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Effects of Calcium on Brazilin-Induced Glucose Transport in Isolated Rat Epididymal Adipocytes

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ABSTRACT. Brazilin increased [^{3}H]2-deoxyglucose uptake in isolated rat epididymal adipocytes. The fact that calcium may be required for the stimulatory effects of insulin on glucose transport suggests that brazilin might also require calcium for its glucose transport-stimulating action. Changes in the concentration of extracellular calcium had no significant effect on brazilin-induced glucose transport. Nifedipine and verapamil decreased brazilin-induced glucose transport, and quin2-AM abolished the effect of brazilin on glucose transport. A23187, however, showed no effect on brazilin action. $^{45}Ca^{2+}$ uptake into adipocytes was not influenced by brazilin treatment, and trifluoperazine significantly inhibited the effect of brazilin on glucose transport. These data suggest that calmodulin and the maintenance of the intracellular calcium concentration, rather than an increase in it, may be essential for the stimulatory action of brazilin on glucose transport. BIOCHEM PHARMACOL **54**;1: 97–101, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. brazilin; glucose transport; calcium; calmodulin; adipocytes

Facilitated glucose transport constitutes a prerequisite step for glucose utilization and is mediated by GLUTs§ [1]. Glucose transporters consist of six isoforms [2], and the major insulin-responsive glucose transporter in adipocytes and skeletal muscle is GLUT4 [3, 4]. Insulin stimulates glucose transport in insulin-responsive tissues via translocation of intracellular glucose transporters (largely GLUT4) to the plasma membrane [5, 6], and this process has been found to be mediated by a fusion mechanism [7].

Because of its widespread action in many cellular pathways, many investigators are of the opinion that calcium may serve as a second messenger in insulin action [8, 9]. However, many reports have demonstrated that, although intracellular calcium may be required for insulin action, it is not the critical factor in signal transduction generated by insulin. Rather than acting as a second messenger, intracellular calcium could participate in the fusion step between plasma membrane and exocytic vesicles that contain glucose transporters [7]. During the process of fusion, membrane properties are changed from a bilayer conformation to a hexagonal conformation, and intracellular calcium and the calcium–calmodulin complex play an important role in this process [10–12]. From the studies on the relationship between glucose transport and intracellular calcium concentration, it was reported that insulin increases the cytosolic calcium content in isolated rat adipocytes [13] and optimum levels of intra-/extracellular calcium exist that may be required to mediate insulin-responsive glucose transport [14, 15]. It has also been found that intracellular calcium chelation results in the inhibition of insulin-induced glucose transport [16]. The calmodulin system is reported to be involved in glucose transport by facilitating the fusion of glucose transporter-containing exocytic vesicles to the plasma membrane [17].

Brazilin, the major component of *Caesalpinia sappan* [18], has been shown previously to have hypoglycemic action in experimental diabetic animals with no changes in plasma insulin level. This is referred to as an extrapancreatic action [19–21].

This paper reports an investigation of the effect of brazilin on glucose transport in isolated rat epididymal adipocytes and the role of calcium and the calcium–calmodulin complex in brazilin-induced glucose transport.

MATERIALS AND METHODS

Experimental Animals

Sprague–Dawley rats were supplied by the Laboratory Animal Center, Seoul National University, Seoul, Korea, and given access to food and water *ad lib*. Rats having body weights of 170–200 *g* were used throughout the experiments.

Reagents

Brazilin monohydrate was obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A., and [^{3}H]2-deoxyglucose (366 GBq/mmol, 9.9 Ci/mmol) was purchased from

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§ *Abbreviations:* 2-DOG, 2-deoxy-D-glucose; GLUT, glucose transporter; KRH, Krebs–Ringer HEPES buffer; and quin2-AM, 2-[(2-bis-[carboxymethyl]amino - 5 - methyl - phenoxy)methyl] - 6 - methoxy - 8 -bis[carboxymethyl]amino quinoline tetrakis-[acetoxy methyl] ester.

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Amersham, Buckinghamshire, U.K. $^{45}CaCl_2$ (37 MBq/mL, 1 mCi/mL) was the product of New England Nuclear, Boston, MA, U.S.A. Insulin, collagenase type II, nifedipine, A23187, quin2-AM, verapamil, and trifluoperazine were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Other reagents were reagent grade.

Isolation of Epididymal Adipocytes

Epididymal adipocytes were isolated as described by Rodbell [22]. The distal portions of the epididymal fat pads were removed and digested for 1 hr at 37° with 1 mg/mL of type II collagenase in Krebs–Ringer HEPES buffer (NaCl 131.5 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.25 mM, NaH_2PO_4 2.5 mM, HEPES 10.0 mM, pH 7.4) supplemented with 1% BSA and 2.5 mM pyruvate. After digestion, cells were liberated from adipose tissues and washed three times with collagenase-free KRH buffer, and then resuspended to adequate cell populations with KRH buffer.

Measurement of Glucose Transport in Adipocytes

The uptake of [^{3}H]2-deoxyglucose was measured in order to estimate glucose transport [23, 24]. Brazilin and other reagents were added to the reaction mixture as indicated in the figure legends, and the uptake of [^{3}H]2-deoxyglucose (0.125 mM, 0.4 μCi) was measured after 3 min of incubation. Cell layers were separated by the oil centrifugation technique with dinonylphthalate. Radioactivity was measured using a liquid scintillation counter in a toluene-base cocktail containing Triton X-100. [^{3}H]2-deoxyglucose trapped in the extracellular spaces was corrected for by subtraction of [^{3}H]2-deoxyglucose uptake in the presence of phloretin.

Measurement of $^{45}Ca^{2+}$ Uptake into Adipocytes

Uptake of calcium into adipocytes was measured using $^{45}CaCl_2$ (1 mM, 0.5 μCi). For measurement of the time-dependent uptake of Ca^{2+}, adipocytes were incubated with or without brazilin for the indicated times in the presence of $^{45}Ca^{2+}$. The reactions were terminated by oil centrifugation as described above. Influx of calcium was measured after incubation with brazilin for 4 hr, followed by a 10-min pulse of $^{45}CaCl_2$ and oil centrifugation. Radioactivity was measured as described above.

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.

RESULTS AND DISCUSSION

The effects of brazilin on glucose transport were studied in isolated rat epididymal adipocytes in order to elucidate the mechanism of its hypoglycemic action. In adipocytes, glucose transport constitutes the prerequisite step of glucose utilization. This experiment showed that brazilin increased glucose uptake in adipocytes at 4 hr of incubation. The optimum concentration of brazilin required to increase glucose transport was 50 μM (Fig. 1A), and the effect was time dependent (Fig. 1B). The decrease in glucose transport by brazilin concentrations in excess of 75 μM was the result of its cytotoxicity to adipocytes *in vitro* (data not shown).

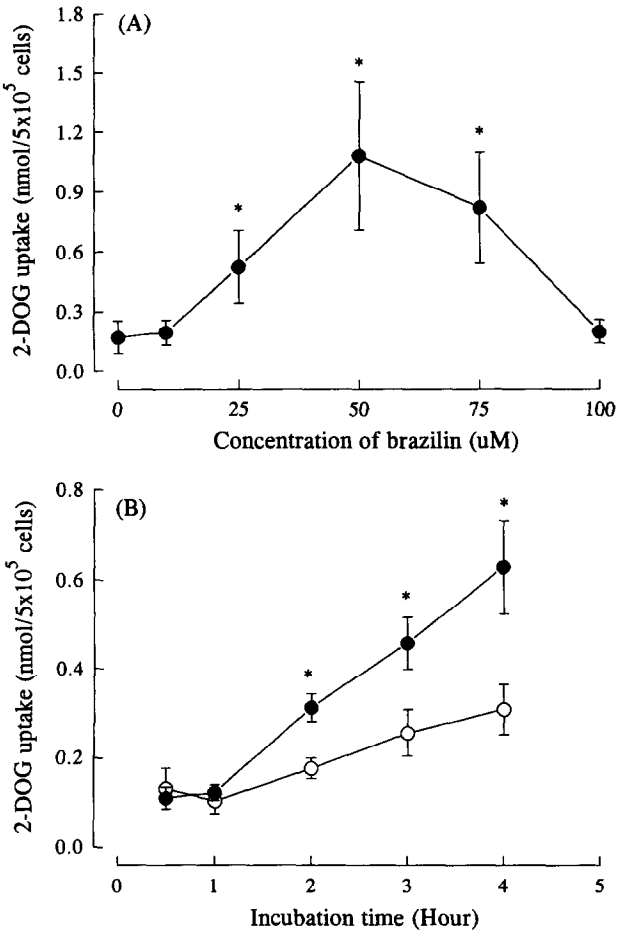

FIG. 1. Effect of brazilin on 2-DOG uptake in isolated rat epididymal adipocytes. (A) Adipocytes were treated with the indicated concentrations of brazilin for 4 hr, and 2-DOG uptake was measured. (B) Adipocytes were treated with (●) or without (○) brazilin (50 μM) for the indicated times, and 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. Key: (*) significantly different from the control group ($P < 0.05$).

It is well known that insulin is the major regulator of glucose utilization through the enhancement of glucose transport, and that calcium acts as a fusogen in the translocation of glucose transporters from an intracellular pool to the plasma membrane [7, 14, 15, 17]. Therefore, we investigated the role of calcium in brazilin-stimulated glucose transport using extra- and intracellular calcium modulators under the same conditions in which insulin stimulates glucose transport.

Figure 2 shows that the various levels of extracellular

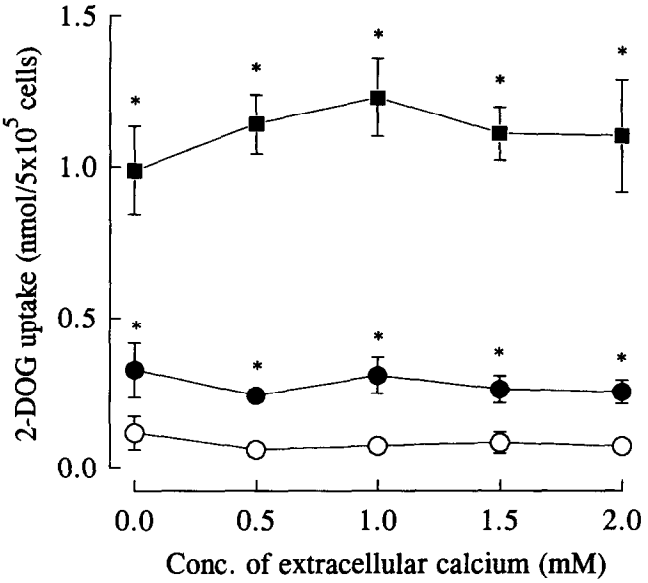

FIG. 2. Effect of extracellular calcium concentrations on 2-DOG uptake induced by brazilin in isolated rat epididymal adipocytes. Adipocytes were treated with (●) or without (○) brazilin (50 μM) for 4 hr or with insulin (25 ng/mL, ■) for the final 30 min in the medium with the indicated extracellular calcium concentrations. 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. Key: (*) significantly different from the control group ($P < 0.05$).

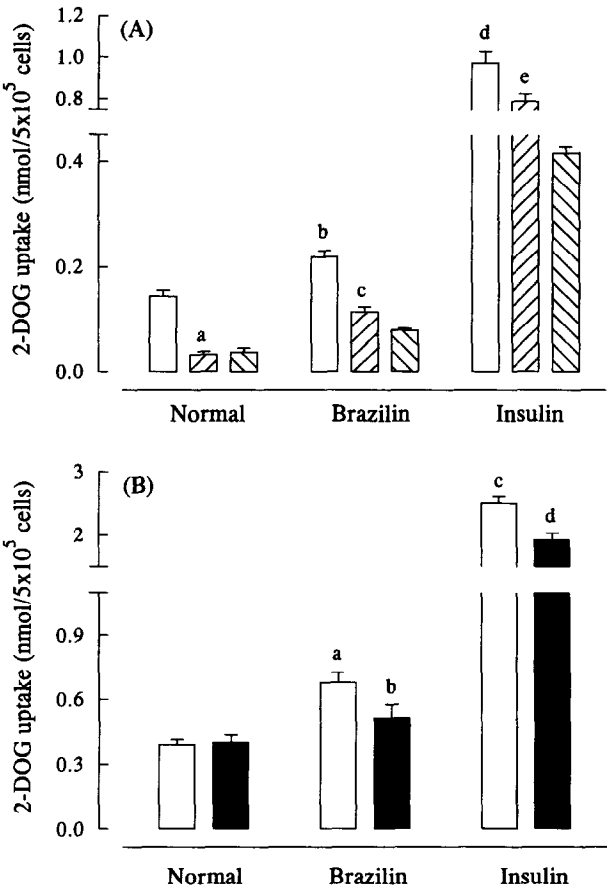

FIG. 3. Effect of nifedipine and verapamil on 2-DOG uptake induced by brazilin in isolated rat epididymal adipocytes. Adipocytes were treated with brazilin (50 μM) for 4 hr or insulin (25 ng/mL) for the final 30 min in the absence (□) or presence of nifedipine (10 μM, ▨; 100 μM, ▧; in panel A) or in the absence (□) or presence (■) of verapamil (100 μM; in panel B) for all incubation periods. 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. In panel A, control vs a, b, d, and e: $P < 0.05$; b vs c, d vs e: $P < 0.05$. In panel B, control vs a and c: $P < 0.05$; a vs b, c vs d: $P < 0.05$.

calcium did not alter the effect of brazilin on glucose transport. Depletion of extracellular calcium by the addition of 1 mM EDTA to calcium-free KRH buffer also did not alter brazilin-induced glucose transport (data not shown). The effect of the movement of extracellular calcium through the voltage-dependent calcium channel on brazilin-induced glucose transport was studied using the calcium channel blockers verapamil and nifedipine. Nifedipine (Fig. 3A) and verapamil (Fig. 3B) inhibited brazilin or insulin-stimulated glucose transport. Nifedipine also markedly inhibited basal glucose transport (Fig. 3A). This is in contrast to the effect of verapamil (Fig. 3B), in which there was no apparent effect on basal glucose transport. This fact suggests that an increase in intracellular calcium may be required for glucose transport stimulated by brazilin or insulin, but basal glucose transport may not be mediated by intracellular calcium elevation. To examine the effect of intracellular calcium on brazilin-stimulated glucose transport, quin2-AM and A23187 were used. Quin2-AM has been used to measure intracellular calcium levels in various cells [25]. In the present study, we used quin2-AM as a chelating agent to deplete intracellular calcium [16]. Chelation of intracellular calcium with quin2-AM nearly abolished the increase in glucose transport induced by either brazilin or insulin (Fig. 4).

A calcium ionophore, A23187, which causes a large elevation in intracellular calcium levels, showed no significant effect on basal and brazilin-induced glucose transport, but decreased insulin-stimulated glucose transport (Fig. 5). This reduction of insulin-induced glucose transport could be caused by excessive calcium influx, which elevates the intracellular calcium concentration above the optimum range for insulin-induced glucose transport [14].

Calcium uptake of adipocytes was measured in order to understand the role of intracellular calcium in glucose transport stimulated by brazilin. As shown in Fig. 6, the cumulative uptake of calcium in a time-dependent manner for 4 hr (panel A) or the influx of calcium for 10 min after a 4-hr incubation with brazilin (panel B) was unchanged. These data suggest that brazilin does not cause calcium influx and may not elevate the intracellular calcium level in adipocytes, and the increase in intracellular calcium did not essentially contribute to the action of brazilin in glucose transport. Inhibition of brazilin-induced glucose transport by nifedipine and verapamil may be caused by some unknown actions of these calcium channel antago-

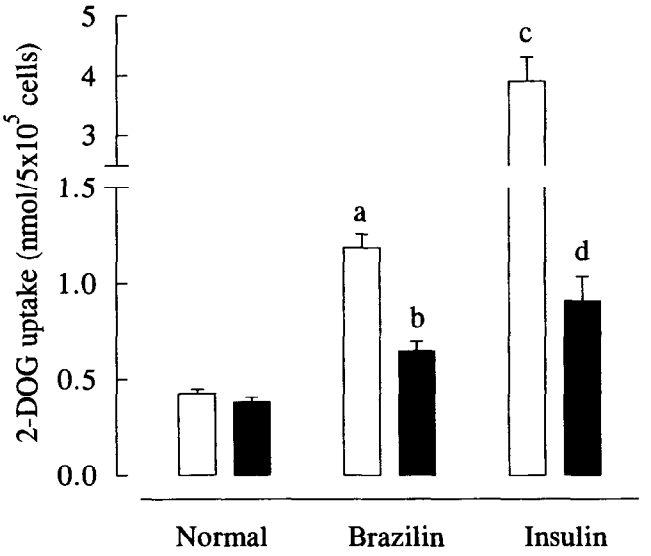

FIG. 4. Effect of quin2-AM on 2-DOG uptake induced by brazilin in isolated rat epididymal adipocytes. Adipocytes were treated with brazilin (50 μM) for 4 hr or insulin (25 ng/mL) for the final 30 min in the absence (□) or presence (■) of quin2-AM (100 μM) for all incubation periods. 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. Key: control vs a and c: $P < 0.05$; a vs b, c vs d: $P < 0.05$.

nists which may not be concerned with the blockade of calcium mobilization [26].

The action of calcium is mediated by binding to calmodulin during the fusion process. Trifluoperazine binds to, and blocks the biological action of, the calcium–calmodulin

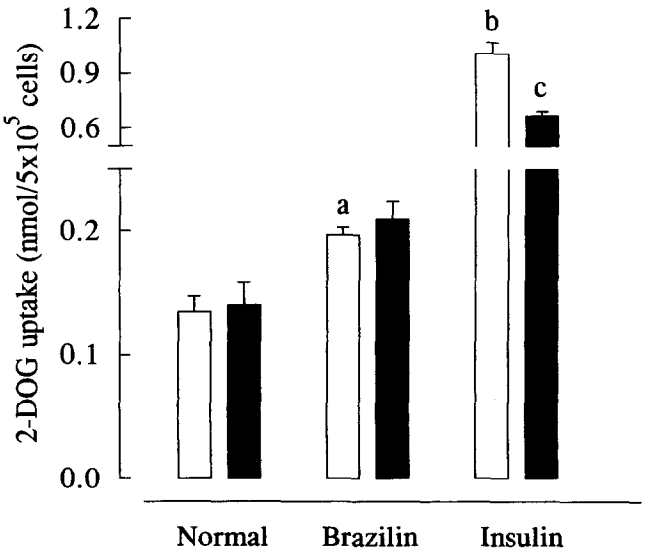

FIG. 5. Effect of A23187 on 2-DOG uptake induced by brazilin in isolated rat epididymal adipocytes. Adipocytes were treated with brazilin (50 μM) for 4 hr or insulin (25 ng/mL) for the final 30 min. A23187 (10 μM, ■) was added for the final 1 hr. 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. Key: control vs a and b: $P < 0.05$; b vs c: $P < 0.05$.

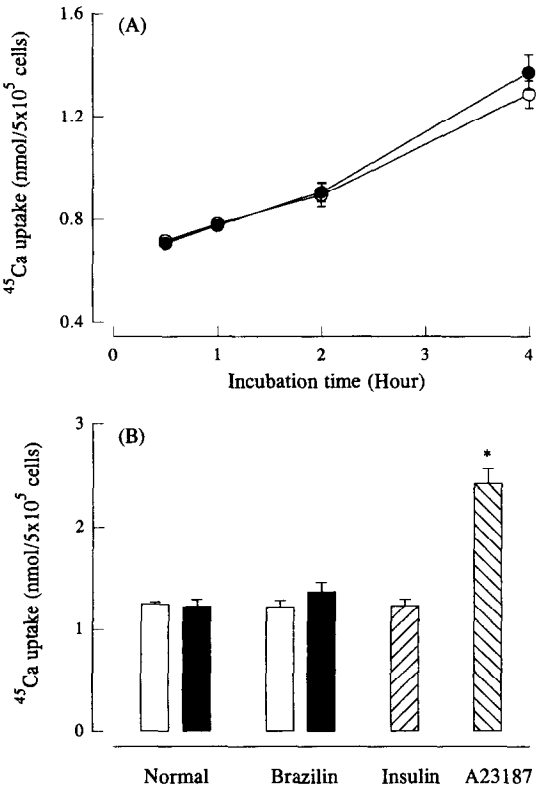

FIG. 6. Effect of brazilin on calcium uptake into isolated rat epididymal adipocytes. (A) Adipocytes were treated with (●) or without (○) brazilin (50 μM) for the indicated periods in the presence of $^{45}CaCl_2$. (B) Adipocytes were treated with brazilin (50 μM) for 4 hr, A23187 (10 μM) and nifedipine (10 μM, ■) for the final 1 hr, and insulin (25 ng/mL) for the final 30 min. $^{45}CaCl_2$ was added 10 min prior to the termination of incubation, and calcium uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. Key: (*) significantly different from the control group ($P < 0.05$).

complex [25]. The calcium–calmodulin system was found to participate in the fusion of exocytic vesicles containing glucose transporters to the plasma membrane [17]. As shown in Fig. 7, trifluoperazine significantly decreased the effects of brazilin and insulin on glucose transport. The level of inhibition of brazilin-induced glucose transport by trifluoperazine was less than that by quin2-AM. This could be the result of the different efficiencies of trifluoperazine and quin2-AM in binding to the calcium–calmodulin complex, or, chelation of intracellular calcium. Considering the fact that intracellular calcium and the calcium–calmodulin complex may be important factors for the insulin-induced translocation of glucose transporters, it can be assumed that brazilin increases glucose transport by stimulation of glucose transporter translocation from intracellular pools to the plasma membrane of adipocytes.

Insulin-responsive glucose transport is mediated largely by the translocation of glucose transporters from intracellular pools to the plasma membrane. This process is mediated by intracellular calcium and the calcium–calmodulin complex, which facilitate the fusion of glucose

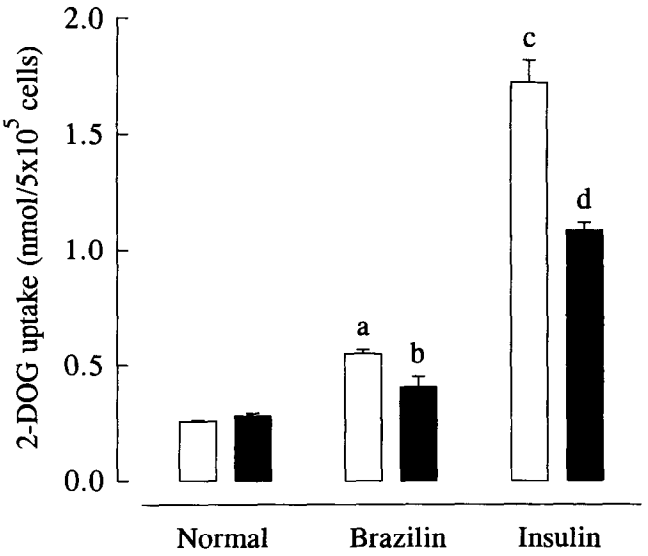

FIG. 7. Effect of trifluoperazine on 2-DOG uptake induced by brazilin in isolated rat epididymal adipocytes. Adipocytes were treated with brazilin (50 μM) for 4 hr or insulin for the final 30 min in the absence (□) or presence (■) of trifluoperazine (100 μM) for all incubation periods. 2-DOG uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3. Key: control vs a, c and d: $P < 0.05$; a vs b, c vs d: $P < 0.05$.

transporter-containing exocytic vesicles to the plasma membrane. The results obtained in this study showed that brazilin increased glucose transport in isolated rat epididymal adipocytes, and that the maintenance of a certain level of intracellular calcium and calmodulin activity appeared to be essential for brazilin-stimulated glucose transport. With respect to the role of intracellular calcium and calmodulin in glucose transporter translocation, the data suggest that brazilin increases glucose transport, at least partially, by the translocation of glucose transporters from intracellular pools to the plasma membrane.

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