

# Evidence against protein kinase B as a mediator of contraction-induced glucose transport and GLUT4 translocation in rat skeletal muscle

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**Abstract** Both insulin and muscle contraction stimulate glucose transport activity. However, contraction stimulation does not involve the insulin signalling intermediate phosphatidylinositol 3-kinase (PI 3-kinase). Protein kinase B (PKB) has recently been identified as a direct downstream target of PI 3-kinase in the insulin signalling pathway. We have examined here whether the two stimuli share PKB as a convergent step in separate signalling pathways. Insulin stimulates both glucose transport, GLUT4 cell-surface content and PKB activity (by 4–6-fold above basal) in a wortmannin-sensitive manner in *in vitro* incubated rat soleus muscles. By contrast, muscle contraction, which stimulates glucose transport and the cell surface content of GLUT4 by 3-fold above basal levels, had no effect on PKB activity. These data demonstrate that PKB is not a mediator of contraction-induced glucose transport and GLUT4 translocation.

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**Key words:** Protein kinase B; Skeletal muscle; Contraction signalling; 2-*N*-4-(1-Azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine; GLUT4

## 1. Introduction

Transport of glucose across the surface membrane of muscle cells is the cardinal step in glucose disposal. Both insulin and contraction stimulate glucose transport in skeletal muscles by increasing the cell surface GLUT4 content. Insulin stimulates glucose transport through a pathway that involves binding to a specific receptor at the cell surface followed by activation of the receptor's intrinsic tyrosine kinase activity. The activated insulin receptor then phosphorylates downstream proteins, including insulin receptor substrates (IRS-1–4). The p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) binds to the activated IRS and thereby activates the lipid kinase of the p110 catalytic subunit. Activation of PI 3-kinase leads to generation of 3-phosphorylated phosphoinositides such as phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate, which act as second messengers (for reviews see [1,2]). By contrast to the insulin-mediated processes, little is known about intracellular signalling mechanisms through which muscle contraction elicits glucose transport and GLUT4 translocation. It has been demonstrated that muscle contraction has no effect on tyrosine phosphorylation of either the insulin receptor or IRS-1 [3]. Furthermore, inhibition of PI 3-kinase with the specific inhibitor wortmannin, which is capable of completely block-

ing insulin action, has no effect on contraction-stimulated glucose uptake and GLUT4 translocation [4–6]. Effects of insulin and contraction on glucose transport [7–10] and GLUT4 translocation [4,11] are additive. Although contraction and insulin both recruit GLUT4 to the plasma membrane and stimulate glucose transport, the signalling pathways seem to be distinct. Thus it is conceivable that contraction stimulates glucose transport either via a pathway entirely different to that of insulin or through common steps downstream of the wortmannin-sensitive PI 3-kinase which is stimulated by insulin.

The serine/threonine kinase PKB (also known as RAC and Akt) has recently been shown to be a downstream target for the PI 3-kinase-generated signals [12–15]. This occurs through activation by the 3-phosphoinositide-dependent kinase (PDK-1) [16,17]. Over-expression of a constitutive active PKB mutant in 3T3-L1 and rat adipose cells increases glucose transport and promotes GLUT4 translocation [18–20]. This does not necessarily implicate PKB in the normal insulin-stimulated pathway leading to glucose transport. It is necessary to show that inhibition of PKB inhibits the insulin stimulus. In an attempt to address this issue Cong et al. transiently transfected rat adipose cells with an inhibitory form of PKB. They reported that this inhibited insulin-stimulated translocation of GLUT4 by 20% [20]. If insulin and contraction share a common step downstream of PI 3-kinase, then PKB may be a candidate for a common mediator of insulin- and contraction-stimulated glucose transport and GLUT4 translocation in skeletal muscles. For this reason we have explored the effect of contraction on activation of PKB in rat skeletal muscles. Our results demonstrate that insulin activates PKB in a wortmannin-sensitive manner. By contrast, muscle contraction did not activate PKB. Therefore PKB can be excluded as a mediator of contraction-induced glucose transport and GLUT4 translocation.

## 2. Materials and methods

### 2.1. Materials

2-*N*-4-(1-Azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine (ATB-[2-<sup>3</sup>H]BMPA, specific activity 10 Ci/mmol) was prepared as described previously [21]. 3-*O*-[<sup>3</sup>H]Methyl-*D*-glucose and [<sup>14</sup>C]*D*-mannitol were from Du Pont-NEM (UK). Protein A-Sepharose CL-4B, protein kinase A inhibitor fragment 6–22 amide, wortmannin, and bovine serum albumin (radioimmunoassay grade) were from Sigma (St. Louis, MO, USA). Thesit was from Boehringer Mannheim (Mannheim, Germany). Insulin was from Novo-Nordisk, Denmark, anti-akt1 (C-20) affinity-purified goat polyclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, protein G Sepharose 4 fast flow from Pharmacia, St. Albans, UK, adenosine [<sup>γ</sup>-<sup>32</sup>P]triphosphate redivue equivalent from Amersham, Little Chal-

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font, UK and P81 cellulose phosphate paper from Whatman, Maidstone, UK.

## 2.2. Insulin and contraction stimulation of *in vitro* incubated rat soleus muscles

Male Wistar rats (Møllegaards Breeding Laboratory, Denmark) weighing 55–65 g were deprived of food overnight, killed by a blow to the neck followed by cervical dislocation. Soleus muscles were rapidly but carefully dissected out avoiding stretching of the muscle fibres. Intact soleus muscles were incubated initially for 10 min in 4 ml oxygenated Krebs-Henseleit bicarbonate buffer (KHB buffer: 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , pH 7.4) containing 2 mM pyruvate, 38 mM mannitol and 0.1% BSA (radioimmunoassay grade). In experiments with wortmannin, this was added to the buffer immediately before use. Then further incubation took place for 20 min in identical medium in the absence or presence of 1 mU/ml insulin and wortmannin. Muscles were then used for measurements of either glucose transport activity, cell surface GLUT4 content or PKB measurements.

The effect of muscle contraction was investigated using a pulse generator as described previously [4]. Briefly, soleus muscles were first preincubated as described above, then mounted on two platinum electrodes positioned 3 mm apart and surrounding the central part of the muscle. The mounted muscle was then immersed in 4 ml oxygenated KHB containing the same constituents as described above for the preincubation phase. Muscles were stimulated to contract for 5 min at 10 Hz with square-wave pulses of 0.5 ms duration and 10 V amplitude. We have previously found that this contraction protocol produced maximal effects on glucose transport and GLUT4 translocation in soleus muscles [4]. Immediately after the contraction period, muscles were removed and treated as described above for the measurement of glucose transport activity. All incubations were carried out at 30°C under continuous gassing with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  in a shaking water bath.

## 2.3. 3-O-Methylglucose (3-OMG) uptake

Muscles used for assessment of glucose transport activity were incubated in 3 ml KHB containing 8 mM 3-O-[ $^3\text{H}$ ]methyl-D-glucose (437  $\mu\text{Ci}/\text{mmol}$ ) and 32 mM [ $^{14}\text{C}$ ]D-mannitol (8  $\mu\text{Ci}/\text{mmol}$ ) for a period of 10 min, at 30°C under continuous gassing (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ). After the final incubation muscles were blotted on filter paper and clamp-frozen in liquid  $\text{N}_2$ . Glucose transport activity was calculated from the intracellular [ $^3\text{H}$ ]3-OMG accumulation using [ $^{14}\text{C}$ ]D-mannitol as the extracellular marker as described previously [22].

## 2.4. Photolabelling of rat soleus muscles

Muscles used for assessment of cell surface GLUT4 content were incubated at 18°C for 8 min in KHB buffer containing 1 mCi/ml ATB-[ $^3\text{H}$ ]BMPA in a dark room. They were then irradiated 2×3 min in a Rayonet RPR 100 photochemical reactor (RPR 3000 lamps). Muscles were turned over between irradiations to achieve maximum exposure. Following irradiation, muscles were immediately blotted, trimmed and clamp-frozen in liquid  $\text{N}_2$ . Photolabelled muscles were processed for cell surface GLUT4 as described previously [22].

## 2.5. Protein kinase B measurements in rat soleus muscle

Muscles were homogenised in 1.25 ml buffer (10 mM Tris, 5 mM EDTA, 255 mM sucrose, 10 mM NaF, 0.2 mM  $\text{Na}_3\text{VO}_4$ , 1 mM sodium molybdate, 1  $\mu\text{g}/\text{ml}$  antipain, aprotinin, pepstatin, leupeptin and 100  $\mu\text{M}$  AEBSEF, pH 7.2). Triton X-100 was added to the homogenates to give a final concentration of 1% Triton X-100, and samples were rotated for 30 min at 4°C. Homogenates were centrifuged at 20000×g for 10 min and the soluble fractions were added to anti-PKB antibody conjugated to protein G sepharose. Immunoprecipitates were rotated for 2 h at 4°C. The immune pellets were then washed twice with homogenisation buffer (containing 1% Triton X-100), and then twice with kinase buffer (50 mM Tris, 10 mM  $\text{MgCl}_2$ , pH 7.5). The washed pellets were added to 40  $\mu\text{l}$  assay buffer containing 50 mM Tris, 10 mM  $\text{MgCl}_2$ , 1 mM DTT, 100 ng PKA inhibitor peptide, and 25  $\mu\text{g}$  protamine sulphate pH 7.4. Samples were maintained for 5 min at room temperature. The reactions were started by adding 5  $\mu\text{l}$  50  $\mu\text{M}$  ATP and 5  $\mu\text{l}$  2  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP to each tube. After 20 min at 30°C, supernatants were removed and 40  $\mu\text{l}$  of 2% SDS added to the pellets. After a further 10 min, this supernatant was

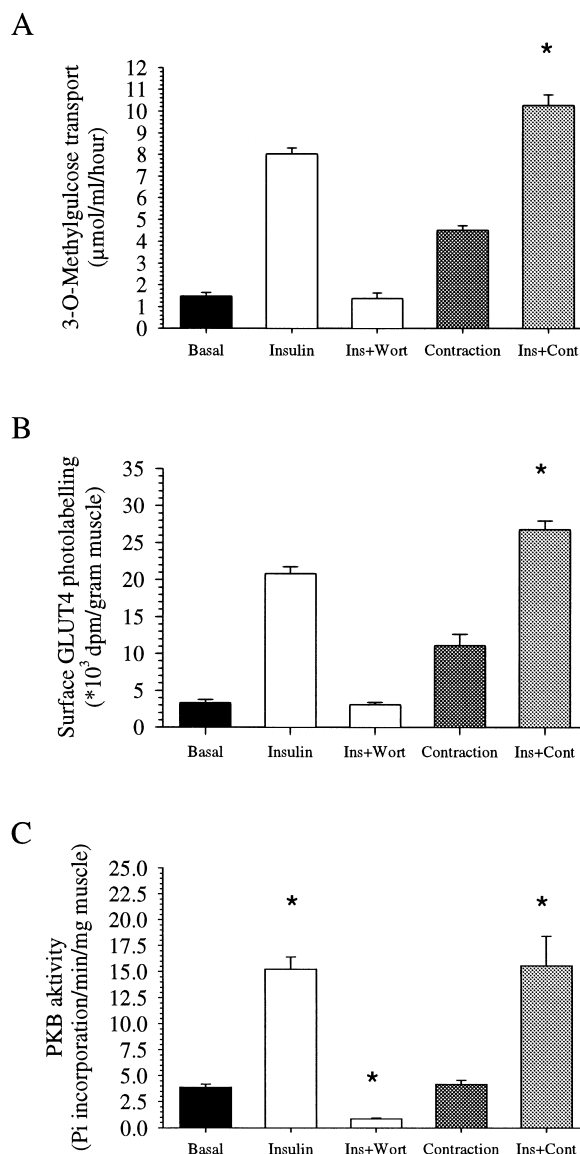


Fig. 1. A: 3-O-Methylglucose transport in *in vitro* incubated rat soleus muscles. Muscles were incubated at 30°C as described in Section 2. Basal: unstimulated muscles; Insulin: incubation with 1 mU/ml insulin for 20 min; Ins+Wort: muscles were preincubated with 1  $\mu\text{M}$  wortmannin for 10 min, then stimulated with 1 mU/ml insulin+1  $\mu\text{M}$  wortmannin for 20 min. Contraction: muscles were preincubated as described in Section 2, then stimulated to contract for 5 min at 10 Hz; Ins+Cont: muscles were incubated with 1 mU/ml insulin for 20 min, then stimulated to contract for 5 min at 10 Hz. Values are mean  $\pm$  S.E.M. ( $n=10-18$ ). \*Significantly different from insulin,  $P<0.001$ . B: ATB-BMPA labelling of rat soleus muscles. Cell surface GLUT4 content was determined as described in Section 2. Muscles were stimulated as described in A. Values are mean  $\pm$  S.E.M. ( $n=5-7$ ). \*Significantly different from insulin,  $P<0.005$ . C: Protein kinase B activity in isolated rat soleus muscles. Muscles were incubated and stimulated as in A. Values are mean  $\pm$  S.E.M. ( $n=6$ ). \*Significantly different from basal,  $P<0.001$ .

removed. The two supernatants were pooled and 20  $\mu\text{l}$  of the combined supernatants were spotted onto a 2 cm×2 cm area of phosphocellulose paper. The phosphocellulose paper was washed five times with 500  $\mu\text{l}$  75 mM phosphoric acid. This was followed by washing with water and then acetone. After drying, the phosphocellulose paper was cut into squares and the radioactivity counted by liquid scintillation counting.

### 3. Results and discussion

#### 3.1. Effect of insulin and contraction on glucose transport activity and cell surface GLUT4 content

As depicted in Fig. 1A,B, insulin at a concentration of 1 mU/ml, which induces a maximal stimulatory effect [22], resulted in a ~6-fold increase above basal levels in both 3-OMG transport and cell surface GLUT4 content in soleus muscles. The effects of insulin on glucose transport activity and cell surface GLUT4 content were completely inhibited by 1  $\mu$ M of the PI 3-kinase inhibitor wortmannin. Muscle contraction accelerated soleus glucose transport activity approximate 3-fold and led to a comparable rise in cell surface GLUT4 content. As shown previously the maximal stimulatory effect of both insulin and muscle contraction was almost completely additive on glucose transport activity and increase in cell surface GLUT4 content [4,7–11]. This indicates that at some level in the signalling pathways, there are different mechanisms leading to the stimulatory actions of insulin and muscle contraction.

#### 3.2. Effect of insulin and contraction on protein kinase B activity

Insulin stimulated PKB activity about 4-fold above basal in vitro incubated rat soleus muscles (Fig. 1C) (basal  $3.9 \pm 0.3$  vs insulin  $15.2 \pm 0.5$  Pi incorporated/min/mg muscle). This is in good agreement with recently published data achieved in rat skeletal muscles after in vivo insulin administration [23]. The increase correlates well with the fold increase in glucose transport activity and cell surface GLUT4 content observed after insulin stimulation (Fig. 1A,B). Addition of 1  $\mu$ M wortmannin, which completely abolished the insulin-induced increase in glucose transport activity and cell surface GLUT4 content, totally prevented the insulin-induced activation of PKB, indicating, as shown previously [13,14,16,24], that the regulation of PKB by insulin is mediated by the wortmannin-sensitive PI 3-kinase. Indeed, wortmannin addition to insulin-stimulated muscle decreased PKB activity even below basal activity (basal  $3.9 \pm 0.3$  vs insulin+1  $\mu$ M wortmannin  $0.8 \pm 0.1$  Pi incorporated/min/mg muscle). However, this is not reflected by a similar reduction in glucose transport activity (basal  $1.5 \pm 0.2$  vs insulin+1  $\mu$ M wortmannin  $1.4 \pm 0.2$   $\mu$ mol/ml/h). One explanation for this effect might be that basal glucose transport activity is partially stimulated due to manipulation of the muscles during the surgical procedure and the in vitro incubation. Additionally, as in 3T3-L1 cells, long incubations may be required before inhibition of signalling by wortmannin results in a reduced level of basal cell surface transporters [25]. In contrast to insulin, maximal electrically stimulated muscle contraction, which increased both glucose transport and cell surface GLUT4 content approximately 3-fold above basal, did not induce activation of PKB above basal activity (basal  $3.9 \pm 0.3$  vs contraction  $4.2 \pm 0.5$  Pi incorporated/min/mg muscle). The combined effect of insulin and contraction also failed to affect PKB activity further than that already activated by insulin alone (insulin  $15.2 \pm 1.2$  vs

insulin+contraction  $15.6 \pm 2.8$  Pi incorporated/min/mg muscle). Our results therefore clearly demonstrate that insulin- and contraction-induced activation pathways do not converge at the level of PKB and that this enzyme is not involved at all in contraction-induced glucose transport and GLUT4 translocation in rat soleus muscle.

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