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Inhibition of Cation Channels in Human Erythrocytes by Spermine

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Abstract In erythrocytes, spermine concentration decreases gradually with age, which is paralleled by increases of cytosolic Ca^{2+} concentration, with subsequent cell shrinkage and cell membrane scrambling. Cytosolic Ca^{2+} was estimated from Fluo-3 fluorescence, cell volume from forward scatter, cell membrane scrambling from annexin V binding and cation channel activity with whole-cell patch-clamp in human erythrocytes. Extracellular spermine exerted a dual effect on erythrocyte survival. At 200 μM spermine blunted the increase of intracellular Ca^{2+}, cell shrinkage and annexin V binding following 48 h exposure of cells at +37°C. In contrast, short exposure (10–30 min) of cells to 2 mM spermine was accompanied by increased cytosolic Ca^{2+} and annexin binding. Intracellular addition of spermine at subphysiological concentration (0.2 μM) significantly decreased the conductance of monovalent cations (Na^+, K^+, $NMDG^+$) and of Ca^{2+}. Moreover, spermine (0.2 μM) blunted the stimulation of voltage-independent cation channels by Cl^- removal. Spermine (0.2 and 200 μM) added to the extracellular bath solution similarly inhibited the cation conductance in Cl^--containing bath solution. The effect of 0.2 μM spermine, but not the effect of 200 μM, was rapidly reversible. Acute addition (250 μM) of a naphthyl acetyl derivative of spermine (200 μM) again significantly decreased basal cation conductance in NaCl bath solution and inhibited voltage-independent cation channels. Spermine is a powerful regulator of erythrocyte cation channel cytosolic Ca^{2+} activity and, thus, cell survival.

Keywords Red blood cell · Ca^{2+} · Phosphatidylserine exposure · Cell volume · Spermine · NASPM · Aging · Channel

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Introduction

Polyamines (putrescine, spermidine and spermine) are present in all eukaryotic cells (Bachrach 1973). Enhanced polyamine concentrations are usually associated with increased cell proliferation and correlated with nucleic acid concentrations in rapidly growing tissues (Bachrach 1973). Cellular polyamine concentrations depend on synthesis (Koenig et al. 1983) or transport across the cell membrane, which is accomplished by an Na^+/spermine antiporter (Kobayashi et al. 1999).

Concentrations of putrescine, spermidine and spermine in erythrocytes were shown to decrease with age (Cooper et al. 1976). The concentration of spermine was found to be 14 μM in "young" cells, 6 μM in "middle-aged" cells and 3 μM in "old" cells (Cooper et al. 1976). In nucleated blood cells (e.g., leukocytes) the intracellular concentration of spermine may reach some 1,000- to 10,000-fold higher values (Cooper et al. 1976). Spermine concentrations have been reported to be low (0.14 μM) in plasma of healthy individuals (Baylin et al. 1980) but to increase in blood during a variety of clinical conditions, including malignancies (Rennert et al. 1976; Russell 1971), cystic fibrosis (Cohen et al. 1976) and psoriasis (Proctor et al. 1975).

Polyamines are highly associated with phospholipids, such as phosphatidylserine and phosphatidylinositol (Toner

et al. 1988), and negatively charged proteins (cytoplasmic amino-terminal domain glutamate residues) of the plasma membrane (Lin et al. 2006; Tassoni et al. 1996).

Polyamines were shown to block the activity of N-type Ca^{2+} channels (Cino and Formenti 2008); several nonselective TRP cation channels, such as TRPM4, TRPM5 and TRPM7 (Kerschbaum et al. 2003; Nilius et al. 2004; Ullrich et al. 2005); and sarcoplasmatic reticulum Ca^{2+}-ATPase of skeletal muscle (Hughes et al. 1994). Spermine is cosecreted with neurotransmitters and neuromodulators (Shaw 1994). The naphthyl acetyl derivative of spermine (NASPM) was shown to block effectively AMPA-type glutamate receptors lacking the $GluR_2$ subunit (Koike et al. 1997). Dual effects of spermine were demonstrated on mitochondrial Ca^{2+} transport (Lenzen et al. 1992). Depending on the experimental conditions, spermine inhibited or accelerated mitochondrial Ca^{2+} uptake but invariably increased mitochondrial Ca^{2+} accumulation (Lenzen et al. 1992). There are two independent spermine binding sites with the mitochondrial membrane (Dalla et al. 1996, 1998). The binding of spermine to one of these sites, the so-called S_1 site, prevents the mitochondrial permeability transition. Instead, binding of spermine to the other site, S_2, is involved in the spermine-dependent mitochondrial import of certain cytosolic enzymes such as casein kinase I and casein kinase II (Bordin et al. 1994; Clari et al. 1994).

In erythrocytes spermine has been shown to inhibit KCl cotransport (Sachs 1994). Polyamines do not modify the activities of the erythrocyte Na-K-ATPase or Ca^{2+}-ATPase (Ballas et al. 1983a). Polyamines regulate erythrocyte adenylate cyclase and acetylcholinesterase (Khan et al. 1990; Kossorotow et al. 1974). Spermine decreases erythrocyte deformability and stabilizes the membrane skeleton, rendering the cells more resistant to fragmentation (Ballas et al. 1983b). The presence of spermine fosters Ca^{2+}-induced microvesiculation but inhibits Ca^{2+}-induced cell membrane scrambling (Bucki et al. 1998). Added extracellularly at high concentrations (100–1,000 μM), spermine exerts antioxidant effects on the erythrocyte cell membrane, reducing lipid peroxidation induced by exogenous H_2O_2 (Orta et al. 2003). Spermine can be oxidized by plasma and cell aminooxidases, resulting in the formation of cytotoxic products, such as aminoaldehydes, NH_3 and H_2O_2 (Bachrach 1970). Recently, tumor cells were shown to be more sensitive than normal cells to the cytotoxic effects of oxidized end products of spermine and spermidine, a property which may be exploited for cancer therapy (Agostinelli et al. 2010). The oxidized end products may further kill intraerythrocytic parasites without lysing the host cell (Ferrante et al. 1983; Rzepczyk et al. 1984).

Spermine may be important in the regulation of erythrocyte aging, which is paralleled by increases in intracellular Na^+ and Ca^{2+}, cell shrinkage, membrane blebbing and breakdown of cell membrane phosphatidylserine asymmetry, all typical features of apoptosis (Aiken et al. 1992; Lew et al. 2007). Moreover, it is well established that erythrocytes may be subject to programmed suicidal cell death or eryptosis (Foller et al. 2009a). Triggers of eryptosis include an increase in intracellular free calcium, which in turn stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface (Lang et al. 2008).

Eryptotic cells are rapidly eliminated from blood circulation (Lang et al. 2008). During eryptosis, Ca^{2+} enters through cation channels (Lang et al. 2008). According to electrophysiological studies, two types of nonselective cation channels permeable for Ca^{2+} are expressed in human erythrocytes: the voltage-gated channel, coupled to an acetylcholine receptor (Baunbaek and Bennekou 2008; Bennekou et al. 2004; Kaestner et al. 1999), and the voltage-independent cation channels, which can be activated by oxidation, osmotic shock and energy depletion (Huber et al. 2001). The voltage-independent channels were shown to be partially related to TRPC6 channels (Foller et al. 2008). Recent data demonstrated the existence of NMDA receptors in rat (Makhro et al. 2010) and AMPA receptors in human (Foller et al. 2009a) erythrocytes. In addition, the presence of subtypes of voltage-dependent Ca^{2+} channels was demonstrated by Western blot analysis in age-fractionated erythrocytes (Romero et al. 2006).

The effects of spermine on erythrocyte channel activity or erythrocyte intracellular Ca^{2+} activity have never been shown. The present whole-cell patch-clamp and fluorescence study thus explored the effect of spermine on cation channels and cytosolic Ca^{2+} activity in human red blood cells.

Materials and Methods

Erythrocytes

Erythrocytes were drawn from healthy volunteers, who signed informed consent, or banked erythrocyte concentrates provided by the blood bank of the University of Tübingen. The study was approved by the ethics commission of the University of Tübingen (184/2,003 V). Patch-clamp experiments were performed at room temperature (22°C). Erythrocytes were washed twice (1,200×*g*, 5 min, 22°C) in physiological saline buffered with 10 mM HEPES (pH 7.4).

Phosphatidylserine Exposure and Forward Scatter

Experiments were performed with erythrocytes incubated for 30 min and 2 days at +37°C in bath solution,

containing (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 0 (0.2 or 200 μM) spermine, 10 HEPES/NaOH (pH 7.4).

Erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$. Cells pretreated with spermine (0.2 μM, 200 μM and 2 mM) were washed with spermine-containing 5 mM $CaCl_2$ Ringer solution. Cells were then stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:50 dilution in 5 mM Ca^{2+}-containing Ringer. After 20 min, samples were washed once and resuspended in 5 mM Ca^{2+}-containing Ringer without or with (0.2 μM, 200 μM or 2 mM) spermine and measured by flow-cytometric analysis (FACS-Calibur; Becton Dickinson, Heidelberg, Germany). Annexin V fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The size (volume) of the cells was determined by forward scatter.

Measurements in control and spermine-treated cells were done at the same cell number (hematocrit), bath saline solutions and instrument settings.

Intracellular Ca^{2+}

Experiments were performed with erythrocytes incubated for 30 min and 2 days at +37°C in bath solution, containing (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 0 (0.2 or 200 μM) spermine, 10 HEPES/NaOH (pH 7.4).

Erythrocytes were washed in Ringer solution (with 0, 0.2 μM, 200 μM and 2 mM spermine) containing 5 mM $CaCl_2$ and loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. Cells were incubated at 37°C for 20 min, washed once and resuspended in 5 mM Ca^{2+}-containing Ringer without or with (0.2 μM, 200 μM and 2 mM) spermine and subsequently analyzed in fluorescence channel FL-1 by FACS analysis. As the analysis does not provide a fluorescence ratio, Fluo-3 does not allow calculation of the cytosolic Ca^{2+} concentration.

Electrophysiology

Patch-clamp whole-cell recordings were performed at room temperature. Patch electrodes were made of borosilicate glass capillaries (150 TF-10; Clark Medical Instruments, Reading, UK) using a horizontal DMZ puller (Zeitz, Martinsried, Germany). Pipettes with high resistance from 12 to 17 MOhm were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (Heka, Lambrecht, Germany). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech, Great Neck, NY) and using Pulse software (Heka). For current measurements, erythrocytes were held at a holding potential (V_h) of −10 mV, and 200-ms pulses from −100 to +100 mV were applied in increments of +20 mV. The original current traces are depicted without filtering (acquisition frequency 3 kHz). Currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (current–voltage relationship). The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between the electrodes were zeroed before sealing. The liquid junction potentials between the bath and pipette solutions and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch (1991). Data were corrected for liquid junction potentials. Recordings were obtained after reaching >10 GΩ seal in on-cell configuration.

The pipette solution consisted of (in mM) 125 Na-gluconate, 10 NaCl, 1 $MgCl_2$, 1 MgATP, 1 EGTA, 10 HEPES/NaOH (pH 7.4). Spermine (0.2 or 200 μM final concentration) was added either acutely after reaching a whole-cell steady-state configuration or intracellularly as a component of the pipette solution (20 nM or 0.2 μM). A naphthyl acetyl derivative of spermine (1-naphthyl acetyl spermine, NASRM) was added acutely (250 μM final concentration).

The NaCl Ringer bath solution contained (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). In the experiments where Cl^- was substituted with gluconate$^-$ the solution contained (in mM) 150 Na-gluconate, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). The KCl, NaCl, NMDG-Cl and $CaCl_2$ bath solutions contained (in mM) 150 KCl, 150 NaCl, 180 NMDG (titrated with HCl to pH 7.4) or 100 $CaCl_2$, 10 HEPES/HCl (for the KCl and NaCl solutions), NMDG or $CaOH_2$ (pH 7.4). Reagents were obtained from Sigma (Deisenhofen, Germany), spermine was from Roth (Karlsruhe, Germany); all were of the highest grade available.

Statistics

Data are expressed as arithmetic means ± SEM, and paired two-tailed *t*-test or ANOVA was employed as appropriate. $P < 0.05$ was considered statistically significant.

Results

In a first series of experiments, FACS analysis was performed to elucidate the effect of extracellular spermine on

Fluo-3 fluorescence, reflecting cytosolic Ca^{2+} activity; annexin V binding, reflecting phosphatidylserine exposure, a measure of membrane scrambling; and foward scatter, reflecting cell volume.

As shown in Fig. 1, short treatment (30 min) with a submillimolar concentration of spermine (200 μM) did not significantly alter cytosolic free Ca^{2+} or forward scatter. Short treatment with 200 μM spermine significantly ($n = 8$–9, $P \leq 0.05$) reduced annexin V binding. In contrast, treatment (30 min) with 2 mM spermine resulted in a marked increase in annexin V binding and Fluo-3 fluorescence accompanied by a decrease in forward scatter, effects pointing to toxicity of millimolar concentrations of spermine (Fig. 1a–c). The same toxic effects were observed when 2 mM spermine was applied for a shorter period (10 min, data not shown).

Oxidative stress induced by treatment with 2 mM H_2O_2 was followed by a significant increase of Fluo-3 fluorescence and annexin V binding as well as a decrease of forward scatter, effects augmented in the presence of 2 mM spermine but not by 200 μM spermine (Fig. 1).

As shown in Fig. 2a, b, 200 μM spermine significantly ($n = 8$, $P \leq 0.05$) blunted annexin V binding of erythrocytes incubated for 48 h at +37°C. No significant effect was observed at physiological 0.2 μM spermine concentration.

The forward scatter was significantly ($n = 9$, $P \leq 0.05$) decreased in the presence of 200 μM spermine. Again, 0.2 μM spermine did not significantly modify forward scatter.

Following incubation for 48 h at +37°C, Fluo-3 fluorescence was significantly lower ($n = 7$, $P \leq 0.05$) in the presence than in the absence of 200 μM spermine (Fig. 2e, f).

The FACs experiments clearly demonstrated an antiaging effect of extracellular applied submillimolar concentration of spermine, an effect paralleled by decreases in $[Ca^{2+}]_i$. Thus, patch-clamp experiments were performed to determine the effect of spermine on the activity of cation channels in human erythrocytes.

Prior to the application of spermine the erythrocyte cation conductance (measured as the inward current slope of the current–voltage [*I–V*] relation shown in Fig. 3a, c) approached some 77 ± 8 pS (Fig. 3b, d). The conductance declined significantly following exposure to spermine-containing Ringer bath solution, reaching values of 55 ± 9 pS ($n = 5$, $P \leq 0.01$) following exposure to 0.2 μM spermine and 32 ± 6 pS ($n = 5$–6, $P \leq 0.05$) following exposure to 200 μM spermine (Fig. 3b, d). It is noteworthy that upon washout currents reappeared completely after 0.2 μM spermine application (Fig. 3d, e), whereas the inhibitory effect of 200 μM spermine was only partially reversed (Fig. 3b).

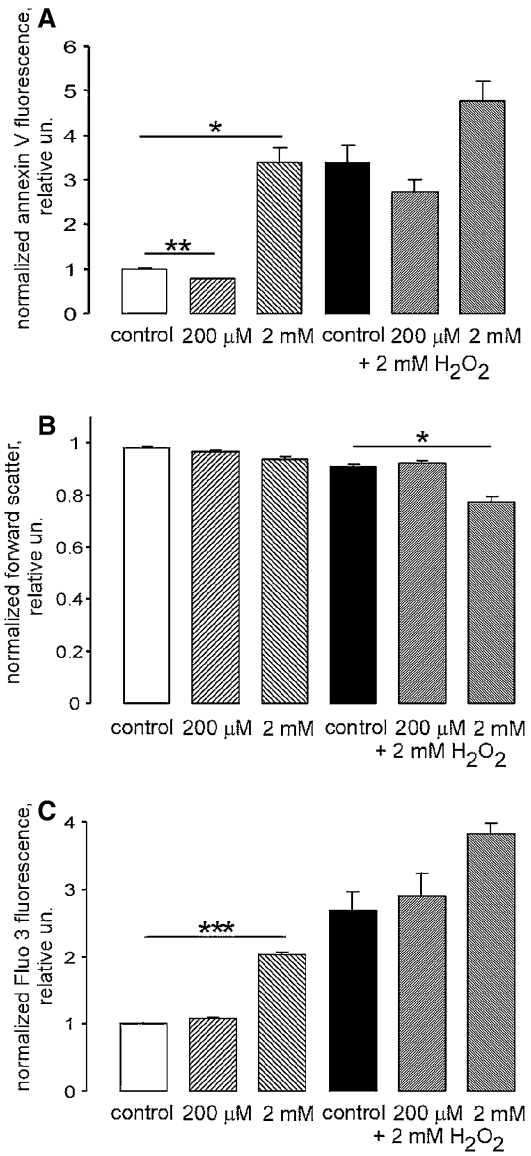

Fig. 1 Effects of short-term spermine (200 μM and 2 mM) and 2 mM H_2O_2 treatment on phosphatidylserine exposure, forward scatter and cytosolic free Ca^{2+} concentration in human erythrocytes. **a** Arithmetic means ± SEM of normalized annexin V fluorescence of human erythrocytes (*white bar*, $n = 9$) exposed for a short time (30 min) to 200 μM spermine (*left hatched bar*, $n = 8$) and 2 mM spermine (*right hatched bar*, $n = 9$) in the absence as well as in the presence of 2 mM H_2O_2 (*black bar*, $n = 6$–7). Significant differences from control (* $P < 0.05$ and ** $P < 0.01$, ANOVA). **b** Arithmetic means ± SEM of normalized forward scatter of human erythrocytes (*white bar*, $n = 9$) exposed for a short time (30 min) to 200 μM spermine (*left hatched bar*, $n = 8$) and 2 mM spermine (*right hatched bar*, $n = 9$) in the absence as well as in the presence of 2 mM H_2O_2 (*black bar*, $n = 6$–7). Significant difference from control (* $P < 0.05$, ANOVA). **c** Arithmetic means ± SEM of normalized Fluo-3 fluorescence of human erythrocytes (*white bar*, $n = 9$) exposed for a short time (30 min) to 200 μM spermine (*left hatched bar*, $n = 8$) and 2 mM spermine (*right hatched bar*, $n = 9$) in the absence as well as in the presence of 2 mM H_2O_2 (*black bar*, $n = 6$–7). Significant difference from control (*** $P < 0.001$, ANOVA)

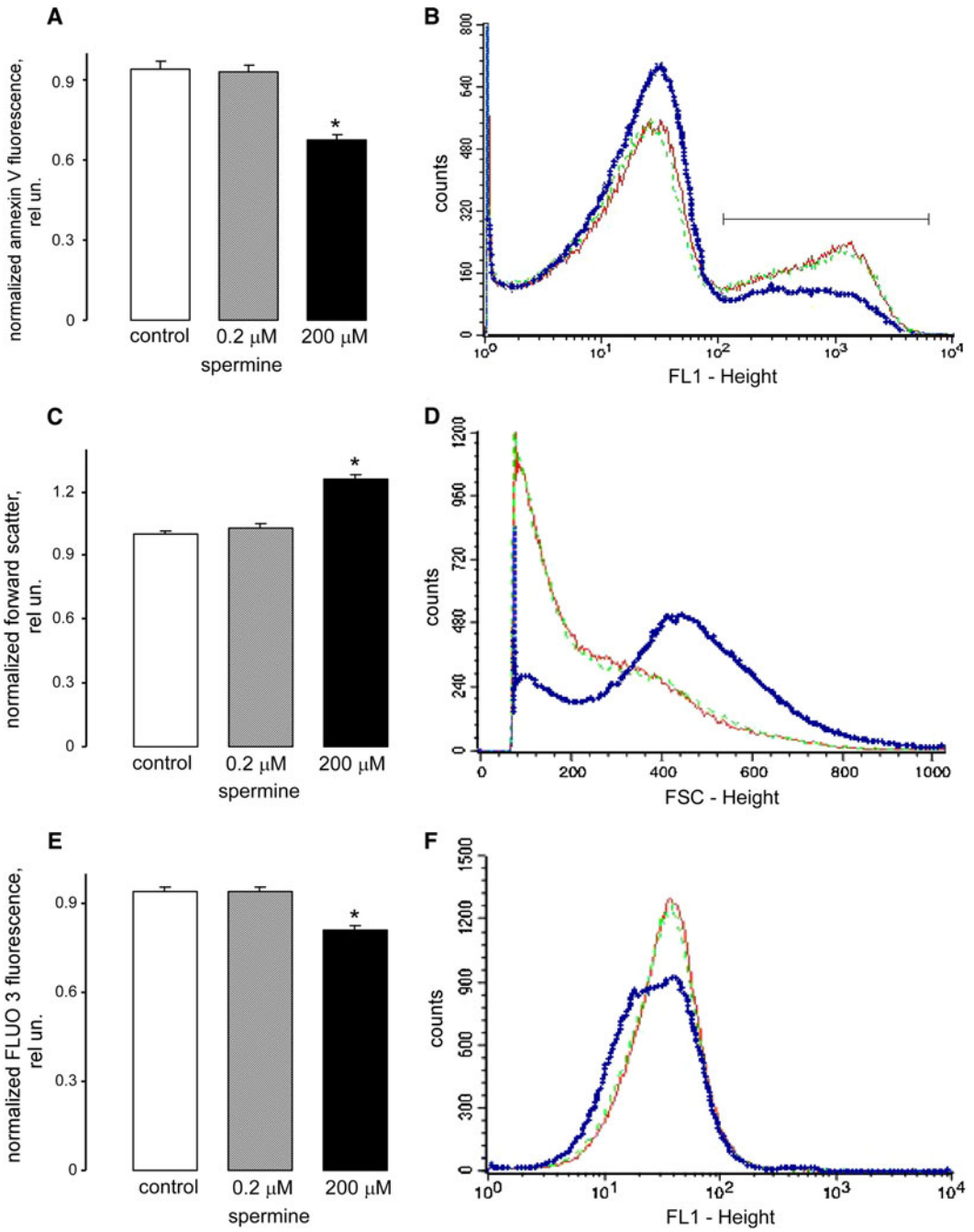

Fig. 2 Effect of spermine (0.2 and 200 μM) treatment on phosphatidylserine exposure, forward scatter and cytosolic free Ca^{2+} concentration. **a** Arithmetic means ± SEM of normalized annexin V fluorescence of control cells after a 48-h treatment with Ringer solution in the absence (*white bar*, $n = 8$) and presence of 0.2 μM spermine (*left hatched bar*, $n = 8$) or 200 μM spermine (*right hatched bar*, $n = 8$). Significant difference from control (* $P < 0.05$, ANOVA). **b** Histogram of annexin V binding in a representative experiment of human erythrocytes exposed for 48 h to isotonic Ringer (*red line*), to Ringer with 0.2 μM spermine added (*green line*) and to Ringer with 200 μM spermine added (*blue thicker line*). **c** Arithmetic means ± SEM of normalized forward scatter of control cells after a 48-h treatment with Ringer solution in the absence (*white bar*, $n = 9$) and presence of 0.2 μM spermine (*left hatched bar*, $n = 9$) or 200 μM spermine (*right hatched bar*, $n = 9$). Significant difference from control (* $P < 0.05$, ANOVA). **d** Histogram of forward scatter in a representative experiment of human erythrocytes exposed for 48 h to isotonic Ringer (*red line*), to Ringer with 0.2 μM spermine added (*green line*) and to Ringer with 200 μM spermine added (*blue thicker line*). **e** Arithmetic means ± SEM of normalized Fluo-3 fluorescence of control cells after a 48-h treatment with Ringer solution in the absence (*white bar*, $n = 7$) and presence of 0.2 μM spermine (*left hatched bar*, $n = 7$) or 200 μM spermine (*right hatched bar*, $n = 7$). Significant difference from control (* $P < 0.05$, ANOVA). **f** Histogram of Fluo-3 fluorescence in a representative experiment of human erythrocytes exposed for 48 h to isotonic Ringer (*red line*), to Ringer with 0.2 μM spermine added (*green line*) and to Ringer with 200 μM spermine added (*blue thicker line*) (Color figure online)

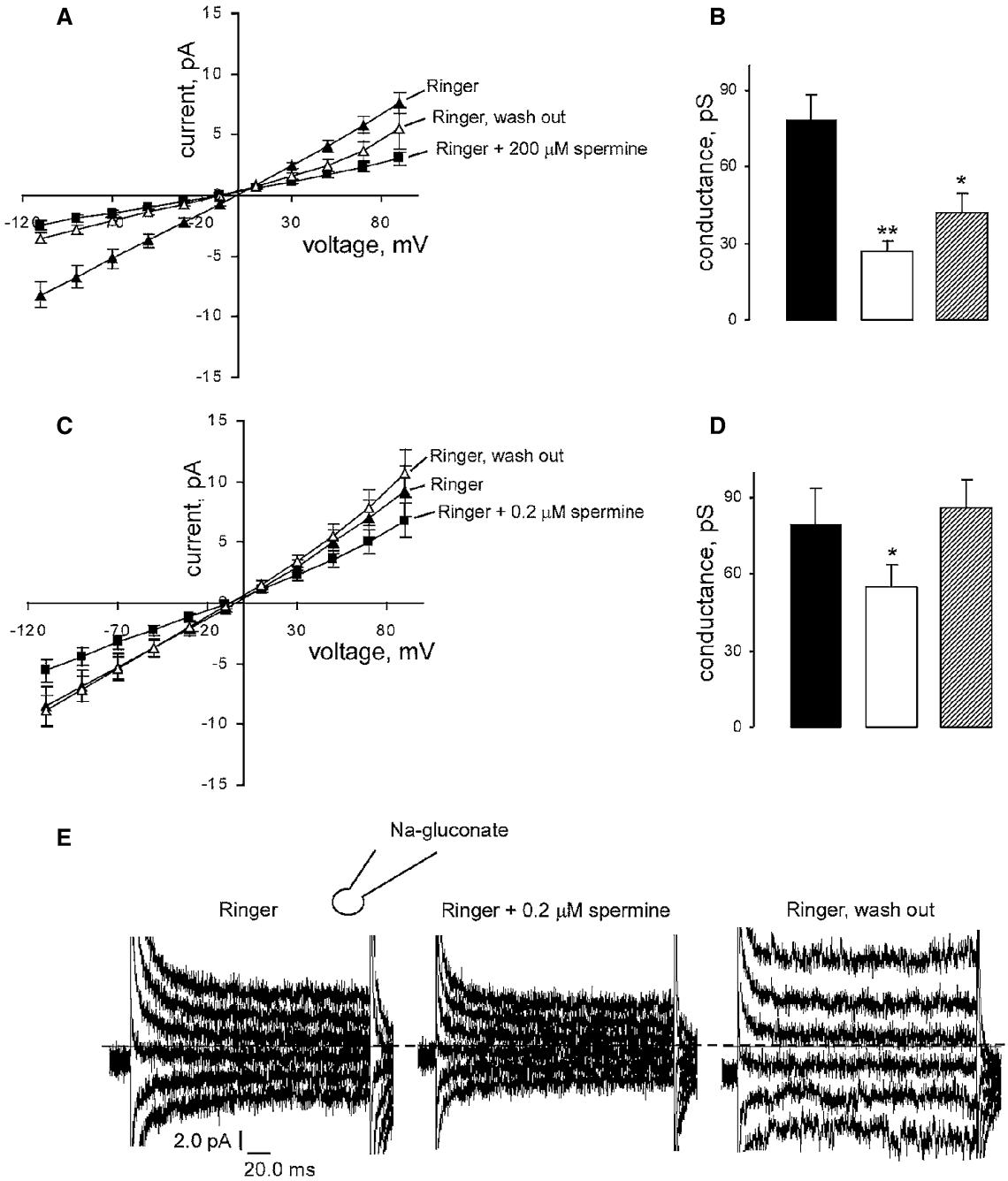

Fig. 3 Acute application of spermine blocks cation conductance in human erythrocytes. **a** Arithmetic means (±SEM, $n = 5$) of the current as a function of voltage (*I–V* relationship) recorded in NaCl Ringer bath solution prior to (*black bar*) and following (*white bar*) acute application of 0.2 μM spermine in NaCl Ringer bath solution and after (*hatched bar*) washout with NaCl Ringer bath solution. **b** Arithmetic means (±SEM, $n = 5$) of the conductance (as calculated for the inward currents by linear regression) prior to (*black bar*) and following (*white bar*) acute application of 0.2 μM spermine in NaCl Ringer bath solution and after (*hatched bar*) washout with NaCl Ringer bath solution. Significant difference from control (* $P \leq 0.05$ and ** $P \leq 0.01$, *t*-test). **c** Arithmetic means (±SEM, $n = 6$) of the current as a function of voltage (*I–V* relationship) recorded in NaCl Ringer bath solution prior to (*black bar*) and following (*white bar*) acute application of 0.2 μM spermine in NaCl Ringer bath solution and after (*hatched bar*) washout with NaCl Ringer bath solution. **d** Arithmetic means (±SEM, $n = 5$) of the conductance (as calculated for the inward currents by linear regression) prior to (*black bar*) and following (*white bar*) acute application of 0.2 μM spermine in NaCl Ringer bath solution and after (*hatched bar*) washout with NaCl Ringer bath solution. Significant difference from control (* $P \leq 0.05$, *t*-test). **e** Whole-cell current tracings (recorded with Na-gluconate pipette, shown with 40-mV increments) of erythrocytes incubated in NaCl Ringer bath solution, following acute application of 0.2 μM spermine and after washout with NaCl Ringer bath solution

The combination of Na-gluconate pipette and NaCl Ringer bath solutions allowed the characterization of cation influx into the cell. Replacing bath Na^+ by the larger and less permeable cation $NMDG^+$ decreased the inward currents and shifted the reversal potentials of the *I–V* relationships to about −70 mV in 180 mM NMDG-Cl bath solution (Fig. 4a). This indicates cation selectivity of the principal whole-cell current fraction in control cells. To further define the cation selectivity of the spermine-sensitive cation conductance, we substituted NaCl Ringer bath solution with isotonic buffered solutions of KCl, NaCl, NMDG-Cl and $CaCl_2$. The *I–V* relations for the cells in NaCl Ringer bath solution, 150 mM KCl, 150 mM NaCl, 180 mM NMDG-Cl and 100 mM $CaCl_2$ in the absence as well in the presence of 0.2 μM spermine in the Na-gluconate pipette solution are shown in Fig. 4a, b. As a result, spermine (0.2 μM) inhibited the inward (cation) conductance irrespective of the charge carrier in extracellular bath solution (Fig. 4c). However, the blocking effect of spermine was more pronounced for monovalent cations (K^+, Na^+, $NMDG^+$) than for divalent Ca^{2+}. Nevertheless, the addition of spermine (0.2 μM) to the pipette decreased the conductance as well in 100 mM $CaCl_2$ bath solution ($n = 3–5$, $P > 0.05$).

Surprisingly, acute application of 200 μM spermine decreased the $NMDG^+$ conductance (Fig. 5a, b). As described above for cation currents in Ringer bath solution, the effect of acute application of 200 μM spermine on the $NMDG^+$ currents was only partially reversible upon spermine removal (Fig. 5c).

The erythrocyte cation conductance was similarly inhibited by addition of spermine to the intracellular pipette solution (Fig. 6a). At 1 mM Ca^{2+}-containing NaCl Ringer bath solution the cation conductance approached 88 ± 8 pS in the absence and 60 ± 5 pS in the presence of 0.2 μM spermine in the intracellular pipette solution.

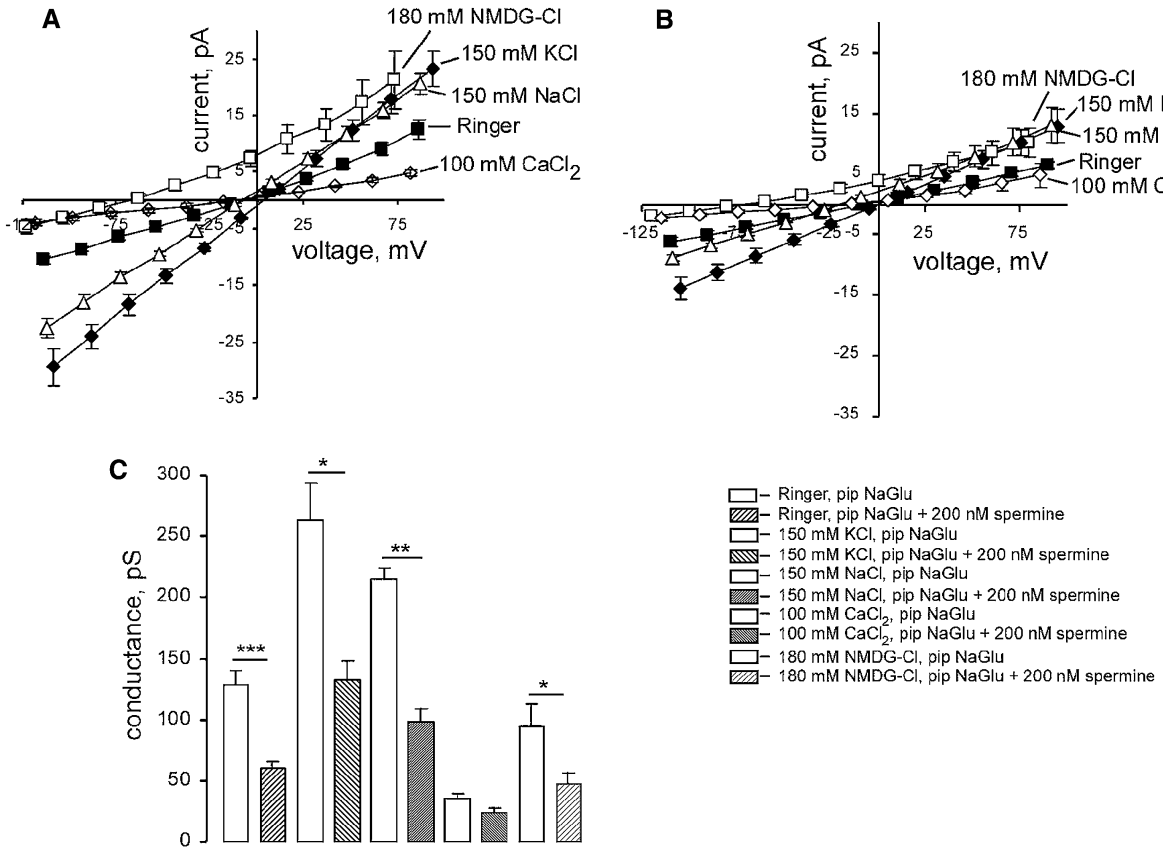

Fig. 4 Nonselective blocking effect of 0.2 μM intracellular applied spermine on cation conductance in human erythrocytes. **a** Arithmetic means (±SEM, $n = 5$) of the current as a function of voltage (*I–V* relationship), recorded with Na-gluconate pipette solution, in human erythrocytes suspended in NaCl Ringer bath solution (*closed squares*), 150 mM KCl bath solution (*closed diamonds*), 150 mM NaCl bath solution (*open triangles*), 100 mM $CaCl_2$ bath solution (*open diamonds*) and 180 mM NMDG-Cl bath solution (*open squares*). **b** As in **a** in the presence of 0.2 μM spermine in the Na-gluconate pipette solution. **c** Arithmetic means (±SEM) of the conductance (as calculated for the inward currents by linear regression) of cells in NaCl-Ringer (Ringer, $n = 9$), 150 mM KCl (KCl, $n = 3$), 150 mM NaCl (NaCl, $n = 3–6$), 100 mM $CaCl_2$ ($CaCl_2$, $n = 3–5$) or 180 mM NMDG (NMDG, $n = 3–6$) bath solution, each without spermine in the Na-gluconate pipette solution (*white bars*) and with 0.2 μM spermine in the Na-gluconate pipette solution (*hatched bars*). Significant differences from control (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, *t*-test)

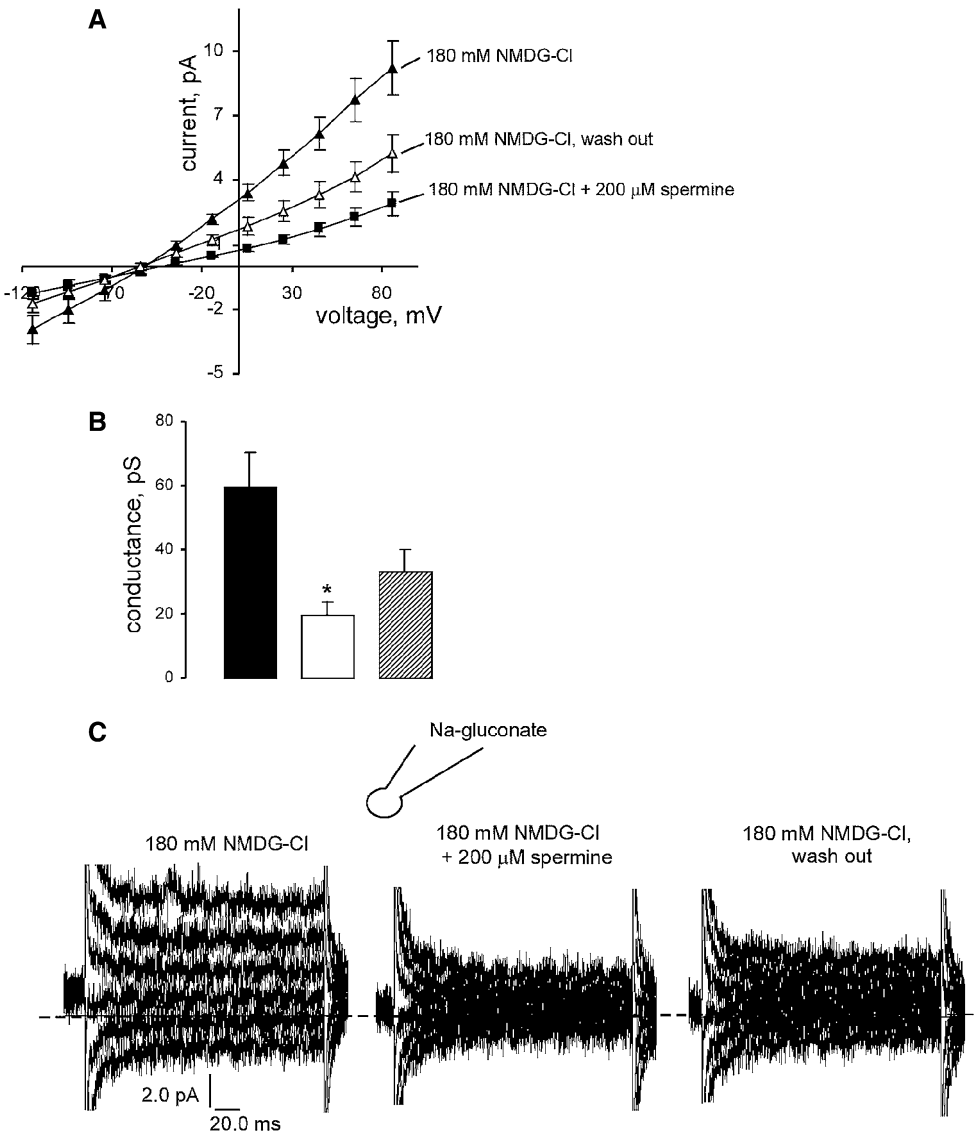

Fig. 5 Acute application of spermine blocks $NMDG^+$ conductance in human erythrocytes. **a** Arithmetic means (±SEM, $n = 5$) of the current as a function of voltage (*I–V* relationship) recorded in 180 mM NMDG-Cl solution prior to (*black bar*) and following (*white bar*) acute application of 200 μM spermine in 180 mM NMDG-Cl solution and after washout (*hatched bar*) with 180 mM NMDG-Cl solution. **b** Arithmetic means (±SEM, $n = 5$) of the conductance (as calculated for the inward currents by linear regression) prior to (*black bar*) and following (*white bar*) acute application of 200 μM spermine in 180 mM NMDG-Cl solution and after washout (*hatched bar*) with 180 mM NMDG-Cl solution. Significant difference from control (* $P \leq 0.05$, *t*-test). **c** Whole-cell current tracings (recorded with Na-gluconate pipette, shown with 40-mV increments) of erythrocytes incubated in 180 mM NMDG-Cl solution, following acute application of 200 μM spermine and after washout with 180 mM NMDG-Cl solution

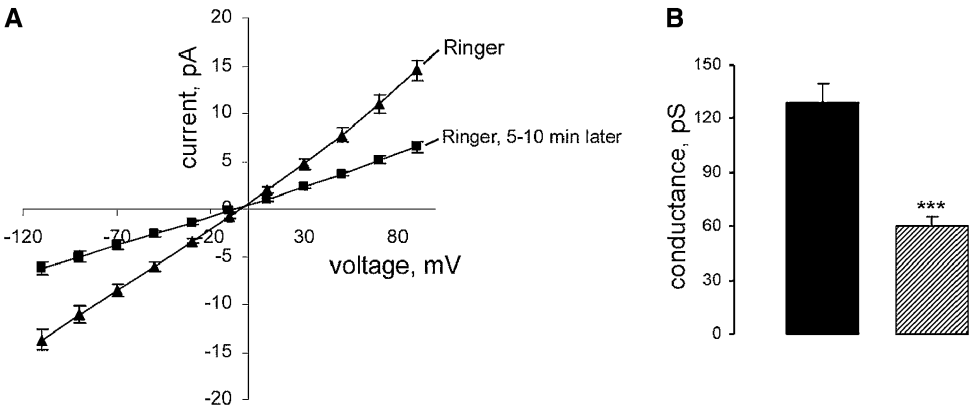

Fig. 6 Inhibition of cation conductance in human erythrocytes by 0.2 μM intracellular spermine in NaCl Ringer bath solution. **a** Arithmetic means (±SEM, $n = 9$) of the current as a function of voltage (*I–V* relationship) recorded in NaCl Ringer bath solution and 0.2 μM spermine-containing Na-gluconate pipette solution immediately after reaching whole-cell configuration (*closed triangles*) and 5–10 min later (*closed squares*). **b** Arithmetic means (±SEM, $n = 9$) conductance (as calculated for the inward currents by linear regression) at the beginning of the experiment (*black bar*) and 5–10 min later (*hatched bar*). Significant difference from control (*** $P \leq 0.001$, *t*-test)

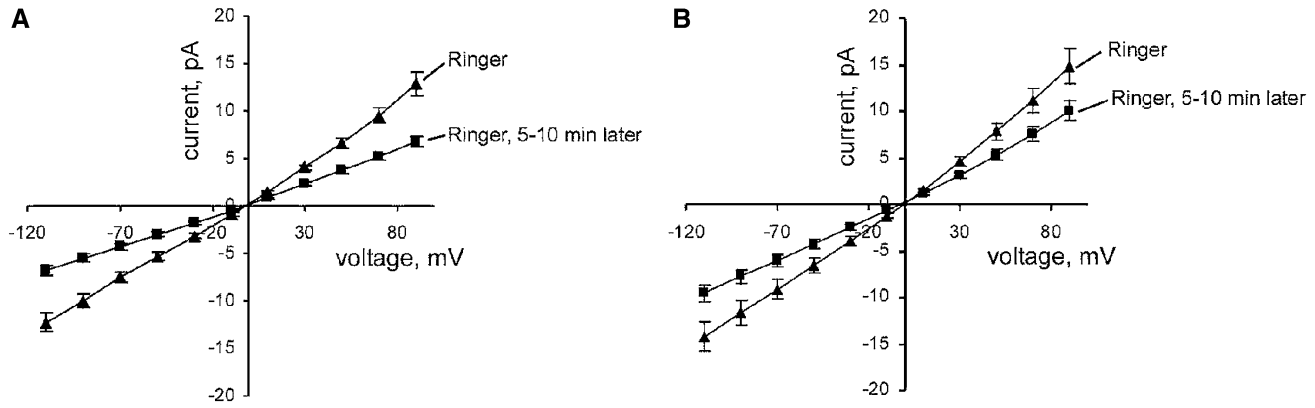

Fig. 7 Low concentrations of intracellular spermine (20 nM) have little blocking effect on cation conductance in human erythrocytes. **a** Arithmetic means (±SEM, $n = 6$) of the conductance as a function of voltage (*I–V* relationship) recorded in 1 mM Ca^{2+}-containing Ringer bath solution and 20 nM spermine-containing Na-gluconate pipette solution: immediately after reaching whole-cell configuration (*closed triangles*) and 5–10 min after (*closed squares*). **b** Arithmetic means (±SEM, $n = 6$) of the conductance as a function of voltage (*I–V* relationship) recorded in 1 mM Ca^{2+}-containing Ringer bath solution and spermine-free Na-gluconate pipette solution: immediately after reaching whole-cell configuration (*closed triangles*) and 5–10 min after (*closed squares*)

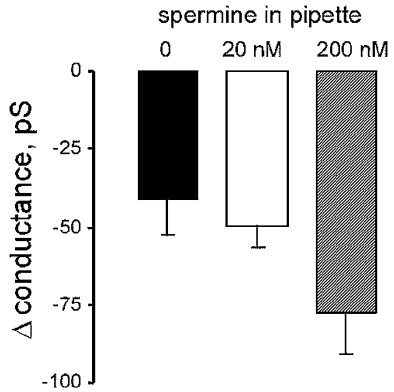

Fig. 8 Additive effect of intracellular spermine and extracellular Ca^{2+} on cation conductance of human erythrocytes in Cl^--containing bath medium. Time-dependent decline in mean (±SEM) conductance (as calculated for the inward currents by linear regression) of cells exposed to 1 mM Ca^{2+}-containing Ringer bath solution without spermine in the Na-gluconate pipette solution (*black bar*, $n = 10$), with 20 nM spermine in (*white bar*, $n = 6$) and with 0.2 μM spermine in the Na-gluconate pipette solution (*gray bar*, $n = 9$)

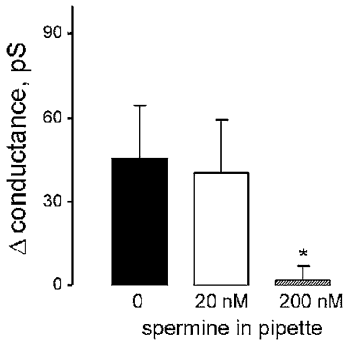

Fig. 9 Concentration-dependent inhibition of Cl^- removal-induced sodium conductance in human erythrocytes by intracellular spermine. Arithmetic means (±SEM) of Cl^--dependent sodium conductance, calculated as a difference of the conductances in Na-gluconate and NaCl bath media in the absence of spermine (*black bar*, $n = 5$), with 20 nM spermine (*white bar*, $n = 5$) and with 0.2 μM spermine in the Na-gluconate pipette solution (*gray bar*, $n = 6$). Significant difference from control (* $P \leq 0.05$, *t*-test)

Lower concentrations of spermine (20 nM) similarly decreased the cation conductance (Fig. 7a). However, there was no significant difference between the conductances recorded without (Fig. 7b) or with 20 nM spermine in Na-gluconate pipette solution, when 1 mM Ca^{2+}-containing NaCl Ringer bath medium was used (Figs. 7a, b, 8), suggesting that the presence of Ca^{2+} in the bath solution also inhibited the cation conductance in human erythrocytes.

We also tested whether intracellular spermine application influenced the nonselective voltage-independent cation channels stimulated by extracellular replacement of Cl^- with gluconate$^-$. The data shown in Fig. 9 demonstrate that 0.2 μM, but not 20 nM, spermine significantly ($n = 6$, $P \leq 0.05$) blunted the Na^+ conductance triggered by Cl^- removal.

Additional patch-clamp experiments, performed with 100 mM $CaCl_2$ bath solution, further confirmed the inhibitory effect of 200 μM spermine on the Ca^{2+} conductance in human erythrocytes. The results (Fig. 10a, b) show low cation conductance values for Ca^{2+} (27 ± 5 pS), suggesting a self-inhibitory effect of calcium in human erythrocytes. The value, however, could be further lowered significantly ($n = 4$, $P \leq 0.05$) by acute application of 200 μM spermine (21 ± 3 nS).

To explore whether the effect of spermine was due to structural properties of the molecule, we tested the naphthyl acetyl derivative of spermine (NASRM), an effective inhibitor of Ca^{2+}-permeable AMPA-type glutamate receptors lacking $GluR_2$ subunit in neurons. As shown in Fig. 11a, b, acute addition of NASPM inhibited the

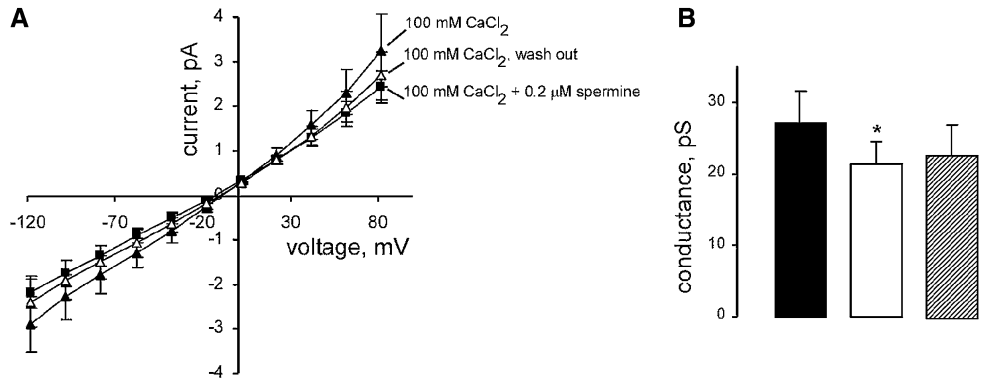

Fig. 10 Acute application of spermine blocks Ca^{2+} conductance in human erythrocytes. **a** Arithmetic means (±SEM, $n = 4$) of the current as a function of voltage (*I–V* relationship) recorded in 100 mM $CaCl_2$ solution prior to (*closed triangles*) and following (*closed squares*) acute application of 200 μM spermine in 100 mM $CaCl_2$ solution and after wahout (*open triangles*) with 100 mM $CaCl_2$ solution. **b** Arithmetic means (±SEM, $n = 4$) of the conductance (as calculated for the inward currents by linear regression) prior to (*black bar*) and following (*white bar*) acute application of 200 μM spermine in 100 mM $CaCl_2$ solution and after washout (*gray bar*) with 100 mM $CaCl_2$ solution. Significant difference from control (* $P \leq 0.05$, *t*-test)

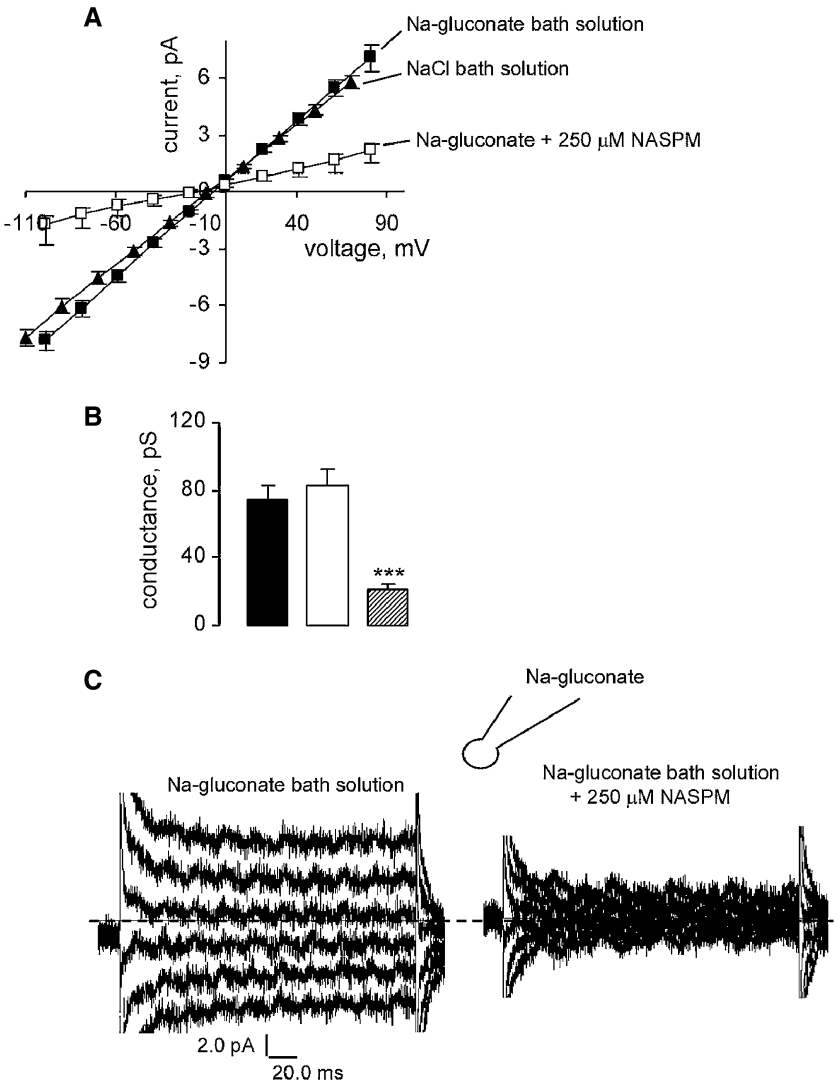

Fig. 11 Inhibition of the cation conductance in human erythrocytes by NASPM. **a** Arithmetic means (±SEM, $n = 10$) of the current as a function of voltage (*I–V* relationship) recorded in NaCl Ringer bath solution (*open triangles*), in Na-gluconate bath solution (*closed squares*) and after (*open squares*) acute application of 250 μM NASPM in Na-gluconate bath solution. **b** Arithmetic means (±SEM, $n = 10$) of the conductance (as calculated for the inward currents by linear regression) recorded in NaCl Ringer bath solution (*white bar*), Na-gluconate bath solution (*black bar*) and after (*gray bar*) acute application of 250 μM NASPM in Na-gluconate bath solution. Significant difference from control (* $P \leq 0.05$ and *** $P \leq 0.001$, *t*-test). **c** Whole-cell current tracings (recorded with Na-gluconate pipette, shown with 40-mV increments) of erythrocytes in Na-gluconate bath solution and following acute application of 250 μM NASPM

nonselective voltage-independent cation channels stimulated by extracellular Cl^- removal. Besides its inhibitory effect on the Cl^--sensitive cation conductance, NASRM also significantly ($n = 9$, $P \leq 0.001$) reduced the basal cation conductance in NaCl Ringer bath solution (75 ± 8 and 21 ± 3 pS, respectively).

Discussion

The present study reveals that a subphysiological (0.2 μM) concentration of spermine significantly inhibits the cation conductance of erythrocytes. Accordingly, physiological cytosolic spermine concentrations impose a tonic inhibition of those channels. The inhibition of the Ca^{2+} channels is expected to decrease Ca^{2+} entry and, thus, cytotolic Ca^{2+} concentration, which was indeed observed.

For inhibition of the cation channels, intracellular concentrations of less than 1 μM are needed, which is below the physiological concentration of some 3–14 μM (Cooper et al. 1976). In nucleated blood cells, such as leukocytes, the intracellular concentration of spermine may reach millimolar levels (Cooper et al. 1976).

Extracellular spermine concentrations required for an effect on channel activity, intracellular Ca^{2+} activity, cell volume and cell membrane scrambling amount to 200 μM, which is several orders of magnitude higher than the plasma concentration (0.14 μM) encountered in healthy individuals (Baylin et al. 1980). However, spermine plasma concentrations in the order of the 200 μM employed in this study have been generated in the treatment of sepsis (Zhu et al. 2009), a disorder with accelerated suicidal erythrocyte death (Kempe et al. 2007). The present study may thus provide a mechanism explaining the beneficial effect of spermine in the treatment of this severe disorder. Moreover, similarly high concentrations of spermine may prove useful for the long-term storage of erythrocytes.

Spermine was shown to bind to negatively charged phospholipids and, thus, can modify the cation conductance of lipid bilayers. However, the literature data for sodium conductance (0.1 M NaCl, pH 7.4) of pure lipid bilayers are 3.6 ± 0.5 nS per $\Omega^{-1} \times cm^{-2}$ (Cala et al. 1982), which allows estimation of the pure lipid sodium conductance of human erythrocytes (which has a 6–8 μm diameter) of $\sim 7.2 \times 10^{-15}$ S. This number is 10^4 times lower than that measured in our experiments. The comparison suggests that the cation conductance of human erythrocytes is in large part due to channel activity.

According to electrophysiology, human erythrocytes express Ca^{2+}-activated K^+ channels with the molecular identity of SK4 (KCNN4) (Foller et al. 2010), voltage-gated (Baunbaek and Bennekou 2008; Bennekou 1993; Kaestner et al. 2000) and voltage-independent (Huber et al. 2001) cation channels. The voltage-independent cation channels were shown to be partially related to TRPC6 channels (Foller et al. 2008). The presence of subtypes of voltage-dependent Ca^{2+} channels was demonstrated by Western blot analysis in membranes of young and senescent erythrocytes (Romero et al. 2006). Nevertheless, voltage-dependent gating, typical for L-type Ca^{2+} channels, has not been detected in the *I–V* profiles of erythrocytes.

The nonselective voltage-dependent cation channels are active only in nonelectrolyte media (Bennekou 1993; Kaestner et al. 1999, 2000) and, thus, do not account for the nonselective voltage-independent cation currents observed in our experiments. Most likely, the cation currents recorded in our experiments are currents of several nonselective voltage-independent cation channels in human erythrocytes.

The precise mechanism by which spermine inhibits cation conductance in human erythrocytes is unknown. However, lack of selectivity and voltage independence of the spermine effect as well as its efficacy from both sides of the cell membrane may be taken as evidence for direct binding of spermine molecules to the channels and/or associated proteins. Spermine has four positive charges at physiological pH, and it can interact electrostatically with organic anions. This property of spermine is common with Ca^{2+} and some heavy metal cations (La^{3+}, Co^{2+}, Gd^{3+}) that were shown to block a number of cation channels (Molgo et al. 1991; Sanchez-Chapula and Sanguinetti 2000; Starushchenko et al. 2002). Our data suggest that the effect of spermine is additive to extracellular calcium. Extracellular Ca^{2+} is again most likely effective by permeation blockade and/or stabilization of gating in "nonconductive" states. As was shown for nonselective, Ca^{2+}-permeable cation channels, the blocking effect of Ca^{2+} on Na^+ conductance is a result of strong binding of Ca^{2+} to highly charged glutamate residues of the channel pore (Corry et al. 2001). Western blot analysis revealed the existence of TRPC3/-6/-7-nonselective, Ca^{2+}-permeable cation channels in human erythrocytes (Foller et al. 2008). A negatively charged glutamate residue in the putative pore-forming loop of TRPC6 and TRPC7 channels (Glu^{598}) and an additional four glutamates (or aspartates) in the central part of the channel pore (Shi et al. 2004) are the most likely candidates for the binding of spermine and Ca^{2+}. Highly positively charged spermine may compete with Ca^{2+} for negatively charged glutamates in the channel pores. However, the inhibitory effect of Ca^{2+} on the channels may also involve Ca^{2+}-dependent activation of signaling components such as kinases and phosphatases (Estevez and Strange 2005).

The AMPA receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX) has previously been shown to inhibit cation channels in human

erythrocytes following Cl^- removal (Foller et al. 2009a). The present study showed that another AMPA receptor antagonist, NASRM, was effective not only in blocking cation currents induced by Cl^- removal but also in significantly blunting the basal cation conductance in Cl^--containing bath solution. Similar to the effect of spermine, the blocking effect of NASRM on cation conductance was voltage-independent, suggesting a similar mechanism of inhibition.

The present results reveal a nonlinear concentration dependence of cation blockage by spermine and NASPM in human erythrocytes (see Figs. 8, 12). Low subphysiological (submicromolar) concentrations of spermine were found to inhibit up to ~30% of the basal current in normal buffered saline, whereas submicromolar concentrations of spermine were required to block ~70% of current. It is further noteworthy that the effect of low (submicromolar) concentrations of spermine was fully reversible, whereas a higher (submillimolar) concentration of spermine was only partly reversible. The data may suggest that there is more than one unspecific binding site for spermine (and its derivative NASPM) on cation channels or proteins associated with the channel proteins and regulating their activity. Alternatively, the data also may suggest the existence of unrevealed nonselective cation channels, also sensitive to spermine, that are different from TRPC3/-6/-7 channels in human erythrocytes.

The effect of spermine may be relevant for erythrocyte senescence, which does involve Ca^{2+}-sensitive mechanisms (Bosman et al. 2005; Kiefer and Snyder 2000). Erythrocyte senescence is paralleled by a decline of cytosolic spermine concentration (Cooper et al. 1976), which is expected to disinhibit the erythrocyte cation channels and, thus, to foster Ca^{2+} entry.

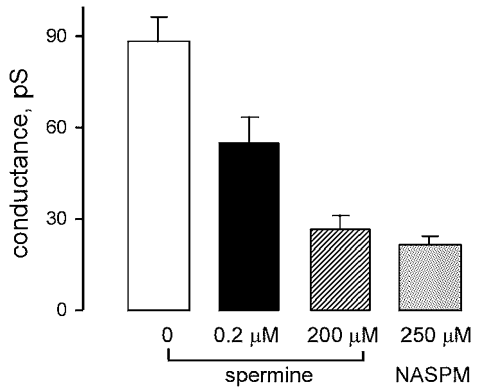

Fig. 12 Nonlinear blocking effect of spermine on cation conductance in human erythrocytes. Arithmetic means (±SEM) of cation conductance in human erythrocytes: prior to (*white bar*) and following (*black bar*) acute application of 0.2 μM spermine, 200 μM spermine (*hatched bar*) or 250 μM NASPM (*dotted bar*)

Enhanced cation channel activity, increase of cytosolic Ca^{2+} concentration and Ca^{2+}-dependent stimulation of K^+ channels with cell shrinkage as well as Ca^{2+}-dependent stimulation of cell membrane scrambling with phosphatidylserine exposure at the erythrocyte surface are hallmarks of eryptosis, the suicidal death of eryrthrocytes (Lang et al. 2008). At least in theory, cellular loss of spermine could foster eryptosis by disinhibiting Ca^{2+} entry. Accelerated Ca^{2+} entry and subsequent stimulation of cell membrane scrambling are triggered in several conditions leading to accelerated eryptosis, including sepsis (Kempe et al. 2007), malaria (Brand et al. 2008; Foller et al. 2009b; Koka et al. 2008a, b, 2009; Lang et al. 2009), iron deficiency (Kempe et al. 2006), hemolytic uremic syndrome (Lang et al. 2006), Wilson disease (Lang et al. 2007), hyperglycemia (Kucherenko et al. 2010) and several hemoglobinopathies (Kuypers 2007; Lang et al. 2008). Moreover, a wide variety of substances trigger eryptosis (Lang et al. 2008; Lui et al. 2007; Mahmud et al. 2009; Niemoeller et al. 2008a, b; Sopjani et al. 2008a, b; Wang et al. 2008), an effect possibly in part involving cellular loss or decreased activity of spermine.

The present observations may further relate to the determination of cation channel activity by patch clamp. Patch-clamp experiments are usually performed in the absence of spermine in the pipette and thus facilitate the detection of cation channels (Bennekou et al. 2004; Browning et al. 2007; Duranton et al. 2002; Egee et al. 1998; Huber et al. 2001; Kaestner and Bernhardt 2002; Lapaix et al. 2008; Vandorpe et al. 2010). While the omission of spermine facilitates the discovery of spermine-sensitive cation channels, it should be kept in mind that the conductance in the absence of cytosolic spermine may be an order of magnitude larger than the conductance at physiological concentrations of spermine.

In conclusion, the present study demonstrates for the first time that spermine at concentrations below those observed in erythrocytes is a powerful inhibitor of erythrocyte cation channels. Moreover, it shows for the first time that spermine inhibits Ca^{2+} entry into erythrocytes. As Ca^{2+} is a main trigger of suicidal erythrocyte death, spermine thus participates in the regulation of erythrocyte survival.

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