Original Paper

Inhibitory Effect of Furosemide on Non-Selective Voltage-Independent Cation Channels in Human Erythrocytes

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Patch-clamp • Cation channels • ATP-depletion • Oxidation • Phosphatidylserine exposure • Eryptosis • Suicidal erythrocyte death

Abstract

Background: Furosemide, a loop diuretic inhibiting the renal tubular $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter, has been shown to decrease cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in platelets and erythrocytes. $[\text{Ca}^{2+}]_i$ in erythrocytes is a function of Ca^{2+} permeable cation channels. Activation of those channels e.g. by energy depletion or oxidative stress leads to increase of $[\text{Ca}^{2+}]_i$, which in turn triggers eryptosis, a suicidal erythrocyte death characterized by cell membrane scrambling. The present study was performed to explore whether furosemide influences the cation channels and thus influences eryptosis. **Methods:** Cation channel activity was determined by whole-cell patch clamp, $[\text{Ca}^{2+}]_i$ utilizing Fluo3 fluorescence and annexin V binding to estimate cell membrane scrambling with phosphatidylserine exposure. **Results:** A 45 min exposure to furosemide (10 and 100 μM) slightly, but significantly decreased cation channel activity and $[\text{Ca}^{2+}]_i$ in human erythrocytes drawn from healthy individuals. ATP-depletion (> 3 hours, +37°C, 6 mM ionosine and 6 mM iodoacetic acid) enhanced the non-selective cation channel activity, increased $[\text{Ca}^{2+}]_i$ and triggered cell membrane scrambling, effects significantly blunted by furosemide (10 - 100 μM). Oxidative stress by exposure to tert-butylhydroperoxide (0.1-1 mM) similarly enhanced the non-selective cation channels activity, increased $[\text{Ca}^{2+}]_i$ and triggered cell membrane scrambling, effects again significantly blunted by furosemide (10 - 100 μM). **Conclusions:** The present study shows for the first time that the loop diuretic furosemide applied at micromolar concentrations (10 - 100 μM) inhibits non-selective cation channel activity in and eryptosis of human erythrocytes.

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Introduction

Furosemide, a sulphonamide derivative, is clinically widely used as a high ceiling loop diuretic [1]. The diuretic effect of furosemide is accomplished by inhibition of the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter in the thick ascending limb [2-6]. Further effects of furosemide include non-competitive inhibition of GABA-A receptor subtypes [1, 7] as well as influence of protein kinase B PKB/Akt [8], mitogen activated protein kinase MAP [9] and cAMP-phosphodiesterase [10]. Furosemide further modifies Na^+/K^+ -ATPase activity by stimulating the Na^+/H^+ -exchanger [11]. At high concentrations furosemide inhibits platelet aggregation [12], water flux via aquaporin-1 water channels [13], adenosine transport in erythrocytes [14], postoperative pain in rats [15] and the new permeability pathways (NPP) induced by the malaria pathogens *plasmodia* in parasitized erythrocytes [16-18]. Moreover, furosemide may counteract apoptosis [5, 8]. Furosemide infusion attenuated ischemia-induced apoptosis and associated gene transcription [8], inhibited Bax translocation to mitochondria [19, 20] and modified RAF/MEK/ERK phosphorylation in Balb/c 3T3 fibroblasts [9]. Exposure of human peripheral blood mononuclear cells to 5 mM furosemide decreased the levels of pro-inflammatory cytokines TNF-alpha and IL-6, mimicking the effect of hydrocortisone [21]. Even higher concentrations of furosemide (10 mM) are cytotoxic [21]. *In vivo* furosemide treatment leads to upregulation of renin [22]. A parallel increase of ubiquitous, membrane-bound angiotensin-converting enzyme [23] may result in enhanced angiotensin II production, with subsequent activation of membrane-bound NADPH-oxidase, increased O_2^- production, inactivation of endothelial NO, increase in ONOO^- , and thus inflammation [24]. Accordingly, high concentrations of furosemide may promote inflammation.

Loop diuretics including furosemide decrease intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) in platelets and erythrocytes from patients with hypertension [25-27]. The lowering effect of loop diuretics on $[\text{Ca}^{2+}]_i$ is considered to result from its effect on the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter activity. The carrier increases cytosolic Cl^- activity above thermodynamic equilibrium across the cell membrane leading to an outwardly directed electrochemical gradient, which in turn fosters Cl^- exit and thus depolarization. The depolarisation increases $[\text{Ca}^{2+}]_i$ by activating voltage-gated Ca^{2+} channels [4]. Diuretic treatment may further enhance $[\text{Ca}^{2+}]_i$ by modifying the gradient for the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger [28].

In human erythrocytes increases of $[\text{Ca}^{2+}]_i$ may stimulate suicidal cell death or eryptosis with cell membrane scrambling and subsequent phosphatidylserine (PS) exposure at the cell surface [29, 30]. Eryptotic cells are rapidly eliminated from circulating blood by macrophages [29, 30]. Eryptosis is triggered by a wide variety of clinical conditions [24, 30-40], chemicals [30, 41-53], energy depletion [54] and oxidative stress [55, 56]. Erythrocytes are lacking intracellular calcium stores (mitochondria and sarcoplasmic reticulum) and an increase in $[\text{Ca}^{2+}]_i$ may result from activation of Ca^{2+} -permeable cation channels.

According to electrophysiological studies two types of non-selective cation channels permeable for Ca^{2+} are expressed in human erythrocytes. The voltage-gated channel, coupled to an acetylcholine receptor [57-59] and the voltage-independent Cl^- -sensitive cation channels that can be activated by oxidation, osmotic shock and energy depletion [60]. The voltage-independent channels were shown to be partially related to TRPC6 channels [61]. Moreover, erythrocytes may express NMDA receptors in the rat [62] and AMPA receptors in man [31]. The presence of subtypes of voltage-dependent Ca^{2+} channels was demonstrated by Western blot analysis in age-fractionated erythrocytes [63] but has not been detected by patch-clamp.

The present study explored whether furosemide at therapeutic serum concentrations (6 – 30 μM [64]) and higher concentrations (100 μM) used for inhibition of new permeability pathways (NPP) in malaria infected erythrocytes [16-18] influences Ca^{2+} permeable cation channels, $[\text{Ca}^{2+}]_i$ and cell membrane scrambling in human erythrocytes. It is indeed shown for the first time that the loop diuretic furosemide applied at micromolar concentrations (10 - 100 μM) decreases cation channel activity, decreases $[\text{Ca}^{2+}]_i$ and protects against cell membrane scrambling following energy depletion and oxidative stress.

Materials and Methods

Erythrocytes

The experiments were carried out with banked erythrocyte concentrates provided by the blood bank of the University of Tübingen. Erythrocytes were washed twice (1200 g, 5 min, 22°C) in physiological saline buffered with 10 mM HEPES (pH 7.4). Metabolically depleted cells were obtained according to the method of Lew [65] by incubation of control cells (> 3 hours, +37°C) in the ATP-depleting medium consisting of (mM): 140 NaCl, 5 KCl, 10 HEPES, 6 iodoacetic acid, and 6 inosine. Furosemide (10, 30 and 100 µM final concentrations) was added as a component of ATP-depleting medium. Oxidative stress was induced by addition of 0.1, 0.3 and 1 mM tert-butyl-hydroperoxide (t-BHP) to the cells in the absence or presence of furosemide (10, 30 and 100 µM).

Electrophysiology

Whole-cell patch clamp recordings were performed at room temperature. The patch electrodes were made of borosilicate glass capillaries (150 TF-10, Clark Medical Instruments) using a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 17 to 20 MΩ were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by 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 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 both electrodes were zeroed before sealing. The liquid junction potentials between 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 [66]. 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 MgCl₂, 1 MgATP, 1 EGTA, 10 HEPES/NaOH (pH 7.4). The NaCl Ringer bath solution contained (in mM): 145 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 5 glucose, 10 HEPES/NaOH (pH 7.4). In experiments, where Cl⁻ was substituted with gluconate⁻, the solution contained (in mM): 150 Na-gluconate and 10 HEPES/NaOH (pH 7.4). The NMDG-Cl, and CaCl₂ bath solutions contained (in mM): 180 NMDG (titrated with HCl to pH 7.4) or 100 CaCl₂ (titrated with CaOH₂ to pH 7.4), 10 HEPES. Reagents were obtained from Sigma (Germany) and were of the highest grade available. All bath solutions used in whole-cell patch-clamp experiments on ATP-depleted furosemide-pretreated cells contained furosemide (30 µM and 100 µM). In the experiments with acutely oxidized cells (20 min, 22°C, 1 mM t-BHP) furosemide (100 µM) was added as a component of the NaCl Ringer bath solution.

Intracellular Ca²⁺

Experiments were performed with ATP-depleted, t-BHP oxidized or with erythrocytes incubated for 45 minutes in the NaCl-containing Ringer bath in the presence or absence of furosemide (10, 30 and 100 µM). Erythrocytes (0.4% suspension) were washed in the appropriate media (with or without 10, 30 and 100 µM furosemide) containing 5 mM CaCl₂ and loaded with 2 µM Fluo-3/AM (Calbiochem, Bad Soden, Germany). The cells were incubated at 37°C for 20 min, washed once and resuspended in 5 mM Ca²⁺-containing Ringer/ATP-depleting bath media (with or without 10, 30 and 100 µM furosemide) and subsequently analyzed in fluorescence channel FL-1 in FACS analysis.

Phosphatidylserine exposure

Cells (0.4 % suspension) were prepared as described above and stained with annexin V-Fluos (Roche, Mannheim, Germany) at a 1:50 dilution in 5 mM Ca²⁺-containing Ringer/ATP depleting bath media in the presence or absence of 10, 30 and 100 µM furosemide. After 20 min, samples were washed once and resuspended in 5 mM Ca²⁺-containing Ringer/ATP depleting media (with or without 10, 30 and 100 µM furosemide) and measured by flow cytometric analysis (FACS-Calibur from 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.

Fig. 1. Short exposure to furosemide reduces $[\text{Ca}^{2+}]_i$ and inhibits cation conductance in human erythrocytes. **A.** Arithmetic means $\pm$ SEM of normalized Fluo-3 fluorescence, reflecting cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), of human erythrocytes (white bar, $n = 11$) exposed for a short time (45 min) to 10 μM furosemide (grey bar, $n = 12$) and 100 μM furosemide (black bar, $n = 12$). * and ** indicate significant difference from control ($p < 0.05$ and 0.01 respectively; t-test). **B.** Histogram of Fluo-3 fluorescence in a representative experiment of human erythrocytes exposed 45 min to Ringer bath solution (solid line), and to 10 μM furosemide (dotted line) or 100 μM furosemide (dashed line) added to Ringer bath solution. **C.** Arithmetic means ($\pm$ SEM, $n = 7$) of the current as a function of voltage (I/V relationship) recorded in NaCl Ringer bath solution prior to (open circles) and following (closed circles) acute application of 100 μM furosemide in NaCl Ringer bath solution. **D.** Arithmetic means ($\pm$ SEM, $n = 7$) of the conductance (as calculated for the inward currents by linear regression) prior to (white bar) and following (black bar) acute application of 100 μM furosemide in NaCl Ringer bath solution. ** indicates significant difference from control ($p \leq 0.01$; paired t-test).

Statistics

Data are expressed as arithmetic means $\pm$ SEM and paired two-tailed t-test or ANOVA were employed as appropriate, $p < 0.05$ was considered statistically significant.

Results

We first examined, whether a short incubation (45 min at 37°C) with furosemide resulted in a decline of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) in human erythrocytes. As apparent from Fluo3 fluorescence (Fig. 1A, B), short exposure to furosemide (10 and 100 μM) indeed reduced in a concentration-dependent manner the basal levels of $[\text{Ca}^{2+}]_i$ in erythrocytes of healthy donors. Whole-cell patch-clamp experiments confirmed the inhibitory effect of furosemide on the cation conductance in human erythrocytes. Acute application of 100 μM furosemide dissolved in NaCl Ringer bath solution had a slight but statistically significant lowering effect on the cation conductance (Fig. 1C, D).

Fig. 2. Effect of furosemide (10 μ M, 30 μ M and 100 μ M) on $[Ca^{2+}]_i$ in metabolically ATP-depleted human erythrocytes. **A.** Arithmetic means $\pm$ SEM of normalized Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), of cells without ATP depletion (dotted bar, $n = 11$), and after 3–7 h incubation with ATP-depletion medium (6 mM inosine, 6 mM iodoacetic acid) in the absence (white bar, $n = 12$) and presence of 10 μ M furosemide (light grey bar, $n = 8$), 30 μ M furosemide (dark grey bar, $n = 4$) or 100 μ M furosemide (black bar, $n = 8$). ** and *** indicate significant difference from ATP-depleted cells in the absence of furosemide ($p < 0.01$ and 0.001 , respectively). # indicates significant difference from control cells without ATP depletion ($p < 0.05$; ANOVA). **B.** Histogram of Fluo-3 fluorescence in a representative experiment with metabolically ATP-depleted human erythrocytes in Ca^{2+} -containing ATP-depletion medium in the absence (solid line), or presence of 10 μ M, 30 μ M and 100 μ M furosemide (dotted and dashed lines respectively).

Since ATP-depletion triggers eryptosis, the next series of experiments explored the effect of furosemide on metabolically (ATP-depleted) cells. Similar to what was observed in energy replete cells, furosemide significantly decreased $[Ca^{2+}]_i$ in ATP-depleted cells (Fig. 2 A, B).

ATP-depletion is assumed to activate the non-selective voltage-independent cation channels. As shown in Fig. 3 (Fig. 3 A, B, E), ATP depletion indeed significantly increased the conductance of all tested cations (Na^+ , Ca^{2+} and $NMDG^+$). In contrast, ATP depletion did not significantly increase the conductance in the presence of furosemide (30 and 100 μ M). In the presence of furosemide, the conductance of ATP depleted cells was not significantly different from control cells suggesting an inhibitory effect of furosemide on non-selective cation channels.

Since increase in $[Ca^{2+}]_i$ is known to stimulate cell membrane scrambling with subsequent phosphatidylserine exposure, additional experiments were performed to elucidate the effect of furosemide on annexin-V binding, which reflects phosphatidylserine exposure at the cell surface. As shown in Fig. 4, ATP depletion enhanced annexin V binding, an effect significantly lowered by furosemide (10, 30, 100 μ M) added to ATP-depleting medium.

A further series of experiments explored the effect of furosemide on the known stimulating effect of oxidative stress on cation channel activity, $[Ca^{2+}]_i$ and cell membrane scrambling. As a result, oxidative stress induced by addition of high (1 mM) or moderate (0.1 and 0.3 mM) concentrations of t-BHP significantly increased $[Ca^{2+}]_i$, an effect significantly blunted by furosemide (10, 30 and 100 μ M) (see Fig. 5).

In acute experiments high (1 mM) concentrations of t-BHP were required to appreciably increase the cation conductance in patch-clamp experiments. Similarly, only high (100 μ M) concentrations of furosemide were tested, as according to flow cytometric experiments they had strong inhibitory effects on Ca^{2+} influx in oxidized cells. The exposure of erythrocytes

Fig. 3. Furosemide blocks non-selective cation conductance in metabolically ATP-depleted human erythrocytes. A. Arithmetic means ($\pm$ SEM, $n = 18$) of the current as a function of voltage (I/V relationship), recorded with Na-gluconate pipette solution, in human erythrocytes (control) suspended in NaCl Ringer bath solution (open triangles), 150 mM Na-gluconate bath solution (closed squares), 100 mM CaCl₂ bath solution (closed triangles) and 180 mM NMDG-Cl bath solution (open squares). B. Arithmetic means ($\pm$ SEM, $n = 10$) of the current as a function of voltage (I/V relationship), recorded with Na-gluconate pipette solution, in metabolically ATP-depleted human erythrocytes suspended in NaCl Ringer bath solution (open triangles), 150 mM Na-gluconate bath solution (closed squares), 100 mM CaCl₂ bath solution (closed triangles) and 180 mM NMDG-Cl bath solution (open squares). C. As in B. in the presence of 30 μ M furosemide in the ATP-depleting medium, NaCl Ringer, 150 mM Na-gluconate, 100 mM CaCl₂ and 180 mM NMDG-Cl bath solutions ($n = 12$). D. As in B. in the presence of 100 μ M furosemide in the ATP-depleting medium, NaCl Ringer, 150 mM Na-gluconate, 100 mM CaCl₂ and 180 mM NMDG-Cl bath solutions ($n = 15$). E. Arithmetic means ($\pm$ SEM, $n = 10 \div 18$) of the conductance (as calculated for the inward currents by linear regression) in control cells without ATP depletion (dotted bars), ATP-depleted cells in the absence (white bars) and presence of 30 μ M furosemide (grey bars) and 100 μ M furosemide (black bars) suspended in NaCl Ringer, 150 mM Na-gluconate, 100 mM CaCl₂ and 180 mM NMDG-Cl bath solutions. * and ** indicate significant difference from ATP-depleted cells without furosemide ($p \leq 0.05$ and 0.01 , respectively), # and ## indicate significant difference from control cells without ATP depletion ($p \leq 0.05$ and 0.01 , respectively; ANOVA).

Fig. 4. Furosemide (10 μ M, 30 μ M and 100 μ M) blunts PS-exposure in ATP-depleted cells. **A.** Arithmetic means $\pm$ SEM of normalized annexin V fluorescence of control cells without ATP depletion (dotted bar, $n = 3$) and cells after 3-7 h incubation with ATP-depleting medium in the absence (white bar, $n = 3$) and presence of 10 μ M furosemide (light grey bar, $n = 6$), 30 μ M furosemide (dark grey bar, $n = 6$), or 100 μ M furosemide (black bar, $n = 6$). * and ** indicate significant difference from the ATP-depleted cells in the absence of furosemide ($p < 0.05$ and 0.01 , respectively) and ### indicates significant difference from control cells without ATP depletion ($p < 0.001$; ANOVA). **B.** Histogram of annexin V-binding in a representative experiment with metabolically ATP-depleted human erythrocytes in Ca^{2+} -containing ATP-depletion medium in the absence (solid line), or presence of 10 μ M, 30 μ M and 100 μ M furosemide (dotted and dashed lines respectively).

to Ringer bath solution containing 1 mM t-BHP increased the cation conductance due to activation of non-selective cation channels, an effect significantly inhibited by furosemide (Fig. 6).

Oxidative stress (0.3 mM t-BHP) further increased annexin-V binding and thus phosphatidylserine exposure, an effect again significantly blunted in the presence of furosemide (10, 30 and 100 μ M) (Fig. 7).

Discussion

The present study discloses a novel anti-eryptotic effect of the clinically widely used loop diuretic furosemide. At therapeutic serum concentrations (10 – 30 μ M furosemide) and at concentrations (100 μ M) used for inhibition of ion channels activated by *Plasmodium falciparum* infection of human erythrocytes [16-18] furosemide inhibited Ca^{2+} permeable non-selective cation channels, thus decreasing cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and counteracting eryptosis of human erythrocytes.

The decrease in $[\text{Ca}^{2+}]_i$ upon loop diuretics treatment was supposed to be due to blockage of the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter. Van Mil et al. [67] showed that under physiological conditions the activity of the $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter accumulates Cl^- in the cells above equilibrium resulting in depolarisation and increases of Ca^{2+} influx via L-type Ca^{2+} channels. Diuretic therapy with furosemide was further shown to increase the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) which could also modify the basal level of $[\text{Ca}^{2+}]_i$ [28].

Neither NCX nor voltage-dependent Ca^{2+} channels have been detected in human erythrocytes by patch-clamp experiments. Channels revealed by patch-clamp technique in human erythrocytes exposed to physiological saline are non-selective cation channels with low whole-cell cation conductance (< 100 pS) and activated upon Cl^- removal, oxidative stress and osmotic shock [60]. A number of different non-selective cation channels could contribute to

Fig. 5. Furosemide (10 μ M, 30 μ M and 100 μ M) inhibits Ca^{2+} uptake in t-BHP (0.1mM, 0.3 mM and 1 mM) treated human erythrocytes. A. Arithmetic means $\pm$ SEM of normalized Fluo-3 fluorescence of human erythrocytes exposed for a short time (45 min, 37°C) to 0.1 mM t-BHP in the absence (white bar, $n = 3$) and presence of 10 μ M, 30 μ M and 100 μ M furosemide (light grey, dark grey and black bars, respectively $n = 3$ each). * and ** indicate significant difference from control ($p < 0.05$ and 0.01 respectively; t-test). B. Arithmetic means $\pm$ SEM of normalized Fluo-3 fluorescence of human erythrocytes exposed for a short time (15 min, 37°C) to 1 mM t-BHP in the absence (white bar, $n = 10$) and presence of 10 μ M furosemide (light grey bar, $n = 8$), 30 μ M furosemide (dark grey bar, $n = 6$) and 100 μ M furosemide (black bars, $n = 3$) respectively. * and *** indicate significant difference from control ($p < 0.05$ and 0.001 , respectively; t-test). C. Arithmetic means $\pm$ SEM of normalized Fluo-3 fluorescence of human erythrocytes exposed for a short time (20 min, 37°C) to 0.1 mM t-BHP in the absence (white bar, $n = 6$) and presence of 10 μ M, 30 μ M and 100 μ M furosemide (light grey, dark grey and black bars, respectively, $n = 6$ each). * and ** indicate significant difference from control ($p < 0.05$ and 0.01 respectively; t-test). D. Histogram of Fluo-3 fluorescence in a representative experiment with t-BHP treated human erythrocytes in Ca^{2+} -containing Ringer bath solution in the absence (solid line), or presence of 10 μ M, 30 μ M and 100 μ M furosemide (dashed or dotted lines respectively).

the total cation conductance observed in erythrocytes. The presence of TRPC3/6/7 channels was confirmed by Western blotting [61]. Most recent data demonstrated the existence of NMDA receptors in rat [62] as well as AMPA receptors [31] and endothelin-B receptors [68] in human erythrocytes.

The whole-cell patch-clamp experiments performed in the present study revealed the absence of voltage-dependence of the cation channels and demonstrated that the channels are blocked by furosemide. They further show that the furosemide sensitive cation channels are induced by ATP-depletion and permeable to all cations tested (Na^+ , Ca^{2+} , NMDG^+). The observation suggests that the loop diuretic furosemide exerts its effect on $[\text{Ca}^{2+}]_i$ in erythrocytes of healthy donors by inhibiting those non-selective cation channels. The fact that furosemide effectively inhibited non-selective cation conductance induced by ATP-depletion, oxidative

Fig. 6. Furosemide blocks cation conductance increase due to oxidative stress. A. Arithmetic means ($\pm$ SEM, $n = 6$) of the current as a function of voltage (I/V relationship) recorded in control cells in NaCl Ringer bath solution prior to (open circles) and after (open squares) acute application (20 min) of 1 mM t-BHP in Ringer bath solution. B. Arithmetic means ($\pm$ SEM, $n = 6$) of the conductance (as calculated for the inward currents by linear regression) in control cells prior to (white bar), and after (black bar) acute application of 1 mM t-BHP in Ringer bath solution. * indicates significant difference from control ($p \leq 0.05$; paired t-test). C. As in A. in the presence of 100 μ M furosemide in the NaCl Ringer bath solution ($n = 7$). D. As in B. in the presence of 100 μ M furosemide in the NaCl Ringer bath solution (hatched bar, $n = 7$).

Fig. 7. Furosemide blunts PS-exposure in human erythrocytes treated with t-BHP. A. Arithmetic means $\pm$ SEM of normalized annexin V fluorescence of the cells treated with 0.3 mM t-BHP (20 min, 37°C) in the absence (white bar, $n = 6$) and presence of 10 μ M furosemide (light grey bar, $n = 6$), 30 μ M furosemide (dark grey bar, $n = 3$), or 100 μ M furosemide (black bar, $n = 6$). *** indicates significant difference from control ($p < 0.001$, t-test). B. Histogram of annexin V-binding in a representative experiment with 0.3 mM t-BHP treated cells. Solid line corresponds to control t-BHP treated cells while dashed and dotted lines show the cells oxidized in the presence of 10 μ M, 30 μ M and 100 μ M furosemide respectively.

stress and Cl^- removal allows us to assume the involvement of the non-selective channels described earlier [60]. At the same time, the experiments showed that acute application of furosemide dissolved in Cl^- containing bath medium also reduced the cation conductance and lowered basal $[\text{Ca}^{2+}]_i$, an observation possibly pointing to inhibition of another type of voltage-independent cation channel not requiring Cl^- removal for activation.

Obviously, the inhibitory effect of furosemide on non-selective cation conductance does not result from blockage of electroneutral $\text{Na}^+, \text{K}^+, 2\text{Cl}^-$ cotransporter activity. The present observations do not, however, define the exact mechanism of action. In theory, furosemide may inhibit the channel directly or by interference with some regulating mechanism. Furosemide was shown to enhance cAMP-phosphodiesterase activity that resulted in a decrease of cAMP concentration [10]. The members of TRPC channel family (TRPC3/4/5/6) have consensus PKA phosphorylation sites [69]. TRPC6 channels (that are also present in human erythrocytes [61]) are phosphorylated by PKA and associated with other cAMP-kinase substrates [70]. Thus, at least in theory, the decline in $[\text{Ca}^{2+}]_i$ and the non-selective cation channels activity in furosemide-treated human erythrocytes could be due to PKA downregulation.

Besides affecting PKA and thus non-selective cation channels activity cAMP could be directly involved in the anti-eryptotic effect of furosemide. It is known that Raf/MEK/ERK signaling may result in the induction or arrest of cell cycle progression, as well as apoptosis [71]. cAMP has been shown to activate TRPC6 channels via PI3K-PKB-MEK-ERK1/2 signaling pathway [72]. At the same time, activation of RAF/MEK/ERK pathway through RAS by calcium ionophore calcimycin (A23187) was shown to induce apoptosis in ocular lens epithelial cells [73]. Furosemide inhibits RAF/MEK/ERK phosphorylation in Balb/c 3T3 fibroblasts [9]. Thus, in theory, the anti-eryptotic effect of furosemide observed in our study might involve the MAPK pathway.

In conclusion, we show for the first time that the loop diuretic furosemide - applied at micromolar concentrations (10 – 100 μM) - effectively blocks the non-selective cation channels and has an anti-eryptotic effect in human erythrocytes.

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