Expression of polycystin-1 enhances endoplasmic reticulum calcium uptake and decreases capacitative calcium entry in ATP-stimulated MDCK cells


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Hooper, K. M., A. Boletta, G. G. Germino, Q. Hu, R. C. Ziegelstein, and M. Sutters. Expression of polycystin-1 enhances endoplasmic reticulum calcium uptake and decreases capacitative calcium entry in ATP-stimulated MDCK cells. Am J Physiol Renal Physiol 289: F521–F530, 2005. First published May 3, 2005; doi:10.1152/ajprenal.00355.2004.—Autosomal dominant polycystic kidney disease (ADPKD) types 1 and 2 arise as a consequence of mutations in the PKD1 or PKD2 genes, encoding polycystins-1 and -2. Because loss of function of either of the polycystins leads to a very similar phenotype and the two proteins are known to interact, polycystins-1 and -2 are probably active in the same pathway. The way in which loss of either polycystin leads to the development of ADPKD remains to be established, but disturbances of cell calcium regulation are likely to play an important role. Here, we demonstrate that polycystin-1, heterologously expressed in Madin-Darby canine kidney cells, had a pronounced effect on intracellular calcium homeostasis. ATP-induced calcium responses in transfection control cells exhibited a double peak and relatively gradual return to baseline. By contrast, cells expressing heterologous polycystin-1 showed a brief, uniphasic peak and an accelerated rate of decay. Heterologously expressed polycystin-1 accelerated endoplasmic reticulum (ER) calcium reuptake and inhibited capacitative calcium entry; we found no effect of the protein on mitochondrial calcium buffering or plasma membrane calcium extrusion. We therefore propose that polycystin-1 accelerated the decay of the cell calcium response to ATP by upregulation of ER calcium reuptake and consequent minimization of the stimulus for capacitative calcium entry. It is possible that cellular dedifferentiation, fluid secretion, and proliferation might therefore arise in ADPKD as a consequence of disturbances in cytoplasmic and ER calcium homeostasis and aberrant capacitative calcium entry.

autosomal dominant polycystic kidney disease; purinergic; calcium signaling; stable transfection; Madin-Darby canine kidney cells

AUTOSOMAL DOMINANT POLYCYSTIC kidney disease (ADPKD) arises as a consequence of loss of function of either polycystin-1 or polycystin-2, the gene products of the PKD1 and PKD2 genes respectively (10a, 22). ADPKD type 2, arising from mutations in the PKD2 gene, typically progresses somewhat less rapidly than ADPKD type 1, but the latter accounts for the majority of affected individuals. The phenotypes associated with either genotype are otherwise very similar, indicating that the two proteins probably function in the same pathway. The disease, which affects 1 in 1,000 individuals, is characterized by the development of multitudes of renal cysts. As these cysts expand, the resultant compression and distortion of normal renal tissue leads to loss of renal function, which typically progresses to end-stage and dialysis dependency by mid- to late adulthood. Polycystin-1 is a very large transmembrane protein, which appears to be active in a number of diverse pathways. The isolated truncated cytoplasmic COOH terminus of polycystin-1 has shown activity in 1) wnt signal transduction pathways (18); 2) AP-1 transcription factor activation (2); 3) heterotrimeric G protein-coupled signal transduction (25); 4) regulation of calcium channel activity (33, 37); 5) in-gel morphogenesis (24); and 6) modulation of ATP-stimulated cell calcium and chloride secretory responses (16, 34). The full-length protein has been shown to 1) regulate the ion channel properties of polycystin-2 (14, 2) promote tubulogenesis and resistance to apoptosis (5, 3) activate the JAKSTAT pathway (3), and 4) regulate G protein-coupled signal transduction (10). Polycystin-1 has been localized to the plasma membrane (6), at the sites of adherens junctions (17) and focal adhesions (35), and also, more recently, in the cilia (39). Polycystin-2 is predominantly seen in the endoplasmic reticulum (ER) (19) but may also be expressed at the cell membrane (13) and apical cilia (39). The cell calcium response to cilial deflection appears to depend on expression of functional polycystin-1 and -2 (23). The observation that polycystin-2 is a nonspecific cation channel with predominant calcium conductance (19) raised the possibility that ADPKD might develop as a consequence of disturbed intracellular calcium homeostasis (30). However, the effects of the polycystins on cell calcium homeostasis, whether acting independently or as a functional complex, are currently incompletely understood. If dysregulation of cell calcium homeostasis is an important mechanism of disease, in view of the fact that ADPKD types 1 and 2 share an almost identical phenotype, it would be predicted that the two proteins should have concordant effects on some aspect of cell calcium regulation. However, from studies of the effects of the polycystins on cell calcium homeostasis it is not clear that the two proteins always have consistent functions in this respect. Although heterozygous loss of polycystin-2 has been linked to a fall in resting cytoplasmic calcium concentration in vascular smooth muscle cells (27), homozygous loss of polycystin-1 activity seemed not to alter resting calcium concentration in collecting duct cells (23). Overexpression of polycystin-2 appeared to increase the cell calcium response to vasopressin in LLC-PK cells (19); we are not aware of any previous studies of heterologously expressed full-length polycystin-1 on stimulated cell calcium responses, but loss of functional polycystin actually caused an increase in...
the thrombin-stimulated cell calcium response in collecting duct cells (23). That polycystin-1 and -2 are required for the cell calcium response to cilial deflection is perhaps the most convincing evidence for a functional concordance in the regulation of cell calcium homeostasis (23).

To achieve a more complete understanding of the role of the polycystins in integrated cell calcium homeostasis, it is clear that more work is required to examine the effects of heterologically expressed proteins and loss of polycystin function on resting and stimulated cell calcium responses in renal epithelia and other cell types.

In earlier studies, we found that expression of the isolated cytoplasmic COOH terminus of polycystin-1 resulted in the prolongation of ligand-activated cell calcium responses (16, 34). Others have presented data consistent with these findings (1). We found that this effect resulted in increased ATP-stimulated chloride secretion and proposed that, in promoting a prosecretory phenotype, the truncated polycystin-1 fragment was acting as a dominant negative with respect to endogenous polycystin-1. The possibility that the cytoplasmic COOH-terminal polycystin-1 truncation might act as a dominant negative has been raised by other studies (31, 38). Consequently, we predicted that full-length polycystin-1 should have the opposite effect to that of the truncation, to abbreviate the ATP-stimulated cell calcium response. In the findings of the current study, we show that this is indeed the case. Our experiments indicate that heterologously expressed full-length polycystin-1 abbreviated the cell calcium response to ATP through a combination of acceleration of ER calcium reuptake and attenuation of capacitative calcium entry.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**

Stably transfected Madin-Darby canine kidney (MDCK)-derived cell lines expressing human polycystin-1 were developed as previously described (5). The original parental cells were MDCK type II. It should be noted that heterologous human polycystin-1 was not expressed at high levels in these cells, because the protein was only detectable after enrichment by immunoprecipitation (5). Native expression of polycystin-1 in these cell lines was only detectable by RT-PCR (data not shown). In the experiments described below, we studied two vector control (F6 and F8) and two polycystin-1-expressing cell lines (C8/68 and G7/36) between passages 4 and 16. Cells were propagated in DMEM/F-12 supplemented with 5% fetal calf serum under dual selection with G418 and Zeocin and were passaged weekly at $1 \times 10^6$ cells/25-cm$^2$ flask. To prepare cells for calcium imaging, glass coverslips were coated with Matrigel (0.45 mg/ml) for at least 1 h at room temperature. Cells were sparsely seeded onto the coated glass coverslips and grown for 3 days in culture. Small clusters of cells were selected for imaging. These islands of cells were chosen to ensure that the same number and density of cells (25 cells/field) was studied in each experiment. The morphology of these groups was similar in all the cell lines studied.

**Fluorescent Cell Calcium Measurements**

The coverslips were placed in a perfusion chamber mounted on the stage of a Nikon Diaphot microscope fitted with dual photomultipliers and illuminated with a xenon light source passed through a monochromator and appropriate filters (Photon Technologies International). The cells were excited at 340 and 380 nm, and emission was measured at 510 nm. Cell fluorescence was measured in response to excitation for 100 ms once every second. Shutter opening was controlled using a Spectroscan device driven by Felix software. Imaging was performed with a long-working-distance Nikon Fluor 40 lens. All perfusion was performed at room temperature at 2 ml/min to ensure rapid turnover of the 0.25-ml bath volume.

**Bath Calcium-Switch Repeat-Stimulation Studies**

Cells were loaded in a 5 μM solution of the cell-permeant acetoxymethyl (AM) ester of the calcium indicator fura 2 (fura 2-AM) at room temperature for 45 min in DMEM/F-12. All fluorescent dyes used in these experiments were made up to a 1 mM stock solution in 10% Pluronic/DMSO. Following incubation, cells were allowed to recover for 30 min in DMEM/F-12. The incubation and recovery media both contained 5 mM probenecid to maximize dye loading through inhibition of dye efflux. Cells were then switched into the perfusion chamber in a zero-calcium EGTA buffer and stimulated with 10 μM ATP for a 30-s pulse in this buffer. Cells were then switched into a calcium-containing buffer for 10 min to refill calcium stores before being stimulated again with a second 30-s pulse of ATP in the presence of bath calcium. This sequence of studies was used to avoid the need for a prolonged zero-calcium washout of calcium-containing buffer.

**Measurement of Intracellular Calcium Stores by Ionomycin**

The magnitude of compartmentalized calcium content can be inferred from the peak in cytoplasmatic calcium concentration in response to the release of this calcium by ionomycin (4). However, we were concerned that the peak might have been rendered very transient as a consequence of concurrent loss of calcium from the cell through the ionomycin-permeabilized plasma membrane. For this reason, we decided to use fura 5F in these experiments, because the rapid response of this indicator made it superior to fura 2 in the capture of short-lived calcium transients. Cells were loaded with 5 μM fura 5F-AM for 45 min at room temperature in the presence of 5 mM probenecid. After being mounted on the microscope stage, cells were perfused with a zero-calcium (200 μM BAPTA) buffer and treated with 10 μM ionomycin at 100 or 460 s post-ATP, to release the total sequestered intracellular calcium stores at these time points. Ionomycin was added at 2 ml/min using a gravity-feed system through a separate perfusion pathway to prevent carryover contamination.

**Manganese Quench Studies**

Because of the duration of these studies and the presence of basal quenching, cells were maximally loaded with 10 μM fura 2-AM for 45 min at room temperature in the presence of 5 mM probenecid. The cells were perfused with nominally calcium-free extracellular buffer containing MnCl$_2$ (which quenches the fura 2 signal on access to the cytoplasm) and treated with 10 μM ATP for 30 s. For these studies, cells were excited at 360 nm, the isosbestic point of fura 2, and the emission was recorded at 510 nm.

**ER Calcium Imaging**

Cells were loaded with 10 μM mag-fura 2-AM for 1 h 15 min at room temperature in the absence of probenecid. Cells were perfused with zero-calcium EGTA extracellular buffer and then permeabilized with saponin (20 μg/ml). Once the mag-fura 2 escaped from the cytoplasm (defined as a decline in the 340-nm signal to one-third of the starting value), the cells were switched into the intracellular buffer. Transfer to intracellular buffer resulted in an increase in ER calcium signal because of activation of the ER calcium ATPase, previously rendered inactive through ATP depletion in the permeabilizing buffer (which contained no ATP). ER calcium release was then induced by treatment with a 30-s pulse of 10 μM inositol...
1,4,5-triphosphate (IP3), followed by restoration of intracellular buffer to terminate the IP3 response and promote ER calcium reuptake.

Assessment of the mitochondrial contribution to ATP-stimulated calcium responses. To determine the contribution made by mitochondrial calcium uptake to the clearance of calcium from the cytoplasm following ER calcium release, ATP responses were measured after treatment with FCCP. FCCP is a mitochondrial membrane-potential uncoupler, which abolishes the driving force for calcium uptake into this organelle (12). In these experiments, coverslips were incubated in 2 μM FCCP for 1 min before a 30-s stimulation with 10 μM ATP. These experiments were performed in the absence of bath calcium (EGTA buffer) to demonstrate calcium release from mitochondria on exposure to FCCP and to preclude potentially confounding effects of calcium entry.

Assessment of the contribution made by plasma membrane calcium transport. We determined that plasma membrane sodium/calcium exchange did not contribute to calcium extrusion because substitution of bath sodium with choline had no effect on the width of the ATP response (data not shown). For a variety of technical reasons, we were unable to establish a protocol to effectively inhibit the other principal extrusion pathway, the plasma membrane calcium ATPase. We were unable to use lanthanum or calmidazolium because they either abolished or markedly inhibited the ATP response in all cell lines (data not shown). Neither vanadate nor alkalinization of the bath appeared to have any effect on plasma membrane calcium ATPase because neither broadened the cell calcium responses (data not shown). As an alternative approach, we used the C18 conjugate of fura 2 as a “near-membrane” calcium probe in the external plasma membrane leaflet to monitor the appearance rate of calcium in the immediate extracellular compartment (9). Cells were loaded with 10 μM C18 fura 2 for 45 min at room temperature. We confirmed the extracellular location of the indicator by demonstrating complete quenching with 5 mM nickel, which cannot cross the cell membrane and therefore had no effect on the fluorescent signal of fura 2-AM-loaded cells. C18 fura 2 calibration curves were constructed in bath calcium concentrations, ranging from 17 to 600 nM, using a concentrate calibration kit from Molecular Probes.

Buffers and Reagents

The buffers used were composed as follows (in mM): extracellular calcium buffer (140 NaCl, 5 KCl, 2 CaCl2, 1.8 MgCl2, 10 HEPES, 5 probenecid, pH 7.4 with NaOH); 0-calcium EGTA extracellular buffer (140 NaCl, 5 KCl, 1.8 MgCl2, 10 HEPES, 0.1 EGTA, 5 probenecid, pH 7.4 with NaOH); 0-calcium BAPTA extracellular buffer (140 NaCl, 5 KCl, 10 HEPES, 0.2 BAPTA, 5 probenecid, pH 7.4 with NaOH); and intracellular buffer (19 NaCl, 125 KCl, 10 HEPES, 1 EGTA, 0.33 CaCl2, 1.4 MgCl2, 3 ATP, pH 7.2 with KOH). The BAPTA and fluorescent dyes were obtained from Molecular Probes, and all other chemicals were from Sigma. All the fluorescent dyes were made up to 1 mM stock solutions in DMSO to aid solubilization. Stock solutions of FCCP (Sigma) were made up to 2 mM in DMSO.

Data Analysis

To present actual cell calcium data, response traces for individual experiments were superimposed (based on the initial upstroke) and plotted as the mean flanked by the SE range using Graph Pad software (Prism). Because the responses were not perfectly synchronous, statistical analysis required manual examination from individual experiments to determine the peak amplitude, time for decay from peak to 80% of peak (tpeak-80%), and the time taken for decay from 80% of peak to 20% of peak (t80-20%). These numerical descriptors were applied as a simple way of quantifying the kinetics of the calcium responses and have no a priori physiological significance. In the manganese quench experiments, the effect of ATP on the rate of manganese entry was determined as the slope for the 1-min interval immediately preceding ATP stimulation subtracted from the slope for the 1-min interval starting 2 min after stimulation. In similar fashion, the rate of decay of the Ca2+ transient induced by FCCP was also expressed as the slope. Comparisons between cell line groups were made by t-test, with significance accepted at the level of P < 0.05.

RESULTS

In a series of concentration-response experiments (Fig. 1), it was determined that 10 μM ATP produced an 80%-of-maximal ATP-stimulated cell calcium response in both control and polycystin-1 expressing cell lines. Because responses in the two groups of cells were very similar, it is unlikely that expression of polycystin-1 was having a dominant effect on the transduction of extracellular calcium.

Fig. 1. Heterologous expression of polycystin-1 abolished the plateau and accelerated the decay of the ATP-stimulated Ca2+ response. Cell lines F6 and F8 are transfection controls for the heterologous polycystin-1-expressing cell lines C8/68 and G7/36. Shown here are the cellular Ca2+ responses to 10 μM ATP, measured using fura 2. A: each trace is a composite of multiple individual experiments, presented as the mean flanked by SE. B: concentration-response curves show that both sets of cell lines exhibited 80% of maximal response at the dose of ATP used in these experiments. C: peak and decay characteristics of curves are described in the bar graphs as tpeak-80% and t80-20%, respectively. In each experiment, the same groups of 25 cells were exposed to ATP twice, first in zero-Ca2+ buffer and again, after 10-min washout perfusion in 2 mM Ca2+ buffer. In the presence of bath Ca2+, the control cells had very obviously prolonged, multiply peaked plateaus and slow decay times compared with the polycystin-1-expressing lines. In the absence of bath Ca2+, the difference between groups in the plateau phase was much less marked. By contrast, prolongation of decay times in control cells compared with polycystin-1-expressing cells remained the same irrespective of bath Ca2+ concentration. F6, n = 6; F8, n = 7; C8/68, n = 6; G7/36, n = 6. *P < 0.05, exact values listed in RESULTS and DISCUSSION.
Effect of Polycystin-1 on Cell Calcium Responses to Stimulation with ATP in the Absence and Presence of Bath Calcium

Store-operated or capacitative calcium entry is thought to make a significant contribution to the latter part of the calcium response (26). Therefore, to examine the contribution made by capacitative calcium entry, ATP responses were studied twice in each preparation, first in the absence of bath calcium, and then in the presence of bath calcium. Following ATP treatment in EGTA buffer, cells were allowed to recover for 10 min in calcium containing buffer before repeat exposure to ATP. As shown in Fig. 1, in the presence of bath calcium, the ATP-stimulated cell calcium responses of the control cell lines F6 and F8 exhibited a broader peak and more gradual decay than the polycystin-1-expressing cell lines C8/68 and G7/36 [tpeak-80% 29.2 (SE 2.8) vs. 10.6 s (1.2 SE), P = 4.44 × 10−2; t50-20% 37.4 (SE 4.8) vs. 22.4 s (SE 1.9), P = 0.001]. The basal fluorescence ratio, given in arbitrary units, was slightly lower in polycystin-1-expressing cells in zero-calcium [control 0.83 (SE 0.02) polycystin-expressing 0.76 (SE 0.02), P = 0.014] but was not significantly different between cell lines in 2 mM calcium buffer [control 0.86 (SE 0.02), polycystin-expressing 0.81 (SE 0.02), P = 0.09]. The peak amplitude of the ATP response was no different between cell lines, irrespective of bath calcium. Manipulation of bath calcium had no effect on the decay rate of the ATP response in either group of cell lines, [t50-20% 0-calcium vs. calcium-containing buffer; control, 36.8 (SE 3.2) vs. 37.4 s (SE 4.8), P = 0.88; polycystin-expressing cells 26.2 (SE 1.8) vs. 22.4 s (SE 1.9), P = 0.26]. However, the peaks of the control cell responses were greatly expanded by replacement of bath calcium, exhibiting a second and often third spike, [tpeak-80% 15.4 (SE 1.8) in zero-calcium vs. 29.2 s (SE 2.8) plus calcium, P = 6.11 × 10−2]. By contrast, the peak calcium response to ATP was always uniphasic in the polycystin-1-expressing cells, being unaffected by bath calcium concentration [tpeak-80% 10.5 (SE 1.0) in zero-calcium vs. 10.6 s (SE 1.2) plus calcium, P = 0.86]. Because of the influence of bath calcium on the control cells, the difference between the two groups of cell lines in the breadth of the peak in 2 mM calcium buffer [tpeak-80% control 29.2 (SE 2.8), polycystin-expressing 10.6 s (SE 1.2), P = 4.44 × 10−9] was less evident in the zero-calcium bath [tpeak-80% control 15.4 (SE 1.8) vs. polycystin-expressing 10.5 s (SE 1.0), P = 0.027].

It therefore appeared that polycystin-1 was influencing cell calcium responses through combined effects on calcium entry (suppression of bath calcium-dependent multiple peaking) and intracellular calcium turnover (bath calcium-independent acceleration of the decay of the calcium response).

Effect of Polycystin-1 on Ligand-Activated Calcium Entry

To assess calcium entry using an independent methodology, the manganese quench approach was used to examine activation of calcium entry pathways. In these experiments, cells were loaded with fura 2-AM and then perfused in a nominally calcium-free bath containing 0.1 mM manganese. Manganese enters the cell via store-operated or capacitative calcium channels and quenches fura 2 fluorescence when excited at the isosbestic wavelength of 360 nm. Therefore, the rate of manganese quench of fura 2 fluorescence provides an index of the degree of activation of calcium entry channels (21). As can be seen in Fig. 2, treatment with ATP resulted in a steepening of the quench slope in the control but much less so in the polycystin-1-expressing cell lines [Δslope; control −887 (SE 137) vs. polycystin-expressing −143 (SE 67) fluorescent units/s, P = 3.86 × 10−5]. Basal quench slopes were no different between cell lines [control −1,208 (SE 102), polycystin-expressing −1,083 fluorescent units/s (SE 88), P = 0.37]. The manganese quench and zero-calcium experiments therefore indicated that polycystin-1 inhibited ATP-stimulated calcium entry, providing a plausible explanation for the narrowed peaks in the calcium response in the polycystin-1-expressing cell lines C8/68 and G7/36. However, the effect of polycystin-1 on calcium entry could not have accounted for the differences between cell lines over the later phase of the response, which was not sensitive to changes in bath calcium.

As depicted diagrammatically in Fig. 3, there were several alternative explanations for the effect of polycystin-1 on the decay phase of the calcium response. The heterologously expressed protein might have promoted more rapid clearing of

![Fig. 2. Overexpressed polycystin-1 inhibited capacitative Ca²⁺ entry following stimulation with ATP. In these experiments, cells were loaded with fura 2 and perfused in a buffer containing 0.1 mM manganese. Under these conditions, manganese entered the cells through plasma membrane Ca²⁺ channels activated by ATP-stimulated ER store depletion. Upon translocation into the cytoplasm, manganese quenched the fura 2 fluorescent signal; (excitation was at the isosbestic wavelength of 360 nm); the rate of quench was therefore proportional to the degree of activation of capacitative Ca²⁺ entry channels. The traces show a representative experiment, where treatment with ATP resulted in a steepening of the quench curve in controls, which was less apparent in polycystin-1-expressing cells. In the bar charts, these data are represented as the mean and SE of the Δslope (the difference between the pre- and post-ATP slopes sampled as indicated by the boxes superimposed on the traces). In response to ATP, there was a pronounced increase in manganese quench in control cells but very little increased quench in the polycystin-1-expressing cells, indicating suppression of capacitative Ca²⁺ entry channel activity by polycystin-1. F8, n = 10; F6, n = 12; C8/68, n = 10; G7/36, n = 9.*P = 3.85 × 10−5 between groups.](http://ajprenal.physiology.org/)}
cytoplasmic calcium following the calcium release phase through 1) enhanced reuptake into some intracellular compartment (most probably the ER); 2) increased buffering within the cell, (possibly by mitochondria); or 3) increased calcium extrusion across the cell membrane. The next series of experiments was designed to examine each of these possible explanations for the effect of polycystin-1.

Use of Ionomycin to Assess the Effect of Polycystin-1 on the Rate of Store Repletion Following a Calcium Release Stimulus

We reasoned that, if polycystin-1 accelerated calcium reuptake into intracellular stores following a calcium release response, then the total calcium content within these stores should recover more rapidly after stimulation with ATP (Fig. 3). Therefore, if total store calcium could be assayed at a defined time soon after ATP exposure, the levels should be greater in cells expressing polycystin-1. Ionomycin can be used to provide an indication of total intracellular calcium stores, because at high concentrations, this agent will render not only the plasma membrane but also intracellular membranes permeable to calcium (4). If applied to fura 2-loaded cells in a zero-calcium bath, ionomycin-induced cell calcium responses can only arise as a consequence of calcium release from within the cell and therefore provide an indication of the total amount of calcium sequestered within membrane-delimited intracellular compartments. The results obtained using this approach are described in Fig. 4. In these experiments, BAPTA was used as the bath calcium buffer because of its greater affinity and more rapid binding of calcium. Fura 5F-AM was chosen as the calcium indicator because of its capacity to report high-amplitude or fleeting calcium transients more effectively than fura 2, as discussed in EXPERIMENTAL PROCEDURES. Under basal, non-stimulated conditions, ionomycin-induced cell calcium responses in cells perfused with a BAPTA-buffered solution were the same in control and polycystin-1-expressing lines (ionomycin-induced peak fluorescence ratio: controls 0.27 (SE 0.03) vs. polycystin-expressing cell lines 0.27 (SE 0.03), $P = \frac{11005}{11005}$). The control ionomycin response approached that of polycystin-expressing cells if given enough time (460 s, bottom), explaining why basal ionomycin responses were no different between cell lines. For the basal ionomycin experiment, F6, $n = 8$; F8, $n = 7$; C8/68, $n = 6$; G7/36, $n = 5$. For the ATP-stimulated experiment, $n = 7$ for each cell line. *$P < 0.05$, exact values given in text.

Fig. 4. Polycystin-1 promoted more rapid refilling of total intracellular sequestered Ca$^{2+}$ stores following treatment with ATP. Cell Ca$^{2+}$ was measured with fura 5F in cells incubated in 0-Ca$^{2+}$ buffer. A: traces are composites depicting the mean and SE. In the traces on the left, ionomycin application resulted in release of sequestered Ca$^{2+}$ into the cytoplasm, detected as a fluorescence peak. These basal responses were the same in all cell lines (B; top), indicating that Ca$^{2+}$ stores were no different at baseline. The traces on the right show the ATP responses, followed by ionomycin responses. The post-ATP ionomycin peaks were greater in the polycystin-1-expressing cells, indicating enhanced reuptake of Ca$^{2+}$ into intracellular stores compared with control. B: bar graphs show that 1) basal ionomycin responses were the same between groups, 2) ATP responses were, if anything, somewhat greater in polycystin-1-expressing cells, and 3) ionomycin responses 100 s after ATP were greater in the polycystin-1-expressing cells ($P = 3.41 \times 10^{-5}$). The control ionomycin response approached that of polycystin-1-expressing cells if given enough time (460 s, bottom), explaining why basal ionomycin responses were no different between cell lines. For the basal ionomycin experiment, F6, $n = 8$; F8, $n = 7$; C8/68, $n = 6$; G7/36, $n = 5$. For the ATP-stimulated experiment, $n = 7$ for each cell line. *$P < 0.05$, exact values given in text.
0.95]. This indicated that, in unstimulated cells, the total compartmentalized intracellular calcium content was not influenced by polycystin-1. The response to ionomycin was considerably attenuated in all cell lines when exposed to the ionophore 100 s after the ATP-induced cell calcium response. This was expected because, in the absence of bath calcium, ATP stimulation would have caused depletion of intracellular calcium stores that could not have been replenished by the usual capacitative entry of calcium from outside the cell. Residual ionomycin peaks were significantly greater in cells expressing polycystin-1 [controls 0.053 (SE 0.007); polycystin-1-expressing cells 0.108 (SE 0.008), \( P = 3.4 \times 10^{-5} \)], indicating that calcium stores were replenished more rapidly in these cells. In these experiments, the ATP peaks preceding exposure to ionomycin were greater in the polycystin-1-expressing cell lines [0.36 (SE 0.02) vs. 0.47 (SE 0.03), \( P = 0.007 \)], indicating that these cells did not simply release less calcium in response to ATP. In a separate series of experiments, cells were subjected to essentially the same protocol, except that ionomycin was applied 460 s following exposure to ATP, i.e., 3 min later than in the original ionomycin experiments. Following this additional 3-min period, the control cells, but not the polycystin-expressing cells, showed an increase in the ionomycin-releasable pool compared with the 100-s ionomycin response [controls from 0.053 (SE 0.007) to 0.074 (SE 0.005), \( P = 0.018 \); polycystin expressors from 0.108 (SE 0.008) to 0.109 (SE 0.01), \( P = 0.92 \)]. This is consistent with the notion that calcium reuptake rates were increased by polycystin-1 but that the somewhat slower calcium uptake rate of control cells was still sufficient to accumulate an equivalent calcium store from available intracellular sources, given a little more time. This would be expected in view of the lack of difference between cell lines in the basal ionomycin responses.

We are proposing that the ionomycin release peak is related to the rate of calcium reuptake following ATP-stimulated release and that increased reuptake explains the decrease in \( I_{80-20\%} \) in polycystin-1-expressing cells. Therefore, it would be predicted that there should be an inverse relationship between the magnitude of the ionomycin peak and the \( t_{80-20\%} \). This is shown to be the case in Fig. 5.

**Direct Measurement of the Effect of Polycystin-1 on the Rate of Recovery of ER Calcium Concentration Following a Calcium Release Stimulus**

The ionomycin approach indicated the total intracellular calcium content of compartmentalized calcium. To complement and corroborate the ionomycin experiments, we performed direct measurement of compartmentalized calcium concentration using the low-affinity calcium indicator mag-fura 2-AM. Because cells had to be permeabilized to minimize signal contamination from cytoplasmic dye (therefore mandating the use of an ATP-containing intracellular buffer), it was not possible to examine receptor-mediated ATP responses. For this reason, we used exogenous IP \(_3\) as the calcium release stimulus, thereby examining the influence of polycystin-1 on ER calcium responses independently of possible effects at the receptor level. As shown in Fig. 6, administration of IP \(_3\) resulted in a prompt fall in compartmentalized calcium concentration, which recovered on washout. It is most likely that we were measuring calcium within the ER, because this is the principal IP \(_3\)-responsive compartment. The \( t_{peak-80\%} \) and \( t_{80-20\%} \) values were again used to describe two components of the ER response, duration of peak depletion and rate of refilling, respectively. Expression of polycystin-1 was associated with a shortened period of IP \(_3\)-induced maximal compartmental depletion \( t_{peak-80\%} \); control 49.9 (SE 1.7) vs. polycystin-expressing 37 s (SE 2), \( P = 3.6 \times 10^{-5} \) and an accelerated rate of refilling \( t_{80-20\%} \); control 38.7 (SE 2.8) vs. polycystin-expressing 29.6 s (SE 1.4), \( P = 0.016 \). As can be seen from the composite response curves in Fig. 6, the fluorescent signal returned to the prestimulus baseline by 100 s in the polycystin-1-expressing cells, whereas the control cells remained significantly below baseline at the 100-s time point and beyond, up to the termination of the experiment. In keeping with this observation, the 100-s-post-IP \(_3\) minus the pre-IP \(_3\) fluorescence values were significantly different from an hypothesized mean of 0 for control but not polycystin-1-expressing cells [control \(-0.031\) (SE 0.007), \( P = 0.0007 \), polycystin-1-expressing 0 (SE 0.006), \( P = 0.97 \)]. The pre-IP \(_3\) baseline fluorescent signal was not significantly different between groups [control 0.64 (SE 0.04) vs. polycystin-1-expressing 0.59 (SE 0.04), \( P = 0.4 \)].

**Examination of the Role of Mitochondrial Buffering of Calcium in the Effect of Polycystin-1**

Because mitochondria can accumulate calcium during peaks in cytoplasmic calcium concentration (11), it was possible that polycystin-1 might have accelerated the decay of the cell calcium response through upregulation of mitochondrial calcium buffering. As seen in Fig. 7, the effect of polycystin-1 to accelerate the decay of the cytoplasmic calcium response to ATP persisted after pretreatment with FCCP to abolish mitochondrial calcium uptake. A small calcium transient was seen after treatment with FCCP, probably due to release from the depolarized mitochondrial compartment. There were no differences between cell lines in the magnitude of the FCCP-
switched at the beginning of the calibration experiments, it was clear that this BAPTA-buffered solution actually had a much greater calcium concentration than that of the lowest calcium (17 nM) standard. In the calibration curve, the difference between cell lines in the near-membrane signal was not so marked, perhaps because of initial washout in the lowest calcium standard.

DISCUSSION

Polycystin-1 and Ligand-Activated Cell Calcium Responses

In the experiments reported in this paper, we used a variety of approaches to examine each of the possible explanations (shown diagrammatically in Fig. 3) for the more rapid decay of the ATP-stimulated calcium response in cells expressing heterologous polycystin-1. Altered calcium entry did not entirely explain the difference in calcium response kinetics, because the decay times of control and polycystin-1 cell lines remained different in the zero-calcium bath. There was no evidence for decreased ER calcium release in the polycystin-1-expressing cells because neither peak ATP-stimulated calcium responses nor total basal intracellular calcium stores were diminished in these cells relative to control. Similarly, increased extrusion of associated calcium transient. If anything, the rate of return of the FCCP transient to baseline was more gradual in the polycystin-1-expressing cells.

Evaluation of Effect of Polycystin-1 on Calcium Export from the Cell Following Treatment with ATP

As described in Fig. 3, polycystin-1 might have accelerated the decay of the ATP-stimulated calcium response through enhanced calcium extrusion across the plasma membrane. We used the near-membrane calcium indicator fura C18 to measure the accumulation of calcium in the immediate extracellular domain. As seen in Fig. 8, the extracellular near-membrane calcium transient appeared shortly after the spike in the intracellular response and peaked during the period of the steepest slope of the decay phase of the intracellular response. If polycystin-1 were accelerating the decay phase through increased calcium extrusion, this would have been expected to appear as an increase in the magnitude of the near-membrane transient. If anything, the near-membrane transient was a little greater in the control cells. Based on the calibration curves, the control cell’s near-membrane calcium was ~500 vs. 150 nM in the polycystin-1 cells in the 25 µM BAPTA-buffered solution used in the ATP stimulation experiments. As solutions were

Fig. 6. Polycystin-1 accelerated refilling of the ER following inositol 1,4,5-triphosphate (IP₃)-stimulated Ca²⁺ release. In these experiments, cells were loaded with the low-affinity Ca²⁺ indicator mag-fura 2. This dye reports Ca²⁺ concentrations in the micromolar range seen in the ER but is insensitive to the nanomolar range seen in the cytoplasm. To ensure complete absence of contamination from cytoplasmic signal, cells were permeabilized with 20 µg/ml saponin and then perfused in an intracellular buffer to wash out cytoplasmic dye, while leaving compartmentalized dye loading intact. A: tracings are composites depicting the mean flanked by the SE. Introduction of 10 µM IP₃ into the bath resulted in a prompt decrease in signal, indicating Ca²⁺ release from the ER. Once the IP₃ was washed out, there was a return toward baseline, indicating ER Ca²⁺ uptake back into this compartment. B: cells overexpressing polycystin-1 (C8/68 and G7/36) had a significantly shortened period of maximal ER Ca²⁺ depletion (shown as _I_peak-80%) and returned toward baseline (shown as _I_80–20%) significantly more rapidly than the control cells. As can be seen in A, at the time when the polycystin-1-expressing cells attained basal levels (at 100 s following stimulation), control cells remained below baseline. F6, n = 11; F8, n = 7; C8/68, n = 7; G7/36, n = 6. *P < 0.05, exact values given in text.

Fig. 7. Mitochondrial Ca²⁺ uptake played no significant role in the effect of polycystin-1 on the ATP response. In these experiments, cells were exposed to the proton ionophore FCCP before stimulation with ATP. A: traces (mean and SE) on the left show the small Ca²⁺ release response to FCCP, followed by the typical ATP-stimulated Ca²⁺ transient, in which the swifter decay in polycystin-1-expressing cells persisted; _I_80–20% data are shown on the right. B: traces, now on a considerably expanded scale, show the FCCP Ca²⁺ transient. The accompanying bars show summary data for the peak and decay rates of the FCCP Ca²⁺ transient. The decay rate is here expressed as the slope, since the small size of the responses precluded accurate determination of _I_80–20%. F6, n = 8; F8, n = 8; C8/68, n = 6; G7/36 n = 9. *P < 0.05, exact values given in text.
We propose that the effect of polycystin-1 to accelerate ER calcium reuptake also accounted for the reduced calcium entry, because capacitative calcium entry appears to be activated in a graded fashion by the degree of ER store depletion (15).

**Polycystin-1 and Basal Cytoplasmic Calcium Concentration**

In our experiments, heterologous expression of polycystin-1 did not significantly alter resting cytoplasmic calcium concentrations in MDCK cells in calcium-containing buffer. The slight lowering of basal cytoplasmic calcium relative to control cells in the zero-calcium bath raised the possibility that polycystin-1 might have activated ER calcium retention in response to depletion of ER stores by incubation in a zero-calcium bath. A similar lack of effect of polycystin-1 on resting cell calcium concentration was evident in studies of the loss of polycystin-1 activity in embryonic renal tubular epithelial cells (23). By contrast, Qian et al. (27) reported that cytoplasmic calcium concentration was diminished in vascular smooth muscle cells from heterozygous polycystin-2 knockout mice. However, because partial loss of polycystin-2 is not associated with a renal phenotype, this effect on resting cell calcium concentration might be restricted to vascular smooth muscle cells and reflect an activity of polycystin-2 independent of the functions of polycystin-1.

**Potential Significance**

Based on our data, loss of polycystin-1 would be predicted to lead to prolonged ligand-activated elevation of cell calcium with reciprocally prolonged depletion of ER calcium concentration. In the following sections, we discuss how these effects might influence processes considered necessary for ADPKD pathogenesis.

**Fluid secretion.** Increased cytoplasmic calcium is a potent stimulus for chloride secretion and inhibition of sodium reabsorption (8). Therefore, one of the functions of polycystin-1 might be to attenuate the net secretory response to ATP. Because the effect of polycystin-1 was evident in cells stimulated with IP3, it is likely that polycystin-1 would similarly attenuate chloride secretion in response to other hormones acting through phospholipase C. If so, then loss of polycystin-1 in the ADPKD cyst epithelium would lead to a prosecretory state through the prolongation of cytoplasmic calcium responses and chloride channel activation following exposure to a number of naturally occurring ligands.

**Proliferation.** In a recent series of studies, Yamaguchi et al. (38) demonstrated that blockade of plasma membrane channels, or chelation of extracellular calcium, reversed the cAMP growth phenotype to an ADPKD type pattern. Although a decline in cytoplasmic calcium was evident in response to either manipulation, these authors showed that the thapsigargin-releasable calcium store was also depleted. Because ER store depletion has been linked to ERK activation (20), ER calcium depletion might have been a stimulus for the reversal of the cAMP response in the studies of Yamaguchi et al. (38). We have shown that polycystin-1 enhances calcium uptake into the ER and would therefore act to minimize ER calcium depletion in response to a variety of release stimuli. Because, in vivo, renal epithelial cells are continuously exposed to endocrine, paracrine, and autocrine stimulation, the calcium concentration of the ER of cells lacking polycystin-1 might be

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**Fig. 8.** External near-membrane Ca\(^{2+}\) responses indicated that polycystin-1 had no effect on Ca\(^{2+}\) extrusion in response to stimulation with ATP. In these experiments, the C18-conjugated form of fura 2 was used to report the concentration of Ca\(^{2+}\) in the immediate vicinity of the external plasma membrane leaflet. A: traces (composites of multiple responses showing mean and SE), fura 2-AM intracellular Ca\(^{2+}\) responses to extracellular ATP are shown superimposed on the C18 near-membrane Ca\(^{2+}\) responses on the same time and fluorescent ratio scales. The increase in cell Ca\(^{2+}\) activated Ca\(^{2+}\) extrusion, with a peak in the near-membrane signal that coincided with the maximum rate of decline of the cell Ca\(^{2+}\) response. There were no differences in the near-membrane responses between cell lines, despite the clearly accelerated rate of decay of the cell Ca\(^{2+}\) response in the polycystin-1-expressing cells (summary data for \(t_{80-20\%}\) shown in B). C: fura C18 signal in the two groups of cell lines in response to a range of bath Ca\(^{2+}\) concentrations. D: representative traces showing that fura C18 was exclusively retained in the external leaflet of the plasma membrane. C18 signal was completely quenched by 5 mM nickel in the bath, whereas, because the cells were impermeable to nickel, bath nickel had no effect on intracellular fura in fura 2-AM-loaded cells. For the C18 experiments, F6, n = 9; F8, n = 9; C8/68, n = 8; G7/36, n = 8. For the fura 2-AM studies, n = 8 for all 4 cell lines. *P < 0.001.
slower to recover and therefore remain depleted to a greater extent than neighboring unaffected cells. This could promote proliferation through ERK activation in cyst epithelia. Our use of ATP as the calcium release stimulus is pathophysiologically relevant, because all the components for purinergic signal transduction are resident in the cystic environment (29).

**Apoptosis.** Our observations raise a possible explanation for the increased apoptosis seen in polycystic epithelia in ADPKD (36). As discussed above, calcium released by the ER can be cleared from the cytoplasm by buffering by intracellular organelles such as the mitochondrion (mitochondrial calcium uptake is closely coupled to ER calcium release) (28). There is evidence that mitochondrial calcium loading is an important mechanism for apoptotic cell death (32). The prolonged elevation of cytoplasmic calcium with every calcium release stimulus in ADPKD cyst epithelia could conceivably result in increased apoptosis through progressive accumulation of calcium within mitochondria.

In conclusion, we suggest that it might be important to consider the interplay between ER and cytoplasmic calcium in future studies of the cell biological consequences of loss of polycystin function in ADPKD.

**REFERENCES**


