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Molecular and Functional Characterization of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP-38)/ Vasoactive Intestinal Polypeptide Receptors in Pancreatic β-Cells and Effects of PACAP-38 on Components of the Insulin Secretory System*

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ABSTRACT

It has been previously demonstrated that pituitary adenylate cyclase-activating polypeptide (PACAP) regulates insulin secretion. PACAP exerts its biological action by binding to at least three different receptor subtypes coupled to different signal transduction mechanisms. The signaling pathways underlying the insulinotropic effect of PACAP involve mainly the activation of adenylate cyclase to form cAMP, which directly and indirectly, through increased intracellular Ca^{+2}, stimulates insulin exocytosis. In the present study we have characterized the functional and molecular expression of PACAP/vasoactive intestinal polypeptide receptors isoforms and subtypes and its isoforms in a β-cell line and in isolated rat pancreatic islets. Although insulinoma cells express the messenger RNA encoding PAC1 (-R and -hop variants), VPAC1 and VPAC2, binding experiments indicate the preponderance of PAC1 over VPAC1–2 receptors. We have also shown that the main signaling pathway of PACAP in β-cells is mediated by adenylate cyclase, whereas the inositol 1,4,5-trisphosphate pathway is almost inactive. Furthermore, we have demonstrated that PACAP exerts long-term effects on β-cells, such as transcriptional regulation of the insulin gene and genes of the glucose-sensing system (GLUT1 and hexokinase 1). (*Endocrinology* **140:** 5530–5537, 1999)

INSULIN SECRETION is regulated by nutritional, neural, and hormonal factors. Peptide members of the glucagon/vasoactive intestinal polypeptide (VIP)/secretin family, such as glucagon-like peptide 1 (GLP-1) are able to influence glucose-induced insulin secretion at very low concentrations and, therefore, are thought to be involved in the physiological regulation of insulin secretion (1). Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide structurally related to the glucagon/VIP/secretin family, showing a high degree of homology with VIP (2). Two isoforms of PACAP, PACAP-27 and PACAP-38, sharing the 27 N-terminal amino acids, are derived from the same 176 amino acids precursors by tissue-specific posttranslational processing (3, 4).

In the rat pancreas, PACAP-like immunoreactivity has been localized in the nerve fibers, around blood vessels, and in the central portion of islets (5). Previous studies demonstrated that PACAP is a potent stimulator of glucose-induced insulin release (6). PACAP exerts its biological action by binding to specific membrane receptors, which can be divided into at least three subtypes: PAC_1 (7–11), is PACAP selective, whereas $VPAC_1$ (12–15) and $VPAC_2$ (16–20) exhibit similar affinities for PACAP and VIP. Several PAC_1 variants, generated by an alternative splicing mechanism, have been identified. The splicing of a 21-amino acid domain in the N-terminal extracellular region modulates the binding and relative potencies of PACAP-27 and PACAP-38 (10), whereas the presence or absence of either one or two cassettes, designated hip and hop, in the third intracellular loop regulates the coupling to adenylate cyclase (AC) and phospholipase C (PLC) (11). $VPAC_1$ are coupled to AC (12, 13, 15, 21), whereas $VPAC_2$ can additionally activate PLC (16–20). PACAP/VIP receptors with distinct transducing systems may subserve different functional assignments. Previous studies demonstrated the presence of PAC_1 and $VPAC_2$ in HIT-T15 and RINm5F insulinoma cells (17, 22, 23).

In the present study we determined the molecular and functional characterization of PACAP receptors isoforms in a rat insulinoma cell line (RIN 1046–38 cells) and analyzed the signal transduction pathways activated by PACAP-38; furthermore we investigated whether the insulinotropic action of PACAP-38 involves modification of the expression of components of the β-cell glucose-sensing system.

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Materials and Methods

Cell cultures, cell treatment, and RNA isolation

RIN 1046–38 cells were cultured in medium 199 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS, 2 mM glutamine, 50 IU penicillin, and 50 μg/ml streptomycin at 37 C in an atmosphere of 95% humidified air-5% CO_2. Cells were seeded in culture medium at a density of 2×10^5 cells/ml in 6-well plates or at a density of 1×10^5 cells/ml in 24-well plates with PACAP-38 (Peninsula Laboratories, Inc., Belmont, CA) in increasing amounts (0.01, 0.1, and 1 pM) in the presence of different glucose concentrations. At the end of the treatment, cells were processed either for RNA extraction by RNA-Fast II (Molecular System, San Diego, CA) or for insulin release experiments. RNA was quantified by UV spectrophotometry and stored in diethylpyrocarbonate-treated water at −70 C until use. Cells were used at passages 19–25, at which glucose responsiveness is maintained (24).

Pancreatic islets were isolated from neonatal rats (5 days old) as previously described (25). Briefly, pancreas were excised, digested with collagenase type V (Sigma, St. Louis, MO), and seeded in 35-mm petri dishes in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS, 2 mM glutamine, 50 IU penicillin, and 50 μg/ml streptomycin at 37 C in an atmosphere of 95% humidified air-5% CO_2. After 6 h, most of fibroblasts, present as contaminating cells in the cultures, were attached to the bottom of the dishes; the supernatant, containing islets and exocrine tissue, was replated in a new dishes. After 24 h, the supernatant containing undigested material and debris was discarded, and fresh medium was added for an additional 24 h. Thereafter, islets were processed for RNA extraction or insulin release experiments.

Analysis of PACAP/VIP-receptor messenger RNAs (mRNAs) by RT-PCR

Primers used to identify PAC_1 receptor mRNAs by RT-PCR were based on the reported sequence for rat PAC_1 hip-hop1 isoform (11) (GenBank accession no. Z23272). Oligonucleotides were synthesized by Pharmacia Biotech (Milan, Italy). Sequences and location of the primers with respect to the initiation of translation were as follows: A1, 5′-CATCCTTGTACAGAAGCTGC-3′ (forward primer, matching the beginning of the third intracellular loop and corresponding to bases 1074–1093); A2, 5′-ACAAATTTAAGACTGAGAGT-3′ (forward primer, matching the beginning of the hip cassette and corresponding to bases 1135–1154); A3, 5′-TCCACCATTACTCTACGGCT-3′ (forward primer, matching the end of the hop cassette and corresponding to bases 1288–1307); and A4, 5′-GGTGCTTGAAGTCCATAGTG-3′ (reverse primer, matching a region of the cytoplasmic tail and corresponding to bases 1527–1546). The following are the sizes of the expected PCR products using primer pair A1/A4: 305 bp for PAC_1, 386 bp for PAC_1-hop2, 389 bp for PAC_1-hip and PAC_1-hop1, and 473 bp for PAC_1-hip-hop1. The predicted PCR products using primer pair A2/A4 are 328 bp for PAC_1-hip and 412 for PAC_1-hip-hop1, whereas those obtained with primer pair A3/A4 are 256 bp for PAC_1-hop2 and 259 bp for PAC_1-hop1 and PAC_1-hip-hop1. To investigate the existence of the 21-amino acid domain in the N-terminal extracellular region of PAC_1 we used two primers external to the exonic cassette encoding for this domain. Oligonucleotide sequences and location of the primers with respect to the initiation of translation were as follows: A5, 5′-CTGCATCTTCAAGAAGGAGC-3′ (forward primer corresponding to bases 159–178); and A6, 5′-CACAAG-CATCGAAGTAGTGG-3′ (reverse primer corresponding to bases 471–490). The sizes of the expected PCR products are 332 and 269 bp in the presence or absence of the cassette, respectively. Primers used to identify $VPAC_1$ mRNA by RT-PCR were based on the reported sequence (13) (GenBank accession no. M86835). Oligonucleotide sequences and locations of the primers with respect to the initiation of translation were as follows: B1, 5′-CACGAGTGTGAGTACCTGCA-3′ (forward primer, corresponding to bases 161–180); and B2, 5′-CGGTCTTCACGGTATTG-TAG-3′ (reverse primer, corresponding to bases 475–494). Primers used to identify $VPAC_2$ mRNA by RT-PCR were based on the reported sequence (18) (GenBank accession no. Z25885). Oligonucleotide sequences and locations of the primers with respect to the initiation of translation were as follows: C1, 5′-GTGCTGGTCAAGGACAGTGT-3′ (forward primer, corresponding to bases 591–610); and C2, 5′-AGGCGAGTTGC-TATCCATG-3′ (reverse primer, corresponding to bases 838–856). The following are the sizes of the expected PCR products: 334 bp for $VPAC_1$ (primer pair B1/B2) and 584 bp for $VPAC_2$ (primer pair C1/C2). To control for the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for rat phosphoglycerate kinase 1 (26) were included in each PCR reaction and generated a 183-PCR product. Oligonucleotide sequences and locations of the primers were as follows: 5′-AGGTGCTCAACAACATGGAG-3′ (forward primer, residues 777–796) and 5′-TACCAGAGGCCACAG-TAGCT-3′ (reverse primer, residues 940–959). Total RNA from RIN 1046–38 or pancreatic islets was treated with ribonuclease-free deoxyribonuclease (Roche Molecular Biochemicals, Milan, Italy) to remove any residual genomic DNA. Single stranded complementary DNAs (cDNAs) were synthesized using total RNA (1 μg), Moloney murine leukemia virus reverse transcriptase (200 U; Roche Molecular Biochemicals, Milan, Italy), and oligo-$(deoxythymidine)_{18}$ primer (100 nM) in presence of deoxy-NTPs (1 mM) and ribonuclease inhibitor (40 U; Roche Molecular Biochemicals) in a final volume of 20 μl. The reaction was terminated by incubation at 70 C for 10 min, and cDNA samples were diluted by adding 80 μl sterile H_2O. For each amplification, 40 μl of a PCR master mix were added to 10 μl diluted cDNA sample to yield the following final concentrations: 1 μM specific primers, 200 μM deoxy-NTPs, 1.25 U *Taq* DNA polymerase (Roche Molecular Biochemicals), and *Taq* buffer containing 1.5 mM $MgCl_2$. PCR was performed on a Perkin-Elmer Corp./Cetus thermal cycler (35 cycles of 95 C for 30 sec, 57 C for 30 sec, and 72 C for 45 sec). At the end of PCR, samples were kept at 72 C for 10 min for final extension and stored at 4 C. Amplification products arising from RT-PCR were separated by electrophoresis (2.0% agarose gel in 0.045 M Tris-borate and 1 mM EDTA buffer) and visualized by ethidium bromide staining. In a preliminary series of experiments, the use of RT-PCR for the identification of PACAP/VIP receptors was validated on the bases of specificity of the primers, and sizes and sequences of the amplification products.

Membrane preparation and displacement studies

RIN 1046–38 cells membranes were prepared following the technique previously described by Andersson *et al.* (22) with minor modifications. Cells were grown to confluence, washed in 50 mM Tris (pH 7.4), and scraped off with a rubber policeman in a buffer containing 50 mM Tris, 1.5 mM $MgCl_2$, and 150 μg/ml bacitracin, pH 7.4. After centrifugation ($1200 \times g$ at 4 C for 15 min), the pellet was resuspended in Tris buffer and homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) at 4 C. The homogenate was than centrifuged at $100,000 \times g$ at 4 C for 30 min, the pellet containing the membranes was resuspended in Tris buffer, the protein content was evaluated by the Bradford method, and aliquots were stored at −80 C until use. For displacement studies RIN 1046–38 membranes were resuspended in a buffer containing 50 mM Tris, 1.5 mM $MgCl_2$, 300 μg/ml bacitracin, and 1% BSA at a protein concentration of 600 μg/ml. Membranes were incubated at 15 C in a final volume of 250 μl with [^{125}I]PACAP-27 (50 pM) in the absence or presence of increasing concentrations of native PACAP-38 (ranging from 0.1 nM to 1 μM). After 1 h, aliquots of the incubation mix were transferred to microtubes and centrifuged at $10,000 \times g$ in a microfuge, the pellets were washed twice, and the microtube tips containing the pellets with the bound radioactivity were cut and counted in a γ-counter. Nonspecific binding, considered to be binding in the presence of 1 μM native PACAP-38, was less than 15% of total binding and was subtracted from each point to obtain the specific binding. Experiments were performed in triplicate.

Cellular cAMP content

Cells were seeded in 24-well plates and washed twice with glucose-free HEPES buffer containing 114 mM NaCl, 25.5 mM $NaHCO_3$, 10 mM HEPES, 2.5 mM $CaCl_2$, 4.7 mM KCl, 1.21 mM KH_2PO_4, 1.16 mM $MgSO_4$, and 0.1% BSA (pH 7.2). Thereafter, cells were incubated for 10 min in a final volume of 250 μl with PACAP-38 (0.1 pM) in the absence or presence of 2.8 mM glucose. The incubation was stopped by adding ice-cold ethanol to a final concentration of 65%; thereafter, cells were scraped off with a rubber policeman and centrifuged at $1200 \times g$ for 15 min. Aliquots of the supernatants were dried with a Speed-Vac (Savant Instruments, Hicksville, NY) and stored at −20 C until analysis of protein content (27) and cAMP RIA.

Measurement of inositol phospholipid (IP) hydrolysis

The rate of IP hydrolysis was estimated by measuring the accumulation of [^{3}H]IP in the presence of Li^+, which blocks the conversion of IP to free inositol. Cells were seeded in six-well plates in medium containing 20 nM myo-[2-^{3}H]inositol (SA, 20 Ci/mmol) to label membrane inositol phospholipids. Cells were washed twice at 37 C with Krebs Henseleit buffer containing 119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, and 0.1% BSA, pH 7.4, with 10 mM glucose and 10 mM LiCl and incubated in the same buffer for 1 h in the presence of PACAP-38 (0.1 pM). The supernatant was removed, the reaction was stopped by adding 1.5 ml ice-cold methanol-H_2O (1:1, vol/vol), and the cells were scraped off with a rubber policeman. After the addition of 0.75 ml chloroform, the cell suspension was centrifuged at 500 × *g* for 2 min. Aliquots (800 ml) of the aqueous phase were applied to columns containing 1 ml Dowex 1x8 resin (100–200 mesh, formate form, Bio-Rad Laboratories, Inc., Hercules, CA), and the phosphate esters were eluted by stepwise addition of formate solution of increasing strength. Inositol monophosphate was eluted with 0.2 M ammonium formate and 0.1 M formic acid. Fractions were collected, and the radioactivity was measured by liquid scintillation counting. Protein was measured by the Bradford method (27).

Insulin release experiments and intracellular insulin content evaluation

For short term experiments, cells were seeded in 24-well plates; thereafter, medium was removed, and cells were washed twice at 37 C for 30 min each time with glucose-free HEPES buffer. Thereafter, cells were incubated for 1 h in the same buffer with PACAP-38 (0.01, 0.1, and 1 pM) in the absence or presence of glucose (1 and 2.8 mM). At the end of the incubation period, aliquots of the supernatant were collected and stored at −20 C for subsequent insulin RIA; cells were extracted overnight at 4 C with a solution of acidified ethanol for intracellular insulin content assay. For long term experiments, PACAP-38 was added to the culture medium for 12 h (renewed after 6 h) in the absence or presence of glucose (6, 12, or 18 mM); 6 mM glucose was the lowest glucose concentration allowing the cells to remain attached during the entire incubation period. Aliquots of the medium were collected after 3, 6, and 12 h for subsequent insulin RIA. Cells were extracted overnight at 4 C with a solution of acidified ethanol for intracellular insulin content assay.

Insulin and cAMP RIA

Insulin was determined using a dextran-charcoal method as previously described (28), with an antiinsulin antibody raised in guinea pig, porcine insulin standard (Sigma), and [^{125}I]insulin from New England Nuclear (Boston, MA).

For cAMP determination, sample were previously acetylated and successively processed for RIA using a commercial kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

Analysis of insulin mRNA expression by Northern blot

Insulin mRNA expression was analyzed after 12 h of treatment with 0.1 pM PACAP-38 in the absence or presence of 6, 12, or 18 mM glucose. Total RNA (20 μg for each condition) was denatured at 65 C and applied to a 1% agarose gel containing 5% (vol/vol) formaldehyde. RNAs were then blotted onto nylon membrane (Schleicher & Schuell, Inc., Keene, NH) using a Posiblot apparatus (Stratagene, La Jolla, CA) and cross-linked. The blots were hybridized with a 450-kb mouse insulin cDNA (provided by Dr. A. L. Notkins, NIH, NIDR, Bethesda, MD) labeled with [α-^{32}P]deoxy-CTP (Amersham Pharmacia Biotech, Arlington Heights, IL) using a random priming procedure with 2 U Klenow polymerase (Boehringer Mannheim, Milan, Italy). The hybridization were carried out overnight at 42 C in 50% formamide, 6 × SSC, 0.5% SDS, and 100 μg/ml single stranded DNA. The membrane were then washed with 1 × SSC (standard saline citrate) and 0.5% SDS at 60 C. Blots were subsequently exposed overnight to Kodak films (Eastman Kodak Co., Rochester, NY) at −80 C with intensifying screens. Densitometric analysis was performed using an image analyzer (Fluor-S, Bio-Rad Laboratories, Inc.).

Analysis of hexokinase (HK) and GLUT1 mRNA by competitive RT-PCR

HK and GLUT1 mRNA expressions were analyzed after 12 h of treatment with 0.1 pM PACAP-38 in the absence or presence of 6, 12, or 18 mM glucose.

Competitive RT-PCR assay was performed by coamplification of increasing amounts of HK or GLUT1 complementary RNA internal standard (i.s.) with a constant amount of wild-type (w.t.) RNA isolated from RIN 1046–38 cells, as previously described, with minor modifications regarding w.t. synthesis (29). Briefly, a 100-bp fragment was introduced into the HK i.s. sequence or the GLUT1 i.s. sequence by PCR-based, site-directed mutagenesis, allowing us to distinguish the amplification products arising from the i.s. RNA from those arising from the w.t. RNA. Known amounts of HK or GLUT1 i.s. complementary RNA were incubated in the presence of a constant amount of w.t. RNA (0.5 μg) isolated from RIN 1046–38 cells. The i.s./w.t. RNA mixtures were reverse transcribed and amplified for 31 cycles (94 C/45 sec, 62 C/45 sec, and 72 C/1 min, with a final extension at 72 C/5 min) by 2.5 U *Taq* DNA polymerase (Promega Corp., Madison, WI) in a mixture containing 2.1 mM $MgCl_2$ and 100 pmol upstream and downstream amplification primers in the presence of 0.5 × 10^6 cpm [γ-^{32}P]ATP-labeled upstream primer (Amersham Pharmacia Biotech, Arlington Heights, IL). The upstream primer was labeled with 10 U T4 kinase (Roche Molecular Biochemicals) and purified on Stratagene purification columns (Stratagene).

Analysis of the PCR-amplified products

PCR products were analyzed on 1% agarose-0.5 × Tris-borate EDTA gel, showing a 574-bp band for HK w.t. or a 674-bp band for HK i.s. and a 475-bp band for GLUT1 w.t. or a 575-bp for GLUT1 i.s. To quantitate the amount of product arising from the amplified i.s. or w.t. RNA, the corresponding ethidium bromide-stained bands were excised from the gel, and the amount of incorporated radioactivity was determined in a β-counter (LKB, Rockville, MD). Gel slices corresponding to lanes containing a control sample (water) were also excised at the sizes of i.s. and w.t. bands to determine the amount of background radioactivity. Data were plotted as the ratio of counts per min incorporated into the w.t. amplification product and the counts per min incorporated into the i.s. amplification product as a function of the known amount of i.s. The plot was analyzed by logarithmic regression, and the extrapolated point of equivalence corresponds to the amount of HK or GLUT1 mRNA present in the unknown sample.

Statistical analysis

Data are presented as the average ± SEM. Statistical analysis was carried out by one-way ANOVA followed by Student's *t* test.

Results

Analysis of PACAP/VIP receptor mRNAs by RT-PCR

To determine the expression of PAC_1 receptor variants in RIN 1046–38 insulinoma cells (Fig. 1A) and in rat pancreatic islets (Fig. 1B), we analyzed their mRNAs by the use of four primer pairs.

Amplification with primer pair A1/A4 (which is external to the site of insertion of the hip/hop cassettes) produces amplification products of about 300 and 390 bp (Fig. 1, A and B, lane 1). Primer pair A2/A4 produced no amplification product (Fig. 1, A and B, lane 2), whereas primer pair A3/A4 produced an amplification product of about 250 bp (Fig. 1, A and B, lane 3), indicating the existence of PAC_1-R and -hop receptor variants. In addition, to investigate whether these variants contained the exon encoding the 21-amino acid domain in the N-terminal extracellular region, we used primers A5/A6, which are external to the site of insertion of this cassette. Amplification with these primers produced a single

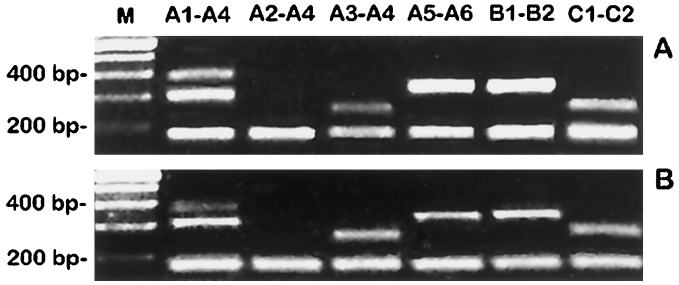

FIG. 1. Ethidium bromide-stained gel electrophoresis of RT-PCR analysis of PAC1, VPAC1, and VPAC2 receptor mRNAs in RIN 1046–38 cells (A) and rat pancreatic islets (B). Total RNA was reverse transcribed and PCR amplified with primer pairs specific for the PAC1 receptor (A1/A4, A2/A4, A3/A4, and A5/A6; lanes 1–4), for the VPAC-1 receptor (B1/B2; lane 5), and for the VPAC-2 receptor (C1/C2; lane 6). To control for the integrity of RNA and for differences attributable to errors in experimental manipulation from tube to tube, primers for rat phosphoglycerate kinase 1 (PGK1) were included in each PCR reaction and generated a 183-bp PCR product. A 100-bp DNA ladder is shown on the *left* of the gel (lane M), with bands labeled in base pairs.

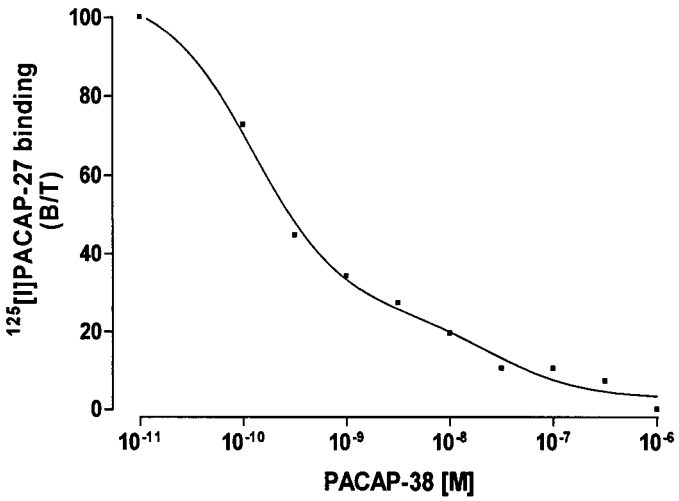

FIG. 2. Competition-inhibition of [^{125}I]PACAP-27 binding to RIN 1046–38 membranes by PACAP-38. Data are plotted as the percentage of tracer radioactivity specifically bound in the absence of competitor and represent the mean of three experiments, performed in triplicate.

amplification product of about 330 bp (Fig. 1, A and B, lane 4), which indicates the presence of the 21-amino acid domain in all of the PAC_1 receptors expressed in both rat insulinoma cells and pancreatic islets.

To determine the mRNA expression of $VPAC_1$ and $VPAC_2$ we used primer pairs B1/B2 and C1/C2, respectively; amplification products obtained with these primers were indicative of the $VPAC_1$ and $VPAC_2$ mRNAs (Fig. 1, A and B, lanes 5 and 6).

Displacement studies

PACAP-38 displaced [^{125}I]PACAP-27 (50 pM) in RIN 1046–38 cells in a concentration-dependent manner within the range of 0.1 nM to 1 μM. IC_{50} was obtained with 0.2 nM PACAP-38 (Fig. 2).

VIP (1 μM) displaced [^{125}I]PACAP-27 (50 pM) by 20%, whereas glucagon (1 μM) did not displace [^{125}I]PACAP-27, demonstrating that the binding was specific (not shown).

Cellular cAMP content

PACAP-38 induced a rapid increase in intracellular cAMP, as demonstrated in Table 1, in both the absence and presence of glucose. The basal intracellular cAMP contents were 198 ± 40 and 350 ± 72 fmol/μg protein, respectively, in the absence and presence of 2.8 mM glucose. When 0.1 pM PACAP was added for 10 min, the intracellular cAMP contents were 602 ± 93 and 1106± 181 fmol/μg protein, respectively, in the absence and presence of 2.8 mM glucose.

Measurement of IP hydrolysis

PACAP-38 did not cause significant changes in IP formation in RIN 1046–38 cells. It is noteworthy that the level of unstimulated IP was very low in this cell line (data not shown).

Insulin release experiments and intracellular insulin content evaluation

Insulin release from RIN 1046 38 cells was 2.16 ± 0.36% of the total insulin content in the absence of glucose; increasing glucose concentrations (1 and 2.8 mM) stimulated insulin release (4.59 ± 0.52% and 4.30 ± 0.72% of the total insulin content, respectively). Short term treatment (1 h) with PACAP-38 increased basal insulin release in a dose-dependent manner (2.94 ± 0.57%, 4.00 ± 0.16%, and 3.38 ± 0.49% of the total insulin content, respectively, at 0.1, 1, and 10 pM); insulin release induced by glucose was also increased by PACAP-38 in a dose-dependent manner (5.28 ± 0.79%, 7.09 ± 0.54%, and 6.46 ± 0.54%, respectively, with 0.01, 0.1, and 1 pM PACAP-38 in the presence of 1 mM glucose; 4.83 ± 0.69%, 7.41 ± 0.49%, and 6.59 ± 0.25%, respectively, with 0.01, 0.1, and 1 pM PACAP-38 in the presence of 2.8 mM glucose; Fig. 3).

Insulin release in the culture medium containing 6 mM glucose reached a maximum after 12 h; in the presence of 0.1 pM PACAP-38, insulin accumulation in the medium was significantly increased after 3 and 6 h (Fig. 4). Insulin accumulation in the medium was not influenced by higher glucose concentrations (12 and 18 mM) in either the absence or presence of PACAP-38 (data not shown).

Table 2 shows that intracellular insulin content was significantly increased by glucose (6, 12, and 18 mM); the addition of 0.1 pM PACAP-38 significantly potentiated the effect of glucose.

TABLE 1. Modifications of intracellular cAMP formation (fmol/mg prot) induced by PACAP-38 in the presence or in the absence of glucose

Glucose (mM)	PACAP-38 (pM)	
	0	0.1
0	198 ± 40	602 ± 93[a]
2.8	350 ± 72	1106 ± 181[a]

Values are the mean ± SEM (n = 3).
[a] $P < 0.001$ *vs.* glucose alone.

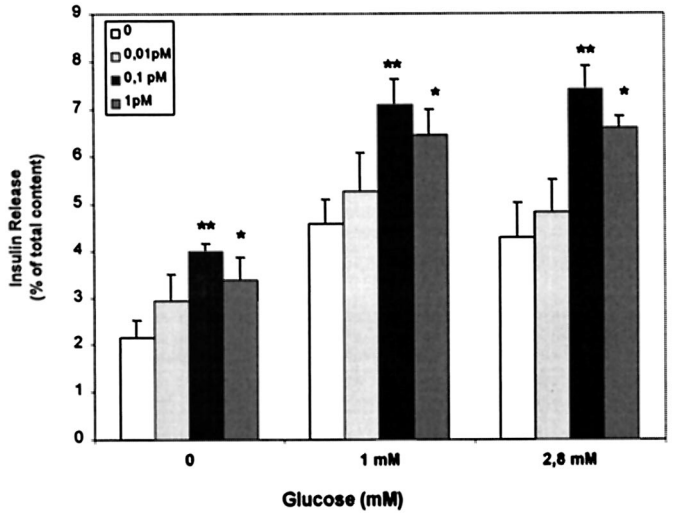

FIG. 3. Insulin release from RIN 1046–38 cells after 1-h incubation with or without PACAP-38 in the presence of different glucose concentrations. Data are expressed as a percentage of intracellular insulin content and represent the mean ± SEM of three independent experiments, performed in triplicate. *, $P < 0.05$; **, $P < 0.01$.

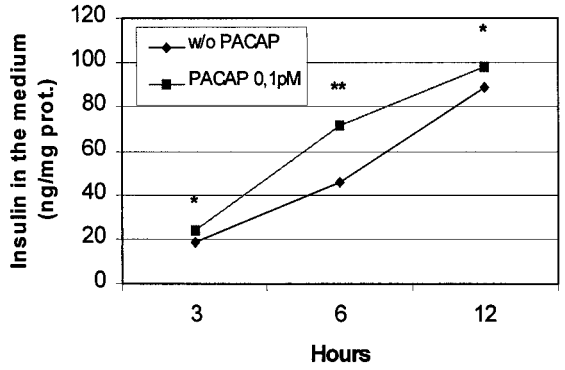

FIG. 4. Insulin accumulation in the medium from RIN 1046–38 cells cultured for 12 h with or without PACAP-38 in the presence of 6 mM glucose. Data represent the mean ± SEM of three independent experiments. **, $P < 0.01$.

TABLE 2. Modifications induced by PACAP-38 after 12 h on intracellular insulin content (nanograms per mg protein)

Glucose (mM)	PACAP-38 (pM)	
	0	0.1
6	9.13 ± 0.43	14.68 ± 0.98[a]
12	14.87 ± 0.71	26.13 ± 1.30[b]
18	15.75 ± 0.85	25.32 ± 1.25[b]

Values are the mean ± SEM (n = 3).
[a] $P < 0.05$ *vs.* glucose alone.
[b] $P < 0.01$ *vs.* glucose alone.

Analysis of insulin mRNA expression by Northern blot

Northern blot analysis of insulin mRNA expression revealed a band at 0.8 kb as expected; glucose alone (6, 12, and 18 mM) increased the expression of insulin mRNA in a dose-dependent manner; the addition of 0.1 pM PACAP-38 increased insulin mRNA expression compared with the control condition with glucose alone (Fig. 6, *upper panel*). Densitometric analysis indicated that PACAP-38 increased insulin

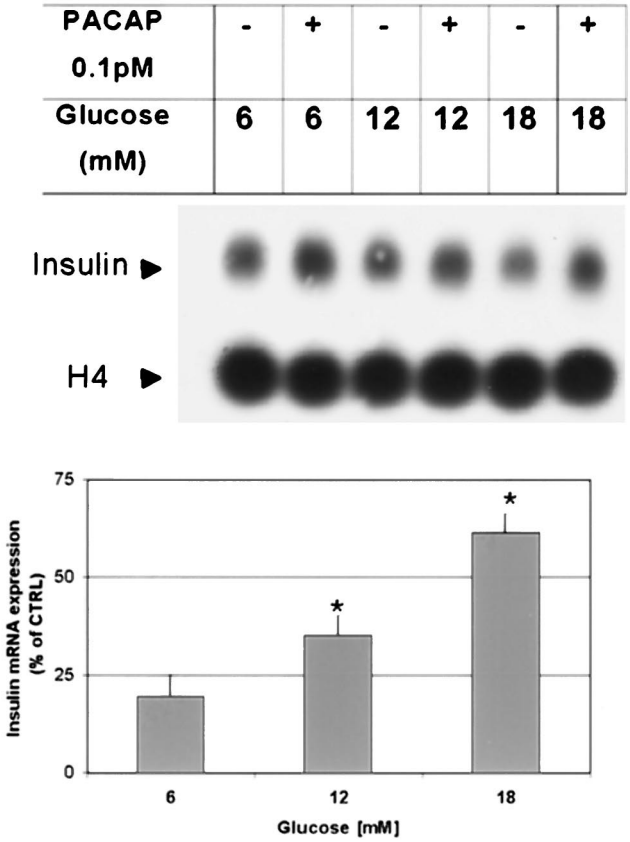

FIG. 5. *Upper panel*, Northern blot analysis of Insulin mRNA expression in RIN 1046–38 cells cultured for 12 h with or without PACAP-38 in the presence of 6, 12, and 18 mM glucose. Data represent the mean of three independent experiments. *Lower panel*, Densitometric analysis. Data represent the percent increment in insulin mRNA expression induced by PACAP compared with the control condition, where the control is insulin mRNA expression in the absence of PACAP at the same glucose concentration.

mRNA expression by 19.31 ± 5.70%, 35.39 ± 4.85%, and 61.35 ± 4.94%, respectively, in the presence of 6, 12, or 18 mM glucose (Fig. 5, *lower panel*).

Analysis of HK and GLUT1 mRNA by competitive RT-PCR

Figure 6A shows that glucose increased the expression of GLUT1 mRNA in a dose-dependent manner; the basal GLUT1 mRNA level in the presence of 6 mM glucose were 7.61 ± 0.08 pg i.s./μg total RNA, whereas in the presence of 12 or 18 mM glucose they were, respectively, 14.16 ± 1.26 and 21.29 ± 1.06 pg i.s./μg total RNA. The addition of 0.1 pM PACAP-38 to 6 or 12 mM glucose produced an increase in GLUT1 mRNA expression compared with that using glucose alone (14.26 ± 1.21 and 17.61 ± 0.83 pg i.s./μg total RNA, respectively); on the contrary, in the presence of 18 mM glucose, PACAP-38 slightly decreased GLUT1 mRNA expression induced by glucose (15.94 ± 0.76 pg i.s./μg total RNA).

Figure 6B shows that glucose increased the expression of HK1 mRNA in a dose-dependent manner. The value of HK mRNA in the presence of 6 mM glucose was 28.82 ± 0.98 pg i.s./μg total RNA, whereas in the presence of 12 or 18 mM

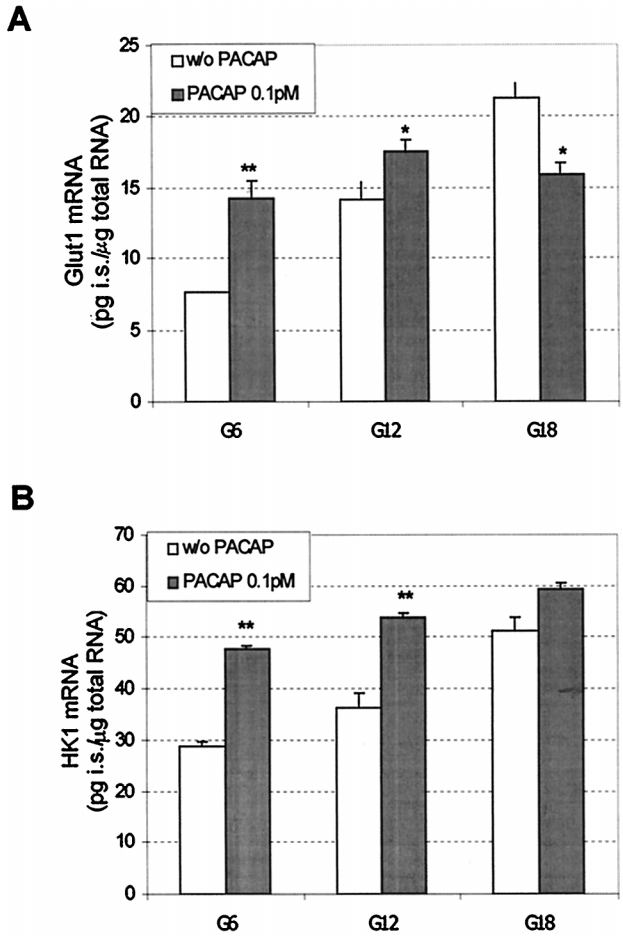

FIG. 6. GLUT1 (A) and HK1 (B) mRNA expression in RIN 1046–38 cells cultured for 12 h with or without PACAP-38 in the presence of 6, 12, and 18 mM glucose. Data represent the mean ± SEM of three independent experiments, performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ (*vs.* glucose alone).

glucose they were, respectively, 36.43 ± 1.55 and 51.19 ± 1.73 pg i.s./μg total RNA. The addition of 0.1 pM PACAP-38 produced an increase in HK1 mRNA expression compared with that using glucose alone, which was more pronounced in the presence of 6 and 12 mM glucose but very weak in the presence of 18 mM glucose (47.69 ± 0.65, 53.91 ± 0.90, and 59.36 ± 1.05 pg i.s./μg total RNA, respectively).

Discussion

To gain insight into the mechanisms by which PACAP exerts its potent insulinotropic effect, we studied the molecular expression and functional properties of the PACAP/VIP receptors, and we investigated the effect of PACAP-38 on components of the glucose-sensing system in a β-cell line (RIN 1046–38 cells). Our data reveal distinct splicing patterns and functional properties of native PAC_1, $VPAC_1$, and $VPAC_2$ receptors in rat insulinoma cells and pancreatic islets. In rat insulinoma cells, the short isoform of PAC_1 receptor (PAC_1-R) is expressed together with the PAC_1-hop splice variant, whereas the PAC_1-hip and hip-hop splice variants are absent; moreover, the 21-amino acid domain is present in all of the PAC_1 receptors expressed. The mRNA expression pattern of PACAP/VIP receptors in pancreatic islets was identical to that in RIN 1046–38 cells. PAC_1-R is the most abundant receptor isoform in the rat central nervous system and pituitary; the PAC_1-hop isoform is predominant in testes, olfactory bulb, and adrenal gland; the PAC_1-hip isoform has been detected at low levels in the olfactory bulb and hippocampus, but it is not expressed in the other tissues. The expression of the hip cassette determines an impairment of the AC stimulation and abolishes PLC stimulation by PACAP. On the other hand, the 21-amino acid deletion in the N-terminal domain determines an increased potency in PLC stimulation by PACAP-27; on the contrary, the insertion of 21 amino acids would lead to impairment of PLC stimulation by PACAP-27. This consequence should not be of relevance, as PACAP-38 is more abundant than PACAP-27 in adult tissues, and therefore, PACAP-38 is the main functional effector. In tissues in which PACAP-27 is more abundant, regulation of alternative splicing of exons encoding the N-terminal region of PAC_1 is a possible manner of tuning PLC stimulation (11). The analysis of receptor isoform expression in RIN 1046–38 cells and isolated rat pancreatic islets shows the presence of the PAC_1-R-hop isoform, but not of the PAC1-hip and hip-hop isoforms, and the binding experiments indicate the presence of high affinity receptors for PACAP (PAC1). The pattern of receptor isoform expression suggests that the main PACAP signal transduction pathway in RIN 1046–38 cells is mediated by AC, as also indicated by studies of intracellular cAMP formation and IP hydrolysis, which show that the inositol 1,4,5-trisphosphate (IP_3) pathway is almost inactive. These data are in agreement with previous results demonstrating a poor increment in IP_3 by PACAP-38 in a different insulinoma cell line (30).

Glucose-induced insulin release is proportional to the rate of glucose metabolism, which, therefore, represents a rate-limiting step. The range of glucose concentrations to which β-cells respond is regulated by the plasma membrane glucose transporters and the glucose-phosphorylating enzyme HKs (31, 32), representing the two major components of the glucose-sensing system (31, 33). Normal, fully differentiated, mature β-cells express mainly GLUT2 and glucokinase (HK4), whereas insulinoma cell lines express preferentially GLUT1 and HK1 (33, 34). Glucose transporter isoforms and hexokinase isoforms are coexpressed in a specific manner, based on the reciprocal biochemical and functional characteristics of each isoform; for example, the high K_m GLUT2 is coexpressed with the high K_m glucokinase, whereas the low K_m GLUT1 is coexpressed with the low K_m HK1, and GLUT4 is coexpressed with HK2 (35). In RIN 1046–38 cells, GLUT1 and HK1 are quantitatively more expressed than GLUT2 and glucokinase, as this cell line looses the high K_m components with time in culture; therefore, RIN 1046–38 cells metabolize glucose using preferentially the low K_m components GLUT1 and HK1 and release insulin at subphysiological glucose concentrations (36).

In the present study we demonstrate that PACAP-38 regulates the expression of GLUT1 and HK1 in RIN 1046–38 cells. GLUT1 and HK1 mRNA expressions are increased by glucose in a dose-dependent manner, and the addition of PACAP-38 at very low concentration significantly enhances this effect. The effect on GLUT1 mRNA may be due to an

increased transcription by cAMP. In fact, a cAMP-responsive element (CRE) has been described in the GLUT1 gene (37) and cAMP is known to increase the GLUT1 transcription rate (38, 39). HK1 mRNA can be regulated by cAMP as previously demonstrated in other cell lines (40). Therefore, it is possible to hypothesize that PACAP-38 regulates GLUT1 and HK1 mRNA expression via the same cAMP-mediated mechanism. Likewise, we have observed that insulin mRNA expression is increased by glucose in a dose-dependent manner and that PACAP-38 potentiates this effect. Insulin gene transcription is increased by cAMP as previously demonstrated in βTC-1 cells (41), suggesting that the same mechanism can be activated by PACAP-38 in RIN 1046–38. Actually, mutational analysis showed that forskolin increases the activity of the proinsulin promotor through a specific CRE present in this gene (42). Furthermore, there is evidence that at least two different nuclear proteins that bind to CRE and the recently cloned CRE-binding protein (CREB) 327/341 are expressed in β-cells (43, 44). Therefore, it is possible to suggest that PACAP-38, after binding to its receptor, activates the cAMP-dependent PKA pathway, leading to phosphorylation of CREB, which, in turn, acts as a third messenger, triggering gene transcription.

It has been demonstrated that transfection of RINr cells with GLUT1 cDNA induces higher insulin mRNA level and intracellular insulin content (45); moreover, islets from mice overexpressing the high affinity yeast HK show increased glucose utilization, insulin synthesis, and insulin secretory capacity (46). Therefore, it is possible to hypothesize that up-regulation of the glucose-sensing components induced by PACAP-38 may secondarily increase the expression of insulin mRNA and insulin secretion.

In conclusion, our results indicate that RIN 1046–38 cells express a specific pattern of PACAP/VIP receptors, and that PACAP-38 binds to these receptors and preferentially activates the cAMP-dependent PKA pathway, triggering insulin secretion through short and long term mechanisms of action. It has been demonstrated that the short term effects of PACAP on insulin secretion are mediated by increased cytoplasmic Ca^{2+} concentration by both voltage-dependent and -independent mechanisms, the latter involving elevation of intracellular cAMP, which, in turn, may stimulate Ca^{2+} release from intracellular stores (47). We suggest that the long term effects of PACAP on insulin secretion are mediated by transcriptional mechanisms involving the cAMP-CRE pathway, leading to transcription of insulin gene and genes of the glucose-sensing system, which, in turn, can further increase insulin biosynthesis and secretion.

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