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A Novel G Protein-Coupled P_2 Purinoceptor (P_{2Y3}) Activated Preferentially by Nucleoside Diphosphates

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SUMMARY

A partial cDNA was isolated by hybridization screening of an embryonic chick brain library for P_{2Y} purinoceptors. After extension to full length, it revealed an open reading frame that encoded a protein, P_{2Y3}, of 328 amino acids that is nearest in sequence identity to the G protein-coupled P_2 purinoceptors obtained by DNA cloning. Expression of P_{2Y3} in cRNA-injected *Xenopus* oocytes confirmed that this cDNA encodes a member of the metabotropic purinoceptor family, with a novel order for the relative activities of nucleotides. At 100 μM concentrations, ADP gave the highest activity, and UTP and UDP were also strongly active. When expressed in the human T cell line Jurkat, P_{2Y3} mediated transient increases in intracellular Ca^{2+} in response to various nucleotides. Again, an unusual agonist rank order was revealed, with uridine nucleotides being more potent than adenosine nucleotides and UDP being the most potent agonist tested (half-maximal concentration, 0.13 μM) and 10-fold more potent than UTP. 2-Methylthio-ATP was of relatively low activity in both systems. The receptor transcript is expressed in brain, spinal cord, kidney, and lung and is highly abundant in the spleen but not in other peripheral tissues that we tested. The results indicated that P_{2Y3} is a previously unknown P_2 purinoceptor subtype with a preference for nucleoside diphosphates.

Extracellular ATP has been established as a mediator of a wide variety of physiological responses, with these effects being transduced through specific cell surface receptors, the P_2 purinoceptors. After the primary subdivision of P_2 purinoceptors into P_{2X} and P_{2Y} types by agonist selectivities (1), further subtypes of this receptor class have been defined on the basis of the rank orders of potency of nucleotide analogues, although this procedure has never been straightforward in the absence of selective antagonists. It was recently recommended that the P_{2X}/P_{2Y} division be used to distinguish between members of this receptor family that are ligand-gated ion channels or metabotropic GPCRs, respectively; accordingly, an official nomenclature of $P_{2Y1}\ldots P_{2Yn}$ has been assigned for the metabotropic P_2 receptors (2). This series will include the previously designated "P_{2Y}" type, for which 2-MeSATP is the most potent agonist, as well as the P_{2T} purinoceptor, for which ADP is the endogenous agonist and ATP is an antagonist, and the P_{2U} purinoceptor, for which ATP and UTP are equipotent. It may also be appropriate to include the receptor activated by dinucleotide phosphates, the "P_{2D} receptor" (3), in the P_{2Y} class.

DNA cloning has revealed the first two receptors of the metabotropic P_2 family, P_{2Y1} and P_{2Y2} (4, 5), the latter being the previously designated P_{2U} type. New pharmacological evidence with more-selective ligands (6, 7) and different tissue sources suggests that additional subtypes exist within, as well as outside, the previously recognized P_2 purinoceptor types, as reviewed by Abbracchio and Burnstock (3); for clear definitions of these, however, there is a need to characterize single forms as obtained by DNA cloning and expression. An additional such form is described here.

We previously reported the cDNA cloning (4) of a P_{2Y1} purinoceptor from chick brain. During the isolation of that cDNA, we purified a second type of clone weakly cross-hybridizing to the P_{2Y1} clone. Here, we describe the amino acid sequence of the encoded protein and its pharmacological properties as shown by expression in *Xenopus* oocytes and in

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ABBREVIATIONS: GPCR, G protein-coupled receptor; 2-MeSATP, 2-methylthio-ATP; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); BSS, basal salt solution; dATPαS, 3′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); RB-2, Reactive Blue-2; RACE, rapid amplification of cDNA ends; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl ester.

mammalian (Jurkat) cells, which have been used to identify this GPCR as a novel P_2 purinoceptor (P_{2Y3}).

Experimental Procedures

Materials. All culture media and reagents were obtained from GIBCO-BRL (Gaithersburg, MD); 2-MeSATP and suramin (8-(3-benzamido-4-methylbenzamido)napthalene-1,3,5-trisulfonic acid) were obtained from Semat Technical Ltd.; dATPαS and [^{32}P]dATPαS were obtained from DuPont-New England Nuclear; and all other nucleotides, adenosine, apyrase (type IV), Fura-2 AM, and fluo-3 AM were obtained from Sigma.

cDNA library screening and DNA sequencing. An embryonic chick whole brain cDNA library was screened with a partial cDNA encoding a chicken RDC1-like sequence as described previously (4). DNA (clone 103) from a weakly hybridizing phage that cross-hybridized with the P_{2Y1} cDNA at low stringency was isolated by standard procedures, and the entire *Eco*RI insert was subcloned into the reciprocal site in M13 mp18 and subjected to sequence analysis (Sanger dideoxy chain termination, Sequenase kit; United States Biochemical, Cleveland, OH).

5′-RACE. Sequence analysis revealed that the isolated λ clone was truncated at a natural *Eco*RI site at the 5′ end. The sequence of this region of the clone was determined by 5′-RACE (8). First-strand cDNA was synthesized from 20 μg of total RNA from Rhode Island 1-day-posthatch chick whole brain using random hexanucleotide primers and was polyadenylated (9). The first and second stage 5′-RACE reactions were performed as described previously (9) using the 103 sequence-specific primers 103Ro (5′-GGCCAGGTTCAGCATGTAGATGGTGGTGCG-3′) and 103Ri (5′-GCCCGTCGACAACGGCATTGAGTGGCAGCCCCAGTAG-3′) respectively. The amplification conditions were as follows: first stage, 94° for 1 min, 42° for 1 min, and 72° for 1 min (three cycles) and 94° for 1 min, 60° for 1 min, and 72° for 1 min (37 cycles); second stage, 94° for 1 min, 60° for 1 min, and 72° for 1 min (40 cycles). The 5′ sequence was determined by direct polymerase chain reaction sequencing of a 1:10 dilution of the second-stage RACE product (GIBCO-BRL).

Generation of a construct containing the full coding region of P_{2Y3}. The complete open reading frame of P_{2Y3} was amplified using *Taq* polymerase (Promega) from chicken genomic DNA with the primers 5′-CGCTTCACCCAGTAAAGAGG-3′ and 5′-GTGGCCTCGTCCACCTAGG-3′ and the following conditions: 94° for 30 sec, 65° for 30 sec, and 72° for 1 min for 35 cycles. Sequence analysis of the cloned amplification product, inserted into pCRScript SK$^+$ (Stratagene, La Jolla, CA) to generate pP_{2Y3}amp, revealed several one-base mutations introduced 3′ to the *Eco*RI site that marked the 5′ end of the λ clone. To generate a corrected construct, pP_{2Y3}amp was digested with *Eco*RI, liberating the vector, and the 5′-fragment of the 103 cDNA was isolated. This was ligated to the original λ clone insert and subcloned into pCR-Script SK$^+$ at the *Srf*I and *Eco*RI sites to generate pP_{2Y3}. The entire 5′ and coding region of the insert of this construct was then sequenced (as before) on both strands to confirm its integrity.

***In vitro* transcription and oocyte expression.** Ovarian lobes were removed surgically from *Xenopus laevis* frogs (14 animals) anesthetized with Tricane (0.1% w/v) and killed by decapitation. Mature oocytes (stages V/VI) were separated and stored at 4° for up to 14 days in modified Barth's solution (4). Both folliculated and defolliculated oocytes were used in this study; defolliculated oocytes were prepared as described previously (10).

Capped cRNA was transcribed *in vitro* from the construct described above, after linearization with *Kpn*I, using T3 RNA polymerase and a riboprobe kit (Promega, Madison, WI) and was then polyadenylated. As described previously (4), cRNA (~50 ng in 50 nl/oocyte) was injected into folliculated (273 cells from nine batches) or defolliculated (206 cells from five batches) oocytes that were maintained in Barth's medium at 18° for 48 hr and thereafter at 4° for ≤12 days. In a few cases, defolliculated oocytes were, instead, nuclear injected (11) with the receptor cDNA in the expression vector noted below but without the level of expression being increased. Ionic currents in injected oocytes were measured using a two-electrode voltage clamp amplifier (Axoclamp 2A). The voltage-recording and current-recording electrodes (1–2-MΩ tip resistance) were filled with 0.6 M K_2SO_4 and 3 M KCl, respectively. Oocytes were superfused at a rate of 5 ml/min (bath volume, 0.5 ml) with a Mg^{2+} amphibian Ringer solution (4) at room temperature.

Functional expression in Jurkat cells. The P_{2Y3} open reading frame was subcloned from pCRScript SK$^+$ into the pRc/CMV vector (InVitrogen, San Diego, CA) as a *Not*I/*Apa*I fragment for expression in mammalian cells. Jurkat cells were cultured and transfected by electroporation as described previously for a P_{2Y1} receptor cDNA (12). Cells stably expressing the construct were cloned by limited dilution. Intracellular calcium concentrations were measured in Fura-2-loaded cells essentially as described previously (12). Then, 1×10^8 cells were loaded with 10 μM Fura-2 AM in 3 ml of BSS medium (125 mM NaCl, 5 mM KCl, 1 mM MgCl, 1.5 mM CaCl, 25 mM HEPES, 5 mM glucose, pH 7.3) containing 1% bovine serum albumin; they were rinsed twice in BSS with 1% bovine serum albumin at 37° and underwent shaking for 30 min before being resuspended in BSS with 0.1% bovine serum albumin at a density of 1×10^6 cells/ml for fluorimetry at 37°. Apyrase (EC 3.6.1.5) was present at 2 U/ml during the period of dye loading. For studies with the antagonist suramin, cells were loaded with 10 mM fluo-3 AM dye (Sigma) because the absorbance spectrum of suramin precludes the use of Fura-2 AM.

Northern hybridization. Northern hybridization was performed as detailed previously (4). The hybridization probe was an antisense 45-base oligonucleotide located at the 5′ end of the P_{2Y3} λ clone sequence (5′-GAAGGTGCACGAGTTCCTCCCCCCCGTGAAGTTGGCCATGCTCAT-3′). The probe was labeled with [^{32}P]dATPαS using terminal deoxynucleotidyl transferase to a specific activity of ~1×10^9 dpm/μg. After hybridization, the filter was washed at 65° in 1× saline sodium citrate/0.1% sodium dodecyl sulfate for 15 min before autoradiography. Exposure was at −70° in the presence of an intensifying screen for 15 days.

Results

Isolation of a novel P_2 purinoceptor cDNA. We previously reported the cloning of a cDNA from chick brain encoding a P_{2Y1} purinoceptor (4). A weakly cross-hybridizing recombinant phage, clone 103, was also purified during that screening. Nucleotide sequence analysis of this clone revealed that it contained a potential open reading frame but was bereft of an initiation codon, being truncated at a natural *Eco*RI site. The sequence upstream of this *Eco*RI site was determined by 5′-RACE (8) and was found to contain two potential initiation codons, the latter of which may be the preferred sequence as it has a guanine base at position 4 and is more similar to an optimal sequence for translation, AC<u>CATG</u>G′ (13). The nucleotide and predicted amino acid sequences (328 amino acids; molecular mass = 37,586 Da from the first methionine) of the clone 103 cDNA are shown in Fig. 1.

Further analysis of the sequence clearly defines this protein as a member of the major family of the GPCRs (i.e., the rhodopsin family) (14). The sequence contains most of the conserved motifs present in such receptors, including three potential asparagine-linked glycosylation sites in the extracellular domains; conserved proline residues within the transmembrane domains; two conserved cysteine residues, in the second and third extracellular loops; and potential phosphorylation sites in the carboxyl-terminal domain. Searches

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                                        *
                                  M  S  M  A  N  F  T  G  G  R  N      11
GGCGCTTCACCCAGTAAAGAGGGACCATGAGCATGGCCAACTTCACGGGGGGGAGGAACT      60

S  C  T  F  H  E  E  F  K  Q  V  L  L  P  L  V  Y  S  V  V      31
CGTGCACCTTCCATGAGGAATTCAAGCAGGTCCTGCTGCCCCTGGTCTACTCAGTGGTGT     120

F  L  L  G  L  P  L  N  A  V  V  I  G  Q  I  W  L  A  R  K      51
TCCTACTGGGGCTGCCACTCAATGCCGTTGTCATTGGGCAGATCTGGCTGGCCCGCAAGG     180

A  L  T  R  T  T  I  Y  M  L  N  L  A  M  A  D  L  L  Y  V      71
CGTTGACCCGCACCACCATCTACATGCTGAACCTGGCCATGGCCGACCTGCTTTATGTCT     240

                     *
C  S  L  P  L  L  I  Y  N  Y  T  Q  K  D  Y  W  P  F  G  D      91
GCTCCCTCCCTCTCCTCATCTACAACTACACCCAGAAGGATTACTGGCCCTTTGGGGACT     300

F  T  C  K  F  V  R  F  Q  F  Y  T  N  L  H  G  S  I  L  F     111
TCACCTGCAAATTCGTCCGCTTCCAGTTCTACACCAACCTGCACGGCAGCATCCTCTTCC     360

L  T  C  I  S  V  Q  R  Y  M  G  I  C  H  P  L  A  S  W  H     131
TCACCTGCATCAGCGTCCAGCGCTACATGGGGATCTGCCACCCCTTGGCCTCGTGGCACA     420

K  K  K  G  K  K  L  T  W  L  V  C  A  A  V  W  F  I  V  I     151
AAAAGAAGGGAAAGAAGCTGACGTGGCTGGTGTGTGCTGCCGTGTGGTTCATCGTCATCG     480

                                                 *
A  Q  C  L  P  T  F  V  F  A  S  T  G  T  Q  R  N  R  T  V     171
CCCAGTGCCTGCCCACCTTTGTCTTCGCCTCCACCGGCACGCAGAGGAATCGCACTGTCT     540

C  Y  D  L  S  P  P  D  R  S  T  S  Y  F  P  Y  G  I  T  L     191
GCTATGACCTGAGCCCCCCGGACCGCTCCACATCCTACTTCCCCTATGGCATCACGTTGA     600

T  I  T  G  F  L  L  P  F  A  A  I  L  A  C  Y  C  S  M  A     211
CCATCACTGGCTTCCTGCTGCCCTTCGCAGCCATCCTGGCCTGCTACTGCAGCATGGCCC     660

R  I  L  C  Q  K  D  E  L  I  G  L  A  V  H  K  K  K  D  K     231
GCATCCTGTGCCAGAAAGACGAGCTGATTGGCTTGGCGGTGCACAAGAAGAAGGACAAGG     720

A  V  R  M  I  I  I  V  V  I  V  F  S  I  S  F  F  P  F  H     251
CCGTGCGCATGATCATCATCGTTGTCATCGTCTTCTCCATCAGCTTCTTCCCCTTCCACC     780

L  T  K  T  I  Y  L  I  V  R  S  S  A  S  L  P  C  P  T  L     271
TCACCAAGACCATCTACCTGATCGTCCGCTCCTCAGCCAGCTTGCCCTGCCCTACCCTGC     840

Q  A  F  A  I  A  Y  K  C  T  R  P  F  A  S  M  N  S  V  L     291
AGGCTTTTGCCATTGCCTACAAGTGCACGCGGCCCTTTGCCAGCATGAACAGCGTCCTCG     900

D  P  I  L  F  Y  F  T  Q  R  K  F  R  E  S  T  R  Y  L  L     311
ACCCCATCCTCTTCTACTTCACCCAGCGCAAGTTTCGTGAGAGCACCCGCTATCTCCTGG     960

D  K  M  S  S  K  W  R  Q  D  H  C  I  S  Y  G  S  *           328
ACAAGATGAGCTCCAAGTGGCGGCAAGACCACTGCATCAGCTACGGCTCCTAGGTGGACG    1020
AGGCCACCTCGGTGTCACCGGGGCTGGGCATGGAGCAATTTGGGTTGAAGCTGCATGGTG    1080
CGGAGATGGGGATGAGCCCAGAGTGCTGCGGGTGCCCCATCTCTGGAGGTGTTGGAGATT    1140
AGATTGGATGGGGCTCTGGGCCC                                          1163
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Fig. 1. Nucleotide and predicted amino acid sequence (single letter code) of clone 103. *Underlined sequences*, the *Eco*RI site that marked the 5′ end of the λ clone and putative transmembrane domains; *, potential sites for N-linked glycosylation; *bold sequences*, potential sites for phosphorylation by protein kinase C. The nucleotide sequence has been deposited in the EMBL database (accession number X98283).

of the GenBank and EMBL databases revealed that the 103-derived sequence is most related to the mouse P_{2Y2} (5) and chicken P_{2Y1} (4) receptors, with which it shares 41% and 39% amino acid sequence identity, respectively (Fig. 2). Other related receptors include opioid, somatostatin, angiotensin, thrombin, and certain chemokine receptors, but none of these share >34% sequence identity with that encoded by clone 103. This degree of relatedness with the known P_{2Y} purinoceptor sequences suggested that this protein is another member of that series and was assigned at a preliminary stage (15) as the P_{2Y3} purinoceptor.

Identification of the cloned cDNA as encoding a P_{2Y} purinoceptor. The identification of P_{2Y3} was confirmed electrophysiologically by expression in *Xenopus* oocytes. However, a unique cytotoxicity to the P_{2Y3} cRNA was found: only a small fraction of folliculated [21 of 273 cells (8%)] and

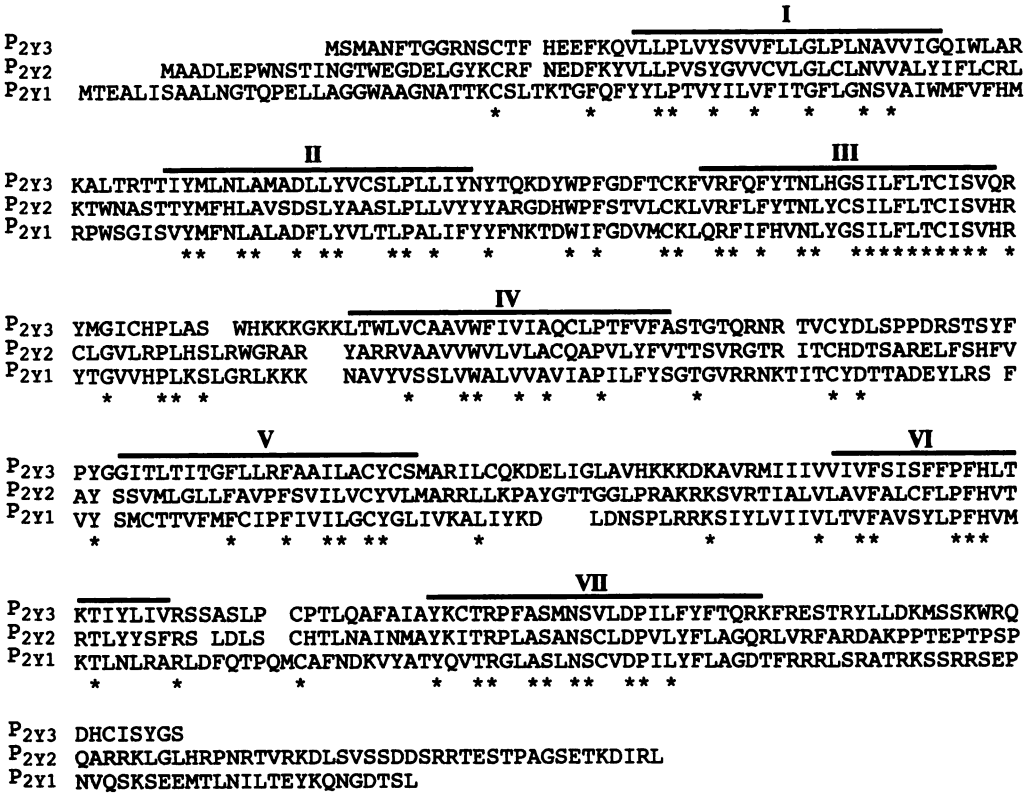

Fig. 2. Comparison of the chicken P_{2Y3} purinoceptor with the chicken (4) P_{2Y1} and mouse (5) P_{2Y2} receptors. Gaps were introduced to optimize the alignment. *, Residues identical in all three receptor sequences. *Overlined and numbered sequences*, transmembrane domains.

of defolliculated [5 of 206 cells (3%)] oocytes survived microinjection with the P_{2Y3} cRNA and receptor expression to show membrane currents with nucleotides under voltage-clamp conditions. Parallel experiments with P_{2Y1} (4), in which oocytes from the same batch were microinjected with the P_{2Y1} cRNA, revealed no such cytotoxicity and demonstrated good response to agonist. Furthermore, the small responses to ADP (<100 nA) of the injected folliculated oocytes were difficult to discriminate from the small responses to ADP mediated by native P_2 purinoceptors on the follicle cell layer (16). Therefore, the results documented here are limited to observations on defolliculated oocytes. In these cases, in the 3% of the oocytes surviving microinjection, the P_{2Y3} receptor seemed to be expressed well because it produced larger and consistent currents. Superfusion of ADP (0.01–1 mM) evoked fast and slow oscillatory inward currents associated with a conductance increase (Fig. 3A), typical of the involvement of the oocyte calcium-activated chloride current (17). Responses to ADP were dose dependent, with an EC_{50} of 62 ± 10 μM (Fig. 3B). Inward currents were also evoked in P_{2Y3}-injected oocytes by UTP, UDP, ATP, and ITP, with CTP displaying low potency and GTP being inactive (Fig. 3C). ADP evoked the largest currents when compared with the others tested, at an equimolar concentration of 100 μM (Fig. 3C and Table 1). AMP and adenosine were not significantly active. Amiloride and the sulfonic acid derivatives suramin and RB-2, which have broad ranges of pharmacological activity, have been shown to competitively block a number of P_2 purinoceptors (18). The ADP response present in oocytes expressing P_{2Y3} was antagonized by RB-2 (100 μM) and by suramin (300 μM), whereas 100 μM amiloride (which blocks an ATP response only in an exceptional case; see Discussion) gave no inhibition (Fig. 3D).

Functional characterization of P_{2Y3} in stably transfected Jurkat cells: mobilization of Ca^{2+}. The P_{2Y3} construct was expressed stably in the Jurkat cell line, which we previously used for the characterization of purinoceptors (12), because it lacks a detectable calcium response to P_2 receptor ligands. Initially, calcium fluxes were not detected in Fura-2-loaded transfected populations. However, calcium transients (albeit very small) were seen when the cells were pretreated with apyrase (data not shown). Transfected Jurkat cell clones were therefore derived by limiting dilution of the transfected population and were screened (by total RNA dot-blot analysis) for expression of the P_{2Y3} mRNA. One of the clones expressing the highest levels of the transcript was chosen for further study. In the Fura-2-loaded cells, the maximal calcium transients detected were not large, with the increase being ~40 nM Ca^{2+} above basal levels (Fig. 4, *top*). This compares with an observed 140-nM increase observed in response to thrombin in these cells (data not shown). Using this clone, calcium transients were detected in response to 100 μM concentrations of ATP, ADP, 2-MeSATP, ATPγS, UTP, and UDP, whereas dATPαS gave a small response, and no response was seen with α,β-methylene ATP or AMP (Table 1). The responses were only discernible when the cells were pretreated with apyrase. Concentration-response curves were constructed for the active ligands (Fig. 4, *middle*, and Table 1). The agonist potency order UDP > UTP > ADP, 2-MeSATP, ATPγS > ATP was found. The general P_2 antagonist suramin was also tested. A 100-μM concentration of suramin competitively antagonized the UDP response, causing a 25-fold shift in the UDP EC_{50} value (Fig. 4, *bottom*) (pA_2 = 5.0 ± 0.1).

Expression pattern of the P_{2Y3} mRNA. The tissue distribution of the P_{2Y3} transcript was determined by Northern

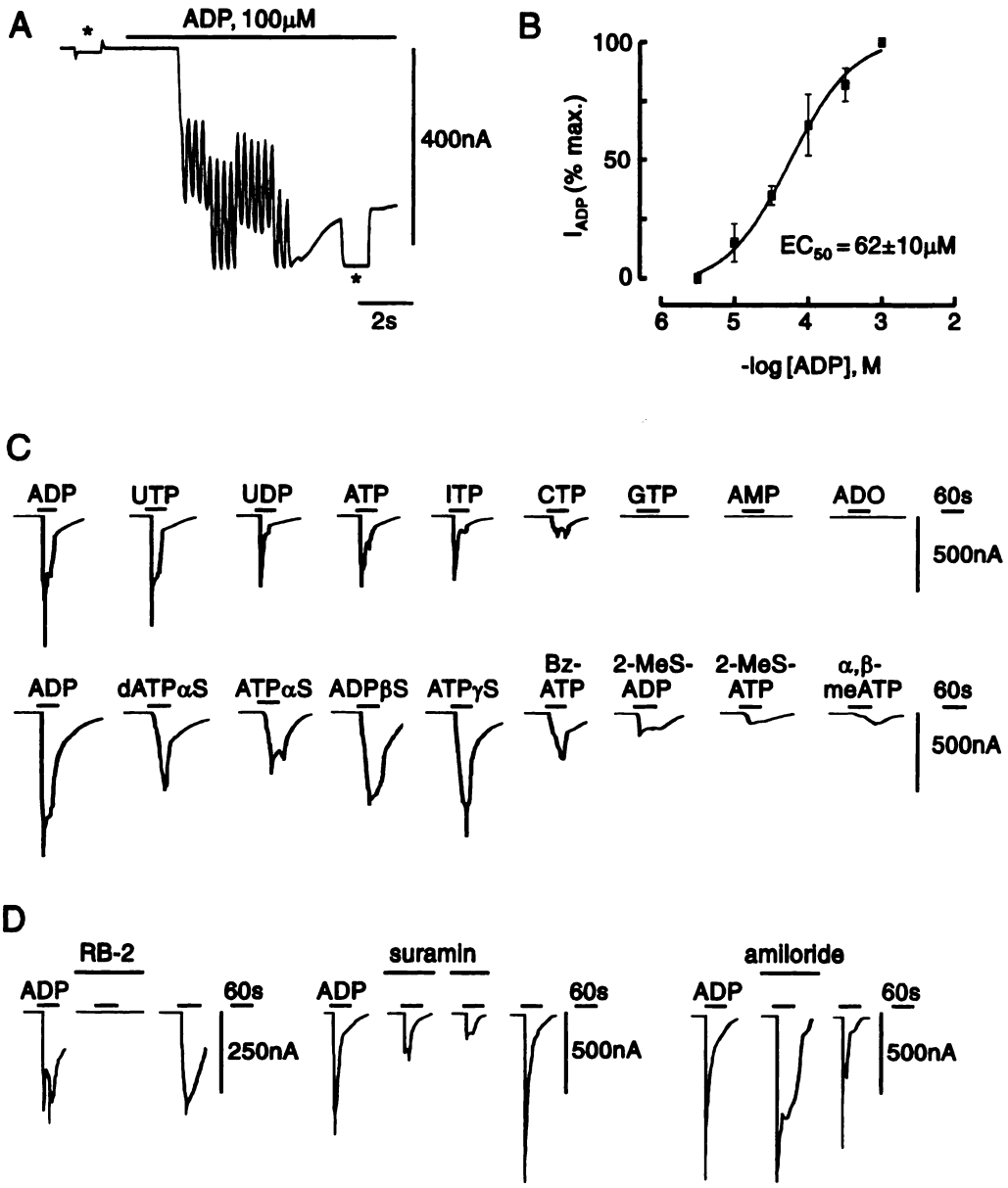

Fig. 3. Responses to ADP and other nucleotides in P_{2Y3} cRNA-injected *Xenopus* oocytes (V_h = −40 mV). A, Fast oscillatory inward current evoked by ADP (100 μM). *, Evoked currents to hyperpolarizing voltage commands (−10 mV for 1 sec, every 10 sec). B, The concentration-response relationship for the ADP-evoked inward current. The curve was fitted and the EC_{50} value was calculated by the software package Prism version 1.03 (GraphPad, San Diego, CA). C, Agonist activity (at 100 μM) of naturally occurring nucleotides and adenosine (*top*) and synthetic nucleotide analogues (*bottom*) compared with ADP. *α,β-meATP*, α,β-methylene ATP; *ADO*, adenosine; *ATPαS*, adenosine 5′-*0*-(1-thiotriphosphate); *ADPβS*, adenosine 5′-*0*-(2-thiodiphosphate); *BzATP*, 2′- and 3′-*0*-(4-benzoylbenzoyl)ATP. D, Reversible antagonism of ADP responses by RB-2 (100 μM) and suramin (100 μM and 300 μM). ADP responses were not antagonized by amiloride (100 μM).

hybridization (Fig. 5). A 2.8-kb transcript was expressed in a variety of tissues. The greatest expression was seen in the spleen; the transcript was also present in the kidney, lung, brain, and spinal cord but was not detectable in the heart, liver, stomach, gastrointestinal tract, or skeletal muscle.

Discussion

We report the isolation and characterization of a recombinant P_2 purinoceptor from the chicken, which has previously undescribed pharmacological characteristics. We designated this receptor as P_{2Y3}, the third member of the family of metabotropic ATP receptors to be identified by DNA cloning (15), on the basis of its structural similarity to the P_{2Y1} and P_{2Y2} purinoceptors (Fig. 2) and its distinct pharmacological properties.

Previously, we characterized recombinant P_2 receptors by transient expression in COS-7 cells and the binding of [^{35}S]dATPαS because that radioligand does not bind with high affinity to the endogenous P_2 receptors of COS cells (11).

TABLE 1

Agonist activity of the P_{2Y3} purinoceptor

In the expression in *Xenopus* oocytes, because of the toxicity seen with P_{2Y3}, the currents at 100 μM agonist concentration [taken as maximal (I_{max}), see Fig. 3C] were compared; these values therefore are relative, taking the value for the most active agonist [$I_{max(ADP)}$] as 1.00. In the stable expression in Jurkat cells, EC_{50} values were calculated from a set of the dose-response curves of the type shown in Fig. 4A. All values are expressed as the mean ± standard error, with the number of determinations indicated in parentheses.

Agonist	Oocyte $I_{max}/I_{max(ADP)}$	Jurkat cells EC_{50}
		μM
UDP	0.56 (1)	0.12 ± 0.05 (4)
UTP	0.78 ± 0.02 (3)	1.7 ± 0.7 (4)
ADP	1.00 (4)	1.7 ± 0.3 (4)
ATP	0.64 ± 0.10 (5)	N.D.[a]
ATPγS	0.86 (1)	13 ± 7 (3)
2-MeSATP	0.27 ± 0.10 (3)	8.1 ± 3.7 (4)
α,β-meATP	0.05 ± 0.02 (3)	Inactive[b]
AMP	0.06 ± 0.03 (3)	Inactive

[a] N.D., not determined.
[b] Consistently no response up to 100 μM.

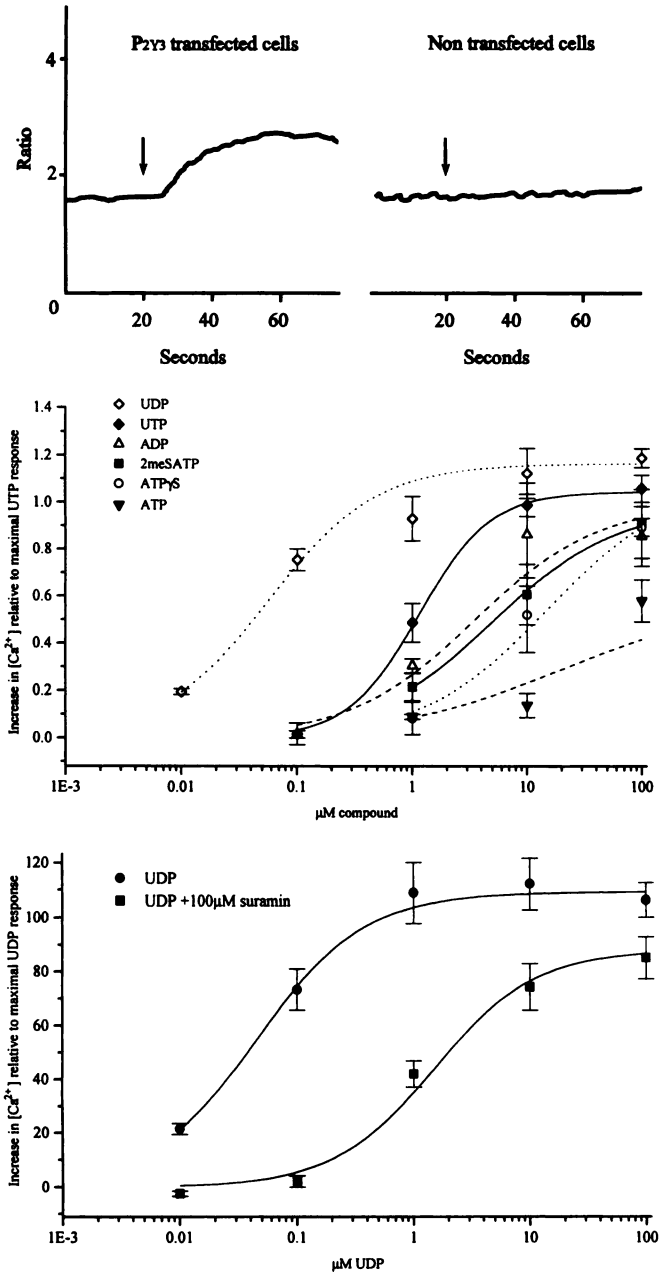

Fig. 4. [Ca^{2+}] responses to P_2 receptor ligands. *Top*, Representative responses to UTP (*arrow*, 100 μM) in Fura-2-loaded cell, transfected or nontranfected with the P_{2Y3} construct. The ratio of the emissions at 510 nm with excitations at 340/380 nm, which is proportional to the intracellular Ca^{2+} concentration increase, is shown. *Middle*, Concentration-response relationship for agonists active at the P_{2Y3} receptor. *Bottom*, Effect of suramin (100 μM) on the UDP concentration-response curve. Increases in intracellular Ca^{2+} concentration are plotted as a percentage of the maximal response, obtained as indicated, with the same batch of dye-loaded cells (four experiments). The curves were fitted and the EC_{50} values were calculated with the Origin graphics program version 3.5 (Microcal, Northampton, MA).

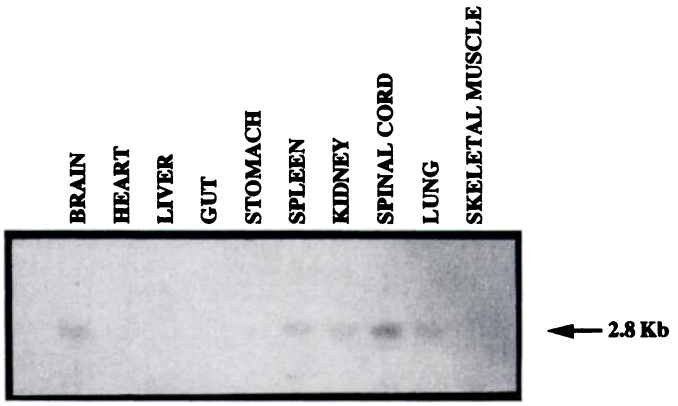

Fig. 5. Expression pattern of the P_{2Y3} receptor transcript. A, Northern blot of total RNA (30 μg, with the exception of spleen, for which 5 μg was loaded) from various adult chicken tissues. *Arrow*, band size was determined using RNA standards (GIBCO-BRL). Bands were absent from the regions of the blot not shown.

However, this radioligand does not bind with high affinity to P_{2Y3}-expressing COS-7 cells (data not shown) and was distinctly less active than ADP on P_{2Y3}-expressing oocytes (Fig. 3C) and even less so in P_{2Y3}-transfected Jurkat cells (data not shown). It was not possible to test labeled ATP, ADP, or UTP as ligands in this system because COS-7 cells have specific binding sites for these compounds resembling those of an endogenous P_{2Y2} receptor. Furthermore, all other clonal cell lines (including the Jurkat cells) that we have tested also possess binding sites for one or more of these ligands.[3] The P_{2Y3} receptor was therefore initially characterized functionally by expression in *Xenopus* oocytes. Data derived thus suggested ADP to be the most active ligand; however, those studies were hampered, as described in Results, by the unusual but consistently found cytotoxicity of the P_{2Y3} mRNA.

Nucleotide responses seen in P_{2Y3}-microinjected follliculated oocytes were pharmacologically distinct from the small native responses to nucleotides that were seen on 40% of noninjected follliculated oocytes; in the latter, ATP, 2-MeSATP, and UTP are more potent than ADP (16). In any case, the process of defolliculation rendered noninjected oocytes inactive to nucleotides (16). A high-threshold (>100 μM) ATP-activated sodium current has been seen in some defolliculated oocytes (19) but is activated by ATP and by no other nucleotide, whereas amiloride and UTP are full antagonists of this unique ATP response. This pharmacological profile differs completely from the observed activity of P_{2Y3}, which was stimulated by a broad range of nucleotides (including ADP, ATP, and UTP) but was spared by amiloride. Thus, responses obtained in P_{2Y3}-microinjected defolliculated oocytes were taken as truly representative of the pharmacological activity of the P_{2Y3} purinoceptor. The combination of fast and slow oscillations of inward current seen (Fig. 3A) indicated that Ca^{2+}-mobilization occurred in different ways, involving a short-latency release or an influx of Ca^{2+} ions followed by a slower release from intracellular stores.

This newly characterized receptor can be clearly differentiated from other metabotropic P_2 purinoceptor subtypes. It is distinct from the originally designated P_{2Y} and P_{2T} purinoceptors as detected on tissues (or blood platelets) because uridine nucleotides were highly active at the P_{2Y3} but are weak agonists or weak antagonists at those P_{2Y} and P_{2T} receptors, respectively (2, 18, 20). Also, ATP is an antagonist of P_{2T} receptors (18) but is seen to be a partial agonist on the P_{2Y3}. The P_{2Y3} receptor is also quite unlike the P_{2Y2} (P_{2U}) receptor, for which (in transfected mammalian cells) ADP, UDP, and 2-MeSATP are partial agonists and ATP and UTP

[3] T. E. Webb and J. Simon, unpublished observations.

are essentially equipotent full agonists (21, 22); all of these features differ strongly in the P_{2Y3} receptor. This receptor is also unlike the "uridine nucleotide receptor" seen, for example, on rat C6–2B cells, which preferentially recognizes pyrimidine nucleotides (23). The higher activity of ADP over ATP seen in both expression systems has been observed at (unclassified) P_2 purinoceptors on rat megakaryocytes (progenitor cells of blood platelets) (24). However, the P_2 purinoceptors on rat megakaryocytes are entirely distinct from P_{2Y3} inasmuch as the former are not activated by UTP and 2-MeSATP is more potent there than ADP (24).

We conclude that P_{2Y3} represents a unique metabotropic P_2 purinoceptor that is distinguishable by a high affinity for nucleoside diphosphates (particularly for UDP) but also a broad selectivity for naturally occurring nucleotides and a susceptibility to the known P_2 purinoceptor antagonists suramin and RB-2. There are differences in some agonist activities when studied in the two expression systems (Table 1), even after allowance is made for the inability to determine concentration-response relationships for most of the oocyte activities, and the potency of ADP was 36-fold lower in the oocyte expression. Differences between the oocyte and mammalian cell expressions are not so uncommon for GPCRs and are understandable because the two systems typically yield different receptor densities (which can modify potencies), because G protein subtypes and affinities may differ between the two cell types, and because the calcium-activated chloride current in the oocyte is a transduction event considerably downstream to the Ca^{2+} mobilization (monitored directly in the cell lines) and is not known to be a native coupling mechanism of G protein-coupled P_2 purinoceptors.

Receptor activity in transfected Jurkat cells was revealed after suppression of the desensitizing effect of basal ATP release with the diphosphatase apyrase. Under these conditions, the receptor was stimulated by UDP and UTP at submicromolar concentrations (Fig. 4, *top*). The true receptor profile for the bovine P_{2Y1} receptor (in terms of Ca^{2+} flux) expressed in this same cell line was also only obtainable after apyrase removal of the desensitizing nucleotides, which can accumulate in the culture medium (12). In the case of the human P_{2Y2} receptor expressed on the human astrocytoma line 1321N1 (21), Ca^{2+} flux measurements were obtainable in the absence of apyrase, but the basal level of inositol phosphates in those transfected cells was very high. However, levels could be reduced to near-normal levels on preincubation with apyrase, again indicating the release of nucleotides from the cells into the culture medium. The other possible distortion of the activity profile, attributable to agonist loss by the nucleotidase commonly found on cell surfaces (25), is not thought to interfere here because the period of contact with the agonist is too short in the calcium release assay used on Jurkat cells and because defolliculated oocytes (which were in Mg^{2+}-free medium) lost their nucleotidase (10). It was, however, difficult to obtain dose-response curves in the oocyte expression because of the cytotoxic effect of the P_{2Y3} receptor (even without agonist application). This effect was not blunted by the presence of apyrase, indicating that it is not caused by chronic excitation by nucleotides in the medium. Slow cell death was found, at a high level, in all sets of oocytes after injection with the P_{2Y3} receptor cRNA. This effect is unique and of unknown origin.

As in the case of the other isolated P_2 purinoceptors, P_{2Y3} does not share significant identity with the adenosine receptors. As noted above, P_{2Y3} shares an equal degree of sequence identity with the P_{2Y1} and P_{2Y2} receptor sequences. It has recently been shown (22) that a number of positively charged residues within transmembrane 6 (His262 and Arg265) and transmembrane 7 (Arg292) are important for agonist potency and specificity in the mouse P_{2Y2} receptor. Positively charged amino acids are also present at these positions in P_{2Y3}, with the conservative substitution of arginine to lysine at the equivalent position within transmembrane 6 of P_{2Y3} (Fig. 2). The RGD (arginine-glycine-aspartate) motif, implicated in integrin binding and found in the P_{2Y2} receptor (5), is absent here, despite sharing UTP responsiveness with that receptor.

In situ hybridization experiments on 1-day-posthatch chick brain have failed to reveal any specific expression of the P_{2Y3} transcript (only a diffuse overall faint labeling), whereas the transcript was detectable (relatively weakly) by Northern hybridization at this stage of development (data not shown). On the basis of these observations, we inferred that in the brain, this receptor is expressed rarely or not at all in neurons; probably, it is expressed on glia (or microglia) or endothelial cells. ATP has been demonstrated to be vasoactive in the mammalian spleen, kidney, and lung (26–28), three tissues in which P_{2Y3} mRNA is well expressed (Fig. 5); in the last two organs, ATP has been implicated in ion transport rate modulation (29, 30). Extracellular ATP also regulates surfactant secretions from lung type II alveolar epithelial cells and airway goblet cells (31, 32). It is clear that the P_{2U} (P_{2Y2}) receptor is involved in many of these cases (21, 27, 33). However, the additional presence of P_{2Y3} pharmacology would not have been recognized in the tests used on the tissues in at least some of these cases. Furthermore, the greater expression of the P_{2Y3} gene in the spleen than elsewhere could perhaps indicate a role in some cells of the immune system. Further pharmacological studies, together with localization of the receptor transcript by *in situ* hybridization in these peripheral sites, should provide clues to the function of the P_{2Y3} purinoceptor. It will also be important to seek the equivalent receptor in mammalian species.

As this article was being submitted for publication, a report of a new P_{2Y}-like receptor sequence was published (34). This sequence (in transfected rat glioma cells) is most active on UTP, with ADP being ~20-fold less potent but equipotent with 2-MeSATP: quite different from P_{2Y3} and with a different tissue mRNA distribution. This shares only 60% amino acid identity with P_{2Y3}, which is much below the chicken-mammal correspondence in the P_{2Y1} receptor. It therefore seems to be a different P_{2Y} subtype.

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