Expression of Two ATP-Gated Ion Channels, P2X₅ and P2X₆, in Developing Chick Skeletal Muscle

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ABSTRACT

Physiological and pharmacological studies have shown that ATP has potent effects on developing chick skeletal muscle. These effects have previously been shown to be developmentally regulated, and the responses were characteristic of activation of the P2X ligand-gated ion-channel family of ATP receptors. Here, using immunohistochemistry, we describe the expression patterns of two members of the P2X receptor family, P2X₅ and P2X₆, during development of skeletal muscle in the chick embryo. These receptors were first expressed at early stages of skeletal muscle development, and expression disappeared immediately before the stage at which fusion of myoblasts to form myotubes occurs. P2X₆ was also demonstrated in nerves supplying developing skeletal muscle, in some dorsal root ganglion cells, and in dorsal and ventral spinal cord. No expression of the other five members of the P2X family were demonstrated in developing skeletal muscle. Dev Dyn 1999;216:442–449.

Key words: ATP; chick embryo; P2X receptors; skeletal muscle development

INTRODUCTION

The role of P2 receptors and their ligands, extracellular nucleotides, is well established in many areas of physiology and pharmacology (Burnstock, 1997). There is also a growing body of evidence that suggests that this type of signalling may be important during embryonic development (Burnstock, 1996). P2 receptors are divided into two families, P2X and P2Y, based on molecular structure, transduction mechanisms, and pharmacological properties (Abbraccio and Burnstock, 1994; Burnstock and King, 1996). The P2X receptors are a family of G protein-coupled receptors. In most cell types, P2Y receptor activation by either ATP, ADP, or UTP leads to increases in [Ca²⁺], a process mediated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the Ca²⁺ mobilising second messenger inositol 1,4,5-trisphosphate, and diacylglycerol (Dubyak and El-Moatassim, 1993). One member of this family, the Xenopus x1-P2Y₉ receptor, is expressed at early stages of neural plate development (Bogdanov et al., 1997). The expression of these P2Y receptors during developmental stages of these divergent species strongly suggests a role for this type of signalling during embryogenesis.

P2X receptors are members of the ligand-gated ion channel superfamily, and activation of these receptors by extracellular ATP elicits a flow of cations (Na⁺, K⁺, and Ca²⁺) across the plasma membrane. To date, seven P2X receptor subtypes (P2X₁₋₇) have been cloned from mammalian species (Burnstock and King, 1996). Although embryonic expression of specific P2X receptor subtypes has not been demonstrated, a number of physiological and pharmacological studies have shown P2X-like responses in embryonic tissues. Responses to ATP have been described in diliary neurons acutely dissociated from day 14 embryonic diliary ganglia (Abe et al., 1995). A transmitter-like action of ATP on patched membranes of myoblasts and myotubes cultured from 12-day chick embryos was first demonstrated by Kolb and Wakelam (1983), and ATP-induced cation influx was later demonstrated in myotubes prepared from 11-day chick embryos (Hägglblad et al., 1985). ATP has a potent depolarising action on myotubes derived from pectoral muscle cultured from day 11 chick embryos (Hume and Höning, 1986), and its physiological and pharmacological properties have been described in a series of articles (Hume and Thomas, 1988; Thomas and Hume, 1990a,b, 1993; Thomas et al., 1991). The sensitivity to extracellular ATP has been tested at various stages of development of different muscles (Wells et al., 1995). At embryonic day 6 (stage 30; Hamburger and Hamilton, 1951), ATP elicits contraction in all muscles tested, but by day 17 (stage 43), none of the muscles contract in response to ATP. These data strongly suggest that in developing chick skeletal muscle, P2X receptors have a role to play during embryonic development. To investigate this further, we looked for P2X receptor immunoreactivity in the developing chick embryo using polyclonal antibodies raised against rat P2X receptor peptides. Immunoreactivity for P2X₅ and P2X₆...
was found in early developing skeletal muscle, and strong labelling persisted until the fusion of myoblasts to form myotubes. P2X₅ immunoreactivity was also observed in dorsal root ganglion cells and in dorsal and ventral spinal cord.

### RESULTS

#### Specificity of the P2X₅ and P2X₆ Antibodies in Chick Tissues

Polyclonal rabbit antisera to the rat P2X₅ and rat P2X₆ receptors were generated by immunising rabbits with a 15 amino acid receptor-specific peptide corresponding to the C-terminal region of each receptor. To establish as far as possible antibody specificity in chick embryo tissues, immunoblotting was performed with stage 30–32 chick embryo crude extracts. A single P2X₅ reactive band of approximately 70 kDa and two P2X₆ reactive bands in the 60–70 kDa range were detected (Fig. 1). These bands were in the expected weight range, as determined by immunoblotting with crude rat tissue extract (Groeschel-Stewart et al., 1999), and membrane fractions of cell lines expressing recombinant P2X₅ or P2X₆ receptors (Oglesby et al., 1999). Although these results suggest that these antibodies are specific for a single protein, they cannot rule out the possibility that these antibodies are detecting chick proteins unrelated to P2X₅ and P2X₆ but that are of molecular weights similar to that of these two receptors. Specificity of the P2X₅ and P2X₆ antibodies on chick embryo tissue sections was therefore checked by preabsorption of the antibodies with the peptides used to generate them. This abolished nearly all immunoreactivity to both antibodies on chick embryo sections (Fig. 2). These data combined with the consistent and tissue-specific staining described below suggest that these antibodies are labelling chick P2X receptors.

#### P2X₅ and P2X₆ Immunoreactivity in Developing Chick Skeletal Muscle

Staining with P2X₅ and P2X₆ antibodies was first seen in the somites of a stage 14 embryo (Fig. 3B and C). Expression at this stage could only be visualised using the tyramide amplification step. Immunohistochemistry with an antibody to chicken myosin (a-PM) on sections from the same embryo showed that staining was confined to the dermamyotome of each somite (Fig. 3A). This skeletal muscle marker was used throughout this study to identify areas of developing skeletal muscle. P2X₅ and P2X₆ immunoreactivity in all developing chick skeletal muscle strengthened from this stage onward, with particularly strong expression seen in the intervertebral muscles (Fig. 3D and E) and in muscle surrounding the developing skeletal elements in the limbs (Fig. 4A and B). Expression of both of these receptors began to disappear at around stage 39 (Fig. 4D and E). At different stages of development, there were temporal and spatial differences in patterns of P2X₅ and P2X₆ expression between different muscle groups. The expression of these receptors was developmentally regulated, and expression and disappearance of these receptors occurred earlier in some muscle groups than in others. This is seen in Figure 4D and E. The rhomboideus superficialis (rs) muscle still expressed P2X₅ and P2X₆ while the underlying latissimus dorsi (ld) muscle had begun to lose expression of both these receptors. Because expression of these receptors seemed to be developmentally regulated, we also wanted to correlate the temporal pattern of expression with a crucial event during skeletal muscle development, the fusion of myoblasts to form multinucleate myotubes. Simultaneous nuclear 4,6-diamidino-2-phenylindole (DAPI) and myoblast (phalloidin) staining of the latissimus dorsi muscle showed that fusion of myoblasts starts at stage 40 (Fig. 5A). At this stage, myoblasts had begun to fuse and their nuclei move to the periphery of the newly formed myotubes, although there were still some unfused myoblasts present (as shown by single centrally located nuclei). By stage 43 (Fig. 5B), all nuclei were located at the periphery, indicating that fusion of myoblasts to form myotubes is complete at this stage. Therefore, the disappearance of P2X₅ and P2X₆ immunoreactivity from skeletal muscle that began at stage 39–40 immediately preceded the process of myotube formation.

#### P2X₅ Immunoreactivity in the Developing Nervous System

P2X₅ was first detected in the developing nervous system at stage 22 in the ventral roots (Fig. 3D). Figure 6A also shows strong expression of P2X₅ in sensory axons in the dorsal roots and in motor axons in ventral roots at stage 28. Expression of P2X₅ was also detected in the dorsal funiculus, which contains the central
axons of dorsal root ganglia neurons, and also in ventral spinal cord. At stage 30, P2X5 immunoreactivity was also detected in a subset of small cells in the dorsal root ganglion (Fig. 6B). P2X5 was also observed in what appeared to be nerve fibres innervating skeletal muscle (Fig. 6C). P2X6 was not detected in the developing nervous system at any stage.

DISCUSSION

The observation that ATP has a potent depolarising action on embryonic chick muscle (Hume and Höning, 1986), and that the sensitivity to extracellular ATP seems to be developmentally regulated (Wells et al., 1995) suggests that ATP may be an important signalling molecule during development of chick skeletal muscle. The finding that a single class of ATP-activated ion channel conducts both cations and anions in the myotube (Thomas and Hume, 1990a) and that the P2 receptors involved show marked desensitisation (Thomas and Hume, 1990b), suggests that the receptor(s) involved are members of the P2X ligand gated ion channel family. In this study, we show using immunohistochemistry that two members of the P2X family are indeed expressed in all developing skeletal muscles of the chick embryo. Specificity of P2X5 and P2X6 antibodies on chick tissues was tested by Western blotting with chick embryo crude protein extracts. A single P2X5 reactive band of approximately 70 kDa was detected, and two P2X6 reactive bands in the 60–70-kDa range were detected. These were in the expected weight range (Grosschel-Stewart et al., 1999; Oglesby et al., 1999), but the two bands detected by P2X6 raises the possibility that this antibody is recognising other P2X receptor subtypes, or proteins unrelated to P2X6. We believe that this is unlikely for several reasons. First, the antibodies were raised against receptor subtype specific peptides, making it unlikely that the P2X6 antibody is binding to other P2X receptor subtypes. Second, P2X6 consistently labelled only skeletal muscle in this study, so if the P2X6 antibody is labelling more than one protein, these proteins would have to be colocalised in this tissue. Finally, preabsorption of both P2X5 and P2X6 antibodies with their respective peptides abolished nearly all immunoreactivity to these antibodies in chick tissues. The two bands detected by the P2X6 antibody on the Western blot could be caused by alternative splicing of P2X6 mRNA generating proteins of different molecular weights. An increasing number of splice variants are being detected for other P2X receptor subtypes (Simon et al., 1997; Brändle et al., 1997; Lé...
Posttranslational modification of the P2X6 protein, by glycosylation, for example, would also result in the detection of proteins with different molecular weights.

Immunoreactivity to P2X5 and P2X6 was first detected in the dermamyotome of stage 14 chick embryos, and expression of P2X5 and P2X6 within skeletal muscle strengthened from this stage onward. Within developing skeletal muscle, there was considerable temporal and spatial overlap in expression of these receptors, raising the possibility that three functionally distinct P2X receptors are present in developing skeletal muscle. Homomeric P2X ion channels made up of either P2X5 or P2X6 subunits alone could exist, or, in areas where these receptors are coexpressed, heteropolymerization of P2X5 and P2X6 subunits could occur to form a third type of ion channel. Heteropolymerization has been shown to occur; coexpression of P2X2 and P2X3 is required to produce the responses to ATP of adult sensory neurons (Lewis et al., 1995), and in rat dorsal root and nodose ganglia, P2X2 and P2X3 immunoreactivity were found to be highly colocalised (Vulchanova et al., 1997). It has also been shown that P2X5 can coassemble with P2X7 to form a novel channel (Torres et al., 1998). Expression of both P2X5 and P2X6 began to disappear from skeletal muscle between stages 39 and 40, the stage at which fusion of myoblasts occurred to form multinucleate myotubes, which suggests a possible role for these receptors in maintaining muscle cells in a myoblastic state.

The expression pattern of the receptors described here bear striking similarities to the developmental pattern of sensitivity of skeletal muscle to ATP described by Wells et al. (1995). That study showed that all skeletal muscle from embryonic day 6 (stage 30) onward contracted in response to extracellular ATP and that by stage 40, the proportion of muscles responding dropped quickly to zero. However, in culture, mononucleate myotubes and recently fused myotubes (which are not contractile) are responsive to ATP (Hume and Thomas, 1988), so as the expression pattern of P2X5 and P2X6 suggests, ATP responsiveness is likely to be present in developing muscles much earlier than stage 30. The study by Wells et al. (1995) also showed reappearance of ATP responsiveness after surgical denervation of the latissimus dorsi muscles of chicks 1–2 days after hatching, suggesting that expression of the ATP receptor(s) involved may be regulated by motor neurons. This would also explain why in vitro, recently fused myoblasts that are not innervated still respond to ATP (Hume and Thomas, 1988). It would be interesting to see whether expression of P2X5 and P2X6 returns after denervation of skeletal muscle in posthatch chicks. Although their patterns of expression make it plausible to assume that P2X5 and P2X6 receptors mediate the responses of developing chick skeletal

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**Fig. 3.** Immunohistochemistry on early chick embryo sections shows P2X5 and P2X6 immunoreactivity in developing vertebral muscle. Embryonic stage is indicated in the bottom left corner of each photograph, and the antibody used is indicated in the top right corner. A: Myosin (a-PM) staining of the myotomes in a longitudinal section of a stage 14 embryo. B: P2X5 immunoreactivity in the dermamyotome of a section adjacent to the one stained for pectoral myosin. C: Staining for P2X5 is also seen at stage 14. D: P2X6 staining is seen in the vertebral muscles (vm) and in the ventral nerve roots (vr). E: P2X6 immunoreactivity in vertebral muscles. Scale bar = 100 µm.
muscle to ATP, the absence of P2X subtype-specific agonists and antagonists make it difficult to say unequivocally whether this is the case.

Possible sources of extracellular ATP required to activate P2X5 and P2X6 are motor neurons and the muscles innervated by them. ATP is known to be costored in synaptic vesicles within presynaptic nerve terminals and to be coreleased with acetylcholine (Silinsky and Hubbard, 1973; Zimmerman, 1978). Muscle cells are also known to secrete substantial amounts of ATP in response to electrical activity (Lindgren and Smith, 1986), and in the chick, synaptic responses can be elicited as early as embryonic day 6 (Landmesser and Morris, 1975). The possibility that extracellular ATP coreleased with ACh may serve as a trophic factor for the developing neuromuscular synapses in Xenopus...
embryos has also been raised (Fu and Poo, 1991; Fu, 1995). Therefore, in addition to acting as a pre- and postjunctional modulator of ACh release and action in mature neuromuscular synapses (for review see Henning, 1997), P2X5 and P2X6 may mediate the transmitter-like action of ATP in developing chick skeletal muscle (Kolb and Wakelam, 1983) and may also play a role in the development of chick neuromuscular synapses.

P2X5 was also found to be expressed on sensory and motor axons in dorsal and ventral roots, respectively. A subset of small cells, possibly glia or neural progenitors in the dorsal root ganglion, also expressed P2X5. P2X5 immunoreactivity was strong in dorsal and ventral spinal cord. This pattern of P2X5 immunoreactivity in the nervous system of the chick embryo correlates well with the expression pattern of P2X5 receptor mRNA in adult rat, where expression was found in motoneurons of the ventral horn and in the dorsal root ganglion (Collo et al., 1996). However, this is the first study that demonstrates expression of P2X5 and P2X6 in skeletal muscle, despite a number of in situ hybridisation and Northern blotting studies that have examined receptor expression in adult rats (Collo et al., 1996; Soto et al., 1996; Garcia-Guzman et al., 1996), thus raising the possibility that P2X5 and P2X6 have specialised roles to play in development of skeletal muscle.

In this article, we show for the first time embryonic expression of specific P2X receptor subtypes, P2X5 and P2X6. These receptors are expressed in a developmentally regulated manner in chick skeletal muscle. Their pattern of expression makes them strong candidates for mediating the responses of myoblasts, myotubes, and developing chick skeletal muscle to extracellular ATP.

Fig. 6. P2X5 expression in the nervous system. Developmental stage is indicated in the bottom left corner. A: Strong staining for P2X5 was seen in the dorsal funiculus (df), the dorsal root (dr), and ventral root (vr). P2X5 expression was also strong in the ventral spinal cord (sc). B: A subset of cells in the dorsal root ganglion (drg) strongly express P2X5. Note also the strong staining in the latissimus dorsi muscle at this stage. C: P2X5 expression in vertebral muscles (vm) and in nerves innervating them (indicated by large arrowhead). vr, ventral root. Scale bar = 100 µm.
(Hume and Thomas, 1988; Wells et al., 1995), but the precise roles for these receptors in skeletal muscle development, and the functional and/or developmental role for P2X$_5$ in the nervous system remain to be investigated.

**MATERIALS AND METHODS**

**Embryos**

Eggs were incubated at 37°C and staged according to the Hamburger and Hamilton series (Hamburger and Hamilton, 1951). Embryos were dehydrated in 20% sucrose in phosphate buffered saline (PBS) before freezing. Early-stage embryos (stages 14–20) were placed on a block of gelatine to facilitate sectioning and frozen in isopentane cooled in liquid nitrogen. Later-stage embryos were fixed onto a cork block with OCT compound. These were also frozen in liquid nitrogen–cooled isopentane. Cryostat sections were cut at 12 µm and collected on gelatinised slides.

**Antibodies and Western Blotting**

Polyclonal rabbit antisera to all rat P2X receptors (subtypes 1–7) were obtained from Roche Bioscience (Palo Alto, CA). In a preliminary investigation, P2X$_5$ (subtypes 1–7) were obtained from Roche Bioscience Antibodies and Western Blotting on gelatinised slides. These were also frozen in liquid nitrogen–cooled isopentane. Cryostat sections were cut at 12 µm and collected on gelatinised slides.

**Immunohistochemistry**

The avidin-biotin technique was used according to the protocol described by Llewellyn-Smith et al. (1992). The slide-mounted sections were fixed in 4% paraformaldehyde and 0.2% of a saturated picric acid solution in PBS for 2 min. To inactivate endogenous peroxidases, the sections were then treated with 50% methanol containing 0.4% hydrogen peroxide for 10 min. Non-specific binding was blocked by incubating for 20 min in 3% normal horse serum in PBS containing 0.05% methio- late. Sections were incubated overnight at room temperature in 5 µg/ml of either P2X$_5$ or P2X$_6$ antibody diluted in 3% normal horse serum in PBS. The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch) used at 1:500 for 30 min followed by the extravidin peroxidase conjugate (Sigma) at 1:1,000 for 30 min. At some stages of development, the tyramide amplification step was used according to the recommendation of the supplier (TSA indirect kit, NEN Life Science Products). In these cases, the concentration of the primary antibodies was reduced to 1 µg/ml. Stained sections were photographed using the Edge R-400 high-definition light microscope (Edge Scientific Instruments Co.) and Kodak TMX 100 colour slide film.

To further ensure specificity of the P2X antibodies in chick tissues, a twofold absorption of the antibodies was performed with their respective peptides before performing immunohistochemistry. Briefly: 1.5 µl of anti-P2X$_5$ or P2X$_6$ at 1 mg/ml were incubated overnight at 4°C with 24 µl of the respective peptide at 5 µg/ml. A further 24 µl of peptide at 5 µg/ml was added the following morning, and the incubation was extended for a further 6 hr. Two hundred microliters of 3% normal horse serum was then added to give 300 µl of a 5 µg/ml concentration of the P2X$_5$ or P2X$_6$ antibodies. This solution was centrifuged for 15 min at 13,000 rpm at 4°C. The supernatant was used for immunohistochemistry on chick embryo sections.

**REFERENCES**


