J. Biochem. 122, 90-95 (1997)

The β_3-Adrenergic Agonist BRL37344 Increases Glucose Transport into L6 Myocytes through a Mechanism Different from That of Insulin[1]

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Received for publication, January 30, 1997

In the present study, we examined the effects of BRL37344, a selective β_3-adrenergic agonist, on glucose transport into L6 myocytes and the results were compared with the effects of insulin. Insulin increased 2-deoxyglucose (2-DG) uptake in a dose-dependent manner, with maximal stimulation at 10^{-7} M. BRL37344 ranging from 10^{-7} to 10^{-5} M also enhanced 2-DG uptake in the absence of insulin. The effects of insulin and BRL37344 were completely additive, suggesting that these two agents enhance glucose uptake by L6 myocytes through different mechanisms. In fact, BRL37344 apparently did not increase tyrosine phosphorylation of cellular proteins in L6 myocytes, whereas insulin stimulated tyrosine phosphorylation of 180-190 and 95 kDa proteins. Furthermore, BRL37344-induced increase in glucose transport was not blocked by wortmannin, an inhibitor of phosphatidylinositol 3-kinase, whereas the insulin-induced effect was completely abolished. When L6 myocytes were incubated with insulin, the content of GLUT4 in the plasma membrane was increased. However, BRL37344 did not affect the GLUT4 content in the plasma membrane. BRL37344 did not increase the V_{max} value for glucose uptake but decreased the K_m value, although insulin increased the V_{max} value. These results suggest that BRL 37344 enhances glucose transport into L6 myocytes through a signaling pathway different from that of insulin and that the mechanism does not involve the translocation of GLUT4, but may be due to an increase in the intrinsic activity of GLUT present in the plasma membrane.

Key words: β_3-adrenoceptor, β_3-agonist, glucose transport, insulin, skeletal muscle.

Skeletal muscle, accounting for a large part of the total body mass, is a major tissue for blood glucose utilization. It is well known that glucose uptake into skeletal muscles is under the regulation of various stimuli, including insulin and muscle contraction (*1-4*). Our previous studies have shown that electrical and chemical stimulation of the ventromedial hypothalamus (VMH), a presumed center of the sympathetic nervous system (*5*), also enhances glucose uptake into skeletal muscles in rats under anesthesia and treatment with muscle relaxant (*6, 7*). The enhanced glucose uptake after VMH stimulation is abolished by treatment of rats with guanethidine, which blocks the postganglionic sympathetic neuronal activity (*7*). These results suggest that sympathetic nerves, as well as insulin and muscle contraction, contribute to the regulation of glucose uptake in skeletal muscles.

Recently, the novel β-adrenoceptor (AR) termed β_3-AR has been identified and characterized (*8-10*). It is considered that the β_3-AR mediates the sympathetic regulation of energy metabolism in adipose tissue and skeletal muscle. In fact, selective agonists for the β_3-AR can mimic the stimulatory effects of the sympathetic nerves on glucose uptake in brown adipose tissue and skeletal muscles in rats *in vivo* (*11, 12*). Furthermore, β_3-agonist directly stimulates glucose uptake in brown adipocytes in primary culture (*13*) and isolated soleus muscle *in vitro* (*11*). Recent pharmacological studies have demonstrated the presence of atypical β-AR in skeletal muscles (*14-18*), as well as in adipose tissues (*8-10*). Molecular studies have also detected mRNA of β_3-AR in soleus muscle of rats by the RT/PCR method (*18*). However, intracellular events leading to the increase in glucose uptake in skeletal muscles in response to β_3-agonists are not yet understood.

L6 myocytes have been used widely for the analysis of the glucose transport system in skeletal muscle (*3, 19-21*). The cell line is a continuous clonal line of myoblasts originally derived from the thigh muscle of neonatal rats (*19*). These cells can divide indefinitely as myoblasts, but if allowed to grow to confluence, they undergo terminal differentiation into multinucleated skeletal muscle myotubes. Myotubes, but not myoblasts, express GLUT4 glucose transporter (muscle/adipocyte type) as well as

[1] This work was supported by grants from The Mitsubishi Foundation, the Uehara Memorial Foundation, the Japan Diabetes Foundation and Senri Life Science Foundation, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: β-AR, β-adrenoceptor; 2-DG, 2-deoxyglucose; DMEM, Dulbecco's modified Eagle's medium; GLUT1, erythrocyte type glucose transporter; GLUT4, muscle/adipocyte type glucose transporter; FCS, Fetal calf serum; IRS-1, major insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; VMH, ventromedial hypothalamus.

GLUT1 (erythrocyte type) and increase glucose transport in response to insulin (*20, 21*). To explore the mechanism by which β_3-agonist enhances glucose uptake in skeletal muscle, we examined the effects of BRL37344, a potent agonist for β_3-AR, on glucose transport into L6 myotubes and the results were compared with the stimulatory effects of insulin. We report here that BRL37344 directly increases glucose uptake into L6 myotubes through a mechanism different from that of insulin.

EXPERIMENTAL PROCEDURES

Materials—2-Deoxy-D-[^{3}H]glucose (2-DG) and [^{14}C]-sucrose were obtained from American Radiolabeled Chemicals. [^{125}I]Protein A was from ICN Radiochemicals. Insulin, wortmannin, and BSA were from Sigma. The β_3-adrenergic agonist, BRL37344, was a generous gift from Dr. M.A. Cawthorne of SmithKline Beecham, Epsom, Surrey, UK. Dulbecco's modified Eagle's medium (DMEM) was from Nissui Pharmaceutical. Fetal calf serum (FCS) was from Whittaker Bioproducts. Antiserum against the C-terminal peptide of GLUT4 was prepared as previously described (*22*). The anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, USA) was kindly provided by Dr. N. Okumura of the Institute for Protein Research, Osaka University, Osaka. The ECL (enhanced chemiluminescence) system was from Amersham.

Cell Culture—The L6 muscle cell line originally developed by Yaffe (*19*) was obtained from Japanese Cancer Research Resources Bank. These cells were grown in monolayers in DMEM containing 2% (v/v) FCS in an atmosphere of 5% CO_2 in air. Myoblasts were maintained in continuous passages by trypsinization of subconfluent cultures, but they were allowed to reach confluence and fusion prior to measurement of glucose uptake.

Measurement of Glucose Uptake—2-DG uptake was measured with differentiated, multinucleated myotubes as described previously (*21*). Briefly, cells grown in 24-well plates were washed three times with a HEPES-buffered saline solution (140 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 2.5 mM $MgSO_4$, and 20 mM HEPES, pH 7.4), and preincubated with plain DMEM for 16 h at 37°C. Hormones were added during the last 3 h of preincubation. The cells were then rinsed twice with the HEPES-buffered saline solution to remove glucose from the medium. After incubation for 20 min in the HEPES-buffered saline containing 0.5% fatty acid-free BSA in the presence or absence of the same hormones, the cells were further incubated with 50 μM 2-DG (0.01 μCi/nmol) in the same solution. 2-DG uptake was allowed to proceed for 6 min, and then terminated by rapid suction and subsequent washing with ice-cold 3 mM $HgCl_2$ in PBS. Contamination by the isotope due to the extracellular space was corrected using [^{14}C]sucrose. The net uptake of 2-DG was expressed in pmol/min/mg protein ± SEM.

In experiments to determine the kinetic parameters of glucose uptake in L6 myocytes, the uptake of glucose was calculated as nmol of glucose/min/mg protein in the presence of different concentrations of glucose. It was assumed that GLUT did not distinguish between glucose and 2-DG. Although this assumption was made for simplicity, the results would not be affected if it were wrong, as long as the relative affinities of the transporter for glucose and 2-DG were unaffected by the treatment with insulin or BRL37344. Kinetic parameters were determined by linear regression of the relationship between the ratio of the uptake rate to the substrate concentration (V/S) and the uptake rate (V) (Eadie-Hofstee plot). In this analysis, the slope of the line gives the apparent K_m, whereas the y-intercept gives the maximal uptake velocity (V_{max}) (*23*).

Detection of Protein Tyrosine Phosphorylation—After incubation with 10^{-7} M insulin or 10^{-5} M BRL37344, cells were taken up in SDS-sample buffer containing 100 μM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Equal volumes of the samples were subjected to SDS-PAGE on 6% linear gels and then transferred to nitrocellulose membranes. The blots were incubated with the anti-phosphotyrosine antibody, followed by the peroxidase-labeled second antibody. Immunostained proteins were visualized using the ECL system.

Western Blot Analysis of GLUT4 in the Plasma Membrane and Microsomal Fractions—Plasma membrane and microsomal fractions were isolated according to the method described previously (*20*). The membrane samples (40 μg of protein/lane) were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose filters. The filters were incubated for 1.5 h with a 1:1,000 dilution of antiserum against the C-terminal peptide of GLUT4, followed by incubation for 1.5 h with [^{125}I]protein A (0.2 μCi/ml). Immunoreactivity was analyzed with a BAS-1000 image analyzer (Fuji Film, Tokyo).

Data Analysis—All values are presented as means ± SEM. Statistical significance was examined by analysis of variance, with post hoc testing by means of Duncan's multiple range test.

RESULTS

Effects of BRL37344 and Insulin on 2-DG Uptake by L6 Myocytes—We first examined the effects of insulin and BRL37344 on the time course of 2-DG uptake into differentiated L6 myocytes. Incubation of the cells with insulin (0.1 μM) increased 2-DG uptake progressively with time, resulting in a 1.6-fold increase over the basal uptake after 20 min, 1.8-fold after 1 h, and the maximum increase of approximately 2.3-fold by 3 h. A similar time course was observed with BRL37344 (10 μM) in the absence of insulin or serum. The increase in 2-DG uptake by BRL37344 became significant after 30 min and reached a maximum level of about 2.2-fold over the basal uptake after 3-h incubation. Although the BRL37344-induced increase in 2-DG uptake was apparent at 1 h, the extent of the increase varied considerably from preparation to preparation of the cells at this time point. Accordingly, the cells treated for 3 h with hormones were used for subsequent experiments.

Figure 1 shows dose-response curves for the actions of BRL37344 and insulin on 2-DG uptake by L6 myocytes. Insulin increased 2-DG uptake in a dose-dependent manner, with maximal stimulation at 10^{-7} M. BRL37344 ranging from 10^{-7} to 10^{-5} M also enhanced 2-DG uptake (Fig. 1). To examine the interaction between the actions of BRL37344 and insulin, L6 myocytes were incubated for 3 h together with the two agents at the maximally effective concentrations, and 2-DG uptake was measured. As shown in Table I, the effects of insulin and BRL37344 were completely additive, suggesting that these two agents

stimulate glucose uptake by L6 myocytes through different mechanisms.

Effects of BRL37344 and Insulin on Protein Tyrosine Phosphorylation in L6 Myocytes—To clarify the difference between the signaling pathways of BRL37344- and insulin-induced glucose uptake by L6 myocytes, we examined the tyrosine phosphorylation of intracellular proteins by BRL37344 and insulin using phosphotyrosine immunoblotting. Figure 2 shows that stimulation of L6 myocytes with 0.1 μM insulin caused an increase in phosphorylation of several proteins. The 180-190 kDa proteins could be the major insulin receptor substrate, IRS-1, and the 95 kDa protein corresponds to the β-subunit of the insulin receptor. These proteins were phosphorylated within 1 min. The level of the 180-190 kDa proteins was reduced almost to the basal level by 30 min (Fig. 2). In contrast, BRL37344 did not stimulate tyrosine phosphorylation, at least with respect to these proteins (Fig. 2).

Effects of Wortmannin on BRL37344- and Insulin-Induced Glucose Transport—We further investigated the effects of wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase) (*24*), on the insulin- and BRL37344-induced increases in glucose transport. The increase in 2-DG uptake by L6 myocytes in response to insulin was completely blocked by pretreatment with wortmannin (Fig. 3), confirming an important role of PI 3-kinase in the signaling pathway of insulin-induced glu-

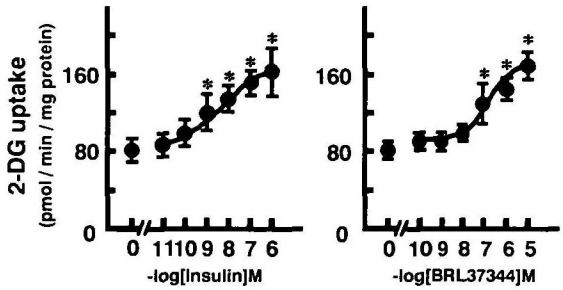

Fig. 1. **Dose-response curves for BRL37344 and insulin action on 2-DG uptake in L6 myotubes.** Fully differentiated L6 myotubes were incubated for 3 h with increasing doses of BRL37344 or insulin, and the 2-DG uptake was measured as described under "EXPERIMENTAL PROCEDURES." Values are means±SEM (n=6-8). *$p<0.05$ *versus* basal.

TABLE I. **Additivity of the effects of BRL37344 and insulin on 2-DG uptake by L6 myotubes.** 2-DG uptake was measured in L6 myotubes in the basal state, after treatment with 10 μM BRL37344 or 0.1 μM insulin, or both BRL37344 and insulin, as described under "EXPERIMENTAL PROCEDURES." Values are means±SEM (n=4-6). *$p<0.05$ *versus* basal state; †$p<0.05$ *versus* insulin alone.

BRL37344	Insulin	2-DG uptake (pmol/min/mg protein)
−	−	81.8±2.8
−	+	190.3±7.0*
+	−	183.0±9.9*
+	+	395.0±2.4*†

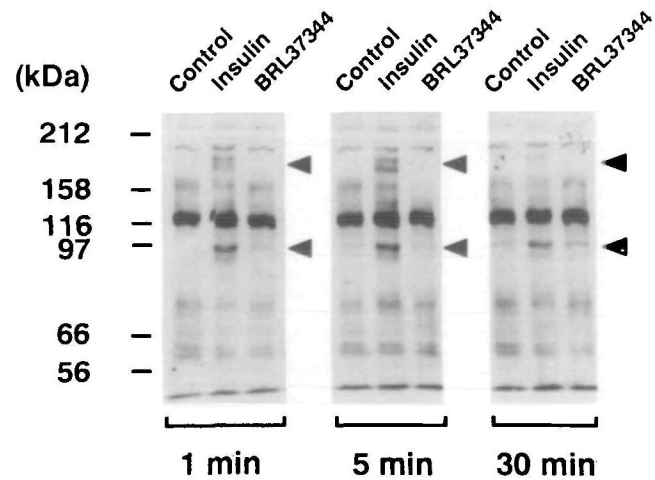

Fig. 2. **Effects of BRL37344 and insulin on tyrosine phosphorylation in L6 myotubes.** L6 myotubes were treated with 10 μM BRL37344 or 0.1 μM insulin for 1-30 min as indicated on the abscissa. Protein tyrosine phosphorylation was detected after analysis of cell lysates by SDS-PAGE and immunoblotting using anti-phosphotyrosine antibody. Molecular size markers (in kDa) are indicated. The arrowheads indicate major phosphoproteins observed after insulin treatment.

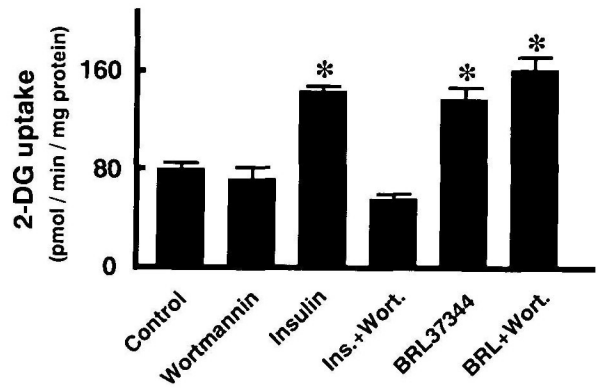

Fig. 3. **Effect of wortmannin on BRL37344- and insulin-induced increase in 2-DG uptake in L6 myotubes.** L6 myotubes were incubated with 10 μM BRL37344 or 0.1 μM insulin for 3 h, and then 2-DG uptake was measured as described under "EXPERIMENTAL PROCEDURES." Wortmannin (Wort.), at the final concentration of 10^{-7} M, was added 5 min before treatment with hormones. Values are means±SEM (n=6-8). *$p<0.05$ *versus* basal (control).

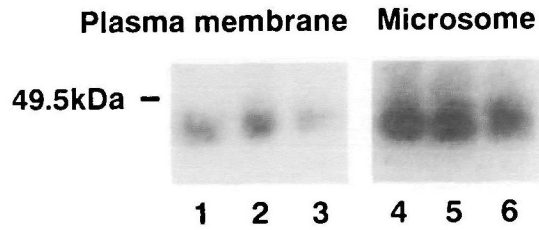

Fig. 4. **Effects of BRL37344 and insulin on GLUT4 contents in the isolated plasma membrane and microsomal fractions from L6 myotubes.** L6 myotubes were incubated for 3 h with either 10 μM BRL37344 or 0.1 μM insulin, and then plasma and microsomal membrane fractions were isolated as described under "EXPERIMENTAL PROCEDURES." Forty micrograms of the membrane samples were subjected to SDS-PAGE and immunoblotted with antiserum specific for GLUT4. A representative autoradiogram of GLUT4 in the membrane fractions is shown. Lanes 1 and 4, control; lanes 2 and 5, insulin-treated; lanes 3 and 6, BRL37344-treated. Reproducible results were obtained in six independent experiments.

TABLE II. **Effects of BRL37344 and insulin on kinetic parameters of glucose uptake in L6 myotubes.** L6 myotubes were treated for 3 h with 10 μM BRL37344 or 0.1 μM insulin, and glucose uptake was measured in the presence of different concentrations of glucose as described under "EXPERIMENTAL PROCEDURES." The rates of glucose uptake (nmol/min/mg protein) were plotted against V/[glucose] ([nmol/min/mg protein]/[mM]). In this analysis, the negative slope represents K_m and the y-intercept corresponds to V_{max}. The lines drawn through the points were calculated by linear least-squares regression ($r>0.90$ in each instance). Values are means ± SEM ($n=5$-6). *$p<0.05$ *versus* basal state (control).

	Control	Insulin	BRL37344
K_m (mM)	3.8±0.1	3.6±0.3	2.6±0.3*
V_{max} (nmol/min/mg protein)	118±3	155±5*	125±5

cose uptake by muscle cells (*25-27*). However, wortmannin did not affect the stimulatory effect of BRL37344 on glucose transport (Fig. 3).

Effects of BRL37344 and Insulin on Subcellular Distribution of GLUT4—To determine if BRL37344 promotes recruitment of GLUT4 to the plasma membrane, we also analyzed the protein content of GLUT4 in the isolated plasma membrane and microsomal fractions after treatment of the cells with BRL37344 or insulin by Western blotting. Figure 4 shows a representative autoradiogram. Insulin increased the GLUT4 content in the plasma membrane by 60%, in agreement with previous studies (*20*). However, a corresponding decrease in the microsomal fraction was barely observed in the present study, probably owing to the higher protein yield and thus much more abundant GLUT4 in the microsomal fraction than in the plasma membrane. In contrast, BRL37344 did not change the GLUT4 content either in the isolated plasma membrane or in the microsomal fraction. The results were well reproduced in six independent experiments.

Effects of BRL37344 and Insulin on Kinetic Parameters for Glucose Uptake—We next examined the kinetic parameters for glucose uptake after treatment of the myocytes with BRL37344 or insulin. The results are shown in Table II. For cells in the basal state, the K_m value was 3.8±0.1 mM and V_{max} was 118±3 nmol/min/mg protein. Insulin increased the V_{max} value to 155±5 nmol/min/mg protein. However, the effect of BRL37344 was restricted to the change in K_m, which decreased significantly to 2.6±0.3 mM.

DISCUSSION

In this paper, we have demonstrated that BRL37344 increases glucose transport into L6 myocytes through a mechanism different from that of insulin. Our major findings are: (1) BRL37344, in the absence of insulin, directly stimulates glucose transport into L6 myocytes in a dose-dependent manner, (2) the stimulatory effects of BRL37344 and insulin on glucose transport are completely additive, (3) BRL37344 enhances glucose transport without stimulating intracellular protein tyrosine-phosphorylation or intermediation of PI 3-kinase, (4) BRL37344 does not promote the translocation of GLUT4 to the plasma membrane, (5) BRL37344 decreases K_m for glucose uptake, without altering V_{max}.

It was previously reported that intravenous infusion of β_3-agonists in rats increases glucose uptake in skeletal muscles (*11, 12*). The increase in glucose uptake in skeletal muscles *in vivo* might be related, at least in part, to an action of insulin, because β_3-agonists potently stimulate pancreatic insulin release (*28, 29*). It should be emphasized, however, that this may not be the only mechanism responsible for the increase in glucose uptake in skeletal muscles after treatment with β_3-agonist. A recent study in this laboratory using isolated soleus muscle demonstrated that BRL37344 increases glucose uptake *in vitro* in the absence of insulin (*11*). The present study has also shown that BRL37344 substantially enhances glucose uptake into L6 myocytes in the absence of insulin. Furthermore, β_3-agonist-induced increase in glucose uptake by the cells was still obvious even when insulin-stimulated glucose uptake was maximally activated (Table I). These results indicate that BRL37344 possesses direct stimulatory action on glucose transport by muscle cells.

BRL37344 has been reported to exhibit a much greater potency on β_3-AR than on β_1- or β_2-AR (*30, 31*). It is thus likely that BRL37344 increases glucose uptake in L6 myocytes through mediation of atypical β-AR or β_3-AR. In fact, recent pharmacological studies have revealed the presence of atypical β-AR in rat skeletal muscle (*14-18*). More recently, mRNA of β_3-AR was detected in rat soleus muscle by the RT/PCR method (*18*). Furthermore, preliminary studies in this laboratory showed that BRL37344-induced glucose uptake in L6 myocytes was resistant to pretreatment with propranolol. In addition, atenolol (a selective β_1-antagonist) and butoxamine (a selective β_2-antagonist) also had no effect on glucose uptake in response to BRL37344. These data suggest that the increase in glucose uptake in L6 myocytes in response to BRL37344 is mediated by atypical β-AR or β_3-AR.

In the present studies, the myocytes were subjected to subacute (3 h) stimulation with hormones, since acute (within 1 h) stimulation did not give maximum responses of glucose transport. Thus, it might be possible that enhanced expression of GLUTs could account for the increase in glucose transport after hormone stimulation. However, this seems to be unlikely, because the total cellular contents of GLUT4 and GLUT1 were unchanged by 3-h treatment of the cells with BRL37344 or insulin (unpublished observation). In addition, if GLUTs were newly synthesized and located in the plasma membrane during 3-h treatment of the cells with hormones, the basal 2-DG uptake would be increased after 3 h. However, the uptake rate of the cells with hormone treatment for 3 h followed by extensive washing was comparable with that of the unstimulated control cells. Collectively, these observations exclude the possibility that *de novo* synthesis of GLUTs, if any, contributes primarily to the increased glucose transport seen after 3-h incubation.

It has been well established that insulin recruits GLUT4 to the plasma membrane (*32, 33*). This is the major mechanism responsible for the insulin-induced increase in glucose transport in muscle cells, as in adipocytes. On the other hand, the recruitment of GLUT1 to the plasma membrane is reported to occur only marginally in L6 myocytes after treatment with insulin (*20*). Although the intracellular signal transduction pathway leading to the recruitment of GLUT4 is not fully understood, recent evidence has revealed some pivotal reactions in the pathway. Insulin causes activation of an intrinsic tyrosine

kinase of the insulin receptor as a result of autophosphorylation of the β-subunit of the receptor and subsequent tyrosine phosphorylation of endogenous substrates including IRS-1 (*34-36*). The tyrosine-phosphorylated IRS-1 then binds to and activates PI3-kinase (*25, 35, 36*). It is believed that downstream events subsequent to the activation of PI3-kinase promote recruitment of GLUT4 to the plasma membrane. In accordance with these notions, treatment of L6 myocytes with insulin caused tyrosine phosphorylation of 180-190 and 95 kDa proteins, which might correspond to IRS-1 and β-subunit of insulin receptor, respectively. In addition, wortmannin, a specific inhibitor of PI3-kinase, blocked the increase in glucose uptake in response to insulin. However, the BRL37344-induced increase in glucose uptake was not accompanied with tyrosine phosphorylation of these proteins, or blocked by wortmannin, suggesting that BRL37344 enhances glucose transport into skeletal muscle through a signaling pathway different from that of insulin.

Muscle contraction is known to produce a large increase in glucose transport *via* an insulin-independent mechanism (*2, 4*). Recent studies have also demonstrated that pretreatment with wortmannin does not block the increase in glucose transport after muscle contraction, excluding the possible involvement of PI3-kinase in contraction-induced increase in glucose transport (*26, 27*). Since muscle contraction stimulates the translocation of GLUT4 from an intracellular pool to the plasma membrane, like insulin action (*37*), it is pertinent to examine whether BRL37344 can recruit GLUT4 protein to the plasma membrane. By using a membrane fractionation method, this study showed that BRL37344 did not increase GLUT4 content in the plasma membrane. Although the fractionation method commonly underestimates the degree of translocation, several lines of evidence support the conclusion that the increase in glucose uptake by L6 myocytes in response to BRL37344 is not likely to be due to the translocation of GLUT4. First, we could only detect the recruitment of GLUT4 in insulin-treated L6 myocytes (Fig. 4), though BRL37344 and insulin could increase glucose transport into L6 myocytes to similar extents (about twofold). In addition, the effect of BRL37344 was completely additive to the effect of insulin on glucose transport, indicating that two different mechanisms were operating in response to BRL37344 and insulin (Table I). Second, BRL37344 decreased the K_m value for glucose transport without affecting V_{max} (Table II). If the translocation were promoted by BRL37344, the V_{max} value would be increased. In fact, insulin increased the V_{max} value significantly. Finally, the increased glucose transport into cultured brown adipocytes in response to the β_3-agonist was not associated with translocation of GLUT4 as assessed by the fractionation method (*13, 38*) and by photoaffinity labeling of cell surface glucose transporters with a membrane-impermeant bismannose derivative (unpublished work in this laboratory). Overall, it seems probable that, in contrast to insulin and muscle contraction, BRL37344 induces an increase in glucose transport without causing the translocation of GLUT4, but it may cause an increase in the intrinsic functional activity of GLUT already present in the plasma membrane. Further studies are needed to clarify the intracellular events that lead to the activation of GLUT after treatment with β_3-agonist.

We thank Dr. M.A. Cawthorne for kindly providing β_3-adrenergic agonist, BRL37344. We also thank Dr. N. Okumura for kindly providing anti-phosphotyrosine antibody and for helpful discussions, and Miss N. Fujimoto for her expert technical assistance. We are grateful to Japanese Cancer Research Resources Bank for providing L6 muscle cell line.

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