DEVELOPMENTAL DYNAMICS 221:331–341 (2001)

Sequential Expression of Three Receptor Subtypes for Extracellular ATP in Developing Rat Skeletal Muscle

MINA RYTEN, ASTRID HOEBERTZ, AND GEOFFREY BURNSTOCK*
Autonomic Neuroscience Institute, Royal Free & University College Medical School, Royal Free Campus, London, United Kingdom

ABSTRACT **In this study, we investigated the expression of the P2X receptor subtypes ($P2X_{1-7}$) during the development of skeletal muscle and in relation to acetylcholine receptors in the rat embryo and pup. By using immunohistochemistry, we showed that three receptor subtypes, $P2X_2$, $P2X_5$, and $P2X_6$, were expressed in developing skeletal muscle. The timing and pattern of receptor expression seemed to be precisely regulated. $P2X_2$, $P2X_5$, and $P2X_6$ were expressed in a sequential manner, which was consistent for all regional muscles tested (intercostal, paravertebral, and lower limb): $P2X_5$ expression appeared first (E15–E18) followed by $P2X_6$ (E16–E18), and finally $P2X_2$ (E18–adult). At no developmental stage did we observe colocalization of $P2X_2$ and acetylcholine receptors. In the case of $P2X_2$ and $P2X_6$, immunoreactivity was found to be widespread, immunopositive cells being apparent throughout the muscle. However, staining for $P2X_5$, both at the beginning and end of expression, was restricted to regions of muscle close to the myotendinous junctions. Because the timing of receptor expression is closely related to key events in skeletal muscle development, notably the generation of secondary myotubes and the redistribution of acetylcholine receptors, it is possible that ATP-signaling by means of P2X receptors could be involved in these processes.** **© 2001 Wiley-Liss, Inc.**

Key words: P2X receptors; ATP; skeletal muscle development; rat embryo; myotube formation; acetylcholine receptors

INTRODUCTION

In recent years, the biological effects of extracellular purine nucleotides acting through P2 receptors have been studied in many cell and tissue types and ATP is now recognized as an important messenger molecule in cell–cell communication (Burnstock, 1997). Thus far, attention has been largely focused on the role of purinergic signaling in mediating changes in short-term cellular activity. However, there is growing evidence that purinergic signaling may also play a role in long-term cellular communication, including cell proliferation, differentiation, and apoptosis (Neary et al., 1996; Abbracchio and Burnstock, 1998). These processes are central to embryonic development, and the specific functions of purine receptor subtypes during skeletal muscle development are the focus of interest in this study.

P2 receptors are classified into two main families, P2X and P2Y, based on molecular structure, transduction mechanisms, and pharmacological properties (Ralevic and Burnstock, 1998). The P2Y receptors are G protein–coupled receptors, which act principally by activating phospholipase C, leading to the formation of inositol 1,4,5-trisphophate and mobilization of intracellular Ca^{2+}. In contrast, P2X receptors are a ligand-gated ion channel family, and activation of these receptors by extracellular ATP elicits a flow of cations (Na^+, K^+, and Ca^{2+}) across the plasma membrane. To date, seven P2X receptor subunits ($P2X_{1-7}$) capable of assembling homo- or heteromultimeric receptors (Torres et al., 1999) have been cloned from mammalian species.

The functional importance of these receptor subtypes is yet to be fully understood, particularly with regard to their potential trophic actions. The $P2X_5$ receptor subunit has been shown to be expressed in the proliferating and differentiating cell layers of stratified squamous epithelial tissues (Gröschel-Stewart et al., 1999) suggesting that ATP signaling by means of the $P2X_5$ receptor may play a role in these processes. Conversely, the $P2X_7$ receptor subunit has been strongly linked to apoptosis (Surprenant et al., 1996; Collo et al., 1997; Gröschel-Stewart et al., 1999). There is increasing evidence to suggest that $P2X_7$ activation can induce apoptosis in several cell types and that this process is dependent on the caspase signaling cascade (Coutinho Silva et al., 1999; Ferrari et al., 1999).

Responses to ATP have been demonstrated in embryonic tissues, notably skeletal muscle. By using the patch clamp recording technique, a transmitter-like action of ATP on the cell membranes of myoblasts and myotubes cultured from 12-day chick embryos was first demonstrated by Kolb and Wakelam (1983). These results were confirmed by Hume and Thomas (Hume and

M. Ryten and A. Hoebertz contributed equally to the work presented in this study.

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

Received 7 November 2000; Accepted 9 March 2001

© 2001 WILEY-LISS, INC.

Thomas, 1988; Thomas and Hume, 1990a,b, 1993; Thomas et al., 1991), who showed that ATP elicits a potent, P2-dependent depolarizing action on cultured chick myotubes. Similar responses, characteristic of P2 receptors, have been demonstrated in the mouse myoblast cell line, C2C12 (Henning et al., 1993; Henning, 1997). In chick muscle, ATP-responses were also shown to be developmentally regulated (Wells et al., 1995). ATP-elicited contractions were most apparent in early development (embryonic day 6 [E6]) and were absent by embryonic day 17. More recently, immunohistochemistry has been used to describe the developmentally regulated expression pattern of two members of the P2X family, $P2X_5$ and $P2X_6$, in the skeletal muscle of the chick embryo (Meyer et al., 1999).

However, the role of ATP and the identity of the P2X receptor subtypes involved in the development of mammalian skeletal muscle are still largely undefined. In this study, we use immunohistochemistry to investigate the expression of P2X receptors and their relationship to acetylcholine receptors (AChRs) in developing rat skeletal muscle. By using polyclonal antibodies raised against the seven different rat P2X receptor peptides (Oglesby et al., 1999), we demonstrate the timing and localization of receptor expression. In summary, we show the sequential and developmental expression of three receptor subtypes, $P2X_2$, $P2X_5$, and $P2X_6$, perhaps indicating that ATP acting by means of P2X receptors may play a key role in skeletal muscle formation.

RESULTS

$P2X_2$, $P2X_5$, and $P2X_6$ Immunoreactivity in Developing Rat Skeletal Muscle

Staining procedures were carried out for all seven of the P2X receptors at all embryonic stages; however, we found immunoreactivity in developing skeletal muscle only for $P2X_2$, $P2X_5$, and $P2X_6$. Control experiments, performed by preabsorbing the antibodies with the corresponding peptides, showed no immunostaining, confirming the specificity of our findings. The expression of these receptors was followed from E12 to postnatal day (P) 21 in various muscles, including the intercostal, paravertebral, and limb muscles. Results from the muscle groups examined showed that the timing of receptor expression was subtype-specific.

Before E15, there was no immunoreactivity for any of the P2X receptors in the developing muscle. Immunoreactivity for $P2X_5$, but not $P2X_2$ or $P2X_6$, began at E15. Staining with $P2X_5$ was restricted to a small number of cells at the ends of the muscles, close to the myotendinous junctions (Fig. 1A). Immunohistochemistry with an antibody against skeletal myosin on sequential sections from the same embryo confirmed that the staining was confined to the areas of developing muscle (Fig. 1B) described above. This skeletal marker was used throughout this study to identify areas of future muscle. Immunoreactivity for $P2X_5$ strengthened during development; by E16 staining was more widespread within muscles already shown to have positive immunoreactivity for $P2X_5$ and began to appear in muscles that had previously been negative (Fig. 1C,D). This stage also marked the appearance of $P2X_6$ immunoreactivity within skeletal muscle. $P2X_6$ staining was strong and extensive, immunopositive cells could be seen throughout muscle blocks and in all muscle groups tested (Fig. 2A). Immunoreactivity for $P2X_5$ and $P2X_6$ was still apparent at E18 (Figs. 2B,C,D, 3), although in the case of $P2X_5$, it had become restricted to the ends of developing muscle fibers (Fig. 6A). By E20, immunoreactivity for both $P2X_5$ and $P2X_6$ within the skeletal muscle had disappeared. However, it was at these final stages of prenatal development, E18 onward, that $P2X_2$ expression began to appear. Initially $P2X_2$ was expressed most strongly in the intercostal (Fig. 4C,D) and paravertebral muscles (Fig. 4B) and was almost entirely absent in the limb musculature (Fig. 4A). $P2X_2$ expression was most marked in the muscles of the lower limb at E20 (Fig. 5A). Immunoreactivity for $P2X_2$ continued for at least 1 week postnatally (Fig. 5B) but had reduced in strength to near adult expression by P14 (Fig. 5C,D).

In summary, we found three P2X receptors to be expressed in developing skeletal muscle, $P2X_2$, $P2X_5$, and $P2X_6$. These receptors were expressed sequentially, $P2X_5$ being the first receptor to be expressed (E15) followed by $P2X_6$ (E16) and finally $P2X_2$ (E18). This sequence of receptor expression was maintained for all muscles tested. Expression of $P2X_5$ and $P2X_6$ was restricted to in utero development, whereas staining for $P2X_2$ continued after birth. Immunoreactivity for the $P2X_5$ and $P2X_6$ receptor subtypes was most transient, E15-E18 and E16-E18, respectively, whereas $P2X_2$ expression was more sustained, being reduced to near adult expression in the second postnatal week (E18-P14).

Double-Labeling for $P2X_2$ and Acetylcholine Receptors

Double-labeling experiments for $P2X_2$ and AChRs, localized with α-bungarotoxin-Texas Red, were carried out at E18, E20, P7, P14, P21, and adult. As described above, $P2X_2$ was expressed from E18 to P14 (Fig. 5). AChR staining was restricted to large membrane clusters at the center of the muscle fibers, i.e., prospective endplate sites (Fig. 6B–D). Consequently, only a subset of clusters is seen in any one section. At no stage did we observe colocalization of $P2X_2$ and AchR clusters (Fig. 6B–D). This finding included adult muscle where endplates (positive for α-bungarotoxin) as well as extrasynaptic regions were $P2X_2$ negative.

$P2X_5$ Immunoreactivity in Cells Located Between Muscle Fibers

Although $P2X_5$ expression in developing skeletal muscle was restricted to the prenatal period, $P2X_5$-immunopositive cells were apparent within the muscle mass at P7. These cells appeared to be in between

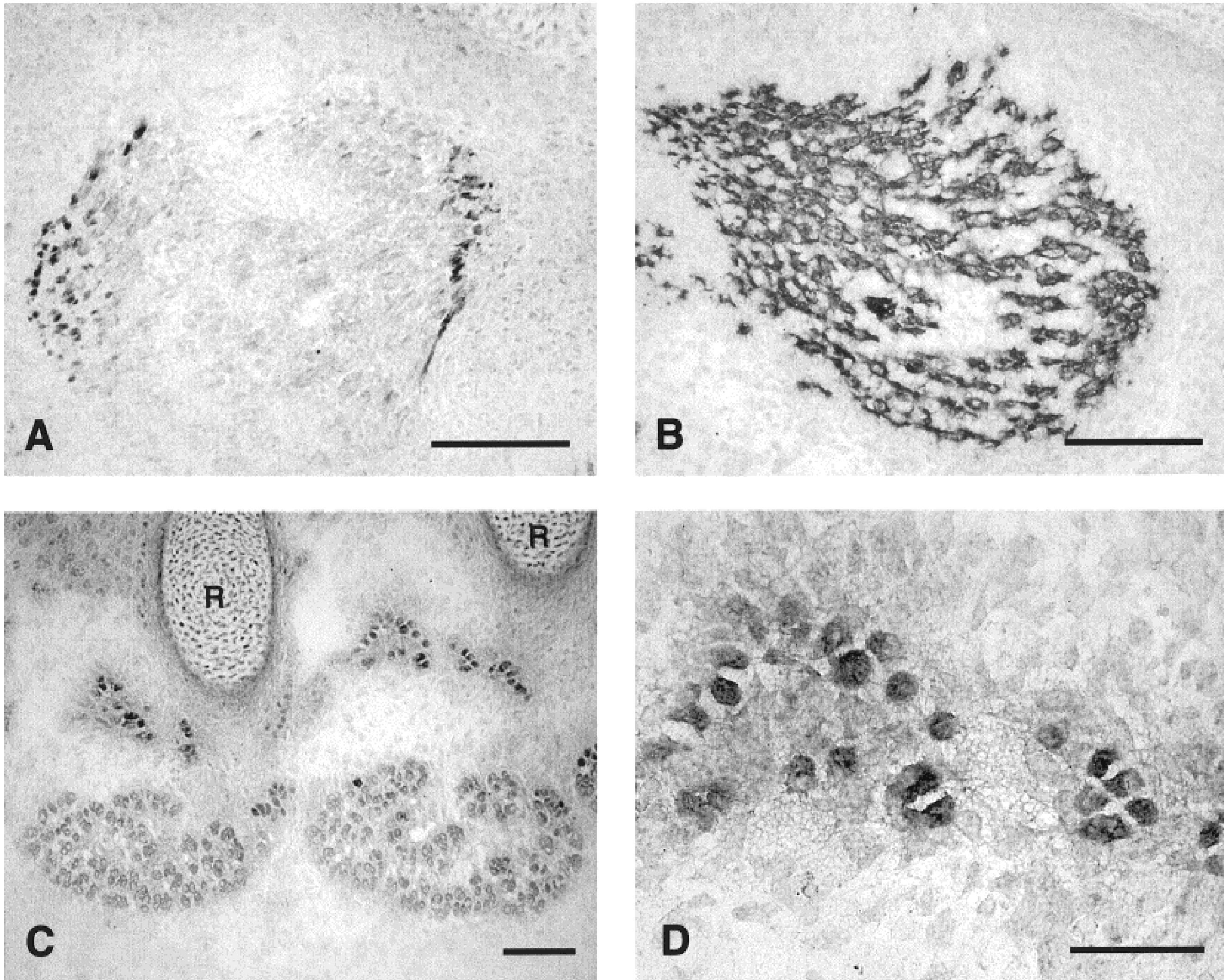


Fig. 1. Expression of $P2X_5$ in rat skeletal muscle in longitudinal sections of embryonic day (E) 15 and E16 embryos. **A:** Immunoreactivity for $P2X_5$ in a muscle block of the developing lower limb at E15 was restricted to a small number of cells at the ends of the muscles close to the myotendinous junctions. **B:** Sequential section showing skeletal muscle myosin staining. **C,D:** $P2X_5$ immunoreactivity, previously absent, began to appear in paravertebral muscles at E16, shown at higher magnification in (D). R, rib cartilage. Scale bars = 100 μm in A–D.

developing muscle fibers. This was confirmed by using double labeling with anti-skeletal muscle myosin (anti-PM) and anti-$P2X_5$, which showed a complete absence of overlapping expression (Fig. 7A). By P21, $P2X_5$ staining both within and outside muscle fibers had largely disappeared. To identify the $P2X_5$-positive cells present at P7, further double-labeling experiments were performed by using anti-neurofilament 200 or anti-smooth muscle actin. In both cases, there was limited coexpression with many cells remaining positive only for $P2X_5$ (Fig. 7B-D). This finding would suggest that $P2X_5$ stained a mixed population of cells at P7 including nerves, smooth muscle cells, and probably endothelial cells.

DISCUSSION

ATP, coreleased with acetylcholine, has been shown to modulate the developing neuromuscular synapses of *Xenopus* embryos (Fu and Poo, 1991; Fu, 1995) and to have direct transmitter-like actions on developing chick skeletal muscle (Kolb and Wakelam, 1983). In chick, ATP has potent P2-dependent, depolarizing actions on myoblasts in vitro (Hume and Honig, 1986) and causes muscular contraction in vivo (Wells et al., 1995). Furthermore, Wells et al. (1995) demonstrate that sensitivity to ATP is developmentally regulated and returns in denervated skeletal muscle. Experiments on the mouse myoblast cell line, C2C12, showing P2-dependent responses, suggest that ATP may also be

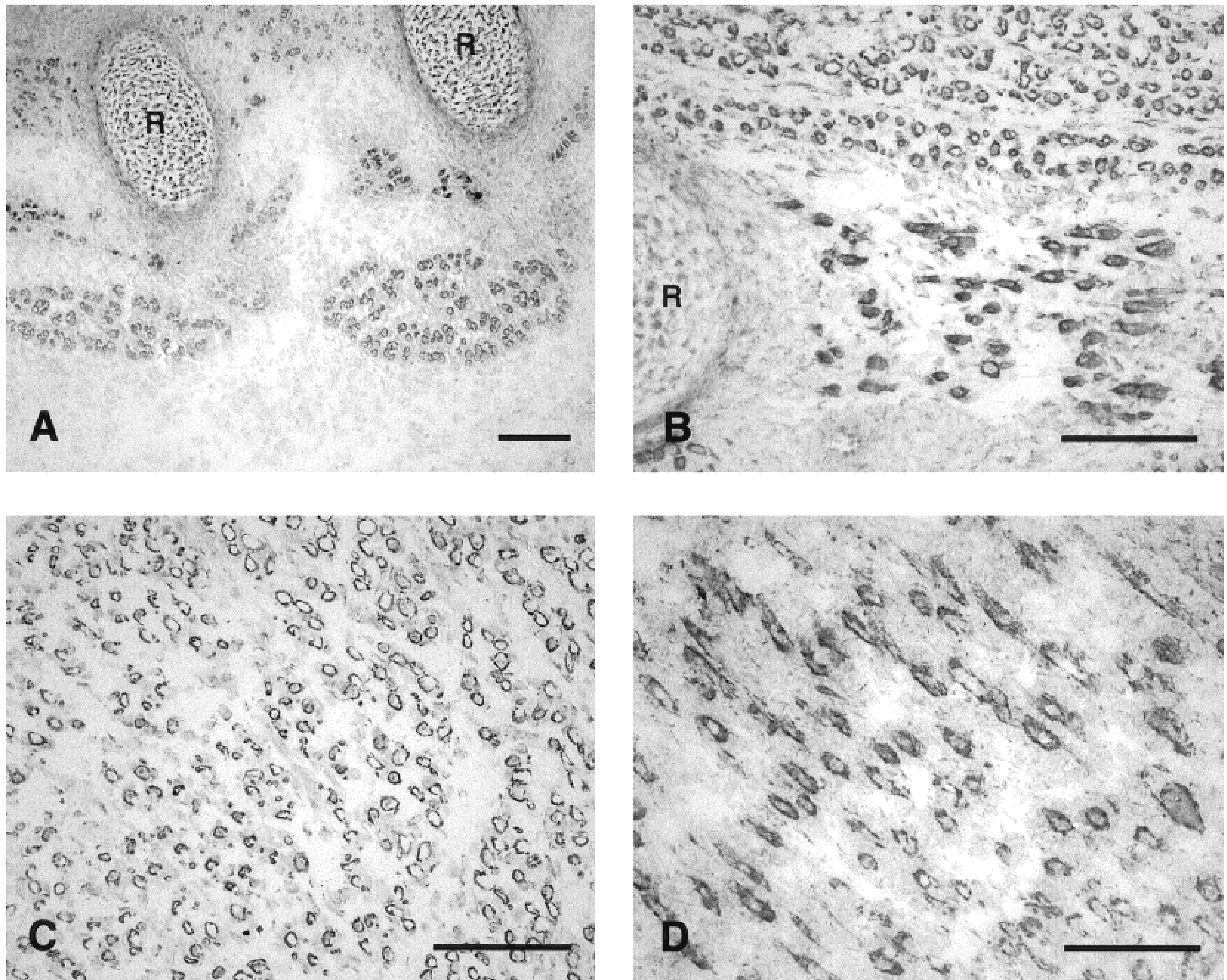


Fig. 2. Expression of $P2X_6$ in rat skeletal muscle at embryonic day (E)16 and E18. **A:** At E16, $P2X_6$ immunoreactivity appeared in all muscle groups tested, shown here in a longitudinal section of the embryo, in the intercostal and paravertebral muscles. **B–D:** Strong positive $P2X_6$ membrane staining of all muscle cells remained until E18, shown in an oblique section of the external intercostal muscles (B), a transverse section of the paravertebral muscles (C) and an oblique section of the lower limb muscles (D) of E18 embryos. R, rib cartilage. Scale bars = 100 μm in A–D.

an important signaling molecule in mammalian skeletal muscle development (Henning et al., 1993).

However, this work was performed before knowledge of the existence, let alone the properties of the P2X receptor subtypes (Ralevic and Burnstock, 1998). Our findings suggest that expression of the P2X receptors could account for the ATP sensitivity of developing skeletal muscle. By using immunohistochemistry, we show developmentally regulated expression of three P2X receptor subtypes, $P2X_2$, $P2X_5$, and $P2X_6$, in rat skeletal muscle. These receptors are expressed in a sequential manner, which was consistent for all muscles tested: $P2X_5$ was expressed first (E15-E18), followed by $P2X_6$ (E16-E18), and finally $P2X_2$ (E18-P14).

The temporal and spatial overlap in the expression of $P2X_2$, $P2X_5$, and $P2X_6$ receptor subunits, which we observed, raises the possibility that both homomeric and heteromeric receptors may be present in developing skeletal muscle. In sensory neurones of the nodose ganglion, $P2X_2$ and $P2X_3$ subunits are coexpressed (Vulchanova et al., 1997). The characteristics of the P2X receptors present on these cells can only be accounted for by the presence of a $P2X_{2/3}$ heteromeric receptor (Lewis et al., 1995). Biochemical studies have shown that 11 heteromeric receptors can be formed by pairwise combination of P2X subunits (Torres et al., 1999). However, of these only $P2X_{2/3}$, $P2X_{1/5}$, $P2X_{4/6}$, and $P2X_{2/6}$ have been demonstrated in functional studies (Lewis et al., 1995; Torres et al., 1998; Lê et al., 1998; King et al., 2000). Further double-labeling studies will be required to determine more closely the extent of P2X receptor coexpression in developing skele-

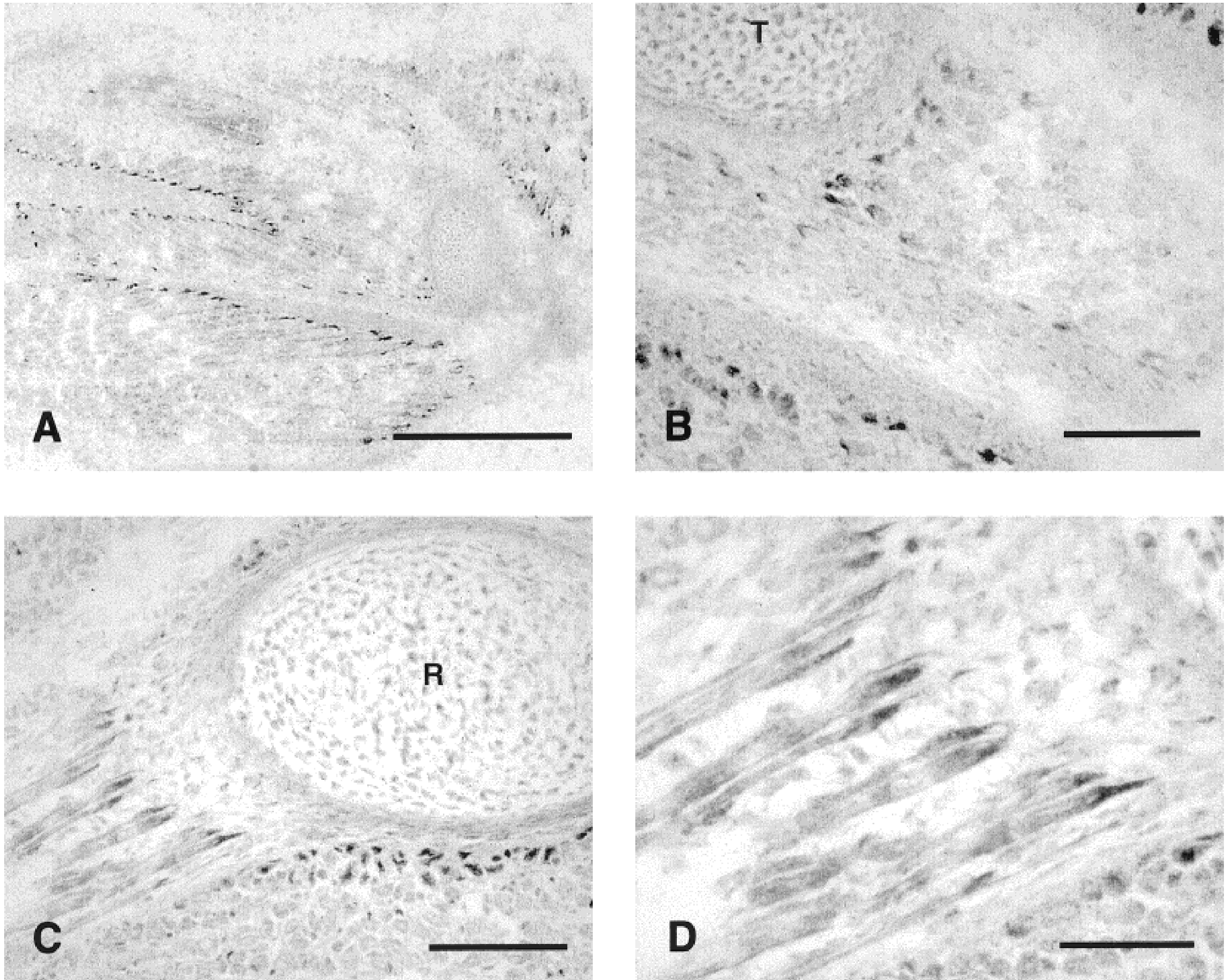


Fig. 3. Expression of $P2X_5$ in rat skeletal muscle at embryonic day (E) 18. At E18, $P2X_5$ immunoreactivity became restricted to cells at the end of the muscles, close to the developing cartilage/bones, shown in longitudinal sections of the lower limb (**A,B**) and external intercostal muscles (**C,D**). T, tibial cartilage; R, rib cartilage. Scale bars = 500 μm in A, 100 μm in B,C, 50 μm in D.

tal muscle. This issue is highlighted by our findings at P7, demonstrating the expression of both $P2X_2$ and $P2X_5$, but whereas $P2X_2$ stains skeletal muscle, $P2X_5$ is expressed on a range of other cell types, including smooth muscle and nerve fibers. However, it is worth noting that the study of Torres et al. (1999) suggests that $P2X_6$ does not form a homomeric receptor, suggesting that any functional role will be in coassembly with either $P2X_2$ or $P2X_5$ subunits.

The developmentally regulated expression of the P2X receptors suggests that the channels formed are of functional significance. The timing of receptor expression seems to be closely related to key events in skeletal muscle development (Fig. 8), notably secondary myotube formation and the establishment of mature neuromuscular junctions. We suggest that ATP signaling by means of $P2X_5$ and $P2X_6$ could be involved in the former event, whereas $P2X_2$ could be related to the latter.

Skeletal muscle cells are formed in two stages: primary myotubes develop first, and are followed after a delay by secondary myotubes, which will form the majority of muscle fibers in the adult tissue (Kelly and Zacks, 1969; Harris, 1981; Ontell and Kozeka, 1984; Ross et al., 1987). Secondary myotubes develop from the middle of the muscle in close association with the neuromuscular junctions of guiding primary myotubes (Duxson et al., 1989). They extend by the asynchronous fusion of myoblasts at their ends (Zhang and McLennan, 1995) to finally attach to the muscle tendons (Duxson and Usson, 1989). Immunohistochemical data from our study suggest the involvement of P2X receptors in

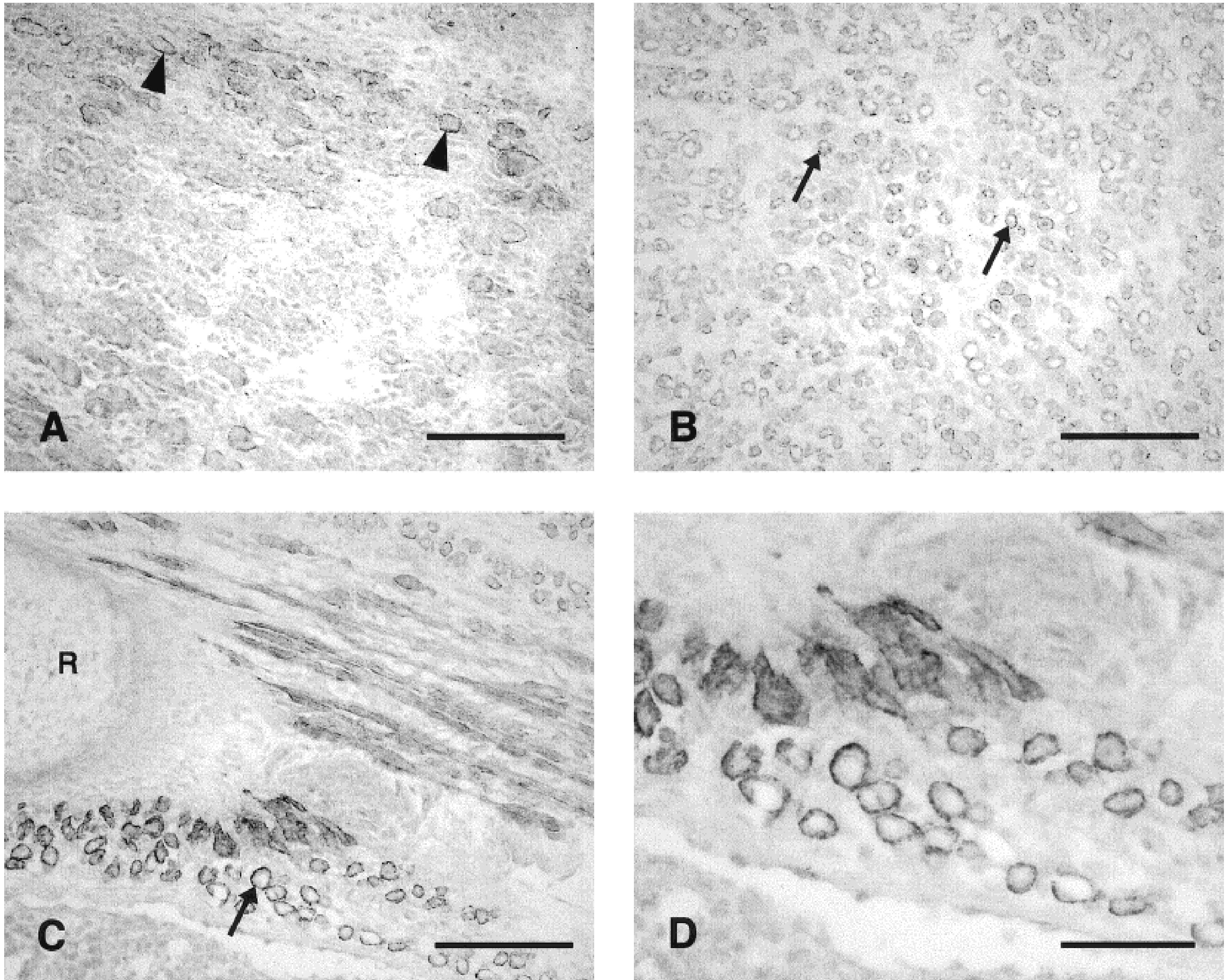


Fig. 4. Expression of $P2X_2$ in rat skeletal muscle at embryonic day (E) 18. **A:** In the lower limb muscle, shown in oblique section, $P2X_2$ immunoreactivity was restricted to a small number of cells (arrowheads). **B–D:** $P2X_2$ immunoreactivity, which was confined to cell membranes, was more widespread in the paravertebral muscles (B) and strongest in the intercostal muscles (C,D), shown in transverse section. R, rib cartilage. Scale bars = 100 μm in A–C, 50 μm in D.

secondary myotube formation. We detected no immunoreactivity for $P2X_5$ (the first receptor to be expressed) during at least the early stages of primary myotube formation (Ross et al., 1987). However, P2X receptors could be involved in the formation of secondary myotubes, which begins at E16 in the intercostal muscles (Kelly and Zacks, 1969), precisely the same time at which there is strongest immunoreactivity for $P2X_5$ and $P2X_6$.

Staining for $P2X_2$ begins in the intercostal muscles at E18, which is the peak of polyneuronal innervation (Dennis et al., 1981). It is well established that nerve-induced activity plays a crucial role in regulating the expression and distribution of receptors and channels on the muscle membrane, notably the acetylcholine receptor. Whereas developing myotubes express clusters of AChRs throughout the membrane, adult skeletal muscle is characterized by the striking concentration of receptors in the postsynaptic membrane and their virtual absence from the extrasynaptic membrane. This reorganization is in part activity-dependent (Vrbova et al., 1995; Sanes and Lichtman, 1999). In the case of rat intercostal muscles, synaptic inputs are first detected at E15 and produce clustering of AChRs by E16 (Dennis et al., 1981). This finding is confirmed in our results which show acetylcholine clusters in prospective endplate regions. However, on the basis of intracellular recordings in response to acetylcholine application (generally considered to be more sensitive than detection by α-bungarotoxin) the extrajunctional AChR density, particularly in regions close to the myotendinous junction, remains high after birth

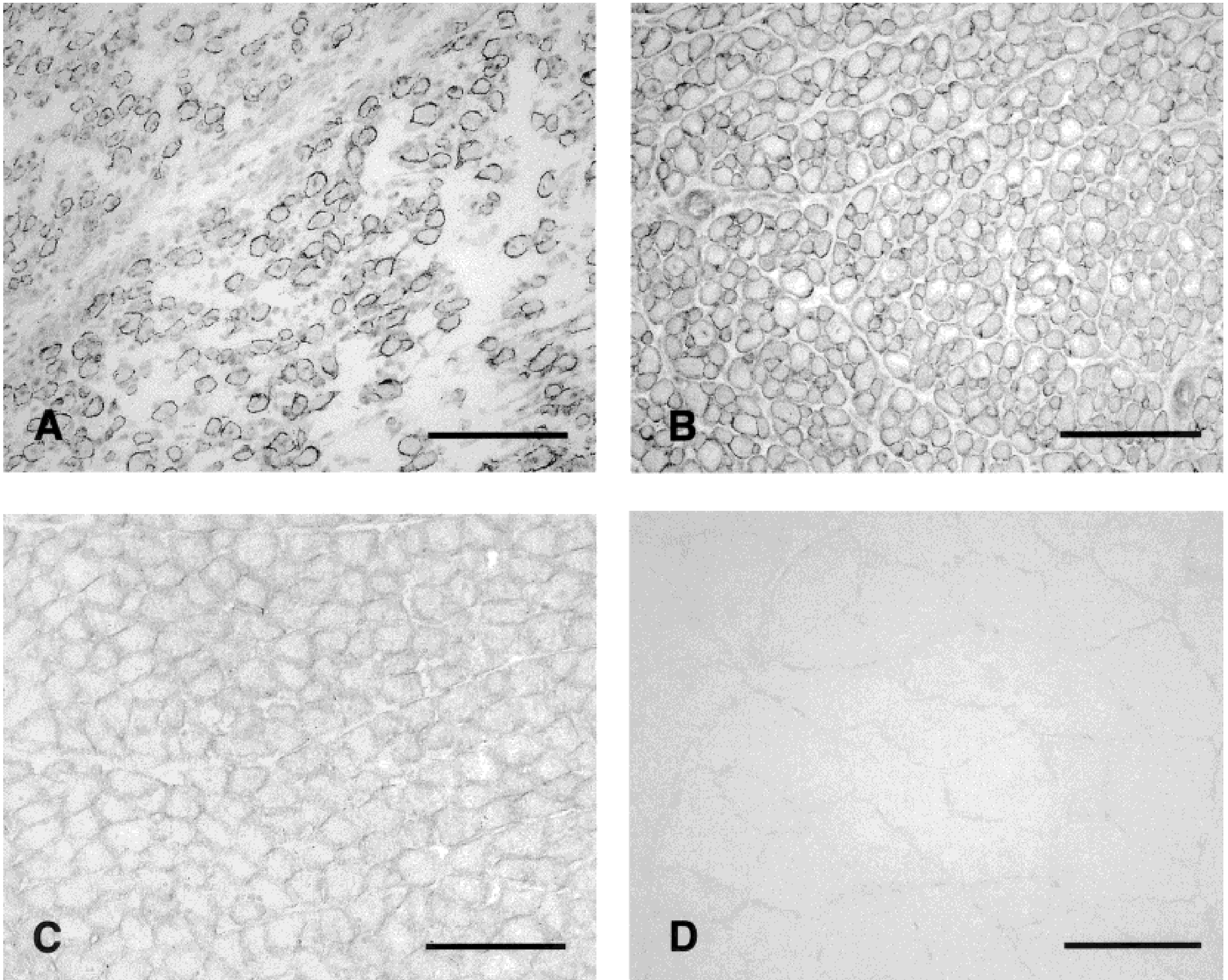


Fig. 5. Expression of $P2X_2$ in rat skeletal muscle at embryonic day (E) 20 and postnatally. **A:** At E20, strong $P2X_2$ immunoreactivity could be seen in the membranes of lower limb muscle cells. **B:** Strong immunostaining of lower limb muscles with $P2X_2$ remained postnatally at P7. **C:** $P2X_2$ immunoreactivity reduced in strength at P14. **D:** In adult tibialis anterior rat muscle immunostaining for $P2X_2$ was almost absent. All transverse sections. Scale bars = 100 μm in A–D.

and only declines during the first postnatal week (Diamond and Miledi, 1962). Changes in the expression of the P2X receptor subtypes, particularly $P2X_2$, mirrors and may even contribute to this process because $P2X_2$ receptor expression is also maintained during the first postnatal week and declines subsequently. In our dual-labeling experiments, extrajunctional AChRs were probably not visible due to the low detection sensitivity of α-bungarotoxin compared with intracellular recording. Interestingly, no expression of $P2X_2$ has been reported in chick skeletal muscle (Meyer et al., 1999). This may reflect differences in the development of mammalian as opposed to avian neuromuscular junctions.

The surprising abundance of these receptors raises the issue of what the potential sources of ATP in this developing system might be. It is well established that ATP is stored within the synaptic vesicles of presynaptic nerve terminals and is coreleased with acetylcholine (Silinsky and Hubbard, 1973). Muscles are also known to secrete substantial amounts of ATP in response to electrical activity (Landmesser and Morris, 1975). However, there are other important nerve-independent sources of extracellular ATP. Osteoblasts and chondrocytes, which are obviously closely associated with developing skeletal muscle, have been shown to be capable of releasing ATP (Bowler et al., 1998; Lloyd et al., 1999) and could be associated with the marked expression of $P2X_5$ at myotendinous junctions.

In conclusion, we show for the first time that three members of the P2X family, $P2X_2$, $P2X_5$, and $P2X_6$, are expressed in developing rat skeletal muscle. These re-

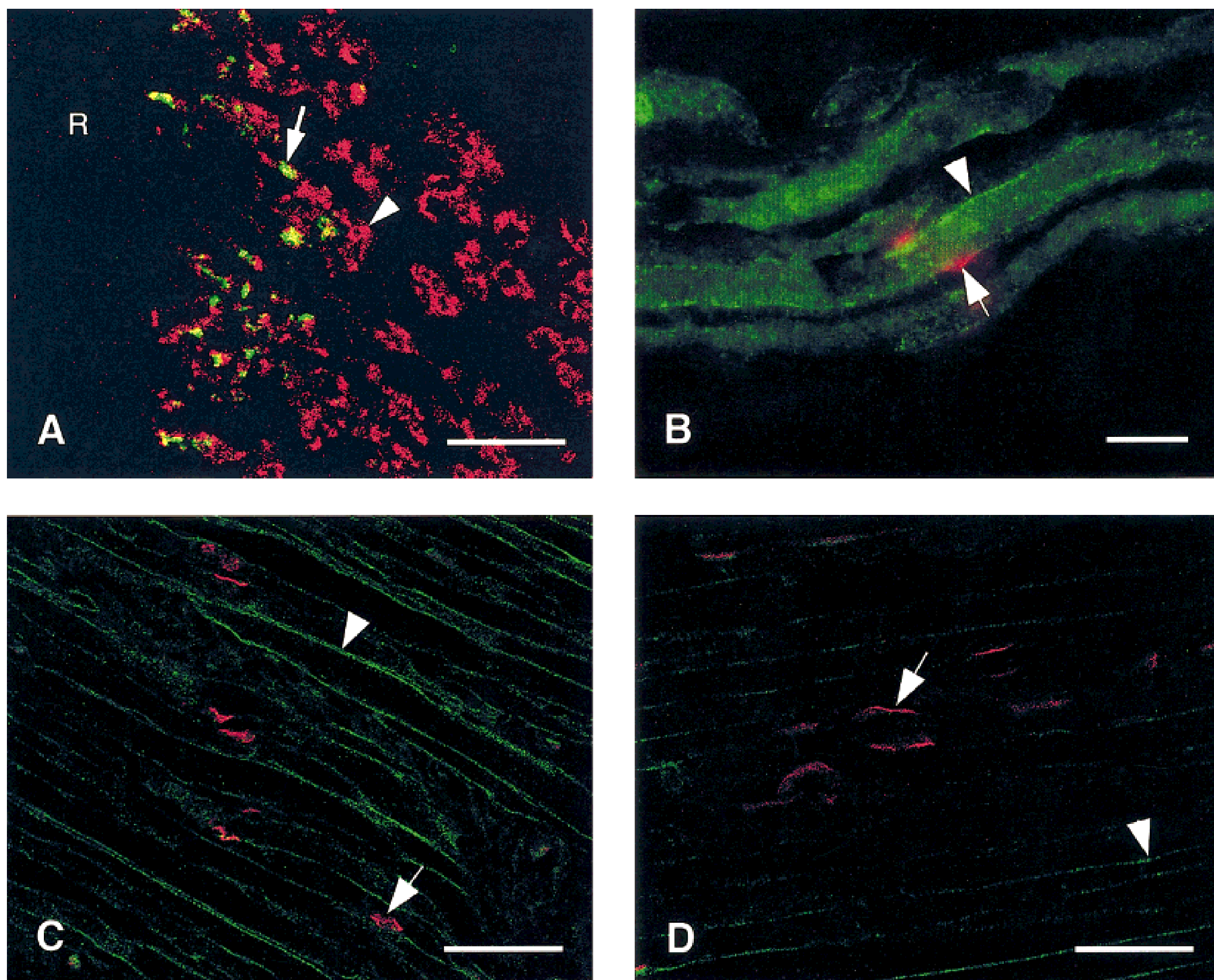


Fig. 6. **A:** Double labeling for $P2X_5$ (green, arrow) and skeletal myosin (red, arrowhead) demonstrated coexpression in areas close to the rib. **B–D:** Double labeling for $P2X_2$ (green, arrowheads) and acetylcholine receptors (AChRs) (red, arrows) on intercostal muscles at embryonic day (E) 20 (B) and tibialis anterior muscle at postnatal day (P) 7 (C) and P14 (D). Although there is strong staining for $P2X_2$ on the muscle membranes, it did not colocalize with AChR-clusters at any of the ages examined. R, rib cartilage. Scale bars = 50 μm in A, 20 μm in B, 100 μm in C,D.

ceptors are expressed sequentially ($P2X_5$, followed by $P2X_6$, and finally $P2X_2$) and appeared to be developmentally regulated. The timing of receptor expression is closely related to key events in skeletal muscle development, notably the generation of secondary myotubes and the redistribution of acetylcholine receptors, suggesting that ATP may have trophic actions on developing mammalian skeletal muscle. However, the precise roles of the P2X receptor subtypes in skeletal muscle development remain to be investigated.

EXPERIMENTAL PROCEDURES

Mating of Rats and Embryo Preparation

For dated pregnancies, female Sprague-Dawley rats in estrus were placed overnight with primed male rats and examined in the morning for the presence of a vaginal plug. The day of finding the plug was designated embryonic day 0 (E0) and the day of birth, postnatal day 0 (P0). Pregnant rats, killed by a rising concentration of CO_2 and confirmed by cervical dislocation, were used to obtain embryos at E12, E14, E15, E16, E18, and E20. Once removed, some embryos were placed decapitated and intact onto a cork block, covered with OCT compound, and frozen in liquid nitrogen-cooled isopentane. Limb buds were dissected from the remaining embryos, mounted on cork blocks, and frozen separately in liquid nitrogen-cooled isopentane. Skeletal muscle samples were also taken from rat pups killed at P7, P14, and P21. Segments of rib cage and lower limb were removed and prepared as previously described. Cryostat sections were cut at 12 μm and collected on gelatinized slides.

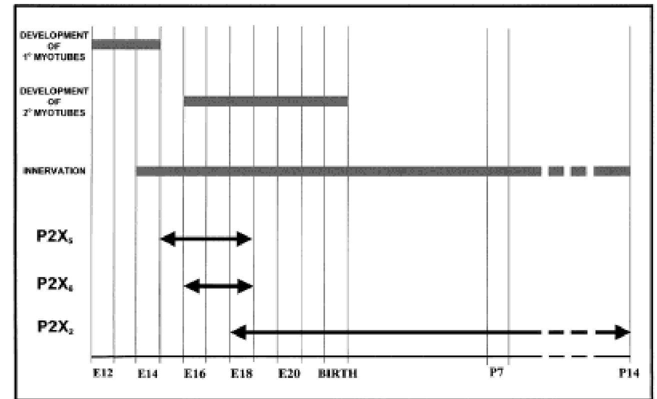
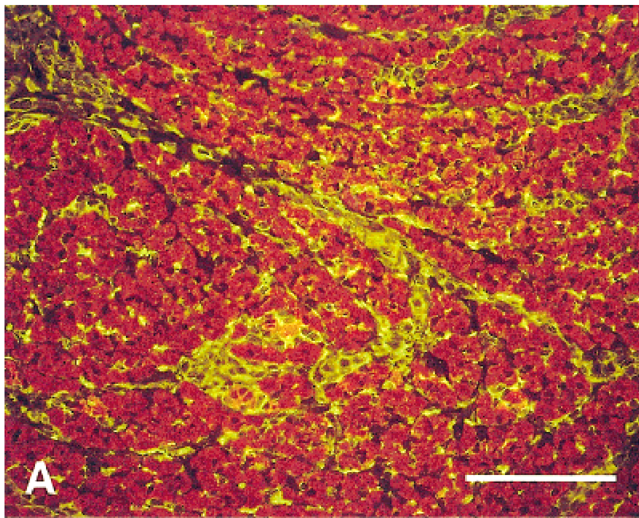


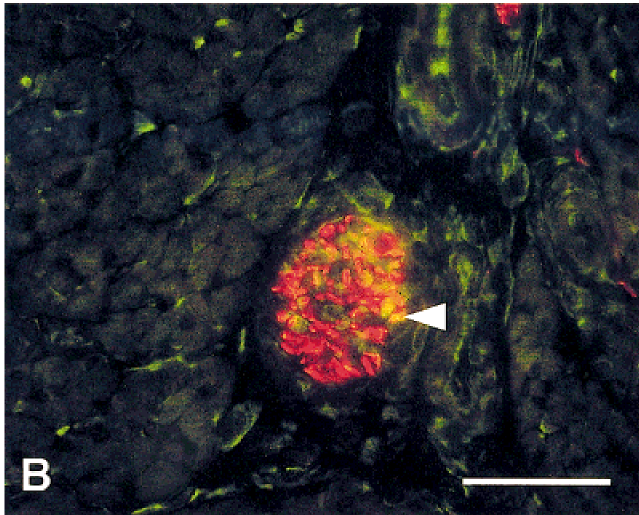
Fig. 8. This figure summarizes the time course of $P2X_{2,5,6}$ receptor expression for the rat intercostal muscles and demonstrates the possible overlaps with key events in skeletal muscle formation, namely development of primary and secondary myotubes and innervation (from the first detection of synaptic inputs to the development of the mature neuromuscular junction). The first receptor to appear is $P2X_5$ at embryonic day (E) 15, which disappears at E18; $P2X_6$ receptor expression starts at E16 and disappears after E18, whereas $P2X_2$ receptor expression only starts at E18 and gets significantly reduced after birth at postnatal day (P) 7.

Longitudinal sections of tibialis anterior muscle were prepared from animals at P7, P14, P21, and adult. Muscles were stretched and pinned on Sylgard, fixed in 4% paraformaldehyde for 2 hr at room temperature, and transferred to 20% sucrose in phosphate-buffered saline (PBS) and left overnight. Tissue was placed longitudinally and embedded in OCT on cork blocks. Cryostat sections were cut at 50 μm and collected in PBS.

Immunohistochemistry

The immunogens used for the production of polyclonal antibodies were synthetic peptides corresponding to the carboxy termini of the cloned rat P2X receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences are as follows. $P2X_1$: amino acids 385–399, ATSSTLGLQENMRTS; $P2X_2$: amino acids 458–472, QQDSTSTDPKGLAQL; $P2X_3$: amino acids 383–397, VEKQSTDSGAYSIGH; $P2X_4$: amino acids 374–388, YVEDYEQGLSGEMNQ; $P2X_5$: amino acids 437–451, RENAIVNVKQSQILH; $P2X_6$: amino





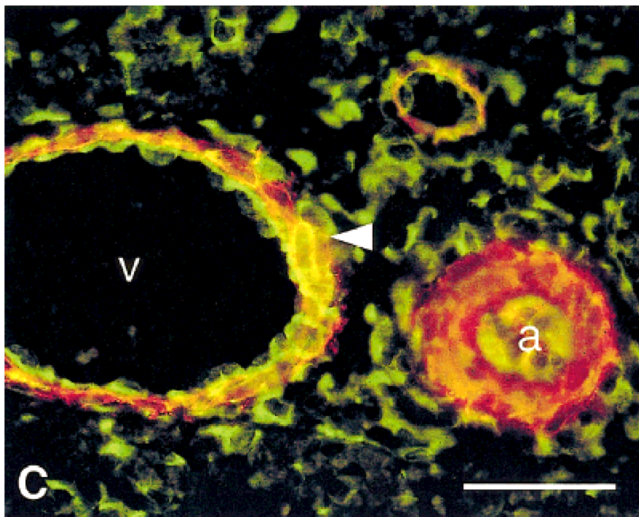


Fig. 7. **A:** At postnatal day (P) 7, double labeling for $P2X_5$ (green) and skeletal muscle myosin (red) showed the presence of $P2X_5$-immunopositive cells exclusively in between muscle fibers. **B:** Double labeling for $P2X_5$ (green) and neurofilament 200 (red) demonstrated limited coexpression (yellow, arrowhead) of these markers in fibers within a nerve bundle. **C:** Double labeling for $P2X_5$ (green) and smooth muscle actin (red) demonstrated limited coexpression (yellow) of these markers in both arteries and veins (arrowhead). Note that in C some $P2X_5$ (green) staining appears to be on endothelial cells. Because skeletal muscle develops from center to periphery, photographs taken from within the muscle bulk (B), which is more mature, demonstrate less immunoreactivity for $P2X_5$ than those taken at the less developed periphery (B). All transverse sections of lower limb muscle (tibialis anterior) at P7. a, artery; v, vein. Scale bars = 100 μm in A, 50 μm in B,C.

acids 357–371, EAGFYWRTKYEEARA; $P2X_7$: amino acids 555–569, TWRFVSQDMADFAIL.

The polyclonal antibodies were raised by multiple monthly injections of New Zealand rabbits with the peptides (performed by Research Genetics, Huntsville, AL). The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO-K1 cells expressing the cloned $P2X_{1-7}$ receptors. The antibodies recognized only one protein of the expected size in the heterologous expression systems and were shown to be receptor subtype-specific (Oglesby et al., 1999). For immunostaining of cryostat sections, the avidin-biotin (ABC) technique was used according to the protocol developed by Llewellyn-Smith et al. (1992, 1993). Air-dried serial sections of the tissues were fixed in 4% formaldehyde and 0.2% of a saturated solution of picric acid in 0.1 M phosphate buffer (pH 7.4) for 2 min. After washing in PBS for 15 min, endogenous peroxidase activity was blocked by treating the sections with 0.4% H_2O_2 and 50% methanol for 10 min. Nonspecific binding sites were blocked by a 20-min preincubation in 10% normal horse serum (NHS) in PBS. Sections were incubated overnight at room temperature in 5 μg/ml of $P2X_{1-7}$ antibody or 2 μg/ml anti-skeletal myosin (Sigma, Poole, UK), in 10% NHS in PBS + 2.5% NaCl at room temperature. Subsequently, sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA) diluted 1:500 in 1% NHS in PBS for 1 hr, and then with ExtrAvidin peroxidase (Sigma) diluted 1:1500 in PBS for 1 hr at room temperature. For color reaction, a solution containing 0.05% 3,3′-diaminobenzidine 0.04% nickel ammonium sulphate, 0.2% β-D-glucose, 0.004% ammonium nitrate, and 1.2U/ml glucose oxidase in 0.1 M PBS was applied for 8 min. Sections were washed three times with PBS after each of the above steps except after the preincubation. Control experiments were carried out with the primary antibody omitted from the staining procedure and the primary antibody preabsorbed with the peptides used to immunize the rabbits, according to the protocol described by Meyer et al. (1999).

For immunofluorescent staining, air-dried serial sections of tissue were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 min. After washing in PBS for 15 min, sections were treated as before with 10% NHS in PBS to block nonspecific binding. Sections were then incubated overnight at room temperature in 5 μg/ml of $P2X_5$ or $P2X_2$ antibody in 10% NHS in PBS + 2.5% NaCl. P2X expression was visualized by using Oregon-green–labeled goat anti-rabbit IgG (Jackson ImmunoResearch Lab) applied for 1 hr at 1:100. Double staining for acetylcholine receptors was performed by further incubation of sections with α-bungarotoxin-Texas Red at 1:1,000 for 1 hr at room temperature. For neurofilament 200 or smooth muscle actin, double staining was performed by incubation of sections with either mouse anti-neurofilament 200 IgG (Sigma) or mouse anti-smooth muscle actin (Sigma) at 1:100 and 1:1,000, respectively, overnight. Staining was visualized by incubating with TRITC-labeled goat anti-mouse antibody (Jackson ImmunoResearch Lab) at 1:100 for 1 hr.

In the case of double staining for $P2X_5$ and skeletal myosin, sections were incubated overnight at room temperature in 1 μg/ml of $P2X_5$ antibody in 10% NHS in PBS + 2.5% NaCl. This step was followed by incubation with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab) diluted 1:500 in 1% NHS in PBS for 1 hr, ExtrAvidin peroxidase diluted 1:1500 in PBS for 1 hr, tyramide amplification for 8 min (Tyramide Amplification Kit, NEN Life Science Products, Boston, MA), and finally streptavidin-fluorescein (Amersham, UK) at 1:200 for 30 min. Sections were washed three times with PBS + Tween (0.05%) after each of the above steps. Staining for skeletal muscle myosin was performed by further incubation of sections overnight in 2 μg/ml rabbit anti-PM in 10% NHS in PBS + 2.5% NaCl, followed by visualization with goat anti-rabbit cy3 (Jackson ImmunoResearch Lab), 1:200 in 1% NHS in PBS for 1 hr. For the longitudinal 50-μm sections, photographs were generated by using confocal microscopy.

ACKNOWLEDGMENTS

The authors thank Tim Arnett, Michelle Bardini, and Ute Gröschel-Stewart for help and technical assistance and Roche Bioscience, Palo Alto, California, for their support. Mina Ryten is funded by University College Medical School and Astrid Hoebertz by the Arthritis Research Campaign.

REFERENCES

Abbracchio MP, Burnstock G. 1998. Purinergic signalling: pathophysiological roles. Jpn J Pharmacol 78:113–145.

Bowler WB, Tattersall JA, Hussein R, Dixon CJ, Cobbold PH, Gallagher JA. 1998. Release of ATP by osteoblasts: modulation by fluid shear forces. Bone 22:3S.

Burnstock G. 1997. The past, present and future of purine nucleotides as signalling molecules. Neuropharmacology 36:1127–1139.

Collo G, Neidhart S, Kawashima E, Kosco Vilbois M, North RA, Buell G. 1997. Tissue distribution of the $P2X_7$ receptor. Neuropharmacology 36:1277–1283.

Coutinho-Silva R, Persechini PM, Bisaggio RD, Perfettini JL, Neto AC, Kanellopoulos JM, Motta Ly I, Dautry Varsat A, Ojcius DM. 1999. P2Z/$P2X_7$ receptor-dependent apoptosis of dendritic cells. Am J Physiol 276:C1139–C1147.

Dennis MJ, Ziskind Conhaim L, Harris AJ. 1981. Development of neuromuscular junctions in rat embryos. Dev Biol 81:266–279.

Diamond J, Miledi R. 1962. A study of foetal and new-born rat muscle fibres. J Physiol (Lond) 162:393–408.

Duxson MJ, Usson Y. 1989. Cellular insertion of primary myotubes and secondary myotubes in embryonic rat muscles. Development 107:243–251.

Duxson MJ, Usson Y, Harris AJ. 1989. The origin of secondary myotubes in mammalian skeletal muscles: ultrastructural studies. Development 107:743–750.

Ferrari D, Los M, Bauer MK, Vandenabeele P, Wesselborg S, Schulze-Osthoff K. 1999. P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett 447:71–75.

Fu WM. 1995. Regulatory role of ATP at developing neuromuscular junctions. Prog Neurobiol 47:31–44.

Fu WM, Poo MM. 1991. ATP potentiates spontaneous transmitter release at developing neuromuscular synapses. Neuron 6:837–843.
Gröschel-Stewart U, Bardini M, Robson T, Burnstock G. 1999. Localisation of $P2X_5$ and $P2X_7$ receptors by immunohistochemistry in rat stratified squamous epithelia. Cell Tissue Res 296:599–605.
Harris AJ. 1981. Embryonic growth and innervation of rat skeletal muscles: I. Neural regulation of muscle fibre numbers. Philos TransR Soc Lond B Biol Sci 293:257–277.
Henning RH. 1997. Purinoceptors in neuromuscular transmission. Pharmacol Ther 74:115–128.
Henning RH, Duin M, den Hertog A, Nelemans A. 1993. Characterization of P2-purinoceptor mediated cyclic AMP formation in mouse C2C12 myotubes. Br J Pharmacol 110:133–138.
Hume RI, Honig MG. 1986. Excitatory action of ATP on embryonic chick muscle. J Neurosci 6:681–690.
Hume RI, Thomas SA. 1988. Multiple actions of adenosine 5'-triphosphate on chick skeletal muscle. J Physiol Lond 406:503–524.
Kelly AM, Zacks SI. 1969. The histogenesis of rat intercostal muscle. J Cell Biol 42:135–153.
King BF, Townsend-Nicholson A, Wildman SS, Thomas T, Spyer KM, Burnstock G. 2000. Coexpression of rat $P2X_2$ and $P2X_6$ subunits in *Xenopus* Oocytes. J Neurosci 20:4871–4877.
Kolb HA, Wakelam MJ. 1983. Transmitter-like action of ATP on patched membranes of cultured myoblasts and myotubes. Nature 303:621–623.
Landmesser L, Morris DG. 1975. The development of functional innervation in the hind limb of the chick embryo. J Physiol (Lond) 249:301–326.
Lê K, Babinski K, Séguéla P. 1998. Central $P2X_4$ and $P2X_6$ channel subunits coassemble into a novel heteromeric ATP receptor. J Neurosci 18:7152–7159.
Lewis C, Neidhart S, Holy C, North RA, Buell G, Surprenant A. 1995. Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432–435.
Llewellyn-Smith IJ, Song ZM, Costa M, Bredt DS, Snyder SH. 1992. Ultrastructural localization of nitric oxide synthase immunoreactivity in guinea-pig enteric neurons. Brain Res 577:337–342.
Llewellyn-Smith IJ, Pilowsky P, Minson JB. 1993. The tungstate-stabilized tetramethylbenzidine reaction for light and electron microscopic immunocytochemistry and for revealing biocytin-filled neurons. J Neurosci Methods 46:27–40.
Lloyd DK, Golding SL, Bowler WB, Dixon CJ, Dillon JP, Gallagher JA. 1999. Regulated ATP release by cultured human articular chondrocytes. Calcif Tissue Int 64:S58.
Meyer MP, Gröschel-Stewart U, Robson T, Burnstock G. 1999. Expression of two ATP-gated ion channels, $P2X_5$ and $P2X_6$, in developing chick skeletal muscle. Dev Dyn 216:442–449.
Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G. 1996. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci 19:13–18.
Oglesby IB, Lachnit WG, Burnstock G, Ford APDW. 1999. Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, $P2X_1$ through $P2X_7$. Drug Dev Res 47:189–195.
Ontell M, Kozeka K. 1984. Organogenesis of the mouse extensor digitorum longus muscle: a quantitative study. Am J Anat 171:149–161.
Ralevic V, Burnstock G. 1998. Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492.
Ross JJ, Duxson MJ, Harris AJ. 1987. Formation of primary and secondary myotubes in rat lumbrical muscles. Development 100: 383–394.
Sanes JR, Lichtman JW. 1999. Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389–442.
Silinsky EM, Hubbard JI. 1973. Release of ATP from rat motor nerve terminals. Nature 243:404–405.
Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). Science 272:735–738.
Thomas SA, Hume RI. 1990a. Permeation of both cations and anions through a single class of ATP-activated ion channels in developing chick skeletal muscle. J Gen Physiol 95:569–590.
Thomas SA, Hume RI. 1990b. Irreversible desensitization of ATP responses in developing chick skeletal muscle. J Physiol (Lond) 430:373–388.
Thomas SA, Hume RI. 1993. Single potassium channel currents activated by extracellular ATP in developing chick skeletal muscle: a role for second messengers. J Neurophysiol 69:1556–1566.
Thomas SA, Zawisa MJ, Lin X, Hume RI. 1991. A receptor that is highly specific for extracellular ATP in developing chick skeletal muscle in vitro. Br J Pharmacol 103:1963–1969.
Torres GE, Haines WR, Egan TM, Voigt MM. 1998. Co-expression of $P2X_1$ and $P2X_5$ receptor subunits reveals a novel ATP-gated ion channel. Mol Pharmacol 54:989–993.
Torres GE, Egan TM, Voigt MM. 1999. Identification of a domain involved in ATP-gated ionotropic receptor subunit assembly. J Biol Chem 274:22359–22365.
Vrbova G, Gordon T, Jones R. 1995. Encounter of motor nerves with muscle fibres. In: Nerve-muscle interaction. London: Chapman and Hall. p 36–41.
Vulchanova L, Riedl MS, Shuster SJ, Buell G, Surprenant A, North RA, Elde R. 1997. Immunohistochemical study of the $P2X_2$ and $P2X_3$ receptor subunits in rat and monkey sensory neurons and their central terminals. Neuropharmacology 36:1229–1242.
Wells DG, Zawisa MJ, Hume RI. 1995. Changes in responsiveness to extracellular ATP in chick skeletal muscle during development and upon denervation. Dev Biol 172:585–590.
Zhang M, McLennan IS. 1995. During secondary myotube formation, primary myotubes preferentially absorb new nuclei at their ends. Dev Dyn 204:168–177.