A novel nucleotide receptor in *Xenopus* activates the cAMP second messenger pathway

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Abstract We describe a *Xenopus* P2Y receptor that shares only weak homology with members of the mammalian P2Y family, being most similar to human P2Y$_{11}$. When activated by nucleotide analogs, it stimulates both calcium and cAMP mobilization pathways, a feature unique, among mammalian P2Y receptors, to P2Y$_{11}$. Activity can be blocked by compounds known to act as antagonists of mammalian P2Y$_{11}$. Genomic synteny between ortholog of P2Y$_{11}$. *Xenopus* P2Y$_{11}$ is transcribed during embryonic development, beginning at gastrulation, and is enriched in the developing nervous system.

Keywords: P2Y$_{11}$; cAMP; Calcium; Embryo; *Xenopus*

1. Introduction

The P2Y family are G protein-coupled receptors activated by extracellular nucleotides such as ATP, ADP and UTP [1]. In mammals, eight P2Y receptors have been cloned and proven to be nucleotide receptors, either activating phospholipase C (P2Y$_1$, P2Y$_2$, P2Y$_4$ and P2Y$_6$) or inhibiting adenylyl cyclase (P2Y$_{12}$, P2Y$_{13}$ and P2Y$_{14}$). Uniquely, P2Y$_{11}$ activates both phospholipase C and adenylyl cyclase [2,3]. In humans P2Y$_{11}$ is selective for ATP [2] but in dogs it is selective for ADP [3].

Two amino acid changes in the sixth transmembrane domain appear to be responsible for this difference [4]. To date, only human and canine P2Y$_{11}$ receptor sequences have been reported. Here, we describe a *Xenopus* GPCR that has low sequence homology to previously described members of the P2Y family yet is responsive to nucleotides, increasing both intracellular calcium concentration and de novo production of cAMP. Synteny between *Xenopus* and mammalian genomes suggests we have identified an ortholog of P2Y$_{11}$. *Xenopus* P2Y$_{11}$ is expressed during embryonic development and is enriched in the nervous system.

2. Materials and methods

2.1. Xenopus studies

*Xenopus laevis* IMAGE clone 3199394 (MRC Gene Service, Cambridge, UK) was fully sequenced (Genbank accession number AM040941). RT-PCR was performed [5] using specific primers for p2y$_{11}$ (5'-TGCTACGAGGAGGAGCAAGAG-3' and 5'-TGGTCTTCAAGGAGGACCTCC-3'), ornithine decarboxylase, brachyury and sox2 [http://www.hhmi.ucla.edu/derobertis/index.html]. Whole mount in situ hybridization was performed as described [6]. Animal caps were isolated from mid-blastulae (stage 8) and incubated in 50% NAM [7], supplemented with either *Xenopus* eFGF (a kind gift of Dr. H. Isaacs) or human Activin A (Sigma), until sibling control embryos had become late-gastrulae (stage 13). Caps were also isolated from mid-blastulae previously injected with mRNA (1 ng) for *Xenopus* noggin and incubated until sibling control embryos had become mid-neurulae (stage 17).

2.2. Activity measurements

Stably expressing pools of 13121N1-*Xenopus* P2Y$_{11}$ cells, were selected with 400 μg/ml Geneticin and propagated in phenol red-free DMEM. cAMP production was determined using a modification of the method described by Salomon et al. [8]. Assays were performed in the presence of adenosine deaminase to prevent activation of endogenous adenosine receptors following ATP breakdown. Cells were pre-incubated for 30 min at 37 °C in the presence of 10 μM Ro 20-1724 and 2 IU/ml adenosine deaminase followed by a 10 min incubation at 37 °C in the presence of agonist. Antagonists were added during the pre-incubation period. Column efficiency was monitored using 14C-cAMP and the data were normalized for loading and column efficiencies. Intracellular calcium release was measured using the Fluorometric Imaging Plate Reader System (Molecular Devices) [9]. Cells were loaded with dye in the presence of 2.5 mM probenecid. Antagonists were pre-incubated for 30 min prior to data collection. Data were normalized for loading efficiency and the peak height of the response was determined.

Nucleotide analogs were prepared as described in [13]. Where indicated, 10 mM stocks of ADP and UDP were treated with hexokinase (15 U/ml) in the presence of glucose for 90 min to remove contaminating triphosphates. Statistical analyses were performed using Student’s t-test (unpaired, two-tailed).

3. Results and discussion

3.1. Identification of Xenopus P2Y$_{11}$

A cDNA encoding a putative P2Y receptor was identified in a search of *X. laevis* EST databases. The predicted 313 amino acid protein is most closely related to its *Xenopus tropicalis* ortholog, with which it shares 91% sequence identity (Fig. 1A). Homology with mammalian P2Y receptor proteins is very low, sharing a maximum sequence identity of only 35% with P2Y$_{11}$. Analysis of the *X. tropicalis* genome (*X. laevis* has not been sequenced) shows that the gene encoding the
The receptor described here is flanked by PPAN (5') and EIF3S4 (3'), with the former transcribed in the same orientation as the receptor and the latter in the opposite direction (Fig. 1B). P2Y11 is flanked by PPAN (5') and EIF3S4 (3') in most mammalian genomes for which there is available sequence, with P2Y11 and PPAN transcribed in the same orientation and EIF3S4 transcribed in the opposite orientation (Fig. 1B). Synteny extends 3' to EIF3S4, including the genes...
Edg5 and DMNT1. Despite the low sequence homology, synteny suggests we have identified the Xenopus ortholog of mammalian P2Y_{11}. To date, only human and canine P2Y_{11} orthologs have been described, although a P2Y_{11}-like sequence has been isolated from the rodent-like lagomorph species Cavia porcellus (ATN, unpublished data), the high GC content of which might explain why rodent P2Y_{11} orthologs have not yet been reported in the literature. In humans, the first six amino acids of P2Y_{11} are encoded on a separate exon, permitting intergenic splicing that creates a PPAN-P2Y_{11} chimeric protein [10]. Xenopus P2Y_{11}, however, is encoded by a single exon. The preference of P2Y_{11} orthologs for ATP or ADP is influenced by the identity of two amino acids located in TM6 [4]; these positions are occupied by histidine (H^{265}) and arginine (R^{268}) in the ATP-preferring human receptor and tyrosine (Y^{265}) and glutamine (Q^{268}) in the ADP-preferring canine receptor (Fig. 1). Xenopus P2Y_{11} has a histidine (H^{241}) and arginine (R^{244}) at these key positions. Seven amino acid residues involved in ATP recognition by the human P2Y_{11} receptor have

Fig. 2. Functional responses of X. laevis P2Y_{11} expressed in 1321N1 cells. (A) The production of cAMP and (B) the release of calcium from intracellular stores was measured in response to 100 μM of each of ATP/S, BzATP, 2MeSATP, 2MeSADP, ATP, ADP and UTP. The response to ATP/S in the presence of indomethacin (INDO; 1 μM) reflects the extent of G_{s}-coupling of Xenopus P2Y_{11}. Hexokinase (HK) has been included to dephosphorylate contaminating ATP and UTP into the corresponding diphosphate. (C) The inhibition of 100 μM 2MeSATP-stimulated cAMP production by several novel and known P2 receptor antagonists, each tested at 500 μM. The data represent the mean responses ± S.E.M. of three independent experiments, each performed in triplicate. Data are expressed as a percentage of the response to ATP/S, the most potent agonist at the human P2Y_{11} receptor. There was no response, in either functional assay, to these nucleotides in the untransfected 1321N1 cell line.

Fig. 3. Xenopus P2Y_{11} is expressed during embryogenesis. (A) RT-PCR analysis of staged Xenopus embryos using gene specific primers for P2Y_{11} and ornithine decarboxylase (ODC) – (control). O = oocytes, 6 = 32 cell stage, 10 = early gastrula, 13 = early neurula, 17 = neurula, 20 = neural tube closure, 30 = early tadpole, 40 = early tadpole – RT, control reaction performed on stage 20 mRNA in the absence of reverse transcriptase. (B) RT-PCR analysis showing that P2Y_{11} is induced in animal caps by Activin, but not by eFGF. Caps were isolated at stage 8 (blastulae) and incubated in either eFGF or Activin A, which induce ventral and dorsal mesoderm, respectively, until stage 13 (late-gastrulae). Expression of brachyury (Bra), an early response gene for both FGF and Activin, and ODC were also determined. (C) RT-PCR analysis showing that P2Y_{11} expression is induced in animal caps by Noggin, a neuralizing factor. Caps were isolated at stage 8 (blastulae) from Noggin-injected embryos and incubated until stage 17 (neurulae). Expression of Sox2, a neural marker, and ODC were also determined.
been identified: $R^{106}$, $F^{109}$, $S^{206}$, $R^{268}$, $R^{307}$, $M^{310}$, within the transmembrane domains, and $E^{186}$, in the second extracellular loop (ECL2) [11]. In Xenopus P2Y$_{11}$, four of these positions are identical ($R^{93}$, $F^{96}$, $S^{182}$ and $R^{244}$), two are conserved ($K^{271}$ and $V^{274}$) and there are two candidate glutamic acid residues ($E^{160}$ and $E^{165}$) in ECL2, which is 11 amino acids shorter than in the human receptor.

3.2. Functional properties of the X. laevis P2Y$_{11}$

A unique feature of the mammalian P2Y$_{11}$ receptor, is its ability to activate both cAMP and calcium second messenger pathways [2,3]. To determine whether this is also true of Xenopus P2Y$_{11}$ this receptor was expressed in 1321N1 human astrocytoma cells, which do not respond to extracellular nucleotides [12,13]. Several different nucleotide analogs including ATP$_7$S, the most potent agonist at the human P2Y$_{11}$ receptor [14], elicit a functional response in both assays (Fig. 2A and B). There is no statistically significant ($P = 0.61$) difference in the cAMP response to ATP$_7$S in the presence or absence of 1 $\mu$M indo- methacin, indicating that the observed response is due to the activation of adenylyl cyclase and not an indirect consequence of calcium increase. Hexokinase-treated ADP increases cAMP production above control levels ($P = 0.0004$), while hexokinase-treated UDP does not ($P = 0.43$), indicating that the diphosphate ADP has significant activity at the Xenopus P2Y$_{11}$ receptor. We tested the ability of several novel and known antagonists of P2Y receptors to inhibit functional responses at Xenopus P2Y$_{11}$ (Fig. 2C). Reactive Blue and Reactive Red are able to fully block the 2MeSATP-induced cAMP response. Suramin and Phenol Red elicit modest blockade and PPADS and Acid Blue are relatively ineffective at the highest concentrations tested. Surprisingly, none of the antagonists tested block the calcium responses elicited by three different agonists: ATP$_7$S (100 $\mu$M), BzATP (100 $\mu$M) and 2MeSATP (100 $\mu$M). Although we do not understand this phenomenon, it has previously been reported that Reactive Blue was able to inhibit ATP-induced cAMP production in HL-60 cells, but was not able to inhibit the ATP-induced calcium response in that cell line [15]. Our data show that the Xenopus receptor described here exhibits functional features unique to the P2Y$_{11}$ subtype of the mammalian P2Y family. When combined with genomic synteny this data provides compelling evidence that we have identified the Xenopus ortholog of mammalian P2Y$_{11}$.

Fig. 4. Whole mount in situ hybridization of staged Xenopus embryos. (A) Vegetal view of an early gastrula showing P2Y$_{11}$ transcripts localized to the mesoderm of the marginal zone. Strongest expression is seen in dorsal mesoderm, above the dorsal blastopore lip (dlp). (B) Section through early gastrula showing expression localized to the involuting dorsal mesoderm. The animal pole (ap) is to the right, vegetal pole (vp) to the left, and dorsal marginal zone at the top. (C) Dorsal view of an early neurula showing localized expression of P2Y$_{11}$ in the neural plate (np). Anterior (ant) is at the top and posterior (post) at the bottom. (D) Dorsal view of a late neurula showing localized expression of P2Y$_{11}$ in the neural tube (nt). Anterior (ant) is at the bottom and posterior (post) at the top. (E) Lateral view of a stage 28 embryo showing that P2Y$_{11}$ transcripts are enriched in the head and dorsal axis. The head is on the left and expression can be seen in the brain, eye, otic vesicle (ov), branchial arches (ba), neural tube, somites and pronephros (pro).
3.3. Developmental expression of the X. laevis P2Y11

Xenopus embryos were collected at different stages of development and poly A+ mRNA purified and reverse-transcribed for RT-PCR. Transcripts for P2Y11 were first detected, at low levels, in early gastrulae (stage 10), with higher levels of expression detected from early neurulae (stage 13) onwards (Fig. 3A). Whole mount in situ hybridization showed that P2Y11 transcripts were localized to the involuting dorsal mesoderm of early gastrulae (Fig. 4A and B), a region of the embryo that is both an important signaling center and an area of extensive cell and tissue movements. Using an animal cap assay we showed that P2Y11 expression is induced by Activin, a dorsal mesoderm inducing factor, but not by eFGF, a ventral mesoderm inducing factor (Fig. 3B), consistent with expression of P2Y11 in the dorsal mesoderm of early gastrulae. At neural plate stages P2Y11 transcripts, like those of P2Y8 [16], are localized to the developing nervous system (Fig. 4C and D) and expression is induced in animal caps by Noggin, a neuralizing factor (Fig. 3C). P2Y11 is expressed throughout the dorsal axis at tailbud stages, with staining in the brain, eye, lens, otic vesicle, brachial arches, spinal cord, notochord, somites, and pronephric kidney (Fig. 2).

Our results are the first to demonstrate a cAMP-coupled nucleotide receptor in Xenopus. Nucleotide signaling via the P2Y11 receptor may take place through both the calcium and cAMP pathways during Xenopus development. The developmental consequences of this dual signaling capability remain to be elucidated.

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