PACAP-mediated sperm–cumulus cell interaction promotes fertilization

Ichiro Tanii¹, Tadashi Aradate¹, Kouhei Matsuda², Akira Komiya³ and Hideki Fuse³

¹Department of Medical Biology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, 930-0194, Japan, ²Department of Biology, Faculty of Science, University of Toyama, Toyama, 930-8555, Japan and ³Department of Urology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, 930-0194, Japan

Correspondence should be addressed to I Tanii; Email: taniibio@las.u-toyama.ac.jp

Abstract

The developing acrosome in spermatids contains pituitary adenylate cyclase-activating polypeptide (PACAP). However, the role of the acrosomal PACAP remains unclear because it has not been detected in mature spermatids and sperm. We reinvestigated whether the sperm acrosome contains PACAP. An antiserum produced against PACAP reacted to the anterior acrosome in epididymal sperm fixed under mild conditions, suggesting that PACAP acts on oocytes and/or cumulus cells at the site of fertilization. Immunolabeling and RT-PCR demonstrated the presence of PACAP type I receptor, a PACAP-specific receptor, in postovulatory cumulus cells. To investigate the role of PACAP in fertilization, we pretreated cumulus–oocyte complexes with the polypeptide. At a low concentration of sperm, the fertilization rate was significantly enhanced by PACAP in a dose-dependent manner. Sperm penetration through the oocyte investment, cumulus layer, and zona pellucida was also enhanced by PACAP. The enhancement was probably due to an enhancement in sperm motility and the zona-induced acrosome reaction, which were stimulated by a cumulus cell-releasing factor. Indeed, PACAP treatment increased the secretion of progesterone from the cumulus–oocyte complexes. These results strongly suggest that in response to PACAP, cumulus cells release a soluble factor that probably stimulates sperm motility and the acrosome reaction, thereby promoting fertilization.

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Introduction

During the fertilization process, sperm penetrate two major extracellular layers surrounding the oocyte, first the cumulus layer, then the zona pellucida. It has been well documented that cumulus cells function as a selective barrier to the movement of sperm toward the oocyte. A failure of implantation and pregnancy may be eventually minimized by blocking morphologically or functionally abnormal sperm from penetrating the cumulus layer. Although a number of studies have suggested that cumulus cells provide soluble factors affecting sperm functions, little is known about the role of the cumulus as a promoting element in fertilization. Cumulus-conditioned medium stimulated the forward movement of sperm (Bradley & Garbers 1983, Westphal *et al.* 1993, Fetterolf *et al.* 1994). Only a few candidates were identified in the conditioned medium as cumulus-derived factors. Prostaglandins, PGE₁, PGE₂, and PGF_{2α}, were detected in the incubation medium of cumulus–oocyte complexes (Gurevich *et al.* 1993, Viggiano *et al.* 1995). Blocking the biosynthesis of prostaglandins with indomethacin resulted in a decrease in the rate of fertilization (Viggiano *et al.* 1995). Progesterone was

identified as another candidate, which induced hyper-activated flagellar movement and the acrosome reaction as well (Roldan *et al.* 1994, Jaiswal *et al.* 1999). Recent studies have shown that chemokines secreted from cumulus cells facilitate the migration of sperm toward the oocyte (Tamba *et al.* 2008). However, the mechanisms by which the secretion is regulated and how the soluble factors promote fertilization remain poorly understood.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal peptide family, which acts as an extracellular signal for various types of cells in the CN and peripheral organs (Arimura 1998, Vaudry *et al.* 2000). The binding of PACAP to a seven-transmembrane receptor activates intracellular signaling by way of a G-protein-coupled system, which leads to multiple cellular responses involving cell proliferation, differentiation, and secretion (Vaudry *et al.* 2000). A high level of PACAP is contained in the testis, comparable to that in the brain (Arimura *et al.* 1991). Previously, PACAP was detected in spermatogenic cells but not in Sertoli cells or Leydig cells (Shioda *et al.* 1994). Because PACAP stimulated the secretion of testosterone in cultured Leydig cells

(Romanelli *et al.* 1997, Rossato *et al.* 1997, El-Gehani *et al.* 2000), it is considered to have a role in the regulation of steroidogenesis in Leydig cells. Although intense immunoreactivity for PACAP was detected in the developing acrosome in the spermatids, curiously, PACAP was undetectable in mature spermatids and epididymal sperm (Shioda *et al.* 1994, Hannibal & Fahrenkrug 1995, Leung *et al.* 1998, Yanaihara *et al.* 1998). Accordingly, PACAP has been considered to disappear from the organelle. These findings raise the question of whether PACAP is released from the acrosome and acts on Leydig cells in the seminiferous tubules. Since the acrosome reaction occurs during penetration of the oocyte investment in the ampulla of the oviduct, PACAP is not likely to be released from the developing acrosome. Therefore, the role of acrosomal PACAP remains to be determined. In this context, we reinvestigated whether PACAP is contained in the sperm acrosome, and examined the role of PACAP in fertilization. The results presented show that the sperm acrosome contains PACAP and raise the possibility that PACAP mediates the interaction between sperm and cumulus cells.

Results

Localization of PACAP in epididymal sperm

When cauda epididymal sperm were fixed under mild conditions, 1% paraformaldehyde for 5 min, the anterior acrosome showed distinct immunoreactivity for PACAP (Fig. 1). The immunoreactivity was colocalized with PNA-lectin staining specific to the anterior acrosome (Fig. 1B and C). Spontaneously acrosome-reacted PNA-negative sperm in the head lost the immunoreactivity for PACAP. The middle piece of the flagellum seemed to be immunoreactive to PACAP. In control experiments, using an antiserum absorbed with PACAP, no immunoreactivity for PACAP was detected in the sperm head with an intact acrosome as indicated by PNA-lectin staining (Fig. 1D–F). The pattern and intensity of the immunoreactivity were the same without fixation. When sperm were fixed with 4% paraformaldehyde for 15 min as previously reported (Yanaihara *et al.* 1998), immunoreactivity was not detected in the sperm head.

Expression and localization of PACAP type I receptor

The presence of PACAP in the acrosome suggests that the target cells for PACAP may exist at the site of fertilization. To determine whether PACAP type I receptor (PAC1R), a PACAP-specific receptor, is present in cumulus cells and/or oocytes, we used a polyclonal antibody raised against its N-terminal extracellular domain (Matsuda *et al.* 2002). Immunoreactivity was found on cumulus cells, especially the outer layer, but not in the oocyte located in the central position of the complex (Fig. 2).

In the control with normal rabbit serum, no clear immunoreactivity was detected in cumulus cells. Immunoreactivity for PAC1R was not detectable in sperm. To demonstrate the expression of *Pac1r* mRNA in postovulatory cumulus cells, RT-PCR was performed with total RNA extracted from cells isolated from the cumulus–oocyte complexes. *Pac1r* mRNA expression was demonstrated in the cumulus cells after ovulation (Fig. 3).

Effect of PACAP on IVF

Based on the presence of PACAP in the anterior acrosome, in addition to the presence of PAC1R in the cumulus cells, PACAP is assumed to function during the passage through the cumulus layer in fertilization. We then examined the effect of exogenous PACAP on the fertilization rate *in vitro*. The cumulus–oocyte complexes were exposed to PACAP at various concentrations, and subsequently inseminated. To reduce the effect of the endogenous PACAP in the acrosome, the experiments were conducted with a lower sperm concentration (20 sperm/ μ l) than that used in conventional IVF (50–100 sperm/ μ l). Since the number of sperm is very small at the site of fertilization *in vivo* (Yanagimachi 1994), the present conditions mimic those *in vivo*. The fertilization rate was determined by counting two-cell embryos as fertilized and was significantly increased ($P < 0.005$) by PACAP at concentrations ranging from 10 nM to 1 μ M, as compared with rates in the control groups without the pretreatment, and the change was dose dependent (Fig. 4).

Fertilization has multiple steps: sperm penetration through the oocyte investment, cumulus layer, and zona pellucida, and finally sperm–oocyte fusion. Then, we examined the effect of the PACAP pretreatment on penetration. When the cumulus–oocyte complexes were pre-incubated with PACAP (1 μ M) prior to insemination, the number of sperm in the perivitelline space was significantly increased ($P < 0.001$) by 1.9-fold relative to that in the control group (Fig. 5). In these experiments, the mean number of the perivitelline sperm in the control groups was 0.97 ± 0.27 .

Effect of PACAP-treated fluid on sperm motility and the acrosome reaction

The efficiency with which sperm penetrate the oocyte investment is attributable to the motility of the sperm. We then prepared the cumulus–oocyte complex-conditioned medium in the presence or absence of PACAP, which is referred to as the PACAP-treated fluid or control-treated fluid respectively, and determined the effect of the cumulus–oocyte complex-derived factors on sperm function. The parameters of sperm motility were analyzed using the computer-assisted semen analysis (CASA) system to determine the effect of PACAP-treated

Figure 1 Immunolocalization of PACAP in the sperm acrosome. (A–C) Double staining with the anti-PACAP antibody (A and C, green) and PNA (B and C, red). Immunoreactivity for PACAP in the acrosome (A–C, arrow heads) was co-labeled with the PNA staining. PNA-negative sperm (A–C, double arrow) showed no distinct immunoreactivity for PACAP. (D–F) Control experiment using the pre-absorbed antibody. Phase-contrast image (D). The acrosome labeled with PNA staining (E) showed no immunoreactivity using the absorbed antibody (F). The arrow (D and E) indicates the cytoplasmic droplet. Scale bar = 10 μ m.

fluid on sperm function. The amplitude of lateral head displacement of sperm was significantly increased ($P < 0.05$) when sperm were mixed with the conditioned medium of the PACAP-treated cumulus–oocyte complexes (PACAP-treated fluid) as compared with the control experiment with the medium in the absence of PACAP (control-treated fluid; Table 1), whereas there were no significant differences between sperm incubated with the PACAP-treated fluid and control sperm in other parameters of motility such as beat cross frequency, straight line velocity, and curvilinear velocity.

The acrosome reaction is another factor influencing penetration. We therefore assessed acrosomal status using an Alexa-Fluor 594-conjugated soybean trypsin inhibitor (SBTI) as previously described (Fukami *et al.* 2003). SBTI goes through the fenestrated plasma and acrosomal membranes during the initial phase of the acrosomal reaction and tightly binds to acrosin, which is located in the inner acrosomal membrane (Tollner *et al.* 2000). When sperm were incubated with the PACAP-treated fluid, a significantly higher percentage of sperm ($P < 0.05$) was shown to undergo the acrosome reaction induced by zona proteins (ZP; 53.3%), whereas when sperm were incubated with the control-treated fluid, an

apparently lower percentage of acrosome-reacted sperm (34.1%) was found (Fig. 6). The PACAP-treated fluid by itself had no effect to induce the acrosome reaction: when sperm were incubated with the PACAP-treated fluid, the percentage showing acrosome loss was $7.36 \pm 1.8\%$, which was not significantly different ($P > 0.05$) from that of sperm incubated with the control-treated fluid, $4.00 \pm 2.7\%$ or with TYH medium, $4.88 \pm 1.4\%$.

Figure 2 Immunolocalization of PAC1R in the cumulus–oocyte complex. (A) Immunoreactivity for PAC1R was found in the outer layer of the cumulus cells. (B) Control experiment using normal rabbit serum. No distinct immunoreactivity was detected in the complex. Scale bar = 50 μ m.

Figure 3 Expression of *Pac1r* mRNA in the cumulus cells. RT-PCR was performed using specific primers for *Pac1r* with an equimolar amount of mRNA from the brain (Br), ovary (Ov), and postovulatory cumulus cells (Cu). Control experiments were conducted with primers for β -actin.

Progesterone and prostaglandins in the cumulus–oocyte-conditioned medium

The results of the IVF experiment and the analysis of sperm motility and acrosome reaction suggested that the cumulus–oocyte complexes, in response to PACAP, release a factor stimulating sperm motility and the acrosome reaction, which accordingly lead to enhanced fertilization. Because progesterone and prostaglandins have been identified as cumulus cell-derived molecules and regulate sperm functions (Osman *et al.* 1989, Viggiano *et al.* 1995, Schaefer *et al.* 1998), they are candidates for this factor. Therefore, we compared the amounts of progesterone and PGE₁ and PGE₂ in the PACAP-treated fluid to those in the control-treated fluid. HPLC showed that, as compared with that in the control-treated fluid, the amount of progesterone was increased in the PACAP-treated fluid (Fig. 7). Moreover, a competitive enzyme immunoassay showed that the amount of progesterone was significantly enhanced ($P < 0.05$) when the cumulus–oocyte complexes were incubated with PACAP (4.88 ± 1.1 nM) as compared with that in the control-treated fluid (3.91 ± 0.87 nM). By contrast, there was no significant difference in the amount of PGE₁ between the PACAP-treated fluid and control-treated fluid ($P > 0.05$): 73.5 ± 9.7 and 64.3 ± 8.7 nM respectively, and no difference either in the amount of PGE₂ between the fluids ($P > 0.05$): 358 ± 53 and 346 ± 13 nM respectively.

Discussion

This study has demonstrated the presence of PACAP in the sperm acrosome. Although PACAP has been detected previously in the developing acrosome of spermatids, its functional roles remained unclear due to a lack of immunoreactivity for PACAP in mature spermatids and epididymal sperm (Shioda *et al.* 1994, Hannibal & Fahrenkrug 1995, Leung *et al.* 1998, Yanaihara *et al.* 1998). The loss of immunoreactivity may be attributable to a masking of antigenic determinants caused by overfixation. In fact, immunoreactivity for PACAP was undetectable when sperm were fixed with 4% paraformaldehyde (PFA) for 15 min. Given that the acrosome contains molecules necessary for the passage of sperm through the oocyte investment, our finding provides a clue as to the role of acrosomal PACAP in fertilization.

Considering the immunolocalization of PAC1R exclusively in the cumulus cells, the cells were presumably a target for acrosomal PACAP. The predominant localization of PAC1R to the outer layer of cumulus cells suggests that the PACAP-mediated interaction between sperm and cumulus cells occurs at this site. A previous study demonstrated the expression of PAC1R in the granulosa cells of preantral follicles and in the cumulus oophorus, proposing a role for PACAP in follicular development (Shioda *et al.* 1996). In addition, the present RT-PCR experiments demonstrated PAC1R expression in the postovulatory cumulus cells, implying another role. Given its immunolocalization in the acrosome, PACAP is most likely to function at the site of fertilization.

The exogenous PACAP facilitated fertilization with a low sperm concentration, demonstrating a role for acrosomal PACAP in fertilization. The enhancement of fertilization was due to efficient sperm penetration through the oocyte investment because an increased number of sperm were found in the perivitelline space when the cumulus–oocyte complexes were pre-incubated with PACAP. Together with previous findings that cumulus-conditioned medium contained factors stimulating sperm motility (Bradley & Garbers 1983, Westphal *et al.* 1993, Fetterolf *et al.* 1994), our results suggest that the cumulus cells, in response to PACAP, secrete one or more factors stimulating the forward movement of sperm for penetration.

The analysis of parameters of sperm motility confirmed this notion. The incubation of capacitated sperm with the PACAP-treated fluid resulted in an enhancement of lateral head displacement. While passing through the oviduct, sperm start to show vigorous movement of the flagellum with an increased amplitude of lateral head displacement and high

Figure 4 Effect of PACAP treatment of the cumulus–oocyte complexes on the fertilization rate. Cumulus–oocyte complexes were pretreated with PACAP at concentrations of 10 nM–1 μ M prior to insemination. The fertilization rate was determined by counting two-cell embryos. PACAP produced a dose-dependent increase in the fertilization rate. ** $P < 0.005$ versus control experiments without the PACAP pretreatment.

Figure 5 Effect of PACAP treatment of the cumulus–oocyte complex on sperm penetration. After the pretreatment with 1 μ M PACAP, the cumulus–oocyte complexes were inseminated. The number of sperm in the perivitelline space was counted after 1 h incubation. The relative number of sperm in the perivitelline space is expressed as a ratio of the number of sperm in the control experiments. The PACAP pretreatment significantly increased the number of perivitelline sperm. *** $P < 0.001$ versus control experiments. In the control experiments, the number of perivitelline sperm was 0.97 ± 0.27 sperm/egg ($n = 6$).

curvature, ‘hyperactivation’, which is considered necessary for penetration (Yanagimachi 1994). Moreover, the zona-induced acrosome reaction was stimulated by the PACAP-treated fluid. Our results indicate that factors contained in the PACAP-treated fluid could endow sperm with hyperactivation-like motility and stimulate the zona-induced acrosome reaction, which increase sperm ability to penetrate through the zona pellucida.

Progesterone is presumably responsible for the stimulation of sperm motility by the PACAP-treated fluid because an increased amount of progesterone was detected when the cumulus–oocyte complexes were treated with PACAP. Studies have demonstrated that human, mouse, porcine, and rabbit cumulus cells synthesize and secrete progesterone (Osman *et al.* 1989, Vanderhyden & Tonary 1995, Chian *et al.* 1999, Yamashita *et al.* 2003, Guidobaldi *et al.* 2008). In addition, human sperm express a cell surface receptor for progesterone (Luconi *et al.* 2004). Based on the finding that a picomolar concentration of progesterone chemotactically attracted human and mouse sperm, presumably by activating a signal transduction pathway in sperm (Teves *et al.* 2006, 2009), the increased amount of progesterone upon the PACAP treatment seems to be sufficient for the chemoattractant activity.

A previous study reported that low-micromolar levels of progesterone can stimulate the acrosome reaction and, furthermore, can enhance the zona-pellucida-induced acrosome reaction (Roldan *et al.* 1994), showing that progesterone is a co-factor for initiation of the reaction. Because progesterone was contained in the PACAP-treated fluid, which stimulated the

zona-induced acrosome reaction, progesterone may be responsible for the increased reaction. Although only nanomolar level of progesterone was detected in the PACAP-treated fluid, the concentration may be sufficient to prime sperm for the subsequent interaction with the zona pellucida. Alternatively, another molecule other than progesterone in the fluid may be a factor responsible for the effect.

It is still controversial where the acrosome reaction occurs on the zona surface or in the cumulus layer, or both. Because a zona component, ZP3, is a potent inducer of the reaction (Bleil & Wassarman 1980), and only acrosome-intact sperm can penetrate the zona pellucida (Yanagimachi 1994, Wassarman *et al.* 2001), it has been believed that sperm undergo the reaction on the surface of the zona pellucida. Based on the expression and localization of PAC1R exclusively on the cumulus cells, acrosomal PACAP is most likely to leak from the acrosome before sperm contact with the zona. This appears contradictory to the above notion, concerning the site of the acrosome reaction. Evidence gathered from *in vivo* and *in vitro* studies support our notion. Both acrosome-intact and acrosome-reacted sperm have been observed in the cumulus layer *in vivo* in various mammals including hamsters and humans by light and electron microscopy (Yanagimachi & Noda 1970, Bedford 1972, Cummins & Yanagimachi 1982). In addition, sperm underwent the acrosome reaction when incubated with mouse and bovine cumulus cells without the zona pellucida (Fukui 1990, Yin *et al.* 2009).

Our findings strongly suggest that PACAP is released from the acrosome while passing through the cumulus layer, and acts on the cumulus cells by way of PAC1R, possibly causing the cells to secrete soluble factors such as progesterone, which helps sperm to complete fertilization. This hypothesis is supported by the observation

Table 1 The effect of the cumulus–oocyte complex-conditioned medium on the parameters of sperm motility.

Test fluid/medium	Parameters of motility			
	ALH	BCF	VSL	VCL
TYH	3.19 ± 0.25	14.1 ± 0.66	22.5 ± 0.92	82.7 ± 5.93
TYH with PACAP	3.30 ± 0.15	14.3 ± 0.68	22.5 ± 1.42	82.6 ± 4.11
Control-treated fluid	3.28 ± 0.17	14.2 ± 0.64	22.8 ± 1.28	87.5 ± 4.09
PACAP-treated fluid	$3.76 \pm 0.15^*$	15.4 ± 0.52	22.3 ± 0.61	91.5 ± 3.31

Suspensions of capacitated sperm were mixed with the cumulus–oocyte complex-conditioned medium (fluid) or TYH medium at a 4:1 ratio and incubated for 30 min at 37 °C. The parameters of sperm motility were analyzed by a computer-assisted semen analysis (CASA). Observations were made from at least 2000 sperm. Values are the mean $\pm$ S.E.M. Explanation of parameters: ALH, amplitude of lateral head displacement (μ m); BCF, beat cross frequency (Hz); VSL, straight line velocity (μ m/s); VCL, curvilinear velocity (μ m/s). *Significantly different compared with all control experiments (TYH, TYH with PACAP, and control-treated fluid) at $P < 0.05$.

Figure 6 Effect of the cumulus–oocytes complex-conditioned medium on the acrosome reaction. Capacitated sperm were incubated with a conditioned medium prepared from the cumulus–oocytes complex in the presence or absence of PACAP, the PACAP-treated fluid, or control-treated fluid respectively and treated with ZP (2.4 ZP/ μ l) for 15 min to induce the acrosome reaction. The acrosome status was monitored using an Alexa-Fluor 594-conjugated SBTI. The PACAP-treated fluid significantly stimulated the zona-induced acrosome reaction. * $P < 0.05$ versus control experiments using the control-treated fluid.

in PAC1R-null mice that the lack of PAC1R resulted in reduced fertility when nullizygous females and wild-type males were mated but not when nullizygous males and wild-type females were mated (Jamen *et al.* 2000). In conclusion, our study emphasizes the importance of the sperm–cumulus cell interaction in fertilization. Recent study has also demonstrated the sperm–cumulus cell interaction mediated by a sperm glycosylphosphatidylinositol (GPI)-anchored protein, NYD-SP8, which is released from the sperm head and binds to the cumulus cells, causing an increase in secretion of progesterone from the cells (Yin *et al.* 2009).

Materials and Methods

Animals

Female (8 weeks) and male (12–16 weeks) mice (ICR strain) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were housed under controlled temperature (25 °C) and lighting (12 h light/day) conditions and allowed free access to food and water. Animal care and experimental protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the University of Toyama. Mice were killed by cervical dislocation.

Immunofluorescence microscopy

The distribution of PACAP was examined by indirect immunofluorescence microscopy, using rabbit antiserum produced against a synthetic PACAP27 as described previously (Matsuda *et al.* 2002). Sperm obtained from the cauda epididymides were fixed with 1% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.0, at room temperature for 5 min. After being

washed in PBS, cells were allowed to attach to glass slides and dried. The sperm were washed again in PBS, treated with a 10% normal goat serum blocking solution (Zymed Laboratories, San Francisco, CA, USA), and then incubated with the anti-PACAP rabbit antiserum diluted 1:1000 with the blocking solution at 4 °C overnight. After being washed in PBS, the sperm were incubated with an Alexa-Fluor 488-conjugated F(ab')₂ of goat anti-rabbit IgG (Molecular Probe, Eugene, OR, USA) diluted 1:2000 with PBS at room temperature for 1 h. Images were taken with a fluorescence microscope (model BZ8000, Keyence, Osaka, Japan). An immunoabsorption test was carried out to examine the specificity of the antiserum as described below. The anti-PACAP antiserum diluted 1:1000 with the blocking solution was incubated with 2 μ M PACAP38 at 4 °C overnight. The specific antibodies reactive to PACAP were spun down and the supernatant containing the nonspecific antibodies was used in the negative control experiments. For a precise examination in the acrosome, double staining was conducted with Alexa-Fluor 569-conjugated PNA-lectin, which predominantly reacts to the anterior acrosome.

Immunolabeling of the PAC1R was performed with an affinity-purified rabbit polyclonal anti-PAC1R antibody (Suzuki *et al.* 2003). Cumulus–oocyte complexes were prepared as described in the next section and fixed with 1% PFA in 0.1 M PB at room temperature for 5 min. After being washed with PBS, the complexes were treated with the blocking solution at room temperature for 1 h, and then incubated with the rabbit anti-PAC1R antibody diluted 1:400 with the blocking solution at room temperature for 2 h. The complexes were thoroughly washed by replacing the solution with PBS five times and then incubated with an Alexa-Fluor 488-conjugated F(ab')₂ of goat anti-rabbit IgG diluted 1:2000 with PBS for 30 min at room

Figure 7 Reverse phase HPLC chromatograms of the cumulus–oocyte complex-conditioned medium. A continuous gradient was used: 100% water to 100% acetonitrile over 40 min at a constant 1 ml/min flow rate. (A) The cumulus–oocyte complex-conditioned medium in the absence of PACAP (control-treated fluid). (B) The cumulus–oocyte complex-conditioned medium in the presence of PACAP (PACAP-treated fluid). Arrows indicate the fraction corresponding to progesterone.

temperature. After being washed with PBS, the complexes were placed on a glass slide and covered with a cover slip. The penetration of the antibodies through the cumulus mass was ascertained using an anti-CD9 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which recognizes an egg surface protein CD9. Under the above-mentioned conditions, the anti-CD9 antibody reacted to the egg. Images were taken with a confocal laser scanning microscope (TCS-SP5, Leica Microsystems, Wetzlar, Germany).

RNA extraction and PCR

Total RNA was extracted from the mouse brain, ovary, and cumulus cells using a Qiagen RNA kit (Qiagen). Cumulus cells were isolated from the cumulus–oocyte complexes that were dispersed with 2 mg/ml of hyaluronidase in the TYH medium. The RNA (0.5 µg) was reverse transcribed using a PrimeScript RT-PCR kit (Takara Bio, Inc., Ohtu, Japan) according to the manufacturer's instructions. PCR was performed using the following primer sets: forward (5'-CAA-GAAGGAGCAAGCCATGTGC-3') and reverse (5'-CAT-CGAAGTAATGGGGGAAGG-3') for mouse *Pac1r*; forward (5'-TGTGATGGTGGGAATGGGTCAGAA-3') and reverse (5'-GCTTCTCTTTGATGTACGCACGATT-3') for mouse β -actin. A 'touchdown' PCR was performed with TAKARA EX Taq HS DNA polymerase (Takara). The initial denaturation (94 °C for 2 min) was followed by a 30 s denaturation at 94 °C; a 30 s initial annealing at 67 °C, which was lowered by 2 °C every two cycles to a 'touchdown' temperature of 59 °C; and a 1 min extension at 72 °C. This in turn was followed by 30 cycles of a 30 s denaturation at 94 °C, 30 s annealing at 59 °C, and 1 min elongation at 72 °C. The PCR products were electrophoresed on 2% (w/v) agarose gel (Takara LO3 agarose) and stained with ethidium bromide.

IVF

TYH medium (Toyoda *et al.* 1971) was used for IVF assays. Cumulus–oocyte complexes were prepared as described below. Eight-week-old female mice were induced to super-ovulate with 10 IU of pregnant mares' serum gonadotrophin (Sigma Chemical) and 10 IU of human chorionic gonadotrophin (hCG, ASKA Pharmaceutical Co. Ltd, Tokyo, Japan), injected i.p. 48 h apart. Both oviducts were excised and placed in mineral oil (Sigma) 15–16 h after the hCG injection. Cumulus–oocyte complexes were released from the ampulla of the oviduct into a 200 µl drop of medium containing PACAP38 at a final concentration of 10 nM–1 µM under mineral oil. In the control, ultrapure water was substituted for the PACAP solution. After a 30 min incubation in the medium containing PACAP, the cumulus–oocyte complexes were transferred into 320 µl of fresh medium without PACAP supplemented with 0.4% BSA. Suspensions of epididymal spermatozoa from 12- to 16-week-old male mice were prepared and then incubated in the medium supplemented with 0.4% BSA for 90 min in a humidified culture chamber (37 °C, 5% CO₂ in air) to allow capacitation. Eighty microliters of the sperm suspension at a concentration of 10⁵ sperm/ml was mixed with the cumulus–oocyte complexes in a 320 µl

drop of the medium. At 24 h after insemination, the fertilization rate was determined by counting two-cell embryos as fertilized. For statistical analysis, we observed 250 or more oocytes in each experimental group, and conducted the experiments at least six times.

Sperm penetration assay

Ovulated cumulus–oocyte complexes were placed in the BSA-free TYH medium containing an anti-CD9 antibody (50 µg/ml, Santa Cruz Biotechnology) to block the sperm–oocyte fusion (Kaji *et al.* 2000). In the experimental group, 1 µM PACAP was contained in the medium. After 30 min incubation, the cumulus–oocyte complexes were transferred into 400 µl of fresh medium with BSA twice. Capacitated cauda epididymal sperm were diluted with TYH medium and placed with the oocytes in a 400 µl drop of TYH medium at a final concentration of 20 sperm/µl. Two hours after insemination, the cumulus–oocyte complexes were treated with hyaluronidase (0.05 mg/ml, type IV-S, Sigma) in PBS to disperse the cumulus cells, and the number of sperm in the perivitelline space was counted under a phase-contrast microscope. To compare data from a matched control, one of the two ampullae of oviducts from three individual mice was used in the experimental group and the other was used in the control. For statistical analysis, we counted the number of sperm in the perivitelline space of 200 or more oocytes in each experimental group, calculated the ratio of the number of sperm in the experimental group to that in the control group in each experiment ($n=6$), and presented the mean ratio.

Preparation of the cumulus–oocyte complex-conditioned medium

One hundred or more cumulus–oocyte complexes were placed in 50 µl of BSA-free TYH medium in the presence or absence of 1 µM PACAP, and incubated for 2 h at 37 °C in a CO₂ incubator. After centrifugation at 12 000 *g* for 20 min, the supernatant was collected and used as the conditioned medium, which is referred to as the PACAP-treated fluid or control-treated fluid.

Sperm motility analysis

To assess the effect of the cumulus–oocyte complex-conditioned medium on sperm motility, the cauda epididymal sperm suspension was split into four 80 µl aliquots and mixed with 20 µl of various test media: 1) the cumulus–oocyte complex-conditioned medium in the presence of PACAP (PACAP-treated fluid); 2) the cumulus–oocyte complex-conditioned medium in the absence of PACAP (control-treated fluid); 3) TYH; and 4) TYH with 1 µM PACAP. After 30 min incubation, the parameters of sperm motility were examined in a CASA using the Sperm Motility Analysis System (SMAS, DITECT Corporation, Tokyo, Japan). For statistical analysis, parameters for at least 2000 individual sperm were measured, and mean values were calculated from the results of six experiments.

Assessment of the acrosome reaction

Capacitated cauda epididymal sperm were mixed with the cumulus–oocyte complex-conditioned medium at a ratio of 4:1, and incubated for 15 min at 37 °C in a CO₂ incubator. ZP were isolated from ovulated eggs of 8–11-week-old mice as previously described (Tanii *et al.* 2001) and used for induction of the acrosome reaction at a concentration of 2.4 ZP/μl. The acrosomal status was monitored using an Alexa-Fluor 594-conjugated SBTI at a concentration of 1 μM as previously described (Tollner *et al.* 2000). After incubation with ZP and SBTI for 15 min, sperm were immobilized on a laminin-coated glass slide, and observed under a fluorescent microscope (BZ-8000, Keyence Corporation, Osaka, Japan). The rate of the acrosome reaction was calculated by dividing the number of Alexa-Fluor 594-positive live sperm by the number of total live sperm. For statistical analysis, mean values of the rate of the acrosome reaction were calculated from the results of four experiments and at least 400 individual sperm were observed in each experimental group.

HPLC

The cumulus–oocyte complex-conditioned medium was fractionated on a C₁₈ column (250×4.6 mm, Mightysil RP-18, Kanto Chemical Co., Inc., Tokyo, Japan) by reverse phase HPLC (LC-10A, Shimadzu, Kyoto, Japan). Aliquots of 45 μl were loaded into the column, running with a continuous gradient, 100% water to 100% acetonitrile, at a constant flow rate (1 ml/min). To confirm the fraction of progesterone, authentic progesterone was also assayed.

Enzyme immunoassay for progesterone and prostaglandins

Concentrations of progesterone and PGE₁ and PGE₂ in the conditioned medium were determined by a competitive immunoassay with commercial kits (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's directions. For statistical analysis, mean values of the amount of each molecule in the conditioned medium were calculated from the results of six experiments.

Statistical analysis

The data are shown as the mean ± S.E.M. The variables were normally distributed, and statistical significance was examined with William's test for multiple comparisons in IVF experiments, and with a two-sided paired *t*-test in experiments on sperm penetration, sperm motility, and acrosome reaction, and in enzyme immunoassays. Values with *P* < 0.05 were considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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