Expression of functional GABA\(_A\) receptors in neuroendocrine gastropancreatic cells

Abstract  Gastropancreatic neuroendocrine cells synthesize large amounts of \(\gamma\)-aminobutyric acid (GABA). This amino acid neurotransmitter appears to be stored in and released from, vesicles similar to small synaptic vesicles. So far, the function of GABA in gastropancreatic, neuroendocrine cells has not been clarified. Previous work suggested that only pancreatic, glucagon-producing \(\alpha_2\) cells contain functional GABA\(_A\) receptors. Using subunit-specific antibodies in sections of human antral mucosa, a human gastrinoma and rat pancreas, we show that expression of GABA\(_A\) receptors is abundant in gastropancreatic, neuroendocrine cells. Using the patch-clamp technique in the whole-cell mode we demonstrate that both the rat insulinoma cell line RIN 38 and the ampiphicrine cell line AR42J express functional GABA\(_A\) receptors, which are characterized by a relatively low benzodiazepine and Zn\(^{2+}\) sensitivity and by an insensitivity to the inverse benzodiazepine agonist 6,7-\(\alpha\)-methoxy-4-ethyl-\(\beta\)-carboline-3-carboxylate (DMCM). In contrast to neurons, activation of GABA\(_A\) receptors leads to a membrane depolarization. This depolarization presumably activates voltage-gated Ca\(^{2+}\) channels, resulting in an increase in cytosolic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_i\), as shown with the fluorimetric dye fura-2. The combination of GABA release, GABA\(_A\) receptor activation and the [Ca\(^{2+}\)]\(_i\) increase could constitute an autocrine mechanism, modulating the release of hormones such as gastrin, insulin and somatostatin.

Key words  Pancreatic \(\beta\) cells \cdot GABA\(_A\) receptor subunits \cdot Ca\(^{2+}\) signalling \cdot GABA secretion \cdot Patch-clamp \cdot Fura-2

Introduction

\(\gamma\)-Aminobutyric acid (GABA) was identified as the main inhibitory neurotransmitter in the mammalian brain, but has recently been shown to be of functional importance also in peripheral tissues such as the gut, urinary bladder, heart, lung, ovary and pancreas (for review see [11]). A number of studies have demonstrated that the GABA concentration in the pancreatic islets is as high as that in the brain and mainly localized to the \(\beta\) cells of the endocrine pancreas (for review see [22]). Recently it has been observed that the 64-kDa antigen associated with the onset of insulin-dependent diabetes mellitus is glutamate decarboxylase (GAD), the main GABA-synthesizing enzyme [3, 20, 31]. These findings indicate that GABA might have an important function in the islets of Langerhans. Interestingly, it has been shown that GABA is stored by gastropancreatic neuroendocrine (NE) cells in vesicles similar to the small synaptic vesicles of neurons [2, 29, 35]. Additionally, GABA can be released by a regulated, Ca\(^{2+}\)-dependent pathway from the NE pancreatic cell line AR42J [1]. These findings strongly indicate that GABA could be released to act on GABA receptors present on either the same cell or on neighbouring cells, thereby modulating the membrane potential of the activated cell. Previous work has suggested that only pancreatic, glucagon-producing \(\alpha_2\) cells contain functional GABA\(_A\) receptors [24]. Activation of these receptors resulted in a hyperpolarization of \(\alpha_2\) cells. Therefore, it
was proposed that GABA, co-released with insulin from pancreatic β cells, may act in a paracrine manner to mediate the glucose-induced inhibition of glucagon release. However, previous studies [24, 30] were hampered by the sole use of an antibody directed against the β2 and β3 subunits. A functional, benzodiazepine-sensitive GABA<sub>A</sub> receptor consists of α, β, and γ subunits (for review see [8]), with the exact subunit combination determining the physiological and pharmacological properties of the receptor [34]. Therefore, we reinvestigated this issue using affinity-purified antibodies recognizing peptide sequences specific for the α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2/3</sub>, and γ<sub>2</sub> subunits using formalin-fixed, frozen sections of human antral mucosa, a human gastrinoma and rat pancreas. Additionally, we investigated the functional properties of GABA<sub>A</sub> receptors in the rat insulinoma cell line RIN 38 and the amphibicrine cell line AR42J, using the patch-clamp technique in the whole-cell mode, to gain insight into the physiological function of these receptors in gastropancreatic, neuroendocrine cells.

Recently, it has been demonstrated that GABA can depolarize immature neurons and glial cells (for review see [7]). This GABA-induced depolarization can activate voltage-gated Ca<sup>2+</sup> channels, leading to an increase of the intracellular Ca<sup>2+</sup> concentration, [Ca<sup>2+</sup>]<sub>i</sub>. Since it has been shown that oscillations of [Ca<sup>2+</sup>]<sub>i</sub> drive the oscillations of insulin secretion in pancreatic β cells (see [18] for review), we investigated the effect of GABA on the [Ca<sup>2+</sup>]<sub>i</sub> of AR42J and RIN 38 cells using Ca<sup>2+</sup> fluorimetric methods.

In the present study we show that a large variety of gastropancreatic NE cells express functional GABA<sub>A</sub> receptors, which could constitute an important modulatory function of GABA for hormonal secretion in gastropancreatic NE cells.

**Materials and methods**

**Immunohistochemistry**

Immunohistochemistry was performed using formalin-fixed, frozen sections of human, antral mucosa, a human gastrinoma and rat pancreas in combination with conventional peroxidase histochemistry [32]. Affinity-purified antibodies recognizing peptide sequences specific for the α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>, α<sub>6</sub>, β<sub>1</sub>, β<sub>2/3</sub>, and γ<sub>2</sub> subunits were used [10, 32]. Staining was abolished by absorption of antiserum with the respective peptide sequences.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

RNA isolation from cell lines was performed by the guanidine thiocyanate/caesium chloride method described elsewhere [20], mRNA was prepared by means of the Poly A tract kit (Promega-Serva, Heidelberg, Germany). The spectrophotometrically determined absorbance ratio (A<sub>260</sub>/A<sub>280</sub>) was usually 1.9 to 2.0. First strand cDNA for thermal amplification was generated by reverse transcription (RT) of mRNA using the M-MLV reverse transcriptase (Gibco, Berlin, Germany). Briefly, about 1 µg mRNA was denatured at 85°C for 5 min, then M-MLV reverse transcriptase solution (200 units) was added and the reaction was carried out for 45 min at 42°C in a final volume of 20 µl Tris-buffer (10 mM, pH 9.0) containing 1 mM dithiobitol (DTT), 6 mM MgCl<sub>2</sub>, 500 mM of each deoxynucleoside 5’-triphosphate (dNTP), 100 pmol random hexamer primer, 20 units recombinant RNAsin (Promega-Serva). Control experiments were carried out replacing M-MLV reverse transcriptase or mRNA with water in the reaction mixture. The RT mix was used directly as a template for thermal amplification in a 1/50 dilution. Amplification of subunit-specific cDNA sequences encoding GABA<sub>A</sub> receptor subunits was performed using the following subunit-specific primer pairs:

- α<sub>1</sub>: ATC GTC TGA CAG CAC GGT TCT GCT GCC ACA ACC ACT GAG CG
- α<sub>2</sub>: TGG CCC TGT CTC AGA TAC AGA/CAG CCA GGA TGA CAC TTG GGT
- α<sub>3</sub>: GCT TGG AGA TCG AGT GAC TGA/GGC AAG TAG GTC GTG ATG AC
- α<sub>6</sub>: GCC AGC ATA GAC ATG GTC TCG/TGG TTG TCA TGG TCA GCA CCG
- β<sub>1</sub>: TGG ACA ATC GAG TGG CAG ACC/GCA CCC CAT TAG GTC CAC ACC
- β<sub>2</sub>: GCC AGC ATA GAC ATG GTC TCG/TGG TTG TCA TGG TCA GCA CCG
- β<sub>3</sub>: TGG ACA ATC GAG TGG CAG ACC/GCA CCC CAT TAG GTC CAC ACC
- γ<sub>2</sub>: TAG GCG TGA GCC CCA CAG TAA/GGA TGT TCT AGC AGG TAC AGC
- γ<sub>3</sub>: CTT CCG GCC GAC ATA GGA GT/CTT GCA GGG ACA GCA GCA TCC TTA
- γ<sub>6</sub>: CAG AAG TGG GTC TTG GCA CCC/CAC CTG CAT TAG TCA CGA

Amplification was carried out in 50 µl 10 mM Tris-Cl buffer, pH 9.0, containing 50 mM KCl, 0.1% Triton X-100, 1.0 mM MgCl<sub>2</sub>, 100 µM of each dNTP, 50 pmol of each primer and 2.5 U Tag DNA polymerase (Promega-Serva) using a Trio Thermocycler (Biometra, Göttingen, Germany). Following an initial denaturing step for 3 min at 95°C the mixtures were maintained at 85°C while adding Taq DNA polymerase (hot start technique). The amplification program consisted of up to 45 cycles with 30 s denaturation at 94°C, 1 min annealing at 60°C and 2 min extending at 72°C with the extending time increased by an increment of 2 s in every cycle. Control amplifications were performed with control RT as the template. The reaction was stopped at different cycles by freezing and the PCR mixtures were stored at 4 to 8°C. Control amplifications of the RT-generated cDNA were performed at consecutive cycles using a rat glyceraldehyde-3-phosphatodehydrogenase (G3PDH) control amplimer set (Clontech, Palo Alto, Calif., USA). Aliquots of the PCR mixtures were analysed by agarose gel electrophoresis using 2% agarose mini gels in TBE (Tris-Borat-EDTA) at standard conditions. Amplification resulted in single bands of the expected size. Identification of the products was confirmed by restriction analysis that resulted in DNA fragments as predicted. No amplification products were detected in control experiments using control RT as the template.

The abundance of the mRNAs of GABA<sub>A</sub> receptor subunits was assessed visually from the signal strength of amplified sequences from different thermal cycles in ethidium-bromide-stained agarose gels.

**Electrophysiology**

For recording of membrane currents, coverslips were transferred to the stage of an upright microscope (Zeiss, Oberkochen, Germany). Cells were maintained at room temperature (22°C) in a recording chamber with continuous perfusion of extracellular buffer. Drugs were added to the perfusate. The standard salt solution (BSS) contained in mM: NaCl 129.0, glucose 5.0, KCl 5.4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub>...