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Adenosine A_{2B} receptors modulate cAMP levels and induce CREB but not ERK1/2 and p38 phosphorylation in rat skeletal muscle cells

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Abstract

The present study examined the existence of the adenosine A_1, A_{2A}, and A_{2B} receptors and the effect of receptor activation on cAMP accumulation and protein phosphorylation in primary rat skeletal muscle cells. Presence of mRNA and protein for all three receptors was demonstrated in both cultured and adult rat skeletal muscle. NECA (10^{-9}–10^{-4} M) increased the cAMP concentration in cultured muscle cells with an EC_{50} of (95% confidence interval) = 15 (5.9–25.1) μM, whereas CGS 21680 (10^{-9}–10^{-4} M) had no effect on cAMP accumulation. Concentrations of [R]-PIA below 10^{-6} M had no effect on cAMP accumulation induced by either isoproterenol or forskolin. NECA resulted in phosphorylation of CREB with an EC_{50} of (95% confidence interval) = 1.7 (0.40–7.02) μM, whereas ERK1/2 and p38 phosphorylation was unchanged. The results show that, although the A_1, A_{2A}, and A_{2B} receptors are all present in skeletal muscle cells, the effect of adenosine on adenylyl cyclase activation and phosphorylation of CREB is mainly mediated via the adenosine A_{2B} receptor.

Keywords: Transcription factor; Mitogen-activated protein kinase; Cyclic AMP response element binding protein; Extracellular signal-regulated protein kinase 1 and 2; Stress-activated protein kinase

Adenosine activates four different adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3 receptors [1]. All of them are G protein coupled. The A_1 and A_3 receptors couple to $G_{i/o}$ proteins that inhibit adenylyl cyclase, whereas the A_{2A} and A_{2B} receptors stimulate adenylyl cyclase via G_s proteins [2,3]. However, adenosine receptors mediate many other effects than changes in cyclic AMP [4,5]. In skeletal muscle, adenosine receptors have been shown to be involved in the regulation of blood flow [6,7], insulin-mediated glucose uptake [8,9], and the regulation of contractile force [10]. In a recent study on human skeletal muscle, the A_1, A_{2A}, and the A_{2B} receptors were found to be present in vascular endothelial and smooth muscle cells, whereas only the A_{2A} and A_{2B} receptors were found in skeletal muscle cells [11]. These data would suggest that there are only G_s-coupled and adenylyl cyclase activating adenosine receptors present in skeletal muscle cells.

The main intracellular pathway allocated downstream of adenosine A_2 receptors is the cAMP/PKA cascade. Stimulation of the adenosine A_2 receptors will lead to activation of adenylyl cyclase and the production of cAMP, causing activation of the cAMP-dependent protein kinase (PKA) which in turn may phosphorylate an array of different proteins, e.g., transcription factors such as the cAMP response element binding protein (CREB) [12], thus leading to changes in gene transcription (for a review see [13]). In addition, cAMP can mediate mitogenic signalling either independent of [14] or dependent on MAPKs [15]. This, however, appears to be very cell specific [16].

Adenosine receptors have also been shown to activate several MAPKs. Schulte and Fredholm [17]

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recently demonstrated in CHO cells expressing the human adenosine receptors that phosphorylation of extracellular regulated kinases 1/2 (ERK1/2) occurred upon stimulation of A_1, A_{2A}, A_{2B}, and A_3 receptors. ERK1/2 has also been found to be stimulated by adenosine receptor activation in other cell types such as endothelial cells, mast cells, and DDT_1MF-2 cells [18–20]. p38, another member of the MAPK family, has been reported to be stimulated by adenosine in DDT_1MF-2 cells via activation of the A_1 receptor [20] and via the A_{2B} receptor in human mast cells [19]. However, the intracellular signalling pathways activated by adenosine receptors in skeletal muscle are still unknown.

In skeletal muscle ERK1/2, p38, and CREB have all been shown to be phosphorylated after contractile activity [21–23], but the physiological consequences of activation are not certain. Growing evidence suggests that activation of ERK1/2 and p38 during muscle contraction leads to an increased intracellular activation of downstream kinases and transcription factors [24]. Moreover, activation of p38 has been implicated in the regulation of glucose uptake in skeletal muscle [25,26]. These pathways have been suggested to participate in the regulation of gene expression and cell growth in skeletal muscle [27,28].

The aim of the present study was to identify the adenosine receptors present in rat skeletal muscle and to determine the effect of adenosine receptor activation on cAMP accumulation as well as on ERK1/2, p38, and CREB phosphorylation.

Materials and methods

Culture media, calf, and horse serum, and culture dishes were all from Life Technologies. 5′-*N*-Ethylcarboxamido-adenosine (NECA), 2-[*p*-(2-carboxy-ethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM 241385) from ICN Biomedicals. (−)-N^6-Phenylisopropyladenosine([R]-PIA), forskolin, isoproterenol, pertussis toxin, and Hepes were from Sigma, MO, USA. Adenosine deaminase was from Böhringer–Mannheim, GmbH, Germany. Phospho-specific antibodies: rabbit anti-P-CREB was from Upstate Biotechnology (Lake Placid, NY, USA) and rabbit anti-P-ERK1/2 and rabbit anti-P-p38 were from New England Biolabs (Beverly, MA, USA). Goat anti-rabbit horseradish peroxidase coupled antibody was from Pierce (Rockford, IL, USA).

Skeletal muscle cell cultures

Ten-day old rat pups were sacrificed by cervical dislocation. Skeletal muscle satellite cells were isolated and cultured as previously described [29]. Fibroblasts were removed by initial pre-plating and by dispase treatment at 96 h after seeding. Experiments were performed on 11–12 days old cultures. All treatment of animals complied with the European Convention for the protection of vertebrate animals used for experiments or other scientific purposes (Council of Europe No. 123, Strasbourg, France, 1985).

RNA isolation

Muscle and brain tissue. RNA was isolated from ~25 mg tissue [white gastrocnemius (WG), extensor digitorum longus (EDL), soleus (SOL), and brain tissue] by a modified guanidinium thiocyanate (GT)–phenol–chloroform extraction method adapted from Chomczynski and Sacchi [30] as described previously [31]. Briefly, the samples were homogenized in a GT solution and extracted by the addition of NaOAc, pH 4.0, DEPC saturated-phenol, and chloroform:isoamyl-OH, followed by centrifugation. RNA was precipitated by the addition of isopropanol and centrifugation. Glycogen was added before precipitation to facilitate localization of the pellet. The pellet was then washed with 75% EtOH and resuspended in 0.1 mM EDTA dissolved in nuclease-free water.

Cultured cells. RNA was isolated from cells by use of TRIzol (Invitrogen) according to the manufacturer's recommendations.

Reverse transcription. The reverse transcription reaction was performed using the Superscript II RNase H^- system (Gibco-BRL) and oligo(dT) primers.

Real time PCR. Specific primers were designed for each of the mRNA sequences of interest. The cDNA sequences specific for rat were obtained from NCBI and The Sanger Centre. Primers were designed by the use of Primer Express v 2.0 (Applied Biosystems) and specificity of the obtained product sequence was confirmed by a search in the NCBI Blast database. Validation of the different PCR product sizes was carried out by 2.5% agarose gel electrophoresis. Furthermore, a dissociation curve analysis was performed to verify the theoretical melting temperature of the product. For details on primers see Table 1. The ABI 7700 real time PCR system was used for relative quantification. Each reaction was composed of 1.2 μl of the diluted cDNA and 7.5 μl of 2× SYBR Green PCR Mastermix (Applied Biosystems). Primers and water were added to give a final reaction volume of 15 μl per well. Each sample was analysed in triplicates. The PCR was carried out by incubation at 50 °C for 2 min followed by activation of the AmpliTaq Gold enzyme at 95 °C for 10 min. PCR cycling was performed as denaturation at 95 °C for 15 s and annealing + elongation at 60 °C for 60 s. A total of 40 cycles were performed.

Data analysis. A threshold fluorescence level was manually chosen in the exponential phase of the amplification where no noise interfered with the signal. The cycle at which the fluorescence rose above the threshold level was defined as the threshold cycle (C_T).

RNA purity. To verify that the PCR products were a result of amplification of cDNA and not DNA, C_T was determined for samples

Table 1
Primer sequences

Receptor subtype	Forward primer (5′ → 3′)	Reverse primer (3′ → 5′)	Product length (bp)
A_1	GGCAACTCCGCCATGAAC	CAGAAAGGTGACCCGGAACTT	66
A_{2A}	CCCTTCATCTACGCCTACAGGAT	CGTGGGTTCGGATGATCTTC	67
A_{2B}	GCATTGGACTGACTCCTTTCCT	TCCCCAGGTTCTGTGCAGTT	73

A_1, adenosine receptor 1; A_{2A}, adenosine receptor 2A; and A_{2B}, adenosine receptor 2B.

where reverse transcription was performed and from samples where no reverse transcription was performed. A difference of at least 2 C_T's was found, corresponding to a 4-fold difference, thus verifying that the PCR products primarily were a result of cDNA amplification.

Western blot

Western blot analysis was performed on homogenates of primary skeletal muscle cell cultures and homogenates of plasma membrane giant vesicles which were isolated from a mix of adult skeletal muscle consisting of type I and type II fibres. Proteins were separated according to their molecular weight by SDS–PAGE. Immunoblotting was conducted using polyvinylidene difluoride membranes (Millipore, Bedford, MA) and the transfer was carried out for 1 h at 0.8 mA/cm^2 membrane, 100 V.

The membranes were blocked with 5% non-fat dried milk in 20 mM Tris, 500 mM NaCl, and 1% Tween 20 (pH 7.5) for 1 h and the membranes were subsequently incubated overnight at +4 °C with polyclonal antibodies to the adenosine receptors (A_1: PA1-041; A_{2A}: PA1-042, and A_{2B}: AB1589P, Affinity Bioreagent, CO, USA). The peptide sequences used for immunization for A_1 and A_{2A} were from the C-terminal end and for A_{2B} from the third extracellular domain. The sequences were as follows: A_1: ^{309}CQPKPPIDEDLPEEKAED^{326}, origin of sequence: rat brain and spinal cord; A_{2A}: ^{373}ES HGDMGLPDVELLSHELK^{391}, origin of sequence: canine brain; and A_{2B}: ^{150}ATNNCTEPWDGTTNES^{165}, origin of sequence: human brain. After washing, the membranes were incubated with a secondary antibody coupled to alkaline phosphatase followed by detection with enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, England). The blots were visualized with densitometric scanning using a densitometer (Molecular Dynamics Storm 840) with digital analysis software (ImageQuaNT).

Experimental procedures for cell cultures used for cAMP determination

Dishes with muscle cells were rinsed twice with Krebs–Ringer buffer (KRB) containing Hepes (25 mM), β-glycerophosphate (50 mM), and glucose (50 mM). Dishes were placed in a temperature-controlled chamber (32 °C) and the cells were incubated in KRB with adenosine deaminase (17 U/ml medium) for 10 min. The adenosine receptor agonists (NECA, CGS 21680, and [R]-PIA) and antagonists (ZM 241385) were added to the cell-culture dishes and incubated for 5 min. Incubation of agonist for 5 min was sufficient since 10 min of incubation with NECA did not increase cAMP accumulation further. Cells were then placed on ice to stop all reactions. The buffer was removed and the cells were rinsed once with ice-cold buffer and then 300 μl of 65% ice-cold ethanol was added. After 5 min of extraction cells were scraped off and transferred to a reaction tube and the dish was rinsed with 100 μl of ethanol. The samples were frozen immediately at −80 °C until the time of analysis.

cAMP assay

For the cAMP analysis, the cell extracts were vacuum dried and diluted in a 0.05 M sodium acetate buffer, pH 5.8, containing 0.02% bovine serum albumin. The concentration of cAMP was assessed using a cAMP enzyme immunoassay system (RPN 225) Biotrak, Amersham Pharmacia Biotech, UK. Standard for the assay was in the range from 12.5 to 3200 fmol/well lyophilized cAMP.

Experimental procedures for cell cultures used for phosphorylation assay

For the protein phosphorylation assay, cell cultures were serum deprived (0.2% horse serum) overnight. Dishes with cell cultures were treated as described above until the introduction of adenosine receptor agonists and antagonists. The adenosine receptor agonists NECA and CGS 21680 (concentrations ranging from 10^{-9} to 10^{-4} M) were added to the cell-culture dishes for 5 min. In experiments using pertussis toxin to downregulate $G_{i/o}$ proteins, pertussis toxin (200 ng/ml of medium) was added 18 h prior to the experiments; cell culture dishes were incubated with NECA (concentrations ranging from 10^{-9} to 10^{-4} M). The dishes were placed on ice to stop the stimulation. The medium was aspirated and 500 μl of ice-cold homogenizing buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM Na-pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 2 mM Na_3VO_4, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM PMSF, 1 mM $MgCl_2 \cdot 6\ H_2O$, 1 mM $CaCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 3 mM benzamidine) was added to the cells. The cells were scraped off, transferred to a reaction tube, and dipped into liquid nitrogen. The samples were stored at −80 °C until the time of analysis.

Phosphorylation assay

The cells were homogenized in Laemmli buffer. Proteins were separated by polyacrylamide gel electrophoresis as described previously [17]. After electrical transfer to PVDF-membranes and blocking in 3% non-fat dried milk in TBS T (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20) phosphorylated proteins were incubated with the phospho-specific antibodies diluted in TBS-T, 5% BSA overnight at 4 °C [rabbit anti-P-ERK1/2 (1:1000), rabbit anti-P-p38 (1:1000), and rabbit anti-P-CREB (1:5000)]. Proteins were visualized using the appropriate goat-anti rabbit secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence kit (Amersham Biosciences, Sweden) according to the manufacturer's instructions.

Statistics

Data were statistically analysed by one-way analysis of variance. EC_{50} values were calculated from dose–response curves using non-linear regression. Data are presented as means ± SEM. Values of $p < 0.05$ were considered statistically significant.

Results

Presence of the adenosine A_1, A_{2A}, and A_{2B} receptor subtypes

The agarose gel analysis verified that mRNA coding for adenosine receptor type A_1, A_{2A}, and A_{2B} exists in samples from primary skeletal muscle cells, rat soleus-, white gastrocnemius-, and EDL muscle (Fig. 1). The same mRNA sequences were present in samples obtained from brain tissue.

Western blot analysis revealed existence of the adenosine A_1, A_{2A}, and A_{2B} receptors in primary skeletal muscle cell culture homogenates as well as plasma membrane giant vesicles derived from a mix of adult rat skeletal muscles with type I and type II fibres (Fig. 2). The observed molecular weights of the adenosine A_1, A_{2A}, and A_{2B} receptors were about 36, 45, and 36 kDa, respectively.

NECA increased cAMP levels and CREB phosphorylation, but not p38 MAPK and ERK1/2 phosphorylation

Incubation of primary skeletal muscle cells with increasing concentrations (10^{-9}–10^{-4} M) of the non-selective adenosine analogue NECA increased ($p < 0.05$)

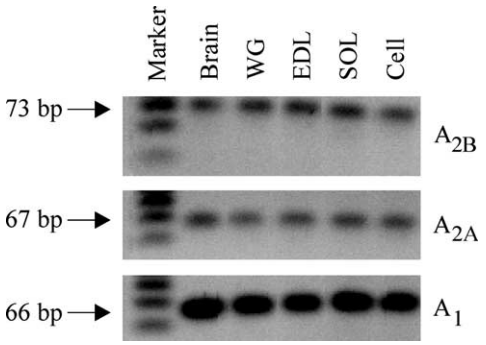

Fig. 1. Presence of mRNA for adenosine A_1, A_{2A}, and A_{2B} receptors in rat skeletal muscle. Ethidium bromide stained agarose gel analysis of PCR products from rat brain, rat muscle (WG, white gastrocnemius; EDL, extensor digitorum longus; and SOL, soleus), and primary muscle cells (Cells).

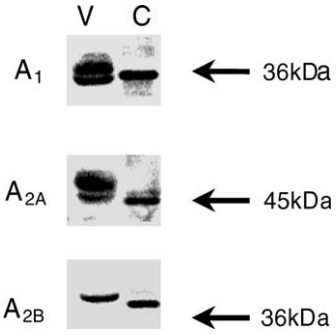

Fig. 2. Presence of adenosine A_1, A_{2A}, and A_{2B} receptors in rat skeletal muscle. Western blot analysis of adenosine A_1, A_{2A}, and A_{2B} receptors in cultured rat skeletal muscle cells and in adult rat skeletal muscle homogenate of mixed fibre types. Column V, samples from plasma membrane giant vesicles derived from adult rat skeletal muscles consisting of type I and type II fibres. Column C, samples from cultured primary rat skeletal muscle cells. Forty micrograms of protein was loaded in each lane.

the concentration of cAMP with an EC_{50} (95% confidence interval) = 15 (5.9–25.1) μM ($N = 6$). Saturation was reached at 100 μM NECA (Fig. 3). When CREB phosphorylation was measured in primary skeletal muscle cells an increase ($p < 0.05$) in phosphorylation was observed with an EC_{50} (95% confidence interval) = 1.7 (0.4–7.0) μM, ($N = 4$; Fig. 4). However, there was no effect ($p > 0.05$) of NECA stimulation on ERK1/2 and p38 phosphorylation ($N = 4$; Fig. 4), even though—as positive control—electrical stimulation of the cultured muscle cells was found to strongly increase ERK1/2 and p38 phosphorylation (data not shown).

Effect of CGS 21680 and ZM 241385 on cAMP levels and CREB phosphorylation

Furthermore, experiments were conducted to investigate if the adenosine A_{2A} and/or A_{2B} receptors were coupled to the G_s-protein/adenylyl cyclase pathway in

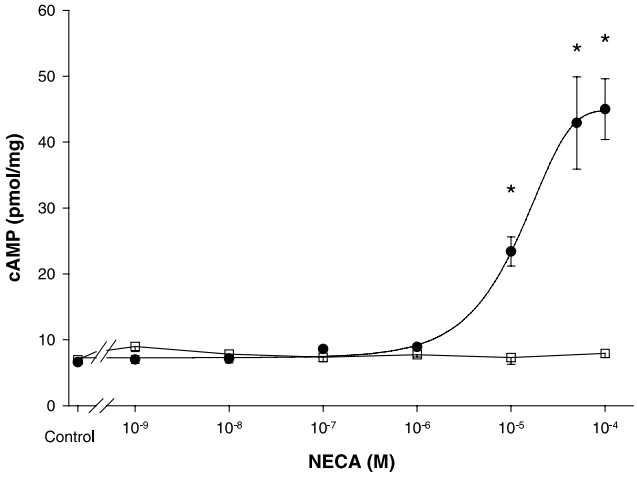

Fig. 3. Effect of NECA and CGS 21680 on cAMP levels in cultured rat skeletal muscle cells. Dishes with primary rat skeletal muscle cells were pre-treated with adenosine deaminase for 10 min and incubated with either the non-specific adenosine receptor agonist NECA or the selective adenosine receptor agonist CGS 21680 for 5 min prior to extraction. cAMP was assessed using a cAMP enzyme immunoassay system. Results are from six experiments, each performed in duplicate. Data are means ± SEM. * indicates significant ($p < 0.05$) difference from control value.

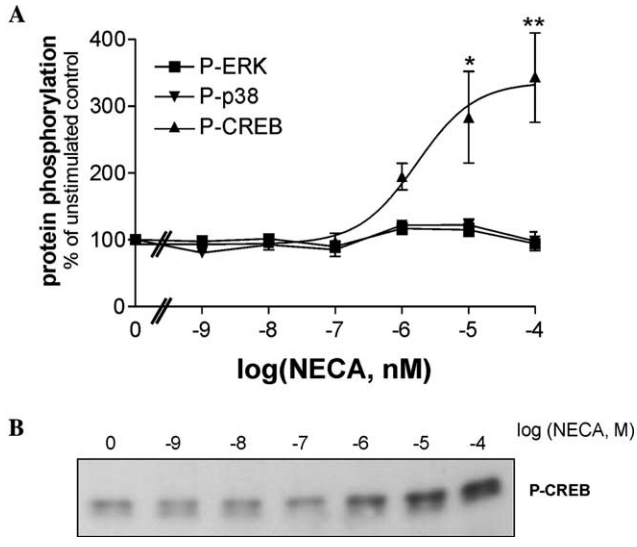

Fig. 4. The effect of NECA stimulation on CREB, ERK1/2, and p38 phosphorylation in primary rat skeletal muscle cells. Dishes with cultured primary rat skeletal muscle cells were serum deprived (0.2% serum) overnight. Cells were pre-treated with adenosine deaminase for 10 min and incubated with the non-specific adenosine receptor agonists NECA for 5 min in concentrations ranging from 10^{-9} to 10^{-4} M. Cells were homogenized in Laemmli buffer. Proteins were separated by polyacrylamide gel electrophoresis and immunoblotted using phospho-specific anti-P-ERK1/2, P-p38, and P-CREB antibodies. Results from four experiments are summarized in (A). Data were normalized (set to 100%) to phosphorylation values of the unstimulated controls. Error bars give SEM. *$p < 0.05$ and **$p < 0.01$ indicate data points being significantly different from control value. In (B) a representative immunoblot for NECA-induced CREB phosphorylation is shown.

skeletal muscle cells. Incubations of skeletal muscle cells with the adenosine A_{2A} receptor specific agonist CGS 21680 in concentrations ranging from 10^{-9} to 10^{-4} M had no effect ($p > 0.05$) on the intracellular levels of cAMP ($N = 6$; Fig. 3) or on the phosphorylation of CREB (not shown). To investigate the effect of adenosine A_2 receptor antagonist ZM 241385, muscle cells were incubated with 1 μM NECA in combination with ZM 241385 in concentrations ranging from 10^{-9} to 10^{-5} M, which did not change cAMP levels significantly ($N = 6$; $p > 0.05$). NECA was added in order to increase ($p < 0.05$) the cAMP levels (from 7.3 ± 0.4 to 9.7 ± 0.5 pmol/mg protein).

Effect of R-PIA on cAMP levels and the effect of pertussis toxin on CREB phosphorylation

In order to study signalling via the $G_{i/o}$ coupled adenosine A_1 receptor, the adenylyl cyclase activator forskolin (1 μM) was added to muscle cells in order to elicit an increase ($p < 0.05$) in the levels of cAMP from 7.8 to 32.0 pmol/mg ($N = 6$). This increase would allow the adenosine A_1 receptor agonist [R]-PIA to decrease the levels of cAMP. However, [R]-PIA did not decrease ($p > 0.05$) the level of cAMP concentrations in primary muscle cells ($N = 6$; Fig. 5) at lower concentrations, whereas cAMP levels were instead increased at higher concentrations. The same type of experiment was done with the addition of the β-adrenergic agonist isoproterenol (1 μM), which increased ($p < 0.05$) the concentration of cAMP from 6.5 to 40.8 mol/mg protein. [R]-PIA, however, did not decrease ($p > 0.05$) the isoproterenol-induced cAMP production in primary muscle cells ($N = 4$; data not shown). The involvement of $G_{i/o}$ protein coupled adenosine receptors in primary skeletal muscle cells could further be excluded by overnight treatment with pertussis toxin, which did not affect NECA provoked CREB phosphorylation (not shown).

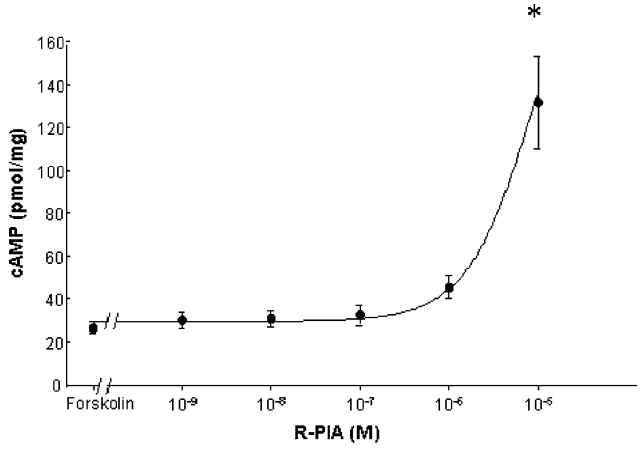

Fig. 5. Effect of R-PIA on cAMP levels in cultured rat skeletal muscle cells after incubation with forskolin. Dishes with primary rat skeletal muscle cells were pre-treated with adenosine deaminase for 10 min and incubated with the adenylyl cyclase stimulator forskolin and the adenosine A_1 receptor agonists R-PIA for 5 min prior to extraction. CAMP was assessed using a cAMP enzyme immunoassay system. Results are from six experiments, each performed in duplicate. Data are means ± SEM. * indicates significant ($p < 0.05$) difference from control value.

Discussion

In the current study, real time PCR and Western blot analysis revealed the existence of mRNA and protein for the adenosine A_1, A_{2A}, and A_{2B} receptors in cultured rat skeletal muscle cells as well as in adult skeletal muscle. Pharmacological experiments on cultured muscle cells showed moreover that activation of the adenosine A_{2B} receptor, but not the adenosine A_1 and A_{2A} receptors, modulates intracellular cAMP levels. Furthermore, the pharmacological profile of CREB phosphorylation with an EC_{50} value in the micromolar range points at a predominant physiological role for adenosine A_{2B} receptors in skeletal muscle cells.

Investigating the physiological and pharmacological effect of adenosine receptors, and especially the adenosine A_{2B} receptor, is difficult because of the lack of selective pharmacological agonists and antagonists. Identification and characterization of receptors in tissues containing more than one receptor sub-type therefore relies on methods of exclusion [32]. In the present study the physiological role of the adenosine A_{2B} receptor was assessed from results from several different approaches.

First, incubation of skeletal muscle cells with NECA showed accumulation of cAMP with concentrations in the micro-molar range. This observation is in agreement with studies on cells only expressing A_{2B} receptors, showing that the EC_{50} for NECA is approximately 2 μM [33–35]. Furthermore, previous studies using CHO cells, over-expressing a single type of adenosine receptors, have shown the order of EC_{50} values for NECA-induced cAMP production to be 26 nM (A_1) = 26 nM (A_{2A}) < 692–2400 nM (A_{2B}) [36–38], confirming the ability of adenosine A_{2B} receptors mediating NECA-induced half maximal effects in the lower micro-molar range [1,32]. In addition, the observed pharmacological profile for CREB phosphorylation mirrored the changes in cAMP production with an EC_{50} in the lower micro-molar range, indicating the presence of functional adenosine A_{2B} receptors.

Second, the adenosine A_{2A} receptor agonist CGS 21680, which shows a high degree of selectivity for rat adenosine receptor A_{2A} compared to A_{2B} [39], neither caused an increase in cAMP levels nor an enhanced CREB phosphorylation. Furthermore, the A_{2A} antagonist ZM 241385 did not lower the NECA induced elevation in cAMP. This indicates that the adenosine A_{2A}

receptor is not functionally coupled to adenylyl cyclase or that the number of receptors is too small to be of any significance for the activation of adenylyl cyclase. These findings suggest that the adenosine A_{2B} receptor is the adenosine receptor of importance in the cAMP/PKA/CREB pathway in rat skeletal muscle.

Third, cAMP levels elevated by forskolin, a non-receptor mediated activator of adenylyl cyclase, or isoproterenol, a β-adrenergic receptor agonist, were not diminished by the adenosine A_1 receptor specific agonist [R]-PIA. The basal level of cAMP was increased 4-fold when forskolin was added, but the levels remained unaltered when [R]-PIA was added in concentrations ranging from 1 nM to 1 μM. As the adenosine A_1 receptor is coupled to $G_{i/o}$ proteins, a decrease in cAMP with nanomolar concentrations of [R]-PIA would have been expected, in view of the findings that CHO cells over-expressing the adenosine A_1 receptor show an EC_{50} for [R]-PIA of 0.4 nM [40]. The finding of a lack of response to A_1 receptor stimulation appears to be true for mature rat skeletal muscle as studies on whole rat muscle homogenate have shown that incubation with [R]-PIA does not lower the isoproterenol induced increase in cAMP [41]. At higher concentrations of [R]-PIA the cAMP concentration was elevated rather than decreased, which strengthens the hypothesis of adenosine A_{2B} receptors mediating cAMP production. [R]-PIA was previously shown to bind and activate adenosine A_{2B} receptors with an affinity [42,43] and potency [44] in the micro-molar range. Although it cannot be excluded that the adenosine A_3 receptor is present in rat skeletal muscle, we were not able to detect it immunohistochemically.

Combined, the above results on cAMP accumulation and CREB phosphorylation indicate a negligible physiological role of the adenosine A_1 and A_{2A} receptors and strongly suggest that the A_{2B} receptor is of importance in the cAMP/PKA/CREB pathway in rat skeletal muscle cells. In contrast to previous studies in other cellular systems, where adenosine A_{2B} receptors were shown to mediate activation of the MAPK ERK1/2 or p38 [17,19,45], we could not detect an increase in MAPK phosphorylation upon NECA stimulation. Adenosine A_{2B} receptor-mediated MAPK activation has been described to be dependent on $G_{q/11}$ proteins [19,45], which may be a pathway that is not functional in primary rat muscle cells. Moreover, cAMP-dependent pathways may also lead to MAPKs activation [15], but they appear to be very cell specific [16] and an increase in cAMP does not necessarily increase MAPK phosphorylation [46].

The present study shows that adenosine can activate adenosine A_{2B} receptors with a concomitant increase in cAMP and activation of CREB. In several different tissues activation of CREB has been found to be associated with an increased expression of several genes and, specifically in skeletal muscle, CREB activation has been shown to be involved in myoblast differentiation [47], the expression of the L-type Ca^{2+} channel [48] and expression of hexokinase II [49]. It is thus likely that adenosine has at least the above-mentioned effects in skeletal muscle and that these effects are mediated via activation of A_{2B} receptors. Moreover, as A_{2B} receptors are low affinity receptors and since physical exercise has been shown to induce a marked accumulation of extracellular adenosine in human skeletal muscle tissue [50], it appears probable that muscular activity is an important stimulus for these pathways.

In conclusion, the results of the present study show that the adenosine A_1, A_{2A}, and A_{2B} receptors are present in both mature as well as in primary rat skeletal muscle cells. Stimulation of the adenosine A_{2B} receptor, but not the A_{2A} or A_1 receptor, activates adenylyl cyclase and mediates CREB-phosphorylation and, thus, the adenosine A_{2B} receptor is likely to be responsible for the physiological functions of adenosine in skeletal muscle.

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