Purinergic Receptors Stimulate $\text{Na}^+/\text{Ca}^{2+}$ Exchange in Pancreatic Duct Cells: Possible Role of Proteins Handling and Transporting Ca^{2+}

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Key Words

ATP • P2 purinergic receptors • $\text{Na}^+/\text{Ca}^{2+}$ exchanger • Ca^{2+} transport • Ca^{2+} absorption • Pancreas • Ca^{2+} sensing receptor • Calbindin • Pancreatitis

Abstract

Most purinergic receptors activate intracellular Ca^{2+} signalling, and in epithelia they stimulate transport of major ions. Aim of the present study on pancreatic ducts was to find whether P2 receptors also regulate cellular Ca^{2+} transport, such as that via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). Since NCX can also be connected with epithelial Ca^{2+} transport, we also investigated expression of some Ca^{2+} -handling/transporting proteins. Expression analysis revealed that pancreatic ducts of rat and human duct cell line CFPAC-1 (also PANC-1 and Capan-1) express the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (splice variants NCX1.3 and 1.7), as well as the Ca^{2+} -sensing receptor and the Ca^{2+} -binding protein calbindin-D 9k. These proteins localize to the luminal and lateral membranes of pancreatic duct epithelia. Ca^{2+} imaging experiments showed that activation of purinergic P2Y and P2X receptors stimulated NCX in human and rat duct cells. Application of ATP to CFPAC-1 monolayers also

stimulated Ca^{2+} transport from the luminal to the basolateral side. Taken together, these results show that pancreatic ducts express a number of Ca^{2+} -handling/transporting proteins and we propose that these together with purinergic receptors play a role in regulation of duct lumen Ca^{2+} content.

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Introduction

Pancreatic ducts are a site of purinergic signalling; they express a variety of different metabotropic P2Y and ionotropic P2X receptors that are regulated by ATP secreted into lumen by acini, or possibly by ATP released from nerve endings or islet cells [1, 2]. These purinergic and also adenosine receptors regulate Cl^- and K^+ channels and $\text{H}^+/\text{HCO}_3^-$ transport, and thus control duct secretion [2-4]. Recent studies in our laboratory showed that nucleotides elicit robust intracellular Ca^{2+} signalling in human duct cells [5]. However, Ca^{2+} signals were inhibited at high nucleotide concentrations, possibly due to unusual stimulation of Ca^{2+} efflux. Since rat pancreatic ducts express $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX1), which is regulated

by secretin, acetylcholine and insulin [6, 7], it was worth considering whether P2 receptors could regulate this exchanger on a single cell level. In addition, NCX may not only be important in regulating intracellular Ca^{2+} signalling, but at the organ level it could be a part of transepithelial Ca^{2+} transport mechanism. For example, in epithelia of the kidney and intestine NCX participates in Ca^{2+} absorption. Other Ca^{2+} -handling/transporting proteins involved are TRPV channels, vitamin D-dependent Ca^{2+} -binding proteins called calbindins (CaBP-D_{28k} and CaBP-D_{9k}), plasma membrane Ca^{2+} -ATPase (PMCA) and calcium sensing receptors (CaR) [8-11]. In pancreas, the normal pancreatic juice contains relatively low Ca^{2+} concentrations (<0.5 mM) [12-14], but except for a few studies on CaR [15, 16] and our studies on NCX [6, 7], very little is known about the mechanism and possible sites of Ca^{2+} transport and expression of Ca^{2+} handling/transport proteins.

The aim of the present study was to elucidate whether ATP and other nucleotides affect Ca^{2+} homeostasis in pancreatic duct cells. Furthermore, in order to determine whether pancreatic ducts are the sites of transepithelial Ca^{2+} transport, we started to map expression of certain Ca^{2+} -handling proteins, and initiated a first study on Ca^{2+} transport. Present study was performed on rat pancreatic ducts and on cells from human pancreatic duct lines CFPAC-1 (also PANC-1 and Capan-1), which are commonly used as models for epithelial transport of human pancreatic ducts [2]. We show that purinergic receptors stimulate NCX in both rat and human duct cells. Pancreatic ducts express NCX1, CaR and CaBP-D_{9k} and their localization and first transport studies show that they can participate in Ca^{2+} homeostasis and regulated transport.

Materials and Methods

Cell culture

All standard chemicals were purchased from Sigma-Aldrich. Pancreatic cell lines CFPAC-1, PANC-1 and Capan-1 (#CRL-1918, CRL-1469 and HTB-79 from ATCC, Manassas) were grown in DMEM or IMDM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells (passages 14 to 53) were grown at 37°C with 5% CO_2 for 5-7 days until confluent on NUNC plastic dishes for RNA and protein extraction, optically-transparent dishes (Willco Wells, Amsterdam or Ibidi, Munich) for Ca^{2+} imaging, and on glass cover slips or permeable membrane support (Costar Snapwells) for immunocytochemistry and transport measurements. As discussed earlier, cells form polarized monolayers and develop primary cilia [5, 17].

Ca^{2+} measurements

Fura-2 (Invitrogen) was used to monitor intracellular Ca^{2+} signals in cultured cells as described earlier [5]. Briefly, cells were loaded with 2 μM Fura-2/AM in control solution containing in mmol/l: Na^+ 145, K^+ 3.6, Ca^{2+} 1.5, Mg^{2+} 1, Cl^- 144, HEPES 10, glucose 5, pH 7.4. In nominally Na^+ -free solutions, NaCl was substituted with N-methyl-D-glucamine. In nominally Ca^{2+} -free solutions, Ca^{2+} was omitted and 5 mmol/l EGTA was added. In some experiments, K^+ concentrations were 0.5 or 20 mM. Fura-2 ratio (340nm/380nm) was used to estimate changes in intracellular Ca^{2+} concentrations (i.e. Ca^{2+} signals) in response to nucleotides. Agonists were carefully added to cells in a standing bath. When bath solution was changed, cells rested at least 15 min before recording started. In each experiment, Ca^{2+} responses from 20-40 single cells were evaluated. Addition of nucleotides caused the peak, plateau-like and dip phases in the Fura-2 ratio. In some experiments *in situ* calibration using 1 $\mu\text{mol}/\text{L}$ ionomycin was performed.

Pancreatic ducts were prepared from female Wistar rats as described previously [18]. Intercalated and small intralobular ducts were obtained by collagenase digestion method; these were too small and fragile for perfusion, compared to ducts used for perfusion in our other studies [7, 19]. Ducts were held by holding pipettes and both luminal and basolateral sides were accessible to bath solutions.

Transepithelial measurements

In one series of experiments CFPAC-1 cells were grown on permeable membranes until monolayer resistance was about $200\ \Omega\text{cm}^2$. Medium was carefully substituted with physiological saline containing 25 mM NaHCO_3 and solution was equilibrated with 5% CO_2 in O_2 (0.5 ml in luminal and 2 ml in serosal compartments) at 37°C . After 30-60 min rest, monolayer was stimulated with luminal and serosal addition on ATP (0.1 mM) and 10 and 40 μl samples were withdrawn for analysis of Ca^{2+} concentrations using QuantiChrom™ Calcium Assay (BioAssay Systems, Hayward, CA). Using the same method, Ca^{2+} concentrations in the physiological saline used for experiments was 1.32 ± 0.05 mmol/l and in culture media it was 1.91 ± 0.10 mmol/l ($n=9$).

RT-PCR analysis, Western blot and immunoprecipitation

Total RNA from confluent duct cells and homogenized rat pancreas was isolated with Total RNA Isolation Reagent from ABgene (Rockford, IL, USA). As control for DNA contamination PCR with β -actin specific primers was performed. RT-PCR was done with OneStep RT-PCR kit (Qiagen) as described earlier [5]. Table 1 show the primers used and the expected fragment lengths for transcripts. Primers were synthesized by MWG Biotech (Ebersberg, Germany) and TAG Copenhagen A/S (Copenhagen, Denmark) and designed using VectorNTI software. Identity of products was confirmed by sequencing (MWG Biotech, Ebersberg, Germany).

For Western blot and immunoprecipitation cultured cells were harvested in ice-cold PBS, centrifuged at $10.000\times g$ at 4°C . Pellets were resuspended and lysed on ice for 15 min in 2 ml ice-cold lysis buffer (10 mmol/L Tris-HCl pH 7.4, 0.25 mmol/L sucrose, 1 mmol/L EDTA, 2% NP-40, Roche protease inhibitor).

Primer set	Forward primer	Reverse primer	Exp. length (bp)
huNCX1	GTTATCGTTCCATATAAAACCATCG	TGCAATGGTGATTACAGTAGAGAGA	330
NCX1.3/1.7	CATTGGCATCATGGAGGTGA	CCAATGGTGCAGCCAAAGT	726/795*
ratNCX1.3/1.7	AGATATCTTTG AAGAAGATGAAAACCTTTCTGTGCA	CCAAGTCGACTTAGAAGCCTTTTATGTGGCA	1368/1436#
huCaR	TACGGCAAGTTTGTCTCTGC	CTTCTGTTTGCATCTCGGCT	411
huCaBP-D _{9k}	GAAGGCGATCCAAAC	TCCATCACCGTTCTT	182
ratCaR	ATCGGCAAGTTTGTCTCTGC	TCGGCTCTACGTTTGTCTTC	413
ratCaBP-D _{9k}	GAAGGCGATCCAAAC	TTCTTGCCACTACCT	128

Table 1. Primer sets for RT-PCR analysis. *NCX1.3 726 bp and NCX1.7 795 bp. #NCX1.3 1368 bp and NCX1.7 1436 bp.

Alternatively, rat pancreas, heart, kidney, spleen, liver, lung, brain and skeletal muscle were excised, washed, homogenized and lysed. Lysates were centrifuged for 10 minutes at 4°C and 10.000x g. For immunoprecipitation (IP) lysate was centrifuged at 15.000x g for 1 hour at 4°C and primary antibody (1-2 µl) was added to 500 µl of lysate and incubated overnight at 4°C in orbital shaker. 100 µl of 50% slurry of protein A/G sepharose beads were added and incubated 1 hour at 4°C. Beads were washed in ice-cold lysis buffer and collected by pulse centrifugation. Immuno-complexes were dissociated from beads at 96° C for 5 minutes in 60 µl sample buffer, collected by centrifugation and stored at -80°C. Cell lysates and immunoprecipitates were boiled in sample buffer and loaded to NuPAGE 10% Bis-Tris SDS gels or SDS-PAGE and blotted onto NuPAGE PVDF membranes. Blots were blocked 1 hour in BSA/NGS in PBS and incubated 1 hour with antibodies (1:1000) directed against CaBP-D_{9k} and NCX (Swant, Bellinzona, Switzerland) and CaR (Affinity BioReagents, Golden, USA), and visualized with ECL Advance Western Blotting Detection Kit (Amersham Biosciences) on Kodak ImageStation 440 CF.

Immunocytochemistry on duct cell lines and rat pancreas

Confluent duct cell lines were fixed in a 4% paraformaldehyde (PFA) for 15 minutes or in methanol at -20°C for 5 minutes. Cryosections from rat pancreas were prepared and fixed as above. PFA-fixed cells or tissues were permeabilized with 0.1% Triton X-100 and 1% BSA in PBS for 10-12 minutes. Samples were blocked for 30 minutes in 3% BSA or 10% FCS in PBS and incubated overnight at 4°C with primary antibodies (1:100)(see above). Secondary antibodies conjugated to Alexa488 (1:400) were added for 45 minutes. In controls primary or secondary antibodies were omitted and scanning was performed at same settings. Nuclei were stained with DAPI (1:400) for 5 minutes. Samples were mounted with 2% n-propylgallat and 90% glycerol. Fluorescence was examined in Leica TCS NT/SP CLSM with 40x or 100x oil objectives.

Statistics

Fura-2 measurements are presented as original recordings of single cells or isolated ducts, or as summaries; *n* refers to the number of experiments in different dishes or ducts. For analysis of responses on cell lines unpaired *t*-test were used; for rat ducts measurements were performed on same preparation ducts and the paired *t*-test was applied. In transport studies, Ca²⁺ concentrations are shown as means±SEM and *t*-test was applied to test difference between lumen and serosal samples.

Results

Ca²⁺ signals in human duct cell lines

In the first series of experiments we tested the effect of increased nucleotide concentrations (repetitive addition) on intracellular Ca²⁺ signals. Fig. 1a depicts a representative experiment with ATP stimulation of CFPAC-1 cells, which do not express functional CFTR (cystic fibrosis transmembrane conductance regulator), but are often used in studying Ca²⁺-activated Cl⁻ transport that can be stimulated by ATP. In our experiments ATP evoked a peak Ca²⁺ response in all cells (represents mainly Ca²⁺ release from intracellular stores), and this was followed by a plateau-like phase of oscillating or smooth-type responses in individual cells (includes Ca²⁺ influx and efflux components) [5, 20]. Re-stimulation with the same concentration of ATP (100 µmol/l) 4-5 min later resulted in a transient inhibition of the Ca²⁺ plateau phase - so called “dip response”. UTP did not cause the dip response with ATP, but Ca²⁺ continued to decrease (Fig. 1b). Adenosine (100 µmol/l) also failed to induce the dip response (*n* = 3) (results not shown). BzATP (2',3'-*O*-benzoylbenzoyl-ATP), a putative P2X7 agonist, elicited the Ca²⁺ peak but not the plateau [5], and therefore could not be used in re-stimulation experiments on duct cell lines.

In another cell line, PANC-1 cells, re-stimulation with ATP caused in a new and less prominent Ca²⁺ peak (Fig. 1c), reflecting Ca²⁺ store release and probably some purinergic receptor desensitization. In contrast, UTP (30 µmol/l) induced significant Ca²⁺ dip (Fig. 1d), similar to that seen in ATP re-stimulated CFPAC-1 cells. At higher UTP concentrations (100 µmol/l), the whole plateau decreased, as reported earlier [5].

Next, we examined cause of the Ca²⁺ dip event. One obvious possibility was that P2 receptor stimulation evoked Ca²⁺-efflux by, for example, Na⁺/Ca²⁺ exchanger (NCX). NCX is an electrogenic transporter, which exchanges 1 Ca²⁺ for 3 or 4 Na⁺ and can operate in two modes referring to cell: Ca²⁺ efflux mode (forward mode)

Fig. 1. Effect of nucleotides on Ca^{2+} signals in CFPAC-1 and PANC-1 cells. Representative original recordings of Fura-2 ratio in cells stimulated twice with ATP or UTP (100 and 30 μM). In every experiment responses of 10 cells are shown: oscillating responses are in colors; poorly and non-oscillating are shown in grey. In CFPAC-1 (a) ATP (100 μM) and (b) UTP (100 μM); in PANC-1 (c) ATP (100 μM) and (d) UTP (30 μM) ($n=4$ to 11).

Fig. 2. Effect of ion substitutions on intracellular Ca^{2+} signals in CFPAC-1 cells. Representative recordings of Fura-2 measurements in CFPAC-1 cells stimulated twice with ATP (100 μM). The bath solutions contained: (a) 0 mM Ca^{2+} ; (b) 0 mM Na^{+} ; (c) 20 mM K^{+} ; (d) 0.5 mM K^{+} ($n=3$ to 7).

and Ca^{2+} -influx mode (reverse mode). We continued further experiments on CFPAC-1 cells, which are homogenous in morphology and cell Ca^{2+} signals, and have been used in anion transport studies [2, 5].

Since NCX and other Ca^{2+} transporters/channels depend on Ca^{2+} gradient across the cell membrane, we tested effects of a nominal 0 mM (zero) Ca^{2+} solution. A representative original recording is shown in Fig. 2a and summarized data in Fig. 3. In zero Ca^{2+} solution ATP evoked the Ca^{2+} peak and plateau significantly lower than in control experiments with 1.5 mM Ca^{2+} and the dip depth decreased significantly (Fig. 1a vs. 2a, 3).

The well established test for NCX activity is to determine the effect of low extracellular Na^{+} on intracellular Ca^{2+} concentrations. Therefore, nominal 0 mM Na^{+} solutions were used and results are shown in Fig. 2b and 3. The zero Na^{+} experiments did not differ from control experiments (145 mM Na^{+}) regarding the Ca^{2+} peak or plateaus after the first stimulation with ATP (Fig. 2b vs 1a). The second ATP addition induced a small dip and cells remained oscillating (Fig. 3). In these conditions Na^{+} gradient would favor Ca^{2+} influx rather than Ca^{2+} efflux.

The widely used NCX inhibitor KB-R7943 (5 to 100 $\mu\text{mol/L}$) was used in pilot studies. The inhibitor significantly inhibited and shortened the dip response in about 40% of

cells, but it also exerted variable effect on the peak and plateau Ca^{2+} response. Further experiments with KB-R7943 were not pursued, as two studies began to question specificity of this inhibitor [21, 22].

Since NCX is electrogenic, membrane potential should affect $\text{Na}^{+}/\text{Ca}^{2+}$ exchange. In order to depolarize cells, bathing solutions were changed from 3.6 to 20 mM K^{+} . This would favor Ca^{2+} influx via NCX; one might also expect inhibition of Ca^{2+} influx through Ca^{2+} permeable channels. Fig. 2c shows an original experiment and Fig. 3 the summary. Overall, 20 mM K^{+} seemed to decrease the Ca^{2+} peak and plateau and increased the dip duration, although no statistical significance was achieved. In contrast, the 0.5 mM K^{+} solution would hyperpolarize cells and favor Ca^{2+} -efflux mode of NCX. In addition, one might also expect increased influx via Ca^{2+} channels - this was seen as larger Ca^{2+} peaks and plateaus (Fig. 2, 3).

Ca^{2+} signals in rat pancreatic ducts

The above experiments indicate that ATP stimulates NCX in human pancreatic duct cells. Since rat pancreatic ducts express NCX [6, 7], we performed key experiments on isolated rat ducts (Fig. 4). In resting ducts, zero Na^{+} increased intracellular Ca^{2+} concentrations via NCX, as also observed earlier [7]. Addition of ATP, UTP or BzATP

Fig. 3. Summary of the Δ Fura-2 ratios under different experimental conditions showing: (a) mean peak; (b) mean plateau; (c) mean dip depth; and (d) mean dip durations ($\pm$ SEM). Numbers refer to the number of experiments; each experiment is average of all cell responses in on dish. Statistical significance ($P < 0.05$) indicated * with regard to control experiments, and # with regards to other experiments in that group.

also evoked Ca^{2+} signals, and as we show earlier, these were dependent on extracellular Ca^{2+} [18]. Here the most important observation is that BzATP grossly increased the effect of zero Na^+ (Fig. 4a). In summary: BzATP in normal Na^+ solution increased the Fura-2 ratio by 0.374 ± 0.123 ; if the same ducts were stimulated in zero Na^+ solution the ratio increased by 1.001 ± 0.289 ; change to a zero Na^+ solution alone increased the ratio by 0.317 ± 0.137 ($n = 4$). Notably, when ATP or UTP were used as agonists, the Ca^{2+} response to zero Na^+ was not significantly affected (Fig. 4b). In summary: ATP in normal solutions increased the ratio by 0.305 ± 0.070 , while ATP in zero Na^+ solution gave 0.214 ± 0.059 change; in zero Na^+ solution alone the Fura-2 ratio changed by 0.243 ± 0.035 ($n = 6$). The following data were obtained with UTP: in normal solutions UTP gave 0.322 ± 0.107 ratio change, while UTP in zero Na^+ solution gave 0.302 ± 0.088 change in the same ducts; zero Na^+ solution alone changed in Fura-2 ratio by 0.246 ± 0.057 ($n = 3$).

Fig. 4. Activation of $\text{Na}^+/\text{Ca}^{2+}$ exchange with nucleotides in isolated rat pancreatic ducts. (a) BzATP ($100 \mu\text{M}$) accentuated increase in intracellular Ca^{2+} evoked by 0 mM Na^+ bath solution. Note that BzATP alone increased intracellular Ca^{2+} (beginning of the black bar). (b) ATP ($100 \mu\text{M}$) did not enhance the zero Na^+ effect. Representative of 4-9 experiments.

Expression of NCX and other Ca^{2+} transporting and binding proteins

To support the functional data we then determined expression of NCX on mRNA and protein level (Fig. 5). RT-PCR analysis confirms expression of NCX in two splice variants NCX1.3 and NCX1.7 in rat pancreas (Fig. 5a). Now we show in CFPAC-1, PANC-1, and Capan-1 cells both splice variants, although NCX1.3 gave stronger band than NCX1.7 (Fig. 5b). Following immunoprecipitation, western blot analysis of the human material shows strongest band at 150-160 kDa (Fig. 5c). Bands of 70, 120 and 150-160 kDa are in agreement with expression of the NCX protein and correspond to the proteolytic fragment, full protein and glycosylated protein, respectively [7].

We also looked for expression of other proteins involved in Ca^{2+} homeostasis. Expression of CaR in PANC-1, CFPAC-1 and Capan-1 was examined with RT-PCR and western blot (Fig. 6). All three cell lines

Fig. 5. Expression of NCX mRNA and protein in rat pancreas and human pancreatic duct cell lines PANC-1 (P), CFPAC-1 (CF) and Capan-1 (CP). (a) RT-PCR on six different pools of rat pancreas RNA with primers specific for NCX1. The two transcripts correspond to splice variants NCX1.3 (1368 bp) and NCX1.7 (1436 bp). (b) RT-PCR on RNA extracted from human duct lines gave two transcripts: NCX1.3 (726 bp) and NCX1.7 (795 bp). (c) Western blot on protein immunoprecipitated with NCX antibody showed bands at ~ 70 kDa and ~150 kDa.

showed CaR transcripts, the strongest seen in Capan-1 cells and weak bands seen in PANC-1 and CFPAC-1 cells (Fig. 6a). Also the rat pancreas had CaR transcripts (Fig. 6b). Western blot on total cell lysates and immunoprecipitates also gave expected bands of ~ 120 kDa (Fig. 6c). CaR expression was verified with western blot on rat pancreas and other tissues (Fig. 6d). CaR was expressed strongly in pancreas and also in spleen, heart, lung and brain and weakly in the kidney. The difference in sizes between the bands found in pancreas and brain, compared to other tissues could be due to either phosphorylation and/or glycosylation.

In next experiments we determined expression of a Ca^{2+} -binding protein, CaBP- D_{9k} . CaBP- D_{9k} transcripts were detected in duct cell lines (Fig. 6e) and rat pancreas (Fig. 6f). Expression of CaBP- D_{9k} protein was detected in pancreas, liver, kidney and lung (Fig. 6g).

Immunolocalization of Ca^{2+} -handling proteins

Using CLSM, NCX, CaR and CaBP- D_{9k} were immunolocalized in human duct cell lines, and they showed similar antibody staining (Fig. 7a, b). NCX1 was clearly localized to plasma membranes and peri-nuclear regions (Fig. 7a), and small clusters were apparent on apical (luminal) and lateral membranes of cells grown on permeable supports (Fig. 7b). NCX has been observed in various organelles including mitochondria and secretory vesicles of various cells [23]. CaR had more diffuse cytoplasmic localization (Fig. 7a), and on permeable supports some localization in plasma membranes and dense clusters was detected (Fig. 7b). CaBP- D_{9k} showed diffuse localization, and in polarized cells some protein was targeted to punctuate regions close to the lateral and luminal plasma membranes (Fig. 7b).

Fig. 6. Expression of CaR (a-d) and CaBP- D_{9k} (e-g) in rat pancreas and human pancreatic duct cell lines PANC-1 (P), CFPAC-1 (CF) and Capan-1 (CP). (a) RT-PCR on RNA extracted from cell lines gave transcripts of 411 bp corresponding to CaR mRNA. (b) RT-PCR on RNA extracted from rat pancreas gave transcripts of 413 bp corresponding to CaR mRNA. (c) Western blot on total lysate and immunoprecipitates gave band of ~120 kDa. (d) Western blot on protein extracted from rat tissues. (e) RT-PCR on RNA from the three cell lines revealed transcripts of the expected size 182 bp in all samples. (f) RT-PCR on RNA rat pancreas showed transcripts of the expected size 128 bp. (g) Western blot on immunoprecipitates from various rat tissues.

Fig. 7. Localization of NCX1, CaR and CaBP-D_{9k}. Human pancreatic duct cell lines were grown on glass (a) and on permeable support (b). In (b) split half-images show scans close the apical membrane (left) and bottom of the cells (right). The middle image is a control image with the primary antibody omitted. Full arrows indicate localization of proteins at apical/luminal aspects of cells; empty arrows indicate localization in the lateral plasma membrane. (c) Expression of NCX, CaR and CaBP-D_{9k} in rat pancreas. NCX and CaBP-D_{9k} localized to the luminal and lateral membranes of ducts (full arrows) and to smaller ducts and acini (empty arrows). CaR localized to luminal membranes of duct cells. In all images green is due to secondary antibody conjugated to Alexa 488 and blue is the nuclear stain DAPI. All scale bars are 20 μ m.

Rat pancreatic tissue was also used for immunofluorescence detection of NCX, CaR and CaBP-D_{9k} proteins (Fig. 7c). The NCX1 was detected in luminal and lateral membranes areas of many ducts. In addition, NCX1 also localized to intercalated ducts, centro-acinar cells and canaliculi, and there was a weak reaction in apical poles of acinar cells. Similar staining was observed with the CaBP-D_{9k} antibody. CaR immunolocalization was slightly different. The protein was detected in the luminal and lateral membranes of intra- and interlobular ducts, but no staining of centro-acinar or acinar cells was detected.

Ca²⁺ transport

Above results on Ca²⁺-handling proteins and stimulation of NCX by P2 receptors indicate that pancreatic duct epithelium might transport Ca²⁺. Hence, we set up new experiments on CFPAC-1 monolayers stimulated with ATP in protocols similar to those used for studying intracellular Ca²⁺ in imaging experiments (Fig. 8). Since the key Ca²⁺-handling proteins are expressed on both luminal and serosal membranes, ATP was applied from both sides. Shortly after the first ATP luminal stimulation, when increase in intracellular Ca²⁺ signals was expected, serosal and luminal Ca²⁺ concentrations were similar. However, with re-stimulation, luminal Ca²⁺ concentrations decreased significantly compared to paired serosal samples, indicating transepithelial Ca²⁺ transport. Serosal ATP also supported Ca²⁺ absorption, except again for the first sample where intracellular Ca²⁺ signalling

Fig. 8. ATP stimulates Ca²⁺ transport. CFPAC-1 monolayers were stimulated with ATP given luminal and serosal (100 μ M) and Ca²⁺ concentrations in luminal and serosal compartments were determined. Data points are means $\pm$ SEM (n=9) and statistical significance (P<0.05) is indicated with * for paired measurements.

may have dominated. Summarizing the data for restimulated conditions, the mean serosal Ca^{2+} concentration was 1.58 ± 0.08 mmol/l, while the luminal concentration was 1.26 ± 0.07 mmol/l ($n=9$, $P=0.0006$). The physiological buffer used on both sides of epithelia was 1.32 ± 0.05 mmol/l Ca^{2+} ($n=9$).

Discussion

The most important finding in this paper is that purinergic P2 receptors stimulate cellular $\text{Na}^+/\text{Ca}^{2+}$ exchange in both human and rat pancreatic ducts, and this may explain inhibitory effects of high nucleotide concentrations on Ca^{2+} signals. P2 receptors also stimulate Ca^{2+} absorption in duct cell monolayers. On a molecular level the NCX1, CaR, and CaBP- D_{9k} were identified. We propose that purinergic receptors and Ca^{2+} -handling/transporting proteins may contribute to the mechanism responsible for regulation of Ca^{2+} absorption in pancreatic ducts.

Function and expression of NCX

In single pancreatic duct cells nucleotides evoke intracellular Ca^{2+} responses with oscillations that may arise from different intracellular Ca^{2+} stores [5], and as we show here these are also affected by conditions that influence Ca^{2+} influx and efflux (Fig. 2). In normal Na^+ solutions, P2 receptors stimulate Ca^{2+} influx via flufenamate-sensitive channels [20] and possibly via NCX (see below). Prolonged nucleotide stimulation, or restimulation, reveals Ca^{2+} efflux (the dip effect). Such stimulation of Ca^{2+} efflux would explain anomalous observation with nucleotide concentrations which inhibit the Ca^{2+} plateau, e.g. high UTP inhibits in CFPAC-1 cells and ATP in PANC-1 cells [5]. We propose that these effects are due to stimulation of the high-capacity Ca^{2+} transporter, NCX1, which turns to the Ca^{2+} efflux (forward mode).

The most straight forward evidence for NCX in CFPAC-1 cells was obtained in experiments where zero extracellular Na^+ solutions increased intracellular Ca^{2+} and thus shortened duration of the dip (Fig. 2b). The nucleotide-stimulated Ca^{2+} influx in zero Na^+ was also very prominent in rat pancreatic ducts (Fig. 4). We argue that these are specific NCX effects; they are not due to inhibition of Na^+/H^+ exchange and cell acidification [6]; they are not due to influx through flufenamate-sensitive cation channels [20] and the electrophysiological response is consistent with electrogenic NCX [6]. In normal Na^+

concentrations and moderate stimulation, NCX could operate in Ca^{2+} influx mode, which is energetically feasible if there is increased Na^+ entry via Na^+ transporters or cation-permeable channels (e.g. TRP channels, P2X), or if the membrane potential is depolarized (e.g. opening of Cl^- channels). We also show that membrane potentials (depolarizing and hyperpolarizing K^+ concentrations) affected NCX transport. This Ca^{2+} -influx mode of NCX transport is now accepted and proposed for other tissues [23–26].

In experiments with zero extracellular Ca^{2+} , there would be decreased Ca^{2+} influx, both in the peak and plateau phases (Fig. 2, 3), through Ca^{2+} transporters such as NCX and P2X4/P2X7 receptors, the latter are expressed in both rat and human and pancreatic ducts [2, 5]. Another possibility is that Ca^{2+} influx could occur via Ca^{2+} permeable channels of the TRP family. Although TRPV5 and TRPV6 are expressed in rat pancreas (in islets and acini), they were not detected in ducts [27–29].

The present work also provides molecular evidence for NCX1 expression in human pancreatic cells and expands on evidence for the rat pancreas [6, 7]. Human duct cells express two splice variants, NCX1.3 and NCX1.7 (Fig. 5). These splice variants are also seen in rat pancreas, acini and β -cells [7, 30]. Western blot and immunolocalization further verified NCX protein expression in human and rat duct cells. Most interestingly, NCX1 was localized on the luminal and lateral membranes of intercalated, intra- and interlobular pancreatic ducts and also in centro-acinar cells of the rat pancreas (Fig. 7c). Similar expression on luminal and lateral membranes was seen in human duct monolayers (Fig. 7). In rat acini weak NCX expression was also detected by immunocytochemistry (Fig. 7c) and earlier by RT-PCR and Western blot [7], but no functionality of NCX1 but rather PCMA was demonstrated in other studies [31]. In pancreatic ducts, the NCX1 localization is different to the kidney, where in the latter NCX1 is predominantly located on the basolateral membrane of distal tubuli [32]. In the intestine, localization of NCX1 is unclear and both basolateral and luminal localizations are reported [25, 33]. Interestingly, in another study western blot revealed $\text{Na}^+/\text{Ca}^{2+}\text{-Li}^+$ exchanger (NCLX) in rat pancreas [34], but Li^+ experiments in ducts were inconsistent with NCLX function [35].

P2 receptors stimulate NCX and Ca^{2+} transport

The finding that purinergic receptors affect NCX is new and further work is needed to explain identity of P2 receptors and their coupling to NCX1. Nevertheless,

we recognize the following patterns. In PANC-1 cells NCX was stimulated with UTP and not ATP using given nucleotide concentrations (Fig. 1), which indicate involvement of P2Y2 receptors. In CFPAC-1 cells, NCX was stimulated better by ATP compared to UTP (Fig. 1), suggesting involvement of P2Y or P2X receptors. Capan-1 cells have similar responses as CFPAC-1 cells (preliminary data). In rat pancreatic ducts the P2X7 receptor agonist, BzATP, had the strongest effect on NCX, while ATP and UTP had no effects (Fig. 4), indicating that here the P2X7 receptor was involved. Duct epithelia have the same P2 receptor profiles [3, 5], but we cannot exclude differences in the quantitative expression of certain P2 receptors and different nucleotide response profiles. Possibly, different intracellular coupling of P2 receptors to NCX or to other transporters/channels might indirectly affect NCX in these duct epithelia.

In rat pancreatic ducts, acetylcholine and secretin inhibit NCX, possibly contributing to increasing intracellular Ca^{2+} concentrations in normal conditions [7]. This up-regulation may potentiate signalling pathways leading to HCO_3^- secretion, which is the primary function of the ducts. P2Y receptors would lead to release of Ca^{2+} from intracellular Ca^{2+} stores and Ca^{2+} influx [18]. P2X4 and P2X7 receptors would also allow Ca^{2+} and Na^+ influx. Thus, it may be important for P2 receptors also to stimulate NCX and Ca^{2+} transport, so that the duct cells do not flood with Ca^{2+} .

In addition, we propose that P2 receptors and NCX not only contribute to keeping Ca^{2+} signalling under control, but that they also contribute to the net transcellular Ca^{2+} transport. The first experiments on CFPAC-1 cells show that ATP stimulates Ca^{2+} absorption, as Ca^{2+} concentration in the lumen decreases and that in serosa increases compared to control solutions (Fig. 8). If we assume that *in vivo* the duct luminal compartment is much smaller than the serosal compartment, then the concentration difference would be much larger.

Expression of Calbindin and Calcium-sensing receptor

Both human and rat pancreatic duct cells express CaBP- D_{9k} at RNA and protein levels (Fig. 6-7). Although CaBP- D_{28k} is reported for β -cells [36], to our knowledge this is the first report showing CaBP- D_{9k} expression in pancreas. Most interestingly, CaBP- D_{9k} localization close

to the plasma membrane domains follows that of NCX1 (Fig. 7), suggesting coordinated function of these two proteins. Thus, pancreas shows some similarities regarding the calbindin expression to parts of the small intestine [8, 11]. In kidney, CaBP- D_{28k} was detected intracellularly and also in the basolateral membrane of the distal nephron [8].

CaR is expressed in a number of Ca^{2+} -transporting epithelia and may be important in sensing of Ca^{2+} and cationic compounds [37]. Whether the operational range of Ca^{2+} concentrations in duct lumen is relevant for this receptor needs to be established. Nevertheless, CaR has been detected on mRNA level in rat pancreas, human biopsy tissue and Capan-1 cells [15, 16]. We confirm these findings, and show that this receptor is also expressed on a protein level in human pancreatic duct cell lines and rat pancreas. Furthermore, we show convincingly CaR localization in these tissues (Fig. 7). Especially in rat pancreas CaR is well localized in the luminal and lateral membrane of larger and small ducts. In two earlier studies [15, 16], weak staining of acini was also reported.

Ca^{2+} homeostasis in pancreas

Taken together, we propose a connection between purinergic stimulation and cellular Ca^{2+} signals and Ca^{2+} transport in rat and human pancreatic duct cells. The functional studies on rat and human duct epithelia showed that nucleotides activate NCX. We also show that these epithelia express the Ca^{2+} -handling/transporting proteins NCX, CaR and CaBP- D_{9k} . Future studies need to clarify whether other Ca^{2+} transporters/channels are also expressed, and define driving forces for net Ca^{2+} transport. Clearly, pancreatic physiology of Ca^{2+} homeostasis needs to be addressed as disturbances thereof may lead to calcium-deposit formation (microlithiasis), duct obstruction and pancreatitis.

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