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P2 nucleotide receptors on C2C12 satellite cells

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Abstract

In developing muscle cells environmental stimuli transmitted by purines binding to the specific receptors are crucial proliferation regulators. C2C12 myoblasts express numerous purinergic receptors representing both main classes: P2X and P2Y. Among P2Y receptors we have found the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ family members while among P2X receptors $P2X_4$, $P2X_5$ and $P2X_7$ were discovered. We have been able to show that activation of those receptors is responsible for ERK class kinase activity, responsible for regulation of cell proliferation pathway. We have also demonstrated that this activity is calcium dependent suggesting Ca^{2+} ions as secondary messenger between receptor and kinase regulatory system. More specifically, we do suspect that in C2C12 myoblasts calcium channels of P2X receptors, particularly $P2X_5$ play the main role in proliferation regulation. In further development of myoblasts into myotubes, when proliferation is gradually inhibited, the pattern of P2 receptors is changed. This phenomenon is followed by diminishing of the $P2Y_2$-dependent Ca^{2+} signaling, while the mRNA expression of $P2Y_2$ receptor reminds still on the high level. Moreover, $P2X_2$ receptor mRNA, absent in myoblasts appears in myotubes. These data show that differentiation of C2C12 cell line satellite myoblasts is accompanied by changes in P2 receptors expression pattern.

Abbreviations: 2MeSADP – 2-methylthio-ADP; BzATP – 3′-0(4-benzoyl)benzoyl ATP; $[Ca^{2+}]i$ – intracellular Ca^{2+} concentration; DMEM – Dulbecco's modified essential medium; ERK – Ras/extracellular signal-regulated kinase; FCS – fetal calf serum; GAPDH – glycerol 3-phosphate dehydrogenase; HS – horse serum; $InsP_3$ – inositol 1,4,5-trisphosphate; PLC – phospholipase C; PPADS – pirydoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid

Introduction

Skeletal muscle satellite cells are precursors of mammalian skeletal muscle multinucleated myotubes. The small population of these satellite cells posses *in vivo* a special ability to respond to myofibers damage. After muscle fiber injury, satellite cells are activated, start to proliferate, become committed to differentiation and fuse to form new myotubes [1–4]. Similar process of myotubes formation can be observed *in vitro* using primary culture of satellite cells [4–6], or satellite myoblast cell cultures [7].

Differentiation of muscle cells is regulated by extracellular growth factors that transmit signals into the cells [3]. Functional studies on satellite cells have shown that MAPK activity is involved in directing myogenesis [8–10]. Extracellular ATP acting trough P2X and P2Y purinergic receptors is also involved in this process [5, 11–13]. $P2X_{1-7}$ receptors are intrinsic ligand-gated ion channels and activation of these receptors by ATP evokes a flow of cations (Na^+, K^+ and Ca^{2+}) across the plasma membrane. P2Y receptors are G-protein coupled receptors linked to activation of phospholipase C (PLC), inositol lipid signalling and the mobilization of intracellular Ca^{2+} (for review see: [14–17]). Within the family of P2Y receptors $P2Y_1$ responds selectively to ADP. 2-methylthio-ADP (2MeSADP) is also a selective agonist of high potency for this receptor, while UTP is not effective. In contrast, $P2Y_2$ receptor responds to ATP and UTP, while 2MeSADP has no effect on its activation. $P2Y_4$ receptor responds to UTP, and $P2Y_6$ to UDP. In the case of $P2Y_{12}$ and $P2Y_{13}$ receptors responding to ADP, their coupling to G_i protein results in inhibition of cAMP formation [16, 18–20].

It is well established that ATP has potent effects on developing skeletal muscle. These effects are developmentally regulated and those responses have been shown to be characteristic for activation of the P2X purinergic receptors [11]. More recently, the expression of specific P2X receptor subtypes during skeletal muscle development [5, 11, 13] and in regenerating skeletal muscles [4] have been demonstrated. Among these receptors, $P2X_5$ and $P2X_6$, and $P2X_2$ and $P2X_5$, have been found to be expressed in chick and rat skeletal muscle development, respectively [5, 11].

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On the contrary, the expression and function of P2Y nucleotide receptors in developing skeletal muscle cells has rarely been studied. In chick muscle, the $P2Y_1$ receptor was implicated in the regulation of acetylcholine receptor [21]. The expression of this receptor was detected during the first 10 days of chick embryonic development [12]. In rat embryonic skeletal muscle cells, Cheung et al. [22] found similar early expression of the $P2Y_1$ receptor, whereas the $P2Y_2$ receptor expression became progressively stronger with development and the $P2Y_4$ receptor expression was similarly high at early and late embryonic days. In contrast, postnatal skeletal muscles from 3-week- or 2-month-old rats demonstrated the down-regulation of $P2Y_4$ receptor expression and $P2Y_1$ as well as $P2Y_2$ receptor expression were detected only in the small population of cells present between muscle fibers, tentatively identified as satellite cells [22].

In this study, using RT-PCR analysis we investigated the presence of P2Y and P2X receptors on cultured mouse satellite C2C12 cells and their involvement in ERK1/2 activation and intracellular Ca^{2+} mobilization. We compared the mRNA expression levels of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2X_{1-7}$ receptors in C2C12 myoblasts and myotubes and demonstrated that in myotubes the expression of all P2Y receptors mRNA decreased, except of $P2Y_2$ mRNA. $P2X_4$, $P2X_5$ and $P2X_7$ receptors are present both in myoblast and myotubes while receptor $P2X_2$ mRNA appears only in myotubes. We suspect that nucleotide can regulate myoblast proliferation and differentiation via both P2X and P2Y receptors, and particularly the ATP-responding $P2X_5$ and $P2Y_2$ receptor seem to be involved in these processes.

Materials and methods

Materials

Dulbecco's modified essential medium (DMEM), fetal calf serum (FCS), horse serum (HS) was from Gibco BRL. TRI reagent, ADP, ATP, UTP, UDP, 2MeSADP, BzATP, BSA, EGTA, Trypsin-EDTA solution, phosphate-buffered solution (PBS), ethidium bromide, agarose and common chemicals were purchased from Sigma Chemical Co. Penicilin was from Polfa Tarchomin Poland. Fura-2/AM was from Molecular Probes, Inc. Pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid (PPADS) was obtained from Research Biochemicals International. Expand RT enzyme was purchased from Roche. Taq PCR Core Kit was obtained from QIAGEN and M-MLV Reverse Transcriptase from Sigma Chemical Co.

Cell culture

C2C12 cells, a murine myoblast cell line was from the American Tissue Culture Collection, Rockville, USA, (ATCC) and was a kind gift from Prof Jerzy Moraczewski, Warsaw University, Warsaw, Poland. Cells were cultured in DMEM with high glucose (4500 g dm^{-3}) and Glutamax I, supplemented with 10% (vv^{-1}) FCS and penicilin (100 UI ml^{-1}) under humidified atmosphere of 5% CO_2 at 37 °C. The cells were passaged when reached state of about 60% confluency, and medium was changed three times a week. For experiments, cells were cultivated in DMEM supplemented with 10% FCS to reach 80% confluence: in 60-mm dishes (for Western blot analysis), in 100-mm dishes (for RT-PCR analysis), on 24-mm glass coverslips in 35-mm dishes (for calcium measurement). Medium was changed 12 h before each experiment. In case of calcium measurements, 30 min before the experiment, cells were washed once with PBS and once with the solution containing: 137 mM NaCl, 2.7 mM KCl, 1-mM Na_2HPO_4, 25 mM glucose, 20 mM HEPES (pH 7.4), 1-mM $MgCl_2$, 1% bovine serum albumin and 2-mM $CaCl_2$ (later referred as standard buffer). Only in experiments performed in the absence of external Ca^{2+}, 500 μM EGTA was added instead of 2 mM $CaCl_2$. In case of differetiation myoblasts to myotubes, cells were grown to 90% confluency and medium was changed to DMEM supplemented with 2% HS, which was changed third and fifth day of culture. Myotubes were harvested for experiments on fifth and seventh day of differentiation.

Measurement of intracellular calcium

Cells on coverslips were washed once with PBS and once with the standard buffer. The cells were incubated at 37 °C for 30 min in the standard buffer with 1 μm Fura-2 AM. Thereafter, the coverslips were mounted in a chamber over a Nikon Diaphot inverted-stage microscope equipped with a fluo × 40/1.3 NA oil-immersion objective lens. Fura-2 digital fluorescence microscopy was used to determine the changes in intracellular calcium levels ($[Ca^{2+}]_i$) [23]. Ludl Lep MAC 5000 filter wheel system loaded with Chroma Inc. Fura-2 filter set was used for illumination of specimens. Images were acquired using Retiga 1300 chilled digital CCD camera (QImaging Inc.). Data processing was carried out using AQM Advance 6 (Kinetic Imaging Inc) and MS Excel software.

Preparation of cell extracts and Western blot analysis of phospho-ERK1 and ERK2

For these experiments cells were plated in 60-mm dishes and were incubated with agonists in DMEM medium at 37 °C for various times. When antagonists were used, they were applied 2 min prior to the addition of agonists. The reaction was stopped by aspiration of the medium, washing once with ice-cold PBS and addition of lysis buffer: 50-mM Tris/HCl pH 7.5, 1% (wv^{-1}) Nonident NP-40, 120-mM NaCl, 25-mM NaF, 40-mM β-glycerol phosphate, 0.1-mM Na_3VO_4, 1-mM phenyl methyl sulfonyl fluoride (PMSF), 1-mM benzamidine. Cell extracts were then centrifuged 12,000 *g* for 30 min at 4 °C and supernatants were transferred to new tubes. Protein concentration was measured by Bradford method [24]. Then samples were mixed with 4 times concentrated Leammli sample buffer: 200-mM Tris/HCl pH 6.8, 2% (wv^{-1}) SDS,

0.4% (wv^{-1}) bromophenol blue, 40% (vv^{-1}) glycerol and 2% (vv^{-1}) 2-mercaptoethanol. After boiling for 5 min, cell lysates were analysed by SDS-PAGE on 10% (wv^{-1}) polyacrylamide gel. Proteins were blotted onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). Immunodetection was performed according to the manufacturer's instructions using rabbit antibodies raised against phosphorylated p44 (ERK1) and p42 (ERK2) (Cell Signalling Technology). Primary antibodies were detected with a horseradish peroxidase conjugated mouse anti-rabbit antibody, and visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Equal amount of protein loaded was detected with anti-actin antibody (Sigma Chemical Co).

Detection of P2Y receptors mRNA and RT-PCR analysis

Total RNA was extracted using TRI reagent. Reverse transcription of total RNA was performed using Expand RT enzyme. Specific primers for PCR reaction were designed using 'DNA Star' software (DNA Star Inc., USA). All primers were based on unique sequences comprising bases in case of: $P2Y_1$: (1100–1123) 5′ GTG GCG TGG TGT ACC CTC TCA AGT 3′ (forward), (1666–1650) 5′ TGG TGG CTC GGG ACA GT 3′ (backward), product length 566 bp (Acc. No. NM_008772); $P2Y_2$: (229–250) 5′ GCT GCC GGT GCG CTG ATG AAC T 3′ (forward), (759–736) 5′ CAC CCC GGG CGT AGT AAT AAA CCA 3′ (backward), product length 530 bp (Acc. No. NM_008773); $P2Y_4$: (549–572) 5′ TGC CCA CCC TCG TCT ACT ACT ATG 3′ (forward), (1016–993) 5′ GAG AAC GGA GCC GAG AAG ATG ACT 3′ (backward), product length 468 bp (Acc. No. NM_02062); $P2Y_6$: (161–177) 5′ CCC GCC GGA CCC TGA CC 3′ (forward), (652–633) 5′ GGC GGG CCA TGC GAC AGT AG 3′ (backward), product length 491 bp (Acc. No. AF298899); $P2Y_{12}$: (342–362) 5′ 5′CTT TGC TGG GCT CAT CAC GAA 3′ (forward), (744–721) 5′ CTT ATC TTT TGG CCT CCT GTT GGT 3′ (backward), product length 401 bp (Acc. No. NM_027571). As a reference control glycerol 3-phosphate dehydrogenase (GAPDH) was used. The estimated product length was 900 bp, unique sequence: (581–600) 5′ ACC ACA GTC CAT GCC ATC AC 3′ (forward), (1032–1013) 5′ TCC ACC ACC CTG TTG CTG TA 3′ (backward) (Acc. No. BC083149). Equal amounts of cDNA were used in parallel experiments. A hot-start PCR protocol was used, involving denaturation at 95 °C, annealing at 57.8 °C ($P2Y_1$, 32 cycles), at 61.6 °C ($P2Y_2$, 34 cycles), at 59.0 °C ($P2Y_4$, 33 cycles), at 60.6 °C ($P2Y_6$, 34 cycles), at 54.9 °C ($P2Y_{12}$, 32 cycles), at 65.0 °C (GAPDH, 32 cycles). PCR products were separated on 1% agarose gel, stained with ethidium bromide and quantified using ImageQuant and MS Excel software.

Detection of P2X receptors mRNA and RT-PCR analysis

Total RNA was extracted using TRI reagent. Reverse transcription of total RNA was performed using M-MLV Reverse Transcriptase. Specific primers for PCR reaction were designed using 'Primer 3' software. All primers were based on unique sequences comprising bases in case of: $P2X_1$: (927–946) 5′ TGG TTG GTA TCA CCA TCG AC 3′ (forward), (1009–1028) 5′ CAG GTT CTT CTC CCC GTA CA 3′ (backward), product length 101 bp (Acc. No. NM_153400); $P2X_2$: (898–917) 5′ CAA CAT TGC AAG CCA GAA GA 3′ (forward), (1012–1032) 5′ TTG TGT GCC AGT TCT GTG AAG 3′ (backward), product length 135 bp (Acc. No. NM_153400); $P2X_3$: (762–781) 5′ CCT TCC TAA CCT CAC CGA CA 3′ (forward), (854–873) 5′ TCC TGC CCA GCA AAC TTA AC 3′ (backward), product length 111 bp (Acc. No. NM_145526); $P2X_4$: (97–116) 5′ GGC TTT CCT GTT CGA GTA CG 3′ (forward), (186–205) 5′ CCA ATG ACG TAA GCC AGG AT 3′ (backward), product length 108 bp (Ac. No. NM_011026); $P2X_5$: (210–233) 5′ ATG GGT GTT TCT GAT AAA GAA GAG 3′ (forward), (338–360) 5′ TGA GAC GGA ATG ACA AAG TCT G 3′ (backward), product length 150 bp (Ac. No. NM_033321); $P2X_6$: (213–232) 5′ GAC TTG GCC CCT CAG ACT TC 3′ (forward), (297–316) TTC ACA AAG TCA GCC ACG TC 3′ (backward), product length 104 bp (Acc. No. NM_011028); $P2X_7$: (127–146) 5′ TTT GCT TTG GTG AGC GAT AA′ (forward), (247–267) GGG AAG GTG TAG TCT GCA GTG 3′ (backward), product length 161 bp (Acc. No. NM_011027); As a reference control glycerol 3-phosphate dehydrogenase (GAPDH) was used. The estimated product length was 900 bp, unique sequence: (581–600) 5′ ACC ACA GTC CAT GCC ATC AC 3′ (forward), (1032–1013) 5′ TCC ACC ACC CTG TTG CTG TA 3′ (backward) (Acc. No. BC083149). Equal amounts of cDNA were used in parallel experiments. A hot-start PCR protocol was used, involving denaturation at 95 °C, annealing at 60 °C (30 cycles), at 65.0 °C (GAPDH, 32 cycles). PCR products were separated on 2% agarose gel, stained with ethidium bromide.

Results

C2C12 myoblasts, a mouse satellite cell line, were differentiated to myotubes by transferring the cells from high (10% FCS) to low (2% HS) serum growth medium. The alterations in the morphology of myoblasts, differentiated to myotubes 7 days after changing the serum growth medium is presented in Figure 1 (Figures 1A and B, respectively).

In order to find out whether the differentiation of C2C12 cells may have an impact on the mRNA expression of P2Y and P2X receptors, the mRNA level of these receptors was measured using RT-PCR method. As a quantitative control mRNA of constitutively active GAPDH gen product was used. Figure 1 shows that all, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ as well as $P2X_4$, $P2X_5$ and $P2X_7$ receptors mRNA were expressed in both myoblasts and myotubes. In myoblasts, the mRNA expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors was strong, while $P2Y_6$ and $P2Y_{12}$ weak, and the

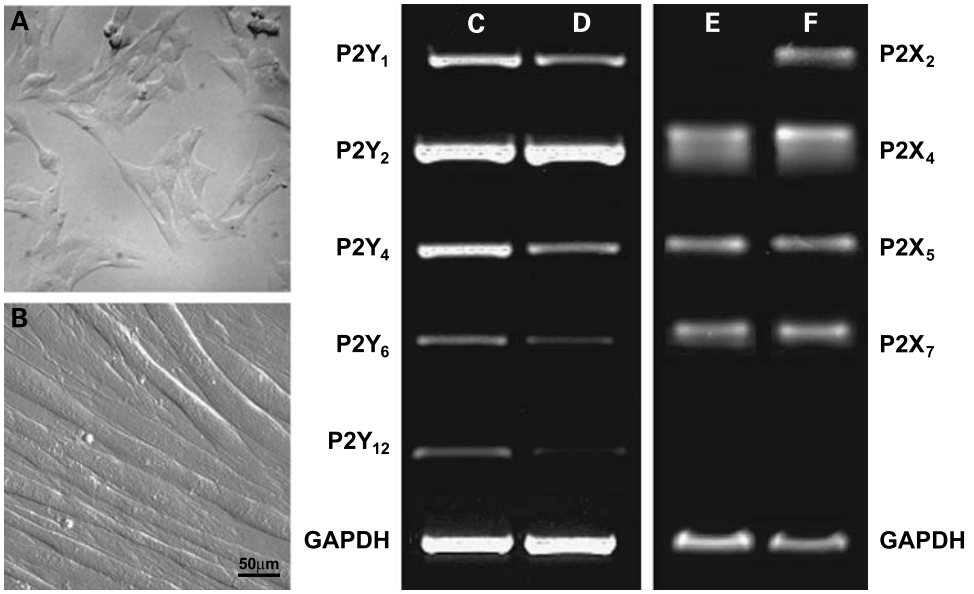

Figure 1. Seven days of C2C12 cells differentiation leads to changes in morphology from myoblast (A) to myotubes (B) as observed in light microscopy (DIC Nomarski). Morphological changes are accompanied by changes in P2Y (myoblasts – C and myotubes – D) and P2X (myoblasts – E and myotubes – F) mRNA receptors expression. GAPDH measured as constitutively active control. For details, see Materials and methods. Experiments were repeated four times.

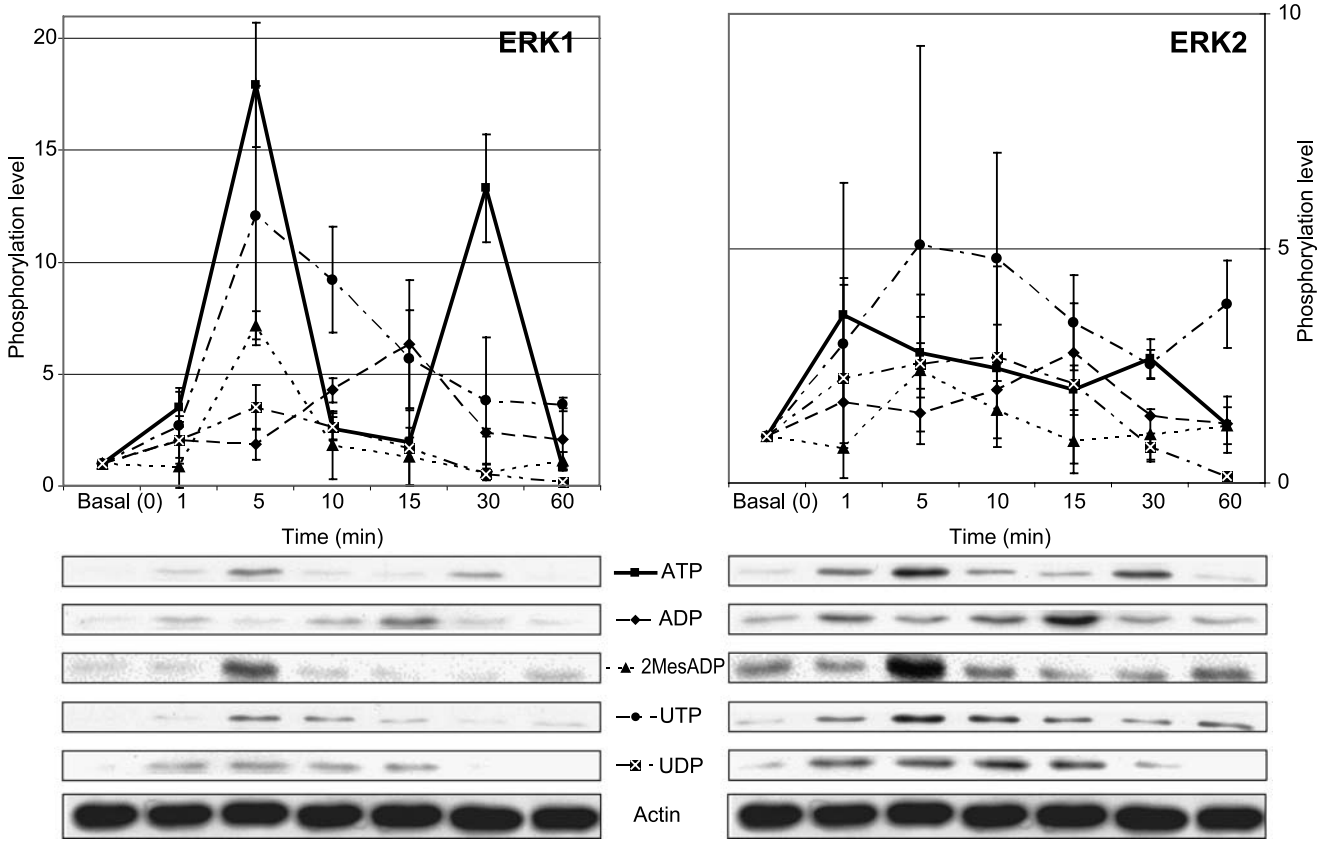

Figure 2. Time-dependent activation of p44/42 ERK1/ERK2. C2C12 myoblasts were grown as described in Materials and methods. The cells were stimulated with specific purinergic receptor ligands (see legend) and ERK activation was measured by immunoblotting (*lower panel*) and quantified by densitometry (*upper panel*). Note, that ERK2 phosphorylation level is generally higher, while ERK1 phosphorylation shows stronger dynamics. Actin was used as a control. Experiments were conducted on three separate occasions. All measurements were made in three repetitions; error *bars* on the plot represent ±S.D. Concentration of used ligands: ATP 100 μM, ADP 10 μM, 2MeS ADP 10 μM, UTP 100 μM and UDP 100 μM.

strongest mRNA expression was found for $P2Y_2$ receptor (Figure 1C). However, after myoblasts differentiation to myotubes, $P2Y_1$ and $P2Y_{12}$ (ADP sensitive), $P2Y_4$ (UTP sensitive) and $P2Y_6$ (UDP sensitive) mRNA expression was distinctly decreased (Figure 1D). Only the level of the ATP sensitive, $P2Y_2$ receptor mRNA expression was not changed (Figure 1C *versus* D). In C2C12 cells mRNA for $P2X_1$, $P2X_3$ and $P2X_6$ receptors was not present (not shown). PX_4, $P2X_5$ and $P2X_7$ mRNA was found both in myblasts and myotubes. $P2X_2$ mRNA was absent in myoblasts and appeared after 7 days of differentiation to myotubes (Figure 1E *versus* F).

To determine whether in myoblasts, P2Y receptors are involved in ERK1/2 activation, the cells were stimulated with ATP (100 μM), ADP (10 μM), 2MeSADP (10 μM), UTP (100 μM) and UDP (100 μM), and phosphorylation status of p44/Erk1 and p42/Erk2 was monitored by Western blot analysis. As shown in Figure 2, all nucleotides induced a transient increase in the level of p42 and p44 phosphorylation and in any case p42/ERK2 phosphorylation (also a basal level) was higher than that of p44/ERK1 (see Western blots). Densitometric analysis of the blots revealed that, within 5–15 min, these compounds induced the highest increase in the phosphorylation level. In comparison to the basal level, representing ERK1/2 phosphorylation in the absence of receptor agonists, changes in phosphorylation were more pronounced in ERK1 (Figure 2, left panel). Stimulation of cells with UDP produced the lowest phosphorylation level of ERKs. In the case of ATP, an additional peak of ERK1 phosphorylation, after 30 min of the cells stimulation was also observed (Figure 2, left panel). The effect of ATP may be explained by the activation of more than one, different P2 receptors.

To examine whether the effect of nucleotides on p44/p42 ERK1/ERK2 activation was Ca^{2+} dependent, myoblasts were placed in calcium-free medium and ERK1/ERK2 activity was measured after 5 min cells treatment with 2MeSADP, UTP and ATP or 15 min with ADP (Figure 3). As shown, extracellular Ca^{2+} removal strongly inhibited ERK1/2 nucleotides activation. These data pointed out the significant role of Ca^{2+} in ERK1/2 activation.

In the subsequent experiments influence of ATP on ERKs activation in C2C12 myotubes was examined. Figure 4A shows that ATP (100 μM) stimulated a rapid and transient increase in the phosphorylation level of the active ERK1/ERK2 in myotubes. Stimulation with ATP reached the highest increase after 5 min. The second peak of ERK activation (characteristic for myoblasts) was not observed and after 30 min, ERK activation returned to the control level.

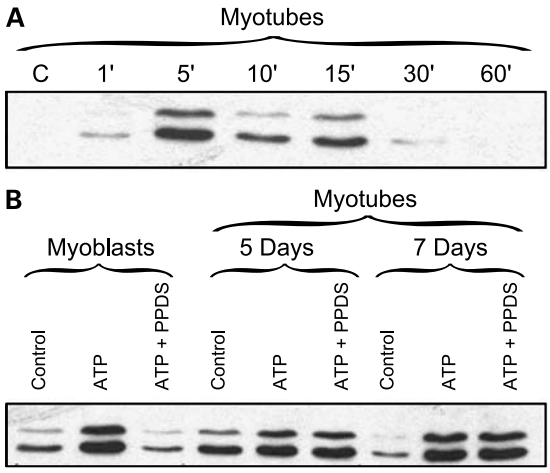

Figure 4. (A) ATP (100 μM) stimulates ERK I/II kinase phosphorylation in myotubes. Phosphorylation measurements were made after 0–60 min of incubation in ATP solution. (B) Effect of 2 min preincubation with PPADS (100 μM) on ERK activation in myoblasts by ATP (100 μM), measured after 5 min of incubation in ligand solution (*left*). Effect of the same treatment with PPDS on myotubes: 5 days in differentiating medium (*center*) and 7 days in differentiating medium (*right*). For details, see Materials and methods. Experiments were repeated three times.

Since ATP can activate $P2Y_2$ and P2X receptors, the cells (myoblasts as well as myotubes) were pretreated 2 min with 100 μM pyridoxal-5-phosphate-6-azophenyl-2′, 4′-disulfonic acid (PPADS) and then treated for 5 min with 100 μM ATP. PPDS is well known antagonist of P2X2 and P2X5 receptors, whereas $P2X_4$ and $P2Y_2$ are insensitive to this compound [14]. Figure 4B shows that preapplication of PPADS resulted in almost complete inhibition of ERKs activation in myoblasts, indicating the role of P2X receptors in this process. On the contrary to myoblasts, PPADS treatment of myotubes differentiated for 5 or 7 days (time after changing culture medium to differentiating medium) did not inhibit ATP-induced ERKs activation

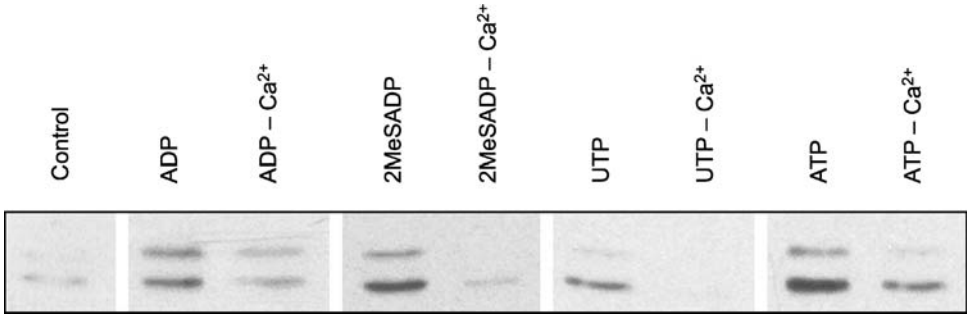

Figure 3. Presence of extracellular calcium is required for effective ERK kinase activation. Control shows ERK phosphorylation level in unstimulated cells. Note that chelation of extracellular calcium by 0.5 mM EGTA in the absence of $CaCl_2$ in medium lowers ERK phosphorylation level slightly higher than that in control cells. For details, see Materials and methods. Ligand concentrations: ATP 100 μM, ADP 10 μM, 2MeS ADP 10 μM and UTP 100 μM. Experiments were repeated three times.

(Figure 4B). Figure 4B data are results of three independent experiments and the level of control ERK phosphorylation are uncomparable between experimental setups.

The effect of ATP on intracellular Ca^{2+} mobilization in C2C12 myoblasts and myotubes is shown in Figure 5. Myoblasts were placed in the medium containing 2-mM $CaCl_2$ (Figure 5A) or in Ca^{2+}-free medium (Figure 5B). Figure 5A (solid line) shows that 100-μM ATP-induced Ca^{2+} elevation started only with an initial peak response. In the presence of PPADS (100 μM) (Figure 5A, dashed line),

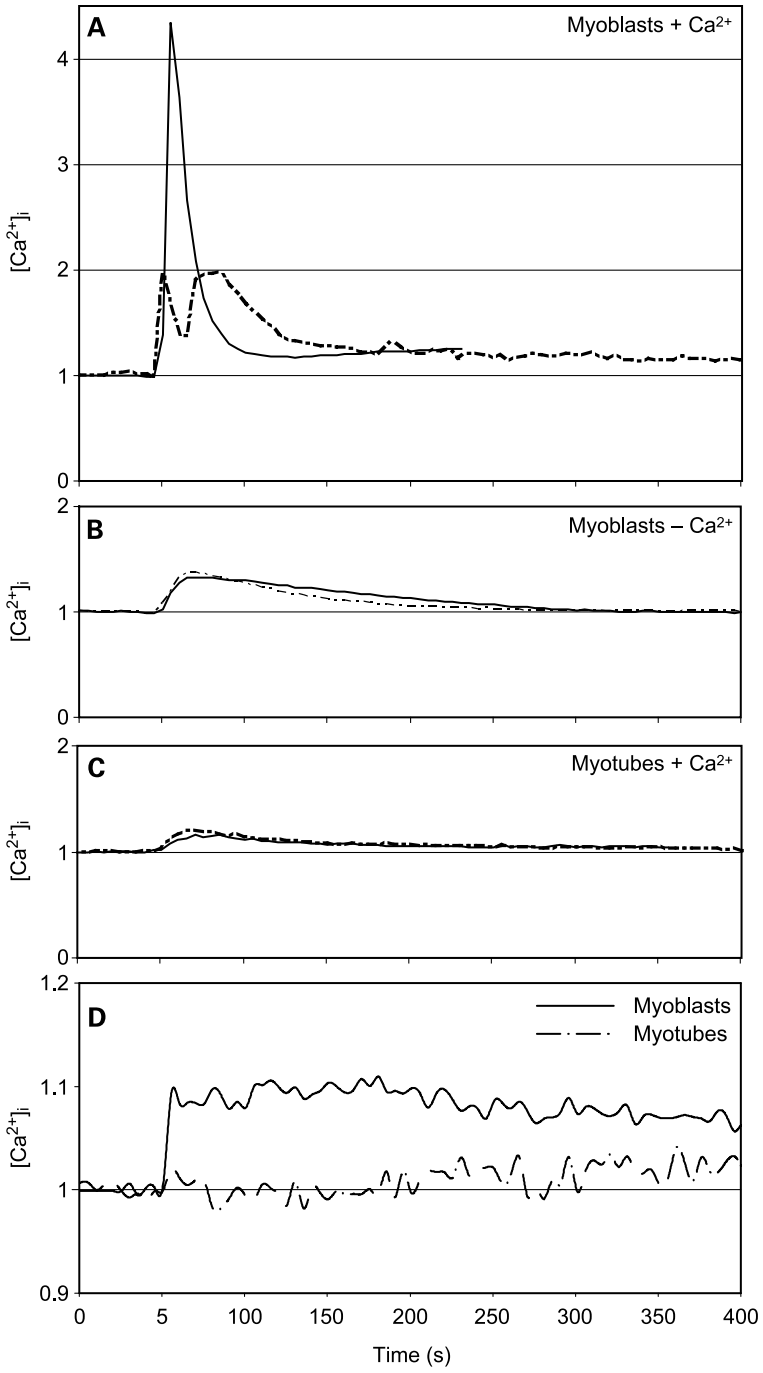

Figure 5. Calcium response of C2C12 myoblasts and myotubes. Plots of $[Ca^{2+}]_i$ measured as 340/380 nm induced fluorescence of Fura-2 ratio changes against time. Each trace in this figure represents the mean ratio value of the Ca^{2+} response. Data shown for one representative experiment of three repetitions performed for each setup. (A) Two-minute preincubation with PPADS (100 μM) inhibits myoblast calcium response to ATP (100 μM) as shown on the plot. Note, that cell calcium response in presence of PPADS (*dashed line*, mean from nine cells) is not only lowered but also biphasic and longer than response observed in absence of PPDS (*solid line*, mean from nine cells). This shape suggests metabotropic nature of response while sharp response is characteristic for ionotropic phenomena. (B) The simillar, slow Ca^{2+} response to 100 μM ATP (*solid line*) observed in myoblasts after extracellular calcium depletion by 0.5 mM EGTA in absence of $CaCl_2$. The response is now independent from PPADS inhibition (*dashed line*); both plots show the mean from nine cells. (C) Myotubes response to ATP is similar to that of myoblasts in absence of extracellular calcium. Responses are long and relatively weak and there is no difference between presence (*dashed line*, mean from five cells) and absence of PPADS (*solid line*, mean from nine cells). (D) Myoblasts and myotubes response to BzATP (100 μM), $P2X_7$ receptor specific agonist. Myoblasts – *solid line* (mean from 16 cells) and myotubes – *dashed line* (mean from four cells). The response to BzATP was so weak that the vertical axis scale on this plot had to be changed.

the kinetic of ATP-evoked Ca^{2+} changes differed from those observed without addition of this compound. In the presence of PPADS, ATP initiated Ca^{2+} response much weaker and biphasic, consistent with the PLC-mediated Ca^{2+} release from the endoplasmic reticulum Ca^{2+} stores (the first phase of calcium response) and capacitative Ca^{2+} enter from the extracellular space (the second phase). Such response is typical for metabotropic-G_q protein coupled receptors [25–27]. Furthermore, the intracellular Ca^{2+} concentration generated by ATP in the presence of PPADS (Figure 5A, dashed line) was at least three times lower than that induced by ATP alone (Figure 5A, solid line). In the absence of extracellular Ca^{2+}, ATP was also able to induce intracellular Ca^{2+} mobilization, resulting from the direct action of inositol 1,4,5-trisphosphate ($InsP_3$) on the endoplasmic reticulum stores. Addition of PPADS did not change this response (Figure 5B). Figure 5C illustrates C2C12 myotubes Ca^{2+} response induced by ATP in the cells pretreated (Figure 5C, dashed line), or not pretreated (Figure 5C, solid line) with PPADS. As it is shown, ATP, both in the absence and the presence of PPADS generated similar elongated Ca^{2+} response, characteristic to metabotropic receptors and P2X receptors antagonist (PPADS) did not influence this process (Figure 5C, solid and dashed lines). ATP-induced Ca^{2+} response was much weaker in myotubes than in myoblasts (Figure 5). Moreover, similar Ca^{2+} response in myotubes was induced by UTP (data not shown).

α, β-methylene ATP (100–600 μM), a $P2X_1/P2X_3$ agonist produced no Ca^{2+} response (data not shown) what is coherent with the lack of mRNA for these receptors both in myoblasts and myotubes. Figure 5D shows the effect of 100-μM 3′-0(4-benzoyl)benzoyl ATP (BzATP) application on myoblasts and myotubes calcium response. BzATP is a specific agonist of the $P2X_7$ receptor, which is 10–100 times more sensitive to this ATP analogue then to ATP itself [14]. While myoblasts clearly responded to addition of BzATP, myotubes did not react at all. The presence of calcium response to BzATP in myoblasts is in agreement with the presence of $P2X_7$ mRNA in those cells whereas in myotubes, the $P2X_7$ receptor seemed not to be coupled to calcium signal. It is worthy adding that response to BzATP was so weak that we have had to change the vertical axis scale to show changes, while all other plots on this figure (Figure 5A–C) have the same scale to make comparison of responses easier.

Discussion

In this study we investigated the presence of the different P2Y receptor subunits on the mouse myoblast satellite cell line, C2C12, and on myoblasts differentiated to myotubes, 7 days after transfer into differentiating medium. Using RT-PCR we have demonstrated that C2C12 myoblasts did express a wide range of P2Y receptors – $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$. After differentiation to myotubes, $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ receptors mRNA expression distinctly decreased. In contrast, the $P2Y_2$ receptor mRNA expression was not affected in myotubes. Differentiation into myotubes did not change the level of $P2X_4$, $P2X_5$ and $P2X_7$ mRNA expression whereas $P2X_2$ mRNA absent in myoblasts appears in myotubes.

These results are generally similar to those presented by Ryten et al. [4], who used primary cultures of satellite skeletal myoblasts maintained in the medium with 5% HS to form myotubes. Similarly to our data, the authors showed the presence of the $P2X_5$ receptor in myoblasts and myotubes, whereas the $P2X_2$ receptor was absent in myoblasts and appeared in myotubes only from fifth day of differentiation. The authors also found that myoblasts contained the $P2Y_1$ receptor which disappeared after 5 days of differentiation, whereas in our experiments the level of its expression was only lowered. The difference is the $P2Y_4$ receptor, which appeared only after plating in Ryten experiments [4], while in our results was present in myoblasts and diminished in myotubes. At all these time points, the $P2Y_2$ receptor was not detectable in Ryten data whereas in our data $P2Y_2$ mRNA both in myoblasts and myotubes was always the most aboundant one. Moreover, in C2C12 myotubes, Henning et al. [28, 29] demonstrated the activity of this metabotropic $P2Y_2$ receptor. Thus, some discrepancy between our results and the results of Ryten et al. [4] may be caused by the different origin of the cultured cells. Nevertheless, both papers demonstrated dynamic expression of P2Y and P2X receptors.

ERK1/2 signalling cascade have been implicated in myoblast proliferation [30, 31]. The present study shows that the treatment of C2C12 myoblasts with ATP, ADP, 2MeSADP, UTP and UDP caused a rapid and transient increase in the level of phosphorylated ERK1/2. This process was fully Ca^{2+} dependent. Application of ATP to myotubes caused also a significant increase in ERK1/2 phosphorylation. Furthermore, the effect of ATP on ERKs activation in myoblasts may be inhibited by preaplication of PPADS, known as an antagonist of $P2X_2$ and $P2X_5$ receptors but not $P2X_4$ and $P2Y_2$ receptors [14, 16, 19, 32]. Such effect of PPADs was not observed in myotubes. Hence, these results suggest that although the $P2Y_2$ receptor is expressed in C2C12 myoblast cells, $P2X_5$ receptors is primarily responsible for the observed ATP effects since $P2X_2$ is absent in myoblasts. On the other hand the myotubes are not PPDS sensitive even though both $P2X_2$ and $P2X_5$ receptor mRNA is present in those cells. Since ERK 1/2 activation is strongly dependent on Ca^{2+}, these results could suggest that in myotubes P2X receptors might be permeable to some other than Ca^{2+} cations [33].

These conclusions are strongly supported by the difference in the kinetic of ATP-evoked Ca^{2+} responses examined in C2C12 myoblasts in the presence and absence of PPADS, or extracellular Ca^{2+}. In the presence of extracellular Ca^{2+} and absence of PPADS, the high and rapid Ca^{2+} response was most probably generated by P2X receptors, particularly by the $P2X_5$ receptor. In general, the Ca^{2+} influx generated by ligand-gated ionotropic receptors is much quicker than that mediated by metabotropic-G_q protein coupled receptors [34]. In addition, the rapid increase in

intracellular Ca^{2+} concentration can inhibit $InsP_3$ receptor and stops the metabotropic receptor action [35, 36]. Thus, one can expect that when ATP stimulates a cell that contains P2X and P2Y receptors, a P2X, no P2Y receptor will be primarily involved in Ca^{2+} mobilization. On the other hand, when P2X receptors are antagonized with PPADS, or when the cells are examined in the absence of extracellular Ca^{2+}, then the $P2Y_2$ receptor activity may still lead to the increase in the intracellular Ca^{2+} concentration. Such ATP-induced Ca^{2+} response difference has been observed herein.

In C2C12 myotubes, ATP generated similar, however very weak Ca^{2+} response in the presence and absence of PPADS, and PPADS did not reduced ATP-induced ERKs activation. These results suggest the lack of P2X receptor activity in these cells. Similarly, in developing chick skeletal muscle, $P2X_5$ and $P2X_6$ receptors were only expressed at early stages of skeletal muscle development. Their expression disappeared immediately before the stage at which fusion of myoblast to form myotubes occurred [11]. Furthermore, as we have already mentioned, in C2C12 myotubes, Henning et al. [28, 29] demonstrated the presence of the metabotropic $P2Y_2$ receptor, responding to ATP and UTP and causing a biphasic rise in intracellular Ca^{2+} concentration by the release of Ca^{2+} from the internal stores and activation of Ca^{2+} entry from the extracellular space.

Thus, it seems that during C2C12 myoblast developing, P2X receptors are the first to be expressed and first to be down regulated. While in C2C12 myoblasts an ATP-sensitive $P2X_5$ receptor is primarily responsible for Ca^{2+} mobilization and ERK1/2 activation, in myotubes this role may play the $P2Y_2$ receptor, which mRNA is still expressed in myotubes. Our data agree with thesis that $P2X_5$ receptor plays the crucial role in regulation of shift between myoblast proliferation and differentiation [4, 5]. However, we should be aware that C2C12 cell line never completely differentiates. Therefore P2Y receptors may be still active in those cells while in primary lines they do disappeared during further differentiation into myofibers [22]. It is also worth adding that we have recently shown that serum withdrawal for 48 h had effects on glioma C6 cells morphology, which changed from fibroblast-like to astrocyte-like appearance. Simultaneously with this differentiation-like process, the $P2Y_1$ mRNA expression strongly decreased and the $P2Y_{12}$ mRNA slightly increased [32, 37]. Hence, we do believe that changing pattern of expression and activity of purinergic receptors may be involved in cell differentiation control and should be further studied in various cell types.

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