Activation of Glycine and Glutamate Receptors Increases Intracellular Calcium in Cells Derived from the Endocrine Pancreas

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ABSTRACT

We studied calcium signaling in a newly described pancreatic cell line, GK-P3, that expresses functional amino acid neurotransmitter receptors. GK-P3 cells express the first strychnine-sensitive glycine receptors reported in a permanent cell line. In addition, GK-P3 cells express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. Both types of amino acid receptors showed electrophysiological and pharmacological behavior similar to their neuronal counterparts. The glycine receptors were permeable to Cl\(^{-}\) and blocked by the selective antagonist strychnine. AMPA receptors showed limited permeability to Ca\(^{2+}\), were blocked by 6-cyano-2,3-dihydroxy-7-nitroquinoxaline, and were potentiated by cyclothiazide. Interestingly, activation of either receptor type increased intracellular Ca\(^{2+}\) measured by digital imaging of Fura-2 fluorescence. These Ca\(^{2+}\) signals were completely blocked by 30 μM La\(^{3+}\), suggesting that the Ca\(^{2+}\) entered the cells largely through voltage-dependent Ca\(^{2+}\) channels. Alterations in the extracellular concentrations of Cl\(^{-}\) and/or HCO\(_3\)\(^{-}\) had only marginal effects on glycine-evoked Ca\(^{2+}\) signals. However, increases in intracellular Ca\(^{2+}\) mediated by AMPA receptors were absent when the extracellular Na\(^{+}\) was replaced with an impermeant cation, N-methyl-D-glucamine. We conclude that activation of ligand-gated cation or anion channels depolarize GK-P3 cells sufficiently to activate their voltage-gated Ca\(^{2+}\) channels leading to increases in intracellular Ca\(^{2+}\) concentration. Thus, glycine and glutamate receptors may regulate Ca\(^{2+}\)-dependent secretory mechanisms in islet cells by altering the membrane potential of these cells. Our data in GK-P3 cells support the growing weight of evidence for a role of amino acid neurotransmitters in pancreatic islets and introduce strychnine-sensitive glycine receptors as a novel target of amino acid neurotransmitter regulation in islets.

Neurons and pancreatic islet cells have much in common. Both types of cells are electrically excitable and both express a number of proteins found in the CNS. Recently, it has become apparent that machinery used by the CNS for rapid ionotropic synaptic transmission also is found in the endocrine pancreas. GABA, released from β cells, has been postulated to activate GABA\(_A\) receptors on α cells and thereby attenuate glucagon secretion when glucose levels rise (Rorsman et al., 1989). Functional AMPA-type GluRs also are present in islets, but their role in islet physiology is not completely understood (Inagaki et al., 1995; Weaver et al., 1996).

The membrane potential of both neurons and islet cells is vital to their physiological function. Because both cell types express voltage-dependent Ca\(^{2+}\) channels, depolarizations of the membrane often lead to increases in [Ca\(^{2+}\)]\(_i\), which initiates hormone and neurotransmitter release and other long term biochemical alterations. Although excitatory (depolarizing) stimuli usually are associated with channels permeable to Na\(^{+}\) ions, anion channels also may depolarize cells under certain conditions. For example, activation of GABA\(_A\) receptors causes depolarization in neurons after excessive inhibitory synaptic inputs (Staley et al., 1995). GABA receptors activation in the gastropancreatic cell line, AR42J, also has been shown to increase [Ca\(^{2+}\)]\(_i\). The exact mechanism by which this occurs has not been completely elucidated. In one case, it was suggested that collapse of the normal Cl\(^{-}\) gradient coupled with outward fluxes of HCO\(_3\)\(^{-}\), which also is

ABBREVIATIONS: CNS, central nervous system; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; RT, reverse transcription; PCR, polymerase chain reaction; nt, nucleotide; GluR, glutamate receptor; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; I-V, current-voltage; [Ca\(^{2+}\)]\(_i\), intracellular calcium concentration.
permeable through GABA receptors, causes the depolarizing response (Staley et al., 1995).

Permanent lines derived from pancreatic islet cells often contain and secrete the appropriate hormone and maintain the electrical excitability of the parent cell types. In addition, some are known to express glutamate and GABA<sub>a</sub> receptors (Rorsman et al., 1989; Von Blankenfeld et al., 1995). However, to our knowledge, naturally expressed functional glycine receptors have not been detected in any permanent cell line previous to this report.

We have characterized the expression patterns, functional properties, and structural features of amino acid receptors expressed in GK-P3 cells, which are derived from pancreatic β cells. These cells were found to express both strychnine-sensitive glycine receptors and AMPA-type GluRs. We used this cell line to examine how both glycine and AMPA receptors regulate [Ca<sup>2+</sup>], using fluorescent imaging with Fura-2. Our studies revealed that the kinetic and ionic permeability properties of glycine and AMPA receptors in GK-P3 cells were similar to those receptors found in neurons. However, we observed that the activation of either strychnine-sensitive glycine receptors, traditionally regarded as inhibitory, or AMPA receptors increased [Ca<sup>2+</sup>]. The Ca<sup>2+</sup> seemed to enter the cells largely through voltage-gated Ca<sup>2+</sup> channels. Thus, because of their ability to depolarize GK-P3 cells, glycine receptors and GluRs may be capable of stimulating or potentiating peptide hormone secretion from islets. In addition, our results suggest that because neither islet glycine receptors nor GluRs are Ca<sup>2+</sup>-permeable, their ability to increase intracellular Ca<sup>2+</sup> depends on their coexpression with voltage-gated Ca<sup>2+</sup> channels and on the ionic gradients present in the cells in which they are expressed.

**Experimental Procedures**

**Materials.** AMPA was obtained from Research Biochemicals (Natick, MA). Kainic acid, NMDA, leupeptin, pepstatin A, and amphotericin B were obtained from Sigma Chemical (St. Louis, MO). CNQX and dizocilpine maleate (MK-801) were obtained from Research Biochemicals. Cyclothiazide was obtained from Dr. Robert Wenthold. Anti-GluRA and anti-GluRD antibodies were purchased from Chemicon International (Temecula, CA). The anti-glycine receptor antibody was a gift from Dr. Heinrich Betz. Alkaline phosphatase-labeled goat anti-rabbit and goat anti-mouse antibodies were purchased from DAKO (Carpinteria, CA). Bicinechonic acid protein assay reagents were purchased from Pierce Chemical (Rockford, IL). All other chemicals were of reagent grade or higher.

**Tissue preparation and culture.** GK-P3 cells were derived from an insulinoma removed from a transgenic mouse expressing a gialine receptor 14) linked to the SV40 large T antigen gene (Jetton TL, et al., 1998). Experiments were performed on GK-P3 cells grown for 6–20 passages in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal bovine serum.

**Electrophysiological methods.** Ionic currents were measured by whole-cell patch-clamp electrophysiological methods using either an Axopatch 200 (Axon Instruments, Foster City, CA) or a Dagan PC-1 Amplifier (Dagan Corporation, Minneapolis, MN). Signals were filtered at 2000 Hz (~3 dB, eight-pole Bessel filter; Frequency Devices, Haverhill, MA), digitized at 5000 Hz using an ITC-16 interface (Instrutech, Great Neck, NY), and recorded on a Macintosh Quadra 800 or IIfx computer. The cells were perfused continuously with an extracellular solution containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.2. In some experiments, an islet external solution was used consisting of 145 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 2 mM glucose, and 10 mM HEPES, pH 7.3. Most currents were measured in conventional whole-cell mode using patch pipettes filled with a solution that consisted of 135 mM CsCl, 1 mM MgCl<sub>2</sub>, 11 mM EGTA, and 10 mM HEPES, pH 7.3. In some cases, electrical access to the cell was obtained using the perforated patch method. In these experiments, patch pipettes were filled with a solution containing 10 mM KCl, 10 mM NaCl, 70 mM K<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES, pH 7.3, and 240 μg/ml amphotericin B. Rapid agonist application experiments were performed by lifting a single cell from the bottom of the culture dish after whole-cell recording was established. The cell was perfused continuously by control external solution through one side of a glass θ tubing pipette. Agonist-containing solution flowed in the other side of the pipette. The solution bathing the cell was exchanged by stepping the application pipette with a piezoelectric manipulator so that the side of the pipette containing agonist solution faced the cell. The time required for complete agonist exchange was ~10 msec. In situations that did not require rapid application, cells remained attached to the bottom of the culture dish for the duration of the recording. In these cases, the cell was continuously bathed in control solution from a flow pipe connected to eight reservoirs. Solution exchange was accomplished by opening a single valve at a time.

For determining HCO<sub>3</sub> permeability through glycine receptors, external solutions were prepared fresh daily as described above except Cl<sup>−</sup> was replaced by HCO<sub>3</sub> at the concentrations of 0 mM HCO<sub>3</sub> and 145 mM Cl<sup>−</sup>, 36 mM HCO<sub>3</sub> and 109 mM Cl<sup>−</sup>, 109 mM HCO<sub>3</sub> and 36 mM Cl<sup>−</sup>, and 145 mM HCO<sub>3</sub> and 0 mM Cl<sup>−</sup>. For calcium permeability experiments with AMPA receptors, the solutions used for perfusion were Na<sup>+</sup> solution, which consisted of 147 mM NaCl, 1.8 mM CaCl<sub>2</sub>, and 5 mM HEPES, pH 7.2, or Ca<sup>2+</sup> solution, which consisted of 92 mM CaCl<sub>2</sub> and 5 mM HEPES, pH 7.2. Current-voltage (I-V) curves were generated by ramping the voltage from −60 mV to 60 mV at a rate of 30 mV/sec first in the absence and then in the presence of agonist. The I-V curve obtained in the absence of agonist then was subtracted from the curve obtained in the presence of agonist to yield the agonist-evoked I-V curve. Permeability ratios were calculated from measured reversal potentials according to the following variants of the Goldman-Hodgkin-Katz equation: \( E_{rev} = RT/F \ln P_{CA}/P_{Na} \), \( E_{rev} = RT/F \ln (P_{CA}/P_{Cs}) \), or \( E_{rev} = RT/F \ln P_{Ca}/P_{Na} \). Ca<sup>2+</sup>/Na<sup>+</sup> permeability ratios (\( P_{Ca}/P_{Na} \)) were calculated by dividing the \( P_{Ca}/P_{Na} \) ratio by \( P_{Cs}/P_{Na} \). Data were analyzed using Igor, Excel, and WingZ computer programs.

La<sup>3+</sup> block of Ca<sup>2+</sup> channels was performed in standard whole-cell mode by stepping from −80 mV to 0 mV. Leak subtraction was performed by estimating the leak current by stepping four times from −80 mV to −100 mV, summing the current from the four steps, and subtracting it from the current measured during the step from −80 mV to 0 mV. The external solution consisted of 25 mM BaCl<sub>2</sub>, 120 mM N-methyl-d-glucamine, and 10 mM HEPES, pH 7.3, in the presence or absence of 30 μM LaCl<sub>3</sub>. The internal solution consisted of 125 mM N-methyl-glucamine, 20 mM tetraethylammonium chloride, 14 mM Tris<sub>2</sub>-phosphocreatine, 11 mM EGTA, 1 mM CaCl<sub>2</sub>, 4 mM Mg-ATP, and 0.3 mM Tris-GTP. The pH was adjusted to 7.2 with methane sulfonic acid.

**Ratiometric imaging.** The experimental setup for ratio imaging studies consisted of a Nikon Diaphot inverted microscope with a 40× oil immersion Fluor objective. Epifluorescence illumination was provided by a 100-W mercury burner. The excitation wavelength was set by bandpass filters of 340 or 380 nm and changed by a Luld high-speed filter wheel. The intensity of illumination was set by neutral density filters, also controlled by the Luld filter wheel. Images were collected through a Dage CCD72 video camera and a DAGE GenII-Sys Image Intensifier. Computer acquisition of the images and control of the filter wheel were provided by the program Simca (Compix), which also was used for analyzing the images. With this system, ratio images were recorded at a speed of ~1 frame/sec.
For imaging studies, cells were cultured on No. 1 glass coverslips for 24–72 hr before being loaded with 2 μM Fura-2 AM ester. Before dilution into islet external, Fura-2 was prepared as a 1 mM stock in dimethyl sulfoxide containing 20% (v/v) Pluronic. The cells were loaded for 30 min at room temperature before being washed twice with islet external and placed in a perfusion chamber. Punctate fluorescence was not observed, indicating that compartmentalization of the dye had not occurred with this loading procedure. Cells were perfused continuously with appropriate external solutions. Agonists and modulators were applied using a flow pipette similar to that described above. Images were collected continuously throughout each experiment. With the help of the imaging software, each cell in a field of view was defined as a region of interest, and the data from that cell were stored separately for later analysis.

Ratio measurements were converted into estimates of [Ca2+]i, according to the equation (Grynkiewicz et al., 1985)

\[
[Ca^{2+}]_i = K_a \times \frac{R - R_{min}}{R_{max} - R} \times \frac{F(380)_{max}}{F(380)_{min}}
\]

The values of \(R_{max}\), \(R_{min}\), and \(F(380)_{min}\) and \(F(380)_{max}\) for our experimental setup were determined using a [Ca2+]i calibration kit purchased from Molecular Probes (Eugene, OR). For most studies, \(R_{min} = 0.35\) and \(R_{max} = 7.0\), but some early studies were done using a different epifluorescent light source that gave \(R_{min}\) and \(R_{max}\) values of 0.2 and 5.0, respectively. These values were rechecked weekly to control for aging of the light source. The accuracy of the calibration also was checked using a wide variety of [Ca2+]i standard solutions provided by Molecular Probes. Although the values for [Ca2+]i seem to be consistent internally, the known difficulties in accurately calibrating ratio measurements and the unknown extent to which Fura-2 itself alters the Ca2+ dynamics inside cells (Neher, 1995) mean that the [Ca2+]i values reported here should be considered estimates.

An Igor macro was used to measure simultaneously the amplitudes of [Ca2+]i signals in a large number of cells. For each cell, a section of base-line signal was analyzed to obtain the average [Ca2+]i values and the standard deviation of the measurement. Then, data points covering an agonist application were analyzed to measure the peak change in [Ca2+]i, which then was defined as \(\Delta[Ca^{2+}]_i\). A cell was defined as having a positive signal if the [Ca2+]i value was greater than the base-line [Ca2+]i, plus one standard deviation. The [Ca2+]i values were converted to log for statistical analysis because otherwise they were not normally distributed about the mean.

Immunological methods. GK-P3 cells were harvested from tissue culture flasks with a cell scraper and pelleted by centrifugation at 2000 rpm for 5 min at 4°C in a Beckman TJ-6 centrifuge. The supernatant solutions were aspirated and the cell pellets were rinsed twice with extraction buffer that consisted of 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.002 mM leupeptin, and 0.002 mM pepstatin A. The pellets then were resuspended in 3 ml of extraction buffer. The resuspended samples were sonicated twice for 15 sec at 30-sec intervals and then centrifuged at 100,000 × g for 1 hr at 4°C. The supernatant fractions were aspirated away, and the crude membrane pellets were rinsed with extraction buffer and resuspended in 200 μl of a buffer consisting of 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, and 0.1% (w/v) SDS. Islet and brain membranes were prepared as described previously (Weaver et al., 1996).

Membrane proteins were solubilized in SDS-sample buffer that consisted of 250 mM Tris, pH 6.8, 500 mM dithiothreitol, and 8% (w/v) SDS. Solubilized membrane proteins were separated on SDS-polyacrylamide gels according to the method of Laemmli (1970). Separated membrane proteins were blotted to nitrocellulose in a blotting buffer that consisted of 25 mM Tris base, 191 mM glycine, and 20% (vol/vol) methanol using a Genie electroblotter (Idea Scientific, Minneapolis, MN) for 1 hr at 1 A. The membrane then was blocked with a solution of 10% (v/v) nonfat dry milk in PBS consisting of 136 mM NaCl, 10 mM KCl, 32 mM NaHPO4, and 5 mM K2HPO4, pH 7.2) for 1 hr and washed three times for 10 min/wash with PBS containing 0.05% (v/v) Tween 20. The blot then was transferred to a multichannel slot blotter (Idea Scientific) and probed for 30 min at room temperature with each of these antibodies (one antibody per slot) diluted in PBS containing 0.5% (v/v) goat serum: anti-GluRA (2 μg/ml), anti-GluRB (1 μg/ml), anti-GluRD (2 μg/ml), and the glycine receptor antibody, mAB4a (1:500). After incubation, each channel was washed with 10 ml of PBS containing 0.05% (v/v) Tween 20. The blot then was removed from the multichannel blotter and washed twice for 10 min/wash in TBST consisting of 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20. The blot was incubated for 30 min with a mixture of alkaline phosphatase-labeled goat anti-rabbit (0.3 μg/ml) and goat anti-mouse (0.3 μg/ml) diluted in TBST containing 0.5% (v/v) goat serum. The blot was washed three times for 10 min/wash with TBST and was developed by adding 0.33 mg/ml nitro blue tetrazolium and 0.17 mg/ml bromochloroindolyl phosphate diluted in a buffer consisting of 100 mM Tris pH 9.5, 100 mM NaCl, and 5 mM MgCl2.

RNA PCR analysis. GK-P3 cells were cultured as described. RT-PCR was performed using a two-step RNA PCR kit (PerkinElmer-Cetus) according to the supplier’s directions. RNA was isolated according to the AGCP method (Chomczynski and Sacchi, 1987) from rat brain, GK-P3 cells, and rat liver and then poly(A)+ selected using the PolyA Tract system (Promega, Madison, WI). Poly(A)+ RNA (100 ng) was used for RT-PCR amplification. Primer pairs were designed to correspond to different exons so that unspliced precursor mRNA, if present, would amplify as larger fragments because of intron or introns. Poly(A)+ RNA was converted to cDNA at 42°C using primers specific to either GluRB (nt 3051-CCATGTGTTAGCGACCTCAAGAGGTCTCC-nt 3080) or GluRC (nt 2933-TAGGCCGGGGCAAGAGGATTPTCAAG-nt 2954) (GluRB and GluRC-specific primers were a gift from R. Emeson). Glutamate receptor sequences then were amplified using a primer to a conserved transmembrane region designed to amplify all of the AMPA receptor subtypes (TM1 corresponding to GluRB “flip” nt 861-TAT-GAAATCTGGATGTGTCAT-nt 1681) and either GluRB or GluRC primers by melting at 95°C for 1 min, annealing at 60°C for 1 min, and extending at 72°C for 2.5 min for 30 cycles. PCR products were separated on 1.2% (w/v) agarose gels, blotted onto Zeta Probe nylon membrane (BioRad) using 0.4 N NaOH, probed with oligomers (100 ng) corresponding to a conserved region in AMPA receptors (GluRB “flip” nt 2635-GCTTCCGGAGTTCTGTC-nt 2651), and detected with autoradiography.

Results

Amino acid receptors in pancreatic GK-P3 cells. We detected functional receptors for glycine, glutamate, and GABA in the pancreatic β cell line GK-P3. Glycine receptors appeared in 98% of the GK-P3 cells tested. However, we failed to observe glycine-elicted currents in the gluta- mate and GABA receptor-containing pancreatic α cell lines (GluRC and α TC-9 (34 cells). Responses to glutamate were seen in 60% of GK-P3 cells, and GABA receptor found in 23% of the cells tested. The average amplitude of currents evoked by various ligands in GK-P3 cells is shown in Table 1. Although cells containing glycine receptors often coexpressed GluRs, GABA receptor-containing pancreatic α cell lines (GluRC and GluRS) appeared in only 1 of the 30 GK-P3 cells tested. Because the properties of GABA receptors in pancreatic cell lines (Tyndale et al., 1994; Von Blankenfeld et al., 1995) have been more examined thoroughly, we focused our attention on characterization of glycine receptors and GluRs in these cells.
Pancreatic receptors are closely related to neuronal receptors. Currents elicited by 100 μM glycine in GK-P3 cells seemed to arise from the glycine receptor associated with chloride channels. These currents were not due to activation of the glycine receptor associated with the NMDA/receptor complex. Glycine-evoked currents were blocked completely by 1 μM strychnine (Fig. 1A) and were insensitive to the NMDA receptor channel blocker MK-801 (Fig. 1B). Currents also were activated by two other agonists for the native glycine receptor: 300 μM taurine and 300 μM β-alanine (Fig. 1C, Table 1). Zinc was an effective antagonist of these receptors (Fig. 1D) with 50 μM Zn²⁺ blocking an average of 88 ± 3% of the currents evoked by 100 μM glycine (10 cells). The concentration-response relation for glycine gave an EC₅₀ value of 90.4 (95% confidence interval, 66.1–123) μM (11 cells) and a Hill coefficient of 2.34 ± 0.28. Ionic substitution experiments were performed in which the extracellular chloride was replaced with HCO₃⁻. The magnitude of the rightward shift in the I-V relation indicated that the Cl⁻/HCO₃⁻ permeability ratio was 5.6 ± 0.4 (four cells), which is identical to that found previously for neuronal glycine receptors (Bormann et al., 1987).

GK-P3 cell glycine receptors also were structurally similar to neuronal glycine receptors. An antibody that recognizes a number of glycine receptor subunits (Pfeiffer et al., 1984) showed immunoreactivity with GK-P3 cells (Fig. 3A). This antibody also recognized a band of similar molecular weight in membrane proteins isolated from rat brain and isolated rat pancreatic islets. Thus, the expression of glycine receptors in GK-P3 cells is not likely an artifact of transformed cell lines but may be indicative of similar receptors in native islet cells. Preliminary analysis of glycine receptor mRNA revealed expression of the α3 and not the α1 or α2 variants (data not shown).

The glutamate-evoked currents we observed in GK-P3 cells showed kinetic properties indicative of the AMPA receptor subtype. Currents evoked by the rapid application of 1 mM glutamate to a single GK-P3 cell showed two components similar to those seen with neuronal AMPA receptors (Fig. 2A). The peak current desensitized rapidly with a single exponential time constant that averaged 7.82 ± 1.22 msec (four cells). Kainate-evoked currents showed no desensitization in these experiments (five cells). The pharmacological properties of the GK-P3 GluRs also indicated that AMPA receptors were the main type of GluRs expressed in these cells. Steady state currents evoked by 300 μM glutamate were potentiated 1.2–29.7-fold by cyclothiazide, a compound that potentiates neuronal AMPA receptors (Yamada and Tang, 1993) (Fig. 2B). The average potentiation of the steady state current in the presence of 50 μM cyclothiazide was 6.9 ± 5.0 fold (six cells). The concentration-response relation for glutamate (Fig. 2B) measured in GK-P3 cells in the presence of the 50 μM cyclothiazide revealed an average EC₅₀ value of 164 μM (range, 35–758 μM) (three cells). In GK-P3 cells, the selective AMPA receptor antagonist CNQX (10 μM) completely blocked the current evoked by 300 μM kainate (four cells) (Fig. 2C). Currents were not observed on application of 30 μM NMDA in the presence of 10 μM glycine in Mg²⁺ free extracellular solution (six GK-P3 cells), indicating a lack of NMDA receptors in GK-P3 cells.

The calcium permeability of AMPA receptors depends on subunit structure and RNA editing (Sommer et al., 1991). Because the levels of intracellular calcium are important for insulin secretion, we examined the calcium permeability of the AMPA receptors expressed in GK-P3 cells. For these experiments, Ca²⁺/Na⁺ permeability ratios (PCa/PNa) were calculated from measured reversal potentials in the presence of either 147 mM external Na⁺ or 92 mM external Ca²⁺. Currents evoked by 300 μM glutamate and 50 μM cyclothiazide had an average reversal potential of −2.39 ± 0.72 mV (seven cells) in the presence of high external Na⁺ and an average reversal potential of −34.97 ± 5.35 mV (seven cells) in the presence of high external Ca²⁺. These measurements yielded an average PCa/PNa of 0.046 ± 0.02 (seven cells). The estimated Ca²⁺ permeability of receptors in GK-P3 cells was somewhat variable, however. Two of nine GK-P3 cells exhibited inward rectification along with higher PCa/PNa values (0.17). This suggests that a subpopulation of GK-P3 cells contain AMPA receptors with greater average Ca²⁺ permeability.

In an immunological survey of GK-P3 cells with subunit-specific anti-GluR antibodies (Petralia and Wenthold, 1992; Wenthold et al., 1992, 1994; Petralia et al., 1994), we detected immunoreactivity with an anti-GluRβ/C antibody (Fig. 3B) but not with either the GluRA or the GluRD antibodies. RT-PCR of mRNA derived from GK-P3 cells showed that mRNA encoding both GluRB and GluRC were expressed (Fig. 3C). These results confirm that the compliment of AMPA receptors expressed in GK-P3 cells is the same as that found in native islets (Weaver et al., 1996).

**Receptor activation regulates [Ca²⁺].** Membrane depolarization is a critical step in the signal transduction mechanism that links serum glucose levels to insulin secretion from pancreatic β cells. Therefore, we examined the ability of glycine and glutamate to alter the membrane potential of GK-P3 cells through actions at their respective receptors. In cases where both whole-cell capacitance and current amplitudes were measured, GK-P3 cells exhibited an average steady state current density of 21 ± 2.7 pA/pF (63 cells) on application of 100 μM glycine, whereas 300 μM glutamate gave an average current density of 1.99 ± 0.32 pA/pF (11 cells). Whole-cell recordings of membrane potential using perforated patch revealed that the application of 300 μM

### Table 1

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Current amplitude (cells positive/cells tested)</th>
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<tbody>
<tr>
<td>Glycine (100 μM)</td>
<td>134 ± 19 pA (74/76)</td>
</tr>
<tr>
<td>β-Alanine (300 μM)</td>
<td>237 ± 111 pA (9/9)</td>
</tr>
<tr>
<td>Taurine (300 μM)</td>
<td>50.9 ± 22.8 pA (6/6)</td>
</tr>
<tr>
<td>Glutamate (300 μM)</td>
<td>37.7 ± 6.7 pA (51/89)</td>
</tr>
<tr>
<td>Kainate (300 μM)</td>
<td>40.4 ± 7.2 pA (23/29)</td>
</tr>
<tr>
<td>AMPA (100 μM)</td>
<td>44.4 ± 23.6 pA (5/8)</td>
</tr>
<tr>
<td>NMDA (30 μM)</td>
<td>82.4 ± 14.9 pA (10/65)</td>
</tr>
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</table>

Reported are the mean ± standard error current amplitude evoked by the indicated agonist in all positive cells identified. The number of positive cells and the total number of cells tested (number positive/number tested) are shown below the amplitudes.
glutamate depolarized these cells an average of 27 ± 6.8 mV (five cells) (Fig. 2D) from their normal resting membrane potential of −50.5 ± 3.6 mV (five cells). This depolarization led to the firing of repetitive action potentials similar to those observed in pancreatic β cells on exposure to high glucose (Rorsman and Trube, 1986). Because of uncertainty in the effects of whole-cell patch-clamp on the normal Cl− gradients in GK-P3 cells, we did not conduct a similar experiment with glycine receptor agonists but instead turned to ratiometric imaging of [Ca2+]i.

In addition to amino acid neurotransmitter receptors, GK-P3 cells harbor functional voltage-sensitive Ca2+ channels. The activation of these channels by membrane depolarization allows influx of significant amounts of Ca2+. Indeed, ratiometric imaging of [Ca2+]i with Fura-2 revealed that depolarization evoked by application of 40 mM K+ to these cells produced marked increases in [Ca2+]i (Table 2). A subpopulation of cells also responded to glutamate (300 μM) with increases in [Ca2+]i, and these responses were greatly potentiated by 50 μM cyclothiazide even in cells that did not respond to glutamate alone (Table 2, Fig. 4). Glycine (100 μM) also increased [Ca2+]i in many GK-P3 cells, presumably by

Fig. 1. Glycine-evoked currents in GK-P3 cells are indicative of neuronal inhibitory glycine receptors. In all cases, currents were recorded under conventional whole-cell voltage-clamp (Vhold = −60). Solid bars, application of agonists. Stippled bars over the current traces, application of antagonists. A, Inward currents evoked by glycine were completely and reversibly blocked by 1 mM strychnine. B, Glycine-evoked currents were completely insensitive to block by the NMDA receptor antagonist, MK-801. C, Currents also were evoked by glycine receptor agonist taurine and β-alanine. D, Zinc (50 μM) blocked currents mediated by GK-P3 glycine receptors.

Fig. 2. AMPA receptors in GK-P3 cells showed properties similar to those of neuronal AMPA receptors. Solid bars, application of agonist. Stippled bars over the current traces, antagonists or modulators. All currents were recorded in standard whole-cell mode with voltage clamped at −60 mV. A, Rapid application of 1 mM glutamate produced currents with rapid desensitization. B, Steady state currents evoked by 300 μM glutamate by the AMPA receptor were potentiated by cyclothiazide. C, Currents evoked by the AMPA receptor agonist kainate (300 μM) were blocked by 10 μM CNQX, a competitive antagonist of neuronal AMPA receptors. D, Application of 300 μM glutamate depolarized GK-P3 cells and elicited action potentials typical of pancreatic β cells. Voltage recordings were made using perforated patch mode at the resting membrane potential, which in this cell was −52 mV.
depolarizing the cells and activating voltage-gated Ca\(^{2+}\) channels. These responses were blocked completely by 1 \(\mu M\) strychnine (Table 2). Thus, under the ionic conditions used here, Cl\(^-\) seemed to flow out of GK-P3 cells on glycine receptor activation, leading to membrane depolarization. To test the role of HCO\(_3\)\(^-\) in setting the equilibrium potential for glycine currents, we measured the size of Ca\(^{2+}\) signals evoked by glycine when the extracellular concentrations of Cl\(^-\) and HCO\(_3\)\(^-\) were altered. There were no significant differences in signal amplitude because the NaCl was incrementally replaced with NaHCO\(_3\) (not shown). Thus extracellular differences in signal amplitude because the NaCl was incrementally replaced with NaHCO\(_3\) (not shown). Thus extracellular differences in signal amplitude because the NaCl was incrementally replaced with NaHCO\(_3\) (not shown).

Islet membranes also contain some membrane proteins isolated from brain (20 \(\mu g\)), GK-P3 cells (30 \(\mu g\)), and isolated rat pancreatic islets (25 \(\mu g\)) probed with an antibody that recognizes glycine receptor subunits, mAAb4a. Bands of similar size (50 kD) were seen in all three preparations. Islet membranes also contain some bands of lower apparent molecular weight. B, Western blots of membrane proteins from brain (20 \(\mu g\)) and GK-P3 cells (30 \(\mu g\)) probed with the subunit-specific anti-GluR antibodies GluRA, GluRB/C, and anti-GluRD.

Results of RT-PCR analysis of GluR expression in GK-P3 cells are illustrated. PCR amplicons were generated from poly(A)\(^+\) RNA from rat brain, GK-P3 cells, and rat liver. RNA was converted to cDNA using primers specific to GluRB or GluRC (3\' end). GluR sequences were amplified using TM1 and GluRB or GluRC primers, separated on a 1.2% (w/v) agarose gel, transferred to nitrocellulose, then probed with GluR-specific oligonucleotides.

**Table 2**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Change in [Ca(^{2+})] (cells positive/cells tested)</th>
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<tbody>
<tr>
<td>Glycine (100 (\mu M))</td>
<td>158 (141–178) nM (193/501)</td>
</tr>
<tr>
<td>Glycine + strychnine (1 (\mu M))</td>
<td>2.1 (1.4–3.2) nM (0/45)</td>
</tr>
<tr>
<td>Glutamate (300 (\mu M))</td>
<td>57.5 (40.7–79.4) nM (41/153)</td>
</tr>
<tr>
<td>Glutamate + cyclosporin (50 (\mu M))</td>
<td>251 (218–288) nM (268/333)</td>
</tr>
<tr>
<td>High K(^+) (40 (\mu M))</td>
<td>652 (575–741) nM (332/347)</td>
</tr>
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To test the postulate that most of the Ca\(^{2+}\) measured in the Fura-2 studies fluxed into the cells through voltage-activated Ca\(^{2+}\) channels, we used an inorganic blocker of these channels, La\(^{3+}\). Increases in [Ca\(^{2+}\)]\(_i\), evoked by glutamate plus cyclothiazide or glycine were completely inhibited in the presence of 30 \(\mu M\) La\(^{3+}\) as were increases in [Ca\(^{2+}\)]\(_i\), in response to high K\(^+\). Ba\(^{2+}\) currents through Ca\(^{2+}\) channels in GK-P3 cells averaged 54.4 ± 0.64 pA (five cells). La\(^{3+}\) (30 \(\mu M\)) reversibly blocked these currents (0.7 ± 0.1% of control, five cells). Currents evoked by glutamate plus cyclothiazide or glycine measured under patch-clamp at −60 mV were completely insensitive to 30 \(\mu M\) La\(^{3+}\). The mean amplitude of currents evoked by 100 \(\mu M\) glycine were 97 ± 50 pA under control conditions and 100 ± 52 pA (six cells) in the presence of 30 \(\mu M\) La\(^{3+}\). The responses to 300 \(\mu M\) glutamate plus 50 \(\mu M\) cyclothiazide averaged 219 ± 48 pA in the absence and 204 ± 47 pA (seven cells) in the presence of 30 \(\mu M\) La\(^{3+}\). These results suggest that increases in [Ca\(^{2+}\)]\(_i\), mediated by AMPA and glycine receptors are likely to secondary activation of voltage-gated Ca\(^{2+}\) channels by membrane depolarization.

**Discussion**

We characterized the amino acid neurotransmitter receptors expressed in a cell line derived from the endocrine pancreas. These cells were used to examine the mechanisms of Ca\(^{2+}\) signaling mediated by these ligand-gated ion channels. The major findings of our work are (1) strychnine-sensitive glycine receptors and AMPA-type GluRs were expressed in the β cell-derived cell line, GK-P3; (2) the structural, pharmacological, and ionic permeability properties of GK-P3 glycine and AMPA receptors were very similar to those of neuronal receptors, (3) activation of glycine receptors, which are anion channels, depolarized GK-P3 cells and activated voltage-gated Ca\(^{2+}\) channels, causing increases in [Ca\(^{2+}\)]\(_i\), (4) AMPA receptor cation channels also were capable of increasing [Ca\(^{2+}\)]\(_i\), through a similar mechanism.

In addition to neuronal cells derived from pluripotent precursors (Turetsky et al., 1993; Younkin et al., 1993), a number of immortalized lines of islet cells have been shown to express functional ligand-gated ion channels. Glutamate receptors have been reported in β cell-like MIN6 cells (Goni et al., 1994), and GABA receptors have been reported in native α and δ cells based on immunological analysis and in α cells based on electrophysiological measurements (Rorsman et al., 1989). GABA receptors also have been detected in other gastropancreatic cell lines, such as AR42J and RIN (Tytendale et al., 1994; Von Blankenfeld et al., 1995). However, the GK-P3 cells represent the first transformed line reported to harbor functional, strychnine-sensitive glycine receptors.

The expression pattern of amino acid receptors in islet cell lines often approximates that of native islets. Previously, we have shown that AMPA receptors are distributed in all islet cells, except somatostatin-containing δ cells, and that kainate receptors are found in αTC cells as well as native α cells (Weaver et al., 1996). Others have found immunoreactivity for GABA receptor subunits in native α cells (Rorsman et al., 1991). Western blots of GK-P3 and islet cell membrane proteins using a glycine receptor antibody revealed proteins of an apparent molecular weight consistent with that of neuro-
nal glycine receptors. These data suggest that glycine receptors expression in GK-P3 cells may be indicative of glycine receptor expression in native pancreatic islets. Previous reports have demonstrated that perfusion of islets with relatively high concentrations of glycine transforms glucose-induced fluctuations of $[\text{Ca}^{2+}]_i$ into sustained increases (Tengholm et al., 1992; Hellman et al., 1994). Our data suggest that these effects may be mediated by islet glycine receptors. Determining the strychnine sensitivity of the effects of glycine on insulin secretion clearly will be an area of interest for further research.

As mentioned, the mouse insulinoma cell line MIN6 has been shown to possess functional ionotropic GluRs (Gono et al., 1994). In contrast to the current results, MIN6 cells were reported to express active AMPA and NMDA receptor types when measured by patch-clamp. Analysis of mRNA also showed transcripts for a variety of GluR subunits, including NMDAR1, NMDAR2D, GluRB, GluRC, and KA2. We did not extensively analyze the expression of all these mRNA species and thus cannot rule out the possibility that GK-P3 cells harbor mRNAs encoding other GluR types. However, the evidence presented here clearly shows that the only functional GluRs detectable in GK-P3 cells belong to the AMPA receptor subtype. Our previous immunological studies on isolated rat islets did not reveal expression of NMDAR1 protein (Weaver et al., 1996), but islets did show GluRB/C and GluRB immunoreactivity very similar to that seen in GK-P3 cells. Thus, it seems that the constellation of GluRs expressed in GK-P3 cells is more similar to native islets than to those found in MIN6 cells and that AMPA receptors and not NMDA receptors are the main type of GluRs in pancreatic islets. Consistent with this proposal, perfusion experiments with whole pancreas have shown that activation of AMPA receptors, but not NMDA receptors, potentiates secretion of insulin (Bertrand et al., 1992) and glucagon (Bertrand et al., 1993).

Glycine and GABA receptors in the CNS generally are considered to be inhibitory because of their selectivity for anions. However, a number of recent observations suggest that in some situations GABA receptor activation also can be excitatory (Staley et al., 1995; Von Blankenfeld et al., 1995). The exact reason for this remains debatable, but the two most attractive hypotheses suggest that depolarizing responses to GABA are caused by variations in the concentration gradient for the permeant anions Cl$^-$ (Misfeld et al., 1986) or HCO$_3^-$ (Staley et al., 1995). The permeability properties of GK-P3 glycine receptors are identical to those of glycine or GABA receptors expressed in spinal neurons (Bormann et al., 1987) and thus represent a convenient system in which this issue can be examined. In many GK-P3 cells, glycine receptor activation depolarizes the cells sufficiently to activate voltage-dependent Ca$^{2+}$ channels, leading to increases in $[\text{Ca}^{2+}]_i$, which blocks Ca$^{2+}$ channels, but not glycine receptor currents, completely inhibited the increase in $[\text{Ca}^{2+}]_i$ normally seen with glycine. By contrast, the extracellular concentration of HCO$_3^-$ had little influence on the Ca$^{2+}$ signals observed in these studies. It seems likely that normal intracellular concentrations of Cl$^-$ and HCO$_3^-$, which were undisturbed during our Ca$^{2+}$ imaging experiments, are sufficient to generate a net efflux of anions on receptor activation. This depolarizes the cell and opens voltage-dependent Ca$^{2+}$ channels, allowing influx of Ca$^{2+}$.

GABA receptor activation in AR42J cells (Von Blankenfeld et al., 1994) likewise has been shown to increase $[\text{Ca}^{2+}]_i$ in AR42J cells. Further investigations into the properties of GK-P3 and AR42J cells may help to reveal the mechanisms by which glycine and GABA can become excitatory neurotransmitters. In the future, it will be important to investigate the expression of strychnine-sensitive glycine receptors in native islets and to attempt to determine whether the anion gradients in native islet cells would cause glycine receptors to function as observed in GK-P3 cells.

Glutamate also was able to depolarize GK-P3 cells enough to increase $[\text{Ca}^{2+}]_i$. Because La$^{3+}$ blocked the responses to

![Fig. 4. Glutamate and glycine receptor activation increased $[\text{Ca}^{2+}]_i$ in GK-P3 cells. Shown are ratiometric measurements of $[\text{Ca}^{2+}]_i$ in Fura-2-loaded GK-P3 cells. Solid bars, agonists were perfused on the cells during the time indicated by the. Stippled bars, perfusion of modulators or antagonists. Each trace, measurements from representative cells in the same field of view to illustrate the variety of response observed. Typically, 20–30 cells were measured at the same time.](image-url)
glutamate, the major component of the signals generated by AMPA receptor activation were probably due to flux of Ca2+ through voltage-gated Ca2+ channels. In other experiments, extracellular Na+ was replaced with the impermeant cation N-methylglucamine, and this also prevented AMPA receptor activation from increasing [Ca2+]i. (Partridge JG and Verdoorn TA, unpublished observations). This result is not particularly surprising based on the low average Ca2+ permeability exhibited by the AMPA receptors in GK-P3 cells. However, human embryonic kidney 293 cells expressing recombinant AMPA receptors that contain an edited GluR2 subunit and exhibit low Ca2+ permeability mediate large increases in [Ca2+]i, as measured by the same assay (Utz and Verdoorn, 1997). Thus, factors other than Ca2+ permeability may dominate the ability of AMPA receptors to alter [Ca2+]i. These factors may include receptor density, the other ion channels that are coexpressed with AMPA receptors in different cell types, and the differential ability of cell types to resist changes in measurable [Ca2+]i, through Ca2+-buffering proteins or by the ability of the cell to extrude Ca2+ from intracellular solutions.

The exact role of amino acid neurotransmitter systems in pancreatic islets remains uncertain. The postulated role of GABA in communicating between α and β cells (Rorsman et al., 1989) remains an important model for exploration of this issue. In this vein, glutamate and glycine could mediate signaling between different cells within an islet in addition to subserving communication between islets and the central nervous system. Recently, we detected high affinity, Na+-dependent glutamate uptake in isolated pancreatic islets, which has the capability of clearing glutamate from extracellular spaces and preventing chronic depolarization of the receptors there (Weaver et al., 1998). Similar processes may facilitate GABA and glycine signaling in islets.

It seems likely that fine tuning of islet hormone secretion under normal physiological conditions may be due in part to factors other than Ca2+ permeability in pathological situations. Indeed, block of GluR desensitization by compounds such as diazoxide, which also is an insulin secretagogue acting at ATP-sensitive K+ channels, may underlie some of the therapeutic benefits of this class of drugs.

Acknowledgments

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Verdoorn CD, Gundersen V, and Verdoorn TA (1998) A high affinity glutamate/ aspartate transport system targets for modifying islet function in pathological situations. Indeed, block of Glur desensitization by compounds such as diazoxide, which also is an insulin secretagogue acting at ATP-sensitive K+ channels, may underlie some of the therapeutic benefits of this class of drugs.


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