Original Paper

A Potassium-Selective Current Affected by Micromolar Concentrations of Anion Transport Inhibitors

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Key Words

Voltage-dependent outwardly rectifying potassium channels • Swelling-activated chloride current • NPPB • DIDS • Niflumic acid • ICl_{swell} • Kv 3.1

Abstract

Background/Aims: In the human genome, more than 400 genes encode ion channels, which are ubiquitously expressed and often coexist and participate in almost all physiological processes. Therefore, ion channel blockers represent fundamental tools in discriminating the contribution of individual channel types to a physiological phenomenon. However, unspecific effects of these compounds may represent a confounding factor. Three commonly used chloride channel inhibitors, i.e. 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid (DIDS), 5-nitro-2-[(3-phenylpropyl) amino]benzoic acid (NPPB) and the anti-inflammatory drug niflumic acid were tested to identify the lowest concentration effective on Cl⁻ channels and ineffective on K⁺ channels. **Methods:** The activity of the above mentioned compounds was tested by whole cell patch-clamp on the swelling-activated Cl⁻ current ICl_{swell} and on the endogenous voltage-dependent, outwardly rectifying K⁺ selective current in human kidney cell lines (HEK 293/HEK 293 Phoenix). **Results:** Micromolar (1–10 μM) concentrations of DIDS and NPPB could not discriminate between the Cl⁻ and K⁺ selective currents. Specifically, 1 μM DIDS only affected the K⁺ current and 10 μM NPPB equally affected the Cl⁻ and K⁺ currents. Only relatively high (0.1–1 mM) concentrations of DIDS and prolonged (5 minutes) exposure to 0.1–1 mM NPPB preferentially suppressed the Cl⁻ current. Niflumic acid preferentially inhibited the Cl⁻ current, but also significantly affected the K⁺ current. The endogenous voltage-dependent, outwardly rectifying K⁺ selective current in HEK 293/HEK 293 Phoenix cells was shown to arise from the Kv 3.1 channel, which is extensively expressed in brain and is involved in neurological diseases. **Conclusion:** The results of the present study underscore that sensitivity of a given physiological

phenomenon to the Cl⁻ channel inhibitors NPPB, DIDS and niflumic acid may actually arise from an inhibition of Cl⁻ channels but can also result from an inhibition of voltage-dependent K⁺ channels, including the Kv 3.1 channel. The use of niflumic acid as anti-inflammatory drug in patients with concomitant Kv 3.1 dysfunction may result contraindicated.

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Introduction

Pharmacological inhibition plays a fundamental role in discriminating the contribution of distinct ion transport systems to measurable phenomena, including macroscopic currents, differences of potential across plasma or epithelial membranes, salt reabsorption or secretion, and cardiac or nerve action potentials. However, unspecific effects of ion transport inhibitors may represent a confounding factor. In this regard, chloride channel blockers are recognized to variably affect (either activate or inhibit) cation currents [1], including those from Na⁺ [2], K⁺ [3, 4], Ca⁺⁺ [5] and nonselective cation [6] channels.

Being involved in the determination of basic homeostatic functions, such as the difference of potential across the plasma membrane and the regulation of cellular volume, K⁺ and Cl⁻ channels always coexist in a same tissue or cell type [7]. However, the discrimination between K⁺ and Cl⁻ currents is not always straightforward. Cl⁻ channel blockers have been reported to exert various effects on K⁺ currents, especially on voltage-dependent (Kv) channels, and specifically on cardiac Ca⁺⁺-independent transient outward current I_{to1}, of which Kv 4.2/4.3 are the underlying channels. I_{to} was found to be blocked by relatively high (2 mM) concentrations of disodium 4-acetamido-4'-isothiocyanato-stilben-2, 2'-disulfonate (SITS) [8]. Accordingly, 4, 4'-diisothiocyano-2, 2'-stilbene-disulfonic acid (DIDS) and SITS (≥ 10 μM) increased the time constant for recovery from inactivation of Kv 4.3 current, while niflumic and flufenamic acid (≥ 30 μM) produced large shifts in the steady state inactivation curve, both effects leading to an apparent inhibition of the current [9]. Tamoxifen (10 μM) and DIDS (10 μM) inhibited a delayed rectifier K⁺ current of ~38 and ~10% respectively in canine colonic myocytes [10]. On the other hand, 5-nitro-2-[(3-phenylpropyl) amino] benzoic acid (NPPB; 25 μM) and niflumic acid (50 μM) enhanced the delayed rectifier K⁺ current but inhibited I_{to} in rat ventricular myocytes [11].

Given that chloride channel blockers can also affect K⁺ channels, and based on the common knowledge that increasing the concentration of a drug can lead to unspecific effects, three commonly used chloride channel blockers (0.1 μM-1 mM DIDS and niflumic acid and 1 μM-1 mM NPPB) were tested to identify the lowest concentration effective on Cl⁻ channels and ineffective on K⁺ channels. The activity of the aforementioned compounds was assayed on the swelling-activated Cl⁻ current I_{Cl,swell} and on the endogenous voltage-dependent, outwardly rectifying K⁺ selective current in a human kidney cell line.

I_{Cl,swell} (also called I_{Cl,vol}) is the ubiquitous cell volume-sensitive chloride current, which is virtually absent or minimally activated in isotonic conditions and is strongly activated in response to extracellular hypotonicity. The biophysical properties of this current include mild outward rectification, iodide over chloride selectivity, and variable inactivation at positive potentials. I_{Cl,swell} is considered a major player in the process of regulatory volume decrease (RVD), a homeostatic reaction that reduces the cellular volume after anisotonic cell swelling. RVD is achieved by the loss of intracellular K⁺, Cl⁻ and osmotically active organic molecules, which then drives an obligated efflux of water from swollen cells, thereby reducing their volume. I_{Cl,swell} has been proposed to induce both the Cl⁻ and organic osmolytes efflux during RVD [12-15].

Voltage-dependent K⁺ (K_v) channels are a large and diverse group of ion channels that includes delayed rectifiers (I_K), A-type (I_A), outward rectifying, inwardly-rectifying, slowly activating α subunits, modifiers and regulatory β subunits. The members of this family of channels present distinctive characteristics in terms of biophysical and pharmacological properties, tissue distribution and disease association. For example, delayed rectifiers (including the *Shaw*-related Kv 3.1/Kv 3.2 channels, which are predominantly expressed in

brain) are either slowly inactivating or non-inactivating, while A-type channels (including the *Shal*-related Kv 4.2/Kv 4.3, which are mainly expressed in brain and heart) are rapidly inactivating [16, 17].

It was found here that micromolar (1–10 μ M) concentrations of DIDS and NPPB could not discriminate between the above-mentioned Cl[−] and K⁺ currents. A preferential block of Cl[−] currents could be obtained either with 0.1–1 mM DIDS, 0.1 mM niflumic acid or after prolonged (5 minutes) exposure to 0.1–1 mM NPPB.

Materials and Methods

Cell culture

Human embryonic kidney (HEK) 293 (CRL-1573, directly obtained from American Type Cell Culture Collection), HEK 293 Phoenix (this is a second-generation retrovirus producer line for the generation of helper free ecotropic and amphotropic retroviruses based on the 293T cell line [18]) and HeLa (human cervical adenocarcinoma, CCL-2, directly obtained from American Type Cell Culture Collection) cells were cultured in Minimum Essential Eagle Medium (Sigma-Aldrich, Austria) supplemented with 10% fetal bovine serum (FBS; Gibco, MA, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 1 mM pyruvic acid (sodium salt). NIH-3T3 cells (mouse fibroblasts, CRL-1658, directly obtained from American Type Cell Culture Collection) were cultured in Dulbecco's modified Eagle's Medium (Sigma-Aldrich, Austria) supplemented with 10% newborn bovine serum (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin.

Mouse primary renal proximal tubular epithelial cells were obtained from CD1 mice by microdissection. In brief, mice were sacrificed by cervical dislocation. Kidneys were immediately removed and placed in ice-cold Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich, Austria). The renal capsule was removed and small slices from the cortex were prepared. Microdissection of proximal tubules from the cortical tissue was performed in cold (4°C) HBSS containing bovine serum albumin (1 mg/ml; Merck, Germany) under a stereomicroscope using forceps. Isolated proximal tubules were transferred into 96 well plates coated with a collagen (1, 3 mg/ml; Sigma-Aldrich) and fibronectin (55 μ g/ml; Sigma-Aldrich) matrix and cultured in Dulbecco's Modified Eagle Medium/F12 (Sigma-Aldrich, Austria) supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 μ g/ml insulin, 5 μ g/ml holo-transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 4 pg/ml T3 and 10 ng/ μ l epidermal growth factor. Epithelial cells from the tubules dissociated from the tubular structure over a span of 5–14 days and attached to the culture dish. Cells were then passaged onto successively larger culture areas for amplification. All experiments were performed in accordance to the guidelines of the "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes" and were approved by the local animal care authorities.

The cells were maintained at 37 °C, 5% CO₂, 95% air and 100% humidity. Except for kidney proximal tubule cells, subcultures were routinely established every second to third day by seeding the cells into 100 mm diameter Petri dishes following trypsin/ethylenediaminetetraacetic acid (EDTA) treatment. Kidney proximal tubule cells were subcultured once weekly and used between passages 4 to 7. HeLa and NIH-3T3 cells were used between passages 2 to 20.

Cell transfection and plasmid vectors

For transfection, NIH-3T3 cells were seeded into 35 mm diameter Petri dishes and grown overnight to ~30% confluence. Cells were transfected with 3 μ g of plasmid DNA and 6 μ l of Metafectene Pro (Biontex, Germany) following the manufacturer's instructions. Control (mock transfected) cells were exposed to the transfection mixture with no plasmid DNA. The plasmid vector pcDNA3.1+P2A-eGFP coding for Kv 3.1 (KCNC1, NCBI Reference Sequence: NM_004976.4) was purchased from GenScript (USA). The self-cleaving 2A peptide (P2A) allows for the simultaneous expression of two individual proteins — Kv3.1 and eGFP — from a single bicistronic mRNA without the production of fusion proteins [19]. For patch-clamp, the single transfected cells could be individuated optically by detecting the fluorescent light emitted by eGFP (excitation maximum, 488 nm; emission maximum, 507 nm). Experiments were performed 24 hours after transfection.

Patch clamp experiments

Cells for electrophysiology were seeded on glass coverslips (diameter, 10 mm) contained in 30 mm diameter Petri dishes and grown overnight. Single cells were selected by phase contrast microscopy and voltage clamped using the whole-cell patch clamp technique as previously described [20-26]. To isolate chloride currents, bath solutions 1 and 2 and pipette solution 1 (Table 1) were used. The seal was realized and cells were initially kept in isotonic bath solution 1. To activate the swelling-dependent chloride current (ICl,swell), a fast exchange of the isotonic with the hypotonic bath solution 2 was accomplished using a perfusion system with a flow rate of 5 ml/min and a bath volume of ~300 µl. The cells were held at 0 mV, and step pulses of 400 ms duration were applied from 0 mV to +40 mV every 20 s to monitor current changes over time. To establish the current-to-voltage (I/V) relationship, step pulses of 500 ms duration were applied from -120 mV to +100 mV in 20 mV increments from a holding potential of 0 mV. The IV relationships were established every 5 minutes. To isolate potassium currents, bath solution 3 and pipette solution 2 (Table 1) were used. To establish the I/V relationship, step pulses of 500 ms duration were applied from -120 mV to +100 mV in 20 mV increments from a holding potential of -60 mV.

The resistance of the glass pipettes filled with a pipette solution and immersed in a bath solution was 3 to 8 MΩ. For data acquisition, EPC-10 and EPC-8 (HEKA Elektronik, Germany) amplifiers controlled by Macintosh computers running the Patch Master (HEKA Elektronik) software were used. The whole-cell configuration was only obtained from stable seals of resistance not lower than 2-3 GΩ in cell-attached configuration (for glass electrodes not subjected to fire polishing). The limits of membrane resistance in whole-cell configuration and leak currents accepted to validate a recording were 1 GΩ and +100 pA at +100 mV, respectively. These values refer to conditions where macroscopic currents were not activated, i.e. in extracellular isotonic solution for the Cl⁻ current and at a holding potential of -60 mV for the K⁺ current. Fast and slow capacitance were compensated and series resistance was monitored throughout the recordings. If the series resistance was higher than twice the resistance of the patch electrode at the beginning of a recording, or exceeded this value any time during a recording, the current measurement was not accepted. All current measurements were filtered at 5 kHz and digitized at 50 kHz. For data analysis, Fit Master (HEKA Elektronik) and Excel (Microsoft, USA) software were used. Where indicated, the current values (pA) were normalized to the membrane capacitance (pF) to obtain the current density (pA/pF), which is a measure of the current magnitude independent from the cell size. The subtraction of currents of leakage was not performed. All experiments were carried out at room temperature. Each independent experimental series was performed rigorously alternating one recording with the selected inhibitor and one recording with the respective vehicle over a time frame of no more than two weeks. The decay of potassium and chloride currents at +100 mV was best fitted with an exponential of the form

$$I(t) = I_{peak} e^{-t/\tau}$$

where I_{peak} is the peak current and τ is the time constant of current inactivation.

Reverse Transcription-PCR (RT-PCR)

Extraction of total RNA from HEK 293, 293 Phoenix, HeLa, NIH-3T3 and primary proximal tubule cells was performed with the All Prep DNA/RNA mini kit (Qiagen, Germany) from cells grown to confluence on

Table 1. Composition (in mM) of extracellular (bath) and intracellular (pipette filling) solutions for whole-cell patch-clamp experiments. Osmotic pressure was adjusted to ~300 mOsm/Kg_{H2O} with mannitol or raffinose as needed, except for bath solution 2, where mannitol was omitted and osmotic pressure was ~260 mOsm/Kg_{H2O}. ATP was added to solutions as a salt of Mg²⁺. EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

	Bath solutions			Pipette filling solutions	
	1 Isotonic, Cl ⁻	2 Hypotonic, Cl ⁻	3 Isotonic K ⁺	1 Cl ⁻	2 K ⁺
Na ⁺	125	125	5.4	Cs ⁺	125
K ⁺			115	K ⁺	115
Ca ²⁺	2.5	2.5	1.8	Mg ²⁺	5
Mg ²⁺	2.5	2.5	0.5	Cl ⁻	10
Cl ⁻	135	135	10	Gluconate	115
Gluconate			115	EGTA	5
HEPES	10	10	5	HEPES	5
Glucose			5	ATP	5
Mannitol	~40		~70	Mannitol	~50
				Raffinose	~10
pH	7.4	7.4	7.4	pH	7.2

one 100 mm diameter Petri dish. Total RNA from human adult brain (Cat # 540005-41, Lot # 0006242610) was purchased from Agilent (USA). One µg of total RNA was used for the reverse transcription reaction with the QuantiTect® reverse transcription kit for cDNA synthesis with integrated removal of genomic DNA contamination (Qiagen). For detecting the Kv 1.1, Kv 1.2, Kv 1.3, Kv 1.4, Kv 1.5, Kv 1.6, Kv 2.1 and Kv 3.1 mRNAs, previously reported primers were used [27]. The β-actin signal was detected by using the following primers: forward, 5'-GGC ATG GGT CAG AAG GAT TC-3'; and reverse, 5'-AGA GGC GTA CAG GGA TAG CAC-3'. These primers span an intron-exon boundary and would disclose contamination from genomic DNA as a band at 740 bp, which was not detected. To verify the identity of the signals that were detected (if any), the bands were extracted from the gel, purified with the MinElute Gel Extraction kit (Qiagen) and Sanger sequenced in the forward and reverse directions with the same primers used for amplification (Microsynth AG, Switzerland).

HEK 293 Phoenix and human adult brain were tested for the expression of Kv 3.1 transcripts. To discriminate between the long and short Kv 3.1 transcripts, a common forward primer (5'-GCC TCG AGA TGG GCC AAG GGG ACG AGA G-3') and two distinct reverse primers (5'-GCC GGA TCC TCA AGT CAC TCT CAC AGC CTC-3' and 5'-GGG GAT CCT TCA GAT CGA CAT GCC TCT AAG-3' for the long and short transcript, respectively) were used.

Western Blot

HEK 293 Phoenix cells seeded in each well of a 6-well plate were washed with phosphate buffered solution (PBS) and scraped in 50 µl of ice-cold lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1X Halt Protease Inhibitor Cocktail, Thermo Scientific, MA, USA) on ice. Cell lysates were centrifuged at 16,000 *xg* at 4 °C for 30 minutes. The supernatant was collected and stored at -80 °C until use. Total protein extracts (≥20 µg) were electrophoresed with constant voltage (120 V) on SDS-PAGE gels (7.5%). Proteins were then transferred for 2 hours onto polyvinylidene fluoride (PVDF) membranes with constant voltage (75 V). The membranes were blocked for 1 hour at room temperature in 5% nonfat dry milk diluted in Tris-buffered saline containing 0.01% Tween 20, pH 7.6 (TBST). Afterwards, PVDF membranes were incubated overnight at 4 °C with primary antibodies diluted in TBST containing 5% nonfat dry milk, washed 3 times in TBST, incubated for 1 hour in the dark and at room temperature with the secondary antibody diluted in TBST containing 5% nonfat dry milk, washed again and kept in TBST. Immunocomplexes were visualized using the ODYSSEY infrared imaging system (LICOR, NE, USA). The mouse monoclonal anti-Kv 3.1 (E-2) antibody (sc-514554, 1:100 dilution) was from Santa Cruz Biotechnology (TX, USA). The goat polyclonal anti-GAPDH antibody (JP_A00191-40, 1:1000 dilution) was from GenScript (NJ, USA). The anti-goat (926-32214, 1:10, 000 dilution) and anti-mouse (926-32210, 1:10, 000 dilution) IRDye 800 CW secondary antibodies were from LICOR. Kv 3.1 signal was normalized for the signal of the housekeeping protein GAPDH. Densitometry was performed with ImageJ 1.46r software (Wayne Rasband, NIH, MD, USA).

RNA Interference

Small interfering (si) RNAs for knocking down the expression of Kv 3.1 (core sequence siRNA #1: 5'-GAA CUC GCU CAA CAU CAU U-3'; siRNA #2: 5'-CGU UCG CAA UGG CAC GCA A-3'; siRNA #3: 5'-CAA GGU AGA GUU CAU CAA G-3') were designed with the siRNA Design Tool of Microsynth. HEK 293 Phoenix cells seeded into 30 mm diameter Petri dishes were co-transfected with 180-360 pmol of siRNAs, 1 µg of pEYFPN1 vector (Clontech, CA, USA) coding for the transfection marker enhanced yellow fluorescent protein EYFP and 24 µl of Metafectene SI (Biontex) following the manufacturer's instruction. Control cells were transfected with the following negative control siRNA (Microsynth), with no homologies with known sequences: 5'-AGG UAG UGU AAU CGC CUU GTT-3'. Functional (patch clamp) or expression (Western blot and semi-quantitative PCR) assays were performed 48-72 h after transfection. For patch clamp, single transfected cells were selected by fluorescence microscopy and voltage clamped using the whole-cell patch clamp technique as described above.

Salts and chemicals

4, 4'-diisothiocyanato-2, 2'-stilbene-disulfonic acid (DIDS; D3514), 5-nitro-2-[(3-phenylpropyl)amino] benzoic acid (NPPB; N4779) and niflumic acid (N0630) were purchased from Sigma-Aldrich. Stock solutions (0.1 M) were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C in the dark. All salts and chemicals used were of *per analysis* grade.

Statistical Analysis

All data were expressed as arithmetic means $\pm$ S.E.M. For statistical analysis, GraphPad Prism software (version 4.00 for Windows, GraphPad Software, CA, USA) was used. Significant differences between data sets were tested by two-tailed, paired or unpaired Student's t test or two-way ANOVA with Bonferroni's *ad hoc* post test, as appropriate. Statistically significant differences between data sets were assumed at $p < 0.05$; (n) corresponds to the number of independent measurements.

Results

Chloride and potassium selective currents in HEK 293/293 Phoenix cells

Endogenous currents resembling the outwardly rectifying and voltage-dependent potassium currents of the Kv channel family can be found in HEK 293 Phoenix cells (Figs. 1A, 2A and 3A). These currents are detectable in the whole-cell configuration of patch clamp, were found in HEK 293 cells but not in HeLa cells, NIH-3T3 cells or primary culture of mouse kidney tubule cells (data not shown).

HEK 293 Phoenix cells kept in an isotonic medium do not manifest Cl^- currents detectable in the whole-cell configuration of patch clamp (Figs. 1B, 2B and 3B, isotonic). However, prominent activation of Cl^- currents is seen after reduction of extracellular osmolarity (Figs. 1B, 2B and 3B, hypo SS).

Micromolar concentrations of DIDS, NPPB and niflumic acid modify the biophysical properties of a potassium selective current

In the range tested (0.0001 – 1 mM), the lowest concentration of DIDS with a statistically significant inhibitory effect on K^+ currents was 0.001 mM (Table 2). At this concentration, the effect of DIDS on Cl^- currents showed considerable variability and was not statistically

Fig. 1. DIDS inhibits a voltage-dependent potassium selective current and the swelling-activated chloride current. A, top, original current recordings obtained from HEK 293 Phoenix cells in whole-cell configuration with pipette solution 2 and bath solution 3 before and after the addition of 1 mM DIDS or the vehicle to the bath solution; bottom, current (pA) to voltage (mV) relationship of K^+ currents before, immediately after (0 min) and 5 min after the addition of DIDS or the vehicle to the bath solution. B, top, original current recordings obtained from HEK 293 Phoenix cells in whole-cell configuration with pipette solution 1 and isotonic bath solution 1, hypotonic bath solution 2 and after the addition of 1 mM DIDS or the vehicle to the hypotonic bath solution; bottom, current (pA) to voltage (mV) relationship of Cl^- currents in isotonic bath solution 1, hypotonic bath solution 2 at the maximal current activation (or steady state, hypo SS), immediately after (0 min) and 5 min after the addition of DIDS or the vehicle to the hypotonic bath solution. Scale of original current recordings of panel B (inset) also applies to A. Numbering of solutions refers to Table 1. (n) indicates the number of cells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, before and 5 min after addition of DIDS or the vehicle to the bath solution, two-tailed paired Student's t test.

Fig. 2. NPPB inhibits a voltage-dependent potassium selective current and the swelling-activated chloride current. A, top, original current recordings obtained from HEK 293 Phoenix cells in whole-cell configuration with pipette solution 2 and bath solution 3 before and after the addition of 1 mM NPPB or the vehicle to the bath solution; bottom, current (pA) to voltage (mV) relationship of K⁺ currents before, immediately after (0 min) and 5 min after the addition of NPPB or the vehicle to the bath solution. B, top, original current recordings obtained from HEK 293 Phoenix cells in whole-cell configuration with pipette solution 1 and isotonic bath solution 1, hypotonic bath solution 2 and after the addition of 1 mM NPPB or the vehicle to the hypotonic bath solution; bottom, current (pA) to voltage (mV) relationship of Cl⁻ currents in isotonic bath solution 1, hypotonic bath solution 2 at the maximal current activation (or steady state, hypo SS), immediately after (0 min) and 5 min after the addition of NPPB or the vehicle to the hypotonic bath solution. Scale of original current recordings of panel B (inset) also applies to A. Numbering of solutions refers to Table 1. (n) indicates the number of cells. *: p<0.05, **: p<0.01, before and 5 min after addition of NPPB or the vehicle to the bath solution, two-tailed paired Student's t test.

Fig. 3. Niflumic acid inhibits a voltage-dependent potassium selective current and the swelling-activated chloride current. A, top, original current recordings obtained from HEK 293 Phoenix cells in whole-cell configuration with pipette solution 2 and bath solution 3 before and after the addition of 1 mM niflumic acid or the vehicle to the bath solution; bottom, current (pA) to voltage (mV) relationship of K⁺ currents before, immediately after (0 min) and 5 min after the addition of niflumic acid or the vehicle to the bath solution. B, top, original current recordings obtained from HEK 293 Phoenix cells in whole-cell configuration with pipette solution 1 and isotonic bath solution 1, hypotonic bath solution 2 and after the addition of 1 mM niflumic acid or the vehicle to the hypotonic bath solution; bottom, current (pA) to voltage (mV) relationship of Cl⁻ currents in isotonic bath solution 1, hypotonic bath solution 2 at the maximal current activation (or steady state, hypo SS), immediately after (0 min) and 5 min after the addition of niflumic acid or the vehicle to the hypotonic bath solution. Scale of original current recordings of panel B (inset) also applies to A. Numbering of solutions refers to Table 1. (n) indicates the number of cells. *: p<0.05, **: p<0.01, before and 5 min after addition of niflumic acid or the vehicle to the bath solution, two-tailed paired Student's t test.

significant (Table 2). The inhibition of K^+ currents determined immediately after exposure of cells to 0.001 mM DIDS was ~8.2% and ~11.0% after 5 minutes (Table 2). These values refer to the peak current and are corrected for the mean % of current variation observed in control cells. In addition, the analysis of τ values revealed a significant acceleration of current inactivation in time (Table 2). The effect on peak current as well as on current inactivation become more pronounced with 0.01 mM DIDS. The increasing inhibition of the peak current observed with DIDS concentrations higher than 0.01 mM (>20% with 1 mM DIDS, Table 2, Fig. 1A and [28] masked the effect on the current inactivation (Table 2).

In the range tested (0.0001 – 1 mM), the lowest concentration of DIDS with a statistically significant inhibitory effect on Cl^- currents was 0.01 mM (~28% and ~29% immediately after and 5 minutes after exposure of cells to DIDS, respectively; Table 2). DIDS concentrations of 0.1–1 mM exhibited a significantly higher inhibitory effect on Cl^- currents compared to K^+ currents (Table 2 and Fig. 1B), that was typically voltage-dependent, i.e. more pronounced on the outward currents, in agreement with previous observations [1]. Also the current inactivation in time was significantly accelerated (Table 2).

In the range tested (0.001 – 1 mM), the lowest concentration of NPPB with a statistically significant inhibitory effect was 0.01 mM. This was observed on both Cl^- and K^+ currents. The inhibition of K^+ currents determined immediately after exposure of cells to 0.01 mM NPPB was ~7% and ~18% after 5 minutes (Table 2) and did not significantly differ from that observed on Cl^- currents (i.e. ~13% and ~37%, respectively; Table 2). Prolonged exposure (5 minutes) to 0.1–1 mM NPPB led to a significantly higher inhibitory effect on Cl^- currents compared to K^+ currents (Fig. 2 and Table 2).

Table 2. Current variation (in %) and time constant of current inactivation (τ , in msec) determined in HEK 293 Phoenix cells immediately after (0 min) and 5 minutes after exposure to the indicated inhibitors or their vehicles. The % of current variation was calculated at +100 mV between peak currents recorded before and immediately after (0 min), as well as before and 5 minutes after exposure of cells to the inhibitor, and was corrected for the average of the corresponding current variation observed in cells treated with the vehicle. (-) indicates a current decrease, (+) indicates a current increase. Being 0.001 mM niflumic acid ineffective on the K^+ current, a lower concentration (0.0001 mM) was only assayed on the Cl^- current. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, n.s.: not significant compared to the vehicle, two-tailed, unpaired Student's t test. §: $p < 0.05$, §§: $p < 0.01$, §§§: $p < 0.001$ compared to the K^+ current, two-way ANOVA with Bonferroni's ad-hoc post-test

Inhibitor	mM	Current variation, %		n inhibitor (vehicle)	τ (msec) inhibitor (vehicle)	
		0 min	5 min		0 min	5 min
K^+ current	1	-22.8±2.5**	-20.9±2.7**	10 (10)	275.4±82.3 ^{n.s.} (249.7±92.7)	223.0±50.0 ^{n.s.} (174.4±63.4)
	0.1	-17.6±2.0**	-18.0±2.4**	17 (14)	103.0±11.9 ^{n.s.} (211.6±58.9)	114.4±13.0 ^{n.s.} (207.6±57.4)
	DIDS	0.01	-16.2±4.4***	9 (9)	68.4±7.5* (151.2±29.5)	41.7±4.9*** (122.7±16.8)
		0.001	-8.2±2.6*	10 (8)	139.4±13.6 ^{n.s.} (177.4±34.3)	106.4±11.5* (190.8±33.8)
		0.0001	-3.2±3.2 ^{n.s.}	10 (9)	90.9±17.7 ^{n.s.} (149.7±46.8)	82.0±12.9 ^{n.s.} (106.7±29.2)
		1	-63.3±3.6***	3 (3)	91.2±40.0 ^{n.s.} (189.0±74.6)	84.0±33.5 ^{n.s.} (151.3±22.0)
	NPPB	0.1	-19.3±3.6***	10 (10)	147.9±18.3 ^{n.s.} (253.3±66.7)	102.8±14.0 ^{n.s.} (214.3±70.2)
		0.01	-7.0±2.2*	5 (4)	131.4±8.8 ^{n.s.} (100.7±21.4)	83.4±8.4 ^{n.s.} (86.5±11.4)
		0.001	+1.0±2.9 ^{n.s.}	6 (6)	100.2±20.9 ^{n.s.} (114.3±24.7)	92.3±18.7 ^{n.s.} (114.2±22.6)
		1	-34.8±5.1***	6 (6)	124.4±57.5 ^{n.s.} (134.5±36.2)	109.7±58.2 ^{n.s.} (179.0±32.9)
Niflumic Acid	0.1	-15.6±3.4**	-13.2±5.0 ^{n.s.}	11 (9)	229.7±38.2 ^{n.s.} (247.5±71.6)	284.9±53.9 ^{n.s.} (236.1±74.6)
	0.01	-7.2±1.8 ^{n.s.}	-10.6±2.4*	11 (11)	245.4±46.5 ^{n.s.} (135.0±18.8)	241.6±54.4 ^{n.s.} (140.0±41.6)
	0.001	-2.3±2.7 ^{n.s.}	-7.2±3.1 ^{n.s.}	9 (9)	213.4±99.5 ^{n.s.} (141.4±12.4)	233.7±111.9 ^{n.s.} (167.0±23.2)
	Cl^- current					
DIDS	1	-77.5±2.9*** §§§	-77.8±3.5*** §§§	5 (5)	6.0±1.8** (221.6±48.4)	11.5±6.3*** (176.3±43.4)
	0.1	-81.0±0.6*** §§§	-96.7±2.2*** §§§	3 (5)	15.1±5.4* (158.8±31.5)	9.4±3.5** (157.8±23.5)
	0.01	-28.3±7.0*	-29.5±6.3 ^{n.s.}	8 (6)	181.3±25.6 (145.2±43.2)	199.1±38.7 (98.6±36.8)
	0.001	-8.1±3.0 ^{n.s.}	-28.8±10.6 ^{n.s.}	11 (7)	178.3±30.2 (192.9±49.7)	185.0±33.9 (227.8±35.8)
	0.0001	-2.1±3.5 ^{n.s.}	-23.4±7.1 ^{n.s.}	3 (3)	186.4±17.0 (256.4±82.5)	233.6±23.0 (177.1±38.7)
	1	-62.9±13.8*	-102±7.4* §	3 (3)	67.9±16.9* (192.0±37.6)	30.7±14.7* (192.0±38.0)
	NPPB	0.1	-32.5±6.0**	5 (4)	189.5±22.7 (142.3±25.9)	184.6±23.5 (172.5±32.8)
		0.01	-12.9±3.8*	9 (8)	209.3±30.2 (149.1±25.0)	225.4±27.9 (142.2±31.5)
		0.001	+3.5±8.9 ^{n.s.}	4 (4)	176.3±19.1 (171.4±74.0)	231.9±42.1 (259.5±99.2)
		1	-66.8±5.7*** §	3 (3)	3736.7±129.4*** (199.0±72.3)	2506.0±509* (228.3±56.5)
Niflumic Acid	0.1	-43.9±8.2*** §	-77.3±5.4* §§	4 (3)	153.8±37.9 ^{n.s.} (175.7±34.0)	74.5±15.4* (242.0±67.0)
	0.01	-15.3±5.2*	-35.2±8.6* §	11 (7)	121.1±25.6 ^{n.s.} (119.9±24.5)	124.8±32.5 ^{n.s.} (108.6±19.1)
	0.001	-2.6±2.5 ^{n.s.}	-22.8±2.9* §	9 (9)	172.3±26.4 (265.3±54.4)	159.1±19.9* (218.3±41.1)
	0.0001	+9.2±6.9 ^{n.s.}	+15.3±7.9 ^{n.s.}	3 (4)	230.4±60.5 (140.1±28.2)	203.2±88.6 (190.5±60.8)

Fig. 4. Kv 3.1 is expressed in HEK 293/HEK 293 Phoenix cells and generates the voltage-dependent outwardly rectifying potassium selective current. A, total RNA was extracted from HEK 293 Phoenix cells, reverse transcribed and subjected to PCR amplification with primers specific for the indicated α subunits of Kv channels. The transcript of the housekeeping gene β -actin was amplified as a control. The image is representative of three independent replicates leading to a same result. B, top: the levels the transcripts of Kv 3.1 and β -actin were detected by RT-PCR in 3 independent samples of total RNA from HEK 293 Phoenix cells transfected for 72 hours with 360 pmol of Kv 3.1 siRNA or control siRNA. To exclude genomic DNA contamination, the PCR reaction was also conducted on samples of total RNA not subjected to the reverse transcription reaction (-RT); bottom: densitometric analysis of the Kv 3.1 signal normalized to the β -actin signal; **: $p < 0.01$, two-tailed, unpaired Student's t-test. C, top: the levels the Kv 3.1 and GAPDH proteins were detected by Western blot in 3 independent samples of total protein extracts from HEK 293 Phoenix cells transfected for 72 hours with 360 pmol of Kv 3.1 siRNA or control siRNA; bottom: densitometric analysis of the Kv 3.1 signal normalized to the GAPDH signal. D, the magnitude of K^+ currents was monitored by whole cell patch-clamp in HEK 293 Phoenix cells transfected with Kv 3.1 siRNA or control siRNA. The current density (pA/pF) to voltage (mV) relationship is shown. (n) indicated the number of cells. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, two-tailed, unpaired Student's t-test. E, total RNA was extracted from the indicated cell lines, reverse transcribed and subjected to PCR amplification with primers specific for transcripts encoding the α subunit of Kv 3.1 channel. β -actin transcript was amplified as an internal control. -RT as in B. The image is representative of three independent replicates leading to a same result. PT, kidney proximal tubule cells in primary culture. F, total RNA was extracted from HEK 293 Phoenix cells, reverse transcribed and subjected to PCR amplification with primers specific either for the short (NM_004976.4) or long (NM_001112741.1) transcripts of Kv 3.1.

of the Kv 3.1 signal normalized to the β -actin signal; **: $p < 0.01$, two-tailed, unpaired Student's t-test. C, top: the levels the Kv 3.1 and GAPDH proteins were detected by Western blot in 3 independent samples of total protein extracts from HEK 293 Phoenix cells transfected for 72 hours with 360 pmol of Kv 3.1 siRNA or control siRNA; bottom: densitometric analysis of the Kv 3.1 signal normalized to the GAPDH signal. D, the magnitude of K^+ currents was monitored by whole cell patch-clamp in HEK 293 Phoenix cells transfected with Kv 3.1 siRNA or control siRNA. The current density (pA/pF) to voltage (mV) relationship is shown. (n) indicated the number of cells. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, two-tailed, unpaired Student's t-test. E, total RNA was extracted from the indicated cell lines, reverse transcribed and subjected to PCR amplification with primers specific for transcripts encoding the α subunit of Kv 3.1 channel. β -actin transcript was amplified as an internal control. -RT as in B. The image is representative of three independent replicates leading to a same result. PT, kidney proximal tubule cells in primary culture. F, total RNA was extracted from HEK 293 Phoenix cells, reverse transcribed and subjected to PCR amplification with primers specific either for the short (NM_004976.4) or long (NM_001112741.1) transcripts of Kv 3.1.

In the range tested (0.0001 – 1 mM), the lowest concentration of niflumic acid with a statistically significant inhibitory effect was 0.001 mM. This was observed on Cl^- but not on K^+ currents. The inhibition of Cl^- currents determined immediately after exposure of cells to 0.001 mM niflumic acid was ~2.6% and ~23% after 5 minutes (Table 2). The last value was significantly different from that observed on K^+ currents (i.e. ~7%, Table 2). Increasing concentrations (0.01-1 mM) of niflumic acid inhibited both Cl^- and K^+ currents; the effect on Cl^- currents was statistically higher, with the exception of 1 mM niflumic acid (5 minutes exposure), which inhibited Cl^- and K^+ currents to a same extent (~80%, Fig. 3 and Table 2).

Molecular identity of the potassium selective current

To identify the channel(s) underlying the voltage-dependent K^+ current, end-point PCR was performed on cDNA from total mRNA of HEK 293 Phoenix cells with primers pairs specific

Fig. 5. Kv 3.1 currents are sensitive to niflumic acid. A, original current recordings obtained from untransfected (mock) or Kv 3.1-transfected (+100 mV) NIH-3T3 cells in whole-cell configuration with pipette solution 2 and bath solution 3 before and 5 min after the addition of 0.1 mM niflumic acid or the vehicle (0.03% DMSO) to the bath solution. The current recording obtained in the presence of niflumic acid is indicated by a black arrowhead. B, Intensity (pA) and C, time constant of inactivation (τ) of current measured at +100 mV in cells expressing Kv 3.1 before (pre), immediately after (0 min) and 5 min after exposure to 0.1 mM niflumic acid or the vehicle. Numbering of solutions refers to Table 1. (n) indicates the number of cells. *: $p < 0.05$, **: $p < 0.01$, before and 5 min after addition of niflumic acid to the bath solution, two-tailed paired Student's t test.

for different members of the Kv family (Kv 1.1, Kv 1.2, Kv 1.3, Kv 1.4, Kv 1.5, Kv 1.6, Kv 2.1 and Kv 3.1). Of these, only Kv 3.1 was reproducibly detected in three independent cDNA samples (Fig. 4A), and the identity of the amplicon was confirmed by Sanger sequencing. A band with size compatible with the Kv 1.3 amplicon was detected in two out of six independent cDNA samples from HEK 293 Phoenix cells, however, Sanger sequencing could not confirm the identity of this PCR product. All the remaining primer couples did not amplify a band of the expected size, also following variation of the annealing temperature between 47 and 61 °C.

To assess if the channel Kv 3.1 is involved in determining the voltage-dependent K⁺ current in HEK 293 Phoenix cells, the expression of Kv 3.1 mRNA was silenced by RNA interference. Three different siRNAs (180-360 pmol) were assayed following a 48-72 h transfection in cells. A 72 h transfection with 360 pmol of equimolar amounts of siRNA #1 and 2 led to the best result, i.e. a Kv 3.1 mRNA silencing of ~49% (Fig. 4B), which corresponded to a Kv 3.1 protein silencing of ~47%, (Fig. 4C) and was utilized for functional testing. In these conditions, the voltage-dependent K⁺ current in HEK 293 Phoenix cells was reduced by ~45% (Fig. 4D), strongly suggesting that Kv 3.1 is the underlying channel. Kv 3.1 was also detected in cDNA samples from HEK 293 cells, but not in NIH-3T3, HeLa or mouse primary proximal tubules cells (Fig. 4E). The weak band detected in HeLa cells is lower than the expected size and is most likely an unspecific PCR product (Fig. 4E). Of the two known Kv 3.1 transcripts, only the short transcript was detected in HEK 293 Phoenix cells (Fig. 4F).

To further confirm the molecular identity of the channel eliciting the voltage-dependent K⁺-selective current in HEK 293/293 Phoenix cells, the short isoform of Kv 3.1 was expressed in a heterologous system, and the biophysical and pharmacological properties of the resulting current were studied and compared to those of the endogenous current measured in HEK 293/293 Phoenix cells. For this, a cell type void of macroscopic endogenous voltage-dependent K⁺-selective current, i.e. NIH-3T3 cells, was chosen. NIH-3T3 cells transiently transfected with a plasmid vector (pcDNA3.1+P2A-eGFP; see Methods for further details) encoding the short isoform of Kv 3.1 and voltage-clamped with intracellular and extracellular solutions suitable for isolating K⁺ currents (Table 1) exhibited a large current which was never observed in mock transfected cells (Fig. 5A). This current is therefore the consequence

of the activity of heterologous Kv 3.1. The biophysical fingerprints of this current were reminiscent of those of the endogenous K^+ current measured in HEK 293/293 Phoenix cells, i.e., voltage-dependent activation at depolarizing potentials, rapid activation kinetic and slow time-dependent inactivation at holding potentials above +40 mV (Fig. 5A). Exposure of cells to 0.1 mM niflumic acid either led to an inhibition of peak current (~17% and ~39% immediately after and 5 minutes after exposure to niflumic acid, respectively, Fig. 5B) or significantly accelerated the inactivation of the current in time (Fig. 5C). These effects were not observed following exposure of cells to the vehicle (Figs. 5B and C). These results show that Kv 3.1 is sensitive to niflumic acid and further support the concept that Kv 3.1 sustains the voltage-dependent K^+ -selective current in HEK 293/293 Phoenix cells.

Discussion

Voltage-gated (Kv), outwardly rectifying K^+ currents of several hundreds of picoamperes detectable with the whole-cell configuration of patch-clamp were reported in human embryonic kidney (HEK 293) [29-32] and HEK 293-derived cell lines ([21, 26] and Figs. 1A, 2A and 3A), and were previously characterized in detail. Shortly, these currents have an activation potential around -20 mV, show a rapid activation kinetic and slow time-dependent inactivation at holding potentials above +40 mV, are blocked by tetraethyl ammonium (TEA) and Cs^+ and are highly selective for K^+ [21]. These currents are active in an isotonic medium (Fig. 1A, 2A and 3A, high K^+) and exhibit a partial and slow spontaneous rundown in time after establishing the whole cell configuration and maintaining a holding potential of -60 mV (11.5% at +100 mV in 5 minutes, Fig. 1A). Exposure to a hypotonic extracellular medium does not further activate these currents but appears to slow their rundown [26].

The chloride currents elicited in HEK 293 Phoenix cells following exposure to an extracellular hypotonic medium (Figs. 1B, 2B and 3B) reproduce the biophysical properties of the swelling-activated chloride current ICl_{swell} , including outward rectification and slow time-dependent inactivation at holding potentials higher than +40 mV [22, 24-26].

Messenger RNA of both the delayed rectifier (I_K) and transient (I_A) outward Kv channel family members were detected in HEK 293 cells [31, 32]. However, the fast activation and little to no inactivation kinetics suggest that delayed rectifier (I_K) channels may be primarily implicated in generating this current. Accordingly, abundant Kv 3.1 mRNA was found in HEK 293 Phoenix cells (Fig. 4A). Interestingly, the same mRNA was found in HEK 293 cells, which possess the voltage-gated K^+ current, but not in HeLa, NIH-3T3 or primary proximal tubule cell cultures (Fig. 4E), where the voltage-gated K^+ current was not detected. Primary proximal tubule, HeLa and NIH-3T3 cells were used as models of epithelial cells of kidney origin, extra-renal epithelial cells and non-epithelial cells, respectively. Therefore, the voltage-gated, outwardly rectifying K^+ current detectable in HEK 293 and HEK 293 Phoenix does not represent a common feature of cells of kidney or epithelial derivation, but rather seems to be characteristic of HEK 293 and HEK 293 Phoenix. The presence of Kv 3.1 protein in HEK 293 Phoenix cells was confirmed by Western blot (Fig. 4C), and Kv 3.1 mRNA silencing (Fig. 4B) was accompanied by a significant (~45%) decrease of the voltage-gated K^+ current (Fig. 4D). Based on these evidences, it is reasonable to assume that Kv 3.1 is the main molecular entity underlying the voltage-gated K^+ current in HEK 293/HEK 293 Phoenix cells, although a minor contribution of other Kv family members cannot be ruled out.

The human voltage-gated K^+ channel Kv 3.1 gene encodes a protein (KCNC1) resembling the *Drosophila* Shaw subfamily channel type, and is predominantly - although not exclusively - expressed in excitable cells [16], where it regulates neuronal excitability [33], neural precursor cell proliferation and differentiation [34] as well as muscle contraction [35], amongst others. Specifically in neurons, the Kv 3.1 biophysical properties (i.e., the positively shifted voltage dependency and very fast deactivation rate after membrane repolarization) can enable fast repolarization of action potentials without compromising action potential initiation and are therefore essential for proper activity of neurons characterized by repetitive firing at high

frequency, such as auditory [36] and fast-spiking inhibitory GABAergic interneurons in the mammalian cortex [37-39]. Also, Kv 3.1 is involved in regulating the duration and amplitude of the presynaptic potential, thus controlling evoked neurotransmitter release [40]. Given their essential role in modulating neuronal activity, reduction of Kv 3.1 channel expression and/or activity has been linked to neurodegenerative disorders such as Alzheimer's disease [41], inherited ataxia [42, 43], schizophrenia [44] and intellectual disability [45]. Recently, a role of Kv 3.1 in causing a distinct form of progressive myoclonus epilepsy called myoclonus epilepsy and ataxia due to potassium channel mutation (MEAK) has been described [46]. Specifically, loss of Kv 3.1 function in inhibitory GABAergic interneurons and cerebellar neurons due to genetic mutation was found to lead to myoclonus and seizures accompanied by ataxia and tremor [46-48]. Positive modulators of Kv 3.1 channels are envisioned as potential therapeutic agents in the treatment of epilepsy, hearing disorders, schizophrenia and cognitive impairments [49].

Besides neurons and skeletal muscle, Kv 3.1 channels are also expressed in various other tissues and cells including B lymphocytes [50], germ cells, lung and testis [16], but not in cell of renal derivation, such as renal inner medullary collecting duct [51], BHK21 [52] or proximal tubule cells (Fig. 4E). This observation points to the fact that HEK 293/293 Phoenix cells possess peculiar properties with respect to other cells of epithelial origin, express channel proteins normally found in excitable cells and, due to the presence of large endogenous Kv currents, are not ideal for the characterization of heterologous Kv channels. However, these cells are readily available, easy to cultivate, guarantee a relatively high throughput when employed for patch clamp and can be used to test the pharmacological properties of molecules targeting Kv 3.1 channels. As previously mentioned, Kv 3.1 is activated upon depolarization in neurons and participates in the re-polarization of membrane potential. In HEK 293/293 Phoenix cells, most likely the membrane potential never reaches values allowing for the activation of Kv 3.1 channel. Therefore, the physiological role of Kv 3.1 in these cells remains undetermined.

The gene encoding Kv 3.1 (NG_041827.1) gives rise to two different transcripts (NM_001112741.1 and NM_004976.4) by alternative splicing. These transcripts encode two distinct Kv 3.1 protein isoforms that differ at their C-termini: a longer isoform (NP_001106212.1, designated in the literature as "isoform 1", "b" or "alpha"), and shorter isoform (NP_004967.1, called "isoform 2", "a" or "beta"). Kv 3.1 alpha was the predominant splice variant found in neurons of the adult brain, whereas Kv 3.1 beta appeared to be the predominant species in embryonic and perinatal neurons, at least in rat. Kv 3.1 beta mRNA was also detected in the adult rat brain, although at a lower levels compared to Kv 3.1 alpha [53]. Both isoforms appear to be functional [54]. Accordingly, in HEK 293/293 Phoenix cells, which originated from embryonic tissue, only the transcript encoding the shorter isoform (Kv 3.1 beta) was detected (Fig. 4F), while only the transcript encoding the longer isoform (Kv 3.1 alpha) was detected in human adult brain (data not shown).

Given the importance of Kv 3.1 channels in human physiology and pathology, a complete understanding of their pharmacological properties is mandatory. Notably, Kv 3.1 current is sensitive to both 4-aminopyridine (IC₅₀ = 29 μM) and TEA (IC₅₀ = 0.2 mM) [16]. Here, it is shown that the chloride transport inhibitor DIDS leads to alteration of Kv 3.1 current properties, as demonstrated by acceleration of the current inactivation kinetics, minor, but significant peak current inhibition at 1 μM and a sizeable (>20%) peak current inhibition at 1 mM (Table 2). The effect of DIDS appeared to be significantly more pronounced on Cl⁻ rather than K⁺ currents only at concentrations ≥0.1 mM (Table 2 and Fig. 1). Similarly, 0.01-1 mM of the chloride channel blocker NPPB led to a significant inhibition of both peak K⁺ and Cl⁻ currents (Fig. 2 and Table 2). The effect of NPPB appeared to be significantly more pronounced on Cl⁻ than K⁺ currents only at concentrations ≥0.1 mM and after prolonged (5 minutes) exposure (Table 2).

Niflumic acid (<https://www.drugbank.ca/drugs/DB04552>, accessed on the 5th of September 2017) is a member of the fenamate class of nonsteroidal anti-inflammatory drugs and was originally developed for the treatment of rheumatic disorders [55]. This drug and

its derivatives are widely used in clinics for the relief of chronic and acute pain conditions [56]. Niflumic acid is also known as an inhibitor of several anion channels, including Ca^{++} -activated [57-59] and swelling-activated [12] Cl^- channels, and was reported to variably affect different membrane ion transport systems, such as anion exchangers [28, 60], the glycine [61], GABA(A) [62] and N-methyl-D-aspartate [63] receptors and T-type calcium channels [64]. The findings presented here show that 0.01-1 mM niflumic acid, despite being more potent on the swelling-activated Cl^- current, also significantly blocked the K^+ current (Table 2 and Fig. 3). The plasma concentration of niflumic acid after administration of a single oral therapeutic dose of 750 mg talniflumate to healthy human volunteers approximates values of 4000-7000 ng/ml (i.e. 14-25 μM) [65, 66], thus falling within the concentration range capable of affecting the activity of Kv 3.1 channels. Therefore, the use of niflumic acid and its prodrugs in the treatment of inflammatory conditions in patients with concurrent pathologies linked to Kv 3.1 dysfunction may result contraindicated.

To our knowledge, this is the first report of an effect of chloride channel blockers on Kv 3.1 currents. The findings presented here indicate that sensitivity of a physiological phenomenon to either NPPB, DIDS or niflumic acid does not necessarily imply an involvement of Cl^- transport but can also be explained by an inhibition of voltage-dependent K^+ currents, and possibly of the Kv 3.1 channel.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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