PHOSPHORYLATION OF FOLLICLE PROTEINS FROM THE TELEOST, *ORYZIAS LATIPES*, IN THE ACTION OF GONADOTROPIN AND FORSKOLIN

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ABSTRACT

Gonadotropin-stimulated fish follicle layers play a major role in inducing oocyte maturation via the stimulated secretion of steroids. In order to determine the molecular mechanism of the action of gonadotropin on fish ovarian follicles, we investigated the effect of pregnant mare serum gonadotropin (PMSG) on the accumulation of cAMP and on the protein phosphorylation in isolated ovarian follicle layers of the teleost, *Oryzias latipes*. Incubation of isolated follicle layers with PMSG resulted in a rapid increase in the production of cAMP. The response could be detected within 5 min after the addition of PMSG. When the homogenate of the follicle layers was incubated with [γ-32P]ATP, four endogenous proteins with Mr of 250 K (P1), 120 K (P2), 94 K (P3) and 75 K (P4) were phosphorylated. The phosphorylation of 94 K protein (P3) was not affected by cAMP, while that of P1, P2 and P4 was stimulated by the addition of cAMP. The enhancement of the phosphorylation by cAMP was low, but was inhibited by the protein kinase inhibitor protein from rabbit skeletal muscle and H8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), an inhibitor of cyclic nucleotide-dependent protein kinases. Phosphorylation of the 94 K protein (P3) was markedly enhanced by Ca2+ and calmodulin, indicating that ovarian follicle layers of the teleost contain an intrinsic calmodulin-dependent protein kinase and that P3 is an endogenous protein substrate for the kinase. The phosphorylation of P1, P2 and P4 was also enhanced by PMSG or forskolin in isolated intact follicle layers which had been prelabeled with [32P]phosphoric acid. In addition, the phosphorylation of P1, P2 and P4 in the [32P]-labeled isolated follicle layers was markedly reduced by prior exposure to PMSG or forskolin, indicating that the phosphorylation of the three proteins is under regulation of PMSG via cAMP. These results altogether strongly indicate that cAMP-dependent phosphorylation of the three proteins (P1, P2 and P4) is involved in the early action of PMSG on ovarian follicles of the fish.

Maturation of fish and amphibian oocytes surrounded by follicle cells can be induced *in vitro* by a pituitary extract or gonadotropin (GTH), or by steroids such as progestins, androgens, and corticosteroids. In amphibians, the pituitary GTH acts on the ovarian follicular tissues, causing them to mature (13, 24, 28, 32, 33, 35).

In the freshwater teleost, *Oryzias latipes*, removal of all follicular constituents resulted in a failure of induced maturation of oocytes in response to GTH, but the GTH-induced maturation of oocytes was not affected by removal of thecal cells and basal lamina from the follicle. Moreover, denuded oocytes were able to mature in the presence of GTH, when cocultured either with thecal cells previously separated from large prevulatory follicles or with post-ovulatory follicles. The results thus indicate that granulosa cells of large pre- and post-
ovulatory follicles are involved in the GTH-induced maturation of oocytes. The denuded oocytes, which are incapable of responding to GTH, can be induced by various steroids to enter meiosis (14). These findings have led to a hypothesis that follicle layer cells respond to GTH by producing steroids, which in turn induce the maturation of oocytes in *O. latipes* (14) as well as in amphibia (23). During the past decade, numerous studies on isolated granulosa cells from various animal species led to an assumption that GTH stimulates the adenylate cyclase system. The assumption was mainly based on the findings that GTH enhances the production of cAMP and that exogenous cAMP exhibits steroidogenic effects similar to GTH in culture systems (4, 5, 9, 12, 26, 29, 30). GTH as well as the receptors for GTH has been detected in ovarian follicles of some animal species, including a teleost (18, 19, 36). However, much less has been known about the effects of GTH on the cyclic nucleotide-dependent protein kinase system, and the detailed mechanism of the hormonal effects on follicle cells remained to be clarified. In the present study, we aimed at elucidating the mechanism of the specific effects of GTH on isolated follicle layers of ovarian follicles of a freshwater teleost, *O. latipes*, by examining whether or not cAMP and cAMP-dependent phosphorylation are involved in the hormonal action.

**MATERIALS AND METHODS**

**Materials**

cAMP, cGMP and ATP were obtained from Boehringer Mannheim GmbH (Mannheim, Germany), 3-isobutyl-1-methylxanthine (IBMX) from Aldrich (Milwaukee, WI, U.S.A.), [γ-32P]ATP (specific activity, 4,000 Ci/mmol) and [32P]phosphoric acid (carrier-free) from ICN Radiochemicals (Irvine, CA, U.S.A.), histones, molecular weight marker proteins and forskolin from Sigma (St. Louis, MO, U.S.A.), H8, a specific inhibitor of cyclic nucleotide-dependent protein kinase, from Seikagaku Kogyo (Tokyo, Japan), pregnant mare serum gonadotropin (Serotropin, PMSG) from Teikoku Zohki (Tokyo, Japan) and Earle's medium 199 (EM 199) from Dainippon Seiyaku (Osaka, Japan). In the present experiments, diluted EM 199 (90% EM 199 containing various reagents) was used. Calmodulin from rabbit testis was a gift from Dr M. Sano (Institute for Developmental Biology, Aichi Prefectural Colony, Aichi, Japan). Protein kinase inhibitor protein from rabbit skeletal muscle was purified by the method of Walsh et al. (40).

**Preparation and Incubation of Follicles**

Sexually mature medkas (*O. latipes*) were purchased from a local fish farm (Yamato-Kohriyama, Nara Prefecture, Japan) and kept at 26±0.5°C under a lighting regime of 14 h of light and 10 h of darkness. Under these conditions, females usually spawn daily just before 1 h of the onset of light. Vitellogenesis and meiotic maturation of individual oocytes occur 48 h after the onset of light. Intact follicles including oocytes were obtained by dissection of ovaries 2.5 h after the onset of light (21.5 h before spawning) in diluted EM 199 (supplemented with 50 mg/l penicillin G and 30 mg/l streptomycin sulfate, pH 7.3 with NaHCO3). Oocytes in the follicles were punctured with a fine forceps and the remaining follicular constituents (follicle layers) were rinsed in the medium. Five to twenty follicle layers were collected for each experiment. Isolated follicle layers were incubated in the wells of plastic culture dishes that contained 100 µl of diluted EM 199 in the continuous presence or absence of PMSG for varying periods of time at 25°C.

In this manuscript, follicle layers represent follicle walls, which include theca cell layer, basal lamina and granulosa cell layer, and a small amount of oocyte membranes.

**Phosphorylation in a Cell-Free System**

Follicle layers from five ovarian follicles were homogenized in 1 ml of cold 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM EGTA with the aid of a Potter-Elvehjem homogenizer. The reaction mixture for studying cyclic nucleotide-dependent phosphorylation of endogenous proteins contained 20 mM Tris-HCl buffer (pH 7.5), 10 mM 2-mercaptoethanol, 20 mM Mg-acetate, 0.5 mM IBMX, 0.5 µM cyclic nucleotide (when added), 1.0 µM [γ-32P]ATP (approximately 10⁶ cpm) and the homogenate (50–100 µg protein) in a final volume of 100 µl. The reaction mixture (100 µl) for the assay of calmodulin-dependent phosphorylation of endogenous proteins comprised 20 µg/ml calmodulin, either 0.4 mM EGTA (minus calcium) or 1.5 mM CaCl₂ (plus calcium), 2.0 µM [γ-32P]ATP (approximately 10⁶ cpm) and the homogenate (50–100 µg protein). After incubation for 1–15 min
at 25°C with constant agitation, the reaction was stopped by the addition of 100 µl of an SDS solution which contained 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and a small amount of bromphenol blue in 0.2 M Tris-HCl buffer (pH 6.7). The mixture was then boiled for 3 min and an aliquot of the sample was subjected to polyacrylamide gel electrophoresis (10% polyacrylamide) in the presence of 0.1% SDS (SDS-PAGE).

**Phosphorylation in Intact Follicle Layers**

Follicle layers dissected as described above and washed in diluted EM 199 medium were first incubated in diluted EM 199 medium in the presence of [32P]phosphoric acid (10 µCi) at 25°C for 30 min and transferred to a non-radioactive medium. The second incubation was then carried out in the presence of 100 IU/ml PMSG or 1.0 µM forskolin or in their absence. In other experiments, isolated follicle layers were first exposed to 100 IU/ml PMSG or 1.0 µM forskolin at 25°C for 30 min and then incubated with [32P]phosphoric acid (10 µCi) for 15 min. After the incubation, follicle layers were removed from the medium by centrifugation and washed twice with diluted EM 199 medium. They were then boiled in 100 µl of the SDS solution as described above. Aliquots, usually 20–30 µl, were subjected to SDS-PAGE.

**Electrophoresis and Autoradiography**

Electrophoresis was carried out in 10% polyacrylamide slab gels in the presence of 0.1% SDS at a constant current of 20 mA for 3 h (21), and the gels were stained in a solution of 0.25% Coomassie brilliant blue (41). Autoradiograms were prepared by exposing the stained gels at −70°C to Fuji X-ray film (RX) under an intensifying screen (Dupon Lightning Plus). The relative amount of protein and radioactivity in the individual bands were estimated by densitometric scanning of strips of the stained gels and autoradiograms with a Shimadzu dual-wavelength TLC scanner. The recorded absorbance was a linear function with respect to protein concentration and radioactivity under the conditions used.

**Other Methods**

Protein kinase was assayed as described in our previous paper (37). In brief, reaction mixtures (100 µl) comprised 50 mM Na phosphate buffer (pH 7.0), 10 mM Mg acetate, 10 mM 2-mercaptoethanol, [γ-32P]ATP (2 × 106 cpm), 0.5 µM cAMP or cGMP (when added) and 1 mg of a histone mixture (type II-S, Sigma). The reactions were initiated by the addition of enzyme and after incubation at 25°C for appropriate periods of time the reaction was terminated by spotting the mixture onto 2.5 cm (diameter) Whatman 3 MM filter discs. The discs were processed as described by Reimann et al. (27). One unit of the enzymic activity was defined as that amount of enzyme which transfers 1 pmol of 32P from [γ-32P]ATP to substrate protein in 5 min under the conditions described above. Concentration of cAMP in tissue extracts was determined by the method of Gilman (8). The preparation of samples for the cAMP-binding assay was performed as described previously (16). Protein was determined by the method of Lowry et al. (22) with bovine serum albumin as a standard.

**RESULTS**

**Effects of PMSG and Forskolin on the Formation of cAMP in Isolated Follicle Layers**

The follicle layers isolated from large preovulatory follicles just before the release of GTH (2.5 h after the onset of light) were incubated for various times in the absence or presence of 100 IU/ml PMSG or 1.0 µM forskolin. The kinetics of production of cAMP during the incubation of follicle layers are shown in Fig. 1A, which shows the increase in the content of cAMP in isolated follicle layers in terms of percentage over the untreated control. The addition of PMSG to the medium caused a significant increase in cAMP levels in the follicle layers at 2–10 min, the rate of increase declined thereafter (Fig. 1A). No further change in cAMP was observed beyond 30 min of the addition of PMSG and the elevated level of cAMP was then maintained as long as 6 h. When whole follicles including oocytes were used instead of the follicle layers, the level of cAMP did not change significantly, and in the case of denuded oocytes the level of cAMP decreased slowly during the incubation period even in the presence of the same concentration of PMSG (data not shown). When a low concentration of forskolin (1.0 µM) was added to the medium, the production of cAMP in the follicle layers was greatly enhanced and this increase continued up to 30 min, indicating the occurrence of a high level of adenylate cyclase activity in these cells (Fig. 1B). A similar
increase in the cellular cAMP level (500±12% increase over the cAMP level of untreated control) was observed when isolated granulosa cells were incubated with the same concentration of forskolin, suggesting that the increased levels of cAMP in the isolated follicle layers are mainly due to the changes in granulosa cells.

These results strongly suggest that as in other species of animals, cAMP and cAMP-dependent phosphorylation systems are involved in the early steps of GTH-stimulated steroidogenesis.

**Phosphorylation of Endogenous Proteins in the Cell-Free System**

In order to assess the ability of cAMP to promote the phosphorylation of specific follicle layer proteins and to detect protein kinase activities in the tissue, homogenate of follicle layers was incubated in the reaction mixture for cyclic nucleotide-dependent phosphorylation of endogenous proteins as described under Materials and Methods. The densitometric scanning of the resultant autoradiograms is shown in Fig. 2. After incubation without cyclic nucleotides, incorporation of $^{32}$P was observed in several bands, which corresponded to apparent $M_r$ of 250 K, 120 K, 94 K and 75 K (Fig. 2a). These proteins are designated as P1, P2, P3 and P4, respectively, in this paper. Phosphorylation of P1, P2 and P4 was enhanced by cAMP (Fig. 2b). The stimulatory effect of cAMP was low, but the protein kinase inhibitor protein from rabbit skeletal muscle and H8, inhibitors for cyclic nucleotide-dependent protein kinases, inhibited the stimulation (Fig. 2, d and e). Cyclic GMP was totally ineffective in the stimulation of the phosphorylation (Fig. 2c). The results suggest that follicle layers contain cAMP-dependent protein kinase(s) and the specific endogenous substrate proteins for the kinase(s). The cAMP-dependent protein kinase activity in homogenate of follicle layers was determined to be quite high (specific activity: 23,000 units per mg of follicle layer protein) with a histone mixture (type II-S) as substrate. In addition to the phosphorylation of above-mentioned proteins, phosphorylation of P3 was increased dramatically in the presence of calmodulin and Ca$^{2+}$ (Fig. 2f). Removal of calmodulin from the reaction mixture caused a decrease in the incorporation of $^{32}$P into the protein (Fig. 2g), indicating that P3 is a specific endogenous substrate for the calmodulin-dependent protein kinase. Alkaline treatment of gels
Phosphorylation of endogenous proteins from follicle layers. The homogenate of follicle layers was incubated in the reaction mixture for cyclic nucleotide-dependent (a–c) or calmodulin-dependent (f–h) phosphorylation of endogenous proteins at 25°C for 2 min, as described under Materials and Methods. After the incubation, an aliquot of the reaction mixture (equivalent to 50 μl) was removed and subjected to SDS-PAGE (10% acrylamide gel), followed by autoradiography. The autoradiograms were scanned with a Shimadzu dual-wavelength TLC scanner. Additions during the incubation were as follows; a, none; b, 0.5 μM cAMP; c, 0.5 μM cGMP; d, 0.5 μM cyclic AMP and 2 μg protein kinase inhibitor protein from rabbit skeletal muscle; e, 0.5 μM cAMP and 5.0 μM H8 (inhibitor for cyclic nucleotide-dependent protein kinase); f, 2 μg calmodulin (plus Ca²⁺); g, 2 μg calmodulin (minus Ca²⁺); h, minus calmodulin, minus Ca²⁺. Molecular weight marker proteins used were α-chymotrypsinogen (26,000), ovalbumin (45,000), bovine serum albumin (68,000), phosphorylase b (94,000), heavy chain of silkworm vitellin (180,000; see reference 37), and sea urchin dynein subunit (320,000; see reference 7).

Before autoradiography caused the disappearance of radioactive bands, suggesting that the amino acids which had been phosphorylated might be principally serine and threonine residues (data not shown).

In another phosphorylation experiment, follicle layers were homogenized after incubation at 25°C for 30 min in the presence or absence of PMSG. When homogenate of follicle layers prepared after the preincubation in the absence of PMSG was subjected to the reaction for cyclic nucleotide-dependent phosphorylation of endogenous proteins, the phosphorylation of P1, P2 and P4 was evident (Fig. 3, lane 1). The relative intensity of each band in this figure differs from that in Fig. 2a, but this is solely due to the fact that in this experiment, the homogenate was incubated in the phosphorylation reaction mixture for a longer period (15 min) to ensure the detection of the phosphorylated bands. By contrast, when the homogenate was prepared from follicle layers after the pretreatment with PMSG, most of the radioactive bands in the autoradiogram disappeared (Fig. 3, lane 2), indicating that the hormonally controlled proteins had been mostly phosphorylated during the preincubation with PMSG. The results together with those of Figs. 1 and 2 provide further evidence for the GTH- or cAMP-dependent phosphorylation of the endogenous proteins, (P1, P2 and P4).

Phosphorylation of Proteins in Intact Isolated Follicle Layers

In order to ascertain that the kinases are actually operating in the cell, isolated intact follicle layers were first incubated with [32P]phosphoric acid to
allow endogenous ATP to be phosphorylated, and then the second incubation was carried out in the presence or absence of PMSG or forskolin. No phosphorylated endogenous proteins were detectable in the prelabeled follicle layers before the second incubation, but upon subsequent exposure to PMSG or forskolin an endogenous proteins corresponding to P4 on SDS-PAGE was rapidly (within 1–2 min) phosphorylated (Fig. 4, A–C). In the absence of the hormone or forskolin P4 was phosphorylated only after 20–30 min. The phosphorylation of P1 and P2 was also observed, although the degree of the phosphorylation was much lower under the conditions used (data not shown).

In order to confirm above results, a second set of experiments was carried out, in which the isolated follicle layers were first exposed to PMSG and then incubated with [32P]phosphoric acid. Most of the proteins whose phosphorylation was hormonally controlled were supposed to be phosphorylated during the preincubation with PMSG. As shown in Fig. 4D, when the follicle layers had been preincubated without PMSG or forskolin (lane 1), phosphorylation of P1, P2 and P4 was evident. On the contrary, when the isolated follicle layers had been preincubated with PMSG (lane 2) or forskolin (lane 3), most of the radioactive bands diminished in intensity, indicating that the phosphorylation of the three proteins (P1, P2 and P4) is under the regulation of PMSG via cAMP and three proteins play an important role in the cAMP-mediated action of the hormone.

We conclude from these results that, in fish follicles, PMSG stimulates cAMP production and phosphorylation of specific proteins in the follicle layers.

**DISCUSSION**

A number of recent studies have been devoted to the mechanism of the action of gonadotropins on ovarian follicles (1, 6, 11, 31). It has been generally accepted that the action of GTH in stimulating steroidogenesis is mediated by cAMP and cAMP-dependent protein kinase systems. In the case of lower vertebrates, such as a freshwater teleost, however, details of the action of GTH still remained to be clarified (25). We have previously reported that forskolin, an activator of adenylate cyclase, and a hemolymph factor have an effect similar to PMSG (15, 16). We suggested that cAMP might be involved in the early stages of the action of GTH in fish follicles. The present experiments were designed to analyze in more detail the mechanism of the action of PMSG on ovarian follicles of the fish.

The data presented in this paper suggest that cAMP and a cAMP-dependent protein kinase system are involved in the response of ovarian follicles to PMSG although we have not directly assayed adenylate cyclase. The ability of PMSG and forskolin to increase the cAMP concentration in isolated follicle layers strongly suggests that the cyclase is present in the ovarian follicle layers and responds to PMSG. Furthermore, cAMP-dependent protein kinase and its endogenous substrates were detected in this tissue, and the phosphorylation of several endogenous substrate proteins was stimulated by both PMSG and forskolin in the intact tissue. The phosphorylation of endogenous substrate proteins occurred prior to the physiological response of the tissue (steroidogenesis: 14, 16) in parallel to the increase in the level of cAMP. Taken together, these data suggest that some biochemical steps, distal to the receptor, to activation of adenylate cyclase and to cAMP-dependent phosphorylation sys-
tem, are involved in steroidogenesis.

It is noteworthy that the three substrate proteins (P1, P2 and P4) were found to be phosphorylated in the intact follicle layers in the presence of PMSG and forskolin. Significant differences were observed in the kinetics and patterns of phosphorylation of the endogenous protein substrates after stimulation by PMSG and forskolin. These proteins were only weakly phosphorylated in unstimulated follicles (Fig. 4). The labeling of P4 protein is obvious as early as 2 min after the addition of PMSG and 1 min after addition of forskolin. P1 protein was also weakly phosphorylated under these conditions. In the presence of forskolin, phosphorylation of P2 was observed in addition to that of P1 and P4, whereas it was not significant in the presence of PMSG. At present it is not yet clear whether or not the phosphorylation of P2 is actually involved in the early step in the action of PMSG.

We have not yet enough information to assess the physiological functions of these proteins, but we assume that these proteins may be components of the cell membranes since most of them were sedimented by low speed centrifugation (data not shown). The results thus suggest that the proteins may function in the membrane fraction of the cells.

GTH-induced steroidogenesis in granulosa cells (ovarian follicles) is generally considered to be initiated by the activation of the adenylate cyclase and cAMP-dependent phosphorylation system after the binding of the hormone to its specific receptors. The increase in the levels of cAMP by itself cannot, however, fully account for the steroidogenic response of the cells to GTH. Recent studies have indicated that calcium plays an important role in GTH-mediated steroidogenesis not only in ovarian
follicle cells, but also in the testis and in the adrenal gland (2, 3, 17, 20, 39). Among the results presented in this paper, it is noteworthy that isolated follicles from O. latipes contain calmodulin-dependent protein kinase(s) and its endogenous substrate protein. Although the cAMP-dependent mechanism is an essential step in the GTH-mediated ovarian steroidogenesis, calcium has also been shown to modulate hormonally stimulated steroidogenesis in ovarian follicles (2, 3, 10, 39). Among the various calcium-dependent enzymes, a calcium-activated, phospholipid-dependent protein kinase (protein kinase C) and calmodulin-dependent protein kinases have been implicated in the GTH-induced production of steroids (2, 3, 10, 34, 38, 39). Although the studies to date suggested important roles of protein kinase C in modulating the steroidogenic activation of gonadal cells (38, 39), role of protein kinase C on the steroidogenic pathway remained to be clarified. At present, under our conditions, the activity of protein kinase C was not detectable in the isolated follicle layers. Recently, several groups of researchers have discussed the importance of calmodulin and calcium in the steroidogenic effect of GTH (10, 38, 39). In the present study, the effect of PMSG on the phosphorylation of the calmodulin-dependent system was not clearly demonstrated in intact cells, but this was probably due to the fact that the concentration of calcium in the incubation medium was not critically controlled, i.e., the reactions were carried out in the presence of excess calcium. It is highly susceptible from the presence of high levels of calmodulin-dependent protein kinase and endogenous substrate protein that the calmodulin-dependent protein kinase system plays an important role in the regulation of GTH-mediated steroidogenesis. The present study provides substantial support for the hypothesis that CAMP and a cAMP-dependent phosphorylation system play an essential role in the GTH-mediated steroidogenesis in O. latipes. Further studies of the physiological functions of endogenous substrate proteins of the kinase are now in progress.

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