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Purinoceptor expression in regenerating skeletal muscle in the *mdx* mouse model of muscular dystrophy and in satellite cell cultures

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ABSTRACT

ATP is an important extracellular signaling molecule mediating its effects by activation of P2X and P2Y receptors. P2 receptors are expressed during muscle development, and recent findings demonstrate that ATP can regulate myoblast proliferation and differentiation in vitro. However, the role of purinergic signaling during regeneration of injured skeletal muscle has not been investigated. To examine this process in a clinically relevant system, we used the mouse model of muscular dystrophy (*mdx*), in which muscle degeneration is rapidly followed by regeneration. The latter process, in vivo muscle regeneration, was the focus of this study, and to study the cellular mechanisms involved in it, a parallel study on normal rat skeletal myoblast cultures was conducted. Using immunohistochemistry, RT-PCR, and electrophysiology, we investigated the expression of the $P2X_{1\text{-}7}$ receptor subtypes and the $P2Y_{1,2,4,6}$ receptors. Experiments in vitro and in vivo demonstrated the sequential expression of the $P2X_5$, $P2Y_1$, and $P2X_2$ receptors during the process of muscle regeneration. The $P2X_5$ and $P2Y_1$ receptors were expressed first on activated satellite cells, and the $P2Y_1$ receptor was also expressed on infiltrating immune cells. Subsequent $P2X_2$ receptor expression on newly formed myotubes showed significant colocalization with AChRs, suggesting a role in regulation of muscle innervation. Thus, this study provides the first evidence for a role for purinergic signaling in muscle regeneration and raises the possibility of new therapeutic strategies in the treatment of muscle disease.

Key words: ATP • P2 receptor • myotube

Adult skeletal muscle fibers are large, terminally differentiated cells containing multiple postmitotic nuclei. However, skeletal muscle shows a remarkable ability to recover from injury. The ability of mammalian skeletal muscle to respond to pathological damage is largely attributed to the presence of a small population of self-renewing muscle precursors, termed skeletal muscle satellite cells. Normally quiescent in response to muscle fiber injury satellite cells are activated, proliferate, and ultimately fuse to repair existing muscle fibers and form new myotubes (1–3). This process must be followed by myotube differentiation and growth

before full muscle function can be regained. Many growth factors have been implicated in the regulation of muscle regeneration, including fibroblast growth factors, insulin-like growth factors, and nitric oxide (3).

In this study, we explore the possibility that ATP might also be involved in regulating muscle regeneration. ATP and other extracellular nucleotides are now widely recognized as important extracellular messenger molecules with diverse biological effects ranging from smooth muscle contraction and synaptic transmission to cell proliferation and differentiation (4). The effects of ATP are mediated by activation of either P2X or P2Y receptors (5). P2Y receptors are G protein-coupled receptors, which act principally by activating phospholipase C, leading to formation of inositol 1,4,5-trisphophate and the mobilization of intracellular Ca^{2+} (5). In contrast, P2X receptors are ligand-gated ion channels 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 mammalian P2X receptor subunits ($P2X_{1-7}$) capable of assembling into homo- or heteromultimeric receptors have been cloned (6).

The expression of purinoceptors on developing skeletal muscle cells is well established. Responses characteristic of P2 receptor activation have been detected on myoblasts and myotubes cultured from embryonic chick muscle and C2C12 myoblasts (7–14). More recently the expression of specific P2X and P2Y receptor subtypes during skeletal muscle development has been demonstrated. $P2Y_1$, $P2X_5$, and $P2X_6$ are expressed in chick skeletal muscle development (15–18), while expression of the $P2X_2$, $P2X_5$, $P2X_6$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors has been demonstrated in rat skeletal muscle development (19, 20).

In the case of the $P2X_5$ and $P2Y_1$ receptors, the functional significance of receptor expression has been explored. Whereas the $P2Y_1$ receptor has been implicated in the regulation of acetylcholine receptor (AChR) and acetylcholinesterase (AChE) expression (17, 21), a role for the $P2X_5$ receptor in the regulation of myoblast activity and differentiation has been demonstrated (22). In vitro experiments show that activation of the $P2X_5$ receptor by ATP (but not adenosine, ADP, or UTP) results in a shift in the balance between myoblast proliferation and differentiation. Rat skeletal satellite cells (in primary culture) exposed to ATP have a reduced rate of proliferation but express markers of differentiation including myogenin and p21 mRNA (22). There is also evidence to suggest that adenosine, acting via purinergic (P1) receptors, may have trophic effects on muscle cells, causing apoptosis of C2C12 myoblasts (23).

There is also growing evidence for the release of ATP from multiple sites in skeletal muscle. As in all tissues, muscle fiber damage will result in the passive release of ATP and other nucleotides from cells. In addition, significant quantities of ATP are coreleased with ACh from motor nerve terminals (24, 25) on nerve activation and may be released from muscle fibers on contraction (26–28). Thus, skeletal muscle, particularly on injury, would be expected to contain high levels of extracellular ATP.

Together these findings suggest that purinergic signaling may play a significant role not only in skeletal muscle formation but also in muscle regeneration. To investigate this possibility, we studied purinoceptor expression and function in response to muscle injury both in vivo and in vitro. The *mdx* mouse model of Duchenne muscular dystrophy (DMD) was used as an example of a clinically relevant form of muscle damage and subsequent regeneration, the latter being the

focus of this study. DMD, the most common and severe of the muscular dystrophies, is caused by mutations in the dystrophin gene that result in the loss of dystrophin protein (29, 30). Dystrophin (as part of the dystrophin-glycoprotein complex) is involved in linking the cytoskeleton to the extracellular matrix, thereby stabilizing the periphery of the muscle fiber during contraction (31, 32). Loss of this protein renders the muscle plasma membrane susceptible to contraction-induced damage (33, 34), which (in the early stages of the disease) is followed rapidly by muscle regeneration. The *mdx* mouse has a genetic defect in a homologous region to that identified in humans and similarly lacks dystrophin protein (35, 30). This results in cycles of muscle fiber degeneration and subsequent regeneration. However, it must be noted that muscle regeneration in *mdx* mice does not result in such marked muscle weakness and premature death as occurs in human DMD patients.

Primary cultures of postnatal rat myoblasts exhibit a process of muscle regeneration in vivo that parallels that seen in vivo. Myoblasts in primary culture proliferate, align, and fuse to form contractile myotubes. This cellular morphogenesis is accompanied by muscle-specific gene expression, including the expression of the myogenic regulatory factors and proteins characteristic of terminal differentiation, such as myosin heavy chain (MHC) and nicotinic AChRs. By using this model of muscle regeneration, we have been able to apply a variety of techniques, including immunocytochemistry, RT-PCR, and patch clamping, to investigate the underlying signaling pathways. Thus, it was possible to investigate the timing, localization, and pharmacology of purinoceptors on satellite cells and myotubes during the process of muscle formation and maturation.

MATERIALS AND METHODS

Tissue preparation

Mdx and control, C57Bl/10 strain, mice were weaned at 3 wk and killed at 3.5 wk, 5 wk, and 2-3 months of age by exposing animals to a rising concentration of CO_2, and death was confirmed by cervical dislocation. The tibialis anterior muscles were removed, placed onto cork blocks, covered with OCT compound, and frozen in liquid nitrogen-cooled isopentane. Cryostat sections were cut, producing transverse sections of the muscle at 12 μm or 7 μm and were collected on gelatinized slides.

Longitudinal sections of tibialis anterior muscle were prepared by pinning muscles on sylgard and then fixing the tissue in 4% paraformaldehyde for 2 h at room temperature. After being rinsed in PBS, muscles were placed longitudinally onto cork blocks, embedded in OCT and frozen in liquid nitrogen-cooled isopentane. Cryostat sections were cut at 50 μm and collected in PBS for staining as floating sections.

Immunohistochemistry (color reaction)

Immunostaining of 12 μm cryostat sections for the $P2X_{1\text{-}7}$ and $P2Y_{1,2,4}$ receptors was performed as described in Ryten et al. (20) and Cheung et al. (19); all sections were counterstained for nuclei with contrast green (Kirkegaard and Perry Laboratories) for 5 min at room temperature. Control experiments were carried out with the primary antibody omitted from the staining

procedure or with the primary antibody preabsorbed with the cognate peptides used to immunize the rabbits.

To make meaningful comparisons between the levels of immunoreactivity for a given receptor, sections were stained in sets containing tissues from control (C57Bl/10) and *mdx* animals at 3.5 wk, 5 wk, and 2-3 months of age and the color reaction was timed and equal for all samples.

Immunohistochemistry (fluorescence)

Air-dried cryostat sections and skeletal myoblasts plated on laminin-coated 8-well LabTek chamberslides (Nunc Life Technologies) were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). After being washed in PBS, nonspecific binding sites were blocked using 10% NHS in PBS or 10% NGS. Due to the greater degree of permeabilization required for immunostaining for nuclear transcription factors, 7 μm thick cryostat sections were used when staining for MyoD and myogenin, and these sections were incubated in 10% NHS + 0.2% Triton X-100. Sections were incubated with primary antibodies, diluted in the respective blocking solutions, overnight at room temperature. The primary antibodies used were rabbit anti-$P2X_2$ or anti-$P2X_5$ (Roche Bioscience), rabbit anti-$P2Y_1$ (Alomone Laboratories), rabbit anti-neurofilament 200 (Sigma), rabbit anti-MyoD1 (Autogen Bioclear), and rabbit anti-myogenin (Autogen Bioclear). These antibodies were used at the following concentrations: anti-$P2X_2$ or -$P2X_5$, 5 μg/ml; anti-$P2Y_1$, 3 μg/ml; anti-neurofilament, 1:100; anti-MyoD1, 1:400; and anti-myogenin 1:400. After being washed in PBS, cells were incubated with fluorescence-labeled secondary antibodies for 1 h at 37°C. Donkey anti-rabbit Cy3 (1:500 in 1% NHS in PBS) or Oregon green-labeled goat anti-rabbit secondary antibodies (Stratech Scientific; 1:100 in 1% NGS in PBS) were applied as appropriate. In some cases, cell nuclei were also stained by incubation with DAPI (Sigma) at 0.6 μg/ ml in PBS for 1 h at room temperature.

Double labeling experiments for $P2Y_1$ and CD11b were conducted as described above, except for an additional 1 h incubation with fluorescein-labeled rat anti-CD11b (Serotec). Immunostaining for $P2X_2$ and AChRs was also performed using a similar protocol, except that sections were incubated for 1 h with Texas Red-labeled α-bungarotoxin (specific marker of the α-subunit of nicotine AChRs). Longitudinal, 50 μm thick sections were examined using confocal microscopy.

Control experiments were carried out with the primary antibody omitted from the staining procedure, and, in the case of the rabbit anti-$P2X_{1-7}$, $P2Y_1$, $P2Y_2$, and $P2Y_4$ antibodies, the primary antibody was preabsorbed with the peptides used to immunize the rabbits.

Quantitative analysis of $P2X_5$ immunoreactivity

To measure the expression of $P2X_5$ receptor staining, the number of nuclei present in $P2X_5$-positive cells was counted in five randomly chosen fields (of 0.2 mm^2) from a minimum of two muscle sections per muscle sample. Muscle samples from at least four animals were examined. Sections were taken from the mid-belly region of the TA muscle, photographed using a digital camera (Leica) attached to a Zeiss Axioplan microscope (Zeiss), and viewed on a screen with a grid applied to aid accuracy.

The total number of nuclei present in each field of view was also counted. This revealed that *mdx* muscle contained a significantly higher number of nuclei as compared with control muscle (204 ± 23 nuclei/ field of view in control, as compared with 353 ± 23 nuclei/ field of view in *mdx* tibialis anterior muscle). To correct for differences in nuclei number, the number of nuclei present in $P2X_5$-positive cells was expressed as a percentage of the total number of nuclei.

Differences in the percentage of nuclei present in $P2X_5$-positive cells in age-matched muscle samples were assessed for significance by one-way ANOVA followed by a post hoc Bonferroni's multiple comparison test.

Tissue culture

Primary cultures of skeletal myoblasts were prepared as described in Ryten et al. (22). Cells were maintained in DMEM supplemented with 10% FCS, 10% normal horse serum (NHS; GIBCO), 0.5% gentamicin, and 0.5% ampicillin (Sigma) (termed growth medium, GM), or DMEM supplemented with 5% NHS (differentiation medium, DM), at 37°C and 5% CO_2. Freshly isolated cells were used for each experiment.

Electrophysiology

Cells were plated at low density in 35 mm plastic culture dishes (Falcon) and maintained in culture for 1–7 days. Culture dishes were placed on the stage of an inverted microscope (Diaphot, Nikon), and cells were visualized under phase contrast at x600 magnification. Culture dishes were perfused with extracellular solution at room temperature, at a rate of 0.5 ml/min, while solution was applied locally to the cell of interest using a microperfusion device (36). Recordings were carried out using the conventional whole cell patch clamp technique (37) or the perforated patch technique. Patch electrodes were fabricated from thin wall borosilicate glass capillaries (Clark Electromedical, GC 150TF) and had a resistance of 2–4 MΩ when filled with "intracellular solution." Membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments), displayed on a chart recorder (Gould TA240), and stored on digital audiotape using a DTR-1204 (Biologic, Claix) recorder for subsequent line analysis. Traces were acquired using Fetchex (pClamp software, Axon Instruments) and plotted using Origin 4 (Microcal).

Concentration effect data were fitted with the Hill equation: $Y = A/[1+(K/X)^n]$ where A is the maximum effect, K is the EC_{50}, and n is the Hill coefficient, using Origin 4 (Microcal). The combined data from a number of cells were fitted, and the results are presented as values ± SE determined by the fitting routine.

Except where modified as indicated, the extracellular solution contained (mM): 154 NaCl, 4.7 KCl, 10 HEPES, 5.6 D-glucose, 1.2 $MgCl_2$, 2.5 $CaCl_2$ adjusted to pH 7.4 with NaOH. In experiments to investigate P2X mediated responses, the intracellular (pipette) solution contained (mM): 56 citric acid, 3 $MgCl_2$, 20 CsCl, 40 HEPES, 0.1 EGTA, 10 TEA Cl, adjusted to pH 7.2 with CsOH (total Cs^+ concentration 170 mM). To investigate P2Y-mediated responses, the pipette solution contained (mM): 56 tripotassium citrate, 25 KCl, 10 NaCl, 35 HEPES, 0.1 K EGTA, 0.26 amphotericin B.

Drugs used

ATP, ADP, UTP, DMPP, Reactive Blue 2, and suramin were purchased from Sigma. PPADS (tetrasodium salt) was supplied by Tocris Cookson Ltd (Bristol).

RT-PCR

Total RNA was extracted from skeletal myoblasts using the SV Total RNA Isolation System (Promega). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham). Reverse transcription was performed using the Moloney murine leukemia virus reverse transcriptase. Primer sequences for $P2X_{1\text{-}7}$ (38) and $P2Y_{1,2,4,6}$ (39-41) were used for amplification reactions, as previously reported. The amplification reaction, performed in the same reaction tube, was conducted under the following conditions: 95°C for 30 s, the relevant annealing temperature for 30 s, and 72°C for 1 min, plus an additional cycle with an elongation time of 5 min. Amplification products were separated by electrophoresis and visualized by ethidium bromide staining. The presence of possible contaminants was investigated in all experiments, using control RT-PCR reactions in which either mRNA had been omitted or heating to 95°C had inactivated the reverse transcriptase.

RESULTS

Histology of *mdx* and control skeletal muscle

The tibialis anterior (TA) muscles of 3.5 wk, 5 wk, and 2-3 months old, *mdx* and age-matched control animals were studied. At all time points, muscle from control animals consisted of mature muscle fibers (Fig. 1*A*), characterized by peripheral my nuclei. Muscle fibers (in each age group) had similar diameters.

In contrast, degenerative and regenerative tissue changes were observed in skeletal muscle samples from *mdx* mice (Fig. 1*A*). As previously reported, muscle fiber degeneration and regeneration were initially episodic (42, 43). At birth, all the limb muscle fibers were relatively normal. However, by 3.5 wk of age histological evidence of muscle fiber degeneration in the TA muscle was obvious. Examination of sections by haematoxylin and eosin staining demonstrated a greatly reduced number of intact fibers and the heavy infiltration of phagocytic cells (Fig. 1*A*).

This acute phase of degeneration was followed by rapid regeneration, such that by 5 wk of age necrotic fibers had been replaced with small diameter myotubes (identified by their central nuclei) and the normal muscle architecture had been largely reestablished (Fig. 1*A*). In adult skeletal muscle samples, there was evidence of concurrent muscle fiber degeneration and regeneration. This resulted in a wide variation in muscle fiber diameter with newly formed, smaller diameter myotubes adjacent to larger, hypertrophied muscle fibers containing central nuclei (Fig. 1*A*).

Expression of P2 receptors in adult *mdx* and control skeletal muscle

Using specific antibodies against the $P2X_{1\text{-}7}$ receptor subunits and the $P2Y_{1,2,4}$ receptors we studied the expression of purinoceptor proteins in adult (2-3 month old) *mdx* and control skeletal

muscle. Control experiments, performed by preabsorbing the antibodies with the corresponding peptides, were used to confirm the specificity of the staining.

In normal, adult skeletal muscle, purinoceptor expression was restricted to vascular and nervous tissue but was absent from skeletal muscle cells. $P2X_1$ and $P2Y_1$ receptor proteins were detected in blood vessels, while the $P2X_5$ receptor was detected in nerve fibers (Fig. 1*B* and *C*). These findings were confirmed by double labeling experiments (Fig. 1*B* and *C*). Colocalization of $P2Y_1$ receptor protein (red) with smooth muscle actin (green) demonstrated expression of this receptor on vascular smooth muscle cells (Fig. 1*B*). Similarly, nerve bundles in both control and *mdx* muscle stained for $P2X_5$, as demonstrated by colocalization of $P2X_5$ receptor protein (red) and neurofilament (green) (Fig. 1*C*).

In contrast, adult *mdx* skeletal muscle was characterized by increased and widespread expression of three purinoceptor proteins, $P2Y_1$, $P2X_5$, and $P2X_2$ (Fig. 2*A*). Whereas $P2Y_1$ immunostaining was seen only in blood vessels in control samples, $P2Y_1$ immunopositive cells were present throughout adult *mdx* muscle. These small, mononuclear cells appeared to be particularly concentrated in areas of muscle necrosis (Fig. 2*A*). Similarly, immunoreactivity for the $P2X_5$ receptor was increased in *mdx*, as compared with adult skeletal muscle. $P2X_5$-positive mononuclear cells and muscle fibers were scattered throughout adult *mdx* skeletal muscle (Fig. 2*A*). A pronounced increase in $P2X_2$ expression was also observed in diseased as compared with control skeletal muscle. No immunoreactivity for $P2X_2$ was detected on any cell type in control muscle samples, but there were many $P2X_2$-immopositive myotubes and muscle fibers present in adult *mdx* skeletal muscle. Immunostaining was apparent as dark patches of immunoreactivity on the plasma membrane (Fig. 2*A*).

Specific purinoceptors are expressed in regeneration as opposed to degeneration of *mdx* muscle

The investigation of *mdx* and control skeletal muscle in 2- to 3-month-old mice demonstrated the increased and widespread expression of purinoceptors in *mdx* skeletal muscle, (as compared with control tissue). However, since degenerating and regenerating muscle fibers co-exist in adult *mdx* muscle, it was unclear whether P2 receptor expression was associated with the former and/or latter process. It is well recognized, both in human DMD and in the *mdx* mouse model, that muscle fiber degeneration and regeneration were initially episodic. We therefore investigated purinoceptor expression in the first cycle of damage and repair. Consistent with previous reports, the first degenerative phase in TA muscle was at 3.5 wk, while muscle regeneration was apparent at 5 wk of age (Fig. 1*A*) (42, 43).

With the use of immunohistochemistry, it was possible to demonstrate that expression of specific purinoceptors in *mdx* skeletal muscle was associated with specific time points, characterized by either degeneration or regeneration (Table 1). Whereas skeletal muscle from 3.5-wk-old *mdx* mice was strongly immunopositive for $P2Y_1$, there was no immunoreactivity for $P2X_2$ and little immunostaining for the $P2X_5$ receptor in this tissue (Fig. 2*B*). As in adult *mdx* tissue, $P2Y_1$ receptor immunostaining was expressed on small, mononuclear cells surrounding necrotic muscle fibers. This would suggest that while $P2Y_1$ receptor expression was associated with muscle degeneration, the $P2X_5$ and $P2X_2$ receptors were not involved in this process (Table 1).

In contrast, at 5 wk of age when muscle regeneration was the predominant process occurring in *mdx* TA skeletal muscle (similar changes occur at different times in other muscles), both the $P2X_5$ and $P2X_2$ receptors were strongly expressed (Fig. 2*C*, Table 1). As noted in adult *mdx* muscle, immunoreactivity for $P2X_5$ was present on mononuclear cells and myotubes, whereas immunoreactivity for $P2X_2$ was restricted to distinct patches on muscle fiber and myotube membranes.

$P2X_5$ receptor expression was quantified in control and *mdx* muscle from 3.5-wk, 5-wk, and 2- to 3-month-old mice. Receptor expression in each muscle sample was assessed by counting the number of nuclei present in $P2X_5$-positive cells and the total number of nuclei in five randomly chosen fields from a minimum of two muscle sections. To correct for differences in nuclei number due predominantly to the age of the mice or the heavy infiltration of immune cells on muscle damage, the number of nuclei present in $P2X_5$-positive cells was expressed as a percentage of the total number of nuclei. With the use of this method of quantifying $P2X_5$ receptor expression, we demonstrated that in no sample did the number of nuclei present in $P2X_5$-positive cells exceed 6% (Fig. 3*A*). However, there was a significant ($P<0.1\%$) increase in $P2X_5$ immunoreactivity in *mdx*, as compared with control skeletal muscle in 5-wk-old and adult mice. At 5 wk, 5.63% ± 0.34% of nuclei were present in $P2X_5$-positive cells in *mdx* muscle, while only 0.47% ± 0.19% of nuclei were present in $P2X_5$-positive cells in control tissue (Fig. 3*A*). There was no significant difference in immunoreactivity for $P2X_5$ in *mdx* skeletal muscle from 5-wk-old, as compared with adult skeletal muscle (Fig. 3*A*).

A subpopulation of activated satellite cells in *mdx* muscle express P2 receptors

Sequential sections of 2- to 3-month-old *mdx* muscles were stained for myogenin, MyoD, $P2X_5$, and $P2Y_1$. These experiments revealed that many $P2X_5$-positive mononuclear cells expressed the myogenic regulatory factors myogenin and MyoD, indicating that these cells were activated satellite cells (Fig. 3*B*, arrows). Furthermore, in some cases $P2X_5$-positive myotubes contained nuclei positive for myogenin, suggesting that these fibers had been recently formed or repaired by satellite cell fusion. However, it was clear that there were also a number of cells that expressed myogenin, MyoD, or $P2X_5$ alone. Thus, it appeared that only a subpopulation of activated satellite cells was positive for $P2X_5$ and that some $P2X_5$-positive cells were not activated satellite cells.

Immunostaining for $P2Y_1$ was present in regions of regenerating muscle (as identified by MyoD or myogenin expression), and it was possible to identify a number of $P2Y_1$-positive cells as activated satellite cells (Fig. 3*B*, arrowheads), though there were fewer of these than $P2X_5$-positive satellite cells. This suggests that, as in the case of $P2X_5$, only a subpopulation of activated satellite cells expresses $P2Y_1$ and that the $P2Y_1$ receptor is expressed by a variety of cell types.

$P2Y_1$ receptor was expressed on inflammatory cells in *mdx* muscle

To determine the identity of $P2Y_1$-positive cells, adult *mdx* muscle was double labeled for $P2Y_1$ (red) and CD11b (green; Fig. 4*A*). CD11b is a marker for a large variety of inflammatory cells, including macrophages and B-lymphocytes. Significant colocalization of $P2Y_1$ and CD11b (yellow) was detected (Fig. 4*A*). However, the fact that some CD11b cells were not

immunopositive for $P2Y_1$ indicated that this receptor was not expressed by all infiltrating inflammatory cells.

Double labeling for $P2X_2$ and AChRs

A number of studies demonstrate abnormalities in the localization and degradation of AChRs on *mdx* muscle from 5 wk of age onwards (43–45). Furthermore, there is growing evidence that $P2X_2$ receptors and AChRs can functionally interact (46-50). Thus, the expression of AChRs and $P2X_2$ receptors was investigated on adult skeletal muscle from control and *mdx* mice (Fig. 4*B*). Double staining experiments performed on muscle from control mice demonstrated largely continuous and well-organized clusters of AChRs at the neuromuscular junctions (NMJs) and the absence of any staining for $P2X_2$ (Fig. 4*B*). In contrast, NMJs in adult *mdx* muscle consisted of numerous clusters of AChRs. Furthermore, $P2X_2$ receptor expression was apparent on muscle fibers, and there were small areas of AChR and $P2X_2$ receptor colocalization (yellow; Fig. 4*B*).

Myoblasts in primary culture express P2 receptors

Cells extracted from neonatal rat skeletal muscle were plated at low density and maintained in differentiation medium for 24 h. As demonstrated by staining for the myogenic transcription factor MyoD, 93.6 ± 2.5% of cells were myoblasts (Fig. 5*A*). These cells were also capable of fusing to form multinuclear myotubes that were positive for muscle-specific proteins, such as skeletal myosin heavy chain (Fig. 5*A*).

Using specific antibodies for $P2X_{1\text{-}7}$ and $P2Y_1$, $P2Y_2$, and $P2Y_4$, we investigated purinoceptor expression on myoblast cultures. Control experiments, performed by preabsorbing the antibodies with the corresponding peptides, were used to confirm the specificity of our findings. We found immunoreactivity for only two of the P2 receptors, $P2X_5$ and $P2Y_1$, on mononuclear cells in these cultures. Using RT-PCR, we were able to confirm the expression of both $P2X_5$ and $P2Y_1$ receptor mRNA in myoblasts (Fig. 5*B*, data for the P2X receptors not shown). RT-PCR experiments also demonstrated the expression of $P2Y_2$, $P2X_4$, $P2X_6$, and $P2X_2$ receptor mRNA.

Patch clamp recordings on myoblasts in primary culture

To investigate the functional properties of the P2 receptors present on myoblasts, we carried out whole cell patch clamp recording. Recordings were only made from small fusiform mononuclear cells. When the recording pipette contained a Cs^+-based solution, rapid application of 10 μM ATP evoked a small, inward current of 9.7 ± 1.6 pA in 80% (17/21) of cells (Fig. 5*C*). In contrast, the nicotinic AChR agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), failed to produce an inward current in 80% (8/10) of cells tested. Responses to ATP reflected a current density of 0.87 ± 0.1 pA/pF and activated and inactivated quickly (Fig. 5*C*). The inward current activated by ATP was inwardly rectifying with a reversal potential close to 0 mV indicative of a nonselective cation conductance. UTP and ADP, which are potent agonists at some types of P2Y receptors failed to evoke any significant response under these recording conditions even at a concentration of 100 μM (Fig. 5*D*). Using perforated patch recording with a K^+-based pipette solution, 4/4 cells responded to ATP with an outward current, which activated with a delay of several seconds (Fig. 5*E*). This would suggest the involvement of a metabotropic receptor and activation of Ca^{2+}-activated potassium channels.

P2 receptor expression and regulation on myotubes

Myoblast cultures, maintained in DM to encourage myotube formation, were investigated 1, 3, 5, and 7 days after plating. Day 1 cultures contained mononucleated myoblasts, as confirmed by immunostaining for MyoD (Fig. 5*A*). However, by day 3 some myoblasts had aligned and fused to form multinuclear myotubes, as confirmed by the expression of skeletal myosin and the presence of three or more nuclei (Fig. 5*A*). This process continued such that by day 5 and 7, large, spontaneously contractile myotubes had formed. Staining procedures were carried out for all seven of the P2X receptors and the $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors at all four time points. We found immunoreactivity only for the $P2X_2$, $P2X_5$, $P2Y_1$, and $P2Y_4$ receptors (Fig. 6*A*). These receptors were expressed sequentially, $P2X_5$ and $P2Y_1$ being expressed first, followed by $P2Y_4$ and $P2X_2$ (Fig. 6*A*; Table 2).

As discussed above, skeletal myoblasts (maintained in DM) stained strongly for the $P2X_5$ and $P2Y_1$ receptors (Fig. 6*A*). Similarly, early myotubes, present on day 3 cultures, expressed the $P2X_5$ and $P2Y_1$ receptors only (Fig. 6*A*; Table 2). However, with increasing time in culture, expression of these receptors on both mononucleated myoblasts and myotubes was greatly reduced. In the case of $P2Y_1$, immunoreactivity on both unfused cells and myotubes had disappeared by day 5 (Fig. 6*A*; Table 2). Similarly in the case of $P2X_5$, there was no immunostaining on unfused cells, and staining of myotubes was restricted to the ends of the cells (Fig. 6*A*). Since myotubes formed in vitro may branch to form multi- as opposed to bipolar cells, it was noted that the tips of all poles of the myotubes stained for $P2X_5$. By day 7, no staining for $P2X_5$ was detectable on any cell type (Fig. 6*A*).

Unlike $P2X_5$ and $P2Y_1$, immunoreactivity for the $P2X_2$ and $P2Y_4$ receptors could only be detected on myotubes (Fig. 6*A*; Table 2) but not on myoblasts. However, whereas immunoreactivity for $P2Y_4$ could be detected on myotubes at all time points (from day 3 until day 7) (Fig. 6*A*; Table 2), $P2X_2$ receptor expression could only be detected from day 5 (Fig. 6*A*; Table 2). Furthermore, unlike $P2Y_4$ receptor expression, immunostaining for the $P2X_2$ receptor was not homogenous (Fig. 6*A*). Although $P2X_2$ was expressed along the whole length of the myotubes, there were clusters or areas of membrane with particularly high receptor staining. This pattern of staining was particularly apparent on the myotubes of day 7 cultures (Fig. 6*A*).

In summary, we were able to demonstrate the sequential expression of four purinoceptors during myotube formation, $P2Y_1$, $P2X_5$, $P2Y_4$, and $P2X_2$ (Table 2). Whereas the $P2Y_1$ and $P2X_5$ were expressed on both mononucleated myoblasts and myotubes, the $P2Y_4$ and $P2X_2$ receptors were only expressed on myotubes (Table 2).

Coexpression of $P2X_2$ and AChRs on myotubes

To determine whether the pattern of $P2X_2$ immunostaining was related to the well-documented clustering of AChRs on myotubes of aneural cultures we double-stained day 5 and 7 cultures for $P2X_2$ receptors and AChRs (Fig. 6*B*). As previously reported, Texas Red-labeled α-bungarotoxin stained clusters of AChRs scattered across the myotube membrane (Fig. 6*B*). Using confocal microscopy to determine the precise location of receptor staining, we were able to demonstrate colocalization of $P2X_2$ (green) and AChRs (red; Fig. 6*B*). Although there were clearly areas of

myotube membrane with significant colocalization (yellow), the same myotube also stained in other areas for $P2X_2$ alone or AChRs alone (Fig. 6*B*).

Patch clamp recordings on myotubes

To avoid complications arising from poor space clamp, recordings were only made from small linear myotubes (<300 μm in length). When recordings were made with a Cs^+-based pipette solution, these cells all responded to ATP with a small, rapidly activating inward current (Fig. 7*A* and *B*). This response was much smaller than that produced by the nicotinic AChR agonist DMPP (Fig. 7*A*). The effect of ATP was concentration-dependent, with an EC_{50} of 2.3 μM (Fig. 7*C*) and could not be replicated by application of either UTP or ADP (at concentrations of 30 μM; Fig. 7*B*). In agreement with the findings of Hume and Hönig (8, we were unable to observe any ATP-activated single channel currents in outside out patches excised from these myotubes. We investigated the pharmacology of the ATP-activated inward current in myotubes. Both suramin and Reactive Blue 2 produced a concentration-dependent, reversible antagonism of the ATP response with IC_{50} values of 3 and 0.97 μM, respectively (Fig. 7*D* and *E*). The most potent antagonist tested was pyridoxal 5-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; Fig. 7*F*). This compound, at a concentration of 0.1μM, produced > 95% inhibition. However, the antagonism developed slowly over the course of 4 min and washed out very slowly, so that after 15 min washing, the response had recovered to <50% of the control value (Fig. 7*F*). As with the satellite cells, recording with a K^+-based solution revealed a slowly activating outward current (Fig. 7*G*), which would suggest the involvement of a metabotropic receptor and activation of Ca^{2+}-activated potassium channels.

DISCUSSSION

In this paper, we have shown for the first time that purinoceptor expression, in particular expression of the $P2X_5$ and $P2X_2$ receptors, is a key feature of muscle regeneration. In normal skeletal muscle, purinoceptor expression was absent from skeletal muscle cells, though present in vascular and nervous tissue. However, three purinoceptor proteins were strongly expressed in adult *mdx* muscle, namely $P2X_2$, $P2X_5$, and $P2Y_1$. To determine more precisely the role of these receptors in the response to muscle injury, we investigated the expression and function of the purinoceptors at different time points in the *mdx* mouse lifetime and in primary postnatal rat myoblast cultures. With the use of RT-PCR, immunohistochemistry, and electrophysiology, these experiments revealed the sequential expression of the $P2X_5$, $P2Y_1$, and $P2X_2$ receptors during the process of muscle regeneration. The $P2X_5$ and $P2Y_1$ receptors were expressed first on activated satellite cells and in the case of $P2Y_1$, a range of immune cells. This was followed by the expression of the $P2X_2$ receptor on newly formed myotubes both in vivo and in vitro. Thus these findings strongly suggest a role for purinergic signaling in the process of skeletal muscle regeneration.

The cause of muscle injury in *mdx* mice, as in DMD patients, appears to be the absence of the subsarcolemmal cytoskeletal protein dystrophin (35, 30). In the *mdx* mouse, the limb muscle fibers are histologically normal postnatally (51) but undergo degeneration soon after weaning (at 3 wk in this study) when motor activity increases. This first phase of muscle damage and the subsequent inflammatory response were characterized by a marked increase in immunoreactivity for the $P2Y_1$ receptor. Double-staining experiments demonstrated that $P2Y_1$ receptor protein was

strongly expressed on leukocytes (as identified by immunoreactivity for CD11b) and leukocyte infiltration could largely account for the increase in $P2Y_1$ receptor expression in damaged muscle. This finding is consistent with past research demonstrating $P2Y_1$ receptor expression on a range of white blood cells (including monocytes, dendritic cells, and thymocytes) and a role for this receptor in the regulation of cytokine release (52-54). Since some cytokines (including IL-6 and LIF) can act as growth factors in damaged skeletal muscle, it is possible that $P2Y_1$ receptor expression may be involved in regulating leukocyte activity and indirectly muscle repair (55. 56).

The regeneration of muscle fibers in *mdx* muscle ensures that muscle function rapidly recovers (42). This process depends on the presence of skeletal muscle satellite cells. On muscle injury, satellite cells are activated, proliferate, and ultimately fuse to repair damaged fibers and form new myotubes. Past research on *mdx* muscle suggests that, while these regenerative processes continue in adult mice, they are most prominent at ~4-5 wk of age, when activated satellite cells are present in the greatest numbers (42, 43, 57). Thus, the increase in $P2X_5$ receptor expression in *mdx* skeletal muscle from 5 wk of age onwards strongly suggests a role for this receptor in muscle regeneration. Immunostaining demonstrated a >10-fold increase in the expression of the $P2X_5$ receptor in *mdx* muscle at 5 wk, as compared with age-matched control tissue and *mdx* muscle at 3.5 wk of age. Although a similar increase in $P2X_5$ receptor expression was also demonstrated in adult *mdx* muscle when regeneration is less obvious, this may be due to a slight overestimate of receptor expression in this tissue. Although correcting for differences in the number of nuclei present in *mdx* and control skeletal muscle was an effective means of accounting for the infiltration of immune cells into *mdx* tissue, it was not an effective means of accounting for differences in muscle morphology when comparing *mdx* tissue from animals of different ages.

Immunolabeling of sequential muscle sections demonstrated that the marked increase in immunoreactivity was due to the expression of the $P2X_5$ receptor on a subpopulation of activated satellite cells (as identified by the expression of the myogenic transcription factors MyoD or myogenin). A number of activated satellite cells were also positive for $P2Y_1$ (though fewer than for $P2X_5$). The absence of significant immunoreactivity for either of these receptors in mononucleated cells in control muscle samples suggested that purinoceptors were not expressed by quiescent satellite cells but only activated satellite cells.

In keeping with the observations in vivo, we found that postnatal mononucleated myoblasts in primary culture expressed both $P2X_5$ and $P2Y_1$ receptor mRNA and protein. Expression of these receptor proteins by satellite cells could account for the P2X- and P2Y-like responses detected by patch clamp recording on addition of ATP. Furthermore, since the cultures investigated were derived from normal rat skeletal muscle, this study strongly suggested that purinoceptor expression in *mdx* skeletal muscle was not directly due to a deficiency in the dystrophin protein, but occurred secondary to muscle regeneration (irrespective of the initial cause of injury). Thus, it seems likely that ATP is one of a number of extracellular signaling molecules, including mechano growth factor, fibroblast growth factors, and insulin-like growth factors, which regulate satellite cell activity (3, 58, 59).

However, there were some differences between myoblasts in culture and in vivo. It should be recognized that whereas all cells in culture expressed P2 receptors, $P2X_5$ and $P2Y_1$ receptor

proteins were expressed by only a subpopulation of myoblasts in *mdx* muscle. This discrepancy could be explained by the fact that high levels of receptor expression may be specific to particular cell states (for example proliferation or differentiation). Thus, in vivo, where satellite cells have not been activated simultaneously or maintained under identical conditions, differential expression of the $P2X_5$ and $P2Y_1$ receptors might be expected.

Recent research demonstrating the role of the $P2X_5$ receptor in muscle formation (22) strongly suggests that the expression of this receptor on activated satellite cells in *mdx* muscle was of functional significance. Activation of the $P2X_5$ receptor on rat skeletal satellite cells in primary culture has been shown to increase the rate of commitment to terminal differentiation and fusion to form multinuclear myotubes (22). Consistent with such a function for the $P2X_5$ receptor, it was observed that newly formed myotubes in culture and small myotubes in vivo (presumably recently regenerated) express this receptor.

Although the functional significance of $P2X_2$ receptor expression in skeletal muscle is not established, the timing of receptor expression in *mdx* muscle may indicate a possible role in the regulation of muscle reinnervation after damage. The absence of dystrophin in *mdx* muscle not only affects muscle architecture but also disrupts the relationship between motor nerve and muscle fiber (43). Abnormalities in the structure and function of neuromuscular junctions include NMJ fragmentation and changes in AChR function and the expression of embryonic-type AChRs. These appear after the first postnatal month and after the onset of muscle damage and repair and become increasingly obvious with age (44, 45). Similarly, expression of the $P2X_2$ receptor in *mdx* muscle began only at 5 wk of age and immunoreactivity for this receptor was strongest in adult *mdx* muscle, when $P2X_2$-immunopositive clusters were detected across the cell membranes of myotubes and muscle fibers. $P2X_2$ receptor expression was also observed on aneural myotubes in vitro, confirming that receptor expression in *mdx* muscle was indeed a feature of muscle regeneration and not degeneration. Furthermore, patch clamp recordings conducted on myotubes in vitro demonstrated responses characteristic of P2X receptors and consistent with the expression of homomeric $P2X_2$ receptors (ATP responses were sensitive to the antagonists, suramin, Reactive Blue 2, and PPADS). Although it is possible that heteromeric $P2X_2/P2X_5$ receptors were present (the $P2X_5$ receptor protein being transiently expressed on myotubes and capable of coassembly with $P2X_2$ subunits), the different distribution patterns and timing of $P2X_2$ and $P2X_5$ receptor subunits makes this unlikely.

Recent studies demonstrating that $P2X_2$ and ACh receptors can interact may suggest a specific role for the $P2X_2$ receptor in the regulation of AChR function and possibly distribution under conditions of abnormal innervation. Coactivation of $P2X_2$ and nicotinic AChRs elicits currents significantly smaller than those predicted on the basis of activation of each channel separately (46–50). Thus, coexpression of $P2X_2$ and AChRs could account for some of the abnormalities in AChR activity observed in *mdx* muscle. In fact, our double-staining experiments demonstrated colocalization of $P2X_2$ and ACh receptors both in *mdx* muscle and in myotube cultures. As previously reported, AChRs were expressed in clusters on the myotube membrane in aneural cultures, and in many cases receptor clusters contained not only acetylcholine but also $P2X_2$ receptors. Similarly, in adult *mdx* muscle, $P2X_2$ receptor protein colocalized with AChRs at small receptor clusters at the NMJ. Since ATP is coreleased with ACh at the NMJ, coactivation of the nicotinic and $P2X_2$ receptors could occur and cause previously observed reductions in the opening time and current amplitude of AChRs in dystrophic muscle (60, 37).

It is important to note that, contrary to the findings presented by Choi et al. (17), coexpression of $P2Y_1$ and ACh receptors at the NMJs could not be demonstrated in either normal or *mdx* muscle at any time point. However, immunocytochemistry and patch clamp recording in vitro did demonstrate the expression of P2Y receptors on myotubes in vitro. Both $P2Y_1$ and $P2Y_4$ receptor protein were detected on myotubes in culture, though not on muscle fibers in *mdx* skeletal muscle. The presence of these receptors in culture is consistent with the findings of Henning et al. (12–14), showing that application of UTP and ADP to C2C12 myotubes can cause an increase in inositol-1,4,5-trisphophate levels and cAMP respectively. Thus, the differences in the expression of P2Y receptors in vivo and in vitro may be, at least in part, related to the absence of dystrophin in *mdx* mice.

In summary, the age-dependent pattern of P2 receptor expression in *mdx* muscle strongly suggests that purinergic signaling plays a key role in the response to muscle injury. The $P2X_2$, $P2X_5$, and $P2Y_1$ receptors were strongly expressed in *mdx* skeletal muscle (as compared with controls), and these receptors were expressed on cells known to be important in muscle regeneration. In particular, the $P2X_5$ and $P2Y_1$ receptors were expressed on activated satellite cells. P2 receptors were also expressed on myotubes/ muscle fibers ($P2X_2$) and on infiltrating immune cells ($P2Y_1$). Since *mdx* muscle is likely to contain high levels of extracellular ATP due to muscle damage, and it is known that ATP and other extracellular nucleotides can affect satellite cell activity, it seems likely that P2 receptor expression in *mdx* muscle is of functional significance in muscle regeneration. Furthermore, strong similarities in the specific subtypes and pattern of P2 receptor expression in regenerating muscle in vivo and in vitro suggest that P2 receptors are part of the normal response to muscle injury. Thus, this study provides the first evidence for a role for purinergic signaling in muscle regeneration in vivo and indicates that purinergic receptors may be therapeutic targets for the treatment of muscle diseases.

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Table 1

Summary of purinoceptor expression in *mdx* skeletal muscle at 3.5 wk, 5 wk and 2-3 months of age.

	3.5 wk predominantly muscle degeneration	5 wk predominantly muscle regeneration	2-3 months muscle degeneration and regeneration
$P2Y_1$	+++	+	++
$P2X_5$	-	+++	+++
$P2X_2$	-	++	+++

In normal skeletal muscle (at all ages), purinoceptor expression was absent from skeletal muscle cells, though present in vascular and nervous tissue. However, skeletal muscle from 3.5-wk-old *mdx* mice, when the predominant process was muscle degeneration, showed strong immunoreactivity for $P2Y_1$. There was no immunoreactivity for $P2X_2$ and little immunostaining for the $P2X_5$ receptor in this tissue. In contrast, at 5 wk of age when muscle regeneration was the predominant process occurring in *mdx* TA skeletal muscle, both $P2X_5$ and $P2X_2$ receptors were strongly expressed. Adult *mdx* skeletal muscle, which is characterized by areas of both muscle degeneration and regeneration, expressed $P2X_2$, $P2X_5$, and $P2Y_1$ receptors. These findings suggest that whereas $P2Y_1$ receptor expression was associated with muscle degeneration, expression of the $P2X_5$ and $P2X_2$ receptors was associated with muscle regeneration; - = no immunoreactivity, + = weak immunoreactivity, ++ = strong immunoreactivity, +++ = very strong immunoreactivity.

Table 2

Summary of purinoceptor expression in cultures of normal rat myoblasts

		DAY 1	DAY 3	DAY 5	DAY 7
MYOBLASTS	$P2Y_1$	+	+	-	-
	$P2X_5$	+	-	-	-
	$P2Y_4$	-	-	-	-
	$P2X_2$	-	-	-	-
MYOTUBES	$P2Y_1$		+	-	-
	$P2X_5$		+	+	-
	$P2Y_4$		+	+	+
	$P2X_2$		-	+	+

P2 receptors are expressed sequentially: $P2Y_1$ and $P2X_5$ were expressed first, followed by $P2Y_4$ and $P2X_2$. Activated satellite cells expressed only the $P2Y_1$ and $P2X_5$ receptors. These receptors were also transiently expressed on myotubes. However, by day 5 there was no expression of $P2Y_1$ on any cell type and $P2X_5$ receptor expression was restricted to the tips of myotubes. On day 7, myotubes expressed only $P2Y_4$ and $P2X_2$. Note that there were no myotubes present in cultures on day 1; - = no immunoreactivity, + = strong immunoreactivity.

Fig. 1

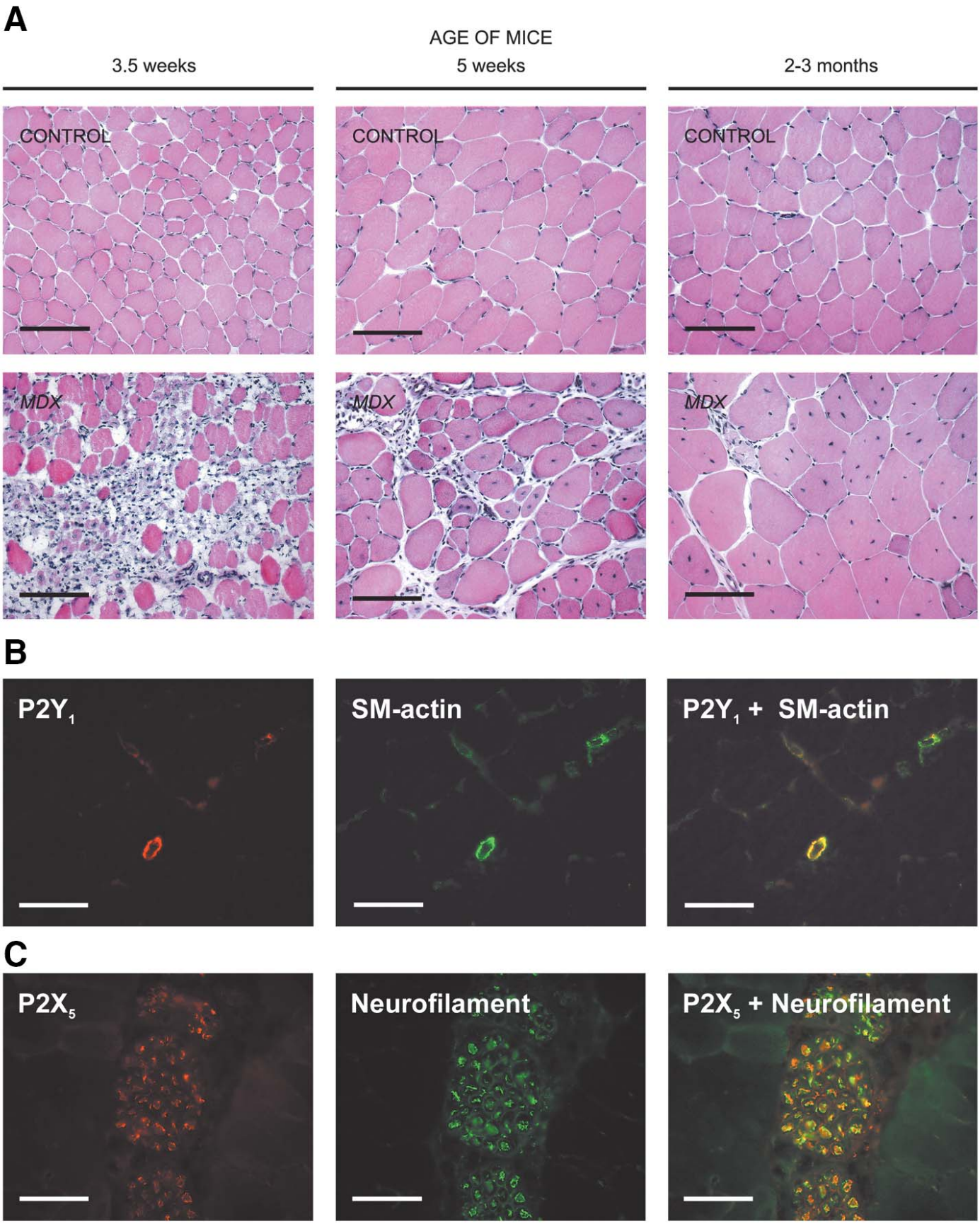
A
AGE OF MICE
3.5 weeks
5 weeks
2-3 months
CONTROL
CONTROL
CONTROL
MDX
MDX
MDX
B
P2Y1
SM-actin
P2Y1 + SM-actin
C
P2X5
Neurofilament
P2X5 + Neurofilament

Figure 1. ***A*****)** Muscle structure in control and *mdx* mice. Haematoxylin and eosin staining of tibialis anterior (TA) muscles from control mice at 3.5 wk, 5 wk, and 2-3 months of age demonstrated the presence of mature muscle fibers characterized by peripheral my nuclei. In contrast, TA muscles from *mdx* mice demonstrated abnormalities in muscle structure at all time points. At 3.5 wk of age, there were many degenerative changes characterized by necrotic muscle fibers and the presence of many immune cells. At 5 wk of age, more normal muscle architecture had been restored, but there were many small diameter fibers and myotubes with central nuclei and small areas of inflammation. Adult *mdx* muscle showed signs of both muscle degeneration and regeneration. ***B*****)** Immunocolocalization of $P2Y_1$ receptors and smooth muscle actin in blood vessels. Sections of TA muscle from 2- to 3-month-old control mice were stained for $P2Y_1$ (red) and smooth muscle actin (green). Double staining for both $P2Y_1$ and smooth muscle actin demonstrated complete colocalization of these proteins (yellow). ***C*****)** Colocalization of $P2X_5$ and neurofilament in nerve bundles. Sections of TA muscle from 2- to 3-month-old control mice were stained for $P2X_5$ (red) and neurofilament 200 (green). Double-staining for both these proteins demonstrated significant colocalization (yellow). Bars = 100 μm.

Fig. 2

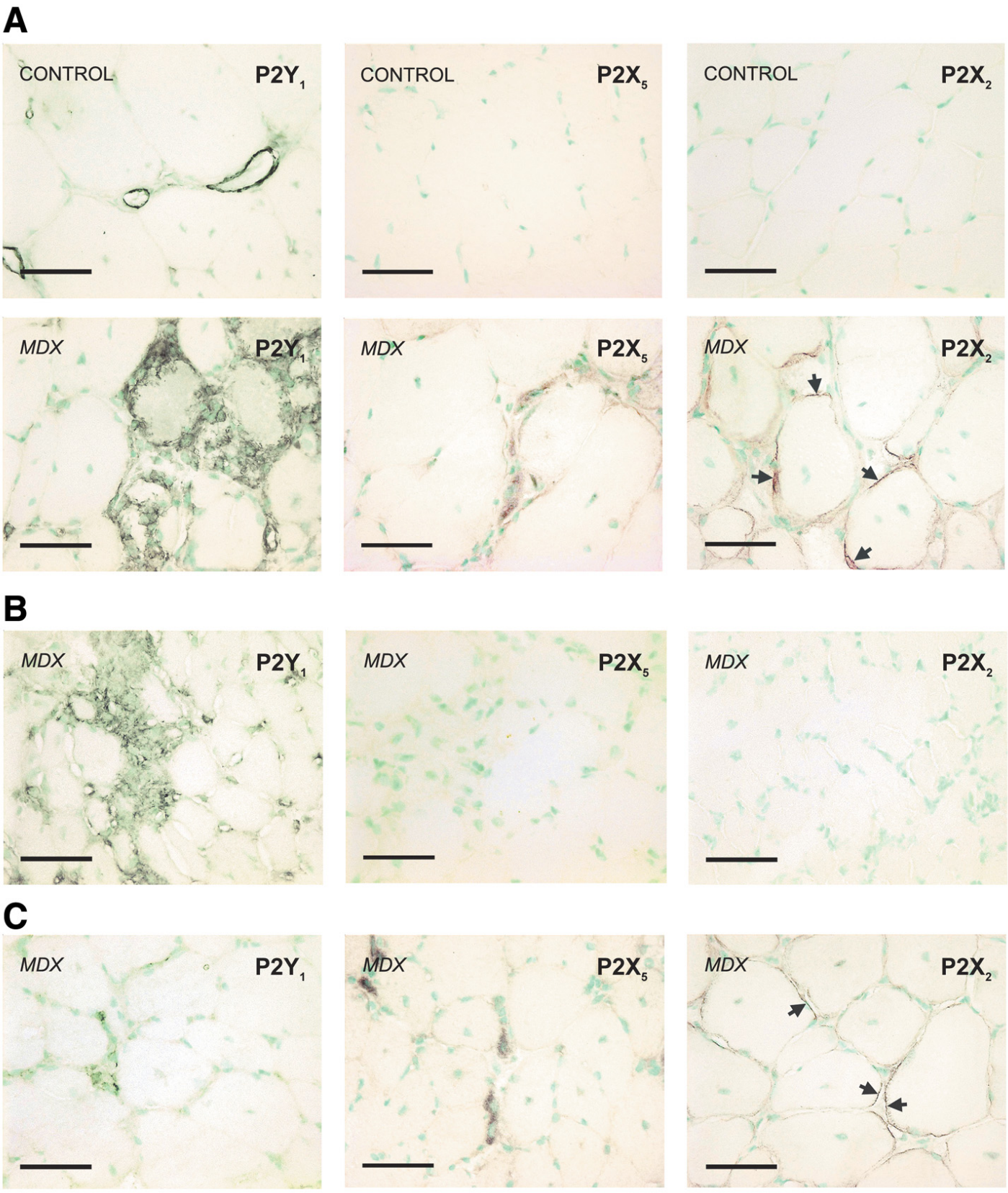
A
CONTROL
P2Y1
CONTROL
P2X5
CONTROL
P2X2
MDX
P2Y1
MDX
P2X5
MDX
P2X2
B
MDX
P2Y1
MDX
P2X5
MDX
P2X2
C
MDX
P2Y1
MDX
P2X5
MDX
P2X2

Figure 2. ***A*****)** Changes in the expression of the $P2Y_1$, $P2X_5$, and $P2X_2$ receptors in adult *mdx* muscle. Sections of TA muscle from 2- to 3-month-old C57Bl/10 (control) and *mdx* mice were stained for $P2Y_1$, $P2X_5$, and $P2X_2$ (black) and counterstained for nuclei (green). Immunoreactivity for $P2Y_1$ was only observed in blood vessels in control muscle samples; many immunopositive mononucleated cells were present in adult *mdx* muscle. Similarly, while immunoreactivity for $P2X_5$ or $P2X_2$ was absent in muscle samples from adult control mice, $P2X_5$-immunopositive cells were scattered throughout *mdx* muscle, and strong immunoreactivity for $P2X_2$ was observed on myotube and muscle fiber membranes (arrows). ***B*****)** Sections of TA muscle from 3.5-wk-old *mdx* mice stained for $P2Y_1$, $P2X_5$, and $P2X_2$ receptors (black) and counterstained for nuclei (green). $P2Y_1$ receptor was strongly expressed during the first phase of *mdx* muscle degeneration. Neither the $P2X_2$ nor $P2X_5$ receptors were expressed at significant levels at this time. ***C*****)** Sections of TA muscle from 5-wk-old *mdx* mice stained for $P2Y_1$, $P2X_5$ and $P2X_2$ receptors (black) and counterstained for nuclei (green). Immunoreactivity for the $P2X_5$ and $P2X_2$ receptors was present at this age in *mdx* muscle, when the predominant process occurring is regeneration. Although $P2Y_1$ receptor was also expressed at this time, immunoreactivity for this receptor appeared to be less widespread compared with that in adult *mdx* muscle. Bars = 100 μm.

Fig. 3

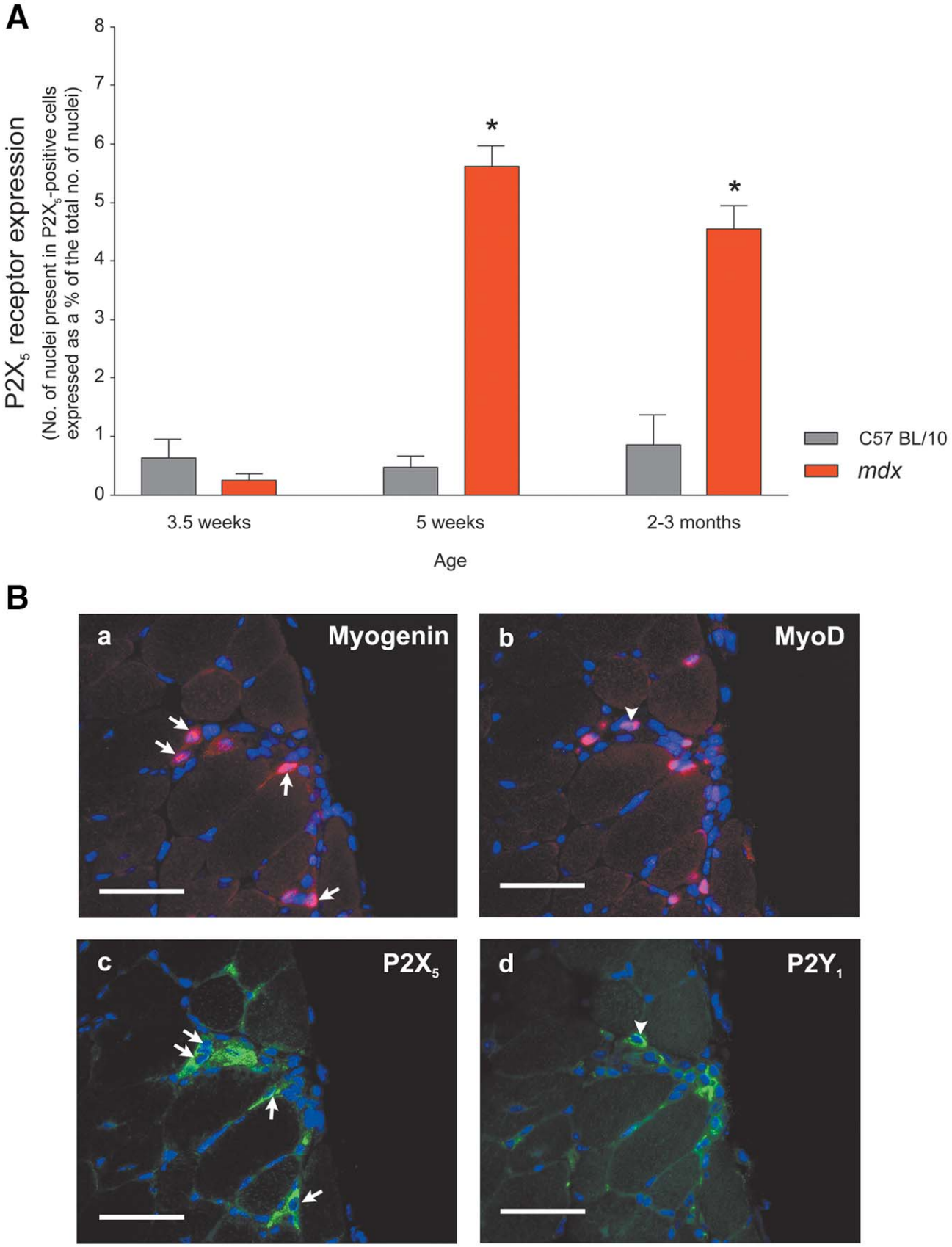
A
P2X5 receptor expression
(No. of nuclei present in P2X5-positive cells expressed as a % of the total no. of nuclei)
0
1
2
3
4
5
6
7
8
*
*
3.5 weeks
5 weeks
2-3 months
Age
C57 BL/10
mdx
B
a
Myogenin
b
MyoD
c
P2X5
d
P2Y1

Figure 3. ***A*)** Quantitative analysis of $P2X_5$ receptor immunoreactivity in control and *mdx* mice. Histogram shows number of nuclei in $P2X_5$-positive cells expressed as a percentage of total number of nuclei. At 5 wk and 2-3 months, there was a significant increase ($P<0.01$) in number of $P2X_5$-positive cells compared with control animals. *Significantly ($P<0.01$) different from age-matched controls. ***B*)** A subpopulation of activated satellite cells in *mdx* muscle at 2-3 months of age express P2 receptors. ***a*)** Immunostaining of *mdx* muscle for the myogenic regulatory factor (MRF), myogenin (red), and the nuclear stain DAPI (blue) confirmed the presence of many activated satellite cells in this tissue. ***b*)** Similarly immunostaining of a sequential muscle section for MRF, MyoD (red), and the nuclear stain DAPI (blue) also demonstrated the presence of many activated satellite cells. ***c*)** Immunostaining of a sequential section of muscle for $P2X_5$ (green) and the nuclear stain DAPI (blue) demonstrated that many of the activated satellite cells present in *mdx* muscle were positive for $P2X_5$ receptor protein (arrows in ***a, b,*** and ***c***). ***d*)** Immunostaining of a sequential section of muscle for $P2Y_1$ (green) and the nuclear stain DAPI (blue) demonstrated that a number of the activated satellite cells present in *mdx* muscle were positive for $P2Y_1$ receptor protein (arrowheads ***b*** and ***d***). Bars = 100 μm.

Fig. 4

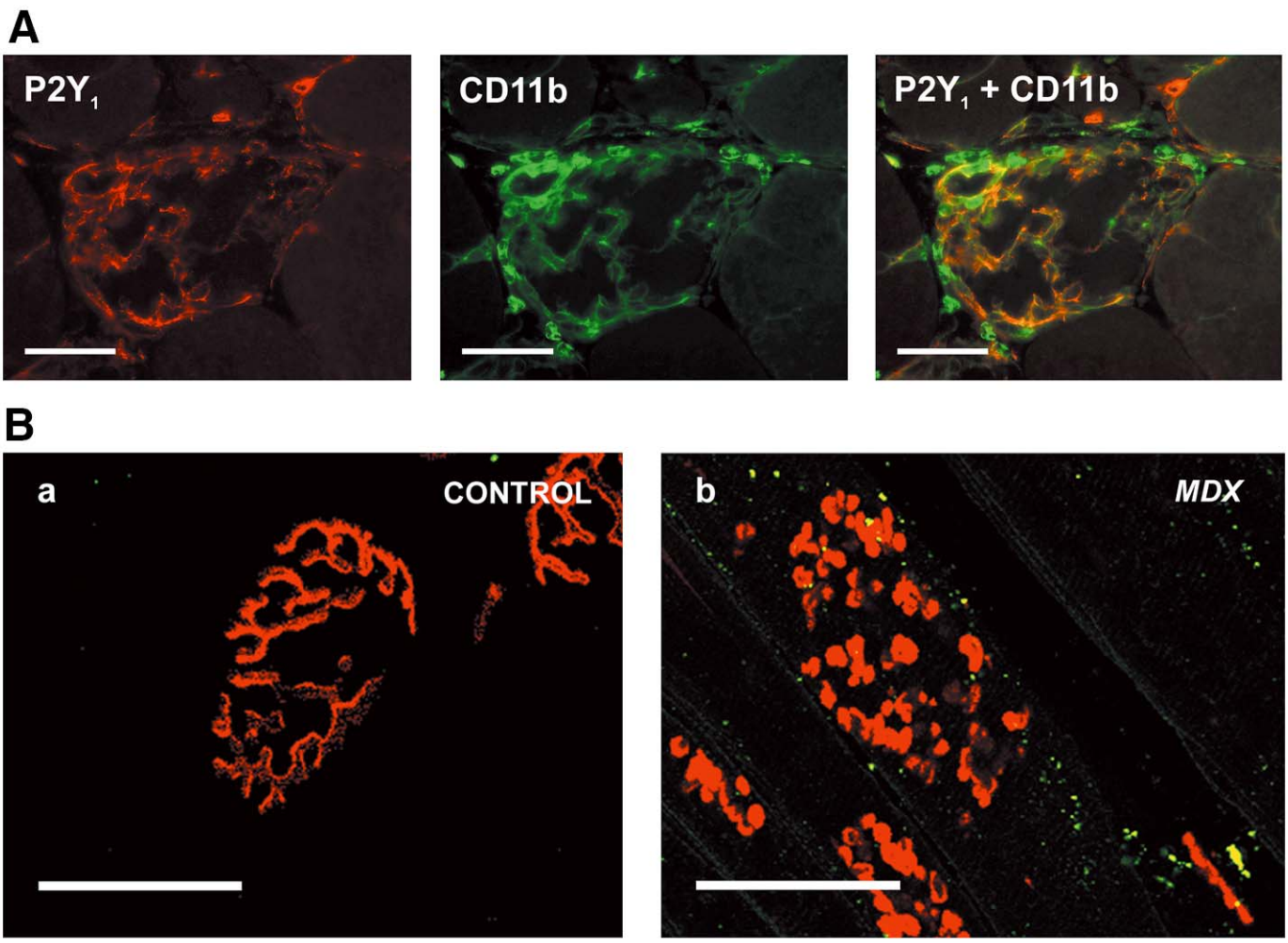

Figure 4. ***A*****)** Subpopulations of inflammatory cells in *mdx* muscle express the $P2Y_1$ receptor. Immunostaining of adult *mdx* muscle for $P2Y_1$ (red) and CD11b (green) (a marker for a variety of immune cells) revealed that there was a significant degree of colocalization of $P2Y_1$ and CD11b (yellow) in adult *mdx* muscle. However, since some cells expressed only CD11b (green) or only $P2Y_1$ (red), it is clear that the $P2Y_1$ receptor was expressed by a subpopulation of inflammatory cells and that not all $P2Y_1$-positive cells were immune cells. ***B*****)** Representative neuromuscular junctions from control and *mdx* skeletal muscle. Longitudinal sections of adult skeletal muscle from control and *mdx* mice were double stained for ACh receptors (red) and $P2X_2$ receptors (green). ***a*****)** Immunostaining of muscle from control mice demonstrated largely continuous and well-organized clusters of AChRs at the NMJs and the absence of any staining for $P2X_2$. ***b*****)** In contrast, NMJs in adult *mdx* muscle consisted of multiple clusters of AChRs and were generally larger. Furthermore, $P2X_2$ receptor expression was apparent on muscle fibers, and there were numerous small areas of AChR and $P2X_2$ receptor colocalization (yellow). Bars = 100 μm.

Fig. 5

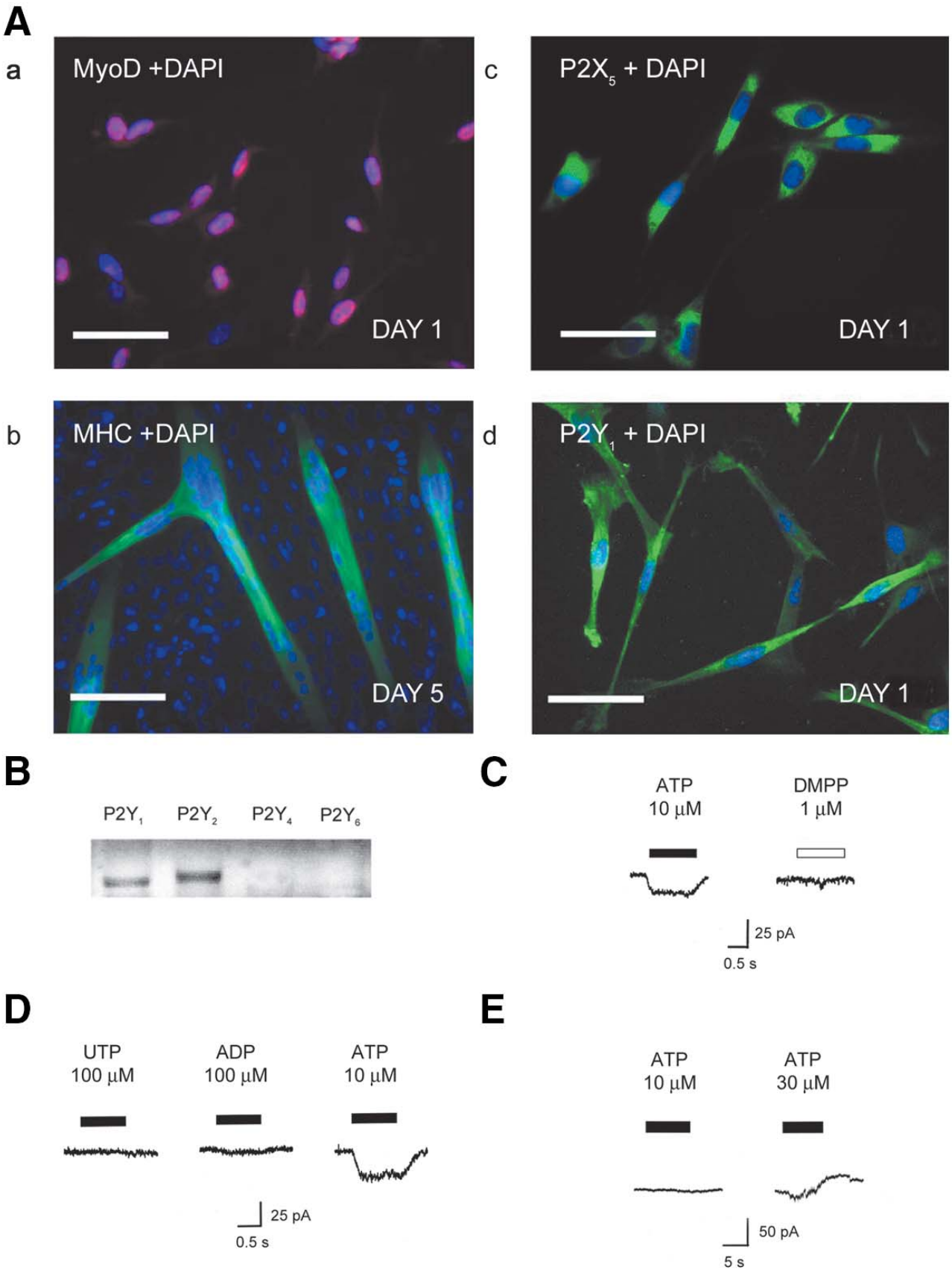
A
a
MyoD +DAPI
DAY 1
c
P2X5 + DAPI
DAY 1
b
MHC +DAPI
DAY 5
d
P2Y1 + DAPI
DAY 1
B
P2Y1
P2Y2
P2Y4
P2Y6
C
ATP
10 μM
DMPP
1 μM
25 pA
0.5 s
D
UTP
100 μM
ADP
100 μM
ATP
10 μM
25 pA
0.5 s
E
ATP
10 μM
ATP
30 μM
50 pA
5 s

Figure 5. ***A*****)** Expression and function of purinoceptors in myoblasts in vitro. ***a*****)** Immunocytochemistry for MyoD (red) and DAPI (blue) demonstrated that day 1 cultures contained 93.6 ± 2.5% myoblasts. Bar = 50 μm. ***b*****)** After 5 days in culture, many myoblasts had fused to form multinuclear myotubes, which stain for myosin heavy chain (green) and DAPI (blue). Bar = 100 μm. ***c*****)** Immunocytochemistry for $P2X_5$ receptors (green) and DAPI (blue) demonstrated that cells maintained in differentiation medium (DM) for 24 h, stained strongly for $P2X_5$ receptor protein. Bar = 50 μm. ***d*****)** Immunocytochemistry for $P2Y_1$ receptors (green) and DAPI (blue) demonstrated that the $P2Y_1$ receptor was expressed by myoblasts in day 1 cultures. Bar = 50 μm. ***B*****)** RT-PCR demonstrates the expression of P2Y receptor mRNA. Both $P2Y_1$ and $P2Y_2$ receptor mRNA was detected, but $P2Y_4$ and $P2Y_6$ were absent. ***C*****)** Application of ATP to myoblasts voltage clamped at – 60 mV using a Cs^+-based electrode solution, produced a rapidly activating inward current. In this and subsequent figures, the bar indicates duration of drug application. Most cells failed to respond to the nicotinic ACh receptor agonist DMPP. ***D*****)** Effect of ATP was not mimicked by the $P2Y_1$ and $P2Y_{2/4}$ receptor agonists ADP and UTP. ***E*****)** When recordings were carried out using the perforated patch technique, and a K^+-based pipette solution, ATP evoked a concentration-dependent outward current, which was sometimes preceded by a small inward current.

Fig. 6

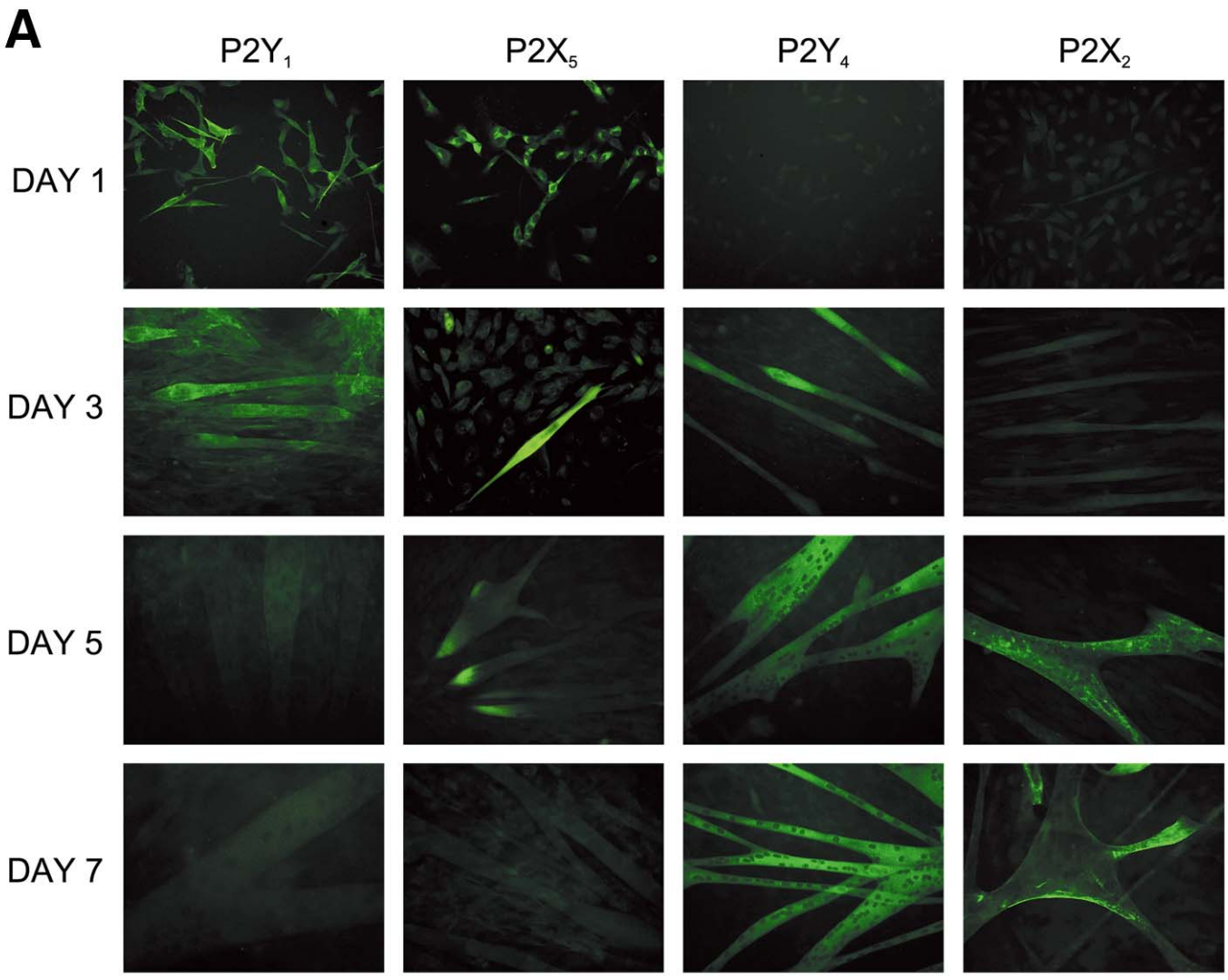
A
P2Y1
P2X5
P2Y4
P2X2
DAY 1
DAY 3
DAY 5
DAY 7
B
AChRs
P2X2
AChRs + P2X2 + DAPI

Figure 6. ***A*****)** Sequential expression of P2 receptors in primary myoblast cultures. At day 1, myoblasts expressed only $P2Y_1$ and $P2X_5$ receptors. By day 3, small myotubes had formed, which additionally expressed $P2Y_4$ receptors. By day 5, there was no expression of $P2Y_1$ on any cell type, $P2X_5$ receptor expression was restricted to tips of myotubes, which now expressed $P2X_2$. On day 7, myotubes expressed only $P2Y_4$ and $P2X_2$ receptors. Bars = 100 μm. ***B*****)** Coexpression of $P2X_2$ and ACh receptors demonstrated using confocal microscopy. Day 5 myotubes were stained with Texas-Red-labeled α-bungarotoxin to label ACh receptors (red), with $P2X_2$ antibody (green), and with the nuclear stain DAPI (blue). ACh receptors (red) and $P2X_2$ receptors (green) were present in clusters across the myotube membranes. Triple-staining for ACh receptors (red), $P2X_2$ receptors (green), and DAPI (nuclear stain, blue) demonstrated colocalization of ACh receptors and $P2X_2$ receptors on myotubes (yellow). However, there were also areas of myotube membrane positive only for ACh receptors or $P2X_2$ receptors. Bars = 50 μm.

Fig. 7

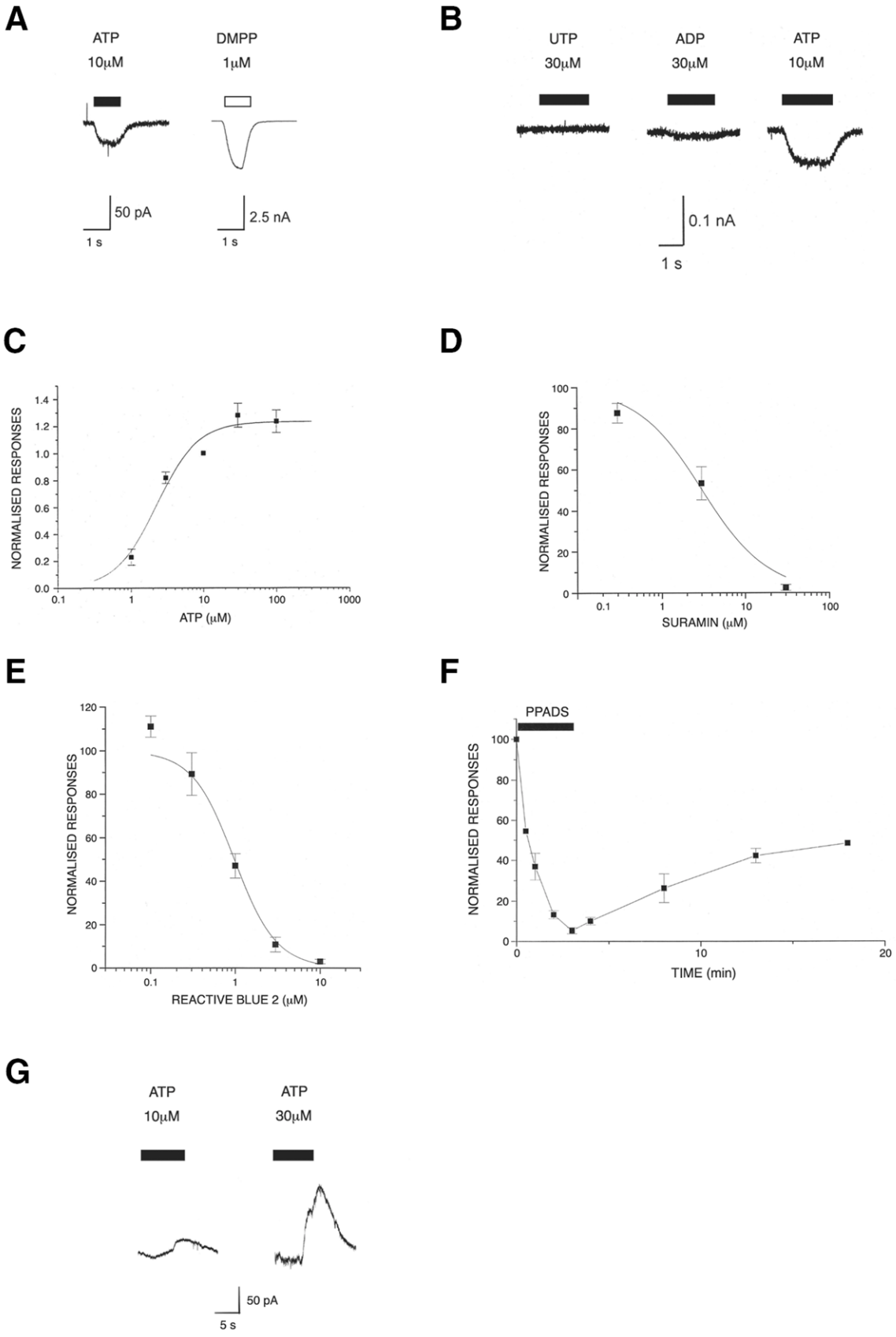
A
ATP
10μM
DMPP
1μM
50 pA
1 s
2.5 nA
1 s
B
UTP
30μM
ADP
30μM
ATP
10μM
0.1 nA
1 s
C
NORMALISED RESPONSES
ATP (μM)
D
NORMALISED RESPONSES
SURAMIN (μM)
E
NORMALISED RESPONSES
REACTIVE BLUE 2 (μM)
F
PPADS
NORMALISED RESPONSES
TIME (min)
G
ATP
10μM
ATP
30μM
50 pA
5 s

Figure 7. Patch clamp recording from myotubes. ***A*****)** Application of ATP to small myotubes voltage clamped at –60 mV using a Cs^+-based electrode solution, produced a small rapidly activating inward current. A much larger current was produced by the nicotinic AChR agonist, DMPP. ***B*****)** Effect of ATP was not mimicked by the P2Y receptor agonists UTP and ADP, even when applied at higher concentrations. ***C*****)** Concentration response curve for inward current activated by ATP. Fitting data with Hill equation gave an EC_{50} of 2.25 ± 0.05 μM. ***D*****)** Concentration effect curve for inhibition of the inward current response to 10 μM ATP by suramin (using a Cs^+-based electrode solution). Points are means ± SE from 3-5 cells. Fitting data to Hill equation gave an IC_{50} value of 3 ± 0.8 μM. ***E*****)** Concentration effect curve for the inhibition of the response to 10 μM ATP by Reactive Blue 2 (using a Cs^+-based electrode solution). Points are mean ± SE from 4 cells. Fitting the data to the Hill equation gave an IC_{50} of 0.97 ± 0.19 μM. ***F*****)** Time course for inhibition of ATP response by PPADS (using a Cs^+-based electrode solution). Responses to ATP (10 μM) were recorded at various time points during the application of 0.1 μM PPADS, and following washout of the antagonist. Points are means ± SE from 3 myotubes. ***G*****)** When myotubes were voltage-clamped using the perforated patch technique and a K^+-based pipette solution, ATP evoked a concentration-dependent outward current, which was often preceded by a much smaller inward current.