Aminoglycosides Increase Intracellular Calcium Levels and ERK Activity in Proximal Tubular OK Cells Expressing the Extracellular Calcium-Sensing Receptor

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Abstract. Aminoglycoside antibiotics (AGAs) are nephrotoxic, with most of the damage confined to the proximal tubule, but the mechanism for cellular toxicity is not clear. It has been previously shown that the extracellular-calcium sensing receptor (CaR) is expressed in intact rat proximal tubule and can be stimulated by the AGA neomycin. To investigate whether CaR could contribute to AGA-induced nephrotoxicity, the acute responses to various AGAs in the proximal tubule-derived opossum kidney (OK) cell line were examined. The presence in OK cells of CaR-related transcripts and protein was demonstrated by northern analyses, reverse transcriptase–PCR, immunocytochemistry, and immunoblotting. OK cells responded to elevated extracellular calcium (Ca\(^{2+}\)) and neomycin but also to gentamicin and tobramycin with an increase in cytosolic [Ca\(^{2+}\)]. Ca\(^{2+}\), neomycin, and gentamicin also activated the extracellular signal-regulated kinases, ERK1 and ERK2. Neomycin-induced ERK activation was both dose- and time-dependent and was attenuated by inhibitors of phosphatidylinositol 3-kinase, phosphatidylinositol bisphosphate (PIP\(_2\))–specific phospholipase C, and MEK1, but not of protein kinase C. Thus, proximal tubular OK cells express a CaR that mediates Ca\(^{2+}\), mobilization and PIP\(_2\)-PLC-dependent ERK activation in response to AGAs and thus could play a role in AGA-induced nephrotoxicity.

Aminoglycoside antibiotics (AGAs) are widely used in the treatment of Gram-negative infections, but their clinical usefulness is reduced by their nephrotoxicity. Renal impairment, defined as an increase in plasma creatinine ≥45 \(\mu\)mol/L during or after therapy, occurs in 10 to 37% of patients and is proportional to dose and duration of administration (1,2). Although renal failure is generally reversible, renal dialysis therapy may be required in patients with severe acute renal damage. Surface membrane binding of AGAs is well characterized (3–5), and cellular uptake occurs by fluid-phase and receptor-mediated endocytosis (6). Nephrotoxicity has therefore been ascribed to endocytosis and sequestration of AGAs to lysosomes, formation of myeloid bodies, and phospholipidosis. Rupture of the lysosomal membrane and release of acid hydrolases follows with subsequent necrotic cell death (1,2). Central to this process is the multiligand endocytic receptor, megalin, possibly in association with cubilin, acting as the receptor for the polybasic AGAs (7,8). However, several observations indicate that drug endocytosis per se may not constitute the main cause of AGA cellular toxicity. For instance, the net cationic charge of the molecule correlates with the nephrotoxic potential of these drugs. This cannot be explained in terms of an increase in the number of secondary lysosomes and phospholipidosis (1). In addition, several drugs such as antihistamines and tetracyclines accumulate into the cytosol of proximal tubular cells, causing myeloid body formation, but significant clinical or experimental nephrotoxicity is rare (9). In addition to the cellular damage and the accompanying fall in GFR, acute gentamicin treatment in rat results in significant hypercalciuria and hypermagnesuria and a rapid dose-related increase in urinary n-acetyl-\(\beta\)-d-glucosaminidase (a brush border marker for early gentamicin toxicity) well before tubular injury and histologic changes take place (10). Moreover, dietary calcium loading reduces the nephrotoxic potential of the drug, but it does not reduce drug accumulation in the renal cortex (11). Finally, chronic treatment of rats with low concentrations of gentamicin induces renal cortical apoptotic, rather than necrotic cell death (3), as would be expected if cell death was due to release of lysosomal hydrolases (12).

An alternative mechanism mediating AGA cellular toxicity has been identified in the cell-surface, G protein-coupled Ca\(^{2+}\)/(polyvalent cation)–sensing receptor (CaR) (13–16). The receptor is present in the kidney, where it regulates urinary salt and water excretion by the distal nephron (17). The CaR has also been localized to the luminal membrane of the proximal tubule (16), but its role and downstream effectors are unknown. Functional studies in which the CaR was overexpressed in Xenopus laevis oocytes and human embryonic kidney (HEK) cells have demonstrated that the receptor responds, not only to changes in extracellular free ionized Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_o\)], but also to various divalent and trivalent cations (i.e., Mg\(^{2+}\) and Gd\(^{3+}\), as well as polycationic compounds, including the AGA neomycin (13,14,18). In nontransfected...
oocytes or HEK cells, CaR agonists do not stimulate inositol phosphate production or elevate intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Thus, the presence of the CaR in the proximal tubule, in conjunction with the ability of neomycin to activate the receptor, suggests that the CaR might be involved in the process of cellular toxicity induced by the AGAs.

A well-characterized cell culture model of the proximal tubule is the opossum kidney (OK) cell, but no previous knowledge of the existence of a CaR in this expression system was available. Therefore, we initially sought in this study to demonstrate the presence in OK cells of an endogenous CaR, similar to that previously identified, and then to examine the effects of CaR agonists on intracellular signaling. Our findings indicate that OK cells express a CaR (OK-CaR) and that AGAs stimulate the receptor through a phosphatidylinositol 4,5 bisphosphate phospholipase C (PIP$_2$-PLC)–dependent pathway.

### Materials and Methods

#### Materials

Various items were obtained from sources described previously (16,19) or from the following sources: ECL and ECLplus reagents were purchased from Amersham International Plc. (Little Chalfont, Bucks, UK). All other chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK).

#### Tissue Cell Culture

Opossum kidney cells (used between passage 12 to 25) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and grown in Minimum Essential Medium Eagle (MEM) supplemented with 10 mM l-Glu; Sigma) supplemented with 10% fetal bovine serum and 50 U/ml penicillin and 50 U/ml streptomycin.

#### Northern Blotting and Reverse Transcriptase-PCR

Total RNA was extracted from OK or cells using TRI-reagent (Helena Biosciences, Sunderland, UK) and mRNA separated using Dynabeads (Dynal AS, Oslo, Norway). Northern blots using 2 to 3 μg of poly(A$^+$) RNA were probed with a full-length CaR [32P]cDNA probe as described previously (14). Blots were washed with moderate stringency at 42°C. For reverse transcriptase–PCR (RT-PCR), the first strand of the mRNA was synthesized using Superscript II (Life Technologies, Paisley, UK) and PCR amplification performed as described previously (20). To avoid genomic DNA amplification, the primer sequences were designed to span one intron based on the human parathyroid sequence (21): forward primer, 5'-CTGCTTTGAGTGTGGAGT-3'; reverse primer, 5'-GAAGATGAGCATGCTGAAGG-3'. As negative and positive controls, the OK cell DNA template was replaced with an equivalent volume of water and with an equivalent amount of RNA reverse-transcribed from rat kidney, respectively. The 759-kb PCR product was visualized with ethidium bromide, and specificity of the reaction was determined by Southern blotting using a [32P]labeled 3.2-kb XhoI-Apal fragment corresponding to the coding region of the rat kidney CaR as described previously (20). Hybridization occurred overnight at 50°C followed by two 30-min washes in 5X SSC at 37°C and then two 60-min washes in 0.1X SSC at 65°C.

### Preparation of OK Cell Membrane Particulate Fractions

Cells were rinsed in phosphate-buffered saline (PBS) for 5 min, scraped from the flasks in lysis buffer containing 12 mM HEPES (pH 7.6), 300 mM mannitol, pepstatin, leupeptin, and PMSF (19), and then repeatedly drawn in and out of a narrow-gauge needle to disrupt the cell membranes. The lysate was centrifuged at 2500 × g for 15 min, and an aliquot of this post-nuclear supernatant was centrifuged at 100,000 × g for 30 min to give a particulate protein pellet. Samples were normalized for protein content by assaying according to the method of Bradford (22). For use as CaR positive controls, particulate fractions were also prepared from rat and opossum kidney. The whole kidneys were homogenized in a hand-held homogenizer using the same buffer as for the OK cells and particulate fractions prepared using the same centrifugation protocol.

#### Immunoblotting and Immunocytochemistry

Immunoblotting was performed as described previously (19). The following antisera were used: monoclonal anti-CaR (1:5000 dilution, raised to amino acids 214 to 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, Bethesda, MD); affinity-purified anti-CaR (1:200 dilution, raised in rabbit to amino acids 214 to 236 of the extracellular domain of the rat kidney CaR [Lofstrand, Inc., Bethesda, MD] and affinity purified as described elsewhere [16]); phosphospecific anti-ERK polyclonal antibody (1:5000 dilution; Promega UK, Southampton, UK); and anti-ERK1 polyclonal antibody (which detects ERK2 with equal intensity; 1:5000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For immunofluorescence, OK cells from at least three separate passages were grown on coverslips to 50 to 80% confluence and fixed with 2% paraformaldehyde solution at RT for 30 min and permeabilized with 0.075% saponin in PBS for 10 min. Indirect immunofluorescence was performed using an anti-CaR polyclonal antibody (1:200 dilution). Antibody binding was detected using an affinity-purified anti-rabbit IgG conjugated with cyanine3 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). As negative control, the primary antibody was preincubated overnight at 4°C with an excess of antigenic peptide before incubation with the slides. Confocal images were taken using an Ultraview confocal optical scanner with a Kr/Ar laser (Perkin-Elmer Life Sciences, Cambridge, UK) mounted on an Olympus microscope IX70 (Olympus, London, UK). Images were acquired with an Ultrapix CCD digital camera and processed using the Ultraview software package.

#### Fura-2 Fluorescence

Cells were grown to 80 to 100% confluence on 15-mm circular glass coverslips (Clark Electromedical; Harvard Apparatus Ltd., Kent, UK). For the measurement of [Ca$^{2+}$], cells were loaded with Fura-2 AM (Molecular Probes, Eugene, OR) (1.25 μM) for 2 h at room temperature in the dark in 20 mM HEPES buffer, pH 7.4, containing 125 mM NaCl, 4 mM KCl, 1.25 mM CaCl$_2$, 1 mM MgSO$_4$, and 1 mM Na$_2$HPO$_4$ and supplemented with 0.1% bovine serum albumin and 5.5 mM glucose. After loading, the cells were washed at 37°C for 20 to 30 min in the same HEPES-buffered solution.

Single-cell microfluorimetric measurements were made using a Nikon (Tokyo, Japan) Diaphot inverted microscope as described in detail by Berrie and Elliott (23). The coverslip with the cells was mounted into a perfusion chamber (volume 150 μl; Warner Instruments, Hamden, CT) with the coverslip acting as the base of the chamber. The chamber apparatus was then placed on the stage of the...
microscope, and the cells were observed through a 40X-oil immersion objective. Fluorescence was excited at 340 and 380 nm (emission wavelength, 500 ± 20 nm) in experiments using Fura-2 AM by means of a spinning filter wheel (40 Hz) (Cairn Research, Faversham, Kent, UK).

Cells were continuously superfused at a flow rate of 3 ml/min with a buffer containing 20 mM HEPES (pH 7.4), 125 mM NaCl, 4 mM KCl, 0.5 mM CaCl₂, 0.5 mM MgCl₂, and 5.5 mM glucose, but without bovine serum albumin (experimental buffer). Experiments were performed at room temperature.

ERK Activation Assay

Cells were grown to 80 to 100% confluence in 35-mm culture dishes and rinsed in PBS for 5 min before equilibration for 20 min in experimental buffer (these and subsequent incubations were conducted at 37°C). Cells were then incubated for 5 min in either experimental buffer alone or in buffer supplemented with various CaR agonists. Alternatively, cells were pretreated for 5 min with various signaling inhibitors before addition of neomycin for an additional 5 min. Where [CaCl₂] was increased to 5 mM, the [NaCl] was reduced accordingly to normalize osmolarity. The cells were then lysed on ice in the following RIPA buffer: 12 mM HEPES (pH 7.6), 300 mM mannitol, 1% (vol/vol) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate supplemented with 1.25 μM peptatin, 4 μM leupeptin, 4.8 μM PMSF, 1 mM EDTA, 1 mM EGTA, 100 μM vanadate, 1 mM NaN₃, and 250 μM sodium pyrophosphate. Lysate was then mixed with 5X Laemmli buffer and boiled for 3 min before immunoblotting using the phospho-specific anti-ERK antibody. The protein equivalency of the samples was always demonstrated by ponceau-staining the blot before immunoblotting. To ensure that ERK protein levels were not altered by the experimental treatments, samples were also immunoblotted against conventional anti-ERK antisera.

Statistical Analyses

Data are presented as means ± SEM, and statistical significance was determined by one-way ANOVA (Tukey post hoc test).

Results

Proximal Tubular–Derived OK Cells Express CaR-Like mRNA and Protein

A full-length CaR [³²P]-labeled cDNA probe detected a series of CaR transcripts of approximately 8, 4.5, 2.5, and 1.5 kb on a Northern blot of OK cells poly(A⁺)RNA (Figure 1A, lane 1). The most prominent transcript at approximately 4.5 kb was also observed in the lane containing poly(A⁺)RNA from whole opossum kidney (lane 2). Reverse transcription and PCR amplification using intron spanning, CaR gene-specific primers amplified a 759 bp sequence from both rat kidney and OK cell mRNA. No products were detected when the DNA template was replaced with water in the PCR reaction (Figure 1B). High stringency Southern analysis using a ³²P-labeled CaR-specific cDNA probe confirmed that the 759-bp PCR products were amplified from genuine CaR transcripts (Figure 1C).

To investigate whether whole opossum kidney contains a CaR-like protein of similar molecular mass to the CaR cloned from rat kidney (14), we immunoblotted kidney particulate proteins from each species against an anti-CaR–specific monoclonal antibody (Figure 2A). The anti-CaR immunoreactivity under reducing conditions was very similar between the rat and opossum kidney fractions (Figure 2A, lanes 2 and 4, respectively), with differences noted in the apparent glycosylation pattern between the two. In addition, when denatured under nonreducing conditions, the CaR-immunoreactive proteins of both species exhibited apparent molecular masses double that observed under reducing conditions (Figure 2A, lanes 1 and 3). This lower electrophoretic mobility is indicative of the disulfide-linked dimerization we reported previously for the rat kidney CaR (19).

The particulate fractions of lysed OK cells also contained CaR-immunoreactive bands (Figure 2A, lane 6, right-hand side) of apparent molecular masses 120, 170, and >200 kD, consistent with the mass of rat kidney CaR species (Figure 2A, lane 5, left-hand side). The binding of the anti-CaR monoclonal antibody to the CaR bands was shown to be specific because it was ablated by preincubation of antibody with excess immunizing peptide (Figure 2A, right-hand side of lane 5 and left-hand side of lane 6). In addition, the anti-CaR immunoreactivity was greater in a sample of OK cell crude membranes (lane 8) than in a protein equivalent sample of OK cell postnuclear supernatant (i.e., total homogenate, lane 7).

These data are supported by immunocytochemical investigation of polyclonal anti-CaR–stained OK cells using scanning laser confocal microscopy to assess the three-dimensional architecture of the CaR signal. This revealed extensive CaR immunoreactivity in OK cells (Figure 2B; X-Y section, parallel to the coverslip) that was most intense at the upper surface of the cells (Figure 2B, X-Z sections, i and ii, perpendicular to the...
plane of the coverslip) but that included some intracellular localization. Substantially less CaR immunoreactivity was observed in the central and basal regions of the cells, and none was observed in the nuclear region (Figure 2B, i and ii). CaR staining in OK cells was shown to be specific, as it was completely ablated by preincubation of the antiserum with immunizing peptide to block specific binding. The specific anti-CaR staining in the OK cells is highlighted by asterisks. Lanes 7 and 8 show that the OK cell CaR-immunoreactivity is enriched in the particulate fraction (lane 7, 30 μg of OK cell postnuclear supernatant; lane 8, 30 μg of OK cell particulate protein; lane 9, 30 μg of particulate protein from whole opossum kidney). Arrowheads on the left indicate the apparent molecular masses of the CaR-reactive species observed. (B) An affinity-purified, rabbit anti-CaR polyclonal antibody (1:200 dilution) detects a CaR-immunoreactive protein in confluent OK cells (labeled a, b, and c; X-Y section, parallel to the plane of the cover-slip). Binding of the primary antibody is detected using a Cyanine3-coupled anti-rabbit secondary antibody (1:400) by confocal fluorescence microscopy. Panels Bi and Bii show X-Z sections (i.e., perpendicular to the plane of the cover-slip) through cells c and a, respectively. The most intense CaR staining is located at the top of the cell, whereas the central and basal regions of the cells exhibit considerably less CaR immunoreactivity. Results are representative of a minimum of three independent experiments.

### Elevated $[Ca^{2+}]_i$ Levels in OK Cells Treated with High $[Ca^{2+}]_o$ or Aminoglycosides

To examine the possible presence of functional CaR-like activity in OK cells, we measured the responsiveness of Fura-2–loaded OK cells (by calculation of the 340/380 nm fluorescence ratio) to elevated $[Ca^{2+}]_o$ and to various AGA agonists. Figure 3A shows that raising $[Ca^{2+}]_o$ from 0.5 mM to 2.5, 5, 1484 Journal of the American Society of Nephrology J Am Soc Nephrol 13: 1481–1489, 2002

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#### Anti-CaR immunofluorescence (X-Y section)

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Figure 2. OK cells express a CaR-like protein. (A) Anti-CaR immunoblot (monoclonal antibody) containing 50 μg of particulate protein from rat (lanes 1 and 2) and opossum kidneys (lanes 3 and 4) denatured under reducing (143 mM β-mercaptoethanol; lanes 2 and 4) or nonreducing conditions (lanes 1 and 3). Lanes 5 and 6 contain 30 μg of particulate protein from whole rat kidney and OK cells, respectively. The right-hand side of lane 5 and left-hand side of lane 6 were separated and probed with anti-CaR antibody preincubated with immunizing peptide to block specific binding. The specific anti-CaR staining in the OK cells is highlighted by asterisks. Lanes 7 and 8 show that the OK cell CaR-immunoreactivity is enriched in the particulate fraction (lane 7, 30 μg of OK cell postnuclear supernatant; lane 8, 30 μg of OK cell particulate protein; lane 9, 30 μg of particulate protein from whole opossum kidney). Arrowheads on the left indicate the apparent molecular masses of the CaR-reactive species observed. (B) An affinity-purified, rabbit anti-CaR polyclonal antibody (1:200 dilution) detects a CaR-immunoreactive protein in confluent OK cells (labeled a, b, and c; X-Y section, parallel to the plane of the cover-slip). Binding of the primary antibody is detected using a Cyanine3-coupled anti-rabbit secondary antibody (1:400) by confocal fluorescence microscopy. Panels Bi and Bii show X-Z sections (i.e., perpendicular to the plane of the cover-slip) through cells c and a, respectively. The most intense CaR staining is located at the top of the cell, whereas the central and basal regions of the cells exhibit considerably less CaR immunoreactivity. Results are representative of a minimum of three independent experiments.
in ERK phosphorylation described in the study can be explained by nonequivalent protein loading or by altered ERK protein expression, as these were assessed by staining membranes with ponceau S and by immunoblotting samples against conventional anti-ERK antisera (data not shown).

Neomycin-induced ERK activation was then studied as a function of time and concentration. Neomycin treatment (5 min) elicited dose-dependent ERK activation that was maximal at approximately 100 μM (Figure 4C). The activation of ERK by neomycin (300 μM) was also time-dependent, with the onset of effect occurring between 1 and 2.5 min after exposure to neomycin (Figure 4D). The peak effect of neomycin was observed at approximately 5 min, and the response was only slightly diminished after 20 min, which indicates that it is not a transient response.

**Neomycin-Induced ERK Activation Involves PIP₂-PLC and PI₃ Kinase but Not PKC**

To examine the signaling pathway(s) involved in the neomycin response, we pretreated the cells with inhibitors to PIP₂-PLC (U73122), MAPK/ERK-activating kinase-1 (MEKI) (PD98059), PI₁ kinase (wortmannin and LY294002), and protein kinase C (PKC) (GF109203X and calphostin C). GF109203X acts by binding to the catalytic domain of all PKC isosforms, whereas calphostin C binds to the regulatory domain blocking 1,2 diacylglycerol–induced activation of the conventional and novel PKC isosforms. The concentration of calphostin C was carefully chosen to fully inhibit PKC without affecting protein kinase A activity (24). Neomycin-induced ERK activation was abolished by inhibition of PIP₂-PLC with U73122 and by inhibition of MEKI with PD98059 (Figure 5). Also, inhibition of PI₁ kinase almost completely ablated neomycin-induced ERK activation (Figure 6), whereas inhibition of PKC had no significant effect on the ERK response (Figure 7).

**Discussion**

AGAs cause structural, functional and metabolic alterations within the renal proximal tubules, but the mechanisms by which they exert these changes are not understood. Accumulating indirect evidence indicates that drug endocytosis may not be related to cell toxicity. CaR distribution and abundance in rat (16) proximal tubules is consistent with the sites of AGA nephrotoxicity, which is mostly confined to the S1 and S2 segments (25); we therefore tested the ability of AGAs to activate the CaR as a possible mechanism for the early events of AGA-induced cell toxicity. We used a proximal tubular–derived cell line model, OK cells. First, to determine whether OK cells express a CaR-like molecule, we performed northern analysis on poly(A⁺) RNA extracted from the OK cells. The two biggest transcripts (4.5 and 8 kb) are approximately the same size as those detected in whole rat kidney (14), and, given that the predicted size for the CaR protein is approximately 120 kD, they could produce a functional CaR. The presence of CaR–related transcripts in OK cells was also confirmed by RT-PCR using intron-spanning primers designed to amplify a region in the putative transmembrane domain of the rat kidney.

**Figure 3.** Effects of various aminoglycoside antibiotics and elevated extracellular Ca²⁺ levels on intracellular free calcium concentration. Cultured opossum kidney cells loaded with 1.25 μM Fura-2 were superfused with a HEPES-buffered medium containing 0.5 mM Ca²⁺, and changes in the emission ratio (340/380 excitation) were measured to assess changes in [Ca²⁺]ᵢ, evoked either by elevating [Ca²⁺]ᵢ from 2.5 to 10 mM or by addition of bradykinin (BK; 200 nM) to demonstrate cell viability. (A) Solid black bars indicate 3-min treatment to reach statistical significance. None of the apparent changes to neomycin (300 μM) could still be observed (n = 4; not shown). Although not shown, other classic CaR agonists, Gd³⁺ and Mg²⁺, were also effective in the activation of the OKCaR.

**Activation of ERK in OK Cells by CaR Agonists**

To examine whether CaR agonists induce other downstream signaling pathways in OK cells in addition to increasing [Ca²⁺]ᵢ, we examined the effects of Ca²⁺ (5 mM), Gd³⁺ (100 μM), neomycin (300 μM), and gentamicin (300 μM) on phosphorylation and thus activation of extracellular signal-regulated kinase (ERK). When OK cells were exposed to each CaR agonist, they exhibited markedly increased ERK phosphorylation/activation, which indicates that the putative OK cell CaR couples to the ERK pathway (Figure 4, A and B). ERK activation by Gd³⁺ (100 μM) was strong but not consistent, as it was seen in seven of the nine experiments, failing therefore to reach statistical significance. None of the apparent changes and 10 mM caused a dose-dependent elevation in [Ca²⁺]ᵢ. The potency of the Ca²⁺ᵢ response is indicated in the log dose-response curve in Figure 3B, and from this we calculated an EC₅₀ value for [Ca²⁺]ᵢ of 2.6 mM. Superfusion with either neomycin (500 μM; n = 4), tobramycin (500 μM; n = 1), or gentamicin (500 μM; n = 2) for 3 min also induced a rapid increase in [Ca²⁺]ᵢ in OK cells (Figure 3C). At concentrations of neomycin as low as 10 μM, smaller elevations in [Ca²⁺]ᵢ could still be observed (n = 4; not shown). Although not shown, other classic CaR agonists, Gd³⁺ and Mg²⁺, were also effective in the activation of the OKCaR.
CaR (13,14). Next, we confirmed that whole opossum kidney does contain a CaR-immunoreactive protein similar in size to the CaR previously characterized in rat kidney (14). We previously reported that renal CaR is seen on immunoblot as a disulfide-linked dimer in the absence of reducing agents (19). Thus, as both the OK CaR and rat kidney CaR exhibited dimeric immunoreactivity in the absence of reducing agents, this further confirms that the monoclonal anti-CaR antibody used here detects the opossum version of the CaR. Particulate fractions of OK cells exhibited anti-CaR immunoreactivity that was essentially similar in size to the whole rat kidney CaR, although less diffuse. Indeed, the OK cell CaR immunoreactivity was strongest in the 170-kD species, which possibly represents the mature, fully-glycosylated CaR. The immunoreactivity of this and the other, fainter bands (120 and 200 kD) was fully ablated by preincubation of the antibody with immunizing peptide indicating specific binding of the antibody. Whole OK cells also stained positively for CaR using a polypeptide anti-CaR antiserum raised to the same peptide sequence as for the monoclonal antibody. Confocal microscopy revealed that the most intense CaR immunoreactivity in OK cells is observed at the upper extremity of the cells, consistent with localization on, or at, the cell membrane. In addition, some intracellular CaR staining was also observed. This is entirely consistent with numerous studies of endogenous CaR cellular localization including keratinocytes (26), rat distal convoluted tubule cells (16), pancreatic acinar cells (27), rat chondrogenic RCJ.C5.18 cells (28), and mouse osteoblastic MC3T3-E1 cells (29). The reason for, or consequence of, such localization remains unclear, but CaR-mediated signals are nevertheless still observed in these cells.

To determine whether the OKCaR exhibits functional activity comparable to that of rat kidney CaR, we examined the sensitivity of the OK cells to classic CaR agonists, namely Ca$^{2+}$o and neomycin (13,14,30). Our results show that both agonists evoked dose-dependent, rapid elevations in [Ca$^{2+}$]i, which is characteristic of the activation of G$\alpha$-coupled, seven-transmembrane receptors. The EC$_{50}$ value for Ca$^{2+}$o in OK cells is very similar to that reported for the previously identified CaR (EC$_{50}$, 2.6 mM and 3 mM, respectively) (13). Together with the molecular evidence, these data strongly suggest that OK cells express a CaR-like molecule with substantial similarity to the receptor previously identified.

Work from our laboratory shows that the AGAs, neomycin, tobramycin, and gentamicin, can all evoke dose-dependent increases in [Ca$^{2+}$], in HEK cells that have been stably transfected with the CaR, but not in non-transfected, non-CaR-expressing cells (Mclarnon et al., unpublished observations). In the current study, using OK cells the same AGAs tested in HEK cells, have produced elevations of [Ca$^{2+}$]i comparable to those evoked by Ca$^{2+}$o. Given that these compounds are strongly charged at the physiologic/experimental pH (1) and therefore cannot cross the plasma membrane, we conclude that neomycin, gentamicin, and tobramycin increase [Ca$^{2+}$]i by
stimulating the endogenous OKCaR, a membrane-bound receptor. The sustained increase in \([Ca^{2+}]_i\) due to an activation of the proximal tubular CaR could account for the cell calcium overload concomitant with the pathogenesis of nephrotic renal tubular injury induced by gentamicin (12).

Stimulation of the CaR by \([Ca^{2+}]_o\) has been recently associated with ERK activation in fibroblasts (31), osteoblasts (32), parathyroid cells, and CaR-transfected HEK cells (33). To explore the possibility that AGAs could do the same in the proximal tubule, we next tested the ability of AGAs to activate the ERK pathway in OK cells. In the current study, \([Ca^{2+}]_o\) and the AGAs, neomycin and gentamicin, induced the activation of the proline-directed protein kinases ERK 1 and ERK 2 with responses which were both highly consistent and statistically significant.

To further demonstrate that neomycin exerts its effect on ERK via a G protein-coupled receptor-mediated pathway, we employed U73122 to inhibit PIP2-PLC and found that it abolished the neomycin response. This result is supported by the time course data, which show that neomycin induces an almost immediate elevation in \([Ca^{2+}]_i\) but a slightly delayed activation of ERK. It has been suggested that AGAs exert their toxic effect by binding to membrane phospholipids (namely phosphatidylinositol) and by inhibiting PIP2-PLC activity in some systems (34). The current study does not support this, finding instead that in OK cells, neomycin stimulates the PLC-IP3 pathway with subsequent activation of ERK and this effect was ablated by the specific PIP2-PLC inhibitor U73122.

The neomycin response was also abolished by the MEK1 inhibitor, PD98059, indicating that its effect on ERK is via the classic MEK1/ERK 1,2 pathway. It has previously been shown that CaR stimulation can result in PKC activation and that CaR activity can indeed be regulated by PKC (35,36). However, two separate PKC inhibitors in the current study failed to...
significantly alter the response of ERK to neomycin, which indicates that the neomycin effect is PKC-independent.

To examine the role of the PI3 kinase pathway in AGA-induced ERK activation, we used two structurally unrelated inhibitors of PI3 kinase, wortmannin and LY294002 (37). Identical effects for the two inhibitors were seen; we therefore identified PI3 kinase as one of the effectors downstream of CaR activation by neomycin. We furthermore determined that neomycin treatment of OK cells induces PI3 kinase-mediated activation of the MEK1/ERK 1,2 pathway. One possible mediator of PI3 kinase-induced MEK/ERK activation is p21cdc42/rac1-activated kinase (PAK) (reviewed in reference 38); future studies could examine whether CaR activates PAK. Regarding the use of these inhibitors, it is worth noting that Davies et al. (37) have recently reported that at concentrations 5 to 33 times greater than those used in the current study, PD98059 and wortmannin exerted no nonspecific inhibitory effects on the activities of a panel of 24 diverse protein kinases. In addition, LY294002 at a concentration 1.7 times greater than used here was specific for PI3 kinase except for an inhibitory effect on casein kinase-2 (37). The impressive selectivity profiles of these compounds (37), together with the fact that they exerted no changes in gross cell morphology (data not shown), indicates that there is good reason to believe that they are acting specifically in the current study. Previous studies have indicated drug accumulation in the cytosol of renal cortical cells as the main cause for AGA nephrotoxicity (1,2). However, the time necessary for AGA uptake and sequestration into the lysosomal compartment, rupture and release of the lysosomal content exceeds that described for early markers of cytotoxicity (6,10). Although it is not disputed that a megalin/cubilin complex might represent the endocytic receptor for polybasic drugs (7), our study indicates that AGAs rapidly stimulate signal transduction pathways common to many Gq-coupled receptors. Megalin lacks known determinants for G protein耦联ens; we therefore exclude the possibility that the observed results could be due to megalin activation. With regards to the nephrotoxicity of neomycin and gentamicin, the current data demonstrate that these agents very rapidly produce intracellular signaling responses in proximal tubule cells that they are known to ultimately damage in vivo. Thus, the question must now be asked whether the AGA-stimulated elevation of $[Ca^{2+}]_i$ and activation of ERK seen here is involved in cell toxicity or whether it occurs independently of it. For instance, can the AGAs alter cell fate in OK cells, and if so, do they exhibit a rank order of potency that matches their nephrotoxic potential. It remains the case that AGA accumulation within the cell most likely results from endocytosis after binding of the AGAs to the proximal tubular apical membrane on either phosphatidylinositol 4,5-bisphosphate molecules (39) or on proteins such as the megalin/cubilin complex (7). It is conceivable however, that parallel binding of AGAs to endocytic receptors together with occupation of intracellular signal-generating CaR could combine to initiate the cell responses that lead to toxicity. Work is currently underway to test the hypothesis that AGAs alter OK cell fate via stimulation of the CaR. It will be necessary to determine whether proximal tubule CaR stimulation promotes apoptotic or necrotic cell death or indeed whether it may actually induce mitogenesis.

Finally, it has long been appreciated that diabetes mellitus protects the rats from gentamicin-induced nephrotoxicity (40). We have recently shown that in the streptozotocin model of diabetes mellitus in rats, renal CaR becomes downregulated after 2 wk of diabetes (41). Although the protective effect could be due to the chronic diabetic hypercalciuria, we would suggest in light of the current data that it may also result from the attenuation of CaR-mediated signaling. The current data indicate that proximal tubular CaR can be activated by AGAs; therefore, it could potentially play a pathologic role in the nephrotoxicity of the aminoglycoside antibiotics. Further work will be required to substantiate such a suggestion; however, such a hypothesis is eminently testable with the combined approach of using protein knockout technology and specific CaR agonists and antagonists.

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