CLENBUTEROL-STIMULATED GLUCOSE UPTAKE ACTIVATES BOTH GS AND GI PATHWAYS THROUGH β2-ADRENOCEPTOR IN MOUSE ISOLATED SOLEUS MUSCLE

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ABSTRACT
β₂-adrenoceptors activated by adrenaline can also couple to both Gs and Gi proteins. The former is associated with an increase in cAMP to illicit the effect of the catecholamine. In the later, β₂-AR induces PKA-catalysed phosphorylation of the receptor, which intends couples to Gi, at high concentration. We proposed that, clenbuterol which stimulates glucose uptake at low concentration and inhibits it at high concentration might have identical signalling pathway as adrenaline. Mouse isolated muscles were pre-incubated in flasks containing 3 ml of Krebs-Henseleit Bicarbonate buffer. After 120 min of pre-incubation, with the appropriate concentration of PTX the muscles were transferred to another incubation flask containing 3 ml of the same buffer and 0.3mCi 2-deoxy[1-¹⁴C]glucose containing varying concentrations of adrenaline, clenbuterol or 1nM insulin with or without PTX concentrations of 100ng/ml for adrenaline and clenbuterol and 0.1 or 1.0ng/ml for insulin, or 1M acetylcholine for 45 minutes. Adrenaline stimulated glucose uptake in isolated mouse soleus muscle at low concentration (10⁻¹⁰M) and inhibited it at high concentration (10⁻⁵M). The effect of the lower concentration was mediated through the β₂-AR coupling to the Gs protein and to the Gi protein at high concentration. Similarly, clenbuterol stimulated glucose uptake in isolated mouse soleus muscle at low concentration (10⁻¹¹M) and inhibited it at high concentration (10⁻⁷M). These effects of high concentration of adrenaline and clenbuterol were supported by the fact that 100ng/ml PTX relieved their inhibitory effects. The higher concentration effect of clenbuterol was additionally supported by the fact that, 1M acetylcholine, relieved the inhibitory effect.

PTX, Pertusis toxin, G-proteins, Guanine nucleotide binding proteins, β-AR, beta adrenoceptor, M₂, muscarinic receptors

INTRODUCTION
Heterotrimeric GDP binding protein (G-protein) plays an important physiological role in many hormones and neurotransmitters (Bourne et al., 1991). Agonists binding to the receptor lead to the interaction with G-protein to exchange GDP for GTP, resulting in dissociation of α₂GTP and βγ subunits (Gill and Mereen, 1978). The heteromeric G-proteins are divided into four main subfamilies according to
the identity of their G\(_\alpha\) subunit G\(_\alpha\), G\(_\alpha\), G\(_\alpha\), and G\(_\alpha\) proteins (Kristiansen, 2004), which are further divided into several isoforms (Cabrera-Vera et al., 2003). All the G\(_\alpha\) family have GTPase intrinsic properties; and also contain sites for NAD\(^+\)-dependent ADP-ribosylation, which is catalysed by cholera toxin (CTX) and/or pertussis toxin (PTX) resulting in the transfer of ADP-ribosyl group from intracellular NAD\(^+\) to the \(\alpha\) subunit of G-protein. This irreversibly modifies G\(_\alpha\) and continuously activates adenylyl cyclase, whereas ADP-ribosylation of G\(_\alpha\) cannot inhibit adenylyl cyclase. Thus both CTX and PTX normally increase adenylyl cyclase activity and raise cAMP levels. The G\(_\alpha\) subfamily (G\(_\alpha\), G\(_\alpha\) and G\(_\alpha\)) and the G\(_\alpha\) are substrates for CTX but resistant to PTX, while G\(_\alpha\) is sensitive to PTX complex (Fields and Casey, 1997).

Adrenaline has been shown to increase and decrease left arterial force. However, the negative inotropic effect was abolished by pertussis toxin (PTX) consistent with mediation through Gi protein (Heubach et al., 2004), but, norepinephrine only increased contractile force, and high concentration prevented the positive inotropic effects but did not affect the negative inotropic effect. The inotropic effect of adrenaline seems to be elicited through binding to two sites of \(\beta_2\)-AR. One site involves the receptor coupling to Gs for which both adrenaline and noradrenaline compete. The other site, recognised by noradrenaline only, couples to Gi.

Both \(\beta_2\) and \(\beta_1\)-AR are involved in the mediation of cardio stimulating effects of adrenaline in human atrium (Gille et al., 1985; Kaumann et al., 1999). Human \(\beta_2\)-AR overexpressed in murine heart has been reported to couple with Gs proteins (Milano et al., 1994) but not with Gi (Xiao et al., 1999) in the absence of agonist, thereby eliciting continuous stimulation.

In addition to coupling to Gs proteins \(\beta_2\)-AR activated by isoproterenol, can also couple to Gi proteins (Daaka et al., 1997). Similarly, isoproterenol both increased and decreased contractility through \(\beta_2\)-AR in the left atrium (Heubach et al., 2003), the cardiodepressant effect of (-) isoproterenol was abolished by pretreatment with pertussis toxin also consistent with mediation through \(\beta_2\)-AR coupled Gi protein (Hasseldine et al., 2003).

In our previous study (Ngala et al., 2008), Clenbuterol stimulated glucose uptake at low concentration (10pM) but inhibited it at a high concentration (100nM). This work was aimed at determining whether clenbuterol-stimulated glucose uptake in isolated soleus muscle was mediated through coupling to Gi and Gs as was the case in the negative and positive inotropic effect on transgenic mouse cardiomyocytes by adrenaline (Heubach et al., 2003). We also determined the effect of acetylcholine on clenbuterol-stimulated glucose uptake and the inhibitory effect of PTX on insulin-stimulated glucose uptake.

**MATERIALS AND METHODS**

**Animals**

Housing and procedure were conducted in accordance with the UK Government Animal (Scientific procedures) Act 1986 and approved by the University of Buckingham Ethical Review Board. Male C57Bl/6 lean and ob/ob mice aged 6-7 weeks, were obtained from Harlan, UK, and fed ad libitum (free access to water and standard mouse chow) prior to use. The animals were maintained in a constant temperature (21±2°C) room, with a fixed 12-hr artificial light/12-hr dark cycle (8am to 8pm).

**Reagents**

cAMP immunoassay Reagent Kit (RPN 225 Lot 94A, Park W208287), was purchased from Amersham Biosciences UK Ltd, and PTX from Tocris UK Ltd, all other reagents were purchased from Sigma Aldrich UK,

**Measurement of glucose uptake**

Measurement of glucose uptake in isolated soleus muscle has previously been described by Ngala et al. (2008). Briefly, Mice were killed by cervical dislocation and the soleus muscles dissected from both hind legs. The muscles were immediately placed in individual 25 ml incubation flasks containing 3 ml of Krebs-Henseleit Bicarbonate buffer (KHB: pH 7.30).
After 120 min of pre-incubation, with the appropriate concentration of PTX the muscles were transferred to another incubation flask containing 3 ml of the same buffer and 0.3mCi 2-deoxy[1-14]C]glucose (working solution of 0.1mCi/ml), containing varying concentrations of adrenaline, clenbuterol or 1nM insulin with or without PTX at concentrations of 100ng/ml for adrenaline and clenbuterol and 0.1 or 1.0ng/ml for insulin, for 45 minutes. Muscles were then digested with 1M NaOH. The 2-deoxyglucose-6-phosphate formed was then precipitated out with Ba(OH)2 and the radioactivity counted. Glucose uptake was then calculated from the count.

cAMP extraction

Mouse isolated soleus muscles were incubated with 100ng/ml PTX for two hours in 25 ml incubation flasks containing 3 ml of Krebs-Henseleit Bicarbonate buffer (KHB: pH 7.30), with 5.5 mM glucose, 0.14% bovine serum albumin and 10 mM HEPES equilibrated with 95% O2: 5% CO2. All flasks were immediately sealed with SubaSeals, transferred to shaking water bath at 37°C and gassed continuously with 95% O2: 5% CO2 through a 21G needle and with a 23G acting as a vent in the SubaSeal. After incubation, muscles were homogenised in 0.5 ml cold 6% trichloroacetic acid at 2-8°C using rota blender RW 20 D2M (Jank & Kunkel, IKA labotecnik) to give a 10% (w/v) homogenate. The homogenates were centrifuged at 2000 x g for 15 min at 4°C. The supernatants were washed 3 times with an equal volume of water-saturated diethyl ether, and the ether layer discarded after each wash. The aqueous extract was lyophilised under stream of nitrogen at 60°C (Meyer et al., 1974). The dried extracts were then dissolved in 1.5 ml of assay buffer (0.05M acetate buffer, pH 5.8) from the cAMP reagent kit.

Measurement of cAMP

cAMP was determined using an enzyme-linked immunos assay Reagent Kit (RPN 225 Lot 94A, Park W208287,) as described by manufacturer (Amersham Biosciences UK Ltd) in acetylation EIA procedure. The 96-well plate was pre-coated with donkey anti-rabbit IgG of rabbit cAMP. The cAMP antiserum (100 ml) was then added to each well followed by 50 ml of 1:200 dilution of the re-dissolved cAMP extract and the plate was incubated at 3-5°C for 60 min. This allowed the cAMP to bind to the antiserum, which was bound to the well. Antiserum that had not bound cAMP from the sample was then able to bind cAMP-peroxidase-conjugate. The amount of peroxidase bound was determined by adding 150 ml enzyme substrate to develop a blue colour of intensity inversely related to the amount of cAMP in the sample. The reaction was terminated with 100 ml of 1M sulphuric acid and the plate read at 450 nm.

Analysis of Data

Results were expressed as means ± SEM. Data were analysed by one-way ANOVA followed by the Bonferroni test for multiple comparison using graph Pad Prism version 4 (Graph pad soft ware, San Diego California). Unpaired Student t-tests were used to assess for significance.

RESULTS

Glucose uptake

Adrenaline induced a significant glucose uptake in isolated soleus muscle at 10^10 M and
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at $10^{-5}$M by 40% and by 58.6% (2.03±0.35 mM base and 3.07±0.45mM and 3.22±0.40 mM) respectively. However, there was an inhibitory effect of 21.6% at $10^{-5}$M (1.60±0.34 mM).

PTX converted adrenaline-inhibited glucose uptake at $10^{-5}$M to a significantly induced glucose uptake of 109%, but at $10^{-9}$M there was no significant effect.

PTX on its own had no significant effect in soleus muscle glucose uptake, however, it converted the 34% clenbuterol-inhibited glucose uptake at $10^{-7}$M to a significant 106% induction. Similarly, PTX had no effect on clenbuterol-induced glucose uptake at $10^{-11}$M.

Acetylcholine alone induced a significant increase in 2DG uptake by 63%. Clenbuterol alone elicited a 51% increase in 2DG uptake at $10^{-11}$M and a 32% decrease at $1x10^{-7}$M. There was no significant difference between acetylcholine-induced 2DG-uptake alone, and $1x10^{-11}$M clenbuterol, or acetylcholine plus $1x10^{-11}$M clenbuterol. However, the $1x10^{-7}$M clenbuterol inhibition was converted to a two fold increase in the presence of acetylcholine.

Fig 2: Rate of 2DG6P formation in isolated mouse soleus muscle induced by adrenaline in the presence and absence of 100ng/ml PTX. Significance value: (n =6) ★p<0.05 ★★p<0.01

Fig 3: Rate of 2DG6P formation in isolated mouse soleus muscle induced by clenbuterol in the presence and absence of 100ng/ml PTX. Significance value: (n =6) ★p<0.05 ★★p<0.01

Fig 4: Rate of 2DG6P formation in isolated mouse soleus muscle induced by clenbuterol in the presence and absence of 1µM Acetylcholine. Significance value: (n =6) ★p<0.05
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1.0 nM insulin, induced a four fold increase in glucose uptake (0.96±0.05mM base and 2.88mM). This was inhibited by 33% by 0.1 ng/ml PTX and 46% by 1.0ng/ml PTX.

DISCUSSION
Human β2-AR overexpressed in transgenic mouse heart can bind adrenaline and isoprenaline in two ways: one coupled to Gs to increase contractility force at low concentration and the other to Gi to suppress contractility force of the heart at higher concentrations (Heubach et al., 2004). Noradrenaline, by contrast couples only to Gs. Similarly, β2-ARs in cell line have been shown to couple to both Gs and Gi with phosphorylation by protein kinase A (PKA) switching the β2-AR towards Gi (Daaka et al., 1997). Conversely, ICI118551 (a specific β2-AR blocker) binding directs β2-AR to Gi-coupling, acting as an agonist and directs to Gs coupling as an antagonist (ligand-directed trafficking) (Gong et al., 2002).

In this study clenbuterol seems to exhibit a similar reaction pattern, increasing 2DG uptake at a lower concentration and inhibiting at a higher concentration (Fig 3), Investigation was therefore conducted to verify if the effects of adrenaline and clenbuterol on 2DG uptake in isolated mice soleus muscle were similar to those reported for heart contractility by adrenaline.

Adrenaline, exhibited a biphasic response on 2DG uptake in the soleus muscle, increasing it at lower concentration and decreasing it at higher concentration (Fig.1), very similar to its ionotropic effect in increasing and decreasing left arterial force in transgenic mouse heart overexpressing human β2-AR at lower concentration (10nM) and at high concentration respectively (10uM). However, the negative inotropic effect was abolished by pertusis toxin (PTX) consistent with mediation through Gi protein (Heubach, 2004). In glucose uptake, the stimulatory effect was not affected by 100ng/ml PTX. However, the inhibitory effect was relieved by 100ng/ml PTX and converted to a stimulatory effect (Fig 2). Similarly the glucose uptake stimulatory effect at lower concentration clenbuterol was not affected by 100ng/ml PTX, but the inhibitory effect at the higher concentration was relieved and converted to a stimulatory effect (Fig 3). It therefore seems possible...
that the glucose uptake stimulatory effect at the lower concentration is mediated through the β2-AR coupled to Gs and the inhibitory effect at the higher concentration is mediated through the β2-AR coupled to Gi.

Studies have also shown that in native rat cardiomyocytes, PTX pre-treatment selectively potentiates the positive ionotropic effect of β2-AR but not β1-AR stimulation, suggesting that β2-AR dually couples to Gs and to Gi the PTX-sensitive inhibitory G proteins (Xiao et al., 1995).

PTX has also been shown to inhibit insulin-stimulated glucose uptake, this is possible because the effect of insulin is not mediated through activation of β2-AR coupled to G-proteins (Zeng et al., 2000) but through PKC, but PKC is inhibited by PTX (Kano et al., 2000). To check whether we could reproduce effects of PTX reported by others, we determined whether PTX inhibited insulin-stimulated glucose uptake, as reported by Kano et al., (2000). PTX inhibited insulin-stimulated glucose uptake was dose depended. 1.0 nM insulin-stimulated glucose uptake was inhibited by 33% by 0.1 ng/ml PTX and 46% by 1.0ng/ml PTX (Fig 5).

The effect of acetylcholine-induced glucose uptake was investigated because M2 and M4 receptors are coupled to Gi and muscarinic receptors are expressed in muscle (Hosey, 1992; Reyes and Jaimovich, 1996; Felder, 1995). Therefore, the inhibitory effect at the high concentration clenbuterol thought to be mediated through coupling to Gi would be reversed by acetylcholine known to mediate its effect through the muscarinic receptors coupled to the Gi protein.

Similar to the effect of PTX on clenbuterol and adrenaline-stimulated glucose uptake at lower and higher concentrations, acetylcholine had no significant effect on clenbuterol-stimulated glucose uptake at the lower concentration. However, at the higher concentration, clenbuterol-inhibited glucose uptake was relieved and converted to a stimulatory effect by the acetylcholine (Fig 4). These effects of PTX and acetylcholine on clenbuterol stimulated supports our hypothesis that clenbuterol elicits glucose uptake through the β2-AR coupled to Gs at lower concentration and through coupling to Gi at higher concentration.

At the higher concentration, clenbuterol elicited an inhibitory effect in cAMP levels consistent with the inhibition of glucose uptake (Ngala et al, unpublished). PTX stimulated an increase in cAMP levels (Fig 6). PTX adds ADP-ribose from intracellar NAD+ to the α subunit of G-protein and this cannot inactivate adenylate cyclase and adenylate production is continuously turned on (Gill and Mereen, 1987). It seems therefore that at high concentration clenbuterol inhibitory effect of cAMP accumulation is overridden by PTX leading to an increase in cAMP and a corresponding increase in glucose uptake.

CONCLUSION
Clenbuterol stimulated glucose uptake in isolated soleus muscle is mediated through the β2-AR coupling to Gs at lower concentration and to Gi at higher concentration similar to the effect of adrenaline which stimulates glucose uptake at the lower concentration and inhibits at high concentration. PTX and acetylcholine abolished clenbuterol glucose uptake inhibitory effect at high concentration and turns it into stimulation. PTX also induced an increase in cAMP and since in its presence, high concentration clenbuterol stimulates glucose uptake implies clenbuterol stimulatory effect is cAMP dependent, therefore cAMP is a secondary messenger in clenbuterol stimulated glucose uptake. Clenbuterol stimulated glucose uptake is therefore mediated through a ligand-directed signalling.

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