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ARTICLE

β_2-Adrenergic activation increases glycogen synthesis in L6 skeletal muscle cells through a signalling pathway independent of cyclic AMP

D. L. Yamamoto · D. S. Hutchinson · T. Bengtsson

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Abstract
Aims/hypothesis In skeletal muscle, the storage of glycogen by insulin is regulated by glycogen synthase, which is regulated by glycogen synthase kinase 3 (GSK3). Here we examined whether adrenergic receptor activation, which can increase glucose uptake, regulates glycogen synthesis in L6 skeletal muscle cells.
Methods We used L6 cells and measured glycogen synthesis (as incorporation of D-[U-^{14}C]glucose into glycogen) and GSK3 phosphorylation following adrenergic activation.
Results Insulin (negative logarithm of median effective concentration [pEC_{50}] 8.2±0.3) and the β-adrenergic agonist isoprenaline (pEC_{50} 7.5±0.3) induced a twofold increase in glycogen synthesis in a concentration-dependent manner. The α_1-adrenergic agonist cirazoline and α_2-adrenergic agonist clonidine had no effect. Both insulin and isoprenaline phosphorylated GSK3. The β-adrenergic effect on glycogen synthesis is mediated by β_2-adrenoceptors and not β_1-/β_3-adrenoceptors, and was not mimicked by 8-bromo-cyclic AMP or cholera toxin, and also was insensitive to pertussis toxin, indicating no involvement of cyclic AMP or inhibitory G-protein (G_i) signalling in the β_2-adrenergic effect on glycogen synthesis. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increased glycogen synthesis 2.5-fold and phosphorylated GSK3 fourfold. Inhibition of protein kinase C (PKC) isoforms with 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrollo(3,4-*c*)-carbazole (Gö6976; inhibits conventional and novel PKCs) or 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide (Gö6983; inhibits conventional, novel and atypical PKCs) inhibited the stimulatory TPA effect, but did not significantly inhibit glycogen synthesis mediated by insulin or isoprenaline. Inhibition of phosphatidylinositol 3-kinase (PI3K) with wortmannin inhibited the effects of insulin and isoprenaline on glycogen synthesis.
Conclusions/interpretation These results demonstrate that in L6 skeletal muscle cells adrenergic stimulation through β_2-adrenoceptors, but not involving cyclic AMP or G_i, activates a PI3K pathway that stimulates glycogen synthesis through GSK3.

Keywords β_2-Adrenoceptor · AMPK · Cyclic AMP · Glycogen · GSK3 · L6 · PI3K · Skeletal muscle

Abbreviations

AICAR	5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside
AMPK	AMP activated protein kinase
B_{max}	maximal binding
G_i	inhibitory G-protein
G_q	$G_q/_{11}$ type G-protein
G_s	stimulatory G-protein
GSK3	glycogen synthase kinase 3
K_D	concentration of ligand required to occupy 50% of the binding sites
pEC_{50}	negative logarithm of median effective concentration
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A

The first two authors contributed equally to this paper.

D. L. Yamamoto · D. S. Hutchinson · T. Bengtsson (✉)
Department of Physiology, The Wenner-Gren Institute,
Arrhenius Laboratories F3, Stockholm University,
SE 10691 Stockholm, Sweden
e-mail: Tore.Bengtsson@zoofys.su.se

D. S. Hutchinson
Department of Pharmacology, Monash University,
Clayton, VIC, Australia

PKC	protein kinase C
pK_i	negative logarithm of the affinity constant K_i
TPA	12-*O*-tetradecanoylphorbol-13-acetate

Introduction

Skeletal muscle contributes to much of the glucose utilisation in the body. Glucose is a primary source of fuel for skeletal muscle cells and enters the cell via glucose transporters. Upon entering the cell, glucose is phosphorylated to glucose 6-phosphate, which has two alternative fates, either to be broken down to produce energy (glycolysis), or to be stored as glycogen (glycogen synthesis). Glycogen synthesis is mediated by glycogen synthase, which is phosphorylated by glycogen synthase kinase 3 (GSK3). Both GSK3 and glycogen synthase are inactivated by phosphorylation. Phosphorylation of GSK3 leads to decreased phosphorylation of glycogen synthase, thereby increasing the activity of glycogen synthase and the rate of glycogen synthesis.

Insulin increases glycogen synthesis twofold in rat skeletal muscle, an effect dependent on phosphatidylinositol 3-kinase (PI3K) [1] and phosphorylation of GSK3 [2]. Exercise-mediated increase of glycogen synthase activity is seen in response to treadmill exercise in vivo and following electrically induced contraction in situ [3]. Regulation of glycogen synthesis by exercise is independent of PI3K [4]. Glycogen synthase activity is also regulated by AMP-activated protein kinase (AMPK) [5], calmodulin-dependent protein kinase [6], protein kinase A (PKA) [7] and protein kinase C (PKC) [8].

We were interested in investigating the involvement of adrenergic stimulation on glycogen synthesis. Adrenaline is a circulatory hormone, which does not normally reach high concentrations and has effects on many tissues, while noradrenaline is a neurotransmitter, which is locally released and can reach relatively high concentrations locally and have local effects. Adrenaline has inhibitory effects on glycogen synthesis in vivo [9]. However, adrenaline and noradrenaline inhibit insulin release in vivo, a subject that has been extensively reviewed [10]. Adrenaline also has inhibitory effects on glycogen synthesis induced by contraction. Blockade of stimulatory G-protein (G_s)-coupled β-adrenoceptors by propranolol abolishes this inhibition [11].

Adrenoceptors are G-protein-coupled receptors, of which there are three major groups: α_1-, α_2- and β-adrenoceptors (all further subdivided into three subtypes per group) with different affinity for the endogenous ligands noradrenaline and adrenaline. α_1-Adrenoceptors couple to $G_q/_{11}$ type G-protein (G_q) and signal via phospholipase C, Ca^{2+} and diacylglycerol. α_2-Adrenoceptors couple to inhibitory G-protein (G_i) to negatively regulate adenylate cyclase and cyclic AMP production, while β-adrenoceptors couple to G_s to positively regulate adenylate cyclase and generate cyclic AMP. Although most well-described β-adrenergic signalling involves cyclic AMP, atypical signalling also occurs, in which the G-protein βγ subunits may initiate crosstalk with other signalling pathways in addition to cyclic AMP [12]. An example of this is the way G-protein-coupled receptors can use PI3K for signalling, possibly by βγ subunits released after G-protein activation [13]; moreover, some β_2-adrenoceptor responses have been shown to be mediated via PI3K [14, 15].

There are many examples of involvement of G-protein-coupled receptors in the regulation of factors involved in glycogen synthesis, e.g. the 5-hydroxytryptamine receptor in skeletal muscle [16], the G_i-coupled opioid receptor in rat heart [17], the G_i-coupled cannabioid 1 receptor in C6 glioma cells [18], and the dopamine receptor in the striatum [19]. In rat epididymal fat cells, β-adrenoceptors, but not α-adrenoceptors increase glycogen synthesis [20]. In the same cells, β-adrenoceptors decrease GSK3 activity leading to increased glycogen, a process that cannot be mimicked by activation of cyclic AMP signalling [21]. And finally, β-adrenergic-mediated GSK3 phosphorylation is also seen in a fibroblast like cell line [7].

We have previously shown that an increase of glucose uptake occurs through α_1- and β_2-adrenoceptors in L6 skeletal muscle cells, and primarily through β_3-adrenoceptors in brown adipocytes [22–26]. It is not known whether activation of adrenergic receptors leads to glucose uptake for use in glycolysis or whether glucose is taken up to be stored as glycogen. The aim of this study was to examine if activation of adrenoceptors is capable of phosphorylating GSK3 and thereby increasing glycogen synthesis in L6 skeletal muscle cells. We also explored the adrenergic signalling pathways leading to glycogen synthesis.

Materials and methods

Materials and reagents All drugs were purchased as indicated here: insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark); D-[U-^{14}C]glucose (specific activity 8.5–13.7 GBq/mmol; Amersham Biosciences, Arlington Heights, IL, USA); A23187, cirazoline, isoprenaline, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), clonidine, wortmannin, pertussis toxin, cholera toxin, 8-bromo-cyclic AMP, 3-(2-ethylphenoxy)-1-[(1,*S*)-1,2,3,4-tetrahydronapth-1-ylamino]-2*S*-2-propanol oxalate (SR59230A) (Sigma-Aldrich, St Louis, MO, USA); 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR; Toronto Research Chemicals, North York, ON,

Canada); 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole, 12-(2-cyanoethyl)-6,7,12, 13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrollo [3,4-*c*]-carbazole (Gö6976), 2-[1-(3-dimethylamino-propyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide (Gö6983), 4-cyano-3-methylisoquinoline (Calbiochem, La Jolla, CA, USA). All cell culture media were purchased from (HyClone, Logan, UT, USA) and supplements from Life Technologies, Paisley, UK. GSK3β and phospho-GSK3α/β (Ser21/9) antibodies were obtained from (Cell Signaling Technology, Beverly, MA, USA). Radio-labelled 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1, 3-dihydro-2*H*-benzimidazol-2-one hydrochloride ([^{3}H] CGP12177A; specific activity 1.11–2.22 TBq/mmol) was from Amersham Biosciences. (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol (ICI118551) was from Imperial Chemical Industries (Wilmslow, Cheshire, UK), and (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy] propyl] amino]ethoxy]-benzamide methanesulfonate salt (CGP20712A) was from Ciba-Geigy, Basel, Switzerland.

Cell culture Rat L6 skeletal muscle cells were grown as described earlier [25]. To differentiate, cells were allowed to reach confluence and the media changed to medium containing 2% (vol/vol) fetal bovine serum for 7 days, with medium changes every second day. Experiments were restricted to cells from passages 2 to 15, and undifferentiated cells were not allowed to grow to more than 60 to 70% confluence.

Whole cell binding L6 cells were grown in 48-well plates and cells were used for experiments following 7 days of differentiation. All experiments were performed at 37°C in a total volume of 100 μl for 1 h. For saturation binding experiments, cells were incubated with either [^{3}H]CGP12177A (20–640 pmol/l) alone or in the presence of 1 μmol/l (−)-propranolol to define non-specific binding. Competition experiments were performed using a range of concentrations of unlabelled drug using 100 pmol/l [^{3}H]CGP12177A and non-specific binding defined by 1 μmol/l (−)-propranolol. Reactions were terminated by rapid aspiration and cells washed twice with ice-cold PBS. Cells were digested (0.2 mol/l NaOH, 50°C, 1 h), contents of the wells transferred to scintillation vials, and radioactivity measured. Protein was determined [27] using BSA as a standard. Data were analysed using non-linear curve fitting (Graph Pad Prism, San Diego, CA, USA) and a one-site model to determine the concentration of ligand required to occupy 50% of the binding sites (K_D) and maximal binding (B_{max}) values, or a one-site competition fit to determine the negative logarithm of the affinity constant K_i (pK_i) values as appropriate.

Glycogen synthesis Glycogen synthesis was assessed by the incorporation of D-[U-^{14}C]glucose into glycogen. L6 cells were serum-starved on day 6 and on day 7, cells were washed twice with warm PBS, and glucose-free DMEM was added with drugs as indicated for 30 min, before adding 37 kBq of D-[U-^{14}C]glucose for 90 min. Cells were then quickly washed with ice-cold PBS and lysed in 0.5 ml of 30% potassium hydroxide for 10 min. Samples were boiled for 20 min and glycogen precipitated with ice-cold ethanol. Samples were filtered through GF/B filters and dried filters transferred to scintillation vials for radioactivity counting. Where inhibitors were used, the time indicated in the results represents the time that cells were preincubated with the inhibitors before addition of the agonists.

Immunoblotting Cells were serum-starved overnight before each experiment on day 7, and were exposed to drugs for the times and concentrations indicated in the figures. Extraction of cells and subsequent western blotting were performed as previously described [25], except that samples were electro-transferred to polyvinylidene difluoride membranes (Hybond-P, pore size 0.45 μm; Amersham Biosciences). The primary antibodies used were GSK3 and phospho-GSK3 antibody diluted 1:1,000, which were detected using a secondary antibody (horseradish peroxidase-linked antirabbit IgG) diluted 1:2,000 and enhanced chemiluminescence (Amersham Biosciences). The blots were exposed to enhanced chemiluminescence films (Hyperfilm; GE Healthcare Europe, Uppsala, Sweden) and quantified on a densitometer (Molecular Dynamics, Sunnyvale, CA USA) using image quant NT software (Molecular Dynamics). Results are expressed as the ratio between phosphorylated and total protein, with the ratio normalised in each experiment to that of control samples. All experiments were performed singly or in duplicate with *n* referring to the number of independent experiments performed.

Analysis of results All results are expressed as mean±SEM of *n* experiments. Data were analysed (Graph Pad Prism) using non-linear curve fitting to obtain the negative logarithm of median effective concentration (pEC_{50}) values. Statistical analysis was determined by paired *t* test where $p<0.05$ was considered significant.

Results

β-Adrenoceptors but not α_1- or α_2-adrenoceptors increased glycogen synthesis in L6 cells Insulin increased glycogen synthesis in a concentration-dependent manner (maximal increase 195±10%, pEC_{50} 8.2±0.3; n=4). The β-adrenergic agonist isoprenaline also increased glycogen synthe-

sis in a concentration-dependent manner (maximal increase 192±8%, pEC_{50} 7.46±0.3; n=4) (Fig. 1). The α_1-adrenergic agonist cirazoline (10 μmol/l, maximal increase 114±7%; n=6) and the α_2-adrenergic agonist clonidine (10 μmol/l, maximal increase 95±9%; n=4) failed to significantly increase glycogen synthesis.

β-Adrenergically mediated glycogen synthesis via the β_2-adrenoceptor To determine which β-adrenoceptor is responsible for isoprenaline-mediated increases in glycogen synthesis, we performed saturation binding experiments. [^{3}H]CGP12177A bound a single β-adrenoceptor in a saturable manner with a K_D of 59.0±18.7 pmol/l and B_{max} of 65.5±5.6 fmol/mg protein (Fig. 2), revealing that no β_3-adrenoceptors were present (the β_3-adrenoceptor has a much weaker affinity for [^{3}H]CGP12177A than β_1/β_2-adrenoceptors [28]). Competition studies revealed pK_i values of 8.45±0.09 for the β_2-adrenoceptor antagonist ICI118551, 5.18±0.11 for the β_1-adrenoceptor antagonist CGP20712A, and 6.03±0.06 for the β_3-adrenoceptor antagonist SR59230A (Fig. 2), showing that the high-affinity site determined via saturation binding is a β_2-adrenoceptor, since the β_1- and β_3-adrenoceptor antagonists competed weakly at this site as compared with the β_2-adrenoceptor antagonist ICI118551. We treated cells with CGP20712A, ICI118551 or SR59230A (100 nmol/l) to investigate the effects on isoprenaline-mediated glycogen synthesis. Only ICI118551 significantly blocked glycogen synthesis (Fig. 3).

The β-adrenergic signalling pathway converged with the insulin signalling pathway in the regulation of glycogen synthesis To investigate whether insulin signalling and adrenergic signalling act through separate signalling pathways, we compared the effects of stimulation with isoprenaline and insulin together with the effects of treatment with isoprenaline and insulin separately. Insulin and isoprenaline both increased glycogen synthesis, but we were unable to show a significant additive effect of the two drugs (Fig. 4).

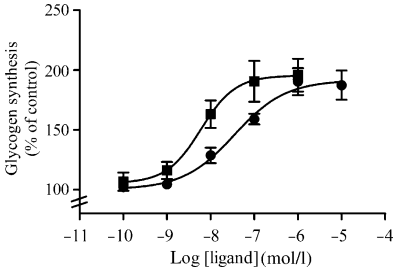

Fig. 1 Concentration–response curve for insulin (*filled squares*) or the β-adrenoceptor agonist isoprenaline (*filled circles*) on glycogen synthesis in differentiated L6 cells. Points show means and vertical lines indicate the SEM of four experiments performed in duplicate

β-Adrenoceptor activation phosphorylated GSK3 To evaluate the involvement of GSK3, we examined phosphorylation of GSK3α on the Ser21 and GSK3β on the Ser9 residues. Insulin, which increases glycogen synthesis in L6 cells, phosphorylated GSK3 3.5-fold, and was used as a positive control in assessing GSK3 phosphorylation. Isoprenaline (Fig. 5) and noradrenaline (n=2, data not shown) phosphorylated GSK3 three- to fourfold, a finding that was stable over the 2-h time period examined. Cirazoline, which like isoprenaline also increases glucose uptake [25], did not phosphorylate GSK3 at any time points examined (Fig. 5).

Glycogen synthesis was not dependent on AMPK AMPK is an energy sensor that is activated by increased AMP levels in the cell. AMPK may be involved in the regulation of systems closely linked to energy regulation, such as glucose uptake and glycogen synthesis. To investigate this, we used an AMPK activator, AICAR. Even though AICAR induces a large increase in glucose uptake [29], it did not affect GSK3 phosphorylation (Fig. 6a); there may, however, be a minor effect on glycogen synthesis (Fig. 6b).

β-Adrenoceptor activation of glycogen synthesis was independent of cyclic AMP We checked the functionality of the system by measuring the production of cyclic AMP in response to isoprenaline. β_2-Adrenoceptor activation by

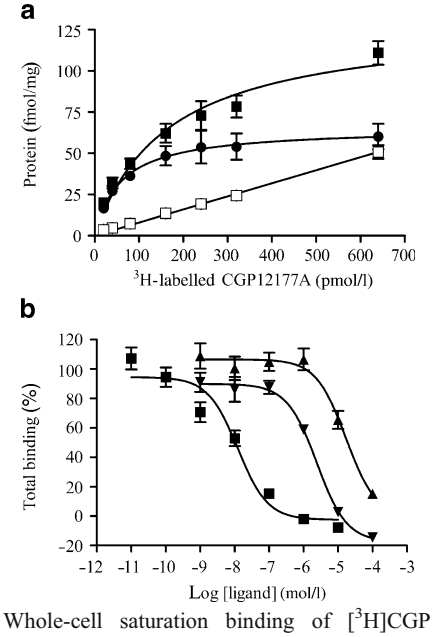

Fig. 2 **a** Whole-cell saturation binding of [^{3}H]CGP12177A to differentiated L6 skeletal muscle cells. Total, *filled squares*; specific, *circles*; non-specific, *open squares*. **b** Competition between [^{3}H]CGP12177A and CGP20712A (*triangles*), ICI118551 (*squares*) or SR59230A (*inverted triangles*) for binding sites in L6 cells. Incubations were performed for 1 h and non-specific binding defined with 1 μmol/l (−)-propranolol. Points show mean±SEM for four experiments performed in duplicate. For competition experiments results are expressed as a % of the maximum specific binding for [^{3}H]CGP12177A in each individual experiment

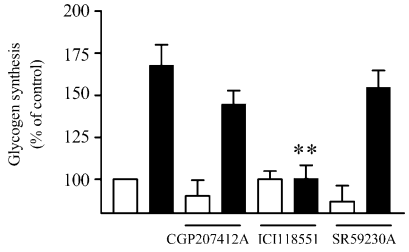

Fig. 3 Glycogen synthesis in response to isoprenaline (*filled bars*; 10 μmol/l, 2 h) in the presence or absence of the β_1-antagonist CGP20712A, the β_2-antagonist ICI118551 or the β_3-antagonist SR59230A (all antagonists were added at 300 nmol/l). The histograms are the mean±SEM of four experiments performed in duplicate. *Open bars*, control. $^{**}p<0.01$

isoprenaline increased cyclic AMP levels in L6 cells (data not shown). We have previously studied the cyclic AMP response to β_2-adrenergic activation in detail [24]. To investigate whether cyclic AMP is capable of stimulating glycogen synthesis, we used a cyclic AMP analogue, 8-bromo-cyclic AMP, and an activator of adenylate cyclase, cholera toxin. No significant effect of cholera toxin on glycogen synthesis was observed. There may have been a minor increase with 8-bromo-cyclic AMP, but not to the magnitude that would explain the isoprenaline response (Fig. 7a). Considering that the same concentrations of 8-bromo-cyclic AMP and cholera toxin increased cyclic AMP levels to a greater degree than isoprenaline [24] and yet did not significantly alter glycogen synthesis levels, we feel confident that increases in cyclic AMP levels do not increase glycogen synthesis. To investigate the involvement of PKA, we used the PKA inhibitor 4-cyano-3-methylisoquinoline ($n=3$). 4-Cyano-3-methylisoquinoline did not significantly affect glycogen synthesis in response to either insulin (increased insulin-mediated glycogen synthesis 6.4%) or isoprenaline (inhibited isoprenaline-mediated glycogen synthesis 16.6%). We also used pertussis toxin, which inhibits the inhibitory action of G_i on adenylate cyclase. Pertussis toxin did not affect insulin or isoprenaline-mediated increases in glycogen synthesis (Fig. 7b).

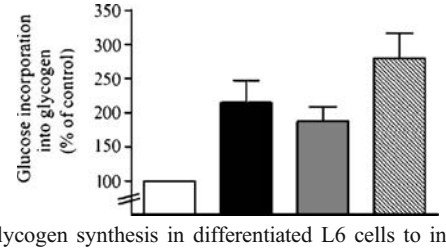

Fig. 4 Glycogen synthesis in differentiated L6 cells to insulin (*grey bars*; 1 μmol/l, 2 h) or isoprenaline (*black bars*; 10 μmol/l, 2 h) alone or in combination (*hatched bars*). The histograms are the mean±SEM of three experiments performed in duplicate. *Open bars*, control

PKC is involved in the phosphorylation of GSK3 but not in the glycogen synthesis response to insulin and isoprenaline TPA activated both conventional and novel PKCs and increased the phosphorylation of GSK3 in a time-dependent manner, although phosphorylation of GSK3 was transient in manner (Fig. 8). It also increased glycogen synthesis 2.5-fold (Fig. 8) and glucose uptake 2.5-fold [25]. The Ca^{2+} ionophore A23187 did not increase GSK3 phosphorylation or glycogen synthesis (Fig. 8) despite increasing glucose uptake [25]. To investigate the role of different isoforms of PKC, we used the PKC inhibitor Gö6976, which inhibits novel and conventional PKCs, and Gö6983, which inhibits novel, conventional and atypical PKCs. The stimulatory effect of TPA on glycogen synthesis was inhibited by both Gö6976 and Gö6983 (Fig. 8). None of the inhibitors affected insulin or isoprenaline-mediated glycogen synthesis (Fig. 9).

β-Adrenergic signalling increased glycogen synthesis via PI3K To investigate the role of PI3K, we first used the PI3K inhibitor LY294002. It significantly inhibited basal glycogen synthesis, but also inhibited the glycogen synthesis response to both insulin and isoprenaline (data not

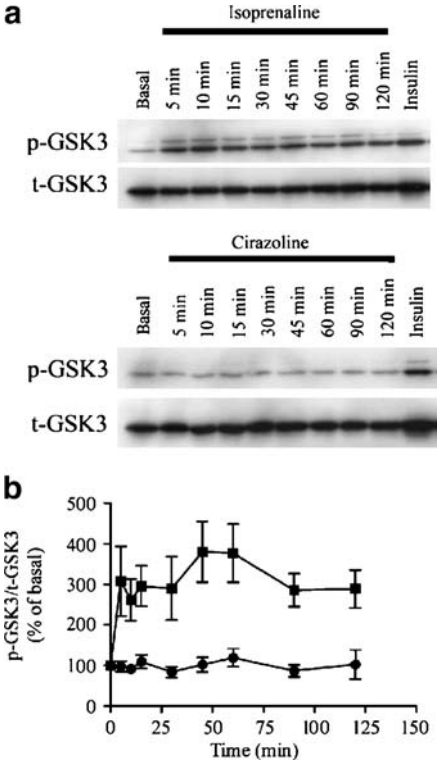

Fig. 5 Representative blots (**a**) of phosphorylated (p) (Ser9/21) and total (t) GSK3 content in differentiated L6 cells after isoprenaline (10 μmol/l) or cirazoline (10 μmol/l) treatment, showing insulin (1 μmol/l, 5 min) as a positive control. The graph (**b**) shows the ratio of phosphorylated GSK3 to total GSK3. *Points* show means and *vertical lines* indicate the SEM of six experiments performed with one replicate each. *Squares*, isoprenaline; *circles*, cirazoline

shown). We then used the PI3K inhibitor wortmannin. Our results show that inhibition of PI3K completely blocked the insulin and partially blocked the isoprenaline response (Fig. 10).

Discussion

Adrenergic effects on glucose metabolism are of great interest in skeletal muscle since the adrenergic system has a considerable influence on whole-body metabolism. Traditionally, skeletal muscle was not thought to be innervated directly by the sympathetic nervous system, but it has now been concluded that the sympathetic nervous system innervates skeletal muscle at two levels, namely in blood vessels (smooth muscle), but also directly in muscle fibres [30]. Interestingly, several studies indicate that stimulation of the sympathetic nervous system increases glucose uptake in skeletal muscle by a mechanism independent of insulin. Furthermore β-adrenergic stimulation increases glucose uptake in rodent skeletal muscle [31–33]. Activation of either α_{1A}- [25, 34] or β_2-adrenoceptors [26, 32, 33] increases glucose uptake in rodent skeletal muscle cells and skeletal muscle cell lines.

The glycogenolytic effects of adrenaline are well-known and β-adrenoceptors through cyclic AMP have been shown to decrease glycogen synthesis in response to insulin in skeletal muscle [35, 36]. In other studies β-adrenergic activation causes phosphorylation of GSK3 in cardiac myocytes [37] and epididymal fat cells [21]. We have shown before that β-adrenoceptors increase glucose uptake via cyclic AMP but also via PI3K [24, 26], and it is possible that adrenergic receptors affect glycogen content in different ways.

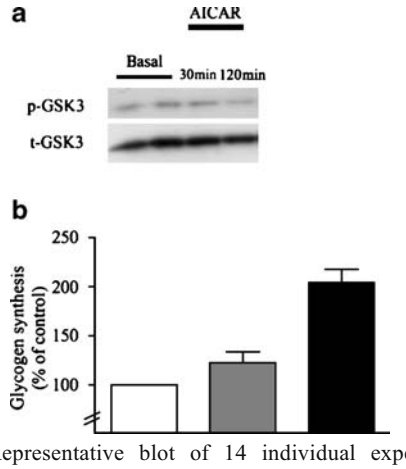

Fig. 6 **a** Representative blot of 14 individual experiments of phosphorylated (p) (Ser9/21) and total (t) GSK3 content in differentiated L6 cells after AICAR (2 mmol/l) treatment. **b** Glycogen synthesis in differentiated L6 cells in response to insulin (*black bar*; 1 μmol/l, 2 h) or AICAR (*grey bar*; 2 mmol/l, 2 h). *Open bar*, control. The histograms are the mean±SEM of four to six experiments performed in duplicate

We have seen here that G_s-coupled β-adrenoceptors induced glycogen synthesis in L6 cells. It is necessary to determine the involvement of the different β-adrenoceptors in the signalling leading to glycogen synthesis. Our results suggest that the β-adrenoceptor identified by radioligand binding fits the pharmacological profile of the β_2-adrenoceptor. Glycogen synthesis studies in the presence of antagonists specific for the different β-adrenoceptors show that the glycogen synthesis response to isoprenaline is mediated by the β_2-adrenoceptor and not by the β_1- or β_3-adrenoceptor (Fig. 3).

In contrast to the G_s-coupled β_2-adrenoceptor, the G_q-coupled α_1-adrenoceptor and the G_i-coupled α_2-adrenoceptors did not stimulate glycogen synthesis. It is interesting that both α_1- and β_2-adrenoceptors increase glucose uptake, but only the β_2-adrenoceptor induced glycogen synthesis. We have previously seen that β_2-adrenoceptors and α_1-adrenoceptors utilise different pathways in increasing glucose uptake in skeletal muscle cells, since α_1-adrenoceptors activate AMPK but β_2-adrenoceptors do not activate AMPK in these cells [29]. In these cells, stimulation of α_1-adrenoceptors thus leads to activation of AMPK and glucose uptake (determined by inhibition of α_1-adrenoceptor-mediated glucose uptake by the AMPK inhibitor compound C [29]), but no activation of glycogen synthesis. In contrast, activation of β_2-adrenoceptors does not activate AMPK, but does increase glucose uptake and phosphorylation of GSK3 and glycogen synthesis.

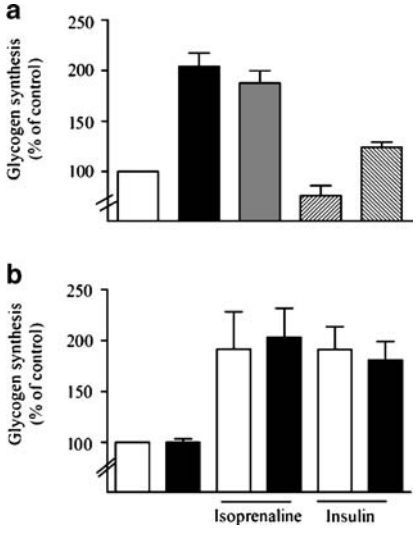

Fig. 7 **a** Glycogen synthesis in differentiated L6 cells in response to insulin (*black bars*; 1 μmol/l, 2 h), isoprenaline (*grey bars*; 10 μmol/l, 2 h), 8-bromo-cyclic AMP (hatched, *right*; 10 μmol/l, 2 h) and cholera toxin (hatched, *centre-right*; 5 μg/ml, 2 h). The histogram shows the mean±SEM of three to four experiments performed in duplicate. **b** The effect of pertussis toxin (*filled bars*; 100 ng/ml, overnight pretreatment) on glycogen synthesis in response to insulin (1 μmol/l, 2 h) and isoprenaline (10 μmol/l, 2 h). The histogram shows the mean±SEM of three experiments performed in duplicate. *Open bars*, control

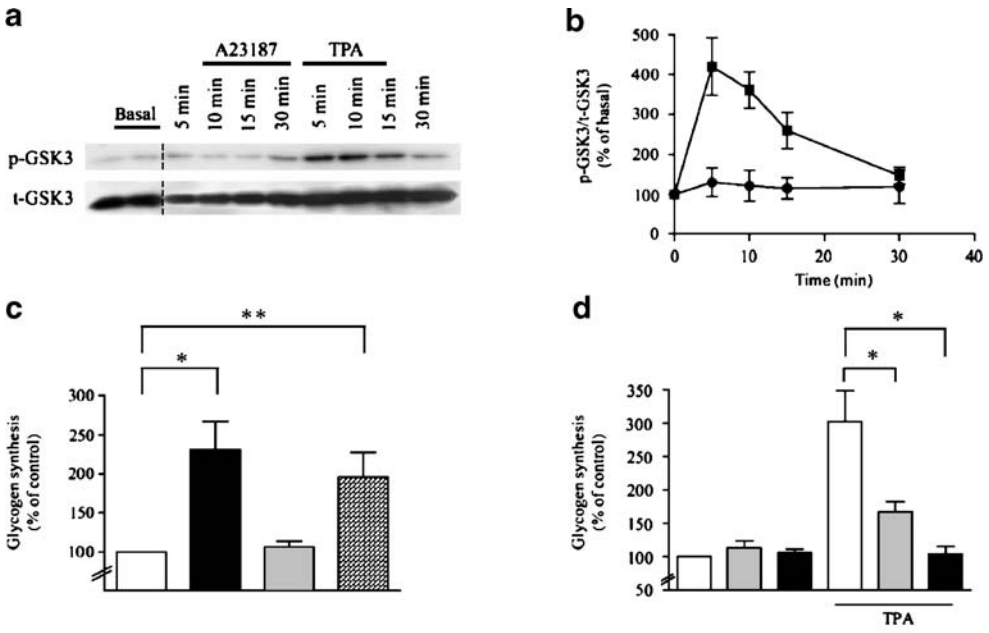

Fig. 8 **a** Representative blot of phosphorylated (p) (Ser9/21) and total (t) GSK3 content in differentiated L6 cells after TPA (100 nmol/l) or A23187 (100 nmol/l) treatment. The graph (**b**) shows the ratio of phosphorylated GSK3 to total GSK3. *Points* show means and *vertical lines* indicate the SEM of five experiments performed. *Squares*, TPA; circles, A23187. **c** Glycogen synthesis in differentiated L6 cells in response to TPA (*black bar*; 100 nmol/l, 2 h) or A23187 (*grey bar*; 100 nmol/l, 2 h) alone or in combination (*checked bar*). *Open bar*, control. The histogram shows the mean±SEM of four to seven experiments performed in duplicate. **p* <0.05 and ***p* <0.01 as analysed by paired Student's *t* test **d** Glycogen synthesis in response to TPA (100 nmol/l, 2 h) in the absence and presence of Gö6976 (*grey bars*; 1 μmol/l, 5 min pretreatment) and Gö6983 (*black bars*; 1 μmol/l, 5 min pretreatment). *Open bars*, control. The histogram shows the mean ±SEM of three experiments performed in duplicate. **p*<0.05 as analysed by paired Student's *t* test

Our results show that isoprenaline treatment phosphorylates GSK3, suggesting that GSK3 is involved in the β-adrenergic signalling leading to glycogen synthesis. Although activation of α_1-adrenoceptors increased glucose uptake, it did not increase phosphorylation of GSK3 or glycogen synthesis. α_2-Adrenoceptors were not involved in glucose uptake, GSK3 phosphorylation or glycogen synthesis. Several reports show that acute β-adrenergic signalling inhibits insulin-mediated glucose uptake [38, 39]. However, there is also evidence that β-adrenergic signalling may potentiate insulin-mediated glucose uptake [40, 41]. Isoprenaline does not inhibit insulin-stimulated glycogen synthesis, as observed in other studies in skeletal muscle [35, 36]. In our study we were unable to show a full additive effect of insulin and isoprenaline on glycogen synthesis; however, a partial additive effect was observed.

An interesting observation is that the pEC_{50} value for insulin-mediated glycogen synthesis (8.2) is approximately 1 logarithm higher than for glucose uptake (7.1±6.9; [25, 26], suggesting that the system is more sensitive to insulin-stimulated glycogen synthesis than to glucose uptake, and that glycogen storage is the prime function of insulin in this system. The pEC_{50} value for isoprenaline is approximately 1.5 lower for glycogen synthesis (7.5) than for glucose uptake (9.0; [26]), suggesting that glucose uptake is an important role for isoprenaline, and that only with higher concentrations of isoprenaline does glycogen synthesis occur. It is therefore likely that these effects will not be seen in vivo with adrenaline released from the adrenal gland, but rather locally in the muscle, where noradrenaline can reach high concentrations near the sympathetic nerve terminal.

AMPK is important in the signalling involved in glucose uptake [42, 43]. AICAR increases glucose uptake in L6 skeletal muscle cells [29, 44]. We have previously shown that only α_1-adrenergic activation phosphorylates AMPK in these cells and AMPK activation contributes to α_1-adrenergic-mediated increases in glucose uptake [29]. The involvement of AMPK in the regulation of glycogen synthesis is still being debated. We have measured the effects of AMPK activation on glycogen synthesis, based on earlier observations showing us that AMPK is phosphorylated by α_1- but not β_2-adrenoceptors [29]. Our results show that AICAR has no effect on GSK3 phosphorylation and negligible effects on glycogen synthesis.

β_2-Adrenoceptors increase cyclic AMP levels to produce most of their metabolic effects. We have previously shown that in L6 cells, β_2-adrenoceptor activation increases cyclic AMP levels, but most of the signal to glucose uptake is through activation of PI3K [24]. It is not optimal to use

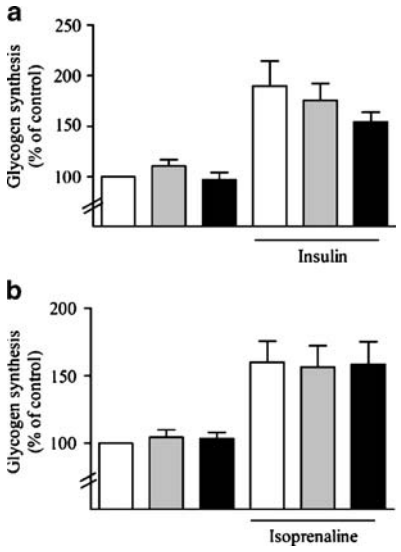

Fig. 9 Glycogen synthesis in differentiated L6 cells in response to (**a**) insulin (1 μmol/l, 2 h) or (**b**) isoprenaline (10 μmol/l, 2 h) in the absence or presence of the PKC inhibitors Gö6976 (*grey bars*; 1 μmol/l, 5 min pretreatment) and Gö6983 (*black bars*; 1 μmol/l, 5 min pretreatment). *Open bars*, control. The histograms show the mean±SEM of four experiments performed in duplicate

forskolin in glucose uptake studies, because it may bind to and inhibit glucose transporters in adipocytes [45] and L6 skeletal muscle cells [46]. Although forskolin greatly increased the cyclic AMP levels in L6 cells [24], we were unable to see a significant increase in glucose uptake [26]. Interestingly, while 8-bromo-cyclic AMP increased cyclic AMP levels in L6 cells and also increased glucose uptake [24], it only had negligible effects on glycogen synthesis. These results are supported by the use of the adenylate cyclase activator, cholera toxin, which increased glucose uptake in L6 cells [24], as well as in brown adipocytes [22], but in our study did not have any effect on glycogen synthesis in L6 cells. β_2-Adrenoceptors can produce effects through G_i-proteins, as exemplified in cardiac tissues [47]. Pertussis toxin, which inactivates G_i, has a small negative

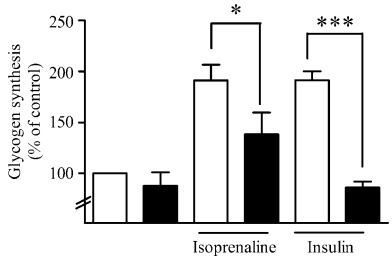

Fig. 10 Glycogen synthesis in differentiated L6 cells in response to insulin (1 μmol/l, 2 h) or isoprenaline (10 μmol/l, 2 h) in the absence or presence of the PI3K inhibitor wortmannin (*filled bars*; 100 nmol/l, 30 min pretreatment). *Open bars*, control. The histogram shows the mean±SEM of three experiments performed in duplicate. *$p<0.05$, ***$p<0.001$ as analysed by paired Student's *t* test

effect on both isoprenaline- and insulin-stimulated glucose uptake [24], but has no effect on glycogen synthesis. These findings suggest that neither cyclic AMP nor G_i are required for glycogen synthesis.

The involvement of atypical PKCs in insulin-mediated glucose uptake has been suggested in skeletal muscle cells [48] as well as in α_{1A}- and β_2-adrenoceptor-dependent glucose uptake in L6 cells [25, 29]. Atypical PKCs may be involved in PI3K dependent signalling leading to glycogen synthesis [49]. We show here that TPA, but not A23187, increased the phosphorylation of GSK3, suggesting that activation of several conventional and novel isoforms of PKC by TPA increases glycogen synthesis. This increase was inhibited by Gö6983, and Gö6976. This suggests that some conventional and novel PKCs are important for signalling to glycogen synthesis. Calcium is not involved, since A23187 does not increase glycogen synthesis. Insulin- and β_2-adrenoceptor-mediated glycogen synthesis is not affected by Gö6976 or Gö6983, despite atypical PKCs being implicated in insulin-, β_2-adrenoceptor- and α_{1A}-adrenoceptor-mediated increases in glucose uptake [25, 29]. This shows that this response is not dependent on PKC. Activation of glycogen synthesis induced by TPA may be mediated by specific isoforms of PKC that are not activated by insulin and isoprenaline.

Our previous studies have shown that β_2-adrenergic stimulation of glucose uptake involves PI3K [24, 26]. Signals from the adrenergic receptors possibly activate PI3K via the $\beta\gamma$ subunit of the G-protein [13, 50], but the exact mechanism whereby adrenoceptors activate PI3K in L6 cells is still under investigation. The glycogen synthesis response to β_2-adrenergic signalling is partially inhibited by two structurally distinct PI3K inhibitors, wortmannin and LY294002. It is clear that insulin- and isoprenaline-mediated increase in glycogen synthesis is dependent on PI3K. The insulin response is fully inhibited by wortmannin, but inhibition of the isoprenaline response was only partial, which may indicate that other pathways are involved in the isoprenaline response that cannot be accounted for by PI3K. The results shown in Fig. 4 support this notion. We suggest that both insulin and β_2-adrenergic signalling activate a signalling pathway that is downstream of PI3K and is capable of increasing glycogen synthesis through GSK3. It is thus possible that noradrenaline release from the sympathetic nervous system can regulate muscle glycogen synthesis locally and thus have a physiological role in glycogen storage.

In conclusion, we have demonstrated that adrenergic signalling increases glycogen synthesis in skeletal muscle through the β_2-adrenoceptor, but not via α_1- or α_2-adrenoceptors. We also show that although certain forms of PKC are able to stimulate glycogen synthesis, they are not involved in β_2-adrenergic signalling leading to glycogen

synthesis. Most importantly, we have demonstrated that this signal is independent of cyclic AMP and probably goes via PI3K.

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