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Pituitary Adenylate Cyclase Activating Polypeptide-Mediated Intracrine Signaling in the Testicular Germ Cells

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Pituitary adenylate cyclase activating polypeptide (PACAP) is found not only in the brain, but is also abundantly expressed in the testicular germ cells. However, the physiological role of testicular PACAP remains unknown. Autoradiographic studies showed a considerable number of PACAP-specific binding sites in the seminiferous tubules. Immunohistochemistry demonstrated PAC_1-receptor (R)-like immunoreactivity (li) in the cytoplasm of round spermatids, aggregated in the acrosome and coexpressed with PACAP-li. Spermatid-enriched fractions were examined for the subcellular localization of PACAP binding sites and PAC_1-R-li. The highest levels of PACAP binding sites and PAC_1-R-li were found in the cytosolic, followed by the nuclear, and the lowest levels in the membrane fraction. The testicular cytosolic PAC_1-R-like protein showed a specific competitive inhibition in the radioreceptor assay for PACAP38 and 27, with a K_i of 0.069 n*M* and 0.179 n*M*, respectively. The addition of PACAP to the cytosol of spermatids only slightly activated adenylate cyclase, while it markedly stimulated the expression and activation of ERK-type mitogen-activated protein kinase (MAPK). In the PAC_1-R-like protein-depleted cytosol, a PAC_1-R-specific agonist, maxadilan, did not activate MAPK, but PACAP and VIP still did. Because $VPAC_2$-R, which binds both PACAP and VIP, is expressed in the testis, the findings suggest that cytosolic $VPAC_2$-R-like proteins are also present and coupled to MAPK. The MAPK activation does not seem to require a heterotrimeric G-protein. Because PACAP and its receptors are coexpressed in the cytoplasm of spermatids, endogenous PACAP may directly interact with the cytosolic PAC_1-R-like protein without the ligand being released into the extracellular space. This possibility is supported by the observation that cytosolic endogenous PACAP in spermatids was co-immunoprecipitated with the cytosolic PAC_1-R. This mechanism may be called "intracrine," and its physiological significance is discussed.

Key Words: Immunohistochemistry; testicular germ cells; mitogen-activated protein kinase; adenylate cyclase; soluble adenylate cyclase; pituitary adenylate cyclase activating polypeptide; vasoactive intestinal peptide; cytosolic receptors; intracrine; prohormone convertase 4 knock-out mouse; growth hormone releasing hormone; secretin.

Introduction

A 38-residue neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP38), was isolated from the ovine hypothalamus based on its ability to stimulate adenylate cyclase in pituitary cell cultures *(1)*. Subsequently, a truncated form with 27 residues, PACAP27, was isolated *(2)*. A radioimmunoassay (RIA) for PACAP38 and PACAP27 demonstrated that PACAP is not only expressed in the hypothalamus, but also in the extra-hypothalamic regions of the brain as well as peripheral organs, including the gut, adrenals, and testis *(3)*. In all tissues, PACAP38 is the major form of PACAP. The rat testis contains a high concentration of PACAP38; its total amount being comparable to that in the brain *(3)*.

Immunohistochemistry (IHC) and *in situ* hybridization histochemistry showed that PACAP and its mRNA are mainly expressed in round and elongated spermatids *(4,5)*. PACAP-like immunoreactivity (-li) was especially strong in the acrosome *(5)*. All other reports have also indicated that testicular PACAP is expressed in the germ cells in a stage-specific manner during spermatogenesis *(6–8)*.

The pituitary membrane fractions have saturable binding sites that are specific for PACAP as demonstrated with a conventional radioreceptor assay (RRA), whereas those from the lung and the gut contain binding sites for PACAP, which are shared with VIP *(9)*. The PACAP binding sites shared with VIP were found to be identical to the VIP-1 and VIP-2 receptors, now called the $VPAC_1$ and $VPAC_2$ receptors, respectively *(10)*. The specific receptor for PACAP,

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PAC_1-R, was cloned by several laboratories *(10)*. PAC_1-R is a typical G-protein-coupled receptor with seven transmembrane domains, and has at least 10 splice variants. Autoradiography with [^{125}I]PACAP27 showed abundant binding sites in the testicular seminiferous tubules that are specific for PACAP, but the binding sites were only demonstrated in some, but not all, seminiferous tubules, suggesting their expression at certain stages of spermatogenesis *(11)*. However, the physiological significance of testicular PACAP and its receptors remains unknown.

On the other hand, in the past we failed to demonstrate saturable specific binding sites for PACAP in rat testicular membranes using a RRA, although the same method showed a clear-cut competitive inhibition curve for PACAP in the membranes from the pituitary, brain, and other tissues *(9)*. In the testicular membrane preparation, the total binding of the radiolabeled ligand was considerably lower than in other tissues, and only a small number of the binding sites were displaced by an excess of the unlabeled ligand. This could be due to occupation of the binding sites with endogenous PACAP, which is abundant in the testis, but no further binding studies with the testicular membrane preparation were conducted for many years. More recently, we generated a rabbit antiserum against PAC_1-R *(12)* and used the antiserum to examine the localization of PAC_1-R in the rat testis and other tissues with IHC. PAC_1-R-li was demonstrated mainly in the round spermatids, with especially strong staining in the acrosome similar to PACAP-li. PAC_1-R-li was absent in spermatocytes or mature spermatozoa *(13)*, indicating that like PACAP, PAC_1-R is also expressed in the germ cells in a stage-specific manner during spermatogenesis. Thus, both PACAP and PAC_1-R in the testis appear to be expressed at the same intracellular site of the germ cells at the same stages of spermatogenesis. These findings have cast a doubt on our preconception that PACAP receptors in the testis are associated with the plasma membrane, and prompted us to systematically examine the subcellular fractions of the testis for the localization of PACAP receptors.

The antiserum generated against PAC_1-R *(12)* enabled us to recognize and isolate a PAC_1-R-like protein in the soluble and solubilized fractions of the rat testes. Here we report that in contrast to the peptide receptors in somatic cells, most, but not all, of PACAP binding sites in the testis were found in the cytosolic fraction of spermatids, but not the membrane fraction. Furthermore, in contrast to the plasma membrane-associated PAC_1-R, which is coupled to adenylate cyclase or phospholipase C, the cytosolic PAC_1-R-like receptors in the testis are primarily coupled to mitogen-activated protein kinase (MAPK). The study further suggests that $VPAC_2$-R in the testis is also present in the cytosolic fraction and coupled to MAPK. From these findings together with the IHC demonstration of coexpression of PACAP and its receptors in the cytoplasm of spermatids, we propose a unique mode of action for PACAP and its receptors in the testicular germ cells that rather resembles the interaction of an intracellular regulatory molecule with its receptors. Finally, the physiological significance of the testicular PACAP and the soluble cytosolic PAC_1-R-like receptors is discussed.

Results

PACAP-li and PAC_1-R-li in Rat Spermatids

Using a rabbit PACAP antibody and a goat PAC_1-R antibody prepared as described previously *(12)*, the localizations of PACAP and PAC_1-R in the rat testicular cells were examined immunohistochemically with a double immunofluorescence labeling method using a confocal microscope. As shown in Fig. 1 A–C, most of the PACAP- and PAC_1-R-li were co-localized in the cytoplasm of spermatids, particularly in the acrosome where they were coexpressed. The results are compatible with our previous findings *(4,5,13)* and those by others *(7)*. The finding suggests that testicular PACAP and its receptors are coexpressed at the same intracellular site of spermatids at the same stage of spermatogenesis. No ring-like distribution of PAC_1-R-li was demonstrated with IHC using a confocal microscope.

Inhibition of [^{125}I]PACAP27 Binding by PACAP or VIP in Membrane Preparations of Rat Brain and Testis Using A Conventional RRA with a Rapid Filtration Method: Effect of Prewashing

We previously failed to demonstrate saturable specific binding sites for PACAP or a clear-cut competitive inhibition curve for the PACAP-specific binding sites in rat testicular membrane preparations with a conventional RRA, because of low specific binding with a high background. However, the same method worked well with other tissues, such as the pituitary and the brain membrane preparations. This could be partly due to occupation of the binding sites in the testicular membrane preparation by endogenous PACAP, because the testis contains abundant PACAP. To examine this possibility, a traditional RRA for PACAP with the rat testicular and brain membrane preparations were conducted in parallel using the same amount of protein and 200 p*M* freshly labeled [^{125}I]PACAP27 *(14)*. Some of the membrane preparations were prewashed three times with a large volume of RRA buffer to dissociate the endogenously bound ligand so that the binding sites would be uncovered *(15)*.

As shown in Fig. 2A, the brain membrane preparations bound a 20-fold greater amount of [^{125}I]PACAP27 than did the testicular membrane preparations, and about 75% of the total binding was displaced by an excess (1 μ*M*) unlabeled PACAP38 or PACAP27, but only a small percentage was displaced by VIP. This suggests that the majority of the binding sites are specific for PACAP, or PAC_1-R type. On the other hand, only a small portion of the total binding sites were displaced by an excess amount of cold PACAP38 or PACAP27 in the testicular membrane preparations. Prewashing slightly reduced total and specific binding for PACAP

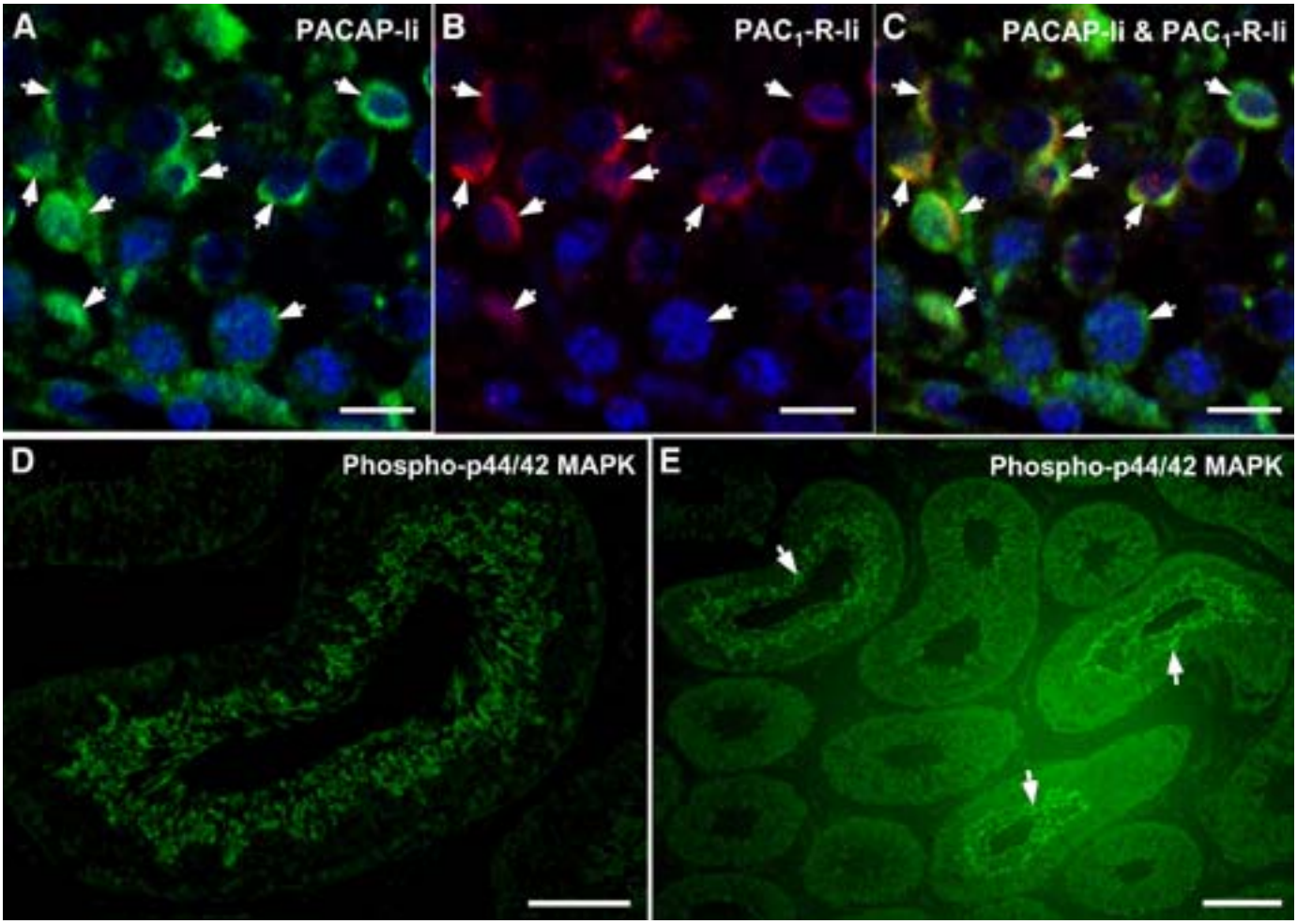

Fig. 1. (A, B, C) PACAP- and PAC_1-R-li in rat spermatids detected with a triple-labeling immunofluorescence method using a confocal microscope. PACAP-li (green) (**A**) is shown with a 488 nm laser beam and PAC_1-R-li (red) (**B**) with a 543 nm laser beam. The nucleus is stained by DAPI (blue), which is shown with a 405 nm laser beam. Panel **C** is the merged image, which shows co-expression of PACAP and PAC_1-R in the spermatids. Most of the PACAP- and PAC_1-R-li are co-localized in the cytoplasm of spermatids, particularly aggregated in the acrosome. (**D, E**) Immunofluorescence IHC of phospho44/42 MAP kinase (Thr^{202}/Tyr^{204}) in the rat testis. Phosphorylated MAPK is found only in the spermatids and more mature germ cells in the periluminal zone of the seminiferous tubules (**D**). Only one third of total seminiferous tubules contain phosphorylated MAPK-positive cells (**E**). Scale bars in panels **A, B, C** are 12 μm. Scale bar in panel **D** is 100 μm and in panel **E** is 220 μm.

and VIP in the brain membrane preparation, while prewashing slightly increased the total and specific binding for PACAP in the testicular membrane preparation. However, the PACAP binding sites, both total and specific, in the testicular membrane preparations were still considerably fewer than those found in the brain membrane preparations. These findings indicate that a few of the PACAP binding sites in the testicular membrane preparations were, in fact, occupied by endogenous PACAP, but the ratio of the specific (displaceable) binding sites to the total binding sites remained unchanged after prewashing in both the brain and the testicular membrane preparations. The small displacement of the bound ligand by an excess amount of PACAP with a high ratio of the non-specific binding to the total binding suggests that a clear-cut competitive inhibition curve for PACAP is difficult to demonstrate with the testicular membrane preparations using a conventional RRA.

Inhibition of [^{125}I]PACAP27 Binding by PACAP or VIP in Subcellular Fractions of Rat Spermatids

The presence of PAC_1-R-li in the cytoplasm of spermatids, as suggested by IHC and a few specific binding sites for PACAP in the membrane fraction, prompted us to examine the PACAP-specific binding sites and the PAC_1-R-like receptors in the subcellular fractions of spermatids. The PAC_1-R antiserum used in this study was generated in a rabbit against a synthetic peptide corresponding to a 25-amino-acid sequence in the intracellular C-terminal domain of PAC_1-R, which is common to all splice variants *(12)*, and has been used successfully for IHC for PAC_1-R-li in the brain

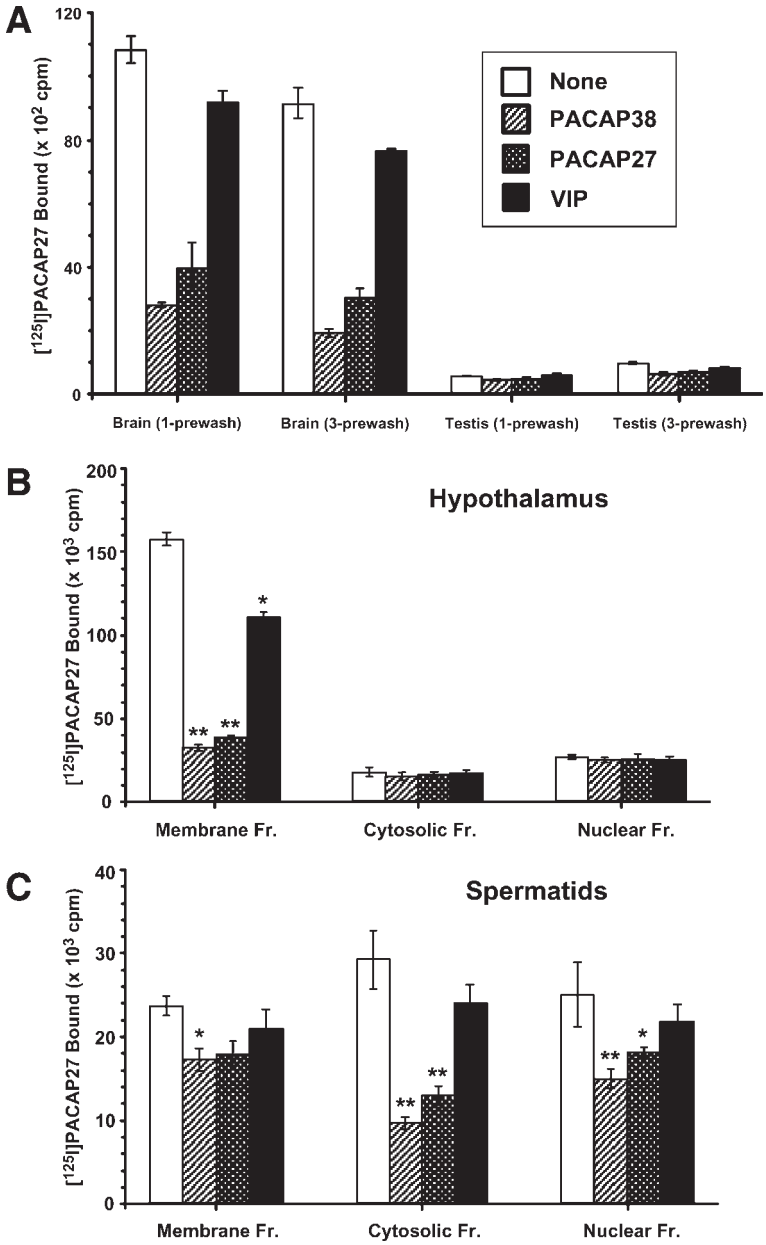

Fig. 2. (**A**) Inhibition of [^{125}I]PACAP27 binding by PACAP38, PACAP27, or VIP in membrane preparations of the rat testis and brain using a conventional RRA with rapid filtration. The membrane proteins were prewashed with a large amount of assay buffer once or three times to dissociate the endogenously bound ligand. Each sample was then incubated with 200 p*M* freshly labeled [^{125}I]PACAP27 with or without 1 μ*M* unlabeled PACAP38, PACAP27, or VIP. Each bar shows the mean bound radioligand with the standard deviation for five determinations. Total binding of [^{125}I]PACAP27 by the brain membrane preparations was high and considerably displaced by 1 μ*M* unlabeled PACAPs, and slightly reduced by VIP, while the binding by the testicular membrane preparations was low and not displaced appreciably by unlabeled peptides. Prewashing did not clearly affect these binding characteristics. (**B, C**) Inhibition of [^{125}I]PACAP27 binding by 10^{-6} *M* PACAP38, PACAP27, or VIP in subcellular fractions of the rat hypothalamus (**B**) and spermatids (**C**). To compare the binding characteristics of membrane, cytosolic and nuclear preparations of the hypothalamus and the spermatid preparation under the same experimental conditions, a novel RRA with receptor immunoprecipitation was used as described in Materials and Methods. Each bar shows the mean and standard deviation of five experiments. *$p < 0.05$ and **$p < 0.01$ compared to the [^{125}I]PACAP27 bound in the absence of unlabeled peptides (none). In the hypothalamic tissues (**B**), a significant displacement was found only in the membrane preparations. On the other hand, in the spermatid preparation, a highly significant displacement of the bound tracer by the unlabeled peptides was demonstrated in the cytosolic fraction, and then a moderate displacement in the nuclear fraction (**C**). The membrane fraction showed the smallest displacement (**C**).

as well as in the testis *(13,16)*. The spermatid-enriched fractions as well as the brain as the control were homogenized and sonicated, and then fractionated into membrane, cytosolic, and nuclear fractions. Digitonin-solubilized nuclear and membrane proteins, and soluble cytosolic proteins from both brain tissues and spermatids, were first incubated with

normal rabbit serum and protein A-Sepharose CL-4B. The precleared fractions were then incubated with 10 μg/mL of rabbit anti-PAC_1-R antibody at 4°C overnight. Protein A Sepharose CL-4B beads were added and incubated for an additional 3–4 h at 4°C to immunoadsorb the receptor protein. The beads were then washed and further incubated with freshly radioiodinated PACAP27 at a concentration of 200 p*M* (350 μCi/μg as determined by a self-displacement experiment) at 4°C overnight. Non-specific binding was determined with 10^{-6} *M* unlabeled PACAP38, PACAP27, or VIP. After centrifugation, the radioactivity of the beads was measured with a gamma-counter. The results are shown in Figs. 2B,C. The solubilized membrane preparations from the brain (hypothalamus) bound considerable [^{125}I]PACAP27, and that the binding was displaced by 1 μ*M* PACAP38 and PACAP27 about 80%, but only slightly by VIP (Fig. 2B). The cytosolic and nuclear fractions of the brain did not show any specific binding (Fig. 2B). In contrast to the brain tissue, in the spermatid fractions, the largest number of specific binding sites for PACAP were found in the cytosolic fraction (about 70% displacement of the bound tracer by an excess of unlabeled PACAP38), and then moderate binding activity was found in the nuclear fraction (Fig. 2C) (about 45% displacement of the bound tracer by an excess of unlabeled PACAP38). The membrane fraction showed the fewest specific binding sites for PACAP (about 25% displacement of the tracer by an excess of PACAP38). The binding was not significantly displaced by an excess of VIP in any fraction.

Competitive Inhibition of Bindings of [^{125}I]PACAP27 by PACAP and VIP in Cytosolic Fractions of Rat Testis

Using the testicular cytosolic PAC_1-R like protein immunoadsorbed onto protein A Sepharose CL-4B beads, we conducted a competitive binding study with increasing concentrations (10^{-13} *M*–10^{-6} *M*) of unlabeled PACAP38, PACAP27 or VIP. The binding of [^{125}I]PACAP27 to the cytosolic PAC_1-R like protein was dose-dependently displaced by unlabeled PACAP38 and PACAP27, but displaced only slightly by 10^{-6} *M* VIP (Fig. 3). The K_i was 0.069 n*M*, 0.179 n*M*, and 2.175 μ*M* for PACAP38, PACAP27 and VIP, respectively. Thus, the binding sites are PAC_1-R type.

Western Blot Analysis of PAC_1-R-li in Subcellular Fractions of Rat Spermatids

Western blot analysis of the subcellular fractions of the rat spermatids and brain (hypothalamus) was performed using the PAC_1-R antiserum *(12)*. The most distinct band, which corresponded to the cloned rat PAC_1-R expressed in CHO cells, was demonstrated in the membrane fraction of rat hypothalamic extracts (Fig. 4). Both the cytosolic and nuclear fractiond showed weak signals. On the other hand, in the subcellular fractions from the spermatid-enriched fraction, the strongest signal was seen in the cytosolic fraction and a less intense band in the nuclear fraction (Fig. 4).

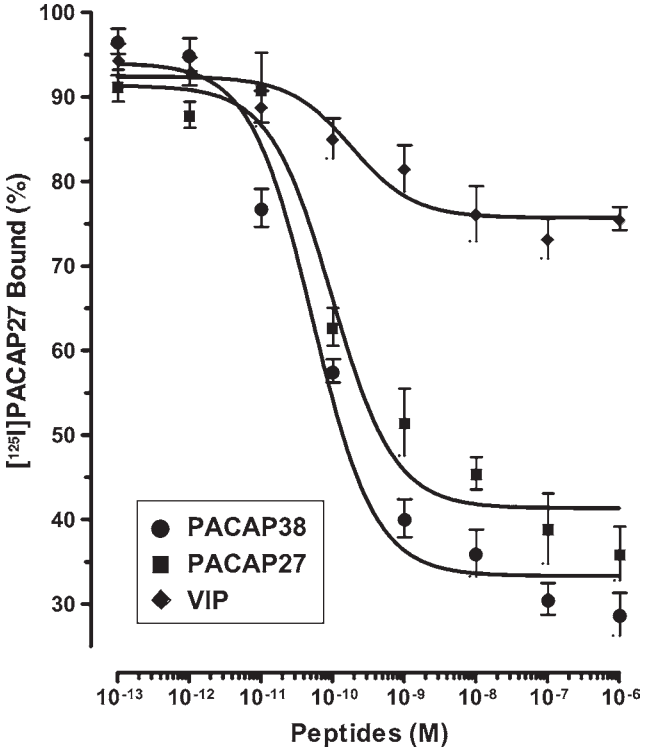

Fig. 3. Competitive inhibition curves of [^{125}I]PACAP27 by PACAP38, PACAP27, and VIP in cytosolic fractions of rat testis. The novel RRA described in Materials and Methods was used with increasing concentrations (10^{-13} *M*–10^{-6} *M*) of unlabeled PACAP38, PACAP27, or VIP with freshly iodinated PACAP27 (200 p*M*, 350 μCi/μg) in the testicular cytosolic PAC_1-R-like proteins immunoprecipitated with PAC_1-R antiserum and pre-absorbed on protein A-conjugated Sepharose CL-4B beads. Each value represents the mean ± SE of five replicates. The spermatid cytosolic PACAP receptor-like binding proteins bind specifically to PACAP38 and PACAP27 in a dose-dependent manner with a high affinity (K_i: 0.069 n*M* and 0.179 n*M* for PACAP38 and PACAP27, respectively). VIP only slightly displaced the bound tracer (K_i: 2.175 μ*M*).

Both bands appeared to migrate slightly faster than the rat PAC_1-R (57 kDa) expressed in the membrane preparations from the spermatids and the brain. The spermatid membrane fraction showed only a very weak band. All of these bands were not found when the antiserum was pre-absorbed with the 25-amino-acid synthetic immunogen. The findings are compatible with the results of the preceding experiment.

Effects of PACAP and VIP on Activation of Soluble Adenylate Cyclase (sAC) in Spermatid-Enriched Fractions

We examined whether the testicular cytosolic PACAP receptor is coupled to adenylate cyclase, as are most splice variants of the membrane-associated PAC_1-R in other tissues. However, the cytosol of the rat spermatids contains soluble adenylate cyclase which is insensitive to forskolin, but is activated by Ca^{2+} and HCO_3^- in the presence of Mn^{2+} *(17)*. The assay conditions for soluble adenylate cyclase included $MnCl_2$ in place of $MgCl_2$ and contained [α-^{32}P]ATP.

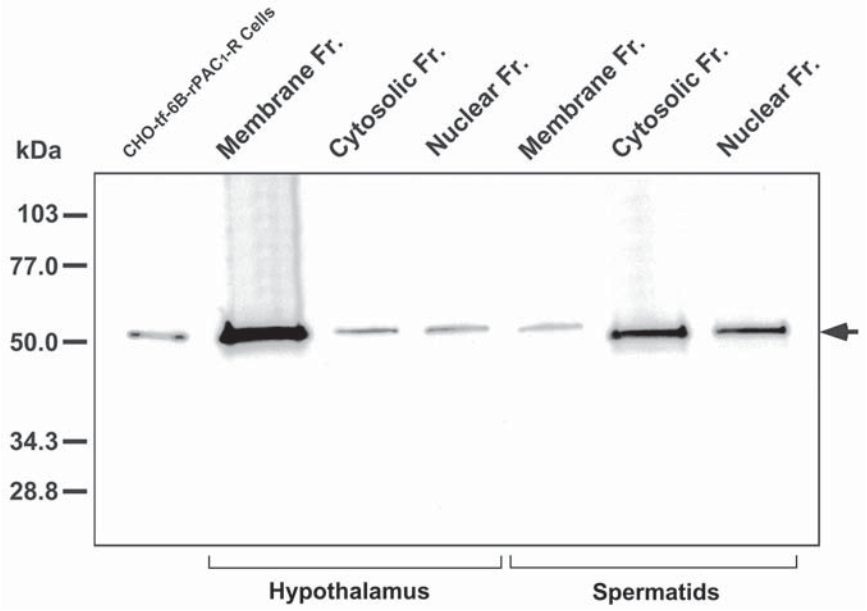

Fig. 4. Western blot analysis of PAC_1-R-li in subcellular fractions of rat hypothalamus and spermatids. In the hypothalamus, the strongest signal is demonstrated in the membrane fraction and a weak signal was also found in the cytosolic and the nuclear fractions. The sizes of these bands correspond to the rat PAC_1-R (57 kDa) expressed in CHO cells. In spermatids, the strongest signal is demonstrated in the cytosolic fraction and a less intense band in the nuclear fraction; both bands migrate slightly faster than the rat PAC_1-R (57 kDa). The spermatid membrane fraction shows only a very weak band corresponding to 57 kDa.

As reported elsewhere, soluble adenylate cyclase in spermatids was dramatically stimulated by $CaCl_2$ and/or $NaHCO_3$, while 10^{-7} *M* PACAP and VIP showed a much smaller effect (Fig. 5A). The magnitude of the response was much smaller than that demonstrated in pituitary cells, neurons, glia, and other types of cells *(18)*. PACAP38 at 10^{-13} *M* had no effect (Fig. 5A). The finding is compatible with other reports that the soluble form of adenylate cyclase present in germ cells is insensitive to any of the known modulators of transmembrane adenylate cyclase activity, such as forskolin and G-protein regulators, and is uniquely modulated by bicarbonate or calcium ions *(17,19,20)*.

Effects of PACAP38 and VIP on MAPK Activation in the Cytosolic Fraction of Rat Testis

Recent evidence indicates that Ras/Rap1, B-Raf, and the MAPKs, also known as ERKs, are expressed during the progression of mammalian spermatogenesis and maturation of spermatozoa *(21,22)*. It has been suggested that the Ras/ERK cascade is a regulatory signal during the development of testicular germ cells *(21,23–26)*. We evaluated whether the interaction of PACAP38 with the cytosolic PAC_1-R like proteins in the testis activates MAPK. The expression of MAPKs (ERK1 and ERK2) and their phosphorylation were analyzed by Western blot analysis using the antibodies that recognize unphosphorylated or phosphorylated MAPK or both. The initial study showed that 10^{-10} *M* PACAP38 stimulated both the ERK1 (p44) and ERK2 (p42) of MAP kinases as early as 10 min, reaching the maximal level at 30 min. PACAP38 stimulated the expression of both unphosphorylated and phosphorylated ERK1 and ERK2 at as low as 10^{-12} *M* or even at 10^{-15} *M* PACAP38 at 30 min (Fig. 5B).

Activated MAPK in the rat testis was also examined immunohistochemically. Phosphorylated MAPK-positive cells were demonstrated in about one third of the total seminiferous tubules. Activated MAPK was found only in the spermatids and more mature germ cells in the periluminal zone of the seminiferous tubules (Figs. 1D, E). The finding suggests that activated MAPK is expressed after PACAP and PAC_1-R-like receptors are expressed in the testicular germ cells.

The activation of MAPKs by PACAP38 was confirmed by measuring the phosphorylating activity of the immunoprecipitates with the MAPK antibody on the MAPK-specific substrate myelin basic protein (MBP) (Fig. 5C); 50 μ*M* of the MEK-1 inhibitor PD98059 abolished the kinase activity of MAPK induced by PACAP38 (Fig. 5C). PACAP27 also activated MAPK, but to a much lesser extent. Unexpectedly, 10^{-10} *M* VIP also activated MAPK (Fig. 5C). Although the rat testis contained a negligible amount of VIP, $VPAC_2$-R mRNA, but not $VPAC_1$-R mRNA, was detected by RT-PCR (data not shown). The expression of $VPAC_2$-R mRNA in the testis has also been reported by others *(27–29)*. The findings suggest that the testicular cytosolic fraction also contains a soluble $VPAC_2$-R which is coupled to MAPK.

The PAC_1-R-like Protein in the Spermatid Cytosol is Coupled to MAPK

To examine whether the PAC_1-R-like protein in the cytosol of the spermatids is indeed coupled to MAPK signaling,

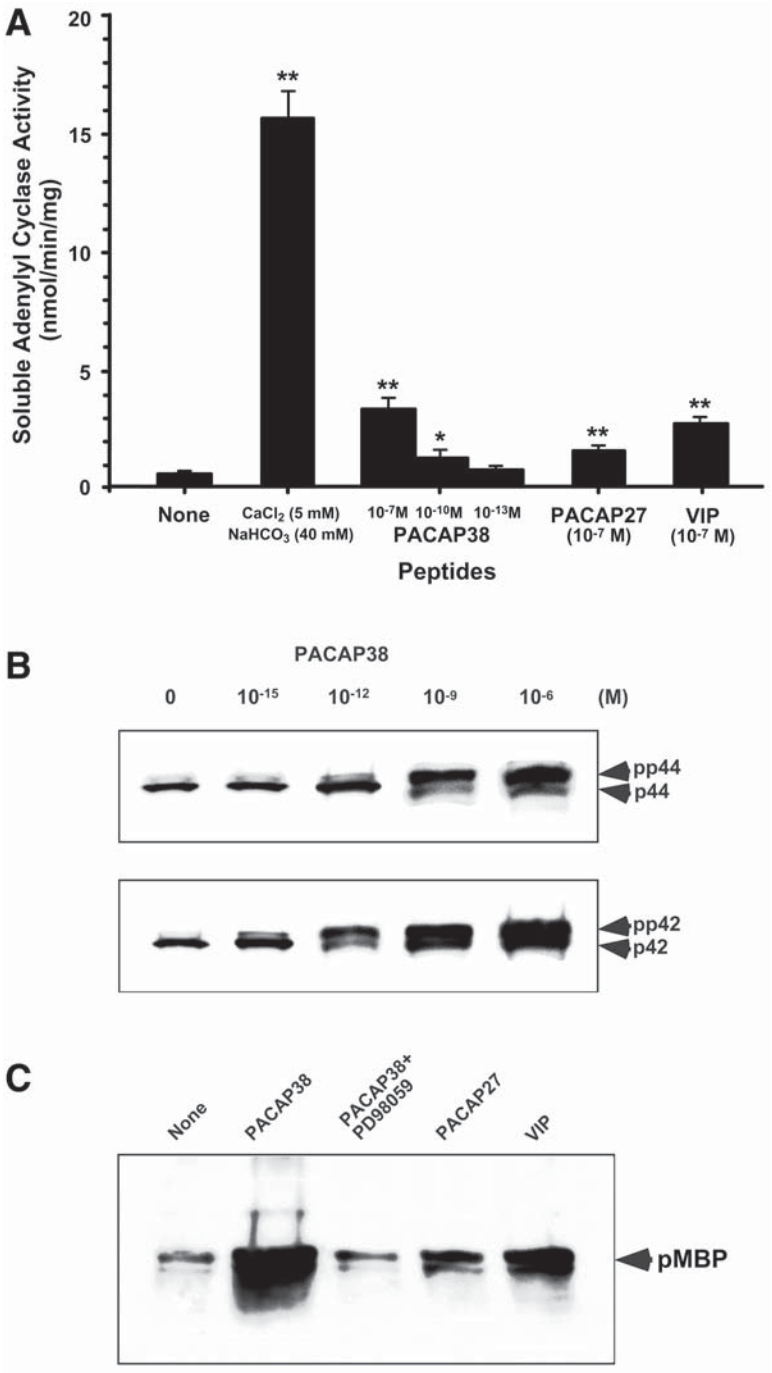

Fig. 5. Effects of PACAP38, PACAP27, or VIP on activation of soluble adenylate cyclase (**A**) and MAP kinase activation (**B, C**) in the cytosolic fraction of rat spermatids. (**A**) The in vitro soluble adenylate cyclase (sAC) assay was performed with 5 m*M* $MnCl_2$ in the presence of 25 m*M* ATP and [α-^{32}P]ATP. Each bar represents the mean ± SE of six replicates. *$p < 0.05$ and **$p < 0.01$ compared to the non-stimulated control (none). Calcium and bicarbonate ions dramatically activate the soluble adenylate cyclase. PACAP38, PACAP27, and VIP only moderately stimulated soluble adenylate cyclase activity at high concentrations. PACAP38 at 10^{-13} *M* had no effect. In the pituitary and brain cell cultures, PACAP and VIP induce a far greater increase in cAMP production. The cytosol of spermatids contains a soluble adenylate cyclase. The findings show that the cytosolic adenylate cyclase in spermatids is relatively insensitive to PACAP and VIP. (**B**) Dose-response effect of PACAP38 on the expression and activation of p44 ERK1 and p42 ERK2 of MAPKs. PACAP38 stimulates the expression or phosphorylation of ERK1 and ERK2 at as low as 10^{-12} *M*. (**C**) The activation of MAPKs by 10^{-10} *M* PACAP38, PACAP27, and VIP in the testicular cytosolic fraction was also determined by measuring the phosphorylating activity of the immunoprecipitates with the MAPK antibody on the MAPK-specific substrate MBP. Fifty micromoles of the MEK inhibitor PD98059 abolished the kinase activity of MAPK induced by PACAP38 on phosphorylation of MBP. Unexpectedly, 10^{-10} *M* VIP also phosphorylated MBP suggesting the presence of a VIP receptor, probably $VPAC_2$-R, in the cytosol.

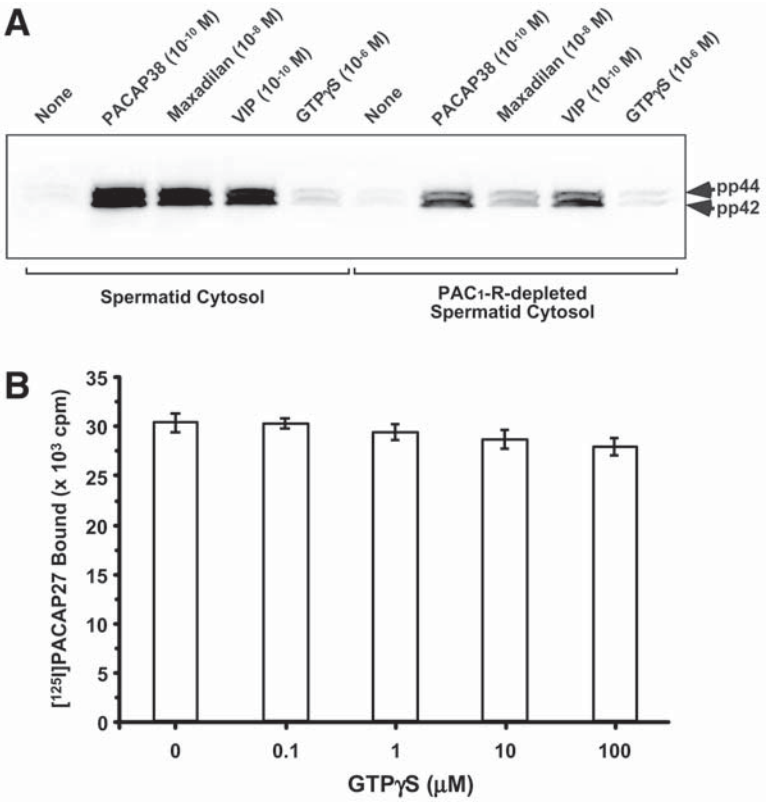

Fig. 6. (**A**) Effects of PACAP, maxadilan, VIP, or GTPγS on MAPK activation in control and PAC_1-R-like protein-depleted cytosolic fraction of rat spermatids. The normal rabbit serum-treated control or PAC_1-R antiserum-treated PAC_1-R-like protein-depleted cytosolic fraction was incubated with PACAP38, maxadilan (a specific ligand for PAC_1-R), VIP, or GTPγS (a nonhydrolyzable GTP analog). PACAP, maxadilan, and VIP all activate MAPKs in the control cytosolic fraction, while GTPγS does not. On the other hand, in PAC_1-R-depleted spermatid cytosol, maxadilan does not activate MAPKs, but PACAP38 and VIP still do. The finding that both PACAP38 and VIP stimulate MAPK in the PAC_1-R-like protein-depleted cytosolic fraction further supports the presence of a $VPAC_2$-R-like protein, which is coupled to MAPK, in the cytosolic fraction of spermatids. (**B**) Inhibitory effect of [^{125}I]PACAP27 binding by the guanine nucleotide GTPγS in soluble cytosolic fractions of the rat testis. Soluble cytosolic proteins were incubated with [^{125}I]PACAP27 in the absence or presence of 0.1–100 μ*M* of GTPγS, which permanently activates heterotrimeric G-protein, at 4°C overnight. The binding of [^{125}I]PACAP27 is not suppressed by GTPγS. The result suggests that the PAC_1-R-like protein is coupled to MAPK independently of a heterotrimeric G-protein.

the PAC_1-R-like protein in the soluble fraction of the spermatid fraction was removed by immunoprecipitation with an excess amount of the PAC_1-R antiserum and adsorbed onto protein A-conjugated Sepharose CL-4B beads. The PAC_1-R-like protein-depleted cytosolic fraction was then incubated with PACAP38, maxadilan (a specific ligand for PAC_1-R), or VIP. PACAP38, maxadilan, and VIP all stimulated the phosphorylation of MAPK (both p42 and p44) in the control spermatid cytosolic fraction (Fig. 6A). The nonhydrolyzable GTP analog GTPγS did not activate MAPK. The binding of [^{125}I]PACAP27 to the cytosolic PAC_1-R-like protein was also not altered by GTPγS either (Fig. 6B). This suggests that MAPK activation by PACAP is G protein-independent. In the PAC_1-R-depleted cytosolic fraction, maxadilan no longer activated MAPK, whereas PACAP38 and VIP still did (Fig. 6A). GTPγS also had no effect. This may indicate that the cytosolic fraction of spermatids indeed contains soluble PAC_1-R-like receptors, and also other receptors that interact with both PACAP38 and VIP, most likely $VPAC_2$-R-like receptors. Therefore, it is conceivable that PACAP interacts with both the cytosolic PAC_1-R and $VPAC_1$-R-like proteins, and VIP interacts with $VPAC_2$-R-like proteins, and both receptors are coupled to MAPK.

Plasma Membrane-Associated PAC_1-R and Cytosolic PAC_1-R-like Proteins Are Coupled to Different Signaling Cascades

The double-labeled fluorescence IHC with a confocal microscope indicated the coexpression of PACAP- and PAC_1-R-like immunoreactivities in the cytoplasm of spermatids. The examination of the subcellular localization of PAC_1-R also indicated that the majority of PAC_1-R-like protein in the testis was present in the cytosolic fraction, but some was also associated with the plasma membrane. Daniel et al. *(30)* reported that a PAC_1-R variant, PAC_1-R(3a), which is coupled to adenylate cyclase and phospholipase C, is expressed in the spermatids and Sertoli cells of the rat testis. We demonstrated that the testicular cytosolic PAC_1-R-like

protein is coupled to MAPK. However, it is still possible that these cytosolic PAC_1-R-like proteins represent an internalized or degraded transmembrane PAC_1-R *(30,31)*. To address this issue, the short-term cultures of an enriched population of spermatids were treated with PACAP or maxadilan, and then both MAPK and cAMP in the cells were determined. Both PACAP and maxadilan induced a clear-cut increase in the intracellular cAMP levels in the spermatid cultures (Fig. 7A). However, maxadilan did not increase and 10^{-10} *M* PACAP38 induced only a slight increase in cAMP when added to the spermatid cytosol (Fig. 7A). The response to 10^{-10} *M* PACAP38 in the spermatid cytosol was comparable to that demonstrated in a different assay system shown in Fig. 5A. On the other hand, MAPK was not activated appreciably when either maxadilan or PACAP was added in the spermatid cultures, while both dramatically activated MAPK when they were added to the cytosol (Fig. 7B). Because the PAC_1-R ligands added to the spermatid cultures may first interact with the plasma membrane-associated receptors of spermatids, they may have interacted with PAC_1-R(3a) associated with the plasma membrane as reported by Daniel et al. *(30)*. On the other hand, these ligands added to the spermatid cytosol may have interacted with the soluble PAC_1-R-like receptors, which are coupled to MAPK. Thus, it is unlikely that the PAC_1-R-like protein in the cytosol shown with IHC and the binding study represents the internalized membrane-associated PAC_1-R. These findings indicate that the plasma membrane-associated PAC_1-R and the cytosolic PAC_1-R-like receptors in the spermatids are coupled to different signaling cascades.

"Intracrine" Mode of Action of PACAP and PAC_1-R-like Protein in The Testis

All the findings shown above suggest that both PACAP and PAC_1-R-like proteins, which are expressed in the cytoplasm of spermatids at nearly the same time during spermatogenesis, may directly interact and stimulate MAPK without the ligand being released into the extracellular space. If so, endogenous PACAP expressed in spermatid cytoplasm may directly bind to the cytosolic PAC_1-R-like receptors;

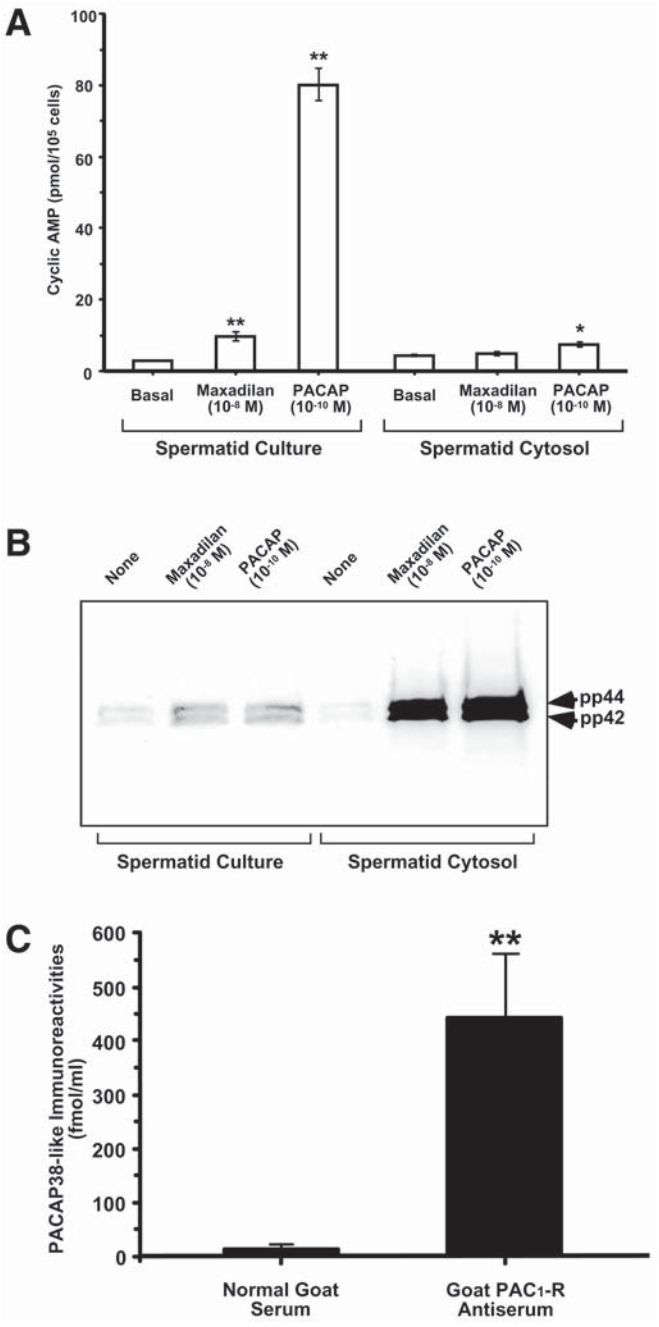

Fig. 7. (**A, B**) Effects of PACAP and maxadilan on cAMP production (**A**) and MAP kinase activation (**B**) in rat spermatid cultures and the cytosol of spermatids. Short-term cultures of spermatid-enriched rat testicular germ cells were treated with PACAP or maxadilan, and then cAMP and MAPK in the cells were determined as described in Materials and Methods. PACAP and maxadilan both induced a considerable increase in the intracellular cAMP levels (**A**), while both induced no or only a slight activation of MAPK in the spermatid cultures (**B**). On the other hand, in the cytosol fraction of spermatids, maxadilan and PACAP induced no or only a slight increase in cAMP (**A**), while both dramatically activated MAPKs (**B**). The findings also suggest that the plasma membraneassociated PAC_1-R and the cytosolic PAC_1-R-like receptors in the spermatids are coupled to different signaling cascades. *$p < 0.05$ and **$p < 0.01$. (**C**) Co-precipitation of endogenous PACAP with immunoprecipitated PAC_1-R-like protein in the rat spermatid cytosol. The rat spermatid cytosolic fraction was treated with normal goat serum or goat anti-PAC_1-R antiserum and immunoadsorbed onto protein A-Sepharose CL-4B beads. The beads were then washed with 0.5% TFA and the TFA was evaporated by lyophilization. The dried residue was dissolved in the RIA buffer and an aliquot was assayed for PACAP38 by RIA. Each value represents the mean ± SE of six replicates/group; **significantly greater ($p < 0.001$) than the normal goat serum-treated sample. The finding shows that a significant amount of PACAP38-li has been specifically co-precipitated with the PAC_1-R–like protein in the spermatid cytosol, indicating that endogenous PACAP directly binds to the PAC_1-R-like proteins in the cytoplasm of spermatids where both PACAP and its soluble receptors are coexpressed. Other findings show that the interaction of PACAP with the cytosolic PAC_1-R-like receptors triggers the expression and activation of MAPKs.

thus, the endogenous bound PACAP can be co-immunoprecipitated with PAC_1-R-like protein by the antibody. To examine this possibility, the cytosolic fraction of the spermatid-enriched fraction was incubated with the PAC_1-R antibody and then immunoadsorbed onto protein A-conjugated Sepharose CL-4B beads. The beads were then washed with dilute trifluoroacetic acid (TFA) to release the endogenous ligand bound to the adsorbed receptors. The TFA extract was then lyophilized and the residue was dissolved in RIA buffer and assayed for PACAP38 by RIA. As shown in Fig. 7C, a considerable amount of endogenous PACAP was co-precipitated with the PAC_1-R antibody, while the same treatment with the normal serum did not co-precipitate PACAP. The finding indicates that endogenous PACAP is indeed bound to the cytosolic PAC_1-R-like protein in spermatids. This may be called an "intracrine" mechanism in contrast with endocrine, paracrine, or autocrine mechanisms, which are mediated by the receptors associated with the plasma membrane, as schematically illustrated in Fig. 8.

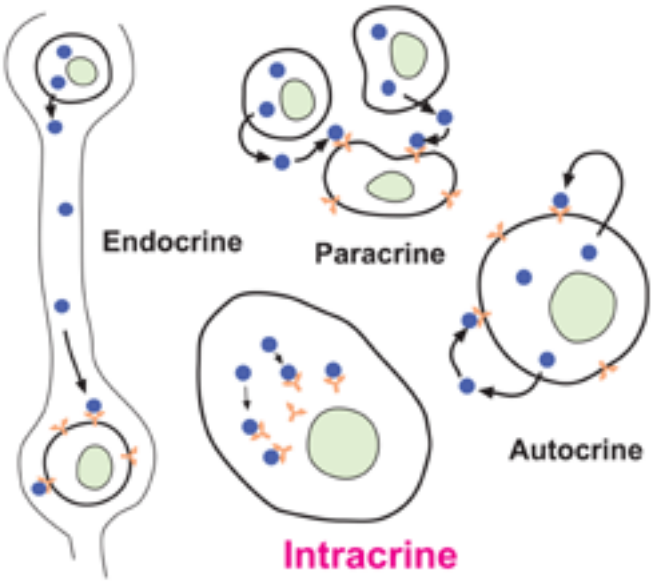

Fig. 8. Schematic illustration of an "intracrine" mechanism compared to "endocrine," "paracrine," and "autocrine" mechanisms. In the endocrine mechanism, the hormone released from the endocrine cell is transported by bloodstream to its target cell where it binds to the membrane-associated receptors. In the paracrine mechanism, the hormone is released into the intercellular space and transported by diffusion or other mechanism to the nearby target cells and interacts with the membrane-associated receptors. In the autocrine mechanism, the hormone released into the intercellular space interacts with the membrane-associated receptors of the cell from which the hormone was released. The interaction of the hormone with the membrane-associated receptors activates an intracellular signaling cascade. On the other hand, in the intracrine mechanism, both the hormone and the receptors are expressed at the same intracellular site and interact directly without the ligand being released into the extracellular space. The interaction of PACAP and PAC_1-R-like receptors in the cytoplasm in spermatids stimulates the expression and activation of MAPK in the intracrine fashion.

Possible Physiological Significance of the Intracrine Action of PACAP and Its Cytosolic PAC_1-R-like-Proteins in Spermatids

Prohormone convertase (PC)4 is specifically expressed in round spermatids, thus its substrates are likely to be expressed in spermatids. Because secretin *(32)* and GHRH *(33,34)* are also known to be expressed in spermatids and they are PACAP paralogs, their precursors are likely to be processed by PC4 in spermatids. If so, all these PACAP-related peptides may be absent in spermatids in PC4-deficient mouse testis. The major phenotype of PC4 null male mice is severely impaired fertility with apparently normal spermatogenesis *(35)*. The absence of a single peptide in spermatids does not seem to impair the fertility, as found for the PACAP null mice *(36–38)*, but the absence of all related peptides in the testicular germ cells, such as expected in PC4-deficient mouse, may impair fertility. This assumption let us to examine if these PACAP-related peptides, particularly secretin and GHRH in spermatids, may function similarly in a cooperative manner. Like PACAP and VIP, the other paralogs GHRH and secretin indeed activated MAPK when added to the spermatid cytosolic fraction. However, another unrelated hypothalamic hormone, LHRH, did not activate MAPK (Fig. 9A).

We have previously demonstrated that PC4, which is specifically expressed in the testicular germ cells *(39,40)*, is the sole processing enzyme for the testicular PACAP precursor *(41–44)*. Because the precursors of PACAP, GHRH, and secretin share similar amino-acid sequences, it is likely that they are all processed by PC4. Therefore, if the intracrine reaction of the testicular PACAP and other related peptides with their cytosolic receptors play a critical role in the expression and activation of MAPK during the round spermatid stage, the absence of testicular PACAP and the related peptides could result in a considerable reduction of MAPK levels in late spermatids and mature spermatozoa. To examine this possibility, we determined MAPK levels in the spermatozoa from PC4 knockout and the wild-type mice. As expected, the MAPK levels in the PC4 homozygous null mouse spermatozoa were drastically reduced compared to the level in the wild-type spermatozoa (Fig. 9B).

Discussion

The results showed that the majority of the PAC_1-R-like binding sites in the rat testis are found in the cytosolic fraction, followed by the nuclear fraction. The PAC_1-R-like proteins in the membrane fraction showed the smallest portion of the total PAC_1-R binding sites (Fig. 2). These findings are consistent with our previous failure to demonstrate the specific binding sites for PACAP with the membrane fraction of the rat testis using a conventional RRA, despite the fact that the same method with the membrane fraction of

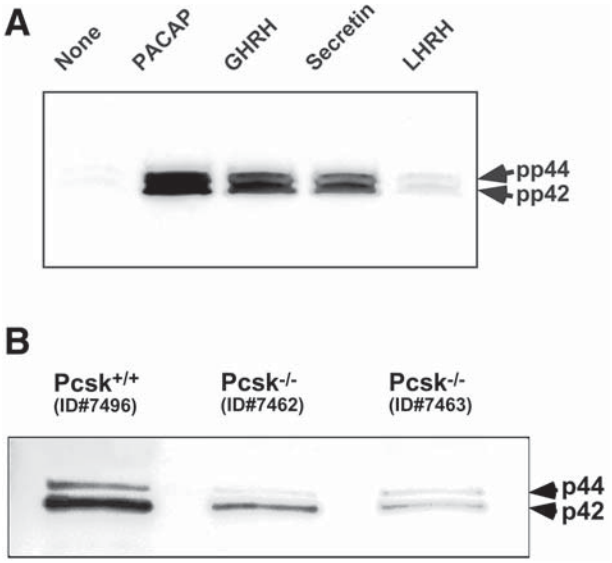

Fig. 9. (**A**) Activation of MAPKs by GHRH, secretin, and PACAP in the rat spermatid cytosolic fraction. GHRH and secretin at 10^{-8} *M* markedly activated MAPK in the rat spermatid cytosolic fraction as did PACAP. LHRH had no effect. This suggests that the testicular GHRH and secretin also interacts with their spermatid cytosolic receptors which are coupled to MAPK. (**B**) Western blot analysis of MAPKs in epididymal spermatozoa of PC4 knockout (*Pcsk*$^{-/-}$) and wild-type (*Pcsk*$^{+/+}$) mice. The levels of MAPKs in the spermatozoa from PC4 knockout mice are considerably reduced compared to those in the wild-type spermatozoa. Because MAPK plays a critical role in capacitation, the acrosome reaction, and fertilization of sperm, the reduced expression of MAPK in the spermatozoa from PC4 knockout mice may account for their severely impaired fertility.

the pituitary, brain, and other peripheral tissues clearly showed a typical competitive inhibition curve for PACAP *(9)*. The current study also showed that the failure was not due to pre-occupation of the PACAP receptors by endogenous PACAP.

On the other hand, the addition of PACAP to the intact spermatid cultures activated adenylate cyclase (Fig. 7A), suggesting the presence of a transmembrane PACAP receptor in the spermatids which is coupled to the cAMP signaling pathway. A new variant of PAC_1-R, PAC_1-R(3a), has been described *(30)*. The PAC_1-R(3a) mRNA is expressed at the highest levels in Sertoli cells and round spermatids, and the receptor is coupled to adenylate cyclase and phospholipase C. The present findings are comparable with this report. The testis also contains somatic cells such as Leydig and Sertoli cells. PACAP increases cAMP levels in Sertoli cells *(45)* and stimulates steroidogenesis in Leydig cells *(46)*. However, the present findings have also shown that the level of membrane-associated PAC_1-R accounts for only a small portion of the total PACAP binding sites in the testis (Fig. 2).

Immunohistochemistry for PAC_1-R-li in the rat testis with a confocal microscope showed the presence of PAC_1-R-like proteins in the cytoplasm of spermatids, aggregated in the acrosome. No clear-cut plasma membrane bound structure was demonstrated for PAC_1-R-li in the spermatids, suggesting that the plasma membrane does not contain a sufficient level of PAC_1-R to be detectable with IHC. The double-labeling IHC showed coexpression of PACAP-li and PAC_1-R-li in the cytoplasm of spermatids, mainly in round spermatids (Fig. 1). Because both PACAP- and PAC_1-R-li are seen only in round and some elongated spermatids *(4,5, 7,13)*, it is assumed that PACAP and its receptors are expressed at the same intracellular site of spermatids at the same stages during spermatogenesis.

The distribution of PAC_1-R-like protein in the subcellular fractions of the testis as determined by Western blot analysis also indicated that the majority of the PAC_1-R protein in the spermatids is in the cytosolic fraction (Fig. 4). A less intense band was found in the nuclear fraction. The membrane fraction showed the least intense band. All of these findings are consistent with the notion that the majority of PAC_1-R-like proteins or receptors in the testicular germ cells are expressed in the cytoplasm of spermatids.

It was noted in the Western blot analysis that the size of the PAC_1-R-like protein associated with the membrane of the hypothalamic tissue and spermatids was identical to the cloned PAC_1-R, 57 kDa. However, the PAC_1-R-like protein in the cytosolic and the nuclear fractions of the testis appeared to be slightly smaller than 57 kDa, although this apparent difference in size remains to be re-confirmed under a more appropriate experimental condition (Fig. 4). Nevertheless, it is possible that PAC_1-R like receptors in the cytosol and nucleus are different from the PAC_1-R associated with the plasma membrane. This possibility was further supported by the finding that the membrane associated PAC_1-R in spermatids is primarily coupled to adenylate cyclase, while the cytosolic PAC_1-R is coupled to the MAPK signaling cascade (Fig. 7A, B).

In the rat testis, the presence of a soluble adenylate cyclase in the cytoplasm has been reported *(19,20)*. The soluble adenylate cyclase was forskolin insensitive, but activated by Ca^{2+} and HCO^- in the presence of Mn^{2+} *(17)*. In the present study, we confirmed that the addition of $CaCl_2$ and $NaHCO_3$ to the testicular cytosolic fraction dramatically activated adenylate cyclase, while PACAP and VIP at a high concentration induced only a small activation (Fig. 5A). On the other hand, the testicular cytosolic PAC_1-R-like proteins are coupled to MAPK (Fig. 5B, C).

The cloned PAC_1-R is a typical G-protein-coupled membrane-associated receptor with seven transmembrane domains *(13)*. It is unknown whether the cytosolic PAC_1-R-like receptors require a G-protein to stimulate MAPK. However, the addition of GTPγS to the cytosolic fraction did not affect MAPK (Fig. 6A) nor suppressed the binding of the radiolabeled PACAP27 to the cytosolic PAC_1-R-like protein (Fig. 6B), suggesting that the cytosolic PAC_1-R like proteins are independent of heterotrimeric G-proteins for

activation of MAPK. However, the possibility of the involvement of a small G-protein, such as Ras/Rap1, in MAPK activation remains to be examined.

The specific binding to PACAP38 and its recognition by the antibody generated against the 25 residue synthetic peptide corresponding to the C-terminal intracellular domain of the cloned PAC_1-R suggest that the structure of the testicular cytosolic PAC_1-R-like receptors resembles that of the cloned PAC_1-R. However, some difference in the transmembrane domains, such as a deletion or modification of the transmembrane amino acids, might exist. The apparently smaller size of the cytosolic PAC_1-R-like protein than the cloned PAC_1-R is compatible with this notion (Fig. 4). Considering the abundance of the PAC_1-R-like protein in the cytosol with a relatively small signal for PAC_1-R mRNA in the testis in this study and others *(28,29,47)*, it is also possible that the cytosolic PAC_1-R is encoded by a different gene from that of the cloned PAC_1-R. In any case, a precise analysis of the structure of the cytosolic PAC_1-R-like protein must await its cloning.

PACAP, its specific agonist maxadilan, as well as VIP markedly stimulated the both expression (unphosphorylated MAPK) and activation (phosphorylated MAPK) of MAPKs in the cytosolic fraction of spermatids (Figs. 5 and 6). Because this reaction takes place in the cytosolic fraction, stimulation of the *de novo* synthesis of the enzyme is unlikely. An increase in the band of unphosphorylated MAPK by PACAP could be due to an increased translation or some post-translational modification of the molecule that is necessary for phosphorylation. On the other hand, removal of PAC_1-R-like protein from the cytosolic fraction by immunoprecipitation did not abolish MAPK stimulation by PACAP or VIP, although its removal did abolish the effect of maxadilan (Fig. 6A). The expression of $VPAC_2$-R mRNA in the testis has been demonstrated *(27,29,47–49)*. Therefore, MAPK activation by VIP in the spermatid cytosolic fraction suggests the presence of a soluble form of $VPAC_2$-R-type receptors in the cytosol that are also coupled to MAPK. Accordingly, it is likely that stimulation of MAPK by PACAP in the testicular cytosolic fraction is mediated via both PAC_1-R- and $VPAC_2$-R-like receptors. On the other hand, under physiological condition, VIP may not participate in MAPK activation in spermatids, because VIP is not expressed in the testis at an appreciable level. The competitive binding assay for the cytosolic PAC_1-R-like receptor with [^{125}I]PACAP27 showed a typical displacement curve with K_i: 0.069 n*M*, 0.179 n*M*, and 2.175 μ*M* for PACAP38, PACAP27, and VIP, respectively (Fig. 3). On the other hand, activation of MAPKs was seen even at as small a concentration as $<10^{-12}$ *M* (Fig. 5B). Although the competitive inhibition analysis of the cytosolic PAC_1-R showed binding sites for PACAP38 with a $K_i < 10^{-10}$ *M*, stimulation of MAPK was evoked by $<10^{-12}$ *M* PACAP38 which differs by two or three orders of magnitude. The apparent discrepancy, however, may be explained by the relative insensitivity of the competitive inhibition analysis in which a relatively large, fixed amount of iodinated PACAP27 (200 p*M*) was used with increasing concentrations of unlabeled PACAP. The method may be unable to detect an extremely high affinity, but possibly a very small number of binding sites. Such high-affinity binding sites could not be demonstrated, unless a stable tracer with an extremely high specific activity was used. Furthermore, the competitive binding assay was performed by using the immunoadsorbed PAC_1-R-like protein with [^{125}I]PACAP27 at 4°C and an overnight incubation. MAPK activation study was conducted in the cytosolic fraction at 30°C for 30 min. The differences in the experimental conditions could also explain some quantitative discordance.

As mentioned before, both PACAP and the PAC_1-R-like protein are expressed at the same intracellular site of spermatids at the same stages of spermatogenesis. Thus, the endogenous PACAP could interact with its receptors directly and stimulate MAPK in an "intracrine" fashion *(50)*. Co-precipitation of endogenous PACAP with the cytosolic PAC_1-R-like protein supports this contention (Fig. 7C). This mode of intracrine action of PACAP and the PAC_1-R resembles the function of intracellular regulatory molecules. This action might also reflect the primitive role of peptides during evolution, before peptides became messengers that carry information between cells. Unlike the plasma membrane-associated adenylate cyclase, which mediates signals between cells, the soluble adenylate cyclase demonstrated in the testicular germ cells may regulate the pathways which are used in unicellular organisms *(19,51)*. It is noteworthy that the catalytic domains of the soluble adenylate cyclase are more closely related to those from cyanobacterial and myxobacterial adenylate cyclase than from any eukaryotic adenylate cyclase. Furthermore, in Gram-positive bacteria, a subset of bacterial signaling peptides interacts with their intracellular receptors, like the testicular PACAP and PAC_1-R-like receptors in the spermatid cytosol *(52)*. Interestingly, PACAP is considered to be one of the most ancient peptides of its size as suggested by the fact that the amino acid sequence of PACAP in a protochordate, tunicate, is nearly identical with that in humans, indicating the conservation of its sequence during 700 million years of evolution *(53)*. Unlike somatic cells, the testicular germ cells appear to retain such a primitive regulatory mode as that found in the unicellular organism.

Because some other PACAP-related peptides, GHRH *(54–56)* and secretin *(32,57)* are also expressed in spermatids and they also stimulate MAPK when added to the spermatid cytosolic fraction, these peptides appear to function similarly to PACAP through their cytosolic receptors in spermatids. Because all of these peptides appear to be expressed in spermatids around the same stage during spermatogenesis, they may stimulate the expression and activation of MAPK in a cooperative manner. Therefore, if all of these peptides are absent, MAPK expression and activation in

spermatids may be impaired. Stimulation of MAPK by interaction of these neuropeptides and their respective receptors in the spermatid cytosol by an intracrine mechanism might be of physiological significance. Because the nuclear functions, including transcription and translation of genes, in the testicular germ cells terminate at the time of nuclear compaction, any regulatory proteins including MAPK required for spermatozoa must be expressed before nuclear compaction. Other reports suggest that MAPK in human spermatozoa is essential for capacitation, the acrosome reaction, and fertilization *(21,58,59)*. Thus, the process of MAPK expression and activation, which takes place during the spermatid stage, may be critical for providing spermatozoa with their normal functions. IHC of the rat testis showed that MAPK-li was expressed in some, but not all, of the seminiferous tubules. MAPK-li was seen in the peritubular region where spermatids, late spermatids, and spermatozoa were found (Fig. 1D, E). On the other hand, PC4 is specifically expressed in the round spermatids *(39,40)* and processes the PACAP precursor *(41,43)*. Indeed, PC4 is the sole processing enzyme for the PACAP precursor *(44)*. Because, the precursors of PACAP, GHRH, and secretin have similar sequences, it is likely that all of these peptides in spermatids are the substrate of PC4. We found that the testis of PC4 homozygous null mice lacks bioactive PACAP *(44)*. It is likely that bioactive GHRH and secretin in spermatids are also absent in PC4 null mice.

One of the co-authors reported that PC4-deficient mice develop severely impaired fertility, without any apparent morphological alteration of spermatogenesis *(35)*. Therefore, it is likely that the lack of testicular PACAP, and possibly other related peptides, leads to a reduced level of MAPK in the spermatids and then spermatozoa, resulting in the severely impaired fertility of the PC4-deficient mice. The present study, in fact, showed a reduced level of MAPK in the spermatozoa from PC4-deficient mice (Fig. 9B).

The current study with PACAP and its soluble cytosolic receptors as a prototypic testicular neuropeptide and its cognate receptor may provide a clue toward understanding the physiological role of PACAP and its related neuropeptides expressed in spermatids. The findings in these studies could also help us to understand the etiology of some types of male infertility and to develop a treatment for it. Furthermore, if the notion presented here is found to be correct, it should also be possible to develop a novel male contraceptive based on the development of a potent and specific antagonist for PC4 which is known to be specifically expressed in the testis.

Materials and Methods

Animals

The experiments were conducted using testes isolated from 6-wk-old male rats of the CD strain (Charles River Laboratories, Wilmington, MA). Six-week-old (250–300 g) rats were used because nearly 90% of their testicular germ cells are spermatids at this age in rats, and both PACAP and PAC_1-R-like receptors were abundantly expressed in spermatids *(30,60)*. All rats were housed under controlled ambient temperature and lighting (6:00 to 18:00 h), and were provided water and laboratory chow *ad libitum*. Under deep anesthesia with sodium pentobarbital (50 mg/kg ip), the animals were sacrificed by decapitation and the testes were collected immediately. For IHC, under deep anesthesia with sodium pentobarbital (50 mg/kg ip), the rats were perfused with 2% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.2) for 30 min transcardially. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Tulane University Health Sciences Center and at the Clinical Research Institute of Montreal, University of Montreal.

Purification of Germ Cells and Short-Term Cultures of An Enriched Population of Rat Spermatids

The initial studies were conducted using whole testis; however, most of subsequent studies were conducted using a spermatid-enriched fraction of the testis, because the majority of both PACAP and PAC_1-like receptors in the testis were found in spermatids. The different spermatogenic cell suspensions from the seminiferous epithelium were recovered from adult male animals by sequential enzymatic dissociation *(61–63)*, using reduced concentrations of collagenase (0.5 mg/mL) and trypsin (0.25 mg/mL). Incubations with trypsin were terminated by adding 0.25 mg/mL soybean trypsin inhibitor (Type I-S; Sigma, St. Louis, MO). Germ cells at defined stages of spermatogenesis were purified by velocity sedimentation of testicular cells at unit gravity on a 2–4% bovine serum albumin (BSA) gradient. The purities of the germ cell populations, determined on the basis of cell size and morphological characterization, were assessed and identified under a differential interference contrast microscope.

For the short-term culture of spermatids, the spermatid-enriched fraction was washed with minimum essential medium (MEM) Alpha medium containing 1% fetal bovine serum (FBS) and 1% antibiotic–antimycotic, and then cultured in a Corning 25 cm^2 flask containing MEM alpha medium supplemented with 10% FBS and 1% antibiotic–antimycotic (Invitrogen, Carlsbad, CA), in a humidified incubator at 32–33°C in 5% CO_2/95% air *(64,65)*. Because germ cells do not adhere to culture plates, cells were recovered by gentle pipetting and washed twice with MEM alpha medium by centrifugation. Cell viability was assessed by Trypan blue exclusion and cell numbers were determined by counting in a hemocytometer.

Spermatozoa of PC4-Deficient and Wild-Type Mice

Spermatozoa were collected from the cauda epididymis and/or vas deferens of two PC4-deficient mice ($Pcsk^{-/-}$) and one wild-type mouse ($Pcsk^{+/+}$) *(35)*, filtered through Nitex

80-μm mesh (Sefar America, Depew, NY), and washed twice in phosphate-buffered saline (PBS) at 4°C. Spermatozoa were further purified by density gradient centrifugation in Percoll according to the procedure of Lessley and Garner *(66)*.

Immunohistochemistry

The frozen testis was cut into sections on a cryostat. Eight-micrometer-thick sections were rinsed in PBS, preincubated for 1 h with 10% normal horse serum in PBS. Thereafter, the sections were incubated with goat anti-PAC_1-R serum, G5, or phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (Cell Signaling Technology, Beverly, MA), in PBS containing 10% normal horse serum for 2 d at 4°C. This was followed by washing in PBS before incubation with a Cy3-labeled donkey anti-goat IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at room temperature. For dual immunostaining, the sections were then incubated with rabbit anti-PACAP antiserum, R12, for 2 d at 4°C. These sections were then incubated with an Alexa 488-labeled anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) for 2 h at room temperature. After washing in PBS, the sections were counterstained for nuclei with 4', 6-diamine-2'-phenylindole dihydrochloride (DAPI) (0.1 μg/mL; Boehringer Mannheim, Mannheim, Germany) for 15 min at room temperature and rinsed. The sections were mounted and examined with a Radiance 2100 confocal microscope (Nippon Bio-Rad Laboratories, Tokyo, Japan).

Preparation of Subcellular Fractions from Spermatid-Enriched Fractions of Rat Testis

The spermatid-enriched fractions as well as the rat hypothalamus were homogenized and sonicated using a Teflon/glass Potter homogenizer and Vibra Cell (Sonics & Materials, Danbury, CT) in 20 m*M* Tris (pH 8.0 at 0°C) containing 0.25 *M* sucrose, 10 m*M* EDTA, 30 m*M* KCl, and a protease inhibitor cocktail [10 μ*M* pepstatin A, 10 μ*M* leupeptin, 4 μg/mL E-64, 1 μg/mL aprotinin, and 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)]. The homogenates were centrifuged at 1,000*g* for 5 min at 4°C. The precipitates (nuclear fractions) were suspended in buffer A (20 m*M* Tris [pH 7.5], 5 m*M* EDTA, 0.15 *M* NaCl, and a protease inhibitor cocktail). The supernatants were centrifuged at 125,000*g* for 60 min at 2°C. The supernatants (soluble cytosolic fractions) were collected and the pellets (membrane fractions) were resuspended in buffer A *(67)*. All fractions were assayed for protein using the Bradford method (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard.

Competitive Inhibition Analysis of PACAP Binding to Membrane Preparations of the Testis and the Brain with and without Prewashing Using A Conventional Radioreceptor Assay (RRA) with Rapid Filtration

The membrane preparations from the rat testis and brain containing 20 μg of protein were used for the RRA in parallel with 200 p*M* freshly labeled [^{125}I]PACAP27 in a total volume of 300 μL containing 50 m*M* Tris (pH 7.4), 5 m*M* $MgCl_2$, 0.5 mg/mL bacitracin, 2 μg/mL PMSF, 200 U/mL Trasylol, and 1.0% BSA at 22°C for 90 min. Because the testis contains abundant PACAP, some of the membrane preparations were prewashed three times with a large amount of assay buffer to dissociate the endogenously bound ligand so that the binding sites could be exposed. The assay was terminated, and bound/free ligands were separated by a rapid filtration method as previously described *(9)*. The radioactivity of peptides bound to the membrane was measured using an automatic gamma-counter. Non-specific binding was determined in the presence of 10^{-6} *M* PACAP38, PACAP27 or VIP.

Immunoadsorption of PAC_1-R-like Proteins and Receptor Binding Analysis with Subcellular Fractions of Rat Spermatids and Hypothalamus—A Novel RRA with Immunoprecipitated Receptors

To determine the specific binding sites for PACAP in the membrane, nuclear, and cytosolic fractions of spermatids and the hypothalamus of the rat for comparison, it was necessary to first solubilize each of these fractions and then determine the binding sites using identical conditions. The membrane and nuclear fractions were diluted with buffer A containing digitonin (Sigma) so that the ratio of digitonin to protein was 5:1. The mixture was gently stirred at 4°C for 1 h and then centrifuged at 125,000*g* for 30 min at 2°C. Digitonin-solubilized nuclear and membrane proteins, and soluble cytosolic proteins were diluted in buffer B (50 m*M* Tris [pH 7.5], 5 m*M* EDTA, 0.1% digitonin, 0.1 m*M* PMSF, and 0.1% BSA) and then incubated with normal rabbit serum (5% in the final concentration) for 1 h at 4°C. They were then pre-cleared with protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech) for 1 h at 4°C. The supernatants were then incubated with 10 μg/mL of the rabbit anti-PAC_1-R antibody (93093-3) *(12)* at 4°C overnight. Protein A-Sepharose CL-4B beads (0.5 mg of IgG/reaction) were then added and incubated for an additional 4 h at 4°C. The beads were washed with buffer B and incubated with 200 p*M* freshly iodinated PACAP27 (350 μCi/μg as determined by a self-displacement experiment) at 4°C overnight. Non-specific binding was determined with a 100-fold molar excess of unlabeled PACAP38, PACAP27, or VIP (American Peptide Co., Sunnyvale, CA). After a brief centrifugation, the radioactivity of the beads was measured with a gamma-counter. The competitive binding experiment was conducted with the protein A-Sepharose CL-4B-trapped PAC_1-R-like protein from the cytosolic fraction of spermatids with 10^{-12}–10^{-6} *M* unlabeled PACAP38, PACAP27, or VIP.

To prepare the PAC_1-R-like protein-depleted cytosol fraction, the testicular cytosolic fractions were diluted (2.5 mg protein/mL) in Tris buffer and incubated with normal rabbit serum (5%) or the PAC_1-R antiserum (5%) at 4°C

overnight to immunoadsorb the PAC_1-R like protein. Then protein A conjugated Sepharose CL-4B beads were added and incubated for 1 h at 4°C. After a brief centrifugation, the supernatant was collected and incubated with PACAP38, maxadilan (a specific ligand for PAC_1-R, VIP, or GTPγS).

Western Blot Analysis of PAC_1-R-li in Subcellular Fractions of Rat Spermatids and Hypothalamus

Fifty micrograms of subcellular proteins from the rat spermatids and hypothalamic extracts in Laemmli sample buffer were subjected to a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and then electroblotted onto a nitrocellulose membrane. The blots were blocked with 5% skim milk and incubated with the rabbit PAC_1-R antibody *(12)*. The final signals were visualized by incubation with a horseradish peroxidase-conjugated donkey anti-rabbit IgG and an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech). To check for specificity, antibodies pre-incubated with 50 μg/mL of their corresponding immunogen and bovine thyroglobulin were used.

Immunoblot Analysis of MEK-Phosphorylated ERK-type MAPK

Five hundred micrograms of soluble cytosolic proteins of the rat testis in buffer C (50 m*M* Tris [pH 7.5], 1 m*M* EDTA, 1 m*M* EGTA, 0.5 m*M* Na_3VO_4, 0.1% β-mercaptoethanol, 1% Triton X-100, 50 m*M* NaF, 5 m*M* $Na_4P_2O_7$, 10 m*M* $C_3H_7O_6PNa_2$, protease inhibitor cocktail, and 1 μ*M* microcystin) were incubated with 10^{-15}–10^{-6} *M* PACAP38 for 10–60 min at 30°C. Then, 10% trichloroacetic acid (TCA) containing 1% pyrophosphate was added, yielding a final TCA concentration of 5% and stopping the reaction. The solution was immediately centrifuged at 15,000*g* for 10 min at 4°C. After washing the pellets twice with buffer C, 50 μg of each sample were subjected to 12% SDS-PAGE. Immunoblotting was performed with 0.5 μg/mL of anti-ERK1 and anti-ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), using an ECL Western blot system. The cytosolic fraction of spermatids treated with the PAC_1-R antiserum (PAC_1-R-depleted cytosol) or normal rabbit serum (control cytosol) were also incubated with 10^{-10} *M* PACAP38, VIP, 10^{-8} *M* maxadilan, or 10^{-6} *M* GTPγS for 10 min at 30°C, and MAPKs were analyzed on immunoblots with phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (Cell Signaling Technology, Beverly, MA). The overnight cultured spermatid germ cells in GIBCO MEM alpha medium (Invitrogen) containing 10% FBS and 1% antibiotic–antimycotic were also incubated with 10^{-10} *M* PACAP38 or 10^{-8} *M* maxadilan for 30 min at 32–33°C. The cells were then lysed for immunoblot analysis of phospho-MAPK as described above. Fifty micrograms of cell lysate from epididymal spermatozoa of the PC4-deficient mouse in Laemmli sample buffer were subjected to a 12% SDS-PAGE, and then electroblotted onto a nitrocellulose membrane. Immunoblotting for phospho-MAPK was performed with p44/42 MAPK antibodies (Cell Signaling Technology), using an ECL Western blot system.

Determination of MAPK Activity in Cytosolic Fractions of Rat Testis

The activity of phosphorylated MAPKs was determined with the MAPK Immunoprecipitation Kinase Cascade Assay Kit (Upstate Biotechnology, Lake Placid, NY). Briefly, 500 μg of cytosolic protein were preincubated with or without 10^{-10} *M* PACAP38, PACAP27, or VIP, and 50 μ*M* PD-98059 (A. G. Scientific, San Diego, CA), an inhibitor of MAPK (MEK-1). After incubation for 30 min at 30°C, the reaction mixture was transferred to a microcentrifuge tube containing an anti-MAPK1/2 agarose conjugate, and incubated for an additional 3 h at 4°C to immunoprecipitate MAPK. The MAPK/agarose immunocomplex was then mixed with substrate buffer containing the ERK-specific substrate MBP, and a cocktail of Mg/ATP was added to start the reaction. After incubation for 30 min at 30°C, 2.5 μg of MBP in the reaction mixture was placed in a Laemmli sample buffer. Each of the samples was electrophoresed through a 12% SDS-polyacrylamide gel and blotted on a nitrocellulose membrane. The membranes were incubated with a phospho-specific MBP antibody and developed with the ECL Western blot detection kit.

Soluble Cytosolic Adenylate Cyclase Assay

The soluble adenylate cyclase assay was performed as described by Levin and Reed *(68)* except that the assay conditions included 5 m*M* $MnCl_2$ in place of $MgCl_2$ and contained 5 m*M* [α-^{32}P]ATP. First, 5 μL of ATP (final concentration 2.0 m*M*) was added to tubes, followed by the addition of 5 μL of $CaCl_2$ (final concentration 5 m*M*) and $NaHCO_3$ (final concentration 40 m*M*), or PACAP38 (final concentrations 10^{-13}–10^{-7} *M*), PACAP27, or VIP (final concentration 10^{-7} *M*). Thereafter, 50 μg of soluble cytosolic proteins from spermatids of rats suspended in 20 μL of binding buffer (75 m*M* Tris/HCl [pH 7.4], 12.5 m*M* $MnCl_2$, and 1 m*M* EDTA) were added. Tubes were kept on ice before preincubation. The binding buffer supplied the divalent cations and buffer required for the AC assay (final concentrations: 5 m*M* $MnCl_2$, 0.4 m*M* EDTA, and 30 m*M* Tris/HCl [pH 7.4]). The assay tubes were placed at 37°C and pre-incubated for 3 min before the addition of the reaction mixture (20 μL) containing (final concentrations) 40 μ*M* [α-^{32}P]ATP (2.5–3.0 μCi/tube), 2.7 m*M* mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU of pyruvate kinase, 1 IU of myokinase, and 0.1 m*M* cAMP. Blank values were obtained by processing tubes without the stimulant. The reactions were conducted at 30°C for 30 min and were terminated by addition of 20 μL of 2.2 *N* HCl. Sixty-five microliters of the reaction fluid were applied onto disposable chromatography columns each filled with 0.325 g of neutral alumina. cAMP was eluted directly into 8 mL

scintillation counting tubes by adding 1 mL of 0.1 *M* ammonium acetate (pH 7.0) into the column reservoir. The recovery of cAMP was reproducibly approx 80%. [^{32}P]cAMP was determined by liquid scintillation counting after the addition of 5 mL of the Ecolume cocktail. Samples were carefully mixed and counted for 1 min. Blank values were <0.01% of total amount of [α-^{32}P]ATP added, and less than 0.5–1.0% of the total substrate added was consumed. Specific activity was determined and expressed as nanomoles of cAMP formed per minute/milligram of protein.

Determination of Adenylate Cyclase Activity

For determination of adenylate cyclase activation, the accumulation of cyclic AMP production in cultured spermatids after incubation with PACAP or maxadilan was also determined. The overnight cultured spermatids in the MEM alpha medium containing 10% FBS and 1% antibiotic–antimycotic were incubated with 10^{-10} *M* PACAP38 or 10^{-8} *M* maxadilan for 20–30 min at 32–33°C. After the incubation, the spermatids were extracted with ethanol (65% at final concentration) and the incubates were centrifuged at 10,000*g* for 10 min at 4°C to pellet the denatured proteins. The supernatant was evaporated using a Speed-Vac Concentrator. The residue was dissolved in the RIA buffer and cAMP was measured by RIA *(14)*.

Data Analysis and Statistics for the Competitive Binding Assay

All experiments were performed at least three times unless otherwise stated. The quantitative data for the magnitudes of adenylate cyclase activation by various stimulants were expressed as the mean ± SE. The results were analyzed with a two-tailed unpaired *t*-test using InStat 3.0a (GraphPad Software, Inc., San Diego, CA). The magnitudes of displacement of [^{125}I]PACAP27 from the cytosolic proteins by PACAP38, PACAP27, and VIP in the competitive inhibition assay were examined with a one-way ANOVA followed by the Tukey–Kramer multiple comparisons test. $p < 0.05$ was considered statistically significant.

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