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Identification of 6H1 as a P_{2Y} Purinoceptor: $P2Y_5$

Tania E. Webb,* Mark G. Kaplan,† and Eric A. Barnard*,[1]

**Molecular Neurobiology Unit, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, United Kingdom; and †Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan 48201*

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We have determined the identity of the orphan G-protein coupled receptor cDNA, 6H1, present in activated chicken T cells, as a subtype of P_{2Y} purinoceptor. This identification is based first on the degree of sequence identity shared with recently cloned members of the P_{2Y} receptor family and second on the pharmacological profile. Upon transient expression in COS-7 cells the 6H1 receptor bound the radiolabel [^{35}S]dATPαS specifically and with high affinity (K_d, 10 nM). This specific binding could be competitively displaced by a range of ligands active at P_2 purinoceptors, with ATP being the most active (K_i, 116 nM). Such competition studies have established the following rank order of activity: ATP ADP 2-methylthioATP α,β-methyleneATP, UTP, thus confirming 6H1 as a member of the growing family of P_{2Y} purinoceptors. As the fifth receptor of this type to be identified we suggest that it be named $P2Y_5$. © 1996 Academic Press, Inc.

P_2 purinoceptors mediate the actions of extracellular nucleotides and are present on a wide range of tissues (1). A number of P_2 purinoceptor subtypes have been defined pharmacologically (P_{2X}, P_{2Y}, P_{2T}, P_{2U}, P_{2D} and P_{2Z}) and include both ligand-gated cation channels and G protein-coupled receptors, for which a recent classification into P2X and P2Y receptors respectively has been proposed (2).

Pharmacological data and the recent application of molecular biology techniques to the characterisation and identification of P2Y receptors has indicated that, as in other receptor families, further heterogeneity exists, as reviewed by Abbracchio and Burnstock (3). To our knowledge four members of the P2Y receptor family have been isolated which share up to 50% amino acid sequence identity but possess differing pharmacological profiles. At the $P2Y_1$ (P_{2Y}) receptor 2-MeSATP has greater activity than ATP and ADP and UTP is inactive (4-6), while at the $P2Y_2$ (P_{2U}) receptor UTP and ATP display approximately equal activity while 2-MeSATP is relatively inactive (7, 8). A third receptor, $P2Y_3$, displays a preference for diphosphate nucleotides (9), while yet another recently cloned member of the P_{2Y} receptor family (10) is selective for uridine nucleotides.

In this report we describe the identification of 6H1, an orphan G protein-coupled receptor cDNA isolated from activated T cells of the chicken (11), as a further member of this metabotropic purinoceptor family which we assign the name $P2Y_5$.

MATERIALS AND METHODS

Materials

All tissue culture reagents were obtained from Gibco BRL. [^{35}S]dATPαS and dATPαS were purchased from Dupont New England Nuclear. 2-MeSATP was purchased from Research Biochemicals Inc. All other nucleotides and reagents were purchased from Sigma. Buffer A has the composition: 50 mM Tris/1 mM EDTA/1 mM EGTA, adjusted to pH 7.4 with HCl, and also contains (as protease inhibitors) 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride, 0.01% (w/v) bacitracin, 0.001% (w/v) soybean trypsin inhibitor and 40 kallikrein inhibition units of aprotinin (12).

[1] To whom correspondence should be addressed. Fax: +44 171 431 1793.

Abbreviations: 2-MeSATP, 2-methylthioATP; α,β-meATP, α,β-methyleneATP; DMEM, Dulbecco's modified Eagle medium; EST, expressed sequence tag; TM, transmembrane.

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Cell Culture and Transfections

COS-7 cells were grown in DMEM supplemented with 10% (v/v) FCS, 2 mM glutamine and 100 units/ml penicillin and 0.1 mg/ml streptomycin, at 37°C under a CO_2/air atmosphere (8%/92% v/v) for 18-24 h prior to transfection. The expression construct pcDNA1/Amp6H1 (10μg) was transfected into COS-7 cells, previously plated at a density of 2 million cells/150 mm diameter dish, by incubating with Lipofectamine^{TM} (60 μg, Gibco BRL) for 6 hrs in a total volume of 9 ml DMEM. 11 ml of DMEM supplemented with antibiotics as above and 20% (v/v) FCS was then added. Media was replaced 24 hrs post transfection. The transfected cells were cultured for a further 48 hrs prior to harvesting in buffer A.

Membrane Preparation

The harvested cells in buffer A were freeze-thawed and further disrupted by homogenisation with a Ultra-Turrax J-25 homogeniser (2 × 15 sec, setting 5, cooling the suspension for 1 min. between pulses). The membranes were collected by centrifugation at 12,000 × g, 30 min in a microcentrifuge at 4°C. The supernatant was discarded, the membranes were resuspended in buffer A (1 ml) by passing through a 21G sterile needle and incubated on ice (30 min) to chelate endogenous divalent cations, destroy labile endogenous ligands and inactivate traces of proteases. The membranes were then centrifuged and washed with buffer A twice, resuspended in buffer A and frozen in liquid nitrogen before storage at −70°C.

Binding Assays

Aliquots of the membrane fraction containing 5-10 μg protein in buffer A were incubated with [^{35}S]dATPαS at a final concentration of 10 nM in the competition binding studies or ranging from 0.5-30 nM in the saturation experiments in a final volume of 0.5 ml. Non-specific binding was defined in all cases with 100 μM dATPαS. After a 60 min incubation at room temperature the membranes harvested by rapid filtration through Whatman GF/C filters (pre-soaked in 20 mM sodium pyrophosphate) and the filters were immediately washed with 3 × 5 ml of iced 50 mM Tris/HCl (pH 7.4) on a vacuum manifold (Millipore). The bound radioactivity was determined using Optiphase 'HiSafe' II (LKB) scintillant by liquid scintillation counting in a Beckman LS counter model 5000CE at a counting efficiency routinely of 95%.

All experiments were carried out in triplicate and were repeated at least three times. Data are expressed as overall mean ± s.e.mean whereas figures show representative plots. Non-specific binding ranged between 5-20% of the total binding. All binding data were analysed using the EBDA-LIGAND computer program (Biosoft, Cambridge, UK; 13). Protein concentrations were determined by the dye binding method (Biorad).

RESULTS

Screening of the GENBANK database with P_2 purinoceptor encoding sequences isolated in our laboratory identified 6H1 as a candidate P_{2Y} purinoceptor. As illustrated in Table 1 the 6H1 sequence displayed over 30% amino acid sequence identity with members of the P2Y purinoceptor family. An alignment of the 6H1 receptor amino acid sequence with the $P2Y_1$ and $P2Y_2$ is shown in Fig. 1 and its structural relatedness to all members of this receptor family is shown in Fig. 2. Sequence conservation is greatest in the TM domains with the exception of TM5 (Fig. 1).

To determine if 6H1 was indeed a P_{2Y} purinoceptor the cDNA was expressed transiently in COS-7 cells and membranes from these cells were used in binding assays with the radioligand [^{35}S]dATPαS, a ligand which we have used previously in the characterisation of the chicken $P2Y_1$ purinoceptor (12, 14). As before (12) there was negligible binding of this ligand to the membranes of mock-transfected COS-7 cells. Specific, high affinity, saturable binding was detected in the

TABLE 1
Percentage Amino Acid Sequence Identities of 6H1 with Members of the P_{2Y} Purinoceptor Family

$P2Y_1$	$P2Y_2$	$P2Y_3$	$P2Y_4$	6H1	
100	34	36	35	32	$P2Y_1$
	100	40	51	33	$P2Y_2$
		100	39	36	$P2Y_3$
			100	34	$P2Y_4$
				100	6H1

$P2Y_1$ (chick), $P2Y_2$ (mouse), $P2Y_3$ (chick), $P2Y_4$ (human); references as in the text and Fig. 2.

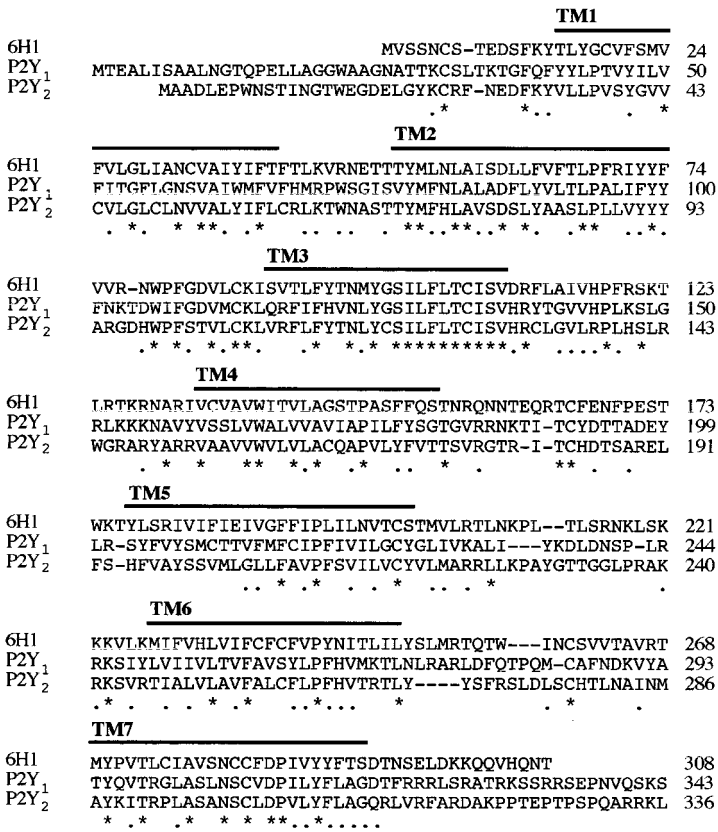

FIG. 1. Alignment of 6H1, the chick $P2Y_1$, and mouse $P2Y_2$ sequences. Residues conserved between all three sequences are marked with an asterisk (*) below the sequence and conservative substitutions are indicated by a dot (.). Spaces inserted to optimize the alignment are indicated by dashes. Predicted transmembrane domains are overlined and numbered. The $P2Y_2$ sequence is truncated at the carboxy-terminal end.

transfected COS-7 cell membranes. Scatchard analysis of the [^{35}S]dATPαS binding to these membranes resulted in a linear plot, and the data best fitted a single binding site model (Fig. 3A). The K_d value was 10.2 ± 1.5 nM and the pseudo-Hill coefficient for these sites was 0.98 ± 0.01 (Hill plot not shown). Expression levels varied between different transfections, with the maximal number of binding sites being in the range of 3 to 12 pmol/mg protein.

The pharmacological profile of this binding site was examined in displacement experiments. A number of purinoceptor ligands were able to compete for the [^{35}S]dATPαS binding sites in the transfected cell membranes (Fig. 3B). ATP displayed greatest affinity for this receptor (K_i = 116 ± 12 nM), with ADP displaying slightly lower affinity at a K_i value of 165 ± 46 nM. 2-MeSATP exhibited ~5-fold lower affinity than ATP with a K_i of 549 ± 66 nM. Very low affinity was displayed by UTP and α,β-meATP which both had K_i values greater than 10,000 nM. Neither adenosine nor AMP were able to displace binding at concentrations of up to 0.1 mM (data not shown). Thus, these data establish the rank order of affinity of the purinergic ligands tested here, in displacing [^{35}S]dATPαS binding from the recombinant receptor, as: ATP ADP 2-MeSATP α,β-meATP, UTP.

DISCUSSION

The relative affinities of the ligands tested at the transiently expressed receptor confirmed the prediction that 6H1 is a purinoceptor. However, from this profile it is clear that it possesses a subtly

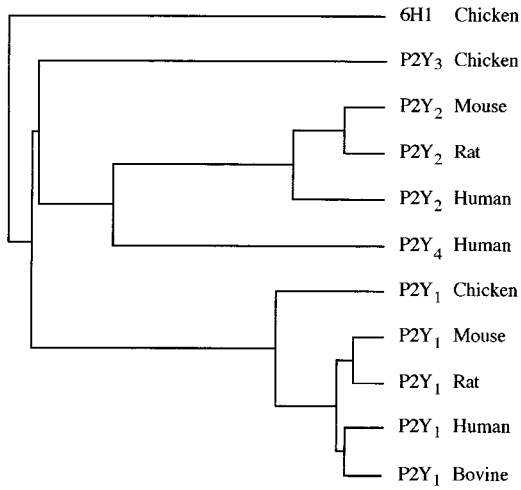

FIG. 2. Relatedness of 6H1 to other members of the P_{2Y} purinoceptor family. The dendogram was generated using the PC/Gene 6.5 CLUSTAL program (18). Sequences were obtained from the EMBL and GENBANK sequence databases. The turkey $P2Y_1$ sequence (5) is not included as there is only one amino acid difference between this and the chicken sequence. Sequences not referenced in the text, $P2Y_1$, mouse and rat (19), $P2Y_1$ human (20), $P2Y_2$ rat (21). $P2Y_3$ was isolated in our laboratory (9).

different pharmacological specificity from known P2Y receptor subtypes. For example, in contrast to the chicken $P2Y_1$ receptor, where the affinity for 2-MeSATP and ATP were in the region of 50 nM (12), the affinity for 2-MeSATP is 10-fold lower at 6H1. In addition, the relative inactivity of UTP clearly distinguishes this receptor from $P2Y_2$, at which UTP is equipotent with ATP (15). Taken together with its sequence divergence, these differences indicate that this receptor is clearly a further subtype of the P_{2Y} purinoceptors rather than being a species homologue of a previously identified receptor. In keeping with the chronological order of publication of P2Y receptors, and the recommended numbering scheme (2), we propose that this receptor be designated $P2Y_5$.

A mutagenesis study on the mouse $P2Y_2$ purinoceptor has implicated charged amino acids in

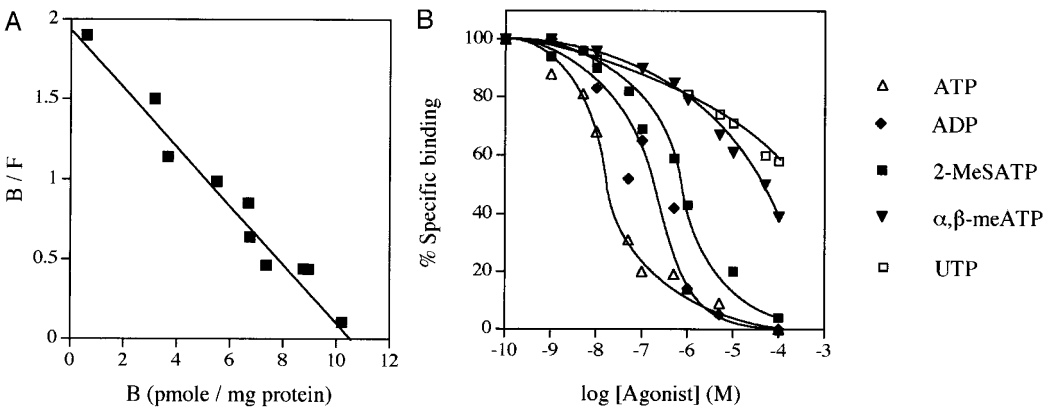

FIG. 3. Pharmacological properties of 6H1. (A) Scatchard plot of the equilibrium specific binding of [^{35}S]dATPαS (0.1 − 30 nM) to COS-7 cell membranes tranfected with the 6H1 receptor cDNA. (B) Competition curves of purinoceptor-active ligands. The results are presented as a percentage of the control specific binding levels. The curves are theoretical (Hill coefficient = 1) for a one-site binding model (which always gave the best fit). In both (A) and (B) the lines were drawn by hand. The data were obtained from a single experiment, where each point is the mean of triplicate determinations and is representative.

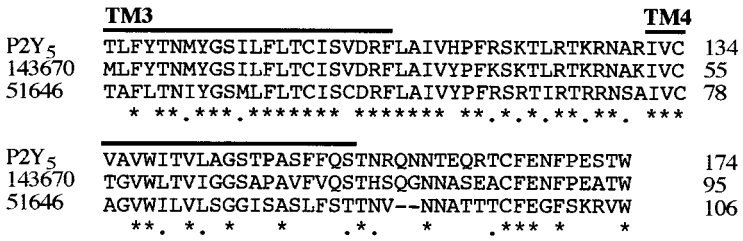

FIG. 4. Alignment of $P2Y_5$ with related ESTs. Equivalent regions of the DNA sequences of 143670 (accession number R76070) and 51646 (accession number H20663) were translated to the first ambiguity, and aligned with the same region of the amino acid sequence of $P2Y_5$. Amino acid numbers are taken from the first residue of the EST sequences and the start methionine of $P2Y_5$.

TM6 (His^{262}, Arg^{265}) and TM7 (Arg^{292}) as important for nucleotide activity at this receptor (15). $P2Y_5$ shares 33% sequence identity with $P2Y_2$ and it is interesting to note that none of these three residues are present in $P2Y_5$. A conservative substitution of His to Asn occurs at the equivalent position in TM6 of $P2Y_5$, while the other two indicated residues are each replaced by Leu.

Within the chicken, expression of the receptor transcript was absent in brain, liver, bursa and blood cells and was only detected at low levels in unstimulated spleen and thymus (11). Transcription was markedly upgraded during T cell activation, suggesting that this receptor does not participate in the initial events of T cell activation (11). Previously, Southern blotting of mammalian genomic DNA did not reveal hybridisation with related sequences (11). However, we have evidence that highly related sequences are present within the human genome. Firstly, the chicken $P2Y_5$ shares sequence identity (mid TM1 to TM7) with a region of intron 17 of the human retinoblastoma susceptibility gene (16). Furthermore, recent database screening of the expressed sequence tag database dbEST (17) with the chicken $P2Y_5$ sequence have identified related expressed human sequences, ESTs 143670 and 51646, isolated from cDNA libraries derived from placenta and infant brain respectively (Fig. 4) by the Washington University-Merck EST Project. Clone 143670 shares 75% amino acid sequence identity with the $P2Y_5$ receptor over the region compared (Fig. 4), indicating that it may be the human equivalent of this receptor. The second clone, 51646, has 63% sequence identity, suggesting that this may be a related receptor.

Thus we have identified an orphan receptor as a novel subtype of P2Y purinoceptor. The role of $P2Y_5$ in T cell activation remains to be determined, as do the functions of the related human sequences.

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