Original Article

Genistein Acutely Stimulates Insulin Secretion in Pancreatic β-Cells Through a cAMP-Dependent Protein Kinase Pathway

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Although genistein, a soy isoflavone, has beneficial effects on various tissues, it is unclear whether it plays a role in physiological insulin secretion. Here, we present evidence that genistein increases rapid glucose-stimulated insulin secretion (GSIS) in both insulin-secreting cell lines (INS-1 and MIN6) and mouse pancreatic islets. Genistein elicited a significant effect at a concentration as low as 10 nmol/l with a maximal effect at 5 μmol/l. The effect of genistein on GSIS was not dependent on estrogen receptor and also not related to an inhibition of protein tyrosine kinase (PTK). Consistent with its effect on GSIS, genistein increases intracellular cAMP and activates protein kinase A (PKA) in both cell lines and the islets by a mechanism that does not involve estrogen receptor or PTK. The induced cAMP by genistein, at physiological concentrations, may result primarily from enhanced adenylate cyclase activity. Pharmacological or molecular intervention of PKA activation indicated that the insulinotropic effect of genistein is primarily mediated through PKA. These findings demonstrated that genistein directly acts on pancreatic β-cells, leading to activation of the cAMP/PKA signaling cascade to exert an insulinotropic effect, thereby providing a novel role of soy isoflavones in the regulation of insulin secretion. *Diabetes* 55:1043–1050, 2006

Soy isoflavones have received widespread attention over the past few years because of their potential for preventing some highly prevalent chronic diseases. Genistein, the primary soy-derived isoflavone, has various biological actions, including a weak estrogenic effect by binding to estrogen receptors (1) and inhibiting protein tyrosine kinases (PTKs) (2). Studies on whether genistein has an effect on diabetes are very limited. Recent studies performed in animals and humans have shown that ingestion of soy protein associated with isoflavones moderates hyperglycemia (3,4), suggesting a beneficial role for soy in diabetes. However, it is not clear whether the beneficial effect of soy protein is due to genistein or other components. Data from recent animal studies suggest an antidiabetic effect of genistein presumably by a hypolipidemic effect (5). However, recent reports demonstrated that soy isoflavone administration lowered plasma glucose, whereas triglyceride levels were unaffected in obese and diabetic animals (6) and postmenopausal women (7). Therefore, although these data suggest that genistein may have a protective role in diabetes, the mechanism underlying these beneficial effects is still largely unknown.

Few data exist on whether genistein has a direct effect on pancreatic β-cells. Several earlier studies demonstrated that genistein stimulates insulin secretion from a clonal pancreatic β-cell line (8) and cultured islets (9,10), whereas other studies have found an inhibitory effect on insulin secretion (11,12). These discrepant data may be the result of variations in the experimental conditions and model used. Nevertheless, the doses used in most of these studies (>20 μmol/l) are well above those concentrations physiologically achievable through dietary means (<5 μmol/l). Therefore, it is still unclear whether genistein, at physiological doses, can act on pancreatic β-cells to modulate insulin secretion. In the present study, we examined whether genistein can directly regulate pancreatic β-cell function through stimulation of insulin secretion. We focused on the acute effects of genistein on insulin secretion, and the cellular signaling related to this effect.

RESEARCH DESIGN AND METHODS

Reagents and materials. Culture media and supplements were from Gibco (Gaithersburg, MD); an insulin radioimmunoassay (RIA) kit was obtained from Crystal Chem (Downer Grove, IL); nitrocellulose membranes were from Schleicher & Schuell (Keene, NH); chemiluminescence detection system, stripping buffer, protein assay, and PTK assay kits were purchased from Pierce (Rockville, IL); small interfering RNA (siRNA) of protein kinase A (PKA) catalytic subunits (PKACα and PKACβ) was from Dharmacon Research (Lafayette, CO); antibodies to PKACα (sc-903), PKACβ (sc-904), and PKA regulatory subunit IIα were from Santa Cruz Biotechnology (Santa Cruz, CA); cAMP enzyme immunoassay kits were obtained from Cayman Chemical (Madison, WI); Chariot protein transfection reagent was bought from Active Motif (Carlsbad, CA); siRNA transfection reagent was from Targeting System (Santee, CA); ICI 182,780 was purchased from Tocris Cookson (Balwin, MO); and other reagents and chemicals were from Sigma (St. Louis, MO).

Cell culture and islet isolation. INS-1 (a generous gift from Dr. Joseph Dillon, The University of Iowa) and MIN6 cells (provided by the Islet Core facility at the University of Virginia) were cultured in RPMI-1640 medium containing 5.5 mmol/l glucose and supplemented with 10% heat-inactivated fetal bovine serum, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 50 μmol/l β-mercaptoethanol, 100 units/ml penicillin, and 100

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GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; IBMX, isobutylmethylxanthine; KRBB, Krebs-Ringer bicarbonate buffer; PDE, phosphodiesterase; PKA, protein kinase A; PKACα and PKACβ, PKA catalytic subunits α and β; PTK, protein tyrosine kinase; RIA, radioimmunoassay; siRNA, small interfering RNA.

μg/ml streptomycin. The medium was changed every other day until the cells became confluent. Mouse islets were isolated from female C57BL/6J mice as described previously (13) and maintained in complete RPMI-1640. Before the experiment, INS-1 and MIN6 cells were preincubated in Krebs-Ringer bicarbonate buffer (KRBB; 129 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 2.5 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3$, 0.1% BSA, and 10 mmol/l HEPES, pH 7.4) containing 1.0 mmol/l glucose at 37°C for 2 h. Mouse islets were preincubated in KRBB containing 2.8 mmol/l glucose for the same duration.

Insulin secretion. INS-1 and MIN6 cells were incubated in KRBB containing various concentrations of glucose with or without stimulating reagents. In some experiments, cells were preincubated with antagonists for 30 min before addition of genistein. Mouse islets (25 islets/tube) were incubated in different glucose concentrations with 1 μmol/l genistein or various concentrations of genistein with 8 mmol/l glucose in 37°C water bath with gentle shaking for 30 min. Insulin secreted in experimental samples was measured by a RIA kit. Our preliminary experiments show that exposure of the cells to genistein for 30 min had no effect on insulin or protein content. All insulin secretion data in the present study were therefore normalized to cellular protein as determined by a protein assay kit.

PTK activity assay. INS-1 cells were exposed to genistein (10 nmol/l–100 μmol/l) or vehicle in the presence of 5.6 mmol/l glucose for 30 min in KRBB at 37°C. PTK activity in cell extracts was measured as previously described (14). The relative fluorescence data were normalized to corresponding protein levels.

Intracellular cAMP assay. INS-1 cells were incubated in KRBB containing different glucose concentrations with 1 μmol/l genistein or various concentrations of genistein with 5.6 mmol/l glucose at 37°C for 20 min. Mouse islets (25 islets/tube) were stimulated with genistein (1 and 5 μmol/l) in the presence of 8 mmol/l glucose in a 37°C water bath with gentle shaking for 20 min. In some experiments, INS-1 cells were preincubated with 0.25 mmol/l isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterases (PDEs), before addition of 2.5 μmol/l genistein or vehicle. Intracellular cAMP levels were determined by an enzyme immunoassay kit as previously described (15). Data were normalized to the protein concentration in samples.

Adenylate cyclase assay. The plasma membranes from INS-1 cells and the mouse islets were isolated by differential centrifugation (15). The islets (50 islets/tube) were stimulated with genistein (1 and 5 μmol/l) or vehicle in the presence of 8 mmol/l glucose at 37°C for 10 min before the membrane preparations. The adenylate cyclase activity assays were as described previously (15).

PKA-specific kinase assay. β-Cells or the islets treated with different agents or vehicle were collected in PBS supplemented with protease (1:500) and phosphatase inhibitor (1:200) cocktails. Protein was extracted and harvested by sonication and centrifugation. The enzymatic activity of PKA in lysates was assessed by measuring phosphorylation of kemptide, a highly specific peptide substrate for PKA, as described previously (15).

Transfection for siRNA construct of PKACα and -Cβ. Exponentially growing MIN6 cells were transfected with 25 pmol mouse-specific siRNA-PKACα plus 25 pmol PKACβ or 50 pmol scrambled control by using a siRNA transfection reagent according to the manufacturer's protocol. After transfection, cells were incubated with complete medium containing 10% fetal bovine serum for 48 h and then with KRBB containing 1 mmol/l glucose for 2 h before stimulation with 2.5 μmol/l genistein or vehicle for 30 min. Supernatants were collected for insulin assay, and cells were either harvested by scraping in PBS for PKA activity assay or collected in lysis buffer for immunoblot analysis.

Antibody transfection. Polyclonal antibodies against PKACα plus -Cβ or a preimmune IgG were transfected into mouse islets using a Chariot reagent according to the manufacturer's protocol. Following 5 h for the islets in culture medium and 2 h in KRBB containing 2.8 mmol/l glucose after transfection, the islets were treated with genistein for 30 min, and the supernatants were assayed for glucose-stimulated insulin secretion (GSIS). Efficacy for neutralization of the target proteins PKACα and -Cβ by the antibodies was confirmed by PKA activity assay.

Immunoblot analysis. Cell extracts were first equalized to the same protein levels and were then subjected to immunoblot analysis as previously described (14,15). Membranes were probed, stripped, and re-probed sequentially with antibodies against PKACα, PKACβ, and PKA regulatory subunit IIα. The protein bands were digitally imaged for densitometric quantitation with a software program (ImageJ; National Institutes of Health).

Statistical analysis. Data were analyzed with one-way ANOVA using the General Linear Model procedure of SAS and expressed as means ± SE. Treatment differences were subjected to a Duncan's multiple comparison test or paired *t* test when designated. Differences were considered significant at $P < 0.05$.

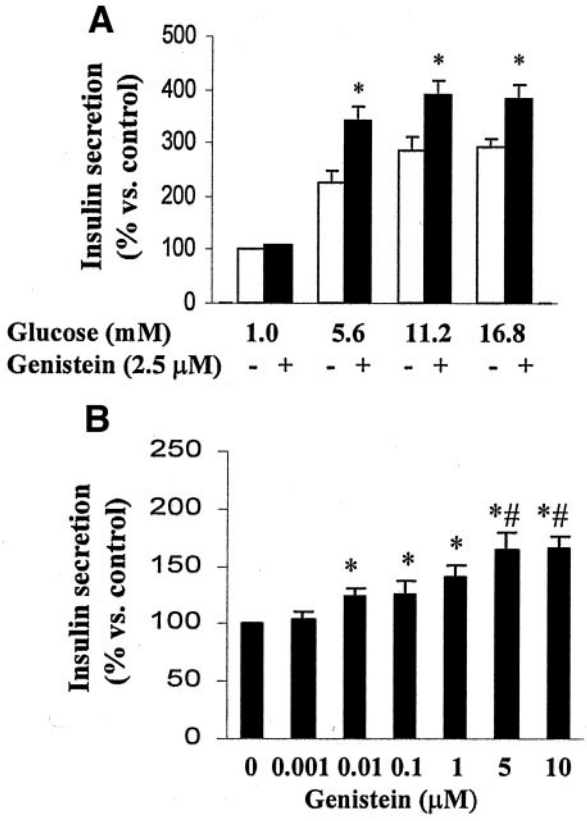

FIG. 1. Genistein potentiates GSIS. INS-1 cells were stimulated with 2.5 μmol/l genistein in the presence of indicated concentrations of glucose (*A*) or with various concentrations of genistein in KRBB containing 5.6 mmol/l glucose (*B*) for 30 min at 37°C. Insulin secreted in culture buffer was determined by a RIA kit. Data were expressed as means ± SE of four to six experiments each in triplicate. *$P < 0.05$ vs. vehicle alone–treated cells, #$P < 0.05$ vs. ≤0.1 μmol/l genistein–treated cells.

RESULTS

Effects of genistein on GSIS. We first examined whether genistein has an effect on GSIS in INS-1 cells. Cells were stimulated with various concentrations of glucose with 2.5 μmol/l genistein or vehicle for 30 min. The basal level of insulin secretion was 380 ± 29 pg · mg^{-1} protein · min^{-1}. As shown in Fig. 1*A*, genistein alone had no effect on insulin secretion but significantly augmented GSIS. Dose-response studies demonstrated that exposure

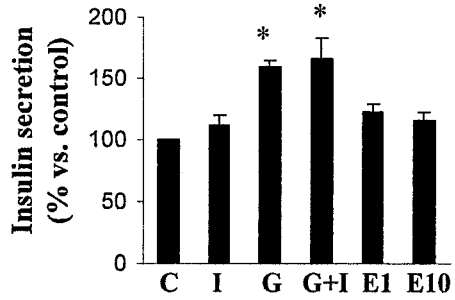

FIG. 2. Effect of genistein on GSIS is not mediated by estrogen receptor. INS-1 cells were preincubated with ICI 182,780 (I, 1 μmol/l) or vehicle for 30 min, followed by stimulation with genistein (G, 2.5 μmol/l), 17β-estradiol (E1, 1 nmol/l; E10, 10 nmol/l), or vehicle (C) in KRBB containing 5.6 mmol/l glucose for 30 min at 37°C. Insulin secreted in buffer was measured. Data were expressed as means ± SE of three experiments each in triplicate. *$P < 0.05$ vs. vehicle alone–treated cells.

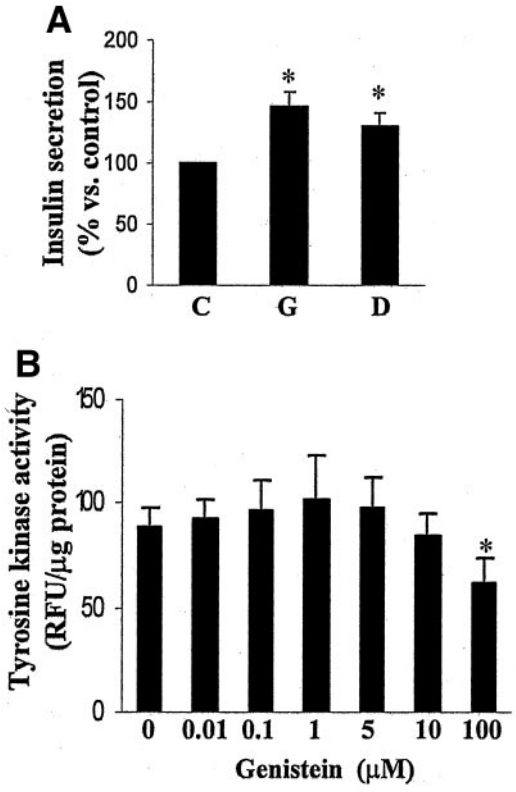

FIG. 3. Effect of genistein on GSIS is independent of PTK. *A*: INS-1 cells were stimulated with genistein (G, 2.5 μmol/l), daidzein (D, 2.5 μmol/l), or vehicle (C) in KRBB containing 5.6 mmol/l glucose for 30 min at 37°C. Insulin secreted in culture buffer was determined. *B*: INS-1 cells were incubated with various concentrations of genistein or vehicle for 30 min. Cells were then lysed, and PTK activity, expressed as relatively quenched fluorescence, was determined. Data were expressed as means ± SE of three experiments each in triplicate. **P* < 0.05 vs. vehicle alone–treated cells.

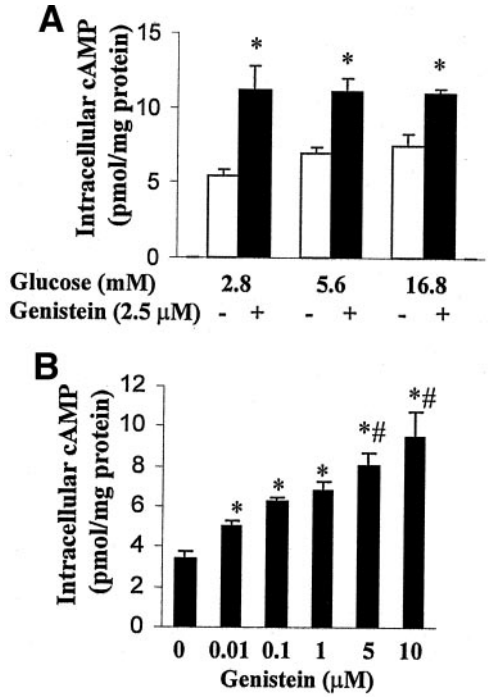

FIG. 4. Genistein stimulates intracellular cAMP accumulation. *A* and *B*: INS-1 cells were stimulated with 2.5 μmol/l genistein in the presence of indicated concentrations of glucose (*A*) or with various concentrations of genistein in the presence of 5.6 mmol/l glucose (*B*) in KRBB for 20 min at 37°C. cAMP was measured, and data were expressed as means ± SE from four to five experiments each in triplicate. **P* < 0.05 vs. vehicle alone–treated cells, #*P* < 0.05 vs. ≤0.1 μmol/l genistein–treated cells.

of the cells to genistein at a concentration as low as 10 nmol/l potentiated GSIS, although a maximal increase was observed at 5 μmol/l genistein (Fig. 1*B*).

Estrogen receptor–independent effect of genistein on GSIS. Because genistein has weak estrogenic effects in some tissues by binding to estrogen receptors (1), we examined whether the genistein effect was mediated through the estrogen receptors. Consistently, genistein increased GSIS by >50%. The estrogen receptor antagonist ICI 182,780 caused no change in genistein-potentiated GSIS (Fig. 2). In addition, exposure of INS-1 cells to 17β-estradiol (1 and 10 nmol/l) for 30 min failed to significantly affect GSIS (Fig. 2). The activity of ICI 182,780 used in this study was validated through our recent study (15). These results suggest that the effect of genistein on insulin secretion is independent of estrogen signaling mechanisms.

Effect of genistein on GSIS is independent of PTK. Because genistein is often used as a PTK inhibitor in studies of PTK-mediated cellular events and because PTK may be involved in regulation of insulin secretion (12), we therefore evaluated whether genistein enhances GSIS by inhibition of PTK in INS-1 cells. We first compared the effect of genistein with that of daidzein, an analog of genistein that is inactive for PTK inhibition, on GSIS. As expected, exposure of cells to genistein for 30 min augmented GSIS. Daidzein was slightly less potent than genistein but increased insulin secretion in a similar way (Fig. 3*A*). We then directly measured PTK activity in cells treated with genistein. Genistein, at the highest concentration used in the present study (10 μmol/l), did not inhibit the basal PTK activity (Fig. 3*B*). Significant inhibition of PTK activity in this study was observed only at 100 μmol/l genistein, consistent with previous findings that PTK inhibition requires higher concentrations of genistein (16).

Genistein stimulates accumulation of intracellular cAMP. Previous studies established that the cAMP signaling pathway plays an important role in insulin secretion (17). We therefore determined whether genistein can induce intracellular cAMP, thereby elevating GSIS. Genistein, in the absence of IBMX, significantly elevated cAMP concentrations both in low and high glucose–cultured INS-1 cells (Fig. 4*A*). Dose-response studies showed that genistein as low as 10 nmol/l elevated intracellular cAMP level, with a maximal increase at ≥5 μmol/l genistein, which was also significantly higher than those induced by ≤0.1 μmol/l genistein (Fig. 4*B*).

Genistein stimulates adenylate cyclase activity. To determine whether genistein elevates cAMP through stimulation of cAMP production and/or inhibition of cAMP hydrolysis, we preincubated the cells with 0.25 mmol/l IBMX before addition of 2.5 μmol/l genistein. As shown in Fig. 5*A*, genistein or IBMX alone increased cAMP levels from 6.2 ± 0.5 to 9.5 ± 1.1 and 10.1 ± 0.7 pmol/mg, respectively. In the presence of IBMX, genistein-stimulated cAMP production was further increased by 85%, suggesting that genistein may at least partially elevate cAMP by activation of adenylate cyclase activity. Genistein (2.5 μmol/l) had no additive effect on adenylate cyclase agonist forskolin-induced cAMP production, whereas IBMX potentiated forskolin-induced cAMP in INS-1 cells (data not shown), confirming a role for adenyl-

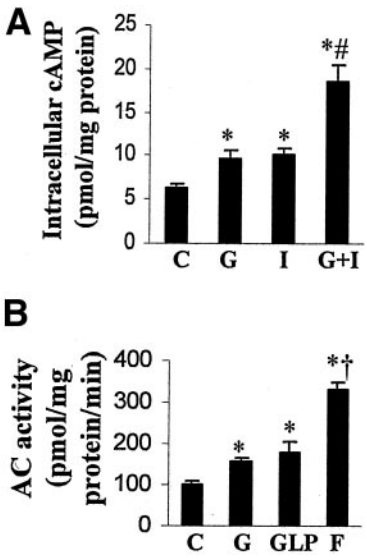

FIG. 5. Genistein stimulates adenylate cyclase activity. *A*: INS-1 cells were preincubated with IBMX (I, 0.25 mmol/l) or vehicle for 5 min, followed by addition of genistein (G, 2.5 μmol/l) or vehicle (C) for 20 min in KRBB containing 5.6 mmol/l glucose at 37°C. *B*: Membrane protein (25 μg) of INS-1 cells was incubated in adenylate cyclase assay mixture with genistein (G, 2.5 μmol/l), GLP-1 (GLP, 10 nmol/l), forskolin (F, 10 μmol/l), or vehicle (C) for 10 min at 37°C. cAMP was measured, and data were expressed as means ± SE from four experiments each in triplicate. *$P < 0.05$ vs. vehicle alone–treated cells, #$P < 0.05$ vs. genistein or IBMX alone–treated cells, †$P < 0.05$ vs. genistein- or GLP-1–treated cells.

ate cyclase in genistein-induced cAMP accumulation in β-cells. The level of genistein-induced cAMP was about one-third of that achieved by exposure to forskolin (9.5 ± 1.1 vs.27.7 ± 3.4 pmol/mg protein) but only 10% lower than that induced by 10 nmol/l glucagon-like peptide-1 (GLP-1) (7–36) amide (10.7 ± 1.4 pmol/mg protein), a potent incretin that activates adenylate cyclase and increases the cAMP concentration in β-cells. We further determined whether genistein activates adenylate cyclase in isolated plasma membranes of INS-1 cells. As shown in Fig. 5*B*, genistein significantly increased adenylate cyclase activity in plasma membranes (15.8 ± 0.9 pmol · mg^{-1} protein · min^{-1}) compared with basal values (9.7 ± 1.0 pmol · mg^{-1} protein · min^{-1}), a change that was comparable with that induced by GLP-1 but was about one-third that achieved by exposure to forskolin (33.1 ± 1.4 pmol · mg^{-1} protein · min^{-1}).

Genistein activates cAMP-dependent PKA activity that is independent of the estrogen receptors and PTK. Because cAMP is a direct activator of PKA, we next investigated whether the elevation of cAMP by genistein is sufficient to activate PKA in INS-1 cells. As shown in Fig. 6*A*, genistein potently stimulated PKA activity. The PKA activity increased in response to increasing concentrations of genistein, with a maximal response observed at 5 μmol/l genistein. To further characterize the cAMP/PKA pathway, INS-1 cells were preincubated with SQ 22536 (10 μmol/l), an adenylate cyclase inhibitor. As shown in Fig. 6*B*, inhibiting the generation of cAMP completely blocked the genistein-induced PKA activity. As expected, SQ 22536 also completely ablated forskolin-augmented PKA activity (data not shown). These data clearly demonstrated that genistein is a potent activator of the cAMP/PKA cascade. Consistently, inhibition of estrogen receptors with ICI 182,780 had no effect on genistein-stimulated PKA (Fig. 6*C*). In addition, daidzein (5 μmol/l) was potent in stimulation of PKA activity, whereas 17β-estradiol (10 nmol/l) had no obvious effect (Fig. 6*C*), suggesting that activation of PKA by genistein is PTK independent and estrogen receptor independent.

Genistein-enhanced insulin secretion is mediated by PKA. Next, we examined whether PKA regulates the genistein-induced insulin secretion. INS-1 cells were pre-

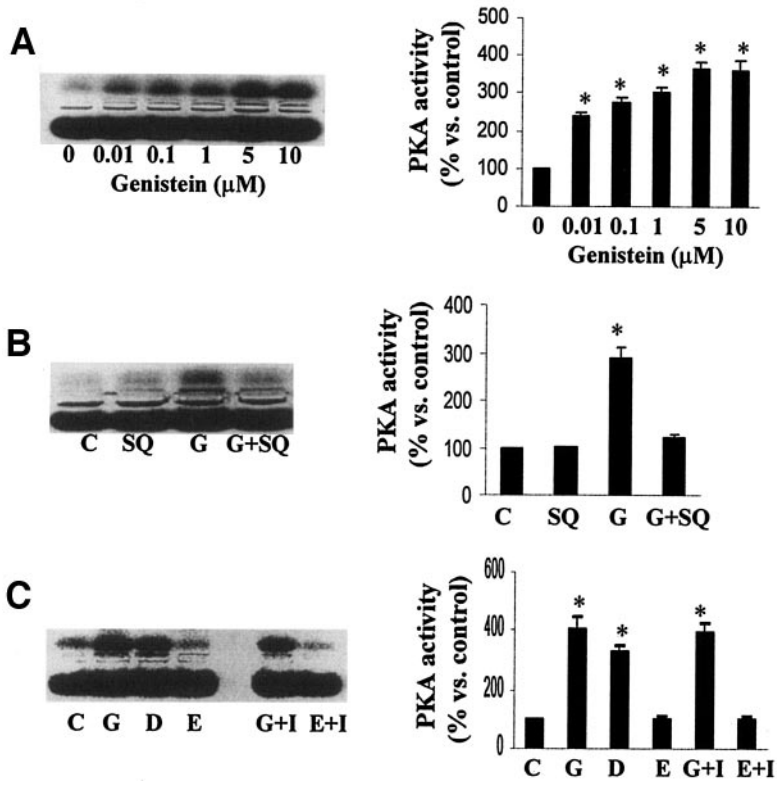

FIG. 6. Genistein stimulates cAMP-dependent PKA activity. *A*: INS-1 cells were stimulated with various concentrations of genistein in KRBB in the presence of 5.6 mmol/l glucose for 30 min at 37°C. *B*: Cells were preincubated with SQ 22536 (SQ, 10 μmol/l) for 30 min, followed by stimulation with genistein (G, 2.5 μmol/l) or vehicle for 30 min at 37°C. *C*: INS-1 cells were preincubated with ICI 182,780 (I, 1 μmol/l) for 30 min, followed by stimulation with genistein (G, 2.5 μmol/l), daidzein (D, 2.5 μmol/l), 17β-estradiol (E, 10 nmol/l), or vehicle (C) for 30 min at 37°C. PKA activity in cell extracts was measured. A representative photograph of the agar gel used for the kemptide assay is shown on the *left*, with phosphorylated kemptide in the *top panel* and unphosphorylated kemptide in the *bottom panel*, and the quantification of the assay on the *right*. The experiment was repeated three times, and data were expressed as means ± SE. *$P < 0.05$ vs. vehicle alone–treated cells.

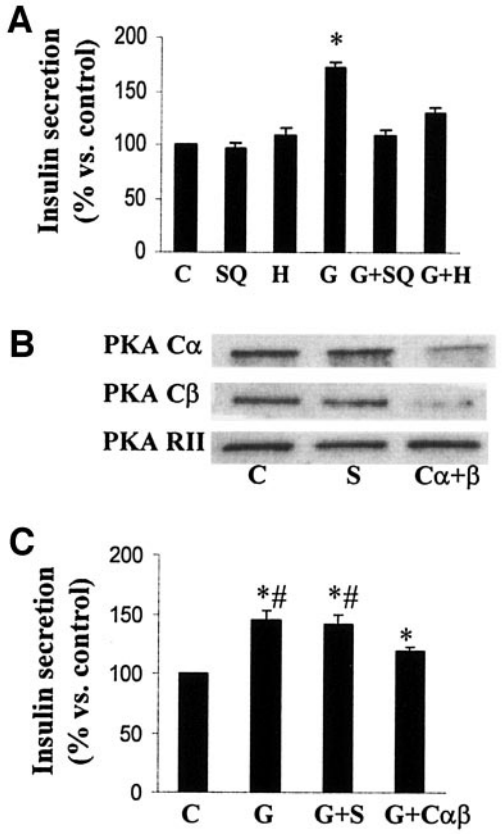

FIG. 7. Effect of genistein on GSIS is mediated through PKA. INS-1 cells were preincubated with SQ 22536 (SQ, 10 μmol/l), H89 (H, 10 μmol/l), or vehicle for 30 min. Cells were then stimulated with genistein (G, 2.5 μmol/l) or vehicle (C) in KRBB in the presence of 5.6 mmol/l glucose for 30 min at 37°C. *A*: Insulin secreted in culture buffer was measured. MIN6 cells transfected with scrambled siRNA (S) or siRNA directed against PKACα and PKACβ (Cαβ) were stimulated with genistein (G, 2.5 μmol/l) in KRBB containing 5.6 mmol/l glucose for 30 min at 37°C. The cell extracts were harvested to analyze the protein expression of PKA (*B*), and supernatants were collected for insulin determination (*C*). The experiment was repeated three times, and data were expressed as means ± SE. **P* < 0.05 vs. vehicle alone–treated cells, #*P* < 0.05 vs. G+Cαβ.

incubated with a specific PKA inhibitor H89 (10 μmol/l) or SQ 22536 (10 μmol/l) for 30 min, followed by stimulation with genistein (2.5 μmol/l) for 30 min. As shown in Fig. 7*A*, SQ 22536 blocked the genistein-induced GSIS by 88% and H89 resulted in 66% inhibition. To further examine the specificity and role of the PKA and also to ensure that the genistein effect was not a species-specific phenomenon, we used siRNA to ablate the catalytic subunits of PKA in mouse MIN6 cells. As shown in Fig. 7*B*, transfection of MIN6 cells with siRNA of PKACα and -Cβ knocked down the PKACα and -Cβ protein expression by 73 ± 3 and 79 ± 6%, respectively, whereas scrambled construct had no effect. As a result, this transfection ablated genistein-induced PKA activity by 64%. Accordingly, ablation of PKA reduced 56% of genistein-augmented GSIS, whereas the scrambled control did not suppress the insulinotropic effect of genistein (Fig. 7*C*). These results further support an important role of the cAMP/PKA pathway in genistein-induced insulin secretion.

Genistein activates the adenylate cyclase/cAMP/PKA cascade and enhances GSIS in mouse pancreatic islets. Finally, we tested whether genistein has a similar effect on the cAMP signaling and insulin secretion in pancreatic islets. As shown in Fig. 8*A*, genistein greatly potentiated GSIS in mouse islets, a similar response to that observed in INS-1 cells, suggesting that in vitro findings may have physiological relevance. In parallel to its effect on insulin secretion, genistein also significantly enhanced adenylate cyclase activity (Fig. 8*B*), elevated cAMP (Fig. 8*C*), and activated PKA (Fig. 8*D*) in the islets. To confirm the role of PKA in the regulation of genistein effect in the islets, we either preincubated the islets with H89 or delivered PKACα plus -Cβ antibodies into the islets with a recently developed protein delivery reagent that has been successfully used in previous studies (18). Preincubation with the PKA inhibitor or transfection of the cells with PKA antibodies significantly attenuated genistein-induced GSIS (Fig. 8*E*) and PKA activity (Fig. 8*F*), whereas preimmune IgG had no effects.

DISCUSSION

The soy isoflavone genistein has various biological functions. Recent studies suggest a potential antidiabetic role for isoflavones in animals and humans (3,4). However, it is unknown whether genistein, at physiological doses, directly acts on pancreatic β-cells to modulate insulin secretion. In the present study, we found that genistein, at physiologically achievable concentrations for individuals consuming soy products, potentiated GSIS both in insulin-secreting cell lines and mouse pancreatic islets. This effect of genistein was not mediated through the estrogen receptor mechanism and also was independent of PTK or NO signaling pathway. However, genistein stimulates intracellular cAMP accumulation, which subsequently activates PKA that at least partially mediates the genistein-augmented GSIS.

Genistein at a concentration as low as 10 nmol/l significantly augmented GSIS in INS-1 cells, although the maximal effect was achieved at 5 μmol/l genistein. The augmentation of GSIS by genistein in the INS-1 cells was maximal at 5.6 mmol/l glucose, with a 50% increase over control, and then gradually declined with the increase of glucose concentration. These results agreed with previous studies demonstrating that glucose-dependent insulinotropic polypeptide (19) and GLP-1 (20) maximally enhance insulin secretion in INS-1 cells at this glucose concentration. The reported serum concentrations of genistein are 0.16–0.89 μmol/l in Japanese men (21,22). However, the serum genistein level in humans consuming three meals per day containing soy milk can reach 4.6 μmol/l (23). Given the fact that the plasma glucose concentration usually changes between 5 and 8 mmol/l under physiological conditions (24), our results are therefore physiologically relevant. The results observed in INS-1 cells were confirmed with mouse MIN6 β-cells and isolated mouse islets, suggesting a non–species-specific effect of genistein and further suggesting that physiologically relevant concentrations of genistein may augment GSIS in vivo and thereby have antidiabetic implications by directly acting on β-cells.

Genistein has weak estrogenic effects by binding to estrogen receptors (1). However, unlike genistein, 17β-estradiol did not significantly alter insulin secretion. Our result is in agreement with previous findings that 17β-estradiol had no effect on basal and stimulated insulin secretion in INS-1 cells overexpressing estrogen receptors (25). In addition, blocking estrogen receptors with ICI 182,780 did not inhibit the cellular response to genistein. It is unlikely that the inability of this agent to block the effect

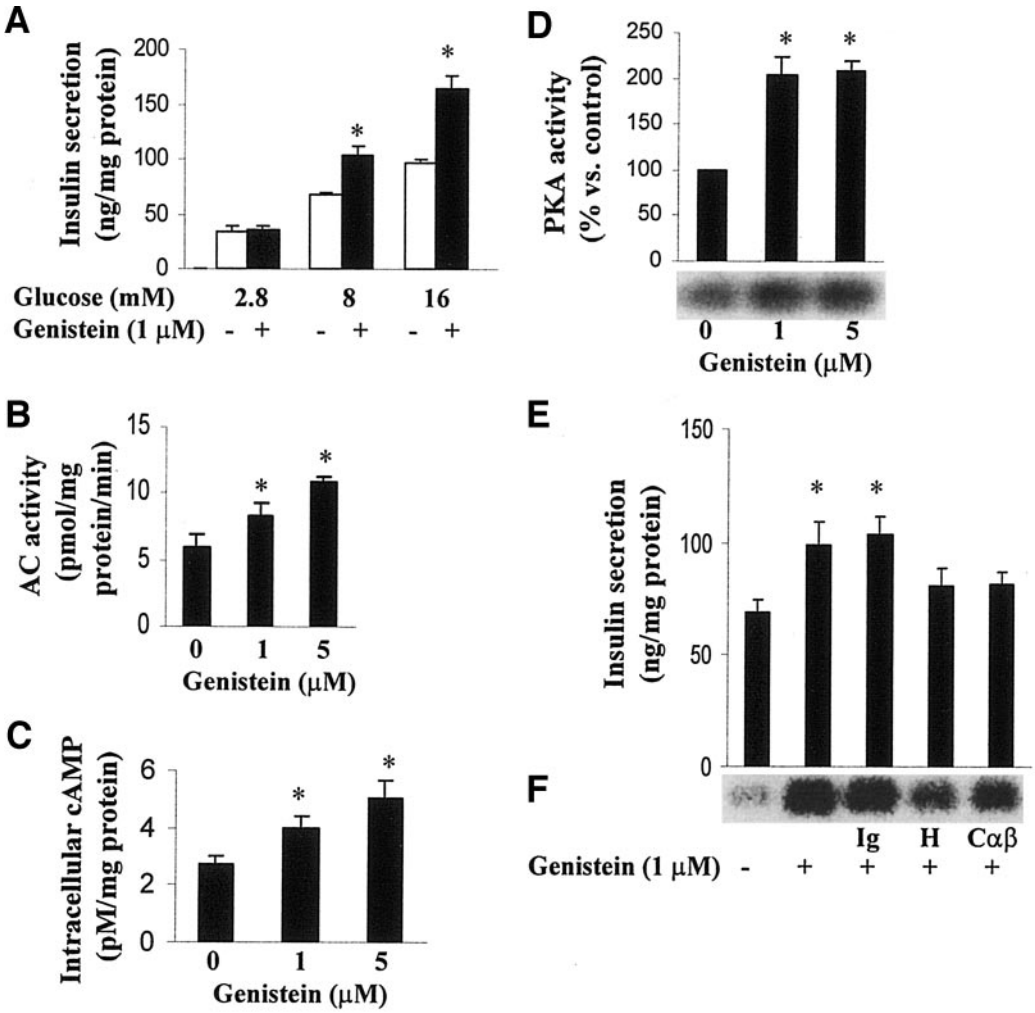

FIG. 8. Genistein activates the adenylate cyclase/cAMP/PKA cascade and potentiates GSIS in pancreatic islets. *A*: Mouse islets were stimulated with genistein (1 μmol/l) in the presence of indicated concentrations of glucose for 30 min at 37°C. Insulin secreted in culture buffer was measured. *B*: The islets were treated with or without genistein for 10 min at 37°C. The adenylate cyclase activity in membrane preparations was determined. *C* and *D*: The islets were stimulated with indicated concentrations of genistein in KRBB containing 8 mmol/l glucose for 20 min (*C*) or 30 min (*D*) at 37°C. cAMP (*C*) and PKA (*D*) activity in the cell extracts from the islets was determined. *E*: The islets were either preincubated with H89 (H, 10 μmol/l) for 30 min or transfected with antibodies against PKACα and PKACβ (Cαβ) or preimmune IgG (Ig). The H89-treated or -transfected islets were stimulated with or without genistein (1 μmol/l) for 30 min at 37°C. Insulin released in culture buffer was measured. *F*: PKA activity was determined from the protein extracts of the treated islets in *E*. Data were expressed as means ± SE from three experiments each in triplicate. *$P < 0.05$ vs. vehicle alone–treated islets.

of genistein on GSIS is due to a lack of efficacy, because we previously reported that, at the same concentration used, ICI 182,780 completely abolished the 17β-estradiol–induced endothelial NO synthase activity (14). These results suggest that the observed insulinotropic effect of genistein is not related to its estrogenic effect.

Genistein is an inhibitor of PTK (2) and is often used to study PTK-mediated signaling events. However, we provide evidence that genistein-induced GSIS is not related to PTK inhibition (8–10) because genistein at the concentrations used in the present study (10 nmol/l–10 μmol/l) failed to inhibit PTK activity. As reported (26), genistein only inhibits PTK at a much higher concentration (100 μmol/l). In addition, daidzein, an analog of genistein that does not inhibit PTK, also significantly increased insulin secretion and stimulated PKA activity, although these effects are less potent than those of genistein as previously observed in other tissues (27), further supporting a PTK-independent effect of genistein. Additionally, these results suggest that genistein should be cautiously used in studies of PTK signal transduction, because increased activation of PKA by genistein may contribute to the observed effects.

cAMP is a central signaling molecule in a variety of cellular systems. It is well established that an elevation of cAMP level and consequent activation of PKA in β-cells plays an important role in incretin-stimulated insulin secretion (28,29). We found in the present study that genistein stimulated cAMP accumulation over the same concentration range as its effect on insulin secretion. We further demonstrated that in a pattern similar to the stimulated cAMP accumulation, genistein potently activated PKA activity, an effect that was blocked by inhibition of adenylate cyclase, suggesting that the elevated cAMP by genistein is essential to stimulate PKA activity, an effect that is also independent of estrogen receptors or PTK inhibition. Our results demonstrated that activation of PKA by genistein in response to cAMP plays an important role in mediating genistein-induced insulin secretion because ablating the production of cAMP or inhibition of PKA attenuated the genistein effect on insulin secretion by 88 and 66%, respectively. The efficacy of inhibitors in our assay was confirmed by demonstrating their ability to block the genistein-stimulated PKA activity (data not shown). This result was confirmed by our observation that transfecting MIN6 cells with both PKACα and PKACβ siRNA or mouse islets with their polyclonal antibodies diminished insulin secretion in response to genistein, whereas the scrambled control or preimmune IgG had no effect, further emphasizing the important role of PKA in mediating genistein effect.

PKA is a heterotetramer composed of a regulatory subunit (R) dimer and a catalytic subunit (C) dimer. It was shown that rat β-cell line expresses both PKACα and -Cβ (29), which was confirmed in the preliminary study. Although it is known that elevation of cAMP causes translocation of PKACβ to the plasma membranes in β-cells (30),

where it may interact with L-type Ca^{2+} channel (31) and thereby may participate in the insulin exocytotic process, it is unclear whether PKACβ primarily regulates rapid insulin secretion, thereby mediating the genistein effect. In addition, our finding that although both SQ 22536 and H89 maximally inhibited PKA, SQ 22536 inhibited genistein-induced insulin secretion to a greater extent than H89, suggesting that a PKA-independent mechanism may exist to contribute to the genistein action, although PKA apparently plays a major role. A PKA-independent pathway involving a cAMP-regulated guanine nucleotide exchange factor (Epac) has recently been suggested to play a role in cAMP stimulation of insulin exocytosis (32,33). Whether genistein also activates Epac remains to be determined.

AC- and cAMP-specific PDEs are the primary enzymes responsible for regulation of cAMP. Recent studies by others suggested that genistein, at pharmacological concentrations, may control the degradation of cAMP by inhibiting PDEs in anterior pituitary cells (34) and airway epithelial cells (35). However, our results suggest that genistein, at physiological concentrations, may elevate cAMP primarily via activation of adenylate cyclase. First, genistein (2.5 μmol/l) effect on cAMP was potentiated in the presence of potent inhibitor of PDEs. Second, unlike IBMX, genistein (2.5 μmol/l) had no additive effect on adenylate cyclase agonist forskolin-induced cAMP accumulation. Third, genistein induced adenylate cyclase activity in plasma membranes of INS-1 cells and the islets. Previous studies have shown that various adenylate cyclase isoforms are expressed in β-cells that are differentially regulated by Ca^{2+}, G-proteins, and protein kinases (36–38). A recent study reported that genistein may enhance cAMP accumulation by modifying α-adrenoceptors in rat brain (39). However, how genistein activates adenylate cyclase in β-cells remains to be clarified.

Besides playing an important role in insulin secretion, it has been recently shown that cAMP has an array of beneficial effects on β-cells, including protection of cells from proinflammatory cytokine- and lipid-induced damage and apoptosis (40,41), stimulation of β-cell survival and proliferation (31,42), and direct regulation of insulin gene expression (43). In addition, activation of the cAMP/PKA pathway in β-cells increases the expression of insulin receptor substrate-2 and activates pancreatic duodenal homeobox-1 protein, a transcriptional factor with essential functions for pancreas development and islet formation (44,45). Given the present results and the previous evidence showing a wide range of roles for cAMP in maintaining β-cell function, it is tempting to speculate that, besides regulation of insulin secretion, genistein may have various beneficial effects on β-cells by targeting the cellular cAMP/PKA pathway.

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