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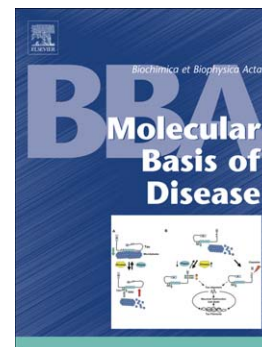
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Deficiency of polycystic kidney disease-1 gene (PKD1) expression increases A₃ adenosine receptors in human renal cells: implications for cAMP-dependent signalling and proliferation of PKD1-mutated cystic cells

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SUMMARY

Cyst growth and expansion in autosomal dominant polycystic kidney disease (ADPKD) has been attributed to numerous factors, including ATP, cAMP and adenosine signalling. Although the role of ATP and cAMP has been widely investigated in PKD1-deficient cells, no information is currently available on adenosine-mediated signalling. Here we investigate for the first time the impact of abnormalities of polycystin-1 (PC1) on the expression and functional activity of adenosine receptors, members of the G-protein-coupled receptor superfamily. Pharmacological, molecular and biochemical findings show that a siRNA-dependent PC1-depletion in HEK293 cells and a PKD1-nonsense mutation in cyst-derived cell lines result in increased expression of the A₃ adenosine receptor via an NFκB-dependent mechanism. Interestingly, A₃ adenosine receptor levels result higher in ADPKD than in normal renal tissues. Furthermore, the stimulation of this receptor subtype with the selective agonist CI-IB-MECA causes a reduction in both cytosolic cAMP and cell proliferation in both PC1-deficient HEK293 cells and cystic cells. This reduction is associated with increased expression of p21^{waf} and reduced activation not only of ERK1/2, but also of S6 kinase, the main target of mTOR signaling. In the light of these findings, the ability of CI-IB-MECA to reduce disease progression in ADPKD should be further investigated. Moreover, our results suggest that NFκB, which is markedly activated in PC1-deficient and cystic cells, plays an important role in modulating A₃AR expression in cystic cells.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary renal disorder (1 in 500-1000 people), and accounts for approximately 10% of all cases of ESRD [1]. It is characterized by progressive formation and enlargement of multiple fluid-filled cysts in both kidneys, and leads to renal failure in approximately 50% of patients in their 5th or 6th decade of life [1,2]. Genetically, ADPKD is caused by loss-of-function mutations in the PKD1 and PKD2 genes, which encode polycystin-1 (PC1) and polycystin-2 [3, 4]. PC1 is a large integral membrane protein required for activation of cation permeable currents [5, 6] and regulation of cell cycle [7], G-protein signaling [8, 9] and perhaps adenylyl cyclase (AC) activity [10], and mutations in the PKD1 gene account for the vast majority of ADPKD cases (~85%), which are those associated with a more severe clinical presentation [1, 2].

AC activity in PKD cells is highly relevant, as intrarenal production of 3':5'-cyclic monophosphate (cAMP) plays a major role in the pathogenesis of ADPKD by stimulating transepithelial secretion, accumulation of cystic fluid and cell proliferation [10]. In particular, the disruption of PC1 could result in enhancement of cAMP levels and induction of cystic cell proliferation via activation of the B-Raf/MEK/ERK pathway [11,12]. Consistently, administration of antagonists of receptors for AC agonists markedly inhibits disease development in ADPKD animal models [13, 14].

It has also been found that cAMP, when it reaches either the apical or basolateral membranes of proximal tubular cells, is converted in part to adenosine [15]. This nucleoside acts as a primordial signaling molecule, which, through the activation of specific surface G-protein-coupled receptors (GPCR) [16], modulates several processes, among which proliferation, survival and apoptosis [17], in numerous cell types, including kidney cells [18, 19].

Although adenosine signaling has already been proposed to be detrimental to ADPKD cyst growth and expansion [20], little is known about the role of adenosine receptors (AR) in the regulation of cAMP production, cAMP-related pathways or cell proliferation in ADPKD cystic cells.

We report here that PC1-depleted HEK293 cells and PKD1-mutated cystic cells express increased levels of AR type 3 (A₃AR), which is associated with, if not sustained by, increased activation of the transcription factor NF-κB. Interestingly, the stimulation of A₃AR with the specific agonist CI-IB-MECA causes a reduction in cell proliferation and in ERK1/2 and S6 kinase activities in cystic cells. These findings pave the way to future investigations into the role of CI-IB-MECA in preventing or slowing renal cyst growth in ADPKD animal models.

MATERIALS AND METHODS

Reagents

D-MEM/F12 and MEM media, Bovine Serum albumin (BSA), N⁶-(3-iodo-benzyl)2-iodo-benzyl-2-chloroadenosine-5'-N-methyluronamide (CI-IB-MECA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), N⁶-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA), 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 201712) and forskolin were purchased from Sigma (Milano, Italy). FBS, Glutamine and antibiotics were obtained from Eurobio (Celbio, Italy), and protease inhibitors from Roche Diagnostics (Monza, Italy). 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) was purchased from Tocris, Ltd, UK. 5-n-(4-methoxyphenyl-carbamoyl) amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine (MRE 3008F20) and N-benzo[1,3] dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]-acetamide (MRE 2029F20) were synthesized by Prof. P.G. Baraldi, Ferrara University, Italy. The radioligand [³H]-1,3-

dipropyl-8-cyclopentyl-xanthine ($[^3\text{H}]$ -DPCPX), specific activity 120 Ci/mmol, was obtained from NEN-Perkin Elmer Life and Analytical Sciences, USA. $[^3\text{H}]$ -4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolo[2,3-a][1,3,5] triazin-5-yl-amino]ethyl ($[^3\text{H}]$ -ZM 241385), specific activity 27.4 Ci/mmol, was obtained from American Radiolabeled Chemicals Inc, Saint Louis, MO, USA. $[^3\text{H}]$ -N-benzo [1,3[dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yl-oxy]-acetamide ($[^3\text{H}]$ -MRE 2029 F20), specific activity 123 Ci/mmol, and $[^3\text{H}]$ -5N-(4-methoxyphenylcarbamoyl) amino-8-propyl-2-(2-furyl) pyrazolo[4,3-e]-1,2,4-triazolo [1,5-c]pyrimidine ($[^3\text{H}]$ -MRE 3008F20), specific activity 67 Ci/mmol, were obtained from Amersham International Chemical Laboratories, Buckinghamshire, UK. Rabbit polyclonal anti-actin, anti- β -tubulin, anti-PC1, anti-A3AR and anti-SV40 Tag antibodies were obtained from Santa Cruz (DBA Italia Srl, Segrate, Italy), while rabbit polyclonal anti-S6 kinase, anti-pS6 kinase, Erk1/2 and pErk1/2 antibodies were obtained from Cell Signaling Technology (Celbio SRL, Italy). Enhanced chemiluminescent substrates for Western blotting (SuperSignalDura or SuperSignalFemto) and HRPconjugated goat anti-rabbit and anti-mouse antibodies were purchased from Pierce (Celbio Srl, Italy).

Cell lines and tissues

HEK293^{pSsiPKD1} and HEK293^{pSuper} cells, which were previously obtained by stable transfection of HEK293 cells with pSsiPKD1 vectors expressing PKD1 siRNA and the pSUPER empty vector, respectively [21], were cultured in MEM 10% FBS. HEK293^{pSsiPKD1} cells were repeatedly monitored for the extent of PC1 depletion, and two clones (a1 and b3) maintaining an approximate 85 % reduction in PC1 were used in the current studies (see the PC1 percent values in inset of Figure 1A and 4B). SV40-transformed human cell lines, one derived from normal (4/5) and two from cystic kidney cell lines [22] (all expressing comparable levels of T antigen, data not shown) were grown in Dulbeccos's modified Eagle's medium/F12, 10% FBS. Cystic cells carry the Q2556X PKD1 mutation: one is heterozygous (9.7), while the

other is hemizygous (9.12), for deletion of the normal allele [22]. Tissue samples of adult kidneys with normal histology were obtained from renal tumor nephrectomy patients (a male 54 yr old and a female 58 yr old). ADPKD kidneys were obtained from ADPKD patients (three males 53, 55 and 58 yr old and a female 55 yr old) who had the typical clinical manifestations and reached endstage renal disease, during transplant nephrectomy. The diagnosis was verified by anatomic examination. Explicit genotypic data were not available. The study protocol was in line with the ethical guidelines of the 1975 Declaration of Helsinki.

Saturation binding experiments to purinergic receptors

Cells were resuspended in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4), homogenized and centrifuged for 30 min at 100,000 g. The membrane pellet was resuspended in the buffer solution used in binding experiments, incubated with 2 IU/ml of adenosine deaminase for 30 min at 37°C and centrifuged for 30 min at 100,000 g. Finally, the membrane suspension (40-60 µg protein/assay) was used in experiments of saturation binding to A₁ A_{2A} A_{2B} and A₃ adenosine receptors that were performed by using [³H]-DPCPX, [³H]-ZM 241385, [³H]-MRE 2029 F20 and [³H]-MRE 3008F20, respectively, according to previously described methods [23].

Real time PCR

Total cytoplasmic RNA was extracted by the guanidinium thiocyanate phenol method, reverted with random primers and A₃ mRNA quantitative real-time RT-PCR assay was carried out using a gene-specific double fluorescently labeled TaqMan MGB probe (minor groove binder) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington Cheshire, UK) as previously reported [24]. The following primer and probe

sequences were used for real-time RT-PCR: A3 forward primer, 5'-ATG CCT TTG GCC ATT GTT G-3'; A3 reverse primer, 5'-ACA ATC CAC TTC TAC AGC TGC CT-3'; A3 MGB probe, 5'-FAM-TCA GCC TGG GCA TC-TAMRA-3' (Applied Biosystems, Monza, Italy). The endogenous control human β -actin kit was used for real-time RT-PCR of the reference gene, and the probe was fluorescent-labeled with VIC (Applied Biosystems). A₃R RNA quantification was obtained by the Δ CT method. Final results, expressed as *N*fold content in A₃AR gene expression relative to the ACTIN gene, were determined as follows: $N(\text{target}) = 2^{-\Delta\Delta\text{CT}}$, as previously described [25].

Measurement of cyclic AMP levels

Cells ($1-2 \times 10^6$) were suspended in 0.5 ml of Krebs Ringer phosphate buffer, containing 1.0 IU adenosine deaminase ml⁻¹, and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor, then pre-incubated for 10 min at 37°C. The cells were also incubated with forskolin (1 μ M) and/or Ro 20-1724 (0.5 mM) to evaluate the adenylyl cyclase activity. Subsequently the effect of typical adenosine agonists was studied by using CHA, NECA and CI-IB-MECA at different concentrations (1 nM-1 μ M) for a further 5 min. In addition, the effects of typical adenosine antagonists such as DPCPX (A₁), SCH 58261 (A_{2A}), MRE 2029F20 (A_{2B}), and MRE 3008F20 (A₃) were evaluated. The reaction was terminated by the addition of 6% trichloroacetic acid cold solution (TCA). The TCA suspension was centrifuged at 2,000 *g* for 10 min at 4°C and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay [26].

Cell cycle and proliferation assays

Cells were plated overnight in 24-well plates at density of 30,000 cells/ml in 10 % FBS-supplemented medium. After plating, cells were starved for 24 h in medium supplemented

with 0.4% BSA, and then cultured for 24 h and 72 h in medium containing 1 % FBS, alone or in combination with CL-IBMECA (1, 10, 100 and 1000 nM), or CL-IBMECA and MRE 8000F20 (1 μ M). MRE 8000F20 inhibitor were added 30 min before treatment with the A3AR agonist CL-IBMECA. Cell proliferation was calculated by direct cell counting after trypan blue staining, using a Burker chamber [27].

Cell cycle analysis was carried out by flow cytometry of propidium iodide-treated cells, using the FACSCalibur Becton Dickinson Immunocytometry System according to the Becton Dickinson procedure [27].

Caspase 3 assay

Caspase 3 activity was evaluated using EnzChek® caspase-3 Assay Kit (Invitrogen). After treatment with 1% FBS alone or in combination with 100 nM CL-IB-MECA for 24h, cells were harvested, lysed and centrifuged according to the manufacturer's instructions. 50 μ l aliquots of supernatant were incubated with 50 μ l of 2X substrate working solution containing 5mM Z-DEVD-R110. Fluorescence was measured every min for 90 min at 520nm by a fluorimeter (VICTOR³ 1420 Multilabel Counter, PerkinElmer). Values were normalized to the amount of protein samples by the Bradford method.

Western Blotting

Cells were collected and lysed in a 1% Triton X-100 solution containing a cocktail of protease inhibitors (Roche, Milan, Italy), and processed for immunoblots as described [28]. For protein phosphorylation analysis, cell lysates were supplemented with 10 mM sodium pyrophosphate and 1 mM sodium vanadate. Total proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis. Cell membranes were prepared as previously reported [27]. Cells and tissues were homogenized (Dounce) and centrifuged at 8000g for 20 min, and the pellet was solubilized (P8 fraction, containing plasma membranes, debris and nuclei), while the

supernatant was subsequently centrifuged at 100,000 g (45 min). The pellet obtained was resuspended in single detergent lysis buffer [27] (P100 membrane fraction). Aliquots of P8 (25 µg) and P100 (50 µg) fractions were analyzed on both 10% and 5% SDS-polyacrylamide gel. Different amounts of proteins (100-20 µg, as indicated) were resolved on gels depending on the detectability of probed protein. Blocked membranes were probed overnight at 4° C with primary antibody, and incubated with the secondary antibody in 5% milk in PBS-T. Finally, proteins were visualized by using the SuperSignalFemto chemiluminescence system (Pierce, Celbio, Milan, Italy). Band intensity was detected by X-ray film scanning and quantitative analysis by a Model GS-700 Imaging Densitometer (BIO-RAD, Milan, Italy).

NFκB luciferase assay and NFκB fluorescence analysis

After 24 h plating in a 6-well plastic plate, cells were transiently transfected with 4 µg of pNFκB-TA-Luc plasmid DNA (Clontech, Celbio Srl, Italy) by the Ca²⁺ phosphate method, and, 6 h post-transfection, were washed and incubated for an additional 20 h in medium supplemented with 1% FBS. After cell treatment with passive lysis buffer (Promega, Madison, WI, USA), 25 µl of cell lysate was added to 50 µl of luciferase substrate (Promega) and the samples were then analysed using a 20/20n luminometer (Turner Biosystems, Sunnyvale, CA, USA). Data were expressed as relative firefly luciferase units (RLUS) normalized by the β-galactosidase units, and as n-fold change with respect to control cells.

Fluorescence analysis was performed on cells transfected with NFκB-GFP construct by using the Ca²⁺ phosphate procedure [25]. NFκB subcellular localization was analysed using a digital fluorescence microscope, and images were captured and recorded using a digital-imaging system [25].

Statistical analysis

A weighted non-linear least-squares curve-fitting program Ligand was used for computer analysis of saturation binding experiments. Analysis of data was performed using Student's t test (unpaired analysis). Differences were considered significant at a value of $p < 0.05$. All data are reported as mean \pm SEM or \pm SD of independent experiments ($n = 3-8$).

RESULTS

A₃AR expression is increased in PC1- deficient cells

We initially investigated the expression of AR in PC1-depleted HEK293 cells (HEK293^{pSsiPKD1}) that were produced by knockdown of PC1 through stable expression of PKD1 siRNA [21], and showed a constant ~85 % reduction in PC1 levels (see PC1 percent values in inset of Figure 1 A and 4 B).

The presence of AR subtypes was examined in membranes by evaluating antagonist radioligand binding to A₁, A_{2A}, A_{2B} and A₃ adenosine receptors. The mean values of receptor density (B_{max}, fmol/mg protein) and affinity (K_D, nM) in HEK293^{pSsiPKD1} and HEK293^{pSuper} control cells are shown Table IA. Both cell types express the four ARs and the highest density values were found for A₃AR (A₃>A_{2A}>A_{2B}>A₁). While affinity values were similar between the different cells (Table IA), A₃AR density resulted 1.7-fold higher in HEK293^{pSsiPKD1} than in control cells ($p < 0.01$) (Figure 1A), indicating a possible link between PC1-knockdown and A₃AR increase.

To further investigate this link, similar experiments were performed in tubular cells (4/5) derived from a normal kidney, and in cystic cells (9.7 and 9.12) derived from a polycystic kidney carrying the PKD1-Q2556X-truncating mutation (Table IB and Figure 1B) [22]. In comparison with HEK293 cells, tubular and cystic cells expressed the four ARs at markedly higher levels (1 or 2 orders of magnitude), while affinity values for A₁, A_{2B} and A₃AR were only slightly increased (Table IB). Density values were higher for A₁AR (A₁>A₃>A_{2A}>A_{2B}). However, in PKD1-mutated heterozygous 9.7 and hemizygous 9.12 cystic cells, A₃AR

densities were 1.47 and 1.76-fold higher than in tubular cells, as detailed by saturation curves and the corresponding Scatchard plot shown in Figure 1C. The increase in A₃AR density was thus associated with PC1 deficiency.

Consistently with increase in A₃AR density, PKD1-mutated cystic cells showed increased A₃AR mRNA levels in comparison with tubular cells (Figure 1D) (1.6 and 2.53-fold increases in levels of RNA, respectively, in 9.12 vs. 4/5 cells, $p < 0.05$). This increase was consistent with that of A₃AR protein levels detected by immunoblotting experiments not only in cystic and HEK293^{pSsiPKD1} cultured cells (Figure 1E), but also in ADPKD kidney tissues (Figure 1F).

These results show that PC1 depletion, either by PKD1 knockdown or by its mutation, is associated with a constant rise in A₃AR expression accompanied by possible functional deficits in PC1-deficient cells.

A₃AR activation causes different decreases in cAMP levels in tubular and cystic cells

It is known that AR occupation is followed by alteration in cAMP levels because A_{2A} and A_{2B} ARs stimulate AC activity whereas A₁ and A₃ ARs inhibit it [16]. Therefore, in order to verify the functional activity of the abovementioned A₃AR increase, cAMP levels were investigated in tubular and cystic cells. Basal cAMP levels, as well as those induced by the presence of either phosphodiesterase inhibitor (RO201712) or direct AC activator (forskolin), alone or in combination, did not differ in 9.7 and 9.12 cystic cells from those in 4/5 tubular cells (Figure 2A). Moreover, cAMP levels in tubular and cystic cells in the presence of RO201712 (Figure 2B) were similarly increased by the treatment with a specific A₂AR agonist (NECA), and reduced by the presence of either A_{2B} or A_{2A} AR antagonists (MRE2029F20 and SCH58261, respectively). Furthermore, in tubular and cystic cells, RO201712 and forskolin-associated cAMP levels were reduced, without statistical significance, by the presence of the specific A₁AR agonist CHA, but not by the specific A₁AR antagonist DPCPX (Figure 2C). In contrast,

in the presence of the specific A₃AR agonist CI-IB-MECA [29], the inhibition of cAMP levels (which was reverted by the addition of the specific A₃AR antagonist MRE3008F20)[26] was smaller in tubular than in cystic cells (1.4, 1.8 and 2.1-fold reductions in 4/5, 9.7 and 9.12 cells, respectively, p<0.05 in 9.7 and 9.12 vs. 4/5 cells) (Figure 2D). Since A₃AR activation is associated with reduction in AC activity, these results are consistent with the increased A₃AR density observed in cystic cells, and strongly support a functional role for A₃AR increase in cystic cells.

A₃AR activation provokes different proliferation patterns in tubular and cystic cells

On the basis of known cAMP inhibition by A₃AR activation and pathogenic roles of intracellular cAMP on PKD cyst growth [10-14], cell proliferation was investigated in cells stimulated with the specific A₃AR-agonist CI-IB-MECA. After both 24 and 72 h incubations, 1% FBS-induced cell proliferation was higher in cystic than in tubular cells (Figure 3A). 24 h treatment with CI-IB-MECA (100 nM) caused an inhibitory effect on cystic cell proliferation (Figure 3B). This inhibition was deduced to be receptor-mediated as it was reverted by addition of the antagonist MRE3008F20. Results of dose-response analyses with 1, 10, and 100 nM CI-IB-MECA treatment for 24 h (Figure 3C) showed that 10 and 100 nM concentrations decreased cell proliferation to a greater extent in cystic than in tubular cells (0.61±0.021 and 0.49±0.13 in cystic vs. 0.75±0.08 and 0.91±0.18 in tubular cells, at 100 and 10 nM respectively, p<0.05, cystic vs tubular cells); the 1 nM concentration did not affect cell proliferation. After the 72h treatment, the inhibitory effect of 10 and 100 nM CI-IB-MECA was still present and greater in cystic than in tubular cells, with a prevalent inhibition at 100 nM concentration (0.64±0.03 in cystic vs. 0.88±0.05 in tubular, p<0.01, Figure 4D). This concentration was also inhibitory in HEK293^{pSsi^{PKD1}} cells (Figure 3E), and therefore 100 nM CI-IB-MECA was used in the subsequent experiments.

Analysis of cell cycle phases showed that the CI-IB-MECA-mediated reduction in cystic cell proliferation by was associated with a small decrease in the S phase and an increase in the G2/M phase, effects which were blunted by the addition of MRE3008F20 (Figure 3F).

As the increase in the G2/M phase may have been dependent on increased apoptosis, analysis of caspase 3 activity was performed. Basal activity was not modified by CI-IB-MECA (Figure 3G).

A₃R activation decreases Erk and S6 kinase phosphorylation in PC1-deficient cells

As cAMP was found to activate ERK and increase ADPKD epithelial cell proliferation [18], we decided to investigate whether the reduction in CI-IBMECA-mediated cell proliferation was associated with reduced ERK1/2 activation in tubular and cystic cells. As expected from the increased proliferation, levels of phosphorylated ERK1/2 (p-ERK) were 1.36-fold higher in cystic than in tubular cells ($p < 0.05$) (Figure 4A). Addition of 100 nM CI-IB-MECA reduced p-ERK levels in cystic but not in tubular cells, and this effect was deduced to be A₃AR receptor-specific as it was reverted by MRE 8000F20. Reduction in phosphorylated ERK1/2 by CI-IB-MECA was confirmed in PC1-depleted HEK293^{pSsiPKD1} cells (Figure 4B).

It has recently been shown that ERK phosphorylates the tumor suppressor TSC2, leading to the disruption of the TSC1–TSC2 complex and ultimately unleashing mTOR kinase activity [30], an important signalling pathway which leads to cystogenesis [31]. In fact, cystic epithelial cells in ADPKD patients exhibit elevated levels of phosphorylated S6 kinase (S6K), the main substrate for mTOR kinase [31]. Therefore the effect of A₃AR activation was also investigated in S6K p70. As expected, the phosphorylated S6K levels were higher in cystic than in tubular cells, and were reduced by CI-IB-MECA (Figure 4C).

The expression of p21^{WAF1}, which plays a central role in the regulation of the cell cycle, is induced by PKD1 [7], and could be implicated in the ERK signal strength [32], was also

investigated by immunoblotting (Figure 4D); the positivity of p21 bands increased in cystic CI-IB-MECA- treated cells.

Together, these results support a modulatory role of A₃AR on cystic cell proliferation.

Inhibition of NFκB activation reverses the A₃AR expression increase

Activation of A₃AR promoter was found to be associated with activation of the transcription factor NFκB in peripheral blood mononuclear cells of arthritis patients [33], and NFκB activity is known to be related to increased cell survival. We therefore investigated the possible association between NFκB and A₃AR levels in cystic cells. These cells showed abnormally increased NFκB activity. In particular, the nuclear fluorescence of transiently transfected NFκB-GFP (Figure 5A and B), as well as the NFκB-dependent promoter activity (Figure 5C), was higher in cystic than in tubular cells. A link between NFκB activation and increased A₃AR gene expression could therefore exist in cystic cells. As similar results were found in PC1-depleted HEK293^{pSsiPKD1} cells (Figure 5D and E), NFκB activation may be imputed to PC1 impairment. This hypothesis is supported by the finding that inhibition of NFκB activity by pre-treatment with parthenolide (inhibitor of the IK kinase) [34] results in reduction of A₃AR mRNA levels (Figure 5F). The increase in A₃AR expression in cystic cells, therefore, is supported by increased NFκB binding activity.

DISCUSSION

Evidence supports the concept that the kidney expresses specific adenosine membrane receptors, that adenosine plays a vital role in the metabolic control of kidney function, and that interstitial adenosine can derive from extracellular breakdown of AMP, cAMP and ATP [19]. Since receptor levels are rate limiting for the actions of adenosine [19], receptor expression is of particular importance in physiological and pathological conditions such as

polycystic kidney disease, especially considering that ATP concentration is elevated in cyst luminal fluid [35].

Here we demonstrate that loss of PC1 in PKD1-mutated cystic cells and in PC1-depleted HEK 293 cells, leads to a specific increase in the expression of the A₃ adenosine receptor subtype, a G-protein-coupled receptor which inhibits AC activity [16]. We therefore focused on changes in cAMP levels and cAMP-dependent processes in tubular and cystic cells. Consistently with increased A₃AR levels, the activation of A₃AR in cystic cells with the selective agonist Cl-IB-MECA resulted in a significant decrease in RO201712 and forskolin-associated cAMP levels, as compared to tubular cells. In contrast, activation of A₁, A_{2A} and A_{2B} receptors, which are similarly co-expressed in these cells, did not yield differences in cAMP levels between cystic and tubular cells. In light of the proliferative response to cAMP of cyst-derived cells [10-14], this alteration represents an interesting characteristic of cystic cells because, as discussed herein, it may lead the way to a novel therapeutic approach for ADPKD. Approaches to reduce kidney cAMP levels in ADPKD have been indeed already taken [2], and the therapy with octreotide, a somatostatin analogue activating Gi-coupled receptors, has been indeed found to be effective in slowing renal volume expansion [36].

In particular, the activation of A₃AR by Cl-IB-MECA is associated with a reduction in cell proliferation, which is prevalent in cystic cells, with a maximal ~35% inhibition. As this inhibitory effect is exerted by nanomolar concentrations (10 and 100 nM) of the agonist and blunted by the selective A₃AR antagonist MRE3008F20, it was shown to be a receptor-mediated effect and associated with the decrease in cAMP. Moreover, the agonist selectivity at this concentration may exclude involvement of A₁AR, which is expressed at high level in both cystic and tubular cells. The loss of inhibitory effect observed at higher doses of Cl-IB-MECA (1000 nM) could be attributed to interaction with other AR subtypes, or to agonist-dependent desensitization-degradation and down-regulation of A₃AR that has been demonstrated by other authors in various cell systems [37].

Earlier studies have demonstrated that micromolar concentrations of A₃AR agonists such as Cl-IB-MECA induce apoptosis in different cell types [17, 38]. In our study, the Cl-IB-MECA-induced reduction in proliferation was not found to be associated with increased cell death by necrosis or apoptosis, since neither the number of trypan blue positive cells (data not shown) nor the activity of caspase 3 were modified by the 100 nM treatment. Instead, cell cycle studies showed that Cl-IB-MECA impairs cell cycle progression by increasing the G₂/M phase in cystic cells. This finding is consistent with the reduction in proliferation provoked by A₃AR activation in A₃AR-transfected CHO cells, which was due to an augmented G₂/M phase [39].

The reduction in cystic cell proliferation by Cl-IB-MECA occurs via modulation of the ERK1/2 pathway by A₃AR-mediated reduction in phosphorylated-ERK levels. This finding is consistent with the stimulatory effect exerted by cAMP on MAPK/ERK signaling in cystic cells [10, 12-14], and is also in line with the observed cell cycle changes. Reduction in ERK activity has, in fact, been implicated in retarding the progression from metaphase to anaphase [32, 40]. However, we cannot exclude the hypothesis that ERK1/2 inactivation by Cl-IB-MECA via A₃AR may be partly cAMP-independent. For instance, the anti-proliferative effect of Cl-IB-MECA and ERK1/2 inactivation in melanoma cells occur, in part, via a PLC/PI3K/AKT pathway [41].

An other important pathway contributing to cystic expansion and epithelial cystic cell proliferation is the mTor signaling; enhanced levels of phospho-mTOR and the downstream effector phospho-S6 ribosomal protein have been indeed observed in cystic cells [31]. Cl-IB-MECA-induced reduction in phosphorylated S6 kinase levels in cystic cells, therefore, provides evidence for anti-proliferative and anti-growth effects of the A₃AR activation in these cells. It is noteworthy that ERK mediates phosphorylation of TSC2/tuberin, leading to dissociation and inactivation of the TSC1/TSC2 complex and activation of mTOR [30]. Therefore, S6 kinase inactivation may result from ERK inactivation. In addition, the

PI3K/AKT pathway is known to activate the mTOR pathway. Consequently, S6 kinase inactivation by CI-IB-MECA may exclude the involvement of AKT in the abovementioned ERK inactivation.

The observation that the increase in A₃AR density is consistent with that in A₃AR protein and mRNA levels strongly indicates that loss of PKD1 gene expression leads to increased expression of the A₃AR gene. PC1 depletion may cause an increase in A₃AR gene expression via cAMP elevation in cystic cells, which might upregulate the A₃AR promoter activity. Enhancement of A₃AR promoter in response to elevation in cAMP has been already demonstrated in rat vascular smooth muscle cells and in the rat mast cell line RBL-2H3 [42]. These results suggest the presence of a generalized mechanism in A₃AR-expressing cells, by which cAMP might control its own level via regulation of genes involved in the modulation of AC activity [42]. However, it has recently been shown that upregulation of A₃AR in peripheral blood mononuclear cells of rheumatoid arthritis patients is directly correlated to an increase in expression of NFκB [33], and there are two NFκB/NFκB1-binding sites in the A₃AR gene promoter [33]. The hypothesis of an association between NFκB activation and A₃AR expression is supported by our results. Marked NFκB nuclear localization and elevated luciferase promoter activity have, in fact, been demonstrated in cystic cells and in two HEK293^{pSsiPKD1} clones, both characterized by increased A₃AR expression. A potential role of NFκB in A₃AR expression is further supported by the finding that NFκB inhibition by parthenolide reduces A₃AR mRNA levels in cystic cells. In addition, these findings strongly indicate that NFκB is activated in PKD1-defective cells, probably contributing to cystic cell proliferation and survival.

In conclusion, our study shows that cystic cells “in vitro” and ADPKD kidneys express increased A₃AR levels, and upon A₃AR activation, the modulation of cAMP and of key signaling proteins leads to inhibition of the growth of cystic cells. The capacity of synthetic A₃AR agonists to inhibit the growth of various tumor cell types, such as melanoma,

colon carcinoma and lymphoma [43-46], as well as renal injury [47], render them potentially useful in the treatment of ADPKD. This is certainly an important issue, worthy of more detailed investigation in the future with animal models of the disease.

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FIGURE LEGENDS

Figure 1

Expression of AR subtypes in human kidney cell lines. Bars show receptor density (B_{max}, fmol/mg protein) of adenosine receptors (A₁, A_{2A}, A_{2B} and A₃) in PC1-depleted HEK293^{pSsiPKD1} and control HEK293^{pSuper} cells (see inset) (A), and in PKD1-mutated cystic 9.7, 9.12 and wild-type tubular 4/5 cells (B). Immunoblot analysis shown in the inset was performed as previously reported on p100 membrane fractions (100 μg/lane) [21]. Bars show mean ± SE of at least three independent experiments in duplicate (**, p<0.01 versus control cells). Data obtained from HEK293^{pSsiPKD1} a and b clones have been combined. Saturation curves of [³H]-MRE 3008F20 binding to A₃AR in 4/5, 9.7 and 9.12 cell membranes, and Scatchard plot of the same data (C). Overall binding parameters including affinity (K_D, nM) are shown in Table I. (D) A₃AR mRNA expression in 9.7 and 9.12 relative to those in 4/5 cells. Levels of A₃AR mRNA were measured by real time RT-PCR. Experiments were performed as described in Materials and Methods. Bars show mean ± SD of three independent experiments in duplicate (*, **, p<0.05 and 0.01 versus control cells). (E and F) A₃AR protein expression in kidney cell lines and tissues. A₃AR levels were measured by immunoblot analysis of total lysates of cultured cells (50 μg/lane) (E), and from membranes of kidney tissue samples (F). Fractions containing plasma membranes, P8 (25 μg/lane) and those containing reticular membranes, P100 (50 μg/lane), were obtained from four ADPKD and two control kidneys, as reported in experimental procedures. Bars show mean ± SD of three independent experiments. A significant ~ 3 fold increase was observed in both PKD1-

deficient cells and ADPKD tissues (** and ***, $p < 0.01$ and < 0.001 versus control cells). Data from cystic and PC1-depleted cells, as those from P8 and P100 fractions, have been combined.

Figure 2

Effect of adenosine receptor subtypes on cAMP levels in 4/5, 9.7 and 9.12 cell lines. A) Basal, RO 201712 (0.5 mM) and forskolin (1 μ M) associated levels of cAMP in 4/5 (white), 9.7 (light grey) and 9.12 cells (dark grey). Modulation of cAMP levels (B) by typical A_2 AR ligands: the non-selective agonists NECA (100 nM), the A_{2A} AR antagonist SCH 58261 (1 μ M) and the A_{2B} AR antagonist MRE 2029F20 (100 nM); (C) by typical A_1 AR ligands: the agonist CHA (100 nM), the antagonist DPCPX (1 μ M); (D) by typical A_3 AR ligands: the agonist Cl-IB-MECA (100 nM), the antagonist MRE 3008F20 (1 μ M), *, $p < 0.05$ of 9.7 and 9.12 vs. 4/5 Cl-IB-MECA-treated cells. Experiments were performed as described in Materials and Methods. Bars represent the means \pm SE of four experiments.

Figure 3

The inhibitory effect of Cl-IB-MECA on cell proliferation. Trypan blue-negative cells were counted after 24 and 72 h culture in 1% FBS, in the absence or presence of the agonist Cl-IB-MECA alone or in combination with the antagonist MRE3008F20. A) After 24 and 72h of culture in 1% FBS, cell proliferation was increased in PKD1-mutated 9.7 and 9.12 cystic cells, as compared to 4/5 tubular cells. Bars show mean \pm SD of at least three independent experiments in triplicate; * and **, $p < 0.05$ and 0.01 vs. tubular cells. B) Inhibitory effect of Cl-IB-MECA (100 nM) on proliferation of 9.7 and 9.12 cells after 24 h treatment. Bars represent mean \pm SD of three triplicate experiments. ° and °°, $p < 0.05$ and 0.01 of cystic vs. tubular untreated cells; * and **, $p < 0.05$ and 0.01 vs. Cl-IB-MECA-untreated cystic cells. The A_3 AR antagonist MRE3008F20 (1 μ M) blunted the Cl-IB-MECA-induced inhibitory effect.

Relative decrease in cell proliferation induced by treatment with increasing concentrations of CI-IB-MECA for 24 h (C) and 72 h (D) in 4/5 and 9.12 cells. E) Relative decrease in cell proliferation induced by 72 h treatment with 100 nM CI-IB-MECA in HEK293^{pSsiPKD1} (clones a and b) and control HEK293^{pSuper} cells. Bars (means \pm SD) express the ratios between cell number in treated versus untreated cells in four triplicate experiments (* and **, $p < 0.05$ and 0.01 in cystic vs. tubular CI-IB-MECA-treated cells and in HEK293^{pSsiPKD1} vs. HEK293^{pSuper} CI-IB-MECA-treated cells). F) Changes in cell cycle phases (G0/G1, S and G2/M) of tubular and cystic cells by CI-IB-MECA treatment. After 24 h culture in presence of 1% FBS and 100 nM CI-IB-MECA alone or in combination with MRE3008F20, cells were incubated with propidium iodide and analysed as described in the Methods section. The % of cells in G0/G1, S and G2/M phases in treated cells were related to those of untreated cells. Bars show mean \pm SD of ratios obtained in three duplicate experiments (one with 4/5 and 9.7 cells, two with 4/5 and 9.12 cells); *, $p < 0.05$ in combined 9.7 and 9.12 cystic vs. 4/5 tubular cells. G) Caspase 3 activity in 4/5, 9.7 and 9.12 cells cultured for 24 h with 1% FBS and 100 nM CI-IB-MECA, relative to untreated 4/5 cells. Bars show the mean \pm SD of three independent experiments in duplicate.

Figure 4

Inhibitory effect of 100 nM CI-IB-MECA on activation of ERK1/2 and P70 S6K in PC1-deficient cells.

Levels of phosphorylated ERK1/2 in 4/5 and 9.12 cells (A) and in HEK293^{pSuper} and HEK293^{pSsiPKD1} cells (B); levels of phosphorylated P70S6K in 4/5 and 9.12 cells (C); p21^{WAF1} levels are also shown (D). PC1 expression levels shown in the inset of part B were obtained as described in the legend of Figure 1 A. Phosphorylated and non-phosphorylated proteins (20 μ g/lane), were detected in total cell lysates of cells, cultured for 24 h with 1% FBS, 100 nM CI-IB-MECA in absence or presence of 1 μ M MRE3008F20, as described in the Methods

section. Levels of phosphorylated proteins were normalized for those of non-phosphorylated ones, and those of p21^{WAF1} were normalized for the content of β -actin. The findings obtained were related to those of untreated control cells and represented as mean \pm SD of three independent experiments. * and ** indicate statistical significance from 4/5 and HEK293^{pSuper} untreated cells ($p < 0.05$ and 0.01). °, indicates statistical significance from 9.12 and HEK293^{pSsiPKD1a} untreated versus CI-IB-MECA-treated cells ($p < 0.05$). °°, indicates statistical significance from HEK293^{pSsiPKD1b} untreated versus CI-IB-MECA-treated cells ($p < 0.01$).

Figure 5

NF κ B activation and A₃AR expression in kidney cell lines. NF κ B-GFP nuclear translocation is shown in tubular and cystic cells (A), and in HEK293^{pS}, HEK293^{pSsiPKD1a} and HEK293^{pSsiPKD1b} cells (D). Cells were grown in 1% FBS on coverslips and, after 24 h transfection with the NF κ B-GFP construct in 1% FBS, were analyzed by fluorescence microscope as described in the Methods section. The percentage of fluorescent cells in the cytoplasm (C) and in the nucleus (N) of 4/5 (n=153), 9.7 (n=146) and 9.12 (n=130) cells are shown. **, $p < 0.01$ of 9.7 and 9.12 vs. 4/5 cells in 3 duplicate experiments. (B). Increase in luciferase promoter activity in NF κ B-pTA-luc-transfected 9.12 (C) and HEK293^{pSsiPKD1} (E) cells, was related to control cells. ** and *** indicate statistical significance from control cells ($p < 0.01$ and 0.001). (F) Parthenolide, an inhibitor of NF κ B activation,²⁹ reduced A₃AR mRNA levels in cystic cells. Cells were cultured for 48 h in 1% FBS and in presence or absence of 50 nM parthenolide. A₃AR mRNA was related to the content of actin mRNA in parthenolide-treated and untreated 4/5, 9.7 and 9.12 cells. The levels of A₃AR mRNA were measured by real time RT-PCR and related to those of actin mRNA as described in Experimental procedures. ** indicates statistical significance from untreated cells ($p < 0.01$).

Table I

Affinity and density of adenosine receptors in PC1-deficient kidney cell membranes

A – HEK 293^{pSuper} and HEK293^{pSsiPKD1} cell membranes

Cells	[³ H]- DPCPX A ₁ R	[³ H]- ZM 241385 A _{2A} R	[³ H]- MRE2029F20 A _{2B} R	[³ H]- MRE3008F20 A ₃ R
293^{pSuper}				
K _D	2.78 ± 0.20	2.40 ± 0.15	1.71 ± 0.18	3.46 ± 0.07
Bmax	13 ± 2 n=3	44 ± 3 n=6	27 ± 2 n=3	154 ± 8 n=8
293^{pSsiPKD1}				
K _D	2.63 ± 0.22	2.55 ± 0.22	1.87 ± 0.23	3.51 ± 0.18
Bmax	12 ± 2 n=3	40 ± 3 n=7	25 ± 2 n=3	263 ± 22** n=9

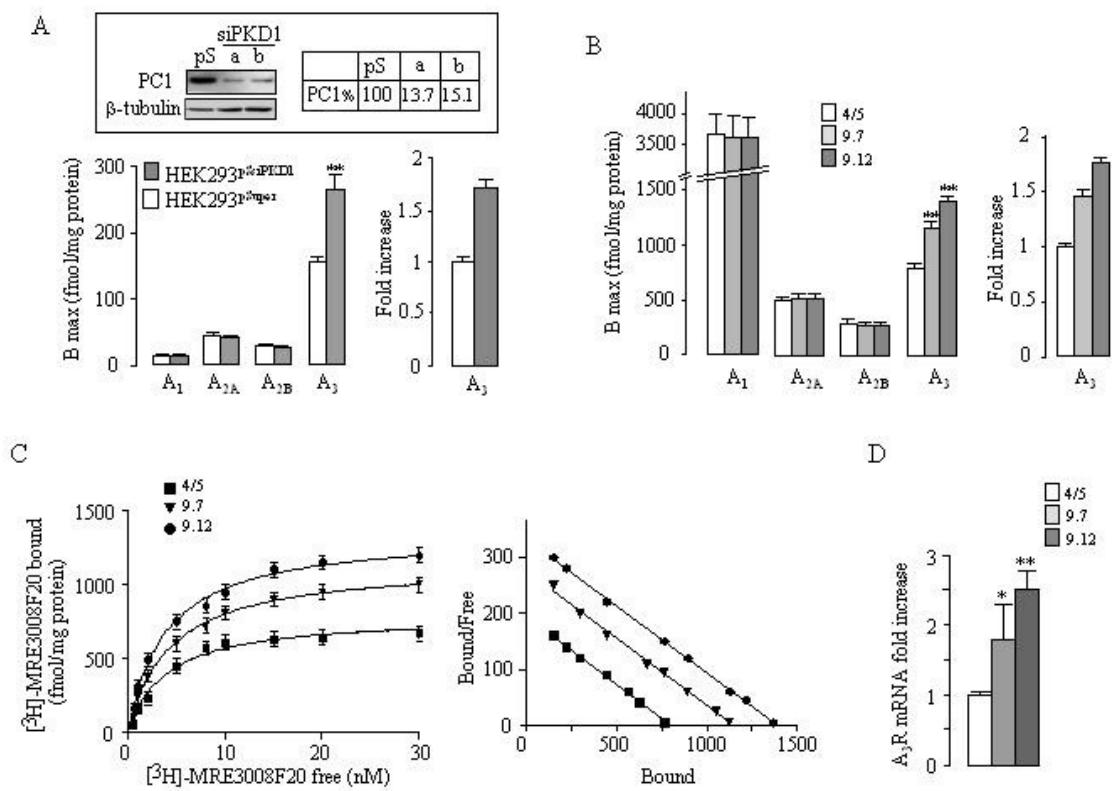
B – Tubular (4/5) and PKD1-mutated cystic (9.07 and 9.12) cell membranes

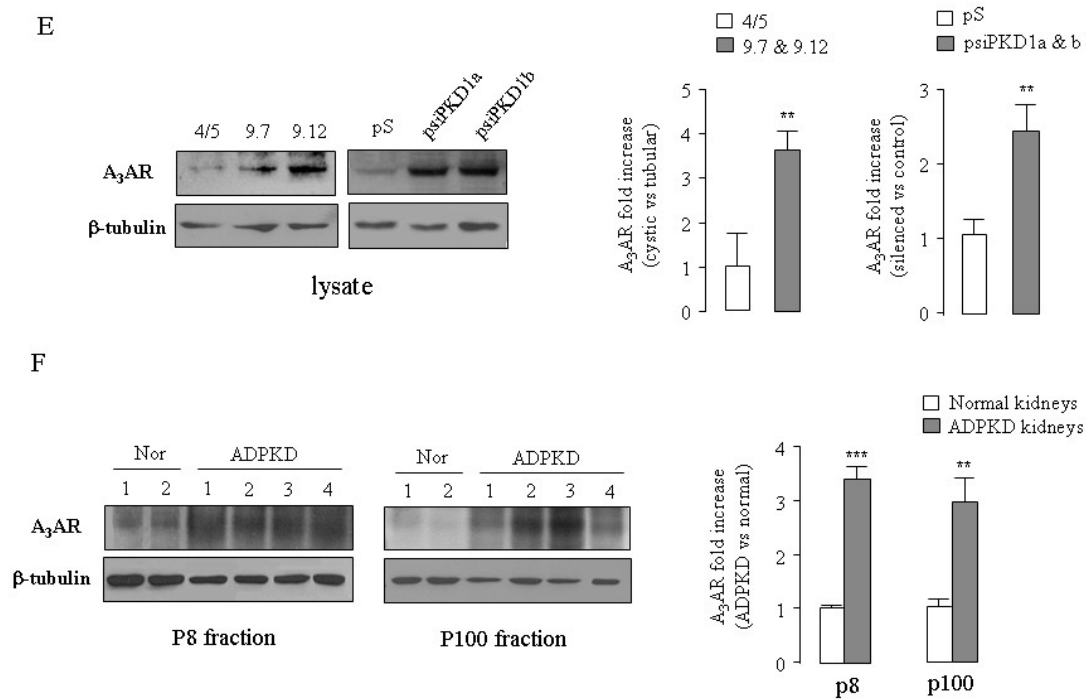
Cells	[³ H]- DPCPX A ₁ R	[³ H]- ZM 241385 A _{2A} R	[³ H]- MRE2029F20 A _{2B} R	[³ H]- MRE3008F20 A ₃ R
4/5				
K _D	5.45 ± 0.21	2.28 ± 0.12	2.75 ± 0.13	4.04 ± 0.12
Bmax	3633 ± 233 n=3	503 ± 23 n=3	287 ± 24 n=3	783 ± 44 n=3
9.7				
K _D	5.39 ± 0.20	2.31 ± 0.11	2.66 ± 0.11	4.22 ± 0.24
Bmax	3587 ± 247 n=3	510 ± 38 n=3	270 ± 21 n=3	1150 ± 58** n=3
9.12				
K _D	5.36 ± 0.13	2.39 ± 0.18	2.85 ± 0.10	4.37 ± 0.19
Bmax	3600 ± 231 n=3	513 ± 35 n=3	277 ± 23 n=3	1383 ± 60** n=3

Binding parameters such as affinity (K_D, nM) and density (Bmax, fmol/mg protein) in HEK293^{pSsiPKD1} and control HEK293^{pSuper} cells (A), and in PKD1-mutated 9.7, 9.12 cells and in

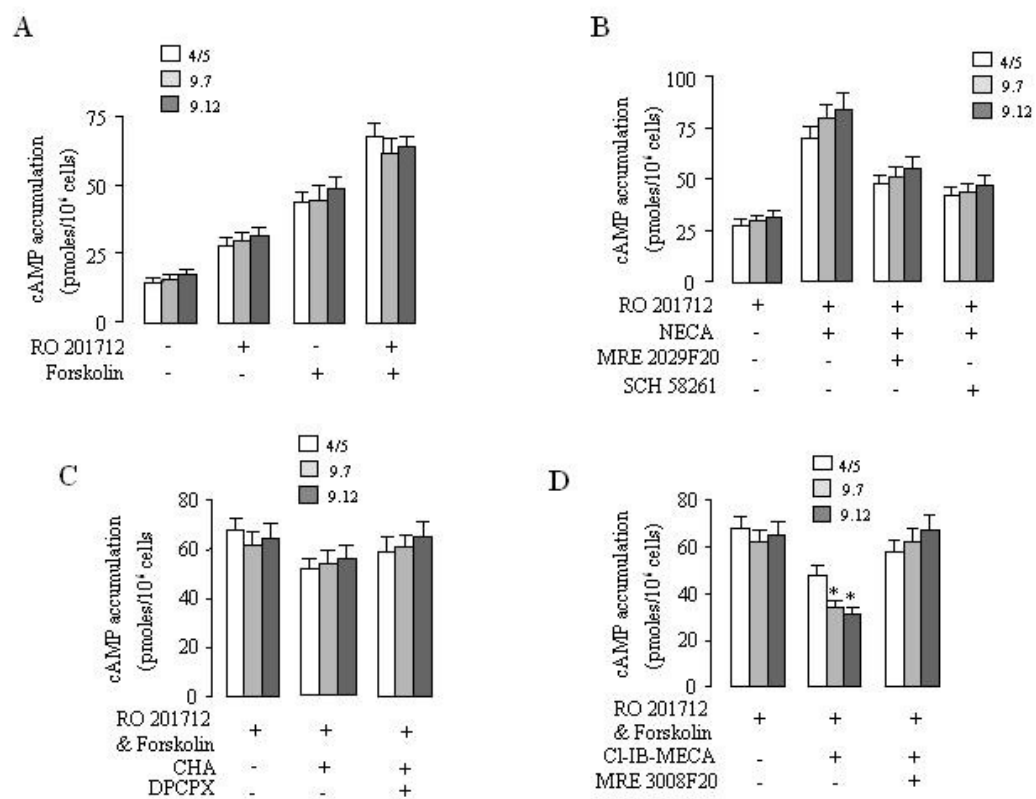
control 4/5 cells (B). n= number of experiments; **, $P < 0.01$ relative to PC1-deficient versus control cells.

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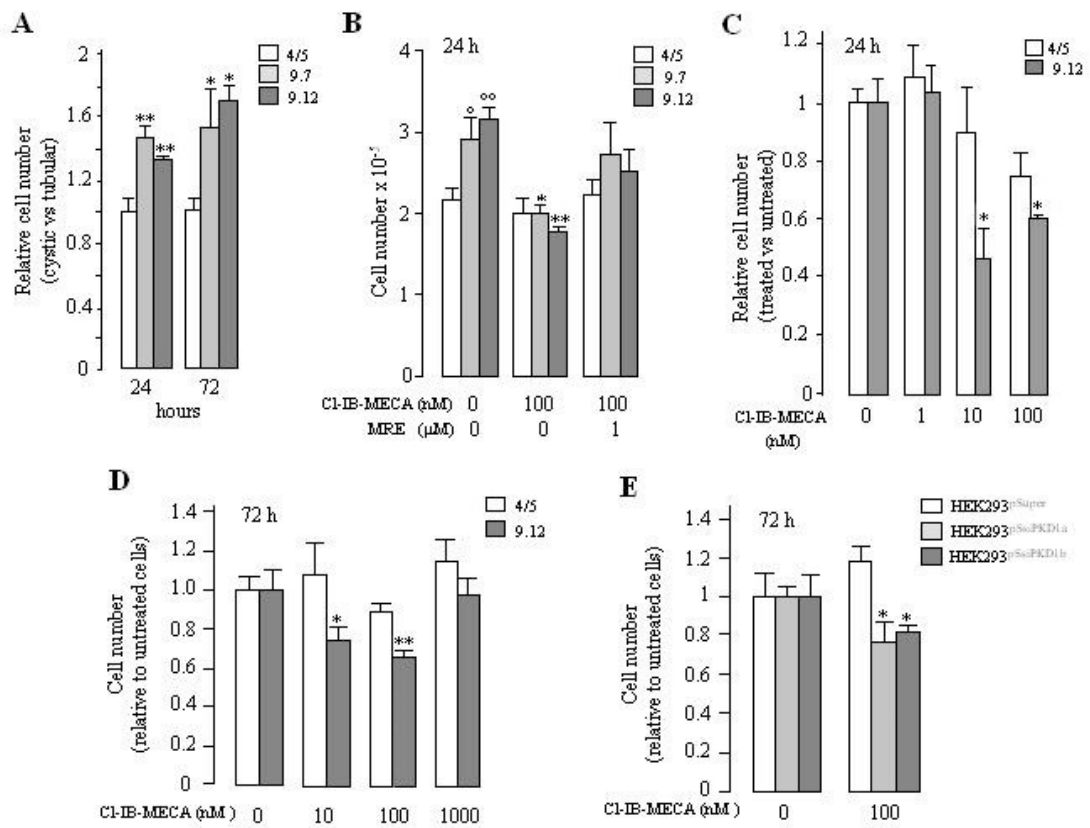


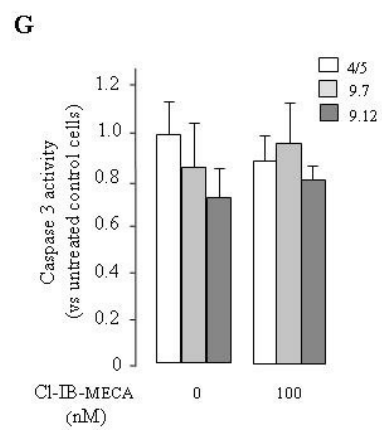
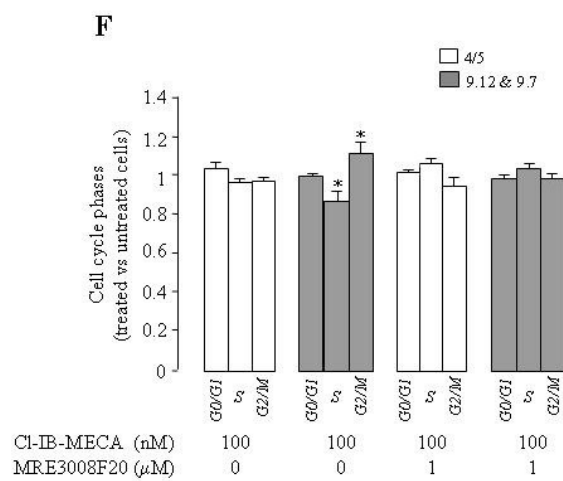


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