Desensitization of human renal D_1 dopamine receptors by G protein-coupled receptor kinase 4

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Background. The D_1 dopamine receptor, expressed in several nephron segments, participates in the regulation of water and electrolyte transport. Because the renal D_1 receptor is desensitized in genetic hypertension, we sought to determine the mechanism(s) of the desensitization of D_1 receptors endogenously expressed in renal proximal tubules.

Methods. The mechanisms involved in the homologous desensitization of the D_1 receptor in human renal proximal tubule cells were studied by measuring the production of cAMP in response to stimulation or inhibition of G protein-coupled receptor kinase (GRK) activity and expression. Protein expression was assessed by immunoblotting.

Results. In human renal proximal tubule cells, the D_1 agonist, fenoldopam, increased cAMP accumulation ($73 \pm 2\%$). Fenoldopam pre-treatment decreased the responsiveness to subsequent fenoldopam stimulation ($t_{1/2} \approx 20$ min) with complete desensitization at 30 minutes. Recovery occurred gradually ($t_{1/2} \approx 20$ min) with full recovery at 60 minutes. Forskolin pre-treatment minimally affected the fenoldopam effect, indicating a minor involvement of protein kinase A in the homologous desensitization process. Because GRKs are involved in the homologous desensitization process, we determined the consequences of inhibition of GRK expression and activity. Heparin, an inhibitor of GRK activity, decreased the expression of GRK2 and GRK4 and attenuated the desensitization of the D_1 receptor ($85 \pm 1\%$). Antisense oligonucleotides (GRK4 > GRK2) blunted the D_1 receptor desensitization. However, the first 20 minutes of homologous desensitization were not affected by either heparin or GRK antisense oligonucleotides.

Conclusion. These studies document the critical role of GRK4, relative to GRK2, in the homologous desensitization of D_1 receptors in renal proximal tubule cells. However, the early phase of homologous desensitization is regulated by a non-GRK-mediated pathway.

Dopamine produced in the kidney, mainly by proximal tubules, is an important autocrine/paracrine regulator of renal sodium transport [reviewed in 1–3]. There are two families of receptors belonging to the superfamily of G protein-coupled receptors (GPCRs) that mediate the actions of dopamine: the D_1-like family (D_1 and D_5) stimulates and the D_2-like family (D_2, D_3, and D_4) inhibits adenylyl cyclase activity [1–3]. Several studies support the notion that the responsiveness of the D_1-like receptor, specifically the D_1 receptor, in the renal proximal tubule and thick ascending limb of Henle is decreased in genetic hypertension [1–3]. The attenuated responsiveness is not caused by a decrease in renal D_1-like receptor density or D_1 expression [1, 2, 4]. Rather, the D_1 receptor is uncoupled from its G protein/effector complex [1, 2, 4–8], similar to that noted with the homologous desensitization of the D_1 receptor (following agonist stimulation) [9–14]. However, in genetic hypertension, the D_1 receptor in renal proximal tubules is desensitized even in the absence of its ligand [1, 2, 4–8]. Nevertheless, an understanding of the mechanisms by which the D_1 receptor is desensitized by its ligand may yield additional insights into the mechanism(s) that govern the ligand-independent desensitization of the D_1 receptor in hypertension.

Desensitization of GPCRs involves several processes including phosphorylation, sequestration/internalization, and degradation of receptor protein [15]. Homologous desensitization occurs via the phosphorylation of GPCRs by a member or members of the GPCR kinase (GRK) family [15–18]. GRKs 1 and 7 belong to the rhodopsin family, GRKs 2 and 3 belong to the β-adrenergic receptor kinase (β ARK) family, and GRKs 4, 5, and 6 belong to the GRK4 family [15–18]. GRKs are serine and threonine kinases that phosphorylate GPCRs in response to agonist stimulation. The phosphorylation of GPCRs, including D_1 receptors, leads to the binding of a member or members of the arrestin family and uncoupling of the receptor from its G protein complex, decreasing its functional response. The phosphorylated GPCR and β-arrestin complex undergoes internalization via clathrin-

coated pits into an endosome, where it is dephosphorylated and recycled back to the plasma membrane, or degraded by lysosomes [9–20].

Several investigators have helped to decipher the pathways involved in D_1 receptor desensitization [9–14]. GRKs play a role in D_1 receptor desensitization in that over-expression of GRK2, GRK3, and GRK5 results in an increase in D_1 receptor phosphorylation but only the over-expression of GRK5 reduces the maximal activation of the D_1 receptor [11]. The role of GRK4 in the homologous desensitization of the dopamine receptor has not been studied. GRK4 has been reported to regulate three GPCRs (luteinizing hormone receptor, metabotropic glutamate receptor, M_2 muscarinic receptor) and has been thought to be expressed mainly in brain, testes, and renal medulla [17, 18, 21–24]. Following D_1 agonist stimulation, D_1 receptors have been shown to be phosphorylated and internalized via clathrin/dynamin-dependent mechanisms [11–14]. However, as most of these studies were performed in cells heterologously over-expressing the D_1 receptor, the mechanisms involved in GPCR regulation may vary from GPCR to GPCR and from cell to cell [13, 25]. Over-expression of GPCRs may lead to coupling to G proteins not seen under "normal" circumstances [26]. Except for the studies in opossum kidney (OK) cells [27, 28], the studies on D_1 receptor desensitization were performed in cells that do not have the same transport mechanisms as those noted in renal proximal tubule cells. Therefore, the current experiments were designed to study mechanisms that may be involved in the homologous desensitization of D_1 receptors in human renal proximal tubule cells. The role of cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) was studied using forskolin, which directly stimulates adenylyl cyclase [28]. The role of GRKs was studied by the use of heparin, an inhibitor of GRK activity [17, 29–31]. The role of the β ARK family of GRKs was studied by using antisense oligonucleotides to GRK2 and the GRK4 family by using antisense oligonucleotides to GRK4.

METHODS

Human kidney

Human kidneys were obtained as fresh surgical specimens from patients (unilateral nephrectomy due to renal carcinoma or trauma) or kidneys not used for transplantation. The experiments were approved by the University Health System Institutional Review Board.

Renal proximal tubule cell culture

Stock cultures of human proximal tubule cells (hPTC) were grown in 75 cm² flasks as reported [8]. Briefly, the cells were grown in serum free medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with

selenium (5 ng/mL), insulin (5 μg/mL), transferrin (5 μg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg mL) and epidermal growth factor (10 ng/mL). The cells were fed fresh growth medium every three days. When confluent, the cells were subcultured for use in experimental protocols using trypsin (0.05%) and ethylenediaminetetraacetic acid (EDTA; 0.02%). For the current studies reported, cells between passages 6 and 8 were used.

Desensitization protocol

Cultures of hPTCs from normotensive subjects were seeded at 10^5 cells/well in 24-well plastic plates. hPTCs were incubated at 37°C in 95% air/5% CO_2 for 72 hours. When the cells were ~80% confluent, the culture media were aspirated and the cells were washed twice with dextrose phosphate-buffered saline (DPBS). After the second wash, the cells were incubated at varying times and concentrations with the D_1 agonist, fenoldopam, at 37°C. At the end of the incubation period, the media were removed and the cells rinsed twice with fresh media. The cAMP responses to subsequent fenoldopam (10^{-6} mol/L, 30 minutes at 37°C) stimulation were then measured.

Resensitization protocol

In additional studies, the cells were treated with fenoldopam (10^{-6} mol/L) for 30 minutes at 37°C. The cells were then washed twice in DPBS prior to testing their responses to fenoldopam (10^{-6} mol/L, 30 minutes at 37°C) at the indicated times.

Effect of heparin

The role of GRKs in the homologous desensitization process was evaluated using heparin, an inhibitor of GRK activity [17, 29–31]. The cells (10^5 cells/24 well plate) were plated and allowed to grow to ~80% confluence. Heparin 10^{-7} mol/L (porcine intestinal mucosa, sodium salt, molecular weight = 3 to 5 kD) entry into the cells was facilitated by co-incubating the cells with lipofectin (5 μg/mL) overnight. The cells were washed twice with DPBS and the desensitization protocol carried out.

Effect of GRK2 and GRK4 antisense oligonucleotides

The role of GRK2 or GRK4 in the homologous desensitization process was evaluated using antisense or scrambled phosphorothioate/propyne oligonucleotides. We chose to study GRK2 based on the report of Tiberi et al [11], and GRK4 based upon our earlier studies indicating the importance of this GRK subtype in the regulation of D_1 receptor function [32]. The cells were seeded on a 24 well plate as described above. The cells were treated with the oligonucleotides mixed with lipofectin (5 μg/mL) for 6 to 12 hours. After changing the media, the cells were incubated in fresh media for a total

incubation time of 48 hours. The cells were washed twice with DPBS and the desensitization protocol carried out.

cAMP measurement

The culture media were aspirated, the cells washed twice with DPBS, and incubated in 400 μL of DPBS containing 1 mmol/L 3-isobutyl-1-methyl xanthine in the presence or absence of the D$_1$ agonist, fenoldopam (total reaction time 30 min). The reaction was terminated by aspirating the media and washing the cells twice with DPBS, and then freezing them at −80°C for at least one hour. The cells were then lysed with 0.1 N HCl and cAMP was measured using a radioimmunoassay as previously reported [5, 8].

Immunoblotting

Well-characterized antibodies to GRK2 and GRK4γ/δ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) [18]. Cell lysates were mixed with Laemmli sample buffer, boiled for 5 minutes, subjected to electrophoresis on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred electrophoretically to nitrocellulose membranes. Non-specific binding was blocked with 5 to 10% nonfat dry milk in Tris/HCl/saline/Tween-20 buffer. The membrane was then probed with the primary antibody (GRK2 diluted 1:750, GRK4 diluted 1:250) for one hour. After three washes, the membrane was incubated with peroxidase-labeled donkey anti-rabbit IgG (Amersham, Arlington Heights, IL, USA) with 5% nonfat dry milk for one hour. In some studies, the antibodies were pre-adsorbed with their respective immunizing peptide (1:5 wt/wt incubated overnight at 4°C). Specific bands were visualized using enhanced chemiluminescence (ECL Western Blotting Detection Kit; Amersham). Chinese hamster ovary cells transfected with the GRK4γ served as positive control for GRK4 studies. The specific bands were quantified using Quantiscan (Biosoft, Ferguson, MO, USA) with the total area arbitrarily set at 100% [8].

Statistical analysis

Data are expressed as mean ± standard error. Significant differences among groups and within groups were determined by one-way analysis of variance (ANOVA) and ANOVA for repeated measures, respectively, followed by the Newman-Keuls or Duncan test. A $P \leq 0.05$ was considered significant.

RESULTS

Time course of homologous desensitization

Individual batches ($N = 3$ to 5) of hPTCs were stimulated with the vehicle or the D$_1$ agonist, fenoldopam (10^6 mol/L), at 0 (control) 20, 25, 30, 60, and 90 minutes, and then washed. cAMP accumulation in response to fenoldopam (10^{-6} mol/L × 30 min) was used to assess D$_1$ receptor responsiveness. In cells pre-treated with the vehicle, fenoldopam (10^{-6} mol/L) uniformly increased cAMP accumulation almost twofold throughout the duration of the study (Fig. 1A). In the cells pre-treated with fenoldopam (10^{-6} mol/L), re-exposure to fenoldopam (10^{-6} mol/L) resulted in a time-dependent decrease in responsiveness to subsequent fenoldopam stimulation (Fig. 1B). For example, a 15-minute pre-treatment with fenoldopam resulted in a decrease in subsequent fenoldopam responsiveness by 27 ± 5%, (t ∼ 20 min) whereas a 30-minute pre-treatment resulted in the maximum reduction in subsequent responsiveness (95 ± 1%); there were no further changes after 60 minutes.

Effect of inhibition of total GRK activity with heparin

Because GRKs are involved in the homologous desensitization of D$_1$ receptors [11, 32], we determined the effect of inhibition of total GRK activity with low molecular weight heparin. Heparin has been reported to inhibit GRK activity with a potency of: GRK5 > GRK6 > GRK4 > GRK2, with IC$_{50}$ (nmol/L) of 1, 15, 154, and >150, respectively [17, 29–31]. Heparin had no effect on cAMP accumulation under basal conditions (Table 1) or with the first time exposure to fenoldopam (Fig. 1A). Heparin also was unable to affect the early time course of the homologous desensitization process such that during the first 20 minutes, the decreased ability of D$_1$ agonist to stimulate cAMP production was indistinguishable from the cells not treated with heparin. However, by 30 minutes, heparin was able to restore up to 85 ± 1% of maximum D$_1$ agonist response (Fig. 2A). The restoration by heparin of the ability of fenoldopam to stimulate cAMP accumulation was noted over a wide range of concentrations (Fig. 2B). Heparin also decreased the expression of GRK2 and GRK4 (Fig. 2C).

Effect of inhibition of GRK2 and GRK4 activity with antisense oligonucleotides

Because heparin does not exclusively inhibit GRK activity, we determined the effect of more selective inhibitors of GRK, namely, antisense oligonucleotides to GRK2 and GRK4. GRK2 and GRK3 decreased the sensitivity but not the efficacy of dopamine to stimulate cAMP accumulation in co-transfection studies in HEK 293 cells [11]. However, GRK3 expression was quite weak in human renal proximal tubule cells (unpublished studies) and, therefore, we chose to study GRK2 and not GRK3. Although GRK5 decreased both the sensitivity and efficacy of dopamine to stimulate cAMP accumulation in co-transfection studies in HEK 293 cells [11], we did not study the effect of GRK5, because the GRK5 band observed in our immunoblots of hPTC was not of the correct molecular size (data not shown). GRK6 was not studied because the D$_1$ agonist, fenoldopam (10^{-6}
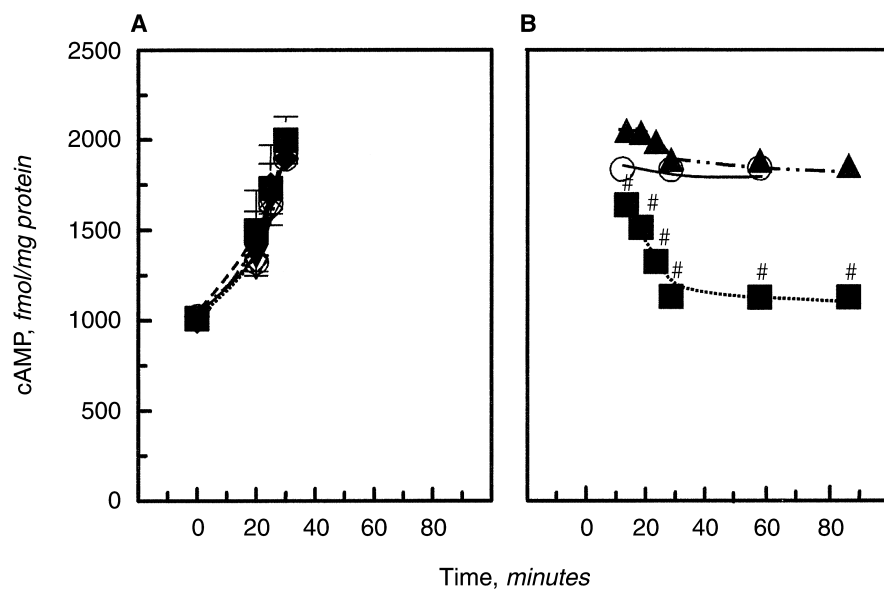
Fig. 1. Time course of fenoldopam-induced cAMP accumulation in human renal proximal tubule cells. (*A*) Time course of fenoldopam-induced cAMP accumulation in human renal proximal tubule cells pre-treated with: (○) vehicle; (■) heparin; (▼) GRK2 antisense oligonucleotide; (▽) GRK2 scrambled oligonucleotide; (◆) GRK4 antisense oligonucleotide; (◇) GRK4 scrambled oligonucleotide. cAMP accumulation was measured after a 10, 20, 25, or 30 minute treatment with fenoldopam 10^{-6} mol/L (○) or a 30 minute treatment with forskolin 10^{-7} mol/L (▽). cAMP accumulations are greater after agonist stimulation that at 0 time, ANVR, Duncan's test. (*B*) Time course of fenoldopam-induced desensitization in human renal proximal tubule cells. Cells were treated with fenoldopam, 10^{-6} mol/L (■), forskolin, 10^{-7} mol/L (▲), or vehicle (○) for 15, 20, 25, 30, 60, or 90 minutes. The cells were washed twice and cAMP accumulation in response to a 30-minute treatment with fenoldopam 10^{-6} mol/L was measured. The vehicle (time controls) in panel B represents cells that were incubated in buffer for the indicated times prior to measuring cAMP accumulation in response to a 30 minute treatment with fenoldopam 10^{-6} mol/L. #$P < 0.05$ vs. cells pre-treated with vehicle or forskolin, ANOVA, Newman-Keuls test, $N = 3$-5/group/time period. Data are mean ± standard error. Standard errors are not depicted when they are smaller than the symbols.

Table 1. Effect of heparin or GRK oligonucleotides on basal cAMP accumulation in human renal proximal tubule cells

Treatment	cAMP accumulation *fmol/mg protein*
Vehicle	1022 ± 9
Heparin	1015 ± 7
GRK2 scrambled oligonucleotides	1008 ± 9
GRK2 antisense oligonucleotides	1001 ± 6
GRK4 scrambled oligonucleotides	1009 ± 9
GRK4 antisense oligonucleotides	1002 ± 7

N is 2/group performed in triplicate. Data are mean ± standard error.

mol/L) had no effect on GRK6 activity (unpublished observations). We have reported that GRK4 is important in the regulation of D₁ receptors [32], and because heparin also inhibited the expression of GRK2 and GRK4 (Fig. 2C), the role of GRK4 on D₁ receptor function was studied. As with heparin, neither scrambled nor antisense oligonucleotides had any effect on cAMP accumulation under basal conditions (Table 1) or the first time exposure to fenoldopam (Fig. 1A). The first 20 minutes of homologous desensitization were not affected by GRK2 or GRK4 antisense oligonucleotides (Fig. 2A). In subsequent time points, antisense but not scrambled oligonucleotides partially restored the stimulatory effect of fenoldopam on cAMP accumulation. However, the effect of antisense GRK4 oligonucleotides (71 ± 1%, $N = 3$) was substantially greater than that afforded by

GRK2 antisense oligonucleotides (45 ± 1%, $N = 3$; Fig. 2A). The improvement in the stimulatory effect of fenoldopam afforded by GRK2 and GRK4 antisense oligonucleotides was evident at all concentrations (Fig. 2B). The ability of heparin to almost completely restore the fenoldopam action was approximated by GRK4 but not GRK2 antisense oligonucleotides (Fig. 2 A, B). The greater restoration of fenoldopam action afforded by GRK4 than GRK2 antisense oligonucleotides (Fig. 2 A, B) was not caused by differential effects on the expression of their respective proteins (Fig. 2D). Thus, the inhibition of GRK2 expression by GRK2 antisense oligonucleotides was similar to the inhibition of GRK4 expression by GRK4 antisense oligonucleotides (Fig. 2D).

Effect of forskolin

Protein kinase A has been reported to affect homologous desensitization of the D₁ receptor by some investigators [27, 33], but not by others [28, 34]. Pre-treatment of the cells with forskolin (10^{-7} mol/L) minimally affected the stimulatory effect of fenoldopam (Figs. 1B and 2B). The effect of forskolin on cAMP accumulation also was not affected by heparin, GRK2, or GRK4 antisense oligonucleotides or fenoldopam pre-treatment (Table 2).

Time course of resensitization

After removal of fenoldopam and washing of the cells, recovery occurred gradually with full recovery at 60 min-
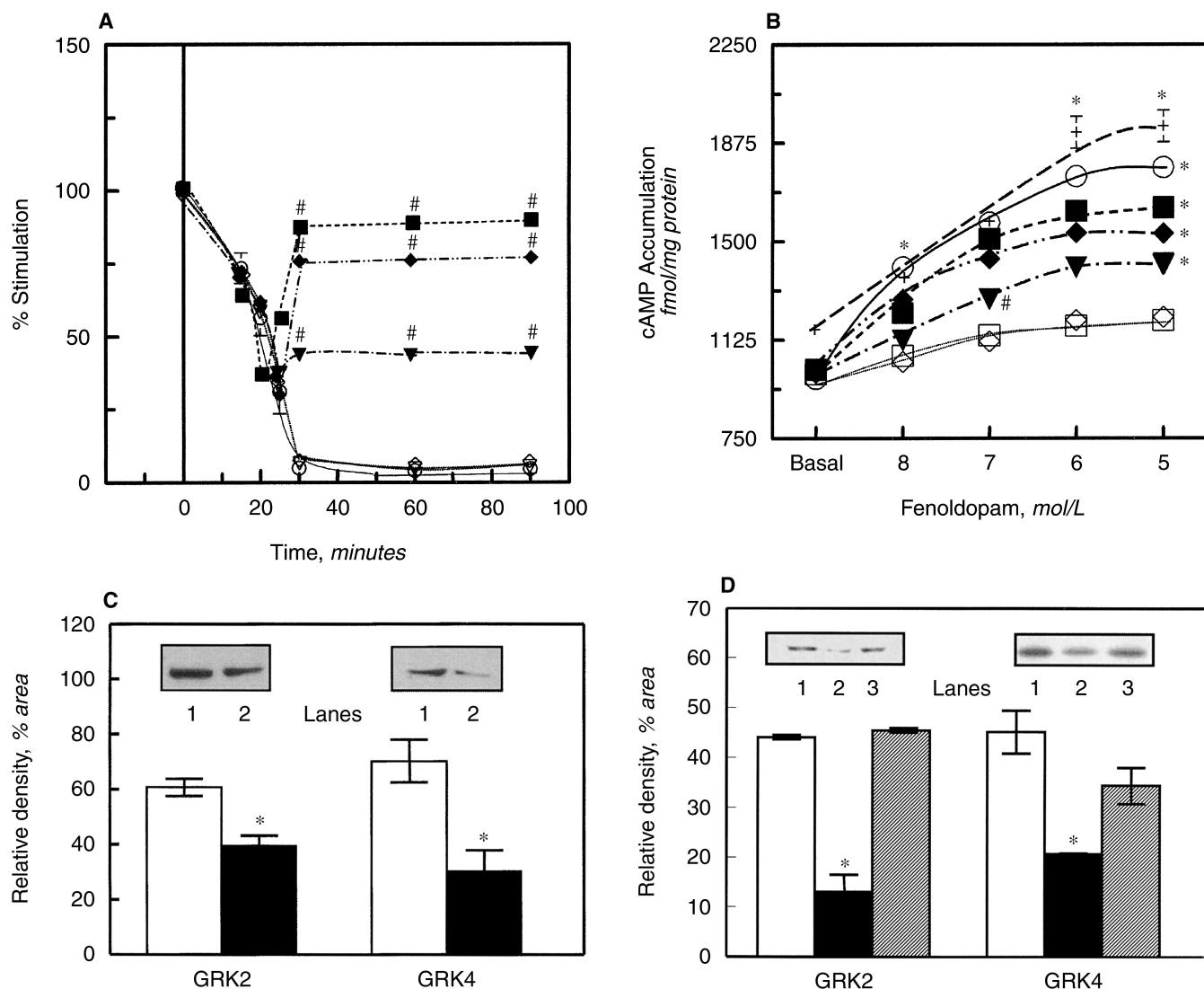
Fig. 2. (A) Effects of heparin, GRK2, and GRK4 oligonucleotides on the time course of fenoldopam-induced desensitization in human renal proximal tubule cells. Cells pre-treated with heparin 10^{-7} mol/L (■) or vehicle (○) were exposed to fenoldopam 10^{-6} mol/L for indicated times (15, 20, 25, 30, 60, 90 min). Symbols are: (▼) GRK2 antisense oligonucleotide; (▽) GRK2 scrambled oligonucleotide; (◆) GRK4 antisense oligonucleotide; (◇) GRK4 scrambled oligonucleotide. The cells were washed twice with DPBS (37°C) and then tested for their response to fenoldopam (10^{-6} mol/L) treatment of 30 minutes, as in Figure 1. Maximum response of 100% is depicted at 0 time. Data are mean ± standard error. Standard errors are not depicted when they are smaller than the symbols. #$P < 0.05$ vs. others, ANOVA, Newman-Keuls test, $N = 3$/group per time period. (*B*) Concentration response curves on the effect heparin, GRK2, and GRK4 oligonucleotides after a 30-minute fenoldopam-induced desensitization in human renal proximal tubule cells. Cells treated with vehicle (◇), GRK4 or GRK2 scrambled oligonucleotides (□), GRK2 antisense oligonucleotides (▼), GRK4 antisense oligonucleotides (◆), or heparin (■) were incubated with fenoldopam (10^{-6} mol/L) for 30 minutes. The cells were washed twice in DPBS (37°C) and then tested for their responses to fenoldopam (10^{-6} mol/L) treatment of 30 minutes. The responses of the cells not pre-treated with any drug (○) or cells pre-treated with forskolin (+) are shown for comparison. Data are mean ± standard error. Standard errors are not depicted when they are smaller than the symbols. cAMP accumulation was increased by fenoldopam to a greater extent in cells pre-treated with heparin and antisense oligonucleotides than in cells pre-treated with vehicle or scrambled oligonucleotides and greater than basal levels ($P < 0.05$, ANVR, Duncan's test). *$P < 0.05$ vs. other groups, #$P < 0.05$ GRK2 antisense vs. other groups, ANOVA, Newman-Keuls test, $N = 2$-3/group/concentration. (*C*) Effects of heparin on the expression of GRK2 and GRK4γ/δ. Symbols are: (□) vehicle; (■) heparin. The cells were treated with heparin and lipofectin as described in the **Methods** section, immunoblotted for GRK2 and GRK4γ/δ, and expression quantified by densitometry. Data are mean ± SE. *$P < 0.05$ vs. vehicle, t test, $N = 3$-4/group. Inset contains representative immunoblots: lane 1, vehicle; lane 2, heparin. (*D*) Effects of GRK2 or GRK4 oligonucleotides on GRK2 and GRK4γ/δ expression, respectively, in renal proximal tubule cells. The cells were treated with (□) vehicle and (■) antisense or (▨) scrambled GRK4 oligonucleotides as described in the **Methods** section. The cells were lysed, immunoblotted for GRK2 and GRK4γ/δ, and expression quantified by densitometry. Data are mean ± SE. *$P < 0.05$ vs vehicle (GRK4) or others (GRK2), ANOVA, Newman-Keuls test, $N = 2$/group. Inset contains representative immunoblots: lane 1, vehicle; lane 2, antisense oligonucleotides; lane 3, scrambled oligonucleotides.

Table 2. Effect of forskolin 10^{-7} mol/L on cAMP accumulation in human renal proximal tubule cells pre-treated with vehicle, fenoldopam, heparin or GRK antisense oligonucleotides

Treatment	cAMP accumulation *fmol/mg protein*
Vehicle ($N = 12$)	6933 ± 249
Fenoldopam ($N = 12$)	5774 ± 351
Heparin ($N = 5$)	5701 ± 380
GRK2 antisense oligonucleotides ($N = 4$)	7035 ± 62
GRK4 antisense oligonucleotides ($N = 7$)	5944 ± 191

N is number of experiments. Data are mean $\pm$ standard error.

utes (t $\sim$ 40 min; Fig. 3B). The rank order potency by which the agents enhanced the resensitization of the D_1 receptor was similar to that noted with the desensitization studies (heparin > GRK4 > GRK2); scrambled oligonucleotides had no effect on the resensitization process (Fig. 3C). However, the enhancement of resensitization was secondary to the prevention of the desensitization process because the cAMP levels prior to the resensitization studies were higher in heparin and GRK4 antisense oligonucleotides than the control or scrambled oligonucleotide-treated cells.

DISCUSSION

There are several novel observations in our study: (*1*) GRK4, a GRK that has been reported to have limited tissue expression [17, 18, 21–24], was expressed in renal proximal tubules; (*2*) GRK4, which has been reported to regulate only a few GPCRs [17, 18, 21–24], regulated D_1 dopamine receptors; (*3*) heparin decreased the expression of GRK2 and GRK4; and (*4*) the initial process of homologous desensitization was not inhibitable by heparin, GRK2, or GRK4 antisense oligonucleotides.

The time course for D_1 receptor homologous desensitization in hPTCs appeared to be similar to those reported for D_1 receptors in the opossum kidney (OK) cell, a kidney cell line [27, 28], C6, glioma cells [12, 14, 35], retina, striatum [36], NS20Y neuroblastoma cells [9], human astrocytoma D384 cells [37] and D_1 receptors expressed in Sf9 [38], and the internalization of D_1 receptors in human embryonic kidney, HEK 293 cells [13], but was more rapid than those reported in SK-N-MC neuroblastoma cells [39] and Chinese hamster ovary cells [33]. However, while it only took one hour for complete resensitization of the D_1 receptor in human renal proximal tubules, the complete resensitization of the D_1 receptor took five to six hours in C6 glioma cells [14] and 24 hours in NS20Y cells [9]. The difference in time course of desensitization and resensitization between the D_1 receptor in human renal proximal tubule cells and the D_1 receptor in other cells could have been due to the use of dopamine in several studies and fenoldopam in other studies, including the current report. However, the partial agonists, fenoldopam and SKF38393 desensitized
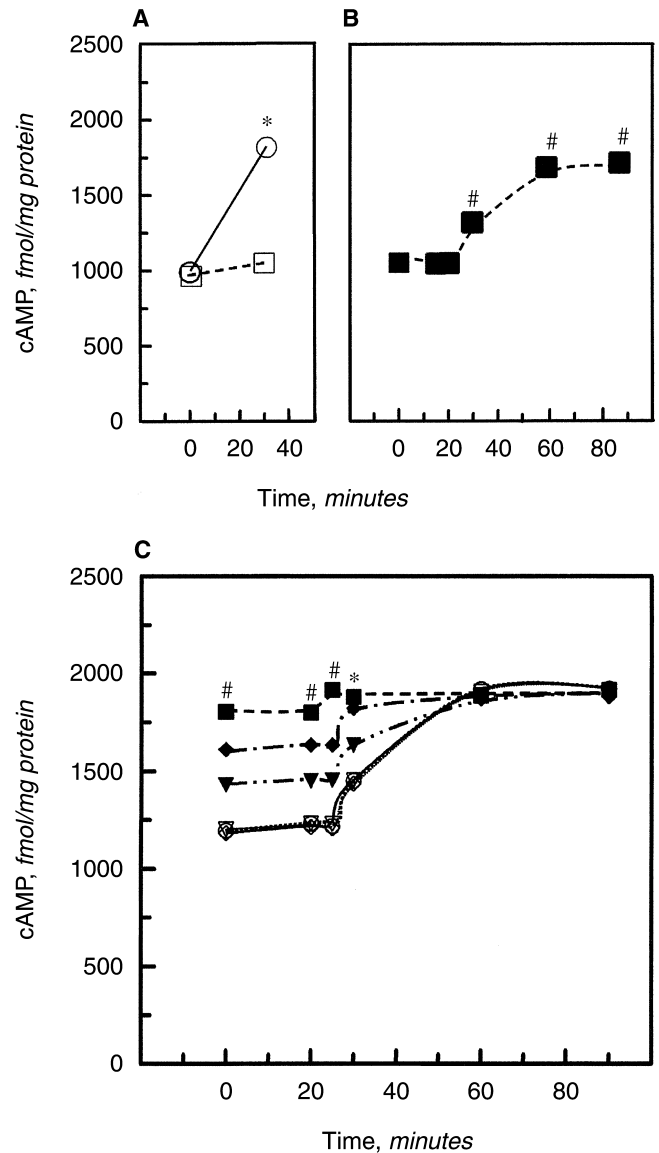

Fig. 3. Time course of fenoldopam-induced resensitization of D_1-like receptor-mediated cAMP accumulation in human renal proximal tubule cells. (*A*) cAMP accumulation in response to fenoldopam (10^{-6} mol/L) was measured after a 30-minute treatment with vehicle (○) or fenoldopam 10^{-6} mol/L (□). *$P < 0.05$ vs. 0 time, t test. (*B*) Cells treated with fenoldopam (■) for 30 minutes were washed twice, incubated in media for 0, 15, 20, 30, 60, or 90 minutes, and then cAMP accumulation in response to a 30-minute treatment with fenoldopam 10^{-6} mol/L was measured. #$P < 0.05$ vs. cells pre-treated with fenoldopam and retested immediately (0 time) or after incubation with media at 15 and 20 minutes, ANVR, Duncan's test, $N = 3$-5/group/time period. (*C*) Cells were pre-treated with vehicle, heparin, GRK2, and GRK4 oligonucleotides as described in the text, and the resensitization process was tested as in panel B. Symbols are: (■) heparin; (◆) GRK4 antisense oligonucleotide; (▼) GRK2 antisense oligonucleotide; (◇) GRK4 scrambled oligonucleotide; (▽) GRK2 scrambled oligonucltotide; (○) vehicle. #$P < 0.05$ vs. other groups, *$P < 0.05$ vs. other groups except GRK4 antisense oligonucleotides, ANOVA, Duncan's test, $N = 2$/group/time period. Complete resensitization was noted in the heparin and GRK4 antisense-treated cells at 30 minutes, ANVR, Duncan's test. Data are mean $\pm$ SE. Standard errors are not depicted when they are smaller than the symbols.

D_1 receptors to the same extent as dopamine in glioma cells, retina, and striatum [35, 36] and less than dopamine in human astrocytoma D384 cells [37]. Although comparison of our data with other reports is hampered by differences in drugs and concentrations used by different investigators, the time course for D_1 receptor homologous desensitization in renal proximal tubules was similar to those reported in many but not all cells. However, the time course of resensitization was at least threefold faster than those noted in other cells, supporting the general consensus that the desensitization/resensitization process is affected by the cellular environment [13, 25].

G protein-coupled receptor kinases have been implicated in the homologous desensitization of GPCRs [11, 13, 15–17]. Because GRKs are involved in the desensitization of D_1 receptors [11, 32], we determined the effect of a non-selective GRK inhibitor, heparin, in the homologous desensitization process [17, 29–31]. Heparin decreased expression of GRK2 and GRK4, and reversed D_1 receptor desensitization by $85 \pm 1\%$. The greater effect of heparin on GRK4 than on GRK2 expression is consistent with the greater sensitivity of GRK4 than GRK2 to the inhibitory effect of heparin on GRK activity [17, 29–31]; however, the mechanism by which heparin inhibits GRK expression remains to be determined.

The non-selective nature of heparin inhibition of GRKs did not allow the determination of the precise role of each member of the GRK family. Thus, in order to determine, selectively, the role of GRK2 or GRK4 in the homologous desensitization of the D_1 receptor, we measured the ability of fenoldopam to stimulate cAMP accumulation after treatment with antisense oligonucleotides to these GRKs. GRK4 antisense was more effective than GRK2 antisense oligonucleotides in attenuating the fenoldopam-induced desensitization, suggesting a greater role for GRK4 in the phenomenon of D_1 receptor homologous desensitization. This is in contrast to the important role of GRK2—but not GRK4—in the homologous desensitization of the β-adrenergic receptor and human muscarinic acetylcholine receptors [20, 24].

Agonist stimulation leads to receptor desensitization and down regulation through at least two pathways (PKA/ protein kinase C [PKC] and GRK). PKA has been reported to phosphorylate the D_1 receptor at Thr268; mutation of this residue minimized the homologous desensitization process [12, 33]. A second pathway involves receptor phosphorylation by GRK and β-arrestin binding leading to homologous desensitization of the receptor [15–17, 29–31]. As demonstrated in neuroblastoma and C6 glioma cells heterologously expressing the D_1 receptor, agonist occupation of the receptor led to receptor phosphorylation, β-arrestin binding, and receptor internalization [12, 13]. These two pathways may work in a synergistic fashion in that receptor phosphorylation by PKA may impair $G\alpha_S$ coupling and enhance GRK phos-

phorylation, β-arrestin binding and receptor desensitization. However, there are reports of the independence of homologous desensitization from PKA activation [28, 34]. In our studies, direct stimulation of adenylyl cyclase activity by forskolin, which leads to the activation of PKA, minimally affected the desensitizing effect of fenoldopam. Thus, in the renal proximal tubule, PKA has a small role in the homologous desensitization of D_1 receptors. D_1 receptors, via calcyon, also increase PKC activity [40] and PKC may participate in the desensitization process. We did not directly test the involvement of PKC in our studies; however, like PKA, PKC is mainly involved in heterologous rather than homologous desensitization [22]. PKC also has been shown to desensitize GPCRs via GRK2 [16, 41] and thus, it is not likely that the GRK independent desensitization of the D_1 receptor is caused by PKC.

The production of cAMP in response to the first time exposure to the D_1 agonist in the first 25 minutes is almost linear (Fig. 1A). However, homologous desensitization is already occurring during this time period (Fig. 2). One explanation for this apparent discrepancy is that during the initial exposure to the ligand, desensitization and recruitment are occurring at the same rate. Recruitment of cytosolic D_1 receptors in the first 15 minutes of agonist stimulation has been described [42]. This recruitment is not evident in the studies of the time course of homologous desensitization, because the recruitment may have already occurred during the first exposure and a limited number of receptors are available for recruitment in the subsequent exposure to the ligand. It also is possible that the slope of the stimulation would have been steeper if homologous desensitization could have been prevented during the first exposure to the ligand. However, pretreatment of the cells of heparin or GRK antisense oligonucleotides did not alter the first time ability of the D_1 agonist to stimulate cAMP production (Fig. 1A). These data indicate that the presence of a GRK-independent process in the early stages of homologous desensitization.

The apparent inability of GRKs to influence the first 20 minutes of the homologous desensitization process is novel. Because GRK-mediated desensitization has been related to clathrin, we suggest that the first 20 minutes of the homologous desensitization process may have been caused by clathrin-independent mechanisms [15, 16, 19]. One such clathrin-independent desensitization process has been ascribed to caveolin in caveolae [43–45]. Caveolae are flask-like membrane invaginations, rich in caveolins and lipids, found in many cell types. Caveolae are thought to be centers of signal transduction, where proteins are endocytosed separately from clathrin-coated pits. One of the integral proteins that coat caveolae is caveolin. Mammals have at least three caveolin genes; caveolin-1 (caveolin-1α, caveolin-1β), caveolin-2 (caveolin-2α, caveolin-2β), and caveolin-3 [43–47]. Caveolin-1

and -2 are expressed in many cell types while caveolin-3 is specific to the muscle [43]. Caveolin-1 is important in the formation of caveolae on plasma membranes [43–47]. In many cell types, caveolin-1 and -2 are co-localized and form hetero-oligomers with caveolin-2 acting as an accessory protein. Several GPCRs have been co-localized with caveolae and caveolin-1 [43–47]. Caveolae with caveolin-1 and caveolin-2 have been described in Madin-Darby canine kidney (MDCK) cells, a distal tubular cell line [48–50]. Although caveolin-1 and caveolae have been described in LLCPK, a porcine renal proximal tubule cell line [51], caveolin-2 (but not caveolin-1) was found in rat and human renal proximal tubules [52; abstract, Yu et al, *Mol Biol Cell* 11:551, 2000]. The D_1 receptor also does not possess the caveolin-1 binding motif [43–47, 53]. However, we found that D_1 receptors and caveolin-2 colocalized in brush border membranes of rat renal proximal tubules (unpublished observations). This is in contrast to that described in MDCK cells where caveolin 1 was at both apical and basolateral surfaces while caveolin-2 was present at the basolateral surface [48–50]. However, caveolin-2 has been shown to form caveolae-like vesicles only in association with caveolin-1 [54]. Therefore, the role of caveolin-2 in the GRK-independent D_1 receptor homologous desensitization process remains to be determined. Interestingly, inhibition of caveolin-2 expression enhanced the stimulatory effect of D_1 receptors on cAMP accumulation in renal proximal tubule cells (unpublished studies).

The role of GRKs in the resensitization process also was evaluated. GRK antisense oligonucleotides and heparin seemed to enhance the resensitization process. As with the homologous desensitization studies, GRK4 antisense oligonucleotides were more potent than GRK2 in regulating the resensitization of the D_1 receptor. However, because the cells treated with heparin and GRK oligonucleotides attenuated the homologous desensitization process, cAMP levels were already higher in the heparin and GRK-antisense oligonucleotides than the vehicle-treated cells. Thus, the inhibition of GRK activity seemingly enhanced the resensitization process because these cells were not as desensitized as the vehicle-treated cells.

In summary, our studies demonstrated that the renal D_1 dopamine receptor exhibited an extremely rapid homologous desensitization and resensitization process following agonist stimulation. The homologous desensitization of the D_1 dopamine receptor in human renal proximal tubules appeared to involve GRKs only in later stages of the homologous desensitization process. GRK4 also was more efficacious than GRK2 in the homologous desensitization of the D_1 dopamine receptor. The mechanism(s) involved in the GRK-independent, homologous desensitization of the D_1 dopamine receptor in the early stage of the process remains to be determined.

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