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Functional expression of human and mouse P2Y12 receptors in *Saccharomyces cerevisiae*☆

Mark H. Pausch[a,*], Margaret Lai[a], Eugene Tseng[a], Janet Paulsen[b], Brian Bates[b], Seung Kwak[c]

[a] *Discovery Neuroscience, Wyeth Research, Princeton, NJ 08543-8000, USA*
[b] *Functional Genomics, Wyeth Research, Cambridge, MA 02140, USA*
[c] *Molecular Genetics, Wyeth Research, Princeton, NJ 08543-8000, USA*

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Abstract

DNA sequences encoding the murine ortholog of the human P2Y12 receptor were cloned. The human and mouse P2Y12 receptors were expressed in a yeast cell-based GPCR expression technology containing chimeric yeast Gα protein (Gpa1) constructs in which the 5 C-terminal amino acids were identical to corresponding sequences from mammalian Gαi/o proteins. LacZ reporter gene assays of agonist-induced activation of the G protein-coupled mating signal transduction pathway revealed murine P2Y12 functional pharmacological properties that closely resembled those exhibited by the human P2Y12 receptor. In NIH3T3 cells, the mouse P2Y12 stimulated calcium uptake monitored in FLIPR via coupling to a Gαq/i3 chimeric protein. Murine P2Y12 mRNA was expressed at high levels in the brain and at lower levels in a variety of peripheral tissues. In situ hybridization analysis indicated glia-specific expression within the brain.

Keywords: Purinergic P2Y12 receptor; Yeast; Chimeric G proteins; Reporter gene assay

Platelets are critical to the maintenance of normal hemostasis. Pathological thrombus formation producing vascular occlusion can result in stroke, myocardial infarction, and unstable angina. Interaction of platelets with sites of vascular damage initiates a cascade of events involving several signaling molecules, including ADP, that lead to blood clot formation [1]. ADP is released from damaged vessels and red blood cells, induces platelet aggregation and shape change, and is also secreted by platelets from dense granules on activation, potentiating the aggregation response induced by other agents. Three types of purinergic receptors mediate the platelet response to ADP: P2X, an ADP-gated ion channel, and two types of G protein coupled receptors: P2Y1, coupled to increases in intracellular calcium via Gαq, and P2Y12, an ADP receptor coupled to inhibition of adenylyl cyclase through Gαi [2–4]. The P2Y12 receptor plays a particularly important role in the process of platelet activation [5], making it an important target for the treatment of disease related to pathological thrombus formation. Indeed, the anti-clotting drug clopidogrel selectively targets the P2Y12 receptor, which is irreversibly inactivated by an active metabolite,

☆ *Abbreviations:* SCD, synthetic complete yeast medium containing D-glucose (Dextrose); 3-AT, 3-aminotriazole; GPCR, G protein-coupled receptor; kb, kilobase; 2MeSATP, 2-methylthio adenosine 5′ triphosphate; 2MeSADP, 2-methylthio adenosine 5′ diphosphate; 2MeSAMP, 2-methylthio adenosine 5′ monophosphate; ADP, adenosine 5′ diphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ADPβS, adenosine 5′-*O*-(2-diphosphate); 2ClATP, 2-chloroadenosine 5′ triphosphate.

* Corresponding author. Fax: +1 732 274 4755.
E-mail address: pauschm@wyeth.com (M.H. Pausch).

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leading to a reduction in clot formation [6]. As well, patients with a rare heritable clotting disorder lack P2Y12 receptors [7,8]. In addition, the P2Y12 receptor may play a role in regulating certain responses of glial cells in the brain. B10 brain microvascular endothelial cells and C6 rat glioma cells expressing purinergic receptors exhibit pharmacological properties comparable to those of platelet P2Y12 receptors [9]. ADP induces chemotaxis of cultured microglia via Gαi/o-coupled P2Y12-like receptors [10].

In an effort to identify murine genes encoding orphan G protein coupled receptors, the sequence of the human orphan GPCR, EBI-2, was used to identify its murine ortholog by querying mouse genomic and cDNA databases. The predicted amino acid sequence was highly similar to those of the reported human P2Y12 sequences. The pharmacological properties of the mouse P2Y12 receptor expressed in a yeast-based GPCR expression technology [11] and in NIH3T3 cells are comparable to the human receptor [12,13]. The mouse P2Y12 receptor mRNA is expressed at high levels throughout the mouse brain and in various peripheral tissues, including those that contain platelets. The cloned murine sequence described herein likely encodes the ortholog of the human P2Y12 receptor.

Materials and methods

Materials. Purinergic ligands and 3-aminotriazole were purchased from Sigma RBI. CPRG was purchased from Boehringer–Mannheim. Mouse genomic DNA and multiple tissue Northern blots were purchased from Clontech. The chemiluminescent β-galactosidase assay kit GAL-SCREEN was purchased from TROPIX.

Identification of genomic sequences encoding the mouse P2Y12 receptor. The Celera mouse genome DNA sequence database was queried with the DNA sequence of the human EBI-2 receptor [US patent 6,060,272], using the BLASTN algorithm [14]. Overlapping genomic sequences were assembled into a single contig using Sequencher (GeneCodes). A similar search of the GenBank identified a complete cDNA sequence encoding the mouse P2Y12 receptor (Accession No. AK013804) [15].

Cloning of the human and mouse P2Y12 receptors. The human P2Y12 protein coding sequences were amplified using oligonucleotides MPO630: (5′-GTAAAGATCTAAAAATG CAAGCCGTCGACAAT CTCACC), MPO631: (5′-GTTACTCGAGTTACATTGGAGTCTCT TCATTTGGGTC) that add *Bgl*II and yeast consensus translational initiation sites to the 5′ end and an *Xho*I site to the 3′ end. The fragment was cloned into corresponding sites in the multicopy yeast expression vector, p426GPD [16], producing plasmid pET64. The *Kpn*I–*Sac*I expression cassette, which contains the GPD1 promoter, P2Y12 coding sequence, and CYC1 terminator, was transferred to corresponding sites in pRS416 [17], forming pET76.

The mouse P2Y12 receptor protein coding sequences were amplified from mouse genomic DNA using oligonucleotides MPO720 (5′-AA GGATCC AAA ATG GAT GTG CCT GGT GTC) and MPO721 (5′-AACTCGAGCTACATTGGGGT CTCTTCGC) that add *Bam*HI to the 5′ end and a *Xho*I site to the 3′ end. The fragment was cloned into corresponding sites in the multicopy yeast expression vector, p426GPD [16] producing pMP344 and into pCDNA3.1 (Invitrogen), forming pCDNA-mP2Y12.

Yeast expression. The P2Y12 yeast expression plasmids were introduced into variants of MPY578fc cells (MAT**a** ura3 his3 trp1 leu2 lys2 ade2 far1::LYS2 fus1::FUS1-HIS3 sst2::SST2-G418^{R} ste2::LEU2) [18] using LiOAc and selected for ura^{+} prototrophy. In order to facilitate coupling of the P2Y12 receptor to G proteins, MPY578fc variant cells express chimeric Gpa1 proteins coupled to the mating signal transduction pathway. The chimeric constructs are expressed from the GPA1 locus and are composed of Gpa1 sequences in which the 5 C-terminal amino acids have been replaced with those of all the mammalian Gα proteins [19]. A multicopy FUS1–LacZ reporter gene plasmid, pMP283, was constructed by transferring a *Pst*I–*Sal*I fragment encoding the FUS1 promoter and FUS1–LacZ fusion protein from pSL307 [20] into corresponding sites in pRS424 [17]. The plasmid was introduced into P2Y12 receptor-containing cells and selected on media lacking trp and ura. The resulting yeast strains were used for further analysis.

Yeast cell-based assay of human and mouse P2Y12 receptor agonist activation. MPY578fc cells that express human and mouse P2Y12 receptor, chimeric Gα protein, and pMP283 were diluted in assay medium (SCD-ura-trp, pH 6.8, 25 mM Pipes, and 1 mM 3-aminotriazole) and dispensed to the wells of 96-well microtiter dishes (5×10^6/ml, 200 μl/well) containing purinergic ligands. The plates were incubated with shaking (600 rpm) at 30 °C for 3 h. Samples (25 μl) were transferred to Wallac B&W isoplates for β-galactosidase assay. An equal volume of GAL-SCREEN lysis and chemiluminescent β-galactosidase assay mixture was added. The plates were incubated for 5–30 min at 30 °C and light emission was measured using a Wallac Victor II. Assays were conducted in quadruplicate and results were plotted using GraphPad Prizm.

Mammalian cell expression and measurement of intracellular calcium. NIH3T3 cells were stably transfected with pCDNA-Gαq/i3 by electroporation and cultured in the presence of neomycin. Stably transfected colonies were detected by RT-PCR. The NIH3T3 Gαq/i3 lines were subsequently stably transfected with pCDNA-mP2Y12 by electroporation and cultured in the presence of neomycin and hygromycin. Cells were plated without selection overnight at 37 °C. FLIPR assays were performed to measure accumulation of intracellular calcium in response to compound treatments using Fluo-4 dye (Molecular Probes) according to manufacturer's (Molecular Devices) instructions.

Northern blotting analysis of mRNA expression. A 400 bp fragment corresponding to positions +70 to +470 with respect to the A in the initiator ATG in the mouse P2Y12 sequence was PCR amplified from pMP344. Similarly, a 1344 bp fragment corresponding to positions +729 to +2073 with respect to the A in the initiator ATG in the human P2Y12 sequence was PCR amplified. The DNA fragments were labeled with [^{32}P]dCTP by random priming (Life Technologies, Rockville, MD) and used as probes to screen according to species a murine multiple tissue Northern (MTN) blot and a human immune system MTN Blot II (Clontech, Palo Alto, CA). The blots were washed at final stringency of 0.5× SSC, 0.5% SDS, at 60 °C and exposed to film. The resulting images were captured electronically for documentation.

In situ hybridization. The distribution of murine P2Y12 receptor mRNA within the mouse brain was assessed in situ as described previously [21]. Briefly, frozen mouse brains from C57/BL6 strain were sectioned on a cryostat (Bright-Hacker, Fairfield, NJ). Coronal as well as para-sagittal 15 μm sections were thaw-mounted on polylysine-coated slides and stored at −70 °C. On the day of experimentation, the slides were postfixed in 4% paraformaldehyde for 1 h. Riboprobe complementary to region +70 to +470 bp of murine P2Y12 was synthesized by an in vitro transcription reaction that incorporated [^{33}P]UTP. Approximately 2 million cpm were applied per slide for hybridization. The sections were incubated at 55 °C overnight, treated with RNase A, and then washed in 0.5× SSC at 65 °C for 1 h. The sections were initially exposed on film and subsequently dipped in emulsion for further analysis.

Results

Cloning of the murine P2Y12 receptor

Queries of the Celera mouse genome DNA sequence database using the DNA sequence of the human orphan GPCR, EBI-2, revealed fragments that once assembled into a single contiguous sequence appeared to encode a presumptive murine ortholog. Comparison of the genomic sequence with a recently reported cDNA sequence identified in a search of the GenBank indicated that the murine genomic sequence encoded the apparent protein coding sequence in a single uninterrupted exon (data not shown). Translation of the single long open reading frame yielded a predicted protein 347 amino acids in length that was 86% identical to the human P2Y12 receptor and 95% identical to the rat P2Y12 receptor (Fig. 1). The similarity between the three proteins is even greater in the transmembrane domains where only four conservative substitutions were found. The greatest divergences were found in the amino terminal extracellular domain and carboxy terminal intracellular domains. The P2Y12 receptors exhibit the greatest degree of similarity to a subset of the P2Y receptors including the UDP-glucose receptor [22] and H963 [23]. Oligonucleotides corresponding to the 5′ and 3′ ends of the predicted protein-coding region were used to amplify fragments encoding human and mouse P2Y12 receptors that were cloned into appropriate mammalian and yeast cell expression vectors.

Pharmacological analysis

The human and murine P2Y12 receptors were expressed in yeast cells modified to permit agonist-induced expression of a β-galactosidase reporter gene [11,18]. Agonist activation of the mouse receptor P2Y12 (Fig. 2A) with various purinergic ligands induces a dose-dependent activation of LacZ activity with pharmacological properties comparable to those of the cloned human P2Y12 receptor (Fig. 2B). The EC50 for ADP stimulation of the human and mouse P2Y12 receptors assayed in yeast were 147 and 258 nM, respectively. These values closely match those reported for the human P2Y12 expressed in *Xenopus* oocytes injected with the human P2Y12 receptor cRNA and coupled to stimulation of coinjected Kir3.1/Kir3.2 inwardly rectifying K+ currents (EC50 = 300 nM) [13].

In NIH3T3 cells, the mouse P2Y12 receptor stably transfected along with a chimeric Gαq/i3 construct produced an EC50 of 27 nM as measured by FLIPR. The rank orders of potency of agonist stimulation of the mouse P2Y12 receptor in both yeast (2MeSADP > ADP > ADPβS > ATPγS) and FLIPR (2MeSADP >

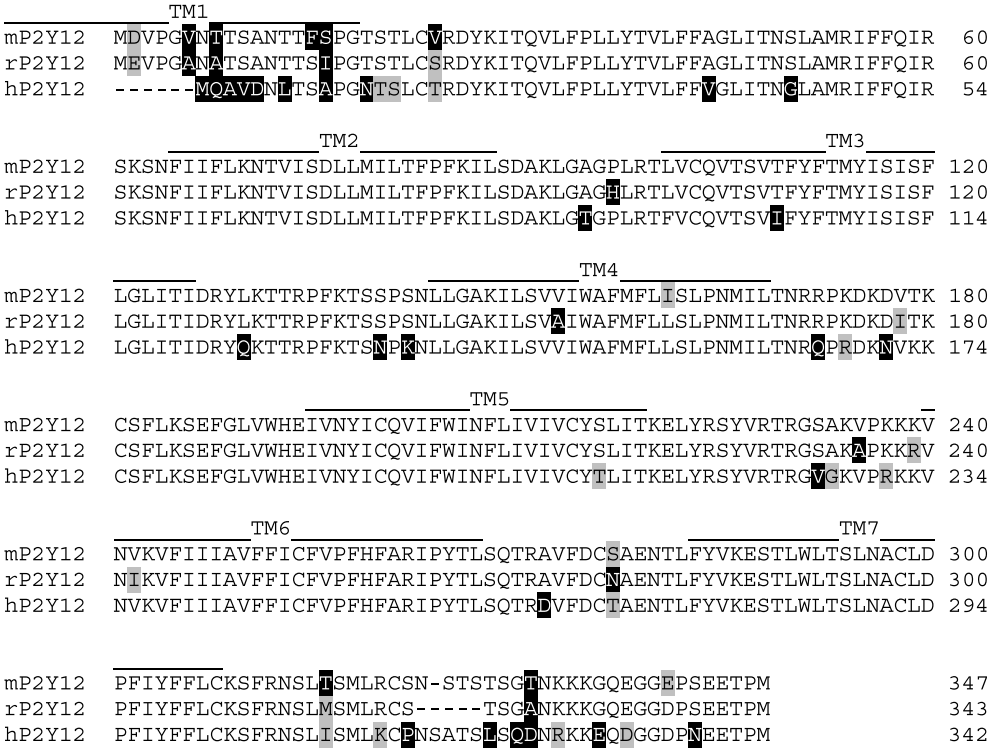

Fig. 1. Amino acid sequence alignments of human, rat, and mouse P2Y12 receptors. Non-conservative replacements are highlighted in black, conserved amino acid replacements are highlighted in grey. Putative transmembrane domains (TM1–7) are underlined. ClustalW was used to perform the alignment.

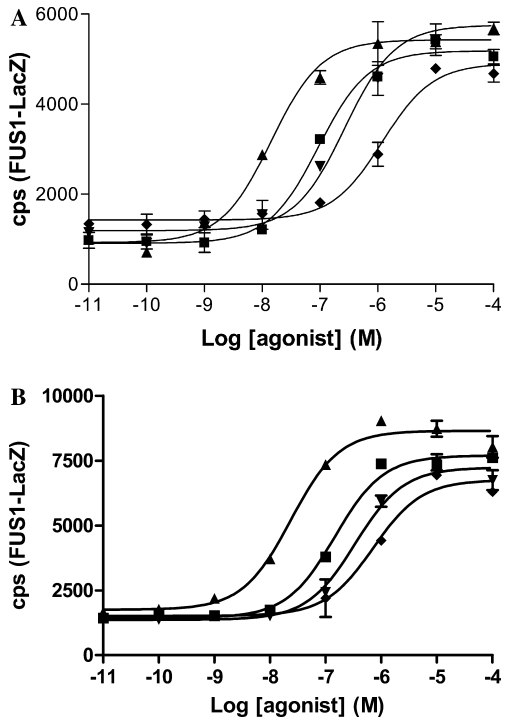

Fig. 2. Pharmacological characterization of human and mouse P2Y12 receptor expressed in yeast cells. (A) MPY578t5 cells containing pMP344 (mouse P2Y12 receptor) were assayed for agonist-induced stimulation of β-galactosidase activity as described in Materials and methods. (B) MPY578o5 cells containing pET64 (human P2Y12 receptor) were assayed for agonist-induced stimulation of β-galactosidase activity as described in Materials and methods. Symbols: square, ADP; triangle, 2MeSADP; inverted triangle, ADPβS; diamond, ATPγS.

ADP = ADPβS > ATPγS) agree well with reported values for the human P2Y12 receptor measured in FLIPR (2MeSADP = 2MeSATP > ADP = ADPβS > 2ClATP > ATPγS) and in adenylyl cyclase inhibition assays (2MeSATP > 2MeSADP > ADP > ATPγS > ADPβS > 2ClATP) [12] (see Fig. 3).

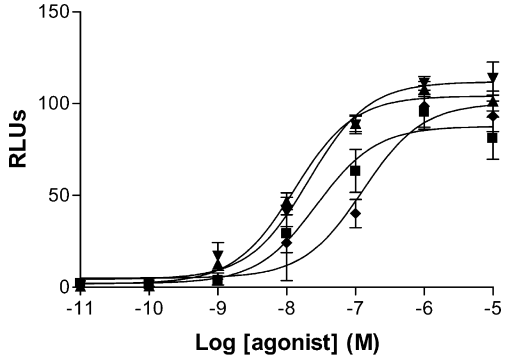

Fig. 3. P2Y12 agonist-induced release of intracellular calcium. NIH3T3 cells expressing the mouse P2Y12 and a chimeric Gαq/i3 were assayed for agonist-induced release of intracellular calcium as described in Materials and methods. Symbols: square, ADP; triangle, 2MeSADP; inverted triangle, ADPβS; and diamond, ATPγS.

Expression analysis

Northern blotting analysis was performed on mRNA extracted from various murine tissues (Fig. 4A). A single prominent 2.4 kb band was detected in brain. Lesser amounts were present in liver, spleen, and testis. A 2.6 kb band was detected as well in heart, lung, and kidney. Notably, spleen RNA contains both the 2.4 and 2.6 kb species. A similar analysis of human P2Y12 mRNA expressed in immune tissues indicates expression of multiple 2.5–3.0 kb species in spleen, lymph node, thymus, peripheral blood leukocytes (PBL), and fetal liver (Fig. 4B).

In situ hybridization of murine P2Y12 anti-sense riboprobe to mouse brain sagittal (Fig. 5A) and coronal

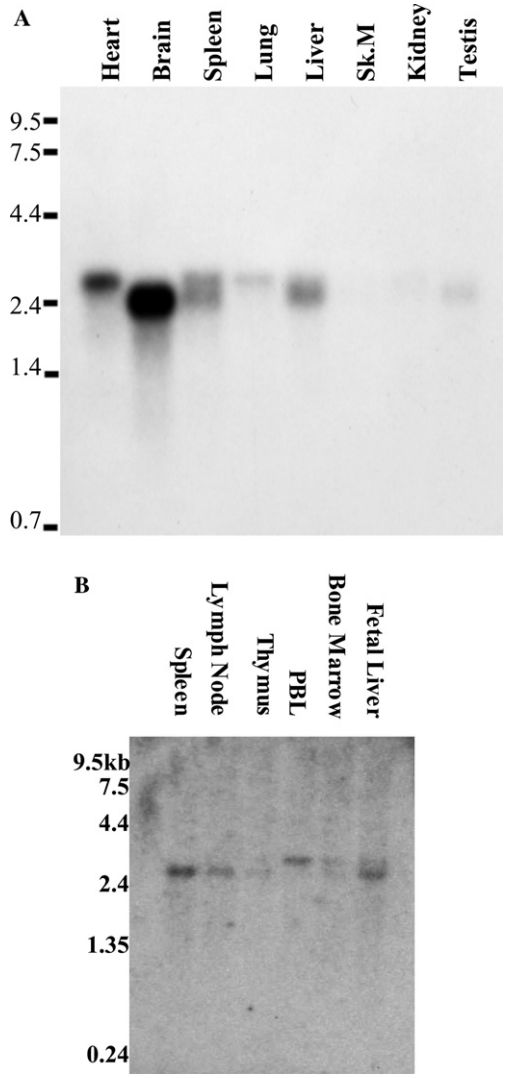

Fig. 4. Northern blotting analysis of human and mouse P2Y12 receptor mRNA expression. (A) Hybridization of a mouse P2Y12 probe to mouse multiple tissue Northern blot (Clontech) is described in Materials and methods. (B) Hybridization of a human P2Y12 probe to human immune tissue Northern blot (Clontech) is described in Materials and methods.

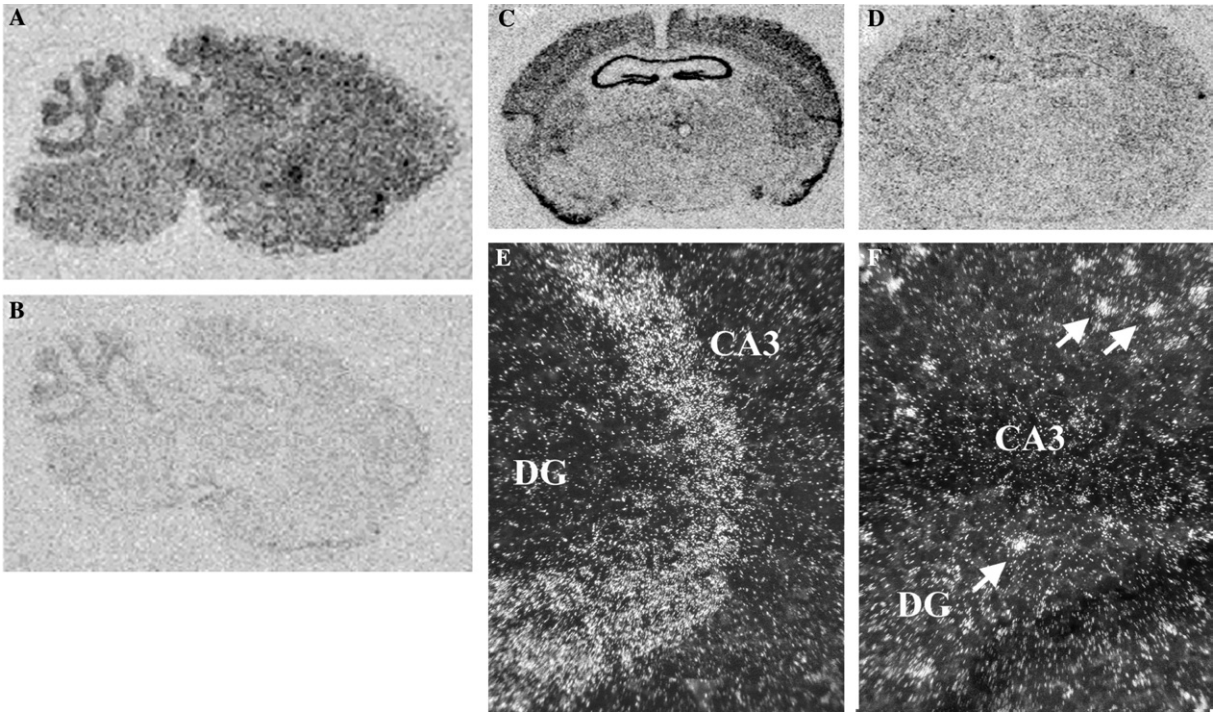

Fig. 5. In situ hybridization analysis of P2Y12 receptor mRNA expression. Hybridization of probes to mouse brain sections is described in Materials and methods. (A) Sagittal section using P2Y12 or (B) P2Y12 mis-sense riboprobe in situ. (C) Neuron-specific mRNA Wave-1 [24] or (D) P2Y12 mRNA signal on coronal plane. (E) Wave1 probe hybridizing to the pyramidal layer of the hippocampus (CA3), (F) EC323 circumvents the pyramidal layer and labels scattered glial cells in the hilus of the dentate guys (DG) and strata moleculare (open arrows).

sections (Fig. 5D) reveals broadly diffuse and uniform signal throughout the brain, while a P2Y12 sense riboprobe produced a negligible response (Fig. 5B). Unlike Wave1 mRNA [24], which is expressed in discrete layers of pyramidal and granule neurons within the hippocampus (Fig. 5C), the P2Y12 receptor signal is in all major fiber tracts including the corpus callosum and brain stem pyramidal tracts, suggesting that the mRNA is in glia. Examination at high magnification of emulsion-dipped slides counter-stained with cresyl violet confirms that murine P2Y12 receptor mRNAs are expressed exclusively in astroglial or microglial cells of the brain and not in neurons (Fig. 5F). In contrast, the WAVE1 riboprobe defines the neuronal cell bodies within the pyramidal layer of the hippocampus (Fig. 5E).

Discussion

In this report, we describe the identification of murine P2Y12 receptor genomic and cDNA sequences and the pharmacological properties of the encoded receptor. Interestingly, the highest degree of similarity between the P2Y12 receptors and other types of GPCRs is to the UDP-glucose receptor [22] and H963 [23], an orphan GPCR. The ligand for the UDP-glucose receptor was identified using a similar yeast-based GPCR expression technology [22] while H963 was cloned using a yeast-based signal sequence trapping technology [23]. These observations suggested that the P2Y12 receptor might be readily examined in our yeast GPCR expression technology. Successful expression in the yeast GPCR expression technology makes possible the use of this powerful genetic system to investigate aspects of P2Y12 receptor ligand binding and G protein coupling selectivity [18].

Our results using the yeast GPCR expression system are in excellent agreement with the three different measures of human P2Y12 pharmacological activity. That the high degree of amino acid sequence identity between mouse, rat, and human P2Y12 receptors suggesting that their pharmacological properties are quite similar is borne out of the close resemblance between rank-orders-of potency for the human and mouse P2Y12 receptors. Several amino acid residues that participate in ligand binding of the purinergic receptors have been mapped in detail [25] and many are conserved in the P2Y12 receptors. The active metabolite of the antithrombotic drug, clopidogrel, blocks P2Y12 receptor function by covalently modifying free cys residues [6]. Of the 10 cys residues in the mouse P2Y12 receptor, seven are highly conserved amongst the purinergic receptors. Two pairs of extracellular cys residues are predicted to be involved in extracellular disulfide bonds in the P2Y1 receptor [25]. In contrast, Cys17 and Cys270 of the human P2Y12 receptor, which correspond to Cys23 and Cys275 in the mouse P2Y12 sequence, are

modified by the reactive thiol reagent *p*-chloromercuribenzene sulfonic acid, making them candidates for modification by the clopidogrel active metabolite [26]. A third cys residue, C200, is found in a position corresponding to T222 of the P2Y1 receptor, a position involved in ligand binding [25]. None of the conservative differences amongst the three P2Y12 receptors are in positions thought to be involved in ligand binding.

The function of the P2Y12 receptor in the platelet response to ADP is well recognized. Less well understood is the role played by the high levels of P2Y12 receptor expressed in glial cells. ADP receptors with pharmacological properties similar to the platelet P2Y12 receptor have been identified in B10 brain endothelial cells and rat C6 glioma cells [9,10]. The Gαi-coupled ADP receptor in C6 cells responds to purinergic agonists with rank order of potency similar to that observed for the human and mouse P2Y12 (2MeSATP = 2MeSADP > ADPβS > 2ClATP = ADP = ATPγS > ATP > UTP) [10]. In the CNS, nucleotides and related compounds are often released in concert with other neurotransmitters and act as signal transduction modulators. It has been reported that treatment of astrocytes with P2Y12 selective agonists produces an increase in arachidonic acid release via a pertussis toxin sensitive G protein-dependent mechanism [27]. ADP-induced arachidonic acid release from astrocytes stimulates glycogenolysis suggesting that the ADP receptor may be involved in regulation of glycogen metabolism in the CNS. Arachidonic acid acts directly on glial glutamate transporters to inhibit uptake of glutamate [28,29], suggesting that glial ADP receptors may regulate neurotransmitter re-uptake.

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