

# GABA<sub>B</sub> receptor activation inhibits exocytosis in rat pancreatic $\beta$ -cells by G-protein-dependent activation of calcineurin

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We have investigated the regulation of hormone secretion from rat pancreatic islets by the GABA<sub>B</sub> receptors (GABA<sub>B</sub>Rs). Inclusion of the specific GABA<sub>B</sub>R antagonist CGP 55845 in the extracellular medium increased glucose-stimulated insulin secretion 1.6-fold but did not affect the release of glucagon and somatostatin. Conversely, addition of the GABA<sub>B</sub>R agonist baclofen inhibited glucose-stimulated insulin secretion by ~60%. Using RT-PCR, transcription of GABA<sub>B</sub>R<sub>1a-c,f</sub> and GABA<sub>B</sub>R<sub>2</sub> subunits was detected in  $\beta$ -cells. Measurements of membrane currents and cell capacitance were applied to single  $\beta$ -cells to investigate the mechanisms by which GABA<sub>B</sub>R activation inhibits insulin secretion. In perforated-patch measurements, baclofen inhibited exocytosis elicited by 500-ms voltage-clamp depolarizations to 0 mV by  $\leq 80\%$  and voltage-gated Ca<sup>2+</sup> entry by only ~30%. Both effects were concentration-dependent with IC<sub>50</sub> values of ~2  $\mu$ M. The inhibitory action of baclofen was abolished in the presence of CGP 55845. The ability of baclofen to suppress exocytosis was prevented by pre-treatment with pertussis toxin and by inclusion of GDP $\beta$ S in the intracellular medium, and became irreversible in the presence of GTP $\gamma$ S as expected for a process involving inhibitory G-proteins (G<sub>i/o</sub>-proteins). The inhibitory effect of baclofen resulted from activation of the serine/threonine protein phosphatase calcineurin and pre-treatment with cyclosporin A or intracellular application of calcineurin autoinhibitory peptide abolished the effect. Addition of baclofen had no effect on [Ca<sup>2+</sup>]<sub>i</sub> and electrical activity in glucose-stimulated  $\beta$ -cells. These data indicate that GABA released from  $\beta$ -cells functions as an autocrine inhibitor of insulin secretion in pancreatic islets and that the effect is principally due to direct suppression of exocytosis.

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Pancreatic islets contain high levels of the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA). GABA is the most important inhibitory neurotransmitter in the mammalian central nervous system (CNS). Three types of GABA-receptors exist. In addition to the ionotropic GABA<sub>A</sub>- and GABA<sub>C</sub>-receptors, which are ligand-gated Cl<sup>-</sup> channels, there are also metabotropic G-protein-coupled GABA<sub>B</sub>-receptors (GABA<sub>B</sub>Rs; Chebib & Johnston, 1999). In neurones, GABA reduces electrical

excitability via both GABA<sub>A</sub>- and GABA<sub>B</sub>-signalling and by a combination of pre- and postsynaptic mechanisms. Several cellular processes are influenced by GABA<sub>B</sub>Rs. Examples include inhibition of the voltage-gated Ca<sup>2+</sup> channels, modulation of the voltage dependence of a transient A-type K<sup>+</sup> current and activation of inwardly rectifying K<sup>+</sup> channels (for review see Kerr & Ong, 1995). In addition, it has recently been reported that baclofen reduces synaptic transmission by inhibition of vesicle priming (Sakaba & Neher, 2003).

By contrast, the physiological roles of GABA in the pancreatic islets are not fully understood. GABA is stored in the  $\beta$ -cell in synaptic-like microvesicles (SLMVs), which

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accumulate GABA by active transport (Thomas-Reetz *et al.* 1993). We have recently demonstrated that GABA is released by regulated  $\text{Ca}^{2+}$ -dependent exocytosis of the SLMVs (Braun *et al.* 2004), and that GABA thus released regulates glucagon secretion from neighbouring  $\alpha$ -cells by activation of  $\text{GABA}_A$ -receptors (Wendt *et al.* 2004). However, there is also evidence suggesting that  $\text{GABA}_B$ Rs are expressed in pancreatic islet cells (Brice *et al.* 2002) and application of the  $\text{GABA}_B$ R agonist baclofen has been reported to inhibit glucose-induced insulin secretion from rat pancreatic islet (Gu *et al.* 1993) and clonal MIN6-cells (Brice *et al.* 2002). Here we have studied the expression and function of  $\text{GABA}_B$ Rs in rat pancreatic islets using a combination of hormone release measurements, RT-PCR analysis of gene transcription, recordings of the cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and single-cell measurements of exocytosis and voltage-gated  $\text{Ca}^{2+}$  currents. We demonstrate that: (1) activation of  $\text{GABA}_B$ Rs selectively modulates insulin secretion from the pancreatic islets and that release of glucagon and somatostatin is not affected; (2) the effect of GABA on insulin secretion involves inhibition of exocytosis at a late stage and is independent of any changes in the intracellular cAMP levels,  $\text{Ca}^{2+}$  influx or  $[\text{Ca}^{2+}]_i$ ; and (3) the inhibitory action involves G protein-dependent activation of the protein phosphatase calcineurin.

## Methods

### Preparation of rat $\alpha$ - and $\beta$ -cells

Pancreatic islets and single  $\alpha$ - and  $\beta$ - cells were prepared from Sprague-Dawley or Wistar rats as previously described for mice (Olofsson *et al.* 2002). The rats were anaesthetized with sodium pentobarbitone (100 mg  $\text{kg}^{-1}$  i.p.) and killed by cervical dislocation or by inhalation of  $\text{CO}_2$  followed by cervical dislocation. All experimental procedures involving animals were approved by the ethical committees in Lund, the City of Hamburg and the University of Copenhagen. After excision of the pancreas, islets were isolated by collagenase digestion and dispersed into single cells essentially as detailed elsewhere (Ämmälä *et al.* 1993). The cells were plated on 35 mm diameter Petri dishes and incubated in a humidified atmosphere for up to 5 days in RPMI 1640 tissue culture medium (Gibco BRL, Life Technologies Ltd, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 i.u.  $\text{ml}^{-1}$  penicillin and 100  $\mu\text{g ml}^{-1}$  streptomycin.

One series of experiments (see Fig. 7) was performed on  $\alpha$ -cells obtained by fluorescence-activated cell sorting (FACS; Josefsen *et al.* 1996). Based on the hormone contents and their glucose sensitivities, we estimate that the preparations contain > 80%  $\alpha$ -cells and < 3%  $\beta$ -cells (Josefsen *et al.* 1996; Gromada *et al.* 1997). The  $\alpha$ -cells could be functionally distinguished from the  $\beta$ -cells

by the presence of a prominent rapidly inactivating  $\text{Na}^+$  current and small cell size (cell capacitance < 2.5 pF).

### Electrophysiology

Patch pipettes were pulled from borosilicate glass (tip resistance 3–4  $\text{M}\Omega$  when filled with the pipette solution), coated with Sylgard and fire-polished before use. The zero-current potential was adjusted before establishment of the seal with the pipette in the bath. The holding potential in all experiments was  $-70$  mV.

Exocytosis was monitored in single  $\beta$ -cells as changes in cell membrane capacitance using either the standard or the perforated-patch whole-cell configuration. The patch-clamp recordings were made using EPC-7 (List Elektronik, Darmstadt, Germany; Figs 3 and 5–7) or EPC-9 (Heka Electronics, Lamprecht/Pfalz, Germany; Figs 4 and 8B) patch-clamp amplifiers. Exocytosis was elicited by 500-ms voltage-clamp depolarizations from  $-70$  to 0 mV. Changes in cell capacitance were detected using in-house software written in Axobasic (Axon Instruments, Union City, CA, USA; Ämmälä *et al.* 1993) or the software Pulse (Heka, version 8.41). During the electrophysiological experiments, the cells were placed in an experimental chamber with a volume of 0.4 ml, which was continuously superfused at a rate of 1.5  $\text{ml min}^{-1}$  to maintain the temperature at 33°C. Experiments commenced when two successive depolarizations or trains of pulses applied at a 1–2 min interval elicited exocytotic responses of the same amplitude ( $\pm 10\%$ ) to ascertain that the observed changes were not simply attributable to spontaneous long-term changes of the secretory capacity. Membrane potential recordings (Fig. 8B) were performed in  $\beta$ -cells in intact rat islets as previously described for mouse islets (Göpel *et al.* 1999).

### Solutions for electrophysiology

The pipette solution for standard whole-cell experiments (Figs 5 and 6) contained (mM): caesium glutamate 125, CsCl 10, NaCl 10,  $\text{MgCl}_2$  1, Hepes 5, EGTA 0.05, MgATP 3, cAMP 0.1 and GTP 0.01; pH was adjusted to 7.15 with CsOH. In perforated-patch whole-cell experiments, the pipette solution contained (mM): NaCl 10, KCl 10,  $\text{MgCl}_2$  1,  $\text{Cs}_2\text{SO}_4$  76 (Figs 3–4 and 7) or 76  $\text{K}_2\text{SO}_4$  (Fig. 8B) and Hepes 5; pH was adjusted to 7.35 with CsOH or KOH. Electrical contact with the cell interior was established by adding 0.24  $\text{mg ml}^{-1}$  amphotericin B to the pipette solution. Perforation required a few minutes and the voltage clamp was considered satisfactory when the series conductance ( $G_{\text{series}}$ ) was constant and exceeded 35–40 nS. The extracellular medium usually contained (mM): NaCl 118,

tetraethylammonium (TEA) chloride 20, KCl 5.6, MgCl<sub>2</sub> 1.2, CaCl<sub>2</sub> 2.6, D-glucose 5 (Figs 3–7) and Hepes 5; pH was adjusted to 7.40 using NaOH. TEA was included to block outward rectifying K<sup>+</sup> currents, which persist even after replacement of intracellular K<sup>+</sup> by Cs<sup>+</sup>. For measuring glucose-induced electrical activity (Fig. 8B), TEA was replaced by an equal concentration of NaCl, and the glucose concentration was increased to 20 mM. Forskolin (2 μM) was included in the extracellular solution in all experiments involving measurements of exocytosis in intact β-cells (perforated-patch recordings) to increase the exocytotic capacity. Calcineurin auto-inhibitory peptide was supplied by Calbiochem (La Jolla, CA, USA) and CGP 55845 was purchased from Tocris (Bristol, UK). All other chemicals were purchased from Sigma.

### Measurements of [Ca<sup>2+</sup>]<sub>i</sub>

The [Ca<sup>2+</sup>]<sub>i</sub> measurements in single cells (Fig. 3) were made using an Axiovert 135 inverted microscope equipped with a Plan-Neofluar × 100, 1.30 NA objective (Carl Zeiss, Oberkochen, Germany) and an Ionoptix (Milton, MA, USA) fluorescence imaging system as described elsewhere (Bokvist *et al.* 1995). The experiments were conducted using the perforated-patch whole-cell configuration with the pipette-filling solution specified above. Prior to the experiments, the cells were loaded with the acetoxymethyl ester of fura-2 (fura-2 AM; 0.2 μM; Molecular Probes, Eugene, OR, USA) for 16–18 min. Calibration of the fluorescence ratios was performed using the standard whole-cell configuration to infuse fura-2 AM with different mixtures of Ca<sup>2+</sup> and EGTA to obtain a known [Ca<sup>2+</sup>]<sub>i</sub>.

[Ca<sup>2+</sup>]<sub>i</sub> in intact islets (Fig. 8A) was recorded by dual-wavelength microfluorimetry using a D104 PTI microfluorimetry system (Monmouth Junction, NJ, USA) as described elsewhere (Olofsson *et al.* 2002). In short, the cells were loaded with 3 μM fura-2 AM in the presence of 0.007% w/v pluronic acid (Molecular Probes) at 37°C for 30–40 min prior to measurements. During the experiment, the islet was continuously superfused with a solution containing (mM): NaCl 140, KCl 3.6, NaHCO<sub>3</sub> 2, NaH<sub>2</sub>PO<sub>4</sub> 0.5, MgSO<sub>4</sub> 0.5, Hepes 5, CaCl<sub>2</sub> 2.6 and glucose 5 or 15; pH was adjusted to 7.4 with NaOH.

### Hormone release measurements

Insulin, glucagon and somatostatin release were determined by radioimmunoassay (RIA) as described elsewhere (Vonen *et al.* 1989; Salehi *et al.* 1999). Briefly, batches of 8–10 islets were pre-incubated in 1 ml of Krebs-Ringer buffer (KRB) supplemented with 1 mM

glucose for 30 min followed by 1 h incubation in 1 ml KRB containing 1 or 20 mM glucose. The specific GABA<sub>B</sub>R agonist baclofen, the GABA<sub>B</sub>R antagonist CGP 55845 and the ATP-dependent K<sup>+</sup> (K<sub>ATP</sub>) channel blocker tolbutamide (Sigma, St Louis, MO, USA) were included in the extracellular medium as indicated. At the end of the incubation, duplicate aliquots (25–100 μl) of the medium were removed and frozen pending the radioimmunoassays.

### RT-PCR

Total RNA from rat islets and from FACS purified rat α- and β-cells was isolated using RNeasy Mini Kit (Qiagen). Total RNA 1 μg was used for reverse transcription using random hexamer primers and Superscript II (Invitrogen). In a negative control Superscript II was omitted from the reaction. PCR was performed under the following conditions: 2 min 94°C followed by 35 cycles of 45 s 94°C, 45 s 54°C or 56°C and 60 s 72°C. Products were analysed on a 1% agarose gel and by sequencing. The following primers were used:

Insulin: 5'CTGTGGATGCGCTTCCTGCC3' and 5'CGGGACTTGGGTGTGTAGAAG3' (expected fragment 158 bp);

Glucagon: 5'ATGCTGGTACAAGGCAGCTGGCAG3' and 5'CAAGTAAGAACTCACATCACTGG3' (expected fragment 297 bp);

\*GABA<sub>B</sub>R<sub>1a-d,f</sub>: 5'GCTGGATGGTTACCACATAG3' and 5'GGTCACAGGAGCAGTGATG3' (expected fragment sizes were 525 bp and 618 bp for a,b,d,f and c, respectively);

GABA<sub>B</sub>R<sub>1a,f</sub>: 5'GCCTGTGGACTATGAGATCG3' and 5'TTCGATTACCTGGCAGTGG3' (expected fragment: 313 bp);

\*GABA<sub>B</sub>R<sub>1c,f</sub>: 5'GTGAGTAGTGATGTTTCAGCG3' and 5'GCTTGATCCTTCTCCATGC3' (expected fragment: 671 bp);

\*GABA<sub>B</sub>R<sub>1d</sub>: 5'CCTTCGATAGAGGTTTGAG3' and 5'CTGGAGGAAGAAACACAATC3' (expected fragment: 506 bp);

GABA<sub>B</sub>R<sub>2</sub>: 5'CAACGACAGCAAGTACATC3' and 5'CAGCTCTGTGATCTTCATTC3' (expected fragment: 357 bp).

Except for GABA<sub>B</sub>R<sub>2</sub> and GABA<sub>B</sub>R<sub>1d</sub>, it was not possible to design primers specific for the individual isoforms. Primer sequences indicated by the asterisks were designed as previously described (Brice *et al.* 2002).

### Data analysis

Results are presented as means ± s.e.m. for the indicated number of experiments. Significant differences were evaluated using Student's *t* test for paired data except for Fig. 1 where Student's *t* test for unpaired data was used.

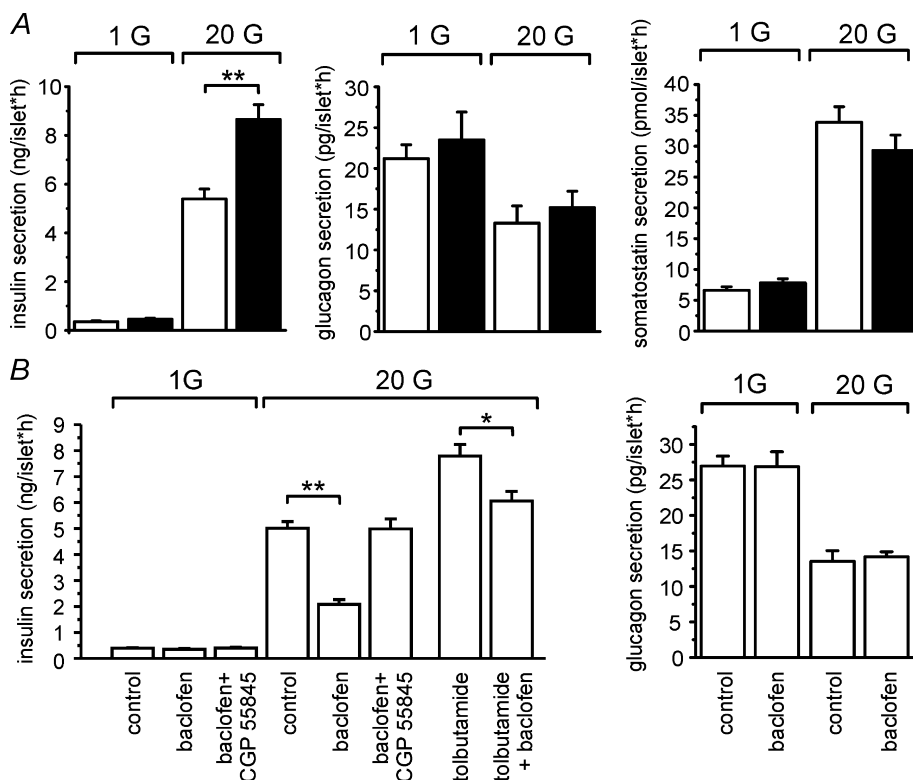
## Results

### GABA<sub>B</sub>R antagonism increases glucose-induced insulin secretion but has no effect on glucagon and somatostatin release

To study the involvement of GABA<sub>B</sub>Rs in the regulation of hormone release from isolated rat pancreatic islets, we used the GABA<sub>B</sub>R antagonist CGP 55845 (Fig. 1A). Increasing glucose from 1 mM to 20 mM stimulated insulin secretion 15-fold. Addition of CGP 55845 resulted in a further 61% enhancement of glucose-induced insulin secretion whereas basal release was not affected. Glucagon secretion was suppressed by glucose (−38%) but it was not affected by GABA<sub>B</sub>R antagonism. Finally, glucose stimulated somatostatin release 5.1-fold. Neither basal, nor glucose-induced somatostatin secretion were affected by the antagonist. It should be noted that the observed effects of CGP 55845 in these experiments are likely to reflect the removal of GABA<sub>B</sub>R-signalling due to endogenous GABA as no exogenous GABA was added.

### The GABA<sub>B</sub>R agonist baclofen inhibits glucose-stimulated insulin secretion

We next tested the effects of the selective GABA<sub>B</sub>R agonist baclofen on insulin and glucagon secretion (Fig. 1B). Basal insulin secretion was unaffected by 10 μM baclofen. In this series of experiments, glucose (20 mM) stimulated insulin secretion 13-fold. The latter effect was partially suppressed (−63%) by baclofen. The inhibitory action of baclofen on glucose-induced insulin secretion was fully antagonized by the GABA<sub>B</sub>R antagonist CGP 55845. It is important that the amount of insulin secreted in the presence of baclofen, CGP 55845 and 20 mM glucose is less than that released from islets exposed to glucose and CGP 55845 (compare Fig. 1A and B). The K<sub>ATP</sub> channel blocker tolbutamide stimulated insulin secretion in excess of that elicited by glucose alone (+55%). Baclofen remained capable of inhibiting insulin secretion under the latter conditions but the effect was limited to a reduction by 23%. Whereas 20 mM glucose inhibited glucagon secretion by ~50%, addition of baclofen had no statistically significant effect on glucagon secretion (Fig. 1B). Taken together,



**Figure 1. Modulation of hormone release from isolated rat pancreatic islets by GABA<sub>B</sub>Rs**

A and B, hormone release from isolated rat pancreatic islets was measured in the presence of 1 mM glucose (1 G) or 20 mM glucose (20 G) in the medium. Data are mean values  $\pm$  s.e. of 10 experiments (\* $P$  < 0.01, \*\* $P$  < 0.001). A, insulin (left panel), glucagon (middle) and somatostatin release (right) in the absence (open bars) or presence (filled bars) of the GABA<sub>B</sub>R antagonist CGP 55845 (10 μM). B, effects of the GABA<sub>B</sub>R agonist baclofen (10 μM), the GABA<sub>B</sub>R antagonist CGP 55845 (10 μM) and the K<sub>ATP</sub> channel blocker tolbutamide (100 μM) as indicated on insulin secretion (left panel) and glucagon secretion (right panel).

the hormone release measurements suggest that GABA<sub>B</sub>R activation selectively modulates insulin secretion and that it has no effect on the release of the two other major islet hormones.

### Expression of GABA<sub>B</sub>Rs in pancreatic islet cells

We explored expression of metabotropic GABA<sub>B</sub>R isoforms in rat islets and purified rat  $\alpha$ - and  $\beta$ -cells (Fig. 2A). We thus confirmed expression of GABA<sub>B</sub>R<sub>1a</sub> and/or GABA<sub>B</sub>R<sub>1b</sub> and GABA<sub>B</sub>R<sub>2</sub> in pancreatic islets. We also observed expression of GABA<sub>B</sub>R<sub>1c</sub> and/or GABA<sub>B</sub>R<sub>1f</sub>. The same expression pattern was observed for whole rat islets, purified rat  $\alpha$ -cells and purified rat  $\beta$ -cells. Both  $\alpha$ - and  $\beta$ -cell cDNA preparations were checked for purity using insulin and glucagon primers in a separate multiplex RT-PCR (Fig. 2B). Insulin and glucagon mRNAs were only detected in the  $\beta$ - and  $\alpha$ -cell fractions, respectively.

### The GABA<sub>B</sub>R agonist baclofen inhibits depolarization-evoked exocytosis in rat $\beta$ -cells

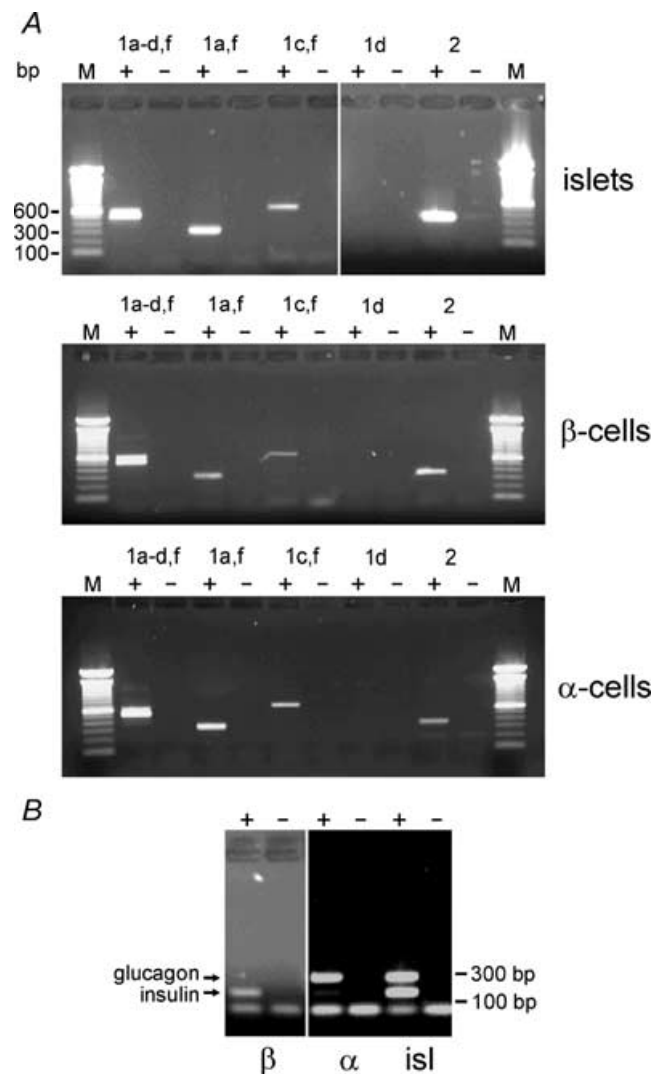
Figure 3A shows parallel recordings of Ca<sup>2+</sup> current ( $I_{Ca}$ ), associated increase in cytoplasmic free Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ) and exocytosis ( $\Delta C_m$ ) in a single rat  $\beta$ -cell in response to 500-ms depolarization from  $-70$  mV to  $0$  mV applied at 2 min intervals. Under control conditions, the depolarization evoked an integrated Ca<sup>2+</sup> current of  $\sim 7$  pC, produced a  $[Ca^{2+}]_i$  increase from a basal concentration of  $0.1 \mu M$  to a peak concentration of  $\sim 1 \mu M$  and a elicited a capacitance increase of  $\sim 80$  fF. Following the addition of  $10 \mu M$  baclofen to activate the GABA<sub>B</sub>R, both the Ca<sup>2+</sup> current and the associated increase in  $[Ca^{2+}]_i$  were moderately ( $\sim 20\%$ ) reduced, whereas exocytosis was more strongly (83%) inhibited. All the effects of baclofen were readily reversible and 4 min after the removal of the agonist, the parameters had returned to their control values.

In a series of six experiments, the average inhibition of exocytosis produced by baclofen amounted to  $79 \pm 14\%$  ( $P < 0.01$ ; Fig. 3B). The effect was associated with an average reduction of the integrated Ca<sup>2+</sup> current ( $Q_{Ca}$ ) of  $28 \pm 9\%$  and a decrease in the amplitude of the depolarization-evoked peak  $[Ca^{2+}]_i$  ( $\Delta[Ca^{2+}]_i$ ) of  $22 \pm 8\%$ . All effects were reversed upon withdrawal of baclofen. The inhibition of exocytosis in  $\beta$ -cells produced by baclofen is comparable to the reduction of neurotransmitter release at the calyx of Held (Takahashi *et al.* 1998; Sakaba & Neher, 2003).

### Dose-dependent inhibition of exocytosis by baclofen

The relationship between baclofen concentration and the amplitude of the exocytotic responses is shown in Fig. 3C. The inhibitory effect of baclofen on exocytosis was

concentration-dependent. Little inhibition of exocytosis was observed at  $0.3 \mu M$  baclofen, and  $10 \mu M$  produced maximal inhibition. Approximating the mean data points to the Hill equation yielded a half-maximal inhibition of  $1.7 \mu M$  and co-operativity factor of 1.1. Our value for the IC<sub>50</sub> of baclofen-induced inhibition of exocytosis in  $\beta$ -cells ( $1.7 \mu M$ ) is close to the  $0.8 \mu M$  obtained for the inhibition of neurotransmitter release in the calyx of Held (Takahashi *et al.* 1998). Similarly, the integrated Ca<sup>2+</sup> current was half-maximally inhibited by baclofen at a concentration of  $1.6 \mu M$  (not shown).



**Figure 2.** RT-PCR on purified  $\alpha$ - and  $\beta$ -cells with specific primers against different GABA<sub>B</sub>R subunits

A, total RNA from rat islets as well as FACS-purified  $\alpha$ - and  $\beta$ -cells was reverse transcribed into cDNA. PCR analysis was performed using specific primers against GABA<sub>B</sub>R<sub>1a-d,f</sub>, GABA<sub>B</sub>R<sub>1a,f</sub>, GABA<sub>B</sub>R<sub>1c,f</sub>, GABA<sub>B</sub>R<sub>1d</sub> and GABA<sub>B</sub>R<sub>2</sub> (+). In the negative control reaction (–) reverse transcriptase was omitted. Molecular standards are shown to the left and the right. B, multiplex RT-PCR on islets (isl) and purified  $\alpha$ - and  $\beta$ -cell with specific primers against insulin and glucagon.

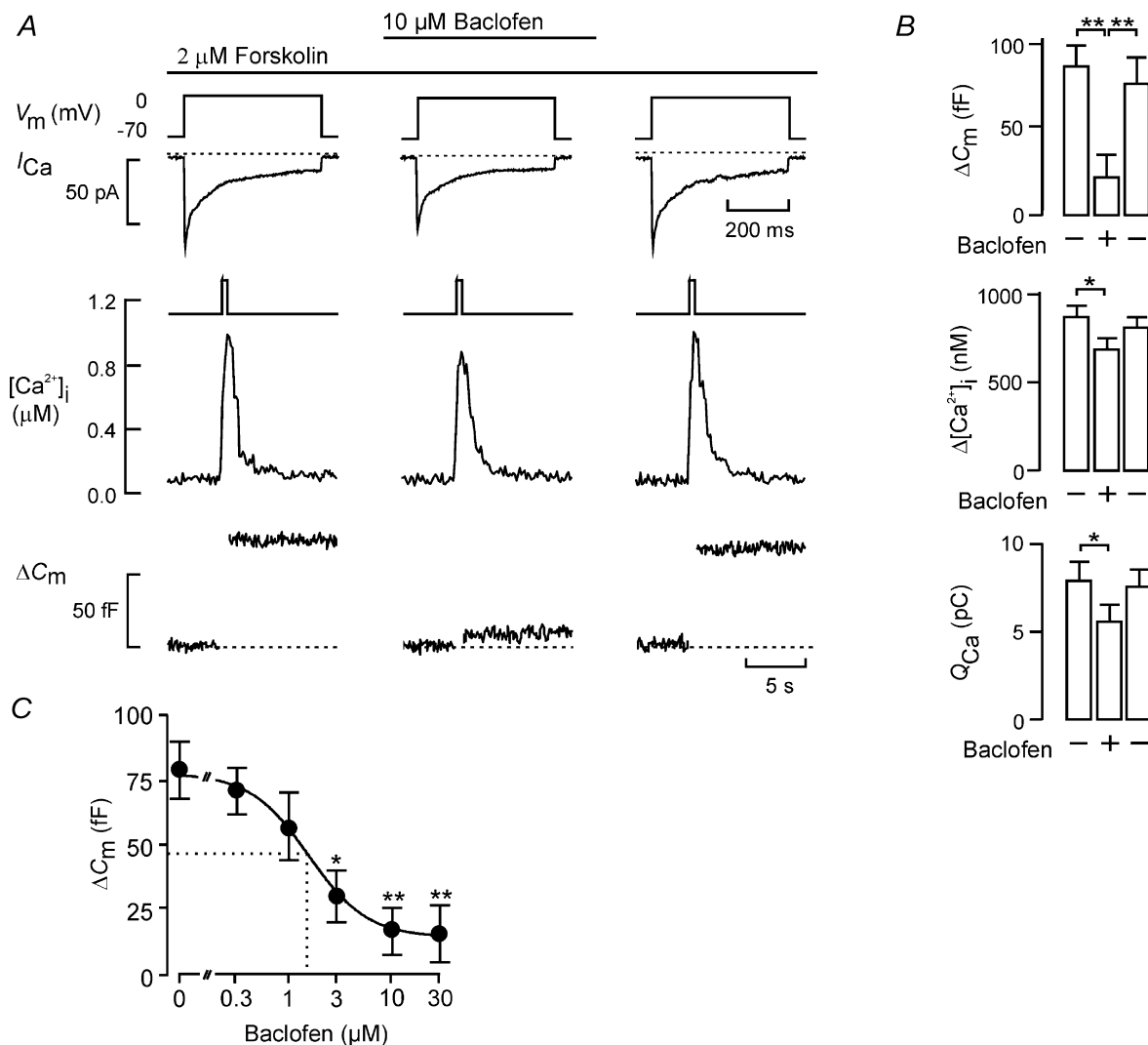
### The inhibitory action of baclofen is antagonized by CGP 55845

We next ascertained that CGP 55845 is capable of antagonizing the inhibitory action of baclofen on exocytosis. Exocytosis was elicited by 500-ms depolarizations from  $-70$  mV to  $0$  mV, applied at 2-min intervals. Figure 4A shows recordings of exocytosis (from left to right) under control conditions, after addition of baclofen, following washout of baclofen, after application of CGP 55845 alone and in the combined presence of

CGP 55845 and baclofen. It can be seen that whereas baclofen in this experiment inhibited exocytosis by  $\sim 70\%$  when applied on its own, it lacked inhibitory action in the presence of the antagonist. Data from a series of seven experiments are summarized in Fig. 4B.

### The inhibitory action of baclofen on exocytosis is mediated by activation of an inhibitory G-protein

The GABA<sub>B</sub> receptor is a member of the large group of G protein-linked membrane receptors. We made use



### Figure 3. Baclofen reduces $Ca^{2+}$ currents, $[Ca^{2+}]_i$ and exocytosis

A, whole-cell  $Ca^{2+}$  current ( $I_{Ca}$ ), cytoplasmic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) and exocytosis ( $\Delta C_m$ ) evoked by membrane depolarizations ( $V_m$ ; 500 ms) from  $-70$  to  $0$  mV using the perforated-patch whole-cell configuration in single rat  $\beta$ -cells. Exocytosis was observed under control conditions, 2 min after addition of baclofen ( $10 \mu M$ ) and 4 min after wash-out of the agonist. The dotted lines indicate the zero current level and the pre-stimulatory capacitance level. B, histograms summarizing effects on changes of cell capacitance ( $\Delta C_m$ ), cytoplasmic  $Ca^{2+}$  levels ( $\Delta[Ca^{2+}]_i$ ) and integrated  $Ca^{2+}$  current ( $Q_{Ca}$ ). Data are mean values  $\pm$  s.e.m. of six cells.  $*P < 0.05$ ;  $**P < 0.01$ . C, dose-inhibition curve for the effect of baclofen on exocytosis. Data are the mean  $\pm$  s.e.m. ( $n = 5-7$ ) of the responses to 500-ms depolarizations from  $-70$  mV to  $0$  mV at concentrations of baclofen between  $0$  and  $30 \mu M$ ;  $*P < 0.01$  and  $**P < 0.001$  versus control (no baclofen). The curve was obtained by fitting the mean data points to the Hill equation. The dotted lines indicate the concentration of baclofen at which exocytosis is reduced by 38% (i.e. exocytosis is 1/1.6 of that observed in the absence of baclofen; cf. Fig. 1).

of the standard whole-cell configuration, which has the advantage of permitting the cell interior to be dialysed by the pipette solution (Hamill *et al.* 1981), to elucidate the involvement of G proteins in the effects of baclofen on exocytosis. Figure 5A demonstrates that the ability of baclofen to inhibit exocytosis is maintained in the standard whole-cell configuration and when the intracellular cAMP levels are clamped at 0.1 mM. In a series of six experiments, 10  $\mu\text{M}$  baclofen reversibly inhibited exocytosis ( $\Delta C_m$ ) elicited by individual 500-ms pulses from  $-70$  mV to 0 mV by  $82 \pm 13\%$  (Fig. 5E). Consistent with the involvement of an inhibitory G protein ( $G_{i/o}$ ), the ability of baclofen to inhibit exocytosis was lost following overnight treatment with 100 ng ml<sup>-1</sup> pertussis toxin (Fig. 5B and E) or when 0.5 mM of the stable GDP analogue GDP $\beta$ S was included in the intracellular solution (Fig. 5C and E). By contrast, when the experiment was conducted in the intracellular presence of 100  $\mu\text{M}$  GTP $\gamma$ S (a stable GTP analogue that causes irreversible activation of the GTP-binding proteins), application of baclofen resulted in permanent inhibition of exocytosis (Fig. 5D and E).

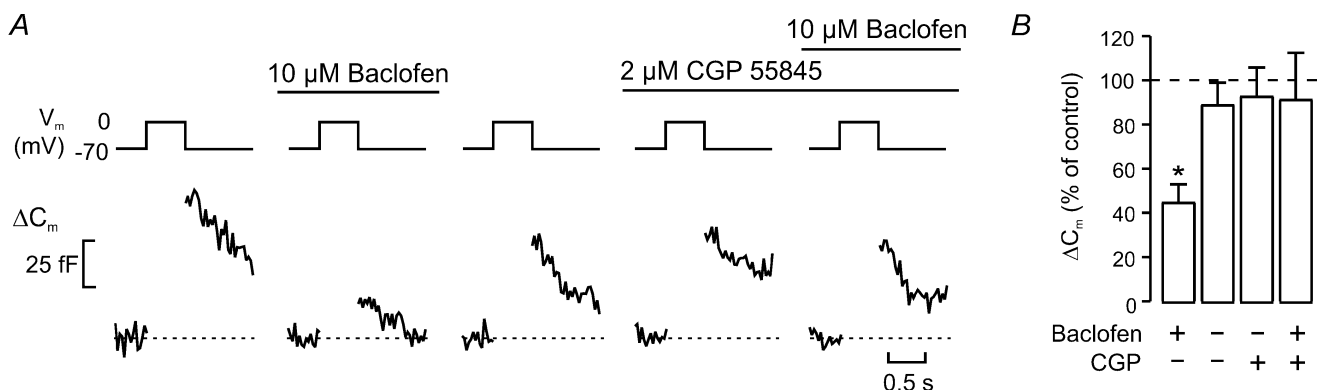
#### Baclofen mediates its inhibitory action on exocytosis via activation of calcineurin

We have previously reported that the inhibitory action of somatostatin, galanin and adrenaline on exocytosis from  $\beta$ -cells is secondary to activation of the serine/threonine protein phosphatase calcineurin (Renström *et al.* 1996a). We explored whether this also applies to baclofen-induced inhibition of exocytosis. Indeed, baclofen failed to suppress exocytosis following inhibition of calcineurin with cyclosporin A (1  $\mu\text{M}$  for  $> 20$  min; Fig. 6A). A similar abolition was observed after intracellular application (through

the patch electrode during whole-cell recordings) of calcineurin autoinhibitory peptide (100  $\mu\text{M}$ ), which is a highly selective inhibitor of calcineurin (Perrino *et al.* 1995) (Fig. 6B). Exocytosis measured after addition of baclofen averaged  $87 \pm 18\%$  ( $n = 5$ ) and  $89 \pm 13\%$  ( $n = 5$ ) of that observed under control conditions when the experiments were conducted in the presence of cyclosporin A and calcineurin autoinhibitory peptide, respectively. Under otherwise identical experimental conditions but in the absence of the calcineurin inhibitors, baclofen suppressed exocytosis by  $\sim 80\%$  (Fig. 5A).

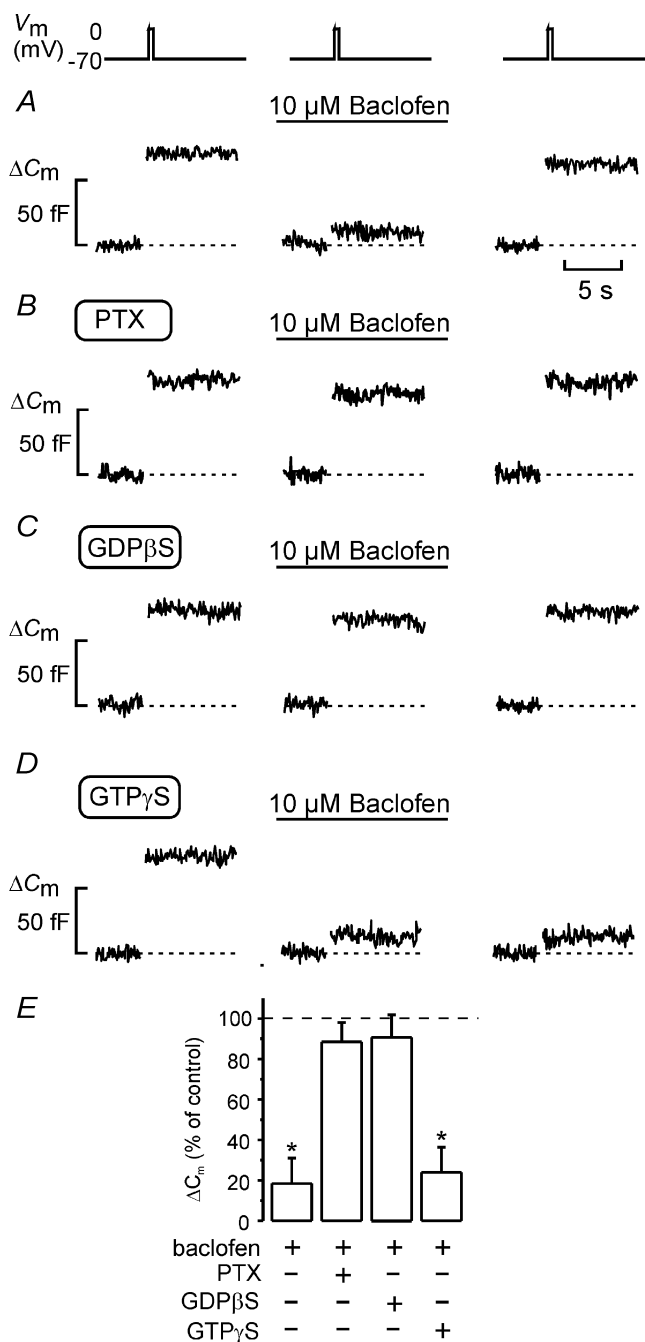
#### Baclofen does not inhibit adrenaline-stimulated exocytosis in $\alpha$ -cells

We have previously reported that somatostatin in addition to its effects on  $\beta$ -cells (Renström *et al.* 1996a) also inhibits exocytosis in  $\alpha$ -cells by a G protein-mediated action involving activation of calcineurin (Gromada *et al.* 2001). Given the similarity between the effects of somatostatin and baclofen in  $\beta$ -cells and the detection of GABA<sub>B</sub>R mRNA in the  $\alpha$ -cell fraction (Fig. 2), we next investigated the ability of baclofen to modulate exocytosis in rat  $\alpha$ -cells exposed to adrenaline (Fig. 7). In a series of five experiments, adrenaline increased exocytosis by  $312 \pm 31\%$  ( $P < 0.01$ ), an effect that was associated with a moderate increase in the integrated Ca<sup>2+</sup> current ( $40 \pm 17\%$ ;  $P < 0.01$ ). Baclofen had no effect on either parameter, and the mean amplitude of the exocytotic responses was  $95 \pm 9\%$  ( $n = 5$ ) of that observed in the presence of adrenaline alone. The failure of baclofen to affect exocytosis is in agreement with the lack of effect of the agonist on glucagon secretion (Fig. 1).



**Figure 4. Baclofen-induced inhibition of exocytosis can be antagonized by CGP 55845**

A, exocytosis ( $\Delta C_m$ ) elicited by 500-ms depolarizations of membrane potential ( $V_m$ ) from  $-70$  mV to 0 mV under control conditions, in the presence of 10  $\mu\text{M}$  baclofen, following the wash-out of baclofen, in the presence of 2  $\mu\text{M}$  CGP 55845 and in the simultaneous presence of 2  $\mu\text{M}$  CGP 55845 and 10  $\mu\text{M}$  baclofen (left to right). Pulses were applied with a 2-min interval. B, exocytotic responses normalized to control (before addition of baclofen) as the mean  $\pm$  s.e.m ( $n = 7$ ; \* $P < 0.01$  versus control).



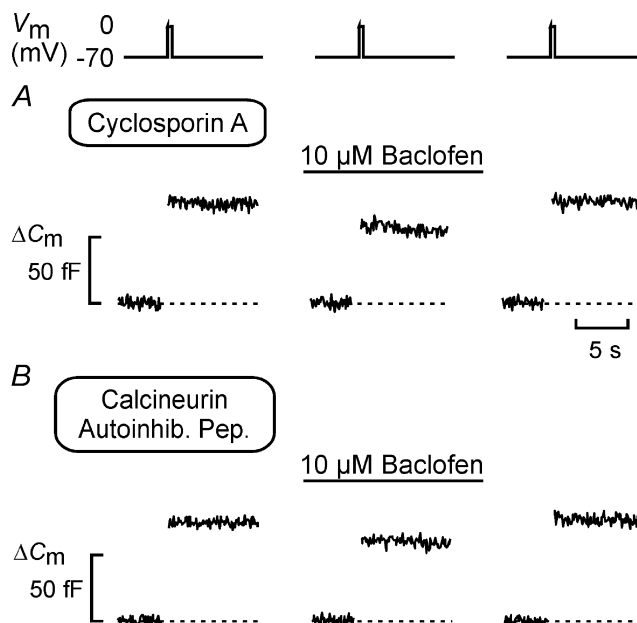
**Figure 5. Baclofen-induced inhibition of exocytosis is mediated by activation of a  $G_{i/o}$  protein**

A–E, effects of 10  $\mu$ M baclofen on exocytosis ( $\Delta C_m$ ) elicited by 500 ms voltage-clamp depolarizations of the membrane potential ( $V_m$ ) from  $-70$  to  $0$  mV applying the standard whole-cell configuration to rat  $\beta$ -cells. Changes in cell capacitance were measured before and 2 min after the addition of baclofen, and 4 min after wash-out of the agonist under control conditions (A), in cells treated for > 20 h with 100 ng ml $^{-1}$  pertussis toxin (PTX; B), with 0.5 mM GDP $\beta$ S included in the pipette solution (C) and in the presence of 0.1 mM intracellular GTP $\gamma$ S (D). Cyclic AMP (0.1 mM) was included in all pipette solutions. E, histogram showing changes in cell capacitance ( $\Delta C_m$ ) normalized to the respective controls (before addition of baclofen). Data are the mean  $\pm$  S.E.M. ( $n = 5$ –6 cells; \* $P < 0.01$ ).

### Baclofen has no effect on $[Ca^{2+}]_i$ or $\beta$ -cell electrical activity in intact islets

To investigate the effect of GABA $_B$ R stimulation on  $Ca^{2+}$  signalling we measured  $[Ca^{2+}]_i$  in glucose stimulated islets before and after the addition of baclofen (Fig. 8A). The agonist was added to the islet in the steady state after a regular  $[Ca^{2+}]_i$  oscillatory pattern had established. In a series of nine experiments, the fura-2 AM fluorescence ( $F$ ) ratio at 350 and 380 nm ( $F_{350}/F_{380}$ ), as a measure of  $[Ca^{2+}]_i$ , was  $0.71 \pm 0.02$  in the presence of 5 mM glucose. In the presence of 15 mM glucose, the average ratio before addition of baclofen was  $0.83 \pm 0.03$ .  $F_{350}/F_{380}$  was  $0.85 \pm 0.03$  4 min after addition of baclofen, and  $0.86 \pm 0.03$  4 min after washout. Oscillations in  $[Ca^{2+}]_i$  were observed in 8 out of 9 islets. The oscillations were temporary in six islets and stable throughout the experiment in two islets (see also Fig. 8A). No consistent correlation between addition of baclofen and the appearance or disappearance of oscillations or the oscillatory pattern was observed.

We also investigated the effect of baclofen on  $\beta$ -cell membrane potential. Recordings were performed in the perforated-patch whole cell configuration on functionally identified  $\beta$ -cells in intact rat islets. Electrical activity



**Figure 6. Baclofen-induced inhibition of exocytosis involves activation of the protein phosphatase calcineurin**

A and B, exocytosis measured as increases in cell capacitance ( $\Delta C_m$ ), elicited by 500 ms voltage-clamp depolarizations of the membrane potential ( $V_m$ ) from  $-70$  to  $0$  mV using the standard whole-cell configuration before and 2 min after the addition of baclofen (10  $\mu$ M), and 4 min after wash-out of baclofen from the medium in cells pre-treated with cyclosporin A (A; 1  $\mu$ M for > 20 min) or when 0.1 mM of the calcineurin autoinhibitory peptide was included in the intracellular solution (B).



was elicited by increasing the glucose concentration of the extracellular medium from 3 to 20 mM. Application of baclofen had no effect on action potential firing and membrane potential (Fig. 8B;  $n = 5$ ). In the same experiments the membrane conductance, principally reflecting  $K_{ATP}$  channel activity, was  $1.24 \pm 0.16$  nS in the presence of 20 mM glucose alone and  $1.35 \pm 0.11$  nS after addition of baclofen (n.s.;  $n = 5$ ).

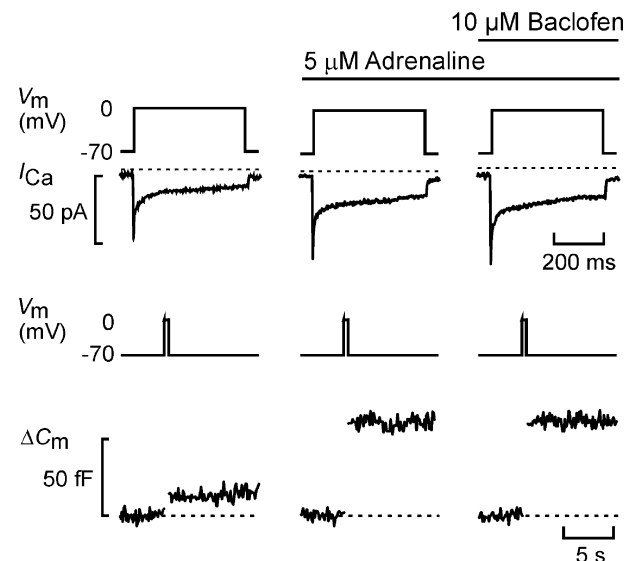
## Discussion

Pancreatic islet cells interact with each other via hormonal and electrical signals. It is well established that the blood sugar-regulating hormones also act as paracrine/autocrine regulators of hormone release from islets (Samols *et al.* 1986). In addition to peptide hormones, islet cells contain and release several other bioactive compounds, including classical neurotransmitters, which constitute potential local signalling molecules (Hayashi *et al.* 2003; Rorsman & Renström, 2003). We have recently demonstrated that  $Ca^{2+}$ -dependent exocytosis of the SLMVs leads to GABA release into the islet interstitium that, via activation of ionotropic GABA<sub>A</sub> receptors in the  $\alpha$ -cells, inhibits glucagon secretion (Braun *et al.* 2004; Wendt *et al.* 2004). In this paper we focus on the possible role of metabotropic GABA<sub>B</sub> receptors in the regulation of pancreatic hormone secretion.

### Endogenous GABA release selectively modulates insulin secretion in intact islets

The GABA<sub>B</sub>R antagonist CGP 55845 when added on its own stimulated glucose-induced insulin secretion whilst not affecting glucagon and somatostatin secretion (Fig. 1A). The fact that the antagonist was effective suggests that biologically active concentrations of GABA are present in the islet interstitium. The observed 1.6-fold enhancement of glucose-induced insulin secretion can be used to estimate the interstitial GABA concentration. As shown in Fig. 3C, exocytosis is influenced by baclofen in a concentration-dependent fashion with an  $IC_{50}$  value of  $\sim 2 \mu M$ . If we assume that insulin secretion in the intact islet and in the presence of the antagonist is equivalent to exocytosis measured in isolated  $\beta$ -cells in the complete absence of GABA, then we can estimate that the average interstitial concentration of GABA experienced by the  $\beta$ -cell *in situ* is equivalent to  $2 \mu M$  baclofen ( $75 \text{ fF}/1.6 = 48 \text{ fF}$ ; see dotted line in Fig. 3C). We have estimated the intravesicular GABA content to be 0.4 amol (Braun *et al.* 2004). A pancreatic  $\beta$ -cell has a volume of  $\sim 1$  pl (estimated using spherical geometry and a diameter of  $12 \mu m$ ). If we assume that the interstitial space is about 10% of the intracellular volume as suggested by electron microscopy (Dean, 1973), then an islet containing 1000

$\beta$ -cells has an interstitial volume of 0.1 nl. An average interstitial concentration of  $1 \mu M$  will accordingly arise if as little as 250 SLMVs are released in the entire islet (i.e. 0.25 SLMV per  $\beta$ -cell). For comparison, the basal rate of GABA release has been estimated to be one vesicle per second (Smismans *et al.* 1997), and the release rate increases up to 10-fold upon stimulation (Braun *et al.* 2004). Even when allowance is made for the fact that baclofen is more potent than GABA (Takahashi *et al.* 1998), these considerations make it likely that physiologically relevant concentrations of GABA do indeed occur in the islet interstitium during  $\beta$ -cell electrical activity. The fact that neither glucagon, nor somatostatin secretion was affected under these conditions suggests that the effect on insulin secretion was indeed mediated by activation of GABA<sub>B</sub>Rs and not unspecific interactions with other effector proteins. The failure to demonstrate an effect of baclofen on hormone release from mouse islets in an earlier study (Gilon *et al.* 1991) possibly reflects the low GABA concentration found in mouse islets (Michalik & Erecinska, 1992). Thus, the importance of GABAergic signalling in islets is likely to vary between different species. It may seem surprising that CGP 55845 has no effect on depolarization-evoked exocytosis (Fig. 4A and B). This we attribute to GABA being quickly diluted/washed away in the experiments on isolated  $\beta$ -cells, whereas it is trapped in the interstitium in the insulin release experiments conducted on intact pancreatic islets.



**Figure 7. Baclofen does not affect adrenaline-stimulated exocytosis in glucagon-secreting  $\alpha$ -cells**

Whole-cell  $Ca^{2+}$  current ( $I_{Ca}$ ) and exocytosis ( $\Delta C_m$ ) evoked by membrane depolarizations ( $V_m$ ; 500 ms) from  $-70$  to  $0$  mV using the perforated-patch whole-cell configuration in single rat  $\alpha$ -cells. Exocytosis was observed under control conditions, 2 min after addition of adrenaline ( $5 \mu M$ ) and in the simultaneous presence of both adrenaline and baclofen ( $10 \mu M$ , applied for 2 min). The dotted lines indicate the zero current level and the pre-stimulatory capacitance level.

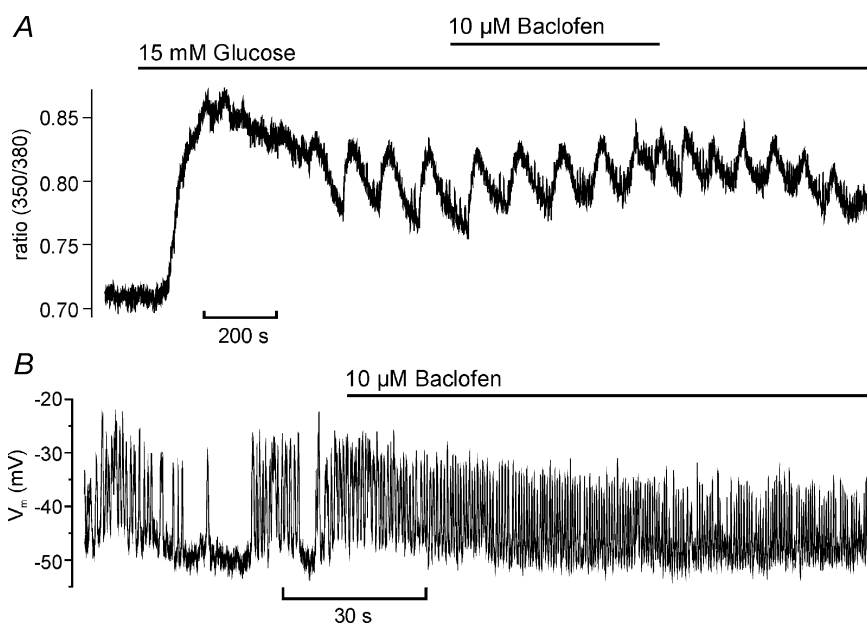
### Mechanisms of baclofen action

It has been reported that baclofen leads to a reduction in the cytoplasmic  $\text{Ca}^{2+}$  concentration in rat  $\beta$ -cells (Gu *et al.* 1993). We did not observe any effect of baclofen on the glucose-induced  $\text{Ca}^{2+}$  signal in islets (Fig. 8A). Accordingly, baclofen had no effect on glucose-induced electrical activity and  $\text{K}_{\text{ATP}}$  currents measured in  $\beta$ -cells in intact islets (Fig. 8B). This suggests that baclofen, unlike other inhibitory agonists (Rorsman *et al.* 1991; Renström *et al.* 1996a), does not inhibit insulin secretion by influencing electrical activity and cytoplasmic  $\text{Ca}^{2+}$  signalling.

In the calyx of Held, acute inhibition of synaptic transmission by baclofen has been found to be principally determined by the suppression of pre-synaptic  $\text{Ca}^{2+}$  entry, and exocytosis itself is unaffected (Takahashi *et al.* 1998). It is unlikely that the small decrease in  $\text{Ca}^{2+}$  current we observe is responsible for the inhibition of exocytosis. Indeed, reducing the  $\beta$ -cell  $\text{Ca}^{2+}$  current by 20–25% only marginally reduces the exocytotic responses (Renström *et al.* 1996b). We therefore conclude that more distal actions are responsible for the suppression of exocytosis in the  $\beta$ -cell. Recently, it has been demonstrated that activation of  $\text{GABA}_\text{B}$ Rs also interferes with the replenishment of the 'readily releasable pool' (RRP) of

synaptic vesicles (Sakaba & Neher, 2003). In the present study, baclofen inhibited exocytosis elicited by individual pulses, which principally reflects release of RRP granules (Renström *et al.* 1997; Barg *et al.* 2001). This suggests that the effect of baclofen is exerted distal to granule priming in  $\beta$ -cells. Given the similarities of exocytosis in various cells, it is possible that a direct effect on the exocytotic process itself also contributes to  $\text{GABA}_\text{B}$ R modulation of synaptic transmission; however, this remains to be confirmed experimentally.

Considering that baclofen appears to inhibit insulin secretion via a direct effect on exocytosis, it was surprising that the  $\text{K}_{\text{ATP}}$  channel blocker tolbutamide (which acts via increasing electrical activity and  $[\text{Ca}^{2+}]_i$ ) partially antagonized the inhibitory effect of baclofen on insulin secretion (Fig. 1B). Sulphonylureas have been shown to stimulate exocytosis in  $\beta$ -cells not only via closure of  $\text{K}_{\text{ATP}}$  channels, but also by a direct effect on insulin granule exocytosis (Eliasson *et al.* 1996). Tolbutamide might therefore counteract the baclofen effect via a direct effect on the secretory machinery. Alternatively, the ability of tolbutamide to antagonize the inhibitory action of baclofen may be apparent. The stimulatory effect of the  $\text{GABA}_\text{B}$  antagonist CGP 55845 on insulin secretion (Fig. 1A) indicates the presence of a substantial concentration of GABA in the



**Figure 8. Baclofen does not affect glucose-induced intracellular  $\text{Ca}^{2+}$  signalling and electrical activity of  $\beta$ -cells**

A,  $[\text{Ca}^{2+}]_i$  was measured in an intact islet by dual-wavelength microfluorimetry using fura-2 AM as the  $\text{Ca}^{2+}$  indicator.  $[\text{Ca}^{2+}]_i$  responses are expressed as fura-2 fluorescence ( $F$ ) ratios ( $F_{350}/F_{380}$ ). The islet was superfused with a solution containing 15 mM glucose to generate the characteristic biphasic  $[\text{Ca}^{2+}]_i$  response. At the time indicated, 10  $\mu\text{M}$  baclofen was included in the bath solution. The trace is representative of nine experiments. B, membrane potential recording from a  $\beta$ -cell in an intact rat islet obtained using the perforated-patch whole-cell configuration. Electrical activity was induced by addition of 20 mM glucose to the bath solution. Baclofen (10  $\mu\text{M}$ ) was applied as indicated by the horizontal line.

islet interstitium. Compared to 20 mM glucose alone, tolbutamide further increased insulin secretion ~1.6-fold, and a similar effect can be expected on GABA release from  $\beta$ -cells (Braun *et al.* 2004). It is therefore conceivable that release of endogenous GABA is sufficient to achieve near saturation of the GABA<sub>B</sub>R and addition of baclofen therefore only leads to a moderate further reduction.

### Baclofen mediates its inhibitory action by activation of the protein phosphatase calcineurin

As cAMP has been shown to enhance Ca<sup>2+</sup>-dependent exocytosis in  $\beta$ -cells by both protein kinase A (PKA)-dependent and PKA-independent mechanisms, it could be argued that suppression of exocytosis by baclofen results from G<sub>i/o</sub>-mediated reduction of adenylate cyclase activity (Kerr & Ong, 1995; Bowery *et al.* 2002). Although we cannot exclude the possibility that baclofen inhibits adenylate cyclase in  $\beta$ -cells, the finding that the inhibitory action of baclofen persisted in standard whole-cell recordings when the cytoplasmic cAMP concentration was clamped at 100  $\mu$ M by inclusion of the nucleotide in the pipette solution, argues that inhibition of cAMP generation is not required for the effect on exocytosis. Our data instead suggest that the ability of baclofen to inhibit Ca<sup>2+</sup>-dependent exocytosis involves activation of the protein phosphatase calcineurin. These results are consistent with the results previously obtained in mouse pancreatic  $\beta$ -cells (Renström *et al.* 1996a), rat  $\alpha$ -cells (Gromada *et al.* 2001), bovine chromaffin cells (Craig *et al.* 2003) and nerve terminals (Hens *et al.* 1998). The data underscore the importance of phosphorylation/dephosphorylation in the control of exocytosis in a variety of secretory cells and suggest that the actions of the inhibitors converge at calcineurin. Clearly, it is now important to identify the molecular target involved. Recent observations made in chromaffin cells suggest that phosphorylation/dephosphorylation of SNAP-25 by PKA/calcineurin determines vesicle release competence (Nagy *et al.* 2004).

### GABA<sub>B</sub>Rs in $\alpha$ -cells

In RT-PCR experiments, expression of GABA<sub>B</sub>R subunit mRNA was detected in both purified  $\beta$ -cells and  $\alpha$ -cells (Fig. 2). However, baclofen neither affected glucagon secretion from isolated islets (Fig. 1) nor adrenaline-stimulated exocytosis in single  $\alpha$ -cells (Fig. 7). We have previously shown that the effects of adrenaline on exocytosis in  $\alpha$ -cells are mimicked by forskolin (Gromada *et al.* 1997), which suggests that the experiments on  $\alpha$ - and  $\beta$ -cells were conducted under comparable conditions. The reason for the discrepancy between gene

expression and function in  $\alpha$ -cells is not clear. Although gene expression is commonly regulated at the level of mRNA synthesis, it is also known to be controlled at more distal stages such as RNA transport and localization, mRNA translation or protein activity. Another possible explanation is that we have only examined exocytosis of glucagon in this study. Therefore we can not rule out the possibility that GABA<sub>B</sub>Rs regulate other processes in  $\alpha$ -cells such as the release of SLMVs (Yamada *et al.* 2001). The exocytosis of these vesicles would contribute little to the capacitance changes (< 1%; cf. Braun *et al.* 2004) and therefore easily escape detection.

### Physiological significance of autocrine suppression of insulin secretion

Pancreatic  $\beta$ -cells are constantly exposed to the micro-environment within the islet. The cells are exposed to the secretions of the neighbouring  $\alpha$ - and  $\delta$ -cells, including the islet hormones glucagon and somatostatin that exert strong effects on insulin secretion (Pipeleers *et al.* 1985a,b). Exocytosis of the insulin granules is associated with the co-release of adenine nucleotides, which have been reported to activate K<sub>ATP</sub> channels and inhibit exocytosis in  $\beta$ -cells (Poulsen *et al.* 1999). It is also surprising that insulin itself has been reported to suppress  $\beta$ -cell electrical activity at concentrations only slightly higher than those found in the plasma (Khan *et al.* 2001; Persaud *et al.* 2002). Here we demonstrate that GABA by activation of GABA<sub>B</sub>Rs inhibits insulin secretion by suppression of exocytosis. Together these mechanisms provide powerful feedback systems safeguarding against hypoglycaemia and may contribute to the pulsatility of insulin secretion *in vivo*.

### References

- Ämmälä C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM & Rorsman P (1993). Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic  $\beta$ -cells. *J Physiol* **472**, 665–688.
- Barg S, Huang P, Eliasson L, Nelson DJ, Obermuller S, Rorsman P, Thevenod F & Renström E (2001). Priming of insulin granules for exocytosis by granular Cl<sup>-</sup> uptake and acidification. *J Cell Sci* **114**, 2145–2154.
- Bokvist K, Eliasson L, Ämmälä C, Renström E & Rorsman P (1995). Co-localization of L-type Ca<sup>2+</sup> channels and insulin-containing secretory granules and its significance for the initiation of exocytosis in mouse pancreatic  $\beta$ -cells. *EMBO J* **14**, 50–57.
- Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI & Enna SJ (2002). International Union of Pharmacology. XXXIII. Mammalian  $\gamma$ -aminobutyric acid (B) receptors: structure and function. *Pharmacol Rev* **54**, 247–264.

- Braun M, Wendt A, Birnir B, Broman J, Eliasson L, Galvanovskis J, Gromada J, Mulder H & Rorsman P (2004). Regulated exocytosis of GABA-containing synaptic-like microvesicles in pancreatic  $\beta$ -cells. *J Gen Physiol* **123**, 191–204.
- Brice NL, Varadi A, Ashcroft SJ & Molnar E (2002). Metabotropic glutamate and GABA (B) receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic  $\beta$ -cells. *Diabetologia* **45**, 242–252.
- Chebib M & Johnston GA (1999). The 'ABC' of GABA receptors: a brief review. *Clin Exp Pharmacol Physiol* **26**, 937–940.
- Craig TJ, Evans GJ & Morgan A (2003). Physiological regulation of Munc18/nSec1 phosphorylation on serine-313. *J Neurochem* **86**, 1450–1457.
- Dean PM (1973). Ultrastructural morphometry of the pancreatic  $\beta$ -cell. *Diabetologia* **9**, 115–119.
- Eliasson L, Renström E, Ämmälä C, Berggren PO, Bertorello AM, Bokvist K, Chibalin A, Deeney JT, Flatt PR, Gabel J, Gromada J, Larsson O, Lindström P, Rhodes CJ & Rorsman P (1996). PKC-dependent stimulation of exocytosis by sulfonylureas in pancreatic  $\beta$ -cells. *Science* **271**, 813–815.
- Gilon P, Bertrand G, Loubatieres-Mariani MM, Remacle C & Henquin JC (1991). The influence of  $\gamma$ -aminobutyric acid on hormone release by the mouse and rat endocrine pancreas. *Endocrinology* **129**, 2521–2529.
- Göpel S, Kanno T, Barg S, Galvanovskis J & Rorsman P (1999). Voltage-gated and resting membrane currents recorded from  $\beta$ -cells in intact mouse pancreatic islets. *J Physiol* **521**, 717–728.
- Gromada J, Bokvist K, Ding WG, Barg S, Buschard K, Renström E & Rorsman P (1997). Adrenaline stimulates glucagon secretion in pancreatic  $\alpha$ -cells by increasing the  $\text{Ca}^{2+}$  current and the number of granules close to the L-type  $\text{Ca}^{2+}$  channels. *J Gen Physiol* **110**, 217–228.
- Gromada J, Hoy M, Buschard K, Salehi A & Rorsman P (2001). Somatostatin inhibits exocytosis in rat pancreatic alpha-cells by  $\text{G}_{i2}$ -dependent activation of calcineurin and depriming of secretory granules. *J Physiol* **535**, 519–532.
- Gu XH, Kurose T, Kato S, Masuda K, Tsuda K, Ishida H & Seino Y (1993). Suppressive effect of GABA on insulin secretion from the pancreatic  $\beta$ -cells in the rat. *Life Sci* **52**, 687–694.
- Hamill OP, Marty A, Neher E, Sakmann B & Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**, 85–100.
- Hayashi M, Yamada H, Uehara S, Morimoto R, Muroyama A, Yatsushiro S, Takeda J, Yamamoto A & Moriyama Y (2003). Secretory granule-mediated co-secretion of L-glutamate and glucagon triggers glutamatergic signal transmission in islets of Langerhans. *J Biol Chem* **278**, 1966–1974.
- Hens JJ, De Wit M, Ghijsen WE, Leenders AG, Boddeke HW, Kismehl R, Wiegant VM, Weller U, Gispen WH & De Graan PN (1998). Role of calcineurin in  $\text{Ca}^{2+}$ -induced release of catecholamines and neuropeptides. *J Neurochem* **71**, 1978–1986.
- Josefsen K, Stenvang JP, Kindmark H, Berggren PO, Horn T, Kjaer T & Buschard K (1996). Fluorescence-activated cell sorted rat islet cells and studies of the insulin secretory process. *J Endocrinol* **149**, 145–154.
- Kerr DI & Ong J (1995). GABA $_B$  receptors. *Pharmacol Ther* **67**, 187–246.
- Khan FA, Goforth PB, Zhang M & Satin LS (2001). Insulin activates ATP-sensitive  $\text{K}^+$  channels in pancreatic  $\beta$ -cells through a phosphatidylinositol 3-kinase-dependent pathway. *Diabetes* **50**, 2192–2198.
- Michalik M & Erecinska M (1992). GABA in pancreatic islets: metabolism and function. *Biochem Pharmacol* **44**, 1–9.
- Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E & Sorensen JB (2004). Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. *Neuron* **41**, 417–429.
- Olofsson CS, Göpel SO, Barg S, Galvanovskis J, Ma X, Salehi A, Rorsman P & Eliasson L (2002). Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic  $\beta$ -cells. *Pflugers Arch* **444**, 43–51.
- Perrino BA, Ng LY & Soderling TR (1995). Calcium regulation of calcineurin phosphatase activity by its  $\beta$  subunit and calmodulin. Role of the autoinhibitory domain. *J Biol Chem* **270**, 340–346.
- Persaud SJ, Asare-Anane H & Jones PM (2002). Insulin receptor activation inhibits insulin secretion from human islets of Langerhans. *FEBS Lett* **510**, 225–228.
- Pipeleers DG, Schuit FC, In't Veld PA, Maes E, Hooghe-Peters EL, Van De Winkel M & Gepts W (1985a). Interplay of nutrients and hormones in the regulation of insulin release. *Endocrinology* **117**, 824–833.
- Pipeleers DG, Schuit FC, Van Schravendijk CF & Van De Winkel M (1985b). Interplay of nutrients and hormones in the regulation of glucagon release. *Endocrinology* **117**, 817–823.
- Poulsen CR, Bokvist K, Olsen HL, Hoy M, Capito K, Gilon P & Gromada J (1999). Multiple sites of purinergic control of insulin secretion in mouse pancreatic  $\beta$ -cells. *Diabetes* **48**, 2171–2181.
- Renström E, Ding WG, Bokvist K & Rorsman P (1996a). Neurotransmitter-induced inhibition of exocytosis in insulin-secreting  $\beta$ -cells by activation of calcineurin. *Neuron* **17**, 513–522.
- Renström E, Eliasson L, Bokvist K & Rorsman P (1996b). Cooling inhibits exocytosis in single mouse pancreatic  $\beta$ -cells by suppression of granule mobilization. *J Physiol* **494**, 41–52.
- Renström E, Eliasson L & Rorsman P (1997). Protein kinase A-dependent and -independent stimulation of exocytosis by cAMP in mouse pancreatic  $\beta$ -cells. *J Physiol* **502**, 105–118.
- Rorsman P, Bokvist K, Ämmälä C, Arkhammar P, Berggren PO, Larsson O & Wahlander K (1991). Activation by adrenaline of a low-conductance G protein-dependent  $\text{K}^+$  channel in mouse pancreatic  $\beta$  cells. *Nature* **349**, 77–79.
- Rorsman P & Renström E (2003). Insulin granule dynamics in pancreatic  $\beta$ -cells. *Diabetologia* **46**, 1029–1045.
- Sakaba T & Neher E (2003). Direct modulation of synaptic vesicle priming by GABA $_B$  receptor activation at a glutamatergic synapse. *Nature* **424**, 775–778.
- Salehi A, Chen D, Hakanson R, Nordin G & Lundquist I (1999). Gastrectomy induces impaired insulin and glucagon secretion: evidence for a gastro-insular axis in mice. *J Physiol* **514**, 579–591.

- Samols E, Bonner-Weir S & Weir GC (1986). Intra-islet insulin-glucagon-somatostatin relationships. *Clin Endocrinol Metab* **15**, 33–58.
- Smismans A, Schuit F & Pipeleers D (1997). Nutrient regulation of  $\gamma$ -aminobutyric acid release from islet  $\beta$ -cells. *Diabetologia* **40**, 1411–1415.
- Takahashi T, Kajikawa Y & Tsujimoto T (1998). G-protein-coupled modulation of presynaptic calcium currents and transmitter release by a GABA<sub>B</sub> receptor. *J Neurosci* **18**, 3138–3146.
- Thomas-Reetz A, Hell JW, Doring MJ, Walch-Solimena C, Jahn R & De Camilli P (1993). A  $\gamma$ -aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic  $\beta$ -cells. *Proc Natl Acad Sci U S A* **90**, 5317–5321.
- Vonen B, Florholmen J, Giaever AK & Burhol P (1989). Radioimmunoassay of somatostatin from isolated rat pancreatic islets. *Scand J Clin Lab Invest* **49**, 135–138.
- Wendt A, Birnir B, Buschard K, Gromada J, Salehi A, Sewing S, Rorsman P & Braun M (2004). Glucose inhibition of glucagon secretion from rat  $\alpha$ -cells is mediated by GABA released from neighboring  $\beta$ -cells. *Diabetes* **53**, 1038–1045.
- Yamada H, Otsuka M, Hayashi M, Nakatsuka S, Hamaguchi K, Yamamoto A & Moriyama Y (2001). Ca<sup>2+</sup>-dependent exocytosis of L-glutamate by  $\alpha$ TC6, clonal mouse pancreatic  $\alpha$ -cells. *Diabetes* **50**, 1012–1020.

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