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Visualization of Progesterone Binding to Plasma Membrane of *Xenopus* Oocytes

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We have previously shown that oocyte maturation is induced by an immobilized progesterone, progesterone-3-carboxymethyloxime - bovine serum albumin conjugate (P-BSA) in *Rana dybowskii*. In this study, we confirmed the maturation inducing activity of P-BSA on *Xenopus* oocyte and examined the binding character of the immobilized progesterone on the surface of *Xenopus* oocytes after removal of the vitelline layer. P-BSA induced maturation of *Xenopus* oocytes but E-BSA failed to do so as observed in *Rana*. Binding of the immobilized progesterone, fluorescein isothiocyanate-labeled progesterone-3-0-carboxymethyloxime-BSA (P-BSA-FITC) on the devitellined oocytes surface was examined by fluorescence confocal microscopy. The binding affinity of P-BSA-FITC to the devitellined oocyte was higher than that of estrogen-BSA-FITC (E-BSA-FITC) or testosterone-BSA-FITC (T-BSA-FITC). The binding disappeared in the presence of excess free progesterone but not in the presence of free estrogen. Maximum binding occurred after two-hours of incubation with P-BSA-FITC at pH 7.5. Stronger binding occurred in oocytes at stage VI than stage IV, and *in vitro* treatment of hCG enhanced the binding. Taken together, these results suggest that a specific receptor for progesterone exists on the plasma membrane of *Xenopus* oocytes and that progesterone acts initially on this putative receptors and triggers generation of membrane-mediated second messengers during the early stage of oocyte maturation in amphibians.

Oocyte maturation is the process by which oocytes arrested in prophase I resume meiosis and complete their first meiotic division. The maturation is stimulated by progesterone, which is synthesized and released by follicle cells in response to pituitary gonadotropins (Fortune et al., 1975). This process involves chromosome condensation, germinal vesicle breakdown (GVBD), nuclear and cytoplasmic division and extrusion of the first polar body (Masui and Markert, 1971; Maller, 1990; Jessus and Ozon, 1993). Several lines of evidence indicate that progesterone interacts initially with the oocyte surface to regulate subsequent events leading to GVBD rather than using a conventional nuclear hormone receptor. Microinjection of progesterone into denuded oocytes failed to induce cell division (Masui and Markert, 1971), but polymer bound steroids could effectively induce maturation of *Xenopus* oocytes. These facts suggest that progesterone interacts with a hormone receptor located at the oocyte surface (Ishikawa et al., 1977; Godeau, et al., 1978). The presence of a progesterone receptor on the surface of oocytes was initially indicated by photoaffinity labeling with synthetic progestin (Sadler and Maller, 1982, 1983) and by radioreceptor binding assays using total plasma membrane fraction in *Xenopus* (Liu and Patino, 1993) and in *Rana pipiens* (Kostellow et al., 1982). However, all these biochemical data could not provide definitive evidence for the progesterone action on oocyte surface. Recently, we reported that P-BSA induced oocyte maturation in *Rana dybowskii* (Bandyopadhyay et al., 1998). Moreover, progesterone caused a rapid and transient increase in membrane-mediated second messengers in oocytes in a manner similar to that produced by protein or peptide hormones when they act through membrane receptors (Chien et al., 1991; Morril et al., 1994; Bandyopadhyay et al., 1998).

Although the amphibian oocyte is the most commonly used model system to study non-genomic actions of steroid, membrane steroid receptors have been also reported in various other systems. For example, progesterone receptors were localized at the mammalian sperm head surface as revealed by P-BSA-FITC and confocal microscopy (Blackmore and Lottanzio, 1991; Aitken et al., 1996). A specific plasma membrane

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receptor was also identified in rat brain using P-BSA in affinity chromatography (Ramirez et al., 1996). Some reports showed the presence of progesterone binding site in luteal cell membranes (Bramley and Menizies, 1994; Rae et al., 1998) and in hepatocytes (Eisen et al., 1997). Estrogen was also known to interact with a membrane binding protein in liver, adrenal, and spleen in rat (Robert et al., 1998). Recently, we have shown that exposure of *Rana dybowskii* oocytes to immobilized progesterone (P-BSA) elicited generation of inositol triphosphate (IP3) and diacylglycerol (DAG), activation of protein kinase C (PKC), and induction of oocyte GVBD (Bandyopadhyay et al., 1998).

The aim of the present study was to ascertain whether the induction of oocyte maturation by progesterone is mediated by a plasma membrane receptor in *Xenopus*. Visualization of the progesterone receptor on oocyte surface was performed by laser confocal microscopy, and some characteristics of the binding was also investigated.

Materials and Methods

Animals

Adult female *Xenopus laevis* were purchased from Nasco (Fort Alkinson, WI) and maintained in glass or plastic boxes. The photoperiod and temperature were kept at 12L:12D and 20℃, respectively. The frogs were fed Nasco Frog Brittle at approximately 1% of their body weight twice a week. Under these conditions, females remain in reproductive condition throughout the year with ovaries containing a heterogeneous population of oocytes.

Hormones and reagents

Progesterone, progesterone-3-O-carboxymethyloxime-BSA (P-BSA, progesterone:BSA = 38:1), progesterone-3-O-carboxymethyloxime-BSA-fluorescein isothiocyanate (P-BSA-FITC, progesterone:BSA = 8:1), estradiol-3-O-carboxymethyloxime-BSA (E-BSA, estradiol:BSA = 32:1), estradiol-3-O-carboxymethyloxime-BSA-fluorescein isothiocyanate (E-BSA-FITC), testosterone-3-O-carboxymethyloxime-BSA (T-BSA, testosterone:BSA = 38:1), and testosterone-3-O-carboxymethyloxime-BSA-fluorescein isothiocyanate (T-BSA-FITC) were purchased from Sigma Chemical Co. (St. Loius, MO).

Procedures to obtain different types of follicular oocytes and culture

Animals were injected with pregnant mare serum gonadotropin (PMSG, Sigma, 50 I.U./animal) 3 d prior to oocyte retrieval. Before surgery, frogs were deeply anesthetized with cold water. A fragment of ovary was surgically removed and placed at 20℃ in Modified Barth's Solution (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes (pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, penicillin G (10 mg/L), and streptomycin sulphate (10 mg/L). Stage of the oocytes was defined according to the classification of Dumont (1972). From intact follicle, theca/epithelial (THEP) layer was peeled off manually by defolliculation and granulosa cell enclosed oocyte (GCEO) was obtained. Denuded oocyte without granulosa cell was obtained by incubating GCEOs in calcium free medium with agitation (Kwon and Lee, 1991). From the denuded oocytes, outer vitelline layer were peeled off with a micro forcep which generated devitellined oocytes. For maturation experiments, the denuded oocytes were incubated in the absence or presence of progesterone-BSA conjugate for 8 h in a shaking incubator at 20℃. At the end of the incubation, the oocytes were treated with 5% trichloroacetic acid and examined for GVBD under a stereomicroscope. Ovarian fragment culture was employed when the effect of hCG(human chorionic gonadotropin) on progesterone binding on oocyte was examined (Kwon et al., 1992). Ovarian fragment containing around 10 follicles was preincubated in the presence of hCG for 1-4 h, and then ovarian follicles were defolliculated, denuded, devitellined, and processed for confocal microscopy.

Laser scanning confocal fluorescence microscopy

Devitellined stage VI oocytes were rinsed extensively in MBS and incubated in the presence of progesterone-BSA-FITC for 2 h in a shaking incubator at 20℃. Confocal microscopy was carried out on a Leica TVS 4D system adapted to Leica IMT. Five devitellined oocytes were incubated in MBS in the presence or absence of 1 μM equivalent steroids of P-BSA-FITC, or 1 μM equivalent steroids of BSA-FITC, T-BSA-FITC or E-BSA-FITC. The oocytes were transferred to wells containing fresh MBS and washed intensively with three changes of fresh MBS. Finally, they were transferred to a petri dish containing fresh MBS. At least three oocytes were then placed onto a small petri dish containing MBS and laser scanning confocal microscopy (Leica, TCS NT, Germany) was performed. The surface of an oocyte was initially visualized using TRITC (tetramethylrhodamine B isothiocyanate) labeled concanavaline A which specifically binds to membrane glycoproteins. TRITC was known to be useful to locate the plasma membrane of an oocyte with FITC labeled steroid-BSA conjugates. Oocytes were visualized using a confocal microscope, with 5X objective and appropriate filters for TRITC and FITC separately. One image of oocyte from two separate experiments was chosen and stored in a computer diskette. Images were printed out with a image printer (FUJIX PICTOGRAPHY 3000). For competitive binding experiments, devitellined oocytes were pre-incubated in the presence of excess unlabeled steroids (10 to 100 folds) for 30 min followed by the addition of 1 μM FITC labeled steroids, and

incubated further for 2 h. Cells were observed by laser scanning confocal microscopy after washing as described above. Preliminary studies showed that binding of P-BSA-FITC (1 μM) was readily visualized after 1 to 2 h of incubation. Hence, the incubation time was fixed for 2 h for all subsequent experiments.

Quantitative analysis of fluorescence on the oocyte surface

Fluorescence intensity in the oocyte surface was determined by densitometry using an image analysis program (TINA). The density of fluorescence was expressed as relative intensity considering the optical density/mm^2 area of BSA-FITC as 1 in each experiment.

Statistics

Most experiments were conducted three times with five oocytes per incubation and at least two oocytes were visualized by laser scanning confocal microscopy. The figures show data from a single representative experiment or the mean ± SEM of data pooled from three independent experiments. Statistical analysis was done by Students' t test, accepting $p < 0.05$ as statistically significant.

Results

Induction of oocyte maturation by BSA-bound steroids

Previously, we have shown that P-BSA which is not permeable to oocyte plasma membrane can induce oocyte maturation in *Rana dybowskii* (Bandyopadhyay et al., 1998). In this study, we examined whether P-BSA and P-BSA-FITC can induce oocyte maturation in *Xenopus*. As expected, 1 μM of P-BSA and P-BSA-FITC induced GVBD of most oocytes examined (>90%), whereas estrogen, E-BSA and E-BSA-FITC failed to do so (Fig. 1). These results demonstrate that P-BSA-FITC is biologically active and can induce GVBD of oocyte probably through a receptor localized on the plasma membrane in *Xenopus*.

Visualization of progesterone binding at the devitellined oocyte surface

We have previously reported that P-BSA-FITC binds to the surface of denuded oocytes in *Rana dybowskii* (Bandyopadhyay et al., 1998). In the present experiment, we examined whether P-BSA-FITC binds to devitellined oocyte surface. After the vitelline layer was removed from denuded oocytes, the devitellined oocytes were incubated with fluorescence labeled immobilized progesterone and examined for the location of this steroid under a confocal microscope. In order to validate the confocal microscopic localization of oocyte surface, the oocytes were incubated with TRITC labeled concanavaline A for 1 h, which was known to specifically recognize the glycoproteins at the plasma

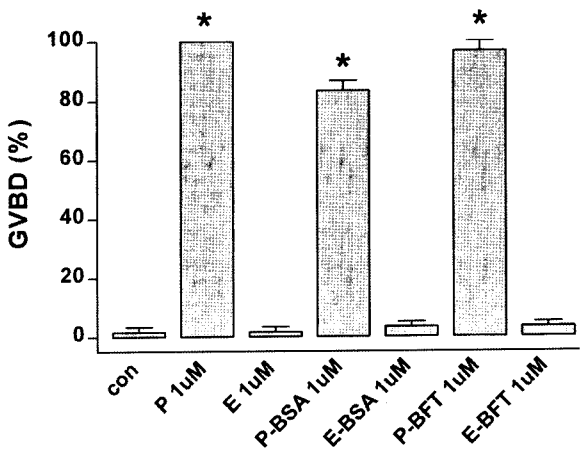

Fig. 1. Induction of oocyte maturation by BSA-conjugated steroids in denuded oocytes. Denuded oocytes were cultured in MBS at 20℃ in the absence (con) or presence of progesterone (P), estradiol (E), or each BSA-conjugated steroids and GVBD was examined after incubation for 8 h. Data represent the mean ± SE of three independent experiments (10 oocytes / experiment). BSA; bovine serum albumin, P-BSA; progesterone conjugated BSA, E-BSA; estradiol conjugated BSA, P-BFT; P-BSA-FITC, E-BFT; E-BSA-FITC. * $p < 0.05$ when compared to control.

membrane, and were observed under a confocal microscope. Strong fluorescence was observed only at the oocyte surface indicating that concanavaline A bound along the periphery of the oocyte (data not shown). For visualization of P-BSA-FITC binding, the devitellined oocytes were incubated in the presence of 1 μM of FITC labeled P-BSA for 2 h and individual oocytes were subjected to laser scanning confocal microscopy. As shown in Fig. 2, the fluorescence at the oocyte surface was visible in the oocyte treated with P-BSA-FITC, whereas it was not or weakly visible in the oocytes treated with BSA-FITC, T-BSA-FITC, or E-BSA-FITC (Fig. 2A). The relative binding intensity of P-BSA-FITC was significantly higher than that of control (BSA-FITC) or other steroids (Fig. 2B). Thus, these results indicated that P-BSA-FITC bound to the oocyte surface and that the binding site was specific for progesterone.

As P-BSA-FITC binds to putative membrane progesterone receptor, free progesterone may competitively interfere with the binding of P-BSA-FITC to oocyte surface. To determine whether P-BSA binding at the oocyte surface was specifically displaceable by the genuine ligand progesterone, oocytes were pre-incubated for 30 min with or without free progesterone or estradiol and incubated further for 2 h in the presence of P-BSA-FITC. The pre-incubation with 10-fold excess of the unlabeled progesterone significantly reduced the P-BSA-FITC induced fluorescence intensity (Fig. 3). This was not changed significantly when the oocytes were pre-incubated with 10-fold excess of estradiol. Thus, competition studies showed that progesterone specifically displaced the P-BSA-FITC induced fluorescence while other steroids did not.

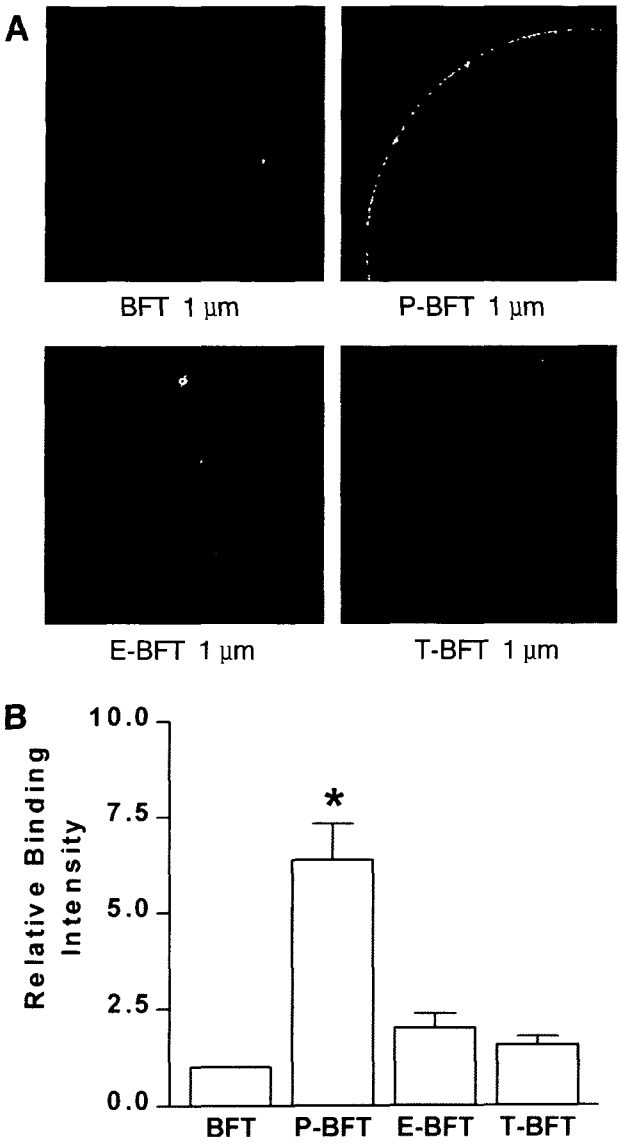

Fig. 2. Comparision of binding intensity among various steroid-BSA-FITC in oocyte. Devitellined oocytes were cultured in MBS containing 1 μM of steroid BSA-FITCs for 2 h. A, Confocal micrograms showing the visualization of steroid-BSA-FITC conjugates on the outside surface of the devitellined oocytes. B, Quantitative analysis of data A. Binding intensities were expressed as relative value when compared to that of BFT treated oocyte. BFT; BSA-FITC, P-BFT; P-BSA-FITC, E-BFT; E-BSA-FITC, TBFT; T-BSA-FITC. $*p < 0.05$ when compared to BFT treated oocyte.

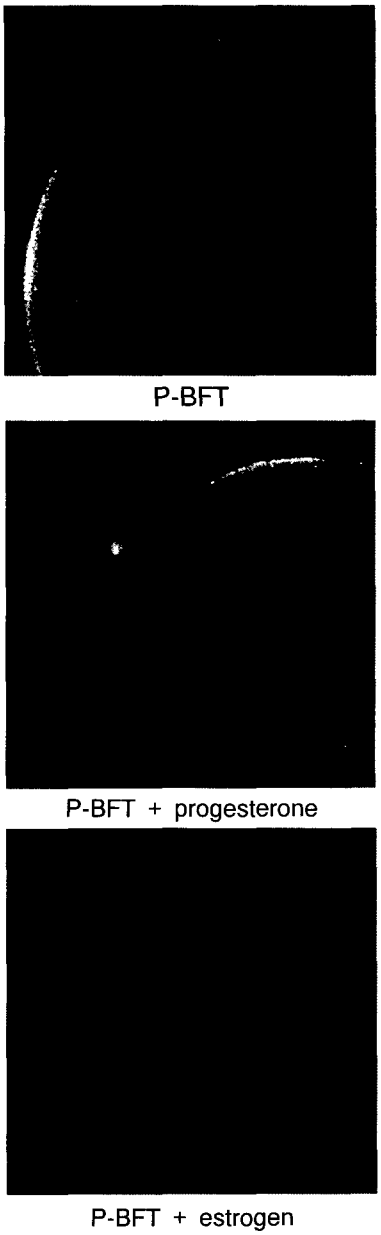

Fig. 3. Displacement of progesterone-BSA-FITC binding by free progesterone in oocyte. Devitellined oocytes were pre-incubated with 10-fold excess of free progesterone or estrogen and then cultured in MBS containing 1 μM of P-BFT for 2 h. visualization of P-BFT conjugates was examined by confocal microscope. BFT; BSA-FITC, P-BFT; P-BSA-FITC, E-BFT; E-BSA-FITC.

Factors affecting the binding of P-BSA-FITC to devitellined oocyte

To identify factors affecting the binding of P-BSA-FITC to progesterone receptor, effect of pH on the binding of P-BSA-FITC to devitellined oocyte was examined in the pH range between 6.0 and 9.0. Optimal binding of P-BSA-FITC to oocyte surface was observed at pH 7.5 (Fig. 4). The binding intensity was low at acidic (pH 6.0-7.0) or alkaline (pH 8.0-9.0) conditions (Fig. 4).

Oocyte maturation is initiated by progesterone, which is synthesized and released by follicle cells in response to pituitary gonadotropins (Fortune et al., 1975). We hypothesized that treatment of gonadotropin (hCG) may affect the affinity of progesterone receptor. Ovarian fragments (follicles) were preincubated in MBS containing 50 IU/ml of hCG for 1-4 h and then follicles were defolliculated, denuded, devitellinated. The devitellinated oocytes were then treated with steroid-BSA-FITC and observed under confocal microscope. As shown in Fig. 5, this treatment increased the binding intensity of P-BSA-FITC to devitellined oocyte when

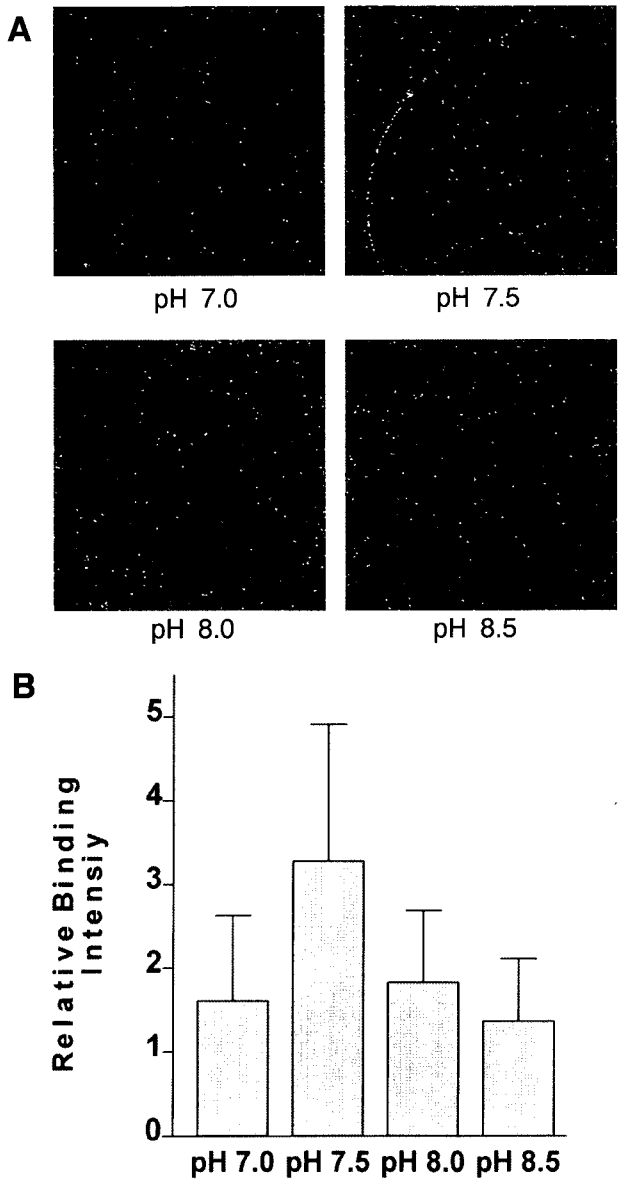

Fig. 4. Effect of pH on the binding of P-BSA-FITC to oocyte. Devitellined oocytes were prepared in MBS at the various pH values and incubated 2 h for microscopy. visualization of P-BFT conjugates was examined by confocal microscope. A, Confocal micrograms showing the visualization of P-BFT conjugates on the outside surface of the devitellined oocytes. B, Quantitative analysis of micrograms.

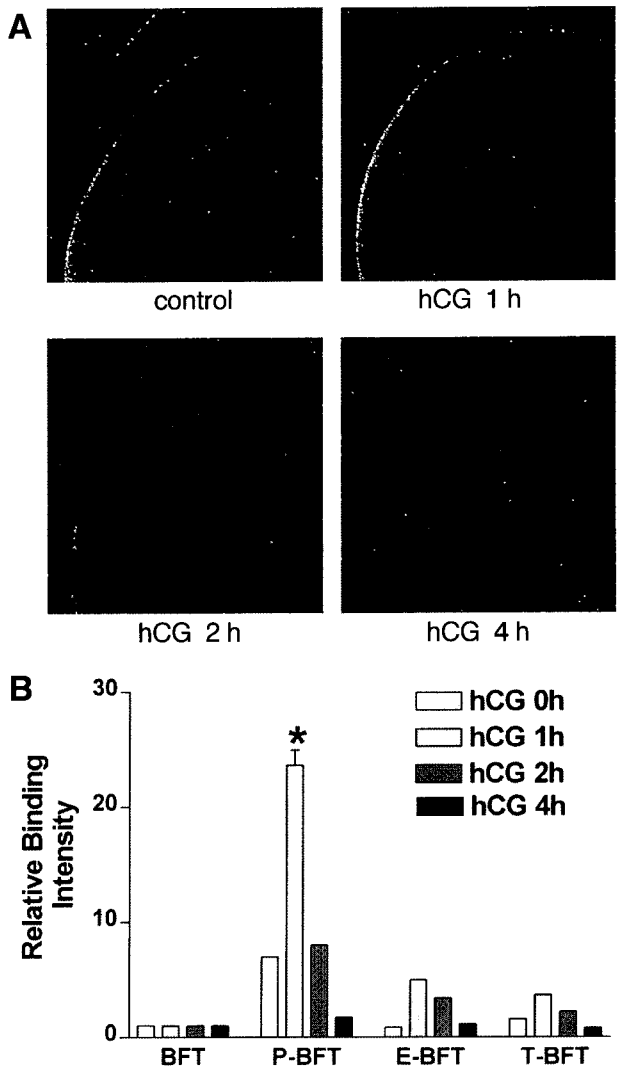

Fig. 5. Effect of hCG treatment on the binding of P-BSA-FITC to devitelline oocyte. Ovarian follicles were preincubated for 1-4 h in MBS containing 50 IU/ml of hCG, and devitellined oocyte were obtained as described in Materials and Methods. A, Confocal microscope micrograms showing the visualization of P-BSA-FITC conjugates on the outside surface of the oocytes. B, Quantitative analysis of micrograms. Data were expressed relative to the levels observed in BFT treated oocyte. $* p < 0.05$ when compared to control.

compared to control(not treated). After 1 h treatment with hCG, the binding intensity of P-BSA-FITC reached maximum levels (Fig. 5B). In contrast, hCG pretreatment caused a negligible effect on E-BSA-FITC or T-BSA-FITC binding intensity (Fig. 5B). These results suggested that hCG might increase the number of progesterone binding sites on oocyte surface.

Changes in binding of P-BSA-FITC during oocyte growth

Stage V and VI oocytes, but not stage IV oocytes, mature in response to progesterone in Xenopus (Sadler and Maller, 1983). We hypothesized that this difference in sensitivity to progesterone was caused by the difference in the number of progesterone membrane receptor. We examined the binding of P-BSA-FITC to small (stage IV) and large (stage VI) oocytes. The binding intensity appeared to be much stronger in Stage VI oocytes than Stage IV oocytes (Fig. 6A). The binding intensity in stage IV oocytes was about a third of that observed in stage VI oocytes (Fig. 6B). Based on these results, we assumed that full grown oocytes might have enough number of progesterone receptor while smaller oocytes had much less number of progesterone receptors on their oocyte surface.

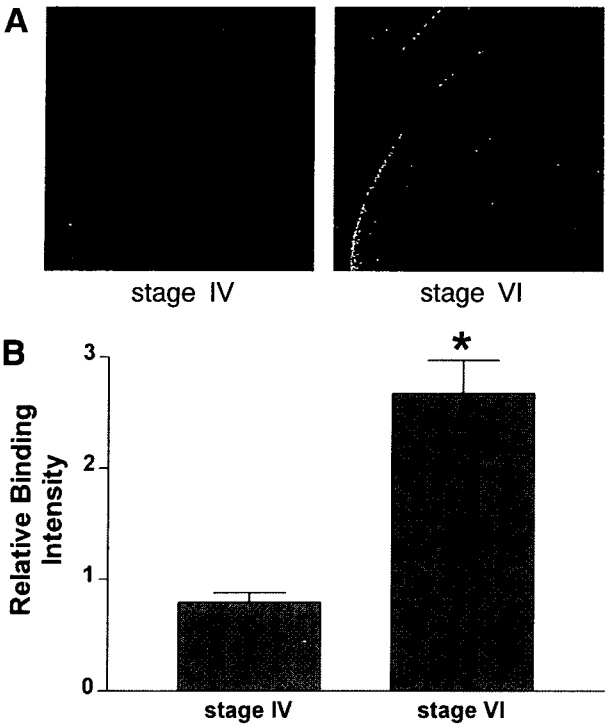

Fig. 6. Changes in the binding of P-BSA-FITC during oocyte growth. Devitellined oocytes were prepared from growing follicles (stage IV, average diameter, 0.9 mm) and full grown follicles (Stage VI, average diameter, 1.2 mm) and localization of P-BFT conjugates was examined. A) Confocal microscope micrograms showing the visualization of P-BFT conjugates on the outside surface of the devitellined oocytes. B) Quantitative analysis of micrograms. Data were expressed relative to the levels observed in stage IV oocyte. $* p < 0.05$ when compared to stage IV.

Discussion

In the present study, we provided direct evidence for the presence of progesterone binding sites on amphibian oocyte surface by visualizing the location of progesterone binding by confocal microscopy. We also demonstrated that the binding activity was specific for progesterone and the interaction of immobilized progesterone with such binding sites induced oocyte maturation. As amphibian oocytes are large (about 1.5 mm diameter) and easy to handle, amphibian oocyte culture system provided a useful model for studying nongenomic action of progesterone. Previously, we have shown that P-BSA-FITC is localized outside the oocyte and can not penetrate the cell in *Rana* (Bandyopadhyay et al., 1998), but the characteristics of that binding was not systematically investigated. In the present study, we have further examined the characteristics of P-BSA-FITC binding at the oocyte surface in detail. In our previous study, we found that there were some non-specific binding of BSA-FITC to oocyte vitelline membrane. Thus, in the present study, we used devitellined oocytes instead of denuded oocytes surrounded by vitelline membrane.

P-BSA and P-BSA-FITC could induce oocyte maturation in *Xenopus* (Fig. 1) as observed in *Rana* (Bandyopadhyay et al., 1998). P-BSA-FITC, which has a large size and hydrophilic nature, could not penetrate the oocyte plasma membrane, and mimicked the action of progesterone on oocyte (Fig. 2). We proposed that progesterone triggered the intracellular signal pathway for oocyte maturation through its membrane receptor. The binding of P-BSA-FITC to oocyte surface *in vitro* exhibited chemical specificity and mimicked the real binding of progesterone to its binding sites or receptors on oocyte surface *in vivo*. This conclusion is supported by the following observations: (i) fluorescence on the oocyte surface was detectable only in the presence of P-BSA-FITC, and (ii) the binding of P-BSA-FITC at the oocyte surface was replaced by progesterone (Fig. 3), but not by other steroids such as estradiol or testosterone. These results indicated that P-BSA-FITC binds to the oocyte surface and that the binding site were specific for progesterone. Optimal binding of P-BSA-FITC to oocyte surface was observed at pH 7.5 and to decrease below and above pH 7.5 (Fig. 4). These results are consistent with that of Liu and Patino (1993), in which optimal binding of progesterone with membrane fraction was obtained between pH 7.0 and pH 7.6. Thus it is evident that the structure of progesterone receptor containing binding epitope is sensitive to pH. It is of interest that the treatment of ovarian follicles with hCG increased the binding intensity of P-BSA-FITC to corresponding devitellined oocyte (Fig. 5). These results suggest that the number or affinity of progesterone receptor to P-BSA-FITC might be increased by hCG. At present, it is unclear how hCG increased the P-BSA-FITC binding activity to devitellined oocytes. Possibly, hCG may act on theca/granulosa cell and indirectly influence on the oocyte rather than directly acting on oocyte to increase the receptor number. Growing oocytes (Stage IV) do not mature in response to progesterone, while full grown oocytes (stage VI) do (Sadler and Maller, 1983). It has been suggested that expression of progesterone receptor is partly responsible for oocyte maturational competency (Kay and Peng, 1991). The binding intensity of P-BSA-FITC to oocyte in early stage is lower than that in full grown oocyte (Fig. 6). Taken together, these results demonstrate that progesterone binding activity increases concomitantly with enhancement of oocyte maturational competence, and support the idea that progesterone binding activity reflects the presence of progesterone receptor activity (Liu and Patino, 1993). However, we can not completely exclude the possibility that cytosolic factors other than maturation inducing substance receptors may also play a role in the regulation (Taylor and Smith, 1987; Bayaa et al., 2000; Tian et al., 2000).

In summary, this study, for the first time, provided a visual evidence for the presence of progesterone binding sites on the amphibian oocyte plasma membrane, and showed that the binding of immobilized proges-

terone to oocyte surface induced oocyte maturation. Visualization of progesterone binding to its receptor on oocytes surface may provide a valuable tool to study non-genomic action of steroids in oocytes.

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