Purinergic Receptors and Calcium Signalling in Human Pancreatic Duct Cell Lines

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Key Words

Purinergic receptors • P2X7 receptor • P2X4 receptor • Pancreatic ducts • ATP release

Abstract

Purinergic receptors regulate various processes including epithelial transport. There are several studies on P2 receptors in pancreatic ducts of various species, but relatively little is known about these receptors in human tissue. The aim of this study was to identify purinergic receptors in human pancreatic duct cell lines PANC-1 and CFPAC-1. Expression of P2 receptors was examined using RT-PCR and immunocytochemistry. Both cell lines, and also Capan-1 cells, express RNA transcripts for the following receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-14 and P2X1, P2X2, P2X4, P2X5, P2X6 and P2X7. Using Fura-2 and single-cell imaging we tested effects of various nucleotide analogues on intracellular Ca^{2+} signals in PANC-1 and CFPAC-1 cells. The cell lines responded to all nucleotides with the following efficiency: $\text{UTP} \geq \text{ATP} = \text{ATP}\gamma\text{S} > \text{BzATP}$. ATP, UTP and $\text{ATP}\gamma\text{S}$ elicited oscillatory responses. BzATP, commonly used to stimulate P2X7 receptors, elicited non-oscillatory and transient Ca^{2+} responses. Ivermectin,

a potentiator of P2X4 receptors, increased Ca^{2+} signals evoked by ATP. The single cell Ca^{2+} measurements indicated functional expression of P2Y2 and other P2Y receptors, and notably expression of P2X4 and P2X7 receptors. Expression of P2Y2, P2X4 and P2X7 receptors was confirmed by immunocytochemistry. This fingerprint of P2 receptors in human pancreatic duct models forms the basis for studying effect of nucleotides on ion and fluid secretion, as well as on Ca^{2+} and tissue homeostasis relevant in physiology and pathophysiology of pancreas.

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Introduction

ATP signalling plays important roles in diverse processes as cell migration, immune defence, cell volume regulation, synaptic transmission and epithelial transport [1-5]. Various epithelia and exocrine glands e.g. airway, inner ear, gastrointestinal tract, liver, kidney, and pancreas, express purinergic receptors belonging to two families: P2Y and P2X [6, 7]. The P2Y receptor family comprises 7-transmembrane spanning G-protein coupled receptors and in epithelia stimulation by ATP/UTP leads to Ca^{2+} release from intracellular stores and regulation of Cl^- and

K⁺ channels [2-4]. The P2X receptor family form ligand-gated cation channels when assembled in homo- or heteromers, and can in epithelia aid Ca²⁺ influx [2, 5].

Rat pancreatic acini cells “secrete” ATP in response to acetylcholine, cholecystokinin, mechanical stimulation and changes in cell volume, but expression of functional P2 receptors on acini is heterogenous and sparse [8, 9]. In contrast, several studies have shown functional P2 receptor expression in pancreatic ducts from rat, guinea pig and dog preparations. For example, native isolated rat ducts seem to express a range of functional P2 receptors, P2Y2, P2Y4, P2X4, P2X7, and respond consistently to ATP stimulation with changes in pH_i, membrane potential, intracellular Ca²⁺ signalling and activation/inhibition of diverse ion-transporters and exchangers [9-11]. Molecular biological approaches (RT-PCR) indicate that rat ducts express P2Y2, P2Y4, P2X4, P2X7 [11], and possibly also P2X1, P2Y1 and P2Y5 receptors [12]. Reports on cultured dog pancreatic duct cells show functional expression of P2Y2 and P2Y11 receptors, which regulate Cl⁻ and K⁺ channels [13-15]. Studies on guinea pig ducts indicated expression of P2Y2 receptors, which regulate secretion [16]. Furthermore, immunohistochemistry on rat pancreas shows expression of P2Y1 and P2Y2 receptors in ductal tissue [17, 18]. Above described studies indicate that in pancreas of several animal species autocrine and paracrine ATP signalling and stimulation of P2 receptors may be important for exocrine functions, such as secretion and tissue homeostasis [19].

Regarding the human pancreas, there are several publications on human duct cell lines reporting effects of the nucleotides ATP/UTP on Cl⁻ and H⁺/HCO₃⁻ transport and “global” Ca²⁺ signalling [20-24]. Exact identification of P2 receptors is important as some P2 receptors could support exocrine secretion (via stimulation of cAMP or Ca²⁺ activated Cl⁻ channels), while others could inhibit exocrine secretion (via inhibition of K⁺ channels), yet others have long-term effects on tissue homeostasis [19]. P2 receptors may be of therapeutic value for improving pancreatic secretion, such as in patients suffering from cystic fibrosis. In respiratory epithelia of patients with cystic fibrosis, P2Y2 receptor agonist has been tested for its ability to rescue fluid secretion by activation of Ca²⁺-activated Cl⁻ channels (see press releases denofosol tetrasodium made by Inspire). In addition, since other cells in pancreas also express P2 receptors which are therapeutic targets, e.g. improvement of insulin secretion of β -cells, it is important to fingerprint P2 receptors in human pancreatic tissue [19].

So far, only two studies have attempted to determine the molecular identity of P2 receptors in human pancreas. In one study the human duct cell line Capan-1 and whole pancreas were analyzed using RT-PCR and the following expression profile was suggested - P2Y1, P2Y2, P2Y4, P2Y6, P2X1, P2X4 and P2X5 receptors, but no P2X6 or P2X7 receptors were found [25]. Using samples from whole human pancreas, Knzli and co-workers [26] showed P2Y1, P2Y2, P2Y6 and P2X7 receptor RNA, and P2Y2 and P2X7 receptors were also identified with western blot. Nevertheless, using immunohistochemistry, P2Y2 and P2X7 receptors were apparently not found in epithelial cells but in endocrine cells, stellate cells and immune cells [26, 27]. It seems then that the P2 receptor expression in human pancreatic duct cells is not yet settled, especially regarding the P2X receptors. Since these receptors may be involved in short-term as well as long-term effects, it is important to determine if they are present on human pancreatic duct epithelium.

The aim of our study was to identify molecular and functional P2 receptor expression in human pancreatic duct cell lines, PANC-1 (and Capan-1) and CFPAC-1. These cell lines are most commonly used for epithelial transport studies and since fresh human ducts are almost impossible to obtain for physiological studies, these cell lines serve as models for human pancreatic duct epithelium [19]. While the PANC-1 (and Capan-1) cells express functional cystic fibrosis transmembrane regulator, CFTR [20, 28, 29], the CFPAC-1 cell line is derived from a cystic fibrosis patient with Δ F508 mutated CFTR [30]. Our experimental approach was to estimate functional expression and molecular identity of P2 receptors by means of single cell Ca²⁺ imaging combined with simple pharmacological approaches, RT-PCR and immunocytochemistry.

Materials and Methods

Cell culture

Pancreatic cell lines were purchased from ATTC (Manassas, VA, USA). PANC-1 (ATTC, #CRL-1469) was grown in Dulbecco’s Modified Eagles Medium (DMEM), CFPAC-1 (#CRL-1918) and Capan-1 (#HTB-79) were grown in Iscove’s Modified DMEM (IMDM), respectively. Cell culture media contained Glutamax, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Some cultures were serum starved with 0.5% FBS for 1-2 days). All cells were grown at 37°C in a humidified atmosphere with 5% CO₂ for 5-7 days until a confluent monolayer was formed. Cells from passage 14 to 53 were used in this study. Cells were grown in NUNC plastic dishes when used for RNA extraction. For fluorescence

Receptor	Forward primer	Reverse primer	Exp. size bp
P2Y1	CGG CTC AAAAAG AAT GCG	GGC CCT CAA GTT CAT CGT TTT C	375
P2Y2	TCT ACT TTG TCA CCA CCA GCG	TTG ATG GCG TTG AGG GTG TG	349
P2Y4	TCG TGC CCA ACC TGT TCT TT	TAAATG GTG CGG GTG ATG TG	304
P2Y6	GCT GCC TGG CTA GTG TGT GT	AAG TAG AAG AGG ATG GGG TCC A	482
P2Y11	CCG AGG CCT GCA TCA AGT GT	ATG TCT GCA AAG CTC GGG CA	310
P2Y12	ATG ATT CTG ACC AAC AGG CAG C	TCA GGG TGT AAG GAA TTC GGG	307
P2Y13	TGA GCA ACAAGG AAG CAA CAC C	TGC TGT GGT CTT TCT CCC TTG C	473
P2Y14	CTG AAA AGT GAA CTG GGA CGG A	TCC AAG CAT ACA TTT GCA GCA G	350
P2X1	TGAATG CTG CCC ACA TGA AG	CCT TGA AGA GGT GAC GGT AG T TG	303
P2X2	CCT CGT CAG GCT ACAACT TCA G	AAG GGT CAC AGG CCA GCT AC	302
P2X3	GGG GAG TTC TGG GCA TTA AG	CAG TTC CCA CTC CCA CAG AAG	303
P2X4 (I)	CCG TGG AGG ATG ACA CAC AC	GGC TCT GTC CAG GTT GCA GT	302
P2X4 (II)	TGG GTC AAC TCT GCT TTT CCC GCA AC	TCT CTG AGG AGA AATGCC AGC TCT GC	294
P2X5	AGC CCA GGG AGA GAA CGT CT	CTG TCC TTG ACG TCC ATC ACA T	360
P2X6	GAA CCA CAA TTC AGC CCC TAC T	GGC TTC TCT ATC CAC ATA CAG CAG	408
P2X7 (I)	TAA AAG TCT TCG GGA TCC GTT T	CTG GTT CAC CAT CCT AAT GTG G	334
P2X7 (II)	AAG TGC GAG TCC ATT GTG GAG CC	AGG ACG TGT CTG GAC AGG ACC A	410

Table 1. Primer sets used for RT-PCR analysis. The expected transcript size (bp) is indicated in the right column.

microscopy, cells were grown in glass bottom dishes (Willco Wells, Amsterdam, Netherlands) or optically transparent plastic dishes from Ibidi (Munich, Germany). All chemicals were purchased from Sigma unless otherwise stated.

Immunocytochemistry

The duct cell lines were cultured to confluence on optically transparent dishes or on coverslips. Samples were rinsed in phosphate buffered saline (PBS) and fixed in methanol at -20°C for 5 min. Subsequently, cells were washed in PBS with 0.1% Triton and autofluorescence was blocked in 0.1 M Tris-glycine. After blocking in 3% BSA, preparations were incubated overnight at 4°C with 1:50 to 1:100 dilutions of various primary antibodies for P2 receptors. The following antibodies were used: polyclonal anti-P2X4 receptor raised against residues 370-388 of rat receptor (Alomone, APR-002); polyclonal anti-P2X7 raised against residues 136-152 of mouse receptor (Alomone, APR-008F); polyclonal anti-P2X7 receptor raised against internal region of human receptor (Santa Cruz, L-20) and anti-P2Y2 receptor raised against human receptor C-terminus (Santa Cruz, C-20). Subsequently, samples were incubated with secondary antibodies (1:400) conjugated to Alexa488 or Alexa568, and with nuclear stain DAPI (1:400). In some samples cells were stained with primary antibody for acetylated α -tubulin (1:5000), in order to mark microtubule and primary cilia. Control samples were prepared without the primary or secondary antibodies. Fluorescence was examined using Leica TCS NT/SP CLSM. Images were analyzed using Leica CLSM software or MetaMorph 5.0 software (Universal Imaging Corporation, West Chester, PA, USA).

Ca²⁺ measurements

The Ca²⁺-sensitive fluorophore Fura-2/AM (Invitrogen) was used to monitor intracellular Ca²⁺ signalling events. Cells were loaded with 2 μ M Fura-2/AM for 30 minutes, gently washed and allowed to rest for at least 30 minutes in a HCO₃⁻-free physiological saline of the following composition (mM): Na⁺ 145, K⁺ 3.6, Ca²⁺ 1.5, Mg²⁺ 1, Cl⁻ 144, HEPES (4-2-

hydroxyethyl-1-piperazineethanesulfonic acid) 10, glucose 5, pH was adjusted to 7.4. Fura-2 was excited at 340 nm and 380 nm and emission at 540±25 nm was captured in the fluorescence microscope imaging setup (Zeiss Axiovert 100 TV equipped with Lambda DG-4 monochromator, CoolSNAP HQ camera and Metamorph 5.0 acquisition system). Regions of interest (ROI) representing single cells were selected on ratiometric fluorescence images (340/380 nm), and the change in ROI fluorescence ratio was used to monitor changes in intracellular Ca²⁺ concentrations (abbreviated as intracellular Ca²⁺ signals or Δ Fura-2 responses) in response to various agonists including ATP, UTP and BzATP (2',3'-O-benzoylbenzoyl-ATP), non-degradable ATP analogue ATP γ S and P2X4 receptor potentiator ivermectin. Addition of agonists was done in standing bath conditions with minimum of mechanical disturbance in order to avoid endogenous ATP release. In some experiments where the bath solution needed to be changed, cells were allowed to rest about 10 min before testing of agonists. Addition of blank probe (saline buffer) to the cells in the chamber was used to check for any disturbances prior to experimenting.

In each experiment 20-40 cells in the field of view were evaluated. Addition of agonists caused an immediate and transient rise in the Fura-2 ratio. The peak value of this rise was defined by the maximum Δ Fura-2 ratio for an average of all ROI in one dish, i.e. one experiment. Following the transient peak, responses decreased to a "plateau-like phase", during which some cells oscillated and others did not. Note that if responses of all cells within each experiment were averaged, then the smooth plateau-like patterns would be more apparent. For simplicity and quantitative comparisons between experiments, we will refer to this phase as the plateau phase. The level of this plateau was determined by averaging Δ Fura-2 ratio values for one minute following end of the peak. For every experiment, responses of 20-40 cells were used to calculate the average Δ peak and Δ plateau values. Also the number of responding and oscillating or non-oscillating cells was determined. In some experiments we performed *in situ* calibration using 1 μ M ionomycin permeabilization. Estimated resting [Ca²⁺]_i in single

duct cells was 50-100 nmol/l and it increased to the peak values of 400-500 nmol/l with ATP/UTP stimulations. These $[Ca^{2+}]_i$ were similar to those observed in rat pancreatic ducts [31]. For analysis, ANOVA or unpaired *t*-test were used, when comparing group of experiments or two set of experiments, respectively; *n* refers to the number of experiments in a series, means and SEM are given in tables and *P*<0.05 was considered statistical significant.

Reverse transcriptase polymerase chain reaction (RT-PCR)

PANC-1, CFPAC-1 and Capan-1 cells were grown as described above for 6-7 days in NUNC petri dishes until confluent and in some cultures FBS was decreased to 0.5% for 1-2 days. Total RNA was isolated with Total RNA Isolation Reagent from ABgene (Rockford, IL, USA) according to the manufacturer's instructions. As negative control for DNA contamination PCR with β -actin specific primers was performed. No DNA contamination was detected (data not shown). RT-PCR was performed with OneStep RT-PCR kit (Qiagen). The amplification parameters were as follows: one cycle at 50°C for 30 min and one cycle at 95°C for 15 min followed by 35-40 cycles at 95°C for 1 min, 55-60°C depending on the specific receptor subtype for 1 min, 72°C for 1 min and finally one cycle at 72°C for 10 min. Subsequently, all reactions were subjected to electrophoresis on 1.2% agarose gels. Table 1 show sequences of the 15 primers used and the expected fragment lengths for their respective transcripts are also listed. 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).

Results

PANC-1 and CFPAC-1 cells in culture

PANC-1 and CFPAC-1 form adherent epithelial monolayers when grown on glass or plastic and show polarization as demonstrated for example by occludin localization [32, 33]. Such preparation of these model cell lines are most often used for studying ion transport and signalling in human pancreas (Introduction), and were appropriate for our study that required single-cell resolution of Ca^{2+} signals. PANC-1 cells, derived from epithelioid carcinoma, form a monolayer of heterogeneous cells with tendency to grow on top of each other when confluent (Fig. 1A). CFPAC-1 cells, derived from a patient with cystic fibrosis expressing deletion in Phe-508 in CFTR, appear homogenous and they form a dense monolayer interrupted by a few void spaces (Fig. 1B). PANC-1 cells seem to have more epithelial-like cytoskeletal arrangement compared to CFPAC-1 cells [34]. In addition, both types of cells form primary cilia on

the apical surface (Fig. 1A, B), similar to rodent and human pancreatic ducts [34].

Ca^{2+} responses to ATP, UTP and BzATP

Fura-2 experiments were carried out in order to characterize and evaluate several P2 agonists and their effect on intracellular Ca^{2+} signals in PANC-1 and CFPAC-1 cells. Responses to agonists were recorded for 4 minutes or longer after addition of agonists. Figure 2A shows original Fura-2 ratio images of PANC-1 cells responding to ATP (100 μ M). The images show marked responses in many cells shortly after stimulation (the peak Ca^{2+} response), then a more heterogeneous oscillating Ca^{2+} signal in many cells (the plateau-like phase – see Methods). Figure 2B shows original images of CFPAC-1 cells during similar ATP stimulation. Likewise, CFPAC-1 cells also demonstrate the Ca^{2+} peak response followed by a plateau phase with pronounced and coordinated oscillations in groups of cells. In general, CFPAC-1 cells were more synchronous in Ca^{2+} oscillations than PANC-1 cells (also see below).

PANC-1 and CFPAC-1 cells were stimulated with various concentrations of nucleotides in order to identify functional P2Y and P2X receptors. Typical Ca^{2+} signals for these agonists given at 100 μ M concentrations are shown as the Fura-2 ratio in Figure 3 and 4. The Ca^{2+} signals evoked by ATP and UTP show two general features: first an immediate rise in intracellular Ca^{2+} followed by the second phase with a lower Ca^{2+} level and sometimes declining response, where many cells showed Ca^{2+} oscillations. Nevertheless, we will denote this latter phase as a “plateau-like” phase. The transient rise (here described as the peak) reflects release of Ca^{2+} from intracellular stores and possibly Ca^{2+} influx. The plateau-like phase reflects Ca^{2+} release from intracellular Ca^{2+} stores, as well as influx and efflux across the plasma membrane via, for example, Ca^{2+} channels, P2X receptors, Na^+/Ca^{2+} exchanger and plasma membrane Ca^{2+} -ATPase. In PANC-1 cell experiments, ATP evoked an immediate Ca^{2+} peak followed by a plateau phase that declined with time (Fig. 3A). Some cells oscillated and some gave a smooth response in Ca^{2+} signals. UTP (Fig. 3C) evoked a more sustained Ca^{2+} plateau phase and more pronounced oscillations in most cells. Figure 3B and D represents similar recordings from CFPAC-1 cells stimulated with 100 μ M agonists. ATP (Fig. 3B) evoked Ca^{2+} signals that were oscillating in synchrony in most cells. Similarly, UTP (Fig. 3D) also evoked an oscillatory response followed by a declining plateau. The ATP analogue ATP γ S is more resistant to breakdown by nucleotidases. When applied to

Fig. 1. Morphology of pancreatic duct cell lines. (A) PANC-1 cells are heterogenous and cell clusters grow on top of other cells as seen in center of the image. (B) CFPAC-1 cells appear more uniform and grow in flat monolayers. Cell lines were stained for nuclei DAPI (blue) and acetylated α -tubulin (green). Note tubulin and primary cilia (full arrows) pointing upwards, and sometimes pointing to the side as seen in the inserts. Bars are 20 μ m.

Fig. 2. Effect of ATP on intracellular Ca^{2+} signals in duct cell lines. Time-lapse Fura-2 ratio images (in pseudocolors) of stimulated PANC-1 (A) and CFPAC-1 (B) cells. Fura-2 loaded cells were stimulated with ATP (100 μ M) at time=0, and selected subsequent fluorescence ratio images from the sequential recording are shown. Time in seconds is given at the top left corner and ratio intensity scale is from 0.9 - 1.4. (A) Many PANC-1 cells respond shortly after addition of ATP (10 sec), and in many cells Ca^{2+} signals oscillate asynchronously. (B) Most CFPAC-1 cells respond immediately (4 sec) and Ca^{2+} signals oscillate synchronously in two groups in the image.

PANC-1 or CFPAC-1 cells (Fig. 3E, F), it evoked Ca^{2+} signals characterized by high responsiveness and oscillations similar to those seen with ATP. Like with the other nucleotides, ATP and UTP, the $\text{ATP}\gamma\text{S}$ -elicited responses seem to fade over time, indicating that also P2 receptor desensitization rather than nucleotide hydrolysis was involved.

In the next series of experiments we tested the effect of BzATP, a reputed P2X7 receptor agonist. In PANC-1 cells BzATP (Fig. 4A) elicited a different Ca^{2+} signal compared to ATP. Here only some of the cells showed sustained and oscillating Ca^{2+} response after the

initial peak. In most other cells, just after the Ca^{2+} peak, the signal declined back to the baseline within 1 minute of stimulation onset. In CFPAC-1 cells the recordings with BzATP (Fig. 4B) were completely different from ATP and UTP responses. BzATP stimulated CFPAC-1 cells responded with a peak Ca^{2+} signal that quickly faded to baseline for all cells. In these concentrations (100 μ M) BzATP had no detrimental effect, such as cell blebbing and lysis on either PANC-1 or CFPAC-1 cells.

In one series of experiments the effect of ivermectin (IVM) treatment prior to ATP stimulation was also investigated (Fig. 4C and D). This was done in order to

Fig. 3. Effect of nucleotide analogues on pancreatic duct cell Ca^{2+} signals. Representative original recordings of Fura-2 fluorescence ratio show agonist-induced Ca^{2+} signals from 10 individual cells in each experiment with either ATP (A and B), UTP (C and D) or $\text{ATP}\gamma\text{S}$ (E and F). Agonists were applied at 100 μM concentrations, and times of applications are indicated with arrowheads. Left panels show recordings from PANC-1 cells and right panels those from CFPAC-1 cells. Cells responding with oscillating signals are given in colours, while non-oscillating cells are presented in grey.

explore whether IVM could potentiate ATP effect on P2X_4 receptors and thus the Ca^{2+} signals. In both PANC-1 and CFPAC-1 cells IVM (10 μM) given prior to ATP (100 μM) significantly increased $\Delta\text{Fura-2}$ ratio for the plateau phase compared to the control (ATP only). There was a similar tendency with IVM effect in the experiments with 30 μM ATP, but not 1 μM ATP (Table 2 and 3). Taken together, these results indicate that IVM can potentiate the P2X_4 receptor activity in both cell lines.

In some experiments we removed extracellular Ca^{2+} and stimulated PANC-1 and CFPAC-1 cells with ATP. Fig. 4E and F show relatively robust and oscillating responses to stimulus that were maintained in both cell lines.

Table 2 and 3 summarize $\Delta\text{Fura-2}$ ratios in PANC-1 and CFPAC-1 cells, respectively, with various

Fig. 4. Effect of P2X receptor activation. Representative original recordings of Fura-2 fluorescence ratio show agonist-induced Ca^{2+} signals from 10 individual cells in each experiment with either BzATP (A and B), ATP in the presence of ivermectin, IVM, (C and D) or ATP given in Ca^{2+} -free solutions (E and F). Agonists were applied at 100 μM concentrations, ivermectin at 10 μM , and times of applications are indicated with arrowheads. Left panels show recordings from PANC-1 cells and right panels those from CFPAC-1 cells.

agonists. The data are given as the peak and plateau $\Delta\text{Fura-2}$ values together with percentages of responding cells and cells responding with Ca^{2+} oscillations. Summarized data reflect variability in Ca^{2+} signals seen in PANC-1 cells (Table 2), as also seen at the single cell level (Fig. 3 and 4). It appeared that PANC-1 cells responded to increasing ATP concentrations (1, 10 and 100 μM in control series and with ivermectin) with increasing Ca^{2+} signals indicated by the mean peak and mean plateau ($\Delta\text{Fura-2}$ ratios), although differences were not statistically significant, most likely due to cellular and P2 receptor heterogeneity (see below). UTP also evoked large changes in Fura-2 ratios. The number of oscillating cells increased with higher UTP concentrations, but interestingly, it seemed that the plateau

Agonist	Cells	Resp. cells	Osc. cells	Mean peak (Δ ratio)	Mean plateau (Δ ratio)
ATP 1 μ M ($n=7$)	145	87%	81%	0.174 ± 0.036	0.112 ± 0.025
ATP 30 μ M ($n=15$)	393	79%	52%	0.288 ± 0.053	0.174 ± 0.039
ATP 100 μ M ($n=23$)	554	92%	78%	0.311 ± 0.039	0.179 ± 0.022
UTP 30 μ M ($n=4$)	88	83%	12%	0.429 ± 0.059	0.297 ± 0.073
UTP 100 μ M ($n=3$)	56	66%	95%	0.256 ± 0.035	0.146 ± 0.016
BzATP 100 μ M ($n=10$)	261	71%	39%	0.208 ± 0.031	$0.090 \pm 0.021^*$
ATP γ S 100 μ M ($n=3$)	60	98%	66%	0.242 ± 0.047	0.172 ± 0.021
ATP 1 μ M + IVM 10 μ M ($n=4$)	98	84%	72%	0.095 ± 0.011	0.039 ± 0.006
ATP 30 μ M + IVM 10 μ M ($n=5$)	212	98%	62%	0.448 ± 0.048	0.310 ± 0.052
ATP 100 μ M + IVM 10 μ M ($n=6$)	140	100%	44%	$0.398 \pm 0.070^*$	$0.295 \pm 0.053^*$

Table 2. Effect of various nucleotide agonists on Ca^{2+} signals in PANC-1 cells. Summary of various parameters measured in Fura-2 experiments in PANC-1 experiments. This table shows the experimental series for each agonist and their respective data. Following each agonist, n refers to a number of experiments conducted in a given series. Mean peak and plateau Δ ratio $\pm$ SEM are calculated for given number of experiments not individual cells (see Methods). Also for each series the total numbers of individual cells analyzed are registered and the percentage of responding cells are calculated from the total number of registered cells. Oscillating cells are given as a percentage of responding cells. ATP concentration series were tested with ANOVA, other agonists were compared to ATP (100 μ M); * indicates $P < 0.05$.

Agonist	Cells	Resp. Cells	Osc. Cells	Mean peak (Δ ratio)	Mean plateau (Δ ratio)
ATP 30 μ M ($n=4$)	110	100%	79%	0.399 ± 0.04	$0.213 \pm 0.031^*$
ATP 100 μ M ($n=11$)	250	99%	86%	0.313 ± 0.037	0.156 ± 0.019
UTP 30 μ M ($n=2$)	46	100%	83%	0.296	0.145
UTP 100 μ M ($n=6$)	133	99%	92%	0.382 ± 0.069	0.231 ± 0.048
BzATP 100 μ M ($n=5$)	123	69%	1%*	0.205 ± 0.028	$0.008 \pm 0.003^*$
ATP γ S 100 μ M ($n=7$)	161	100%	87%	0.343 ± 0.052	0.144 ± 0.021
ATP 100 μ M + IVM 10 μ M ($n=6$)	131	100%	54%*	0.401 ± 0.022	$0.216 \pm 0.014^*$

Table 3. Effect of various nucleotide agonists on Ca^{2+} signals in CFPAC-1 cells. Summary of Δ ratio, number of cells analyzed, as well as percentage of responding and oscillating cells. Similar conditions and analysis as in Table 2.

Ca^{2+} decreased with the higher UTP concentrations, possibly due to an increased Ca^{2+} efflux (see discussion). The BzATP (100 μ M) plateau phase value was significantly lower than that evoked with ATP (100 μ M). Overall in PANC-1 cells, at equimolar nucleotide concentrations, the apparent rank order for agonist potency as judged by the plateau and the peak responses was $\text{UTP} \geq \text{ATP} = \text{ATP}\gamma\text{S} > \text{BzATP}$. With regard to the responsiveness of PANC-1 cells, UTP (100 μ M) and BzATP (100 μ M) gave a lower percentage of responding cells compared to ATP (100 μ M). PANC-1 cells also showed a rather large variation in the agonists' ability to evoke oscillatory responses - 12% to 95% of the responding cells were oscillating, depending on the nucleotide concentration.

Compared to PANC-1 cells, CFPAC-1 cells were generally more responsive to nucleotide analogues and they oscillated more synchronously (Table 3, Fig. 3). All of the agonists tested evoked large peak Δ Fura-2 ratios similar in magnitude, but the plateau responses differed. Interestingly, ATP in high concentration (100 μ M) evoked significantly lower plateau than low concentration of ATP (30 μ M). BzATP (100 μ M) resulted in Ca^{2+} signals with a clear peak response, but near absence of a plateau phase and lack of oscillations (also see Fig. 4). At equimolar concentrations, the apparent rank order for agonists was $\text{UTP} \geq \text{ATP} = \text{ATP}\gamma\text{S} > \text{BzATP}$ as judged by the plateau and the peak responses. Except for BzATP, almost all cells responded to given agonists with oscillations.

Fig. 5. P2 receptors in human pancreatic duct cell lines. RT-PCR analysis on expression of (A) P2Y and (B) P2X receptors in PANC-1 cells (P), CFPAC-1 cells (CF) and Capan-1 cells (CP). The primer sets used gave expected fragment lengths (see Table 1). Representative gels for at least three independent determinations.

Fig. 6. Immunolocalization of P2X4, P2X7 and P2Y2 receptors in PANC-1 cells (top images) and CFPAC-1 cells (bottom images). P2X4 receptor (green) distribution in PANC-1 and CFPAC-1 cells (A and B). P2X7 receptor (green) from Alamone (C and D) and from Santa Cruz (red)(E and F) localization in PANC-1 and CFPAC-1 cells, respectively. P2Y2 receptor (red) localization in PANC-1 and CFPAC-1 cells (G and H). Overlay images also shows nuclear stain (blue). Inserts in images F and H show marked staining in some CFPAC-1 cells. The scale bar is 20 µm in all images.

RNA expression of various purinoceptors in duct cell lines

The above mentioned Fura-2 experiments gave clear indications that PANC-1 and CFPAC-1 cells express functional purinergic receptors of both the P2X and P2Y families. These observations led us to use RT-PCR analysis in order to further clarify the P2 receptor profile. Primers for detection of all the known P2 receptors were designed and RT-PCR was carried out on total RNA isolated from PANC-1, CFPAC-1 and also Capan-1 cells. All the P2Y receptors tested, i.e. P2Y1, 2, 4, 6, 11-14 turned out with cDNA transcripts of the expected size (Fig. 5A, Table 1). Regarding the P2X receptors tested, not all showed positive results. P2X3 receptors could not be identified in the given cell lines (results not shown). For PANC-1 and CFPAC-1 cells

P2X1, P2X2, P2X4, P2X5, P2X6 and P2X7 transcripts of expected size were detected (Fig. 5B, Table 1). The P2X7 primer set I gave the expected fragment of 334 kb and unspecific band of 400 bp. The P2X7 primer set II gave expected fragment length of 410 bp. In addition to RT-PCR analysis on PANC-1 and CFPAC-1 cells, we also used another human duct cell line Capan-1 and found P2 receptor expression similar to the other two cell lines (Fig. 5A and B). The identity of mRNA for the most controversial P2 receptors, including P2X4 and P2X7 was confirmed by sequencing.

Localization of P2 receptors

Some key P2 receptors were also localized in duct cell lines and results are shown in Fig. 6. Immunofluores-

cence ascribed to the P2X4 receptor was detected in intracellular vesicles within both PANC-1 and CFPAC-1 cells (Fig. 6A and B). In some PANC-1 cells it seems that also the plasma membrane was labeled. One P2X7 antibody (Alomone) showed predominant intracellular localization in CFPAC-1 cells and PANC-1 cells and some plasma membrane marking in top-growing PANC-1 cells (Fig. 6C and D). Another P2X7 antibody (Santa Cruz) marked CFPAC-1 cells more sparingly, except for a few cells that showed intense vesicular staining (Fig. 6F). In contrast, PANC-1 cells showed intense intracellular and plasma membrane staining (Fig. 6E). P2Y2 antibody also localized intracellularly in both cell lines and in CFPAC-1 cells again occasionally a few cells showed marked staining in intracellular vesicles (Fig. 6G and H).

Discussion

This is the first detailed molecular, immunocytochemical and functional analysis of purinergic receptor expression in human pancreatic duct cell lines, based on cell lines commonly used for ion-transport studies. Our Fura-2 experiments and expression at the mRNA and protein levels show that PANC-1 and CFPAC-1 (and Capan-1) human pancreatic duct cells express a number of functional purinergic receptor subtypes from both the P2Y and P2X families.

Fura-2 measurements on PANC-1 and CFPAC-1 cells gave different Ca^{2+} signals depending on the nucleotide agonist and cell line. The pharmacological approach revealed functional differences in responses regarding the shape of the Ca^{2+} signals and the percentage of cells responding. Both cell lines responded to all nucleotides tested with the following efficacy: $\text{UTP} \geq \text{ATP} = \text{ATP}\gamma\text{S} > \text{BzATP}$; and these nucleotide effects were reflected in both the initial and sustained intracellular Ca^{2+} responses. This potency range is similar to our earlier observations on rat pancreatic ducts [11, 35]. Interestingly, in PANC-1 cells maximal concentrations of UTP evoked lower plateau Ca^{2+} signal than submaximal concentrations of UTP. In case of CFPAC-1 cells, similar phenomenon was observed with high concentrations of ATP (Table 2 and 3). These effects may be due to receptor-specific stimulation of Ca^{2+} efflux via $\text{Na}^+/\text{Ca}^{2+}$ exchange (manuscript in preparation), and may explain the relatively large variation in the concentration effects of nucleotides.

Regarding the individual P2 receptor types, we draw the following conclusions from our Fura-2 studies. In PANC-1 and CFPAC-1 cells, addition of ATP, UTP or

ATP γ S evoked a transient Ca^{2+} signal followed by a sustained plateau phase above the baseline level. Since UTP evokes large Ca^{2+} responses in both cell lines, it is very likely that P2Y receptors (especially P2Y2 and P2Y4) are functionally expressed. In fact, the P2Y4 receptor was cloned from the human pancreas, although the receptor localization was unknown then [36]. Sensitivity to ATP γ S may indicate presence of P2Y11 or P2Y1 and P2Y2 receptors [6]. The ATP analogue, BzATP, is characterized by its ability to activate Ca^{2+} signals mainly from P2X7, but it may also stimulate other receptors such as P2X1, P2X3 and P2Y11 [5, 6]. Both PANC-1 and CFPAC-1 cells responded in a very distinct way to BzATP. The intracellular Ca^{2+} signals evoked by BzATP generally comprised only a transient Ca^{2+} signal that faded within 30-60 sec to baseline values. The P2X1 receptors desensitize fast [5], and it is not likely that they would contribute to this relatively slow Ca^{2+} transient. Oscillatory and sustained Ca^{2+} responses of some PANC-1 cells may though indicate involvement of P2Y11 receptors. Thus, the distinct features of BzATP-activated Ca^{2+} signals indicate prevalent expression of functional P2X7 receptors. Both cell lines had lower percentage of cells responding to BzATP compared to ATP, i.e. PANC-1 cells responded 71% versus 87% and CFPAC-1 cells responded 69% versus 99% to the two agonists, respectively.

The anti-parasitic agent ivermectin (IVM) has been shown to augment Ca^{2+} currents through ATP activated P2X4 receptor channels. IVM does not directly activate P2X4, but only has a modulating action, and it does not modulate P2X2, P2X3 or P2X7 receptors [37, 38]. In our present study we found that IVM (10 μM), added before stimulation with ATP (100 μM), significantly increased the plateau Ca^{2+} response compared to ATP alone. Therefore, the functional data with IVM indicate that both PANC-1 and CFPAC-1 cells express functional P2X4 receptors. This is supported by our RT-PCR and immunolocalization data. Present data are then in agreement with the report on functional and molecular P2X4 receptor expression in rat duct cells [11]; and the RNA expression profile in Capan-1 human duct cell line [25].

In terms of individual cell Ca^{2+} responses, CFPAC-1 cells showed an overall higher percentage of responding and oscillating cells than PANC-1. Furthermore, the Ca^{2+} signals in CFPAC-1 appeared to be much more synchronous. These responses may be related to the morphology of CFPAC-1 cells, which seem homologous in shape and size and forming clearly denser and more closely coupled monolayer compared to PANC-1.

PANC-1 cells are more heterogeneous in cell size, form and monolayer appearance and cell-cell contact (Fig. 1). Correspondingly, number of responding cells and oscillating Ca^{2+} signals were more variable in PANC-1 cells. Most likely, the heterogeneous phenotype also manifests itself in P2 receptor expression. Naturally, this phenomenon will arise in experiments carried out on single cell level, such as in this study, compared to whole organ/whole tissue level studies. Similar heterogeneity was also recently reported regarding expression of adenosine receptors in PANC-1 and CFPAC-1 cells [39]. Another example of functional P2 receptor heterogeneity was also observed in rat pancreatic acini and in parotid acini [2, 40, 41].

One notable observation was that the Ca^{2+} response in many cells, especially CFPAC-1 cells, oscillated. UTP and ATP were most efficient in eliciting Ca^{2+} oscillations and over the range tested, nucleotide concentrations did not seem to have effect on the frequency pattern. In contrast, BzATP was not very good at eliciting Ca^{2+} oscillations in PANC-1 cells, and in CFPAC-1 cells none oscillated. Since UTP (and ATP) stimulates P2Y receptors and BzATP stimulates P2X7 receptors (and perhaps P2Y11 receptors in some PANC-1 cells), the oscillatory pattern observed indicate involvement of functional P2Y receptors rather than P2X receptors. Also removal of extracellular Ca^{2+} does not abolish Ca^{2+} oscillations with ATP or UTP, indicating that Ca^{2+} influx through P2X receptors is not involved (Fig. 3). Similar Ca^{2+} oscillations in response to ATP and UTP have been reported for rat pancreatic duct cells [39] and dog pancreatic duct cells [15]. Indeed oscillatory Ca^{2+} responses have been observed for a number of different cells and with various agonists [42], particularly in rat pancreatic acini where frequency and intensity depend on agonist [43-45]. It is proposed that such oscillations arise from interrelated Ca^{2+} release from different Ca^{2+} stores: InsP_3 -, cADPribose- and NAADP-sensitive stores [42, 46]. The alternative theory to the store model is that oscillations reflect local fluctuations in ATP hydrolysis, synthesis and release, as for example proposed for the Ca^{2+} wave propagations in astrocytes [47, 48]. Such model appears unlikely in duct cells, as we observed oscillations with supposedly non-degradable agonist $\text{ATP}\gamma\text{S}$. In addition, it is not likely that these effects are due to adenosine receptors, as in these pancreatic duct cells lines adenosine evokes very sparse Ca^{2+} signals with different pattern compared to nucleotides [39].

In order to determine the molecular identity of P2 receptors in duct cell lines RT-PCR analysis was per-

formed on total RNA extracted from PANC-1 and CFPAC-1 cells. The data showed that both PANC-1 and CFPAC-1 cells expressed transcripts for P2Y1, P2Y2, P2Y4, P2Y6, P2Y11-14 and P2X1, P2X2, P2X4, P2X5, P2X6 and P2X7 receptors. Also analysis on Capan-1 cells shows the same P2 receptor signature. Although there is some disagreement about certain P2X receptors (see below), these results are overall in accordance with a recent study on one human pancreatic duct line, i.e. Capan-1 cells that were also found to express P2Y1, P2Y2, P2Y4 and P2Y6 receptors [25]. In addition to the studies mentioned above, the present study shows transcripts for P2X2, P2X6 and P2X7 receptors and also for P2Y11-14 receptors. The P2Y12-P2Y13 receptors may not have been detected in our functional studies due to their coupling to cAMP signalling rather than Ca^{2+} signalling. Nevertheless, the function of these receptors in regulation of ion transport should be considered. Furthermore, the role of the P2Y14 receptor, which is UDP-glucose receptor expressed in cells of the nervous system, is unexplored in pancreas.

Present immunocytochemical experiments also support our RT-PCR and functional data regarding P2Y2 and P2X4 and P2X7 receptors, although one should take specificity and receptor localization with reservation. We show expression of P2Y2 receptor in both PANC-1 and CFPAC-1 cells, and a few CFPAC-1 cells showed marked accumulation of fluorescence (Fig. 6). The data with P2Y2 receptor then agrees with immunocytochemistry data on rat pancreatic ducts [17, 18]. Until now, P2X4 and P2X7 receptors were not detected in rat or human pancreatic duct material [18, 26]. Present study shows P2X4 receptor immunofluorescence in both cell lines (Fig. 6A and B). Presumed receptor localization to intracellular vesicles has also been observed in a number of preparations which used antibodies and recombinant receptor expression [18, 49, 50]. Guo and coworkers [49] found that P2X4 receptors, which undergo rapid endocytosis, reside in intracellular compartments in HEK293 cells and these compartments also mark with lysosomal markers. Apparently, lysosomal versus plasma membrane marking can be affected by method of fixation. Two different antibodies for the P2X7 receptor used in the present study showed similar reaction in PANC-1 cells (Fig. 6E and G). In CFPAC-1 cells, however, much fewer cells were showing reaction with Santa Cruz P2X7 antibody. Again the marked punctuate intracellular localization of receptor compared to weaker plasma membrane expression agrees with other studies using antibodies and recombinant receptor expression [18, 49, 51], and some

localization agrees with endoplasmatic reticulum [49].

Notably, until now evidence for the P2X7 and also P2X4 receptors was still not settled for human pancreas. Using whole human pancreas Künzli and co-workers [26, 27] reported P2X7 mRNA and protein, and immunohistochemistry showed P2X7 receptors in islet cells, immunoreactive cells and stellate cells, but apparently not on duct or acinar cells. Similar immunohistochemical localization was seen in another study on rat pancreas [17, 18]. Also Szücs and co-workers failed to detect P2X7 in Capan-1 cells (human duct cell line) and in the normal human pancreas RNA control reaction [25]. Nevertheless, two other groups [11, 12] have molecularly and functionally identified P2X4 and P2X7 receptors in rat pancreatic ducts. Thus, it seemed unlikely that these receptors should not be expressed in human pancreas. Therefore, in the present study we used several P2X7 primer sets, some also used for P2X4 and P2X7 detection in human parotid acini [52], sequenced the products, and we show clearly that PANC-1, CFPAC-1 and Capan-1 all contain transcripts for P2X4 and P2X7 receptors. We also detected P2X4 and P2X7 receptors by immunofluorescence (see above). Most importantly, these molecular data are supported by our functional data, where we studied effects of BzATP and ivermectin on Ca^{2+} signals, and conclude that human duct cells have similar functional P2X4

and P2X7 receptors. Interestingly, both types of receptors are also detected in human parotid acini and other salivary glands [2, 52].

In conclusion, our functional and molecular data on P2 receptor expression in human pancreatic cell lines PANC-1 and CFPAC-1 show that they express a number of P2Y and P2X receptors. They express P2Y2 receptors, but also other P2Y receptors that may regulate ion and fluid transport. Most notably, human ducts express P2X4 and P2X7 receptors, which may have short- and long-term effects. The exact polarity of receptors and target proteins they affect, such as Cl^- versus K^+ channels, will require more refined preparation of human pancreatic ducts. Nevertheless, similar to rat pancreas, one would expect that in human pancreas, ductal P2 receptors participate in modulation of fluid transport and possibly also in tissue homeostasis

Acknowledgements

The projects were supported by the Danish Science Research Council (272-05-0420). MRH was supported by the University of Copenhagen Ph.D. stipend. The technical assistance of Ms A. V. Olsen is greatly acknowledged.

References

- 1 Burnstock G: Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* 2007;87:659-797.
- 2 Novak I: ATP as a signaling molecule - the exocrine focus. *News Physiol Sci* 2003;18:12-17.
- 3 Leipziger J: Control of epithelial transport via luminal P2 receptors. *Am J Physiol Renal Physiol* 2003;284:F419-F432.
- 4 Schwiebert EM, Zsembery A: Extracellular ATP as a signaling molecule for epithelial cells. *Biochim Biophys Acta* 2002;1615:7-32.
- 5 North RA: Molecular physiology of P2X receptors. *Physiol Rev* 2002;82:1013-1067.
- 6 Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA: International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 2006;58:281-341.
- 7 Khakh BS, North RA: P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 2006;442:527-532.
- 8 Sørensen CE, Novak I: Visualization of ATP release in pancreatic acini in response to cholinergic stimulus. Use of fluorescent probes and confocal microscopy. *J Biol Chem* 2001;276:32925-32932.
- 9 Yegutkin GG, Samburski SS, Jalkalen S, Novak I: ATP-consuming and ATP-generating enzymes secreted by pancreas. *J Biol Chem* 2006;281:29441-29447.
- 10 Henriksen KL, Novak I: Effect of ATP on intracellular pH in pancreatic ducts involves P2X7 receptors. *Cell Physiol Biochem* 2003;13:93-102.
- 11 Hede SE, Amstrup J, Christoffersen BC, Novak I: Purinoceptors evoke different electrophysiological responses in pancreatic ducts. P2Y inhibits K^+ conductance, and P2X stimulates cation conductance. *J Biol Chem* 1999;274:31784-31791.
- 12 Luo X, Zheng W, Yan M, Lee MG, Muallem S: Multiple functional P2X and P2Y receptors in the luminal and basolateral membranes of pancreatic duct cells [see comments]. *Am J Physiol* 1999;277:C205-C215.
- 13 Nguyen TD, Moody MW, Savard CE, Lee SP: Secretory effects of ATP on nontransformed dog pancreatic duct epithelial cells. *Am J Physiol* 1998;275:G104-G113.
- 14 Nguyen TD, Meichle S, Kim US, Wong T, Moody MW: P2Y11, a purinergic receptor acting via cAMP, mediates secretion by pancreatic duct epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G795-G804.
- 15 Jung SR, Kim K, Hille B, Nguyen TD, Koh DS: Pattern of Ca^{2+} increase determines the type of secretory mechanism activated in dog pancreatic duct epithelial cells. *J Physiol* 2006;576:163-178.
- 16 Ishiguro H, Naruse S, Kitagawa M, Hayakawa T, Case RM, Steward MC: Luminal ATP stimulates fluid and HCO_3^- secretion in guinea-pig pancreatic duct. *J Physiol (Lond)* 1999;519:551-558.

- 17 Coutinho-Silva R, Parsons M, Robson T, Lincoln J, Burnstock G: P2X and P2Y purinoceptor expression in pancreas from streptozotocin-diabetic rats. *Mol Cell Endocrinol* 2003;204:141-154.
- 18 Coutinho-Silva R, Parsons M, Robson T, Burnstock G: Changes in expression of P2 receptors in rat and mouse pancreas during development and ageing. *Cell Tissue Res* 2001;306:373-383.
- 19 Novak I: Purinergic receptors in the endocrine and exocrine pancreas. *Purinergic Signal* 2008;(doi 10.1007/s11302-007-9087-6).
- 20 Zsembery A, Strazzabosco M, Graf J: Ca^{2+} -activated Cl^- channels can substitute for CFTR in stimulation of pancreatic duct bicarbonate secretion. *FASEB J* 2000;14:2345-2356.
- 21 Galletta LJ, Zegarra-Moran O, Mastrocola T, Wohrle C, Rugolo M, Romeo G: Activation of Ca^{2+} -dependent K^+ and Cl^- currents by UTP and ATP in CFPAC-1 cells. *Pflügers Arch* 1994;426:534-541.
- 22 Chan HC, Cheung WT, Leung PY, Wu LJ, Chew SB, Ko WH, Wong PY: Purinergic regulation of anion secretion by cystic fibrosis pancreatic duct cells. *Am J Physiol* 1996;271:C469-C477.
- 23 Namkung W, Lee JA, Ahn W, Han W, Kwon SW, Ahn DS, Kim KH, Lee MG: Ca^{2+} activates cystic fibrosis transmembrane conductance regulator- and Cl^- -dependent HCO_3 transport in pancreatic duct cells. *J Biol Chem* 2003;278:200-207.
- 24 O'Reilly CM, O'Farrell AM, Ryan MP: Purinoceptor activation of chloride transport in cystic fibrosis and CFTR-transfected pancreatic cell lines. *Br J Pharmacol* 1998;124:1597-1606.
- 25 Szucs A, Demeter I, Burghardt B, Ovari G, Case RM, Steward MC, Varga G: Vectorial bicarbonate transport by Capan-1 cells: a model for human pancreatic ductal secretion. *Cell Physiol Biochem* 2006;18:253-264.
- 26 Kunzli BM, Berberat PO, Giese T, Csizmadia E, Kaczmarek E, Baker C, Halaceli I, Buchler MW, Friess H, Robson SC: Upregulation of CD39/NTPDases and P2 Receptors in Human Pancreatic Disease. *Am J Physiol Gastrointest Liver Physiol* 2007;292:G223-G230.
- 27 Kunzli BM, Nuhn P, Enjyoji K, Banz Y, Smith RN, Csizmadia E, Schuppan D, Berberat PO, Friess H, Robson SC: Disordered pancreatic inflammatory responses and inhibition of fibrosis in CD39-null mice. *Gastroenterology* 2008;134:292-305.
- 28 Kopelman H, Gauthier C, Bornstein M: Antisense oligodeoxynucleotide to the cystic fibrosis transmembrane conductance regulator inhibits cyclic AMP-activated but not calcium-activated cell volume reduction in a human pancreatic duct cell line. *J Clin Invest* 1993;91:1253-1257.
- 29 Steagall WK, Kelley TJ, Marsick RJ, Drumm ML: Type II protein kinase A regulates CFTR in airway, pancreatic, and intestinal cells. *Am J Physiol* 1998;274:C819-C826.
- 30 Schoumacher RA, Ram J, Iannuzzi MC, Bradbury NA, Wallace RW, Hon CT, Kelly DR, Schmid SM, Gelder FB, Rado TA, Frizzell RA: A cystic fibrosis pancreatic adenocarcinoma cell line. *Proc Natl Acad Sci USA* 1990;87:4012-4016.
- 31 Hug M, Pahl C, Novak I: Effect of ATP, carbachol and other agonists on intracellular calcium activity and membrane voltage of pancreatic ducts. *Pflügers Arch* 1994;426:412-418.
- 32 Hollande E, Salvador-Cartier C, Alvarez L, Fanjul M: Expression of a wild-type CFTR maintains the integrity of the biosynthetic/secretory pathway in human cystic fibrosis pancreatic duct cells. *J Histochem Cytochem* 2005;53:1539-1552.
- 33 Madden ME, Sarraz MP, Jr.: Morphological and biochemical characterization of a human pancreatic ductal cell line (PANC-1). *Pancreas* 1988;3:512-528.
- 34 Nielsen SK, Møllgård K, Clement CA, Thorsteinsson RI, Yoder BK, Pedersen LB, Novak I, Christensen ST: Characterization of primary cilia and hedgehog signaling during development of the human pancreas and in human pancreatic duct cancer cell lines. *Dev Dynamics* 2008; in press.
- 35 Christoffersen BC, Hug MJ, Novak I: Different purinergic receptors lead to intracellular calcium increases in pancreatic ducts. *Pflügers Arch Eur J Physiol* 1998;436:33-39.
- 36 Stam NJ, Klomp J, van de Huevel M, Olijve W: Molecular cloning and characterization of a novel orphan receptor (P2P) expressed in human pancreas that shows high structural homology to the P2U purinoceptor. *FEBS Lett* 1996;384:260-264.
- 37 Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA: Allosteric control of gating and kinetics at P2X(4) receptor channels. *J Neurosci* 1999;19:7289-7299.
- 38 Priel A, Silberberg SD: Mechanism of ivermectin facilitation of human P2X4 receptor channels. *J Gen Physiol* 2004;123:281-293.
- 39 Novak I, Hede SE, Hansen MR: Adenosine receptors in rat and human pancreatic ducts stimulate chloride transport. *Pflügers Arch* 2007;456:437-447.
- 40 Novak I, Nitschke R, Amstrup J: Purinergic receptors have different effects in rat exocrine pancreas. Calcium signals monitored by Fura-2 in confocal microscope. *Cell Physiol Biochem* 2002;12:83-92.
- 41 Tannetti L, Gibbons SJ, Talamo BR: Expression and trans-synaptic regulation of P2x4 and P2z receptors for extracellular ATP in parotid acinar cells. Effects of parasympathetic denervation. *J Biol Chem* 1998;273:26799-26808.
- 42 Berridge MJ, Bootman MD, Roderick HL: Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 2003;4:517-529.
- 43 Osipchuk YV, Wakui M, Yule DI, Gallacher DV, Petersen OH: Cytoplasmic Ca^{2+} oscillations evoked by receptor stimulation, G-protein activation, internal application of inositol trisphosphate or Ca^{2+} : Simultaneous microfluorimetry and Ca^{2+} dependent Cl^- current recording in single pancreatic acinar cells. *EMBO J* 1990;9:697-704.
- 44 Thorn P, Gerasimenko O, Petersen OH: Cyclic ADP-ribose regulation of ryanodine receptors involved in agonist evoked cytosolic Ca^{2+} oscillations in pancreatic acinar cells. *EMBO J* 1994;13:2038-2043.
- 45 Cancela JM, Gerasimenko OV, Gerasimenko JV, Tepikin AV, Petersen OH: Two different but converging messenger pathways to intracellular Ca^{2+} release: the roles of nicotinic acid adenine dinucleotide phosphate, cyclic ADP-ribose and inositol trisphosphate. *EMBO J* 2000;19:2549-2557.
- 46 Petersen OH: Ca^{2+} signalling and Ca^{2+} -activated ion channels in exocrine acinar cells. *Cell Calcium* 2005;38:171-200.
- 47 Bowser DN, Khakh BS: Vesicular ATP is the predominant cause of intercellular calcium waves in astrocytes. *J Gen Physiol* 2007;129:485-491.
- 48 Iadecola C, Nedergaard M: Glial regulation of the cerebral microvasculature. *Nat Neurosci* 2007;10:1369-1376.
- 49 Guo C, Masin M, Qureshi OS, Murrell-Lagnado RD: Evidence for functional P2X4/P2X7 heteromeric receptors. *Mol Pharmacol* 2007;72:1447-1456.
- 50 Royle SJ, Qureshi OS, Bobanovic LK, Evans PR, Owen DJ, Murrell-Lagnado RD: Non-canonical YXXGPhi endocytic motifs: recognition by AP2 and preferential utilization in P2X4 receptors. *J Cell Sci* 2005;118:3073-3080.
- 51 Amstrup J, Novak I: P2X7 receptor activates extracellular signal-regulated kinases ERK1 and ERK2 independently of Ca^{2+} influx. *Biochem J* 2003;374:51-61.
- 52 Brown DA, Bruce JI, Straub SV, Yule DI: cAMP potentiates ATP-evoked calcium signaling in human parotid acinar cells. *J Biol Chem* 2004;279:39485-39494.