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Insulin Secretion Is Controlled by mGlu5 Metabotropic Glutamate Receptors

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ABSTRACT

Recent evidence suggests that metabotropic glutamate (mGlu) receptors are involved in the regulation of hormone secretion in the endocrine pancreas. We report here that endogenous activation of mGlu5 receptors is required for an optimal insulin response to glucose both in clonal β-cells and in mice. In clonal β-cells, mGlu5 receptors were expressed at the cell surface and were also found in purified insulin-containing granules. These cells did not respond to a battery of mGlu5 receptor agonists that act extracellularly, but instead responded to a cell-permeant analog of glutamate with an increase in $[Ca^{2+}]_i$ and insulin secretion. Both effects were largely attenuated by the mGlu5 receptor antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP). MPEP and its structural analog, (*E*)-2-methyl-6-styryl-pyridine (SIB-1893), reduced the increase in $[Ca^{2+}]_i$ and insulin secretion induced by glucose in clonal β-cells, whereas a mGlu1 receptor antagonist was inactive. mGlu5 knockout mice showed a defective insulin response at all times after a glucose pulse (1.5 g/kg, i.p.), whereas wild-type mice treated with MPEP (10 mg/kg, i.p.) showed a selective impairment in the late phase of insulin secretion in response to glucose challenge. Mice injected with MPEP or lacking mGlu5 receptors also showed a blunted glucagon response to an insulin challenge. We conclude that insulin secretion is under the control of mGlu5 receptors both in clonal β-cells and in vivo. Drugs that modulate the function of mGlu5 receptors might affect glucose homeostasis by altering the secretion of pancreatic hormones.

A growing body of evidence suggests that glutamate, the major excitatory neurotransmitter in the central nervous system, acts as a signaling molecule in peripheral tissues (Gill and Pulido, 2001; Skerry and Genever, 2001; Hinoi et al., 2004). In α- and β-cells of the endocrine pancreas, glutamate is stored in glucagon- or insulin-containing granules (Yamada et al., 2001; Hoy et al., 2002; Hayashi et al., 2003) and, once secreted, might act extracellularly to regulate hormone secretion (Moriyama and Hayashi, 2003; Uehara et al., 2004). In addition, glutamate has been implicated as a putative intracellular messenger coupling glucose metabolism to insulin secretion in β-cells (Maechler and Wollheim, 1999; MacDonald and Fahien, 2000; Hoy et al., 2002; Maechler et al., 2002). The molecular mechanisms underlying the action of glutamate in the endocrine pancreas are only partially elucidated. Glutamate receptors can either form membrane ion channels (AMPA, NMDA, or kainate receptors), or couple to G proteins ("metabotropic glutamate receptors" or mGlu receptors). The mGlu receptor family includes eight subtypes subdivided into three groups based on their sequence homology, pharmacological profile, and transduction pathways. Group I includes mGlu1 and mGlu5 receptors, which are coupled to G_q proteins; their activation stimulates polyphosphoinositide hydrolysis into inositol 1,4,5-trisphosphate and diacylglycerol, with ensuing release of intracellular calcium and activation of protein kinase C; group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7, and mGlu8)

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; SIB-1893, (*E*)-2-methyl-6-styryl-pyridine; DHPG, (*S*)-3,5-dixydroxyphenylglycine; CPCCOEt, 7-hydroxyiminocyclopropan[*b*]-chromen-1*a*-carboxylic acid ethyl ester; DMEM, Dulbecco's modified Eagle's medium, RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; AM, acetoxymethyl ester; PI-3-K, phosphatidylinositol-3-kinase; SIB-1757, 6-methyl-2-(phenylazo)-3-pyridinol; MK-801, 5*H*-dibenzo[*a*,*d*]cycloheptene-5,10-imine (dizocilpine maleate); GAD, glutamate decarboxylase.

receptors are coupled to G_i proteins in heterologous expression systems (Pin and Duvoisin, 1995; De Blasi et al., 2001). AMPA receptors are expressed by α- and β-cells of the islet of Langerhans, and their activation stimulates insulin secretion (Gonoi et al., 1994; Inagaki et al., 1995; Weaver et al., 1996). In addition, both AMPA and kainate—but not NMDA—stimulate insulin and glucagon secretion from perfused pancreas (Bertrand et al., 1992, 1993). A role for mGlu receptors in the physiology of pancreatic endocrine cells is now emerging but is still unclear. Brice et al. (2002) could detect the expression of mGlu3 and mGlu5 receptors in clonal α- and β-cells and the expression of mGlu3, mGlu4, mGlu5, and mGlu8 receptors in pacreatic islets. They also showed that pharmacological activation of group I and II mGlu receptors enhances, whereas activation of group III mGlu receptors reduces, glucose-stimulated insulin secretion from clonal β-cells. More recently, Uehara et al. (2004) reported that mGlu2/3 and mGlu5 receptor antibodies stain synaptic terminals and blood vessels in the pancreas, but not islet cells. These authors found that α- and β-pancreatic cells express mGlu4 receptors, the activation of which inhibits glucagon secretion by lowering cAMP formation (Uehara et al., 2004). In apparent contrast with these findings, Tong et al. (2002) found that the only group III receptor expressed by rat pancreatic islets is the mGlu8 receptor, which negatively regulates glucagon secretion. Further studies are clearly required to unravel the role of pancreatic mGlu receptors in the regulation of insulin or glucagon secretion. Moving from the original article by Brice et al. (2002), we now report that 1) clonal pancreatic β-cells express mGlu5 receptors, 2) these receptors are found not only in the cell membrane, but also in purified insulin-secreting granules, 3) *endogenous* activation of mGlu5 receptors is permissive to glucose-stimulated intracellular Ca^{2+} release and insulin secretion, and 4) mice lacking mGlu5 receptors or treated with a selective mGlu5 receptor antagonist show a blunted insulin response to glucose.

Materials and Methods

Material. 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), [^{3}H]MPEP (specific activity, 25 Ci/mmol), SIB-1893, (*S*)-3,5-dixydroxyphenylglycine (DHPG), 7-hydroxyiminocyclopropan[*b*]chromen-1*a*-carboxylic acid ethyl ester (CPCCOEt), and quisqualate were purchased from Tocris Cookson Ltd (Bristol, UK); The rat insulin-^{125}I Biotrak Assay kit was from Amersham Pharmacia (Milano, Italy). The human glucagon-^{125}I radioimmunoassay kit was from Pantec (Torino, Italy). Fluo4-acetoxymethyl ester (AM) was from Molecular Probes (Burlington, ON, Canada). All other drugs and chemicals were purchased from Sigma (Milano, Italy). Our studies involving animal experimentation were approved by the Italian Ministry of Health following the criteria of the Italian National Research Council.

Cell Cultures. MIN6 cells were furnished by one of the authors (G.Y.R.). βTC-6 cells were purchased from the American Type Culture Collection (Manassas, VA), and all studies were performed with cultures at 30 to 35 passages. Cells were cultured in DMEM containing 15% fetal calf serum, 4.5 g/l glucose, 1.5 g/l $NaHCO_3$, 2 mM glutamine, 100 U/ml penicillin, and 0.05 mg/ml streptomycin.

RT-PCR and Western Blot Analysis. RT-PCR analysis of mGlu receptor mRNA was carried out as described previously (Storto et al., 2000a). Western blot analysis (Storto et al., 2000b) was performed using the following antibodies: anti-mGlu1a, anti-mGlu2/3 and anti-mGlu5 receptors, anti-insulin receptor (β-subunit), anti-IRS-1 (polyclonal, 1 μg/ml; Upstate Biotechnology, Charlottesville, VA); anti-phospho-Akt and anti-Akt (polyclonal, 1:1000, Cell Signaling Technology Inc. (Beverly, MA); anti-vGLUT1 and -2 (monoclonal, 1:1000; Synaptic Systems (Goettingen, Germany). Immunostaining was revealed by ECL (Amersham).

Preparation of Secretory Vesicles. Dense core secretory vesicles from MIN6 cells were prepared after infection with phogrin. Enhanced green fluorescent protein-expressing adenovirus, and immunoisolation using anti-GFP antibodies were performed essentially as described previously (Varadi et al., 2002).

Confocal Analysis of mGlu5 Receptors in Insulinoma Cells. βTC-6 and MIN6 cells were grown for 3 days onto chamber slides and then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The autofluorescence was quenched by incubation for 30 min in 50 mM NH_4Cl, 50 mM glycine in PBS, and nonspecific interactions were prevented by treatment with blocking solution (0.05% saponin and 0.5% BSA in PBS) for 30 min at room temperature. Cells were incubated with anti-mGlu5 receptor antibody (1:600), overnight at 4°C in blocking solution. The chamber slides were then incubated with blocking solution containing Cy3-conjugated anti-rabbit (1:800; Sigma) for 1 h at room temperature. Each incubation step was carried out in the dark and followed by careful washes with PBS (three times for 3 min each). After immunostaining, the coverslips were mounted on slides with Mowiol 4-88, and observed with an UltraView Confocal Imaging System (PerkinElmer Life and Analytical Sciences, Boston, MA). Ten groups of optical *Z*-section serial slices from each experiment were taken with 1-μm *Z*-steps from the top to the bottom of the specimen.

Real-Time Confocal Analysis of Intracellular Ca^{2+}. βTC-6 and MIN6 cells were cultured in glass-bottomed culture dishes (MaTek Corp, Ashland, MA) in DMEM, washed two times with PBS, loaded with 5 μM fluo4-AM in Krebs buffer containing 3 mM glucose for 30 min in the dark at 37°C. Cells were washed two times with Krebs buffer and maintained at 37°C in a temperature-controlled perfusion chamber (PerkinElmer Life and Analytical Sciences) placed on the stage of an inverted microscope (Nikon, Tokyo, Japan). Glucose (25 mM) or mGlu receptor agonists were added to the cells through the perfusion chamber; when present, MPEP was added 10 min before glucose or L-glutamate γ-methyl ester. Confocal images of $[Ca^{2+}]$ were acquired with the Ultraview analysis system through a 60× oil immersion objective (Nikon) with an excitation beam (488 nm) produced by an argon ion laser tube and transferred to a videotape recorder. The release of intracellular calcium was recorded as frames of change in fluorescence images (from green to red) of 256 × 256 pixels, every 10 s for 500 s. Calcium concentration was estimated according to the in vivo calibration method proposed by Kao et al. (1989) using the following equation: $[Ca^{2+}]_i = K_d\,(F - F_{min})/(F_{max} - F)$, where F_{min} is the fluorescence in presence of EGTA and F_{max} is the maximum fluorescence obtained with 10 μM ionomycin and 10 mM $CaCl_2$; K_d is the dissociation constant of Fluo4-AM (345 nM).

Measurement of [^{3}H]MPEP Binding in Intact βTC-6 Cells. Cells cultured in 24-well plates were incubated in Locke's solution, pH 7.4, for 30 min. [^{3}H]MPEP was added and cultures were additionally incubated for the indicated times either at 4°C or at 37°C. Nonspecific binding was determined in the presence of 300 μM MPEP. Incubations were terminated by three rapid washings in ice-cold buffer, and cells were then left in 0.5 N NaOH at 37°C for 0.5 to 1 h. Radioactivity in cell lysates was measured by scintillation spectrometry. Concentrations ranging from 0.26 to 30 nM [^{3}H]MPEP were used for the determination of the maximal density (B_{max}) and the K_d in cultures incubated at 4°C. For association experiments, cultures were incubated with 3 nM [^{3}H]MPEP for 0.5 to 45 min. For dissociation experiments, nonradioactive MPEP (300 μM) was added after a 10-min labeling with 3 nM [^{3}H]MPEP and incubations were terminated after additional 0.5 to 15 min.

Measurement of Extracellular Glutamate. Analysis of glutamate was performed by precolumn derivatization with *o*-phthalaldehyde and mercaptoethanol followed by high-performance liquid chromatography with fluorescence detection as described previously (Storto et al., 2000b).

Measurements of Insulin Secretion in Cell Cultures. MIN6 and βTC-6 cells were maintained for 2 h in DMEM glucose-free medium, washed, and preincubated in Krebs buffer containing 3 mM glucose for 30 min, and then incubated in presence of glucose, L-glutamate, MPEP, SIB-1893, CPCCOEt, DHPG, quisqualate, or L-glutamic acid γ-methyl ester for additional 30 min at 37°C. When present, mGlu receptor antagonists were applied 10 min before glucose or L-glutamic acid γ-methyl ester. The amount of insulin secreted extracellularly was assayed using the insulin-^{125}I rat Biotrak Assay kit (Amersham).

In Vivo Studies. Glucose-stimulated insulin secretion was studied in wild-type (+/+), heterozygous (+/−), and mGlu5 knockout (−/−) mice. Heterozygous mGlu5 receptor knockout mice (129-Gprc1e^{tmt1rod}) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice heterozygous for the targeted mutation were intercrossed to homozygosity. Thus, all mice (+/+, +/−, and −/−) were generated by heterozygous breeding and +/+ mice were used as controls. Mice were kept under environmentally controlled conditions (ambient temperature, 22°C; humidity, 40%) on a 12-h light/dark cycle with food and water ad libitum. Mice were identified by PCR analysis on tail samples after birth. Primers for the genotyping of knockout mice were from The Jackson Laboratory. The absence of mGlu5 receptors in knockout mice was confirmed by Western blot analysis carried out in the cerebral cortex when they were killed. Wild-type, heterozygous, and mGlu5 knockout mice were deprived of food for 12 h and then injected with glucose (1.5 g/kg, i.p.). MPEP (10 mg/kg, i.p.) was injected 10 min before glucose. All drugs were dissolved in saline. Blood samples were collected at different times after glucose injection in tubes containing 40 mM EDTA. Samples were centrifuged at 2000*g* for 5 min at 4°C and plasma was used for measurements of insulin plasma levels by the Biotrak Assay kit (Amersham). Total blood was used for the determination of glucose levels. Additional groups of mice were also deprived of food for 12 h and then challenged i.p. with 5 IU/kg human insulin with or without MPEP (10 mg/kg, i.p., injected 10 min before insulin) for the assessment of plasma glucagon levels measured by radioimmunoassay kit (Pantec) and plasma glucose levels. Wild-type and mGlu5 knockout mice not injected with glucose or MPEP were also used for the assessment of insulin receptors (β-subunit) and IRS-1 in the liver, fat tissue, and skeletal muscles.

Isolation of Hepatocytes and Assessment of Insulin-Stimulated Phosphatidylinositol-3-kinase Pathway. Hepatocytes were isolated from the liver of wild-type or mGlu5 knockout mice as described previously (Krause et al., 2002) and then incubated in suspension in a Ringer-HEPES buffer. Cells were stimulated for 2 to 15 min with 100 μM bovine insulin (Sigma). Activation of the PI-3-K pathway was assessed by measuring the levels of phospho-Akt by immunoblotting (see above).

Results

Western blot analysis of mGlu5 receptors showed two immunoreactive bands at 135 to 145 kDa, of which the upper one corresponded to the receptor monomer because it was absent in the cerebral cortex of mGlu5−/− mice (Fig. 1A). We could detect the expression of mGlu5 receptors in the rat or mouse pancreas, as well as in βTC-6 and MIN6 insulinoma cells, where mGlu1 and mGlu2/3 receptors were absent (Fig. 1A, and data not shown). The mGlu5 receptor was not present in the pancreas of mGlu5−/− mice (Fig. 1A), supporting the specificity of immunoblots. RT-PCR analysis showed the expression of mGlu5, but not mGlu1, mRNA in insulinoma cells (Fig. 1B). Confocal analysis showed that the mGlu5 receptor was mainly localized both at the cell surface of βTC-6 and MIN6 cells under "resting" conditions; agonist exposure was followed by a rapid and transient internalization of surface mGlu5 receptors (Fig. 1D). It is noteworthy that we could also detect the mGlu5 receptor in purified preparations of insulin-containing granules (Fig. 1A), which also expressed the selective vesicular glutamate transporters v-Glut1 and v-Glut2 (Fig. 1C).

To examine whether endogenous activation of mGlu5 receptors was involved in the β cell's response to glucose, we used the drug MPEP, which behaves as a negative allosteric modulator of mGlu5 receptors (Gasparini et al., 1999; Pagano et al., 2000). [^{3}H]MPEP binding to intact βTC-6 cells incubated at 4°C revealed a single population of recognition sites with low nanomolar affinity (Fig. 2A). These sites may correspond to surface mGlu5 receptors. Specifically bound [^{3}H]MPEP was displaced by SIB-1757 and SIB-1893, which behave as negative allosteric modulators of mGlu5 receptors (Varney et al., 1999), but not by the NMDA channel blocker MK-801 (Fig. 2B). [^{3}H]MPEP binding in intact βTC-6 cells was influenced by the temperature. Nonspecific binding was 30 to 35% at 4°C, but as high as 65 to 70% at 37°C (see Fig. 2C). This suggests that a large amount of [^{3}H]MPEP either binds nonspecifically to the plasma membrane or diffuses inside the cells at physiological temperature. Binding association was rapid, reaching saturation after 10 to 15 min at both 4° and 37°C (not shown). Dissociation at 4°C was nearly maximal after 1 min and was slower at 37°C (maximal dissociation after 5 min) (Fig. 2C). Exposure of βTC-6 and MIN6 cells to 25 mM glucose raised $[Ca^{2+}]_i$ by more than 4-fold, as assessed by real-time confocal microscopy. This increase was markedly reduced by MPEP (1 μM) (Fig. 3, A and B), which also lowered insulin secretion in response to 25 mM glucose (Fig. 3C). The action of MPEP was mimicked by the noncompetitive mGlu5 receptor antagonist SIB-1893 (10 μM) (Fig. 3C), but not by the noncompetitive mGlu1 receptor antagonist CPCCOEt (10 μM; data not shown). We also tested responses to a number of mGlu5 receptor agonists including L-glutamate (0.2–10 mM), quisqualate (100 μM), and 3,5-dihydroxyphenylglycine (100 μM). None of these drugs had any effect on $[Ca^{2+}]_i$ and insulin secretion when applied alone or in the presence of 25 mM glucose (data not shown). In contrast, the cell-permeant glutamate analog L-glutamate γ-methyl ester acted as a secretagogue in insulinoma cells, confirming a previous report (Maechler and Wollheim, 1999). Low concentrations of L-glutamate γ-methyl ester (200 μM), which were inactive per se, enhanced responses to increasing concentrations of glucose (data not shown). At higher concentrations (5–10 mM), L-glutamate γ-methyl ester increased both $[Ca^{2+}]_i$ (Fig. 3, A and B) and insulin secretion (Fig. 3C) in βTC-6 cells, and its action was antagonized by 1 μM MPEP. These findings indicate that activation of intracellular mGlu5 receptors contribute to stimulate insulin secretion, although a role for surface mGlu5 receptors might have been masked by the micromolar amounts of glutamate spontaneously released from βTC-6 and MIN6 cells (extracellular glutamate levels after 30 min of incubation in Krebs buffer were 1.5 ± 0.2 and 11.2 ± 0.4 μM, respectively, $n = 6$).

To examine the function of mGlu5 receptors in vivo, we measured insulin secretion in mice after a parenteral administration of glucose (1.5 g/kg, i.p.). Systemic injection of MPEP (10 mg/kg, i.p.) did not affect the peak insulin response to glucose but significantly reduced the late phase of insulin secretion (30–120 min after the glucose pulse) (Fig. 4A). Parallel measurements of blood glucose levels showed a

sustained hyperglycemia in mice treated with MPEP (Fig. 4B). Insulin and glucose levels were also assessed in mGlu5−/− mice challenged with glucose. These mice showed a blunted insulin response to glucose at all times (Fig. 4A), whereas heterozygous mGlu5+/− mice behaved similarly to wild-type mice (data not shown). Despite the lower insulin levels, no abnormalities in blood glucose levels were found in mGlu5 knockout mice compared with wild-type mice (Fig. 4B). This could not be ascribed to an increased responsiveness to the low levels of insulin because mGlu5+/+ and mGlu5−/− mice did not differ either for the expression of insulin receptors in the liver, skeletal muscle, and fat tissue (Fig. 5A), or for the ability of insulin to stimulate the PI-3-K pathway (assessed by immunoblot analysis of phosphorylated Akt) in isolated hepatocytes (Fig. 5B). However, mGlu5−/− mice showed lower basal plasma glucagon levels and a blunted glucagon response to an insulin challenge compared with wild-type mice (Table 1). Glucagon secretion

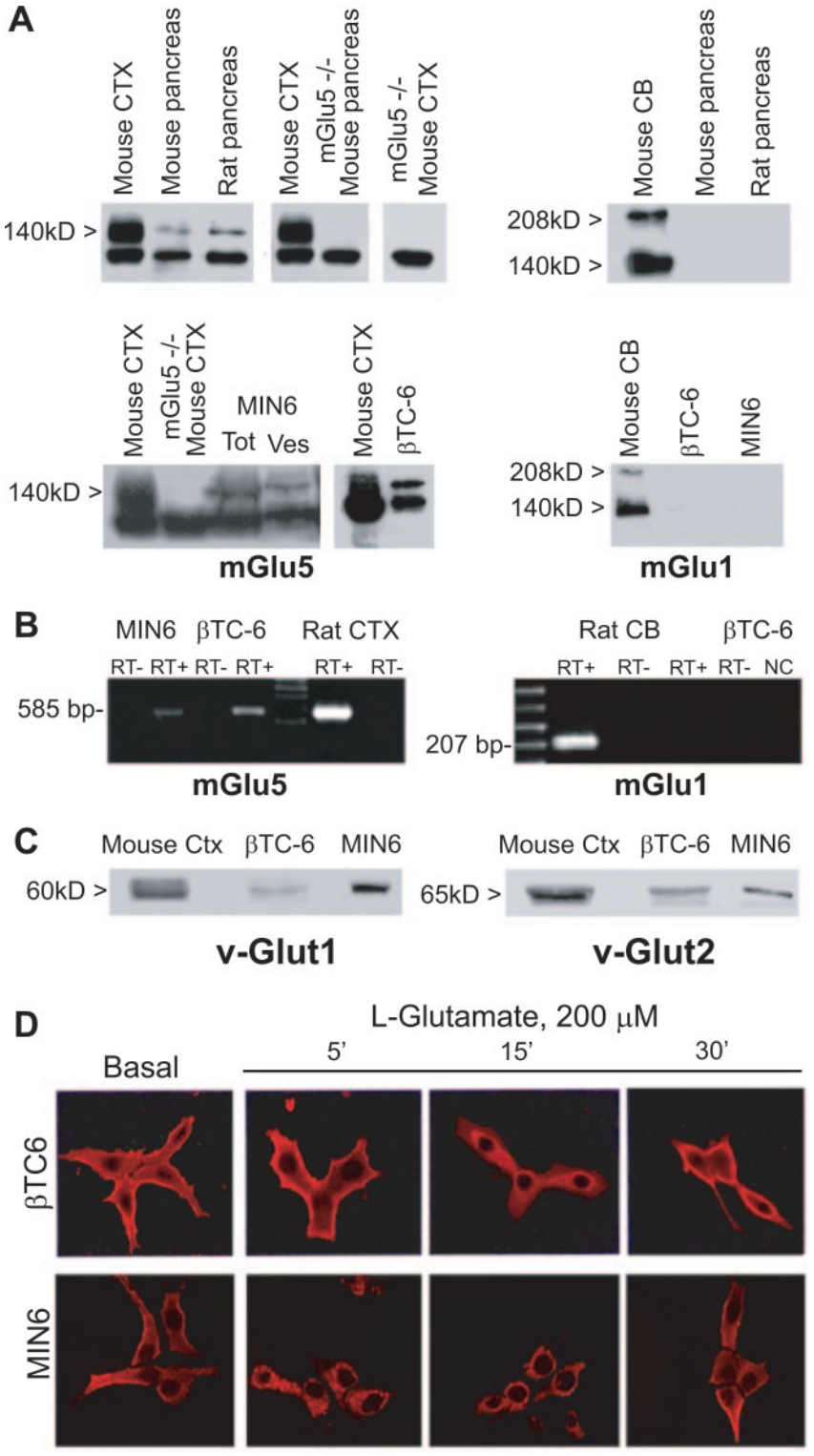

Fig. 1. Immunoblots of mGlu5 and mGlu1 receptors in the rodent pancreas, βTC-6 and MIN6 insulinoma cells, and purified secretory granules from MIN6 cells are shown in A; note the lack of the receptor (upper band at 140 kDa) in tissues from mGlu5 knockout mice (−/−). Tot, total protein extracts from MIN6 cells; Ves, protein extracts from purified insulin-containing vesicles of MIN6 cells. Immunoblots of mouse cerebral cortex (CTX) and cerebellum (CB) are also shown; RT-PCR analysis of mGlu5 and mGlu1 receptor mRNA is shown in B; immunoblots of the specific vesicular glutamate transporters v-Glut1 and v-Glut2 are shown in C; confocal analysis of the mGlu5 receptor in insulinoma cells is shown in D; note that the receptor is expressed both at the cell surface and intracellularly (outside the nuclear region) under control conditions (basal). Surface receptors translocate inside the cells within 15 min after exposure to the agonist L-glutamate (200 μM).

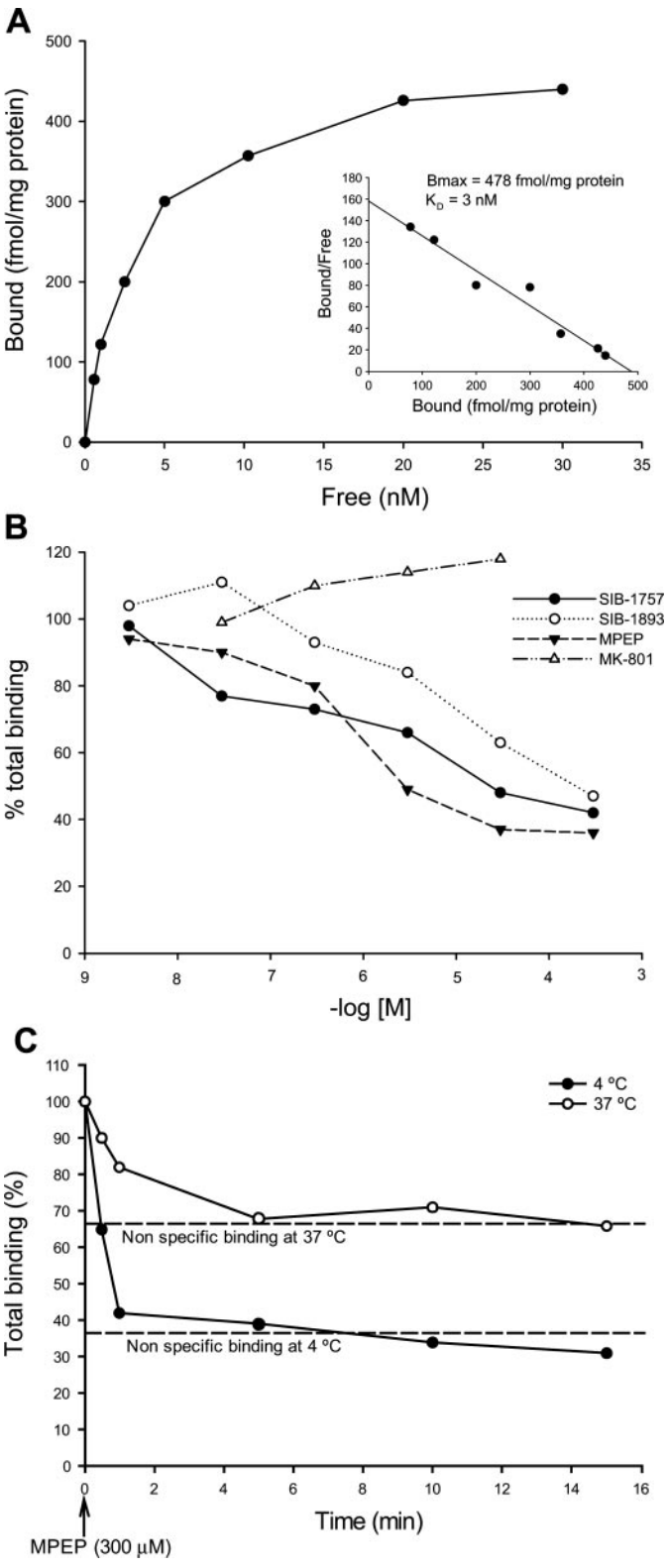

Fig. 2. [^{3}H]MPEP binding in intact βTC-6 cells. The saturation isotherm at 4°C is shown in A. A representative Scatchard plot is shown in the inset. Analysis was repeated twice with similar results. Displacement of specifically bound [^{3}H]MPEP (3 nM) is shown in B, where values are expressed as percentage of total binding and were calculated by a representative experiment performed in triplicate. Dissociation of [^{3}H]MPEP binding at 4°C and 37°C is shown in C. Note that nonspecific binding (defined as the binding remaining in the presence of 300 μM MPEP) was much higher at 37°C. Values were calculated from a representative experiment performed in triplicate.

was also reduced in wild-type mice injected with MPEP but to a lower extent compared with untreated mGlu5−/− mice (Table 1). There was no difference in the ability of insulin to reduce plasma glucose levels between mGlu5+/+ mice, mGlu5+/+ mice treated with MPEP, and mGlu5−/− mice (Table 1). This suggests that the reduced glucagon secretion does not result from changes in insulin-induced hypoglycemia.

Discussion

We confirmed the original finding of Brice et al. (2002) that clonal β-cells express mGlu5 receptors. A substantial portion of these receptors was localized on the cell surface, as suggested by the distribution of mGlu5 immunoreactivity and by the relatively high specific [^{3}H]MPEP binding in intact βTC-6 cells incubated at 4°C (i.e., under conditions in which [^{3}H]MPEP should not easily diffuse across the membranes). Surface mGlu5 receptors showed the typical behavior of G-protein coupled receptors; i.e., they translocated inside the cell upon agonist exposure. Inhibition of surface mGlu5 receptors may well contribute to the effect of MPEP or SIB-1893 on glucose-stimulated $[Ca^{2+}]_i$ increase and insulin secretion. For example, activation of ionotropic glutamate receptors in isolated pancreatic β-cell (Inagaki et al., 1995) or in MIN-6 cells (Gonoi et al., 1994) enhances both $[Ca^{2+}]_i$ and insulin secretion. However, the existence of an additional mechanism was suggested by the use of receptor agonists in our study. Classic mGlu5 receptor agonists that act extracellularly (such as L-glutamate, quisqualate, or DHPG) failed to induce increases in $[Ca^{2+}]_i$ or insulin secretion in βTC-6 or MIN-6 cells. In contrast, Brice et al. (2002) found that DHPG could amplify glucose-stimulated insulin secretion in clonal β-cells. The lack of activity of extracellular agonists in our hands might be explained with the micromolar concentrations of ambient glutamate, which can saturate or desensitize mGlu5 receptors. Extracellular glutamate levels were not measured by Brice et al. (2002). It is noteworthy that a cell-permeant ester of glutamate (L-glutamate γ-methyl ester) was able to increase intracellular Ca^{2+} and insulin secretion (Maechler and Wollheim, 1999) and its action was largely reduced by MPEP. The low specific [^{3}H]MPEP binding in intact βTC-6 cells incubated at 37°C suggests that MPEP, which is a highly lipophilic drug, may diffuse inside the cells. Thus, pharmacological data suggest that an intracellular mGlu5 receptor participates in processes regulating insulin secretion. This is a real possibility because intracellular mGlu5 receptors have been found on nuclear membranes, and their activation mediates intranuclear Ca^{2+} changes (O'Malley et al., 2003). It is noteworthy that we found that insulin-containing granules isolated from clonal β-cells expressed mGlu5 receptors in addition to the specific vesicular glutamate transporters, v-Glut1 and v-Glut2. Thus, at least some intracellular mGlu5 receptors are localized in structures that are directly involved in insulin secretion. Previous studies have shown that the glucose metabolized by β-cells generates glutamate as a by-product of the tricarboxylic acid cycle and that a membrane-permeant glutamate analog sensitizes the glucose-evoked insulin secretion (Maechler and Wollheim, 1999). Overexpression of the enzyme glutamate decarboxylase (GAD)-65, which transforms glutamate into GABA, reduces glucose-stimulated insulin secre-

tion in β cells (Rubi et al., 2001). It is noteworthy that glutamate uptake into insulin granules is required for the stimulation of insulin secretion. Drugs that inhibit vesicular glutamate transport suppress the glutamate-stimulated insulin secretion without affecting insulin release at basal conditions (Maechler and Wollheim, 1999; Hoy et al., 2002; Eto et al., 2003). Although a number of mechanisms have been proposed—including a reduced consumption of ATP by the vacuolar-type H^+-ATPase or the induction of granule swelling, which facilitates granule fusion with plasma membrane (Maechler and Wollheim, 1999)—how glutamate functions in β cells is still unknown. Our findings that MPEP and its congener SIB-1893 inhibit glucose-stimulated $[Ca^{2+}]_i$ increase and insulin secretion and that mGlu5 receptors are expressed by insulin-containing granules may shed new light onto the mechanism whereby glutamate regulates insulin secretion in clonal β-cells. We speculate that the glutamate generated from glucose metabolism in clonal β-cells exceeds the clearance capacity of GAD-65 and is actively transported inside the insulin-containing granules. There, glutamate can interact with the N-terminal binding domain of the mGlu5 receptor (De Blasi et al., 2001), which, according to the receptor topography, should face the vesicular lumen. Receptor activation would stimulate polyphosphoinositide hydrolysis and intracellular Ca^{2+} release around the vesicles, thus providing a highly localized mechanism contributing to insulin secretion in response to glucose. Moreover, the locally high concentrations of Ca^{2+} in the vicinity of vesicles may also go on to provoke Ca^{2+} -induced Ca^{2+} release from the vesicles, consistent with the expression of type 1 ryanodine receptors on these organelles (Mitchell et al., 2003). At the same time, the glutamate released extracellularly from the secretory

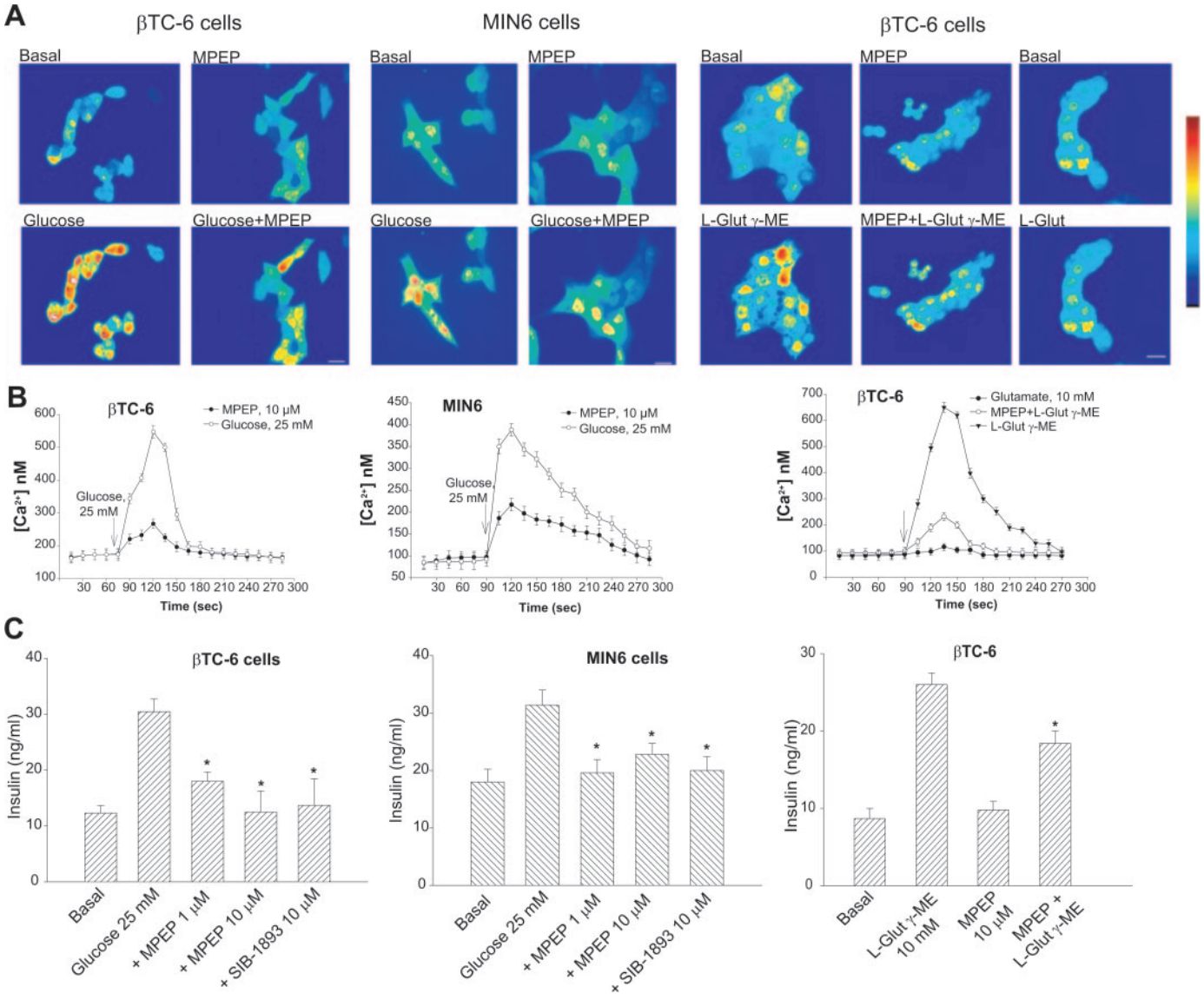

Fig. 3. Pharmacological blockade of mGlu5 receptors reduces the increase in free $[Ca^{2+}]_i$ and insulin secretion induced by glucose or L-glutamate γ-methyl ester (L-Glut γ-ME) in βTC-6 or MIN6 cells. Real-time confocal analysis of intracellular Ca^{2+} in fluo4-loaded insulinoma cells stimulated with glucose or L-Glut γ-ME is shown in A and B. Under resting conditions, cells were perfused with oxygenated Krebs/Henseleit's buffer containing 3 mM glucose. Cells were challenged with 25 mM glucose or 10 mM L-glutamate (L-Glut) or L-Glut γ-ME. MPEP was applied 10 min before the challenge. Scale bars, 7 μm in βTC-6 cells and 5 μm in MIN6 cells. Extracellular insulin levels in insulinoma cells treated for 30 min with glucose or L-Glut γ-ME are shown in C. Data are means ± S.E.M. of six to nine determinations. *, $p < 0.05$ (one-way analysis of variance + Fisher's protected least significant difference), if compared with glucose or L-Glut γ-ME alone.

granules may act as an autocrine/paracrine factor interacting with surface ionotropic and metabotropic receptors localized on neighbor cells (see the hypothetical model in Fig. 6). An intrinsic implication of this model is that glutamate acts as a putative coupling factor in cells that respond to metabolic activation with an increased vesicular secretion. In this context, intracellular mGlu5 receptors might have a pivotal role by "sensing" the glutamate that escapes the clearing mechanisms and becomes available for intracellular signaling. Although the model is attractive, it cannot yet be extended to

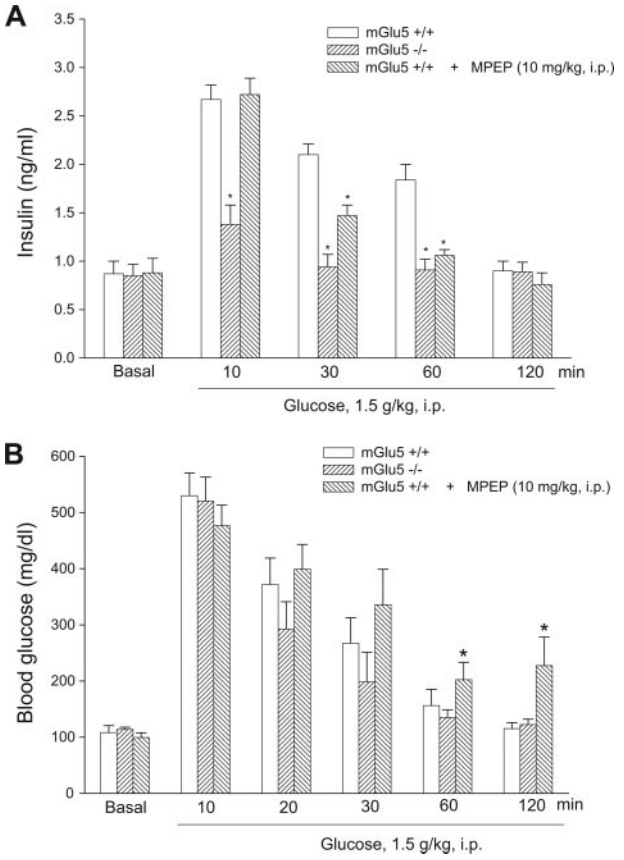

Fig. 4. mGlu5 receptors are involved in glucose-stimulated insulin secretion in mice. Plasma insulin levels and blood glucose levels in mice parenterally injected with glucose are shown in A and B, respectively. MPEP was applied 10 min before glucose. Values are means ± S.E.M. of 6 to 12 determinations. *, $p < 0.05$ (one-way analysis of variance + Fisher's protected least significant difference) if compared with mGlu5 +/+ mice.

TABLE 1

Plasma glucagon and glucose levels in wild-type or mGlu5 knockout mice deprived of food for 12 h and then challenged with insulin and/or MPEP

Values are means + S.E.M. of four to six determinations. $P < 0.05$ (one-way analysis of variance + Fisher's protected least significant difference) versus the group from the same strain of mice treated with saline (*), mGlu5+/+ mice treated with insulin alone (#), or the respective groups of mGlu5+/+ mice (§).

	Glucagon	Glucose
	pmol/l	*mg/dl*
mGlu5+/+ mice		
Saline	149 ± 18	105 ± 11
MPEP (10 mg/kg, i.p.)	102 ± 12*	117 ± 10
Insulin (5 IU/kg, i.p.)	365 ± 48*	29 ± 2*
Insulin + MPEP	220 ± 28*#	26 ± 1*
mGlu5−/− mice		
Saline	110 ± 7§	104 ± 7
Insulin (5 IU/kg, i.p.)	170 ± 23*§	28 ± 2*

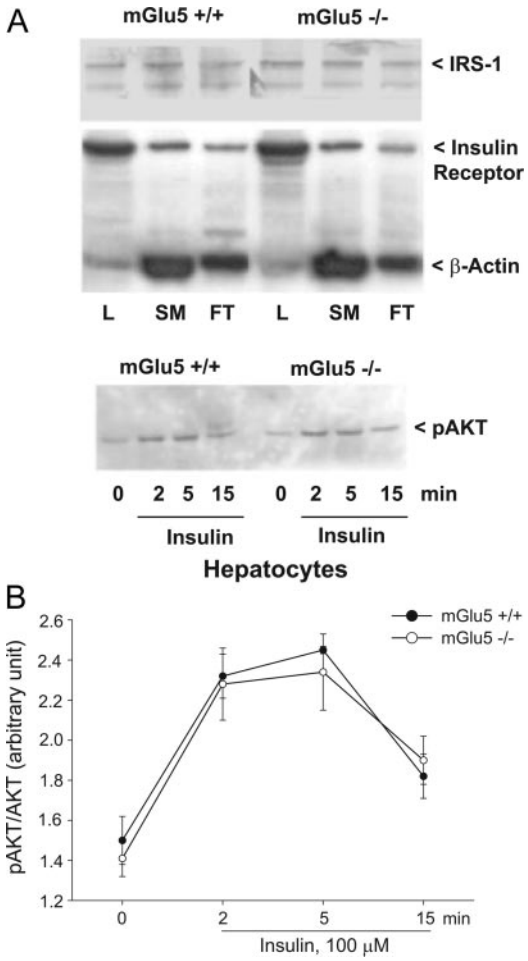

Fig. 5. Representative immunoblots showing no difference in the expression of IRS-1 and insulin receptors (β-subunit) in the liver (L), skeletal muscle (SM), and fat tissue (FT) of wild-type and mGlu5 knockout mice is shown in A; insulin activated PI-3-K pathway (phospho-Akt) in hepatocytes isolated from wild-type or mGlu5 knockout mice is shown in B. Values (means ± S.E.M.) were normalized by the expression of nonphosphorylated Akt and were calculated from five determinations.

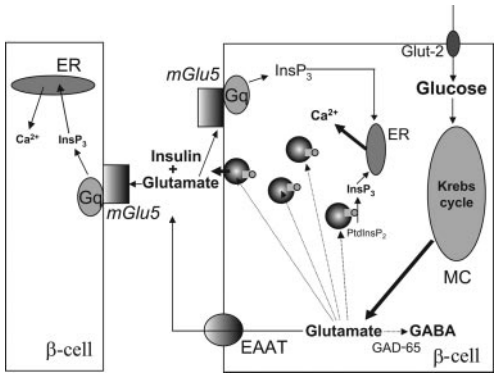

Fig. 6. Hypothetical model showing the potential role of mGlu5 receptors in the regulation of insulin secretion. The glutamate produced by glucose metabolism that exceeds the clearance capacity of GAD-65 or other mechanisms penetrates the insulin granules, where it activates mGlu5 receptors. Receptor activation, and the stimulation of vesicle-associated phospholipase C, might generate calcium transients around the vesicles, thus stimulating insulin secretion. The amount of glutamate secreted extracellularly from the vesicles might interact with surface mGlu5 receptors contributing to the autocrine and paracrine regulation of β-cell function. Glut-2, type-2 glucose transporter of pancreatice β-cells; MC, mitochondria; EAAT, excitatory amino acid transporters; PydInsP_2, phosphatidylinositol-4,5-bisphosphate.

the physiological regulation of insulin secretion. Despite the high density of mGlu5 receptors in βTC-6 cells (see the B_{max} of [^{3}H]MPEP binding), evidence for the existence of mGlu5 receptors in pancreatic β-cells is still lacking. Uehara et al. (2004) found that mGlu5 receptors are present in pancreatic islets, but they are localized on blood vessels and synaptic terminals rather than in islet cells. In addition, there is no evidence that vesicular glutamate transporters are present on insulin secreting vesicles of pancreatic β-cells, although indirect evidence suggests that glutamate uptake into secretory granules is necessay for the stimulation of insulin secretion (see above). Without additional data, our in vivo findings do not demonstrate a direct role of mGlu5 receptors on insulin and glucagon secretion in pancreatic islet cells. Mice injected with MPEP showed a selective impairment of the late phase of glucose-stimulated insulin secretion, whereas mGlu5 knockout mice showed a severe impairment of all phases of insulin secretion. In addition, both MPEP-injected mice and mGlu5 knockout mice showed a blunted glucagon response to an insulin challenge in spite of an unchanged insulin-induced hypoglycemia. In the absence of a direct demonstration that mGlu5 receptors are localized on islet cells, our data are difficult to explain. mGlu5 receptors localized on pancreatic blood vessels might be involved in the regulation of insulin and glucagon secretion because the impaired vascularization of the endocrine pancreas in type-A vascular endothelial growth factor knockout mice causes a reduced glucose tolerance (Lammert et al., 2003). On the other hand, the effect we have seen might involve mGlu5 receptors in glucose-responsive neurons of the hypothalamic ventromedial nucleus, which control glucagon secretion (Miki et al., 2001).

Regardless of the mechanism(s), our in vivo data suggest that activation of mGlu5 receptors supports the secretion of pancreatic hormones and that drugs that are currently under development for neurologic or psychiatric disorders, such as mGlu5 receptor enhancers or negative modulators, may affect glucose homeostasis.

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