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PACAP Stimulates Transcription of c-Fos and c-Jun and Activates the AP-1 Transcription Factor in Rat Pancreatic Carcinoma Cells

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Pituitary Adenylate Cyclase Activating Peptide (PACAP) strongly induces proliferation of the rat pancreatic carcinoma cell line AR4-2J via interaction with the G-protein coupled type 1 PACAP/VIP (PV1) receptor. RT-PCR analysis revealed that this mitogenic effect of PACAP is preceded by a rapid and transient increase of transcription of the protooncogene c-fos and to a lesser extent of c-jun. Transcriptional activation is abolished by a specific PACAP antagonist and by inhibitors of PKC and PKA. In parallel to c-fos/c-jun induction, PACAP rapidly activates the heterodimeric transcription factor AP-1, as shown by electrophoretic mobility shift assay. These findings demonstrate that signal transduction of a growth-stimulating G-protein-coupled receptor involves the c-fos/c-jun/AP-1 cascade, a pathway mainly linked to classical growth factor receptor tyrosine kinases.

The gut-brain peptide Pituitary Adenylate Cyclase Activating Peptide (PACAP) exists in two molecular forms of 27 and 38 amino acids (1) and is closely related to vasoactive intestinal peptide (VIP). PACAP is expressed in several neuronal and nonneuronal tissues and interacts with two classes of receptors: The type-2 PACAP/VIP (PV2) receptor (2), formerly designated VIP receptor, accepts PACAP and VIP as high affinity ligand and mediates the effects of PACAP on the exocrine pancreas (3,4) or on smooth muscle cells of the gut (5–7), mainly via cAMP dependent mechanisms. The type-1 PACAP/VIP (PV1) receptor (2,8) exclusively binds PACAP with high affinity, but not VIP or any other related peptide (9–11). This receptor subtype is expressed on neuronal cells in the CNS (10,11), on intestinal nerve fibers (7,12) pituitary cells (13,14) adrenal cells (15) and couples to the cAMP- as well as to the IP_3/Ca^{2+}-signalling pathway. Besides the actions of PACAP as secretagogue, regulator of intestinal motility and neuromodulatory peptide, ligand occupation of the PV1 receptor is also linked to a strong mitogenic effect in a number of cellular systems (16–18) including the rat pancreatic acinar cell line AR4-2J. This cell line expresses a high number of PV1 receptors (9) and proliferates in response to PACAP, as shown recently (16). The present study investigates whether the growth-stimulating effect of PACAP on AR 4-2J cells involves transcriptional regulation of c-fos and c-jun and functional activation of the AP-1 transcription factor complex.

EXPERIMENTAL CONDITIONS

Materials. Sources of materials are given in brackets: Cell culture media (Biochrom); ^{32}P-labelled nucleotides (Amersham); enzymes and other reagents for cDNA synthesis and PCR (GIBCO-BRL); Protein detection kit (BioRad); PACAP[1–38] and PACAP[6–38] (Saxon Biochemicals); H-7 and staurosporine (Biomol); Primers for PCR were custom-synthesized by Biometra.

Cell culture. AR4-21 cells were cultured with DMEM containing 10% fetal calf serum, as described (4). Prior to stimulation, cells were serum deprived for 24 h.

Cell proliferation assay. Serum starved cells were incubated with peptide or buffer for 24 h, 48 h and 72 h. Then, 0.5

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Abbreviations: PACAP, pituitary adenylate cyclase activating peptide; VIP, vasoactive intestinal peptide; EMSA, electrophoretic mobility shift assay; PV1, type-1 PACAP/VIP receptor; PV2, type-2 PACAP/VIP receptor; PKA, protein kinase A; PKC, protein kinase C.

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μCi [^{3}H]-thymidine (Amersham) was added for 4 hours. Tracer incorporation was terminated by removal of all supernatant, followed by washing twice with 1 ml 100% MeOH for 5 min. Then, cells were treated with 1 ml chilled 10% TCA for 5 min and washed twice with distilled water for 5 min. Lysis was carried out by addition of 1 ml chilled 0.3 M NaOH for 15 min. Samples were neutralized with an equal volume of 0.3 M HCl and submitted to β-counting. Experiments were performed in triplicate.

RNA isolation and cDNA synthesis. For rapid and reproducible preparation of total RNA, the QIAshredder and RNeasy kits were used following the manufacturers instructions (Qiagen). After determination of the RNA content, samples were adjusted to 0.5 μg/μl RNA and 2–3 μg of heat denaturated (75°C, 3 min) total RNA was submitted to reverse transcription (30 μl) using 200 units M-MLV reverse transcriptase/1 μg total RNA. Reverse transcription was carried out at 37°C for 60 min in the presence of 60 units RNAse inhibitor (RNAsin, Promega). cDNA synthesis was terminated by heating to 95°C for 5 min.

RT-PCR. Routinely, 6 μl of the cDNA were submitted to PCR (30 μl) using 0.75 units Taq-Polymerase and a primer concentration of 0.5 μM at 1.5 mM $MgCl_2$. Amplification of c-fos and c-jun was carried out using the following primer sets: c-fos, 5′CCT GTC AAC ACA CAG GAC TT 3′ (sense, position 260) and 5′CTG CAA GAT CCC CAA TGA CC 3′ (sense, position 762) and 5′AGG TCC ACA TCT GGC ACA GA 3′ (antisense, position 1015); c-jun, 5′GCT TCT CTA GTG CTC CGT AA 3′ (sense, position 1747), and 5′ TCT AGG AGT CGT CAG AAT CC 3′ (antisense, position 2483) 5′ CAA CCA GAC GGG AGG GAC TA 3′ (antisense, position 2174). PCR was carried out as follows: 95°C, 1 min; 52°C, 1.5 min; 72°C, 1.5 min for 16 cycles (Thermocycler 9600; Perkin Elmer). After removal of 8 μl of PCR products for PAGE analysis, a second run was performed for additional 4 cycles. This was repeated when necessary. The identity of amplified products was verified by means of nested PCR and restriction analysis. For control, an amplimer set (Clontech) amplifying the rat enzyme glyceraldehyde 3-phosphate dehydrogenase (GPDH) was used.

Electrophoretic mobility shift assay (EMSA). Stimulated or unstimulated AR4-2J cells (2×10^6 cells) were washed with PBS and homogenized in 200 μl 50 mM Tris/HCl, pH 8.0 supplemented with 10 mM CHAPS, 150 mM NaCL, 2 mM EDTA, 5 mM NaF, 10 μM $NaVaO_4$, 1 mM DTT, 20% glycerol and the protease inhibitors aprotinin, leupeptin, pepstatin (all 10 μg/ml) and 0.5 mM PMSF. Upon incubation on ice for 10 min, cell lysates were centrifugated for 5 min, 10,000 g, 4°C. After protein determination (Coomassie dye assay, BioRad), supernatants were adjusted to identical protein concentrations (2 μg/μl). 1 μl of the ^{32}P-endlabelled DNA probe CGC TTG ATG ACT CAG CCG GAA (Promega) containing the AP-1 consensus sequence was added to 5 μl of the supernatant preincubated for 10 min with 4 μl incubation buffer (50 mM Hepes/HCl, pH 7.8, 250 mM KCl, 5 mM EDTA, 25 mM spermidine, 50% glycerol), 2 μl BSA (10 mg/ml), 2 μl polydI-dC (1 mg/ml), 2 μl DTT (50 mM) and 4 μl H_2O. For control, an excess of unlabelled AP-1 sequence was added. After 20 min incubation at room temperature, samples were mixed with 1/10 volume of PAGE-loading buffer (50% glycerol, 0.1% bromphenolblue) and submitted to native PAGE (5% Acrylamide/Bisacrylamide). Gels were dried and exposed to X-ray film (Hyperfilm, Amerham).

Statistics. Data were expressed as mean ± SD. Statistical significance of thymidine incorporation data was determined using the Whitney matched pair test.

RESULTS AND DISCUSSION

When administered for 48 hours, PACAP[1–38] and PACAP[1–27] maximally stimulated the uptake of [^{3}H]-thymidine into serum starved AR4-2J cells (figure 1). The increase of thymidine incorporation upon PACAP treatment amounted to 300% compared to AR4-2J cells treated with PBS and up to 10% compared to FCS-treated cells. Both molecular forms of PACAP revealed similar potency and efficacy in terms of AR4-2J cell proliferation. In contrast, VIP had no effect on AR4-2J cell proliferation. This mitogenic effect of PACAP on AR4-2J cells is of similar or even higher magnitude compared to EGF (data not shown), gastrin or the recently discovered gly-extended gastrin (16,19). Since VIP was without effect on AR4-2J cell proliferation, it can be concluded that the growth promoting effect of PACAP is mediated via the PV1 receptor. Similar results have been published recently (16).

To investigate whether PACAP-induced cell proliferation is preceded by the activation of the heterodimeric transcription factor complex AP-1 and by transcriptional induction of the AP-1 constituents c-fos and c-jun, mRNA levels of these proto-oncogenes were analyzed by RT-PCR. As shown in figure 2, incubation of AR4-2J cells with 10 nM PACAP[1–38] strongly increased the amount of c-fos mRNA within 30 min. The peak level of c-fos mRNA persisted for 30 min followed by a rapid increase within 120 min. Upon preincubation of 200 ng/ml of the transcriptional inhibitor actinomycin D for 2 h, no increase of c-fos mRNA levels was observed (data not shown), indicating that c-fos is subject to transcriptional induction by PACAP. In contrast to c-fos,

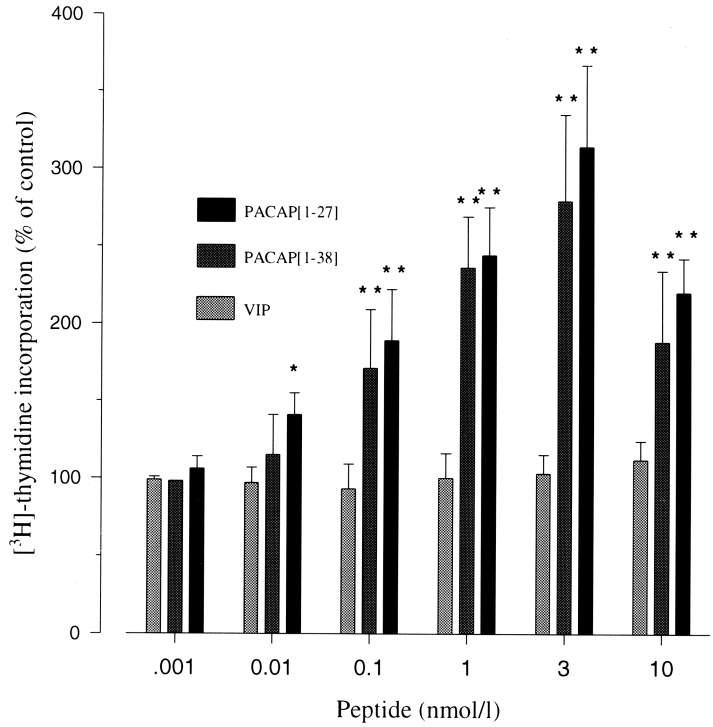

FIG. 1. Effect of PACAP[1–38], PACAP[1–27] and VIP on [^{3}H]-thymidine uptake into AR4-2J cells. Serum starved AR4-2J cells were incubated for 48 h with increasing concentrations of PACAP[1–38], PACAP[1–27] and VIP or with PBS for control. Cell proliferation was determined by means of [^{3}H]-thymidine incorporation. Results are expressed in percentage of control (= 100%). Data are shown as mean ± SD from 8 experiments performed in duplicate. Statistical significance is indicated: **, $p < 0.005$; *, $p < 0.02$.

mRNA levels of c-jun are constitutively elevated in AR4-2J cells and only moderately increased in response to PACAP stimulation. This suggests that transcriptional induction of c-fos by PACAP is one mechanism leading to increased AP1 activity whereas induced c-jun expression contributes only marginally to this process. The PACAP effect on c-fos transcription is linked to the PV1

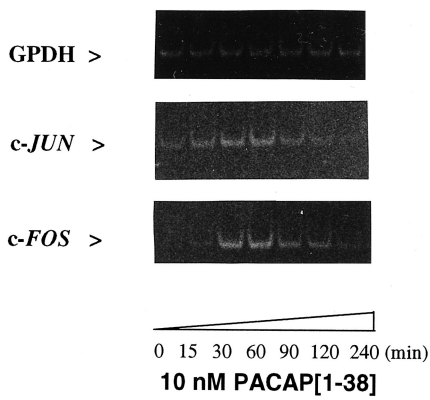

FIG. 2. Time course of the effect of PACAP[1–38] on c-*fos* and c-*jun* mRNA levels in AR4-2J cells. After AR4-2J cells were incubated with 10 nM PACAP[1–38] for the indicated time periods and total RNA was submitted to RT-PCR using appropriate primers for c-*fos* and c-*jun* and GPDH as control. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide.

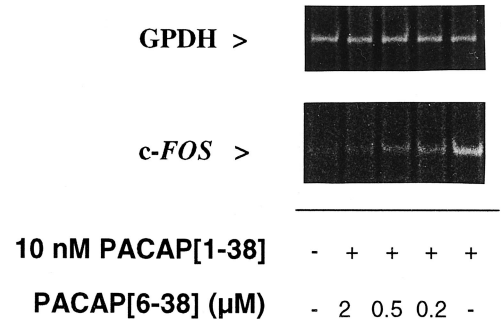

FIG. 3. The PACAP antagonist PACAP[6–38] blocks the PACAP[1–38]-stimulated c-*fos* expression in AR4-2J cells. AR4-2J cells were incubated with 10 nM PACAP[1–38] for 30 min in the presence or absence of 2 μM, 0.5 μM and 0.2 μM PACAP[6–38]. Total RNA was submitted to RT-PCR with primers for c-*fos* and GPDH. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide.

receptor as shown by the lack of effect of VIP (data not shown) and by the inhibitory effect of the PV1 receptor antagonist PACAP[6–38] which strongly and dose-dependently reduced the stimulatory effect of PACAP[1–38] on c-fos expression (figure 3).

In order to establish which signal transduction pathways downstream to the second messengers cAMP and IP_3/Ca^{2+} are involved, inhibitors of protein kinase C (PKC) and protein kinase A (PKA) were characterized for their capacity to abolish PACAP-induced c-fos expression. As shown in figure 4, the inhibitors H-7 (10 μM) and staurosporine (1 μM) abolished the effect of PACAP on c-fos expression (IC_{50} values for H7 and staurosporine were 1 μM and 0.2 μM, respectively). These data suggest that transcriptional induction of c-fos by PACAP depends on multiple effector systems that in concert trigger c-fos transcription. The inhibitory actions of the PKC inhibitor H-7 and of staurosporine which equally affects PKC and PKA probably reflect the contribution of PKA- as well as PKC-dependent effector systems, i.e. CREB phosphorylation (20) and Elk1/TCF activation (21) via the MAP kinase cascade, respectively. This pathway has been extensively investigated in terms of signal transduction of classical growth factors, but recently it became evident that G-protein coupled receptors are similarly linked to the MAP kinase cascade via ras-dependent and ras-independent mechanisms (22,23). Recruitment of MAP kinase by PACAP has been shown for PACAP in AR4-2J cells (R. Günther, unpublished results) and in PC12h cells (24).

The PACAP signal in AR4-2J cells involves activation of the AP-1 transcription factor complex. As demonstrated by electrophoretic mobility shift assay (figure 5), administration of 10 nM

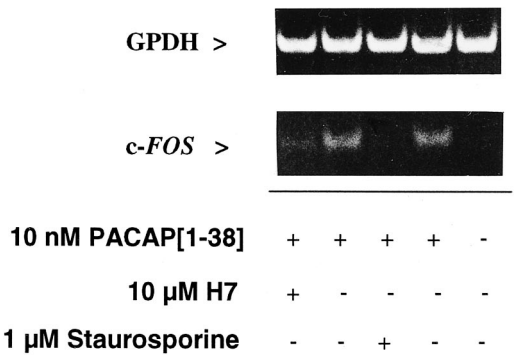

FIG. 4. Inhibition of the stimulatory effect of PACAP[1–38] on c-*fos* expression by protein kinase C inhibitors. Cells were incubated with 10 nM PACAP[1–38] in the presence or absence of the protein kinase C inhibitors H-7 (10 μM) and staurosporine (1 μM). Total RNA was submitted to RT-PCR with primers for c-*fos* and GPDH. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide.

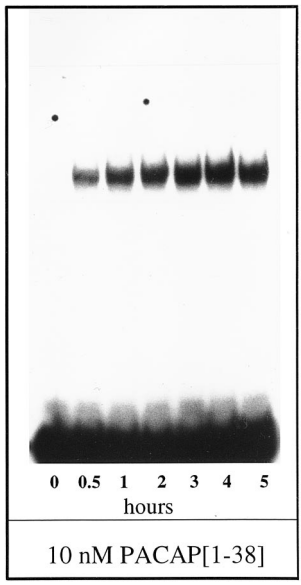

FIG. 5. Effect of PACAP on AP-1 DNA binding activity. Cell extracts from AR4-2J cells treated with 10 nM PACAP[1–38] for various time periods were submitted to EMSA using an AP-1 consensus sequence. Separation of labelled AP-1/DNA complexes was carried out by means of native PAGE. A representative result from three independent experiments is shown.

PACAP[1–38] produced a band consisting of the AP-1 protein complex bound to the labelled AP-1 consensus oligonucleotide. No band was observed in the presence of an excess of unlabelled DNA probe (not shown) indicating the specificity of the protein-DNA complex. The increased DNA binding activity of AP-1 in AR4-2J cells became evident after 30 min, was maximal at 1–2 h and persisted for several hours. These data indicate that the PACAP induced activation of AP-1 binding not only depends on the stimulation of c-fos transcription but probably involves also activation of the constitutively expressed cJUN protein. As has been shown recently, activation of AP-1 generally requires postranslational modifications of the C-terminal part of cJUN, i.e. via phosphorylation and dephosphorylation (25–27).

Our results show that ligand occupation of the G protein-coupled PV1 receptor leads to proliferation of AR4-2J cells. Furthermore, PACAP binding to the PV1 receptor involves transcriptional induction of c-fos and activation of the transcription factor AP-1. These early signalling events are recruited by PACAP to a similar extent compared to classical growth factors that activate tyrosine kinase receptors.

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