**PRGI: A Novel Early-Response Gene Transcriptionally Induced by Pituitary Adenylate Cyclase Activating Polypeptide in a Pancreatic Carcinoma Cell Line**

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**ABSTRACT**

The rat pancreatic carcinoma cell line AR4-2J was screened for growth-associated genes linked to the mitogenic effect of the novel gut brain hormone, pituitary adenylate cyclase activating polypeptide (PACAP). Using the mRNA differential display technique, we identified and sequenced an unknown rat gene, PACAP-responsive gene 1 (PRGI), which is highly homologous to gly96, a novel murine gene of unknown function. The PRGI cDNA sequence of 1.1 kb encodes a 160-amino acid protein. Using targeted PCR, the gene structure of PRGI, constituting 0.6 kb of the promoter region, and the DNA coding region, including a single 107-bp intron, were established from rat genomic DNA. In AR4-2J cells, PACAP(1-38) increased PRGI mRNA levels up to 10-fold in a rapid (30 min), transient (3-6 h), and dose-dependent (ED50 < 1 μM) fashion. The growth-stimulating gastrointestinal hormones cholecystokinin and gastrin showed a similar degree of PRGI induction, and the PACAP-related peptides vasoactive intestinal peptide and secretin were without effect. The transcriptional inhibitor actinomycin D, various protein kinase C inhibitors, and the calmodulin inhibitor W-7 strongly reduced PRGI induction by PACAP, whereas the translation inhibitor cycloheximide potentially increased PRGI mRNA levels in unstimulated and PACAP-stimulated cells. Feedback-mediated hyperplasia of the rat exocrine pancreas induced by oral treatment of rats with the protease inhibitor camostat (FOY-305) was preceded by a 15-fold transient elevation of PRGI mRNA levels. These data suggest that PRGI is an early-response gene linked to PACAP-induced growth of AR4-2J cells as well as to hyperplasia of the rat exocrine pancreas in vivo.

**INTRODUCTION**

PACAP, a novel gut brain peptide belonging to the secretin-glucagon-VIP family, is expressed widely in neuronal and gastrointestinal tissues of several mammalian species (1—4). Both molecular forms of PACAP, PACAP(1-38) and PACAP(1-27), exert their biological actions via at least three G protein-coupled receptors that differ in structure, ligand specificity, and tissue distribution. The PV1 receptor (5, 6) exclusively binds PACAP(1-38) and PACAP(1-27) as high-affinity ligands and is expressed predominately in the brain, the adrenal medulla, and various cell lines (7-10), including the rat pancreatic carcinoma cell AR4-2J (11). The PACAP/PVIP type 2 and 3 receptors (12, 13), formerly designated VIP-1 and VIP-2 receptors, respectively, do not discriminate between PACAP and VIP as ligands and are expressed in liver (14), pancreas (15), lung (7), intestinal smooth muscle cells (16), and various tumor cell lines (17).

Besides its role as a secretagogue in the exocrine pancreas and pituitary (18, 19), as a neuromodulatory peptide in the central nervous system, adrenal gland, and intestinal nerve fibers (8, 20) or as regulator of intestinal motility (16, 20, 21), PACAP has been shown to exert a growth-promoting effect on neuronal cells (22) and epithelial tumor cells such as rat pancreatic carcinoma AR4-2J cells (23). Similar to other mitogenic gut brain hormones such as gastrin or CCK (24), this effect includes increased transcription of the proto-oncogenes c-fos and c-jun as well as activation of the heterodimeric transcription factor AP-1 (6). However, although activation of AP-1 certainly represents an early key event linked to growth factor-mediated AR4-2J cell proliferation, many other effectors and probably yet unidentified growth-related genes are involved in the induction of this process.

To identify those growth-related genes inducible by PACAP in AR4-2J cells via the PV1 receptor, the mRNA differential display procedure (25) was used. Hereby, a novel early-response gene, PRGI, was identified and analyzed in terms of its expression and its relation to the induction of cell growth.

**MATERIALS AND METHODS**

Materials. Suppliers of the following chemicals were: cell culture media, Biochrom (Berlin, Germany); VIP, PACAP, CCK, and secretin, Saxon Biochemicals (Hanover, Germany); QIAshredder, RNAeasy, and QIAEX DNA-gel extraction kits, Qiagen (Hilden, Germany); primers for mRNA differential display (Gene-ExScreen) and for all PCR applications, Biometra (Gottingen, Germany); M-MLV RT, dNTPs, and Taq polymerase, Life Technologies, Inc. (Eggenstein, Germany); RNAsin and b-galactosidase assay, Promega (Madison, WI); TA-cloning kit, Invitrogen (De Schelp, Netherlands); [α-32P]dATP and redipthine kit, Amersham (Braunschweig, Germany); α-35S-labeled dATP, Hartmann Scientific (Braunschweig, Germany); PMA, calphostinC, staurosporine, and H-7, Biomol (Hamburg, Germany); forskolin, Sigma Chemical Co. (Deisenhofen, Germany); CAT-ELISA, Boehringer Mannheim (Mannheim, Germany); and GAPDH RT-PCR amplifier set, Clontech (Heidelberg, Germany).

Culture and Preparation of AR4-2J Cells. AR4-2J cells were grown for 24 h on 60-mm culture dishes in 5 ml DMEM containing 10% FCS. After serum deprivation for 16 h, cells (2 × 10^5—5 × 10^6) were treated with various stimulants or with vehicle alone for various time periods. All experiments were performed at least in duplicate.

Isolation of Total RNA. For rapid and reproducible preparation of total RNA, the QiAshredder and RNAeasy kits (Qiagen) were used following the manufacturer’s instructions. After determination of the RNA content using a Gen-Quant UV photometer (Pharmacia), samples of total RNA were adjusted to 0.1 μg/μl RNA. Three-μl aliquots were analyzed by agarose gel electrophoresis and ethidium bromide staining. Additional quantitative analysis of the mRNA content was carried out by means of RT-PCR of GAPDH as control.

First-Strand cDNA Synthesis. Total RNA (2–3 μg) was heat denatured (75°C, 3 min) and subjected to oligo(T)-primed (1 μM) reverse transcription

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5. The abbreviations used are: PACAP, pituitary adenylate cyclase activating polypeptide; VIP, vasoactive intestinal peptide; PV1, PACAP/PVIP type 1; CCK, cholecystokinin; RT, reverse transcriptase; dNTP, deoxynucleotide triphosphate; PRGI, PACAP-responsive gene 1; PMA, phorbol 12-myristate-13-acetate; UT, untranslated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyl transferase; PKC, protein kinase C; ARE, A/T-rich element; poly(A), polyadenyl acid; M-MLV, Moloney murine leukemia virus.


7. Nucleotide sequences of PRGI gene and mRNA have been deposited in the European Molecular Biology Laboratory data bank (accession numbers X96437 and X96438, respectively).
using 200 units M-MLV RT/1 μg total RNA. Reverse transcription (final volume, 30 μl) was carried out at 37°C for 60 min in the presence of 60 units RNAse inhibitor (RNAsin; Promega), 200 μM dNTPs (Life Technologies, Inc.), and 1 mM DTT. cDNA synthesis was terminated by heating to 95°C for 5 min. The quality of cDNA was checked by PCR analysis of GAPDH, which was the most suitable control (see below).

mRNA Differential Display. Heat-denatured (75°C, 3 min) total RNA (0.6 μg) was incubated (total volume, 60 μl) with 1 μM of 1 of 12 different dodecameric anchored oligo(T) primers (dT12MN; M - A, G or C), 20 μM dNTPs, 120 units RNAse inhibitor (RNAsin), 1.5 mM MgCl2, 0.5% Triton X-100, 0.02% DTT, and 900 units M-MLV RT for 90 min at 37°C. Thereafter, first-strand cDNA was heated to 95°C for 5 min, and 2 μl of the cDNA mixture were incubated with 0.2 μM of 1 of 26 decameric arbitrary primers (Biometra), 1 μM of the corresponding oligo(T) primer, 2 μM dNTPs, 3 μCi [α-32P]dATP, 1.5 mM MgCl2, and 1 units Taq polymerase (total volume, 20 μl). Low-stringency PCR was run as follows: 95°C for 45 s; 60 cycles of 95°C for 45 s, 60°C for 1 s, 72°C for 10 s; and a 10-min extension at 72°C. Thereafter, overlapping PCR products were generated using the following degenerated primers: PRGJ, sense 1, 5′-GGG CAT AGO 'IT!' AGO 0-3' (positions 323—300); PRGJ, antisense 1, 5′-GCC TOC GAC ACA CD' TCT TCA GCC-3' (positions 94—119 referring to the PRGJ cDNA sequence in Fig. 1A); U2 primer, 5′-GGN CCN TAC TCG COT AAC CAC C-3' (positions 1-21) and a specific reverse primer, 5′-ATG GCN GAR OAR GGN GTh TCG CAG G-3' (positions 298-322); 5′-ATG TGC CAY TCN CGN AAC CAY fT-3' (positions 1-20) and a primer concentration of 0.5 μM at 1.5 mM MgCl2. Amplification parameters were: 95°C for 60 s; 25 cycles of 56°C for 45 s and 72°C for 70 s, 60°C for 2 mm, 72°C for 90 s; and a 10-min extension at 72°C (Thermocycler 9600; Perkin-Elmer Corp.). After a 5-μl aliquot of PCR products was verified by means of nested PCR and DNA sequencing. For quantitation, PCR was carried out with limited cycle numbers, hereby ensuring linearity of amplification. All reactions were performed in parallel.

Quantitation of PCR Products. Fifteen μl of PCR products were diluted with 35 μl Tris-EDTA buffer (10 mM Tris, 1 mM EDTA; pH 7.40) and subjected to ionic exchange chromatography on a MiniQ column (Pharmacia) using the SMART microchromatography apparatus (Pharmacia). For separation of PCR products, a linear gradient (0—100%; 10 min) of 1 M NaCl in Tris/EDTA buffer was quantified by means of peak integration (absorbance, 260 nm) using a concentration standard for calibration. GAPDH mRNA was used as internal standard, because mRNA levels did not change more than ±15% in response to various stimuli (i.e., PACAP, EGF, CCK, and FCS) incubated at different concentrations with AR4–2J cells for up to 48 h. External control samples proving stable, noninduced GAPDH expression were incorporated in each assay. Ratios (PRGJ/GAPDH) of the corresponding peak areas were calculated for each sample and used for quantitative calculations and comparisons.

Northern Hybridization. The PRGJ cDNA (positions 1—1011) was 32P-labeled using the rediprime labeling kit (Amersham). Twenty μg denatured total RNA from AR4–2J cells were separated on 1% agarose gels containing 2 μg formamide. After staining with ethidium bromide for RNA visualization, gels were subjected to capillary transfer onto a nylon membrane for 16 h at room temperature. Then, transferred RNA was immobilized by means of UV irradiation, and membranes were prehybridized (65°C, 15 min) and hybridized (65°C, overnight) in 0.5 M Na2HPO4-NaH2PO4, 1 mM EDTA, 1% BSA, and 7% SDS and washed twice (65°C, 10 min) in 40 mM Na2HPO4-NaH2PO4, 1 mM EDTA, 0.5% BSA, and 5% SDS; twice (65°C, 10 min) in 40 mM Na2HPO4-NaH2PO4, 1 mM EDTA, and 1% SDS; and briefly in 2× SSC. Blots were exposed for 24—48 h at −70°C to an X-ray film using an intensifier screen. Autoradiographs were evaluated densitometrically and quantified using the Whole Band Analysis software (Bioimage).

PCR of Genomic DNA. For further structural characterization of PRGJ, genomic DNA was prepared from AR4–2J cells using QIAamp tissue kit (Qiagen). Two μg of genomic DNA were subjected to PCR using the following primers: for the 5′-flanking region, forward (F1), 5′-GAA TAG CTG CTC GGG NNT KCT CCT C-3′ (positions 589—566) and reverse (R1), 5′-GGG TTC TAC GGG AAC ACA TAT CCT CAC C-3′ (positions 1017—1004). PCR (final volume, 30 μl) was carried out using 0.75 units Taq polymerase at a primer concentration of 1 μM and 2.5 mM MgCl2. Amplification parameters were: 95°C for 45 s; 25 cycles of 60°C for 45 s and 72°C for 45 s; and a 10-min extension at 72°C (Thermocycler 9600; Perkin-Elmer Corp.). PCR products were purified, reamplified, and sequenced as described above. For preparation of a PCR product encompassing the full-length cDNA of PRGJ, PCR was performed using a specific forward primer 5′-ATG TGC CAT CGC AAT GCC ACC CAC C-3′ (positions 1—20) and a specific reverse primer 5′-AGA CAG CCG CCC TCT GAC AA-3′ (positions 1011—992). Amplification parameters were: 95°C for 60 s; 25 cycles of 56°C for 45 s and 72°C for 45 s; and a 10-min extension at 72°C (Thermocycler 9600; Perkin-Elmer Corp.). PCR products were purified, reamplified, and sequenced as described above.

Transfection with the CAT Reporter Gene and Assay for CAT Activity. AR4–2J cells (5 × 104 to 5 × 105) were harvested and resuspended in 400 μl DMEM containing 5% FCS. Cells were transiently transfected with 10 μg of a PRGJ promoter construct of the CAT basic vector (Promega) by means of electroporation (Celljet electroporation instrument; Eurogentech) at a pulse of 1000 V, 1200 μF for 30-40 ms. The CAT vector contained the CAT reporter gene linked to a SphI-B fragment of the 3′-flanking region of the PRGJ gene. This insert was generated by PCR using the forward primer 5′-GAA TAG CTG CTC GGG AGT GTC CCT C-3′ (positions 589 to 566) extended by a 5′ PstI restriction side and the reverse primer 5′-AAT TGG CAT GCC ACC CAC C-3′ (positions 1 to 21) and reverse (R2), 5′-TGG CAT TTA GAA TCC CCG CAT TTT T-3′ (positions 489—461). PCR (final volume, 30 μl) was carried out using 0.75 units Taq polymerase at a primer concentration of 1 μM and 2.5 mM MgCl2. Amplification parameters were: 95°C for 5 min; 35 cycles of 95°C for 70 s, 60°C for 2 min, 72°C for 90 s; and a 10-min extension at 67°C (Thermocycler 9600; Perkin-Elmer Corp.). PCR products were purified, reamplified, and sequenced as described above.
Induction of Pancreatic Hyperplasia in Rats and Tissue Preparation.

Male Wistar rats (180—200 g) were gavage fed twice a day with 200 mg/kg body weight of the synthetic proteinase inhibitor FOY-305 (provided kindly by Prof. H. F. Kern, Institute of Cell Biology, University of Marburg, Germany) suspended in water (50 mg/ml). Rats were sacrificed according to the following time schedule: 0, 1, 6, and 24 h and 4 and 10 days. Plasma samples for CCK determination were collected, pancreata were removed rapidly, dissected, and transferred immediately to liquid nitrogen, and wet weights were determined. For homogenization, small pieces of deep-frozen tissues were ground with a pestle while keeping frozen with liquid nitrogen. Then, the homogenized tissue was lysed in the QiaShredder lysis buffer and total RNA was isolated, as described above. Protein content was determined using the Bio-Rad dye reagent, DNA was quantified using fluorescent dye H-33258 (Hoechst), and amylase was determined as described (26).

RESULTS

Identification and Sequencing of PRGJ. Duplicate preparations of total RNA from AR4–2J cells either incubated with 10 nM PACAP(1—38) for 6 h or grown in parallel without PACAP were subjected to at least two independent runs of mRNA differential display. Hereby, 100–150 individual bands were displayed for each primer combination exhibiting a high degree of congruency between all four samples. Besides these PCR products generated from identically expressed genes, some bands seemed to be expressed differently in response to PACAP(1—38). Among these, some products lacked the poly(A) signal (see below), and others were shown not to be inducible by PACAP in confirming experiments and were therefore excluded from further analysis.

However, one primer combination produced a band present in stimulated cells but to a much lesser extent in unstimulated cells, as shown in Fig. 1a. Upon excision and reamplification with the same primer set, a product of 280 bp was obtained, as shown in Fig. 1b. The other two bands revealed sequences not derived from mRNA. DNA sequencing of this PCR product designated PRGJ revealed a 3'-UT sequence containing the polyadenylation signal AATAAAA, located 15 bp upstream from the 5' end of the poly(A) tail. This DNA sequence could be aligned by >80% to the recently isolated novel murine gene of unknown function, g1y96 (27). To verify the differential expression of PRGJ, high-stringency RT-PCR was performed using the sequence-specific primers PRG1 sense 2 and PRG1 antisense 2, which anneal to the sequenced 3'-UT part of PRG1. As shown in Fig. 1c, the original cDNA used for the differential display procedure revealed a 150-bp PCR band present in both PACAP-stimulated samples but to a much lesser extent in the unstimulated samples.

RT-PCR with the 16–32-fold degenerated primers U1, U2, and U3 that anneal to 5'-located parts of PRG1 and sequence-specific downstream primers (D1 and D2) generated four PCR products (U1-D1, U2-D1, U2-D2, and U3-D2) that were subjected to DNA sequencing in both directions. Fig. 2A shows the 1063-bp cDNA sequence constituting a coding region of 483 bp that corresponds to a 160-amino acid protein (Fig. 2B). Using the forward primer F2 and the primer PRG1 antisense 2, a 1012-bp PCR product was generated encompassing the entire cDNA sequence. Sequencing of this PCR product confirmed the sequence data obtained from the overlapping PCR products mentioned above.

To elucidate the PRG1 gene structure, AR4–2J cell genomic DNA was targeted using specific PRG1 sense and antisense primers that anneal to both ends of the PRG1 coding region (forward F2, reverse R1, and reverse R2) and a degenerated sense primer homologous to the g1y96 5' flanking region (forward R2). Hereby, 600 bp of the PRG1 5' flanking region were cloned and sequenced, as shown in Fig. 2C. In addition, the coding region of the PRG1 gene was cloned and sequenced, including a single 107-bp intron centered within (Fig. 2D).

Analysis of PRG1 Gene Expression in AR4–2J Cells. As demonstrated by Northern hybridization (Fig. 3a) or by quantitative RTPCR (Fig. 3b), PRG1 mRNA levels in AR4–2J cells were elevated in a time-dependent manner by PACAP(1–38) at a dose of 10 nm. Within 20–40 min, the mRNA level of PRG1 was increased 5–10-fold and reached a peak level after 1 h. Then, PRG1 mRNA levels decreased slowly and returned to baseline after 4–8 h. A similar time course of PRG1 induction was observed in PACAP-stimulated PC12h rat pheochromocytoma cells also expressing PV1 receptors (data not shown).

In contrast, the rat insulinoma cell line RINm5F lacking the PV1 receptor revealed no increase in PRG1 expression when stimulated with PACAP (data not shown).

Fig. 1. mRNA differential display from PACAP-stimulated or unstimulated AR4–2J cells. a, mRNA samples from AR4–2J cells that have been treated without or with 10 nM PACAP(1–38) for 6 h were subjected to the mRNA differential display procedure following the original protocol (25). Amplified 32P-labeled products from two independent experiments were subjected to denaturing DNA sequencing gels. +, PACAP addition; —, PBS addition; ++, differentially displayed band. b, after band excision, PCR fragments were reamplified with the corresponding primers, subjected to agarose gels, and stained with ethidium bromide. The 280-bp fragment is indicated ( ); the other two bands represent PCR products not derived from mRNA. c, specific primers (PRG1, sense 2 and PRG1, antisense 2) were used for PCR of the same first-strand cDNA to verify the differential expression of the identified gene; GAPDH was used to adjust equal amounts of cDNA. Lanes 1 and 2, PBS treatment; Lanes 3 and 4, 6 h treatment with PACAP(1–38); Lane 5, plasmid (TA-cloning vector) containing the 280-bp fragment.
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Fig. 2. DNA and amino acid sequence of PRGI. A, cDNA sequence of PRGI obtained from four overlapping RT-PCR products spanning the coding region and the entire 3' UTR segment. AREs are underlined. The coding sequence is highlighted by boldface. The start and stop codons and the poly(A) signal AATAAA are indicated by italics. B, protein sequence of PRGI and comparison with murine gly96; **, amino acid identity. C, sequence of the 0.6-kb fragment of the PRGI promoter obtained from PCR-targeted rat genomic DNA using primers forward F1 and reverse R1. Potential binding sites for transcription factors (NF-κB, AP-2, myc/max, SPl) are double-underlined. D, sequence of the single 107-bp intron centered within the coding region obtained from PCR-targeted rat genomic DNA using primers forward F2 and reverse R2. Numbers refer to the flanking ends of the cDNA.

Fig. 3. Stimulation of PRGI expression by PACAP in AR4-2J cells. mRNA levels of PRGI from AR4-2J cells were analyzed by means of Northern hybridization (a) using a 32P-PRGI cDNA probe (positions 1–101) or by means of RT-PCR (b) and using the primers PRGI, sense 1 and antisense 1 (c). a, incubation with 10 nM PACAP(1–38) for 0, 1, 2, 3, 4, 6, and 9 h. The PRGI mRNA is displayed as a 1.2-kb band (inset, top). To confirm equal amounts of RNA, rRNAs were shown in the lower panel of the inset. b, incubation with 10 nM PACAP(1–38) for 0, 10, 20, 30, 60, 90, and 180 min. c, the transcriptional inhibitor actinomycin D or the translational inhibitor cycloheximide were administered at 400 ng/ml and 100 ng/ml, respectively, to AR4–2J cells before incubation without or with 10 nM PACAP(1–38) for 45 min. For quantification, the autoradiography of Northern hybridization was evaluated densitometrically, and RT-PCR products were subjected to ionic exchange chromatography (SMART MiniQ PC 3.2/3; Pharmacia) and quantified by OD260 peak area integration. Calculation of PRGI mRNA levels was carried out after correction for GAPDH control in each sample. Results are expressed as arbitrary units of OD260 (absorbance)-peak area; basal value set = 1. Data shown are means (n = 4); bars, SE. Insets (a–c) show a representative Northern blot (a) and representative ethidium bromide-stained polyacrylamide gels.
inhibitor cycloheximide for 30 min increased mRNA levels of PRG1 in PACAP-stimulated and nonstimulated cells (Fig. 3c).

The stimulatory effect of PACAP(1–38) on the PRG1 transcription in AR4–2J cells was dose dependent, as shown in Fig. 4a. Maximal stimulation by PACAP(1–38) was observed at a dose of 30 nm, and the half-maximal effect occurred at 0.3 nm, compared with PACAP(1–27), PACAP(1–27), and CCK (all 10 nm) which stimulated PRG1 expression with similar efficacy (400–500% over basal). In contrast, the PACAP-related peptide VIP (100 nm) and the phorbol ester PMA (0.1 μg/ml) revealed only a weak effect (Fig. 4b).

As shown in Fig. 5, administration of the highly selective PKC inhibitor calphostin C (20 μM) reduced the effect of PACAP on PRG1 expression by more than 50%. The less-selective PKC inhibitors H7 (20 μM) and staurosporine (2 μM) almost entirely abolished the effect of PACAP(1–38). Furthermore, a strong inhibitory effect was also observed when the calmodulin inhibitor W-7 (20 μM) was administered. In contrast, the tyrosine kinase inhibitor genistein or the tyrosine phosphatase inhibitor orthovanadate revealed no inhibition of PRG1 induction by PACAP (data not shown).

To confirm promoter activity of the 5′ flanking region of the PRG1 gene, a 589-bp fragment of this region was joined to the CAT reporter gene (CAT basic vector; Promega) and AR4–2J cells were cotransfected with the CAT vector and a β-galactosidase vector. As shown in Fig. 6, administration of 10 nm PACAP(1–38) for 6 h led to a 4–6-fold increase of the CAT reporter gene expression in AR4–2J cells transfected with the CAT vector containing the 589-bp promoter fragment. A similar increase of CAT expression was observed when incubating AR4–2J cells with 10 nm CCK, whereas VIP was ineffective at 10 nm. No inducible CAT gene

**Fig. 4.** Dose-dependent stimulation of PRG1 expression by PACAP(1–38) in AR4–2J cells and comparison with other peptides or PMA. Increasing concentrations of PACAP(1–38) (a) or 10 nm PACAP(1–38), PACAP(1–27) and CCK, 100 nm VIP and 100 ng/ml PMA, respectively (b), were added to AR4–2J cells for 45 min. Quantitation of PRG1 PCR products was carried out as described above. Results are expressed as arbitrary units of OD260 (absorbance)-peak area; basal value set = 1. Data shown are means (n = 3); bars, SE. Inset, a representative ethidium bromide-stained polyacrylamide gel. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 5.** Influence of various PKC inhibitors and a calmodulin inhibitor on PACAP-stimulated PRG1 expression in AR4–2J cells. AR4–2J cells were incubated with 10 nm PACAP(1–38) in the absence or presence of the PKC inhibitors staurosporine (2 μM), calphostin C, and H-7 or the calmodulin inhibitor W-7 (all 20 μM). Quantitation of PRG1 PCR products was carried out as described above. Results are expressed as arbitrary units of OD260 (absorbance)-peak area; basal value set = 1. Data shown are means (n = 4); bars, SE. Inset, a representative ethidium bromide-stained polyacrylamide gel.
expression was observed in AR4–2J cells transfected with the CAT vector lacking the 589-bp insert (data not shown).

**PRG1 Gene Expression in FOY-305-induced Hyperplasia of the Rat Pancreas.** To demonstrate a growth-promoting role of PRG1 in vivo, hyperplasia of the rat exocrine pancreas was induced by oral treatment of rats with the protease inhibitor FOY-305 for various time periods. Pancreatic mRNA levels of PRG1 were determined by means of quantitative RT-PCR. Hereby, it was demonstrated that PRG1 mRNA levels are strongly increased 1 h after 1 h of FOY-305 administration (Fig. 7). After 6 h of FOY-305 treatment, PRG1 mRNA levels increased more than 15-fold compared with untreated controls. In pancreata from animals treated with FOY for longer time periods (12 and 24 h and 4 and 10 days), no significant increase in PRG1 mRNA levels was observed. For comparison, quantitative RT-PCR revealed that expression of c-fos mRNA occurred earlier than PRG1, exhibiting a maximum at 1 h with a rapid decline thereafter (data not shown).

Hyperplasia of pancreata was confirmed by determination of wet weight, protein, DNA, and amylase content. As shown in Table 1, pancreatic weight increased by 80% after 4 days, paralleled by a 110% increase in the protein content. The DNA content was also augmented significantly after 4 days and reached a maximum after 10 days (30% over control). Amylase content was elevated more than 3-fold after 10 days.

**DISCUSSION**

In AR4–2J cells, mRNA differential display identified an unknown PACAP-inducible gene exhibiting high structural similarity (>80% when comparing nucleotide sequences) to a recently described novel murine gene, gly96, of unknown function (27). The highest degree of sequence variation (>30%) was found mainly in the 3′-UT region. However, five A/T-rich parts (positions 686, 700, 739, 796, and 808) of the 3′-UT region are present in both PRG1 (see Fig. 2A) and gly96, respectively. These A/T-rich parts consist of heptameric or octameric AREs with the sequence motifs TATTTAT or TATTTATT and represent mRNA degradation signals (28) commonly found in the 3′-UT region of genes with a short half-life, i.e., early-response genes such as c-fos (29, 30). Thus, the presence of AREs in the 3′-UT region of PRG1 may indicate rapid degradation, a feature that is compatible with its putative role as an early-response gene.

PRG1 encodes a protein of 160 amino acids, compared with the 153-amino acid murine protein gly96. This 3′ extension is due to a U→C base shift at cDNA position 460. In PRG1 and gly96, two glycosylation sites as well as a stretch of hydrophobic amino acids between positions 86 and 101 are similarly present, the latter presumably representing a transmembrane domain. Alternatively, this hydrophobic region might favor the formation of a disulfide bridge between the two single cysteine residues at positions 2 and 96 that are conserved between gly96 and PRG1. Because sequence analysis (DNAsis software) of the N-terminal part of PRG1 did not identify a functional
signal peptide, the cysteine at position 2 might indeed provide a sulfhydryl group for disulfide bridge formation.

Besides the TATA box, the 0.6-kb promoter region of the PRG1 gene contains several important putative consensus sequences for binding of transcription factors. Similar to the gly96 gene (27), these include three potential binding sites, (G)GGA/GNTTCC (31), for the NF-κB transcription factor (32); one myc/max site (33); and one SP1 binding site (34) that overlaps with the NF-κB site proximal to the transcription initiation point. Furthermore, two sequences related to the binding site CCC/GCA/060C of the AP-2 transcription factor (35) were identified.

PRG1 represents an early-response gene in AR4–2J cells, as indicated by the rapid (15–30 min) and transient (3–6 h) elevation of the PRG1 mRNA content in response to PACAP treatment. Obviously, this is due to an increased transcription of the PRG1 gene, as shown by the inhibitory effect of the transcriptional inhibitor actinomycin D. Furthermore, PRG1 mRNA seems to be down-regulated by de novo synthesized protein, as indicated by the finding that the translational inhibitor cycloheximide strongly increased PRG1 mRNA levels. This down-regulation might be attributable to rapid degradation, as could be expected from the presence of various AREs (see above) in the 3′-UT region of the PRG1 mRNA.

The stimulatory effect of PACAP on PRG1 transcription requires the activity of PKC and calmodulin, as shown by the inhibitory actions of the PKC inhibitors H7, staurosporine, or calphostin C and the calmodulin inhibitor W7, respectively. Involvement of PKC could be anticipated by the presence of several potential NF-κB binding sites in the PRG1 promoter, because activation of NF-κB in many cell types depends substantially on the activity of certain subtypes of PKC (36). Recently, the PKC ζ isoform was shown (37, 38) to be crucial for phosphorylation and ultimately for degradation of IκB, the negative modulator of NF-κB (39). Interestingly, ζ-PKC represents a PKC isoform insensitive to treatment with phorbol esters in various cells, thereby providing a possible explanation for the weak effect of PMA on PRG1 transcription in AR4–2J cells. In contrast to PKC, tyrosine kinase activity seems to be less important for transcriptional induction of PRG1, because neither the widely efficient tyrosine kinase inhibitor genistein nor the tyrosine phosphatase inhibitor orthovanadate had any effect on PACAP-induced PRG1 expression (data not shown).

In AR4–2J cells, regulation of PRG1 transcription by PACAP depends on the PV1 receptor, because VIP and secretin had no effect on PRG1 expression (data not shown). PACAP activates the PV1 receptor with high affinity (22, 23). Furthermore, VIP activates the PV2 receptor with lower affinity. The stimulatory signal transduction by five splicing variants of the PACAP receptor (Nature (Lond.), 365: 170–175, 1993).

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A NOVEL PACAP-INDUCIBLE EARLY-RESPONSE GENE


PRG1: A Novel Early-Response Gene Transcriptionally Induced by Pituitary Adenylate Cyclase Activating Polypeptide in a Pancreatic Carcinoma Cell Line

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