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Modulation of single cardiac Na^+ channels by cytosolic Mg^{++} ions

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Abstract. Elementary Na^+ currents were recorded in inside-out patches excised from cultured neonatal rat heart myocytes in order to study the influence of cytosolic Mg^{++} and other bivalent cations present at the cytoplasmic membrane surface on cardiac Na^+ channel gating. Exposing the cytoplasmic membrane surface to a Mg^{++}-free environment shortened the open state of cardiac Na^+ channels significantly. τ_{open} declined to $62 \pm 2\%$ of the value obtained at 5 mmol/l Mg_i^{++}. Other channel properties including the tendency to reopen and the elementary current size either changed insignificantly within a 10% range or remained completely unchanged. An almost identical change of τ_{open} can be caused by switching from a Mn^{++} (5 mmol/l) containing internal solution to a Mn^{++}-free internal solution. But τ_{open} failed to significantly respond to a variation in internal Ni^{++} from 5 mmol/l to 0 mmol/l. The same response to internal Mg^{++} withdrawal was obtained with (−)-DPI-modified, non-inactivating Na^+ channels, indicating that the exit rate from the open state remains as sensitive to cytosolic Mg^{++} variations as in normal Na^+ channels with operating inactivation.

Key words: Na^+ channel gating – Internal bivalent cations – Negative surface charges – DPI-modification

Introduction

Cardiac excitability can be effectively modulated by bivalent cations. As in neuronal tissues (Yamamoto et al. 1984; Worley et al. 1986), external Ca^{++} and Mg^{++} block elementary Na^+ currents in cardiac Purkinje cells (Sheets et al. 1987) and isolated myocytes (Nilius 1988), with a dissociation constant in the millimolar range. Ca^{++} and Mg^{++} most probably compete with Na^+ for the entrance in the Na^+ pore, thereby transiently occluding the latter with very fast kinetics. The resultant fast flicker blockade is manifested as a decrease in elementary current size (Sheets et al. 1987; Nilius 1988) and underlies the well-established membrane-stabilizing, inhibitory action of external bivalent cations. The interaction of Ca^{++} and Na^+ with the Na^+ channel can be modelled by the four barrier Eyring rate theory model and provides insights into the principles of cationic permeation through the open pore. Another influence of bivalent cations on excitability arises from their ability to modify the voltage dependence of steady state Na^+ inactivation (Weidmann 1955; Frankenhaeuser and Hodgkin 1957). The latter action may emerge predominantly from a shielding of fixed negative surface charges.

Cytosolic bivalent cations act predominantly by modulating the voltage-dependent gating of Na^+ channels, at least in the physiologically relevant potential range negative to E_{Na}. However, a significant activation was shown in reconstituted neuronal Na^+ channels to require an internal Ca^{++} concentration in the millimolar range (Cukierman et al. 1988). Clearly, this largely excludes the possibility that physiological Ca^{++} fluctuations in the cytosol, which are associated with the normal cellular function, might influence Na^+ channels and, thus, cardiac excitability.

The present patch clamp experiments with neonatal rat heart myocytes dealt primarily with the influence of cytosolic Mg^{++} ions on cardiac Na^+ channels. Mg^{++} represents the most abundant cation in the cytosol of living cells including myocardium. It was shown to be capable of blocking L-type Ca^{++} channels in myocytes (Agus et al. 1989) and several K^+ conductances including $K^+_{(ATP)}$ channels (Horie et al. 1987) at a physiologically relevant concentration. By determining open state and closed state kinetics in normal and non-inactivating cardiac Na^+ channels, the role of cytosolic bivalent cations for the gating process was defined on the single channel level.

Methods

Elementary Na^+ currents were recorded in inside-out patches with an LM-EPC 5 amplifier by applying the

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standard patch clamp technique (Hamill et al. 1981). The patches were excised from short-time (18–24 h) cultured neonatal rat heart myocytes. The culture and the handling of the cardiocytes have already been described in detail (Kohlhardt et al. 1986). Briefly, the cells were kept in an isotonic K^+ saline buffered with EGTA in order to improve patch stability during an equilibration in the initial 10 minute duration cell-attached configuration. After patch excision, another equilibration of 10 min duration was expected until Na^+ channel activity was recorded in cell-free conditions. Elementary Na^+ currents were triggered by rectangular command impulses of 70–120 ms duration at a rate of 0.67 Hz by stepping the patches from a holding potential between −100 mV and −125 mV to test potentials between −65 mV and −45 mV. The patch clamp recordings were filtered at 1 kHz, digitized with a sampling rate of 5 kHz and stored on floppy discs. The dead time under these recording conditions was 0.2 ms.

After correcting for leakage and residual capacity currents, open and closed kinetics were analyzed by employing the 50% unitary currents methods (Colquhoun and Sigworth 1983). Probability density functions were constructed from non-overlapping single channel events and yielded τ_{open} and τ_{closed}. By neglecting the first bin of 0.4 ms, they were based on an unweighted fit (least square method), i.e. each bin was considered to be of identical significance. To fit late and, therefore, rare openings in normal Na^+ channels with operating inactivation, open time probability density functions were fitted by lumping several bins with a certain minimum of events arbitrarily chosen to be four. The analysis of reopening properties was complicated by the fact that each patch contained more than one Na^+ channel. Therefore, sequential events do not necessarily reflect the activity of one individual Na^+ channel. A count of sequential openings will, consequently, yield only a rough estimate of channel reopening properties.

By adding 3×10^{-6} mol/l (−)-DPI to the pipette solution, modified Na^+ channel activity was evoked. The analysis of τ_{open} and τ_{closed} was based on activity sweeps with an $P_0 > 0.1$. The arguments that this bias eliminates Na^+ channels with normal kinetics not affected by DPI have been presented in detail elsewhere (Kohlhardt et al. 1989a). Moreover, a maximal likelihood analysis (Colquhoun and Hawkes 1983) revealed that high open probability sweeps without overlapping events will reflect the repetitive activity of an individual channel. Burst analysis in (−)-DPI-modified Na^+ channels was based on the bimodal closed time distribution determined individually in each experiment. Gaps within bursts can be discriminated from gaps between bursts by introducing a certain critical gap time which is dependent on the ratio $\tau_{closed(1)}$ to $\tau_{closed(2)}$ and was calculated from the geometric mean of both closed time constants.

τ_{open} and τ_{closed} as will be given in the results are an overestimation of the true values since no attempt was made to correct for missed events. This error is meaningless unless gating properties are defined quantitatively and expressed in terms of rate constants.

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

Solutions (composition in mmol/l)

A. Isotonic K^+ saline (facing the cytoplasmic membrane surface of the inside-out patches): K^+ aspartate 120; KCl 20; Na^+ pyruvate 5; $MgCl_2$ 5 (or 1; or 0); glucose 20; HEPES 10; EGTA 2; pH 7.4. B. Pipette solution (facing the external membrane surface): NaCl 150; $CaCl_2$ 0.03; $MgCl_2$ 1; HEPES 10; pH 7.4. Temperature (controlled by a Peltier element): 19 °C or 9 °C.

Compounds

(−)-DPI was freshly dissolved in dimethylsulfoxide and diluted in pipette solution to give a final concentration of 3×10^{-6} mol/l (dimethylsulfoxide concentration 0.2%).

Results

A first series of experiments with normal, i.e. inactivating Na^+ channels provided evidence that the gating process can be significantly influenced by changes of the cytosolic Mg^{++} concentration. For methodological reasons, the inside-out patches were excised in a Mg^{++}-rich (5 mmol/l) solution referred to as control condition since the exposure to a Mg^{++}-free environment was poorly tolerated and reduced, in most of the cases, the patch life time to a few minutes. Therefore, the reversibility of the Mg^{++} withdrawal was not tested. Figure 1 illustrates results from an inside-out patch studied at 9 °C before and after switching to a Mg^{++}-free cytoplasmic solution. The open time distribution could be described in both cases by a single exponential. τ_{open} (at −65 mV) decreased from 1.44 ms at 5 mmol/l internal Mg^{++} to 0.88 ms at 0 mmol/l. A similar shortening of the open state was obtained in Mg^{++} withdrawal experiments at 19 °C. No significant changes in burst activity occurred. As evaluated from the frequency distribution of sequential openings and with the precautions mentioned in the Methods section the mean number of sequential openings decreased by 10% or less, in the experiment shown in Fig. 1 from 5.5 to 5.0. It was, therefore, not surprising to see that I_{Na} decay kinetics in reconstructed macroscopic Na^+ currents (not shown) were not affected by internal Mg^{++} removal. Ultralong opening sequences not fitting the single exponential frequency event distribution (see Fig. 1 C) could also be observed in Mg^{++}-free conditions. This suggests that Na^+ channels preserve their capability to switch occasionally in such a particular activity mode (Patlak and Ortiz 1985; Kohlhardt et al. 1988) in the absence of internal bivalent cations.

In an attempt to exclude the possibility that, perhaps by a mechanical alteration of the patch, the cytosolic solution change might artifactually affect the gating of Na^+ channels, open time probability density functions were analyzed in 3 inside-out patches kept throughout at 5 mmol/l Mg^{++} and exposed to a sham superfusion. Apart from a 5% change, τ_{open} remained unaffected.

A significant change of the open state kinetics required the complete Mg^{++} withdrawal from the cytosolic envi-

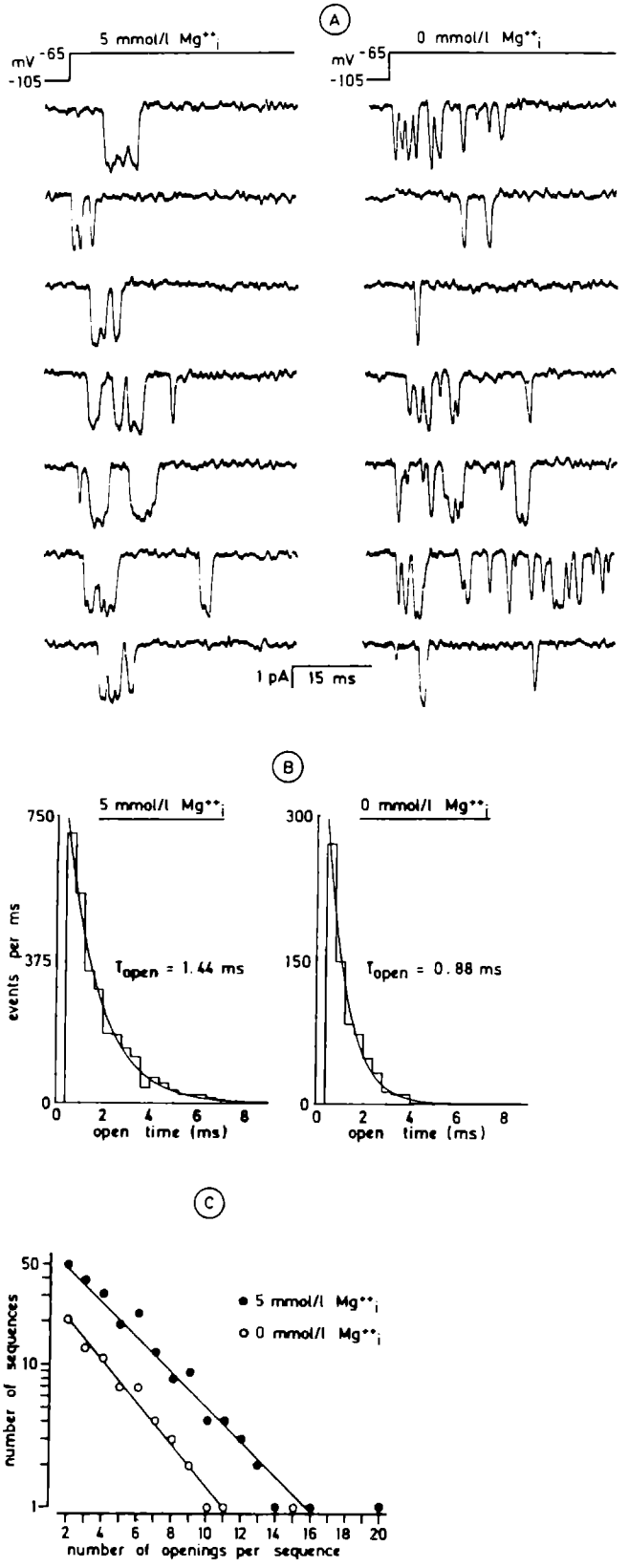

Fig. 1 A–C. The influence of cytosolic Mg^{++} variations on Na^+ channel gating. **A** Inside-out recordings of elementary Na^+ currents, selected activity sweeps at 5 mmol/l Mg_i^{++} (left) and at 0 mmol/l Mg_i^{++} (right). **B** Open time probability density functions. The histograms were constructed from 1285 events at 5 mmol/l Mg_i^{++} (left) and from 345 events at 0 mmol/l Mg_i^{++} (right). By disregarding the first bin of 0.4 ms, they could be best fitted by $N(t)=1013 \exp(-t/0.00144)$ and by $N(t)=494 \exp(-t/0.00088)$, respectively. **C** Frequency event distribution of the number of sequential Na^+ channel openings at 5 mmol/l Mg_i^{++} (*filled circles*) and at 0 mmol/l Mg_i^{++} (*open circles*). The regression lines are fitted by eye. Note the sequence with 20 opening events at 5 mmol/l Mg_i^{++} and the sequence with 15 opening events at 0 mmol/l Mg_i^{++} not fitting the respective unimodal distributions. Patch 617IO; holding potential −105 mV, test potential −65 mV; temperature 9 °C

ronment. Figure 2 shows that a decrease in the Mg^{++} concentration from 5 mmol/l to 1 mmol/l was less effective and, as likewise studied in a potential range between −65 mV and −55 mV, decreased τ_{open} to only 84±2% ($n=5$) of the initial control value at 5 mmol/l Mg^{++}. Switching from 5 mmol/l Mg^{++} to 0 mmol/l Mg^{++}, however, caused a decrease of τ_{open} to 62±2% ($n=5$). That such a modulation of open state kinetics is not specifically related to Mg^{++} became apparent in another series of experiments when Mn^{++} served instead of Mg^{++} as the only cytoplasmic bivalent cation. Switching from 5 mmol/l Mn^{++} to a Mn^{++}-free cytosolic environment caused a quantitatively very similar effect and led to a reduction of τ_{open} to 45% of the initial control value.

Removal of other bivalent cations from the cytoplasmic membrane surface was found to be less effective, if at all. This was evidenced in two inside-out patches exposed initially to Ni^{++} (5 mmol/l) as the only bivalent cation. Figure 3 demonstrates that Ni^{++} withdrawal left τ_{open} almost unchanged. Additional support that Ni^{++} cannot fully substitute other bivalent cations in modulating Na^+ channel gating arises from a comparison of τ_{open} at 19 °C and the same test potential (−60 mV) in the cytoplasmic presence of 5 mmol/l Ni^{++} and in the cytoplasmic presence of 5 mmol/l Mg^{++}: τ_{open} was 1.21±0.05 ms ($n=8$) with Mg^{++} but 1.00±0.03 ms ($n=3$) with Ni^{++}.

Experiments with (−)-DPI-modified Na^+ channels revealed, first of all, that the modulating effect of cytoplasmic Mg^{++} on open state kinetics is not restricted to cardiac Na^+ channels with intact inactivation. There are several lines of evidence indicating that an interaction of the S-enantiomer of DPI 201-106 with a channel-associated binding site (Romey et al. 1987) eliminates Na^+ inactivation (Kohlhardt et al. 1986). This is associated with a several-fold increase of open time. A change of the cytosolic Mg^{++} concentration from 5 mmol/l to 0 mmol/l caused, again, a significant shortening of the open state (Fig. 4). As an average at 9 °C and −45 mV, τ_{open} declined from 5.98±0.59 ms to 3.54±0.65 ms ($n=3$), i.e. it decreased to 58±6.2% of the initial control value. It is important to note that this quantitative response agrees with the respone of a normal Na^+ channel with operating inactivation. The unimodal open time density function does not necessarily persist after Mg^{++} removal. In 1 out of 4 experiments, the best fit of the open time histogram was biexponential (Fig. 5 A) suggesting that Na^+ channels may attain two conducting states in this particular condition: a dominating short-lasting one ($\tau_{open(1)}=$ 1.02 ms) and a several-fold longer open state ($\tau_{open(2)}=$ 7.07 ms). It was surprising to see that $\tau_{open(2)}$ coincides with the value for τ_{open} found in the same experiment in the presence of Mg^{++}. In addition, Mg^{++} removal led

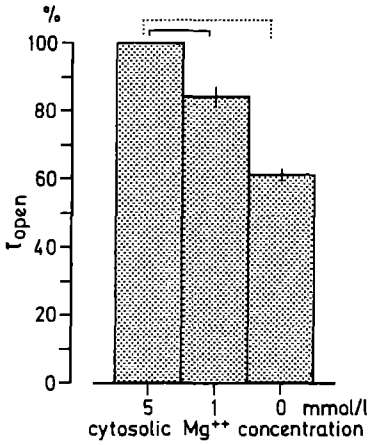

Fig. 2. Percent changes of τ_{open} in response to an internal Mg^{++} concentration change from 5 mmol/l to 1 mmol/l ($n=5$) and from 5 mmol/l to 0 mmol/l ($n=5$). *Vertical bars* in the columns indicate SEM

consistently to a shortening of the mean burst time to $73\pm2.5\%$ ($n=3$) of the control value at 5 mmol/l Mg^{++}.

Figure 4 also shows that internal Mg^{++} concentration changes fail to influence i_{unit}. This result was expected since recent observations in rat brain type II Na^+ channels (Pusch et al. 1989) indicate that internal Mg^{++} can interfere only with cationic movements through Na^+ channels when they are outwardly directed, i.e. at membrane potentials positive to E_{Na}.

(−)-DPI-modified Na^+ channels offer the opportunity for analyzing closed time kinetics. Persisting burst activity during the whole membrane depolarization in activity sweeps without superpositions (see Fig. 4) indicate with a reasonable likelihood the repetitive opening of an individual Na^+ channel whose closing becomes manifest as gaps between opening events. The closed time probability density functions depicted in Fig. 5 B show, first of all, that the bimodal event distribution which is characteristic for (−)-DPI-modified Na^+ channels remains after Mg^{++} withdrawal. Both $\tau_{closed(1)}$ and $\tau_{closed(2)}$ were found to increase. On average, the former time constant rose to $169\pm17.8\%$ of the control but the latter time constant varied only insignificantly to $93\pm20.5\%$ ($n=3$). In 2 out of 3 experiments, the relative contribution of closing events governed by $\tau_{closed(2)}$ rose slightly in the absence of cytosolic Mg^{++}.

These gating changes were accompanied by a reduction of open probability (P_0). Since $I_{Na}=i\cdot N\cdot P_0$ (where i means unitary current size and N the number of Na^+ channels in the patch), peak I_{Na} reflects the moment where NP_0 attains its maximum during membrane depolarization. I_{Na} was reconstructed from ensemble averages each based on 80 sweeps and taken just before and after switching from 5 mmol/l to 0 mmol/l internal Mg^{++}. Internal Mg^{++} removal reduced NP_0 to $40\pm8.5\%$ ($n=3$) in normal Na^+ channels and to $42\pm8.9\%$ ($n=3$) in (−)-DPI-modified Na^+ channels. This basically agrees with the response of reconstituted neuronal Na^+ channels when exposed to a Ca^{++}-free internal environment (Cukierman et al. 1988) and can be likewise explained with a modulation of the P_0-E_m relationship reported to be shifted to a more positive potential range in the absence of cytosolic bivalent cations. A systematic analysis of this relationship was complicated by the fact that most of the inside-out patches tended to deteriorate after internal Mg^{++} withdrawal which reduced their life time to a few minutes.

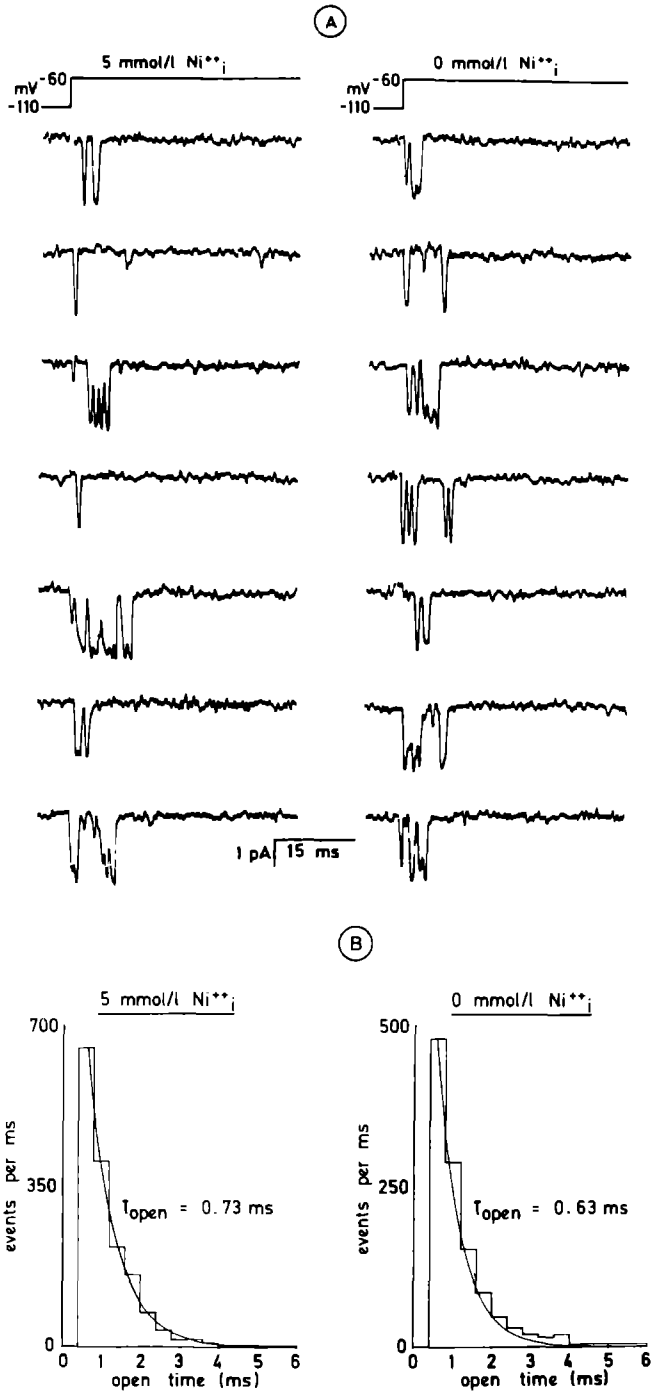

Fig. 3 A, B. The influence of cytosolic Ni^{++} variations on Na^+ channel gating. **A** Inside-out recordings of elementary Na^+ currents, selected activity sweeps at 5 mmol/l Ni_i^{++} (left) and at 0 mmol/l Ni_i^{++} (right). **B** Open time probability density functions. The histograms were constructed from 818 events at 5 mmol/l Ni_i^{++} and from 562 events at 0 mmol/l Ni_i^{++}. By disregarding the first bin of 0.4 ms, they could be best fitted by $N(t)=1537\exp(-t/0.00073)$ and by $N(t)=1204\exp(-t/0.00063)$, respectively. Patch 624IO; holding potential −110 mV, test potential −60 mV; temperature 19 °C

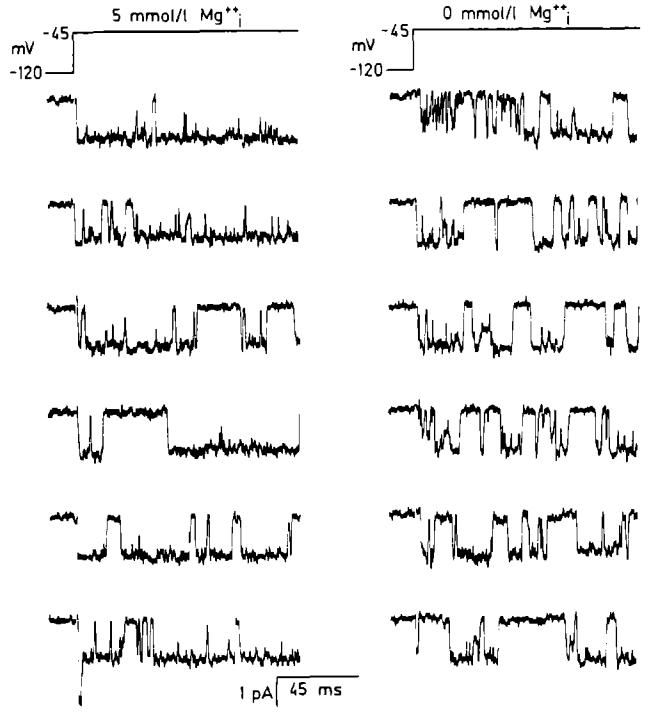

Fig. 4. The influence of cytosolic Mg^{++} variations on (−)-DPI-modified Na^+ channels. Inside-out recordings of elementary Na^+ currents; shown are selected activity sweeps with a $P_0 > 0.1$ (see methods). Substate openings (see row 3 and 4, right) can also happen in the presence of Mg_i^{++} and are, therefore, not specifically related to Mg^{++} withdrawal. Patch 616IO; holding potential −120 mV, step potential −45 mV; temperature 9 °C

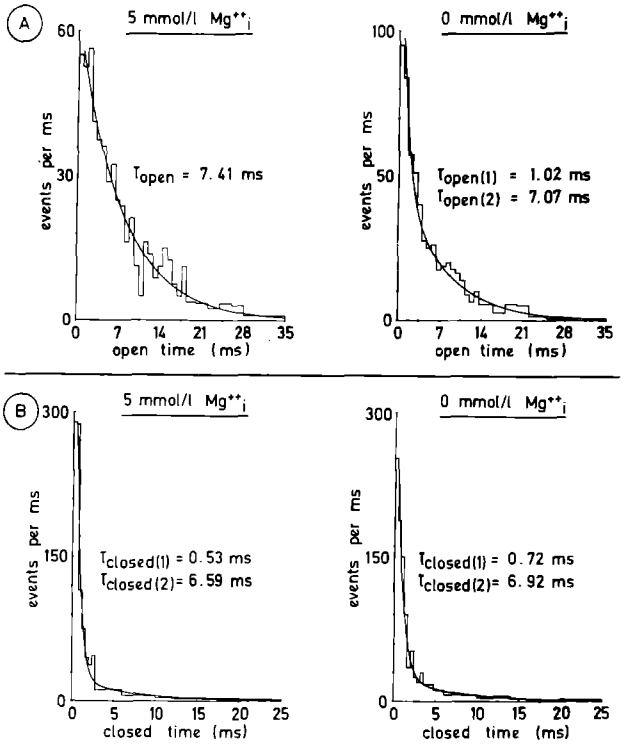

Fig. 5. **A** The influence of internal Mg^{++} withdrawal on open state kinetics of (−)-DPI-modified Na^+ channels. The open time probability density functions were constructed from 484 events at 5 mmol/l Mg_i^{++} and from 483 events at 0 mmol/l Mg_i^{++}. By disregarding the first bin of 0.4 ms, they could be best fitted by $N(t) = 63 \exp(-t/0.00741)$ and by $N(t) = 131 \exp(-t/0.00102) + 52 \exp(-t/0.00707)$, respectively. **B** Changes in closed state kinetics of (−)-DPI-modified Na^+ channels after internal Mg^{++} withdrawal. The closed time probability density functions were constructed from 482 events at 5 mmol/l Mg_i^{++} and from 467 events at 0 mmol/l Mg_i^{++}. By disregarding the first bin of 0.4 ms, they could be best fitted by $N(t) = 751 \exp(-t/0.00053) + 25 \exp(-t/0.00659)$ and $N(t) = 406 \exp(-t/0.00072) + 26 \exp(-t/0.00692)$, respectively. Patch 616IO; holding potential −120 mV, step potential −45 mV; temperature 9 °C

Discussion

The present inside-out patch clamp experiments demonstrate that bivalent cations including Mg^{++} and Mn^{++} acting at the cytoplasmic membrane surface are able to modulate the gating kinetics of cardiac Na^+ channels. In the absence of cytosolic Mg^{++} and Mn^{++}, normal and non-inactivating Na^+ channels leave the conducting state faster and attain, as evidenced with (−)-DPI-modified channels, the conducting state slower than in the presence of these bivalent cations. Clearly, bivalent cations at either membrane surface modulate Na^+ channel gating in exactly the opposite fashion. As shown in BTX-modified Na^+ channels reconstituted in planar bilayers with Ba^{++}, the presence of bivalent cations at the external side of the membrane decreased the opening rate and increased the closing rate (Cukierman and Krueger 1990). This agrees with the reaction of adult cardiac (−)-DPI-modified Na^+ channels where τ_{open} was shown to be a function of external Ca^{++} and steeply decreases with elevated Ca^{++} concentrations in order to exhibit saturation (Nilius 1988).

The voltage-dependent gating process of Na^+ channels can be modulated by external (Frankenhaeuser and Hodgkin 1957) and internal (Cukierman et al. 1988) Ca^{++}. According to the surface-potential theory of Frankenhaeuser and Hodgkin (1957), Ca^{++} and other bivalent cations bind or adsorb to fixed negative surface charges. These charges are provided by phospholipids in the headgroups of the plasma membrane and are symmetrically located at both the external and internal membrane surface. Since surface charges contribute to the electric field across the membrane, their screening by bivalent cations alters this field which can be sensed by the voltage sensor of the Na^+ channel. By determining single channel exit rates from the conducting and the non-conducting state, the modulation influence of internal bivalent cations could be directly demonstrated in the present experiments. This agrees with the capability of internal Ca^{++} to shift the $P_0 - E_m$ relationship along the voltage axis (Cukierman et al. 1988). As Ca^{++} was found in reconstitution experiments to retain its effectiveness when Na^+ channels are incorporated in artificial membranes formed by neutral phospholipids and lacking surface charges, Cukierman et al. (1988) assumed that shifts of the $P_0 - E_m$ relationship induced by Ca^{++} in this particular situation can only be produced by a direct interaction of Ca^{++} with the Na^+ channels. A more complicated situation seems to exist in biological membranes with charge-carrying phospholipids. If the surface charge mechanism is predominantly involved in gating changes,

the exit rates from both the conducting and the non-conducting state would be expected to respond in a quantitatively identical manner when the internal Mg^{++} concentration changes. In (−)-DPI-modified Na^+ channels, τ_{open} increased due to an internal Mg^{++} change from 0 mmol/l to 5 mmol/l by the same factor (being on average 1.7) as $\tau_{closed(1)}$ due to an internal Mg^{++} change from 5 mmol/l to 0 mmol/l. $\tau_{closed(2)}$ was found to react differently. $\tau_{closed(2)}$ represents a second, long-living closed state usually detectable in non-inactivating Na^+ channels irrespective of the tool employed for modification, (−)-DPI, BTX (Keller et al. 1986) or protein reagents (Kohlhardt et al. 1989 b). It should be emphasized that the determination of the closed state kinetics and in particular the estimation of $\tau_{closed(1)}$ may be seriously complicated by the limitations in the recording conditions. Thus, the differential Mg^{++} sensitivity of both closed states remains to be firmly established.

When compared with Mg^{++}, internal Ni^{++} variations were less effective in modulating τ_{open}. This might support the hypothesis that Mg^{++} not only acts by screening surface charges but also by influencing the Na^+ channel directly. This implies that the putative binding site at or near the cytoplasmic Na^+ channel surface is capable of discriminating among several bivalent cations. As evidenced with Ca^{++} (Cukierman et al. 1988), Na^+ channels seem symmetrically organized in this respect and likewise expose at their external side a similar binding site to the surrounding bulk solution.

To consider the possible functional significance of the modulating effect of internal Mg^{++}, it is important to note that living cells including cardiac tissue effectively control the intracellular free Mg^{++} concentration in order to maintain a level which is close to 1 mmol/l. A significant variation in response to physiologically relevant influences on cellular function still remains to be elucidated. A major Mg^{++} fraction forms with ATP the chelated Mg-ATP complex and represents a potential source of free Mg^{++} ions. Circumstantial evidence has been presented (Kirkels et al. 1989; Murphy et al. 1989) that a depletion of ATP stores in hypoxic myocardium is associated with a rise in intracellular free Mg^{++} exceeding the physiological level several-fold. Since a five-fold cytosolic Mg^{++} variation from 1 mmol/l to 5 mmol/l proved to be less effective in modulating the open state kinetics of cardiac Na^+ channels, even significant internal Mg^{++} fluctuations are conceivable without major changes of the Na^+-dependent excitation process in heart muscle. Nevertheless, a cytosolic Mg^{++} rise in ischemic myocardium can have a secondary effect on cardiac excitability since L-type Ca^{++} channels have been reported to become significantly blocked at elevated internal Mg^{++} (Agus et al. 1989) will reduce the refractory period.

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