Poly-L-aspartic Acid Enhances and Prolongs Gentamicin-mediated Suppression of the CFTR-G542X Mutation in a Cystic Fibrosis Mouse Model*

Received for publication, August 29, 2008, and in revised form, December 22, 2008 Published, JBC Papers in Press, January 9, 2009, DOI 10.1074/jbc.M806728200

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Aminoglycosides such as gentamicin have the ability to suppress translation termination at premature stop mutations, leading to a partial restoration of protein expression and function. This observation led to studies showing that this approach may provide a viable treatment for patients with genetic diseases such as cystic fibrosis that are caused by premature stop mutations. Although aminoglycoside treatment is sometimes associated with harmful side effects, several studies have shown that the co-administration of polyanions such as poly-L-aspartic acid (PAA) can both reduce toxicity and increase the intracellular aminoglycoside concentration. In the current study we examined how the co-administration of gentamicin with PAA influenced the readthrough of premature stop codons in cultured cells and a cystic fibrosis mouse model. Using a dual luciferase readthrough reporter system in cultured cells, we found that the co-administration of gentamicin with PAA increased readthrough 20–40% relative to cells treated with the same concentration of gentamicin alone. Using a Cftr−/− hCFTR-G542X mouse model, we found that PAA also increased the in vivo nonsense suppression induced by gentamicin. Following the withdrawal of gentamicin, PAA significantly prolonged the time interval during which readthrough could be detected, as shown by short circuit current measurements and immunofluorescence. Because the use of gentamicin to suppress disease-causing nonsense mutations will require their long term administration, the ability of PAA to reduce toxicity and increase both the level and duration of readthrough has important implications for this promising therapeutic approach.

Previous studies have shown that aminoglycosides such as gentamicin and amikacin can suppress translation termination at disease-causing premature stop (nonsense) mutations and partially restore the expression of functional proteins in mammalian cells (for a review, see Ref. 1). In particular, gentamicin has been shown to suppress nonsense mutations and partially restore protein expression in mouse models of Duchenne muscular dystrophy (2) and cystic fibrosis (CF) (3, 4). However, the use of aminoglycosides is commonly associated with serious side effects, including nephrotoxicity and ototoxicity (5, 6). The high dose of gentamicin (34 mg/kg) initially used to demonstrate readthrough in mouse models also resulted in serum concentrations that were far in excess of their maximum clinically recommended levels (2–4). More recently, we demonstrated that a lower dose of gentamicin (5 mg/kg) produced peak serum concentrations in a CF mouse model that were within the accepted clinical range for these compounds (4). Functional CFTR protein was produced under those conditions, as shown by immunofluorescence and short circuit current measurements. However, the level of suppression obtained was significantly less than was observed with higher doses. Consistent with the efficacy of these clinically relevant doses in mice, small clinical trials have suggested that gentamicin can suppress premature stop mutations in patients with Duchenne muscular dystrophy (7) and CF (8–10). However, negative results have also been obtained in other clinical trials for both CF (11) and Duchenne muscular dystrophy (12). These discrepancies suggest that further refinement of aminoglycoside-based treatment strategies is needed.

Several approaches have been investigated to reduce aminoglycoside toxicity (6). Among these, one of the most potent protectants against the renal toxicity associated with these compounds is poly-L-aspartic acid (PAA). The co-administration of PAA with gentamicin has been shown to provide a significant level of protection against aminoglycoside-induced nephrotoxicity in rats, as measured by the absence of functional and pathological changes caused by lysosomal phospholipidosis in proximal tubular cells. Because phospholipidosis results from the intralysosomal accumulation of aminoglycosides and their binding to the acidic head groups of phospholipids in the lysosomal membrane, it was proposed that PAA exerts its protective effect by forming complexes with gentamicin following their protonation within lysosomes, thus preventing their membrane association (13–16).

In this report, we show that the co-administration of PAA with gentamicin induced a higher level of suppression of a readthrough reporter in cultured cells than gentamicin alone. The co-administration of PAA with gentamicin also resulted in...
an increased and prolonged level of suppression of the CFTR-G542X nonsense mutation in a CF mouse model. Because it is well documented that the co-administration of PAA with gentamicin reduces the toxicity of aminoglycosides (13–16), these results suggest that the co-administration of these compounds may alleviate the major limitation of this therapeutic approach while enhancing its efficacy.

EXPERIMENTAL PROCEDURES

Cell Culture and Dual Luciferase Readthrough Assays—HEK293T cells maintained as monolayer cultures were grown in Dulbecco's modified Eagle's medium with 4.5 gm/liter d-glucose, 584 mg/liter l-glutamine, and 110 mg/liter sodium pyruvate supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Gentamicin was from Abbott Laboratories (North Chicago, IL). Poly-l-aspartic acid (sodium salt; molecular weight, 8,900–10,300; catalogue number P-5387) was from Sigma.

For dual luciferase assays, HEK293T cells were split into 96-well plates and treated with different concentrations of gentamicin with or without PAA for the indicated time period. The cells were transfected with the dual luciferase UGA readthrough reporter (pDB691) or CGA sense control reporter (pDB690) using FuGENE 6 (Roche Applied Science). Each well was transfected with 0.75 μl of FuGENE 6 and 0.25 μg of dual luciferase constructs, and cells were grown for an additional 24 or 48 h before harvesting for assays. Dual luciferase assays were performed using a dual luciferase assay kit (Promega Corp.), and light emission was quantitated using a Berthold Lumat LB9507 luminometer. The dual luciferase reporters used to monitor readthrough of stop codons in mammalian cells have been described previously (17, 18). The percentage of readthrough was defined as the firefly/Renilla luciferase activity (nonsense) divided by firefly/Renilla luciferase activity (sense) × 100. All of the results are expressed as the means ± S.D. The Student’s t test was used for statistical analysis.

Measurement of Intracellular Gentamicin Concentrations—Gentamicin was extracted from HEK293T cells treated with gentamicin ± PAA by a sodium hydroxide extraction method (19). Briefly, the cells were grown in 10-cm culture dishes in the presence of gentamicin (± PAA) at different concentrations for 24 h. The cells were washed three times with PBS to remove any remaining extracellular gentamicin. 300 μl of PBS was then added to the dish, and the cells were homogenized. The homogenizer was rinsed with another 300 μl of PBS, which were pooled with the original homogenate. An equal volume of 2 M sodium hydroxide was added to the homogenate, and the mixture was incubated at 70°C for 20 min. The samples were centrifuged at 13,000 × g for 20 min, and the supernatant was collected and neutralized to pH 7.0 prior to measuring the gentamicin and protein concentrations. Gentamicin concentrations were determined using a fluorescence polarization immunnoassay, whereas protein concentrations were measured using a dye binding assay (Bio-Rad).

Mouse and Treatment Protocols—The CFTR-G542X mice used in this study contained the Cfr<sup>pm1Cam</sup> knock-out (20) and expressed a human CFTR transgene with the G542X premature stop mutation (3, 4, 21) (referred to as Cfr<sup>−/−</sup> hCFTR-G542X mice). Control mice were C57BL/6J (referred to as wild type or Cfr<sup>+/+</sup> mice) or Cfr<sup>pm1Cam</sup> knock-out mice (22) (referred to as Cfr<sup>−/−</sup> mice). Transcription of the hCFTR-G542X transgene was driven by the rat intestinal fatty acid-binding protein promoter. Gentamicin and PAA were dissolved separately in PBS. Treatments consisted of subcutaneous injections of 5 mg/kg gentamicin alone or 5 mg/kg gentamicin plus 70 mg/kg PAA delivered in the hind limb once daily for the time periods indicated. The treatment of all mice was initiated 16 days after birth; the mice were weaned 23 days after birth. Because of the high incidence of intestinal blockage in CF mice (22), all of the mice were maintained on a liquid diet (Peptamen® complete elemental diet; Nestlé) after weaning. The animal protocols used in this work were reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Short Circuit Current Measurements—Transepithelial short circuit current measurements were carried out using an Ussing chamber under conditions previously described (3, 4, 21). Forskolin (10 μM) was added to both the mucosal and serosal solutions, and the short circuit current was continuously monitored for ≥10 min to ensure that a sustained response was obtained. Glybenclamide (200 μM) was then added to both the mucosal and serosal solutions to block the forskolin-activated CFTR short circuit current. In all experiments, the current measurements obtained immediately before and 10 min after forskolin addition were used to calculate the current change in each sample.

Immunofluorescence—Immunofluorescence experiments were carried out as previously described (3, 4, 21). CFTR-specific antisemur 4562 was raised against an antigen that included hCFTR NBD1 and a portion of the R domain (hCFTR amino acids 521–828).

RESULTS

Treatement of Cultured Cells with PAA and Gentamicin Induces More Readthrough than Gentamicin Alone—Previous studies have shown that PAA protects against the renal toxicity associated with aminoglycosides. Surprisingly, this protection was accomplished even though PAA increases the intracellular aminoglycoside concentration (13–16). However, it is unknown how the co-administration of PAA with aminoglycosides affects the suppression of nonsense mutations. To examine this question, we used a dual luciferase readthrough reporter system in cultured HEK293T cells. In this system, the Renilla and firefly luciferase reporter genes are located upstream and downstream of an in-frame UGA stop codon, respectively (17, 18) (Fig. 1A). The cells were transfected with the relevant dual luciferase plasmids, cultured for 24 h, and treated with gentamicin ± PAA for another 24 h. They were then harvested and lysed for readthrough assays.

Treatment of HEK293T cells with gentamicin induced a dose-dependent increase in readthrough (Fig. 1B), as previously reported using various cell-based systems (23–26). In preliminary experiments, the optimal gentamicin to PAA ratio was determined (data not shown). We found that treatment of cells for 24 h with 0.5 mM gentamicin plus 6.5 μM PAA (a gentamicin to PAA molar ratio of ~77:1) stimulated readthrough of the
UGA nonsense codon 1.4-fold more than the same concentration of gentamicin alone, a significant increase ($p < 0.01$). Treatment of cells with 1 mM gentamicin plus 13 μM PAA resulted in a 1.2-fold increase in readthrough compared with gentamicin alone, again a significant increase ($p < 0.01$).

We next asked whether the increased readthrough observed when cells are treated with gentamicin plus PAA correlated with an increased intracellular gentamicin concentration. HEK293T cells were incubated in the presence of 0.5 or 1.0 mM gentamicin ± PAA for 24 h. The cells were then washed, and the amount of intracellular gentamicin was quantitated using fluorescence polarization immunoassay. We found that cells treated with gentamicin plus PAA contained 1.2–1.3-fold more of the aminoglycoside than cells treated with gentamicin alone (Fig. 2A), which represented a significant increase ($p < 0.01$).

This suggested that the intracellular gentamicin concentration increased in proportion to the amount of gentamicin in the culture medium. Further analysis of these data revealed a very strong correlation between the readthrough level and the intracellular gentamicin concentration (correlation coefficient, 0.998; Fig. 2B). These results indicate that the co-administration of gentamicin with PAA leads to a higher level of intracellular gentamicin than treatment with gentamicin alone, leading to increased readthrough of the UGA nonsense codon.

We next asked whether the enhanced suppression of nonsense mutations was maintained following extended treatments with gentamicin plus PAA. The cells were grown in continuous culture in medium containing 1 mM gentamicin ± PAA for 1, 8, or 16 days, and dual luciferase readthrough reporters were transfected into the cells 24 h before harvesting. At the end of the treatment period the cells were harvested, and dual luciferase readthrough assays were carried out. We found that a similar level of gentamicin-induced readthrough was sustained after 1, 8, or 16 days (Table 1). A significant 1.3–1.4-fold increase in readthrough was also observed in cells grown in the

![FIGURE 1. PAA enhances gentamicin-induced readthrough of the UGA nonsense codon in HEK293T cells. A, diagram of dual luciferase readthrough reporter constructs. The stop codon and surrounding context are indicated by the expanded sequence. B, quantitation of readthrough induced by 0.5 and 1 mM gentamicin (−6.5 and 13 μM PAA, respectively). The $p$ values were calculated using the Student’s $t$ test. $p$ values of < 0.05 were considered significant and are indicated by an asterisk.](image1)

![FIGURE 2. Intracellular concentration of gentamicin measured in HEK293T cells treated with gentamicin or gentamicin plus PAA. A, gentamicin levels measured in HEK293T cells treated with 0.5 mM gentamicin (± 6.5 μM PAA) or 1 mM gentamicin (± 13 μM PAA). The gentamicin concentration in each sample was determined using fluorescence polarization immunoassay. B, correlation between percentage readthrough and intracellular concentration of gentamicin. $p$ values of < 0.05 were considered significant and are indicated by an asterisk.](image2)

| TABLE 1 Persistence of readthrough stimulation by PAA in HEK293 cells |
|---------------------------------|
| Treatment time | Gentamicin* | Gentamicin + PAA* | Fold stimulation | $p$ value |
|----------------|-------------|------------------|-----------------|-----------|
| 1 day          | 4.30 ± 0.22 | 5.45 ± 0.24      | 1.27            | < 0.01    |
| 8 days         | 4.10 ± 0.35 | 5.21 ± 0.49      | 1.27            | < 0.01    |
| 16 days        | 4.09 ± 0.34 | 5.57 ± 0.23      | 1.36            | < 0.01    |

*The percentage of readthrough is expressed as the means ± standard deviation.

*1 mM gentamicin, 13 μM PAA (as indicated).
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FIGURE 3. Quantitation of readthrough persistence after gentamicin treatment (±PAA) is ended. A, experimental protocols to quantitate UGA readthrough in HEK293T cells after gentamicin treatment (±PAA). HEK293T cell were treated with 1 mM gentamicin (±13 μM PAA) for 24 h. The culture medium was then replaced with fresh medium without gentamicin (±PAA) for another 24- or 48-h chase period. B, quantitation of readthrough from HEK293T cells after 24-h chase period. C, quantitation of readthrough from HEK293T cells after 48-h chase period. The percent readthrough values obtained using protocols 3 or 4 were significantly larger than the percent readthrough values obtained using protocol 2 (p values <0.05; indicated by an asterisk). Similarly, the percent readthrough value obtained using protocol 4 was significantly larger than the percent readthrough value obtained with protocol 3 (p values <0.05; indicated by †).

presence of gentamicin plus PAA after each time period (p value < 0.01). These results indicate that readthrough promoted by gentamicin (or gentamicin plus PAA) can be sustained in cultured cells over an extended growth period.

We next asked whether the co-administration of gentamicin plus PAA extended the time period during which readthrough occurred after gentamicin was removed from the culture medium. HEK293T cells were treated with the four protocols shown in Fig. 3A. Protocol 1 was a control without any treatment. In protocol 2, the cells were initially grown in culture medium with 1 mM gentamicin for 24 h and then changed to fresh medium without gentamicin and grown for an additional 24 or 48 h. In protocol 3, the cells were treated with 1 mM gentamicin plus 13 μM PAA for 24 h, then changed to fresh medium without either compound, and grown for an additional 24 or 48 h. In protocol 4, the cells were grown in culture medium with 1 mM gentamicin plus 13 μM PAA for 24 h, then changed to fresh medium without gentamicin (but with 13 μM PAA), and grown for an additional 24 or 48 h. In all four protocols, the dual luciferase plasmid constructs were transfected into the cells when the medium was changed to initiate the chase period. After the 24- or 48-h chase period, the cells were harvested, and dual luciferase assays were carried out to monitor the level of readthrough. Fig. 3B shows readthrough after the 24-h chase period, whereas Fig. 3C shows readthrough following the 48-h chase. In both cases, treatment with gentamicin plus PAA (protocol 3) increased readthrough by 20% more than gentamicin alone (protocol 2), a significant increase (p value < 0.05). A further 10% increase in readthrough was observed when PAA was present during the chase period (protocol 4). This latter result represented a significant increase when compared with not only to gentamicin alone (protocol 2; p value < 0.05) but also when compared with gentamicin plus PAA (protocol 3; p value < 0.05). When taken together, these results indicate that the elevated readthrough resulting from the co-administration of gentamicin plus PAA persisted following the removal of gentamicin from the culture medium.

PAA Enhances Gentamicin-induced Readthrough in Cftr−/− hCFTR-G542X Mice—We previously reported that once daily subcutaneous injections of 5 mg/kg gentamicin or 15 mg/kg amikacin resulted in suppression of the hCFTR-G542X mutation and a partial restoration of CFTR protein and function in Cftr−/− hCFTR-G542X mice (4). Notably, those doses also resulted in peak serum aminoglycoside levels within the accepted clinical range. However, the readthrough afforded by either aminoglycoside at those low dosages was weaker than the readthrough observed with higher doses. Because our in vitro results indicated that the co-administration of gentamicin plus PAA enhanced suppression, we next investigated whether PAA could enhance the readthrough of a nonsense mutation by a low dose of gentamicin in Cftr−/− hCFTR-G542X mice.

Several studies have previously shown that the co-administration of gentamicin with PAA at molar ratios ranging from 1.5:1 to 8:1 significantly reduced gentamicin-induced nephrotoxicity while increasing the intracellular gentamicin concentration in the renal cortex (13–15). Based on these results, we chose to examine the effect of PAA on gentamicin-induced readthrough in our CF mouse model using a gentamicin:PAA molar ratio of 1.5 to 1. The CFTR protein is a cAMP-activated chloride channel, and transepithelial chloride conductance attributable to CFTR can be monitored in intestinal tissues mounted in an Ussing chamber following the addition of the cAMP agonist forskolin. To systematically examine the effect of PAA on readthrough of the hCFTR-G542X nonsense mutation, Cftr−/− hCFTR-G542X mice were included in six different treatment groups as shown in Fig. 4A. Treatments consisted of subcutaneous injections of 5 mg/kg gentamicin alone or 5 mg/kg gentamicin plus 70 mg/kg PAA delivered once daily for a 4-day treatment period, the mice were sacrificed, and intestinal short circuit current measurements were carried out. Groups 1–3 contained Cftr−/− hCFTR-G542X mice that were treated as indicated for 14 days. Group 1 contained control mice that were left untreated for 14 days. Group 2 contained mice treated with gentamicin alone for 14 days, whereas group 3 contained Cftr−/− hCFTR-G542X mice that were administered gentamicin plus PAA for 14 days. Following the 14-day treatment period, the mice were sacrificed, and intestinal short circuit current measurements were carried out. Groups 4–6 contained Cftr−/− hCFTR-G542X mice that were again treated for 14 days as described above, followed by a 4-day chase period before assays were performed. Group 4 mice were treated with gentamicin for 14 days and then maintained without treatment for another 4 days. Group 5 mice were given gentamicin plus PAA for 14 days and then maintained without treatment for 4 days. Group 6 mice were given gentamicin plus PAA for 14 days and then administered PAA alone for the next 4 days.
As controls, both wild type mice (Cfr<sup>+/+</sup>, group 8) and Cfr knockout mice without the hCFTR-G542X transgene (Cfr<sup>−/−</sup>, group 10) were treated with gentamicin plus PAA for 14 days with continuing administration of PAA for another 4 days. These mice were then sacrificed for cAMP-stimulated short circuit current measurements, and the results were compared with untreated mice of the same genotype (groups 7 and 9, respectively).

The results of short circuit current measurements from the 10 different treatment groups of Cfr<sup>−/−</sup> hCFTR-G542X, wild type mice, and Cfr<sup>−/−</sup> mice are shown in Fig. 4B. A summary and statistical analysis of short circuit currents from the 10 different treatment groups are shown in Table 2. The results from group 1 (untreated Cfr<sup>−/−</sup> hCFTR-G542X mice) revealed that 11% of samples (3/28) exhibited cAMP-stimulated short circuit currents, resulting in an average current of only 0.2 μA/cm<sup>2</sup>. The infrequent response observed in untreated Cfr<sup>−/−</sup> hCFTR-G542X mice may be attributable to a low baseline level of endogenous readthrough of the hCFTR-G542X transgene, because these currents are not observed in Cfr<sup>−/−</sup> mice that do not carry the transgene, as shown below and discussed in a previous report (21). For the mice of group 2 treated with gentamicin, we detected a cAMP-stimulated short circuit current in 35% of samples (9/26), with an average current of 0.7 μA/cm<sup>2</sup>. The average short circuit current of group 2 is significantly larger than group 1 (p value < 0.05). In group 3 mice treated with gentamicin plus PAA, we detected a cAMP-stimulated short circuit current in 44% of samples (8/18) with an average current of 1.1 μA/cm<sup>2</sup>. The magnitude of this average current is also significantly larger than group 1 (p value < 0.05). There was not a significant difference between the average short circuit currents measured in groups 2 and 3.

The mice in group 4 were treated with gentamicin for 14 days, left untreated for 4 days, and then sacrificed for short circuit current measurements. We detected a cAMP-stimulated short circuit current in 8% of samples (1/12) with an average current of 0.13 μA/cm<sup>2</sup>, a result very similar to the untreated mice of group 1. This result suggested that most of the CFTR protein synthesized during the gentamicin treatment period was degraded during the 4 days without treatment.

We observed a strikingly different result with the mice of group 5, which were treated with gentamicin and PAA for 14 days, left untreated for 4 days, and then sacrificed. We observed a cAMP-stimulated short circuit current in 35% of samples (6/17) with an average current of 1.2 μA/cm<sup>2</sup>. These average short circuit currents were significantly larger than the untreated mice of group 1 and the mice of group 4 (both p values < 0.05).

### TABLE 2

|                | Cfr<sup>−/−</sup> hCFTR-G542X | Cfr<sup>+/+</sup> | Cfr<sup>−/−</sup> |
|----------------|-----------------------------|-----------------|-----------------|
|                | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | Group 6 | Group 7 | Group 8 | Group 9 | Group 10 |
| Treatment (14 days) | None   | Gent   | Gent PAA | Gent  | Gent PAA | Gent  | Gent PAA | Gent  | Gent PAA | Gent  |
| Positives/total   | 3/28   | 9/26   | 8/18   | 1/12  | 6/17    | 9/18  | 14/15   | 13/14 | 0/8     | 0/8   |
| Positives (%)     | 11     | 35     | 44     | 8     | 35      | 50    | 93      | 93    | 0       | 0     |
| Mean current (μA/cm<sup>2</sup>) | 0.2    | 0.7    | 1.1    | 0.13  | 1.3     | 2.1   | 5.0     | 5.9   | 0       | 0     |
| p value (relative to group 1)<sup>a</sup> | <0.05  | <0.05  | <0.05  | <0.05 | <0.05   | <0.05 | <0.05   | <0.05 | <0.05   | <0.05 |
| p value (relative to group 4)<sup>b</sup> |       |        |        |       |         |       |         |       |         |       |
| Wild type current (%) | 4      | 14     | 22     | 3     | 24      | 42    | 100     | 118   | 0       | 0     |

<sup>a</sup> p values of mean currents measured in groups 2–6 are relative to the mean current measured in group 1.

<sup>b</sup> p values of mean currents measured in groups 5 and 6 are relative to the mean current measured in group 4.
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FIGURE 5. hCFTR immunofluorescence in submucosal glands of intestinal tissues from mice treated with gentamicin (± PAA). Samples from the duodenum of Cfr −/− hCFTR-G542X mice were incubated with either preimmune serum or CFTR-NBD1 serum. After incubation of the sample with a fluorescent secondary antibody, the samples were visualized by fluorescence microscopy. Treatments consisted of subcutaneous injections of 5 mg/kg gentamicin alone or 5 mg/kg gentamicin plus 70 mg/kg PAA delivered once daily in the hind limb for the time periods indicated. The chase periods were carried out with no treatment or with subcutaneous injections of 70 mg/kg PAA alone.

The mice of group 6 were treated with gentamicin and PAA for 14 days and then PAA alone for 4 days before sacrifice. We detected a cAMP-stimulated short circuit current in 50% of samples (9/18) from these mice with a mean current of 2.1 μA/cm². The mean short circuit current obtained with group 6 was also significantly different from the untreated mice of both group 1 and the mice of group 4 (both p values < 0.05), but the mean current obtained for group 6 was not significantly different from group 5. When considered together, these results indicate that the co-administration of PAA significantly increases the average cAMP-stimulated short circuit current induced by gentamicin in hCFTR-G542X mice. In addition, PAA significantly prolonged the duration of CFTR function resulting from in vivo nonsense suppression after gentamicin treatment was terminated.

As controls, the wild type mice of group 8 were treated with gentamicin plus PAA for 14 days followed by a 4-day treatment with PAA alone. In these mice, we found that 93% of samples (14/15) showed a cAMP-stimulated short circuit current with a mean current of 5.9 μA/cm². Because these values were not significantly different from the untreated wild type mice in group 7, we conclude that the administration of gentamicin and PAA did not alter the magnitude of cAMP-stimulated transepithelial chloride currents resulting from the endogenous mouse CFTR protein. Finally, group 10 contained Cfr −/− mice that were treated with gentamicin plus PAA for 14 days and then PAA alone for another 4 days. A CAMP-stimulated short circuit current was not observed in any samples from these mice, similar to the results observed with group 9 (untreated Cfr −/− mice). Together, these results confirmed that the CFTR activity that appears following gentamicin (or gentamicin plus PAA) treatment is dependent upon the presence of the hCFTR-G542X transgene.

Following the different treatment protocols described above, mice were also sacrificed and intestinal tissues were harvested for immunofluorescence staining with hCFTR-specific antibodies. Previous studies with this mouse model found that treatment with gentamicin resulted in the appearance of hCFTR protein primarily at the apical surface of epithelial cells of submucosal glands in the duodenum. Representative immunofluorescence staining from groups 3, 4, 5, and 6 are shown in Fig. 5. No hCFTR protein could be detected in any samples incubated with preimmune serum. Similarly, hCFTR protein expression was not observed using the hCFTR-specific antiserum in group 4 mice, which were treated with gentamicin alone for 14 days followed by 4 days without treatment. However, hCFTR protein expression was observed in mice from group 3 that were treated with gentamicin plus PAA without a chase period. Similarly, mice from group 5 that were treated with gentamicin plus PAA for 14 days and then left without treatment for 4 days showed hCFTR protein staining, as did the mice from group 6 that were treated with gentamicin plus PAA for 14 days and then PAA alone for 4 days. Consistent with the functional studies described above, these results indicate that PAA prolonged the duration of in vivo nonsense suppression that occurred after gentamicin treatment was terminated.

DISCUSSION

Aminoglycoside uptake into mammalian cells is mediated primarily by megalin, a multi-ligand endocytic receptor that is particularly abundant in the proximal tubules of the kidney and the hair cells of the inner ear (27, 28). It has been estimated that 90% of internalized aminoglycosides accumulate within the lysosome, where they induce lysosomal phospholipidosis by inhibiting the activity of phospholipases in the lysosomal membrane (29–31). This defect in lysosomal lipid homeostasis is followed by cellular necrosis and post-necrotic cell regeneration. In addition, roughly 10% of aminoglycosides shuttle to the Golgi complex, where they transport past the endoplasmic reticulum-Golgi intermediate compartment into the endoplasmic reticulum. From there, translocation into the cytosol occurs. Once in the cytosol, association with various targets such as the outer mitochondrial membrane can initiate additional cascades of events leading to renal proximal tubule injury. Such interactions may explain recent reports of mitochondrial localization, free radical formation, and alterations in mitochondrial function (32).

Protection of cells from aminoglycoside toxicity could theoretically be achieved by one of two mechanisms: by blocking aminoglycoside uptake or by preventing their pathological effects once inside the cell. Because PAA increases the renal cortical accumulation of aminoglycosides, it must reduce aminoglycoside toxicity by the latter mechanism. It has also been
shown that PAA binds gentamicin in vitro through electrostatic interactions (33). This suggests that PAA may induce a redistribution of aminoglycosides away from membranes (where many of the cytotoxic effects occur) through the formation of PAA-gentamicin complexes. This conclusion is consistent with the previous observation that the co-administration of aminoglycosides with PAA increased the total aminoglycoside level in crude extracts from the renal cortex of rats, whereas the level in the brush border and basolateral membrane fractions was significantly reduced (16).

Consistent with previous studies showing that the co-administration of aminoglycosides with PAA result in higher aminoglycoside levels in the rat renal cortex than are observed in rats treated with the aminoglycoside alone (13–16), we found that the co-administration of PAA increased the intracellular concentration of gentamicin in cultured cells. Aminoglycosides like gentamicin promote readthrough of nonsense mutations by binding to the decoding site located in helix 44 of 18 S rRNA within the small (40 S) ribosomal subunit (34). In cultured cells, we observed a strong correlation between the intracellular gentamicin concentration and the extent of nonsense codon suppression (correlation coefficient, 0.998). This linear correlation suggests that a similar fraction of the total accumulated aminoglycosides is accessible for ribosome binding when the aminoglycoside concentration is increased in the presence of PAA.

Our results with Cfr−/− hCFTR-G542X mice suggest that the higher level of accumulated intracellular gentamicin also remains accessible for ribosome binding in vivo, because the co-administration of gentamicin with PAA had a stimulatory effect on readthrough of the hCFTR-G542X mutation. Treatment of these mice with 5 mg/kg gentamicin alone restored 14% of the normal intestinal transepithelial cAMP-activated short circuit current observed in wild type mice. In contrast, 22% of the wild type short circuit current was observed when 5 mg/kg gentamicin was co-administered with 70 mg/kg PAA. These results are consistent with our in vitro readthrough results and strongly suggest that PAA co-administration increases the intracellular aminoglycoside concentration accessible to ribosomes in the intestinal tissues of Cfr−/− hCFTR-G542X mice.

We also found that the co-administration of gentamicin with PAA extended the time period during which CFTR activity could be detected after gentamicin treatment was terminated. When Cfr−/− hCFTR-G542X mice were administered gentamicin for 14 days and then left for 4 days without treatment, we found that the CFTR protein and activity was reduced to a negligible level (3% of the mean wild type cAMP-activated short circuit current). This is in sharp contrast to the 14% of wild type currents measured immediately after the 14-day treatment period. Because readthrough of the CFTR-G542X mutation probably ceases shortly after the gentamicin treatment is terminated, this loss of CFTR activity probably reflects the turnover of the hCFTR protein synthesized during the treatment period. Importantly, the co-administration of gentamicin with PAA maintained higher levels of hCFTR activity for a longer time. The mean short circuit current measured was 24% of the wild type level 4 days after gentamicin, and PAA co-administration was terminated. More strikingly, we observed 42% of wild type currents when PAA was included during both the gentamicin treatment and the chase period. These results demonstrate that the co-administration of gentamicin with PAA not only enhances the suppression of the hCFTR-G542X nonsense mutation in Cfr−/− hCFTR-G542X mice but also extends the time period during which CFTR activity and protein can be detected following the termination of gentamicin treatment.

When considered in the context of gentamicin suppression of nonsense mutations that cause genetic diseases, three significant consequences can be attributed to the co-administration of gentamicin with PAA. First, PAA helps prevent gentamicin-induced nephrotoxicity (13–16) and ototoxicity (35). Second, it has been estimated that the restoration of 10–35% of normal CFTR activity may be needed to reach the necessary threshold of activity required to provide a significant phenotypic improvement in CF patients (36). Our results indicate that the co-administration of PAA with gentamicin increases the total amount of CFTR protein and activity restored from the mutated gene, thus improving the chances that the therapeutic threshold of CFTR protein and activity is reached. Finally, the prolonged CFTR protein and activity observed following the co-administration of gentamicin with PAA (particularly when PAA was also present during the chase period without the aminoglycoside) suggests that it slows the elimination of gentamicin from the cytosol, thus extending the time period during which readthrough occurs. Each of these consequences provides important therapeutic benefits over treatment strategies that depend on gentamicin alone and suggest that the co-administration of gentamicin with PAA may provide an important new strategy to treat genetic diseases caused by nonsense mutations.

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