Purinergic regulation of CFTR and Ca\(^{2+}\)-activated Cl\(^{-}\) channels and K\(^{+}\) channels in human pancreatic duct epithelium

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Wang J, Haanes KA, Novak I. Purinergic regulation of CFTR and Ca\(^{2+}\)-activated Cl\(^{-}\) channels and K\(^{+}\) channels in human pancreatic duct epithelium. Am J Physiol Cell Physiol 304: C673–C684, 2013. First published January 30, 2013; doi:10.1152/ajpcell.00196.2012.—Purinergic agonists have been considered for the treatment of respiratory epithelia in cystic fibrosis (CF) patients. The pancreas, one of the most seriously affected organs in CF, expresses various purinergic receptors. Studies on the rodent pancreas show that purinergic signaling regulates pancreatic secretion. In the present study we aim to identify Cl\(^{-}\) and K\(^{+}\) channels in human pancreatic ducts and their regulation by purinergic receptors. Human pancreatic duct epithelia formed by Capan-1 or CFPAC-1 cells were studied in open-circuit Ussing chambers. In Capan-1 cells, ATP/UTP effects were dependent on intracellular Ca\(^{2+}\). Apically applied ATP/UTP stimulated CF transmembrane conductance regulator (CFTR) and Ca\(^{2+}\)-activated Cl\(^{-}\) (CaCC) channels, which were inhibited by CFTRinh-172 and niflumic acid, respectively. The basolaterally applied ATP stimulated CFTR. In CFPAC-1 cells, which have mutated CFTR, basolateral ATP and UTP had negligible effects. In addition to Cl\(^{-}\) transport in Capan-1 cells, the effects of 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-EBIO) and clotrimazole indicated functional expression of the intermediate conductance K\(^{+}\) channels (IK, KC\(_{\text{a},3.1}\)). The apical effects of ATP/UTP were greatly potentiated by the IK channel opener DC-EBIO. Determination of RNA and protein levels revealed that Capan-1 cells have high expression of TMEM16A (ANO1), a likely CaCC candidate. We conclude that in human pancreatic duct cells ATP/UTP regulates via purinergic receptors both Cl\(^{-}\) channels (TMEM16A/ANO1 and CFTR) and K\(^{+}\) channels (IK). The K\(^{+}\) channels provide the driving force for Cl\(^{-}\)-channel-dependent secretion, and luminal ATP provided locally or secreted from acini may potentiate secretory processes. Future strategies in augmenting pancreatic duct function should consider sidedness of purinergic signaling and the essential role of K\(^{+}\) channels.

TMEM16A; ANO1; KC\(_{\text{a},3.1}\); IK; P2Y2; HCO\(_{3}^{-}\) transport; K\(^{+}\) transport

THE PANCREAS IS ONE OF THE MOST SERIOUSLY AFFECTED ORGANS IN CYSTIC FIBROSIS (CF) AND 80–90% OF THE PATIENTS DEVELOP PANCREATIC INSUFFICIENCY AT OR SOON AFTER BIRTH. THIS COMPLEX MULTIFACTORIAL DISEASE IS CORRELATED WITH MUTATIONS IN THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR), WHICH ENCODES FOR CL\(^{-}\) CHANNELS THAT ARE EXPRESSED IN MANY EPITHELIUM, INCLUDING PANCREATIC DUCTS. SOME OF THE THERAPEUTICAL APPROACHES ARE ACTIVATION OF ALTERNATIVE NON-CFTR CL\(^{-}\) CHANNELS, AS WELL AS INCREASING THE DRIVING FORCE FOR SECRETION BY ACTIVATING K\(^{+}\) CHANNELS (7, 43). SOME OF THE PHYSIOLOGICAL ACTIVATORS OF THESE CHANNELS WOULD BE PURINERGIC RECEPTORS. URIDINE 5'-TRIPHOSPHATE (UTP) ANALOGS FOR P2Y2 RECEPTORS HAVE BEEN CONSIDERED IN THE TREATMENT OF RESPIRATORY EPITHELIUM IN CF PATIENTS AND EYE EPITHELIUM IN DRY EYE DISEASES, ALTHOUGH ONLY DRUGS DEVELOPED FOR THE LATTER DISEASE WILL BE USED CLINICALLY (2, 3, 8, 26, 35, 59). REGARDING PANCREATIC DUCTS, IT IS IMPORTANT TO IDENTIFY CL\(^{-}\) AND K\(^{+}\) CHANNELS AND THEIR REGULATION BY PURINERGIC RECEPTORS. THEREBY, WE COULD ESTIMATE WHETHER ACTIVATION OF SUCH CHANNELS WOULD BRING THE SECRETORY RESPONSE THAT INVOLVES ALSO OTHER TRANSPORTERS AND RESULTS IN SECRETION OF HCO\(_{3}^{-}\) RICH FLUID, WHICH IS A CENTRAL FUNCTION OF A HEALTHY DUCT EPITHELIUM.

FOR THE PANCREAS, THERE IS EVIDENCE THAT AUTOINN/PARACRINE ATP RELEASE, E.G., FROM ACINI, REGULATES PANCREATIC DUCTS (16, 36, 49). STUDIES ON RODENT DUCTS AND HUMAN DUCT CELL LINES SHOW THAT THEY EXPRESS A NUMBER OF DIFFERENT METABOTROPIC P2Y AND IONOTROPIC P2X RECEPTORS (18, 20, 29, 50). AS IN OTHER EPITHELIUM, PREVAIL P2 RECEPTORS, THEIR LOCALIZATION AND SIGNALING TO DIFFERENT IOCHANNLES/TANSPORTERS WILL DETERMINE THE OVERALL PHYSIOLOGICAL RESPONSE (37).

MOST P2 RECEPTORS ELEVATE INTRACELLULAR Ca\(^{2+}\); THUS ONE WOULD EXPECT THE DOWNSTREAM ACTIVATION OF Ca\(^{2+}\)-ACTIVATED ION CHANNELS, SUCH AS Ca\(^{2+}\)-ACTIVATED Cl\(^{-}\) CHANNELS (CaCC) AND K\(^{+}\) CHANNELS. IN RODENT, DOG, AND HUMAN PANCREATIC CELLS, ATP/UTP STIMULATES Ca\(^{2+}\)-DEPENDENT Cl\(^{-}\) TRANSPORT, WHICH IS COUPLED TO HCO\(_{3}^{-}\) TRANSPORT (5, 12, 13, 20, 23, 32, 34, 50, 60). THE MOLECULAR IDENTITY OF CaCC HAS BEEN INVESTIGATED AND DISCUSSED, AND A NUMBER OF CANDIDATES WERE PROPOSED, INCLUDING SOME MEMBERS OF THE BESTRPHINS AND TMEM16/ANOCTAMIN CHANNEL FAMILY (10). THE AVAILABLE FUNCTIONAL STUDIES ON THE IDENTITY OF SUCH CaCC WERE PERFORMED ON HUMAN PANCREATIC DUCT CELL LINE CFPAC-1, WHICH HAS ΔF508 IN CFTR. THERE IS EVIDENCE FOR BOTH TMEM16A (ANO1) AND Best-1 CHANNEL ACTIVITY THAT COULD BE STIMULATED BY UTP (4, 30).

THE Ca\(^{2+}\)-ACTIVATED K\(^{+}\) CHANNELS EXPRESSED IN Pancreatic ducts include TMEM16A (ANO1) and the BK channel can be regulated by both P2Y2 and P2Y4 receptors (20, 21), although basolateral P2Y2 stimulation inhibits the channel and may explain the findings that basolateral UTP decreases flux and possibly HCO\(_{3}^{-}\) secretion in guinea-pig ducts and Capan-1 cells (23, 50). IN CONTRAST, STUDIES ON Pancreatic ducts and cell lines shown that ATP/UTP INCREASES K\(^{+}\) FLUXES/CURRENTS POSSIBLY THROUGH IK CHANNELS (12, 13, 21, 25, 34, 60).

THE AIM OF THE PRESENT STUDY WAS TO ELUCIDATE WHETHER PURINERGIC RECEPTORS ON THE LUMINAL AND BASOLATERAL MEMBRANES OF Pancreatic ducts stimulate Cl\(^{-}\) and/or K\(^{+}\) channels, identify these, and evaluate whether activities of such channels are coordinated to allow transepithelial ion transport. Furthermore, we wished to identify especially Cl\(^{-}\) channels on molecular and functional levels. For this purpose we used a human adenocarcinoma duct cell line, Capan-1 cells, which have
receptors for hormones and transmitters and ion transport characteristics of "normal" duct cells (53). In addition, we used PANC-1 cells and CFPAC-1 cells, the latter derived from a CF patient with the most common CFTR mutation, deletion of phenylalanine 508 (44). The experimental approach included Ussing chamber experiments on epithelial monolayers, as well as RT-PCR, quantitative PCR analysis, and immunocytochemistry. This study shows that luminal ATP/UTP stimulates both CaCC and CFTR, as well as IK channels, and in particular, IK channel activation potentiates the effect of ATP. The basolateral ATP stimulates predominantly CFTR and IK channels. The stimulatory effects of ATP and UTP are dependent on intracellular Ca2+. Furthermore, Capan-1 cells express relatively high levels of CFTR and TMEM16A.

MATERIALS AND METHODS

Cell culture. All standard chemicals were purchased from Sigma-Aldrich unless otherwise stated. Pancreatic cell lines were purchased from ATCC (Manassas, VA). PANC-1 (no. CRL-1469) was grown in DMEM and CFPAC-1 (no. CRL-1918) and Capan-1 (no. HTB-79) were grown in Iscove's modified DMEM. Cell culture media contained Glutamax, 10% (20% for Capan-1) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO2. Cells from passage 12 to 25 were used in this study. Cells were seeded at a density 200,000 – 400,000 cells/cm² on collagen-coated Snapwells (no. 3407; Corning) for electrophysiological measurements or Transwells (no. 3460; Corning) for immunocytochemistry and on Nunc 10-cm culture dishes for RNA preparation.

Ussing chamber measurements. Confluent monolayers were mounted in Ussing chambers and allowed to stabilize for at least 30 min. Electrophysiological measurements in the open-circuit mode were carried out as detailed in our recent work (53). In brief, the transepithelial potential, Vₑₑ, was monitored continuously and the epithelium was pulsed with intermittent 19 µA/cm² pulses at 5-s intervals (Pulse Generator 5.1; Physiologische Institut in Freiburg), and the transepithelial resistance, Rₑₑ, could be calculated from voltage deflections in Vₑₑ. The equivalent short-circuit current, Iₑₑ, was calculated from Vₑₑ and Rₑₑ, according to Ohm’s law. Vₑₑ is expressed as apical with respect to basolateral side, and increase in Vₑₑ denotes increase in the negative direction. Inward currents are given a positive sign. Data were digitized and recorded via Power Lab, Chart 5 (ADI Instruments). Control and test measurements were on the same monolayer, and monolayers were washed twice and allowed to stabilize for 30 min before further measurements were conducted.

The apical compartments were filled with solutions containing 145 mM Na⁺, 3.6 mM K⁺, 1.5 mM Ca²⁺, 1.0 mM Mg²⁺, 145 mM Cl⁻, 10 mM HEPES, 2 mM phosphate, and 5 mM glucose; the pH was 7.4 and the solution was gassed with air. Basolateral side solutions were similar in composition except that 25 mM NaCl was replaced by NaHCO₃ and the pH was equilibrated to 7.4 with 5% CO₂ in O₂. The temperature was kept constant at 37°C during all experiments. Agonists/antagonists, ATP, UTP, BAPTA-AM, 5, 6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-BLB1), niflumic acid, CTFRinh-172, and clotrimazole were added to the luminal or basolateral bath at the indicated concentrations. The usual response to a purinergic agonist was a transient change in Vₑₑ and Iₑₑ, which we define as the peak, and a more sustained response after ~5 min, which we define as the plateau.

RT-PCR and real-time RT-PCR. Cells were cultured to confluence and then RNA was isolated with RNaseasy Mini Kit (74104; Qiagen) according to manufacturer’s instruction. The RNA was treated with DNase I (RNase free DNase set, 79254; Qiagen) to avoid any DNA contamination. One micromgram extracted RNA was used per reaction mixture in Qiagen OneStep RT-PCR kit (210212) analysis with amplification parameters as follows: one cycle at 50°C for 30 min and one cycle at 95°C for 15 min followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and finally, one cycle at 72°C for 10 min. Subsequently, all transcripts were subjected to electrophoresis on agarose gels (1%).

For real-time analysis, cDNA was synthesized based on 5 µg RNA template per reaction using the RevertAid First Strand cDNA synthesis kit (K1622; Fermentas) with oligo (dT)18 primers and RevertAid M-MuLV reverse transcriptase and then purified using GenElute PCR Clean-Up Kit (NA1020; Sigma). The purified cDNA was quantified by absorbance at 260/280 nm, and 100 ng were used as template for each PCR reaction. The PCR reactions were run using LightCycler 480 SYBR Green I Master (04707516001; Roche) according to the manufacturer’s instructions for a LightCycler 480 (Roche). The parameters were as follows: 5 min at 95°C followed by 45 cycles of 10 s at 95°C, 1 min at 55°C, and 30 s at 72°C, and then melting curve of 5 s at 95°C, 1 min at 65°C, and continuous to 97°C, and then cooling for 10 s at 40°C. Table 1 shows primers used; these were synthesized with MWG-Biotech or TAG Copenhagen A/S (Copenhagen, Denmark). Four housekeeping genes, 18S ribosomal RNA (18s rRNA), β-actin, β-glucuronidase (GUSB), and glutaminyl-tRNA synthetase (QARS), were used for normalization.

Immunocytochemistry. Detailed methods for immunocytochemistry are described elsewhere (53). Briefly, confluent monolayers on Transwells were fixed in 4% paraformaldehyde for 15 min at room temperature. After being washed with PBS, monolayers were treated with 0.1 M TRIS-glycine (pH 7.4) for 15 min, then rinsed in PBS, and blocked with 5% BSA for 30 min. Subsequently, preparations were incubated with 1:100 or 1:200 dilutions of the primary antibodies recognizing CFTR (MAB1660; R&D Systems) or TMEM16A (SP31 ab64085 or ab53212; Abcam) overnight at 4°C. Then, preparations were incubated with a secondary antibodies conjugated to Alexa488 (Invitrogen). DAPI (1:200) was used for nuclear staining and Texas Red-X phalloloid (T7471; Invitrogen) was used to mark actin. Fluorescence was examined with 40 × 1.2 NA objective in Leica TCS SX 5 MP confocal laser scanning microscope (CLSM; Leica Microsystems, Heidelberg, Germany). Images were analyzed using Leica LAS software and exported as TIFF files to CorelDRAW for composite picture. Except for cropping, no image manipulation was used.

Western blot. Protein lysates were made by adding lysis buffer (50 mM Tris base, 0.25 M NaCl, 5 mM EDTA, 1% Triton X-100, and 4 mM NaF) to the Capan-1 and CFPAC-1 cells. Cell lysates were centrifuged at 15,000 g for 15 min to obtain whole cell lysate samples. This buffer and method were used to prepare both cell lysates and Table 1. Primer sets used for RT-PCR and real-time analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Product Length, bp</th>
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<tr>
<td>CFTFR508 FW</td>
<td>GATCCACTGGAGCACGCGAAG</td>
<td>168</td>
</tr>
<tr>
<td>CFTFR508 RW</td>
<td>CATCATGAGAAGACACCAAGATG</td>
<td></td>
</tr>
<tr>
<td>CFTFR COOH-terminal FW</td>
<td>GTGGAGTAGTGCGGAGATT</td>
<td>158</td>
</tr>
<tr>
<td>CFTFR COOH-terminal RW</td>
<td>ATGGAGAGTGCCGCGATTT</td>
<td></td>
</tr>
<tr>
<td>TMEM16A FW</td>
<td>TCCAAAGATCTTCCGCGCCGTT</td>
<td>305</td>
</tr>
<tr>
<td>TMEM16A RW</td>
<td>TAGAGGTGTCGGGCGGTT</td>
<td></td>
</tr>
<tr>
<td>18S rRNA FW</td>
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<td>150</td>
</tr>
<tr>
<td>18S rRNA RW</td>
<td>GCACATCCTCGGATTTCTGTC</td>
<td></td>
</tr>
<tr>
<td>β-ACTIN FW</td>
<td>GTCCATCAAGAGAGGAAGATG</td>
<td>300</td>
</tr>
<tr>
<td>β-ACTIN RW</td>
<td>AATGGAGAGGTCGGATGTT</td>
<td></td>
</tr>
<tr>
<td>GUSB FW</td>
<td>ACCTGAGGCTGCGGATGTT</td>
<td>200</td>
</tr>
<tr>
<td>GUSB RW</td>
<td>AGAGTCGGATGATGTCG</td>
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</tr>
<tr>
<td>QARS FW</td>
<td>CCTCTATGAGCCCAGCTTCCAG</td>
<td>200</td>
</tr>
<tr>
<td>QARS RW</td>
<td>GATTGGGCTCTCGAGACGCG</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primer sets used for human pancreatic duct cell lines in RT-PCR and real-time analysis. CFTR, cystic fibrosis transmembrane conductance regulator; 18s rRNA, 18S ribosomal RNA; GUSB, β-glucuronidase; QARS, glutaminy-l-tRNA synthetase.
membrane microdomain-enriched samples. To obtain the membrane microdomain enriched samples, the cell lysate was centrifuged at 200,000 g for 1 h (Beckman Ultracentrifuge Ti 70.1 Rotor) to pellet the Triton-X insoluble membrane fraction. The membrane pellet was washed with 250 mM potassium bromide and recentrifuged. The following pellet was then washed in 100 mM Na2CO3 (pH 11) and centrifuged for a last time. All solutions contained 1X protease inhibitor (S-8820; Sigma). Western blot samples were reduced by gentle heating to 37°C in the presence of 50 mM dithiothreitol for 15 min. Equal amounts of protein for all samples (7 μg/lane) were added and run on precast gels from Invitrogen. The membrane was blocked overnight at 4°C in 0.5% milk powder and 1% BSA. Primary antibodies (1:500) for actin (A-3853; Sigma) and TMEM16A (ab53212 and ab64085) were added in blocking buffer for 1 h. The mouse/rabbit secondary antibody conjugated to horseradish peroxidase (1:2,000) was added in blocking buffer for 1 h. Enzyme substrate was added, and blots were viewed on a Fusion FX Vilber Lourmat, where also intensities were determined.

Statistics. Data are shown as means ± SE, and n denotes number of experiment on different preparations. For Ussing chamber data, control and test measurements with inhibitors were made on the same monolayer and Student’s paired t-test was applied, and P < 0.05 was accepted as significant and denoted with asterisks. BAPTA experiments were performed on different tissues and nonpaired t-test was applied. One-way ANOVA was used to compare effects of various inhibitors and Cl− channel mRNA levels among different cell lines. For real-time PCR, relative quantification (2−ΔΔCt) was used (28); the level of transcripts was normalized to housekeeping genes and then normalized to the expression in PANC-1 cells for comparison among the cell lines. Data were analyzed in Origin or Microsoft Office Excel.

RESULTS

Electrophysiological responses of Capan-1 monolayers to purinergic agonists. Several purinergic agonists evoke electrophysiological responses in Capan-1 cells (53), and these cells express several types of P2 receptors, i.e., P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14, P2X1, P2X2, and P2X4–X7 (18). In the present study, we have used ATP as a prototype agonist that would stimulate both P2Y and P2X receptors and UTP as a prototype agonist for P2Y2 and P2Y4 receptors. After presenting the basic responses to these two agonists obtained in the present study, we will focus further experiments on elucidating molecular identities of the ion channels underlying these electrophysiological responses.

In the present series of experiments, nonstimulated Capan-1 cell monolayers displayed resting Rse of 356 ± 19 Ω·cm2, Vse of −1.0 ± 0.1 mV, and Isc of 2.9 ± 0.3 μA/cm2 (n = 64). Stimulation of the luminal and basolateral purinergic receptors led to characteristic electrophysiological responses that were relatively reproducible in repeated applications (data not shown). Representative recordings with ATP (100 μM) and UTP (100 μM) are shown in Fig. 1–5 and summary of data is given in Table 2. Notably, both ATP and UTP induced large peak changes in Vse, although the effects of UTP were smaller. In addition, UTP also had much smaller effects on the plateau phase of the response. On the other hand, basolateral ATP had significant and relatively large plateau changes. Original recordings show that as Vse (and Isc) was rising to its peak, there was a large transient decrease in Rse. That is, Rse decreased by 234 ± 23 Ω·cm2 following apical ATP application and by 191 ± 20 Ω·cm2 following basolateral ATP (n = 31); the Rse decrease for apically applied UTP was 113 ± 23 and 130 ± 20 Ω·cm2 for basolaterally applied UTP (n = 11).

Effects of ATP and UTP are dependent on intracellular Ca2+. Most of the P2 receptors act via intracellular Ca2+ signaling pathways (except for P2Y11–14), and therefore in one series of experiments, we investigated the effect of intracellular Ca2+ on purinergic signaling. Capan-1 cell monolayers were preincubated for 30 min with the Ca2+ chelator BAPTA-AM (25 μM, applied to both apical and basolateral side). The data are presented as representative recordings with or without BAPTA-AM (Fig. 1, A–D), and summary of the changes in Vse and Isc evoked by ATP/UTP (Fig. 1, E–H). With BAPTA-AM preincubation, the peak response to apical or basolateral ATP and UTP was significantly reduced by 60–80%. Also, ATP- and UTP-induced plateau responses were diminished after Ca2+ chelation (data not shown).
Table 2. Electrophysiological responses of Capan-1 cells to ATP and UTP

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Peak V_e, mV</th>
<th>ΔV_e, μA/cm²</th>
<th>Plateau V_e, mV</th>
<th>ΔV_e, μA/cm²</th>
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<tbody>
<tr>
<td>ATP ap (n = 31)</td>
<td>-3.3 ± 0.2</td>
<td>14.8 ± 0.9</td>
<td>-0.6 ± 0.05*</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td>ATP bl (n = 31)</td>
<td>-3.5 ± 0.3</td>
<td>13.5 ± 1.0</td>
<td>-1.2 ± 0.1</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>UTP ap (n = 11)</td>
<td>-2.4 ± 0.4*</td>
<td>15.8 ± 3.7</td>
<td>-0.3 ± 0.1</td>
<td>0.3 ± 0.2NS</td>
</tr>
<tr>
<td>UTP bl (n = 11)</td>
<td>-1.5 ± 0.3</td>
<td>11.5 ± 3.3</td>
<td>-0.2 ± 0.1</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

Summary of changes in transepithelial potential (ΔV_e) and short-circuit current (ΔI_sc) induced by apically (ap) and basolaterally (bl) applied purinergic agonists ATP and UTP (100 μM) to Capan-1 monolayers in Ussing chamber recordings. Both ATP and UTP led to characteristic peak and plateau responses in V_e (and I_sc). However, the plateau responses induced by UTP were significantly smaller compared with those by ATP (unpaired t-test). All responses were significantly different to nonstimulated values except those marked with NS. *Peak response to ATP bl was significantly smaller than that to ATP ap (paired t-test); †peak response to ATPap was significantly larger compared with UTPbl (paired t-test).

**Effect of ATP on Ca²⁺-activated Cl⁻ channels in Capan-1 monolayers.** The next task was to find to what extent the electrogenic transport responses induced by a wide-acting purinergic agonist ATP were due to opening of Cl⁻ channels. Since human pancreatic duct cells express functional CaCC (see Introduction), one may expect that ATP elevated intracellular Ca²⁺, which would regulate such channels. Thus a CaCC inhibitor, niflumic acid, was used to study ATP responses. The data are shown as original V_e recordings for single experiments (Fig. 2, A and B) and summarized changes in V_e and I_sc evoked by ATP before and after niflumic acid (Fig. 2, C and D). Niflumic acid (100 μM) applied to the apical side inhibited the peak response of apical ATP by 42 and 61%, as calculated from ΔV_e and ΔI_sc, respectively (n = 5; Fig. 2A). Also, part of the fast change in I_sc preceding the peak V_e and I_sc was inhibited by niflumic acid. The peak response of basolaterally applied ATP was, however, not affected by niflumic acid (n = 5; Fig. 2B). With respect to the plateau phase, niflumic acid also inhibited 40–60% of the responses to both apical and basolateral ATP (n = 5).

**Effect of ATP on CFTR Cl⁻ channels in Capan-1 monolayers.** To evaluate the effect of ATP on another key Cl⁻ channel in pancreatic duct epithelia, the CFTR Cl⁻ channel inhibitor CFTRinh-172 was tested (10 μM, apical; n = 5). Figure 3 shows representative original recordings and summary of ΔV_e and ΔI_sc. CFTRinh-172 decreased the apical ATP peak and plateau responses by 59 and 71%, while the basolateral ATP peak and plateau responses were inhibited by 67 and 68%, respectively, as calculated from ΔV_e (n = 5). A similar extent of inhibition was deduced from the I_sc data, i.e., 72 and 73% inhibition of the apical ATP peak and plateau responses, while 77 and 67% inhibition of the basolateral ATP peak and plateau responses. Notably, also the fast I_sc decrease before the peak V_e with ATP stimulation was diminished with CFTRinh-172.

Since none of the Cl⁻ channel inhibitors alone completely inhibited the ATP effects, niflumic acid and CFTRinh-172 were used together in one series of experiments. The peak response to ATP applied from the apical and basolateral sides were inhibited by 60 and 74%, while the plateau responses were decreased by 72 and 86% when both inhibitors were applied on the apical side simultaneously (calculated from ΔV_e; n = 5). Thus the inhibitors did not have additive effects in these experiments.

**Effect of ATP on CFPAC-1 cell monolayers.** To further reveal effects of ATP stimulation on CaCC with CFTR, we made use of CFPAC-1 cells, which have a ΔF508 mutation in CFTR. Monolayers formed by CFPAC-1 cells had R_e 112 ± 5 Ω-cm² (n = 7). Representative original recordings and summary of ΔV_e and ΔI_sc are shown in Fig. 4. Apically applied ATP caused a fast and transient response in V_e, followed by a secondary slower response. The change of V_e was from −0.4 ± 0.2 mV to −1.3 ± 0.3 mV and then to the plateau value −0.5 ± 0.2 mV (n = 7). The change in I_sc was from 3.7 ± 1.3 μA/cm² to 12.2 ± 2.5 μA/cm² and then to 4.1 ± 1.3 μA/cm², respectively. Importantly, niflumic acid inhibited V_e responses by 78% and I_sc responses by 81% (n = 6; Fig. 4, E and F). Basolaterally applied ATP did not have significant effects on either V_e or I_sc in these cells (Fig. 4). Similarly, basolateral UTP had almost no effect, while luminal UTP had similar effects as ATP (n = 3). Note that V_e and I_sc responses...
in CFPAC-1 monolayers were much smaller than those in Capan-1 monolayers.

Effect of UTP on CaCC and CFTR Cl⁻ channels in Capan-1 cell monolayers. Since many epithelia seem to be regulated by P2Y2 receptors, the relation between purinergic signaling and Cl⁻ channels in Capan-1 monolayers was further studied with the P2Y2 (and P2Y4) receptor agonist UTP. Figure 5 shows representative original recordings of UTP and summary of $V_{te}$ and $I_{sc}$ evoked by UTP above the basal nonstimulated values. Niflumic acid (apical, 100 μM) inhibited the peak response of apical UTP by 64%, while the peak response of basolaterally applied UTP was reduced.
by 39% (calculated from ΔVic; n = 7; Fig. 5, C and E). CFTRinh-172 (apical, 10 μM) decreased the apical UTP peak response by 46% (calculated from ΔVic; n = 9; Fig. 5, D and F). However, the basolateral UTP peak response was not significantly inhibited by CFTRinh-172 (n = 9; Fig. 5, D and F). Notably, the plateau responses to UTP were too small (Table 2), and therefore effect of inhibitors could not be evaluated.

Expression of Cl− channels in pancreatic duct cell lines. To support the functional study with Ussing chambers, the expression of Cl− channels was tested in three human pancreatic adenocarcinoma duct cell lines (Capan-1, PANC-1, and CFPAC-1) using RT-PCR. The two former cell lines express functional CFTR, whereas CFPAC-1 cells have deletion in phenylalanine 508 in CFTR that causes misfolded protein and defective trafficking to the plasma membrane. Thus two primer pairs were chosen to detect CFTR, one pair was specific for the phenylalanine 508 region (denoted CFTR-F508), while the other one recognized a region in the COOH terminus of CFTR (denoted CFTR COOH terminus). CFPAC-1 showed no bands using CFTR-F508 primers, while the other two cell lines showed expected transcripts. The primer pair for CFTR COOH terminus produced specific bands in all three cell lines. Thus CFTR mRNA was expressed in Capan-1 and PANC-1, but CFPAC-1 cell line had the mutated CFTR (Fig. 6A).

One candidate for CaCC is TMEM16A (see Introduction). Using specific primers, we could demonstrate on the RNA level this Cl− channel in all the chosen cell lines (Fig. 6A). To quantify the expression, RNA from Capan-1, CFTR-1, and PANC-1 was also subjected to real-time PCR, and the results are shown in Fig. 6, C and D. Figure 6B shows the results for transcripts for control primers 18S rRNA (150 bp), β-actin (300 bp), GUSB (200 bp), and QARS (200 bp). Quantitative analysis shows that among the three cell lines, Capan-1 cells have the highest level of transcripts for both CFTR and TMEM16A, i.e., about five times higher than CFPAC-1 cells.

Expression of TMEM16A in Capan-1 (and CFPAC-1) cells was further analyzed by Western blotting on cell lysates and membrane microdomain enriched fractions. Figure 6, E and F, shows that two antibodies tested detect TMEM16A protein, with actin as loading control. TMEM16A was detected at the expected size (∼130 kDa) and clearly the concentration is highest in the membrane microdomains. This finding is in agreement with report of Sones et al. (48) who showed that TMEM16A was present in lipid raft-enriched fractions in myocytes and functional properties of the channel are influenced by lipid raft integrity. The SP31 antibody, which gave very clear bands (Fig. 6E), was tested in another study using the TMEM16A overexpression system, small interfering RNA protocols, and functional patch-clamp readout (51). In our study, notably, Capan-1 cells express more protein than CFPAC-1 cells, with proteins levels being 2.5 times higher, relative to actin in whole cell lysate (Fig. 6E).

Further, using immunocytochemistry we detected localization of CFTR and TMEM16A in Capan-1 and CFPAC-1 (Fig. 7) and this supported the RT-PCR and Western blot results. CFTR was localized to the apical site of the Capan-1 monolayer in unstimulated and stimulated monolayers (Fig. 7, C and D). In CFPAC-1 cells, which are heterogenous, CFTR staining was weaker and more cytosolic (Fig. 7E). In Capan-1 cells, the TMEM16A/ANO1 signals were diffuse in cytosol and close to plasma membranes (Fig. 7A). However, following apical ATP stimulation, many cells showed increased apical localization of TMEM16A (Fig. 7B).

Effect of ATP on K+ channels in Capan-1 monolayers. In addition to Cl− channels, it is most likely that K+ channels also contribute to transepithelial ion transport in pancreatic ducts. Thus we studied the ATP regulation on Ca2+-activated K+ channels. Intermediate conductance K+ channels (denoted IK and IKCa,3.1 and coded by KCN(V/N)4) have been detected both in rat pancreatic ducts and in one human pancreatic duct cell line HPAF (12, 19, 21). We applied the IK channel blocker clotrimazole on the basolateral side of Capan-1 monolayers. The blocker totally abolished Vte and Ite effects normally evoked by either apical or basolateral ATP (30 μM; n = 8; Fig. 8). That is, the peak Ite with apical ATP of 23.6 ± 2.3 μA/cm2 decreased to −0.9 ± 0.3 μA/cm2 when clotrimazole was included. The same treatment decreased basolateral ATP response from 18.4 ± 3.0 to −2.6 ± 0.7 μA/cm2 (n = 8; Fig. 8). As seen in the Vte traces, Vte was abolished or even became slightly positive, a phenomenon that requires further investigations. In further experiments we found that apically applied clotrimazole had similar effects on nucleotide-stimulated Vte and Ite. With apical clotrimazole pretreatment, the Ite decreased from 23.4 ± 6.0 to −0.9 ± 0.2 μA/cm2 for apically applied ATP/UTP, while basolateral ATP/UTP induced Ite decreased from 25.5 ± 6.1 to −1.3 ± 0.9 μA/cm2 (n = 4).

In the next series of experiments, we tested the IK channel opener DC-EBIO on the apical side and the results are shown in Fig. 9. In control experiments, apical ATP (100 μM) alone could transiently increase Vte by −3.3 ± 0.2 mV and Ite by 14.8 ± 0.9 μA/cm2 (n = 31). DC-EBIO significantly increased the effect of apical ATP, both on Vte by −5.1 ± 0.3 mV and Ite by 24.2 ± 4.9 μA/cm2 (n = 5), and again, there was a large initial fall in Rtc. The plateau response to ATP was also augmented by DC-EBIO (Fig. 9): ΔVte was −0.6 ± 0.1 mV.
ATP REGULATES Cl⁻ AND K⁺ CHANNELS IN PANCREATIC DUCTS

A

B

C

D

E

F

CFTR-F508

CFTR C-terminal

TMEM16A

18SrRNA

β actin

GUSB

QARS

M CA CF PA

M CA CF PA

M CA CF PA

M CA CF PA

M CA CF PA

M CA CF PA

M CA CF PA

M CA CF PA

M CA CF PA

CFTR C-terminal

TMEM16A

Relative expression (2⁻ΔΔCT)

Relative expression (2⁻ΔΔCT)

E

F

CA CF CAₘ CFₘ

CA CF CAₘ CFₘ

CA CF CAₘ CFₘ

CA CF CAₘ CFₘ

CA CF CAₘ CFₘ

CA CF CAₘ CFₘ

CA CF CAₘ CFₘ

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CA CF CAₘ CFₘ
and ΔI_{sc} was 1.4 ± 0.2 μA/cm² (n = 31) for ATP alone, while ΔV_{te} was −2.1 ± 0.1 mV and ΔI_{sc} was 6.0 ± 0.7 μA/cm² (Fig. 9; n = 5) for ATP and DC-EBIO together. Furthermore, after the combined stimulation of apical DC-EBIO and ATP, basolaterally applied DC-EBIO and ATP could not elicit further effects (Fig. 9, A and B). Similar results were found for DC-EBIO and apically applied UTP (Fig. 9, E–H; n = 6). The peak response of apically applied UTP was increased for ΔV_{te} from −2.4 ± 0.4 mV (Table 2; n = 11) to −5.3 ± 0.2 mV with DC-EBIO (Fig. 9G; n = 6), and ΔI_{sc} increased from 15.8 ± 3.7 to 31.3 ± 6.7 μA/cm² (Table 2 and Fig. 9H). Additionally, the negligible plateau response to UTP (Table 2) was significantly augmented by DC-EBIO; e.g., ΔV_{te} was −2.2 ± 0.2 mV and ΔI_{sc} was now 5.3 ± 0.3 μA/cm² (Fig. 9, G and H; n = 6), which was ~10 times bigger than that with UTP alone. Thus, most importantly, DC-EBIO almost doubled the peak responses of ATP/UTP and amplified the plateau responses by 5–10 times. In addition, we tested the effect of basolateral ATP and UTP together with basolaterally applied DC-EBIO and these seemed to have similar effects as the luminal application. We have concluded above that the effect of apical applied ATP/UTP was dependent on CFTR and CaCC (Figs. 2–4) and that the apical DC-EBIO given at the same time greatly amplified the stimulation of apical ATP/UTP. Thus one may ask whether opening of K⁺ channels might increase the driving force on basally active Cl⁻ channels. Therefore, we performed experiments on Capan-1 cells stimulated only with DC-EBIO and with/without Cl⁻ channel inhibitors. Figure 10A shows represen-
tative recordings for apical DC-EBIO (100 μM) response in Capan-1 cells: $V_{te}$ was changed from baseline $-0.3 \pm 0.2$ mV to peak $-1.5 \pm 0.4$ mV and then to plateau $-1.3 \pm 0.3$ mV (Fig. 10, A and B; $n = 8$). If the changes were represented by $I_{sc}$, the values were $1.2 \pm 0.5$, $4.4 \pm 0.9$, and $3.7 \pm 0.7$ μA/cm$^2$, respectively (Fig. 10, C and D; $n = 8$). These responses were significantly smaller than with ATP alone or in conjunction with DC-EBIO. Nevertheless, niflumic acid (100 μM, apical) and CFTRinh-172 (10 μM, apical) totally inhibited the sustained effect of apical DC-EBIO (Fig. 10).

**DISCUSSION**

In the present study, we demonstrate some important aspects of electrolyte transport regulation by purinergic receptors in human pancreatic duct cells. The most important findings are as follows: 1) Capan-1 cells functionally express two distinct types of Cl$^-$ channels, the cAMP-activated CFTR Cl$^-$ channel and the Ca$^{2+}$-activated Cl$^-$ channel, which is consistent with TMEM16A/ANO1, although other CaCC cannot be excluded; 2) electrogenic responses to ATP and UTP rely on intracellular Ca$^{2+}$; 3) apical ATP/UTP opens both CFTR and CaCC, and basolateral ATP regulates secretion predominantly through CFTR; and 4) ATP/UTP also regulate K$^+$ (IK, KCa3.1) channels, which provide the driving and potentiating force for Cl$^-$ secretion.

Stimulation of human pancreatic duct cells with extracellular ATP/UTP from the apical and basolateral sides leads to complex intracellular Ca$^{2+}$-dependent electrophysiological responses. These are characterized by fast peak and then plateau responses in $V_{te}$ and $I_{sc}$, and we will argue that both Cl$^-$ and K$^+$ channels are involved in these events. First, the purinergic regulation from the apical membrane and then from the basolateral membrane will be considered.

Effects of purinergic receptors have often been evaluated by monitoring intracellular Ca$^{2+}$, and we have shown that the effects
of ATP/UTP were greatly reduced by chelating intracellular Ca\(^{2+}\) (Fig. 1). In a recent study, we also show that electrophysiological responses to ATP/UTP resemble responses to ionomycin but not to the cAMP mediators secretin and forskolin (53). Therefore, we focused on the Ca\(^{2+}\)-activated channels in our study. In CFPAC-1 cells, both TMEM16A and bestrophin 1 have been identified (4, 30). These cells do not express CaCC very strongly (see below) and therefore let us first consider Capan-1 cells. In Capan-1 cells, the peak responses in \(I_{sc}\) and \(V_{te}\) induced by apically applied ATP and UTP were inhibited by niflumic acid (Fig. 2). This compound is not highly specific for TMEM16A and a search for more specific inhibitors by high-throughput screening is ongoing, although best candidates, e.g., TE16A\(_{high}\)-AO1, may have different potencies depending on cells or function tested (31, 45, 58). We find that Capan-1 cells express high levels of TMEM16A (ANO1) and this channel is shown for the first time on both mRNA and protein levels on pancreatic duct cells that are good models for human duct epithelium (53). Most importantly, TMEM16A immunoreactivity was greatest in the membrane microdomain fraction (Fig. 6) and upon apical ATP stimulation, the protein relocated to the apical membrane (Fig. 7A). Two studies support our finding. Similar relocalization of TMEM16A was recently reported for bronchial epithelial cells stimulated with interleukin-4 (45). A recent study on myocytes shows that TMEM16A was enriched in lipid rafts, and currents are modified by cholesterol-depleting agents (48). Nevertheless, we cannot exclude that other CaCC are present in pancreatic ducts.

Capan-1 cells also express relatively high levels of CFTR (compared with CFPAC-1 and PANC-1 cells), and we show that this channel is inhibited by CFTRinh-172 when the monolayers are stimulated by ATP or UTP added to the apical sides. Activation of CFTR may seem unexpected, as ATP and UTP, equally potent stimulators of P2Y2 (and P2Y4) receptors, lead to intracellular Ca\(^{2+}\) signals and electrophysiological effects in rodent and human duct cells (see above) (36). In accordance, several studies show that CFTR is stimulated by signaling involving PLC, PKC, and other kinases and these may operate via P2Y2 receptors (11, 22, 27, 37, 40). However, we cannot totally exclude that small contribution of P2Y11 receptors and A\(_{2A/2B}\) receptors that are expressed in Capan-1 cells and could stimulate cAMP/PKA signaling (1, 18, 33, 39, 50, 54, 56).

CFPAC-1 cells express ∆F508 CFTR, which exhibits impaired intracellular processing and delivery to the plasma membrane and thus loss of CFTR-mediated anion secretion. Notably, CFPAC-1 cells express relatively low quantities of TMEM16A (Fig. 6), and some ATP-stimulated CaCC activity has been detected in patch-clamp studies (13). Therefore, it is likely that TMEM16A is stimulated by apical ATP in CFPAC-1 cells, as indicated by a marked sensitivity to niflumic acid. However, the response is relatively small and transient compared with that in Capan-1 cells (Figs. 2 and 4). The simplest interpretation is that TMEM16A is not sufficient to maintain secretion. That agrees with several studies that show that CaCC currents and CaCC-driven secretion were not upregulated in pancreatic ducts of transgenic CF mice (15, 42, 55).

Basolaterally applied ATP and UTP in Capan-1 monolayers have somewhat different effects and sensitivity to Cl\(^{-}\) channel inhibitors. Clearly, P2 receptors were stimulated by ATP open CFTR rather than CaCC (especially in the peak phase). This is confirmed by CFPAC-1 data, where basolateral ATP and UTP have hardly any effects. In Capan-1 cells, basolateral UTP is not sensitive to the CFTR inhibitor, implying possibly a different P2 receptor and/or signaling pathway compared with ATP. Most importantly, basolateral (and apical) UTP has only very transient effects on \(V_{te}\) and \(I_{sc}\), and a negligible plateau response would indicate that prolonged secretion process could not be maintained. In fact, basolateral UTP (and P2Y2 receptors) inhibit big conductance \(K^{+}\) channels (known as BK, Slo1, KCa1.1, or KCNMA1) and thus secretion (20, 21, 23). This can be overcome by stimulation of IK (Fig. 9).

ATP supports best the plateau phase of secretory response in Capan-1 cells, and the inhibitor study shows that both CFTR and CaCC channels are required. Such sustained \(V_{te}\) and \(I_{sc}\) effects have also been observed with secretin and forskolin (53), and the plateau responses were more dependent on HCO\(_{3}^{-}\) provided from the basolateral side (6). Previous studies on rat pancreatic ducts also showed that HCO\(_{3}^{-}\} is necessary for normal electrophysiological responses (including activation of \(K^{+}\) channels; Ref. 38). Even though the present study cannot provide the direct evidence for HCO\(_{3}^{-}\) secretion, the basolateral to apical HCO\(_{3}^{-}\) gradient used would favor HCO\(_{3}^{-}\) transport to the apical side, and both CFTR and TMEM16A/ANO1 may be involved in this process. Recent studies raise again the possibility that these channels can be permeable to HCO\(_{3}^{-}\) (24, 41).

An important part of our study shows that \(K^{+}\) channels, most likely IK (i.e., KCa3.1), are involved in transepithelial responses to ATP and UTP. The first line of evidence is that clotrimazole, probably acting from intracellular sides (57), totally inhibits the ATP effects on \(V_{te}\) and \(I_{sc}\) (Fig. 8). In a parallel study, where we investigated molecular signatures of pancreatic \(K^{+}\) channels, we also confirmed molecular identity of IK on both apical and basolateral membranes of Capan-1 monolayers (19). Similar ATP/UTP-mediated effects on IK channels have also been observed in human pancreatic duct cell line HPAF (12) and isolated dog pancreatic duct cells (25). We speculate that if IK channels were contributing to negative \(V_{te}\) directly, then the conductance of
the basolateral K+ channels would have to be greater than that of the luminal channels. Nevertheless, we propose that the luminal IK channels could also contribute to the driving force for anion secretion and also K+ secretion, as pancreatic juice contains \(~10\) mmol/l K+ (46).

The second line of evidence for functional IK channels is the DC-EBIO experiments. EBIO analogs have been used in many studies on respiratory and intestinal epithelia (9, 17, 43, 47). In the absence of other agonist, i.e., ATP/UTP, DC-EBIO itself has small, plateau-like stimulating effects on \(V_m\) and \(I_{sc}\) of Capan-1 epithelium, which were sensitive to Cl\(^-\) channel inhibitors. It has been suggested that EBIO analogs can stimulate IK as well as calmodulin-activated TMEM16A channels (52). However, we propose that these drugs do not need to stimulate Cl\(^-\) channels directly; it may be sufficient to open IK channels and thus increase the driving force for Cl\(^-\) efflux through basally active Cl\(^-\) channels.

Thus both clotrimazole and DC-EBIO data show that active IK channels are prerequisite for anion efflux mediated by Cl\(^-\). P2 receptors activate both Cl\(^-\) and K+ channels. Most importantly, extra stimulation of K+ channels by DC-EBIO amplifies responses to ATP and especially to UTP (Fig. 9). That is, the peak \(I_{sc}\) response is increased by about factor of 2, while the plateau response is increased by a factor of 5 to 10 when ATP/UTP is applied together with DC-EBIO.

Taken together, this study shows function and expression of TMEM16A in human duct cells and we propose that this may be the CaCC that is responsible for the P2 receptor-stimulated response, especially by the luminal P2 receptors. This channel seems not to be upregulated in CFPAC-1 cells, which respond modestly to luminal ATP. CFTR is also stimulated by ATP on the luminal and basolateral membranes. Thus, for the optimal secretory response both CFTR and TMEM16A are necessary, which may explain why the pancreatic duct epithelium is affected so extensively by CF. Furthermore, the K+ channel, presumably IK, seems essential for maintenance and amplification of the driving force for Cl\(^-\) channels.

In conclusion, the luminal P2 receptors activate Cl\(^-\) and K+ channels leading to electrogenic secretory events that will have to be followed by other transporters and exchangers on both plasma membranes to eventuate in HCO\(_3\)\(^-\)-rich secretion. This scenario underpins the physiological role of luminal ATP that is secreted by acini and implies this as an amplification system for regulation of pancreatic secretion. Regarding the therapeutic approach to improve pancreatic secretion, along with CFTR rescue, delivery of luminal nucleotides and activators of Cl\(^-\) or K+ channels is a challenging problem.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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