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Monovalent cation (MC) current in cardiac and smooth muscle cells: regulation by intracellular Mg^{2+} and inhibition by polycations

[1]Sergey I. Zakharov, [1]Tarik Smani, [1]Endri Leno, [2]Regina Macianskiene, [2]Kanigula Mubagwa & *,[1]Victoria M. Bolotina

[1]Boston University School of Medicine, Boston, Massachusetts, MA 02118, U.S.A. and [2]University of Leuven, Leuven, Belgium

1 Previously we have described a monovalent cation (MC) current that could be unmasked by the removal of extracellular divalent cations in vascular smooth muscle cells (SMC) and cardiac myocytes, but specific and potent inhibitors of MC current have not been found, and the mechanism of its intracellular regulation remains obscure.

2 Here we show that small MC current is present in intact cells and could be dramatically up-regulated during cell dialysis. MC current in dialyzed cells strongly resembled monovalent cation current attributed to Ca^{2+} release-activated Ca^{2+}-selective (CRAC) channels, but its activation did not require depletion of Ca^{2+} stores, and was observed when the cells were dialyzed with, or without BAPTA.

3 Intracellular free Mg^{2+} inhibits MC current with $K_d = 250$ μM.

4 Extracellular (but not intracellular) spermine effectively blocked MC current with $K_d = 3-10$ μM, while store-operated cations (SOC) channels and capacitative Ca^{2+} influx were not affected.

5 Spermine effectively inhibited MC current-induced SMC depolarization, and prevented Ca^{2+} paradox-induced vascular contracture.

6 Both, MC and SOC currents were inhibited by 2-aminoethoxydiphenyl borate (2-APB).

7 It is concluded that MC current could be regulated by intracellular Mg^{2+}, and low concentrations of extracellular spermine could be used to discriminate it from SOC current, and to assess its role in cellular function.

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CRAC, Ca^{2+} release-activated Ca^{2+} current; MC, current, monovalent cation current; SMC, smooth muscle cell; SOC, channel, store-operated cation channel; SpM, spermine; SpD, spermidine

Introduction

A whole-cell monovalent cation (MC) current that can be unmasked by the removal of extracellular divalent cations has been found in a variety of excitable and non-excitable cells (Mubagwa *et al*., 1997; Zakharov *et al*., 1999b; Bosteels *et al*., 1999; Hagiwara *et al*., 1992; Voets *et al*., 1996; Sabovcik *et al*., 1995; 1996; Arellano *et al*., 1995; Zhang *et al*., 1998). In cardiac and smooth muscle cells, the signature features of the MC current are: (1) the absence of inactivation, (2) selectivity for monovalent cations, (3) inhibition by various extracellular divalent cations (including Ca^{2+} with an IC_{50} between 60 and 250 μM in different preparations), (4) inhibition by trivalent cations, and (5) lack of inhibition by dihydropyridine receptor antagonists, and blockers of various K^+ channels. Under physiological conditions, the MC current is thought to participate in regulation of resting membrane potential (Zakharov *et al*., 1999a; Bae *et al*., 1999). Under pathological conditions, the current may be responsible for the so called 'Ca^{2+} paradox' phenomenon (Zimmerman & Hulsmann, 1966) in smooth muscle cells (SMC) and cardiac myocytes (when reduction in extracellular Ca^{2+} concentration triggers pathological Na^+ influx, membrane depolarization and secondary Ca^{2+} overload that result in significant injury of the cells) (Zakharov *et al*., 1999b; Bosteels *et al*., 1999). The MC current was also recently shown to be modulated by metabolic inhibition and by different oxidants (Macianskiene *et al*., 2001). In spite of the important physiological and pathological role of MC current, the nature of underlying channels and the mechanism of their regulation are far from being understood. Potent and specific inhibitors of MC channels have not yet been found, which makes it very hard to distinguish these channels from some other non-inactivating channels capable of conducting monovalent cations. In non-excitable cells some monovalent cation currents have recently been considered to reflect specific Ca^{2+} release activated Ca^{2+} (CRAC) currents that under divalent cation-free conditions can switch from Ca^{2+} to monovalent cation conducting mode (Lepple-Wienhues & Cahalan, 1996; Braun *et al*., 2001; Rychkov *et al*., 2001; Mignen & Shuttleworth, 2001), but this possibility was questioned in some most recent

*Author for correspondence at: Vascular Biology Unit, Boston University School of Medicine, ECRC, X-704, 650 Albany Street, Boston, Massachusetts, MA 02118, U.S.A.;
E-mail: vbolotina@medicine.bu.edu

studies (Prakriya & Lewis, 2002; Bakowski & Parekh, 2002). Thus, new experimental approaches and new specific inhibitors are needed to distinguish the true MC channels (that do not conduct Ca^{2+}) from Ca^{2+} selective channels (that readily conduct Ca^{2+} if it is present, but switch to the monovalent-conducting mode in divalent cation-free conditions). Here we present new evidence on the mechanism of intracellular regulation of MC channels in cardiac myocytes and in vascular SMC. Because pharmacological inhibition of MC channels could be the mechanism underlying the cardioprotective action of some polyamines (Busselen, 1991), we have tested their effects on MC current and found that spermine is a potent extracellular inhibitor and could be used to discriminate MC current from some other Na^+ and Ca^{2+}-conducting channels. Polyamines as inhibitors of MC current could be an important new tool for further studies of the physiological and pathological role of MC channels in cellular function. Preliminary data have been presented as an abstract (Zakharov *et al*., 2002).

Methods

Adult rat cardiac myocytes (rCM)

Ventricular myocytes were isolated from the hearts of male Sprague–Dawley rats (200 g) as previously described (Communal *et al*., 1998). All anaesthetic and surgical procedures used fall within the guidelines of local Animal Ethics Committee. Briefly, animals were anaesthetized and heparinized, and their hearts aseptically removed into ice-cold modified cardioplegic (KB) solution, and then retrograde-perfused with regular Tyrode's solution for 5 min at 37°C. The perfusion solution was switched to a Ca^{2+}-free solution for 6 min, and then to a Ca^{2+}-free solution containing 0.02% protease and 0.06% collagenase A. After perfusion, cells from left ventricle were released by shaking the tissue, filtered and allowed to settle (40 min) in KB solution. The cells were then resuspended in DMEM, laid over 60 μg ml^{-1} BSA to separate ventricular myocytes from nonmyocytes, and allowed to settle for 15 min. The cells were then resuspended in DMEM with 2 mg ml^{-1} BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin, and plated on glass coverslips coated with laminin. The cells were kept in an incubator at 37°C, and were used during the period of 12–72 h after isolation. Only cardiac myocytes that preserved a typical rod shape were used for experiments.

Vascular SMC

SMC from rabbit aorta and rabbit pulmonary artery were isolated as we previously described (Zakharov *et al*., 1999b; Smani *et al*., 2001). Freshly isolated cells were stored at 5°C until used during the next 6 h.

Human platelets were prepared and studied as described before (Trepakova *et al*., 1999).

Electrophysiological studies

Whole-cell currents were recorded using perforated, or dialyzed patch clamp technique as described before (Zakharov *et al*., 1999b; Bosteels *et al*., 1999). Briefly, amphotericin B (240 μg ml^{-1}) was added to the pipette solution and whole-cell recording was performed only when the access resistance of less than 20 MΩ was achieved (usually 10–20 min after obtaining of a giga-seal). For experiments with cell dialysis, standard whole-cell technique was used and the current was monitored starting immediately after breaking into the cell. Data were recorded with an Axopatch 200a, filtered at 2 kHz, sampled at 5 kHz, and analysed using pCLAMP8 software. Heat-polished glass pipettes were used with tip resistance of 1–2 MΩ. Cell capacitance and series resistance were compensated. The whole-cell current was monitored at holding potential of −80 mV in cardiac myocytes and −60 mV in SMC. Ramp depolarization (from −120 to +80 mV and back to −120 mV, 4-s long, applied every 10 s) was used to assess the changes in the I/V relationship. All experiments were performed at 20–22°C.

There are certain technical limitations that may interfere with the studies of the currents that depend on cell dialysis. Indeed, during the first few minutes of cell dialysis, (a) the liquid junction potential between cytoplasm and pipette solution is changing, (b) K^+ currents are getting inhibited by Cs^+ in the pipette, (c) reversal potential of the currents changes because of the changes in intracellular concentration of different ions. Luckily, these factors cannot account for the slow time-dependent up-regulation of the inward MC current (when measured at −80 mV for CM and −60 mV for SMC), which allowed us to study and describe its dependence on intracellular Mg^{2+}.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} measurements in human platelets were done as described before (Trepakova *et al*., 1999).

Solutions and drugs

For whole-cell recordings the same standard bath solution was used in SMC and cardiac myocytes (in mM): NaCl 140, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Glucose 11, NaH_2PO_4 1, HEPES (pH 7.4 with NaOH) 10. Extracellular Ca^{2+}/Mg^{2+}-free solution was made by omitting Ca^{2+} and Mg^{2+} and adding EGTA (100 μM) to the standard bath solution. In perforated patch experiments the following pipette solution was used (in mM): CsAspartate 100, CsCl 40, NaCl 4.5, EGTA 0.1, HEPES (pH 7.2 with CsOH) 10. For membrane potential measurements K^+ was used instead of Cs^+ in the bath and pipette solutions. For experiments with intracellular dialysis the basic (chelators-free) pipette solution contained (in mM): CsGlutamate 130, TEA 10, HEPES (pH 7.2 with CsOH) 10. To this basic solution the following additions were made depending on the type of experiments (in mM): (1) in standard experiments – $MgCl_2$ 1, EGTA 1, Na_2ATP 5, (2) for BAPTA dialysis – BAPTA 12, $CaCl_2$ (free Ca^{2+} < 5 nM) 0.9, (3) for studies of the effects of intracellular free Mg^{2+} – from 3 μM to 1 mM $MgCl_2$ was added. Nifedipine (10 μM) was used in the bath solution to suppress L-type Ca^{2+} current in cardiac myocytes. Nifedipine and 2-APB were diluted in DMSO, and added to the bath solutions before each experiment (the residual concentration of DMSO was <0.1%). Polyamines were dissolved in bath solution before the experiment. All drugs were obtained from Sigma.

Data analysis

Results were expressed as mean ± standard error; *n* represents the number of cells tested. Statistical significance was evaluated using paired Student *t*-test ($P<0.05$ was considered significant).

Dose-response curves were obtained by fitting the Hill equation to the average data. Each data point in the figures represents the mean ± s.e.mean per cent of maximum MC current from *n* cells (as indicated). The data are fitted with Hill equation: $I = 100(1 + K_d/[C]^{nHill})^{-1}$, where K_d is a measure of the affinity constant, [C] is concentration of inhibitor, and n_{Hill} is the Hill coefficient. Because only a limited number of concentrations of inhibitors could be tested on a given cell, K_d values were estimated from the above fittings to average data rather than being the mean of individual values obtained from each cell.

Results

Intracellular regulation of MC current

The monovalent cation (MC) current with biophysical properties that we have described previously in SMC and cardiac myocytes (Zakharov *et al*., 1999b; Mubagwa *et al*., 1997) was studied and compared in intact cells (under perforated patch-clamp conditions when intracellular environment, including divalent cations and other components of the cytoplasm have been preserved intact), and in dialyzed cells (under standard whole-cell patch clamp conditions, when the cells were dialyzed with the pipette solutions of different compositions).

In intact cells we found that when extracellular Ca^{2+} and Mg^{2+} are removed, MC current could be unmasked with an amplitude of -0.40 ± 0.1 pA/pF (at -80 mV, $n=12$) in cardiac myocytes, and -0.7 ± 0.2 pA/pF (at -60 mV, $n=10$) in SMC, and it does not change over time during up to 1 h of the recording (Figure 1A, open symbols). In contrast, when the cells were dialyzed, a slow time-dependent up-regulation of the MC current was observed (Figure 1A, closed symbols). Interestingly, the inward component of MC current showed significantly stronger up-regulation than the outward component, resulting in a pronounced inward rectification of the whole-cell current (Figure 1B, also see Figures 3B and 5A) which was not observed under perforated-patch conditions (Figure 1B). After 30–50 min of cell dialysis the amplitude of the inward MC current peaked at the level of -5.6 ± 1.1 pA/pF ($n=19$) in cardiac myocytes and -12.4 ± 1.0 pA/pF ($n=7$) in SMC, which reflected a more than 10 fold increase in the amplitude in comparison with the MC current in intact cells. Figure 2A summarizes the differences in the amplitude of the inward MC current recorded from the intact cardiomyocytes (a) and those dialyzed with a standard intracellular solution (b) in the beginning and after 30+ min of the experiment. It is important to mention that we found the same MC current developed during cell dialysis in the presence of divalent cations, and could be unmasked by the removal of extracellular divalents at any time ($n=10$). The time-dependent up-regulation of the MC current during cell dialysis strongly suggests that activation of the MC current could be regulated by some intracellular component(s), or process that could be affected by cell dialysis.

First we tested if chelation of intracellular Ca^{2+} could be a reason for the up-regulation of the MC current. The amplitude of MC current (Figure 2A) was compared in cardiac myocytes dialyzed with solutions containing either 12 mM BAPTA (c), or no chelators at all (d). After 30+ min of cell dialysis we found no significant differences in MC

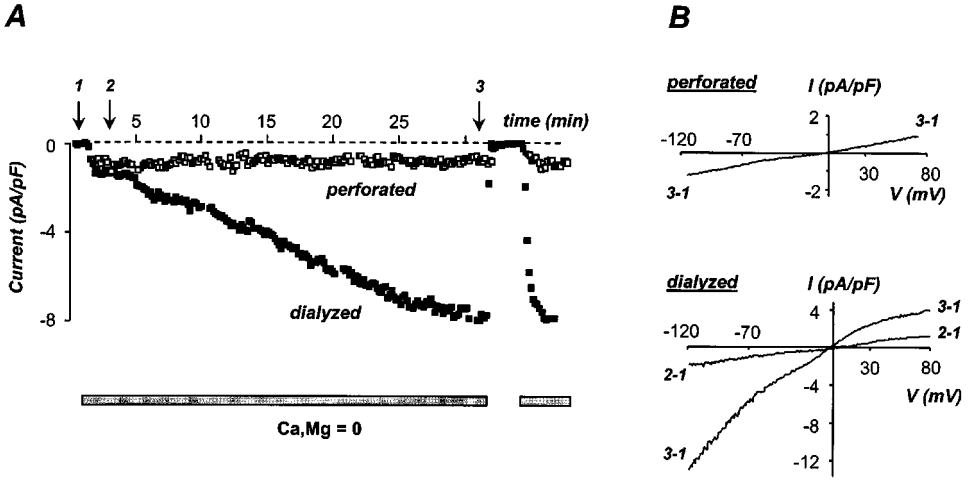

Figure 1 MC current in the absence of extracellular Ca^{2+} and Mg^{2+} in perforated and dialyzed rat ventricular myocytes during the time-course of the experiment. (A) The superposition of the time course of changes in inward whole-cell current density (pA/pF) recorded at holding potential of -80 mV in perforated (open symbols) and dialyzed (closed symbols) cardiac myocytes. The recording started right after the formation of the sufficient electrical access into the cell (perforated patch conditions), or after the 'physical breaking into the cell' (whole-cell dialysis conditions). In both cases, after about 1 min, extracellular Ca^{2+} and Mg^{2+} were removed unmasking the basal MC current which either remained stable, or showed dramatic up-regulation with time. Divalent cations (2 mM Ca^{2+} and 1 mM Mg^{2+}) were readmitted after 32 min of the experiment (for a couple of minutes) to verify that inward current is indeed extracellular divalent cation-sensitive MC current. (B) The current–voltage relationships of MC current are shown at corresponding times (2 and 3) of the experiments on A (in perforated and dialyzed cells). The basal non-MC current (1), that could be observed before divalent cations are removed, was subtracted from each trace.

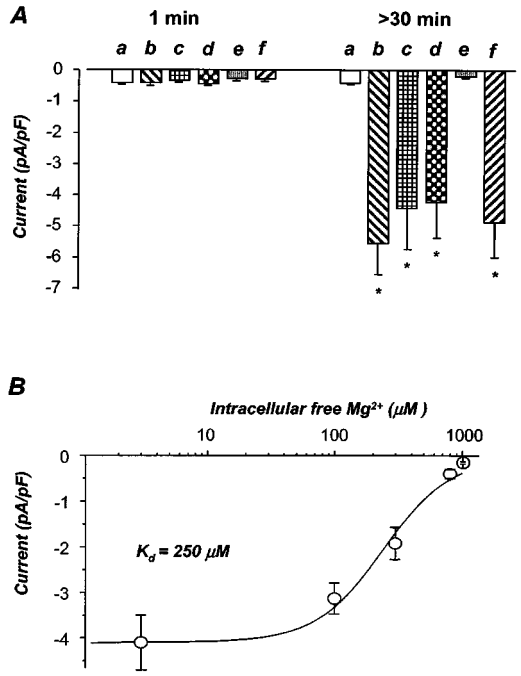

Figure 2 The effect of cell dialysis, Mg^{2+} and other intracellular components on the up-regulation of MC current in rat ventricular myocytes. Summary data showing the density of MC current (at −80 mV) at the beginning of the experiment (1 min) and 30–45 min after (>30 min), when MC current reaches its maximum. Statistically significant increase in current amplitude over the course of the experiment is shown by asterisk (*$P<0.001$). Each bar is an average of 5–21 experiments. (A) The bars show the summary data from MC current recording under the following experimental conditions, when either perforated patch clamp technique (a), or whole-cell dialysis (b–f) was used. Intracellular (pipette) solution contained (in mM): 130 CsAspartate, 20 TEA-Cl, 5 HEPES (chelators-free solution), plus the following components: *Bars* a,b: 1 $MgCl_2$, 1 EGTA and 5 Na_2ATP; *Bar* c: 12 BAPTA, 0.9 Ca^{2+} (free $Ca^{2+}<5$ nM); *Bar* d: chelator-free solution with nothing else added, *Bar* e: 5 MgATP, *Bar* f: 500 μM spermine. (B) Dose-dependent inhibition of the maximum MC current that developed after >30 min of cell dialysis with chelators-free solution (130 mM CsAspartate, 20 mM TEA-Cl, 5 mM HEPES) with different concentrations of free Mg^{2+} added. $K_d=250$ μM. Each point is an average of 3–7 experiments. The fit was done using Hill equation as described in Methods.

current, which appeared to be equally up-regulated independently on whether intracellular Ca^{2+} was buffered, or not. Additional pretreatment of the cells with 5 μM thapsigargin (an inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase, that is known to deplete intracellular Ca^{2+} stores and to cause activation of store-operated current) did not affect the MC current recorded 1 min after breaking into the cell, nor its up-regulation during cell dialysis (not shown). Thus, an up-regulation of the MC current occurred in the presence, or absence of intracellular Ca^{2+} buffering, suggesting that depletion of Ca^{2+} stores is not required. Next, we tested if intracellular ATP could be involved into regulation of the MC current, which brought an interesting paradigm. Indeed, we found that the time-dependent up-regulation of MC current is present in cardiac myocytes dialyzed with solution containing 5 mM Na_2ATP (Figure 2A, bars b), but it is totally absent when 5 mM MgATP is used instead (Figure 2A, bars e). These results could indicate that intracellular free Mg^{2+}, rather than ATP itself, may play an important role in this phenomenon. In order to test the possible role of free Mg^{2+} in intracellular regulation of the MC current, the maximum MC current amplitude was compared in cells dialyzed with solutions containing different amount of free Mg^{2+} (without exogenously added ATP). Figure 2B shows that when intracellular free Mg^{2+} concentration was increased, there was a dose-dependent inhibition of MC current with a K_d of 250 μM. Although a similar detailed analysis of the effects of intracellular Mg^{2+} was not carried out in SMC, the typical upregulation of the MC current was always observed in SMC when dialyzed with low-Mg^{2+} solutions, suggesting that in SMC there is a similar regulation of MC current by intracellular Mg^{2+}. Thus, Mg^{2+} (in its free form, or bound to ATP) served as intracellular inhibitor of the MC current, and depletion of free Mg^{2+} during cell dialysis is probably one of the major reasons for the dramatic up-regulation of MC current.

Inhibition of MC current by extracellular polyamines

The next goal of our study was to find a potent extracellular inhibitor of the MC current, that could help us further identify this current and allow its pharmacological separation from store-operated and other Na^+- and Ca^{2+}-conducting currents.

Since extracellular divalent and trivalent cations have been shown to inhibit MC current (Zakharov *et al*., 1999b; Mubagwa *et al*., 1997), we tested if some other extracellular polycations (like polyamines) could produce similar effects. Figure 3A shows that extracellular application of spermine (SpM, 100 μM), that carries four positive charges, completely inhibited the inward MC current that could be unmasked by the removal of extracellular Ca^{2+} and Mg^{2+} in dialyzed cardiac myocytes. The effect of SpM was fast and easily reversible upon washing it away (Figure 3A). Examples of the I/V relationships of MC current in the absence and presence of SpM are shown in Figure 3B). SpM inhibited the MC current in cardiac myocytes in a dose-dependent manner with K_d (SpM) = 10 μM (Figure 3C). Importantly, SpM effectively inhibited not only the MC currents that had been up-regulated during cell dialysis (Figure 3), but also the MC current present in intact cells (without intracellular dialysis). Figure 4A,B show the examples of the time-course of SpM-induced inhibition of MC current and corresponding I/V relationships in intact SMC (when perforated patch-clamp technique was used, and only small basal MC current could be unmasked similar to that illustrated in Figure 1A for cardiac myocytes). Similarly to cardiac myocytes, the effect of extracellular SpM on the MC current in SMC was fast, reversible and concentration-dependent. However, SpM was slightly more effective in SMC (K_d (SpM) = 3 μM, Figure 4C) compared to cardiac myocytes. In SMC we also tested the effect of spermidine (SpD, carrying three positive charges) and putrescine (with two positive charges), and found that spermidine also inhibited the MC current, but with much less potency ($IT > K_d$(SpD) = 70 μM, Figure 4C), while putrescine was without any effect at concentrations up to 100 μM ($n=3$, not shown). Importantly, intracellular application of SpM (100–500 μM in the dialysis solution) did not affect the up-regulation and amplitude of the MC current in cardiac myocytes (bars F in Figure 2). These data indicate that SpM

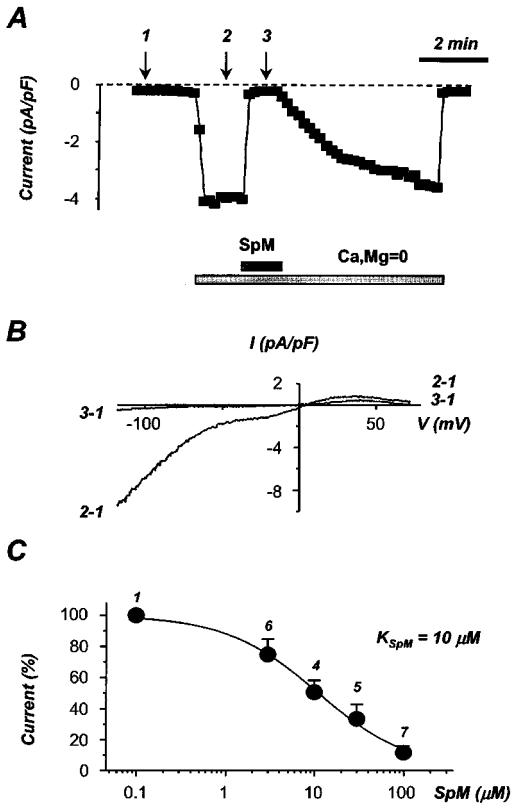

Figure 3 The effect of extracellular spermine on MC current in rat ventricular myocytes. (A) A typical example of inward MC current (recorded 30 minutes after the beginning of cell dialysis at holding −80 mV) unmasked by the removal of extracellular Ca^{2+} and Mg^{2+}, and inhibited by extracellular application of 100 μM spermine (SpM). (B) Corresponding current-voltage relationship before (1) and after (2) the removal of extracellular divalent cations, and after application of spermine (3), as specified in A. (C) Summary data showing the average per cent of MC current (at −80 mV) in the presence of different concentrations of extracellular spermine (SpM). Each data point represents the mean±s.e.mean current (per cent of the maximum current in each cell) from *n* cells (as indicated). The best fit was generated using Hills equation with $K_{SpM}=10$ μM and $n_{Hill}=0.84$ (see Methods for details).

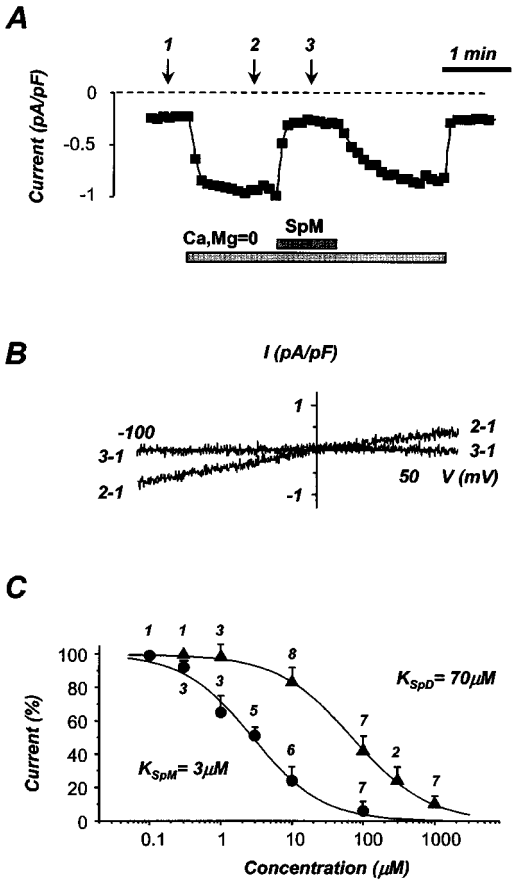

Figure 4 The effect of extracellular polyamines on MC current in vascular SMC. (A) A typical example of inward MC current (recorded using perforated patch clamp technique at holding −60 mV) unmasked by the removal of extracellular Ca^{2+} and Mg^{2+}, and inhibited by extracellular application of 100 μM spermine (SpM). (B) Corresponding curernt-voltage relationship before (1) and after (2) the removal of extracellular divalent cations, and after application of spermine (3), as specified in A. (C) Summary data showing the average per cent of MC current (at −60 mV) in the presence of different concentrations of extracellular spermine (SpM) and spermidine (SpD). Each data point represents the mean± s.e.mean current (per cent of the maximum current in each cell) from *n* cells (as indicated). The best fit was generated using Hill equation with $K_{SpM}=3$ μM, $n_{Hill}=0.85$, and $K_{SpD}=70$ μM, $n_{Hill}=0.83$.

may be used as an effective extracellular, but not intracellular inhibitor of the MC channels.

Inhibition of MC current with 2-aminoethoxydiphenyl borate (2-APB)

Looking for the other potential and helpful inhibitors of the MC current, we have also tested 2-aminoethoxydiphenyl borate (2-APB), which was originally introduced as an inhibitor of IP_3 receptor (Maruyama *et al*., 1997), but later has been widely used to inhibit store-operated cation channels and capacitative Ca^{2+} influx in a variety of cell preparations (Ma *et al*., 2000; Dobrydneva & Blackmore, 2001; Gregory *et al*., 2001; Iwasaki *et al*., 2001; Prakriya & Lewis, 2001). Figure 5 shows that extracellular application of 100 μM 2-APB produced fast and reversible inhibition of the inward MC current that developed during cell dialysis in both cardiac myocytes and SMC. The effect of 2-APB was dose-dependent with $K_d=30$ μM.

Extracellular SpM, but not 2-APB could be used to discriminate MC from store-operated channels

Monovalent cation-selective MC channels are clearly different from store-operated cation (SOC) channels (that are specifically activated upon the depletion of intracellular Ca^{2+} stores and conduct Ca^{2+} and other divalent cations), but both types of channels may coexist in the same cell, and can be both activated during cell dialysis in divalent cation-free conditions. Previous investigations did not distinguish them (Kerschbaum & Cahalan, 1998), in part because the experimental tools for separation of MC and SOC channels are lacking. SOC channels do not appear to be present in adult rat cardiomyocytes, and in this preparation the MC channels can be studied without any contamination from SOC currents. However, in other cells, like SMC, where the two channel types coexist, it is important to be able to

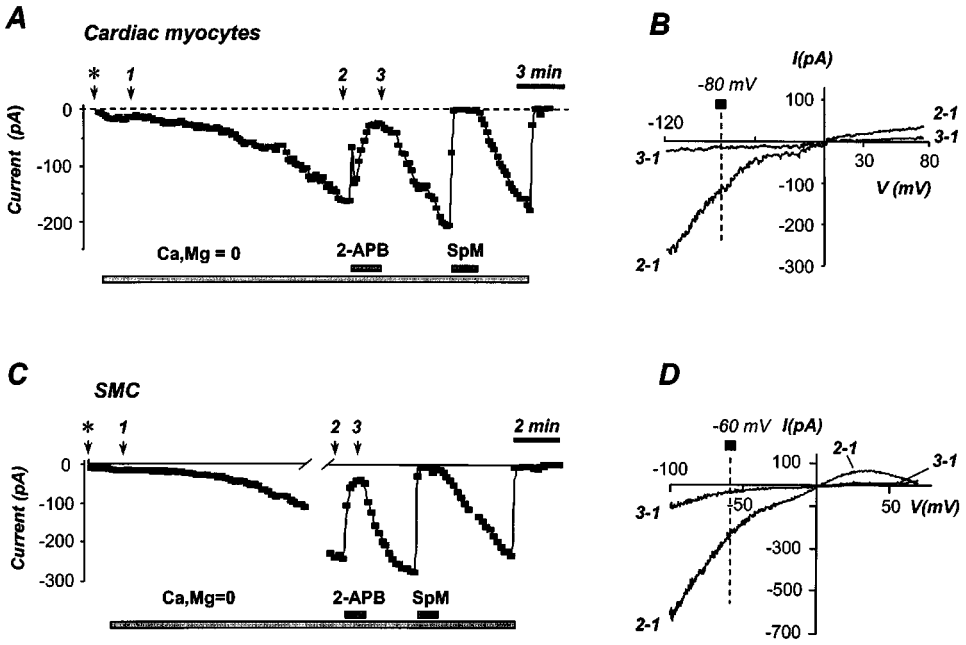

Figure 5 The up-regulation of the whole-cell MC current in divalent cation-free conditions and its inhibition by 2-APB and spermine in dialyzed cardiac myocytes (A) and SMC (B). (A,C) The development of inward current was monitored at -80 mV in cardiac myocytes (A) and -60 mV in SMC (C). The time of breaking into the cell is shown by asterisk (*). Extracellular Ca^{2+} and Mg^{2+} were removed ($Ca^{2+}/Mg^{2+}=0$) about 1 min after the beginning of dialysis with the pipette containing BAPTA (12 mM) and estimated free Ca^{2+} ~ 5 nM. 2-APB (100 μM) and spermine (SpM, 100 μM) were added at the times specified on the figure. Representative of >5 experiments of each kind. (B,D) The current-voltage relationships of MC current before (2) and after (3) application of 2-APB (100 μM) from the experiments shown on A and C panels, accordingly. The basal current (at time 1) is subtracted from each trace.

separate them. It has been previously shown that extracellular SpM does not affect voltage-gated Na^+, Ca^{2+} and K^+ channels (Williams, 1997a), but nothing is known about its possible effects on the SOC channels. Since both MC and SOC channels are present in vascular SMC, both conduct Na^+, and both could be activated during cell dialysis with BAPTA, and as we have just found out both could be inhibited by 2-APB, it was important to test if extracellular SpM could help to discriminate the whole-cell MC and SOC currents. Figure 6 shows the summary I/V relationships of the SOC currents that were activated during SMC dialysis with 12 mM BAPTA in the presence of 2 mM extracellular Mg^{2+}. Previously we have shown that SOC channels are responsible for the major part of the whole-cell current recorded under these experimental conditions (Trepakova *et al*., 2001), and MC current is mostly suppressed when extracellular divalent cations are present (Zakharov *et al*., 1999b). Application of 2-APB (100 μM) inhibited SOC current at all membrane potentials. In contrast, extracellular application of 100 μM SpM, which we have shown to inhibit 95% of the MC current in SMC (Figure 5C), was without an effect on the SOC current (Figure 6B). Figure 6C summarizes these data ($n=5-9$).

To further confirm that extracellular SpM has no effect on store-operated Ca^{2+} influx, we tested its effects in human platelets, which we have previously shown to have SOC channels and capacitative Ca^{2+} influx that is similar to that in SMC (Trepakova & Bolotina, 2002; Trepakova *et al*., 2001). Figure 7A shows examples of the capacitative Ca^{2+} influx, which resulted from the activation of SOC channels by a depletion of Ca^{2+} stores with thapsigargin. As with the SOC current in SMC, the capacitative Ca^{2+} influx in platelets was inhibited by 2-APB, but was insensitive to extracellular SpM (100 μM). Figure 7B summarizes these data ($n=5$).

Thus, spermine appeared to be a specific inhibitor of MC current and had no effect on SOC current and capacitative Ca^{2+} influx, while 2-APB was an effective inhibitor of both types of channels. Thus, extracellular SpM, but not 2-APB could be used to discriminate MC and SOC channels in different cell preparations.

Effect of SpM on the MC current-induced depolarization and SMC contraction during Ca^{2+} pararox

Having identified SpM as a novel effective inhibitor of MC current, we tried to use it to obtain additional evidence on the role of MC channels in regulation of membrane potential and in the Ca^{2+} paradox phenomenon in vascular SMC. Previously we have proposed (Zakharov *et al*., 1999b) that activation of MC current by the removal of extracellular divalent cations causes membrane depolarization and Na^+ influx, which could induce Ca^{2+} overload and contraction of the cells when extracellular Ca^{2+} is readmitted (the Ca^{2+} paradox). First we tested if SpM could prevent depolarization of SMC caused by MC current. Figure 8 shows that indeed SpM (100 μM) not only effectively inhibited the inward MC current (I/V relationships in A and time courses in B), but also reverses depolarization (C) caused by the removal of extracellular divalent cations in SMC. Importantly, the time course of the effects of SpM on the inward MC current was

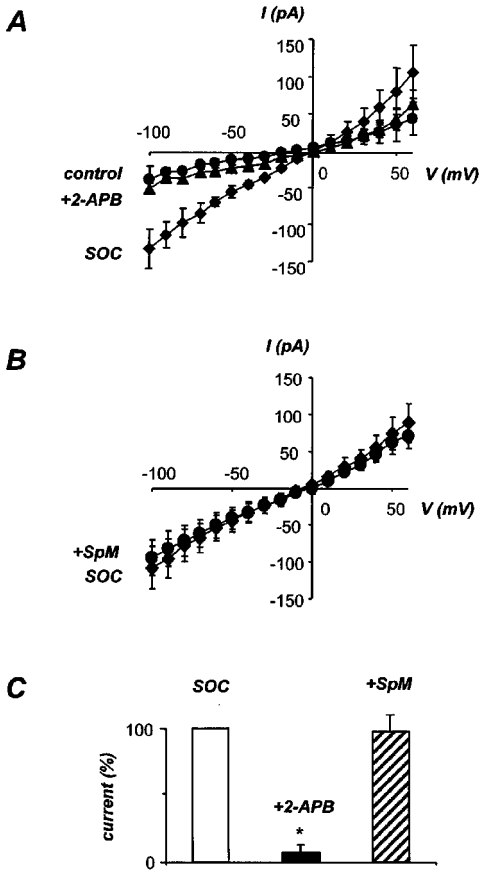

Figure 6 Effect of 2-APB and spermine on store-operated cation (SOC) current in vascular SMC. (A) The current-voltage relationships (summarized from 5–9 cells) in control, after the development of SOC current (SOC), and after the addition of 2-APB (+2-APB, 100 μM). (B) The current-voltage relationships (summarized from six cells) of the SOC current in the absence (SOC) and presence of SpM (+SpM, 100 μM). (C) Summary data (from seven experiments) showing the effects of 2-APB (100 μM) and SpM (100 μM) on the whole-cell SOC current (measured at −60 mV). The peak SOC current in each experiment before application of the drugs was counted as 100%.

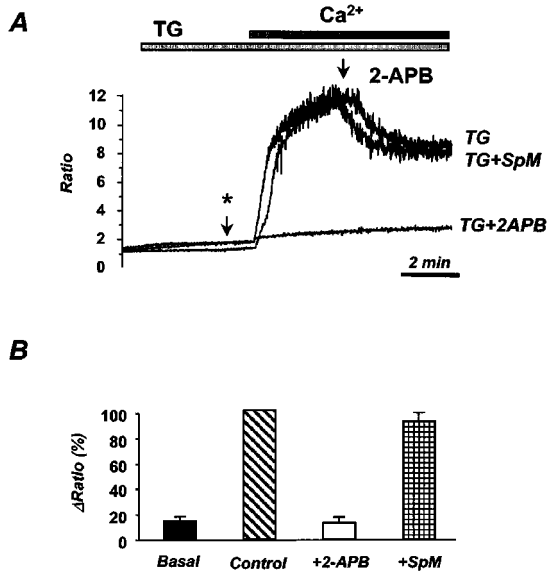

Figure 7 Store-operated Ca^{2+} influx is inhibited by 2-APB, but not spermine in human platelets. (A) A typical example of changes in intracellular [Ca^{2+}] during thapsigargin (TG, 2 μM) application in the absence of extracellular Ca^{2+}, followed by Ca^{2+} (2 mM) readmission. 2-APB (100 μM), or spermine (100 μM) were added 1 min before Ca^{2+} readmission (shown by asterisk). In control experiment and in the presence of spermine (which did not change the amplitude of Ca^{2+} influx), the effect of 2-APB was also tested after full development of Ca^{2+} influx (at the time specified by the arrow). Basal Ca^{2+} influx was measured in the absence of thapsigargin. (B) Summary data from 5 experiments similar to A.

identical to that on MC current-induced SMC depolarization (Figure 8B,C). Next, we tested if SpM could prevent SMC contraction induced by Ca^{2+} paradox conditions in the rings of rabbit aorta. Figure 9A shows that addition of Ca^{2+} to the vessel after the period of extracellular divalent cation removal, caused sustained contraction, which was absent when SpM (500 μM) was present in the bath (Figure 9B). These results confirmed that MC current plays an important role in vascular contraction that could be triggered under Ca^{2+} paradox conditions. They also show that SpM could be used as an important tool in the studies of the role of the MC channels in physiological and pathological function in the cardiovascular system.

Discussion

MC current and its regulation by intracellular Mg^{2+}

While studying the monovalent cation (MC) current that is unmasked by the removal of extracellular Ca^{2+} and Mg^{2+} in SMC and cardiac myocytes, we have noticed that the current had a small and stable amplitude during the course of a long-lasting experiment when the perforated patch-clamp technique was used for whole-cell current recording. In contrast, the amplitude of the MC current increased dramatically over time when the standard whole-cell patch clamp technique was used. Since the latter technique is associated with a dialysis of the cells with pipette solution, this observation led to the hypothesis that the amplitude of MC current could be regulated by some cytoplasmic components that may be washed away during cell dialysis. When studying cardiomyocytes, we have previously used a standard intracellular solution containing 1 mM EGTA, 1 mM $MgCl_2$ and 5 mM Na_2ATP, which insured long-lasting recordings of large amplitude MC current (Bosteels *et al.*, 1999). Here we tried to vary the composition of intracellular solution, and found that neither EGTA, nor ATP is involved in the time-dependent up-regulation of MC current. Indeed, when very simple 'buffer-free' intracellular solution was used (no added EGTA, ATP, Ca^{2+} or Mg^{2+}), the amplitude of MC current increased to the same extent as with the standard intracellular solution, consistent with neither Ca^{2+} buffering, nor the presence/absence of ATP being essential for MC current up-regulation. Since the presence of ATP (5 mM of its Na^{+} salt) in standard solution did not prevent the development of MC current (Figure 1B), it was surprising to find that addition of 5 mM MgATP to the 'buffer-free' solution completely eliminated the up-regulation phenomenon (Figure 2A). This finding led us to further study the effect of intracellular free Mg^{2+} on the MC current. To test the possible role of free Mg^{2+} in intracellular inhibition of MC current, we tried to

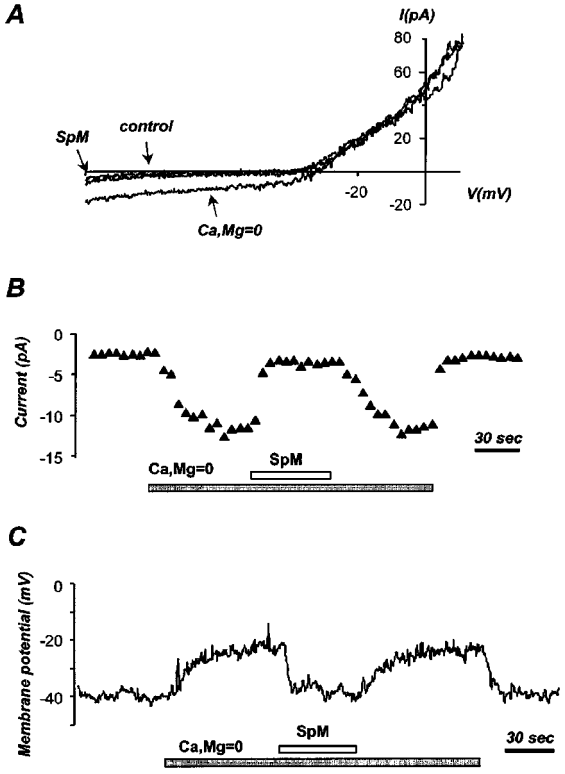

Figure 8 SpM inhibits MC current and MC current-induced depolarization in intact SMC. (A) A typical example of the I/V relationship in intact SMC (perforated patch-clamp mode) in control, after the development of MC currents in Ca, Mg = 0, and after addition of SpM (100 μM). Representative of five experiments. (B) The time course of the changes in inward MC current (at -60 mV) in experiment shown in A. (C) The time course of the changes in membrane potential following the development of MC current (in Ca, Mg = 0), and after SpM (100 μM was applied in the same SMC as in A and B (representative of three experiments).

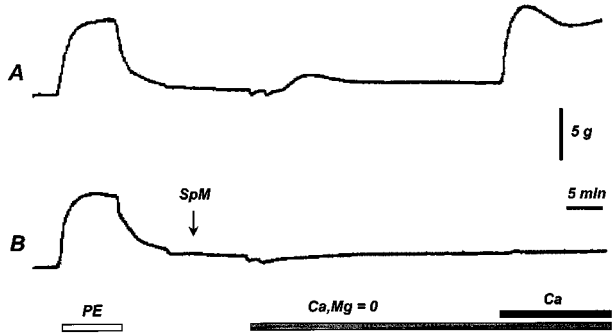

Figure 9 Prevention of Ca^{2+} paradox by SpM. Representative tracings of tension recorded in the rings of rabbit aorta during their transient exposure to phenylephrine (PE, 0.1 μM), followed by the continuous exposure to extracellular Ca, Mg = 0 (with 1 mM EGTA), followed by readmission of extracellular Ca^{2+} (3 mM). In B, SpM (500 μM) was added to the bath solution at the time specified and was continuously present after that. Representative of four experiments.

avoid the use of buffers in the pipette solution during the whole-cell current recordings. Increasing the free Mg^{2+} concentration in 'buffer-free' intracellular solution inhibited the amplitude of MC current ($K_d = 250$ μM). These results and calculations of the amount of free Mg^{2+} in our solutions explain our initial observations. Indeed, under perforated patch-clamp conditions (which do not affect the physiological divalent cation concentrations inside the cell), the MC current was small and did not change with time. The dramatic up-regulation of the MC current when the standard solution was used for intracellular dialysis is consistent with a calculated free Mg^{2+} of 30 μM. This up-regulation was prevented when intracellular solution with 5 mM MgATP (estimated free $Mg^{2+} = 750$ μM) was used, or when $MgCl_2$ was added to an ATP-free internal solution. All these data suggest that free Mg^{2+} rather than ATP is a regulator of the MC current.

The molecular mechanism of intracellular Mg^{2+}-induced inhibition of MC current is not clear, and could involve either direct inhibition of the channel itself (binding within the pore region), or through affecting some closely associated regulatory proteins. The block produced by intracellular Mg^{2+} is not unique to the channels responsible for the MC current. Intracellular Mg^{2+} blocks inward rectifying K^+ channels (Matsuda *et al*., 1987; Vandenberg, 1987) and NMDA channels (Johnson & Ascher, 1990). Some other channels, which contrary to the MC current are permeable to Ca^{2+} under physiological conditions, can also carry monovalent cation currents upon the removal of extracellular divalent cations. Monovalent cation currents through these channels can also be inhibited by intracellular Mg^{2+}. For example, intracellular Mg^{2+} was shown to produce a strong voltage-dependent inhibition of the monovalent cation current that was initially attributed to the Ca^{2+} release-activated Ca^{2+} (CRAC) channels of Jurkat cells in the absence of extracellular divalent cations (Kerschbaum & Cahalan, 1998), but later was shown to be mediated by another store-independent Mg^{2+}-inhibitable cation (MIC) channels (Prakriya & Lewis, 2002). Similar Mg^{2+}-induced inhibition (resulting in a strong inward rectification) was reported for L-type Ca^{2+} current (Kuo & Hess, 1993). A magnesium-nucleotide-regulated metal ion current (MagNum) (Nadler *et al*., 2001), that was attributed to LTRPC7 (a new member of the TRP family of channels), was shown to be inhibited when cells were dialyzed with 1 mM of intracellular free Mg^{2+}, or MgATP, but not 2 mM Na_2ATP. It is important, to emphasize that experimentally it could be hard to distinguish the effects of free Mg^{2+} from those of MgATP, because ATP (known to be one of the major buffers for Mg^{2+} within the cell) could affect MC and other cation currents indirectly through changing the free intracellular Mg^{2+} concentration. On the other hand, there may be an incomplete depletion of intracellular ATP even in cells dialyzed without exogenous ATP, since the nucleotide can still be formed by the cell metabolism and lead to the formation of MgATP. In experiments using metabolic inhibitors, the effect of removing extracellular divalent cations was increased (Macianskiene *et al*., 2001), a result which could suggest some regulation of MC current by the remaining ATP.

It is hard to determine precisely the free Mg^{2+} concentration physiologically present inside the cells, since this cation is readily bound to a variety of intracellular components, including ATP, but the best available measurements and estimates showed that concentration of free (unbound) intracellular Mg^{2+} could be in the range of 0.5–1 mM (Lüthi *et al*., 1999; Romani & Scarpa, 2000), and MC current regulation by free intracellular Mg^{2+} could be feasible. The

indirect effect of ATP on Mg^{2+}-induced intracellular block of ion channels could be especially important during some pathological conditions when the concentration of intracellular free Mg^{2+} could change, for example as a result of the change in cytoplasm concentration of ATP and/or its 'Mg^{2+}-buffering' capacity.

Polyamines as new extracellular inhibitors of MC current. Possible role in cardioprotection

Our search for new and effective inhibitors of MC current resulted in the finding that extracellular polyamines (spermine and spermidine) reversibly inhibit the MC current. Spermine (with four positive charges) was much more effective (with $IC_{50}=3$ and 10 μM in SMC and cardiac myocytes, respectively) than the closely related compound spermidine (with three positive charges and $IC_{50}=70$ μM in SMC). Interestingly, putrescine (with two positive charges) was without any significant effect. Together with our previous findings showing that extracellular Ca^{2+} inhibits MC current with $IC_{50}=250$ μM (Zakharov *et al*., 1999b), these data are consistent with the idea that different polycations can inhibit the MC current from outside the cell. Their potency seems to depend, at least in part, on their positive charge, but other molecular properties (e.g. their three-dimensional structure) could be involved. It is important to emphasize that our data show that SpM effectively inhibited the MC channels only from outside, but not from inside of the cell. Other polycations (antibiotics neomycin and gentomycin) have been shown to inhibit MC current in cardiac myocytes, but their potency was very low and millimolar concentrations of these drugs were needed to produce a significant effect (Bosteels *et al*., 1999). On the other hand, trivalent cations like Dy^{3+}, Gd^{3+} and La^{3+}, were shown to completely inhibit MC current when applied extracellular at 100 μM (Bosteels *et al*., 1999), but these heavy metals are known to inhibit many other types of cation channel, and cannot be viewed as selective inhibitors of MC current.

Recently, polyamines were found to modulate the activity of several different classes of ion channels (for review see Williams, 1997a, b), but most channels appeared to be highly sensitive only to intracellular, and not extracellular polyamines. For example, polyamines were shown to be intracellular, but not extracellular physiological regulators of ATP-sensitive (K^+_{ATP}) and other inward rectifier K^+ channels (Lopatin *et al*., 1994; Ficker *et al*., 1994; Fakler *et al*., 1995). The only channels found so far that are highly sensitive to extracellular polyamines are N-methyl-D-aspartate and nicotinic acetylcholine receptors (Anis *et al*., 1990; Scott *et al*., 1993) and nucleotide-gated channels (Lu & Ding, 1999), which are clearly different from MC channels. Extracellular SpM has been also shown to inhibit Ba^{2+} current through L-type Ca^{2+} channels in intestinal SMC, but only at very high concentrations (EC_{50} about 1 mM) (Gomez & Hellstrand, 1995). In the present study we showed that store-operated nonselective cation channels in SMC and capacitative Ca^{2+} influx in human platelets are insensitive to 100 μM SpM (which completely inhibits MC current). Thus, MC channels can be easily distinguished from SOC channels by their sensitivity to inhibitory action of extracellular spermine, which could become an excellent tool for further studies of MC channels and their role in physiological processes and pathological conditions.

The molecular mechanism by which extracellular polyamines and other polycations (like Ca^{2+} and Mg^{2+}) inhibit MC channels is presently unclear, but it is attractive to speculate that inhibitory action is a result of their ability to enter the extracellular pore region of the MC channel and bind there (thus preventing permeation of monovalent cation), as was proposed for nicotinic acetylcholine receptor channel (Bixel *et al*., 2001) and voltage-gated Na^+ channel (Huang & Moczydlowski, 2001; Yakehiro *et al*., 2001). A similar mechanism has been proposed earlier for intracellular regulation of inward rectifier K^+ channels (Lopatin *et al*., 1995), and polyamines were shown to enter the channel through an intracellular vestibule.

Polyamines (spermine and spermidine) and other polycationic compounds (e.g. the aminoglycoside antibiotics gentamicin and neomycin) have been shown to inhibit Ca^{2+} paradox in guinea-pig and rat hearts (Godicke *et al*., 1992; Busselen, 1991), but the exact mechanism of action has previously remained unknown. Contractions that could be induced by Na^+/Ca^{2+} exchange were not affectd by extracellular polyamines (Busselen, 1991), consistent with Na^+/Ca^{2+} exchange not being responsible for cardioprotective effect of polyamines. In view of the findings of the present study, it is possible that an inhibition of the MC current by polyamines is the mechanism underlying their cardioprotective effect.

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References

ANIS, N., SHERBY, S., GOODNOW, JR. R., NIWA, M., KONNO, K., KALLIMOPOULOS, T., BUKOWNIK, R., NAKANISHI, K., USHERWOOD, P., ELDEFRAWI, A. & ELDEFRAWI, M. (1990). Structure-activity relationships of philanthotoxin analogs and polyamines on n-methyl-D-aspartate and nicotinic acetylcholine receptors. *J. Pharmacol. Exper. Ther.,* **254,** 764–773.

ARELLANO, R.O., WOODWARD, R.M. & MILEDI, R. (1995). A monovalent cationic conductance that is blocked by extracellular divalent cations in Xenopus oocytes. *J. Physiol. (Lond.),* **484,** 593–604.

BAE, Y.M., PARK, M.K., LEE, S.H., HO, W.K. & EARM, Y.E. (1999). Contribution of Ca^{2+}-activated K^+ channels and non-selective cation channels to membrane potential of pulmonary arterial smooth muscle cells of the rabbit. *J. Physiol.,* **514,** 747–758.

BAKOWSKI, D. & PAREKH, A.B. (2002). Monovalent cation permeability and Ca^{2+} block of the store-operated Ca^{2+} current I_{CRAC} in rat basophilic leukemia cells. *Pflügers Arch. Eur. J. Physiol.,* **443,** 892–902.

BIXEL, M.G., WEISE, C., BOLOGNESI, M.L., ROSINI, M., BRIERLY, M.J., MELLOR, I.R., USHERWOOD, P.N.R., MELCHIORRE, C. & HUCHO, F. (2001). Location of the polyamine binding site in the vestibule of the nicotinic acetylcholine receptor ion channel. *J. Biol. Chem.,* **276,** 6151 – 6160.

BOSTEELS, S., MATEJOVIC, P., FLAMENG, W. & MUBAGWA, K. (1999). Sodium influx via a non-selective pathway activated by the removal of extracellular divalent cations: possible role in the calcium paradox. *Cardiovasc. Res.,* **43,** 417 – 425.

BRAUN, F.J., BROAD, L.M., ARMSTRONG, D.L. & PUTNEY, JR. J.W. (2001). Stable activation of single Ca^{2+} release-activated Ca^{2+} channels in divalent cation-free solutions. *J. Biol. Chem.,* **276,** 1063 – 1070.

BUSSELEN, P. (1991). Polyamines and the calcium paradox in rat hearts. *J. Mol. Cell. Cardiol.,* **23,** 237 – 247.

COMMUNAL, C., SINGH, K., PIMENTEL, D.R. & COLUCCI, W.S. (1998). Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the β-adrenergic pathway. *Circulation,* **98,** 1329 – 1334.

DOBRYDNEVA, Y. & BLACKMORE, P. (2001). 2-Aminoethoxydiphenyl borate directly inhibits store-operated calcium entry channels in human platelets. *Molec. Pharmacol.,* **60,** 541 – 552.

FAKLER, B., BRANDLE, U., GLOWATZKI, E., WEIDEMANN, S., ZENNER, H.-P. & RUPPERSBERT, J.P. (1995). Strong voltage-dependent inward rectification of inward rectifier K^+ channels is caused by intracellular spermine. *Cell,* **80,** 149 – 154.

FICKER, E., TAGLIALATELA, M., WIBLE, B.A., HENLEY, C.M. & BROWN, A.M. (1994). Spermine and spermidine as gating molecules for inward rectifier K^+ channels. *Science,* **266,** 1068 – 1072.

GODICKE, J., JACOBSON, L., LULLMAN, H. & MULDER, G. (1992). The polycationic compound gemtamicin inhibits the calcium paradox in guinea-pig hearts. *Acta Physiol. Scand.,* **144,** 349 – 354.

GOMEZ, M. & HELLSTRAND, P. (1995). Effects of polyamines on voltage-activated calcium channels in guinea-pig intestinal smooth muscle. *Pflügers Arch. Eur. J. Physiol.,* **430,** 501 – 507.

GREGORY, R.B., RYCHKOV, G. & BARRITT, G.J. (2001). Evidence that 2-aminoethyl diphenylborate is a novel inhibitor of store-operated Ca2+ channels in liver cells, and acts through a mechanism which does not involve inositol trisphosphate receptors. *Biochem. J.,* **354,** 285 – 290.

HAGIWARA, N., IRISAWA, H., KASANUKI, H. & HOSODA, S. (1992). Background current in sino-atrial node cells of the rabbit heart. *J. Physiol.,* **448,** 53 – 72.

HUANG, C.J. & MOCZYDLOWSKI, E. (2001). Cytoplasmic polyamines as permeant blockers and modulators of the voltage-gated sodium channel. *Biophys. J.,* **80,** 1262 – 1279.

IWASAKI, H., MORI, Y. & HARA, Y. (2001). 2-Aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channels,* **7,** 429 – 439.

JOHNSON, J.W. & ASCHER, P. (1990). Voltage-dependent block by intracellular Mg2+ of N-methyl-D-aspartate-activated channels. *Biophys. J.,* **57,** 1085 – 1090.

KERSCHBAUM, H.H. & CAHALAN, M.D. (1998). Monovalent permeability, rectification, and ionic block of store-operated calcium channels in Jurkat T lymphocytes. *J. Gen. Physiol.,* **111,** 521 – 537.

KUO, C.-C. & HESS, P. (1993). Block of the L-type Ca^{2+} channel pore by external and internal Mg^{2+} in rat phaechhromocytoma cells. *J. Physiol.,* **466,** 683 – 706.

LEPPLE-WIENHUES, A. & CAHALAN, M.D. (1996). Conductance and permeation of monovalent cations through depletion-activated Ca2+ channels(Icrac) in jurkat T cells. *Biophys. J.,* **71,** 787 – 794.

LOPATIN, A.N., MAKHINA, E.N. & NICHOLS, C.G. (1994). Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature,* **372,** 366 – 369.

LOPATIN, A.N., MAKHINA, E.N. & NICHOLS, C.G. (1995). The mechanism of inward rectification of potassium channels: ''long-pore plugging'' by cytoplasmic polyamines. *J. Gen. Physiol.,* **106,** 923 – 955.

LU, Z. & DING, L. (1999). Blockade of a retinal cGMP-gated channel by polyamines. *J. Gen. Physiol.,* **113,** 35 – 43.

LÜTHI, D., GÜNZEL, D. & MCGUIGAN, J.A.S. (1999). Mg-ATP binding: its modification by spermine, the relevance to cytosolic Mg^{2+} buffering, changes in the intracellular ionized Mg2+ concentration and the estimation of Mg2+ by 31P-NMR. *Experimental Physiol.,* **84,** 231 – 252.

MA, H.-T., PATTERSON, R.L., VAN ROSSUM, D.B., BIRNBAUMER, L., MIKOSHIBA, K. & GILL, D.L. (2000). Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels. *Science,* **287,** 1647 – 1651.

MACIANSKIENE, R., MATEJOVIC, P., SIPIDO, K., FLAMENG, W. & MUBAGWA, K. (2001). Modulation of the extracellular divalent cation-inhibited non-selective conductance in cardiac cells by metabolic inhibition and by oxidants. *J. Mol. Cell. Cardiol.,* **33,** 1371 – 1385.

MARUYAMA, T., KANAJI, T., NAKADE, S., KANNO, T. & MIKOSHIBA, K. (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-permeable modulator of Ins(1,4,5)P3-induced Ca2+ release. *J. Biochem. (Tokyo),* **122,** 498 – 505.

MATSUDA, H., SAIGUSA, A. & IRISAWA, H. (1987). Ohmic conductance through the inwardly rectifying K channel and blocking by internal Mg2+. *Nature,* **325,** 156 – 159.

MIGNEN, O. & SHUTTLEWORTH, T.J. (2001). Permeation of monovalent cations through the noncapacitative arachidonate-regulated Ca^2+(ARC) channels in HEK293 cells – comparison with endogenous store-operated channels. *J. Biol. Chem.,* **276,** 21365 – 21374.

MUBAGWA, K., STENGL, M. & FLAMENG, W. (1997). Extracellular divalent cations block a cation non-selective conductance unrelated to calcium channels in rat cardiac muscle. *J. Physiol. (Lond.),* **502,** 235 – 247.

NADLER, M.J.S., HERMOSURA, M.C., INABE, K., PERRAUD, A.-L., ZHU, Q., STOKES, A.J., KUROSAKI, T., KINET, J.-P., PENNER, P., SCHARENBERG, A.M. & FLEIG, A. (2001). LTRPC7 is a Mg-AR+ATP-regulated divalent cation channel required for cell viability. *Nature,* **411,** 590 – 595.

PRAKRIYA, M. & LEWIS, R.S. (2001). Potentiation and inhibition of Ca2+ release-activated Ca2+ channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP3 receptors. *J. Physiol.,* **536,** 3 – 19.

PRAKRIYA, M. & LEWIS, R.S. (2002). Separation and characterization of currents through store-operated CRAC channels and Mg(2)-inhibited cation (MIC) channels. *J. Gen. Physiol.,* **119,** 487 – 507.

ROMANI, A.M. & SCARPA, A. (2000). Regulation of intracellular magnesium. *Front Biosc,* **5,** D720 – D734.

RYCHKOV, G., BRERETON, H., HARLAND, M.L. & BARRITT, G.J. (2001). Plasma membrane Ca^{2+} release-activated Ca^{2+} channels with a high selectivity for Ca^{2+} identified by patch-clamp recording in rat liver cells. *Hepatology,* **33,** 938 – 947.

SABOVCIK, R., LI, J., KUCERA, P. & PROD'HOM, B. (1995). Permeation properties of a Ca^{2+}-blockade monovalent cation channel in the ectoderm of the chick embryo: pore size and multioccupancy probed with organic cations and Ca^{2+}. *J. Gen. Physiol.,* **106,** 149 – 174.

SABOVCIK, R., KUCERA, P. & PROD'HOM, B. (1996). Characterization of whole-cell Ca^{2+}-blockade of monovalent cation current in isolated ectodermal cells of chick embryo. *Pflügers Arch. Eur. J. Physiol.,* **431,** 599 – 607.

SCOTT, R.H., SUTTON, K.G. & DOLPHIN, A.C. (1993). Interactions of polyamines with neural ion channels. *Trends Neurosci.,* **16,** 153 – 159.

SMANI, T., IWABUCHI, S., LOPEZ-BARNEO, J. & URENA, J. (2001). Differential segmental activation of Ca2+-dependent CI- and K+ channels in pulmonary arterial myocytes. *Cell Calcium,* **29,** 369 – 377.

TREPAKOVA, E.S. & BOLOTINA, V.M. (2002). Comparison of the store-operated channels and the effects of nitric oxide on capacitative Ca^{2+} influx in platelets, smooth muscle cells and Jurkat cells. *Membrane Cell Biol.,* **19,** 49 – 56.

TREPAKOVA, E.S., COHEN, R.A. & BOLOTINA, V.M. (1999). Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase-dependent refilling of Ca^{2+} stores. *Circulation Res.,* **84,** 201 – 209.

TREPAKOVA, E.S., GERICKE, M., HIRAKAWA, Y., WEISBROD, R.M., COHEN, R.A. & BOLOTINA, V.M. (2001). Properties of a native cation channel activated by Ca^{2+} store depletion in vascular smooth muscle cells. *J. Biol. Chem.,* **276,** 7782–7790.

VANDENBERG, C.A. (1987). Inward rectification of a potassium channel in cardiac ventricular cells depends on internal magnesium ions. *Proc. Nat. Acad. Sci.,* **84,** 2560–2564.

VOETS, T., DROOGMANS, G. & NILIUS, B. (1996). Membrane currents and the resting membrane potential in cultured bovine pulmonary artery endothelial cells. *J. Physiol.,* **497,** 95–107.

WILLIAMS, K. (1997a). Interactions of polyamines with ion channels. *Biochem. J.,* **325,** 289–297.

WILLIAMS, K. (1997b). Modulation and block of ion channels: a new biology of polyamines. *Cell Signal,* **9,** 1–13.

YAKEHIRO, M., FURUKAWA, Y., KOIKE, T., KIMURA, E., NAKAJIMA, K., YAMAOKA, K. & SEYAMA, I. (2001). Novel mechanism of blocking axonal Na+ channels by three macrocyclic polyamine analogues and two spider toxins. *Brit. J. Pharmacol.,* **132,** 63–72.

ZAKHAROV, S.I., SMANI, T., MACIANSKIENE, R., MUBAGWA, K. & BOLOTINA, V.M. (2002). Regulation of the hidden monovalent cation current by intracellular Mg^{2+} extracellular polyamines and 2-APB. *Biophys. J.,* 624A.

ZAKHAROV, S.I., COHEN, R.A. & BOLOTINA, V.M. (1999a). The role of nonselective cation currents in regulation of membrane potential in vascular smooth muscle cells. In *Endothelium-dependent Hyperpolarization,* ed Vanhoutte, P.M., pp. 243–251. Amsterdam: Harwood Academic Publishers.

ZAKHAROV, S.I., MONGAYT, D.A., COHEN, R.A. & BOLOTINA, V.M. (1999b). Monovalent cation and L-type Ca^{2+} channels participate in calcium paradox-like phenomenon in rabbit aortic smooth muscle cells. *J. Physiol. (Lond.),* **514,** 71–81.

ZHANG, Y., MCBRIDE, D.W. & HAMILL, O.P. (1998). The ion selectivity of a membrane conductance inactivated by extracellular calcium in Xenopus oocytes. *J. Physiol.,* **508,** 763–776.

ZIMMERMAN, A.N.E. & HULSMANN, W.C. (1966). Paradoxical influence of calcium ions of the permeability of the cell membranes of isolated rat hearts. *Nature,* **211,** 646–647.

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