Contribution of the extracellular cyclic AMP–adenosine pathway to dual coupling of β2-adrenoceptors to Gs and Gi proteins in mouse skeletal muscle.

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Abbreviations
ADO, adenosine; AC, adenylyl cyclase; β-AR, β-adrenoceptor; CGS, CGS-15943; Clen, clenbuterol; COPD, chronic obstructive pulmonary disease; DMSO, dimethyl sulfoxide; d-tc, d-tubocurarine; EDL, extensor digitorum longus; Fen, fenoterol; FSK, forskolin, GRK, G protein-coupled receptor kinases; GPCR, G protein-coupled receptor; GsPCR, stimulatory G
protein-coupled receptor; GiPCR, inhibitory G protein-coupled receptor; PKA, protein kinase A; PTX, pertussis toxin.
Abstract

$\beta_2$-adrenoceptor ($\beta_2$-AR) agonists increase skeletal muscle contractile force via activation of Gs protein/ adenylyl cyclases (AC) and increased generation of cAMP. Herein, we evaluated the possible dual coupling of $\beta_2$-AR to Gs and Gi proteins and the influence of $\beta_2$-AR/Gs-Gi/cAMP signaling cascade on skeletal muscle contraction. Assuming that increment of intracellular cAMP is followed by cAMP efflux and extracellular generation of adenosine, the contribution of extracellular cAMP-adenosine pathway on the $\beta_2$-AR inotropic response was also addressed. The effects of clenbuterol/fenoterol ($\beta_2$-AR agonists), forskolin (AC activator), cAMP/8-Br-cAMP and adenosine were evaluated on isometric contractility of mouse diaphragm muscle induced by supramaximal direct electrical stimulation (0.1 Hz, 2 ms duration). Clenbuterol/fenoterol (10-1000 $\mu$M), 1 $\mu$M forskolin, 20 $\mu$M rolipram induced transient positive inotropic effects that peaked 30 min after stimulation onset, declining to 10-20% of peak levels in 30 min. The late descending phase of $\beta_2$-AR agonist inotropic effect was mimicked by either cAMP or adenosine and abolished by preincubation of diaphragm with pertussis toxin (PTX, Gi signaling inhibitor) or the organic anion transporter inhibitor probenecid, indicating a delayed coupling of $\beta_2$-AR to Gi protein which depends on cAMP efflux. Remarkably, the PTX-sensitive $\beta_2$-AR inotropic effect was inhibited by the A1 adenosine receptor antagonist DPCPX and ecto-5'-phosphodiesterase inhibitor AMPCP, indicating that $\beta_2$-AR coupling to Gi is indirect and dependent on A1 receptor activation. The involvement of extracellular cAMP-adenosine pathway in $\beta_2$-AR signaling would provide a negative-feedback loop that may limit GsPCR positive inotropism and potential deleterious effects of excessive contractile response.
Introduction

A key physiological mechanism to increase skeletal muscle contractile force relies on circulating hormones that elevate intracellular cAMP. Endogenous sympatomimetic amines or synthetic β-adrenoceptors (β-AR) agonists, such as non-selective isoproterenol (Bowman and Nott, 1969; Andrade-Lopes et al., 2011) or β2-AR selective salbutamol (Easton et al., 2008) increase muscle contraction force via activation of stimulatory G protein (Gs) coupled receptors (GPCR) which in turn activates adenylyl cyclases (AC). The sequential increase in intracellular cAMP (Bowman and Nott, 1969; Cairns and Dulhunty, 1993; Andrade-Lopes et al., 2011) and activation of PKA result in phosphorylation of ryanodine receptor and increased Ca^{2+} release from the sarcoplasmic reticulum (Reiken et al., 2003; Lynch and Ryall, 2008), that culminates in potentiation of muscle contraction. However, in many cells, β-AR/Gs/AC-mediated responses is affected by promiscuous coupling of β-AR to other Gα subunits, especially with the inhibitory Gαi subfamily as reported in Sf9 insect cells overexpressing β2-AR (Wenzel-Seifert and Seifert, 2000) and HEK293 cells (Daaka et al., 1997). Also, secondary coupling of β2-AR to Gi proteins in rodent (Xiao et al., 1999; Kuschel et al., 1999) and human (Kilts et al., 2000) heart muscle cells has been associated with a cardioprotective effect of β2-AR agonists, via attenuation of Gs-mediated inotropic response.

In fact, dual coupling of β2-AR with Gs and Gi proteins has already been reported in rat skeletal muscle (Gosmanov et al., 2002) and in L6 myogenic cell line (Nevzorova et al., 2006) but the precise pathways implicated in this signaling shift and its physiological significance on regulation of skeletal muscle contraction remain unknown.

In skeletal muscle, β-AR-dependent increase of intracellular cAMP is followed by the efflux of cyclic nucleotide via probenecid-sensitive transporters (Godinho and Costa, 2003). Within the large superfamily of ATP-binding cassette transporters, three members of the subfamily of multidrug resistance protein (MRP) are able to transport cAMP - MRP4, MRP5,
and MRP8 (for review, see Sager and Ravna, 2009) two of them are expressed in skeletal muscle -MRP4 and MRP5 (Knauer et al., 2010).

Considering that, once outside the muscle fiber, cAMP is sequentially metabolized to AMP and adenosine by ecto-phosphodiesterases (ecto-PDE) and ecto-nucleotidases (ecto-NT) (Chiavegatti et al., 2008), and the expression of Gi-coupled A₁ and A₃ adenosine receptors in skeletal muscle tissue (Lynge and Hellsten, 2000; Zheng et al., 2007), an indirect coupling of β₂-AR to Gi protein via activation of adenosine receptors would have physiological significance. Our working hypothesis is that the extracellular adenosine, generated as consequence of activation of β₂-AR/Gs/AC signaling, may influence the initial positive inotropic response, by autocrine stimulation of adenosine receptors coupled to Gi protein.

Thus, in the present study we have employed the *in vitro* mouse diaphragm preparation to evaluate the effect of continuous β₂-AR activation on skeletal muscle contraction, focusing on its coupling to distinct G proteins and the possible influence of *extracellular cAMP-adenosine pathway* on β₂-AR-mediated positive inotropic response.

**Methods**

**Animals**

All animal procedures were approved by the Institutional Research Ethics Committee (protocols 0011/08 and 0072/11) at Universidade Federal de São Paulo and have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All experiments were performed using diaphragm strips isolated from 90 to 120-day-old male Swiss mice from the institutional animal care facility.

**Isometric contraction of mouse diaphragm muscle**
Mice were killed by cervical dislocation, and diaphragm strips were prepared according to Souccar et al., 2005) with modifications (Andrade-Lopes et al., 2011). Briefly, diaphragm strips with rib were quickly dissected and placed in Tyrode solution (135 mM NaCl; 5 mM KCl; 1 mM MgCl₂·6 H₂O; 15 mM NaHCO₃; 2 mM NaH₂PO₄·H₂O; 2 mM CaCl₂·2 H₂O; 11 mM glucose, pH 7.4). The rib was gently pinned to a holder fixed in the bottom of a organ bath filled with 2 mL of Tyrode solution and a segment of central diaphragm tendon attached to a PowerLab® force transducer (ADInstruments, Sydney, Australia), which was maintained at 30°C and continuously gassed with 95% O₂/5% CO₂. Isometric twitch contraction was elicited by electrical stimulation of muscle strips through silver electrodes, with 0.1 Hz frequency, 2 ms duration, and supra-maximal voltage (Grass Stimulator S88, Rhode Island, USA), under optimal tension. D-tubocurarine (10 µM) was added to the solution in order to block neuromuscular transmission and prevent double stimulation of the muscle from remaining axon stub. After 20-30 min stabilization, muscle length was readjusted to give an optimal twitch tension and 30 min later the effect of drugs was investigated, as described below. Data were collected and analyzed using the Powerlab® Chart® 5 software (ADInstruments, Sydney, Australia). Muscle wet weight was determined at the end of each experiment and contraction amplitudes were normalized as g of tension per g of wet tissue and expressed as percentage of baseline values, which were taken as 100%.

**Evaluation of inotropic effect of drugs on mouse diaphragm preparation**

The time course of 1-1000 nM clenbuterol or 1-1000 nM fenoterol effects was evaluated on the amplitude of diaphragm isometric contraction. To determine whether β₂-AR agonists were able to activate Gi protein, the effect of 1 µM clenbuterol or fenoterol on diaphragm contraction were evaluated in muscle strips pre-incubated for 1 h with pertussis toxin (PTX, 1 µg/mL) or vehicle (Tyrode contained 1 mg/mL bovine serum albumin), and compared to those of 1 µM forskolin, an direct activator of AC. Finally, the involvement of
“extracellular cAMP-adenosine pathway” on clenbuterol-induced inotropic effect was analyzed in diaphragm strips pre-treated for 30 min with the organic anion transporter inhibitor probenecid (100 μM), CGS-15943 (100 nM; a nonselective adenosine receptor antagonist), DPCPX (50 nM; a selective A1 adenosine receptor antagonist) and compared to those obtained with 10 μM cAMP, 100 μM 8-Br-cAMP, 1 μM adenosine or 20 μM rolipram.

**Drugs and chemical reagents**

4-[3-(cyclopentyloxy)-4-methoxyphenyl] pyrrolidin-2-one (rolipram) was purchased from Tocris Bioscience, Minneapolis, USA. Adenosine (ADO), adenosine 3′,5′-cyclic monophosphate (cAMP), 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS-15943), 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP), clenbuterol hydrochloride, dimethyl sulfoxide, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), fenoterol hydrobromide, forskolin, α,β-Methyleneadenosine 5′-diphosphate sodium salt (AMPCP), pertussis toxin (PTX), probenecid, d-tubocurarine hydrochloride and all other chemicals were purchased from Sigma, St. Louis, MO, USA.

**Statistical analysis**

Data were presented as mean ± S.E.M. Statistical significance was tested by Student’s ‘t’ test or one-way ANOVA with Newman-Keuls multiple comparison post hoc test using Graph Pad Prism software for Windows (version 5.01, San Diego, CA, USA). Differences were considered significant at \( P < 0.05 \).

**Results**

**β2-adrenoceptor agonists induce transient positive inotropic effect on skeletal muscle contraction force**

Figures 1A and B show the time course of 1-1000 nM clenbuterol effects on contraction amplitude of mouse diaphragm. The \( \beta_2 \)-AR agonist induced a concentration-
dependent increase in contractile force that peaked 30 min after stimulation onset. Interestingly, while 1 to 100 nM clenbuterol were able to progressively increase diaphragm contraction force by up to 43% of basal value (figure 1A and C), the maximal positive inotropic effect was attenuated at concentrations higher than 100 nM, reaching 123% of basal twitch contraction (100%), at 1 μM (figure 1B and C).

The transient inotropic effect of clenbuterol was mimicked by the short-acting β2-AR agonists fenoterol (figure 2), which increased by up to 32% the diaphragm contractile force at 30 nM. The maximum inotropic effect of fenoterol was obtained ~35-40 min after stimulation onset and, as observed with clenbuterol (figure 1B and C), concentrations higher than 30 nM were less efficacious in bioactivity (figure 2B and C).

Attenuation of positive inotropic effect induced by β2-adrenoceptor agonists involves activation of pertussis toxin-sensitive Gi protein.

To further investigate whether attenuation of the maximum inotropic response observed at high concentration of agonists was due to coupling of β2-AR to Gi protein, the effects of clenbuterol and fenoterol on skeletal muscle contraction were evaluated in diaphragm strips pre-incubated for 1 h with PTX (1.0 μg/ mL). As shown in figure 3A, PTX potentiated the inotropic effect of 1 μM clenbuterol restoring it to the maximum potentiation seen at 100 nM (43%, figure 1B).

PTX also potentiated the inotropic effect of 1 μM fenoterol (figure 3B), which reached the maximum response seen at 30 nM (figure 2B). Moreover, PTX significantly attenuated the late descending phase of inotropic effect induced by high concentration of clenbuterol or fenoterol (figures 3A-B), indicating that a PTX-sensitive Gi protein mediates the delayed attenuation of β2-AR inotropic effect.

Interestingly, the transient inotropic effect triggered by β2-AR agonists was mimicked by direct activation of AC with forskolin (figure 3C). Furthermore, as observed under β2-AR
stimulation, pre-treatment of diaphragm with PTX also abolished the descending phase of forskolin inotropic effect, indicating that activation of PTX-sensitive Gi protein by β2-AR in diaphragm is triggered by signaling events downstream of AC activation. The selective inhibitor of cAMP phosphodiesterase 4 rolipram also induced a transient positive inotropic effect in diaphragm preparation (figure 4A), supporting the involvement of cAMP in both ascending and descending phases of β2-AR-mediated positive inotropic response. In addition, considering that rolipram selectively inhibits degradation of cAMP, with no effect on other cyclic nucleotides such as cGMP, cCMP or cUMP (Reinecke et al., 2011), this result associated to those of forskolin demonstrate that on a Gi-related effect of β2-AR agonists depends exclusively on cAMP signaling pathway.

β2-adrenoceptor coupling to Gi protein involves extracellular cAMP-adenosine pathway and activation of adenosine receptors

Taking into account that β2-AR-dependent increase of intracellular cAMP is followed by efflux of cyclic nucleotide to interstitial space (da Costa, Jr. et al., 2001; Chiavegatti et al., 2008), the possible implication of extracellular cAMP-adenosine pathway on the biphasic pattern of inotropism induced by β2-AR agonists was assessed by analyzing the effect of cAMP, adenosine and 8-Br-cAMP on muscle contraction.

Surprisingly, while cAMP induced a negative inotropic effect, its cell-permeable and PDE-resistant analog 8-Br-cAMP induced a sustained positive inotropic effect that persisted for at least 2 h (figure 4B), indicating that final inotropic effect depends on the site of cyclic nucleotide action: the extracellular or the intracellular compartment.

As observed with cAMP, incubation of diaphragm preparation with adenosine also resulted in a negative inotropic effect (figure 5A). The delayed onset of cAMP and adenosine action overlapped with the descending phase of forskolin- (figure 5A) and β2-AR-induced
inotropic effects (figure 3A and B), indicating that extracellular cAMP and its metabolite adenosine reproduce the activation of Gi protein induced by β2-AR agonists. In fact, pre-incubation of diaphragm strips with PTX abolished the negative inotropic effect of cAMP (figure 5B), showing that extracellular cAMP is able to activate a Gi protein signaling pathway. More importantly, the negative inotropic effect of cAMP (figure 5C) and the descending phase of clenbuterol-induced inotropic effect (figure 6A) were inhibited by pre-incubation of diaphragm with the non-selective adenosine receptor antagonist CGS-15943. Furthermore, the A1 receptor antagonist DPCPX completely inhibited the negative inotropic effect of either clenbuterol (figure 6B) or adenosine (figure 6C), demonstrating that β2-AR coupling to Gi is indirect, via activation of A1 adenosine receptors.

Finally, the connection between β2-AR stimulation, the efflux of cAMP and its extracellular degradation into adenosine is shown in figure 7. Pre-incubation of diaphragm with the organic anion transporter inhibitor probenecid (figure 7A), in a concentration that inhibits by 75% the efflux of cAMP (Chiavegatti et al., 2008), or with the ecto-5’nucleotidase inhibitor AMPCP (figure 7B) prevented the descending phase of positive inotropic effect elicited by clenbuterol. AMPCP also abolished the cAMP negative inotropic effect. All together, these results show that β2-AR coupling to Gi depends on the efflux of cAMP via probenecid-sensitive transporter and extracellular generation of adenosine, which schematically illustrated in figure 8.

**Discussion**

The present study provides strong evidences for a dual coupling of β2-AR to Gs and Gi protein at skeletal muscle, with a significant impact on muscle inotropic state. More importantly, the secondary coupling of β2-AR to Gi protein seems to be indirect and
dependent on extracellular degradation of cAMP into adenosine, which in turn activates muscle A1 adenosine receptors.

By studying the time course of clenbuterol and fenoterol effects on diaphragm twitch contraction, here we show that the well established β2-AR positive inotropism (Bowman and Nott, 1969) is attenuated after prolonged exposure (> 30 min) to agonists (Figures 1 and 2). At high concentrations of β2-AR agonists, the decline phase of inotropic effect was so intense that it abolished the positive inotropism.

The ability of PTX to attenuate the descending phase of β2-AR positive inotropism (figure 3) supports the idea of dual coupling of β-AR to Gs and Gi proteins, which appears to depend on the concentration and/or exposure time to agonists. At low concentrations, β2-AR agonists induce a more sustained increase in twitch contraction (figures 1A, 2A), supporting the preferential coupling of β2-AR to Gs protein in skeletal muscle (Andrade-Lopes et al., 2011). On the other hand, a secondary coupling of β2-AR to Gi protein would be accountable for either the descending phase of β2-AR positive inotropic response, observed after persistent (≥ 30 min) activation of β2-AR with low to intermediary concentrations of agonists or reduced inotropic response at high concentration of clenbuterol or fenoterol (≥ 100 nM). The coupling of β2-AR to Gi can be used to explain previous puzzling results obtained by McCormick et al (2010) showing that extremely high concentrations of clenbuterol (5-150 μM) reduced the contraction force of mouse EDL and soleus muscles.

Dual coupling of β2-AR to Gs and Gi proteins has been reported in several cells and tissues including cardiac myocytes from different species (Communal et al., 1999; Xiao et al., 1999; Hasseldine et al., 2003), mouse pulmonary artery (Wenzel et al., 2009), cultured endothelial cells and rat carotid artery (Ciccarelli et al., 2007). However, the precise mechanism underlying the switch of β2-AR from Gs to Gi signaling remained obscure because it was primarily based on disruption of Gi signaling by PTX or immunoprecipitation.
of GTP-Gi protein complexes induced by β2-AR agonists (Daaka et al., 1997; Ciccarelli et al., 2007; Wenzel et al., 2009; Liu et al., 2009; Wang et al., 2011).

A plausible explanation for reduced coupling of β2-AR to Gs would be receptor desensitization and reduced receptor efficacy in stimulating AC (Bunemann et al., 1999). This phenomenon depends on sequential phosphorylation of agonist-activated receptor by specific GPCR kinases (GRK) (Ferguson et al., 1998) and interaction of phosphorylated receptor with β-arrestin, which results in dissociation of receptor from G protein (for review, see Whalen et al., 2011). However, in the present study, the contribution of GRK-dependent receptor desensitization on the descending phase of β2-AR inotropism was discarded based on the ability of forskolin (a direct AC activator that bypasses β2-AR; figure 3C) and rolipram (a selective PDE4 inhibitor; figure 4A) to induce similar biphasic inotropic effects. In fact, by showing that descending phase of forskolin inotropic effect is also inhibited by PTX (figure 4A), our data indicate that β2-AR/Gi coupling is indirect and dependent on signaling events downstream of AC activation.

Therefore, taking into account previous studies from our group showing that β2-AR agonists and forskolin are able to sequentially increase cAMP efflux from skeletal muscle cells and the extracellular adenosine level (Godinho and Costa-Jr, 2003; Chiavegatti et al., 2008), we evaluate the possible contribution of the extracellular cAMP-adenosine pathway to the biphasic inotropic effect of β-AR agonists. In support to the above hypothesis, incubation of diaphragm with cAMP resulted in a PTX-sensitive negative inotropic effect (figure 5B). Remarkably, the time-course of cAMP effect resembled the late attenuation phase of β2-AR positive inotropism. In fact, the delayed descending phase of clenbuterol/fenoterol/forskolin inotropic effect presented here coincides with the increment of extracellular cAMP induced by β-AR agonist isoproterenol or forskolin in cultured skeletal muscle or in rat skeletal muscle (Godinho and Costa-Jr, 2003; Chiavegatti et al., 2008). Furthermore, Gi-dependent
effects of clenbuterol and cAMP were mimicked by adenosine and inhibited by adenosine receptor antagonists CGS-15943 (non-selective) DPCPX (A₁-selective) (figure 5C and 6A-C), indicating that cAMP effect depends on activation of adenosine receptor. More importantly, selective inhibition of descending phase of clenbuterol inotropic effect by either probenecid or ecto-5′-nucleotidase inhibitor AMPCP (figure 7A-B), which definitively support the indirect coupling of β₂-AR to Gi, via extracellular cAMP-adenosine pathway, illustrated in figure 8.

In view of the expression of distinct adenosine receptors subtypes (A₁, A₂A, A₂B and A₃) coupled to Gi/Gs proteins in skeletal muscle (Ren and Stiles, 1994; Lynge and Hellsten, 2000; Reading and Barclay, 2001; Thong et al., 2007), the interstitial formation of adenosine via ecto-5′-PDE and ecto-5′-nucleotidase (Hellsten and Frandsen, 1997; Chiavegatti et al., 2008) may allow the autocrine regulation of skeletal muscles function. This is of special clinical importance because β₂-AR agonists, used for treatment of asthma or chronic obstructive pulmonary disease (COPD), transiently improve isometric and isotonic contractility of respiratory skeletal muscle, including the diaphragm (Van Der Heijden et al., 1998), which is compromised in patients with COPD (Ottenheijm et al., 2007). Besides, under pathological conditions, increased β₂-AR-Gi coupling could result in impaired contraction function, as observed in muscle-specific Gs deficient mice (Chen et al., 2009).

Although the cross-talk between β₂-AR and adenosine receptors have been described in several cell types, the involvement of extracellular cAMP-adenosine pathway on the β₂-adrenergic signaling has been overlooked. This new level of GPCR cross-talk may explain the inhibitory effect of adenosine on isoproterenol-induced activation of AC in rat cardiac membranes (LaMonica et al., 1985), adipocytes (Elks et al., 1987) and smooth muscle (Gerwins et al., 1990) cells lines. In addition, the extracellular cAMP-adenosine pathway would elucidate why in rat perfused heart, catecholamines increases extracellular adenosine
formation, which in turn prevents full mechanical responsiveness to β-AR stimulation (Dobson et al., 1986). In stress conditions, like hypoxia or ischemia, increased extracellular adenosine levels is responsible for a cardioprotective effects, which, at least in part, involve activation of Gi-coupled adenosine A1 and A3 adenosine receptors (Safran et al., 2001; Du et al., 2012).

As observed in heart, β-AR improves skeletal muscle twitch contraction by increasing intracellular Ca\textsuperscript{2+} availability (Lynch and Ryall, 2008). However, long lasting elevation of cytosolic Ca\textsuperscript{2+} may have deleterious consequences, involving activation of the Ca\textsuperscript{2+}-dependent proteases (Gissel, 2005; Verburg et al., 2009). Again, extracellular adenosine exerts protective effect in skeletal muscle, mainly via activation of A1 and A3 subtype receptors (Zheng et al., 2007). Thus, the cross-talk between Gs-coupled β2-AR and Gi-coupled adenosine receptors presented here possibly provides a feedback mechanism for a fine control of intracellular cAMP levels which results in attenuation of β responsiveness and modulation of skeletal muscle contraction.

In summary, this study add a new view to the actual concept of promiscuous receptor/G protein coupling by showing the indirect coupling of β2-AR to Gi protein, which depends on the activation of adenosine receptors. In skeletal muscle, this extracellular arm of cAMP signaling illustrated in figure 8 provides a negative-feedback loop, which may limit GsPCR response and possible harmful exacerbation of muscle contraction. In fact, considering the increased generation of extracellular adenosine during muscle contraction (Lynge et al., 2001), “extracellular cAMP-adenosine pathway” may influence many other aspects of muscle physiology via activation of postsynaptic adenosine receptors, including those associated to the regulation of skeletal muscle carbohydrate metabolism (Hespel and Richter, 1998) and muscle proteolysis (Gissel, 2005; Bergantin et al., 2011). Thus, in skeletal muscle, the extracellular cAMP–adenosine pathway may function as a feedback mechanism.
able to modulate the cAMP signaling events initiated by other endogenous substances through activation of G-protein-coupled receptors, such as adrenoceptors, calcitonin gene-related peptide, are able to induce the efflux of cAMP (Godinho and Costa-Jr).

Actually, it is important to emphasize that efflux of cAMP/cGMP efflux seems to be a widespread signaling mechanism (Hofer and Lefkimmiatis, 2007; Sager and Ravna, 2009) reported in vascular smooth muscle cells (Dubey et al., 1996), cardiac fibroblasts (Dubey et al., 2001), oviduct cells (Cometti et al., 2003), kidney (Jackson and Raghvendra, 2004; Dubey et al., 2010), adipose tissue (Strouch et al., 2005) gastrointestinal tract (Giron et al., 2008), human placenta explants (Biondi et al., 2010), astrocytes and microglial cells (Verrier et al., 2011). Thus, extracellular cAMP-adenosine pathway may represent a general autocrine and/or paracrine mechanism that indirectly modulates the signaling triggered by distinct receptors coupled to Gs proteins, qualifying cyclic nucleotides as extracellular third messengers, and extracellular cAMP-adenosine signaling pathway as potential pharmacological target for therapeutic intervention. Definitely, additional studies will be necessary to determine the contribution of other extracellular cyclic nucleotides to GPCR signaling cascade.

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Authorship Contributions

Participated in research design: Duarte, Menezes-Rodrigues and Godinho.

Conducted experiments: Duarte, Menezes-Rodrigues and Godinho.
Performed data analysis: Duarte, Menezes-Rodrigues and Godinho.

Wrote or contributed to the writing of the manuscript: Godinho
References


Footnotes

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Figure Legends

Figure 1. Clenbuterol potentiates skeletal muscle contraction force in a bimodal concentration-response manner. Mouse diaphragm muscles (n=3-4) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage) in the presence of 1-100 nM (A) or 100-1000 nM (B) clenbuterol (Clen). (C) Maximal positive inotropic effect of clenbuterol (1-1000 nM). Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%; A and B, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of agonist. (a) Significantly different from basal value; (b) significantly different from 100 nM group; (P < 0.05; ANOVA followed by Newman-Keuls multiple comparison test).

Figure 2. Fenoterol potentiates skeletal muscle contraction force in a bimodal concentration-response manner. Mouse diaphragm muscles (n=3-5) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage) in the presence of 1-30 nM (A) or 30-1000 nM (B) fenoterol (Fen). (C) Maximal positive inotropic effect of fenoterol (1-1000 nM). Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%; A and B, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of agonist. (a) Significantly different from basal value; (b) significantly different from 30 nM group; (P < 0.05; ANOVA followed by Newman-Keuls multiple comparison test).

Figure 3. Pertussis toxin potentiates the positive inotropic effect of β2-AR agonists and forskolin in mouse diaphragm. Mouse diaphragm muscles (n=3-4) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage). The effect of 1 μM clenbuterol (Clen, A), 1 μM fenoterol (Fen, B) and 1 μM
forskolin (FSK, C) on diaphragm twitch contraction were evaluated in muscle strips pre-incubated for 1 h with pertussis toxin (PTX, 1 μg/mL) or vehicle. Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of agonist.

Figure 4. Effect of rolipram, 8-Br-cAMP and cAMP on mouse diaphragm contraction.
Mouse diaphragm muscles (n=3-4) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage) in the presence of 20 μM rolipram (A) 100 μM 8-Br-cAMP and 10 μM cAMP (B). Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of drugs.

Figure 5. cAMP elicited a negative inotropic response via Gi-dependent activation of adenosine receptors. Mouse diaphragm muscles (n=3-6) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage) in the presence of 1 μM forskolin (FSK), 10 μM cAMP or 1 μM adenosine (ADO) (A). The effect of 10 μM cAMP on diaphragm twitch contraction was evaluated in muscle strips pre-incubated for 1 h with pertussis toxin (PTX, 1 μg/mL) or vehicle (B) or for 30 min with non-selective adenosine receptor inhibitor CGS-15943 (CGS, 100 nM) or vehicle (C). Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of cyclic nucleotide.
Figure 6. Descending phase of clenbuterol inotropic effect on mouse diaphragm involves activation of $A_1$ adenosine receptor. Mouse diaphragm muscles ($n=3-4$) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage). The effect of 10 nM clenbuterol on diaphragm twitch contraction was evaluated in muscle strips pre-incubated for 30 min with (A) non-selective adenosine receptor antagonist CGS-15943 (CGS, 100 nM) or vehicle (B) and selective $A_1$ adenosine receptor antagonist DPCPX (50 nM) or vehicle. The effect of 10 $\mu$M ADO on diaphragm twitch contraction was evaluated in muscle strips pre-incubated for 30 min with selective $A_1$ adenosine receptor inhibitor DPCPX (50 nM) or vehicle (C). Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of agonist or cyclic nucleotide.

Figure 7. Inhibition of either organic anion transporters with probenecid or ecto-5’nucleotidase with AMPCP abolished the descending phase of positive inotropic responses elicited by clenbuterol or the negative inotropic responses elicited by cAMP. Mouse diaphragm muscles ($n=3-6$) were submitted to electric transmural stimulation (0.1 Hz frequency; 2 ms duration, and supra-maximal voltage). The effect of 10 nM clenbuterol on diaphragm twitch contraction was evaluated in muscle strips pre-incubated for 30 min with (A) 100 $\mu$M probenecid or vehicle, (B) 100 $\mu$M AMPCP or vehicle. The effect of 10 $\mu$M cAMP on diaphragm twitch contraction was evaluated in muscle strips pre-incubated for 30 min with 100 $\mu$M AMPCP or vehicle (C). Amplitude of twitch contraction was expressed as mean ± e.p.m of basal value (100%, dashed horizontal line) obtained in the presence of d-tubocurarine before incubation of agonist or cyclic nucleotide.
**Figure 8. Indirect cross-talk of Gs-coupled β2-AR and Gi-coupled adenosine receptors.**

Activation of β2-AR by agonist induces a Gs-dependent activation of AC. The increase of intracellular cAMP is followed by cyclic nucleotide efflux via multidrug resistance-related proteins (MRP) and sequential conversion to AMP and adenosine by ecto-phosphodiesterase and ecto-nucleotidase. The extracellular adenosine may act on A1 adenosine receptors to modulate activity of Gi-sensitive adenylyl cyclases isoforms (ACV and ACVI). This pathway may provide an important negative-feedback loop that may limit β2-AR stimulation and possibly deleterious effects of increased skeletal muscle contractile response (based on model presented by Chiavegatti *et al.*, 2008). The signaling steps, labeled by numbers (① to ⑤), were analyzed in the present study using the following drugs: ① Rolipram, as a selective cAMP specific phosphodiesterase 4 inhibitor; ② Probenecid, as organic anion transporter inhibitor; ③ AMPCP, as ecto-5’nucleotidase inhibitor; ④ CGS 15943 and DPCPX, as non-selective and A1-selective adenosine receptor inhibitors; and ⑤ PTX, which catalyzes ADP-ribosylation of Gαi subunit, impairing its interaction with the receptor.
Figure 1

**A**

![Graph showing contraction (% of basal) over time (min) for different concentrations of Clen (1 nM, 10 nM, 30 nM, 100 nM)](image)

**B**

![Graph showing contraction (% of basal) over time (min) for different concentrations of Clen (1 nM, 300 nM, 1000 nM)](image)

**C**

![Bar chart showing contraction (% basal) for different concentrations of Clembuterol (0 nM, 1 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1000 nM)](image)
Figure 2
Figure 3

(A) Clen

(B) Fen

(C) FSK
Figure 4

A

Ropiram

Contraction (% of basal)

Time (min)

120

115

110

105

100

95

-30

0

30

60

90

120

100 μM 8-Br-cAMP

10 μM cAMP

B

Drug

Contraction (% of basal)

Time (min)

115

110

105

100

95

90

-20

0

20

40

60

80

100
Figure 5
Figure 6
Figure 7

A

B

C

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Figure 8