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Molecular cloning and functional expression of *Xenopus laevis* oocyte ATP-activated P2X4 channels[1]

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Abstract

All cells contain mechanosensitive ion channels, yet the molecular identities of most are unknown. The purpose of our study was to determine what encodes the *Xenopus* oocyte's mechanosensitive cation channel. Based on the idea that homologues to known channels might contribute to the stretch channels, we screened a *Xenopus* oocyte cDNA library with cation channel probes. Whereas other screens were negative, P2X probes identified six isoforms of the P2X4 subtype of ATP-gated channels. From RNase protection assays and RT-PCR, we demonstrated that *Xenopus* oocytes express P2X4 mRNA. In expression studies, four isoforms produced functional ATP-gated ion channels; however, one, xP2X4c, had a conserved cysteine replaced by a tyrosine and failed to give rise to functional channels. By changing the tyrosine to a cysteine, we showed that this cysteine was crucial for function. We raised antibodies against a *Xenopus* P2X4 C-terminal peptide to investigate xP2X4 protein expression. This affinity purified anti-xP2X4 antibody recognized a 56 kDa glycosylated *Xenopus* P2X4 protein expressed in stably transfected HEK-293 cells and in P2X4 cDNA injected oocytes overexpressing the cloned P2X4 channels; however, it failed to recognize proteins in control, uninjected oocytes. This suggests that P2X4 channels and mechanosensitive cation channels are not linked. Instead, oocyte P2X4 mRNA may be part of the stored pool of stable maternal mRNA that remains untranslated until later developmental stages.

Keywords: Masked maternal RNA; Mechanosensitive; P2X; Pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid; Purinoceptor; *Xenopus* oocyte

1. Introduction

Mechanosensitive (MS) channels, ion channels whose open probability responds to membrane deformation, are found in bacteria, fungi, animal and plant cells [1]. *Xenopus laevis* oocytes have an abundance of what appears to be a single type of mechanosensitive cation channel; it is electrophysiologically well characterized but so far has eluded biochemical characterization [2]. The impetus for

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[1] The nucleotide sequence data reported in this paper have been deposited in the GenBank database under accession Nos. AF308148 (xP2X4a), AF308149 (xP2X4b), AF308150 (xP2X4c), AF308151 (xP2X4d), AF308152 (xP2X4e) and AF308153 (xP2X4f).

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this research was to try to clone these MS channels by homology cloning from a *Xenopus* oocyte cDNA library, using DNA probes that, from a variety of criteria, might be possible candidates for the *Xenopus* MS channel.

In *Caenorhabditis elegans* the mechanosensory receptor neurons responsible for detecting gentle touch have been linked genetically to numerous genes, two of which, mec4 and mec10, are putative ion channels based on their homology to the epithelial Na channels (ENaC) [3]. P2X channels also fall within this broad family of ion channels, having a structural motif of two hydrophobic segments and a large extracellular domain [4,5].

Diverse electrophysiological studies have implicated nucleotide-gated cation channels at least tenuously with mechanosensitivity. An ATP-sensitive cation channel in rat hepatoma cells is activated by membrane stretch [6,7] and defolliculated *Xenopus* oocytes expressing P2X3 RNA exhibit small inward currents upon a gentle mechanical stimulus [8]. Recently, P2X4 has been demonstrated to be involved in sheer stress-mediated calcium influx in aortic endothelial cells [9].

Although repeated probing of a *Xenopus* oocyte cDNA library under low stringency hybridization conditions yielded no clones when mec4 and rat ENaC DNA were tried, six P2X clones were isolated from the library with a mixture of rat P2X1 and P2X2 DNA. This paper describes the molecular and electrophysiological characterization of these *Xenopus* P2X4 clones.

2. Materials and methods

2.1. Molecular cloning of Xenopus P2X4 receptor cDNA

1.7×10^5 plaques from a *X. laevis* oocyte λgt10 cDNA library [10] was screened concurrently with the *Pst*I fragment of rat P2X1 [11] and *Hin*dIII fragment of rat P2X2 [12] which encompassed all of the coding region and most of the 3′ untranslated region (UTR) of these cDNAs excluding the poly(A) sequences. The gel purified probe DNAs were labeled with ^{32}P-dCTP by random hexamer priming, pooled and used at 4×10^5 cpm/ml to probe filters overnight in hybridization solution (6×SSC, 5×Denhardt's, 0.5% SDS, 50% formamide) at 42°C and washed at low stringency with 5×SSC+0.2% SDS at 42°C. The resulting six positive cDNAs were subcloned into the *Eco*RI site of pBS KS+ vector (Stratagene) and sequenced on both strands by dideoxy sequencing. Some phage contained two *Eco*RI fragments and PCR was performed on phage DNA (forward primer 5′-CAGAGAAATTGATCACAATGTGTC-TCCCGG-3′; bases 861–890 in the *Xenopus* P2X4 sequence (Fig. 1); reverse primer λgt10F; Sigma) to determine the relationship of the fragments and to recover the second fragment for sequencing.

For electrophysiological experiments, the five full length cDNAs were subcloned into the eukaryotic expression vector pcDNAI/Amp (Invitrogen, San Diego, CA) using a common *Kpn*I site (Fig. 1), resulting in identical 200 bp 3′ UTRs.

2.2. Electrophysiology

Defolliculated *Xenopus* oocytes were prepared as described by Bertrand et al. [13] and 1–3 ng of cDNAs coding for either *Xenopus* p2x4 (xP2X4a-xP2X4f) or rat p2x4 [14] were injected into the nucleus of oocytes. Oocytes were incubated at 19°C for 48–72 h before recording with two-electrode voltage clamp. To measure ATP-evoked currents in oocytes, we used two-microelectrode voltage clamp [13] at 22–24°C (amplifier built by A. Sherman, McGill University), superfusing with control solution or agonist solutions at 10–20 ml/min; switching among solutions was done manually. Currents were sampled at 100–350 Hz on-line using the program PATCHKIT (Alembic Software, Montreal). Recording electrodes had 10–15 μm tips and were filled with 3 M KCl. External solution contained 96 mM NaCl, 2 mM KCl, 1 mM NaH_2PO_4, 1 mM $BaCl_2$, 10 mM HEPES and 1 μM atropine; pH was adjusted with NaOH to 7.4–7.5. ATP (Sigma) and pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS; from RBI) were dissolved in sterile water and aliquots were kept frozen at −20°C. Voltage ramp protocols were at 333 mV/s. Current-voltage (I-V) curves were fit by eye to a polynomial function using Origin 4.1 graphics software (MICROCALTM Software).

2.3. Mutagenesis

The double-stranded, site-directed mutagenesis protocol was carried out using the Chameleon mutagenesis kit (Stratagene) as per manufacturer's instructions. Briefly, a selection primer and a mutagenic primer were both annealed to a denatured double-stranded plasmid, either xP2X4f-pcDNAI/Amp or xP2X4c-pcDNAI/Amp. The primers were extended with DNA polymerase and the newly synthesized strand carrying the selection and mutagenic primer was closed with DNA ligase. Any remaining parental plasmid was linearized with *Nhe*I, followed by transfection into competent XLmutS *Escherichia coli*. The DNA was recovered from overnight cultured cells and put through a second round of *Nhe*I linearization and transfection into XL1-blue *E. coli* cells. The colonies were screened for the mutation by restriction digestion analysis and sequenced to confirm the presence of the mutation. The selection primer X18 (5′-CAACGCAAGCGCGCTTCTAGCT-3′) was created to mutate the unique restriction site, *Nhe*I, in the pcDNAI/Amp vector to a different sequence coding for the unique restriction site, *Bss*HI. The mutagenic primers were X9 (5′-ACCTGTGAAATTTTTGCA-3′; bases 490–507) used on xP2X4c to revert Y160 to C, X19 (5′-GAAAGTCTATAGAATCCGCTTTGACAT-3′; bases 954–980; destroys a *Bam*HI site) used on xP2X4f to convert G317 to R, and X20 (5′-CAGTAAAAACATATGAAATTTTTGCATGG-3′; bases 482–510; creates a new *Nde*I site) used on xP2X4f to convert the C160 to Y.

2.4. RNase protection assays

To obtain devitellinated oocytes, stage VI oocytes were first defolliculated by 3 h treatment with 2 mg/ml collagenase (Sigma) in calcium-free OR2 (82 mM NaCl, 2.5 mM KCl, 1 mM $NaHPO_4$, 1 mM $MgCl_2$, 5 mM HEPES pH 7.4) solution. Defolliculated oocytes were rinsed repeatedly in OR2, exposed to hypertonic solution (2× concentrated OR2) and the vitelline membrane was manually removed with forceps. RNA was purified by the guanidine thiocyanate acid-phenol method [15]. Four different RNA preparations were tested, derived from: (1) defolliculated, devitellinated oocytes, (2) oocytes with intact follicle cell layer, (3) the transparent vascularized epithelial sac which surrounds each ovarian lobe minus the folliculated oocytes and (4) the entire ovarian lobe with folliculated oocytes at various stages I–VI and the vascularized sac surrounding them. The RNA was quantitated spectrophotometrically at absorbance 260 nm, checked for integrity on agarose gels by observing for the ribosomal RNA and stored in DEPC-treated water at −20°C.

The RNase protection assays were according to [16]. ^{32}P-Radiolabeled antisense RNA probes were synthesized in vitro from linearized plasmids containing subcloned portions of *Xenopus* P2X4 (*Ava*II-xP2X4c, bases 1211–1439, and *Sac*II-xP2X4e, bases 2791–3052). The *Ava*II-xP2X4c probe was 307 nucleotides long and protected 229 nucleotides and the *Sac*II-xP2X4e probe was 318 nucleotides long and protected 256 nucleotides. As a control for RNA loading a *Xenopus* histone probe was synthesized from *Bst*EII linearized H4-pSP70. This H4 probe was 220 bases long and protected a number of different size fragments. Each probe was purified on a 5% polyacrylamide-8 M urea gel before use. Individual sample RNAs (4 or 20 μg) were combined with each probe at 200 000 cpm, and coprecipitated with 1 M ammonium acetate plus 65% ethanol. The pellet was resuspended in hybridization buffer (80% deionized formamide, 40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA), denatured for 5 min at 85°C and hybridized overnight at 60°C. The remaining single-stranded RNA was digested with RNase T1 and the protected RNA:RNA duplexes were denatured and run on a 5% polyacrylamide-8 M urea gel. Gels were exposed to film at −70°C for 3–25 h.

2.5. RT-PCR

1 μg total RNA isolated from defolliculated, devitellinated oocytes (above) was reverse transcribed with oligo(dT) priming of first strand DNA synthesis (Perkin-Elmer GeneAmp RNA PCR kit). Subsequently the entire coding region was amplified by PCR using the primers X7 (5′-AGCGAATTCTAGGGACGGCTGCTGTGGG-3′; bases 12–39) and X8 (5′-GTCGAATTCGGTTGCGTCTTTGGTCAAGGG-3′; bases 1185–1215) which were synthesized to include an *Eco*RI restriction site to facilitate subcloning into pBS KS+ vector.

2.6. Tissue culture: stable transfection of Xenopus P2X4 into HEK-293 cells

HEK-293 cells were stably transfected with *Xenopus* P2X4 receptors by cotransfecting 3 μg xP2X4f-pcDNAI/Amp and 1 μg pGK-puro (plasmid conferring puromycin resistance) as a calcium phosphate precipitate on 2×10^6 HEK-293 cells. The cells were grown in normal medium (10 ml DMEM (Gibco) plus 10% fetal calf serum with streptomycin and penicillin) for 3 days and then selected with 2 μg/ml puromycin for 10 days and 0.5 μg/ml puromycin for another 7 days. Individual colonies were picked from different plates, trypsinized, cells were counted and then expanded from single cells in 96-well dishes under constant 0.5 μg/ml puromycin selection.

2.7. Generation of antisera

Based on the previous success of antipeptide antisera generated against the C-termini of P2X family members [17,18], rabbit antiserum, BSYN 291, was raised by Biosynthesis (Lewisville, TX) against a peptide conjugated to keyhole limpet hemocyanin. The peptide CYREKKYKYVEDYDELVGSE represents amino acids 368–386 of the C-terminus of *Xenopus* P2X4, plus a cysteine for coupling. The IgG fraction of the antiserum was purified on a DEAE-affigel blue column and then affinity purified on a peptide agarose column which was made by coupling the peptide through its free sulfhydryl group to an iodoacetyl-agarose column (Sulfolink, Pierce, Rockford, IL) as per manufacturer's instructions. The purified antibody is referred to as anti-*X*-P2X4.

2.8. In vitro transcription-translation

The *Xenopus* (xP2X4f-pcDNAI/Amp), rat P2X4 (rP2X4-pcDNAI [14]) and a control plasmid coding for luciferase (Promega) were transcribed and translated in a coupled reticulocyte lysate system (TnT, Promega). The reaction was primed with 1 μg DNA and the ^{35}S-methionine-labeled peptides (1 μl) were resolved on an 8% polyacrylamide gel by SDS-PAGE. The peptides were transferred to nitrocellulose and either exposed to X-ray film for 3 days or probed as a Western with anti-*X*-P2X4 antibody as described below.

2.9. Western blots on oocytes, HEK-293 and rat olfactory bulb membrane extracts

Ten oocytes were resuspended in 100 μl oocyte lysis buffer (150 mM NaCl, 10 mM K_2HPO_4 pH 7.4, 1% Triton X-100 supplemented with the following protease inhibitors: 500 μM PMSF, 10 mM benzamidine, 0.2 units/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, 200 μg/ml soybean trypsin inhibitor) and homogenized by repeated pipetting. The cell lysate was centrifuged in a microfuge at

Table 1
Nucleotide and amino acid sequence differences for six *Xenopus* P2X4 cDNAs and four RT-PCR products

Base No.:	44	90	93	197	252	449	494	924	954	964
Consensus:	T	C	G	C	G	T	G	T	G	G
xP2X4a	T	**T**	G	C	**A**	T	G	**C**	G	G
xP2X4b	T	C	G	C	G	T	G	T	G	G
xP2X4c	T	C	**A**	C	G	**C**	**A**	T	**A**	G
xP2X4d	T	C	G	C	G	T	G	T	G	**A**
xP2X4e	**C**	C	G	**T**	G	T	G	T	G	G
xP2X4f	T	**T**	G	C	**A**	T	G	T	G	G
rtp1	T	**T**	G	C	**A**	T	G	T	G	G
rtp2	T	**T**	G	C	**A**	T	G	T	G	G
rtp7	T	C	G	nd	nd	T	G	T	G	G
rtp8	T	**T**	G	nd	nd	T	G	T	G	G
Amino acid No.:	10	25	26	61	79	145	160	303	313	317
Consensus:	GTG	ATC	AAG	ACA	ACG	GTA	TGT	GAT	ATG	GGG
	Val	Ile	Lys	Thr	Thr	Val	Cys	Asp	Met	Gly
Variation:	G**C**G	AT**T**	AA**A**	A**T**A	AC**A**	G**C**A	**T**AT	GA**C**	AT**A**	**A**GG
	Ala	Ile	Lys	Ile	Thr	Ala	Tyr	Asp	Ile	Arg

15 000 rpm for 20 min at 4°C and the middle layer was removed. 10–50 μg protein were used for the Western blots.

Six 100 mm diameter dishes of HEK-293 cells grown to 80% confluence were washed twice with phosphate-buffered saline, drained and the cells were scraped into 1 ml of hypotonic buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA containing the above protease inhibitors). The cells were lysed by polytron, centrifuged for 10 min at 5000×*g* at 4°C and the pellet was discarded. The supernatant was centrifuged in a TLX 120.2 rotor for 60 min at 60 000 rpm at 4°C. The pellet was resuspended in oocyte lysis buffer and 2–50 μg were used in Western blots. The olfactory bulbs from two rats were suspended in 1 ml of hypotonic buffer and processed as above for HEK-293 cells.

All extracts were quantitated by the Lowry DC assay (Bio-Rad) with bovine serum albumin as the protein standard. Proteins were resolved on 10% polyacrylamide gel by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was blocked overnight with 5% skim milk powder in TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl and 0.1% Tween 20). Membranes were probed with the affinity purified anti-*X*-P2X4 or anti-rat P2X4 antibody at 1.5 μg IgG/ml blocking buffer. The immunoreactive bands were visualized by enhanced chemiluminescence using a horseradish peroxidase-conjugated secondary anti-rabbit IgG (ECL, Amersham).

3. Results

3.1. Cloning of the six Xenopus P2X4 cDNAs

Low stringency screening of a *Xenopus* oocyte cDNA library with rat P2X1 and rat P2X2 probes identified six positive clones. None of the six *Xenopus* cDNA clones were siblings based on differences in the lengths of 5′ and 3′ UTRs, as well as numerous scattered nucleotide differences (Table 1). The cDNAs ranged in size from 1.6 to 3.0 kb with the differences due to the length of the 3′ UTRs which were 0.4 kb in xP2X4f, 0.95 kb in xP2X4c and 1.8 kb in xP2X4e. The presence of multiple alternate polyadenylated messages is not unusual, for example,

```
                  M  S  R  D  G  C  C  G  Q  V  Y  S  C    13
GAGCCGCACTGAGCCATGTCTAGGGACGGCTGCTGTGGGCAAGTGTACAGCTGC    54

 L  F  D  Y  D  T  P  R  I  A  L  I  K  S  R  K  I  G    31
TTATTCGACTACGACACTCCGCGAATAGCGCTCATTAAGAGCAGGAAGATCGGA   108

 L  L  N  R  F  I  Q  L  G  I  L  A  Y  V  I  G  W  V    49
CTCCTCAACAGGTTTATCCAGCTGGGAATCCTCGCTTATGTCATAGGGTGGGTT   162

 F  I  W  E  K  G  Y  Q  E  F  D  T  V  V  S  S  V  T    67
TTTATCTGGGAGAAAGGCTACCAGGAGTTTGATACAGTGGTCAGTTCTGTGACT   216

 S  K  V  K  G  V  V  V  T  N* T  T  E  L  G  V  K  I    85
TCTAAAGTGAAGGGAGTGGTTGTGACCAACACAACAGAACTGGGTGTCAAGATC   270

 W  D  V  A  D  Y  I  I  P  A  Q  E  E  N  A  V  F  V   103
TGGGATGTGGCAGATTACATTATACCAGCTCAGGAGGAAAATGCCGTCTTTGTT   324

 M  T  N  L  I  L  T  Q  N* Q  T  Q  G  H  Ç  P  E  L   121
ATGACCAATTTAATCCTTACCCAGAATCAAACCCAAGGTCACTGCCCTGAGTTA   378

 P  E  T  S  F  Ç  S  K  E  Q  P  Ç  T  P  G  Y  V  G   139
CCTGAAACCAGTTTCTGTTCCAAAGAACAACCCTGTACCCCTGGTTATGTGGGC   432

 K  Q  S  N  G  V  Q  T  G  K  Ç  V  P  Y  N* S  T  V   157
AAACAAAGCAACGGTGTACAGACTGGAAAGTGTGTGCCCTACAATTCTACAGTA   486

 K  T  Ç  E  I  F  A  W  Ç  P  V  E  N* D  T  H  V  P   175
AAAACCTGTGAAATTTTTGCATGGTGTCCAGTGGAAAATGATACTCATGTGCCA   540

 D  P  A  F  L  N  G  A  E  N* F  T  V  L  I  K  N  N   193
GATCCAGCATTTCTGAATGGGGCTGAAAACTTTACAGTTCTAATAAAGAATAAT   594

 I  W  Y  P  K  F  Q  V  S  K  R  N  I  L  S  N* I  S   211
ATCTGGTATCCAAAATTCCAAGTCTCCAAACGAAACATATTGTCCAATATCAGC   648

 S  S  Y  L  K  T  Ç  Q  Y  D  K  V  N  H  P  F  Ç  P   229
AGTAGTTACCTCAAAACGTGCCAGTATGATAAAGTGAACCACCCATTCTGTCCA   702

 I  F  R  L  G  N  I  V  K  E  A  G  E  S  F  S  D  M   247
ATATTTCGACTCGGCAACATAGTAAAAGAAGCAGGAGAGTCTTTCAGTGACATG   756

 A  V  Q  G  G  V  M  G  I  Q  I  N  W  N  Ç  D  L  D   265
GCTGTTCAGGGAGGAGTGATGGGGATACAAATCAACTGGAACTGTGACCTGGAC   810

 R  K  L  T  Y  Ç  V  P  K  Y  S  F  R  R  L  D  N  R   283
CGGAAACTTACATACTGTGTGCCAAAATACTCATTCCGCCGCTTGGACAACAGA   864

 E  I  D  H  N* V  S  P  G  Y  N  F  R  F  A  K  Y  Y   301
GAAATTGATCACAATGTGTCTCCCGGCTATAATTTCAGGTTTGCTAAATATTAT   918

 K  D  S  N  G  V  E  S  R  T  L  M  K  V  Y  G  I  R   319
AAGGATAGTAACGGTGTTGAATCCAGAACTCTGATGAAAGTCTATGGGATCCGC   972

 F  D  I  L  V  F  G  T  A  G  K  F  D  I  I  P  T  M   337
TTTGACATCCTGGTTTTTGGAACAGCGGGGAAATTCGACATTATACCTACAATG  1026

 I  N  I  G  S  G  A  A  L  F  G  V  A  T  V  L  C  D   355
ATTAACATTGGCTCCGGCGCTGCCTTGTTTGGAGTGGCAACTGTGCTGTGTGAT  1080

 M  I  V  F  H  F  F  K  K  R  H  Y  Y  R  E  K  K  Y   373
ATGATCGTCTTCCATTTCTTTAAGAAAAGACATTACTACCGAGAAAAGAAATAC  1134

 K  Y  V  E  D  Y  D  E  L  V  G  S  E  C  G  S  N  P   391
AAATATGTGGAAGATTATGATGAACTGGTTGGCAGTGAATGTGGATCAAACCCT  1188

TGACCAAAGACGCAACCGGATTGGACCCTTTTAGTTAGTAATCGTTAAATAACA  1242
AATTATATCATGTTGTTGGGACTGCTTTCCTGGAAATCTCTCCACCAACGACGA  1296
TACAATTGTACAATTAATGTCCCTTGTACAGCAATGTGAATTGAGGATTGTGTT  1350
CTTGAAGGGCATTAGGTAGATATTTCTCCATTCTCTGTACCTGTTTGGTACCAC  1404
TTATTGGAGCAAGGAAATCTGTTGTCTGAAATGTGGATCCTCGCAAGACACCTG  1458
GACCCCTGTGGAGGCTAAAATAAAGTCCATGAAATTTACCATTC-poly(A)78  1502
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Fig. 1. Primary structure of the *Xenopus* oocyte P2X4 channel. Nucleotide and deduced amino acid sequences of isoform xP2X4f. The two putative transmembrane domains are underlined. Between these, in the extracellular loop, the ten conserved cysteines are undermarked and the seven putative N-linked glycosylation sites (N-X-S/T) are marked with an asterisk. The polyadenylation signal in the 3′ UTR is in bold and underlined and the *Kpn*I site used for subcloning all isoforms into pcDNAI/Amp is in italics and underlined.

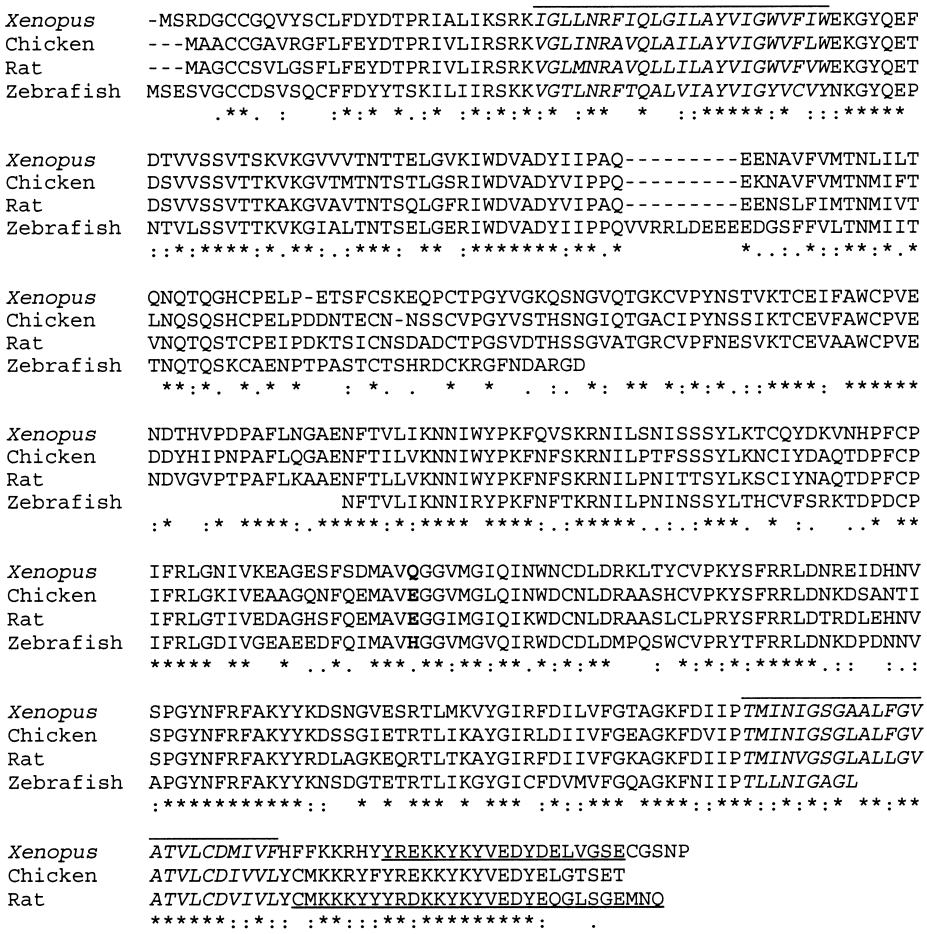

Fig. 2. Alignment of different classes of P2X4 amino acid sequences. The amino acid sequence alignment of mammalian, bird, amphibian and fish P2X4. The number of amino acids and the GenBank accession numbers for these sequences are: rat 388 (AAA99777), chicken 384 (CAB56283), *Xenopus* 391 (AF308153) and zebra fish, an incomplete deduced amino acid sequence made by translating two partial cDNA sequences (AW133550 and AW115797). Underlined amino acids represent the C-terminal peptides used to generate the *Xenopus* (BSYN291) and rat [18] antisera. As in Fig. 1 the two transmembrane domains are overlined. Asterisks below the alignments indicate identity in all sequences while a colon or period indicates decreasing degrees of amino acid conservation between the four sequences. The bolded residues (Q, E, E, H for the four organisms) are mentioned in Section 3 in relation to PPADS.

four species of mRNA (1.8/2.6/3.6/4.2 kb) are detected on Northern blots by rat P2X1 and both the 1.8 and 2.6 kb mRNAs differ in the length of the 3′ UTR [11].

A BLAST search of GenBank protein sequences revealed that the deduced amino acid sequence of the *Xenopus* protein is clearly P2X4 with high identity scores for the chicken, human, rat and mouse P2X4 amino acid sequences. The nucleotide and amino acid sequences of the shortest cDNA clone, xP2X4f, are shown in Fig. 1. Clone xP2X4b was truncated at the 5′ end and because it lacked the first seven amino acids was not characterized further. The other five cDNAs contained a 1173 nucleotide open reading frame which encodes a protein of 391 amino acids with a predicted molecular mass of 44 350 Da. A comparison with all seven cloned rat P2X gene family members, P2X1–P2X7, shows that the *Xenopus* P2X4 has 67% amino acid identity with the 388 amino acid rat P2X4 channel, whereas the other P2X genes were lower with a range of between 43 and 51% amino acid identity. In the six *Xenopus* P2X4 clones, there were ten nucleotide changes within the protein coding region (Table 1). Six resulted in ami-

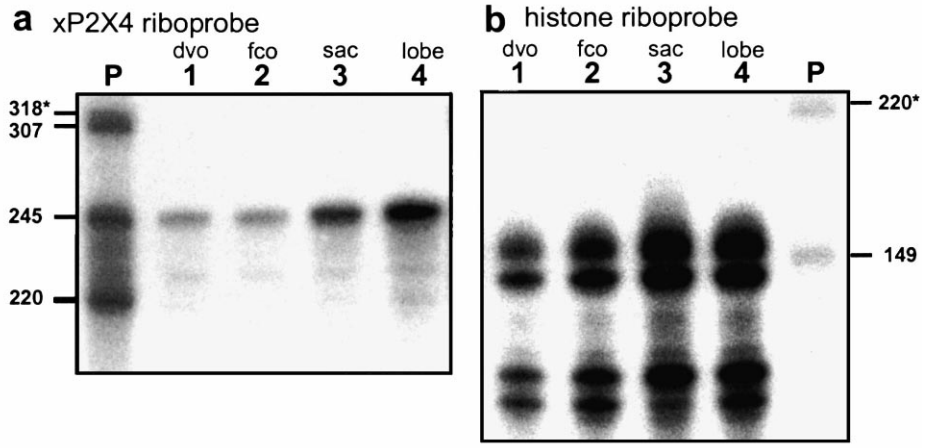

Fig. 3. Detection of *Xenopus* P2X4 mRNA in oocytes and surrounding tissues by RNase protection assay. RNase protection assays performed on total cellular RNA (20 μg A and 4 μg B) extracted from defolliculated, devitellinated oocytes (lane 1), oocytes with intact follicle cell layer (lane 2), the transparent vascularized epithelial sac which surrounds each ovarian lobe minus the folliculated oocytes (lane 3) and the entire ovarian lobe with folliculated oocytes at various stages I–VI and the vascularized sac surrounding them (lane 4). 200 000 cpm of each riboprobe ((a) the 318 nucleotide *Xenopus* P2X4 riboprobe (asterisk in lane P; protects 256 nucleotides), (b) the 220 nucleotide *Xenopus* histone H4 riboprobe (asterisk in lane P; protects multiple sizes of RNA) was used in the reactions and the gels were exposed to X-ray film for 25 h (a) or 3 h (b). The riboprobes were used at 2000 cpm/lane as size markers (lane P, size in nucleotides as indicated).

no acid substitutions, four were silent. Additionally, there were a variety of nucleotide changes in the 3′ UTR of the six isoforms (data not shown).

A comparison of orthologues across classes indicates a high degree of conservation between fish, amphibian, bird and mammalian P2X4 genes ranging from 58 to 76% amino acid identity (Fig. 2). This includes many of the N-linked glycosylation sites and in particular the ten extracellular cysteines that are conserved across the entire group of P2X isoforms, from P2X1 to P2X7 [4,5].

3.2. Expression of P2X4 mRNA in oocytes

To determine that P2X4 mRNA was present in oocytes, we performed RNase protection assays on total RNA isolated from defolliculated and devitellinated oocytes. The precaution of manually removing the vitelline membrane from defolliculated oocytes ensured that there would be no contaminating follicular cells in the RNA preparation (similar to [19]). Four different RNA preparations were tested, derived from: (1) defolliculated, devitellinated oocytes, (2) oocytes with intact follicle cell layer, (3) the transparent vascularized sac which surrounds each ovarian lobe minus the folliculated oocytes and (4) the entire ovarian lobe with folliculated oocytes at various stages I–VI and the vascularized sac surrounding them. The results from $n=4$ independent assays (using probes from two different portions of *Xenopus* P2X4, bases 2791–3052 and 1211–1439) show that P2X4 RNA was present in the oocytes, and the amount in defolliculated, devitellinated and in folliculated oocytes was similar (compare lanes 1 and 2 in Fig. 3a). Apparently the vascular epithelial tissue also contains P2X4 RNA (lane 3, Fig. 3a). To control for RNA loading, we also measured *Xenopus* histone H4 mRNA. Based on quantitation of RNA absorbance at 260 nm, the quantity of total cellular RNA was equal in each hybridization reaction, although the histone probe data suggest that the level of mRNA may differ among the preparations. However, it is clear that P2X4 mRNA is present in much smaller amounts than histone mRNA since more total RNA (20 μg) and a longer exposure (25 h) were required to detect the P2X4 as compared to the 4 μg and 3 h exposure required for detection of the multiple strongly protected histone bands (Fig. 3a,b, respectively).

To determine whether oocytes from a single frog contain transcripts for more than one isoform, we isolated total RNA from defolliculated/devitellinated

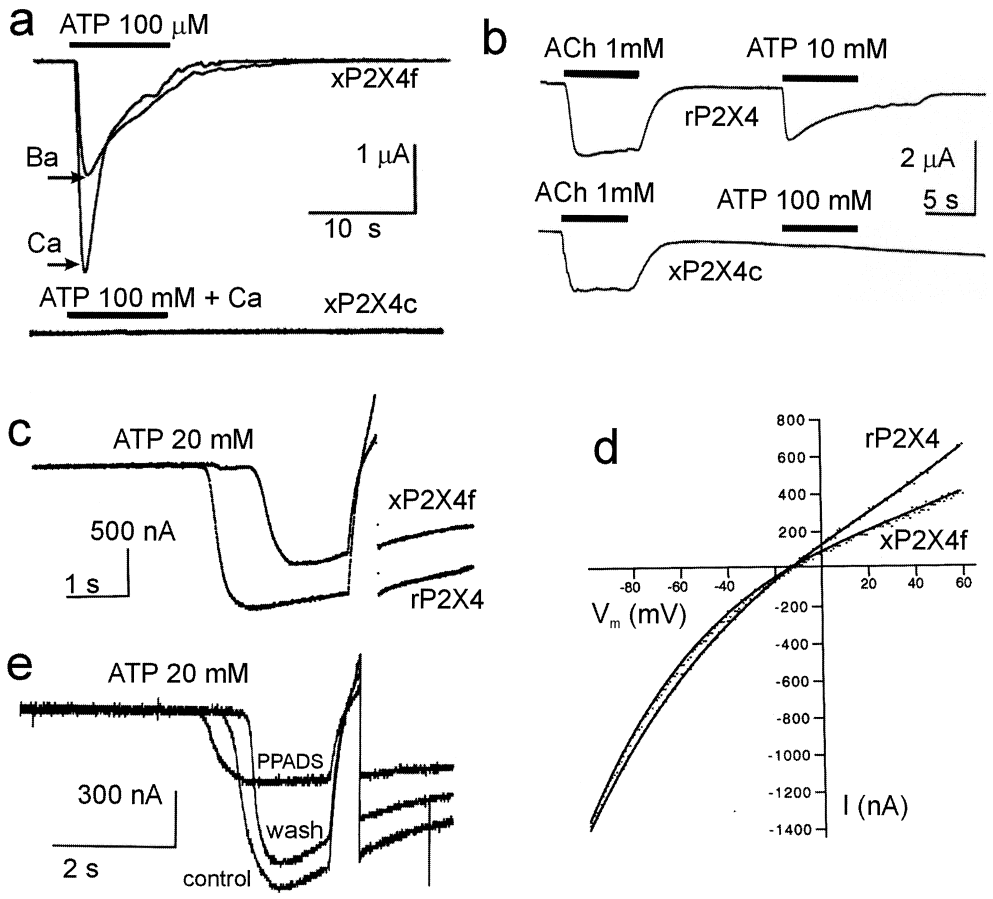

Fig. 4. Electrophysiology of P2X4 channels. (a) Functional expression of xP2X4 clones in *Xenopus* oocytes. (b) Coinjection of α4β2 nAChR and rat P2X4 or *Xenopus* P2X4 (xP2X4c) clones in *Xenopus* oocytes. (c,d) Comparison of rectification for rat P2X4 and *Xenopus* P2X4 (xP2X4f) ATP-gated ion channels; in d, the *Xenopus* current is scaled to the maximum inward rat current at −100 mV. (e) Block of *Xenopus* P2X4 (xP2X4f) ATP-gated channels by PPADS.

oocytes obtained from separate females, reverse transcribed into cDNA, amplified it with PCR and then sequenced the products. Our results indicated that xP2X4b (rtp7, Table 1) and xP2X4f (rtp1, rtp2, rtp8, Table 1) are found in *Xenopus* oocytes and that a frog can harbor both sequences simultaneously. The origin of the multiple isoforms could be allelic polymorphism, polyploidy, and/or library construction artifacts.

3.3. Functional expression of Xenopus P2X4 cDNAs

Since individual oocytes contain mRNA for P2X4 receptors, we used two-electrode voltage clamp techniques to determine whether these cells had endogenous ATP-gated currents. In recording from over 100 control oocytes obtained from seven different females, none responded to rapid application of 100–300 μM ATP. This suggests that *Xenopus* oocytes do not have endogenous ATP receptors, even though they contain mRNA for P2X4 receptors.

Next, we investigated whether these xP2X4 genes were capable of giving rise to ATP-gated channels when overexpressed in *Xenopus* oocytes. We found that 2–5 days after nuclear injection with cloned *Xenopus* P2X4 cDNAs, oocytes had ATP-evoked currents; when holding the membrane potential at −90 mV, these ATP-gated currents were 1–2 μA in amplitude when we applied 100 μM ATP (see Fig. 4; Table 2b). When recording with 1 mM Ca^{2+} in the perfusion fluid, the ATP-evoked current had a rapidly decaying component which disappeared when

the Ca^{2+} was replaced with Ba^{2+} (Fig. 4a). We attribute the rapidly decaying early component to Ca^{2+}-activated Cl^- currents intrinsic to *Xenopus* oocytes. A comparison of the rat and *Xenopus* P2X4 (clone xP2X4f) revealed that both activated rapidly and desensitized slowly in Ba^{2+}-containing solutions. The current-voltage relationship was roughly linear in its zero current region, it reversed at about −10 mV and showed slight inward rectification (Fig. 4c,d); however, *Xenopus* P2X4 channels rectified more strongly than those of rat P2X4, particularly in the outward direction.

PPADS, a commonly used antagonist of the P2 purinergic channels, blocked both the rat P2X4 ([14], but see [20]) and *Xenopus* P2X4 channels (Fig. 4e). Like Fig. 4c these raw data traces include ramp currents used to generate I-V curves which have not, however, been replotted in this case. With 20 μM PPADS, ATP-activated current recorded from xP2X4f channels reversibly decreased to about 50% but I-V curves had the same fundamental shape and the reversal potential was unchanged, indicating that PPADS did not alter the permeation properties of the channel. The glutamine residue, Q250, is not conserved, since in birds and mammals it is glutamate, and in fish it is histidine (see Fig. 2). In mammals, the glutamate has been identified as contributing to agonist binding [5,20] but the *Xenopus* result suggests that it is not essential for a PPADS response.

Expression of four of the five isoforms led to ATP-gated currents; only xP2X4c (Table 2a; bottom trace of Fig. 4a) failed to produce functional channels. To rule out the possibility that the oocytes were incapable of expressing channels after nuclear injection of xP2X4c, we coinjected oocytes with cDNAs for rat P2X4 or *Xenopus* xP2X4c together with cDNAs for a neuronal nicotinic acetylcholine receptor (nAChR) subtype α4β2 (Fig. 4b). All oocytes coinjected with the nAChR α4β2 plus ratP2X4 cDNAs produced both ACh-evoked (ACh, 1 μM) and ATP-evoked currents ($n = 20$) (top trace, Fig. 4b); however, oocytes coinjected with the nAChR α4β2 plus *Xenopus* xP2X4c cDNAs produced only ACh-evoked currents; we observed no ATP-evoked currents from these in ten of ten oocytes (e.g. Fig. 4b, bottom trace), ruling out the possibility that the lack of ATP-gated currents when expressing xP2X4c was related to poor expression of ion channels in these oocytes.

Unlike other P2X channels which have a cysteine at position 160, xP2X4c contains a tyrosine at this position. To determine whether this C to Y substitution accounts for the failure of xP2X4c cDNA to produce functional ATP-gated channels when overexpressed in *Xenopus* oocytes, we mutated xP2X4c, changing Tyr at 160 to a Cys ($xP2X4c_{Y160C}$) and expressed it in oocytes. All oocytes overexpressing $xP2X4c_{Y160C}$ had ATP-gated currents similar in appearance to those produced by xP2X4f cDNA. Furthermore, in a corollary experiment, we changed C160 in xP2X4f to a Tyr ($xP2X4f_{C160Y}$) and observed that no oocytes which overexpressed $xP2X4f_{C160Y}$ had functional ATP-gated channels. These experi-

Table 2

(a) Comparative effectiveness of 100 μM ATP for oocytes overexpressing various P2X4 isoforms

P2X4 isoforms	ATP-activated current
Rat P2X4	++++
xP2X4a, xP2X4e, xP2X4f	+++
xP2X4d	+
xP2X4c	−
$xP2X4c_{Y160C}$	+++
$xP2X4f_{C160Y}$	−
$xP2X4f_{G317R}$	+

(b) Comparison of electrophysiological trials for xP2X4f and xP2X4c

	100 μM ATP in Ca^{2+} OR2	100 μM ATP in Ba^{2+} OR2	10 μM ATP in Ba^{2+} OR2
xP2X4f	1–6 μA ($n = 8$)	0.6–2.5 μA ($n = 10$)	0.4–1.0 μA ($n = 10$)
xP2X4c	not detectable ($n > 20$)	not detectable ($n > 20$)	not detectable ($n > 20$)

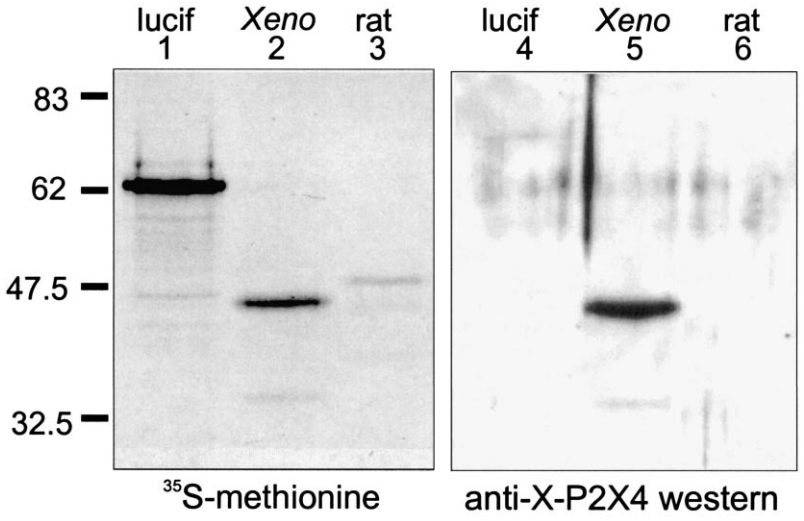

Fig. 5. Detection of in vitro translated P2X4 protein by ^{35}S-methionine incorporation and by Western blot. The cDNAs for *Xenopus* P2X4 (xP2X4f-pcDNAI/Amp), rat P2X4 (rP2X4-pcDNAI [14]) and a control plasmid coding for luciferase (Promega) were transcribed and translated in a coupled reticulocyte lysate system (TnT, Promega). The reaction was primed with 1 μg DNA and the ^{35}S-methionine-labeled proteins (1 μl) were resolved on an 8% polyacrylamide gel by SDS-PAGE. The peptides were transferred to nitrocellulose and either exposed to X-ray film for 3 days (lanes 1, 2, 3) or probed with anti-*X*-P2X4 antibody (lanes 4, 5, 6) as described in Fig. 6. Luciferase (lanes 1, 4), *Xenopus* P2X4 (lanes 2, 5) and rat P2X4 (lanes 3, 6). The mass of the proteins was determined by comparison to prestained molecular weight marker proteins (New England Biolabs) shown on the left.

ments suggest that a C at position 160 is essential for the expression of functional ATP-gated channels. Clone xP2X4d did not consistently express, but ATP-gated currents were obtained in some batches of oocytes (Table 2a). The only difference in xP2X4d from the consensus was an extracellular R317 instead of G317. Introduced into xP2X4f-pcDNA/Amp by site-specific mutagenesis, G317R resulted in the clone behaving like xP2X4d (Table 2a).

3.4. Westerns

Although electrophysiological controls showed no ATP-sensitive P2X channels in defolliculated oocytes, our working hypothesis was that *Xenopus* P2X4 monomers contribute not to an ATP-gated channel, but to a stretch-gated channel complex. P2X channels can form functional heteromers [21] with properties distinct from the respective homomultimers. We thought, therefore, that the stretch channels might be an ATP-insensitive complex that incorporated the xP2X4c isoform. An antibody was therefore raised to detect *Xenopus* P2X4 channels in situ.

The specificity of anti-*X*-P2X4 was tested in Western blots (Figs. 5 and 6). Membrane preparations were made from HEK-293 cells that had been co-transfected with xP2X4f-pcDNAI/Amp DNA and a puromycin-resistant plasmid and then selected for stable resistance to the antibiotic. Of the seven puromycin-resistant clones tested, two produced a strong novel immunoreactive band at approx. 56 kDa (one of these is shown in Fig. 6a, lane 2; $n=4$). Western blots also detected this 56 kDa protein in cell extracts made from oocytes incubated for 1.5 days after nuclear injection of xP2X4f-pcDNAI/Amp (Fig. 6a, lane 6; $n=3$). The 56 kDa protein was absent in uninjected oocytes (Fig. 6a, lane 5; $n=5$).

In vitro transcription-translation of *Xenopus* xP2X4f-pcDNAI and rat P2X4-pcDNA gave rise to 45 kDa and 48 kDa ^{35}S-labeled peptides (Fig. 5, lane 2 and 3, respectively; $n=6$). The *Xenopus* p2X4 in vitro translation product was detected by the anti-*X*-P2X4 antibody (Fig. 5, lane 5; $n=2$); however, the 48 kDa rat protein was not (Fig. 5, lane 6; $n=2$). The observed 45 kDa size of *Xenopus* P2X4 is similar to the calculated molecular mass of 44 350 Da. The larger 56 kDa protein produced in vivo, as seen in Westerns of P2X4 transfected HEK-293 and P2X4-injected oocytes (Fig. 6a, lanes 2 and 6), is likely due

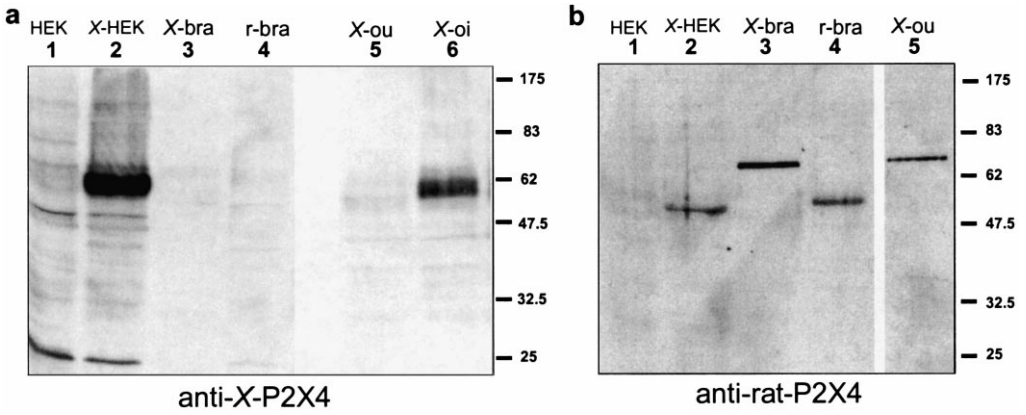

Fig. 6. Detection of *Xenopus* and rat P2X4 protein by Western blot using (a) anti-*Xenopus* and (b) anti-rat P2X4 antisera. The Westerns were probed with affinity purified anti-C-terminal P2X4 peptide antisera (1.5 μg IgG/ml). Lanes 1–4 were cell membrane extracts from: 1, HEK-293, 15 μg; 2, HEK-293 stably transfected with *Xenopus* P2X4f, 15 μg; 3, *Xenopus* brain, 5 μg; 4, rat olfactory bulb, 5 μg; and lanes 5 and 6 were 10 μg of crude cell extracts of *Xenopus* oocytes which were either nuclear injected with xP2X4f isoform (lane 6) or uninjected (lane 5). Enhanced chemiluminescence with horseradish peroxidase-conjugated anti-rabbit secondary antibody was used to develop the Western. The mass of the proteins was determined by comparison to prestained molecular weight marker proteins (New England Biolabs) shown on the right.

to N-linked glycosylation; a number of putative N-X-(S/T) sites are shown in Fig. 1. For P2X1, a similar increase in mass was attributed to glycosylation, based on the difference between an in vitro translated 45 kDa product and a 60 kDa in vitro translation product made in the presence of microsomes [5] which is similar to the 62 kDa endogenous receptor [11].

The specificity of anti-*X*-P2X4 was also tested by peptide competition assays in which peptide was preincubated for 30 min with antibody in a 170:1 molar ratio and then tested on a Western blot. The C-terminal peptide successfully removes the 56 kDa signals on Westerns done on the xP2X4f-transfected HEK-293 cells and the xP2X4f cDNA injected oocytes (data not shown).

A recent report, using an antibody generated against the entire cytoplasmic C-terminus of rat P2X4 (Fig. 2), identified rat olfactory bulb as a tissue source rich in P2X4 protein [18]. This rat P2X4 antibody (kindly provided by P. Seguela) was used in Western blots and cross-reacted with the *Xenopus* P2X4 protein, but with an apparently lower affinity compared to the *Xenopus* P2X4 antibody (compare lane 2 in both Fig. 6a,b; $n=2$). The rat P2X4 antibody detected the expected rat olfactory bulb P2X4 (Fig. 6b, lane 4; $n=4$) at 58 kDa [18]. The *Xenopus* P2X4 antibody did not cross-react with the rat P2X4 protein in the olfactory bulb extracts (Fig. 6a, lane 4; $n=2$) even when 50 μg was tested, in keeping with the inability to detect in vitro translated rat P2X4 protein (Fig. 5, lane 6; $n=2$). Although the C-terminal peptides used to create the two antibodies had some highly conserved stretches of amino acids (Fig. 2), our observation of the apparent lower affinity of rat versus *Xenopus* P2X4 antibodies and the inability

Table 3
Summary of endogenous P2X4 signals from devitellinated oocytes and follicle cells

	P2X4 cDNA in expression library	Endogenous P2X4 mRNA, from RT-PCR	Endogenous P2X4 mRNA, by RNase protection assay	Endogenous P2X4 protein (56 kDa) by immunoblot	Endogenous P2X4 purinoceptor responses
Devitellinated oocytes	Yes	Yes	Yes	No	No[a]
Follicle cells	–	Not tested	Unlikely[b]	No	No [19]

[a]Present study, plus [30].
[b]Compare intensity of signals in Fig. 4a, lanes 1, 2 (noting that the control (Fig. 4b, lanes 1, 2) indicated *more*, not less, RNA in the follicle-plus-oocyte cell lanes).

of the *Xenopus* P2X4 antibody to detect the rat protein suggest that the few species-specific sequence differences contribute substantially to the epitopes recognized by these two antibodies.

Since the rat P2X4 antibody could detect the *Xenopus* P2X4 protein, we used it to independently test for the presence of endogenous P2X4 protein in oocytes. These Westerns confirmed what was found by anti-*X*-P2X4 Westerns, namely that there is no detectable 56 kDa protein in the uninjected oocyte extracts. There was, however, a strongly immunoreactive peptide at 71 kDa (Fig. 6b, lane 5; $n=4$).

Because P2X4 is a major ATP-activated channel in the mammalian brain [5,14,18,20], we immunoblotted *Xenopus* brain membrane extracts and tested the blots with both *Xenopus* and rat antibodies (lane 3 in Fig. 6a ($n=4$) and Fig. 6b ($n=4$)) and were surprised to discover no 56 kDa P2X4 protein. A Western, probed with anti-*X*-P2X4 antibody, which had 10-fold more frog brain membrane extract (50 μg) did show peptides at 48 and 28 kDa. Since incubation with excess C-terminal *X*-P2X4 peptide did not reduce the intensity of these bands, they were likely not P2X4. The rat antibody did, however, as in the oocyte immunoblot, strongly react with an unknown 71 kDa protein from *Xenopus* brain (Fig. 6b, lane 3).

4. Discussion

Though invertebrates show no signs of having purinoceptors (e.g. nematode and insect nucleotide databases reveal no P2X sequences), diverse purinoceptors must have been well established in vertebrates at a relatively early stage. Here, we have molecularly cloned and functionally expressed the first amphibian P2X4 ATP-gated channel. This helps consolidate the emerging picture that early vertebrates already had multiple P2X receptors, since for amphibians, there are also sequence fragments in GenBank (BE509086 and AW148097) for another P2X-purinoceptor type, P2X7. For fish, there is now molecular/functional evidence for P2X3 [22,23] plus fragmentary evidence for P2X4 (see Fig. 2). In addition to P2X, there is pharmacological evidence for P2U and P2Y receptors in amphibians [19].

The absence of an immunoreactive 56 kDa P2X4 protein in uninjected *Xenopus* oocytes suggests that, in spite of the presence of message in the ooplasm, channels with P2X4 subunits are not expressed in stage V and VI oocytes (see Table 3). P2X4 protein was also not expressed in follicle cells, an observation in line with the presence of P2Y and P2U but not of P2X pharmacological activity in voltage-clamped follicle cells [19]. Therefore it is unlikely that our hypothesis – namely that P2X4 is a component of the *Xenopus* oocyte mechanosensitive cation channel – is correct. Moreover, as previously reported [24], we tested by patch clamp (using rigorously stereotyped mechanostimulus regimes and 'blinded' data analysis) whether the *Xenopus* P2X4 channels expressed in LM-TK$^-$ cells yield a novel mechanosensitive current and found that erratic mechanosensitive currents occurred at almost the same frequency in experimental and control patches.

A surprising result here was the multiplicity of P2X4 isoforms isolated from an oocyte cDNA library based on a single female ovary [10]. However, multiple nucleotide substitutions have also recently been found in the cloning of *X. laevis* Requiem genes from a different oocyte cDNA library [25]. The multiple isoforms may result from allelic polymorphism or artifacts due to library construction (reverse transcriptase lacks proofreading functions) or library amplification (proofreading mistakes during the repeated amplification and propagation of the phage library). Additionally, polyploidy is not ruled out as a possible explanation. *X. laevis* is a pseudo-tetraploid species with 36 chromosomes, almost double the 20 chromosomes of *Xenopus tropicalis* [26]. Duplicated loci in *Xenopus* can remain active, with isozymes showing differential tissue expression or development-specific patterns [27].

During *Xenopus* oogenesis most of the maternal mRNA is sequestered into storage messenger ribonucleoprotein particles in association with an RNA histone like protein FRGY2 [28]. Many of these oocyte mRNAs remain untranslated until they are required during, say, meiotic maturation or after fertilization [29]. Since both RNase protection and RT-PCR confirmed what the oocyte expression library indicated, namely that mRNA for P2X4 was present in defolliculated/devitellinated oocytes, we presume that it was part of the pool of masked mRNA. Masked mRNAs have short poly(A) tails and when recruited for translation, undergo cytoplasmic lengthening of the

poly(A) tail [29]. Studies on recruitment of masked mRNA identified two *cis*-acting elements critical for cytoplasmic poly(A) addition: the hexanucleotide, AAUAAA (a sequence also required during the initial polyadenylation and cleavage in the nucleus), and a U-rich element called a cytoplasmic polyadenylation element (CPE). The xP2X4e isoform of *Xenopus* P2X4 which had the longest 3′ UTR (1.8 kb), contained both a CPE signal UUUUUAU (at base 2604) and tandem AAUAAA sequences (at bases 2661 and 2670). Neither the small xP2X4f nor the medium sized xP2X4c alternatively polyadenylated isoforms of P2X4 contained a CPE sequence and xP2X4c also lacked the conventional AAUAAA despite evidence of a poly(A) tail in the cDNA clone.

Because the P2X4 isoform is a major ATP-activated cation channel in mammalian brain tissue [5,14,18,20], we were surprised at its apparent absence in *Xenopus* brain. The fact that anti-rat P2X4 but not anti-*X*-P2X4 detected a 71 kDa protein in *Xenopus* oocyte and brain may be a fortuitous cross-reaction to a very different protein with an epitope like the rat P2X4 peptide sequence. A BLAST search with the rat antigen sequence did not point to other purinoceptors (e.g. P2Y or P2U). We cannot rule out that the 71 kDa protein is a novel isoform of P2X4, such as a splice variant resulting in a larger peptide (e.g. a human P2X4 splice variant has been documented; GenBank accession No. CAB39333), although arguing against this, anti-*X*-P2X4 did not detect the 71 kDa and there was no RT-PCR evidence for a larger P2X4 cDNA product.

Assuming that our interpretation is correct and P2X4 mRNA is masked in stage V and VI *Xenopus* oocytes, it should prove interesting to monitor for the first signs of expression of channel subunits during embryogenesis.

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