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Biochemical and Biophysical Research Communications 297 (2002) 71–77

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Aminoglycoside antibiotics induce pH-sensitive activation of the calcium-sensing receptor

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Received 2 August 2002

Abstract

The aminoglycoside antibiotic (AGA) neomycin is a known agonist of the extracellular calcium-sensing receptor (CaR). To test whether other AGA drugs stimulate the CaR, we studied the relative effects of four AGAs on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using CaR-transfected human embryonic kidney (HEK)-293 cells. Gentamicin, tobramycin, and neomycin evoked dose-dependent increases in $[Ca^{2+}]_i$ with EC_{50} values of 258, 177, and 43 μM, respectively, in CaR-transfected, but not in non-transfected cells. Kanamycin was ineffective at doses <1 mM. Thus, AGAs stimulate the CaR with a rank order of potency that correlates positively with the number of their attached amino groups. The CaR is expressed on the apical surface of renal proximal tubule cells, which is also the site of AGA endocytosis and nephrotoxicity. In the current study, reducing extracellular pH from 7.4 to 6.9, to mimic the luminal pH of the proximal tubule, enhanced the sensitivity of the CaR to tobramycin, suggesting that the AGAs may be more potent CaR agonists in the proximal tubule than elsewhere. This pH effect was not observed when stimulating CaR with the non-ionizable agonist, Gd^{3+}, suggesting that the enhanced AGA effect is due to increased ionization of the drug. Thus, we show that a number of AGA drugs are capable of CaR activation and that their potency most likely relates to the number of their amino side chains and to their pH-dependent charge characteristics. The contribution of CaR activation to the pharmacological/toxicological effects of these AGAs remains to be determined.

Keywords: Intracellular calcium; Calcium receptor; Intracellular pH; Fura-2 AM

The extracellular Ca^{2+}-sensing receptor (CaR) is a class III, G protein-coupled receptor that is responsible for, amongst other things, the suppression of parathyroid hormone (PTH) secretion, and renal calcium reabsorption during hypercalcemia [1]. Functional studies in vitro aimed at measuring receptor-mediated increase in intracellular calcium concentration ($[Ca^{2+}]_i$) have demonstrated that the CaR responds, not only to changes in extracellular free ionized Ca^{2+} concentration ($[Ca^{2+}]_o$), but also to various di- and trivalent cations (e.g., Mg^{2+} and Gd^{3+}), as well as polycationic compounds, including the aminoglycoside antibiotic (AGA), neomycin [2,3]. AGAs are widely used in the treatment of Gram-negative infections, but their use is associated with nephrotoxicity in some patients [4], an effect characterized by internalization of the drug into renal proximal tubular cells, with vacuolization and extensive cellular necrosis [4,5]. An AGA-binding protein present in the proximal tubule is megalin and this has been proposed to provide the entry mechanism for AGAs into the cell [6]. More recently, we have localized the neomycin-responsive CaR also to the luminal membrane of the proximal tubule [7]. However, its physiological function(s) there and its putative involvement in AGA toxicity remain unclear [8].

The aim of the present study was to evaluate the capacity of the known AGAs to activate the receptor in HEK-293 cells stably expressing the human parathyroid CaR. We report that several AGAs, in addition to neomycin, potently stimulate the CaR in HEK-293 cells, that the number of amino side chains on the AGAs

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)02133-2

correlates with their rank order of potency for the receptor and that the acidic conditions present in the proximal tubule lumen enhance the CaR's sensitivity to AGA challenge.

Methods

Cell culture. HEK-293 cells, stably transfected with human parathyroid CaR [9], were a gift from Dr. E.F. Nemeth (NPS Pharmaceuticals, Salt Lake City, UT, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, Scotland, UK) supplemented with 10% heat-inactivated fetal calf serum (Hyclone-Perbio, Helsingborg, Sweden), 10 mM L-glutamine (Invitrogen), with or without 200 mg/ml hygromycin B (Boehringer–Mannheim, Lewes, Sussex, UK).

Preparation of cell membrane particulate fractions. After rinsing in PBS, cells were scraped into lysis buffer containing 12 mM Hepes (pH 7.6), 300 mM mannitol, 1 mM *N*-ethylmaleimide, pepstatin, leupeptin, and PMSF [10] and drawn repeatedly in and out of a narrow gauge needle. The lysate was centrifuged at 2500*g* for 15 min and the resulting post-nuclear supernatant was centrifuged at 100,000*g* for 30 min. The resulting particulate protein pellets were normalized for protein content and equivalent protein loading was confirmed by staining with Coomassie blue (not shown). Alternatively, particulate fractions were obtained from whole rat kidney for use as a positive control. Immunoblotting was then performed as described previously [10]. The antisera used included a monoclonal anti-CaR antibody (ADD; raised to amino acids 214–235 of the extracellular domain of the human parathyroid CaR, supplied by NPS Pharmaceuticals, Salt Lake City, UT, and Drs. Allen Spiegel and Paul Goldsmith, Metabolic Diseases Branch, NIDDK/NIH) and an anti-megalin serum (A55; 1:5000 dilution, a gift from Dr. M. Marino, Department of Endocrinology, University of Pisa, Italy) that cross-reacts with human megalin [11].

Fluorescence measurements. Cells grown to 70–100% confluence on circular 15 mm glass coverslips were incubated for 1–2 h at room temperature with either fura-acetoxymethyl ester (1.25 μM; for $[Ca^{2+}]_i$ quantification) or 7-bis(carboxyethyl)-5-carboxyfluorescein (1.25 μM BCECF-acetoxymethyl ester; for pH_i measurement) (Molecular Probes, Eugene, OR, USA) in buffer containing (mM) Hepes (20, pH 7.4), NaCl (125), KCl (4), $CaCl_2$ (1.25), $MgSO_4$ (1), NaH_2PO_4 (1), glucose (5.5), and 0.1% bovine serum albumin.

Microfluorimetric measurements were made using a system based on a Nikon Diaphot inverted microscope as described in detail elsewhere [12]. Cells were mounted in a perfusion chamber (volume 300 μl; Warner Instruments, Hamden, CT, USA), with the coverslip forming the chamber floor, and observed through a 40× oil-immersion objective. Fluorescence was excited at 350 and 380 nm for fura-2 (emission wavelength 500 ± 20 nm) and at 436 and 488 nm for BCECF (emission wavelength 526 ± 12 nm) by means of a spinning filter wheel (40 Hz; Cairn Research, Kent, UK).

The cells were superfused continuously (3 ml/min) with control solution containing (mM) Hepes, pH 7.4 (20), NaCl (125), KCl (4), $CaCl_2$ (0.5), $MgCl_2$ (0.5), and glucose (5.5), and experiments performed at room temperature. The AGAs (obtained from Sigma–Aldrich) were each dissolved directly in the above control solution, except for kanamycin, which was first dissolved in distilled water (10 mM). Neomycin was a mixture of neomycin B (85%) and C (15%) both having six amino groups. Gentamicin consisted of gentamicin C1 (<45%), C1a (<35%), and C2 (<30%) (each with five amino groups) whilst tobramycin and kanamycin had five and four amino groups, respectively. $GdCl_3$ was also initially dissolved in distilled water (1 M). In experiments using sodium acetate, isotonicity was maintained by lowering the [NaCl] of the perfusate solution accordingly.

Data analysis. Dose–response curves for individual experiments were fitted using the Graph Pad Prism (Version 3.02) software package. Responses were normalized to the maximal response elicited by each individual cell. The mean EC_{50}'s for each agonist (±SEM) were calculated as the mean of the EC_{50} values obtained from individual experiments.

Results

CaR and megalin expression in CaR-transfected and non-transfected HEK-293 cells

We first confirmed the expression of CaR protein in CaR-transfected HEK-293 cells and the absence of CaR protein from non-transfected HEK cells (Fig. 1A). The two broad bands of CaR immunoreactivity of approximate molecular masses 120–170 and 240–310 kDa that we observed in the CaR-HEK cells correspond exactly to monomeric and dimeric CaR species, respectively [10]. We then examined the relative expression of a second AGA-binding protein [13], namely megalin, in CaR-HEK and non-transfected HEK cells. We observed megalin immunoreactivity in rat kidney particulate fractions as a protein band of approximate molecular mass 450–470 kDa, consistent with the expected size for megalin protein [11] (Fig. 1B, upper panel). However, no such bands were observed in the HEK or CaR-HEK sample lanes. Following overexposure of the immunoblot, very weak megalin-immunoreactive bands were

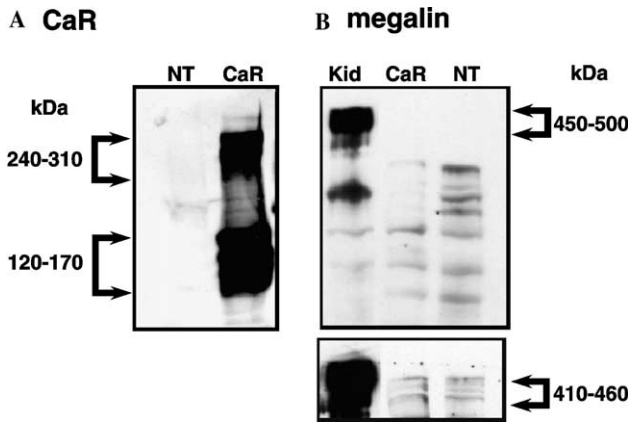

Fig. 1. CaR and megalin immunoreactivity in CaR HEK-transfected and non-transfected cells. (A) Fifty μg of whole cell lysate from non-transfected (NT) and calcium receptor-transfected (T) HEK-293 cells probed with an anti-CaR monoclonal antibody. Approximate molecular weights of the CaR species are indicated by arrows by left. (B) Rat kidney particulate fraction (25 μg; positive control) and 20 μg membrane particulate fractions from CaR-transfected (T) and non-transfected (NT) HEK-293 cells were probed with an anti-megalin polyclonal antibody (dilution 1:5000) and exposed to light-sensitive film briefly (i) or for a longer exposure time (ii). Overexposure reveals faint signal of the same intensity in both CaR-transfected and non-transfected cells. Approximate molecular weights of the immunoreactive bands are indicated by arrows.

observed in the HEK-293 cells; however, these immunoreactive species (410–460 kDa) were slightly smaller than those observed in the rat kidney sample and their intensities were considerably lower. Whilst it is unclear whether these faint bands represent megalin protein or non-specific staining, these data establish that, in HEK cells, megalin abundance is either extremely low or completely absent and is, in either case, the same in both CaR-HEK and non-transfected HEK cells.

Aminoglycosides as agonists of the CaR

The AGAs gentamicin, tobramycin, neomycin, and kanamycin were applied to CaR-HEK cells to produce a series of dose–response curves, as shown in Fig. 2. Non-transfected, non-CaR-expressing HEK-293 cells failed to respond to any of the AGA agonists tested ($n = 3$ for each drug, data not shown).

Application of neomycin to CaR-transfected HEK-293 cells produced sharp increases in $[Ca^{2+}]_i$ on application, typically consisting of a rapid spike, followed by a decrease in $[Ca^{2+}]_i$ towards baseline, characteristic of G protein-coupled receptor activation. The response to each AGA was measured as the amplitude of the initial $[Ca^{2+}]_i$ spike and normalized to the maximal response in each individual cell. A maximal response was seen with gentamicin at 500 μM, whereas with tobramycin and neomycin the maximum response was attained at 250 and 100 μM, respectively. The calculated EC_{50} values for gentamicin, tobramycin, and neomycin were 257.7 ± 30.2 μM ($n = 6$), 176.6 ± 16.3 μM ($n = 5$), and 42.6 ± 5.3 μM ($n = 3$), respectively. These data are summarized in Table 1. Kanamycin proved a poor agonist for the CaR, since it only elicited increased $[Ca^{2+}]_i$ at concentrations of 1 mM and above ($n = 5$) (not shown).

Effect of pH on tobramycin-elicited CaR activation

In the proximal tubule, the luminal fluid has a pH of 6.8–6.9, as opposed to the pH on the basolateral side of the cell, which is ~7.4. We therefore examined the effect of lowering extracellular pH (pH_o) on the CaR response to AGA challenge. We chose tobramycin for this analysis, since it is a single molecular species, while neomycin and gentamicin represent mixtures of neomycin B and C and gentamicin C1, C1a, and C2, respectively (see Methods). To reduce inter-experiment variability, we compiled complete dose–response curves for tobramycin at the two pH values using the same cells. Fig. 3A shows a representative trace of tobramycin response at pH 6.9 and 7.4 while Fig. 3B shows the mean dose–response relationships obtained from three independent experiments. Lowering the pH_o, produced a leftward shift in the dose–response curve to tobramycin (EC_{50},

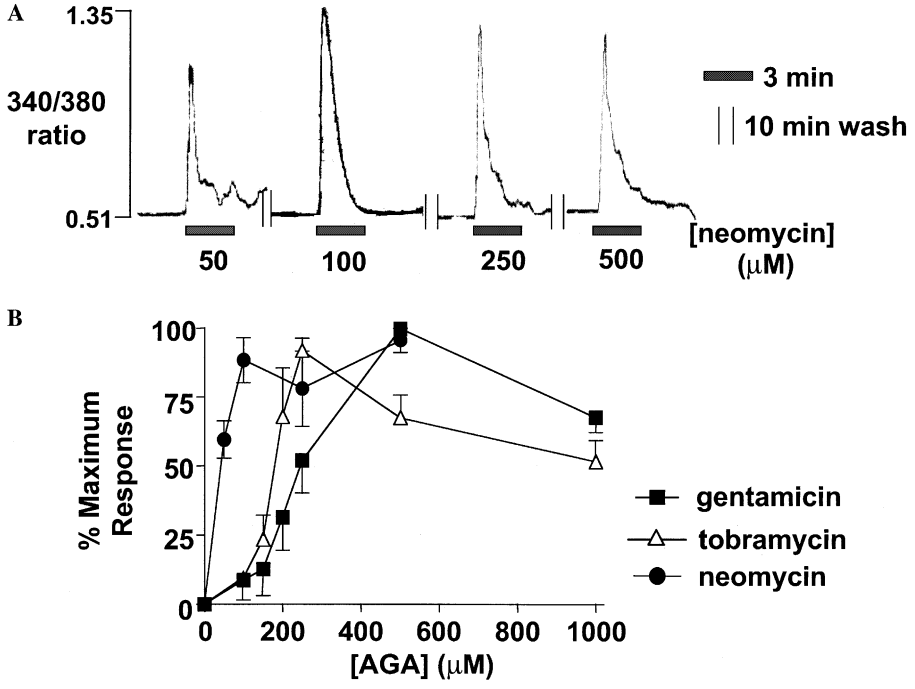

Fig. 2. AGAs increase $[Ca^{2+}]_i$ in CaR-transfected HEK cells. (A) Representative traces of neomycin-evoked increases in $[Ca^{2+}]_i$ (340:380 fluorescence ratio) in fura-2-loaded HEK-293 cells stably transfected with the human parathyroid CaR. Similar results were obtained for other AGAs tested (i.e., gentamicin and tobramycin). Cells were treated with each agonist concentration for 3 min and allowed to recover for 10 min between agonist exposures. The perfusate contained 0.5 mM Ca_o^{2+} and 0.5 mM Mg^{2+}. Non-CaR-transfected, non-CaR-expressing cells did not respond to any of the agonists tested. (B) Mean concentration–response curves for gentamicin, tobramycin, and neomycin-dependent increase in $[Ca^{2+}]_i$ in HEK-293 cells transfected with the CaR. EC_{50} values for gentamicin, tobramycin, and neomycin were 258 ± 30 μM ($n = 6$), 177 ± 16 μM ($n = 5$), and 43 ± 5 μM ($n = 3$), respectively.

Table 1
Aminoglycoside-induced CaR activation in CaR-transfected HEK-293 cells

AGA	pK_a [25]	Net charge at pH 7.4 [26]	$EC_{50} \pm SEM$ (N)	Maximal effective concentration (μM)	N amino groups [15]	Nephro-toxic potential [21,22]
Neomycin	7.8	+4.37	42.6 ± 5.3 (3)	100	6	++++
Tobramycin	6.7, 8.3, 9.9	+3.10	176.6 ± 16.3 (5)	250	5	++
Gentamicin	8.2	+3.46	257.7 ± 30.2 (6)	500	5	+++
Kanamycin	7.2	–	–	>1000	4	+

The potency of the different AGAs on CaR activation correlates with the net cationic charge, the number of amino groups, and the nephrotoxic potential of the different drugs.

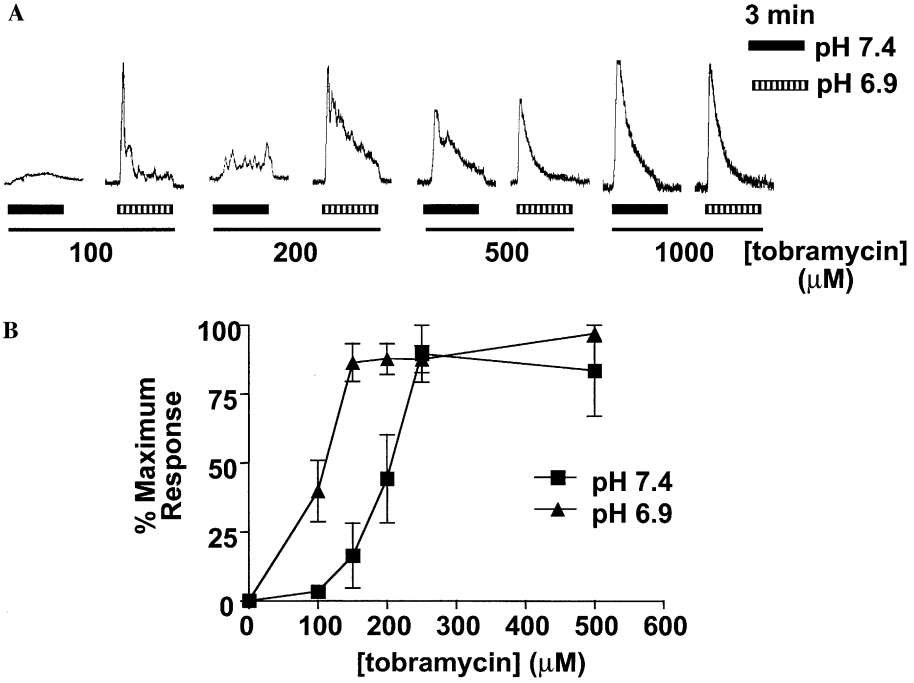

Fig. 3. Stimulation of the CaR with tobramycin at two different pH values. (A) Representative trace of tobramycin-dependent increase in $[Ca^{2+}]_i$ (fura-2 fluorescence) at pH 7.4 and 6.9 in CaR-transfected HEK-293 cells. Note that, when the same dose was applied, the CaR response to tobramycin was consistently larger at pH 6.9 than pH 7.4. (B) Mean concentration–response curves for tobramycin-induced increase in intracellular calcium at pH 7.4 (square) and pH 6.9 (triangle) ($n = 3$) in HEK-293 cells transfected with the CaR. Cells were perfused with different tobramycin concentrations (100–500 μM) at pH 7.4 and pH 6.9 for 3 min. Each individual agonist dose was followed by a 30 min wash in control solution and the pH_o was changed 10 min prior to tobramycin application. At pH_o 7.4 and 6.9, EC_{50} values were 195.8 ± 16.3 and 106.1 ± 10.7 μM, respectively.

195.8 ± 16.3 μM, pH 7.4, and 106.1 ± 10.7 μM, pH 6.9; $n = 3$), corresponding to a 1.8-fold increase in CaR potency. In contrast, the effect of Gd^{3+} (5–20 μM) on CaR activation was unaffected by lowering pH_o (Fig. 4; EC_{50}, 7.7 ± 0.6 μM, pH 7.4, and 8.8 ± 1.0 μM, pH 6.9; $n = 3$).

Effects of changing pH_o and of tobramycin administration on pH_i

Extracellular pH changes are typically reflected by changes in intracellular pH (pH_i), although such effects vary widely in extent and speed between different cell types. Alterations in pH_i may in turn affect agonist-evoked $[Ca^{2+}]_i$ signals [14]. We therefore employed the pH-sensitive fluorescent dye BCECF to test whether reduced pH_o and/or application of tobramycin effects significant changes in pH_i. Reduction of pH_o from 7.4 to 6.9 produced a small, gradual intracellular acidosis over the 10 min exposure period (Fig. 5), with a total acidification of 0.21 ± 0.03 pH units ($n = 8$). Application of 200 μM tobramycin at pH 7.4 failed to produce any detectable change in the pH_i ($\Delta pH_i = 0.02 \pm 0.02$ pH units, $n = 4$; Fig. 5). As a control, application of 20 mM sodium acetate produced a rapid and very large intracellular acidification of 1.18 ± 0.09 pH units ($n = 8$).

Discussion

Functional studies using native CaR-expressing parathyroid tissue [15], as well as work with heterolo-

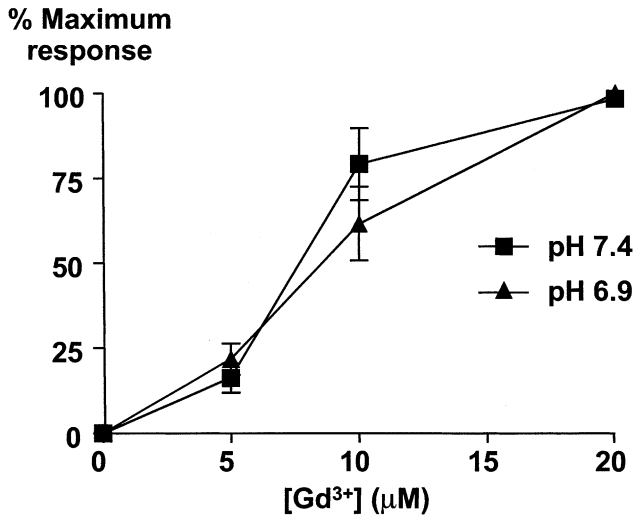

Fig. 4. Gd^{3+} activation of the CaR at two different pH values. Mean concentration–response curves for Gd^{3+} (5–20 μM)-dependent increase in $[Ca^{2+}]_i$ at pH 7.4 (7.7 ± 0.6 μM) and pH 6.9 (8.8 ± 1.0 μM) ($n = 3$) in HEK-293 cells transfected with the CaR. Data were obtained from protocols similar to those shown in Fig. 4 but with Gd^{3+} replacing tobramycin as the CaR agonist. Each individual agonist dose was followed by a 30 min wash control solution. EC_{50} values for Gd^{3+} were 7.7 ± 0.6 μM at pH 7.4 and 8.8 ± 1.0 μM at pH 6.9 ($n = 3$).

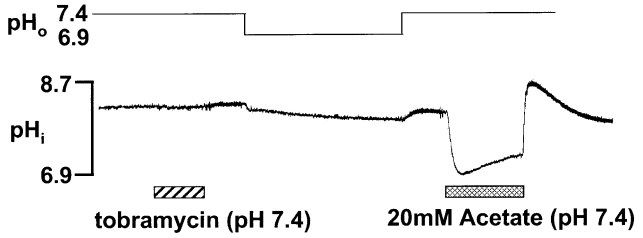

Fig. 5. Measurement of intracellular pH in a CaR-transfected HEK-293 cell loaded with BCECF. Tobramycin administration did not significantly alter pH_i ($\Delta pH_i = 0.02 \pm 0.02$, $n = 4$). Acidifying pH_o from 7.4 to 6.9 caused a small acidification of pH_i ($\Delta pH_i = 0.21 \pm 0.03$, $n = 8$), while 20 mM sodium acetate evoked a large and rapid intracellular acidification ($\Delta pH_i = 1.18 \pm 0.09$, $n = 8$).

gous expression systems [2,3,16], have shown that CaR can be stimulated by the AGA neomycin, in a manner characteristic of G protein-coupled receptor activation. To better understand the pharmacology of the AGAs as CaR agonists, we measured the CaR-mediated increase in $[Ca^{2+}]_i$ evoked by several known AGAs in HEK-293 cells stably transfected with the human parathyroid CaR [9]. This receptor shares >90% similarity with the human kidney CaR [17] and all the available data show no significant functional differences between the two receptor subtypes [18].

Single-cell fluorescence experiments showed that all AGAs tested acted as agonists of the CaR, although only weakly in the case of kanamycin. The $[Ca^{2+}]_i$ response to CaR activation was characterized by a rapid, spike-like increase in $[Ca^{2+}]_i$, indicative of Ca^{2+} release from internal stores. This was followed by a fall in $[Ca^{2+}]_i$ to a slightly elevated (plateau) level, possibly reflecting the entry of extracellular Ca^{2+}. Given that we detected no activation of the CaR by AGAs in non-CaR-expressing cells, our data indicate that these effects of polycations are mediated by stimulation of the CaR.

The EC_{50} for activation of the receptor by neomycin in the present study was 43 μM, in good agreement with previous observations in dispersed bovine parathyroid cells (70 μM) [19], CaR-transfected HEK-293 cells (30 μM) [20], and *Xenopus laevis* oocytes (60 μM) [2]. Gentamicin and tobramycin also elicited CaR-mediated Ca_i^{2+} mobilization. The efficacy of the latter two compounds was less than that of neomycin, with EC_{50} values of 258 and 177 μM, respectively. Kanamycin-elicited Ca_i^{2+} responses only at concentration $\geqslant 1$ mM. Since neomycin B and C both have six amino groups, tobramycin and the gentamicin isomers (C1, C1a, and C2) five, and kanamycin four [15], we can correlate the number of amino groups attached to the AGA to their relative potencies as agonists for the CaR (Table 1). Although not specifically investigated in the current study, it is interesting that the rank order of potency of the AGAs as agonists for the CaR also positively correlates with their nephrotoxic potential, i.e., neomycin > gentamicin ⩾ tobramycin > kanamycin [4,21,22] (Table 1).

Most AGA nephrotoxicity occurs in the proximal tubule, where the CaR is located on the luminal membrane [7]. In a recent study, we have shown that in CaR-expressing, proximal tubular-derived opossum kidney (OK) cells, neomycin and gentamicin increase $[Ca^{2+}]_i$ and stimulate the extracellular signal-regulated kinases, ERK1 and ERK2 [8], indicating that, in this cell system, the AGAs act via a G_q protein-coupled receptor [8]. Our current study confirms previous findings and indicates that the G protein-coupled receptor in question is the calcium-sensing receptor.

Next we found that on decreasing pH_o from 7.4 to 6.9, to reflect the pH of the proximal tubular lumen (pH 6.8–6.9) [23], the sensitivity of the CaR to tobramycin almost doubled. Since changes in pH_o generally cause changes in pH_i, it was important to rule out changes in pH_i as a possible cause of the increase in AGA sensitivity. Direct measurement of pH_i showed relatively small and slow changes in pH_i upon extracellular acidification. In addition, "acid-loading" cells to acidify pH_i did not mimic the effect of reducing pH_o. This indicates that the effect of reducing pH_o is direct rather than mediated via changes in pH_i.

There are two possible explanations for the enhanced sensitivity of the CaR to tobramycin at pH 6.9. The first is that the effect could be due to a direct alteration of receptor structure, in particular the structure of the ion-

sensing region, by pH. The presence of several histidine residues in the amino terminus of the receptor supports the hypothesis of a pH effect on receptor function. Thus, we have estimated the isoelectric point (p*I*) for the extracellular domain (amino acids 1–613) of the human parathyroid CaR [9], calculated using the following approximation:

$$\mathrm{p}I = \sum_i n_i \mathrm{p}K_{\mathrm{a}}^i \Big/ \sum_i n_i,$$

where n_i is the number of amino acid side chains and $\mathrm{p}K_{\mathrm{a}}^i$ their corresponding pK_{a} values [24]. Thus, we estimate that the p*I* of the ECD of CaR is between 7.1 and 8. This implies that, at pH 6.9 and 7.4, the ECD would have a small positive net charge and that acidification of the buffer from pH 7.4 to 6.9 would alter the net charge of the ECD and therefore could affect the CaR's affinity for its agonists. However, as the sensitivity of the CaR to Gd^{3+} (whose net charge is independent of pH_o) was not affected by lowering pH_o, we conclude that such a change in pH_o does not alter the agonist sensitivity of the receptor per se. The second possible explanation is that the pH_o change alters the charge on the AGA itself thereby altering its potency as an agonist. Tobramycin has five amino groups, with pK_{a} values of 6.7, 8.3, and 9.9 [25]. Using these pK_{a} values, together with the value of net charge for tobramycin at pH 7.4, namely +3.1, [26], we estimate that lowering the pH_o from 7.4 to 6.9 would change the net cationic charge of the drug from +3.1 to +4.2. Such an increase in net positive charge could account for the enhanced CaR response to tobramycin at the lower pH value. We cannot rule out the possibility that the small intracellular acidification present during incubation in pH 6.9 buffer contributes to the enhanced CaR responsiveness to tobramycin. However, as the responses to Gd^{3+} at pH_o 6.9 (that is, in the presence of a small intracellular acidification) were equivalent to those obtained at pH 7.4, we would suggest that intracellular acidification alone does not alter the CaR-induced Ca_i^{2+} mobilization. Together, the current data suggest that the enhanced AGA response seen at acidic pH_o results from enhanced drug ionization rather than a specific effect on the ECD of the CaR. Whatever the mechanism, the data clearly indicate that AGA stimulation of the CaR will be enhanced by the prevailing proximal tubular pH.

The CaR is also expressed in some arteries, either in the perivascular nerve network [27] or in the smooth muscle cells [28] and treatment with CaR agonists can relax preconstricted vessels [27,29]. In canine cerebral arteries, preconstricted with either KCl or oxyhemoglobin (which is proposed to cause cerebrovascular spasm following subarachnoid hemorrhage), AGAs relax the vessels with a rank order of potency equivalent to that observed here for their effects on the CaR [29]. Whether the AGAs relax the vessels via stimulation of the CaR or by an alternative mechanism remains to be determined.

Megalin is a ~600 kDa membrane protein present in the endocytic pathway of renal proximal tubule cells that binds calcium and polybasic drugs such as AGAs and delivers them to the lysosomes [4,13]. Thus, megalin has been proposed to be an AGA receptor [30,31]. However, it remains unclear whether megalin-mediated internalization of AGAs in proximal tubular cells represents the sole determinant of AGA toxicity. As we show here, HEK-293 cells express little or no megalin, which is not surprising given their embryonic, mesenchymal origin. Thus, in light of the current data and our previous study in OK cells [8], it will be interesting to ascertain whether CaR signals also contribute to AGA-induced nephrotoxicity.

In conclusion, the current study demonstrates that the CaR is a target for various AGAs and that their potency for the receptor relates to the number of their attached amino acids and to their pH-dependent charge. Thus, the pharmacological/toxicological effects of the AGAs could potentially involve activation of the CaR. Further studies will be necessary to determine whether CaR activation plays any part in AGA-elicited nephrotoxicity or vasodilatation, or in hearing damage.

Acknowledgments

This study was supported by a grant from the Wellcome Trust. S.M. was a BBSRC CASE student supported by Sanofi-Synthelabo. D.H. was a recipient of a National Kidney Research Fund prize studentship and D.T.W. is a National Kidney Research Fund, Career Development Fellow. We thank Dr. E. Nemeth (NPS Pharmaceuticals, Salt Lake City, UT, USA) for the generous gift of the CaR-transfected HEK-293 cells. We also thank NPS Pharmaceuticals and Drs. Allen Spiegel and Paul Goldsmith (Metabolic Diseases Branch, NIDDK/NIH) for the CaR antibody and Dr. M. Marino for the megalin antiserum.

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