

Glucose Inhibition of Glucagon Secretion From Rat α -Cells Is Mediated by GABA Released From Neighboring β -Cells

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γ -Aminobutyric acid (GABA) has been proposed to function as a paracrine signaling molecule in islets of Langerhans. We have shown that rat β -cells release GABA by Ca^{2+} -dependent exocytosis of synaptic-like microvesicles. Here we demonstrate that GABA thus released can diffuse over sufficient distances within the islet interstitium to activate GABA_A receptors in neighboring cells. Confocal immunocytochemistry revealed the presence of GABA_A receptors in glucagon-secreting α -cells but not in β - and δ -cells. RT-PCR analysis detected transcripts of α_1 and α_4 as well as β_{1-3} GABA_A receptor subunits in purified α -cells but not in β -cells. In whole-cell voltage-clamp recordings, exogenous application of GABA activated Cl^- currents in α -cells. The GABA_A receptor antagonist SR95531 was used to investigate the effects of endogenous GABA (released from β -cells) on pancreatic islet hormone secretion. The antagonist increased glucagon secretion at 1 mmol/l glucose twofold and completely abolished the inhibitory action of 20 mmol/l glucose on glucagon release. Basal and glucose-stimulated secretion of insulin and somatostatin were unaffected by SR95531. The L-type Ca^{2+} channel blocker isradipine evoked a paradoxical stimulation of glucagon secretion. This effect was not observed in the presence of SR95531, and we therefore conclude that isradipine stimulates glucagon secretion by inhibition of GABA release. *Diabetes* 53:1038–1045, 2004

In the mammalian central nervous system (CNS), γ -aminobutyric acid (GABA) is the most important inhibitory neurotransmitter. Three types of GABA receptors have been identified: GABA_A and GABA_C receptors are ligand-gated Cl^- channels, while GABA_B receptors are G protein coupled (1). In the CNS, application of GABA reduces excitability by a combination of GABA_A and GABA_B receptor activation, leading to mem-

brane repolarization, reduced Ca^{2+} influx, and suppression of neurotransmitter release.

Outside the CNS, GABA and GAD, the enzyme catalyzing the formation of the neurotransmitter, are present at high levels in the pancreatic β -cells (2,3). GAD65 is the most abundant, if not the only, isoform of GAD in human islets (4) and has been implicated in the etiology of autoimmune type 1 diabetes (5). At the time of diagnosis, ~80% of type 1 diabetic patients have autoantibodies against GAD65 (6). The physiological roles of GABA and GAD65 in pancreatic islets are unknown. GABA can, via the formation of succinic semialdehyde and succinic acid, be introduced into the tricarboxylic acid cycle and has therefore been suggested to serve as an energy source within the β -cell (7). Others have hypothesized that GABA, by analogy to its role in the CNS, functions as a paracrine signaling molecule that conveys messages from the β -cells to surrounding cells in the islet (7–9).

In the β -cell, GAD65 and GABA are associated with synaptic-like microvesicles (SLMVs), which are distinct from the insulin-containing large dense-core vesicles (LDCVs) (10,11). SLMVs are smaller in diameter (~90 nm) than LDCVs (~300 nm) (12) and accumulate GABA by active transport (11). We have developed a patch clamp-based technique to investigate GABA secretion from single β -cells with high temporal resolution. This technique involves the expression of GABA_A receptors at a high density in the β -cell using adenoviral vectors. We have thus been able to demonstrate that GABA is released by voltage- and Ca^{2+} -dependent exocytosis of SLMVs from β -cells (12). In this study we have focused on the influence of endogenously released GABA on islet hormone secretion. We demonstrate that GABA, following its exocytotic release, travels over sufficient distances to activate GABA_A receptors in neighboring cells, that functional GABA_A receptors are present in α -cells, and that GABA receptor antagonism selectively affects glucagon release.

RESEARCH DESIGN AND METHODS

Islet preparation. Pancreatic islets and single islet cells were prepared from Sprague Dawley or Wistar rats as described previously (13). In experiments involving detection of vesicular GABA release (Fig. 1B–D), cells were coinfecting with adenoviruses expressing the α_1 and β_1 subunits of the GABA_A receptor (12) and cultured for ~24 h. All experimental procedures involving animals were approved by the ethical committees in Lund, the city of Hamburg, and the University of Copenhagen.

Electrophysiology. The electrophysiological measurements were conducted in the standard whole-cell configuration. α -Cells were identified on the basis of their small size (<4 pF as compared with >4 pF for β -cells) and the

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CNS, central nervous system; FACS, fluorescence-activated cell sorter; GABA, γ -aminobutyric acid; LDCV, large dense-core vesicle; SLMV, synaptic-like microvesicle.

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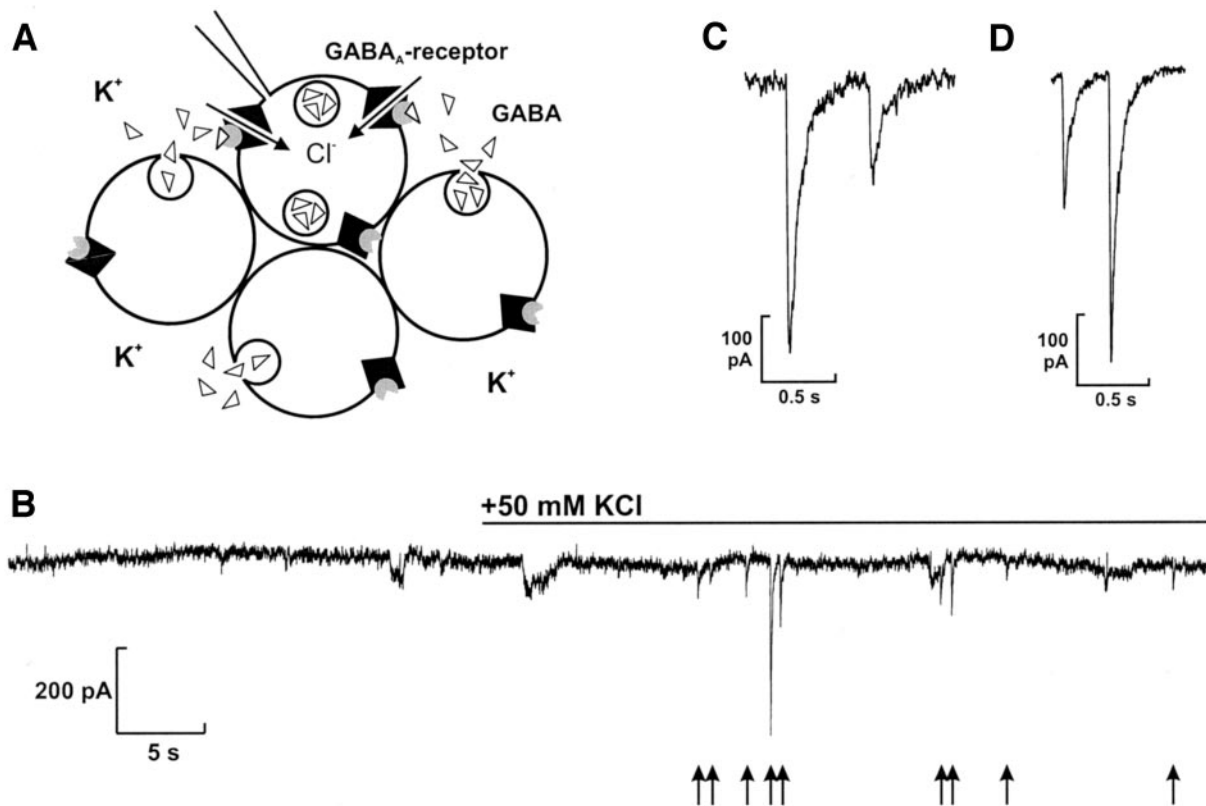


FIG. 1. Ability of GABA to function as a paracrine signaling molecule. **A:** Schematic picture showing the principles of the experiments. Membrane currents are recorded from a cell voltage clamped at -70 mV and dialyzed with 10 mmol/l EGTA. Exocytosis from surrounding cells is induced by depolarization with elevated K^+ . **B:** Whole-cell recording from a voltage-clamped cell in a cluster of rat islet cells infected with adenovirus expressing GABA $_A$ $\alpha_1\beta_1$ receptors. Addition of K^+ (50 mmol/l) is indicated by the horizontal bar. Inward current transients triggered by GABA release are indicated by arrows ($n = 4$). **C:** Examples of current transients from **B** on an extended time base. **D:** Examples of current transients due to exocytosis of GABA-containing SLMVs, recorded from an isolated cell. Exocytosis was elicited by intracellular dialysis with 3 μ mol/l free Ca^{2+} .

inactivation properties of their voltage-gated Na^+ channels (14,15). The experiments were performed either at $32^\circ C$ (Fig. 1) or at room temperature (Fig. 4).

Solutions. The standard extracellular solution consisted of (in mmol/l) 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4 with NaOH), and 1 – 5 glucose. In the experiment shown in Fig. 1B–C, pentobarbital (0.05) and forskolin (2 μ mol/l) were included to increase the amplitude of the GABA-activated Cl^- currents (16) and to elevate intracellular cAMP levels to stimulate GABA release (12), respectively. When the cells were depolarized with 50 mmol/l KCl (Fig. 1), the concentration of NaCl was correspondingly decreased. For the electrophysiological recordings of endogenous GABA $_A$ receptor activity (Fig. 4), 20 TEA-Cl was substituted for an equal concentration of NaCl. The pipette solution (intracellular solution) used for all measurements was composed of (in mmol/l) 125 CsCl, 30 CsOH, 10 EGTA, 1 $MgCl_2$, 5 HEPES (pH 7.15 with HCl), and 3 Mg-ATP. With this intracellular solution, activation of Cl^- channels will result in Cl^- efflux at negative membrane potentials, thus giving rise to an inward current (downward deflection). In the experiments displayed in Fig. 1D, 9 mmol/l $CaCl_2$ was added to the above intracellular solution to increase the intracellular free concentration of Ca^{2+} to 3 μ mol/l.

Immunofluorescence. Intact rat islets were fixed with the pH-shift/formaldehyde method and permeabilized with 5% Triton X-100. Normal donkey serum at 5% was used to block unspecific binding. The islets were incubated with antibodies directed against insulin ($1:200$ dilution; Eurodiagnostica, Malmö, Sweden), glucagon ($1:200$; Dako, Älvsjö, Sweden), somatostatin ($1:200$; Biogenesis, Poole, U.K.), and the $\beta_{2/3}$ subunits of the GABA $_A$ receptor ($1:100$, clone 62-3G1; Upstate Biotechnology, Lake Placid, NY) at $4^\circ C$ overnight. Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used to detect the labeled sites. Immunofluorescence was then detected using a confocal microscope (Zeiss 510; Zeiss, Göttingen, Germany).

RT-PCR. Total RNA was extracted from fluorescence-activated cell sorter (FACS)-purified (17) rat α - and β -cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) and used for reverse transcription using random hexamer primers

and Superscript II (Invitrogen, Karlsruhe, Germany). Total RNA from human brain (Clontech, Heidelberg, Germany) was used as positive control. The predicted sizes for the bands in the positive control were α_1 363 bp, α_2 407 bp, α_3 505 bp, α_4 246 bp, α_5 589 bp, α_6 177 bp, β_1 270 bp, β_2 342 bp, and β_3 379 bp; the α s and β s correspond to previously identified GABA $_A$ receptor subunits. PCR amplifications were performed under standard conditions running 40 cycles. Primer sequences are available on request (sewing@lilly.com).

Western blot. Islet homogenate was separated by SDS-PAGE on 8% acrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight with the anti-GABA $_A\beta_{2/3}$ antibody (2 μ g/ml, clone 62-3G1; Upstate Biotechnology). After 1 h incubation with horseradish-peroxidase-coupled secondary antibody ($1:20,000$ dilution), the blots were developed by using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Cheshire, U.K.) and visualized on X-ray films.

Hormone release measurements. Insulin, glucagon, and somatostatin release was determined by radioimmunoassay as described elsewhere (18,19). Briefly, batches of 8 – 10 islets were preincubated in 1 ml of Krebs-Ringer buffer (KRB) supplemented with 1 mmol/l glucose for 30 min followed by 1 h incubation in 1 ml Krebs-Ringer buffer containing 1 or 20 mmol/l glucose. The specific GABA $_A$ receptor antagonist SR95531 (10 μ mol/l; Sigma, St. Louis, MO) or the specific Ca^{2+} channel antagonists isradipine (2.5 μ mol/l; kindly provided by J. Striessing, Innsbruck, Austria), SNX482 (0.1 μ mol/l; Peptides International, Louisville, KY), and ω -conotoxin GVIA (0.1 μ mol/l; Alomone Labs, Jerusalem, Israel) were included as indicated. At the end of the incubation, duplicate aliquots (25 – 100 μ l) of the medium were removed and frozen pending the radioimmunoassay.

Statistical analysis. Data are given as means \pm SE. Statistical significances were evaluated using Student's t test.

RESULTS

Intercellular GABA signaling between pancreatic islet cells. We have previously demonstrated that GABA

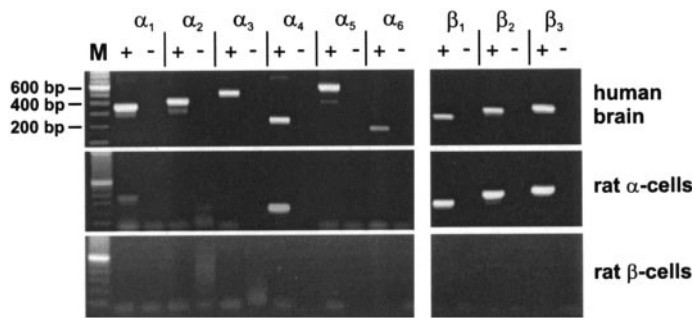


FIG. 2. RT-PCR on purified α - and β -cells with specific primers against different GABA_A receptor subunits. Total RNA from FACS-purified α - and β -cells was reverse transcribed into cDNA, and PCR analysis was performed with specific primers for the α_{1-6} and β_{1-3} subunits (+). In the negative control reaction (-), reverse transcriptase was omitted. Total RNA from human brain was reverse transcribed into cDNA and used as a positive control. Molecular standards are shown to the left.

can be released from rat β -cells by Ca²⁺-dependent exocytosis of SLMVs (12). Here we investigate whether GABA released from one cell can activate GABA receptors in neighboring cells, a requirement for GABA to function as a paracrine regulator. To this end, patch-clamp recordings were applied to cells within small islet cell clusters infected with adenovirus expressing GABA_A receptor Cl⁻ channels. Secretion was stimulated by adding 50 mmol/l K⁺ to the extracellular medium. This leads to membrane depolarization, opening of voltage-gated Ca²⁺ channels, and exocytosis in the unclamped cells. The membrane potential of the patch-clamped cell was kept at -70 mV throughout the experiment, which is too negative for Ca²⁺ channel activation and voltage-dependent GABA release (12). Furthermore, intracellular free Ca²⁺ was clamped to nonexocytotic levels by including 10 mmol/l EGTA in the pipette-filling solution dialyzing the cell interior. Any recorded GABA-activated Cl⁻ currents must accordingly result from GABA being released by the surrounding unclamped cells as illustrated schematically in Fig. 1A.

Figure 1B shows the holding current before and during stimulation with high extracellular K⁺. Before stimulation,

the holding current was stable. Following addition of 50 mmol/l K⁺ to the extracellular medium, a series of nine fast current transients were observed (arrows). Examples of these current transients are shown in Fig. 1C on an expanded time base. Figure 1D shows examples of GABA-activated Cl⁻ currents recorded in a single cell in which exocytosis was stimulated by dialyzing the interior of the voltage-clamped cell with a Ca²⁺-EGTA buffer containing 3 μ mol/l free Ca²⁺ instead of the Ca²⁺-free medium. The events shown in Fig. 1C-D show a great resemblance. We analyzed the activation of the current spikes and their durations by measuring the $t_{10-90\%}$ (i.e., the time it takes for the current to increase from 10 to 90% of its maximal amplitude) and half-width (the time the current exceeds the half-maximal amplitude). Mean values for $t_{10-90\%}$ and the half-width of 15 ± 2 and 53 ± 5 ms ($n = 9$), respectively, were obtained. These values are similar to those recorded from β -cells in which exocytosis was stimulated by intracellular application of 3 μ mol/l Ca²⁺ ($t_{10-90\%}$ 12.6 ± 0.7 ms, half-width 30 ± 1 ms [12]). We conclude that GABA released from one cell can diffuse to neighboring cells and that the concentration of the neurotransmitter remains sufficiently high to activate GABA_A receptors in these cells.

GABA_A receptor subunits are expressed in α -cells but not in β - or δ -cells. Ionotropic GABA_A receptors have been reported to be endogenously expressed in rat (20) and human (21) pancreatic islets. We used RT-PCR to determine the expression pattern of endogenous GABA_A receptors in FACS-purified α - and β -cells from rat, utilizing specific primers for the α_1 - α_6 and the β_1 - β_3 subunits of the GABA_A receptor. The α_1 , α_4 , β_1 , β_2 , and β_3 subunits were expressed in rat pancreatic α -cells, whereas none of the subunits investigated were detected in β -cells (Fig. 2). The identity of all PCR products was confirmed by sequencing. We verified that the GABA_A receptor transcripts detected with RT-PCR translated into protein by performing Western blot on rat islet homogenate. As shown in Fig. 3A, an antibody directed against the $\beta_{2/3}$ subunits detected a protein with a molecular weight of ~ 60 kDa, close to the

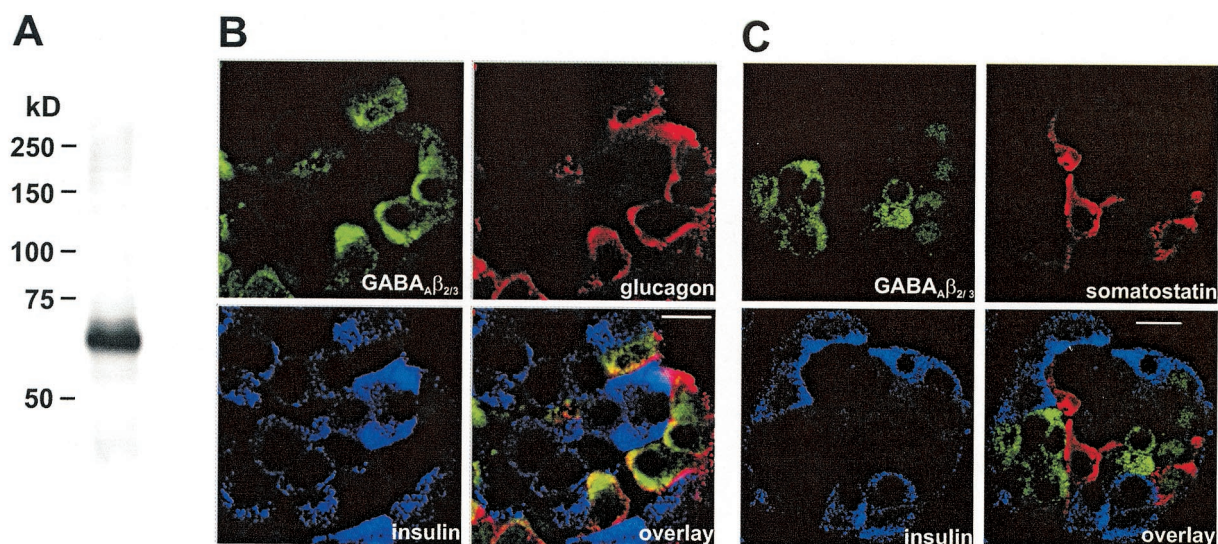


FIG. 3. Western blot and confocal immunocytochemistry with a GABA_A receptor-specific antibody. A: Western blot on rat islet homogenate (~ 150 islets) using a specific antibody against the $\beta_{2/3}$ subunits of the GABA_A receptor. B and C: Confocal immunocytochemistry of isolated rat islets detecting the $\beta_{2/3}$ subunits of the GABA_A receptor, insulin, and glucagon or somatostatin and overlay as indicated. Scale bars: 10 μ m.

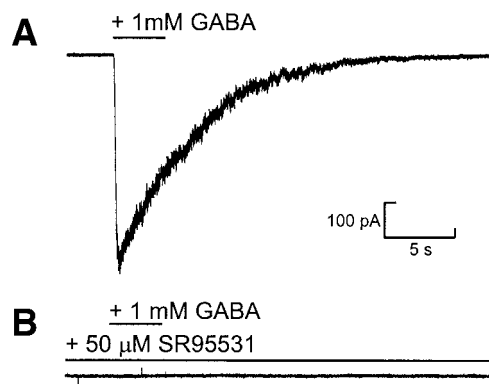


FIG. 4. Electrophysiological measurements of endogenous GABA_A receptors. Cl⁻ current elicited by application of 1 mmol/l GABA to an α -cell under control conditions (A) and in presence of the GABA_A receptor antagonist SR95531 (B).

expected size (57 kDa) (22). Confocal immunocytochemistry using the same antibody revealed the presence of GABA_A receptors in the glucagon-containing α -cells (Fig. 3B), whereas no $\beta_{2/3}$ immunoreactivity was observed in β -cells (Fig. 3B–C) or δ -cells (Fig. 3C).

Electrophysiological measurements of endogenous GABA_A receptors. We next applied whole-cell patch-clamp measurements to confirm the presence of functional GABA_A receptors in α -cells. Application of 1 mmol/l GABA to functionally identified (see RESEARCH DESIGN AND METHODS and below) α -cells elicited inward Cl⁻ currents (Fig. 4A). In this particular experiment, the amplitude of the current was unusually large, reaching a peak amplitude of approximately -600 pA. The amplitude of the GABA-evoked Cl⁻ current was extremely variable and ranged between -20 pA and -600 pA, with an average of -138 ± 93 pA ($n = 6$). The current elicited by GABA was blocked by the specific GABA_A receptor antagonist SR95531 (Fig. 4B). As expected for α -cells (23), the capacitance of the cells containing GABA-activated currents averaged 3.0 ± 0.3 pA ($n = 6$), significantly less than the 4.7 ± 0.2 pF ($n = 31$) obtained for β -cells ($P < 0.001$). No inward Cl⁻ currents could be elicited in β -cells (not shown).

Effect of endogenous GABA release on pancreatic hormone release. α -Cells are electrically active (24) and secrete glucagon in response to membrane depolarizations and opening of voltage-dependent Ca²⁺ channels (17). Because GABA_A receptors are present in α -cells, it is expected that exocytosis of GABA-containing SLMVs from β -cells will hyperpolarize the α -cells with resultant suppression of action-potential firing and glucagon secretion. This is not easily studied using electrophysiological techniques, but by using the GABA_A receptor antagonist

SR95531, some insight into the significance of the intrinsic GABAergic signaling in the islets can nevertheless be obtained. Pancreatic hormone secretion was measured in isolated intact rat pancreatic islets at 1 and 20 mmol/l glucose in the absence and presence of 10 μ mol/l SR95531. It should be noted that by using an antagonist, we explore the role of endogenous GABA and no exogenous GABA was applied in these experiments. As seen in Table 1, including SR95531 in the extracellular medium had no effect on the release of insulin or somatostatin, irrespective of the glucose concentration. By contrast, glucagon secretion at 1 and 20 mmol/l glucose was stimulated approximately two- to threefold in the presence of the antagonist. Importantly, whereas glucagon secretion was reduced by $\sim 50\%$ under control conditions when raising glucose to 20 mmol/l, this effect was abolished in the presence of SR95531.

Ca²⁺ channel antagonists reveal paracrine interactions between islet cells. Table 2 summarizes the effects of the L-type Ca²⁺ channel blocker isradipine (2.5 μ mol/l), the N-type Ca²⁺ channel antagonist ω -conotoxin GVIA (100 nmol/l), and the R-type Ca²⁺ channel blocker SNX482 (100 nmol/l) on insulin, glucagon, and somatostatin secretion at 1 and 20 mmol/l glucose in isolated rat pancreatic islets. Isradipine had no effect on insulin, somatostatin, or glucagon release measured at 1 mmol/l glucose. Glucose-induced insulin secretion was inhibited by $>80\%$ by the L-type channel blocker, whereas there was no significant effect on somatostatin release. By contrast, SNX482 failed to affect insulin secretion, but produced an $\sim 50\%$ reduction in glucose-stimulated somatostatin secretion. SNX482 likewise lacked effect on glucagon release measured at 1 mmol/l glucose.

In the presence of 1 mmol/l glucose, ω -conotoxin GVIA (0.1 μ mol/l) inhibited glucagon secretion by $\sim 50\%$, similar to the inhibition produced by raising glucose to 20 mmol/l. Importantly, neither isradipine nor SNX482 affected glucagon secretion at 1 mmol/l glucose. Addition of isradipine in the presence of 20 mmol/l glucose resulted in a paradoxical stimulation of glucagon release. Figure 5 summarizes glucagon release measured at 20 mmol/l glucose under control conditions and in the presence of 2.5 μ mol/l isradipine, 0.1 μ mol/l SNX482, and 10 μ mol/l of the GABA_A receptor antagonist SR95531. These data have been normalized to glucagon secretion measured at 1 mmol/l glucose. This analysis reveals that, whereas glucagon secretion in the presence of SR95531 and 20 mmol/l glucose exceeds that observed at 1 mmol/l glucose, the release of the hormone measured in the presence of a combination of 20 mmol/l glucose and 2.5 μ mol/l isradipine was $\sim 80\%$ of that observed at 1 mmol/l glucose. We propose that isradipine stimulates glucagon release by

TABLE 1
Effect of the GABA_A receptor antagonist SR95531 on hormone release from isolated rat islets

Glucose (mmol/l)	Insulin (ng · islet ⁻¹ · h ⁻¹)		Somatostatin (pmol · islet ⁻¹ · h ⁻¹)		Glucagon (pg · islet ⁻¹ · h ⁻¹)	
	1	20	1	20	1	20
Control	0.37 ± 0.03	6.17 ± 0.3	5.05 ± 0.5	24.48 ± 1.1	27.18 ± 2.1	15.31 ± 1.2
10 μ mol/l SR95531	0.32 ± 0.06	6.01 ± 0.2	5.50 ± 0.2	26.1 ± 1.6	46.72 ± 3.8*	51.04 ± 4.0*†

Data are means \pm SE of 10 experiments. * $P < 0.001$ vs. control; †NS vs. 10 μ mol/l SR95531 at 1 mmol/l glucose.

TABLE 2
Effect of Ca^{2+} channel antagonists on hormone release from rat islets

Glucose (mmol/l)	Insulin ($\text{ng} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$)		Somatostatin ($\text{pmol} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$)		Glucagon ($\text{pg} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$)	
	1	20	1	20	1	20
Control	0.49 ± 0.04	10.39 ± 0.7	4.30 ± 0.6	17.82 ± 2.3	36.85 ± 2.3	19.79 ± 1.2
2.5 $\mu\text{mol/l}$ isradipine	0.47 ± 0.05	$1.72 \pm 0.2^*$	3.65 ± 0.5	14.06 ± 1.3	$34.10 \pm 2.5^\ddagger$	$28.98 \pm 3.6\&$
0.1 $\mu\text{mol/l}$ SNX482	0.42 ± 0.04	8.87 ± 0.9	2.92 ± 0.3	$9.37 \pm 1.3^\ddagger$	39.75 ± 3.9	23.91 ± 3.3
0.1 $\mu\text{mol/l}$ ω -conotoxin GVIA	0.44 ± 0.04	9.40 ± 0.6	3.44 ± 0.5	14.13 ± 2.6	$17.03 \pm 2.9^*$	16.06 ± 1.1

Data are means \pm SE of 8 experiments. Hormone release was measured at 1 and 20 mmol/l glucose in the absence and presence of isradipine, SNX482, and ω -conotoxin GVIA as indicated. Statistical significance is evaluated against the control at the same glucose concentration. * $P < 0.001$; $^\ddagger P < 0.01$; $^\ddagger\text{NS}$; $\& P < 0.05$.

inhibiting the release of a paracrine regulator from the β -cell. Given the observation that isradipine was ineffective when added to islets already exposed to SR95531, it appears that the compound mediating the effect is GABA.

DISCUSSION

We have recently developed an electrophysiological assay based on the overexpression of GABA_A receptor Cl^- channels that allows us to detect exocytosis of single GABA-containing vesicles from β -cells (12). In this study we have applied the assay to clusters of islet cells (Fig. 1A). As the experimental conditions were designed to prevent any exocytosis from the patch-clamped cell, the Cl^- current transients recorded must result from GABA exocytosis from neighboring cells. The experiment therefore suggests that GABA is indeed capable of traveling in the interstitium between islets cells. Close inspection of the observed current spikes revealed that they rose with the same velocity as those recorded in single cells, where the current transients result from GABA being released from the same cell. This indicates that the receptor cell must be sitting very close to the cell in which GABA release occurred. Indeed, electron microscopy reveals that the interstitial space between neighboring islet cells is very small (25), which facilitates paracrine regulation.

GABA_A receptors are heteromultimeric channels typically composed of two α , two β , and a varying third subunit. In RT-PCR experiments performed on FACS-

purified islet cells, we found that rat α -cells express the α_1 and α_4 subunit as well as β_{1-3} (Fig. 2). Although RT-PCR is not a quantitative method, the intensity of the band for the α_4 subunit suggests that this is the predominant α subunit in α -cells. Receptor combinations containing the α_4 subunit are found extrasynaptically in hippocampus and thalamus (26) and characteristically exhibit a higher affinity for GABA (27). This argues that release of GABA may activate GABA_A receptors even beyond the immediate vicinity of the release sites, which facilitates GABAergic signaling within the islet. The experiment shown in Fig. 1B was performed in cells transfected with $\alpha_1\beta_1$ receptors, which have a lower GABA sensitivity than receptors containing the α_4 subunit (11 $\mu\text{mol/l}$ [28] vs. 0.5–2.6 $\mu\text{mol/l}$ [27]). The extent of intercellular GABAergic signaling might accordingly be even stronger than suggested by the present data.

To date, most functional experiments on GABAergic signaling in pancreatic islets have been conducted using application of exogenous GABA (29,30) or agonists such as muscimol (7). GABA_A receptor Cl^- channels inactivate (i.e., the channels enter a nonconducting state) within seconds when exposed to GABA concentrations of ≥ 30 $\mu\text{mol/l}$ (31). Thus, in studies using application of high concentrations of GABA to affect hormone release, a current could flow through the channels only for a brief period immediately after the addition of the agonist. Accordingly, during most of the experiment, both membrane potential and secretion are likely to be unaffected. A way around this problem is to use an antagonist against the GABA_A receptor instead and study the influence of endogenous GABA on pancreatic hormone release. However, bicuculline, which has been used to this end in the past has promiscuous pharmacological properties and inhibits, for example, Ca^{2+} -activated K^+ channels (32) in addition to the GABA_A receptors, which may lead to unspecific effects (M.B., A.W., unpublished data). In this study we have instead used the selective antagonist SR95531 and thereby found that, at least in rat islets, only glucagon secretion is modulated by GABA_A receptor activation. This observation is corroborated by the finding that GABA_A receptors are only detected in pancreatic α -cells. The fact that neither somatostatin nor insulin secretion were affected by the antagonist, although the electrophysiology of δ - and β -cells is similar to that of the α -cells, confirms that the action of this compound is selective.

Surprisingly, addition of SR95531 stimulated glucagon secretion measured at 1 mmol/l glucose. This might indicate that GABA is present at biological concentrations

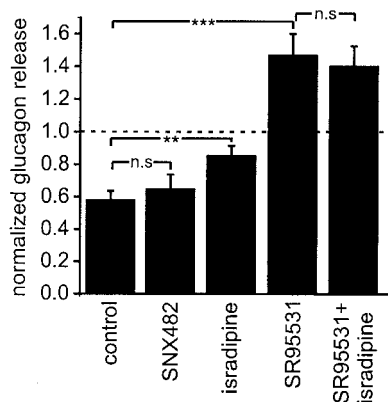


FIG. 5. Effect of the Ca^{2+} channel blockers SNX482 and isradipine and the GABA_A receptor antagonist SR95531 on glucagon secretion from isolated rat islets. Glucagon secretion was measured in the presence of 20 mmol/l glucose alone or after including 0.1 $\mu\text{mol/l}$ SNX482, 2.5 $\mu\text{mol/l}$ isradipine, or 10 $\mu\text{mol/l}$ SR95531 as indicated. Data are normalized to glucagon release at 1 mmol/l glucose and are means \pm SE of 8–10 experiments.

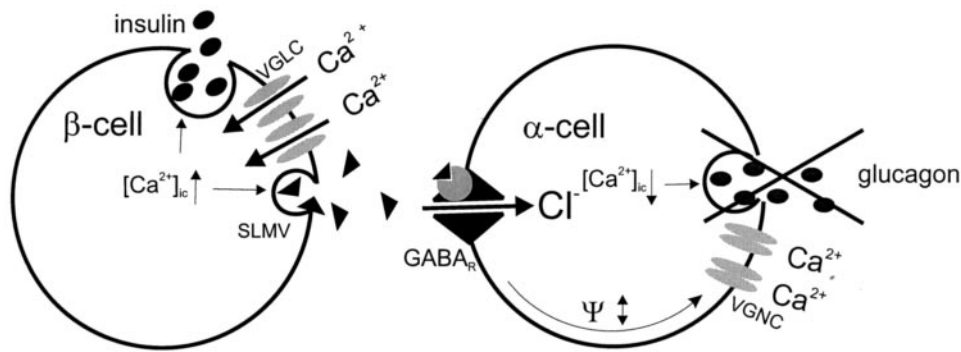


FIG. 6. Model for GABAergic regulation of glucagon secretion. See text for details. $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; $GABA_R$, $GABA_A$ receptor Cl^- channels; $VGLC$, voltage-gated L-type Ca^{2+} channels; $VGNC$, voltage-gated N-type Ca^{2+} channels; ψ , membrane potential; \downarrow , an increase or decrease in ion concentration or membrane potential (depolarization/hyperpolarization).

already at low glucose concentrations. Indeed, it has been estimated that 25% of the total β -cell content of GABA (21 pmol per 1,000 β -cells) is released per hour regardless of the glucose concentration (33). This corresponds to a net release rate of 0.4 amol of GABA, equivalent to approximately one GABA-containing SLMV, per second and cell. Our studies on exocytotic GABA release performed on single cells did not provide evidence for this relatively high basal release rate (12). However, additional signaling cascades may be operative in intact islets. For example, it has been proposed that GABA release from the β -cells is controlled by glutamate, cosecreted with glucagon from the α -cells, rather than by glucose directly (25). It should be pointed out, however, that even millimolar amounts of glutamate have no detectable effect of GABA release in our hands (12). Alternatively, basal GABA release may reflect passive leakage mediated by an uncharacterized transporter. The relative contributions of basal and stimulated release to GABAergic signaling remain to be elucidated.

In this study we demonstrate that hormone release from α -, β -, and δ -cells depends differentially on Ca^{2+} influx through different types of Ca^{2+} channels. Thus glucose-induced insulin secretion is almost completely blocked by the L-type Ca^{2+} channel blocker isradipine. Glucose-induced somatostatin release is principally due to SNX482-sensitive R-type Ca^{2+} channels, while glucagon secretion is dependent on ω -conotoxin-sensitive N-type Ca^{2+} channels (Table 2). Interestingly, isradipine stimulated glucagon secretion in the presence of a maximally inhibitory concentration of glucose, although this Ca^{2+} channel antagonist had no effect when release of the hormone was stimulated by hypoglycemia. This observation is easiest to explain if glucagon secretion was suppressed at high glucose concentrations by a compound released from the β -cells. Theoretically, any substance released from the β -cell could exert such an inhibitory action; possible candidates include insulin itself, zinc, ATP, islet amyloid polypeptide, etc. (34). Indeed, both insulin (35) and zinc (36) have been suggested to suppress glucagon release. It is pertinent that isradipine had no additional stimulatory effect in the presence of SR95531. The data therefore suggest that a significant part of the inhibitory action of glucose in isolated rat islets is mediated by GABAergic mechanisms. They also argue that exocytosis of GABA-containing SLMVs, like insulin-containing LDCVs, depends on Ca^{2+} entry via L-type Ca^{2+} channels. This could either reflect a requirement of such channels for glucose-induced electrical activity (37) or exocytosis of SLMVs being directly coupled to Ca^{2+} entry via these channels. Our

single-cell measurements of GABA release using β -cells infected with $GABA_A$ receptors have clearly established that exocytosis of SLMVs is controlled by Ca^{2+} entry via voltage-gated Ca^{2+} channels (12), but the molecular identity of the channel remains to be determined. However, given that L-type Ca^{2+} channels account for the largest part (60%) of the whole-cell Ca^{2+} current with little (<10%) contribution by both R- and N-type Ca^{2+} channels (A.W., M.B., P.R., unpublished results), it seems reasonable to conclude that the L-type Ca^{2+} channels are indeed important for exocytosis of the GABA-containing SLMVs.

Figure 6 summarizes schematically the molecular players and nature of interactions involved. Glucose-induced electrical activity of the β -cell leads to opening of voltage-gated L-type Ca^{2+} channels with subsequent Ca^{2+} influx and Ca^{2+} -dependent exocytosis of both insulin-containing LDCVs and GABA-containing SLMVs. GABA thus released diffuses in the narrow space between the release site and the neighboring α -cells, where it activates $GABA_A$ receptor Cl^- channels. The rate of release is one vesicle per second in the absence of glucose but increases 5- to 10-fold during electrical activity. Each event elicits a rapidly activating current that lasts >100 ms. Because the α -cells have a high input resistance (few active channels), the activation of the $GABA_A$ receptor Cl^- channels will clamp the α -cell membrane potential to the Cl^- equilibrium potential (E_{Cl}). Depending on the intracellular Cl^- concentration, opening of Cl^- channels will suppress electrical activity by either hyperpolarizing or depolarizing the α -cell. In the former case, the membrane potential becomes too negative for action potential generation. In the latter case, the membrane potential becomes so positive that the ion currents involved in action-potential generation undergo voltage-dependent steady-state inactivation. The reduction in action-potential firing leads to reduced activation of N-type Ca^{2+} channels and reduced exocytosis of the glucagon-containing secretory granules in the α -cell. In addition to N-type Ca^{2+} channels, α -cells are also equipped with L-type Ca^{2+} channels (17) that open during depolarization and contribute to the increases in cytoplasmic free Ca^{2+} concentration (38,39). However, these channels contribute little to exocytosis under basal conditions (17,40), but their importance increases dramatically under certain physiological situations. For example, L-type Ca^{2+} channels account for 80% of glucagon secretion under experimental conditions associated with enhanced protein kinase A activity (17).

We acknowledge that paracrine regulation by GABA may not be the only mechanism influencing glucagon

release. Release of Zn^{2+} from the β -cells has recently been proposed to suppress glucagon release in neighboring α -cells in rat islets (36). Studies using a novel somatostatin receptor antagonist have shown that somatostatin inhibits glucagon secretion in rat islets (41). We point out that these mechanisms are not mutually exclusive, and it is indeed possible that all these mechanisms operate in parallel in α -cells in situ. The finding that SR95531 completely reverses the inhibitory effect of glucose on glucagon secretion strongly suggests, however, that GABAergic signaling plays an important role in the metabolic control of glucagon secretion in intact rat pancreatic islets. In addition, pancreatic β -cells contain metabotropic GABA_B receptors (42), thus providing yet another GABAergic regulatory mechanism within the islet. This aspect will be addressed at length elsewhere (M.B., A.W., K.B., A.S., S.S., J.G., P.R., unpublished observations). Interestingly, mouse islets contain much less GABA (2), and GABAergic mechanisms such as those we discuss here are consequently likely to be less important in such islets. This argues that other mechanisms regulate glucagon secretion in mouse islets (see for example ref. 43). It is now essential to establish whether human α -cells also are equipped with GABA_A receptor Cl^- channels and, if so, to clarify their role in the control of glucagon secretion. Circumstantial evidence in support of an important role for GABAergic mechanisms in human islets comes from the finding that these islets contain high levels of both GABA and GAD65.

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