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Effect of Chloride Channel Inhibitors on Cytosolic Ca^{2+} Levels and Ca^{2+}-Activated K^+ (Gardos) Channel Activity in Human Red Blood Cells

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Abstract DIDS, NPPB, tannic acid (TA) and AO1 are widely used inhibitors of Cl^- channels. Some Cl^- channel inhibitors (NPPB, DIDS, niflumic acid) were shown to affect phosphatidylserine (PS) scrambling and, thus, the life span of human red blood cells (hRBCs). Since a number of publications suggest Ca^{2+} dependence of PS scrambling, we explored whether inhibitors of Cl^- channels (DIDS, NPPB) or of Ca^{2+}-activated Cl^- channels (DIDS, NPPB, TA, AO1) modified intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and activity of Ca^{2+}-activated K^+ (Gardos) channel in hRBCs. According to Fluo-3 fluorescence in flow cytometry, a short treatment (15 min, +37 °C) with Cl^- channels inhibitors decreased $[Ca^{2+}]_i$ in the following order: TA > AO1 > DIDS > NPPB. According to forward scatter, the decrease of $[Ca^{2+}]_i$ was accompanied by a slight but significant increase in cell volume following DIDS, NPPB and AO1 treatments. TA treatment resulted in cell shrinkage. According to whole-cell patch-clamp experiments, TA activated and NPPB and AO1 inhibited Gardos channels. The Cl^- channel blockers further modified the alterations of $[Ca^{2+}]_i$ following ATP depletion (glucose deprivation, iodoacetic acid, 6-inosine), oxidative stress (1 mM t-BHP) and treatment with Ca^{2+} ionophore ionomycin (1 μM). The ability of the Cl^- channel inhibitors to modulate PS scrambling did not correlate with their influence on $[Ca^{2+}]_i$ as TA and AO1 had a particularly strong decreasing effect on $[Ca^{2+}]_i$ but at the same time enhanced PS exposure. In conclusion, Cl^- channel inhibitors affect Gardos channels, influence Ca^{2+} homeostasis and induce PS exposure of hRBCs by Ca^{2+}-independent mechanisms.

Keywords Erythrocyte · DIDS · NPPB · Tannic acid · AO1 · Gardos channel

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Introduction

Cl^- channels can be subdivided into five subclasses as follows: cAMP-, Ca^{2+}-, cell volume-, voltage-activated and ligand-gated. The subtypes differ in electrophysiological and regulatory characteristics as well as their sensitivity to Cl^- channel blockers. The stilbene disulfonate derivative 4,4′-diisothio-cyanatostilbene-2,2-disulfonic acid (DIDS) and the diphenylamine-2-carboxylate derivative 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) are known as universal nonspecific blockers that inhibit Ca^{2+}-activated and volume-regulated Cl^- channels, anion exchangers (DIDS) and K^+-Cl^- cotransporters (NPPB). Gallotannins inhibit Ca^{2+}-activated Cl^- channels (CaCC) (Namkung et al. 2010).

Cl^- channel inhibitors have been shown to influence apoptosis. In cortical neurons Cl^- channel inhibitors (DIDS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid [SITS], NPPB) were shown to prevent apoptotic cell shrinkage and mildly attenuated cell death induced by apoptotic stimuli (stauporine, C2-ceramide, serum deprivation) but had no effect on caspase activation and/or DNA

fragmentation (Wei et al. 2004). In cardiomyocytes volume-sensitive Cl^- channel blockers (IAA-94, DIDS) abolished the apoptotic effects of doxorubicin through PI_3K, Akt and Erk 1/2 (d'Anglemont de Tassigny et al. 2008). H_2O_2-induced apoptosis of cardiomyocytes was inhibited by NPPB and DIDS, which decreased Cl^- channel open probability in those cells (Malekova et al. 2007). In erythroleukemia cell lines NPPB prevented cisplatin-induced apoptosis, inducing Cl^- channel (CIC-3) expression (Xu et al. 2011). Gallotannins are known to attenuate apoptosis by inhibition of CaCCs (Namkung et al. 2010). In addition, tannins were shown to inhibit a number of eukaryote protein kinases: cAMP, cAK, CDPK, PKC, MLCK, MAPK and epidermal growth factor tyrosine kinase (Wang et al. 1996; Polya et al. 1995; Yang et al. 2006).

Similar to nucleated cells, mature human red blood cells (hRBCs) are equipped with a set of different Cl^- channels that are mostly silent and nondetectable in patch-clamp experiments of untreated and noninfected hRBCs (Dyrda et al. 2010; Huber et al. 2005). In hRBCs, DIDS inhibits the Cl^- conductive pathway mediated by capnophorin (band 3 protein) (Freedman et al. 1994), nonselective cation conductance sensitive to Cl^- removal (Huber et al. 2001; Duranton et al. 2002) and shear-induced K^+, Na^+ and Ca^{2+} permeability (Johnson and Tang 1993). Tannic acid (TA) inhibits anion and nonelectrolyte (glycerol and erythritol) permeability with an increase of erythrocyte fragility (Hunter 1960). NPPB inhibits anion and cation conductance via new permeability pathways in *Plasmodium* parasitized hRBCs (Duranton et al. 2003).

Similar to apoptosis of nucleated cells, hRBCs may enter suicidal cell death or eryptosis, which is characterized by phosphatidylserine (PS) exposure, cell shrinkage and membrane blebbing (Lang et al. 2008; Aiken et al. 1992; Nguyen et al. 2011). Eryptosis is triggered by an increase of $[Ca^{2+}]_i$ (Lang and Qadri 2012). Eryptosis is stimulated by a large number of small molecules (Abed et al. 2012a, b; Bottger et al. 2012; Felder et al. 2011; Firat et al. 2012; Ganesan et al. 2012; Gao et al. 2012; Ghashghaeinia et al. 2011, 2012; Jilani et al. 2012, 2013; Kucherenko and Lang 2012; Lang et al. 2011, 2012a, b; Lang and Qadri 2012; Lupescu et al. 2012a, b, c; Polak-Jonkisz and Purzyc 2012; Qadri et al. 2011a, b, c; Qian et al. 2012; Shaik et al. 2012a, b; Vota et al. 2012; Weiss et al. 2012; Zappulla 2008; Zbidah et al. 2012a, b; Zelenak et al. 2012b).

The rate of suicidal erythrocyte death is sensitive to cellular K^+ content and cell volume (Lang et al. 2003; Schneider et al. 2007). Accordingly, eryptosis of oxidized and ionomycin-treated hRBCs is inhibited by the Cl^- channel inhibitors NPPB and niflumic acid (Myssina et al. 2004).

The present study explored the impact of Cl^- channel inhibitors on Ca^{2+} homeostasis, Gardos channel activity and PS exposure in hRBCs.

Materials and Methods

Red Blood Cells

Banked hRBC concentrates were provided by the blood bank of the University of Tübingen. Cells were washed twice (1,200×*g*, 5 min, 22 °C) with NaCl Ringer bath solution containing (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). Metabolically depleted cells were obtained according to the method of Lew (1971) by incubation of control cells (>3 h, +37 °C) in ATP-depleting medium consisting of (mM) 140 NaCl, 5 KCl, 10 HEPES, 6 iodoacetic acid, 6 inosine. Oxidative stress was induced by 1 mM *tert*-butyl-hydroperoxide (*t*-BHP) treatment (15 min, +37 °C). The Ca^{2+} ionophore ionomycin (1 μM; 15 min, +37 °C) was applied to enhance $[Ca^{2+}]_i$ independently of the cell Ca^{2+}-permeable channel activity. Cl^- channel inhibitors (10 μM DIDS, 100 μM NPPB, 10 μM TA, 20 μM AO1) were added to control cells or to hRBCs treated by oxidation, ATP depletion or ionomycin.

Electrophysiology

Patch electrodes were made of borosilicate glass capillaries (150 TF-10; Clark Medical Instruments, Lacey Green, UK) using a horizontal DMZ puller (Zeitz, Martinsried, Germany). Pipettes with high resistance from 17 to 20 MOhm were connected via an Ag–AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech, Longmont, CO) and using Pulse software (HEKA). For current measurements, RBCs were held at a holding potential (V_h) of −10 mV; and 11 pulses of 200 ms duration (from −100 to +100 mV) were applied with +20 mV increments. Currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (current–voltage relationship) and recorded at 22 °C in fast whole-cell, voltage-clamp mode, with a 3 kHz low-pass filter. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solutions and between 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. After >10 GOhm seal formation, the membrane was ruptured by additional suction.

For whole-cell recording, the pipette solutions consisted of (in mM) 145 KCl, 1.2 $MgCl_2$, 1.15 $CaCl_2$, 10 HEPES, 1 EGTA, 2 Na_2ATP (pH 7.4 with KOH) or 120 K-gluconate,

5 KCl, 1.2 $MgCl_2$, 2 MgATP, 2 EGTA, 2.05 $CaCl_2$, 10 HEPES (pH 7.4 with KOH). 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 gluconate pipette solution was combined with gluconate bath solution, the latter contained (in mM) 150 Na-gluconate, 5 mM K-gluconate, 5 glucose, 10 HEPES/NaOH (pH 7.4).

Cells with pronounced stable activity of Gardos channels were acutely perfused with the bath solutions containing Cl^- channel inhibitors (10 μM DIDS, 100 μM NPPB, 10 μM TA, 20 μM AO1, final concentrations). At the end of each experiment the SK4 channel blocker TRAM-34 (1 μM) was added.

Chemicals (NPPB, DIDS, TA, TRAM-34) were obtained from Sigma (Taufkirchen, Germany) and were of the highest grade available. AO1 (De La Fuente et al. 2008) was a kind gift of Dr. Karl Kunzelmann (University of Regensburg, Regensburg, Germany). DMSO was used as a solvent for AO1, NPPB and TRAM-34. TA and DIDS were dissolved in deionized, purified water.

Intracellular Ca^{2+} and Forward Scatter

Experiments were performed with native, ATP-depleted, t-BHP-oxidized or ionomycin-treated hRBCs (see above) in the presence or absence of the Cl^- channel inhibitors. Cells (0.4 % suspension) were washed in the appropriate media (with or without the corresponding Cl^- channel inhibitor) containing 5 mM $CaCl_2$ and loaded with 2 μM Fluo-3/AM (Calbiochem, Bad Soden, Germany). Then, cells were incubated at 37 °C for 20 min, washed once and resuspended in 5 mM Ca^{2+}-containing Ringer/ATP-depleting bath medium (with or without the corresponding Cl^- channel inhibitor). Cells were analyzed by forward scatter, and Fluo-3 intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

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 Ca^{2+}-containing Ringer/ATP depleting bath medium in the presence or absence of 10 μM TA or 20 μM AO1. After 20 min, samples were washed once and resuspended in 5 mM Ca^{2+}-containing Ringer/ATP depleting medium (with or without 10 μM TA and 20 μM AO1) 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.

Statistics

Data are expressed as arithmetic means ± SEM, and the paired two-tailed t test was employed as appropriate, with $p < 0.05$ considered statistically significant.

Results

In the present study we tested the most widely used Cl^- channel inhibitors (DIDS, NPPB) and CaCC inhibitors (DIDS, NPPB, TA, AO1). We first checked whether a short treatment with Cl^- channel inhibitors affected Ca^{2+} homeostasis in hRBCs. Exposure of the cells for 15 min to 10 μM DIDS, 100 μM NPPB, 10 μM TA or 20 μM AO1 resulted in a decrease of cytosolic Ca^{2+} ($[Ca^{2+}]_i$). The potency of the Cl^- channel inhibitors for $[Ca^{2+}]_i$ reduction was TA > AO1 > DIDS > NPPB (Fig. 1a). A decrease in $[Ca^{2+}]_i$ (observed for AO1-, DIDS- and NPPB-treated cells) was accompanied by a slight (~5 %) but significant increase of cell volume, pointing to a Ca^{2+}-dependent decline of basal Gardos channel activity. In contrast, volume decreased following treatment with TA, suggesting activation of the Gardos channel even though TA treatment resulted in an ~50 % decrease of $[Ca^{2+}]_i$ compared to untreated hRBCs (Fig. 1b).

Whole-cell patch-clamp experiments were performed to explore the effect of the Cl^- channel blockers on the Gardos channel activity. Since the basal activity of the Gardos channels in untreated hRBCs is low due to low $[Ca^{2+}]_i$, we added $CaCl_2$ to K^+-containing pipette bath solutions. As shown in Fig. 2a, b, the outward current due to K^+ efflux via Ca^{2+}-activated K^+ channels was increased in the cells loaded with Ca^{2+} (31 μM intracellular free Ca^{2+}) upon acute exposure to 10 μM TA. The same effect was observed when intra- and extracellular Cl^- was substituted for gluconate. Perfusion of the cells with the SK4 channel inhibitor TRAM-34, followed by TA treatment, blocked the outward current (Fig. 2c, d). Thus, we conclude that short exposure of hRBCs to 10 μM TA induced an increase (~30 and ~96 % for Cl^- and gluconate bath media, respectively) in Gardos channel activity.

Acute 10 μM DIDS (dissolved in Cl^--containing bath medium) was followed by an increase (up to ~35 %) in Gardos channel activity (Fig. 3a, b). However, no effect of DIDS was observed when Cl^- was eliminated from the bath media (Fig. 3c, d). In contrast, NPPB and AO1 rapidly inhibited the basal Gardos channel activity recorded in hRBCs in both Cl^- and gluconate Ca^{2+}-containing pipette solutions (Figs. 4, 5).

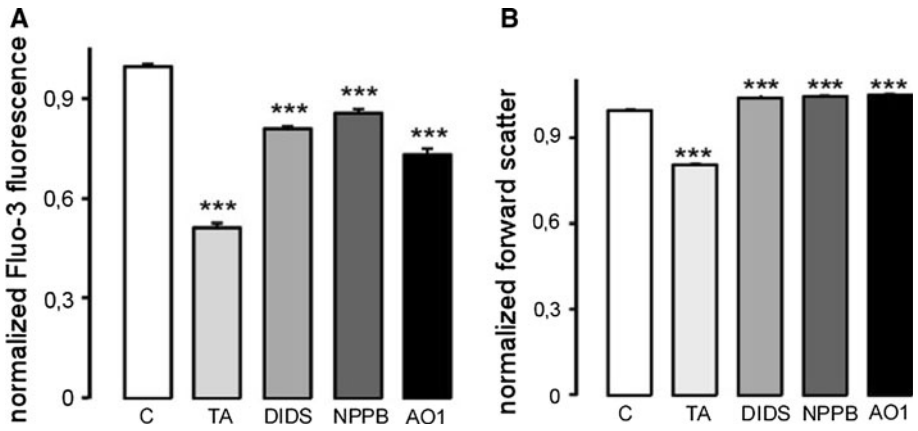

Fig. 1 Short exposure to Cl^- channel inhibitors reduces $[Ca^{2+}]_i$ and affects hRBC volume. **a** Arithmetic means ± SEM of normalized Fluo-3 fluorescence, reflecting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in hRBCs (*white bar*, $n = 11$) exposed for a short time (15 min) to 10 μM tannic acid (*TA*; *light gray bar*, $n = 6$), 10 μM DIDS (*gray bar*, $n = 6$), 100 μM NPPB (*dark gray bar*, $n = 11$) and 20 μM AO1 (*black bar*, $n = 10$). ***Significant difference from control ($p < 0.001$, *t* test). **b** As in **a** for normalized forward scatter that reflects hRBC volume changes

The next series of experiments explored whether the Cl^- channel inhibitors affected $[Ca^{2+}]_i$ in metabolically depleted (ATP-depleted), oxidized (t-BHP) and Ca^{2+} ionophore (ionomycin)-treated hRBCs. As shown in Fig. 6a, Cl^- channel inhibitors (TA, NPPB, AO1) significantly blunted Ca^{2+} uptake in hRBCs induced by ATP depletion. The most effective inhibitors (TA, NPPB) blocked Ca^{2+} influx and reduced $[Ca^{2+}]_i$ to levels lower than those observed in untreated hRBCs. In contrast, DIDS enhanced Ca^{2+} uptake in hRBCs.

TA, DIDS and AO1 exerted a significant antioxidative effect, blunting Ca^{2+} influx induced by 1 mM t-BHP treatment. DIDS was the most effective antioxidant among the Cl^- channel inhibitors tested (Fig. 6b).

The Cl^- channel inhibitors further reduced the ionomycin-induced increase in hRBCs $[Ca^{2+}]_i$ (Fig. 6c). TA was the most effective inhibitor of Ca^{2+} entry in ionomycin-treated hRBCs and decreased Ca^{2+} uptake by more than 80 %. DIDS (~1 % blockage) was almost ineffective.

No correlation was found between the ability of Cl^- channel blockers to decrease Ca^{2+} entry and to counteract PS scrambling in hRBCs. AO1, which was highly effective at decreasing $[Ca^{2+}]_i$ and blocking Gardos channel activity, actually enhanced PS exposure in hRBCs (Fig. 7a). TA, which was most effective at reducing $[Ca^{2+}]_i$ and increasing Gardos channel activity, dramatically (~15-fold) increased Annexin-V binding in hRBCs (Fig. 7b).

Discussion

Cl^- channel inhibitors (DIDS, SITS, NPPB) were shown to prevent apoptotic cell shrinkage and attenuate cell death induced by apoptotic stimuli in cortical neurons and cardiomyocytes (Wei et al. 2004; d'Anglemont de Tassigny et al. 2008). Under the conditions of increased $[Ca^{2+}]_i$ (oxidation, Ca^{2+} ionophore treatment) NPPB and niflumic acid blunted PS scrambling and extended the life span of hRBCs (Myssina et al. 2004). Since PS scrambling is regulated by $[Ca^{2+}]_i$, we assumed that the antiapoptotic effect of NPPB and niflumic acid was due to modulation of intracellular Ca^{2+} activity induced by Cl^- channel inhibitors.

In the present study we show that the commonly used Cl^- blockers (DIDS, NPPB, TA, AO1) reduced $[Ca^{2+}]_i$ after a short treatment in physiological saline and blunted Ca^{2+} uptake in ATP-depleted, oxidized and ionomycin-treated cells. Interestingly, the efficiency of the blockers for $[Ca^{2+}]_i$ reduction varied. TA was most effective at decreasing hRBC $[Ca^{2+}]_i$ upon acute application, ATP depletion and ionomycin treatment, whereas DIDS displayed the highest protection against oxidation. In contrast to the other Cl^- channel blockers tested, DIDS increased Ca^{2+} uptake in metabolically depleted cells. Our results are reminiscent of earlier observations (Diakov et al. 2001) that DIDS activates two types of endogenous cation conductance in the cell membrane of *Xenopus laevis* oocytes.

Modulation of hRBC $[Ca^{2+}]_i$ was not the only effect of the Cl^- channel blockers that might affect the erythrocyte life span. We found that the blockers, acutely added to the cells, modified the activity of the Gardos channels in hRBCs. AO1, which was the second most effective at decreasing $[Ca^{2+}]_i$, inhibited Gardos channel activity. In contrast, TA, which again effectively reduced $[Ca^{2+}]_i$, enhanced Gardos channel activity.

The tested Cl^- channel blockers belong to different chemical classes (Fig. 8) and may, in addition to Cl^- channel inhibition, exert different effects on hRBCs. Possibly, the blockers affect $[Ca^{2+}]_i$ and Gardos channel

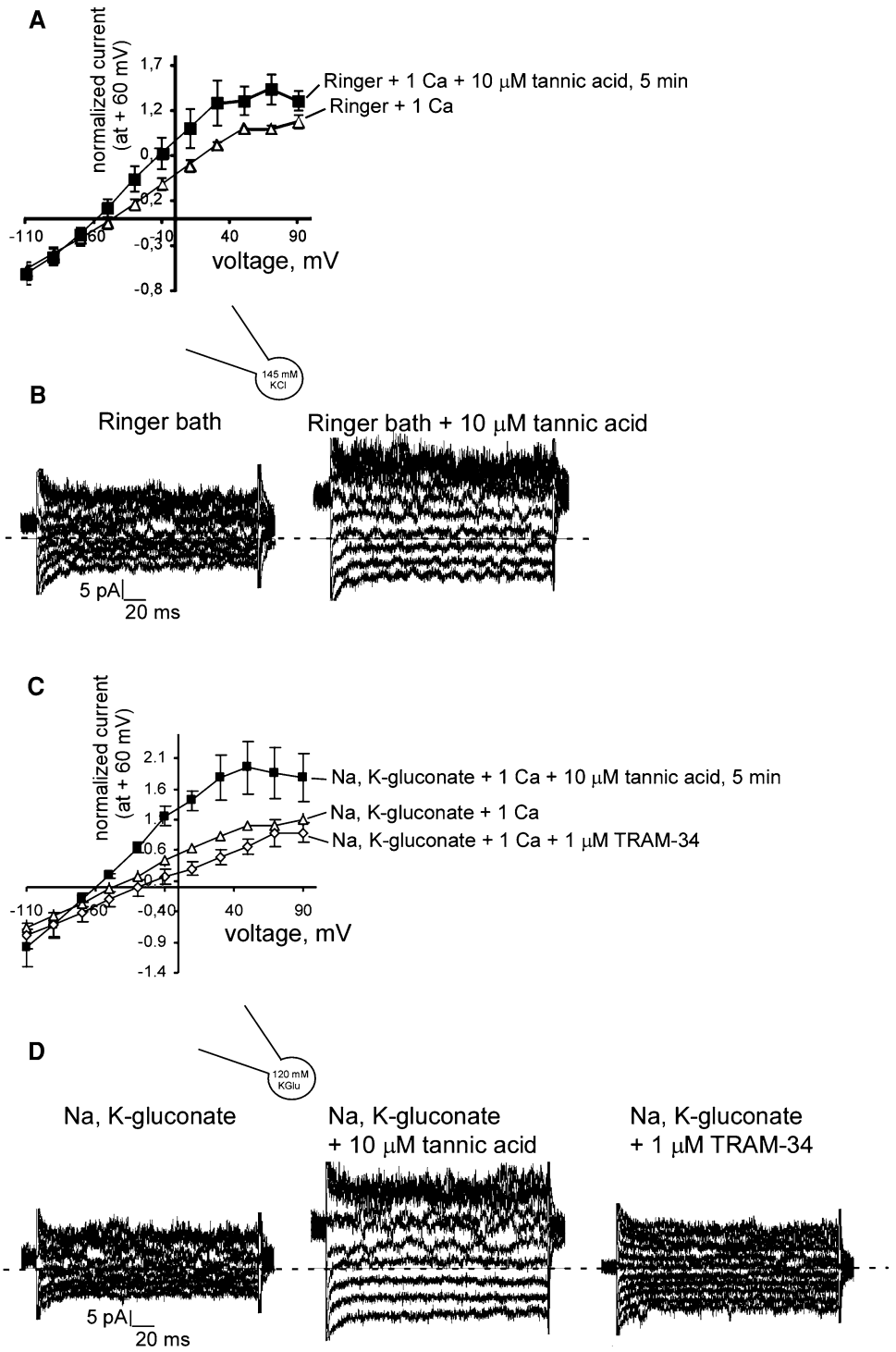

Fig. 2 Tannic acid (TA) activates the Gardos channel in hRBCs. **a** Normalized ($I_{60\ mV}$) current–voltage (*I–V*) relationships of control hRBCs, recorded with KCl pipette solution, in NaCl-containing Ringer bath solution before (*open triangles*, $n = 4$) and after (*closed squares*, $n = 4$) acute application of 10 μM TA, 5 min incubation. **b** Whole-cell patch-clamp recordings of the Gardos channel activity of hRBCs, recorded with KCl pipette solution, in NaCl-containing bath solution at the beginning and after acute application of 10 μM TA, 5 min incubation. **c** Normalized ($I_{60\ mV}$) *I–V* relationships of control hRBCs, recorded with K-gluconate pipette solution, in Na-gluconate bath solution before (*open triangles*, $n = 3$), after acute 5 min incubation with 10 μM TA (*closed squares*, $n = 3$) and after acute 1-μM TRAM-34 application. **d** Whole-cell patch-clamp recordings of the Gardos channel activity of cells, recorded with K-gluconate pipette solution, in Na-gluconate bath solution at the beginning, after acute 5 min incubation with 10 μM TA and after acute 1 μM TRAM-34 application

activity in hRBCs via modulating the activity of intracellular enzymes. TA was shown to inhibit rat liver cAMP and PKC kinases (Polya et al. 1995; Radcliffe et al. 1971) at concentrations lower than those used for inhibition of CaCCs (Namkung et al. 2010). The Gardos channels in hRBCs are the hSK4 (KCNN4) isoform of the small-conductance Ca^{2+}-activated K^+ channel (Hoffman et al. 2003). The channel activity is known to be modulated by cAMP and PKA (Pellegrino and Pellegrini 1998) and by PKC (Del Carlo et al. 2003; Rivera et al. 2002). Thus, the cell shrinkage and enhanced PS exposure observed in TA-treated hRBCs may be, at least in theory, a result of

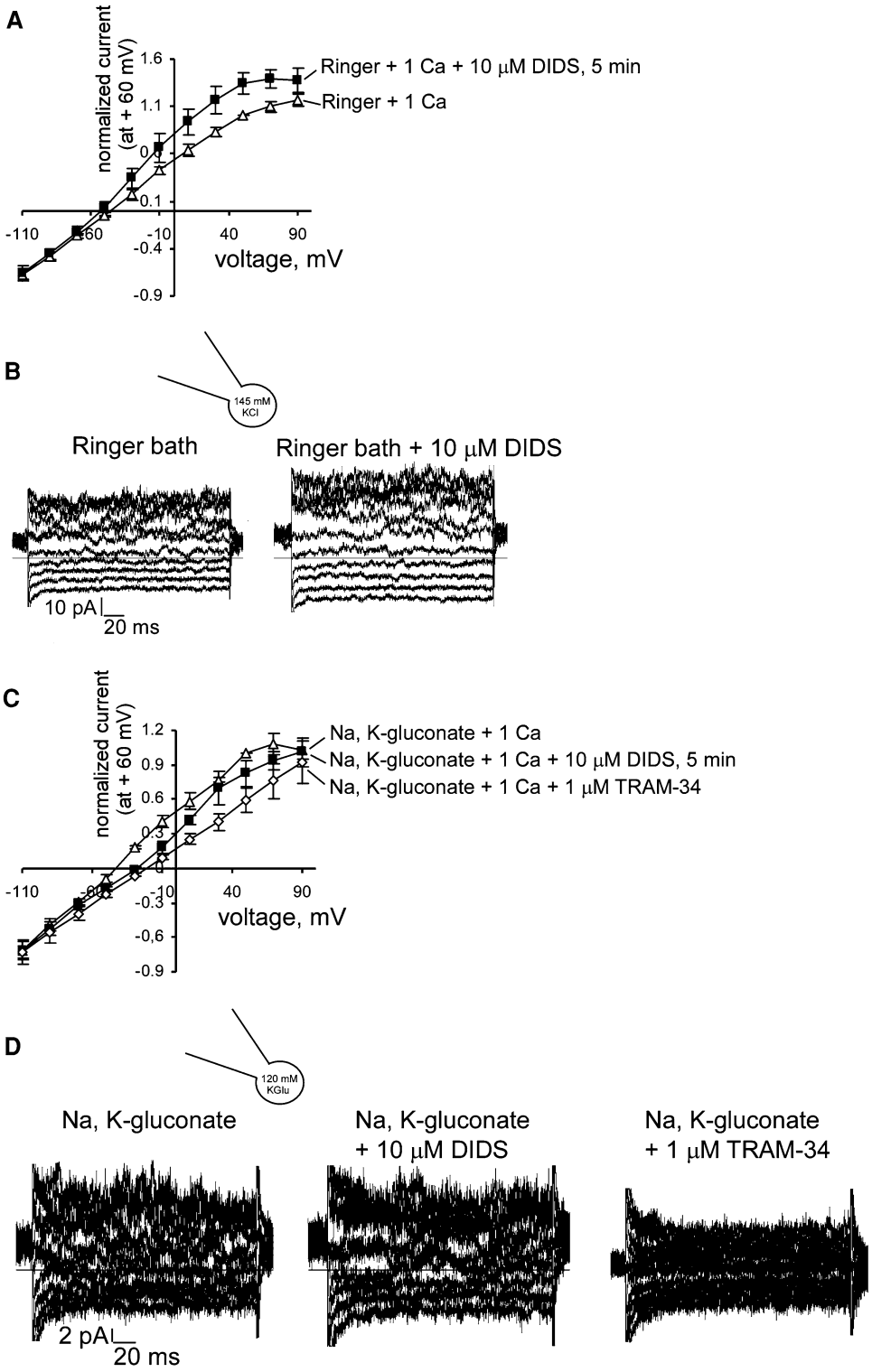

Fig. 3 DIDS does affect the Gardos channel activity in hRBCs. **a**, **b** As in Fig. 2a, b for 10 μM DIDS ($n = 5$). **c**, **d** As in Fig. 2c, d for 10 μM DIDS ($n = 4$)

Ca^{2+}-independent activation of the Gardos channels due to PKC/cAMP modulation by TA.

PKC and PKA are also known to regulate nonselective cation channel activity (Del Carlo et al. 2003; Saleh et al. 2009; Meves 2006). Activation of the voltage-independent, nonselective, Ca^{2+}-permeable, Cl^--sensitive cation channels (Huber et al. 2001) may trigger PS exposure in hRBCs (Lang et al. 2008). Removal of Cl^- from the bath medium (substitution with gluconate) increases $[Ca^{2+}]_i$ and enhances PS scrambling in hRBCs

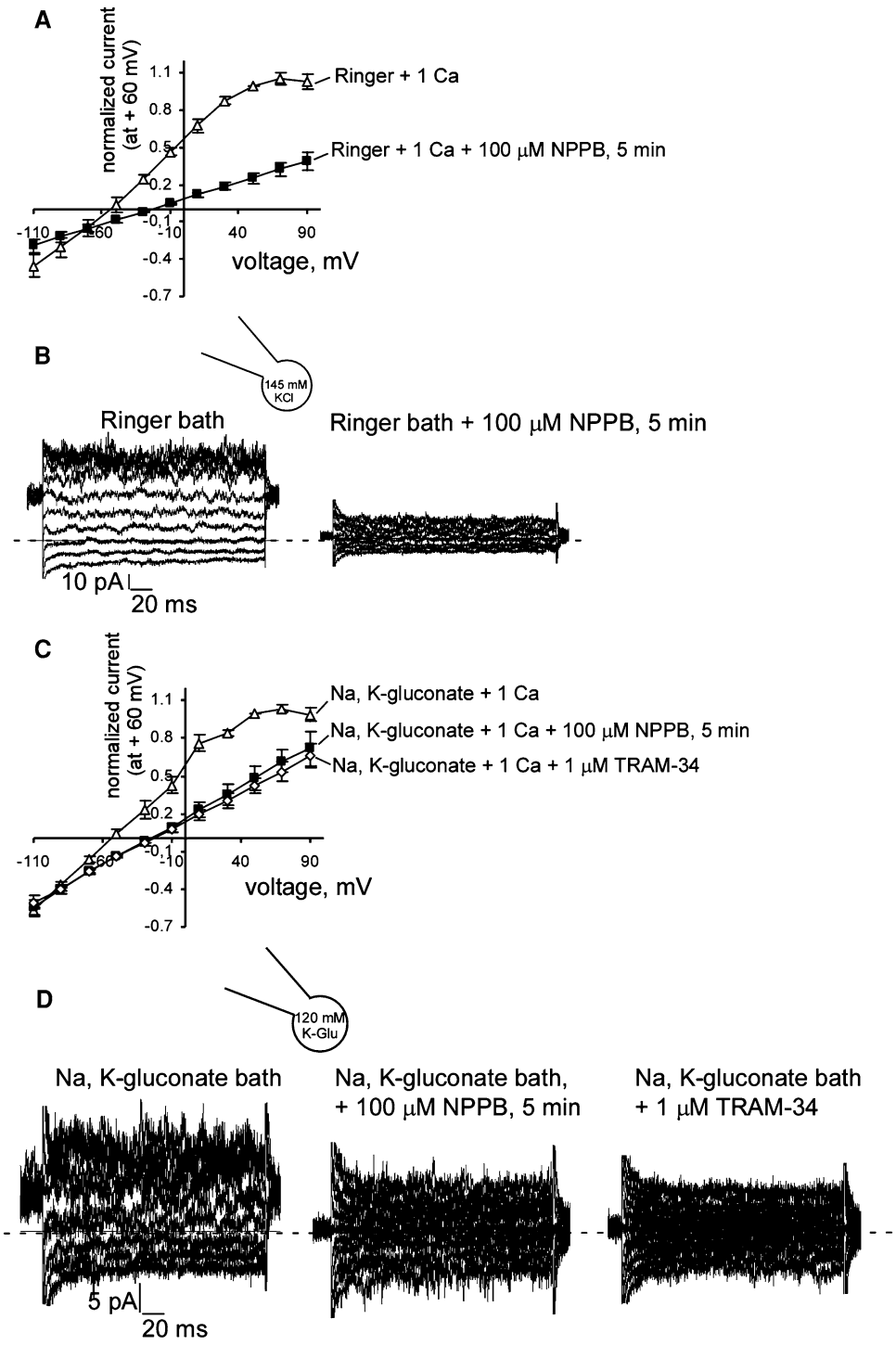

Fig. 4 Acute NPPB application blocks the Gardos channel in hRBCs. **a**, **b** As in Fig. 2a, b for 100 μM NPPB ($n = 4$). **c**, **d** As in Fig. 2c, d for 100 μM NPPB ($n = 3$)

(Lang et al. 2003, 2008; Schneider et al. 2007). Thus, in theory, blockage of Cl^- fluxes could result in nonselective cation channel activation and $[Ca^{2+}]_i$ increase. However, additional mechanisms must be taken into consideration, such as impaired Ca^{2+} extrusion and increased intracellular Ca^{2+} binding (e.g. calmodulin-dependent).

It should be further considered that Ca^{2+} sensitivity of PS scrambling could be modified by ceramide (Lang et al. 2010). Moreover, eryptosis is sensitive to a variety of kinases including AMP-activated kinase AMPK (Foller et al. 2009), cGMP-dependent protein kinase (Foller et al. 2008), Janus-activated kinase JAK3 (Bhavsar et al. 2011), casein kinase (Kucherenko et al. 2012; Zelenak et al. 2012a), p38 kinase

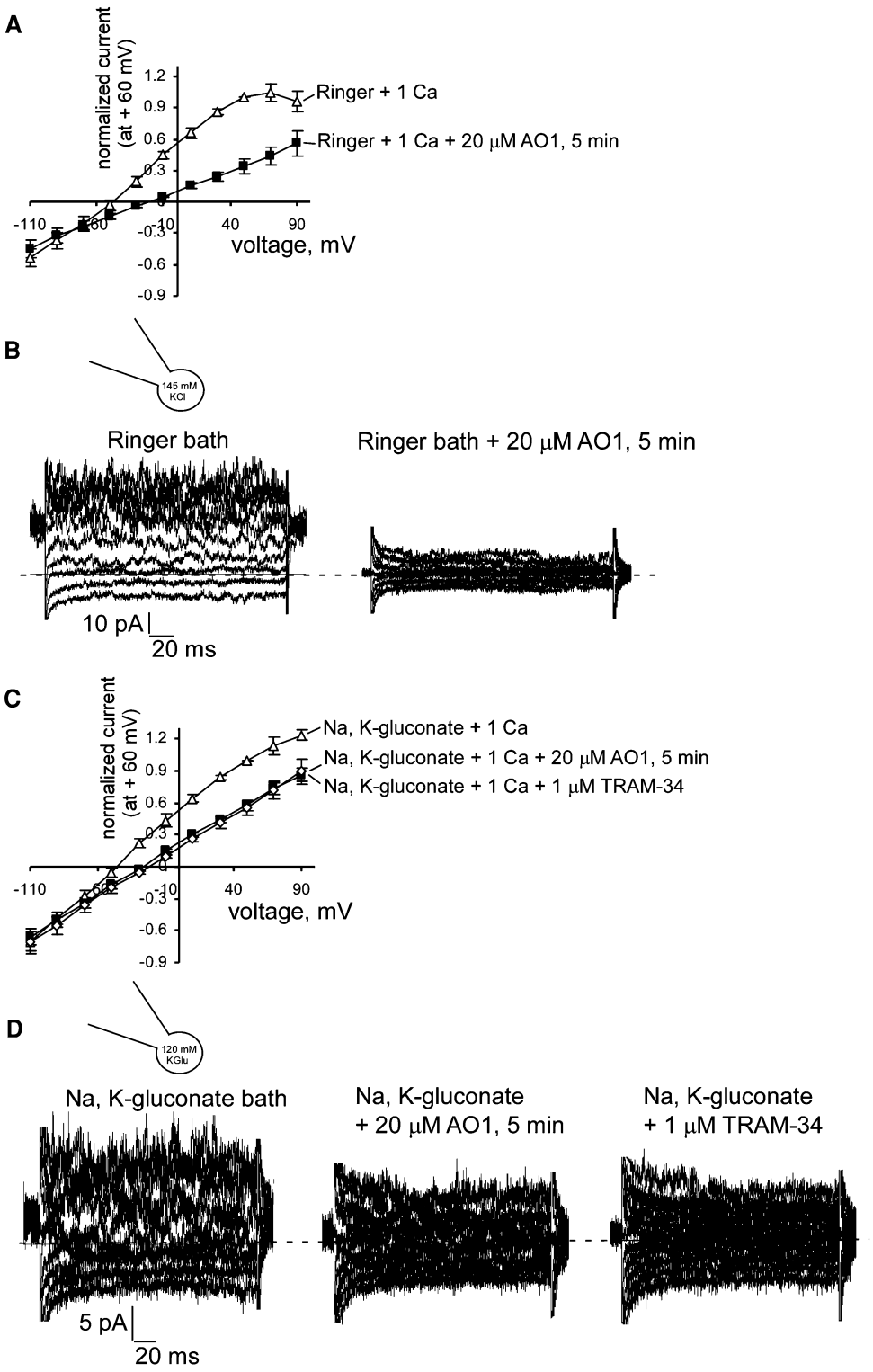

Fig. 5 AO1 inhibits the Gardos channel in hRBCs. **a**, **b** As in Fig. 2a, b for 20 μM AO1 ($n = 5$). **c**, **d** As in Fig. 2c, d for 20 μM AO1 ($n = 6$)

(Gatidis et al. 2011), PAK2 kinase (Zelenak et al. 2011) as well as sorafenib- (Lupescu et al. 2012d) and sunifinib- (Shaik et al. 2012a) sensitive kinases.

Eryptosis participates in the pathophysiology of several clinical conditions (Lang et al. 2008) including diabetes mellitus (Calderon-Salinas et al. 2011; Maellaro et al. 2011), renal insufficiency (Myssina et al. 2003), phosphate depletion (Birka et al. 2004), hemolytic uremic syndrome (Lang et al. 2006), sepsis (Kempe et al. 2007), fever (Foller et al. 2010), malaria (Bobbala et al. 2010; Lang et al. 2009; Siraskar et al. 2010), sickle cell disease (Lang et al. 2009), iron deficiency (Kempe et al. 2006), Wilson's disease

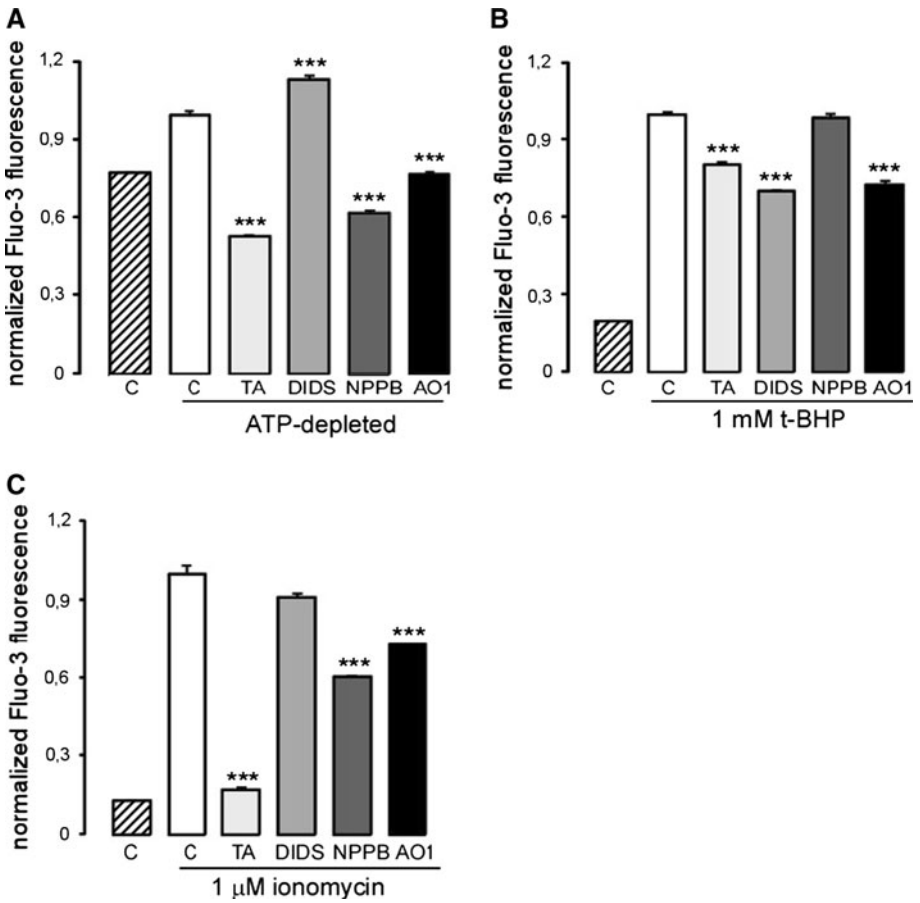

Fig. 6 Effect of Cl^- channel inhibitors on $[Ca^{2+}]_i$ in metabolically ATP-depleted, oxidized and ionomycin-treated hRBCs. **a** Arithmetic means ± SEM of normalized Fluo-3 fluorescence of control cells after 3- to 7-h incubation with ATP-depletion medium (6 mM inosine, 6 mM iodoacetic acid) in the absence (*white bar*, $n = 12$) and presence (*light gray bar*, $n = 8$) of 10 μM tannic acid, 10 μM DIDS (*gray bar*, $n = 8$), 100 μM NPPB (*dark gray bar*, $n = 8$) and 20 μM AO1 (*black bar*, $n = 7$). Control untreated cells are shown as *hatched bar* ($n = 12$). ***Significant difference from ATP-depleted cells in the absence of inhibitors ($p < 0.001$). **b** Arithmetic means ± SEM of normalized Fluo-3 fluorescence of 1 mM t-BHP (15 min, 37 °C) oxidized hRBCs in the absence (*white bar*, $n = 6$) and presence (*light gray bar*, $n = 6$) of 10 μM tannic acid, 10 μM DIDS (gray bar, $n = 6$), 100 μM NPPB (*dark gray bar*, $n = 6$) and 20 μM AO1 (*black bar*, $n = 7$). Control untreated cells are shown as *hatched bar* ($n = 6$). ***Significant difference from t-BHP oxidized hRBCs ($p < 0.001$). **c** Arithmetic means ± SEM of normalized Fluo-3 fluorescence of 1 μM ionomycin (15 min, 37 °C)−treated hRBCs in the absence (*white bar*, $n = 4$) and presence (*light gray bar*, $n = 4$) of 10 μM tannic acid, 10 μM DIDS (*gray bar*, $n = 4$), 100 μM NPPB (*dark gray bar*, $n = 4$) and 20 μM AO1 (*black bar*, $n = 4$). Control untreated cells are shown as *hatched bar* ($n = 4$). ***Significant difference from ionomycin-treated hRBCs in the absence of inhibitors ($p < 0.001$)

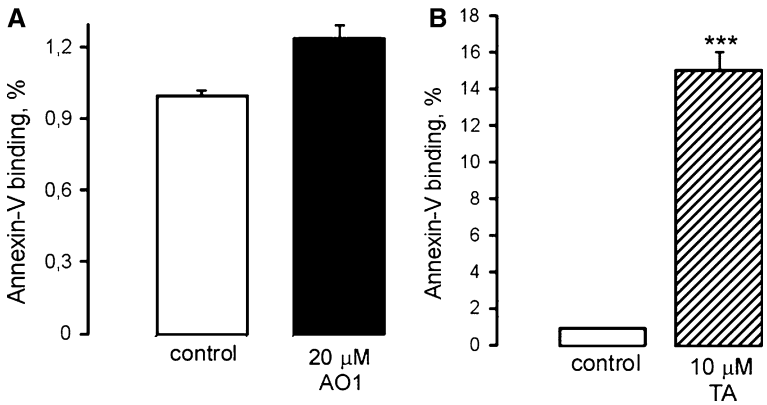

Fig. 7 Effect of tannic acid (TA) and AO1 on phosphatidylserine exposure in hRBCs. **a** Arithmetic means ± SEM of phosphatidylserine exposure, measured as Annexin-V binding, of hRBCs in the absence of AO1 (*white bar*, $n = 6$) and after a short treatment (15 min, 37 °C) with 20 μM AO1 (*black bar*, $n = 6$). **b** As in **a** for 10 μM TA–treated cells (*striped bar*, $n = 3$). ***Significant difference from control hRBCs ($p < 0.001$)

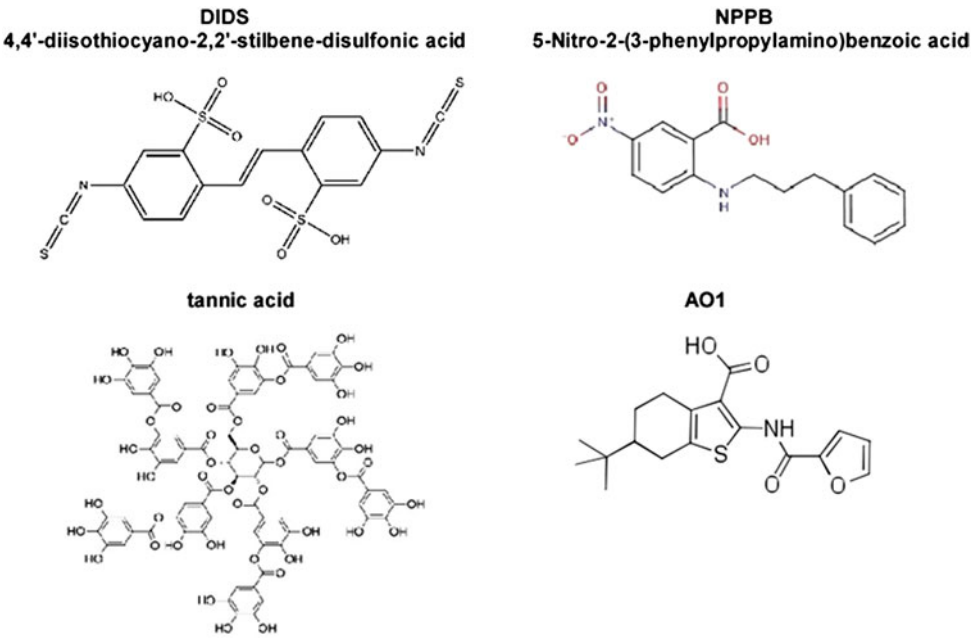

Fig. 8 Chemical structures of the Cl^- channel inhibitors used in the study

(Lang et al. 2007) and possibly metabolic syndrome (Zappulla 2008).

In conclusion, all tested Cl^-- and Ca^{2+}-activated Cl^- channel blockers (DIDS, NPPB, TA, AO1) in the concentrations used for Cl^- channel inhibition affect Gardos channel activity, influence Ca^{2+} homeostasis and induce PS exposure by a Ca^{2+}-independent mechanism in hRBCs. Thus, none of them could be referred to a specific Cl^- channel blocker, and their side effects should be considered when used for Cl^--transport studies or when these or related substances are used in therapy.

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Conflict of interest We report no conflict of interest.

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