

# ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X<sub>5</sub> receptor on satellite cells

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**A**TP is well known for its role as an intracellular energy source. However, there is increasing awareness of its role as an extracellular messenger molecule (Burnstock, 1997). Although evidence for the presence of receptors for extracellular ATP on skeletal myoblasts was first published in 1983 (Kolb and Wakelam), their physiological function has remained unclear. In this paper we used primary cultures of rat skeletal muscle satellite cells to investigate the role of purinergic signaling in muscle formation. Using immunocytochemistry, RT-PCR, and electrophysiology, we demonstrate that the ionotropic P2X<sub>5</sub> receptor is present on satellite cells and that activation of a P2X

receptor inhibits proliferation, stimulates expression of markers of muscle cell differentiation, including myogenin, p21, and myosin heavy chain, and increases the rate of myotube formation. Furthermore, we demonstrate that ATP application results in a significant and rapid increase in the phosphorylation of MAPKs, particularly p38, and that inhibition of p38 activity can prevent the effect of ATP on cell number. These results not only demonstrate the existence of a novel regulator of skeletal muscle differentiation, namely ATP, but also a new role for ionotropic P2X receptors in the control of cell fate.

## Introduction

The first report of the transmitter-like action of ATP on the cell membranes of myoblasts and myotubes (Kolb and Wakelam, 1983) was published prior to the discovery and cloning of the P2X receptor family (for review see Ralevic and Burnstock, 1998). It is now well established that ATP can exert its effects via activation of either P2X or P2Y receptors (for review see Ralevic and Burnstock, 1998). P2X receptors are ligand-gated ion channels and activation of these receptors by extracellular ATP elicits a flow of cations (Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup>) across the plasma membrane. To date, seven mammalian P2X receptor subunits (P2X<sub>1-7</sub>) capable of assembling into homo- or heteromultimeric receptors have been cloned (Torres et al., 1999). In contrast, P2Y receptors are G protein-coupled receptors, which act principally by activating phospholipase C, leading to formation of inositol 1,4,5-trisphosphate and the mobilization of intracellular Ca<sup>2+</sup> (Ralevic and Burnstock, 1998). Although,

a role for P2Y receptors in mediating the trophic effects of ATP is widely recognized (Neary et al., 1996; Abbracchio and Burnstock, 1998), P2X receptors are still largely viewed as mediators of short-term, fast cell-cell communication. However, recent studies demonstrating P2X<sub>7</sub>-dependent apoptosis (Coutinho-Silva et al., 1999; Humphreys et al., 2000) and P2X<sub>5</sub> expression in the differentiating cell layers of stratified squamous epithelial tissues (Gröschel-Stewart et al., 1999), suggest that P2X receptors could also mediate trophic effects, including cell proliferation, differentiation, and apoptosis.

These processes are of key importance in skeletal muscle regeneration. Because skeletal myofibers are terminally differentiated, the regeneration of skeletal muscle is largely dependent on a small population of resident, quiescent cells, termed satellite cells (Perry and Rudnicki, 2000; Seale and Rudnicki, 2000). In response to muscle damage, satellite cells proliferate, become committed to differentiation, exit the cell cycle, and fuse to form multinucleated myotubes. As in muscle development, the MyoD family of basic-helix-loop-helix transcription factors (known as myogenic regulatory factors) is required for the commitment and differentiation of satellite cells during regeneration. This process is also closely tied to cell cycle activity (Lassar et al., 1994; Walsh and

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Perlman, 1997; Perry and Rudnicki, 2000). Proliferating skeletal satellite cells express high levels of MyoD and Myf5, but on commitment to terminal differentiation these transcription factors are downregulated, and myogenin expression increases. This is followed by activation of the cell cycle arrest protein, p21, and permanent exit from the cell cycle. The differentiation program is then completed with the activation of muscle specific proteins, such as myosin heavy chain (MHC)\* and fusion to form multinuclear myotubes (Walsh and Perlman, 1997; Perry and Rudnicki, 2000; Seale and Rudnicki, 2000).

It has long been recognized that the processes of skeletal satellite cell proliferation and differentiation are mutually exclusive (Lassar et al., 1994; Walsh and Perlman, 1997). Understanding the signaling systems involved in directing these cells between proliferation and differentiation is of key importance in developing new therapeutic strategies to treat muscle injury and disease. Many factors, including fibroblast growth factors, insulin-like growth factors, and interleukin-6 cytokines, have already been implicated in the control of satellite cell activity (Hawke and Garry, 2001). In the case of insulin-like growth factors and fibroblast growth factors, the MAPKs have been shown to be involved in mediating these effects (Campbell et al., 1995; Weyman and Wolfman, 1998; Conejo and Lorenzo, 2001; Adi et al., 2002). In particular, extracellular signal-regulated protein kinases (ERKs) 1/2 and p38 MAPK activity has been shown to be involved in directing myogenesis. Whereas a number of studies have demonstrated the importance of the ERK 1/2 pathway in myoblast proliferation (Bennett and Tonks, 1997; Jones et al., 2001), activation of p38 isoforms has been shown to stimulate commitment to differentiation and myotube formation (Chun et al., 2000; Wu et al., 2000; Zetser et al., 1999).

Recent publications demonstrating the expression of ATP receptor subunits (P2X<sub>2</sub>, P2X<sub>5</sub>, and P2X<sub>6</sub>) during skeletal muscle development (Meyer et al., 1999b; Ruppelt et al., 2001; Ryten et al., 2001) suggest that ATP might also act as a trophic factor in skeletal muscle formation. In this paper we used primary cultures of neonatal, rat, skeletal muscle satellite cells to study the role of purinergic signaling in muscle regeneration. We present evidence for the regulated expression of the ionotropic P2X<sub>5</sub> receptor protein and mRNA on satellite cells and demonstrate that activation of a P2X receptor inhibits proliferation of satellite cells. Because the processes of myoblast proliferation and differentiation are mutually exclusive (Lassar et al., 1994; Walsh and Perlman, 1997), we also tested the ability of ATP to potentiate differentiation and investigated some of the intracellular mechanisms that might be responsible for mediating these effects.

## Results

### ATP can inhibit satellite cell proliferation under differentiating (low serum) conditions

It is well recognized that satellite cell differentiation, is potentiated by transferring cells from high serum (growth me-

dia [GM]) to low serum (differentiation media [DM]) (Daniels et al., 2000). The effect of ATP on cell proliferation was determined for cells plated at low density (to prevent myotube formation) and maintained under both conditions (GM and DM). Although cells proliferated more slowly in DM as compared with GM, under both conditions the cell number increased significantly after 24 h (DM, 140% ± 10.0%; GM, 404% ± 10.5% of cell number on plating). At a concentration of 10–100 μM, ATP significantly inhibited (*P* < 5%) the expected increase in the number of satellite cells. At 24 h, ATP reduced the total number of cells present to 67.1% ± 4.3% of that seen in untreated cultures (Fig. 1 A, red). However, ATP had no significant effect on cells maintained in GM (Fig. 1 A, black). ATP acted in a concentration-dependent manner and completely inhibited the expected increase in cell number (even after 72 h) when applied at a maximal concentration of 500 μM (Fig. 1 B). However, at no point did addition of ATP, at any concentration, result in a fall in cell number below that at plating (i.e., Fig. 1 B, 100%). This suggests that ATP did not cause cell death, but inhibited cell proliferation. This interpretation was confirmed using BrdU incorporation studies. Addition of 100 μM ATP resulted in a 35% ± 4.5% reduction (as compared with control) in the percentage of cells staining for BrdU, indicating that cells exposed to ATP had a reduced proliferation rate (Fig. 1 C, a and b).

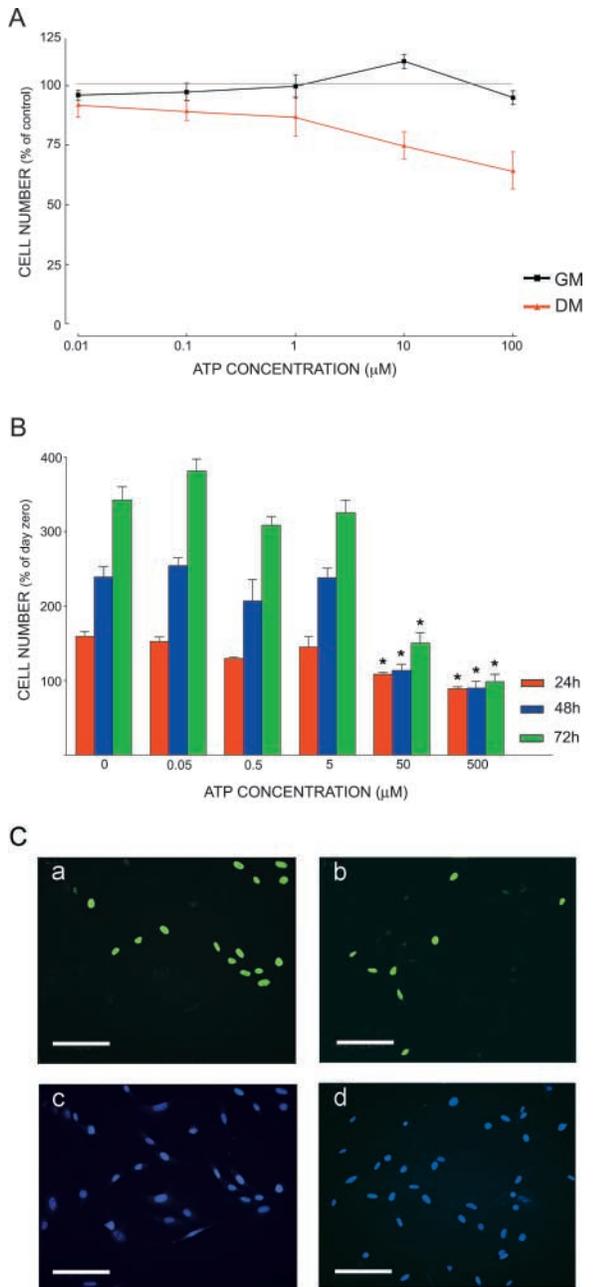
### The effect of ATP on satellite cell proliferation at 24 h cannot be replicated by ADP, UTP, or adenosine and is inhibited by pyridoxal 5-phosphate-6-azophenyl-2',4'-disulfonic acid

In order to determine which receptors were involved in mediating the effect of ATP on satellite cell proliferation, we applied a variety of agonists and antagonists, each specific to a different range of purinoceptors. ATP, ADP, UTP, and α-β-methylene ATP were applied to cells (maintained in DM) at 1–100-μM concentrations. ATP significantly inhibited cell proliferation, whereas the P2Y receptor agonists, ADP, an agonist at P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors (Ralevic and Burnstock, 1998), and UTP an agonist at P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> receptors (Ralevic and Burnstock, 1998), did not inhibit cell proliferation (Fig. 2 A). In fact, UTP significantly increased the cell number (Fig. 2 A). The P2X<sub>1</sub> and P2X<sub>3</sub> selective agonist, α-β-methylene ATP, had no effect on cell number.

ATP can be hydrolyzed to adenosine and this is also an important signaling molecule. Consequently, we tested the effect of adenosine at 1–100-μM concentrations on satellite cell number (Fig. 2 A). Unlike ATP, adenosine application did not reduce cell number at 24 h. However, when adenosine was applied for 72 h at a concentration of 100 μM, a greater concentration than required to demonstrate an ATP effect, a reduction in proliferation was observed (Fig. 2 C). This adenosine effect (100 μM) was inhibited by 8-(p-sulfophenyl)-theophylline (8-SPT) (30 μM), but not dipyrindamole, suggesting that this effect was due to activation of a P1 receptor and not secondary to the uptake of adenosine (Fig. 2 C).

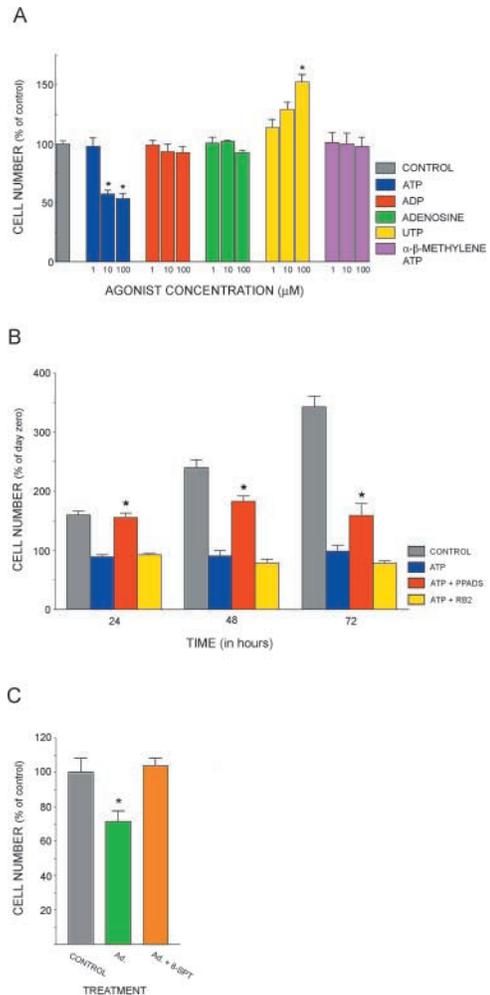
The antiproliferative activity of ATP measured at 24 h could not be inhibited by the P2 receptor antagonist reactive

\*Abbreviations used in this paper: 8-SPT, 8-(p-sulfophenyl)-theophylline; DM, differentiation media; ERK, extracellular signal-regulated protein kinase; GM, growth media; JNK, c-Jun NH<sub>2</sub>-terminal kinase; MHC, myosin heavy chain; NGS, normal goat serum; NHS, normal horse serum; PPADS, pyridoxal 5-phosphate-6-azophenyl-2',4'-disulfonic acid; RB2, reactive blue 2.



**Figure 1. ATP inhibits satellite cell proliferation.** (A) ATP significantly reduced ( $P < 5\%$ ) the number of satellite cells when maintained in DM (red) for 24 h (by  $32.9\% \pm 4.3\%$ , when ATP was applied at  $100 \mu\text{M}$ ), but had no significant effect on cells maintained in GM (black). (B) ATP was applied to cells maintained in DM and cell number assessed 24, 48, and 72 h after application. At all three time points, ATP at concentrations of  $50\text{--}500 \mu\text{M}$  reduced the number of cells. However at no point did ATP cause a reduction in cell number below that at plating (i.e.,  $100\%$ ). Values significantly different from respective controls are denoted by \* ( $P < 5\%$ ). (C) BrdU incorporation assay. (a) BrdU immunostaining (green) demonstrated cell proliferation in control cultures (24 h). (b) Application of  $100 \mu\text{M}$  ATP for 24 h significantly ( $P < 5\%$ ) reduced the proportion of BrdU-positive cells. (c and d) Similar numbers of cells were present in control and ATP-treated cultures (DAPI staining, blue). Bars,  $100 \mu\text{m}$ .

blue 2 (RB2) (preapplied for 20 min at  $50 \mu\text{M}$ ), but was fully inhibited by pyridoxal 5-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (preapplied for 20 min at  $10 \mu\text{M}$ )

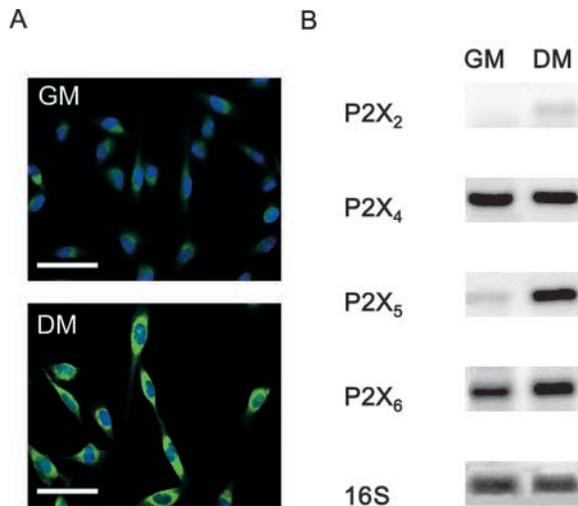


**Figure 2. ATP inhibits satellite cell proliferation by activation of a P2X receptor.** (A) The inhibitory effect of ATP on cell number at 24 h was not replicated by adenosine, ADP, UTP, or  $\alpha$ ,  $\beta$ -methylene ATP. In fact, UTP significantly increased cell number. Values significantly different from control ( $P < 5\%$ ) are denoted by \*. (B) Preapplication of PPADS ( $10 \mu\text{M}$ ) for 20 min, but not RB2 ( $50 \mu\text{M}$ ) completely inhibited the effect of ATP at 24 h. PPADS produced a partial inhibition at 72 h, probably due to the break down of ATP to adenosine and subsequent activation of a P1 receptor. Values significantly different from ATP application alone on respective days ( $P < 5\%$ ) are denoted by \*. (C) Application of  $100 \mu\text{M}$  adenosine (Ad) resulted in a significant ( $P < 5\%$ ) reduction in cell number at 72 h. This could be inhibited by preapplication of the P1 receptor antagonist, 8SPT ( $30 \mu\text{M}$ ). Values significantly different from control ( $P < 5\%$ ) are denoted by \*.

(Fig. 2 B). At 72 h, the effect of ATP was only partially inhibited by this antagonist.

### Satellite cells express P2X receptors and the expression of P2X<sub>5</sub> receptor protein and mRNA is regulated by serum conditions

We investigated the presence of different P2X receptor subunits on satellite cells maintained in growth and differentiating media by using selective antibodies to P2X<sub>1-7</sub>. We were unable to detect significant immunoreactivity for any of these receptors in cells maintained in GM. In contrast, cells maintained in DM were strongly immunopositive for the P2X<sub>5</sub> receptor subunit only (Fig. 3 A). Using

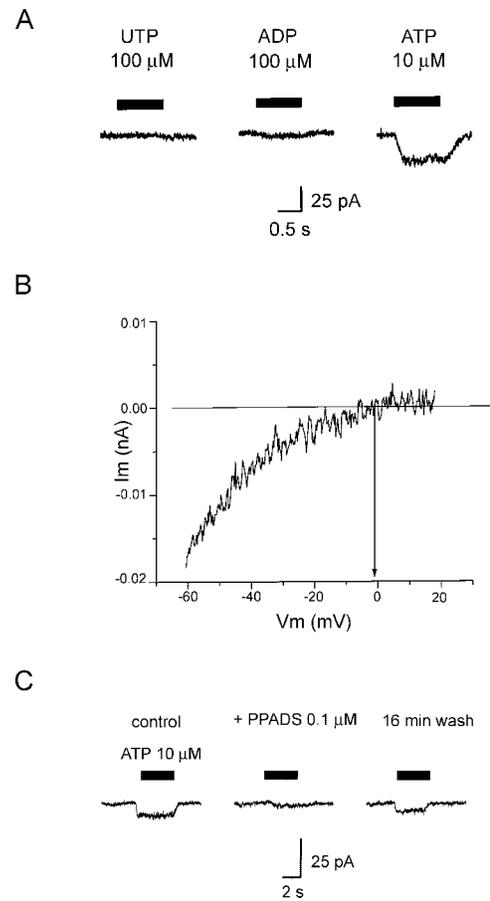


**Figure 3. Regulation of P2X receptor protein and mRNA in satellite cells in vitro.** (A) Immunocytochemistry for P2X<sub>5</sub> (green) and DAPI (blue) demonstrated that cells maintained in DM for 24 h stained more strongly for P2X<sub>5</sub> than those maintained in GM. Note that photographs were taken using identical exposure times. Bar, 50  $\mu$ m. (B) Semiquantitative RT-PCR was used to investigate the expression of P2X receptor mRNA in satellite cells. Cells maintained in DM, expressed higher levels of P2X<sub>5</sub> receptor mRNA compared with cells maintained in GM. Similarly P2X<sub>2</sub> receptor mRNA was only expressed on cells in DM. P2X<sub>4</sub> and P2X<sub>6</sub> receptor mRNA was also expressed by skeletal satellite cells, but expression was not effected by changes in medium.

semiquantitative RT-PCR, we were able to demonstrate increased expression of P2X<sub>5</sub> receptor mRNA (Fig. 3 B). RT-PCR experiments also provided evidence for the expression of P2X<sub>2</sub>, P2X<sub>4</sub>, and P2X<sub>6</sub> receptor mRNA (Fig. 3 B). P2X<sub>2</sub> receptor mRNA was expressed only at a very low level in cells maintained in DM, whereas P2X<sub>4</sub> and P2X<sub>6</sub> receptor mRNA was expressed at similar levels in cells maintained under both conditions. Because the only P2X receptors to be differentially expressed were P2X<sub>2</sub> and P2X<sub>5</sub>, this would implicate these receptors in the differential response to ATP of cells maintained in growth or differentiation medium and consequently the antiproliferative effects of ATP.

#### ATP activates a cation conductance in satellite cells

We further investigated the action of ATP on skeletal muscle satellite cells using electrophysiology. Cells were plated at low density and voltage clamped using the whole cell patch clamp technique. Rapid application of 10  $\mu$ M ATP to voltage clamped cells evoked a small, sustained inward current of  $9.7 \pm 1.6$  pA ( $n = 22$ ). These responses reflected a current density of  $0.87 \pm 0.1$  pA/pF and activated and inactivated quickly (Fig. 4 A). In contrast, UTP and ADP, which are potent agonists at some subtypes of P2Y receptors (at a concentration of 100  $\mu$ M), failed to evoke any significant response. The response to ATP was associated with an increase in membrane conductance. The current voltage relationship showed pronounced inward rectification and a reversal potential close to 0 mV (Fig. 4 B; mean reversal potential  $-3.8 \pm 4.7$  mV,  $n = 8$ ) indicative of a nonselective cation conductance. The purinocep-

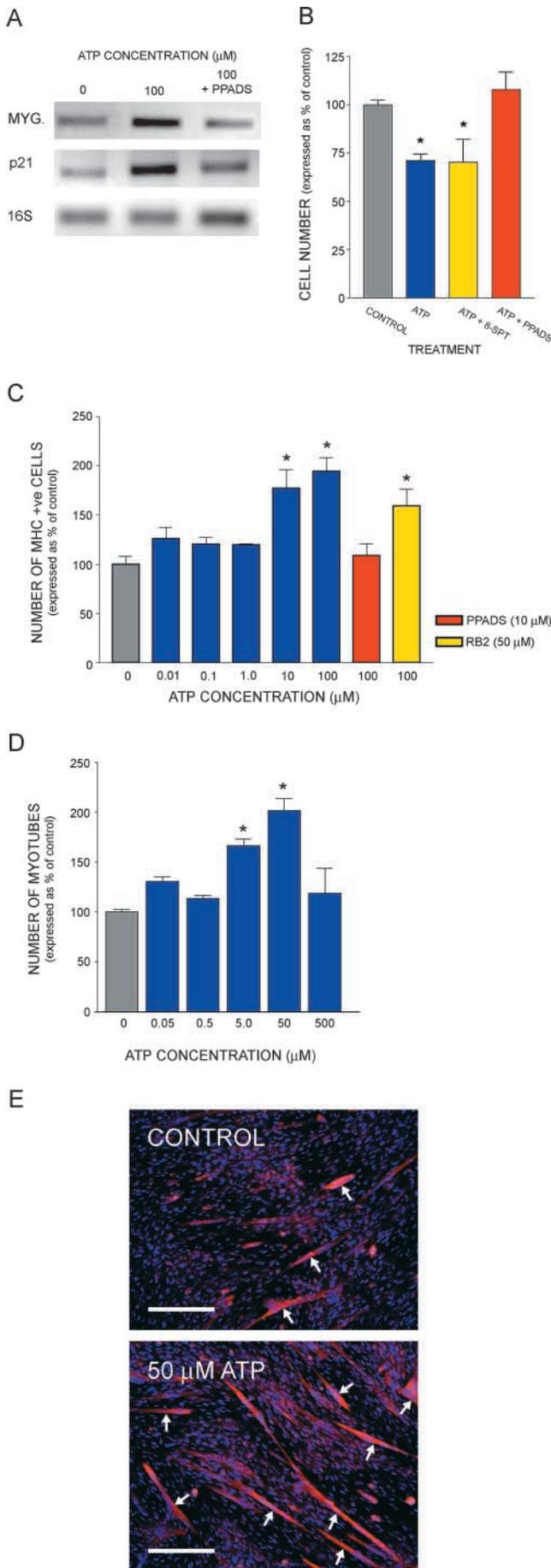


**Figure 4. Nucleotide responses of myoblasts recorded under whole-cell voltage clamp at  $-60$  mV.** (A) Whereas 10  $\mu$ M ATP evokes a rapidly activating, sustained inward current, a tenfold higher concentration of the P2Y receptor agonists UTP and ADP failed to evoke a response. (B) The current-voltage relationship for the ATP activated current reveals inward rectification and a reversal potential close to 0 mV. (C) The response to 10  $\mu$ M ATP is inhibited by the purinoceptor antagonist PPADS, and recovers slowly on washing out the antagonist.

tor antagonist PPADS produced a potent inhibition of the response to ATP (Fig. 4 C), which reversed only very slowly on washout.

#### ATP application increased the expression of markers of terminal differentiation and these effects could be inhibited by preapplication of the purinoceptor antagonist PPADS

Because the processes of skeletal satellite cell proliferation and differentiation are mutually exclusive (Lassar et al., 1994; Walsh and Perlman, 1997), ATP might be expected not only to inhibit satellite cell proliferation, but potentiate differentiation. Addition of 100  $\mu$ M ATP increased the expression of myogenin and p21, as judged by semiquantitative RT-PCR (Fig. 5 A). Preapplication of 10  $\mu$ M PPADS for 20 min inhibited this effect (Fig. 5 A). Increased expression of p21 by skeletal muscle satellite cells would be expected to result in irreversible cell cycle exit, such that removal of ATP would not rescue cells. In fact we found that treatment of satellite cell cultures with 100  $\mu$ M ATP for only 1 min (Fig. 5 B), was sufficient to cause



a significant reduction in cell number at 72 h ( $28.8\% \pm 3.2\%$ ,  $P < 5\%$ ). Preapplication of PPADS (red), but not 8-SPT, inhibited this effect (Fig. 5 B), confirming that the effects of ATP are due primarily to P2 and not P1 receptor activity. Furthermore, ATP application significantly increased ( $P < 5\%$ ) the number of cells expressing MHC ( $194.3\% \pm 14.6\%$  when 100  $\mu\text{M}$  ATP applied for 24 h) (Fig. 5 C) and the number of myotubes formed in 24 h (Fig. 5, D and E) ( $201\% \pm 12.1\%$  when 50  $\mu\text{M}$  ATP applied). The increased MHC expression produced by ATP (100  $\mu\text{M}$ ) could be inhibited by preapplication of PPADS (10  $\mu\text{M}$ ), but not RB2 (50  $\mu\text{M}$ ).

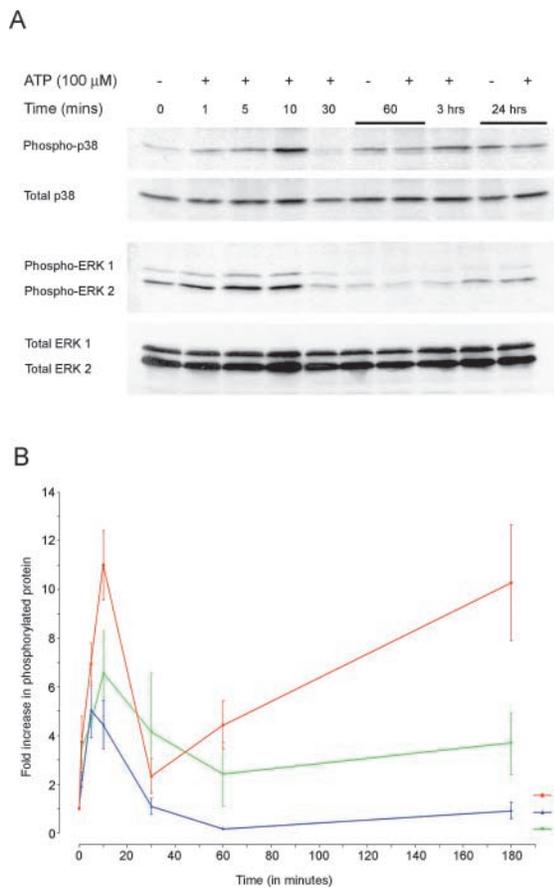
### ATP application increased the phosphorylation of p38 and ERK 1/2

MAPK signalling cascades have been implicated in the regulation of myogenesis. Whereas the ERKs have been implicated in myoblast proliferation (Bennett and Tonks, 1997; Jones et al., 2001), the p38 pathway has been implicated in myoblast differentiation (Chun et al., 2000; Wu et al., 2000; Zetser et al., 1999). We used antibodies specific for the active (phosphorylated) and inactive (nonphosphorylated) forms of the p38 and ERK 1/2 proteins, to assess these signaling pathways at a range of time points (Fig. 6, A and B).

Consistent with previous studies (Wu et al., 2000; Zetser et al., 1999), we found that maintaining cells in DM in itself (without the addition of ATP) had significant effects on the p38 MAPK pathway. Even in untreated cultures, the levels of phosphorylated p38 significantly ( $P < 5\%$ ) increased when cultures were maintained in DM for  $>24$  h ( $7.2 \pm 1.9$ -fold increase in p38 phosphorylation at 24 h relative to time zero). However, as reported by Wu et al. (2000), we found no significant difference in ERK 1/2 phosphorylation relative to time zero when cells were maintained in DM for 24 h ( $2.0 \pm 0.8$ -fold increase in ERK 1 phosphorylation at 24 h).

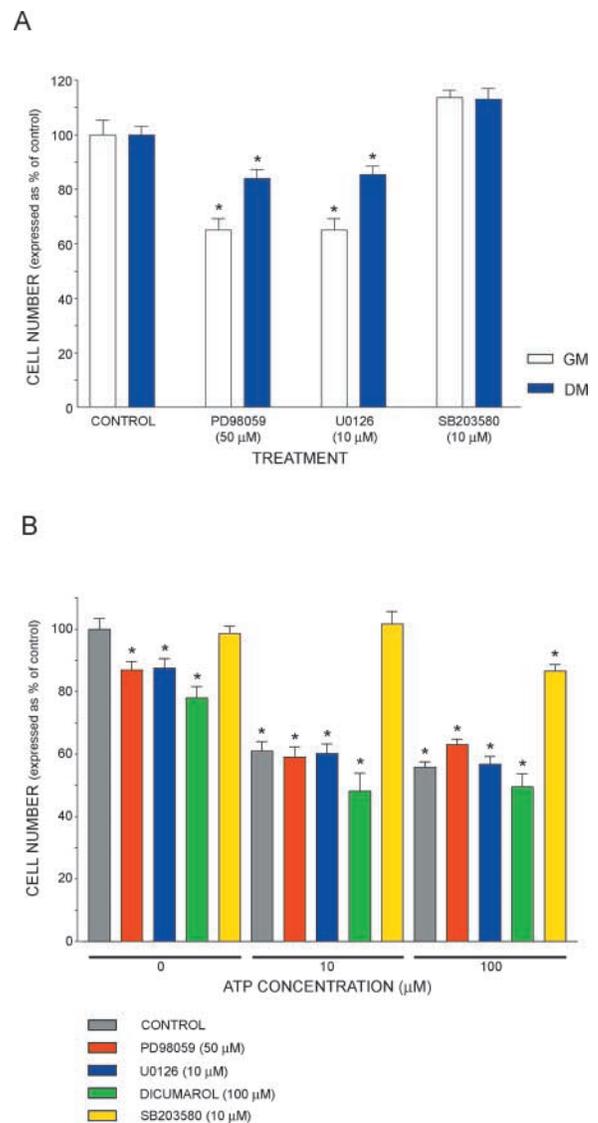
### Figure 5. Treatment of satellite cells with ATP increases the expression of markers of differentiation.

(A) Semi-quantitative RT-PCR demonstrated increased expression of myogenin and p21 mRNA in cells treated with 100  $\mu\text{M}$  ATP for 24 h. Pre-application of PPADS inhibited the increase of both myogenin and p21. The ribosomal protein, 16S was used as an internal control. (B) Application of 100  $\mu\text{M}$  ATP for 1 min, followed by replacement with fresh media without ATP, was sufficient to cause a significant ( $P < 5\%$ ) reduction in cell number (of  $28.8\% \pm 3.2\%$  compared with untreated controls) at 72 h. This effect could be fully inhibited by PPADS (10  $\mu\text{M}$ ), but not 8-SPT (30  $\mu\text{M}$ ). (C) ATP application for 24 h caused a concentration-dependent increase in the number of cells positive for MHC. The effect of ATP (100  $\mu\text{M}$ ) could be inhibited by preapplication of PPADS (10  $\mu\text{M}$  for 20 min), but not RB2 (50  $\mu\text{M}$ ). Values significantly different from control ( $P < 5\%$ ) are denoted by \*. (D) Treatment of confluent cultures with 0.05–50  $\mu\text{M}$  ATP for 24 h resulted in a concentration-dependent increase in myotube formation. Above a concentration of 50  $\mu\text{M}$ , the strong anti-proliferative effect of ATP and consequent reduction in cell density, reduced the number of myotubes formed. Values significantly different from control ( $P < 5\%$ ) are denoted by \*. (E) Treatment of confluent cultures with 50  $\mu\text{M}$  ATP for 24 h resulted in significantly increased ( $P < 5\%$ ) numbers of myotubes ( $201\% \pm 12.1\%$ ) (arrowheads). Myotubes are visible in cultures stained with DAPI for nuclei (blue) and skeletal myosin (red), as multinuclear cells, strongly immunopositive for myosin. Bars, 200  $\mu\text{m}$ .



**Figure 6. ATP application to satellite cells maintained in DM causes a rapid and transient increase in phosphorylation of p38 and ERK1/2.** (A) Western blotting demonstrated that application of 100  $\mu\text{M}$  ATP to cultures increased the levels of phosphorylated p38 and ERK 1/2. Relative to time zero, ATP caused a significant ( $P < 5\%$ ) increase in the levels of ERK 1/2 phosphorylation at 1, 5, and 10 min, but not 30 min. Similarly, ATP caused a significant increase in the levels of phosphorylated p38 at 5 and 10 min, but not 30 min. In both the cases, the peak of phosphorylation was at 10 min and the effect of ATP was transient. Consequently there was no significant difference in the levels of phosphorylated p38 and ERK 1/2 in treated and control cultures 1 h after ATP application. It should also be noted that in untreated cultures, the levels of phosphorylated p38 had significantly increased at 1 h and 24 h, but there was no significant change in the level of ERK1/2 phosphorylation over the same time period. ATP did not have any effect on the levels of total p38 or ERK 1/2. Representative Western blots are shown from one set of treated cells. (B) The levels of phosphorylated p38, ERK 1 and ERK 2 from three independent experiments were analyzed using densitometry and plotted as fold increases in phosphorylated protein, compared with the levels at time 0. Phosphorylated p38 is plotted in red, ERK 1 in blue and ERK 2 in green.

Despite the basal activities of the MAPK pathways, we found that treatment with ATP caused a rapid and significant increase in the levels of phosphorylated p38 and ERK 1/2. Application of 100  $\mu\text{M}$  ATP to cultures maintained in DM caused a significant increase ( $P < 5\%$ ) in p38 phosphorylation at 5 and 10 min ( $6.9 \pm 0.9$ - and  $11.0 \pm 1.4$ -fold increase in p38 phosphorylation at 5 and 10 min, respectively, relative to time zero) (Fig. 6, A and B). Similar but less dramatic changes were also seen with ERK 1/2 phosphorylation. The fold increases in ERK 1/2 were  $4.5 \pm 1.0$  and  $6.5 \pm 1.8$ , respectively at 10 min (Fig. 6, A and



**Figure 7. Inhibitors of the MAPK pathways have effects on satellite cell number, but only the inhibitor of p38 $\alpha$  and p38 $\beta$  activation (SB203580) blocked the effects of ATP.** (A) PD98059 (50  $\mu\text{M}$ ), U0126 (10  $\mu\text{M}$ ) and SB203580 (10  $\mu\text{M}$ ) were applied to satellite cells, maintained in GM or DM for 12–16 h, the medium changed and cell number assessed at 24 h. Inhibitors of ERK 1/2 activation (PD 98059 and U0126) caused a significant reduction ( $P < 5\%$ ) in cell number, when cells were maintained in GM or DM. This effect was greater on cells maintained in GM, than those in DM. Application of U0126 caused a  $34.84\% \pm 4.01\%$  decrease in cell number at 24 h, when cells were maintained in GM, as compared with an  $11.73\% \pm 1.64\%$  decrease, when cells were maintained in DM. SB203580 application had no significant effect on cell number when cells were maintained in GM or DM. Values significantly different from the relevant control (DM or GM) ( $P < 5\%$ ) are denoted by \*. (B) Inhibitors of the ERK 1/2, p38, and JNK MAPK signaling pathways (PD98059, U0126, SB203580 and dicumarol) were tested for their ability to inhibit the effect of ATP on satellite cell number. PD98059 (50  $\mu\text{M}$ ), U0126 (10  $\mu\text{M}$ ), dicumarol (100  $\mu\text{M}$ ), and SB203580 (10  $\mu\text{M}$ ) were preapplied for 30 min in the case of PD98059 and U0126, and 1 h in the case of dicumarol and SB203580, before application of ATP. As previously demonstrated application of 10 or 100  $\mu\text{M}$  ATP caused a significant reduction in cell number. These effects were not inhibited by PD98059, U0126, or dicumarol. However, SB203580 an inhibitor of p38 $\alpha$  and p38 $\beta$  activation completely prevented the effect of 10  $\mu\text{M}$  ATP and partially prevented the effect of 100  $\mu\text{M}$  ATP. Values significantly different from control ( $P < 5\%$ ) are denoted by \*.

B). These effects were transient, such that 1 h after application, there was no significant difference in p38 or ERK 1/2 phosphorylation between treated and untreated cultures (Fig. 6 A). After 3 h, cultures treated with ATP had levels of p38 phosphorylation, which were not significantly different from the levels of p38 phosphorylation in untreated cultures after 1 or 24 h (Fig. 6 A).

### Inhibition of p38 activity prevented the ATP-dependent reduction in cell proliferation

In order to determine whether the trophic effects of ATP were dependent on activation of MAPK signaling pathways, we used a variety of specific inhibitors of MAPK activity. We found that application of specific inhibitors of MEK1 (responsible for ERK activation), PD95809, and U0126 (Alessi et al., 1995; Favata et al., 1998), resulted in a decrease in cell number (Fig. 7 A). This effect was greater on cells maintained in GM than on cells maintained in DM. At 24 h, U0126 (10  $\mu$ M) reduced the total number of cells present to  $65.16\% \pm 4.01\%$  of that seen on treated cultures, when cells were maintained in GM, as compared with  $88.27\% \pm 1.64\%$ , when cells were maintained in DM (Fig. 7 A). In contrast, SB203580, the specific inhibitor of p38 $\alpha$  and p38 $\beta$  MAPKs (Lee et al., 1994; Cuenda et al., 1995), had no significant effect on cell number when cells were maintained in either GM or DM (Fig. 7 A).

These inhibitors were also tested for their ability to prevent the effects of ATP on satellite cells (Fig. 7 B). As previously demonstrated, ATP caused a significant reduction in cell number at 24 h when applied at concentrations of 10–100  $\mu$ M. The effect of 10  $\mu$ M ATP on cell number ( $61.90\% \pm 3.15\%$  of that seen on untreated cultures) was completely inhibited by preapplication of SB203580 (Fig. 7 B), but not by preapplication of PD98059 (50  $\mu$ M), U0126 (10  $\mu$ M), or dicumarol (a specific inhibitor of c-Jun NH<sub>2</sub>-terminal kinase (JNK) activity, when applied at a concentration of 100  $\mu$ M) (Cross et al., 1999; Krause et al., 2001) (Fig. 7 B). Similarly, only SB203580 inhibited the effects of 100  $\mu$ M ATP at 24 h. In this case, the effects of ATP on cell number were partially inhibited (Fig. 7 B).

## Discussion

Although Kolb and Wakelam's (1983) demonstration of the transmitter-like effects of ATP on chick skeletal muscle cultures prompted a number of studies on the effects of ATP and other nucleotides on myotubes, there has been little further research on myoblasts. In this paper, we use primary cultures of rat skeletal muscle satellite cells to identify not only the P2 receptor subtypes expressed by myoblasts, but to demonstrate that activation of these receptors can regulate muscle formation. ATP acts on cells maintained in low-serum conditions to inhibit proliferation and potentiate the expression of markers of differentiation including myogenin and p21. Surprisingly, we found that these effects were mediated by an ionotropic P2X receptor, containing the P2X<sub>5</sub> receptor subunit and involved activation of the MAPK signaling cascades.

Primary rat satellite cells *in vitro* proliferate, commit to differentiation and fuse to form myotubes in a manner anal-

ogous to muscle formation *in vivo*. This has allowed us to investigate the effects of ATP on skeletal muscle regeneration in an easily accessible and well-characterized system. Using this model, we found that ATP had significant effects on satellite cell activity. Application of 100  $\mu$ M ATP increased the expression of myogenin and the cell cycle arrest protein p21 at 24 h, and these effects could be blocked using PPADS. Expression of these nuclear proteins has been shown to be necessary for terminal differentiation of myoblasts. Myogenin-null mice, in accordance with the appearance of myogenin at the onset of differentiation, die perinatally due to a severe deficiency of differentiated muscle fibers (Hasty et al., 1993; Nabeshima et al., 1993). As would be expected, upregulation of these transcription factors by ATP: (a) potentiated terminal differentiation of satellite cells, as judged by the expression of MHC and myotube formation; and (b) inhibited satellite cell proliferation. Consistent with evidence that ATP increased expression of p21, we found that the effect of ATP was irreversible. Incubating cells with media containing 100  $\mu$ M ATP for 1 min, followed by replacement with fresh medium still produced a reduction in cell number.

The antiproliferative effects of ATP at 24 h could not be replicated by application of adenosine, ADP, UTP, or  $\alpha$ - $\beta$ -methylene ATP, and were inhibited by PPADS, but not RB2. However, PPADS is effective at P2Y<sub>1</sub> receptors (Schachter et al., 1996, 1997); as ADP (a P2Y<sub>1</sub> receptor agonist) had no effect on cell number, this receptor is unlikely to be involved. Therefore, antagonism by PPADS would implicate a P2X receptor (P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, or P2X<sub>5</sub>) (Lambrecht, 2000). The fact that the P2X<sub>1</sub>- and P2X<sub>3</sub>-selective agonist,  $\alpha$ - $\beta$ -methylene ATP, was found to have no significant effect on cell number, suggests that the receptor involved contained the P2X<sub>2</sub> or P2X<sub>5</sub> receptor subunits. However, RB2 an antagonist at the P2X<sub>2</sub>, but not the P2X<sub>5</sub> receptor subunit (King et al., 1997; Wildman, S.S., S.G. Brown, M. Rahman, G. Burnstock, J. Urwin, and B.F. King. 2002. Joint Meeting: The Physiological Society, Scandinavian Physiological Society, and Deutsche Physiologische Gesellschaft. 81st Annual Meeting. 31 [Abstr.]), could not inhibit the effects of ATP on cell number. Therefore, although we cannot rule out the possibility of a novel, yet to be cloned receptor, this data points strongly to the involvement of the P2X<sub>5</sub> receptor.

P2Y receptors are also known to be present in skeletal muscle (Parr et al., 1994; Meyer et al., 1999a; Choi et al., 2001) and the increase in cell number on UTP application could be explained by activation of a P2Y<sub>2</sub> or P2Y<sub>4</sub> receptor. Because prolonged application of adenosine produced a significant reduction in cell number, which could be inhibited by preapplication of the P1 antagonist, 8-SPT, the presence of a P1 receptor was also demonstrated. However, the effect of ATP applied for 1 min, could only be inhibited by preapplication of PPADS and not 8-SPT. These results suggest that although P1 and P2Y receptors are expressed by satellite cells, a P2X receptor is primarily responsible for the effects of ATP studied in this paper.

The antiproliferative and differentiating effects of ATP could be considered counterintuitive. An obvious, but by no means the only, source of ATP under physiological condi-

tions is damaged myofibers. However, muscle trauma, far from inhibiting satellite cell proliferation, activates quiescent satellite cells to reenter the cell cycle and proliferate. Trauma also results in the release of many other trophic factors, including hepatocyte growth factor and FGFs, known to be capable of promoting satellite cell proliferation (Hawke and Garry, 2001). Therefore, purinoceptor signaling is likely to be only one of many active systems *in vivo*. Maintenance of cells in high-serum media (GM), when increased levels of trophic factors are likely to be present, could be considered analogous to these conditions. We found that ATP had no effect on cell number even at a concentration of 100  $\mu$ M, when cells were maintained in GM. Furthermore, under low-serum conditions, ATP not only inhibited cell proliferation, but also potentiated differentiation. This would suggest that the ATP effects studied in this paper relate predominantly to late events in muscle regeneration, when the levels of other trophic factors have fallen and controlled release of ATP from motoneurons (Redman and Silinsky, 1994; Silinsky and Redman, 1996) and muscle activity (Smith, 1991; Cunha and Sebastiao, 1993; Hellsten et al., 1998) can influence satellite cell activity.

One way of varying the response of satellite cells to ATP is to regulate receptor expression. We found that whereas cells maintained in GM expressed no P2X receptor proteins, cells maintained in DM expressed high levels of the P2X<sub>5</sub> receptor subunit. Furthermore, maintaining cells in low-serum medium increased the expression of P2X<sub>5</sub> receptor mRNA and stimulated the expression of P2X<sub>2</sub> receptor mRNA. Because the only P2X receptor to be differentially expressed (on the basis of protein and mRNA) was P2X<sub>5</sub>, this would implicate this receptor in the differential response of cells maintained in growth or differentiation medium to ATP and consequently the antiproliferative effects of ATP. Certainly the pharmacological profile observed (see above) is consistent with this suggestion.

The mechanism by which P2X<sub>5</sub> receptor activation produced effects on satellite cell activity was also investigated. We were able to demonstrate that ATP application to satellite cells produced a significant and rapid increase in the phosphorylation of p38 and, to a lesser extent, ERK 1/2. However, only inhibition of p38 $\alpha$  and p38 $\beta$  activation blocked the P2X-dependent effects on cell number. These results are consistent with previous studies demonstrating the importance of the p38 MAPK signaling pathway in myogenesis. p38 can directly activate the MEF2 transcription factors in skeletal muscle (Zetser et al., 1999; Wu et al., 2000), and together with the MyoD family, MEF2 transcription factors are necessary for the differentiation of myoblasts (Black and Olson, 1998; Molkenstein et al., 1995). Thus, overexpression of p38 isoforms or upstream activators stimulate myogenesis, whereas inhibition of p38 activation prevents formation of myotubes (Wu et al., 2000; Zetser et al., 1999).

P2X<sub>5</sub> receptor activation results in a rapid and transient flow of cations across the plasma membrane. Thus, like all ionotropic receptors, P2X<sub>5</sub> has been viewed as a mediator of transient cellular events. However, recent research on various ligand-gated ion channels, including P2X<sub>7</sub> (a member of the P2X receptor family), demonstrates that ionotropic receptors interact with cytoskeletal and signaling proteins

(Sheng and Pak, 2000; Kim et al., 2001) and can produce long-term effects on cellular activity. A number of proteins capable of activating intracellular signalling pathways have been shown to interact with the P2X<sub>7</sub> receptor, such as phosphatidylinositol 4-kinase (Kim et al., 2001), and it is known that this receptor can activate the p38, ERK 1/2 and JNK MAPK signaling cascades (Humphreys et al., 2000; Panenka et al., 2001). Furthermore, activation of the P2X<sub>7</sub> receptor has been shown to produce caspase-dependent apoptosis in various cell types, including microglia, dendritic, and mesangial cells (Schulze-Lohoff et al., 1998; Coutinho Silva et al., 1999; Ferrari et al., 1999). Thus, the results presented in this paper suggest that activation of the P2X<sub>5</sub> receptor can produce changes in MAPK signaling pathways by analogous mechanisms.

Although in this paper we have only investigated satellite cells *in vitro*, recent immunohistochemical and *in situ* localization studies demonstrate that P2X receptors, notably P2X<sub>5</sub>, are also expressed during skeletal muscle formation *in vivo* (Meyer et al., 1999b; Ruppelt et al., 2001; Ryten et al., 2001). P2X<sub>2</sub>, P2X<sub>5</sub>, and P2X<sub>6</sub> receptor expression have been demonstrated in developing rat and chick skeletal muscle (Meyer et al., 1999b; Ruppelt et al., 2001; Ryten et al., 2001). In chick, P2X<sub>5</sub> receptor mRNA and protein was detected not only on myotubes, but also muscle precursor cells in the dermamyotome (Meyer et al., 1999b; Ruppelt et al., 2001). These results suggest that signaling through a P2X receptor containing the P2X<sub>5</sub> subunit may be important in muscle formation *in vivo*, whether in development or regeneration of skeletal muscle. Similarly, immunohistochemical localization of P2X<sub>5</sub> in the differentiating cell layers of stratified squamous epithelial tissues suggest a general function for P2X<sub>5</sub> receptor-mediated signaling in regulating the balance between cell proliferation and differentiation in a variety of systems.

In summary, we demonstrate in this paper a novel role for ATP in the regulation of skeletal muscle formation. Under differentiating conditions, ATP acts on satellite cells to inhibit proliferation and increase the rate of differentiation. These effects are mediated by upregulation of myogenin and the cell cycle regulator p21. Surprisingly, the effect of ATP is due to the activation of an ionotropic P2X receptor, containing the P2X<sub>5</sub> receptor subunit. Although a role for P2X<sub>5</sub> in cell differentiation has been proposed, this is the first functional evidence. Thus these results not only open the way for a new therapeutic target in the treatment of myogenic disorders, but also a new role for P2X receptors in the control of cell cycle and fate.

## Materials and methods

### Tissue culture

Primary cultures of skeletal muscle satellite cells were prepared using a protocol adapted from Daniels et al. (2000). Briefly, skeletal muscle was dissected from the hindlimbs of 1- to 2-d-old, neonatal Sprague Dawley rats and finely chopped using a McIlwain Tissue Chopper. After digestion in trypsin and collagenase (Sigma-Aldrich), the resulting suspension was filtered using a 200  $\mu$ m mesh and centrifuged. The pellet was resuspended and the resulting cell suspension preplated to remove fibroblasts. Cells were then replated on 1% gelatin-coated dishes and maintained for 1 to 2 days in DME supplemented with 10% FCS, 10% normal horse serum (NHS; GIBCO-BRL), 0.5% gentamicin, and 0.5% ampicillin (termed GM; Sigma-Aldrich). Skeletal satellite cells were then selectively detached

using 0.05% Dispase II (Roche), preplated to remove fibroblasts, and then finally plated at  $10^4$  cells/cm<sup>2</sup> on laminin-coated coverslips or plasticware as appropriate. Cells were maintained in GM or DME supplemented with 5% NHS (DM), at 37°C and 5% CO<sub>2</sub>. Freshly isolated cells were used for each experiment.

### Electrophysiology

Whole-cell patch clamp recordings were carried out as described previously by Zhong et al. (1998). Agonists were applied rapidly by microperfusion from a four-barrel manifold controlled by computer-driven solenoid valves. Data were acquired using pClamp v8 software (Axon Instruments), and plotted using Origin v4.1 (Microcal). The current-voltage relationship for the ATP activated current was obtained by subtracting the current response to a ramped change in voltage (−60 to +20 mV, 0.6 s) in the absence of agonist from that recorded in the presence of ATP.

### Immunocytochemistry

Skeletal satellite cells were plated on laminin-coated glass coverslips or laminin-coated 8-well LabTek chamberslides (Nunc Life Technologies) for immunocytochemistry. In both cases, cells were fixed for 20 min in 4% paraformaldehyde. After washing in PBS, cells were permeabilized and non-specific binding sites were blocked using 10% normal goat serum (NGS) + 0.2% Triton X-100 in PBS. Fixed cells were incubated with primary antibodies, diluted in 10% NGS + 0.2% Triton X-100 in PBS, overnight at room temperature. The primary antibodies used were rabbit anti-P2X<sub>1-7</sub> (Roche); rabbit anti-P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> (Alomone Laboratories); MF20 (University of Iowa Developmental Studies Hybridoma Bank); and rabbit antiskeletal myosin (Sigma-Aldrich). These antibodies were used at the following concentrations: rabbit anti-P2X<sub>1-7</sub>, 5 µg/ml; MF20, 1:100 of hybridoma supernatant; and rabbit antiskeletal myosin, 1:100. After washing 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-labelled goat anti-mouse/rabbit secondary antibodies (Strattech 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-Aldrich) at 0.6 µg/ml in PBS for 1 h at room temperature. Control experiments were carried out with the primary antibody omitted from the staining procedure and, in the case of the rabbit anti-P2X<sub>1-7</sub> antibodies, the primary antibody preabsorbed with the peptides used to immunize the rabbits.

### Drugs used

ATP, ADP, UTP, α-β-meATP (lithium salt), RB2, 8-SPT, and 3,3'-methylenebis-(4-hydroxy-coumarin) (dicumarol) were purchased from Sigma-Aldrich. PPADS (tetrasodium salt) was supplied by Tocris Cookson Ltd. The kinase inhibitors PD98059, U0126, and SB203580 were purchased from Calbiochem.

### Cell counting

Cell number was assessed directly, after trypsin treatment, by staining with trypan blue for viability and counting using a haemocytometer, or indirectly by DNA quantification using the Fluoreporter Blue Fluorometric ds-DNA Quantification kit (Molecular Probes).

### MHC expression

The number of cells positive for MHC was assessed by first staining cultures with MF20 (see above) and then counting the number of immunoreactive cells in five random fields on three separate coverslips for each treatment.

### Myotube assay

Skeletal satellite cells were plated at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> on laminin-coated glass coverslips and maintained in GM overnight. After treatment for 24 h, cells were fixed in 4% paraformaldehyde for 20 min. Myotubes were scored for five random fields on three separate coverslips for each treatment.

### Data analysis

Cell/myotube number was expressed as a percentage of that at time zero or as a percentage of the cell/myotube number in the untreated, control wells after the same time in culture. Each experiment was conducted at least three times with separate preparations of satellite cells. Test and control cultures were run in parallel on each occasion. Differences in cell/myotube number were assessed for significance using a paired, two-tailed Student's *t* test.

### BrdU incorporation assay

Skeletal satellite cells were plated at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> on laminin-coated glass coverslips and maintained in DM overnight. Cells were

then treated with ATP and maintained for 12 h, prior to addition of BrdU (at a concentration of 50 µM). After a further 12 h, coverslips were washed with PBS to remove nonadherent cells and the remaining cells fixed with 95% ethanol. Cells were treated with 2 M hydrochloric acid, washed with PBS and incubated with 10% NGS + 0.2% Triton X-100 in PBS for 1 h. The cells were then incubated with mouse anti-BrdU antibody, 1:1,000 in 10% NGS in PBS (Sigma-Aldrich) overnight and staining visualized using Oregon green-labelled goat anti-mouse secondary antibody (Strattech Scientific). Nuclei were stained using DAPI. The percentage of proliferating cells (nuclei immunopositive for BrdU/nuclei staining for DAPI) was scored for five random fields on three separate coverslips for each treatment.

### RT-PCR

Total RNA was extracted from skeletal satellite cells using the SV Total RNA Isolation System (Promega). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech). Reverse transcription was performed using the Moloney murine leukemia virus reverse transcriptase. Primer sequences for P2X<sub>1-7</sub> (Shibuya et al., 1999), p21 (Wong et al., 2000), and myogenin and 16S (Ogilvie et al., 2000) were used for amplification reactions, as reported previously. 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. To normalize mRNA levels between samples, PCR reactions were performed for 16 s (ribosomal protein). Amplification products were separated by electrophoresis and visualized by ethidium bromide staining. According to densitometric analysis of the PCR products (BioRad Multi-Imager software), sample loading was adjusted for subsequent PCR reactions for P2X<sub>1-7</sub>, p21, and myogenin. The presence of possible contaminants was investigated in all experiments, using control RT-PCR reactions in which, either mRNA had been omitted or the reverse transcriptase had been inactivated by heating to 95°C.

### Protein preparation and Western blotting for the MAPK proteins

Skeletal satellite cells were plated at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> on laminin-coated dishes and maintained in DM overnight. Cells were then treated with 100 µM ATP for time periods ranging from 0 min to 24 h. After treatment with ATP, cells were quickly rinsed twice with ice-cold PBS, collected in PBS, and pelleted at 13,000 g for 10 min. Cells were then lysed in a buffer containing 20 mM Tris, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 1 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 1% Triton X-100, 1 µg/ml pepstatin A, 4 µM leupeptin, 30 µg/ml aprotinin, and 0.1 mg/ml AEBF (Calbiochem) for 20 min on ice. The resulting homogenate was centrifuged at 13,000 g for 10 min. The protein concentration was determined using the modified Lowry procedure (Peterson, 1983).

Samples containing equal amounts of protein were subjected to SDS-PAGE using 11% acrylamide and transferred to nitrocellulose filters. Filters were incubated with a blocking solution containing 20 mM Tris, pH 7.7, 137 mM NaCl, 0.1% Tween 20 (TTBS), and 5% nonfat dry milk for 1 h at room temperature, rinsed in TTBS, and then incubated for 1 h at room temperature with specific primary antibodies. The antibodies used were all diluted in 5% bovine serum albumin in TTBS at the following concentrations: rabbit anti-ERK 1/2, 1:5,000 (Cell Signaling Technology); rabbit anti-phospho-ERK 1/2, 1:2,000 (Santa Cruz Biotechnology); rabbit anti-p38, 1:1,000 (Cell Signaling Technology); and mouse anti-phospho-p38, 1:1,000 (Cell Signaling Technology). After three rinses in TTBS, filters were incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit or anti-mouse IgG diluted in TTBS (1:20,000 or 1:10,000, respectively) (Amersham Life Sciences). Filters were washed three times in TTBS, and proteins were detected by enhanced chemiluminescence (Amersham Life Sciences). Results were analysed using densitometry (Bio-Rad Multi-Imager software).

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