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Cyclosporin A Stimulation of Glucose-Induced Insulin Secretion in MIN6 Cells*

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ABSTRACT

Effects of the immunosuppressant cyclosporin A (CsA), a specific inhibitor of Ca2+/calmodulin-dependent protein phosphatase (PP2B), were examined with regard to the induction of insulin secretion from MIN6 cells, a glucose-responsive cell line derived from mouse insulinoma. CsA had no effect on basal insulin secretion from MIN6 cells, but did increase glucose-, tolbutamide-, and KCl-induced insulin secretion. Treatment of the cells with CsA resulted in a dose-dependent increase in insulin secretion, which was maximal at 3 μM. CsA inhibited PP2B activity in a dose-dependent manner, and the increase in insulin secretion correlated with the decrease in PP2B activity. In 32P-labeled cells, treatment with CsA for 30 min increased phosphorylation of synapsin I-like protein by 50 ± 5.7%. As revealed by one-dimensional phosphopeptide mapping of 32P-labeled synapsin I-like protein, treatment with CsA for 30 min increased phosphorylation of site II of synapsin I-like protein by 59 ± 8%, which is phosphorylated by calmodulin kinase II. Messenger RNAs, which hybridize with complementary DNAs of calcineurin A and B subunits from rat brain, were detected in MIN6 cells. Western blot analysis showed a 61-kDa band, which interacts with rat brain calcineurin A antibody. Similar increases in secretagogue-induced insulin secretion with CsA were observed for HIT-T15 cells. These results suggest that CsA stimulates glucose-induced insulin secretion by inhibiting the activity of PP2B, an event that may be involved in mechanisms governing glucose-induced insulin secretion via dephosphorylation of synapsin I-like protein in MIN6 cells. (Endocrinology 137: 5255-5263, 1996)

SEVERAL intracellular second messenger systems of β-cells of pancreatic islets are involved in stimulus-induced insulin secretion through variation in states of protein phosphorylation (1, 2). Although much attention has been directed to the role of protein kinases, little is known of the role of protein phosphatases in insulin secretion. Although effects of serine/threonine protein phosphatases 1 and 2A on insulin secretion were examined using the specific inhibitor, okadaic acid, the roles of protein phosphatases have remained an open question (3, 4).

Calcineurin was first discovered to be a calmodulin (CaM)-binding protein and an inhibitor of the CaM-sensitive cyclic nucleotide phosphodiesterase (5). Calcineurin can function as a CaM-stimulated phosphoprotein phosphatase (6). Protein phosphatase 2B (PP2B) is structurally analogous to calcineurin and is widely distributed in various tissues. PP2B is a heterodimer composed of two subunits, A subunit with CaM-binding and catalytic domains and B subunit with four EF-hand Ca2+-binding sites like CaM. PP2B is inhibited by immunosuppressants such as cyclosporin A (CsA) and FK506, and these agents bind to intracellular receptor proteins termed immunophilins, the complexes of which in turn bind to and inactivate PP2B (7). The secretion of ACTH from AtT20 cells (pituitary corticotrope tumor cells) was reported to increase by inhibition of PP2B activity (8), whereas inhibition of PP2B activity resulted in a decrease in amylase secretion from rat pancreatic acinar cells (9). Studies on the effects of CsA and FK506 on insulin secretion in response to various stimulants suggested that these agents have negative effects on insulin secretion (10-12). PP2B has been identified in rat pancreatic islets (13), but whether or not the enzyme is involved in insulin secretion has remained unknown.

A central event in insulin secretion from β-cells of pancreatic islets in response to glucose is the increase in intracellular Ca2+ ([Ca2+]i) concentrations (14). Glucose is taken up into β-cells through glucose transporter-2 (GLUT-2) and is then metabolized by various enzymes. Increase in intracellular ATP resulting from glucose metabolism leads to closure of ATP-sensitive K+ channels, membrane depolarization occurs, voltage-dependent Ca2+ channels open, and there is an increase in cytoplasmic free Ca2+ concentrations. However, the pathway involving the elevated [Ca2+]i leading ultimately to insulin secretion is unclear.

Many effects of Ca2+ are mediated through Ca2+-binding proteins, in which Ca2+/CaM-dependent protein kinases and phosphatases are involved. It has been suggested that CaM kinase II plays an important role in neurotransmitter release in the brain via phosphorylation of synapsin I, which was first discovered as one major substrate for cAMP-dependent protein kinase (cAMP-kinase) (15). Synapsin I has four known phosphorylation sites, one site for cAMP-kinase and CaM kinase I on the amino-terminal domain and two sites for CaM kinase II and one site for proline-directed protein kinase on the carboxyl-terminal domain. The dephosphorylated form of synapsin I binds to synaptic vesicles and to actin filament with a high affinity. Although synapsin I phosphorylated by cAMP-kinase or CaM kinase I has no...
apparent effect on the regulation of neurotransmitter release, when it is phosphorylated by CaM kinase II, it dissociates from synaptic vesicles and actin filament and promotes neurotransmitter release (15).

In previous studies on MIN6 cells, a mouse insulinoma cell line from transgenic mice (16), synapsin I-like protein has been detected (17), and this protein has properties similar to those of synapsin I in the brain; phosphorylation by CaM kinase II correlated well with stimulation of insulin secretion. This observation suggested that CaM kinase II is involved in insulin secretion via phosphorylation of the protein. Although synapsin I is dephosphorylated by P2P2B (18), less is known of the involvement of P2P2B in insulin secretion. The present study was designed to determine whether P2P2B is indeed involved in insulin secretion. Using CsA and MIN6 cells, we found that inhibition of P2P2B activity enhances secretagogue-induced insulin secretion and correlates with increases in the phosphorylation of synapsin I-like protein.

Materials and Methods

Reagents and chemicals

The following reagents and chemicals were obtained from the indicated sources: DMEM, Nissui Pharmaceutical Co. (Tokyo, Japan); PBS, JRH Biosciences (Lenexa, KS); [γ-32P]ATP, [125I]protein A, and [32P]orthophosphate, DuPont-New England Nuclear (Boston, MA); BSA and tolbudatamide, Sigma Chemical Co. (St. Louis, MO); CsA, Sandoz Pharmaceuticals (Tokyo, Japan); calyculin A and silver stain kit, Wako Pure Chemical Industries (Osaka, Japan); 2-(N-morpholino) ethanesulfonic acid and fura-2/acetoxymethyl ester, Dojindo Laboratories (Kumamoto, Japan); protein A-Sepharose CL-4B, Pharmacia-LKB Biotechnology (Piscataway, NJ); Phadebein insulin, Kabi Pharmacia Diagnostics (Uppsala, Sweden); glass-bottom microwells, MatTek Corp. (Ashland, MA); and HIT-T15 cells (passage 57), American Type Culture Collection (Rockville, MD). CaM was purified from bovine brain (19). The polyclonal antibodies against CaM kinase II (20), synapsin I (21), and calcineurin A (22) were prepared as described. Calcineurin (22) and myristoylated alanine-rich C kinase substrate (MARCKS) (23) were purified from rat brain, as described. Preparation and characterization of the polyclonal anti-MARCKS antibody will be described in detail elsewhere. CaM kinase II (24) and the catalytic subunit of cAMP-kinase (25) were purified from rat brain. Other chemicals were of analytical grade.

Cell culture

MIN6 cells, a mouse insulinoma cell line (16) obtained from Dr. J. Miyazaki were cultured in DMEM supplemented with 25 mM glucose containing 15% heat-treated FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml 2-mercaptoethanol, and maintained at 5% CO2 and air at 37°C as described (16). HIT-T15 cells were cultured in RPMI 1640 medium with 10% heat-treated FBS at 3% CO2 and air at 37°C as described (10). Three or four days before experiments, 1–2×10⁶ cells were plated on a 35-mm Petri dish (Nunc, Roskilde, Denmark).

Insulin secretion

In all experiments, cells in the medium containing a vehicle without drugs were used as controls. The cells were preincubated at 37°C for 30 min in Krebs-Ringer HEPES (KRH) with 3 mM glucose containing 128 mM NaCl, 5 mM KCl, 2.7 mM CaCl₂, 1.2 mM MgSO₄, 1 mM Na₂HPO₄, and 20 mM HEPES (pH 7.4). CaA was dissolved in 100% ethanol and was added to KRH, producing a final concentration of ethanol below 0.1%. Following preincubation, the media were removed, and the cells were incubated at 37°C for the indicated time in 1 ml KRH with a vehicle containing the agent. Control cells received an equal volume of the vehicle. After incubation, the media were collected and centrifuged at 12,000×g for 2 min, then the supernatants were used for insulin assay. The concentrations of insulin in the supernatant were determined by a double antibody RIA (26) using insulin assay kits (Phadebein insulin, Kabi Pharmacia Diagnostics). The unit of insulin was based on the instructions in the assay kit used for the RIA. Cells in the dish were solubilized in a homogenizing buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 75 mM NaCl, and 0.05% Triton X-100. After sonication in a Branson Sonifier 250 (Danbury, CT), the solutions were centrifuged at 15,000×g for 5 min, and aliquots (10 μl) were used to determine protein content (27). The amount of insulin was corrected for protein concentration.

Because HIT cells express a considerable level of GLUT-1 with a relatively low Michaelis Menton constant (Kₘ) for glucose and respond to a much lower glucose concentration than do pancreatic islets (28), the cells were preincubated for 30 min in KRH without glucose. Insulin secretion from the cells was then measured after incubation for 30 min with various stimulants in the presence and absence of 3 mM CsA, in the same manner as for MIN6 cells. Because the amount of insulin secreted per cell decreases gradually with continuous passages (29), the value of insulin secretion was expressed as percent of the value without glucose and CsA. For this study, we used passages 57–60 of HIT-T15 cells.

Preparation of 32P-labeled substrates

Protein substrates were phosphorylated with [γ-32P]ATP and the catalytic subunit of cAMP-kinase from rat brain, as described (30), but with some modifications. The reaction mixture for phosphorylation of substrates contained 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 0.5 mM EGTA, 0.6 mg/ml BSA, 0.4 mg/ml casein, 0.1 mg/ml of the catalytic subunit of cAMP-kinase, and 5 μM [γ-32P]ATP (20,000 cpm/μm) in a final volume of 1 ml. The mixture was incubated at 30°C for 30 min and then at 60°C for 15 min. Following incubation, the protein was precipitated with 560 mg/ml (NH₄)₂SO₄. After 30 min on ice, the protein was pelleted by centrifugation at 15,000×g for 5 min and washed four times with 80% (NH₄)₂SO₄ to exclude free 32P. The precipitate was dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), and dialyzed overnight against the buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM DTT, and 10% glycerol. Radioactivity of the dialyzed solution was measured in a liquid scintillation counter (Alolka, Tokyo, Japan) (about 20,000 cpm/μl).

Protein phosphatase assay

Following preincubation, in the manner described above for insulin secretion, MIN6 cells were incubated for 60 min in 1 ml of KRH with 25 mM glucose in the presence of various concentrations of CsA. At the end of 60 min, the media were removed, the cells were quickly frozen in liquid N₂, then solubilized at 0°C in 250 μl of the homogenizing buffer with 0.1 mM leupeptin, 75 μM pepstatin A, 50 μg/ml soybean trypsin inhibitor, and 1 mM DTT. After sonication in a Branson Sonifier, the insoluble materials were removed by centrifugation at 15,000×g for 5 min. The cell-lysates were used to measure P2P2B activity and aliquots (10 μl) were analyzed for protein content (27). Dephosphorylation of 32P-labeled casein was done as described (8), but with slight modifications. Each assay contained 50 mM HEPES (pH 7.5), 1 mg/ml BSA, 1 mM DTT, 1 mM CaCl₂, 3 μM CaM, 100 μg/ml 32P-labeled casein (about 50,000 cpm) and 100 nm calyculin A and no Mg²⁺ to inhibit protein phosphatases 1 and 2A (31) and protein phosphatase 2C, respectively, in a total volume of 25 μl. Protein phosphatase activity was measured in the presence and absence of 100 μM trifluoperazine (TFP), which binds to CaM and prevents CaM from binding to CaM⁺/CaM-dependent enzymes (CaM-binding proteins) (8). Phosphatase activity was determined by the difference in the amount of 32P released from 32P-labeled casein in the presence and absence of TFP and expressed as percent of control value. The reaction was initiated by the addition of 5 μl enzyme solution to each assay, incubated at 30°C for 15 min, and then terminated by the addition of 25 μl 40% TCA and 50 μl 6 mg/ml BSA. After 10 min on ice, the samples were microfuged for 10 min and aliquots (80 μl) of the supernatants were analyzed using a liquid scintillation counter to quantify the amount of free 32P present.

Immunoprecipitation of 32P-proteins

MIN6 cells in a 35-mm dish were washed once with phosphate-serum-free DMEM containing 5.6 mM glucose and labeled in 1.0 ml of the medium containing carrier-free [32P]orthophosphate (0.25 mCi/ml),
as described (17). After labeling for 5 h, the cells were washed once in KRH and preincubated at 37 °C for 30 min with 3 mM glucose in KRH. Following preincubation, the cells were incubated at 37 °C for the indicated time with 25 mM glucose in the presence and absence of 3 μM CsA in KRH. After incubation, the media were aspirated, and the cells were quickly frozen on liquid N2. The cells were then homogenized in 400 μl 50 mM HEPES (pH 7.5), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 0.1 mM leupeptin, 15 μg pepstatin A, 50 μg/ml soybean trypsin inhibitor, and 1 mM DTT. The insoluble materials were removed by centrifugation at 15,000 × g for 15 min. To the supernatant fraction we added 0.1% SDS. The solution was incubated at 4 °C for 4 h with antibodies to Calmodulin kinase II (6 μg IgG protein) and synapsin I (6 μg IgG protein) and 50 μl protein A-Sepharose CL-4B suspension (50% vol/vol). Following incubation, the immuno-complex immobilized on protein A was pelleted by centrifugation at 12,000 × g for 2 min and washed three times with RIPA solution containing 50 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, 0.5% Triton X-100, 10 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 4 mM EGTA, and 0.1% SDS. After the first centrifugation, the supernatant was used for immunoprecipitation of 32P-labeled MARCKS. Immunoprecipitation of 32P-labeled MARCKS was done in the same manner using the antibody to MARCKS (10 μg IgG protein). Immunoprecipitates were eluted from the protein A-Sepharose CL-4B by adding the SDS-sample buffer (32), then boiled for 5 min and subjected to SDS-PAGE in 10% polyacrylamide, followed by autoradiography and analysis using a Bio-Image analyzer (BA 100, Fuji Film Co., Tokyo, Japan).

32P-peptide mapping

The phosphorylation sites of synapsin I-like protein were determined by proteinolysis of 32P-labeled synapsin I-like protein, as described (17). Following SDS-PAGE, the bands of synapsin I-like protein were cut out from the gel and incubated at room temperature for 1 h with 1 ml 125 mM Tris-HCl buffer (pH 6.8) containing 0.1% SDS. Pieces of the gels were placed into wells of polyacrylamide gel. Proteins were cleaved with *Staphylococcus aureus* V8 protease (3 μg/ml) in a stacking gel and separated by SDS-PAGE in 15% polyacrylamide, followed by autoradiography and analysis using a Bio-Image analyzer. As control, purified rat brain synapsin I was phosphorylated by either purified rat brain CaM kinase II or cAMP-kinase and analyzed, as described above.

RNA blotting

Total cellular RNA was purified from rat brain, mouse brain (CS7BL/6), and MIN6 cells by acid guanidinium thiocyanate-phenol-chloroform extraction. The complementary DNA (cDNA) of rat calcineurin A was obtained by the documented method (33). The cDNA of rat calcineurin B was kindly provided by Prof. T.R. Soderling. For RNA transfer blots, 5 and 20 μg total RNA from rat and mouse brains and from MIN6 cells, respectively, were denatured with formaldehyde, electrophoresed on 1% agarose gel, and transferred to nylon membrane. Hybridizations were carried out at 42 °C for 16 h in 5 × SSC, 50% formamide, 2 × Denhardt's solution, 0.02% SDS, 100 μg/ml sonicated and denatured salmon testis DNA, 0.1% N-lauroyl sarcosine, and 1 × 106 cpm/ml 32P-labeled for each cDNA probe in that order. The nylon membranes were then washed in 2 × SSC and 0.1% SDS for 10 min, 1 × SSC and 0.1% SDS for 10 min, and then for 1 h at 55 °C, 60 °C, and 65 °C, in that order before autoradiography.

Western blotting of proteins extracted from cultured MIN6 cells

All procedures were carried out at temperature below 4 °C for identification of the A subunit of P2P2. MIN6 cells were plated in a 100-mm dish and used at the stage of subconfluency. Cells in the dishes were harvested by trypsinization and precipitated by centrifugation at 900 × g for 3 min. The precipitated cell homogenates were solubilized with 20 mM imidazole, 50 mM HEPES (pH 7.5), 0.1% Triton X-100, 4 mM EGTA, 4 mM EDTA, 1 mM leupeptin, 75 μg pepstatin A, 50 μg/ml soybean trypsin inhibitor, and 1 mM DTT in a Teflon glass homogenizer. The homogenate was centrifuged at 20,000 × g for 30 min. After applying the supernatants to diethylaminoethyl (DEAE)-cellulose column chromatography with a linear gradient of NaCl (20–400 mM), protein phosphatase activity was measured in the same manner as described above in the presence of Ca2+/CaM and EGTA. The active fractions were collected and concentrated by Amicon ultrafiltration with a PM-10 membrane (Amicon Corp., Danvers, MA). Mouse brain calcineurin was partially purified on a CaM-Sepharose affinity column after DEAE-cellulose column chromatography. The concentrated solution was assayed for P2P2 and used for immunoblotting of the enzyme from MIN6 cells. Aliquots (20 μg) of proteins from MIN6 cells, partially purified calcineurin (3 μg) from mouse brain, and rat brain calcineurin (2 μg) as a control were electrophoresed by SDS-PAGE in 10% acrylamide and electrophoretically transferred to nitrocellulose membrane at 60 V for 4 h by Trans Blot (Bio-Rad, Richmond, CA). Following incubation of the membrane for 2 h with 4.5% (wt/vol) skim milk in Tris-based saline (TBS) containing 0.15 μM NaCl and 20 mM Tris-HCl (pH 7.5) to block non-specifically binding sites, it was incubated overnight in TBS containing 4.5% skim milk with the polyclonal rat calcineurin A antibody diluted 1:200 (10 μg IgG/ml). After washing four times in TBS containing 0.1% Nonidet P-40 (NP-40) at room temperature for 10 min, the membrane was incubated with 32P-labeled antibody for 1 h in TBS containing 0.1% NP-40 at room temperature and then washed four times in TBS containing 0.1% NP-40 and in TBS for 60 min. Immunoreactive proteins were detected by autoradiography. The specificity of the antigen-antibody reaction was controlled using the anti-calcineurin A subunit IgG (10 μg/ml) in TBS containing 4.5% skim milk preincubated at 4 °C for 1 h with an excess of calcineurin (10−6 mol/liter).

Other methods

[Ca2+]i in MIN6 cells was measured using fura-2 fluorescence, as described (34). The viability of MIN6 cells was assessed using trypan blue as described (34).

Statistical analysis

Values were expressed as means ± sem. Statistical analysis was performed using one-way ANOVA plus Duncan's multiple range test. A P < 0.05 was considered to have statistical significance.

Results

Effects of CsA on insulin secretion from MIN6 cells

We made use of a glucose-responsive cells, MIN6, to investigate effects of CsA on basal and stimulus-induced insulin secretion. After incubating the cells for 30 min with 3 mM glucose, 25 mM glucose, 0.37 mM tolbutamide, and 56 mM KCl in the presence and absence of 3 μM CsA, the concentrations of insulin were measured. In the case of incubation with 3 mM glucose, exposure to CsA led to no detectable increase in insulin secretion. In case of incubation with secretagogues, insulin secretion from the cells exposed to 3 μM CsA significantly increased, compared with control (Fig. 1). Thus, CsA does affect stimulus-induced insulin secretion. To confirm which concentration of CsA would have the greatest influence on glucose-induced insulin secretion, we examined the cells after incubation for 60 min with 25 mM glucose in the presence of various concentrations of CsA. As shown in Fig. 2A, CsA increased the glucose-induced insulin secretion in a dose-dependent manner. Exposure to CsA above 1 μM led to a significant increase in insulin secretion. Incubation with 3 μM CsA led to a maximal insulin secretion, and the ED50 was about 1.3 μM. Similar results were obtained for tolbutamide-stimulated insulin secretion (data not shown). The basal insulin secretion with 3 mM glucose did not increase in the presence of any concentration of CsA.

Dose-dependent inhibition of P2P2 with CsA

We then asked whether CsA would have an inhibitory effect on P2P2 in MIN6 cells. In parallel with examinations...
Effects of CsA on insulin secretion from MIN6 cells. MIN6 cells were preincubated for 30 min with 3 mM glucose in KRH and then incubated with 3 and 25 mM glucose, 0.37 mM tolbutamide, and 56 mM KCl in KRH in the presence and absence of 3 μM CsA. Insulin content in incubated medium was measured by RIA after 30 min incubation. Values are means ± SE (n = 6–8). *P < 0.01 vs. control. □, no CsA; ■, 3 μM CsA.

of insulin secretion, PP2B activity in the cells was measured using 32P-labeled casein after the cells had been incubated for 60 min with 25 mM glucose in the presence of various concentrations of CsA. The specific activity of PP2B in MIN6 cells was about 10% (1.3 ± 0.3 pmol·min⁻¹·mg⁻¹ and 12.8 ± 0.8 pmol·min⁻¹·mg⁻¹ for MIN6 cells and rat brain, respectively, n = 4). As shown in Fig. 2B, treatment with CsA resulted in a dose-dependent inhibition of PP2B activity. When incubated with CsA above 500 nM, PP2B activity was inhibited with a median inhibitory concentration (IC₅₀) of about 0.9 μM. Thus, the increase in insulin secretion correlated well with the inhibition of PP2B activity. These results suggest that PP2B is involved in negatively regulatory mechanisms for glucose-induced insulin secretion. Because 3 μM CsA had the maximal effect on insulin secretion, this concentration of CsA was used in the following experiments.

CsA inhibits voltage-dependent Ca²⁺ channels (35). Because Ca²⁺ influx through voltage-activated L-type Ca²⁺ channels serves as the major stimulatory pathway in insulin secretion from the cells, we examined the effects of CsA on [Ca²⁺]ᵢ concentrations using fura-2. For this we measured changes in [Ca²⁺]ᵢ in MIN6 cells by stimulation with 56 mM KCl. CsA had no apparent effects on changes in [Ca²⁺]ᵢ in MIN6 cells (data not shown).

**Time course of glucose-induced insulin secretion in the presence and absence of 3 μM CsA**

To further examine the effects of CsA on glucose-induced insulin secretion, insulin secretion from MIN6 cells was measured for the indicated time with 25 mM glucose in the presence and absence of 3 μM CsA (Fig. 3). Treatment with the agent resulted in no notable change on total insulin secretion within 10 min, but there was a significant total insulin secretion at 30 min compared with control. Insulin secretion from the cells was almost equal to that in control from 30–120 min (Fig. 3). Treatment with CsA for 120 min had no evident effect on cell viability, as assessed using trypan blue (data not shown). On the other hand, basal insulin secretion did not increase in the presence of 3 μM CsA (data not shown).

**Effects of CsA on insulin secretion from HIT-T15 cells**

Insulin secretion from HIT-T15 cells, another β-cell line, was earlier examined (10). The treatment of the cells with CsA for 16 h decreased insulin secretion from the cells. To investigate the acute effects of CsA, we examined insulin secretion from the cells for 30 min in the presence and absence of 3 μM CsA under the same conditions as for MIN6 cells (Table 1). At passage 59, insulin secretion from the cells with 0, 3, and 25 mM of glucose was 103, 161, and 190 μU·ml⁻¹·mg⁻¹, respectively. In contrast to the previous study (10), treatment of HIT-T15 cells with CsA for 30 min mark-
E1111tro

25 mM Glucose
25 mM Glucose + CsA

**TIME (MIN)**

**Fig. 3.** Time course of glucose-induced insulin secretion from MIN6 cells in the presence and absence of 3 μM CsA. MIN6 cells were incubated for indicated time with 25 mM glucose in KRH in the presence and absence of 3 μM CsA. Insulin content in incubated medium was measured by RIA. Values are means ± SE (n = 6). *, P < 0.01 vs. control. O, no CsA; *, CsA.

**TABLE 1.** Effects of CsA on insulin secretion with various secretagogues in HIT-T15 cells

<table>
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<th>P-value</th>
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<tr>
<td>Glucose (0 mM)</td>
<td>100 ± 5</td>
<td>111 ± 17</td>
<td></td>
</tr>
<tr>
<td>Glucose (3 mM)</td>
<td>175 ± 16</td>
<td>468 ± 20*</td>
<td></td>
</tr>
<tr>
<td>Glucose (25 mM)</td>
<td>192 ± 19</td>
<td>574 ± 32*</td>
<td></td>
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<tr>
<td>Tolbutamide (0.37 mM)</td>
<td>239 ± 22</td>
<td>677 ± 39*</td>
<td></td>
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<tr>
<td>KCl (56 mM)</td>
<td>200 ± 11</td>
<td>635 ± 38*</td>
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*a HIT-T15 cells were preincubated for 30 min in KRH without glucose and then incubated for 30 min with 0, 3, and 25 mM glucose, 0.37 mM tolbutamide, and 56 mM KCl in KRH in the presence and absence of 3 μM CsA. Insulin content in incubated medium was measured by RIA. Values are means ± SE (n = 4–5) and are expressed as percent of control value, which was obtained from cells incubated without glucose and CsA. Statistical analysis was performed with values between the presence and absence of CsA. *, P < 0.01 vs. value in the absence of CsA.

Effects of CsA on secretagogue-induced insulin secretion (Table 1). CsA had no apparent effects on insulin secretion from HIT-T15 cells incubated in the absence of glucose (Table 1).

Effects of CsA on the phosphorylation of CaM kinase II and synapsin I-like protein in MIN6 cells

To investigate which proteins are involved in glucose-induced insulin secretion, as targets for PP2B, we examined synapsin I-like protein, MARCKS, and CaM kinase II (Fig. 4). To search for a possible relationship between glucose-induced insulin secretion and phosphorylation of these proteins in MIN6 cells treated with 3 μM CsA, we measured the in situ incorporation of [32P]phosphate. For this, MIN6 cells were preincubated for 5 h in the presence of [32P]phosphate to label the ATP pool and then stimulated for 5–60 min with 25 mM glucose in the presence and absence of 3 μM CsA. The phosphorylation levels of synapsin I-like protein and CaM kinase II increased by 50 ± 5.7% and 29 ± 4.4%, respectively (P < 0.01) (Fig. 4, A and C). In contrast, we observed no significant increase in the phosphorylation of MARCKS with 3 μM CsA (Fig. 4B). These results suggest that CsA prevents dephosphorylation of synapsin I-like protein and CaM kinase II by inhibiting PP2B activity, thereby increasing glucose-induced insulin secretion.

In neurons, synapsin I, a synaptic vesicle-associated phosphoprotein involved in neurotransmitter release, has four known phosphorylation sites. Phosphorylation of two sites (site II) in the carboxyl-terminal domain by CaM kinase II
allows synapsin I to dissociate from actin filament and synaptic vesicles, and there is an increase in neurotransmitter release (15). To determine the phosphorylated sites of the protein in MIN6 cells, phosphopeptide mapping of 32P-labeled synapsin I-like protein was done by limited digestion of the protein with V8 protease, followed by one-dimensional SDS-PAGE. As shown in Fig. 5, 10- and 30-kDa bands corresponding to sites I and II, respectively, were obtained (Fig. 5A). Following treatment with 3 μM CsA for 30 min, phosphorylation of site II increased by 59 ± 8% (P < 0.01) (Fig. 5B), and phosphorylation of site I (phosphorylated by cAMP-kinase or CaM kinase I) also increased by 39 ± 7% (P < 0.05) (Fig. 5B).

Identification of PP2B in MIN6 cells

To determine whether PP2B is expressed in MIN6 cells, the messenger RNA (mRNA) levels of PP2B were examined by RNA blot analysis. We noted the expression of mRNA for the A subunit of PP2B in MIN6 cells at 55°C (Fig. 6A) and 65°C (data not shown). However, when mRNA was washed out at 65°C, mRNA from MIN6 cells for the calcineurin A subunit was almost undetectable, whereas mRNA from rat and mouse brains tightly hybridized with cDNA from rat brain (Fig. 6B). A moderate level of mRNA for the calcineurin B subunit was also expressed in the cells (Fig. 6C). These results suggest that mRNA of PP2B from MIN6 cells weakly binds to cDNA of the calcineurin A subunit from rat brain, and that the calcineurin B subunit is common to both tissues.

We carried out Western blot analysis using the polyclonal antibody to rat brain calcineurin A. The enzyme preparation was purified by DEAE-cellulose column chromatography. As shown in Fig. 7A, one major peak of the protein phosphatase activated by Ca2+/CaM appeared, and active fractions of the peak were collected and used for Western blot analysis. As shown in Fig. 7B, the major band of a 61-kDa polypeptide of MIN6 cells (lane 6) migrated at the same position as those for the calcineurin A subunit of rat and mouse brains (lanes 4, 5). These bands of 61 kDa disappeared when the calcineurin A antibody was absorbed with an excess amount of calcineurin (lanes 7-9). Minor bands of 49 and 59 kDA in MIN6 cells and mouse brain, respectively, also disappeared by preincubation of the antibody with an excess amount of calcineurin. Therefore, the proteins may be degraded products of the 61-kDa protein. On the other hand, two bands of about 10 and 10.5 kDA did not disappear by treatment of the antibody with calcineurin, indicating that these proteins are not related to the calcineurin A subunit. Density of the band of 61 kDa was almost equal between mouse and rat brain, whereas it was much weaker for the enzyme of MIN6 cells than that of rat brain, on the basis of the protein amounts applied, which were corrected for activity. Thus, immunoreactivity of the enzyme of MIN6 cells seems to be weaker than that of the brain enzyme.
CsA stimulates glucose-induced insulin secretion in MIN6 cells in a dose-dependent manner, and similar results are seen with tolbutamide and KCl-induced insulin secretion. Because CsA is a specific inhibitor for calcineurin, the stimulatory effects of CsA on insulin secretion would be exerted through inhibition of PP2B. PP2B, similar to calcineurin, was present in MIN6 cells (Figs. 6 and 7). PP2B may possibly act as a regulatory factor for insulin secretion in MIN6 cells.

Gagliardino et al. (13) reported that calcineurin was detectable in rat pancreatic islets, using a polyclonal antibody to calcineurin. In contrast, the monoclonal antibody to calcineurin A could not recognize calcineurin in the pancreas, whereas the monoclonal antibody to calcineurin B showed immunoreactivity in the tissue (36). In the present study, Western blot analysis by use of the polyclonal antibody to calcineurin A revealed that the immunoreactive band at the same position with calcineurin A of rat brain had a much lesser density, on the basis of protein application of the same activity. Furthermore, Northern blot analysis revealed that mRNA of PP2B in MIN6 cells more loosely binds to cDNA of calcineurin A of rat brain and is therefore washed out at 65°C (Fig. 6B), although mRNA of PP2B in MIN6 cells migrated at the same position as that of rat calcineurin A (Fig. 6A). Thus, PP2B in MIN6 cells is similar, but not identical to calcineurin in rat brain, although both enzymes are Ca²⁺/CaM-dependent. Because MIN6 cells are derived from mouse insulinoma and the sequence of calcineurin A of mouse brain is unknown, we also asked whether this is caused by tissue or species differences. Northern and Western blot analysis revealed that the mouse brain seems to express the same calcineurin as that in rat brain. In view of differences in immunoreactivities with polyclonal and monoclonal antibodies to brain calcineurin, it is assumed that there are not species but tissue-specific differences in the amino acid sequences of the enzymes. On the other hand, calcineurin B is common to both tissues. Thus, it seems that an isoform of calcineurin distinct from rat brain calcineurin is expressed in MIN6 cells and acts as Ca²⁺/CaM-dependent PP2B.

Pancreatic β-cells are thought to arise from neuroectoderm and have properties common to those in the brain (37-41). It was reported that synaptic vesicle-associated proteins in the brain such as synaptotagmin (39), vesicle-associated membrane protein 2 (40), and synaptosomal associated protein of 25 kDa (41) occur in secretory granules of pancreatic islets, findings that suggested that the mechanism of the release of neurotransmitters from synaptic vesicles in the brain is in common with that of insulin secretion from secretory granules in pancreatic islets. We earlier reported that synapsin I-like protein occurs in MIN6 cells, and immunoreactivity and phosphopeptide mapping were similar to synapsin I in brain, and that the protein may be involved in insulin secretion via its phosphorylation by CaM kinase II (17). In the present study, we obtained evidence that the inhibition of PP2B activity with CsA increases the phosphorylation of synapsin I-like protein with a concomitant increase in glucose-induced insulin secretion. Thus, the phosphorylation level of synapsin I-like protein is elevated not only by activation of CaM kinase II but also by the inhibition of PP2B activity, events that result in an increase in insulin secretion.

The treatment of MIN6 cells with CsA for 30 min increased the autophosphorylation of CaM kinase II (Fig. 4A). Because PP2B cannot dephosphorylate phosphorylated CaM kinase II (42), PP2B may be involved in dephosphorylation of CaM kinase II via regulation of PP1 activity by dephosphorylation of inhibitor I, a heat-stable molecule (43). On the other hand,
synapsin I-like protein is phosphorylated with CaM kinase II and dephosphorylated with PP2B. Therefore, the inhibition of PP2B with CsA is considered to lead to an increase in phosphorylation of the protein.

The biphasic secretion of insulin is usually observed in response to glucose (44). The first phase is a transient initial peak, and the second phase is a gradually rising and lasting peak of insulin secretion. Because CsA had no apparent effect on glucose-induced insulin secretion and on phosphorylation of synapsin I-like protein during the first 10 min, PP2B may be involved in the second phase. In this sense, it may be of interest that defects in the second phase of insulin secretion are observed in transgenic islets with the elevated level of CaM in β-cells (45, 46). These results suggest that over-expressed CaM activates PP2B more than does CaM kinase II.

It has been reported that CsA inhibits insulin secretion (10–12). In contrast to the previous study (10), we found that treatment of HIT-T15 cells for 30 min with CsA increased secretagogue-induced insulin secretion. The differences between these results are not explainable, but in their experiments, a higher concentration of CsA was used for a longer period (10, 11). Because pancreatic islet cells are relatively vulnerable to damage with CsA, CsA may act as a toxic agent for the cells. In relation to this point, it was reported that a long-term exposure of pancreatic islet cells to CsA decreases insulin mRNA (47) and inhibits voltage-dependent calcium channels (35). It should be noted that in the present study, CsA did not alter the viability of MIN6 cells and had no apparent effect on the basal insulin secretion from MIN6 cells, which means that the effects of CsA on MIN6 cells are probably not toxic. It will be necessary to examine effects of CsA on insulin secretion from β-cells of pancreatic islets, which express PP2B (48), however, it is extremely difficult to isolate pure β-cells that are not contaminated with α- and δ-cells. This mixture of cells may lead to misinterpretations of the results, because glucagon from α-cells and somatostatin from δ-cells stimulate and inhibit, respectively, insulin secretion from β-cells. Instead of β-cells, we used HIT-T15 cells, which are a pure cell line, and we examined effects of CsA on insulin secretion. As shown in Table 1, CsA stimulated the secretagogue-induced insulin secretion from HIT-T15 cells. Thus, the effects of CsA were considered to be in common in insulin secretory cells.

In contrast to MIN6 cells, treatment of HIT-T15 cells with CsA increased insulin secretion in the presence of 3 mM glucose. Because HIT-T15 cells express GLUT-1 with a low Km for glucose and respond to a much lower glucose concentration than do pancreatic islets (28), glucose at a lower concentration has the potential to stimulate insulin secretion and therefore to activate PP2B by elevating [Ca^{2+}]i concentrations in HIT-T15 cells. Indeed, CsA without glucose had no effects on insulin secretion from cells. In contrast, MIN6 cells has similar properties to pancreatic islets with regard to the response to glucose.

When we examined the effects of FK506, another specific inhibitor for PP2B, on insulin secretion of MIN6 cells, FK506 had no effects on basal and glucose-induced insulin secretion. MIN6 cells may not express FK506-binding proteins sufficient to inhibit PP2B activity.

To our knowledge, this is a first report to show that CsA stimulates glucose-induced insulin secretion. We proposed in our previous study that the phosphorylation of synapsin I-like protein by CaM kinase II is involved in the stimulation of insulin secretion (17). In the present study, we showed that the inhibition of dephosphorylation by PP2B with CsA increases phosphorylation of the protein and thereby stimulates secretagogue-induced insulin secretion. Protein kinases as well as protein phosphatases probably have important roles in regulating insulin secretion.

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