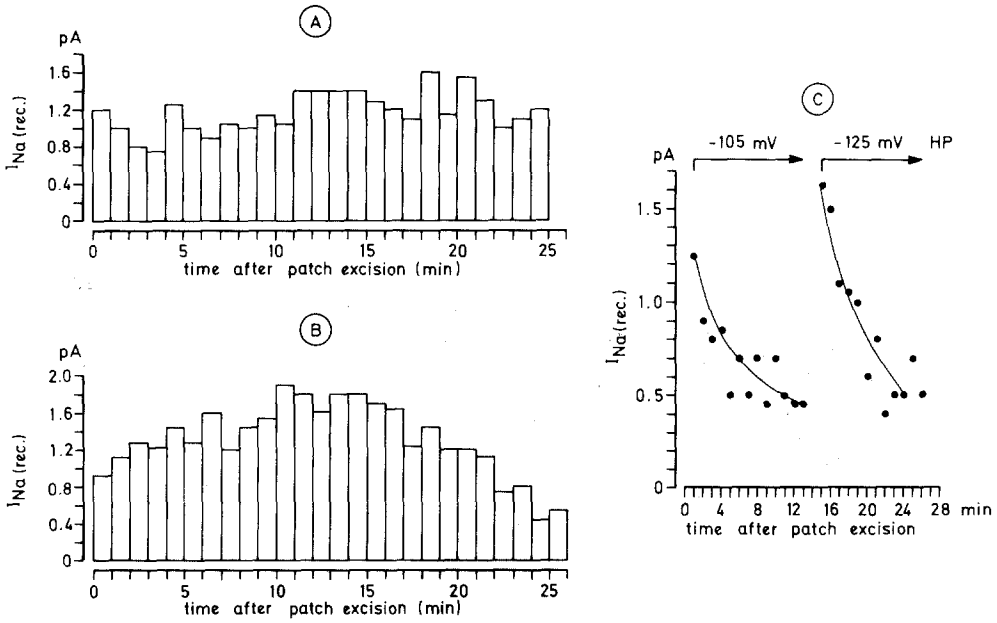

Fig. 2. NP_o profiles of Na^+ channels in inside-out patches. (*A*) and(*B*) Momentary I_{Na} changes in two individual patches (5621O and 5601O; test potential −60 mV). (*C*) Time-dependent decrease in peak I_{Na} at two differential holding potentials (−105 and −125 mV, respectively). Patch 5671O; test potential −60 mV

the experiment illustrated in Fig. 3 from 0.70 to 0.88 msec, amounting to 22 ± 3% (n = 6) on average. In interpreting this result, a methodological limitation should be regarded inherent in comparing data obtained in cell-attached with those in cell-free recording conditions and emerging from an erroneous reference potential. The assumption that the resting potential of K^+-depolarized cardiocytes amounts to 0 mV (which serves as reference potential throughout for the test potential) ignores a ± 5-mV deviation and will introduce, consequently, a shift in reference potential after patch excision with a concomitant difference in test potential between either recording condition.

Despite this uncertainty, the prolongation of the conductive state was a consistent observation, suggesting that patch excision can directly modulate open-state kinetics. Other kinetic properties such as reopening with an even stronger voltage dependence (Kunze et al., 1985) remained almost unaffected. The mean number of openings per sequence in the experiment depicted in Fig. 3, for example, was (at −55 mV) 3.63 in the cell-attached mode but 3.34 in the inside-out mode. Accordingly, the decay kinetics of reconstructed I_{Na} remained unaffected (Fig. 3*C*), a result which provides another argument against a relevant discrepancy of the test potential in both recording conditions. The mean number of openings per sequence changed by a factor of 0.95 ± 0.09 (n = 7) and τ_{decay} of I_{Na} by a factor of 1.01 ± 0.06 (n = 7).

Very similar results were obtained in eight patches excised in a K^+saline with 140 mmol/liter Cl^-, indicating that major kinetic properties of Na^+ channels are not affected by Cl^- concentration changes.

To assess the influence of artificial anions, the cytoplasmic effect of an isotonic KF solution was studied. Open-time probability density functions revealed, again, an increase of τ_{open} and the persistence of the unimodal frequency event distribution (Fig. 4). But Fig. 4 also demonstrates that reopening and I_{Na} decay kinetics remained no longer unaffected: the mean number of sequential openings (at −65 mV) rose from 2.83 to 3.29 and τ_{decay} from 2.2 to 3.3 msec. Nevertheless, I_{Na} decay could be best fitted by a single exponential. As a total from four experiments, the mean number of openings rose by a factor of 1.23 ± 0.04 and τ_{decay} by a factor of 1.32 ± 0.06.

A retarded Na^+ inactivation has been reported in voltage-clamped axons internally perfused with F^--containing solutions (for review *see* Meves, 1978). Comparing these data with the present results suggests, however, a much stronger responsiveness of axonal Na^+ inactivation to F^-.

Does Na^+ Channel Gating Change with Time in Cell-Free Conditions?

The less pronounced gating changes observed on patch excision in a quasi-physiological anionic milieu distinctly contrast to recent observations in

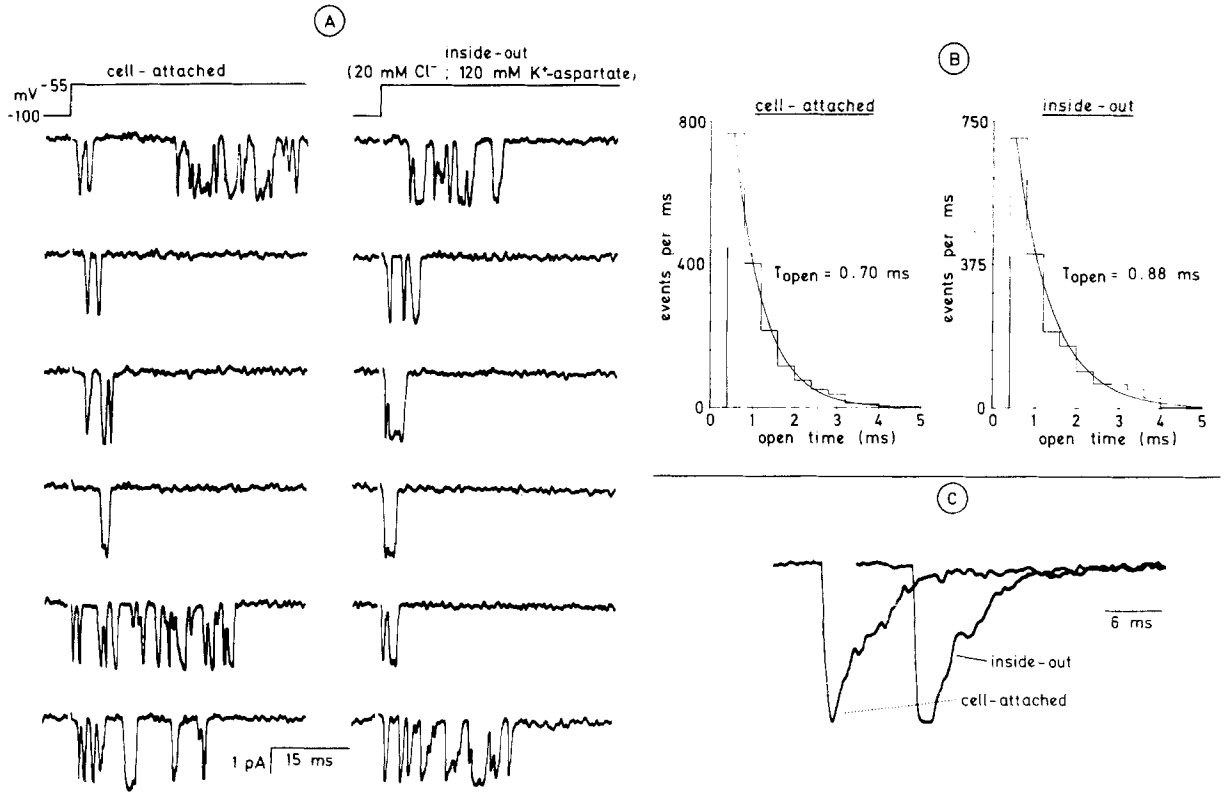

Fig. 3. Na^+ channel activity in the natural environment and after patch excision into a solution with a quasi-physiological anionic composition (20 mmol/liter Cl^-, 120 mmol/liter aspartate). (*A*) Consecutive recordings of elementary Na^+ currents. (*B*) Open-time probability density functions from single-channel events as collected before and after patch excision during sampling periods each of 8 min in duration. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 1726 \exp(-t/0.0007)$ and by $N(t) = \exp(-t/0.00088)$, respectively. (*C*) Reconstructed macroscopic Na^+ currents in normalized form from the same sampling periods as in *B*. I_{Na} decay could be best fitted by a single exponential; τ_{decay} 2.9 msec. Patch 544CA/IO; test potential −55 mV

adult cardiac Na^+ channels from guinea pig myocytes (Nilius, 1988) where, under similar environmental conditions, a particular activity mode may develop. A possible explanation for this discrepancy could be that ultralong openings and increased burst activity represent kinetic properties which require a longer exposure of isolated Na^+ channels to cell-free conditions and could be, therefore, not yet detectable during an early phase of, lets say, 10 min. This point was addressed in a systematical time-dependent analysis in 10 long-living inside-out patches with lifetimes of up to 50 min. As shown in Fig. 5, no evidence was obtained that prolonged cell-free recording conditions evoke additional changes of open-state kinetics. Specifically, open-time probability density functions remained monoexponentially distributed (Fig. 5*A*). This largely excludes that a second, long-lasting open state may be attained. The initial increase in τ_{open} described above was accomplished during the first 8 min after patch excision, at least in the cytoplasmic presence of Cl^-. In the F^- experiments, however, the steady state occurred somewhat later. Similarly, I_{Na} decay kinetics tended to be retarded with time, but it should be emphasized that the increase in τ_{decay} was only weak and statistically not significant (*see* Fig. 5*C*).

Reopening proved to be rather insensitive to prolonged cell-free conditions, too. In some experiments (*see* Fig. 6), however, a particular activity mode appeared during a later stage characterized by the occurrence of ultralong opening sequences. They can be easily detected in a histogram analysis because they do not fit the monoexponential frequency event distribution of the number of openings per sequence. Ultralong opening sequences cannot be *a priori* considered as being specifically related to prolonged cell-free conditions. Na^+ channels in their natural environment, i.e., in cell-attached recording conditions, also switch occasionally to this particular mode (Kohlhardt, Fichtner & Fröbe, 1988). The incidence of ultralong opening sequences was time independent. For example, their relative contribution to opening sequences amount to 1.5% during the middle-age stage, but 2.1% during the later stage of the experiment depicted in Fig. 6. That this type of burst activity is unlikely to reflect a

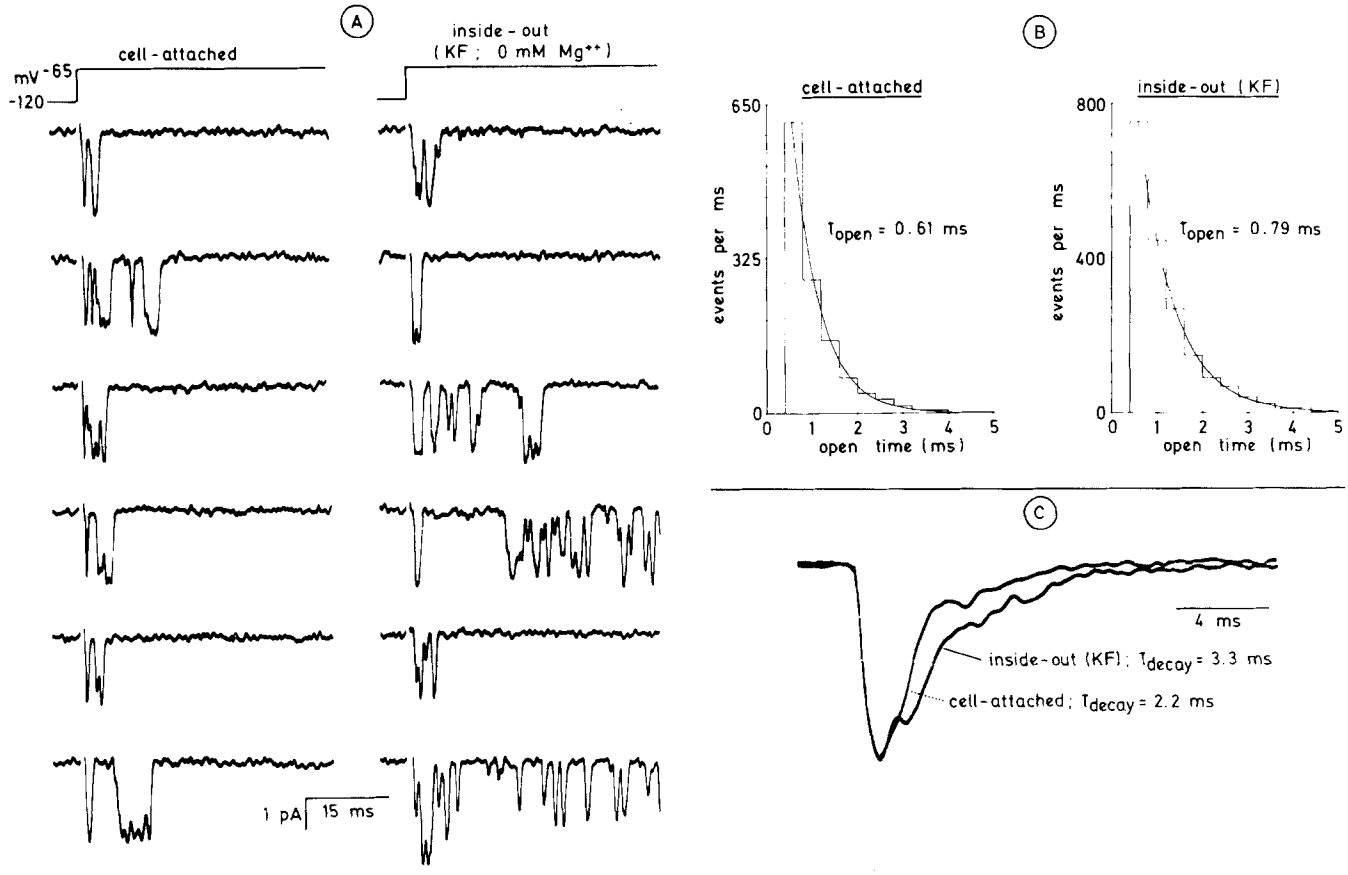

Fig. 4. Na^+ channel activity in the natural environment and after patch excision into a F^--containing solution. (*A*) Consecutive recordings of elementary Na^+ currents. (*B*) Open-time probability density functions constructed from single-channel events collected before and after patch excision during sampling periods each of 8 min in duration. By disregarding the first bin of 0.4 msec, the histograms could be best fitted by $N(t) = 1555 \exp(-t/0.00061)$ and $N(t) = 1586 \exp(-t/0.00079)$, respectively. (*C*) Superimposed reconstructed macroscopic Na^+ currents in normalized form from the same sampling periods as in *B*. I_{Na} decay was best fitted by a single exponential; τ_{decay} was 2.2 msec in the cell-attached mode and 3.3 msec after patch excision. Patch 562CA/IO; test potential −65 mV

particular open state becomes readily apparent from the open time during those episodes. It ranged between 0.61 and 1.15 msec (at −65 mV) and was close to τ_{open} (0.79 msec) of the whole ensemble. Ultralong episodes always ceased spontaneously within some 10 msec after the onset of the command impulse (*see* inset of Fig. 6) and never persisted during the whole 70 msec of membrane depolarization.

Low Temperature Accentuates F^- Effects

Although patch excision in an artificial anionic milieu did influence Na^+ channel gating, only minor quantitative changes occurred when compared with results of Horn and Vandenberg (1986) in neuronal Na^+ channels or, more importantly, of Kirsch and Brown (1989) likewise in neonatal rat heart Na^+ channels. The transition in a F^--containing cytoplasmic environment evoked in those studies a significant channel activation including a prolongation of the conductive state and an increase in channel reopening which is mirrored in a several-fold slowing of I_{Na} decay kinetics. One cannot exlude that distinctly different experimental conditions, namely a temperature of 19°C in the present experiments but of 9–11°C in those of Kirsch and Brown (1989) account for this discrepancy. To test this hypothesis, a series of four inside-out patches with a cytoplasmic F^- environment and initially kept at 19°C was cooled to 9°C. Cooling most prominently increased burst activity. In the experiment illustrated in Fig. 7, the mean number of openings per sequence rose (at −65 mV) from 3.44 to 4.66 without the occurrence of ultralong opening sequences. Burst-like activity persisted for several tens of milliseconds during membrane depolarization and, in a considerable fraction

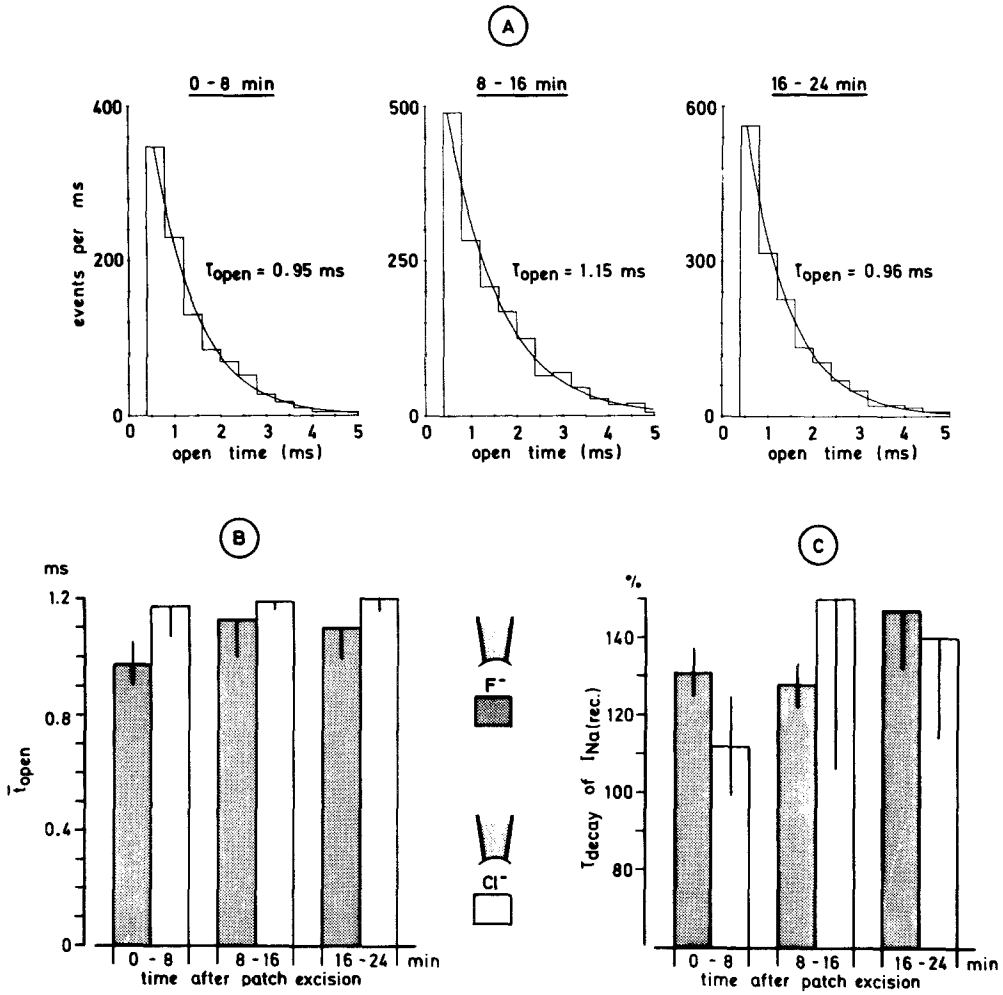

Fig. 5. Time-dependent changes of open-time probability functions (*A*), $\bar{t}_{open}$ (*B*) and I_{Na} decay kinetics (*C*) after exposing Na^+ channels to cell-free conditions. The open-time probability density functions shown in *A* were constructed from an individual patch and could be best fitted by a single exponential with time constants as indicated (patch 56010; test potential −60 mV). The shadowed columns in *B* and *C* represent mean values of four inside-out experiments exposed to 140 mmol/liter F^- (test potential −65 mV); the light columns symbolize mean values of 10 inside-out experiments exposed to 140 mmol/liter Cl^- (test potential −50 mV). Vertical bars indicate SEM. In *C*, 100% refers to the value of τ_{decay} obtained before patch excision in the cell-attached mode

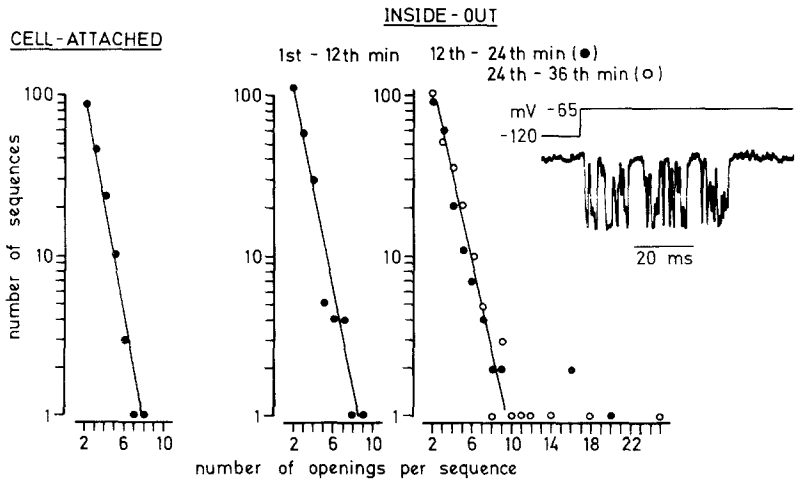

Fig. 6. Reopening during cell-attached and inside-out recording conditions. Frequency event distributions of the number of openings per sequence are shown. Note that a monoexponential fit can be only obtained during later stages (12^{th}–24^{th} min and 25^{th}–36^{th} min, respectively) by disregarding sequences with the largest number of openings. One of them is demonstrated in the inset. Patch 562IO; test potential −65 mV; cytoplasmatically exposed to 20 mmol/liter Cl^- and 120 mmol/liter aspartate

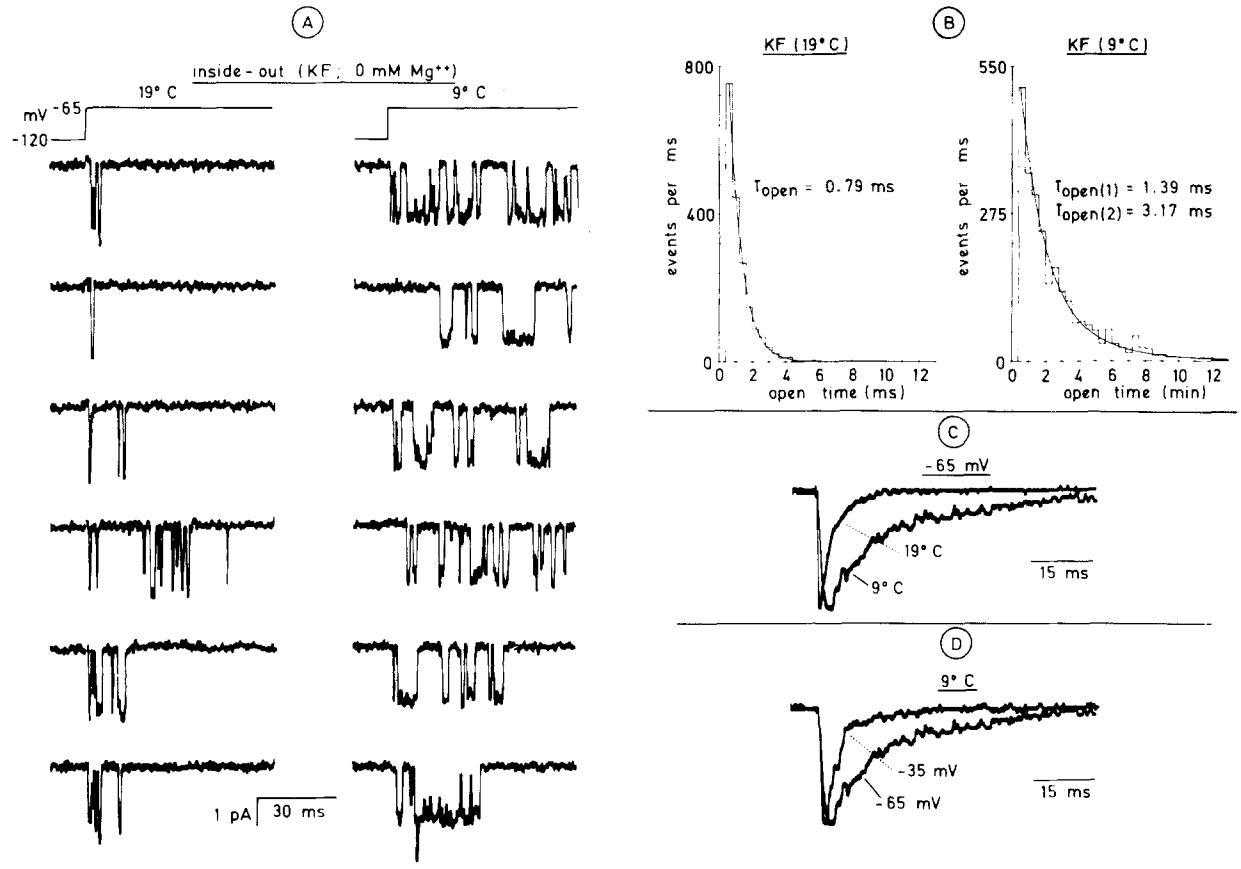

Fig. 7. Na^+ channel activity in the cytoplasmic presence of 140 mmol/liter F^- at 19°C and after cooling to 9°C. (*A*) Consecutive records of elementary Na^+ currents. (*B*) Open-time probability density functions constructed from single-channel events. By disregarding the first bin of 0.4 msec, the histograms could be best fitted, at 19°C, by $N(t) = 1586 \exp(-t/0.00079)$ and, at 9°C, by $N(t) = 534 \exp(-t/0.00138) + 165 \exp(-t/0.000317)$. (*C*) and (*D*) Superimposed, reconstructed macroscopic Na^+ currents in normalized form before and after cooling (*C*). *D* demonstrates the change in I_{Na} decay kinetics in response to a depolarizing shift in test potential; τ_{decay} was 12.9 msec at −65 mV but 3.2 msec at −35 mV. Patch 5621O; test potentials −65 and −35 mV

of activity sweeps, was only terminated by back-clamping the membrane to the holding potential. The other remarkable effect was a change of the open-time frequency event distribution from unimodal to bimodal, thus, indicating the occurrence of a second open state with a longer dwell time. The ratio $\tau_{open(2)}/\tau_{open(1)}$ was found to range between 1.8 and 3.3. The relative proportion of second open-state events can be calculated from the area under both exponentials of the open-time density function. As a result of four experiments, the ratio of second open-state events to first open-state events varied strongly between 2.63 and 0.23, i.e., the longer lasting open state may dominate in one individual patch but not in another one. This large variance suggests a heterogeneous channel responsiveness to F^- which also explains why the second open state remained undetectable in three other experiments.

In order to stress the idea that the particular experimental condition, namely the cytoplasmic F^- presence combined with a lowered temperature but not simply cooling facilitates burst-like activity, the response of Na^+ channels to the same decrease in temperature from 19 to 9°C but facing to a cytoplasmic anionic milieu containing 20 mmol/liter Cl^- plus 120 mmol/liter aspartate was examined. Under the latter conditions, burst-like activity not only proved rather insensitive to cooling (mean Q_{10} of the mean number of openings per sequence: 1.06 ± 0.07) but, much more importantly, reacted in the opposite way and tended to be reduced. Consequently, when compared at −60 mV, the mean number of openings per sequence amounted to 3.00 ± 0.15 and, thus, took a significantly smaller value than in the presence of F^- (3.50 ± 0.11). In three out of eight inside-out patches, the open-time probability density functions changed from an unimodal to a bimodal distribution similar to the F^- experiments, but the relative contribution of openings governed by $\tau_{open(2)}$ was much smaller.

It is, therefore, tempting to assume that the combined influence of F^- with hypothermia modulates Na^+ inactivation. But in contrast to the action of other modifying agents such as BTX or protein reagents, Na^+ inactivation remains operative. This was tested by evaluating the voltage dependence of τ_h. In fact, τ_{decay} of the reconstructed macroscopic I_{Na} still responded to a positive potential shift from -65 to -35 mV with a decline (see Fig. 7*B*).

Discussion

As the present patch-clamp experiments with neonatal rat heart myocytes demonstrate, cardiac Na^+ channels largely preserve major elementary properties such as open-state kinetics or burst kinetics after isolation from their natural, cellular environment when a quasi-physiological anionic milieu faces their cytoplasmic surface under cell-free conditions. This indicates, first of all, that the detachment of the sarcolemma from the cytoskeleton which is caused by patch excision and may be followed by some structural changes of the membrane (for review *see* Vusse, van Bilsen & Reneman, 1989) does not necessarily impair Na^+ channel activity. But it is not justified to conclude from the uniform activity mode seen under cell-attached and inside-out recording conditions that Na^+ channels would be definitely insensitive to cellular metabolic events.

Many of them, including rat heart Na^+ channels, are proven to be a good substrate for phosphorylation by cAMP-dependent protein kinase (Gordon et al., 1988) which enables them to respond quite sensitively to interventions that are accompanied by an elevated cellular cAMP level with a reduction in open probability (for review *see* Catterall, 1988). In addition, as found in cell-free recording conditions, several metabolites of the glycolytic pathway such as 2.3 diphosphoglycerate and glyceroaldehydephosphate (Kohlhardt, Fichtner & Fröbe, 1989) or the degradation product of the physiological membrane constituent phosphatidylcholine, lysophosphatidylcholine, (Burnashev et al., 1989) enhance burst activity. Exposing cardiocytes to micromolar concentrations of the peptide angiotensin II was recently reported to produce a very similar response (Nilius, Tytgat & Albitz, 1989) which seems to be mediated by an intracellular cascade of molecular reactions, most probably due to stimulating phosphoinositide hydrolysis and a consecutive activation of protein kinase C. This sensitivity of reopening seems to contrast, but in a rather superficial view, to the uniform channel-gating mode in both the cell-attached and the inside-out recording conditions. In reality, however, the channels *in situ* sensed the intracellular space of cardiocytes kept under "normal" conditions, i.e., conditions which should prevent the formation of the ischemic degradation product lysophosphatidylcholine and where the phosphoinositide hydrolysis remained unstimulated. The physiological significance of the metabolites of glycolysis seems intimately related to the so far unresolved problem whether their concentration may attain an effective range in the vincinity of the cytoplasmic channel surface.

The mechanism underlying the reduced exit rate from the open state and, specifically, the link between the cell-free recording conditions and this kinetic alteration awaits further clarification. The prolongation of the conductive state develops irrespective of the anionic composition at the cytoplasmic membrane surface and was also observed in Na^+ channels from GH_3 cells (Horn & Vandenberg, 1986) facing to F^- or aspartate and, as the present experiments confirm, in neonatal cardiac Na^+ channels exposed to F^- (Kirsch & Brown, 1989). Surprisingly, Na^+ channels from adult cardiocytes need a much longer exposure until long-lasting openings occur (Nilius, 1988), whilst their neuronal relatives from the rat brain possess open-state kinetics which are apparently resistant to patch excision (Kirsch & Brown, 1989).

Increased burst activity occurred in the present experiments only on patch excision in a F^--containing solution. Obviously, it is the cytoplasmic presence of this artificial anion which evokes enhanced burstiness but not the cell-free conditions *per se*. In reviewing Na^+ inactivation, Meves (1978) summarized I_{Na} data from voltage-clamped axons internally perfused with artificial solutions in order to demonstrate a theoretically significant controversy to the classical data of Hodgkin and Huxley (1952): in the presence of F^-, τ_h is clearly larger than predicted from their experiments at the same temperature and voltage which were performed in the absence of F^-. Meves (1978) already presented the explanation that fluoride salts slow inactivation. Consequently, Na^+ channels will attain their inactivated, absorbing (Aldrich, Corey & Stevens, 1983) state later during membrane depolarization and are, therefore, capable of enhanced burst activity.

The F^- action formally resembles, to some extent, the influence of the metabolites just mentioned above which likewise facilitate reopening. Since gating in cooled channels operates with lower rates, it is tempting to speculate that the accentuation of F^- action seen after a decrease in temperature from 19 to 9°C is somehow related to these particular conditions. Na^+ channels from GH_3 cells, however, respond already sensitively at 16°C (Horn & Vandenberg, 1986). It is interesting to note that F^-, or

more precisely the AlF_4 complex which is easily formed by the aluminium contamination of the employed glassware (Sternweis & Gilman, 1982) can interfere with some membrane-associated proteins including phosphatases and G-proteins. By mimicking the action of GTP, AlF_4 may basically activate the whole family of G-proteins (Bigay et al., 1985) including G_s- and G_i-proteins. Their regulatory influence on enzymatic activities of adenylate cyclase or phosphodiesterase causes secondarily a modulation of ionic channel activity but G-proteins were recently identified to be also capable of interferring directly with ionic channels. Although their significance specifically for voltage-dependent Na^+ channels needs further clarification, at least the G_s-protein cannot be involved in enhanced burst activity. This becomes readily apparent from observations in whole-cell clamped cardiocytes where β_1-adrenergic stimulation remains ineffective to alter the I_{Na} decay kinetics (Ono, Kiyosue & Arita, 1989). Schubert et al. (1989) presented evidence for a direct modulating effect of the activated G_s-protein on cardiac Na^+ channels consisting in a reduction of open probability and burst activity. Whether cardiac Na^+ channels may react sensitively to the activated G_i-protein thereby switching to enhanced burst activity as seen in the cytoplasmic F^- presence remains open. Another and probably more attractive hypothesis arises from the assumption that the Na^+ channel protein is intrinsically sensitive to F^- ions or the AlF_4 complex. If this holds true, F^- or the AlF_4 complex could bind to a superficially located, easily accessible amino acid residue in the cytoplasmic channel surface to promote finally burst activity.

The underlying retardation of the inactivation process seems to be a kinetic response which is specifically related to F^- since other channel modifiers including naturally occurring toxins or drugs follow in interacting with Na^+ channels strictly the all-or-none principle, meaning that binding of these agents inevitably removes inactivation. Merely in the latter case, a Markovian reaction scheme consisting of open and closed configurations but devoid of the absorbing inactivated state can model the channel kinetics.

Although voltage-gated Na^+ channels are basically capable of moving laterally, this diffusion seems to be rather differently pronounced and is apparently related to a particular type of excitable membrane. Using the fluorescence bleaching recovery of I_{Na}, Angelides et al. (1988) obtained evidence in neurons that Na^+ channels located in the cell body are free to diffuse within the soma with a lateral diffusion coefficient, D, in the order of magnitude of 10^{-9} cm^2 sec^{-1}. Na^+ channels installed in the axon hillock show a comparatively much lower mobility; D was found to range between 10^{-10} and 10^{-11} cm^2 sec^{-1}. The restricted mobility seems to reflect a structural pecularity of the axonal region, namely, a cytoskeletal attachment of the surface membrane. In frog skeletal muscle, however, no evidence for lateral mobility could be obtained; the upper limit of D was estimated to be 10^{-12} cm^2 sec^{-1} (Stühmer & Almers, 1982).

Phenomena such as run-up and run-down of Na^+ channel activity as seen in the present experiments sometimes in cell-free conditions might be suggestive for lateral mobility if it is assumed that the spontaneously increasing or decreasing I_{Na} emerges from corresponding changes in the number of functioning Na^+ channels within the patch. This hypothesis basically implies that cardiac Na^+ channels may invade into or leave the patch area under voltage control, i.e., the portion freely spanning the lumen of the patch pipette in order to leave or to enter the other portion of the patch which is tightly wall attached. This ignores physical interactions between the glass wall of the pipette and the channel protein not yet defined precisely but conceivable to exist that, by creating adhesive forces, might well immobilize the channels in the wall-attached region of the membrane. Since run-up and run-down proceeds with a rather low rate which would correspond to a value for D in the order of magnitude of 10^{-10} cm^2 sec^{-1}, rim currents as indicating that particular phase during lateral diffusion when a channel is going to pass the borderline between the wall-attached and the free-patch area should occur, but were not observed.

Another and even major problem in interpreting run-up and run-down of Na^+ channel activity is related to the fact that both phenomena only appeared occasionally. Consequently, if actually existent, lateral Na^+ channel movements will not be principally allowed but would require particular and perhaps artificial conditions being no general feature of the isolated cardiac membrane.

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