Published in final edited form as:

Free Radic Biol Med. 2011 April 1; 50(7): 832–840. doi:10.1016/j.freeradbiomed.2010.12.027.

Dopamine D₁ receptor-mediated inhibition of NADPH oxidase activity in human kidney cells occurs via protein kinase A-protein kinase C cross-talk

Peiying Yu¹, Weixing Han², Van Anthony M. Villar¹, Hewang Li¹, Francis B. Arnaldo³, Gisela P. Concepcion³, Robin A. Felder⁴, Mark T. Quinn⁵, and Pedro A. Jose¹

¹Center for Molecular Physiology Research, Children's National Medical Center, and Department of Pediatrics, George Washington University School of Medicine, Washington, DC

²Department of Cardiovascular Medicine, Anhui Medical University, Hefei, Anhui, P.R. China

³Marine Science Institute, College of Science, University of the Philippines, Diliman, Quezon City 1101, Philippines

⁴Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, VA

⁵Department of Immunology and Infectious Diseases, Montana State University, Bozeman, MT

Abstract

Dopamine cellular signaling, via the D₁ receptor (D₁R), involves both protein kinase A (PKA) and protein kinase C (PKC), but the PKC isoform involved has not been determined. Therefore, we tested the hypothesis that the D₁R-mediated inhibition of NADPH oxidase activity involves cross-talk between PKA and specific PKC isoform(s). In HEK-293 cells heterologously expressing human D₁R (HEK-hD₁), fenoldopam, a D₁R agonist, and phorbol-12-myristate-13-acetate (PMA), a PKC activator, inhibited oxidase activity in a time- and concentration-dependent manner. The D₁R-mediated inhibition of oxidase activity (68.1±3.6%) was attenuated by two different PKA inhibitors, H89 (10 μmol/L) (88±8.1%) and Rp-cAMP (10 μmol/L) (97.7±6.7%), and two different PKC inhibitors, bisindolylmaleimide I (1 μmol/L) (94±6%) and staurosporine (10 nmol/L) (93±8%), which by themselves, had no effect (n=4–8/group). The inhibitory effect of PMA (1 μmol/L) on oxidase activity (73±3.2%) was blocked by H89 (100±7.8%) (n=5–6/group). The PMA-mediated inhibition of NADPH oxidase activity was accompanied by an increase in PKCθ^{S676}, an effect that was also blocked by H89. Fenoldopam (1 μmol/L) also increased PKCθ^{S676} in HEK-hD₁ and human renal proximal tubule (RPT) cells. Knockdown of PKCθ with siRNA in RPT cells prevented the inhibitory effect of fenoldopam on NADPH oxidase activity. Our studies demonstrate for the first time that cross-talk between PKA and PKCθ plays an important role in the D₁R-mediated negative regulation of NADPH oxidase activity in human kidney cells.

© 2010 Elsevier Inc. All rights reserved.

Address correspondence to, Peiying Yu, MD, Center for Molecular Physiology Research, Children's National Medical Center, 111 Michigan Ave, NW, Washington, DC 20010, Tel: 202-476-5715, Fax: 202-476-3298.
Weixing Han, co-first author

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

NADPH oxidase; protein kinase A; protein kinase C; D₁ dopamin receptor

Introduction

Dopamine is an important regulator of blood pressure, sodium balance, and renal and adrenal function through the peripheral dopaminergic system (1,2). Dopamine receptors are grouped into two subfamilies: D₁-like receptors (D₁R and D₅R) stimulate adenylyl cyclases, while D₂-like receptors (D₂R, D₃R, and D₄R) inhibit adenylyl cyclases. D₁-like receptors also stimulate phospholipase C (PLC) and activate protein kinase C (PKC) in many cells, including neural and renal proximal tubule (RPT) cells (3–8). D₂-like receptor signaling may also involve protein kinase A (PKA) and PKC pathways (9–12).

The interaction between PKA and PKC pathways is important in the regulation of G protein-coupled receptor (GPCR) signaling (3,6,13–16). Phorbol-12-myristate-13-acetate (PMA), a direct activator of PKC, stimulates adenylyl cyclase activity and enhances agonist-induced cAMP production (15). In addition, activation of PKC with PMA stimulates intracellular cAMP production in human eosinophils, in the absence of any agonist (17). Dopamine, via D₁R, decreases renal sodium transport and increases sodium excretion, in part, by activation of PKA- and PKC-dependent and independent mechanisms (1–6). Norepinephrine, via the α_1 -adrenergic receptor (α_1 AR), and angiotensin II, via the angiotensin type I receptor (AT₁R), also increase PKC activity in many cells, including RPT cells, but increase, rather than decrease, renal sodium transport (18,19). The differential effect of dopamine and α_1 AR or AT₁R on sodium transport may occur because α_1 AR (18,20), AT₁R (19,21) and dopamine D₁-like receptors activate different PKC isoforms (4,14,16).

PKC isoforms are classified into four subgroups based on their requirements for Ca²⁺ and phospholipids for activation: classical PKCs (cPKCs: α , β and γ), novel PKCs (nPKCs: ϵ , δ , θ and η), atypical PKCs (aPKCs: ζ , ι and λ), and PKC μ , also called PKD which consists of PKD1 and PKD2/3 (22,23). AT₁R-mediated stimulation of renal sodium transport is associated with an increase in the levels of PKC α , PKC ϵ , PKC δ , and PKC ζ (19,21,24) in the plasma membrane, while α_1 AR-mediated stimulation of renal sodium transport is associated with increased levels of PKC δ and PKC ζ (25,26). In contrast, dopamine receptor-mediated inhibition of renal sodium transport is associated with an increase in membrane levels of PKC α , PKC ϵ (14), and PKC θ (4), but a decrease in renal membrane of PKC δ (4). Although D₁-like receptors have been reported to increase membrane PKC ζ levels in RPT cells from hypertensive rats (4) and in an opossum kidney cell line (16), D₁-like receptor stimulation actually decreases PKC ζ in RPT cells from normotensive rats (4). Thus, the PKC isoforms that are differentially regulated by AT₁R and α_1 AR on the one hand and D₁-like receptors on the other are PKC δ and PKC θ , respectively.

Overproduction of reactive oxygen species (ROS) plays an important role in the pathogenesis of cardiovascular diseases, including hypertension, heart failure, and atherosclerosis (27). NADPH oxidase enzymes are major sources of ROS in the cardiovascular system and the kidney (27). Several lines of evidence have shown that PKC isoforms are involved in the regulation of NADPH oxidase activity (28–32). PKC δ is involved in the phosphorylation and translocation of p47phox from cytosol to membrane, which is required for the subsequent activation of several NADPH oxidase isoforms (28). PKC α - and δ -dependent activation of NADPH oxidase is one of the mechanisms responsible for oxalate-induced oxidative injury of RPT cells (29). Angiotensin II has been reported to increase superoxide production, in part, by stimulation of PKC/PLD2 activity (30).

EXP3179, a metabolite of the AT₁R antagonist losartan, inhibits the activity of NADPH oxidase and PKC, specifically, PKC α and PKC β (31). However, PKA has also been reported to inhibit NADPH oxidase activity (32).

We and others have reported that both D₁R and D₅R inhibit NADPH oxidase activity in vascular smooth muscle and RPT cells (33–36). Vascular smooth muscle cells and renal RPT cells express all subtypes of dopamine receptors but only the D₁-like receptors (D₁R and D₅R in human or D_{1a} and D_{1b} in rodents) have been reported to have antioxidant property (27,33). We have previously reported that in HEK-293 cells heterologously expressing the human D₅R, stimulation of D₅R inhibits NADPH oxidase activity, which is PKA-independent, partly PLD dependent, and partly due to interference with the distribution and assembly of NADPH oxidase components (34). Jackson et al. have also reported that PMA increases D₁R but decreases D₅R signaling (15). Therefore, it is necessary to use cell lines that heterologously and exclusively express D₁R or D₅R to distinguish the mechanisms of involved in D₁R or D₅R activation because there are no commercially available D₁-like receptor agonists or antagonists that can distinguish D₁R from D₅R. We tested the hypothesis that a specific PKC isoform may be involved in the D₁R-mediated inhibition of NADPH oxidase activity. We hypothesized further that phosphorylated PKC θ may be involved because this PKC isoform is activated by D₁-like receptors, but not by AT₁R and α_1 AR. Because PKC can augment D₁R function but can decrease D₅R function (15), studies were performed in HEK-293 cells heterologously expressing human D₁R (HEK-hD₁) or human D₅R (HEK-hD₅). We also studied the effect of PKC activation in human RPT cells which express all the subtypes of dopamine receptors in order to determine if the results obtained in an expression system (HEK-293) are relevant in cells that endogenously express these receptors. We now report that D₁R but not D₅R exerts its negative effect on NADPH oxidase activity by increasing the phosphorylation of PKC θ ^{S676}, which is PKA-dependent.

For the materials used in this report, please see the supplementary file

Methods

Cell Treatment

D₁R and D₅R density and cAMP response to D₁-like receptor stimulation, in stably transfected HEK-hD₁ and HEK-hD₅ cells, were reported previously (34,35). To demonstrate that the D₁R inhibits NADPH oxidase activity, HEK-hD₁ cells, pre-starved in serum-free MEM for 1 hour, were incubated for 20 min with vehicle, the D₁-like receptor agonist, fenoldopam (1.0 μ mol/L/20 min), or the D₁-like receptor antagonist, SCH 23390 (5.0 μ mol/L, added to the cells 5 min prior to the addition of fenoldopam). To determine if PKA or PKC mediates the inhibition of NADPH oxidase activity by D₁R, pre-starved HEK-hD₁ cells were pre-treated for 40 min with vehicle, two different PKA inhibitors (H89 [10 μ mol/L] or Rp-cAMP [50 μ mol/L]), or two different PKC inhibitors, (bisindolylmaleimide I [1 μ mol/L] or staurosporine [10 nmol/L]). In HEK- D₅ studies, fenoldopam and H89 were used. The pre-treated cells were then incubated for an additional 20 min with vehicle, the PKA activator Sp-cAMP (10 μ mol/L), PMA (1.0 μ mol/L), fenoldopam (1 μ mol/L) alone, or in combination with the PKA or PKC inhibitors. In the concentration-dependent experiments, the cells were treated with fenoldopam or PMA (10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , and 10^{-5} mol/L) for 20 min. In the time-course experiments, the cells were treated with fenoldopam (1 μ mol/L) or PMA (1 μ mol/L) for 0, 20, 40, 60 and 120 min. The temperature of all incubations was set at 37°C. After treatment, the cells were rinsed, and the cell membranes were isolated (36).

Measurement of NADPH oxidase activity by lucigenin chemiluminescence

NADPH oxidase activity was measured using lucigenin chemiluminescence in the presence of NADPH (36). Whole cell membranes were prepared as described in “Methods” in the supplementary file. Equal amounts of cell membranes were incubated with lucigenin (5 $\mu\text{mol/L}$) for 10 min at 37°C in a final volume of 0.9 ml assay buffer. Dynamic tracings were recorded for 180 sec (Autolument Plus LB953, EG&G Berthold, Germany) following the injection of 0.1 ml of NADPH (final concentration, 100 $\mu\text{mol/L}$) (36). The activity was expressed as arbitrary light units per 50 μg protein and converted to percent of control. In some experiments NADPH oxidase activity was measured using a luminometer (MicroWin 2000). The activity was expressed as relative light units per mg protein and converted to percent of control.

siRNA knockdown of PKC θ in human RPT cells

Human RPT cells were seeded in 6-well plates (for immunoblotting) or in 60 mm dishes (for oxidase assay) at a density of 2×10^5 /well or dish at day 1 and then transfected with vehicle, scrambled RNA, or PKC θ -specific siRNA at day 2, as described previously (36). Cells were treated with or without drug and harvested at day 4 and assayed for NADPH oxidase activity, as described above. Protein levels were quantified by immunoblotting.

Statistical analysis

Data are expressed as mean $\pm$ standard error. Significant difference between two groups was determined by Student's t-test and factorial ANOVA followed by Newman-Keuls test for groups >2 . $P < 0.05$ was considered significant.

Results

D $_1$ R inhibits NADPH oxidase activity in HEK-hD $_1$ cells

Since our studies utilized HEK-293 cells, we confirmed that the various Nox isoforms (Nox1, Nox2, Nox3, Nox4 and Nox5) were expressed endogenously in these cells (Figure S1). To test the specificity of the inhibitory effect of D $_1$ R on NADPH oxidase activity, non-transfected HEK-293 cells, HEK-293 cells transfected with the empty vector (HEK-EV), and HEK-293 cells heterologously expressing hD $_1$ R (HEK-hD $_1$) were treated with vehicle or the D $_1$ -like receptor agonist, fenoldopam (1 $\mu\text{mol/L}/20\text{min}$) and NADPH oxidase activity was measured. As shown in Figure 1A, fenoldopam inhibited NADPH oxidase activity in HEK-hD $_1$ cells ($33 \pm 8.2\%$) but not in HEK-293 ($10.5 \pm 3.6\%$) or HEK-EV cells ($7 \pm 7.3\%$) (compared to vehicle). In addition, the inhibitory effect of fenoldopam on NADPH oxidase activity in HEK-hD $_1$ cells was concentration- and time-dependent (supplementary Figure S2 and supplementary Table S1). Furthermore, the inhibitory effect of fenoldopam on NADPH oxidase activity ($37 \pm 5.0\%$ inhibition) was blocked by the D $_1$ -like receptor antagonist, Sch23390 (5 μM) ($16 \pm 6.2\%$), which by itself had no effect ($2 \pm 5.7\%$) (Figure 1B).

D $_1$ R-mediated inhibition of NADPH oxidase activity involves both PKA and PKC in HEK-hD $_1$ cells

Dopamine has been reported to inhibit NADPH oxidase activity through PKA and PKC pathways in rat vascular smooth muscle cells (33,34) that endogenously express both D $_1$ R and D $_5$ R (D $_{1A}$ and D $_{1B}$ in rodents) (1–3). In the current study, the inhibitory effect of D $_1$ R, independent of D $_5$ R, on NADPH oxidase activity (Fen= $68.1 \pm 3.6\%$ of control, set at 100%) was almost completely prevented by two different PKA inhibitors, H89 (10 $\mu\text{mol/L}$) (Figure 2A), and Rp-cAMP (50 $\mu\text{mol/L}$) (supplementary Figure S3), which by themselves had no effect on oxidase activity (H89= $88 \pm 8.1\%$ and Rp-cAMP= $97.7 \pm 6.7\%$ of control) in HEK-hD $_1$ cells. The inhibitory effect of D $_1$ R on NADPH oxidase activity was also reversed by

two different PKC inhibitors, bisindolylmaleimide I and staurosporine, which by themselves, also had no effect (bisindolylmaleimide I = $89.04 \pm 3.4\%$, and staurosporine = $98 \pm 4.5\%$ of control) (Figure 2B and supplementary Table S2).

Activation of PKC inhibits NADPH oxidase activity in HEK-hD₁ cells

To evaluate the effect of PKC activation on NADPH oxidase activity, HEK-EV and HEK-hD₁ cells were treated with PMA (1 μ M/20 min), and NADPH oxidase activity was measured. PMA significantly increased NADPH oxidase activity in HEK-EV cells ($121.6 \pm 6.2\%$ vs. control = $100 \pm 3.0\%$). In contrast, PMA significantly decreased NADPH oxidase activity in HEK-hD₁ cells ($80.5 \pm 2.8\%$ vs. $100 \pm 4.9\%$) (Figure 3A), indicating that direct activation of PKC inhibits oxidase activity in HEK-hD₁ but not HEK-EV cells. We next determined the concentration- and time-dependent effect of PMA on NADPH oxidase in HEK-hD₁ cells. The cells were treated with PMA at various concentrations for 20 min and PMA (1 μ mol/L) at various durations, as indicated. As shown in Figure 3B and supplementary Table S3, NADPH oxidase activity was decreased by PMA in a concentration- and time-dependent manner. To confirm that the effect of PMA on oxidase activity was mediated by PKC, the PKC inhibitor bisindolylmaleimide I (1 μ M) was used. Indeed, the suppressive effect of PMA on NADPH oxidase activity ($77.3 \pm 3.4\%$ of control) was prevented by bisindolylmaleimide I ($97.3 \pm 8.9\%$ of control) (Figure 3C).

Activation of PKC decreases NADPH oxidase activity through cross-talk with PKA in HEK-hD₁ cells

To determine if the effect of PMA on NADPH oxidase activity involves PKA signaling, cells were treated with a PKA inhibitor, H89. The inhibitory effect of PMA on oxidase activity ($73 \pm 3.2\%$ of control) was prevented by the addition of H89 ($100 \pm 7.8\%$ of control), which by itself had no effect on NADPH oxidase activity ($104 \pm 7.4\%$ of control) in HEK-hD₁ cells (Figure 4A and supplementary Table S4).

To determine if activation of PKC by PMA is isoform-specific, we studied the abundance of phosphorylated PKC isoform in HEK-hD₁ cells treated with PMA (1 μ mol/L for 20 min). PMA decreased the level of cytosolic PKC, but increased the membrane abundance of serine-phosphorylated PKC α , PKC ϵ , and PKC η and threonine-phosphorylated PKC θ in HEK-hD₁ cells (Figure 4B). A similar pattern has been reported for the PMA-mediated translocation of PKC isoforms without taking into account their phosphorylation status (37). In contrast, the serine-phosphorylated PKC θ ^{S676} was increased by PMA in both cytosol and membrane, whereas PKC δ ^{S645} was increased in the membrane but not in the cytosol fraction. Additionally, the PMA-mediated increase in phosphorylated PKC θ ^{S676} and PKC δ ^{S645} was prevented by the PKA inhibitor H89 (Figure 4C) and (Figure 4D).

To determine the PKC isoforms involved in D₁R signaling, HEK-hD₁ cells were treated with fenoldopam (D₁R agonist), Sch23390 (D₁R antagonist), the PKA inhibitor Rp-cAMP, the PKC inhibitor bisindolylmaleimide I, or inhibitor combinations. Whole cell lysates were used to determine PKC θ ^{S676} protein because it was increased in both cytosol and membrane fractions (Figure 4B) of the cells. PKC θ ^{S676} was increased by fenoldopam ($153 \pm 11.3\%$ of control) in whole cell lysates, an effect that was abolished by the D₁R antagonist Sch23390 ($97.1 \pm 9.2\%$ of control) (Figure 5A). The increase in PKC θ ^{S676} induced by fenoldopam was prevented by the PKA inhibitor Rp-cAMP ($112 \pm 7.7\%$ of control) (Figure 5B, **left panel**) and the PKC inhibitor bisindolylmaleimide I ($112 \pm 5.4\%$ of control) (Figure 5B, **right panel**), which by themselves had no effect.

To determine if D₅R can also increase PKC θ ^{S676}, we assessed the effect of a D₁-like receptor agonist, fenoldopam, and a PKA inhibitor, H89, on the protein level of PKC θ ^{S676} in

HEK-293 cells heterologously expressing D₅R (HEK-D₅) (Figure S7, supplementary file). Stimulation of D₅R with fenoldopam did not significantly increase PKC θ ^{S676} ($P>0.05$, ANOVA, Duncan's test) (Fenoldopam=108.2 $\pm$ 2.7 vs. control=100 $\pm$ 1.5 % change). However, the PKA inhibitor H89, by itself or with fenoldopam significantly decreased PKC θ ^{S676} (H89=82.4 $\pm$ 6.8 and H89+fenoldopam =77.8 $\pm$ 6.8 % change, $P<0.05$, control vs. H89 and H89+fen, $n=3$ /group, ANOVA, Duncan's test).

To determine the relevance of our findings to cells that endogenously express functional D₁R and D₅R, we studied the effect of D₁-like receptor stimulation in human RPT cells. In these cells, we found that stimulation of D₁-like receptors increased the phosphorylated PKC θ ^{S676} (Supplementary Figure S6), but not the phosphorylated PKC δ ^{S645} (data not shown). To determine which of the D₁-like receptor (D₁R or D₅R) is responsible for the fenoldopam-mediated increase in PKC θ ^{S676} abundance in human RPT cells, we knocked down of D₅R protein expression using siRNA specific for D₅R in human RPT cells (Figure S8–S9, supplementary file), which decreased D₅R protein levels by 45% (45 $\pm$ 6.7 % vs. control siRNA=100 $\pm$ 0.73 %, $P<0.05$, $n=3$, t -test) (Figure S8). Knockdown of D₅R did not prevent the fenoldopam-mediated stimulation of PKC θ ^{S676} ($P<0.05$, Fen vs. control, H89 and H89+Fen, ANOVA, Duncan's test) (Figure S9 and Table S6 in the supplementary file).

The involvement of PKC θ ^{S676} in D₁R signaling in HEK-hD₁ cells was also studied by confocal microscopy. Fenoldopam (1 μ mol/L/20 min) increased the colocalization of PKC θ and PKC θ ^{S676}, an effect that was attenuated by the PKA inhibitor H89, which by itself had no effect (supplementary Figure S10).

The D₁-like receptor-mediated inhibition of NADPH oxidase activity is abrogated by the knockdown of PKC θ

To demonstrate further that PKC θ plays an important role in D₁-like receptor-mediated negative regulation of NADPH oxidase activity, the expression of PKC θ was knocked-down by PKC θ -specific siRNA in human RPT cells. Knockdown of PKC θ , which decreased PKC θ protein by 70% (Figure 6A), prevented the inhibitory effect of fenoldopam (1 μ mol/L/20 min) on NADPH oxidase activity (Figure 6B and supplementary Table S6).

Discussion

The current studies provide firm evidence that D₁R inhibits NADPH oxidase activity in HEK-hD₁ cells heterologously expressing hD₁R but not hD₅R. These studies are consistent with previous reports using rat vascular smooth muscle and human RPT cells, which endogenously express both D₁-like receptors, D₁R and D₅R (33,36). Although the inhibitory effect of D₁-like receptors on NADPH oxidase activity has been reported to be mediated by PKA- and PKC-dependent pathways (33,34,36), the role of cross-talk between PKA and various PKC isoforms in D₁R-mediated oxidase inhibition has not been determined. We now report that D₁R inhibits NADPH oxidase activity by increasing the PKA-dependent phosphorylated PKC θ ^{S676}. The current studies show that direct activation of PKC with PMA decreases NADPH oxidase activity in a time- and concentration-dependent manner in HEK-hD₁ cells. PMA, a direct activator of PKC, induces the translocation of conventional and novel PKC isoforms from cytosol to membranes (37). In the current studies, PMA also induced the translocation of serine-phosphorylated PKC α , PKC δ , PKC θ , PKC η , and PKC ϵ and threonine-phosphorylated PKC θ from cytosol to membranes in HEK-hD₁ cells. In addition, the PMA-mediated increase in membranes of PKC θ ^{S676} and PKC δ ^{S645}, but not the other phosphorylated PKC isoforms, was attenuated by the PKA inhibitor H89 in HEK-hD₁ cells. PKC θ , a Ca²⁺-independent novel PKC isoform (37), has been implicated in T cell receptor signal transduction (38). PKC θ is the only PKC isoform involved in the formation of a membrane-signal complex when the T cell comes into contact with a stimulator cell

(39). In addition, PKC θ is constitutively phosphorylated at the activation-loop (threonine 538) and turn-motif (serine 676) sites in T cells (40). PKC θ activity is also involved in aromatic hydrocarbon receptor signal transduction and nicotinic acetylcholine receptor (nAChR) cluster formation (41,42). The inhibitory effect of PKC and PKA on NADPH oxidase activity in our studies is similar to that recently reported for Nox 1 in HEK-293 cells (43). We now report, for the first time, that D₁R-mediates the inhibition of NADPH oxidase activity via a cross-talk between PKA/PKC that is linked to PKC θ . In contrast, the increase in ROS production in the renal medullary thick ascending limb of diabetic rats and adipocytes of obese, insulin-resistant mice has been reported to be PKC-dependent but involves PKC δ rather than PKC θ (44,45). PKC α and PKC ζ may also be involved in the PKC-mediated activation of NADPH oxidase in rat mesangial cells (46,47). PKC α and PKC δ contribute to NADPH oxidase activation in a pig kidney cell line (LLC-PK1) (48). Thus, the PKC-mediated inhibition or activation of NADPH oxidase is PKC isoform-dependent and tissue- and cell-specific. Activation of PKC with phorbol ester generally results in increase NADPH-oxidase activity (44–48). In HEK-293 cells heterologously expressing human D₁R, we found that PMA decreases NADPH oxidase activity. This indicates that PMA, in the presence of D₁R, exerts a strong antioxidant effect PMA has been reported to stimulate D₁R but inhibit D₅R signaling in HEK-293 cells heterologously expressing human D₁R or D₅R (15). PKC can also potentiate forskolin- and dopamine-induced cAMP formation in a concentration-dependent manner *in vitro* (49). Therefore, the activation of D₁R by PMA along with elevated cAMP concentration (and PKA activity) can synergistically activate PKC (3,6,15). An activated D₁R can also directly stimulate PLA₂ (50). The activation of PLA₂ increases the release of arachidonic acid that is converted to 20-hydroxy-eicosatetraenoic acid (20-HETE) and stimulates PKC activity (50). Therefore, the PKC-mediated decrease in NADPH oxidase activity in HEK-hD₁ cells may be due to activation of D₁R, resulting in a positive feedback.

In RPT cells, the activation of D₁-like receptors (D₁R and D₅R in human and D_{1A} and D_{1B} in rodents) stimulates PKC θ and PKC ζ and inhibits PKC δ . In addition, D₁-like receptor stimulation induces the translocation of specific PKC isoforms from cytosol to membrane (α , β , and ϵ) and membrane to cytosol (δ) (3,4,14,17). We and others have reported previously that D₁R-mediated stimulation of PLC can be a result of PKA and PKC activation in epithelial cells (3–6,14,15). We have also reported that intrarenal arterial infusion of two different D₁-like receptor agonists, SKF38393 and fenoldopam, for 10 min increases PKC θ protein in rat RPT membranes (4). In the present study, activation of D₁R (fenoldopam, 1 μ mol/20 min) did not significantly increase PKC θ protein in HEK-hD₁ cells. The difference between our previous *in vivo* study and the current *in vitro* study may lie in the inability of D₁-like receptor agonists to distinguish between the two D₁-like receptors, D₁R and D₅R. In the current *in vitro* study in which the D₁R but not the D₅R is expressed, D₁R stimulation increases membrane PKC θ ^{S676}. This effect is exerted at the D₁R because it is prevented by a D₁R antagonist. In contrast, D₅R stimulation with the D₁-like receptor agonist fenoldopam does not increase PKC θ ^{S676} in HEK-D₅ cells. Additionally, knockdown of D₅R in RPT cells does not prevent the fenoldopam-mediated increase PKC θ ^{S676} protein level in human RPT cells. The D₁R-mediated increase in the membrane PKC θ ^{S676} is mediated via PKA, because the effect is prevented by pre-treatment with the PKA inhibitor H89. These results suggest that the D₁R-mediated increase in PKC θ ^{S676} abundance is PKA-dependent. PKC θ ^{S676} has been reported to have a limited effect on kinase activity but may negatively regulate other aspects of PKC θ function, at least as related to NF- κ B activation in Jurkat T-cells (40). Nevertheless, the fact that knockdown of PKC θ with PKC θ -specific siRNA prevented the inhibitory effect of fenoldopam on oxidase activity in HEK-hD₁ cells confirms the important role of PKC θ in D₁R-mediated inhibition of NADPH oxidase activity.

In summary, our studies demonstrate that the D₁R-mediated inhibition of NADPH oxidase activity is via the phosphorylated PKC isoform, PKC θ^{S676} in HEK-hD₁ and human RPT cells and that the D₁R-mediated increase in the phosphorylated PKC θ^{S676} involves the PKA pathway. In contrast, the D₅R-mediated inhibition of NADPH oxidase activity is independent of PKA and PKC and is rather due to interference with the distribution and assembly of NADPH oxidase components and stimulation of PLD (34).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

These studies were supported in part by grants from the National Institutes of Health (HL023081, HL074940, DK039308, HL068686, HL092196, and RR020185).

References

1. Jose PA, Eisner GM, Felder RA. Renal dopamine receptors in health and hypertension. *Pharmacol Ther.* 1998; 80:149–182. [PubMed: 9839770]
2. Hussain T, Lokhandwala MF. Renal dopamine receptors and hypertension. *Exp Biol Med* (Maywood). 2003; 228:134–142. [PubMed: 12563019]
3. Yu PY, Eisner GM, Yamaguchi I, Mouradian MM, Felder RA, Jose PA. Dopamine D1A receptor regulation of phospholipase C isoform. *J Biol Chem.* 1996; 271:19503–19508. [PubMed: 8702641]
4. Yao LP, Li XX, Yu PY, Xu J, Asico LD, Jose PA. Dopamine D1 receptor and protein kinase C isoforms in spontaneously hypertensive rats. *Hypertension.* 1998; 32:1049–1053. [PubMed: 9856972]
5. Kansra V, Chen C, Lokhandwala MF. Dopamine causes stimulation of protein kinase C in rat renal proximal tubules by activating dopamine D1 receptors. *Eur J Pharmacol.* 1995; 289:391–394. [PubMed: 7621915]
6. Gomes P, Soares-da-Silva P. Dopamine acutely decreases type 3 Na⁺/H⁺ exchanger activity in renal OK cells through the activation of protein kinases A and C signaling cascades. *Eur J Pharmacol.* 2004; 488:51–59. [PubMed: 15044035]
7. Snyder GL, Fienberg AA, Haganir RL, Greengard P. A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (Mr 32 kDa)/protein phosphatase-1 pathway regulates dephosphorylation of the NMDA receptor. *J Neurosci.* 1998; 18:10297–10303. [PubMed: 9852567]
8. Sahu A, Tyeryar KR, Vongtau HO, Sibley DR, Undieh AS. D5 dopamine receptors are required for dopaminergic activation of phospholipase C. *Mol Pharmacol.* 2009; 75:447–453. [PubMed: 19047479]
9. Yao L, Fan P, Jiang Z, Gordon A, Mochly-Rosen D, Diamond I. Dopamine and ethanol cause translocation of epsilon PKC associated with epsilon RACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways. *Mol Pharmacol.* 2008; 73:1105–1112. [PubMed: 18202306]
10. Morris SJ, Van-Ham II, Daigle M, Robillard L, Sajedi N, Albert PR. Differential desensitization of dopamine D2 receptor isoforms by protein kinase C: the importance of receptor phosphorylation and pseudosubstrate sites. *Eur J Pharmacol.* 2007; 577:44–53. [PubMed: 17869243]
11. Li Z, Yu C, Yu Han, Ren H, Weibin Shi W, Fu CJ, Duofen He DF, Huang L, Yang CM, Wang XK, Zhou L, Asico LD, Zeng CY, Jose PA. Inhibitory effect of D1-like and D3 dopamine receptors on norepinephrine-induced proliferation in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol.* 2008; 294:H2761–H2768. [PubMed: 18441198]
12. Sharma A, Waly M, Deth RC. Protein kinase C regulates dopamine D4 receptor-mediated phospholipid methylation. *Eur J Pharmacol.* 2001; 427:83–90. [PubMed: 11557258]

13. Silva GB, Ortiz PA, Hong NJ, Garvin JL. Superoxide stimulates NaCl absorption in the thick ascending limb via activation of protein kinase C. *Hypertension*. 2006; 48:467–472. [PubMed: 16894053]
14. Nowicki S, Kruse MS, Brismar H, Aperia A. Dopamine-induced translocation of protein kinase C isoforms visualized in renal epithelial cells. *Am J Physiol Cell Physiol*. 2000; 279:C1812–C1818. [PubMed: 11078696]
15. Jackson A, Sedaghat K, Minerds K, James C, Tiberi M. Opposing effects of phorbol-12-myristate-13-acetate, an activator of protein kinase C, on the signaling of structurally related human dopamine D1 and D5 receptors. *J Neurochem*. 2005; 95:1387–1400. [PubMed: 16313517]
16. Efendiev R, Bertorello AM, Pedemonte CH. PKC- β and PKC- ζ mediate opposing effects on proximal tubule Na⁺,K⁺-ATPase activity. *FEBS Lett*. 1999; 456:45–48. [PubMed: 10452527]
17. Ezeamuzie CI, Taslim N. Reactive oxygen species mediate phorbol ester-stimulated cAMP response in human eosinophils. *Eur J Pharmacol*. 2006; 543:174–180. [PubMed: 16814765]
18. Baines AD, Drangova R, Ho P. Role of diacylglycerol in adrenergic-stimulated ⁸⁶Rb uptake by renal proximal tubules. *Am J Physiol*. 1990; 258:F1133–F1138. [PubMed: 2337144]
19. Houillier P, Chambrey R, Achard JM, Froissart M, Poggioli J, Paillard M. Signaling pathways in the biphasic effect of angiotensin II on apical Na/H antiport activity in proximal tubule. *Kidney Int*. 1996; 50:1496–1505. [PubMed: 8914015]
20. Balboa MA, Insel PA. Stimulation of phospholipase D via α 1-adrenergic receptors in Madin-Darby canine kidney cells is independent of PKC α and - ϵ activation. *Mol Pharmacol*. 1998; 53:221–227. [PubMed: 9463479]
21. Boesch DM, Garvin JL. Age-dependent activation of PKC isoforms by angiotensin II in the proximal nephron. *Am J Physiol Regul Integr Comp Physiol*. 2001; 281:R861–R867. [PubMed: 11507002]
22. Sim AT, Scott JD. Targeting of PKA, PKC and protein phosphatases to cellular microdomains. *Cell Calcium*. 1999; 26:209–217. [PubMed: 10643559]
23. Yan X, Walkiewicz M, Carlson J, Leiphon L, Grove B. Gravin dynamics regulates the subcellular distribution of PKA. *Exp Cell Res*. 2009; 315:1247–1259. [PubMed: 19210988]
24. Karim Z, Defontaine N, Paillard M, Poggioli J. Protein kinase C isoforms in rat kidney proximal tubule: acute effect of angiotensin II. *Am J Physiol*. 1995; 269:C134–C140. [PubMed: 7631740]
25. Wang XD, Kiang JG, Atwa MA, Smallridge RC. Evidence for the involvement of protein kinase C isoforms in α -1 adrenergic activation of phospholipase A2 in FRTL-5 thyroid cells. *J Invest Med*. 1996; 44:566–574.
26. Liedtke CM, Cole T, Ikebe M. Differential activation of PKC- δ and - ζ by α 1-adrenergic stimulation in human airway epithelial cells. *Am J Physiol*. 1997; 273:C937–C943. [PubMed: 9316415]
27. Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? *Am J Physiol Regul Integr Comp Physiol*. 2005; 289:R913–R935. [PubMed: 16183628]
28. Bey EA, Xu B, Bhattacharjee A, Oldfield CM, Zhao X, Li Q, Subbulakshmi V, Feldman GM, Wientjes FB, Cathcart MK. Protein kinase C δ is required for p47phox phosphorylation and translocation in activated human monocytes. *J Immunol*. 2004; 173:5730–5738. [PubMed: 15494525]
29. Thamilselvan V, Menon M, Thamilselvan S. Oxalate-induced activation of PKC- α and - δ regulates NADPH oxidase-mediated oxidative injury in renal tubular epithelial cells. *Am J Physiol Renal Physiol*. 2009; 297:F1399–F1410. [PubMed: 19692488]
30. Choi H, Leto TL, Hunyady L, Catt KJ, Bae YS, Rhee SG. Mechanism of angiotensin II-induced superoxide production in cells reconstituted with angiotensin type 1 receptor and the components of NADPH oxidase. *J Biol Chem*. 2008; 283:255–267. [PubMed: 17981802]
31. Fortuño A, Bidegain J, Robador PA, Hermida J, López-Sagaseta J, Belouqui O, Díez J, Zalba G. Losartan metabolite EXP3179 blocks NADPH oxidase-mediated superoxide production by inhibiting protein kinase C: potential clinical implications in hypertension. *Hypertension*. 2009; 54:744–750. [PubMed: 19687351]

32. Kim JS, Diebold BA, Babior BM, Knaus UG, Bokoch GM. Regulation of Nox1 activity via protein kinase A-mediated phosphorylation of NoxA1 and 14-3-3 binding. *J Biol Chem*. 2007; 282:34787–34800. [PubMed: 17913709]
33. Yasunari K, Kohno M, Kano H, Minami M, Yoshikawa J. Dopamine as a novel antioxidative agent for rat vascular smooth muscle cells through dopamine D₁-like receptors. *Circulation*. 2000; 101:2302–2308. [PubMed: 10811599]
34. Yang Z, Asico LD, Yu P, Wang Z, Jones JE, Escano CS, Wang X, Quinn MT, Sibley DR, Romero GG, Felder RA, Jose PA. D5 dopamine receptor regulation of reactive oxygen species production, NADPH oxidase, and blood pressure. *Am J Physiol Regul Integr Comp Physiol*. 2006; 290:R96–R104. [PubMed: 16352863]
35. Yu P, Yang Z, Jones JE, Wang Z, Owens SA, Mueller SC, Felder RA, Jose PA. D₁ Dopamine receptor signaling involves caveolin-2 in HEK-293 cells. *Kidney Int*. 2004; 66:2167–2180. [PubMed: 15569306]
36. Han W, Li H, Villar VAM, Pascua AM, Dajani MI, Wang X, Natarajan A, Quinn MT, Felder RA, Jose PA, Yu P. Lipid rafts keep NADPH oxidase in the inactive state in human renal proximal tubule cells. *Hypertension*. 2008; 51(part 2):481–487. [PubMed: 18195159]
37. Saito N, Kikkawa U, Nishizuka Y. The family of protein kinase C and membrane lipid mediators. *J Diabetes Complications*. 2002; 16:4–8. [PubMed: 11872358]
38. Hayashi K, Altman A. Protein kinase C theta (PKC θ): a key player in T cell life and death. *Pharmacol Res*. 2007; 55:537–544. [PubMed: 17544292]
39. Monks CR, Kupfer H, Tamir I, Barlow A, Kupfer A. Selective modulation of protein kinase C- θ during T-cell activation. *Nature (London)*. 1997; 385:83–86. [PubMed: 8985252]
40. Liu Y, Graham C, Li A, Fisher RJ, Shaw S. Phosphorylation of the protein kinase C-theta activation loop and hydrophobic motif regulates its kinase activity, but only activation loop phosphorylation is critical to in vivo nuclear-factor- κ B induction. *Biochem J*. 2002; 361(Pt 2): 255–265. [PubMed: 11772397]
41. Ahmed S, Shibasaki M, Takeuchi T, Kikuchi H. Protein kinase C θ activity is involved in the 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced signal transduction pathway leading to apoptosis in L-MAT, a human lymphoblastic T-cell line. *FEBS J*. 2005; 272:903–915. [PubMed: 15691325]
42. Miles K, Wagner M. Overexpression of nPKC θ is inhibitory for agrin-induced nicotinic acetylcholine receptor clustering in C₂C₁₂ myotubes. *J Neurosci Res*. 2003; 71:188–195. [PubMed: 12503081]
43. Krovjarski Y, Debbabi M, Bachoual R, Périani A, Gougerot-Pocidalo MA, El-Benna J, My-Chan Dang P. Phosphorylation of NADPH oxidase activator 1 (NOXA1) on serine 282 by MAP kinases and on serine 172 by protein kinase C and protein kinase A prevents NOX1 hyperactivation. *FASEB J*. 2010; 24:2077–2092. [PubMed: 20110267]
44. Yang J, Lane PH, Pollock JS, Carmines PK. PKC-dependent superoxide production by the renal medullary thick ascending limb from diabetic rats. *Am J Physiol Renal Physiol*. 2009; 297:F1220–F1228. [PubMed: 19741016]
45. Talior I, Yarkoni M, Bashan N, Eldar-Finkelman H. Increased glucose uptake promotes oxidative stress and PKC- δ activation in adipocytes of obese, insulin-resistant mice. *Am J Physiol Endocrinol Metab*. 2003; 285:E295–E302. [PubMed: 12857675]
46. Wei XF, Zhou QG, Hou FF, Liu BY, Liang M. Advanced oxidation protein products induce mesangial cell perturbation through PKC-dependent activation of NADPH oxidase. *Am J Physiol Renal Physiol*. 2009; 296:F427–F437. [PubMed: 19019916]
47. Xia L, Wang H, Munk S, Kwan J, Goldberg HJ, Fantus IG, Whiteside CI. High glucose activates PKC- ζ and NADPH oxidase through autocrine TGF- β ₁ signaling in mesangial cells. *Am J Physiol Renal Physiol*. 2008; 295:F1705–F1714. [PubMed: 18815221]
48. Thamilselvan V, Menon M, Thamilselvan S. Oxalate-induced activation of PKC- α and - δ regulates NADPH oxidase-mediated oxidative injury in renal tubular epithelial cells. *Am J Physiol Renal Physiol*. 2009; 297:F1399–F1410. [PubMed: 19692488]
49. Schinelli S, Paolillo M, Corona GL. Modulation of dopamine- induced cAMP production in rat striatal cultures by the calcium ionophore A23187 and by phorbol-12- myristate-13-acetate. *Brain Res. Mol. Brain Res*. 1994; 21:162–166. [PubMed: 8164517]

50. Grider JS, Ott CE, Jackson BA. Dopamine D1 receptor-dependent inhibition of NaCl transport in the rat thick ascending limb: mechanism of action. *Eur J Pharmacol.* 2003; 473:185–190. [PubMed: 12892837]

Figure 1A

Figure 1B

Figure 1. Effect of D₁-like receptor agonist and antagonist on NADPH oxidase activity

A. Cells [HEK-293, HEK-293 expressing human D₁R (HEK-hD₁) and empty vector (HEK-EV)] were treated for 20 min at 37°C with vehicle (Con), or the D₁-like receptor agonist, fenoldopam (Fen, 1 μmol/L). Membranes were prepared and oxidase activity was measured in the presence of 5 μmol/L lucigenin and 100 μmol/L NADPH as described in “Methods”. Values are mean ± SEM (n = 6/group). *P<0.001 vs. control, *t*-test

B. HEK-hD₁ cells were treated for 20 min at 37°C with vehicle (Con), fenoldopam (Fen, 1 μmol/L/20min), the D₁-like receptor antagonist, Sch23990 (Sch, 5 μmol/L), alone or in combination (S+F), as described in “Methods”. Membranes were assayed for NADPH oxidase activity (Arbitrary Light Units per 50 μg protein). The left panel shows the dynamic tracings of oxidase-dependent chemiluminescence recorded over a period of 180 seconds; right panel shows the oxidase activity expressed as % of control. Values are the mean ± SEM (n=5/group). *P<0.01 vs. others, ANOVA, Newman-Keuls test

Figure 2. Effect of PKA and PKC inhibitors on NADPH oxidase activity in HEK-hd₁ cells
 HEK-hd₁ cells were pre-incubated for 40 min at 37°C with vehicle, PKA inhibitor H89 (10 μmol/L), two different PKC inhibitors, bisindolylmaleimide I (Bis, 1 μmol/L), or staurosporine (St, 10nmol/L), and then were treated for 20 min at 37°C with vehicle (Con), fenoldopam (Fen, 1 μmol/L), or inhibitor combinations (H89+Fen) (**A**) and (Bis+Fen, St+Fen) (**B**), as described in “Methods”. Membranes were assayed for oxidase activity (Arbitrary Light Units per 50 μg protein). The left panel (**2A**) and the upper panels (**2B**) show dynamic tracings of oxidase-dependent chemiluminescence recorded over a period of 180 seconds; the right panel (**2A**) and the lower panel (**2B**) show oxidase activity expressed as % of control. Values are mean ± SEM (n=4–8/group). *P<0.01 vs. others, ANOVA, Newman-Keuls test

Figure 3A

Figure 3B

Figure 3C

Figure 3. Effect of PKC activator and inhibitor on NADPH oxidase activity in HEK-hd₁ cells
A. Effect of PMA on NADPH oxidase activity in HEK-EV and HEK-hd₁ cells. HEK-EV and HEK-hd₁ cells were treated with the PKC activator PMA (1 μmol/L) for 20 min at 37°C, as described in “Methods”. Membranes were prepared and assayed for oxidase activity (Arbitrary Light Units per 50 μg protein). The left panel shows the dynamic tracings of oxidase-dependent chemiluminescence recorded over a period of 180 seconds; the right panel shows oxidase activity expressed as % of control. Values are mean ± SEM (n=8/group). *P=0.007 vs. control in HEK-EV and **P=0.004 vs. control in HEK-hd₁, *t*-test

B. Concentration- and time-dependent effect of PMA on membrane NADPH oxidase activity. In the concentration-dependent experiments, HEK-hD₁ cells were treated for 20 min at 37°C at the indicated concentrations of PMA. In the time-dependent experiments, cells were treated at 37°C with PMA (1 μmol/L) for the indicated times. Membranes were prepared and assayed for oxidase activity (Arbitrary Light Units per 50 μg protein). The upper panels show the dynamic tracings of oxidase-dependent chemiluminescence recorded over a period of 180 seconds; the lower panels show oxidase activity expressed as % of control. Values represent the mean ± SEM (n=6/group). *P<0.01 vs. all concentrations except 10⁻⁹M, #P<0.05, vs. others (time), ANOVA, Newman-Keuls test

C. Effect of PKC inhibitor on PMA-induced increase in NADPH oxidase activity. HEK-hD₁ cells pre-incubated for 40 min at 37°C with vehicle, or a PKC inhibitor bisindolylmaleimide I (Bis, 1 μmol/L) were incubated for an additional 20 min at 37°C with vehicle (Con), PMA (1 μmol/L), or drug combination (Bis+PMA) as described in “Methods”. Membranes were prepared and assayed for oxidase activity which is expressed as % of control. Values represent the mean ± SEM (n=6/group). *P<0.05 vs. others, ANOVA, Newman-Keuls test

Figure 4A

Figure 4B

Figure 4C

Figure 4D

Figure 4. Effect of PKA inhibitor on PMA-induced decrease in NADPH oxidase activity and phosphorylated PKC isoforms

A. Effect of PKA inhibitor on PMA-induced decrease in NADPH oxidase activity. HEK-hD₁ cells pre-incubated for 40 min at 37°C with vehicle or a PKA inhibitor H89 (10 μ mol/L) were incubated for an additional 20 min at 37°C with vehicle (Con) or PMA (1 μ mol/L), as described in “Methods”. Membranes were prepared and assayed for oxidase activity using a luminometer. Oxidase activity is expressed as % of control. Values are mean $\pm$ SEM (n=5–6/group). *P<0.01 vs. others, ANOVA, Newman-Keuls test

B. Effect of PMA on the phosphorylation of PKC. HEK-hD₁ cells pre-incubated for 40 min at 37°C with vehicle or PKA inhibitor H89 (10 μ mol/L) were incubated for an additional 20 min at 37°C with vehicle, PMA (1 μ mol/L) or drug combination (H+P). The cells were then fractionated into cytosol and membrane, as described in “Methods”. Protein loading was verified by Ponceau-S staining in Figure S4, supplementary file. The proteins were probed with specific anti-phospho-PKC isoforms, as indicated. N=3/group.

C–D. Graphic representation of the effect of PMA on PKC θ ^{S676} (Figure 4C) and PKC δ ^{S645} (Figure 4D) proteins in the cells studied in Figure 4B. In the some cases, cell lysates were used for immunoblotting because PKC θ ^{S676} protein was increased in both cytosol and membrane fractions. The immunoblots of PKC θ ^{S676} and PKC δ ^{S645} (upper band) from

Figure 4B were semi-quantified, as described in “Methods”. Values are mean $\pm$ SEM (n= 4–6/group). *P<0.05 vs. others, ANOVA, Newman-Keuls test

Figure 5A

Figure 5. Effect of D₁R stimulation on PKCθ^{S676} protein

Cell lysates were prepared and used for immunoblotting following drug treatment. Protein loading was verified by Ponceau-S staining, Figure S5, supplementary file.

A. Effect of fenoldopam on PKCθ^{S676}. HEK-hD₁ cells were treated for 20 min at 37°C with vehicle (Con), D₁-like receptor agonist fenoldopam (Fen, 1 μmol/L), or D₁-like receptor antagonist Sch23990 (Sch, 5 μmol/L) added to cells 5 min prior to the addition of fenoldopam or drug combination (Sch+Fen). The cell lysates were probed with anti-PKCθ^{S676}, and the immunoreactive bands semi-quantified, as described in “Methods”. Values are mean ± SEM (n=4–6/group). *P< 0.05 vs. others, ANOVA, Newman-Keuls test

B. Effect of fenoldopam and PKA and PKC inhibitors on PKCθ^{S676}. HEK-hD₁ cells pre-incubated for 40 min at 37°C with vehicle, PKA inhibitor Rp-cAMP and (50 μmol/L) (left panel) or PKC inhibitor bisindolylmaleimide I (Bis, 1 μmol/L) (right panel) were incubated

for an additional 20 min at 37°C with vehicle (Con), fenoldopam (1 μ mol/L), or drug combinations (Rp+Fen, Bis+Fen), as described in “Methods”. The cell lysates were probed with anti-PKC θ^{S676} , and the immunoreactive bands were semi-quantified as described in “Methods”. Values are mean $\pm$ SEM (n=4–6/group). *P< 0.05 vs. others, ANOVA, Newman-Keuls test

Figure 6A

Figure 6B

Figure 6. Knockdown of PKCθ with PKCθ-specific siRNA in human RPT cells

A. Effect of PKCθ-specific siRNA on PKCθ in human RPT cells. Human RPT cells were transfected with control siRNA (siCon) or specific PKCθ siRNA (si PKCθ) for 48 hr as described in “Methods”. Cell lysates were immunoblotted with anti-PKCθ monoclonal antibody. The immunoreactive bands were semi-quantified (siCon=209.94±4.5, siPKCθ=51±8.9 density units). Values are mean ± SEM (n=3/group). *P< 0.05, t-test

B. Effect of PKCθ knockdown on D₁-like receptor-mediated inhibition of NADPH oxidase activity in human RPT cells. Human RPT cells were transfected with vehicle (Con), siCon, or siPKCθ as in Figure 6A. Membranes were prepared and assayed for oxidase activity using a luminometer. Oxidase activity is expressed as % of control. Values are mean ± SEM (n=3–6/group). *P<0.01 vs. respective controls, ANOVA, Newman-Keuls test