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EFFECTS OF Mg^{2+} ON BASAL AND β-ADRENERGIC-STIMULATED DELAYED RECTIFIER POTASSIUM CURRENT IN FROG ATRIAL MYOCYTES

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SUMMARY

1. The effects of internal Mg^{2+} ions on the delayed rectifier potassium current (I_K) of bull-frog atrial myocytes were studied using the whole-cell configuration of the patch-clamp technique with a perfusable patch electrode.

2. Initial variations in I_K amplitude were dependent on $[Mg^{2+}]_i$. With $[Mg^{2+}] > 1$ mM, the amplitude of I_K usually decreased after initiating the whole-cell recording configuration (run-down); with $[Mg^{2+}]_i < 1$ mM, I_K usually increased (run-up). Mg^{2+} blocked I_K with an apparent half-maximal effect of 0·6 mM $[Mg^{2+}]_i$.

3. The basal free $[Mg^{2+}]_i$, indicated by the amplitude of I_K before run-up or run-down, was estimated from the relationship between $[Mg^{2+}]_i$ and I_K to be 0·8 mM.

4. The amplitude of both the activation curve and the instantaneous voltage–current relationship was decreased by increasing $[Mg^{2+}]_i$. Under these conditions, the voltage dependence of I_K was not affected.

5. The rate of activation of the current at +40 mV was slowed by increasing $[Mg^{2+}]_i$ with little effect on the rate of deactivation at −50 mV. This is in contrast to the effects of isoprenaline, which speeded activation and slowed deactivation.

6. Isoprenaline increased I_K on average by about 2·5 pA/pF, whether I_K had previously run down or not, and regardless of $[Mg^{2+}]_i$. The reversibility of isoprenaline was partially inhibited at $[Mg^{2+}]_i < 1$ mM.

7. It is concluded that Mg^{2+} affects I_K via several mechanisms that might include a Mg^{2+}-dependent phosphatase.

INTRODUCTION

Recently, the involvement of magnesium ions (Mg^{2+}) in the modulation of the electrical activity of a number of cell types, especially cardiac cells, has received considerable attention (White & Hartzell, 1989). In the heart, several ion channels are sensitive to the concentration of Mg^{2+} present at the intracellular face of the membrane. In particular, inward rectification of a family of potassium channels (I_{K1}, $I_{K,ACH}$, $I_{K,ATP}$) is due to a voltage-dependent block of the outward current by

intracellular Mg^{2+} ions entering the permeation pathway (Horie & Irisawa, 1987, 1989; Horie, Irisawa & Noma, 1987; Vandenberg, 1987; Matsuda, Saigusa & Irisawa, 1987; Matsuda, 1988; Ishihara, Mitsuiye, Noma & Takano, 1989). In the absence of Mg^{2+}, these channels do not rectify and the current–voltage relationship is linear. Furthermore, intracellular Mg^{2+} can block the cardiac calcium current carried by calcium ions in frog ventricle if the channels have been previously phosphorylated by the cyclic AMP-dependent protein kinase A (White & Hartzell, 1988) and alter the kinetics of basal calcium current carried by barium ions (Hartzell & White, 1989). Mammalian cardiac calcium channels, however, do not appear to require the phosphorylation step to be modulated by intracellular Mg^{2+} (Agus, Kelepouris, Dukes & Morad, 1989). Some very important aspects of magnesium homeostasis, such as the exact intracellular concentration of Mg^{2+} and whether and how it is regulated, are still unanswered. $[Mg^{2+}]_i$ appears to be in the submillimolar or millimolar range and may be regulated in part by a sodium-dependent process, which may be a Na^+–Mg^{2+} exchange (see Hartzell & White, 1989).

We have previously reported that the delayed rectifier potassium current (I_K) in bull-frog heart is sensitive to Mg^{2+} in the submillimolar range (Duchatelle-Gourdon, Hartzell & Lagrutta, 1989). Similar results have also been reported by Tarr, Trank & Goertz (1989). In these studies, we suggested that intracellular Mg^{2+} blocked I_K in a voltage-independent manner. This suggests that, unlike the effects of Mg^{2+} on inwardly rectifying channels where Mg^{2+} enters the permeation pathway, the effects of Mg^{2+} on I_K are via a different mechanism. Furthermore, it has been suggested that the stimulatory effects of isoprenaline on I_K are dependent on prior run-down of I_K (Hume, 1985; Harvey & Hume, 1989). Here we present a more detailed analysis of the effects of intracellular magnesium on cardiac I_K, as well as new insights into the effects of Mg^{2+} ions on β-adrenergic stimulation of I_K. We propose that I_K may be regulated by Mg^{2+} by several processes including a phosphorylation–dephosphorylation-dependent mechanism.

METHODS

The methods were the same as previously published (Duchatelle-Gourdon *et al.* 1989). Cells were dissociated from bull-frog (*Rana catesbeiana*) atria by collagenase and trypsin incubation and mechanical dissociation.

Table 1 gives the composition of the solutions used. The cells were superfused with either control or isoprenaline-containing extracellular solution by positioning the cell in front of 250 μm Teflon (Clay Adams) capillaries. The standard intracellular solution contained 1 mM free $[Mg^{2+}]_i$. Intracellular solutions containing various free $[Mg^{2+}]$ at a constant [MgATP] were constructed from the Godt & Lindley (1982) program. Changes in the solution perfusing the cell was achieved using a polyethylene capillary in the whole-cell electrode as described in Fischmeister & Hartzell (1987). TTX, isoprenaline and adenosine 3′5′ cyclic monophosphate (cyclic AMP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Adenosine cyclic 3′,5′-(Rp)-phosphorothioate (Rp-cAMPS), an analogue of cyclic AMP and competitive inhibitor of the cyclic AMP-dependent protein kinase A, was a gift from Dr Ira Cohen (Stony Brook, NY, USA).

All experiments were carried out at room temperature (21–24 °C). The whole-cell variation of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1986) was used to record membrane currents. Patch electrodes made of soft glass capillaries (Drummond Scientific Co., Broomall, PA, USA) were pulled on an horizontal puller (Sutter Instrument Co., Novato, CA, USA) and had resistances of 1–1·6 MΩ. The uncompensated series resistance was usually < 2 MΩ. The

currents were recorded with a List EPC-7 amplifier. Average cell capacity was 70 ± 1.5 pF ($n = 135$). On-line analysis of the amplitude of the calcium and potassium currents was performed with software developed by Bill Goolsby (Emory University, Atlanta, GA, USA). Current waveforms were also stored on a 1220 VCR (Panasonic Co., Secaucus, NJ, USA) for further analysis. Voltage clamp protocols and nomenclature have been described elsewhere (Duchatelle-Gourdon *et al.* 1989).

TABLE 1. Intra- and extracellular solutions (mM)

	Intracellular solutions						Bath
Free Mg^{2+}	0·1	0·3	1	1·5	2	3	~ 1·8
KCl	107	117	118	117	116	114	2·5
NaCl	—	—	—	—	—	—	115
$MgCl_2$	2·75	3	4	4·75	5·45	6·8	1·8
$CaCl_2$	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	1·8
K_2EGTA	5	5	5	5	5	5	—
Na_2K_2ATP	4·55	3·3	2·8	2·7	2·7	2·6	—
HEPES	—	—	—	—	—	—	10
PIPES	10	10	10	10	10	10	—
Glucose	—	—	—	—	—	—	5
Na-pyruvate	—	—	—	—	—	—	5
Na-creatine-P	5	5	5	5	5	5	—
TTX	—	—	—	—	—	—	0·3
pH	7·15	7·15	7·15	7·15	7·15	7·15	7·4

E_H is the holding potential, E_C the clamped pulse potential and E_T the tail potential. For routine evaluation, Ca^{2+} and K^+ currents were evoked by pulses from $E_H = -80$ mV to $E_C = 0$ mV for 12 s ($E_T = E_H$) given every 20 s.

I_K was measured as the maximal outward current activated during the pulse minus $I_{200\,ms}$. I_{Ca} was measured as the maximal peak inward current minus $I_{200\,ms}$ ($I_{200\,ms}$ gives a rough estimation of the leak current for each pulse, before the activation of I_K). For a detailed description of the activation and instantaneous current–voltage curves protocols, see Simmons, Creazzo & Hartzell, (1986) and Duchatelle-Gourdon *et al.* (1989).

In the text, the 'basal' condition will refer to the absence of either isoprenaline or cyclic AMP stimulation. The results are expressed as means ± standard error unless otherwise noted.

RESULTS

Run-down of I_K

Figure 1*A* illustrates the behaviour of the delayed rectifier current (I_K) after breaking into a frog cardiac atrial cell to initiate the whole-cell recording with intracellular solution containing 3 mM-free Mg^{2+}. The amplitude of I_K decreased (ran down) and stabilized after ~ 6 min (see also Duchatelle-Gourdon *et al.* 1989; Harvey & Hume, 1989; Tarr *et al.* 1989). If the patch membrane was ruptured in the presence of solutions containing 0·3 or 0·1 mM [Mg^{2+}], I_K did not run down, but instead usually increased, reaching a steady state in < 10 min (Fig. 1*B*). In addition, the current at 200 ms and sometimes the holding current at −80 mV changed in the same direction as I_K but the changes were smaller (see Figs 2 and 3 for examples). These changes presumably reflect effects of Mg^{2+} on I_{K1}. Figure 1*C* shows the average change in I_K amplitude, from patch break until steady state was reached, as a function of free [Mg^{2+}] present in the patch pipette at the time of patch rupture. A value of 0

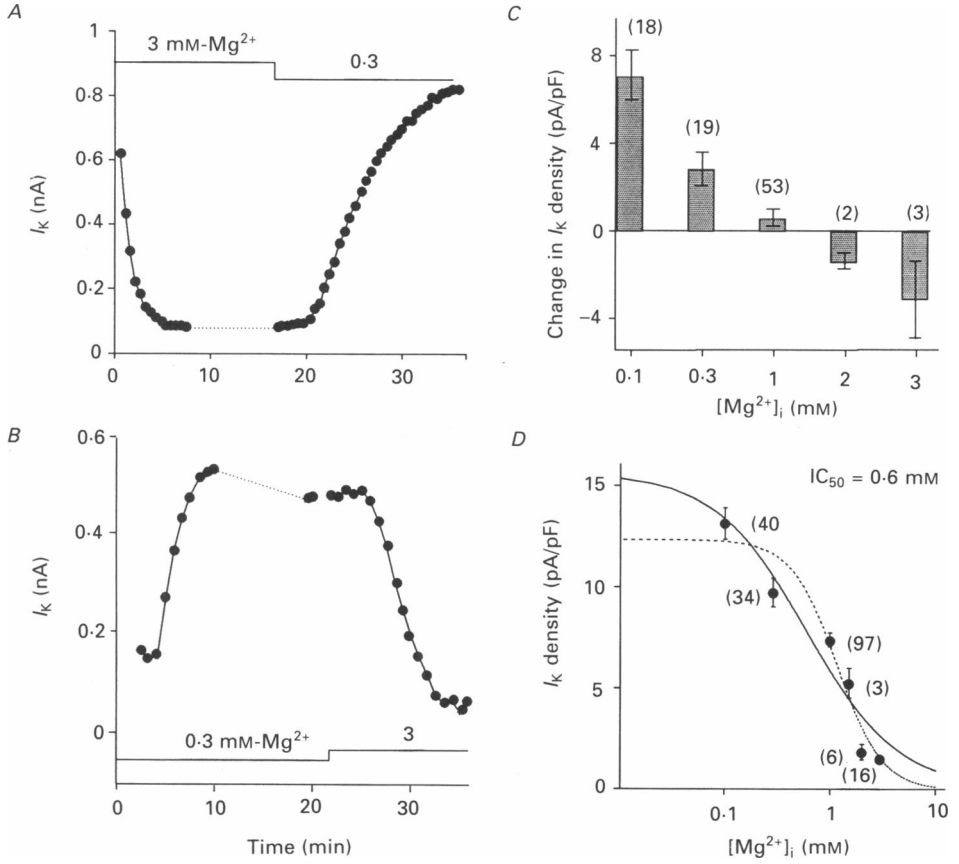

Fig. 1. Effect of $[Mg^{2+}]_i$ ion basal I_K amplitude measured as described in Methods. *A* and *B*, plots of steady-state I_K amplitude at 0 mV as a function of time. *A*, the cell was initially perfused with 3 mM $[Mg^{2+}]_i$, then with 0·3 mM $[Mg^{2+}]_i$ as illustrated. Patch was ruptured at $t = 0$. *B*, the cell was initially perfused with 0·3, then with 3·0 mM $[Mg^{2+}]_i$. *C*, change in I_K amplitude at 0 mV as a function of free Mg^{2+} in the intracellular solution, as percentage change in amplitude from first record (t = 20–90 s after patch break) to steady state (t = 8–10 min). Filled bars represent the average change in initial I_K amplitude for several experiments (n). *D*, concentration–response curve of I_K current density at 0 mV as a function of $[Mg^{2+}]_i$. ●, averaged values for I_K current density at different $[Mg^{2+}]_i$ from experiments where $[Mg^{2+}]_i$ was changed by internal perfusion, fitted to equations of the form:

$$I_K \text{ density} = 15{\cdot}8/(1+(0{\cdot}57/[Mg^{2+}]_i)^1),$$

for a one-site model (continuous line) or

$$I_K \text{ density} = 12{\cdot}3/(1+(1{\cdot}05/[Mg^{2+}]_i)^2),$$

for a two-site model (dotted line). Number of experiments for each $[Mg^{2+}]_i$ are shown on the curve.

indicates that the amplitude of I_K remained constant over the first 10 min of recording. To investigate the effects of Mg^{2+} more thoroughly, we used a system for changing the solution in the patch electrode as shown in Fig. 1*A* and *B*. Lowering $[Mg^{2+}]_i$ from 3 to 0·3 mM caused an increase in I_K (Fig. 1*A*). Increasing $[Mg^{2+}]_i$ from

0·3 to 3 mM caused a decrease in I_K (Fig. 1*B*). From experiments such as these, the relationship between Mg^{2+} concentration and I_K amplitude was obtained (Fig. 1*D*). Means of data points were fitted to saturation kinetics:

$$dI_K = dI_{K,max}/1 + (IC_{50}/[Mg^{2+}]_i)^n,$$

where the Hill coefficient, n, could reflect the number or co-operativity of Mg^{2+} binding sites. The goodness of fit was similar for $n = 1$ or 2. Standard error of the fit was 1·39 for $n = 1$ and 1·35 for $n = 2$ and the 95% confidence intervals overlapped, and if n is allowed to be a free parameter in the fit procedure, $n = 1·7$. The two fits are shown in Fig. 1*D*. IC_{50}, the $[Mg^{2+}]_i$ necessary to block 50% of I_K was 0·6 mM assuming a one-site model and 1·1 mM assuming a two-site model. An estimation of the resting $[Mg^{2+}]_i$ in atrial myocytes can be derived from this analysis. If we assume that the intra-electrode solution needs some time to diffuse into the cell, the amplitude of I_K immediately after initiating the whole-cell configuration should indicate the free $[Mg^{2+}]_i$ present in the cell before the patch membrane was broken. In 135 cells tested, I_K density was $6·59 \pm 0·2$ pA/pF, giving an estimated free $[Mg^{2+}]_i$ of 0·8 mM under basal conditions for a one-site model and 1 mM for a two-site model.

Effects of Mg^{2+} on the electrophysiological properties of I_K

Current–voltage relation

Several mechanisms could explain the effects of Mg^{2+} on the amplitude of I_K. The first one we considered was that Mg^{2+} ions blocked the outward current flowing through I_K channels by a voltage-dependent block of the permeation pathway analogous to the block of other inwardly rectifying I_K channels. Inward K^+ current should be less affected, because K^+ ions could force Mg^{2+} ions out of the channel mouth when they are going inward and Mg^{2+} would be less strongly drawn towards the membrane at negative voltages (Horie *et al.* 1987; Matsuda *et al.* 1987; Vandenberg, 1987; Matsuda, 1988; Horie & Irisawa, 1989; Ishihara *et al.* 1989).

The average instantaneous current–voltage (I–V) relations in 0·1 mM (▲), 0·3 mM (●) and 1 mM (■) $[Mg^{2+}]_i$ are shown in Fig. 2*B*. The cells were voltage-clamped from $E_H = -80$ mV to $E_C = +40$ mV for 12 s to fully activate I_K, then repolarized to various E_T levels from +20 to −120 mV for 15 s before returning to E_H for 20 s (Fig. 2*A*). The differences between the initial amplitude of the tails recorded during E_T arise from the differences in driving force at E_T as all the channels available in the membrane have been activated during $E_C = +40$ mV. Plotting the amplitude of the tail currents *versus* E_T gave the instantaneous I–V curve in Fig. 2*B*. The net amplitude of the current at each potential was decreased by increasing $[Mg^{2+}]_i$, with no change in the reversal potential (see Duchatelle-Gourdon *et al.* 1989 for an illustration in 0·3 and 1 mM $[Mg^{2+}]_i$). If the curves were normalized to the current recorded at 0 mV in each $[Mg^{2+}]_i$, there was no statistical difference, showing that the rectification properties of the delayed rectifier were not modified. These data show that Mg^{2+} block is not measurably voltage dependent. For higher concentrations of Mg^{2+}, the currents were too small to allow reliable determination of instantaneous I–V relation (as well as activation curves).

Steady-state activation

The decrease in I_K produced by high $[Mg^{2+}]_i$ could be due to a decrease in the maximal current that can be activated or to shifts in the activation curve. The activation curves obtained for different cells are presented in Fig. 2*C* and *D*. The cells

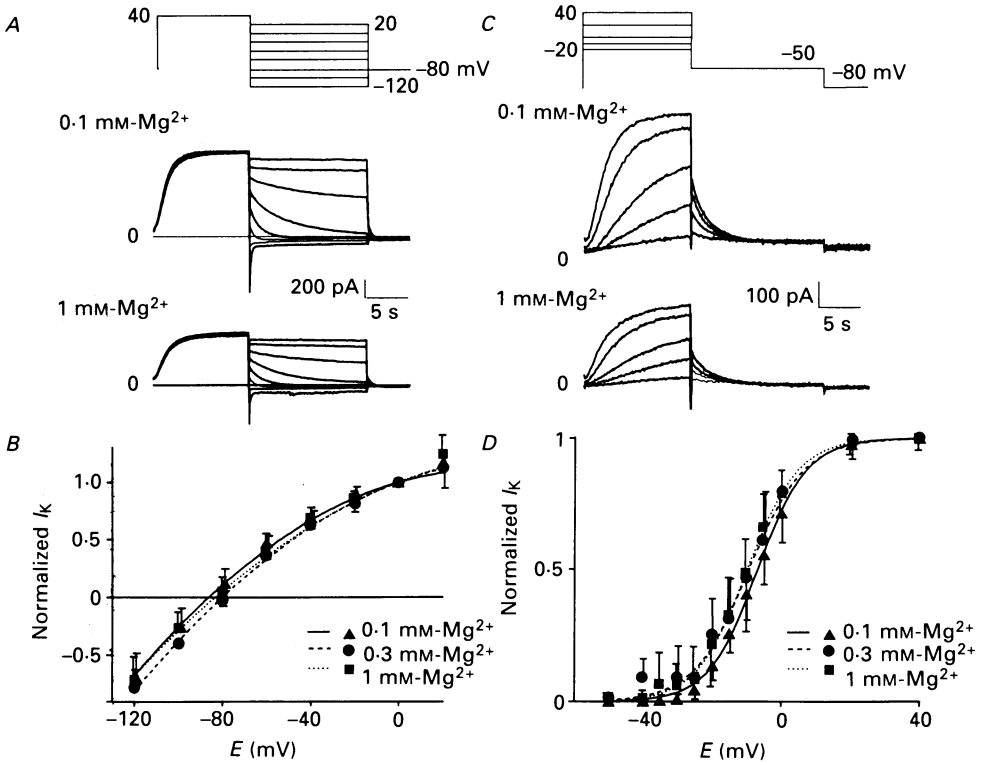

Fig. 2. Average effect of $[Mg^{2+}]_i$ on I_K current–voltage relation and steady-state activation. *A*, instantaneous current–voltage protocol. I_K was fully activated at $E_C = +40$ mV, then repolarized to various E_T levels. $E_H = -80$ mV as shown. Current traces for 0·1 mM (top trace) and 1 mM (bottom trace) $[Mg^{2+}]_i$ are from the same cell. *B*, current–voltage relations for I_K. Peak amplitude of I_K tail ($E_T = +20$ to -80 mV), or its net amplitude ($E_T = -100$ and -120 mV) was plotted as a function of E_T after normalization to the tail amplitude at 0 mV. Normalized current–voltage relations for I_K obtained by averaging data from individual experiments using $[Mg^{2+}]_i$ at 0·1 mM ($n = 8$), 0·3 mM ($n = 3$), and 1 mM ($n = 6$) are shown. Mean data points were fitted to a second-order polynomial. *C*, steady-state activation protocol. I_K was activated to various E_C level from $E_H = -80$ mV, then repolarized to $E_T = -50$ mV as shown. Current traces for 0·1 mM (top trace) and 1 mM (bottom trace) $[Mg^{2+}]_i$ are from the same cell. *D*, steady-state activation curves for I_K. The net amplitude of I_K tails was normalized to the tail from +40 mV and plotted as a function of E_C in each $[Mg^{2+}]_i$. Averaged data from individual experiments using $[Mg^{2+}]_i$ at 0·1 mM ($n = 6$), 0·3 mM ($n = 6$), and 1 mM ($n = 14$) are shown. Mean data points were fitted to a Boltzmann distribution of the form $I_K = 1/\{1+\exp[(V_N - E_C)/S]\}$, where V_N is the half-maximal activation and S is the slope factor. For 0·1 mM $[Mg^{2+}]_i$, $V_N = -6{\cdot}85$, $S = 7{\cdot}04$. For 0·3 mM $[Mg^{2+}]_i$, $V_N = -9{\cdot}42$, $S = 7{\cdot}93$. For 1 mM $[Mg^{2+}]_i$, $V_N = -9{\cdot}86$, $S = 7{\cdot}33$. ▲, 0·1 mM $[Mg^{2+}]_i$; ●, 0·3 mM $[Mg^{2+}]_i$, ■, 1 mM $[Mg^{2+}]_i$. Error bars designate S.D.

were first voltage-clamped to various E_C levels, from -50 to $+40$ mV for 12 s, then repolarized to a constant E_T of -50 mV for 15 s before returning to E_H for 20 s (Fig. 2*C*). The differences between the initial amplitude of the tails recorded during E_T arise from the differences in the number of channels activated during E_C, as the

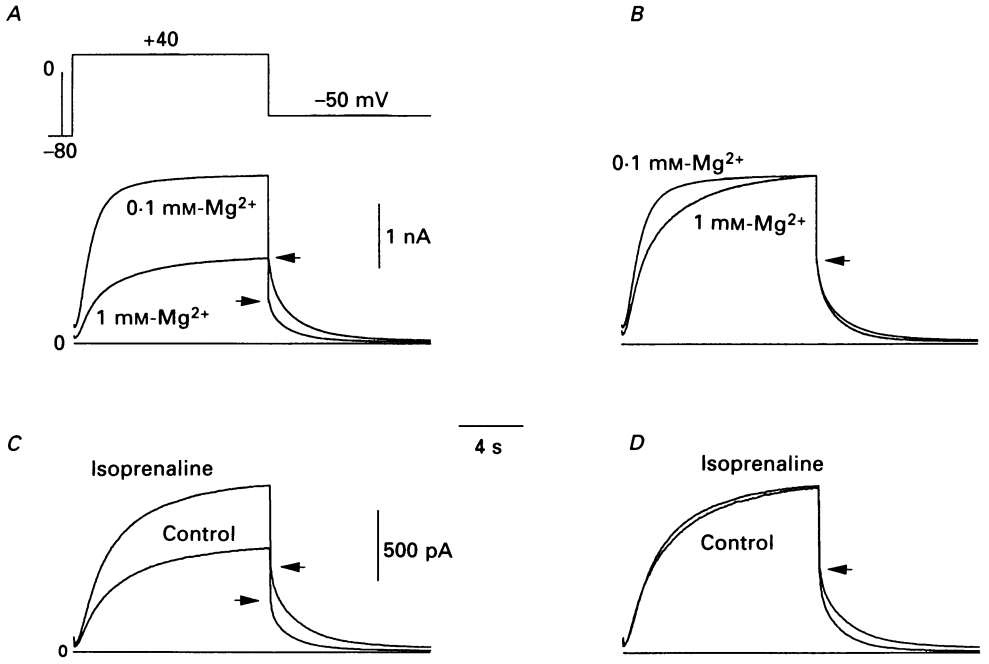

Fig. 3. Effects of $[Mg^{2+}]_i$ and isoprenaline on I_K kinetics. *A*, I_K current traces elicited by a depolarization to $E_C = +40$ mV from $E_H = -80$ mV, comparing the effect of 0·1 mM *versus* 1 mM $[Mg^{2+}]_i$ (top), and the effect of isoprenaline (1 μM) *versus* control conditions (bottom). For comparison traces have been superimposed. *B*, normalized current traces. Arrows indicate the maximum amplitude of I_K tails (instantaneous I_K at -50 mV).

driving force during E_T is constant. Plotting the initial amplitude of the tail current *versus* E_C gave the activation curve. The activation threshold of the current was not modified by Mg^{2+}, but the amplitudes of the currents were decreased by higher $[Mg^{2+}]_i$, as seen for the instantaneous *I–V* curve (not shown, but see Duchatelle-Gourdon *et al.* 1989 for an illustration). When the activation curves were normalized to the maximal tail current (Fig. 2*D*), the curves were almost identical. The absence of shift in the activation curve argues against a large charge screening effect of the Mg^{2+} ions at the intracellular face of the membrane (Frankenhaeuser & Hodgkin, 1957).

Kinetics

Figure 3 shows the effect of lowering $[Mg^{2+}]_i$ from 1 to 0·1 mM on I_K kinetics and compares it to the effect of isoprenaline. In Fig. 3*A*, I_K was elicited by a 12 s pulse to $+40$ mV after a 200 ms pre-pulse to 0 mV to inactivate most of the calcium

current. The interpulse duration was 5 ms. In the presence of 0·1 mM $[Mg^{2+}]_i$, I_K activated more rapidly than in the presence of 1 mM $[Mg^{2+}]_i$. This is better seen when the current waveforms are normalized to the current at the end of the 12 s pulse (Fig. 3*B*). The increase in the instantaneous current at +40 mV in 0·1 mM-Mg^{2+} is probably due to suppression of part of I_{K1} rectification by 1 mM $[Mg^{2+}]_i$. The normalized tails were very similar, suggesting that the deactivation parameters were not significantly modified.

These effects of Mg^{2+} were quite different from the effects of isoprenaline (Fig. 3*C* and *D*). Like low $[Mg^{2+}]_i$, isoprenaline speeded I_K activation, but in addition, it also slowed deactivation tails considerably. These effects of isoprenaline on I_K kinetics are the same as reported by Giles, Nakajima, Ono & Shibata (1989).

We decided not to quantify the activation time constants because the currents were not always easily fitted by a classical single Hodgkin–Huxley component raised to the second power when they were elicited by a step from −80 mV. However, it is usually possible to fit the currents to single Hodgkin–Huxley components when they are elicited from −50 mV (see Hume *et al.* 1986; Simmons *et al.* 1986; Matsuura *et al.* 1987; Clay, Hill, Roitman & Shrier, 1988; Giles *et al.* 1989), indicating that several deep closed states might be required to fully describe I_K in heart. The presence of several types of I_K channels contributing to the whole-cell current has also been shown (Llano, Webb & Bezanilla, 1988; Mazzanti & DeFelice, 1988) and could explain unsatisfactory fits from $E_H = -80$ mV.

Effects of isoprenaline in low $[Mg^{2+}]_i$

Isoprenaline-induced current and reversibility

We have previously demonstrated that β-adrenergic stimulation increased I_K via a cyclic AMP-dependent phosphorylation in the presence of 1 mM $[Mg^{2+}]_i$ (Duchatelle-Gourdon *et al.* 1989). However, the possibility that the stimulatory effect of isoprenaline was dependent on prior run-down of I_K induced by Mg^{2+} was not investigated. Figure 4*A* presents a typical experiment where isoprenaline was tested in different $[Mg^{2+}]_i$. When isoprenaline was applied in the presence of 1 mM $[Mg^{2+}]_i$ for a short period of time (3–4 min), an increase of about 60% in I_K amplitude was observed, as we have already reported (Duchatelle-Gourdon *et al.* 1989). When isoprenaline was removed from the extracellular solution, I_K decreased to the basal level in ~ 5 min. When $[Mg^{2+}]_i$ was lowered to 0·1 mM, I_K increased and reached a steady-state level of about 250% the initial current over a period of 10 min. Under these conditions, isoprenaline exposure increased I_K about 20% relative to the new steady-state level. Surprisingly, when isoprenaline was washed out, the current did not return to its basal value even after 20 min. The effect of isoprenaline was at least partly persistent. Upon returning to 1 mM $[Mg^{2+}]_i$, I_K decreased to a level comparable to the level recorded soon after patch-break. A brief application of isoprenaline increased I_K in the same way as it did the first time in 1 mM $[Mg^{2+}]_i$, and the effect was again rapidly reversible. Similar results were obtained in twelve cells. Figure 4*B* summarizes the effects of isoprenaline on I_K density in different $[Mg^{2+}]_i$. The increase in I_K density stimulated by isoprenaline was relatively independent of $[Mg^{2+}]_i$, although obviously the percentage stimulation was decreased with decreasing $[Mg^{2+}]_i$ as the basal I_K increased. The extent of reversibility of isoprenaline (10 min

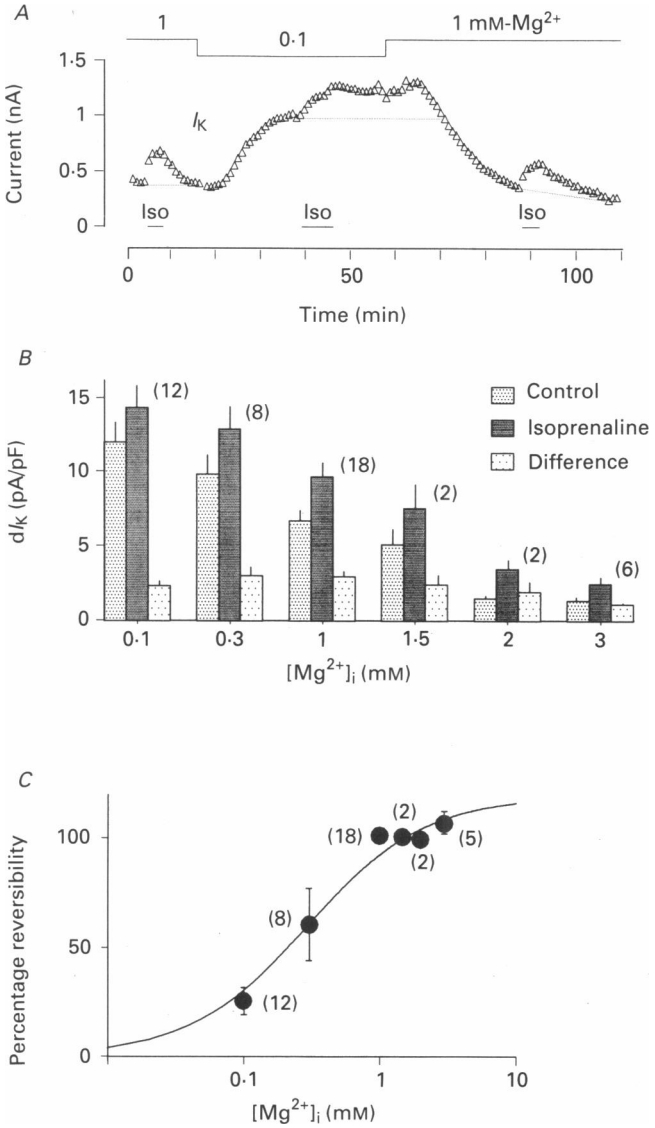

Fig. 4. Interactive effects of $[Mg^{2+}]_i$ and isoprenaline on I_K. *A*, plot of I_K steady-state amplitude at 0 mV (△) as function of time. Isoprenaline (Iso, 1 μM) was applied at the times indicated. $[Mg^{2+}]_i$ was changed from 1 to 0·1 mM and back to 1 mM, as shown. *B*, average experiments as in *A*, with different $[Mg^{2+}]_i$. Shaded bars represent averages of individual experiments (n as shown). The net isoprenaline stimulation ($I_{K,Iso}$ density − $I_{K,Control}$ density) is about the same between 0·1 and 2 mM $[Mg^{2+}]_i$. *C*, concentration–response curve of the reversibility of the effects of isoprenaline as function of $[Mg^{2+}]_i$, expressed as $I_{K,wash}$ density × 100/$I_{K,iso}$ density. Data points were fitted to the Michaelis–Menten equation: percentage reversibility = 119 + $[Mg^{2+}]_i$/0·29 × $[Mg^{2+}]_i$.

after removing isoprenaline from the extracellular solution) greatly depended on $[Mg^{2+}]_i$ (Fig. 4*C*).

Two major points in these experiments are worth emphasizing. (i) For $[Mg^{2+}]_i$ ranging between 0·1 and 2 mM, the isoprenaline-stimulated current is $2·5 \pm 0·6$ pA/pF (Fig. 4*B*). (ii) The lower the $[Mg^{2+}]_i$, the less reversible was the isoprenaline

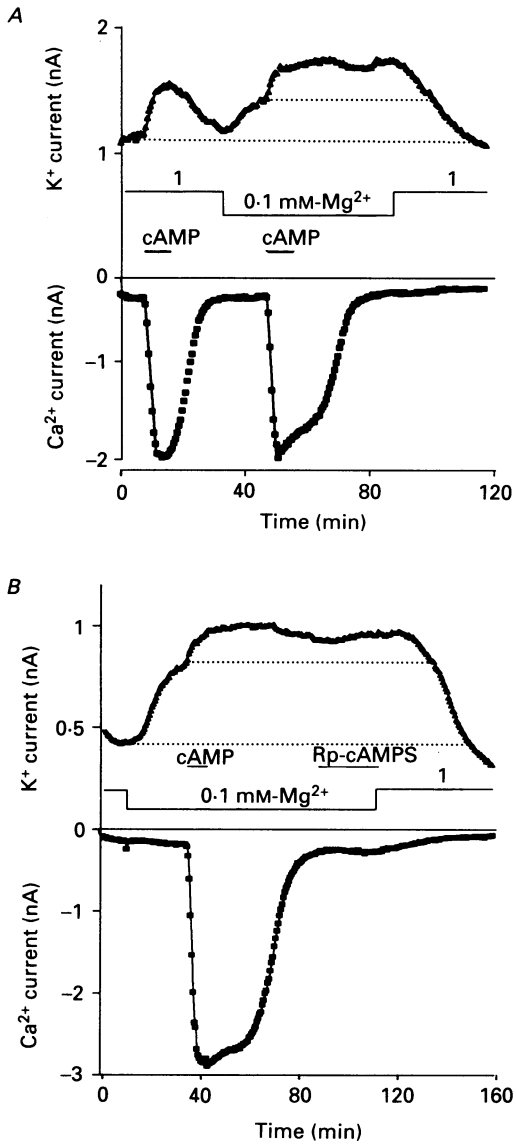

Fig. 5. Interactive effects of $[Mg^{2+}]_i$ and cyclic AMP. *A*, plot of I_K steady-state amplitude (▲) and I_{Ca} peak current (■) at 0 mV as function of time. Cyclic AMP (30 μM) was applied at the times indicated. $[Mg^{2+}]_i$ was changed from 1 to 0·1 mM and back to 1 mM, as shown. *B*, similar plot illustrating that Rp-cAMPS (200 μM) a competitive inhibitor of cyclic AMP binding on protein kinase, does not reduce I_K when stimulated with isoprenaline in $[Mg^{2+}]_i = 0·1$ mM.

stimulation (Fig. 4*C*). After 10 min of washing of isoprenaline in 0·1 mM $[Mg^{2+}]_i$, the current was still 76 ± 20 % of the current in the presence of isoprenaline ($n = 12$), whereas in 1–3 mM $[Mg^{2+}]_i$, the effects of isoprenaline were fully reversed (2 ± 17 %, $n = 25$).

Cyclic AMP-induced current and reversibility

One explanation of the effects of $[Mg^{2+}]_i$ on wash-out of isoprenaline in Fig. 4*A* is that Mg^{2+} affects cyclic AMP concentration. To test this, we repeated the experiment of Fig. 4*A* with internal perfusion of 30 μM-cyclic AMP (Fig. 5*A*). The results were virtually the same as obtained with isoprenaline exposure. The current stimulated by cyclic AMP was similar in 1 and 0·1 mM $[Mg^{2+}]_i$ (3·38 ± 0·5 pA/pF, $n = 7$, and 3·75 ± 0·59 pA/pF, $n = 10$, respectively). In 0·1 mM $[Mg^{2+}]_i$, the effect of cyclic AMP was at least partly persistent (after 15 min washing out intracellular cyclic AMP, 50 ± 16 % of the cyclic AMP-stimulated current was reversed, $n = 10$). In the same set of experiments, 15 min after washing out cyclic AMP in 1 mM $[Mg^{2+}]_i$ I_K was actually lower than the control I_K level by 37 ± 20 % ($n = 6$). The reason for this decrease below basal levels may be due to the long recording times involved. Nevertheless, the difference in wash-out between 0·1 and 1 mM $[Mg^{2+}]_i$ was 87 %, which was comparable to that seen with isoprenaline. The lower part of Fig. 5*A* shows the changes in I_{Ca} recorded simultaneously in the same cell. Although the stimulation of I_{Ca} washes out more slowly in 0·1 mM than in 1 mM $[Mg^{2+}]_i$, the effects of cyclic AMP on I_{Ca} are fully reversible in both 1 *and* 0·1 mM $[Mg^{2+}]_i$. Similar results were found with isoprenaline (not shown).

These observations suggest a role for Mg^{2+} in the cyclic AMP-dependent phosphorylation of I_K. One possibility is that in $[Mg^{2+}]_i < 1$ mM, I_K might be more sensitive to cyclic AMP than I_{Ca} and that low concentrations of cyclic AMP remaining after wash-out might be sufficient to elevate I_K. This explanation is rendered unlikely by our previous results showing that I_{Ca} and I_K have similar sensitivity to cyclic AMP (Duchatelle-Gourdon *et al.* 1989). Nevertheless, this possibility was tested as shown in Fig. 5*B*. In 0·1 mM $[Mg^{2+}]_i$, intracellular application of 200 μM-Rp-cAMPS, an analogue of cyclic AMP that is a competitive inhibitor of the cyclic AMP-dependent protein kinase A, did not reduce I_K. This suggests that persistent elevation of I_K by cyclic AMP in low $[Mg^{2+}]_i$ does not require continued protein kinase A activity. This concentration of Rp-cAMPS inhibited the isoprenaline-stimulated I_K by more than 80 % in 1 mM $[Mg^{2+}]_i$ (not shown).

DISCUSSION

The present results expand the observations we recently reported (Duchatelle-Gourdon *et al.* 1989). In that paper, we presented evidence that the run-down of the delayed rectifier current of frog cardiac atrial cells could be explained by the amount of Mg^{2+} that was present in the patch electrode at the initiation of whole-cell recording (see also Tarr *et al.* 1989). The run-down of I_K has been a subject of concern. Hume (1985) and Harvey & Hume (1989) suggested that the ability of isoprenaline to increase I_K was related to the prior run-down of the current, because they found

that isoprenaline had no effects in non-perfused cells where I_K had not run-down. We present here a more detailed study of this phenomenon as well as new evidence that Mg^{2+} ions can affect I_K in several, and apparently unrelated, ways.

Mg^{2+} affects I_K in basal conditions

I_K is very sensitive to $[Mg^{2+}]_i$ in basal conditions (non-stimulated by isoprenaline or cyclic AMP). Increasing $[Mg^{2+}]_i$ from 0·1 to 3 mM blocks I_K. This range is within the values given for the resting free intracellular Mg^{2+} by various techniques (see Levy, Murphy, Raju & London, 1988; White & Hartzell, 1989). The sensitivity of I_K to $[Mg^{2+}]_i$ is about one order of magnitude less than that found by Horie & Irisawa (1989) for muscarinic K^+ channels. If we assume that the cells are not significantly perfused within the first minute after breaking the patch membrane, the amplitude of I_K could be a good indicator of the free $[Mg^{2+}]_i$ present in the intact cells. This gives an estimated value of 0·8 mM free $[Mg^{2+}]_i$ for a one-binding-site model or 1 mM for a two-site model. This opens the possibility that these channels can respond to small variations in the free Mg^{2+} level as these values are very close to the IC_{50} estimated for each model.

There are several mechanisms by which Mg^{2+} ions could regulate delayed rectifier K^+ channels: (i) Mg^{2+} could block the channels via a voltage-dependent block similar to the one reported for the inward rectifier K^+ channels (Hoire *et al.* 1987; Matsuda *et al.* 1987; Vandenberg, 1987; Matsuda, 1988; Horie & Irisawa, 1989; Ishihara *et al.* 1989); (ii) Mg^{2+} could affect the surface charge at the intracellular face of the membrane and alter the transmembrane potential (screening effect, Frankenhauser & Hodgkin, 1957); (iii) Mg^{2+} could bind allosterically to the channels and modify their gating properties; or (iv) Mg^{2+} could regulate an intermediate enzyme or protein that, in turn, modifies the K^+ channels.

Current–voltage and activation protocols in different $[Mg^{2+}]_i$ have evidently ruled out the first two possibilities. Even though I_K amplitude is strongly dependent on $[Mg^{2+}]_i$, there are no differences in the shape of the *I–V* or activation curves among the various $[Mg^{2+}]_i$ tested. This shows that the rectification, gating properties, selectivity of the channels, and the transmembrane potential that the channels experience are not significantly perturbed. This confirms our previous findings as well as those of Tarr *et al.* (1989). The remaining two possibilities could be very difficult to separate experimentally because single I_K channels have been very difficult to record (see Duchatelle-Gourdon & Hartzell, 1990).

Mg^{2+} alters the effects of isoprenaline on I_K

An important question was whether isoprenaline could stimulate delayed rectifier currents that had not previously run down. It turned out that isoprenaline could increase I_K even after it ran up in low $[Mg^{2+}]_i$. For $[Mg^{2+}]_i$ between 0·1 and 1·5 mM, the isoprenaline-induced current is relatively independent of $[Mg^{2+}]_i$, about 2·5 pA/pF (see Fig. 4*B*). The effects of isoprenaline are partly persistent if the $[Mg^{2+}]_i$ is low.

Mg^{2+} and isoprenaline-induced current

The effects of Mg^{2+} and isoprenaline on I_K were additive, at least within the range of $[Mg^{2+}]_i$ examined, and the effect of isoprenaline was independent of $[Mg^{2+}]_i$. The effects of lowering $[Mg^{2+}]_i$ and applying isoprenaline on activation kinetics are similar, increasing the rate of activation, but their effects on deactivation are quite different. Whereas changing $[Mg^{2+}]_i$ does not affect the deactivation tails, isoprenaline slows them. There are three possible mechanisms one could use to explain the effects of Mg^{2+} and isoprenaline on I_K: (i) Mg^{2+} and isoprenaline affect two different populations of channels; (ii) Mg^{2+} and isoprenaline could act on two different sites on the same channel; or (iii) the β-adrenergic receptors or the channels could be compartmentalized, so that only a fraction of I_K channels is affected by isoprenaline. It is tempting to suppose that there are two different populations of channels, one sensitive to $[Mg^{2+}]_i$ and responsible for the basal current and one recruited by isoprenaline, because of the results that we have obtained in single-channel experiments (Duchatelle-Gourdon & Hartzell, 1990). We could record a channel having the characteristics of a delayed rectifier only if the β-adrenergic system had been previously activated. Under basal conditions, single delayed rectifier channels were very rare. This suggests that the channel responsible for basal I_K was too small or too rapid to be resolved.

If we assume that the channels sensitive to isoprenaline are not the same as the ones present in basal conditions, one important question remains. Which channel(s) underlies the basal delayed rectifier current? This question has been a matter of some controversy over the years. When electrophysiological investigations are performed on multicellular preparations, it is clear that the cardiac outward current has several components (see Carmeliet & Vereecke, 1979, for a review; Shrier & Clay, 1986). Whole-cell recording of I_K in single cardiac cells apparently established the single-component nature of the delayed rectifier (Simmons *et al.* 1986, Hume *et al.* 1986; Matsuura Ehara & Imoto, 1987; Clay *et al.* 1988), but single-channel recordings have demonstrated various species of delayed rectifier K^+ channels. Understanding the relationship between single-channel and macroscopic current continues to pose an interesting challenge as three channel types have been reported to contribute to the cardiac (Mazzanti & DeFelice, 1988) and the squid giant axon (Llano *et al.* 1988) delayed rectifier.

Mg^{2+} and isoprenaline reversibility

It is clear that the effects of isoprenaline are mainly mediated through the phosphorylation of the K^+ channels by protein kinase A (Duchatelle-Gourdon *et al.* 1989; Giles *et al.* 1989; Harvey & Hume, 1989; Yazawa & Kameyama, 1990), and it is reasonable to assume that the wash-out of isoprenaline reflects the dephosphorylation of the channels. In view of the fact that the reversibility of the effects of isoprenaline on I_K is dependent on $[Mg^{2+}]_i$ and that neither cyclic AMP nor protein kinase A appear to be involved in this poor reversibility of isoprenaline on I_K in low $[Mg^{2+}]_i$, the following possibilities remain. (i) Mg^{2+} ions could regulate the K^+ channels directly. With $[Mg^{2+}]_i < 1$ mM the conformation of K^+ channels could be modified, rendering them poor substrates for the phosphatases that usually

dephosphorylate them under higher $[Mg^{2+}]_i$ conditions. (ii) The phosphatases themselves may require Mg^{2+} to dephosphorylate the K^+ channels. This latter possibility predicts that K^+ channels could be substrates for a Mg^{2+}-dependent phosphatase such as one described by Cohen (1989).

In contrast to I_K, I_{Ca} measured in these conditions (in the presence of K^+ currents) is not persistently stimulated by isoprenaline in 0·1 mM $[Mg^{2+}]_i$. Since we have shown that this is not due to different sensitivities of I_{Ca} and I_K for cyclic AMP (Duchatelle-Gourdon *et al.* 1989) or persistent protein kinase A activation, this suggests that the dephosphorylation process is different for the two channels.

It is then possible to imagine that modulation of I_K in basal conditions by $[Mg^{2+}]_i$ could also be via a phosphatase. 'Basal I_K' could, in fact, be a phosphorylated state of the channel. Lowering the $[Mg^{2+}]_i$ could inhibit a Mg^{2+}-dependent phosphatase allowing a net phosphorylation of the channels and an increase in the current. The possible role of protein kinases in regulating basal I_K (for example kinase C or another protein kinase) is currently under study.

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