Ca^{2+} signalling in human proximal tubular epithelial cells deficient for cystinosin

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ABSTRACT

Nephropathic cystinosis is an autosomal recessive lysosomal storage disorder caused by loss-of-function mutations in the CTNS gene coding for the lysosomal cystine transporter, cystinosin. Recent studies have demonstrated that, apart from cystine accumulation in the lysosomes, cystinosin-deficient cells, especially renal proximal tubular epithelial cells are characterized by abnormal vesicle trafficking and endocytosis, possible lysosomal dysfunction and perturbed intracellular signalling cascades. It is therefore possible that Ca^{2+} signalling is disturbed in cystinosis, as it has been demonstrated for other disorders associated with lysosomal dysfunction, such as Gaucher, Niemann-Pick type C and Alzheimer's diseases. In this study we investigated ATP-induced, IP$_3$-induced and lysosomal Ca^{2+} release in human proximal tubular epithelial cells derived from control and cystinotic patients. No major dysregulation of intracellular Ca^{2+} dynamics was found, although ATP-induced Ca^{2+} release appeared slightly sensitized in cystinotic cells compared to control cells. Hence, these subtle changes in Ca^{2+} signals elicited by agonists may contribute to the pathogenesis of the disease.

1. Introduction

Nephropathic cystinosis is a lysosomal storage disorder caused by mutations in the CTNS gene that encodes the lysosomal cystine transporter cystinosin [1]. Cystinosis is characterized by intralysosomal storage of cystine leading to cystine accumulation in all organs and tissues. The first affected organ is the kidney. Cystinotic patients present with generalized proximal tubular dysfunction called renal Fanconi syndrome, resulting in the urinary loss of proteins, amino acids, glucose and other solutes [2]. Glomerular dysfunction is also present starting from an early age and is manifested by intermediate and high molecular weight proteinuria and pathological changes in the glomeruli visible in biopsy material [3].

Current therapy of cystinosis is based on a life-long cysteamine treatment [4]. Cysteamine can enter the lysosome, where it reacts with cystine forming products that can exit into the cytoplasm using alternative transporters. The lysosomal storage of cystine is therefore reduced. Started at an early age, cysteamine therapy prevents extra-renal complications and delays the kidney disease progression [5]. However, it is not effective against the main proximal tubular pathology, the renal Fanconi syndrome and the eventual development of kidney damage. Accordingly, it has been hypothesized that cystine accumulation is not the only mechanism of the disease pathogenesis, and that cystinosin might have other functions apart from cystine transport.

The role of endoplasmic reticulum (ER) stress and of the unfolded protein response (UPR) in cystinosis has been revealed recently [6]. UPR signalling is a complex pathway that regulates cell survival in stress conditions and can be caused by the loss of luminal Ca^{2+} from the ER [7]. Previous studies demonstrated elevated levels of oxidized glutathione (GSSG) in cystinotic cells [8–10]. It has been reported that GSSG can increase the Ca^{2+} release from the ER and from the sarcoplasmic reticulum into the cytosol via ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate (IP$_3$) receptors (IP$_3$R) [11,12]. Moreover, GSSG was also shown to affect

some isoforms of the sarco-/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) pumps that are responsible for filling the ER stores with Ca^{2+} [13]. Therefore, it is possible that ER Ca^{2+} signalling is affected in cystinosis and may contribute to the pathological process.

Acidic compartments, such as late endosomes and lysosomes, represent another type of intracellular Ca^{2+} stores. Although the capacity of the acidic stores is lower than that of the ER, they play an important function in the regulation of endosomal functioning and fusion and can trigger Ca^{2+} release from larger stores amplifying the signalling cascade [14,15]. The loading of the acidic Ca^{2+} stores is dependent on the luminal pH, but the exact mechanism of their filling remains to be elucidated. The release of Ca^{2+} from the acidic stores is mediated by several channels expressed on the surface of these organelles, including the two-pore channels (TPC1/2/3) that respond to nicotinic acid adenine dinucleotide phosphate (NAADP) [16]. TPCs were shown to play a role in Ca^{2+}-induced Ca^{2+} release through the recruitment of IP_3Rs and RyRs [17]. To date, it has not been investigated whether lysosomal Ca^{2+} signalling or Ca^{2+} stores are affected in nephropathic cystinosis.

A study of the molecular mechanisms of the pathogenesis revealed an altered morphology and function of endosomal and lysosomal compartments in the murine model PTECs deficient for cystinosin [18]. Lysosomes appeared to be abnormally enlarged and lysosomal degradation of proteins was partly impaired. Early studies have demonstrated that lysosomal acidification was unaffected in cystinotic cells, although more recent research questioned these findings [19]. Interestingly, the impaired lysosomal function was demonstrated to be linked to deficient acidic Ca^{2+} stores rather than to lysosomal pH abnormalities in similar pathologies such as Gaucher, Niemann-Pick type C or presenilin deficiency in familial Alzheimer's disease [20–22].

It is therefore important to investigate the ER and the acidic Ca^{2+} stores in cells deficient for cystinosin, particularly the cells suffering the most and causing the earlier and most characteristic clinico-pathological feature of the disease, the renal Fanconi syndrome. In this work we screened for possible alterations in Ca^{2+} signalling in cultured human proximal tubular epithelial cells derived from healthy donors and cystinotic patients.

2. Materials and methods

2.1. Cellular models

The study of the molecular mechanisms of the pathogenesis of cystinosis requires suitable *in vitro* models. To study possible abnormalities in Ca^{2+} signalling, we used cultured conditionally immortalized proximal tubular epithelial cells (ciPTEC) lines obtained from healthy donors and cystinotic patients. The cell lines have been established from living kidney cells exfoliated into the urine providing therefore a non-invasive method of obtaining kidney cell cultures bearing known mutation of the *CTNS* gene leading to nephropathic cystinosis [23]. The cell lines were immortalized using a temperature-sensitive SV40-TERT viral system and subcloned to obtain lines derived from single cells. We have selected 4 control (PT34.8, PT10.5, PT33.5 and PT14.4) and 4 cystinotic (PT2.1, PT46.2, PT53.3 and PT13.5) ciPTEC lines previously characterized by our group [23]. Two of the cystinotic cell lines were bearing a homozygous 57-kb deletion that affects a large part of the *CTNS* gene and results in a complete absence of cystinosin expression at both the mRNA and protein level. The third cell line was compound heterozygous for the 57-kb deletion and the mutation: c.del18_21GACT (p.T7FfsX7) in exon 3, while the fourth cell line was compound heterozygous for the mutations: c.518-519delCA (p.Y173X) in exon 8 and c.1015G > A (p.G339R) in exon 12 of the

CTNS gene. All four cell lines were derived from patients with a severe clinical form of the disease.

2.2. Cell culture

The cells were grown in DMEM-HAM's F12 culture medium (Lonza) supplemented with 10% foetal bovine serum (Gibco), 50 IU/ml penicillin and 50 mg/ml streptomycin (Lonza). The medium was additionally supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 40 pg/ml tri-iodothyronine, 36 ng/ml hydrocortisone and 10 ng/ml EGF (all from Sigma).

2.3. ATP-induced Ca^{2+} release in intact cells

Control and cystinotic ciPTEC lines were grown on flat-bottom 96-well plates to 80% confluency. Before the experiment, the cells were washed once with modified Krebs solution (130 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 12 mM glucose and 11 mM HEPES, pH 7.3). Ca^{2+}-free Krebs solution was made identically, but containing no Ca^{2+} and supplemented with 30 mM EGTA. The cells were then loaded with Fura-2 AM (Sigma) (1.25 μg/ml in modified Krebs solution) for 30 min in the dark at room temperature. The cells were washed once with modified Krebs solution and incubated in 100 μl of the same medium for 30 min in the dark to hydrolyze the Fura-2 AM ester. Afterwards, the cells were incubated in EGTA-containing Ca^{2+}-free Krebs solution for 40 s and stimulated with ATP (0.25, 1, 10 and 100 μM in Ca^{2+}-free Krebs solution) at room temperature. The $[Ca^{2+}]$ in the cytosol was recorded with a FlexStation 3 Multi-Mode Microplate Reader from Molecular Devices using the excitation wavelengths of 340 and 380 nm (5 reads per well). Emission was measured at a wavelength of 510 nm. The 340/380 ratio corresponds to the free $[Ca^{2+}]$ in the cytosol. The presented results are the average of 3–4 independent experiments for each cell line.

2.4. Lysosomal Ca^{2+} release

Lysosomal Ca^{2+} measurements in control and cystinotic ciPTEC lines were performed using the protocol described above. Cells were first treated with 2 μM thapsigargin for 2 min to exclude the contribution of the ER Ca^{2+} stores. Ca^{2+} release from the acidic stores was then induced by adding 200 μM L-glycyl-L-phenylalanine 2-naphthylamide (GPN) in Ca^{2+}-free Krebs solution. The amplitude of the Ca^{2+} peak corresponds to the amount of Ca^{2+} stored in the lysosomal compartments disrupted by the GPN reagent.

2.5. IP_3-induced Ca^{2+} release in permeabilized cells

The measurements were performed as described previously [24]. Control and cystinotic ciPTEC lines were plated in 12-well cell-culture plates and grown to confluency. The plates were placed on a thermostated plate at 30 °C on a shaker. The culture medium was aspirated and the cells were permeabilized by incubation in a solution containing 120 mM KCl, 30 mM imidazole (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA and 20 μg/ml saponin for 10 min. The cells were then loaded for 45 min in 120 mM KCl, 30 mM imidazole (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 0.44 mM EGTA, 10 mM NaN_3 and 150 nM free $^{45}Ca^{2+}$ (0.3 MBq/ml). The cells were then washed twice in an efflux medium containing 120 mM KCl, 30 mM imidazole (pH 6.8), 1 mM EGTA and 2 μM thapsigargin. One ml of efflux medium was then added and replaced every 2 min. The indicated concentrations of IP_3 or 10 μM of the Ca^{2+} ionophore A23187 were added for 2 min after 8 min of efflux. Eight minutes later, all $^{45}Ca^{2+}$ remaining in the stores was released by incubation with 1 ml of 2% sodium-dodecyl-sulphate for 30 min The $^{45}Ca^{2+}$ present
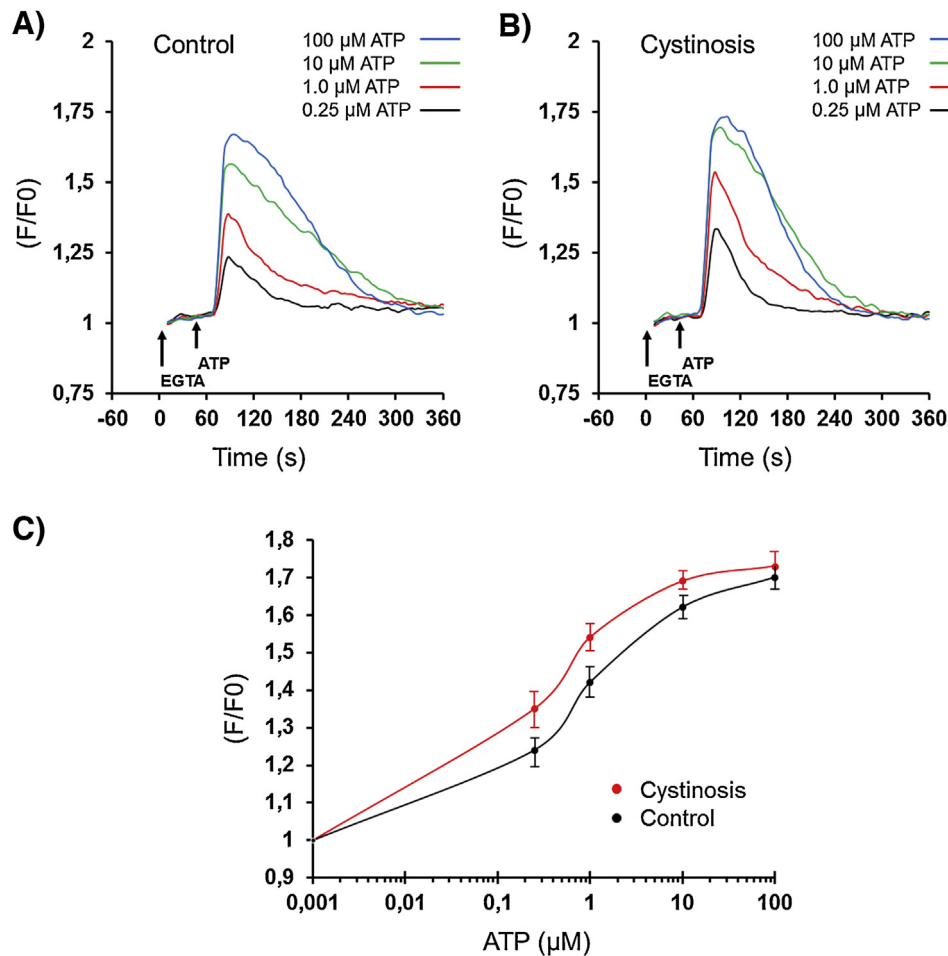
Fig. 1. ATP-induced Ca^{2+} release in control and cystinotic ciPTEC lines. Cells were exposed to the indicated [ATP] after the addition of 3 mM EGTA. The increase of the Fura-2 F340/F380 ratio in response to ATP was determined to assess the release of Ca^{2+} from the ER. (A) A representative experiment showing Ca^{2+} release triggered by different [ATP] in a control cell line. Values were plotted as F/F0. (B) A representative experiment showing Ca^{2+} release triggered by different [ATP] in a cystinotic cell line. Values were plotted as F/F_0. (C) The maximal increase (delta ratio) of the signal induced by each ATP concentration is plotted. The data are fitted with the Hill equation. Shown are the averages of 4 control and 4 cystinotic cell lines and 3–4 independent measurements were done for each concentration for each cell line. The results are presented as means ± SEM.

in each of the effluent samples and in the lysed sample was measured using a liquid scintillation counter. The time course of the $^{45}Ca^{2+}$ wash-out was then calculated by summing in retrograde order the amount of radioactivity remaining in the stores at the end of the efflux and the radioactivity collected during the successive time intervals. The Ca^{2+} release was plotted as fractional loss, i.e. the amount of $^{45}Ca^{2+}$ leaving the cells in 2 min, normalized to the amount of $^{45}Ca^{2+}$ present in the cells at that time. Concentration-response curves were fitted using OriginPro 8 software using the Hill equation.

2.6. RNA isolation and quantitative real time PCR

Total RNA was isolated from the 4 cystinotic and 4 control PTEC cell lines using the RNeasy mini kit (QIAGEN, Valencia, CA, USA). RNA was eluted in 30–50 μl of nuclease-free water and stored at −80 °C until further use. cDNA was synthesized from 1 μg of total RNA with the SuperScript III RT kit (Invitrogen, Waltham, MA, USA). qPCR reactions were carried out for IP_3R gene (*ITPR3*, transcript ENST00000374316, Forward primer: 5′-TGGGGCACAACATCTAC-3′, Reverse primer: 5′-CTTTGTTATGCCGAGCCA- 3′) and the reference gene *GAPDH* (transcript ENST00000229239, Forward primer: 5′-GAGTCAACGGATTTGGTCGTAT-3′, Reverse primer: 5′-CAGGAGGCATTGCTGATGATCT-3′) using SYBR green with ROX in

the StepOnePlus RT-PCR system (Applied Biosystems, Carlsbad, CA, USA).

2.7. Statistical analysis

An unpaired Student's *t*-test was used to test for significant differences between means. Statistical values were expressed as mean ± SEM and a 2-tailed P value < 0.05 was considered significant.

3. Results

3.1. ATP-induced Ca^{2+} release is slightly potentiated in cystinotic cells

Cytosolic Ca2+ signals were monitored in 4 control and 4 cystinotic ciPTEC lines using the ratiometric fluorescent Ca2+ dye Fura-2. Baseline Ca^{2+} levels were very similar between control and cystinotic cells. We further assessed the Ca^{2+} release from the ER in the 4 control and 4 cystinotic ciPTEC lines after stimulation with extracellular ATP, an agonist coupled to intracellular IP_3 production and Ca^{2+} release from intracellular stores. ATP-induced Ca^{2+} release in control and cystinotic cells was determined in the absence of extracellular Ca^{2+} to exclude the contribution of an influx of extra-
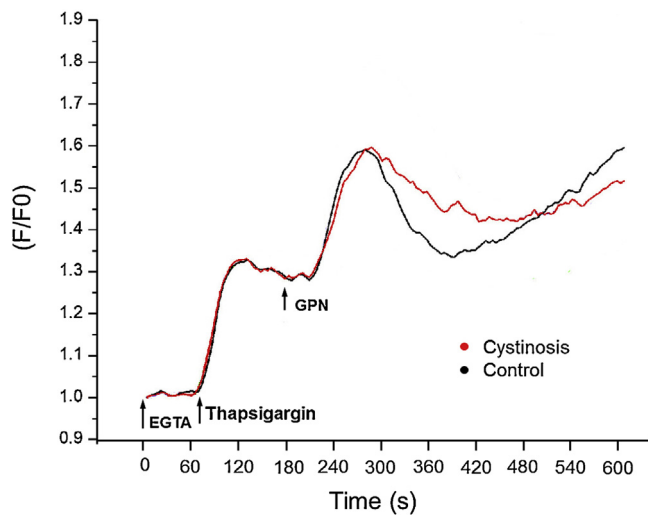
Fig. 2. Lysosomal Ca^{2+} content in control and cystinotic ciPTEC lines. After the addition of 3 mM EGTA, the cells were first exposed to 2 μM thapsigargin and then with 200 μM GPN. The increase of the Fura-2 F340/F380 ratio after the addition of GPN corresponds to the release of lysosomal Ca^{2+}. No difference was observed between control (black) and cystinotic cells (red). Averaged data from 5 independent experiments in representative control and cystinotic cell lines are shown.

cellular Ca^{2+}. Fig. 1A and B depict a typical experiment for PT33.5, a control cell line, and PT2.1, a cystinotic cell line, showing cytosolic Ca^{2+} levels as F/F0 values. We quantified the maximal amplitude of the Ca^{2+} release in response to increasing concentrations of ATP. We found that ATP-induced Ca^{2+} release was slightly potentiated in cystinotic cells (Fig. 1C). The EC_{50} for ATP-induced Ca^{2+} release decreased from 0.66 μM in control cells to 0.42 μM in cystinotic cells, although the statistical significance has not been reached (p = 0.189 in a 2-tailed t-test). No difference in Hill coefficient was observed: the Hill coefficient was 0.699 in control cells and 0.769 in the cystinotic cells. Also the Ca^{2+} release by a maximal [ATP] was not significantly different.

3.2. The acidic Ca^{2+} stores were unaffected in cystinotic cells

We next investigated whether the acidic Ca^{2+} stores are altered in cystinosis. To that end, control and cystinotic ciPTEC lines were pre-incubated with 2 μM thapsigargin to exclude the input of Ca^{2+} fluxes from the ER stores and then treated with 200 μM GPN. The cleavage of GPN in the lysosomes leads to an osmotic disruption of these organelles and the release of Ca^{2+} into the cytoplasm, where it can be detected by the Fura-2-AM dye. We did not observe any difference in lysosomal Ca^{2+} content between control and cystinotic cells using the described method (Fig. 2). The average height of the GPN-induced Ca^{2+} release was 0.329 (SD ± 0.046) for control and 0.311 (SD ± 0.040) for cystinotic cells (p = 0.537). Also the Ca^{2+} content of the thapsigargin-sensitive stores was the same in control and cystinotic cells.

3.3. IP_3-induced Ca^{2+} release was unaffected in cystinotic cells

To assess the Ca^{2+} release from the ER stores in control and cystinotic ciPTEC lines, we used a previously described method making use of a radioactive Ca^{2+} probe [24]. Cells were permeabilized with saponin and the non-mitochondrial Ca^{2+} stores were loaded to steady-state with $^{45}Ca^{2+}$. The cells were then incubated in an efflux medium, and the $^{45}Ca^{2+}$ efflux was measured as the amount of radioactivity measured in subsequent fractions of medium exchanged every 2 min. The re-uptake of $^{45}Ca^{2+}$ was prevented by keeping its free concentration low (by the presence

of 1 mM EGTA), by the presence of 2 μM of thapsigargin to block the Ca^{2+} pump, and by the absence of Mg-ATP to fuel the Ca^{2+} pumps. After 8 min of efflux, 1, 3, 10, 30 or 100 μM IP_3 or 10 μM of the Ca^{2+} ionophore A23187 were added for 2 min. The addition of these agents resulted in a more rapid loss of Ca^{2+} (Fig. 3A,B). The effect of increasing concentrations of IP_3 was tested using this protocol. The Ca^{2+} release was quantified from the difference in fractional loss (%/2 min) obtained in the presence and absence of IP_3. The concentration-response curves for control and cystinotic ciPTEC lines are shown on Fig. 3C. Data from 3 independent experiments demonstrated no difference in Ca^{2+} release in response to stimulation with increasing doses of IP_3. Furthermore, there was no significant difference regarding the IP_3R3 (ITPR3) gene expression at the mRNA level between the control and cystinotic cell lines used in the study (Fig. 3D).

4. Discussion

Several lines of evidence suggested the possibility of disturbed Ca^{2+} signalling in cystinosis. Several pathological features observed in cystinosis are common in other disorders where Ca^{2+} signalling is affected. For instance, ER stress and the UPR can be associated with disturbed ER Ca^{2+} stores, and lysosomal dysfunction can be caused by a decreased luminal Ca^{2+} content, as it has been demonstrated for other disorders with lysosomal dysfunction [20–22]. Despite the significant progress made in the understanding of the pathogenesis of cystinosis, it has not been investigated whether Ca^{2+} signalling is affected in this disease or not.

In the current study, we explored the possibility of altered ER and lysosomal Ca^{2+} signalling using human PTEC lines being the most representative of the disease pathology. We used several cell lines derived from different healthy donors and cystinotic patients. Analysis of the data indicated that the ATP-induced Ca^{2+} release was slightly potentiated in cystinotic cells since the concentration-response curve was shifted to the left. However the significance of this observation for the pathophysiology of cystinosis is uncertain. Further analysis of the IP_3-induced Ca^{2+} release from permeabilized cells revealed no difference between the control and the cystinotic cells. This observation indicates that the IP_3R was not affected in cystinotic cells. This was further confirmed by the expression of IP_3R3 (ITPR3) gene in both control and cystinotic cell lines which revealed no significant difference. The potentiation of the ATP-induced Ca^{2+} release could possibly be attributed to an altered ATP receptor expression or its phosphorylation in cystinosin-deficient cells. Alternatively, IP_3 production or breakdown could be affected, or the rate of Ca^{2+} extrusion from the cells could be affected.

No difference in lysosomal Ca^{2+} content or in the size of the thapsigargin-sensitive Ca^{2+} stores could be detected between control and cystinotic cells. Measurement of the lysosomal Ca^{2+} content provided the data on the total amount of Ca^{2+} in the acidic stores that could be disrupted by GPN. However, the endolysosomal system appears to be severely perturbed in cystinosis, with the lysosomes being significantly enlarged and clustered. Moreover, the method provides no information on the Ca^{2+} content of the vesicles that could not be reached by GPN, such as recycling endosomes and autophagosomes, or of vesicles that are unable to fuse due to disrupted endosomal function. It could be worthwhile to measure the luminal $[Ca^{2+}]$ in the whole range of endosomal vesicles by labelling them with a Ca^{2+}-sensitive fluorescent dye using a fluorescent microscope.

Interestingly, a link between the lysosomal Ca^{2+} signalling and autophagy has been demonstrated as the lysosomal Ca^{2+} release during starvation through mucolipin 1 caused local activation of calcineurin and dephosphorylation of transcription factor EB (TFEB), which triggered lysosomal and autophagic gene expres-
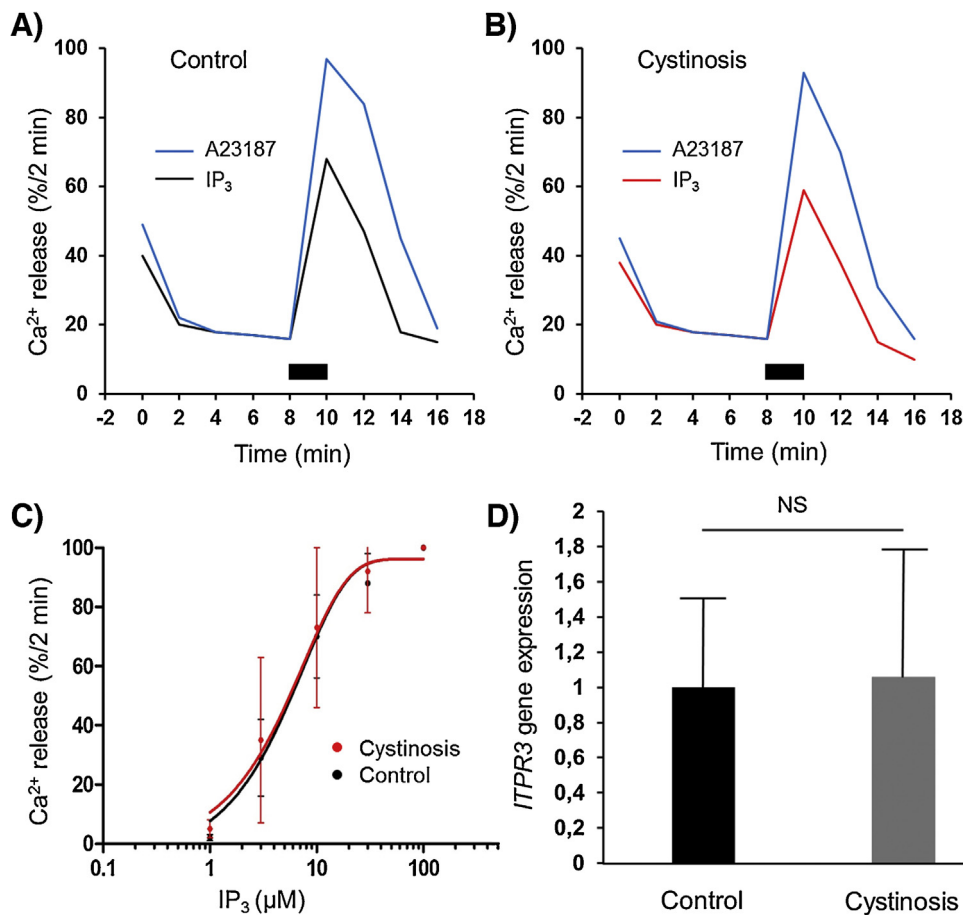
Fig. 3. IP$_3$-induced Ca^{2+} release in permeabilized ciPTEC lines. Effect of 10 μM IP$_3$ or 10 μM A23187 on the Ca^{2+} release plotted as fractional loss (%/2 min), i.e. the amount of ^{45}Ca^{2+} leaving the cells in 2 min normalized to the amount of ^{45}Ca^{2+} present in the cells at that time. The average data of three independent experiments from representative (A) control and (B) cystinotic cell lines are shown, The time frame in which IP3 or A23187 were added is indicated with a black box. (C) Concentration-response curves for control (black) and cystinotic (red) ciPTEC lines fitted with the Hill equation. The Ca^{2+} release induced by 100 μM IP$_3$, a supramaximal concentration, was set at 100%. The results are presented as means ± SEM. D) IP$_3$R3 (ITPR3) gene expressions in control and cystinotic cell lines. Data were normalized to GAPDH and the average expression of control cell lines was set at 1.0.

sion in the nucleus [25]. Recently, TFEB expression was found to be reduced in human cystinotic ciPTEC cells associated with the abnormal translocation to the nucleus. Enhancing endogenous TFEB activity by genistein or overexpression of exogenous TFEB, both stimulated the delayed endocytic cargo processing and significantly decreased cystine concentrations in these cells [26]. Since the lysosomal Ca^{2+} store appears to be intact in cystinosis, it would be interesting to test other components of this signalling pathway, namely, mucolipin 1 expression and calcineurin activation.

In conclusion, our work demonstrated no major abnormality of ER and lysosomal Ca^{2+} signalling associated with cystinosin deficiency in human proximal tubular epithelial cells. Some minor alterations, like in the Ca^{2+} responses triggered by extracellular agonists, may contribute to the disease pathogenesis.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgments

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