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Translocation of two glucose transporters in heart: effects of rotenone, uncouplers, workload, palmitate, insulin and anoxia

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Abstract

Our previous studies on the acute regulation of glucose transport in perfused rat hearts were extended to explore further the mechanism of regulation by anoxia; to test the effects of palmitate, a transport inhibitor; and to compare the translocation of two glucose transporter isoforms (GLUT1 and GLUT4). Following heart perfusions under various conditions, glucose transporters in intracellular membranes were quantitated by reconstitution of transport activity and by Western blotting. Rotenone stimulated glucose uptake and decreased the intracellular contents of glucose transporters. This indicates that it activates glucose transport via net outward translocation, similarly to anoxia. However, two uncouplers of oxidative phosphorylation produced little or no effect. Increased workload (which stimulates glucose transport) reduced the intracellular contents of transporters, while palmitate increased the contents, indicating that these factors cause net translocation from or to the intracellular pool, respectively. Relative changes in GLUT1 were similar to those in GLUT4 for most factors tested. A plot of changes in total intracellular transporter content vs. changes in glucose uptake was roughly linear, with a slope of -0.18. This indicates that translocation accounts for most of the changes in glucose transport, and the basal pool of intracellular transporters is five times as large as the plasma membrane pool.

Keywords: Glucose transport; GLUT1; GLUT4; Rotenone; Workload; Palmitate; (Rat heart)

1. Introduction

Glucose transport is stimulated by insulin by means of the net outward translocation of glucose transporters from an intracellular pool to the plasma membrane [1]. This regulatory mechanism appears to be a general one for the short-term control of glucose transport. Previously, we employed membrane fractionation techniques that had been used to demonstrate translocation in response to insulin in heart [2], and showed that anoxia also stimulates transport via translocation [3]. Glucose transport is also stimulated by outward translocation in response to hyperosmolarity in adipocytes [4], phorbol esters in fibroblasts [5] and adipocytes [6], workload and methylglucose in heart [7], exercise in skeletal muscle [8,9], catecholamines in heart [10], and elevated pH in a liver cell line [11]; and inhibited by inward translocation in response to glucocorticoids in adipocytes [12] and fibroblasts [13]. However, some lines of evidence (reviewed in Ref. [14]) suggest that there is also regulation of the intrinsic activity of transporters.

In the work described here, we extended our previous studies [3] in three ways. First, we explored the nature of the stimulation by anoxia by examining effects of an inhibitor and uncouplers of oxidative phosphorylation. Second, we examined the effects a negative regulatory factor, metabolism of palmitate [15,16]. Since greater inhibition by palmitate was observed when glucose transport was stimulated in response to increased perfusion pressure [16] (referred to as increased 'workload'), we tested the effects of palmitate at both normal (60 torr) and elevated (120 torr) pressures. Third, we compared the translocation of GLUT4, the major transporter isoform in heart [17,18], and GLUT1, the minor form. In our earlier studies [3], we employed antibodies that probably recognized only GLUT1.

We previously measured changes in transporter content in both a plasma membrane fraction and an intracellular

Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HSP, high-speed pellet; ANOVA, analysis of variance.

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membrane (high-speed pellet, HSP) fraction. However, because the plasma membrane preparation was less reproducible, had lower yields of membrane protein, and had a relatively low specific activity of transporters, it was necessary to combine homogenates from sets of three hearts for each membrane fractionation. In the work reported here, we wished to examine multiple conditions within single experiments while avoiding long delays between the perfusions and the preparation of membrane fractions. Therefore the experiments were restricted to one heart per condition, and we prepared only the high-speed pellet fractions. Since decreases of transporters in the HSP fraction in response to insulin or anoxia are accompanied by increases in the plasma membrane fraction [3], the assay of only the former appears to be a valid means of assessing translocation.

Preliminary accounts describing some of this work have been presented [19,20].

2. Experimental procedures

2.1. Materials

Male Sprague-Dawley rats were from Harlan. Antibodies against a peptide within the carboxyl terminus of GLUT1 were from East Acres Biologicals. Affinity-purified antiserum against the carboxyl-terminal 19 amino acids of GLUT4 was a gift of Gustav Lienhard, Dartmouth Medical School. Rotenone, 2,4-dinitrophenol, FCCP, palmitic acid (sodium salt), and bovine albumin were from Sigma. Sources of radioisotopes and other chemicals were as listed previously [3,21].

2.2. Heart perfusion

Hearts were perfused by the Langendorff procedure and glucose uptake monitored as described previously [3], except as noted below. Perfusions were carried out for 24 min, with aliquots of the perfusate sampled at 3 min intervals.

For experiments with rotenone and uncouplers, perfusions were initially carried out in the absence of the reagents in order to verify normal beating of the heart. After 6 min of recirculating perfusion, the reagent (in 25 μl ethanol) was added to the perfusion buffer (25 ml). Control hearts received ethanol alone at 6 min.

For experiments with palmitate and increased workload, solutions of albumin (20%, w/v) were prepared in the perfusion buffer. A probe sonicator was used to solubilize palmitate (10 mM) in the albumin solution. Albumin, with or without palmitate, was then diluted 6.67-fold with perfusion buffer to give 3% albumin and, where present, 1.5 mM palmitate. These solutions were passed through 0.45 μm filters to remove small amounts of insoluble material. The albumin preparation employed ('essentially fatty acid free') contained fatty acid impurities of less than 0.005%, giving a final concentration in the perfusions of less than 5 μM fatty acid. The perfusion pressure was either kept at the normal 60 torr or increased to 120 torr, as indicated.

In our earlier studies [3], as well as in many of the experiments reported here, the perfusion buffer was oxygenated by bubbling the gas mixture into a buffer reservoir beneath the heart. To avoid foaming of perfusion buffer containing albumin, two alternatives were used. In the first (which was used for the experiments with fed rats), the recirculating system contained a coiled length (250 cm) of silastic tubing (o.d. 2 mm). This tubing was contained within a flask that was gassed with the 95% O_2, 5% CO_2 mixture; oxygen could diffuse through the tubing and into the perfusion buffer. For the experiments with fasted rats, we employed a perfusion apparatus (Radnoti Glass Technology) in which the perfusion buffer flows across a large surface area in the oxygenating chamber. Thus, bubbling the liquid is unnecessary.

For each set of conditions the order of treatments was varied in the different experiments to average out possible effects of the timing of the perfusions and/or membrane preparations.

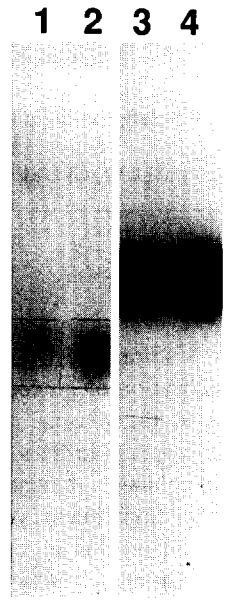

Fig. 1. Autoradiograms of rat heart membrane proteins labeled with antibodies against GLUT1 and GLUT4. Membrane proteins were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose paper, and labeled with antibodies and ^{125}I-protein A. The antibodies were specific for portions of the GLUT1 or GLUT4 carboxyl-terminal sequences. High-speed pellet membranes (50 μg protein) were from control hearts (lanes 2 and 4) or from hearts treated with 2 μM rotenone (lanes 1 and 3). Lanes 1–2 were labeled with anti-GLUT1 and lanes 3–4 with anti-GLUT4. The fine horizontal and vertical lines resulted from marking the autoradiograms before cutting out the corresponding portions of the nitrocellulose paper for gamma counting. The counting gave relative labeling (rotenone vs. control) of 0.69 for GLUT1 and 0.82 for GLUT4.

2.3. Preparation of intracellular membranes

At the end of each perfusion, the heart was flushed with 6 ml of ice-cold 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5). It was then trimmed, blotted, weighed, suspended in 15 ml of the same buffer, minced, and left on ice until all perfusions were complete. Hearts were then homogenized and high-speed pellet membrane fractions prepared by differential centrifugation as described by Watanabe et al. [2]; this fraction is enriched in the intracellular pool of glucose transporters. Marker enzymes (ouabain-sensitive *p*-nitrophenylphosphatase, 5′-nucleotidase, and UDP-galactose:*N*-acetylglucosamine galactosyltransferase) were assayed as previously described [3].

2.4. Quantitation of glucose transporters in intracellular membranes

Glucose transporter contents were determined by two methods. The first was reconstitution of stereospecific glucose uptake in liposomes, using proteins obtained after treatment of the membranes with 0.3% cholate [3]. In some cases the uptake was assayed for 30 min instead of 2 min, as was done previously [3]; this approximately doubles the stereospecific uptake. Small amounts of excess uptake of D-glucose over L-glucose observed with protein-free liposomes [3] were subtracted before calculation of the stereospecific uptake due to glucose transport. Some assays were performed which used either a less pure preparation of D-[^{14}C]glucose or liposomes prepared from soybean phospholipids instead of egg phospholipids, and the appropriate corrections for these were determined. The membranes prepared in a given experiment were reconstituted and assayed in parallel, and the stereospecific uptakes normalized to the appropriate control membranes. Typically the reconstitutions were performed twice and the uptakes relative to controls averaged.

The second measure of transporter content was Western blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and labeled with antibodies and ^{125}I-protein A as described [3], except that antibodies specific for GLUT1 or GLUT4 were employed. All membrane fractions from a perfusion experiment were run on the same gel, and labeling of each was compared to the appropriate control. Typically the Western blotting was performed at least twice and the relative labeling results averaged.

Because the absolute values determined in these two types of measurements were variable (depending, for example, on the freezing and thawing of the membranes and on the decay of ^{125}I), all values were compared to those for membranes from control hearts of the same experiment, and relative rather than absolute values are displayed in Figs. 2, 4, and 6–7. Since about 80% of the HSP transporters are GLUT4, and relative changes in GLUT1 were similar to changes in GLUT4 for all conditions tested, the GLUT4 assay is a good measure of total transporter content, in addition to the reconstitution assay. Therefore we averaged, for each experiment, the ratios obtained by these two techniques as a composite measure of total transporters. These composite ratios, as well as the separate reconstitution and GLUT4 ratios, were then analyzed over all experiments.

2.5. Statistics

Data were analyzed using both parametric (*t*-test; analysis of variance (ANOVA)) and nonparametric (Wilcoxon test; sign test) methods. In general, the results of the two classes of test were consistent, though the nonparametric tests usually indicated significance with lower confidence (i.e., higher *P* values). For ANOVA for the experiments with workload and palmitate, the data were analyzed as a 3-factor design with repeated measures on two factors (workload and palmitate) within the experiment, with the third factor (fasting status) remaining constant throughout the experiment [22].

2.6. Other assays

Protein was determined using Pierce's BCA* Protein Assay Reagent, with bovine albumin as a standard.

3. Results

3.1. Labeling of GLUT1 and GLUT4

Our previous studies [3] used three methods to quantitate transporters in membrane fractions. One was Western blotting using antibodies raised against the human erythrocyte glucose transporter. These probably recognized primarily the minor (GLUT1) form, since the carboxyl-terminal portion of the protein (which is distinct in the various isoforms) is the most antigenic portion. In the experiments described here, we used antibodies against peptides from the carboxyl-terminal sequences of GLUT1 and GLUT4, and thus could quantitate the two forms individually.

Fig. 1 illustrates the labeling of the two forms in the HSP membranes. The antibodies against GLUT1 and GLUT4 labeled bands with apparent molecular masses of about 44 and 50 kDa, respectively. The heart membranes were labeled much more strongly by anti-GLUT4 than by anti-GLUT1. In contrast, human erythrocyte membranes (which contain only GLUT1) were labeled very strongly by anti-GLUT1 but not at all by anti-GLUT4 (not shown).

3.2. Effects of rotenone

Rotenone blocks oxidative phosphorylation by inhibiting NADH dehydrogenase. Since we had shown that anoxia stimulates glucose uptake in the perfused heart at least in

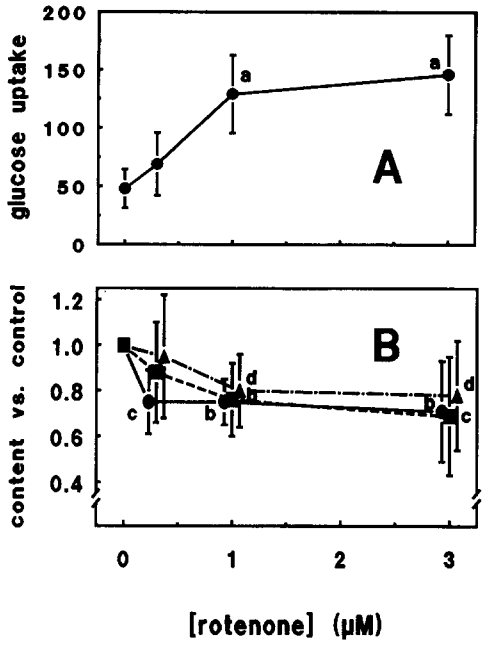

Fig. 2. Effects of rotenone on glucose uptake and transporter content in intracellular membranes. Isolated rat hearts were perfused by the Langendorff procedure in the presence of 0, 0.3, 1, or 3 μM rotenone. Following perfusions, HSP fractions were prepared and glucose transporter contents analyzed. (A) glucose uptake (μmol/h per g wet weight). (B) glucose transporter contents, relative to contents in membranes from control hearts. Contents were assessed by reconstitution of glucose transport activity (▲) or by Western blotting using anti-GLUT1 (●) or anti-GLUT4 (■). Some points in B have been displaced slightly along the x-axis for clarity of presentation. Results are expressed as means ± S.D. from 4 to 7 experiments (rotenone-treated uptakes and translocation results) or from 11 experiments (control uptakes). Since values are relative to controls, no error bar is indicated for 0 rotenone. Lower-case letters indicate values significantly different from controls by t-test: a, $P < 0.025$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.0005$. The points designated by letters (except for GLUT1 at 0.3 and 1 μM) were also significantly different from control ($P < 0.05$) by the Wilcoxon test.

part via translocation of glucose transporters from intracellular membranes (found in the HSP fraction) to the plasma membrane [3], we tested whether rotenone would also do so. Fig. 2A shows that rotenone stimulated glucose uptake about 3-fold, with the effect nearly maximum at 1 μM.

HSP membranes were prepared from rotenone-treated hearts and their contents of glucose transporters measured by reconstitution of transport activity and by Western blotting with antibodies against GLUT1 and GLUT4 (Fig. 2B). The reconstitution assay determines the total volume of liposomes that take up glucose, which in turn depends upon the total number of transporters rather than their rates. Since GLUT4 is about 80% of the total [17,18], this assay should primarily reflect the level of GLUT4. All three assays revealed decreases (20–30%) in the content of transporters in the HSP membranes in response to rotenone, consistent with a translocation of glucose transporters from this fraction to the plasma membranes. The concentration dependence for the translocation agreed with that for the stimulation of glucose transport. An example of the decreased labeling of GLUT1 and GLUT4 in response to rotenone is shown in Fig. 1.

At each rotenone concentration, the relative content of GLUT1 did not differ significantly from that of GLUT4; thus, the two transporter forms seem to be translocated to about the same extent, relative to their initial contents. Since GLUT4 is the major form, the absolute number of transporters translocated is larger for GLUT4 than for GLUT1.

Activities of marker enzymes (galactosyltransferase, ouabain-sensitive p-nitrophenylphosphatase, and 5′-nucleotidase) in the HSP fractions were unaffected by the rotenone treatment (data not shown). This indicates that the changes in transporter content were not artifactual consequences of changes in membrane fractionation in rotenone-treated hearts.

3.3. Effects of uncouplers

The fact that rotenone affected glucose uptake and transporter distribution similarly to anoxia suggests that both act by decreasing oxidative phosphorylation. To test this idea, we examined the effects of two uncouplers of oxidative phosphorylation, dinitrophenol and FCCP. Uncouplers were reported to stimulate sugar uptake in the perfused heart [23,24], as well as in diaphragm [25] and soleus [26,27] muscle.

As shown in Fig. 3, we observed no significant stimulation of glucose uptake by uncouplers; in fact, FCCP inhibited uptake at the highest concentrations tested. In some of these experiments, glucose transporters in HSP membranes were quantitated (Fig. 4). For dinitrophenol, the data show no evidence of translocation. For FCCP, there was a trend toward depletion of HSP transporters at the higher concentrations. However, this apparent translocation was accom-

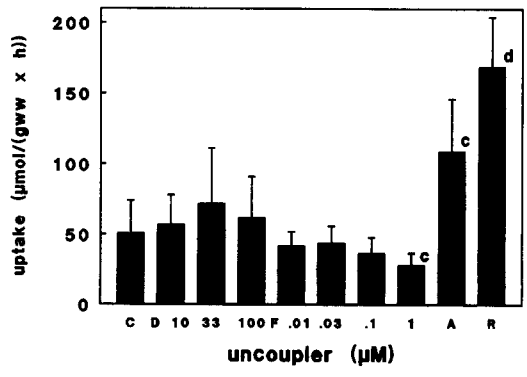

Fig. 3. Effects of uncouplers on glucose uptake. Hearts were perfused in the presence of various concentrations of uncouplers, under anoxic conditions, or in the presence of rotenone, and glucose uptake measured. C, control; D, dinitrophenol at 10, 33, and 100 μM; F, FCCP at 0.01, 0.03, 0.1, or 1 μM; A, anoxia; R, rotenone at 3 μM. Results are means ± S.D. from 3 to 7 (uncouplers, anoxia, rotenone) or 15 (control) experiments. Letters indicate values significantly different from controls, as in Fig. 2.

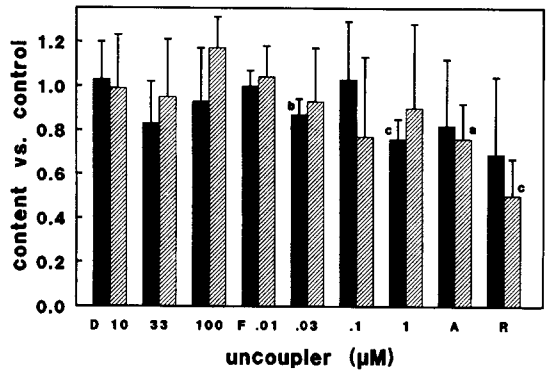

Fig. 4. Effects of uncouplers on transporter contents in intracellular membranes. HSP fractions were prepared from some of the hearts in the experiments of Fig. 3, and their contents of glucose transporters measured by reconstitution (solid bars) or by Western blotting using anti-GLUT4 (hatched bars). Results are relative to controls, and are means ± S.D. from 3 or 4 experiments. Labels on the *x*-axis are as in Fig. 3. Letters indicate values significantly different from controls, as in Fig. 2.

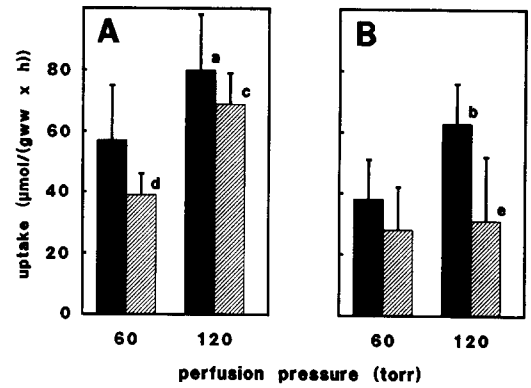

Fig. 5. Effects of palmitate and workload on glucose uptake. Hearts were perfused at 60 or 120 torr in the absence (solid bars) or presence (hatched bars) of 1.5 mM palmitate, and glucose uptake measured. (A) fed rats. (B) fasted rats. Uptake at 120 torr significantly greater (by *t*-test) than at 60 torr: a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.0005$. Uptake at 1.5 mM palmitate significantly less than in the absence of palmitate: d, $P < 0.05$; e, $P < 0.005$. Uptakes designated by letters were also significantly different ($P < 0.05$) from their corresponding controls by one or both of the nonparametric tests.

panied by a decrease rather than an increase in glucose uptake (Fig. 3). GLUT1 measurements in these experiments also did not reveal any strong evidence of translocation (not shown). In contrast, anoxia and rotenone treatment in these experiments both increased glucose uptake (Fig. 3) and decreased intracellular transporters (Fig. 4).

3.4. Effects of workload and palmitate on glucose uptake

Initial experiments testing these factors used rats fed ad libitum. The concentration of palmitate (1.5 mM) was similar to concentrations producing substantial inhibitions of glucose uptake and 3-*O*-methylglucose transport in earlier experiments [16]. Effects of workload and palmitate on glucose uptake are shown in Fig. 5A. The increased perfusion pressure increased uptake by about 40% in the absence of palmitate and by 80% in its presence. The former increase was smaller than reported previously (2.8- to 7-fold effects on glucose uptake or methylglucose efflux [7,16,28]). Palmitate inhibited uptake at both pressures. The inhibition at 60 torr (about 30%) is similar to that seen at normal perfusion pressures in earlier studies (19–29%, Refs. [15,16]). However, the decrease (14%) was not significant at 120 torr; in contrast, Neely et al. [16] observed 85% inhibition by 1.7 mM palmitate at 100 torr.

Because the earlier studies had used rats which had been fasted overnight [16], we performed additional experiments with fasted rats (Fig. 5B). The increased perfusion pressure, in the absence of palmitate, again increased glucose uptake (by about 65%). At 60 torr, palmitate again produced a modest (25%) inhibition. However, at 120 torr, palmitate produced a large (50%) decrease in glucose uptake, giving values similar to those obtained at 60 torr in the presence of palmitate. The inhibition was much larger than for fed rats at 120 torr (Fig. 5A).

3.5. Effects of workload and palmitate on intracellular glucose transporter contents

Intracellular membranes prepared from the hearts in the above experiments were analyzed for their contents of glucose transporters. Fig. 6 shows the composite measure of total transporters, obtained by averaging the reconstitu-

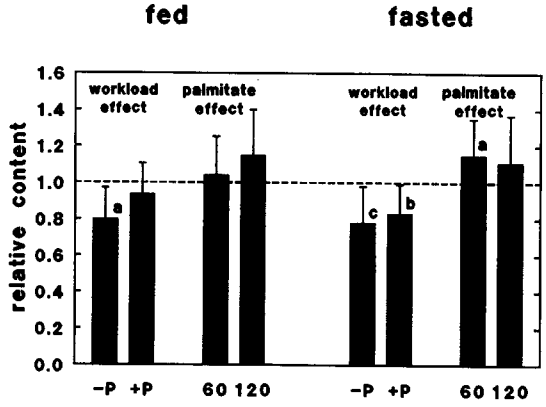

Fig. 6. Effects of palmitate and workload on glucose transporter content in intracellular membranes. Composite ratios of contents in treated vs. control membranes, based on both the reconstitution and GLUT4 assays, were determined for each experiment and averaged over all experiments. Results are means ± S.D. for 4–5 experiments with fed rats (left half of figure) or 5–8 experiments with fasted rats (right half). The first two bars of each half show the effect of workload (perfusion at 120 torr vs. 60 torr), either in the absence (first and fifth bars) or presence (second and sixth bars) of 1.5 mM palmitate. The second two bars of each half show the effects of 1.5 mM palmitate (plus vs. minus palmitate), either at 60 torr (third and seventh bars) or 120 torr (fourth and eighth bars). Mean ratios significantly different from 1.0 by *t*-test: a, $P < 0.05$; b, $P < 0.025$; c, $P < 0.005$. The same effects were also significant ($P < 0.05$) by one or both of the nonparametric tests.

tion and GLUT4 results. GLUT1 was also measured (not shown).

As shown at the left in each half of the figure, increased workload decreased the intracellular content of transporters, both in the presence and absence of palmitate and for both fed and fasted rats. The average decrease over all experiments was 17%. The decrease was significant ($P < 0.05$) for three of the four composite measurements shown in the figure, as well as for several of the measurements (reconstitution, GLUT1, GLUT4) not shown. ANOVA for the complete data set (fed and fasted rats, both with and without palmitate) yielded significant decreases as measured by reconstitution ($P < 0.025$), GLUT4 ($P < 0.01$), and the composite reconstitution-GLUT4 data ($P < 0.0005$). These results indicate a net outward translocation of transporters in response to workload, as previously reported [7].

As shown at the right in each half of Fig. 6, palmitate increased the intracellular content of transporters. For the composite data shown, the increase was relatively small (averaging 12% over all experiments), and was significant only for fasted rats at 60 torr. However, it was also significant by the following measures: reconstitution, fasted rats at 60 torr ($P < 0.05$ by t-test, Wilcoxon test, and sign test); GLUT1, fed rats at 120 torr ($P < 0.05$ by t-test and sign test) and fasted rats at 60 torr ($P < 0.005$ by t-test, $P < 0.05$ by sign test). Changes in GLUT4 were significant only at the $P < 0.1$–0.2 level (120 torr, both fed and fasted rats). ANOVA for the complete data set yielded significant increases as measured by GLUT4 ($P < 0.05$), GLUT1 ($P < 0.01$), and the composite reconstitution-GLUT4 data ($P < 0.025$). These results indicate that the inhibition of glucose transport produced by fatty acid metabolism arises at least in part from a net movement of glucose transporters (both GLUT1 and GLUT4) from the plasma membrane to intracellular membranes.

For all of the conditions tested (workload and palmitate effects for both fed and fasted rats), relative changes in GLUT1 were not significantly different from those in GLUT4, with the exception of the palmitate effect at 60 torr for fasted rats. In this case GLUT1 was increased 34 ± 20% compared to 5 ± 22% for GLUT4 (mean ± S.D., $n = 5$).

3.6. Relative translocation of GLUT1 and GLUT4 in response to insulin and anoxia

Studies with adipocytes and skeletal muscle have revealed differential translocation of GLUT1 and GLUT4 [9,29–38]. As noted above, in heart GLUT1 and GLUT4 are translocated similarly in response to rotenone (Fig. 2), workload, and palmitate. We also compared their translocation in response to insulin, anoxia, and insulin plus anoxia. Our previous studies of the effects of insulin and anoxia [3] indirectly demonstrated translocation of both transporter forms, since the antibodies probably recognized

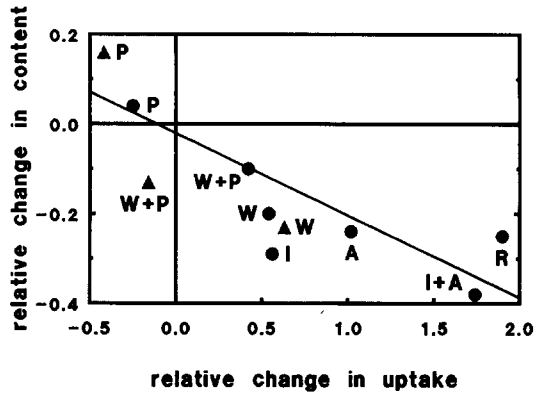

Fig. 7. Comparison of translocation to effects on glucose uptake. Effects of various perfusion conditions are expressed relative to hearts perfused at 60 torr in the presence of oxygen and absence of palmitate. For each treatment, the absolute values of glucose uptake (in μmol/g wet weight per h) were averaged for treated and for control hearts; the ratio of these averages, minus 1, is plotted on the x-axis. Negative values correspond to inhibition and positive values to activation. Effects on transporter content in HSP membranes are plotted on the y-axis. Results from GLUT4 and reconstitution assays (relative to control hearts from the same experiments) were averaged, when both values were available; otherwise the single value (GLUT4 or reconstitution) was used. These relative contents were then averaged over all experiments, and the average, minus 1, plotted. Negative values correspond to decreased contents relative to controls, and positive values to increased contents. ●, hearts from fed rats; ▲, hearts from fasted rats. Treatments, with number of experiments in parentheses: I, insulin (22); A, anoxia (9); I+A, insulin plus anoxia (7); R, rotenone (1 or 3 μM) (12); W, workload (perfusion at 120 torr) (4–7); P, palmitate (1.5 mM) (4–5); W+P, workload plus palmitate (4–7). The diagonal line is a fit to the data for fed rats only, which takes into account the control values by including seven additional points having the coordinates 0, 0.

only GLUT1, while the reconstitution and cytochalasin B binding measured total transporters (mostly GLUT4). Here we demonstrated this directly using specific antibodies.

For treatment with insulin, the depletion of GLUT1 in the intracellular membranes (45 ± 24%, $n = 11$) was greater than that of GLUT4 (31 ± 11%). This difference was significant ($P < 0.05$) by the Wilcoxon test, but by a t-test it was significant only at the $P < 0.1$ level. The decrease in GLUT1 was also greater than that of total transporters, as measured by reconstitution (28 ± 20%); the latter assay should reflect primarily GLUT4. For the case of anoxia, the decrease in GLUT1 (33 ± 16%, $n = 5$) was also greater than that in GLUT4 (22 ± 27%) and in reconstituted activity (20 ± 27%), but these differences were not statistically significant. When insulin and anoxia were combined, GLUT1 and GLUT4 both decreased by about 40%.

3.7. Additive effects of insulin and anoxia

The stimulation of glucose uptake by the combination of insulin and anoxia (2.86 ± 1.11-fold, $n = 5$) was significantly greater than that due to insulin alone (1.88 ± 0.57-fold, $n = 11$) ($P < 0.05$ by t-test, $P < 0.025$ by Wilcoxon

test). It was also larger than that produced by anoxia alone (2.13 ± 0.49-fold, $n = 5$), but in this case the differences were only significant at the $P < 0.1$ or $P < 0.15$ level, respectively, using the same two tests. When the average of the GLUT4 and reconstitution assays was used as a composite measure of total transporters, more translocation was seen for insulin plus anoxia (35 ± 9%) than for insulin alone (30 ± 13%) or anoxia alone (21 ± 22%). However, these differences were not statistically significant.

3.8. Comparison of translocation and effects on glucose uptake

Fig. 7 compares changes in intracellular transporter content (composite reconstitution and GLUT4 results) to changes in glucose uptake. All data were relative to a single control condition, perfusion at 60 torr in the absence of palmitate. Thus, the data for workload and palmitate differ from those shown in Fig. 6, where the effects of the two factors were considered separately. In general, the results show a linear trend. A fit to the data for fed rats using linear regression (diagonal line in the figure) gave a correlation coefficient of −0.89 and a slope of −0.18.

4. Discussion

The results presented here extend previous studies by ourselves [3] and others on the acute regulation of glucose transport in heart. The effects which we observed all occurred within the 24 min perfusion period, and thus likely are due to changes in the distribution or activity of existing transporters rather than to changes in their numbers.

4.1. Effects of rotenone

As shown in Fig. 2A, rotenone produces a large stimulation of glucose uptake by perfused hearts, in agreement with results for isolated heart cells [39]. This was accompanied, with the same dose dependence, by a decrease in the content of HSP glucose transporters (both GLUT1 and GLUT4) (Fig. 2B). These results indicate that the stimulation is produced, at least in part, by the translocation of intracellular glucose transporters to the plasma membrane.

The effects of rotenone on glucose uptake and transporter distribution are similar to those of anoxia [3], suggesting that both stimulate transport by blocking oxidative phosphorylation. Thus, the signalling pathway involved in the stimulation of glucose transport by anoxia may respond to the metabolic consequences of oxygen deficiency, rather than directly to the decreased oxygen.

The respiratory chain inhibitors cyanide and azide previously have been observed to stimulate methylglucose transport in diaphragm [25], soleus muscle [27], avian erythrocytes [40], and Clone 9 cells (a liver cell line) [41]. However, in adipocytes they did not affect transport in the absence of insulin [42]. Recently, it was reported that the increased uptake in avian erythrocytes [43] and Clone 9 cells [44] is due to activation of GLUT1 in the plasma membrane, without translocation [1]. These results differ from ours, where translocation (of both GLUT1 and GLUT4) is clearly involved.

Another recent study tested the effects of one or more hours of treatment with hypoxia or rotenone on deoxyglucose uptake in L6 myotubes [45]. Effects of hypoxia required the synthesis of GLUT1, in contrast both to the rapid translocation of GLUT1 and GLUT4 which we observed, and to the translocation of GLUT4 seen in skeletal muscle [46]. Effects of rotenone, while largely insensitive to cycloheximide, appeared to involve translocation of GLUT1 only, which also differs from our results. The same study also found rotenone-dependent translocation of GLUT1, but not GLUT4, in 3T3-L1 adipocytes. The authors pointed out the possible divergence of regulatory mechanisms between the relatively immature L6 myotubes and adult skeletal muscle. Our observations for heart more closely resemble the latter.

4.2. Effects of uncouplers

In contrast to the large effects of rotenone on glucose uptake and transporter distribution, we saw no stimulation of uptake (Fig. 3), and little change in HSP transporter contents (Fig. 4), in response to uncouplers. Previously, the uncouplers salicylate [23,24] and dinitrophenol [24] had been reported to stimulate glucose uptake in perfused heart, while FCCP stimulated uptake in cardiac cells [47]. Various uncouplers also stimulated glucose transport in skeletal muscle [25–27], avian erythrocytes [40,43], and thymocytes [48], but not in adipocytes [42]. We cannot account for the disagreement between our results and the earlier heart studies [23,24]. However, it should be noted that in those studies the effects of uncouplers were relatively weak, with stimulation of glucose uptake by only 20–45% (compared to 2- [25] to 8-fold [27] increases in muscle). Moreover, in one of them [23], dinitrophenol increased uptake during the first 15 min of perfusion, but decreased it during the next 15 min.

It was suggested [39] that while increased energy demand would be expected to increase glucose transport, a decrease in ATP that is too rapid would not allow this to occur, since translocation is itself ATP-dependent [49].

[1] In Ref. [44], our previous results [3] were described as if they supported a similar mechanism in heart: "The stimulation of glucose transport by hypoxia in rat myocardium is associated with only a minimal increase in the abundance of glucose transporters in the plasma membrane." However, as we noted on p. 19453, the observed increase of 50% in the plasma membrane fraction is likely an underestimate of the true increase in the plasma membrane, since there probably is cross-contamination with intracellular membranes.

Some studies with muscle [25,50] have found biphasic effects of dinitrophenol, with decreases in the stimulation of transport beyond a certain concentration. Our results with FCCP suggest movement of transporters from the intracellular pool (Fig. 4) but insufficient energy for their incorporation in the plasma membrane, such that glucose uptake is not activated (Fig. 3).

Because of the potential problem of ATP depletion, we tested dinitrophenol and FCCP over 10- and 100-fold ranges of concentrations, respectively (Fig. 3), but saw little effect on transport (although in nearly all cases the uncouplers clearly affected the hearts, as indicated by reduction or even complete cessation of the heart contractions [2]). Also, in preliminary experiments, we found that uncouplers (33 μM dinitrophenol or 30 nM FCCP) depleted ATP no more than did anoxia (Wheeler, T.J. and Fell, R.D., unpublished data). This suggests that the lack of transport stimulation by uncouplers was not simply due to a catastrophic depletion of ATP.

Thus, disruption of oxidative phosphorylation by anoxia or rotenone produced large effects on glucose uptake and transporter distribution, while uncouplers produced only small effects on uptake in previous studies of heart and none in ours. This difference suggests that a change in oxidation-reduction state might be a regulatory signal, since anoxia and rotenone produce a more reduced state, while uncouplers produce a more oxidized state.

4.3. Effects of workload and palmitate

In the experiments presented in Fig. 6, we explored the mechanisms by which workload increases, and palmitate decreases, glucose uptake. These phenomena are likely to be of physiological importance in allowing the heart to increase glucose metabolism when there is an increased energy demand, and to reduce glucose metabolism when fatty acids are being utilized. With respect to the former, it has been shown that the increase in uptake is related to increased coronary (rather than intraventricular) pressure [51]. With respect to the latter, glucose uptake in the human heart is inversely correlated with free fatty acid levels [52]. Elevated fatty acids may cause decreased glucose utilization and impaired cardiac function in diabetes [53] and in reperfusion after ischemia [54].

We demonstrated that increased workload causes a translocation of glucose transporters (both GLUT1 and GLUT4) from the intracellular pool. This agrees with the changes in total glucose transporters measured by Zaninetti et al. [7], who used a much different membrane fractionation procedure. We also found that palmitate, which inhibits glucose uptake, increases the intracellular contents of both transporter forms. Thus, as for glucocorticoids [12,13], an inhibitory factor can reduce glucose transport via net inward translocation. In contrast to the regulation in heart, palmitate stimulates glucose transport in adipocytes, and causes outward translocation of GLUT4 [55].

It was difficult to measure significant increases in HSP transporter content in response to palmitate, in part because of the large size of the intracellular pool compared to the plasma membrane pool (see below). This limits the degree to which the former can be increased by even a large decrease in the latter. While many of the individual measurements did not reveal significant changes, the data taken as a whole strongly support translocation; ANOVA for the complete data set showed significant increases for GLUT1, GLUT4, and the composite reconstitution-GLUT4 data.

Hearts from fasted rats (Fig. 5B) were more sensitive to palmitate inhibition than hearts from fed rats (Fig. 5A). This may be an adaptation to fasting, with glucose being spared for use by the brain. However, we did not see a correlation between the measured extent of translocation and the inhibition of glucose uptake.

4.4. Relative translocations of GLUT1 and GLUT4

For the various conditions tested, we generally observed relative changes in intracellular GLUT1 that were not significantly different from those in GLUT4. In the case of insulin treatment, the data support a larger relative decrease in GLUT1 than in GLUT4. This is the same pattern as was observed in adipocytes, where the relative depletion of GLUT1 was larger (by 24 to 400%) than that of GLUT4 [29–33].

Previously we showed, using antibodies that probably recognized only GLUT1, that disappearance of transporters from the HSP membranes in response to insulin or anoxia was accompanied by an increase in the plasma membrane [3]. Therefore it is likely that rotenone and workload, which decrease HSP GLUT1, also increase plasma membrane GLUT1. In contrast, skeletal muscle under basal conditions appears to have a greater fraction of its GLUT1 located in the plasma membrane, such that plasma membrane levels are not increased significantly by insulin or exercise, and changes in intracellular GLUT1 are difficult to measure [9,36–38,56]. While GLUT1 is mostly in myocytes in heart [57], in skeletal muscle it is largely in perineurial sheaths [56,57].

A question relevant to the relative translocations of GLUT1 and GLUT4 is their intracellular locations. A recent study [18] found that 20% of total cardiac GLUT1 could be recovered in vesicles adsorbed with anti-GLUT4. Since much of the GLUT1 may be in the plasma mem-

[2] This should not have prevented the delivery of glucose to the heart tissue, which in the Langendorff preparation is due to the retrograde perfusion of the aorta rather than to the ventricular contractions. Similar inhibitions of contraction were observed in response to anoxia and rotenone, where glucose uptake was increased. We did not notice that the uncouplers produced any abnormalities in perfusion pressure or flow suggestive of ischemia.

brane, this indicates that a large fraction of intracellular GLUT1 is colocalized in vesicles with GLUT4. Our observation of similar relative changes in GLUT1 and GLUT4 are consistent with such colocalization.

4.5. Insulin and anoxia in combination

Two lines of evidence from skeletal muscle support the idea that exercise and hypoxia translocate a pool of glucose transporters which is distinct from the insulin-regulated pool. First, insulin and exercise produce additive effects on glucose transport (e.g., Ref. [58]), while hypoxia produces effects which are additive with insulin but not with exercise [46]. Second, an intracellular membrane fraction can be isolated whose transporter content is reduced in response to insulin but not to exercise [9]. In agreement with this idea, we observed that insulin and anoxia produced additive effects on glucose uptake (about 3-fold stimulation, compared to about 2-fold for either factor alone), as seen by others (e.g., Ref. [23]) and in our earlier studies [3]. The translocation of glucose transporters was also greater for insulin plus anoxia than for either stimulus alone, as well as for rotenone. If the insulin-sensitive pool were more enriched in GLUT1, this would also account for the greater insulin-dependent depletion of GLUT1 than of GLUT4 which we observed. However, rotenone stimulated glucose uptake as much as insulin plus anoxia. Assuming that rotenone and anoxia recruit from the same pool, this suggests that our perfusion conditions did not produce the maximum possible effect of anoxia. The difficulty of achieving completely anoxic conditions during perfusions has been noted previously [59]. Studies of the effects of insulin and rotenone in combination would provide a further test of the hypothesis of distinct pools.

4.6. Translocation vs. changes in uptake

Fig. 7 compares changes in HSP transporter contents to changes in glucose uptake under various conditions. If translocation were the major determinant of changes in uptake, the points would be expected to lie near a line passing through the origin, as we observed.

If the intracellular membrane fraction is not significantly contaminated with plasma membranes, the slope of such a plot is determined by the relative sizes of the intracellular and plasma membrane pools of transporters. The observed slope in Fig. 7 (-0.18) indicates that the basal plasma membrane pool is about $1/5$ the size of the basal intracellular pool. Thus, the greatest possible increase in the latter would be about 18%, when the entire plasma membrane pool would move inward. This is consistent with the relatively small increases (about 12%) which we observed in the presence of palmitate (Fig. 6). Conversely, under the maximum stimulatory conditions which we observed (about a 200% increase in uptake), about 35% of the intracellular pool would move outward, increasing the plasma membrane pool to about 45% of the total.

In an immunocytochemical study [60], GLUT4 was labeled, after whole body fixation, in hearts from rats which had been fasted or given insulin and treadmill exercise. For the fasted rats, virtually no GLUT4 was found in the plasma membrane. In contrast, the increase in intracellular GLUT4 in response to palmitate (Fig. 6) indicates that there were significant levels of plasma membrane GLUT4 in the basal state of our perfusions. However, for stimulated hearts, labeling of plasma membrane GLUT4 increased to 42% of the total, which is in excellent agreement with the 45% calculated above.

Various lines of evidence have indicated changes in intrinsic activity of glucose transporters [12]. If such changes were occurring in heart in addition to translocation, they would produce deviations from a linear relationship in Fig. 7. The deviations from the regression line which we observed were similar to the experimental errors in the two measurements. Thus, there is no strong evidence for regulation of the intrinsic activity of the heart glucose transporters by the factors which we studied.

There has been considerable interest in whether insulin regulates transporter intrinsic activities. Cell surface labeling studies with adipocytes have found that increases in GLUT4 can largely account for the stimulation of glucose transport [30,34]. In our studies, the point in Fig. 7 for insulin treatment lies to the left of the regression line, whereas an increase in intrinsic activity would produce a shift to the right. The leftward shift could occur if glucose transport is not entirely rate-limiting for uptake [61].

In summary, the results indicate that in heart, translocation of glucose transporters can account for changes in glucose transport in response to various factors. GLUT1 and GLUT4 appear to move in parallel from or to an intracellular pool that is normally much larger than the plasma membrane pool. The regulatory features in heart differ in several respects from those seen in skeletal muscle and other types of cells.

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References

[1] Simpson, I.A. and Cushman, S.W. (1986) Annu. Rev. Biochem. 55, 1059–1089.
[2] Watanabe, T., Smith, M.M., Robinson, F.W. and Kono, T. (1984) J. Biol. Chem. 259, 13117–13122.

[3] Wheeler, T.J. (1988) J. Biol. Chem. 263, 19447–19454.
[4] Toyoda, N., Robinson, F.W., Smith, M.M., Flanagan, J.E. and Kono, T. (1984) J. Biol. Chem. 261, 2117–2122.
[5] Kitagawa, K., Nishino, H. and Iwashima, A. (1985) Biochem. Biophys. Res. Commun. 128, 1303–1309.
[6] Muhlbacher, C., Karnielli, E., Schaff, P., Obermaier, B., Mushack, J., Rattenbuber, E. and Häring, H.U. (1988) Biochem. J. 249, 865–870.
[7] Zaninetti, D., Greco-Perotto, R. and Jeanrenaud, B. (1988) Diabetologia 31, 108–113.
[8] Fushiki, T., Wells, J.A., Tapscott, E.B. and Dohm, G.L. (1989) Am. J. Physiol. 256, E580–E587.
[9] Douen, A.G., Ramlal, T., Rastogi, S., Bilan, P.J., Cartee, G.D., Vranic, M., Holloszy, J.O. and Klip, A. (1990) J. Biol. Chem. 265, 13427–13430.
[10] Rattigan, S., Appleby, G.J. and Clark, M.G. (1991) Biochim. Biophys. Acta 1094, 217–223.
[11] Hakimian, J. and Ismail-Beigi, F. (1991) J. Membr. Biol. 120, 29–39.
[12] Carter-Su, C. and Okamoto, K. (1985) J. Biol. Chem. 260, 11091–11098.
[13] Horner, H.C., Munck, A. and Lienhard, G.E. (1987) J. Biol. Chem. 262, 17696–17702.
[14] Czech, M.P., Clancy, B.M., Pessino, A., Woon, C.-W. and Harrison, S.A. (1992) Trends Biochem. Sci. 17, 197–201.
[15] Randle, P.J., Newsholme, E.A. and Garland, P.B. (1964) Biochem. J. 93, 652–665.
[16] Neely, J.R., Bowman, R.H. and Morgan, H.E. (1969) Am. J. Physiol. 216, 804–811.
[17] Calderhead, D.M., Kitagawa, K., Lienhard, G.E. and Gould, G.W. (1990) Biochem. J. 269, 597–601.
[18] Kraegen, E.W., Sowden, J.A., Halstead, M.B., Clark, P.W., Rodnick, K.J., Chisholm, D.J. and James, D.E. (1993) Biochem. J. 295, 287–293.
[19] Wheeler, T.J. (1990) FASEB J. 4, A1021.
[20] Wheeler, T.J., Fell, R.D., Cole, D. and Hauck, M.A. (1991) FASEB J. 5, A1190.
[21] Wheeler, T.J. and Hauck, M.A. (1985) Biochim. Biophys. Acta 818, 171–182.
[22] Cody, R.P. and Smith, J.K. (1987) Applied Statistics and the SAS Programming Language, 2nd Edn., pp. 166–176, Elsevier, New York.
[23] Morgan, H.E., Randle, P.J. and Regen, D.M. (1959) Biochem. J. 73, 573–579.
[24] Randle, P.J., Newsholme, E.A. and Garland, P.B. (1964) Biochem. J. 93, 652–665.
[25] Randle, P.J. and Smith, G.H. (1958) Biochem. J. 70, 490–500.
[26] Chaudry, I.H. and Gould, M.K. (1970) Biochim. Biophys. Acta 196, 327–335.
[27] Kohn, P.G. and Clausen, T. (1971) Biochim. Biophys. Acta 225, 277–290.
[28] Neely, J.R., Liebermeister, H. and Morgan, H.E. (1967) Am. J. Physiol. 212, 815–822.
[29] Zorzano, A., Wilkinson, W., Kotliar, N., Thoidis, G., Wadzinski, B.E., Ruoho, A. and Pilch, P.F. (1989) J. Biol. Chem. 264, 12358–12363.
[30] Calderhead, D.M., Kitagawa, K., Tanner, L.I., Holman, G.D. and Lienhard, G.E. (1990) J. Biol. Chem. 265, 13800–13808.
[31] Weiland, M., Schűrmann, A., Schmidt, W.E. and Joost, H.G. (1990) Biochem. J. 270, 331–336.
[32] Gibbs, E.M., Calderhead, D.M., Holman, G.D. and Gould, G.W. (1991) Biochem. J. 275, 145–150.
[33] Piper, R.C., Hess, L.J. and James, D.E. (1991) Am. J. Physiol. 260, C570–C580.
[34] Holman, G.D., Kozka, I.J., Clark, A.E., Flower, C.J., Saltis, J., Habberfield, A.D., Simpson, I.A. and Cushman, S.W. (1990) J. Biol. Chem. 265, 18172–18179.
[35] Vogt, B., Mushack, J., Seffer, E. and Häring, H.-U. (1991) Biochem. J. 275, 597–600.
[36] Douen, A.G., Ramlal, T., Cartee, G.D. and Klip, A. (1990) FEBS Lett. 261, 256–260.
[37] Goodyear, L.J., Hirshman, M.F., Smith, R.J. and Horton, E.S. (1991) Am. J. Physiol. 261, E556–E561.
[38] Goodyear, L.J., Hirshman, M.F. and Horton, E.S. (1991) Am. J. Physiol. 261, E795–E799.
[39] Haworth, R.A. and Berkoff, H.A. (1986) Circ. Res. 58, 157–165.
[40] Wood, R.E. and Morgan, H.E. (1969) J. Biol. Chem. 244, 1451–1460.
[41] Mercado, C.L., Loeb, J.N. and Ismail-Beigi, F. (1989) Am. J. Physiol. 257, C19–C28.
[42] Kono, T., Robinson, F.W., Sarver, J.A., Vega, F.V. and Pointer, R.H. (1977) J. Biol. Chem. 252, 2226–2233.
[43] Diamond, D.L. and Carruthers, A. (1993) J. Biol. Chem. 268, 6437–6444.
[44] Shetty, M., Loeb, J.N., Vikstrom, K. and Ismail-Beigi, F. (1993) J. Biol. Chem. 268, 17225–17232.
[45] Bashan, N., Burdett, E., Guma, A., Sargeant, R., Tumiati, L., Liu, Z. and Klip, A. (1993) Am. J. Physiol. 264, C430–C440.
[46] Cartee, G.D., Douen, A.G., Ramlal, T., Klip, A. and Holloszy, J.O. (1991) J. Appl. Physiol. 70, 1593–1600.
[47] Haworth, R.B., Hunter, D.R. and Berkoff, H.A. (1985) Arch. Biochem. Biophys. 239, 191–199.
[48] Reeves, J.P. (1975) J. Biol. Chem. 250, 9413–9420.
[49] Kono, T., Suzuki, K., Dansey, L.E., Robinson, F.W. and Blevins, T.L. (1981) J. Biol. Chem. 256, 6400–6407.
[50] Korbl, G.P., Sloan, I.G. and Gould, M.K. (1977) Biochim. Biophys. Acta 465, 93–109.
[51] Takala, T.E.S., Kainulainen, H., Komulainen, J. and Ruskoaho, H. (1991) Mol. Cell. Cardiol. 23, 381–385.
[52] Wisneski, J.A., Gertz, E.W., Neese, R.A., Gruenke, L.D., Morris, D.L. and Craig, J.C. (1985) J. Clin. Invest. 76, 1819–1827.
[53] Wall, S.R. and Lopaschuk, G.D. (1989) Biochim. Biophys. Acta 1006, 97–103.
[54] Lopaschuk, G.D., Spafford, M.A., Davies, N.J. and Wall, S.R. (1990) Circ. Res. 66, 546–553.
[55] Hardy, R.W., Ladenson, J.H., Henriksen, E.J., Holloszy, J.O. and McDonald, J.M. (1991) Biochem. Biophys. Res. Commun. 177, 343–349.
[56] Marette, A., Richardson, J.M., Ramlal, T., Balon, T.W., Vranic, M., Pessin, J.E. and Klip, A. (1992) Am. J. Physiol. 263, C443–C452.
[57] Doria-Medina, C.L., Lund, D.D., Pasley, A., Sandra, A., and Sivitz, W.I. (1993) Am. J. Physiol. 265, E454–E464.
[58] Nesher, R., Karl, I.E. and Kipnis, D.M. (1985) Am. J. Physiol. 249, C226–C232.
[59] Williamson, J.R. (1966) J. Biol. Chem. 241, 5026–5035.
[60] Slot, J.W., Geuze, H.J., Gigengack, S., James, D.E. and Lienhard, G.E. (1991) Proc. Natl. Acad. Sci. USA 88, 7815–7819.
[61] Morgan, H.E., Henderson, M.J., Regen, D.M. and Park, C.R. (1961) J. Biol. Chem. 236, 253–261.