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EDITORIAL

β_2-Adrenoceptor-mediated regulation of glucose uptake in skeletal muscle—ligand-directed signalling or a reflection of system complexity?

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Abstract The capacity of G protein-coupled receptors (GPCRs) to activate multiple G protein isoforms and additional effectors such as β-arrestins has become a well-established paradigm and provides the basis for developing drugs that preferentially activate beneficial signalling pathways. There are many published examples of ligand-directed signalling, and recent studies have provided direct evidence that different agonists stabilise distinct GPCR conformations. This field is rapidly evolving, but a key question is whether signalling bias observed in heterologous cell expression systems can be translated to physiological systems of therapeutic relevance. The paper by Ngala et al. in this issue of the journal addresses the capacity of agonists acting at the β_2-adrenoceptor to engender signalling bias in relation to glucose uptake in isolated skeletal muscle, an area of considerable potential interest in targeting insulin-independent pathways for the treatment of type 2 diabetes. The authors show that clenbuterol and BRL37344 have opposite effects on glucose uptake, despite both having agonist actions at β_2-adrenoceptors. This study underlines some of the obstacles associated with studies in a complex physiological system but nonetheless highlights the need to consider signalling bias in the relevant target tissue when developing novel drugs.

Activation of G protein-coupled receptors (GPCRs) often results in multiple signalling outputs via receptor coupling to one or more G proteins, Gα or Gβγ signalling and coupling to G protein-independent effectors such as β-arrestins (reviewed in Evans et al. 2010). Furthermore, drugs that block one effector pathway may stimulate alternative pathways (Kenakin and Miller 2010; Sato et al. 2007; Sato et al. 2008), and two or more agonists may display bias for different cellular responses—a phenomenon variously known as ligand-directed signalling (LDS), biased agonism or functional selectivity (Blattermann et al. 2012; Busnelli et al. 2012; Emery et al. 2012; Evans et al. 2011; Gregory et al. 2012; Nijmeijer et al. 2012; Tschammer et al. 2011). Recent elegant studies have provided robust evidence that LDS reflects the stabilisation of distinct active receptor conformations by different agonists (Kahsai et al. 2011; Liu et al. 2012; Mary et al. 2012; Wootten et al. 2013). This concept is an important innovation in drug design, as one can theoretically tailor ligands to have desirable drug effects with less unwanted side effects.

The capacity of agonists to promote signalling bias at the β_2-adrenoceptor (AR) is the subject of a paper in this issue of the journal (Ngala et al. 2013). Although LDS has been described extensively in an expanding number of heterologous cell systems expressing recombinant GPCRs, there are relatively few studies in physiological systems that express endogenous receptors (Allen et al. 2011; DeWire et al. 2013; Pradhan et al. 2010; Violin et al. 2010) and even fewer in tissues. The importance of the paper by Ngala et al. is that it addresses cAMP and glucose uptake responses mediated by endogenous β_2-ARs in preparations of mouse isolated soleus muscle. Although β_2-ARs are predominantly Gαs-coupled, they do display additional promiscuous coupling to Gαi/o proteins. Ngala et al. suggest that the agonists adrenaline and clenbuterol display LDS, as they promote greater coupling to Gαi/o than noradrenaline or BRL37344.

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Biased coupling of the β_2-AR to Gαi/o and Gαs has been demonstrated previously in rat cardiomyocytes (Woo et al. 2009). Fenoterol is a β_2-AR selective partial agonist with two chiral centres. The S,R and R,R isomers of fenoterol both stimulate cardiomyocyte contractility and these responses are blocked by the β_2-AR selective inverse agonist ICI118551. The R,R isomer has higher potency than the S,R isomer, and the S,R but not the R,R response shows a clear leftward shift following treatment with the Gαi/o inhibitor pertussis toxin (PTX). Whereas contractility is reduced by Gαi/o coupling, ERK1/2 phosphorylation in response to (S,R)-fenoterol was blocked by PTX treatment, indicating a *dependence* on Gαi/o coupling. Interestingly, (R,R)-fenoterol-stimulated ERK1/2 phosphorylation is unaffected by PTX, suggesting a Gαs-mediated or alternative mechanism. Another key aspect of this study is that differential Gα coupling was demonstrated directly by subtype-specific immunoprecipitation of activated Gα subunits labelled with [γ-^{32}P]GTP-azidoanilide. (S,R)-fenoterol stimulates activation of Gαi2 substantially more than the R,R isomer, whereas (R,R)-fenoterol produces a threefold higher activation of Gαs than (S,R)-fenoterol. Overall, these results provide convincing evidence, in a system endogenously expressing β_2-ARs, that the two fenoterol stereoisomers stabilise conformations of the β_2-AR that have distinct coupling properties, and indicate direct interactions with Gαs and Gαi/o.

In contrast to these findings, another recent study showed that reduction in the contractility of skeletal muscle (isolated from mouse diaphragm) over time or in response to high concentrations of clenbuterol or fenoterol was not due to changes in direct β_2-AR coupling to Gαs or Gαi/o (Duarte et al. 2012). Instead, bell-shaped time courses or concentration–response curves reflected cAMP accumulation and *efflux* from myocytes, followed by conversion of the cAMP to adenosine and activation of Gαi/o-coupled adenosine A1 receptors. The primary objective of this paper by Duarte et al. was not the examination of LDS, but the observed responses are reminiscent of those seen by Ngala et al. in mouse soleus muscle, and thus we cannot exclude the possibility of indirect Gαi/o mechanisms in the present study.

Glucose uptake in isolated soleus muscle measured by Ngala et al. (2013) and reported in previous publications (Ngala et al. 2008, 2009) is complicated by atypical concentration–response curves that have sharp peaks (or troughs) occurring at single agonist concentrations. Glucose uptake in response to BRL37344 or clenbuterol shows a peak at 10 pM, and the curve for adrenaline shows a peak at 100 pM; however, responses to low concentrations of BRL37344 and clenbuterol are present in β_2-AR knockout mice and are not inhibited by the β_1-AR or β_3-AR antagonists CGP20712A or SR59230A (Ngala et al. 2009). The interpretation of these studies is not clear but they emphasise that some of these agents may have off-target effects unrelated to actions at β-ARs. The present study has therefore focused on β_2-AR-mediated responses that occur with higher agonist concentrations and are abolished in knockout mice or in the presence of the β_2-AR inverse agonist ICI118551. Clenbuterol (100 nM) and adrenaline (1 μM) both inhibit glucose uptake relative to basal levels, whereas BRL37344 (10 nM) and noradrenaline (1 μM) stimulate glucose uptake. Higher concentrations of BRL37344 (100 nM and 1 μM) decrease glucose uptake relative to the peak at 10 nM. The new study shows that clenbuterol (100 nM) also reduces cAMP accumulation, and that inhibitory effects on glucose uptake and cAMP accumulation are converted to stimulatory responses following treatment with PTX. Similarly, the reduced glucose uptake at higher BRL37344 concentrations is restored to the peak response seen at 10 nM by pretreatment with PTX. Whereas clenbuterol reduces the cAMP content of isolated muscle, BRL37344 has no effect, which may be due to roughly equivalent coupling to Gαs and Gαi/o. Overall, both clenbuterol and BRL37344 promote coupling of the β_2-AR to Gαi/o, but this appears to be more pronounced for clenbuterol than for BRL37344. The observed pattern of glucose uptake also indicates that adrenaline promotes greater coupling to Gαi/o than noradrenaline.

Several lines of evidence implicate a cAMP-PKA pathway in β_2-AR-mediated glucose uptake in the isolated muscle preparations. Ngala et al. (2013) show that treatments known to increase intracellular cAMP, namely forskolin (1 μM) and the phosphodiesterase IV inhibitor rolipram (10 μM), both increase glucose uptake. In combination, BRL37344 (1 nM) and rolipram (0.5 μM) promote significant glucose uptake whereas neither BRL37344 nor rolipram produces a response at these lower concentrations. Third, despite the lack of measurable *global* cAMP accumulation, BRL37344-stimulated glucose uptake is substantially reduced by the PKA inhibitors 4CM and ALX-151-014. Increased glucose uptake in the presence of PTX is therefore likely to reflect increased cAMP production following inhibition of Gαi/o coupling. The BRL37344 data suggest that skeletal myocyte β_2-ARs form signalling complexes that facilitate interaction with distinct downstream pathways; although a population of β_2-ARs couple to Gαi/o, there must be receptor complexes that signal via PKA and downstream pathways leading to glucose uptake.

It is interesting to note that both clenbuterol and BRL37344 promote cAMP accumulation in CHO-K1 cells overexpressing the β_2-AR (Baker 2010), with maximal responses of 95 and 80 % relative to isoprenaline, and pEC_{50} values of 9.2 and 6.9, respectively. For glucose uptake in the isolated muscle preparations, Ngala et al. (2013) point out that "100 nM clenbuterol behaves like adrenaline, whereas 10 nM BRL37344 is more like noradrenaline". This is

noteworthy, as the effect of these drugs on glucose uptake tracks with the degree of amplification, or intrinsic efficacy, seen in CHO-K1 cAMP assays. In other words, the concentrations of adrenaline and clenbuterol required for a half maximal cAMP response are 63- and 19-fold lower than K_D values determined from binding experiments, noradrenaline is intermediate with ninefold amplification, and BRL37344 shows little amplification with an intrinsic efficacy of 2.3 (Baker 2010). Thus, agonists with a higher capacity to stabilise active conformations of the β_2-AR appear to promote greater coupling to Gαi/o in the isolated muscle system. The difference between overexpressed β_2-ARs in CHO-K1 cells and endogenous receptors in skeletal myocytes may be due solely to receptor abundance or may in addition reflect the cellular architecture and abundance of other signalling and scaffolding proteins in the two cell types. The stoichiometry of β_2-ARs relative to Gαs or Gαi/o isoforms would certainly be affected substantially by a large difference in receptor abundance. The findings of Ngala et al. (2013) suggest that a greater proportion of β_2-ARs in skeletal muscle couple to Gαi/o proteins and that this population predominates in the presence of high efficacy agonists such as adrenaline and clenbuterol.

The question of whether these findings indicate authentic LDS is difficult to ascertain. Bias can be unambiguously demonstrated when two ligands exhibit a reversal of efficacy or potency between two pathways, that is, drug A has higher efficacy/potency than drug B for pathway 1, but a lower efficacy/potency than drug B for pathway 2 (Kenakin 2011; Kenakin and Miller 2010). However, until recently, such findings have not been quantified, and there are also examples where drugs do not display reversal of potency or efficacy, but clearly promote distinct receptor conformations (Galandrin et al. 2008). LDS can be examined and quantified by generating bias plots and calculating bias factors (Evans et al. 2011; Kenakin and Christopoulos 2012; Kenakin et al. 2012; Rajagopal et al. 2011; Stallaert et al. 2011); however, these analytical approaches rely on the comparison of responses that adhere to classical sigmoidal concentration–response curves. In complex physiological systems such as the isolated muscle preparations used here, the concentration–response relationships are not sigmoidal, indeed they display multiple peaks and troughs, and cannot therefore be assessed quantitatively by current methods. An alternative approach is to measure responses to potentially biased agonists in the presence of selective signalling protein inhibitors. Ngala et al. (2013) show that glucose uptake in response to BRL37344 (10 nM) is blocked by inhibitors of PI3K (wortmannin, LY294002) and p38 MAPK (SB203580) but not by inhibitors of MKK1 (upstream of Erk1/2), AMPK or nitric oxide synthase. In contrast to the BRL37344 responses, wortmannin and SB203580 had no effect on the inhibition of glucose uptake by clenbuterol (100 nM). This approach is most informative, however, when both agonists are *stimulating* the measured response, whereas in the skeletal muscle preparation, clenbuterol is inhibiting glucose uptake via Gαi/o coupling and *reduced* cAMP accumulation. It is not surprising that blocking downstream signalling components had no effect in the absence of an upstream stimulus (increased cAMP), especially given that neither wortmannin nor SB203580 inhibited basal glucose uptake.

Ngala et al. (2013) reasoned that relative coupling to Gαi/o may be due to differential phosphorylation of the β_2-AR by the different agonists, and it was confirmed that clenbuterol (100 nM) does cause significantly more phosphorylation at Ser355/356 than BRL37344 (10 nM). It has previously been suggested (Baker et al. 2003) that β_2-AR conformations induced by the full agonist isoprenaline and the partial agonist salbutamol differ in their capacity for phosphorylation and desensitisation of responses, and that the conformation of the β_2-AR that is phosphorylated and possibly interacting with additional proteins has a lower affinity for antagonists than the non-phosphorylated state. These mechanisms may be at play here as well, although it is difficult to resolve such questions in complex physiological systems.

The development or validation of drugs that stimulate beneficial pathways whilst having no effect or inhibiting potentially detrimental signalling pathways is an attractive concept. It may be possible to determine activity profiles that correlate positively or negatively with clinical efficacy, and it will be interesting to see whether this profiling can be done successfully in recombinant systems with high receptor abundance or whether it must be augmented by the use of primary cell systems or isolated tissues expressing endogenous receptors (Appleton et al. 2013; Baker et al. 2011). There are very few studies demonstrating LDS with native receptors either ex vivo or in vivo. Despite some limitations, the work described by Ngala et al. (2013) shows that agonists can behave in a qualitatively different manner in a complex ex vivo system, and therefore represents welcome progress in this field.

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