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Research Article

Characterization and Expression of ATP $P2X_4$ Receptor From Embryonic Chick Skeletal Muscle

Xuenong Bo,[1,2] Min Liu,[1] Ralf Schoepfer,[2] and Geoffrey Burnstock[1]*

[1]*Autonomic Neuroscience Institute, Royal Free and University College Hospital School of Medicine, London, UK*

[2]*Wellcome Laboratory for Molecular Pharmacology and Department of Pharmacology, University College London, London, UK*

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ABSTRACT Previous pharmacological experiments have indicated the existence of ATP P2X receptors in chick embryonic skeletal muscles. In this study we cloned a $P2X_4$-like cDNA encoding a protein of 385 amino acids, which shares 75% and 76% identity with rat and human $P2X_4$ receptors, respectively. Functional studies of this $cP2X_4$ receptor expressed in *Xenopus* oocytes showed that ATP induced a fast inward current, which was partially desensitized upon prolonged application of ATP. The ATP-induced currents were concentration-dependent, with an EC_{50} of 9.5 μM. Adenosine 5′-*O*-(thio)triphosphate and 2-methylthioATP very weak agonists. α,β-methyleneATP was almost inactive. In contrast to their potentiating effects on recombinant rat $P2X_4$ receptors, both suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid partially blocked ATP-induced currents. TrinitrophenylATP was able to block ATP-induced response completely, with an IC_{50} of 4.7 μM. Northern blot and RT-PCR analysis showed that $cP2X_4$ mRNAs were mainly expressed in skeletal muscle, brain, and gizzard of day 10 chick embryos. Lower levels of expression were also detected in liver, heart, and retina. Whole-mount in situ hybridization showed that $cP2X_4$ mRNAs were expressed in the brain, spinal cord, notochord, gizzard, and skeletal muscle. The physiological functions of $cP2X_4$ receptors in embryonic skeletal muscle remain unclear at present. Drug Dev. Res. 53:22–28, 2001. © 2001 Wiley-Liss, Inc.

Key words: purinoceptor; P2X receptor; ATP; skeletal muscle

INTRODUCTION

Extracellular ATP induces biological effects via two types of receptors, fast ligand-gated ion channel receptors (P2X), and G-protein-coupled receptors (P2Y) [Abbracchio, and Burnstock, 1994]. P2X and P2Y receptor subtypes have been identified in many cell types and found to mediate a variety of biological activities [Burnstock, 1997; Ralevic and Burnstock, 1998]. So far, seven P2X receptor subtypes (or subunits) have been cloned from mammalian tissues [MacKenzie et al., 1999]. Recently, we cloned a novel P2X receptor from chick embryonic skeletal muscle [Bo et al., 2000], which is tentatively named $cP2X_8$ receptor.

Rat $P2X_4$ receptor was cloned in our laboratory from rat hippocampus cDNA library [Bo et al., 1995]. It was also cloned from superior cervical ganglion cDNA library [Buell et al., 1996], whole brain cDNA libraries [Séguéla et al., 1996; Soto et al., 1996], and pancreatic islet cDNA library [Wang et al., 1996]. Rat $P2X_4$ receptor has wide tissue distribution in both central nervous tissues and peripheral tissues [Bo et al., 1995; Buell et al., 1996; Soto et al., 1996; Xiang et al., 1998]. Its expression has been

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*Correspondence to: Geoffrey Burnstock, Autonomic Neuroscience Institute, Royal Free and University College Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, UK.
E-mail: g.burnstock@ucl.ac.uk.

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detected in epithelia of submandibular glands, where $P2X_4$-like receptor-mediated currents have been observed, indicating the existence of possible functional homomeric receptors [Buell et al., 1996]. The formation of functional heteromeric receptors of $P2X_4$ with $P2X_6$ in *Xenopus* oocytes has been reported [Lê et al., 1998]. The $P2X_{4+6}$ receptors showed different functional properties from both homomeric $P2X_4$ and $P2X_6$ receptors, such as increased sensitivity to α,β-methyleneATP and to the antagonist suramin. However, the existence of native $P2X_{4+6}$ receptors remains to be confirmed.

It was observed many years ago that ATP could activate cation channels in cultured chick myoblasts and myotubes [Kolb and Wakelam, 1983]. Later, Hume and Honig [1996] showed that ATP induced depolarization and contraction in chick myotubes. The existence of P2X receptors in the embryonic chick skeletal muscle is conspicuous. Two years ago, we started to clone the P2X receptors from embryonic chick skeletal muscle. Using RT-PCR, we obtained two cDNA fragments: one encodes a partial sequence of the above-mentioned $cP2X_8$ receptors, while the other encodes a partial sequence closely related to $P2X_4$ receptors. The full-length $P2X_4$-like cDNA was cloned and the encoded receptor protein was expressed in *Xenopus* oocytes for functional studies. The expression of its mRNA transcripts in chick embryonic tissues was studied with Northern blot hybridization, RT-PCR analysis, and whole-mount in situ hybridization. A similar cDNA was cloned from embryonic chick heart and brain [Ruppelt et al., 1999]; however, an alignment of these two encoded proteins revealed that two amino acid residues are different. In this article, we report the full-length $cP2X_4$ cDNA sequence, including the $polyA^+$ tail, more functional characterization data on recombinant $cP2X_4$ receptor, and its expression in chick embryonic tissues.

MATERIALS AND METHODS

Isolation of cDNA Clone

$PolyA^+$ mRNAs were isolated from skeletal muscles of six day-10 White Leghorn chick embryos using FastTrack 2.0 kit (Invitrogen BV, Leek, The Netherlands). Two degenerated oligonucleotide primers (sense: ACCTGTGAGATSTBKRSYTGGTGCCC, and anti-sense: ARTRHKTGGCDRWCCTGAARTTGTASC) based on the sequences of the seven cloned rat P2X receptors were ordered from Sigma-Genosys (Cambridge, UK). First-strand cDNAs were synthesized with oligo(dT) and random primers using Superscript II RNase H^- reverse transcriptase (Life Technologies, Inchinnan Business Park, UK). Touch-down PCR was performed on the first-strand cDNAs using the degenerated primers. The annealing temperature was reduced from 65°C to 55°C in 20 cycles (–0.5°C per cycle) and a further 30 cycles were run at an annealing temperature of 55°C. PCR fragments of the predicted sizes were purified and subcloned into pCR II using TOPO TA Cloning kit (Invitrogen BV) and sequenced. Two fragments related to P2X receptors were identified.

To isolate the full-length clone of the P2X receptor cDNA, a chick embryonic skeletal muscle cDNA library was constructed using a kit (Stratagene Europe, The Netherlands) [Bo et al., 2000]. The cDNA library was screened with ^{32}P-labeled probe derived from the PCR fragment. The probe was labeled using Prime-IT II kit (Stratagene Europe). Hybridization was carried out at 42°C in a buffer containing 20% formamide. Low stringency washing was controlled by the final wash in 1 × SSPE at 45°C. Positive clones were subjected to secondary screening. Totally isolated clones were obtained and the phagemid pBluescript SK(-) containing cDNA inserts was excised from Lambda ZAP II vector and transformed into *E. coli* for amplification. cDNA inserts were sequenced in both directions.

Electrophysiology

To study the electrophysiological and pharmacological properties of the encoded receptor, cRNA was synthesized and injected into defolliculated *Xenopus* oocytes (23 nl of cRNA at 100 μg/ml). Injected oocytes were analyzed in the two-electrode voltage clamp configuration as described previously [Bo et al., 1995].

Northern Blot Analysis

$PolyA^+$ mRNAs were isolated from fresh tissues of day 10 chick embryos using the FastTrack 2.0 kit. $PolyA^+$ mRNAs (3 μg) were size-separated through a 1.5% agarose gel containing formaldehyde and transferred onto nylon membranes (Hybond N, Amersham-Pharmacia Biotech, Amersham, UK). The cDNA of the coded region was radiolabeled as described above and used as probe. Hybridization was carried out in a buffer with 50% formamide overnight at 42°C and the stringency was controlled by a final wash of the membrane in 0.3 × SSPE at 65°C. The membrane was exposed to X-ray film for 3 days.

RT-PCR

The first-strand cDNAs were synthesized with Superscript II RNase H^- reverse transcriptase from 0.8 μg $polyA^+$ mRNAs. A sense primer with the sequence of AGAGCTGCTTCCCACTGCGTG and an anti-sense primer with a sequence of GGTCTCACAGCAGGGTCACAG were used to amplify the cDNAs. PCR was performed using the touch-down method with an initial annealing temperature of 70°C and a reduction of 0.5°C per cycle for the first 10 cycles. The second stage was performed with an annealing temperature of 65°C for 20 cycles. PCR products were identified in a 1.5% agarose gel.

Whole-Mount In Situ Hybridization

The first 400 bp of the cDNA open reading frame was subcloned into pCRII vector (Invitrogen). Both sense and antisense cRNA probes were synthesized with the DIG-RNA labeling kit (Roche Diagnostics, Lewes, UK). Day 3, day 4, and day 6 chick embryos were fixed in 4% paraformaldehyde overnight. Whole-mount in situ hybridization was performed according to the protocol described by Nieto et al. [1996]. Prehybridization was carried out in a buffer containing 50% formamide at 55°C for 4 h, followed by overnight hybridization at 55°C for day 3 and day 4 embryos and 3 days for day 6 embryos. Final wash was in 2 × SSC with 50% formamide at 55°C for 2 × 60 min. Hybridization signals were revealed with anti-DIG-alkaline phosphatase antibody staining (DigNucleic Acid Detection Kit, Roche). Whole embryos were photographed and then embedded in gelatin-albumin gel. Sections (100 μm thick) of the embryos were cut with a vibratome.

RESULTS

Cloning of the cP2X$_4$ Receptor

RT-PCR with degenerated primers produced a cDNA fragment of 427 bp. Alignment of this cDNA fragment with the cloned P2X receptors showed that it was closely related to P2X$_4$ receptors. Screening of the embryonic chick skeletal muscle cDNA library identified two full-length cDNA clones with 1,803 bp (including the polyA$^+$ tail) (Fig. 1). The open reading frame encodes a protein of 385 amino acids (Fig. 1). The calculated molecular weight of the protein is 43.4 kDa. The P2X receptor sequence has the two putative transmembrane domains and the 10 conserved cysteine residues in the extracellular loop like all other cloned P2X receptors. The deduced amino acid sequence of cP2X$_4$ shares 75% identity with rat and mouse P2X$_4$, and 76% with human P2X$_4$. The alignment of their amino acid sequences is shown in Figure 2. For other receptors, the percentage of identity is as follows: 46% with rat P2X$_1$, 38% with rat P2X$_2$, 42% with rat P2X$_3$, 44% with rat P2X$_5$, 43% with rat P2X$_6$, 29% with P2X$_7$, and 53% with chick P2X$_8$. The high percentage of identity of the encoded protein with the P2X$_4$ receptors indicates this receptor is an ortholog of P2X$_4$ receptors.

Functional Characterization of Recombinant Receptors

Application of ATP on *Xenopus* oocytes injected with cP2X$_4$ RNA produced a fast inward current (Fig. 3A), which partially desensitized the receptors. The degree of desensitization depended on the concentration

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   1 CC ATG GCT GCG TGC TGC GGG GCT GTG CGC GGC TTC CTC TTC GAG TAC GAC ACC CCC CGC ATC GTG CTG ATC CGC     74
   1     M   A   A   C   C   G   A   V   R   G   F   L   F   E   Y   D   T   P   R   I   V   L   I   R     24

  75 AGC CGC AAA GTC GGG CTG ATC AAC CGC GCC GTG CAG CTC GCC ATC CTC GCC TAC GTG ATC GGC TGG GTC TTT CTG    149
  25  S   R   K   V   G   L   I   N   R   A   V   Q   L   A   I   L   A   Y   V   I   G   W   V   F   L     49

 150 TGG GAG AAG GGC TAC CAG GAA ACA GAT TCC GTG GTC AGT TCT GTA ACC ACG AAG GTG AAA GGG GTA ACG ATG ACA    224
  50  W   E   K   G   Y   Q   E   T   D   S   V   V   S   S   V   T   T   K   V   K   G   V   T   M   T     74

 225 AAC ACA TCC ACC TTG GGA TCC AGG ATC TGG GAT GTA GCT GAC TAC GTC ATC CCT CCC CAG GAG AAG AAC GCT GTG    299
  75  N   T   S   T   L   G   S   R   I   W   D   V   A   D   Y   V   I   P   P   Q   E   K   N   A   V     99

 300 TTT GTC ATG ACC AAC ATG ATA TTC ACA CTG AAC CAG AGC CAA AGC CAC TGC CCA GAG CTT CCA GAT GAT AAC ACA    374
 100  F   V   M   T   N   M   I   F   T   L   N   Q   S   Q   S   H   C   P   E   L   P   D   D   N   T   124

 375 GAG TGC AAC AAC AAC AGC AGC TGC GTT CCA GGA TAT GTC AGC ACC CAC AGC AAT GGT ATC CAG ACC GGG GCG TGC    449
 125  E   C   N   N   N   S   S   C   V   P   G   Y   V   S   T   H   S   N   G   I   Q   T   G   A   C   149

 450 ATA CCG TAC AAC AGC AGC ATC AAG ACC TGT GAA GTC TTT GCA TGG TGC CCT GTG GAG GAT GAC TAT CAC ATA CCC    524
 150  I   P   Y   N   S   S   I   K   T   C   E   V   F   A   W   C   P   V   E   D   D   Y   H   I   P   174

 525 AAT CCG GCA TTC TTG CAA GGA GCT GAG AAC TTC ACC ATT CTG GTA AAG AAC AAC ATT TGG TAT CCC AAG TTC AAC    599
 175  N   P   A   F   L   Q   G   A   E   N   F   T   I   L   V   K   N   N   I   W   Y   P   K   F   N   199

 600 TTC AGC AAG CGA AAC ATC CTC CCC ACT TTC AGT TCC TCT TAT CTC AAG AAC TGC ATC TAT GAT GCC CAG ACA GAT    674
 200  F   S   K   R   N   I   L   P   T   F   S   S   S   Y   L   K   N   C   I   Y   D   A   Q   T   D   224

 675 CCC TTC TGC CCT ATC TTT CGT CTG GGG AAA ATA GTT GAA GCT GCA GGG CAG AAC TTC CAG GAA ATG GCT GTG GAG    749
 225  P   F   C   P   I   F   R   L   G   K   I   V   E   A   A   G   Q   N   F   Q   E   M   A   V   E   249

 750 GGT GGG GTC ATG GGG CTG CAG ATC AAC TGG GAC TGC AAC CTC GAC AGA GCT GCT TCC CAC TGC GTG CCC AAG TAT    824
 250  G   G   V   M   G   L   Q   I   N   W   D   C   N   L   D   R   A   A   S   H   C   V   P   K   Y   274

 825 TCC TTC CGG CGC CTG GAC AAC AAG GAC TCT GCC AAC ACT ATC TCA CCT GGG TAC AAC TTC AGG TTT GCA AAA TAC    899
 275  S   F   R   R   L   D   N   K   D   S   A   N   T   I   S   P   G   Y   N   F   R   F   A   K   Y   299

 900 TAC AAG GAT AGT AGT GGC ATT GAA ACA CGA ACG CTT ATC AAA GCT TAT GGC ATC CGC CTT GAT ATC ATA GTG TTT    974
 300  Y   K   D   S   S   G   I   E   T   R   T   L   I   K   A   Y   G   I   R   L   D   I   I   V   F   324

 975 GGA AAG GCA GGA AAA TTT GAT GTA ATT CCT ACC ATG ATT AAC ATT GGC TCT GGC TTA GCG CTG TTT GGA GTG GCA   1049
 325  G   K   A   G   K   F   D   V   I   P   T   M   I   N   I   G   S   G   L   A   L   F   G   V   A   349

1050 ACA GTG CTG TGT GAC ATC GTT GTC CTG TAC TGC ATG AAA AAG AGG TAC TTC TAT CGG GAG AAG AAG TAC AAA TAC   1124
 350  T   V   L   C   D   I   V   V   L   Y   C   M   K   K   R   Y   F   Y   R   E   K   K   Y   K   Y   374

1125 GTG GAG GAT TAT GAA CTG GGC ACA AGT GAG ACA TAA GGAAGACTCCTGACCTCCTGCTGCTGCTGTGAATGTTACTGTGACCCTGC 1210
 375  V   E   D   Y   E   L   G   T   S   E   T   *

1211 TGTGAGACCTACCTCTCCTTTGGAAGGAAGGGGGAACAGAACCTAAGAAAGGGACCTTCTTCATTACTCATCACCTTTTGCTGGCCAGAACCAAAGAGGA 1310

1311 GAACCGGATCTGCTCCTTGTCTGTTCTTTCCAGGTTAACTGCACCAAGCCCATACAGAGCCTTGTGGCTTCGTGCCAGACATCTGCACTAGCAAGCCTGG 1410

1411 GACAGGGACTGCCCCGTCCTTGTCATTTCAGCAGTCCCTTCCCTGAGCTCACTGGTAGTAGCAGTGAAGCCCTTGAAGAGGCACAGACTCTTCCTTAGAG 1510

1511 GGAACATGGCCCATGACATGTGTTGCCTACCCCTGGGAGAGCAGTCTCTTGCTGTCAGGGATCACGATGCTTAGGTGAGACCGCCTGCTATTTAATTGCA 1610

1611 TCTCCAAGCTCTAGTGGCCACAGCTTGAGACAAAGCAGTGTCTGATTCCATTGTGCTATGGTAAATAGCTTACTGGCTACAGGATTTCTTATCTGTTTTG 1710

1711 TCTGGCTAATACCACTACACTGGAATTCGTTTCTCATAAAATGTTACTCTCTACCAGCTCTCTTGGAGTCCTGCAAAAAAAAAAAAAAAAAAAA 1803
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Fig. 1. cDNA sequence of cP2X$_4$ receptor and the deduced amino acid sequence. The nucleotide sequence of cP2X$_4$ cDNA has been submitted to the GenBank database with accession number AF218449.

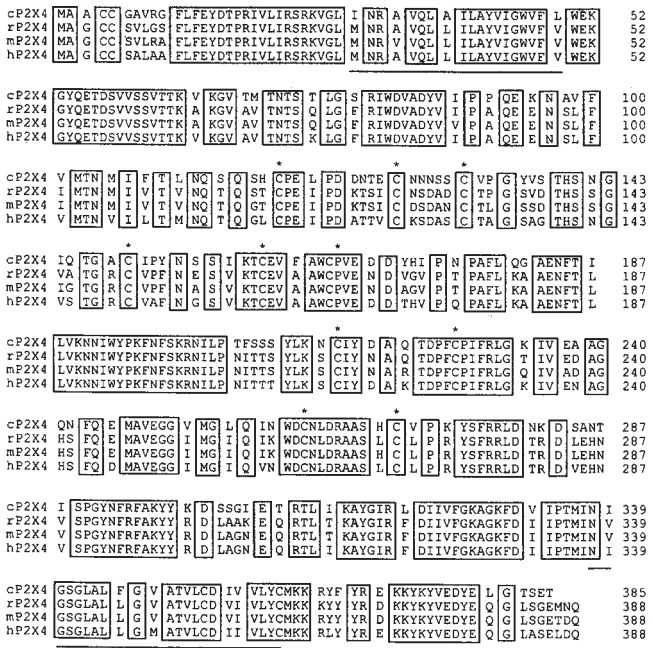

Fig. 2. Alignment of the deduced amino acid sequence of cP2X$_4$ receptor with rat, mouse, and human P2X$_4$ receptors. The two putative transmembrane domains are indicated by bars underneath. The ten conserved cysteine residues on the extracellular loop are marked by an asterisk.

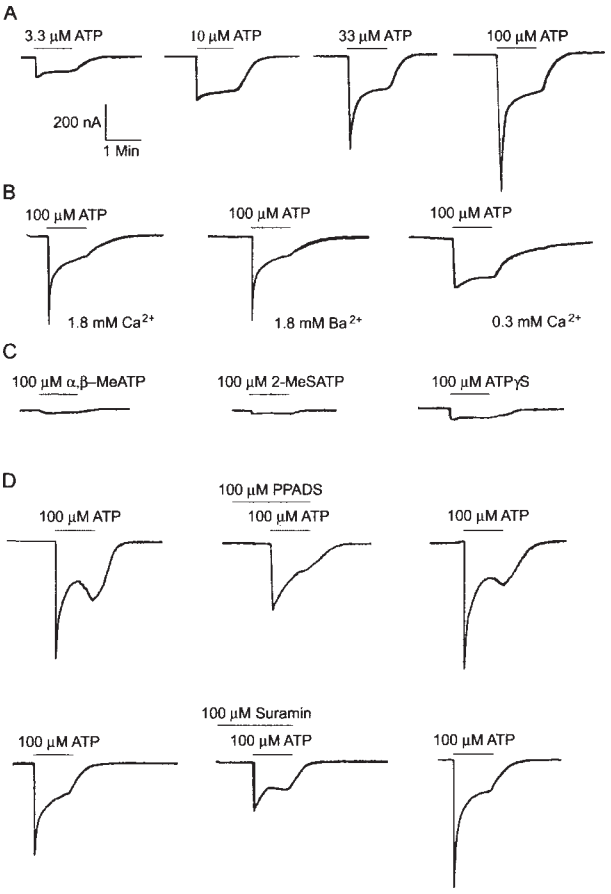

Fig. 3. Functional expression of recombinant cP2X$_4$ receptors in *Xenopus* oocytes. **A**: Inward currents induced by different concentrations of ATP in cP2X$_4$ cRNA injected *Xenopus* oocytes in 1.8 mM Ca^{2+} medium. **B**: Comparison of the currents induced by 100 μM ATP in 1.8 mM Ca^{2+}, 1.8 mM Ba^{2+}, and 0.3 mM Ca^{2+} medium. **C**: Currents induced by 100 μM α,β-methyleneATP (α,β-MeATP), 2-methylthioATP (2-MeSATP), and ATPγS. **D**: Currents induced by ATP (100 μM) were partially blocked by 100 μM PPADS or suramin.

of ATP used. With 3.3 and 10 μM ATP, the spikes were usually small; at higher concentration of ATP the desensitization was much more obvious. The recovery time from desensitization also depended on the concentration of ATP applied, ranging from 5 min at 3.3 μM to 15 min at 100 μM. Construction of a concentration–response curve for ATP (not shown) yielded an EC_{50} value of 9.5 ± 1.3 μM.

Replacement of Ca^{2+} in the perfusion buffer with Ba^{2+} did not change the characteristics of the ATP-induced inward currents (Fig. 3B). However, when Ca^{2+} concentration in the perfusion buffer was reduced from 1.8 mM to 0.3 mM, the ATP-induced currents were much smaller, with the obvious disappearance of the fast-desensitizing component (Fig. 3B).

In addition to ATP, many other ATP receptor-active compounds were also tested. 2-MethylthioATP and adenosine 5′-*O*-(thio)triphosphate (ATPγS) were weak agonists for cP2X$_4$ receptors, inducing inwards currents of about 20–60 nA at 100 μM, which were much smaller than that of ATP (average of 600 nA at 100 μM) (Fig. 3C). α,β-MethyleneATP was even weaker than 2-methylthioATP (Fig. 3C). No detectable response was recorded from β,γ-methyleneATP, UTP, GTP, TTP, ADP, AMP, adenosine, and diadenosine tetraphosphate at concentrations up to 100 μM.

Suramin or pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), each at 100 μM, did not produce any detectable response in oocytes expressing cP2X$_4$ receptors. Both were shown to partially block ATP-induced currents (Fig. 3D). A recently identified P2X receptor antagonist, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) [Mockett et al., 1994; Virginio et al., 1998], could completely block the ATP-induced currents (Fig. 4). Construction of a concentration-inhibition curve yielded an IC_{50} value of 4.74 ± 0.14 μM for TNP-ATP.

Tissue Distribution

Northern blot hybridization analysis revealed the expression of cP2X$_4$ mRNA transcripts of about 1.9 kb in day 10 chick embryonic tissues (Fig. 5A). The skeletal muscle, brain, and gizzard showed the highest level of expression. Lower levels of expression were detected in

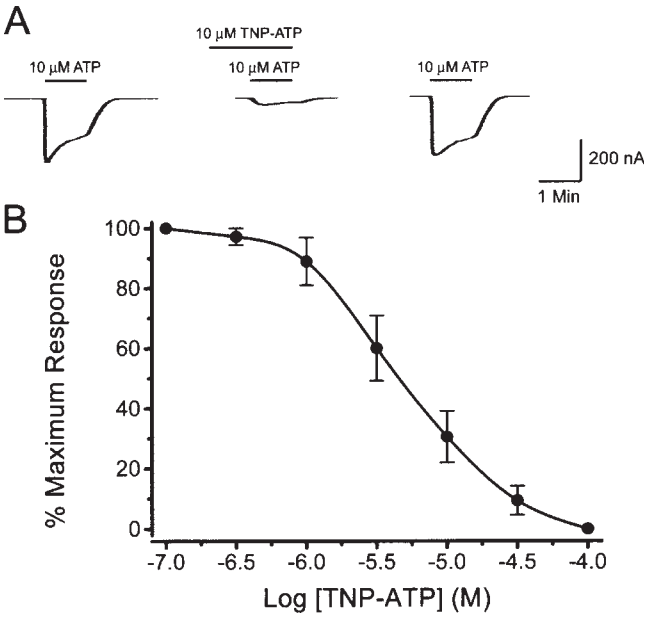

Fig. 4. Inhibition of ATP-induced currents via recombinant $cP2X_4$ receptors in *Xenopus* oocytes by trinitrophenylATP (TNP-ATP). **A**: Inhibition of 10 μM ATP-induced current by 10 μM TNP-ATP. **B**: Concentration–inhibition curve of ATP-induced currents by TNP-ATP, with an IC_{50} of 4.74 ± 0.14 μM.

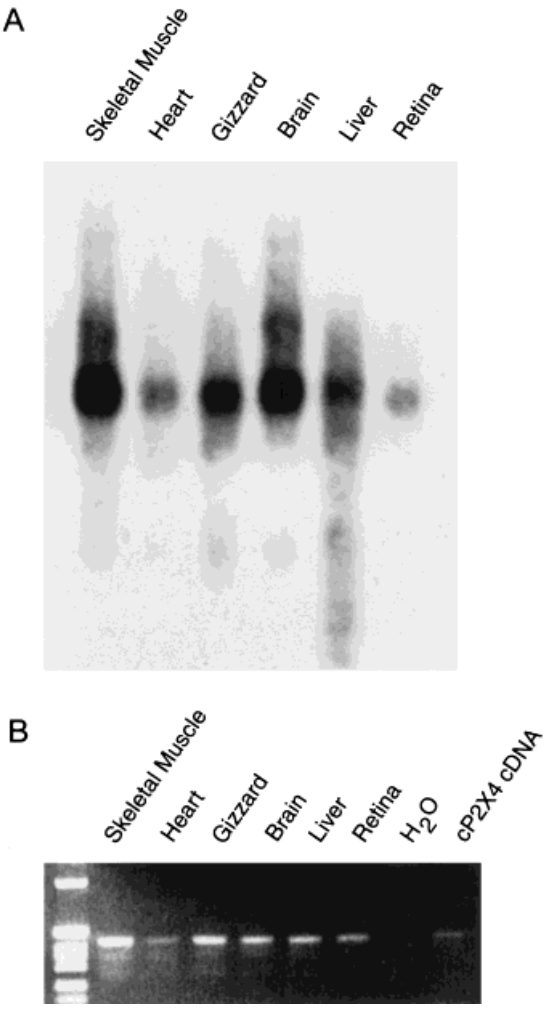

Fig. 5. Expression of $cP2X_4$ receptor mRNA in different embryonic (day 10) chick tissues detected by Northern blot hybridization (**A**) and RT-PCR (**B**). **A**: For Northern blot analysis, 3 μg of $polyA^+$ RNA was loaded per lane in a 1.5% agarose gel and the X-ray film was exposed for 3 days. **B**: Touch-down PCR was run for 30 cycles. $cP2X_4$ cDNA (0.3 pg) was used as positive control. Molecular marker, 1 kb ladder from Promega Ltd.

liver, heart, and retina. RT-PCR assays revealed a similar pattern of expression (Fig. 5B).

Whole-mount in situ hybridization on day 3, day 4, and day 6 chick embryos showed a wide distribution of $cP2X_4$ mRNA transcripts (Fig. 6). The signals generated were lower compared with that of $cP2X_8$ probes. In day 3 and day 4 embryos, hybridization signals were observed in the brain and the ventral part of the spinal cord. They were also present in notochord, esophagus, gizzard, and heart (Fig. 6). In day 6 embryo, faint signals in myotomes and premuscle mass were observed; however, they were not as strong as that detected with $cP2X_8$ probes at the same stage of development.

DISCUSSION

The $P2X_4$ receptor is the most widely distributed receptor of the P2X family. It is also the most abundant in the central nervous system. Following the cloning of rat $P2X_4$ receptors [Bo et al., 1995], the human and mouse orthologs of $P2X_4$ receptors have also been cloned from brain cDNA libraries [Garcia-Guzman et al., 1997; Townsend-Nicholson et al., 1999]. A partial $P2X_4$ cDNA sequence was isolated from rabbit osteoclast cDNA library [Naemsch et al., 1999]. Last year, a chick $P2X_4$ cDNA was cloned from embryonic chick heart and brain [Ruppelt et al., 1999] (GenBank accession number Y18008). Alignment of the deduced amino acid sequence of Clone Y18008 with our clone revealed two different sites in the sequence. When the amino acid sequence of Clone Y18008 is aligned with rat and human $P2X_4$ receptors, it is shown that Y18008 has one amino acid residue missing at position 128 of rat and human $P2X_4$ receptor sequences. In our clone that amino acid, an asparagine, does exist, which means the chick $P2X_4$ receptor polypeptide chain should have 385 amino acids instead of 384. Another different site is at amino acid 326 of the $P2X_4$ receptor; rat, human, mouse, and our chick $P2X_4$ clones all show that this residue is a conserved lysine, while clone Y18008 shows a residue of glutamate, which is due to the difference of a single nucleotide: a G in Clone Y18008 and an A in our clone.

Functional study of homomeric $cP2X_4$ receptors expressed in *Xenopus* oocytes revealed that $cP2X_4$ recep-

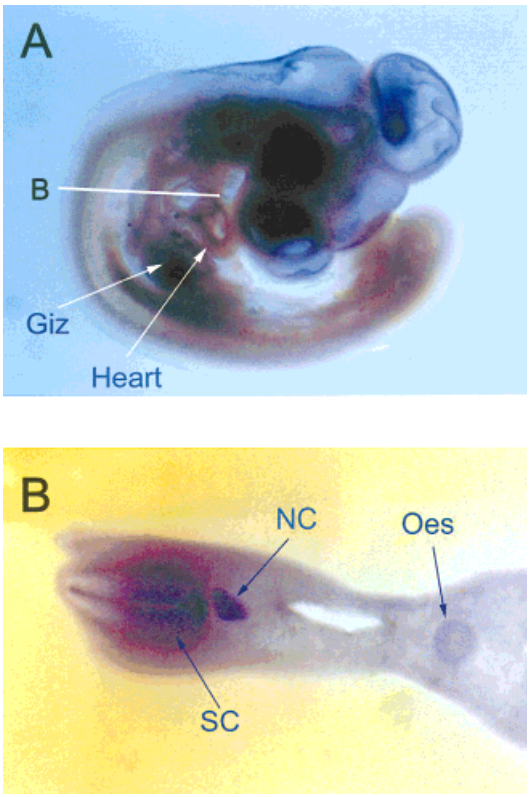

Fig. 6. Whole-mount in situ hybridization of day 4 chick embryo with digoxigenin-labeled cP2X$_4$ RNA probe. **A**: Lateral view of the whole embryo (Giz, gizzard). **B**: Section over the middle part of the embryo showing the expression of cP2X$_4$ RNA in spinal cord (SC), notochord (NC), and esophagus (Oes).

tors share many electrophysiological and pharmacological properties with rP2X$_4$ receptors, such as partial desensitization and insensitivity to α,β-methyleneATP. The potency of ATP in activating cP2X$_4$ receptors is quite close to that of rP2X$_4$ receptor (10 μM) and also close to the cP2X$_4$ reported by Ruppelt et al. [1999] (13.7 ± 2.1 μM). For both chick and rat P2X$_4$ receptors, ATP was the most potent among the known P2X receptor agonists. However, 2-methylthioATP and ATPγS are much less potent on cP2X$_4$ than on rP2X$_4$.

The commonly used P2X receptor antagonists, suramin, PPADS, and Reactive blue 2 all potentiated the ATP-induced currents via rP2X$_4$ receptors, whereas they blocked the hP2X$_4$ receptors [Garcia-Guzman et al., 1997]. Mouse P2X$_4$ receptors expressed in *Xenopus* oocytes were potentiated by the three antagonists [Townsend-Nicholson et al., 1999], whereas mP2X$_4$ receptors expressed in HEK 293 cells showed a different profile: they were effectively blocked by PPADS, but were still insensitive to suramin [Jones et al., 2000]. The difference in sensitivity to PPADS is probably because of the longer preincubation of PPADS before application of ATP in the later study. Chick P2X$_4$ receptors were shown to be partially blocked by suramin and PPADS, which is different from both the rP2X$_4$ and hP2X$_4$ receptors. The structural determinants controlling the antagonist sensitivity have been explored before. A mutant of rP2X$_4$ produced by replacing amino acid 249 from glutamate to lysine was shown to be readily and irreversibly antagonized by PPADS [Buell et al., 1996]. However, this mutant was still not sensitive to suramin, indicating these two antagonists bind to different structures of the receptor protein. In another experiment, chimeras of rat and human P2X$_4$ receptors were generated to identify the domains responsible for the antagonist sensitivity [Garcia-Guzman et al., 1997]. It was also suggested that PPADS and suramin binds to different domains of the P2X$_4$ receptors.

TNP-ATP has been used as a fluorescent label for ATP binding sites in proteins [Mockett et al., 1994]. It has also been found to be a potent P2X receptor antagonist [Mockett et al., 1994; Virginio et al., 1998; Thomas et al., 1998]. Homomeric P2X$_1$ and P2X$_3$, and heteromeric P2X$_{2/3}$ receptors are especially sensitive to TNP-ATP, with IC$_{50}$ values of 1–7 nM. Much higher concentrations of TNP-ATP were required to block P2X$_2$, P2X$_4$, and P2X$_7$ receptors, with IC$_{50}$ values >2 μM [Virginio et al., 1998]. Homomeric cP2X$_4$ receptors were blocked by TNP-ATP with an IC$_{50}$ value of 4.74 μM, about three times lower than the IC$_{50}$ value obtained on rP2X$_4$ receptors expressed in HEK293 cells (15.2 μM) [Virginio et al., 1998]. On the other hand, cP2X$_8$ receptors are highly sensitive to TNP-ATP: 10 nM TNP-ATP completely blocked 10 μM ATP-induced inward currents (data not shown). This might be due to the finding that cP2X$_8$ receptor has similar electrophysiological and pharmacological properties to P2X$_1$ and P2X$_3$ receptors, such as fast desensitization and sensitivity to α,β-methyleneATP [Bo et al., 2000]. An IC$_{50}$ value for cP2X$_8$ receptor could not be obtained due to the prolonged recovery after the activation of the receptor.

With Northern blot hybridization and RT-PCR the expression of cP2X$_4$ mRNA transcripts was detected in all the tissues tested in day 10 chick embryos. Whole-mount in situ hybridization of day 3, 4, or 6 embryos also confirmed the expression of cP2X$_4$ mRNAs in a variety of organs. Although our cP2X$_4$ receptor was cloned from the skeletal muscle, the electrophysiological and pharmacological properties of the native P2X receptors in embryonic skeletal muscle are very much like those of cP2X$_8$ receptors [Bo et al., 2000]. The physiological function of cP2X$_4$ receptors in embryonic skeletal muscle remains to be elucidated. In embryonic chick neural retina, ATP receptor was reported to regulate the synthesis of DNA [Sugioka et al., 1999]. It is not clear whether P2X4 receptor is involved in such a process, although cP2X$_4$ RNA is highly expressed in embryonic chick retina.

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