Molecular mechanisms of contraction-regulated cardiac glucose transport

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Insulin and contraction are the most important regulators of glucose utilization in cardiac muscle. In contrast with insulin, the intracellular signalling elements of contraction have remained unexplored. In the present studies, adult rat ventricular cardiomyocytes were electrically stimulated to perform rhythmic contractions to permit the determination of potential sites of convergence of contraction and insulin signalling to glucose transport. The participation of phosphoinositide 3-kinase (PI-3K) in Ca^{2+}- and contraction-stimulated 3-*O*-methylglucose transport was suggested by the great sensitivity of this process towards the PI-3K inhibitors wortmannin and LY294002 and by the presence of PI-3K activity in anti-phosphotyrosine immunoprecipitates from contracted cells. Initial signalling events of insulin action, including receptor kinase activation, the tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 and the recruitment of PI-3K to IRS-1 and IRS-2, were found not to be involved in contraction-mediated signalling. However, immunoprecipitation of p85α revealed a markedly enhanced tyrosine phosphorylation of an unknown co-precipitated 200 kDa protein in response to both stimuli. It is concluded that contraction-regulated cardiac glucose transport involves the activation of PI-3K in response to upstream signalling pathways different from that of insulin.

Key words: cardiomyocytes, insulin receptor substrate proteins, phosphoinositide 3-kinase, signal transduction.

INTRODUCTION

Contractile activity and insulin are the most potent and physiologically important factors regulating glucose utilization in muscle tissue [1]. The acceleration of glucose transport is achieved in both cases by a redistribution of the glucose transporter isoform GLUT4 from intracellular tubulovesicular structures clustered in the *trans*-Golgi region [2] to the plasma membrane [3,4]. The insulin signalling pathway leading to elevated glucose transport has been elucidated in detail [1,5]. In contrast, the intracellular elements linking the mechanical stimulus of contraction to the increased disposal of muscle glucose have remained poorly understood, although it has been suggested that the contraction-stimulated signalling might be initiated by Ca^{2+} release from sarcoplasmic reticulum, leading to the activation of other signalling intermediates [1,6].

The mechanism of glucose transport induced by insulin or the contraction of skeletal muscle has been the subject of numerous studies, showing that the lipid kinase phosphoinositide 3-kinase (PI-3K) has a critical role in the stimulation of glucose uptake by insulin. Activation of the enzyme triggers the activation of the serine kinases Akt/protein kinase B (PKB) and of the atypical protein kinase C (PKC) isoforms PKCλ and PKCξ [7–11]; studies with dominant-negative as well as constitutively active mutants of these enzymes have shown that these steps contribute to the stimulation of GLUT4 translocation by insulin. In contrast, the stimulation of glucose uptake by skeletal muscle contraction occurs via pathways independent of PI-3K and Akt/PKB [3,12–16]. Recent studies suggest that 5′-AMP-activated protein kinase might be part of the signalling mechanism by which contraction regulates glucose transport [17,18]. These mechanistic differences between the stimulation of glucose transport by contraction as opposed to insulin, at least in skeletal muscle, are further supported by the observation that the effects of the two stimuli on glucose transport [19] and GLUT4 translocation [3,20] are additive. Moreover, in skeletal muscles of obese Zucker rats and high-fat-fed Wistar rats, contractile activity elevates glucose uptake, although insulin stimulation is severely impaired in these animals [21,22]; GLUT4 vesicle pools have been separated that respond independently to exercise and insulin respectively [23].

In contrast with skeletal muscle, much less research has focused on contraction-mediated glucose uptake in cardiac muscle, possibly owing to experimental difficulties in contracting isolated cardiac myocytes. In a previous study from this laboratory [4], we approached this problem by using a contraction chamber permitting the electrical stimulation of the cells in suspension with defined contraction protocols, at constant temperature and under visual control. In that study, electrical stimulation of isolated ventricular cardiomyocytes at a frequency of 5 Hz increased both the GLUT4 content in the plasma membrane and glucose transport. Furthermore, the effects of insulin and contraction on glucose transport activation were not additive, suggesting convergence of the activation mechanisms and/or the existence of a glucose transporter pool in the heart that is recruitable by insulin and contraction alike. However, the signalling pathways mediating this effect, and the possible implications for the diabetic heart, have remained unexplored.

The present study was initiated to determine whether contraction-mediated signalling to GLUT4 might be related to the insulin signalling cascade, thus defining convergent steps in the two signalling pathways. By applying electrical stimulation to isolated adult rat ventricular myocytes, this issue was investigated at the level of the insulin receptor, insulin receptor substrate (IRS)-1, IRS-2, PI-3K and Akt/PKB kinase, which are known to be important elements of the insulin signalling pathway leading to elevated glucose transport in all target tissues [1,5].

EXPERIMENTAL

Materials

3-*O*-[^{14}C]Methyl-D-glucose, L-[1-^{14}C]glucose and ^{125}I-Protein A were purchased from Amersham Pharmacia Biotech (Freiburg,

Abbreviations used: ECL, enhanced chemiluminescence; IRS, insulin receptor substrate; PI-3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; SH2, Src homology 2.

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Germany). Reagents for SDS/PAGE were supplied by Amersham Pharmacia Biotech and by Sigma (Deisenhofen, Germany). Collagenase was from Serva (Heidelberg, Germany) and BSA (fraction V, fatty-acid-free) was obtained from Boehringer Mannheim (Germany). Protein A–trisacryl (GF-2000) and Protein G–agarose were products from Pierce (Oud Beijerland, The Netherlands). Wortmannin, the ionophore A23187 and goat anti-mouse IgG1–agarose were from Sigma, and LY294002 was purchased from Calbiochem-Novabiochem (Bad Soden, Germany). All other chemicals were of the highest analytical grade commercially available and were supplied by Sigma, by Merck (Darmstadt, Germany) and by Fluka (Neu-Ulm, Germany).

Antibodies

Alkaline-phosphatase-conjugated and horseradish-peroxidase-conjugated anti-phosphotyrosine antibodies (RC20) and monoclonal anti-p85α antibody, raised against the C-terminal Src homology 2 (SH2) domain of human p85α (p85α^{CSH2}), were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit polyclonal antibodies recognizing phospho-Thr^{308} of Akt were obtained from New England Biolabs (Beverly, MA, U.S.A.). Rabbit polyclonal antiserum raised against a glutathione S-transferase fusion protein corresponding to the N-terminal SH2 domain of human p85α (p85α^{NSH2}) was kindly supplied by Dr P. R. Shepherd (London, U.K.) and rabbit polyclonal antiserum raised against a glutathione S-transferase fusion protein corresponding to the SH3 domain of p85β was generously provided by Dr T. Asano (Tokyo, Japan). Broad-specificity polyclonal anti-p85 antibody (p85^{PAN}) and polyclonal anti-(IRS-2) serum were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Polyclonal anti-(IRS-1) serum was a gift from Dr J. A. Maassen (Leiden, The Netherlands). Monoclonal anti-(insulin receptor) antibody (clone 29B4) was a product of Calbiochem-Novabiochem. Horseradish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies were from Promega (Mannheim, Germany) and monoclonal anti-phosphotyrosine (PY20)–agarose conjugate was from Santa Cruz Biotechnology (Heidelberg, Germany).

Isolation and electrical stimulation of ventricular cardiomyocytes

Male Wistar rats (280–340 g) were used in all experiments. Ca^{2+}-tolerant cardiac myocytes were isolated by perfusion of adult rat heart with collagenase as detailed previously [24]. The final cell suspension was incubated for 60 min until further use in Hepes buffer [130 mM NaCl/4.7 mM KCl/1.2 mM KH_2PO_4/25 mM Hepes/5 mM glucose/2 % (w/v) BSA (pH 7.4), equilibrated with oxygen] containing $MgSO_4$ and $CaCl_2$ (final concentrations 1 mM) at 37 °C in a rotating water-bath shaker. Cell viability was judged by determination of the percentage of rod-shaped cells and averaged 90–97 % under all incubation conditions. Electric field stimulation was performed in a chamber originally described by Rose and Kammermeier [25]. A 4 ml aliquot of the cell suspension was transferred to the chamber, in which the cells were stirred continuously at 37 °C throughout the experiment and stimulated for different durations with biphasic 5 Hz pulses 160 V in amplitude and 40 μs in duration. This contraction protocol has previously been shown to avoid confounding effects on glucose transport due to hypoxic conditions within the chamber or changes in the energy state of the cells, because it excludes any changes in the cellular ATP content or the oxygen tension within the chamber [4].

Cell treatment and determination of 3-*O*-methylglucose transport

After preincubation for 60 min, cardiomyocytes (4×10^5 cells/ml) were either left untreated or incubated with wortmannin (for 20 min) or with LY294002 (for 15 min) at the concentrations indicated in the Figure legends and were subsequently contracted or not for 5 min at 5 Hz. For experiments with the ionophore A23187, cells were prepared in nominally Ca^{2+}-free Hepes buffer and preincubated with or without wortmannin (50 nM, for 20 min) or LY294002 (10 μM, for 15 min). Incubation was then continued for 15 min in the absence or presence of 20 μM A23187 with or without the addition of 100 μM $CaCl_2$ and/or 350 nM insulin. The transport assay was performed as outlined previously [24], with the non-metabolizable glucose analogue 3-*O*-[^{14}C]methyl-D-glucose and L-[1-^{14}C]glucose to correct for simple diffusion and extracellular trapping of radioactivity.

Immunoprecipitation

Freshly isolated cardiac myocytes were left untreated, contracted at 5 Hz or incubated with 350 nM insulin for different durations and solubilized in ice-cold radioimmunoprecipitation buffer [50 mM Tris/HCl (pH 7.4)/1 % (v/v) Nonidet P40/0.25 % (v/v) sodium deoxycholate/150 mM NaCl/1 mM EGTA/1 mM PMSF/1 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin/1 mM Na_3VO_4/1 mM NaF] for 2 h at 4 °C with gentle rotation. For immunoprecipitation with monoclonal anti-p85α antibody and anti-(insulin receptor) antibody, cells were solubilized in ice-cold lysis buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 20 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4, 0.3 mM PMSF, 10 mM benzamidine, 15 μM pepstatin and 1 % (v/v) Triton X-100. Insoluble material was removed by centrifugation. Antibodies were precoupled to a mixture of Protein A and Protein G-beads for 2 h at 4 °C, except anti-(IRS-2) and anti-(insulin receptor), which were preadsorbed to Protein A–trisacrylic beads and goat anti-mouse IgG1–agarose respectively. The adsorbed antibody was then added to the cell supernatant and incubated for 16 h at 4 °C with gentle rotation. The immunopellet was collected by centrifugation, washed three times with ice-cold PBS, pH 7.4, or lysis buffer and used for Western blot analysis.

Immunoblotting

The immunoadsorbed proteins were solubilized in Laemmli sample buffer, resolved by SDS/PAGE on 8–18 % (w/v) horizontal gradient gels and transferred to a nitrocellulose filter (Protran BA 85; Schleicher and Schuell, Dassel, Germany) or a PVDF filter (Immobilon P; Sigma) in a semi-dry blotting apparatus. Filters were blocked and incubated with the appropriate primary antibody; after extensive washing, proteins were detected with ^{125}I-Protein A (0.3 μCi/ml), horseradish-peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) or alkaline-phosphatase-conjugated or horseradish-peroxidase-conjugated anti-phosphotyrosine antibodies and alkaline phosphatase substrate or ECL respectively. Blots were analysed by laser scanning densitometry; quantification was performed on a SPARCstation (Sun Microsystems) with BioImage whole-band analysis software. ^{125}I-Protein A blots were detected and analysed on a Fujix BAS 1000 BioImaging analyser by using image analysis software.

Measurement of PI-3K activity

Freshly isolated cardiac myocytes (10^6 cells) were left untreated, contracted at 5 Hz or incubated with 350 nM insulin for 5 min, then lysed in 1 ml of ice-cold lysis buffer [30 mM Tris/HCl (pH

7.4)/1 % (v/v) Nonidet P40/150 mM NaCl/1 mM EDTA/10 % (v/v) glycerol/10 mM $MgCl_2$/1 mM Na_3VO_4/1 mM NaF containing protease inhibitors] (Complete™; Boehringer Mannheim) for 2 h at 4 °C with gentle rotation. For immunoprecipitations, lysates (1 mg of protein) were made 0.5 % (v/v) in Nonidet P40 and incubated for 4 h at 4 °C with 10 μl of anti-phosphotyrosine antibody precoupled to Protein G–agarose beads. The immunoprecipitates were then washed and assayed for PI-3K activity as described elsewhere [26,27].

Statistical analysis

All results are expressed as means ± S.E.M. The significance of reported differences was evaluated by using the null hypothesis and *t* statistics for paired or unpaired data. Corresponding significance levels are indicated.

RESULTS

Contraction-stimulated and Ca^{2+}-stimulated glucose uptake in cardiomyocytes is sensitive to both wortmannin and LY294002

The use of two structurally unrelated inhibitors of the catalytic activity of PI-3K, namely wortmannin and the benzopyran-4-one compound LY294002, in different cell preparations [3,12,13,28–33] and the introduction of dominant-negative mutants of the regulatory p85 subunit of the enzyme lacking the binding site for the catalytic p110 subunit into Chinese hamster ovary cells [34] and 3T3-L1 adipocytes [35] have served to identify PI-3K as a key signalling intermediate of the insulin-induced stimulation of glucose transport and transporter translocation. To define potential sites of convergence between the signalling pathways of cardiac contraction and insulin, we first evaluated the participation of PI-3K in contraction-stimulated glucose transport activation. Field stimulation was applied to freshly isolated adult rat cardiomyocytes for 5 min at a frequency of 5 Hz, preincubated with or without wortmannin or LY294002, followed by determination of the initial influx (10 s) of 3-*O*-methylglucose. Electrical stimulation significantly increased 3-*O*-methylglucose transport from 173.5 ± 16.9 to 318.8 ± 29.6 pmol/10 s per 10^6 cells (84 % increase) (Figure 1). Pretreatment of the cells for 20 min with different wortmannin concentrations inhibited contraction-regulated glucose transport activity in a dose-dependent manner, with a 62 % decrease in the presence of 10 nM wortmannin; at 50 nM, transport was already completely blocked. As shown in Figure 1, the inhibitory action on contraction-mediated glucose transport was not due to an effect on basal glucose transport because the use of wortmannin concentrations 4-fold higher than those needed to achieve a complete inhibition failed to alter basal glucose transport (Figure 1). Preincubation of the cells for 15 min with 10 or 50 μM LY294002 significantly decreased the incremental increase in glucose transport due to contraction by 24 % and 56 % respectively (Figure 1, inset).

Contraction-stimulated glucose uptake might be triggered by the release of sarcoplasmic Ca^{2+} release, a well-known event during muscle contraction. To test this hypothesis, 3-*O*-methylglucose transport was analysed in cardiac myocytes prepared in nominally Ca^{2+}-free medium with the Ca^{2+} ionophore A23187 in the absence or presence of 100 μM $CaCl_2$. As shown in Figure 2, the addition of A23187 alone did not modify the basal hexose transport rate. However, 3-*O*-methylglucose transport increased significantly from 361.3 ± 35.0 to 509.1 ± 59.8 pmol/10 s per 10^6 cells when cardiomyocytes were treated with the ionophore in the presence of 100 μM $CaCl_2$ for 15 min. Preincubation of cells with either wortmannin (50 nM, for 20 min) or LY294002 (10 μM, for 15 min) completely blocked the effect of Ca^{2+} on glucose transport in the A23187-treated cells (Figure 2). Thus both contraction and the release of endogenous Ca^{2+} stimulate glucose uptake in a wortmannin- and LY294002-sensitive fashion, suggesting the involvement of PI-3K in this process.

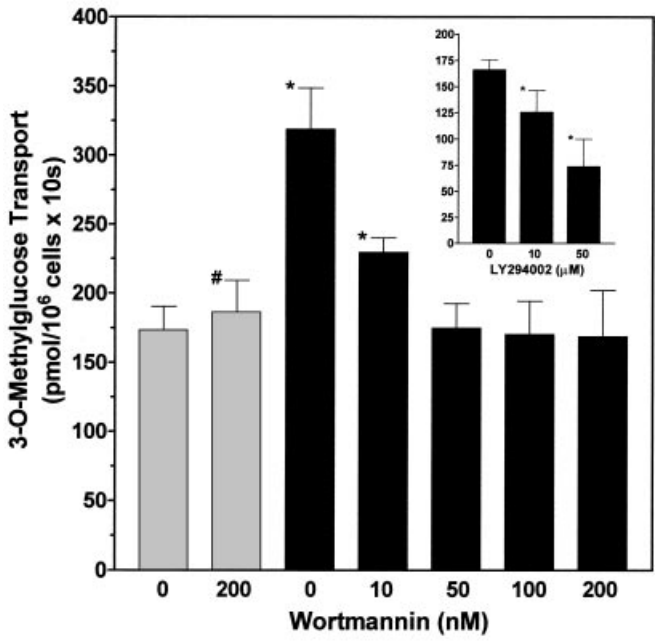

Figure 1 Contraction-stimulated glucose transport in ventricular cardiomyocytes is sensitive to both wortmannin and LY294002

Freshly isolated rat cardiac myocytes (4×10^5 cells/ml) were either left untreated or preincubated for 20 min with the indicated concentrations of wortmannin at 37 °C. Cells were transferred to the contraction chamber and left untreated (grey bars) or electrically stimulated (black bars) to contract at a frequency of 5 Hz for 5 min. Thereafter, 3-*O*-methylglucose transport was determined as outlined in the Experimental section. Inset: incremental increase in 3-*O*-methylglucose transport of contracted cells that were preincubated for 15 min with or without the indicated doses of LY294002 at 37 °C. The glucose transport rates presented are means ± S.E.M. for three to five individual experiments, each performed in triplicate. *Significantly different from uncontracted control at $P < 0.05$; #not significantly different from untreated control.

Contraction does not induce the tyrosine phosphorylation of insulin receptor and complex formation between p85α and IRS-1 or IRS-2

We examined whether contraction-stimulated signalling involves the activation of the insulin receptor. Freshly isolated cardiac myocytes (10^6 cells) were left untreated or stimulated either by contraction (5 Hz, for 2 or 5 min) or with insulin (350 nM, for 2 min). Insulin receptor immunoprecipitates from total cell lysates were then analysed for tyrosine phosphorylation of the receptor's β subunit by immunoblotting with anti-phosphotyrosine antibodies. As depicted in Figure 3, contraction of cardiomyocytes *in vitro* for different durations did not result in enhanced tyrosine phosphorylation of the β subunit in comparison with basal cells. In contrast, insulin exerted a marked stimulatory action on the tyrosine phosphorylation of the insulin receptor.

Because the regulation of PI-3K activity by insulin is achieved by interaction of the enzyme with tyrosine-phosphorylated IRS proteins [36,37], we next investigated the recruitment of the p85α adapter subunit of PI-3K to IRS-1 or IRS-2. In initial experi-

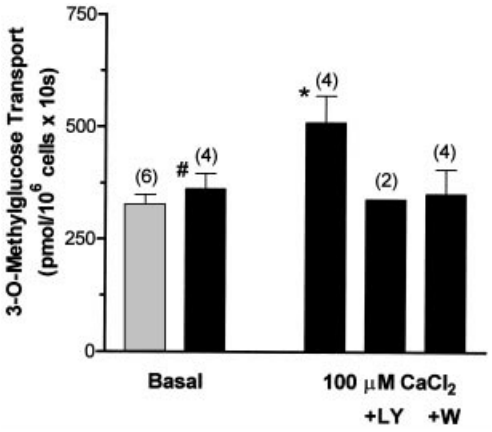

Figure 2 Ca^{2+}-stimulated glucose uptake in cardiomyocytes is sensitive to both wortmannin and LY294002

Isolated rat cardiac myocytes (4×10^5 cells/ml) were prepared in nominally Ca^{2+}-free Hepes buffer, preincubated with or without LY294002 (10 μM, for 15 min) or wortmannin (50 nM, for 20 min) as indicated. Incubation was then continued for an additional 15 min in the absence (grey bars) or presence (black bars) of 20 μM Ca^{2+} ionophore A23187 with or without the addition of 100 μM $CaCl_2$. Thereafter, the initial influx of 3-*O*-methylglucose, representing carrier-mediated glucose transport was determined over a 10 s assay period as outlined in the Experimental section. Results are presented as total transport rates and are means $\pm$ S.E.M. for individual experiments, with the number indicated in parentheses. *Significantly different from corresponding basal control at $P < 0.05$; **$P < 0.005$; #not significantly different from basal control without A23187. Abbreviations: W, wortmannin; LY, LY294002.

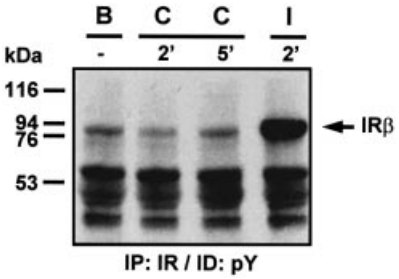

Figure 3 Cardiac muscle contraction does not induce tyrosine phosphorylation of the insulin receptor's β subunit

Freshly isolated rat ventricular cardiomyocytes (10^6 cells) were either left untreated (B) or stimulated by contraction [5 Hz, 2 and 5 min (indicated by 2′ and 5′ respectively)] (C) or with insulin (350 nM, 2 min) (I). After cell lysis, the insulin receptor (IR) was immunoadsorbed (IP), immune complexes were solubilized in Laemmli sample buffer and subjected to separation by SDS/PAGE on gradient gels. Immunodetection (ID) was then performed with horseradish-peroxidase-conjugated anti-phosphotyrosine antibody (pY) and ECL. The positions of the insulin receptor's β subunit (IRβ) and molecular mass markers are indicated at right and left respectively. A representative autoradiogram is shown.

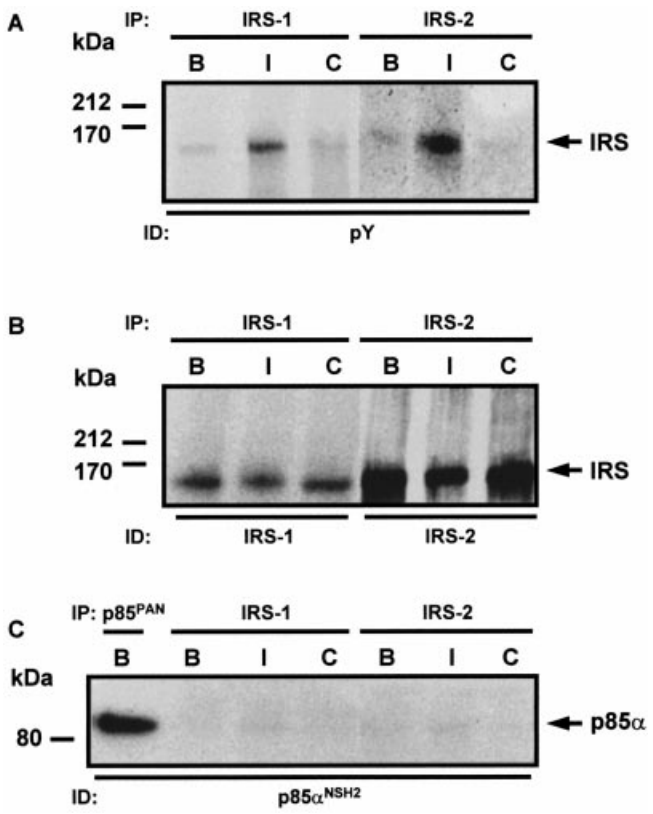

Figure 4 Cardiac contraction does not induce complex formation between p85α and IRS-1 or IRS-2

Cardiomyocytes (10^6 cells) were either left untreated or stimulated for 5 min by contraction at 5 Hz or with 350 nM insulin. After cell lysis, IRS-1 and IRS-2 immunoprecipitates (IP) were subjected to SDS/PAGE and transferred to nitrocellulose. (**A**) The filters were then cut apart and the upper parts were analysed with (ID) horseradish-peroxidase-conjugated anti-phosphotyrosine antibody (pY) to detect the tyrosine phosphorylation of IRS-1 and IRS-2. (**B**) Equal loading was ensured by reprobing the stripped filters with polyclonal antisera against the IRS proteins and ^{125}I-Protein A or ECL. (**C**) The lower parts of the filters were immunoblotted with polyclonal anti-p85α^{NSH2} serum and ^{125}I-Protein A. Abbreviations: B, basal; I, insulin; C, contraction. Representative experiments out of three are shown. The positions of molecular mass markers are indicated at the left, and those of IRS proteins and p85α ar indicated at the right.

ments, the polyclonal anti-p85α^{NSH2} serum, raised against a glutathione S-transferase fusion protein corresponding to the N-terminal SH2 domain of human p85α, was characterized for its ability to detect the p85α adapter subunit in rat cardiac and skeletal muscle as well as in different cell lines and was found consistently to detect a prominent immunoreactive band at approx. 85 kDa in extracts from all tissues and in a p85^{PAN} immunoprecipitate from cardiac myocytes (results not shown). It is worth noting that the anti-p85α^{NSH2} serum used in these experiments has been shown to recognize exclusively the α isoform of the p85 adapter subunit without any cross-reactivity with the β isoform [33].

IRS-1 and IRS-2 were then immunoadsorbed from cell lysates of cardiomyocytes after activation by field stimulation or incubation with insulin, then analysed for co-precipitating p85α by immunoblotting with the polyclonal anti-p85α^{NSH2} serum. As shown in Figure 4(B), comparable amounts of IRS-1 and IRS-2 were precipitated from basal and treated cardiac myocytes. Immunoblotting with anti-phosphotyrosine antibodies indicated that both IRS-1 and IRS-2 were tyrosine phosphorylated after treatment with insulin, but not in response to contraction (Figure 4A). Remarkably, the anti-p85α serum revealed no significant amount of p85α recruited to the IRSs in response to both contraction and insulin (Figure 4C). Consistent with this result, no p85α adapter subunit was detected in IRS-1 immunoprecipitates from cardiac muscle lysates of Wistar rats stimulated *in vivo* with insulin for 20 min (I. Uphues, personal communication). The experiments were therefore repeated by Western blotting of co-precipitated p85α with monoclonal anti-p85α^{CSH2} antibody, which was directed against the C-terminal SH2 domain of human p85α. In agreement with the results described above, no significant amount of p85α was recruited to the IRSs in response to both stimuli (results not shown).

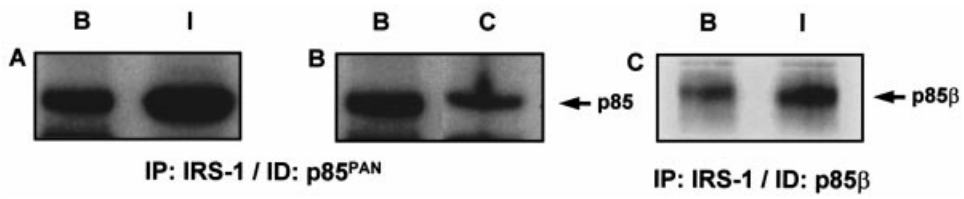

Figure 5 Differential effects of contraction and insulin on the interaction between p85 and IRS-1

(**A**, **B**) IRS-1 immunoprecipitates (IP) from lysates of untreated, insulin-stimulated (350 nM, for 5 min) (**A**) or contracted (5 Hz, for 5 min) (**B**) cardiac myocytes (2×10^6 cells) were subjected to separation by SDS/PAGE; Western blot analysis was performed with anti-p85^{PAN} serum and ^{125}I-Protein A. (**C**) IRS-1 was immunoadsorbed from basal and insulin-treated heart lysates. After separation by SDS/PAGE, immunodetection (ID) was performed with polyclonal anti-p85β serum and ECL. Representative autoradiograms are shown. The positions of p85 and p85β are indicated.
Abbreviations: B, basal; I, insulin; C, contraction.

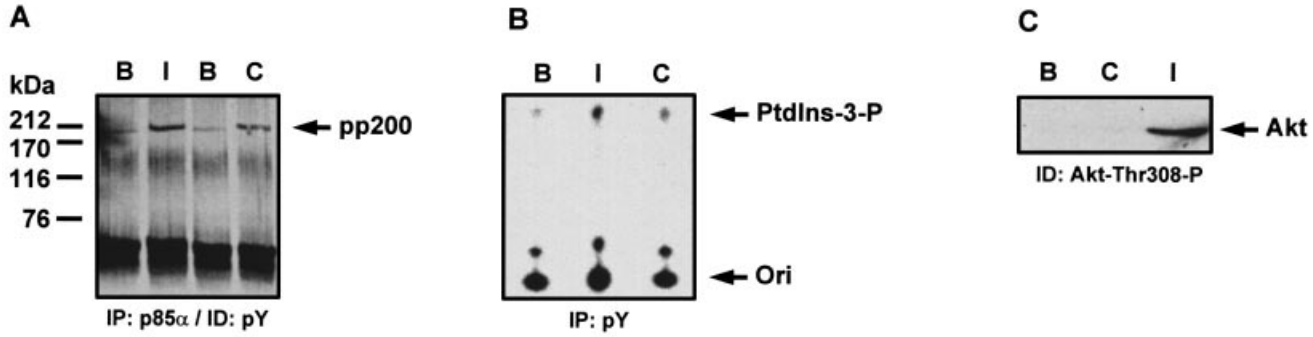

Figure 6 Cardiac contraction induces the recruitment of a 200 kDa tyrosine-phosphorylated protein to p85α and enhances PI-3K activity *in vitro*

(**A**) Adult rat ventricular cardiomyocytes (10^6 cells) were either left untreated or stimulated by contraction (5 Hz, for 5 min) or with insulin (350 nM, for 5 min). After cell lysis, p85α was immunoprecipitated (IP) with monoclonal anti-p85α^{CSH2} antibody and immune complexes were subjected to SDS/PAGE on gradient gels. Western blot analysis (ID) was then performed with alkaline phosphatase-conjugated anti-phosphotyrosine antibody (pY). Substrate was added for appropriate colour development and detected signals were quantified with BioImage whole band analysis software. Shown is a representative Western blot of four to six separate experiments. Abbreviations: B, basal; I, insulin; C, contraction. The migrations of the 200 kDa phosphoprotein (pp200) and of molecular mass markers are indicated at right and left respectively. (**B**) Adult rat ventricular cardiomyocytes (10^6 cells) were either left untreated or stimulated by contraction (5 Hz, for 5 min) or with insulin (350 nM, for 5 min) and lysed. Anti-phosphotyrosine immunoprecipitates were collected from the cell lysates and analysed for co-precipitating PI-3K activity by determining the incorporation of ^{32}P into phosphatidylinositol. Reaction products were extracted and detected by TLC followed by autoradiography on a Fujix BAS 1000 BioImaging analyser. Abbreviation: Ori, origin. (**C**) Adult rat ventricular cardiomyocytes (10^6 cells) were either left untreated or stimulated by contraction (5 Hz, for 5 min) or with insulin (350 nM, for 5 min) and lysed. Total cell lysates (20 μg per lane) were resolved by SDS/PAGE and blotted. Phosphorylation of Akt was determined by immunoblotting with phosphospecific antibodies recognizing phospho-Thr^{308} of Akt. The immunoblot shown is representative of five experiments performed independently.

However, when IRS-1 immunoprecipitates from whole cell lysates of untreated, insulin-stimulated (350 nM, for 5 min) or contracted (5 Hz, for 5 min) cardiomyocytes were immunoblotted with broad-specificity anti-p85^{PAN} antibody (Figures 5A and 5B) or polyclonal p85β antiserum (Figure 5C), p85 protein was found to be recruited rapidly to IRS-1 in response to insulin but not contraction.

Contraction induces the recruitment of a 200 kDa tyrosine phosphorylated protein to p85α and enhances PI-3K activity in anti-phosphotyrosine immunoprecipitates

The p85α adapter subunit has been reported to mediate the stimulation of PI-3K activity by insulin in different cell lines [36], whereas it seems to be unrelated to this function in heart, at least at the level of IRS-1 and IRS-2 (Figure 5). Similarly, contraction-mediated PI-3K activation is not regulated by the recruitment of p85α to one of these IRS proteins (Figure 5). We therefore investigated whether cardiac p85α alternatively associates with additional tyrosine phosphoproteins. The p85α adapter subunit was immunoadsorbed from cell lysates of control, insulin-treated or contracted cardiomyocytes. Western blot analysis of co-precipitated tyrosine-phosphorylated proteins with anti-phosphotyrosine antibody revealed the co-precipitation of a phosphoprotein of approx. 200 kDa (pp200) in association with p85α (Figure 6A), which was clearly distinguishable from IRS-1 and IRS-2 with apparent molecular masses of approx. 160–170 kDa (results not shown). The phosphorylation of pp200 was enhanced by both insulin and contraction (Figure 6A), making it an attractive candidate for mediating cardiac PI-3K activation.

To test this hypothesis, we next analysed anti-phosphotyrosine immunoprecipitates for co-precipitating PI-3K activity *in vitro*. As shown in Figure 6(B), both contraction (5 Hz, for 5 min) and insulin (350 nM, for 5 min) induced a significant increase in PI-3K activity, although with different magnitudes. Insulin enhanced PI-3K activity by 3.81 ± 0.55-fold ($P < 0.001$, $n = 5$), whereas contraction stimulated it 1.39 ± 0.24-fold ($P = 0.01$, $n = 5$). On the basis of these results, it is tempting to speculate that the contraction-induced tyrosine phosphorylation of pp200 contributes to the activation of PI-3K, thereby stimulating glucose uptake in cardiac muscle.

To elucidate the potential convergence of cardiac contraction and insulin signalling at an additional level downstream of PI-3K, we assessed the effect of the two stimuli on the activity of Akt/PKB kinase, a protein distal to PI-3K that might function in the regulation of GLUT4 translocation [7,8]. For this assay we used a phosphospecific (Thr^{308}) antibody that recognizes the protein only in its phosphorylated (active) state. As presented in Figure 6(C), the phosphorylation of Akt was markedly stimulated in response to insulin; however, essentially no effect on the phosphorylation of Akt was observed after contraction.

DISCUSSION

Here we have studied the mechanisms of contraction-stimulated glucose uptake in cardiac muscle. A key finding of this study is that contraction-stimulated glucose uptake in the heart is sensitive to inhibition by the two structurally unrelated PI-3K inhibitors wortmannin and LY294002. Furthermore, the observed inhibition of glucose transport by wortmannin was in the low nanomolar range, clearly pointing to an involvement of PI-3K in cardiac contraction-regulated glucose uptake. This situation is in contrast with numerous studies performed in skeletal muscle, in which inhibition of PI-3K does not affect contraction-stimulated GLUT4 translocation and glucose transport, even at concentrations of wortmannin that completely block insulin-stimulated glucose uptake [3,12,13]. To substantiate our finding we therefore studied the effects of the two PI-3K inhibitors on Ca^{2+}-stimulated glucose transport in Ca^{2+}-ionophore-treated cardiomyocytes. Muscle contraction is associated with a large increase in cytosolic Ca^{2+} levels. Several studies, including the present one, have demonstrated a stimulation of glucose transport and GLUT4 translocation on increasing cytosolic Ca^{2+} levels in cardiac and skeletal muscle [38–41], whereas inhibitors of the release of Ca^{2+} from sarcoplasmic reticulum block the effects of Ca^{2+}-releasing agents on skeletal muscle glucose transport [39,42]. These observations strongly indicate that contraction-stimulated glucose uptake is triggered by the increase in cytosolic Ca^{2+} levels. Further, the inhibition of Ca^{2+}-induced glucose uptake by both wortmannin and LY294002 reported here and the lack of this effect in skeletal muscle [42] support our idea of mechanistic differences in contraction-stimulated glucose uptake between cardiac and skeletal muscle.

To identify components of contraction-stimulated signalling in the heart, we determined whether this process shows characteristics that are more common in the insulin-signalling system. The stimulation by insulin of glucose transport via translocation of the glucose transporter GLUT4 involves the binding of insulin to its cognate receptor, leading to autophosphorylation of the receptor's β subunit [5]. The activated insulin receptor then tyrosine-phosphorylates several substrate proteins, including members of the IRS family such as IRS-1 and IRS-2 [37]. A critical step in insulin signalling towards glucose uptake is the recruitment of the regulatory subunit of PI-3K to tyrosine-phosphorylated IRS proteins, thereby activating the lipid kinase of the catalytic p110 subunit [36,37]. In contrast with the insulin signalling cascade, cardiac muscle contraction does not result in the phosphorylation of the insulin receptor's β subunit, an observation that is in line with studies performed on skeletal muscle [43,44]. In addition, we did not observe the tyrosine phosphorylation of IRS-1 or IRS-2 or the formation of complexes between the regulatory p85 subunit and IRS-1 or IRS-2 in contracted cardiac myocytes.

This raises the possibility that PI-3K activity results from recruitment of the regulatory subunit to other tyrosine-phosphorylated proteins. Indeed, we observed an increase in PI-3K activity in anti-phosphotyrosine immunoprecipitates obtained from contracted cardiac myocytes. It should be noted in this context that the increase in PI-3K activity in insulin-stimulated cells is much greater than that in contracted cells. It is plausible that this is due to the co-precipitation of PI-3K bound to tyrosine-phosphorylated IRS-1 and IRS-2 from insulin-stimulated cells.

How, then, is PI-3K activated in response to contraction? A putative candidate is the tyrosine-phosphorylated 200 kDa protein (pp200) that co-precipitated with p85α in both insulin-treated and contracted cardiomyocytes. Interestingly, other studies reported the binding of a 190 kDa protein to p85, not identical with IRS-1 or IRS-2, in response to the stimulation of 3T3-L1 adipocytes with arsenite [45]. Furthermore, in rat hepatocytes a 195 kDa protein was suggested to be a novel substrate of the insulin receptor kinase [46]. Although it is too early to conclude that pp200 is indeed involved in the regulation of glucose uptake in cardiac muscle, several observations are in favour of this suggestion. Here one should note that several isoforms and splice variants of the regulatory subunit of PI-3K have been reported [33,47]. At this moment it is not completely clear to what extent these isoforms contribute to insulin-mediated responses; however, studies performed in Chinese hamster ovary and Cos-1 cells suggest that p85α mediates the stimulation of PI-3K activity by insulin, whereas kinase complexes containing p85β seemed to be less sensitive [36,47]. When analysing anti-(IRS-1) immunoprecipitates from insulin-stimulated cardiac muscle, we observed co-precipitation of the p85β subunit rather than p85α. Although our previous studies suggest a significant role for IRS-1 and its associated PI-3K activity in glucose transport [48,49], GLUT4 vesicles prepared from insulin-stimulated cardiomyocytes contain only p85α, not p85β (A. Kessler, M. D. Ouwens, M. Till and J. Eckel, unpublished work). As our previous studies have suggested the existence of identical insulin- and contraction-recruitable GLUT4 transporter pools in cardiac myocytes [4], pp200 is a likely candidate for recruiting p85α to GLUT4 vesicles to stimulate glucose transport in response to both insulin and contraction. Interestingly, contraction of the cardiomyocytes was found not to activate Akt/PKB kinase despite the activation of PI-3K. This might be explained by the difference in PI-3K compartments regulated in response to contraction as opposed to insulin, as also seen from a much more limited amount of PI-3K activity associated with phosphotyrosine proteins after contraction (see Figure 6B). Our results clearly exclude the involvement of Akt/PKB from the regulation of cardiac glucose uptake in response to contraction. Two recent reports have described a slight activation of Akt in rodent [50] and human [51] skeletal muscle by exercise; however, the precise role of Akt kinase for signalling to GLUT4 remains unclear. Alternatively, PKCξ and PKCλ have been shown to be regulated by the PI-3K pathway and to be required for insulin-stimulated glucose transport [9–11]. Studies are currently under way to elucidate the potential involvement of different PKC isoforms in contraction-regulated cardiac glucose transport.

In summary, the present study indicates that, in cardiac myocytes, contraction-stimulated glucose transport is mediated by PI-3K, independently of activation of the insulin receptor–IRS-1/IRS-2 signalling pathway. We speculate that this process involves the activation of a specific tyrosine kinase in response to the release of Ca^{2+} from the sarcoplasmic reticulum, which phosphorylates pp200, leading to the recruitment and activation of PI-3K.

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