Characterization of P₂-purinoceptor mediated cyclic AMP formation in mouse C2C12 myotubes

Robert H. Henning, Marry Duin, Adriaan den Hertog & Adriaan Nelemans

Department of Pharmacology/Clinical Pharmacology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands

1 The formation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) and inositol(1,4,5)trisphosphate (Ins(1,4,5)P₃), induced by ATP and other nucleotides was investigated in mouse C2C12 myotubes.

2 ATP (100 µM) and ATPγS (100 µM) caused a sustained increase in cyclic AMP content of the cells, reaching a maximum after 10 min. The cyclic AMP content reached a maximum in the presence of 100 µM ATP, followed by a decline at higher ATP concentrations. ATP-induced cyclic AMP formation was inhibited by the P₂-purinoceptor antagonist, suramin.

3 Myotubes hydrolysed ATP to ADP at a rate of 9.7 ± 1.0 nmol mg⁻¹ protein min⁻¹. However, further hydrolysis of ADP to AMP and adenosine was negligible.

4 The cyclic AMP formation induced by ADP (10 µM–1 mM) showed similar characteristics to that induced by ATP, but a less pronounced decline was observed than with ATP. ADP-induced cyclic AMP formation was blocked by suramin, while cyclic AMP formation elicited by adenosine (10 µM–1 mM) was insensitive to suramin.

5 The ATP analogue, α,β-methylene-ATP also induced a suramin-sensitive cyclic AMP formation, while 2-methylthio-ATP and the pyrimidine, UTP, did not affect cyclic AMP levels.

6 Stimulation of the myotubes with ATP or UTP (10 µM–1 mM) caused a concentration-dependent increase in the Ins(1,4,5)P₃ content of the cells. ADP (100 µM–1 mM) was less effective. Adenosine did not affect Ins(1,4,5)P₃ levels.

7 Incubation of the cells with UTP (30 µM–1 mM) inhibited the ATP- and ADP-induced cyclic AMP formation, suggesting that stimulation of the 'nucleotide' type P₂-receptor inhibits P₂-purinoceptor mediated cyclic AMP formation in C2C12 myotubes. In contrast, UTP (30 µM–1 mM) enhanced adenosine-induced cyclic AMP formation.

8 Adenosine-sensitive P₁-purinoceptors activating cyclic AMP formation were found in C2C12 myotubes. Further, a novel P₂-purinoceptor is postulated, sensitive to ATP, ADP and ATPγS, which also activates the formation of cyclic AMP in C2C12 myotubes.

Keywords: Cyclic AMP; Ins(1,4,5)P₃; P₂-purinoceptor; P₁-purinoceptor; cross-talk; nucleotide receptor; C2C12 myotubes

Introduction

It is known that adenosine 3':5'-cyclic monophosphate (cyclic AMP) affects the nicotinic acetylcholine receptors (AChRs) of skeletal muscle and myotubes. An acute increase in intracellular cyclic AMP accelerates desensitization of these receptors (Albuquerque *et al.*, 1986; Miles *et al.*, 1987) most likely via cyclic AMP-dependent phosphorylation of the δ-subunit of the nicotinic AChR (Miles *et al.*, 1989; Haganir & Greengard, 1983; Changeux, 1991). A sustained increase in cyclic AMP level induced up-regulation of nicotinic AChRs (Betz & Changeux, 1979), mediated by transcriptional (Fontaine *et al.*, 1987; Moss *et al.*, 1991), and post-transcriptional (Green *et al.*, 1991) effects on nicotinic AChR biosynthesis. Only a few primary messengers possibly involved in the regulation of the cyclic AMP level of skeletal muscle have been established. Calcitonin gene-related peptide, a peptide that is co-released with acetylcholine (ACh) at the neuromuscular junction (Changeux, 1991), elevates intracellular levels of cyclic AMP (Mulle *et al.*, 1988) and induces the related desensitization (Takami *et al.*, 1986) and up-regulation (Fontaine *et al.*, 1987) of nicotinic AChRs.

Another co-transmitter with ACh at the neuromuscular junction, ATP (Silinsky, 1975), activates phosphoinositide signalling in skeletal myotubes by stimulation of P₂-purinoceptors (Häggblad & Heilbronn, 1988). In C2C12 myotubes, this signalling pathway is activated through the 'nucleotide' type P₂-purinoceptor, which also responds to UTP (Henning *et al.*, 1992). It has been shown that ATP affects the cyclic AMP level via stimulation of P₂-purinoceptors in hepatic and

aortic cells (Okajima *et al.*, 1987; Tada *et al.*, 1992). It is also known that adenosine, the hydrolysis product of ATP, modulates cyclic AMP levels through stimulation of P₁-purinoceptors (Londos *et al.*, 1980). The contribution of ATP and derivatives in the regulation of cyclic AMP and the interaction with the phospholipase C (PLC) pathway was studied by measuring the formation of cyclic AMP and inositol(1,4,5)tris-phosphate (Ins(1,4,5)P₃) in C2C12 myotubes.

Methods

Cell culture

C2C12 cells, a murine myoblast cell line (Yaffee & Saxel, 1977) were obtained from the American Tissue Type Collection, Rockville, U.S.A. and cultured in 9.6 cm² plastic wells as described by Henning *et al.* (1992). Myotubes were used 7 days after initiating myoblast fusion.

Cyclic AMP measurement

The cyclic AMP content of the myotubes was assessed by mass measurement using a radioligand binding assay. Before the experiment, the cells were washed three times with a buffer of the following composition (mM): NaCl 125, KCl 6, CaCl₂ 1.2, MgCl₂ 2.5, NaH₂PO₄ 1.2, glucose 11, HEPES 10

(pH 7.4). The reaction was started by the addition of one of the nucleotides and stopped by addition of trichloroacetic acid (TCA: 5%, 350 μ l) after removing the buffer. Experiments were conducted at 20°C. The TCA was extracted (three times) with water-saturated diethylether, the samples were neutralized with KOH and stored at -40°C. Cyclic AMP mass was determined essentially according to Brown *et al.* (1971). To reach equilibrium, 50 μ l sample was incubated for at least 2 h at 4°C with 1.7 mg bovine adrenal binding protein and 10 μ l [3 H]-cyclic AMP (190 nM; specific activity: 40 Ci mmol $^{-1}$) in a buffer of 50 mM Tris-HCl and 4 mM EDTA (pH 7.4) in a final volume of 300 μ l. The incubation was terminated by adding a charcoal suspension (Norit A special, 3.5 g l $^{-1}$), mixing, and subsequent centrifugation to remove excess [3 H]-cyclic AMP, all at 4°C. The radioactivity in the supernatant was determined by scintillation counting. A standard curve for determination of cyclic AMP mass was constructed using ether-extracted TCA solution.

Adenine nucleotides

Single wells (9.6 cm 2) containing 7 days differentiated C2C12 myotubes were incubated at 20°C for 5 or 10 min with 2 ml extracellular medium containing ATP 300 μ M. Adenine nucleotides (adenosine, AMP, ADP and ATP) in the extracellular medium were separated by high-performance liquid chromatography (h.p.l.c.), using a Zorbax SAX Bioseries column (Du Pont, Wilmington, DE, U.S.A.) and detected by light absorption at 259 nm. Separation of adenine nucleotides was accomplished by use of a programmed gradient of NH $_4$ H $_2$ PO $_4$ in water at a flow rate of 1.5 ml min $^{-1}$. Injection of samples (100 μ l) was followed by a 2-min flow of water and a 14 min flow of linearly increasing NH $_4$ H $_2$ PO $_4$ (1 M; pH 3.7) in water to a final concentration of 40% (v/v). Retention times of adenosine, AMP, ADP and ATP were 2, 6, 8 and 12 min, respectively.

Ins(1,4,5)P $_3$ measurement

Experiments were conducted in a similar way to those described for cyclic AMP. The samples were assayed for Ins(1,4,5)P $_3$ content by a radioligand binding assay as described in detail by Den Hertog *et al.* (1992).

Data analysis

Data are presented as mean $\pm$ s.e.mean unless stated otherwise and were considered statistically different at $P < 0.05$ (unpaired Student's *t* test).

Drugs

Adenosine 3':5'-cyclic monophosphate (cyclic AMP), adenosine, adenosine 5'-diphosphate (ADP), adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) and α,β -methylene-adenosine 5'-triphosphate (α,β -MeATP) were obtained from Boehringer, Mannheim, Germany. Adenosine 5'-triphosphate (ATP), adenosine 5'-monophosphate (AMP) and uridine 5'-triphosphate (UTP) were obtained from Serva, Heidelberg, Germany. Suramin was obtained from Bayer, Leverkusen, Germany. 2-(Methylthio)-adenosine 5'-triphosphate (2-Me-SATP) was obtained from Research Biochemicals Inc., Natick, Mass., U.S.A. and pertussis toxin (PTx) from Sigma, St. Louis, U.S.A. [2,8- 3 H]-adenosine 3':5'-cyclic monophosphate ([3 H]-cyclic AMP) and D-[2- 3 H]-inositol 1,4,5-trisphosphate were obtained from Du Pont-New England Nuclear U.S.A.

Results

ATP and cyclic AMP formation

The basal cyclic AMP content of the C2C12 myotubes, as measured by radioligand binding assay, was 10.5 ± 0.9 pmol

mg $^{-1}$ protein ($n = 72$). The myotubes responded to application of ATP (100 μ M) with a slowly developing but marked increase in cyclic AMP content, becoming manifest after 30 s. A further increase in cyclic AMP content was found after prolonged stimulation with ATP (up to 60 min), approaching a maximum at about 10 min (Figure 1a). Application of the stable ATP analogue ATP γ S (100 μ M) also induced an increase in cyclic AMP content of the cells, showing a similar time course as observed with ATP (not shown).

The formation of cyclic AMP in myotubes stimulated with different ATP concentrations was used to plot a concentration-response curve. The cyclic AMP content increased until a maximum was reached at 100 μ M ATP, followed by a decline at higher concentrations. Consequently, the concentration-response curves obtained after 5, 10 and 30 min ATP stimulation were bell-shaped (Figure 1b). Pretreatment (15 min) of the cells with the P $_2$ -purinoceptor antagonist, suramin (500 μ M) prevented the increase of cyclic AMP content of the cells as evoked by ATP (Figure 1b).

To investigate whether the slow increase in cyclic AMP content could be caused by ATP derivatives, hydrolysis of ATP was investigated by measuring the accumulation of the adenine nucleotides ATP, ADP, AMP and adenosine in the incubating medium in the presence of C2C12 myotubes. During incubation ATP (300 μ M; 10 min) was hydrolysed to ADP at 9.7 ± 1.0 nmol mg $^{-1}$ protein min $^{-1}$. Further hydrolysis of ADP to AMP or adenosine was not observed (Table 1).

An action of ADP on the cyclic AMP level can be expected in view of its formation from ATP in the incubation medium (Table 1). Stimulation of the cells with ADP (10 μ M–1 mM) induced an increase in cyclic AMP with a similar time course as observed for ATP (not shown). The concentration-response curve obtained at optimal stimulation periods (10 min), showed a small decline at a high agonist concentration (Figure 2a). The ADP-induced increase in cyc-

Figure 1 The effect of ATP on cyclic AMP formation in C2C12 myotubes. (a) Time-dependent increase in cyclic AMP content upon stimulation with ATP (100 μ M). (b) Concentration-dependent changes in cyclic AMP content of the myotubes measured after 5 min (∇), 10 min ($\circ$) and 30 min ($\square$) stimulation. The increase in cyclic AMP content observed after 10 min was blocked by suramin (500 μ M, $\bullet$). Data are expressed as pmol mg $^{-1}$ protein (mean $\pm$ s.e.) and were obtained from 4 or more experiments.

Table 1 Hydrolysis of extracellular ATP by C2C12 myotubes

	Medium without cells (nmol/well)	5 min (nmol/well)	Incubation 10 min (nmol/well)	Hydrolysis (nmol mg ⁻¹ protein min ⁻¹)
ATP	545 ± 8	497 ± 4	436 ± 43	+ 9.7 ± 1.0
ADP	34 ± 4	88 ± 2	129 ± 27	- 9.6 ± 0.8
AMP	20 ± 5	19 ± 1	35 ± 16	- 0.6 ± 0.3
Adenosine	<0.5	<0.5	<0.5	-
Total	599 ± 10	604 ± 5	594 ± 33	

Single wells containing differentiated C2C12 myotubes were incubated with 600 nmol of ATP in 2 ml medium (300 μ M) for 5 or 10 min. Control experiments were performed in wells in the absence of myotubes (medium without cells). Data are expressed as nmol of recovered nucleotides (mean $\pm$ s.e., $n = 3$). Rate of hydrolysis was calculated by pooling data from 5 and 10 min incubation ($n = 6$). Average protein content per well was 1.06 ± 0.10 mg protein/well.

lic AMP content was blocked by suramin (500 μ M; Figure 2a). It was found that ADP was about equipotent to ATP as judged by the onset of the ascending phase of their respective concentration-response curves (Figures 1b, 2a).

Although hydrolysis of ATP to adenosine was not detected in the incubation medium (Table 1), adenosine might be produced at the cell surface very near its receptor. The presence of P₁-purinoceptors on the myotubes was investigated by measuring the change of cyclic AMP content evoked by adenosine. Stimulation with adenosine (10 μ M–1 mM) induced the formation of cyclic AMP with a similar time course as observed for ATP. However, adenosine-induced cyclic AMP formation was not affected by suramin and reached a lower maximal level compared to that induced by ATP or ADP (Figure 2b).

By convention, a purinoceptor sensitive to ATP and ADP, has to be classified as a P₂-purinoceptor. To examine whether this receptor can be recognized as one of the established P₂-purinoceptor subclasses, the action of ATP analogues was studied. The selective P_{2X}-agonist, α,β -MeATP, evoked a concentration-dependent increase in cyclic AMP content of the myotubes; this response was also blocked by suramin (Figure 2c). The concentration-response curve of α,β -MeATP ascended at a similar agonist concentration as found with ATP and ADP, but in the presence of α,β -MeATP con-

sistently lower values of cyclic AMP were found compared to ATP and ADP (Figures 1b, 2a,c). The selective agonist for the P_{2Y}-purinoceptor, 2-MeSATP did not affect cyclic AMP levels (Figure 2c). To examine whether the ATP-evoked increase in cyclic AMP content was mediated by the nucleotide type P₂-purinoceptor present in C2C12 myotubes (Henning *et al.*, 1992), the action of the pyrimidine, UTP, on cyclic AMP formation was examined. It was found that stimulation of the myotubes with UTP (10 μ M–1 mM) did not affect their cyclic AMP content (Figure 2d). To investigate whether ATP- and ADP-induced formation of cyclic AMP was mediated through the same purinoceptor, simultaneous stimulation of the myotubes by these compounds was examined. Stimulation of the cells (10 min) by ATP (100 μ M) and ADP (100 μ M) respectively induced a similar formation of cyclic AMP (89.6 ± 8.3 pmol mg⁻¹ protein and 82.4 ± 9.7 pmol mg⁻¹ protein; $n = 4$). However, formation of cyclic AMP elicited by simultaneous addition of the nucleotides was significantly diminished (36.5 ± 2.1 pmol mg⁻¹ protein, $n = 4$).

Ins(1,4,5)P₃ formation

In C2C12 myotubes, both ATP (10 μ M–1 mM) and UTP (10 μ M–1 mM) induced a four fold increase in the Ins(1,4,5)P₃ content of the cells after 45 s stimulation (Table 2). In contrast to the generation of cyclic AMP, ADP (10 μ M–1 mM) was less effective in producing Ins(1,4,5)P₃ than ATP, while adenosine was ineffective (Table 2). Further, it was observed that the maximal increase of Ins(1,4,5)P₃ elicited by ATP (1 mM) and UTP (1 mM) were not additive (3.36 ± 0.81 pmol mg⁻¹ protein; $n = 4$).

Inhibition of P₂-purinoceptor-mediated cyclic AMP formation

The decline in formation of cyclic AMP at high ATP concentrations was further examined in ATP-stimulated myotubes pretreated for 14 h with pertussis toxin (PTx; 100 ng ml⁻¹). Pretreatment of the cells with PTx enhanced the maximum formation of cyclic AMP, but did not affect the decline in cyclic AMP formation at higher ATP concentrations (Figure 3).

Inhibition of cyclic AMP formation at higher ATP concentrations might be caused by simultaneous stimulation of the 'nucleotide' receptor. UTP stimulated the nucleotide receptor (Table 2; Henning *et al.*, 1992), but did not affect cyclic AMP formation in contrast to ATP (Figure 2d). Therefore, this compound was used to study the effect of 'nucleotide' receptor stimulation on cyclic AMP formation in the myotubes. When added simultaneously, UTP (30 μ M–1 mM) progressively decreased the cyclic AMP formation in myotubes elicited with an optimal concentration of ATP or ADP (100 μ M; Figure 4a,b). In contrast, UTP enhanced the adenosine (100 μ M) induced cyclic AMP formation (Figure 4c). The inhibitory effect of UTP on ATP-induced cyclic AMP formation was further studied by constructing concentration-re-

Figure 2 Concentration-dependent changes in cyclic AMP content of C2C12 myotubes after 10 min stimulation with different nucleotides. (a) The increase in cyclic AMP content induced by ADP (○) was blocked by suramin (500 μ M; ●). (b) The increase in cyclic AMP content induced by adenosine (○) was not affected by suramin (500 μ M; ●). (c) The increase in cyclic AMP content induced by α,β -MeATP (○) was blocked by suramin (500 μ M; ●); 2-MeSATP (□) did not affect cyclic AMP content. (d) The cyclic AMP content was not changed in the presence of UTP. Suramin was added 15 min prior to stimulation. Data are expressed as pmol mg⁻¹ protein (mean $\pm$ s.e.) and were obtained from 4 or more experiments.

Table 2 The increase in Ins(1,4,5)P₃ content of C2C12 myotubes following stimulation by various nucleotides

Agonist concentration	ATP	Increase in Ins(1,4,5)P ₃ (pmol mg ⁻¹ protein)			
		UTP	ADP	Adenosine	
10 μ M	0.52 $\pm$ 0.07	-0.05 $\pm$ 0.12	-0.12 $\pm$ 0.07	-	
30 μ M	1.69 $\pm$ 0.13	1.02 $\pm$ 0.09	0.26 $\pm$ 0.07	-	
100 μ M	2.90 $\pm$ 0.12	2.10 $\pm$ 0.10	0.44 $\pm$ 0.11	0.07 $\pm$ 0.13	
300 μ M	3.34 $\pm$ 0.29	2.68 $\pm$ 0.14	1.05 $\pm$ 0.13	-0.19 $\pm$ 0.30	
1 mM	3.41 $\pm$ 0.12	3.22 $\pm$ 0.06	3.01 $\pm$ 0.10	-0.10 $\pm$ 0.14	

Myotubes were stimulated for 45 s in the presence of the nucleotide concentrations indicated. Data are expressed as the increase in Ins(1,4,5)P₃ content over basal level (1.06 $\pm$ 0.12 pmol mg⁻¹ protein, mean $\pm$ s.e., $n \geq 4$).

Figure 3 The effect of pretreatment of C2C12 myotubes with pertussis toxin (PTx) on ATP-induced formation of cyclic AMP. Stimulation of the cells with ATP for 10 min in control cells (○) and after pretreatment with PTx (100 ng ml⁻¹) for 14 h (●). Data are expressed as pmol mg⁻¹ protein (mean $\pm$ s.e.) and were obtained from 4 or more experiments.

sponse curves to ATP at various UTP (100 μ M–1 mM) concentrations, showing a progressive decrease in the ATP-induced formation of cyclic AMP, without an apparent shift in the concentration-response curve (Figure 5).

Discussion

Extracellular ATP caused a relatively slow formation (10 min) of cyclic AMP in C2C12 myotubes, which was characterized by a prominent bell-shaped concentration-response relationship. Application of adenosine also induced a concentration-dependent increase in cyclic AMP formation, which was characterized by a regular concentration-response relationship. For several reasons, however, it seems likely that cyclic AMP formation induced by ATP in C2C12 myotubes is mediated via P₂-purinoceptors, rather than caused by stimulation of P₁-purinoceptors by its hydrolysis product, adenosine. This is supported by the finding that the ATP-induced cyclic AMP formation was completely blocked by the P₂-purinoceptor antagonist, suramin (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989; Henning *et al.*, 1992), while adenosine-induced cyclic AMP formation was insensitive to suramin. Further, the formation of cyclic AMP was also observed with the stable ATP analogue, ATP γ S. Moreover, during the incubation period, negligible amounts of AMP and adenosine were generated, in contrast to ATP hydrolysis to ADP. These observations strongly suggest that ATP-induced formation of cyclic AMP is caused by stimulation of a P₂-purinoceptor in C2C12 myotubes, while adenosine-induced cyclic AMP formation is presumably mediated through an A₂-type P₁-purinoceptor as found in other cells (Londos *et al.*, 1980).

The nucleotides ATP or ADP showed a similar potency with respect to the formation of cyclic AMP. The action of both nucleotides was not additive and was blocked by suramin. Formation of cyclic AMP was also induced by the P_{2X}-purinoceptor agonist, α , β -MeATP. However, since α , β -

Figure 4 Concentration-dependent effect of UTP on cyclic AMP formation induced by adenine nucleotides. UTP and adenine nucleotides were added simultaneously. UTP (30 μ M–1 mM) evoked a progressive decline in cyclic AMP formation of myotubes stimulated with ATP (100 μ M; a) and ADP (100 μ M; b). The cyclic AMP formation was enhanced in myotubes stimulated with adenosine (100 μ M; c). Cells were stimulated for 10 min. Data are expressed as pmol mg⁻¹ protein (mean $\pm$ s.e.) and were obtained from 4 or more experiments.

MeATP did not exceed ATP or ADP potency with respect to cyclic AMP formation, the ATP-sensitive receptor cannot be classified as P_{2X}-purinoceptor (Cusack & Hourani, 1990). Moreover, the P₂-purinoceptor was not sensitive to 2-Me-SATP or UTP, excluding the involvement of P_{2Y}-purinoceptors (Cusack & Hourani, 1990) and the nucleotide type P₂-purinoceptor (O'Connor *et al.*, 1991). Other P₂-purinoceptors, i.e. P_{2T}- and P_{2Z}-purinoceptors have to be excluded in view of the agonistic action of both ATP and ADP on cyclic AMP formation (Cusack & Hourani, 1990). Thus, it is concluded that a novel type of P₂-purinoceptor is linked to the cyclic AMP formation in C2C12 myotubes. This P₂-

Figure 5 Inhibition of ATP-induced cyclic AMP formation by UTP. The concentration-dependent cyclic AMP formation evoked by ATP at 10 min (○) was progressively inhibited by UTP 100 μM (Δ), 300 μM (◻) and 1 mM (▽). Both nucleotides were added simultaneously. Data are expressed as pmol mg⁻¹ protein (mean ± s.e.) and were obtained from 4 or more experiments.

purinoceptor of C2C12 myotubes resembles the P₂-purinoceptor mediating cyclic AMP formation in bovine aortic smooth muscle cells (Tada *et al.*, 1992). The ATP-induced cyclic AMP formation in the aortic cells, however, was not inhibited by suramin, suggesting the presence of different P₂-purinoceptor subtypes in bovine aortic cells and C2C12 myotubes.

The concentration-dependent ATP-induced cyclic AMP formation showed a substantial decline at high agonist concentrations. This decline was not caused by activation of a PTx-sensitive G_i-protein at higher agonist concentrations. C2C12 myotubes have been shown to possess a nucleotide type P₂-purinoceptor (Henning *et al.*, 1992) activating the PLC pathway, besides the P₂-purinoceptor mediating cyclic AMP formation. Simultaneous stimulation of the myotubes with UTP, not affecting cyclic AMP formation, inhibited the ADP- and ATP-induced formation of cyclic AMP. The effect of progressively higher concentrations of UTP on the ATP-induced concentration-response relationship for cyclic AMP formation was characterized by a decrease in the maximum effect without an apparent shift in the curve. This observation demonstrates that the action of UTP in reducing the ATP-induced response cannot be explained solely by a competitive antagonism of UTP on the P₂-purinoceptor mediating the ATP-induced cyclic AMP formation. The inhibition of the P₂-purinoceptor-mediated cyclic AMP formation by UTP might be related to the stimulation of the PLC pathway by this compound. A similar Ins(1,4,5)P₃ formation was produced by UTP and ATP in the myotubes, while ADP was less effective. The cyclic AMP content of myotubes optimally stimulated by ATP or ADP but inhibited by maximal concentrations of UTP, was similar to that of myotubes stimulated with the maximal ATP concentration. However, cyclic AMP formation induced by maximum ADP concentrations was inhibited to a lesser extent. These observations suggest that the activation of the PLC pathway via the nucleotide receptor inhibits P₂-purinoceptor-mediated cyclic AMP for-

mation. In contrast to the reduction of P₂-purinoceptor mediated cyclic AMP formation found in the presence of UTP, enhancement of adenosine-induced cyclic AMP formation by UTP was observed, possibly also dependent on nucleotide-receptor mediated activation of the PLC pathway. At this stage, the involvement of nucleotide receptor-mediated PLC activation in either the inhibition of P₂-purinoceptor-mediated cyclic AMP formation or stimulation of P₁-purinoceptor-mediated cyclic AMP formation by UTP remains speculative. PLC-activated modulation of the cyclic AMP pathway has been studied extensively in non-skeletal muscle cells and is attributed to the activation of the phosphorylating enzyme protein kinase C (PKC) (Houslay, 1991). Activation of PKC is associated with stimulation (Garte & Belman, 1980; Goureau *et al.*, 1990) as well as with inhibition of cyclic AMP formation, due to phosphorylation of different components of the cyclic AMP pathway (Katada *et al.*, 1985; Bell & Brunton, 1986; Irvine *et al.*, 1986; Yoshimasa *et al.*, 1987; Abou-Samra *et al.*, 1987; Convents *et al.*, 1989). Modulation of cyclic AMP formation by receptor-mediated PLC activation, however, may also be dependent on mechanisms other than PKC-mediated phosphorylation, such as Ca²⁺-dependent activation of phosphodiesterase (Boyajian & Cooper, 1990) or involvement of different heteromeric G protein subunits (cf. Birnbaumer, 1992). The opposite action of UTP on P₁- and P₂-purinoceptor-activated cyclic AMP formation in C2C12 myotubes would suggest that the inhibitory effect of UTP is not exerted on adenylate cyclase or phosphodiesterase. However, multiple isoenzymes, possessing different sensitivity to the UTP-activated mechanism may be present in these cells. Therefore, to establish the mechanisms involved in the modulation of cyclic AMP formation by UTP in C2C12 myotubes needs further study.

It has been shown that cyclic AMP regulates important cellular functions in myotubes, including desensitization (Albuquerque *et al.*, 1986; Miles *et al.*, 1987) and expression (Betz & Changeux, 1979; Fontaine *et al.*, 1987; Moss *et al.*, 1991; Green *et al.*, 1991) of nicotinic AChRs. In this study we identified two receptor types, an ATP-sensitive P₂-purinoceptor mediating cyclic AMP formation, and a P₁-purinoceptor sensitive to adenosine, both mediating the generation of cyclic AMP. So far, only calcitonin gene-related peptide had been identified as a possible primary messenger in the regulation of intracellular levels of cyclic AMP in myotubes (Mulle *et al.*, 1988; Changeux, 1991). As ATP is excreted from the motor nerve endings in conjunction with acetylcholine (Silinsky, 1975), the existence of multiple purinoceptors coupled to the cyclic AMP pathway suggests that a primary messenger role for ATP needs to be considered.

In summary, P₁-purinoceptor-induced cyclic AMP formation was established in C2C12 myotubes. Further, a novel ATP- and ADP-sensitive P₂-purinoceptor is proposed, also activating the formation of cyclic AMP. Activation of these cellular pathways by ATP in C2C12 myotubes suggests that this co-transmitter may serve as a primary messenger to regulate intracellular signalling in skeletal muscle.

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