Increase of cAMP upon release from prophase arrest in surf clam oocytes

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Summary

Surf clam (*Spisula solidissima*) oocytes are spawned at the prophase I stage of meiosis, and they remain arrested at this stage until fertilization. Full oocyte meiosis reinitiation, first evidenced by germinal vesicle breakdown (GVBD), may be induced by artificial activators mimicking sperm, such as high K^+ or serotonin. Previous reports indicated that treatments thought to increase the level of oocyte cAMP inhibited sperm- or serotonin-induced, but not KCl-induced, GVBD in clam oocytes. These observations extend the well known requirement for a drop in occyte cAMP levels in mammalian, amphibian or starfish oocytes and support the view that such a drop is universally important throughout the animal kingdom. We have re-examined the cAMP dependency of GVBD in clam oocytes and found that various treatments that raise oocyte cAMP levels did not, surprisingly, affect either KCl- or serotonin-induced GVBD. Such treatments, however, inhibited GVBD upon insemination of the oocytes, but this was due to the failure of sperm to fuse/penetrate the oocytes; thus, it was not an inhibition of oocyte activation as such. Direct measurements of oocyte cAMP levels after activation by serotonin, KCl or sperm showed that, contrary to expectations, there is a rise in cAMP levels before GVBD. Using SQ22536, an adenylyl cyclase inhibitor, the increase in oocyte cAMP level was partly prevented and GVBD proceeded, but with a significant retardation, indicating that the normal cAMP rise facilitates GVBD. Our work sheds light on the diversity of upstream pathways leading to activation of MPF and provides a unique model whereby the onset of meiosis reinitiation is associated with an increase, not a decrease, in oocyte cAMP levels.

Key words: cAMP, Meiotic maturation, Surf clam, Fertilization, Prophase arrest

Introduction

Oocyte meiotic maturation is an extensively studied process in the animal kingdom. During the course of gametogenesis, fully grown oocytes generally arrest at prophase I (germinal vesicle stage) of meiosis. Mostly, meiosis is reinitiated upon hormonal stimulation, and results, firstly, in GVBD and extrusion of polar bodies, with a secondary arrest usually occurring at either metaphase I or metaphase II of meiotic maturation, which is later released by fertilization. In well known amphibians and mammals, progression from prophase I to metaphase II is initially promoted by a surge in progesterone or luteinizing hormone (LH) levels, respectively. Accumulated evidence indicates that the release from prophase I arrest is accompanied by a decrease in oocyte intracellular levels of cAMP, which is thought of as a major prophase-I-arresting factor in both animal groups. Indeed, several observations support the view that the observed decrease in cAMP levels, in both amphibian and mammalian oocytes, is not only required but is also sufficient to trigger further meiotic progression [(Cicirelli and Smith, 1985; Maller, 1985) for *Xenopus laevis*] [(Vivarelli et al., 1983; Schultz et al., 1983; Abergdam et al., 1987) for mouse].

The current view is that cAMP-dependent phosphorylation by protein kinase A (PKA) regulates the activity of one or more proteins, which are still to be identified, one of which may be MPF (Meijer and Arion, 1991; Rime et al., 1992; Matten et al., 1994), that would maintain prophase I arrest as long as they are in their phosphorylated form. A decrease in cAMP levels would result in their dephosphorylation, and hence, in the release from prophase I block. This view is supported by the observations that injecting prophase I oocytes with the catalytic subunit of protein kinase A prevents or retards GVBD, whereas injecting the regulatory subunit, on the contrary, is sufficient to trigger GVBD and further meiotic maturation [(Maller and Krebs, 1977; Huchon et al., 1981) for *Xenopus laevis*] [(Bornslaeger et al., 1986) for mouse]. Moreover, maintaining high oocyte cAMP levels by incubation in the presence of either forskolin (6 β-[β′-(piperidino)propionyl]-, hydrochloride), an adenylyl cyclase activator, or IBMX (3-isobutyl-1-methylxanthine), a phosphodiesterase inhibitor, prevents spontaneous or progesterone-induced meiotic maturation in mammalian and amphibian oocytes, respectively [(Schultz et al., 1983; Urner et al., 1983; Sato and Koide, 1984) for mouse] [(Schorderet-Slatkine and Baulieu, 1982) for *Xenopus laevis*].

One notable exception to this general rule is the invertebrate ophiuroid echinoderm, *Amphipholis kochii*, in which the isolated prophase I oocytes may be stimulated to undergo meiosis reinitiation upon the addition of forskolin (Yamashita,

1988). The interpretation of this unusual observation is, however, limited as the natural, presumably hormonal, trigger for meiosis reinitiation in this species is not known. In addition, whether or not the forskolin treatment mimicks the normal physiological process remains to be established. Interestingly, it has recently been shown, in two species of nemertean worms, that their oocytes could be induced to undergo meiotic maturation from prophase I with various agents raising their intracellular cAMP (Stricker and Smythe, 2001). Also, serotonin-stimulated meiosis reinitiation could be triggered in the absence of external Ca^{2+} (and in the absence of MAP kinase activation) and was accompanied by a required increase of cAMP (Stricker and Smythe, 2001). This serotonin-stimulated meiotic reinitiation, however, uses a signalling pathway that differs from the physiological process (i.e. spontaneous maturation upon release in Ca^{2+}-containing seawater) which requires Ca^{2+} fluxes and MAP kinase activation (Stricker and Smythe, 2001). Nevertheless, these observations highlight the fact that increased cAMP may stimulate, rather than inhibit, meiotic maturation, at least in some species, whether or not this is the normal physiological process.

However, in another echinoderm (starfish), it has been shown that 1-methyl-adenine-induced meiotic maturation of oocytes is accompanied by a decrease in cAMP which, if altered, does not prevent but at least retards meiotic progression (Mazzei et al., 1981; Meijer and Zarutskie, 1987). Thus, there appears to be an almost universal decrease in oocyte cAMP, which is sufficient to trigger the release of oocytes from prophase I arrest or at least positively affects it. However, echinoderms and vertebrates belong to deuterostome animals and, as such, have a quite different developmental regulation than the more primitive protostome animals such as annelids and molluscs. For example, oocytes from deuterostome animals rely on intracellular Ca^{2+} stores for their initial activation, whereas oocytes from protostome animals require external sources of Ca^{2+} for their activation (Jaffe, 1983; Colas and Dubé, 1998). Little is known, however, about the possible involvement of cAMP in the regulation of meiotic maturation, especially in those protostome species that are normally fertilized at the prophase I stage.

The arrested oocytes of surf clam, a bivalve mollusc, are released at the prophase I stage, and fertilization normally reinitiates the full meiotic maturation process, up to formation of pronuclei without a secondary arrest in metaphase I. Artificial activation may be induced through the use of compounds, such as ionophore or high K^+ seawater (Allen, 1953; Schuetz, 1975), and by the neurohormone serotonin (Hirai et al., 1988), which raises intracellular Ca^{2+} concentration, presumably through binding to endogenous specific receptors. It has been briefly mentioned, without providing detailed experimental results, that cAMP-raising treatments, such as incubating oocytes in the presence of forskolin or IBMX, prevent induction of GVBD by sperm or 5-HT (serotonin, 5-hydroxytryptamine), but not by KCl (Sato et al., 1985). However, previous attempts to actually measure cAMP levels during the course of meiotic maturation in surf clam oocytes were inconclusive (Adeyemo et al., 1987). These observations nevertheless led to the suggestion that oocytes from protostome animals were also relying on decreased intracellular cAMP to achieve meiotic maturation.

The aim of the present work was to re-examine the involvement of cAMP in triggering meiotic maturation in surf clam oocytes. It also aimed at better characterizing the signaling pathway utilized by putative serotonin receptors that radioligand-binding studies had characterized as pharmacologically atypical and different from all known mammalian serotonin receptors (Krantic et al., 1993). We report that, contrary to previous reports, treatments of oocytes with forskolin, IBMX, or both, have no inhibitory effect on meiotic maturation induced by high K^+ or serotonin. We further show that, contrary to expectations, intracellular cAMP indeed increases upon triggering activation and that altering this increase slows down the meiotic maturation process. Our work establishes that release from prophase I arrest in oocytes is not universally dependent on, or accompanied by, decreased cAMP and that alternative pathways exist, with protostome animals providing this new original model. The implications of these findings for a better general understanding of the regulation of meiotic maturation and MPF activation are discussed.

Materials and Methods

Animals, chemicals and solutions

Specimens of surf clams (*Spisula solidissima*) were collected at Iles-de-la-Madeleine (Quebec, Canada) from mid June to late July. Gametes were obtained and handled as described by Allen (Allen, 1953). IBMX, an inhibitor of phosphodiesterase, and dbcAMP (N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate), a membrane-permeable cAMP analog that activates cAMP-dependent protein kinases, BSA (bovine serum albumin), TME-ScAMP (mono-succinyl adenosine 3′,5′-cyclic monophosphoric acid tyrosine methyl ester) and cAMP were purchased from Sigma Chemical Co (St Louis, MO). ^{125}I was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, Qué, Canada). 8-bromo-cAMP (8-bromoadenosine-3′,5′-cyclophosphate sodium), a membrane-permeable analog of cAMP, Sp-cAMPs (Sp-Adenosine 3′,5′-cyclic monophosphothioate triethylamine), a potent membrane-permeable activator of cAMP-dependent protein kinase I and II, SQ 22,536, an adenylyl cyclase inhibitor and forskolin were purchased from Research Biochemicals International (Natick, Massachussetts). Stock solutions of IBMX and forskolin were prepared at 5 and 10 mg/ml in DMSO (dimethyl sulfoxide), respectively. Dibutyryl-cAMP (dbcAMP), 8-bromo-cAMP, and Sp-cAMPs were prepared at 185 mM, 21 mM, and 2 mM in artificial sea water (ASW), respectively. [^{125}I]-TME-ScAMP (^{125}I-cAMP, 2.0′-monosuccinyl cAMP tyrosine methyl ester) was iodinated by the chloramine-T method (Brooker et al., 1979). Anti-cAMP antibody (CV-27) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD) through the National Hormone and Pituitary Program (NHPP).

Artificial sea water (ASW) and calcium-free sea water (CaFSW) were prepared according to the Marine Biological Laboratory (MBL) formulae (Cavanaugh, 1975) with the addition of 2 mM HEPES (N-2-hydroxyethylpiperazine-N″-2-ethane sulfonic acid), pH 8.0, and 2mM ethylene glycol bis (β-aminoethyl ether) *N,N*′-tetraacetic acid (EGTA) for the CaFSW. GA (glucamine acetate) buffer was prepared with 250 mM N-methyl glucamine, 250 mM potassium gluconate, 50 mM HEPES and 10 mM EGTA, and adjusted to pH 7.4 with glacial acetic acid. GA-formol solution was prepared by mixing formaldehyde (37% v/v) with GA buffer at approximately 10% v/v in solution and was mixed 1:1 with the oocytes to be fixed.

Fertilization or artificial activation of oocytes

Oocytes were washed several times by sedimentation and resuspended in ASW as a 1% (v/v) suspension. K^+ activation was performed by adding known amounts of isotonic KCl (0.52 M) to obtain the final

desired concentrations (1-52 mM). Serotonin was prepared as a 1 mM stock solution in ASW and used at a final concentration of 5 μM. Ammonium chloride (NH_4Cl) was used at a final concentration of 10 mM by adding 1 M stock solution in ASW, adjusted to pH 8.0 just prior to use, to oocyte suspensions. Fertilization was achieved by adding a 10,000- or 50,000-fold dilution of 'dry sperm' maintained at 4°C until use. Lower concentrations of oocytes (0.2%) were used for fertilization experiments. The percentage of GVBD was determined under the light microscope by randomly counting ~100-200 fixed oocytes per sample. In some experiments, the oocyte DNA was stained with the fluorescent dye Hoechst 33258 and observed under the Leitz Diaplan fluorescence microscope for the cytological observations.

Measurement of cAMP by RIA

Conditions for the preparation of oocytes are described in the legends of each figure. Briefly, tubes containing oocytes were rapidly centrifuged to pellet oocytes and discard ASW. These tubes were rapidly transferred to a container filled with liquid nitrogen. The tubes collected in this fashion were then stored at –80°C if the extraction of cAMP were not to be performed immediately. For homogenization and extraction of cAMP, frozen samples were left for incubation at –20 °C for 30 minutes in the presence of 0.5 ml 90% ethanol. Near the end of 30 minutes, oocytes suspended in ethanol were transferred to a Kimble and Kontes tissue homogenizer on ice and were homogenized for one to two minutes. A volume equivalent to 1/200 of the whole homogenate was taken out to evaluate the extent of homogenization under light microscopy. After transferring the homogenate to an Eppendorf tube, the homogenization tube was washed using 0.5 ml 90% EtOH and added to the Eppendorf tube. After an additional 30 minutes at –20°C, tubes were centrifuged (12,000 ***g***) for 15 minutes at 4°C. The supernatant was separated from the pellet and either immediately evaporated in a SpeedVac concentrator or kept at –80°C for later use. The pellets of centrifuged homogenates were either solubilized in 0.5 NaOH or kept at 4°C for later determinations of protein content by Detergent-Compatible (DC) protein assay (BIO-RAD).

Intracellular cAMP levels were measured by radioimmunoassay (Brooker et al., 1979). Samples and standards were incubated with the anti-cAMP antibody and [^{125}I]-TME-ScAMP (25 000 cpm/100 μl) in phosphate-buffered saline (PBS: 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.5) at 4°C for 20 hours. A pre-precipitated second antibody preparation containing 1% normal rabbit serum and 2% goat anti-rabbit antibody in PBS was then added, and samples were centrifuged (750 ***g***) after six hours at 4°C. Radioactivity in the pellets was measured in a Gamma counter. Results are reported as picomoles cAMP per mg protein. The sensitivity of the assay was 5 pmoles per tube. Inter- and intra-assay coefficients of variation were less than 10%.

Statistical analysis

All the cAMP measurements that required statistical analysis were compared with the cAMP measurements of control groups at corresponding times by ANOVA (analysis of variance, $P<0.05$). Multiple comparisons were performed using either the Dunnet's method (comparisons of each treatment versus control) or the Student-Newman-Keuls method (comparisons of each treament at indicated times with each other) as indicated. Significantly different measurements are depicted by asterisks (*).

Results

Raising cyclic AMP levels does not inhibit serotonin- or KCl-induced GVBD

It has been reported that cAMP-raising treatments, more specifically the incubation of oocytes in the presence of forskolin and IBMX at 5 μg/ml (12 μM) and higher concentrations, strongly inhibit the maturation of *Spisula* oocytes induced by sperm or serotonin but not by KCl (Sato et al., 1985). In our first set of experiments we tried to reproduce and further characterize this inhibition. Surprisingly, when forskolin was tested up to a concentration of 50 μg/ml (120 μM), and IBMX up to 25 μg/ml (115 μM), neither chemicals alone had any detectable effect on GVBD induced by 5-HT or KCl, contrary to previous reports (data not shown). We therefore examined whether adding various combinations of both chemicals would affect GVBD. Fig. 1A,B shows the results of such experiments which, once again, did not reveal any significant inhibitory effect of various combinations of both forskolin- and IBMX- on KCl- or 5-HT-induced GVBD. Other experiments in which the concentration of IBMX was increased up to 1 mM while using a fixed concentration of forskolin (60 μM) similarly showed no inhibitory effect on 5-HT-induced GVBD (data not shown). We extended the duration of pretreatment with both chemicals for up to one hour, measured the oocyte cAMP levels and examined their effect on subsequent 5-HT-induced GVBD. As shown in Fig. 1C, the cAMP concentrations (pmoles cAMP/mg protein) were not significantly different for oocytes incubated with either forskolin or IBMX alone, as compared to control untreated oocytes. However, the oocytes treated with both chemicals had significantly increased their cAMP concentration, compared to control oocytes (Fig. 1C; Dunnet's method, $P<0.05$), but this nevertheless resulted in normal GVBD upon the addition of 5-HT (Fig. 1D). It was also noted that prolonged incubations (>three hours) of oocytes with IBMX and forskolin, without the other usual activating agents, never resulted in GVBD (data not shown).

In many systems, membrane-permeable cAMP analogs such as dbcAMP, 8-Bromo-cAMP and Sp-cAMPs have been demonstrated to be potent activators of cAMP-dependent protein kinases. In Figure 2, we tested their possible inhibitory effect on 5-HT-induced GVBD of *Spisula* oocytes. Dibutyryl cAMP was unable to inhibit GVBD when concentrations up to 5 mM were tested (Fig. 2A), even though this compound was previously reported to inhibit 5-HT-induced GVBD by 24% at 1 mM (Sato et al., 1985). Similarly, other cAMP analogs such as 8-bromo-cAMP at concentrations up to 5 mM (Fig. 2B), or Sp-cAMPs at concentrations up to 100 μM (Fig. 2C), showed no inhibitory effect on GVBD triggered by 5-HT. When similarly tested, these chemicals did not have an inhibitory effect on KCl-induced GVBD (data not shown)

Interestingly, upon insemination in the presence of IBMX and forskolin, there was a pronounced inhibition of GVBD (Fig. 3), as previously reported (Sato et al., 1985). This concentration of the two chemicals had been shown to be ineffective in inhibiting GVBD induced by 5-HT or KCl (Fig. 1A,B). This inhibition of GVBD upon insemination was further characterized by determining the percent incorporation of sperm pronuclei into the oocytes observed under the fluorescence microscope after staining DNA with Hoechst dye 33258. After a normal insemination, most oocytes showed condensed maternal chromosomes and a decondensed male pronucleus (Fig. 4A; after 30 minutes of insemination), and polar bodies were visible by 60 minutes (Fig. 4B). However, most oocytes showed intact GV following insemination in the presence of IBMX and forskolin and even 60 minutes after insemination (Fig. 4C,D). Though there were visible sperm heads bound to the oocytes, they remained at the periphery of the oocytes and were not

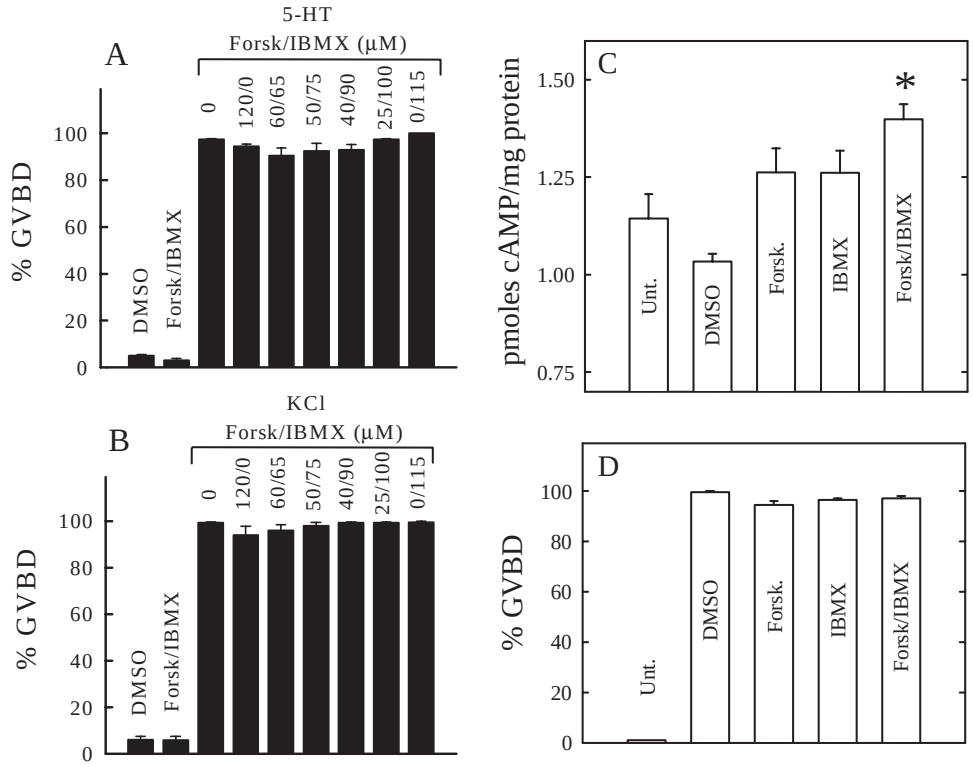

Fig. 1. Effect of forskolin and IBMX pretreatments on GVBD and cAMP levels in *Spisula* oocytes. In (A) and (B), oocytes were pretreated with various combinations of forskolin and IBMX at indicated concentrations for 15 minutes before adding 5-HT (5 μM, A) or KCl (45 mM, B). DMSO, DMSO vehicle alone; Forsk/IBMX: Forskolin and IBMX (65/60 μM) without any activating agent. GVBD was scored after 20 minutes. Mean results (±s.e.m.) of four experiments are shown. In (C) and (D), the effect of incubating oocytes for one hour in the presence of forskolin (120 μM), IBMX (115 μM) or a combination of both (60/65 μM) on oocyte cAMP concentration (A) and on subsequent 5-HT-induced GVBD (B) is shown. Unt, oocytes left untreated; DMSO, oocytes treated with the DMSO vehicle alone (Panel C) and to which 5-HT was added (Panel D). Asterisk (*), significantly different from untreated oocytes. Mean results (±s.e.m.) of three experiments are shown.

incorporated into them (Fig. 4C,D). The state of sperm incorporation in oocytes at different times after insemination in the presence or absence of IBMX and forskolin was further examined. 30 minutes after addition of the sperm suspension, 96% of control oocytes had undergone GVBD, with at least one sperm pronucleus incorporated, whereas in oocytes inseminated in the presence of IBMX and forskolin, almost none underwent GVBD, and they were generally devoid of an incorporated male pronucleus (<10%, not shown). This indicates that fertilization and incorporation of sperm pronuclei, rather than GVBD, are inhibited by IBMX and forskolin.

Transient increase in cAMP levels upon 5-HT- or KCl-induced GVBD

The resting oocyte cAMP concentration varied from 1.00±0.073 to 1.54±0.13 pmoles cAMP/mg protein (±s.e.m.) depending on the batch of females. The overall average value was 1.14±0.17 (n=16). We further analyzed the change in oocyte cAMP concentration occuring during the first few minutes after the addition of 5-HT. Contrary to expectations, the oocyte cAMP increases very rapidly after the addition of 5-HT (Fig. 5). In control untreated oocytes, the cAMP rise starts within two minutes, reaches a plateau between 5-10 minutes and then the cAMP slowly declines, thus establishing that the increase in cAMP occurs prior to GVBD (at 10 minutes) and remains higher than in unactivated oocytes throughout this period (Fig. 5A). The peak increase observed for oocyte cAMP was between 20-40% the initial resting level (1.09±0.016 pmoles cAMP/mg protein±s.e.m.) of unactivated oocytes (Fig. 5A). When the same experiment was performed with oocytes pre-incubated in the presence of forskolin and IBMX, a slightly higher (30-50% increase) in cAMP over the resting level (1.12±0.04 pmoles cAMP/mg protein ±s.e.m.) was seen within the same period after adding 5-HT, but the subsequent decrease after GVBD was less obvious. The kinetics of GVBD was unaffected (Fig. 5B).

The increase of cAMP concentration in 5-HT-activated oocytes was rather unexpected and might suggest the involvement of a G-protein-coupled receptor positively affecting the adenylyl cyclase. Unlike 5-HT-induced activation, oocyte activation by excess K^+ is not receptor mediated but rather involves, probably, a depolarization that opens up the voltage-gated Ca^{2+} channels (Dubé, 1988). To test whether this increase in cAMP concentration was restricted to 5-HT-activated oocytes, we compared the effect of KCl activation with that of 5-HT activation on the cAMP concentration. Interestingly, a similar transient increase in cAMP concentration was also observed in KCl-activated oocytes (Fig. 5C), thus establishing that this rise is not strictly receptor mediated but is linked to some downstream step(s) of a common activating pathway. Activation is accompanied by an increase of pHi in *Spisula* oocytes, and this increase can be mimicked by incubations of oocytes in the presence of NH_4Cl which, however, does not result in GVBD (Dubé and Eckberg, 1997). To test whether the increased level of cAMP could be caused by this rise of pHi, we tested the effect of incubating oocytes in the presence of NH_4Cl on their cAMP level and observed no significant changes (Fig. 5C). We also verified whether the addition of 5-HT or excess K^+ to oocytes in Ca^{2+}-free seawater, a condition that precludes any Ca^{2+} influx and hence does not result in GVBD, would nevertheless affect the cAMP levels of oocytes. When either 5-HT or excess K^+ was added to oocytes in Ca^{2+}-free seawater, no significant changes in cAMP levels could be detected (not shown). This indicates that the rise in cAMP is not immediately linked to any early

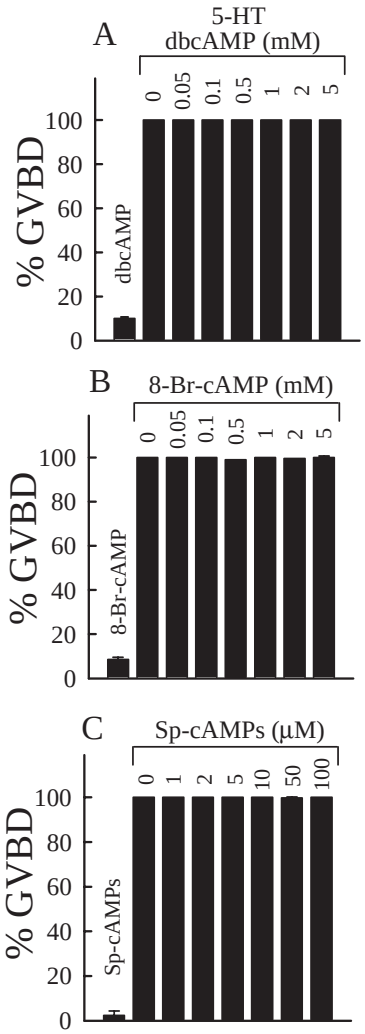

Fig. 2. Effect of dbcAMP, 8-bromo-cAMP or Sp-cAMPs pretreatment on 5-HT-induced GVBD. Oocytes were pretreated using various indicated concentrations of dbcAMP (A), 8-bromo-cAMP (B) or Sp-cAMPs (C) for 15 minutes prior to the addition of 5-HT (5 μM). dbcAMP, dbcAMP alone; 8-Br-cAMP, 8-bromo-cAMP alone; Sp-cAMPs, Sp-cAMPs alone. Mean results (±s.e.m.) of four experiments are shown.

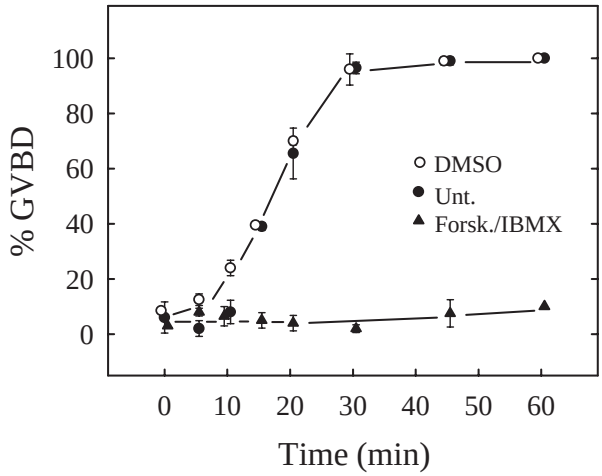

Fig. 3. Effect of IBMX and forskolin on sperm-induced GVBD. Oocytes were either left untreated (●, Control) or pre-incubated in presence of 65 μM IBMX and 60 μM forskolin (▲, Forsk/IBMX) or an equivalent amount of DMSO (○, DMSO), for 15 minutes prior to insemination at time t=0. Aliquots were sampled at indicated times for determination of percentage GVBD. Mean results (±s.e.m.) of two separate experiments are shown.

event such as a ligand binding to its receptor or initial membrane depolarization. Instead, it might be linked to the early Ca^{2+} influx or some other step(s) required for oocyte activation. Finally, we examined this latter possibility by testing whether the oocyte cAMP levels are sensitive to a graded series of Ca^{2+} influxes, below or above the required threshold for activation, induced by a graded series of added K^+ (Dubé, 1988). Fig. 5D illustrates that no increase of oocyte cAMP could be detected with any concentration of K^+ lower than those resulting in GVBD (≤2% v/v of added KCl 0.52 M). This suggests that the rise in cAMP is not very sensitive to any enhanced Ca^{2+} influx that is lower than that required for the commitment of oocytes to proceed to GVBD.

Cyclic AMP increases upon fertilization

Even though excess K^+- or 5-HT-induced oocyte activation mimick sperm-induced activation almost perfectly, we wanted to verify whether a normal fertilization would also result in increased oocyte cAMP, despite a previous report stating that no detectable changes occurred under this condition (Adeyemo et al., 1987). On the contrary, Figure 6 shows that fertilization indeed results in an early and steady increase of cAMP before GVBD. Despite the slower kinetics of GVBD, owing to imperfect synchrony of oocytes upon fertilization (20±11 at 20 minutes, 86.5±3.5 at 30 minutes, %GVBD±s.e.m.; Fig. 6), this rise in cAMP surpasses that seen after the addition of 5-HT (Fig. 5). This is related to the absence of a detectable secondary decrease over the monitored period, which may be due to this poorer synchrony of activation of fertilized oocytes (Fig. 6). Therefore, under this condition, the exact kinetics of the rise in oocyte cAMP cannot be perfectly assessed. The measured cAMP cannot originate from spermatozoa as most of them were washed out prior to oocyte sampling, and the remaining ones accounted for very few cells. Moreover, in some experiments in which insemination did not result in a successful fertilization, as evidenced by GVBD, no change in cAMP could be detected (not shown). This confirms that the rise in oocyte cAMP does not only occur during artificial activation but also during the normal process of fertilization in *Spisula* oocytes.

Effect of SQ 22,536 on oocyte maturation

The observed increase in oocyte cAMP might be crucial in the cascade of events leading to GVBD and may be mediated through cAMP-dependent phosphorylation. Abolishing this increase, or preventing it by lowering the basal cAMP level, might in fact delay or even inhibit GVBD. To test this hypothesis, we treated oocytes with SQ 22,536, a potent adenylyl cyclase inhibitor. A pre-incubation of oocytes with SQ 22,536 (1 mM) for one hour before the addition of 5-HT or excess K^+ resulted in GVBD, but with much slower

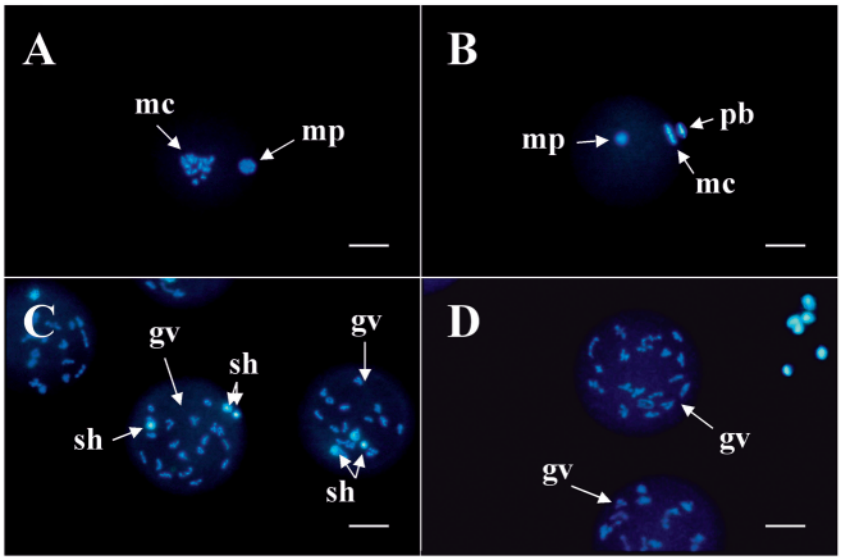

Fig. 4. Effect of IBMX and forskolin on sperm incorporation into the oocytes. (A) Control fertilized oocyte after 30 minutes (metaphase I of meiosis) showing alignment of maternal chromosomes (mc) and a decondensed male pronuleus (mp). (B) Control fertilized oocyte 60 minutes (metaphase II of meiosis) after the addition of sperm, showing a polar body (pb), maternal chromosomes (mc) and a decondensed male pronucleus (mp). (C) Oocytes 30 minutes after insemination in the presence of IBMX (65 μM) and forskolin (60 μM), showing an intact germinal vesicle (gv) and undecondensed sperm heads (sh) at the periphery of the oocyte. (D) Similarly treated oocytes 60 minutes after insemination, still showing intact germinal vesicles and the absence of any male pronucleus in their cytoplasm.

kinetics than that seen with control untreated oocytes (a 50% GVBD of 16.5 minutes in pre-incubated cells compared with 9.5 minutes in the control, i.e. ~75% delay; Fig. 7A). Even though GVBD was significantly retarded in the presence of SQ22,536, the teated oocytes could proceed, albeit slowly, to subsequent steps of meiotic maturation, including extrusion of polar bodies (not shown). Moreover, washing out SQ22,536 after prolonged incubations of oocytes resulted in the re-establishment of normal kinetics of meiotic maturation (not shown), indicating that SQ22,536 had no overall toxicity on cell viability. This suggests that inhibition of adenylyl cyclase, and possibly interfering with the normal increase of cAMP, alters the normal program of oocyte activation. This is further supported by the results presented in Fig. 7B, which show that, although pre-incubating the oocytes with SQ 22,536 does not significantly affect the resting cAMP level of unactivated oocytes, it significantly inhibits the normal rise of cAMP seen after the addition of 5-HT, thus strongly suggesting that this lower rise in cAMP is related to the observed delayed GVBD (Fig. 7B). This indicates that the rise of cAMP might indeed

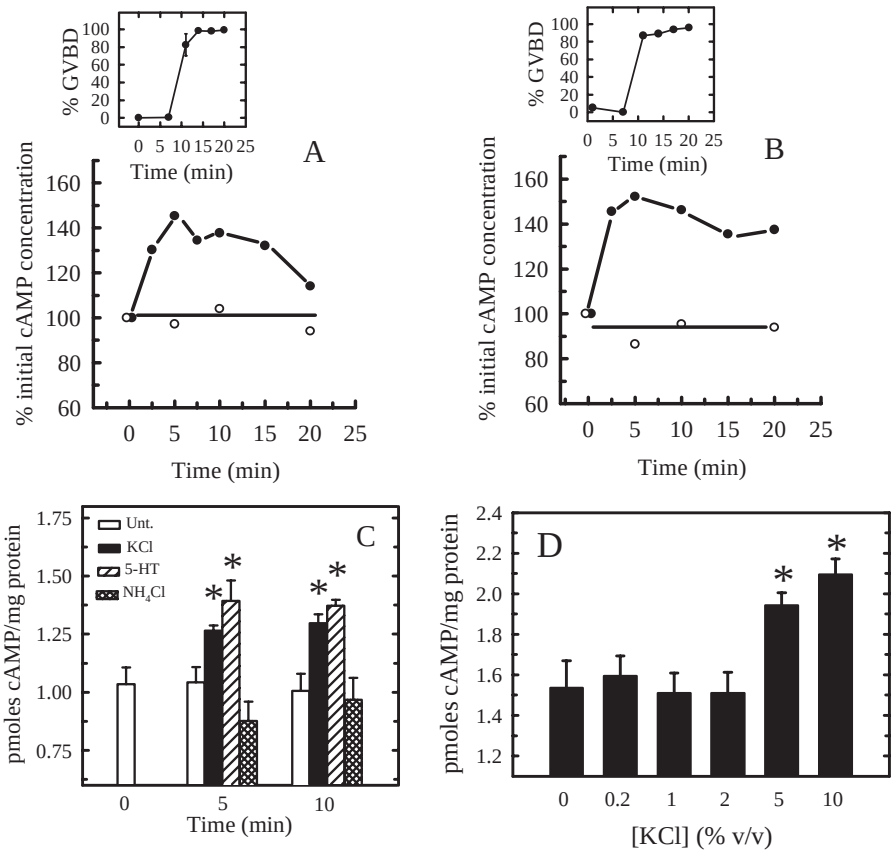

Fig. 5. Effect of 5-HT, NH_4Cl or KCl on the cAMP concentration of *Spisula* oocytes. (A) Oocytes were either treated with 5-HT (5 μM, ●) or left untreated (○). Aliquots were removed at different times and processed for cAMP determinations, and the results are expressed as a percentage of the initial cAMP concentration of untreated oocytes. (B) A similar experiment using oocytes pre-incubated in the presence of forskolin and IBMX (60/65 μM) for 15 minutes prior to the addition of 5-HT. The insets depict the corresponding time course of GVBD (±s.e.m.) for sampled oocytes. Mean results of three experiments are shown. (C) shows the effect of adding 5-HT (5 μM), KCl (10% v/v) or NH_4Cl (10 mM) on oocyte cAMP concentration at indicated times, as compared with untreated oocytes. Percentages of GVBD scored 15 minutes after adding 5-HT, KCl or NH_4Cl were 100%, 100% and 0% percent, respectively. Mean results (±s.e.m.) of three experiments are shown. (D) shows the effect of adding K^+ on oocyte cAMP concentration. Various amounts of isotonic KCl (0.52 M) were added to oocytes to obtain the final concentrations shown (% v/v). Oocytes were sampled 10 minutes later for determination of cAMP concentration, as described in the Materials and Methods. GVBD, as scored after 15 minutes, had occurred only at KCl concentrations of 5 and 10% (v/v), in 86% and 100% of the oocytes, respectively, with all lower KCl concentrations resulting in less than 2% GVBD. Mean results (±s.e.m.) of three experiments.

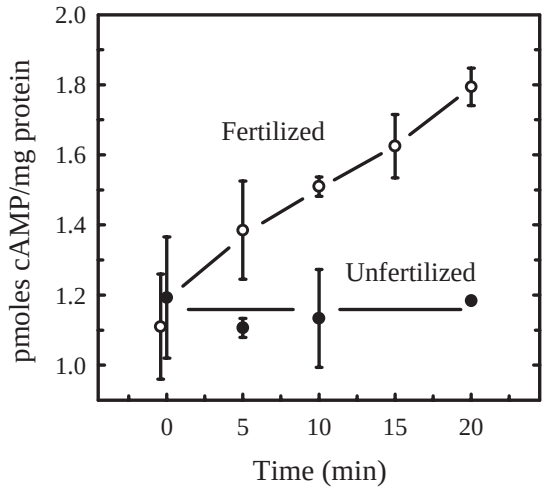

Fig. 6. Effect of fertilization on cAMP concentration in *Spisula* oocytes. Oocytes (0.2% v/v) were divided in two lots, which were either inseminated at time t=0 (●, Fertilized) or not (○, Unfertilized). At indicated times, oocytes were sampled for determinations of cAMP concentration, as described in Materials and Methods. GVBD was less than 2% in unfertilized oocytes. Mean results (±s.e.m.) of two experiments are shown.

play a substantial causal role in the steps leading to full meiosis reinitiation.

Discussion

Cyclic AMP increases during the first minutes of activation in *Spisula* oocytes

It has been well documented that cAMP exerts negative effects on the release from prophase I arrest in oocytes of many animal species, including mammals, *Xenopus* and starfishes (Faure et al., 1998; Ferrell, 1999; Heikinheimo and Gibbons, 1998; Dekel, 1996). Artificially elevating cAMP by treatments that activate adenylyl cyclase or inhibit PDEs (phosphodiesterases) or by injecting the catalytic subunit of PKA were shown to block meiosis resumption in these oocytes. Furthermore, direct measurements of cAMP upon release from the prophase block have proven that cAMP indeed decreases prior to GVBD (Meijer and Zarutskie, 1987). It has been reported that increasing cAMP by forskolin and/or IBMX, or incubations in the presence of dbcAMP, in *Spisula* oocytes similarly blocked GVBD induced by 5-HT or spermatozoa (Sato et al., 1985). This led to the view that a decrease in oocyte cAMP might be a universally required step for the release from prophase arrest.

In this report, we show that cAMP-raising treatments do not inhibit 5-HT- or KCl-induced GVBD in *Spisula* oocytes, even when using the same compounds at concentrations ten times higher than those previously reported inhibitory (Sato et al., 1985). Other cAMP analogs tested, dbcAMP, 8-bromo-cAMP and Sp-cAMPs, were similarly inefficient in inhibiting GVBD, including dbcAMP, even at concentrations ten times higher than those reported to be inhibitory (Sato et al., 1985). The reason for the discrepancy between our results and the previous ones is unclear. Sperm-induced GVBD is blocked in the presence of IBMX and forskolin, but we have shown that this inhibition is at the level of sperm-oocyte fusion/incorporation.

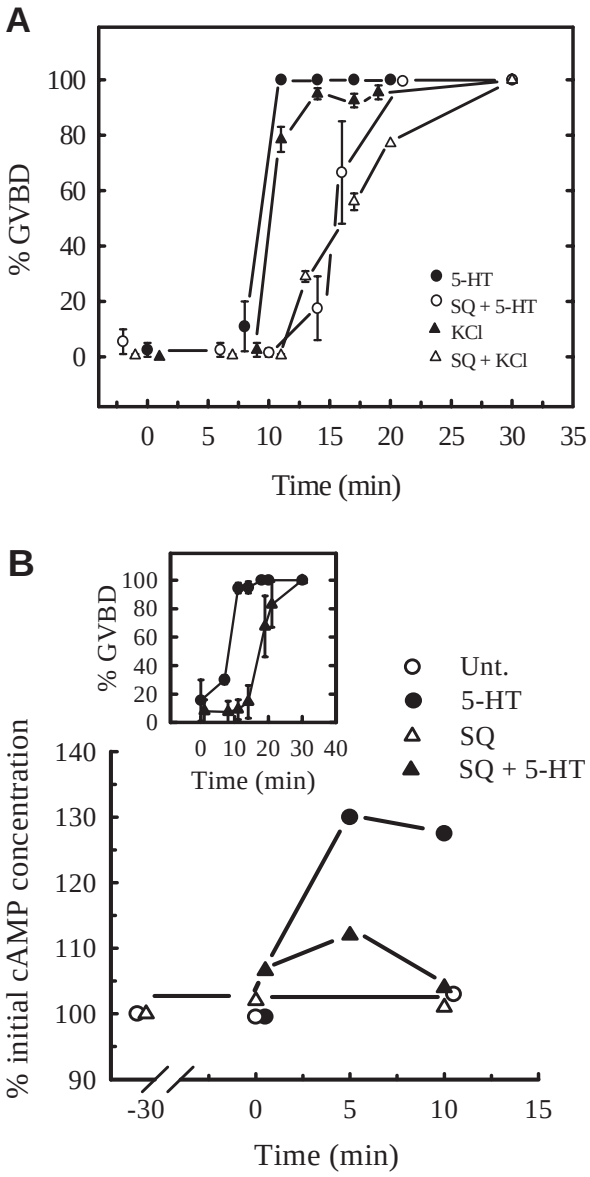

Fig. 7. Effect of pretreatment of oocytes with SQ 22,536 on cAMP concentration and the time course of GVBD. (A) Oocytes were either pretreated with 1 mM SQ 22,536 (open symbols, ○,) or left untreated (filled symbols, ●, ▲) for one hour prior to the addition of 5-HT (5 μM, ●, ○) or KCl (45 mM, ▲, △), at time 0. At indicated times, oocytes were fixed for determination of the percentage of GVBD. Mean results (±s.e.m.) of two experiments are shown. (B) Oocytes were divided in two lots, one to which SQ 22,536 (1 mM) was added (△) while the other was left untreated (○). One hour later, 5-HT (5 μM) was added to an aliquot of both SQ 22,536-treated (▲) and -untreated oocytes (●). At indicated times, samples of oocytes were frozen and later processed for determinations of cAMP concentration expressed as a percentage of the initial cAMP concentration of oocytes at the beginning of the experiment. The inset depicts the corresponding time course of GVBD after addition of 5-HT, in both groups of oocytes. Mean results of two experiments are shown.

We thus interpret this inhibition as an effect on the sperm itself, which becomes unable to fertilize the oocyte when raising its cAMP content. One likely possibility is that the acrosome

reaction is somewhat affected by this treatment, perhaps prematurely triggered, as this process is generally associated with increased cAMP levels (Breitbart and Spungin, 1997). However, further investigations would be required to confirm this hypothesis.

Surprisingly, in addition, direct measurements of oocyte cAMP showed that it increases by 20% to 40% very soon after triggering meiosis reinitiation in *Spisula* oocytes by 5-HT, KCl or sperm, in strong contrast with all other animal species so far studied. Our results contradict previous attempts that failed to detect any significant changes in oocyte cAMP levels after fertilization (Adeyemo et al., 1987). This could be due to the experimenter's use of a less sensitive technique to measure cAMP. However, under the conditions they used (oocyte concentrations of 10% v/v), poor fertilizations and/or synchrony might have resulted, as it has been established that fertilization rates decrease dramatically above oocyte concentrations of 0.5%, in this species (Clotteau and Dubé, 1993). It should be noted that the observed rise in cAMP after addition of 5-HT cannot be taken as indicative that the putative 5-HT receptor present on oocytes (Krantic et al., 1991; Krantic et al., 1993) is of a G_s-protein-coupled type as a similar rise of cAMP is seen in KCl-activated oocytes, which proceed through GVBD not by a receptor-mediated process but presumably because of the opening of voltage-gated Ca^{2+} channels (Colas and Dubé, 1998). Taken altogether, these results reveal an unsuspected rise of oocyte cAMP at the onset of release from prophase I arrest, which is in contrast with what occurs in all other animal species so far studied.

Is the rise in oocyte cAMP required for GVBD?

As mentioned above, the presence of forskolin and IBMX are not inhibitory but, instead, they stimulate the normal rise in cAMP seen after activation by KCl or serotonin. However, even prolonged incubations in the presence of these chemicals (>three hours) did not result in GVBD unless another activating agent was subsequently added to the oocytes. This indicates that the rise of cAMP is not sufficient to trigger GVBD.

To test whether the rise of cAMP was required for GVBD, oocytes were incubated with an adenylyl cyclase inhibitor, SQ 22,536. Whereas the basal level of cAMP in resting oocytes was not significantly altered by this inhibitor, it considerably reduced the rise of cAMP normally seen after the addition of 5-HT (Fig. 7B). This treatment did not prevent GVBD induced by either KCl or 5-HT but significantly retarded it in both cases. We conclude that the rise in cAMP, if not absolutely required for GVBD, at least facilitates it and contributes to the normal kinetics of meiotic maturation. Since the inhibitor did not compeletely abolish the cAMP rise, we cannot rule out the possibility that in the complete absence of any increased cAMP, GVBD might have been completely prevented. Notwithstanding this limitation, the rise in cAMP reported here is positively correlated to the onset of GVBD, which is unique, to our knowledge, in the animal kingdom, with the exception of brittle star oocytes in which forskolin triggers GVBD (Yamashita, 1988). In the latter species, however, the physiological trigger for the release from prophase I arrest is not known, and it is uncertain to what extent the activation by forskolin mimicks the normal process.

The rise in cAMP is neither Ca^{2+} nor pH sensitive but tightly coupled to activation

Two well known ionic changes accompany *Spisula* oocyte activation, namely, an increased Ca^{2+} influx raising the internal Ca^{2+} concentration, which is absolutely required for GVBD to occur (Dubé, 1988; Colas and Dubé, 1998), and a 0.4 U increase of pHi, driven by an Na^+/H^+ exchanger, which is dispensable for GVBD (Dubé and Eckberg, 1997). In order to test whether either of these two ionic processes could be causally related to the rise of cAMP, we performed several experimental manipulations known to alter Ca^{2+} or pHi and verified their effects on cAMP levels.

Adding 5 to 52 mM K^+ to oocyte suspensions promotes Ca^{2+} influxes to increasing amplitudes beyond and above a threshold level that results in GVBD (Dubé, 1988). We did not observe any changes in cAMP levels in oocytes that did not reach the threshold for GVBD, indicating that the rise in cAMP is not especially sensitive to moderate Ca^{2+} rises. Similarly, artificially increasing the pHi with NH_4Cl, at or above the level reached by activated oocytes, but without inducing GVBD (Dubé and Eckberg, 1997), did not affect the level of oocyte cAMP (Fig. 7A), suggesting that the normal rise in pHi is unlikely to be causally related to the observed rise of cAMP. Interestingly, the inverse might be possible; for example, the increase in pHi might be caused by increased cAMP if the *Spisula* oocyte Na^+/H^+ exchanger were, for example, of the beta type which is activated by cAMP (Malapert et al., 1997). Moreover, when the rise of cAMP is partly inhibited by SQ 22,536, the observed retardation in GVBD is reminiscent of that observed when the pHi rise is directly prevented either by amiloride derivatives or Na^+-free seawater (Dubé and Eckberg, 1997). Thus, the rise in cAMP does not seem specifically Ca^{2+}- nor pHi-sensitive but rather appears as an all-or-none process tightly coupled to the 'activated state' of oocytes committed to undergo GVBD. Interestingly, this is similar to the all-or-none overall increase in protein phosphorylation observed under identical experimental conditions (Dubé et al., 1991), a process that may be itself, at least partly, related to increased cAMP and enhanced activity of PKA.

How can a rise in cAMP be involved in the steps leading to MPF activation and GVBD?

The key biochemical process for achieving GVBD is the activation of MPF, a cdc2-cyclin complex that must undergo tyrosine dephosphorylation of cdc2 by the phosphatase cdc25 to be active. Although this process is most probably a universal convergent point in the release from prophase arrest, there appears to be considerable variation in the upstream events leading to active MPF from one species to another.

A drop in oocyte cAMP is associated with release from prophase arrest by reducing PKA activity which, in turn, is thought to maintain the arrest by phosphorylation of a regulatory susbtrate, which remains to be identified. In starfish oocyte maturation, cAMP seems to negatively affect the activation of MPF through mik1, wee1 and cdc25 (Meijer and Arion, 1991), although a decrease in cAMP alone is insufficient to trigger oocyte activation (Meijer et al., 1989). In *Xenopus* oocytes, the cascade leading to MPF activation is thought to involve an early phosphorylation of a cytoplasmic

polyadenylation element binding factor (CPEB), which in turn induces c-mos synthesis and accumulation (Mendez et al., 2000) followed by the activation of MAP-kinase and MPF (Nebreda et al., 1993; Posada et al., 1993; Shibuya and Ruderman, 1993). However, along this sequence of events, there are positive feedback loops (Matten et al., 1996), which make the identification of causal effects difficult. The nature of the early link between PKA and c-mos translation is not known, but there appears to be no effect of PKA on c-mos accumulation once MAP kinase is activated (Faure et al., 1998). On the other hand, the accumulation of cyclin B1 is more dependent upon reduced PKA activity (Frank-Vaillant et al., 1999).

However, in *Spisula* oocytes as opposed to *Xenopus* oocytes, there is no need for new protein synthesis for completion of meiosis I (Hunt et al., 1992), and thus no accumulation of c-mos appears to be involved. In more closely related nemertean oocytes, meiosis reinitiation (from prophase I to metaphase I) can be induced by serotonin through an increase in cAMP, and without any Ca^{2+} fluxes or MAP kinase activation as occur during the physiological process of spontaneous maturation upon release of oocytes in Ca^{2+}-containing seawater (Stricker and Smythe, 2001). In contrast, in *Spisula* oocytes, release from prophase arrest (up to full meiotic maturation) absolutely requires external Ca^{2+}, whether KCl, serotonin or a normal fertilization (Colas and Dubé, 1998) triggers it. Indeed, in *Spisula* oocytes, activation by serotonin seems to perfectly mimic fertilization, and no differences in signalling pathways used by these two modes of activation have been detected so far. The discrepancy between nemertean and *Spisula* oocytes might be related to the fact that the former secondarily arrests at metaphase I, whereas the latter do not. In light of current knowledge, it is thus difficult to speculate about which process could be positively affected by increased cAMP and enhanced PKA activity to promote cell cycle re-entry in *Spisula* oocytes. The temporal sequence of events seems to involve an early Ca^{2+} rise (Dubé, 1988) and an increase in cAMP (this work), a slightly later activation of MAP kinase followed by MPF activation (Shibuya et al., 1992; Walker et al., 1999). Further investigations will be required to establish the causal relationships between these various events and to identify any specific substrate phosphorylated by PKA that may contribute to oocyte activation.

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