Novel role for polycystin-1 in modulating cell proliferation through calcium oscillations in kidney cells

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Abstract. Objectives: Polycystin-1 (PC1), a signalling receptor regulating Ca$^{2+}$-permeable cation channels, is mutated in autosomal dominant polycystic kidney disease, which is typically characterized by increased cell proliferation. However, the precise mechanisms by which PC1 functions on Ca$^{2+}$ homeostasis, signalling and cell proliferation remain unclear. Here, we investigated the possible role of PC1 as a modulator of non-capacitative Ca$^{2+}$ entry (NCCE) and Ca$^{2+}$ oscillations, with downstream effects on cell proliferation. Results and discussion: By employing RNA interference, we show that depletion of endogenous PC1 in HEK293 cells leads to an increase in serum-induced Ca$^{2+}$ oscillations, triggering nuclear factor of activated T cell activation and leading to cell cycle progression. Consistently, Ca$^{2+}$ oscillations and cell proliferation are increased in PC1-mutated kidney cystic cell lines, but both abnormal features are reduced in cells that exogenously express PC1. Notably, blockers of the NCCE pathway, but not of the CCE, blunt abnormal oscillation and cell proliferation. Our study therefore provides the first demonstration that PC1 modulates Ca$^{2+}$ oscillations and a molecular mechanism to explain the association between abnormal Ca$^{2+}$ homeostasis and cell proliferation in autosomal dominant polycystic kidney disease.

INTRODUCTION

Increases in cytosolic concentration of Ca$^{2+}$ ions [Ca$^{2+}$], regulate virtually all cellular processes, including events such as survival, proliferation and apoptosis, which decide the fate of each cell (Berridge et al. 2000). These functions are disturbed in autosomal dominant polycystic kidney disease (ADPKD), one of the most common inherited diseases in humans (1 in 500 to 1 in 1000), and one that accounts for about 10% of end-stage renal failure. The primary phenotype

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of ADPKD is the progressive expansion, in both kidneys, of multiple fluid-filled cysts, which gradually replace normal renal tissue and lead to end-stage renal failure and dialysis dependency by mid-to-late adulthood (Gabow 1990). The systemic nature of the disease is determined by a number of extrarenal manifestations (from hepatic and brain cysts to heart valve and cerebrovascular abnormalities) that accompany the kidney cysts (Gabow 1990) and indicate the complex role of ADPKD causative gene products (Ong & Harris 2005).

In approximately 85% of ADPKD cases, the disease arises as a consequence of loss of function and missense mutations of the \textit{PKD1} gene, which encodes polycystin-1 (PC1), with the remaining cases being caused by mutations in polycystin-2-encoding \textit{PKD2} gene (PC2) (Mochizuki et al. 1996; Harris 1999). The phenotypes associated with either genotype are otherwise very similar, indicating that the two proteins function with the same pathways. PC1 and PC2 are the prototype members of a new family of integral membrane proteins that play important roles in a variety of biological processes, including fertilization, ion translocation and mechanotransduction (Delmas 2004). PC1 (around 450 kDa and 11 transmembrane domains) acts as an orphan receptor mainly involved in control of epithelial cell population growth (Bhunia et al. 2002; Kim et al. 2004; Li et al. 2005; Manzati et al. 2005), migration (Nickel et al. 2002; Polgar et al. 2005), differentiation (Aguiari et al. 1998; Puri et al. 2004) and apoptosis (Boca et al. 2006). In association with PC2, a Ca^{2+}-regulated Ca^{2+}-permeable non-selective cation channel (Hanaoka et al. 2000; González-Perrett et al. 2001; Koulen et al. 2002), PC1 is required for regulation of the cell cycle (Bhunia et al. 2002) and activation of cation permeable currents (Vandorpe et al. 2001; Babich et al. 2004; Pelucchi et al. 2006); it is also thought to be involved in regulation of G-protein signalling (Parnell et al. 1998; Delmas et al. 2002).

Our group has recently found that expression of a membrane-targeted PC1 C-terminus in kidney HEK293 cells increases cell response to ATP-evoked [Ca^{2+}], (Aguiari et al. 2003) and serum-evoked cell proliferation (Manzati et al. 2005). It has also recently been reported that the treatment of PKD-cystic cells with molecules causing sustained increases in [Ca^{2+}] may reverse their mitogenic response (Yamaguchi et al. 2006). Therefore, the aim of the present work was to determine the mechanism by which PC1 modulates Ca^{2+} levels, considering the possibility that a reduction in endogenous PC1 by small-interference RNA might affect Ca^{2+} mobilization and thus induce a growth-stimulated phenotype characteristic of PKD cells. Our results, obtained by employing pharmacological tools on kidney cell lines depleted of endogenous PC1 by PKD1 RNA interference or in PKD1 mutated cystic cell lines, demonstrate that down-regulation of PC1 leads to elevation in Ca^{2+} oscillations by stimulating non-capacitative Ca^{2+} entry (NCCE). Such oscillatory signals trigger nuclear factor of activated T cell (NFAT) activation and other Ca^{2+}-dependent signalling pathways, leading to increased cell proliferation. Furthermore, the inhibitory effects of exogenously expressed PC1 on Ca^{2+} oscillations, as well as proliferation of HEK293 cells, indicate PC1 as a physiological modulator of NCCE, which plays a crucial role on basal and evoked [Ca^{2+}] levels. These findings pave the way to future investigations on involvement of such channels in both the physiology and pathophysiology of kidney cells.

MATERIALS AND METHODS

Reagents
Dulbecco’s modified Eagle’s medium/F12 and minimum essential medium media, G418 antibiotic, bovine serum albumin (BSA), 1-(beta-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride (SKF96365), cyclosporin A, gadolinium and anti-FLAG M2 mouse
monoclonal antibody were obtained from Sigma-Aldrich (Milano, Italy), foetal bovine serum (FBS) was obtained from Eurobio (Celbio, Milan, Italy) and selective cell-permeable inhibitors of protein kinase C (PKC)-α and PKC-β1 (Ro-320432 and hispidin, respectively), were purchased from Calbiochem (La Jolla, CA, USA) and protease inhibitors were from Roche Diagnostics (Monza, Italy). Rabbit polyclonal antitubulin and antirabbit rhodamine- and FITC-conjugated antibody were obtained from Santa Cruz (DBA Italia Srl, Segrato, Italy), while rabbit polyclonal antip27 antibody was obtained from Cell Signalling Technology (Celbio SRL, Italy). Enhanced chemiluminescent substrates for Western blotting (SuperSignalDura or SuperSignalFemto) and horseradish peroxidase-conjugated goat antirabbit and antimouse antibodies were purchased from Pierce (Celbio S.r.l., Milan, Italy). The pSUPER RNAi System was obtained from OligoEngene (Seattle, WA, USA). (R,S)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-N,N-di[2-(2,3,4trimethoxyphenyl)ethyl] acetamid mesylate (LOE908) was kindly provided by Boehringer Ingelheim (Ingelheim, Germany).

Production of siRNA expressing vectors
Two siRNAs (a and b) for PC1 were constructed according to a published method (Brummelkamp et al. 2002). Briefly, 64 nt primers were designed to include a 19-nt PKD1 sequence, its complement, a spacer region, 5′ BglII site and 3′ HindIII site.

The forward PKD1a primer sequence: 5′-GATCCCCGACAAGCAGTCCTGACCTTC-AAGAGAGGTCAGGGACTGCTTGTCGTTTTAAA-3′ and the reverse primer sequence: 5′-AGCTTTTCCAAAAACGACAAGCAGTCCCTGACCTCTCTTGAAGGTCAAGGACTGCTTGTGGGG-3′.

The forward PKD1b primer sequence: 5′-GATCCCCCTTCAACGGGAGCCGCGTTCAGAGACCGCCTCCGGCTGAAATTTTTGGAAA-3′ and the reverse primer sequence: 5′-AGCTTTTCCAAAAACCTCAACGGGAGCCGCGTTCAGCTTGTGAAACCTTAAGGG-3′. The target PKD1 sequence was substituted by a scramble nucleotide sequence as control: 5′-TTCTCCGAACGTGTCACGT-3′. Annealed double strands of DNAs were cloned into the pSUPER plasmid vector following the Oligoengene procedure.

Cells, stable transfection, cell cycle and proliferation assays
HEK293 cells were maintained and cultured as previously described (Manzati et al. 2005). Wild-type (pSUPER) and recombinant constructs (pSiScramble, pSiPKD1 a and b) were co-transfected with pCDNA3 (ratio 1 : 10, respectively) by the calcium phosphate method, in HEK293 cells (Pelucchi et al. 2006), and G418-resistant clones (HEK293pSUPER, HEK293pSiScramble and HEK293pSiPKD1), and were screened by Western blotting analysis, through detection of endogenous PC1 protein using 7e12 monoclonal antibody (Qian et al. 2003). PKD1 RNA levels were analysed by real-time RT-PCR, as previously reported (Aguiari et al. 2004). PKD1 RNA quantification was obtained by the ΔCT method. Transcription of the ACTIN housekeeping gene was used as endogenous RNA control for normalization and the ΔCT was then calculated (ΔCT = CT(target gene) – CT(actin gene)). Final results, expressed as N-fold content in PKD1 gene expression relative to the ACTIN gene, were determined as follows: $N_{(target)} = 2^{-\Delta\Delta CT}$, as previously described (Aguiari et al. 2004).

Cell cycle analysis was done with the use of flow cytometry of propidium iodide-treated cells, using the FACSCalibur Becton Dickinson Immunocytometry System. Previously described SV40-transformed human cell lines (one derived from normal and two from cystic kidney cell lines) (Nauli et al. 2006), were grown in Dulbecco’s modified Eagle’s medium/F12, 10% FBS. Cystic cells carry the Q2556X PKD1 mutation: one is heterozygous, while the other is hemizygous, for deletion of the normal allele (Nauli et al. 2006). After plating at low density
(30 000 cells/mL) in 24-well plates, cell proliferation was measured by direct cell counting (Manzati et al. 2005), and by 24 and 48 h incubation with [3H] thymidine (1 µCi/mL).

**Transient transfection and immunofluorescence analysis**

HEK293pSUPER and HEK293pSsPKD1 cells were cultured on 24-mm coverslips for 24 h then were transiently transfected with full-length FLAG-tagged mouse PC1 (Grimm et al. 2003) by Ca2+ phosphate precipitation. After 3 days of transfection, non-fixed cells were washed twice with phosphate-buffered saline (PBS) buffer and incubated at room temperature for 1 h with anti-FLAG M2 mouse monoclonal antibody at a dilution of 1 : 200, in a solution containing 0.2% gelatin. After three washes with PBS, cells were treated with a 0.2% gelatin PBS solution that contained secondary antirabbit rhodamine-conjugated antibody at room temperature in the dark for 1 h. After three washes in PBS, cells were analysed using a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ, USA), excitation and emission filter wheels (Sutter Instrument Company, Novato, CA, USA), and piezoelectric motoring of the Z stage (Physik Instrumente, GmbH and Co., Karlsruhe, Germany).

**Ca2+ measurement**

Measurements of FBS-evoked calcium levels were performed in cells grown on coverslips and transfected with recombinant cytoplasmic aequorin cDNA (Aguiari et al. 2003). Seventy-two hours after aequorin reconstitution, cells on coverslips were transferred into the perfusion chamber of a luminometer and, following 1 min of perfusion, stimulated with 1% FBS. The relationship between photon emission \( \frac{L}{L_{\text{max}}} \) and Ca2+ concentration allows conversion by computer algorithm of light released into the specimen. \( L \) and \( L_{\text{max}} \) are the instant and maximal rates of light emission, the former having been obtained after 1% FBS stimulation and the latter brought about by cell lysis with 100 µm digitonin (Manzati et al. 2005).

Cytoplasmic Ca2+ oscillations were measured in Fura-2AM-loaded cells (Manzati et al. 2005). Cells, grown on coverslips, were starved ON with BSA, loaded with Fura-2AM (4 µm/30 min/37 °C), and transferred to the thermostated stage of a Zeiss Axiovert 200 inverted microscope equipped with a Sutter filterwheel and 340/380 excitation filters. Cells were stimulated either with 1% FBS alone or with other molecules for 15 min or 30 min, acquiring 1 image every 1 s or 3 s, respectively. Signals were computed into relative ratio units of the fluorescence intensity of different wavelengths (340/380 nm). Ca2+ oscillation amplitude was calculated by measuring Ca2+ concentration in Fura-2AM-loaded cells, as previously described (Manzati et al. 2005).

Calibration was carried out using MetaFluor software, according to the equation \[ [\text{Ca}^{2+}] = K_d \times (S_2/S_0) \times (R - R_{\text{min}})/(R_{\text{max}} - R), \]

where \( K_d = 224 \text{ nm} \), \( R_{\text{max}} \) and \( R_{\text{min}} \) were determined at the end of each experiment in KRB/Ca2+/1 µm ionomycin, and KRB/1 mm EGTA/ionomycin, respectively.

**NFAT and PKC-α fluorescence analysis**

Cells were transfected with either NFAT–green fluorescent protein (GFP) or PKC-α-GFP constructs by using the Ca2+ phosphate procedure (Manzati et al. 2005). NFAT and PKC-α subcellular localization was analysed using a digital fluorescence microscope, and images were captured and recorded using a digital-imaging system (Manzati et al. 2005).

**NFAT luciferase assay**

After 24 h plating in a 6-well plastic plate, cells were transiently transfected with 4 µg of pNFAT-TA-Luc plasmid DNA by the Ca2+ phosphate method, and 6 h post-transfection, they 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 then the samples were analysed using a 20/20 luminoimeter (Turner Biosystems, Sunnyvale, CA, USA).

Statistical analysis
Where applicable, statistical errors of averaged data were given as means ± SD or SEM and statistical significance was assessed by Student’s *t*-test.

RESULTS

Relationship between PC1 depletion by RNA interference and proliferation of HEK293 cells

A variable, but consistent down-regulation of PC1 was accomplished in HEK293pSsiPKD1 cell clones obtained by stable transfection with pSUPER plasmid expressing PKD1 siRNAs (Fig. 1a). An average of more than ~50% reduction in PC1 protein was found in clones transfected by either the *a* or *b* PKD1 siRNA-cloned sequence, as compared to those transfected with the empty vector or that expressing the scramble nucleotide sequence, as controls [Fig. 1b, PC1/β-tubulin: 0.70 ± 0.09 and 0.55 ± 0.5 in HEK293pSsiPKD1 *a* and *b* clones (pSsiPKD1), respectively, versus 1.52 ± 0.11 and 1.46 ± 0.34 in control HEK293pSUPER and HEK293pSiScramble clones (pS and pSsc); *P* < 0.01 and *P* < 0.05, respectively, versus pS and *P* < 0.05 versus pSc]. This inhibition of PC1 by siRNA was found to be selective as neither variations in β-tubulin were observed, nor PC1 levels altered, in clones expressing the control vectors. Moreover, extent of PC1 inhibition was consistent with the reduction in PKD1 RNA, as shown in HEK293pSsiPKD1 *a* cells by real-time RT-PCR analysis (Fig. 1c).

Because the loss of PC1 in kidney cysts is associated with proliferation of epithelial cells, and HEK293 cells overexpressing the putative dominant negative PC1 C-terminus have been shown to increase cell proliferation (Manzati *et al*. 2005), HEK293pSsiPKD1 stably transfected cells were assessed for possible changes in cell proliferation. PC1 inhibition did indeed cause a significant increase in 1% FBS-induced cell proliferation, as illustrated by the examples shown in Fig. 1d. This increase was detectable both in [3H] thymidine uptake after 24 and 48 h incubation, and after 3 and 5 days of culture (106 667 ± 5700 versus 62 333 ± 8500 and 65 100 ± 11 300 after 3 days of culture, and 203 333 ± 6500 versus 126 667 ± 5600 and 120 600 ± 17 800 after 5 days, respectively, in HEK293pSsiPKD1 cells versus HEK293pSUPER and HEK293pSiScramble control cells; *P* < 0.01 and *P* < 0.001 after 3 and 5 days, respectively). Furthermore, proliferation increase in HEKpSsiPKD1 *a* and *b* cells was found to be smaller than that observed in *b*3 cells, which showed the lowest PC1 levels (Fig. 1e), thus indicating a relationship between degree of PC1 suppression and cell proliferation.

PC1 expression reduces calcium oscillation

The effect of PC1 silencing on [Ca^{2+}]i evoked by FBS, at 1% concentration used in cell proliferation studies, was initially investigated in HEKpSsiPKD1 clone *a*1 through both aequorin and Fura-2AM experiments.

After transfection with Ca^{2+}-sensitive photoprotein aequorin, a slight, although non-significant, increase in serum-induced cytoplasmic Ca^{2+} peak was found in HEKpSsiPKD1 cells, as compared to control cells (bars of Fig. 2a). Nevertheless, it is apparent from representative traces of Fig. 2a that Ca^{2+} levels were more sustained and oscillating in HEKpSsiPKD1 cells. These differences were confirmed in single-cell Ca^{2+}-imaging experiments (Fig. 2b). Representative traces from
HEK293\textsuperscript{SUPER} and HEK293\textsuperscript{psiPKD1} cells revealed a pattern of changes in FBS-evoked Ca\textsuperscript{2+} levels that was characterized by oscillation frequency that was consistently higher in HEK293\textsuperscript{psiPKD1} cells. The oscillation increase was observed in 81.5% of cells (n = 76). When the analysis was performed in different HEK\textsuperscript{psiPKD1} clones, mean oscillation frequency increased ∼2-fold as compared to control cells (9.78 versus 5.52 and 5.36 in HEK293\textsuperscript{psiPKD1} and HEK\textsuperscript{psiScramble} cells, respectively, the mean values in a 15-min period, P < 0.01) (Fig. 2c), suggesting a relationship between reduced PC1 expression and increased Ca\textsuperscript{2+} oscillation. Oscillation amplitude, expressed as Ca\textsuperscript{2+} concentration, was not different in HEK293\textsuperscript{psiPKD1} cells (98.05 ± 29.12 nm and 102.00 ± 20.89 nm in HEK293\textsuperscript{psiPKD1} and HEK293\textsuperscript{psiPKD1} cells, respectively, Fig. 2d), lending further support to the hypothesized association between PC1 deficiency and oscillation frequency. Because the difference in oscillation frequency at 10% FBS concentration was markedly reduced (from about 2 in 1% FBS to about 1.4-fold in 10% FBS, Fig. 2e), further experiments were performed in 1% FBS. Moreover, because no significant differences were
observed between HEK293pSUPER and HEK293pSiScramble cells, further experiments were mainly performed using HEK293pSUPER cells.

Consistent with increase in proliferation observed in HEK293pSiPKD1 cells, oscillation frequency was higher in HEK293pSiPKD1 clones with higher proliferation rates (Fig. 2f), strongly indicating the relation between Ca2+-oscillation frequency and cell proliferation. The b3 clone, which exhibited greatest oscillation and proliferation associated with the most pronounced PC1 down-regulation (Fig. 1e), was used in the further experiments.

To test the hypothesis of a role for PC1 in regulating Ca2+-oscillatory patterns, the latter were examined in HEK293pSiPKD1 cells transiently transfected with a cDNA plasmid expressing mouse full-length PC1-containing N-ter FLAG and C-ter HA tags (Grimm et al. 2003). Exogenous expression of mouse PC1 (mPC1) was demonstrated by anti-FLAG immunoblotting in total

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Figure 2. PKD1 gene silencing increases FBS-induced Ca2+-oscillation frequency in HEK293 cells. (a) Representative cytoplasmic Ca2+ levels from control HEK293pSUPER 1 (pS, blue trace) and HEK293pSsiPKD1 a1 (red trace) cells. Ca2+ levels were measured in cells transfected with the Ca2+-binding photoprotein cytAEQ. Where indicated, cells were challenged with 1% FBS. Bars represent the peak evoked by FBS stimulation (averages ± SEM, P = 0.057) in control (blue) and HEK293pSsiPKD1 (red) clones. (b) FBS increased Ca2+ oscillation frequency in PKD1-specific siRNA expressing cells. Representative Ca2+ signals from an individual control pS (blue trace) and HEK293pSsiPKD1 a1 cell (red trace) loaded with Fura-2-AM and stimulated with 1% FBS. Average distribution of Ca2+ oscillation frequency (± SD, white parts of the bars) from HEK293pSUPER 1 (n = 66 cells, four experiments) and HEK293pSsiPKD1 a1 cells (61 = cells min period, four experiments) in 15 min after FBS stimulation. (c) Bars indicate the average Ca2+ oscillation frequency in four HEK293pSUPER, three HEK293pSsiScramble (black bar) and four HEK293pSsiPKD1 different clones. The data are the means ± SEM of five experiments (P < 0.01 versus pS, unpaired Student’s t-test). (d) Average (± SD) of Ca2+ oscillation amplitude in three HEK293pSUPER (blue bar) and five HEK293pSsiPKD1 (red bar) clones. Amplitude is expressed as Ca2+ concentration, measured as described in the Materials and Methods section. (e) Calcium oscillations (average ± SD) evoked by treatment with 0%, 1% and 10% FBS in three HEK293pSUPER (blue bars) and four HEK293pSsiPKD1 (red bars) clones. (f) Cell proliferation increase as a function of Ca2+ oscillation frequency in four different HEK293pSsiPKD1 clones relative to the HEK293pSUPER 1 control clone.
lysates of both HEK293pSUPER and HEK293pSsiPKD1 mPC1-transfected cells, and confirmed in at least 20% of cells by anti-FLAG immunofluorescence (inset of Fig. 3), indicating that mPC1 was not silenced by specific human PKD1 siRNA. mPC1 expression caused a clear reduction in oscillation frequency in some cells (Fig. 3a), leading to an average 30% reduction (Fig. 3b, left). Notably, a similar reduction was also observed in PC1-transfected HEK293pSUPER control cells (Fig. 3a, right). Combined, these findings suggest that exogenous expression of PC1 is capable of reducing Ca^{2+} oscillation in HEK293 kidney cells. Furthermore, stable transfection of mouse PC1 in HEK293 cells caused a statistically significant reduction in cell proliferation (Fig. 3c). After

Figure 3. Exogenous expression of PC1 reduces Ca^{2+} oscillation frequency in HEK293 cells. (a) Representative Ca^{2+} oscillation patterns from individual HEK293pSsiPKD1 (red) and HEK293pSUPER (blue) cells before (upper traces) and after (lower traces) transient transfection with the mouse PC1 expressing plasmid (mPC1). Cells were grown on coverslips, transfected with the plasmid DNA as described in the Materials and Methods section, loaded with Fura-2-AM after 48 h and stimulated with 1% FBS, as described in Fig. 2. Inset: the expression of the mouse PC1 was confirmed by Western blotting and immunofluorescence analysis, as shown in HEK293pSUPER (pS) and HEK293pSsiPKD1 (pSsiPKD1) transfected cells, the latter showing that mPC1 is not silenced by human PKD1 siRNA. Cells were lysed and total extracts were analysed by immunoblotting with the anti-FLAG M2 mouse monoclonal antibody recognizing the FLAG-tagged mouse PC1, as described in the Materials and Methods section. The antibody identified a band of about 400 kDa in only mPKD1 cDNA-transfected HEK293pSUPER and HEK293pSsiPKD1 cells. The ~200 kDa band was deemed to be aspecific as present in all samples. For immunofluorescence analysis, cells were fixed and treated with M2 antibody, as described in the Materials and Methods section. Upper and lower panels: contrast phase and fluorescence images. Staining of plasma membranes was indicated by arrows, mainly at cell–cell interactions. (b) Average percent reduction in Ca^{2+} oscillation frequency in a 15-min period obtained after transient transfection with the mouse PC1 expressing plasmid of HEK293pSsiPKD1 clone b_2 (red bars; n = 122 cells, five experiments) and HEK293pSUPER cells (blue bars, n = 60, three experiments). (c) Reduction in cell proliferation in HEK293 cells stably expressing the full length mouse PC1. pCDNA3 stably transfected control cells (white bar) and cells stably transfected with the full length mouse PKD1 cDNA plasmid (grey bar) were grown for 3 days in presence of 1% FBS. Data are expressed as the average values (± SD; *P<0.05 unpaired Student’s t-test) obtained from two control and four PC1-transfected clones that were plated as described in the Materials and Methods section.
3 days of 1% FBS treatment, mean cell proliferation was reduced almost by half in four different PC1-transfected clones (371,833 ± 171,660 versus 766,666 ± 28,284 cells in transfected versus untrasfected cells, respectively, \( P < 0.05 \)). This finding demonstrates that PC1-dependent reduction in Ca\(^{2+}\) oscillations is strongly associated to reduction in cell proliferation.

Calcium oscillation increases in human PC1-deficient cystic cells

Enhancement of proliferation by PKD1 RNA interference suggests that HEK293pSsiPKD1 cells behave in a similar fashion to ADPKD cyst cells, and an increase in cell proliferation and frequency of Ca\(^{2+}\) oscillations in cells derived from PKD1 cysts was therefore expected. In order to test this hypothesis immortalized cell lines from kidney cysts, which are heterozygous and putatively hemizygous for the Q2556X PKD1 mutation (Nauli et al. 2006), were analysed and results were compared to those obtained with PKD1 wild-type non-cystic cells.

After 1% FBS treatment for 24 h, cell cycle analysis showed that S and G\(_2\)/M phases in hemizygous and heterozygous PC1-mutant cystic cells were higher than in wild-type cells, thereby demonstrating a higher proliferation rate of the cystic cells (Fig. 4a). Consistently in these cells, FBS-evoked Ca\(^{2+}\) oscillations were increased (Fig. 4b). In particular, average oscillation frequency over a 15-min period was 9.35 ± 1.36 and 7.36 ± 1.16 in cystic versus 3.904 ± 0.04 in normal cells (\( P < 0.05 \), wild-type versus either hemizygous or heterozygous cells) (bars in Fig. 4b).
Inhibitors of NCCE blunt both increased oscillation and cell proliferation

In order to ascertain the type of channel involved in the increase in FBS-evoked Ca\textsuperscript{2+} oscillations in HEK293\textsuperscript{pSsiPKD1} clones, Ca\textsuperscript{2+} influx was evaluated in the presence of inhibitors of CCE and NCCE (Berridge 2002) (Fig. 5a). The frequency of FBS-evoked Ca\textsuperscript{2+} oscillations in HEK293\textsuperscript{pSsiPKD1} remained unaffected by the presence of 1 \textmu m Gd\textsuperscript{3+}, sufficient to block CCE (Mignen et al. 2003a). However, oscillations were markedly affected by 5 \textmu m Gd\textsuperscript{3+}, which also completely inhibits NCCE (Mignen et al. 2003a), in both HEK293\textsuperscript{pSsiPKD1} and HEK293\textsuperscript{pSUPER} control cells. Interestingly, treatment with 100 n\textmu m SKF96365, a putative inhibitor of receptor-operated Ca\textsuperscript{2+} entry with NCCE blocker activity at this low concentration (Moneer et al. 2003), provoked markedly greater reduction in oscillation frequency in HEK293\textsuperscript{pSsiPKD1} more than in HEK293\textsuperscript{pSUPER} cells. In particular, treatment of HEK293\textsuperscript{pSUPER} cells with either 1 \textmu m Gd\textsuperscript{3+} or 100 n\textmu m SKF96365 caused a 38% or 50% reduction in Ca\textsuperscript{2+} oscillations, respectively; conversely,

![Figure 5](image_url). NCCE-specific inhibitors blunt the effect of the PKD1 gene silencing or PKD1 nonsense mutation on FBS-induced increase in Ca\textsuperscript{2+} oscillations. Effect of gadolinium (Gd\textsuperscript{3+}) and SKF96365 on Ca\textsuperscript{2+} oscillation frequency. Representative Ca\textsuperscript{2+} traces from individual HEK293\textsuperscript{pSUPER} (blue lines) and HEK293\textsuperscript{pSsiPKD1} cells (red lines) loaded with Fura-2-AM and treated with the indicated blocker and 1% FBS, are shown. The 1 \textmu m Gd\textsuperscript{3+} did not affect Ca\textsuperscript{2+} oscillations activated by 1% FBS in HEK293\textsuperscript{pSsiPKD1} cells; 5 \textmu m Gd\textsuperscript{3+} and 100 n\textmu m SKF96365 markedly reduced Ca\textsuperscript{2+} oscillations. Inset bars represent the average distribution of Ca\textsuperscript{2+} oscillation frequency (± SD) from HEK293\textsuperscript{pSUPER} cells (1 \textmu m Gd\textsuperscript{3+}: n = 45 cells; 5 \textmu m Gd\textsuperscript{3+}: n = 50 cells; 100 n\textmu m SKF96365: n = 52 cells) and from HEK293\textsuperscript{pSsiPKD1} cells (1 \textmu m Gd\textsuperscript{3+}: n = 54 cells; 5 \textmu m Gd\textsuperscript{3+}: n = 42 cells; 100 n\textmu m SKF96365: n = 49 cells) in a 15-min period after FBS stimulation. Results are representative of three independent experiments. Blue and red bars represent percentage reductions (± SD) produced by each treatment in HEK293\textsuperscript{pSUPER} (blue) and HEK293\textsuperscript{pSsiPKD1} cells (red) compared to untreated cells.

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in HEK293pSsiPKD1 cells, 1 μM Gd^{3+} did not affect Ca^{2+} oscillations, while SKF96365 caused an 82% reduction (see percentage values in Fig. 5). Ca^{2+} oscillations were also reduced by 2.5 times in HEK293pSUPER (64%) than in HEK293pSsiPKD1 (25%) by treatment with 30 μM LOE908, another NCCE blocker (Moneer & Taylor 2002) (data not shown), suggesting involvement of NCCE activity in abnormal Ca^{2+} oscillations seen in PC1-deficient cells.

Consistently with the results on Ca^{2+} oscillations, 5 μM Gd^{3+}, but not 1 μM Gd^{3+}, 100 nM SKF96365 and 30 μM LOE908 markedly reduced the FBS-induced cell proliferation in HEK293pSsiPKD1 clones (Fig. 6a). As shown in the summary graph of Fig. 6a (showing percentage values), reductions in proliferation caused by different treatments highlighted the strong inhibition caused by NCCE blockers in HEK293pSsiPKD1 cell proliferation (67% to 61%), in comparison to the effect in control cells, which was observed to be four times lower (15%).

In order to confirm inhibition also in cyst-derived cells, the effects of NCCE channel blockers were examined in PKD1 Q2556X-mutated cell lines. As expected, cell proliferation was higher in cystic cells than in wild-type cells (152 888.6 ± 18 151 and 130 553.3 ± 10 210 versus 105 777.5 ± 4337, in heterozygous and hemizygous versus wild-type cells, P < 0.05) (Fig. 6b). Treatment with either 100 nM SKF96365 or 30 μM LOE908 NCCE blockers did not modify proliferation of wild-type cells, although it did reduce that of PKD1 heterozygous and hemizygous cells (Fig. 6b). Overall, these results confirm the association between abnormal increase in Ca^{2+} oscillations and cell proliferation in ADPKD tubular cells, ascribing the regulatory effect of PC1 mainly to NCCE.
Increase in NFAT activity in HEK293pSsiPKD1 cells is associated with cell proliferation

It has been reported that oscillations are an efficient way of differentially coding Ca\(^{2+}\) signals and that frequency of oscillations may modulate gene transcription (Dolmetsch \textit{et al.} 1998). NFAT is a Ca\(^{2+}\)-dependent transcription factor expressed in many cells, including those of the kidney (Puri \textit{et al.} 2004), which is regulated by frequency of Ca\(^{2+}\) oscillations (Tomida \textit{et al.} 2003). We, therefore, investigated whether the FBS-induced oscillations observed in HEK293 PKD1-suppressed cells affected NFAT signalling. We monitored NFAT translocation to the nucleus in HEK293pSUPER and HEK293pSsiPKD1 cells expressing a GFP-tagged NFAT2. Prior to FBS treatment (0 min of Fig. 7a, left), NFAT-GFP was present in cytoplasmic regions of both HEK293pSsiPKD1 and HEK293pSUPER cells, whereas its nuclear translocation following 1% FBS treatment was clearly faster in HEK293pSsiPKD1 than in HEK293pSUPER cells. Thus, Ca\(^{2+}\) oscillations induced rapid and constant NFAT nuclear translocation in HEK293pSsiPKD1. This faster activation was also observed in PC1-mutant cystic cells (Fig. 7a, right). Moreover, HEK293 cells were

![Figure 7](image-url)
transiently transfected with an NFAT-promoter luciferase-reporter plasmid, and luciferase activity was assessed after FBS treatment. Following 2 days of stimulation, ~3.5-fold increase in luciferase activity in HEK293pSsiPKD1 cells as compared to HEK293pSUPER cells was observed (Fig. 7b). This suggests that gene expression is modulated by the effect of PC1 depletion on the oscillatory pattern of Ca^{2+} responses and is further supported by the observation that SKF96365 treatment, which blocked HEK293pSsiPKD1 cell proliferation, also reduced the NFAT-dependent luciferase activity (Fig. 7c).

In order to test whether the FBS-induced NFAT activation contributed to the increase in proliferation of HEK293pSsiPKD1 cells, the cell proliferation rate was analysed in the presence of cyclosporin A (CsA), a typical inhibitor of the Ca\(^{2+}\)-calmodulin-dependent serine/threonine protein phosphatase calcineurin, which dephosphorylates and activates NFAT (Puri et al. 2004). FBS-induced increase in HEK293pSsiPKD1 cell proliferation was completely inhibited by CsA (Fig. 7d), supporting the hypothesis of a relationship between the reduction in PC1 levels and increase in both Ca^{2+} oscillations and NFAT-dependent cell proliferation in HEK293pSsiPKD1 cells.

Cytoplasmic Ca\(^{2+}\) levels and PKC-\(\alpha\) activity

Over all, these data suggest a model in which the impairment of PC1 signalling in PC1-deficient cells leads to elevation of serum-evoked Ca\(^{2+}\) oscillations, thus activating calcineurin and NFAT signalling and leading ultimately to cell proliferation. This hypothesis is further supported by the measurement of basal Ca\(^{2+}\) levels which, in the absence of serum, are higher in HEK293pSsiPKD1 than in control cells (140.78 ± 27.4 versus 85.92 ± 8.19, respectively). This is consistent with basal Ca\(^{2+}\) levels observed in HEK293 cells expressing the isolated cytoplasmic carboxyl terminus of PC1 (Manzati et al. 2005). As observed for glutamate-induced intracellular Ca\(^{2+}\) oscillations in astrocytes, which involve activation of conventional PKC (Codazzi et al. 2001), only serum-induced Ca\(^{2+}\) oscillations lead to Ca\(^{2+}\) levels sufficient to activate the calcium-dependent PKC-\(\alpha\) isoform (Fig. 8a). Serum treatment was indeed accompanied by a prominent recovery of PKC-\(\alpha\)-GFP fluorescence in the membrane compartments of HEK293pSsiPKD1 cells, while diffuse fluorescence in control cells indicated that the kinase remains confined to cytoplasm.

Because the PKC-\(\alpha\)-specific inhibitor Ro-320432, and not the PKC-\(\beta\) inhibitor hispidin, markedly reduced growth of HEK293pSsiPKD1 cell population (Fig. 8b); cell proliferation increase was also shown to be dependent on a PKC-\(\alpha\) pathway. As we previously observed in HEK293 cells expressing the PC1 C-terminal tail (Manzati et al. 2005), in HEK293pSsiPKD1 cells this increase was also accompanied by a marked reduction of the growth inhibitory protein p27\(^{kip1}\) (Fig. 8c, upper part). Consistently, p27\(^{kip1}\) was similarly reduced in PKD1 Q2556X-mutated hemizygous cystic cells (Fig. 8c, lower part). Moreover, treatment of HEK293pSsiPKD1 cells with either 5 \(\mu\)M Gd\(^{3+}\), 100 nM SKF96365 or 30 \(\mu\)M LOE908, which reduces cell proliferation, led to increased p27 levels (Fig. 8d).

The present findings, therefore, provide evidence that lack of balance in PC1 levels caused by a reduction in wild-type protein, as well as by PC1 C-tail overexpression, plays a critical role for Ca\(^{2+}\) signalling pathways with similar effects on gene expression and subsequently on cell cycle regulation.

DISCUSSION

Our study demonstrates for the first time that PC1 controls Ca\(^{2+}\) oscillations and provides a molecular mechanism to explain association of abnormal Ca\(^{2+}\) homeostasis and increased cell proliferation in ADPKD, caused by PKD1 mutations. We have shown that either inhibition of
PC1 expression by RNA interference in HEK293 cells or loss of PC1 expression in ADPKD kidney cystic cells increases oscillatory activity of cytoplasmic Ca\(^{2+}\), evoked by low serum concentrations. Consistently, we have also found that expression of exogenous PC1 reduces the frequency of serum-evoked Ca\(^{2+}\) oscillations not only in PC1-deficient HEK293\(^{pSsiPKD1}\) cells, but also in HEK293 control cells. The main conclusion of this study is therefore that PC1 is required for negative control of Ca\(^{2+}\) oscillation in kidney-derived cells. The implication is that PC1 exerts a profound influence on Ca\(^{2+}\) signalling by controlling Ca\(^{2+}\) oscillation induced by serum, with a crucial role in cell proliferation. Cell growth studies have shown that cell proliferation in PC1-deficient HEK293 cells is inversely related to the level of PC1 expression which, in turn, is inversely related to the Ca\(^{2+}\) oscillation frequency.

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Ca^{2+} oscillation and cell proliferation

Intracellular Ca^{2+} homeostasis appears to be altered in HEK293pSsiPKD1 cells with basal Ca^{2+} levels higher than those of controls. Increased Ca^{2+} levels have already been observed in the HEK293 cells expressing the dominant negative PC1 C-terminus (Puri et al. 2004; Manzati et al. 2005). These results therefore indicate that loss of PC1 function may be associated with increase in basal Ca^{2+} levels, which may contribute to increased proliferation of these cells. This hypothesis is also supported by the observation that overexpression of TRPV6 slightly increases intracellular Ca^{2+} levels and Ca^{2+}-dependent proliferation of HEK293 cells (Schwarz et al. 2006). However, in primary cultures of ADPKD cystic cells reduction in basal Ca^{2+} levels has been documented, imputing abnormal proliferation of cystic cells to this alteration (Yamaguchi et al. 2006). Even though characterization of molecular lesions in primary ADPKD cultures has not been reported, these differences in basal Ca^{2+} levels may be related to the different type of cell culture. Nevertheless, in the light of these contrasting results it is difficult to explain the PKD-dependent abnormal proliferation with abnormal basal Ca^{2+} levels.

Interestingly, in HEK293pSsiPKD1 cells, serum treatment that increased cell proliferation, did not produce a significant increase in evoked Ca^{2+} levels (Fig. 2a), but rather a clear change (2-fold statistically significant increase) in frequency of FBS-induced Ca^{2+} oscillations. Notably, frequency of Ca^{2+} oscillation is also increased in cystic cells either heterozygous or hemizygous for the Q2556X PKD1. Because both cystic cell lines show increased FBS-evoked Ca^{2+} oscillations, these may be a cause of aberrant proliferation in these cells as well as in HEK293pSsiPKD1 cells. It must, however, be considered that the present study was performed in immortalized cells. Although these are derived from normal and cyst-lining epithelia and maintain many features of tubule of origin, immortalized cells are poorly differentiated and polarized, particularly when cultured under subconfluent conditions such as those used for a proliferation study. Therefore, the precise role of abnormal Ca^{2+} oscillations on cyst formation and cell population growth in the kidney remains to be ascertained.

The finding that HEK293 cells expressing mouse PC1 showed reductions in both Ca^{2+} oscillation and cell proliferation, provides evidence for the role of Ca^{2+} oscillations in the PC1-dependent control of cell proliferation. In the light of these observations therefore growth suppression activity of PC1 may be explained by its inhibitory action on Ca^{2+} oscillations as was observed in both normal and HEK293pSsiPKD1 cells exogenously expressing the mouse PC1.

Ca^{2+} channel activity and cell proliferation

Ca^{2+} entry that supports [Ca^{2+}]), cycling, predominantly occurs through store-operated or a CCE pathway (Bird & Putney 2005). Nevertheless, in many cell types, including HEK293 cells, extracellular Ca^{2+} entry and Ca^{2+} oscillations also result from NCCE, whose activation is independent of store depletion (Mignen et al. 2001).

Non-capacitative Ca^{2+} entry activity appears to be crucial for HEK293pSsiPKD1 cell proliferation. Ca^{2+} oscillations in HEK293pSsiPKD1 cells are insensitive to 1 μm Gd^{3+}, but markedly inhibited by the NCCE blocker, which also inhibited cell proliferation. Moreover, in the absence of external Ca^{2+}, no difference was observed in 1% evoked Ca^{2+} oscillations between HEK293pSsiPKD1 and HEK293pSUPER control cells (3.34 ± 0.31 versus 2.58 ± 0.9 oscillations in 15 min/cell, respectively), consistently with a role of NCCE activity on the oscillation increase. Therefore, the PC1 loss leads to deregulation of the NCCE pathway, strongly associating increased Ca^{2+} oscillations and proliferation to abnormal activity of NCCE channels. Consistently, both SKF96365 and LOE908 are able to reduce proliferation in PKD1-mutated heterozygous and hemizygous cystic cells, but not wild-type kidney tubule cells. Ca^{2+} influx through NCCE channels is therefore necessary for the increased [Ca^{2+}]), oscillatory response and cell proliferation in PC1-deficient cells.
On the basis of these considerations, we hypothesize that PC1 controls an as yet uninvestigated activity of NCCE channels. In HEK293 cells, NCCE has been shown to be associated to the activity of the arachidonate-regulated Ca²⁺ channel (ARC channel) (Mignen et al. 2003a), but because we have observed that calcineurin activity is required for the FBS-induced proliferation increase in HEK293pSsiPKD1 cells (Fig. 7d), and because ARC channels are turned off by calcineurin activation (Mignen et al. 2003b), these channels do not seem to be the target of the PC1 control. On the other hand, an arachidonate-activated NCCE channel activity, which displays biophysical properties different from the ARC channels of HEK293 cells, has been reported in endothelial cells (Fiorio Pla & Munaron 2001). Interestingly, this NCCE activity is required for the promotion of cell proliferation activated by peptidic growth factors like the basic fibroblast growth factor (Fiorio Pla & Munaron 2001). This NCCE activity is also involved in peripheral and localized Ca²⁺ events, probably leading to the involvement of differential patterns of Ca²⁺-dependent proteins and genes (Tomatis et al. 2007), including calcineurin and PKC pathways, abnormally activated in HEK293pSsiPKD1 cells. This activity may lead, in addition to increased cell proliferation, to the abnormal polarity, migration and apoptosis typically observed in ADPKD cystic cells (Ong & Harris 2005).

Ca²⁺ oscillations and signalling

The results of our studies suggest that a loss of PC1 in HEK293pSsiPKD1 cells promotes a serum-induced proliferation increase by inducing Ca²⁺ entry and subsequent activation of the calcineurin/NFAT pathway. As reported in other cell types (Lipskaia & Lompre 2004), NFAT activation in HEK293pSsiPKD1 cells promotes cell cycle progression in association with a reduction in the activity of cyclin kinase inhibitors. Consistently, cyclosporin A treatment, which, by inhibiting calcineurin, inhibits NFAT dephosphorylation and its nuclear translocation (Hogan et al. 2003), blocks the PC1-dependent increase in proliferation (Fig. 7d). Here, we also show that once NCCE-mediated Ca²⁺ entry is blocked by SKF96365 treatment in HEK293pSsiPKD1 cells, NFAT activation is markedly reduced. Thus, as previously demonstrated in other cell types (Dolmetsch et al. 1998; Tomida et al. 2003), activation of NFAT in HEK293pSsiPKD1 appears to be linked to increase in Ca²⁺ oscillation, possibly accompanied by the release of Ca²⁺ from intracellular stores and activation of store-operated Ca²⁺ entry, as proposed in cells expressing the PC1 C-terminus (Puri et al. 2004). Because this peptide appears to act as a dominant negative (Manzati et al. 2005; Xu et al. 2007), NFAT activation in these cells is consistent with that found in HEKpSsiPKD1 cells. HEK293pSsiPKD1 cell proliferation requires PKC-α activation and p27 down-regulation (see Fig. 8), as in cells expressing the PC1 C-terminus that show reduced levels of both p27Kip and p21Waf associated to increased cell proliferation (Manzati et al. 2005). This observation is consistent with previous findings showing that exogenous expression of full-length PC1 in kidney cells induces cell cycle arrest in G₀/G₁ by up-regulating p21Waf expression through the JAK-STAT pathway (Bhunia et al. 2002). It has also been reported that calcineurin and NFAT activity is required in keratinocytes for the expression of p21 and p27, which contribute to growth arrest of these cells (Santini et al. 2001). This supports the postulated cross-talk between calcineurin, p21 and p27. In HEK293pSsiPKD1, this cross-talk may either be dysfunctional or p27 gene down-regulation may result by one or more different Ca²⁺-dependent signalling pathways activated by PC1 depletion (see Fig. 8).

Furthermore, the association between PKC-α membrane translocation and Ca²⁺ oscillation is consistent with the activation of PKC by glutamate-induced Ca²⁺ waves in astrocytes (Codazzi et al. 2001).

Because NFAT nuclear translocation occurs in HEK293pSsiPKD1 and control cells only after serum treatment, the role of basal Ca²⁺ again appears to be irrelevant in the proliferation increase of
PC1-deficient cells, while the role of Ca\textsuperscript{2+} oscillations appears to be crucial. These results provide new insight into the molecular and physiological mechanism of Ca\textsuperscript{2+} oscillations in PKD1-like kidney cells and in promoting NFAT-dependent kidney cell proliferation.

In summary, our results establish that the molecular suppression of endogenous PC1 in HEK293 cells and PKD1 cystic cells leads to abnormal Ca\textsuperscript{2+} oscillations and has a profound influence on Ca\textsuperscript{2+} signalling and cell proliferation, as illustrated in Fig. 9. At present, it is impossible to say how many steps may lie between PC1 and the NCCE channel. Because PC1 has been implicated in gene expression control by activating a variety of transcription factors including the NFAT investigated here, NCCE modulation may result from changes in gene expression, or in post-translational modification (Li et al. 2005). Moreover, either a direct or indirect interaction between PC1 and NCCE channel may exist. PC1 may, in fact, constitute Ca\textsuperscript{2+}-permeable channel activities either by interacting with PC2 in the PC1/PC2 receptor-ion channel (Hanaoka et al. 2000), or independently of PC2 (Babich et al. 2004).

In conclusion, our findings suggest that PC1 may function as a controller of Ca\textsuperscript{2+} release in response to extracellular stimuli also through the NCCE pathway, thus regulating the Ca\textsuperscript{2+}-mediated signal transduction involved in cell proliferation through the NFAT pathway. Moreover, because NCCE channel blockers may reduce the PC1 suppression-dependent [Ca\textsuperscript{2+}]\textsubscript{i} oscillation as well

Figure 9. A hypothetical model illustrating the role of PC1 on the agonist-evoked NCCE channel activity and effects of Ca\textsuperscript{2+} oscillations (Ca\textsuperscript{2+}Os) on NFAT pathway and cell proliferation. In HEK293pSUPER and tubular kidney control cells, PC1 negatively modulates NCCE activity through a still undefined direct or indirect mechanism (dotted bar); Ca\textsuperscript{2+} oscillations are dependent on both CCE and NCCE activities as shown by comparable inhibitory effects of 1 \( \mu \)M Gd\textsuperscript{3+} and SKF96365 on Ca\textsuperscript{2+} oscillations and cell proliferation (Figs 5 and 6). Loss of PC1 function causes an increase in NCCE channel activity and in Ca\textsuperscript{2+} oscillations thus leading to an increase in Ca\textsuperscript{2+}-dependent NFAT activation and cell proliferation.
as cell proliferation, these channels could be potential therapeutic targets for treatment of the abnormalities observed in cystic cells.

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