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RESEARCH PAPER

Skeletal muscle expresses the extracellular cyclic AMP–adenosine pathway

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Background and purpose: cAMP is a key intracellular signalling molecule that regulates multiple processes of the vertebrate skeletal muscle. We have shown that cAMP can be actively pumped out from the skeletal muscle cell. Since in other tissues, cAMP efflux had been associated with extracellular generation of adenosine, in the present study we have assessed the fate of interstitial cAMP and the existence of an extracellular cAMP-adenosine signalling pathway in skeletal muscle.
Experimental approach: cAMP efflux and/or its extracellular degradation were analysed by incubating rat cultured skeletal muscle with exogenous cAMP, forskolin or isoprenaline. cAMP and its metabolites were quantified by radioassay or HPLC, respectively.
Key results: Incubation of cells with exogenous cAMP was followed by interstitial accumulation of 5′-AMP and adenosine, a phenomenon inhibited by selective inhibitors of ecto-phosphodiesterase (DPSPX) and ecto-nucleotidase (AMPCP). Activation of adenylyl cyclase (AC) in cultured cells with forskolin or isoprenaline increased cAMP efflux and extracellular generation of 5′-AMP and adenosine. Extracellular cAMP-adenosine pathway was also observed after direct and receptor-dependent stimulation of AC in rat extensor muscle *ex vivo*. These events were attenuated by probenecid, an inhibitor of ATP binding cassette family transporters.
Conclusions and implications: Our results show the existence of an extracellular biochemical cascade that converts cAMP into adenosine. The functional relevance of this extracellular signalling system may involve a feedback modulation of cellular response initiated by several G protein-coupled receptor ligands, amplifying cAMP influence to a paracrine mode, through its metabolite, adenosine.
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Keywords: G-protein-coupled receptors; cAMP; adenosine; adenylyl cyclase; skeletal muscle; ecto-phosphodiesterase

Abbreviations: AC, adenylyl cyclase; AMPCP, α,β-methylene-adenosine-5′-diphosphate; DPSPX, 1,3-dipropyl-8-*p*-sulphophenylxanthine; ecto-NT, ecto-nucleotidase; EDL, extensor digitorum longus; EHNA, erythro-9-amino-β-hexyl-α-methyl-9*H*-purine; IBMX, 3-isobutylmethylxanthine; PDE, phosphodiesterase

Introduction

Intracellular cAMP is a crucial signalling molecule in the transduction pathways of several membrane receptors coupled to stimulatory G proteins at skeletal muscle and at neuromuscular synapses. For example, when calcitonin gene-related peptide is released by motor neurons at the synaptic cleft, there is a postsynaptic increment of intracellular cAMP through activation of adenylyl cyclase (AC; Poyner *et al*., 2002). Intracellular increase of cAMP induced by activation of AC accelerates desensitization of muscle nicotinic receptors and β-adrenoceptors through mechanisms involving cyclic AMP-dependent protein kinase (Paradiso and Brehm, 1998; Swope *et al*., 1999; Lanuza and Gizaw, 2006; Vaughan *et al*., 2006), modulates acetylcholinesterase expression (da Costa *et al*., 2001; Rossi *et al*., 2003; Xie *et al*., 2007) and regulates glucose uptake after stimulation of β2-adrenoceptors (Nevzorova *et al*., 2006).

In a previous report, we demonstrated that direct- or receptor-dependent activation of AC induced a transient increase of intracellular cAMP in cultures of rat skeletal muscle that peaked 5 min after the onset of stimulation. The subsequent decrease in cAMP content did not correlate with degradation of the cyclic nucleotide as all experiments were performed with phosphodiesterase (PDE) inhibition. On the other hand, the reduction of intracellular cAMP was followed by the appearance of cAMP in the extracellular

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medium and associated with an efflux mechanism, as extracellular accumulation of cAMP was inhibited by probenecid (Godinho and Costa, 2003), a non-selective inhibitor of organic anion transporters.

Demonstration of cAMP efflux from skeletal muscle cells is consistent with the recent identification of three proteins of the ATP-binding cassette transporter family that mediate cAMP efflux: MRP4 (Chen *et al*., 2001; van Aubel *et al*., 2002), MRP5 (Jedlitschky *et al*., 2000; Suzuki *et al*., 2000; van Aubel *et al*., 2002; Wielinga *et al*., 2003) and MRP8 (McAleer *et al*., 1999; Tammur *et al*., 2001; Guo *et al*., 2003). More importantly, MRP5 (Kool *et al*., 1997; Belinsky *et al*., 1998; Suzuki *et al*., 2000) and MRP8 (McAleer *et al*., 1999; Tammur *et al*., 2001) are expressed in skeletal muscle.

Although we have demonstrated cAMP efflux in cultured skeletal muscle (Godinho and Costa, 2003), the physiological relevance of this cyclic nucleotide in the extracellular space is unknown. In the present study, we evaluated a possible fate of cAMP in the extracellular compartment of skeletal muscle cells. Our experiments showed that, once outside the cell, cAMP acts as a substrate of an extracellular biochemical cascade that leads to adenosine production, opening a new perspective of signals modulating both neuromuscular junction and skeletal muscle physiology.

Materials and methods

All animal procedures were in accordance with the ethical principles in animal research adopted by the Biomedical College of Animal Experimentation and approved by the Ethical Committee for Animal Research of the Universidade Federal de São Paulo.

Tissue-cultured skeletal muscle fibres

Primary skeletal muscle cultures were obtained from hind-limb myoblasts/satellite cells of newborn rats as originally described by Furlan and Godinho (2005). Briefly, 3×10^5 myoblasts were seeded onto collagen-coated 35-mm dishes in 2 ml of Dulbecco's modified Eagle's Medium supplemented with 15% fetal calf serum and 40 $\mu g\,ml^{-1}$ gentamicin at 37 °C in humidified atmosphere of 90% air and 10% CO_2. On the third day and every other day, the medium was replaced by DMEM supplemented with 10% horse serum and 2% fetal calf serum. All the experiments were performed on 7- to 8-day-old differentiated skeletal muscle cultures. All culture media and reagents were obtained from Gibco, Invitrogen, Carlsbad, CA, USA.

Analysis of the intra- and extracellular accumulation of cAMP

Cultured skeletal muscle fibres were rinsed three times with Krebs-bicarbonate-buffered solution (containing 25 mM $NaHCO_3$, 116 mM NaCl, 0.8 mM KH_2PO_4, 2.5 mM $MgCl_2$, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 2 $mg\,ml^{-1}$ D-glucose; pH 7.4) and incubated with 3-isobutylmethylxanthine (IBMX), a non-selective inhibitor of intra- and extracellular PDE, in a concentration that completely inhibits cAMP degradation (1 mM). After 15 min, cultures were stimulated with 1–300 μM forskolin or vehicle in 800 μl Krebs at 37 °C for 30 min. Culture dishes were transferred to an ice-cold bath and the medium was collected to determine extracellular cAMP. To quantify intracellular cAMP, cultured fibres were scraped using additional 800 μl cold Krebs buffer containing 4 mM EDTA and transferred to microfuge tubes and both medium and cell samples were immediately boiled for 15 min, vortexed for 1 min and centrifuged for 10 min at 20 000 *g* to denature PDE enzymes. This procedure does not lead to cAMP degradation (Godinho and Costa, 2003) and is sufficient to extract the intracellular nucleotides. cAMP content was determined in the supernatant (50 μl) using a [^{3}H]cAMP-assay kit (GE Healthcare LifeSciences, Fairfield, CT, USA) and expressed as picomoles of cAMP per culture dish. Total cAMP generated was always considered to be the sum of intra- and extracellular values. Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co., St Louis, MO, USA.

Determination of adenine-containing compounds by HPLC

To quantify cAMP metabolites produced by cultured skeletal muscle fibres, samples obtained from control and treated cultures were derivatized by reaction with 10% chloracetaldehyde in sodium citrate buffer (100 mM, pH 4.0) for 60 min at 80 °C to produce fluorescent etheno-compounds (Levitt *et al*., 1984). Fluorescent etheno-derivatives were injected on a HPLC system (Shimadzu, Japan) equipped with a C18 column (150 mm × 4.6 mm, 5 μm) and an LC-10A fluorescent detector. Mobile phase A (0.01 M phosphate buffer, pH 3.0) was maintained for 2 min, followed by a methanol gradient (0–30%) for 15 min (flow = 0.8 $ml\,min^{-1}$). Quantification of cAMP, adenosine, 5′-AMP and ATP from samples was achieved by interpolation of peak areas on the respective standard curve of fluorescent adenosine (Fluka Chemical Co., Buchs, Switzerland), 5′-AMP, ATP and cAMP, using GraphPad Prism for Windows software (version 3.0, San Diego, CA, USA).

Analysis of extracellular degradation of cAMP in cultured skeletal muscle fibres

The existence of an enzymatic system responsible for extracellular degradation of cAMP was investigated by incubating skeletal muscle cultures with exogenous cAMP (60 pmol per dish), in the presence or absence of IBMX (1 mM) at 37 °C. After 30 min, the incubation medium was collected, cells were rinsed and the cAMP was extracted using Krebs containing 0.1 mM IBMX. All samples were immediately boiled and cAMP content was determined using a [^{3}H]cAMP-assay kit. In the next experiments, IBMX was used in a lower concentration (0.1 mM) to allow the measurement of cyclic nucleotide metabolites.

To evaluate the existence of ecto-PDE and ecto-5′-nucleotidase (ecto-NT) activities, cultures were pre-incubated with 0.1 mM IBMX for 15 min and treated with exogenous cAMP (1–100 nmol per dish) at 37 °C. Experiments were performed in the presence or absence of the following inhibitors of adenosine metabolism: 50 μM uridine (adenosine uptake inhibitor), 0.1 μM iodotubericidine (adenosine kinase inhibitor)

and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA; adenosine deaminase inhibitor). The same protocol was used to analyse extracellular degradation of the fluorescent analogue of cAMP, by incubating the cells with 100 nmol per dish etheno-cAMP (ε-cAMP).

In another set of experiments, cultures were pretreated with 0.1 mM IBMX, 50 μM uridine, 0.1 μM iodotubericidine and 10 μM EHNA and incubated with 10 μM isoprenaline or 10 μM forskolin at 37 °C. After 30 min, the extracellular medium and the cells were collected, boiled and used for determination of the adenine-containing compounds by HPLC.

Analysis of extracellular accumulation of cAMP at rat skeletal muscle

The existence of cAMP efflux *in situ* was investigated using isolated extensor digitorum longus (EDL) muscle from adult male Wistar rats. Muscles were carefully removed and transferred to tubes containing 1.5 ml Tyrode solution (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 15 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$ and 11 mM glucose, pH 7.4). After 30 min, the muscles were treated for 30 min with 0.1 mM IBMX, 10 μM EHNA, 0.1 μM iodotubericidine and 50 μM uridine, in the absence or presence of 100 μM probenecid, and subsequently incubated with 10 μM isoprenaline or 10 μM forskolin or vehicle for 30 min at 37 °C under 10% CO_2 atmosphere. EDL and incubation media obtained from each incubation step were boiled for 15 min and kept frozen at −70 °C until determination of purine and cAMP contents.

Statistics

Data were presented as mean ± s.e.mean. Statistical significance was tested by Student's '*t*'-test or one-way ANOVA with Newman–Keuls or Tukey *post hoc* test using GraphPad Prism software. Differences were considered significant at $P<0.05$.

Results

Analysis of cAMP efflux in cultured skeletal muscle fibres

We have recently shown that stimulation of cultured skeletal muscle AC with 10 μM forskolin induces a transient increase in intracellular cAMP that is followed by an increase in cyclic nucleotide at the incubation medium (Godinho and Costa, 2003). To determine the possible correlation between the intracellular generation and the efflux of cAMP, cultured skeletal muscle fibres were incubated with 1–300 μM forskolin in the presence of 1 mM IBMX. After 30 min incubation, forskolin increased intracellular cAMP in a dose-dependent manner. Maximum effect was obtained with 100 μM forskolin, which increased by 28-fold the basal levels of intracellular cAMP (Table 1). Intracellular accumulation of cAMP was followed by a proportional increment of extracellular cAMP, which corresponded to 16–24% of total cAMP. The generation/efflux ratio was maintained even at a very high concentration of forskolin (300 μM) that was not able to sustain formation of cAMP, indicating that cAMP efflux directly depends on intracellular generation of cyclic nucleotide.

Extracellular degradation of cAMP results in increased generation of adenosine outside the cultured muscle fibre

To investigate the possible extracellular conversion of cAMP to 5′-AMP, skeletal muscle cultures were incubated with exogenous cAMP (60 pmol per dish), in the presence or absence of 1 mM IBMX, a non-selective PDE inhibitor that crosses cell membranes. Incubation of cells with cAMP did not change the basal intracellular cyclic nucleotide content (17.7 ± 5.1 pmol per dish). On the other hand, pre-incubation of 1 mM IBMX increased the intracellular cAMP content, corroborating previous studies from our laboratory showing a basal generation of cAMP that is unmasked by the PDE inhibitor (Godinho and Costa, 2003).

When cells were treated with exogenous cAMP alone for 30 min, the amount of cyclic nucleotide recovered in the incubating medium represented 73% of the amount added (Figure 1). Whereas IBMX alone did not alter the basal level of cAMP in the extracellular medium of control cultures (14.9 ± 1.5 pmol per dish), when incubation of cyclic nucleotide was performed in the presence of IBMX, the recovery of cAMP in the medium corresponded to the total amount added, indicating expression of IBMX-sensitive ecto-PDE in skeletal muscle cultures. The basal intracellular cAMP was not changed by the addition of exogenous cyclic nucleotide demonstrating that cAMP does not enter the cell (data not shown).

Table 1 Effect of forskolin in the intra- and extracellular accumulation of cyclic AMP

Forskolin (μM)	*Total (pmol per dish)*	*Intracellular, pmol per dish (% of total)*	*Extracellular, pmol per dish (% of total)*
0	10.80 ± 1.71	10.80 ± 1.71 (100)	ND
1	36.74 ± 2.65	30.68 ± 1.75 (83.2)	6.16 ± 0.91 (16.8)
3	82.07 ± 8.16	68.61 ± 7.10 (83.6)	13.45 ± 1.11 (16.4)
10	212.69 ± 12.95	174.05 ± 8.73 (81.9)	38.64 ± 4.52 (18.1)
30	281.32 ± 22.58	235.11 ± 18.67 (83.6)	46.21 ± 3.91 (16.4)
100	395.02 ± 61.58	305.78 ± 54.75 (77.5)	89.24 ± 6.83 (22.6)
300	241.08 ± 13.70	184.56 ± 11.97 (76.4)	57.52 ± 2.73 (23.9)

Abbreviation: ND = not detected.

Intra- and extracellular cAMP contents were determined in control or forskolin-treated cultures pre-incubated with 1 mM IBMX, as described under 'Materials and methods'. Data are expressed as mean ± s.e.mean. ($n=4$) content (pmol per dish) or as percentage of total cAMP content (intra- + extracellular cAMP).

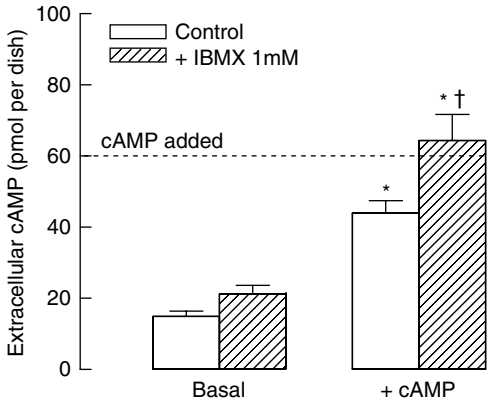

Figure 1 3-Isobutylmethylxanthine (IBMX) reduces extracellular degradation of exogenous cAMP. Tissue-cultured skeletal muscle cells, pretreated with the nonspecific phosphodiesterase (PDE) inhibitor (IBMX), were incubated with exogenous cAMP (60 pmol per dish) for 30 min at 37 °C. The cAMP was measured using a [^{3}H]cAMP-assay kit. Each bar represents mean ± s.e.mean ($n=4$). *Significantly different from basal values ($P<0.01$); †significantly different from control values ($P<0.05$).

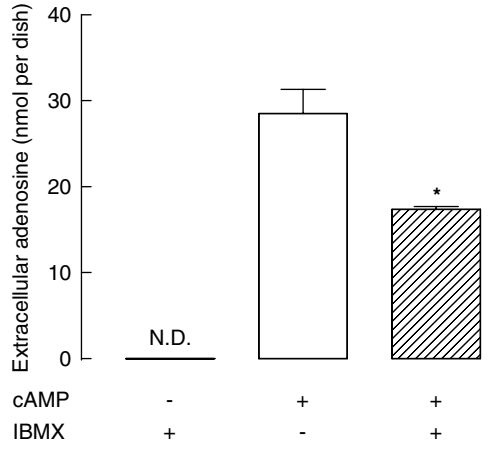

Figure 2 Treatment of cultured skeletal muscle cells with exogenous cAMP results in extracellular generation of adenosine. Tissue-cultured skeletal muscle cells were incubated with exogenous cAMP (100 nmol per dish) for 30 min at 37 °C in the presence or absence of 0.1 mM 3-isobutylmethylxanthine (IBMX). The cAMP was measured using a [^{3}H]cAMP-assay kit. Each bar represents mean ± s.e.mean ($n=3$). *Significantly different from basal values ($P<0.05$). N.D. = not detected.

The existence of an extracellular metabolic pathway for cAMP was substantiated by detection of adenosine in the extracellular compartment when cultured skeletal muscle cells were incubated with exogenous cAMP (100 nmol per dish; Figure 2). After 30 min, the amount of adenosine found in the incubation medium increased from undetectable levels ($\leqslant$0.75 pmol per dish) to almost 30 pmol per dish. In addition, pretreatment of cells with 0.1 mM IBMX reduced the extracellular generation of adenosine by almost 40%, indicating that adenosine resulted from cAMP was hydrolysed by ecto-PDEs.

Pretreatment of cultures with the adenosine metabolism inhibitors 10 μM EHNA, 50 μM uridine and 0.1 μM iodotubericidine increased by 41% the amount of adenosine recovered

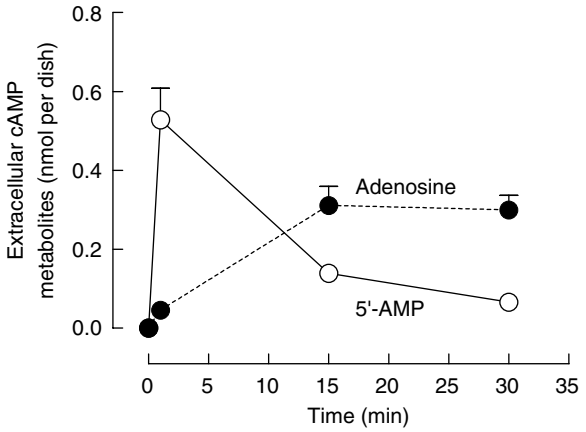

Figure 3 Extracellular degradation of cAMP is followed by sequential generation of 5′-AMP and adenosine. Tissue-cultured skeletal muscle cells were incubated with exogenous cAMP (1 nmol per dish) at 37 °C in the presence of 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). Adenine compounds were detected by HPLC. Values represent the mean ± s.e.mean ($n=3$).

at the incubation medium. These results indicate that once produced, adenosine acts as a substrate to extracellular transporters or enzymatic systems, leading to underestimation of adenosine produced at the extracellular space. To compensate for this problem, the following experiments were performed using adenosine metabolism inhibitors.

Extracellular degradation of cAMP is followed by subsequent generation of 5′-AMP and adenosine

Considering that 5′-AMP is the intermediary metabolite of adenosine synthesis, we analysed the levels of both 5′-AMP and adenosine in the incubation medium after treatment of cells with exogenous cAMP (1 nmol per dish) for up to 30 min. As shown in Figure 3, the metabolite 5′-AMP was immediately formed in the extracellular space, with maximum levels achieved after 1 min. After 15 and 30 min of incubation, 5′-AMP levels were reduced, whereas adenosine detected after 1-min stimulation increased sevenfold, after 15 and 30 min, indicating the successive conversion of exogenous cAMP to 5′-AMP and adenosine.

The existence of an extracellular cAMP–adenosine pathway in skeletal muscle cultures was corroborated by the effect of the ecto-PDE inhibitor 1,3-dipropyl-8-*p*-sulphophenylxanthine (DPSPX) or the ecto-NT inhibitor α,β-methyleneadenosine-5′-diphosphate (AMPCP) on the extracellular generation of adenosine. As shown in Figures 4 and 5, 100 μM DPSPX increased by 22-fold the extracellular accumulation of cAMP and attenuated by 42% the generation of adenosine. On the other hand, 30 and 80 μM AMPCP reduced by 36 and 63%, respectively, the formation of adenosine generated after cAMP incubation in control cultures, demonstrating definitively an extracellular biochemical pathway that metabolizes cAMP.

Finally, extracellular formation of adenosine from cAMP was validated by incubation of cultured skeletal muscles with the fluorescent derivative, ε-cAMP (100 nmol per dish). As

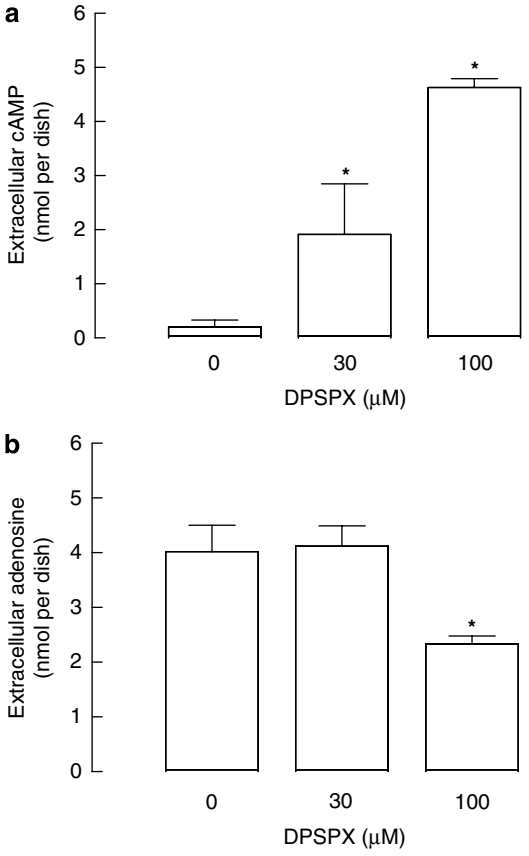

Figure 4 Specific inhibition of ecto-phosphodiesterase (PDE) by 1,3-dipropyl-8-*p*-sulphophenylxanthine (DPSPX) attenuates extracellular degradation of cAMP. Tissue-cultured skeletal muscle cells were pretreated with the selective ecto-PDE inhibitor DPSPX and incubated with exogenous cAMP (30 nmol per dish) for 30 min at 37 °C in the presence of 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). Extracellular cAMP (**a**) and adenosine (**b**) were detected by HPLC. Each bar represents the mean ± s.e.mean ($n = 5$). *Significantly different from control values ($P < 0.05$).

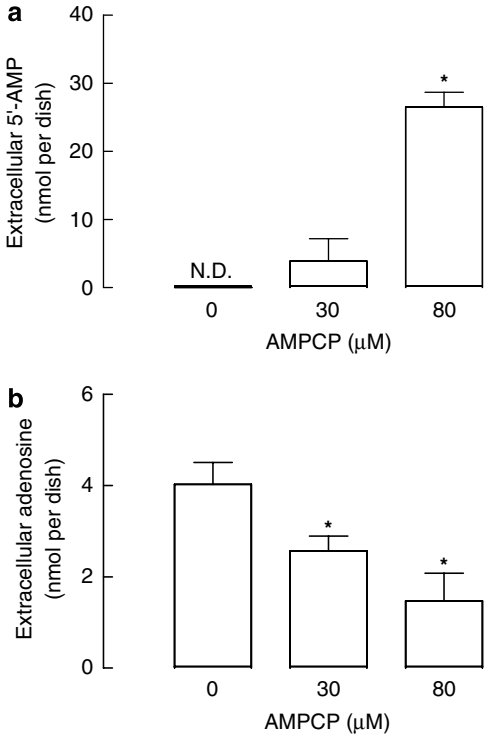

Figure 5 Specific inhibition of ecto-nucleotidase (ecto-NT) by α,β-methylene-adenosine-5′-diphosphate (AMPCP) attenuates extracellular adenosine generation. Tissue-cultured skeletal muscle cells were pretreated with the selective ecto-NT inhibitor AMPCP and incubated with exogenous cAMP (30 nmol per dish) for 30 min at 37 °C in the presence of 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). Extracellular 5′-AMP (**a**) and adenosine (**b**) were detected by HPLC. Each bar represents the mean ± s.e.mean ($n = 5$). *Significantly different from control values ($P < 0.05$). N.D. = not detected.

illustrated in Figure 6, after 5 min incubation, ε-cAMP declined to 40% of the total amount added, which was followed by a more gradual fall reaching 20% of the total amount added in 20 min. Reduction of ε-AMP coincided with the appearance of ε-adenosine in the medium. After 5 and 10 min incubation, 22 and 44% of ε-cAMP were recovered as ε-adenosine, respectively, demonstrating that extracellular cAMP is an important source of interstitial adenosine.

Direct- or receptor-dependent activation of AC induces efflux of cAMP and subsequent extracellular generation of 5′-AMP and adenosine

To investigate whether activation of AC is able to induce efflux of cAMP and extracellular generation of 5′-AMP and adenosine, muscle cultures were incubated for 30 min at 37 °C with the β-adrenoceptor agonist isoprenaline (10 μM) or forskolin (10 μM) in the presence of 0.1 mM IBMX and adenosine metabolism inhibitors. Both intra- and extracellular levels of 5′-AMP increased after direct- or receptor-dependent activation of AC. As shown in Figure 7, intracellular 5′-AMP was increased by 64 and 74% after incubation with forskolin and isoprenaline, respectively, in comparison to the control values. Isoprenaline increased by 82% the extracellular 5′-AMP, whereas forskolin doubled 5′-AMP levels in the extracellular medium compared with control cultures.

In addition, incubation of cultures with forskolin increased by 67% the total content of 5′-AMP (intra- + extracellular levels) compared with control. Similar results were obtained with isoprenaline-treated cultures, where 5′-AMP total content was increased by 74%. Approximately 90% of the total 5′-AMP generated after AC stimulation was found in the intracellular compartment, and the remainder 10% was detected outside the cell.

Extracellular adenosine was not detectable in control cultures. However, stimulation of cells with forskolin or isoprenaline for 30 min, markedly increased the adenosine in the medium (Figure 8).

To evaluate the existence of cAMP efflux *in situ*, EDL muscles were treated with isoprenaline or forskolin. Preincubation of muscles with IBMX and adenosine metabolism inhibitors did not alter the intra- or extracellular content of cAMP (data not shown). Besides, adenosine and 5′-AMP were

not detected in the extracellular medium. In contrast, both isoprenaline and forskolin stimulated the intracellular accumulation of cAMP by 2.6- and 11.6-fold, respectively, in comparison to the basal levels. In consequence, as shown in Figure 9, the extracellular content of the cyclic nucleotide increased by 9- and 35-fold in the medium of the muscles treated with isoprenaline and forskolin, respectively.

Finally, we evaluated the effect of an inhibitor of organic anion transport on the extracellular accumulation of the cyclic nucleotide following *in situ* stimulation of AC. Pre-incubation with probenecid reduced by 75 and 57% the extracellular cAMP increase induced by isoprenaline and forskolin, respectively, with no effect on cAMP synthesis, demonstrating the presence of a probenecid-sensitive transporter that pumps cAMP out of skeletal muscle fibres.

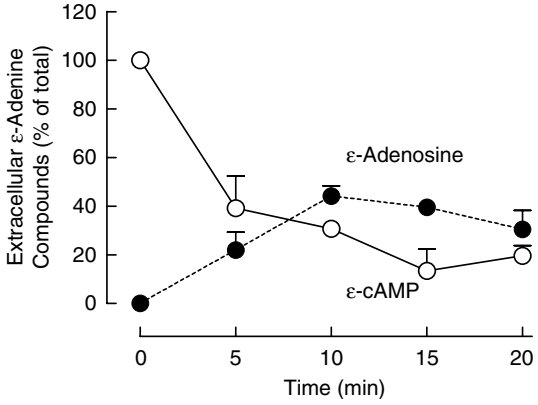

Figure 6 Extracellular degradation of fluorescent cAMP (ε-cAMP) is followed by the accumulation of etheno-adenosine (ε-adenosine). Tissue-cultured skeletal muscle cells were incubated with the etheno fluorescent analogue of cAMP (100 nmol per dish) at 37 °C in the presence of 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). The adenine compounds were detected by HPLC. Each value represents the mean ± s.e.mean ($n=4$).

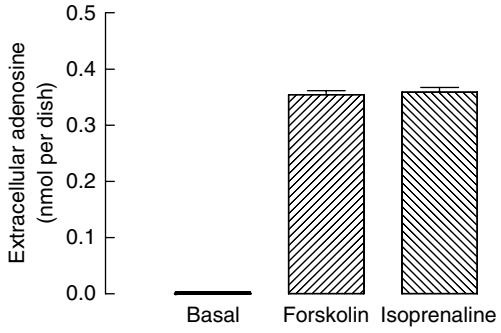

Figure 8 Receptor-mediated or direct stimulation of adenylyl cyclase (AC) induces extracellular generation of adenosine. Tissue-cultured skeletal muscle cells were treated with the AC activator forskolin (10 μM) or with the β-adrenoceptor agonist isoprenaline (10 μM) for 30 min at 37 °C in the presence of 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). Extracellular adenosine was detected by HPLC. Each bar represents the mean ± s.e.mean ($n=5$).

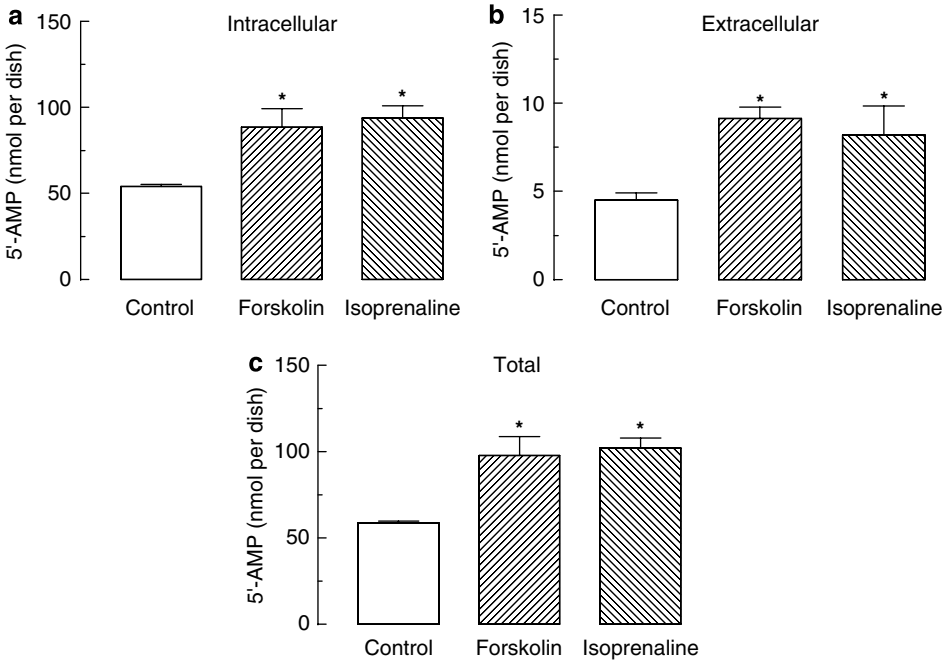

Figure 7 Receptor-mediated or direct stimulation of adenylyl cyclase (AC) increases extracellular 5′-AMP. Tissue-cultured skeletal muscle cells were treated with the AC activator forskolin (10 μM) or with the β-adrenergic agonist isoprenaline (10 μM) for 30 min at 37 °C in the presence of 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). Intra- (**a**) and extracellular (**b**) 5′-AMP were detected by HPLC. The total amount of 5′-AMP produced was considered to be the sum of intra- and extracellular values and is also shown (**c**). Each bar represents the mean ± s.e.mean ($n=5$). *Significantly different from control values ($P<0.05$).

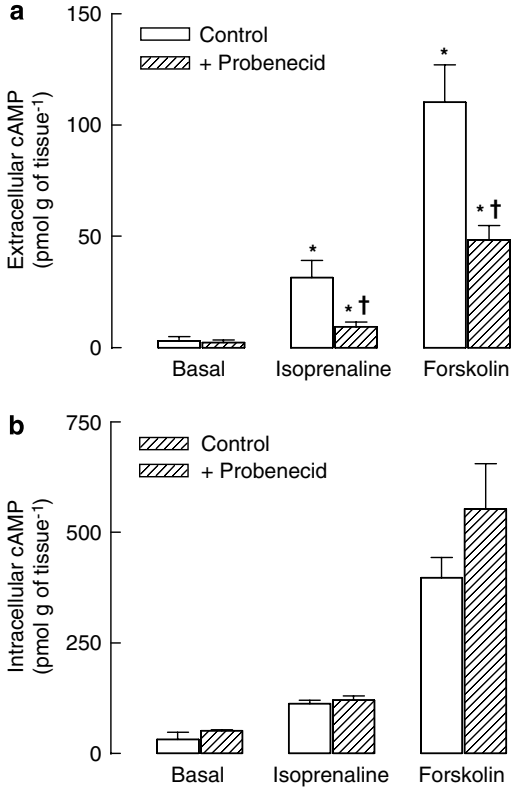

Figure 9 Receptor-mediated or direct stimulation of adenylyl cyclase (AC) *in situ* increases extracellular cAMP. Extensor digitorum longus (EDL) muscles were kept in Tyrode buffer for 30 min at 37 °C and treated with 50 μM uridine, 0.1 μM iodotubericidine, 0.1 mM 3-isobutylmethylxanthine (IBMX) and 10 μM erythro-9-amino-β-hexyl-α-methyl-9*H*-purine (EHNA). After 30 min, muscles were subsequently with the AC activator forskolin (10 μM) or with the β-adrenoceptor agonist isoprenaline (10 μM) for 30 min at 37 °C in the presence or absence of 100 μM probenecid 100 μM. Extra- (**a**) and intracellular (**b**) cAMP measured using a [^{3}H]cAMP-assay kit. Each bar represents the mean ± s.e.mean ($n=8$). *Significantly different from basal values ($P<0.01$); †significantly different from control values ($P<0.05$).

Discussion

Many physiological processes of vertebrate skeletal muscle are regulated by cAMP-dependent intracellular signalling pathways, including muscle differentiation (Dubinsky and Fischbach, 1990; Naro *et al*., 1999), metabolism (Fagher *et al*., 1986; Roberts and Summers, 1998) and contraction (Reading *et al*., 2003). When examining the influence of receptor-dependent activation of AC and cAMP signalling cascade on skeletal muscle acetylcholinesterase, we found a secretory transport system able to actively pump the cAMP out of the cells (da Costa *et al*., 2001; Godinho and Costa, 2003). Although first demonstrated 40 years ago by Davoren and Sutherland (1963), cAMP efflux had received very little attention until 1998, when its extracellular signalling was verified by Mi and Jackson (1998).

cAMP egress from muscle cells could be considered an alternative mechanism to limit cyclic AMP signalling. However, this idea is questionable, taking into account the efficient degradation of cAMP by intracellular PDEs, the high energetic cost of pumping cAMP out of cell, the consumption of cellular adenine pool and a possible function of extracellular cAMP, which was evaluated and confirmed in the present study. Here, we show evidence for the existence of an extracellular arm of cAMP signalling in skeletal muscle known as extracellular cAMP–adenosine pathway, elicited by activation of AC followed by the subsequent efflux of the cyclic nucleotide and extracellular generation of adenosine.

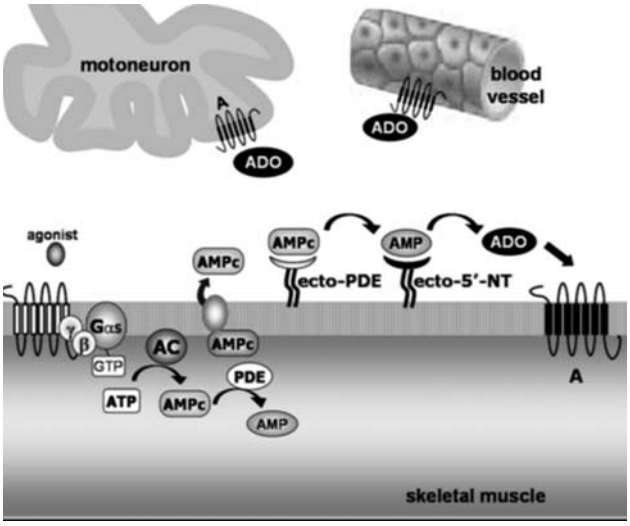

Figure 10 Schematic model of the extracellular arm of cAMP signalling pathway in skeletal muscle. cAMP generated after adenylyl cyclase (AC) stimulation may be degraded by intracellular phosphodiesterases (PDEs) or transported from skeletal muscle fibres. Outside the cell, cAMP is degraded to 5′-AMP and adenosine by ecto-PDE and ecto-NT, respectively. The effector molecule adenosine can then activate specific receptors at the muscle fibre itself or at other cells to modulate skeletal muscle and/or neuromuscular physiology.

Using cultured skeletal muscle as a model, the present results showed that direct activation of AC with forskolin increased extracellular accumulation of cAMP in a concentration-dependent manner and proportionally to the increment of intracellular cyclic nucleotide. The highest rate of cAMP efflux (6 pmol ml^{-1} min^{-1}) was obtained when AC was maximally activated by 100 μM forskolin, indicating that cAMP efflux rate depends on the intracellular cyclic nucleotide availability and is not saturated even at the maximal stimulation of AC. cAMP export in skeletal muscle cells has a profile similar to that observed in avian erythrocytes (Brunton and Mayer, 1979) and rodent preglomerular microvessels (Jackson and Mi, 2000). Indeed, cAMP egress seems to be an ubiquitous process as it has been demonstrated in numerous other tissues and cells (for a review, see Bankir *et al*., 2002; Hofer and Lefkimmiatis, 2007).

Our results also showed that extracellular cAMP is a target to ecto-PDE, as its content is reduced in parallel to the increment of extracellular 5′-AMP and adenosine. Both extracellular hydrolysis of cAMP and accumulation of adenosine were prevented by IBMX, a non-selective inhibitor of both intra- and extracellular PDEs. The existence of extracellular PDEs (ecto-PDEs) in muscle cells was substantiated by inhibition of cAMP degradation with DPSPX, a PDE inhibitor that is negatively charged at physiological pH and hence does not cross cell membranes (Tofovic *et al*., 1991).

Although ecto-PDEs had been described in many tissues and cells, such as liver (Smoake *et al*., 1981), neuronal cells (Rosenberg and Dichter, 1989; Rosenberg *et al*., 1994), lymphocytes (Goding *et al*., 1998), oviduct cells (Cometti *et al*., 2003), adipocytes (Zacher and Carey, 1999), kidney (Jackson and Raghvendra, 2004; Jackson *et al*., 2007), to our knowledge, it is the first time that the extracellular PDE activity is demonstrated in skeletal muscle cells.

In fact, another extracellular enzyme, ecto nucleotide pyrophosphatase/phosphodiesterase is able to hydrolyse phosphodiester bonds (Stefan *et al*., 2005); however cAMP is not a substrate for ecto nucleotide pyrophosphatase/phosphodiesterase. Besides, IBMX and DPSPX are not able to inhibit its phosphodiesterase activity (Picher and Boucher, 2000).

The correlation between extracellular degradation of cAMP and formation of adenosine observed in rat primary muscle cultures is in agreement with the expression of ecto-NTs described in chicken (Delgado *et al*., 1997) and mouse skeletal muscle (Martínez-Martínez *et al*., 1998) and C2C12 cultured cells (Ling *et al*., 2005). However, in the previous studies, this cell-surface enzyme was essentially associated with the extracellular metabolism of ATP, considered to be the only relevant source of adenosine outside the cell (Cunha *et al*., 1996; Delgado *et al*., 1997; Martínez-Martínez *et al*., 1998). In the present study, we show several findings that strengthen the theory of cAMP as an alternative extracellular source of adenosine: (a) in basal conditions, 5′-AMP and adenosine are not detected in the extracellular medium, appearing only after incubation of cultured cells with exogenous cAMP, (b) extracellular 5′-AMP accumulation occurs almost instantaneously after exogenous cAMP is added to the medium, which may be explained by extracellular enzymatic degradation of cAMP, (c) the interstitial accumulation of adenosine correlated with the disappearance of 5′-AMP from the medium, (d) specific inhibition of ecto-PDE and ecto-NT with DPSPX and AMPCP, respectively, reduces cAMP degradation and adenosine accumulation in the extracellular medium. Taking into account the extracellular accumulation of fluorescent adenosine after incubation of muscle cells with the fluorescent analogue of cAMP, ε-cAMP our results change the concept of ATP as the unique extracellular source of adenosine in skeletal muscle. The existence of cAMP–adenosine pathway at skeletal muscle *in vivo* is consistent with the efflux of cAMP detected in EDL muscle presented here and with our unpublished observation showing increased blood cAMP and AMP in mice treated orally with β-adrenoceptor agonists.

The amount of extracellular adenosine generated (0.4 μM) in response to direct or receptor-dependent activation of AC is sufficient to activate adenosine receptors expressed in the sarcolemma (Lynge and Hellsten, 2000). By activating $G\alpha_s$-coupled adenosine A_{2A} and A_{2B} receptors, adenosine may induce positive inotropic effects (Reading *et al*., 2003), potentiate insulin and catecholamine effects on glucose uptake (Hespel and Richter, 1998) and increase the desensitization of nicotinic receptors induced by carbachol (Pitchford *et al*., 1992). Furthermore, adenosine seems to protect slow-twitch muscle from fatigue through A_1 receptors (Reading and Barclay, 2001). Thus, in skeletal muscle, the extracellular cAMP–adenosine pathway may function as a feedback mechanism able to modulate the cAMP signalling events initiated by other endogenous substances through activation of G-protein-coupled receptors, such as adrenoceptors, calcitonin gene-related peptide and even adenosine receptors.

In view of the reduced muscle interstitial volume *in vivo*, the extracellular concentration of adenosine measured in our culture model is probably underestimated, because it was obtained in a rather large volume (1 ml). Thus, it is possible that in addition to autocrine effects on muscle, paracrine actions of adenosine contribute to adjust muscle contraction activity and metabolism. For example, acting on A_1 and A_{2A} receptors at the vascular smooth muscle, adenosine may increase muscle blood flow and thus nutrient availability (Poucher, 1996; Ray *et al*., 2002). Besides, adenosine has presynaptic effects, either facilitating or inhibiting the release of acetylcholine from the motor nerve through A_1 (Correia-de-Sá *et al*., 1991; De Lorenzo *et al*., 2004) and A_{2A} (Correia-de-Sá *et al*., 2000; Baxter *et al*., 2005) receptors, respectively (Correia-de-Sá *et al*., 1996).

Figure 10 summarizes the sequential steps of extracellular cAMP–adenosine pathway in the skeletal muscle, triggered by activation of cell-surface receptors coupled to G_s protein, indicating the possible paracrine sites of adenosine action. Briefly, activation of G_s-coupled receptors present in skeletal muscle fibres causes an intracellular increase of cAMP. Although most of the cyclic nucleotides are inactivated by intracellular PDEs, part of the cAMP produced overflows to the extracellular environment by probenecid-sensitive transporters. Outside the cell, cAMP is degraded by an enzymatic cascade of ecto-PDE and ecto-NTs to form adenosine, an endogenous ligand for A_1 and A_2 adenosine receptors, expressed in muscle fibres, nerve terminal and blood vessels.

In summary, our results demonstrate that, in skeletal muscle, cAMP, assumed to be exclusively an intracellular second messenger, may extend its influence as a paracrine signalling molecule, acting through its metabolite adenosine. Further investigation will be required to determine the precise role of this pathway in the physiology and pharmacology of skeletal muscle.

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