Early Expression of a Novel Nucleotide Receptor in the Neural Plate of Xenopus Embryos*

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Extracellular ATP functions as a neurotransmitter and neuromodulator in the adult nervous system, and a signaling molecule in non-neural tissue, acting either via ligand-gated ion channels (P2X) or G-protein-coupled receptors (P2Y). ATP can cause an increase in intracellular Ca\(^{2+}\) in embryonic cells and so regulate cell proliferation, migration, and differentiation. We have isolated a Xenopus cDNA encoding a novel P2Y receptor, XIP2Y, which is expressed abundantly in developing embryos. Recombinant XIP2Y responds equally to all five naturally occurring nucleoside triphosphates (ATP, UTP, CTP, GTP, and ITP), which elicit a biphasic 1-dependent Cl\(^{-}\) current (I\(_{\text{Cl,Ca}}\)) where the second phase persists for up to 60 min. XIP2Y also causes a continuous release of Ca\(^{2+}\) and a low level persistent activation of I\(_{\text{Cl,Ca}}\) in Xenopus oocytes through the spontaneous efflux of ATP. mRNAs for XIP2Y are expressed transiently in the neural plate and tailbud during Xenopus development, coincident with neurogenesis. This restricted pattern of expression and novel pharmacological features confer unique properties to XIP2Y, which may play a key role in the early development of neural tissue.

Receptors for extracellular nucleotides (P2 receptors) are found on the cell surface of all higher animal tissues, where they regulate a broad range of physiological processes (1). These receptors have been categorized into two major groups (P2X and P2Y), based on their pharmacological and electrophysiological properties, as well as their molecular structure (2, 3). P2X receptors are members of the ligand-gated ion channel superfamily, while P2Y receptors have seven transmembrane domains and belong to the G-protein-coupled-receptor superfamily. To date, seven P2X (P2X\(_1\)–7) and seven P2Y (P2Y\(_1\)–7) receptors have been cloned from mammalian and avian species (3). Different P2Y receptors show preferential selectivity for purine and pyrimidine nucleotides; whereas P2Y\(_1\) is responsive to ATP but not UTP (4), P2Y\(_2\) is equally responsive to both ATP and UTP (5) and P2Y\(_4\) is responsive to UTP and much less to ATP (6, 7). p2y\(_3\) and P2Y\(_6\) are selective for nucleoside diphosphates (8, 9), while p2y5 and P2Y\(_7\) bind ATP with a greater avidity than UTP (10, 11). In most cell types P2Y receptor activation results in the hydrolysis of phosphatidylinositol (4,5)-bisphosphate to the Ca\(^{2+}\)-mobilizing second messenger inositol (1,4,5)-triphosphate and diacylglycerol, a process catalyzed by PLC-\(\beta\) (12).

Cell-cell interactions are crucially important during early embryonic development, providing the impetus to establish and maintain different cell fates, regulate morphogenesis, and control cell differentiation (13). While most of the current evidence suggests that secreted polypeptide growth factors, such as the fibroblast growth factor and transforming growth factor-\(\beta\) superfamily, are the major intercellular signaling molecules in embryonic development (14), there is some evidence to suggest a role for nonpeptide signaling molecules such as serotonin, noradrenaline, and dopamine (15). For example, in the fruit fly Drosophila melanogaster, a serotonin receptor is expressed in even parasegments at the cellular blastoderm, an expression pattern similar to that of the pair-rule gene fushi-tarazu (16). Although the role of this receptor in Drosophila development is currently unknown, it must be functioning is suggested by the presence of ligand at the same stage of development. In Xenopus neurulae, while \(\alpha\)-adrenergic receptor antagonists and inhibitors of dopamine \(\beta\)-hydroxylase inhibit neuronal differentiation, exogenous noradrenaline or dopamine can increase the number of neurons differentiating in neural cultures (17). This suggests that endogenous noradrenaline, acting via \(\alpha\)-adrenergic receptors, is part of the mechanism controlling neuronal differentiation in the central nervous system. Very little is known about the roles of extracellular ATP in early embryonic development, but this molecule has been shown to cause an increase in intracellular Ca\(^{2+}\) \((\text{Ca}^{2+})_2\) concentration in a number of embryonic cell types, including dissociated cells from early chick embryos (18), early embryonic chick otocyst (19) and retina (20), cultured astrocytes from embryonic rat spinal cord (21), cultured neurons from embryonic rat brain (22), myotubes cultured from embryonic chick (23), and a murine myoblast cell line (24). Increases in Ca\(^{2+}\) are known to regulate cell proliferation, migration, and differentiation (15, 25), all important processes during embryonic development. The P2 receptor(s) responsible for most of these ATP-activated developmental signals have not been identified, and none have been cloned, although the response of embryonic chick retina is thought to be mediated by a P2Y receptor responsive to UTP (20).

In this study, we have isolated a cDNA encoding a novel P2Y

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‡ Following the Purines July 6–9, 1996 meeting (Milan, Italy), the IUPHAR nomenclature committee has recommended that the mammalian and non-mammalian P2 receptors already cloned should be distinguished by the use of upper and lowercase alphanumerics (e.g. chick p2y3 but human P2Y3).
receptor, XlP2Y, that is expressed during neurulation in Xenopus embryos. XlP2Y is equally responsive to all five naturally occurring nucleoside triphosphates (ATP, UTP, CTP, GTP, and ITP) and, when expressed in Xenopus oocytes, exhibits an unusually long response to agonists. Our results show that expression of this receptor occurs transiently during embryonic development, being coincident with the phase of primary neurulation, and suggest that this receptor may play an important role in the early development of neural tissue in Xenopus laevis.

**EXPERIMENTAL PROCEDURES**

**Reverse Transcriptase-Polymerase Chain Reaction**—Degenerate oligonucleotide primers based on the sequence of transmembrane domains III and VII of chick P2Y1 (4) and murine P2Y2 (5) were used to amplify fragments of approximately 574 bp from cDNA synthesized from Xenopus neurula total RNA. The forward primer was 5'-GCAGCATCCT(G/C)TTCCTCAC(G/C)TGCAT-3' (amino acid sequence SILFLTCI), and the reverse primer was 5'-CCC(G/A/T)GCCAGGAAGTAGAG(G/T/C)(G/C)GG-3' (amino acid sequence P(M/I/V)LYFLAG). The polymerase chain reaction (PCR) amplification conditions were 94 °C for 60 s, 55 °C for 45 s, 72 °C for 60 s for 30 cycles, followed by 72 °C for 10 min. PCR fragments were subcloned into the pCRII TA cloning vector (Invitrogen) and sequenced by the dideoxy chain termination method.

**Library Screening**—The Xenopus P2Y PCR fragment was used as a probe to screen 7.3 x 10^5 recombinant phage of a Xenopus stage 17 (mid-neurula) cDNA library in λgt10 (26). Hybridization was performed at 65 °C in buffer containing 4 x SSC, 50 mM EDTA, 5 x Denhardt’s conditions.
solution, 1% SDS, 0.1 mg/ml salmon sperm DNA (Sigma). Final washing of membranes was at 65°C in 0.2× SSC, 0.1% SDS. The cDNA of the longest positive clone was subcloned into pBluescript II KS(−) (Stratagene) and sequenced by the dye-deoxy chain termination method.

Embryos—Unfertilized eggs were obtained from females of X. laevis previously injected with 500 units of human chorionic gonadotrophin (Inserm). They were fertilized with a piece of macerated testis, dejellied in 2% cysteine hydrochloride (Sigma), reared in 10% Barth’s solution (Barth’s solution is 110 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 7.5 mM Tris-HCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 50 μg/liter gentamycin, adjusted to pH 7.45) and staged according to Nieuwkoop and Faber (27). Exogastrulae were generated by incubating embryos in full-strength Barth’s solution throughout development. Embryos were then UV-irradiated at the vegetal pole 35 min after fertilization. Dissections were carried out in full-strength Barth’s solution, using forceps and electroejaculated sharpened tungsten needles.

Northern Blots—Total RNA was isolated from staged embryos and embryo fragments, separated on formaldehyde-agarose gels, and transferred by Hybond N† (Amersham) using standard techniques (28). Blots were sequentially probed, under high stringency, with random primed probes for XIP2Y and Xenopus histone H4, the latter acting as a loading control.

Whole Mount in Situ Hybridization—Whole mount in situ hybridization was performed on albino embryos as described previously (29), with the exception that an RNase digestion step was not included, CHAPS buffer was replaced by maleic acid, and BPURM (Boehringer Mannheim) was used as a substrate. Sense and antisense digoxigenin (Boehringer Mannheim) labeled probes for XIP2Y were made by transcription of the initial PCR clone either with T7 (antisense) or SP6 (sense) RNA polymerase according to the manufacturer’s instructions.

In Vitro Transcription and Translation—A 2.361-kilobase fragment of XIP2Y was subcloned into the pRN3 transcription vector (30), and capped synthetic RNA was transcribed with T3 RNA polymerase using the relevant Megascript kit (Ambion). For detection of the XIP2Y translation product, XIP2Y in pRN3 was added to an aliquot of a TNT-T3-coupled reticulocyte lysate system (Promega) along with 40 μCi of P[35S]Methionine (Amersham). As controls, either water or a luciferase cDNA provided by the manufacturers was added to parallel reactions. The subsequent translation products were separated by SDS-PAGE on a mini-gel system (Bio-Rad) according to the manufacturer’s instructions, and prepared for fluorography by immersion in EN'HANCE (DuPont NEN).

Oocyte Preparation, Oocyte Injections, and Electrophysiology—Xenopus oocytes (stages V and VI) were plucked off the inner ovarian epithelial lining with fine forceps and stored (at 4°C) in Barth’s solution, 1% SDS, 0.1 mg/ml salmon sperm DNA (Sigma), g/liter gentamycin, adjusted to pH 7.45) and incubated at 18°C for 49 h in Barth’s solution to allow full expression of this receptor. Control oocytes were injected with either sterile water (40 nl) or an antisense RNA for chick P2Y (40 nl, 1 mg/ml) and incubated under the same conditions.

Nucleotide-activated currents (I_{tRNA}) were recorded from injected oocytes held under voltage-clamp (V_m = −40 mV) using a twin-electrode amplifier (Axoclamp 2A). I_{tRNA} is routinely used as a reporter current for the activation of phospholipase C, IP₃ production, and release of Ca²⁺ by G-protein-coupled receptors, including P2Y subtypes (33). The voltage-clamping and current-recording electrodes (1–2 megohms tip resistance) were filled with 0.6 M KSO₄ and 3 M KCl, respectively. Oocytes were superfused (5 ml/min) with Ringer’s salt solution (at 18°C) containing (mM): NaCl, 110; KCl, 2.5; HEPES, 5, CaCl₂, 1.8; adjusted to pH 7.45. Nucleotides (Sigma and Research Biochemicals International) were added to the superfuse at the concentrations given in the text for periods of 60–180 s, followed by a period of washout of 10 min. Evoked responses were recorded on magnetic tape using a DAT recorder (Sony 1000ES) and displayed using a pen recorder (Gould). For the concentration-response curve for ATP, data were normalized to the amplitude of responses obtained using ATP (1 μM), which gave submaximal responses. A submaximal standard was chosen because it was only possible to test 3–4 concentrations of ATP or other nucleotides on one oocyte, given the long duration (approximately 40–60 min) of each response. Pharmacological drugs are expressed as the mean of three observations from separate oocytes.

RESULTS

Cloning and Sequence of Xenopus Embryonic P2Y Receptor—Using degenerate oligonucleotide primers based on the P2Y₁ and P2Y₂ sequences encoding the highly conserved transmembrane domains III and VII, we used reverse transcriptase-PCR to amplify P2Y sequences from cDNA made from an RNA pool extracted from X. laevis neurulae. A 560-bp fragment was identified among the resultant products, and sequencing suggested that it encoded a novel P2Y receptor. This PCR fragment was used to screen a X. laevis neurula (stage 17) cDNA library, and a number of positive clones were identified, the largest insert size being 2.361 kilobases. Sequence analysis of this insert revealed an open reading frame of 1611 bp, but the closest fit to the Kozak translation initiation consensus sequence (34) is met by the sequence surrounding a second ATG 15 bp downstream (Fig. 1). Translation from this second ATG would produce a protein of 532 amino acids, which is somewhat larger than the 308–377 amino acids described for P2Y₁–7. This is the consequence of a relatively long carboxyl-terminal tail of 216 amino acids (Fig. 1), compared with the 16–67 amino acids for P2Y₁–7. The long carboxyl-terminal tail includes a number of potential phosphorylation sites for protein kinase C (C), protein kinase A (X), calmodulin-dependent kinase (X), OSK3 (X4), and tyrosine kinase (X). There is also a single site for phosphorylation by protein kinase C in the third intracellular loop. To confirm that XIP2Y does indeed encode a protein with a long carboxyl-terminal tail, we have produced the translation product in a combined in vitro transcription-translation system and analyzed it by SDS-PAGE (Fig. 2). XIP2Y has an M_r of approximately 56–57 × 10⁵ compared with the predicted M_r of 61 × 10⁵. Although XIP2Y migrates faster than predicted from its amino acid sequence, which is not uncommon for proteins separated by SDS-PAGE, it is still significantly slower than is observed with other P2Y receptors.

Hydropathy analysis of the predicted XIP2Y receptor protein revealed the presence of seven putative transmembrane domains, a feature characteristic of G-protein-coupled receptors. The NH₂-terminal domain contains two potential asparagine-linked glycosylation sites and a cysteine that is conserved in all the known members of the P2Y family. A single conserved cysteine is also found in each of the three putative extracellular loops, these four cysteines probably forming two disulfide bonds. A cysteine in the carboxyl-terminal tail, which is conserved in many G-protein-coupled receptors, may be a membrane-anchoring palmitoylation site. The amino acid sequence...
of XIP2Y was compared with the sequences of the seven previously reported P2Y receptors, and a total of 26 amino acids were found to be absolutely conserved (Fig. 3). We note a high degree of homology between XIP2Y and P2Y1–6 in TM III and between XIP2Y and both P2Y2 and P2Y4 in TM VII. XIP2Y is most closely related to the UTP receptors P2Y4 (62% identical) and P2Y2 (56% identical) and least related to P2Y7 (26% identical). However, neither P2Y2 nor P2Y4 possess a carboxyl terminus of a similar length as XIP2Y, suggesting that XIP2Y is not the Xenopus homologue of these mammalian receptors.

Four positively charged amino acids (His 273, Arg 276, Lys 300, and Arg 303) reported (35) to play a role in P2Y2 receptor activation by ATP and UTP (36) and the seven putative transmembrane domains (bars) of XIP2Y. Note the highly conserved sequence in TM III (SILFLTCSI) and the strong homology between XIP2Y and the UTP receptors P2Y2 and P2Y4 in TM VII (YKVTRPLASANCI). The alignment was made using CLUSTAL W; only sequences between the highly conserved NH2-terminal cysteine and the end of TM VII were included in the analysis (amino acid numbers are indicated at the end of the alignment). Proteins aligned to XIP2Y are chick P2Y1 (X73288), human P2Y2 (U07225), chick P2Y3 (X98283), human P2Y2 (X97058), and human P2Y4 (U41070). Gaps (-) were introduced to maximize the alignment, and only non-conserved residues are indicated (*), as are the four positively charged amino acids reported to play a role in P2Y2 receptor activation by ATP and UTP (36) and the seven putative transmembrane domains (bars) of XIP2Y.

Temporal Expression and Tissue Distribution of XIP2Y—Embryos of X. laevis were collected at different stages over a period of 3 days post-fertilization. Total RNA was prepared from these embryos, and the temporal pattern of XIP2Y transcription was determined by Northern blotting (Fig. 4A). XIP2Y was undetectable during the earliest stages of development, from fertilization (stage 1) to onset of gastrulation (stage 10), but a single transcript of approximately 3.5 kilobases was detected at the beginning of neurulation (stage 13). Transcript levels remained high throughout neurulation (stages 13–20) before dropping to a lower level at tadpole stages. This lower level was maintained until stage 40, the last embryonic stage that we have examined. Since it took these embryos approximately 9 h to progress from stage 10 to stage 13, we have determined the time point at which XIP2Y expression is initiated by collecting embryos at 1-h intervals following the initiation of gastrulation at stage 10. A Northern blot of total RNA prepared from these embryos shows that XIP2Y transcripts can first be detected 7 h after the onset of gastrulation, and reaches maximal levels by 8 h (Fig. 4B). XIP2Y expression is therefore initiated during the later phases of gastrulation.

The regional distribution of XIP2Y was determined by dissecting Xenopus neurulae (stage 17) into dorsal anterior, dorsal posterior, and ventral regions. Northern blot analysis of total RNA isolated from these regions showed that XIP2Y transcripts are most abundantly expressed in dorsal regions of the embryo, which differentiates into neural tissues, notochord, and somites, although transcripts can also be detected ventrally.

Cloning and Expression of XIP2Y

Fig. 3. Alignment of the amino acid sequence of XIP2Y with P2Y<sub>2</sub>–<sub>7</sub>. The alignment was made using CLUSTAL W; only sequences between the highly conserved NH<sub>2</sub>-terminal cysteine and the end of TM VII were included in the analysis (amino acid numbers are indicated at the end of the alignment). Proteins aligned to XIP2Y are chick P2Y1 (X73288), human P2Y2 (U07225), chick P2Y3 (X98283), human P2Y2 (X97058), and human P2Y4 (U41070). Gaps (-) were introduced to maximize the alignment, and only non-conserved residues are indicated (*), as are the four positively charged amino acids reported to play a role in P2Y2 receptor activation by ATP and UTP (36) and the seven putative transmembrane domains (bars) of XIP2Y.
expression was reduced in embryos irradiated with UV light during the first cell cycle (Fig. 4C), a treatment that reduces the development of dorsal tissues (36). XIP2Y expression was also reduced in embryos cultured in a high salt solution during gastrulation (Fig. 4C). Under these conditions the mesoderm does not involute under the ectoderm, and the expression of several neural tissue-specific, but not mesoderm-specific, genes are reduced (37). These results suggest that XIP2Y is most abundantly expressed in the developing neural tissue during the process of neurulation, when the open neural plate folds to form the neural tube. To confirm this suggestion, we analyzed embryos by whole mount in situ hybridization using a digoxigenin-labeled probe for XIP2Y. Transcripts were detected first at stage 13–14 in an arc corresponding to the anterior ridge of the neural plate (Fig. 5A), subsequently spreading throughout the neural plate (Fig. 5B). After neural tube closure XIP2Y was no longer detectable in caudal regions of the neural tube but was detectable in neural tissue emerging from the tailbud (Fig. 5C).

Functional Expression of XIP2Y in Xenopus Oocytes—Defolliculated oocytes injected with XIP2Y RNA responded to low concentrations of ATP (1 μM and greater) with a biphasic current, where the second phase persisted for 40–60 min after brief superfusion (60–180 s) of agonists (Fig. 6, A and B). Because of these prolonged responses, it was necessary to leave a period of 60 min between ATP applications to evoke responses of similar amplitude and without sign of receptor desensitization. This basic feature of XIP2Y receptor activation is markedly different from the pattern of agonist activation of other recombinant P2Y receptors (e.g. P2Y1–3) that have been expressed in oocytes, where responses are shorter (1–3 min) and reproducible within 20 min of the first agonist application (4, 5, 8). ATP responses at XIP2Y were gradually reduced, then abolished, in Ca2+-free conditions but returned when extracellular Ca2+ was restored, indicating XIP2Y mobilized Ca2+ ions, which was replenished from an extracellular pool. ATP responses were reduced by thapsigargin (100 μM), which activates and then desensitizes IP3 receptors and inhibited by the chloride channel (ICl,Cl) blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (60 μM), indicating XIP2Y activation elevated PLC-β production of IP3 to release Ca2+ and open Ca2+-activated chloride channels that carried a biphasic current.

Half-maximal activation (in terms of the EC50) by ATP required 103 nM for the first phase of current and 80 nM for the second slower phase (Fig. 6B). The Hill co-efficient (nH) was approximately 1, indicating one molecule of ATP is necessary to activate the receptor. XIP2Y was also activated by all naturally occurring nucleoside triphosphates (CTP, GTP, ITP, and UTP) but not by inorganic triphosphates and diphosphates (trisodium trimetaphosphate, pentasodium triphosphate, and sodium pyrophosphate; all 100 μM), confirming the requirement for a nucleotide and not just a phosphate chain. The rank order of potency for nucleoside polyphosphates was (at 100 μM): ADPβS > ATP = CTP = GTP = ITP = UTP > ATP–S > ADP = β,γ-methylene ATP > AMP = 2-methylthio-ATP > α,β-methylene ATP = 2',3'-O-(4-benzoylebenzoyl)-ATP. XIP2Y was weakly stimulated by diadenosine polyphosphates and adenosine, where (at 100 μM): ATP > ADP > ApA > ApA > ApA > ApA > adenosine. On the basis of structure/activity relationship, XIP2Y required a nucleotide and either triphosphate chain or a diphosphate chain with a phosphoro- thioate or methylenephosphonate extension, but did not tolerate substitution at the C-2 position on the adenine base.

Oocytes expressing XIP2Y showed a persistent, strongly rectifying inward current (Ii), which reversed to outward current at −35 mV, then reverted to an inward current at −10 mV (Fig. 6, C and D). This persistent current was not found in control oocytes. The amplitude of Ii was inhibited by the P2 receptor antagonist suramin (1–100 μM) with an IC50 value of 27 ± 4 μM (Fig. 6C), indicating a low level activation by endogenous ATP and suggesting Ii was, in part, Ii,Cl,Cl. Measurement of ATP release by the firefly assay showed a rate of release of 2 ± 0.25 nmol/h/oocyte. Since there is little enzymatic breakdown of extracellular ATP by oocytes (38), the basal efflux of ATP may continuously stimulate XIP2Y and elevate Ca2+, to persistently

![Fig. 4. Northern blot analysis of XIP2Y expression in Xenopus embryos.](http://www.jbc.org/)

![Fig. 5. Spatial expression of XIP2Y in Xenopus embryos.](http://www.jbc.org/)

Whole mount in situ hybridization of staged Xenopus embryos showing expression of XIP2Y in the neural plate and tailbud. A, stage 14. B, stage 17. C, stage 28 tailbud. Ant, anterior; Post, posterior; NP, neural plate.
activate $I_{Cl, Ca}$.

This persistently activated $I_{Cl, Ca}$ had a significant impact on the resting membrane potential and input resistance of oocytes expressing XIP2Y receptors. In comparison to un.injected (control) oocytes, XIP2Y oocytes were depolarized by more than 30 mV and their input resistance lowered by as much as 4-fold (see Table I). The resting membrane potential ($E_m$) of XIP2Y-oocytes lay close to the reversal potential for chloride ions (oocyte $E_{Cl} = -24$ mV) (39), indicating that an ATP-activated chloride conductance was a major factor in determining $E_m$. Suramin (100 μM) significantly increased $E_m$ and input resistance of XIP2Y oocytes, blocking XIP2Y receptors and preventing their activation by a persistent ATP efflux. The electrical properties of XIP2Y oocytes in the presence of suramin closely matched $E_m$ and input resistance of oocytes expressing other P2X subtypes, including chick P2Y$_1$ and a P2Y$_2$-like subtype found in rat cortical astrocytes (40). Values for $E_m$ and input resistance for oocytes expressing either rat P2X$_3$ or rat P2X$_4$ were significantly higher than XIP2Y oocytes, even in the presence of suramin (Table I). These P2X receptors show a higher concentration threshold for ATP activation and also desensitize rapidly; accordingly, the impact of ATP efflux appears to be negligible. Differences in the input resistance of oocytes expressing P2X subtypes and uninjected oocytes may reflect the damage caused to the membrane by the intracellular injection of P2 receptor transcripts.

**DISCUSSION**

We have isolated a cDNA for a G-protein-coupled receptor for extracellular nucleotides (P2Y receptor) that is expressed during early embryonic development in *X. laevis*. To our knowledge this is the first receptor of this class to be cloned in amphibians, and the first vertebrate P2Y shown to be expressed during early embryonic development.

From expression studies in defolliculated *Xenopus* oocytes, XIP2Y possessed several unique pharmacological features when compared with previously described recombinant P2Y$_{1-7}$ subtypes. The first major feature involved the duration of biphasic responses to agonists, some 40–60 min for the second phase with any of the naturally occurring nucleoside triphosphates. These biphasic responses were considerably longer than the 1–3 min observed following expression of P2Y$_1$, P2Y$_2$, and p2y3 in *Xenopus* oocytes (4, 8, 41, 42). Prolonged membrane currents evoked by XIP2Y activation were carried mainly by $I_{Cl, Ca}$, based on their sensitivity to 5-nitro-2-(3-phenylpropylamino)benzoic acid (43) and thapsigargin (44), and long term dependence on extracellular Ca$_{2+}$ to help replenish Ca$_{2+}$ stores. The long duration of XIP2Y responses may not necessarily reflect the situation in the neural plate for a number of reasons, including receptor density, agonist concentration, and receptor/signaling cross-talk. We are currently investigating this issue in neural plate-derived cells.

A second unique feature of XIP2Y is the broad agonist selectivity, where all of the naturally occurring nucleoside triphosphates (ATP, CTP, GTP, ITP, and UTP) proved equally effective. None of the previously described (4–11) recombinant P2Y receptors (P2Y$_{1-7}$) are stimulated by all five nucleotides, while P2Y$_4$ is the only other P2 receptor to be stimulated by ATP and UTP equally (5). Since defolliculated oocytes are devoid of native P2Y or P2X receptors (31, 32), it is unlikely that stimulation of an endogenous receptor contributed to this broad selectivity. A third distinguishing feature of XIP2Y is a low level of continuous activation, probably by the basal efflux of ATP from oocytes, although a constitutive activation of XIP2Y without the need of an agonist cannot be ruled out. The rate of
spontaneous release of ATP was 2 nmol/h, although the local concentration of ATP at the surface of the oocyte may be higher. We found the threshold for activation of a macroscopic whole-cell current was in the region of 10 nM, but small differences in the resting conductance of the oocyte membrane may occur at ATP concentrations lower than this level. Webb and colleagues (8) suggested that chick p2y3 expressed in Jurkat cells may be activated by ATP efflux from the host cell, since this P2Y receptor remains desensitized until an ecto-ATPase, apyrase, is added to the bathing medium. A similar desensitization was observed for bovine P2Y1 expressed in Jurkat cells, and relaxed by the addition of apyrase to the bathing medium (45). In a similar vein, Nakamura and Strittmatter (46) found that human P2Y1, expressed in oocytes, is activated transiently and relaxed by the addition of apyrase to the bathing medium. However, although there is an early response to these signals, it may be the case that all recombinant P2 receptors are partially activated/desensitized in most expression systems, but the prolonged responses of XIP2Y make this feature more noticeable. The persistent activation of XIP2Y expressed in oocytes considerably depolarized these cells, and we are currently looking for a similar effect in neural plate-derived cells.

In increases in Ca^{2+}, are a common response to ATP stimulation in many cell types (12), and increases in Ca^{2+}, are thought to play important roles in regulating cell proliferation, migration and differentiation (15, 25). In the Urodole amphibian Pleurodeles waltl, reagents that cause an increase in Ca^{2+}, promote neural development in ectoderm isolated from early gastrulae. Treating gastrulae ectoderm with either caffeine or ryanodine has been reported to cause a transitory (10–20 min) release of Ca^{2+} from intracellular stores, the ectoderm subsequently differentiating neurons and glia (47). Similarly, reagents such as the lectin concanavalin A and phorbol esters, which can induce neural development in amphibian ectoderm (48, 49), also increase Ca^{2+}, levels (47). In contrast, preloading gastrulae ectoderm with the Ca^{2+} chelator, BAPTA, suppresses neural differentiation in response to dorsal mesoderm, the source of endogenous neuralizing signals (47). These results demonstrate the potential importance of signaling pathways that modulate Ca^{2+}, levels in neural development. It is of great interest that XIP2Y is expressed in the developing neural plate, and that it can induce prolonged (40–60 min) cellular responses, including increases in Ca^{2+}.

In amphibians, the neural plate forms in the dorsal ectoderm as a result of inductive signals released by the underlying dorsal mesoderm, a process initiated during gastrulation (50).
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