Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates adenylate cyclase and promotes proliferation of mouse primordial germ cells

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SUMMARY

During migration and for about 2 days after their arrival in the gonadal ridges, primordial germ cells (the embryonic precursors of gametes of the adult animal) proliferate actively. Certain growth factors, such as stem cell factor and leukemia inhibitory factor, seem to be essential for survival, proliferation and possibly differentiation of mouse primordial germ cell in vivo and/or in vitro. Similarly, increase in intracellular cAMP is followed by a marked enhancement of primordial germ cell proliferation, at least in culture. In the present study, we show that pituitary adenylate cyclase-activating polypeptides (PACAP-27 and PACAP-38), two neuropeptides of the secretin-glucagon-vasoactive intestinal polypeptide-GH-releasing hormone family, stimulate in vitro proliferation of mouse primordial germ cells, bind to primordial germ cells and gonadal somatic cells (possibly to type I PACAP receptor) and activate adenylate cyclase in the same cells. Moreover, PACAP-like immunoreactivity was found in gonadal ridges, mostly on germ cell surface. In conclusion, evidence is provided that PGC proliferation can be stimulated by certain bioactive polypeptides, thus suggesting a novel regulatory role for such compounds in early gonad development.

Key words: primordial germ cells, PACAP, fetal gonad, mouse, adenylate cyclase

INTRODUCTION

The two forms of pituitary adenylate cyclase-activating polypeptide (PACAP-27 and PACAP-38) were originally isolated from the rat hypothalamus (Miyata et al., 1989). On the basis of sequence similarity, PACAP were included in the secretin-glucagon-vasoactive intestinal polypeptide-GH-releasing hormone family of peptides (for a review, see Arimura, 1992a). Two types of high-affinity receptors for PACAP have been identified: type I, which specifically binds to both PACAPs, and type II, which the two peptides share with vasoactive intestinal peptide (VIP). At least five splice variants of type I receptor have been demonstrated, which include either one or both, or none of two 84-bp cassettes, designated ‘hip’ and ‘hop’ (Spengler et al., 1993). The hip cassette encodes a 29 amino acid insert, hop1 a different 28 amino acid insert and hop2 an insert identical to hop1 but lacking a single serine residue (the hip cassette can occur together with hop1 to give rise to a hip-hop insert). Type I receptor isoforms are differentially coupled to adenylate cyclase and phospholipase C (Spengler et al., 1993), while type II receptors are coupled to adenylate cyclase only (Miyata et al., 1989; Deutsh and Sun, 1992). The presence of both PACAP and their receptors in various organs and tissues (for reviews, see Arimura, 1992a,b), suggests that such peptides may have multifunctional roles, including that of a hypophysiotropic hormone, neuromodulator, vasoregulator and regulator of secretion. This view is also supported by the ability of PACAP to increase cAMP accumulation in various cell types in culture (pituitary cells, Miyata et al., 1989; PC12 cells, Deutsch and Sun, 1992; Sertoli cells, Heindel et al., 1992; monocytes and macrophages, Chedeville et al., 1993; osteblast-like cells, Suzuki et al., 1994), and by the recent finding that VIP (which share a 68% sequence omology and type II receptor with PACAP), acts as growth factor on early postimplantation embryo (Gressens et al., 1993). Interestingly, very high levels of PACAP are found in the testis (Arimura et al., 1991), while type I PACAP receptor is present in testicular germ cells, especially spermatogonia and spermatocytes (Shivers et al., 1991; Shioda et al., 1994).

Further to previous studies of ours showing that cAMP-mediated mechanisms are involved in the proliferative regulation of mouse primordial germ cells (PGCs, the precursors of sperm and eggs of the adult animal) (De Felici et al., 1993; Dolci et al., 1993), the present study was designed to investigate a possible involvement of PACAP in such mechanisms. We report here that PACAP stimulates in vitro proliferation of mouse PGCs, binds to PGCs and gonadal somatic cells (possibly to type I receptor) and activates adenylate cyclase in...
such cells. Moreover, PACAP-like immunoreactivity was present in gonadal ridges.

Besides describing for the first time that a bioactive peptide is able to stimulate PGC proliferation, our data suggest that neuropeptides and/or related compounds may have novel, intriguing roles in gonad development and physiology.

MATERIALS AND METHODS

Compounds

Ovine PACAP-27 and PACAP-38, and VIP were purchased from Peninsula Laboratories (St. Helens, Merseyside, UK) or from Calbiochem (California, USA). Ovine CRF and bovine thyroglobulin were purchased from Calbiochem and Sigma (St Louis, MO, USA), respectively. Concentrated (100x) stocks of peptides, dissolved in M2 culture medium (De Felici and Siracusa, 1982), containing 5 mg/ml BSA, were divided into aliquots and stored at -20°C. Forskolin (Sigma) was dissolved at 10 mM in ethanol, stored at -20°C and used at 20 μM. 3-isobutyl-1-methylxanthine (MIX) (Sigma) was freshly prepared in M2 at 3 mM and employed at 300 μM.

PGC isolation, purification and culture

Embryos were obtained from CD-1 mice (Charles River, Italy) at the 11th and 12th day of pregnancy (10.5-11.5 days post coitum, dpc). Monodispersed PGC-containing cell suspensions were obtained from the dorsal mesentery and urogenital ridges of 10.5 and 11.5 dpc embryos and were seeded (about 0.5-1 embryo equivalent/well) in 96-well Falcon tissue culture plates (3072), as previously described (De Felici and Dolci, 1991). PGCs with minor somatic cell contamination (purity about 70-80%) were obtained by EDTA-mechanical treatment of 11.5 dpc gonadal ridges (De Felici and McLaren, 1983) and seeded in 96-well plates containing TMCs feeder layers (De Felici and Dolci, 1991). Cultures were carried out in modified MEM, supplemented with 5% horse serum and 2.5% heat-inactivated fetal calf serum (ICN Biomedical Ltd, Oxford, UK) (De Felici and Dolci, 1991). Before seeding and after 1-3 days of culture, PGCs were identified and counted by alkaline phosphatase (APase) staining, as reported in De Felici and Dolci (1989, 1991). (RT)-PCR for PACAP receptor and intracellular cAMP dosage (see below) were performed using PGCs and somatic cells purified by MiniMACS magnetic separation system (Miltenyi Biotec GmbH, Germany); such method results in highly purified PGCs and somatic cells (purity >90%) (Pesce and De Felici, 1995).

BrdU labelling

5'-Bromo-2'-deoxyuridine (BrdU) labelling was carried out using the Amersham cell proliferation kit (Amersham Italia SRL, Milano), following the procedure reported in Dolci et al. (1991).

(RT)-PCR

Total RNA was extracted by the RNAzol(B) method (Cinna Biotec Laboratories, Houston, TX, USA) according to the manufacturer’s instructions. Reverse transcription was performed on 1 μg of total RNA using AMV reverse transcriptase (Promega Corporation, Madison, WI, USA) and oligo(dT) as a primer. cDNA was then amplified using two primers (PAC-IS1 and 453-40P) specific for the isoforms of type I PACAP receptor, giving final amplified products of 302 and 387 bp (Spengler et al., 1993). As an internal control, two primers specific for the amplification of the cDNA of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT), giving an amplified product of 570 bp, were used (Rossi et al., 1993).

Cell autoradiography

Cells obtained from 11.5 dpc gonadal ridges by EDTA-mechanical treatment and suspended in MEM + 1 ng/ml BSA (see Materials and Methods) (about 4.5x10^5 cells in 150 μl aliquots), were placed in 1 ml Eppendorf tubes containing 30 μl of ^3H-PACAP-27 (about 500,000 cts/minute, specific activity 2,200 Ci/mmol) (NEN DuPont GmbH, Dreieich, Germany) with or without 1000-fold excess of unlabelled PACAP-27. Incubation was carried out for 60 minutes at room temperature, after which time cells were washed three times by centrifugation at 800 revs/minute for 10 minutes. Cell pellets were finally resuspended in M2 and 20 μl aliquots transferred to poly-L-lysine coated glass slides. Cells were then fixed with 4% glutaraldehyde or 4% paraformaldehyde in PBS, and after APase staining (paraformaldehyde-fixed cells only) were dried in air overnight. Labelled cells were then exposed to Kodak NTB2 photographic emulsion for 30 days, developed, fixed and counterstained with hematoxylin (glutaraldehyde-fixed cells). Cells were examined at ×630 or ×1000 and, where possible, grain counts were performed on 250 consecutive cells. Background counts determined from adjacent cell-free areas varied from 0-5 grains and were subtracted from cellular counts.

RIA for cAMP

cAMP was measured by RIA as previously described (Dolci et al., 1993). Briefly, cells (approximately 1-2x10^4 cells/sample) were rinsed with cold PBS, proteins precipitated with 10% trichloroacetic acid and supernatants extracted with 5 volumes water-saturated diethylether; samples were acetylated before the assay following the procedure of Harper and Broker (1976). Values were normalized to the μg of proteins present in the sample. Protein content was measured by the method of Lowry et al. (1951) using BSA as standard.

Immunohistochemistry

Gonadal ridges from 11.5-12.5 dpc embryos (n=5), were immersion-fixed in periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) for 3-8 hours at 0-4°C. Tissues were washed overnight in PBS containing 7-10% sucrose and frozen blocks were prepared using aluminum foil moulds (Ferri et al., 1987). Cryosections (4-10 μm) were collected on poly-L-lysine-coated slides, treated with unconjugated avidin and biotin for background reduction (blocking kit, from Vector, Burlingame, CA, USA) and stained by biotin-avidin amplified immunofluorescence (Jackson, West Grove, PA, USA). Primary antibodies used were: 1:400-1:1500 rabbit anti-PACAP (kindly provided by Dr A. Arimura), which recognizes both PACAP-27 and PACAP-38, and VIP were purchased from Calbiochem (California, USA). Ovine PACAP-27 and PACAP-38, and VIP were purchased from Calbiochem and Sigma (St Louis, MO, USA), respectively. Concentrated (100x) stocks of peptides, dissolved in M2 culture medium (De Felici and Siracusa, 1982), containing 5 mg/ml BSA, were divided into aliquots and stored at -20°C. Forskolin (Sigma) was dissolved at 10 mM in ethanol, stored at -20°C and used at 20 μM. 3-isobutyl-1-methylxanthine (MIX) (Sigma) was freshly prepared in M2 at 3 mM and employed at 300 μM.

RESULTS

PACAP stimulates PGC proliferation in culture

The effect of PACAP-27 and PACAP-38 on PGC proliferation in culture is shown in Fig. 1. One day after addition of either PACAP, a significant increase in PGC numbers was
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seen, both in monodispersed cell suspensions obtained from 10.5-11.5 dpc PGC-containing tissues (Fig. 1A,B) and in purified 11.5 dpc PGCs cultured on TM4 cell feeder layers (Fig. 1C). Such effects of PACAP was concentration-dependent, with an ED50 of 15-17 nM (Fig. 2). It should be noticed here that the two culture systems used are able to sustain PGC growth for at least 2-3 days, then PGCs are rapidly depleted after the third day (De Felici and Dolci, 1991; De Felici et al., 1993). In the present study, PACAP was unable to prevent such phenomenon (data not shown).

In order to verify that PACAP acts as a mitogenic factor for germ cells, 1 day cultures of monodispersed cell suspensions from 11.5 dpc PGC-containing tissues were incubated for 2 hours with 5-bromo-2'-deoxyuridine (BrdU) and the number of PGCs that had incorporated BrdU counted. The results showed that PACAP significantly increased the number of PGCs in S phase in comparison to control (Fig. 3).

PGCs and gonadal somatic cells express PACAP type I receptor

Complementary DNA (cDNA) to the PACAP-specific (type I) receptor mRNA has been recently cloned (Hashimoto et al., 1993; Hosoya et al., 1993; Morrow et al., 1993). Besides PACAP-R, four isoforms of such receptor, designated PACAP-R-hip, PACAP-R-hop1, PACAP-R-hop2 and PACAP-R-hop-hop1, have been isolated (see Introduction). The presence of splice variants in the gonadal ridges was studied by reverse transcriptase (RT)-PCR using a pair of specific oligonucleotides located 56 bp upstream and 247 bp downstream of the site of insertion of the hip/hop cassette (see Materials and Methods). The expression of two such type I PACAP receptor variants in 10.5 and 11.5 dpc PGCs and in 11.5 dpc gonadal somatic cells is shown in Fig. 4 (lanes 6, 8, 10, 12).
Amplification produced two major bands of the expected sizes for PACAP-R form (303 bp) and for the PACAP-R hip or PACAP-R hop form (387 bp), indistinguishable with these primers. Such transcripts were absent in the amnion and liver of embryos of the same ages (Fig. 4, lanes 2, 4). The same amplification products were observed when whole ovaries and testes from 14.5-16.5 dpc fetuses were analysed (data not shown).

When 11.5 dpc PGCs and gonadal somatic cells were examined after exposure to 125I-labelled PACAP, glutaraldehyde fixation and autoradiography, we found that about 50% PGCs and most somatic cells (>90%) were labelled. Somatic cells were more heavily labelled than PGCs, although some heterogeneity of labelling was observed (Figs 5, 6). Parallel autoradiography performed on cells incubated in the presence of 1,000-fold excess unlabelled PACAP showed no significant difference in grain distribution between cells and background. Since APase activity was lost after glutaraldehyde fixation, in such experiments PGC identification was based on morphological criteria alone: PGCs are much larger and less intensely stained by hematoxylin than somatic cells (Fig. 5).

Paraformaldheyde fixation followed by APase staining greatly reduced 125I-PACAP labelling of cells (to levels slightly above background, not shown).

PACAP increases intracellular cAMP in PGCs and gonadal somatic cells

A time course of cAMP level changes in 11.5 dpc PGCs and gonadal somatic cells purified by MiniMACS, following exposure to 300 nM PACAP-38 in the presence of 300 μM MIX, is reported in Fig. 7. The results showed that the peptide caused accumulation of cAMP in PGCs and somatic cells with similar kinetics. Highest levels (about 5- and 20-fold increase in PGCs and somatic cells, respectively), were seen at the 15 minute time point after neuropeptide addition. Similar results (not shown) were obtained with PACAP-27 on such MiniMACS purified cells and when either peptides were used on cells purified by EDTA-mechanical treatment (see Materials and Methods).

PACAP-like immunoreactivity

Immunostaining revealed PACAP-like immunoreactivity in a number of cells within gonadal ridges (Fig. 8A,B). No significant staining was observed with the VIP-antiserum in the same
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region (not shown). Double staining of the same sections with TG-1 (an antibody used to identify mouse PGCs, Donovan et al., 1986; Fig. 8C) showed wide colocalization with PACAP-like immunoreactivity (Fig. 8B,C), thus indicating that most of PACAP immunoreactive cells were PGCs. Those TG-1-negative cells showing PACAP-like immunostaining (Fig. 8B, arrows), and viceversa (Fig. 8, arrowheads), might be somatic cells and PGCs, respectively, bearing low amounts of bound PACAP. Heterogeneity in the ability to bind PACAP by gonadal somatic and germ cells was indeed suggested by the autoradiographic experiments (see above).

DISCUSSION

We have previously shown that agents that increase intracellular cAMP (i.e. dibutyryl cAMP and forskolin) markedly stimulate PGC proliferation in culture (De Felici et al., 1993). We also found that a number of naturally occurring compounds that in various cell systems induce intracellular cAMP increase, such as FSH, prostaglandin E2 and F2α, ACTH, calcitonin and T3, were ineffective (De Felici et al., 1993 and our unpublished results). To obtain further insight on physiological compounds that might be able to stimulate PGC proliferation by cAMP-dependent mechanisms, we have now tested the effect of PACAP-27 and PACAP-38, two neuropeptides known to stimulate adenylate cyclase via specific cell receptors. The term neuropeptide encompasses a wide variety of biologically active peptides, including the recently discovered PACAP, whose site of synthesis or putative targets are not restricted to the nervous system. Besides neurotransmitter, neuroregulator, endocrine, paracrine and autocrine functions, most such peptides have been shown to act as potent growth

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**Fig. 7.** Time course of cAMP accumulation in 11.5 dpc PGCs (○) and gonadal somatic cells (▲) exposed to 300 nM PACAP-27 in the presence of 300 μM MIX (for details, see Materials and Methods). Results are the means of three replicates performed in one experiments and are representative of a total of two experiments. Control PGCs (△) and gonadal somatic cells (●) were exposed to MIX only.

**Fig. 8.** Immunostaining showing PACAP-like immunolabeled cells in 11.5 dpc mouse gonadal ridges (A,B). Double immunofluorescence of the same section with biotin-avidin-Cy3 for PACAP (B) and FITC-conjugated anti-mouse IgM for TG-1 (C) reveals that most PACAP-positive cells are also TG-1 immunoreactive and were thus identified as germ cells. Arrows in B indicate PACAP-like positive, TG-1-negative cells; arrowheads in C indicate TG-1-positive, PACAP-negative cells. Preabsorption of the PACAP antiserum with PACAP-27 greatly or completely inhibited immunostaining (D, similar area in a different sample). Scale bar in A = 20 μm; B-D = 10 μm.
factors and have been involved in a variety of normal and abnormal biological processes, including development and tumorigenesis (Jackson et al., 1988; Sethi and Rozengurt, 1991a,b; Silen and Gardner, 1993).

Despite the importance of primordial germ cells as precursors of gametes of the adult animal, little is known about growth factors and molecular mechanisms controlling their proliferation and differentiation (for a review, see De Felici and Pesce, 1994).

The results reported in the present paper point out that PACAP might be an important regulator of PGC proliferation and possibly of other functions of the embryonic gonad. Our data clearly demonstrate that PGCs and gonadal somatic cells express specific (type I) PACAP receptors, that such receptors are able to bind PACAP and that binding is followed by intracellular cAMP increase, possibly due to adenylate cyclase activation, and by stimulation of in vitro proliferation of mouse PGCs in a concentration-dependent manner. Moreover, immunostaining showed that PACAP or related molecules are present in mouse gonadal ridges, mostly on PGC surface, although their origin remains to be established. As far as we know this is the first direct evidence that embryonic cells, namely germ cells and gonadal somatic cells, possess neuropetide receptors and, most important, that neuropetide is present in the gonadal ridges in vivo and can stimulate PGC proliferation in vitro. Moreover, a mitogenic effect of PACAP has not been described before. Interestingly, we found that two forms of type I PACAP receptor, PACAP-R and PACAP-R hip/hop, are expressed by both PGCs and gonadal somatic cells. According to Spengler et al. (1993), both isoforms possess similar abilities to stimulate adenylate cyclase and phospholipase C. Furthermore, the hop cassette encodes a consensus motif for phosphorylation by protein kinase C, possibly contributing to a fine-tuned regulation of receptor function. These isoforms of PACAP receptor are also present in ovaries and testes of 14.5-16.5 dpc fetuses, when germ cell mitosis has ceased. This suggests that the expression of PACAP receptors is not downregulated when female germ cells enter meiosis or male germ cells undergo G1 arrest, and that PACAP might control various processes of gonad development.

The present data support our previous results showing that cAMP-dependent mechanisms are involved in the regulation of PGC proliferation (De Felici et al., 1993). Growth factors essential for PGC growth in culture, such as stem cell factor (SCF), leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), have been recently identified (for a review, see De Felici and Pesce, 1994). Studies performed in a variety of cell types have shown that SCF and bFGF exert their action through receptors with intrinsic tyrosine kinase activity, while the action of LIF is mediated by heterodimerization between a low affinity receptor (LIFR) and gp130, followed by tyrosine phosphorylation of both subunits and of various cytoplasmic proteins (for reviews, see Besmer, 1991; Jaye et al., 1992; Hirano et al., 1994). None of these factors, however, seems to be able to directly promote PGC proliferation. In fact, while nothing is known about the possible mechanisms of action of bFGF on PGCs, it is likely that SCF and LIF act as survival factors by preventing PGC apoptosis (Dolci et al., 1991; Godin et al., 1991; Pesce at al., 1993). Recent results demonstrate that LIFR is present on the surface of mouse PGCs, suggesting a direct action of LIF on PGCs (Cheng et al., 1994). In this regard, the present results indicate that PACAP is a direct activator of PGC adenylate cyclase and that PGC proliferation results from such activation. On the other hand, our data do not rule out the possibility that in the culture systems employed the action of PACAP on PGCs is indirect or modulated by compounds produced by cell monolayers and/or by gonadal somatic cells on which PGCs need to be cultured. We indeed found that PACAP increases intracellular cAMP level in TM4 cells (unpublished observations) and gonadal somatic cells (Fig. 7) and that the latter express type I PACAP receptor (Fig. 4), bind 125I-PACAP (Figs 5, 6) and possibly include PACAP-like immunopositive cells (Fig. 8).

Studies are currently being carried out to investigate possible effects of PACAP on gonadal somatic cells, to elucidate interactions between cAMP and other cAMP-independent pathways activated by the growth factors reported above, and to determine the origin of PACAP present in the gonads of the mouse embryo.

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