Modulation of the voltage-dependent K\(^+\) current by intracellular Mg\(^{2+}\) in rat aortic smooth muscle cells

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Abstract

**Objective:** Intracellular magnesium ions (Mg\(^{2+}\)) are important in the regulation of a wide range of cellular metabolic processes and modulation of a variety of ion channels. Mg\(^{2+}\) deficiency has been implicated in the aetiology of various cardiovascular diseases. However, potential targets and mechanisms of action of Mg\(^{2+}\) in the cardiovascular system remain poorly understood. We therefore investigated the effect of Mg\(^{2+}\) on the voltage-gated K\(^+\) (KV) channels in rat aortic myocytes (RAMs).

**Methods:** KV currents (I\(_{KV}\)) were investigated in single RAMs isolated from adult Wistar rat thoracic aorta using the whole-cell patch clamp technique. Changes in the vascular reactivity were also assessed in endothelium-denuded rat aortic rings loaded with Mg\(^{2+}\).

**Results:** An increase in Mg\(^{2+}\) caused several significant effects on I\(_{KV}\): (1) slowed down kinetics of activation at high (10 mM) Mg\(^{2+}\); (2) caused inward rectification at positive membrane potentials; (3) shifted the voltage-dependent inactivation, but not steady-state I\(_{KV}\) activation; (4) the effect of Mg\(^{2+}\) on I\(_{KV}\) inactivation was enhanced in the presence of intracellular ATP. Selective changes in the voltage-dependent characteristics predict a significant inhibition of the whole-cell steady-state I\(_{KV}\) ("window current"), resulting in membrane depolarisation and enhanced tissue excitability. An increased sensitivity to KCl and the inhibitors of the I\(_{KV}\), tetraethylammonium and 4-aminopyridine (4-AP), was observed in Mg\(^{2+}\)-loaded aortas, confirming this hypothesis.

**Conclusion:** Our results demonstrate that intracellular magnesium can act as a potent modulator of the KV channel function in vascular smooth muscle cells in the vasculature in the physiological range of membrane potentials, representing a novel mechanism for the regulation of KV channel activity in the vasculature.

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1. Introduction

Magnesium (Mg\(^{2+}\)) is the second most abundant intracellular cation which is involved, both directly (as a cofactor of numerous enzymes) or indirectly (via MgATP), in the regulation of intracellular metabolic processes. Hypomagnesia and dietary Mg\(^{2+}\) deficiency have been implicated in the aetiology of multiple cardiovascular diseases, while Mg\(^{2+}\) supplementation is beneficial in the reduction of blood pressure and stabilisation of cardiac arrhythmias and acute myocardial infarction [1]. Interestingly, a significant accumulation of intracellular Mg\(^{2+}\) (Mg\(^{2+}_{\text{ii}}\)) in erythrocytes was observed in hypertensive patients in comparison to normal subjects, whereas no significant difference in serum ionised Mg\(^{2+}\) was found [2], suggesting a possible abnormal handling of Mg\(^{2+}\) in human hypertension. In addition, Mg\(^{2+}\) concentration can be significantly increased in response to endogenous vasoconstrictors in vascular smooth muscle cells (VSMCs) [3,4], indicating that Mg\(^{2+}\) might also be involved in the regulation of agonist-induced contraction of VSMCs. However, potential targets, which are affected by changes
in Mg$^{2+}$ and mechanisms of its action in the cardiovascular system, remain unclear.

Ion channels, which are essential for the function of the cardiovascular system, represent one possible target for Mg$^{2+}$. Indeed, an increase in Mg$^{2+}$ suppresses voltage-dependent Ca$^{2+}$ channels (VDCCs) [5–7], directly blocks inward rectifier K$^+$ channels [7,8], and stimulates large conductance Ca$^{2+}$-activated K$^+$ (BKCa) channels [9]. The role of Mg$^{2+}$ in the regulation of voltage-dependent K$^+$ (Kv) channels, which contribute to the control of both cardiac function and VSMC excitability, has been limited to a few descriptive reports. In the heart, dialysis of amphibian atrial myocytes with 1–10 mM Mg$^{2+}$ caused voltage-independent block of Kv currents [10,11]. Qualitatively similar suppression of Kv currents was demonstrated in canine and rabbit arterial SMCs [12]. Although the mechanism of inhibition has not been clarified in these reports, it was apparently different to the voltage-dependent block of heterologously expressed Kv channels by Mg$^{2+}$ that occurs at positive membrane potentials (>+40 mV) [13–16].

The main aim of this study was to investigate the effect of Mg$^{2+}$ on the whole-cell Kv channel current ($I_{Kv}$) in adult rat aortic myocytes (RAMs). RAMs were chosen because they express a homogeneous population of Kv channel currents ($I_{Kv}$) that play a key role in the regulation of resting membrane potential and excitability of aortic smooth muscle [18], thus representing a useful model to study mechanisms of Kv channel regulation in the vasculature. This paper describes a novel selective effect of Mg$^{2+}$ on $I_{Kv}$ voltage-dependent characteristics which leads to the inhibition of whole-cell steady-state $I_{Kv}$, in the physiological range of membrane potentials and increased excitability of VSMCs.

2. Methods

Male Wistar rats (225–300 g) were humanely killed and the thoracic aorta was removed, cleaned, and cut into rings which were either used for cell isolation or tension measurements. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Composition of solutions

The composition of physiological salt solution (PSS) was (mM): 130 NaCl, 5 KCl, 1.5 CaCl$_2$, 1.2 MgCl$_2$, 10 HEPES, and 10 glucose, pH=7.2 (NaOH). The pipette solution contained (mM): 110 KCl, 10 NaCl, 0.5 MgCl$_2$, 10 HEPES, 10 EGTA, and 0.5 CaCl$_2$, pH=7.2 (KOH). Different Mg$_{2+}$ concentrations were achieved by adding corresponding amounts of MgCl$_2$ to the pipette solution. Corresponding changes in osmolarity were less than 10% and were neglected. Free [Ca$^{2+}$] (8–10 mM) and [Mg$^{2+}$] were calculated using Maxchelator software (Stanford University, USA).

The composition of Krebs solution was (mM): 118 NaCl, 25 NaHCO$_3$, 4.9 KCl, 1.2 KH$_2$PO$_4$, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 11.7 glucose. Since the Mg$^{2+}$ loading procedure (modified from Ref. [19]) required the complete removal of Na$^+$ to eliminate Na$^+$-dependent Mg$^{2+}$ efflux via the Na$^+$–Mg$^{2+}$ exchanger, one of the major mechanisms of Mg$^{2+}$ extrusion from the cell [20], HEPES-based buffers were utilised. Although changes in Mg$_{2+}$ have not been directly measured, an increase up to 300% from the basal level of ~0.5 mM Mg$^{2+}$ could be anticipated [21]. The Na$^+$-containing buffer had composition (mM): 140 NaCl, 6 KCl, 2 CaCl$_2$, 1.2 MgCl$_2$, 5 HEPES, 11.7 glucose, pH=7.2 (KOH). In the Na$^+$-free buffer, NaCl was replaced with N-methyl-D-glucamine (NMDG; pH=7.2, HCl). In the Mg$^{2+}$-loading buffer 30 mM MgCl$_2$ and 110 mM NMDG were used.

Basic chemicals and reagents were purchased from BDH Merck, Fisher or Sigma (all UK).

2.2. Cell isolation and electrophysiological recordings

Single RAMs were isolated from aortic rings (width ~1–1.5 mm) using collagenase (Type XI) and papain (both at 1 mg/ml) as previously described in detail [17,18].

Cells were placed in a chamber (100–200 μl) and whole-cell $I_{Kv}$ (sampled at 10 kHz and filtered at 2 kHz) were recorded using the standard patch-clamp technique at room temperature as previously described [17,22]. Cell membrane capacitance ($C_m$) was determined from the area under capacitance transients (filtered at 50 kHz and sampled at 200 kHz) elicited by a 10-mV hyperpolarising step. To allow for equilibration of the pipette solution with cell interior, all recordings were started 5 min after establishing the whole-cell configuration. In addition, 1 μM paxilline and 10 μM glybenclamide were added to the PSS to block BKCa and ATP-sensitive K$^+$ channels, respectively. Holding potential was ~80 mV.

2.3. Isometric tension measurements and Mg$^{2+}$-loading protocol

Isolated endothelium-denuded aortic rings (~2–2.5 mm width) were mounted in an organ bath under 1g resting tension and equilibrated for 60 min in Krebs solution, bubbled with 95% O$_2$/5% CO$_2$ at 37 °C. Endothelium was removed by gentle rubbing of the vessel lumen with a horse hair (verified by the absence of relaxation to 10 μM acetylcholine). Before commencing the experimental protocol, each preparation was stimulated with three applications of 2.5 μM phenylephrine (PE) followed by wash out with Krebs. Tension (expressed in grams) was measured using a Biegestab K30 isometric force transducer (Hugo Sack Elektronik, Germany), MacLab/4s interface and Chart v3.6/s software (ADI Instruments, UK). Data were sampled at 40 Hz.

To compare the effect of increased Mg$^{2+}$ on excitability of intact rat aortas, three cumulative concentration responses
to high K⁺ (KCl), tetraethylammonium chloride (TEA), 4-
aminoipyridine (4-AP), and PE (performed in the Na⁺-free buffer unless stated otherwise) were measured before
(Control), after a 30-min incubation in the Mg²⁺-loading buffer (Mg²⁺-loaded), and after a 30-min washout (Recovery) in the same aortic preparation. Since we found that the recovery following washout with a Na⁺-free solution was incomplete after maximal KCl-induced contraction (not shown), washouts between agent-induced concentration responses were therefore performed with the Na⁺-containing or Krebs solutions, as indicated, to restore the basal level. We also found that 100 µM imipramine, a selective inhibitor of Na⁺-Mg²⁺ exchanger [19,23], irreversibly blocked both aortic contraction and IKᵥ in single RAMs (not shown) and therefore has not been used in this study. The Kv channel inhibitors TEA and 4-AP were used since they selectively block the IKᵥ in adult RAMs [17,18].

Data are presented as mean±S.E.M. and statistically compared using Student’s unpaired t test with P<0.05 considered to be significant unless otherwise stated.

3. Results

3.1. Effect of intracellular Mg²⁺ on IKᵥ

RAMs were dialysed with a pipette solution containing 0.5, 5, or 10 mM MgCl₂ ([MgCl₂]₀) and families of IKᵥ were recorded in response to 200-ms step depolarisation (Fig. 1A). The peak amplitude of IKᵥ at each membrane potential was derived from a single exponential fit of the current activation kinetics [17], expressed as a current density and plotted against the test potential (Fig. 1B). This comparison shows that an increase in Mg²⁺ caused no significant inhibition of the current in the negative voltage range, but induced a marked inward rectification at positive membrane potentials. Moreover, a significant slow down of the current activation kinetics was observed in cells dialysed with 10 mM MgCl₂ compared to those in the presence of 0.5 and 5 mM MgCl₂ (0.0001<P<0.05 between +10 and +100 mV, Fig. 1C). In addition, a significant decrease in the maximal conductance of IKᵥ (calculated from the Boltzmann fit of the current–voltage (I–V) relationships) was also observed in the presence of 10 mM MgCl₂ (0.049±0.005 nS/pF, n=20) compared to that in 0.5 mM (0.079±0.009 nS/pF, n=12) and 5 mM (0.073±0.005 nS/pF, n=19; Fig. 1D).

3.2. Mg²⁺-dependent changes in the voltage-dependent characteristics of IKᵥ

The effect of Mg²⁺ on the steady-state activation was compared using the analysis of the normalised conductance–voltage relationship with the standard Boltzmann function (Fig. 2). No significant difference in the half-activation potential (Vₐ) or the slope factor (kₐ) was observed at different Mg²⁺ levels (Table 1).

The effect of Mg²⁺ on inactivation of IKᵥ was investigated with the availability protocol described in Fig. 3A. As can be seen from the representative recordings, the IKᵥ amplitude at the test potential measured following the same conditioning potential was progressively decreased when RAMs were dialysed with 5 and 10 mM MgCl₂ in the pipette compared to that in 0.5 mM (Fig. 3A, arrows). Overall, an increase of Mg²⁺ from 0.5 mM to 5 and 10 mM caused a marked effect on IKᵥ availability shifting it towards more negative membrane potentials by ~5 and 12 mV.

\[
\frac{g_{Kv}}{G_{max}} = \frac{1}{1 + \exp(V_m-V_a)/(k_a)}
\]

where gₖᵥ, is IKᵥ conductance at each membrane potential (Vₘ) derived as a ratio of the peak IKᵥ over the difference between the test Vₘ and the K⁺ equilibrium potential equal to ~83 mV. Gₘₐₓ, Vₐ, and kₐ are the maximal whole-cell IKᵥ conductance, the half-activation potential, and the slope factor, respectively.

Fig. 1. Effect of Mg²⁺ on IKᵥ. (A) Families of IKᵥ in RAMs dialysed with 0.5 (Cₐₘ=18.6 pF), 5 (Cₐₘ=14.1 pF) and 10 (Cₐₘ=15.2 pF) mM MgCl₂. Note that traces are only shown between −40 and +100 mV in 20-mV increments. (B) and (C) Mg²⁺-dependence of I–V relationships and kinetics of activation (time constants derived from single exponential fit) of IKᵥ. (D) Mg²⁺-dependent changes in maximal conductance calculated from I–V shown in panel (B). The peak IKᵥ at each test potential was corrected off-line for a leak current (calculated from the mean slope resistance between −90 and −60 mV) and fitted with Boltzmann function:
Inactivation $k$ described in Methods) is also indicated in brackets. $\text{pH}=7.2$ unless indicated otherwise. Numbers of cells studied shown in brackets. Free $[\text{Mg}^2+]_i$ (calculated as 390 mM near symbols). Normalised conductance was fitted with the Boltzmann function similar to that described in the legend to Fig. 1 with mean parameters given in Table 1 (solid lines). Dashed lines show $V_h$ and $V_a$ values.

Changes in $[\text{Mg}^2+]_i$ in the presence of 1 mM MgATP had practically no effect on the mid-point of $I_{\text{Kv}}$ activation; however, $k_a$ values were significantly increased compared to those in ATP-free solutions (Table 1). The $I_{\text{Kv}}$ availability measured in the presence of 4 mM MgCl$_2$ was also significantly shifted to more negative membrane potentials by a similar degree to that observed in the presence of 10 mM MgCl$_2$ alone (Table 1). It is noteworthy that, when RAMs were dialysed with 0.5 mM MgCl$_2$ or with 1 mM MgATP/0.2 mM MgCl$_2$ (giving similar free $[\text{Mg}^2+]_i=0.33$ and 0.32 mM respectively), mean $V_h$ and $V_a$ values were also similar (Table 1), indicating that observed shifts in the $I_{\text{Kv}}$ inactivation were determined by changes in $[\text{Mg}^2+]_i$. In addition, no significant difference in the current activation kinetics was observed under these conditions (not shown).

The $[\text{Mg}^2+]_i$-dependent effect on $I_{\text{Kv}}$ inactivation was not mimicked by an increased concentration of protons achieved via adjusting the pH of the pipette solution containing 0.5 mM MgCl$_2$ to 6.2 instead of 7.2 (Table 1).

3.3. Effect of $[\text{Mg}^2+]_i$ on the steady-state open probability of $I_{\text{Kv}}$

The selective effect of $[\text{Mg}^2+]_i$ on $I_{\text{Kv}}$ inactivation, but not activation, can potentially affect the number of functional channels opened at rest by altering the steady-state open probability of the whole-cell $I_{\text{Kv}}$ current [24]. To evaluate the effect of $[\text{Mg}^2+]_i$ on the whole-cell steady-state $I_{\text{Kv}}$, the changes in $V_h$ and $V_a$ were plotted against the calculated free $[\text{Mg}^2+]_i$ (Fig. 4A). The nearly linear dependence of the shifts on $[\text{Mg}^2+]_i$ allows the prediction of $V_h$ and $V_a$ at any [Mg$^{2+}$]i in order to calculate the whole-cell steady-state open probability of $I_{\text{Kv}}$ ($P_{\text{open}}$). The $P_{\text{open}}$ was calculated as

$$P_{\text{open}} = mh,$$

where $m$ and $h$ represent theoretical fractions of the steady-state activated and inactivated $I_{\text{Kv}}$ at each membrane potential derived from equations similar to those described in the legends to Figs. 1 and 3, respectively (see Ref. [24] for details). Since no significant changes in the slope factors were found (Table 1), $k_a$ and $k_h$ values in different $[\text{Mg}^2+]_i$ were averaged and used in calculations. Fig. 4B illustrates a decrease in $P_{\text{open}}$ of $I_{\text{Kv}}$ with increased $[\text{Mg}^2+]_i$.

Both the sensitivity of $I_{\text{Kv}}$ and degree of inhibition of the steady-state $I_{\text{Kv}}$, by $[\text{Mg}^2+]_i$ were increased in the presence of 1 mM MgATP (Fig. 4C). It is noteworthy that $P_{\text{open}}$ was affected by changes in $[\text{Mg}^2+]_i$ mostly in the negative voltage range, while at potentials positive to 0 mV the $P_{\text{open}}$ was decreased.

Table 1

<table>
<thead>
<tr>
<th>$[\text{Mg}^2+]_i$ (mM)</th>
<th>$[\text{Mg}^2+]_i$ (mM)</th>
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<tbody>
<tr>
<td>0.5 mM MgCl$_2$</td>
<td>5 mM MgCl$_2$</td>
<td>10 mM MgCl$_2$</td>
<td>1 mM MgATP</td>
<td>1 mM MgATP</td>
<td>0.5 mM MgCl$_2$, pH=6.2</td>
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<tr>
<td>($[\text{Mg}^2+]_i=0.33$ mM)</td>
<td>($[\text{Mg}^2+]_i=3.48$ mM)</td>
<td>($[\text{Mg}^2+]_i=7.3$ mM)</td>
<td>($[\text{Mg}^2+]_i=0.32$ mM)</td>
<td>($[\text{Mg}^2+]_i=2.79$ mM)</td>
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<tr>
<td>Activation</td>
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<tr>
<td>$V_h$ (mV)</td>
<td>8.2±1.7 (12)</td>
<td>2.9±1.7 (19)</td>
<td>7±2.6 (20)</td>
<td>8.3±5.4 (5)</td>
<td>10±4.2 (5)</td>
</tr>
<tr>
<td>$V_a$ (mV)</td>
<td>14±0.5 (12)</td>
<td>13.5±0.5 (19)</td>
<td>13.2±0.8 (20)</td>
<td>18.6±1.3 (5)$^b$</td>
<td>17.5±1.4 (5)$^b$</td>
</tr>
<tr>
<td>$k_a$ (mV)</td>
<td>-44.8±1.6 (6)</td>
<td>-50.4±1.9 (19)</td>
<td>-56.6±3 (13)*</td>
<td>-44.2±2.5 (11)</td>
<td>-56.8±3.3 (7)**</td>
</tr>
<tr>
<td>$k_h$ (mV)</td>
<td>8.9±0.6 (6)</td>
<td>10±0.4 (19)</td>
<td>9.9±0.5 (13)</td>
<td>9.5±0.6 (11)</td>
<td>9.6±0.6 (7)</td>
</tr>
<tr>
<td>$A$</td>
<td>0.15±0.01 (6)</td>
<td>0.17±0.02 (19)</td>
<td>0.13±0.2 (13)</td>
<td>0.26±0.03 (11)</td>
<td>0.15±0.03 (7)</td>
</tr>
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</table>

$V_h$ and $V_a$ are half-activation and half-inactivation potentials and $k_a$ and $k_h$ are slope factors for activation and inactivation dependencies, respectively. $A$ denotes a fraction of noninactivating current. pH=7.2 unless indicated otherwise. Numbers of cells studied shown in brackets. Free $[\text{Mg}^2+]_i$ (calculated as described in Methods) is also indicated in brackets.

* Significant difference in $V_h$ between 10 mM MgCl$_2$ and 0.5 (P<0.023, two-tail t test) and 5 (P<0.041, one-tail t test) mM, respectively.

** Significant changes in $V_a$ in two different ATP-containing solutions (0.006<P<0.01).

§ Significant difference in $k_h$ between ATP-free and ATP-containing solutions (0.001<P<0.018).
relatively independent of Mg$_{2+}^+$ approaching the level determined by the noninactivating fraction of $I_{Kv}$.

3.4. Effect of Mg$_{2+}^+$ on membrane potential in single RAMs

The analysis shown in Fig. 4B suggests that an increase in Mg$_{2+}^+$ should result in membrane depolarisation of RAMs. Therefore, the effect of 0.2 and 4 mM MgCl$_2$ in the pipette solution containing 1 mM MgATP on the cell membrane potential ($V_m$) was investigated in single RAMs. Since cell dialysis with both pipette solutions caused a gradual decrease in the membrane potential in the current clamp mode (as shown in examples in Fig. 5A), changes in the rate of membrane depolarisation during cell dialysis with different Mg$_{2+}^+$ were monitored. Fig. 5A compares time-dependent changes in $V_m$ recorded 20 s after breakthrough into the cell interior in two RAMs dialysed with 0.2 or 4 mM MgCl$_2$. Overall, cells dialysed with higher Mg$_{2+}^+$ had a more positive membrane potential which decreased faster than that in RAMs dialysed with low Mg$_{2+}^+$. Irregular fluctuations of $V_m$ were also observed under both conditions. To analyse quantitatively the difference in the time course of $V_m$, $V_m$ values at 20, 60, 90, 120, 150, and 180 s were averaged in 16 (0.2 mM MgCl$_2$) and 15 (4 mM MgCl$_2$) RAMs and plotted in Fig. 5B. At 20 s, no significant difference in the mean $V_m$ was observed ($-31.9 \pm 4.2$ mV in 4 mM vs. $-39.3 \pm 4.1$ mV in 0.2 mM MgCl$_2$). However, at 60 and 90 s, cells dialysed with high Mg$_{2+}^+$ were significantly more depolarised than RAMs dialysed with 0.2 mM. Additionally, the difference between mean $V_m$ values after 120 s of cell dialysis was marginally significant ($P < 0.031$, one-tail $t$ test). It is noteworthy that another 6 RAMs (3 cells in each pipette solution) were not included in this analysis due to their low $V_m$ immediately after breakthrough ($12.8 \pm 4.6$ mV and $9.4 \pm 2.8$ mV in 0.2 and 4 mM MgCl$_2$, respectively).

3.5. Effect of Mg$_{2+}^+$-loading on excitability of endothelium-denuded rat aortic preparations

The significant Mg$_{2+}^+$-dependent inhibition of the steady-state $I_{Kv}$ at negative membrane potentials associated with membrane depolarisation described above should result in increased excitability of aortic smooth muscles. To evaluate this possibility, contractile responses to various concentrations of KCl were examined in endothelium-denuded rat aortic preparations before and after tissue loading with Mg$_{2+}^+$ as described in Methods. Fig. 6A shows a representative example of the experiment performed on the same aortic ring. Before Mg$_{2+}^+$-loading, an increase in KCl concentration caused a marked contraction at 20 mM (Fig. 6Aa), whereas after incubation in high MgCl$_2$ solution, sensitivity to KCl was significantly increased, eliciting contraction at as low as 10 mM and reaching nearly
maximal contraction at 20 mM KCl (Fig. 6Ab). The effect of Mg^{2+}-loading was fully reversible (Fig. 6Ac). The comparison of the mean absolute and normalised values of tension (Fig. 6Ba and Bb, respectively) clearly shows a significantly enhanced responsiveness of aortic preparations to low concentrations of KCl (between 10 and 20 mM) after Mg^{2+}-loading. It is noteworthy that no significant changes in the maximal contraction were observed after Mg^{2+}-loading in the Na^{+}-free buffer, although a significant increase in the maximal tension was seen upon returning to Na^{+}-containing solution (Fig. 6B). Such enhancement was not found when KCl concentration responses were recorded in the Na^{+}-free buffer during recovery (Fig. 6Bc and Bd), indicating a possible Na^{+}-dependence of this increase in the maximal tension.

If increased excitability of the rat aorta in Mg^{2+}-loaded preparations is due to decreased I_{Kv}, then Kv channel inhibitors should mimic the effect of KCl. Therefore, the effect of different concentrations of TEA and 4-AP (Kv channel inhibitors which block I_{Kv} in RAMs in millimolar range [18]) was compared using the experimental protocol described in Methods. Contraction caused by I_{Kv} inhibitors was reversibly enhanced after loading the tissue with Mg^{2+} (Fig. 7A and B) in a manner similar to that for KCl. To account for differences in the size and force in individual preparations, concentration responses to both inhibitors were expressed as a percentage of maximal contraction induced by 2.5 μM PE in Krebs. Notably, maximal contractions to 2.5 μM PE measured in Krebs between concentration responses to the I_{Kv} inhibitors were not significantly different being equal to 1.3±0.07, 1.4±0.09, and 1.3±0.08 g (n=12), suggesting therefore that the Na^{+}-removal and Mg^{2+}-loading procedures did not significantly alter tissue contractility throughout the experiment. A comparison of TEA and 4-AP concentration responses demonstrates a significant increase in the tension in response to low concentrations of both inhibitors after the Mg^{2+}-loading procedure (Fig. 7C and D). During recovery, the effects of TEA and 4-AP were significantly diminished at low concentrations, but increased at higher doses, although this effect was only significant at 5 mM 4-AP when compared to the control value.

To investigate whether increased contractility of SMCs is responsible for the enhanced contraction described above, the effect of α1-adrenoreceptor agonist PE was studied. Since PE also causes membrane depolarisation and activation of VDCCs [18], experiments were
Fig. 6. Effect of Mg\(^{2+}\)-loading on KCl-induced contraction of rat aorta. (A) Contraction to different KCl concentrations (indicated in mM) measured in a representative preparation before (a) and after (b) Mg\(^{2+}\)-loading and after washout with the Na\(^+\)-containing buffer (c). (Ba) and (Bb) Tension–concentration responses to KCl expressed in grams and normalised to the maximal contraction at 100 mM KCl, respectively \((n=8–12, 3\) rats). (Bc) and (Bd) illustrate the full reversibility of the effect of Mg\(^{2+}\) loading on KCl responses in the Na\(^+\)-free buffer recorded in a separate set of experiments \((n=16, 4\) rats). Note the lack of potentiation of contraction at high KCl during the recovery in the absence of external Na\(^+\). Washouts (30 min) between KCl concentration responses were performed in Krebs. *, **, and *** indicate 0.012\(\leq P < 0.046\), 0.007\(\leq P < 0.005\), and 0.0001\(\leq P < 0.0001\), respectively. Recovery in the Na\(^+\)-containing buffer was significantly different from both control and Mg\(^{2+}\)-loaded preparations between 15 and 100 mM (Ba) and between 15 and 40 mM (Bb) KCl (0.0001\( < P \leq 0.006\)). All statistical comparisons in this and subsequent figures were performed using paired t test.

Fig. 7. Effect of Mg\(^{2+}\)-loading on TEA- and 4-AP-induced contraction. (A) and (B) Representative recordings in different concentrations of TEA (A) and 4-AP (B; as indicated in mM by arrows, each applied for 10 min) performed as described in Methods. Experiments were carried out in the presence of 1 \(\mu\)M paxilline (10 min pretreatment) to block the BK Ca conductance. (C) and (D) Comparison of TEA- and 4-AP-induced contraction (expressed as percentage of the 2.5 \(\mu\)M PE-induced contraction) measured in 8 rings from 4 rats. *, **, and *** indicate significant difference between control and Mg\(^{2+}\)-loaded preparations at \(P \leq 0.02\), 0.001\( < P < 0.005\), and \(P < 0.0002\), respectively. Recovery was significantly different from Mg\(^{2+}\)-loaded rings for TEA at 0.5–2 mM \(P < 0.001\) and for 4-AP at 0.5 mM \(P < 0.005\).
performed both in the absence and presence of the L-type VDCC inhibitor diltiazem (2 μM). The comparison shows that Mg2+-loading significantly enhanced contraction to low PE concentrations in the absence (Fig. 8A) but not in the presence of diltiazem (Fig. 8B). Notably, contraction to higher concentrations of PE in Mg2+-loaded preparations was reduced in the presence of the inhibitor (Fig. 8B) compared to no significant effect in the absence of diltiazem (Fig. 8A). In addition, following recovery, PE-induced contraction (recorded in the presence of diltiazem and probably caused by PE-induced Ca2+ release from intracellular stores) was significantly suppressed (Fig. 8B). Consecutive PE concentration responses performed without Mg2+ loading, but in the presence of diltiazem, demonstrated only a relatively small suppression of contraction during the 3rd application of the agonist (Fig. 8C). This effect was significant between 20 and 1000 nM when compared to the two previous PE concentration dependencies.

4. Discussion

In this paper, we have characterised the modulation of Kv channels by Mg2+ in VSMCs. An increase in Mg2+ produced several significant effects on the IKv: (i) slowed down kinetics of activation at high (10 mM) Mg2+; (ii) caused inward rectification at positive membrane potentials; (iii) shifted voltage-dependent inactivation, but not steady-state IKv activation, to more negative voltages; (iv) the effect of Mg2+ on IKv inactivation was enhanced in the presence of intracellular ATP; and (v) the cell membrane was depolarised more rapidly in high Mg2+. In addition, we demonstrated the enhanced sensitivity of Mg2+-loaded rat aortic preparations to membrane depolarisation by KCl and to IKv inhibitors TEA and 4-AP.

A significant slow down of the kinetics of IKv activation was observed only when cells were dialysed with the pipette solution containing 10 mM MgCl2, when a significant inhibition of the current was seen (Fig. 1). The slow down of IKv activation kinetics mimicked the effect observed in bullfrog atrial myocytes but at much lower concentrations of Mg2+ (1 mM) [11]. Conversely, an acceleration of the current kinetics in frog atrial cells dialysed with 10 mM Mg2+ was found [10]. The effect of Mg2+ on IKv kinetics is unlikely to be a result of a direct block of the channel since no significant effect of blocking cations on the activation rate of expressed Kv channels was previously observed [15]. Although the mechanism of this effect remains to be clarified, it is likely to be mediated by a Mg2+-dependent process.

The inward rectification of IKv in high Mg2+ closely mimics the voltage-dependent block of cloned Kv channels [13–16] and could share a similar mechanism. However, occurring at very positive voltages, this effect is unlikely to have a great impact on vascular contractility. It is noteworthy that increased Mg2+ caused a similar type of rectification of the cloned Kv2.1 channel [16], which was proposed to be a molecular correlate of the IKv in RAMs [18].

By contrast, the differential effect on the steady-state activation and inactivation of IKv in RAMs can affect the whole cell steady-state open probability in the physiological range of membrane potentials, and thus excitability of VSMCs [24]. A classical explanation of shifts in voltage-
dependencies of an ion current, when the extra- or intracellular concentration of divalent cations is changed, is their interaction with negative charges in the close vicinity of the voltage sensor, leading to similar shifts in both activation and inactivation dependencies [25]. Indeed, similar leftward shifts in the activation and inactivation dependencies of the Kv current in increased Mg\textsuperscript{2+} were demonstrated in the giant squid axon. The magnitude of the shifts was increased in the presence of ATP and explained by the addition of new phosphorylated charged sites which interact with Mg\textsuperscript{2+} [26]. Although the presence of ATP increased the shift in the I\textsubscript{Kv} inactivation in RAMs, no significant changes in V\textsubscript{0} values were observed, arguing against similar interactions in these cells. In addition, an increase in intracellular proton concentration did not significantly affect the voltage-dependent characteristics of I\textsubscript{Kv} in RAMs (Table 1), whilst similar changes in the extracellular pH caused a significant shift in the activation of both heterologously expressed Kv channels and Kv currents in ventricular myocytes [27–29]. Additionally, no significant effect of Mg\textsuperscript{2+} on the Kv activation was found in amphibian atrial myocytes [10,11]. However, the effect of Mg\textsuperscript{2+} on Kv inactivation has not been investigated in these reports. Nevertheless, all the evidence argues against direct interactions of Mg\textsuperscript{2+} with fixed negative surface charges. Although the molecular mechanism of Mg\textsuperscript{2+}-induced changes in the I\textsubscript{Kv} inactivation in RAMs has yet to be identified, the ATP-dependence of the effect (this study) and modulation of I\textsubscript{Kv} inactivation by the protein kinase C inhibitor bisindolylmaleimide I [30] are indicative of the involvement of intracellular signalling cascades. To verify this hypothesis, further experimentation is necessary.

To evaluate the effect of Mg\textsuperscript{2+} on I\textsubscript{Kv} in the physiological range of membrane potentials in RAMs, changes in the whole-cell steady-state open probability of I\textsubscript{Kv} (also called “window current”) were assessed using a theoretical approach previously described [24]. This analysis predicts that an increase in Mg\textsuperscript{2+} progressively reduces I\textsubscript{Kv} between −60 and 0 mV and this effect was markedly enhanced in the presence of physiological levels of intracellular MgATP (Fig. 4). Such sensitivity of I\textsubscript{Kv} to changes in Mg\textsuperscript{2+} in the voltage range close to the cell resting potential can favour membrane depolarisation and increase tissue excitability, leading to increased contractility due to increased Ca\textsuperscript{2+} entry via VDCCs. The increased rate of membrane depolarisation during cell dialysis with high Mg\textsuperscript{2+} in single RAMs (Fig. 5) and increased sensitivity to KCl (which causes vasoconstriction entirely via the voltage-dependent pathway) and to the I\textsubscript{Kv} inhibitors TEA and 4-AP in Mg\textsuperscript{2+}-loaded intact aortas (Figs. 6 and 7) strongly supports this conclusion. Although the effect of Mg\textsuperscript{2+} on VDCCs has not been directly investigated, such enhanced sensitivity of Mg\textsuperscript{2+}-loaded tissue is unlikely to be mediated by changes in VDCC activity since no significant differences in the maximal contraction, which would be expected if Ca\textsuperscript{2+} entry is increased, were observed before and after tissue loading with Mg\textsuperscript{2+} (Fig. 6). In addition, Mg\textsuperscript{2+} generally inhibits VDCCs [5,6,31], which would have an opposite effect on the tissue excitability.

The elevated contraction caused by KCl or I\textsubscript{Kv} blockers is unlikely to originate from increased Ca\textsuperscript{2+} release from intracellular stores or increased contractility since PE, in the presence of diltiazem, did not cause an increase in tension in Mg\textsuperscript{2+}-loaded preparations (Fig. 8B). Moreover, the PE-induced contraction was significantly decreased under these conditions. Such a marked decrease in tension is likely to be the result of the Mg\textsuperscript{2+} loading and not due to time-dependent changes in tissue contractility because only a small suppression of PE-induced contraction was observed under control conditions (Fig. 8C). Although from our data it is difficult to speculate about the exact mechanism of this force reduction, the inhibitory effect of Mg\textsuperscript{2+} on both IP\textsubscript{3} and ryanodine receptors, which may be at least partly responsible for the decrease in tension in the presence of the VDCC blocker diltiazem, has been previously described [32,33]. An involvement of Mg\textsuperscript{2+}-dependent enzyme-mediated modulation (e.g. phosphorylation or dephosphorylation) downstream to the activation of α\textsubscript{1}-adrenoreceptors, which may contribute to the long-lasting inhibition of contraction following the recovery from Mg\textsuperscript{2+} loading, cannot be excluded. Nevertheless, the enhanced PE-induced contraction in the absence of diltiazem (Fig. 8A) points towards increased tissue excitability which does not contradict the results obtained with KCl and the I\textsubscript{Kv} inhibitors. It is noteworthy that, like KCl (Fig. 6Bc), no potentiation of PE-induced contraction upon recovery was found (Fig. 8A), indicating that the significant increase in the maximal KCl-induced tension observed in the Na\textsuperscript{+}-containing buffer (Fig. 6Bb) may require the presence of extracellular Na\textsuperscript{+}. Although further experiments are necessary to clarify this question, it is possible that intracellular Na\textsuperscript{+} accumulation, resulting from Mg\textsuperscript{2+} extrusion via Na\textsuperscript{+}-Mg\textsuperscript{2+} exchanger [20], may cause elevation of Ca\textsuperscript{2+} via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger working in the reversed mode.

Finally, the involvement of BK\textsubscript{Ca} currents is also unlikely since: (1) BK\textsubscript{Ca} (recorded in the presence of 200 nM free Ca\textsuperscript{2+}) was not affected by an increase in Mg\textsuperscript{2+} to 5 mM in RAMs (not shown); (2) increased contractility to TEA and 4-AP was observed in the presence of the BK\textsubscript{Ca} inhibitor paxilline (Fig. 7); and (3) BK\textsubscript{Ca} do not significantly contribute to the regulation of aortic excitability [18].

In conclusion, our results demonstrate that Mg\textsuperscript{2+} can act as a potent modulator of the Kv channel function in VSMCs in the physiological range of membrane potentials, representing a novel mechanism for the regulation of Kv channel activity in the vasculature.

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References


