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Molecular Cloning and Characterization of a Novel ATP P2X Receptor Subtype from Embryonic Chick Skeletal Muscle*

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We have cloned a new P2X ligand-gated ion channel receptor from embryonic chick skeletal muscle, which is tentatively named as chick $P2X_8$ ($cP2X_8$) receptor. The cloned cDNA encodes a protein with 402 amino acids. Electrophysiological study of the recombinant $cP2X_8$ receptor expressed in *Xenopus* oocytes showed that 10 μM ATP induced a fast inward current followed by rapid and long lasting desensitization in medium containing 1.8 mM Ca^{2+}. In medium with 0.3 mM Ca^{2+} ATP induced a bi-phasic response as follows: a slower inward current succeeded the initial fast one. 2-Methylthio-ATP, α,β-methylene-ATP, and adenosine 5′-*O*-(thio)triphosphate were potent agonists, whereas ADP was a very weak agonist. ATP-induced currents were blocked by 100 μM suramin and pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid. Northern blot analysis and reverse transcription-polymerase chain reaction showed that $cP2X_8$ RNA transcripts were mainly expressed in skeletal muscle, brain, and heart of Day 10 chick embryos. A moderate level of expression was also detected in gizzard and retina. Whole mount *in situ* hybridization showed that $cP2X_8$ RNA transcripts were expressed mainly in neurotube, notochord, and stomach in Day 3 embryos. In Day 4 and Day 6 embryos, the $cP2X_8$ RNA transcripts were highly expressed in the myotome and premuscle mass. The physiological role of this receptor in the establishment of the skeletal muscle innervation will be studied.

ATP is co-released with acetylcholine from the motor nerve endings (1) and potentiates the acetylcholine-induced response in adult neuromuscular junction (2, 3), although the underlying mechanism is not yet fully understood. It has been observed that external application of ATP can induce cation influx and increase of intracellular Ca^{2+} concentration (4). The latter is partially due to the release of calcium from intracellular stores via second messenger mechanisms. In cultured chick myotubes, ATP was reported to induce the formation of inositol triphosphate via a G protein-coupled receptor (5). In mouse C2C12 myotubes, P2Y receptor subtypes mediated activation of the phospholipase C pathway (6) and the formation of cAMP (7). The expression of $cP2Y_1$ subtype has been demonstrated in chick myotome (8).

Apart from the P2Y receptor-mediated responses via G proteins, ATP was reported to activate cation channels in cultured chick myoblasts and myotubes (9). A detailed study by Hume and Honig (10) showed that ATP induced depolarization and contraction in chick myotubes. However, the depolarization declined during prolonged application of ATP and did not recover for at least 20 min after the removal of ATP. This was recognized as a unique feature of this P2X receptor and has not been observed previously for the known P2X receptor subtypes. A study on the contractile responses of embryonic skeletal muscle to ATP during development revealed that the response disappeared after Day 17 (11). However, denervation of the muscle in newly hatched chicks led to the reappearance of ATP responsiveness, which suggests that the expression of ATP receptors is regulated by innervation.

So far, cDNAs for seven P2X receptor subtypes (or subunits) have been cloned (12). All known P2X receptor subunits can form functional homomeric ATP-gated ion channels (13). The seven P2X receptor subtypes have different tissue distributions. For example, $P2X_3$ and $P2X_2$ receptor subtypes are highly expressed in sensory neurons (14, 15), whereas the $P2X_1$ and $P2X_2$ receptor subtypes appear to be the main ones in smooth muscle cells (16–18). In adult skeletal muscle, most P2X receptor subtypes were reported absent, although a moderate level of $P2X_7$ mRNA was detected in human skeletal muscle using Northern blot hybridization (19). A human P2XM receptor, which shares 80% identity with rat $P2X_6$ receptor, was reported to be predominantly expressed in skeletal muscle (20). However, no functional study has been carried out on this subtype. The pharmacological character of the ATP-induced response in chick embryonic skeletal muscle, *i.e.* the prolonged desensitization, is not present in any of the cloned P2X receptors, suggesting the existence of another P2X receptor subtype in this tissue. This receptor subtype may play an important role in establishment of the skeletal muscle innervation. Therefore, it is of great interest to clone and characterize this receptor subtype and study its role in skeletal muscle innervation.

EXPERIMENTAL PROCEDURES

Extraction of Poly(A)$^+$ mRNA—Skeletal muscles from the leg and chest of six Day 10 White Leghorn chick embryos were isolated and pooled. The FastTrack 2.0 kit (Invitrogen BV, Leek, The Netherlands) was used to isolate the poly(A)$^+$ mRNA according to the manufacturer's protocol.

PCR[1] Cloning—Two degenerated oligonucleotide primers (sense, ACCTGTGAGATSTBKRSYTGGTGCCC, and antisense, ARTRHKTGGCDRWCCTGAARTTGTASC) based on the sequences of the seven cloned rat P2X receptors were designed and ordered from Sigma. First strand cDNAs were synthesized with oligo(dT) and random primers using Superscript II RNase H$^-$ reverse transcriptase (Life Technolo-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF205066.

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[1] The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase; bp, base pair; RT-PCR, reverse transcription-polymerase chain reaction; PPADS, pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid; ATPγS, adenosine 5′-*O*-(thio)triphosphate.

gies, Inc.). Touch-down PCR was performed on the first strand cDNAs using the degenerated primers. The annealing temperature was reduced from 65 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 with a cycle sequencing kit and ABI 377 sequencer from Applied Biosystems Inc.

Construction of cDNA Library—A cDNA library construction kit from Stratagene Europe (The Netherlands) was used. cDNAs were synthesized from 5 μg of poly(A)$^+$ mRNA and inserted into Lambda ZAP II vectors that contain the sequence of pBluescript SK(−) phagemid vector. The total plaque-forming units obtained was 5.77×10^6 with insert sizes ranging from 0.7 to 9 kb (estimate of 20 excised inserts from randomly selected clones).

Isolation of Full-length cDNA—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, The Netherlands), and unincorporated nucleotides were removed with a QIAquick Nucleotides Removal kit (Qiagen Ltd., Crawley, UK). 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 pBluesrcipt SK(−) containing cDNA inserts was excised from Lambda ZAP II vector with a helper phage and transformed into *Escherichia coli* for amplification. cDNA inserts were sequenced in both directions.

Electrophysiology—cRNA was synthesized as run-off transcripts. *Xenopus* oocytes (stage V) were isolated and treated with collagenase (type V, Sigma) (33 μg/ml, shaken lightly for 45 min at room temperature). Oocytes were then defolliculated manually and injected (Drummond Injector) with 23 nl of cRNA (approximately 5 μg/ml). The oocytes were incubated in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 15 mM Tris-HCl, pH 7.4, supplemented with 50 IU/ml penicillin, and 50 μg/ml streptomycin) at 19 °C for 2 days. Injected oocytes were analyzed in the two-electrode voltage clamp configuration (TEC05 amplifier, NPI Electronics, Tamm, Germany) perfused with NFR (115 mM NaCl, 2.5 mM KCl, 10 mM Hepes, pH 7.2, supplemented with 1.8 mM $CaCl_2$ unless otherwise noted). Data were recorded simultaneously to a chart recorder and to a computer hard disc (CELLworks, NPI Electronics, Tamm, Germany). For chart-like digital data recording, data were typically filtered at 100 Hz, digitized at 300 Hz (oversampling), and reduced to 5 Hz before storage. Application of perfusion solutions as well as clamp voltage was computer-controlled.

Northern Blot Analysis—Poly(A)$^+$ mRNAs were isolated from fresh tissues of Day 10 chick embryos using the FastTrack 2.0 kit from Invitrogen. Poly(A)$^+$ mRNAs (3 μg) were size-separated through a 1.5% agarose gel containing formaldehyde and transferred onto nylon membranes (Hybond N, Amersham Pharmacia Biotech). A 400-bp cDNA fragment starting from the start cordon 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 (Life Technologies, Inc.) from 0.8 μg of poly(A)$^+$ mRNAs. A sense primer with the sequence of CATGCCATGGGGCAGGTGTCCTGGAAG and an antisense primer with a sequence of GGCTTGGATCTTTTCTCCAC were used to amplify the cDNAs. PCR was performed using the touch-down method with an initial annealing temperature of 70 °C and a drop of 0.5 °C per cycle (10 cycles for the first stage). The second stage was performed with an annealing temperature of 65 °C for 20 cycles. After PCR reaction, 10 μl of reaction mixture was withdrawn and run in a 1.5% agarose gel.

Whole Mount in Situ Hybridization—The first 530 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 Molecular Biochemicals). 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.* (21). 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 twice for 60 min. Hybridization signals were revealed with anti-DIG-alkaline phosphatase antibody staining (DigNucleic acid detection kit, Roche Molecular Biochemicals). Whole embryos were photographed and then embedded in gelatin-albumin gel. Sections of the embryos (100 μm thick) were cut with a vibratome.

RESULTS

Cloning of the cP2X$_8$ Receptor—RT-PCR with degenerated primers produced a cDNA fragment of 421 bp. Alignment of this cDNA fragment with the cloned P2X receptors showed that it was related to this receptor family. Screening of an embryonic chick skeletal muscle cDNA library identified three full-length cDNA clones with 4308 bp (including the poly(A)$^+$ tail) (Fig. 1). The open reading frame encodes a protein of 402 amino acids. The calculated molecular mass of the protein is 45.1 kDa. We have tentatively named this protein chick P2X$_8$ (cP2X$_8$) receptor. The cP2X$_8$ receptor sequence has the two putative transmembrane domains, and the 10 conserved cysteine residues in the extracellular loop are considered as characteristics for this gene family. The identity of the amino acid sequence of cP2X$_8$ with other cloned rat P2X receptors is 43% for P2X$_1$, 39% for P2X$_2$, 43% for P2X$_3$, 53% for P2X$_4$, 59% for P2X$_5$, 47% for P2X$_6$, and 24% for P2X$_7$. The low percentage of identity of cP2X$_8$ with other P2X receptors indicates this receptor is a new member of the P2X receptor family. Rat P2X$_5$ receptor (22) and a partial human P2X receptor which was believed to be the human homologue of rP2X$_5$ (23) are two of the closest to cP2X$_8$ among the cloned P2X receptors. The alignment of their amino acid sequences is shown in Fig. 2. The percentage of identity of cP2X$_8$ with hP2X$_5$ is 59% by direct alignment; however, the percentage should be increased to 62% if the missing segment in hP2X$_5$ is excluded.

Expression of Recombinant Receptors—Xenopus oocytes injected with cP2X$_8$ RNA yielded consistently robust instant currents after ATP application with an onset that is typical for ligand-gated ion channels (within the limits of the oocyte whole cell configuration). Application of 10 μM ATP induced a large and fast inward current with an average amplitude of 600 nA, which fully desensitized the receptors (Fig. 3*A*). The desensitization of cP2X$_8$ receptor showed a prolonged period for recovery (run-down), only 7% of initial amplitude in 5 min after the removal of ATP, 31% in 30 min, and 65% in 1 h (Fig. 3*A*). Therefore, it was impossible to build a concentration-response curve of ATP and calculate the EC$_{50}$ value. Application of a lower concentration of ATP, such as 0.1 μM, could induce a small inward current of about 20–30 nA, which was also followed by significant run-down.

Replacement of Ca^{2+} in the perfusion buffer with Ba^{2+} did not change the character of the ATP-induced inward currents. However, when the Ca^{2+} concentration in the perfusion buffer was reduced from 1.8 to 0.6 or 0.3 mM, the oocytes became unstable. Application of ATP in 0.3 mM Ca^{2+} medium for 90 s produced a bi-phasic response curve, an initial fast depolarization, and partial desensitization followed by a slow increase of inward current. The current slowly recovered after the removal of ATP but remained below the rest level until restoration of 1.8 mM Ca^{2+} medium (Fig. 3*B*). If ATP was not removed, the current continued to increase, and finally the oocyte died.

In addition to ATP, several other ATP receptor active compounds were also tested. The average amplitude of inward currents induced by 10 μM 2-methylthioATP was about the same as that by ATP. α,β-Methylene-ATP and ATPγS were also potent agonists, but at 10 μM the inward currents induced by these two compounds were smaller than that by ATP (Fig. 3*C*). ADP at 100 μM produced a very small, but detectable, inward current (data not shown). β,γ-Methylene-ATP, UTP, GTP, TTP, AMP, adenosine, and diadenosine tetraphosphate did not produce detectable responses at concentrations up to 100 μM.

Suramin and PPADS, both at 100 μM, did not cause any detectable response in oocytes expressing cP2X$_8$ receptors.

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   1 CTGCTGTCTGCTCCAGCCCTCAGTAAAGAGAGCTGGGAGA ATG GGG CAG GTG TCC TGG AAG GGT TTA TTT CTA TCG CTT TTT GAT   85
   1                                           M   G   Q   V   S   W   K   G   L   F   L   S   L   F   D   15

  86 TAC AAA ACA GAG AAG TAC GTC ATT GCG AAG AAC AAG AAG GTT GGG ATT CTC TAT CGA GTG GTG CAG CTC TCC ATC  160
  16  Y   K   T   E   K   Y   V   I   A   K   N   K   K   V   G   I   L   Y   R   V   V   Q   L   S   I   40

 161 CTG GCT TAC CTG GTG GGG TGG GTG TTT GTT GTC AAG AAA GGC TAT CAG GAC ACG GAC ACA TCC CTG CAG AGC TCT  235
  41  L   A   Y   L   V   G   W   V   F   V   V   K   K   G   Y   Q   D   T   D   T   S   L   Q   S   S   65

 236 GTC ATC ACC AAA CTG AAA GGG GTG GCG TTC ACC AAC ACC TCG GAG CTG GGG GAG AGG CTG TGG GAT GTT GCA GAC  310
  66  V   I   T   K   L   K   G   V   A   F   T   N   T   S   E   L   G   E   R   L   W   D   V   A   D   90

 311 TAT GTC ATC CCT CCA CAG GGT GAA AAC GTC TTC TTT GTC ATG ACA AAT TTG ATT GTG ACC CCA AAC CAG AGA CAA  385
  91  Y   V   I   P   P   Q   G   E   N   V   F   F   V   M   T   N   L   I   V   T   P   N   Q   R   Q  115

 386 ACC ACG TGT CCT GAG AGT GTA AAC ATT CCT GAT GCC TTG TGT CAC CAG GAC GAA GAC TGC CCT GAA GGG CAA GCA  460
 116  T   T   C   P   E   S   V   N   I   P   D   A   L   C   H   Q   D   E   D   C   P   E   G   Q   A  140

 461 GTG GTG GCT GGT AAT GGG GTT AAG ACT GGC CGT TGT TTG AAA GAC AGG GAC AGC ATC AGA GGT TCT TGT GAG GTA  535
 141  V   V   A   G   N   G   V   K   T   G   R   C   L   K   D   R   D   S   I   R   G   S   C   E   V  165

 536 TTG GCC TGG TGC CCA GTG GAG AAA AGA TCC AAG CCC AAG AAA CCA CTT CTC GCC AGT GCA GAA AAC TTC ACT GTT  610
 166  L   A   W   C   P   V   E   K   R   S   K   P   K   K   P   L   L   A   S   A   E   N   F   T   V  190

 611 TTC ATC AAG AAC TCG ATC CGG TTC CCC AAG TTT AAA TTC TCC AAG ATG AAT GTG CTG GCC ACC AAC AAT GAG TCC  685
 191  F   I   K   N   S   I   R   F   P   K   F   K   F   S   K   M   N   V   L   A   T   N   N   E   S  215

 686 TAC CTA AAG ACC TGC CAC TAC AGC ATG GAG CAT CCC TAC TGC CCC ATC TTC CTT CTG GGG AAC ATC GTC AGA TGG  760
 216  Y   L   K   T   C   H   Y   S   M   E   H   P   Y   C   P   I   F   L   L   G   N   I   V   R   W  240

 761 ACT GGG AAC AAC TTT CAG GAA ATG GCT TTG GAG GGT GGT GTG ATA GGA ATT CAG ATT GAA TGG AAC TGT GAC CTT  835
 241  T   G   N   N   F   Q   E   M   A   L   E   G   G   V   I   G   I   Q   I   E   W   N   C   D   L  265

 836 GAT CAA GCC CCT TCT GAA TGT AAT CCC CAC TAT TCT TTT AGC CGT CTG GAT AAC AAG TTT GCA GAA AAG TCT GTC  910
 266  D   Q   A   P   S   E   C   N   P   H   Y   S   F   S   R   L   D   N   K   F   A   E   K   S   V  290

 911 TCT TCT GGG TAC AAC TTC AGG TTT GCT AAA TAT TAC CGG GAT GCC AAA GGG ATT GAG TAC CGG ACA CTC TTT AAA  985
 291  S   S   G   Y   N   F   R   F   A   K   Y   Y   R   D   A   K   G   I   E   Y   R   T   L   F   K  315

 986 GCA TAT GGA ATC CGT TTT GAT GTG ATG GTG AAT GGC AAG GCA GGG AAA TTT AAC ATC ATT CCC ACT ATC ATC AAT 1060
 316  A   Y   G   I   R   F   D   V   M   V   N   G   K   A   G   K   F   N   I   I   P   T   I   I   N  340

1061 ATC GGT TCA GGA TTA GCT CTC ATG GGA GCG GGA GCT TTC TTT TGT GAC CTG GTG CTG CTC TAT CTG ATT AAA AAG 1135
 341  I   G   S   G   L   A   L   M   G   A   G   A   F   F   C   D   L   V   L   L   Y   L   I   K   K  365

1136 AGT AAC TTT TAT CGA GGC AAA AAG TAT GAG GAA GTA AAG TCC AGT TCC AGG AAG TCA TTA ACT AGC CCT ACT CTG 1210
 366  S   N   F   Y   R   G   K   K   Y   E   E   V   K   S   S   S   R   K   S   L   T   S   P   T   L  390

1211 AAT GGG AGT CAG AGC CCA GAC CAA CTT GGT GGG CTC TAG ACCTGGTGATGGGTCTGTCAACTGGAATCACATTCCAGGAAAACTAC 1296
 391  N   G   S   Q   S   P   D   Q   L   G   G   L   *

1297 ACAGGTGTGAAATGACACTGGGAATGGGAGCTGGAGCTACCCCAGATCAGATCCATCATCTATTATTGGGATTTCCAGAGGAAAAATATCCTTTAAAGA 1395
1396 ACACCCTATTTTCCCCGAGACGTTCTTGAACTTGCAAAACCTGTGTTCATCACTATGGATTTATAAAAGGATTTTGTAAGAGGAAAAAGAAGGCACCAA 1494
1495 ATGAATGTTTTTATATCAACTGATAGATAGATCTGTGCCACCATTATCCTGTGGCTGTGCTTTGCTTCCAAGTGCAAAAGAGAAGTGTTGTGAAACGCC 1593
1594 ACTGATGTTTTCAGGAGGGTCGTGGCCAACAATCAACTCATCTCCTGCTCCGCTGGCTTGCAGCTGCAGCTCCCAGCTCTTGCTTTTGTTATTTGCAAG 1692
1693 ATTAGATATAAAGCAGCAGGACTGCTCTTGTCAGGACGTGCAATGAAACGCAGCATTGCAGAATTGCAGCAACTGTGTCCAGAAGAAAGTGGTTAATTA 1791
1792 TCCTGTACTGCCCAATTACTCTTATTGCCCTGTTTTTGTTGTAATCATCAGAGCTAGGAGCGGTGCTCCTCCAGGAAAAGCCTCTACAACTACTGGAGC 1890
1891 AAGGTGCTCTGCTCGTCTCTCCTGGGAATGTCCACAGCAGAACCTGCCACAGCTCTGAGTGGAGAATCAGTTTCAGCCCCGGCTGGCTCCCAGCTACCT 1989
1990 CATCTGCTACACAGATTCTCCATTTATAGTTATATCCAAATAACCATTATTTCAGAGGACAGAAAAGATAACAGCATGCAGAGGAGTATTTTATGGACA 2088
2089 GGGAGGGTATTTTAGCATACTGAAAAGCAACGTGGCACTGAGCGTGTGCATGGAGATAGTCCTCATTTCTCCTTTCTTGGTTAGTGGTTGGTTTCAGTT 2187
2188 GCTAAGAATTGGTCTTTCTGCGTTGCTTACCAATTGCTCATGGTGGGGAGAGATCTGGACAGGCATTTCACGTTCCTGCCATTTTTTGTTGTACCTTGG 2286
2287 AAGTATCAGTAGTGCTGTGGAGATTGAAAAGCGAAACTCTGTCAGGTCAAACAGACTTCGCTGGGTTCTTCCAAATGTAACAGAAGGAAAAAGAAGACA 2385
2386 GACATCCACTGTTCCAACACTTCAGTATCTTCCAAGAATCACAGGTGCTTTGTTGTTTCTCAAGAGAAAACTAAGTCACTGCAACCCATTTGGTGAATT 2484
2485 TCTGTGGGGATGCAATTACTCTAAGAGGAGGAAAACACTTACAAAATAATGTTATTTTCTTTTAAAGTTTGGATTTGCTTGAGCTGACAGGAACTGACT 2583
2584 CACTAAGGCGGTAGGTGAATATTCCTCTCTTTGAAATATTAGTCCTTTCACAGGGTTTAAAGGCCTCCTGCTGCAGCGTCCTTGCCAGGACAGTTGTAG 2682
2683 AGAGGATGGCATTGCAGGTATCTGCTGTGATCTGCTGTTATATGCAGATGGAATCAGCATGCAACACCTACCTCTGCTTTAATTTCTCAAAAGCATTTG 2781
2782 TCCTGAGCAAGGTTTTCTTTTTTGTAAATGAAAATTCACCTTTGCTAGAAGTGGAAAGCTCCCAGTTCAGTCATTAATAGCAAATCCCACCCAGTCCAG 2880
2881 TCTACTCTCTCTGCTCCTGCTTCTGAGCAGATGAGAGTAAATACCAACTGGTCAAACATCTAGCTAGAAAATAGAATTAAACACTGGAGTGATAGTAAT 2979
2980 TTAGGATAAGGTATGTACACCTGGTGCTGTATTTACACTGCAATTCTCCTCTTTGACCTAGAACTCTTGACAGATATGCAGCACCCTGCATATAATGGA 3078
3079 CAGATGACTATCATGGACTACTGGAACTACCAGTGAAAAAACACAGACCTTTTTCTATCATGGCCTAATTGTATTCAAATAGTTTCTAGATGGTAAAAC 3177
3178 ACTTAGTAAACCCCACACGTGACGTCAGGCCAAGGTGCAACTCAGAGACCAGACAGCTCACCATAACATGTAAAGCTGACTGTGCCCAACTGAAGCCAA 3276
3275 TGCTTCAACTTGACAGAATGATGTCCTTGTTAAACAACCAAAGCTTTTTCCCAAATGCAAGTCTTCAGGGGCTTCAGTGCCCCCAGAACTCACGAGAAT 3375
3376 ATCTCCCTCAGTCACACCATTCATTTGCACTTAATAACAATTGCAGTTAATAAAACATAAACAGCTTTCCTCTCAGGATCTTGTTTGATATTTCTTTTT 3474
3475 TTTTTTTTTTTTTGTATTTGAAGTACAGCAATAATGTTTGTCACGTTGGGGCAGTGCTCTGTTAAGACTCTGCTGTAAAACATGTTACTTAGGTTGCAC 3573
3574 ATGCCAGTTTCCTTTGTGATACACACTTATGCAAAACAATTCAATTAAGAGTTCAGAAGACTTTGTAGTATTTAGCAGAGTTAATAGTACACGTGTCTA 3672
3673 TAGATACCAATTAATGTGCAAAAAGCAGCAGATGGCATGAAAAAGCATACTAATGAGGGAGTTAAAACCTTGACTGTTTTTTCTTCTCTGAAAAAGAAA 3771
3772 CATGAGTTAGGCAATGCTGACAGCACCAACCCCCCCTTTTTGTACAACCCTGCGTTTTGCCAGAACTCAGTTTTTCTGTAAAATATTTTTGAATACAAA 3870
3871 ACCTCATGTTGTAAAGTCTTTGTATAACAAACAGGTAATATTTTATAGCGTTCTGGTGAAGCGACGACCGACCCAAAGACCAGACAGCCAGATGGCACA 3969
3970 AGGTGTGGTTGTAGAATATGTGACAAAACCCTGTCATTGCACAAGAGTTTGCTTGCTGCTTTCAGATGGAAGGCACTAAACAGCGTTTGCATAAGCAGC 4068
4069 TCCAGAGCAAAGTGACTTAAACTGCCATAGAGGGTGATCAGAAGTTCCTTTCAGATTCCGTCAGACACAGAGTACCAAAACAATTGTAACAGCTGCAGA 4167
4168 AAAAAGCCAGGGGCTGTAATAAGCGGGTTCAGTACAGACTGTCTTGCAGGTAATTAGACACTGCGTGCATTTGAGTGCCTGTATTTCACTGGACACAAC 4266
4267 GGTATTAAAACAGAGTGATTGACTTGGGAAAAAAAAAAAAAA 4308
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FIG. 1. **The complete cDNA sequence of cP2X$_8$ receptor and the deduced amino acid sequence.**

However, they almost completely blocked ATP-induced currents (Fig. 3*D*). The presence of expressed cP2X$_8$ receptors in the oocytes was confirmed by the appearance of ATP responsiveness 15 min after the removal of the antagonists. PPADS at 1 μM was able to block partially the ATP-induced currents, whereas 10 μM PPADS produced significant blockade. Nevertheless, it is not possible to calculate the IC$_{50}$ values of antagonists due to the prolonged desensitization.

Tissue Distribution—Northern blot hybridization analysis revealed the expression of cP2X$_8$ RNA transcripts of about 4.3–4.6 kb in Day 10 chick embryos tissues (Fig. 4*A*). The skeletal muscle and brain showed the highest level of expression, followed by heart, retina, and gizzard. No band was detectable in the lane loaded with liver mRNA. RT-PCR assays revealed a similar pattern of expression (Fig. 4*B*). However, a very low level of cP2X$_8$ RNA was detected in the liver.

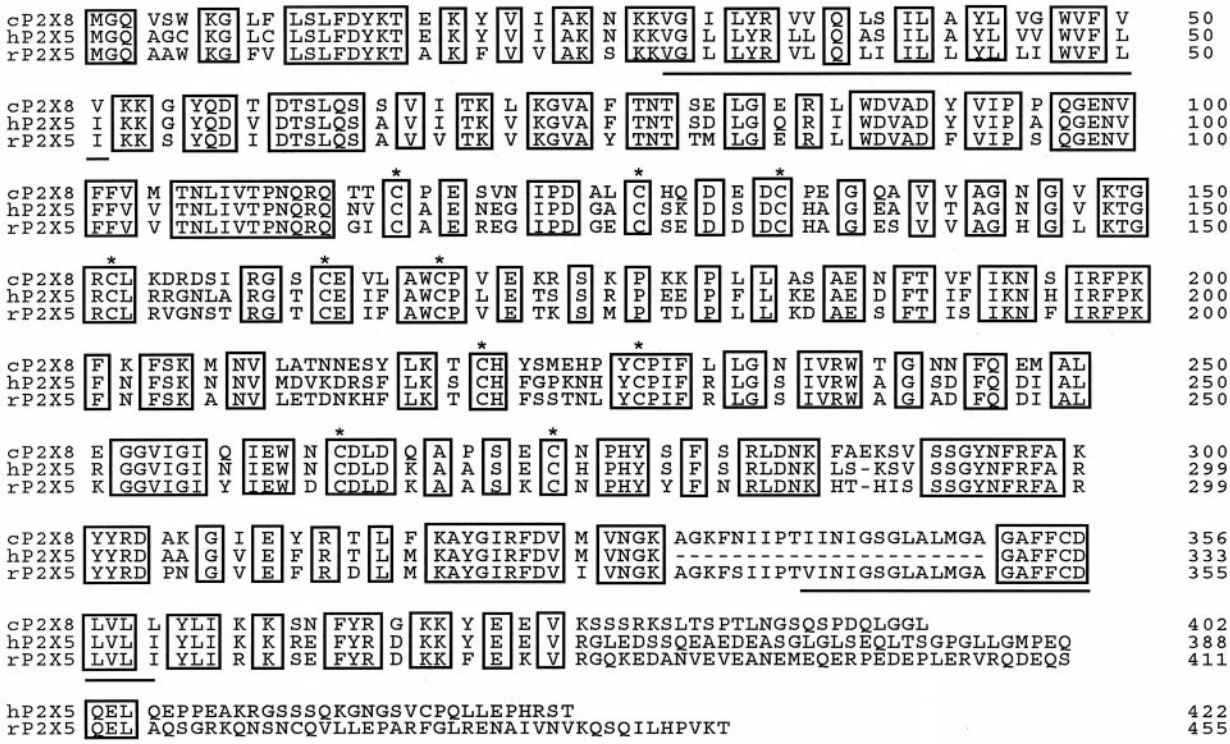

FIG. 2 **Alignment of the deduced amino acid sequence of cP2X$_8$ receptor with rP2X$_5$ and hP2X$_5$ receptors.** The two putative transmembrane domains are indicated by *bars underneath.* The 10 conserved cysteines on the extracellular loop are marked by *. The identity of cP2X$_8$ receptor amino acid sequence with both rat and human P2X$_5$ receptors is 59%.

Whole mount *in situ* hybridization on Day 3, Day 4, and Day 6 chick embryos showed discrete distribution of cP2X$_8$ RNA transcripts (Fig. 5). In Day 3 embryo, the hybridization signals were observed in the brain and the ventral part of the neural tube. They were also present in retina, notochord, stomach, duodenum, and heart (Fig. 5, *panel I*). In Day 4 embryos, the hybridization signals were still present in the structures shown in Day 3 embryos (Fig. 5, *panel II*). However, the distinctive difference was the appearance of myotome, which was labeled by the probe (Fig. 5, *panel II, C* and *D*). In Day 6 embryos, the hybridization signal in myotome was still strong (Fig. 5, *panel III*). Premuscle mass appeared in the wing bud, which showed high level expression of cP2X$_8$ receptor RNA. The hybridization signal was no longer obvious in the neural tube, and no signal was detectable in the notochord at this stage (Fig. 5, *panel III, D*). In parallel experiments using sense probes, no hybridization signal was detected in the embryos.

DISCUSSION

Although seven P2X receptor subtypes have been cloned, previous electrophysiological and pharmacological studies indicated the existence of another subtype in the embryonic chick skeletal muscle cells (10, 11). In this study, we have cloned a new P2X cDNA from embryonic chick skeletal muscle. We propose to name the novel P2X receptor cloned from chick embryos in this study as cP2X$_8$ receptor. Recombinant cP2X$_8$ receptor showed a pharmacological behavior that matches that observed in isolated chick myotubes. The deduced amino acid sequence of this receptor protein shares the common features of the P2X receptor family, like the presence of two putative transmembrane domains and the 10 conserved cysteine residues on the extracellular loop. The cP2X$_8$ receptor has a low identity of amino acid sequence with most other members of the P2X receptor family, which is another feature of P2X receptor family. Although hP2XM was reported to be highly expressed in skeletal muscle, it shares only 44% identity with the cP2X$_8$ receptor. In 1998, a mouse P2XM homologue was reported (24) which shares an identity of 93% with the rat P2X$_6$ receptor, and they are quite likely to be P2X$_6$ receptor homologues.

We have also cloned a chick P2X$_4$ cDNA at the same time from the same chick embryonic skeletal muscle cDNA library,[2] which is identical to the one recently cloned from embryonic chick heart (25). The chick P2X$_4$ and rat P2X$_4$ receptors share an identity of 75%, suggesting that the difference between avian and mammalian P2X receptor homologues should be close to this percentage. The cP2X$_8$ receptor shares an identity of 59% with rP2X$_5$. The polypeptide chain of cP2X$_8$ receptor protein is significantly shorter than rP2X$_5$ (402 *versus* 445 amino acids). The most striking difference in amino acid sequences between these two receptor subtypes lies in the C termini, where they share almost no homology. If the C termini were excluded, the percentage of identity would be increased to 70%, which does raise the question whether cP2X$_8$ is a homologue of rP2X$_5$. However, it should be noted that the C termini of P2X receptors are the most variable parts among the subtypes, which may play an important role in designating the differences among the subtypes. Taken together, the significant differences in their polypeptide length, in their C-terminal sequences, in the electrophysiological and pharmacological characteristics of the recombinant receptors, and in their tissue distribution pattern indicate that cP2X$_8$ is not a homologue of rP2X$_5$.

The cP2X$_8$ receptor shares 62% identity with hP2X$_5$ if the missing exon is excluded. In a sense, cP2X$_8$ is slightly closer to hP2X$_5$ than to rP2X$_5$. The C-terminal of cP2X$_8$ is 42 amino acids shorter than that of hP2X$_5$ and shares 21% identity (40% if the extra 42 amino acids are excluded), which may result in quite different electrophysiological and pharmacological prop-

[2] X. Bo, R. Schoepfer, and G. Burnstock, manuscript in preparation.

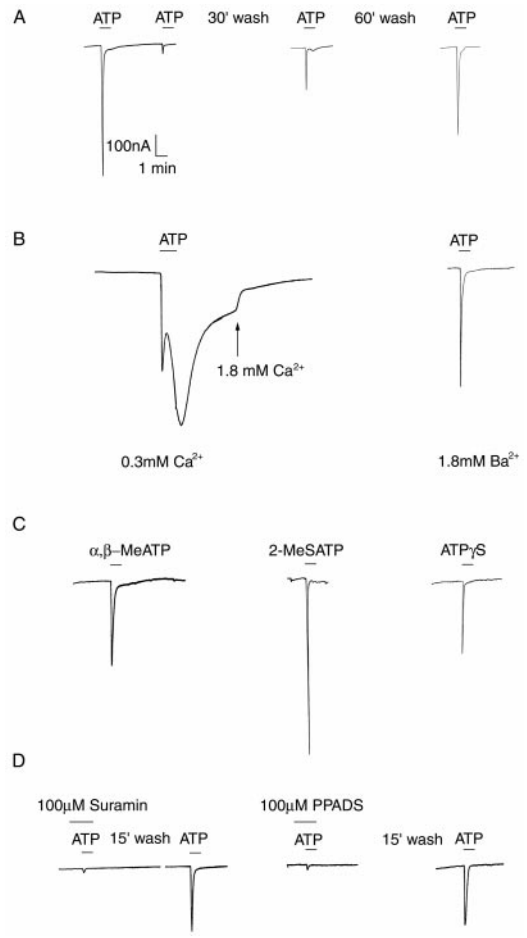

FIG. 3. **Functional expression of recombinant $cP2X_8$ receptors in *Xenopus* oocytes.** *A,* inward currents induced by 10 μM ATP in $cP2X_8$ cRNA-injected *Xenopus* oocytes in 1.8 mM Ca^{2+} medium showing the feature of currents and the slow recovery process; *B,* the bi-phasic currents induced by 10 μM ATP in 0.3 mM Ca^{2+} medium and ATP-induced current in 1.8 mM Ba^{2+} medium; *C,* α,β-methylene-ATP- (α,β-*MeATP,* 10 μM), 2-methylthio-ATP- (*2-MeSATP,* 10 μM), and ATPγS (10 μM)-induced currents in 1.8 mM Ca^{2+} medium; *D,* blockade of ATP (10 μM)-induced currents by suramin and PPADS.

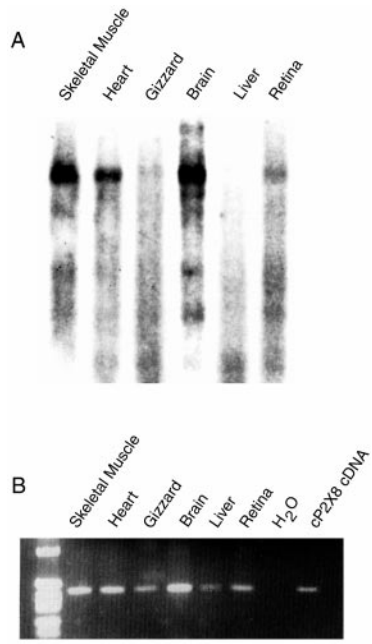

FIG. 4. **Expression of $cP2X_8$ receptor mRNA in different embryonic (Day 10) chick tissues detected by Northern blot hybridization (*A*) and RT-PCR (*B*).** For Northern blot analysis, 3 μg of poly(A)$^+$ RNA was loaded per lane in a 1.5% agarose gel, and the x-ray film was exposed for 3 days. Touch-down PCR was run for 30 cycles. $cP2X_8$ cDNA (0.3 pg) was used as positive control. Molecular marker, 1-kb ladder from Promega Ltd.

erties in these two receptor proteins. As a full-length functional $hP2X_5$ receptor has not been cloned, the comparison of their electrophysiological and pharmacological characteristics cannot be carried out.

It should be noted that $hP2X_5$ and $rP2X_5$ receptors also have significantly different C termini. For other P2X subtypes like $P2X_1$, $P2X_3$, $P2X_4$, and $P2X_7$ receptors, the rat and human homologues have the same number of amino acids in their polypeptide chains and share identities of 80–93%. Their C termini also share a high level of identity ranging from 75 to 88%. $hP2X_5$ also have a different pattern of tissue distribution from $rP2X_5$. It would be interesting to see whether a real $hP2X_5$ exists or not.

It is possible that $rP2X_5$, $hP2X_5$, and $cP2X_8$ receptors are all derived from the same ancient gene. During evolution, alternative splicing has created three receptors with significantly different C termini, and consequently, they have acquired different electrophysiological and pharmacological properties and have different tissue distribution and biological functions. Further cloning and genomic structure analysis of P2X receptors in different species will shed more light on this issue.

Recombinant $cP2X_8$ receptors expressed in *Xenopus* oocytes showed rapid desensitization after being activated by ATP. They were also sensitive to α,β-methylene-ATP and could be blocked by suramin and PPADS. These features are quite similar to those of $rP2X_1$ and $rP2X_3$ and can be included in one subgroup of the P2X receptor family. $rP2X_5$ and $rP2X_2$ subtypes share many common features like α,β-methylene-ATP insensitivity, non-desensitization, and blockade by suramin and PPADS. These two members may be included in another subgroup, whereas $P2X_4$ and $P2X_6$ subtypes fall into a third group (insensitivity to α,β-methylene-ATP, non-blockade by suramin and PPADS, and partial desensitization).

A unique feature of $cP2X_8$ receptor is its prolonged period of recovery from desensitization. A similar phenomenon was observed by Hume and Honig (10) when studying the ATP-induced currents in chick embryonic myotubes. In a later study, it was found that a full recovery of ATP responsiveness took 7 h at 37 °C (26). Preincubation of myotubes with a glycosylation inhibitor, tunicamycin, prevented the recovery from desensitization, indicating that the recovery process is due to the insertion of new P2X receptors into the membrane (26). It was assumed that after the opening of P2X receptor-coupled channels, they became inactivated and could not recover. The inactivated receptors might be internalized and re-processed to be inserted into membrane again. The exact mechanism for such a phenomenon is unclear at the moment. It is also unclear why $cP2X_8$ receptors behave in such a way.

Another feature of $cP2X_8$ receptor-coupled ion channel is that prolonged ATP application in low Ca^{2+} perfusion medium produced a rapid inward current followed by a slower current, which did not reach a peak until the application of ATP was stopped. Continuous application of ATP would finally cause the death of oocytes. A similar phenomenon has been reported on recombinant rat $P2X_4$ receptors expressed in *Xenopus* oocytes and HEK 293 cells in Ca^{2+}-free medium (27). It was suggested that the slower second currents were due to the change of ion selectivity during sustained exposure to ATP, and the occurrence and kinetics were regulated by Ca^{2+} concentration. If extracellular Ca^{2+} concentration dropped significantly in path-

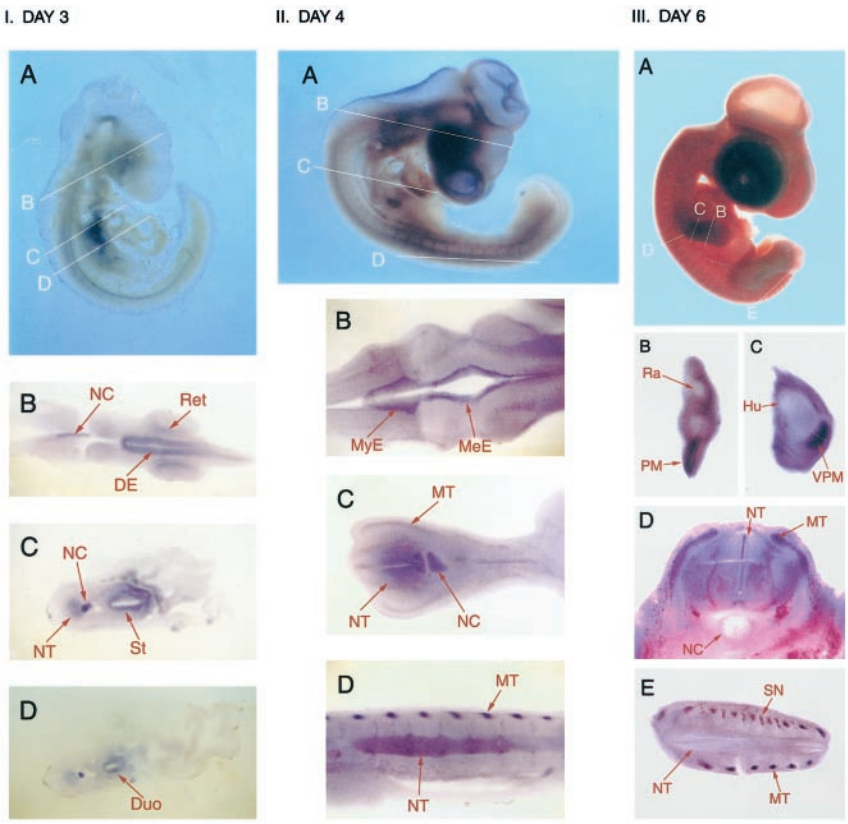

FIG. 5. **Whole mount *in situ* hybridization of chick embryos with digoxigenin-labeled cP2X$_8$ RNA probe.** *Panel I, Day 3*, *A,* lateral view of the whole embryo; *B,* section over upper part of the body showing the expression of cP2X$_8$ RNA in the brain (diencephalon, *DE*), retina (*Ret*), and notochord (*NC*); *C,* section over middle part of the embryo showing the expression of cP2X$_8$ RNA in neurotube (*NT*), notochord, and stomach (*St*); *D,* section over middle-lower part of the embryo showing the expression of cP2X$_8$ RNA in neurotube, notochord, and duodenum (*Duo*). *Panel II, Day 4*, *A,* lateral view of whole embryo; *B,* section over the brain showing the expression of cP2X$_8$ RNA in mesencephalon (*MeE*) and myelencephalon (MyE); *C,* section over the middle part of the embryo showing the expression of cP2X$_8$ RNA in myotome (*MT*), neurotube (*NT*), and notochord (*NC*); *D,* longitudinal section along lower spine showing the expression of cP2X$_8$ RNA in myotome and neurotube. *Panel III, Day 6, A,* lateral view of whole embryo; *B,* transverse section of wing bud across radius (*Ra*) and ulna showing the expression of cP2X$_8$ RNA in premuscle mass (*PM*); *C,* transverse section of wing bud across humerus (*Hu*) showing the expression of cP2X$_8$ receptor in ventral premuscle mass (*VPM*); *D,* transverse section over the spine showing the expression of cP2X$_8$ RNA in myotome; *E,* longitudinal section along lower spine showing the expression of cP2X$_8$ RNA in myotome. *SN*, spinal nerve.

ological conditions, sustained release of ATP from nerve terminals or damaged cells may lead to cell death. rP2X$_7$ receptors have also been reported to change ion selectivity upon prolonged exposure to 2′,3′-(*O*)-(4-benzoylbenzoyl)ATP in low divalent cation concentration, which leads to cytolysis following the formation of large pores on membrane (28). It may be worthwhile to explore further the relationship between cP2X$_8$ receptor activation and cell death under pathological conditions such as hypoxia.

The recombinant cP2X$_8$ receptors expressed in *Xenopus* oocytes and P2X receptors in cultured myotubes (29) showed similarity in pharmacological profiles. ATP, ATPγS, and 2-methylthio-ATP are potent agonists, and ADP is a weak agonist. Other nucleotides, di-adenosine tetraphosphate, β,γ-methylene-ATP, AMP, and adenosine are inactive. The only difference is that α,β-methylene-ATP is a potent agonist for the cP2X$_8$ receptor, whereas it failed to elicit inward currents in myotubes. The difference might be because the native P2X receptors are heteromultimers combining P2X$_8$ and other P2X receptor subunits. cP2X$_4$ was expressed in embryonic skeletal muscle and was insensitive to α,β-methylene-ATP. We tried to co-express cP2X$_4$ and cP2X$_8$ receptors in *Xenopus* oocytes; however, because a much higher amount of cRNA for cP2X$_4$ was required to produce detectable responses, the expression of cP2X$_8$ receptors was suppressed. Therefore, the recorded responses were typical of cP2X$_4$ receptors. Fine-tuning of the co-expression of these two receptor subtypes may resolve the problem.

Northern blot hybridization and RT-PCR revealed that cP2X$_8$ receptors were highly, but not exclusively, expressed in embryonic skeletal muscle. They were also expressed in the brain, heart, gizzard, and retina in Day 10 embryos. These tissues mainly consist of excitable cells, indicating the role of ATP as a fast transmitter in these tissues. The expression of cP2X$_8$ in liver was very low. Whole mount *in situ* hybridization revealed more details of tissue distribution of cP2X$_8$ receptors during development. In an earlier stage of development (Day 3 and Day 4), they are predominantly expressed in nerve tissues such as brain and neurotube. In Day 6 embryos the expression in nerve tissues is no longer so distinctive, although the expression in skeletal muscle becomes very obvious. A further detailed study on the expression of cP2X$_8$ receptor during the whole embryo developmental process is being carried out. The physiological significance of cP2X$_8$ receptors in the innervation of skeletal muscle during development is unknown and will be studied. Information about P2X receptors in chick tissues is very limited. The P2X$_8$ receptor is the only subtype that has been identified in chick tissues and characterized in pharmacological and electrophysiological experiments. More studies on P2X receptors in chick tissues are required considering the importance of chick embryos as a tool for the study of the development.

In conclusion, we have cloned and characterized a novel subtype of P2X receptor from embryonic chick skeletal muscle, which we have tentatively termed cP2X$_8$. The electrophysiological and pharmacological features of the recombinant cP2X$_8$ receptor are very close to the ATP-induced responses in cultured chick myotubes. cP2X$_8$ receptors are highly expressed in embryonic chick skeletal muscle, but they are also expressed in other tissues such as brain, neurotube, heart, gizzard, and retina. The expression of cP2X$_8$ receptors in developing skeletal muscle may play a significant role in the establishment of the innervation of skeletal muscle.

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