A common trafficking route for GLUT4 in cardiomyocytes in response to insulin, contraction and energy-status signalling

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Summary

A new mouse model has been developed to study the localisation and trafficking of the glucose transporter GLUT4 in muscle. The mouse line has specific expression of a GFP and HA-epitope-tagged version of GLUT4 under the control of a muscle-specific promoter. The exofacial HA-tag has enabled fluorescent labelling of only the GLUT4 exposed at the external surface. A distinction between sarcolemma labelling and transverse-tubule labelling has also been possible because the former compartment is much more accessible to intact anti-HA antibody. By contrast, the Fab fragment of the anti-HA antibody could readily detect GLUT4 at the surface of both the sarcolemma and transverse tubules. Here, we have used this mouse model to examine the route taken by cardiomyocyte GLUT4 as it moves to the limiting external membrane surface of sarcolemma and transverse-tubules in response to insulin, contraction or activators of energy-status signalling, including hypoxia. HA-GLUT4-GFP is largely excluded from the sarcolemma and transverse-tubule membrane of cardiomyocytes under basal conditions, but is similarly trafficked to these membrane surfaces after stimulation with insulin, contraction or hypoxia. Internalisation of sarcolemma GLUT4 has been investigated by pulse-labelling surface GLUT4 with intact anti-HA antibody. At early stages of internalisation, HA-tagged GLUT4 colocalises with clathrin at puncta at the sarcolemma, indicating that in cells returning to a basal state, GLUT4 is removed from external membranes by a clathrin-mediated route. We also observed colocalisation of GLUT4 with clathrin under basal conditions. At later stages of internalisation and at steady state, anti-HA antibody labeled-GLUT4 originating from the sarcolemma was predominantly detected in a peri-nuclear compartment, indistinguishable among the specific initial stimuli. These results taken together imply a common pathway for internalisation of GLUT4, independent of the initial stimulus.

Key words: GLUT4 trafficking, Insulin, AMPK signalling, Cardiomyocytes, Sarcolemma, Transverse tubules

Introduction

Skeletal muscle tissue represents the most significant site of post-prandial glucose disposal and therefore plays a vital role in whole-body glucose homeostasis (Katz et al., 1983). Dysregulation of muscle-mediated glucose removal from the circulation is central to insulin resistance and type II diabetes (Defronzo et al., 1985). Although heart muscle is not a major site of glucose disposal, recent clinical studies have revealed that reduced insulin sensitivity is a risk factor for mortality in individuals with coronary heart failure (Swan et al., 1997; Doehner et al., 2005). In addition, it has been shown that measurements of insulin-stimulated glucose disposal are prognostic markers of coronary heart failure (Paolisso et al., 1999; Doehner et al., 2005). Heart muscle glucose transport shares many similarities to that occurring in skeletal muscle and is known to be associated with the translocation of the GLUT4 glucose transporter isoform from intracellular reservoir compartments to the limiting membranes of the sarcolemma and the transverse-tubule system of the cardiomyocytes (Fischer et al., 1997). Using immunogold labelling of GLUT4 in heart cells, Slot et al. reported insulin stimulation of GLUT4 translocation to both sarcolemma and the transverse-tubules membranes (Slot et al., 1997), whereas Davey et al. reported some selectivity in translocation to the transverse-tubules (Davey et al., 2007).

The signalling processes that lead to GLUT4 translocation in heart are complex and may depend upon the combined or related signalling pathways that operate through activated Akt, AMPK, PKCs and Ca^{2+}/calmodulin-dependent protein kinases (Lajoie et al., 2004; Kemi et al., 2007). Insulin action on glucose transport in heart appears to be primarily mediated by the Akt pathway (Matsui et al., 2006). The extent to which these pathways are inter-related and crosstalk with each other in heart muscle cells has been investigated (Bertrand et al., 2006). The effects of short-term exercise may be related to increased contractile activity and to the associated signalling. However, in experimental situations, short-term heart contractile activity appears to be linked to incompletely characterised signalling steps that are partially separate and distinct from AMPK signalling (Kolter et al., 1992; Yang and Holman, 2004). Similar considerations apply to exercise and contraction-regulated signalling in skeletal muscle (reviewed by Sakamoto and Goodyear, 2002; Sakamoto et al., 2003; Sakamoto and Holman, 2008).

As GLUT4 is translocated to the surface of at least two distinct cell membrane surfaces of the sarcolemma and the transverse tubules

in heart muscle cells, it is important to establish whether these separate signalling pathways lead to separate activation of GLUT4 translocation at these compartments. Studies adopting biochemical and subfractionation approaches to determine GLUT4 localisation in cardiomyocytes have led to the proposal of the presence of distinct intracellular pools of GLUT4, which may be differentially mobilised depending on the signalling pathway stimulated (Fischer et al., 1997). In this skeletal muscle system, insulin action has been proposed to lead to increases in both sarcolemma and transverse-tubule GLUT4, whereas contractile activity has been proposed to lead to additional recruitment of GLUT4 from the endosomal, TfR-positive pool, preferentially to the sarcolemma (Lemieux et al., 2000). Because of the high density of myofibrils present in heart and skeletal muscle, subfractionation of membranes is a particularly challenging technique and a high proportion of desired membranes can be lost or mixed. Therefore, studies using subfractionation methods to study trafficking in these tissues can be unreliable for quantitative analysis and the results are difficult to interpret in terms of GLUT4 trafficking from separate intracellular compartments.

In skeletal muscle, light and electron microscopy have been used to investigate the possibility of distinct recruitment of GLUT4 from separate intracellular compartments following the stimulation of initially distinct signalling (Cushman et al., 1998; Lauritzen et al., 2006; Lauritzen et al., 2008a). The notion of differential regulation of GLUT4 recruitment to the sarcolemma and transverse-tubules is further supported by microscopy studies on skeletal muscle from animal models of insulin resistance. In denervated muscle and muscle from fat-fed animals, reductions in GFP-GLUT4 translocation are reported to occur mainly at the transverse-tubule system (Lauritzen et al., 2008b).

As use of light microscopy techniques and GFP-tagged GLUT4 has shed useful information on the use of GLUT4 compartments in skeletal muscle, we have in the study described here, investigated GLUT4 compartmental localisation and trafficking in cardiomyocytes using confocal microscopy. A mouse model has been used in which a GLUT4 fusion protein with C-terminal GFP and HA-epitope in the primary exofacial loop (HA-GLUT4-GFP) has been expressed under the control of a muscle-specific promoter. As the construct that we have used has an exofacial epitope tag that will bind antibody only when the epitope is exposed at the surface, we have been able to quantitatively assess and compare the levels of recruitment of GLUT4 to the surface of the sarcolemma and the transverse tubules. This has clear advantages over analysis of the GFP signal alone. In addition, the availability of the exofacial HA-tag has allowed us to determine the route of internalisation of GLUT4. We have been able to trace the uptake and subsequent internal localisation of HA antibody that is initially bound at the cell surface. Our study reveals that, unlike the skeletal muscle system described above, cardiomyocytes have a common trafficking route for GLUT4 that is stimulated by insulin, contraction and energy-status signalling.

Results

GLUT4 translocation to the sarcolemma and transverse tubules in cardiomyocytes

In basal unstimulated cardiomyocytes, HA-GLUT4-GFP has a punctate and uniform distribution throughout the cell interior with a particular concentration at the perinuclear region. GFP is present only at very low levels at the sarcolemma and the exofacial HA epitope is not available to bind antibody (Fig. 1). GLUT4 does not appear to be aligned along regions that would correspond to the transverse tubules.

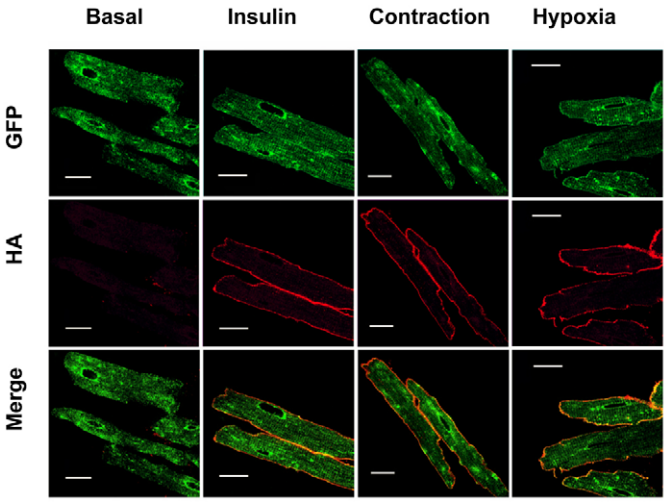

Fig. 1. Translocation of HA-GLUT4-GFP to the sarcolemma of insulin-, contraction- and hypoxia-stimulated cardiomyocytes. Isolated cardiomyocytes expressing HA-GLUT4-GFP were maintained in a basal unstimulated state or incubated with 30 nM insulin for 30 minutes, electrically induced to contract for 5 minutes or incubated under hypoxic conditions for 15 minutes. All cells were viewed in approximately the same focal plane. Bars, 20 µm. GFP (top panels) was detected between 505 and 530 nm, HA antibody (middle panels) was detected using Alexa-633 secondary antibody between 657 and 753 nm. Merged images are in the bottom panels. Results shown are from representative images from ~25 examined cells in five experiments.

When the cardiomyocytes were stimulated with insulin, with electrically induced contraction or by hypoxia (Fig. 1), then the GFP signal from the GLUT4 appeared to show more alignment along transverse tubules. The change in distribution of GFP to the sarcolemma surface was hard to visualise because the GFP signal becomes very thin when it becomes dispersed in the plane of the sarcolemma. In order to detect the translocation to the cell sarcolemma surfaces more readily, we have taken advantage of the availability of the exofacial HA-tag. We have found that when using whole HA antibody detection in non-permeabilised cells, the HA-epitope is only detected at the sarcolemma and not at the transverse-tubule membrane. This specificity in labelling is due to several factors that include the large size of the intact anti-HA antibody and the use of fixation of the cells before the incubation with the antibody.

To more readily detect the exposure of the exofacial HA epitope at both the sarcolemma and the transverse-tubule surfaces, we used a Fab fragment of the antibody. Cells were fixed after the incubation with the Fab fragment. The profile of the GFP and anti-HA signals within an intracellular region of interest (ROI) within a non-stimulated basal cell is diffuse (Fig. 2A). By contrast, in cells that have been stimulated with insulin, the GFP and anti-HA signals are clearly defined and localised transversely within the ROI (Fig. 2B). Cells stimulated by electrically induced contraction (Fig. 2C) and by hypoxia (Fig. 2D) also have strong transverse alignment of the GFP with the anti-HA signals that are similar to those produced following the insulin stimulation.

The signals in the ROI have been averaged along each transverse tubule (in the direction of the *y*-axis) and are presented as intensity plots below the fluorescence data (Fig. 2). This profile averaging efficiently eliminates single-pixel noise and random peaks caused by scattered GLUT4 vesicles. It is clear from these plots that the

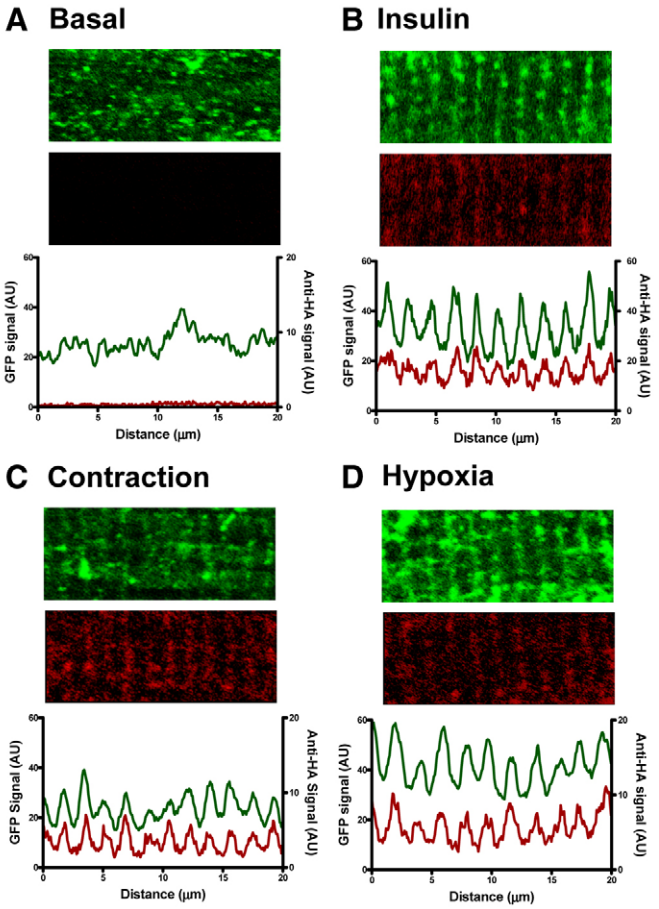

Fig. 2. Translocation of HA-GLUT4-GFP to the transverse tubules of insulin-, contraction- and hypoxia-stimulated cardiomyocytes. Isolated cardiomyocytes expressing HA-GLUT4-GFP were either maintained in the basal unstimulated state (A) or incubated with 30 nM insulin for 30 minutes (B), electrically induced to contract for 5 minutes at 100 V, 10 Hz, pulse width 1 msecond (C) or incubated under hypoxic conditions for 15 minutes (D). Data from the transverse-tubule GFP (top panels) and HA labelling (middle panels) were analysed in regions of interest (defined as described in Materials and Methods) as transverse intensity profiles (lower panels). Results shown are from representative images from ~25 examined cells in three experiments.

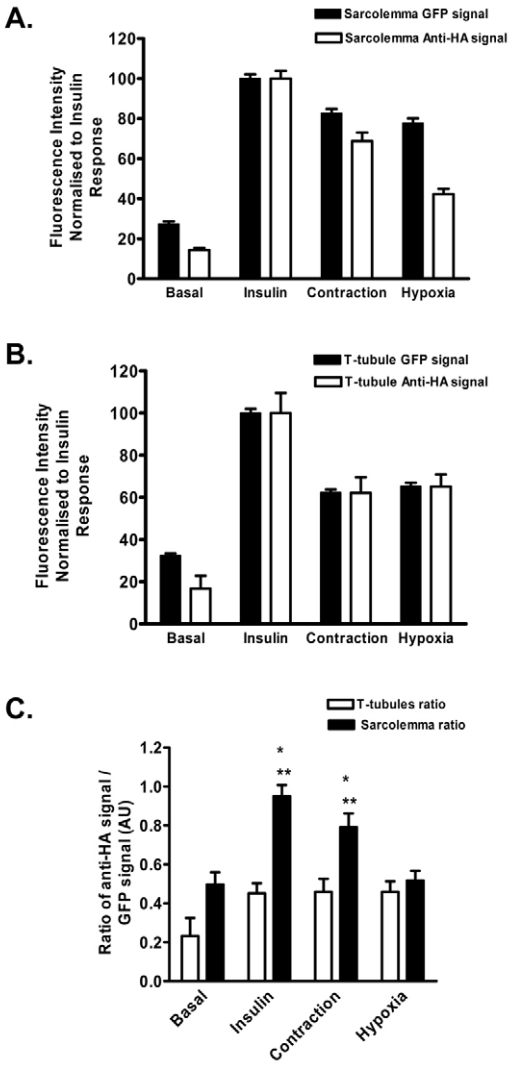

Fig. 3. Quantitative analysis and comparison of GLUT4 translocation to sarcolemma and transverse-tubule membranes of cardiomyocytes. Average GFP and anti-HA fluorescence intensity was determined from regions representing the sarcolemma and transverse-tubule membrane (as defined in the Materials and Methods). (A) Sarcolemma GFP (black bars) and anti-HA (white bars) signals measured under insulin, contraction and hypoxia stimulation. All fluorescence signals were normalised to the maximum values obtained under insulin stimulation. (B) Transverse-tubules GFP (black bars) and anti-HA (white bars) signals measured under insulin, contraction and hypoxia stimulation. (C) Ratio of anti-HA to GFP signals at sarcolemma and transverse tubules. Error bars are s.e.m. of 24-27 sarcolemma values and 40-90 transverse-tubules values from seven to ten cells per condition, from a single representative experiment. *P<0.05 versus basal; **P<0.05 versus hypoxia.

pattern of anti-HA signal very closely matches the pattern of the GFP signal. These data suggest that both signals are specific and correspond to a specific transverse GLUT4 distribution. This conclusion is supported by the calculation of Pearson's colocalisation coefficients. A non-specific Fab fragment was included as a negative control to ensure that signals were not a result of Fab fragments being 'trapped' by the fixation procedure. The co-localisation coefficients were significantly different from the non-specific control for all the conditions studied.

Use of the Fab fragment enabled analysis and comparison of sarcolemma and transverse-tubule labelling within the same cells. The patterns of sarcolemma labelling as recorded from the GFP and Fab anti-HA signals (our unpublished data) are similar to those obtained using the intact anti-HA antibody (Fig. 1).

Quantification of GLUT4 translocation responses

In order to compare the levels of translocation of GLUT4 induced by stimulations of different signalling pathways, both the GFP and anti-HA signals at the sarcolemma and the transverse tubules were quantified (Fig. 3). In comparison with the insulin response, contraction and hypoxia give rise to smaller responses at the sarcolemma (Fig. 3A) and the transverse tubules (Fig. 3B). No selectivity is apparent for the stimulated translocation response at the sarcolemma versus the transverse-tubules. The absolute values for the increases in the anti-HA and GFP at the sarcolemma and the transverse tubules cannot be directly compared because the possibility of different access of antibody to the exofacial surface of GLUT4 at these locations and to the differences in surface area

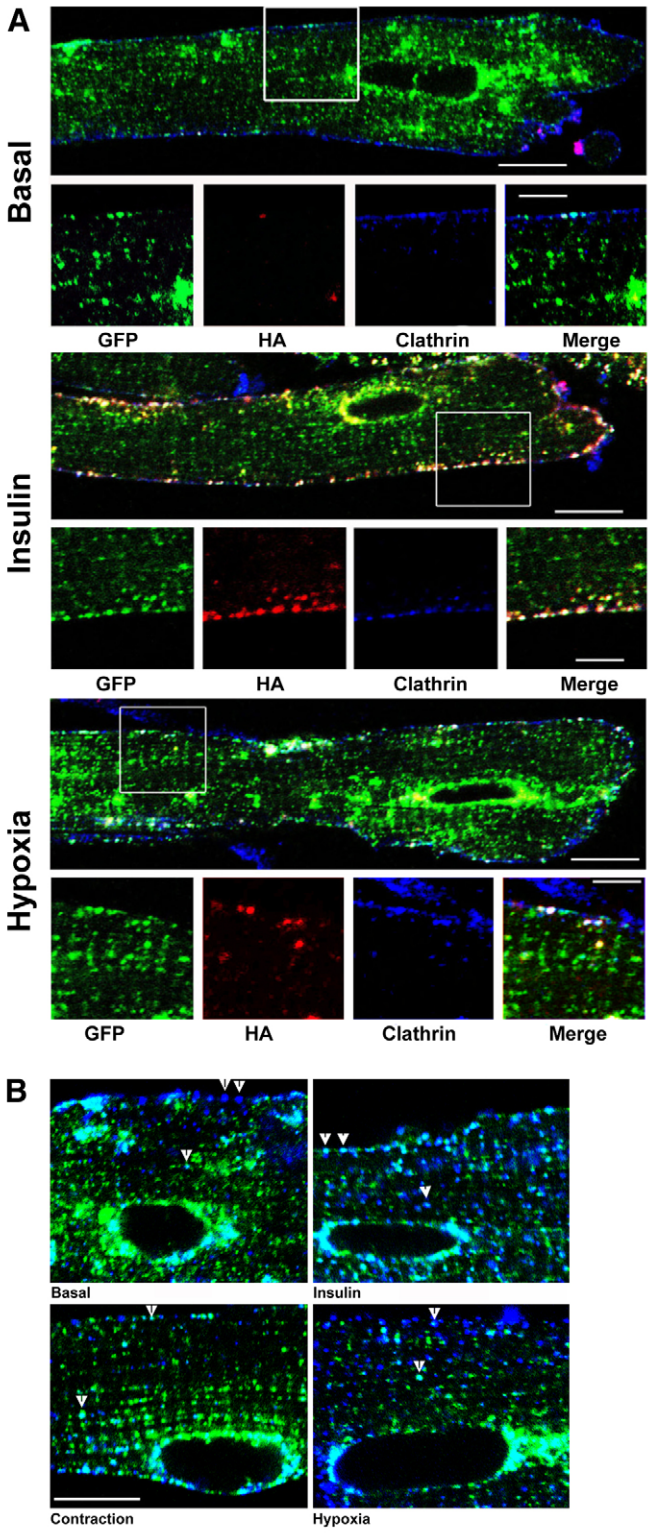

Fig. 4. Colocalisation of internalising GLUT4 with clathrin. (A) Isolated cardiomyocytes expressing HA-GLUT4-GFP were maintained in the unstimulated, basal state (top panels), stimulated with 10 nM insulin (middle panels) or in hypoxic buffer for 15 minutes (bottom panels). During treatment, cells were incubated with anti-HA antibody (5 μg/ml) at 37°C for 15 minutes. Following removal of the stimulus and washing to remove excess antibody, cells were incubated for 0-40 minutes. Images shown are from 10-minute time points. Results shown are representative images. All cells were viewed in approximately the same focal plane. Bars, 10 μm in large images; 5 μm in smaller images. (B) Similar data were obtained with anti-clathrin antibody, for which Alexa-568 conjugated goat anti-rabbit IgG antibody was used. Arrowheads indicate colocalisation of the GFP tag and clathrin. Results shown are representative of ~20 cells per condition examined in three experiments.

in the selected regions of interest that have been selected for analysis. The transverse-tubule network has been estimated to make up 66% of the total external membrane area of cardiomyocytes (Soeller and Cannell, 1999).

At the sarcolemma the insulin- and contraction-stimulated conditions lead to a greater increase in the anti-HA signal than the GFP signal (Fig. 3B). The increase in the anti-HA signal is approximately twice the increase in the GFP signal for these stimulating conditions and the change in anti-HA signal reaches a maximum of approximately sevenfold for the insulin stimulation (Fig. 3B). However, at the sarcolemma location and following the hypoxia stimulation, the rises in GFP and anti-HA signals are similar (both approximately threefold). This differential effect can be seen most readily by comparing the ratios of anti-HA to GFP (Fig. 3C), and it suggests that insulin and contraction signalling may have a greater stimulatory effect on the reaction, possibly vesicle fusion, that leads to full exposure of the HA-epitope tag. This effect of insulin and contraction signalling is not evident at the transverse tubules.

Internalisation of GLUT4 and return to the basal state following insulin, contraction and hypoxia stimuli.

In order to investigate the process of internalisation of GLUT4 mobilised to the sarcolemma by different specific stimuli, cardiomyocytes were stimulated and live cells were incubated with anti-HA antibody. This was used to pulse-label the GLUT4 at the sarcolemma via the HA-epitope. Cells were subsequently subjected to washing steps and reversal conditions to return the cells to an unstimulated basal state.

Upon removal of the stimulus, anti-HA staining at the sarcolemma became punctate, as shown clearly by the red anti-HA-antibody signal (Fig. 4A). The appearance of this punctate anti-HA staining was very rapid (within 1 minute) after the removal of the stimulus. This change is associated with an increase in anti-HA antibody detected at intracellular locations, including the peri-nuclear region. The punctate appearance of the anti-HA signal at the sarcolemma contrasts to images obtained during stimulations as in Fig. 1, where anti-HA staining appears as a contiguous signal at the cell surface.

When insulin- and hypoxia-stimulated cardiomyocytes are returning to a basal state, the GLUT4 becomes co-localised with clathrin at the sarcolemma (Fig. 4). The zoomed images (small insets in Fig. 4A) indicate colocalisation of anti-clathrin antibody (blue) with both anti-HA-antibody- (red) and the GLUT4-GFP signal (green). Clathrin-positive vesicles containing GLUT4-GFP, but not associated with anti-HA antibody are also evident at, adjacent to and further from the sarcolemma (Fig. 4A, insulin and hypoxia panels). In cardiomyocytes that are maintained in the basal state during the same labelling procedures, very little anti-HA antibody is taken up. However, GLUT4 that colocalises with clathrin is also evident at the sarcolemma (Fig. 4, basal panels). We have also found extensive colocalisation of GLUT4-GFP with clathrin at the sarcolemma, transverse tubules and perinuclear regions in the basal state and following the insulin, contraction and hypoxia stimulations (but using a different anti-clathrin antibody) (Fig. 4B).

Full internalisation of anti-HA antibody labelled-GLUT4 following the insulin, contraction and hypoxia stimuli occurs with indistinguishable time courses and is complete within 40 minutes for reversal of all three stimulation conditions. In all cases, the internalised anti-HA-tagged GLUT4 becomes predominately localised to the perinuclear area (Fig. 5). Antibody-tagged GLUT4 is also present at other locations in the cell. For example, some of the tagged GLUT4 remains close to the sarcolemma, although it is not possible to distinguish between internalising GLUT4 and GLUT4 that has recycled following processing through the perinuclear region. At full internalisation and the recovered basal state, neither the anti-HA-antibody signal nor the GFP is found associated with transverse tubules. This finding is consistent with the lack of strong alignment of GFP-GLUT4 along the transverse tubules in cells that have been continuously maintained in the basal state (Fig. 2A).

Discussion

Use of a new mouse model to study GLUT4 translocation

In this study we have used a new method to investigate the subcellular localisation and trafficking of GLUT4 in cardiomyocytes. A transgenic mouse line in which an HA-epitope-tagged and GFP version of GLUT4 has been expressed under the control of a muscle specific promoter has been used (I. Lisinski, V.A.L., D. R. Yver, O. Gavrilova, H. Al-Hasani and S.W.C., unpublished). Recombinant GLUT4 is expressed only in skeletal muscle and heart muscle, but not in adipose tissue. The mouse model allows the GLUT4 translocation to be studied in a physiological setting and in fully differentiated muscle cells.

The inserted HA-epitope is located in the first exofacial loop of GLUT4. This location of the epitope means that it is only accessible to antibody at the external surface of cells. It therefore allows a quantification of the levels of translocation and external surface exposure of GLUT4. This has advantages compared with the visualisation of the GFP alone as the GFP-tagged versions of GLUT4 can accumulate just below the surface membranes of cells, and it is often difficult to distinguish whether GLUT4 vesicles have fused with the limiting membrane and whether GLUT4 is fully exposed in a functional form. Exofacial epitope-tagged versions of GLUT4 have been previously used to study the translocation of GLUT4 in adipocyte (Lampson et al., 2000; Karylowski et al., 2004) and muscle cell lines (Wang et al., 1999; Antonescu et al., 2008), and in primary adipose cells (Lizunov et al., 2005). The trafficking of the C-terminally tagged GLUT4 in all these studied cases appears to closely parallel the trafficking of endogenous GLUT4. The localisation of HA-GLUT4-GFP in the cardiomyocytes mouse model closely parallels the localisation of endogenous GLUT4 in rat cardiomyocytes (Yang et al., 2002).

We have found that the exofacial exposure of GLUT4 at the surface of the sarcolemma can be selectively studied if the intact anti-HA antibody is used in fixed cells. This selectivity is due to the inability of the large intact antibody to penetrate the transverse-tubule spaces and to the tendency of the fixation procedure to narrow these spaces. Such restrictions in accessibility to the transverse tubules have been previously described for an antibody raised towards an exofacial epitope of the Na^+-K^+-ATPase β subunit (McDonough et al., 1996). It is particularly clear using the whole antibody in this way that in the basal state the levels of insertion of GLUT4 into the sarcolemma membrane are extremely low and barely detectable. This technique, using a method that does not involve disruption or extensive manipulation of the cells, therefore confirms that in cardiomyocytes very large increases in GLUT4 translocation occur.

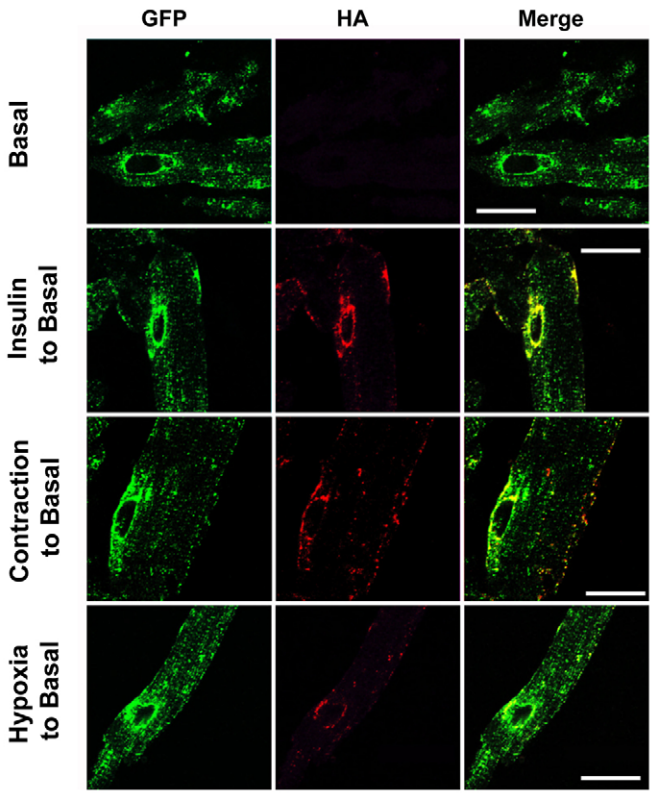

Fig. 5. Reversal of stimulation and internalisation of sarcolemma-tagged GLUT4 in cardiomyocytes. Isolated cardiomyocytes were either maintained in the unstimulated basal state (top panels), incubated with 10 nM insulin for 30 minutes (second row panels), electrically induced to contract (100 V, 10 Hz, pulse width 1 ms) for 5 minutes (third row panels) or incubated under hypoxic conditions for 15 minutes (bottom panels). During these treatments, cells were incubated with anti-HA antibody (5 μg/ml) at 37°C for 15 minutes. Following removal of the stimulus and washing to remove excess antibody, cardiomyocytes were incubated for a further 40 minutes to return to the basal state. All cells were viewed in approximately the same focal plane. Bars, 20 μm. Results shown are representative of ~25 cells examined in three experiments.

Translocation of GLUT4 to sarcolemma and transverse-tubule membranes

As is the case in skeletal muscle cells, heart muscle cells have two distinct locations, the sarcolemma and the transverse-tubule systems, at which GLUT4 can become accessible at the external surface. The transverse-tubule system greatly extends the membranous network of the cell and provides a large surface area over which glucose transport can be facilitated. In cardiac muscle the transverse tubules have approximately twice the surface area of the sarcolemma (Soeller and Cannell, 1999; Clark et al., 2001).

To measure translocation to the surfaces of the sarcolemma and the transverse-tubule system, we have used the Fab fragment of the anti-HA antibody in non-fixed cells. Because of the unknown extent of relative labelling efficiency in these two systems, we cannot directly compare the levels of translocation. However, by normalising to the insulin signal, we can compare the effects of the different signalling initiators on the final steps of translocation to

these systems. We find that insulin- and contraction-induced signalling produce essentially indistinguishable patterns of translocation to these systems. The insulin effect is more potent and produces a greater translocation, but this may be a technical issue associated with a decline in the contraction-induced signalling intermediates between the time of contraction induction and subsequent incubations. The fold changes in translocation to the transverse-tubule system appear approximately twofold lower than translocation to the sarcolemma, but estimating this fold change depends on the accuracy of recording the low basal level of translocation at these two locations. The changes in translocation at the sarcolemma induced by hypoxia produce a slightly but significantly different pattern of exposure of the HA-epitope tag to those produced by insulin- or contraction-induced translocation. The levels of translocation as detected with the HA-epitope tag exposure are significantly lower than those detected by alterations in the alignment of the GFP signal with this membrane. This suggests that, although the routes of GLUT4 vesicle trafficking are similar, the details of the kinetics of trafficking may differ. We have previously reported that whereas insulin action and contraction-induced stimulation lead to increases in the exocytosis of GLUT4 in cardiomyocytes, stimulation of the AMPK pathway by means of hypoxia, mitochondrial inhibition (Yang and Holman, 2005) or metformin action (Yang and Holman, 2006) lead to inhibition of endocytosis. These differences in the kinetics of trafficking may lead to a lower level of exposure of the HA-epitope relative to the GFP in the case of reduced endocytosis. It is possible that because exocytosis and fusion are lower, and occurring at the basal or unstimulated rates, non-fused vesicles at the sarcolemma may accumulate to a greater extent.

Internalisation of GLUT4 by a clathrin-dependent route

The routes and time courses for internalisation of GLUT4 are indistinguishable following the stimulations by insulin action, electrically induced contraction or hypoxia. The internalisation has been followed using the intact anti-HA antibody that, under the conditions used, preferentially associates with GLUT4 that is exposed at the sarcolemma membrane. Following reversal of these stimuli, the anti-HA antibody which attaches to GLUT4 very rapidly, within 1-2 minutes, appears just below the cell surface and becomes punctuate in appearance, suggesting the formation of internalising vesicles. These internalising vesicles are highly associated with clathrin and the anti-HA signal from GLUT4 colocalises with clathrin. These data are consistent with an immunogold study of GLUT4 in cardiomyocytes (Slot et al., 1991), and with a recent study on GLUT4 and clathrin within a 100 nm TIRF zone at the surface of 3T3-L1 cells (Huang et al., 2007).

Over longer (40 minute) periods of internalisation, HA-antibody-tagged GLUT4 becomes mainly associated with a perinuclear compartment, suggesting that long-range movements of GLUT4 occur in cardiomyocytes. The GLUT4 does not appear to relocate to localised satellite reservoirs. A recent study using time-lapse fluorescent confocal microscopy of quadriceps muscle in situ (Lauritzen et al., 2008a), led to the conclusion that translocation of GLUT4 to the sarcolemma and transverse tubules in response to insulin occurred locally and without long-range translocation of GLUT4 vesicles. Instead, localised static GLUT4 reservoirs present at the membranes are replenished as the insulin signal wanes and GLUT4 is internalised. However, we do not see clear evidence for such patches or satellite reservoirs of GLUT4 in basal cardiomyocytes or cardiomyocytes in which the HA antibody is internalised. This difference may be because of the relatively small size of the cardiac muscle cells compared with the long quadricep muscle fibres used with the gene injection technique. In skeletal muscle cells, a more localised translocation may have evolved as an adaptation in these longer multinucleate cells.

Much of the GLUT4 that is present in the perinuclear storage compartment of cardiomyocytes also colocalises with clathrin. The requirement for clathrin association in this location may be associated with vesicle budding reactions at the trans-Golgi network. Whether GLUT4 is associated with clathrin in the basal state has become a somewhat controversial issue. It has recently been reported that in 3T3-L1 cells, GLUT4 that is internalised in the basal state is not associated with a clathrin-dependent internalisation, but rather is associated with a non-clathrin, cholesterol-dependent, nystatin-inhibitable route (Blot and McGraw, 2006). Although we cannot rule out the possibility that a similar route for internalisation occurs in cardiomyocytes, it is evident that a large, but difficult to quantify, proportion of GLUT4 is associated with clathrin in the basal state. Furthermore, use of the HA antibody in the basal state indicates that those very few GLUT4 vesicles that are HA-tagged in the basal state, also associate with clathrin. Quantification of this colocalisation is difficult in this case because of the very low levels of HA-associated GLUT4. GLUT4 internalisation routes may differ between cell types and cell lines (Antonescu et al., 2008). In the future, it will be important to determine, more quantitatively, the proportion of GLUT4 that is internalised by the clathrin-dependent and clathrin-independent cholesterol-dependent route in heart muscle cells.

Convergence of signalling and a common GLUT4 trafficking route

A common trafficking route for GLUT4 that can be regulated by several separate signalling pathways could be considered to be consistent with the possibility that the separate signals lead to a single point of convergence with the GLUT4 trafficking pathway. Recent signalling studies in skeletal muscle have led to the postulation that the Rab-GAP proteins AS160 and TBC1D1 act as points of convergence for signalling from insulin, contraction, and energy-status signalling (reviewed by Sakamoto and Holman, 2008). Induction of signalling through these initially distinct pathways has been shown to lead to increased phosphorylation of AS160 and TBC1D1 (Kramer et al., 2006; Geraghty et al., 2007; Thong et al., 2007; Chavez et al., 2008). However, a single point of convergence cannot account for all aspects of stimulated GLUT4 translocation. Signalling via insulin and contraction in heart leads to increased exocytosis of GLUT4, whereas signalling via AMPK leads to reduced endocytosis of GLUT4 (Yang and Holman, 2005; Yang and Holman, 2006). These kinetic differences appear to be consistent with increased exposure of the HA-epitope tag at the sarcolemma surface in response to insulin and contraction, but not to hypoxia. We therefore propose that a common trafficking route exists for GLUT4 in cardiomyocytes that can be stimulated at different points and that these stimulatory responses produce subtle changes in the kinetics of movement along this route. Kinetic differences in GLUT4 translocation rates rather than differences in the translocation route per se may also account for the additivity of insulin- and hypoxia-mediated, but not insulin- and contraction-mediated, glucose transport responses observed in cardiomyocytes (Yang and Holman, 2005).

The HA-GLUT4-GFP mouse model that we have studied here is an important development as it has allowed analysis of GLUT4

distributions and translocations in cardiac muscle cells that are extremely difficult to study quantitatively using procedures that involve cell disruption. The epitope tagging technique has been previously applied to cell lines and the extension of this approach to a mouse model system should greatly facilitate future studies on insulin resistance. Such studies could address whether selective changes occur at distinct intracellular GLUT4 compartments in insulin-resistant heart cells.

Materials and Methods

Reagents

All chemicals, unless otherwise stated, were sourced from Sigma (Poole, UK). Type II collagenase was from Worthington Biochemicals (Lakewood, NJ, USA). Fatty acid-free bovine serum albumin was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Insulin was from Novo Nordisk (Bagsværd, Denmark).

Antibodies

Anti-HA antibody was from Covance (Berkeley, CA, USA). Anti-Clathrin antibodies were obtained from Sigma and Cell Signaling (Danvers, MA, USA). Mouse IgG isotype control was from Abcam (Cambridge, UK). All secondary antibodies were supplied by Invitrogen Molecular Probes (Paisley, UK). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA, USA). Fab fragments were generated using ImmunoPure Fab Preparation Kit from Pierce, following the manufacturer's instructions. Briefly, Anti-HA antibody (Covance) and mouse IgG isotype control (Abcam) were subjected to papain digestion for 5 hours at 37°C. Fab fragments were subsequently purified by binding undigested antibody and Fc fragments to a Protein A column. Collected fractions were pooled, concentrated and dialysed against phosphate buffered saline (PBS) pH 7.2 (12.5 mM Na_2HPO_4, 154 mM NaCl) containing 0.1% BSA. The concentrations of final solutions were determined by absorbance at 280 nm.

Transgenic mice

As detailed elsewhere (I. Lisinski, V.A.L., D. R. Yver, O. Gavrilova, H. Al-Hasani and S.W.C., unpublished), lines of C57Bl/6 mice were prepared homozygously expressing an HA-GLUT4-GFP transgene with a creatine kinase promoter. In brief, cDNA for HA-GLUT4-GFP (Dawson et al., 2001) was cloned into pBSMCK-polyA, which contains the muscle creatine kinase promoter (Bruning et al., 1998). The transgene was injected into fertilised eggs of FVB/N mice. These were implanted into C57B1/6 foster mothers and the presence of the transgene in the offspring was assessed by PCR amplification. According to all in vivo criteria, these mice exhibit normal growth, feeding behaviour, glucose homeostasis and reproduction. All procedures used in transgenic mouse generation were conducted in accordance with National Institutes of Health guidelines as approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases. The study and procedures for cardiomyocytes isolation conformed to UK Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act and were approved by the University of Bath Animal Care and Use Committee.

Cardiomyocyte isolation and stimulation

Cardiomyocytes from adult male (5-6 weeks, 22-25 g) transgenic mice expressing HA-GLUT4-GFP were prepared by collagenase digestion by use of a method previously described for rat cardiomyocytes (Eckel and Reinauer, 1980; Fischer et al., 1996) but with the inclusion of 20 mM inosine in the medium. Cell suspensions were adjusted to ~10% cytocrit in Krebs-Ringer-HEPES (KRH) buffer (6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM $NaHPO_4$, 1.4 mM $MgSO_4$, 1 mM $CaCl_2$, 128 mM NaCl, 10 mM HEPES pH 7.4) supplemented with 0.5% (w/v) fatty acid-free bovine serum albumin.

Cardiomyocytes were stimulated with insulin at 30 nM for 30 minutes. Stimulation by hypoxia was induced by incubation in KRH buffer with 0.5% fatty-acid free BSA that had been deoxygenated for 60 minutes and pre-gassed with nitrogen for a further 30 minutes. The nitrogen atmosphere was maintained throughout subsequent incubations. For electrical induction of contraction, 1 ml aliquots of cell suspensions at 37°C, ~10% cytocrit, were placed in 19-mm diameter polystyrene dishes. A dish lid with attached electrodes was dipped into the cell suspension. Cells were stimulated at exercise levels of contraction for 5 minutes at 100 V, with pulse duration of 1 ms and frequency of 10 Hz. Contraction was monitored under a microscope.

Selective immunofluorescent labelling of sarcolemma GLUT4

Stimulated cardiomyocytes were fixed in 4% paraformaldehyde for 20 minutes at room temperature and incubated in blocking buffer (3% goat serum and 1% BSA in PBS) for 30 minutes at room temperature. For detection of HA-GLUT4-GFP at the sarcolemma surface, cells were labelled overnight at 4°C with 1 μg/ml whole anti-HA antibody in blocking buffer. Cells were washed to remove unbound antibody and then incubated for 60 minutes at room temperature with Alexa-633 conjugated goat anti-mouse IgG secondary antibody at 4 μg/ml in blocking buffer. Finally, cells were washed in PBS and mounted in Vectashield mounting solution.

Immunofluorescent labelling of sacrolemma and transverse-tubule GLUT4

To label HA-GLUT4 at both the sarcolemma and the transverse tubules, 5 μg/ml HA-antibody Fab fragment or non-specific Fab fragment was incubated with the cells for 15 minutes at 37°C. For insulin and hypoxia stimulated cells, Fab fragments were added during the stimulus. For contraction stimulated cells, cells were first stimulated, and then labelled with Fab fragment for 15 minutes at 37°C. After stimulation (and labelling with Fab fragment), cells were washed with KRH buffer, fixed in 4% paraformaldehyde for 20 minutes at room temperature and incubated with blocking buffer containing 0.1% saponin for 30 minutes at room temperature. Saponin-permeabilised cells were then incubated for 60 minutes at room temperature with Alexa-633 conjugated goat anti-mouse secondary antibody at 4 μg/ml in blocking buffer. This allowed association of the secondary antibody with the Fab fragments bound to HA-GLUT4 at intracellular sites. Finally, cells were washed with PBS and mounted in Vectashield mounting medium.

Pulse labelling and internalisation of GLUT4

Cells were stimulated and subsequently pulse labelled with 5 μg/ml anti-HA whole antibody for 15 minutes at 37°C. For insulin- and hypoxia-stimulated cells, antibody was added during the stimulus. For contraction-stimulated cells, cells were first stimulated and then exposed to anti-HA antibody. Following removal of the initial stimulus, insulin-stimulated cells were washed through KRH buffer (pH 6.0) to strip insulin from its receptor. Contraction- and hypoxia-stimulated cells were transferred to basal oxygenated buffer. Unbound antibody was removed from all cells by washing with KRH buffer containing 0.5% fatty acid-free BSA. The cardiomyocytes were incubated to return to a basal state in KRH buffer with 0.5% fatty acid-free BSA at 37°C for specific times (0, 5, 10, 15 and 30 minutes). Internalisation was considered complete after 40 minutes.

Following the internalisation incubations, cells were washed with KRH buffer, fixed with 4% paraformaldehyde for 20 minutes at room temperature and then incubated with blocking buffer containing 0.1% saponin for 30 minutes at room temperature. The permeabilised cells were then incubated for 60 minutes at room temperature with Alexa-633 conjugated goat anti-mouse IgG secondary antibody at 4 μg/ml in blocking buffer.

For immunofluorescent labelling of clathrin, cells were washed to remove unbound secondary antibody and then incubated overnight at 4°C with anti-clathrin antibodies from Sigma diluted 1/40 in blocking buffer containing 0.1% saponin. Cells were subsequently incubated with Alexa-546-conjugated donkey anti-goat IgG secondary antibody. The same procedure was carried out with anti-clathrin antibody from Cell Signaling at 2.5 μg/ml but instead using Alexa-546 conjugated goat anti-rabbit secondary antibody. Finally, cells were washed in PBS and mounted in Vectashield mounting medium.

Confocal microscopy and image analysis

Confocal microscopy was performed on a Zeiss LSM 510 META microscope with 63× 1.4 NA oil-immersion objective. GFP, anti-Clathrin (Alexa-546) and anti-HA (Alexa-633) fluorescence were excited at 488 nm, 543 nm and 633 nm, respectively, and detected with corresponding channels 505-530 nm, 560-615 and 657-753 nm. Pinhole was set at one Airy disc, images were digitised at 16 bits with a pixel size of 0.07 μm. Gain and offset were optimised to fill the dynamic range and were kept constant for all images acquired for quantitative fluorescence measurements. For analysis, images were exported from a Zeiss LSM Data server (Carl Zeiss MicroImaging) to ImageJ (National Institutes of Health).

For colocalisation measurements, GFP and anti-HA signals were correlated and Pearson coefficients determined for cells labelled with anti-HA and non-specific Fab fragments. Regions of interest (ROIs) at the transverse tubules were selected to include intracellular areas of the cardiomyocyte, excluding sarcolemma and perinuclear regions. ROIs at the sarcolemma were carefully selected in order to exclude signals from adjacent transverse tubules. Single pixel noise was removed with ImageJ despeckle filter, and intensities of GFP and anti-HA signals were analysed and correlated using the Red-Green Correlator plugin. Pearson's coefficients were measured for one to three regions of five to seven cells for each condition.

Relative amount of HA-GLUT4-GFP exposed at transverse tubules and sarcolemma was estimated from measurements of GFP and anti-HA fluorescence signals. Profiles were calculated for regions of transverse tubule and sarcolemma, and averaged along the corresponding membrane (~100 lines/profiles per ROI) to increase signal-to-noise ratio. Sarcolemma ROIs were rotated such that membrane was parallel to the *y*-axis of the profile. Membrane regions were selected such that peak width corresponded to a diffraction limited image of a membrane. This was estimated from the full width half maximum of a point spread function (~7 pixels=0.5 μm). Profiles data were exported to Origin (MicroCal) and peak intensity and peak integrals (area under the peak) were automatically measured using a peak analysis subroutine. Peaks of a width significantly larger than 0.5 μm were excluded from analysis as they were likely to represent membrane regions with associated large intracellular GLUT4-containing compartments. Peak intensity and integrated value were adjusted for non-specific

background measured as a baseline level within the cell. For sarcolemma regions, peaks were adjusted for background difference inside and outside of the cell. For transverse-tubules peak values were averaged for 10-20 individual tubules within three ROIs per cell. For sarcolemma regions, peaks were averaged for three to five ROIs per cell. At least 10 cells were processed for each condition. All data presented as a mean value±standard error of the mean.

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