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Role of Ca^{2+} in the Stimulation of Glucose Transport by Insulin in Adipocytes

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We investigated the role of Ca^{2+} and protein kinases/phosphatases in the stimulatory effect of insulin on glucose transport. In isolated rat adipocytes, the simple omission of $CaCl_2$ from the incubation medium significantly reduced, but did not abolish, insulin-stimulated 2-deoxy glucose (2-DG) uptake. Pre-loading adipocytes with intracellular Ca^{2+} chelator, 5,5'-dimethyl bis (*o*-aminophenoxy)ethane-N,N,N'N' tetraacetic acetoxymethyl ester (5,5'-dimethyl BAPTA/AM) completely blocked the stimulation. Insulin raised intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) about 1.7 times the basal level of 72 ± 5 nM, and 5,5'-dimethyl BAPTA/AM kept it constant at the basal level. This correlation between insulin-induced increases in 2-DG uptake and $[Ca^{2+}]_i$ indicates that the elevation of $[Ca^{2+}]_i$ may be prerequisite for the stimulation of glucose transport. Studies with inhibitors (ML-9, KN-62, cyclosporin A) of Ca^{2+}-calmodulin dependent protein kinases/phosphatases also indicate an involvement of intracellular Ca^{2+}. Additional studies with okadaic acid and calyculin A, protein phosphatase-1 (PP-1) and 2A (PP-2A) inhibitors, indicate an involvement of PP-1 in insulin action on 2-DG uptake. These results indicate an involvement of Ca^{2+}-dependent signaling pathway in insulin action on glucose transport.

Key Words: Insulin, Ca^{2+}, PP-1, 2-DG uptake, Adipocyte, 5,5'-dimethyl BAPTA/AM

INTRODUCTION

Insulin stimulates glucose transport in adipose tissue and skeletal muscle primarily by promoting the translocation of glucose transporters from an intracellular pool to the plasma membrane (Karnieli et al, 1981; Kono et al, 1982; Simpson et al, 1983; Holman et al, 1994). Insulin binding to its receptor activates tyrosine kinase, leading to autophosphorylation of tyrosine residues (Karlsson et al, 1979; White & Kahn, 1989). Despite the intensive efforts to elucidate the mechanism of insulin action, the intermediary steps between the activation of the insulin receptor tyrosine kinase and the stimulation of glucose transport, in particular, the nature of the intracellular signal(s) involved, have not been clearly defined yet.

Many previous studies have dealt with the possible involvement of Ca^{2+} in the signal transduction of insulin on the stimulation of glucose transport in adipocytes (Bonne et al, 1977, 1978; Pershadsingh & McDonald, 1984; Draznin et al, 1987; Klip & Ramal, 1987; Pershadsingh et al, 1987a, 1987b; Kelly et al, 1989), however, it remains controversial and elusive. Some of those studies (Bonne et al, 1977, 1978; Pershadsingh & McDonald, 1984; Pershadsingh et al, 1987a, 1987b) have shown a correlation between the elevation of $[Ca^{2+}]_i$ and the stimulation of glucose transport by insulin. However, low $[Ca^{2+}]_i$ and also high $[Ca^{2+}]_i$ have been found to be associated with the diminished responsiveness to insulin (Draznin et al, 1987a). Furthermore, other studies (Klip & Ramal, 1987; Kelly et al, 1989) have failed to find a causal relationship between $[Ca^{2+}]_i$ and the stimulatory effect of insulin on glucose transport. Thus, the possible involvement of $[Ca^{2+}]_i$ in the intermediary

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steps as an intracellular signal for insulin action on glucose transport needs further investigations.

The activation of the receptor tyrosine kinase by insulin binding couples to multiple intracellular signal transduction pathways through protein phosphorylation/dephosphorylation cascades by activating serine/threonine specific kinases/phosphatases (Lawrence, 1992; Saltiel, 1996). Other ligands acting through receptor tyrosine kinase such as epidermal growth factor, platelet-derived growth factor (PDGF) and basic fibroblast growth factors (bFGFs), have been found to cause a rapid rise in $[Ca^{2+}]_i$ (Moolenaar et al, 1986; Mogami & Kohima, 1993; Lovisolo et al, 1997). Furthermore, specific inhibitors of tyrosine kinase has been shown to abolish Ca^{2+} influx induced by PDGF (Ma et al, 1996) and bFGFs (Munaron et al, 1995). Thus, phosphorylation/dephosphorylation of intracellular target proteins by Ca^{2+}-calmodulin regulated pathway subsequent to the receptor tyrosine kinase activation may be involved in the stimulation of glucose transport by insulin.

This study was to test whether or not the intracellular Ca^{2+} is involved in the signal transduction of insulin on the stimulation of glucose transport in adipocytes and, if it is, to identify the subsequent biochemical steps of which Ca^{2+}-calmodulin dependent protein kinase(s)/phosphatase(s) involved, leading to phosphorylation/dephosphorylation of target proteins in the transducing cascade of insulin action. The role of intracellular Ca^{2+} in the insulin-signaling was tested by simple removal of extracellular Ca^{2+} in the incubation medium, as did in most previous studies (Bonne et al, 1977, 1978), and then rigorously by clamping $[Ca^{2+}]_i$ at physiological level with the use of intracellular Ca^{2+} chelator BAPTA/AM or at sub-physiological level with 5,5'-dimethyl BAPTA/AM (Tsien, 1980; Davis et al, 1989; Van Der Zee et al, 1989). The possibility of the involvement of protein kinases/phosphatases were investigated with the use of their putative selective inhibitors.

METHODS

Materials

Collagenase was purchased from Worthington Biochemical Corp. (Freehold, NJ), 2-deoxy-D-[1-^{3}H] glucose from Amersham Corp. (Arlington Heights, IL), BAPTA/AM, 5,5'-dimethyl BAPTA/AM, Ca^{2+} standard kit and fura-2/AM from Molecular Probes Inc. (Eugene, OR), ML-9 from Biomol Biochem Corp. (Plymouth Meeting, PA), KN-62 from LC laboratories (San Diego, CA), calyculin A and okadaic acid from RBI (Natick, MA), forskolin from Calbiochem (La Jolla, CA), cyclosporin A from Sandoz (Basle, Switzerland), and insulin and other standard chemicals from Sigma Chemical Co. (St. Louis, MO).

Isolation of rat adipocytes

Adipocytes were prepared from the epididymal fat pads of male 100~125 g Wistar rats by the collagenase digestion method of Rodbell (1964). Cells were washed 3 times and resuspended at a dilution of 10^7 cells/ml in KrebsRinger/HEPES incubation medium containing 130 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 1.3 mM $CaCl_2$, 10 mM $NaHCO_3$, 20 mM HEPES, 2 mM glucose and 20 mg/ml bovine serum albumin, pH 7.4. In some experiments, $CaCl_2$ was omitted from the medium to exclude extracellular Ca^{2+}.

2DG uptake

Aliquots (100 μl) of the cell suspension were pipetted into polystyrene 75×12 mm tubes and incubated without shaking at 37°C for 30 min. Insulin (100 nM) was added for 30 min prior to the measurement of 2-DG uptake. Other testing agents, such as 5,5'-dimethyl BAPTA/AM, ML-9, calyculin A, cyclosphorin A, okadaic acid, and forskolin, were present at a final concentration indicated in each experiment for 30 min prior to the addition of insulin. Uptake measurements were initiated by the addition of 2-deoxy-[^{3}H]glucose to a final concentration of 0.1 mM (100 μCi/mmol) and terminated after 3 min by the addition of 400 μl of cold incubation medium containing 20 μM cytochalasin B and 100 μM phloretin. Aliquots (200 μl) of the cell suspension were pipetted into 400 μl microcentrifuge tubes containing 100 μl of silicone oil and centrifuged for 30 sec at 15,000 xg. The tubes were cut through the oil layer, and the radioactivity associated with the cells was measured by scintillation counting. Non-carrier mediated transport was also assessed in parallel incubations containing 20 μM cytochalasin B and it was subtracted from each determination.

Measurement of $[Ca^{2+}]_i$

Cytosolic Ca^{2+} concentration in a single adipocyte was measured by monitoring the fluorescence of fura-2/AM loaded cells using Attofluor Digital Fluorescence Microscopy (Atto Instruments, Inc., Rockville, MA). Adipocytes attached to the poly-L-lysine coated plates, was loaded with fura-2/AM (2 μM) for 30 min. Following the calibration of the machine using calcium standard, the changes of $[Ca^{2+}]_i$ were measured before and after the addition of insulin with or without pre-incubation with 5,5'-dimethyl BAPTA/AM (25 μM; 30 min). The ratio of fura-2 fluorescence emitted at 510 nm was imaged while adipocytes were alternatively illuminated at 334 and 380 nm. $[Ca^{2+}]_i$ was determined by the ratio images of adipocytes calibrated with that of Ca^{2+} standard.

Statistics

Values are presented as mean±SE. The significance of the difference between means was assessed by Student's unpaired *t*-test. Differences were considered significant at $P<0.05$.

RESULTS

Ca^{2+} dependency of insulin effect on glucose transport

The simple omission of $CaCl_2$ from the incubation medium did not affect the basal rate of 2-DG uptake by the isolated adipocytes (data not shown). The insulin-stimulated 2-DG uptake was significantly influenced by the presence/absence of extracellular Ca^{2+}. Insulin (100 nM) increased 2DG uptake 10.5± 0.67 fold the basal level in the presence of Ca^{2+} ($P<0.01$), but its effect was significantly reduced to 50% in the absence of Ca^{2+} ($p<0.01$; Table 1).

Pre-incubation of the cells prior to the addition of insulin with a potent intracellular Ca^{2+} chelating agent, 5,5'-dimethyl BAPTA/AM (25 μM), completely blocked the stimulatory effect of insulin on 2-DG uptake regardless of the presence or absence of Ca^{2+} in the medium (Table 1). BAPTA/AM (25 μM) reduced, but not completely inhibited the effect of insulin on glucose transport. However, preincubation of the cells with A23187 (1 μM), a calcium ionophore, to raise $[Ca^{2+}]_i$ further (in the presence of extracellular Ca^{2+}) did not alter the magnitude of insulin-stimulated glucose transport. The basal rate of 2-DG uptake was only slightly affected by these Ca^{2+} chelators and an ionophore (data not shown).

Table 1. Effect of intracellular Ca^{2+} chelators, 5,5-dimethy BAPTA/AM and BAPTA/AM, and Ca^{2+} ionophore A23187 on insulin-stimulated 2-deoxy glucose (2-DG) uptake by the isolated rat adipocytes in the presence or absence of extracellular Ca^{2+}

	2-DG uptake		
	N	$+Ca^{2+}$	$-Ca^{2+}$
Insulin only	14	10.5±0.67	4.9±0.39*
Insulin+5,5-dimethyl BAPTA/AM	6	1.4±0.76†	0.3±0.04†
Insulin+BAPTA/AM	10	7.9±0.98†	ND
Insulin+A23187	6	10.2±0.67	ND

Results represent the mean±SE of 6~14 experiments, each performed in triplicate. Cells were pre-incubated with 5,5'-dimethyl BAPTA/AM (25 μM), BAPTA/AM (25 μM) or A23187 (1 μM) for 30 min prior to the addition of insulin (100 nM). 2-DG uptake represents an increase in ratio to the basal transport rate (in the absence of insulin). ND, not determined; *, $P<0.05$ versus $+Ca^{2+}$; †$P<0.05$ versus Insulin only.

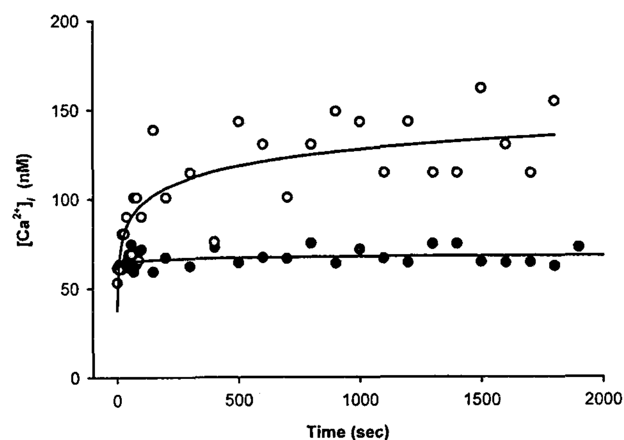

Fig. 1. Typical pattern of changes in cytoplasmic Ca^{2+} concentration in a single adipocyte after an exposure of insulin (100 nM) with (closed circle) or without (open circle) pre-incubation of 5,5'-dimethyl BAPTA/AM (25 μM). Insulin was added to the incubation medium at time zero. Results represent the interpolation of each spot.

Furthermore, by using Attofluor system, we could detect the changes of $[Ca^{2+}]_i$ in a single adipocyte (Fig. 1). Cytosolic Ca^{2+} was around 72±5 nM at basal state and was increased about 1.7 times to 120 ±5 nM ($P<0.01$) within a minute upon the addition of insulin. Pre-incubation with 5,5'-dimethyl BAPTA/AM completely blocked the increase of $[Ca^{2+}]_i$ in response to insulin, maintaining the level (66±1 nM) which was not significantly different from the basal value ($P=0.3$).

Involvement of protein phosphorylation/dephosphorylation in the mediation of insulin action

The biochemical nature of subsequent Ca^{2+}-calmodulin modulated activity of specific kinase(s)/phosphatase(s) leading to phosphorylation/dephosphorylation of target proteins in the transducing cascade of insulin action on glucose transport was examined by using various inhibitors (Table 2). Preincubation of the adipocytes with 100 μM of ML-9, a putative inhibitor of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK; Hidaka & Kobayashi, 1992), reduced the insulin-stimulated 2-DG uptake by 40%: the ratio of 2-DG uptake to the basal rate was decreased from 10.5±0.67 to 6.2±0.98 ($P<0.05$; Table 2). A specific inhibitor of multifunctional Ca^{2+}-calmodulin dependent protein kinase II, KN-62 (Li et al, 1992), also reduced the ratio by 30% ($P<0.05$, Table 2).

Addition of okadaic acid, known as a putative potent inhibitor of PP-1 and PP-2A (Li et al, 1992), inhibited insulin-stimulated 2-DG uptake by about 30% (7.6±0.99; $P<0.05$) at 100 nM and by 70% (4.0±0.42; $P<0.05$) at 1 μM. On the other hand, calyculin A (100 nM), another type of PP-1 and PP-2A inhibitor (Cohen et al, 1990), blocked the stimulatory effect of insulin on glucose transport completely, reducing the ratio of 2-DG uptake to the basal rate (1.2±0.18; $P<0.01$). A specific PP-2B inhibitor cyclosporin A (10 μM; Ikeuchi et al, 1992), blocked the insulin action on 2-DG uptake by about 60% (4.3±0.09; $P<0.01$). On the other hand, forskolin (50 μM), an adenylate cyclase activator, also completely inhibited insulin-stimulated glucose transport and the ratio of 2-DG uptake was not different from the control value of 1.0 (1.1±0.24; $P>0.5$).

Table 2. Effect of protein phosphatase/kinase inhibitors and forskolin on insulin-stimulated 2-deoxy glucose (2-DG) uptake by the isolated rat adipocytes

	N	2-DG Uptake
Insulin only	14	10.5±0.67
Insulin+ML-9	5	6.2±0.98*
Insulin+KN-62	5	7.4±0.34*
Insulin+Okadaic acid 100 nM	5	7.6±0.99*
1 μM	5	4.0±0.42*
Insulin+Calyculin A	14	1.2±0.18*
Insulin+Cyclosporin A	8	4.3±0.09*
Insulin+Forskolin	7	1.1±0.24*

Result represents the mean±SE of 5~14 experiments, each performed in triplicate. 2-DG uptake represents an increase in ratio to the basal transport rate (in the absence of insulin). Cells suspended in Krebs Ringer/HEPES buffer containing 1.3 mM Ca^{2+} were pre-incubated with test agents in the following final concentration for 30 min prior to the addition of 100 nM insulin: ML-9, 100 μM; KN-62, 50 μM; calyculin A, 100 nM; cyclosporin A, 10 μM; forskolin 50 μM. *, $P<0.05$ versus Insulin only.

DISCUSSION

Insulin binding to its receptor on the plasma membrane activates receptor tyrosine kinase and initiates cascades of sequential activation of target molecules involved in insulin signal transduction in adipocytes. Its effect culminates in the stimulation of glucose transport by the translocation of vesicles containing glucose transporters from the intracellular pool to the plasma membrane (Karnieli et al, 1981; Kono et al, 1982; Simpson et al, 1983; Holman et al, 1994). However, what occurs between the receptor activation and the translocation of glucose transporters has not yet been completely defined.

As stated above, there have been many controversies over the role of Ca^{2+} in mediating insulin action in adipocytes (Bonne et al, 1977, 1978; Pershadsingh & McDonald, 1984; Draznin et al, 1987a, 1987b; Klip & Ramal, 1987; Pershadsingh et al, 1987a, 1987b; Kelly et al, 1989). In the present study, the omission of $CaCl_2$ from the medium reduced insulin-stimulated 2-DG uptake without affecting a basal rate (Table 1), suggesting a possible involvement of Ca^{2+} in the mediation of insulin action. However, only a partial reduction, not a complete abolishment, of stimulatory effect of insulin in the

absence of extracellular Ca^{2+}, obscures the definite role of Ca^{2+} in insulin action.

To test further if an increase in $[Ca^{2+}]_i$ is essential for insulin-stimulated glucose transport, we incubated the isolated rat adipocytes with 5,5'-dimethyl BAPTA/AM before insulin stimulation. The tetrakis (acetoxymethyl) ester of 5,5'-dimethyl BAPTA rapidly enters cells where it is hydrolyzed to yield an impermeable tetracarboxylate form of 5,5'-dimethyl BAPTA, which functions as intracellular Ca^{2+} buffer and stabilizes $[Ca^{2+}]_i$ at around 50 nM (Davis et al, 1989; Van DerZee et al, 1989). The addition of 5,5'-dimethyl BAPTA/AM entirely abrogated insulin-stimulated increases in 2-DG uptake (Table 1) as well as that in $[Ca^{2+}]_i$, maintaining it at the basal level (Fig. 1). Although direct measurements of $[Ca^{2+}]_i$ were not made, the blockade of insulin-stimulated glucose transport by buffering intracellular Ca^{2+} has been shown in adipocytes using another type of chelator Quin-2 (Pershadsingh et al, 1987a & 1987b). On the other hand, BAPTA/AM whose affinity for Ca^{2+} (dissociation constant = 100 nM; Tsien, 1980) is lower than that of 5,5'-dimethyl BAPTA/AM, only partially inhibited the effect of insulin on 2-DG uptake (Table 1). Therefore, the present results strongly suggest a functional correlation between increases in 2-DG uptake and $[Ca^{2+}]_i$ by insulin and support the notion that an increase in $[Ca^{2+}]_i$ is fundamental for the stimulation of glucose transport by insulin in adipocytes. In view of our present results, discrepancies on the dependence of insulin action on Ca^{2+} in the previous studies with simple omission of extracellular Ca^{2+} are most likely to be attributed to an incomplete control of $[Ca^{2+}]_i$ upon the stimulation with insulin.

Calcium ionophore A23187, known to increase $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, did not significantly change the basal rate of 2-DG uptake in our experiments (data not shown). It has also been reported that increased cytoplasmic Ca^{2+} by the ionophores does not enhance glucose transport in insulin sensitive cells (Grinstein & Erlij, 1976; Bonne et al, 1977). Thus, an increase in $[Ca^{2+}]_i$ appears to be necessary, but not sufficient for the stimulation of glucose transport by insulin, suggesting that an another Ca^{2+}-independent signal pathway(s) might be activated in parallel with Ca^{2+}-calmodulin dependent pathway.

Insulin has been shown to increase $[Ca^{2+}]_i$ in various types of cells including adipocytes (Draznin et al, 1987b), hepatocytes (Benzeroual et al, 1997), neurons (Jonas et al, 1997), and vascular smooth muscle cells (Bkaily et al, 1992; Yoo et al, 1997). The mechanism by which insulin increases free $[Ca^{2+}]_i$ is not clear in the present study. Pershadsingh and McDonald (1984) have proposed that insulin inhibits the Ca^{2+}-Mg^{2+}-ATPase on adipocyte plasma membrane and thus prevents the efflux of Ca^{2+} from the cell. Draznin et al (1987a) have showed that insulin increases $[Ca^{2+}]_i$ at least in part by promoting Ca^{2+} influx to the cell through Ca^{2+} channels on the plasma membrane. Partial reduction in insulin-stimulated 2-DG uptake by simple omission of $CaCl_2$ from the medium (Table 1) in our present study and previous studies by others (Bonne et al, 1977, 1978) can be explained by a decreased rise in $[Ca^{2+}]_i$ upon insulin stimulation as a consequence of decreased Ca^{2+} influx in the absence of extracellular Ca^{2+}.

The raised $[Ca^{2+}]_i$ is likely to mediate the insulin effect through activating Ca^{2+}-calmodulin dependent protein kinases/phosphatases. It has been shown that a calmodulin inhibitor trifluoperazine blocks the stimulation of hexose uptake by insulin (Shechter, 1984). In the present study, effects of various inhibitors of protein kinases and phosphatases on insulin-stimulated 2-DG uptake were examined (Table 2). ML-9, an inhibitor of Ca^{2+}-calmodulin dependent MLCK, significantly inhibited insulin-stimulated 2-DG uptake as reported by other researchers (Inoue et al, 1993). Thus, intracellular free Ca^{2+} increased by insulin enhances glucose transport possibly through the activation of MLCK. However, multiple intracellular effects of ML-9 (Inoue et al, 1993; Begum, 1995) including the activation of mitogen-activated protein kinase, make this possibility difficult to be conclusive. A specific inhibitor of multifunctional Ca^{2+}-calmodulin dependent protein kinase (CaM kinase II), KN-62 (Li et al, 1992), inhibited 30% of insulin- stimulated 2-DG uptake. In addition, K252a, which has a broad specificity for protein kinases (Hidaka & Kobayashi, 1992), completely blocked insulin stimulated 2-DG uptake in cultured adipocytes (unpublished observation), supporting that some type(s) of protein kinases is involved in the cascade of insulin action.

Recently, it has been demonstrated that insulin activates Ser/Thr PP-1 by phosphorylating at the site-1 on its regulatory subunit in mammalian skeletal muscle (Begum, 1995). In our experiments, calyculin A and okadaic acid, which are known as PP-1 and

PP-2A inhibitors (Cohen et al, 1990; Begum, 1995), inhibited the stimulatory effect of insulin on 2-DG transport (Table 2). Okadaic acid, whose IC_{50} for PP-1 and PP-2A are approximately 300 nM and 1 nM, respectively (Dent, 1990), blocked insulin action by 30% at 100 nM and 70% at 1 μM (Table 2). Calyculin A (100 nM) was more effective in inhibiting insulin action on 2-DG uptake than okadaic acid (Table 2). Calyculin A has the same inhibitory potency to PP-2A as okadaic acid but has 10-100 times higher potency to PP-1 than the latter (Bioalojan & Takai, 1988; Ishihara et al, 1989a; Cohen et al, 1990). Considering such differential inhibitory potencies of the two inhibitors, it is likely that PP-1 activation is involved in the stimulation of glucose transport by insulin.

It has been shown that in adipocytes, major PP-1 is a glycogen-associated form of the enzyme which is a heterodimer composed of a regulatory subunit and a catalytic subunit (Inoue et al, 1993). Insulin is known to activate PP-1 by phosphorylating site-1 of the regulatory subunit (Sutherland et al, 1993; Begum, 1995). Phosphorylation of regulatory subunit at site-2 causes a release of catalytic subunit into the cytosol, inhibiting the activity of PP-1 (Ishihara et al, 1989b). The released catalytic subunit of PP-1 is inactivated by the binding of heat stable cytoplasmic protein, inhibitor-1 (I-1; Cohen et al, 1977; Hubbard & Cohen, 1989; Ishihara et al, 1989b). Phosphorylation of I-1 at a specific Thr residue leads to bind to the catalytic subunit of PP-1 causing an inhibition of PP-1 activity (Hubbard & Cohen, 1989). Cyclic AMP is known to phosphorylate I-1 as well as site-2 on regulatory subunit of PP-1 and thereby inactivate the enzyme (Cohen et al, 1977; Hubbard & Cohen, 1989; Ishihara et al, 1989b). In the present study, forskolin, a direct activator of adenylate cyclase, blocked insulin-stimulated 2-DG uptake completely (Table 2), again, indicating possible involvement of PP-1 in the mediation of insulin action. It is known that the site-2 on regulatory subunit of PP-1 and I-1 are the major endogenous substrates for Ca^{2+}-calmodulin dependent type 2B protein phosphatase (PP-2B; Cohen et al, 1977; Hubbard & Cohen, 1989). In addition, I-1 remains in a phosphorylated form in the absence of elevated PP-2B activity (Wera & Hemmings, 1995). We tested the effect of PP-2B blockage on insulin-stimulated 2-DG uptake. Cyclosporin A, a specific PP-2B inhibitor (Ikeuchi et al, 1992), inhibited insulin action by 60% (Table 2). Another type of PP-2B inhibitor FK506 also inhibited insulin action around 50% (data not shown). These inhibitors reduced but not completely blocked the insulin-stimulated 2-DG uptake, suggesting that another protein kinases such as MLCK, CaM kinase II, or a Ca^{2+}-independent protein kinase(s) in addition to PP-2B may be necessary for full activation of PP-1. Thus, based on the effects of forskolin and PP-2B inhibitors, we propose that insulin activates PP-1 both directly by phosphorylating at site-1 on regulatory subunit and indirectly by dephosphorylating I-1 via the activation of PP-2B cascade.

In summary, our results show that an elevation of $[Ca^{2+}]_i$ is essential but not sufficient for insulin action on glucose transport and a series of Ca^{2+}-calmodulin dependent protein kinase/phosphatase reaction are involved. A tentative cascade of protein phosphorylation/dephosphorylation in insulin action on glucose transport according to our data is shown in Fig. 2. Insulin is proposed to enhance glucose

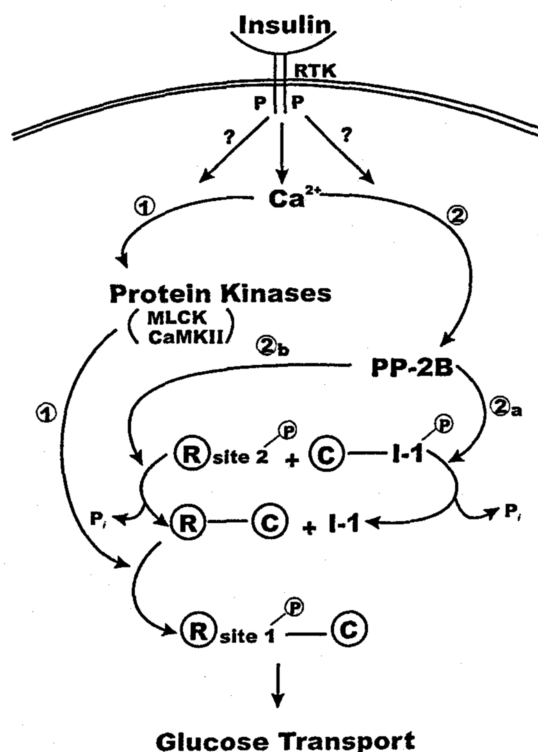

Fig. 2. Hypothetical scheme of insulin action on glucose transport. RTK: receptor tyrosine kinase; MLCK: myosin light chain kinase; CaMKII: multifunctional Ca^{2+}-calmodulin dependent protein kinase; R and C: regulatory and catalytic subunit of protein phosphatase 1, respectively; PP-2B: protein phosphatase 2B; I-1: inhibitor 1.

transport by activating PP-1 through Ca^{2+}-dependent pathways. First, an elevated $[Ca^{2+}]_i$ may phosphorylate the site-1 of PP-1 regulatory subunit by activating Ca^{2+}-dependent protein kinases such as MLCK and/or CaM kinase II (Fig. 2, ①). Secondly, an elevated $[Ca^{2+}]_i$ may activate Ca^{2+}-calmodulin dependent protein phosphatase PP-2B which causes dephosphorylation of I-1 (Fig. 2, ②a) and the regulatory subunit of PP-1 at site-2 (Fig. 2, ②b), resulting in activation of PP-1. However, understanding of the interaction between elevation of $[Ca^{2+}]_i$ and activation of PP-1 in insulin signal transduction requires further studies.

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