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Molecular and Functional Characterization of Human $P2X_2$ Receptors

KEVIN J. LYNCH, EDWARD TOUMA, WENDE NIFORATOS, KAREN L. KAGE, EDWARD C. BURGARD, TIM VAN BIESEN, ELIZABETH A. KOWALUK, and MICHAEL F. JARVIS

Neurological and Urological Diseases Research, Abbott Laboratories, Abbott Park, Illinois

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ABSTRACT

P2X receptors are a family of ATP-gated ion channels. Four cDNAs with a high degree of homology to the rat $P2X_2$ receptor were isolated from human pituitary and pancreas RNA. Genomic sequence indicated that these cDNAs represent alternatively spliced messages. Northern analysis revealed high levels of human $P2X_2$ ($hP2X_2$) message in the pancreas, and splice variants could be detected in a variety of tissues. Two cDNAs encoded functional ion channels when expressed in *Xenopus* oocytes, a receptor structurally homologous to the prototype rat $P2X_2$ receptor (called $hP2X_{2a}$) and a variant containing a deletion within its cytoplasmic C terminus (called $hP2X_{2b}$). Pharmacologically, these functional human $P2X_2$ receptors were virtually indistinguishable, with the P2X receptor agonists ATP, 2-methylthio-ATP, 2′ and 3′-*O*-(4-benzoylbenzoyl)-ATP, and ATP5′-*O*-(3-thiotriphosphate) being approximately equipotent (EC_{50} = 1 μM) in eliciting extracellular Ca^{2+} influx. The P2 receptor agonists α,β-methylene ATP, adenosine, adenosine 5′-*O*-(2-thiodiphosphate), and UTP were inactive at concentrations up to 100 μM. Both $hP2X_{2a}$ and $hP2X_{2b}$ receptors were sensitive to the P2 receptor antagonist pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid (IC_{50} = 3 μM). In contrast to the analogous rat $P2X_2$ and $P2X_{2b}$ receptors, the desensitization rates of the $hP2X_{2a}$ and $hP2X_{2b}$ receptors were equivalent. Both functional forms of the human $P2X_2$ receptors formed heteromeric channels with the human $P2X_3$ receptor. These data demonstrate that the gene structure and mRNA heterogeneity of the $P2X_2$ receptor subtype are evolutionarily conserved between rat and human, but also suggest that alternative splicing serves a function other than regulating the desensitization rate of the human receptor.

Extracellular nucleotides have been found to modulate a variety of physiological processes. ATP evokes fast excitatory responses from neuronal cells, affects vascular tone, and may act as an autocrine and/or paracrine agent in the neuroendocrine system (Ralevic and Burnstock, 1998). The effects of ATP are mediated through two classes of cell surface receptors, a G protein-coupled, heptahelical family called P2Y receptors, and a family of ligand-gated ion channels called P2X (Abbracchio and Burnstock, 1994; Fredholm et al., 1997).

There are seven known members of the P2X family of ATP-gated ion channels cloned from rat tissues, designated $P2X_{1-7}$. These receptor subtypes differ with respect to tissue distribution, as well as to pharmacologic and kinetic profiles (Brake et al., 1994; Valera et al., 1994; Bo et al., 1995; Chen CC et al., 1995; Collo et al., 1996; Surprenant et al., 1996). The P2X receptor proteins share ~30 to 50% sequence identity and are 379 to 595 amino acids in length. Structurally, the receptors are characterized by two transmembrane (TM) domains with both amino and carboxyl ends being located cytoplasmically (Brake et al., 1994; Valera et al., 1994). The extracellular domains of these receptors are glycosylated and contain 10 conserved cysteine residues that participate in intrachain disulfide linkages (Newbolt et al., 1998). When expressed in heterologous systems, the P2X receptors form nonselective cation channels with differing sensitivities to ATP and other P2 receptor agonists, as well as to antagonists such as pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and suramin (reviewed in Soto et al., 1997). The receptors fall into two categories with respect to channel kinetics, with $P2X_1$ and $P2X_3$ forming relatively fast-desensitizing channels, and $P2X_2$, $P2X_4$, and $P2X_7$ forming channels having a slower rate of desensitization (Soto et al., 1997). Although expression of a single P2X subtype in heterologous systems is sufficient to create functional ion channels, it has been demonstrated that subunits from different P2X subtypes can associate to form heteromeric channels with unique characteristics (Lewis et al., 1995; Lê et al., 1998, 1999; Torres et al., 1998). Evidence has been most convincing for the association of $P2X_2$ and $P2X_3$ subunits,

ABBREVIATIONS: TM, transmembrane; α,β-meATP, α,β-methylene ATP lithium salt; PPADS, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid; 2-meSATP, 2-methylthio-ATP tetrasodium; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate) tetralithium salt; BzATP, 2′ and 3′-*O*-(4-benzoylbenzoyl)-ATP tetraethylammonium salt; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate) trilithium salt; GSP, gene-specific primer; fluo-3-AM, fluo-3-acetoxymethyl ester; RT-PCR, reverse transcription-polymerase chain reaction; kb, kilobase; RACE, rapid amplification of cDNA ends.

with the properties of the resulting heteromeric channel closely resembling those of native channels found in small-diameter nociceptive neurons of sensory ganglia (Lewis et al., 1995; Thomas et al., 1998).

The $P2X_2$ receptor subtype originally was isolated from PC12 rat pheochromocytoma cells (Brake et al., 1994). It has been found to be expressed in a variety of organs of the vascular, nervous, neuroendocrine, and sensory systems. Alternatively spliced messages for the $P2X_2$ receptor have been isolated from rat and guinea pig tissue, and functional studies have suggested that alternative splicing of the receptor serves to modulate the response of the receptor to prolonged agonist exposure (Housley et al., 1995; Brandle et al., 1997; Simon et al., 1997; Koshimizu et al., 1998b; Parker et al., 1998).

This report describes the isolation of novel human P2X receptor cDNAs. Based on structure as well as on pharmacologic profiles, it is concluded that they represent human homologs of the $P2X_2$ receptor. The human $P2X_2$ gene, as well as several splice variants of the receptor message, has been isolated, and the tissue expression and functional activity of these variants have been characterized.

Experimental Procedures

Materials. ATP, 2-methylthio-ATP tetrasodium (2-meSATP), $\alpha\beta$-methylene ATP dilithium (α,β-meATP), suramin hexasodium, and PPADS were obtained from Research Biochemicals International (Natick, MA). 2′ and 3′-*O*-(4-benzoylbenzoyl)-ATP tetraethylammonium salt (mixed isomers, BzATP), ADP5′-*O*-(3-thiotriphosphate) tetralithium salt (ATPγS), ADP, and UTP were obtained from Sigma Chemical Co. (St. Louis, MO). ADP5′-*O*-(2-thiodiphosphate) trilithium salt (ADPβS) and G418 sulfate were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Dulbecco's modified Eagle's medium (with 4.5 mg/ml glucose and 4 mM L-glutamine) and fetal bovine serum were obtained from Hyclone Laboratories, Inc. (Logan, UT). Dulbecco's PBS (with 1 mg/ml glucose and 3.6 mg/l Na pyruvate, without phenol red), hygromycin, Lipofectamine, restriction enzymes, and buffers were obtained from Gibco BRL Life Technologies (Gaithersburg, MD). Fluo-3-acetoxymethyl ester (fluo-3-AM) was obtained from Molecular Probes (Eugene, OR). α-[^{32}P]UTP was obtained from Amersham, Inc. (Arlington Heights, IL).

Identification of Human $P2X_2$ Expressed Sequence Tag and Generation of Partial cDNA Clones. The TBLASTN database search tool (National Center for Biotechnology Information, Washington, DC) (Altschul, 1993) was used with the predicted amino acid sequence of the rat $P2X_2$ receptor (GenBank accession no. 1352688) to search the Lifeseq database (Incyte Pharmaceuticals, Inc., Palo Alto, CA) for human DNA sequences that would code for similar polypeptides. A sequence was identified from a cDNA clone derived from human fetal colon tissue that encoded a polypeptide with homology to a region of the rat $P2X_2$ receptor.

Primers were designed to the noncoding sequence of this cDNA to enable 5′-rapid amplification of cDNA ends (RACE) procedures in an attempt to identify the missing coding sequence. Using polyA+ RNA derived from human pituitary tissue (Clontech, Palo Alto, CA), 5′-RACE reactions were performed using the RACE system (Gibco BRL Life Technologies) and nested amplifications using the supplied primers in combination with the following gene-specific primers (GSP): GSP1, 5′-ATGAATGTTAGCAAGATCCA-3′; GSP2, 5′-CAUCAUCAUCAUCACCCCGACGGAAGTCAGAG3-′; GSP3, 5′-CCTGTCCATGCACAATGACG-3′. Products were cloned into the vector pCRII using the TA cloning system (Invitrogen, Carlsbad, CA) and sequenced using the PRISM dye terminator reagents and an ABI 310 genetic analyzer (Perkin-Elmer Applied Biosystems Division, Foster City, CA).

Isolation of the Human $P2X_2$ Gene. A polymerase chain reaction (PCR) primer pair was designed and synthesized based on the sequence compiled from the Incyte (Incyte Pharmaceuticals, Inc.) clone and partial 5′-RACE product, which yielded a product of 339 bases when amplifying human genomic DNA (sense, 5′-TCCTTCCTGTGCGACTGGATCTTG-3′; antisense, 5′-CAAACCTTTGGGGTCTGTGGGTG-3′). Using these primers, a P1 bacteriophage library screen was performed (Genome Systems, Inc., St. Louis, MO). Two clones containing the human $P2X_2$ gene were obtained and sequenced as above. The P1 phagemid DNAs were prepared for sequencing with Qiagen (Chatsworth, CA) Maxiprep plasmid purification system using a protocol supplied by the manufacturer. To facilitate sequencing, one of the clones was digested with the restriction enzyme *Bam*HI and subcloned into the pBluescript II SK+ vector using the pCRscript cloning system (Stratagene, La Jolla, CA).

Isolation of Human $P2X_2$ cDNAs. Using the genomic sequence information surrounding the predicted initiation and termination codons of the human $P2X_2$ message, oligonucleotide primers were designed and synthesized to enable reverse transcription (RT)-PCR of the intact open reading frame of the mRNA. A consensus translation initiation signal was designed into the sense primer to optimize expression (Kozak, 1984). First-strand cDNA was synthesized from polyA+ RNA derived from pituitary gland tissue (1 μg; Clontech), using 10 pmol random hexamer primer, and Superscript II reverse transcriptase (Gibco BRL Life Technologies). A proofreading thermostable polymerase (cloned *Pfu* DNA polymerase; Stratagene) was used in the amplification to ensure high-fidelity amplification. The reaction mixture consisted of: 2 μl of cDNA, 10 pmol of each primer; $hP2X_2$ sense (5′-CCACCATGGCCGCCGCCCAGCCCAAGTA-3′); $hP2X_2$ antisense (5′-GGAAAGGAGCTCAGAGTTGAGCCAAACC-3′); and 1× *Pfu* reaction buffer and 200 μM 2′-deoxynuceleoside 5′-triphosphates. The reaction was preheated to 80°C before addition of the polymerase, after which the reaction was cycled 35 times under these conditions: 94°C for 15 s, 60°C for 20 s, and 72°C for 5 min. After cycling, the reaction was incubated for 10 min at 70°C. The reaction products were separated on a 0.8% agarose gel and products of ~1.5 kilobases (kb) were excised and purified via the Qiaquick (Qiagen) gel purification system. The products were subcloned into the pCRscript II+ vector and sequenced. To create the $hP2X_{2a}$ message, an internal *Kpn*I site was used to splice nucleotides 1 to 666 of $hP2X_{2d}$ with nucleotides 595 to 1349 of $hP2X_{2c}$. The coding sequences of the clones were found to be identical with the predicted exons of the human $P2X_2$ genomic sequence isolated from the P1 library.

Northern Analysis. An α-[^{32}P]UTP radiolabeled antisense RNA probe was synthesized from the $hP2X_{2a}$ message using the T3 promoter of pCRscript vector and reagents from the Maxiscript in vitro transcription kit (Ambion, Inc., Austin, TX). A commercially prepared membrane Multiple Tissue Northern blot (Clontech) was probed overnight at 72°C in hybridization buffer (NorthernMax; Ambion). The blot was washed at 72°C in high-stringency buffer (NorthernMax). The hybridized membranes were analyzed on a Storm phosphoimager (Molecular Devices, Sunnyvale, CA).

RT-PCR Analysis of Tissues. PolyA+ RNAs from human tissues were obtained from Clontech. RNA (0.5 μg) was used in random hexamer-primed reverse transcriptase reactions using Thermoscript RT-PCR reagents (Gibco BRL Life Technologies). PCR primers were designed corresponding to nucleotides 2246 to 2269 (sense) and 3733 to 3710 (antisense) of the $hP2X_2$ gene. One-tenth of each RT reaction was used in PCR amplification reactions that also included 10 pmol of each primer, 1.5 mM $MgSO_4$, 200 μM 2′-deoxynucleoside 5′-triphosphates, 1× PCR× amplification buffer (Gibco BRL Life Technologies), 0.5 × PCR× enhancer (Gibco BRL Life Technologies), and 2.5 U platinum *Taq* polymerase (Gibco BRL Life Technologies). After initial incubation at 95°C for 5 min, the reactions were cycled 35 times under the following conditions: 94°C for 15 s, 55°C for 20 s, and 68°C for 1 min. The products were analyzed by agarose gel electro-

phoresis. To identify the products, they were isolated, purified (Qiaquick; Qiagen), and directly sequenced.

Cloning of the Rat $P2X_2$ and Human $P2X_3$ Receptors. The rat $P2X_2$ and $P2X_{2b}$ receptor cDNAs were isolated through RT-PCR reactions from oligo(dT)-primed cDNA synthesized from total brain polyA+ RNA (Clontech). Primers were designed based on the published sequence for the rat $P2X_2$ receptors (GenBank accession nos.: rat $P2X_2$, U14114; rat $P2X_{2b}$, Y10473). The primers used were $rP2X_2$ sense, 5′-CACCATGGTCCGGCGCTTGGCCCGGGGC-3′; $rP2X_2$ antisense, 5′-TCAAAGTTGGGCCAAACCTTTGGGGTCCG-3′. The PCR amplification was set up using *Pfu* polymerase essentially as described above, and the reactions were cycled 35 times under the following conditions: 94°C for 20 s, 65°C for 20 s, and 72°C for 4 min. Reaction products were separated by agarose gel electrophoresis, and the major products of approximately 1.5 and 1.3 kb were isolated and cloned into the pCRscript vector. Inserts were sequenced and found to be identical with the published sequences for the rat $P2X_2$ (Brake et al., 1994) and $P2X_{2b}$ receptors (Simon et al., 1997).

For the human $P2X_3$ cloning, PCR reactions were performed on oligo(dT)-primed cDNA derived from polyA+ RNA of human pituitary tissue using primers (sense, CACCATGAACTGCATATC CGACTTC; antisense, CTAGTGGCCTATGGAGAAGGC) and *Pfu* polymerase essentially as described above for the full-length $P2X_2$ receptor. The amplification conditions were 94°C for 15 s, 52°C for 20 s, and 72°C for 3.5 min. The major product of 1.2 kb was subcloned into pCRscript, and several clones were sequence-verified. The sequence was found to be essentially identical with that reported by (Soto et al., 1997) (GenBank accession no. Y07683). The exception is at amino acid residue 126, where arginine was encoded; the published sequence encodes proline at this position.

Electrophysiology of $hP2X_2$ Receptors. Adult female frogs (*Xenopus laevis*) were anesthetized with 0.2% tricaine, and sections of one ovary were removed. Oocytes were denuded of overlying follicle cells by agitation for 1 to 2 h in low-Ca^{2+} Barth's solution containing 88 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, and 10 mM Na-HEPES (pH 7.55) plus 2 mg/ml collagenase (Sigma Chemical Co.; type IA) and antibiotics. Selection of stage V and VI oocytes was begun after ~50% of the cells were denuded. Oocytes were maintained at 16 to 19°C in normal Barth's solution containing 90 mM NaCl, 1.0 mM KCl, 0.66 mM $NaNO_3$, 0.74 mM $CaCl_2$, 0.82 mM $MgCl_2$, 2.4 mM $NaHCO_3$, 2.5 mM Na-pyruvate, 10 mM Na-HEPES (pH 7.55) plus antibiotics. Cytoplasmic injections of 50 nl of 1 ng/nl cRNA or intranuclear injections of 12 nl of 1 ng/nl cDNA encoding $hP2X_2$ receptors were given within 24 h of preparation. For $hP2X_2$ and $hP2X_3$ coinjections ($hP2X_{2/3}$), equal concentrations of both cRNAs were premixed before a single 50-nl injection. The standard recording solution contained 96 mM NaCl, 2.0 mM KCl, 1.8 mM $BaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM Na-pyruvate, and 5.0 mM Na-HEPES (pH 7.4). For long agonist applications, the recording solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, and 10 mM Na-HEPES (pH 7.3) (Brandle et al., 1997). Ca^{2+}-free saline was used to minimize the contribution of endogenous calcium-dependent chloride channels to the ATP response.

Two-electrode voltage-clamp recordings were performed 1 to 5 days after injection. Electrodes (1–2 MΩ) were pulled from borosilicate glass and filled with 120 mM KCl. Responses to ATP and α,βme-ATP were routinely recorded while the oocyte membrane was voltage-clamped at −60 mV. Currents were recorded using an Axo-Clamp 2A amplifier, digitized, and analyzed using pClamp software (Axon Instruments, Foster City, CA). Oocytes were perfused with recording solution at a rate of 3.5 ml/min. Agonists were applied using a solenoid-driven drug application pipette positioned close to the oocyte in the perfusion chamber. To investigate possible differences in desensitization among receptor subtypes, voltage-clamped oocytes were exposed to 300 μM ATP for 60 s. Desensitization was quantified as the percentage current remaining at the end of the application relative to peak current amplitude. Desensitization time constants (τ) were calculated using a Chebyshev curve-fitting algorithm in pClamp software (Axon Instruments). For antagonist studies, the P2X receptor antagonist PPADS was bath-applied for at least 2 min before being coapplied with agonist through the drug pipette.

Receptor Expression in Mammalian Cells. Human $P2X_2$ cDNAs were transferred into the mammalian expression vector pIRES(hyg) (Clontech) using a *Not*I and partial *Bam*HI restriction digest of the cDNA in pCRscript and the corresponding sites of the pIRES vector. The resulting plasmids were sequenced and introduced into the 1321N1 human astrocytoma cells via transfection with Lipofectamine (Gibco BRL Life Technologies). Stable transformants were selected by resistance to hygromycin. Surviving individual colonies were isolated and screened for P2 receptor activity. The clones exhibiting the largest ATP-induced response were selected for additional characterization. Cells expressing recombinant P2X receptors were maintained at 37°C in Dulbecco's modified Eagle's medium with 4.5 mg/ml^{-1} glucose and 4 mM L-glutamine), 10% fetal bovine serum, and 100 $\mu g/ml^{-1}$ hygromycin in a humidified 5% CO_2 atmosphere.

Ca^{2+} Flux Measurements. The pharmacologic characterization of the $P2X_2$ receptors was performed on the basis of agonist-mediated increases in cytosolic calcium concentration. A fluorescent calcium-chelating dye (fluo-3) was used as an indicator of the relative levels of intracellular calcium in a 96-well format using the Fluoresence Imaging Plate Reader (Molecular Devices, Sunnyvale, CA). Cells were grown to confluence and loaded with the fluo-3-AM (2 μM) in Dulbecco's PBS for 1 to 2 h at room temperature. Before the assay, each 96-well tissue culture plate was washed three times with Dulbecco's PBS to remove extracellular fluo-3-AM. Agonists were added 10 s after the start of the experimental run. Antagonists were added 10 s after the start of the experimental run, followed by agonists after a 180-s interval (t = 190 s). Fluorescence data were collected at 1- to 5-s intervals throughout each experimental run. Dose–response data were analyzed using a four-parameter logistic Hill equation in GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Results

In an attempt to identify novel human P2X receptors, a search was performed on the Lifeseq (Incyte Pharmaceuticals, Inc.) database using the rat $P2X_2$ amino acid sequence as a query. The search identified one entry that had significant homology to the query and no identity to other known human or rat P2X receptors. Because the position of this sequence with respect to that of the rat $P2X_2$ sequence predicted that this was a partial cDNA, a series of antisense primers were designed to enable identification of the balance of the predicted open reading frame. The sequence was extended using 5′-RACE reactions. However, this approach was unsuccessful at generating products with open reading frames encoding a full-length P2X receptor. Hybridization screens of cDNA libraries also did not identify clones containing sequences encoding the N terminus of the receptor.

The human $P2X_2$ receptor gene was isolated by a PCR screen of a P1 phagemid library in an attempt to characterize the remaining receptor coding sequence. As shown in Fig. 1, the human $P2X_2$ gene contains 11 exons, retaining the structural rules for intron/exon boundaries (Mount, 1982). The gene structure is highly conserved between human and rat (Brandle et al., 1997).

Based on the human $P2X_2$ genomic sequence and on the size and structure of the rat $P2X_2$ open reading frame, primers were designed that encompassed the initiation and termination codons of the putative human $P2X_2$ receptor message. RT-PCR reactions were performed using polyA+-

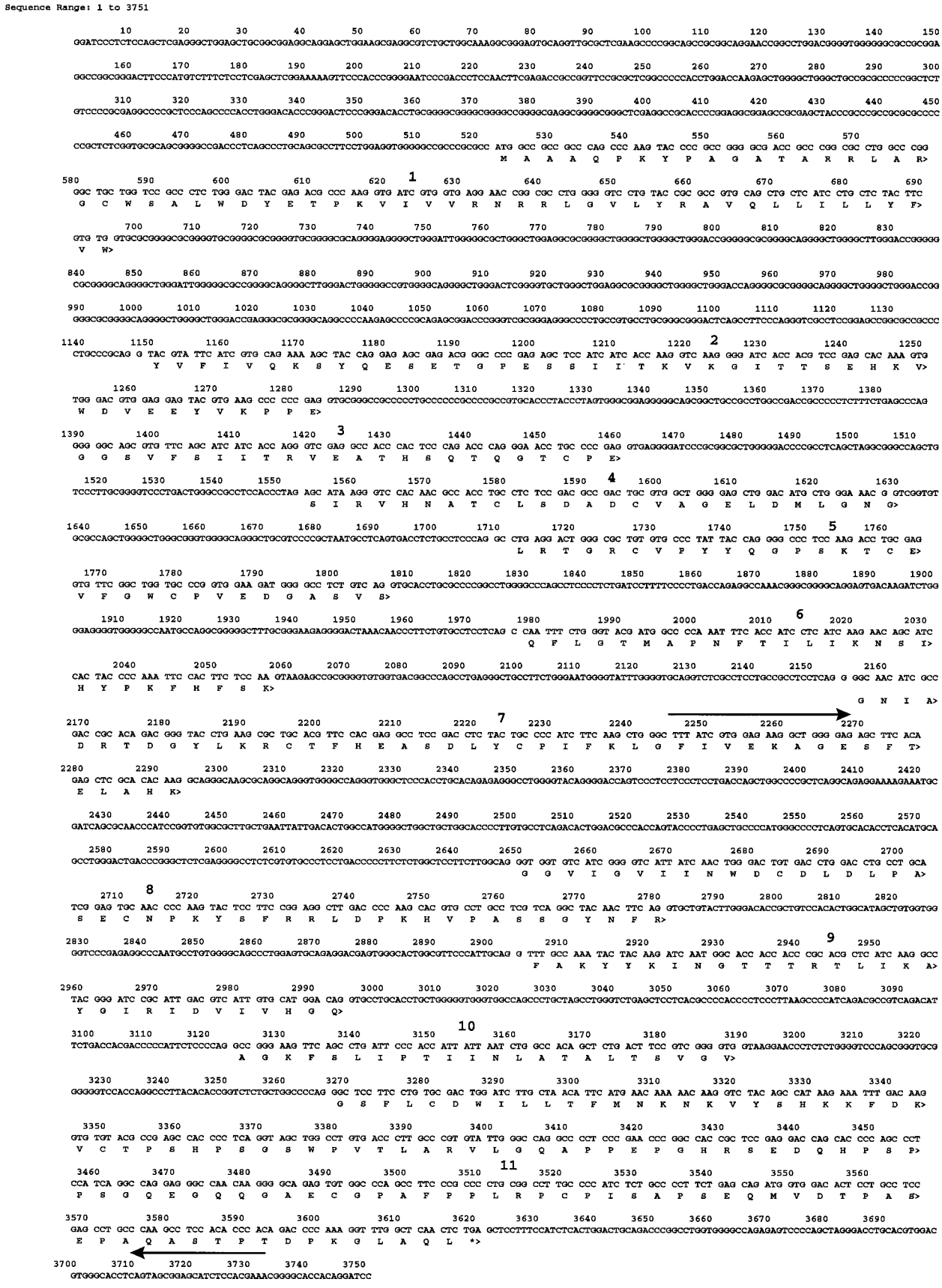

Fig. 1. Genomic organization of the human $P2X_2$ gene. Eleven exons are blocked to phase, whereas the amino acid sequence is shown below the corresponding nucleotide sequence. The positions of the primers used in RT-PCR analysis of tissues are indicated by arrows. The sequence has been deposited into the GenBank database under accession no. AF190826.

enriched RNA from human pituitary tissue, generating a series of products from 1.2 to 1.4 kb in length. These products were cloned and screened by sequencing. Three predominant messages were isolated initially. These sequences were compared with the $P2X_2$ genomic structure to deduce the origin of the structure and to verify the sequence. One message (called $P2X_{2b}$) is formed through the use of an internal splice site within exon 11, deleting a 201-bp sequence from the coding sequence (Fig. 2A). This deletion is in the identical position with that of the functional splice variant shown to be produced from the rat $P2X_2$ gene (Brandle et al., 1997). The second variant (called $hP2X_{2c}$) is formed by the in-frame deletion of exon 3 from the coding sequence (Fig. 2B). The third variant (called $hP2X_{2d}$) is formed from the inclusion of intron 10 in the message (Fig. 2C). Because the intron continues the open reading frame, this results in a 26-amino acid insertion within the predicted second TM region of the receptor. This variant also has been detected in the rat; however, the rat intron contains a termination codon, thereby encoding a truncated receptor (Housley et al., 1995). Several other $P2X_2$ cDNAs were isolated that contained deletions of several exons, resulting in drastically truncated open reading frames (data not shown). Based on the lack of function of the $hP2X_{2c}$ form (see below), it was deemed unlikely that these cDNAs would encode functional receptors and they were not characterized further.

Initial cloning efforts failed to identify a message encoding a receptor homologous to the prototypic rat $P2X_2$ receptor, e.g., that having an intact C-terminal tail and no other deletions or insertions in exon sequences as predicted from the genomic sequence (Brake et al., 1994). To determine whether such a polypeptide would create a functional ATP-gated ion channel, we constructed a cDNA using portions of two of the $hP2X_2$ variants. This message, called $hP2X_{2a}$, was generated by splicing the 5′ half of the $hP2X_{2d}$ message to the 3′ end of the $hP2X_{2c}$ message, using a common *Kpn*I restriction enzyme site. The resulting message encodes a 471-amino acid polypeptide with 68% identity with the prototype rat $P2X_2$ receptor (Fig. 3). The sequence identity of the predicted extracellular domains of the human and rat $P2X_2$ receptors is especially high. Within this region of the human polypeptide are three consensus sites for *N*-linked glycosylation at residues 133, 194, and 310. There also are three sites in the rat sequence, and two of these (at positions 194 and 310) are conserved positionally between the species. The N-terminal cytoplasmic domain of the human $P2X_{2a}$ receptor is 12 amino acids longer than the rat homolog, but it is similar in length and sequence to the guinea pig $P2X_2$ receptor (human, MAAAQPKYPAGATAR. . . and guinea pig, MAATHPKAPTAQRLR. . .) (Parker et al., 1998). Conversely, the C- terminal tail of the human $P2X_{2a}$ receptor

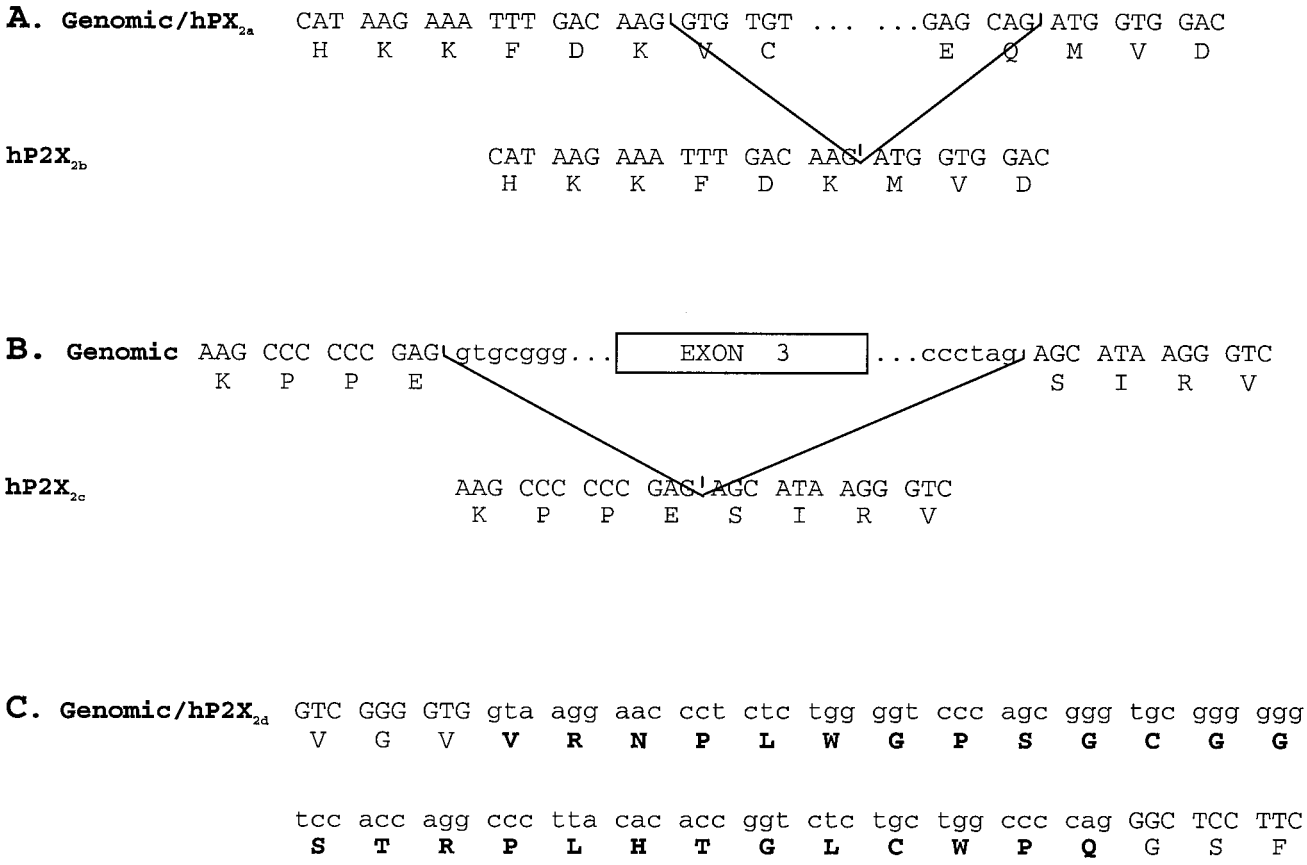

Fig. 2. Splice variants of the human $P2X_2$ message. A, the $hP2X_{2b}$ variant formed by splice donor and acceptor sites lying within exon 11. The resulting message removes 201 bp (67amino acids) from the C-terminal domain of the receptor. B, removal of exon 3 from the $P2X_2$ message forms the $hP2X_{2c}$ variant. This deletes 24 amino acids from the putative extracellular domain of the receptor. C, the $hP2X_{2d}$ form is created by inclusion of intron 10 in the mRNA (lower case nucleotide sequence). This creates an insertion of 26 amino acids to the predicted polypeptide sequence (bold) in the predicted second TM region of the receptor. The sequences have been deposited into the GenBank database under accession numbers AF190822 hP2X2a, AF190823 hP2X2b, AF190824 hP2X2c, and AF190825 hP2X2d.

lacks 12 amino acids when compared with the rat and guinea pig receptors.

Northern analysis using a radiolabeled $P2X_2$ riboprobe detected a major mRNA species of ~2.4 kb in the pancreas (Fig. 4A). Extended exposures of the membrane detected faint bands of the same size in heart and brain (data not shown). Another faint band of ~5.0 kb was detected in all tissues except lung. A third, larger species was detected in the brain. Several tissues were analyzed by RT-PCR to examine distribution of the various $P2X_2$ splice variants. A set of primers was designed to flank exons 10 and 11 (see Fig. 1). This allowed identification of the $hP2X_{2b}$, $hP2X_{2d}$, and $hP2X_{2a/c}$ products (products arising from the $hP2X_{2a}$ and $hP2X_{2c}$ form are identical). Results shown in Fig. 4B indicate that four expected primary products were obtained in all tissues except spinal cord, from which only two products were amplified. Direct sequencing of the products determined that the following were present (in descending order of size): the $hP2X_{2d}$ form (879 bp), the $hP2X_{2a/c}$ form (801 bp), and the $hP2X_{2b}$ form (600 bp). The primary products detected in the spinal cord were derived from the $hP2X_{2d}$ and $hP2X_{2b}$ messages.

Four human and two rat $P2X_2$ messages were injected into *Xenopus* oocytes for functional characterization. Short (10 s) applications of 10 to 30 μM ATP were used for current–voltage relationship and antagonist studies. These applications produced nondesensitizing currents in oocytes expressing either the $hP2X_{2a}$ or the human $hP2X_{2b}$ receptor splice variant. Current–voltage relationships in oocytes expressing either $hP2X_{2a}$ ($n = 5$) or $hP2X_{2b}$ ($n = 3$) receptors were inwardly rectifying and reversed near 0 mV (Fig. 5C), indicating activation of a nonselective cation conductance. The P2X receptor antagonist PPADS was used to determine antagonist sensitivity of the $hP2X_{2a}$ and $hP2X_{2b}$ receptors. PPADS (10 μM) completely blocked ATP-evoked responses in oocytes expressing either $hP2X_{2a}$ ($n = 3$) or $hP2X_{2b}$ ($n = 2$) receptors, but had poor reversibility during wash. A high concentration of the ATP analog α,βme-ATP (100 μM) produced no inward currents in oocytes injected with either receptor subtype, consistent with previous reports for the rat $P2X_2$ receptor (Brake et al., 1994). In contrast to $hP2X_{2a}$ and $hP2X_{2b}$ receptors, ATP (30 μM) failed to evoke a current response in oocytes injected with $hP2X_{2c}$ or $hP2X_{2d}$ cRNA.

Longer applications (60 s) of a high concentration of ATP (300 μM) elicited slowly desensitizing inward currents in oocytes expressing either $hP2X_{2a}$ (desensitization $\tau = 106 \pm 27$ s; $n = 10$) or $hP2X_{2b}$ ($\tau = 102 \pm 11$ s; $n = 9$) (Fig. 5A). At the end of the 60-s ATP application, current had decayed to $84 \pm 2\%$ of peak ($n = 11$) for $hP2X_{2a}$, and to $84 \pm 1\%$ of peak ($n = 11$) for $hP2X_{2b}$ receptors. Similarly, applications of 300 μM ATP for 60 s to oocytes expressing prototype rat $P2X_2$ receptors produced slowly desensitizing responses ($\tau = 115 \pm 21$ s; $n = 5$), which decayed to $65 \pm 3\%$ of peak ($n = 5$) by the end of the application. However, as described previously (Simon et al., 1997), rat $P2X_{2b}$ receptors exhibited more rapid ($\tau = 17 \pm 1$ s; $n = 6$) and complete ($14 \pm 1\%$ of peak; $n = 6$) desensitization (Fig. 5B). Although the human $P2X_{2b}$ form is the structural homolog of the rat $P2X_{2b}$ receptor (see Fig. 3), obvious differences exist with respect to the rate and extent of desensitization.

In addition to characterization of homomeric $hP2X_2$ receptor channels, the ability of $hP2X_{2a-d}$ to form heteromeric receptors with $hP2X_3$ was investigated. Expression of the

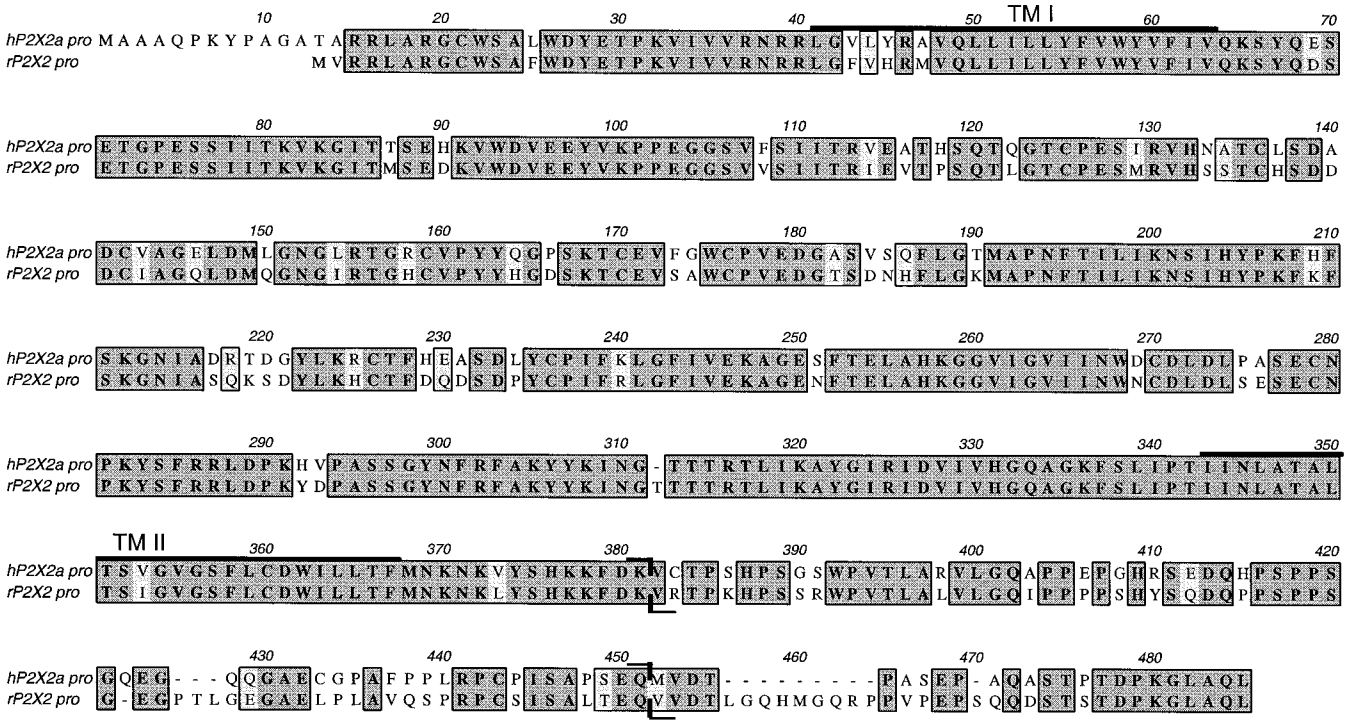

Fig. 3. Alignment of the human and rat prototypic $P2X_2$ receptor polypeptides. Analysis performed using the ClustalW algorithm and the BLOSUM 30 scoring matrix in MacVector 6.0 (Oxford Molecular Group, Oxford, UK). Identical residues are blocked and darkly shaded, and similar residues are blocked and lightly shaded. Predicted TM domains are illustrated by solid bars. The boundaries of the region deleted in the $hP2X_{2b}$ form of each species are illustrated by vertical bars ( ).

heteromeric receptor was confirmed by the appearance of a new phenotypic receptor having characteristics of both $hP2X_2$ and $hP2X_3$ receptors. α,β-meATP was used as an agonist at these receptors to eliminate activation of any $hP2X_2$ homomeric receptors that also may have been expressed. In oocytes expressing either $hP2X_{2a/3}$ ($n = 3$) or $hP2X_{2b/3}$ ($n = 3$) receptors, α,β-meATP (10 μM) evoked inward currents that did not desensitize in the presence of agonist (Fig. 5D), consistent with activation of the heteromeric receptor. For $hP2X_{2c/3}$- and $hP2X_{2/3}$-coinjected oocytes, α,β-meATP evoked rapidly desensitizing inward currents that were indistinguishable from $hP2X_3$-mediated currents (data not shown), indicating that neither a functionally active $hP2X_2$ nor a functionally active heteromeric $hP2X_{2/3}$ receptor was formed in these cells.

Pharmacologic characterization of the functional $hP2X_2$ receptors was performed using Ca^{2+} influx measurements of 1321N1 human astrocytoma cells stably expressing either the $hP2X_{2a}$ or the $hP2X_{2b}$ subtype. Native 1321N1 cells have been shown previously to be unresponsive to extracellular nucleotides (Parr et al., 1994). Figure 6A shows representative fluorimetric traces of untransfected 1321N1 cells, as well as these cells stably expressing either the $hP2X_{2a}$ or the $hP2X_{2b}$ receptor in response to 10 μM ATP. Untransfected 1321N1 cells show no endogenous response to ATP, indicating a lack of functional P2X or P2Y response in this cell type. The pharmacologic profiles of cells expressing the $hP2X_{2a}$ or $hP2X_{2b}$ subtype were found to be virtually identical, as shown in Fig. 6 and Table 1. The agonists ATP, BzATP, ATPγS, and 2-meSATP all had approximately the same potency toward either receptor form, with apparent EC_{50} values of ~1 μM (Table 1). ADPβS was weakly active for both receptors, with an EC_{50} of 30 to 40 μM. The other ATP analogs, as well as UTP, were inactive. The P2 antagonist PPADS was active, with an IC_{50} value of 5 μM. Suramin was a poor antagonist for either human $P2X_2$ receptor variant in 1321N1 cells, as measured by either fluorimetric means ($IC_{50} > 100$ μM) or by electrophysiologic recordings ($IC_{50} > 50$ μM). However, in electrophysiologic recordings from oocytes, suramin was found to be a functional antagonist for both $hP2X_{2a}$ and $hP2X_{2b}$ receptors ($IC_{50} = 6$ μM). The reason for this apparent cell-specific effect on suramin potency at this

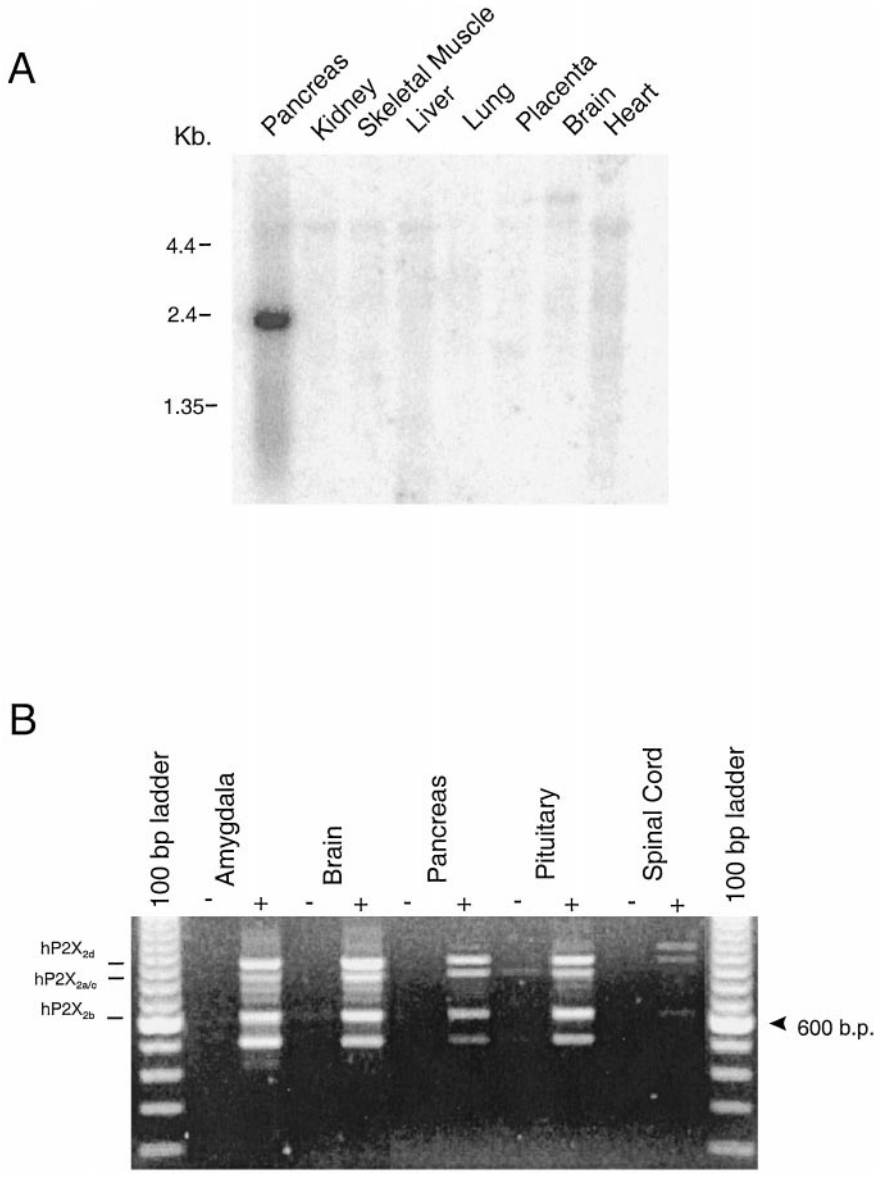

Fig. 4. Tissue distribution of human $P2X_2$ RNA. A, Northern analysis using the full-length $hP2X_{2a}$ probe. Size markers shown are shown on the left and expressed in kilobases (Kb.). B, RT-PCR of various human tissues with $P2X_2$-specific primers. Reactions were performed with (+) or without (−) reverse transcriptase. The products arising from the $hP2X_{2d}$, $hP2X_{2b}$, and $hP2X_{2a/c}$ are labeled. A 100-bp ladder (Gibco BRL Life Technologies) is indicated by the high-intensity 600-bp band.

receptor is unclear. In all other respects, the human $P2X_2$ receptors exhibited a similar rank order potency of prototypic P2 agonists and activity of PPADS to that observed previously for the rat $P2X_2$ subtype (Brake et al., 1994; Bianchi et al., 1999).

Discussion

This report describes the cloning and characterization of a novel human P2X receptor gene and cDNAs. Pharmacologic and structural properties indicate that the gene is the human homolog of the previously identified rat $P2X_2$ subtype (Brake et al., 1994). Comparisons of the human $P2X_{2a}$ and prototype rat $P2X_2$ receptors demonstrate a highly conserved structure, with the most variability occurring in the N- and C-cytoplasmic domains. The design of the sense primer for RT-PCR cloning of the full-length human $P2X_2$ receptor was based on the proximity of the predicted initiation methionine in the human genomic sequence to the initiation methionine of the

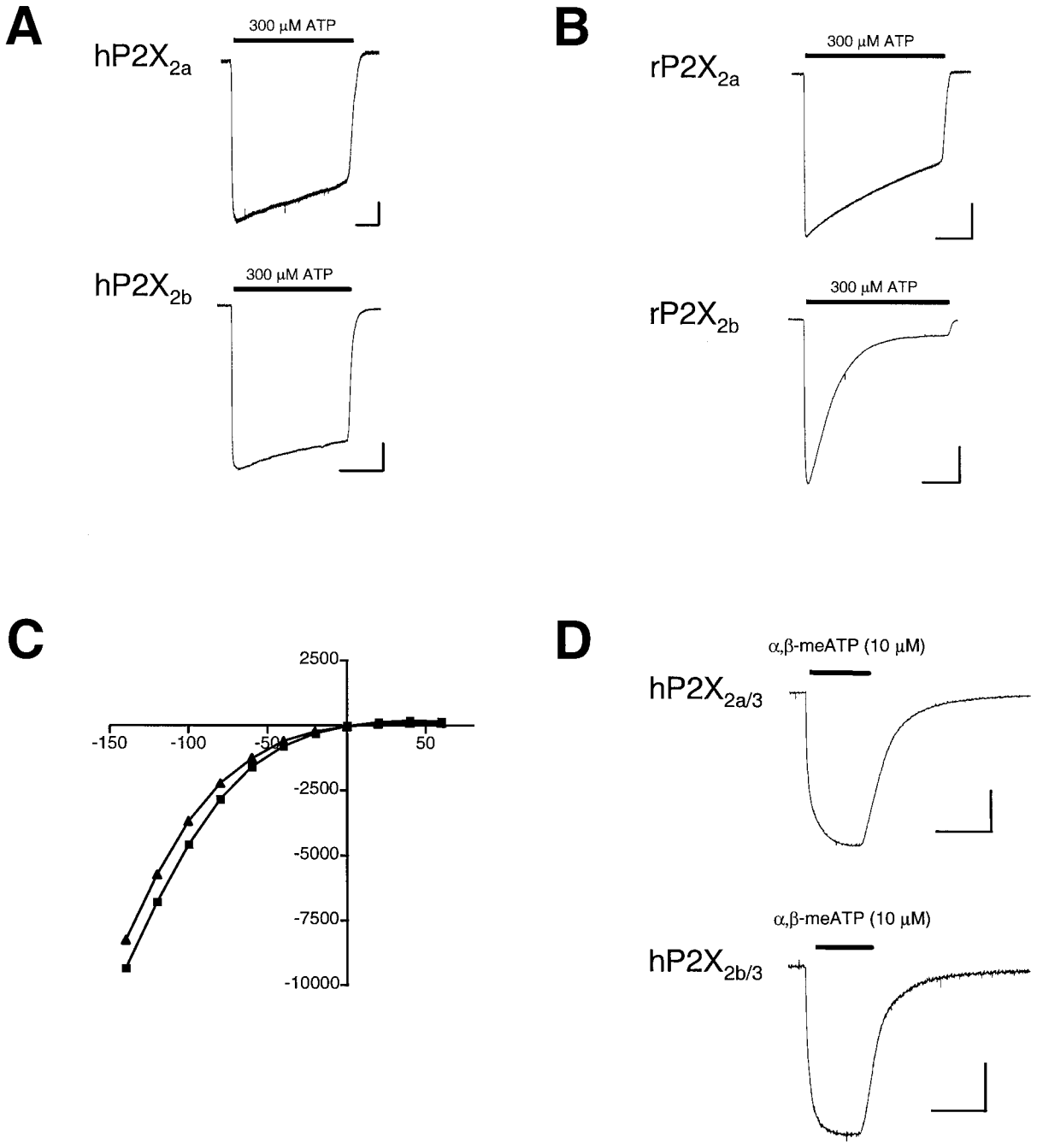

Fig. 5. Functional expression of $P2X_2$ receptors in oocytes. A, representative inward currents evoked by 60-s applications of 300 μM ATP in oocytes expressing either $hP2X_{2a}$ (top) or $hP2X_{2b}$ (bottom) homomeric receptors. Desensitization time constants were similar between $hP2X_{2a}$ and $hP2X_{2b}$. B, inward currents evoked as in A in oocytes expressing either $rP2X_{2a}$ (top) or $rP2X_{2b}$ (bottom) homomeric receptors. Significantly faster desensitization was seen in oocytes expressing $rP2X_{2b}$ receptors. C, current–voltage relationships for currents evoked by 30 μM ATP in oocytes expressing either $hP2X_{2a}$ ■ or $hP2X_{2b}$ ▲ receptors. D, representative inward currents evoked by 10 μM α,β me-ATP in oocytes expressing either $hP2X_{2a/3}$ (top) or $hP2X_{2b/3}$ (bottom) heteromeric receptors. For A, B, and D, cells were voltage-clamped at −60 mV. Agonist application is denoted by the bar. For A and B, calibration bars are 1000 nA and 20 s, except $hP2X2_a$, which is 400 nA and 20 s. For D, calibration bars are 400 nA and 10 s.

aligned rat sequence. Although this creates an N-terminal region for the human P2X$_2$ receptors that is 12 residues longer than that of the rat receptor, the structure is very similar to that of the guinea pig P2X$_2$ receptor (which has a 9-amino acid extension relative to the rat receptor) (Parker et al., 1998).

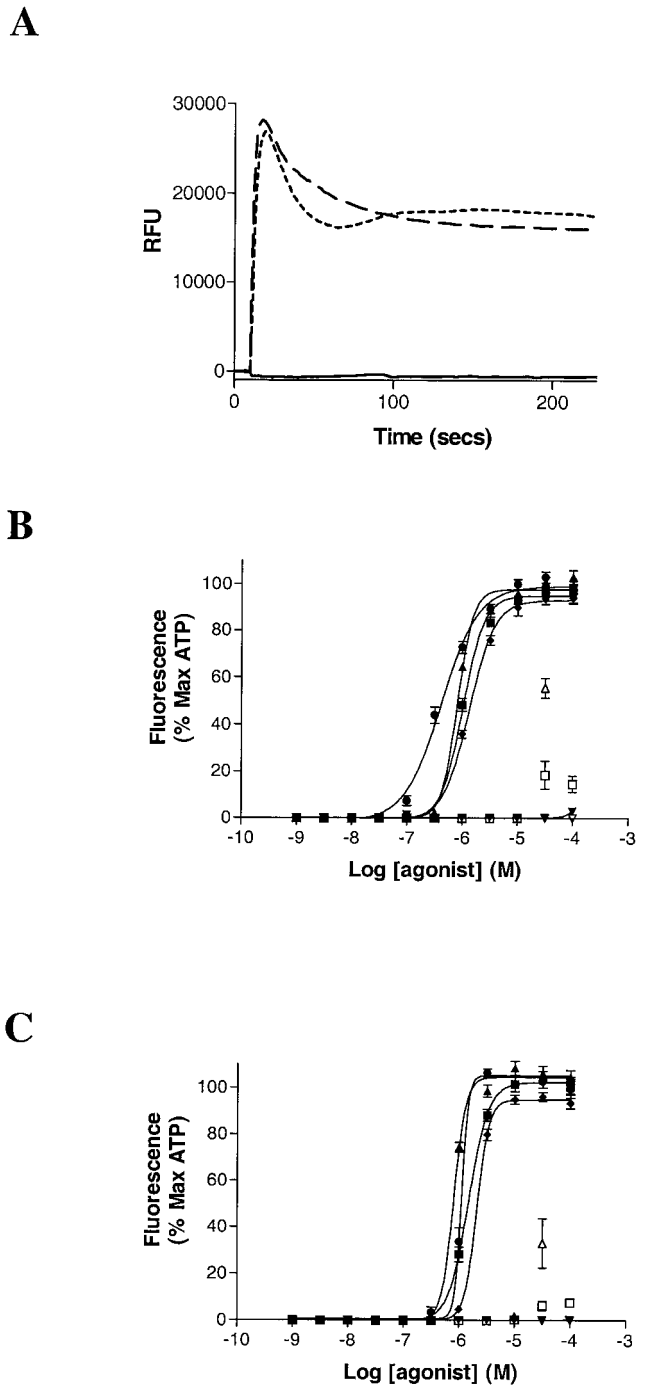

Fig. 6. A, fluorimetric measurement of calcium levels in 1321N1 cells (solid line) and 1321N1 cells expressing the hP2X$_{2a}$ (dashed line) or hP2X$_{2b}$ (dotted line) receptors, as indicated in *Experimental Procedures*. RFU, relative fluorescence units. ATP (10 μM) was added 10 s into run. Dose–response curves of agonists determined by fluorimetric analysis of 1321N1 cells expressing the hP2X$_{2a}$ (B) or hP2X$_{2b}$ (C) receptors. Agonists are indicated as ATP (■), 2-meSATP (▲), α,β-meATP (▼), ATPγS (♦), BzATP (●), ADP (□), ADPβS (△), and UTP (▽).

The intron/exon structure of the P2X$_2$ gene was found to be highly conserved between rat and human, following the general structure found with other P2X receptor genes (Brandle et al., 1997; Souslova et al., 1997). Several recent reports have described the identification of splice variants arising from the rat P2X$_2$ gene (Housley et al., 1995; Brandle et al., 1997; Simon et al., 1997). In addition to the prototype receptor identified initially (Brake et al., 1994), only one of the splice variants, the rat P2X$_{2b}$ receptor (alternatively called P2X$_{2\text{-}2}$) (Brandle et al., 1997; Simon et al., 1997), has been shown to form functional channels in heterologous expression systems. This message has a 207-bp deletion in the intracellular C terminus compared with the prototype receptor. Similar to the rat P2X$_2$ receptor, the human P2X$_2$ gene also gives rise to additional message heterogeneity, as demonstrated in this work. One of the human splice variants initially isolated was found to be a counterpart of the rat P2X$_{2b}$ variant, with a deletion of the identical region of this receptor, and also produced a functional ATP-gated ion channel.

Because a cDNA encoding a form of the human P2X$_2$ receptor analogous to the prototype rat receptor was not found initially, this variant was constructed from the other forms using recombinant techniques and was called hP2X$_{2a}$. Subsequently, the naturally produced P2X$_{2a}$ subtype was isolated from pancreas RNA. Although Northern analysis under high-stringency conditions indicates a high level of P2X$_2$ message in the pancreas, results of RT-PCR studies of different tissues demonstrated similar levels of splice variants in pancreas compared with other tissues examined. Additional Northern analysis of the pancreatic mRNA was performed using a riboprobe consisting of the 201-bp region of the P2X$_2$ gene exon 11, which is deleted in the P2X$_{2b}$ message. This probe also recognized the 2.4-kb message that was detected by the full-length probe (data not shown), indicating that the major P2X$_2$ message in pancreas is not the C-terminal deletion (P2X$_{2b}$) form. This does not identify definitively the pancreas message as the P2X$_{2a}$ form, however, because both the P2X$_{2c}$ and the P2X$_{2d}$ variants also contain the 201-bp sequence. Experiments using Northern analysis are underway to identify conclusively the message detected in the pancreas RNA. One explanation for the apparent discrepancies in P2X$_2$ message levels as determined by Northern and RT-PCR is that the structure of the P2X$_2$ RNA interferes with reverse transcription of the message. This is suggested by the failure in isolating the complete message in early RACE procedures and hybridization screens.

Two of the human P2X$_2$ clones isolated in the present

TABLE 1
Functional potencies of human P2X$_2$ receptor agonists and antagonists

pEC$_{50}$[a]	hP2X$_{2a}$	hP2X$_{2b}$
ATPγS	5.87 ± 0.02	5.68 ± 0.01
ATP	5.99 ± 0.01	5.83 ± 0.02
2-meS-ATP	6.09 ± 0.02	6.1 ± 0.02
BzATP	6.38 ± 0.03	5.96 ± 0.42
α,β-meATP	>4	>4
ADP	>4	>4
ADPβS	4.51 ± 0.11	4.38 ± 0.12
UTP	>4	>4
PPADS[b]	5.46 ± 0.62	5.65 ± 0.04

[a] Ligand potencies were determined in three or more replicate assays.
[b] PPADS pIC$_{50}$ was determined in the presence of 5 μM ATP.

work, the $hP2X_{2c}$ and $hP2X_{2d}$, forms, were unable to form either functional homomeric channels or functional heteromeric channels with the human $P2X_3$ subunit. The $P2X_{2c}$ form splices out exon 3 at predicted intron/exon boundaries, and the $P2X_{2d}$ form may represent an incompletely processed stable intermediate. Interestingly, a splice variant of the human $P2X_5$ receptor (GenBank no. U49396) also has the corresponding region of the putative extracellular domain missing. A rat cDNA analogous to the human $P2X_{2d}$ form, which also retains intron 10, was described by Housley et al. (1995). The role of the various $P2X_2$ receptor mRNAs is unclear. It is possible that the nonfunctional receptor messages may represent splicing artifacts that were detected because of the exquisite sensitivity of the RT-PCR technique. If the nonfunctional messages are artifacts, they appear to make up a high percentage of the total cell $P2X_2$ mRNA in most tissues, as judged by relative levels of products in RT-PCR analysis (Fig. 4B). The role of these transcripts, which do not form functional ion channels, is unknown at this time.

The rat $P2X_{2b}$ receptor (alternatively called $P2X_{2\text{-}2}$) exhibits a more rapid desensitization on prolonged application of agonist compared with the prototypic $P2X_2$ receptor (Brandle et al., 1997; Simon et al., 1997). Expression of different levels of the respective rat $P2X_2$ forms has been proposed as a mechanism for modulating the cellular response to ATP in rat tissues such as the pituitary gland (Koshimizu et al., 1998b). Mutagenesis studies of the cDNAs have implicated certain regions of the rat $P2X_2$ C terminus that are deleted in the $P2X_{2b}$ form as being important to imparting the slow-desensitizing phenotype of the full-length receptor (Koshimizu et al., 1998a). In contrast to studies with the rat $P2X_2$ variants, the desensitization rates of the two analogous human forms were found to be nearly identical. The similar desensitization characteristics exhibited by the human $P2X_{2a}$ and $P2X_{2b}$ receptors indicate that regions other than those exclusive to the $P2X_{2a}$ form must play a role in conferring the slow-desensitizing phenotype to the human receptors. Studies using chimeras of the desensitizing rat $P2X_1$ receptor and nondesensitizing rat $P2X_2$ receptor have demonstrated that the nondesensitizing P2X phenotype appears to be dominant; both TM regions of rat $P2X_1$ receptor were required to confer desensitization to a rat $P2X_2$ receptor, whereas only one TM domain of rat $P2X_2$ creates a nondesensitizing rat $P2X_1$ receptor (Werner et al., 1996). This is exemplified in the behavior of heteromeric $P2X_{2/3}$ channels (Lewis, 1995; this study), in which the nondesensitizing characteristic of $P2X_2$ predominates. Structures within the rat $P2X_{2b}$ variant that are not present in the human $P2X_{2b}$ receptor may therefore promote a desensitizing phenotype in the absence of the extended C-terminal tail. The putative TM domains of the rat and human receptors are primarily conserved; however, the C-terminal domains of human and rat receptors do differ in that the rat receptor has an additional 10 residues present in both the $P2X_2$ and $P2X_{2b}$ forms. Conversely, the N terminus of the human receptor is longer than that of the rat receptor by 12 residues. Differences in these or other structures of the rat and of the human receptors may account for the apparent differences in the desensitization kinetics of the splice variants of the two species.

Because there is no apparent difference in the kinetics of desensitization among the functional forms of the human $P2X_2$ receptor, there may be an additional role for these two forms of the same receptor. A candidate proline-rich site similar to a consensus SH3 binding domain exists within the $P2X_{2a}$ C terminus (Yu et al., 1994). This may serve a functionally unique role for the $P2X_{2a}$ form, such as directing subcellular localization, as has been shown for a similar region of the epithelial Na^+ channel (Rotin et al., 1994). Association of P2X receptors with cellular proteins has been suggested in a recent study that described an effect of the state of the actin cytoskeleton on $P2X_1$ receptor function (Parker, 1998). Phosphorylation of the rat $P2X_2$ receptor C terminus also has been implicated in controlling its function (Chow and Wang, 1998). Interestingly, the protein kinase A phosphorylation site at Ser^{431}, which is found to negatively regulate function of the rat $P2X_2$ receptor, is not conserved in the human receptor (Pro^{440}). Other potential phosphorylation sites do exist in the human C terminus, including Ser^{374} and Thr^{383} (Kennelly and Krebs, 1991). It is possible that the heterologous systems used in this study do not provide the cellular component(s) necessary to enable functional differentiation of the human $P2X_2$ variants. These factor(s) may be present in cells naturally expressing these receptors. Additional studies comparing the characteristics of the human and rat $P2X_2$ splice variants are needed to examine the physiologic function of this alternative splicing event.

To our knowledge, this is the first demonstration that a splice variant of the $P2X_2$ subtype can form heteromeric receptors with $P2X_3$ subunits. These human heteromeric receptors behave in a manner similar to that observed for the rat $P2X_{2/3}$ receptors (Lewis et al., 1995). Thus, the human $P2X_{2/3}$ receptors exhibit the kinetics of $P2X_2$ homomeric receptors, but the pharmacologic profile of $P2X_3$ receptors. The ATP-mediated responses of small-diameter nociceptive neurons of rat sensory ganglia resemble those produced by $P2X_{2/3}$ heteromeric receptors in recombinant systems (Thomas et al., 1998). Whether heteromeric receptors exist in human sensory neurons and which form(s) of $P2X_2$ receptor participate in heteromer formation is the subject of future investigation.

The presence of P2 receptors in the pancreas is not unexpected, because extracellular ATP has been implicated in the function of the neuroendocrine system. ATP induces secretion of hormones, including prolactin and leuteinizing hormone from cells of the pituitary gland (Carew et al., 1994; Chen ZP et al., 1995; Nunez et al., 1997; Villalobos et al., 1997). In addition, because ATP is coreleased with hormones such as insulin, prolactin, and leuteinizing hormone, as well as with catecholamines from adrenal chromaffin cells, it may act as a paracrine regulator of hormone release in these tissues (Leitner et al., 1975; Chen ZP et al., 1995; Tomic et al., 1996; Hollins and Ikeda, 1997; Nunez et al., 1997). Human $P2X_2$ cDNA was isolated from pituitary RNA (this study), and the $P2X_2$ receptor RNA and protein have been detected in rat pituitary tissue (Brake et al., 1994; Housley et al., 1995; Tomic et al., 1996; Vulchanova et al., 1996). There is evidence for the action of purinergic receptors on insulin-secreting β cells of the pancreas (reviewed in Hillaire-Buys et al., 1994). The existence of high levels of $P2X_2$ message in this human organ suggests a role for purinergic modulation of pancreatic function.

This work characterizes a novel human P2X receptor gene with structural and functional properties identifying it as the human homolog of the previously characterized rat $P2X_2$

receptor. Like the rat gene, the human $P2X_2$ gene produces multiple transcripts in a variety of tissues. Unlike the functional rat $P2X_2$ receptors, the two functional human receptors were found to be virtually identical in pharmacologic and electrophysiologic characteristics. The different characteristics exhibited by receptor splice variants from the two species underscore the importance of future studies to further examine the role of the various $P2X_2$ forms in purinergic signaling.

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Send reprint requests to: Kevin J. Lynch, Ph.D., Abbott Laboratories, 100 Abbott Park Rd., Abbott Park, IL 60064. E-mail: Kevin.Lynch@abbott.com