Pituitary adenylate cyclase-activating polypeptide (PACAP) is an islet substance serving as an intra-islet amplifier of glucose-induced insulin secretion in rats

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1. We examined whether pituitary adenylate cyclase-activating polypeptide with 38 or 27 residues (PACAP-38 or PACAP-27) serves as an intra-islet regulator of glucose-induced insulin secretion in rats. PACAP antiserum specific for PACAP-38 and PACAP-27 was used to neutralize the effect of endogenous PACAP in islets. PACAP release from islets was bioassayed using the response of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single β-cells, monitored by dual-wavelength fura-2 microfluorometry. Expression of PACAP mRNA was studied by reverse transcription-polymerase chain reaction (RT-PCR), while expression of PACAP was studied by metabolic labelling and immunoblotting. Localization of PACAP receptors was studied immunohistochemically.

2. High glucose-stimulated insulin release from isolated islets was attenuated by PACAP antiserum but not by non-immune sera.

3. The islet incubation medium with high glucose (Med) possessed a capacity, which was neutralized by PACAP antiserum, to increase $[Ca^{2+}]_i$ in β-cells. PACAP antiserum also neutralized the $[Ca^{2+}]_i$-increasing action of synthetic PACAP-38 and PACAP-27, but not that of vasoactive intestinal polypeptide (VIP) and glucagon.

4. Both Med and synthetic PACAP increased $[Ca^{2+}]_i$ in β-cells only in the presence of stimulatory, but not basal, glucose concentrations. In contrast, ATP, a substance that is known to be released from β-cells, increased $[Ca^{2+}]_i$ in β-cells at both basal and stimulatory glucose concentrations.

5. Expression of PACAP mRNA and biosynthesis of PACAP-38 were detected in islets and a β-cell line, MIN6.

6. Immunoreactivity for PACAP-selective type-I receptor was observed in islets.

7. $[Ca^{2+}]_i$ measurements combined with immunocytochemistry with insulin antiserum revealed a substantial population of glucose-unresponsive β-cells, many of which were recruited by PACAP-38 into $[Ca^{2+}]_i$ responses.

8. These results indicate that PACAP-38 is a novel islet substance that is synthesized and released by islet cells and then, in an autocrine and/or paracrine manner, potentiates and arouses β-cell responses to glucose, thereby amplifying glucose-induced insulin secretion in islets.

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Insulin secretion is initiated by glucose and potentiated by neurotransmitters and hormones (Henquin, 1994). It is well established that glucose-induced insulin release from pancreatic β-cells takes place with greater efficiency in islets or cell clusters than in single cells (Pipeleers, 1987; Henquin, 1994). This assembly-dependent potentiation is thought to be mediated, at least in part, by a humoral factor released from islet cells (Pipeleers, 1987; Henquin, 1994). However, a physiologically relevant potentiator of islet origin has yet to be identified, though a potential role for glucagon and ATP, the substances released from α-cells and co-released with insulin from β-cells, respectively, has been suggested (Leitner, Sussman, Vatter & Schneider, 1975; Pipeleers, 1987; Henquin, 1994).

Pituitary adenylate cyclase-activating polypeptide in the 38 (PACAP-38) or 27 residue form (PACAP-27) is a new member of the secretin/glucagon/VIP (vasoactive intestinal polypeptide) family (Miyata *et al.* 1989; Arimura & Shioda, 1995). Four types of PACAP receptors have been cloned and characterized: the PACAP type-I receptor (PACAPR-I) (Spengler *et al.* 1993) and its variant with different transmembrane domain IV (PACAPR TM4) (Chatterjee, Sharmaj & Fisher, 1996) are specific for PACAP, while the PACAP type-II/VIP_1 receptor (PACAPR-II) (Ishihara, Shigemoto, Mori, Takahashi & Nagata, 1992) and PACAP type-III/VIP_2 receptor (PACAPR-III) (Lutz, Sheward, West, Morrow, Fink & Harmer, 1993; Inagaki *et al.* 1994) are shared equally with VIP (Arimura & Shioda, 1995). It has been shown that PACAP-38 and PACAP-27 potentiate glucose-induced insulin release *in vivo* in mouse (Fridolf, Sundler & Ahren, 1992), in isolated rat pancreas and islets (Kawai, Ohse, Watanabe, Suzuki, Yamashita & Ohashi, 1992; Yada *et al.* 1994), and in insulinoma HIT cells (Klinteberg, Karlsson & Ahren, 1996; Straub & Sharp, 1996). PACAP-38 and PACAP-27 also increase cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat β-cells, and the $[Ca^{2+}]_i$ increase mediates the insulin release (Yada *et al.* 1994). These effects are exerted by PACAP-38 and PACAP-27 at concentrations as low as 10^{-14} to 10^{-13} M (Yada *et al.* 1994), exhibiting an outstanding potency. Furthermore, the PACAP immunoreactivity is localized in pancreatic islets and nerve fibres (Fridolf *et al.* 1992; Yada *et al.* 1994).

The present study was designed to examine whether PACAP serves as an intra-islet regulator of glucose-induced insulin secretion. We studied the effect of a specific antiserum for PACAP-38 and PACAP-27 on glucose-induced insulin release from rat islets, the release of PACAP from glucose-stimulated islets, and the expression and biosynthesis of PACAP in islets and a β-cell line, MIN6. The effect of PACAP on rat β-cells that were glucose unresponsive was also investigated. For the release study, since a reliable radioimmunoassay of PACAP has not been established and, moreover, the concentrations of PACAP that can stimulate insulin release (10^{-14} to 10^{-13} M) are below the detection limit by conventional assay methods, we adopted a bioassay system using the $[Ca^{2+}]_i$ response of single β-cells, which senses 10^{-14} to 10^{-13} M levels of PACAP (Yada *et al.* 1994) and is therefore the most sensitive detection system available.

METHODS

Preparation of islets and single islet β-cells

Islets of Langerhans were isolated from Wistar rats aged 8–12 weeks by collagenase digestion. Animals were anaesthetized by intraperitoneal injection of pentobarbitone at 80 mg kg^{-1}. The abdomen was opened, and collagenase (3 mg ml^{-1}) dissolved in 5 ml of 5 mM Ca^{2+}-containing standard Krebs–Ringer bicarbonate buffer (KRB) solution was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. The rats were killed by cervical dislocation. The pancreas was dissected out and incubated at 37 °C for 17 min. Islets were collected and either used for insulin release experiments or dispersed into single cells in Ca^{2+}-free KRB. The single cells were plated on coverslips and maintained in short-term culture for up to 3 days in standard Eagle's minimal essential medium containing 5·6 mM glucose supplemented with 10% fetal bovine serum (FBS), 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin at 37 °C in a 95% air–5% CO_2 atmosphere.

Measurement of insulin release

Measurement of insulin release was carried out as previously described (Yada *et al.* 1994). Briefly, groups of 30 islets, isolated from Wistar rats 8–12 weeks of age, were first incubated for 1 h in KRB with 2·8 mM glucose. Islets were then incubated at 37 °C for 30 min with test agents in 1 ml of KRB. Insulin concentration was determined by radioimmunoassay.

Solutions

Insulin release experiments were carried out in KRB composed of (mM): NaCl, 121·7; KCl, 4·4; KH_2PO_4, 1·2; $CaCl_2$, 2·0; $MgSO_4$, 1·2; $NaHCO_3$, 23·0; at pH 7·4, supplemented with 0·1% bovine serum albumin (BSA) and equilibrated against a mixture of 95% O_2–5% CO_2 at 37 °C. For single cell experiments carried out in an open chamber, KRB containing 10 mM Hepes and a reduced HCO_3^- (5·0 mM) was used to maintain pH stably at 7·4.

Antibodies and chemicals

A rabbit polyclonal PACAP antiserum (No. 88121-6) (Koves, Arimura, Somogyvari-Vigh, Vigh & Miller, 1990; Koves, Arimura, Gorcs & Somogyvari-Vigh, 1991) and a rabbit polyclonal PACAP receptor antiserum (No. 93093-2) (Arimura & Shioda, 1995) were gifts from Dr A. Arimura, Tulane University Hebert Center, LA, USA. This receptor antiserum was raised against a synthetic peptide corresponding to the C-terminal 25-amino acid fragment (Lys^{411}–Ala^{435}) of PACAPR-I, and has been confirmed to react with a molecule of 57 kDa, the size of PACAPR-I, by Western blot analysis (Arimura & Shioda, 1995). Synthetic PACAP-38 and PACAP-27 were from American Peptide Co. (Sunnyvale, CA, USA) and Peptide Institute (Osaka, Japan). VIP was from Peptide Institute and glucagon from Sigma. All peptides were dissolved at 10^{-4} M in distilled water with 0·1% BSA and stored at −80 °C until use. Adenosine 5′-triphosphate (ATP) was from Sigma. A guinea-pig insulin antiserum was from Dako Corp. (Carpinteria, CA, USA).

Culture of cell lines

A murine insulinoma cell line, MIN6, was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose supplemented with 15% FBS in 5% CO_2–95% air atmosphere. A

phaeochromocytoma cell line, PC12, was cultured similarly but with 5·6 mM glucose, 5% FBS and 10% horse serum.

Measurement and imaging of $[Ca^{2+}]_i$ and selection of β-cells

Measurement and imaging of $[Ca^{2+}]_i$ were carried out by previously described procedures (Yada *et al.* 1994) with slight modification. Briefly, single islet cells and PC12 cells on coverslips were mounted in an open chamber and superfused in KRB, and $[Ca^{2+}]_i$ was measured by dual-wavelength fura-2 microfluorometry using an intensified charge-coupled device (ICCD) camera, and the ratio image was produced by the Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). In experiments with single islet cells, data were taken only from the β-cells that were confirmed either immunocytochemically using anti-insulin antiserum or by morphological and physiological criteria as reported previously (Yada, Hamakawa & Yaekura, 1995).

Bioassay of PACAP release

Between 50 and 400 islets were incubated with 8·3–16·7 mM glucose in 100–200 μl KRB. At 60 min, following cooling and centrifugation at 100 *g* for 1 min, the medium supernatant was collected and stored on ice for up to 2 h until use. It was diluted at 1:100 in KRB with or without PACAP antiserum (1:10000), so that the final concentration of glucose was adjusted to 8·3 mM, and applied to single islet cells subjected to $[Ca^{2+}]_i$ measurements under superfusion conditions.

mRNA analysis

Total RNA was prepared from rat islets and MIN6 cells, and was reverse transcribed using a random primer. PCR (polymerase chain reaction) was performed using a sense primer (5′-AGGACG GAAACCCGCTGC-3′) and an antisense primer (5′-ACT ACCCTAT TCGCCGTC-3′). Thirty cycles (94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min) were used to amplify a 391 bp fragment. PCR products were resolved on 2% agarose gel and stained with ethidium bromide.

De novo protein synthesis

Six hundred islets were isolated and cultured for 1 day in standard Eagle's minimal essential medium with 10% FBS. The islets and MIN6 cells were incubated in Met/Cys-free medium for 30 min, followed by incubation with 100 μCi [^{35}S]Met/Cys (Amersham Co., Arlington Heights, IL, USA) for 4 h. After washing, the cells were treated with 1 ml NP40 lysis buffer: 100 mM NaCl, 0·5% NP40, 1 mM EDTA, 10 mM Tris-HCl (pH 7·5), 0·5 units aprotinin and 1 mM phenylmethylsulphonic fluoride (PMSF). The lysate was centrifuged (12000 *g*, 30 min, 4 °C) to remove debris and precleared with protein A-sepharose for 1 h. PACAP antiserum was then added, followed by incubation at 4 °C for 1 h. Following addition of protein A-sepharose, total immunoprecipitates were eluted in Laemmli's SDS–PAGE sample buffer (62·5 mM Tris–HCl (pH 6·8), 2% (w/v) SDS, 0·005% (w/v) Bromophenol Blue, 7% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol) and subjected to 4–20% SDS–PAGE slab gel. The gel was autoradiographed on Fuji film.

Immunoblotting

MIN6 cells were washed three times with phosphate-buffered saline (PBS) and then lysed in 1 ml NP40 lysis buffer. NP40 lysates were centrifuged at 12000 *g* for 30 min at 4 °C. The supernatant was eluted in Laemmli's SDS–PAGE sample buffer. The proteins were subjected to 15% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with PACAP antiserum at a 1:10000 dilution. Immunoreactive proteins were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG and the ECL system (Amersham Co.).

Immunostaining

For immunohistochemical study of PACAP receptors, the rat pancreas was removed immediately after perfusion fixation with 4% paraformaldehyde. Ten micrometre sections of pancreas were cut and picked up on gelatin-coated slides. Sections were incubated with PACAPR-I antiserum at a dilution of 1:5000, followed by immunostaining with the avidin–biotin complex. Subsequently, they were developed with diaminobenzidine-4 HCl. Sections were counterstained with haematoxylin. Methods for immunocytochemical staining of single islet cells with insulin antiserum are as reported previously (Yada *et al.* 1994).

RESULTS

Attenuation of insulin release from islets by PACAP antiserum and bioassay of PACAP release from islets

Stimulation of insulin release from isolated rat islets by 8·3 mM glucose, under static incubation conditions, was substantially attenuated (26·2%) by the PACAP antiserum (anti-P) specific for PACAP-27 and PACAP-38 (Koves *et al.* 1990, 1991), whereas control rabbit sera were without effect (Fig. 1). Thus, a fraction of the glucose-induced insulin release from islets was mediated by a mechanism that was neutralized by the specific PACAP antiserum. We therefore examined the release of PACAP from glucose-stimulated islets by a bioassay system using the $[Ca^{2+}]_i$ response of single β-cells, which can detect subpicomolar levels of PACAP (Yada *et al.* 1994). The supernatant of the medium in which isolated rat islets were incubated with high glucose (8·3–16·7 mM), at a 1:100 dilution (denoted 'Med'), evoked an increase in $[Ca^{2+}]_i$ in single β-cells under superfusion conditions in the presence of 8·3 mM glucose (Fig. 2*A*). At a 1:10000 dilution, islet incubation medium was without effect (data not shown). When Med was mixed with the PACAP antiserum, its $[Ca^{2+}]_i$-increasing capacity was either abolished or markedly reduced (Fig. 2*A*). The results indicated that the glucose-stimulated islets release a β-cell-stimulating factor that is neutralized by the PACAP antiserum.

Specificity of PACAP antiserum

It has been shown that the PACAP antiserum used in the present study reacts with PACAP-38 and PACAP-27 but not with VIP, glucagon, secretin and other structurally related peptides, or with somatostatin (Koves *et al.* 1990, 1991). The specificity of the PACAP antiserum was further examined in single rat β-cells. The antiserum counteracted the $[Ca^{2+}]_i$-increasing effects of synthetic PACAP-38 and PACAP-27 at 10^{-9} M (Fig. 2*C*) and 10^{-13} M (data not shown) in β-cells, and those at 10^{-9} M in PC12 cells (Fig. 2*B*) that possess PACAP receptors (Deutscht & Sun, 1992). Thus, the β-cell-stimulating capacity of Med and that of synthetic PACAP were antagonized by the PACAP antiserum in essentially the same manner (Fig. 2*C vs. A*). In contrast, the antiserum failed to affect the $[Ca^{2+}]_i$ responses to 10^{-9} M VIP (Fig. 2*D*), the peptide exhibiting the highest homology with PACAP (Arimura & Shioda, 1995), and those to 10^{-9} M glucagon (Fig. 2*E*), an islet hormone released from α-cells.

Furthermore, in the presence of the antiserum, the $[Ca^{2+}]_i$ responses to 8·3 mM glucose remained unaltered (Fig. 2*F*), indicating that the antiserum does not influence the glucose handling by β-cells. These results reconfirmed the specificity of the PACAP antiserum for PACAP-38 and PACAP-27.

Comparative effects of islet incubation medium (Med), synthetic PACAP and ATP on $[Ca^{2+}]_i$ in β-cells

It is known that ATP is co-released with insulin from β-cells and that ATP stimulates β-cells via purinergic receptors (Leitner *et al.* 1975; Henquin, 1994). Accordingly, ATP has been considered as a candidate for the intra-islet potentiator of the β-cell activity and insulin release. We therefore examined the comparative effects of Med and ATP on $[Ca^{2+}]_i$ in β-cells. Med, administered in the presence of 2·8 mM glucose, failed to increase $[Ca^{2+}]_i$, whereas it subsequently increased $[Ca^{2+}]_i$ after the glucose concentration had been raised to 8·3 mM (Fig. 3*A*). Virtually identical results were obtained with synthetic PACAP-38 at 10^{-13} M (Fig. 3*B*) and 10^{-9} M (data not shown). In contrast, ATP at 10^{-6} M increased $[Ca^{2+}]_i$ in the presence of 2·8 and 8·3 mM glucose in a similar manner, while ATP at 10^{-8} M had little effect on $[Ca^{2+}]_i$ at both glucose concentrations (Fig. 3*C*). The same results were obtained in those cells in which only a single concentration of ATP was examined, and the ATP concentration–$[Ca^{2+}]_i$ response relationship with 2·8 mM glucose and that with 8·3 mM glucose were similar (data not shown). Thus, ATP increased $[Ca^{2+}]_i$ in β-cells in a virtually glucose-independent manner, in clear contrast to the glucose-dependent actions of Med and PACAP. Taken together, the β-cell-stimulating factor released from glucose-stimulated islets, the action of which is glucose dependent and neutralized by the specific PACAP antiserum, is very likely to be PACAP-38 and/or PACAP-27.

Expression of PACAP mRNA and biosynthesis of PACAP by islet cells and MIN6 cells

We then asked whether PACAP is produced by islet cells. PACAP mRNA was detected in rat islets and a β-cell line, MIN6, by reverse transcription-polymerase chain reaction (RT-PCR) methods using primers corresponding to the sequence for PACAP (Ogi, Kimura, Onda, Arimura & Fujino, 1990) (Fig. 4*A*). Biosynthesis of PACAP was studied by metabolic labelling with [^{35}S]Met/Cys in isolated rat islets (data not shown) and those after culture in which proteins of neural origin are excluded (Fig. 4*B*), as well as in MIN6

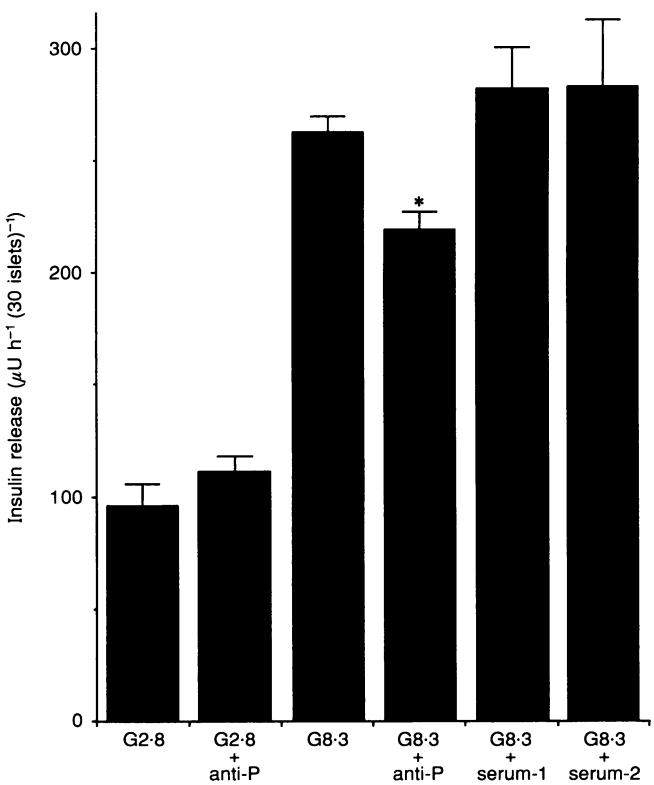

Figure 1. Attenuation of glucose-stimulated insulin release from rat islets by PACAP antiserum

Stimulation of insulin release from 30 islets by 8·3 mM glucose (G8·3), under static incubation conditions, was attenuated by specific PACAP antiserum (anti-P) at a 1:10 000 dilution (G8·3 + anti-P), whereas it was unaffected by control rabbit sera (G8·3 + serum-1 and G8·3 + serum-2). Insulin release at 2·8 mM glucose (G2·8) was unaffected by PACAP antiserum (G2·8 + anti-P). Values are means ± S.E.M. of 11 experiments for G2·8, G8·3 and G8·3 + anti-P groups, 7 experiments for G8·3 + serum-1, and 5 experiments for G2·8 + anti-P and G8·3 + serum-2. * $P < 0·001$ *versus* G8·3, $P < 0·005$ *versus* G8·3 + serum-1 and $P < 0·02$ *versus* G8·3 + serum-2 by Student's *t* test. No significant difference ($P > 0·05$) between G2·8 and G2·8 + anti-P, and between G8·3, G8·3 + serum-1 and G8·3 + serum-2.

cells. In every case, one of the proteins immunoprecipitated specifically with the PACAP antiserum showed an apparent molecular mass of around 5 kDa, corresponding to that of synthetic PACAP-38. Other proteins with molecular masses around 21 and 14 kDa were also immunoprecipitated. These proteins may represent the PACAP precursor with 176 amino acids and its processing product, respectively, as previously suggested in neuroblastoma cells (Suzuki *et al.* 1993). The metabolic labelling was relatively more intense in islets than in MIN6 cells. Immunoblotting with the PACAP antiserum, carried out in MIN6 cells, showed a single protein band, the molecular size of which corresponded to that of synthetic PACAP-38 but not PACAP-27 (Fig. 4*C*). These results reveal the expression of PACAP mRNA and *de novo* synthesis of PACAP-38 in islet cells and a β-cell line, MIN6.

Localization of PACAP receptors in pancreatic islet cells

We next examined the localization of PACAP receptors in islets using the PACAPR-I antiserum. Immunoreactivity was observed in the entire region of rat islets (Fig. 5), but not when the antiserum was preabsorbed with the antigen (data not shown). Since the antiserum used in the present study was raised against the fragment common for PACAPR-

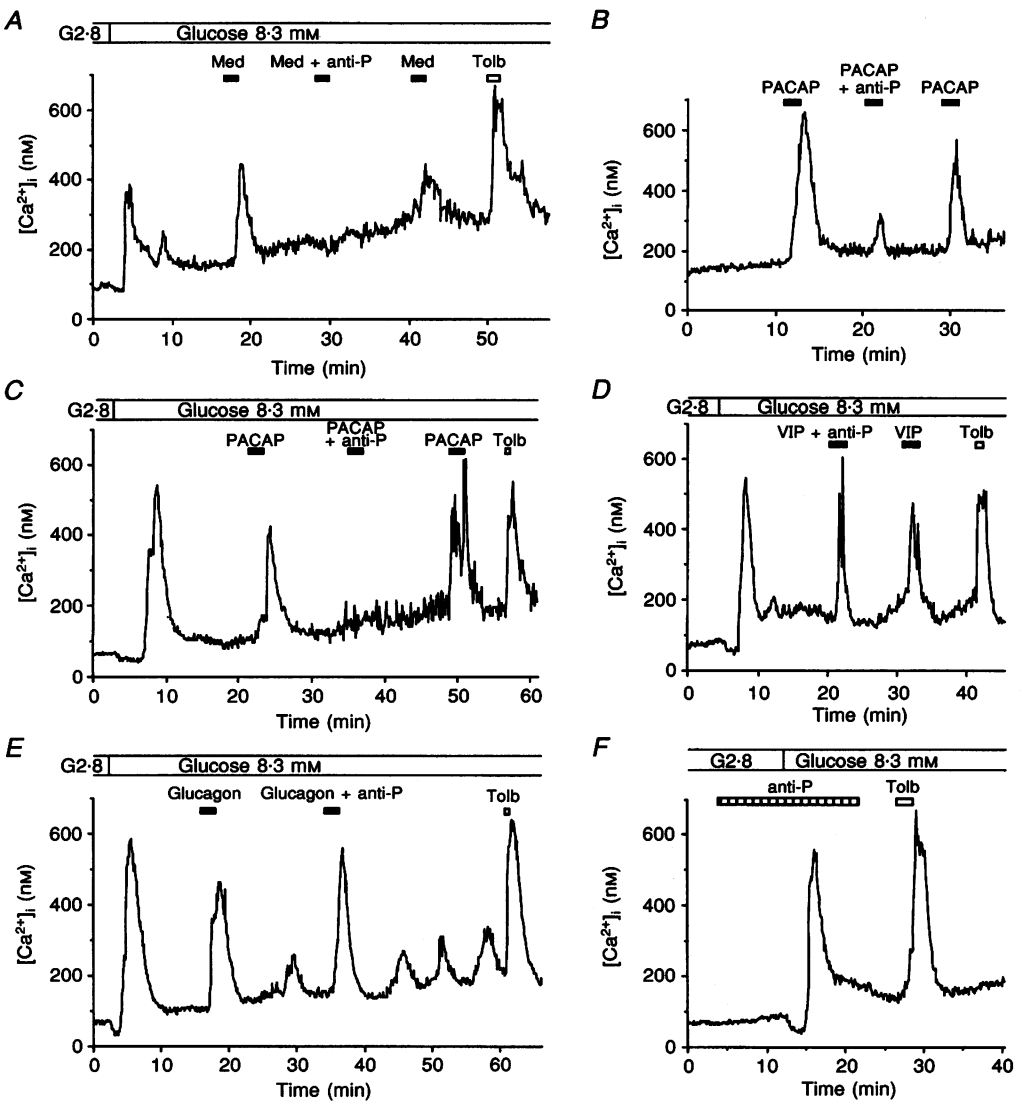

Figure 2. Bioassay of PACAP release from glucose-stimulated islets using $[Ca^{2+}]_i$ responses in single β-cells and specific PACAP antiserum

A, a 1:100 dilution of the medium in which 200 islets were incubated with 16·7 mM glucose (Med) evoked increases in $[Ca^{2+}]_i$ in single rat β-cells in the presence of 8·3 mM glucose, whereas Med mixed with PACAP antiserum (anti-P) (1:10 000) was without effect. *B–F*, specificity of PACAP antiserum. Anti-P (1:10 000) inhibited $[Ca^{2+}]_i$ responses to 10^{-9} M synthetic PACAP-38 in PC12 cells (*B*) and β-cells (*C*), but not those to 10^{-9} M VIP (*D*), 10^{-9} M glucagon (*E*) and 8·3 mM glucose (*F*) in β-cells. Anti-P had no effect on subsequent $[Ca^{2+}]_i$ responses to 300 μM tolbutamide (Tolb). The glucose concentration in *B* was 5 mM. Representative results from 18, 7, 7, 8, 8 and 16 similar experiments are shown in *A–F*, respectively. G2·8, 2·8 mM glucose.

I and PACAPR TM4, our results indicate the localization of PACAPR-I and/or PACAPR TM4 in islet cells.

PACAP recruits glucose-unresponsive β-cells into $[Ca^{2+}]_i$ responses

Heterogeneity of pancreatic β-cells with respect to electrical, metabolic and secretory responses to glucose has been observed both *in vitro* and *in vivo*, and evidence of the existence of glucose-unresponsive β-cells has been obtained (Pipeleers, 1987; Giordano, Bosco, Cirutit & Meda, 1991). Since PACAPR-I and/or PACAPR TM4 were demonstrated in virtually all islet cells (Fig. 5), a possible effect of PACAP on glucose-unresponsive β-cells was examined. The $[Ca^{2+}]_i$ measurement combined with subsequent immunocytochemical staining with insulin antiserum revealed rat β-cells that failed to respond to glucose with an increase in $[Ca^{2+}]_i$ (Fig. 6), the triggering signal for insulin release (Ämmälä, Eliasson, Bokvist, Larsson, Ashcroft & Rorsman, 1993). PACAP-38 at concentrations as low as 10^{-13} M, administered about 10 min after glucose stimulation, induced $[Ca^{2+}]_i$ responses in ten of seventeen glucose-unresponsive, immunocytochemically confirmed β-cells (59%) (Fig. 6). In control experiments, among fourteen cells in which glucose had not increased $[Ca^{2+}]_i$ for the initial 10 min period of stimulation, only two cells (14%) subsequently exhibited an increase in $[Ca^{2+}]_i$ during the following 10–25 min period of glucose stimulation. These results indicate that a substantial portion of glucose-unresponsive β-cells was recruited by PACAP-38 into $[Ca^{2+}]_i$ responses. Similar results were obtained with PACAP-27 (data not shown).

We also examined whether treatment with PACAP could convert glucose-unresponsive cells into responsive cells. Among forty-five β-cells examined under control conditions without PACAP, thirty-four cells (76%) responded to glucose with an increase in $[Ca^{2+}]_i$ (glucose-responding cells),

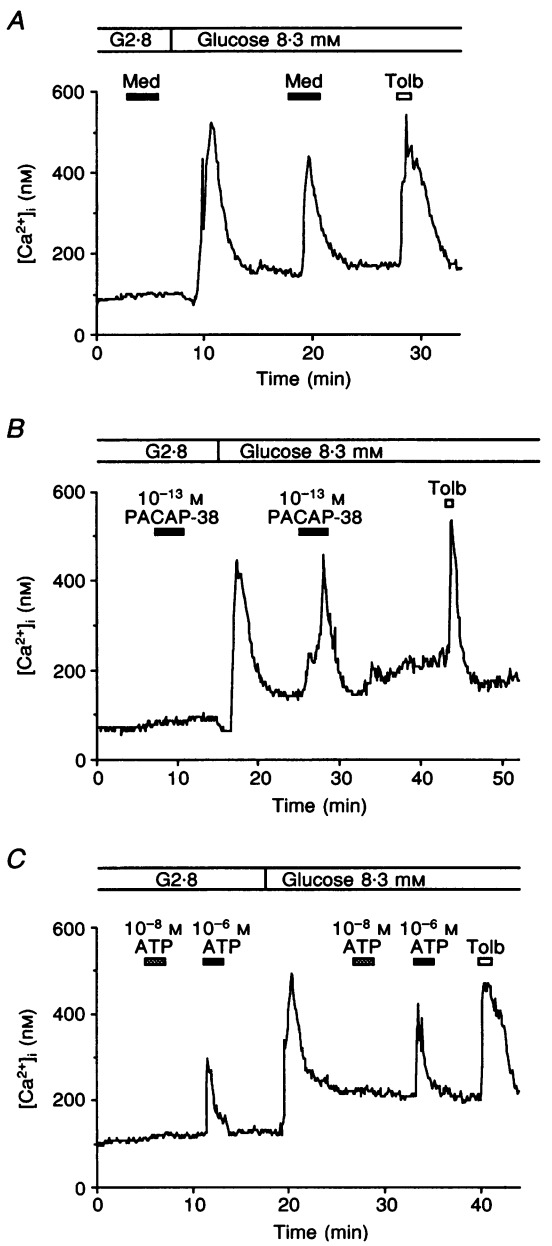

Figure 3. Comparative effects of islet incubation medium (Med), synthetic PACAP and ATP on $[Ca^{2+}]_i$ in β-cells

A 1:100 dilution of the islet incubation medium (Med) (*A*) and 10^{-13} M synthetic PACAP-38 (*B*) failed to increase $[Ca^{2+}]_i$ in single rat β-cells in the presence of 2·8 mM glucose (G2·8), but subsequently increased $[Ca^{2+}]_i$ after the glucose concentration was raised to 8·3 mM. *C*, ATP at 10^{-6} M increased $[Ca^{2+}]_i$ in single rat β-cells in the presence of 2·8 and 8·3 mM glucose in a similar manner. ATP at 10^{-8} M was without effect on $[Ca^{2+}]_i$ at both glucose concentrations. $[Ca^{2+}]_i$ responses to 300 μM tolbutamide (Tolb) were also obtained. Representative results from 3, 7 and 9 similar experiments are shown in *A*, *B* and *C*, respectively.

while eleven cells (24%) failed to respond to glucose during a stimulation period of 25 min (glucose-unresponsive cells). Under experimental conditions where cells were stimulated with glucose plus 10^{-13} M PACAP-38, the population of glucose-responding cells was increased to 91% (32 of 35 cells) and that of glucose-unresponsive cells decreased to 9% (3 of 35 cells). Thus it appears that glucose-unresponsive β-cells were partly converted into responding cells by treatment with PACAP-38.

DISCUSSION

Synthesis and release of PACAP-38 by islet β-cells and their function to amplify glucose-induced insulin secretion

In the present study, to explore a role of PACAP in the regulation of glucose-induced insulin secretion by rat pancreatic islets, we used an antiserum specific for PACAP-38 and PACAP-27 and a supersensitive PACAP bioassay system using the $[Ca^{2+}]_i$ response of single β-cells. We have demonstrated that glucose-induced insulin release from isolated islets is attenuated by the PACAP antiserum, and that glucose-stimulated islets release a β-cell-stimulating factor that is neutralized by the PACAP antiserum. The specificity of the antiserum used in the present study was reconfirmed in single β-cells: the PACAP antiserum counteracted the $[Ca^{2+}]_i$-increasing ability of synthetic PACAP-38 and PACAP-27, but not that of glucagon and VIP, and the PACAP antiserum had no effect on the β-cell $[Ca^{2+}]_i$ response to glucose on which the effectiveness of potentiators depends. These results indicate that PACAP-like activity is released by glucose-stimulated islets, and then acts on β-cells to potentiate glucose-induced insulin release.

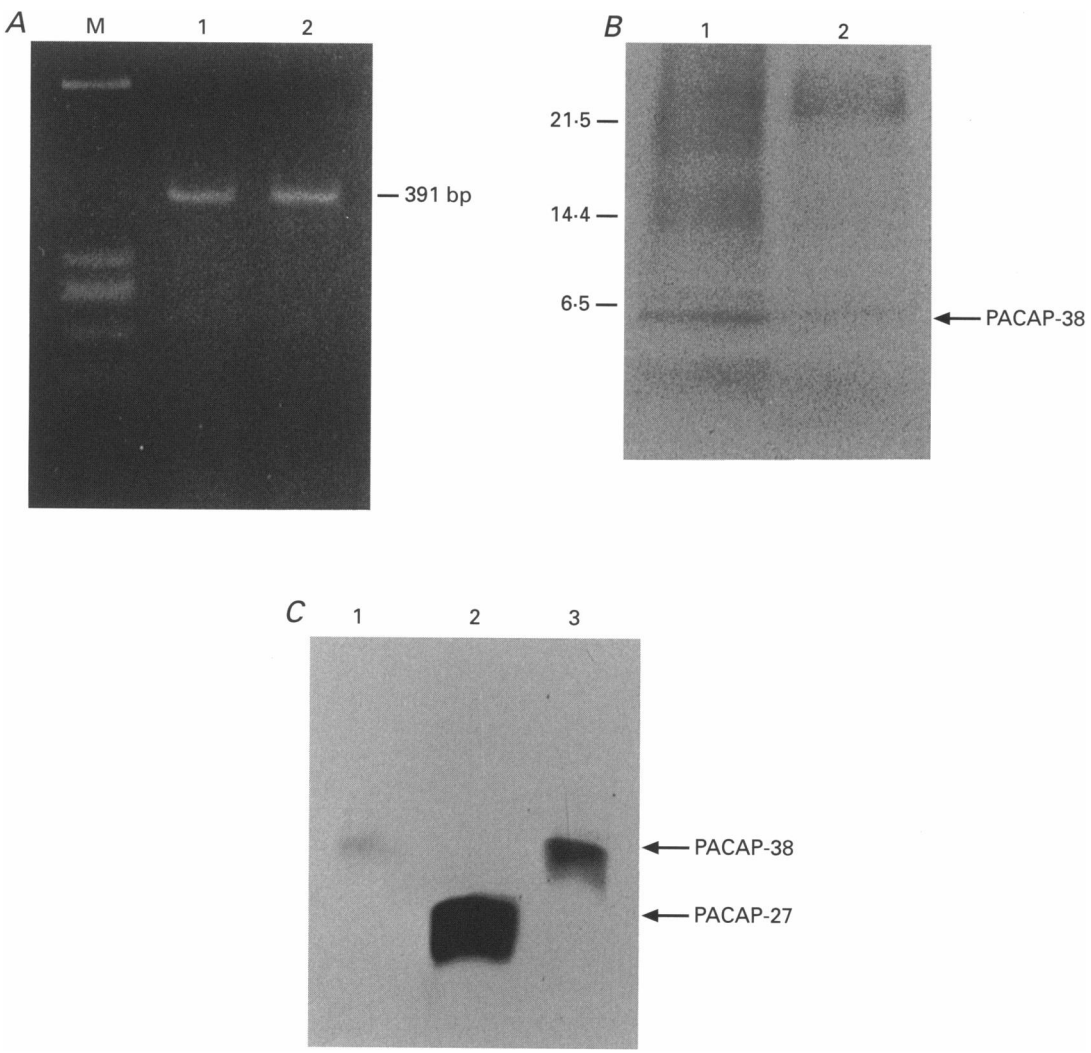

Figure 4. Expression of PACAP mRNA, biosynthesis and presence of PACAP in islets and MIN6 cells

A, RT-PCR analysis of total RNA from rat islets (lane 1) and MIN6 cells (lane 2). DNA size markers in lane M. *B*, biosynthetic labelling in rat islets (lane 1) and MIN6 cells (lane 2). Molecular size markers, in kDa, at left. The arrow shows the position of standard PACAP-38. *C*, immunoblotting with the PACAP antiserum. MIN6 cells (lane 1), synthetic PACAP-27 (lane 2) and synthetic PACAP-38 (lane 3).

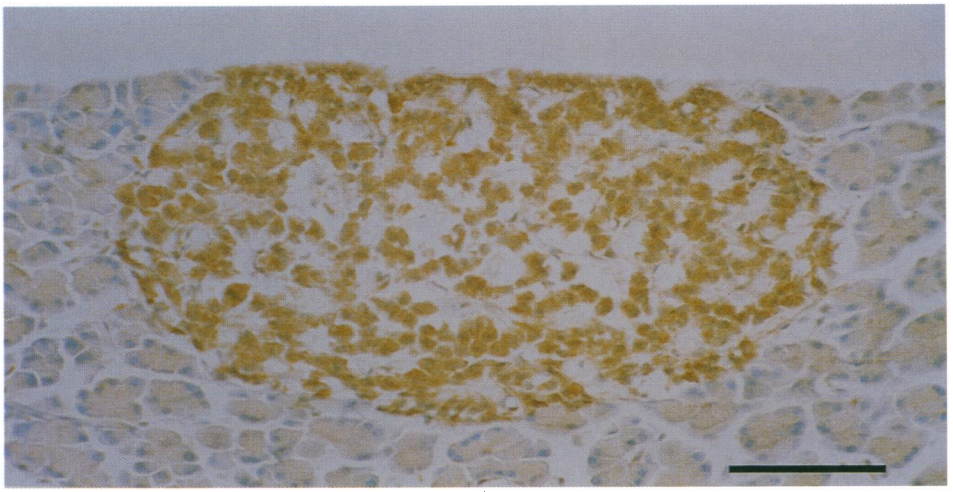

Figure 5. Localization of PACAP receptors in islet cells

Immunohistochemical staining of rat pancreas with PACAPR-I antiserum. Immunoreactivity was localized in islets. Bar indicates 100 μm.

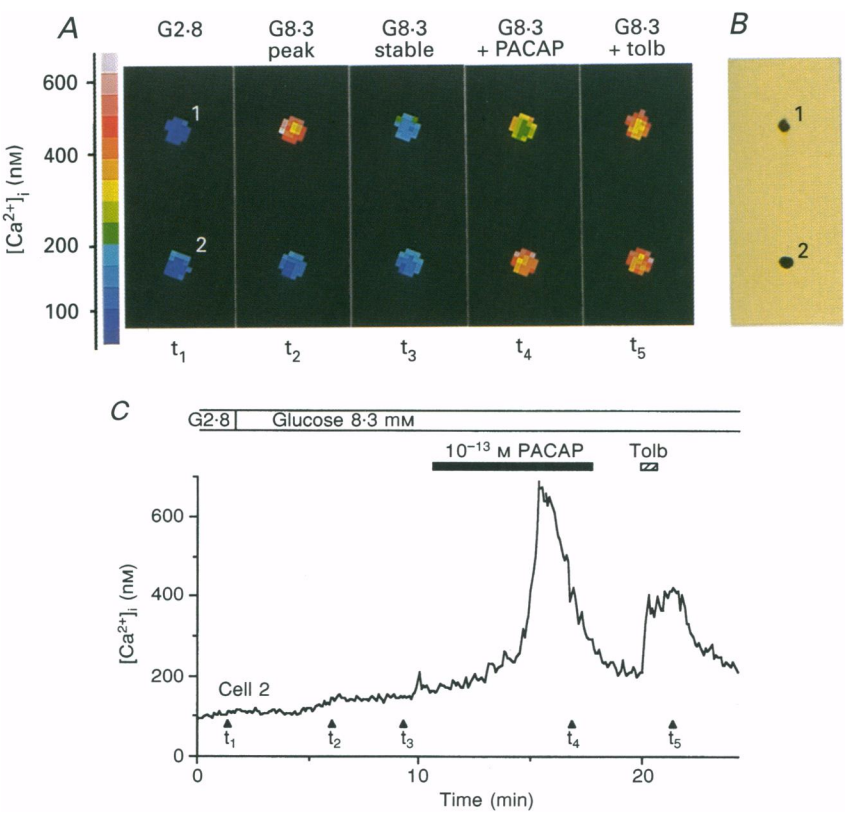

Figure 6. Recruitment of glucose-unresponsive β-cells into $[Ca^{2+}]_i$ responses by PACAP

A, $[Ca^{2+}]_i$ imaging in single islet cells. *B*, subsequent immunocytochemical staining of the same cells with insulin antiserum. The glucose-responsive cell 1 and glucose-unresponsive cell 2 were confirmed to be β-cells by their positive staining with insulin antibody and $[Ca^{2+}]_i$ responses to 300 μM tolbutamide. *C*, temporal $[Ca^{2+}]_i$ profile of the glucose-unresponsive β-cell, cell 2. Triangles, marked t_1–t_5, indicate sequential time points when pictures in *A* were taken. PACAP-38 at 10^{-13} M restored the $[Ca^{2+}]_i$ response in the glucose-unresponsive β-cell, cell 2 (*A* and *C*). Results in cell 2 are representative of 10 glucose-unresponsive, immunocytochemically confirmed β-cells. G2·8, 2·8 mM glucose; G8·3, 8·3 mM glucose.

In the protein biosynthesis experiments, the major protein immunoprecipitated with the PACAP antiserum exhibited an apparent molecular mass of around 5 kDa, corresponding to that of synthetic PACAP-38. These results were obtained in freshly isolated islets, in cultured islets in which proteins of neural origin are excluded, and in a β-cell line, MIN6. In MIN6 cells, immunoblotting with the PACAP antiserum exhibited a single protein band corresponding to synthetic PACAP-38, but not PACAP-27. These findings reveal that islet cells, most probably including β-cells, synthesize PACAP *de novo* and that the major molecular species is PACAP-38. Our previous finding that PACAP immunoreactivity is localized in rat islets suggests the storage of this peptide in islet cells (Yada *et al.* 1994).

PACAP receptor and signal transduction in β-cells

The present immunohistochemical study has demonstrated the localization of PACAPR-I-like immunoreactivity in islets. It was recently reported that PACAPR TM4, a PACAPR-I variant with different transmembrane domain IV, is the primary, if not exclusive, PACAP-selective receptor expressed in islet cells (Chatterjee *et al.* 1996). The receptor antiserum used in the present study was raised against the fragment common for PACAPR-I and PACAPR TM4. Therefore, our immunostaining data using this antiserum appear to reflect the localization of PACAPR TM4, if not exclusively, in islets. Islet β-cells are activated by PACAP at 10^{-14} to 10^{-8} M while they are activated by VIP at 10^{-10} to 10^{-8} M, indicating that PACAP is three to four powers of ten more potent (Yada *et al.* 1994). It is therefore suggested that the effects of PACAP in the picomolar concentration range are mediated by PACAP-selective receptors, most probably by PACAPR TM4. On the other hand, the effects of PACAP in the nanomolar range could also involve PACAPR-III expressed in β-cells (Inagaki *et al.* 1994; Usdin, Bonner & Mezey, 1994), the receptor shared equally with VIP.

PACAPR-I, -II and -III can stimulate the Gs subtype of GTP-binding proteins and adenylyl cyclase (Arimura & Shioda, 1995). In HIT insulinoma cells in which PACAPR-III is expressed (Inagaki *et al.* 1994), PACAP stimulates cAMP-regulated Ca^{2+} entry and $[Ca^{2+}]_i$ increase (Leech, Holz & Habener, 1995; Klinteberg *et al.* 1996; Straub & Sharp, 1996). In contrast, PACAPR TM4, when expressed in Chinese hamster ovary (CHO) cells, was shown not to stimulate adenylyl cyclase or phospholipase C but to stimulate Ca^{2+} influx through L-type Ca^{2+} channels and increase $[Ca^{2+}]_i$ (Chatterjee *et al.* 1996), the processes that trigger insulin release in response to PACAP in islets (Yada *et al.* 1994). The concentration–response curve for the PACAP-induced $[Ca^{2+}]_i$ increase in β-cells exhibits two peaks at 10^{-13} and 10^{-9} M (Yada *et al.* 1994). Taken together, the stimulation of islet β-cells by PACAP appears to involve more than a single signalling pathway. The PACAP signal transduction mechanisms involved in the stimulation of pancreatic β-cells and insulin release remain to be further elucidated.

Recruitment of glucose-unresponsive β-cells by subpicomolar PACAP

Regarding the issue of the glucose-unresponsive β-cell, it is of particular importance to distinguish glucose-unresponsive β-cells from non-β-cells. In the present study, we have combined $[Ca^{2+}]_i$ measurements in single islet cells with subsequent immunocytochemical staining of the same cells with anti-insulin antibody, and demonstrated, in a definitive way, the presence of glucose-unresponsive β-cells that failed to respond to glucose with $[Ca^{2+}]_i$ increase. It is notable that PACAP, an islet substance, at concentrations as low as 10^{-13} M restored $[Ca^{2+}]_i$ responses in these glucose-unresponsive β-cells. It was previously reported that β-cells are rendered glucose competent by glucagon-like peptide-1(7–37), an intestinal hormone, at 10^{-8} M (Holz, Kuhtreiber & Habener, 1993).

Autocrine route of PACAP in islets and its suggested physiological role

Islet endocrine cells are composed of β-, α-, δ- and pp-cells. It is currently not known from which cells PACAP could be released. However, PACAP is localized in rat islets, including the central portion exclusively occupied by β-cells (Yada *et al.* 1994). The present study revealed PACAP gene transcripts and PACAP-38 biosynthesis in MIN6 cells, as well as in islets. Thus, it is likely that PACAP-38 is produced and released, at least, by β-cells. Since PACAP-38 also directly targets β-cells, PACAP-38 appears to operate an autocrine function in β-cells. In addition, the immunoreactivity for PACAP (Yada *et al.* 1994) and that for PACAPR TM4/PACAPR-I (Fig. 5) are observed not only in the centre but also at the periphery of rat islets where α-cells are abundant. Therefore, PACAP may also be released from α-cells and participate in the paracrine interaction between α- and β-cells and/or in the autocrine regulation of α-cells. PACAP-immunoreactive nerve fibres, demonstrated around and within rat and mouse pancreatic islets (Fridolf *et al.* 1992; Yada *et al.* 1994), appear to be additional sources of PACAP.

Based on these findings we hypothesize that PACAP-38 is a novel islet hormone serving as an autocrine and/or paracrine regulator, wherein this peptide is released by glucose-stimulated islet cells and acts on islet β-cells, resulting in amplification of glucose-induced insulin secretion. This is achieved both by potentiation of the glucose response in responsive β-cells (Fig. 2*C*) and by recruitment of glucose-unresponsive β-cells into the processes of $[Ca^{2+}]_i$ increase (Fig. 6) and, in all likelihood, insulin release. The PACAP-mediated auto-amplification of glucose-induced insulin secretion by islets may be of significance for the understanding of the impaired glucose-induced insulin secretion characteristically associated with non-insulin-dependent diabetes mellitus (NIDDM) (Pfeifer, Halter & Porte, 1981).

Ämmälä, C., Eliasson, L., Bokvist, K., Larsson, O., Ashcroft, F. M. & Rorsman, P. (1993). Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *Journal of Physiology* **472**, 665–688.

Arimura, A. & Shioda, S. (1995). Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interactions. *Frontiers in Neuroendocrinology* **16**, 53–88.

Chatterjee, T. K., Sharmaj, R. V. & Fisher, R. A. (1996). Molecular cloning of a novel variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor that stimulates calcium influx by activation of L-type calcium channels. *Journal of Biological Chemistry* **271**, 32226–32232.

Deutscht, P. J. & Sun, Y. (1992). The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *Journal of Biological Chemistry* **267**, 5108–5113.

Fridolf, T., Sundler, F. & Ahren, B. (1992). Pituitary adenylate cyclase-activating polypeptide (PACAP): occurrence in rodent pancreas and effects on insulin and glucagon secretion in the mouse. *Cell and Tissue Research* **269**, 275–279.

Giordano, E., Bosco, D., Cirutit, V. & Meda, P. (1991). Repeated glucose stimulation reveals distinct and lasting secretion patterns of individual rat pancreatic B cells. *Journal of Clinical Investigation* **87**, 2178–2185.

Henquin, J. C. (1994). Cell biology of insulin secretion. In *Joslin's Diabetes Mellitus*, ed. Kahn, C. R. & Weir, G. C., pp. 56–80. Lea & Febigar, Philadelphia, USA.

Holz, G. G., Kuhtreiber, W. M. & Habener, J. F. (1993). Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7–37). *Nature* **361**, 362–365.

Inagaki, N., Yoshida, H., Mizuta, M., Mizuno, N., Fujii, Y., Gonoi, T., Miyazaki, J. & Seino, S. (1994). Cloning and functional characterization of a third pituitary adenylate cyclase-activating polypeptide receptor subtype expressed in insulin-secreting cells. *Proceedings of the National Academy of Sciences of the USA* **91**, 2679–2683.

Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K. & Nagata, S. (1992). Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* **8**, 811–819.

Kawai, K., Ohse, C., Watanabe, Y., Suzuki, S., Yamashita, K. & Ohashi, S. (1992). Pituitary adenylate cyclase activating polypeptide stimulates insulin release from isolated perfused rat pancreas. *Life Sciences* **50**, 257–261.

Klinteberg, K., Karlsson, S. & Ahren, B. (1996). Signaling mechanisms underlying the insulinotropic effect of pituitary adenylate cyclase-activating polypeptide in HIT-T15 cells. *Endocrinology* **137**, 2791–2798.

Koves, K., Arimura, A., Gorcs, T. G. & Somogyvari-Vigh, A. (1991). Comparative distribution of immunoreactive pituitary adenylate cyclase activating polypeptide and vasoactive intestinal peptide in rat forebrain. *Neuroendocrinology* **54**, 159–169.

Koves, K., Arimura, A., Somogyvari-Vigh, A., Vigh, S. & Miller, J. (1990). Immunohistochemical demonstration of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, in the ovine hypothalamus. *Endocrinology* **127**, 264–271.

Leech, C. A., Holz, G. G. & Habener, J. F. (1995). Pituitary adenylate cyclase-activating polypeptide induces the voltage-independent activation of inward membrane currents and elevation of intracellular calcium in HIT-T15 insulinoma cells. *Endocrinology* **136**, 1530–1536.

Leitner, J. W., Sussman, K. E., Vatter, A. E. & Schneider, F. H. (1975). Adenine nucleotides in the secretory granule fraction of rat islets. *Endocrinology* **96**, 662–677.

Lutz, E. M., Sheward, W. J., West, K. M., Morrow, J. A., Fink, G. & Harmer, A. J. (1993). The VIP_2 receptor: Molecular characterization of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Letters* **334**, 3–8.

Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L., Culler, M. D. & Coy, D. H. (1989). Isolation of a novel 38-residue hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochemical and Biophysical Research Communications* **164**, 567–574.

Ogi, K., Kimura, C., Onda, H., Arimura, A. & Fujino, M. (1990). Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP). *Biochemical and Biophysical Research Communications* **173**, 1271–1279.

Pfeifer, M. A., Halter, J. B. & Porte, D. Jr (1981). Insulin secretion in diabetes mellitus. *American Journal of Medicine* **70**, 579–588.

Pipeleers, D. (1987). The biosociology of pancreatic B cells. *Diabetologia* **30**, 277–291.

Spengler, D., Waeber, C., Pantalom, C., Holsboer, F., Bockaert, J., Seeburg, P. H. & Journot, L. (1993). Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **356**, 170–175.

Straub, S. G. & Sharp, G. W. G. (1996). A wortmannin-sensitive signal transduction pathway is involved in the stimulation of insulin release by vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide. *Journal of Biological Chemistry* **271**, 1660–1668.

Suzuki, N., Harada, M., Kitada, C., Ohkubo, S., Matsumoto, H., Watanabe, T., Coy, D. H., Tsuda, M., Arimura, A. & Fujino, M. (1993). Production of immunoreactive pituitary adenylate cyclase activating polypeptide (PACAP) by human neuroblastoma cells, IMR-32: detection and characterization with monoclonal and polyclonal antibodies against different epitopes of PACAP. *Journal of Biochemistry* **113**, 549–556.

Usdin, T. B., Bonner, T. I. & Mezey, E. (1994). Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. *Endocrinology* **135**, 2662–2680.

Yada, T., Hamakawa, N. & Yaekura, K. (1995). Two distinct modes of Ca^{2+} signalling by ACh in rat pancreatic β-cells: concentration, glucose dependence and Ca^{2+} origin. *Journal of Physiology* **488**, 13–24.

Yada, T., Sakurada, M., Ihida, K., Nakata, M., Murata, F., Arimura, A. & Kikuchi, M. (1994). Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet β-cells. *Journal of Biological Chemistry* **269**, 1290–1293.

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