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Cloning, Expression, Signaling Mechanisms, and Membrane Targeting of $P2Y_{11}$ Receptors in Madin Darby Canine Kidney Cells

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ABSTRACT

The $P2Y_{11}$ receptor is hypothesized to link to both G_s and G_q, although this proposition is based on expression and separate assays of G_s and G_q function in different cell types [*J Biol Chem* 1997;**272:**31969–31973]. We have cloned and characterized a canine $P2Y_{11}$-like ($cP2Y_{11}$) receptor from cultured Madin Darby canine kidney (MDCK-D1) cells. When $cP2Y_{11}$ receptors are expressed in canine thymocyte (CF2Th) cells that normally lack functional purinergic responses, ADPβS stimulates phosphatidylinositol (PI) hydrolysis, Ca^{2+} mobilization, and cAMP accumulation. Pharmacologic analysis indicates that the stimulation of cAMP production is direct and not a result of eicosanoid synthesis, activation of PKC, or elevation of cell Ca^{2+}. The rank order of potency for stimulation of PI hydrolysis by $cP2Y_{11}$ receptors (adenosine 5′-(2-*O*-thio) diphosphate = 2-methylthio-ADP ≥ 2-methylthio-ATP ≫ ADP > ATP) differs from that of $hP2Y_{11}$ receptors. Microscopic examination of MDCK-D1 cells expressing carboxyl-terminal green fluorescent protein (GFP)-tagged $cP2Y_{11}$ ($cP2Y_{11}$-GFP) receptors indicates primarily basolateral (BL) targeting. BL addition of 200 μM ADPβS to confluent monolayers of MDCK-D1 cells produces an increase in short circuit current (I_{sc}) (11.6 ± 1.6 $\mu A/cm^2$) whereas apical addition of agonist has no effect, confirming targeting of functional endogenous $P2Y_{11}$ receptors to the BL surface. In contrast, when either $cP2Y_{11}$ or $cP2Y_{11}$-GFP is overexpressed in MDCK-D1 cells, the sensitivity of I_{sc} to BL agonist increases by nearly 2 orders of magnitude, as if receptor density normally limited agonist potency; moreover, apical addition of ADPβS now produces an increase in I_{sc} but with low potency. The data support the BL localization of $cP2Y_{11}$ receptors and receptor coupling to changes in I_{sc} in MDCK-D1 cells except in cases in which receptors are overexpressed; receptor overexpression leads to altered sensitivities and sites of coupling to physiologic responses.

P2Y purinergic receptors are members of the G protein-coupled receptor (GPCR) family. Six mammalian P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{12}$) have been cloned and characterized. Madin Darby canine kidney (MDCK-D1) cells, a well differentiated renal epithelial cell line, express three of these P2Y subtypes: $P2Y_1$, $P2Y_2$, and $P2Y_{11}$. Nucleotide agonists of P2Y receptors may play important roles in cell-cell communication within the kidney, such as that among epithelial, endothelial, and smooth muscle cells in the control of renal microcirculation (Middleton et al., 1993; Navar et al., 1996). Epithelial cells, including MDCK cells in monolayer culture, are polarized and the localization of particular receptor subtypes to the basolateral or apical membrane surface probably helps to determine the functional capabilities of a receptor subtype in vivo. However, studies aimed at defining precise functional roles for purinergic receptors in vivo have been hampered by the lack of subtype-selective agonists and antagonists. Currently, cloning and heterologous expression studies provide the best method for defining nucleotide-mediated, receptor-specific cell-signaling events.

A number of laboratories have examined nucleotide-mediated signaling in MDCK-D1 cells. Stimulation of MDCK-D1 cells by P2Y agonists results in the hydrolysis of phosphoinositides (PI) (Yang et al., 1997), activation of phospholipase D (Balboa et al., 1994) and $cPLA_2$ (Xing et al., 1997), alter-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; MDCK-D1, Madin-Darby canine kidney cells; PI, phosphatidylinositide; AA, arachidonic acid and metabolites; I_{sc}, short-circuit current; AM, acetoxymethyl ester; RT, reverse transcriptase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; IP, inositol phosphates; 2 MT, 2-methylthio; ADPβS, adenosine 5′-(2-*O*-thio) diphosphate; GFP, green fluorescent protein; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

ations in the uptake and release of arachidonic acid and metabolites (AA) (Firestein et al., 1996) and the stimulation of cAMP formation (Post et al., 1996, 1998). When grown to confluence, MDCK monolayers also respond to P2Y agonists with an activation of K^+ channels (Paulmichl et al., 1991) and an increase in short circuit current (I_{sc}) (Simmons, 1982; Zegarra-Moran et al., 1995), reflective of net ion transport.

Although the hydrolysis of PI and mobilization of intracellular Ca^{2+} are signaling events typically mediated by G_q activation, the stimulation of cAMP production in MDCK-D1 cells seems to be a more complex process involving at least two separate pathways. One involves a cyclooxygenase-*dependent* conversion of AA to prostaglandin E_2 and the subsequent activation of EP receptors that couple to G_s. The other pathway is likely to involve coupling of purinergic receptors directly to G_s and thence to the stimulation of adenylyl cyclase. It is clear that ATP and other nucleotides can increase cAMP levels by a cyclooxygenase-*independent* mechanism (Post et al., 1996, 1998). Of the cloned P2Y subtypes, only $P2Y_{11}$ receptors seem capable of stimulating cAMP formation via such a cyclooxygenase-*independent* mechanism (Communi et al., 1997). However, the evidence for $P2Y_{11}$ receptors coupling to G_s and G_q is based on independent expression of cloned receptors in two different heterologous cell types (Communi et al., 1997).

To determine the signaling capabilities and the localization of $cP2Y_{11}$ receptors in polarized epithelial cells, we have cloned this receptor from MDCK-D1 cells and expressed it in a species-specific $P2Y_{11}$-null cell [in canine thymocytes (CF2Th)] and in MDCK-D1 cells. We find that the human and canine $P2Y_{11}$ clones share 70% identity in primary amino acid sequence, with key differences in loci implicated in nucleotide binding and stabilization. The relevance of these differences is suggested by the substantially different nucleotide selectivity in promoting PI hydrolysis and cAMP observed between the human and canine clones. We also find that the $P2Y_{11}$ receptors of MDCK-D1 cells seem to be functional on the basolateral surface of confluent monolayers and that the majority of overexpressed green fluorescent protein (GFP)-tagged receptors also localize to the basolateral domain. When $cP2Y_{11}$ receptors or $cP2Y_{11}$-GFP receptors are overexpressed, apical addition of agonist will also cause an enhancement in ion flux but with a concentration-dependence that is 3 orders of magnitude less sensitive than that caused by basolateral agonist. Thus, the data indicate that overexpression of receptors can produce spatial distributions of receptor that may not faithfully recapitulate normal targeting and associated physiologic function. Furthermore, conclusions from previous studies of membrane localization of overexpressed GPCRs in polarized cells may need to be re-examined and evaluated using functional analyses.

Experimental Procedures

Materials. Suppliers of reagents were as follows: myo-D-[^{3}H]inositol (80 Ci/mmol), PerkinElmer Life Science Products (Boston, MA); indo-1/AM, 5′-(3-*O*-thio) triphosphate, and ADPβS, Calbiochem (La Jolla, CA); 2 MT-ATP, 2 MT-ADP, Sigma/RBI (Natick, MA); all other nucleotides and bases, Sigma-Aldrich (St. Louis, MO); agarose, FMC Bioproducts (Rockland, ME); Ecoscint, National Diagnostics (Atlanta, GA); Trizol, RNase H^- reverse transcriptase, dNTPs, TOPO TA cloning vector, and random hexamers, Invitrogen (Carlsbad, CA); RNase-free DNaseI, Roche Molecular Biochemicals (Summerville, NJ); Advantage cDNA Polymerase mix, Marathon cDNA amplification kit, and pEGFP-N2 vector, CLONTECH (Palo Alto, CA); Poly(A)Pure mRNA isolation kit and phenol/chloroform/isoamyl alcohol, Ambion (Austin, TX); QIAquick Gel Extraction Kit, QIAGEN (Valencia, CA); transwell filters (12 mm wide, 0.45 μm pore), Millipore (Bedford, MA); and primers, Operon Technologies (Alameda, CA). PA317 packaging cells were a gift from Dr. Robert Nicholas (University of North Carolina at Chapel Hill, Chapel Hill, NC); pLXSN was a gift from Dr. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA).

Cell Culture. MDCK-D1 (a subclone of MDCK cells) and CF2Th cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum as described previously (Post et al., 1998; Zambon et al., 2000). Two to three days before experimentation, cells were detached with a trypsin-EDTA solution and plated into six- or 12-well plates. Cultures were 75 to 90% confluent at the time of second messenger assays. Short circuit currents were measured using cells grown to confluence for 5 to 7 days in 12-mm transwell filters plated in 24-well plates.

$cP2Y_{11}$ Receptor Cloning. MDCK-D1 cells were grown in 175 cm^2 flasks to approximately 80% confluence. Trizol was used to extract total RNA from which messenger RNA was subsequently isolated using an Ambion Poly(A)Pure mRNA isolation kit according to the manufacturer's instructions. One microgram of MDCK-D1 mRNA was reverse transcribed, double stranded, and blunt ended, after which adaptors were ligated to the terminal blunt ends using the Marathon cDNA amplification kit. The 5′ end of the $P2Y_{11}$ receptor was amplified using touchdown PCR [95°C, 1 min, five cycles (94°C, 30 s; 72°C, 2 min), five cycles (94°C, 30 s, 70°C 2 min), 30 cycles (94°C, 30 s; 66°C 2 min)] with a 5′ adapter primer (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and 3′ $P2Y_{11}$ specific primer (5′-GGTGGACGATGCCCAGGTAGCGGTTTA-3′). The 3′ end of the message was amplified by nested PCR using a 5′ $P2Y_{11}$ specific primer (5′-CAACGGCCTGGCCTTCTACCG-3′) and the 3′ adaptor primer (5′-CCATCCTAATACGACTCACTATAGGGC-3′) using the same PCR conditions as above. For the 3′ fragment from rapid amplification of cDNA ends, a second nested PCR was conducted using a 5′ nested $P2Y_{11}$ primer (5′-CTACCGCTTTGTGACGCGGGAGCA-3′) and a 3′ nested adaptor primer (5′-ACTCACTATAGGGCTCGAGCGGC-3′). To obtain the full-length coding region, primers were designed based on 5′(5′-CAGGTCTGGGCTCTGGG ACTAGCA-3′) and 3′(5′-CCCATTCTGCTGCTGGTCTCA-3′) untranslated regions that flanked the coding region. PCR using these primers and double stranded cDNA resulted in amplification of a single band that contained the full-length coding region of $cP2Y_{11}$. All PCR fragments were run on 1% agarose gels, excised, purified using a QIAGEN gel extraction kit, cloned into pCR 2.1-TOPO cloning vector ($cP2Y_{11}$-topoTA), and sequenced.

Reverse-Transcriptase Polymerase Chain Reaction. Ten micrograms of total RNA was treated with 2 U of RNase-free DNaseI for 1 h at 37°C in a solution of 0.1 M sodium acetate, 5 mM $MgSO_4$, pH 5.0. RNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. RNA was then reverse transcribed in 20 μl with 4 μl of 5× RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$) together with 0.09 A_{260} units of random hexamers, 10 mM dithiothreitol, 800 μM dNTP, and 200 U of Moloney murine leukemia virus RT. After a 1-h incubation at 37°C, each reaction was stopped by incubating samples at 70°C for 15 min. Two microliters of reverse-transcribed RNA was added to a 50-μl PCR reaction tube containing 400 nM forward (5′-CTACCGCTTTGTGACGCGGGAGCA-3′) and reverse (5′-GGTAGGGCACATAGGAGCTGGCGTA-3′) $P2Y_{11}$ specific primers (designed to amplify region 150–833), PCR buffer (40 mM Tricine-KOH, pH 9.2 at 25°C, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 mg/ml BSA) 0.2 mM dNTPs, 1 μl of Advantage cDNA polymerase mix (CLONTECH). Temperature cycling proceeded as follows: 94°C, 1 min; 35 cycles (94°C, 30 s; 69°C, 3 min), 68°C, 10 min. PCR products were then

subjected to gel electrophoresis on a 1% agarose gel. Control reactions, which omitted RT, resulted in no PCR products.

Construction of the $P2Y_{11}$-GFP Chimeric Receptor. The full-length coding region of the $cP2Y_{11}$ receptor was amplified by PCR using $cP2Y_{11}$-topoTA as a template with a 5′ primer containing a *Kpn*I site (5′-TGGTACCGGGCAGGTCTGGGCTCTGGG-3′) and a 3′ primer with a *Bam*HI site and a mutated stop codon (5′-CTGGATCCAGGGGACTAGGGCTCCACGT-3′). The product was then ligated into a *Bam*HI and *Kpn*I-cut pEGFP-N2 vector.

Retroviral Expression. $cP2Y_{11}$ and $cP2Y_{11}$-GFP were subcloned into the *Eco*RI/*Bam*HI sites and *Eco*RI/*Hpa*I sites of the retroviral expression vector pLXSN, respectively (Miller and Rosman, 1989). Constructs were then transfected into murine PA317 retroviral packaging cells by Ca^{2+} phosphate transfection (Comstock et al., 1997). Virus was removed and used to infect CF2Th and MDCK-D1 cells. Cells were selected for at least 2 weeks with 0.5 mg/ml G418 before use in assays.

Intracellular Ca^{2+}Measurements. CF2Th cells expressing MDCK-D1 $P2Y_{11}$ receptors were grown overnight on 22-mm glass coverslips. Cells were washed twice with HEPES-buffered saline 130 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Na^+-HEPES, pH 7.4 at 37°), then incubated in 2 ml of HEPES-buffered saline containing 1 μM indo-1/AM at 37°C for 30 min. Cells were suspended in fresh medium and fluorometric measurements in fields of 6 to 10 cells were collected using the DX-100 System (Solamere Technology, Salt Lake City, UT) and an inverted Nikon Diaphot microscope (Nikon, Melville, NY). The field was excited at 385 nm, the emission ratio was collected at 405 nm and 495 nm, and data were analyzed and plotted with Maclab software (see Meszaros et al., 2000a).

Phosphoinositide Hydrolysis Assay. Cells were loaded with myo-D-[2-^{3}H]inositol (2.5 μCi/ml) for 12 h. Thirty minutes before agonist stimulation, 10 mM LiCl was added. Cells were then stimulated for 10 min with agonists and reactions were stopped by aspiration of medium and addition of ice-cold methanol/HCl (50% MeOH/0.1 M HCl). Total inositol phosphates were separated from [^{3}H]inositol by chromatography on Dowex-1-formate (Meszaros et al., 2000a). Content of [^{3}H]IPs was assessed by liquid scintillation spectrometry.

Measurement of cAMP Accumulation. Before treatment of cells, growth medium was removed and cells were equilibrated for 30 min at 37°C in serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES buffer (DMEM/HEPES, pH 7.4). Subsequently, cells were incubated in fresh DMEM/HEPES, before addition of a phosphodiesterase inhibitor and agonists, as described in the figure legends. Unless otherwise indicated, incubations with agonist were conducted for 10 min at 37°C in the presence of 200 μM isobutylmethylxanthine, a cyclic nucleotide phosphodiesterase inhibitor. Incubations were terminated by placing on ice followed by aspiration of medium and addition of 7.5% trichloroacetic acid (TCA). TCA extracts were frozen (−20°C) until assay. Intracellular cAMP levels were determined by radioimmunoassay of TCA extracts after acetylation. cAMP data are expressed as picomoles of cAMP per microgram of acid-precipitable protein [as determined by the method of Bradford (1976)].

Fluorescence Microscopy of $cP2Y_{11}$-GFP Expression in MDCK-D1 Cells. Cells (2×10^5) were plated on 35-mm plates (MatTek, Ashland, MA) and grown for 5 days with a medium change every other day. Cells were viewed by confocal microscopy using a MRC-1024 laser-scanning confocal system (Bio-Rad Spectroscopy Group, Cambridge, MA) coupled to a Zeiss Axiovert 35 M microscope (Carl Zeiss Inc., Thornwood, NY). Images were viewed using a 40× objective with a krypton-argon laser exciting at 488 nm. Fluorescence was detected at 522 nm. Monolayers were approximately 20 μM thick and optical sections were 0.36 μM thick.

Short Circuit Current (I_{sc}) Measurements. MDCK-D1 cells either overexpressing $cP2Y_{11}$ or GFP (control) were seeded at 1×10^5 cells onto 0.6-cm^2 transwell filters. Cells were grown for 4 to 7 days with media changes every other day. Transwell filters were mounted in Ussing chambers modified for use with cultured cells (Dharmsathaphorn et al., 1984). Only cultures with resistances exceeding 1000 Ω/cm^2 were used in the experiments. Measurements were carried out in Ringer's solution (140 mM Na^+, 5.2 mM K^+ 1.2 mM Ca^{2+}, 0.8 mM Mg^{2+}, 119.8 mM Cl^-, 25 mM HCO_3^-, 2.4 mM H_2PO_4, 0.4 mM HPO_4^{2-}, and 10 mM glucose), which was maintained at 37°C and constantly bubbled with 95% O_2/5% CO_2. Measurements of I_{sc} were made under continuously short-circuited conditions using an automatic voltage clamp.

Data Analysis. Data analysis and curve fitting were performed using Prism software (GraphPad, Inc., San Diego, CA). Concentration-dependence curves were fit to a single component sigmoidal dose-response formula. Data shown on phosphoinositide hydrolysis are the mean ± range of duplicate determinations from a representative experiment repeated at least three times with similar results. IP data are presented as cpm of [^{3}H]IPs or as hormonal effect divided by total [^{3}H]inositol incorporation (measured by addition of 0.5% Triton X-100 to washed cells, a value of ~200,000 cpm). For cAMP studies, data shown are the mean ± S.E. of triplicate samples from a representative experiment that was repeated at least three times with similar results. Differences between treatment groups were analyzed by Student's t test. Values of $P < 0.05$ were considered significant.

Results

Deduced Amino Acid Sequence; Distribution of Transcripts in Canine Tissues. MDCK-D1 cells were used as a source of mRNA from which a canine $P2Y_{11}$ receptor was cloned by PCR and rapid amplification of cDNA ends. The deduced amino acid sequence and its comparison to that of the human ($hP2Y_{11}$) (Communi et al., 1997) receptor are shown in Fig. 1A. The $cP2Y_{11}$ receptor shares 70% amino acid identity with the $hP2Y_{11}$ clone. The most notable differences between the two $P2Y_{11}$ clones occur at the amino and carboxy-terminal regions, the third extracellular loop region, and in key residues implicated in nucleotide binding and stabilization (Fig. 1B) (Erb et al., 1995). Phylogenic analysis indicates that the $cP2Y_{11}$ receptor clone is clearly distinct from human $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors but is most homologous to $hP2Y_{11}$ receptors (Fig. 1C).

RT-PCR analysis indicated that mRNA for the $cP2Y_{11}$ receptor is expressed in the cerebellum, brain cortex, spleen, and liver of the dog (Fig. 2). We did not detect the expression of $cP2Y_{11}$ receptor mRNA in the canine kidney cortex or medulla, suggesting that expression of $P2Y_{11}$ receptors in vivo may be in only a limited number of renal cells.

Coupling to Transmembrane Signaling. Stable expression of $cP2Y_{11}$ receptors in CF2Th cells conferred on the cells the capacity to respond to P2Y agonists with an increased in phosphoinositide (PI) hydrolysis and IP production (Fig. 3A). ADPβS was most potent, with an EC_{50} value of ~5 μM. The rank order of potency of these agonists was ADPβS = 2 MT-ADP ~ 2 MT-ATP ≫ ADP > ATP; uridine derivatives (UTP, UDP) and AMP were inactive. The potency of agonists in stimulating PI hydrolysis mediated by expressed $cP2Y_{11}$ receptors differs from that reported for the human $P2Y_{11}$ receptor (ATP > 2 MT-ATP ≫ ADP ≥ 2 MT-ADP) (Communi et al., 1997), suggesting the likely importance of structural differences between the clones isolated from the two species (Fig. 1).

We were able to ligate the cDNA of GFP in frame to the 3′ end of the $cP2Y_{11}$ receptor containing a mutated stop codon.

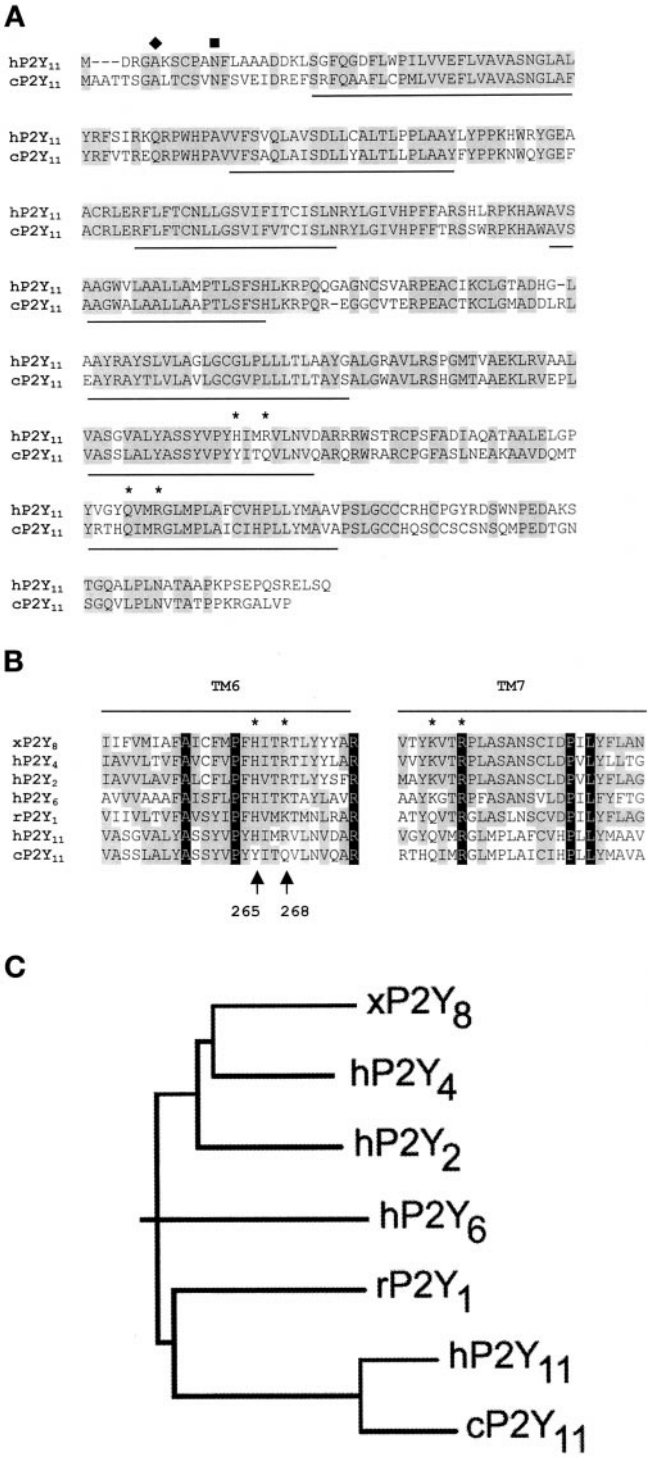


Fig. 1. Comparison of the canine $P2Y_{11}$ receptor with other P2Y receptors. A, sequence alignment of canine and human P2Y11 receptors. Shown here is a CLUSTALW alignment (Thompson et al., 1994) of the inferred amino acid sequences of canine and human $P2Y_{11}$ receptors (**c**$P2Y_{11}$, **h**$P2Y_{11}$). Putative transmembrane spanning domains are underlined. Conserved residues are shaded in dark gray; similar residues are shaded in light gray. ■, putative *N*-myristoylation site; ♦ putative *N*-glycosylation site; *, residue implicated in nucleotide binding and stabilization. B, cP2Y11 sequence homology with other P2Y receptors—CLUSTAL W alignment of the sixth and seventh transmembrane helices (TMH) of $rP2Y_1$, $hP2Y_2$, $hP2Y_4$, $hP2Y_6$, $xP2Y_8$, $hP2Y_{11}$, and $cP2Y_{11}$ receptors. Canine $P2Y_{11}$ receptors are the only P2Y receptors that have two uncharged residues, ^{265}Tyr and ^{268}Glu, in two sites of TMH 6, which have been implicated in nucleotide binding and stabilization. C, phylogenic rooted tree analysis of TMH domains 2 to 7 from P2Y receptors.

We used this construct ($cP2Y_{11}$-GFP) to generate stably expressing CF2Th cells that we also analyzed for their capacity to respond to purinergic agonists with increased PI hydrolysis. These studies indicated that the fluorescent protein traffics to the membrane and signals in a manner similar to the untagged receptor: the GFP-tagged $cP2Y_{11}$ receptor displayed EC_{50} values and a rank order of potency of purines for PI hydrolysis similar to those of $cP2Y_{11}$ receptors (Fig. 3B). Thus, the chimeric receptor recognized ligands and coupled to G_q in a manner similar to the wild-type receptor.

Several P2Y antagonists were screened for their capacity to inhibit $cP2Y_{11}$-mediated PI hydrolysis in $cP2Y_{11}$-expressing CF2Th cells. Of the antagonists tested, only suramin, a nonselective P2Y antagonist, inhibited $cP2Y_{11}$-mediated PI hydrolysis (Fig. 4). A variety of $P2Y_1$-selective inhibitors (adenosine 2′,5′-diphosphate, adenosine 3′,5′-diphosphate, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid 4-sodium) were ineffective, as would be predicted for a $P2Y_{11}$ receptor.

Using $cP2Y_{11}$-expressing CF2Th cells loaded with indo-1/AM, we were able to determine whether IP production couples to the mobilization of stored Ca^{2+} in these cells. As anticipated, purine-stimulated IP formation coupled to an increase in the ratio of indo-1 fluorescence at 405 nm and 495 nm (Fig. 5). Among P2Y agonists, ADPβS was more efficacious and potent in mobilizing intracellular Ca^{2+} levels than was ADP; ADPβS exhibited a threshold for Ca^{2+} mobilization between 1 and 10 nM (Fig. 5, A and B). The removal of extracellular Ca^{2+} and the addition of EGTA had minimal effects on the capacity of ADPβS to stimulate Ca^{2+} mobilization, indicating that the majority of Ca^{2+} mobilized is released from intracellular stores (data not shown). Control cells expressing GFP alone did not respond to ADPβS but did respond to thapsigargin (see inset to Fig. 5C), confirming the presence of releasable Ca^{2+} stores.

We also assayed the capacity of native and $cP2Y_{11}$-expressing CF2Th cells to accumulate cAMP in response to purinergic agonists. Native CF2Th cells exhibited virtually no cAMP accumulation in response to ADPβS. In $cP2Y_{11}$-expressing

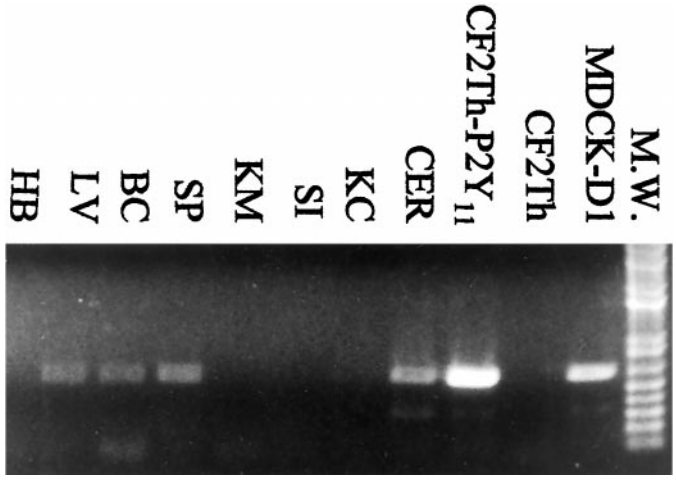
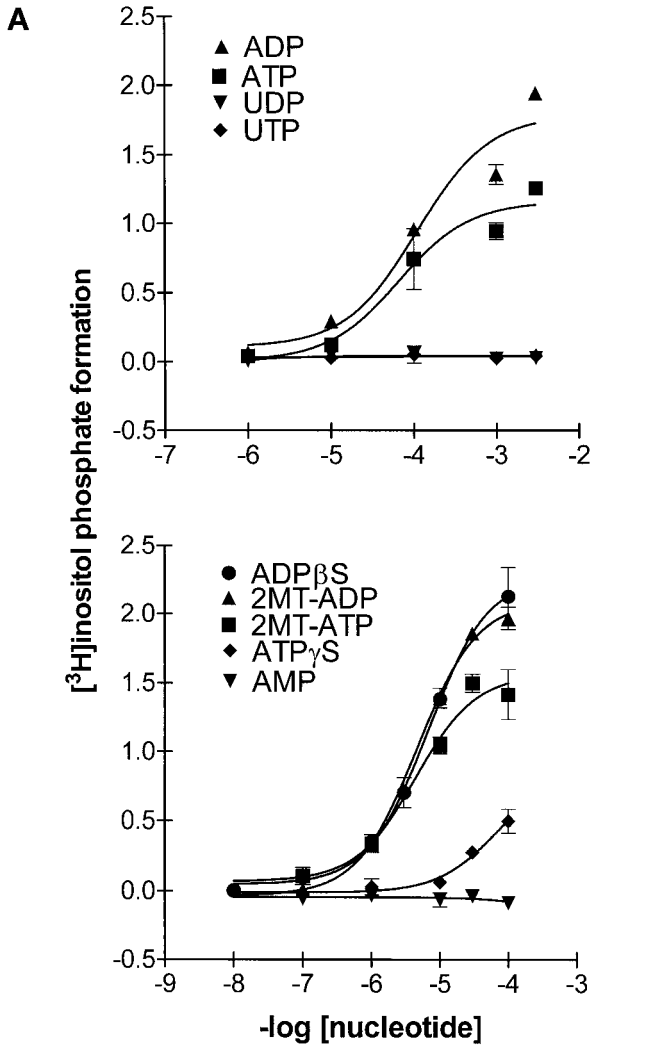


Fig. 2. Localization of $cP2Y_{11}$ transcripts. Total RNA (200 ng) were reverse-transcribed and used as a template for PCR with primers amplifying a 595-base pair fragment $cP2Y_{11}$ receptor. Transcripts were found in cerebellum (CER), spleen (SP), brain cortex (BC), and liver (LV). Transcripts were not found in kidney cortex (KC), small intestine (SI), kidney medulla (KM), or hindbrain (HB).



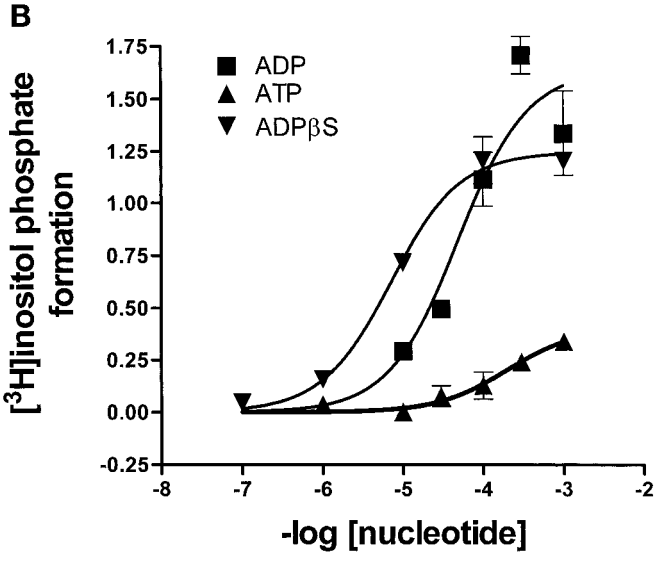


Fig. 3. $cP2Y_{11}$ and $cP2Y_{11}$-GFP induced inositol phosphate formation. $cP2Y_{11}$ receptors (A) and chimeric $cP2Y_{11}$-GFP receptors (B) were stably expressed in CF2Th cells. Agonist-sensitive inositol phosphate production was assessed as described under *Experimental Procedures*. Data ([^{3}H]IP formation as fraction of [^{3}H]inositol incorporation) in A are mean ± range of duplicate determinations from a representative experiment that was replicated at least three times with similar results; data in B are mean ± S.E., $n = 3$.

CF2Th cells responses were readily apparent (Fig. 6): ADPβS, 2 MT-ATP and 2 MT-ADP were roughly equipotent, with EC_{50} values near 1 μM; ADP (EC_{50} ~10 μM) was more potent than ATP (EC_{50} ~60 μM). This order of potency is in accord with that for stimulation of IP production in response to activation of this receptor (Fig. 3).

The cells' cAMP response to ADPβS was inhibited by the P2Y receptor antagonist, suramin, as expected (Fig. 6B). Moreover, the response to ADPβS was relatively unaffected by indomethacin, the cyclooxygenase inhibitor (Fig. 6B), indicating that cAMP accumulation in response to activation of $cP2Y_{11}$ receptors does not result from the intermediate formation of eicosanoids and their subsequent autocrine activation of EP receptors coupled to G_s. We also tested the possibilities that the effect of ADPβS to stimulate cAMP accumulation is secondary to the elevation of intracellular Ca^{2+} or to the activation of protein kinase C (PKC), both of which can activate some isoforms of adenylyl cyclase (Sunahara et al., 1996). Inhibition of PKC activity with GFX and buffering of Ca^{2+} transients with BAPTA-AM, at concentrations and times shown previously to be effective (Meszaros et al., 2000a,b), did not significantly alter the response to ADPβS (see inset to Fig. 6B). The $cP2Y_{11}$-GFP construct, expressed in CF2Th cells, also supported cAMP accumulation in response to ADPβS (data not shown).

As a group, these data indicate that transfection of CF2Th cells with a single receptor, the $cP2Y_{11}$ receptor, confers on CF2Th cells responses that are caused by coupling to both G_q and G_s. In addition, results with ADPβS and ADP demonstrate the relative sensitivities of the three assays of signal transduction: Ca^{2+} mobilization > cAMP accumulation > PI hydrolysis.

Fluorescence Localization of $cP2Y_{11}$-GFP in MDCK-D1 Cells. We expressed $cP2Y_{11}$-GFP receptors in MDCK-D1 cells and used confocal fluorescence microscopy to

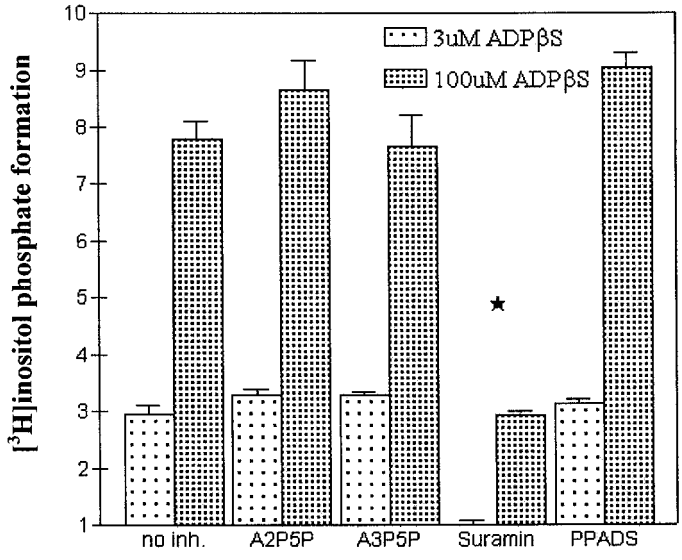


Fig. 4. Effects of P2Y inhibitors on $cP2Y_{11}$-mediated PI hydrolysis. Several P2 antagonists were screened for their capacity to inhibit $cP2Y_{11}$-induced PI hydrolysis in $cP2Y_{11}$-expressing CF2Th cells. Antagonists (at 100 μM) were added 15 min before addition of ADPβS (at 3 and 100 μM). Data ([^{3}H]IP formation as fraction of [^{3}H]inositol incorporation) are the mean ± S.E.; $n = 3$. *, $P < 0.002$; significant difference compared with appropriate control. Of the antagonists screened, only suramin, a nonselective P2Y antagonist, was able to inhibit $cP2Y_{11}$-induced PI hydrolysis.

determine the location of expressed receptors (Fig. 7). By this technique, $cP2Y_{11}$-GFP receptors seemed to localize primarily in areas of cell-cell contact and below (basolateral membranes), with a very low but detectable level above (apical to) this region, as indicated by the fluorescent densities in the z-plane (Fig. 7, bottom). Thus, some receptors appear in apical membranes although the basolateral localization predominates.

Determination of I_{sc} in MDCK-D1 Cells. As an MDCK cell culture becomes confluent, it organizes into a polarized monolayer with relatively high electrical resistance. We employed this property, coupled with our finding of the basolateral predominance of overexpressed $cP2Y_{11}$-GFP receptors (Fig. 7B), in an attempt to correlate receptor localization with function. Specifically, we assessed the capacity of a P2Y agonist, added either apically or basolaterally, to stimulate short circuit current (I_{sc}) responses. Addition of ADPβS (200 μM) to the basolateral surface of control (not transfected) MDCK-D1 cells caused a rapid, large and sustained increase in I_{sc}, to 11.6 ± 1.6 μA/cm^2 (mean ± S.E., n = 4); addition of ADPβS to the apical surface was without effect (Fig. 8). Repetition of these experiments in Cl^--free buffer abolished the effect of basolateral agonist (data not shown). These data suggest that the endogenous $P2Y_{11}$ receptors of MDCK-D1 cells are functionally expressed on the basolateral surface of native MDCK-D1 cells. Moreover, in this location, activated $P2Y_{11}$ receptors couple to a signaling pathway that stimulates a short circuit current response reflective of net chloride transport.

When GFP alone was overexpressed in these cells, the pattern of only basolateral responsiveness to ADPβS was maintained (Fig. 8). However, when functional receptors, either $cP2Y_{11}$ or $cP2Y_{11}$-GFP, were overexpressed, basolateral responsiveness to 200 μM ADPβS was slightly (although insignificantly) diminished and apical addition of ADPβS now caused a large and slowly developing response (Fig. 8). A concentration-dependence curve (Fig. 9) helped to clarify

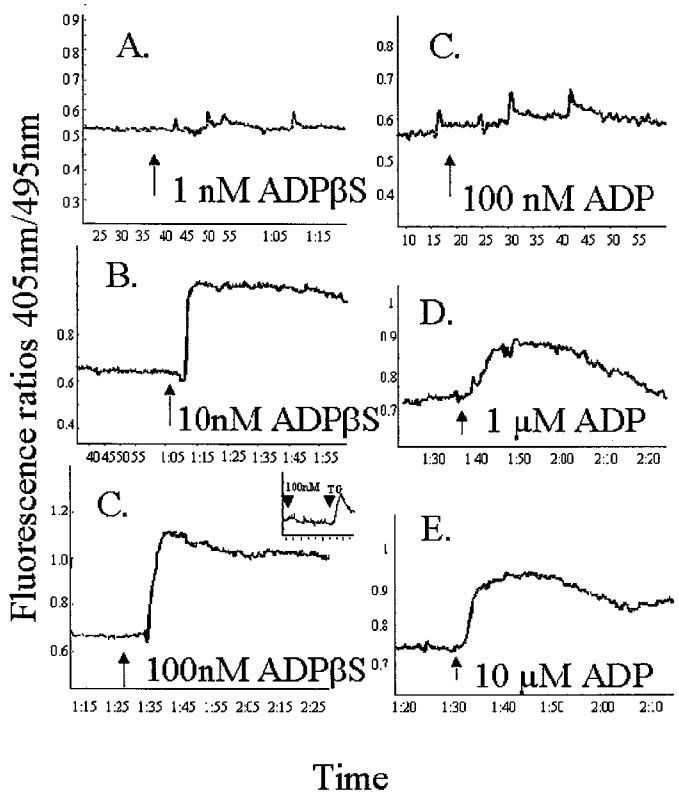


Fig. 5. Mobilization of intracellular Ca^{2+} in $cP2Y_{11}$ expressing CF2Th cells. Ca^{2+} transients were assessed in $cP2Y_{11}$-expressing CF2Th cells using Indo-1/AM as described under *Experimental Procedures*. C, inset, shows the lack of response of control (no $cP2Y_{11}$ receptor) CF2Th cells; the effect of thapsigargin (TG, 5 μM) confirms the storage of releasable Ca^{2+} in these cells. Intracellular free Ca^{2+} (ordinate) is represented as the ratio of emissions at 405 and 495 nm. Abscissa values are in minutes: seconds.

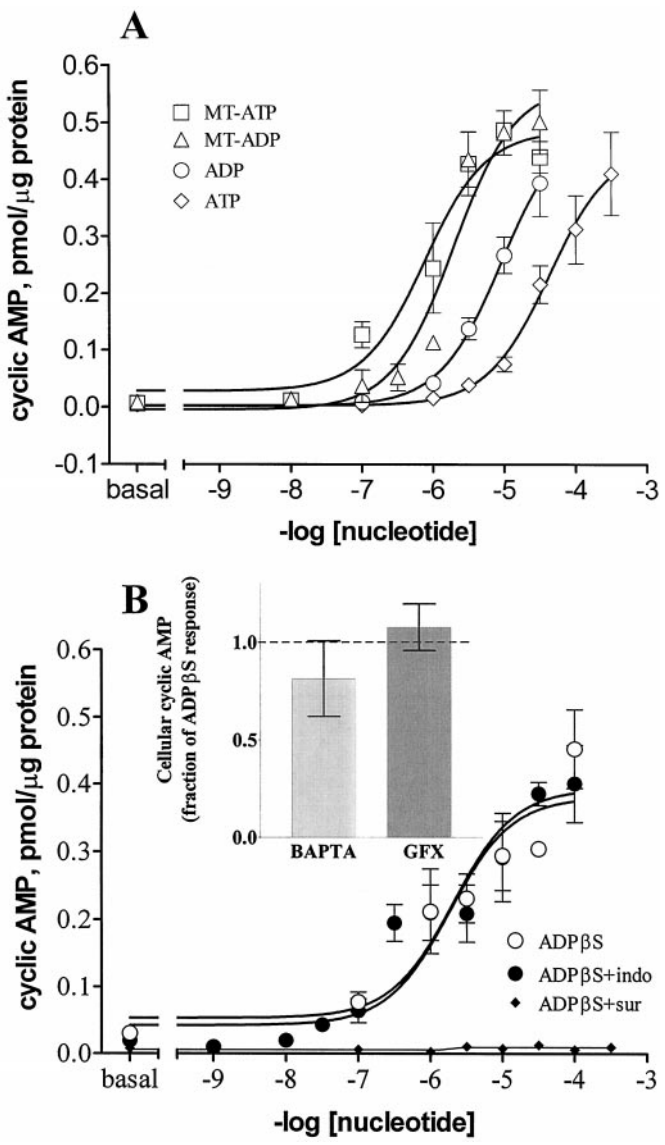


Fig. 6. Characterization of the effect of purinergic agonists to stimulate cAMP formation in CF2Th cells. A, concentration-dependences to multiple purinergic agonists; B, pharmacologic analysis of the effect of ADPβS. □, control response to ADPβS. Suramin (sur, 100 μM) added immediately before ADPβS (♦) fully inhibited the response. Indomethacin (indo, 2 μM) added 25 min before ADPβS (●) did not significantly alter the response. B, inset, lack of significant effect of the PKC inhibitor GFX (GF-109203X, 10 μM) added 30 min before challenge with 10 μM ADPβS ($P > 0.5$) and BAPTA-AM (30 μM, added 30 min before challenge with 10 μM ADPβS; $P > 0.13$). Data are the mean ± S.E. of triplicate samples from replicate experiments (n = 2 or 3) except for MT-ADP (n = 1) and experiments with BAPTA-AM and GFX (n = 6). The capacity of purinergic agonists to stimulate cAMP accumulation in CF2Th cells expressing $cP2Y_{11}$ receptors was assessed as described under *Experimental Procedures*.

these results. In control MDCK-D1 cells, basolateral ADPβS stimulated I_{sc} with an EC_{50} value of ~3 μM. After the overexpression of GFP-tagged $cP2Y_{11}$ receptor, basolateral ADPβS was more potent, more efficacious, and produced a biphasic response: ADPβS stimulated a large increase in I_{sc} with an apparent EC_{50} value of ~0.06 μM; increasing concentrations of ADPβS inhibited the I_{sc} response with an apparent EC_{50} of ~3 μM (Fig. 9A). In contrast, in overexpressing cells, the increase in I_{sc} in response to apical ADPβS was monophasic and as large as that caused by basolateral ligand but required a higher concentration of ligand than the basolateral response, with an EC_{50} value of ~60 μM, 3 orders greater than on the basolateral side of the same cells (Fig. 9B). Thus, the capacity of apical ligand to stimulate I_{sc} in transfected cells is apparently caused by overexpression of the $cP2Y_{11}$ receptor.

Discussion

MDCK-D1 cells express multiple P2Y receptor subtypes that are capable of activating several intracellular events including PI hydrolysis, Ca^{2+} activation, cAMP formation and transepithelial ion flux (Balboa et al., 1994; Xing et al., 1997; Yang et al., 1997; Post et al., 1998). Post et al. (1996, 1998) showed that the stimulation of cAMP formation in MDCK-D1 cells by certain nucleotides is sensitive to cyclooxygenase inhibition, suggesting that G_q-coupled P2Y receptors can link *indirectly* to the G_s pathway via the release and conversion of arachidonic acid to prostaglandin E_2 and the subsequent stimulation of G_s-coupled EP receptors. However, some nucleotides that stimulate cAMP formation in MDCK-D1 cells display little to no sensitivity to cyclooxygenase inhibition, suggesting that these cells also express a P2Y receptor that couples *differently*, possibly *directly,* to the G_s pathway (Post et al., 1996). Of the cloned P2Y receptors expressed in MDCK-D1 cells ($P2Y_1$, $P2Y_2$, and $P2Y_{11}$), $P2Y_{11}$ receptors are the only receptors known to stimulate cAMP formation in an indomethacin-*insensitive* manner (Lustig et al., 1993; Webb et al., 1993; Communi et al., 1997). Further studies have revealed that, among the P2Y agonists, 2 MT-ATP-, ADPβS- and ADP-stimulated increases in cAMP are much less sensitive to indomethacin than those induced by ATP and UTP (Torres et al., 2000). However, because ADPβS and ADP reportedly have little or no efficacy at $hP2Y_{11}$ receptors (Communi et al., 1997), we hypothesized that the canine $P2Y_{11}$ receptor might have a different pharmacological selectivity from that of the human receptor. To test this hypothesis, we have cloned the canine $P2Y_{11}$ receptor from MDCK-D1 cells and expressed it in a species-specific cell line, CF2Th cells. In addition, we used the $cP2Y_{11}$ clone to assess regional localization of the $P2Y_{11}$ receptor and its effects on transepithelial ion flux in polarized MDCK-D1 cells.

Alignment of $cP2Y_{11}$ receptors with other P2Y receptors reveals four positively charged basic residues in the sixth and seventh transmembrane spanning domains, ^{262}His, ^{265}Arg/Lys, ^{289}Lys, and ^{292}Arg (Fig. 1A, *), that are conserved among all P2Y receptors and have been implicated in nucleotide binding and/or stabilization (Erb et al., 1995). Mutations of ^{262}His and ^{265}Arg to the Leu in the murine $P2Y_2$ receptor results in a 400- to 800-fold decrease in the potency of ATP and UTP. Sequence alignment of the $cP2Y_{11}$ receptor with other P2Y receptors indicates $cP2Y_{11}$ receptors have two uncharged residues (^{265}Tyr and ^{268}Glu) at the homologous positions in TM6. Studies are underway to determine whether ^{265}Tyr and ^{268}Glu are responsible for the different agonist selectivities between the two $P2Y_{11}$ clones, such that the $cP2Y_{11}$ receptor preferentially recognizes ADPβS and MT-ADP, as opposed to the preferential recognition of triphosphate derivatives by the $hP2Y_{11}$ receptor.

The capacity of $hP2Y_{11}$ receptors to couple to both PI hydrolysis and cAMP formation was previously suggested by expression of the receptors in two different cell lines, with one mode of coupling being assessed in each cell line (Communi et al., 1997). Our previous work with $cP2Y_2$ receptors, however, has demonstrated that coupling of a receptor to multiple effector pathways is very dependent on the differentiated properties of the cell in which receptor is expressed (Zambon et al., 2000). We show here that activation of the $cP2Y_{11}$ receptor causes both PI hydrolysis/Ca^{2+} mobilization and cAMP generation even when the receptor is expressed in a single cell type. One could argue that coupling of the $cP2Y_{11}$ receptor to G_q produces the primary response and that cAMP accumulation results secondarily from eicosanoid production or from Ca^{2+}-dependent events such as activation of a Ca^{2+}/calmodulin-sensitive adenylyl cyclase activity or activation of

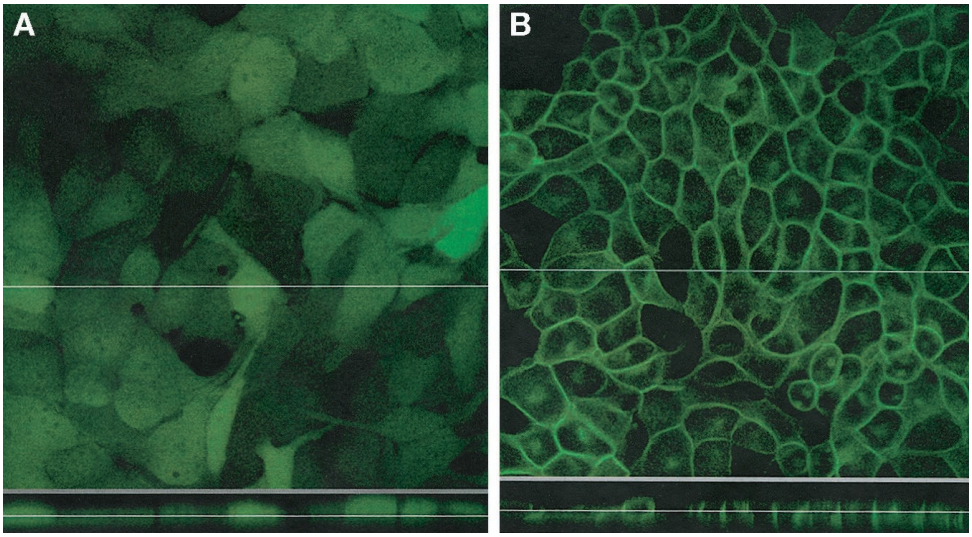


Fig. 7. Localization of $cP2Y_{11}$-GFP receptors in polarized MDCK-D1 cells. Confocal images of MDCK-D1 cells expressing either GFP (A) or $cP2Y_{11}$-GFP receptors (B). The upper images in each pair are fluorescence in the x-y plane. The small lower image is fluorescence of the z-plane, taken at the thin white lines bisecting the upper images. The white lines thus indicate the plane of the other image in relation to the one. In the lower part of B, apical surface is upper surface (above the white line).

PKC, which in turn can activate certain isoforms of adenylyl cyclase (Sunahara et al., 1996). Indeed, Qi et al. (2001) found that such indirect effects of Ca^{2+} and PKC seem to account for variable but substantial portions of the effect of extracellular ATP on cAMP accumulation in Chinese hamster ovary and 1321N1 cells into which hP2Y$_{11}$ receptors are stably expressed: down-regulation of PKC reduced the ATP response by 60 to 80% in both cell types; chelation of intracellular Ca^{2+} decreased the ATP response by 45% in 1321N1 cells but was without effect in Chinese hamster ovary cells (Qi et al., 2001). Even allowing for the possibility that down-regulation of PKC by long-term exposure to phorbol ester might also alter expression of multiple components of the response pathway, one must agree with Qi et al. that mobilization of intracellular Ca^{2+} potentiates the capacity of ATP to stimulate cAMP accumulation in these cells. Expressing the cP2Y11 receptor in CF2Th cells, we obtain a different result: pharmacologic inhibitors of eicosanoid synthesis (indomethacin), the intracellular Ca^{2+} transient (BAPTA-AM), and PKC activity (GFX) are without significant effect on cAMP elevation in response to ADPβS (Fig. 6B). Thus, cP2Y11 receptors, expressed into the milieu of CF2Th cells, seem to couple directly to both G_q and G_s.

Interestingly, the G_q and G_s linkages exhibit different sensitivities: Ca^{2+} mobilization > cAMP generation > PI hydrolysis. It is quite possible that the stoichiometries and affinities of components within the G_q and G_s pathways differ, as may the levels of signal amplification, giving rise to such differences. The greater sensitivity of Ca^{2+} mobilization compared with the promotion of PI hydrolysis is somewhat surprising because Ca^{2+} mobilization probably occurs as a consequence of PI hydrolysis and the generation of inositol 1,4,5-trisphosphate. Our observed order of sensitivity may reflect assay sensitivity. Some of the difference may also reflect the

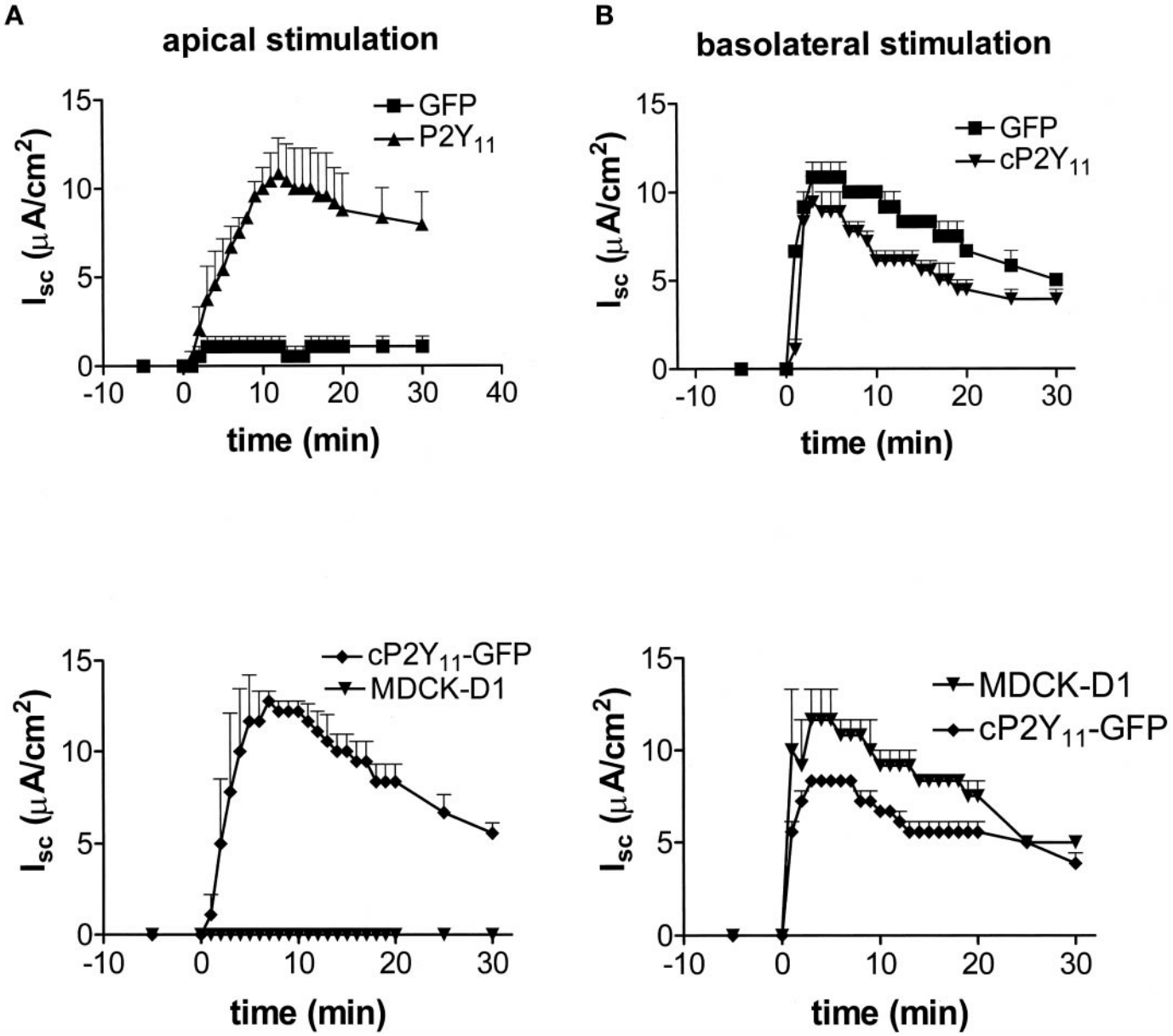


Fig. 8. Chloride secretory response induced by activation of cP2Y$_{11}$ receptors. Parental MDCK-D1 cells and MDCK-D1 cells expressing either cP2Y$_{11}$, cP2Y$_{11}$-GFP, or GFP alone were grown to confluence on transwell filters for 4 to 7 days and mounted in Ussing chambers. At zero time, cells were stimulated with 200 μM ADPβS added either apically (A) or basolaterally (B) and I_{sc} (μA/cm^2) monitored. Data are mean ± S.E., n = 4.

extent to which the cell's capacity to generate IPs exceeds what is needed to cause Ca^{2+} mobilization, as well as the fact that we assess total IPs, not just inositol 1,4,5-trisphosphate. Compartmentation of the IP signal with local mobilization of Ca^{2+} could also lead to an apparent dissociation between Ca^{2+} mobilization and global IP production in the cell cultures. With respect to the lesser sensitivity of cAMP accumulation, it is possible that CF2Th cells express an adenylyl cyclase isoform that is inhibited by high levels of Ca^{2+} (e.g., type V or VI), thus contributing to the lower apparent potency of agonists in promoting cAMP formation. This speculation is consistent with our preliminary studies of the expression of adenylyl cyclase isoforms in the cells.

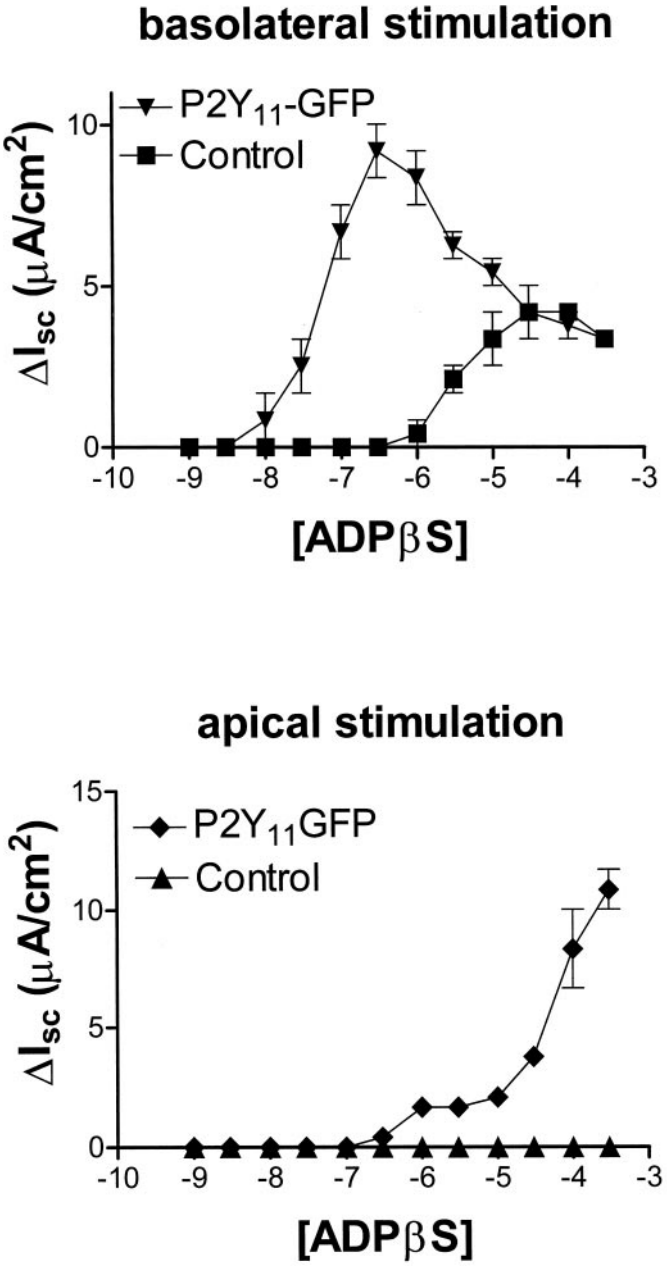


Fig. 9. Dependence of I_{sc} response on [ADPβS]. Parental MDCK-D1 cells and MDCK-D1 cells expressing $cP2Y_{11}$-GFP were mounted in Ussing chambers as described in the legend to Fig. 8 and cumulative concentration-dependence studies were performed with ADPβS, measuring the maximal change in I_{sc} at each concentration. Data are mean ± S.E., $n = 4$.

It is worth noting that Ca^{2+} transients activated by ADPβS stimulation remain elevated longer in time compared with transients activated by ADP. This may be caused by hydrolysis of ADP by extracellular nucleotidases that are unable to hydrolyze ADPβS as effectively. The increased potency and efficacy of ADPβS compared with ADP in all assessments of signaling could thus be caused in part by the extracellular stability of ADPβS. In any event, we conclude that the canine $P2Y_{11}$ does have a pharmacological specificity (ADPβS = 2 MT-ADP ~ 2 MT-ATP ≫ ADP > ATP) that differs from that of the human $P2Y_{11}$ receptor (ATP > 2 MT-ATP ≫ ADP ≥ 2 MT-ADP) (Communi et al., 1997).

Another focus of our study was to determine whether $cP2Y_{11}$ receptors target to a specific membrane domain in polarized cells. Our data in this regard reveal an interesting difference between endogenous and overexpressed receptors. When we stimulated the basolateral side of parental MDCK-D1 cells with ADPβS, we measured an increase in I_{sc}, whereas apical stimulation had no effect. This implies that $cP2Y_{11}$ receptors are targeted to the basolateral membrane, in agreement with the predominant localization of $cP2Y_{11}$-GFP receptors to the basolateral surface. However, MDCK-D1 cells in which $cP2Y_{11}$ and $cP2Y_{11}$-GFP receptors were overexpressed acquired apical responsiveness; that is, apical ADPβS now stimulated I_{sc}. This apical response was less sensitive, by about 3 orders of magnitude, than the basolateral response in the same cells. Leakage of ligand (apical to basolateral) does not explain the effect of apical ADPβS, because a high apical concentration of ligand (200 μM ADPβS) produces no response in control cells (see Fig. 8). Rather, the apical response is, apparently, a consequence of the overexpression of the $cP2Y_{11}$ receptor. Indeed, we do observe slight, but detectable, levels of fluorescence on the apical membranes of cells overexpressing $cP2Y_{11}$-GFP. Perhaps these receptors couple abnormally, producing the slow and less sensitive (but large) response to apical ADPβS that we observe. In our view, these data emphasize the dangers of drawing conclusions from studies of cells in which overexpression of proteins may alter normal cell physiology. By comparison, the preponderance of basolateral localization of $cP2Y_{11}$-GFP and the presence of only a basolateral stimulation of I_{sc} in control MDCK-D1 cells suggest that the native receptors are functionally compartmented to the basolateral domain. We speculate that overexpression of $cP2Y_{11}$ receptors results in mistargeting of receptors to the apical membrane, perhaps as a consequence of saturation of the basolateral membrane regions into which these receptors can be sorted.

A further effect of overexpression of $cP2Y_{11}$ receptors is an increase in the apparent potency of basolateral ADPβS by nearly 2 orders of magnitude. This suggests that receptor number limits the initial interaction of agonist with the cells and that another signaling component limits the extent of the I_{sc} response. The appearance of an inhibitory component of responsiveness (i.e., basolateral ADPβS first stimulates and then inhibits I_{sc}) suggests that overexpression produces an additional mode of coupling or modulation that does not occur in native MDCK-D1 cells.

In summary, we have cloned the canine $P2Y_{11}$ receptor from MDCK-D1 cells and expressed it in canine thymocyte (CF2Th) cells. Although $cP2Y_{11}$ and $hP2Y_{11}$ receptors share ~70% amino acid identity, they have strikingly different

pharmacological selectivities that are most likely attributable to differences in amino acids located in TMH6, which have been implicated in nucleotide binding and stabilization. The $cP2Y_{11}$ receptor couples to G_q and G_s-linked pathways when expressed in CF2Th cells, suggesting that the $P2Y_{11}$ receptor is responsible for the cyclooxygenase-*independent* stimulation of cAMP formation by nucleotides previously observed in MDCK-D1 cells (Post et al., 1998). The stimulation of a short circuit current by basolateral but not apical ADPβS in native MDCK-D1 cells implies that $P2Y_{11}$ receptors target to the basolateral membrane in parental MDCK-D1 cells, a result supported by confocal microscopic assessment of the predominant localization of GFP-tagged receptors. However, overexpression of $cP2Y_{11}$ or $cP2Y_{11}$-GFP receptors results in an enhanced I_{sc} response to basolateral stimulation and a response to apical ADPβS; the apical response possibly corresponds to the small apical fluorescent signal seen with $cP2Y_{11}$-GFP expression. Overexpression of $cP2Y_{11}$ receptors also alters the sensitivity and shape of the concentration-dependence relationship of I_{sc} to basolateral addition of ADPβS. The data thus support the basolateral localization of $P2Y_{11}$ receptors and receptor coupling to I_{sc} in MDCK-D1 cells, except in cases in which receptors are overexpressed; receptor overexpression leads to altered sensitivities, modes, and sites of coupling to physiologic responses.

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