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Brazilin Inhibits Activities of Protein Kinase C and Insulin Receptor Serine Kinase in Rat Liver

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Hypoglycemic action of brazilin was found to be based on the improvement of peripheral glucose utility, and this action might be correlated with the insulin action pathway. In the present study we investigated the effect of brazilin on the insulin receptor autophosphorylation, protein kinase C (PKC), protein phosphatase and insulin receptor serine kinase in order to confirm whether the hypoglycemic mechanism is concerned with insulin action pathway. Brazilin was found to inhibit PKC and insulin receptor serine kinase, which are involved in the regulation of insulin signal pathway. But any significant effect was not shown on insulin receptor tyrosine kinase activity, autophosphorylation and phosphatase activity. These findings suggest that brazilin might enhance insulin receptor function by decreasing serine phosphorylation, which might mediate hypoglycemic effect of brazilin

Key words : Brazilin, Insulin receptor, Autophosphorylation, Protein kinase C, Serine kinase, Liver

INTRODUCTION

Brazilin, the main component of *Caesalpinia sappan*, has been examined for its effects on diabetic complications, such as lens aldose reductase inhibitory effect (Moon *et al.*, 1985) and its improving action on erythrocyte deformability (Moon *et al.*, 1989). Brazilin has also been shown to enhance glucose metabolism in soleus muscles from streptozotocin-induced diabetic rats and in adipose tissues from diabetic KK-mice (Moon *et al.*, 1990, Lee *et al.*, 1993). These findings suggest that the hypoglycemic action of brazilin might be based on the stimulation of peripheral glucose utilization, possibly through the modulation of the postreceptor events of insulin (Lee *et al.*, 1993). The β-subunit of the insulin receptor has intrinsic kinase activity and phosphorylates itself. It is well recognized that these autophosphorylation and kinase actions are the early steps in the signal transduction pathway of insulin (Ellis *et al.*, 1986) and that the insulin signal could be modulated by protein kinase C (PKC) (Tavare *et al.*, 1991), protein phosphatase (Sale, 1992) and insulin receptor serine kinase (Smith *et al.*, 1988a). To examine whether brazilin affects the insulin action pathway, we investigated the effects of brazilin on the kinase activity of insulin receptors and other possible modulating processes.

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MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (180~220 g) were obtained from the Experimental Animal Breeding Center of Seoul National University and allowed food and water ad libitum. Brazilin was obtained from Aldrich Chemicals, Milwaukee, USA. A micropartition system was purchased from Amicon Co, Danvers, USA. Radiolabeled [^{32}P]ATP (3.7 MBq/100 ul) and [^{125}I]-insulin (370 KBq/100 ul) were obtained from Amersham, Buckinghamshire, England, and New England Nuclear, USA, respectively. Other reagents were obtained from Sigma Chemicals, St. Louis, USA.

Preparation of partially purified insulin receptors

Partially purified insulin receptors were prepared by the method of Kasuga (Kasuga *et al.*, 1981), modified as follows. Rats were anesthetized with urethane (1 g/kg body weight) and hepatic blood was perfused out with phosphate buffered saline (pH 7.4). The perfused liver was homogenized with 4 volumes of cold homogenization buffer (0.25 M Sucrose, 1 μg/ml aprotinin, 0.1 mg/ml bacitracin, 1 mM PMSF, 2 μM pepstatin A, 2 μM leupeptin, 50 mM HEPES, pH 7.6) at 4°C. The

homogenate was centrifuged at 10,000 g for 20 min at 4°C and the pellet was resuspended in washing buffer (homogenization buffer without sucrose). The resuspension was centrifuged at 100,000 g for 90 min at 4°C. The pellet was solubilized for 120 min at 4°C by continuous stirring in solubilizing buffer (washing buffer containing 1% Triton X-100). This preparation was centrifuged at 100,000 g for 90 min at 4°C, and the insoluble fraction removed. The supernatant was applied to a wheat germ agglutinin (WGA)-agarose column, which was extensively washed at 4°C with column washing buffer (10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 100 mM NaF, 1 μg/ml aprotinin, 0.1% Triton X-100, 1 mM PMSF, 20 mM HEPES, pH 7.6). Bound glycoproteins, including insulin receptors, were desorbed with 0.3 M of N-acetylglucosamine. In the preparations used for phosphatase activity and the dephosphorylation assay, phosphatase inhibitors were excluded from all the purification buffers.

Insulin binding assay

Aliquots of the eluates were incubated with [^{125}I]-insulin (20,000 cpm, final concentration: 2 ng/ml) at 4°C for 16 hrs in the presence or absence of unlabeled insulin (100 ng/ml). The bound insulin was precipitated by polyethylene glycol and bovine-γ-globulin (Cuatrecasas, 1972). The insulin receptor yield was estimated from the radioactivity of the precipitates.

Phosphorylation of exogenous substrate

A partially purified insulin receptor, which was incubated with various concentrations of insulin and brazilin for 4 hrs at 4°C, was mixed with exogenous substrate (copolymer Glu:Tyr=4:1, final concentration: 2 mg/ml). Phosphorylation was initiated by the addition of [^{32}P]ATP, $MnCl_2$ and $MgCl_2$ at final concentrations of 10 μM (2~5 μCi/nmole), 2 mM and 12 mM, respectively. After 15 min at 20°C the reaction was stopped by blotting the reaction mixture on a 2×2 cm^2 3MM Whatman filter paper and soaking the paper immediately in 10% TCA solution containing 0.01 M sodium pyrophosphate, 100 mM NaF, 2 mM sodium vanadate, and 5 mM EDTA (Pike *et al.*, 1986). The papers were washed with 75 mM phosphoric acid three times and dried. The radioactivity of the papers was measured.

Autophosphorylation

Partially purified insulin receptors were incubated with various concentrations of insulin and brazilin for 16 hrs at 4°C. Phosphorylation was initiated by the addition of reaction mixture calculated to give final concentrations of 6 mM $MnCl_2$ and 10 μM [^{32}P]ATP (10~15 μCi/nmole). After incubation for 30 min at 4°C the reaction was terminated by boiling for 5 min in Laemmli sample buffer (pH 6.8) containing 10 mM ATP, 100 mM DTT, 10 mM EDTA, 1% SDS, 10% glycerol, 0.05% bromphenol blue and 0.5 M Tris base. Phosphorylated proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gel. After electrophoresis, the gel was dried and autoradiographed.

Protein kinase C activity

To measure PKC activity, a modification of the method of Cooper et al. (Huang *et al.*, 1991) was used. The perfused liver was homogenized in 4 volumes of extraction buffer (5 mM EDTA, 10 mM EGTA, 1 mM DTT, 2 mM PMSF, 20 μg/ml leupeptin, 0.02% Triton X-100, 20 mM Tris, pH 7.5) at 4°C and centrifuged at 100,000 g for 1 hr at 4°C The supernatant was applied to a DEAE-cellulose column and the column washed with washing buffer (0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10% glycerol, 20 mM Tris, pH 7.5). PKC was eluted with a 0~4 M NaCl gradient and incubated with brazilin for 4 hr at 4°C. The PKC was then incubated for 15 min at 37°C in 50 mM Tris buffer (pH 7.5) containing 16 μM PMA, 177 μM phosphatidylserine, 75 μM peptide (Lys-Arg-Thr-Leu-Arg-Arg), 1 mM $CaCl_2$, 15 mM $MgCl_2$, 1 mM DTT, 50 μM [^{32}P]ATP (0.6 μCi/nmole). The reaction was terminated with ice cold TCA (final concentration: 10%). The samples were centrifuged and the supernatants spotted onto Whatman P-81 paper. The papers were washed three times with 75 mM phosphoric acid, dried and their radioactivity measured.

Phosphatase activity

Aliquots from eluates, solubilized membrane and cytosol were pre-incubated with brazilin at 4°C and then incubated with 10 mM *p*-nitrophenol phosphate for 15 min at 30°C in various buffers containing 0.5 mM $MgCl_2$. The reaction was stopped by the addition of 0.1 M EDTA and 0.2 N NaOH and the absorbance was measured at 410 nm (Tonks *et al.*, 1988). The buffer systems used were 200 mM acetate buffer (pH 5.5), 50 mM HEPES (pH 7.4), 50 mM phosphate buffer (pH 6.5, pH 8.0), and 10 mM diethanolamine (pH 9.5).

Dephosphorylation

The method of Gruppuso *et al.* (1992) was modified. Partially purified insulin receptors were incubated with 100 nM insulin for 16 hrs at 4°C and then mixed with 10 μM [^{32}P]ATP (10~15 μCi/nmole) and 6 mM $MnCl_2$. After 40 min at 4°C non-reacted [^{32}P]ATP was removed by a micropartition system and isotope-free buffer containing 10 mM $MgCl_2$, 1 mM ATP, 1 mM PMSF and 10 μM brazilin was added. After the in-

dicated time, samples were boiled for 5 min in Laemmli sample buffer and subjected to 7.5% SDS-PAGE. The gel was dried and autoradiographed.

Phosphoamino acid analysis

The method of Smith *et al.* (1988b) was modified. The region of interest of the polyacrylamide gel was excised and reswollen with bicarbonate buffer (0.05 M NH_4HCO_3, 1% SDS, 1% 2-mercaptoethanol). The radioactive material was eluted by 23 mM electricity. The eluate was lyophilized and hydrolyzed in 6 N HCl for 90 min at 110°C. The hydrolysate was lyophilized, redissolved in deionized water, lyophilized, and applied to a high performance thin layer chromatography (HPTLC) plate. The plate was developed with a mixture of PrOH and water (2.1:1, v/v). Phosphoamino acid standards were spotted, detected by staining with Cd/ninhydrin reagent (1% ninhydrin in 100 ml of acetone containing 15 ml of cadmium acetate [0.6% Cd acetate in 50% acetic acid]) and [^{32}P]-labeled phosphoamino acids were identified by autoradiography.

Statistical analysis

Values of three independent experiments were expressed as mean±SE. Values which showed $P<0.05$ by Student's t-test were regarded as significantly different.

RESULTS AND DISCUSSION

Insulin receptors in solubilized membranes were partially purified using WGA-agarose chromatography. This method reportedly yields a maximum recovery of insulin receptors with approximately a 20-fold purification (Freidenberg *et al.*, 1985). As shown in Fig. 1, the eluate from the WGA-agarose column demonstrated insulin-stimulated phosphorylation of a 95 kDa protein. This is the same molecular weight as the β-subunit of the insulin receptor and the profile of insulin binding activity from each fraction was consistent with that of phosphorylation. This confirmed that the 95 kDa protein isolated was the β-subunit of the insulin receptor.

The possible role of phosphorylation in mediating the action of insulin is supported by the finding that the insulin receptor expresses insulin-stimulable kinase activity directed toward its own β-subunit as well as toward tyrosine residues of exogenous substrate (Petruzzelli *et al.*, 1983, Klein *et al.*, 1986). To determine if brazilin could affect the insulin action pathway, partially purified insulin receptors were incubated with brazilin at 4°C and tyrosine kinase activity and insulin receptor phosphorylation were assayed. The tyrosine kinase activity toward exogenous substrate was not affected significantly by 1 and 10 μM brazilin, but was decreased by 100 μM brazilin (Fig. 2). As shown in Fig. 3, autophosphorylation of the β-subunit of the insulin receptor (95 kDa) was not affected by brazilin. Interestingly, brazilin potently diminished the phosphorylation of a 170 kDa protein, which was phosphorylated without any influence of insulin. This 170 kDa protein must be phosphorylated by an unknown kinase or kinases co-purified with the insulin receptors. Similar 170 kDa protein bands were found in WGA-purified extracts and phosphotyrosine was the only phosphoamino acid detected in this band. Since this

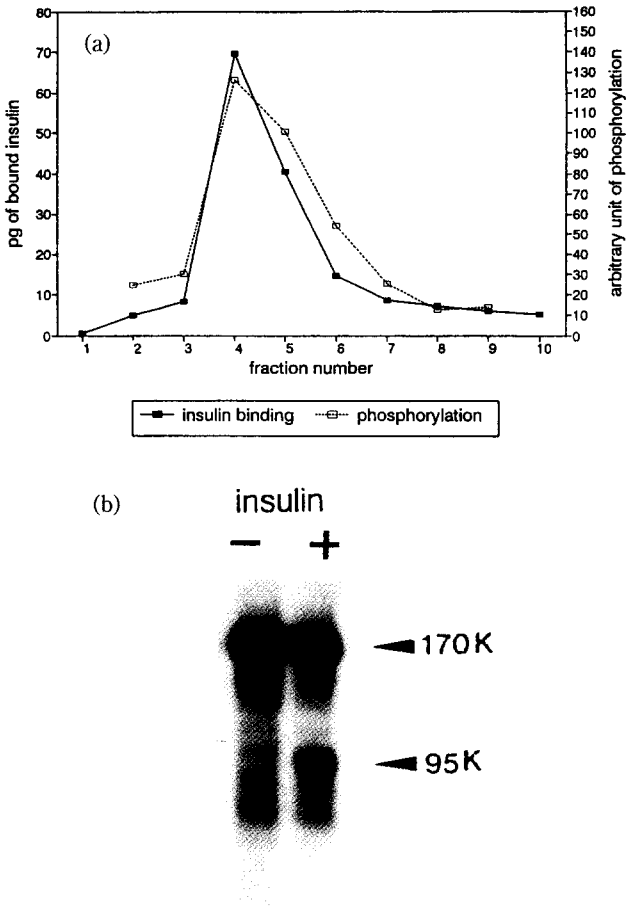

Fig. 1. Identification of partially purified insulin receptors. Aliquots of each fraction from a WGA-agarose column were incubated with 100 nM insulin for 16 hr at 4°C and then mixed with 10 μM [^{32}P]ATP (10~15 μCi/nmole) and 6 mM $MnCl_2$. After 30 min incubation, samples were boiled for 5 min in Laemmli sample buffer and applied to SDS-PAGE. The gel was dried and autoradiographed. The intensities of 95 kDa were measured by densitometer (Biomed Instrument Inc., USA). For insulin binding assays, aliquots of each fraction were incubated with [^{125}I]-insulin (20,000 cpm) at 4°C for 16 hr in the presence or absence of unlabeled insulin (100 ng/ml). The complexes of insulin and receptor were precipitated with 11% PEG and 0.07% bovine-γ-globulin. The radioactivity of the precipitates was calculated in pg of bound insulin.

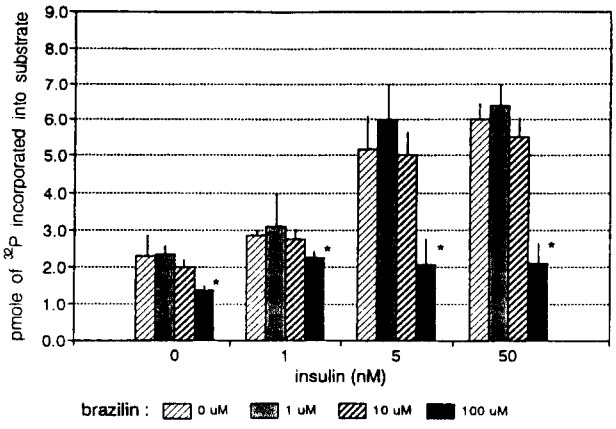

Fig. 2. Effects of brazilin on tyrosine kinase activity of partially purified insulin receptors. Partially purified insulin receptors were incubated for 16 hr at 4°C with various concentrations of insulin and brazilin and then mixed with copolymer (Glu:Tyr=4:1, final conc. 2 mg/ml) at 22°C. After 10 min incubation, the reaction reagents (10 μM ATP[^{32}P], 2~5 μCi/nmole, 2 mM $MnCl_2$ and 12 mM $MgCl_2$) were added, and incubated for indicated time intervals. The reaction mixture was spotted on Whatman No.3 filter paper (2×2 cm^2) and the paper immediately soaked in a 10% TCA bath. After 3 washes with 75 mM phosphoric acid, radioactivity was measured with scintillation counter.

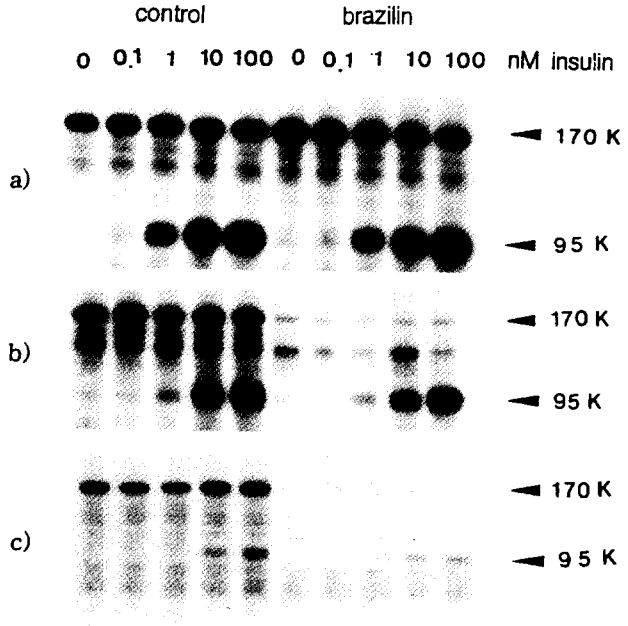

Fig. 3. Effects of brazilin on autophosphorylation of partially purified insulin receptors. Partially purified insulin receptors were incubated for 16 hr at 4°C with various concentrations of insulin and brazilin [a) for 1 μM, b) for 10 μM, and c) for 100 μM] and then mixed with 10 μM [^{32}P] ATP (10~15 μCi/nmole) and 6 mM $MnCl_2$. After 30 min, samples were boiled for 5 min in Laemmli sample buffer and applied to SDS-PAGE. The gel was dried and autoradiographed.

band was not immunoprecipitated by anti-insulin receptor antibodies, the results indicate that this protein band is not a component of insulin receptors (Kadowaki *et al.*, 1984). Because phosphorylation of the protein is stimulated by treatment with Epidermal Growth Factor (EGF) (Freidenberg *et al.*, 1985), the protein was thought to be EGF. The band disappeared after high fat feeding (Watarai *et al.*, 1988). There is no clear explanation for the function of this protein and it is considered that some effects of brazilin on the 170 kDa protein might play a role in controlling cellular events.

We predicted that the effect of brazilin might be similar to that of insulin. However, although brazilin affected phosphorylation of the 170 kDa protein, insulin receptor tyrosine kinase activity and autophosphorylation was not increased. This finding suggested that brazilin does not affect insulin receptor function directly but that it may affect the control factors for insulin receptor phosphorylation such as PKCs, phosphatases, and insulin receptor serine kinases.

It is possible that insulin receptor stimulation might increase PKC activity because diacylglycerol (DAG) has been found to be increased in insulin-stimulated cells (Farese *et al.*, 1985a, Farese *et al.*, 1985b). Phorbol ester increased basal glucose uptake but inhibited insulin-stimulated glucose transport and insulin binding in rat adipocytes (Kirsch *et al.*, 1985). Moreover, it has been shown that phorbol ester can antagonize the action of insulin on glycogen synthase and tyrosine aminotransferase (Roach *et al.*, 1983, Takayama *et al.*, 1984), inhibit insulin receptor kinase activity (Obermaier *et al.*, 1987) and stimulate the phosphorylation of insulin receptors (Jacobs *et al.*, 1983). It is not certain whether PKC is the crucial signal of insulin receptor activation, but it is likely that PKC might be involved in the insulin action pathway and might play a rôle in mediating the action of insulin. In fact, insulin receptors were phosphorylated on seryl-residue by phorbol ester and this seryl-phosphorylation decreased the kinase activity of insulin receptors (Duronio *et al.*, 1990). As shown in Fig. 4, brazilin decreased PKC activity in the present study. It might be that brazilin affects insulin receptor serine phosphorylation and kinase activity through the modulation of PKC activity.

Phosphatases can modulate the lifetime of phosphorylated insulin receptors (Sale, 1992, Gruppuso *et al.*, 1992) and therefore inhibition of phosphatases may enhance the action of insulin. It is well known that hydrogen peroxide and vanadate have insulin-like effects through the inhibition of dephosphorylation of insulin receptors (Heffetz *et al.*, 1992). As a first step, we investigated the effect of brazilin on cytosolic phosphatase activities specified with various pH ranges. The results showed that brazilin has no effect on the

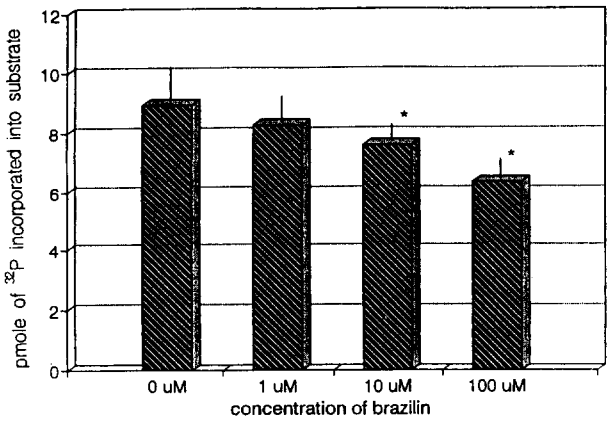

Fig. 4. Effects of brazilin on PKC activity. PKC purified using a DEAE-cellulose column was preincubated with brazilin (0~100 μM) for 4 hr at 4°C and then further incubated in Tris buffer (16 μM PMA, 177 μM phosphatidylserine, 1 mM $CaCl_2$, 75 μM peptide [Lys-Arg-Thr-Leu-Arg-Arg], 15 mM $MgCl_2$, 1 mM DTT, 50 μM [^{32}P]ATP, 0.6 μCi/nmole, 50 mM Tris, pH 7.5) for 15 min at 37°C. Ice cold TCA (10%) was then added. After centrifugation, the supernatants were spotted onto Whatman P-81 paper and radioactivity was measured with a liquid scintillation counter. Asterisks indicate significant differences ($p<0.05$, Student's t-test) from 0 μM group.

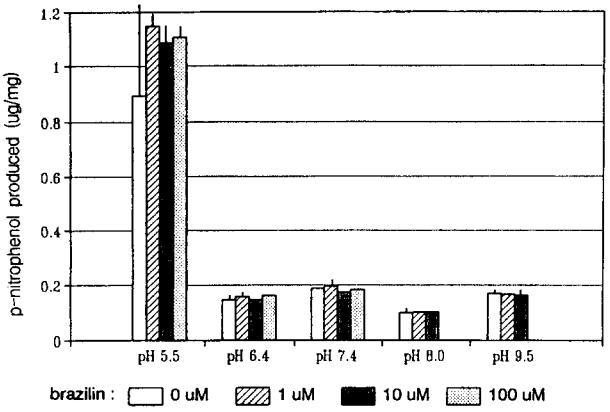

Fig. 5. Effects of brazilin on cytosolic phosphatase activity. Aliquots from cytosol were incubated with various concentrations of brazilin for 4 hrs at 4°C followed by incubation with 10 mM *p*-nitrophenol phosphate for 15 min at 30°C in various buffer systems. The reaction was stopped by the addition of 0.1 M EDTA and 0.2 N NaOH. Absorbance was measured at 410 nm.

activities of these phosphatases (Fig. 5). We next examined membrane phosphatases. As phosphatase activity was found to be low in the membrane, the dephosphorylation rate of insulin receptor was investigated in the absence of phosphatase inhibitors. Phosphorylated insulin receptors spontaneously dephosphorylated with time and the rate was not affected by treatment with brazilin (Fig. 6).

Phosphoamino acid analysis showed that insulin receptors were phosphorylated at the tyrosyl- and seryl-

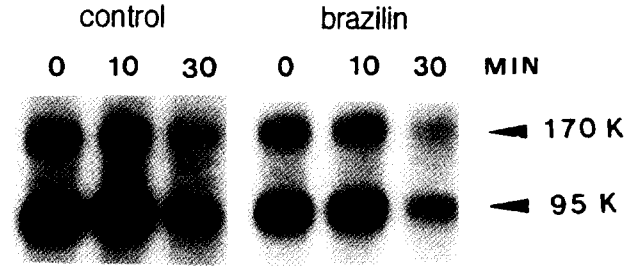

Fig. 6. Effects of brazilin on the dephosphorylation rate of partially purified insulin receptors. Insulin receptors were partially purified using a WGA-agarose column with buffer systems which contained no phosphatase inhibitors. The partially purified insulin receptors were incubated with 100 nM insulin for 16 hr at 4°C and then mixed with 10 μM [^{32}P]ATP (10~15 μCi/nmole) and 6 mM $MnCl_2$. Forty min later [^{32}P]ATP was removed by a micropartition system (Amicon Co.), and isotope-free buffer (10 mM $MgCl_2$, 1 mM PMSF, 1 mM ATP) and brazilin (10 μM) were added. After the indicated times, samples were boiled for 5 min in Laemmli sample buffer and subjected to SDS-PAGE. The gel was dried and autoradiographed.

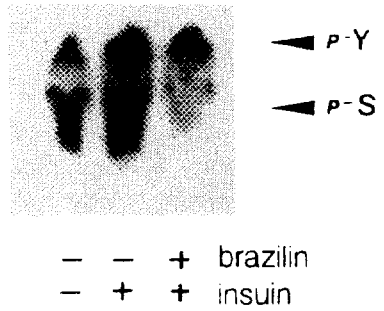

Fig. 7. Phosphoamino acid analysis of 95 kDa protein. A 95 kDa protein was eluted from SDS-PAGE and hydrolyzed in 6 N HCl at 110°C for 90 min. The hydrolysate was spotted on HPTLC and developed with a propanol:water (2.1:1) system. The HPTLC plate was dried, autoradiographed, and visualized with ninhydrin (p-Y:phosphotyrosine, p-S:phosphoserine).

residues (Fig. 7). Phosphorylation at the two residues was increased by insulin treatment, but phosphorylation at the seryl- residue was markedly inhibited by brazilin. It was recently reported that insulin receptors had serine kinase activity in vitro in addition to tyrosine kinase activity (Sale, 1992, Smith *et al.*, 1988b). It is believed that insulin receptor serine kinase is closely related to insulin receptor tyrosine kinase (Smith *et al.*, 1988a, Chou *et al.*, 1987, Smith *et al.*, 1989, Baltensperger *et al.*, 1992) and that this serine kinase activity inhibits or controls insulin receptor function. Therefore, we suggest that brazilin might enhance insulin receptor function through the inhibition of insulin receptor serine kinase.

We have previously reported that the hypoglycemic action of brazilin is dependent upon the enhancement

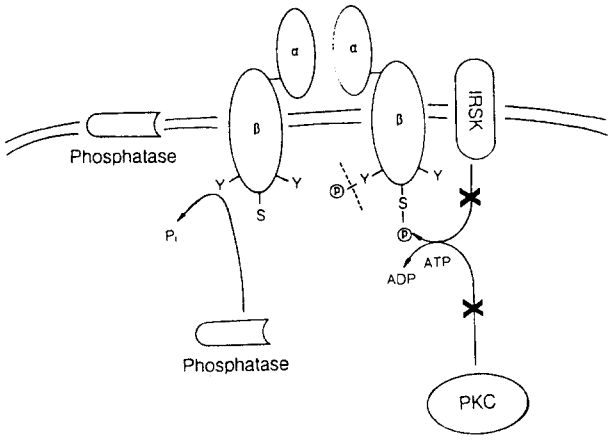

Fig. 8. A schematic model for the effect of brazilin on the insulin receptor and its regulation systems. Insulin receptor tyrosine kinase activity and phosphorylation were not affected. The regulatory systems for insulin receptor phosphorylation were modulated by brazilin. Brazilin, however, did not affect the dephosphorylation of insulin receptors by cellular or intrinsic phosphatases. PKC and insulin receptor serine kinase activity was inhibited by brazilin.

of cellular glucose utilization, and that this might be due to the modulation of the insulin action pathway (Moon *et al.*, 1990, Lee *et al.*, 1993). In the present study, we did not find any significant effect of brazilin on autophosphorylation and tyrosine kinase activity of partially purified insulin receptors. However, brazilin inhibited PKC and insulin receptor serine kinase, proteins which are regarded as possible modulators of insulin receptor function. Brazilin also inhibited the phosphorylation of a 170 kDa protein, the function of which has not yet been elucidated. As shown in Fig. 8, brazilin might modulate the function of insulin receptors by the inhibition of PKC and insulin receptor serine kinase, and this biochemical modulation may be one of the mechanisms underlying its enhancement of cellular glucose utilization.

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