Suppressive action of pituitary adenylate cyclase activating polypeptide (PACAP) on proliferation of immature mouse Leydig cell line TM3 cells

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ABSTRACT

We aimed in the current study to understand the participation of PACAP in stage-specific Leydig and Sertoli cell functions. For this purpose, clonal cell lines TM3 (Leydig) and TM4 (Sertoli) cells, derived from the testis of immature BALB/c mice, were used. PACAP-specific receptors were detected in TM3 cells, but not in TM4 cells, which were characterized as PAC1 (type I) receptors. Stimulation of cAMP accumulation and testosterone secretion were observed in TM3 cells during 1 ~ 2 h treatment with PACAP38 (10^{-10} ~ 10^{-7} M) or PACAP27 (10^{-11} ~ 10^{-7} M). After around 10 h treatment with 10^{-11} ~ 10^{-7} M PACAP38 or PACAP27, proliferation of TM3 cells was suppressed in time- and dose-dependent manners, which was confirmed by real-time cell electronic sensing (RT-CES) system and phase-contrast microscopy. At 6 h after the addition of PACAP38, the percent cell population in G2/M phases increased significantly, while that in S phase showed significant decrease with little change in G0/G1 phases. The results revealed that PACAP exerts, in addition to early stimulatory effect on cAMP formation-steroidogenesis, sustained suppressive effect on cell proliferation in TM3 cells by controlling progression of the cell cycle. The suppressive action of PACAP on proliferation in TM3 cells supports the stage-specific participation of the peptide in differentiation of immature mouse Leydig cells.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide originally isolated from ovine hypothalamic tissues for its ability to stimulate adenylate cyclase (24). On the basis of structure similarity, the peptide is known to belong to the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family (24). Two biologically active forms with 38 (PACAP38) and 27 (PACAP27) amino acid residues have been identified (25). PACAP27 corresponds to the first 27 residues of PACAP38 and exhibits 68% sequence homology with VIP (25).

Highly abundant PACAP was first demonstrated in the rat brain and testis by radioimmunoassay and the total PACAP immunoreactivity (PACAP-IR) in both testes was found to be almost two times greater than that in the whole brain, which exceeded the concentrations of any other peptides found in the testis (3). Subsequent several studies have confirmed the localization of PACAP-IR (11, 15, 28, 34) and PACAP mRNA (11, 14, 16, 28) in the testis of the rat in various stage of age. On the other hand, two high affinity binding sites for PACAP have been demonstrated (9), and in the adult rat testis, PACAP27 binding sites were localized in germinal cells (29).
These findings provided a strong support for a crucial role of PACAP in testicular physiology in the rat. In fact, there have been carried out various studies analyzing PACAP functions in the testis, mostly with tissues or cells of the adult rat testis. The results showed or suggested that the peptide is implicated in spermatogenesis (11, 28, 34) and steroidogenesis (7, 26, 27) in the rat testis. However, some contradictory results are seen among them, and the contradiction is supposed to be attributable largely to species and age differences of the animals used as the sources of experimental tissues and cells. PACAP was also suggested to contribute to growth and differentiation of peritubular and interstitial cells of the immature rat testis (15).

The testis comprises several distinct cell types functioning under complex interacting networks. Although identification of the function of individual cell type is essential for understanding of the whole aspects of the testis functions, it is not easy to obtain well defined experimental systems for its purpose. We aimed in the current study to understand the participation of PACAP in stage-specific Leydig and Sertoli cell functions. For this purpose, we used established clonal cell lines TM3 (Leydig) and TM4 (Sertoli) cells derived from the testis of immature (11 ~ 13 day-old) BALB/c mice (21, 22). Real-time cell electronic sensing (RT-CES) system (33) was advantageously applied to obtain the results that revealed a unique suppressive effect of PACAP on proliferation of TM3 cells. The current result provided evidence to support the participation of PACAP in proliferation and differentiation in immature mouse Leydig cells.

MATERIALS AND METHODS

Cell culture. TM3 or TM4 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in a mixture of Ham’s F12 medium and Dulbecco’s modified Eagle medium [DMEM/F12 (1/1,v/v)] (GIBCO, Grand Island, NY, USA), supplemented with 1.2 g/L NaHCO₃, 5% horse serum (GIBCO) and 2.5% fetal calf serum (Dainippon Sumitomo Pharma, Osaka, Japan) in Falcon #3014 flask at 37°C in an atmosphere of 5% CO₂ in air at 100% humidity. On day 3 ~ 4, the culture medium was replaced with Dulbecco’s PBS(−) medium containing 0.1% trypsin and 0.02% EDTA. The medium was then removed, and the cells were kept at room temperature for 3 min and suspended in fresh culture medium. The cell suspension was centrifuged (800 rpm, 5 min, 20°C), and the precipitated cells were resuspended in fresh culture medium. All the following experiments were carried out in the same culture medium.

PACAP binding assay. TM3 or TM4 cells (0.25 × 10⁶ ~ 3 × 10⁶) suspended in culture medium (0.5 mL) were incubated for 2 h at 15°C with [¹²⁵I]-PACAP27 (Bachem Peninsula Laboratories, San Carlos, CA, USA) (~ 10,000 cpm) and a given concentration of PACAP38, PACAP27 or VIP (Peptide Institute, Osaka, Japan). The suspension was centrifuged (13,000 rpm, 15 min, 4°C). Radioactivity of the precipitate was measured with a gamma-counter (Wallac 1470 WIZARD; Wallac Oy, P.O.Turku, Finland). Nonspecific binding of [¹²⁵I]-PACAP27 was determined by counting radioactivity of cells to which an excess amount of PACAP27 (2 × 10⁻⁶ M) was added. Kᵦ and Bₘₐₓ were calculated according to Scatchard plot analysis (5, 18). All the assays were performed in duplicate.

Affinity cross-linking. TM3 cells were homogenized in 20 volumes of 0.32 M sucrose with a Teflon-glass homogenizer and the homogenate was centrifuged (20,000 × g, 25 min, 4°C). Binding and cross-linking of [¹²⁵I]-PACAP27 to the membranous fraction was carried out according to the method of Buscail et al. (5) with a minor modification. Briefly, the membranes were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing [¹²⁵I]-PACAP27 (~ 300,000 cpm), 2 mM MgCl₂, 1% ovalbumin, 100 kIU/mL aprotinin and 0.5 ng/mL bacitracin and incubated for 80 min at 15°C with or without 10⁻⁶ M PACAP27. The membrane suspension was centrifuged (20,000 × g, 25 min, 4°C) and the pellet was washed twice with ice-cold PBS(−) and reconstituted with an 100-fold volume of 100 mM disuccinimidyl suberate, prepared immediately before use, which was incubated for 30 min at 4°C. The [¹²⁵I]-PACAP27 affinity-labeled membranes were then washed twice with ice-cold PBS(−) and kept for 60 min at 50°C in an equal volume of electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, containing 5% SDS, 20% glycerol and 0.005% bromophenol blue). After centrifugation (105,000 × g, 60 min, 4°C), the supernatant was subjected to SDS-PAGE with a 2–15% linear gradient polyacrylamide separating gel at 4°C. The gel was then dried and analyzed by an autoradiography with Cronex film (Du Pont, Wilmington, DE, USA) exposed for 7 days at −80°C.

Gene expression. Total RNA in TM3 or TM4 cells at 24 h incubation was extracted with a RNeasy
Mini Kit (QIAGEN, Hilden, Germany) and its template cDNA was prepared by a Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Quantitative RT-PCR with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Forster, CA, USA) was carried out using the template cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems) and Assays-on-Demand Gene Expression Products [FAM Probe, Mtrp-pending, PAC1 (Adcyap1r1) Assay ID: Mm00431680, PACAP (Adcyap1) Assay ID: Mm00437433] (Applied Biosystems) comparing with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (VIC Probe, Rodent GAPDH Control Reagents; Applied Biosystems) gene expression according to the manufacturer’s instructions. The TaqMan PCR was performed in a 96 well optical tray (Applied Biosystems). Total RNA extraction from mouse whole brain (BALB/c, 6 weeks, male) was used as positive control, and its cDNA was synthesized in the same manner as mentioned above.

**HPLC analysis.** TM3 or TM4 cells collected at 48 h incubation were suspended in homogenate buffer (2), sonicated (20 Hz, 15 s × 4) and centrifuged (10,000 × g, 15 min, 4°C). The supernatant and the incubation medium were lyophilized, respectively, and stored at −80°C until use. HPLC was carried out on a reverse phase RESOURCE RPC column (1 mL) (Pharmacia Biotech, Uppsala, Sweden) at a flow rate of 1 mL/min with eluent of 10% CH3CN/0.1% trifluoroacetic acid (TFA) (0 ~ 5 min) and 10 ~ 50% CH3CN/0.1% TFA (5 ~ 45 min). The eluates were collected every minute. Radiommoassays for PACAP27 and PACAP38 were carried out using rabbit anti-PACAP38 (1–15) serum (Yanaihara Institute, Shizuoka, Japan) and [33P]-PACAP27 according to the method previously described (6).

**Measurement of cAMP.** TM3 or TM4 cells (1 × 105) were incubated in culture medium in Falcon #3047 multiwell plate for 24 h at 37°C in an atmosphere of 5% CO2 in air at 100% humidity. The medium was then replaced with fresh medium containing 10−3 M 3-isobutyl-1-methylxanthine (IBMX) (0.5 mL) and the cells were incubated for 30 min at 37°C. Culture medium containing 10−13 M ~ 10−6 M PACAP (0.5 mL, each) was added to the flask and the cells were incubated for a designed period at 37°C. The medium was aspirated and the cells were suspended in 65% ice-cold ethanol containing 10−3 M IBMX, and then centrifuged (3,000 rpm, 15 min, 4°C). The supernatant was transferred to fresh tubes and dried with a vacuum oven at 60°C. Content of cAMP in the cell extract was assayed using a cAMP enzyme immunoassay system (GE Healthcare Bioscience, Buckinghamshire, England). All the assays were performed in duplicate.

**Measurement of testosterone.** TM3 cells (3 × 105) were grown to 80% confluence in a Falcon #3004 dish for 5 days. The medium was replaced with fresh culture medium containing 10−13 M ~ 10−7 M PACAP 27 (1.2 mL each), and the cells were incubated for 0.5 ~ 4 h at 37°C in an atmosphere of 5% CO2 in air at 100% humidity. After incubation, the medium (1 mL) was transferred to a fresh tube, which was shaken with diethylether (10 mL) for 15 min. The organic layer was evaporated in a vacuum oven at 30°C. Content of testosterone in the extract was assayed using a testosterone enzyme immunoassay system (Cayman, Ann Arbor, MI, USA). All the assays were performed in duplicate.

**RT-CES system.** A suspension of TM3 cells (10,000 cells/0.1 mL) was added to the wells of E-plate of RT-CES system (ACEA Bioscience, San Diego, CA, USA) (33), and the cells were cultured for 24 h at 37°C, when PACAP38 was added. Changes in cell status were monitored and quantified by detecting sensor electrical impedance every 30 min during 72 h. Cell index (CI) was derived to represent cell status based on the measured electrical impedance (33). A “normalized cell index” is calculated by dividing the cell index at a given time point by the cell index at 0 h point.

**Phase-contrast microscopic analysis.** TM3 cells (3 × 105) were cultured in Falcon #3004 dish for 24 h, when culture medium containing PACAP38 (final concentration 10−9 M or 10−7 M) was added and the cells were incubated at 37°C while being monitored by a culture cell viewing microscope system (SRM-100; Nikon, Tokyo, Japan) and photographed with a phase contrast microscope installed CCD camera.

**Flow cytometric analysis.** TM3 cells (4 × 105) were cultured for 24 h, when the culture medium was replaced with fresh medium containing PACAP38. After incubation for a given period, the cells were separated and resuspended in PBS(−). The suspension was then centrifuged (1,000 rpm, 5 min, 20°C). The cell pellet was resuspended in PBS(−) containing 0.1% Triton X-100, 1% RNase and 50 μg/mL...
propidium iodide and incubated for 30 min at room temperature. The stained cells were measured for cell cycle kinetics analysis by a FACS Calibur flow cytometry (Becton-Dickinson, San Jose, CA, USA) and cell cycle analysis was done using a Mod Fit software (Becton-Dickinson).

**Statistics.** Statistical analysis performed using Student’s *t*-test. All data were expressed as mean ± S.E. A probability of *P* < 0.05 was considered significant.

**RESULTS**

**Specific binding of [125]PACAP27 to TM3 and TM4 cells**

[125]PACAP27 binding to TM3 cells (0.5 × 10⁶ ~ 3 × 10⁶) was in a cell number-dependent manner (Fig. 1A). While TM4 cells showed no binding ability to the labeled peptide under the same condition (Fig. 1B). Expression of PAC1 mRNA was detected only in TM3 cells at nearly same level as that in mouse whole brain (Fig. 2). [125]PACAP27 binding to TM3 cells was inhibited by both PACAP38 and PACAP27 in parallel manners, more efficiently by PACAP38 than PACAP27, with IC₅₀ values of 3.95 × 10⁻⁹ M and 1.75 × 10⁻⁸ M, respectively. VIP at concentrations up to 10⁻⁶ M did not inhibit [125]PACAP27 binding to TM3 cells (Fig. 3). Scatchard plot analysis of the binding revealed the existence of only one class of the binding sites in TM3 cells under the condition used (Fig. 4). Kᵰ and Bₘᵦ values of the labeled peptide binding to TM3 cells were estimated to be 3.85 × 10⁻⁸ M and 2.63 × 10⁻¹² mol/cell, respectively. PACAP38 binding affinity to TM3 cells was approximately 4.4 times as high as that of PACAP27.

The cross-linking of [125]PACAP27 to its binding protein in the solubilized membranes of TM3 cells followed by SDS-PAGE revealed a band having a molecular mass of 62 kDa. The protein band was not detected on SDS-PAGE in the presence of 10⁻⁶ M PACAP27.

**PACAP mRNA and PACAP in TM3 and TM4 cells**
PACAP mRNA was detected neither in TM3 nor in TM4 cells. HPLC analysis of culture medium of
TM3 cells did not identify any components related to PACAP.

**Effect of PACAP on cAMP production in TM3 cells**
With $10^{-7}$ M PACAP27, cAMP concentration in TM3 cells reached the highest peak after 1 h, which then declined (Fig. 5A). The dose-response curves of PACAP27 and PACAP38 for cAMP accumulation in TM3 cells were almost superimposable and the efficacies of the two peptides were at the same levels (Fig. 5B). VIP was about 200 times less potent than the two PACAP peptides in cAMP accumulation activity in TM3 cells. Control: 70.2 pmol/well. PACAP38: 191.5 pmol/well at $10^{-10}$ M, 442.8 pmol/well at $10^{-9}$ M, 547.2 pmol/well at $10^{-8}$ M, 455.7 pmol/well at $10^{-7}$ M and 498.1 pmol/well at $10^{-6}$ M. PACAP27: 101.7 pmol/well at $10^{-11}$ M, 218.4 pmol/well at $10^{-10}$ M, 468.8 pmol/well at $10^{-9}$ M, 551.7 pmol/well at $10^{-8}$ M, 447.3 pmol/well at $10^{-7}$ M and 459.4 pmol/well at $10^{-6}$ M.

**Effect of PACAP on testosterone secretion in TM3 cells**
Both PACAP27 and PACAP38 at $10^{-9}$ M significantly stimulated secretion of testosterone in culture medium at 1 h and 2 h during 4 h incubation (Fig. 6A).
RT-CES system analysis

CI values of TM3 cells as determined on the RT-CES system were linearly correlated with cell numbers in the range between 5,000 and 80,000 cells ($r^2 = 0.996$) (Fig. 7). Fig. 8A shows TM3 cell proliferation curves up to 72 h treatment with PACAP38 at different doses of PACAP38, which clearly showed suppression of proliferation of the cell. PACAP38 at $10^{-10}$ ~ $10^{-7}$ M suppressed cell proliferation of TM3 cells in a dose-dependent manner by 24, 36, 48 and 72 h treatment with PACAP (Fig. 9). CI (percents of control) at 24 h: 77.6% at $10^{-9}$ M, 54.7% at $10^{-8}$ M and 51.9% at $10^{-7}$ M. CI (percents of control) at 48 h: 91.9% at $10^{-10}$ M, 80.8% at $10^{-9}$ M, 45.9% at $10^{-8}$ M and 39.4% at $10^{-7}$ M. PACAP27 also suppressed cell proliferation in the same manner. On the other hand, during early period of treatment of TM3 cells with PACAP38 or PACAP27 at $10^{-11}$ ~ $10^{-7}$ M, normalized CI increased transitorily, reaching maximum at 4 h (Fig. 8B). Normalized CI at 4 h (control 1.2): 1.9 at $10^{-9}$ M and 1.7 at $10^{-7}$ M.

Phase-contrast microscopic analysis

After 1 h treatment with $10^{-9}$ M or $10^{-7}$ M PACAP38, TM3 cells showed morphological changes, such as spreading and formation of cell-clusters. The morphological changes reached maximum after 3 ~ 5 h (Fig. 10A). Treatment with $10^{-9}$ M and $10^{-7}$ M PACAP38 for 24 h and 48 h caused remarkable suppression of cell proliferation as compared with controls (Fig. 10B). There were not observed by PACAP treatment cell shrinkage, nuclear or and cytoplasmic condensation, irregularity in shape and detaching from culture dish as seen in the case of cytotoxicity or apoptosis.

Fig. 8 Dynamic monitoring of TM3 cell proliferation on RT-CES system. (A) Time response curves of CI in TM3 cells treated with different concentrations of PACAP38 during 72 h. (B) Time response curves of normalized CI in TM3 cells treated with different concentrations of PACAP38 during 16 h. The data are expressed as mean ± SE (n = 4).

Fig. 9 Dose-response curves of CI in TM3 cells at different time of treatment with $10^{-11}$ - $10^{-7}$ M PACAP38. The data are expressed as mean ± SE (n = 4). **$P < 0.01$, *$P < 0.05$
Flow cytometric analysis

Fig. 11 shows time-dependent changes of the cell cycle kinetics of TM3 cells in G₀/G₁ phases (A), S phase (B) and G₂/M phases (C) in the presence of 10⁻⁹ M or 10⁻⁷ M PACAP38. The peptide increased percent population of the cells in G₂/M phases sig-
nificantly at 3 h. Cells in G₂/M phases at 3 h (control 8.7%): 13.0% at 10⁻⁷ M and 12.2% at 10⁻⁹ M. Cells in G₂/M phases at 6 h (control 9.0%): 14.4% at 10⁻⁷ M and 14.5% at 10⁻⁹ M. Cells in G₂/M phases at 24 h (control 8.9%): 12.5% at 10⁻⁷ M. On the other hand, significant (P < 0.01) reduction in percent cell population in S phase was observed at 6 h. Cells in S phase at 6 h (control 18.2%): 13.3% at 10⁻⁷ M and 12.6% at 10⁻⁹ M. Cells in S phase at 24 h (control 30.2%): 26.0% at 10⁻⁷ M and 27.8% at 10⁻⁹ M. The percent population of TM3 cells in G₀/G₁ phases showed little change at 3 h and 24 h treatment with PACAP38.

DISCUSSION

There has been demonstrated the existence of the two high affinity binding sites for PACAP, one with a greater affinity for PACAP27 than for VIP, i.e. PAC1 (type I) receptors (50 ~ 68 kDa) (6, 23, 29, 30) and the other with similar affinities for PACAP27 and VIP, i.e. VPAC (type II) receptors (55 ~ 71 kDa) (9, 10, 29, 30, 31). The two PACAP binding sites distributed in different manners in the rat tissues (29). Autoradiographic evidence demonstrated the presence of type I PACAP27 binding sites on germinal cells in the rat testis (29), while other data showing that PACAP acts through VPAC receptors in the rat testis were also presented (17). Heinl et al. mentioned the existence of PACAP receptors on cultured rat (age 14 ~ 60 day-old) Sertoli cells (12). We clearly showed the presence of PAC1 PACAP receptors on TM3 cells and not on TM4 cells, supporting possible role of PACAP on Leydig cells, but not on Sertoli cells, of the mouse specifically in immature stage. On the other hand, PACAP mRNA expression and PACAP production were under detectable levels in both TM3 and TM4 cells. Banks et al. have claimed that circulating PACAP is available to the components of the basal compartment of the testis, including Leydig cells (4). The current results demonstrate the existence of PACAP specific receptors and the undetectable concentration of PACAP in TM3 cells supported the paracrine or/and endocrine pathways for the actions of PACAP on Leydig cells of the mouse in immature stage.

An increase in cAMP levels in Leydig cells is known to be associated with an increase in steroidogenesis (7, 26). In the current study, both PACAP38 (10⁻¹⁰ M ~ 10⁻⁷ M) and PACAP27 (10⁻¹¹ M ~ 10⁻⁷ M) stimulated basal testosterone secretion in almost identical manners in TM3 cells. The stimulation was preceded by the increase of cAMP levels in the cells. Dibutryl cAMP mimicked the stimulation of testosterone secretion. According to previous studies by others, the stimulatory effect of PACAP on steroidogenesis was seen in dispersed rat fetal Leydig cells (7), but not in Leydig cells of adult rat (8, 26). On the other hands, a conflicting result has been presented showing the effect of PACAP on testosterone production by adult rat Leydig cells, though not through cAMP activation pathway (27). The current result obtained with the clonal cell line of immature mouse testis origin provided an additional evidence to support the stage-specific stimulatory effect of PACAP on cAMP formation and steroidogenesis in Leydig cells of immature mouse. Predictably, TM4 Sertoli cells did not responded to either of the PACAP forms.

RT-CES system is a device recently developed, which made it possible to monitor real-time morphological changes in living cells (1, 33, 35). Namely, using the device, changes in cell status such as cell number, viability, morphology and adherence can be monitored and quantified by detecting sensor electrical impedance. In fact, the system had been proved to be utilized to measure minute changes in cellular morphology as a result of ligand-dependent G protein-coupled receptors activation (35). In the current study, we took advantage of the system to monitor continuously the effect of PACAP on TM3 cells during 72 h after the addition of 10⁻¹¹ M ~ 10⁻⁷ M PACAP38 or PACAP27. The results indicated an early dose-dependent peaks of CI increase, reaching maximum at 4 h. The two forms of PACAP showed almost identical effects. During the period, the activation of cAMP production and steroidogenesis were simultaneously observed in TM3 cells. Mather et al. have confirmed that exogenous testosterone has no effect on TM3 cell growth (22). The doubling time of TM3 cells has been reported to be 14 ~ 15 h (21). Such an increase of CI during the early period of PACAP treatment is less, if any, attributable only to increase of the number of TM3 cells. Actually, apparent morphological changes, mainly cell spreading, were observed by phase-contrast microscopic analysis, in parallel with the increase of CI. Accordingly, it is conceivable that the significant increase of CI seen during the initial 4 h of PACAP treatment mainly reflected the morphological changes in TM3 cells as a result of G protein-coupled receptor activation as Yu et al. has proved (35). After 10 h treatment with PACAP, the increase of CI was significantly suppressed time- and dose-dependently as compared with control. The number of cells was obviously reduced by incu-
bation with either $10^{-11}$ M $\sim 10^{-7}$ M PACAP38 or PACAP27. Phase-contrast microscopic photographs demonstrated obviously suppression of proliferation of TM3 cells and confirmed that the suppression of CI was due to neither cytotoxicity nor apoptosis.

At 6 h after the addition of $10^{-9}$ M or $10^{-7}$ M PACAP38, the percent cell population in $G_2/M$ phases increased significantly, while that in S phase showed a significant decrease with little change in the percent cell population in $G_0/G_1$ phases, indicating the effect of PACAP to arrest TM3 cells from the cell cycle. Significant increase in TM3 cell population in $G_2/M$ phases was already observed at 3 h treatment with PACAP. Such patterns of changes in the cell cycle kinetics was also seen at 24 h.

The effect of PACAP on cell proliferation of neuroblastoma cells has been reported. Leliévré et al. (19) demonstrated the differential effects of PACAP on stimulation and suppression of cultured neuroblastoma cell proliferation. The PACAP action was biphasic, with stimulation at subnanomolar doses ($10^{-15}$ M $\sim 10^{-12}$ M) and inhibition at higher doses ($10^{-10}$ M $\sim 10^{-8}$ M). Waschek et al. also described differential PACAP action, stimulatory and inhibitory, on neuroblastoma cell proliferation depending on the peptide concentration used (32). The differential actions of PACAP were suggested to be mediated by multiple subsets of receptors which differentially couple to MAP kinase and PKA signaling pathway (19). Such dose-dependent differential actions of PACAP were not observed in the current study with TM3 cells.

Using clonal cell line TM3 cells, it was revealed that PACAP exerts, in addition to early stimulatory effects on cAMP production-steroidogenesis, sustained suppressive effect on cell proliferation by controlling progression of the cell cycle in immature mouse Leydig cells. TM3 cell cycle arrest was observed already during the activation period of cAMP production and steroidogenesis by PACAP treatment. Important roles of cAMP pathway in differentiation have been suggested in neuronal cells (13, 20). The existence of a sequential functional relation between the PACAP-induced activation of cAMP production and steroidogenesis and subsequent sustained proliferation suppression in the current stage-specific Leydig cells remains to be clarified. Lu et al. has claimed that PACAP inhibits mitosis in cultured cortical precursor cells and enhances neuronal differentiation, thus serving as a signal triggering the transition from proliferation to differentiation (20). The current results obtained with TM3 cells support the stage-specific participation of PACAP in differentiation of immature mouse Leydig cells.

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