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Effects of Brazilin on GLUT4 Recruitment in Isolated Rat Epididymal Adipocytes

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ABSTRACT. The effects of brazilin on glucose transport into isolated rat epididymal adipocytes were investigated. Brazilin increased [^{3}H]2-deoxy-D-glucose uptake, which was characterized by an increase in V_{max} with no effect on the K_m value. Phenylarsine oxide, which inhibits the translocation of glucose transporters, decreased brazilin-stimulated glucose transport to the basal level. The inhibition of phosphatidylinositol 3-kinase (PI3-kinase) with wortmannin also blocked brazilin-stimulated glucose transport. Western blot analysis with an anti-GLUT4 antibody revealed that brazilin increased the translocation of GLUT4 from intracellular pools to the plasma membrane. Brazilin, in combination with phorbol ester, showed an additive effect on glucose transport. The stimulating effect of phorbol ester on glucose transport was inhibited by staurosporine, but the effect of brazilin remained unchanged. Protein kinase C activity was not influenced by brazilin treatment. The inhibition of protein synthesis showed no effect on brazilin-stimulated glucose transport, and GLUT4 content in the total membrane fraction was not altered as a result of treatment with brazilin for 4 hr. Metabolic labeling of GLUT4 with [^{35}S]methionine showed that *de novo* synthesis of GLUT4 was not induced by brazilin. These data suggest that brazilin may increase glucose transport by recruitment of GLUT4 from intracellular pools to the plasma membrane of adipocytes via the activation of PI3-kinase. However, the effect of brazilin may not be mediated by GLUT4 synthesis and protein kinase C activation. BIOCHEM PHARMACOL **58**;11:1705–1712, 1999.

KEY WORDS. brazilin; adipocyte; protein kinase C; GLUT4 translocation; phenylarsine oxide; wortmannin; glucose transport

Glucose transport is mediated by the family of glucose carriers called GLUT†, which is composed of six isoforms [1, 2]. Of these isoforms, only GLUT4 is responsible for insulin-stimulated glucose transport in tissues such as skeletal muscles and adipocytes [3, 4], and it is expressed only in those tissues [5, 6]. Insulin-induced glucose transport is mediated primarily by the translocation of GLUT4 from intracellular storage sites to the plasma membrane [7, 8]. Furthermore, increases in GLUT4 intrinsic activity [9, 10] and gene expression [11, 12] also have been identified as factors that enhance glucose transport. Although a number of reports have been published regarding the possible pathways responsible for increased glucose transport, the signal transduction pathways from the insulin receptor to the glucose transport system are not understood fully at present.

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† *Abbreviations*: GLUT, glucose transporter; 2-DOG, 2-deoxy-D-glucose; IRGT, insulin-responsive glucose transporter; KRH buffer, Krebs-Ringer HEPES buffer; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PAO, phenylarsine oxide; PI3-kinase, phosphatidylinositol 3-kinase; and DAG, diacylglycerol.

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The role of PKC in glucose transport has been studied by a number of investigators [13–15]. Insulin induces the release of DAG and inositol glycan in a variety of cell types, and the addition of phospholipase C increases glucose transport in isolated rat adipocytes [16–19]. The stimulation of PKC activity, however, may not be the sole mechanism for insulin-induced glucose transport, since glucose transport induced by PMA is lower than that observed in response to insulin treatment [20–22], and, in addition, the inhibition of PKC activity does not fully reduce insulin-induced glucose transport to basal levels [14, 23].

PAO, a reagent that specifically binds to sulfhydryl groups, inhibits insulin-induced glucose transport by reducing the translocation of glucose transporters [14]. However, the nature and the location of the SH-containing group that is sensitive to PAO and insulin, have not been identified, but the location has been inferred to be downstream from the insulin receptor step in the insulin signal transduction pathway [24]. In 1994, a report by Cheatham *et al.* [25] revealed a critical role for PI3-kinase in the glucose transport pathway, since the inhibition of PI3-kinase with wortmannin or LY294002 blocks insulin-induced glucose transport.

Brazilin [(6a*S-cis*)7,11b-dihydrobenz[*b*]indeno-[1,2-*d*]py-

ran-3,6a,9,10(6*H*)-tetrol], the major component of *Caesalpinia sappan* [26], shows hypoglycemic action in experimental diabetic animals, and this hypoglycemic effect is the result of increased glucose metabolism, including glucose uptake into soleus muscles and adipocytes [27–29]. Our previous study showed that brazilin-induced glucose transport is inhibited by quin 2-AM, an intracellular Ca^{2+} chelator, and by trifluoperazine, a Ca^{2+}/calmodulin complex inhibitor [30]. This provides support for the hypothesis that brazilin may increase glucose transport by increasing the translocation of the glucose transporter, which requires the Ca^{2+}/calmodulin complex as a fusogen [31, 32].

The goal of the present study was to investigate the effects of brazilin on GLUT4 translocation and expression in isolated rat epididymal adipocytes to better understand the hypoglycemic mechanism of brazilin.

MATERIALS AND METHODS

Experimental Animals

Sprague–Dawley rats were supplied by the Laboratory Animal Center. The rats were given access to food and water *ad lib*. Animals with body weights of 170–200 g were used throughout the experiments.

Materials

Brazilin monohydrate was obtained from the Aldrich Chemical Co. [^{3}H]2-DOG (366 GBq/mmol, 9.9 Ci/mmol) and [γ-^{32}P]ATP (110 TBq/mmol, 3000 Ci/mmol) were purchased from Amersham; [^{35}S]methionine (41.11 TBq/mmol, 1111 Ci/mmol) was purchased from ICN. Anti-GLUT4 monoclonal antibody was obtained from Genzyme and rabbit anti-mouse IgG antibody from the Sigma Chemical Co. Protein A beads, wortmannin, and a protein kinase C assay kit were obtained from Gibco. En^{3}Hance was purchased from DuPont–NEN, and X-ray film from AGFA. Porcine insulin, collagenase type II, PMA, staurosporine, and other reagents were obtained from the Sigma Chemical Co.

Isolation of Epididymal Adipocytes

Epididymal adipocytes were isolated as described by Rodbell [33]. Briefly, epididymal fat pads were removed and digested for 1 hr at 37° with 1 mg/mL of collagenase type II in KRH buffer (131.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.25 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 10.0 mM HEPES, pH 7.4) supplemented with 1% BSA and 2.5 mM pyruvate. After digestion, cells that were liberated from adipose tissues were washed three times with collagenase-free KRH buffer and then resuspended in KRH to give adequate cell populations.

Measurement of Glucose Transport

The uptake of [^{3}H]2-DOG was used as a measure of glucose transport [34, 35]. Brazilin and other reagents were added as indicated in the figure legends, after which the uptake of [^{3}H]2-DOG (0.125 mM, 0.4 μCi) was measured over a 3-min interval. Cell layers were separated by oil centrifugation with dinonylphthalate, and [^{3}H]2-DOG trapped in the extracellular spaces was corrected for by subtraction of the [^{3}H]2-DOG uptake in the presence of phloretin. Under these experimental conditions, the transported 2-DOG was immediately 96% phosphorylated at the 6-position, and the phosphorylation was not the rate-limiting step (data not shown).

PKC Assay

PKC activity was measured with the PKC assay kit essentially as recommended by the manufacturer, as follows. Adipocytes were treated with brazilin and other reagents as indicated in the figure legends, and then were washed three times with PBS. Adipocytes were homogenized with a Teflon homogenizer in extraction buffer (20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg/mL of aprotinin, 25 μg/mL of leupeptin, pH 7.5), and centrifuged for 5 min at 15,000 rpm in microcentrifuge tubes. PKC activity in the supernatant was assayed using the PKC substrate (250 μM acetyl myosin basic protein) in phosphatidylserine liposomes in the presence or absence of 100 μM PMA. [γ-^{32}P]ATP (100 μM, 25 μCi/mL) was pulsed for 5 min at room temperature; the PKC substrates were adsorbed to phosphocellulose paper and washed with 1% phosphoric acid. Radioactivity incorporated into the PKC substrates was determined by liquid scintillation counting.

Subcellular Fractionation of Adipocytes

The plasma membrane fraction and the low-density microsomal membrane fraction were prepared from the incubated cells by the differential ultracentrifugation method described by McKeel and Jarett [36], with minor modifications [37]. Adipocytes were treated as indicated in the figure legends. Cells were washed twice with TES buffer (20 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, and 255 mM sucrose, pH 7.4) and homogenized with a Teflon pestle at room temperature. All steps following the homogenization were carried out at 4° using the same buffer. Each homogenate was centrifuged at 16,000 g_{max} for 15 min. The supernatant was retained for preparation of the low-density microsomal membrane fractions, and the fat cake was discarded. The pellet was resuspended and centrifuged at 1000 g_{max} for 10 min. The supernatant was centrifuged at 16,000 g_{max} for 15 min, and the pellet was resuspended for preparation of the plasma membrane fraction. The plasma membrane fraction was obtained by centrifugation at 110,000 g_{max} for 70 min on a discontinuous sucrose gradient in 20 mM Tris–HCl and 1 mM EDTA, pH 7.4. The densities used were 1.0592, 1.1270, 1.1533, 1.1764, and 1.2375 g/mL. The plasma membrane band was washed twice by resuspension in TES buffer, followed by centrifugation at 48,000 g_{max} for 45 min, and was resuspended to a final concentration of 1 mg protein/mL.

The low-density microsomal membrane fraction was

obtained from the initial supernatant by centrifugation at 210,000 g_{max} for 75 min. The pellet, which constituted the microsomal membrane fraction, was washed once and was resuspended to a final concentration of 1 mg protein/mL. Total membrane fractions were prepared by centrifugation of the initial supernatants, which had been obtained by the centrifugation of adipocyte homogenate at 1000 g_{max} for 10 min, for an additional 15 min at 210,000 g_{max}.

The cross-contamination of plasma membrane fractions and microsomal membrane fractions was examined by assay of 5′-nucleotidase and NADPH-cytochrome *c* reductase, which are the marker enzymes of the plasma membrane and microsomal membrane [7, 8]. The plasma membrane fractions contaminated with less than 5% microsomal membrane and the microsomal membrane fractions contaminated with less than 10% plasma membrane were used in the following experiments.

Electrophoresis and Immunoblotting

Electrophoresis was performed according to Laemmli [38] with 10% slab gels at a constant current of 15 mA/gel. Proteins were transferred to PVDF papers in a buffer consisting of 20% methanol, 192 mM glycine, and 25 mM Tris, pH 8.3. Following the transfer, the papers were blocked with 5% nonfat dry milk in PBS for 1 hr at room temperature and then incubated with anti-IRGT monoclonal antibody [39, 40] under the same conditions. Transfer was confirmed by Coomassie staining of the gel after the electroblot. Antigen–antibody complexes were detected with rabbit anti-mouse IgG antibody labeled with alkaline phosphatase and visualized with the alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Metabolic Labeling

Adipocytes were dispersed in methionine-free Dulbecco's modified Eagle's medium and treated with 50 μM brazilin or 25 ng/mL of insulin for 4 hr at 37° in the presence of 100 μCi [^{35}S]methionine [41, 42]. The cells were washed with PBS three times and solubilized by means of an ultrasonic processor (GEX-400, Branson) in solubilization buffer (50 mM Tris, 1% Tween 20, 10 mM EDTA, 1 mM EGTA, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Cell extracts were centrifuged at 7000 g_{max} for 5 min at 4°, and the radioactivity of the supernatant was measured. The volume of each supernatant was adjusted to have an equal amount of radioactivity per unit volume. Proteins were immunoprecipitated with anti-IRGT antibody at 4°, and protein A beads were added. Immunoprecipitates were washed serially with BSA buffer (1% BSA in high salt buffer), high salt buffer (2 mM EDTA, 1% Tween 20, 500 mM NaCl in PBS), SDS buffer (0.5% SDS in high salt buffer), and PBS, and subjected to SDS–PAGE on a 10% slab gel. Gels were treated with fixing solution (30% methanol, 10% acetic acid) and rocked in En^{3}Hance for 1 hr, after which autoradiography was carried out.

Statistical Analysis

Values of three independent experiments were expressed as means ± SEM. Values that showed $P < 0.05$ by Student's *t*-test were regarded as significantly different, except for those indicated otherwise in the legend.

RESULTS AND DISCUSSION

It has been reported that the hypoglycemic action of brazilin may be due largely to stimulation of glucose transport and metabolism in insulin target organs, such as adipocytes and skeletal muscles [27–29]. The translocation of GLUT4 is mediated by the fusion of the plasma membrane and exocytic vesicles that contain GLUT4, in which intracellular calcium and the Ca^{2+}–calmodulin complex play an important role, causing conformational changes in each membrane compartment to facilitate the fusion process [31]. In isolated rat epididymal adipocytes, chelation of intracellular calcium by quin2-AM and blockage of the Ca^{2+}–calmodulin complex by trifluoperazine were found to inhibit the action of brazilin on glucose transport [32]. This suggests that brazilin may increase the translocation of GLUT4 from intracellular pools to the plasma membrane. The present study involved experiments that were designed to investigate the effect of brazilin on GLUT4 translocation and gene expression. In addition, the involvement of the PKC and PI3-kinase pathways also was examined to elucidate the mechanism of action of brazilin.

Effect of Brazilin on GLUT4 Translocation

To elucidate the mechanism by which brazilin stimulates glucose transport, we investigated the kinetic behavior of glucose transport induced by brazilin. As shown in Fig. 1, brazilin increased the V_{max} of glucose transport 2-fold over basal uptake, but the K_m value was unchanged (Table 1). This suggests that brazilin may increase glucose transporters in the plasma membrane via a translocation mechanism or increase total glucose transporter contents in storage sites, while leaving the intrinsic activity of glucose transporters in the plasma membrane unaltered.

It has been reported that PAO blocks the translocation of glucose transporters to the plasma membrane in adipocytes and also inhibits insulin-induced glucose transport in a concentration-dependent manner [14]. As shown in Fig. 2A, PAO inhibited brazilin-induced glucose transport. This suggests that brazilin may increase glucose transport by stimulating the translocation of glucose transporters from intracellular pools to the plasma membranes of isolated adipocytes.

To investigate the mechanism of brazilin-induced translocation of GLUT4 from intracellular pools to the plasma membrane, wortmannin was employed as a PI3-kinase

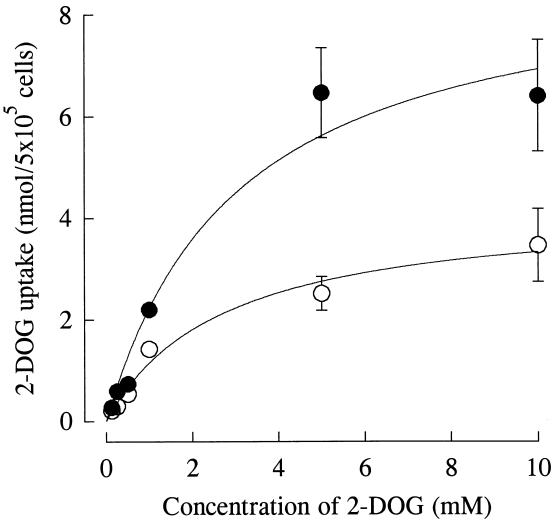

FIG. 1. Effect of brazilin on the kinetic behavior of 2-DOG uptake in isolated rat epididymal adipocytes. Adipocytes were incubated with (●) or without (○) 50 μM brazilin for 4 hr in the presence of 0.125, 0.25, 0.5, 1.0, 5.0, or 10.0 mM 2-DOG, and 2-DOG uptake was measured as described in Materials and Methods. Curves were fitted with Sigma-Plot® according to the Michaelis–Menten equation. Values are means ± SEM of triplicate determinations from three independent experiments.

inhibitor. Wortmannin inhibits GLUT4 translocation and glucose transport in adipocytes [25, 43]. As shown in Fig. 2B, wortmannin inhibited basal, insulin-stimulated, and brazilin-stimulated glucose transport. Treatment with wortmannin reduced the brazilin-induced glucose uptake to a level below that of the basal uptake.

This result may have been caused by the experimental conditions of this study, which involved incubation periods of 4 hr. The basal glucose uptake may be increased slightly by mechanical agitation, which can result in the stimulation of translocation of the glucose transporter [9]. The results obtained, however, indicated that brazilin stimulates GLUT4 translocation through PI3-kinase activation.

The detection of GLUT4 by western blot in both plasma membranes and microsomal membranes showed that GLUT4 translocation from intracellular pools to the plasma membrane was stimulated by both insulin and brazilin (Fig. 2C). By densitometric analysis of western blots, it was found that brazilin increased the GLUT4 level in the plasma membrane by 1.7-fold over the basal level

TABLE 1. Effect of brazilin on the kinetic behaviors of 2-DOG uptake in isolated rat epididymal adipocytes

Group	V_{max} (nmol/10^5 cells/min)	K_m (mM)
Control	1.41 ± 0.14	8.9 ± 2.5
Brazilin, 50 μ M	3.02 ± 0.62*	10.2 ± 5.7

Data from Fig. 1 were transformed by the Lineweaver-Burk equation. V_{max} and K_m values were calculated with Sigma-Plot®. Values are means ± SEM of triplicate determinations from three independent experiments.

*Significantly different from the control group ($p < 0.05$).

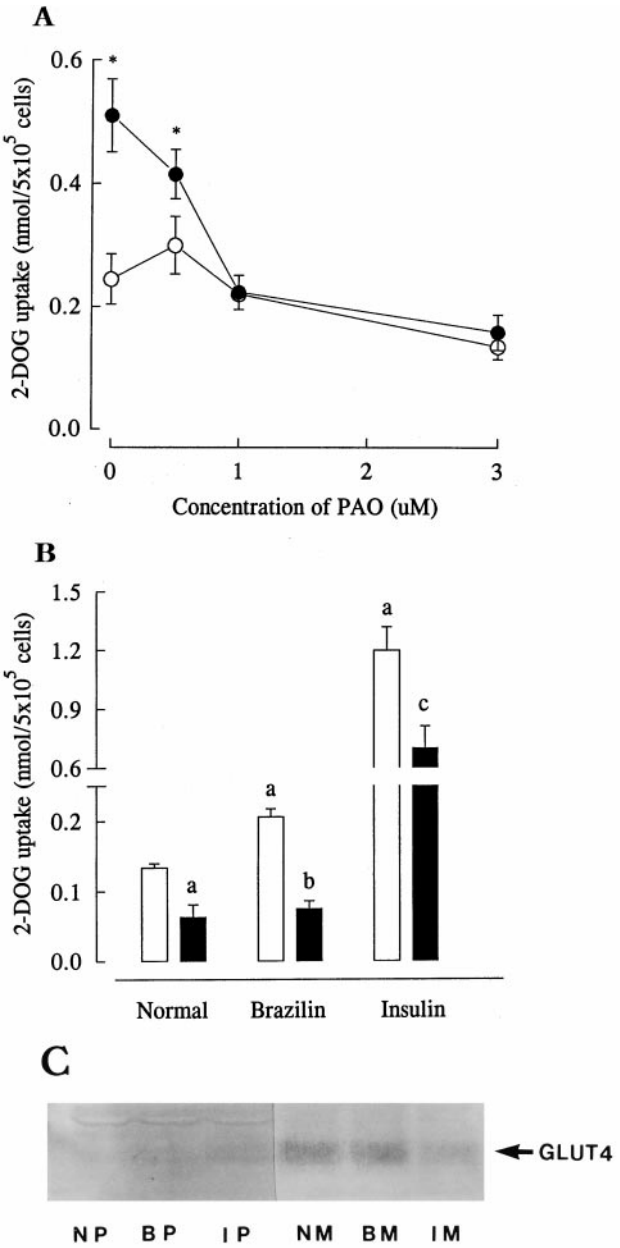

FIG. 2. Effect of brazilin on GLUT4 translocation from the intracellular pool to the plasma membrane in isolated rat epididymal adipocytes. (A) Adipocytes were treated with (●) or without (○) brazilin (50 μM) for 4 hr, followed by treatment with PAO for an additional 1 hr at the indicated concentrations. Values are means ± SEM of triplicate determinations from three independent experiments. Key: (*) significantly different from the control group ($P < 0.05$). (B) Adipocytes were treated with 50 μM brazilin for 4 hr followed by the addition of wortmannin (25 nM, ■) and incubation for an additional 50 min. Values are means ± SEM of triplicate determinations from three independent experiments. Key: (a) significantly different from the control group ($P < 0.05$); (b) $P < 0.05$ for brazilin alone versus brazilin with wortmannin; (c) $P < 0.05$ for insulin alone versus insulin with wortmannin. (C) Western blot of GLUT4. Adipocytes were treated with brazilin (50 μM) for 4 hr or with insulin (25 ng/mL) during the final 30 min, and subcellular fractionations were performed. Thirty micrograms of protein of each fraction per well was subjected to SDS–PAGE. GLUT4 was detected with anti-IRGT antibody as described in Materials and Methods.

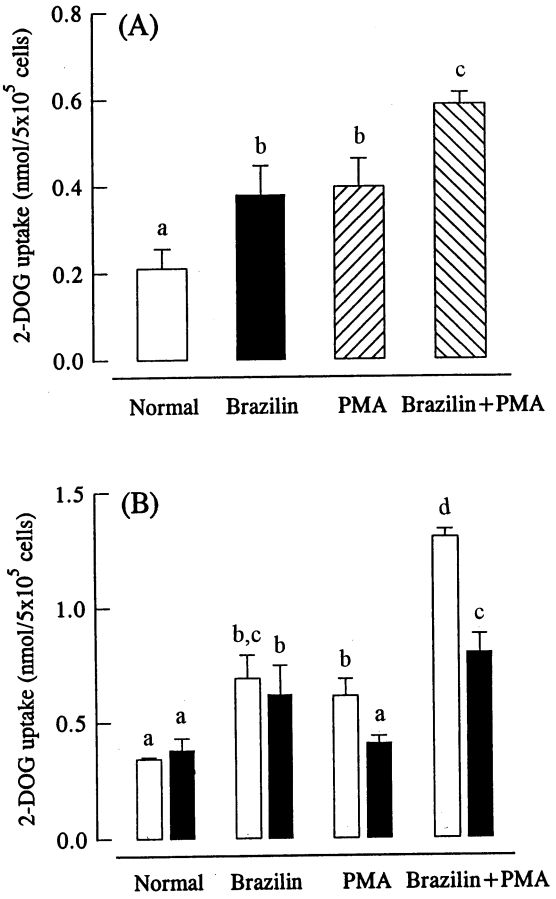

FIG. 3. Effect of PMA and brazilin on 2-DOG uptake in isolated rat epididymal adipocytes. (A) Adipocytes were treated with brazilin (50 μM) for 4 hr with or without PMA (200 nM) for the final 50 min. Results are means ± SEM of triplicate determinations from three independent experiments. (B) Inhibition of brazilin- and PMA-stimulated 2-DOG uptake by staurosporine. Adipocytes were preincubated in the presence (■) or absence (□) of staurosporine (1 μM) for 10 min and then treated with brazilin (50 μM) for 4 hr with or without PMA (200 nM) for the final 50 min. Values are means ± SEM of triplicate determinations from three independent experiments. Groups bearing the same letter are homogeneous subsets whose highest and lowest means do not differ, as analyzed by one-way ANOVA (Duncan's $P > 0.05$).

and decreased the GLUT4 level in low-density microsomal membrane by 0.8-fold. Insulin also increased the GLUT4 level in plasma membrane by 2.5-fold over the basal level and decreased the GLUT4 level in low-density microsomal membrane by 2-fold. These findings indicate that the increase in glucose transport induced by brazilin is the result of the enhancement of GLUT4 translocation from intracellular pools to the plasma membrane.

Effect of Brazilin on PKC Activity and Glucose Transport

PMA-induced PKC activation stimulates GLUT4 translocation, which is followed by the increase of glucose transport into adipocytes [44]. In view of this fact, PKC activity

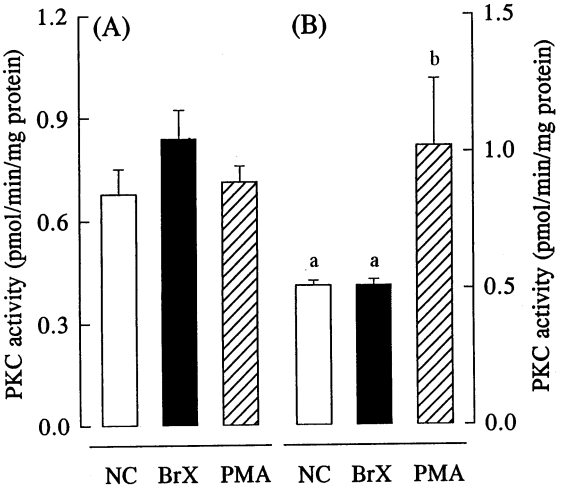

FIG. 4. Effects of brazilin and PMA on PKC activity in isolated rat epididymal adipocytes. Adipocytes were treated with brazilin (50 μM, BrX) for 4 hr or with PMA (200 nM, PMA) for the final 50 min. Then PKC activities were measured with (A) or without (B) stimulation by 100 μM PMA as described in Materials and Methods. Values are means ± SEM of triplicate determinations from three independent experiments. Groups bearing the same letter are homogeneous subsets whose highest and lowest means do not differ, analyzed by one-way ANOVA (Duncan's $P > 0.05$).

and glucose transport were investigated to elucidate the mechanism of brazilin-induced glucose transport. PMA, a PKC activator, increased glucose transport in a concentration-dependent manner, showing a maximal effect at 100

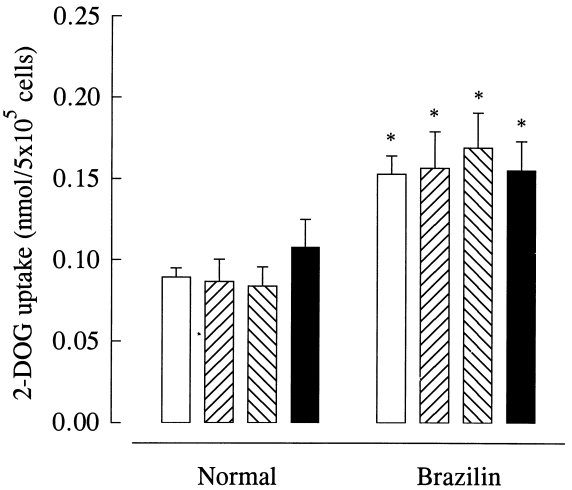

FIG. 5. Effects of protein synthesis inhibitors on brazilin-stimulated 2-DOG uptake in isolated rat epididymal adipocytes. Adipocytes were treated with 50 μM brazilin for 4 hr in the absence (open columns) or presence of 5 μM actinomycin D (hatched columns slanting to the right), 15 μM cycloheximide (hatched columns slanting to the left), and 5 μM puromycin (closed columns) for all incubation periods. 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM of triplicate determinations from three independent experiments. Key: (*) significantly different from the control group ($P < 0.05$).

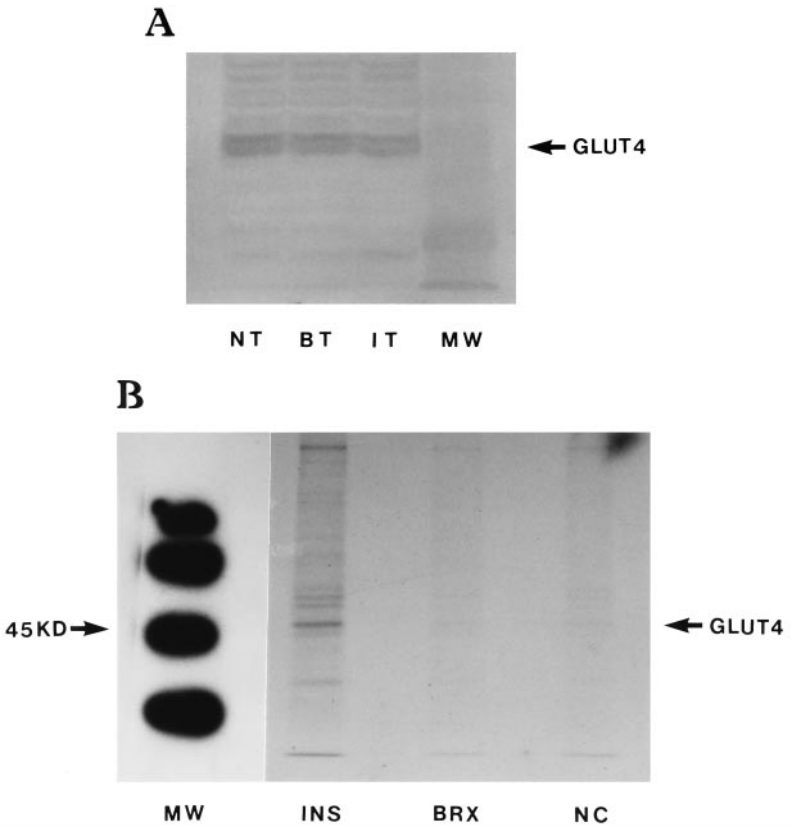

FIG. 6. Effect of brazilin on GLUT4 synthesis in isolated rat epididymal adipocytes. (A) Adipocytes were treated with brazilin (50 μM) for 4 hr or with insulin (25 ng/mL) for the final 30 min, and the total membrane fraction was obtained. Thirty micrograms of protein of each fraction per well was subjected to SDS–PAGE. GLUT4 was detected with anti-IRGT antibody as described in Materials and Methods. Abbreviations: NT, control total membrane; BT, brazilin-treated total membrane; IT, insulin-treated total membrane; and MW, molecular weight marker. (B) Adipocytes were treated with brazilin (50 μM) or insulin (25 ng/mL) for 4 hr in the presence of [^{35}S]methionine for all incubation periods. Cells were sonicated, immunoprecipitated with anti-IRGT antibody, subjected to SDS–PAGE, and fluororadiographed as described in Materials and Methods.

nM in isolated rat epididymal adipocytes; the effect of PMA was linear for up to 2 hr (data not shown). Both brazilin and PMA showed a 2-fold increase in glucose transport over basal uptake, and simultaneous treatment with both brazilin and PMA caused a 3-fold increase (Fig. 3A). To understand the reason for this difference, the effects of staurosporine on glucose transport stimulated by brazilin and PMA were investigated. Staurosporine, a PKC inhibitor, already has been found to inhibit the PMA-induced translocation of glucose transporter [45]. As shown in Fig. 3B, staurosporine inhibited PMA-induced glucose transport, but brazilin-stimulated glucose transport remained unaffected. The additive effect of brazilin and PMA on glucose transport also was inhibited by staurosporine, but it was not inhibited all the way to the basal level. Rather, the effect was equal to the level of brazilin-induced glucose transport. This fact indicated that the effect of brazilin is not dependent on PKC activity.

Because staurosporine has been reported to lack specificity, inhibiting kinases other than PKC, and we wanted to understand whether the additive effect of brazilin and PMA on glucose transport was a consequence of enhancement of PKC activity, changes in PKC activity induced by brazilin treatment were determined directly.

PKC activity was measured in cell lysates obtained from adipocytes, which had been treated with brazilin for 4 hr or with PMA for 50 min under two different experimental conditions. In the first experiment, PKC in cell lysates was activated further by the addition of PMA to the reaction buffer. Under these conditions, it was possible to determine whether PKC was inhibited or not. Further activation of PKC was not induced by the addition of PMA to the PMA-pretreated cell lysate (Fig. 4A). Brazilin showed no significant effect on PKC activity under these conditions. In a subsequent experiment, in which PMA was not added to the reaction buffer, PMA induced a 2-fold increase in PKC activity, but brazilin showed no effect (Fig. 4B). The relatively low stimulating effect of PMA on PKC activity may be due to the omission of affinity-chromatographic purification. Since brazilin showed no effect on PKC activity, further experiments, including affinity purification of PKC from cell lysates, were not performed.

The data we obtained suggested that the effect of brazilin on glucose transport in isolated adipocytes is mediated by recruiting GLUT4 to the plasma membrane via the activation of PI3-kinase, not by affecting protein synthesis, including GLUT4 synthesis.

Effect of Brazilin on GLUT4 Biosynthesis

The stimulation of GLUT4 translocation by brazilin was the result of an increase in the V_{max} for glucose transport, as previously described. Therefore, the effect of brazilin on GLUT4 expression was investigated in this study to determine if this might serve as an alternative explanation for the increase in V_{max} induced by brazilin.

Actinomycin D, a transcriptional protein synthesis inhibitor, showed no effect on brazilin-induced glucose trans-

port. Furthermore, cycloheximide and puromycin, translational protein synthesis inhibitors, also had no effect on the action of brazilin (Fig. 5). Western blot analysis of total cellular GLUT4 also showed that a 4-hr treatment with brazilin failed to increase the content of immunoreactive GLUT4 protein in adipocytes (Fig. 6A). These results suggested that glucose transport mediated by brazilin may not be attributable to stimulation of protein synthesis.

The effects of insulin and brazilin on GLUT4 expression at the translational level were investigated further by the metabolic labeling of GLUT4 with [^{35}S]methionine. Because [^{35}S]methionine was pulsed in the incubation period and proteins were immunoprecipitated with anti-GLUT4 antibody, [^{35}S]methionine-labeled protein could be recognized as GLUT4 that had been newly synthesized in that period [41, 42]. A 4-hr treatment with insulin caused an increase in the synthesis of GLUT4, as shown in Fig. 6B. Brazilin, however, showed no effect on the synthesis of GLUT4 protein as measured by metabolic labeling with [^{35}S]methionine under our experimental conditions.

The data obtained herein are consistent with brazilin causing an increase in glucose transport by recruiting GLUT4 to the plasma membrane via the activation of PI3-kinase. Additionally, the action of brazilin may not be related to protein synthesis, including GLUT4 synthesis.

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