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Extracellular ATP activates ERK1/ERK2 via a metabotropic $P2Y_1$ receptor in a Ca^{2+} independent manner in differentiated human skeletal muscle cells

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Abbreviations:
PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate
A3P5P, adenosine 3′,5′-diphosphate
XAC, xanthine amine congener
U73122, 1-(6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl-1H-pyrrole-2,5-dione
U73343, 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione
2-MeSATP, 2-methylthioadenosine 5′-triphosphate
2-MeSADP, 2-methylthioadenosine 5′-diphosphate
CPK, creatine phosphokinase
CP, creatine phosphate

ABSTRACT

ATP is released at the neuromuscular junction to regulate development and proliferation. The sequential expression of P2X and P2Y receptors has been correlated to these effects in many species and cell lines. We have therefore investigated ATP mediated signalling in differentiated primary human skeletal muscle cells. ATP was capable to trigger Ca^{2+} transients in these cells via P2Y receptors which were not attributable to Ca^{2+} influx via P2X receptors. Instead, ATP propagated the formation of inositol phosphate (IP) with an EC_{50} of 21.3 μM. The Ca^{2+} transient provoked by ATP was abrogated roughly 75% by the phospholipase C (PLC) inhibitor, U73122. Interestingly, the ryanodine sensitive Ca^{2+} pool was not involved in ATP triggered Ca^{2+} release. On mRNA level and by a pharmacological approach we confirmed the presence of the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Substantially, ATP activated IP formation via a $P2Y_1$ receptor. In addition, ATP elicited extracellular signal regulated kinase (ERK)1/2 phosphorylation in a time and concentration dependent manner, again mainly via $P2Y_1$ receptors. The ATP mediated ERK1/2 phosphorylation was strictly dependent on phospholipase C and PI3 kinase activity. Importantly, ATP mediated ERK1/2 phosphorylation was Ca^{2+} independent. This observation was corroborated by the finding that conventional protein kinase C inhibitors did not suppress ATP triggered ERK1/2 phosphorylation. Taken together, these observations highlight the importance of ATP as a co-neurotransmitter at the neuromuscular junction via dual signalling, i.e. IP_3 receptor mediated Ca^{2+} transients and Ca^{2+} insensitive phosphorylation of ERK1/2.

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ERK1/2, extracellular signal-regulated kinase 1/2
PLC, the phospholipase C
IP_3, inositol 1,4,5-triphosphate
IP, inositol phosphate
RB, reactive blue 2
$[Ca^{2+}]_i$, intracellular calcium
MRS2179, 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate
LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one

1. Introduction

Extracellular ATP participates in important biological processes like neurotransmission in the peripheral and central nervous system, the regulation of cardiac function, platelet aggregation, immune response and pain [1–3]. Neurotransmitter release, as far as we understand it today, is the release of a cocktail of signalling compounds which act on pre- and post-synaptic membranes. In the nerve terminals from vertebrate skeletal muscles, ATP is stored and released together with acetylcholine in a ratio of ~1:5 [4]. This implies that physical exercise is accompanied by the pulsatile release of ATP.

P2 purinoceptors form the principal extracellular target for ATP [3]. P2X receptors are ligand gated ion channels, which lead to a flux of cations (Na^+, K^+ and Ca^{2+}) across the plasma membrane when activated [5]. P2Y receptors are G protein coupled receptors, which mainly activate phospholipase C, leading to the formation of inositol 1,4,5-triphosphate (IP_3) and mobilization of intracellular calcium ($[Ca^{2+}]_i$) (for reviews see [3,5]). To date, seven isoforms of P2X receptors ($P2X_{1–7}$) and eight cloned subtypes of the P2Y family: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ [6,7] and $P2Y_{14}$ [8] have been identified.

The pattern of purinergic receptor expression on skeletal muscle varies with species and developmental stage. The sequential expression of the $P2X_2$, $P2X_5$ and $P2X_6$ receptors on developing rat skeletal muscle and the expression of the $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors on developing chicken myoblasts have been shown in great detail [9–12]. All these observations confirm a role of P2X receptors during skeletal muscle development. Immediately after birth, the expression level of P2X receptors in mammalians is reduced and functionally often not detectable [12,13]. The $P2X_7$ receptor was found to be present in moderate amount by Northern blotting [14].

In addition to the ionotropic P2X receptors, ATP may also exert P2Y receptor mediated signalling in skeletal muscle cells. ATP modifies chick and mouse myotubes towards higher performance of muscle contraction by increasing the expression of acetylcholine receptor and acetylcholine esterase. These effects were attributable to a $P2Y_1$ receptor [15]. Recently, differentiated skeletal muscle C2C12 cells were shown to be positive for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on RNA level [13]. Data on the P2Y receptor expression on human skeletal muscle cells are limited. A detailed characterisation of purinergic receptor neurotransmission is considerably complicated by the existence of different subtypes of receptors, present in tissue or on primary cells. Nevertheless, with respect to signalling, especially excitation–contraction coupling differentiated human skeletal muscle cells are suitable model system to investigate purinergic signalling comparable to intact muscle [4,9,10]. On the transcriptional level, the $P2Y_1$ and $P2Y_2$ receptors were identified in human skeletal muscle [16,17]. Functionally, Cseri et al. showed that ATP is capable to stimulate an increase in $[Ca^{2+}]_i$ in human myotubes [18]. The individual receptors responsible for these observations were elucidated as P2X receptors, as the response was strictly dependent on extracellular Ca^{2+}.

Recent findings have also highlighted a Ca^{2+} influx via P2X receptors into C2C12 cells which contributed to the phosphorylation of ERK1/2 in a differentiation dependent manner [13]. There is little known about P2Y receptor signalling in human skeletal muscle but there is clear evidence that physical exercise enhances the phosphorylation of ERK1/2 [19]. Therefore, we investigated the ATP triggered signalling cascades in differentiated human skeletal muscle cells using different methods. The extracellular application of ATP evoked a Ca^{2+} transient which was mediated by P2Y receptors. This signalling cascade also leads to the activation of ERK1/2, which suggests that extracellular ATP participates in skeletal muscle plasticity.

2. Materials and methods

ATP, adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), 2-methylthioadenosine 5′-triphosphate (2-MeSATP), 2-methylthioadenosine 5′-diphosphate (2-MeSADP), adenosine 3′, 5′-diphosphate (A3P5P), creatine phosphokinase (CPK), creatine phosphate (CP), xanthine amine congener (XAC), reactive blue 2 (RB), 1-(6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl-1*H*-pyrrole-2,5-dione (U73122), 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), L-glutamine, penicillin, streptomycin, gentamycin, dexamethasone, amphotericin B und insulin were obtained from Sigma–Aldrich GmbH (Vienna, Austria). Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) were purchased from

Calbiochem (San Diego, CA). The [^{3}H]myo-inositol was supplied by Perkin-Elmer Life Sciences Inc. (Boston, MA, USA). 2′-Deoxy-N^6-methyl adenosine 3′,5′-diphosphate (MRS2179) was purchased from Tocris (Avonmouth, UK).

2.1. Purity of nucleotides

In order to eliminate contamination with diphosphates, ATP, 2-MeSATP and UTP stock solutions were pre-treated in a buffer (2.5 mM $MgCl_2$, 50 mM HEPES/NaOH, pH 7.3) with 20 U/ml creatine phosphokinase (CPK) and 10 mM creatine phosphate at room temperature for 90 min and 180 min for UTP, respectively [20]. Similarly, ADP, 2-MeSADP and UDP were treated with 20 U/ml yeast hexokinase in the same buffer to remove contaminating triphosphates [21]. In some experiments 2 U/ml CPK, 5 mM CP and 2.5 mM Mg^{2+} were supplemented in the assay buffer.

2.2. Cell culture

The waste material (200–400 mg) of skeletal muscle biopsies from fifteen healthy individuals was used for isolation of human satellite cells. The procedure was approved by the local ethics committee according to the code of Ethic of the World Medical Association. The tissue was treated according to a previous protocol [22] and the skeletal muscle cells finally seeded in growth medium (GM; Ham's F12 medium supplemented with 15% fetal calf serum 10 ng/ml EGF, 200 ng/ml insulin, 400 ng/ml dexamethasone, 0.5 mg/ml fetuin, 0.5 mg/ml BSA, 7 mM glucose, 4 mM glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin, 2.5 μg/ml amphotericin B). At a confluency of approximately 60% the cells were switched from GM to differentiation medium (DM; DMEM supplemented with 5% horse serum, and 4 mM glutamine, 100 ng/ml insulin, 0.1 μg/ml gentamicin). Cells were kept at 37 °C under 5% CO_2.

For Ca^{2+} imaging and electrophysiology, undifferentiated cells were reseeded on 25 mm glass cover slips, coated with fibronectin (12.5 μg/ml). Adherent cells were exposed to DM to promote fusion of myogenic satellite cells to myotubes [22]. The cells were typically used after 6–10 days of differentiation.

2.3. RT-PCR

Total RNA from differentiated human skeletal muscle cells was isolated, treated with DNAse I and reverse transcribed into cDNA. Aliquots of the cDNA were used as a template for PCR amplification with specific primers for human P2Y receptors according to Adrian et al. [23] and Vonend et al. [24]. The specific amplification reactions were done with Taq DNA Polymerase. All enzymes were from Roche (Indianapolis, IN, USA).

2.4. Ca^{2+} fluorescence measurements

Differentiated human skeletal muscle cells grown on cover slips were used for Ca^{2+} imaging, as described previously [22]. For dye loading, the cells were incubated in Tyrode's solution (137 mM NaCl, 5.6 mM glucose, 5.4 mM KCl, 2.2 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 10 mM HEPES/Na, 1.8 mM $CaCl_2$, pH 7.4) supplemented with 0.025% pluronic acid and 10 μM Fura2-AM at 37 °C for 45 min. Unloaded dye was washed out and the cover slips were placed on a Nikon fluorescence microscope thermostated at 26 °C. Fluorescence intensity of Fura2 was monitored at an emission wave length of 510 nm with alternative excitation at 340 and 380 nm using an imaging system from VisiTech (Sunderland, UK). Calibration of Fura2 fluorescence signals to calculate $[Ca^{2+}]_i$ values were done according to Grynkiewicz et al. [25]. The K_d of Ca^{2+} for Fura2 was assumed to be 224 nM (R_{min} was found to be 0.499, R_{max} 4.56 and β 4.81).

Fluorescence photometry was also performed in cell suspension using a Hitachi F-4500 photometer [26]. Differentiated human skeletal muscle cells were loaded with 10 μM Fura2-AM in Tyrode's solution supplemented with 0.025% pluronic acid for 45 min at 37 °C. The cells were then washed three times and resuspended in Tyrode's solution. The cell suspension was kept in darkness until use.

2.5. Electrophysiology

Whole cell patch experiments were performed using a HEKA EPC-9 patch clamp amplifier with the pulse software package (HEKA Elektronik, Lambrecht, Germany). Series resistance compensation and leak subtraction were not used. Patch pipettes were pulled from PG10150 glass capillaries (WPI, Berlin, Germany) to a resistance of 2.5–5 MΩ. Intracellular (pipette) solution contained 135 mM KCl, 11 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes/NaOH, 4.2 mM ATP, pH 7.2. Tyrode's solution was used as the extracellular bath solution.

Experiments were done within a period of 6–10 days after changing the culture medium from GM to DM. Application of substances to the cells was performed by a superfusion system with a seven-channel perfusion pipette (List-electronic, Darmstadt, Germany), driven by a valve bank (TSE, Bad Homburg, Germany) with solution exchange times of less than 500 ms. Cells were constantly rinsed during measurements. Electrical stimulation was mimicked by a solution with elevated $[K^+]$, reduced $[Na^+]$, (depolarisation solution HK: Tyrode's solution with 60 mM KCl and 80 mM NaCl) and Ca^{2+} free assay conditions were obtained by omitting Ca^{2+} and addition of 0.5 mM EGTA. Such stimulated skeletal muscle cells were chosen for electrophysiological experiments when they responded with contraction.

2.6. Phospholipase C assay

Differentiated human skeletal muscle cells were kept in an inositol free medium (Medium 199) supplemented with 2.5 mCi/ml of myo-[^{3}H]inositol for 24 h. Free radioactivity was removed with washing buffer (20 mM HEPES/NaOH, 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 0.02% (w/v) BSA, pH 7.4) and subsequently incubated in the same buffer supplemented with 10 mM LiCl at 37 °C and the indicated drugs. The reaction mixture was removed and the assay stopped by adding 750 μl of ice-cold 20 mM formic acid. The samples were held at 4 °C for 1 h and the inositol monophosphate fraction was collected by anion exchange chromatography as described by Berridge et al. [27]. The samples were applied to an anion exchange column (AG-1-X8; BioRad, Hercules, CA) and the [^{3}H]inositol phosphates (IP) were

eluted with 5 ml of 0.1 M formic acid/0.2 M ammonium formate. The eluted radioactivity was quantified by liquid scintillation counting.

2.7. ERK1/2 phosphorylation and Western blot analysis

Cells were stimulated with ATP in the absence and presence of various compounds for the indicated times. Control incubations were carried out with the solvent dimethyl sulfoxide, which was never above a final concentration of 0.1%. Dimethyl sulfoxide neither affected the basal levels of ERK1/2 phosphorylation nor the response to agonists (data not shown). The incubations were terminated by rapidly rinsing the cells with ice-cold phosphate buffered saline, followed by freezing the cells in liquid nitrogen. After rapid thawing, the cells were lysed (50 mM Tris, 40 mM β-glycerophosphate, 100 mM NaCl, 10 mM EDTA, 10 mM *p*-nitrophenol phosphate, 1 mM Na_3VO_4, 10 mM NaF, 1% Nonidet P-40, 0,1% SDS, 1 mM phenylmethylsulfonyl fluoride, 250 units/ml aprotinin, 40 μg/μl leupeptin, pH 7.4). The lysate was centrifuged at 10,000 × *g* for 10 min and the supernatant was dissolved in Laemmli sample buffer. Aliquots of the protein (5–15 μg) were resolved on a SDS-polyacrylamide gel (10% or 12%) and transferred to nitrocellulose membranes. A phospho-specific ERK1/2 antibody and a corresponding antibody directed against ERK1/2 were purchased from Cell Signalling® (MA, USA). Using horseradish-peroxidase conjugated secondary antibodies, the immunoreactive bands were visualized by enhanced chemiluminescence detection system.

The intensity of the protein bands was quantified using the scion imaging software® (www.scioncorp.com) and normalized to the control.

3. Miscellaneous procedures and statistics

Experiments were carried out in triplicates and repeated twice. The data are given as mean ± S.D., if not otherwise stated. Statistical significance was determined by Student's *t*-test or for multiple comparisons with ANOVA and post hoc Scheffe's test. Statistical significance was considered at a $p < 0.05$. Data were fitted using a least square algorithm provided by the Sigmaplot® software according to the respective formulas. Protein concentration was determined by Coomassie-blue kit from BioRad® (Munich, Germany) or alternatively by the bicinchoninic acid assay (Sigma–Aldrich, Vienna, Austria) using bovine serum albumin as a protein standard.

4. Results

4.1. ATP induced Ca2+ transients in differentiated human skeletal muscle cells

To reveal if ATP can induce an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), Fura2 loaded differentiated human skeletal muscle cells were investigated using the Ca^{2+} imaging technique (Fig. 1). Myotubes were identified by a depolarisation induced increase of $[Ca^{2+}]_i$ in the absence of extracellular

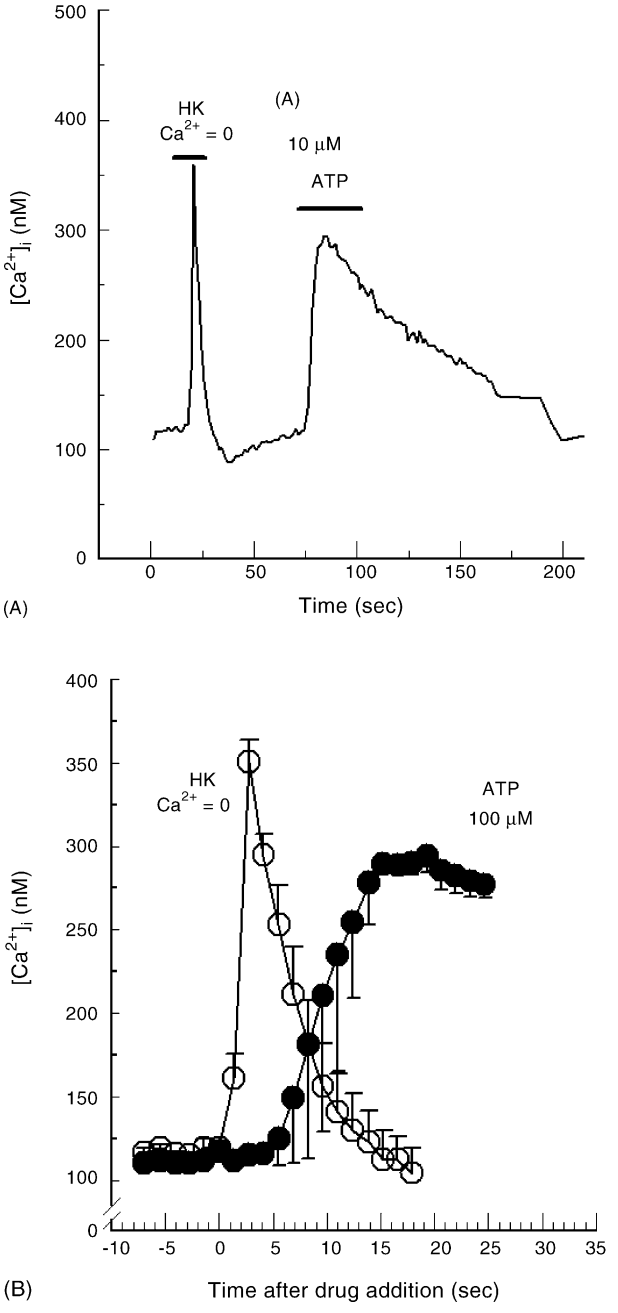

Fig. 1 – ATP induced Ca^{2+} transient in differentiated human skeletal muscle cells. Differentiated human skeletal muscle cells were loaded with 10 μM Fura2-AM to monitor the intracellular changes in Ca^{2+} concentration. (A) The cells were depolarised (Tyrode solution with 60 mM KCl; HK) in the absence of extracellular Ca^{2+} (Ca^{2+} = 0). Subsequently, a Ca^{2+} transient was evoked by the application of 100 μM ATP. A representative experiment is depicted. (B) The onset of the rise in intracellular Ca^{2+} concentration under conditions given in panel (A) was analysed in detail. The time point of drug application (HK, open circles; ATP, closed circles) was set zero. The symbols and error bars represent the mean and standard deviation (n = 6).

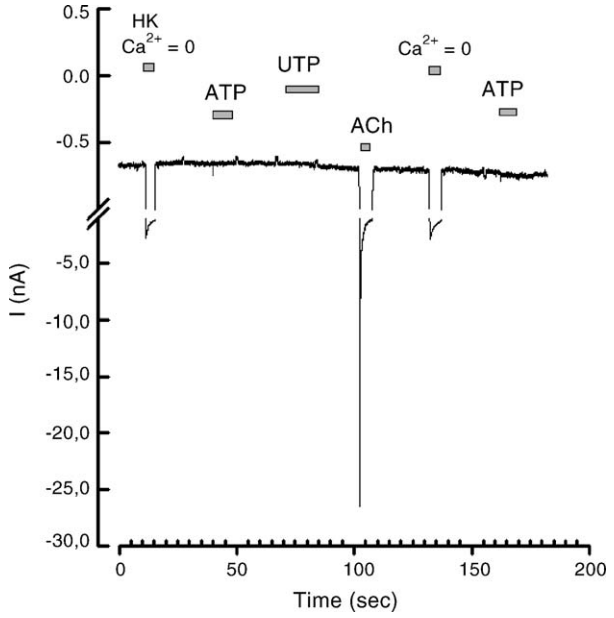

Fig. 2 – Whole cell path clamp measurement of differentiated human skeletal muscle cells. Human skeletal muscle cells were grown on cover slips and kept in a Tyrode bath solution. Currents were measured in the whole cell configuration at a holding potential of −80 mV. A high potassium solution lacking Ca^{2+} (HK, Ca = 0), 100 μM ATP, 100 μM UTP or 10 μM acetylcholine (ACh) were sequentially applied. A representative current trace is shown, the experiment was repeated twice.

Ca^{2+}, indicating a functional skeletal muscle type EC-coupling [22,28]. The broadness of this transient was dependent on the duration of the application of the depolarisation solution. Upon ATP administration myotubes also responded with a Ca^{2+} transient (Fig. 1). The kinetic analysis of the onset of the ATP-induced change in $[Ca^{2+}]_i$ showed a delay of approximately 4 s compared to the depolarisation induced Ca^{2+} transients (Fig. 1B). This delay suggests the formation of a second messenger via a G-protein coupled receptor instead of a cation influx via a P2X receptor. In order to confirm this assumption, membrane currents were measured with the whole cell patch clamp technique.

For whole cell patch clamp experiments, multinucleated myotubes were used, which contracted upon exposure to high potassium solution in the absence of extracellular Ca^{2+}. Unequivocally, the neurotransmitter acetylcholine triggered a huge inward current in differentiated human skeletal muscle cells (Fig. 2). P2X receptors are activated mainly via ATP, however show a much lower current amplitude in the range of 100–200 pA [2]. Even at a 10-fold magnification in scaling of the y-axis, no inward current was elicited by the extracellular application of ATP (Fig. 2). Independent, whether ATP or UTP was used no membrane current was detectable. UTP per se is not expected to trigger a P2X receptor mediated cation influx, however, a nucleoside diphosphokinase activity may convert ADP into ATP utilizing UTP [29,30]. The results depicted in Fig. 2 exclude such a possibility, as UTP failed to evoke a membrane current response. Thus, these findings confirm the absence of P2X receptors on differentiated human skeletal muscle cells and suggest that the ATP induced rise of $[Ca^{2+}]_i$ is indeed mediated by P2Y receptors (cf. Fig. 1).

4.2. *Pharmacological characterisation of P2Y receptors on differentiated human skeletal muscle cells*

In order to confirm the existence of P2Y receptors on differentiated human skeletal muscle cells one would expect that ATP facilitates the synthesis of inositol phosphates (IP's) in a time and concentration dependent manner. The successive exposure to increasing concentrations of ATP indeed resulted in an accumulation of IP's with an EC_{50} of 21.3 ± 6.6 μM (Fig. 3A). The kinetics of the ATP stimulated IP-production revealed a fast initial phase with a time constant of 0.17 ± 0.04 min (Fig. 3B). The IP-production proceeded in a second phase which showed a quasi linear increase parallel to the basal IP-formation (with a time constant of 9.46 ± 1.0 min) which may be explained by the fact that the accumulation of IP was analysed in the presence of 10 mM LiCl, an inhibitor of inositol phosphatases.

Although we had carefully treated the ATP stock solutions with an ATP regenerating system, it cannot be excluded that nucleotides are degraded when added to the cells. We have therefore exposed the cells to an ATP regenerating system (Fig. 3C) but failed to observe any difference on the ATP induced IP-formation. Taken together, these data indicate that the carry over of the ATP regenerating system from the stock solution is sufficient to suppress nucleotide hydrolysis within the indicated exposure times.

The plateau of maximal IP-production was reached at a concentration of 100 μM ATP (Fig. 3A). To evaluate the receptor specificity of the IP-formation, we compared the ability of various adenine and uridine nucleotides to promote the generation of the second messenger in myotubes (Fig. 4). The selective $P2Y_1$ receptor agonists 2-MeSADP and 2-MeSATP stimulated IP accumulation similar to ATP, however, the activation with 2-MeSATP was not significant. Nevertheless, this pharmacological profile indicates the functional expression of a $P2Y_1$ receptor (Fig. 4). Furthermore, the uridine nucleotides UTP and UDP which act as agonists on $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors stimulated the formation of IP's comparable to ATP (Fig. 4). Therefore, most likely, IP-production by ATP was obtained via $P2Y_1$, $P2Y_2$, or $P2Y_6$ receptors. Although the uridine nucleotide selective $P2Y_4$ receptor is not activated by ATP, it might also contribute to IP-formation when activated by UTP.

The receptor specificity of antagonists to P2Y receptors is to some extent promiscuous and overlapping [3,31]. Nevertheless, we used a range of antagonists to substantiate evidence for the above mentioned P2Y receptor subtypes.

The uridine nucleotides UTP and UDP stimulated the formation of IP which indicates the existence of $P2Y_4$ and/or $P2Y_6$ receptors (Fig. 4). Suramin, which has no effect on these receptors, was used as an internal control and had no effect on the IP-accumulation triggered by 100 μM UTP (Fig. 5A). In contrast 100 μM PPADS, a selective $P2Y_1$ antagonist with less potency at the $P2Y_4$ and $P2Y_6$ receptor, reduced the UTP stimulated IP-production by $35.8 \pm 11.3\%$.

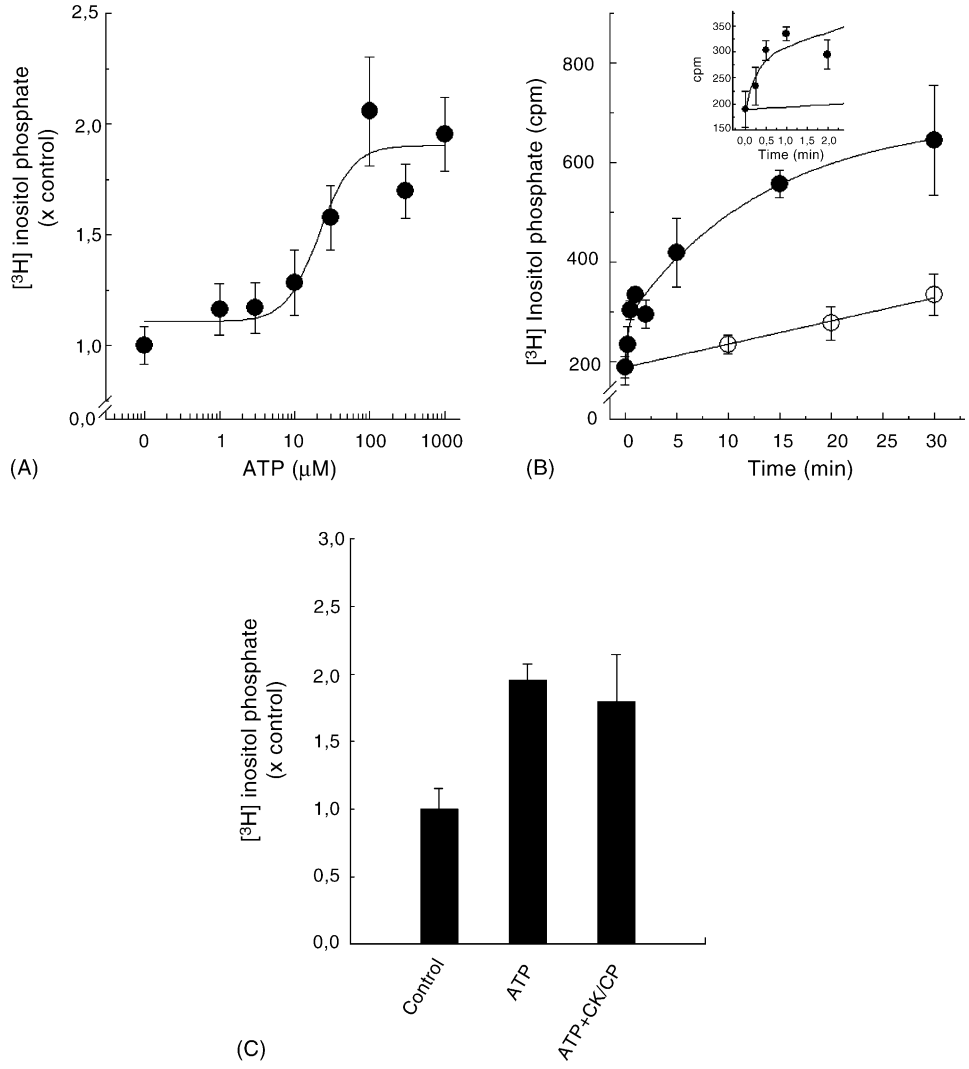

Fig. 3 – ATP dependent accumulation of inositol phosphates in differentiated human skeletal muscle cells. (A) Differentiated human skeletal muscle cells (2–4 × 10^5 cells) were loaded with [^{3}H]myo-inositol (2.5 μCi/ml) overnight. Thereafter cells were exposed to increasing concentrations of ATP for 30 min which was added 20 min after the addition of 10 mM LiCl. The cells were lysed and [^{3}H]-IP collected from anion exchange chromatography as described in Section 2. The data points represent the mean of three independent experiments which were carried out in duplicates. (B) The accumulation of [^{3}H]-IP was monitored for 30 min in the absence (open squares) and presence of 100 μM ATP (filled squares). The insert resolves the initial burst of [^{3}H]-IP formation. (C) The increment in 100 μM ATP dependent [^{3}H]-IP formation was observed in the absence and presence of an ATP regenerating system (25 μg/ml creatine kinase and 4 mM creatine phosphate; CK/CP). The data represent the mean ± S.D. of triplicates of a representative experiment which was repeated twice.

The ATP induced IP-formation was significantly reduced by the non-selective $P2Y_1$ and $P2Y_2$ receptor antagonist suramin by more than 90% ($p < 0.005$; Fig. 5B). The $P2Y_1$ receptor antagonists PPADS and A3P5P have no or little efficacy on the $P2Y_2$ receptor [3,21,31]. PPADS and A3P5P inhibited the ATP induced IP-formation by only 60%. Accordingly, ATP triggered IP-accumulation is mediated via the $P2Y_1$ and $P2Y_2$ receptors.

To further strengthen these findings we carried out RT-PCR from differentiated human skeletal muscle cells. Similar to the observations of Banachewicz et al. in mouse C2C12 myotubes, we confirmed the presence of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on mRNA level (data not shown) [13].

As shown in Fig. 3C, a substantial hydrolysis of ATP did not occur under our experimental conditions. Nevertheless, it is possible that the degradation product adenosine may account in part for IP-formation. To control for this possibility, the IP-formation was analysed in the presence of a saturating concentration of the universal adenosine receptor antagonist XAC (Fig. 5B). The application of 10 μM XAC was not capable to abrogate the ATP induced IP-accumulation. Importantly, these results verify that under the experimental conditions used, ATP hydrolysis and adenosine generation had no significant impact on IP-generation.

4.3. *Characterisation of the ATP induced Ca^{2+} transient*

The main Ca^{2+} release mechanism in skeletal muscle is mediated by the ryanodine receptor type 1 (reviewed in Ref.

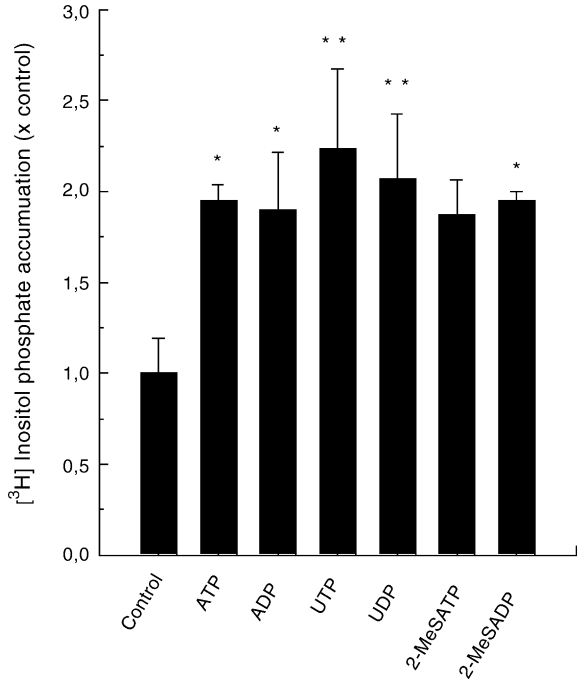

Fig. 4 – Nucleotide dependent inositol phosphate formation in differentiated human skeletal muscle cells. Under experimental conditions identical to Fig. 3A differentiated human skeletal muscle cells were kept in the absence (basal) or presence of the indicated nucleotides at a concentration of 100 μM. The bars indicate the mean ± S.D. of triplicates of a representative experiment which was repeated twice. Asterisks indicate statistical significance vs. control ($^{*}p < 0.05$; $^{**}p < 0.005$).

[32]). Hence, we compared the ability of ATP to trigger a Ca^{2+} transient in differentiated human skeletal muscle cells in the absence and presence of 15 μM ryanodine (Fig. 6). As can be seen from the data summarized in Fig. 6E, a change in the ATP induced Ca^{2+} transient via the ryanodine receptor was not observed. In accordance with our observations described in Fig. 3, the ATP induced Ca^{2+} transient was strictly dependent on the activity of PLC (Fig. 6C and E). Thus, the PLC inhibitor U73122 suppressed the ATP induced Ca^{2+} mobilization by 75%. Importantly, the administration of EGTA to the extracellular Tyrode solution was not sufficient to abolish the ATP induced Ca^{2+} transient, although the resting $[Ca^{2+}]_i$ was markedly reduced. The amplitude of the Ca^{2+} response was not significantly altered by the chelation of the extracellular Ca^{2+} (Fig. 6E). In a similar manner Fura2 loaded differentiated human skeletal muscle cells were not responsive to 100 μM ATP when preincubated for 10 min with 10 μM Xestospongin C, an inhibitor of the IP3 receptor (ratio increment 0.11 ± 0.1; $n = 3$; $p > 0.05$ versus control). Again, these data confirm that a P2X receptor is not involved in the Ca^{2+} response triggered by the extracellular application of ATP.

4.4. *P2Y receptor mediated ERK1/2 activation*

In a recent observation in C2C12 cells, Banachewicz et al. have shown that ERK1/2 is activated via various P2Y receptor

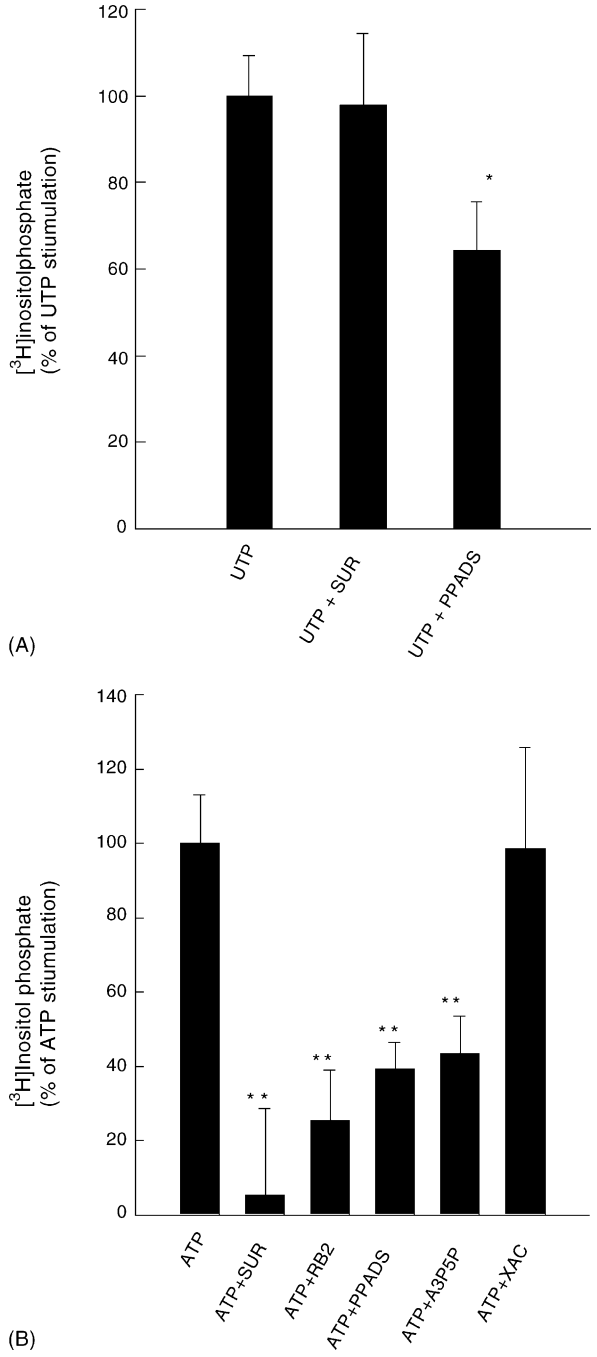

Fig. 5 – Inhibition of the ATP or UTP induced inositol phosphate accumulation by P2Y receptor antagonists. (A) In differentiated human skeletal muscle cells the accumulation of [^{3}H]-IP was stimulated by 100 μM UTP in the absence and presence of 100 μM suramin (SUR) or 100 μM PPADS. (B) The accumulation of [^{3}H]-IP was also triggered by ATP (100 μM), in the absence and presence of 100 μM suramin (SUR), 100 μM reactive blue (RB), 100 μM PPADS, 100 μM A3P5P or 10 μM XAC. The bars indicate the mean ± S.D. of three to seven experiments carried out in triplicates. The statistical significance was calculated with ANOVA and post hoc Scheffe's test to confirm specific inhibition of ATP or UTP induced [^{3}H]myo-InsP formation ($^{*}p < 0.04$; $^{**}p < 0.005$).

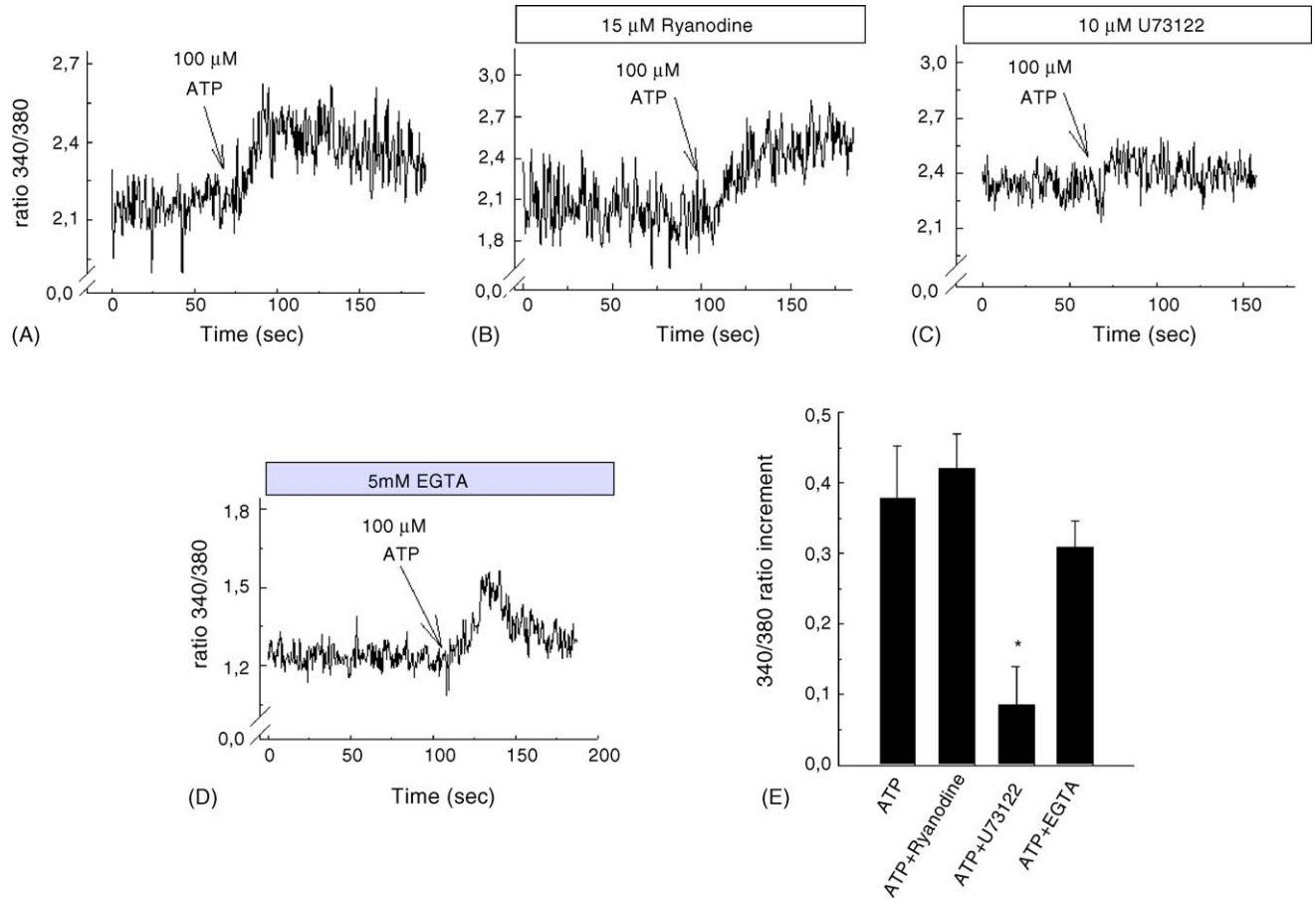

Fig. 6 – ATP induced Ca^{2+} transients are strictly dependent on phospholipase C activity. In mass suspension, differentiated human skeletal muscle cells (1, 5–3, 5×10^5 cells) loaded with Fura2-AM were exposed to 100 μM ATP, in the absence (A) and presence of 15 μM ryanodine (B), 10 μM U73122 (C) or 5 mM EGTA (D). The cells were preincubated with ryanodine 15 min, with U73122 and EGTA 5 min before the assay was started. (E) Summary of the individual experiments exemplified in panel (A)–(D). The bars represent the mean ± S.D. of four to seven experiments. The indicated statistical significance compares ATP induced Ca^{2+} release with that in the presence of U73122 which was obtained by ANOVA and post hoc Scheffe's test ($^*p < 0.05$).

agonists [13]. This is of importance since mitogen-activated protein kinases have been implicated in exercise-induced regulation of gene expression in skeletal muscle (reviewed in Ref. [19]). We have therefore analysed the phosphorylation of ERK1/2 in the presence of increasing concentrations of ATP which was significantly stimulated above 10 μM ATP (Fig. 7). The kinetics of the ERK1/2 phosphorylation revealed a bell-shaped activation, which peaked after 30 min of incubation with ATP (Fig. 7B). The ATP induced phosphorylation of ERK1/2 was propagated in the same concentration range as the IP-accumulation (cf. Figs. 3A and 7A). We have not further investigated the ATP analogues already tested for IP-formation, as this has been shown already in a similar system [13].

In order to link the phosphorylation of ERK1/2 to an individual upstream receptor, differentiated skeletal muscle cells were incubated in the presence of ATP and various P2Y receptor antagonists (Fig. 8). The non-selective P2Y receptor antagonist, RB was capable to prevent ATP triggered ERK1/2 phosphorylation above basal level (Fig. 8). However, ERK1/2 phosphorylation was already inhibited by RB under basal conditions, which is unclear at the moment. Interestingly, the ATP stimulated ERK1/2 phosphorylation was not completely prevented by RB. Thus, the difference between the two conditions was not significant, but a possible involvement of a P2Y receptor other than $P2Y_1$ receptor might be possible.

The $P2Y_1$ receptor selective antagonists MRS2179 and A3P5P abrogated the ATP triggered ERK1/2 phosphorylation by 85% and 60%, respectively (Fig. 8). This inhibitory pattern reflects the different potencies of the two antagonists. While the IC_{50} for MRS2179 is 100 nM that for A3P5P is 5.6 μM [21,33].

Early observations have shown that ERK1/2 activation via P2Y receptor stimulation (using ATP or UTP) is inhibited by chelation of intracellular Ca^{2+} and protein kinase C down-regulation [34,35]. In differentiated human skeletal muscle cells ATP mediated ERK1/2 phosphorylation was not dependent on intracellular Ca^{2+} elevation (Fig. 9). This is mainly based on the observation that pre-incubation of the skeletal muscle cells with BABTA-AM or the inositol triphosphate receptor inhibitor, Xestospongin C, did not prevent ATP induced ERK1/2 phosphorylation. In Ca^{2+} flux measurements similar to that provided in Fig. 6, both compounds were

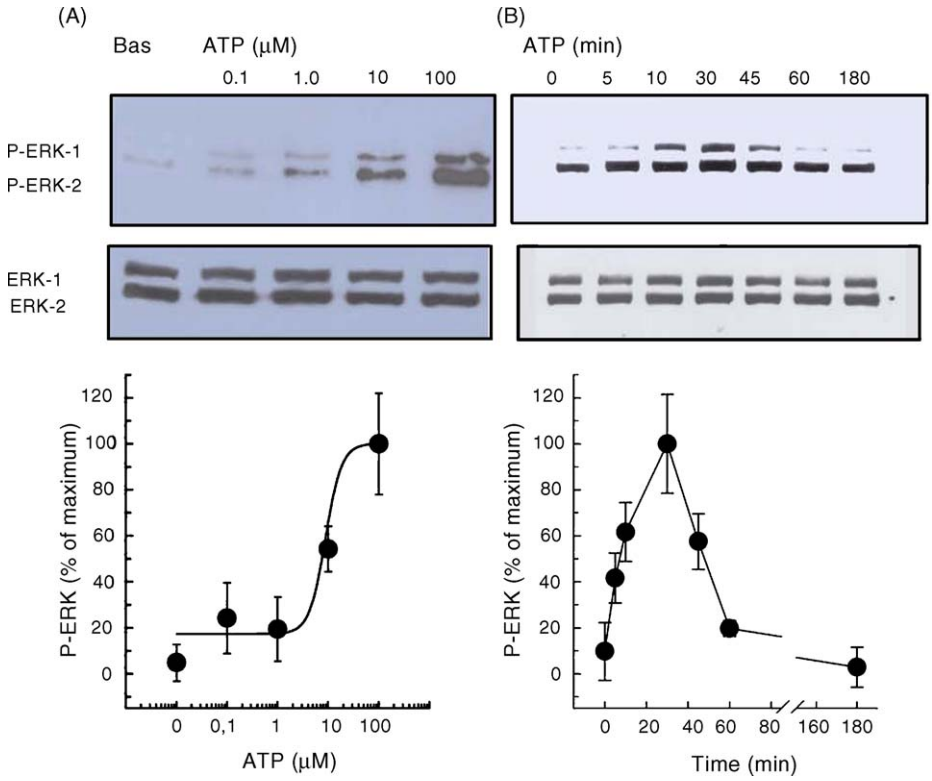

Fig. 7 – ATP dependent phosphorylation of ERK1/2. (A) Western blot analysis of phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 in differentiated human skeletal muscle cells stimulated with increasing concentrations of ATP for 5 min. The intensity of the phosphorylated ERK1/2 was blotted vs. ATP concentration and gave rough estimate for the EC_{50} of 15.4 ± 3.4 μM. (B) Time course of phosphorylated ERK1/2 and total ERK1/2 in differentiated human skeletal muscle cells exposed to 100 μM ATP. The intensities of the protein bands were quantified using the Scion imaging software®. The symbols and error bars represent the mean ± S.D. (n = 3–5).

efficient in reducing the ATP triggered Ca^{2+} transient. Loading of the cells with 3 μM BAPTA-AM led to a reduction in the resting Ca^{2+} concentration as depicted for extracellular application of EGTA (cf. Fig. 6D). Nevertheless, such cells responded barely to 100 μM ATP (ratio increment 0.18 ± 0.16; n = 3; $p > 0.05$ versus control). Conversely, the PLC inhibitor, U73122, which also suppressed ATP mediated Ca^{2+} release (Fig. 6C), completely abrogated ERK1/2 activation (Fig. 9). Moreover, U73122 alone suppressed basal ERK1/2 phosphorylation significantly ($p < 0.01$). Although this effect is not clear at the moment we assume that it is not due to a Ca^{2+} release mechanism, because the basal phosphorylation of ERK1/2 is not altered by BABTA or Xestospongin C. Nevertheless, we have performed control experiments with the inactive PLC inhibitor analogue, U73343 in order to exclude an unspecific effect. The basal phosphorylation of ERK1/2 was not altered by 10 μM U73343 (fold of basal: 1.02 ± 0.17, n = 8). Accordingly, the ATP stimulated phosphorylation of ERK1/2 was not inhibited by 10 μM U73343 (fold of basal: 100 μM ATP, 1.8 ± 0.24, n = 4; 100 μM ATP plus 10 μM U73343, 1.67 ± 0.34, n = 4; $p > 0.05$).

In the line of these findings the inhibitor of the Ca^{2+}-dependent protein kinase C isoforms, GF109203X (1 μM) was not capable to significantly inhibit ATP-mediated ERK1/2 activation (data not shown). Similarly, staurosporine (500 nM) failed to prevent ATP-mediated ERK1/2 activation (data not shown).

In contrast, preincubation of the differentiated human skeletal muscle cells with the PI3 kinase inhibitor LY294002 or wortmannin significantly abolished the ATP mediated ERK1/2 phosphorylation (Fig. 10).

5. Discussion

ATP is an important co-neurotransmitter in the central nervous system [1–3]. However, little is known about the extracellular role of ATP on the neuromuscular junction of human skeletal muscle. Here, we provide evidence that ATP initiates a P2Y receptor mediated Ca^{2+} signal in differentiated human skeletal muscle cells (Figs. 1 and 6). Independent of this Ca^{2+} signal, ATP also triggers phosphorylation of ERK1/2, mainly via a $P2Y_1$ receptor (Figs. 7 and 8).

Extracellular ATP on cultured skeletal muscle cells has been proposed to have transmitter-like action [36]. It has been shown that the extracellular application of ATP stimulates an increase of $[Ca^{2+}]_i$ which was dependent on the developmental state of the skeletal muscle cells [13,18]. In contrast to our findings, Cseri et al. measured ATP induced inward currents in developing human skeletal muscle cells with more than five central nuclei. They attributed this current to a P2X receptor. Nevertheless, the individual P2X receptor was not characterized. The expression of P2X receptors seems to undergo

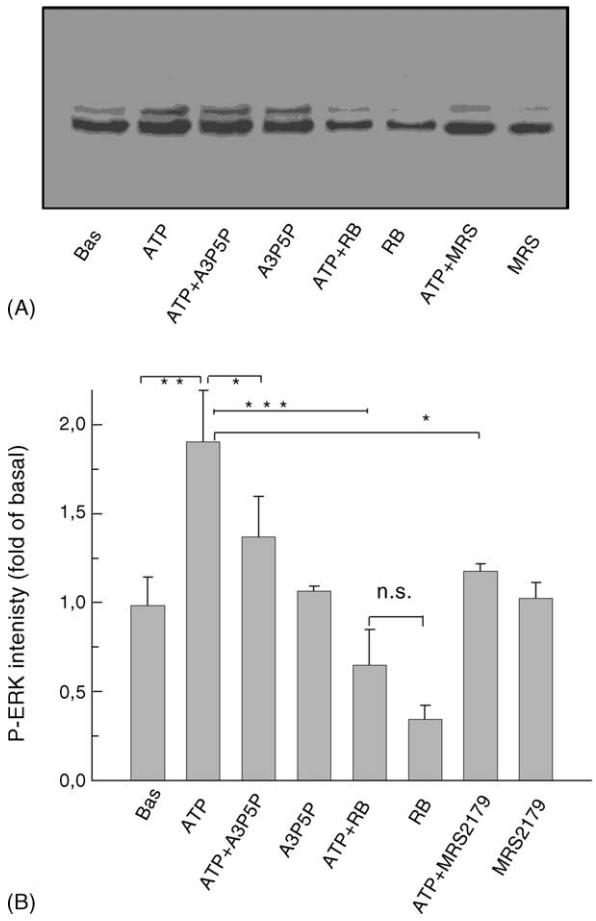

Fig. 8 – Inhibition of ATP dependent ERK1/2 stimulation by P2Y receptor antagonists. (A) Differentiated human skeletal muscle cells were pre-incubated for 20 min in the absence and presence of A3P5P (100 μM), reactive blue 2 (RB; 100 μM) or MRS2179 (1 μM). Subsequently, the probes were stimulated with 100 μM ATP or buffer (Bas) and analysed for phosphorylated ERK1/2. (B) The intensity of the phosphorylated ERK1/2 protein bands were normalized to control and presented as mean ± S.D. ($n = 4$); n.s. denotes not significant ($^{*}p < 0.05$; $^{**}p < 0.03$; $^{***}p < 0.001$).

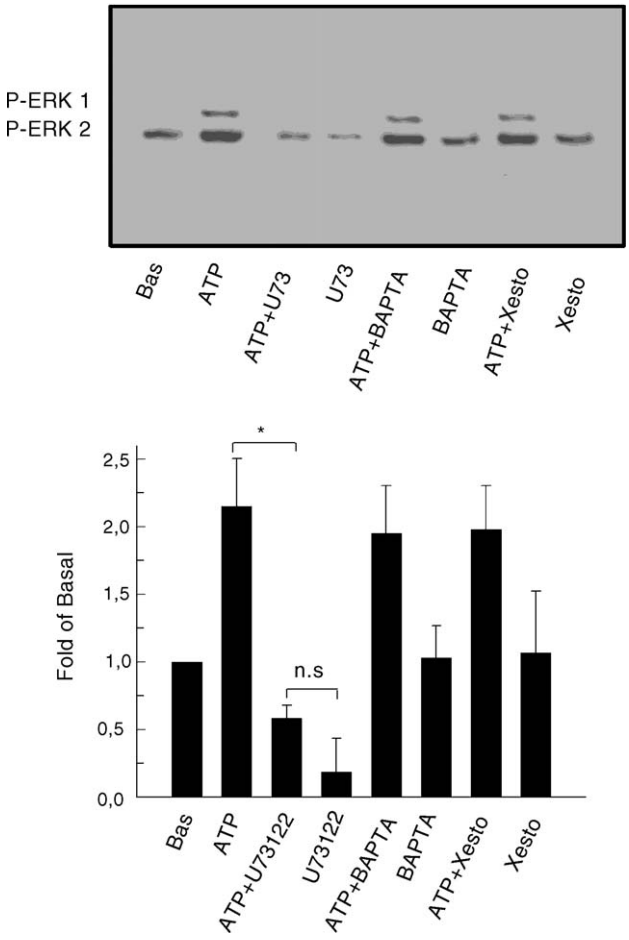

Fig. 9 – ATP-induced ERK1/2 phosphorylation was dependent on phospholipase C (PLC) but independent on Ca^{2+}. (A) Differentiated human skeletal muscle cells were treated with 10 μM U73122, 3 μM BAPTA/AM (BAPTA) and 10 μM Xestospongin C (Xesto) for 20 min, followed by stimulation with 100 μM ATP or buffer (Bas). Phosphorylated ERK1/2 is depicted. (B) Quantification of the phosphorylated ERK1/2 bands are expressed as fold stimulation of the control (Bas); mean ± S.D. ($n = 3$). Asterisk denotes statistical significance ($^{*}p < 0.0005$); n.s. denotes not significant.

temporal changes and the current density was found to be higher in earlier stages of differentiation in human skeletal muscle cells and mouse C2C12 cells [13,18]. Although, we also used myotubes of rather early stages in our patch clamp experiments, we failed to detect P2X receptor carried currents (Fig. 2). Possibly, our experiments were biased by the functional definition of a differentiated human skeletal muscle cell. Our measurements were done in differentiated human myotubes which were exclusively responsive to depolarisation in the absence of extracellular Ca^{2+}, as this is a proof for intact and mature excitation–contraction coupling. In cells elected under such conditions, the participation of a cation influx via a P2X receptor could be excluded in whole cell patch clamp experiments. Nevertheless, acetylcholine generated a robust cation influx, while ATP or UTP failed to induce an inward current. Therefore, we conclude that functional P2X receptors are absent in differentiated human skeletal muscle cells.

Our RT-PCR experiments identified $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on differentiated human skeletal muscle cells (data not shown). These findings were confirmed by functional assays measuring the accumulation of inositol phosphates by extracellular nucleotides. The existence of a $P2Y_1$ receptor was confirmed by the stimulation of IP-production using the selective $P2Y_1$ receptor agonists 2-MeSADP and 2-MeSATP [3] and by the inhibition of the ATP induced IP-production by the selective $P2Y_1$ receptor antagonist A3P5P [21]. Equimolar concentrations of ATP produced a similar IP signal compared to 2-MeSADP and 2-MeSATP.

UDP, an agonist on $P2Y_6$ receptors [3], also lead to an accumulation of IP's suggesting the existence of this receptor subtype on differentiated human skeletal muscle cells. Since

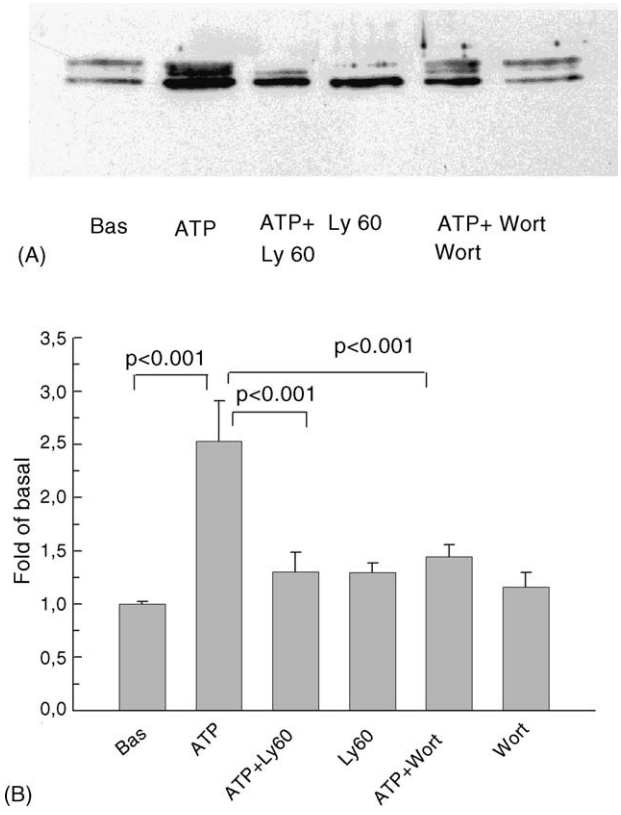

Fig. 10 – ATP-induced ERK1/2 phosphorylation is dependent on PI3 kinase. (A) Differentiated human skeletal muscle cells were exposed to the PI3 kinase inhibitors Ly294002 (Ly60) at 60 μM or 200 nM wortmannin (Wort) for 1 h, prior to treatment with 100 μM ATP or buffer (Bas). (B) Quantification of the phosphorylated ERK1/2 band is expressed as fold stimulation of the control (Bas); mean ± S.D. (n = 3).

ATP and UTP can potentially activate the $P2Y_2$ receptor [3], we used a selection of antagonists to identify the $P2Y_2$ receptor pharmacologically.

The IP-production stimulated by UTP, an agonist at $P2Y_2$ as well as $P2Y_4$ receptors [3], was not reduced by 100 μM suramin. PPADS, however, did have an inhibitory effect on UTP. Considering that suramin blocks $P2Y_2$ receptors but is inactive at $P2Y_4$ receptors [37] and PPADS, which inhibits $P2Y_4$ receptors by 30–73% [37,38], is inactive at $P2Y_2$ receptors [38], we conclude that $P2Y_4$ receptors exist on differentiated human skeletal muscle cells.

Two different sets of Ca^{2+} release kinetics have been described in skeletal muscle cells [39]: a fast Ca^{2+} transient lasting less then five seconds, associated with the ryanodine receptor and excitation–contraction coupling, and a slow Ca^{2+} signal for the duration of up to 20 s, associated with the IP_3 receptor. The kinetics of the ATP evoked Ca^{2+} transient clearly corresponds to the slow Ca^{2+} signal. This, together with the fact that the PLC inhibitor, U73122 and Xestospongin C virtually abolished the ATP-induced Ca^{2+} signal, corroborates the hypothesis that the Ca^{2+} signal induced by ATP derives mainly from IP_3 sensitive Ca^{2+} pools (Fig. 6). Conversely, a preincubation with ryanodine was not able to abrogate the ATP evoked Ca^{2+} transient.

At this stage the physiological role of this calcium increase can only be speculated on. While the ryanodine receptor stimulated Ca^{2+} release is coupled to muscle contraction, the slow Ca^{2+} wave seems to act as a second messenger for signal transduction and gene transcription [39]. The action of ATP via a P2Y receptor as a modulator of gene expression has been shown on chick and mouse myotubes [15]. Although the action of ATP as a neurotransmitter on skeletal muscle has been known for 20 years [36], there is still great uncertainty as to its effects on its target organ. While there are a number of studies demonstrating a role of ATP as a modulator of cell proliferation and myotube formation, the effect of ATP on fully differentiated skeletal muscle tissue has not been studied in detail yet. Since ATP is co-released with acetylcholine in adult muscle tissue [4], alternatively a release mechanism via autocrine or paracrine pathway may be discussed, there must clearly be a role for ATP at differentiated skeletal muscle cells [40]. Differentiated skeletal muscle cells do not proliferate, nevertheless, the rate of protein synthesis is very much dependent on the workload of the muscle. Hence, there must be a signal into the nuclei which regulates the transcription of genes. One of these signalling pathways could be activated by ATP as demonstrated in this study. ATP activates ERK1/2 and this activation is dependent on the PI3-kinase activity. The connection between PI3 kinase and ERK1/2 activity, however, is unclear.

The phosphorylation of ERK1/2 seems to be a downstream effect of the PI3 kinase activity, because the PI3 kinase inhibitor Ly294002 (IC_{50}: 1.4 μM) reduced the ERK1/2 phosphorylation by ATP significantly (Fig. 10) [41]. This observation is corroborated by a second PI3 kinase inhibitor, wortmannin, which also prevented ATP triggered ERK1/2 phosphorylation significantly (Fig. 10). Besides its antagonistic effects on P2Y receptors, RB also blocks the PI3 kinase activity with an IC_{50} of 4 μM in rat C6 glioma cells [42]. This effect may help to explain our observation, that already under basal conditions, RB reduced ATP induced ERK1/2 phosphorylation (Fig. 8).

Consistent with the finding that ATP can activate PI3 kinase in skeletal muscle is the observation that in mouse C2C12 skeletal muscle cells, P2Y receptors are able to stimulate glucose uptake and thereby provide energy for muscle activity [43]. Other trophic factors, like agrin and neurilins, are released from the motor neuron to initiate and maintain the protein pattern expressed at the surface of skeletal muscle cells [44–47]. Whereas these factors seem to provide a qualitative signal to express specific proteins of the neuromuscular junction, ATP may act as a signal for metabolic adaptation related to neuromuscular activity.

The activation of ERK1/2 during cycling exercise and marathon running was reported [48,49]. Under conditions of one-leg cycling only the exercised leg showed ERK1/2 activation, indicating that a local rather than a systemic factor leads to the phosphorylation of ERK1/2 [48]. The authors conclude from their data that contraction is sufficient to activate ERK1/2. Interestingly, in exercising individuals the onset of ERK1/2 phosphorylation is detected after 10 min and peaks after 30 min [48]. A very similar time course is observed upon exposure of differentiated human skeletal muscle cells to ATP

(Fig. 7). By pharmacological means, the Ca^{2+} independent phosphorylation of ERK1/2 by ATP was pinpointed to activation of a $P2Y_1$ receptor. In conclusion, our data highlight the importance of ATP as an extracellular ligand at the neuromuscular junction.

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