Localization and function of group III metabotropic glutamate receptors in rat pancreatic islets

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Received 15 October 2001; accepted in final form 30 January 2002

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receptor-like protein was identified, and high levels of the receptor were found in the pancreas (6). In addition, islet cells appear to display mGlu2 and mGlu3 receptor immunoreactivity (14). This prompted us to search for the expression of mGlu receptors in the pancreatic islets.

We now report, for the first time, that group III mGlu receptors are expressed in rat islets, that mGlu8 receptors are expressed by glutamatergic α-cells, and that activation of group III mGlu receptors inhibits glucagon release from isolated islets. These findings are consistent with the idea that islet mGlu8 receptors are autoreceptors and support the possibility that α-cells utilize glutamate as an autocrine- and/or paracrine-like chemical transmitter, resulting in mGlu8 receptor-mediated inhibition of glucagon secretion.

METHODS

Tissue. Adult female rats (Sprague-Dawley; 150–200 g) were euthanized by CO2 inhalation and then decapitated. The Animal Care and Use Committee of SUNY Downstate Medical Center approved this procedure. The pancreas was removed and washed with oxygenated (95% O2-5% CO2) Krebs solution of the following composition (mM): 121 NaCl, 5.9 KCl, 2.5 CaCl2, 14.3 NaHCO3, 1.3 NaH2PO4, 1.2 MgCl2, and 1.27 glucose.

RNA isolation and RT-PCR. Total RNA (5 μg) from rat pancreas, freshly isolated islets (see Isolation of islets), and brain, prepared using the TRIZol reagent (Ref. 15; Life Technologies, Gaithersburg, MD), was reverse transcribed at 42°C for 1 min, and 72°C for 2 min for 35 cycles, followed by a final extension step at 72°C for 7 min. In controls, reverse transcriptase was omitted. The results of this amplification were used to ensure successful mRNA isolation without the use of genomic DNA contamination. The PCR products were resolved in 2% agarose gel with ethidium bromide. The PCR products were subcloned into EcoRI-digested pGEM-T vector (Promega) for sequencing via dye termination cycle sequencing (ABI Pyramid Automated Sequencer, Perkin Elmer). The best match of the sequences was determined using the gapped BLAST and PSI-BLAST programs.

Immunocytochemistry. The pancreas was fixed for 24 h with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, the tissue was washed in phosphate-buffered saline (PBS), cryoprotected overnight (at 4°C) in PBS containing 30% sucrose, embedded in OCT (TissueTek), frozen with liquid N2, and sectioned (10 μm) using a cryostat (Leica). To locate mGlu8 receptor protein in the tissue by immunocytochemistry, preparations were incubated with 4% normal horse serum with Triton X-100 (0.5%) in PBS for 30 min. The preparations were then exposed overnight to guinea pig polyclonal antibodies generated against a peptide corresponding to amino acids 894–908 of rat and human mGlu8 receptor (diluted 1:1,000; Chemicon International, Temecula, CA; Ref. 26). After a wash with PBS, the preparations were incubated with donkey anti-guinea pig secondary antibodies coupled to Rhodamine Red-X (RRX; Jackson ImmunoResearch Laboratories, West Grove, PA) or fluorescein isothiocyanate (FITC; Jackson), diluted 1:500, for 3 h. The preparations were washed again (3× PBS), and then the tissues were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA).

Double-label immunocytochemistry was used to identify the cells that display mGlu8 receptor-like immunoreactivity. Double labeling was made possible by using primary antibodies raised in different species in conjunction with species-specific secondary antibodies (donkey anti-rat, donkey anti-mouse, donkey anti-guinea pig (Kirkegaard and Perry, Gaithersburg, MD), donkey anti-goat (Jackson ImmunoResearch Labs, diluted 1:500) coupled to contrasting fluorophores (FITC or RRX, as in Immunocytochemistry). Primary antibodies were against glucagon (guinea pig polyclonal, diluted 1:500, Linco Research), glutamate (mouse monoclonal, diluted 1:500, Instar), insulin (mouse monoclonal, diluted 1:2,000, Chemicon International), pancreatic polypeptide (PP; affinity-purified rabbit polyclonal, diluted 1:1,000), somatostatin (affinity-purified rabbit polyclonal, diluted 1:1,000, Instar), synaptophysin (mouse monoclonal, diluted 1:200, Sigma), or DNPI (rabbit polyclonal, diluted 1:1,500), which has been renamed, vesicular glutamate transporter 2 (VGLUT2; Ref. 7). The antibody to VGLUT2 was raised to a bacterial fusion protein containing the cytoplasmic COOH terminus of the rat protein. By Western blot analysis, the antibody recognizes VGLUT2, but not vesicular glutamate transporter 1 (VGLUT1), stably expressed in PC12 cells (7).

Control sections, which were used to determine the level of nonspecific staining, included incubating sections without primary antibody and/or blocking the primary antibody by preincubation (24 h) with the corresponding peptide (10–20

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR Product Size</th>
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<tbody>
<tr>
<td>mGlu4</td>
<td>Forward</td>
<td>5'-TCATTTTCTCTTTCTGTTCCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GACATTCTACATACATCGAGAC-3'</td>
</tr>
<tr>
<td>mGlu6</td>
<td>Forward</td>
<td>5'-GATTGTAGGGAGTTCGACGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTGGTGTACGTGGATCAGAGG-3'</td>
</tr>
<tr>
<td>mGlu7</td>
<td>Forward</td>
<td>5'-GAATCTGTGAAAAATTGAGACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TTAGGGGATCCAGATATCAG-3'</td>
</tr>
<tr>
<td>mGlu8</td>
<td>Forward</td>
<td>5'-CAGGGGTCTTAAAACTCAGAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CAGGGGTCTTAAAACTCAGAGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5'-CAGGGGTCTTAAAACTCAGAGG-3'</td>
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mGlu, metabotropic glutamate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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islets were preincubated for 30 min in 500 μH9262 (oxygenated Krebs) solution and 2.8 mM D-glucose. The preincubation medium was aspirated and discarded. The islets were washed thoroughly with Krebs solution containing 2.8 mM D-glucose, 5 mg/ml BSA, and 1 mM 3-isobutyl-1-methylxanthine (IBMX) and various substances as indicated. At the end of the incubation, islets were transferred into 250 μl of 50 mM sodium acetate with 20 mM EGTA and homogenized. Islet extracts were spun at 13,000 g for 20 min, and the supernatant was retained and stored at −20°C until assayed. The total cAMP content of islets was determined as picomoles cAMP per milliliter by using a cAMP-enzyme immunoassay kit from Assay Designs (Ann Arbor, MI).

**Chemicals and drugs.** (R,S)PPG, CPPG, and (S)-3,4-dicarboxyphenylglycine (DCPG) were obtained from Tocris Cookson (Ballwin, MO). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical (St. Louis, MO).

**Statistical analysis.** Data are expressed as means ± SE, with differences between groups determined by Student’s t-test.

**RESULTS**

**Group III mGlu receptor expression in the rat pancreas.** Group III mGlu receptor expression in the pancreas was analyzed utilizing RT-PCR. As expected, the brain was positive for mGlu4 (Fig. 1B, lane 1), mGlu6 (Fig. 1B, lane 2), mGlu7 (Fig. 1B, lane 3), and mGlu8 expression (Fig. 1A, lane 2), with expected bands at 340, 363, 321, and 440 bp, respectively. The pancreas (Fig. 1C) and isolated islets (Fig. 1D) were also positive for mGlu8 receptor expression (Fig. 1, C and D, lane 5), whereas no expression was detected in these tissues for mGlu4 (Fig. 1, C and D, lane 2), mGlu6 (Fig. 1, C and D, lane 3), or mGlu7 (Fig. 1, C and D, lane 4) receptors. RT-PCR analysis was confirmed after we subcloned and sequenced the PCR products. These results suggest that the pancreas, and specifically the islets, express only the gene encoding mGlu8 receptors, whereas the brain expresses the genes encoding all four group III mGlu receptor subtypes.

**Localization of mGlu8 receptor immunoreactivity in the pancreas.** If mGlu8 receptors were present in the pancreas, then, in other areas where these receptors exist, the pancreas would be expected to contain sites that can be demonstrated with mGlu8 receptor-selective antibodies (12). In both the pancreatic islets (Fig. 1E) and ganglia (Fig. 1F), a population of cells displayed mGlu8 receptor immunoreactivity. In general, immunolabeling was punctate and localized to cell bodies. In addition, mGlu8 receptor immunoreactivity was found on numerous nerve fibers (Fig. 1F), suggesting that mGlu8 receptors are located presynaptically. No immunoreactivity was found in control sections that were processed without the primary antiserum (not illustrated). Moreover, Western blotting with the antibody against mGlu8 showed one labeled band of protein in rat pancreas membrane (Fig. 1G). The labeled protein had an apparent molecular mass of 100 kDa, which is in agreement with the molecular mass deduced from the cDNA sequence of mGlu8.
Identification of mGlu8 receptor-immunoreactive islet cells. Further studies were done to chemically identify the islet cells with mGlu8 receptor immunoreactivity. Three nonoverlapping groups of islet cells were identified. One group displayed glucagon immunoreactivity (Fig. 2, A–C) and 100% of glucagon-immunoreactive α-cells contained mGlu8 receptor. The other groups contained insulin (Fig. 2, D–F) or pancreatic polypeptide immunoreactivity (Fig. 2, G–I), and subsets of these cells expressed mGlu8. In contrast, mGlu8 receptor immunoreactivity was not found in somatostatin-containing cells (Fig. 2, J–L).

mGlu8 receptor immunoreactivity is displayed by islet cells expressing a glutamatergic phenotype. In the CNS, several studies have shown that group III mGlur receptors are expressed by glutamatergic neurons and
Fig. 2. Characterization of mGlu8 receptor-immunoreactive islet cells. 

A–I: mGlu8 receptor immunoreactivity (A, D, G, arrows) is displayed by all glucagon (Gluc)-containing α-cells (B and C, arrows) and subsets of cells containing insulin (E and F, arrows) or pancreatic polypeptide (PP; H and I, arrows). J–L: mGlu8 receptor immunoreactivity is not found in somatostatin (SOM)-containing cells (K and L). Markers = 10 μm.
function as autoreceptors that regulate the physiological release of glutamate (24). This prompted us to determine whether mGlu8-immunoreactive islet cells express a glutamatergic phenotype.

mGlu8 receptor immunoreactivity was displayed by islet cells that contained glutamate (Fig. 3, A–C) and synaptophysin (not illustrated), a marker for synaptic-like vesicles. Moreover, these cells expressed VGLUT2 (Fig. 3, D–F), which was found in all glutamate-immunoreactive islet cells (Fig. 3, G–I). In addition, glutamate/VGLUT2-immunoreactive cells contained glucagon (Fig. 3, J–L), suggesting that mGlu8 is expressed by glutamatergic α-cells.

VGLUT2 is associated with secretory granules in pancreatic islet cells. To elucidate the role of glutamate in pancreatic islets, we localized VGLUT2 in isolated islets employing postembedding electron microscopic immunocytochemistry with immunogold. We focused on the localization of VGLUT2 in cells situated near the border of the islets, because the majority of VGLUT2-positive cells were found in this region.

Analysis at the electron microscopic level confirmed the presence of high levels of VGLUT2 in a subset of islet cells. Cells were identified as glucagon-producing α-cells by their abundance of round granules of high density and a narrow halo (2). As shown in Fig. 4, A and B, the VGLUT2-reacting gold particles were localized in the secretory granules, along or near the granule membrane. Essentially no gold particles were found on control grids incubated in the absence of primary antibodies (Fig. 4, C and D).

To determine whether the location of gold particles over secretory granules was significant, the distribution of gold particles on immunostained grids was compared with that of an array of standard dots superimposed on the electron micrographs of the isolated islets. Although the standard dots were arrayed in a regular pattern, the pattern was random with respect to the structures in the micrographs. The dots served as the distribution that would have been expected if the gold particles had randomly precipitated over the tissue. Structures under the dots were identified and scored, as were structures under the gold particles. The number of immunogold particles over secretory granules (943 ± 306.3; n = 7) was significantly greater (P < 0.01) than that expected for a random distribution (50 ± 7.1; n = 7), whereas that over structures in the cytoplasm was not (210.5 ± 35.7 vs. 323.8 ± 39.1; n = 7). Thus the VGLUT2 is localized to the secretory granules of islet cells.

Activation of mGlu8 receptors inhibits glucagon release and cAMP production. To assess whether the expressed mGlu8 receptors in pancreatic α-cells cells are functional, we determined the effects of the group III-specific agonist (R,S)PPG (9) on glucagon release and cAMP production in isolated islets.

Addition of (R,S)PPG (40 nM) to the incubation solution significantly reduced glucagon release from 1.5 ± 0.19 to 0.9 ± 0.13 pg-islet \(^{-1}\) min \(^{-1}\) (P < 0.05) (Fig. 5A). In the presence of the group III-specific antagonist CPPG (100 μM; Ref. 29), the inhibitory effect of (R,S)PPG was significantly reduced (87 ± 2.4%; n = 8), suggesting that the inhibitory response to (R,S)PPG is mediated primarily by activation of group III mGlu receptors.

To study the possible negative coupling between mGlu8 and the adenylate cyclase-cAMP system (5, 24) in the inhibition of glucagon release, we examined the effects of (R,S)PPG and the group III mGlu receptor agonist DCPG (28) on forskolin-induced accumulation of cAMP. Forskolin (10 μM), a direct activator of adenylate cyclase, significantly increased (P < 0.05; n = 7) cAMP accumulation in isolated islets in the presence of the phosphodiesterase inhibitor IBMX (Fig. 5B). Both (R,S)PPG (40 nM; n = 6) and DCPG (20 nM; n = 7) significantly (P < 0.05) inhibited forskolin-stimulated cAMP production, and the group III mGlu receptor antagonist CPPG (100 μM) was effective in significantly (P < 0.01; n = 7) preventing the inhibition of both agonists on forskolin-induced potentiation (Fig. 5B).

DISCUSSION

The present study demonstrates, for the first time, that group III mGlu receptors are found in pancreatic islets. With the use of RT-PCR analysis, rat islets were shown to express the gene encoding mGlu8. Expression of mGlu8 was corroborated by immunocytochemical data localizing mGlu8 receptor protein in the islet cells. Moreover, functional studies demonstrated that islet group III mGlu receptors might play a role in modulating pancreatic endocrine secretion.

mGlu8 receptors were primarily expressed by α-cells, although a subset of insulin- and PP-secreting cells also appeared to display mGlu8 receptor immunoreactivity. In addition to islets, mGlu8 receptor immunoreactivity was also found on neural elements within the pancreas. Pancreatic nerve cell bodies and fibers displayed intense mGlu8 receptor immunoreactivity; therefore, group III mGlu receptors may be involved in modulating synaptic transmission in pancreatic ganglia. The presence of mGlu8 in all α-cells suggested that activation of group III mGlu receptors might affect glucagon release.

Application of the group III mGlu receptor agonist (R,S)PPG significantly reduced glucagon secretion in isolated islets, and the effect of (R,S)PPG was inhibited by the group III mGlu receptor antagonist CPPG. Thus islet mGlu8 receptors appear to be functional and negatively modulate the release of glucagon when activated. The inhibitory effect of mGlu8 receptors on glucagon release could be, at least in part, attributed to its interfering with the adenylate cyclase/cAMP system, since both (R,S)PPG and DCPG significantly inhibited forskolin-stimulated cAMP production in isolated islets. In addition, the effects of agonists were suppressed by CPPG. Taken together, these findings suggest that glutamate, through the activation of mGlu8 receptors located on the α-cells, inhibits adenylate cyclase, thereby decreasing glucagon secretion.

To date, very few selective group III mGlu receptor agonists have been identified, and only (R,S)PPG and

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Fig. 3. mGlu8 receptor-immunoreactive α-cells display a glutamatergic phenotype. A–F: mGlu8 receptor immunoreactivity (A and D, arrows) is displayed by glutamate-containing islet cells (B and C, arrows). These cells also express the vesicular transporter 2 (VGLUT2; E and F, arrows). G–I: glutamate-immunoreactive islet cells (G, arrow) express VGLUT2 (H and I, arrows). J–L: VGLUT2-immunoreactive islet cells (J, arrow) contain glucagon (K and L, arrows); therefore, α-cells are glutamatergic and express mGlu8 receptors. Markers = 10 μm.
Fig. 4. Immunogold localization of VGLUT2 in ultrathin sections of isolated rat islets. A–D: round granules of high density and a narrow halo (arrows) are found in pancreatic α-cells. The black dots (A and B) are gold particles (6 nm), which identify the granules as containing VGLUT2. The majority of the gold particles are localized on or near the membrane region of the granules. Note that there is some diffusion of the gold particles. Gold particles are not found on the granules of α-cells in control sections incubated without primary antibody (C and D). N, nucleus. Markers = 200 nm.

Fig. 5. Effects of group III mGlu receptor agonists on glucagon release and forskolin-stimulated cAMP production in isolated islets. A: glucagon release in islets incubated with the group III mGlu receptor agonist (R,S)-4-phosphonophenyglycine [(R,S)PPG; 40 nM], in the absence or presence of the group III mGlu receptor antagonist (R,S)-cyclopropyl-4-phosphonophenylglycine (CPPG; 100 µM). Values are means ± SE of 8 determinations. *P < 0.05. B: effects of (R,S)PPG (40 nM) and (S)-3,4-dicarboxyphenylglycine (DCPG; 20 nM) on forskolin (10 µM) -stimulated cAMP production in islets in the absence or presence of CPPG (100 µM). After preincubation, 15 size-matched islets were incubated for 30 min in Krebs solution containing glucose (2.8 mM) and 3-isobutyl-1-methylxanthine (IBMX; 1 mM) and various drugs as indicated in the graph. Values are means ± SE of 6–7 determinations. *P < 0.05 compared with control (IBMX) or (R,S)PPG; **P < 0.05 compared with forskolin or CPPG.
DCPG have been reported to have any degree of selectivity for mGlu8 (9, 28). Nevertheless, despite the lack of selective ligands, it seems likely that mGlu8 is involved, because islets only express the gene encoding mGlu8 receptors and both (R,S)PPG and DCPG are highly potent in this system. The potency of (R,S)PPG and DCPG to produce a response in isolated islets appears to be greater than in mGlu8 receptor-expressing cells (28, 29). An increase in the efficiency of an agonist to elicit an effect is commonly attributed to an increase in the receptor reserve for the agonist (23). Whether there is a large receptor reserve at pancreatic mGlu8 receptors has not yet been determined; however, understanding the mechanisms associated with the supersensitivity of mGlu8 receptors in the islets could be relevant to the pathophysiology of diabetes.

Islet cells that expressed mGlu8 receptors displayed a glutamatergic phenotype. mGlu8 receptor-positive cells contained both glutamate and the vesicular glutamate transporter VGLUT2, which were found co-localized in the same cells. VGLUT2 is one of two types of vesicular glutamate transporter expressed in central glutamatergic neurons (7, 8), the other being brain-specific Na\(^{+}\)-dependent inorganic phosphate cotransporter, renamed VGLUT1 (Refs. 1 and 27). Because the VGLUT accumulates glutamate into synaptic vesicles (1, 7, 8, 27), VGLUT is a selective marker of glutamatergic cells.

Glutamate is released from \(\alpha\)TC6 cells, clonal mouse pancreatic \(\alpha\)-cells, through a Ca\(^{2+}\)-dependent exocytotic mechanism (33). Immunoelectron microscopy revealed a large number of synaptophysin-positive clear vesicles in these cells. From these findings, it was concluded that \(\alpha\)TC6 cells, like central glutamatergic neurons (1, 7, 8, 27) and pinealocytes (10), store glutamate in synaptophysin-containing vesicles (33). In support of this hypothesis, \(\alpha\)TC6 cells, as well as \(\alpha\)-cells in rat islets, have recently been found to express VGLUT2 (10). However, whether VGLUT2 is located in synaptophysin-containing vesicles was not determined.

In the present study, we confirmed that \(\alpha\)-cells in rat islets express VGLUT2. Interestingly, VGLUT1 immunoreactivity is also displayed by a subset of pancreatic \(\alpha\)-cells (Q. Tong and A. L. Kirchgesnner, unpublished observations). Therefore, \(\alpha\)-cells are probably capable of storing and secreting glutamate. The presence of punctate VGLUT2 immunoreactivity in the cytoplasm of these cells suggested that VGLUT2 might localize to vesicles. Labeling with gold particles in isolated islets showed that VGLUT2 was localized in the secretory granules of a subset of islet cells, presumably the glucagon-producing \(\alpha\)-cells, because they contained an abundance of round granules of high density and a narrow halo. In addition, the majority of VGLUT2-reacting gold particles were found along or near the secretory granule membrane, although some diffusion of gold particles did occur. This finding supports the idea that VGLUT2 transports glutamate into secretory granules and that glutamate might play a role in the regulation of glucagon processing. Whether glutamate is localized in secretory granules in islet cells is not yet known; however, d-aspartate is stored in secretory granules in pheochromocytoma PC12 cells (22).

Although the physiological role of glutamate in the islets is not yet fully understood, glutamate appears to regulate the secretion of islet hormones by way of its binding to both ionotropic (3, 13, 17, 31, 32) and mGlu receptors. Glutamate acting via ionotropic receptors exerts an excitatory action and stimulates both insulin and glucagon secretion (13, 31). In this study, we showed that glutamate acting via the group III mGlu8 receptor exerts an inhibitory action on glucagon release. Moreover, mGlu8-positive islet cells display a glutamatergic phenotype. In the CNS, the mGlu8 receptor is known to couple to G\(_{i}\) and function as an autoreceptor to negatively modulate the release of glutamate when activated (24). As in the CNS, group III mGlu receptors in \(\alpha\)-cells may also provide negative feedback to limit further release of glutamate under normal (and/or pathological) conditions of glutamate release. In this way, glutamate may act as an autocrine- and/or paracrine-like chemical transmitter, resulting in inhibition of glucagon synthesis. Because lack of suppression of glucagon secretion can cause postprandial hyperglycemia in subjects with type 2 diabetes (25), group III mGlu receptor agonists could be of value in the treatment of these patients.

In summary, the results from this study have shown that 1) pancreatic islets express the gene encoding mGlu8; 2) mGlu8 receptor immunoreactivity is displayed primarily by \(\alpha\)-cells, which display a glutamatergic phenotype; and 3) activation of group III mGlu receptors inhibits glucagon release and forskolin-stimulated cAMP production in isolated islets. Expression of mGlu8 in \(\alpha\)-cells that contain glutamate and VGLUT2 suggests that this subtype may act as an autoreceptor to inhibit glutamate release. The presence of both ionotropic and mGlu receptors in the islets allows glutamate to play complex roles in endocrine secretion.

We thank Drs. Robert H. Edwards and Robert T. Fremeau for providing the antibody to VGLUT2, Drs. Jeffrey Conn and Min-tsaI Liu for helpful discussions, and Wei Quan for assistance with electron microscopy.

This work was supported by National Institutes of Health Grant NS-35951 and The American Diabetes Association (to A. L. Kirchgesnner).

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