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Bumetanide hyperpolarizes Madin-Darby canine kidney cells and enhances cellular gentamicin uptake via elevating cytosolic Ca^{2+} thus facilitating intermediate conductance Ca^{2+} -activated potassium channels

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Abstract

Loop diuretics such as bumetanide and furosemide enhance aminoglycoside ototoxicity when co-administered to patients and animal models. The underlying mechanism(s) is poorly understood. We investigated the effect of these diuretics on cellular uptake of aminoglycosides, using Texas Red-tagged gentamicin (GTTR), and intracellular/whole-cell recordings of Madin-Darby Canine kidney (MDCK) cells. We found that bumetanide and furosemide concentration-dependently enhanced cytoplasmic GTTR fluorescence by ~60%. This enhancement was suppressed by La^{3+} , a non-selective cation channel (NSCC) blocker, and by K^{+} channel blockers Ba^{2+} and clotrimazole, but not by tetraethylammonium (TEA), 4-aminopyridine (4-AP) or glipizide, nor by Cl^{-} channel blockers diphenylamine-2-carboxylic acid (DPC), niflumic acid (NFA), and $\text{CFTR}_{\text{inh}}-172$. Bumetanide and furosemide hyperpolarized MDCK cells by ~14 mV, increased whole-cell I/V slope conductance; the bumetanide-induced net current I/V showed a reversal potential (V_r) ~-80 mV. Bumetanide-induced hyperpolarization and I/V change was suppressed by Ba^{2+} or clotrimazole, and absent in elevated $[\text{Ca}^{2+}]_i$, but not affected by apamin, 4-AP, TEA, glipizide, DPC, NFA or $\text{CFTR}_{\text{inh}}-172$. Bumetanide and furosemide stimulated a surge of Fluo-4-indicated cytosolic Ca^{2+} . Ba^{2+} and clotrimazole alone depolarized cells by ~18 mV and reduced I/V slope with a net current V_r near -85 mV, and reduced GTTR uptake by ~20%. La^{3+} alone hyperpolarized the cells by ~-14 mV, reduced the I/V slope with a net current V_r near -10 mV, and inhibited GTTR uptake by ~50%. In the presence of La^{3+} , bumetanide caused negligible potential or I/V change. We conclude that NSCCs constitute a major cell entry pathway for cationic aminoglycosides; bumetanide enhances aminoglycoside uptake by hyperpolarizing cells that increases cation influx driving force; and bumetanide-induced hyperpolarization is caused by

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Disclosures

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elevating the intracellular Ca^{2+} and thus a facilitation of the intermediate conductance Ca^{2+} -activated K^+ channels.

Keywords

aminoglycoside; ototoxicity; loop diuretics; membrane potential; ion current; chloride channels; cytosolic calcium

Loop diuretics, such as bumetanide or furosemide, enhance the ototoxicity of aminoglycoside antibiotics when these two classes of drugs are co-administered to mammals including humans [1–3]. Current limited knowledge leads us to hypothesize that this synergism involves pharmacological summations and pharmacodynamic interactions.

Loop diuretics themselves are ototoxic at high doses, and can cause either a temporary, or in some cases, a permanent hearing loss in patients. In animal models, these drugs induce edema in the stria vascularis, a decrease of the endocochlear potential and a temporary loss of auditory function [4, 5]. Loop diuretic-induced edema in the stria vascularis is dependent on Na^+ extrusion into the intra-strial space by Na^+/K^+ -ATPase activity at the basolateral membranes of marginal cells and the presence of Na^+ in perilymph [6, 7]. Loop diuretics are well known inhibitors of $\text{Na}^+/\text{2Cl}^-/\text{K}^+$ cotransporter (NKCC) [8]. Therefore, loop diuretic-induced edema is thought to be due to an imbalance between the NKCC and Na^+/K^+ -ATPase activities in stria marginal cells, but the exact mechanism is not clear. Whether the pharmacotoxicology of loop diuretics is additive with aminoglycoside ototoxicity remains substantiated.

Cochlear and vestibular hair cells, as well as kidney proximal tubule cells are particularly susceptible to aminoglycoside toxicity once the drug enters the cell, leading to cell death and subsequent deafness, vestibular deficits and/or kidney dysfunction [2, 9–11]. Aminoglycosides are lipid-insoluble multivalent cations that can rapidly enter sensory hair cells via their mechanoelectrical transduction (MET) channels and less rapidly by endocytosis at the apical membrane [12–14]. On the other hand, aminoglycoside influx through hair cell basolateral membrane appears negligible [14–16]. Systemically-administered aminoglycosides predominantly cross the multi-layered, cell-based blood-labyrinth-barrier (BLB) in the stria vascularis to enter cochlear endolymph and hair cells to exert their ototoxicity [13, 16]. Loop diuretics have long been known to increase the entry of systemically-administered aminoglycosides into cochlear endolymph [17], suggesting that loop diuretics enhance the trafficking of aminoglycosides across the stria BLB. Based on this mechanism, one plausible pharmacotherapeutic strategy to prevent the aminoglycoside-induced ototoxicity is to selectively block aminoglycoside trafficking across the stria BLB while maintaining serum levels of aminoglycosides for bactericidal activity. Understanding the cellular mechanisms for trafficking aminoglycosides across the stria BLB is a prerequisite for developing this strategy.

The most direct pathway between the vasculature and endolymph is trafficking across the stria BLB. This barrier is composed at minimum of two cell layers: capillary endothelial cells and marginal cells, but little is known about what regulates aminoglycoside trafficking across their cellular membranes. One potential mechanism is endocytosis and exocytosis [14, 18]. Another is permeation through non-selective cation channels (NSCCs) such as the MET channel and TRP channels, as for fluorescent dyes like FM1-43 [3, 12, 19, 20]. Aminoglycoside permeation by NSCC is also supported by our studies on Madin-Darby canine kidney (MDCK) cells and other kidney epithelial cell lines using Texas Red-tagged gentamicin (GTTR). When endocytosis was suppressed by low temperature, cellular uptake

of GTTR was still present; and the uptake of GTTR was inhibited by high K^+ -induced depolarization and by TRPV channel blockers [21, 22]. GTTR uptake is also correlated to expression of functional TRPV4 [23]. The entry of GTTR into hair cells can be stimulated by TRPA1 agonists [20], further supporting the hypothesis that aminoglycosides can permeate NSCCs with a sufficiently large pore diameter. Like native gentamicin, systemic GTTR enters cochlea and is toxic to hair cells [13, 24].

MDCK cells are a widely-used epithelial cell line with many similarities to stria marginal cells in function and expression of NKCCs, Na^+/K^+ -ATPase and ion channels [25]. The present study used MDCK cells as a cell model to identify cellular mechanisms underlying loop diuretic modulation of cellular uptake of aminoglycosides.

We hypothesized that loop diuretics enhance cellular uptake of aminoglycosides by: (i) activating aminoglycoside-permeant ion channels and/or (ii) hyperpolarization-potential of the electrophoretic driving force for cationic aminoglycoside influx through permeant ion channels. We tested this hypothesis by measuring cytosolic GTTR fluorescence and by analyzing membrane potentials and currents. Our data support the (ii) that bumetanide and furosemide enhance cellular uptake of aminoglycosides through NSCCs by hyperpolarizing MDCK cells via activation of intermediate conductance Ca^{2+} -activated K^+ -channels (IK). This activation of IK represents a novel pharmacological action of loop diuretics in addition to its known inhibition on $Na^+/K^+/Cl^-$ co-transporters and aquaporins [26].

Methods

Conjugation and Purification of GTTR

An excess of gentamicin (in K_2CO_3 , pH 10) was mixed with Texas Red (TR) succinimidyl esters (Invitrogen, CA) [27] and, from the reaction mixture, the conjugate (GTTR) was separated from unconjugated gentamicin and potential contamination of unreacted TR by reversed phase chromatography using C-18 columns (Burdick and Jackson, Muskegon, MI) [21, 28]. The isolated GTTR conjugate was aliquoted, lyophilized, and stored desiccated, in the dark at $-20^\circ C$ until required.

Cell Culture

MDCK cells were cultured in antibiotic- and phenol red-free Dulbecco's minimal essential medium (MEM, Invitrogen, CA) with 10% fetal bovine serum at $37^\circ C$ with 5% CO_2 . These cells were a subclone of the parental cell line initially used to reduce the heterogeneity [21]. For GTTR experiments, cells were seeded into 8-well cover-glass chambers (ISC BioExpress) at 3000 cells/well and grown for 24 h, when cells had become ~90% confluent and had developed tight junctions. For electrophysiological experiments, cells were seeded into glass-bottomed 35 mm Petri dish for ~24 h allowing the cells to attach to the bottom glass and grow to ~10% or 90% confluent for single-cell patch recordings or sharp electrode conventional intracellular recordings, respectively.

GTTR Uptake and Pixel Intensity Analysis

MDCK cells cultured to ~50% confluent (18 – 22 h after plating) on chambered cover-slips were washed 3 times with aerated normal extracellular solution (NES) containing (in mM): NaCl 138, KCl 5, $CaCl_2$ 1.6, $MgCl_2$ 1.2, Na-HEPES 5, HEPES 6, glucose 7.5, with pH 7.4 and osmolarity of 300 mOsm L^{-1} , and then treated with GTTR ($5\text{ }\mu\text{g ml}^{-1}$ in NES) for 30 s as the control. We confirmed previously that the GTTR fluorescent signal in MDCK cells is proportionate to the incubation duration and dose; and that a 30 s incubation at room temperature minimized GTTR uptake by endocytosis [21, 28]. Cellular loading of GTTR was also tested with pretreatments of (i) bumetanide or furosemide ($1\text{--}100\text{ }\mu\text{M}$) in NES for

3 – 10 min, (ii) ion channel blocking agents such as La^{3+} , Ba^{2+} , TEA, 4-AP, clotrimazole, DPC, NFA etc. for 3 min, (iii) ion channel blocking agents with 10 μM bumetanide for 3 more min, immediately followed by 30 s incubation with GTTR (5 $\mu\text{g}/\text{ml}$) in each pretreatment solution. After GTTR incubation, cells were washed three times in NES, then fixed and delipidated with 4% formaldehyde plus 0.5% Triton X-100 (FATX) for 45 min [21]. Following fixation, cells were thoroughly rinsed with phosphate buffered saline (PBS, Invitrogen, CA) prior to confocal imaging.

All GTTR specimens were imaged directly on the chambered cover-slip using a Bio-Rad MRC 1024 ES laser scanning confocal system attached to a Nikon Eclipse TE300 inverted microscope. GTTR images were acquired using 1024 $\times$ 1024 pixel box size using a 60x lens (n.a. 1.4) with an xy resolution = 230 nm and xz resolution = 440 nm. All specimens from the same experiment were imaged at the same laser intensity and gain settings. Representative images from each experiment were identically prepared using Adobe Photoshop. Optical sections from each experimental set were manually segmented for cytoplasmic pixel intensity determination (ImageJ, NIH) (supplemental figure 1 [sFig. 1]). GTTR fluorescence is largely localized in the cytosol when compared to nucleolus and the nucleoplasm, with the typical pixel intensity of 184 ± 17.7 , 175 ± 30.4 and 125 ± 25.8 ($p < 0.001$, $n = 46$) in arbitrary unit, and occupies 58%, 5% and 37% of the total pixels (area), respectively. To minimize error, quantification of GTTR uptake was limited to the cytosol (cellular pixels minus nuclear pixels). The intensity mean and S.E. of the mean was normalized against the standard (control data) in the same experimental set. Student's t -test was used to determine any significant difference between treatment groups.

Imaging Analysis of Intracellular Ca^{2+}

MDCK cells at ~50% confluency on 18 mm round glass coverslips were preloaded with the fluorescent dye Fluo-4 (4.6 μM in culture media) for 60 min at room temperature. The coverslip was clamped into a magnetic chamber system (Chamlide CMB, Quorum Canada); excess Fluo-4 was washed away and the chamber was filled with 1 ml normal extracellular solution (NES). Images were acquired on a Hamamatsu OrcaER CCD camera every 5 sec over 15 min with a Yokogawa CSU10 spinning disk confocal system with focus drift correction (Nikon TE2000 PFS). Laser-based 488-nm excitation was combined with filter based emission detection (500–550 nm). Image data acquisition under control conditions began after locating cells (Fig. 8A) akin to those used for electrophysiology. The final concentration of bumetanide (10 μM) application was achieved by replacing 0.5 ml NES with an equal volume of 20 μM bumetanide in NES (Fig. 8B). Fiji/ImageJ software was used for data quantitation and analysis.

RT-PCR

Total RNA was extracted from MDCK cells using RNeasy Mini kit (Qiagen), and first-strand cDNA was synthesized from 5 μg of total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). For PCR, 0.5 μl of the reverse-transcribed cDNA was used in each reaction catalyzed by AmpliTaq DNA polymerase (Applied Biosystems). The sequence information of the primers used is available in Supplementary data (sFig. 6). The PCR condition used is the initial 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min for all reactions. PCR was performed in an Applied Biosystems thermal cycler. PCR products were analyzed by electrophoresis in 2% agarose gel.

Intracellular and Tight-seal Whole-cell Recordings

Conventional intracellular recordings were conducted on 90% confluent MDCK cells in a glass-bottomed 35-mm culture dish continuously superfused with a HEPES-buffered NES

(see above for composition) at 35 C, the same solution for GTTR uptake analysis and whole-cell recording. A molded silicon rubber block was placed in the culture dish to form a bathing chamber sized <0.5 ml in the dish center. For comparison, HCO_3^- -containing Krebs solution was used for some cells, composed of (in mM): NaCl 125, KCl 5, CaCl_2 1.6, MgCl_2 1.2, NaH_2PO_4 1.2, NaHCO_3 19, glucose 8.3, and saturated with 95% O_2 and 5% CO_2 at 35 C (pH 7.4). Using sharp microelectrode filled with 2 M KCl or K^+ -acetate with a tip resistance of 60–120 M Ω , intracellular penetration was obtained with assistance of a micromanipulator (MP-1 Narishige, Japan). The membrane potential (V_m) was monitored by an Axoclamp 2B preamplifier (Molecular Device, Inc. USA) in current-clamp bridge mode. No current injection was employed. Electrical signals were recorded on a PC computer equipped with pClamp9 software (Molecular Device) using sampling intervals of 2 ms. The initial V_m was determined by the voltage jump at the penetration plus ~5 min stabilization thereafter, and confirmed by the voltage jump upon dislodge of the electrode as we did previously [29].

Conventional and perforated patch whole-cell voltage- and current-clamp experiments were conducted mostly on single or small clustered two or three MDCK cells at room temperature with assistance of Axopatch 1-D preamplifier, Digidata 1322A digitizer and pClamp9 software. The method of conventional whole-cell recording was similar to that published previously [30, 31]. Briefly, the glass micropipette of ~1 m tip (4 – 7 M Ω) was filled with a normal internal solution (NIS) containing (in mM): K-gluconate 130, NaCl 10, CaCl_2 2.0, MgCl_2 1.2, HEPES 10, EGTA 5 (free Ca^{2+} =118 nM) and glucose 7.5 (pH7.2 and 290 mOsm l^{-1}). For an elevated Ca^{2+} internal solution (hiCaIS), the EGTA was removed, CaCl_2 was reduced to 2 μM and 8 mM K-gluconate was added to bring up osmolality back to 290 mOsm l^{-1} . A “salt bridge” was routinely used for the reference electrode constructed with Ag/AgCl wire in 4% agar/2 M KCl gel. The liquid junction potential of the electrodes filled with NIS and hiCaIS was 14.3 and 15.1 mV, respectively, in the NES bathing solution, calculated by a tool in pClamp9 software. The liquid junction potential value was used to correct V_m values during off-line data analysis or in setting of on-line correction for conventional whole-cell recording experiments [32].

Whole-cell configuration was achieved by applying negative pressure to rupture the membrane at the tip of an electrode pipette when a giga-ohm seal was obtained. For perforated patch, the pipette was back-filled with a freshly-made solution of nystatin (200 $\mu\text{g ml}^{-1}$) or gramicidin (60 $\mu\text{g ml}^{-1}$) in the NIS; in the latter case, the conducting pores are permeable to monovalent cations (K^+ , Na^+ etc.) but not to Cl^- [33]. Successful whole-cell configuration was indicated by an access resistance between 10 – 20 M Ω for conventional and 20 – 80 M Ω for perforated patch. The access resistance (R_a) was compensated off-line as we described previously [31]. The input capacitance (C_{input}) was not compensated online in order to monitor the access resistance change and to off-line calculate the C_{input} [30]. The current and voltage signals data were filtered at 5 kHz (–3 dB) and digitally recorded in a computer at sampling intervals of 10, 20, 100 μs as needed. A simultaneous gap-free recording was carried out by a Minidigi digitizer and Axoscope 9.2 software at a sampling interval of 50 ms. The input conductance (G_{input}) was measured by 20 mV step-induced steady-state current or the I/V slope between –40 and –60 mV.

Drug Application

Drugs at known concentrations or bathing solutions with different ionic compositions were applied by superfusion of cells within the petri dish. The distance from the tube mouth to the recorded cell was ~150 μm in whole-cell experiments. The exchange of solutions was achieved in seconds by switching the solution reservoirs without affecting the superfusion speed or bath temperature; but for in intracellular recording setting, it took ~30 s for new solution to reach the bath and 2 min to complete the solution exchange. The drugs used in

this study include acetylcholine (ACh), bumetanide (BUM), CFTR_{inh}-172 (CF172, a gift from Cystic Fibrosis Foundation, USA)[34], clotrimazole (CLT), chromanol 293B, diphenylamine-2-carboxylic acid (DPC), Fluo-4 (Invitrogen, USA), furosemide (FUR, Hospira Incorp, USA), glipizide (Glip), gramicidin, niflumic acid (NFA), nitrendipine (NTDP), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), all from Sigma, USA, unless indicated otherwise.

Results

Loop Diuretics Increase Cellular Uptake of GTTR

Exposure of MDCK cells to bumetanide increased intracellular GTTR fluorescence in most cells (n=24 wells from 6 experiments) and this enhanced fluorescence was bumetanide concentration-dependent (Fig. 1A,C). The GTTR fluorescence was maximally increased by 100 μ M bumetanide to $170 \pm 15\%$ of control values. Higher concentrations of bumetanide caused more variable fluorescence intensities, probably due to nonspecific actions on the cells. Furosemide also increased cytoplasmic GTTR fluorescence in a dose-dependent manner (Fig. 1B,C). Hill equation fitting of the data in Figure 1C and D revealed an EC₅₀ of 0.51 ± 0.066 and 1.6 ± 0.58 μ M for bumetanide and furosemide, respectively. Therefore, we used sub-maximum dose of 10 μ M bumetanide and 30 μ M furosemide for quantitative analysis of their membrane actions.

To test whether bumetanide-enhanced fluorescence was due to a decrease in intracellular Cl⁻ level caused by bumetanide inhibition of NKCC [8], GTTR uptake was compared between 3 min and 10 min pre-incubation of bumetanide. Results showed no significant difference between these two treatment groups (-13.1 ± 3.2 vs. 12.8 ± 3.9 mV, n=6 each, $p > 0.05$; see discussion).

La³⁺ Inhibits GTTR Uptake and NSCC Conductance

We previously reported that the non-selective cation channel (NSCC) blocker La³⁺ (0.05 – 5 mM) reduced GTTR uptake [22]. In this study, we observed that La³⁺ at 1 mM inhibited GTTR uptake by $48 \pm 2.1\%$ (Fig. 2). La³⁺ inhibition on GTTR uptake was concentration-dependent in the range of 0.05 to 1 mM; 5 mM La³⁺ caused a slightly lower inhibition ($41 \pm 4.2\%$, $p > 0.05$ when compared to 1 mM). In the presence of 0.1 and 1 mM La³⁺, 10 μ M bumetanide had no significant effect on cellular uptake of GTTR, indicating that bumetanide failed to overcome La³⁺ inhibition of GTTR uptake.

In conventional intracellular recording experiments, La³⁺ (0.1 and 1 mM) hyperpolarized the majority of cells in a dose-dependent manner (-3.7 ± 0.75 and -14 ± 4.7 mV, n=3 and 8, respectively; Fig. 3A), suggesting that a La³⁺-sensitive NSCC conductance played a substantial role in determining V_m in these subconfluent MDCK cells.

In whole-cell recordings from single or small clusters of tightly-coupled MDCK cells (5 cells), 0.1 – 5 mM La³⁺ consistently reduced the slope conductance in entire range of whole-cell I/V curves (Fig. 3D), which was also associated with a hyperpolarization in V_m (zero current potential at the I/V plot, Fig. 3D). Moreover, in the presence of La³⁺, 10 μ M bumetanide induced no significant changes in the I/V curves (see below for I/V change and hyperpolarization by bumetanide alone). The I/V curve of La³⁺-sensitive net current reversed its polarity at -9.2 ± 4.1 mV (n=6), consistent with blockade of NSCCs. The input conductance (G_{input}) was reduced by 1 mM La³⁺ from 0.83 ± 0.41 nS to 0.40 ± 0.25 nS in single cells (n=6, $p < 0.01$, paired *t*-test). Multiple cell (2–5 cells) clusters showed a higher control G_{input} and a bigger La³⁺-induced G_{input} reduction (e.g., 5 cells in Fig. 3B–D). Conversely, in the presence of bumetanide, adding La³⁺ exerted similar inhibition of I/V

slope ($\Delta G_{\text{input}} = -0.31 \pm 0.18 \text{ nS}$) as it did when La^{3+} was given alone ($-0.33 \pm 0.12 \text{ nS}$, $n=3$ each, $p>0.05$).

Taken together, these results support the hypothesis that the NSCC is a major entry route responsible for ~50% of cellular uptake of aminoglycosides. The failure of loop diuretics to overcome La^{3+} blockade of the NSCCs suggests that the diuretics have little, if any, direct stimulation of the NSCCs, the primary pathway of GTTR uptake. As furosemide ($10 \mu\text{M}$) has been reported to hyperpolarize MDCK cells by 11 mV [35], we hypothesized that loop diuretics share this hyperpolarizing effect and, when the aminoglycoside-permeant NSCCs are open, cellular hyperpolarization will increase the electrical driving force and facilitate an influx of the cationic aminoglycosides. The electrophysiological experiments below test this hypothesis and sought to identify the mechanism(s) that underlies loop diuretic-induced hyperpolarization.

Bumetanide Hyperpolarizes Cells and Effects of Selected Channel Blockers on V_m and GTTR Uptake

There is a prominent discrepancy in literature regarding the baseline membrane potential of MDCK cells. The MDCK cell membrane potential was reportedly substantially less negative in HEPES-buffered solution (lacking HCO_3^- , -20 to -30 mV) than that in HCO_3^- -containing solution ($\sim -50 \text{ mV}$) [36, 37], and that it was interpreted as that MDCK cells in HEPES buffer lacked K^+ -conductance and the V_m is solely set by Cl^- conductance. Our initial observations showed otherwise (see below). We measured and compared the V_m in five different conditions (Table 1): The results in Table 1 indicate that the V_m level was not significantly different between any two recording conditions. In conditions where cells were superfused with HEPES buffered NES, elevated K^+ (from 5 to 20 mM) caused $8.9 \pm 1.0 \text{ mV}$ depolarization in all 21 cells tested, which was not significantly different from the cells bathed in Krebs solution ($7.81 \pm 0.90 \text{ mV}$, $n=7$, $p>0.05$), indicating a similar background K^+ conductance existing in cells under these different conditions. Henceforth, we show V_m data without distinguishing among the recording conditions unless otherwise noted.

A phenomenon worth to mention is that a slow V_m oscillation often appeared in cells recorded in Krebs solution with sharp electrode (sFig. 2). The oscillation showed peak-to-peak amplitude of 4–19 mV and a cycle length of 6–8 min which often made a quantitative measurement of drug responses impossible thus we had to limit the use of this method.

Superfusion of $10 \mu\text{M}$ bumetanide hyperpolarized the majority of cells tested ($-13.6 \pm 1.6 \text{ mV}$, ranging from 2–45 mV, $n=42$; Fig. 4). Furosemide ($30 \mu\text{M}$) also hyperpolarized cells ($-11.6 \pm 6.6 \text{ mV}$, $n=10$, sFig. 3A), similar to previous reports [35]. Hyperpolarization occurred after ~2 min exposure, often with a fast initial phase followed by a sustained, sometimes oscillatory, phase until the termination of drug exposure. Washout with drug-free solution yielded a slow recovery to control V_m (Fig. 4). No continuing increase in hyperpolarization amplitude was observed with prolonged application of bumetanide (up to 15 min), suggesting that any reduction of intracellular $[\text{Cl}^-]_i$ by the loop diuretic did not significantly contribute to this hyperpolarization. Repeated drug application typically caused decreasing amplitude of responses even after washout periods >10 min. Thus, attempts to determine bumetanide dose-hyperpolarization response relationship was unsuccessful due to the rundown of the responses upon repeated applications and a large variability in the induced hyperpolarization among cells.

Bumetanide-induced hyperpolarization was sensitive to selected K^+ -channel blockers but not to agents affecting Cl^- currents (Fig. 4). Co-application of 1 mM Ba^{2+} , a non-specific K^+ -channel blocker, or $10 \mu\text{M}$ clotrimazole, a blocker selective for intermediate conductance Ca^{2+} -activated K^+ -channel (IK), consistently and reversibly suppressed

bumetanide-induced hyperpolarization (Fig. 4A). On the other hand, bumetanide-induced hyperpolarization was not significantly affected by other channel blockers, including 50 nM apamin for small conductance Ca^{2+} -activated K^{+} -channel (SK), 10 mM tetraethylammonium (TEA) for large conductance Ca^{2+} -activated K^{+} -channels (BK), 10 mM 4-aminopyridine (4-AP, not shown) for voltage-gated K^{+} -channels (K_V), 0.3 mM diphenylamine-2-carboxylic acid (DPC) non-selectively for anion channels, 10 – 100 μM NFA for Ca^{2+} -activated Cl^{-} -channels, and 10 – 30 μM CFTR_{inh}-172 (CF172) for CFTR Cl^{-} -channels, nor by a bath solution with Cl^{-} reduced to 60 mM ($n=3-7$ in each case).

Several channel blockers alone induced membrane potential changes (Fig. 5. and Table 2). Of note, 1 mM Ba^{2+} and elevated (20 mM) K^{+} consistently induced a 5 – 20 mV depolarization while chloride channel blockers caused a small (2 – 5 mV) or negligible hyperpolarization, supporting a notion that the K^{+} electrochemical force is a major contributor of the V_m negativity in MDCK cells whereas the Cl^{-} electrochemical force normally contributes a few mV depolarization in the membrane potential. Moreover, IK blockers clotrimazole and nitrendipine [38] depolarized the majority (~80%) of cells tested with variable amplitudes (2 – 31 mV) whereas little V_m change was observed in response to TEA, 4AP, glipizide and chromanol 293B, suggesting a significant contribution of IK conductance to the membrane potential of the subconfluent MDCK cells. Similar to that reported previously [39], ACh induced a 2 – 26 mV hyperpolarization in the majority of cells tested (sFig. 4, $n=6$ of 8 cells). The ACh-hyperpolarization was often short-lasting (< 2 min), changing to a depolarization during sustained ACh exposure (> 2 min). This ACh-induced hyperpolarization was largely blocked by 10 μM clotrimazole or 1 μM nitrendipine and unmasked a larger depolarization (sFig. 4), substantiating the notion that an IK activation pathway exists in MDCK cells. It is interesting to note that in the presence of 10 μM bumetanide, 10 μM clotrimazole could no longer induce a significant depolarization (Fig. 4A, $n=4$), suggesting a reciprocal antagonism between these two classes of drugs (see more information below).

The effects of these selected channels blockers on GTTR uptake were also consistent with their V_m effect: agents causing depolarization tended to reduce cytosolic GTTR fluorescence whereas agents causing hyperpolarization enhanced GTTR fluorescence (Fig. 5). Ba^{2+} (1 mM), clotrimazole (10 μM) and nitrendipine (1 μM , not shown) significantly reduced GTTR uptake. As expected, NFA hyperpolarized the cells and enhanced GTTR uptake (Fig. 5 and Table 2). Agents with no V_m effect exerted no action on GTTR uptake.

Taken together, the above results suggest that bumetanide enhances GTTR uptake by hyperpolarizing cells via activation of a K^{+} conductance, probably the IK channels. The whole-cell voltage-clamp experiments below further tested this hypothesis.

Bumetanide Activates a K^{+} -Conductance that is Sensitive to IK-Channel Blockers

Bumetanide (10 μM) induced an outward current (5 – 56 pA) at a holding potential of ~-40 mV and an increase in whole-cell I/V slope in the majority of cells tested (Fig. 6). The G_{input} was increased from 0.88 ± 0.38 nS to 1.40 ± 0.25 nS ($n=12$, $p<0.01$, paired t -test) or net increase of 0.48 ± 0.42 nS near -50 mV. The bumetanide-induced net current (Net I_{bum}) I/V curves showed a mild outward rectification with a reversal potential at -80 ± 8.2 mV, suggesting activation of a K^{+} -conductance. In 0.5 – 5 s step voltage-command induced currents, the bumetanide-induced net current showed little time-dependent changes during the pulses. The activation of whole-cell conductance was partially reversible after drug-free wash for > 5 min. The responses exhibited significant rundown with repeated application of bumetanide.

Addition of 10 μM clotrimazole (CLT, $n=4$), the selective blocker for IK, quickly reversed the bumetanide-induced whole-cell I/V change (Fig. 7) [38, 40]. Similar inhibition was also observed by 1 mM Ba^{2+} and 1 μM nitrendipine ($n=3$ each). On the other hand, 10 mM TEA, 4-AP and 300 μM DPC did not significantly change the bumetanide-induced I/V changes ($n=3$ for each drug). Conversely, in the presence of 10 μM clotrimazole or 1 mM Ba^{2+} , 10 μM bumetanide no longer induced a significant change of the I/V relation or a very small response (sFig. 5, $n=6$ for each case). It is interesting to note that, in the presence of bumetanide or furosemide, clotrimazole failed to block the K^+ conductance to a level below the control and depolarize the cell above its base level (Fig. 4 and 7, sFig. 3); and conversely, in the presence of clotrimazole or Ba^{2+} , bumetanide no longer induced a significant K^+ -conductance and a hyperpolarization (sFig. 5, Fig. 4 and 7). This reciprocal antagonism may suggest that bumetanide/furosemide and clotrimazole/ Ba^{2+} act at a common site – perhaps at the gating of the IK channel – in a competitive manner. Results from GTTR uptake experiments (see below, Fig. 9) are consistent with this hypothesis.

Furosemide (30–100 μM) also activated I/V slope (sFig. 3, by 0.44 ± 0.62 nS, $n=4$) with a net current I/V reversal potential at -81 ± 4 mV. Similar to bumetanide, furosemide-induced conductance was suppressed by 10 μM clotrimazole as well as 1 mM Ba^{2+} but not by TEA or 4-AP ($n=2$). In the presence of 10 μM clotrimazole, furosemide (30 μM) failed to induce a significant change in whole-cell I/V relation ($n=4$).

Bumetanide-induced hyperpolarization Is Associated with a Surge of Intracellular Ca^{2+}

In the next step, we tested the hypothesis that loop diuretics activate a surge of intracellular Ca^{2+} to indirectly facilitate the IK conductance (Fig. 8). First, in cells loaded with fluo-4, 10 μM bumetanide caused a significant elevation of the intracellular fluorescence, indicating a surge of the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Fig. 8A,B). The $[\text{Ca}^{2+}]_i$ signal often showed spiky fluctuations under both control and stimulated conditions. On average of tens of cells, the control $[\text{Ca}^{2+}]_i$ signal exhibited a stable level above the extracellular background with a slow rundown likely due to photobleaching, since the rundown speed was associated with the exposure of excitation laser (strength and duration, data not shown). The bumetanide-stimulated surge of signal had a fast onset, reaching its peak about 2 times the control level in less than 20 s and then slowly decayed afterwards (Fig. 8B). (Fig. 8B). Furosemide (30 μM) showed similar effect on the $[\text{Ca}^{2+}]_i$ signal (23 cells from one plate). Second, when loaded with 2 μM Ca^{2+} via the internal solution of the recording pipette, the cells all showed a hyperpolarized membrane potential, compared to those recorded with the normal internal solution (Table 1). In these cells, bumetanide and furosemide failed to induce a hyperpolarization in all cells tested ($n=5$, Fig. 8C), suggesting that the loop diuretics-induced surge of $[\text{Ca}^{2+}]_i$ is masked by a high $[\text{Ca}^{2+}]_i$, or the drug-induced increase in $[\text{Ca}^{2+}]_i$ no longer be able to cause an additional opening of IK and hyperpolarization due to the saturated effect of high $[\text{Ca}^{2+}]_i$.

Enhanced Cellular Uptake of GTTR by Bumetanide is Inhibited by IK Blockers

Based on these electrophysiological results, we tested the effect of IK channel blockers on bumetanide-enhanced GTTR uptake (Fig. 9). In the presence of 1 mM Ba^{2+} or 10 μM clotrimazole, bumetanide was no longer able to enhance cellular GTTR uptake. Interestingly, bumetanide plus clotrimazole or nitrendipine, GTTR fluorescence levels were not lower than those of control cells (GTTR alone; compare to Fig. 5), again indicative of a reciprocal antagonism between IK blockers and bumetanide. On the other hand, K^+ -channel blockers 10 mM TEA, 4-AP and 3 μM glipizide and Cl^- -channel blockers 300 μM DPC, 100 μM NFA and 10 μM CF172 did not inhibit bumetanide-enhanced GTTR uptake ($n=3$ –6 in each case).

Gene Expression of Selected TRP Channels, NKCC1 and Na/K-ATPase

To identify any possible phenotype changes in the MDCK cells used in this study, gene expression of selected TRP channels, Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) and Na/K ATPase was determined with RT-PCR (sFig. 6). RNA transcripts of TRPV4, TRPA1 and NKCC1 were detected at high levels. Clear bands for TRPV1, TRPV2, and the alpha-1 subunit of Na⁺/K⁺ ATPase were also detected, although the size of TRPV2 band (~500 bp) was inconsistent with the predicted size of 380 bp. Since the gene sequence information was obtained from the predicted sequence of the dog genome, it is possible that additional bases are inserted in canine TRPV2. A band for TRPV3 was also detected but at a very low level, consistent with its typical expression in neural tissues. The results are consistent with previous reports for MDCK cells [22, 23, 25].

Discussion

The present study sought to clarify the cellular mechanism underlying enhanced cellular uptake of aminoglycosides by loop diuretics. Data showed that application of bumetanide or furosemide increased cellular uptake of GTTR in a dose-dependent manner. Moreover, we for the first time demonstrated that bumetanide and furosemide hyperpolarize MDCK cells, increasing the electrical driving force for influx of cationic aminoglycosides through NSCCs. Remarkably, we found that these loop diuretics hyperpolarize the cells mainly, if not solely, via stimulating an increase in cytosolic Ca²⁺, thus to activate the intermediate conductance Ca²⁺-activated K⁺-channels (IK). This is a surprising novel finding on these diuretics pharmacology.

This novel effect of loop diuretics is supported by the following main results: 1) Bumetanide and furosemide hyperpolarize the majority of subconfluent and single cells, and prolonged application did not increase the amplitude of hyperpolarization. 2) Bumetanide-induced hyperpolarization was attenuated by Ba²⁺ and IK blocker clotrimazole but not by apamin, TEA, 4-AP, low Cl⁻ and selected non-specific and specific Cl⁻-channel blockers. 3) Bumetanide activated a whole-cell conductance which I/V exhibited a reversal potential near the calculated K⁺ equilibrium potential (-86 mV). The activated I/V component was suppressed by adding 10 μM clotrimazole. 4) Bumetanide/furosemide stimulated a quick surge of cytosolic Ca²⁺ that slowly subsided over the 5 min of drug application. Cells with loaded high Ca²⁺ showed a hyperpolarized membrane potential and became unresponsive to these diuretics. 5) In the presence of clotrimazole or Ba²⁺, bumetanide could no longer activate the IK current or enhance GTTR uptake.

These findings add a novel aspect to our understanding of the loop diuretic enhancement of aminoglycoside toxicity, as well as a new pharmacological action of the loop diuretics.

Membrane Potential and its Determinant Channels in MDCK Cells

It was widely believed that the membrane potential of MDCK cells is determined by K⁺ and Cl⁻ conductances and their transmembrane concentration gradients [36, 41]. Our extended observations show both agreements and discrepancies with previous reports.

First, the cells used in the present study show a 10 – 20 mV depolarization in response to extracellular exposure to 20 mM K⁺ or 1 mM Ba²⁺ and only a few mV of hyperpolarization in response to Cl⁻ channel blockers NFA and DPC, supporting the contribution of K⁺ and Cl⁻ conductances to membrane potentials in control conditions. On the other hand, the mean value of V_m of -44 mV for cells in HEPES buffer (NES, Table 1), is substantially more negative than that (-22 mV) reported for MDCK cells in HEPES buffer solution that lacks HCO₃⁻ ion [36], and is not significantly different from that of cells in HCO₃⁻-rich buffer in this study or in the literature [35, 42]. The reason for this discrepancy is not fully understood

but the culture duration could be a factor. The cells in this study grown for 24 hours in culture dishes rather than one week in other studies (e.g., [36]). Many cells in our experiments were actively proliferating (Fig. 9 and sFig. 1). It is known that the IK channel plays a key role in cell proliferation [43] and is inversely expressed to the degree of differentiation [44]. We did observe that the IK blocker, clotrimazole, depolarized most cells for up to 32 mV, suggesting a robust background activation of IK in the MDCK cells we used.

Second, previous reports showed that MDCK cells exhibit transcription signals and single channel activity of the TEA-sensitive big conductance Ca^{2+} -activated K^+ channels (BK) [41, 45, 46]. However, we observed only a small (2–4 mV) depolarization by 10 mM TEA in a small portion of cells tested, indicating only a small contribution of this channel to the membrane potential. We did not pursue the reasons for this discrepancy; however, we however, we have an explanation based on that MDCK cells consist of two classes [47]. One class of cells called C7, which resembles the principal cells in the kidney collecting duct, expresses very few BK channels, whereas another class of cells called C11, which mimic the intercalated cells, contains abundant BK channels. The MDCK cells used in this study are from a subclone of the cell line, likely the C7 class. Also, single MDCK cells lack cell-cell contact, that might have suppressed BK expression, as reported previously [48].

Third, in contrast to a report that MDCK type I cells constitutively express functional CFTR channel [49], we failed to see any membrane potential response to $\text{CFTR}_{\text{inh}}-172$, a potent CFTR blocker [34]. Therefore, the channel identity observed here for DPC- and NFA-sensitive Cl^- conductance and in earlier studies [35, 42, 50] remain to be clarified. This clarification will be challenging, as determination of the molecular identity responsible for the Cl^- conductance has proven to be particularly difficult [51, 52].

Finally, and most importantly, we demonstrated that La^{3+} or Gd^{3+} caused a hyperpolarization in the majority of cells under both conventional intracellular and whole-cell recording conditions (Table 2, Fig. 3), suggesting a substantial contribution of NSCC conductance to the V_m by counteracting the hyperpolarizing K^+ current in these cells. It is now known that a large superfamily of channels, the TRP channels, is the mediator of most NSCC currents that sense a variety of physical and chemical stimuli. Indeed, TRPV1, TRPV2, TRPV3, TRPV4 and TRPA1 gene transcripts were identified with TRPV4 and TRPA1 at high levels in the MDCK cells used (sFig. 6). As La^{3+} and Gd^{3+} block a number of TRP channel species [53], the exact channel species for the La^{3+} - and Gd^{3+} -sensitive current observed remains to be clarified. Considering that La^{3+} induces a hyperpolarization which theoretically increases the driving force for cationic aminoglycoside influx, the La^{3+} actually induces inhibition on GTTR uptake (Fig. 2), supporting that GTTR influx must substantially go through the La^{3+} -sensitive channels.

TRP Channels as a Primary Pathway for GTTR Entry into Cells

GTTR permeation through mechanotransduction (MET) and TRP channels has been repeatedly demonstrated [13, 20, 22, 23]. However, the permeant mechanism remains unclear as the estimated end-on diameter of GTTR (1.47 nm) is larger than the reported pore size of the hair cell MET (1.25 ± 0.08 nm)[54] and TRP channels (TRPA1, 1.1–1.38 nm [55]; TRPV1, ~1.0 nm, but may open to 1.4 nm [56]). One plausible explanation is that channel pore sizes are underestimated. Alternatively, the pores are transiently dilated during GTTR entry [13]; however, this requires substantiation.

Based on our findings and others, we speculate that TRPV4 plays a major role, while TRPA1 and TRPV1 have little, if any, role in mediating GTTR uptake in MDCK cells. TRPA1-encoding mRNA is highly expressed in MDCK cells (sFig. 6). TRPA1 agonists

have been demonstrated to mediate GTTR entry into mouse cochlear hair cells even when the MET channels are dysfunctional [20]. However, the TRPA1 channel is normally quiescent at the resting membrane potential until activated by deep cold ($<17^{\circ}\text{C}$) or pungent agonists [57]. These features suggest that TRPA1 is unlikely to play a significant role in mediating the GTTR influx in the present experiments.

The mRNA of TRPV1 is weakly expressed in MDCK cells (sFig. 6), although we have previously reported that GTTR uptake by MDCK cells can be inhibited or enhanced by compounds that modulate TRPV1 channel activity [22]. However, these modulators are no longer considered highly specific for TRPV1 over other TRPV channels [58]. TRPV1 channels are also activated by heat above 43°C , much higher than that in our experiments, and so this channel is unlikely to play a major role in mediating the GTTR uptake.

TRPV4 mRNA is abundantly expressed in MDCK cells (sFig. 6); TRPV4 immunofluorescence was preferentially localized at the basolateral membrane of other kidney epithelial cell-lines, and GTTR uptake is potentiated by TRPV4 overexpression in the kidney cell-lines whereas the TRPV4-dependent GTTR uptake was abolished by a point mutation within the crucial pore region of the channel [23]. TRPV4 is known to be activated by warm temperatures ($27\text{--}34^{\circ}\text{C}$) [57]. Our single channel recording from cell-attached patch in MDCK cells in physiological solutions at 22°C revealed a channel activity with characteristics of the TRPV4 channels [59]. These evidences support a major role for TRPV4 in mediating the observed GTTR uptake by MDCK cells.

Bumetanide-induced Activation of IK vs. Inhibition of NKCC

Loop diuretics are well known inhibitors of $\text{Na}^{+}/\text{K}^{+}/2\text{Cl}^{-}$ cotransporter NKCC [8] expressed by MDCK cells (sFig. 6)[8]. Inhibition of this co-transporter is expected to reduce intracellular Cl^{-} concentration and shift E_{Cl} negatively. When a significant Cl^{-} conductance exists, negative shift of E_{Cl} should result in a hyperpolarization [60]. We believe that the observed hyperpolarization by bumetanide or furosemide was little, if any, related to its inhibition of NKCC. The reasons are four-fold: First, the induced hyperpolarization often showed an abrupt onset, and prolonged application of diuretics did not progressively increase the amplitude of hyperpolarization (Fig. 4), which is inconsistent with the expected time course of the $[\text{Cl}^{-}]_{\text{i}}$ change by co-transport inhibition. Second, the induced hyperpolarization was not inhibited by Cl^{-} channel blockers, DPC, NFA and CFTR_{inh}-172, rather it was suppressed by K^{+} channel blockers Ba^{2+} and IK blocker clotrimazole. Third, the hyperpolarization was associated with an increase, not a decrease, in conductance and with a net current that reversed its polarity at a potential near the calculated E_{K} (-86 mV). Finally, the EC_{50} s of bumetanide and furosemide for GTTR uptake showed a much smaller difference between bumetanide and furosemide (Fig. 1) than that reported for NKCC1 inhibition ($0.33\text{ }\mu\text{M}$ vs. $23\text{ }\mu\text{M}$, [61]).

In agreement with our observations, Lang and co-workers [35] observed that a reduction of extracellular $[\text{Cl}^{-}]$ to half of the control caused only less than 2 mV depolarization, suggesting that Cl^{-} conductance of subconfluent MDCK cells was small in control conditions. Besides, the possible E_{Cl} negative shift and hyperpolarization due to diuretic-induced NKCC inhibition would be counteracted by its concomitant reduction of Na^{+} influx and the resulting downturn of electrogenic $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity. Therefore, the net change of V_{m} by NKCC1 inhibition is expected to be negligible.

Indirect Mechanism of IK Activation by Bumetanide

We investigated how loop diuretics activate the IK conductance in MDCK cells with a hypothesis based on a previous report [36]. Praetorius et al. (2003) reported that gene

transcripts for IK channels (*K_{Ca3.1}* or *SK4*) were detected in MDCK cells, and bending of the primary cilium or mechanically pressing the apical membrane triggers a transient cytosolic Ca^{2+} surge followed, with a long latency, by an IK-activation and hyperpolarization in MDCK cells [36]. The responses are blocked by Gd^{3+} and Ba^{2+} . Similarly, we observed that bumetanide/furosemide activated a surge and lasting increase in fluorescent signal of $[\text{Ca}^{2+}]_i$ (Fig. 8A,B), and that bumetanide induced a Ba^{2+} -sensitive hyperpolarization with a long latency. Moreover, loading cells with high Ca^{2+} (2 μM) abolished loop diuretic-induced hyperpolarization (Fig. 8C), supporting the hypothesis that the diuretics indirectly activate IK by increasing the cytosolic Ca^{2+} .

Bumetanide-induced hyperpolarization was blocked by La^{3+} and Gd^{3+} , typically indicative of involvement of TRP channels [53]. As mentioned above, TRPV1, TRPV2, TRPV4 and TRPA1 gene transcripts were identified in MDCK cells (sFig. 6), and TRPV channels are all highly Ca^{2+} permeable and are candidate channels of bumetanide-activated Ca^{2+} influx, and the entry of Ca^{2+} may further trigger Ca^{2+} -activated Ca^{2+} release from intracellular stores. Indeed, La^{3+} has long been known to effectively block a highly Ca^{2+} -permeable NSCC in the MDCK cells [62]. In this study, a small depolarization preceded bumetanide/furosemide-induced hyperpolarization in some cells (Fig. 4, sFig. 3A) and the reversal potential of the bumetanide/furosemide-induced net current was consistently 5 – 10 mV less negative than the calculated E_K (Fig. 6 and 7, sFig. 3), suggestive of a concomitant activation of NSCC conductance. Moreover, in the presence of CLT or Ba^{2+} , bumetanide/furosemide activated a small conductance in I/V, especially in the positive voltage domain (Fig. 7, sFig. 3 and 6). The diuretics-activated net current had a reversal potential near -40 mV, suggesting an activated cation current mixed with a residual K^{+} -current. In this regard, it is possible that the bumetanide-activated NSCCs may contribute directly to GTTR uptake if the activated NSCCs are GTTR-permeable. However, based on the data shown in Fig. 4, 7 and 9, such direct contribution, if any, should be minimal since 10 μM CLT just completely off-set the effect of 10 μM bumetanide on GTTR uptake as well as on the membrane potential.

Taken together, we suggest that loop diuretics may activate some Ca^{2+} -permeable TRP channels, as a causal step prior to IK activation. Identification of the exact TRP channel species responsible for bumetanide-activated Ca^{2+} influx/release will be a major challenge since specific blockers for most individual TRP channels have yet to be identified and multiple TRP channels are likely implicated in the cross-membrane traffic of the aminoglycoside.

Significance

Here we demonstrated that loop diuretics stimulate cytosolic Ca^{2+} surge, thus activate IK channels, hyperpolarize the MDCK cells and then enhance the cellular uptake of GTTR. Beyond this novel action of loop diuretics, our data support the hypotheses that NSCCs, likely TRPV4 channels, are a major aminoglycoside entry pathway into MDCK cells. This cellular mechanism offers a new explanation for the synergistic nephrotoxicity of these two classes of drugs in clinic use [63]. More importantly, as systemically co-administered loop diuretic enhance aminoglycoside concentration in the endolymph and subsequent ototoxicity [1, 2, 17], our results represent a potential mechanism of how loop diuretics facilitate aminoglycoside trafficking through the strial BLB.

Initially, aminoglycosides must first penetrate through the endothelial cell layer lining the strial capillaries [16, 24]. More than ten TRP channels, including TRPV4, are identified in vascular smooth muscles and endothelial cells [64]. We have demonstrated that IK and TRP channels are highly expressed in endothelial and smooth muscle cells of cochlear arterioles, which play a key role in vasomotor regulation that controls the cochlear blood flow [38, 65, 66]. It is plausible that this mechanism enables loop diuretics to enhance aminoglycoside

loading of the strial vascular endothelium and subsequent clearance into the intrastrial space. Second, strial marginal cells, the single layer of cells between the intrastrial space and endolymph, express TRP channels, especially TRPV4 on their apical (endolymphatic) surface [23, 67]. If functional IK (or SK4, $K_{Ca3.1}$) channels are identified in these cells, we can expect this cellular mechanism would facilitate aminoglycoside loading of marginal cells, then clearance into endolymph and subsequent uptake by hair cells. These assumptions warrant investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4-AP	4-aminopyridine
ACh	acetylcholine
BK	big conductance Ca^{2+} -activated K^{+} -channel
BLB	blood-labyrinth-barrier
BUM	bumetanide
C_{input}	membrane input capacitance
CF172	CFTR inhibitor CFTR _{inh} -172 or 2-thioxo-4-thiazolidinone analog
CLT	clotrimazole
DPC	Diphenylamine-2-carboxylic acid or N-phenylanthranilic acid
FUR	furosemide
G_{input}	membrane input conductance
GTTR	Texas Red-tagged gentamicin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IK	intermediate conductance Ca^{2+} -activated potassium channel
K_v	voltage-gated K^{+} -channel, MDCK, Madin-Darby canine kidney
MET	mechanoelectrical transduction
NES	normal extracellular solution
NFA	niflumic acid
NIS	normal internal solution
NKCC	$Na^{+}/K^{+}/2Cl^{-}$ cotransporter
NTDP	nitrendipine
TEA	tetraethylammonium
V_m	membrane potential
V_r	reversal potential

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Fig. 1. Bumetanide and furosemide enhance GTTR fluorescence in MDCK cells in a concentration-dependent manner

A and **B**: Representative confocal images at increasing doses of the loop diuretics bumetanide (BUM) and furosemide (FUR). **C**: Dose-dependency plots of normalized pixel intensity means of cytoplasmic GTTR fluorescence. Hill equation fits revealed an EC_{50} of $0.51 \pm 0.067 \mu\text{M}$ and Hill number 1.1 ± 0.15 for bumetanide, and $1.6 \pm 0.58 \mu\text{M}$ and 0.99 ± 0.25 for furosemide. Each data point represents a mean of fluorescence intensity from 8 – 16 wells, with >250 cells per well.

FIG. 2. La^{3+} blocks GTTR uptake and bumetanide-enhancement of GTTR uptake

A: Representative confocal images of GTTR fluorescence in the absence and presence of La^{3+} (0.1, 1 mM), bumetanide (BUM, 10 μM) and both agents. **B:** Bar graphs of normalized fluorescence intensity means show that bumetanide significantly increased GTTR fluorescence compared to the control (GTTR only), and La^{3+} dose-dependently inhibited GTTR fluorescence by up to 48%. In the presence 0.1 and 1 mM La^{3+} , bumetanide did not increase in GTTR uptake. $\theta p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Each column represents a mean of fluorescence intensities from 4 or 8 wells.

Fig. 3. La^{3+} hyperpolarizes MDCK cells by blocking a non-selective cation conductance (NSCC), which is not affected by bumetanide

A: Sharp electrode intracellular recording showing La^{3+} induced a hyperpolarization in concentration-dependent manner. **B:** Gap-free record of a whole-cell current at -35 mV holding potential. Vertical deflections (bottom truncated) were caused by ramp- (*a, c, f, g, j*) and step- (*b, d, e, h, i*) commands. **C:** Whole-cell currents (*b, d, e* in **B**) induced by a step protocol in 20 mV increment, depicting that La^{3+} caused amplitude reduction of steady-state currents with little change by adding bumetanide. **D:** Ramp-constructed I/V curves were from averaged traces at times (*a, c, f*) noted in **B** in the absence and presence of La^{3+} and La^{3+} plus bumetanide ($10 \mu\text{M}$), depicting that 1 mM La^{3+} caused a conductance (slope) reduction in the whole range of voltage command (G_{input} from 2.04 to 0.66 nS). The La^{3+} -induced net current (subtraction of *c* - *a*) showed an I/V relation with a negative (inhibition of) slope conductance of -1.38 pS at -50 mV and a reversal potential (V_r) at -11 mV . Adding bumetanide caused a small outward change in I/V curve at very positive potential domain. The membrane potential (zero current potential) shifted from -25 mV to -44 mV by La^{3+} and to -45 mV in La^{3+} plus bumetanide.

Fig. 4. Bumetanide hyperpolarizes MDCK cells, which is attenuated by Ba²⁺ and clotrimazole but not by apamin, TEA and the agents that affect the Cl⁻ current

A: Current-clamp recordings depict that non-selective K⁺-channel blocker 1 mM Ba²⁺ and IK blocker clotrimazole (CLT) suppressed the bumetanide (BUM, 10 μ M)-induced hyperpolarization, but apamin and TEA, blockers for Ca²⁺-activated SK and BK channels respectively, had no effect on the hyperpolarization. **B:** DPC (non-selective anion channel blocker, 0.3 mM), 60 mM low Cl⁻ media, 10 μ M CFTR_{inh}172 (CF, CFTR antagonist), and niflumic acid (NFA, Ca²⁺-activated Cl⁻-channel blocker, 0.1 mM) exerted no significant effect on the bumetanide-induced hyperpolarization.

Fig. 5. Ba²⁺, clotrimazole, but not TEA and Cl⁻ channel blockers, depolarize cells and inhibit the GTTR uptake

A: Typical membrane potential traces from conventional intracellular recordings depict that Ba²⁺ and clotrimazole (CLT; in mM) depolarized the cells, whereas TEA had little effect. DPC and NFA caused a few mV hyperpolarization in about 30% of cells tested. **B:** Bar graph showing the collective data of the membrane effects. **C:** Column bars show that only Ba²⁺ and CLT significantly inhibited cytoplasmic fluorescence of GTTR (normalized means, * p<0.05). Chloride channel blockers NFA, but not DPC and CFTR172, significantly enhanced the GTTR uptake (p<0.05). **D:** Representative confocal images for C.

Fig. 6. Bumetanide activates a conductance with an I/V relation indicating a K^+ current
A: Gap-free recording of the holding current at -35 mV. Vertical deflections were caused by ramp- (*b, c, f*) and step- (*a, d, e*) commands. **B:** Ramp-constructed I/V curves are from traces at times (*b, c*) noted in *A*. Note that $10 \mu\text{M}$ bumetanide caused a conductance (slope) increase in the whole range of voltage command (G_{input} from 0.624 to 1.74 nS). The bumetanide-induced net current ($c - b$, Net I_{BUM}) showed an I/V relation with a weak outward rectification and a reversal potential at -81 mV, suggesting an activation of a K^+ current. The membrane potential (zero current potential) hyperpolarized from -38 mV to -65 mV. **C:** Step command-induced whole-cell currents (*a* and *d* in *A*) showed that bumetanide increased the steady-state current amplitudes (I_{ss}) in all levels tested. Bumetanide caused no significant change in membrane input capacitance (30 pF).

Fig. 7. Clotrimazole inhibits bumetanide-activated increase in K^+ conductance

A: Gap-free whole-cell current recorded at a holding potential (HP) -40 mV. The large deflections (1 – 9) were induced by ramp commands for I/V curve construction. **B:** I/V curves constructed in the absence and presence of bumetanide (BUM; $10 \mu\text{M}$) or bumetanide plus $10 \mu\text{M}$ clotrimazole (CLT). Each curve (*a*, *b*, *c*) was an average of those indicated by numbers in the brackets. Note that the input conductance (G_{input}) was increased from 0.39 to 0.57 nS and the zero-current potential hyperpolarized from -35 to -47 mV. Adding clotrimazole reversed the bumetanide-induced I/V change to near the control level. **C:** I/V plot of the net current induced by bumetanide alone (Net I_{BUM}) and by clotrimazole (Net I_{CLT}) in the presence of bumetanide. Note that these two net currents are almost symmetrical (mirror imaged) to the abscissa with a reversal potential near -70 mV, indicating similar ion currents got off-set when the two drugs are co-applied.

Fig. 8. Bumetanide increased intracellular Ca^{2+} concentration and furosemide and bumetanide fail to induce a hyperpolarization in cells loaded with $2 \mu\text{M Ca}^{2+}$

A: Representative confocal images in the absence (Control) and presence of bumetanide (BUM) taken at the moments indicated by * and ** in **B**. **B:** Time-lapse plots of the fluo-4 fluorescence intensity ratio (mean $\pm$ SE, non-cell background subtracted, $n=52$ cells from 3 plates). **C:** Typical membrane potential recording in 0 current-clamp mode showed that the cell loaded with 2 mM Ca^{2+} exhibited a hyperpolarized resting membrane potential and little or no voltage responses to $30 \mu\text{M}$ furosemide (FUR) or $10 \mu\text{M}$ bumetanide (BUM) ($n=5$).

Fig. 9. Bumetanide enhanced GTTR uptake is inhibited by IK blockers

A and B: Representative confocal images in the absence and presence of bumetanide (BUM) and a combination of bumetanide with clotrimazole (CLT; 10 μ M) or nitrendipine (NTDP, 1 μ M). **C:** Column graphs of normalized means of cytoplasmic GTTR fluorescence intensity. Each column represents a mean of cells of 8 wells. $^{\theta}$ p>0.05, * p<0.05, ** p<0.01.

Table 1

Membrane potential in five recording conditions

Recording method	Condition Number*	Solution in Electrode	Bathing solution	V _m ± SE*	n
Conventional intracellular recording	1	2 M KCl	Krebs	-47.1 ± 2.85	13
	2	2 M KCl	NES	-39.3 ± 1.56	18
	3	2 M K-acetate	Krebs	-44.1 ± 3.26	20
	4	2 M K-acetate	NES	-44.9 ± 1.44	13
Whole-cell tight seal	Merge of above			-44.2 ± 1.25	64
	5	NIS (K-gluconate)	NES	-44.2 ± 1.54	62
	6	HiCaIS (2 μM [Ca] _i)	NES	-66.3 ± 3.64	13
	Merge of above			-44.6 ± 0.97	126

* p > 0.05 between any two recording conditions, Students *t*-test. NES, normal external solution; NIS, normal internal solution; HiCaIS, high Ca²⁺ (2 μM) internal solution.

Table 2

Membrane potential and effects of selected agents

Treatment Agents	Mean $\Delta V_m \pm SE$ (mV)	n	Main ion channel action of the agent	References
K ⁺ (20 mM)	+8.7 $\pm$ 0.8	28	Increase K ⁺ -conductance, Positively shift E _K	[29]
Ba ²⁺ (1 mM)	+18.3 $\pm$ 2.23	9	K ⁺ -channel blocker	[36, 68, 69]
CLT (10 μ M)	+18.3 $\pm$ 3.25	11	IK blocker	[36, 70, 71]
nitrendipine (1 μ M)	+8.92 $\pm$ 2.75	10	IK blocker, I _L -blocker	[38]
TEA (10 mM)	+1.6 $\pm$ 0.42	9	BK blocker	[40, 44]
4-AP (10 mM)	-0.5 $\pm$ 0.866	6	K _V blocker	[72]
chromanol 293B	+0.25 $\pm$ 2.2	6	IsK blocker	[68]
Glipizide (3 μ M)	0.4 $\pm$ 1.2	6	K _{ATP} blocker	[73]
Acetylcholine (3 μ M)	-21 $\pm$ 2.77	6	IK activation (IP3/Ca ²⁺ -release)	[38, 39]
La ³⁺ (1 mM)	-13.8 $\pm$ 4.74	8	NSCC, TRP blocker	[52]
Gd ³⁺ (50 μ M)	-3.84 $\pm$ 1.77	6	NSCC, TRP blocker	[52]
DPC (300 μ M)	-2.63 $\pm$ 1.83	8	Anion channel blocker	[35, 74]
NFA (100 μ M)	-4.1 $\pm$ 3.32	8	I _{Cl-Ca} blocker, Kidney CLC-K activator	[75, 76]
CFTR-172 (10 μ M)	-0.5 $\pm$ 0.261	4	CFTR blocker	[34, 48]