Purinergic Receptors and Calcium Signalling in Human Pancreatic Duct Cell Lines

Mette R. Hansen*, Simon Krabbe* and Ivana Novak

Department of Biology, August Krogh Building, University of Copenhagen, *contributed equally

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 Ca2+ signals in PANC-1 and CFPAC-1 cells. The cell lines responded to all nucleotides with the following efficiency: UTP ≥ ATP = ATPγS > BzATP. ATP, UTP and ATPγS elicited oscillatory responses. BzATP, commonly used to stimulate P2X7 receptors, elicited non-oscillatory and transient Ca2+ responses. Ivermectin, a potentiator of P2X4 receptors, increased Ca2+ signals evoked by ATP. The single cell Ca2+ 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 Ca2+ and tissue homeostasis relevant in physiology and pathophysiology of pancreas.

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 Ca2+ release from intracellular stores and regulation of Cl- and
Ca²⁺-activated Cl⁻ channels (see press releases denufosol for its ability to rescue fluid secretion by activation of cystic fibrosis, P2Y2 receptor agonist has been tested cystic fibrosis. In respiratory epithelia of patients with pancreatic secretion, such as in patients suffering from P2 receptors may be of therapeutic value for improving others have long-term effects on tissue homeostasis [19].

Stimulation of P2 receptors may be important for exo-

mal species autocrine and paracrine ATP signalling and

regulated 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 denufosol 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, Künzli 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 ΔF508 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 ATCC (Manassas, VA, USA). PANC-1 (ATCC, #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 μg/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
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 μM 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 exited 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 ΔFura-2 responses) in response to various agonists including ATP, UTP and BzATP (2´,3´-O-benzoylbenzoyl-ATP), non-degradable ATP analogue ATPγS 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 ΔFura-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 ΔFura-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 Δpeak and Δplateau values. Also the number of responding and oscillating or non-oscillating cells was determined. In some experiments we performed in situ calibration using 1 μM ionomycin permeabilization. Estimated resting [Ca²⁺] in single cells varied from 159 to 200 nM and were similar in all experiments.

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

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

P2 Receptors in Pancreatic Ducts

---

**Cell Physiol Biochem 2008;22:157-168**

In situ calibration using 1 μM ionomycin permeabilization. Estimated resting [Ca²⁺] in single cells varied from 159 to 200 nM and were similar in all experiments.

---
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\(^{2+}\)] 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 epitheliod carcinoma, form a monolayer of heterogenous 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 μM). The images show marked responses in many cells shortly after stimulation (the peak Ca\(^{2+}\) response), then a more heterogenous 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 μM 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 μM 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\(_7\)S is more resistant to breakdown by nucleotidases. When applied to
PANC-1 or CFPAC-1 cells (Fig. 3E, F), it evoked Ca\textsuperscript{2+} signals characterized by high responsiveness and oscillations similar to those seen with ATP. Like with the other nucleotides, ATP and UTP, the ATP\textsubscript{γ}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\textsuperscript{2+} signal compared to ATP. Here only some of the cells showed sustained and oscillating Ca\textsuperscript{2+} response after the initial peak. In most other cells, just after the Ca\textsuperscript{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\textsuperscript{2+} signal that quickly faded to baseline for all cells. In these concentrations (100 μM) 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
explore whether IVM could potentiate ATP effect on P2X4 receptors and thus the Ca\(^{2+}\) signals. In both PANC-1 and CFPAC-1 cells IVM (10 \(\mu\)M) given prior to ATP (100 \(\mu\)M) 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 \(\mu\)M ATP, but not 1 \(\mu\)M ATP (Table 2 and 3). Taken together, these results indicate that IVM can potentiate the P2X4 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 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 \(\mu\)M 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
Ca\(^{2+}\) decreased with the higher UTP concentrations, possibly due to an increased Ca\(^{2+}\) efflux (see discussion). The BzATP (100 μM) plateau phase value was significantly lower than that evoked with ATP (100 μM). 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 UTP ≥ ATP = ATP\(_{\gamma S}\) > BzATP. With regard to the responsiveness of PANC-1 cells, UTP (100 μM) and BzATP (100 μM) gave a lower percentage of responding cells compared to ATP (100 μM). 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 ΔFura-2 ratios similar in magnitude, but the plateau responses differed. Interestingly, ATP in high concentration (100 μM) evoked significantly lower plateau than low concentration of ATP (30 μM). BzATP (100 μM) 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 UTP ≥ ATP = ATP\(_{\gamma S}\) > BzATP as judged by the plateau and the peak responses. Except for BzATP, almost all cells responded to given agonists with oscillations.

### 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 Δratio ± 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 μM); * indicates P<0.05.

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

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

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Cells</th>
<th>Resp. Cells</th>
<th>Osc. Cells</th>
<th>Mean peak (Δ ratio)</th>
<th>Mean plateau (Δ ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP 30 μM (n=4)</td>
<td>110</td>
<td>100%</td>
<td>79%</td>
<td>0.399 ± 0.04</td>
<td>0.213 ± 0.031*</td>
</tr>
<tr>
<td>ATP 100 μM (n=11)</td>
<td>250</td>
<td>99%</td>
<td>86%</td>
<td>0.313 ± 0.037</td>
<td>0.156 ± 0.019</td>
</tr>
<tr>
<td>UTP 30 μM (n=2)</td>
<td>46</td>
<td>100%</td>
<td>83%</td>
<td>0.296</td>
<td>0.145</td>
</tr>
<tr>
<td>UTP 100 μM (n=6)</td>
<td>133</td>
<td>99%</td>
<td>92%</td>
<td>0.382 ± 0.069</td>
<td>0.231 ± 0.048</td>
</tr>
<tr>
<td>BzATP 100 μM (n=5)</td>
<td>123</td>
<td>69%</td>
<td>1%*</td>
<td>0.205 ± 0.028</td>
<td>0.008 ± 0.003*</td>
</tr>
<tr>
<td>ATP(_{\gamma S}) 100 μM (n=7)</td>
<td>161</td>
<td>100%</td>
<td>87%</td>
<td>0.343 ± 0.052</td>
<td>0.144 ± 0.021</td>
</tr>
<tr>
<td>ATP 100 μM + IVM 10 μM (n=6)</td>
<td>131</td>
<td>100%</td>
<td>54%*</td>
<td>0.401 ± 0.022</td>
<td>0.216 ± 0.014*</td>
</tr>
</tbody>
</table>
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.

Localization of P2 receptors

Some key P2 receptors were also localized in duct cell lines and results are shown in Fig. 6. Immunofluorescence
ence 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 Ca2+ signals depending on the nucleotide agonist and cell line. The pharmacological approach revealed functional differences in responses regarding the shape of the Ca2+ signals and the percentage of cells responding. Both cell lines responded to all nucleotides tested with the following efficacy: UTP ≥ ATP = ATP γS > BzATP; and these nucleotide effects were reflected in both the initial and sustained intracellular Ca2+ 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 Ca2+ 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 Ca2+ efflux via Na+/Ca2+ 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 γS evoked a transient Ca2+ signal followed by a sustained plateau phase above the baseline level. Since UTP evokes large Ca2+ 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 γS may indicate presence of P2Y11 or P2Y1 and P2Y2 receptors [6]. The ATP analogue, BzATP, is characterized by its ability to activate Ca2+ 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 Ca2+ signals evoked by BzATP generally comprised only a transient Ca2+ 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 Ca2+ transient. Oscillatory and sustained Ca2+ responses of some PANC-1 cells may though indicate involvement of P2Y11 receptors. Thus, the distinct features of BzATP-activated Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ responses, CFPAC-1 cells showed an overall higher percentage of responding and oscillating cells than PANC-1. Furthermore, the Ca2+ 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: InsP3-, 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 ATP\(_7\)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 performed 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, immunoneactive 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 Szuics 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 Ca2+ 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


