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Tannins, novel inhibitors of the volume regulation and the volume-sensitive anion channel

Original Paper

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Abstract The volume-sensitive outwardly rectifying anion channel (VSOR) is a key component of volume regulation system critical for cell survival in non-isosmotic conditions. The aim of the present study was to test the effects of four tannin extracts with defined compositions on cell volume regulation and VSOR. Preparation I (98% of hydrolysable tannins isolated from leaves of sumac *Rhus typhina L.*) and Preparation II (100% of hydrolysable tannins isolated from leaves of broadleaf plantain *Plantago major L*) completely and irreversibly abolished swelling-activated VSOR currents in HCT116 cells. Both preparations profoundly suppressed the volume regulation in thymocytes with half-maximal effects of 40.9 μg/ml and 12.3 μg/ml, respectively. The inhibition was more efficient at lower concentrations but reverted at higher doses due to possible non-specific membrane-permeabilizing activity. Preparations III and IV (54,7% and 54.3% of hydrolysable tannins isolated, respectively, from roots and aboveground parts of Fergana spurge *Euphorbia ferganensis B.Fedtch*) inhibited VSOR activity in a partially reversible manner and suppressed the volume regulation with substantially higher half-maximal doses of 270 and 278 μg/ml, respectively, with no secondary reversion at higher doses. Hydrolysable tannins represent a novel class of VSOR channel inhibitors with the capacity to suppress the cell volume regulation machinery.

Keywords *Tannins – plant polyphenols – thymocytes – cell volume regulation – volume-sensitive anion channel*

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INTRODUCTION

In order to survive in constantly changing osmotic conditions caused by intensive physiological processes (breathing, food intake, fluid secretion and absorption, filtration and urine formation, etc.) and pathologies (inflammation, edema, trauma, ischemia and hypoxia), living cells developed elaborate volume regulatory mechanisms. The volume-sensitive outwardly rectifying anion channel (VSOR) is a key component of the cellular volume regulation system and of some other physiological and pathophysiological processes including cell proliferation, migration and apoptosis (Akita & Okada, 2014; Hoffmann et al., 2014; Okada et al., 2009). VSOR is the main pathway for the efflux of anions from swollen cells and, in cooperation with the Ca-activated potassium channels, provides a reduction of the intracellular osmotic pressure during the active phase of volume regulation upon the hypoosmotic stress called the Regulatory Volume Decrease (RVD) (Akita et al., 2011; Akita & Okada, 2014; Delpire & Gagnon, 2018; Hoffmann et al., 2014; Okada et al., 2006; Pedersen et al., 2016). Recent studies have demonstrated that the LRRC8 family proteins constitute the molecular

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basis of VSOR (Qiu et al., 2014; Voss et al., 2014), structurally organized into a hexameric pore (Deneka et al., 2018; Kasuya et al., 2018; Kefauver et al., 2018). However, pharmacology of this biologically important ion channel remains poorly explored. Thus far, a number of structurally divers compounds including stilbene derivatives, etacrynic acid analogs and flavonoids have been shown to suppress the activity of VSOR in a voltage-dependent and independent manner (Okada et al., 2019; Xue et al., 2018).

Tannins are structurally heterogenous polyphenols, which bind to proteins and can trigger their precipitation (Mavlyanov et al., 2001). Tannins are secondary metabolites and constitute a part of the plants' defense system against pathogens and insect's invasion. In addition, these substances exhibit a wide spectrum of biological activities such as antimicrobial (Scalbert, 1991); antioxidant, (Rice-Evans et al., 1995, 1996); anti-inflammatory (Terra et al., 2007; Xue et al., 2018); neuroprotective (Behravan et al., 2014). Tannins, along with other polyphenolic compounds, have been considered to be responsible for the health benefits of red wine and green tea, possibly by inhibiting the activity of the Ca-activated chloride channels (CaCCs) (Namkung et al., 2010). Penta-m-digalloyl-glucose, a hydrolysable tannin extracted from the Chinese gallnut, was demonstrated to inhibit the cystic fibrosis transmembrane conductance regulator protein (CFTR), a chloride channel activated by intracellular cAMP (Wongsamitkul et al., 2010). Tannic acid was shown to inhibit CaCCs formed by TMEM16A and B (Cruz-Rangel et al., 2015; Namkung et al., 2010, 2011) and TMEM16F (Szteyn et al., 2012), which is consistent with an antidiarrhoeic activity of tannin-containing extracts reported earlier (Galvez et al., 1991). Tannic acid also blocks L-type Ca-channels (Zhu et al., 2016) and the maxi-anion channel (Woll et al., 1987). The latter is known to be swelling-activated (Okada et al., 2018, 2019; Sabirov & Merzlyak, 2012; Sabirov et al., 2016), although it operates mostly when cells are metabolically deprived, whereas VSOR is the major contributor to the swelling-activated plasmalemmal conductance at normal intracellular ATP levels. Tannins were never considered as modulators of the cell volume regulation system and its constituent components. Here, we tested four tannin preparations of plant origin on their effects on the cell volume regulation in rat thymocytes and the VSOR channel activity in HCT116 human colon cancer cells.

MATERIALS AND METHODS

Substances

The plants were collected from the Tashkent environs during the flowering stage and taxonomically identified by Dr. Gnatchenko E.V. of the Institute of Botany of the Academy of Sciences of Uzbekistan. Preparation of tannin extracts was performed essentially as described previously (Islambekov et al., 1994; Olchowik-Grabarek et al., 2017) and their composition was determined by a combination of preparative column chromatography (silica gel, polyamide), quantitative paper chromatography and spectral methods as described elsewhere (Abdulladzhanova et al., 2001).

Preparation I was obtained from the leaves of sumac (*Rhus typhina* L.) and contained: 3,6-bis-*O*-di-*O*-galloyl-1,2,4-tri-*O*-galloyl-β-D-glucose (74%); 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (10%); 1,4,6-tri-*O*-galloyl-β-D-glucose (5%); 2,3-di-*O*-galloyl-β-D-glucose (2%); 2-*O*-galloyl-β-D-glucose (2%); 3-*O*-galloyl-β-D-glucose (2%); 6-*O*-galloyl-β-D-glucose (2%); gallic acid (1%); rutin (1%); quercetin (0.5%); kaempferol (0.5%).

Preparation II was obtained from the leaves of broadleaf plantain (*Plantago major* L.) and contained: diester of hexahydroxydiphenoyl-1-(*O*-2-*O*-galloyl-β-D-glucopyranosido)-1-(*O*-β-D-xylopyranoside (30.1%); diester of hexahydroxydiphenoyl-1-(*O*-β-D-glucopyranosido)-2-(*O*-4-*O*-galloyl-β-D-glucopyranoside) (27.9%); quercetin-3-*O*-(2″, 6″di-*O*-galloyl-3″-*O*-*p*-coumaroyl)-β-D-glucopyranoside (25.4%); kaempferol-3-*O*-(2″,3″-di-*O*-galloyl-6″-*O*- coumaroyl-β-D-glucopyranoside (16.6%).

Preparation III was obtained from the roots of Fergana spurge (*Euphorbia ferganensis B.Fedtch*.) and contained: 1-*O*-galloyl-2,4-valoneoyl-4,6-hexahydroxydiphenoyl-β-D-glucose (48%); gallic acid (15%); digallic acid (10%); ellagic acid (9%); terchebin (8%); 2,3-digalloyl-β-D-glucose (6.7%); quercetin-3-*O*-rutinoside (2.3%); myricetin (0.8%); iso-myricitrin (0.2%).

Preparation IV was obtained from the aboveground part of Fergana spurge (*Euphorbia humifusa Willd.*) and contained: 1-*O*-galloyl-4,6-hexahydroxydiphenoyl-β-D-glucose (35%); quercetin (17%); ellagic acid (10.8%); 3-*O*-galloyl-4,6-hexahydroxydiphenoyl-β-D-glucose (8.3%); 1,2,3-tri-*O*-galloyl-β-D-glucose (7%); gallic acid (7%); geraniin (2.5%); quercetin-3-*O*-rhamnoside (4%); quercetin-3-*O*-galactoside (3.2%); kaempferol-3-*O*-glucoside (2.7%); 1-*O*-galloyl-6-*O*-bis-galloyl-2,4-valoneoyl-β-D-glucose (1.5%); kaempferol (1%).

The tannin preparations were added from concentrated stock solutions in dimethylsulfoxide (DMSO). Final concentration of DMSO did not exceed 0.1%, and at this concentration, the solvent did not significantly affect the records.

Solutions

The normal Ringer solution contained (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 11 HEPES, 5 glucose (pH 7.4, adjusted with NaOH, 290 mOsm/kg-H_2O). The H-buffer contained (mM): 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 glucose (pH 7.4, adjusted with NaOH, 40 mOsm/kg-H_2O). Hypotonic solutions were prepared by mixing the Ringer solutions with H-buffer in a ratio of 3:4 (vol/vol). The pipette solution for whole-cell experiments contained (in mM): 125 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 3 Na_2ATP, 5 HEPES (pH 7.4 adjusted with CsOH), 10 EGTA, and 50 mannitol (pCa 7.65; 320 mOsm/kg-H_2O).

Cells

Human colon tumor cell line, HCT116, was cultured in DMEM supplemented with 10% of fetal bovine serum and antibiotics (100 U/ml penicillin plus 100 mg/ml streptomycin) at 37°C and 5% CO_2. For patch-clamping, the cells were cultured in suspension under mild stirring during 3–5 h.

All animal experiments were conducted in accordance with the ARRIVE guidelines and approved by the Bioethics Committee of the Institute of Biophysics and Biochemistry. The isolation of thymic lymphocytes (thymocytes) was performed as described previously (Kurbannazarova et al., 2003, 2008, 2011; Sabirov et al., 2013). Briefly, the 6–8 weeks old rats, kept in vivarium on an average diet, were anaesthetized with halothane or diethyl ether and painlessly euthanized by cervical dislocation. The thymi were dissected and carefully washed with an ice-cold normal Ringer solution. The thymi were then minced using fine forceps and passed through a 100 µm-nylon mesh. The suspension was centrifuged at 1000 g for 5 min; the pellet was washed two times with the normal Ringer solution and resuspended in this medium at a cell density of 100 x 10^6 cells/ml. The cell suspension was kept on ice for ≤ 5 h and contained no more than 5% of damaged cells as assayed by trypan blue exclusion.

Cell Volume Measurements

Cell volume changes under non-isosmotic conditions were recorded by light transmittance measurement as described previously (Kurbannazarova et al., 2003, 2008, 2011). Briefly, 900 ml of the normal Ringer or hypotonic solutions was added to the 1.5 cm^3 glass cuvette thermo stated with a water jacket and equilibrated for 10 min. An aliquot (100 ml) of cell suspension was added to this medium to yield the final cell density of 10 x 10^6 cells/ml. The light transmittance was measured at 610 nm (band-pass filter) using a photometer MKMF-01 (Russia). The output signal was amplified by U5-11 amplifier (Russia), digitized at 100 Hz using a USB sensor interface GO!Link and recorded by Logger Lite software (Vernier, Beaverton, OR).

The parameter *RVD* was calculated using the following equation (1):

$$RVD=(T_{max}-T_{15})/(T_{max}-T_0)*100\% \quad (1)$$

where T_0 and T_{max} are the initial and maximal light transmittances, and T_{15} is the light transmittance measured 15 minutes after the onset of hypotonic stress. *RVD* = 100 for complete recovery of the cell volume to the initial level, and *RVD* = 0 when volume regulation is fully suppressed. Under control conditions, *RVD* usually had values of 60–90% depending on the cells condition, osmotic gradient, temperature and other experimental conditions.

Electrophysiology

Patch electrodes were fabricated from borosilicate glass capillaries using a micropipette puller (PP-830, Narishige, Japan) and had a tip resistance of 3–5 MΩ when filled with pipette solution. Fast and slow capacitive transients were routinely compensated for. For whole-cell recordings, the access resistance did not exceed 10 MΩ and was always compensated for by 80%. Membrane currents were measured with an EPC-9 patch-clamp system (Heka-Electronics, Lambrecht/Pfalz, Germany). The membrane potential was controlled by shifting the pipette potential (*Vp*) and is reported as *Vp* for whole-cell recordings. Currents were filtered at 1 kHz and sampled at 5–10 kHz. Data acquisition and analysis were done using Pulse + PulseFit (Heka-Electronics). Liquid junction potentials were calculated using pCLAMP 8.1 (Molecular Devices, Sunnyvale, CA) algorithms and were corrected off-line when appropriate. All experiments were performed at room temperature (23–25 °C).

Data analysis

The dose-response data were approximated using a Hill equation of the following form:

$$RVD = RVD_{min} + (RVD_{max} - RVD_{min})/(1 + (C/IC_{50})^h) \quad (2)$$

Here: RVD_{min} and RVD_{max} are the minimal and maximal values of *RVD*, *C* – concentration of the substance (µg/ml), IC_{50} – concentration of the substance rendering a half-maximal inhibitory effect (µg/ml), *h* – Hill coefficient.

Data were analyzed using Origin 8 (OriginLab Corporation, Northampton, MA, USA). Pooled data are given as means ± SEM of *n* observations. Comparisons between the two experimental groups were made using the unpaired two-sample Student's t-test. The data of Fig. 3d and 4d were analyzed using both unpaired two-sample (comparison at different voltages) and one-sample (comparison with control) t-test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

Tannin preparations inhibit thymocyte volume regulation under hypoosmotic stress

Blockers of VSOR channel are expected to suppress the regulatory volume decrease phase of cellular response to the hypoosmotic stress. In order to test this possibility, we employed the immature thymic lymphocytes which possess fully functional volume regulation machinery (Arrazola et al., 1993; Kurbannazarova et al., 2003; Soler et al., 1993). We have previously shown that thymocytes express the VSOR channels with the same biophysical and pharmacological profile as other cell types, and that VSOR blockers completely abolish

RVD in these cells (Kurbannazarova et al., 2011; Sabirov et al., 2013). Therefore, we supposed that tannin preparations might affect the volume regulation in thymocytes.

In our experiments, thymocytes, when challenged with hypoosmotic stress, first rapidly swelled (passive response) and then gradually restored their volume toward an initial level (active response; Fig. 1a). The parameter *RVD* as defined by Equation (1) (see Experimental section) ranged from 66% to 93% and averaged at 79.6 ± 1.9% (n = 17).

When added to the hypoosmotic medium, tannin preparations I and II exhibited a profound suppressive effect on the thymocyte volume regulation (Fig. 1a,b). For preparation I, an inhibition at lower doses was gradually lost as the amount of the added preparation was increased above 30 μg/ml (Fig. 1a,c). Since the maximal swelling also declined at high doses, we supposed that high concentrations of preparation I were detrimental for cellular plasma membrane. A similar biphasic action was also observed for preparation II (Fig. 1b,d). The half-maximal concentrations and Hill coefficients for the inhibiting phase of the dose-response curves (solid circles and solid lines in Fig. 1c,d) were as follows: preparation I (*IC50* = 40.9 ± 7.2 μg/ml; h = 0.96 ± 0.2) and preparation II (*IC50* = 12.3 ± 8.1 μg/ml; h = 0.59 ± 0.294).

In contrast to the first two preparations, preparations III and IV did not affect the maximal swelling and did not display the secondary damaging phase on the dose-response curves (Fig. 2). The half-maximal concentrations and Hill coefficients for the inhibiting phase of the dose-response curves (solid circles and solid lines in Fig. 2c,d) were as follows: preparation III (*IC50* = 270 ± 77 μg/ml; h = 0.63 ± 0.15) and preparation IV (*IC50* = 278 ± 43 μg/ml; h = 0.65 ± 0.07).

Comparison of the *IC50* values suggested that preparations I and II are more efficient inhibitors of the cell volume regulation than preparations III and IV.

Tannin preparations block the swelling-induced anion conductance

Tannins were never considered as volume-regulated anion channel blockers. In order to test this possibility, we employed direct electrophysiological assessment of the VSOR channel activity in human colorectal cancer HCT116 cells, which have been used recently for molecular identification of the VSOR channel proteins (Qiu et al., 2014; Voss et al., 2014).

In our experiments, we filled the patch-pipettes with slightly hypertonic (by ~30 mOsm/kg-H_2O) solution to induce cellular swelling as described previously (Kurbannazarova et al., 2011; Sabirov et al., 2013). Upon attaining the *whole-cell* configuration, cells gradually swelled as could be observed visually under phase-contrast microscopy. The cellular swelling was accompanied by a robust activation of the macroscopic currents with outward rectification and inactivation at large depolarizing positive potentials (Fig. 3a,b), a phenotypical landmark of the VSOR anion channel (Okada, 1997).

When preparations I and II were added to the flow chamber at a dose of 31 μg/ml and 41 μg/ml, respectively (at these doses, their effect on RVD was maximal, see Fig. 1c,d), we observed almost instant suppression of the macroscopic currents (Fig. 3a,b). The effect was essentially irreversible. The ionic currents were suppressed at both positive and negative potentials (Fig. 3c,d) suggesting that the channel blockage is voltage-independent. Voltage-independence together with the irreversibility of blockage may indicate a strong, possibly covalent, interaction of the tannins with the channel protein.

Preparations III and IV also exhibited suppressive effects on the macroscopic swelling-induced conductance. However, in contrast to the preparations I and II, current inhibition by preparations III and IV was slower (Fig. 4a,b) and partially reversible. The currents in the presence of these preparations were decreased more efficiently at positive potentials (Fig. 4c,d) than at negative voltages. Reversibility of the inhibition together with voltage-dependency may suggest an open-channel blockage mechanism: applied positive voltage drives the negatively charged polyphenolic compounds applied from the extracellular side into the channel lumen.

DISCUSSION

Thus, we have demonstrated that polyphenolic tannins of plant origin represent a novel class of VSOR channel inhibitors. This activity may contribute to the well-documented beneficial health effects of *polyphenol-rich food* and drink products.

In our experiments, preparations (I and II) were more efficient inhibitors of VSOR and volume regulation system compared to the preparations III and IV. Since preparation II consisted exclusively of hydrolysable tannins, and the content of hydrolysable tannins in preparation I reached 98%, we inferred that hydrolysable tannins represent a novel class of VSOR channel inhibitors. Certainly, the tannin preparations used in these experiments have rather complex composition, and thus, the individual tannins might be more effective and selective modulators of the VSOR chloride channel.

Preparations III and IV contained less overall tannin content (54.7% and 54.3%, respectively) and were less effective. Possibly, other types of polyphenols, which constituted a large part of these preparations may have weakened the inhibitory effects of hydrolysable tannins of preparations III and IV.

It should be noted that 3,6-bis-O-digalloyl-1,2,4-tri-O-galloyl-β-D-glucose, which is the major component of Preparation I, was recently shown to form anion-selective channels in lipid bilayers (Borisova et al., 2019). This effect may explain the secondary rising phase on the dose-response curve on the Figure 1a,c, suggesting that these newly formed pores functionally replace the VSOR channel by serving as a pathway for anion efflux. Since Preparation II also displayed a biphasic effect of RVD, one may suppose that some of its

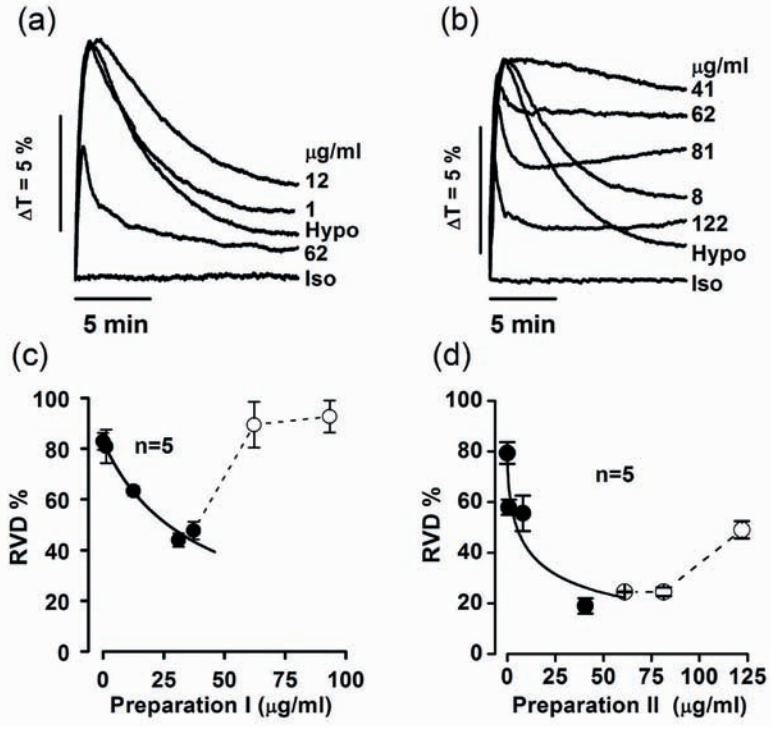

Figure 1: Dose-dependent effects of Preparations I and II on the thymocyte volume regulation under hypoosmotic stress. (a, b) Representative recordings of light transmittance changes. (c, d) Dose-response curves; the solid lines are fits to the equation (2) with half-maximal concentrations and Hill coefficients given in the text.

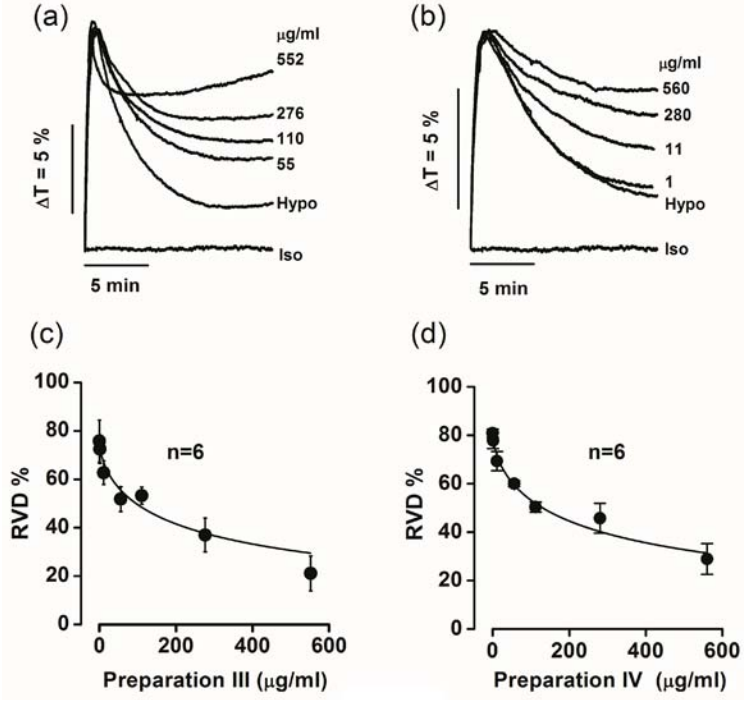

Figure 2. Dose-dependent effects of Preparations III and IV on the thymocyte volume regulation under hypoosmotic stress. (a, b) Representative recordings of light transmittance changes. (c, d) Dose-response curves; the solid lines are fits to the equation (2) with half-maximal concentrations and Hill coefficients given in the text.

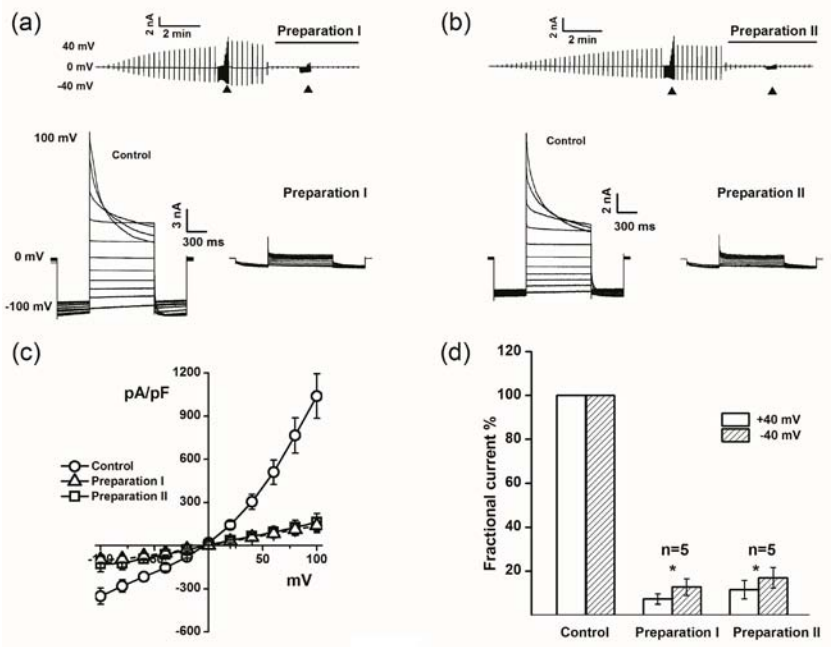

*Figure 3: Inhibition of VSOR currents by Preparations I and II. (a, b) The time course of whole-cell current activation in response to cell swelling. Currents were elicited by application of alternating test-pulses from 0 to ±40 mV every 15 s. Arrowheads (▲) denote the time points where the step-pulses from −100 to +100 mV in 20 mV increments were applied to test the voltage-dependence of the macroscopic conductance. (c) Instantaneous current-to-voltage relationships measured at the beginning of test-pulses from recordings similar to those shown in (a) and (b); n = 5 for Preparation I and n = 4 for Preparation II. (d) Fractional currents measured at +40 mV (open bars) and −40 mV (hatched bars). *Significantly different from control values at p < 0.05.*

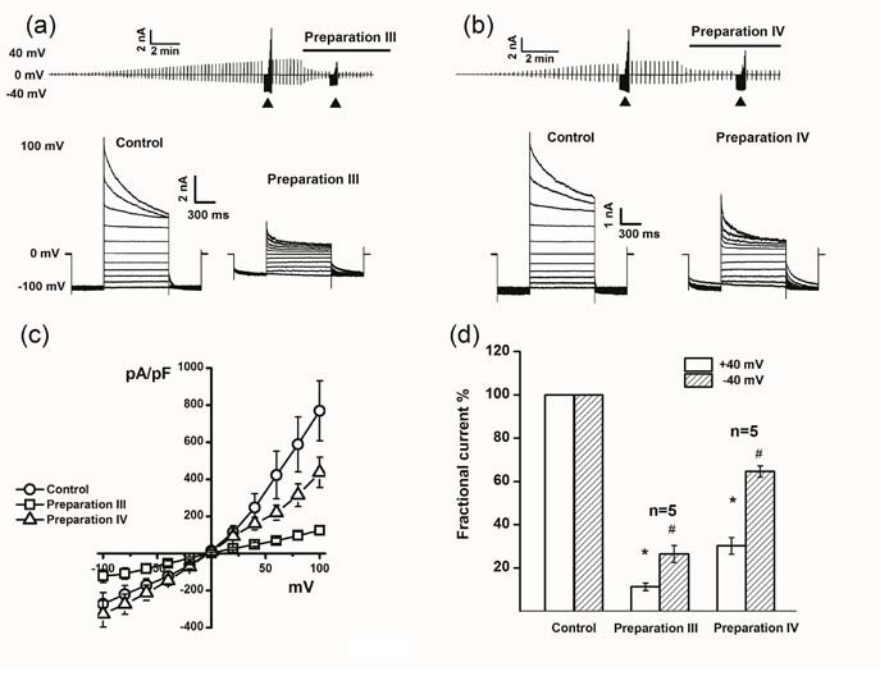

*Figure 4: Inhibition of VSOR currents by Preparations III and IV. (a, b) The time course of whole-cell current activation in response to cell swelling. Currents were elicited by the application of alternating test-pulses from 0 to ±40 mV every 15 s. Arrowheads (▲) denote the time points where the step-pulses from −100 to +100 mV in 20 mV increments were applied to test the voltage-dependence of the macroscopic conductance. (c) Instantaneous current-to-voltage relationships measured at the beginning of test-pulses from recordings similar to those shown in (a) and (b); n = 5 for Preparation III and n = 4 for Preparation IV. (d) Fractional currents measured at +40 mV (open bars) and −40 mV (hatched bars). *Significantly different from control values at p < 0.05. #Significantly different at p < 0.05 from values measured at negative voltages.*

components could also act as pore formers on lipid matrix of the cellular plasma membrane.

What kind of pharmacological effects could be anticipated for VSOR-inhibitory hydrolysable tannins? It is known, that the most effective and selective blocker of VSOR channel, 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB), exhibits a great beneficial effect in the reversible middle cerebral artery occlusion (rMCAO) model in adult rats (Han et al., 2014; Zhang et al., 2008) and in neonatal mouse hypoxic-ischemic brain injury (Alibrahim et al., 2013; Wong et al., 2018). The drug also protected cardiomyocytes from injury induced by hyperglycemia (Wang et al., 2017). Given the well-known beneficial effects of the tannin-containing plant extracts in cerebral ischemia and stroke (Behravan et al., 2014), our results would suggest that tannins antagonizing activity of the VSOR anion channel could be beneficial in protecting brain tissues, and possibly, the heart, during ischemic/hypoxic injury.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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