Immunogold labeling study of the distribution of GLUT-1 and GLUT-4 in cardiac tissue following stimulation by insulin or ischemia

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Davy KA, Garlick PB, Warley A, Southworth R. Immunogold labeling study of the distribution of GLUT-1 and GLUT-4 in cardiac tissue following stimulation by insulin or ischemia. Am J Physiol Heart Circ Physiol 292: H2009–H2019, 2007. First published December 22, 2006; doi:10.1152/ajpheart.00663.2006.—Whereas glucose transporter 1 (GLUT-1) is thought to be responsible for basal glucose uptake in cardiac myocytes, little is known about its relative distribution between the different plasma membranes and cell types in the heart. GLUT-4 translocates to the myocyte surface to increase glucose uptake in response to a number of stimuli. The mechanisms underlying ischemia- and insulin-mediated GLUT-4 translocation are known to be different, raising the possibility that the intracellular destinations of GLUT-4 following these stimuli also differ. Using immunogold labeling, we describe the cellular localization of these two transporters and investigate whether insulin and ischemia induce differential translocation of GLUT-4 to different cardiac membranes. Immunogold labeling of GLUT-1 and GLUT-4 was performed on left ventricular sections from isolated hearts following 30 min of either insulin, ischemia, or control perfusion. In control tissue, GLUT-1 was found predominantly in myocytes, distributed between sarcolemmal and T tubule membranes (1.84 m0.84 and 4.01 m0.27 golds/μm2). GLUT-4 was found predominantly in myocytes, distributed between sarcolemmal and T tubule membranes (1.84 m0.49 and 1.54 m0.33 golds/μm2) and intracellular vesicles (127 m18 golds/μm2). Insulin increased T tubule membrane GLUT-4 content (2.8 m0.4 golds/μm2, P < 0.05) but had less effect on sarcolemmal GLUT-4 (1.72 m0.53 golds/μm2). Ischemia induced greater GLUT-4 translocation to both membrane types (4.25 m0.84 and 4.01 m0.27 golds/μm2, respectively P < 0.05). The localization of GLUT-1 suggests a significant role in transporting glucose across the capillary wall before myocyte uptake via GLUT-1 and GLUT-4. We demonstrate independent spatial translocation of GLUT-4 under insulin or ischemic stimulation and propose independent roles for T-tubular and sarcolemmal GLUT-4.

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IN THE HEART, it has been proposed that glucose transporter 1 (GLUT-1) is primarily responsible for basal glucose uptake, whereas the insulin-regulatable GLUT-4 translocates to myocyte surface membranes to increase glucose transport in response to various stimuli, including insulin and ischemia (19). This translocation can be induced by two independent pathways: insulin stimulation leads to GLUT-4 translocation via a phosphatidylinositol 3-kinase (PI3-kinase)-mediated pathway (6, 8), and ischemia induces GLUT-4 translocation via a PI3-kinase-independent mechanism, most likely mediated by AMP-activated protein kinase (AMPK) (21, 29). Whereas GLUT-4 translocation under these stimuli is well established, the exact membrane locations to which GLUT-4 moves remain unidentified. In the heart, immunogold labeling has shown that, under control conditions, GLUT-4 is distributed in and around the sarcolemmal and T tubule membranes (30), but the relative responsiveness of GLUT-4 at these two locations to insulin or ischemia has not previously been addressed. Since glucose entering the myocyte via the sarcolemma or T tubules may serve different roles, GLUT-4 translocation to these two sites may be independently regulated.

Much of the current understanding of GLUT-1 and GLUT-4 localization and behavior comes from studies using centrifugation and Western blotting (9, 23, 26, 31, 38). Whereas these data provide basic information on GLUT distribution and translocation, the interpretation of such data is limited by the fact that subcellular fractionation cannot currently be used to distinguish GLUTs arising from the various membranes and cell types in a crude tissue homogenate (sarcolemma, T tubule membrane, intercalated disk, and capillary endothelial cell membranes, etc.). Confocal microscopy has also been used to analyze GLUT-transporter location in isolated myocytes, but while it provides more detailed distribution information, the conclusions that can be drawn are limited by the resolution of the confocal microscope (7, 41) and the fact that the cells are not part of an intact beating heart.

 Whereas the predominant energy substrate for the heart during normal metabolism is fatty acids, glucose metabolism becomes more prevalent during insulin stimulation (42) and ischemia (41). The interplay between glucose and fatty acid metabolism is well defined (32, 35); however, in isolated heart studies that investigate the control of glucose uptake, the effect of competing energy substrates is rarely examined.

In this study, therefore, we use immunogold labeling and stereological analysis to 1) visualize and quantify GLUT-1 and GLUT-4 distribution in cardiac tissue and the changes induced by competing energy substrates and 2) determine whether insulin stimulation and ischemia induce differential spatial translocation of GLUT-4.

MATERIALS AND METHODS

Reagents

Perfusion buffer chemicals were obtained from BDH (Poole, UK). The antibodies used were as follows. The primary antibodies were affinity-purified rabbit anti-GLUT-1 (Alpha Diagnostic International) and monoclonal anti-GLUT-4 IgG (Acris, DPC Biemann); polyclonal chicken anti-Na+/K+-ATPase IgG (Chemicon International) was used as a membrane marker. For Western blotting, the following

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secondary antibodies were used: for GLUT-1, anti-rabbit IgG, horse-radish peroxidase (HRP)-linked whole antibody (Amersham Biosciences); for GLUT-4, anti-mouse IgG, HRP-linked whole antibody (Amersham Biosciences); for Na⁺/K⁺-ATPase, HRP-linked goat anti-chicken secondary antibody (Sigma-Aldrich). For immunogold labeling, the following secondary antibodies were used: for GLUT-1, goat anti-rabbit 10-nm colloidal gold (BB International); for GLUT-4, goat anti-mouse 10-nm colloidal gold and goat anti-mouse 15-nm colloidal gold (BB International).

Animals

Male Wistar rats (250–300 g; n = 6/group), fed ad libitum with regular animal feed, were used for all experiments. All animals experiments were carried out in accordance with Home Office regulations as detailed in the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986, HMSO (London); Project License PPL 70/4992; personal license (K. Davey) PIL 70/16538.

Surgery and Perfusion Protocol

Rats were anesthetized with pentobarbitone sodium (120 mg/kg ip) and heparinized via the femoral vein (200 IU). Hearts were excised immediately immersed in ice-cold Krebs-Henseleit buffer. Hearts were cannulated and perfused under a constant pressure of 100 cmH₂O. The basic perfusion fluid was a phosphate-free Krebs-Henseleit buffer containing 11 mM glucose, having the following ionic composition (in mM): 144.0 Na⁺, 2.5 Ca²⁺, 1.2 SO₄²⁻, 25.0 HCO₃⁻, and 0.5 Na₂EDTA. To investigate the effects of competing substrate on myocardial glucose handling, extra groups of hearts were perfused with 10 mM glucose and 0.4 mM oleate (BSA bound), as described in the next section. The buffer was gassed with 95% O₂-5% CO₂ at 37°C using a membrane oxygenator to prevent frothing. Six groups of hearts were perfused in the Langendorff mode; these were insulin treated, ischemic, or control, with either glucose as sole energy substrate or with glucose and oleate in combination. Hearts were perfused for a baseline period of 60 min before being exposed to 30 min of either insulin stimulation (100 IU/l insulin in Krebs buffer at 10% of coronary flow via a side arm to give a final insulin concentration of 10 IU/l), ischemia (zero flow, normothermic), or a control perfusion period. At the end of these experiments, hearts were perfusion fixed for 5 min with 2% formaldehyde + 0.2% GLUT-araldehyde in Krebs-Henseleit buffer (pH 7.4) before immunogold electron microscopic analysis. A further group of aerobically perfused control hearts was then perfused and freeze clamped before GLUT-1 and -4 analysis by Western blotting.

Transmission electron microscopy of cryosections. Sections were examined and micrographs were obtained using a JEOL JEM-1200 EXII transmission electron microscope at an accelerating voltage of 80 kV. For gold particles to be seen, a magnification of 15,000 to 20,000 was needed.

Analysis of immunogold labeling. To evaluate antibody specificity and to identify cellular areas of preferential labeling for GLUT-1 and GLUT-4, a relative labeling index (RLI) was calculated, following the method of Mayhew et al. (25). With this stereological approach, all GLUT-1 and GLUT-4 gold labeling was assessed by comparing the observed distribution of gold particles to that which would have been expected with a random distribution for each membrane and compartment, normalized with respect to compartment size or membrane length.

RLI calculations. To calculate RLI for cellular membranes, test lines were superimposed on the micrographs (see Fig. 1). For each membrane present, the number of gold particles associated with it was counted to give nᵩ, the observed labeling. The length of the membrane with which these gold particles were associated was then assessed by counting the number of intersections the test lines made with the membrane, to give I, the observed intersections. The expected distribution, nₑ, that expected if all labeling was random, was calculated by using the following equation (25):

\[ nₑ = \left( \frac{tᵫ}{tP} \right) \times I \]

where tᵫ is total number of observed particles and tᵫ is total number of intersections.

RLI was then calculated using the following equation:

\[ RLI = \frac{nᵩ}{nₑ} \]

To calculate RLI for cellular compartments, test grids were applied to the micrographs. Gold particles were counted for each cellular compartment present to give observed labeling, nᵩ, as before. Compartment size was then estimated by counting the number of times a compartment intersected the join of two grid lines to give the observed number of points, P. The expected distribution, nₑ, was then calculated with the following equation (25):

\[ nₑ = \left( \frac{tᵫ}{tP} \right) \times P \]

where tᵫ is total number of points.

RLI was then calculated using the following equation:

\[ RLI = \frac{nᵩ}{nₑ} \]

RLIs were evaluated to assess the distribution of gold labeling across tissue sections. In randomly labeled membranes/compartments, RLI ≤1, whereas in preferentially labeled membranes/compartments, RLI >1 (25).
Analysis of GLUT Distribution Using Western Blotting

Differential centrifugation of sarcolemmal and endosomal membranes. Tissue (0.10–0.15 g) was taken from the left ventricle and used for subcellular fractionation by using the methods developed by Fuller et al. (14). Following the homogenization process described previously, homogenates underwent a series of sequential centrifugations, as follows: 1) 100 g for 10 min; 2) 5,000 g for 10 min; 3) 20,000 g for 30 min; 4) 50,000 g for 30 min; and 5) 100,000 g for 60 min. The precipitates from each of the centrifugation steps provided five fractions: fractions 1–3 corresponded to plasma membranes, and fractions 4–5 corresponded to intracellular membranes; 100% of cardiac membranes was recovered in this membrane fractionation protocol because no fractions were discarded.

Proteins were reconstituted in sodium dodecylsulfate sample buffer, and Western blot analyses were performed on aliquots from each of these fractions.

**Gel electrophoresis and immunoblotting procedure.** Electrophoresis was carried out according to the method of Laemli (22), modified by Fuller (14). To enable a direct comparison between our Western blotting and immunogold labeling data, we used the same affinity-purified GLUT-1 and monoclonal GLUT-4 antibodies for both techniques. Images were captured by using a fixed camera and Image Grabber 24.1.1 on an Apple Macintosh computer. Image contrast was adjusted to maximize the density of the blots, which were subsequently quantified by using Image Quant 5. A background correction was made, and the density of each of the five blots was evaluated for each of the antigens under investigation. The relative densities were then used to give a percentage of the protein in the sarcolemma (blots 1–3) compared with the percentage in endosomal membranes (blots 4 and 5). After images were obtained, blotting integrity was confirmed by staining polyvinylidene difluoride membranes with 0.25% Coomassie brilliant blue in 10% acetic acid-50% methanol.

**Statistical Analysis**

All data are expressed as means ± SE. Data were analyzed using Student’s t-test for unpaired data, with a Bonferroni correction. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Preliminary Data Establishing Labeling Specificity**

To determine the amount of labeling caused by nonspecific binding of the secondary antibodies, controls, which consisted of labeling of sections with the gold-conjugated secondary antibodies in the absence of the specific primary antibodies, were included in each incubation. Micrographs from these control sections are shown in Fig. 1, A and B; no gold labeling is observed, confirming secondary antibody specificity. In control-perfused hearts, significant labeling of GLUT-1 was seen in the membranes of the capillary endothelium (RLI = 1.719) when compared with the remaining membranes, where labeling was randomly distributed (sarcolemmal RLI = 0.652; T tubule RLI = 0.428). GLUT-1 labeling was only found to be specifically associated with cell membranes and no intracellular compartments. In control-perfused hearts, the membranes of the capillary endothelium showed no preferential labeling for GLUT-4, the RLI (0.058) being characteristic of a random distribution. The RLI of GLUT-4 in sarcolemmal and T tubule membranes was 2.651 and 2.460, respectively, indicating specific GLUT-4 labeling at these sites. In the cardiac myocytes, significant labeling of GLUT-4 was also identified in small vesicles in the cytosol (RLI = 14.771), whereas the remaining compartments were labeled randomly (mitochondrial RLI = 0.00; myofibril RLI = 0.093; and residual RLI = 0.300). These results show that GLUT-4 distribution was specifically associated with these intracellular vesicles and not randomly distributed across cellular compartments.

**Distribution of GLUT-1 and GLUT-4 in Cell Fractions As Shown by Western Blot Analysis**

Under control conditions, the majority of GLUT-1 (86 ± 6%) was found in the plasma membrane fractions (Fig. 2). In contrast, the majority of GLUT-4 (71 ± 8%) was found in the endosomal membrane fraction, with only 28 ± 8% associated with the plasma membrane fraction. We also found that 78 ±
4\% \text{ of } \text{Na}^+\text{-K}^+\text{-ATPase} \text{ was associated with the plasma membrane fractions, since } \text{Na}^+\text{-K}^+\text{-ATPase} \text{ is known to be mainly present in the sarcolemma; this indicates appropriate separation of the membranes by differential centrifugation.}

Intracellular Distribution of GLUT-1 and 4 by Immunogold Labeling, and the Effect of Insulin, Ischemia, and Buffer Composition

In control-perfused hearts, significantly more GLUT-1 was found to be located in the capillary endothelial cell membranes than in either the sarcolemmal or T tubule membranes (2.60 ± 0.41 vs. 0.27 ± 0.10 and 0.55 ± 0.21 golds/μm, respectively, \( P < 0.01; \) Figs. 3, A and B, and 4). These data indicate that 76\% of the total GLUT-1 in cardiac tissue was located in the capillary endothelium, with the remainder located in the myocyte membranes. It was not possible to distinguish intracellular from membrane GLUT-1 in the capillary endothelial cells due to their thinness. There was no evidence of GLUT-1 being present in intracellular vesicles in myocytes in any of the sections examined. This distribution was similar in all experimental groups and was unaffected by insulin stimulation, ischemia, or buffer substrate composition (Fig. 3, C and D, and Fig. 4).

In control cardiac myocytes, GLUT-4 was localized in intracellular pools, in the T tubules, and in the sarcolemmal membranes (Figs. 5, A–C, 6, and 7). Under high magnification, the intracellular GLUT-4 labeling could be seen to lie within small membrane-bound vesicles (Fig. 5C). Quantitatively, the majority of the GLUT-4 was found to reside in these intracellular vesicles (127 ± 18 golds/μm²), with the remainder evenly distributed between the sarcolemmal and the T tubule membranes (1.84 ± 0.49 and 1.54 ± 0.33 golds/μm, respec-
Fig. 3. Representative electron micrographs of cardiac GLUT-1 distribution. Very little labeling for GLUT-1 was observed in the myocyte itself (glucose-perfused heart; A), whereas there was significant GLUT-1 labeling in the capillary (glucose-perfused heart; B) Insulin (glucose + oleate-perfused heart; C) and ischemia (glucose-perfused heart; D) had no effect on GLUT-1 distribution. Myocyte GLUT-1 labeling is indicated by solid arrows and capillary GLUT-1 labeling by arrowheads. S, sarcolemma; C, capillary; Co, collagen.
very little GLUT-4 was found to be associated with the capillary endothelium.

In hearts perfused with glucose as sole substrate, insulin stimulation induced the translocation of GLUT-4 from intracellular vesicles (labeling decreased from 127 ± 18 to 86 ± 6 golds/μm²; P < 0.05, Fig. 6) to the T tubules (labeling increased from 1.54 ± 0.33 to 2.80 ± 0.36 golds/μm²; P < 0.05, Fig. 7). However, insulin stimulation had no effect on GLUT-4 content at the sarcolemmal membrane (Fig. 6). In contrast, ischemia caused a marked translocation of GLUT-4 to both the sarcolemmal (from 1.84 ± 0.49 to 4.25 ± 0.84 golds/μm²; P < 0.05) and T tubule membranes (from 1.54 ± 0.33 to 4.01 ± 0.27 golds/μm²; P < 0.05), with a concomitant decrease in intracellular vesicle GLUT-4 from 127 ± 18 to 101 ± 15 golds/μm² (P < 0.05, Figs. 6 and 7).

The addition of oleate to control-perfused hearts resulted in the internalization of GLUT-4 to intracellular vesicles (from 127 ± 18 to 175 ± 11 golds/μm²; P < 0.05) by relocation from the sarcolemmal (from 1.84 ± 0.49 to 0.51 ± 0.18 golds/μm²; P < 0.05) and T tubule membranes (from 1.54 ± 0.33 to 0.51 ± 0.10 golds/μm²; P < 0.05, Figs. 6 and 7). Insulin stimulation in these oleate-perfused hearts caused the translocation of GLUT-4 from intracellular vesicles to both sarcolemmal (from 0.51 ± 0.18 to 1.07 ± 0.12 golds/μm²; P < 0.05) and T tubule (from 0.51 ± 0.10 to 2.45 ± 0.28 golds/μm²; P < 0.05) membranes, resulting in a distribution similar to that seen following insulin stimulation in hearts that had been perfused with glucose alone. Ischemia also caused a significant translocation of GLUT-4 to both the T tubules and the sarcolemma in oleate-perfused hearts; the extent of translocation to these membranes (3.69 ± 0.74 and 3.88 ± 0.56 golds/μm², respectively) was markedly greater than that observed under insulin stimulation and comparable to that observed in glucose-only-perfused hearts.

**Double-Labeling Studies of GLUT-1 and GLUT-4**

Double labeling was not used for quantitative studies due to a decreased labeling efficiency; however, this method enabled the different locations of the two transporters to be examined in the same sections. The results are shown in Fig. 8. These studies confirm that the majority of GLUT-1 is located in the capillary endothelium, whereas GLUT-4 is found as the predominant GLUT transporter in the cardiac myocytes (Fig. 8A). A small amount of GLUT-4 can be seen localized to the sarcolemma following basal perfusion and after insulin stimulation (Fig. 8A and B). GLUT-4 is also present at intracellular locations from where it translocates to the sarcolemma and T tubule membranes; no GLUT-1 was observed within any myocyte compartments sampled (Fig. 8C).

**DISCUSSION**

Using immunogold labeling, we have demonstrated that GLUT-1 is found predominantly in the cardiac capillary endothelium, whereas the majority of GLUT-4 is found in the myocytes. This distribution would suggest that, in addition to its known role in basal myocyte glucose uptake, GLUT-1 also has a significant role in transcapillary glucose transport. We have also demonstrated that whereas ischemia induces significant translocation of GLUT-4 to both sarcolemmal and T tubule membranes equally, insulin stimulation induces significantly greater GLUT-4 translocation to the T tubule membranes than it does to sarcolemma. This differential regulation of GLUT-4 translocation suggests that insulin and ischemia have distinct target membranes for the redistribution of GLUT-4 and that glucose entering the myocyte via these two membrane routes may perform different roles.

**Methodological Considerations**

Whereas immunogold labeling has distinct advantages in terms of GLUT localization when compared with Western blotting, there are limitations associated with this technique. We found low labeling densities, but this is not unusual for proteins that are not highly expressed in relation to other cellular components (15, 16). Slot et al. (30) have also studied GLUT-4 distribution in rat myocardium, and they found higher labeling densities for their control tissues than we report here. There are, however, methodological differences between the

**Fig. 4.** Distributions of GLUT-1 in membranes and the effects of 30 min of insulin perfusion (100 IU/l at 10% flow) or zero-flow global ischemia on this distribution in hearts perfused with glucose only or glucose and oleate-containing buffer. Data are shown as means (n = 6) ± SE. *Significantly different from labeling density in sarcolemmal and T tubule membranes (P < 0.05).
two studies that could account for these differences. For example, Slot et al. (30) fasted their control animals, which could conceivably affect transporter distribution. We found a lack of clear definition of the sarcolemma at the magnifications used to show the overall distribution of the transporters along the plasma membrane; this particular problem has, however, been encountered by other investigators (10, 30), and, when labeling was investigated at higher magnifications, delineation of membranes was clearly visible (Figs. 3C and 5, C and D). Tissue samples can only be lightly fixed to ensure immunoreactivity; consequently, they are fragile during the sectioning process, and the endothelial cells are prone to tearing away

![Fig. 5. Representative electron micrographs of cardiac GLUT-4 distribution. Labeling of GLUT-4 was observed in T tubule or sarcolemmal membranes (arrowheads), or in submembrane vesicles (solid arrows). A: GLUT-4 labeling in and around T tubules in a glucose-only control heart. B: GLUT-4 labeling in and around the sarcolemma from a glucose-only control heart. C: high magnification of a GLUT-4 vesicle in a glucose-only control heart. Insulin caused GLUT-4 translocation to the T tubules membranes only in glucose-perfused hearts (D), and to a lesser extent, to sarcolemmal membranes in hearts perfused with glucose and oleate (E). Ischemia resulted in an increase in both sarcolemmal and T tubule GLUT-4 content in hearts perfused with glucose alone (F) or glucose and oleate (G). TM, T tubule membrane; IV, intracellular vesicle.](image-url)
from the myocytes. Nevertheless, the presence of collagen (connective tissue) between the endothelial cells and the myocyte surface indicates that the labeling seen on the myocyte surface corresponds to myocyte sarcolemma and not endothelial cell membranes. In immunoelectron microscopy, nonspecific labeling is frequently a problem (30). We have accounted for this by using RLI to confirm that the compartments under study had been labeled specifically. In addition to this approach, we have included control sections for each of our samples in which the primary antibody was omitted to ensure that nonselective binding of gold-conjugated secondary antibodies was not occurring (Fig. 1, A and B). We also determined experimentally the dilution of our primary antibody that gave minimal background labeling; this was likely to reduce specific labeling concomitantly. Nevertheless, the distribution of GLUT-4 in our stimulated cells is comparable with that reported by Slot et al. (30) for stimulated cells and within the range predicted by Griffiths (16) from theoretical calculations. Thus our results show the same pattern found by others using this technique, giving us confidence that we have shown specific labeling of the transporters in cardiac myocytes and are able to determine the localization of GLUT-1 and GLUT-4 by this method.

To ensure maximal glucose transporter responses to stimulatory conditions, supraphysiological concentrations of insulin (10 U/l) and a 30-min period of total, global ischemia were used in our protocols; these are in the range of those used by other workers in the field (28, 31) and allow direct comparisons to be made. Although the ischemic duration we have used is prolonged, no overt signs of necrosis were apparent in the micrographs from ischemic tissue. Although not physiological, the studies indicate maximal responses to insulin and ischemia, and it should therefore be noted that the extent of GLUT-4 translocation that occurs within the (patho)physiological ranges of these stimuli is likely to be more subtle.

GLUT-1 Localization

Quantification of GLUT-1 distribution using immunogold labeling indicates that it is predominantly localized to the capillaries, with only 24% of the total cardiac GLUT-1 contained in the myocytes themselves. The thinness of the endothelial cells in the lightly fixed cryosections meant that it was not possible to further resolve the location of the GLUT-1 within cells. Physical separation of these compartments by cell fractionation and Western blotting, however, provided this information, showing 86 ± 6% of GLUT-1 to be present at the cell surface. Although there could potentially be some cross-contamination of membrane fractions in the separation technique, since no evidence of GLUT-1 labeling was found intracellularly in myocytes, we calculate that 14% of total cardiac GLUT-1 is present inside capillary endothelial cells, 62% is in the capillary endothelial membrane, and the remaining 24% lies in myocyte membranes.

To the best of our knowledge, there have not been any previous immunogold studies that have successfully localized GLUT-1 in cardiac tissue, although such experiments have been attempted (17). Although this may be due to the antib
ies used, it is also possible that labeling for GLUT-1 was sought only in myocytes, where the labeling density will be low, whereas the capillary membranes were overlooked. Previous quantification of GLUT-1 in cardiac tissue has been by Western blotting of membrane fractions from whole tissue homogenates (9, 23, 26, 31, 38) or from immunofluorescence studies (11, 77). All of this previous work provides evidence for the localization of GLUT-1 in myocytes and concludes that, under both basal and stimulated conditions, the majority of GLUT-1 is located in plasma membrane fractions, as we confirm with our own Western blotting data; however, we, for the first time, demonstrate which plasma membranes in particular GLUT-1 resides in.

Because all methods of detecting GLUT-1 are indirect, absolute numbers of cardiac GLUTs are unknown. To date, only the ratios of GLUT-1 and GLUT-4 have been calculated and reveal that ~30% of the total transporters in the myocyte are GLUT-1 (12). Whereas our studies do not enable us to confirm this ratio of the two isoforms, our data indicate the presence of a further GLUT-1 pool not previously considered in cardiac tissue.

Although GLUT-1 is known to exist in endothelial cells (2, 24), the relative abundance of GLUT-1 and GLUT-4 in capillaries as opposed to myocytes has not been assessed. Previous work has identified GLUT-1 in cardiac arterioles (7) but the presence or absence of GLUT-1 in smaller vessels was not investigated. In the heart, it has been shown that the interstitial glucose concentration is only 50% of that in arterial blood (18). The cardiac capillary membrane therefore represents a significant barrier to glucose, and transcapillary transport, mediated by GLUT-1, may limit glucose availability to the myocyte (18). This would potentially have implications for the myocyte if capillary GLUT-1 were to redistribute in response to prevailing physiological conditions. Since we were unable to distinguish intracellular and membrane GLUT-1 in the capillary visually, we were unable to determine whether transendothelial GLUT-1 redistribution occurred in response to insulin or ischemia. We have previously assessed by Western blotting that acute ischemia does not cause GLUT-1 redistribution in isolated rat hearts (31), although GLUT-1 translocation has been reported by other groups following insulin stimulation (12), ischemia (9, 40), and hypoxia (7).

Our data indicate that only one-quarter of the total GLUT-1 found in the heart works in parallel with GLUT-4 to transport glucose into the myocyte; the remainder transports glucose from the capillary into the interstitial space acting in series with the myocyte transporters. In light of these findings, and what is known of GLUT-1 distribution in the brain and other tissues (10, 11, 39, 42), where similar relative distributions have also been observed, it is possible that the general role of the ubiquitous GLUT-1 is to extract glucose from the capillaries.

**GLUT-4 Distribution and Translocation**

Our GLUT-4 immunogold localization data confirm the findings from previous GLUT-4 immunogold analysis of cardiac tissue performed by Slot et al. (30) demonstrating GLUT-4 labeling at or near the sarcolemmal membrane, T tubules, and intercalated discs. Whereas the fact that GLUT-4 translocates to cardiac membranes in response to insulin and ischemia is well established, we demonstrate, for the first time,
which cardiac membranes GLUT-4 translocates to. Furthermore, we demonstrate that insulin and ischemia induce a different pattern of GLUT-4 redistribution to these membranes. Whereas insulin stimulation led to a significant translocation of GLUT-4 from intracellular pools to the T tubules, it had a smaller effect on GLUT-4 distribution at the sarcolemma. In contrast, ischemia resulted in a much greater, but equal, translocation of GLUT-4 to both the T tubule membranes and sarcolemma.

GLUT-4 translocation occurs via two different pathways, with insulin-mediated translocation occurring via PI3-kinase (8) and ischemia-mediated translocation occurring via AMPK (29). It is therefore likely that the PI3-kinase pathway exerts its effect primarily on movement of GLUT-4 to the T tubule membranes, with less of an effect on sarcolemmal GLUT-4, whereas AMPK causes redistribution to both membranes. Although it has been proposed that some of the downstream intermediates in these pathways may converge (20, 33), our findings would suggest that the response to each of the two stimuli remains distinct.

Insulin-stimulated GLUT-4 translocation has been widely investigated in cardiac tissue by using immunofluorescence and Western blotting (8, 12, 19, 27). Although these studies quantify GLUT-4 translocation following insulin stimulation, they do not differentiate between the different plasma membrane types within the myocyte. The only previous cardiac immunogold study demonstrates GLUT-4 translocation equally to both sarcolemmal and T tubule membranes (30). In their study, however, the authors used both insulin stimulation and increased contraction (by exercise) to induce GLUT-4 translocation; in the heart, contraction-stimulated GLUT-4 translocation is known to occur via the AMPK pathway. This addition of contraction stimulation could account for the higher GLUT-4 in sarcolemmal membranes seen in these studies compared with our insulin-only stimulated hearts. Our data support previous findings in skeletal muscle, where insulin-mediated glucose uptake occurred at the T tubules, only with no increase in GLUT-4 seen at the sarcolemmal membrane (13). This observation is also in agreement with other immunofluorescence studies performed in skeletal muscle cells (1, 4, 36).

We propose that the mechanisms underlying GLUT-4 translocation in response to insulin and ischemia are not only functionally distinct, as was previously known, but that the responses they produce are spatially distinct and that this new spatial information could provide an insight into the roles of GLUT-4 at these two cellular sites. Whereas the main role of the T tubules is to provide the rapid ionic exchange necessary for excitation-contraction coupling (5), glucose delivery via the T tubules would be the most direct route for delivering glucose to hexokinase located at the mitochondria (37) and its subsequent use in contraction, glycolysis, and glycogen synthesis. All these processes are moderated by both insulin and ischemia and are reflected in the responsiveness of T-tubular GLUT-4 to these stimuli. Whereas glucose entering the cell via sarcolemmal GLUT-4 may also contribute to these roles, we suggest that this “sarcolemmal glucose” may have an additional specialist role. The dependence of cardiac ion pumps on ATP derived specifically from glycolysis has been previously demonstrated (3). We suggest that the externalization of sarcolemmal GLUT-4 that we observe during ischemia occurs to meet the increased ATP demand of the sarcolemmal ion pumps (34). Since insulin has relatively little effect on myocardial energy demand or ionic homeostasis, providing increased glycolytic ATP to the sarcolemma would serve no purpose. This is consistent with the decreased sensitivity of sarcolemmal GLUT-4 to insulin that we describe.

Under normoxic conditions, we have demonstrated significant internalization of GLUT-4 in the presence of fatty acids. Insulin stimulation overrode this effect by translocating GLUT-4 from intracellular pools to the T tubules, and to a lesser extent, the sarcolemma. In hearts perfused with glucose only, insulin was seen to have no effect on sarcolemmal GLUT-4, whereas in hearts perfused with additional oleate, insulin increased GLUT-4 representation at the sarcolemma (although remaining lower than that observed in glucose-perfused hearts). Omission of oleate in the perfusate therefore masked this subtle effect of insulin on sarcolemmal GLUT-4 that occurs under more physiological conditions. Sarcolemmal GLUT-4 may therefore play some role in general cellular glucose metabolism. This highlights the importance of appreciating the interplay between competing energy substrates when investigating myocardial glucose metabolism. Under the extreme stimulus of ischemia, where glycolysis is essential for cell survival, this interplay becomes less important, and GLUT-4 translocates to both membrane locations to the same extent, irrespective of the presence of fatty acids.

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