Metabotropic Glutamate Receptor Type 4 Is Involved in Autoinhibitory Cascade for Glucagon Secretion by α-Cells of Islet of Langerhans

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In islets of Langerhans, L-glutamate is stored in glucagon-containing secretory granules of α-cells and cosecreted with glucagon under low-glucose conditions. The L-glutamate triggers secretion of γ-aminobutyric acid (GABA) from β-cells, which in turn inhibits glucagon secretion from α-cells through the GABA A receptor. In the present study, we tested the working hypothesis that L-glutamate functions as an autocrine/paracrine modulator and inhibits glucagon secretion through a glutamate receptor(s) on α-cells. The addition of L-glutamate at 1 mmol/l; (R,S)-3,4-dicarboxyphenylglycine (DCPG), and (S)-3,4-dicarboxyphenylglycine (DCPG), specific agonists for class III metabotropic glutamate receptor (mGluR), at 100 μmol/l; and (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) at 50 μmol/l inhibited the low-glucose-evoked glucagon secretion by 87, 81, 73, and 87%, respectively. This inhibition was dose dependent and was blocked by (R,S)-cyclopropyl-4-phosphonophenylglycine (CPPG), a specific antagonist of class III mGluR. Agonists of other glutamate receptors, including kainate and quisqualate, had little effectiveness. RT-PCR and immunological analyses indicated that mGluR4, a class III mGluR, was expressed and localized with α- and F cells, whereas no evidence for expression of other mGluRs, including mGluR8, was obtained. L-Glutamate, PPG, and ACPT-I decreased the cAMP content in isolated islets, which was blocked by CPPG. Dibutyryl-cAMP, a nonhydrolyzable cAMP analog, caused the recovery of secretion of glucagon. Pertussis toxin, which uncouples adenylate cyclase and inhibitory G-protein, caused the recovery of both the cAMP content and secretion of glucagon. These results indicate that α- and F cells express functional mGluR4, and its stimulation inhibits secretion of glucagon through an inhibitory cAMP cascade. Thus, L-glutamate may directly interact with α-cells and inhibit glucagon secretion. Diabetes 53:998–1006, 2004

L-Glutamate, an excitatory neurotransmitter in the central nervous systems, plays important roles in fast synaptic transmission and neuronal plasticity (1,2). For glutamate-evoked neurotransmission, neuronal cells develop glutamatergic systems: exocytosis of L-glutamate, signal reception through various glutamate receptors, and signal termination through plasma membrane glutamate transporters. The islet of Langerhans, a pancreatic minia
ture endocrine organ for homeostasis of blood glucose, possesses glutamatergic systems similar to those in the central nervous system (3): L-glutamate is stored in glucagon-containing secretory granules through vesicular glutamate transporters, and it is cosecreted with glucagon from α-cells under low-glucose conditions (4,5). The released L-glutamate may bind to the glutamate receptor expressed on islet cells, resulting in the occurrence of the glutamatergic response of islet cells, although the glutamatergic response is not fully understood (3,6–12). Then, the glutamate signals may be terminated by sequestration of L-glutamate through Na+-dependent glutamate transporter (13).

In pioneering works, (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type ionotropic glutamate receptors, mainly GluR2/3, were found to be expressed in α- and β-cells (6–8), and their stimulation was reported to enhance the secretion of insulin under high-glucose conditions (6,7,14). AMPA and kainate, but not N-methyl-d-aspartate, were also shown to stimulate glucagon secretion from perfused pancreas (15). However, under low-glucose conditions, L-glutamate secreted from α-cells stimulates an AMPA-type receptor and selectively triggers γ-aminobutyric acid (GABA) secretion, with a lower effect on insulin secretion in isolated islets and clonal β-cells (4). Because GABA is stored in synaptic-like microvesicles, distinct secretory vesicles from insulin granules, in β-cells (16), the glutamatergic response was explained as being caused by enhanced exocytosis of GABA-containing synaptic-like microvesicles (4). Because α-cells express the GABA A receptor, a chloride channel, and its stimulation modulates the membrane potential, causing inhibition of glucagon secretion (17–19), glutamatergic signaling from α-cells and subsequent GABAergic
signaling from β-cells seem to be involved in the inhibition of glucagon secretion (3).

Recently, Tong et al. (11) reported that islet α-cells express metabotropic glutamate receptor type 8 (mGluR8), and that its stimulation by agonists (R,S)-phosphonophenylglycine (PPG) and (S)-3,4-dicarboxyphenylglycine (DCPG; at around the nmol/l level) strongly inhibits glucagon secretion under low-glucose conditions. This report is of special interest because if mGluR8 actually functions in islet α-cells, the mGluR-mediated signaling cascade should be another signaling pathway for the inhibition of glucagon secretion, which may support the involvement of L-glutamate signaling in the inhibition of glucagon secretion.

mGluRs belong to a big family of glutamate receptors and are classified into three distinct groups (classes I, II, and III) according to their sequence homology, second messenger coupling, and agonist selectivity (20). Class I receptors consist of mGluR1- and -5, which are linked to phosphatidylinositol hydrolysis/Ca2+ signal transmission. Class II receptors (mGluR2 and -3) and class III receptors (mGluR4, -6, -7, and -8) are coupled with inhibitory G-proteins (G\textsubscript{i}) protein, and thus their stimulation results in the inhibition of adenylate cyclase and a decreased cellular cAMP level. A pioneering group has reported negative results as to the expression of mGluRs in clonal β-cells (21). However, Brice et al. (12) recently detected expression of the mGluR4, -4, -5, and -8 gene (using RT-PCR) and expression of the mGluR3 and 5 proteins (using immunoblotting) in isolated islets and clonal α- and β-cells. Taking the observation of Tong et al. (11) into consideration, it is clear that there is no consensus as to the expression of mGluRs in islets. To further investigate the glutamatergic signaling cascade through mGluR, it is necessary to establish which type of mGluR is actually expressed in islet α-cells.

In the present study, we examined the expression of mGluRs by means of a combination of procedures, including pharmacological techniques, RT-PCR, immunoblotting, and immunohistochemistry with pancreas and purified islets of rat. Here we report the evidence for functional occurrence of mGluR4, but not other mGluRs (including mGluR8), in islets. mGluR4 is present in α-cells and F cells that secrete pancreatic polypeptide, and its stimulation inhibits the secretion of glucagon through an inhibitory cAMP cascade. The present results provide the molecular basis for the occurrence of an L-glutamate-mediated auto-inhibitory mechanism for glucagon secretion by islet α-cells.

RESEARCH DESIGN AND METHODS

Preparation. Islets of Langerhans were isolated from male Wistar rats at 7–8 postnatal weeks by the collagenase digestion method combined with discontinuous Ficoll gradient centrifugation (22). Islets were then handpicked and suspended in a bicarbonate-buffered Hank’s solution supplemented with 0.2% BSA. In some experiments, islets (500 pieces) were washed with 20 mmol/l MOPS-Tris (pH 7.0) containing 0.3 mol/l sucrose, 5 mmol/l EDTA, 5 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml antipain, and 5 μg/ml pepstatin A and then extensively homogenized. The homogenate was centrifuged at 800g for 10 min, and the resultant supernatant was centrifuged at 140,000g for 1 h. RT-PCR. Total RNA extracted from isolated islets (1 μg) was transcribed into cDNA in a final volume of 20 μl of a reaction buffer containing 0.2 mmol/l each deoxynucleotide triphosphate (dNTP), 10 mmol/l dithiothreitol, 25 pmol of random octamers, and 200 units of Moloney murine leukemia virus reverse transcriptase (Amersham). After 1 h incubation at 42°C, the reaction was terminated by heating at 90°C for 5 min. For PCR amplification, the 10-fold-diluted synthesized cDNA solution was added to the reaction buffer containing 0.6 mmol/l total dNTP (150 μmol/l each dNTP), 25 pmol of primers (see Fig. 1A), and 1.5 units of AmpliTag-Gold DNA polymerase (Perkin Elmer). A total of 35 temperature cycles were conducted, as follows: denaturation at 94°C for 30 s, annealing at a specific temperature shown in Fig. 1A for 30 s, and extension at 72°C for 1 min. The amplification products were analyzed by agarose gel electrophoresis. The sequences of the oligonucleotides used as primers were based on the published sequences of various mGluRs. DNA sequencing was performed by the chain-termination method (23).

Expression of mGluR4. Rat mGluR4 cDNA, kindly provided by Dr. S. Nakashishi (24), was subcloned into the EcoRI-Xhol site of expression vector pcDNA3.1 (Invitrogen, San Diego, CA). The resultant construct was used to transfec COS7 cells by lipofection using TransIt reagent (Mirus, Madison, WI). COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS as described previously (25). Membrane fraction was prepared as described above.

Antibodies. Site-specific rabbit polyclonal antibodies against rat mGluR4 were produced as follows: DNA fragments encoding MSCKGWAWWVAR LPCLLLSLXAPWPPSLGKPGRPMNS (24) were amplified by PCR and cloned into the EcoRI site of expression vector pGEXX3 (Amersham Pharmacia Biotech) to form glutathione S-transferase (GST) fusion plasmids. DNA fragments encoding the NH\textsubscript{terminal} region of mGluR8, MVCEGKLRASCPCF FLITAKFYWLTMMQGRHT3QETYA (GenBank accession no. U63288) (26), were also amplified and cloned into pGEXX3 as described above. After transformation of the GST fusion proteins, they were purified on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) and then injected into a rabbit with complete adjuvant two times with a 2-week interval. The DNA fragments encoding the above-mentioned polypeptides of the NH\textsubscript{terminal} region of mGluR4 and -8 were also cloned into the Xhol and EcoRI site of expression vector phAD/Img-His B (Invitrogen). After transformation of the plasmid containing the DNA fragments, the six histidine-tagged polypeptides were expressed in a E. coli high-expression strain through a Ni-NTA column (Qiagen, Tokyo) according to the manufacturer’s manuals. The mouse monoclonal antibodies against glucagon were from Progen. The rat monoclonal antibodies against somatostatin, mGluR2/3, mGluR5, and mGluR8 were from Chemicon. The guinea pig polyclonal antibodies against insulin and rat pancreatic polypeptide were from Biosynthesis and Linco Research, respectively. Alexa Fluor 488-labeled anti-mouse IgG and Alexa Fluor 488-labeled goat anti-rabbit IgG were from Molecular Probes.

Western blotting. Membrane fraction was suspended in SDS sample buffer and then subjected to SDS gel electrophoresis. Immunoblotting was performed according to the published protocol, with visualization by enhanced chemiluminescence amplification (Amersham Pharmacia Biotech) (4).

Immunohistochemistry. Indirect immunofluorescence microscopy was performed as described previously, using an Olympus FV-300 confocal laser microscope (25).

Assays. Isolated islets (20 pieces per assay) or cultured cells (4.0 × 10\textsuperscript{5} cells/dish) were washed three times with DMEM and then incubated in Ringer’s solution containing 10 mmol/l HEPES (pH 7.4), 0.2% BSA, and glucose at the specified concentration for 1 h at 37°C. Then, the islets or cultured cells were transferred to 2 ml of the above Ringer’s solution containing glucose at the specified concentration. At the times indicated, samples (10 μl) were taken, and the amount of glucagon and cAMP was quantified with enzyme immunoassay kits obtained from Amersham Pharmacia Biotech and Yamanouchi (Japan), respectively, according to the manufacturers’ manuals. For measurement of cAMP, islets were extensively homogenized with 20 mmol/l MOPS-Tris (pH 7.0) containing 0.3 mol/l sucrose, 5 mmol/l EDTA, 5 μg/ml leupeptin, 5 μg/ml chymostatin, 5 μg/ml antipain, and 5 μg/ml of pepstatin A and then centrifuged at 140,000g for 1 h. The resultant supernatant was used for assay.

Statistics. Statistical significance between sets of data were assessed using Student’s t-test.

Materials. Agonists and antagonists for GluRs listed in Table 1 were purchased from Tocris Cookson (Avonmouth, U.K.). Other chemicals were of the highest grade commercially available.

RESULTS

Pharmacological evidence for involvement of class III mGluR(s) in the inhibition of glucagon secretion. As the first step of this study, we investigated whether mGluRs are involved in the regulation of glucagon secretion, using a pharmacological approach (Table 1).

L-Glutamate at 1 mmol/l inhibited low-glucose–dependent glucagon secretion, as glucose secreted at 16.7 mmol/l subtracted from that secreted at 3.3 mmol/l glucose, by 87%. L-Glutamate at 100 μmol/l was inhibitory, with 59% less effectiveness than that at 1 mmol/l. L-Glutamate is also

DIABETES, VOL. 53, APRIL 2004 999
inhibitory to the arginine-evoked secretion of glucagon: arginine at 15 mmol/l enhanced secretion of glucagon 2.3-fold at 3.3 mmol/l glucose, and arginine-enhanced secretion of glucagon was inhibited by 79% upon treatment of L-glutamate at 1 mmol/l. PPG and DCPG (agonists for class III mGluRs) at 100 \( \mu \)mol/l also inhibited glucagon secretion by 81 and 73%, respectively. The inhibition was dose dependent: at 40 nmol/l both agonists were not effective, the effectiveness being 50% at 40 \( \mu \)mol/l. At 100 \( \mu \)mol/l, no further inhibition was observed. Moreover, it was found that (1\( S \),3\( R \),4\( S \))-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I; another agonist for class III mGluR) at 50 \( \mu \)mol/l inhibited glucagon secretion by 87%. The L-glutamate–, PPG–, DCPG–, and ACPT-I–evoked inhibition of glucagon secretion was blocked by (\( R \),\( S \))-cyclopropyl-4-phosphonophenylglycine (CPPG), an antagonist for class III mGluRs (29), at 100 \( \mu \)mol/l. Essentially the same glucagon secretion was observed when CPPG itself was included in the assay mixture. (\( S \),3\( R \),4\( S \))-2-(2-carboxycyclopropyl)glycine (LY341495) is an antagonist with a \( K_i \) value of 57 nmol/l for mGluR8, which is a ~1,000-fold higher affinity than that for mGluR4 (30). The compound moderately blocked the L-glutamate–evoked inhibition of glucagon secretion at 30 \( \mu \)mol/l, but it did not block inhibition at all at 300 nmol/l. (\( S \)-3,5-dihydroxyphenylglycine [(\( S \))-3,5-DHPG] and quisqualate (specific agonists for class I mGluR) at 1 mmol/l or (\( 2 \),\( S \),\( 3 \),\( S \))-2(carboxycyclopropyl)glycine (L-CCG-I) at 100 \( \mu \)mol/l and (\( \pm \))-1-amino-1-cyclopentene-\((\text{trans})\)-3,1,3-dicarboxylic acid (\( \text{trans} \)-ACPD) at 1 mmol/l (agonists for class II mGluRs) (20,33,34) were only slightly effective (Table 1). AMPA and kainate (agonists for ionotropic glutamate receptors) at 0.5 mmol/l also slightly affected glucagon secretion (Table 1). These results suggest that class III mGluRs, most probably mGluR4, are involved in the inhibition of glucagon secretion.

**Expression and localization of mGluR4 in islets.** Subsequently, we examined which types of mGluRs are expressed in islets. Among the known mGluRs examined, RT-PCR analysis indicated the amplification of RNA frag-
ments of mGluR4 (Fig. 1). The amplified products covered all predicted sequences. The sequence of the amplified RNA fragment was identical to the corresponding sequence of mGluR4. On the other hand, no amplification of RNA fragments for other mGluRs was observed, although the same probes could amplify the predicted gene fragments from brain sources (Fig. 1). In particular, three different sets of primers for mGluR8 did not give any positive products for islet RNA, although these primers gave amplified products with the expected sizes and sequences for brain RNA (Fig. 1). Essentially the same results were obtained with the various amplification protocols used with an annealing temperature of 60 or 65°C (data not shown). These results led us to conclude that the mGluR4 gene is actually expressed in the islets, but expression of the genes of other mGluRs is negative or under the detection limit of our assay.

Similar RT-PCR analyses were performed using RNA prepared from exocrine pancreas. Fragments of mGluR3 and -5 with the expected sizes and sequences were amplified, whereas no amplification of the mGluR4 gene was observed (Fig. 1). Thus, in contrast with isolated islets, the exocrine part of pancreas shows different expression of mGluRs.

To detect the expression of mGluR4 at the protein level, we prepared site-specific antibodies against mGluR4. The mGluR4 antibodies recognized a 100-kDa polypeptide of authentic mGluR4 expressed in COS7 cells (Fig. 2A, left panel). The immunoreactivity disappeared when antigenic polypeptides (1 mg) were included during the immunoreaction. Dot blot analysis indicated that the mGluR4 antibodies recognized the NH2-terminal region of mGluR4, but they did not recognize the counterpart of mGluR8 (Fig. 2A, right panel). These results gave credence to the immunological specificity of the mGluR4 antibodies used in the study. The mGluR4 antibodies recognized a single 100-kDa polypeptide in islet membranes as well as brain membrane (Fig. 2B). The immunoreactivity disappeared when antigenic polypeptides (1 mg) were included during the immunoreaction. These results demonstrated the presence of the mGluR4 protein in islets. Double-labeling indirect immunofluorescence microscopy revealed that mGluR4 immunoreactivity is confined to islets but not to exocrine parts of pancreas (Fig. 2C). mGluR4 immunoreactivity is colocalized with glucagon and with pancreatic polypeptide but not with insulin or somatostatin (Fig. 2C). Treatment with antigenic peptides caused the mGluR4 immunoreactivity to disappear (data not shown). These results indicate that mGluR4 is present in α- and F cells but not in β- and δ-cells.

Consistent with the absence of mRNA of other mGluRs in islets, neither antibodies against mGluR2/3 nor those against mGluR5 recognized any polypeptides of islets on immunoblotting, whereas these antibodies recognized counterparts of brain membranes (Fig. 3A). Consistently, anti-mGluR2/3 and -mGluR5 antibodies did not immunostain any islet cells, but they immunostained synaptic terminals and blood vessels in pancreas as well as brain sections (Fig. 3B). As for mGluR8, mGluR8 antibodies did not recognize any polypeptides of islets on immunoblotting (Fig. 3A). After immunohistochemistry, mGluR8 antibodies slightly immunostained islet cells (Fig. 3B), although the immunoreactivity did not disappear even in the presence of antigenic peptides (data not shown). These results indicated that the level of mGluR2/3,
mGluR4, or mGluR8 in islets is, if anything, very low or under the detection limit of our assay.

Intracellular signaling pathway for the inhibition of glucagon secretion. Because mGluR4 is a class III mGluR, its stimulation may inhibit adenylate cyclase through activation of Gi protein, followed by a decrease in a Ca²⁺ level, resulting in the inhibition of glucagon secretion. To obtain convincing evidence for functional coupling between mGluR4 and adenylate cyclase, the effects of dibutyryl cAMP (DBcAMP; a nonhydrolyzable cAMP analog) and pertussis toxin (PTX; a specific uncoupler of adenylate cyclase and Gi) were investigated. DBcAMP treatment abolished the glutamate- and PPG- or ACPT-I–evoked decreases in glucagon secretion (Fig. 4). PTX also blocked the glutamate- and PPG- or ACPT-I–dependent decreases in glucagon secretion and cAMP content (Figs. 4 and 5). Because it is known that islet α-cells contain PTX-sensitive G₂₅ protein (35,36), these results strongly suggest that mGluR4 and adenylate cyclase are functionally coupled through Gi in α-cells.

DISCUSSION
In this study, we showed that L-glutamate inhibits glucagon secretion from isolated islets under low-glucose conditions. AMPA and kainate did not stimulate glucagon secretion under low-glucose conditions. These results support our proposal that glutamatergic signaling is

**TABLE 1**
The effects of L-glutamate and agonists of GluRs on low-glucose-dependent glucagon secretion

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration (µmol/l)</th>
<th>Glucagon release (ng · 20 islets⁻¹ · 30 min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.7→16.7 (basal release)</td>
<td>1,000</td>
<td>0.51 ± 0.23*</td>
</tr>
<tr>
<td>16.7→3.3 (100% control)</td>
<td>1,000</td>
<td>1.67 ± 0.17</td>
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<tr>
<td>+ L-glutamate</td>
<td>1,000</td>
<td>0.66 ± 0.19*</td>
</tr>
<tr>
<td>+ L-glutamate + 100 µmol/l CPPG</td>
<td>1,000</td>
<td>2.43 ± 0.16*</td>
</tr>
<tr>
<td>+ L-glutamate + 300 µmol/l LY341495</td>
<td>1,000</td>
<td>0.69 ± 0.12*</td>
</tr>
<tr>
<td>+ L-glutamate + 30 µmol/l LY341495</td>
<td>1,000</td>
<td>1.62 ± 0.15 NS</td>
</tr>
<tr>
<td>+ PPG</td>
<td>0.04</td>
<td>1.65 ± 0.13 NS</td>
</tr>
<tr>
<td>+ PPG</td>
<td>0.1</td>
<td>1.49 ± 0.18 NS</td>
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<tr>
<td>+ PPG</td>
<td>1</td>
<td>1.34 ± 0.16†</td>
</tr>
<tr>
<td>+ PPG</td>
<td>10</td>
<td>1.10 ± 0.20‡</td>
</tr>
<tr>
<td>+ PPG</td>
<td>100</td>
<td>0.73 ± 0.10*</td>
</tr>
<tr>
<td>+ PPG + 100 µmol/l CPPG</td>
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<td>2.38 ± 0.20‡</td>
</tr>
<tr>
<td>+ DCPG</td>
<td>0.02</td>
<td>1.72 ± 0.13 NS</td>
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<tr>
<td>+ DCPG</td>
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<tr>
<td>+ DCPG</td>
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</tr>
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<td>+ DCPG + 100 µmol/l CPPG</td>
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<td>2.25 ± 0.21‡</td>
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<tr>
<td>+ ACPT-I</td>
<td>1</td>
<td>1.61 ± 0.13 NS</td>
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<tr>
<td>+ AMPA</td>
<td>500</td>
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</tr>
<tr>
<td>+ Kainate</td>
<td>500</td>
<td>1.33 ± 0.15†</td>
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Data are means ± SE, n = 4. Isolated islets (20 pieces per assay) were first incubated with a solution containing 16.7 mmol/l glucose, and then transferred to a solution containing 3.3 mmol/l glucose to induce glucagon secretion in the presence or absence of the listed compounds at the indicated concentrations. After incubation for 30 min, the medium was carefully sampled and the amount of glucagon was determined. Glucagon secretion with 16.7 mmol/l glucose under the detection limit of our assay.

Because mGluR4 is a class III mGluR, its stimulation may inhibit adenylate cyclase through activation of Gi protein, followed by a decrease in a Ca²⁺ level, resulting in the inhibition of glucagon secretion. To obtain convincing evidence for functional coupling between mGluR4 and adenylate cyclase, the effects of dibutyryl cAMP (DBcAMP; a nonhydrolyzable cAMP analog) and pertussis toxin (PTX; a specific uncoupler of adenylate cyclase and Gi) were investigated. DBcAMP treatment abolished the glutamate- and PPG-evoked decreases in glucagon secretion (Fig. 4). PTX also blocked the glutamate- and PPG–dependent decreases in glucagon secretion and cAMP content (Figs. 4 and 5). Because it is known that islet α-cells contain PTX-sensitive G₂₅ protein (35,36), these results strongly suggest that mGluR4 and adenylate cyclase are functionally coupled through Gi in α-cells.

**FIG. 2.** Expression and localization of mGluR4 protein in islets. A: Left panel: The immunological specificity of mGluR4 antibodies was proven with mGluR4-expressing COS7 cells. Membrane fractions of control COS cells (lane 1) and mGluR4-expressing COS7 cells (100 µg each) (lanes 2 and 3) were solubilized and subjected to SDS-PAGE, followed by immunoblotting with anti-mGluR4 antibodies (lanes 1 and 2) or the same antibodies plus 1 mg antigenic peptide (lane 3). Right panel: Dot blot analysis. Polypeptides of His-tagged NH₂-terminal region of mGluR4 (lane 1) and mGluR8 (lane 2) at 2 µg each were spotted onto nitrocellulose sheets. One portion was stained with Amidoschwartz 10B (Merck). Other portions were decorated with antibodies against mGluR4 or mGluR8, as indicated, and the immunological reaction was visualized with enhanced chemiluminescence. B: Western blotting of brain membrane fraction (50 µg) (lanes 1 and 3) and islets (100 µg) (lanes 2 and 4) was performed with mGluR4 antibodies (lanes 1 and 2) or the same antibodies plus 1 mg antigenic peptide (lanes 3 and 4). C: Immunohistochemical localization of mGluR4 in islets. Sections of pancreas were doubly immunostained with antibodies against mGluR4 and glucagon, mGluR4 and insulin, mGluR4 and somatostatin, or mGluR4 and pancreatic polypeptide, and then they were observed under a confocal laser microscope. Bar = 10 µm.
involved in the inhibition of glucagon secretion (3,4). Furthermore, l-glutamate-evoked inhibition of glucagon secretion was blocked on treatment with CPPG, indicating possible involvement of class III mGluR in this inhibitory process. We then investigated which mGluR is responsible for the inhibition. We found that mGluR4 is the major mGluR in the islet of Langerhans, and it is involved in l-glutamate-evoked inhibition of glucagon secretion. Four lines of evidence support this conclusion. At first, pharmacological analysis suggested the functional involvement of class III mGluRs in the inhibition of low-glucose-stimulated glucagon secretion. Second, RT-PCR analysis demonstrated the presence of mRNA of mGluR4. Third, we prepared antibodies specific to mGluR4 and demonstrated the presence of the mGluR4 polypeptide in islets. Finally, immunohistochemical analysis indicated that mGluR4 is present in α- and F cells but not δ- and β-cells, supporting the results of our pharmacological analysis.

Our results are partly consistent with previous observations reported by two groups (6,21): no expression of mGluRs was detected in β-cells and clonal β-cells. Brice et al. (12) also detected amplification of a gene fragment of mGluR4 after RT-PCR, although they did not perform immunological identification. They also reported amplification of mRNA fragments of other mGluRs (mGluR3, -5, and -8), although no expression of these mGluRs was observed in our case. At present, we do not know the reason for the apparent discrepancy. It should be stressed, however, that the mGluR3 and -5 genes are expressed in the exocrine parts of pancreas (Figs. 1 and 3). Thus, the presence of acinar cells in an islet preparation may be expected to lead to amplification of the gene fragments of these mGluRs.

More importantly, we could not confirm the expression and localization of mGluR8 in islet α-cells. Even with the same DNA probes for RT-PCR analysis (Fig. 1A) (11), we could not detect any expression of the mGluR8 gene. Although the mGluR8 antibodies slightly immunostained islets, as judged by fluorescent microscopy, the immunoreactivity was observed throughout the islets, especially in β-cells (Fig. 3), and did not disappear when excess amounts of antigenic peptides were included during immunoreaction. Consistent with these observations, Western analysis with mGluR8 antibodies did not reveal any

**FIG. 2. Continued**
polypeptide from isolated islets (Fig. 3). These results strongly suggest that immunoreaction by mGluR8 antibodies is artificial in nature, and the concentration of mGluR8 protein in H9251-cells is under the detection limit or very low as compared with that of mGluR4.

In addition, Tong et al. (11) reported that DCPG and PPG at 20 and 40 nmol/l had the maximum inhibitory effect on glucagon secretion. The values are actually too low compared with the above-mentioned abilities of authentic mGluR8 or even authentic mGluR4 of rat (27,28), and they are far from the concentrations required for the inhibition of glucagon secretion (Table 1). We showed that the concentrations giving 50% effectiveness of DCPG and PPG were ~28 and ~19 μmol/l, respectively, which are around the $K_i$ value of CPPG with respect to recombinant mGluR4 expressed in cultured cells or rat tissues (37). ACPT-I most potently inhibited glucagon secretion (Table 1). Moreover, LY341495 did not affect the $L$-glutamate-evoked inhibition of glucagon secretion at 300 nmol/l, a several-fold higher concentration of $K_i$ value for mGluR8 (30). These results further support the involvement of mGluR4 but not mGluR8 in the inhibition of glucagon secretion. Thus, the observation of Tong et al. (11) is incorrect.

Stimulation of mGluR4 causes inhibition of glucagon secretion. mGluR4 is a class III mGluR and is thus believed to be negatively coupled with adenylate cyclase. As expected, the addition of $L$-glutamate, PPG, or ACPT-I decreased the cellular cAMP level, which was inhibited by CPPG and PTX (Fig. 5). The involvement of DBcAMP also led to the recovery of the PPG-evoked inhibition of secretion of glucagon (Fig. 4). As for the cellular cAMP level, essentially the same responses to $L$-glutamate and CPPG were observed in αTC6 cells, which are clonal α-cells (S.U., Y.M., unpublished observation). CPPG resulted in enhanced glucagon secretion and cAMP content (Table 1, Fig. 5), suggesting that endogenous $L$-glutamate released from isolated islets tonically acts through an inhibitory cascade. Although PTX is expected to show the same effect as CPPG, the compound did not enhance either glucagon secretion or cAMP content (Figs. 4 and 5).

FIG. 3. mGluR2/3, mGluR5, and mGluR8 were not detected in islets. A: Western blotting of brain membrane fraction (50 μg) (lane 1) and islets (100 μg) (lane 2) was performed with the listed antibodies. B: Sections of cerebrum (left panels) and pancreas (right panels) were immunostained with antibodies against mGluR2/3, mGluR5, and mGluR8 and then observed under a fluorescence microscope. Right panels: An islet is located at the center position. Immunoreactive blood vessels (middle right panel) and parts of nerve terminal of innervated neurons (bottom right panel) were marked. Bar = 10 μm.
reason for the apparent ineffectiveness of PTX is unknown at present but seems to be caused by decreased viable cells during incubation over a long period. Overall, we concluded that signaling pathways from mGluR4 to Gi and adenylate cyclase operate in α-cells. In vivo, the following signaling cascade can be expected to operate: under low-glucose conditions, L-glutamate is secreted by α- or F cells. Then, the released L-glutamate may bind to the mGluR4 on
α-cells so as to inhibit glucagon secretion through an inhibitory cAMP cascade. The mGluR4-mediated signaling pathway will provide the molecular basis for chemotherapeutics for hyperglycemia, one of the symptoms for type 2 diabetes.

In conclusion, islet α-cells express functional mGluR4 as a major mGluR. L-Glutamate itself may function as an autocrine transmitter and inhibit glucagon secretion by way of the mGluR4-mediated inhibitory cascade. The glutamatergic signaling also triggers GABA secretion from β-cells, and the released GABA in turn inhibits glucagon secretion by way of the GABAA receptor. Thus, the L-glutamatergic system may inhibit glucagon secretion through two distinct signaling pathways, and it may constitute a novel regulatory mechanism of glucagon secretion in the islet of Langerhans.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas—Advanced Brain Science Project from the Ministry of Education, Science, Sports and Culture of Japan, and the Umami Research Foundation. S.Y. was supported by a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

We thank Prof. S. Nakanishi (Kyoto University School of Medicine) for the kind supply of mGluR4 cDNA.

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