Lixazinone Stimulates Mitogenesis of Madin-Darby Canine Kidney Cells

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Polycystic kidney diseases (PKD) are characterized by excessive proliferation of renal tubular epithelial cells, development of fluid-filled cysts, and progressive renal insufficiency. cAMP inhibits proliferation of normal renal tubular epithelial cells but stimulates proliferation of renal tubular epithelial cells derived from patients with PKD. Madin-Darby canine kidney (MDCK) epithelial cells, which are widely used as an *in vitro* model of cystogenesis, also proliferate in response to cAMP. Intracellular cAMP levels are tightly regulated by phosphodiesterases (PDE). Isoform-specific PDE inhibitors have been developed as therapeutic agents to regulate signaling pathways directed by cAMP. In other renal cell types, we have previously demonstrated that cAMP is hydrolyzed by PDE3 and PDE4, but only PDE3 inhibitors suppress proliferation by inhibiting Raf-1 activity (Cheng J, Thompson MA, Walker HJ, Gray CE, Diaz Encarnacion MM, Warner GM, Grande JP. Am J Physiol Renal Physiol 287:F940–F953, 2004.) A potential role for PDE isoform(s) in cAMP-mediated proliferation of MDCK cells has not previously been established. Similar to what we have previously found in several other renal cell types, cAMP hydrolysis in MDCK cells is directed primarily by PDE4 (85% of total activity) and PDE3 (15% of total activity). PDE4 inhibitors are more effective than PDE3 inhibitors in increasing intracellular cAMP levels in MDCK cells. However, only PDE3 inhibitors, and not PDE4 inhibitors, stimulate mitogenesis of MDCK cells. PDE3 but not PDE4 inhibitors activate B-Raf but not Raf-1, as assessed by an *in vitro* kinase assay. PDE3 but not PDE4 inhibitors activate the ERK pathway and activate cyclins D and E, as assessed by histone H1 kinase assay. We conclude that mitogenesis of MDCK cells is regulated by a functionally compartmentalized intracellular cAMP pool directed by PDE3. Pharmacologic agents that stimulate PDE3 activity may provide the basis for new therapies directed toward reducing cystogenesis in patients with PKD. Exp Biol Med 231:288–295, 2006

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Introduction

Polycystic kidney diseases (PKD) are characterized by formation and expansion of multiple cystic lesions (cystogenesis), which eventually destroy kidney structure and function (1, 2). Renal cyst formation and enlargement is the result of a highly coordinated interaction among three central pathologic processes: increased rate of proliferation of renal tubular epithelial cells, fluid accumulation within the cyst cavity, and extracellular matrix modification (3, 4).

Mechanisms underlying cystogenesis have not been completely defined. *In vitro* studies have demonstrated that tubular epithelial cells derived from patients with PKD proliferate in response to cAMP agonists, whereas tubular epithelial cells derived from patients without PKD are growth inhibited by cAMP agonists (4–6). These studies provide evidence that the cAMP-protein kinase A (PKA) signaling pathway may play an important role in both proliferation and fluid secretion by tubular epithelial cells in patients with PKD (4–6). Madin-Darby canine kidney (MDCK) cells provide an excellent *in vitro* model of cystogenesis. When cultured in a matrix of polymerized type I collagen (7, 8), MDCK cells form spherical, monolayered, fluid-filled cysts. MDCK cysts in collagen exhibit epithelial cell proliferation, fluid accumulation, and matrix remodeling (8), processes similar to those observed in tubular epithelial cells cultured from patients with PKD. *In vitro* and *in vivo* studies have demonstrated that cyst formation and expansion can be strikingly accelerated by stimulating the production of intracellular cAMP, which increases the rate of cell proliferation and the rate of transepithelial fluid secretion into the cysts (6).

This aberrant proliferative response to cAMP agonists observed in tubular epithelial cells obtained from patients with PKD has been the focus of recent investigations (5). It is recognized that the effect of cAMP agonists on mitogenesis is cell type–specific (5). In cultured normal renal tubular epithelial cells, cAMP decreases proliferation

through inhibition of the Ras-Raf-1-MEK-ERK pathway at the level of Raf-1 (6). In cultured tubular epithelial cells obtained from patients with PKD, cAMP stimulates proliferation through activation of B-Raf rather than Raf-1 (6). Based on these considerations, it is possible that relative expression and/or localization of B-Raf versus Raf-1 may determine whether cAMP or cAMP agonists stimulate or inhibit proliferation (9). In support of this hypothesis, transfection of cells that normally do not express B-Raf with a constitutively active B-Raf construct converts cAMP from an inhibitor to an activator of ERK (6).

Intracellular cAMP levels are regulated by a balance between activities of synthesizing enzymes, adenylate cyclase and catabolizing enzymes, the 3′,5′-nucleotide phosphodiesterases (PDE) (10, 11). In most cells and tissues, the capacity for cAMP synthesis is far less than the capacity to hydrolyze cAMP by PDE and is one of the key factors determining turnover of cAMP (12, 13). This is consistent with the observations that even a small change in PDE isozyme activity can have a profound effect upon cAMP-PKA signaling; therefore, PDE isozymes must be tightly regulated (14, 15).

We have previously shown that in rat tubular epithelial cells and glomerular mesangial cells (MCs), cAMP hydrolysis is directed by PDE3 and, to a greater extent, PDE4 (15, 16). Although both PDE3 and PDE4 inhibitors elevate cAMP levels and activate PKA to a similar extent, we found that only PDE3 inhibitors suppress mitogenesis of tubular epithelial cells or MCs; PDE4 inhibitors are without significant effect on mitogenesis (15, 16). A potential role for PDE isoforms in regulation of mitogenesis of MDCK cells, which proliferate in response to cAMP, has not previously been defined. Based on these considerations, we sought to determine whether: (i) cAMP hydrolysis in MDCK cells is directed primarily by PDE3 and PDE4; (ii) PDE3 and PDE4 inhibitors differentially regulate MDCK mitogenesis; (iii) PDE3 and PDE4 inhibitors differentially regulate Raf-1 versus B-Raf activity in MDCK cells; and (iv) cell cycle regulatory proteins are differentially regulated by PDE3 and PDE4 inhibitors.

Materials and Methods

Materials. [^{3}H]-thymidine was purchased from DuPont/New England Nuclear Research Products (Boston, MA). Primary antibodies for B-Raf, p21, p27, and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Raf-1 antibody was obtained from BD Transduction Laboratories (San Diego, CA). Protein A agarose was obtained from Santa Cruz Biotechnology. Histone H1 was obtained from Calbiochem (La Jolla, CA). Other reagents and supplies were obtained through standard commercial suppliers.

Cell Culture. Wild-type MDCK cells, obtained at passage 53 from the American Type Culture Collection (ATCC; Manassas, VA), were grown and maintained as monolayer cultures at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. MC cultures were obtained as described previously (17, 18).

PDE Activity. PDE activity was determined by incubating cell extracts in a volume of 110 μl reaction mixture containing (final concentration) 10 m*M* $MgSO_4$, 2 m*M* EGTA, 0.1% bovine serum albumin, 15 m*M* Tris-HCl (pH 7.4), and 0.5 μ*M* [^{3}H]cAMP as substrate plus PDE inhibitors (15, 19). The reaction was stopped by placing the mixture in dry ice and samples were then boiled to destroy enzymatic activity. King Cobra venom was added to cleave PDE affected mononucleotides to nucleosides, which were separated by anion exchange columns (Sephadex QAE 25; Pharmacia, LKB, Inc., Piscataway, NJ). Column flow-through was then evaluated for inhibition as compared to basal values. PDE3 or PDE4 activity was determined as basal cAMP-PDE inhibitable by 3 μ*M* lixazinone (a PDE3 inhibitor) or 3 μ*M* rolipram (a PDE4 inhibitor). At this concentration, these selective inhibitors cause maximal inhibition of corresponding PDE isoforms.

Assay for cAMP. MDCK cells were incubated with the adenylate cyclase activator forskolin (FSK) and PDE inhibitors in 24-well culture dishes for 60 mins. The reactions were terminated with 5% trichloroacetic acid (TCA) (final concentration). Cells were collected by scraping with cell lifters and the mixture incubated on ice for 30 mins. The TCA-precipitated protein was extracted with water-saturated ether, and the cAMP content was measured using radioimmunoassay (RIA) as previously described (19, 20).

Transfection Studies. PKA activation was measured using the PathDetect *In Vivo* Signal Transduction Pathway *trans*-Reporting System (Stratagene, La Jolla, CA). MDCK cells were plated into 24-well culture dishes at 4×10^4 cells/well in DMEM supplemented with 10% FBS. Twenty-four hours after plating, cells were co-transfected with a firefly luciferase reporter vector (pTRE-Luc), a control *Renilla* luciferase reporter vector, and the trans-activator plasmid pFA2-CREB. Transfections were performed using FuGENE 6 Transfection Reagent (Roche Molecular Biochemical, Indianapolis, IN), according to the manufacturer's instructions. Eighteen hours after transfection, FSK and PDE inhibitors were added. Control cells received vehicle only. Cells were rinsed and lysed at the indicated time point as described in Results. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI).

Measurement of [^{3}H]-Thymidine Incorporation. MDCK cells were plated into 24-well culture dishes at 5×10^4 cells/well and grown for 24–48 hrs in DMEM containing 10% FBS. Cells were rendered quiescent for 24 hrs in DMEM without FBS and treated with FSK and PDE inhibitors. Control cells were treated with equal volume of

vehicle only. After 20 hrs, cells were treated with methyl-[^{3}H]-thymidine (1 μCi/ml) for 4 hrs. Cells were then washed three times with 10% TCA and once with water and lysed by addition of 0.2 *N* NaOH. Radioactivity on the disks was determined by liquid scintillation counting. Incorporation of [^{3}H]-thymidine was used as a measure of the rate of mitogenic synthesis of DNA.

Western Blot Analysis. Quiescent MDCK cells were treated with FSK and PDE inhibitors, as described above. After incubation, cells were rinsed, harvested, and subjected to sonication (three cycles of 10 secs each, 8 μm amplitude) in 1× lysis buffer (Cell Signaling Technology Inc., Beverly, MA). The homogenates were centrifuged at 10,000 *g* for 10 mins at 4°C. Protein concentration was determined by the method of Lowry *et al.* (21). Lysates were denatured for 5 mins at 100°C in SDS loading buffer and equal amounts of lysate proteins (~100 μg) were subjected to SDS-PAGE in the Criterion system (Bio-Rad Laboratories Inc., Hercules, CA). Electrophoresis was performed at a constant current (200 mA/gel), followed by transfer to PVDF membranes (Bio-Rad Laboratories). The membranes were blocked with 1× casein in Tris-buffered saline (TBS) containing 0.5% Tween 20 and incubated with primary antibodies followed by HRP-conjugated secondary antibodies. The blots were then visualized by exposure to x-ray film using ECL Western Blotting Detection Reagents (Amersham Biosciences Corp., Piscataway, NJ).

Raf-1 and B-Raf Kinase Assay. Cells were treated with FSK and PDE inhibitors for 5 mins, lysed, and immunoprecipitated with 5 μg of anti-Raf-1 or anti-B-Raf antibodies overnight. The Raf-1 and B-Raf kinase activity in immunoprecipitates was measured *in vitro* using Raf-1 and B-Raf Kinase Cascade Assay Kits (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer's instructions. Briefly, after 30 mins incubation at 30°C in a buffer (500 μ*M* ATP; 75 m*M* $MgCl_2$; 0.4 μg MEK-1 and 1μg MAP kinase2/ERK2; 20 m*M* MOPS, pH 7.2; 2.5 m*M* β-glycerol phosphate; 5 m*M* EGTA; 1 m*M* sodium orthovanadate; and 1 m*M* dithiothreitol), the MAP kinase2/Erk2 substrate myelin basic protein (MBP), and [γ-^{32}P] ATP were added and incubated at 30°C for 10 mins. Then, 25 μl of each reaction was spotted onto P81 phosphocellulose squares, and the squares were washed three times with 0.75% phosphoric acid and once with acetone. The level of [^{32}P] incorporation into MBP was determined by liquid scintillation counting.

***In Vitro* Kinase Assays.** p44/42 MAP Kinase Assay Kits (Cell Signaling Technology) were used to measure ERK kinase activity, according to manufacturer's instructions. Briefly, after treatment with FSK and PDE inhibitors, MDCK cells were rinsed, harvested, and sonicated four times for 5 secs each in 1× lysis buffer plus 1 m*M* PMSF. Samples were microcentrifuged for 10 mins at 4°C, and protein concentration of the supernatants was determined as described above. Two-hundred-microliter cell lysate containing approximately 200 μg total protein was added to 15 μl of resuspended immobilized phospho-p44/42 MAP Kinase (Thr202/Tyr204) Monoclonal Antibody and incubated with gentle rocking overnight at 4°C. After samples were microcentrifuged for 30 secs at 4°C, pellets were washed twice with 1× lysis buffer and twice with 1× kinase buffer. The washed pellets were suspended in 50 μl 1× kinase buffer supplemented with 200 μ*M* ATP and 2 μg Elk-1 fusion protein and incubated for 30 mins at 30°C. Reactions were terminated with 25 μl 3× SDS sample buffer. Samples were boiled for 5 mins, vortexed, microcentrifuged for 2 mins, then loaded (30 μl each) on SDS-PAGE gels. Samples were analyzed by Western blotting, as described above.

Histone H1 Kinase Assays for Cyclin Activity. Following treatment with FSK and PDE inhibitors, cells were lysed in 1× lysis buffer and protein concentration determined as described above. Equal amounts of lysate protein (200 μg) were immunoprecipitated with antibodies specific for cyclin D1 and cyclin E. The immune complexes were collected with protein G plus agarose and washed twice with 1× lysis buffer. Complexes were resuspended and washed twice with kinase buffer (50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 1 m*M* DTT), then resuspended in 50 μl kinase buffer containing 2 μg histone H1, 200 μ*M* ATP, and 10 μCi (γ-^{32}P)-ATP (3000 Ci/m*M*), and incubated at 30°C for 30 mins. After incubation, 25 μl of 3× SDS loading buffer was added and the samples were boiled and electrophoresed on a 12% SDS-PAGE gel. The gels were dried, and incorporation of ^{32}P was visualized by autoradiography and quantitated with a Kodak image analysis system (Kodak Digital Science Image Station 440CF; Eastman Kodak Co., Piscataway, NJ).

Statistical Analysis. Data presented are representative of at lease three independent experiments performed in duplicate or triplicate, as indicated in the figure legends. Groups or pairwise comparisons were evaluated by Student's *t* test; *P* values of <0.05 were considered statistically significant.

Results

cAMP Hydrolysis in MDCK Cells is Directed by PDE3 and PDE4. cAMP-PDE profiles in MDCK cells have not been previously defined. The activities of cAMP-PDE were assayed in extract of MDCK cells prepared as described in Materials and Methods. We found that both PDE3 and PDE4 are present in MDCK cells. The cAMP-PDE activity in MDCK cells was largely attributable to PDE4, only approximately 15% of total PDE activity was attributable to PDE3 (Fig. 1A).

The cAMP content was measured using radioimmunoassay. A transfection-based assay was employed to determine the role of PDE3 or PDE4 inhibitors on PKA activation (17). Rolipram (a PDE4 inhibitor) markedly increased cAMP levels (+825%); this increase is 57% of cAMP accumulation caused by the potent adenylate cyclase

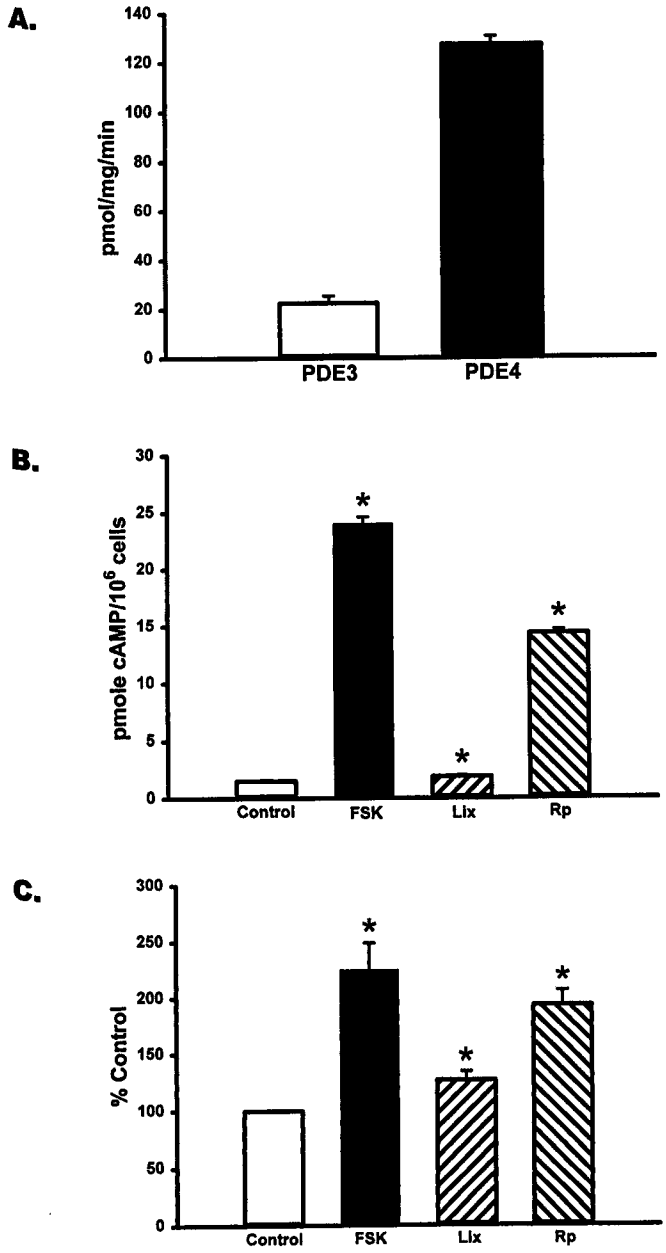

Figure 1. cAMP hydrolysis in MDCK is directed by PDE3 and PDE4. (A) PDE activity in MDCK cells. PDE3 or PDE4 activity in extract of MDCK cells was determined as basal cAMP-PDE inhibitable by 3 μM lixazinone (a PDE3 inhibitor) or 3 μM rolipram (a PDE4 inhibitor). (B) Effect of PDE inhibitors on intracellular cAMP content. Quiescent MDCK cells were treated with 10 μM potent adenylate cyclase agonist forskolin (FSK), 10 μM lixazinone (Lix), or 10 μM rolipram (Rp) for 60 mins. cAMP content was measured using RIA. (C) Effect of PDE inhibitors on PKA activation. Quiescent MDCK cells were treated with 10 μM FSK, 10 μM Lix, or 10 μM Rp, PKA activation was assessed by a transfection-based *in vivo* kinase assay. Values represent means ± SE ($n = 3$). $^*P < 0.05$ vs. control.

agonist forskolin. Lixazinone (a PDE3 inhibitor) only modestly increased cAMP levels (+22%; Fig. 1B). Both lixazinone and rolipram activated PKA. However, rolipram activated PKA to a greater extent than lixazinone (+27%). PKA activation by rolipram (+94%) was similar to that of forskolin (+125%) (Fig. 1C).

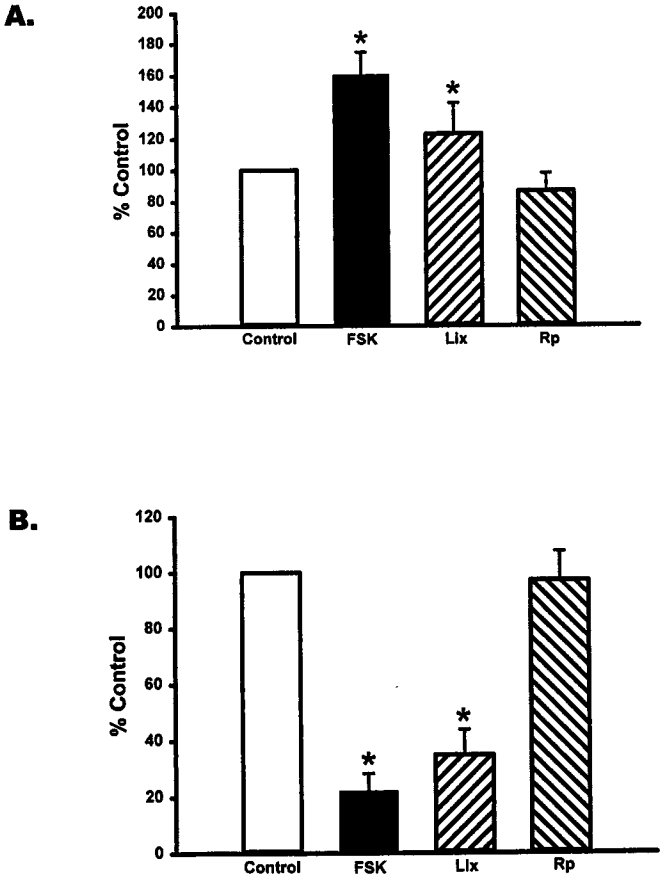

Figure 2. PDE3 inhibitors stimulate MDCK cell proliferation, but suppress rat MC proliferation. (A) Quiescent MDCK cells were treated with 10 μM FSK, 10 μM Lix, or 10 μM Rp for 72 hrs, and [^{3}H]-thymidine incorporation was measured using liquid scintillation counting. (B) MCs were treated with 10 μM FSK, 10 μM Lix, or 10 μM Rp for 24 hrs, and [^{3}H]-thymidine incorporation was measured using liquid scintillation counting. Values represent means ± SE ($n = 3$). $^*P < 0.05$ vs. control.

PDE3 Inhibitors, but Not PDE4 Inhibitors, Stimulate MDCK Cell Proliferation. The PDE3 inhibitor lixazinone significantly stimulated proliferation of MDCK cells (Fig. 2A). Although the majority of cAMP-PDE activity in MDCK cells was attributable to PDE4, and rolipram activates PKA and increases cAMP content to a much greater extent than lixazinone, rolipram had no significant effect on thymidine uptake by MDCK cells (Fig. 2A). However, in accord with our previous studies, identical concentrations of forskolin and lixazinone potently inhibited thymidine uptake by MCs (Fig. 2B; Ref. 15). Cilostamide, a structurally distinct PDE3 inhibitor, stimulated MDCK proliferation to an extent similar to that observed with lixazinone (data not shown).

PDE3 and PDE4 Inhibitors Differentially Regulate B-Raf Kinase Activity. There is considerable evidence that cAMP regulates mitogenesis in a cell type–specific manner through modulation of Raf-1 or B-Raf activity (17, 22, 23). We therefore sought to test the hypothesis that PDE3 inhibitors stimulate mitogenesis of MDCK cells through stimulation of Raf-1 or B-Raf activity.

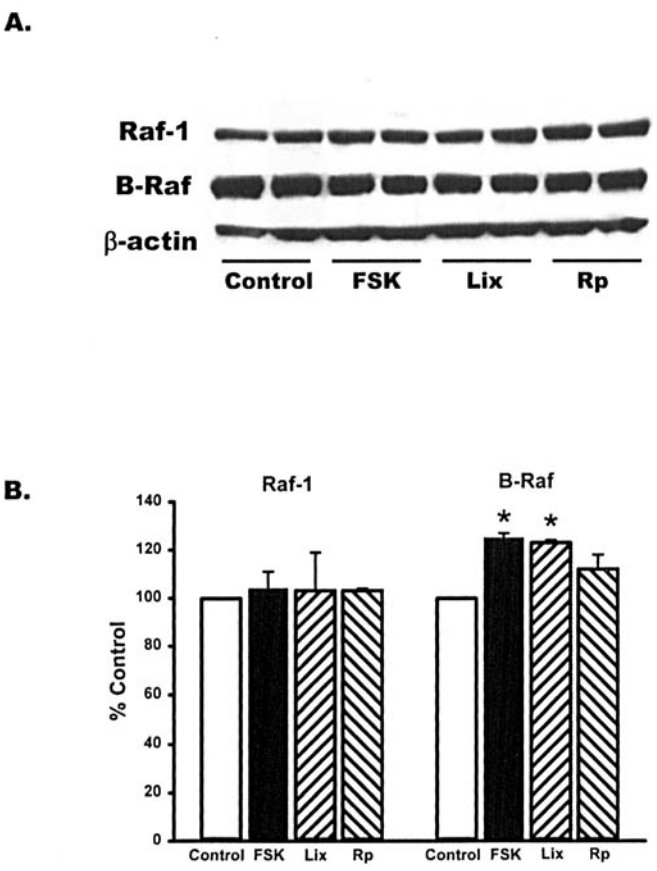

Figure 3. (A) MDCK cells express both Raf-1 and B-Raf. Quiescent MDCK cells were treated with 10 μ*M* FSK, 10 μ*M* Lix, or 10 μ*M* Rp for 8 hrs. Raf-1 and B-Raf levels were assessed by Western blot analysis. (B) PDE3 inhibitors activate B-Raf kinase activity without altering Raf-1 kinase activity in MDCK cells. Quiescent MDCK cells were treated with 10 μ*M* FSK, 10 μ*M* Lix, or 10 μ*M* Rp for 5 mins, lysed, and immunoprecipitated with anti-Raf-1 or anti-B-Raf antibodies. The Raf-1 and B-Raf kinase activity in immunoprecipitates was measured *in vitro* using Raf-1 and B-Raf Kinase Cascade Assay Kits (Upstate Biotechnology), according to the manufacturer's instructions. Values represent means ± SE ($n = 3$). *$P < 0.05$ vs. control.

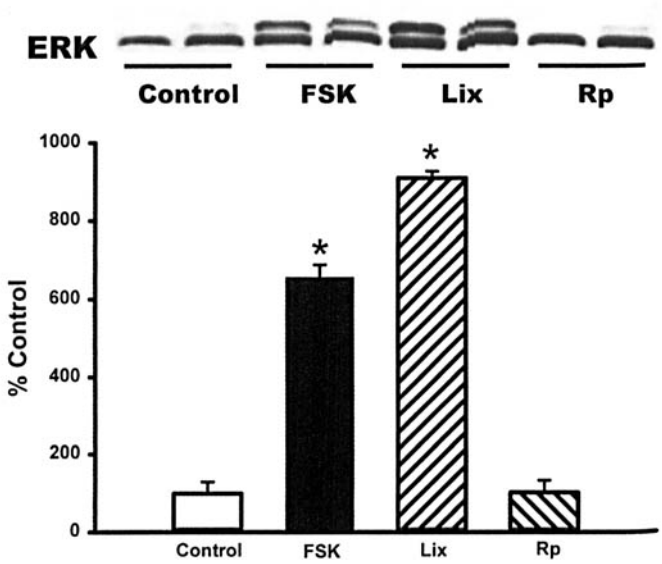

Figure 4. PDE3 inhibitors activate ERK kinase. Quiescent MDCK cells were treated with 10 μ*M* FSK, 10 μ*M* Lix, or 10 μ*M* Rp for 1 hr, and p44/42 MAP Kinase Assay Kits (Cell Signaling Technology) were used to measure ERK kinase activity, according to the manufacturer's instructions. Values represent means ± SE ($n = 3$). *$P < 0.05$ vs. control.

MDCK cells express both B-Raf and Raf-1, as assessed by Western blotting. Neither PDE3 nor PDE4 inhibitors alter B-Raf or Raf-1 levels (Fig. 3A). However, the mitogenic effect of lixazinone and forskolin on MDCK cells is associated with a significant induction of B-Raf activity, as assessed by an *in vitro* kinase assay (Fig. 3B). Rolipram, which had no effect on MDCK mitogenesis, had no effect on B-Raf activity. Neither lixazinone nor rolipram altered Raf-1 kinase activity (Fig. 3B).

PDE3 Inhibitors Stimulate MDCK Proliferation Through Activation of the ERK Pathway. We next sought to determine whether lixazinone or rolipram differentially modulated the ERK signaling pathway. As expected, lixazinone stimulated ERK activity (+810%) and rolipram had no significant effect on ERK activity (Fig. 4). The ERK inhibitor U0126 strongly blocked both basal and lixazinone-stimulated thymidine uptake by MDCK cells (Fig. 5). Based on these findings, we conclude that the ERK pathway may play a role in regulation of MDCK mitogenesis.

PDE3 and PDE4 Inhibitors Differentially Regulate Cell Cycle-Regulatory Proteins. Cell cycle progression is directed via sequential activation of the G1 cyclins D and E (24, 25). We therefore sought to determine whether lixazinone or rolipram differentially regulated G1 cyclin activity. Lixazinone stimulated both cyclin D (+83%) and cyclin E (+58%) activity to a similar extent with forskolin (+98% for cyclin D, +59% for cyclin E), as

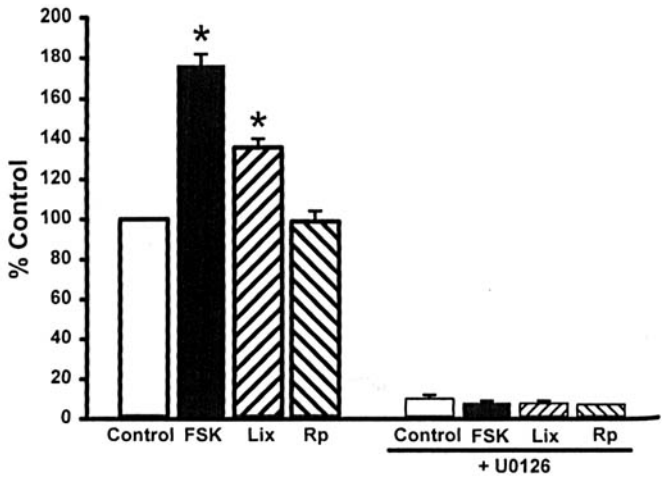

Figure 5. The ERK signaling pathway is involved in PDE3 inhibitor-stimulated MDCK proliferation. Quiescent MDCK cells were treated with the ERK inhibitor U0126 (25 μ*M*) for 30 mins before addition of 10 μ*M* FSK, 10 μ*M* Lix, or 10 μ*M* Rp for 72 hrs. [^{3}H]-thymidine incorporation was measured using liquid scintillation counting. Values represent means ± SE ($n = 3$). *$P < 0.05$ vs. control.

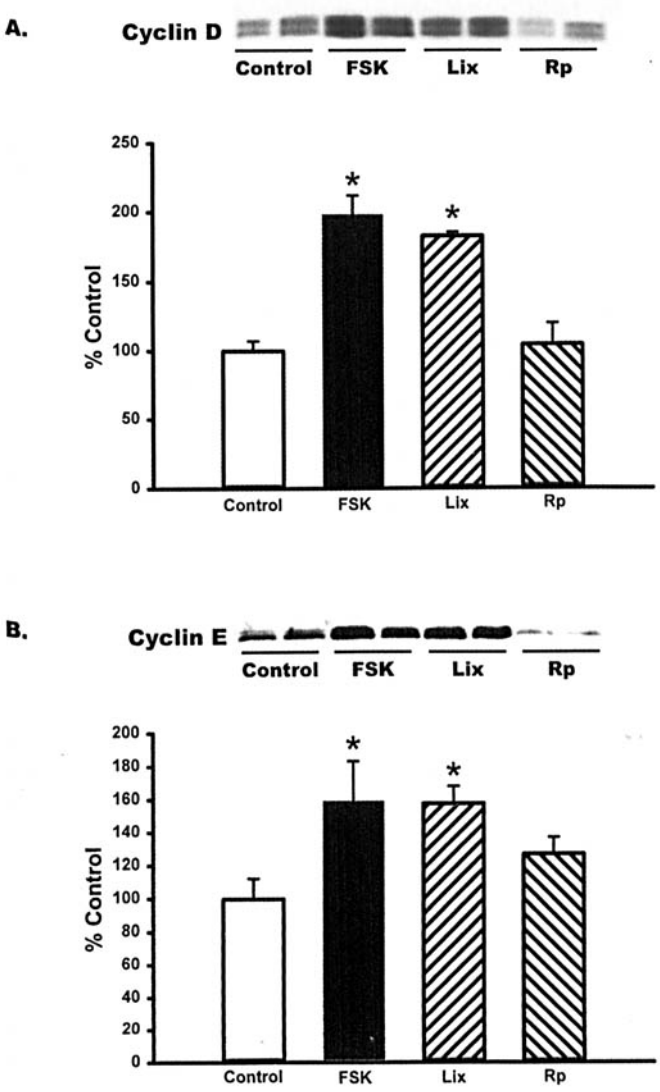

Figure 6. PDE3 and PDE4 inhibitors differentially regulate cell cycle-regulatory proteins. Quiescent MDCK cells or MCs were treated with 10 μM FSK, 10 μM Lix, or 10 μM Rp for 4 hrs. Equal amounts of lysate protein (200 μg) were immunoprecipitated with antibodies specific for cyclin D1 and cyclin E. Cyclin D kinase (A) and cyclin E kinase (B) activity were measured by histone H1 kinase assay. Values represent means ± SE ($n = 3$). $^*P < 0.05$ vs. control.

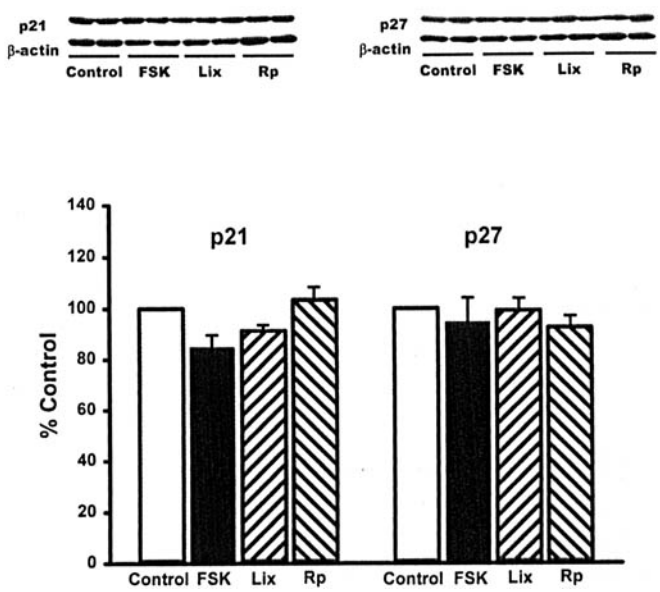

Figure 7. Neither PDE3 nor PDE4 inhibitors alter cell cycle-inhibitory proteins p21 or p27 expression. Quiescent MDCK cells were treated with 10 μM FSK, 10 μM Lix, or 10 μM Rp for 8 hrs. p21 and p27 levels were assessed by Western blot analysis. Values represent means ± SE ($n = 3$). $^*P < 0.05$ vs. control.

assessed by histone H1 kinase assay. However, rolipram had no significant effect on cyclin D or cyclin E kinase activity (Fig. 6A and B). Based on these considerations, we conclude that lixazinone promotes cell cycle progression through activation of cyclin D and cyclin E kinase activity.

PDE inhibitors do not alter cell cycle-inhibitory proteins p21 and p27 expression. Activity of cyclin-cdk complexes is regulated by cdk inhibitor proteins that bind to and inhibit cyclin-cdk activity. We previously reported that in MCs, the suppressive effect of lixazinone on proliferation is, at least in part, through upregulation of p21 (17). p21 and p27 levels in MDCK cells were assessed by Western blot analysis. Neither rolipram nor lixazinone significantly altered expression of p21 or p27 (Fig. 7).

Discussion

It is recognized that the effects of cAMP agonists on mitogenesis is cell type–specific (26). Nonhydrolizable analogs of cAMP or stimuli of adenylate cyclase inhibit proliferation of MCs (27, 28), fibroblasts (29), and vascular smooth muscle cells (27). We have previously demonstrated that the PDE3 inhibitors lixazinone and cilostazol, but not the PDE4 inhibitor rolipram, suppress folic acid induced proliferation of rat tubular epithelial cells *in vivo* (16). However, adenylate cyclase agonists such as forskolin, 8-bromo-cAMP, arginine vasopressin, prostaglandin E_1, adrenomedullin, and non-specific PDE inhibitors (1-methyl-3-isobutylxanthine, caffeine, and theophylline) stimulate proliferation of tubular epithelial cells obtained from patients with PKD (30–33) and MDCK cells (6, 30), which were used as an *in vitro* model of cystogenesis.

In our current studies, we found that cAMP hydrolysis in MDCK cells is directed primarily by PDE4, with only 15% of total cAMP-PDE activity attributable to PDE3. In MDCK cells, PDE4 inhibitors are more effective than PDE3 inhibitors in promoting intracellular cAMP accumulation and activating PKA. However, only PDE3, and not PDE4, inhibitors stimulate thymidine uptake. In normal tubular epithelial cells, only 21% of total cAMP-PDE activity is attributable to PDE3, and in cultured MCs, only 33% of total cAMP PDE activity is directed by PDE3 (16, 28). Nevertheless, in both cell types, only PDE3, and not PDE4, inhibitors suppress thymidine uptake (16, 28). Furthermore, we have previously demonstrated that an intracellular pool of cAMP directed by PDE4 suppresses reactive oxygen species generation in cultured MCs (15). These studies provide strong support for the notion that mammalian cells

contain functionally compartmentalized intracellular pools of cAMP that are differentially regulated by PDE isoforms. We demonstrate that mitogenesis, in different cell types, may be positively or negatively regulated by an intracellular pool of cAMP directed by PDE3. A potential role for PDE3 in regulation of mitogenesis of nonrenal cell types has yet to be established.

We demonstrate that both positive and negative regulation of mitogenesis by PDE3 inhibitors occurs through crosstalk with the Ras-Raf-MEK-ERK signaling pathway. Both B-Raf and Raf-1 are expressed in MDCK cells. The proliferative effect of PDE3 inhibitors on MDCK cells is associated with activation of B-Raf, not Raf-1. We have previously demonstrated that the suppressive effect of PDE3 inhibitors on mitogenesis of MCs is associated with PKA-dependent phosphorylation of Raf-1 on the inhibitory sites serine 43 and serine 259 and with reduced phosphorylation on serine 338, a site associated with Raf-1 activation (17). PDE4 inhibitors had no effect on Raf-1 phosphorylation (17). Structurally distinct PDE3 inhibitors stimulate mitogenesis of MDCK cells, indicating that this effect is not caused by a nonspecific action of lixazinone on B-Raf signaling. The suppressive effect of PDE3 inhibitors on MC proliferation is associated with inhibition of both Raf-1 and B-Raf kinase activity (17). It has been postulated that the relative expression of B-Raf versus Raf-1 may dictate whether cAMP stimulates or inhibits mitogenesis (34). In some cell types expressing B-Raf as the predominant Raf isoform, cAMP stimulates mitogenesis through stimulation of the ERK pathway (35, 36). The ability of cAMP to stimulate mitogenesis may be related to relative levels of B-Raf and Raf-1 or to the ability of B-Raf to form complexes with docking proteins (9, 37). We demonstrate that, in different cell types, an intracellular pool of cAMP directed by PDE3 may positively or negatively regulate mitogenesis through modulation of B-Raf and Raf-1 activity (17). Further studies are needed to define potential mechanisms underlying this specific effect of PDE3 but not PDE4 inhibitors on mitogenesis of MDCK cells and other cell types.

We found that PDE3 inhibitors stimulated the ERK pathway. Specific ERK inhibitors blocked basal and PDE3 inhibitor–stimulated thymidine uptake by MDCK cells, indicating that the ERK signaling pathway is involved in PDE3 inhibitor-stimulated proliferation of MDCK cells. Potential sites of crosstalk between the cAMP-PKA pathway await further definition.

In addition to interacting with the MAPK pathways, studies have shown that cAMP agonists may regulate proliferation through other pathways, including cell cycle proteins (17, 38–40). During mitogenesis, G1 to S phase transition is regulated through activation of cyclin D, which is activated in the early to middle part of G1, and cyclin E, which is activated in mid-G1 (41, 42). We demonstrate that, in MDCK cells, PDE3 but not PDE4 inhibitors activate both cyclin D and cyclin E, as assessed by histone H1 kinase assay. In MCs, the inhibitory effects of PDE3 inhibitors are associated with suppression of cyclin D and cyclin E kinase activity (17).

Mitogenesis is negatively regulated by specific cell cycle inhibitor proteins. The cell cycle inhibitor p27 is highly expressed in quiescent MCs (43) and p27 levels decline when MCs undergo proliferation (44). Quiescent MCs express low levels of the cell cycle inhibitor p21 (43). However, p21 appears to limit the proliferative response of some renal cell types to injury (45). p21 may protect cells against apoptotic cell death by preventing caspase 3 activation (46). We found that cultured MDCK cells express both p21 and p27. Neither rolipram nor lixazinone affected expression of p21 or p27 in MDCK cells.

In summary, we demonstrate that cAMP hydrolysis in MDCK cells is directed by PDE4 and, to a much lesser extent, PDE3. However, PDE3 but not PDE4 inhibitors stimulate mitogenesis of MDCK cells through activation of B-Raf and ERK. Cyclin D and cyclin E kinase activation are also involved in PDE3 inhibitor-stimulated MDCK cell proliferation. MDCK cells, like renal tubular epithelial cells derived from patients with PKD, proliferate in response to cAMP agonists. We propose that therapeutic intervention directed toward augmentation of PDE3 activity may inhibit the aberrant proliferative activity of tubular epithelial cells in PKD and that MDCK cells may provide a model system for testing this hypothesis.

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