Activation of Acetyl-CoA Carboxylase by a Glutamate- and Magnesium-Sensitive Protein Phosphatase in the Islet β-Cell

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Acetyl-CoA carboxylase (ACC) catalyzes the formation of malonyl-CoA, a precursor in the biosynthesis of long-chain fatty acids, which have been implicated in physiological insulin secretion. The catalytic function of ACC is regulated by phosphorylation (inactive)−dephosphorylation (active). In this study we investigated whether similar regulatory mechanisms exist for ACC in the pancreatic islet β-cell. ACC was quantitated in normal rat islets, human islets, and clonal β-cells (HIT-15 or INS-1) using a [^{14}C]bicarbonate fixation assay. In the β-cell lysates, ACC was stimulated by magnesium in a concentration-dependent manner. Of all the dicarboxylic acids tested, only glutamate, albeit ineffective by itself, significantly potentiated magnesium-activated ACC in a concentration-dependent manner. ACC stimulation by glutamate and magnesium was maximally demonstrable in the cytosolic fraction; it was markedly reduced by okadaic acid (OKA) in concentrations (<50 nmol/l) that inhibited protein phosphatase 2A (PP2A). Furthermore, pretreatment of the cytosolic fraction with anti-PP2A serum attenuated the glutamate- and magnesium-mediated activation of ACC, thereby suggesting that ACC may be regulated by an OKA-sensitive PP2A-like enzyme. Streptavidin-agarose chromatography studies have indicated that glutamate- and magnesium-mediated effects on ACC are attributable to activation of ACC's dephosphorylation; this suggests that the stimulatory effects of glutamate and magnesium on ACC might involve activation of an OKA-sensitive PP2A-like enzyme that dephosphorylates and activates ACC. In our study, 5-amino-imidazolecarboxamide (AICA) riboside, a stimulator of AMP kinase, significantly inhibited glucose-mediated activation of ACC and insulin secretion from isolated β-cells. Together, our data provide evidence for a unique regulatory mechanism for the activation of ACC in the pancreatic β-cell, leading to the generation of physiological signals that may be relevant for physiological insulin secretion. *Diabetes* 50:1580–1587, 2001

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ACC, acetyl-CoA carboxylase; AICA, 5-amino-imidazolecarboxamide; BSA, bovine serum albumin; DTT, dithiothreitol; GAPP, glutamate-activated protein phosphatase; OKA, okadaic acid; PP2, protein phosphatase type 2 ; PP2A, spontaneously active PP2; PP2Ac, C subunit of PP2A; SAMS peptide, His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg.

Glucose-induced insulin secretion from pancreatic β-cells involves altered handling and redistribution of ions (e.g., Ca^{2+}) as well as the generation of other second messengers, such as cyclic nucleotides and lipid hydrolytic products of phospholipase A_2, D, or C (1,2). Some of the known actions of these modulators include regulation of various protein kinases indigenous to pancreatic islet β-cells. Several earlier studies have demonstrated localization of such kinases in normal rat islets as well as clonal β-cells. Some of these include Ca^{2+}-, Ca^{2+}/calmodulin-, cAMP-, and phospholipid-dependent protein kinases (3,4,). Using relatively selective inhibitors of these kinases, it has been possible to document critical regulatory roles for islet endogenous protein phosphorylation in insulin exocytosis (3,4). The phosphorylation status of proteins is regulated through the balance of activity by protein kinases and phosphatases, which induce the addition and removal, respectively, of phosphate from these proteins (3–6). Although several studies have focused on the identification and characterization of protein kinases in islets, very little is known about the localization and regulation of phosphoprotein phosphatases in β-cells.

Based on biochemical properties, serine/threonine phosphatases are divided into two major groups (5,6). The first group consists of type 1 phosphatases that are inhibited by two heat-stable proteins, termed inhibitors 1 and 2, and preferentially dephosphorylate the β subunit of phosphorylase kinase. The second group of phosphatases (type 2; PP2) is resistant to inhibitors 1 and 2 and catalyzes the dephosphorylation of the α subunit of phosphorylase kinase. PP2 is further subdivided into three types: spontaneously active PP2 (PP2A), Ca^{2+}-dependent PP2 (known as PP2B), and Mg^{2+}-dependent PP2 (known as PP2C). At low nanomole-per-liter concentrations, okadaic acid (OKA) specifically inhibits PP2A (K_i = 0.2 nmol/l); this property thus discriminates PP2A from other phosphatases. Several trimeric holoenzyme forms of PP2A have been characterized recently (5,6). PP2A consists of a 36-kDa catalytic subunit (C subunit) complexed with a 65-kDa structural A subunit. This dimer associates with a regulatory B subunit of varying mass (54–130 kDa). The C subunit of PP2A (PP2Ac) undergoes posttranslational modifications, including phosphorylation (at Tyr-307) and carboxyl methylation (at Leu-309). Such modification steps appear to influence the catalytic activity of PP2A (5,7,8). Recently,

we immunologically identified PP2A in clonal β-cells, normal rat islets, and human islets (7). We demonstrated further that PP2Ac undergoes methylation at its COOH-terminal leucine and that the carboxyl methylation of PP2Ac is associated with a modest but significant increase in its catalytic activity (7); this finding is compatible with published evidence by Favre et al. (8).

The catalytic function of acetyl-CoA carboxylase (ACC), a lipogenic enzyme, is regulated by phosphorylation-dephosphorylation. ACC is inactivated by phosphorylation at multiple Ser residues (-79, -1,200, and -1,215) by AMP kinase (9). It is reactivated upon dephosphorylation by a PP2A-like activity. Using isolated hepatocytes, Gaussin et al. (10) described a predominantly cytosolic, magnesium- and glutamate-activated protein phosphatase (GAPP) that dephosphorylates and activates ACC. Based on its subcellular distribution and sensitivity to PP2A inhibitors, GAPP appears to be similar to PP2A (10). Although such regulatory mechanisms for ACC activation have been described in other cell types, very little is known about whether similar mechanisms exist for ACC regulation in the pancreatic β-cell. Furthermore, this is an important area of investigation because there is some debate surrounding the putative roles of long-chain fatty acids in β-cell function (11–16). Therefore, the present study was undertaken to verify whether specific components of the lipogenic-signaling cascade mediated by ACC are regulated by physiological modulators of insulin secretion. We report that a glutamate-and magnesium-sensitive PP2A-like protein phosphatase (similar to the GAPP described in hepatocyte literature) dephosphorylates and activates ACC in the islet β-cell, and that a stimulator of AMP kinase (e.g., 5-amino-imidazolecarboxamide [AICA] riboside) inhibits glucose-stimulated ACC activation and concomitant insulin secretion from isolated β-cells.

RESEARCH DESIGN AND METHODS

Materials. [^{14}C]Bicarbonate (50 mCi/mmol) and [γ-^{32}P]ATP (60 Ci/mmol) were purchased from NEN-DuPont (Boston, MA). SAMS peptide (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) was synthesized by Neosystem (Strasbourg, France). A second preparation of SAMS peptide was generously provided by Prof. Louis Hue (Hormone and Metabolic Research Unit, Institute of Cellular Pathology, Université Catholique de Louvain, Brussels, Belgium). Acetyl-CoA, AICA riboside, streptavidin-agarose, biotin, trypsin, trypsin inhibitor, various dicarboxylic acids, and the protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Anti-PP2A serum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antisera directed against ACCα were generously provided by Dr. Lee Witters (Dartmouth Medical School, Hanover, NH). SDS-PAGE supplies and authentic molecular weight protein standards were obtained from Bio Rad (Hercules, CA). All other reagents were of analytical grade and of the highest purity available.

Human and rat pancreatic islets and clonal β (HIT-T15 and INS-1) cells. Pancreatic islets were isolated from male SD rats (300–400 g body wt) by the collagenase digestion method, as described in our earlier publications (7,17,18). Islets were manually picked twice under stereo microscopic control to avoid any contamination by exocrine tissue. HIT-T15 cells were provided by Dr. Paul Robertson (Pacific Northwest Research Institute, Seattle, WA), and INS-1 cells were provided by Claes Wollheim (University of Geneva, Geneva, Switzerland). Human islets were provided by the Islet Isolation Core, Washington University School of Medicine (St. Louis, MO).

Isolation of subcellular fractions from β-cell homogenates. Subcellular fractions from insulin-secreting β-cells were isolated by a differential centrifugation procedure, as previously described (7,17,18). All procedures were carried out at 4°C unless stated otherwise. Briefly, homogenates were prepared in 230 mmol/l mannitol, 70 mmol/l sucrose, and 5 mmol/l HEPES buffer (containing 1 mmol/l EGTA, 1 mmol/l dithiothreitol [DTT], and 2.5 μg/ml each of leupeptin and pepstatin; pH 7.4). The homogenates were spun at 600*g* for 5 min to obtain crude nuclear pellet. The resulting supernatant was centrifuged at 105,000*g* for 60 min to yield a pellet (referred to as postnuclear pellet) and supernatant (referred to as cytosol). The nuclear and postnuclear pellets were washed twice with the homogenization medium and resuspended in a suitable volume of the buffer for biochemical studies.

Quantitation of ACC activity. ACC activity was assayed in β-cell homogenates or subcellular fractions by the [^{14}C]bicarbonate fixation assay. The reaction mixture (200 μl total vol) consisted of 60 mmol/l HEPES (pH 7.5), 1.2 mmol/l DTT, 300 μmol/l acetyl-CoA, 3 mmol/l sodium citrate, bovine serum albumin (BSA; 1 mg/ml final concentration), and β-cell protein, as indicated in the text. Magnesium and dicarboxylic acids were included in the appropriate concentrations noted in the text. The reaction was initiated by the addition of [^{14}C]sodium bicarbonate (18 mmol/l final, 2 μCi per tube) and was carried out at 37°C for different time intervals as described in text. It was terminated by the addition of perchloroacetic acid. The contents of the tube were mixed vigorously and left on ice for 15 min, then spun at 10,000 rpm for 8–10 min in an Eppendorf centrifuge. Next, 200 μl of the supernatant was transferred into a scintillation vial and dried under mild heat. Residue in the vial was dissolved in 400 μl distilled water, and radioactivity was quantitated by scintillation spectrometry. The specific activity of ACC was expressed as nanomoles of product formed per minute per milligram of protein.

Isolation of phosphorylated ACC by streptavidin-agarose affinity chromatography. The phosphorylated ACC was isolated from HIT and INS cells using a streptavidin-agarose matrix because ACC is a biotinylated protein and binds specifically to streptavidin (12,19). Briefly, HIT and INS cell cytosolic proteins were phosphorylated using [γ-^{32}P]ATP as phosphoryl donor (16); phosphorylated proteins were then incubated with streptavidin-agarose (previously equilibrated in 50 mmol/l Tris-HCl [pH 7.5], 500 mmol/l NaCl, 100 μmol/l sodium fluoride, 2 mmol/l EDTA, 10 mmol/l 2-mercaptoethanol, 0.02% sodium azide, and 10% glycerol, as previously described) (12,19). Following extensive washing, streptavidin-agarose–bound proteins were separated by SDS-PAGE (5%), and labeled proteins were identified using a Storm 860 Phosphoimager (Molecular Dynamics). In some experiments, bound proteins were eluted using a medium containing biotin (2 mmol/l), and the radioactivity in the eluates was quantitated by scintillation spectrometry. Alternatively, biotin-elutable proteins were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an antiserum directed against NH_2-terminal 15–amino acid residues of the ACCα subunit. Because earlier studies (12) have shown that this antiserum does not recognize the HIT cell enzyme, we used this antiserum only in studies involving immunological detection of ACC in INS cells. In studies involving dephosphorylation of ACC in HIT and INS cells, we used the streptavidin-agarose affinity chromatography (see RESULTS).

Quantitation of AMP kinase activity. AMP kinase activity was assayed using ammonium sulfate precipitates of β-cell lysates that were prepared as follows. HIT or INS cells were homogenized in a buffer consisting of 50 mmol/l Tris-HCl (pH 8.0); 250 mmol/l sucrose; 5 mmol/l sodium pyrophosphate; 50 mmol/l sodium fluoride; 1 mmol/l each of EDTA, EGTA, benzamidine, and DTT; 0.2 mmol/l phenylmethylsulfonyl fluoride; and 5 μg/ml trypsin inhibitor. Homogenates were centrifuged at 3,000*g* for 5 min, and the resulting supernatant was centrifuged at 100,000*g* for 40 min to obtain the cytosolic fraction. Ammonium sulfate (209 mg/ml) was added slowly to the cytosolic fraction and agitated gently at 4°C. The solution was left on ice for 30 min and then centrifuged at 28,000*g* for 20 min; the resulting pellet was reconstituted in homogenization buffer and used in the enzyme assays (see below).

AMP kinase activity was assayed using SAMS peptide as the substrate (20). The β-cell lysates were incubated in a buffer (100 mmol/l HEPES [pH 7.0], 200 mmol/l NaCl, 5 mmol/l $MgCl_2$, 20% glycerol, and 200 μmol/l [γ-^{32}P]ATP mol/l) and in the absence or presence of 200 μmol/l AMP. Enzyme reaction was carried out at 30°C for 10 min and terminated by the addition of phosphoric acid (5% final concentration). Aliquots of the assay mix were applied to phosphocellulose paper strips, and after extensive washing, the radioactivity associated with filters was quantitated by scintillation spectrometry.

Trypsin or heat treatment of membrane fractions. HIT cell total particulate fraction (5 mg protein/ml) was incubated with trypsin (0.1 mg/ml final concentration) at room temperature for 15 min. After this incubation, further digestion by trypsin was prevented by adding a trypsin inhibitor (0.5 mg/ml final concentration). Samples were left on ice until use. To rule out the possibility that the trypsin inhibitor alone did not affect ACC phosphatase activity, we conducted control experiments in which a trypsin inhibitor was added (0.5 mg/ml final concentration) to the reaction mixture. In experiments involving determination of heat sensitivity of the ACC-phosphatase inhibitor, the membrane fraction was heated at 95°C for 10 min, after which the contents of the tube were left on ice until use.

Insulin-release experiments. Insulin-release studies were carried out as described earlier (21). The amount of insulin released was quantitated using an enzyme-linked immunosorbent assay kit purchased from Crystal Chemical

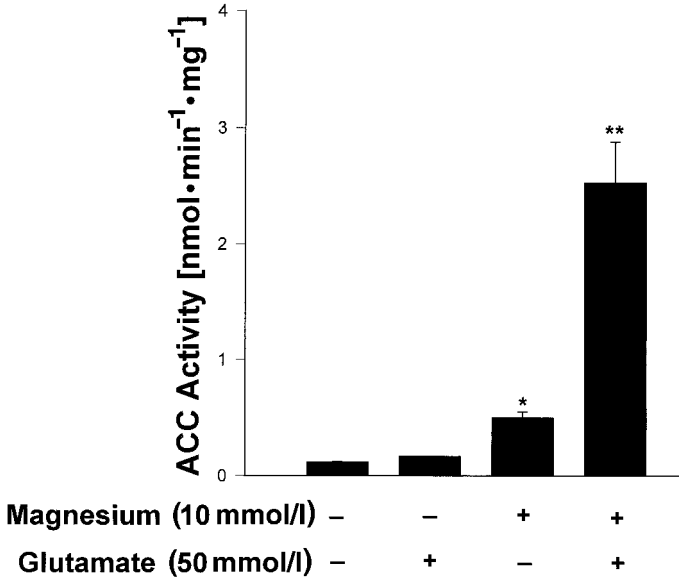

FIG. 1. Glutamate- and magnesium-stimulated ACC activity in HIT cell cytosol. ACC activity was assayed in HIT cell cytosolic fraction in the presence of 10 mmol/l magnesium chloride and 50 mmol/l glutamate, as indicated. Data are expressed as nanomoles of the product formed per minute per milligram of protein and are means ±SE from 15 experiments. Such stimulatory effects of glutamate and magnesium of ACC activity were also demonstrable in normal rat islets, human islets, and INS cell cytosolic fractions. *P < 0.05; **P < 0.001 vs. control (i.e., in the absence of any added magnesium and glutamate).

(Chicago, IL), and was expressed as nanograms of insulin released per milliliter of the incubation medium. Incubation conditions and details of concentrations of various agonists and antagonists of insulin release are provided in the text.

Other methods. The protein concentration in samples was assayed by a dye-binding assay described by Bradford (22), using BSA as a standard. In all experiments, data are expressed as means ± SE for the number of experiments stated in the figure legends. The statistical significance of the differences between experimental groups was determined by Student's t test. Values of $P < 0.05$ were considered significant.

RESULTS

Glutamate- and magnesium-dependent stimulation of ACC activity in insulin-secreting cells. ACC activity was assayed in HIT cell cytosolic fraction by the [^{14}C]bicarbonate fixation assay (see RESEARCH DESIGN AND METHODS) in the presence of various combinations of glutamate and magnesium. In contrast to glutamate, magnesium stimulated ACC activity significantly. Interestingly, however, a combination of glutamate and magnesium synergistically stimulated ACC activity (Fig. 1). The stimulatory effects of glutamate and magnesium on ACC activity were also demonstrable in the cytosolic fraction derived from normal rat (SD and Wistar) islets, human islets (additional data not shown), and INS-1 cells (see below).

Specificity of magnesium and glutamate effects on ACC. To examine the specificity of magnesium ion effects, ACC activity was assayed in a HIT cell cytosolic fraction in the presence of various divalent cations (5 mmol/l). Of all the divalent cations tested, only magnesium stimulated ACC activity significantly. Manganese (5 mmol/l) also stimulated ACC activity relatively modestly compared with magnesium. In contrast, cobalt and zinc were inhibitory. The effects of divalent cations were in the following rank order: magnesium > manganese > calcium = control > cobalt = zinc. Additional studies using HIT cell lysates indicated that the effects of magnesium on ACC activity were concentration-dependent (0–10 mmol/l) (Fig. 2*A*). The degree of stimulation (less than twofold) of this activity by 10 mmol/l manganese was not significantly different from that demonstrable at 5 mmol/l. Further, other divalent cations elicited no stimulatory effects on ACC activity when tested in the concentration range of 0–20 mmol/l (additional data not shown).

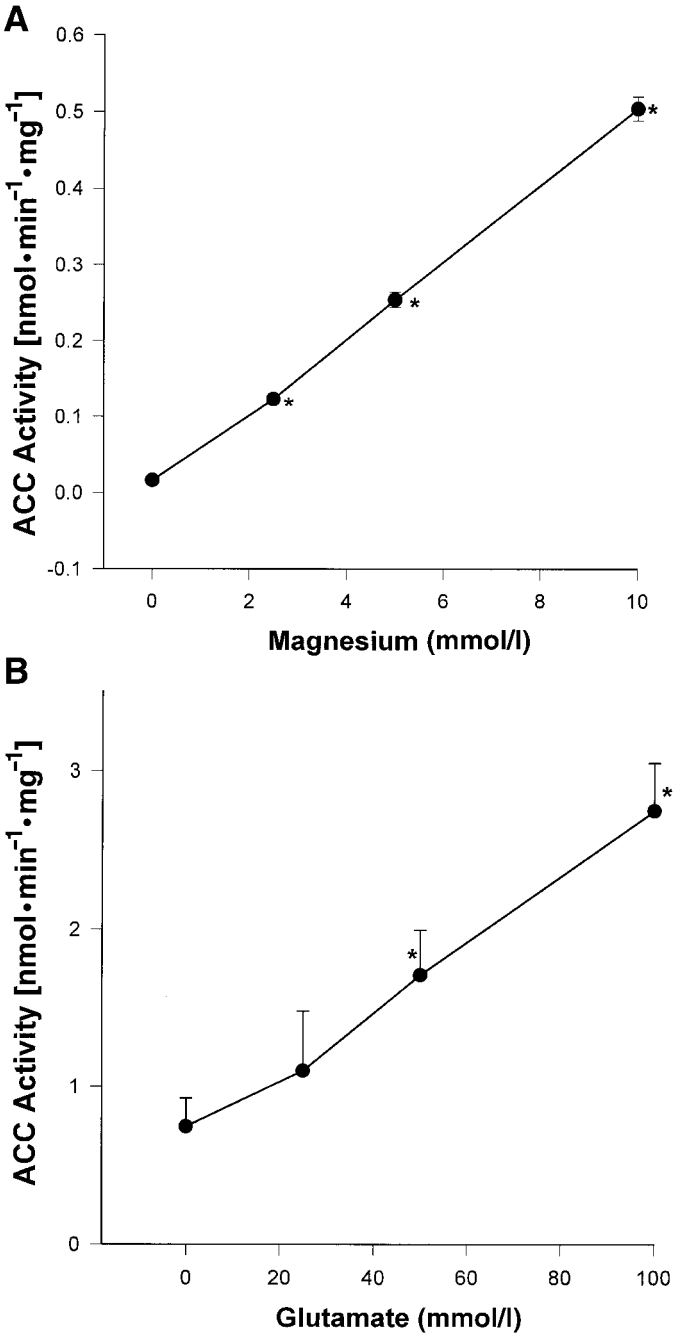

FIG. 2. *A*: Magnesium concentration–dependent stimulation of ACC activity. ACC activity was assayed in HIT cell cytosolic fraction as a function of magnesium concentration (0–10 mol/l), as indicated. Data are means ± SE from three experiments. *P < 0.05 vs. control (i.e., in the absence of any added magnesium). *B*: Glutamate concentration–dependent stimulation of ACC activity. ACC activity was assayed in HIT cell cytosolic fraction as a function of glutamate concentration (0–100 mmol/l) in the presence of a fixed concentration (5 mmol/l) of magnesium. Data are means ± SE from three experiments. *P < 0.01 vs. control.

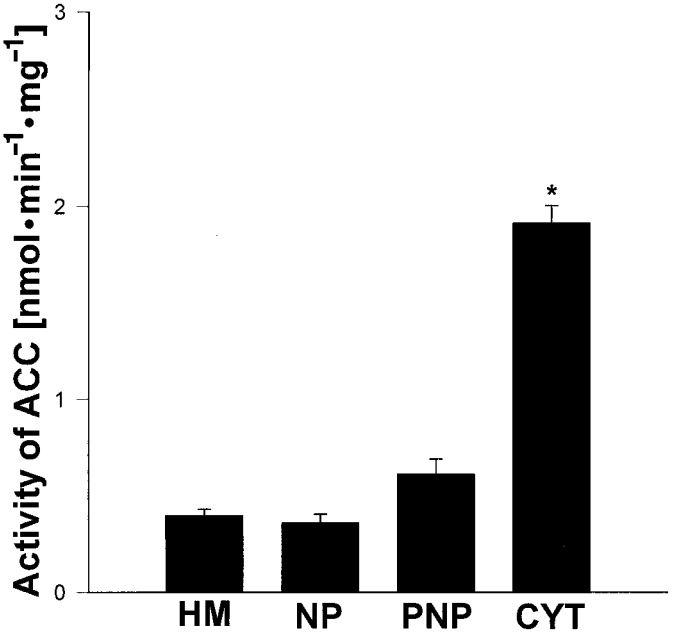

FIG. 3. Activation by glutamate and magnesium of ACC activity in HIT cell subcellular fractions. HIT cell subcellular fractions were isolated by differential centrifugation, as described in RESEARCH DESIGN AND METHODS. Glutamate- and magnesium-activated ACC activity was determined in various HIT cell fractions as indicated. Data are means ± SE from three individual fractionations done in triplicate. *$P < 0.001$ vs. homogenates. HM, homogenate; NP, nuclear pellet; PNP, post nuclear pellet; CYT, cytosol.

To determine the specificity of glutamate effects, ACC activity was assayed in HIT cell homogenates in the presence of various dicarboxylic acids (50 mmol/l each) and 5 mmol/l magnesium. Of all the three dicarboxylic acids tested (glutamate, succinate, and oxalate), only glutamate significantly augmented magnesium-stimulated ACC activity. Succinate augmented the magnesium-stimulated ACC activity by 1.42 ± 0.12−fold ($n = 3$ determinations). Oxalate (50 mmol/l) had no effect on this activity (not shown). Glutamate and succinate (50 mmol/l each) by themselves had no demonstrable effects on ACC activity under similar experimental conditions (additional data not shown). ACC activity was then measured in the HIT cell cytosolic fraction in the presence of a fixed concentration of magnesium (5 mmol/l) but with varying concentrations of glutamate (0–100 mmol/l). These studies indicated a concentration-dependent stimulation of magnesium-sensitive ACC activity by glutamate (Fig. 2*B*). A broad-range concentration curve showed that the degree of activation by succinate was similar at 50 mol/l (1.4-fold) and 100 mmol/l (1.7-fold; additional data not shown). Together, data from these studies (Figs. 1 and 2) indicate that glutamate and magnesium specifically stimulate ACC activity.

Subcellular distribution of glutamate and magnesium-stimulated ACC: identification of a membrane-associated inhibitor of the cytosolic GAPP. The above data suggested that a glutamate and magnesium-sensitive ACC regulatory factor or enzyme is present in insulin-secreting cells. In subsequent studies, we investigated the subcellular localization of such a putative factor/protein. To address this, HIT cell homogenates were subjected to a differential centrifugation (see RESEARCH DESIGN AND METHODS). The magnesium- and glutamate-activated ACC activity was then assayed in homogenates, the nuclear pellet, the postnuclear pellet, and the cytosolic fraction (Fig. 3). The resulting data suggested that the magnesium- and glutamate-stimulated ACC activity is predominantly localized in the cytosolic fraction. In reconstitution assays, we observed a membrane protein concentration–dependent (0–20 μg protein per assay) inhibition of the cytosolic glutamate- and magnesium-activated ACC. Maximal inhibition was demonstrable at 10 μg membrane protein (Fig. 4), indicating that a membrane-associated ACC inhibitory factor/protein may be present in insulin-secreting cells.

We then examined whether this factor is resistant to heat treatment (95° for 10 min) and observed that heat treatment significantly augmented the ability of this putative membrane-associated factor to inhibit ACC activity. For example, a 75 ± 8% ($n = 6$ determinations) inhibition of the glutamate- and magnesium-mediated activation of ACC was demonstrable in the presence of heat-treated membrane fraction, in contrast to the 41 ± 2% ($n = 6$ determinations) inhibition observed in the presence of native membrane protein. Next, we determined the sensitivity of this factor to trypsin (see RESEARCH DESIGN AND METHODS) and observed that trypsin treatment of the membrane fraction markedly attenuated its inhibitory potency (36 ± 8% inhibition in the presence of native membrane vs. 68 ± 18% in the presence of trypsin-treated membrane; $P < 0.001$; $n = 6$ determinations in each case; additional data not shown). Together, these data suggest localization of a heat-resistant (and even heat-activated) trypsin-sensitive membrane-associated inhibitor of glutamate- and magnesium-activated ACC phosphatase in the pancreatic β-cell.

Magnesium- and glutamate-sensitive ACC activating factor is akin to PP2A. We then investigated whether the

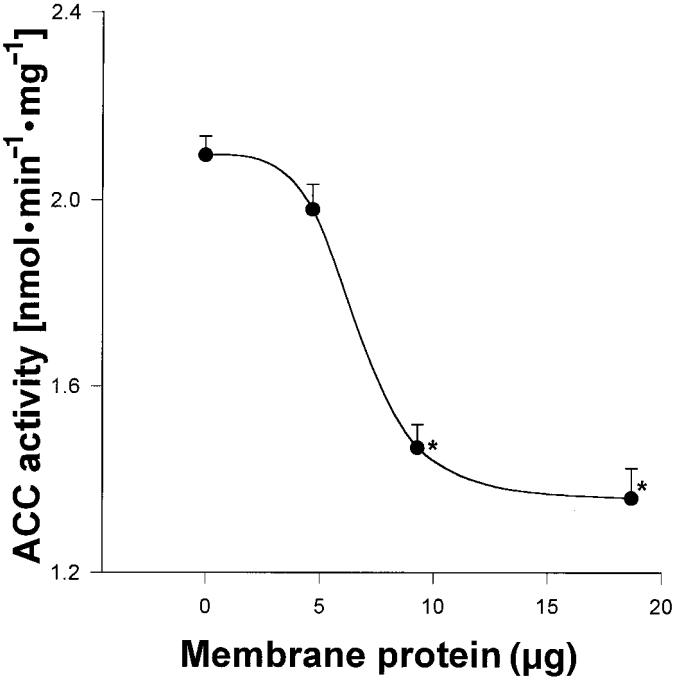

FIG. 4. Inhibition by particulate fraction of glutamate- and magnesium-stimulated ACC activity in HIT cell cytosol. Glutamate- and magnesium-activated ACC activity was quantitated in the HIT cell cytosolic fraction in the presence of increasing concentrations of HIT cell particulate fraction, as indicated in the text. Data are means ± SE from a representative of three experiments carried out in triplicate. *$P < 0.05$ vs. control (i.e., in the presence of no membrane added).

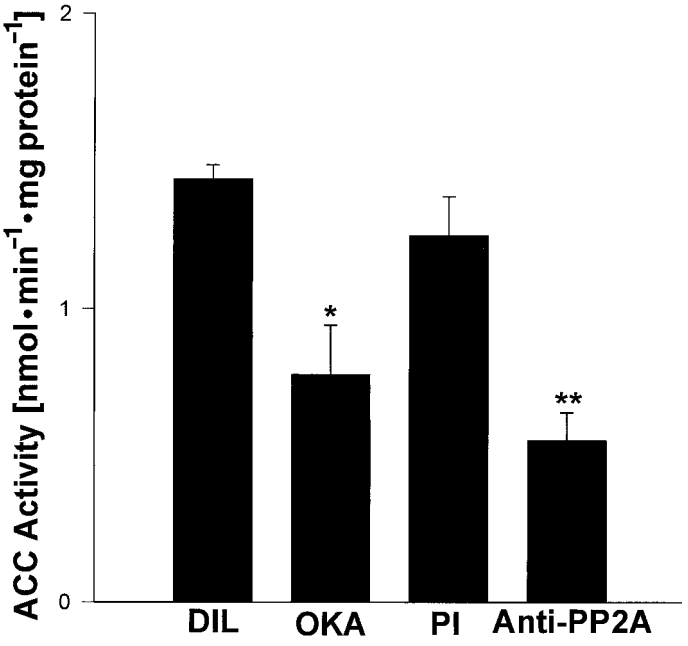

FIG. 5. Magnesium- and glutamate-activated factor may be akin to PP2A. INS cell cytosolic fraction was preincubated with OKA (10 nmol/l for 15 min) or an antiserum directed against PP2Ac (1:500 dilution overnight at 4°C) before assaying ACC activity in the presence of glutamate and magnesium, as described in the text. Glutamate- and magnesium-activated ACC activity was quantitated in these preparations as described in RESEARCH DESIGN AND METHODS. Data are means ± SE from three experiments carried out in triplicate. *P < 0.01 vs. diluent alone; **P < 0.01 vs. activity demonstrable in the presence of preimmune serum alone. DIL, diluent; PI, preimmune serum; Anti-PP2A, treated with an antiserum directed against PP2A.

magnesium and glutamate stimulation of ACC activity is attributable to activation of ACC via dephosphorylation mediated by a protein phosphatase. Localization of such a phosphatase has recently been described in hepatocytes (referred to as GAPP) (10). To verify this, the INS cell cytosolic fraction was preincubated with either OKA (10 nmol/l for 15 min), a known inhibitor of PP2A, or an antiserum directed against PP2Ac (1:500 dilution, overnight at 4°C) before assaying ACC activity in the presence of magnesium and glutamate. We observed that the ability of glutamate and magnesium to stimulate ACC activity was markedly reduced in the cytosolic fractions treated with either the PP2Ac antiserum (−57%) or OKA (−48%) (Fig. 5). These data provide additional evidence to suggest that glutamate- and magnesium-stimulated ACC activation might involve dephosphorylation of ACC catalyzed by an OKA-sensitive PP2A-like enzyme. To test this further, the effects of glutamate and magnesium on the dephosphorylation of ACC were determined, as shown below.

Glutamate- and magnesium-mediated dephosphorylation of ACC in insulin-secreting cells. Two isoforms of ACC (ACCα [265 kDa] and ACCβ [280 kDa]) have been shown to undergo phosphorylation in multiple cell types. Extant studies have demonstrated that ACCα is the predominant isoform in insulin-secreting cells (13,14), even though ACCβ has been localized in HIT-T15 cells (12). To isolate ACC from cell lysates, we used streptavidin-agarose chromatography because ACC is a biotinylated protein and binds to streptavidin-agarose (12,19). In the first set of studies, INS cell lysates were absorbed onto streptavidin-agarose; after extensive washing, bound proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with an antiserum raised against the NH_2-terminal 15 amino acids of ACCα. Data in Fig. 6*A* show that the bound protein is immunoreactive to this antiserum, indicating that the 265-kDa protein represents ACCα. Similar data were obtained using an antiserum directed against the COOH-terminal 15 amino acids of ACCα (data not shown). In subsequent studies, HIT cell lysate proteins were phosphorylated using [γ-^{32}P]ATP, and the phosphorylated proteins were absorbed onto streptavidin-agarose. After extensive washing, bound proteins were eluted with biotin (see RESEARCH DESIGN AND METHODS), separated by SDS-PAGE, and identified by phosphoimaging. The data in Fig. 6*B* indicate that two HIT cell phosphoproteins with apparent molecular weights of 265 and 280 kDa bound to streptavidin. These data confirm localization of both the α and β isoforms of ACC in HIT cells (12). Furthermore, magnesium and glutamate markedly promoted the dephosphorylation of each of these proteins. In addition, quantitation of the radioactivity in biotin eluates (Fig. 6*C*) demonstrated a >60% reduction in the radiolabeling of ACC isoforms in the presence of glutamate and magnesium. Thus, the data in Fig. 6 clearly demonstrate magnesium- and glutamate-mediated dephosphorylation and activation of ACC in the islet β-cell.

Lack of effect of glutamate and magnesium on ACC kinase activity. To examine the possibility that magnesium and glutamate inhibit the phosphorylation of ACC in addition to stimulating its dephosphorylation, we investigated the effects of magnesium and glutamate on AMP kinase activity, which phosphorylates and inactivates ACC (15). As indicated in Fig. 7, no significant effects of glutamate and magnesium on AMP kinase activity were demonstrable at all time points of enzyme activity determination (0–10 min). Together, these findings suggest that the stimulatory effects of glutamate and magnesium on ACC activity are primarily attributable to activation of ACC phosphatase activity rather than inhibition of its kinase activity.

Glucose-stimulated ACC activation and subsequent insulin secretion are inhibited by AICA riboside. To examine a possible relationship between ACC activation and insulin secretion, we studied the effects of stimulatory concentrations of glucose on ACC activity and insulin secretion in INS-1 cells. In these experiments we also included AICA riboside, which inhibits ACC activity by stimulating AMP kinase (9,15). Data in Fig. 8*A* show that glucose-induced ACC activity in INS-1 cells was markedly reduced by the addition of 1 mmol/l AICA riboside to the incubation medium. In companion experiments, we also observed that glucose-stimulated insulin secretion was significantly attenuated in the presence of AICA riboside (Fig. 8*B*). These data show that inhibition of ACC activation results in the inhibition of glucose-stimulated insulin secretion, thus establishing a possible link between glucose-mediated ACC activation and insulin secretion; these results are compatible with those from extant studies (13–15) (see DISCUSSION).

DISCUSSION

The current studies were designed to determine whether ACC activity is acutely regulated by phosphorylation-dephosphorylation in the pancreatic β-cell and, if so, to

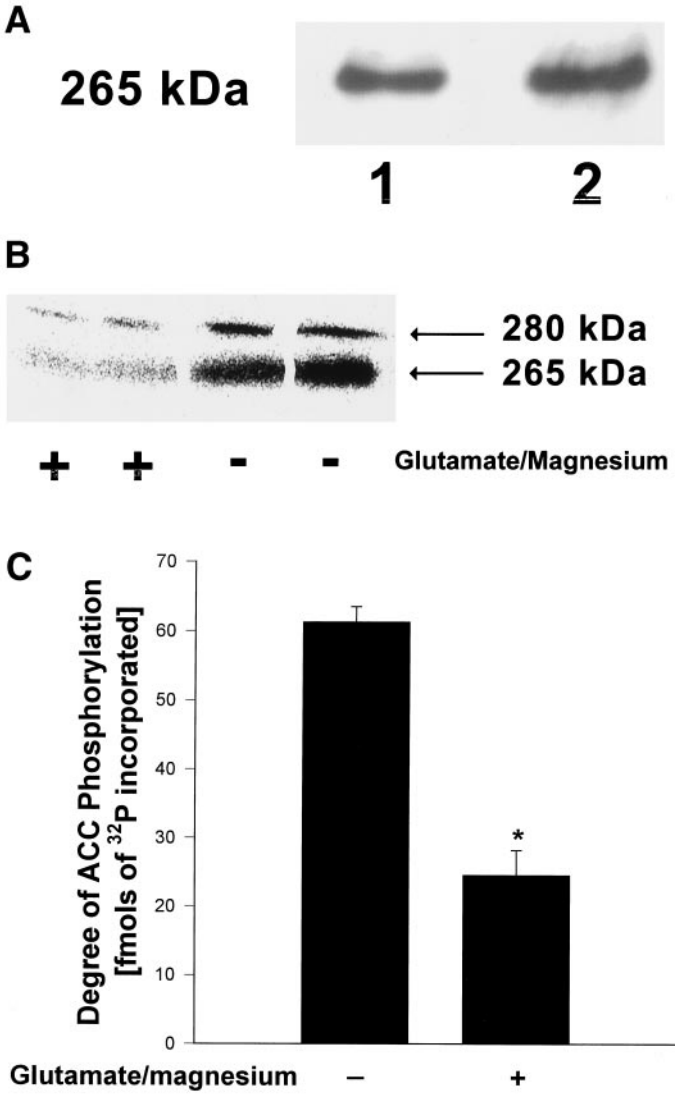

FIG. 6. Dephosphorylation by magnesium and glutamate of ACC in HIT and INS cells. *A*: INS cell cytosolic proteins were applied to streptavidin-agarose column (see RESEARCH DESIGN AND METHODS). After extensive washing, bound proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, which was then probed using an antiserum directed against ACCα (265 kDa). Data are representative of three individual preparations of INS cell lysates carried out in duplicate (marked as 1 and 2 in the figure). *B*: HIT cell cytosolic proteins were phosphorylated in the presence of [γ-^{32}P]ATP and in the absence or presence of 10 mmol/l magnesium and 50 mmol/l glutamate, as indicated. After this, labeled proteins were incubated with streptavidin-agarose, and unbound proteins were removed by extensive washing using column buffer (see RESEARCH DESIGN AND METHODS). Bound, biotinylated proteins were dissociated from streptavidin-agarose matrix using column buffer containing biotin (2 mmol/l); eluted proteins were separated by SDS-PAGE (5% acrylamide), and labeling intensities of phosphorylated proteins were detected by phosphoimaging (see RESEARCH DESIGN AND METHODS). These data demonstrate labeling of two 265- and 280-kDa molecular weight proteins, representing ACCα and ACCβ. Furthermore, a marked reduction in the labeling of these proteins in the presence of glutamate and magnesium was demonstrable. Data are representative of three individual preparations of HIT cell lysates carried out in duplicate. *C*: HIT cell cytosolic proteins were phosphorylated using [γ-^{32}P]ATP in the absence or presence of glutamate (50 mmol/l) and magnesium (10 mmol/l), as in *B*. After this, labeled proteins were incubated with streptavidin-agarose, and unbound proteins were removed by extensive washing using column buffer (see RESEARCH DESIGN AND METHODS). Bound, biotinylated proteins were dissociated from streptavidin-agarose matrix using column buffer containing biotin (2 mmol/l), and radioactivity in the eluates was quantitated by scintillation spectrometry. Data are means ±SE from eight determinations in each case. *P < 0.001 vs. degree of phosphorylation demonstrable in the control.

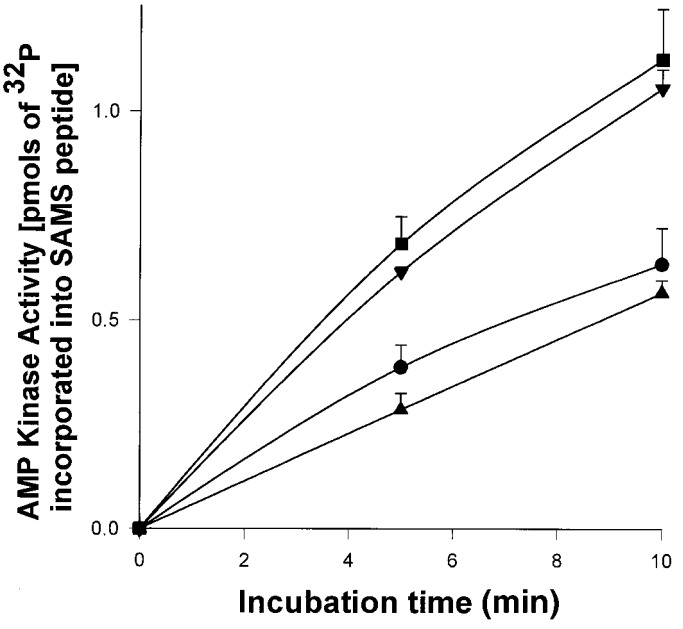

FIG. 7. Lack of effects magnesium and glutamate on AMP kinase activity in insulin-secreting cell. AMP kinase activity was assayed in INS cell cytosolic fraction using SAMS peptide as the substrate in the presence of [γ-^{32}P]ATP as phosphoryl donor and AMP (200 μmol/l) as a function of time, as indicated in the figure. A mixture of glutamate (50 mmol/l) and magnesium (10 mmol/l) was also included in the assays, as indicated. Data are representative of three individual experiments. ●, diluent alone; ■, +AMP; ▲, +glutamate/magnesium; ▼, +glutamate/magnesium plus AMP.

determine the relevance of these findings to physiological insulin secretion. Our data suggested the following: *1*) physiological concentrations of glutamate and magnesium activate ACC in lysates of clonal β-cells and in normal rat islets and human islets; *2*) such activation is significantly attenuated by low nanomolar concentrations of OKA or by pretreatment of cell lysates with anti-PP2A; *3*) glutamate and magnesium stimulate the dephosphorylation of ACC, with no discernable effects on AMP kinase activity; and *4*) AICA riboside, an activator of AMP kinase, significantly reduced glucose-stimulated ACC activity and concomitant insulin secretion from isolated β-cells.

Several studies have been devoted to the identification and quantitation of protein kinases and phosphatases in pancreatic β-cells (3,4). Indeed, the availability of specific inhibitors, such as OKA, has permitted examination of the roles of these phosphatases in β-cell signaling. Recent studies from our laboratory have suggested multiple mechanisms for the regulation of protein phosphatases in the pancreatic islet β-cell. These include regulation by ceramides (24) and by methylation of its COOH-terminal leucine (7,23). The current studies identify an additional regulatory mechanism for protein phosphatases in the islet β-cell: localization of a protein phosphatase that belongs to the PP2A subfamily of protein phosphatases. Also, the glutamate- and magnesium-stimulated ACC phosphatase that we have identified in the β-cell bears several similarities with the hepatocyte enzyme that has been described in the literature (10). Both of these activities *1*) are predominantly cytosolic, *2*) are specifically stimulated by magnesium and glutamate, *3*) are inhibited by OKA and by an antiserum directed against the catalytic subunit of PP2A, *4*) mediate the dephosphorylation of the α subunit

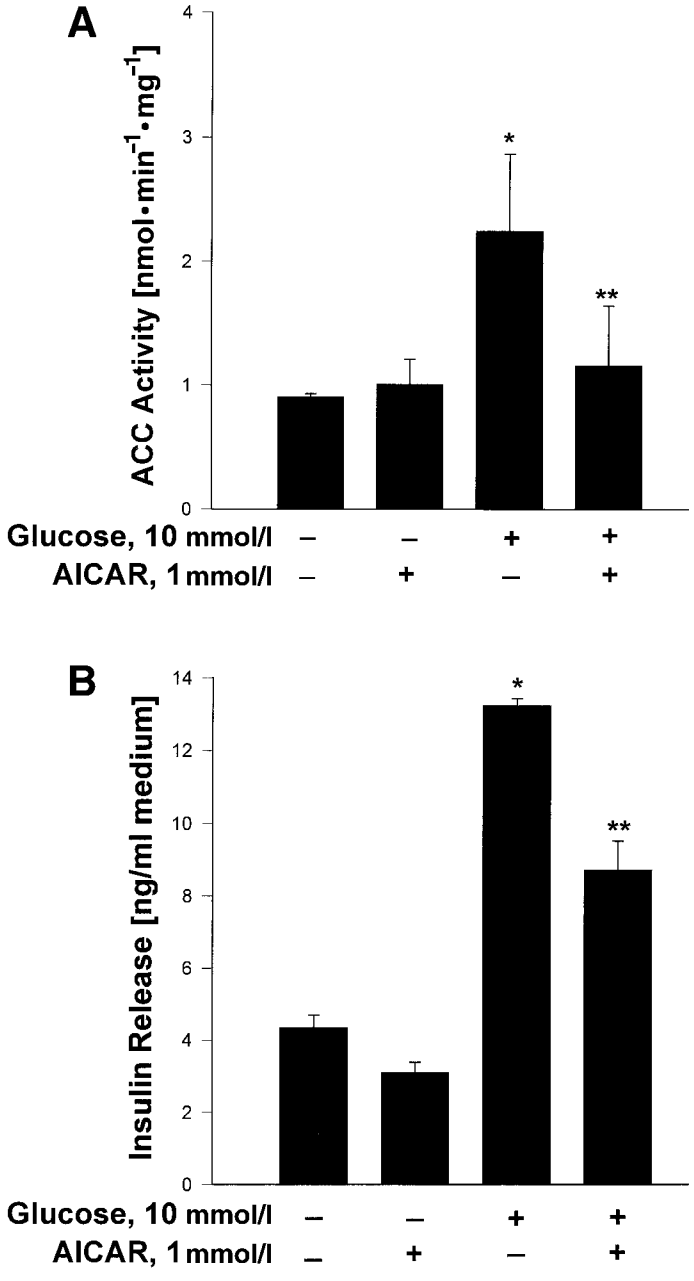

FIG. 8. Inhibition by AICA riboside of glucose-induced ACC activity (*A*) and insulin secretion (*B*) from INS-1 cells. *A*: INS cells were incubated in the presence of 0 or 10 mmol/l glucose for 45 min. Cells were lysed quickly and ACC activity was measured as indicated in the text. Data are means ± SE from three experiments carried out in triplicate. **P* < 0.001 vs. control; ***P* < 0.01 vs. activity demonstrable in the presence of glucose (10 mmol/l) alone. *B*: INS cells were incubated in the presence of 0 or 10 mmol/l glucose for 45 min, as described above. Insulin released into the incubation medium was quantitated by enzyme-linked immunosorbent assay (see RESEARCH DESIGN AND METHODS). Data are means ± SE from three experiments. **P* < 0.0001 vs. no glucose; ***P* < 0.05 vs. release demonstrable in the presence of 10 mmol/l glucose. AICAR, AICA riboside.

of ACC (current study) or a synthetic peptide corresponding to the phosphorylation site of ACC, and *5*) mediate the dephosphorylation and activation of ACC. Along similar lines, recent studies by Vavvas et al. (25) have demonstrated a similar magnesium- and glutamate-sensitive PP2A-like enzyme that mediates regulation of ACC activation in the skeletal muscle. Our current data in insulin-secreting cells, taken together with extant data in hepatocytes and skeletal muscle, clearly identify GAPP as one of the signaling proteins involved in the acute regulation of ACC.

Our data also suggest that the β-cell GAPP is significantly inhibited by a heat-activated trypsin-sensitive protein/peptide localized in the total particulate fraction of the pancreatic β-cell. Several extant studies have demonstrated, however, that PP2A is resistant to the inhibitory effects of heat-resistant inhibitors 1 and 2 (5,6). Therefore, it is likely that this putative factor/protein could represent an as-yet-unidentified ACC regulatory factor in the β-cell. Such regulatory factors of ACC have been reported in other cell types as well. For example, Witters et al. (26) have identified a heat-stable activator of ACC in rat liver extracts that was later identified as a factor similar to 5′-GMP. Based on its sensitivity to trypsin, we concluded that this putative membrane-associated factor identified in our current studies represents a peptide/protein. It remains to be seen whether the activity of this peptide/protein is modulated by insulin secretagogues in the β-cell, thereby contributing to physiological control of insulin secretion.

Our data confirm earlier studies with respect to the localization of different isoforms of ACC insulin-secreting cells (12–14). ACCα is localized in INS cells (13,14), whereas both the α and β forms of ACC are present in HIT-T15 cell lysates (12). We also report phosphorylation of each of these isoforms in HIT cells, the dephosphorylation of which was induced by magnesium and glutamate. Even though our current data implicate a GAPP-mediated activation of ACC, we do not rule out other effects of glutamate that are independent of its effects on ACC-phosphatase. For example, recent studies of Boone et al. (27) have identified additional regulatory mechanisms for glutamate-induced activation of ACC. Using purified preparations of ACC, these investigators demonstrated that glutamate also stimulates the polymerization of ACC. These data clearly indicate direct effects of glutamate (i.e., as an allosteric ligand for dephosphorylated ACC) on ACC.

We also demonstrated that AICA riboside, a stimulator of AMP kinase, inhibits glucose-stimulated ACC activation and insulin secretion. These data are compatible with the observations of Zhang and Kim (13,14). More recent studies by Salt et al. (15) have also demonstrated AICA riboside–induced inhibition of glucose-stimulated insulin secretion from INS-1 cells and isolated rat islets. Interestingly, those researchers observed no inhibition by AICA riboside of glucose-induced insulin secretion from HIT-T15 cells, presumably because ZMP (the monophosphate of AICA riboside) is metabolized much more rapidly in HIT cells compared with INS-1 cells and normal rat islets (15).

Lastly, in the context of a putative role for glutamate in physiological insulin secretion, recent studies by Maechler and Wollheim (28) have suggested a second messenger role for glutamate in glucose-induced insulin secretion. These investigators provided experimental evidence to suggest generation of intramitochondrial glutamate by glucose. However, MacDonald and Fahien (29) recently disputed such a possibility. Clearly, additional studies are needed to further define the precise roles of glutamate in physiological release of insulin from the islet β-cell, especially those pathways involving activation of the β-cell GAPP.

In summary, we report localization of a magnesium- and glutamate-sensitive protein phosphatase in insulin-secret-

ing cells that dephosphorylates and activates ACC. This phosphatase appears to be similar to PP2A. Our findings provide evidence for a unique mechanism for the acute regulation of ACC in the pancreatic β-cell, leading to the generation of physiological signals that may be needed for physiological insulin secretion.

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