Aminoglycoside-mediated rescue of a disease-causing nonsense mutation in the V2 vasopressin receptor gene in vitro and in vivo

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Many human diseases are caused by inactivating mutations in specific G-protein-coupled receptors (GPCRs). In about 10% of these cases, a premature stop codon leads to the generation of a truncated, functionally inactive receptor protein. In this study, we tested the hypothesis that such GPCR mutations can be functionally rescued in vitro and in vivo by treatment with aminoglycoside antibiotics, which are known for their ability to suppress premature termination codons. As a model system, we studied a mutant V2 vasopressin receptor (AVPR2) containing the inactivating E242X nonsense mutation which mimics human X-linked nephrogenic diabetes insipidus (XNDI) when introduced into mice via gene targeting techniques. Studies with cultured mammalian cells expressing the E242X mutant receptor showed that G418 (geneticin) was by far the most potent aminoglycoside antibiotic capable of suppressing the E242X nonsense codon. Strikingly, G418 treatment increased AVP-mediated cAMP responses in cultured kidney collecting duct cells prepared from E242X mutant mice in vitro, and significantly improved the urine-concentrating ability of E242X mutant mice in vivo. This is the first study demonstrating that G418 (aminoglycosides) can ameliorate the clinical symptoms of a disease-causing premature stop codon in a member of the GPCR superfamily.

INTRODUCTION

G-protein-coupled receptors (GPCRs) represent an extraordinary large family of cell surface receptors involved in almost all physiological functions. Activating and inactivating mutations in specific GPCR genes are responsible for many human diseases, including hypo- and hyperthyroidism, male-limited precocious puberty, retinitis pigmentosa and X-linked nephrogenic diabetes insipidus (XNDI) (1).

Current therapies of diseases caused by GPCR mutations rely virtually exclusively on treating the clinical symptoms. However, the development of therapeutic strategies aimed at restoring receptor function in a more direct fashion would be highly desirable. For example, a recent study showed that non-peptide V2 vasopressin receptor (AVPR2) antagonists can restore proper cell surface localization to intracellularly retained mutant AVPR2s known to cause human XNDI (2).

About 5–10% of disease-causing GPCR mutations represent nonsense mutations which lead to the generation of truncated, non-functional receptor proteins. In XNDI patients, for example, 18 different nonsense mutations have been identified in the AVPR2 gene (3).

Interestingly, aminoglycoside antibiotics are able to suppress premature stop codons, thereby permitting protein translation to continue to the normal end of the gene. This phenomenon is most likely due to the interaction of the aminoglycosides with ribosomes, reducing the usual stringency of codon-anticodon pairing (4). Recent in vitro and in vivo studies have shown, for

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Human Molecular Genetics, 2004, Vol. 13, No. 9 © Oxford University Press 2004; all rights reserved
example, that aminoglycoside antibiotics can suppress premature stop codons in the cystic fibrosis transmembrane regulator (CFTR) and dystrophin genes (5–7). In this study, we tested the hypothesis that aminoglycoside antibiotics might be potentially useful in the treatment of diseases caused by premature stop codons in specific GPCRs. As a model system, we studied a mutant AVPR2 containing the E242X nonsense mutation (8). Physiologically, AVPR2s are virtually exclusively expressed in cells of the kidney collecting duct system, where they play a fundamental role in facilitating water reabsorption (9). When expressed in cultured cells, the E242X mutant receptor is completely devoid of functional activity (10). We recently used gene targeting technology to generate mutant mice containing the E242X mutation (11).

In the present study, we initially demonstrated using cultured mammalian cells expressing the E242X mutant receptor that G418 was by far the most potent aminoglycoside capable of suppressing the E242X nonsense codon in vitro. We then examined whether G418 was able to enhance cAMP-mediated responses in cultured kidney collecting duct cells from E242X mutant mice. We also studied whether G418 treatment was able to reduce the severity of the XNDI symptoms displayed by the heterozygous E242X mutant mice in vivo.

Our findings indicate that G418 can functionally rescue, at least partially, the E242X nonsense mutation, both in vitro and in vivo. This is the first report demonstrating that an aminoglycoside can ameliorate the clinical symptoms of a disease-causing premature stop codon in a member of the GPCR superfamily.

RESULTS

Aminoglycoside-mediated rescue of E242X function in vitro

We recently generated a mouse model of XNDI containing a premature stop codon (E242X) in the AVPR2 gene (11) (Fig. 1A). Initially, we examined whether aminoglycosides were able to suppress the E242X stop mutation in vitro. COS-7 cells transiently expressing wild-type or E242X mutant AVPR2s were incubated with or without 108 µM (75 µg/ml) G418 for 24 h prior to cAMP assays. This concentration of G418 was found to be optimal to suppress AVPR2 nonsense mutations in COS-7 cells in previous experiments (12). In the absence of G418, AVP led to a pronounced increase in cAMP levels in cells expressing the wild-type receptor but was completely inactive in cells expressing the E242X mutant receptor (Fig. 1B). Strikingly, G418 treatment led to the appearance of a robust arginine vasopressin (AVP)-induced cAMP response in E242X-expressing cells (Fig. 1B, Table 1).

The AVP EC50 value for this response was almost identical to that seen with wild-type receptor-expressing cells (Table 1).

So, Emax and EC50 values were determined for each construct in order to determine if G418 treatment could rescue the function of the E242X mutant receptor in vitro. The wild-type AVPR2 construct was used as a control for this study. The results are shown in Table 1. As expected, the wild-type AVPR2 construct led to a significant increase in cAMP levels in response to AVP treatment (Fig. 1B). G418 treatment led to a small but significant increase in cAMP levels in response to AVP treatment (Fig. 1B). The results are shown in Table 1.

Also, Emax values and Bmax values remained almost unchanged following G418 treatment of wild-type AVPR2 transfected COS-7 cells (Table 1). In concert with the observed gain of function in E242X transfected cells, a small but significant number of 3H-AVP binding sites appeared after G418 treatment.
at millimolar concentrations. Very similar results were obtained when the compounds tested were unable to rescue E242X function, even when incubation with G418 for longer than 42 h or shorter than 24 h led to a significant reduction in AVP-induced cAMP responses. G418 exposure for longer than 42 h or shorter than 24 h led to a significant reduction in AVP-induced cAMP responses.

We next carried out western blotting studies in order to confirm that the G418-mediated rescue of E242X function was in fact dependent on the generation of full-length AVPR2s. Lysates prepared from COS-7 cells transiently expressing wild-type or E242X mutant AVPR2s were incubated with an antibody directed against the C-terminus of the murine AVPR2. Immunoprecipitates were subjected to SDS–PAGE and immunoblotting using a monoclonal antibody directed against the HA epitope tag present at the N-terminus of the AVPR2 protein. As shown in Figure 2, the wild-type AVPR2 migrated at 40–45 kDa, corresponding to the mature receptor monomer (13), and also gave several higher molecular weight multimeric complexes, probably corresponding to receptor oligomers. As expected, no specific immunoreactive bands were observed with samples prepared from E242X- or GFP-transfected cells. G418 treatment (108 μM) for 24 or 72 h prior to harvesting had no significant effect on the expression levels of the wild-type AVPR2. However, G418 incubation of E242X-transfected COS-7 cells resulted in the appearance of the mature full-length AVPR2 monomer migrating at 40–45 kDa and several higher molecular weight bands, as observed with the wild-type AVPR2. In E242X-transfected cells, G418 treatment also led to the appearance of a pronounced lower molecular mass band (~32–35 kDa), which most probably corresponds to intracellularly trapped immature AVPR2s (13). The longer G418 incubation time (72 versus 24 h) led to increased levels of full-length AVPR2s (Fig. 2).

**G418 is the most effective aminoglycoside in suppressing the E242X nonsense codon**

There are only very few studies which have systematically compared the efficiency of individual aminoglycosides to suppress premature termination codons (14). We therefore decided to study the ability of eight additional aminoglycosides (amikacin, gentamicin, hygromycin B, kanamycin A, neomycin, paromomycin, streptomycin and tobramycin), together with G418 (as a reference), to suppress the E242X nonsense codon in *vitro*. COS-7 cells transiently expressing the E242X mutant receptor were incubated with increasing concentrations of antibiotics for the last 24 h prior to cAMP assays. As shown in Figure 3, G418 was clearly more potent (about one order of magnitude) and about 2-fold more efficacious than gentamicin and paromomycin in restoring function (AVP-mediated increases in cAMP) to the E242X mutant receptor. All other compounds tested were unable to rescue E242X function, even at millimolar concentrations. Very similar results were obtained with a CHO cell clone stably expressing the E242X mutant receptor (data not shown).

**Short exposure to G418 rescues the function of the E242X mutant receptor in vitro**

To study the effect of G418 exposure time on the efficiency of the G418-mediated functional rescue of the E242X mutant receptor, two different experimental protocols were used. First, E242X-transfected COS-7 cells were incubated with G418 for 24–48 h prior to cAMP assays. As shown in Figure 4A, G418 incubation for ~30 h prior to cAMP assays resulted in maximum rescue of E242X function. G418 exposure for longer than 42 h or shorter than 24 h led to a significant reduction in AVP-induced cAMP responses.

We next repeated the same set of experiments using a CHO cell line stably expressing the E242X mutant receptor. In this case, incubation with G418 for 24–48 h prior to cAMP assays resulted in a consistently high efficiency of rescue of E242X function (Fig. 4A), probably due to the stable expression of the E242X mutant receptor.

In the second set of experiments, COS-7 cells transiently expressing the E242X mutant receptor were incubated with G418 (108 μM) for 3–45 h, starting 18 h after transfection. The medium was then replaced with normal G418-free cell culture medium was then replaced with normal G418-free cell culture.
Figure 3. Efficiency of various aminoglycosides in rescuing the function of the E242X mutant receptor transiently expressed in COS-7 cells. COS-7 cells transiently expressing the E242X mutant receptor were treated with different aminoglycosides for 24 h prior to cAMP assays. Cyclic AMP responses to 100 nM AVP were determined as described in Materials and Methods. Amikacin, hygromycin B, kanamycin A, noremycin, streptomycin and tobramycin had no significant effects on AVP-induced cAMP formation, even at millimolar concentrations. Data are presented as means ± SEM of three or four independent experiments, each carried out in triplicate.

medium, and cAMP assays were performed 48 h after addition of G418. Consistent with the experiments shown in Figure 4A, the efficiency of rescue of E242X function was dependent on the G418 exposure time (Fig. 4B). Surprisingly, a rather short G418 exposure time of only 3–6 h, followed by a 42–45 h G418-free cultivation period, resulted in robust AVP-induced cAMP responses. Similar results were obtained with CHO cells stably expressing the E242X construct, except that the efficiency of rescue was less pronounced at short G418 exposure times (1–6 h; Fig. 4B).

E242X mRNA is stably expressed in XNDI mutant mice

All eukaryotes possess the ability to detect and degrade mRNA harbouring premature stop codons (15). To test whether E242X mRNA is subjected to nonsense-mediated mRNA decay in vivo, RT–PCR studies were performed with mRNA prepared from kidneys of female mice heterozygous for the E242X mutation (‘heterozygous E242X mice’). cDNA coding for wild-type and E242X mutant AVPR2s were amplified as described in Materials and Methods. Interestingly, in addition to the published mouse AVPR2 cDNA sequence (16), several cDNAs (both wild-type and mutant receptor alleles) contained a deletion of codon 9. Inspection of the intron/exon borders indicated the existence of an additional splice acceptor site three bases downstream of the original site (Supplementary Material, Fig. S2). Studies with transiently transfected COS-7 cells showed that the AVPR2-ΔAla9 receptor had a similar AVP potency as the AVPR2 receptor containing codon 9 (K. Sangkuhl and T. Schöneberg, unpublished data).

For the determination of allele frequency (E242X versus wild-type AVPR2) in the mRNA pool, cDNAs were subcloned into the pCR 2.1-TOPO vector and analyzed as described in Materials and Methods. If nonsense-mediated mRNA decay has no significant impact on E242X stability one would expect a 50:50 ratio between E242X and wild-type mRNA levels. However, sequence analysis revealed that 24.8 ± 13.8 and 75.2 ± 13.8% of the cDNA clones coded for the E242X mutant and the wild-type allele, respectively (12 heterozygous mice were used and 24 cDNA clones per mouse were analyzed). The variation in allele frequency (range between 5 and 50% E242X mRNA versus wild-type mRNA) may be due to individual differences in the efficiency of the nonsense-mediated mRNA decay or due to skewed X-chromosome inactivation (17). In any case, our data indicate that E242X mRNA is rather stable in vivo. To test whether G418 has an influence of E242X mRNA stability in vivo, E242X female mice were injected i.p. once a day for 7 days with G418 solution (14 mg/kg, see below). Analysis of five heterozygous mice (24 cDNA clones per mouse) revealed that 7-day G418 treatment had no significant effect on the E242X:wild-type mRNA ratio (24.4 ± 11.4 and 75.6 ± 11.4%, respectively).

G418-mediated rescue of E242X function in cultured kidney collecting duct cells

We next tested whether G418 was able to suppress the E242X stop codon in primary kidney IMCD cell cultures from heterozygous adult female E242X mice which exhibit the characteristic symptoms of human XNDI. These experiments could not be carried out with hemizygous male E242X ∼/y pups, because these mutant mice died during the first postnatal week and AVPR2 expression levels are very low even in neonatal wild-type mice (11).

Kidney cells were maintained in a hyperosmolar medium for 4 days in order to select for IMCD principal and intercalated cells (18). As shown in Figure 5, kidney cells from wild-type female mice responded to AVP (100 nM) administration with a 2- to 3-fold increase in intracellular cAMP levels. The magnitude of this response was significantly reduced after treatment with 108 μM G418 for 24 h prior to cAMP assays. As expected, IMCD cells from heterozygous E242X mice showed a ∼50% reduction in AVP-mediated cAMP production. Strikingly, G418 (108 μM) incubation for 24 h led to a significant increase in AVP-induced cAMP formation (Fig. 5).

Pharmacokinetic behavior of G418 in mice

Little is known about the pharmacokinetic behavior of G418 in mice after parenteral application. To determine G418 concentrations in plasma and kidneys after intraperitoneal application, we used a commercially available fluorescence polarization immunoassay (TDxFLX® System) for gentamicin, taking advantage of the fact that the antibody used in this kit cross-reacts with G418. First, different concentrations of G418 (4.5–144 μM) added to plasma were measured and compared with the standard concentrations of gentamicin provided with the test kit. G418 showed ∼20% cross-reactivity when compared with similar concentrations of gentamicin, which allowed us to construct a standard curve for G418 (Fig. 6 inset) in order to determine G418 concentrations in plasma and kidney extracts.

Female adult mice were injected with G418 (14 mg/kg i.p.) and blood plasma samples and kidneys were taken from killed mice after different time intervals. This G418 dose was chosen because it was tolerated well and higher doses (e.g. 70 mg/kg i.p.) caused significant side effects. As shown in Figure 6, the...
plasma concentration of G418 was maximal < 30 min after injection (26.1 ± 2.3 μM; shorter time points were not measured). G418 was no longer detectable in the plasma 2 h after injection. In kidney extracts, as observed with plasma, high G418 levels (69.7 ± 10.2 μM) were found at the 30 min time point. However, substantial concentrations of G418 (18.8 ± 2.9 μM) were detectable in kidney extracts even 4 h after injection (Fig. 6).

G418-mediated rescue of E242X function in a mouse model of XNDI

We next wanted to examine whether G418 was also able to suppress the E242X premature stop mutation when administered to adult female heterozygous E242X mice, an in vivo model of human XNDI (11). Adult female wild-type (+/+) and heterozygous E242X mice (−/+) were treated with G418 for 7 days (one single dose per day; 14 mg/kg i.p.). This dosage was chosen because it resulted in a kidney concentration (Fig. 6) that was sufficient to rescue the E242X mutant receptor in in vitro assays (Fig. 3). A considerably higher dose of G418 (70 mg/kg i.p., LD50, α = 140 mg/kg; see Table 2) resulted in toxic side effects (cachexia) in both heterozygous and wild-type mice. The corresponding control groups were injected with solvent only. At the end of the 7-day injection period, mice were killed and urine samples were collected directly from the bladder. Urine osmolalities were measured using a vapor pressure osmometer.

As shown in Figure 7A, wild-type mice showed a mean urine osmolality of 709 ± 212 mosm/kg. As reported previously (11), heterozygous E242X mice displayed a pronounced reduction in urine osmolality (202 ± 68 mosm/kg; Fig. 7A), indicating that these mutant mice are impaired in concentrating their urine. The 7-day G418 treatment had no significant effect on urine osmolalities in wild-type mice (710 ± 144 mosm/kg). Strikingly, G418 treatment resulted in an ~50% increase in urine osmolalities in heterozygous E242X mice (305 ± 34 mosm/kg; Fig. 7A).

To study urine-concentrating abilities under more challenging conditions, adult female wild-type and heterozygous E242X mice were treated with G418 (or solvent) as described above but deprived of water for the last 24 h prior to urine collection. As expected, both wild-type and heterozygous E242X mice responded to water restriction with a pronounced increase in urine osmolalities (Fig. 7B; wild-type: 2399 ± 321 mosm/kg, heterozygous: 644 ± 89 mosm/kg), as compared...
Table 2. Comparison of aminoglycoside toxicity and efficacy to suppress the E242X stop codon. The efficacy of the indicated aminoglycosides to suppress the E242X stop codon in transfected mammalian cells (this study) and their toxicity in mice are compared. The efficacy of AVP-induced cAMP-formation is expressed as fold over basal following aminoglycoside treatment (incubation with 70 μM aminoglycoside 24 h prior assay; data were taken from the experiments shown in Fig. 3). The toxicity data (LD_{50} values) were taken from material safety data sheets prepared by the indicated companies.

<table>
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<tr>
<th>Aminoglycoside</th>
<th>Efficacy in suppressing the E242X stop codon (fold over basal)</th>
<th>Toxicity (mouse) LD_{50} (i.v. application, mg/kg)</th>
<th>Reference</th>
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<td>Roche, Phytotechnology Lab. LLC</td>
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<td>Paromomycin</td>
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<td>Tobramycin</td>
<td>—</td>
<td>72</td>
<td>Genus®Sicor Pharmaceuticals Inc.</td>
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*No AVP-induced cAMP generation even after treatment with maximum aminoglycoside concentration (2 mM).

with the corresponding non-thirsted mice (Fig. 7A). G418 treatment slightly decreased the urine-concentrating ability of wild-type mice (1879 ± 80 mosm/kg). In contrast, G418 treatment significantly increased urine osmolalities in heterozygous E242X mutant mice by ~100% (1327 ± 115 mosm/kg), indicating that G418 was able to (partially) rescue E242X function in vivo.

DISCUSSION

Mutations occurring in specific GPCRs are the cause of more than two dozen of human diseases. Interestingly, about 5–10% out of such disease-causing mutations represent nonsense mutations leading to truncated receptor proteins (3). Aminoglycoside antibiotics, mostly gentamicin, have been shown to suppress clinically relevant premature stop codons in in vitro cell systems and in two in vivo models with variable efficiency (5–7, 19–23). In this study, we tested the hypothesis that aminoglycoside antibiotics might be potentially useful in the treatment of diseases caused by the presence of premature termination codons in GPCRs. As a model system, we studied an AVPR2 nonsense mutation (E242X) that causes XNDI in humans (8). To generate a mouse model of this disease, we recently used gene targeting technology to introduce the E242X mutation into the mouse genome (11). It has been shown that the stop codon and its surrounding context significantly influence the ability of different aminoglycosides to suppress termination (24). Bedwell and coworkers (24) demonstrated that read-through levels for the UAG(N) tetranucleotide termination signals were generally less than was observed for the UGA(N) tetranucleotide signals. By screening a degenerate oligonucleotide library, a consensus sequence –CA(A/G)(N(U/C/G))CA– was found most efficient when located downstream of a UAG stop codon (25). Based on these comprehensive studies and the –UAG CGG GCA– stop codon sequence in the E242X mice (Fig. 1A), we would expect an intermediate efficiency in read-through.

We initially carried out in vitro studies to examine the ability of G418 to suppress the E242X stop mutation in cultured COS-7 and CHO cells transiently or stably expressing the E242X mutant receptor. Following G418 treatment, these cells gained the ability to respond to AVP challenge with robust increases in intracellular cAMP levels. In G418-treated COS-7 cells expressing the E242X mutant receptor, for example, AVP was able to induce cAMP responses with similar potency as in cells expressing the wild-type AVPR2 (Fig. 1, Table 1). In addition, western blotting studies showed that G418 treatment of E242X-expressing COS-7 cells led to the appearance of full-length AVPR2s (Fig. 2). Taken together, these data indicate that G418 is able to suppress the E242X stop codon in cultured transfected cells. However, the western blotting studies (Fig. 2) also showed that a major portion of the full-length AVPR2s generated by G418-mediated suppression of the E242X stop codon were not folded properly, as indicated by a strong 32–35 kDa immunoreactive band characteristic of immature AVPR2s that are retained intracellularly (13). A likely explanation for this finding is that these misfolded receptors contain amino acids at position 242 that impair proper receptor folding and trafficking. 3H-AVP binding studies carried out with intact E242X-expressing COS-7 cells also supported the concept that only a minor fraction of the full-length AVPR2s generated by G418-mediated suppression of the E242X stop codon is trafficked to the cell surface (Table 1). Similar results were obtained previously with two other mutant AVPR2s containing the W200X and R337X premature stop codons (12). It should be of interest to examine whether ‘chemical chaperons’ or AVPR2 ligands which are able to stabilize the AVPR2 structure (2) can further increase G418-dependent cell surface expression of full-length AVPR2s.

Despite the relatively small number of properly folded full-length AVPR2s resulting from G418-mediated suppression of the E242X stop codon, G418 treatment of cultured mammalian cells expressing the E242X mutant receptor resulted in a rather efficient rescue of receptor function, as discussed above. This observation indicates that even a relatively small number of AVPR2s can mediate robust cAMP responses, probably due to the highly efficient amplification of the receptor signal by the downstream components of the CAMP signalling pathway. Restoration of relatively small numbers of full-length AVPR2s may therefore also be of potential therapeutic benefit in XNDI patients carrying the E242X mutation.
In this study, we also systematically investigated the ability of different aminoglycoside antibiotics to suppress the E242X stop mutation in vitro, using transfected mammalian cells and AVP-mediated increases in intracellular cAMP levels as a functional readout (Fig. 3). These studies showed that G418 was at least 10 times more potent than gentamicin and paromomycin in suppressing the E242X stop mutation (all other aminoglycosides tested were completely inactive in this regard). On the other hand, mouse LD50 measurements indicate that G418 displays a similar toxicity as most other clinically used aminoglycosides (Table 2). It should be noted that acute toxicity data do not reflect the complete spectrum of toxic side effects. G418 is not clinically approved probably because safety studies on dogs have shown that a single oral dose of G418 (50–1000 mg/kg) results in ulceration of the oral and vaginal mucosa in dogs. These alterations appear to be specific since similar lesions have not been reported in animals treated with any other aminoglycoside antibiotics (26). Again, in this study G418 was used as proof of principle because it was the most potent aminoglycoside. Several previous (24,27) and our studies have clearly shown that the ability to overread stop codon differs between various aminoglycosides. Since there is no correlation between the read-through efficiency of the different aminoglycosides and their acute toxicity (Table 2), the two properties appear to be dissociable. Figure 8 shows that the chemical structures of G418 and gentamicin are very similar and differ in only three hydroxyl groups. The clinical application of aminoglycosides has been restricted to their use as antibiotics but no effort has been made to optimize their ability to suppress stop codons in eukaryotic cells. Systematic structural modification of the G418 scaffold, e.g. by combinatorial chemistry, may therefore lead to less toxic compounds which maintain the ability to suppress premature stop codons with high efficiency.

We also examined whether G418 was also able to restore function to the E242X mutant receptor in mice carrying the E242X mutation. Unfortunately, hemizygous male pups containing the E242X mutation died within the first week after birth, probably due to hypernatremic dehydration (11). On the other hand, female mice heterozygous for the E242X mutation were viable and displayed an XNDI-like phenotype, characterized by reduced urinary concentrating ability of the kidney and polyuria. RT–PCR studies indicated that the kidneys of heterozygous E242X mice contained relatively high levels of E242X mRNA. For these reasons, we used heterozygous E242X female mice for all G418 rescue experiments.

We first studied the effect of G418 on AVP-mediated cAMP responses in cultured kidney inner medullary collecting duct (IMCD) cells from wild-type and heterozygous E242X mutant mice. Consistent with previous findings (11), IMCD cells from heterozygous E242X mice showed an ~50% reduction in AVP-mediated cAMP production (Fig. 5). Since one of the two X-linked alleles is normally inactivated, in a random fashion during early development, one half of the IMCD cells in the heterozygous mutant mice is predicted to express the wild-type AVPR2, whereas the other half is predicted to express the inactive E242X mutant receptor. The existence of two distinct populations of IMCD cells is therefore the most likely reason for the observed ~50% reduction in cAMP responses in kidney cell preparations from heterozygous E242X mice. Strikingly, G418 (108 µM) treatment led to a pronounced increase in AVP-induced cAMP formation in IMCD cells from heterozygous E242X mice (Fig. 5). As already discussed above, studies with...
transfected COS-7 cells showed that stimulation of even rather low numbers of wild-type AVPR2s can lead to robust cAMP responses. It is therefore likely that the G418-mediated generation of small numbers of full-length AVPR2s in the E242X-expressing IMCD cells allows these cells, previously unresponsive to AVP, to contribute to the overall cAMP response.

In contrast to the G418-mediated increases in AVP-dependent cAMP responses observed with IMCD cells from heterozygous E242X mice, G418 treatment of IMCD cells from wild-type mice led to significantly reduced cAMP responses (Fig. 5). One possible explanation for this somewhat surprising finding is that G418 impairs AVPR2/cAMP signaling in wild-type cells in a yet unknown fashion. On the other hand, compensatory changes may have occurred in E242X-expressing IMCD cells that have increased the overall sensitivity of the AVPR2/cAMP signaling cascade, thus masking any potential inhibitory effects of G418 on AVPR2 signaling.

To examine whether G418 was able to improve the urine-concentrating ability of E242X mutant mice in vivo, wild-type and heterozygous E242X female mice were injected i.p. once a day for 7 days either with solvent alone (white bars) or G418 solution (14 mg/kg, gray bars). At the end of the 7-day injection period, mice were sacrificed and urine samples were collected from the bladder. Wild-type and heterozygous E242X female mice were treated as described above but were deprived of water for the last 24 h before urine collection. Urine osmolalities were measured in duplicate using a vapor pressure osmometer. Data are given as means ± SEM (n = 7–11 mice per group). The unpaired two-site Student’s t-test was used for statistical evaluation.

Figure 7. G418 treatment improves the urine-concentrating ability of E242X mutant mice in vivo. (A) Wild-type and heterozygous E242X female mice were injected i.p. once a day for 7 days either with solvent alone (white bars) or G418 solution (14 mg/kg, gray bars). At the end of the 7-day injection period, mice were sacrificed and urine samples were collected from the bladder. (B) Wild-type and heterozygous E242X female mice were treated as described above but were deprived of water for the last 24 h before urine collection. Urine osmolalities were measured in duplicate using a vapor pressure osmometer. Data are given as means ± SEM (n = 7–11 mice per group). The unpaired two-site Student’s t-test was used for statistical evaluation.

Figure 8. Chemical structures of G418 and gentamicin. The chemical structures of G418 and gentamicin are shown. G418 contains three additional hydroxyl groups which are marked with arrows.
prior to urine collection. As expected, the solvent-injected, thirsted control mice (wild-type and E242X mutant mice) showed a pronounced increase (~2- to 3-fold) in urine osmolalities (Fig. 7B), as compared with non-thirsted control mice (Fig. 7A). G418 treatment of thirsted wild-type resulted in an ~10–20% reduction in urine osmolalities, perhaps due to some non-specific toxic effect of G418 on kidney function (also see the discussion above regarding the results obtained with cultured IMCD cells). In striking contrast, G418 treatment of thirsted heterozygous E242X mice led to an ~100% increase in urine osmolalities, consistent with a partial rescue of E242X function in vivo. It is likely that the restoration of AVPR2 function in the E242X-expressing kidney cells masks any potential non-specific toxic effects that G418 may have on the urine-concentrating ability of the kidney.

Pharmacokinetic measurements carried out with G418-injected wild-type mice indicated that G418 accumulated in kidney tissue (Fig. 6). It is likely that this effect contributes to the relatively efficient rescue of E242X function that can be observed after G418 treatment of E242X mutant mice in vivo.

In summary, we have shown that a nonsense mutation (E242X) in the mouse AVPR2, a prototypical GPCR, can be partially rescued, in a time- and concentration-dependent manner, with the aminoglycoside antibiotic, G418, in both transient and stable expression systems. Importantly, G418 treatment also increased AVP-mediated cAMP responses in IMCD cells prepared from E242X mutant mice, and was able to clearly improve the urine-concentrating ability of E242X mutant mice in vivo. This is the first study demonstrating that G418 (aminoglycosides) can ameliorate the clinical symptoms of a disease-causing premature stop codon in a member of the GPCR superfamily. Since the ability of aminoglycoside antibiotics to suppress premature stop codons and their cytotoxicity are not tightly correlated, the search for G418 derivatives that maintain their capacity to suppress premature stop codons but have reduced toxicity should be of considerable clinical interest.

MATERIALS AND METHODS

RT–PCR and cloning of wild-type and mutant AVPR2 genes

For the cloning of the mouse wild-type and E242X mutant AVPR2 cDNAs, we first prepared mRNA from kidneys of a heterozygous female E242X mutant mouse, using the Oligotex™ direct mRNA kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized by reverse transcriptase (Invitrogen, Leek, The Netherlands) using an oligo-dT primer as recommended by the manufacturer. Wild-type and E242X mutant AVPR2 cDNAs were amplified via PCR using the following sense and antisense primers (primer pair-1): 5′-CGCC GCG AGA TCT CCC ATG ATC TGT GGT TCT ACC ACG-3′ / 5′-GCG GTT AAC TCA GGA GGG GTG TGT ATC CTT CAT CAG AGA-3′. The resulting 1135 bp PCR products were first subcloned into the pCR2.1–TOPO vector (Invitrogen) and then cloned, as EcoRI fragments, into the polylinker of the mammalian expression plasmid pcDps, resulting in AVPR2- and E242X-pcDps. In addition, the wild-type and E242X mutant AVPR2s were tagged with an N-terminal nine-amino acid epitope (YPYDVPDYA) derived from the influenza virus hemagglutinin protein (HA-tag), which was introduced after the initiating Met codon using a PCR-based mutagenesis strategy. To monitor the transfection efficiency, a mammalian expression plasmid (pEGFP-C1 vector, Clontech, Palo Alto, CA, USA) coding for the green fluorescent protein was used.

For the screening of AVPR2 splice variants, mRNA was prepared from wild-type mice as described above and subjected to RT–PCR using oligo-dT primer for first-strand cDNA synthesis and primer pair-2 for PCR amplification: 5′-CCA CAC CAT GAT CGT GGT GTC TAC C-3′ / 5′-CAC AAA GGG GGG TCT TTC CAG AGG A-3′. PCR products were subcloned into the pCR2.1-TOPO vector and cut with EcoRI/PstI.

Cell culture, transfection and functional assays

COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 7% CO2 incubator. For functional assays, COS-7 cells were transiently transfected using LipofectAMINE (Invitrogen). Cyclic AMP accumulation assays were performed in 12-well plates (2 × 105 cells/well) and cells were transfected with a total amount of 1 μg of DNA/well and 2.5 μl of LipofectAMINE/well. After 48 h cells were prelabeled with 2 μCi/ml of [3H]adenine (31.7 Ci/mmol; Perkin Elmer, Life Science Products, Belgium) and incubated overnight. For cAMP assays, transfected cells were washed once in serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma, Taufkirchen, Germany), followed by incubation in the presence of the indicated AVP (Sigma) concentrations for 1 h at 37°C. Reactions were terminated by aspiration of the medium and addition of 1 ml of 5% trichloroacetic acid. The CAMP content of cell extracts was determined by anion exchange chromatography as described (28).

Unless stated otherwise, G418 (G418, Invitrogen) was added to transfected COS-7 cells 48 h after transfection. Cyclic AMP assays were carried out 24 h later (72 h after transfection).

To establish CHO-K1 cell lines stably expressing the E242X mutant receptor, E242X-pcDps was cotransfected (50:1) with the resistance plasmid pcDNA4 (Invitrogen) using FuGene (Roche, Mannheim, Germany). Single colonies resistant to zeocin (300 μg/ml; Invitrogen) were isolated and maintained in Ham’s F12 medium supplemented with 10% FBS at 37°C in a humidified 5% CO2 incubator. Positive clones were selected by a functional screen examining AVP-induced CAMP-accumulation after 24 h of G418 incubation (75 μg G418 disulfate/ml = 108 μM).

For radioligand binding studies, COS-7 cells were split into 12-well plates (2 × 105 cells/well) and transfected as described above. Saturation binding assays were performed on intact cells. Incubations were carried out for 3 h at 4°C in a 1 ml volume with six different concentrations (0.08–20 nM) of [3H]-AVP (64 Ci/mmol; Perkin Elmer, Life Science Products). Non-specific binding was defined as binding in the presence of 2 μM AVP. Attached cells were solubilized and harvested with 1 ml 0.1 M NaOH after washing three times with ice-cold PBS (2 mM Ca2+, 1 mM Mg2+) containing 0.1% BSA. Binding data
were analyzed using a non-linear curve-fitting procedure (GraphPad Software, San Diego, CA, USA).

**Primary culture of kidney collecting duct cells**

Primary cultures of IMCD cells were prepared essentially as described by Mooren and Kinne (18). Adult mice were killed by decapitation, and kidneys were removed and chopped into small pieces. Tissue was digested in Hank’s buffer (Biochrom, Berlin, Germany) containing 0.2% hyaluronidase (Roche), 0.2% collagenase (Biochrom) and 0.001% DNase I (Sigma) at 37°C for 90 min. The cells were then centrifuged, washed three times with PBS, and seeded in six-well plates. Cells were maintained in DMEM containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin and adjusted to 600 mosmol/l by the addition of 100 mM NaCl and 100 mM urea, at 37°C in a humidified 7% CO₂ incubator. This medium allows the selective growth of IMCD principal and intercalated cells (18).

**Antibody generation and western blot protocols**

A polyclonal antibody was raised in rabbits against a KLH-conjugated synthetic peptide corresponding to the C-terminal 22 amino acids of the mouse AVPR2. The antibody (anti-mouse AVPR2) was purified by affinity chromatography on a peptide column according to standard procedures and tested for specificity in an indirect enzyme-linked immunosorbent assay and in immunofluorescence studies (data not shown).

COS-7 cells were transfected with FuGene (Roche) and the indicated constructs (10 μg of plasmid DNA/100 mm dish). About 72 h later, cells were washed twice with PBS and treated with 1 ml of lysis buffer (250 mM NaCl, 2.5 mM EDTA, 0.9% desoxycholate, 1% Nonidet P-40, 0.2% SDS, 25 mM Tris–HCl, pH 7.4). After vigorous vortexing and solubilization under rotation overnight at 4°C, cell debris was removed by centrifugation. Four milliliters of PBS and 1 μg/ml of the anti-mouse AVPR2 antibody (see above) were added to the supernatants containing solubilized receptor protein. Following incubation of samples at 4°C overnight at constant rotation, 60 μl of 10% (w/v) protein A-Sepharose beads (Sigma) were added, and samples were incubated for 2 h at 4°C. Sepharose beads were pelleted (2000 g for 3 min) and washed twice with 1 ml of washing buffer A (600 mM NaCl, 0.1% Triton X-100, 1% Nonidet P-40, 50 mM Tris–HCl, pH 7.4) and twice with 1 ml of washing buffer B (300 mM NaCl, 10 mM EDTA, 100 mM Tris–HCl, pH 7.4). Pellets were boiled with 40 μl of SDS sample buffer, and proteins were separated via SDS–polyacrylamide gel electrophoresis (10%). After electrotransfer onto nitrocellulose the filter was probed with a biotin-labeled anti-HA monoclonal antibody (12CA5, 0.5 μg/ml in PBS/0.1% Tween 20, Roche). A peroxidase-labeled streptavidin conjugate (1:10 000 in PBS/0.1% Tween 20) and the ECL system (Amersham Bioscience, Buckinghamshire, UK) were used to detect the bound anti-HA antibody.

**Mouse strain containing the E242X mutation and mouse genotyping**

The generation and initial characterization of the mouse strain containing the E242X nonsense mutation have been reported previously (11). Briefly, the mouse AVPR2 gene was inactivated in ES cells by introducing the E242X mutation into the AVPR2 coding region. The E242X mutation generated a novel NheI site which was used for genotyping purposes (see below). To remove the loxP-flanked neo selection cassette from the genome of the AVPR2 mutant mice, homozygous male EIIa-cre mice were bred with female heterozygous E242X mutant mice. The resulting E242X mutant mice colony were back-crossed for 12 generations onto the 129/sv mouse background. Animals were maintained in a controlled animal facility with 25°C room temperature, 60% humidity, and a 12 h light/12 h dark cycle.

Mice were genotyped by PCR analysis of mouse tail DNA. The two PCR primers used flanked the newly created NheI/E242X mutation (primer pair-3): 5’-ATC TGC CGC CCT ATG CTG GCA TAC-3’/5’-CAC AAT CAC TAG TGT CAT CCT CAC-3’ (94°C for 1 min/62°C for 1 min/72°C for 2 min, 35 cycles). The resulting PCR products were digested with NheI and separated on 2% agarose gels (note that only the fragment containing the E242X mutation contains an NheI cleavage site).

**Determination of plasma and kidney concentrations of G418**

Mice were injected with G418 (14 mg/kg mouse i.p.) and plasma samples were prepared from citrate blood at different time periods after G418 administration. In parallel, kidneys from G418-treated mice were collected and disintegrated in 200 μl water with an ultrasound sonicator. To measure plasma and kidney extract concentrations of G418, a commercially available fluorescence polarization immunoassay for gentamicin (TDxFLx® System, Abbott) was used, taking advantage of the fact that the antibody used in this kit cross-reacted with G418.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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REFERENCES


