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Multiple signalling pathways involved in β_2-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells

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1 β-adrenoceptor (AR) agonists increase 2-deoxy-[^{3}H]-D-glucose uptake (GU) *via* β_2-AR in rat L6 cells. The β-AR agonists, zinterol (β_2-AR) and (−)-isoprenaline, increased cAMP accumulation in a concentration-dependent manner ($pEC_{50} = 9.1 \pm 0.02$ and 7.8 ± 0.02). Cholera toxin (% max increase 141.8 ± 2.5) and the cAMP analogues, 8-bromo-cAMP (8Br-cAMP) and dibutyryl cAMP (dbcAMP), also increased GU (196.8 ± 13.5 and 196.4 ± 17.3%).

2 The adenylate cyclase inhibitor, 2′,5′-dideoxyadenosine (50 μM), significantly reduced cAMP accumulation to zinterol (100 nM) ($109.7 + 35.0$ to $21.6 + 4.5$ pmol well^{-1}), or forskolin (10 μM) (230.1 ± 58.0 to 107.2 ± 26.3 pmol well^{-1}), and partially inhibited zinterol-stimulated GU (217 ± 26.3 to 176.1 ± 20.4%). The protein kinase A (PKA) inhibitor, 4-cyano-3-methylisoquinoline (100 nM), did not inhibit zinterol-stimulated GU. The PDE4 inhibitor, rolipram (10 μM), increased cAMP accumulation to zinterol or forskolin, and sensitised the GU response to zinterol, indicating a stimulatory role of cAMP in GU.

3 cAMP accumulation studies indicated that the β_2-AR was desensitised by prolonged stimulation with zinterol, but not forskolin, whereas GU responses to zinterol increased with time, suggesting that receptor desensitisation may be involved in GU. Receptor desensitisation was not reversed by inhibition of PKA or Gi.

4 PTX pretreatment (100 ng ml^{-1}) inhibited insulin or zinterol-stimulated but not 8Br-cAMP or dbcAMP-stimulated GU. The PI3K inhibitor, LY294002 (1 μM), inhibited insulin- (174.9 ± 5.9 to 142.7 ± 2.7%) and zinterol- (166.9 ± 7.6 to 141.1 ± 8.1%) but not 8 Br-cAMP-stimulated GU. In contrast to insulin, zinterol did not cause phosphorylation of Akt.

5 The results suggest that GU in L6 cells involves three mechanisms: (1) an insulin-dependent pathway involving PI3K, (2) a β_2-AR-mediated pathway involving both cAMP and PI3K, and (3) a receptor-independent pathway suggested by cAMP analogues that increase GU independently of PI3K. PKA appears to negatively regulate β_2-AR-mediated GU.
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Abbreviations: [^{3}H]-2-DG, 2-deoxy-[^{3}H]-D-glucose; CR, concentration–response curve; G_i proteins, inhibitory guanosine triphosphatases; GLUT, glucose transporter; G_S proteins, stimulatory guanosine triphosphatases; GU, glucose uptake; pEC_{50}, negative log EC_{50}; PI3K, phosphatidyl inositol-3 kinase; pK_B, negative log K_B

Introduction

Several reports have demonstrated insulin-independent stimulation of glucose uptake (GU) in response to adrenoceptor (AR) stimulation, suggesting that ARs may represent an alternative way to increase GU in the absence of insulin. In the rat skeletal muscle cell line L6, GU is increased by activation of β_2-ARs (Nevzorova *et al*., 2002), the predominant β-AR subtype expressed in these cells (Nagase *et al*., 2001; Nevzorova *et al*., 2002). β_2-ARs are coupled to Gs and adenylate cyclase and utilise cAMP and protein kinase A (PKA) to produce the metabolic effects of catecholamines.

Adrenergic agonists also increase GU in skeletal muscle, white adipose tissue and heart (Abe *et al*., 1993; Liu & Stock, 1995; Liu *et al*., 1996). In addition, adrenaline-stimulated translocation of the insulin-sensitive glucose transporter 4 (GLUT4) has been shown in skeletal muscle (Han & Bonen, 1998). In primary brown adipocytes, stimulation of β_3-ARs increases GU in a cAMP-dependent manner (Shimizu *et al*., 1998; Chernogubova *et al*., 2004). In addition, β_1- and to a smaller extent α_1-ARs compensate for the lack of β_3-AR in brown adipocytes from β_3-AR knockout mice, and increase GU *via* cAMP/PKA/PI3K or PI3K/PKC respectively (Chernogubova *et al*., 2005). On the other hand, some studies show adrenaline-mediated inhibition of insulin-stimulated GU in skeletal muscle (Lee *et al*., 1997). In a number of cell types, it has been demonstrated that the inhibitory effect of cAMP on GU occurs in cells that express GLUT4, and possibly involves cAMP-dependent inhibition of intrinsic activity of the transporter (Lawrence *et al*., 1992; Piper *et al*., 1993; Reusch *et al*., 1993). In contrast, cAMP upregulates GLUT1 gene

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expression and protein synthesis in 3T3-L1 cells, L6 myoblasts and in a human choriocarcinoma cell line (Vinals *et al*., 1997; Ogura *et al*., 2000; Imamura *et al*., 2001; Fong *et al*., 2004; Chiou & Fong, 2005). Therefore, cAMP appears to be capable of both positive and negative modulation of GU with the effect being highly dependent on cell type.

L6 cells are widely used to study insulin-dependent or independent mechanisms of GU and can be differentiated into skeletal muscle-like myotubes, that express the GLUT4 (Mitsumoto *et al*., 1991; Mitsumoto & Klip, 1992). Increases in GU can be produced in L6 cells by β-AR agonists independently of insulin (Tanishita *et al*., 1997), and we recently demonstrated in pharmacological and molecular studies that the β-AR-subtype involved in those responses is the β_2-AR (Nevzorova *et al*., 2002). The signalling mechanisms mediating β_2-AR stimulated GU in L6 cells have not been extensively studied. The present study examined the role of cAMP in GU, and also its involvement in β_2-AR-mediated stimulation of GU in L6 myotubes.

Methods

Cell culture

L6 cells were obtained from ATCC. The cells were grown and differentiated for 7 days as described previously (Tanishita *et al*., 1997; Nevzorova *et al*., 2002). For GU experiments, cells were grown and differentiated in 24-well cell culture dishes, and for cAMP experiments cells were grown and differentiated in 12-well or 96-well cell culture dishes. Cells were serum-starved overnight before each experiment.

Measurement of cAMP using FlashPlate radioimmunoassay

The assay is based on competition between unlabelled cAMP in the test samples and [^{125}I]-cAMP for the anti-cAMP antibody embedded in the solid scintillant-coated wells of the 96-well plate. The cells were pretreated with antagonists or vehicle for 15 min, and then with agonists for further 30 min in 96-well plates containing phenol red-free medium with FBS (0.1%) in the presence of 3-isobutyl methyl-xanthine (IBMX; 2 mM). For time course experiments, the cells were pretreated with agonists for up to 2.5 h in the absence of IBMX, and then treated with agonists in the presence of IBMX in phenol red-free medium for further 30 min. The reaction was stopped by aspiration of the medium and the cells were digested with 0.5 M HCl. An aliquot of each sample was transferred to the FlashPlate (Perkin Elmer Life Sciences, Inc., Boston, MA, U.S.A.) containing assay buffer (0.05 M NaAc, 0.0008 M ethylenediaminetetraacetic acid (EDTA); pH 6.2). [^{125}I]-cAMP was diluted 1 : 100 in tracer diluent (0.5 M NaAc, 0.012 M $CaCl_2$, pH 6.2), and added to all wells in the FlashPlate. The plate was sealed and incubated at room temperature overnight prior to reading on the Top Count™ Microplate Scintillation Counter (Packard Instruments, Meriden, CT, U.S.A.). The assay was used to measure cAMP accumulation in response to β_2-AR agonists in the presence or absence of antagonists and time course experiments.

Measurements of cAMP using [^{3}H] assay system

This assay was used to measure cAMP levels in response to zinterol or forskolin in the presence or absence of the cell-permeable adenylate cyclase inhibitor, 2′,5′-dideoxyadenosine (ddA), or the selective PDE4 inhibitor, rolipram. Cells were pretreated with inhibitors for 30 min, and treated with agonists for further 30 min in serum-free DMEM in the absence of IBMX in 12-well plates. The medium was aspirated and reactions stopped by addition of 70% ethanol containing 4 mM EDTA. The samples were assayed as per manufacturer's protocol (Amersham Biosciences UK Ltd, U.K.).

2-Deoxy-[^{3}H]-D-GU assay

GU was measured using [^{3}H]-2DG (Tanishita *et al*., 1997; Nevzorova *et al*., 2002). Cells were serum-starved overnight and stimulated with agonists for 2.5 h in serum-free DMEM containing 25 mM glucose. To measure time-dependent responses, drugs were added at 30 min intervals for up to 2.5 h. Cells were washed and incubated with agonists in serum- and glucose-free DMEM for further 30 min, with 50 nM [^{3}H]-2DG added for the last 15 min. Cholera toxin (1 μg ml^{-1}) was added to the cells only once, and response was measured after 3 h. The reaction was terminated by aspirating the medium and washing cells with ice-cold HEPES-buffered saline (pH 7.4). Cells were digested with 0.2 M NaOH, then mixed with EcoLite scintillation liquid (Costa Mesa, CA, U.S.A.) and counted on Wallac WinSpectral™ 1414 liquid scintillation counter (Skudtek Scientific Pty. Ltd, Mt. Waverley, VIC, Australia).

Detection of Akt and acetyl CoA carboxylase phosphorylation

Phosphorylation of acetyl CoA carboxylase (ACC) in response to zinterol (100 nM) in the absence or presence of PKA inhibitor, 4-cyano-3-methylisoquinoline (4CM) (100 nM), and Akt phosphorylation in response to insulin or zinterol were detected using Western blotting. For ACC phosphorylation, L6 myotubes were stimulated with zinterol in the presence or absence of the inhibitor for 5 min. For Akt detection, cells were stimulated for 5 min with insulin, or 3 h with zinterol. Samples were harvested in prewarmed (65°C) sodium dodecyl sulfate sample buffer containing 50 mM dithiothreitol, sonicated, boiled for 3 min, and separated on 5% acrylamide gel (or 10% for Akt) for 2 h at 100 V. The proteins were transferred on to Hybond-C extra nitrocellulose (Amersham Biosciences UK Ltd, U.K.) or PVDF membrane (Millipore, Australia), and probed with primary antibodies: phosphor-acetyl-CoA carboxylase (Ser79), (dissolved 1 : 1000 in 5% BSA), phospho-Akt (Ser473) and total Akt (1 : 2000 in 5% BSA) (Cell Signalling Technology, Inc., Danvers, MA, U.S.A.). This was followed by incubation with the secondary anti-rabbit IgG HRP-linked antibody (1 : 2000 in 5% BSA) (Cell Signalling Technology, Inc.) for 1 h at room temperature. Proteins were detected by exposure to Hyperfilm ECL film (Amersham).

Drugs and reagents

The authors would like to thank Dr G. Anderson (Ciba-Geigy AG Switzerland) for (±)-CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl) 1H-imidazole-2-

y1)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulfonate). Drugs and reagents were purchased as follows: EcoLite scintillation liquid, ddA, 4CM, rolipram (Calbiochem, Kilsyth, VIC, Australia), [^{125}I]-cAMP (adenosine 3′,5′-cyclic phosphoric acid, 2′-*O*-succinyl [^{125}I]iodotyrosine methyl ester-), 2-deoxy-[^{3}H]-D-glucose (Perkin Elmer Life Sciences, Inc., Boston, MA, U.S.A.), IBMX, cholera toxin from *Vibrio cholerae,* bovine insulin, (−)-isoprenaline, forskolin, dibutyryl cAMP (N^6,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate), 8-bromo-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt), LY294002, dithiothreitol (Sigma Chemical Co., St Louis MO, U.S.A.), (±)-ICI 118551, (erythro-DL-1(7-methylin-dian-4-yloxy)-3-isopropylamino-butan-2-ol) (Imperial Chemical Industries Wilmslow, Cheshire, U.K.), sodium dodecyl sulfate (GibcoBRL, MD, U.S.A.).

Statistics

All experiments were performed in duplicate, and the results were expressed as mean ± s.e.m. The responses to agonists were calculated as percentage above the basal levels, which were set to 100%. The statistical significance of differences between the untreated and agonist-treated groups were analysed by Student's paired *t*-test, one-way ANOVA or two-way ANOVA. All results were analysed in GraphPad PRISM v. 4.00 for Windows (GraphPad Software Inc.).

Results

Characterisation of receptor-mediated cAMP responses in L6 cells

The general β-AR agonist (−)-isoprenaline significantly increased cAMP levels in a concentration-dependent manner (Figure 1a), and this effect was inhibited by the selective β_2-AR antagonist ICI 118551 (100 nM) (two-way ANOVA, $P<0.0001$, $n=4$). The selective β_1-AR antagonist, CGP 20712A (100 nM), failed to inhibit the response to isoprenaline, showing that isoprenaline-stimulated increases in cAMP were mediated by β_2- and not β_1-ARs. The selective β_2-AR agonist zinterol, previously shown to increase GU in L6 cells (Nevzorova *et al*., 2002), also significantly increased levels of cAMP in a concentration-dependent manner (one-way ANOVA, $P<0.0001$, $n=4$; Figure 1a). Zinterol-stimulated cAMP accumulation was abolished by ICI 118551 (from 100 to 16.8 ± 1.1%, Student's *t*-test, $P<0.05$, $n=3$). Insulin failed to increase cAMP levels above basal (one-way ANOVA, $P=1.0$, $n=4$; Figure 1b).

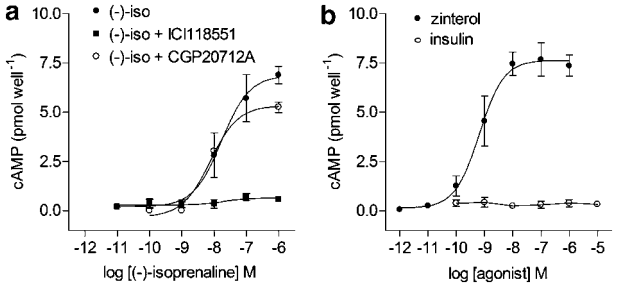

Figure 1 cAMP accumulation in L6 myotubes following exposure to insulin, or the β-AR agonists (−)-isoprenaline or zinterol. In (a) the (−)-isoprenaline CR curve is markedly shifted to the right by the β_2-AR antagonist ICI 118551 (100 nM) (two-way ANOVA, $P<0.0001$, $n=4$), but is not affected by the β_1-AR antagonist CGP 20712A (100 nM) ($P=0.3$, $n=2$). In (b) the β_2-AR agonist zinterol significantly increased cAMP levels in a concentration-dependent manner (one-way ANOVA, $P<0.0001$, $n=4$), whereas insulin did not significantly affect cAMP levels ($P=1.0$, $n=4$).

GU was significantly increased by insulin (% max increase 272.3 ± 39.1, Student's *t*-test, $P<0.01$, $n=6$) or zinterol (190.2 ± 25.5, $P<0.05$, $n=6$) (Figure 2a), and also by the direct Gs activator cholera toxin (141.8 ± 2.5, $P<0.01$, $n=3$) and the cell-permeable cAMP analogues, 8-bromo-cAMP (8Br-cAMP) (196.8 ± 13.5, $n=4$) and dibutyryl cAMP (dbcAMP) (196.4 ± 17.3, $n=4$), although the concentrations required to produce these effects were very high (1 mM) (Figure 2b). The cell-permeable adenylate cyclase inhibitor ddA (50 μM) partially inhibited GU-stimulated by zinterol (from 217 ± 26.3 to 176.1 ± 20.4; Student's *t*-test, $P=0.02$, $n=6$) but not insulin (from 162.4 ± 18.8 to 175.1 ± 6.0, $P=0.59$, $n=2$) (Figure 2c), and significantly inhibited the cAMP levels in response to zinterol (100 nM) (from 109.7 + 35.0 to 21.6 + 4.5 pmol well^{-1}; Student's *t*-test, $P<0.05$, $n=5$) (Figure 2d), or the direct adenylate cyclase activator, forskolin (10 μM) (from 230.1 ± 58.0 to 107.2 ± 26.3 pmol well^{-1}; Student's *t*-test, $P<0.05$, $n=3$) (Figure 2e).

The selective inhibitor of phosphodiesterase PDE4, rolipram (10 μM), sensitised the GU response to zinterol, resulting in higher responses to lower concentrations of the agonists (Figure 3a), but had no significant effect on insulin responses

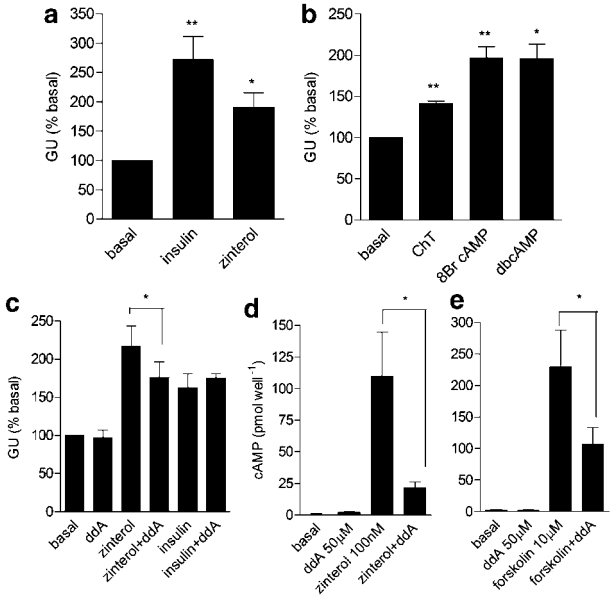

Figure 2 [^{3}H]-2-DG uptake (GU) in L6 myotubes was increased (a) by insulin (10 μM) (Student's *t*-test, $P<0.01$, $n=6$) or zinterol (100 nM) ($P<0.05$, $n=6$), as well as (b) by the direct Gs activator, cholera toxin (ChT, 1 μg ml^{-1}) ($P<0.01$, $n=3$) and the cell-permeable cAMP analogues, 8-Br-cAMP (1 mM) ($P<0.01$, $n=4$) or dbcAMP (1 mM) ($P<0.05$, $n=4$). In (c) the adenylate cyclase inhibitor, ddA (50 μM) partially inhibited GU stimulated by zinterol ($P=0.02$, $n=6$), but had no significant effect on either basal ($P=0.79$, $n=5$) or insulin (1 μM)-stimulated GU ($P=0.59$, $n=2$). In (d and e) cAMP levels in response to zinterol (100 nM) or forskolin (10 μM) were significantly inhibited by ddA ($P<0.05$, $n=3$–5).

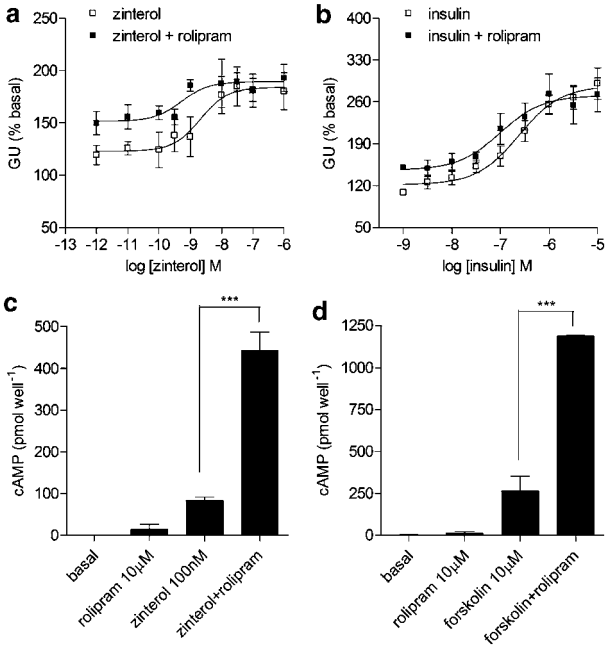

Figure 3 The selective PDE4 inhibitor, rolipram (10 μM), (a) enhanced the [^{3}H]-2-DG (GU) response to zinterol, causing higher responses at low concentrations of zinterol (two-way ANOVA and Bonferroni post-test, $P<0.01$, $n=4$). pEC_{50} values in the absence (8.7 ± 0.5) or presence (9.3 ± 0.5) of rolipram was not significantly different (Student's t-test, $P=0.4$, $n=4$). In (b) rolipram had no significant effect on insulin responses (two-way ANOVA, $P=0.06$, $n=4$). In (c) and (d) cAMP accumulation in response to zinterol (100 nM) or forskolin (10 μM) was significantly augmented by the presence of rolipram (10 μM), (Student' t-test, $P<0.001$, $n=3$–5).

(Figure 3b). The effect appears to be mainly due to an increase in basal GU with rolipram, which was increased by $35.3\pm12.6\%$ (Student's t-test, $P<0.05$, $n=5$). In addition, cAMP accumulation in response to zinterol (100 nM) (Student's t-test, $P<0.01$, $n=4$) or forskolin (10 μM) ($P<0.01$, $n=3$) was significantly increased by the presence of rolipram (Figure 3c and d). Rolipram had no significant effect on basal levels of cAMP ($P=0.5$, Student's t-test, $n=3$) (Figure 3c and d).

Effect of PKA inhibition on zinterol- or insulin-stimulated GU

The selective PKA inhibitor 4CM (60 and 100 nM) failed to inhibit the response to zinterol, but shifted the concentration–response (CR) curve to the left, at concentrations of both 60 nM (two-way ANOVA, $P<0.01$, $n=5$) or 100 nM (two-way ANOVA, $P<0.001$, $n=5$) (Figure 4a). The pEC_{50} values of zinterol were significantly increased from 7.5 ± 0.2 in the absence of 4CM to 8.6 ± 0.3 and 8.8 ± 0.2 in the presence of 4CM (60 and 100 nM respectively) (one-way ANOVA, $P<0.0001$, $n=5$). 4CM (60 or 100 nM) had no significant effect on the insulin CR curve (two-way ANOVA, $n=3$, $P=0.2$ and 0.4, respectively), or on the pEC_{50} values (one-way ANOVA, $P=1.0$, $n=3$), which were 6.9 ± 0.3 in the absence of 4CM, and 6.9 ± 0.3 and 7.0 ± 0.3 in the presence of 4CM (60 and 100 nM, respectively) (Figure 4b), demonstrating that the effect of 4CM on responses to zinterol did not result from a nonspecific sensitising action. However, 4CM (100 nM) inhibited zinterol-stimulated phosphorylation of ACC at Ser79 (Figure 4c), a conversion known to be dependent on

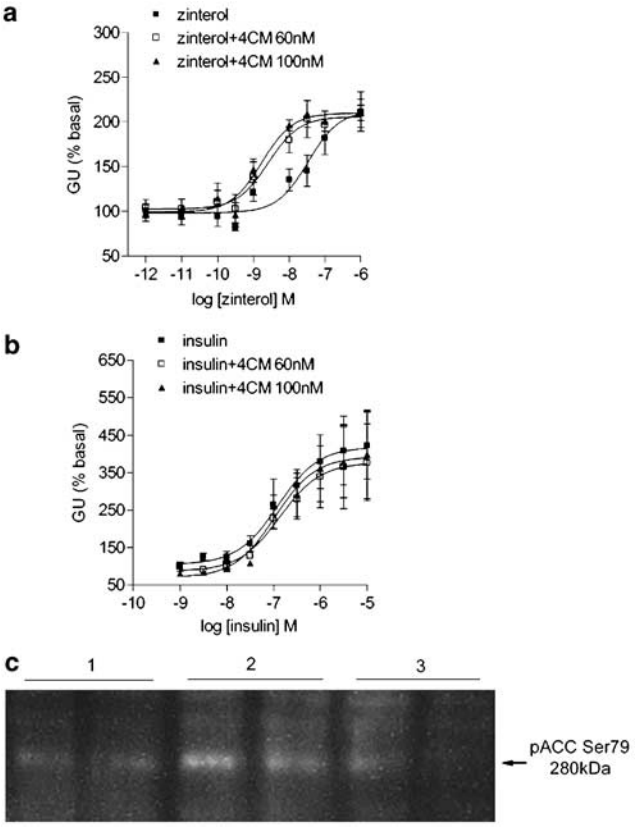

Figure 4 The selective PKA inhibitor, 4CM, shifted the [^{3}H]-2-DG (GU) CR curve to zinterol (a) to the left at 60 nM (two-way ANOVA, $P<0.01$, $n=5$) and 100 nM ($P<0.001$, $n=5$). pEC_{50} values for zinterol were increased from 7.5 ± 0.2 to 8.6 ± 0.3 and 8.8 ± 0.2 in the presence of 4CM at 60 nM and 100 nM, respectively (one-way ANOVA, $P<0.0001$, $n=5$). In (b) 4CM had no significant effect on the insulin CR curve (two-way ANOVA, $P=0.2$ and $P=0.4$, $n=3$) or pEC_{50} values (one-way ANOVA, $P=1.0$, $n=3$). In (c) inhibition of zinterol-induced phosphorylation of acetyl Co-A carboxylase (ACC) at Ser79 by 4CM demonstrating that PKA was inhibited by the concentrations used. The blot was obtained using protein from L6 myotubes and shows: (1) pACC in unstimulated cells, (2) pACC in cells treated with zinterol (100 nM), (3) pACC in cells treated with zinterol + 4CM (100 nM).

PKA activation in L6 cells (D. Hutchinson, personal communication). This demonstrates that 4CM at the concentrations used did inhibit PKA activation. In addition, 4CM partially inhibited 8Br-cAMP-stimulated GU (data not shown).

Effect of pertussis toxin pretreatment on GU

Pretreatment with the Gi/Go inhibitor, PTX (100 ng ml^{-1}, overnight) partially inhibited GU stimulated by zinterol (Figure 5) (from 190.1 ± 7.5 to 155.7 ± 7.0, two-way ANOVA, $P<0.0001$, $n=6$), but also slightly inhibited the response to insulin (from 271.2 ± 15.2 to 232.2 ± 12.1, two-way ANOVA, $P<0.05$, $n=5$) (Figure 5a and b). The GU response to dbcAMP or 8Br-cAMP was not significantly affected by PTX pretreatment ($P=0.5$, $n=2$–4) (Figure 5c and d).

Effect of PI3K inhibition on GU

The inhibitor of PI3K, LY294002 (1 μM) significantly inhibited GU in response to insulin (from 174.9 ± 5.9 to $142.7\pm2.7\%$,

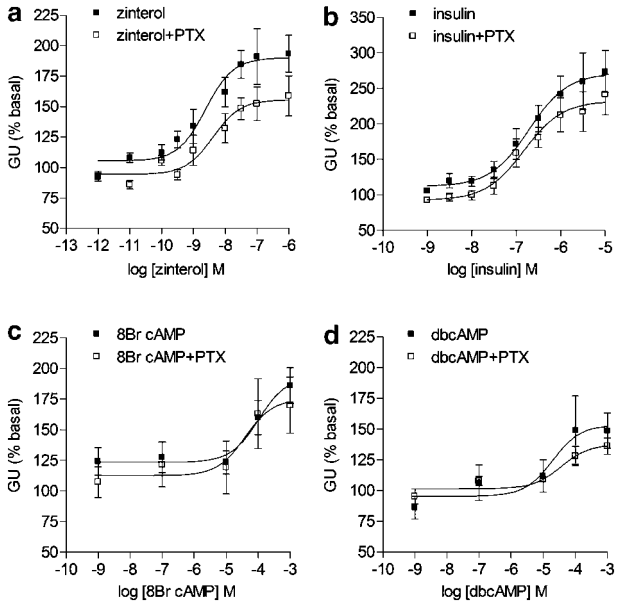

Figure 5 Zinterol-stimulated [^{3}H]-2-DG uptake (GU) (a) was significantly reduced in L6 cells treated with PTX (100 ng ml^{-1} for 16 h), ($P<0.0001$, $n=6$). PTX pretreatment also inhibited insulin-stimulated GU (b) ($P<0.05$, $n=5$) but had no inhibitory effect on GU responses to either 8-bromo cyclic AMP (c) ($P=0.5$, $n=5$) or dibutyryl cyclic AMP (d) ($P=0.5$, $n=3$). Statistical significance was tested using two-way ANOVA.

Student's *t*-test, $P<0.01$, $n=4$) and also zinterol (from 166.9±7.6 to 141.1±8.1%, $P<0.001$, $n=5$), but not that to 8Br-cAMP (144.6±9.8 to 143.9±8.6%, $P=0.9$, $n=5$) (Figure 6a), suggesting that PI3K was involved in the insulin or zinterol pathway, but not in the receptor-independent pathway, stimulated by 8Br-cAMP.

Phosphorylation of Akt

Akt was phosphorylated at Ser473 by insulin after 5 min, but not zinterol even after 3 h of stimulation with the agonist (Figure 6b). Zinterol failed to cause phosphorylation of Akt at any time point up to 3 h (the results are not shown), suggesting that β_2-AR-mediated GU does not involve activation of Akt, believed to be an important step in the insulin pathway.

cAMP and GU time course

Time course experiments carried out to examine the GU responses to zinterol ($P<0.0001$, $n=4$) or insulin ($P<0.05$, $n=4$) showed an increase after 30 min stimulation, which continued to rise for up to 3 h (Figure 7a and b). In contrast, zinterol-stimulated cAMP accumulation was maximal after 30 min stimulation but then fell with further stimulation, indicating desensitisation of β_2-AR (Figure 7c). Forskolin-stimulated cAMP accumulation was maximal at 60 min and failed to significantly desensitise with further stimulation (Figure 7d), indicating that the decline in the response to zinterol was not due to desensitisation of adenylate cyclase. Desensitisation of the cAMP response to zinterol was not reversed by the PKA inhibitor, 4CM (two-way ANOVA, $P=0.8$, $n=3$), or by pretreatment with PTX (two-way ANOVA, $P<0.05$, $n=2$) (Figure 8a and b).

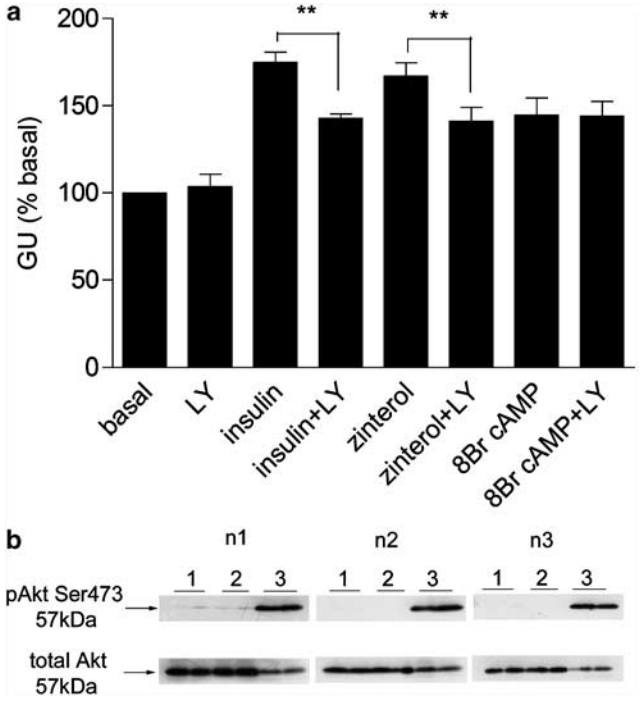

Figure 6 In (a) [^{3}H]-2-DG uptake (GU) in response to insulin (1 μM) or zinterol (1 μM) was significantly inhibited by the presence of PI3K inhibitor LY294002 (1 μM) (Student's *t*-test, $P=0.01$ and $P<0.001$, $n=4$–5), whereas the response to 8Br-cAMP (1 mM) was not affected by LY294002 ($P=0.9$, $n=5$). In (b) Western blot analysis showed that phosphorylation of Akt at Ser473 occurred in response to insulin (10 μM; 5 min stimulation) but not zinterol (100 nM; 3 h stimulation). Total Akt was detected in all samples. The labels are: basal (1), zinterol (2) and insulin (3). Blots from three separate experiments are shown.

Discussion

The findings presented here confirm that insulin-independent stimulation of GU can be achieved by activation of Gs-coupled β_2-AR (Nevzorova *et al.*, 2002), as well as by mechanisms not involving the receptor, such as direct activation of Gs by cholera toxin, or stimulation of cells using cell-permeable cAMP analogues. However, it appears that the signalling mechanisms involved in the receptor-dependent or receptor-independent pathways have distinct characteristics.

Since the β_2-AR is a Gs-coupled receptor (Strosberg, 1993), that utilises the cAMP/PKA (Walsh *et al.*, 1968) pathway to produce most of its metabolic effects, studies were carried out to demonstrate that the β_2-AR in L6 myotubes was functionally coupled to cAMP accumulation. In L6 myotubes, cAMP levels were increased in a concentration-dependent manner by incubation with either (−)-isoprenaline or zinterol, and in addition, (−)-isoprenaline- and zinterol-induced cAMP accumulation was inhibited by the selective β_2-AR antagonist ICI 118551 (Roberts & Summers, 1998), but not by the selective β_1-AR antagonist CGP 20712A (Kaumann & Lynham, 1997). These results show that, in L6 myotubes, cAMP levels are increased by β_2-AR stimulation, and that β_1-ARs, although expressed at low levels in these cells (Nevzorova *et al.*, 2002), do not contribute significantly to the increases in cAMP levels produced by (−)-isoprenaline. Furthermore, insulin had no effect on cAMP levels. The increase in cAMP levels that occurred following stimulation of

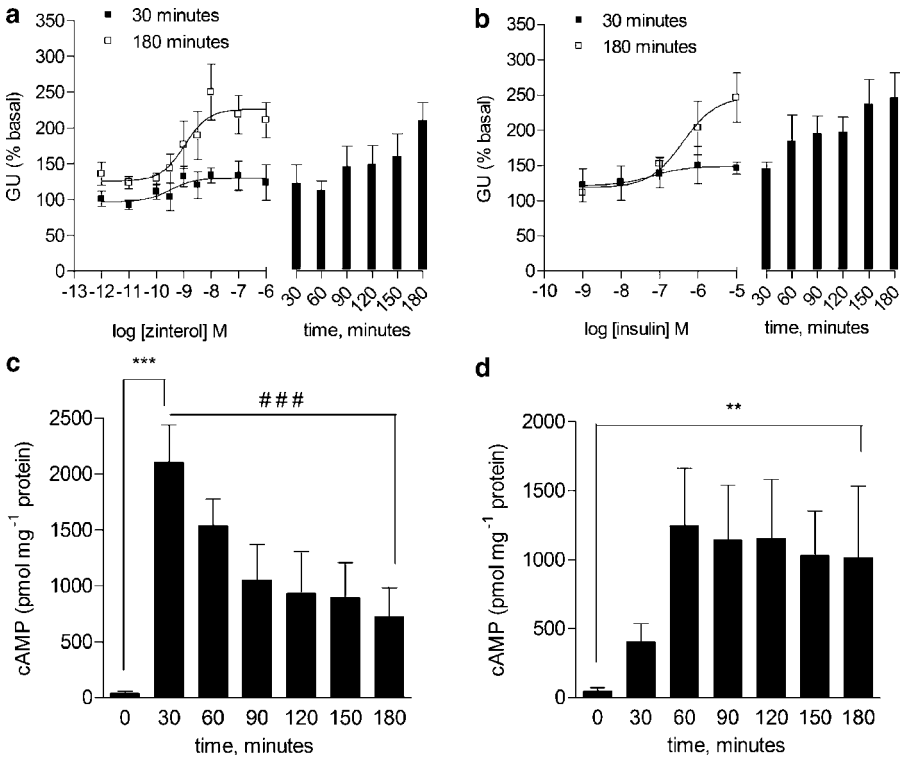

Figure 7 CR curves for (a) zinterol (two-way ANOVA, $P<0.0001$, $n=4$) and (b) insulin ($P<0.05$, $n=4$) showing [^{3}H]-2-DG uptake (GU) responses at 30 and 180 min, together with responses to (a) zinterol (1 μM) or (b) insulin (10 μM) at different time points showing the progressive increase in GU with time. In contrast, (c) zinterol (100 nM) produced a marked rise in cAMP accumulation after 30 min (Student's *t*-test, $^{***}P<0.0001$, $n=4$) but then a progressive decrease at times up to 180 min (one-way ANOVA, $^{\#\#\#}P<0.0001$, $n=4$). The desensitisation of the cAMP response does not result from a desensitisation of adenylate cyclase since forskolin-stimulated cAMP accumulation (d) was (one-way ANOVA, $^{**}P<0.01$, $n=3$) maximal by 60 min, and was maintained up to 180 min.

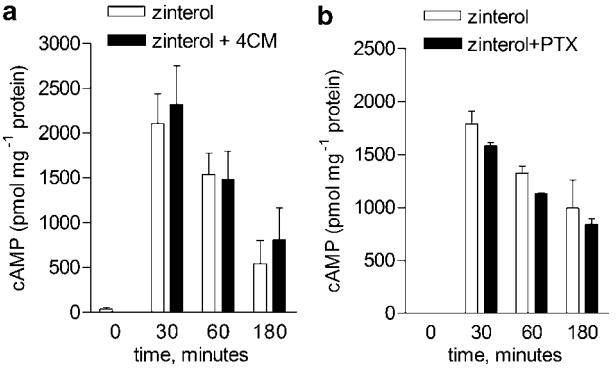

Figure 8 cAMP accumulation in response to zinterol in the presence or absence of (a) the PKA inhibitor, 4CM (100 nM), at 30, 60 and 180 min. 4CM did not affect zinterol-stimulated cAMP accumulation, or the desensitisation associated with continuous receptor stimulation (two-way ANOVA, $P=0.8$, $n=3$) suggesting that desensitisation did not follow PKA phosphorylation of the β_2-AR. In (b) the cAMP time course in response to zinterol following pretreatment with pertussis toxin (PTX, 100 ng ml^{-1}; 16 h). Zinterol-mediated increases in cAMP were significantly lower after PTX pretreatment (two-way ANOVA, $P<0.05$, $n=2$).

the β_2-AR was inhibited by the selective adenylate cyclase inhibitor ddA, which also inhibited forskolin-stimulated cAMP accumulation, indicating that the production of cAMP by β_2-AR stimulation occurred through activation of adenylate cyclase.

cAMP phosphodiesterases (PDE) break down cAMP (Fimia & Sassone-Corsi, 2001), but the cAMP-specific PDE4 isoform is also involved in the regulation of β_2-AR signalling, by a mechanism facilitated by interaction with β-arrestins (Bolger *et al*., 2003; Brunton, 2003; Conti *et al*., 2003). The β-arrestins recruit PDEs to agonist-activated receptors so targetting cAMP breakdown to areas of localised PKA activity at the cell membrane (Perry *et al*., 2002). Thus, PDEs involved in desensitisation of β_2-AR alter signalling by degradation of cAMP and by inhibition of the receptor interaction with Gs (Perry *et al*., 2002). In the present study the selective PDE4 inhibitor, rolipram (Barad *et al*., 1998), augmented cAMP accumulation in response to zinterol or forskolin, and sensitised the GU responses to zinterol, suggesting that the effect of rolipram on GU resulted from higher levels of cAMP in response to zinterol. Rolipram failed to increase the maximal response to zinterol, possibly due to saturation of glucose transporters.

In most systems, cAMP elevation activates PKA (Walsh *et al*., 1968; Taylor, 1989), which phosphorylates a number of cellular proteins (Tasken & Aandahl, 2003). In the present study, the PKA inhibitor, 4CM, failed to inhibit zinterol-stimulated GU, but inhibited GU stimulated by 8Br-cAMP (data not shown). Treatment with 4-CM did, however, inhibit ACC phosphorylation by zinterol, which is PKA-dependent in L6 cells. However, surprisingly, a leftward shift was observed in the CR curve to zinterol (but not to insulin) in the presence of 4CM, suggesting that PKA may have a negative regulatory role in β_2-AR-mediated GU. Since cAMP can inhibit PI3K and Akt activation (Kim *et al*., 2001; Wang *et al*., 2001), and GLUT4-mediated GU (Lawrence *et al*., 1992; Piper *et al*.,

1993), it is possible that PKA in L6 cells has a negative regulatory role on GU. This could explain why inhibition of PKA potentiated the responses to zinterol. On the other hand, insulin had no effect on cAMP accumulation, which would explain why the PKA inhibitor failed to affect GU responses to insulin. However, the main finding of this experiment was that PKA does not appear to be directly involved in the stimulation of GU by β_2-AR.

In our previous study, the involvement of PI3K in β_2-AR-stimulated GU was suggested by the partial inhibition by wortmannin (Nevzorova *et al*., 2002). In the present study, we have confirmed this finding using another PI3K inhibitor (LY294002) that is structurally unrelated to wortmannin. PI3K is necessary for stimulation of GU by insulin (Alessi & Downes, 1998; Taha & Klip, 1999). PI3K generate phospholipids that participate in several cellular functions, including glucose metabolism, cell survival and differentiation (Tsakiridis *et al*., 1995). Although class I PI3K are mostly controlled by receptor tyrosine kinases, including the insulin receptor (Vanhaesbroeck & Waterfield, 1999), GPCRs have also been shown to utilise PI3K for their signalling, possibly through $\beta\gamma$ subunits of G proteins (Bacqueville *et al*., 2001; Brock *et al*., 2003; Wymann *et al*., 2003). For example, it has been shown that β_2-AR activation is associated with survival of cardiac myocytes in a PI3K and Akt-dependent manner (Communal *et al*., 1999; Singh *et al*., 2001; Zhu *et al*., 2001). PI3K is also involved in regulation of β_2-AR internalisation, facilitated by association with β-AR kinase-1 (Naga Prasad *et al*., 2001; 2002), and compartmentalisation of β_2-AR signalling through Gs (Jo *et al*., 2002). Furthermore, PI3K has been demonstrated to be involved in α_1-, β_1- and β_3-AR-mediated GU in brown adipocytes (Chernogubova *et al*., 2004; 2005), as well as in α_1-AR-mediated GU in L6 cells (Hutchinson & Bengtsson, 2005) and white adipocytes *in vitro* (Cheng *et al*., 2000), indicating that involvement of PI3K in GU is a general phenomenon, and is not only part of the insulin signalling, which mediates GU. Therefore, it appears that cAMP is not the only signalling mechanism involved in β_2-AR-dependent GU, and that PI3K plays a significant role in this process. However, the inhibition of insulin- and zinterol-stimulated GU by LY294004 was partial, suggesting that there is another pathway that is independent of PI3K. cAMP also appears to be a contributor to β_2-AR-mediated GU, since adenylate cyclase inhibition partially reduced the response to zinterol. In addition, it has been shown that the Cbl/TC10 pathway is necessary for insulin-stimulated GU and translocation of GLUT4 (Baumann *et al*., 2000; Chiang *et al*., 2001; Watson *et al*., 2001). Furthermore, zinterol-mediated GU was partially reduced by PTX, suggesting an involvement of Gi. Since cardiac β_2-AR activate PI3K *via* Gi (Communal *et al*., 1999; Xiao *et al*., 1999; Zhu *et al*., 2001; Jo *et al*., 2002; Hasseldine *et al*., 2003), it is likely that β_2-AR-mediated increases in GU involve PI3K activation *via* Gi.

To determine whether there was a direct role of cAMP in GU, two cell-permeable cAMP analogues, 8Br-cAMP and dbcAMP were tested. Both analogues caused increases in GU, although high concentrations were required to achieve significant responses, comparable with those to zinterol. The difference may be due to localisation of receptor-stimulated cAMP accumulation within specific microdomains (Kuschel *et al*., 2000; Steinberg & Brunton, 2001; Brunton, 2003), whereas the cAMP analogues are likely to be spread throughout the cell so that high concentrations are required to achieve the right concentrations at particular locations of the cell. The direct activator of Gs protein, cholera toxin, also significantly increased GU. An adenylate cyclase inhibitor inhibited cAMP accumulation to zinterol or forskolin, but only partially inhibited GU to zinterol, suggesting a role for cAMP in β_2-AR-mediated GU. These experiments indicate that direct activation of Gs produces only a modest GU response. Likewise, while cAMP analogues can stimulate GU, only part of the receptor-mediated response is inhibited by an adenylate cyclase inhibitor. To explore cAMP-independent GU, we first tested the importance of Gi/Go proteins by pretreating L6 cells with PTX. PTX inhibited (18%) zinterol-stimulated GU, but also caused a significant reduction (14%) in the response to insulin. PTX has been previously reported to partially inhibit insulin-stimulated GU in adipocytes and soleus muscle (Kanoh *et al*., 2000). The insulin receptor tyrosine kinase is thought to phosphorylate Giα2, releasing G$\beta\gamma$ subunits that in turn activate PI3K. Also, transgenic mice expressing a constitutively active Q205L Giα2 display increased basal and insulin-stimulated GU in skeletal muscle (Song *et al*., 2001). In contrast to β_2-AR or insulin-mediated stimulation of GU, direct stimulation of GU by the cAMP analogue, 8Br-cAMP, was not inhibited by either LY294002 or PTX, showing that PI3K or Gi were not involved and indicating a different mechanism from that utilised by β_2-AR.

In conclusion, activation of β_2-AR increases cAMP in L6 myotubes, and although increases in GU could be produced by membrane-permeant cAMP analogues, zinterol-stimulated GU was only partially inhibited by inhibition of adenylate cyclase. The results suggest that β_2-AR-mediated increases in GU involve both cAMP and PI3K. The mechanism of activation of PI3K by β_2-AR probably involves signalling through Gi and/or G$\beta\gamma$ subunits that are activated following desensitisation. Furthermore, receptor-independent GU stimulation by cAMP analogues does not involve Gi or PI3K and therefore appears to be distinct from that utilised by the β_2-AR. The mechanism of cAMP analogue-activated GU may involve GLUT1 synthesis and/or activation of glucose transporters already present in the plasma membrane.

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