Antagonism of endogenous putative P2Y receptors reduces the growth of MDCK-derived cysts cultured in vitro

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P2Y receptor agonists results in the hydrolysis of phosphoinositides (46), activation of MAP kinase and phospholipases (23), alteration in the uptake and release of arachidonic acid (47), and stimulation of cAMP formation by adenylyl cyclase (AC) (35). In these cultured cells, cAMP regulates Cl− ion secretion, as well as Na+−K+−ATPase activity (33, 34). When suspended in collagen gel and in the presence of the cAMP-forming AC stimulant forskolin, MDCK type 1 cells rapidly proliferate and form rounded epithelial microcysts (10, 17). The cells are polarized, with their apical cell surface facing the lumen of microcysts and their basolateral surface having direct access to the collagen gel (10). We have added P2 receptor antagonists to the extracellular collagen matrix and exploited to determine whether the long-term presence of these agents in the cyst lumen could interrupt ATP-mediated signaling at the apical membrane of these cells and alter the rate of microcyst formation and expansion. We describe the effects of various P2 receptor antagonists on the rates of type 1 MDCK cyst growth in culture.

METHODS

Cells and cell culture. Type 1 MDCK cells were generously provided by Professor D. Sheppard (University of Bristol, UK). These cells possess cAMP-stimulated apical membrane Cl− ion channels (19). Type 1 MDCK cells were cultured in MDCK medium (a 1:1 mixture of DMEM and Ham's F-12 nutrient medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin; all from Life Technologies, Paisley, UK) at 37°C in a humidified atmosphere of 5% CO2.

Cyst growth. To grow cysts, MDCK cells were cultured in collagen gels in the presence of the cAMP-generating agent forskolin using a modification of the method of Grantham and colleagues (10). Cells were separated using 0.25% (wt/vol) trypsin for 30 min at 37°C, diluted with MDCK medium to form a suspension of 2 × 10⁶ cells/ml, and then aliquoted into individual wells of a 24-well plate (0.1 ml/culture. Reagents. Forskolin, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), RB2 (reactive blue 2), Coomassie brilliant blue G (BBG), suramin, 3′-O-(4-benzoyl)benzoyladenosine-5′-triphosphate (BzATP), adenosine 5′-O-[3-thio-triphosphate] (ATPγS), adenosine 5′-O-[2-thiodiphosphate] (ADPβS), 2′-deoxy-N6-methyladenosine-3′,5′-bisphosphate (MRS 2179), zinc chloride, and apyrase were purchased from Sigma-Aldrich (Poole, Dorset, UK); PD98059 and U0126 were purchased from Calbiochem (Nottingham, UK). Stock solutions were prepared by diluting powdered reagents with distilled water, except forskolin, which was dissolved in ethanol; PD98059 and U0126 were dissolved in DMSO. All were stored at −20°C. Stock solutions were diluted in MDCK medium to achieve final concentrations immediately before use. Precautions against light-sensitive breakdown were observed when using PPADS.

Cell viability assay. MDCK cells were seeded onto 96-well plates at a density of 5 × 10⁴ cells/well and cultured overnight at 37°C in a humidified atmosphere of 5% CO2. The following day, after MDCK cells had adhered to the plate, zinc chloride was added to the MDCK media to achieve final concentrations over the range 100 μM-100 mM. MDCK cells were incubated with zinc chloride for 2 days, and then a cell titre-glo cell viability assay (Promega, Southampton, Hampshire, UK) was used following the manufacturer’s instructions. Luminescence was detected using a Luminescence plate-reader spectrometer LS50B (PerkinElmer, Beaconsfield, Bucks, UK).

Immunoblotting. MDCK cysts were harvested from collagen gels by centrifugation (12,000 g, 5 min) and then washed three times in Dulbecco’s PBS (Life Technologies, Paisley, UK). The resulting pellet was resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS with 10% protease inhibitors, Sigma-Aldrich), and 0.2 mM dithiothreitol (Promega). Protein concentration was determined by spectrophotometry. Fifty micrograms of protein were electrophoresed on 12% SDS-PAGE gels and then transferred to Hybond ECL-nitrocellulose membrane (Amersham Biosciences, Buckingham, UK) using a Bio-Rad semidry transfer apparatus. Membranes were blocked with 3% milk in PBS containing 0.05% Tween 20 for 1 h and probed overnight with either anti-ERK1/2 (Cell Signaling Technology) or anti-phospho-ERK1/2 antibody (Cell Signaling Technology) diluted in 0.01% milk in PBS. A peroxidase-linked donkey anti-rabbit IgG and visualizer Western blot detection kit (Upstate Cell Signaling Solutions, Dundee, UK) were used, and images were captured using a Bio-Rad Multi-imager (Bio-Rad, Hemel Hempstead, Herts, UK). Immunoblotting and immunohistochemistry were tried with anti-human and anti-rat P2 receptor antibodies in an attempt to identify P2 receptor protein in these MDCK cells, but unfortunately there was no cross-reactivity of these antibodies with canine P2 receptors.

RT-PCR. MDCK cysts were harvested from collagen gels by centrifugation (12,000 g, 5 min) and then washed three times in Dulbecco’s PBS (Life Technologies). The resulting pellet was resuspended in TRizol reagent (Invitrogen, Paisley, UK) and passed through a pipette several times to ensure a homogeneous suspension. RNA was extracted using TRizol/chloroform extraction and isopropyl alcohol precipitation. The final pellet was air dried and resuspended in RNAse-free distilled water. RNA concentration and purity were determined by spectrophotometry. One microgram of total RNA was reverse transcribed with 0.5 μg oligo(dT)-18 primer and superscript II RNAse H− reverse transcriptase using the manufacturer’s protocol (Invitrogen).

Copy DNA transcripts were used as a template with the PCR Core System I (Promega). Each PCR reaction contained 5.0 pmol of forward primer, 5.0 pmol of reverse primer 1.5 mM MgCl2, 500 μM each of dATP, dCTP, dGTP, dTTP, 0.5 units of Taq polymerase, and
1× PCR buffer in a 20-μl reaction. The cycling parameters were initial denaturing at 95°C for 3 min, 30 cycles of denaturing at 95°C for 30 s, annealing for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min using a Hybaid PCR Sprint thermal cycler (Hybaid, Middlesex, UK). See Table 1 for primer sequences and sequence accession numbers.

Statistics. Results are expressed as means ± SE of n observations for a total of three experiments per treatment. One-way ANOVA was used to compare data sets, and differences were considered statistically significant if P < 0.05.

RESULTS

In all experiments (unless stated otherwise), established cysts were exposed to either an antagonist or agonist of P2 receptors for a continuous period that started at day 6 of culture and extended to day 12. Photographs (Fig. 1) and measurements of cyst size were taken at 3-day intervals until day 12, when experiments were terminated.

Effects of P2 receptor antagonists. As the first step in investigating the role of P2 receptors on renal cyst growth, we determined the ability of different P2 receptor antagonists to influence cyst size. Most antagonists at a concentration of 100 μM reduced cyst growth rate from a control mean expansion rate of 1.06 ± 0.07 nl/day (n = 77) to a minimum expansion rate of 0.35 ± 0.08 nl/day (n = 40), although it was not possible to entirely arrest cyst enlargement (Fig. 2). The rank order of potency was RB2 > suramin > PPADS > MRS2179 > BBG. In nontreated (forskolin-only) cysts, a growth rate of 1.06 ± 0.07 nl/day was recorded. Incubation of MDCK cysts with 100 μM RB2 consistently diminished the rate of cyst growth by 68% to 0.35 ± 0.08 nl/day (P < 0.001) (Fig. 2). Suramin (100 μM) also reduced cyst growth rate by 51% to 0.53 ± 0.07 nl/day (P < 0.001). PPADS (100 μM) reduced cyst growth rate to 0.74 ± 0.11 nl/day (P = 0.01), and the P2Y1-selective antagonist MRS 2179 reduced cyst growth rate to 0.76 ± 0.12 nl/day (P = 0.05) (Fig. 2). BBG (100 μM) had no significant effect on the rate of cyst growth. Since the most potent antagonists were RB2 and suramin, we exposed established cysts to either an increased concentration of these antagonists (1 mM) or to a combination of 100 μM RB2 and 100 μM suramin (Fig. 3A). In this set of experiments, untreated (forskolin-only) cysts, a growth rate of 1.06 ± 0.07 nl/day was recorded. Incubation of MDCK cysts with 100 μM RB2 consistently diminished the rate of cyst growth by 68% to 0.35 ± 0.08 nl/day (P < 0.001) (Fig. 2). Suramin (100 μM) also reduced cyst growth rate by 51% to 0.53 ± 0.07 nl/day (P < 0.001). PPADS (100 μM) reduced cyst growth rate to 0.74 ± 0.11 nl/day (P = 0.01), and the P2Y1-selective antagonist MRS 2179 reduced cyst growth rate to 0.76 ± 0.12 nl/day (P = 0.05) (Fig. 2). BBG (100 μM) had no significant effect on the rate of cyst growth. Since the most potent antagonists were RB2 and suramin, we exposed established cysts to either an increased concentration of these antagonists (1 mM) or to a combination of 100 μM RB2 and 100 μM suramin (Fig. 3A). In this set of experiments, untreated (forskolin-only) cysts, a growth rate of 0.84 ± 0.12 nl/day (n = 27) (Fig. 3A). The combination of RB2 and suramin reduced cyst growth rate by 88% to 0.10 ± 0.01 nl/day (P < 0.001). However, a greater effect on cyst size was seen with both 1 mM RB2, when cyst growth rate was reduced by

![Fig. 1. Sample photomicrographs showing the progressive enlargement of single Madin-Darby canine kidney (MDCK) cysts cultured in control media (10 μM forskolin only; A), or in the presence of 100 μM reactive blue 2 (RB2) (B) or 100 μM suramin (C). Scale bars = 100 μm.](http://ajprenal.physiology.org/)}
products for metabotropic P2Y1 and P2Y2 receptors, showing RT-PCR which P2 receptors were expressed. We detected PCR P2Y receptors; based on available sequences, we checked by (Fig. 3A).

Increased expression in early-stage cysts, P2Y6, P2Y11 receptors, and ionotropic P2X7 receptors (Fig. 4).

It has been reported previously that MDCK cells express P2Y receptors; based on available sequences, we checked by RT-PCR which P2 receptors were expressed. We detected PCR products for metabotropic P2Y1 and P2Y2 receptors, showing increased expression in early-stage cysts, P2Y6, P2Y11 receptors, and ionotropic P2X7 receptors (Fig. 4).

Effects of P2 receptor agonists. Since ATP and its derivatives could be broken down before affecting cyst growth, stable (nonhydrolyzable) analogs of ATP and ADP were used to examine the effects of P2 agonists on cyst growth. ATPγS (100 μM) or ADPβS (100 μM) was added to the medium on day 6; however, there was no significant change in the growth rate of MDCK cysts (Fig. 5). Nontreated cysts had a growth rate of 1.06 ± 0.07 nl/day compared with 1.12 ± 0.18 nl/day with ATPγS (n = 24) and 1.15 ± 0.18 nl/day with ADPβS (n = 29) (Fig. 5A). In addition, there was no cyst formation by MDCK cells when incubated with either ATPγS (100 μM) or ADPβS (100 μM) from day 0 to day 12 in the absence of forskolin (data not shown).

As mentioned previously, BzATP, originally thought to be a selective P2X7 receptor agonist, also antagonizes P2Y1 and P2Y4 receptors, as well as being a potent agonist at P2Y11 receptors (39, 42). At 100 μM, BzATP reduced cyst growth rate by 54.3% (to 0.49 ± 0.04 nl/day, n = 27, P < 0.001) (Fig. 5), whereas, at 1 mM, it reduced growth rate by 84% (to 0.13 ± 0.02 nl/day, n = 23) (P < 0.001) (Fig. 3A). Increasing concentrations of BzATP from 0.01 μM to 1 mM show a stepwise reduction in cyst growth rate (Fig. 5B).

Extracellular ATP is required for MDCK cyst growth. The ATPase, was used to test whether cyst growth depended on the presence of extracellular ATP. In this set of experiments, untreated cysts had a mean growth rate of 0.67 ± 0.01 nl/day (n = 36) (Fig. 6). In parallel with untreated cysts, established cysts were first treated with 10 U of apyrase/well from day 6 to day 12 (n = 13), where media containing apyrase (10 U) was replaced every 2 days. However, this caused no significant reduction in MDCK cyst growth. Treatment of established cysts (n = 20) with a higher concentration of apyrase (20 U) from day 6 to day 12 did reduce cyst growth rate by 46% (P = 0.01), with a mean growth rate of 0.36 ± 0.01 nl/day (Fig. 6). Furthermore, 10 U of apyrase/well from day 0 for 12 days, with replacement of the medium and enzyme every 2 days, reduced 91% to 0.07 ± 0.02 nl/day (P < 0.001), and with suramin (1 mM), which reduced cyst growth rate by 84% to 0.13 ± 0.02 nl/day (P < 0.001).

An inhibitory action of Zn2+ ions has been described for nucleotide responses at P2Y2 and P2Y4 receptors (42). Similarly, BzATP has been shown to act as an antagonist at P2Y1 (39) and P2Y4 receptors (42); therefore, both substances were tested for their effects on cyst growth. Zn2+ ions (1 mM) reduced cyst growth rate by 95% (to 0.04 ± 0.01 nl/day, n = 27) (P < 0.001) (Fig. 3A). Cyst size on day 12 was reduced from an untreated control cyst size of 5.59 ± 0.73 to 0.86 ± 0.12 nl (Fig. 3A). At this concentration, 1 mM zinc is not toxic to MDCK cells (Fig. 3B). BzATP (1 mM) reduced cyst growth rate by 84% (to 0.13 ± 0.02 nl/day, n = 23) (P < 0.001) (Fig. 3A).

It has been reported previously that MDCK cells express P2Y receptors; based on available sequences, we checked by RT-PCR which P2 receptors were expressed. We detected PCR products for metabotropic P2Y1 and P2Y2 receptors, showing increased expression in early-stage cysts, P2Y6, P2Y11 receptors, and ionotropic P2X7 receptors (Fig. 4).
cyst size by 51% to a growth rate of 0.33 ± 0.01 nl/day (P = 0.01).

**Active ERK is required for MDCK cyst enlargement.** Activation of P2Y receptors can increase intracellular Ca\(^{2+}\) concentration and stimulate cAMP production, which are important signaling events for the ERK cascade. Postulating that activation of ERK is required for growth of MDCK-derived cysts, cysts were grown in the presence of either PD98059 or U0126 (Fig. 7A), inhibitors of MEK, which is an upstream activator of ERK. For this set of experiments, untreated (forskolin-only) cysts had a mean growth rate of 1.26 ± 0.16 nl/day (n = 28). The presence of inhibitors of ERK activation caused a 90% reduction in cyst growth: PD98059 reduced the growth rate to 0.12 ± 0.02 nl/day (n = 36) and U0126 reduced the growth rate to 0.13 ± 0.02 nl/day (n = 41 P < 0.001).

These inhibitors were dissolved in DMSO, which had no significant effect on cyst growth.

We examined the expression of active (phosphorylated) ERK in cultured MDCK cells grown as a proliferating monolayer, and in MDCK-derived cysts on days 6, 9, and 12. Immunoblot analysis of cell lysates using antibodies against phosphorylated ERK showed that its abundance increased in proliferating MDCK cells and in MDCK cysts, which continued to increase over the time course of the experiment up to day 12 compared with total ERK (Fig. 7B).

**DISCUSSION**

We have confirmed that MDCK cells form rounded cysts when grown in collagen gel and stimulated with forskolin, as
growth. Furthermore, increased intracellular Ca\(^{2+}\) retention and cell proliferation, both of which augment cyst size (8). Our main findings are also activate Ca\(^{2+}\) and subsequent Ca\(^{2+}\) release from intracellular stores, may also activate Ca\(^{2+}\)-sensitive Cl\(^{-}\) channels and Cl\(^{-}\) secretion (8). Our main findings are 1) the rate of cyst growth was inhibited significantly when relatively unselective P2Y receptor antagonists were added to the growth medium, or when locally released ATP was removed from the growth medium by adding the ATP-hydrolyzing enzyme apyrase; and 2) MDCK cysts express high levels of phosphorylated ERK and cyst growth can be significantly reduced by selective inhibitors of ERK activation. These observations suggest that endogenous P2 receptors may be involved in the control of cyst growth and expansion in this cell model of renal cyst formation.

A variety of P2 receptor subtypes are expressed along the normal rat renal tubule (2, 3, 37) and are also expressed in the cyst-lining cells of the Han:SPRD cy/\(^{+}\) rat (36). Moreover, mRNA transcripts for P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 receptors are also known to be expressed in cultured MDCK cells (7, 25). We have identified mRNA transcripts for P2Y1, P2Y2, P2Y6, P2Y11, and P2X7 receptors, although we were limited by the availability of canine P2 receptor sequence information on P2Y4 and most P2X receptors, which may have escaped detection in our studies. Reports of P2X receptor expression in MDCK cells are generally lacking, but given our finding of P2X7 receptor mRNA, and from observations in other renal epithelia, it is likely that MDCK cells do express multiple P2X receptor subtypes on both apical and basolateral cell membranes (30). However, the role of P2X receptors may be less significant, because the broad-spectrum P2X receptor antagonist BBG had no inhibitory effect on cyst growth. It seems unlikely that P1 receptors are expressed by MDCK cells, because the application of adenosine to the apical or basal surfaces of MDCK cells in cultured monolayers failed to increase short-circuit current (\(I_{sc}\)) (6). Thus, for the purpose of this study, the predominant P2 receptor population is probably represented by the P2Y receptor subtypes found in MDCK cells.

In the present study, we added either antagonists or agonists to the extracellular medium. RB2 and suramin were the most potent of the P2 receptor antagonists at reducing cyst growth, but these two compounds are nonspecific antagonists of the P2Y and P2X class of receptors and provide no clue as to their target receptor in MDCK cells. RB2 is an inhibitor of recombinant P2Y1, P2Y4, P2Y6, and P2Y11 receptors, and suramin is an inhibitor of recombinant P2Y1, P2Y2, and P2Y11 receptors (40). Suramin has also been shown to block ADP\(_{rS}\)-stimulated cAMP production in MDCK-D1 cells (a subclone of the parent strain used in our study) via P2Y1 receptor inhibition (35). However, suramin is an inhibitor of G\(_{i}\) protein \(\alpha\)-subunits (12) and, where cAMP-dependent ion channels are involved, the results obtained with this compound must be treated cautiously. RB2 is not known to inhibit G\(_{i}\) and has previously been reported to inhibit ATP-induced \(I_{sc}\) in MDCK cells (48). In contrast to the inhibitory actions of RB2 and suramin, the antagonist BBG (Coomassie brilliant blue G) was ineffective in reducing cyst growth in MDCK-cultured cells. This compound is a nonselective antagonist at P2X receptors and at the concentration we used should block completely the nondesensitizing P2X2, P2X4, P2X5, and P2X7 receptor subtypes (5, 14). Moreover, the P2X receptor agonist \(\alpha\)-\(\beta\)-methylATP (meATP), which potently activates fast-desensitizing P2X1 and P2X3 receptor subtypes and weakly activates the P2X2,4,5,6.
receptor subtypes, is reported to have no effect on $I_{\text{sc}}$ in MDCK cell monolayers (48). This observation, together with the lack of an effect of BBG in reducing the size of MDCK cysts, seems to indicate that P2X receptors are not functionally important in MDCK cells and that the antagonists that reduce cyst growth (including RB2 and suramin) target principally P2Y receptor subtypes. Based on this interpretation, we tried more selective P2Y receptor antagonists. The P2Y1 receptor antagonists MRS 2179 and PPADS each inhibited cyst growth to the same extent and at concentrations that block fully the recombinant P2Y1 receptors (40). However, MRS 2179 and PPADS were approximately twofold less effective at reducing the size of MDCK cultured cysts compared with RB2 and suramin used in equivalent concentrations, suggesting that more than just the P2Y1 receptor is involved. Interestingly, the P2Y1 and P2Y4 receptor antagonist BzATP was as effective as RB2 or suramin at reducing cyst growth, and twice as effective as MRS 2179 and PPADS. The increased inhibitory action of BzATP compared with MRS 2179 and PPADS support involvement of P2Y1 and P2Y4 receptor subtypes. Further evidence for involvement of multiple P2Y receptor subtypes comes from the action of extracellular Zn$^{2+}$, which inhibits recombinant P2Y2 and P2Y4 receptors, and inhibited MDCK cyst growth to the same extent as RB2 and suramin (at equimolar concentrations). However, it
was not possible to determine whether zinc had other pharmacological effects on MDCK cells, apart from its inhibitory action on P2Y2 and P2Y4 receptors, although it was clearly not toxic at the concentration we used. Indeed, the inhibitory activity of Zn2+ ions (used to target P2Y2 and P2Y4 receptors) matched the inhibitory effect of BzATP (used to target P2Y1 and P2Y4). This suggested to us that a combination of P2Y1, P2Y2, and P2Y4 receptors is involved in reducing cyst growth. The same conclusion can be drawn from the inhibitory action of RB2 (active at P2Y1 and P2Y4) and suramin (active at P2Y1 and P2Y2), which produced similar reductions in cyst growth. In addition to these P2Y receptor subtypes, the possibility of involvement of P2Y6 and P2Y11 receptors, which are also present in MDCK cells (7, 25), could be neither confirmed nor excluded.

It is worth noting that inhibition of ERK activation and inhibition of P2Y1, P2Y4, P2Y6, and P2Y11 receptors with RB2 and suramin were equally effective in reducing cyst growth. Activity of ERK has previously been linked to tubular cell proliferation (16), and ERK is upregulated in the Han:SPRD rat model of ADPKD (20). Furthermore, both ATP and UTP are potent activators of ERK in human intestine (38), and P2Y receptor-mediated activation of ERK via a cAMP-dependent pathway has also been shown to increase proliferation of dendritic cells (21). In cultured normal human kidney cells, cAMP inhibits the ERK signaling pathway, but in cultured ADPKD cells derived from cysts it stimulates this pathway (45). The signal that switches cAMP from a nonmitogenic to a mitogenic stimulus in ADPKD cells is unknown.

To complement the antagonist studies, the actions of metabolically stable analogs of ATP were also investigated. The phosphorothioate derivatives ADPβS and ATPγS were selected as slowly metabolized ATP agonists, because they are highly resistant to breakdown by ectoATPases. Although ADPβS activates P2Y1 and P2Y11 receptors, and ATPγS activates P2Y1, P2Y2, P2Y4, and P2Y11 receptors, neither of these ATP analogs stimulated (or inhibited) the rate of cyst growth. Similarly, BzATP, which is an agonist at P2Y2 and P2Y11 receptors, failed to augment cyst growth, although it did display inhibitory activity. The inhibitory effect of BzATP suggested that it could readily access the P2Y receptor population expressed by MDCK cysts and that this would also have been true for ATPγS and ADPβS. However, the inability of ATPγS and ADPβS to stimulate cyst growth might indicate that locally released ATP is already maximally stimulating the P2Y receptor population. This possibility is consistent with the observation that addition of the ATP-degrading enzyme apyrase to the growth medium significantly reduced cyst size in forskolin-stimulated MDCK cells.

The mechanism of local ATP release in MDCK cells is unknown, although hydrostatic pressure is known to release ATP from the epithelium of urinary bladder (9) and ureters (15) and from other types of epithelia in response to mechanical distortion (27). If similar pressure-sensitive mechanisms occur in the apical membrane of MDCK cells, and the presence of TRP channels such as the polycystin proteins, TRPP1 and TRPP2 (29) and mechanosensory TRPC channels (4) in MDCK cells would support such mechanisms, the formation of cysts by luminal secretion of Cl− ions and fluid accumulation may lead to local release (apically and/or basolaterally) of ATP and autocrine or paracrine activation of P2Y receptors, resulting in positive feedback. This is consistent with the finding that

Fig. 6. ATP depletion reduced growth rate of MDCK microcysts. ATP was removed from the media bathing the cysts using apyrase from day 0 or from day 6. Apyrase was added to the culture medium, which was replaced every 2 days until day 12, when the experiment was terminated (n = 13–36). 10U apyrase from day 0 and day 6; 10U of apyrase from day 0 and day 6 onward, respectively, 20U apyrase from day 6; 20U of apyrase from day 6 onward. *P = 0.01.
20 U of apyrase were needed to reduce growth of established cysts (day 6), whereas 10 U had little effect, perhaps because ATP release exceeded ATP breakdown.

**Conclusion.** In summary, our results suggest an important role for P2Y, rather than P2X, receptor subtypes in cyst formation by MDCK cells, but that the agonists and antagonists currently available cannot ascribe this to one or more specific P2Y receptor subtypes. It is known that MDCK cells can express several P2Y receptor subtypes (7, 25); the effect of P2Y receptor antagonism on cyst growth as well as the effect of removing ATP with apyrase suggest that cyst expansion is stimulated and/or maintained by ATP acting via P2Y receptors. The ATP may be released by stretch or cell damage to activate these P2Y receptors, which may then stimulate Cl⁻ and fluid secretion. If ATP signaling and P2Y receptor activity are important in cyst development in this model, they could have more general therapeutic potential in preventing or slowing renal cyst growth.

Fig. 7. A: inhibition of the ERK pathway using PD98059 or U0126 significantly reduced cyst growth. ERK pathway inhibitors were added to the culture medium on day 6, and medium was replaced every 2 days until day 12, when the experiment was terminated. PD98059 or U0126 was dissolved in DMSO, but an equivalent volume of DMSO with no dissolved inhibitors added to the culture medium had no effect on growth of MDCK microcysts (n = 28-41). **P < 0.001. B: MDCK microcysts were harvested from collagen gels by centrifugation, and in the presence of active (phosphorylated)-ERK1/2 compared with total ERK1/2 was measured by immunoblot. Phospho-ERK1/2 activity increased over the time course on days 6 and 9 and was maximal on day 12 compared with nonphosphorylated ERK1/2.
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