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TRPV1 regulators mediate gentamicin penetration of cultured kidney cells

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Abstract

Transient receptor potential (TRP) receptors are, typically, weakly-selective calcium-permeant cation channels that transduce environmental stimuli. Both kidney epithelial and inner ear sensory cells express TRPV1, are mechanosensors and accumulate the aminoglycoside antibiotic gentamicin. Recently, we showed that Texas Red-conjugated gentamicin (GTTR) enters kidney cells via an endosome-independent pathway. Here, we used GTTR to investigate this non-endocytotic mechanism of gentamicin uptake. In serum-free buffers, GTTR penetrated MDCK cells within 30 seconds and uptake was modulated by extracellular, multivalent cations (Ca^{++} , La^{+++} , Gd^{+++}) or protons. We verified the La^{+++} modulation of GTTR uptake using immunocytochemical detection of unconjugated gentamicin. Membrane depolarization, induced by high extracellular K^{+} or valinomycin, also reduced GTTR uptake, suggesting electrophoretic permeation through ion channels.

GTTR uptake was enhanced by the TRPV1 agonists, resiniferatoxin and anandamide, in Ca^{++} -free media. Competitive *antagonists* of the TRPV1 cation current, iodo-resiniferatoxin and SB366791, also enhanced GTTR uptake independently of Ca^{++} , reinforcing these antagonists' potential as latent agonists in specific situations. Ruthenium Red blocked GTTR uptake in the presence or absence of these TRPV1-agonists and antagonists. In addition, GTTR uptake was blocked by RTX in the presence of more physiological levels (2 mM) of Ca^{++} . Thus gentamicin enters cells via cation channels, and gentamicin uptake can be modulated by regulators of the TRPV1 channel.

Keywords

aminoglycosides; cytoplasmic drug uptake; non-endocytotic uptake; TRP channel

INTRODUCTION

There are now more than twenty members of a newly-described group of membrane proteins that perform both as receptors and ion channels - the transient receptor potential (TRP) family. They are non-selective, calcium-permeant cation channels, and most are non-voltage-gated (Benham et al., 2002; Inoue et al., 2003; Vennekens et al., 2002; Voets et al., 2003) with a few exceptions (Hofmann et al., 2003; Nilius et al., 2003). They are involved in calcium homeostasis, especially in non-electrically active cells (Launay et al., 2002; Riccio et al., 2002; Schlingmann et al., 2002). Of particular interest is that individual TRPs appear to be the mediators of most, if not all, environmental stimuli including heat (Guler et al., 2002; Smith et al., 2002; Story et al., 2003), cold (Thut et al., 2003; Xu et al., 2002), acidity (Story et al.,

2003; Tominaga et al., 1998), fluid flow (Tsiokas et al., 1999), divalent cation concentrations (Schlingmann et al., 2002), odorants (Wuttke et al., 2000), osmolarity (Grimm et al., 2003; Xu et al., 2003), contact (Goodman et al., 2003; Mutai et al., 2003), taste (Hofmann et al., 2003), and sound (Corey et al., 2004; Mutai et al., 2003; Zheng et al., 2003). Signal transduction by a TRP channel involves calcium entry into the cell and hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) at specific binding sites within the receptor channels. In some cases, binding is inhibitory, and hydrolysis of PIP₂ activates the channel (Runnels et al., 2002; Vellani et al., 2001). In other instances, binding of PIP₂ is required for current (Prescott et al., 2003). Our research has led us to consider another, more nefarious, role for TRP channels.

Aminoglycoside antibiotics are powerful drugs used for serious medical situations, such as treatment of Gram-negative infections (e.g., meningitis), and prophylaxis against infection in pre-mature infants, burn patients, and in high-risk surgeries (Begg et al., 1995; de Lalla, 1999; Jackson, 1984). In addition, gentamicin has recently been shown to cause read-through of premature stop codons that produce such genetic diseases as cystic fibrosis and lysosomal storage disease (Keeling et al., 2002; Schulz et al., 2002). This treatment results in production of functional proteins and partial relief of disease.

Unfortunately, aminoglycosides are both nephro- and ototoxic, causing kidney failure and permanent hearing losses in a significant fraction of patients (de Jager et al., 2002; Kahlmeter et al., 1984; Leehey et al., 1993). Despite decades of investigation, the incidence of oto- and nephrotoxicity resulting from the clinical (and veterinary) use of aminoglycoside antibiotics continues to be high. Current efforts to ameliorate these toxic side effects, such as intracellular inhibitors of caspase-3, c-Jun kinase, iron chelators, free oxygen radicals or calpains (see review by Rybak et al., 2003), largely attempt to block the effects of aminoglycosides after the drug has entered the affected cells.

In contrast, our approach is to determine the mechanism of aminoglycoside uptake into cells in order to target drug penetration into cells. The mechanism(s) by which the aminoglycoside antibiotic gentamicin enters cells still remains unclear. We are using the aminoglycoside gentamicin covalently linked to the fluorophore Texas Red (GTTR) as a probe to study these mechanisms. Several recent reports have focused on the endocytotic uptake of aminoglycosides by kidney cells and sensory hair cells (Hashino et al., 1997; Sandoval et al., 2004). Kidney epithelia share a number of other characteristics with inner ear sensory hair cells, including: epithelial polarity, tight junctions, pharmacological sensitivity to aminoglycoside antibiotics and mechanosensitivity (Bagger-Sjoberg et al., 1988; Corey et al., 1983; Forge, 1985; Gonzalez-Mariscal et al., 2000; Humes, 1999; Kuhn et al., 1975; Miller, 1985; Nauli et al., 2004; Tiedemann et al., 1983).

In the accompanying report, we characterized and validated an endosome-independent mechanism by which gentamicin appears to cross the plasma membrane directly into the cytoplasm and then into intra-nuclear compartments (Myrdal et al., 2005). Recent reports of TRPV1 in both inner ear hair cells and the kidney (Cortright et al., 2001; Zheng et al., 2003), as well as the existence of well-characterized agonists and antagonists for this receptor led to exploration of the potential role of TRPV1 in gentamicin penetration of these target cells. In this report, we show that regulators of this vanilloid receptor, TRPV1, mediate the uptake of Texas Red-labeled gentamicin into the Madin-Darby canine kidney distal tubule cell line (MDCK).

Materials and Methods

MDCK cells were used as a model system to determine if non-endocytotic GTTR uptake is modulated by conditions known to produce or modify a cation current, in general, and the TRPV1 channel, in particular. We tested membrane depolarization, cation channel blockers, alteration of pH and extracellular calcium, as well as known agonists and antagonists for TRPV1. All materials were from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Conjugation

The conjugation of gentamicin to Texas Red (TR) succinimidyl esters (Molecular Probes, OR) was done as described previously (Myrdal et al., 2005). The conjugate (GTTR) was isolated by reversed-phase chromatography, aliquoted, dried, and stored dessicated in the dark at -20°C until required.

Cell culture

Canine kidney distal tubule MDCK cells were a gift from Dr. David Ellison (OHSU), but are also commercially available. Cells were routinely cultured in antibiotic and phenol red-free Dulbecco's minimal essential medium (MEM α , Invitrogen, CA) with 10% fetal bovine serum (FBS) and kept at 37°C with 5% CO_2 , 95% air. In order to maintain an epitheloid morphology, cells are occasionally cloned, picking cobblestone islands with smooth edges. For testing, cells were seeded into 8-well coverglass chambers (ISC BioExpress) at 3000 cells/well and grown for 5 days, when they had become subconfluent, columnar and had time to develop tight junctions.

Experimental Procedures

Cells were washed three times with the buffer to be used in the particular experiment, treated with GTTR and the experimental variable for 30 or 60 seconds at 20°C (precluding endocytosis). Following treatment, cells were rinsed three times with buffer then fixed and delipidated with 4% formaldehyde plus 0.5% Triton X-100 (FATX) for 45 minutes. Following fixation, cells were rinsed with phosphate buffered saline (PBS, Invitrogen, CA) for at least 4–6 times or until foaming in the suction pipette ceased. This extensive washing both removed fixative and detergent, and also insured that cells from all experimental protocols were in the same ionic environment when imaged. This avoided any potential influence of specific ions on GTTR fluorescence. In contrast to previous experiments (Myrdal et al., 2005), no FBS was present in the treatment media, allowing for more rapid uptake of the antibiotic-based GTTR. Antibiotics are bound by serum proteins that can retard cellular uptake of antibiotics (unpublished data; Bongard et al., 1993; Kessel et al., 1999).

Membrane depolarization

MDCK cells were washed with Hank's buffered salt solution (HBSS; Invitrogen, CA), then placed into buffers of varying potassium concentrations (5.8, 15.8, 45.8, and 146 mM K^+). HBSS was mixed with equiosmolar KCl/HBSS to produce the required K^+ concentrations. Cells were treated with 1 $\mu\text{g}/\text{mL}$ GTTR for 1 minute, then washed and fixed as described above. Alternatively, cells were washed in HBSS and treated with 5 $\mu\text{g}/\text{mL}$ GTTR with or without 10 $\mu\text{g}/\text{mL}$ valinomycin for 1 minute, then washed and fixed with FATX as described above.

Extracellular trivalent cations

Cells were washed with HBSS, then placed into buffers containing varying concentrations of gadolinium (Gd^{+++} , 0.5, 5, 50 mM) or lanthanum (La^{+++} , 0.05, 0.5, 5 mM), each at pH 7.3. Cells were treated with 5 $\mu\text{g}/\text{mL}$ of GTTR for 30 seconds, then washed and fixed as above.

Extracellular calcium

Cells were washed with PBS and placed into buffers containing varying concentrations of calcium, obtained by mixing HBSS with equimolar calcium chloride, each at pH 7.3. Cells were treated with 5 $\mu\text{g}/\text{mL}$ of GTTR for 30 seconds, then washed and fixed as above.

Extracellular pH

Cells were washed with saline, then treated with 5 $\mu\text{g}/\text{mL}$ of GTTR in saline buffers of varying pH for 30 seconds, then washed and fixed as above. Sodium hydroxide and hydrochloric acid were used to alter pH. These experiments were performed in three different saline buffers: (i) PBS (no calcium), (ii) a mixture of one part PBS and one part HBSS for a final calcium concentration of 0.32 mM, and (iii) a mixture of three parts PBS to one part HBSS for a final calcium concentration of 0.97 mM.

TRPV1 agonists and antagonists

Cells were washed three times with Ca^{++} -free saline (0.9% NaCl), then treated with 5 $\mu\text{g}/\text{mL}$ GTTR and one of the following for 30 seconds: TRPV1 agonists (resiniferatoxin [RTX]; or anandamide [AND]; Alexis Biochemicals, San Diego, CA); or antagonists (iodo-RTX, or SB 366791; Tocris, Ellisville, MO). Cells were then washed in saline and fixed as above. (No EGTA was present in the saline to bind residual calcium, as the cells would have detached from the coverglasses during treatment and subsequent washing. Thus there was, undoubtedly, a small amount of calcium during treatment.)

Ruthenium Red

Cells were washed three times with Ca^{++} -free 0.9% NaCl, then treated with 5 $\mu\text{g}/\text{mL}$ GTTR and 100 μM Ruthenium Red (RR) with or without one of the TRPV1 agonists or antagonists described above.

Immunocytochemistry

MDCK cells grown on 8-well chambered coverslips to 30–40% confluency were incubated with 5 or 300 $\mu\text{g}/\text{mL}$ unconjugated gentamicin for 30 seconds at 20°C, in the presence of 0, 0.05, 0.5 or 5 mM La^{+++} . Cells were rinsed twice with PBS, fixed with FATX, and rinsed 3 times with PBS. Cells were immunoblocked in 10% goat serum in PBS for 30 minutes, and then incubated with 50 $\mu\text{g}/\text{mL}$ rabbit anti-gentamicin IgG (American Quaaalex, San Clemente, CA) for 1 hour. After washing with 1% goat serum in PBS, cells were further incubated with 20 $\mu\text{g}/\text{mL}$ Alexa-488-conjugated goat-anti-rabbit IgG antisera (Molecular Probes, Eugene, OR) for 45 minutes, washed, post-fixed with 4% FA for 15 minutes, and washed again. For immunocytochemical controls, cells untreated with gentamicin were immunoprocessed identically as experimental cells. All wells were imaged using confocal microscopy (see below).

Confocal Microscopy

Specimens were observed using a $\times 60$ lens (N.A. 1.4), on a Nikon TE 300 inverted microscope. Confocal images were collected on a Bio-Rad 1024 ES scanning laser system. Bio-Rad *.pic files were converted to *.tif files, and prepared for publication using Adobe Photoshop (v.7). For sets of images to be compared, the same confocal settings (laser intensity, iris, gain, offset) were used. These were each set up to obtain the best dynamic range for that image set. Thus, intensity values are relative, and should only be compared within an image set. Each type of experiment was done multiple times ($n \geq 3$) to confirm trends. Numerical quantification of fluorescence-based images is difficult to validate. Optical sections from cultured cell layers is

subject to natural variation among cells, which is exacerbated when images are obtained at high magnification and with a narrow depth of focus.

Results

Modulation of Membrane Potential

If cationic GTTR penetrates cells via cation channels down an electrochemical gradient, a reduction of the electrical potential difference across the plasma membrane could reduce GTTR uptake. Increases in extracellular potassium serially decrease the negative intracellular resting potential (Gitter, 1993; Zenner, 1986), and reduce the transmembrane cationic driving force into the cell. Cells treated at 5.8 mM K^+ show bright cytoplasmic and intra-nuclear GTTR fluorescence (Fig. 1A). When K^+ concentrations were increased from 15.8 mM to 146 mM, a clear and considerable decrease in GTTR uptake into cells was observed (Fig. 1B–D).

Valinomycin, a potassium ionophore, also reduces the electrical potential difference across the plasma membrane (Crider et al., 2003; Ren et al., 2001). Valinomycin treatment decreased the uptake of GTTR compared to control cells (Fig. 1E, F). These data suggest that the positive charge of the polyamine gentamicin facilitates the electrophoretic passage of the molecule through cation channels towards the electrically-negative interior of the cells.

Extracellular trivalent cations

Gadolinium—Gadolinium blocks calcium-permeant, mechanosensitive cation channels (Kondoh et al., 2003; Trebak et al., 2002; Urbach et al., 1999). In the absence of Gd^{+++} , GTTR uptake was high (Fig. 2, A1), but serially decreased with increasing concentrations (0.5, 5, or 50 mM) of Gd^{+++} (Fig 2, A2–A4).

Lanthanum—Lanthanum also blocks non-selective cation channels (Gillo et al., 1996; Walker et al., 2002). When no La^{+++} was added, GTTR uptake was high (Fig. 2, B1), but serially decreased with increasing concentrations of La^{+++} (Fig 2, B2–B4). Similarly, in immunofluorochemical experiments, the intensity of immunofluorescence serially decreased with increasing (0.05, 0.5 and 5 mM) La^{+++} concentrations (Fig 2, C1–C4; D1–D4), verifying modulation of the distribution of GTTR by La^{+++} .

The data from both Gd^{+++} and La^{+++} experimental sets are consistent with the hypothesis that gentamicin uptake can occur through non-selective cation-permeant channels into the cytoplasm (Hellwig et al., 2004).

Non-Specific TRPV1 modulators

Calcium—Extracellular calcium desensitizes the TRPV1 channel, and shortens the inward current induced by agonists (Caterina et al., 1997; Tominaga et al., 1998). Changes in extracellular calcium altered GTTR uptake. When no calcium was added, GTTR uptake was low (Fig. 3, A1), but increased at 0.16 mM calcium (Fig 3, A3). As calcium concentrations increased above 0.16 mM, GTTR uptake decreased (Fig 3, A4–A7). These data are consistent with earlier observations that calcium reduces cation movement through TRPV1 channels. Alternatively, calcium might compete with gentamicin uptake through calcium-permeant cation channels into the cytoplasm.

Protons—A slightly acidic environment (pH 5–6.5) enhances current through TRPV1 channels (Caterina et al., 1997; Hellwig et al., 2004). Changes in extracellular pH altered GTTR uptake (Fig 3). Although three different buffers with the varying calcium concentrations were used, the effect of pH was the same and only the PBS (no calcium added) data are shown. At pH 5 (and to a lesser extent at pH 6) there was increased uptake of GTTR (Fig. 3, B2, B3), consistent with the reported pH range of proton stimulation of inward current through the

TRPV1 channel. At pH 4, uptake was lower (Fig. 3, B1). Increasingly basic conditions reduced uptake (Fig. 3, B4–B7). The effects of both calcium and protons on GTTR uptake are consistent with the possibility that TRPV1 channels play a role in the penetration of gentamicin into the cytoplasm of kidney cells.

Specific TRPV1 Regulators

A number of chemical entities have been described which bind to TRPV1 receptors, specifically competing with capsaicin, the canonical TRPV1 agonist. These include agonists, which themselves induce a cation current through TRPV1, and antagonists, which produce no currents, but which competitively block an agonist effect. We tested two of each.

TRPV1 agonists—Resiniferatoxin (RTX) is a potent TRPV1 agonist that induces a transient inward current that is desensitized in the presence of calcium (Acs et al., 1996). We tested the effect of RTX on GTTR penetration of cells to determine whether an agent that opens this channel to a cation current could enhance GTTR uptake. In calcium-free saline with 5×10^{-9} M RTX, GTTR uptake was significantly increased (Fig. 3, C2) over the control (Fig. 3, C1). At the higher dose of 5×10^{-8} M RTX, uptake was increased over control cells, but to a lesser extent (Fig. 3, C3), and at 5×10^{-7} M RTX, there was little or no change over control cells (Fig. 2, C4 and C1, respectively). The decrease in GTTR effect at higher doses might be explained by agonist desensitization due to the residual calcium present (see below).

Anandamide (AND) is an endogenous cannabinoid and a TRPV1 agonist that produces a transient inward cation current and competes with both RTX and capsaicin for binding (Olah et al., 2001). It was tested for its effect on GTTR uptake using the same protocol as for RTX. Consistent with its reported weaker binding to TRPV1 (Toth et al., 2003), AND required higher doses to produce increases in GTTR uptake. At 10^{-6} M and 10^{-5} M AND, GTTR uptake was increased, although not to the level seen with RTX (Fig. 3, C5 and C6, respectively). At 10^{-4} M AND, GTTR uptake showed little or no increase over controls (Fig. 3, C7 and C1, respectively). These data show that TRPV1 channel agonists stimulate gentamicin uptake in nominally Ca^{++} -free media in a manner similar to their reported stimulation of cation currents (Numazaki et al., 2003).

TRPV1 antagonists—Two specific TRPV1 antagonists, SB366791 and iodo-RTX, were also tested. Both competitively reduce the binding of known TRPV1 agonists, and block the cation current induced by specific agonists (Gunthorpe et al., 2004; Wahl et al., 2001). Surprisingly, both SB366791 and iodo-RTX enhanced GTTR uptake in calcium-free saline. At doses from 10^{-7} M to 10^{-5} M, SB366791 serially increased GTTR uptake (Fig. 3, D2–D4). The effect of I-RTX, which binds to TRPV1 with a higher affinity than SB366791 (Davis et al., 2001; Fowler et al., 2003), was more dramatic (Fig. 3, D5–D7). At 10^{-5} M I-RTX the GTTR fluorescence was well over the upper limit of the available 0 to 255 gray scale when using parameters optimized for comparison with the other images in this figure. With both of these molecules, increased doses of these specific antagonists increased uptake (in contrast to the TRPV1 agonists).

To ensure that agonist or antagonist-induced increases in uptake of GTTR was not due to toxicity or increased permeability, we treated cells with hydrolyzed TR at the highest doses shown for both RTX and I-RTX. Neither I-RTX or RTX induced TR penetration into the cytoplasm (Fig. 3, F1, F2).

Ruthenium Red—Ruthenium Red (RR) is a non-competitive TRPV1 antagonist that blocks numerous cation channels. Cells treated with 10^{-5} M RR alone (Fig. 3, E1) took up less GTTR than controls (Fig. 3, D1). The same dose of RR also blocked GTTR increases stimulated by

RTX, AND, SB366791, and I-RTX (Fig. 3, E2–E5, respectively), although the AND effect was not completely blocked. Blockade of GTTR uptake by RR further demonstrated the involvement of cation channels in the penetration of GTTR into the cytoplasmic compartment of MDCK cells. More specifically, it demonstrated that the alteration of GTTR uptake by TRPV1 modulators was directly at the channel level, and not due to a downstream effect.

Effect of calcium on RTX and I-RTX regulation of GTTR uptake

Calcium rapidly desensitizes the RTX-stimulated TRPV1 channel (Acs et al., 1996). MDCK cells were treated with GTTR in normal (138 mM NaCl) saline, or saline with 0.16 mM or 2.0 mM calcium. In each of these solutions, cells received either no other treatment, 5×10^{-9} M RTX, or 10^{-5} M I-RTX. As in Figure 3, GTTR uptake was higher at 0.16 mM Ca^{++} (Fig. 4, A2) than at either no calcium added (Fig. 4, A1) or at 2.0 mM (Fig. 4, A3). As also seen in Figure 3, both 5×10^{-9} M RTX (Fig. 4, B1) and 10^{-5} M I-RTX (Fig. 4, C1) increased GTTR uptake when in saline (no added calcium buffer). But, in both 0.16 or 2.0 mM added calcium buffers (Fig. 4, B2, B3), RTX reduced GTTR uptake compared to controls at the same calcium levels, while I-RTX still caused increased GTTR uptake in the presence of calcium. These RTX results are consistent with calcium-induced desensitization of the TRPV1 response to its specific agonists, as described previously (Numazaki et al., 2003). The contrasting influence of calcium on the I-RTX effect may contribute to understanding its unexpected induction of GTTR uptake.

Discussion

Rapid GTTR uptake by MDCK cells was modulated by membrane depolarization, extracellular cations (Ca^{++} , La^{+++} , Gd^{+++}) or protons. GTTR uptake was enhanced by TRPV1 agonists in Ca^{++} -free media, and also by reported TRPV1 antagonists independently of Ca^{++} . Ruthenium Red blocked GTTR uptake in the presence or absence of these TRPV1-agonists and antagonists. More significantly, GTTR uptake was significantly reduced by RTX in the presence of physiological levels of Ca^{++} . Immunofluorescence of unconjugated gentamicin verified the reduction in GTTR uptake by one of the modulators (La^{+++}).

Gentamicin (average MW = 469) and the conjugate GTTR (MW = approx 1100) are much larger in size than the cations generally envisioned permeating TRP channels. However, a large body of evidence demonstrates that many factors besides size influence permeation of a particular species into a specific channel. These factors include hydration state/hydration energy (Barry et al., 1999; French et al., 1985; Gong et al., 2002; Qu et al., 2000), electrostatic interactions of the permeant with side groups of amino acid residues within the pore (Guidoni et al., 1999), and hydrogen bond exchanges between the permeant and amine side groups which have formed conformational hydrogen bonds with other side groups in the channel pore (Tikhonov et al., 1999). Furthermore, there are numerous reports of large organic cations (including fluorescent dyes) permeating various cation channels, including TRP channels, in inner ear hair cells and transfected kidney cells, with evidence that ionic size is only one of the factors predicting permeability (see discussions in Corey et al., 2004; Gale et al., 2001; Hellwig et al., 2004; Meyers et al., 2003; Steyger et al., 2003). Those studies suggest that aminoglycosides, and possibly other polyamine and cationic compounds can permeate cation channels. The data presented in this report provides evidence that fluorescently-labeled gentamicin enters cells via cation channels, and that this penetration can be mediated by regulators of TRPV1, the vanilloid receptor.

Reduction of Membrane Potential

The difference between intracellular potassium concentrations ($[\text{K}]_i$) and extracellular potassium ($[\text{K}]_o$) is largely responsible for the electrical potential difference across the plasma

membrane. TRP channels, including TRPV1, are not voltage-gated (Benham et al., 2002; Inoue et al., 2003; Vennekens et al., 2002; Voets et al., 2003). Previous studies have shown that aminoglycosides block cation channels of inner ear hair cells which have negative to the inside resting potentials, but not depolarized hair cells (Kroese et al., 1989). Increasing concentrations of $[K]_o$ also depolarizes cells (Gitter, 1993; Zenner, 1986), and in this study reduced GTTR uptake. The toxic effect of high K^+ is unlikely during a brief exposure at room temperature, and if so would more likely have produced increased (but non-specific), penetration of GTTR down its concentration gradient into the cell. The potassium ionophore valinomycin also equilibrates $[K]_i$ and $[K]_o$, and similarly reduced GTTR. Thus, the simplest explanation for this reduction of GTTR uptake is that the reduced electrical potential difference across the plasma membrane reduces the electrical driving force for the cationic GTTR to cross the plasma membrane. These data support a model in which gentamicin enters cells electrophoretically via cation channels.

Extracellular trivalent cations

Both La^{+++} and Gd^{+++} reduced GTTR and gentamicin uptake as expected from physiological inhibition of non-selective cationic TRP channel function in previous studies (Gillo et al., 1996; Kondoh et al., 2003; Trebak et al., 2002; Walker et al., 2002). The dose dependent reduction of GTTR uptake by La^{+++} was confirmed by a similar reduction in the fluorescent intensity of immunolabeled gentamicin.

Calcium—The calcium dilution series shown in Figure 3(A1–A7) shows that extracellular calcium influences GTTR uptake into cells. A very low level of calcium is necessary for uptake. It is likely that when extracellular calcium concentrations are lower than intracellular calcium concentrations, calcium-permeant channels close to protect cells. Even physiological levels (~1.25–1.8 mM) are somewhat inhibitory compared to lower levels. This does not, however, suggest that physiological levels of calcium do not permit significant uptake of GTTR (see Myrdal, et al., 2005), but rather that lower extracellular calcium levels allow greater uptake while higher levels of calcium reduce uptake. This could be due to either (i) the two polycations competing for the same channel, (ii) the calcium regulating the open time of the relevant channels, or a combination of both. The enhanced uptake of gentamicin at lower levels of extracellular calcium mimics the greater open probability of TRP channels and inner ear cation transduction channels at lower calcium concentrations (Caterina et al., 1997; Corey et al., 1983; Crawford et al., 1991; Koplas et al., 1997; Ricci et al., 1997). Thus, the data further implicate TRP channels as a route for gentamicin entry into cells.

TRPV1 Agonists

Protons, as well as specific agonists that bind to, and compete for, the TRPV1 binding site, produce cation currents through those channels (Vellani et al., 2001). Enhanced uptake of GTTR was observed at pH 5 and reduced at more basic pH levels. The TRPV1 channel cation current is also maximal at pH5 and reduced at more acidic, and particularly more basic pH levels (Vellani et al., 2001). Alternatively, increased protonation at acidic pH could lead to enhanced gentamicin uptake (Lesniak et al., 2003). However, we observed a decreased level of GTTR uptake at pH 4 compared to pH 5, suggesting that increased protonation alone does not increase uptake. Cisplatin is another oto- and nephrotoxic polyamine that induces sensory hair cell death. Environmental acidity enhances cisplatin-induced oto- and nephrotoxicity in vivo (Bertolero et al., 1982; Tanaka et al., 2003), although whether this was due to the protonation, enhanced uptake or reactivity of the cisplatin molecule to DNA remains to be determined.

In addition to protons, we found that the specific agonists RTX and anandamide both stimulated GTTR uptake in calcium-free media, with a relative effectiveness consistent with their known affinities for the receptor binding site (Olah et al., 2001). For both agonists, higher

concentrations of agonist were less effective, suggesting agonist-induced closing or blockage of the channel. This is consistent with the known desensitization of agonist induced currents (Acs et al., 1997) which occurs when agonists are tested in the presence of calcium. Although our experiments were done in nominally calcium-free buffer, we were unable to use a chelator such as EDTA, because EDTA caused cell islands to detach from the coverglasses and then not be available for observation.

TRPV1 Antagonists

There are several TRPV1 antagonists that compete with capsaicin or RTX for binding to the TRPV1 receptor. Iodo-RTX binds with high affinity. It induces no current in treated cells, and blocks RTX- or capsaicin-induced currents (Wahl et al., 2001). SB366791 shows similar effects, but with a lower affinity for the binding site than I-RTX (Davis et al., 2001; Fowler et al., 2003). Surprisingly, both of these antagonists significantly increased GTTR uptake, with iodo-RTX strikingly more effective at the 10^{-5} M dose, consistent with the relative binding affinities of these two molecules. Unlike the agonists RTX and anandamide, no “desensitization” of GTTR uptake was observed at higher concentrations using these antagonists, i.e., higher doses of antagonists induced greater GTTR uptake.

These antagonists (I-RTX and SB366791) generally compete with RTX for the binding site on TRPV1 (to negate the agonist effect of RTX). However, 10^{-5} M I-RTX has a latent agonistic effect when bound to TRPV1 during noxious heat stimulation (Petho et al., 2004). Thus, there may also be a direct, as yet unexplained, synergistic effect within the receptor channel in which the gentamicin molecule itself alters the antagonist-receptor interaction, or the receptor conformation, that facilitates (calcium-insensitive) GTTR entry (see Fig. 4). Gentamicin is known to bind to PIP₂; and PIP₂ binding to TRPV1 inhibits the cation current (Chuang et al., 2001; Prescott et al., 2003; Schacht, 1979; Williams et al., 1987). Gentamicin could bind to and then remove PIP₂ from its TRPV1-binding site, thus opening the TRPV1 channel.

Agonists and Antagonists in the presence of Ca⁺⁺

In Figure 3, we show that 5×10^{-9} M RTX, which stimulates GTTR uptake in Ca⁺⁺-free saline, reduced GTTR uptake in the presence of both low and high doses of Ca⁺⁺ compared to controls at the same Ca⁺⁺ concentrations. Indeed, the combination of 2 mM Ca⁺⁺ and 5×10^{-9} RTX greatly reduced GTTR uptake. This effect was not observed with I-RTX. These observations are consistent with the apparent “desensitization” seen in Figure 3 at higher doses of RTX (but not with I-RTX). This suggests that aminoglycoside penetration of these cells can be either increased or reduced by these specific regulators of the TRPV1 channel.

Non-specific blockade of GTTR uptake

The non-competitive cation blocker Ruthenium Red reduces GTTR uptake, and blocks the stimulatory effect of both agonists and antagonists, further supporting the conclusion that gentamicin enters cells via one or more cation channels. This also shows that the effect of the specific agonists and antagonists is directly on the cation channels, and not an indirect effect downstream or on some other molecular entity.

Significance

All receptors in the growing TRP family are well documented as cation channels. The function we describe here is a significant departure from the conventional wisdom that these channels are only atomic cation-permeant, but, instead, they also allow the entry of larger molecules like gentamicin, as reported and discussed previously for less toxic compounds (Corey et al., 2004; Gale et al., 2001; Hellwig et al., 2004; Meyers et al., 2003). Using fluorescently-labeled gentamicin, and both specific and non-specific modulators of TRPV1, we have provided

evidence that that this channel (and/or others with close functional homology) can enable gentamicin entry into the cytoplasm of MDCK cells. In other studies, we show that these regulators also modulate gentamicin uptake into inner ear hair cells, the other major cell type susceptible to aminoglycoside toxicity. Blocking aminoglycoside penetration into cells through ion channels offers the possibility of pharmacologically preventing aminoglycoside-induced oto- and nephrotoxicity.

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Abbreviations

AND	anandamide
FATX	4% formaldehyde plus 0.5% Triton X-100
FBS	fetal bovine serum
Gd⁺⁺⁺	gadolinium
GT	gentamicin
GTTR	gentamicin conjugated to Texas Red
HBSS	Hank's buffered salt solution
I-RTX	iodo-resiniferatoxin
La⁺⁺⁺	lanthanum
MDCK	Madin-Darby canine kidney cell line derived from distal tubule
MEMα	Dulbecco's minimal essential medium
N.A	numerical aperture
PBS	phosphate buffered saline
PIP₂	phosphatidylinositol-4,5-bisphosphate

RR	Ruthenium Red
RTX	resiniferatoxin
TR	Texas Red
TRP	transient receptor potential (channels)

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Figure 1.

Membrane depolarization reduces GTTR uptake. (A–D) Cells treated with increasing extracellular K⁺ concentrations show serially declined GTTR uptake at higher concentrations. (E, F) Cells treated with the potassium ionophore valinomycin (F) show much reduced GTTR uptake compared to control cells (E). Scale bar = 10 µm.

Figure 2.

Gadolinium and lanthanum reduce GTTR uptake. A1–4) Cells treated with increasing Gd⁺⁺⁺ concentrations show serially decreased GTTR uptake at higher concentrations. B1–4) Cells treated with increasing La⁺⁺⁺ concentrations show serially decreased GTTR uptake at higher concentrations. Cells treated with 5 µg/mL (C1–4) or 300 µg/mL (D1–4) unconjugated gentamicin, with or without La⁺⁺⁺, prior to fixation and gentamicin immunofluorochemistry, also show serially decreased gentamicin uptake with increasing La⁺⁺⁺ concentrations. Scale bar = 10 µm.

Figure 3.

Modulation of GTTR uptake into cytoplasmic and intra-nuclear compartments by calcium, pH, TRPV1 agonists and antagonists. A1–A7) Cells treated with varying Ca⁺⁺ concentrations show increasing GTTR binding from 0 to 0.16 mM Ca⁺⁺ with serially declining binding at higher concentrations. B1–B7) Cells treated in PBS at varying pH, as indicated, show maximal GTTR binding at pH 5 (B2), with significantly decreased binding in more basic buffers (B3–B6), and greatly reduced GTTR uptake at pH 4 (B1) and pH 10 (B7). C1 and D1) GTTR alone. C2–4) Cells treated with TRPV1 agonist resiniferatoxin (RTX) show stimulation of GTTR uptake at 5 × 10⁻⁹ M, with declining stimulation at higher RTX concentrations. C5–7) Cells treated with TRPV1 agonist anandamide (AND) also show stimulation of GTTR uptake at 10⁻⁶ M and

10^{-5} M with little or no stimulation at 10^{-4} M. D2–4) Cells treated with antagonist SB366791 show increasing stimulation of GTTR uptake at increasing concentrations (10^{-7} to 10^{-5} M). D5–7) Cells treated with antagonist iodo-RTX (I-RTX) also show increasing stimulation of GTTR uptake at increasing concentrations (10^{-7} to 10^{-5} M). E1–5) Cells treated with GTTR and 100 μ M Ruthenium Red (RR) alone, or with agonists or antagonists at their most effective tested doses. E1) RR alone shows decreased GTTR uptake compared to the control (D1). E2–5) RR blocked enhanced GTTR uptake induced by all agonists and antagonists tested, with only a partial effect on anandamide stimulation (E3). F1) No fluorescence is present in the cytoplasmic compartment when hydrolyzed TR is added with 10^{-5} M I-RTX. F2) No fluorescence occurs in the cytoplasmic compartment when hydrolyzed TR is added with 5×10^{-7} M RTX. Scale bar in D7 = 20 μ m, and applies to all image panels.

Figure 4.

Attenuation of RTX-stimulated GTTR uptake by calcium. MDCK cells were treated with GTTR in 138 mM saline with no calcium (A1, B1, C1), with 0.16 mM (A2, B2, C2), or 2.0 mM calcium (A3, B3, C3). Cells received no other treatment (A1–3), 5×10^{-9} M RTX (B1–3), or 10^{-5} M I-RTX (C1–3). A1–3) Cells incubated with 0.16 mM calcium had enhanced GTTR uptake (A2) compared to cells incubated with 0 or 2 mM calcium (A1, A3 respectively). B1–3) Cells treated with RTX in the absence of calcium had enhanced GTTR uptake (B1) compared to cells treated with RTX in the presence of 0.16 mM calcium (B2); both had much greater GTTR uptake than cells incubated with RTX and 2.0 mM calcium (B3). C1–3) I-RTX increased GTTR uptake at all concentrations of calcium compared to control cells treated in calcium only buffers (A1–3). Scale bar in I = 20 μ m applies to all image panels.