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Biochemical and Biophysical Research Communications 301 (2003) 657–664
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Expression of polycystin-1 C-terminal fragment enhances the ATP-induced Ca^{2+} release in human kidney cells

Gianluca Aguiari,[a,1] Michelangelo Campanella,[b,1] Elisa Manzati,[a] Paolo Pinton,[b] Manuela Banzi,[a] Sabrina Moretti,[c] Roberta Piva,[a] Rosario Rizzuto,[b] and Laura del Senno[a,*]

[a] *Department of Biochemistry and Molecular Biology, Section of General Pathology, University of Ferrara, V.L. Borsari 46, 44100 Ferrara, Italy*
[b] *Department of Experimental and Diagnostic Medicine, Section of General Pathology, University of Ferrara, Ferrara, Italy*
[c] *Department of Advanced Biomedical Sciences and Therapies, Section of Hematology, University of Ferrara, Ferrara, Italy*

Received 16 December 2002

Abstract

Polycystin-1 (PC1) is a membrane protein expressed in tubular epithelia of developing kidneys and in other ductal structures. Recent studies indicate this protein to be putatively important in regulating intracellular Ca^{2+} levels in various cell types, but little evidence exists for kidney epithelial cells. Here we examined the role of the PC1 cytoplasmic tail on the activity of store operated Ca^{2+} channels in human kidney epithelial HEK-293 cell line. Cells were transiently transfected with chimeric proteins containing 1–226 or 26–226 aa of the PC1 cytoplasmic tail fused to the transmembrane domain of the human Trk-A receptor: TrkPC1 wild-type and control Trk truncated peptides were expressed at comparable levels and localized at the plasma membrane. Ca^{2+} measurements were performed in cells co-transfected with PC1 chimeras and the cytoplasmic Ca^{2+}-sensitive photoprotein aequorin, upon activation of the phosphoinositide pathway by ATP, that, via purinoceptors, is coupled to the release of Ca^{2+} from intracellular stores. The expression of TrkPC1 peptide, but not of its truncated form, enhanced the ATP-evoked cytosolic Ca^{2+} concentrations. When Ca^{2+} assays were performed in HeLa cells characterized by Ca^{2+} stores greater than those of HEK-293 cells, the histamine-evoked cytosolic Ca^{2+} increase was enhanced by TrkPC1 expression, even in absence of external Ca^{2+}. These observations indicate that the C-terminal tail of PC1 in kidney and other epithelial cells upregulates a Ca^{2+} channel activity also involved in the release of intracellular stores.

Keywords: Polycystin-1; Polycystin-2; TrkPC1 chimeric receptors; Epithelial cells; HEK 293 cells; HeLa cells; Protein expression; Intracellular Ca^{2+}

Recent studies show that intracellular calcium signalling is important in kidney development, and defects in this pathway appear to be the basis of cyst formation in autosomal dominant polycystic kidney disease (ADPKD) [1–3]. This results from a loss of function of either of two novel integral membrane proteins, polycystin-1 (PC1) or polycystin-2 (PC2), codified by PKD1 or PKD2 genes [4–6], which are mutated in 85% or 15% of all ADPKD cases, respectively [4]. The disease is characterized by cyst formation in the kidney and other organs, and results in end-stage renal failure in 50% of affected individuals [7].

During the past two years growing evidence has emerged that polycystins are ion channels or regulators of ion channels [3].

PC1 (4303 aa) has a large N-terminal extracellular region (approximately 3000 aa) encompassing several domains typical of cell–cell, cell–matrix interaction, followed by a complex transmembrane region with 9–11 transmembrane domains and a 226-aa C-terminal cytoplasmic tail [4]. The cytoplasmic portion, by interacting with various proteins, including PC2 [8,9], regulates several important signalling pathways [10–12], and its deletion suffices to cause cyst formation [4]. Based on its predicted structure, PC1 is postulated to be

* Corresponding author. Fax: +39-0532-202723.
E-mail address: sen@unife.it (L. del Senno).
[1] These two authors contributed equally to this work.

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doi:10.1016/S0006-291X(02)03011-5

a plasma membrane nonkinase type receptor involved in cell–cell/matrix interaction.

PC2 (968 aa) is likely a member of the TRP-like superfamily of channel proteins [13] predicted to have six transmembrane domains and N-terminal and C-terminal intracellular ends; the protein is expressed abundantly in the endoplasmic reticulum (ER) membrane [14]. The site of interaction with PC1 is located in the C-terminal tail [8,9].

Based on these structural and functional features, and on the close similarity of polycystic disease symptoms, irrespective of the causative gene, PC1 and PC2 are predicted to be components of a receptor/channel complex involved in the control of membrane transport along a common pathway with Ca ions as prevalent/early intracellular signal [2]. In CHO cells, overexpression of PC1 and PC2 results in translocation of the latter to the plasma membrane where the molecules co-assemble to produce a new Ca^{2+} permeable nonselective cation current [15]. In addition, overexpression of PC1 C-terminal tail upregulates a Ca^{2+} permeable nonspecific Ca^{2+} channel in *Xenopus* oocytes [16,17] and in 293-EcR cells [17]: the elevated cation conductance correlates with increased Ca^{2+} entry and elevation of intracellular Ca^{2+}.

Also PC2 overexpression in heterologous systems can reconstitute a Ca^{2+} permeable cation channel in human syncytiotrophoblasts [18] and in *Xenopus* oocytes [19]. Futhermore, recent studies show that PC2, expressed in the endoplasmic reticulum (ER) of epithelial cells, is a functional Ca^{2+}-activated high conductance channel that is permeable to divalent cations and mediates intracellular Ca^{2+} release through its own channel activity [20]. It is possible that this channel activity requires association with other proteins including PC1 [20]. Therefore, although growing evidence is emerging on the cation channel activities attributed to PC1 and PC2 in heterologous systems, by contrast, little information is available regarding the role of PC1 in the regulation of Ca^{2+} homeostasis in kidney epithelial cells.

In this study human epithelial kidney HEK-293 and nonkidney HeLa cells were used for expression of PC1 peptides and intracellular Ca^{2+} measurements were performed by using the bioluminescent protein aequorin, a Ca^{2+}-dependent luciferase that produces light (466 nm) upon oxidation of coelenterazine [21]. The cloning of one of the members of this protein family made it possible to transiently transfect cell with cDNAs encoding aequorin or its chimeras, thus allowing measurement of Ca^{2+} in specific cellular compartments, such as the cytoplasm and the nearby mitochondrial membrane.

We now report that the cytoplasmic C-terminal tail of PC1 expressed as a transmembrane fusion protein, leads to increased levels of ATP-evoked cytosolic Ca^{2+}, even in absence of extracellular calcium; this suggests that the increase in cytosolic Ca^{2+} results from release of intracellular stores. The observation provides further evidence for the concept that the coupling of ER Ca^{2+} release channels to cell surface channel receptors may be a generalized principle in nature.

Materials and methods

TrkPC1 clone generation. Trk-A-polycystin-1 chimeric receptors used in transfection experiments were: (1) Trk0, containing extracellular and transmembrane regions plus 6 aa of the cytoplasmic tail of Trk-A [22] (the NGF receptor) plus 18 random aa; (2) $TrkPC1_{1–226}$, constructed by Trk0 with the whole PC1 cytoplasmic tail of 226 aa [13], instead of the 18 random aa; and (3) $TrkPC1_{26–226}$, containing the extracellular and transmembrane regions plus 31 aa of intracellular tail of Trk-A, and the last 200 aa of PC1 (Fig. 1A). $PC1_{1–226}$ and $PC1_{26–226}$ were generated by PCR, using a pfu *Taq* polymerase (Stratagene); PKD1 RNA was obtained from K562 erythroleukemic cells, which express PC1 RNA and protein [23], and reverse transcribed with a primer (nt 13,150, 5′CTAGAAACCGTCCAATACTGCTGTG TCCTTC3′), located in the 3′ untranslated region of the gene, by SuperScript first strand synthesis system for RT-PCR (Invitrogen). $PC1_{1–226}$ was amplified with a *Hin*dIII-tailed forward primer (nt 12,228, 5′CACAAAGCTTGAGTCCTGGCACCTGTCACCC3′) and with a *Eco*RI-tailed reverse primer (nt 12,965, 5′CACAGAATTC TAATACTGAGCGGTGTCCACT3′), located downstream of the canonic stop codon. $PC1_{26–226}$ was amplified with a *Bsp*HI-tailed primer (nt 12,303, 5′CACATCATGACAGCTGTTATTCTCCGCTGG CGC3′) and the 12,965 *Eco*RI-tailed primer (nucleotide numeration begins at the start site of PKD1 cDNA—GenBank L 33243). The Trk-A regions were amplified from a plasmid containing the whole cDNA sequence of human Trk-A [22] (a gift from Dr. S. Alema', Istituto di Biologia cellulare, CNR, Rome). One Trk-A PCR fragment was produced. for $TrkPC1_{1–226,}$ using a forward primer (pCDNA3f 5′CTGCTTA CTGGCTTATCGAAA3′) located close to the *Eco*RI site of the pCDNA3 sequence, and a *Hin*dIII-tailed reverse primer located at nt 1407 of Trk-A (5′CACAAAGTCCGTTTCTCCGTCCA CATTT3′). A different Trk-A PCR fragment was produced for $TrkPC1_{26–226}$, using the forward primer pCDNA3f and a Trk-A downstream primer (nt 1479, 5′CACATCATGACAATGTCATGA AATGCA GGGACATGGC). The PCR fragments were purified with an agarose gel extraction kit (Invitrogen), cut with specific restriction enzymes (Biolabs), and sub-cloned in pBlueScript vector. Trk0 was produced by digestion of pBlueScript-Trk-A (pCDNA3f-1407) with *Eco*RI–*Xho*I and cloning in pCDNA3 (Invitrogen). $TrkPC1_{1–226}$ was constructed by ligation of Trk-A *Eco*RI–*Hin*dIII to $PC1_{1–226}$ *Hin*dIII–*Eco*RI restriction fragments, and the ligation product was subsequently cut with *Eco*RI and then subcloned in pCDNA3. $TrkPC1_{26–226}$ was produced by ligation of Trk-A *Eco*RI–*Bsp*HI to $PC1_{26–226}$ *Bsp*HI–*Eco*RI restriction fragments, cut with *Eco*RI, and finally cloned in pCDNA3 vector.

The clones were sequenced to exclude presence of mutations induced by PCR. Several primers were constructed to amplify recombinant clones and to generate about 500 bp PCR fragments that were sequenced using an automatic sequencer (Pharmacia).

Western blot analysis. After 48–72 h transfections with recombinant clones, HEK-293 cells, grown in 5 cm plates, were washed twice with PBS containing protease cocktail inhibitors (Roche), scraped, collected by centrifugation, and lysed for 30 min at 4 °C in single detergent lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, and 1% v/v Triton X-100) with protease inhibitors. One hundred μg of total lysate was electrophoresed on 8% SDS–polyacrylamide gel. The proteins were electroblotted to a nitrocellulose paper for 2 h at room temperature in 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. The proteins were blocked overnight at 4 °C with 5% nonfat dried milk in PBS–T (PBS with 0.05% Tween 20) and incubated at room temperature with 1 μg/ml of anti-N-ter Trk-A polyclonal antibody (Upstate) in PBS-T

for 2 h. After incubation with primary antibody, the filter was washed four times in PBS–T, incubated with secondary anti-rabbit antibody (1:100.000, Pierce) for 1 h at room temperature in PBS–T, and washed again for four times in PBS–T. Chimeric proteins were visualized with SuperSignal West Pico Chemiluminescent detection system (Pierce).

Glycosylation analysis. Transiently transfected HEK-293 cells were lysed in single detergent lysis buffer with complete protease inhibitor mixture, and 100 μg of each lysate was treated with 500 units of endoglycosidase H (Endo H) and *N*-glycosidase F (PNGase F) for 1 h following the manufacturer's protocols (Biolabs). The deglycosylated proteins were then analysed by SDS–PAGE and immunoblot.

Subcellular fractionation. Stably transfected HEK-293 cells (10^7) were washed twice in pre-chilled (4 °C) PBS supplemented with a complete protease inhibitor mixture, scraped, and collected by centrifugation at 4 °C. Cells were homogenized in homogenization buffer (0.179 M sucrose, 0.75 mM KCl, 19.2 mM NaCl, 10 mM Tris–HCl, pH 7.4, and 1 mM EDTA) containing a cocktail of protease inhibitors and centrifuged at 500*g* for 10 min to remove cell debris. The supernatant was layered on top of a 11.5 ml discontinuous density gradient spanning the range of 0.2–1.43 M sucrose in 10 mM Tris–HCl, pH 7.4, and protease inhibitor [24]. The subcellular organelles were fractionated by overnight centrifugation at 100,000*g* at 4 °C; 600 μl fractions were collected from the top to the bottom of the gradient and 15 μl of this solution was loaded on a 4–12% polyacrylamide mini gel (ICN), electrophoresed, and blotted on nitrocellulose filter. Filters were analysed by immunoblot with anti-N-ter-PC2 polyclonal antibody, as previously described ([14,25] and manuscript in preparation), and with antibodies specific for protein markers of subcellular membranes and structures: β_1 Integrin for plasma membrane, Actin for cytoskeleton (Upstate), and Calnexin for endoplasmic reticulum (Santa Cruz).

Transfection. HEK-293 and HeLa cells were grown up to 60% confluency on 13 mm in diameter circular glass coverslips (BDH) in DMEM 50% F12 medium supplemented with 10% FBS. One h before transfection, fresh medium was added to the cells which were then co-transfected with cytosolic aequorin (aequorin-cyt) cDNA [24] and each recombinant plasmid: Trk0, TrkPC$1_{1–226}$, and TrkPC$1_{26–226}$ (ratio 1:3 μg, respectively), using calcium phosphate procedure.

FACS analysis. HEK-293 cells transiently transfected for 72 h with the plasmids pCDNA3, Trk0, TrkPC$1_{1–226}$, and TrkPC$1_{26–226}$, grown up to 80% confluency, were washed twice with 10 ml PBS containing complete protease inhibitors, scraped, and collected by centrifugation (1200*g*) at room temperature for 10 min. The cells (10^6) were incubated in 3 ml PBS with 5 μg of anti-Trk-A monoclonal antibody H 10 (provided from Dr. Natali, Istituto Regina Elena, Rome) for 1 h at room temperature and then washed twice at room temperature in 5 ml PBS solution. Cells treated with anti-Trk-A H 10 and untreated cells (control) were incubated in 3 ml PBS containing secondary anti-mouse Ig FITC (1:1000) for 30 min at room temperature and washed four times with PBS. Finally, the cells were analysed with a flow cytometer (FACScan Becton and Dickinson).

Calcium measurement. After 48–72 h transfection, 5 μM coelenterazine (the prosthetic group of aequorin) was added to the medium to reconstitute the active form of this protein. Two hours after reconstitution, coverslips with the cells were perfused at 37 °C with KRB buffer (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4, and supplemented with 1 mM

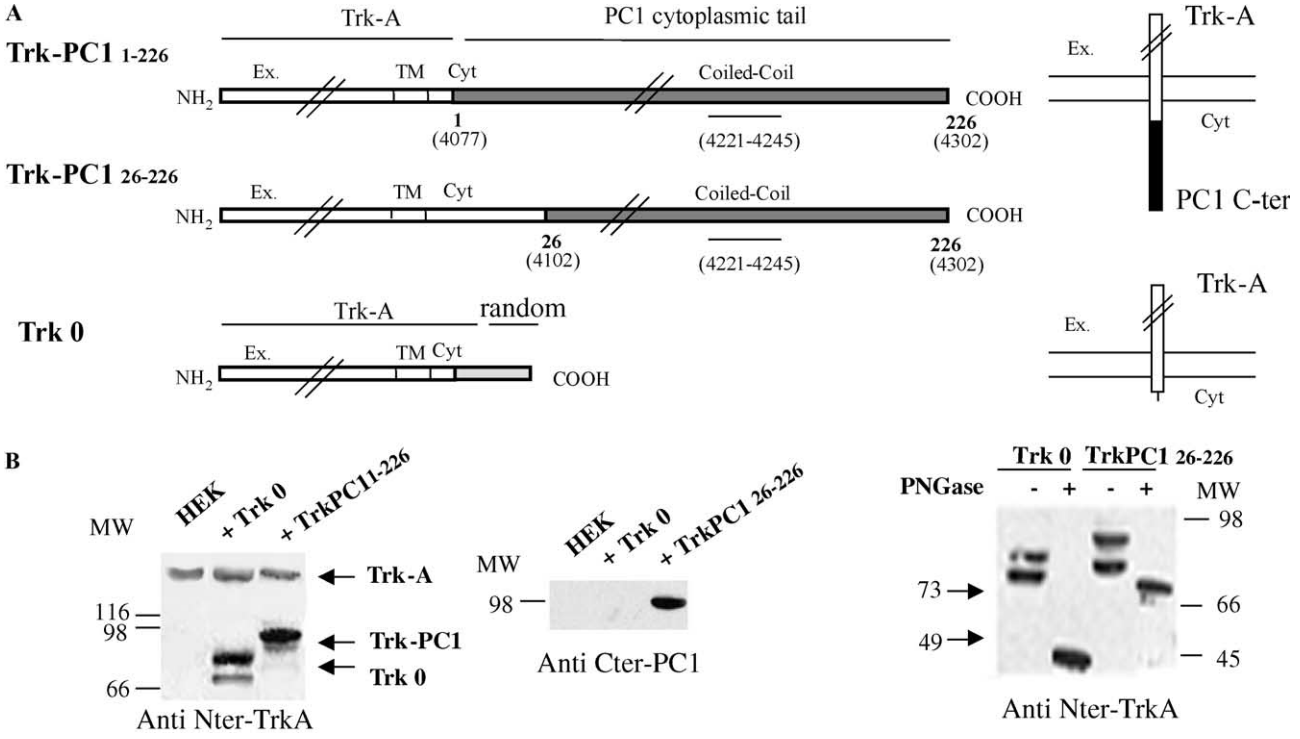

Fig. 1. Expression analysis of Trk0 and TrkPC1 recombinant clones. (A) Scheme of the constructs. The white boxes represent Trk-A portions, while the grey boxes indicate the 226 aa PC1 cytoplasmic C-ter tail (TrkPC$1_{1–226}$), 200 aa PC1 tail (TrkPC$1_{26–226}$) or a 18 aa random sequence (Trk0). TM and cyt indicate the transmembrane domain and the residual cytoplasmic portion of receptors, respectively. Coiled-coil indicates the putative PC2 interaction region. (B) Immunodetection of the recombinant receptors. Western blots of total cell lysates (100 μg) were analysed with a polyclonal antibody recognizing the N-ter peptide of Trk-A. Both the endogenously expressed Trk-A protein of approximately 140 kDa and the chimeric proteins of about 80 and 90 kDa in size, respectively, were detected in Trk0 and TrkPC1 transfected cells (left and right of the figure). The monoclonal anti-C-ter PC1 antibody (middle) identified a 90 kDa band only in TrkPC1 transiently transfected HEK-293 cells. PNGase treatment caused a reduction of the approximately 80–90 kDa recombinant protein sizes to values close to 49 or 73 kDa unglycosylated expected size, for Trk0 and TrkPC1, respectively.

$CaCl_2$) in a perfusion chamber of the measuring instrument (Luminometer). After approximately 1 min of perfusion, HEK-293 and HeLa cells were stimulated with 10 μM ATP and 100 μM histamine, respectively. Calcium ions, released into the cellular cytoplasm, bind aequorin molecules which emit photons that are captured by a luminometer. Using a computer algorithm, the relationship between photon emission (L/L_{max}) and Ca^{2+} concentration allows the conversion of the light release into calcium concentration [26]. L and L_{max} are the instant and the maximal rate light emission. L is obtained after cell stimulation, while L_{max} is produced by cell lysis with 100 μM digitonin [26]. The calcium release from the intracellular stores and from plasma membrane was measured by initially perfusing the cells in KRB buffer with 100 μM EGTA and then by stimulating with ATP. Under such conditions the increase of the $[Ca^{2+}]$ was due only to the calcium release from intracellular stores. Next, Ca^{2+} was reintroduced into extracellular medium and the rise of $[Ca^{2+}]$ was due to the Ca^{2+} influx through the plasma membrane [27].

Statistical analyses. All data are presented as means ± SEM. Statistical comparisons were made using t tests for paired and unpaired samples, as appropriate. Statistical significance was taken as $p < 0.05$.

Results

Characterization of TrkPC1 chimeric proteins in transfected HEK-293 cells

To study the effect of PC1 cytoplasmic peptides in human epithelial cells, we transfected kidney HEK-293 cells with PC1 chimeric receptors (Fig. 1A) containing the cytoplasmic tail of PC1 fused to the transmembrane and extracellular domains of Trk-A that have previously been shown to target cytoplasmic domains to the plasma membrane [28]. First, we confirmed the expression of the chimeric TrkPC1 and control Trk0 truncated peptides (Fig. 1B): the anti-Trk-A polyclonal antibody showed a prevalent band of approximately 80 and 90 kDa, in Trk0 control and $TrkPC1_{1–226}$ transfected cells, respectively, which were absent in the pCDNA3 transfected cells. The fainter immunoreactive band of approximately 140 kDa observed in all lines was consistent with the endogenously expressed Trk-A protein. The same results were obtained with the $TrkPC1_{26–226}$ peptide (data not shown). As expected, the positivity to the anti-PC1 Ab was observed only in cells transfected with the PC1 chimeric peptide. We showed that chimeric proteins were in glycosylated forms: the PNGase treatment markedly reduced the Trk0 and $TrkPC1_{26–226}$ protein molecular weight (49 and 73 kDa are the expected unglycosylated protein sizes, respectively). These data established that in HEK-293 cells chimeric proteins reached the endoplasmic reticulum.

Detection of TrkPC1 proteins at plasma membranes

The localization at plasma membranes of TrkPC1 peptides was established by subcellular fractionation of transiently transfected HEK-293 cells. As shown in Fig. 2A, in $TrkPC1_{26–226}$ transfected cells, the chimeric protein was found in the P8 plasma membrane fraction, while it was faintly detectable in the P100 microsomal fraction and undetectable in the S100 cytoplasmic frac-

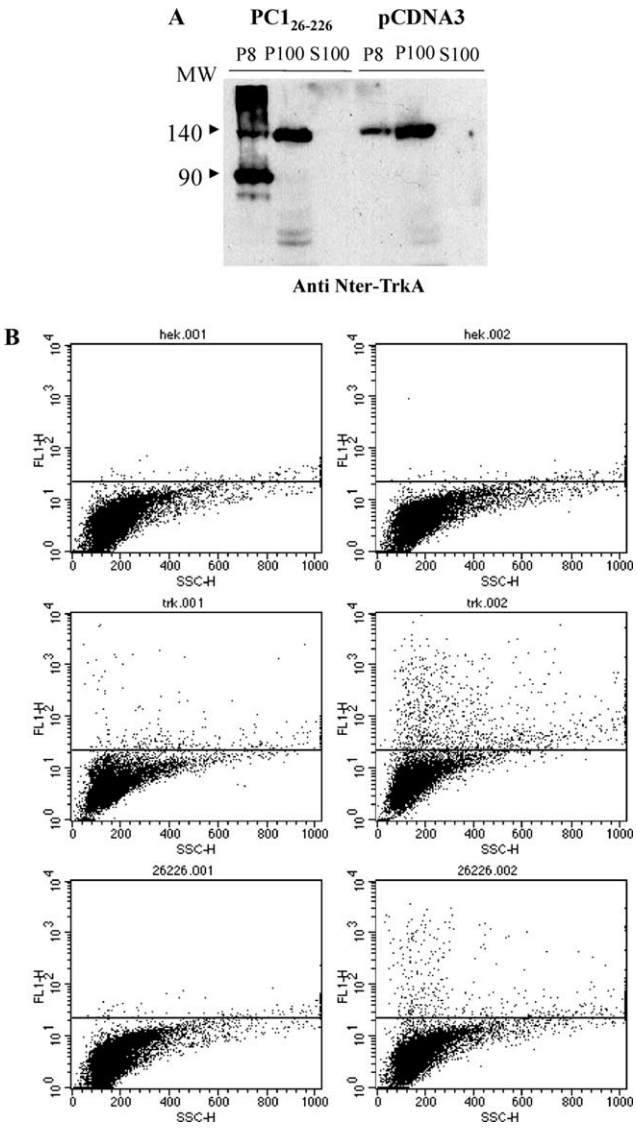

Fig. 2. Localization of chimeric receptors at the plasma membrane. (A) Subcellular fractions of HEK-293 cells transiently transfected with recombinant TrkPC1 and wild-type pCDNA3 plasmids were analysed by Western blot with the polyclonal anti-N-ter Trk-A antibody. A band of about 90 kDa, corresponding to the glycosylated chimeric receptor, was detected mainly in P8 (plasma membrane), faintly in P100 (microsomes), and was absent in S100 (cytosol) fractions of the $TrkPC1_{26–226}$ transfected cells. In pCDNA3 transfected cells, only the high molecular weight band corresponding to endogenous Trk-A was detected. (B) FACS analysis of unfixed transiently transfected HEK-293 cells using a monoclonal anti-N-ter Trk-A antibody. Plots on the left represent control cells (only treated with anti-mouse Ig FITC), while plots on the right represent cells treated also with the primary anti-N-ter Trk-A antibody. The cells expressing the truncated Trk0 (middle) and chimeric $TrkPC1_{26–226}$ (bottom) showed a fluorescence increase compared to cells transfected with wild-type pCDNA3 (upper) and with the transfected cells treated only with the secondary antibody.

tion. The Trk-A 140 kDa band was prevalent in the P100 microsomal fraction of both chimera and pCDNA3 transfected cells.

The immunostaining of intact, unfixed transiently transfected HEK-293 cells with a monoclonal anti-N-ter Trk-A antibody (Fig. 2B) revealed comparable intensities of Trk-A signal at the cell surface, whether the epitope was borne on $TrkPC1_{(26–226)}$ (bottom part) or by Trk0 control (central part), but a very low signal in pCDNA3 transfected cells (upper part). These data established that in transiently transfected cells, chimeric proteins were correctly oriented in the plasma membrane.

Increase in cytoplasmic Ca^{2+} in HEK-293 cells upon stimulation with ATP

It is well known that extracellular ATP elevates the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) by mobilizing Ca^{2+} from internal stores via activation of purinoceptors in the plasma membrane [29], and also in HEK-293 cells [30]. Thus, we co-transfected HEK-293 cells with PC1 chimeric clones and cytoplasmic aequorin cDNA to assess the effect of extracellular ATP signalling on $[Ca^{2+}]_c$. The results of a typical experiment are shown in Fig. 3A. ATP increased $[Ca^{2+}]_c$ to a peak value of 1.7 μM in $TrkPC1_{1–226}$ and 1.2 μM in Trk0 transfected control cells. Data from different experiments are summarized in Fig. 3B. The average elevation of $[Ca^{2+}]_c$ was significantly greater in $TrkPC1_{1–226}$ transfected cells, as compared with Trk0 and pCDNA3 transfected control cells (1.43 ± 0.04 μM in $TrkPC1_{1–226}$ vs 1.05 ± 0.08 in Trk0, $p < 0.01$). Ca^{2+} levels elicited in cells expressing $TrkPC1_{1–226}$ were of a magnitude comparable to the levels observed in $TrkPC1_{26–226}$ expressing cells (1.48 ± 0.08 vs 1.43 μM). A sustained plateau, due to Ca^{2+} entry, was also more apparent in TrkPC1 transfected than in control cells. These data showed that the transient expression of the PC1 tail induced an approximately 40% increase in intracellular Ca^{2+} levels, suggesting that a release from the intracellular stores contributed to the formation of the increased ATP-evoked calcium release.

Increase in $[Ca^{2+}]_c$ in HeLa cells upon stimulation with histamine

Since the stimulated cytoplasmic Ca^{2+} concentration in HEK-293 appears to be low compared to other epithelial cells, we performed Ca^{2+} assay experiments in

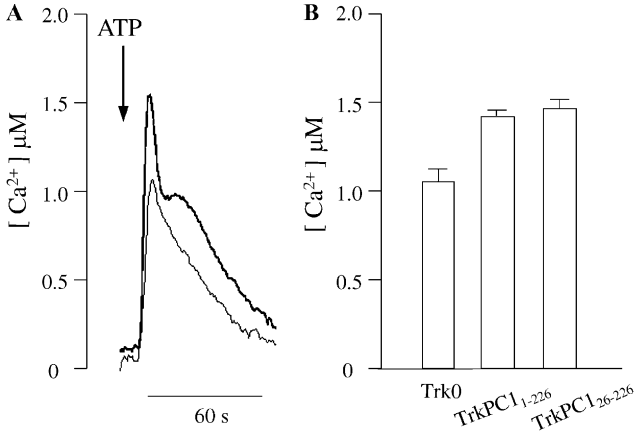

Fig. 3. The Ca^{2+} response to extracellular ATP is increased in TrkPC1 transfected HEK-293. (A) Representative traces of ATP-evoked increase in $[Ca^{2+}]_c$ in $TrkPC1_{26–226}$ (bold line) and Trk0 expressing cells. Cells, grown on coverslips, were co-transfected with Trk0 and aequorin-cyt, or TrkPC1 and aequorin-cyt (ratio 3:1), as described under Materials and methods. After coelenterazine reconstitution, cells were perfused in KRB buffer containing 1 mM $CaCl_2$ for 60 s and then stimulated with 10 μM ATP. The calcium concentration was determined by a luminometer. (B) Summary of Ca^{2+} response to ATP. Data (means ± SEM) are from five experiments at least in triplicate.

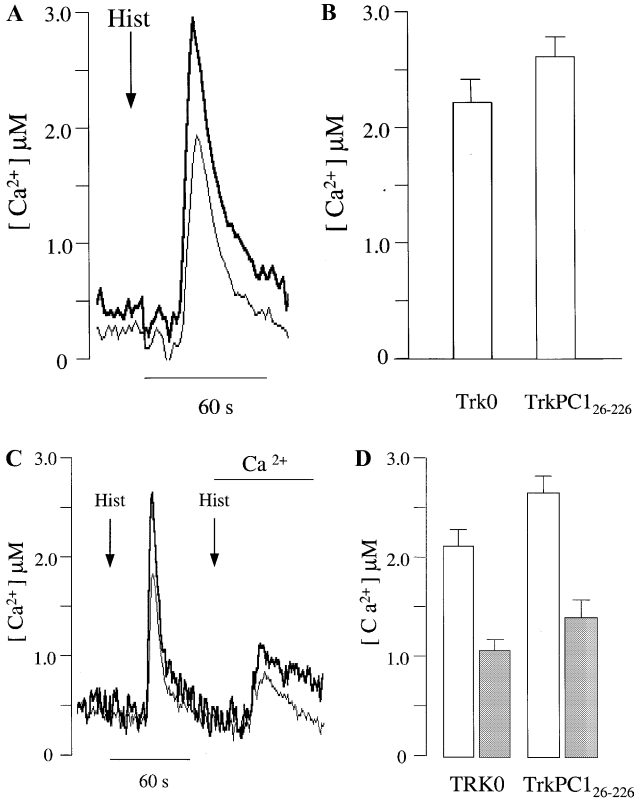

Fig. 4. The Ca^{2+} response to extracellular histamine is increased in TrkPC1 transfected HeLa cells. (A) Representative traces of histamine-evoked increase in $[Ca^{2+}]_c$ in $TrkPC1_{1–226}$ (bold line) and Trk0 expressing cells. Cells, treated as described in the legend of the previous figure, were stimulated with 100 μM histamine (Hist), and the summary of Ca^{2+} response to histamine is shown in B (data—means ± SEM—are from four experiments at least in triplicate). (C) Cells were perfused in KRB buffer containing 100 μM EGTA and after 60 s of perfusion, cells were stimulated with 100 μM histamine. Where indicated, EGTA was substituted by 1 mM Ca^{2+}. (D) Data (means ± SEM) are from four experiments in triplicate.

HeLa cells which, upon stimulation with histamine, another agonist coupled to the generation of inositol trisphosphate, leads to $[Ca^{2+}]_c$ rise up to 2 μM values [27]. The results of a typical experiment are shown in Fig. 4A: histamine increased $[Ca^{2+}]_c$ to a peak value of 2.98 μM in $TrkPC1_{26–226}$ and 1.96 μM in Trk0 transfected control cells. The average elevation in $[Ca^{2+}]_c$ was again greater in $TrkPC1_{26–226}$ than in Trk0 transfected cells (2.6 ± 0.18 vs 2.2 ± 0.2, Fig. 4B). The PC1 tail expression induced an approximately 20% increase in intracellular Ca^{2+} levels.

Furthermore, even when calcium from the extracellular medium was removed, the histamine-evoked change in $[Ca^{2+}]_c$ was greater in $TrkPC1_{26–226}$ transfected cells compared to Trk0 cells (Fig. 4C). The average elevation in $[Ca^{2+}]_c$ was significantly greater in $TrkPC1_{26–226}$ than in Trk0 transfected cells (2.69 ± 0.22 vs 2.08 ± 0.141, $p < 0.05$). The addition of 1 mM Ca^{2+} and the agonist to the external medium was accompanied by a substantial increase in $[Ca^{2+}]_c$ which was not significantly greater in $TrkPC1_{26–226}$ than in Trk0 transfected cells (1.39 ± 0.148 vs 1.04 ± 0.22, Fig. 4D). The results indicate that a release from the intracellular stores contributed to the formation of the increased calcium transients also in nonkidney epithelial cells.

Discussion

Almost all epithelial cells express purinoceptors [29] and kidney tubular epithelia, via these receptors, respond to nucleotides with increases in cytoplasmic calcium concentration $[Ca^{2+}]_c$ [29–33]. In fact, these receptors, via heterotrimeric G proteins, are coupled to the generation of inositol trisphosphate (Ins3P) [34] and may form/activate Ca permeable nonselective Ca channels. We report that the expression of the $TrkPC_{1–226}$ and $TrkPC_{26–226}$ fusion proteins, encoding the C-terminal intracellular tail of PC1, fused to the Trk-A transmembrane chimera, enhances an ATP-evoked Ca^{2+} release in human kidney epithelial HEK-293 cells. This increase has biphasic kinetics, a peak mostly due to the release of stored Ca^{2+}, and a plateau due to the entry of Ca^{2+} through the plasma membrane channels activated by the depletion of internal stores. The PC1-induced increase is also present in human epithelial HeLa cells. The fact that the increase is apparent in the absence of external Ca^{2+} demonstrates that the release of Ca^{2+} derives from intracellular stores. Expression of Trk0 control peptide, with a random small intracellular tail, fails to produce such an increase, despite normal surface expression of this protein. Taken together, the data suggest that the PC1 domain potentiates the activity of a Ca^{2+} store release channel in epithelial cells.

Previous studies have shown that the expression of the carboxy tail of PC1 regulates multiple signalling pathways when expressed as transmembrane fusion proteins in host cells [10–12]. In *Xenopus* oocytes the expression of $CD16\text{-}PKD1_{115–226}$ upregulates an endogenous Ca^{2+}-permeable cation channel, whose properties, to some degree, are similar to those of PC2 [16]: the expression of PC1 tail augments Ca^{2+} influx and consequently global oocyte $[Ca^{2+}]$ increases. However, when the experiments were done in nominally Ca^{2+} free bath, basal intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ was indistinguishable in PC1 expressing and in control oocytes, whereas subsequent bath shift to 10 mM Ca^{2+} increased $[Ca^{2+}]_i$ greatly in PC1 expressing oocytes compared to control. Similar results were obtained in 293 cells stably transfected with the same PC1 chimeric protein under the control of an ecdysone-responsive promoter [17]. Our results provide further observations, because the present study was performed in kidney epithelial cells with an approach that allowed the examination of the effect of PC1 cytoplasmic tail on store-operated Ca^{2+} release activated by passive Ca^{2+} store depletion using Ins3P.

Taking into account previous findings showing that, in vitro, the C-termini of PC1 and PC2 potentially interact [8,9], and, in vivo, the coexpression of full-length PC1 and PC2 produces channel activity in CHO [15], it is highly possible that our data result from interactions of the two polycystins. This hypothesis fits well with recent observations demonstrating that PC1 and PC2 co-localize to the lateral cell borders in transgenic kidney cells [35], and that PC2, in LLC-PK porcine kidney cell lines, behaves as a calcium-activated high conductance ER channel that is permeable to divalent cations [20]: increased levels of intracellular calcium activate PC2-mediated release of calcium from intracellular stores.

It is possible that the TrkPC1 fusion protein might upregulate the activity of PC2 or other distinct cation channels. Interestingly, the studies in oocytes [17] showed that cation current and Ca^{2+} uptake were greatly reduced in oocytes expressing CD16.7-PKD1 with ADPKD1-associated missense mutations, which disrupted the putative coiled coil domain of interaction of PC1 with that of PC2 [8,9]. Furthermore, when both the C-terminal cytoplasmic tails of PC1 and PC2 were coexpressed in oocytes, cation current was suppressed, suggesting that the cytoplasmic tails of PC1 and PC2 interact in vivo, and that functional PC2 molecules are essential for the cation activity.

Other reported data showed that PC1 is required to bring PC2 to the plasma membrane and co-assemble a polycystin complex enabling PC2 to function as an ion channel [15]. We have found that in TrkPC1-transfected HEK-293 cells, PC2 is clearly detectable and mainly expressed in the endoplasmic reticulum (Fig. 5A). Moreover, when anti-PC2 was used in immunohistochemistry of HEK-293 cells, the positivity was mostly

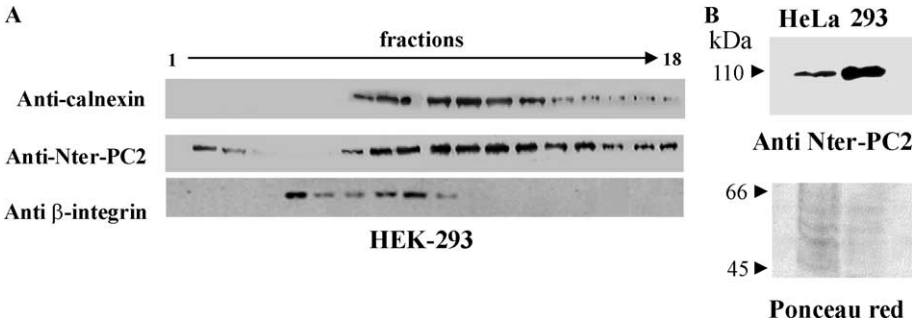

Fig. 5. PC2 expression in HEK 293 and HeLa cells. (A) Lysates from HEK-293 were fractionated through a sucrose gradient and analysed as described under Materials and methods. Fifteen μl of 600 μl fractions was loaded on each lane. PC2 mainly comigrates with calnexin, a marker of endoplasmic reticulum membranes. (B) HEK-293 and HeLa cells were probed with an anti-PC2 rabbit polyclonal antibody (dilution 1:1000). PC2 and approximate molecular weights of the Ponceau red stained bands are indicated by arrows.

perinuclearly localized, as in endoplasmic reticulum, while an anti-PC1 antibody mainly recognized the plasma membrane (data not shown), where TrkPC1 was also located. Therefore, the complex between the two polycystins should be co-assembled in subdomains where plasma and endoplasmic reticulum membrane may interact, as previously hypothesized [20].

On the other hand, few PC2 molecules at the plasma membrane, interacting with TrkPC1 molecules, may also produce an increase in Ca^{2+} influx, contributing to the evident plateau level observed in TrkPC1-transfected HEK-293 cells, as presented in Fig. 3A.

We have also observed that TrkPC1 induced an increase in the histamine-evoked Ca^{2+} release of HeLa cells, but it was lower than the ATP-evoked Ca^{2+} release in HEK-293 cells (20% increase in HeLa vs 40% increase in HEK-293 cells). This observation may derive from a different PC2 expression of the cells; consistent with this hypothesis, we found more immunodetectable PC2 in HEK 293 than in HeLa cells (Fig. 5B). In addition, although both HeLa and HEK-293 cells are epithelial, only HEK-293 cells have a ductal function. Therefore, it is possible that the putative PC1–PC2 complex channel, although present in both cell types, could display a different spatial organization with respect to different populations of Ins3P receptors or different compartmentalized Ca^{2+} stores [34].

In conclusion, these findings, for the first time, provide direct evidence that PC1 contributes to the activity of a Ca^{2+} selective internal channel. Our data support a central role for PC1 protein in the global $[Ca^{2+}]_c$ rise evoked upon store depletion.

Acknowledgments

We thank Dr. S. Alemà for kindly providing us with Trk-A cDNA plasmid, and Dr. P.G. Natali and Dr. P.C. Harris with the anti-Trk-A and anti-PC1 monoclonal antibodies, respectively. We thank the Ferrara Università, Telethon-Italy (Grants E 1250, 1285, and GTF01011), Italian Association for Cancer Research (AIRC), Human Frontier Science Program, Italian Space Agency (ASI), Italian University Ministry (MIUR, COFIN 2002), and National Research Council (CNR).

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