Université de Montréal

Increase of cyclic AMP upon release from prophase arrest in the oocytes of Surf clam, *Spisula solidissima*

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Ce mémoire de maîtrise intitulé:

«Increase of cyclic AMP upon release from prophase arrest in the oocytes of Surf clam, *Spisula solidissima* »

présenté par:

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Résumé

La reprise de la méiose des ovocytes en prophase I chez la palourde, *Spisula solidissima*, est normalement déclenchée par la fécondation mais peut aussi l'être par l'ajout de KCl ou de sérotonine. Les ions K^+ provoquent une dépolarisation membranaire permettant l'ouverture de canaux calciques, tandis que la sérotonine se lie à des récepteurs spécifiques généralement couplés à des protéines G. Celles-ci sont elles-mêmes souvent associées à la voie de signalisation de l'AMPc. Des études précédentes indiquent que les traitements des agents qui augmentent le niveau d'AMPc basal de l'ovocyte inhibent la rupture de la vésicule germinative induite par la sérotonine ou le sperme, mais pas par le KCl. Notre étude a pour but de clarifier l'intervention potentielle de l'AMPc au cours de la fécondation, et l'activation artificielle par le KCl ou la sérotonine.

Les techniques d'observation cytologique par microscopie à fluorescence et par radioimmunoessai permettent de montrer que des pré-traitements des ovocytes à la forskoline, à l'IBMX ou à une combinaison des deux produits, n'inhibent pas la reprise de la méiose induite par le KCl ou la sérotonine. De même, les pré-traitements des ovocytes avec d'autres analogues d'AMPc n'inhibent pas l'activation par le KCl ou la sérotonine. La rupture de la vésicule germinative par l'insémination est inhibée en présence d'une combinaison de forskoline et IBMX, en accord avec des études précédentes, mais cette inhibition est dûe à une inhibition de la fusion/incorporation des deux gamètes et non pas d'une inhibition de l'activation de l'ovocyte. Contrairement à ce qui est admis, les mesures directes du niveau d'AMPc de l'ovocyte avant et après l'activation par la sérotonine ou le KCl nous montrent une augmentation du niveau de l'AMPc avant la rupture de la vésicule germinative et non pas une baisse.

L'étude sur les altérations de conditions expérimentales menant à un changement d'influx de Ca^{2+} ou du pH interne de l'ovocyte nous indiquent que l'augmentation de l'AMPc de l'ovocyte est un processus tout-ou-rien couplé à l'activation menant à la rupture de la vésicule germinative et indépendant de la quantité d'influx de Ca^{2+} et du pH interne.

Contrairement aux autres études précédentes, on a aussi détecté une augmentation du niveau d'AMPc après la fécondation. En utilisant le SQ 22,536, un inhibiteur d'adénylate cyclase, on a pu restreindre l'augmentation du niveau d'AMPc, retarder le temps de la rupture de la vésicule germinative ce qui est un indice que l'augmentation d'AMPc facilite la rupture de la vésicule germinative mais n'est pas indispensable à l'activation.

En conclusion, notre étude clarifie l'implication limitée de l'AMPc dans l'activation des ovocytes de palourde et offre un nouveau modèle où la reprise de la méiose et l'activation du M-phase promoting factor sont associés avec une augmentation, pas une baisse, du niveau de l'AMPc de l'ovocyte.

SUMMARY

Oocytes of the marine invertebrate *Spisula solidissima* are naturally arrested at prophase I of meiosis, and resume meiosis upon fertilization. Different artificial agents such as KCl or serotonin can also trigger the resumption of meiosis in *Spisula* oocytes. Excess K^+ ions trigger an influx of external Ca^{2+} presumably through the opening of Ca^{2+}-channels, whereas serotonin binds to the specific receptor generally coupled to a G-protein. Fertilization, as well as activation by KCl or serotonin, requires the presence of external Ca^{2+} to allow oocytes to resume meiosis. The influx of Ca^{2+} ions then triggers downstream phosphorylations leading to germinal vesicle breakdown.

The nascent protein synthesis is not required for the germinal vesicle breakdown and the 1st polar body extrusion in *Spisula* oocytes. Protein phosphorylation, however, seems to be directly involved in the event leading to germinal vesicle breakdown. The protein phosphorylation is thus responsible for the M-phase promoting factor activation and subsequent phosphorylation of the lamin which is directly linked to germinal vesicle breakdown in *Spisula* oocytes. The activation of M-phase promoting factor by different stimui (serotonin, KCl, sperm, calcium ionophore A23187, etc.) requires the influx of external calcium, since no germinal vesicle breakdown occurs in the absence of external calcium. In addition, the Ca^{2+}-channel blocker verapamil inhibits KCl-induced germinal vesicle breakdown in presence of external calcium. Therefore, it is possible that the early events that precede activation of M-phase promoting factor are calcium-related, possibly a calcium-calmodulin-dependent kinases-related phosphorylation. However, the exact sequence of events starting from increased calcium influx and leading to M-phase promoting factor activation is not completely solved with several gaps to be filled and several missing links remaining to be uncovered.

Interestingly, an involvement of cyclic AMP, an important regulator of metabolic pathways in many systems through the downstream phosphorylation of the cyclic AMP-dependent kinases, was reported in the maturation of *Spisula* oocytes

induced by serotonin or sperm (Sato *et al.*, 1985). This report suggested the possibility of cAMP-dependent kinases-related phosphorylation being responsibe for inhibiting serotonin- or sperm-induced germinal vesicle breakdown in *Spisula* oocytes. Similar results were shown already in other systems such as starfish, mouse, or frog oocytes where the decrease in cyclic AMP, hence the decrease in cyclic AMP-related phosphorylation is apparently required for the occurrence of germinal vesicle breakdown with a normal timing.

Our study involves the investigation of the possible involvement of cyclic AMP in the resumption of meiosis in *Spisula* oocytes induced by sperm, serotonin or KCl. The pre-treatment of oocytes with compounds known to increase intra-oocyte cyclic AMP level did not inhibit serotonin- or KCl-induced germinal vesicle breakdown, contrary to what has been reported. Moreover, the actual levels of cyclic AMP in oocytes were measured at different times after the activation and showed an increase after the addition of serotonin, KCl, or sperm. This increase in cyclic AMP, delayed by adenylyl cyclase inhibitor SQ 22,536, seemed to be independent of pH or the level of Ca^{2+} influx, and closely coupled to the germinal vesicle breakdown. Finally, insemination in presence of the cyclic AMP-raising compounds IBMX and forskolin does not result in germinal vesicle breakdown, as previously reported, but this is due to the failure of sperm fusion/incorporation and is not an inhibition of oocyte activation as such. Our results thus suggest an interesting new mechanism whereby cyclic AMP increases after the activation of surf clam oocyte maturation.

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1.0 INTRODUCTION

1.1 General introduction

The mechanism underlying cell cycle control has been extensively studied over the past years using oocytes, owing to the advantages that they provide over somatic cells: high synchrony and the natural state of arrest during the cell cycle. In fact, somatic cells are difficult to handle and much time and efforts are required to synchronize them. In contrast to somatic cells, oocytes are naturally arrested at certain stages of meiosis and resume meiosis upon fertilization or hormonal stimulation, which provides a great advantage to study the molecular signal transduction mechanisms by which cells control the arrest and the release of their cycle. Oocytes of *Spisula solidissima*, a marine invertebrate of the class Bivalvia, phylum Mollusca, are particularly advantageous in this respect, because they are available in large numbers and do not undergo spontaneous activation upon isolation. The eggs are about 55 μm in diameter and retain a fairly large germinal vesicle of about 31 μm (Allen, 1953). They are arrested at prophase I of meiosis at the time of spawning, and resume meiosis followed by mitotic cell division upon fertilization. The first visible event is germinal vesicle breakdown (GVBD) which is an indicator of the resumption of meiosis. This GVBD is followed by the first and the second polar body extrusions, and subsequent entry into the mitotic cell cycles.

1.1.1 Fertilization and parthenogenic activation in *Spisula* oocyte

Fertilization and parthenogenic activation follows a similar trend in their mechanism during the first minutes of activation in *Spisula* oocytes. The oocytes enter the M-phase 10 min following the activation and complete meiosis I at ~35 min and meiosis II at 50 min. The first mitotic division, observed only in the fertilized eggs, occurs at 80 min (Hunt *et al.*, 1992). The timing of oocyte maturation varies greatly between oocytes of different species. For example, the African clawed frog *Xenopus laevis* oocyte takes hours to reach GVBD after progesterone stimulation.

Mouse oocytes also take several hours to complete spontaneous activation. Another interesting and crucial difference to be aware of is the absence of secondary arrest in *Spisula* oocytes. Unlike *Xenopus* or mouse oocytes that arrest again at metaphase-II following the resumption of meiosis, *Spisula* oocytes complete meiosis following parthenogenic activation and fertilization occurs at prophase-I. Parthenogenic agents for the *Spisula* oocyte include excess potassium ions, UV-light, calcium ionophore A23187, TPA (12-O-tetradecanoyl-13-phorbol acetate), tharpsigargin, and the microinjection of inositol trisphosphate (Allen, 1953; Schuetz, 1975; Bloom *et al.*, 1988; Dubé, 1992; Dubé *et al.*, 1987; Eckberg *et al.*, 1987). All these agents, however, require the presence of external calcium ions to trigger GVBD in *Spisula* oocytes.

1.1.2 Implication of cyclic AMP in *Spisula* oocyte maturation: summary and purpose of this study

One of the major differences between oocytes of different species comes from the time at which fertilization (entry of sperm) occurs. Prophase-arrested oocytes of mouse and *Xenopus* undergo maturation following a LH (luteinizing hormone) surge or progesterone release respectively, and arrest again to await fertilization at metaphase-II. Even some clam species such as *Ruditapes* oocytes undergo secondary arrest at metaphase-I following stimulation by serotonin. The *Spisula* oocytes, however, are arrested at prophase-I and fertilization occurs at this stage. For some cell and molecular biologists, these differences are crucial advantages that give an excellent opportunity to study the signal transduction pathways by which cells control their cycles, by comparing oocytes of different species and how they differ in the regulation of their cycle. In this report, we will take advantage of these facts and try to compare the results obtained from different species to study the possible implication of cyclic AMP (cyclic adenosine monophosphate) on the resumption of meiosis of *Spisula* oocytes. We will focus on the early events, from activation until GVBD, in *Spisula* oocytes. First, we will begin with some of the earlier efforts to unveil the signal transduction pathways involved in the resumption of meiosis,

beginning with *Spisula* oocytes. Second, we will bring about the possible implication of cyclic AMP in the signaling pathways of the resumption of meiosis in *Spisula* oocytes (our results), and compare it to well known systems such as *Xenopus*, mouse, or starfish oocytes. We will end this report by suggesting some of the roles for cyclic AMP in the resumption of meiosis of *Spisula* oocytes.

1.2 Molecular events involved early in the maturation of *Spisula* oocytes

1.2.1 Calcium and GVBD

1.2.1.1 The requirement for the initial Ca^{2+} influx in *Spisula* oocyte activation

Ca^{2+} plays a pivotal role in the activation of *Spisula* oocytes. The pioneering work by Allen (1953) showed that the presence of external Ca^{2+} is indispensable for the resumption of meiosis triggered by sperm, KCl, or UV-light. Upon activation, there exists an immediate influx of Ca^{2+} in *Spisula* oocytes. Measurements of $^{45}Ca^{2+}$ showed an increase in the uptake of Ca^{2+} by the oocytes artificially activated with either serotonin (Krantic *et al.*, 1991) or KCl (Dubé *et al.*, 1987). Excess K^+ ions trigger an influx of external Ca^{2+}, presumably through the opening of Ca^{2+}-channels, which is sensitive to the channel-blocker verapamil up to 1 to 3 minutes after the addition of KCl (Carrol and Eckberg, 1986). Serotonin (5-HT), on the other hand, seems to involve a PLC (phospholipase-C)-mediated pathway to trigger the Ca^{2+} influx (Colas and Dubé, 1998).

1.2.1.2 Internal Ca^{2+} stores and the involvement of phospholipase-C-mediated pathway in *Spisula* oocyte

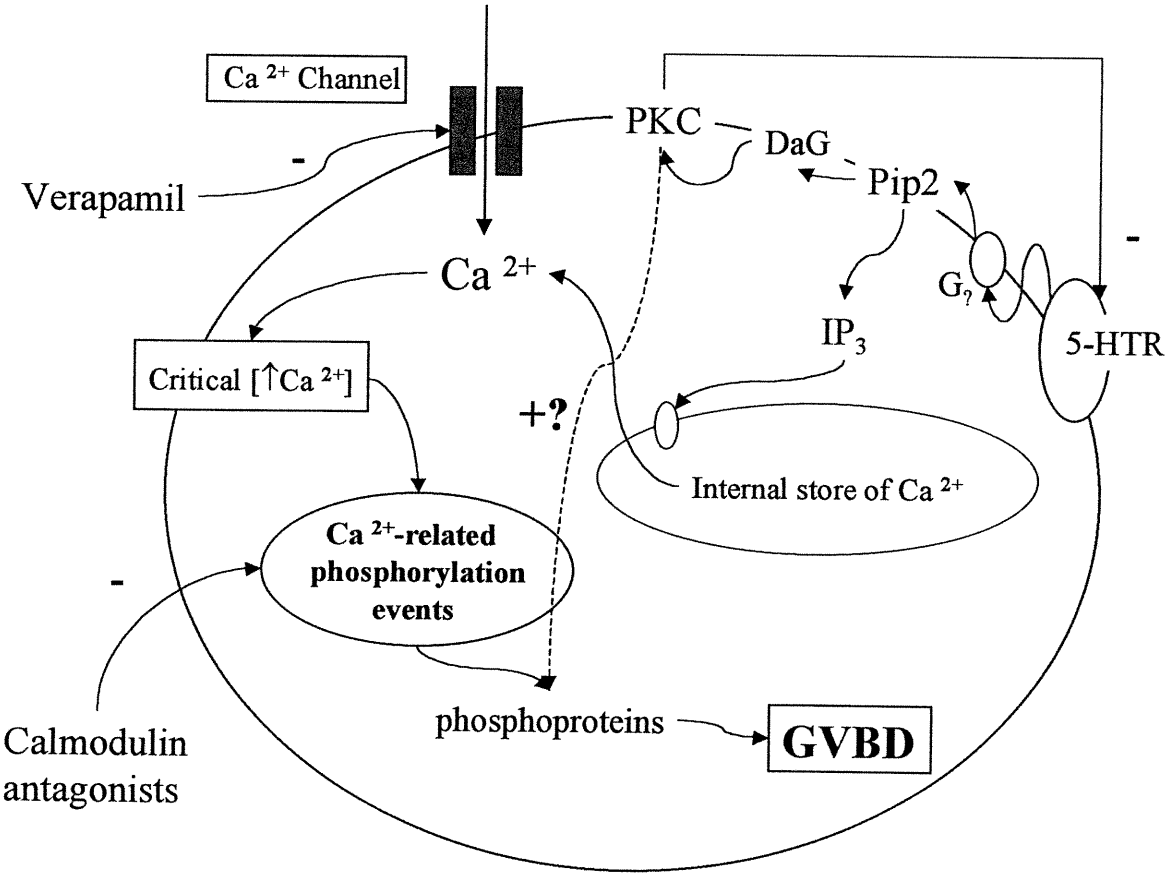
The existence of internal stores of Ca^{2+} has been postulated for the oocytes of this invertebrate. The fact that the calcium ionophore A23187 alone or microinjection of IP_3 can induce GVBD, along with the fact that the inhibitor of Ca^{2+}-ATPase thapsigargin induces GVBD (Dubé, 1992), support this view. The involvement of the

PLC-pathway in the activation of clam oocytes has been the subject of many experiments. Subthreshold levels of KCl, though insufficient alone to trigger GVBD, allow the entry of some Ca^{2+} ions and act synergistically with TPA, a protein kinase C activator, to trigger GVBD with a normal timing (Dubé *et al.*, 1987). TPA alone can activate oocytes, albeit slowly, without any detectable Ca^{2+} uptake (Dubé *et al.*, 1987) even though the presence of external Ca^{2+} is required (Eckberg *et al.*, 1987). The fact that IP_3 injection can trigger GVBD suggests that the mechanism leading to GVBD involve the production of IP_3 and DAG (diacyl glycerol) and the activation of protein kinase C (Bloom *et al.*, 1988). The additional finding that H-7 (1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine), or retinol, both C-kinase antagonists, can inhibit sperm and TPA-induced GVBD further suggests the involvement of PLC pathway leading to IP_3 and DAG production, although the specificity of C-kinase inhibitors has been questioned (Eckberg *et al.*, 1987). Although the cross-talk between the early Ca^{2+} influx and the PLC pathway needs to be further elucidated, it seems likely that fertilization involves the PLC pathway leading to the entry of the Ca^{2+} necessary and responsible for the activation of *Spisula* oocytes.

1.2.1.3 Downstream effect of the early Ca^{2+} influx

What then, could be the immediate downstream effect(s) of the initial Ca^{2+} influx? A calmodulin antagonist, added up to 1 minute after stimulation, inhibits GVBD indicating that calmodulin-dependent events follow the initial Ca^{2+} influx (Carroll and Eckberg, 1986). It seems thus that the initial Ca^{2+} influx triggers downstream events via CaM-kinases (calcium-calmodulin-dependent kinases)-related phosphorylations causing a subsequent phosphorylation of phosphoproteins implicated in the cascade leading to GVBD. Figure 1 summarizes a signal transduction pathway involved in the activation of *Spisula* oocytes, describing postulated functions of Ca^{2+} and the involvement of the PLC-pathway in the resumption of meiosis.

Figure 1. Hypothetical model of early events involved in the resumption of meiosis in *Spisula* oocytes


Ca 2+ Channel
PKC
DaG
Pip2
Verapamil
-
Ca 2+
G?
IP3
5-HTR
-
Critical [↑Ca 2+]
+?
Internal store of Ca 2+
Ca 2+-related phosphorylation events
-
phosphoproteins
GVBD
Calmodulin antagonists

1.2.2 Protein synthesis, protein phosphorylation, and oocyte maturation

The *in vivo* profile of phosphorylated proteins after fertilization or artificial activation seems to be well conserved, regardless of the activating agent used (Clotteau and Dubé, 1996). This suggests that, although utilizing different pathways, the resulting GVBD require the phosphorylation of similar or identical proteins. The block to this phosphorylation process by 6-DMAP (6-dimethylaminopurine) inhibits GVBD upon activation by KCl in a reversible and a dose-dependent manner, further emphasizing the close relation between the phosphorylation process and the meiotic resumption leading to GVBD (Dubé *et al.*, 1991). Considering the short time taken for the activated oocytes to reach GVBD (about 10 minutes), prolonged and sophisticated mechanisms for the activation are unlikely in vertebrates. Indeed, Longo *et al.* (1991) reported that GVBD and the 1st polar body formation is independent of newly synthesized proteins, as confirmed by pretreatment with emetine (protein synthesis inhibitor) in fertilized or A-23187-activated oocytes. Since protein synthesis is not required for completion of meiosis I (Hunt *et al.*, 1992), it seems that the mechanism leading to GVBD mainly involves post-translational modifications of key phosphoproteins.

1.2.2.1 Maturation promoting factor in *Spisula* oocyte

The MPF (M-phase promoting factor) is a complex formed of Cdc2-kinase and cyclin B (Labbé *et al.*, 1989), first found by transferring the cytoplasm of maturing oocyte to another immature oocyte which caused a maturation of the immature oocyte (Masui and Markert, 1971). During the mitotic cell cycles, inactive Cdc2-kinase is joined by newly synthesized cyclin B and then undergoes a series of phosphorylations and dephosphorylations on key threonine and tyrosine residues that activate cdc2 kinase for its M-phase specific functions (Draetta et al., 1988, 1989; Gould and Nurse, 1989; Murray and Kirschner, 1989; Solomon et al., 1990; reviewed in Nurse, 1990). In *Spisula* oocytes, it has been shown that they synthesize new proteins upon activation, two of which were identified as cyclins (Rosenthal *et al.*, 1980, Evans *et al.*, 1983). Both cyclin A and B levels were determined by quantitative densitometry and it was shown that, oocytes lack any detectable cyclin A protein but do contain a stockpile of cyclin B protein which must have been made earlier in oogenesis (Westendorf *et al.*, 1989). Since *Spisula* oocytes already contain a stockpile of cyclin B that are complexed with Cdc2-kinase (Westendorf *et al.*, 1989), the control of its activity must depend on the phosphorylation of its key residues. However, the cascade leading to the activation of MPF and GVBD from the early entry of Ca^{2+} in *Spisula* oocyte remains poorly understood. Nevertheless, MPF remains one of the key proteins implicated in the process of GVBD, as shown by one of its functions to phosphorylate lamins which are directly linked to GVBD (Dessev and Goldman, 1988; Dessev *et al.*, 1991).

The fertilization relieves the masking of preexisting stockpiles of cyclin B to a more dispersed, soluble form (unmasking), possibly in response to the post-fertilization increase in cytoplasmic pH (Westendorf *et al.*, 1989). In the light of the fact that inhibiting protein synthesis by emetine does not inhibit GVBD and the 1st polar body formation, these authors further suggested that this unmasking of maternal cyclin B protein, which undergoes association with Cdc2-kinase to result in functional

MPF, drives cells into meiosis I. It is interesting to note that, in *Spisula* oocytes, increase in pH alone does not trigger GVBD, but the phosphorylation inhibitor 6-DMAP inhibits GVBD (Dubé *et al.*, 1991). The possible events leading to the functional MPF and subsequent GVBD thus seem to be more complex, involving a possible regulation by the pattern of phosphorylation on certain key threonine and tyrosine residues in Cdc2-kinase (Draetta and Beach, 1988; Meijer and Arion, 1991).

1.2.2.2 Early phosphorylation of phosphoproteins in activated *Spisula* oocytes

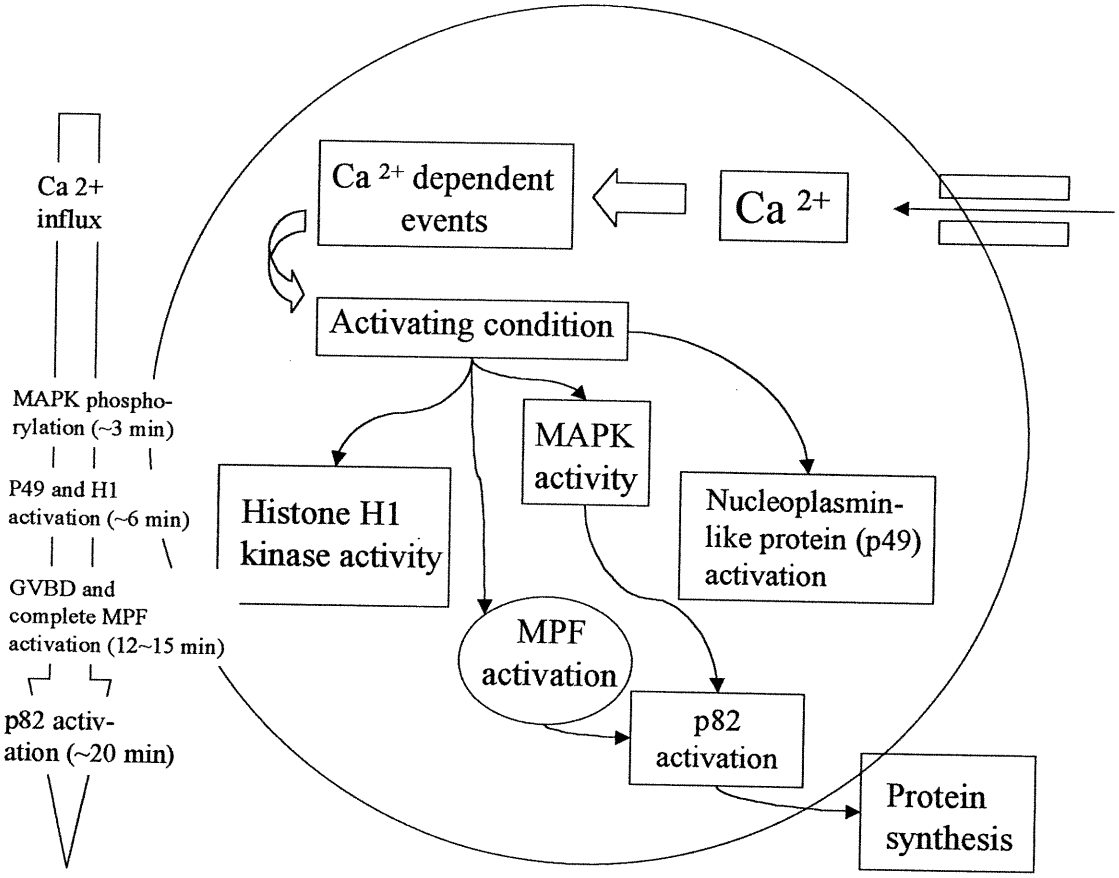
Many proteins undergo major phosphorylations upon activation in *Spisula* oocytes. K^+ activation induces early phosphorylation of 3 major proteins, p42, p49, and p56 (Clotteau and Dubé, 1996). Of these proteins, p42 is a MAP kinase (MAPK, mitogen-activated protein kinase, Shibuya *et al.*, 1992), p49 a nucleoplasmin-like protein (Herlands and Maul, 1994), and p56 a cyclin B (Dubé *et al.*, 1991). The phosphorylation of these proteins clearly precedes the complete dephosphorylation of tyrosine residue of Cdc2-kinase (Shibuya *et al.*, 1992), which occurs only after GVBD. This raises an interesting point of how MPF could promote GVBD if its complete activation occurs after the GVBD. In their report, Shibuya *et al* (1992) hypothesized that the partial activation of MPF might be sufficient to trigger the GVBD. Along with this, the histone H1 activity seems to be detected as early as 6 minutes post-activation (Walker *et al.*, 1999), supporting the hypothesis by Shibuya *et al* (1992). *In vitro* analysis have shown that, in the absence of Ca^{2+}, MAPK activation does not occur, and EGTA prevents p82 hyperphosphorylation but not H1 kinase activity (Katsu *et al.*, 1999). Blocking MPF with $p27^{kip1}$ (specific cdk inhibitor) does not affect MAPK, suggesting that MAPK clearly precede MPF activation, as already shown by Shibuya *et al* (1992) in *in vivo* conditions. It seems thus that MPF activity is regulated by Ca^{2+}-independent event(s) that lie downstream of MAPK activation that requires the Ca^{2+}, at least *in vitro*. *In vivo*, however, MPF activation as well as GVBD does not occur in the absence of Ca^{2+} influx, thus suggesting that some of the Ca^{2+}-dependent steps have been bypassed during the preparation of cell-free in vitro oocyte

extracts. The diagram representing phosphorylation of major proteins and its relative time respect to each other is shown in figure 2.

1.3 Cyclic AMP and the oocyte maturation

It thus appears that the mechanisms of clam oocyte activation are controlled through a series of complex phosphorylation and dephosphorylation rather than via newly synthesized proteins. In this respect, cyclic AMP represents a better candidate for the role of reinitiating meiosis in clam oocytes, since cyclic AMP-dependent kinases (PKAs) are implicated in cell cycle control in many cell types. Cyclic AMP is produced by the hormone acting through stimulation of adenylyl cyclase, and acts as an important regulator of metabolic pathways in many systems through the downstream phosphorylation of the PKAs. The role of cyclic AMP has been continuously studied in model systems such as echinoid, amphibian, and mammalian oocytes, and considerable evidence has accumulated suggesting a crucial role of cyclic AMP as a negative regulator in oocyte maturation. Interestingly, cyclic AMP-raising treatments were reported to inhibit GVBD by spermatozoa or 5-HT in clam oocytes also (Sato *et al.*, 1985). Unfortunately, there were no follow-up reports supporting this report, which might have been crucial in understanding the molecular basis of activation in this species. Thus, for the sake of comparative analysis, we will now review the literature on the function of cyclic AMP in maturation of other well-known oocyte systems, such as mammalian, amphibian and echinoid.

Figure 2. Some of the proteins undergoing a major phosphorylation early in the resumption of meiosis in *Spisula* oocytes. Following the initial Ca^{2+} entry and subsequent Ca^{2+}-dependent events that lender the oocyte in the "activating condition," MAPK, p49, and H1 histone kinase activation precedes the GVBD and the complete MPF activation. P82 activation is regulated by both MAPK and MPF phosphorylation, and the protein synthesis follows the p82 activation.

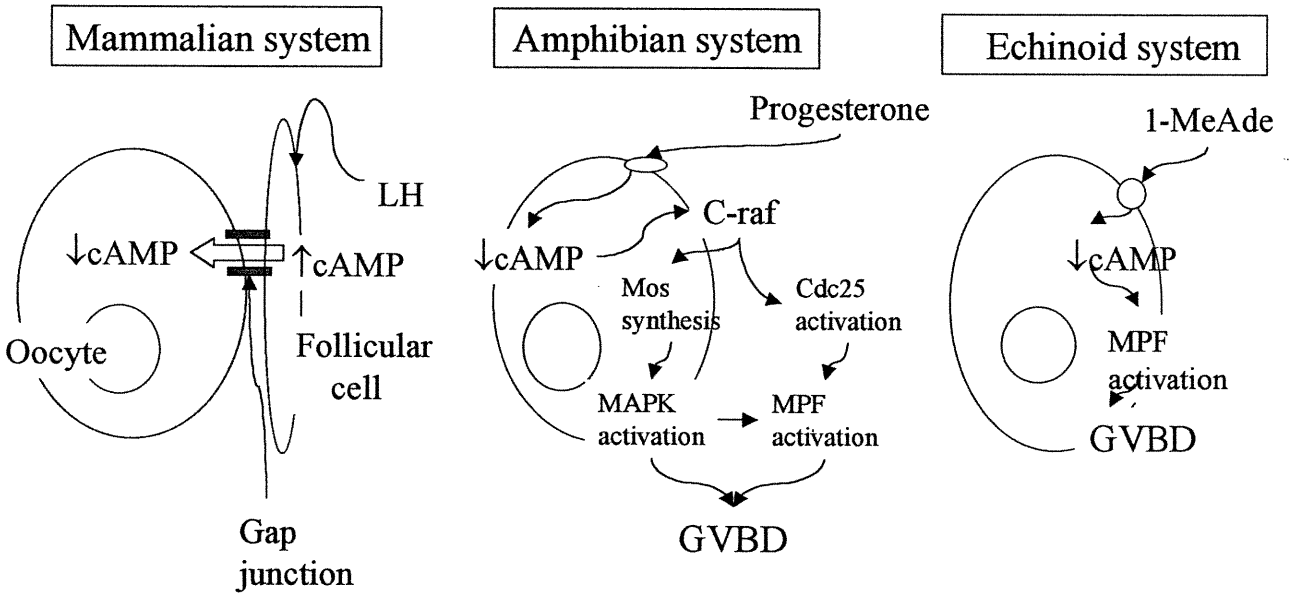

Ca 2+ influx
MAPK phospho-rylation (~3 min)
P49 and H1 activation (~6 min)
GVBD and complete MPF activation (12~15 min)
p82 activ-ation (~20 min)
Ca 2+ dependent events
Ca 2+
Activating condition
MAPK activity
Histone H1 kinase activity
Nucleoplasmin-like protein (p49) activation
MPF activation
p82 activation
Protein synthesis

1.3.1 Cyclic AMP and the mammalian oocyte maturation

1.3.1.1 Negative influence by cyclic AMP upon resumption of meiosis in mammals

It is generally accepted that under the influence of the follicular environment, the immature mammalian oocyte is maintained in meiotic arrest by the production of cyclic AMP in the granulosa cells (Homa, 1995, and references therein). Cyclic AMP produced in the granulosa or cumulus cells is thought to be capable of passing through gap junctions and maintain meiotic arrest in the oocytes. Denuded, cumulus-free oocytes undergo spontaneous maturation. This spontaneous maturation is blocked when oocytes are incubated with compounds that elevate intraoocyte cyclic AMP level. For example, the diterpene forskolin, an activator of adenylyl cyclase, inhibits GVBD of cumulus-free oocytes *in vitro* (Sato and Koide, 1984). Spontaneous GVBD is also inhibited in the presence of dibutyryl cyclic AMP (Cho *et al.*, 1974). Moreover, neither LH or FSH (follicle-stimulating hormone) in a wide range of concentrations acted directly on the oocytes or indirectly through the follicle cells to initiate maturation in oocytes incubated with dbcAMP (Nekola and Smith, 1975). Meiotic resumption is prevented not only by high levels of cyclic AMP following incubation with cyclic AMP analogs, but with inhibitors of cyclic AMP degradation. Spontaneous resumption of meiosis is efficiently blocked by PDE (phosphodiesterase) inhibitors (Dekel *et al.*, 1981). Furthermore, microinjection of PKI (PKA inhibitor) into the oocyte induces GVBD and PKAc (catalytic subunit of PKA) prevents GVBD (Bornslaeger *et al.*, 1986), emphasizing the negative influence by cyclic AMP upon resumption of meiosis. This function of cyclic AMP in the resumption of meiosis seems to apply not only to mouse oocytes but extends to other mammals as well, such as bovine and rabbit oocytes (Rose-hellekant and Bavister, 1996; Richard *et al.*, 1997; Yoshimura *et al.*, 1992). The putative role of cyclic AMP of the pre-ovulatory period in mammalian follicle is depicted in figure 3.

Figure 3. Cyclic AMP negatively regulates oocyte maturation in three different oocyte systems. In some mammalian systems, there exists an opposite compartmental change of the cAMP concentration between the follicular cells and the oocyte. In some amphibian and echinoid systems, oocyte cAMP concentration decreases following progesterone or 1-MeAde stimulation of the ooctye.


Mammalian system
LH
↓cAMP
↑cAMP
Oocyte
Follicular cell
Gap junction
Amphibian system
Progesterone
C-raf
↓cAMP
Mos synthesis
Cdc25 activation
MAPK activation
MPF activation
GVBD
Echinoid system
1-MeAde
↓cAMP
MPF activation
GVBD

1.3.1.2 Compartmentalized changes in the level of cyclic AMP in the granulosa cells and the oocytes upon maturation; action of PDEs

The level of oocyte cyclic AMP decreased significantly during the time in which oocytes become committed to resuming meiosis (Schultz *et al.*, 1983). This decrease in oocyte cyclic AMP is not observed in oocytes inhibited from resuming meiosis by IBMX (isobutyl methylxanthine). Interestingly, this decrease apparently occurred before or during a period of time in which follicle and cumulus cell cyclic AMP were increasing (Schultz *et al.*, 1983). It is reported that GVBD can be induced by injecting the cyclic AMP derivative dbcAMP into the follicular anthrum (Tsafriri *et al.*, 1972), by transient exposure of follicles to 8-Bromo-cAMP (Hillensjo *et al.*, 1978), dbcAMP or IBMX (Dekel *et al.*, 1981), and by the addition of forskolin to cultured follicle-enclosed oocytes (Dekel, 1986). In addition, the hormone LH responsible for triggering meiotic resumption in vivo has classically been associated with stimulation of adenylyl cyclase activity (Marsh, 1975). Thus it can be explained that the induction of oocyte maturation by LH which leads to the increased cyclic AMP level in the follicle cells should lead to a drop in the supply of cyclic AMP to the oocyte. When the supply of cyclic AMP from the somatic compartment is blocked, oocyte levels of cyclic AMP level off through the action of PDE and the maturation takes its course (Tsafriri *et al.*, 1996). Using probes specific for different isoforms of PDEs, it recently has been suggested that PDE4 expression is specific for the follicular cells while PDE3 is expressed predominantly in oocytes (Tsafriri *et al.*, 1996). Using specific blockers, inhibition of PDE3 blocks resumption of meiosis, while inhibition of PDE4 causes an increase in cyclic AMP in the granulosa cell compartment causing a meiotic resumption similar to that induced by LH (Conti et al., 1998). This further supports the compartmentalized changes in the level of cyclic AMP occuring during meiotic maturation in the granulosa cells and the oocytes.

1.3.2 Cyclic AMP and the amphibian oocyte maturation

1.3.2.1 Cyclic AMP decreases in progesterone-treated *Xenopus* oocyte

Xenopus laevis oocyte maturation is by far the most intensely studied, and best understood system probably because of easy access to different experimental conditions, of the large size of eggs, and of the lag period which precedes GVBD. In view of this, a large body of data from many different approaches has accumulated over the years to reveal the signaling cascade that initiates maturation in this species. Progesterone, synthesized and released by follicle cells in response to pituitary hormones, causes a decrease in cyclic AMP levels within minutes. It is reported that physiological concentrations of progesterone selectively inhibit membrane bound adenylyl cyclase activity (Finidori-Lepicard *et al.*, 1981). In consequence, exposure to progesterone causes a drop in cyclic AMP level. Several different results have been reported regarding the drop in cyclic AMP level after progesterone induction of meiosis resumption in *Xenopus* oocytes. A 10 ~ 50% drop in cyclic AMP levels during maturation (Speaker and Butcher, 1977; Bravo *et al.*, 1978; Maller *et al.*, 1979; Schorderet-Slatkine *et al.*, 1982), an increase in cyclic AMP levels (Morrill *et al.*, 1981), or no change (Schorderet-Slatkine *et al.*, 1978) have been reported. This apparent discrepancy might be due to the difficulty of detecting slight variation in cyclic AMP concentration (Cicirelli and Smith, 1985). Cicirelli and Smith (1985) also reported a 20% decrease in the cyclic AMP content of *Xenopus* oocytes during the first 2 ~50 min following progesterone addition, after minimizing the variability by selecting oocytes of the same size and normalizing data from several females.

1.3.2.2 Cyclic AMP-raising treatments inhibit GVBD in *Xenopus* oocytes

In *Xenopus laevis* oocytes, two inhibitors of cyclic AMP phosphodiesterase, theophylline and papaverine, inhibit maturation induced by different stimuli by inhibiting protein synthesis, probably via a cyclic AMP-dependent protein kinase (Bravo *et al.*, 1978). Cholera toxin and forskolin, irreversible and reversible

activators of adenylyl cyclase, respectively, inhibit progesterone-induced meiosis resumption by increasing cyclic AMP level (Schorderet-Slatkine *et al.*, 1978; Schorderet-Slatkine and Baulieu, 1982). In addition, Maller and Krebs (1977) have shown that both the regulatory (R) subunit and inhibitor protein (I) of cAMP-dependent protein kinase induce maturation when injected into the oocyte, while the catalytic (C) subunit inhibited maturation, further demonstrating the negative function of cycilc AMP in *Xenopus* oocyte meiosis resumption. These results clearly indicate the function of cyclic AMP in the *Xenopus* oocyte maturation as a negative regulator. Putative signal pathway of *Xenopus* oocyte maturation is shown in figure 3b.

1.3.2.3 Protein synthesis and possible role for cyclic AMP in *Xenopus* oocyte maturation

The decrease in cyclic AMP is followed by a lag period of a few hours. During the first half of the lag period, protein synthesis is required for GVBD. One key protein synthesized during this period is the Mos (cellular homolog of the Moloney murine sarcoma viral oncogene) oncoprotein. The decrease in the intracellular level of cyclic AMP, which in turn inactivates cyclic AMP-dependent protein kinase and stimulates the transduction of c-mos mRNA (Gebauer and Righter, 1997). Unlike clam oocyte, *Xenopus* oocyte requires synthesis of Mos-protein for the resumption of meiosis. This Mos is a MAPK kinase kinase (MAPKKK), and apparently the only protein that must be synthesized for GVBD to occur. The injection of high doses of bacterially expressed Mos into oocytes in the presence of the protein synthesis inhibitor cyclohexamide induces GVBD (Yew *et al.*, 1992). However, a second protein, a novel type of cyclin called cyclin B1, is synthesized during the first half of the pre-GVBD lag period (Nebreda *et al.*, 1995). Thus the oocyte maturation leading to GVBD seems to implicate a complex regulation in synthesis and accumulation of these two proteins.

The progression of *Xenopus* oocytes into M-phase is accompanied by the nearly simultaneous activation of p42 MAPK and Cdc2/cyclin B (Nebreda and Hunt, 1993).

The regulation of *Xenopus* oocyte maturation by Mos protein seems to implicate the activation of MPF by MAPK cascade (Gebauer and Righter, 1997, and references therein). It has been reported that p42 MAPK activation is essential for the activation of Cdc2 kinase via injection of Mos protein (Huang and Ferrell, 1996). In addition, c-mos proto-oncogene blocks cyclin degradation through MAPK activation, thus causing histone H1 kinase activity to remain high (Abrieu *et al.*, 1996). These results place Mos and MAPK activation upstream of Cdc2 kinase activation, and suggest that the MAPK cascade regulate the activity of MPF. The level of cyclin B1 is directly regulated by the initial progesterone stimulation and does not depend on increased Cdc2 kinase activity. Also, Mos accumulation depends on the Cdc2 kinase unlike cyclin B1 accumulation where inhibition of PKA is sufficient to allow cyclin B1 accumulation when Cdc2 kinase activation is inhibited by $p21^{cip1}$ (a well known inhibitor of cdk/cyclin complexes). When Cdc2 kinase is blocked by $p21^{cip1}$, Mos accumulation does not occur, thus arguing that the Mos/MAPKK/MAPK pathway is under the control of Cdc2 kinase activity (Frank-Vaillant *et al.*, 1999). These results suggest that the mechanism leading to GVBD is regulated by a complex interaction of the kinases involved, and the activation of MPF might be dependent upon both synthesis and accumulation of Mos and cyclin B1 proteins in *Xenopus* oocytes.

It has been suggested that Mos protein synthesis is under the direct control of PKA, such that elevated levels of PKA inhibit Mos protein synthesis (Matten *et al.*, 1994). The injection of the C-subunit of cAMP-dependent protein kinase prevented progesterone induced synthesis of endogenous Mos as well as downstream MPF and MAP kinase activation. The C-subunit of cyclic AMP-dependent protein kinase does not prevent MAP kinase activation by injected Mos, but can inhibit MPF and Cdc25 activation, which suggests that Mos acts at more than one site in the meiotic maturation of *Xenopus* oocytes (Matten *et al.*, 1994). However, it was later found that PKA does not exert any inhibitory effect on Mos translation induced by exogenous Mos-injection (Faure *et al.*, 1998), arguing against the current model of Mos sunthesis control. Rather, it has been suggested that PKA exert its negative effect on c-raf (serine/threonine kinase downstream of Ras that activates MAPKK/MAPK cascade.

Ras belongs to a superfamily of small GTP-binding proteins that act as a molecular switch in the transduction of many extracellular signals). Upon progesterone stimulation, the activity of PKA decreases, and c-raf activates MAPK activation leading to c-mos translation, and cdc25 activation leading to MPF activation in a parallel and separate pathway (Faure *et al.*, 1998). Another postulated action of PKA in *Xenopus* oocyte is upon cdc25, a phosphatase activating MPF by dephosphorylation of tyrosine residue. It has been shown that, PKAc causes a potent block in the electrophoretic mobility shift of cdc25 that is associated with phosphatase activation (Matten *et al.*, 1994). Also, increasing cyclic AMP totally prevents the action of cdc25 microinjected into the oocyte (Rime *et al.*, 1994). However, the exact influence of PKA remains obscure, and awaits further investigation.

1.3.3 The role of cyclic AMP in echinoid systems

Unlike *Xenopus* oocytes, the induction of meiosis I does not require protein synthesis in starfish oocytes. Forskolin and Sp-cAMPs delay 1-methyl-adenine (1-MA)-induced GVBD (Meijer and Zarutskie, 1987; Meijer *et al.*, 1989) but do not inhibit it. 1-MA induces a drop of 10-30 % in cyclic AMP levels, but reversing this drop or increasing cyclic AMP levels only retarded the hormone-dependent period thus resulting in a slower GVBD (Meijer and Zarutskie, 1987). It seems that part of the dephosphorylation process required for the functional activation of MPF, namely tyrosine residues of Cdc2-kinase, in forskolin-treated oocytes, is delayed and incomplete (Meijer and Arion, 1991). Meijer and Arion proposed that a double pathway trigger G2/M transition. A *dis-inhibitory pathway* that inactivates the cAMP-dependent inhibitory pathway and provides "permissive" conditions that are necessary but not always sufficient for maturation, and an *activating pathway* that is negatively influenced by cyclic AMP through cyclic AMP-dependent phosphorylation.

1.4 The possible role of cyclic AMP in the maturation of *Spisula* oocytes

The potential involvement of cyclic AMP in *Spisula* oocytes was first investigated by Sato *et al.* (1985) who showed that compounds that increase intracellular cyclic AMP (forskolin, IBMX, and dibutyryl-cAMP) inhibited GVBD induced by sperm and serotonin, but not by KCl. In their report, Sato *et al.* suggested the arrest of meiosis at the dictyate-state might be regulated by intracellular cyclic nucleotides. However, the detailed mechanism of this inhibitory effect exerted by treatments reported to increase intracellular cAMP is still lacking. The fact that such compounds could inhibit serotonin activation may suggest the possibility of a putative serotonin receptor coupled to a G-protein negatively bound to adenylyl cyclase, thus resulting in a decrease of cAMP level in response to serotonin addition. This leads to the necessity of determining the changes in actual levels of intracellular cyclic AMP in response to 5-HT, required to further characterize the molecular aspect of serotonin activation. In this section, we will review the characteristics of the putative serotonin receptor found in *Spisula* oocyte, and the classification of currently recognized serotonin receptors found in other systems.

1.4.1 Serotonin receptor found in *Spisula* oocytes

The neurohormone serotonin was shown to induce gamete shedding when injected in the gonad of both male and females, and to induce GVBD when applied externally to the isolated oocytes (Hirai *et al.*, 1984). A pharmacological analysis of the serotonin-induced meiosis reinitiation further confirms that the oocyte response to serotonin is specific, and accompanied by a transient increase of Ca^{2+} uptake (Krantic *et al.*, 1991), comparable to the uptake observed in the fertilized *Barnea* oocytes (Dubé and Guerrier, 1982). This suggested that serotonin would best mimic the sperm-activated resumption of meiosis via a physiological, receptor-mediated mechanism (Dubé, 1996), whereas KCl-induced activation would not so properly reflect the normal sperm-induced activation in *Spisula* oocytes. Using oocyte membrane preparations in binding assays, the putative receptors involved in the

maturation process were later proposed to be a novel type with atypical properties when compared to other mammalian receptors (Krantic *et al.*, 1993). Characterizing the signal transduction used by this receptor thus seems to be the prerequisite in understanding the molecular mechanism involved in the resumption of meiosis.

1.4.2 Classification of serotonin receptors

The currently recognized serotonin receptors are classified in a review by Martin and Humphrey (1994), where they report the existence of as many as seven classes of 5-HT receptors on the basis of operational, transductional, as well as structural criteria for definitive receptor characterization. Among them, only the 5-HT_1, 5-HT_2, and 5-HT_3 receptor classes are well defined. The 5-HT_1 receptor class is characterized as 7-transmembrane, G-protein coupled receptors encoded by intronless genes, which are linked preferentially to the inhibition of adenylyl cyclase. At least five 5-HT_1 receptor subtypes are now recognized, viz. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1E} and 5-HT_{1F} (Martin and Humphrey, 1994). The 5-HT_2 receptor class is a 7-transmembrane, G-protein linked receptor encoded by genes possessing introns and exons, and preferentially coupled to PLC. The 5-HT_2 receptor class now comprises three distinct receptor subtypes, being 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (Martin and Humphrey, 1994). The 5-HT_3 receptors are ligand-gated cation channels and structurally and functionally distinct from other 5-HT receptor types (Martin and Humphrey, 1994). The 5-HT_4 receptors are positively coupled to adenylyl cyclase in brain tissue (Dumuis *et al.*, 1988). The 5-HT_5, 5-HT_6, and 5-HT_7 receptors were identified recently and await further characterization of their respective pharmacological profiles (Martin and Humphrey, 1994).

1.5 The objective of the study

Despite the lack of demonstrable evidence for the negative role of cAMP on *Spisula* oocyte activation, the relevant literature on vertebrate species supports the report by Sato et. al. (1985) where cAMP blocked the resumption of serotonin- and

cAMP on the maturation of *Spisula* oocytes, in addition to pharmacological studies pointing to the involvement of serotonin receptor-mediated calcium events in the oocyte (Krantic et al., 1991; Colas and Dubé 1998), led us to hypothesize that the decrease in the concentration of cAMP might not be the contributing factor of the meiosis reinitiation.

To test this working hypothesis, a series of experiments was planned to measure fluctuations of oocyte cAMP content during meiosis resumption, and to study the effect of cAMP-raising treatments on the GVBD. Our specific aim was to investigate the possible involvement of cyclic AMP in the resumption of meiosis in *Spisula* oocytes induced by sperm, serotonin or KCl. Using *in vivo* cellular observations and fluorescence microscopy, we have tested different chemical stimuli, known to increase intracellular cyclic AMP level, for their effect on the activation process. Also, the actual levels of cyclic AMP in oocytes were measured by radioimmunoassay (RIA) at different times after serotonin addition to test its possible involvement in the activation process and gain additional informationon the transductional mechanism used by activated receptors. The implications of these findings for a better understanding of the regulation of meiotic maturation and MPF activation in *Spisula* oocytes are discussed.

2.0

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

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

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Note: Linda Lefièvre is a pH.D. student at McGill university. She has collaborated in this research by introducing the author with more efficient method of cyclic AMP assay, and comments for this article. Dr. Claude Gagnon is her supervisor at McGill university, and his comments on this article are also recognized.

SUMMARY

Surf clam (*Spisula solidissima*) oocytes are spawned in the external medium at the germinal vesicle stage and they remain arrested at this prophase I stage of meiosis until fertilization. Full oocyte meiosis reinitiation, first evidenced by germinal vesicle breakdown (GVBD) followed by extrusion of two polar bodies, may be induced by artificial activators mimicking sperm, such as high K^+ seawater that causes a Ca^{2+} influx through opening of voltage-dependent Ca^{2+} channels, or by serotonin through a recptor-mediated pathway. Previous reports indicated that various treatments, thought to increase the level of oocyte cAMP, were inhibitory, in clam oocytes, for sperm- or serotonin-induced, but not KCl-induced, GVBD. It thus appeared that a drop in cAMP was important for triggering meiosis reinitiation in clam oocytes, as it is well known, especially in mammalian and amphibian oocytes. We have reexamined the cAMP dependency of GVBD in clam oocytes and found that incubations of oocytes in presence of either IBMX, a phosphodiesterase inhibitor, or forskolin, an adenylyl cyclase activator, or both, up to concentrations ten times higher than those previously reported inhibitory, were surprisingly without effect on both KCl- or serotonin-induced GVBD. Other cAMP-raising treatments were similarly without effect on GVBD. Some of these treatments, however, inhibited GVBD upon insemination of the oocytes, as previously reported, but this effect was due to the failure of sperm to fuse/penetrate the oocytes and thus cannot be considered as an inhibition of oocyte activation as such. Direct measurements of oocyte cAMP levels before and after activation by serotonin or KCl showed that, contrary to expectations, there is a transient increase in cAMP levels before GVBD. Experimental alterations of Ca^{2+} influx or intracellular pH (pHi) of oocytes indicated that the rise in oocyte cAMP is specifically neither Ca^{2+}- nor pHi-dependent, but appears as an all-or-none process tightly linked to full oocyte activation leading to GVBD. Contrary to earlier reports, a rise of oocyte cAMP was also detected after normal fertilization. Using SQ22536, an adenylyl cyclase inhibitor, the increase in oocyte cAMP level, upon addition of serotonin, was lowered but this nevertheless resulted in GVBD, but with a significant retardation suggesting that the normal cAMP rise, if not absolutely required, is at least

facilitating GVBD. In conclusion, our work clarifies the involvement of cAMP in meiosis reinitiation of surf clam oocytes and provides a new unusual model whereby meiosis reinitiation and M-phase promoting factor (MPF) activation are associated with a rise, not a drop, of oocyte cAMP.

Introduction

Oocyte meiotic maturation is an extensively studied process in the animal kingdom. During the course of gametogenesis, fully-grown oocytes generally become arrested at prophase I (germinal vesicle stage) of meiosis. Most often, meiosis is reinitiated upon hormonal stimulation, and first results 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, respectively, by progesterone or luteinizing hormone (LH) surge. Accumulated evidences indicate that the release from prophase I arrest is accompanied by a decrease in oocyte intracellular levels of cAMP (adenosine 3', 5'-cyclic monophosphate), 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 in both amphibian and mammalian oocytes (Cicirelli and Smith, 1985 ; Maller, 1985 for *Xenopus laevis* ; Vivarelli *et al.*, 1983 ; Schultz *et al.*, 1983 ; Abergdam *et al.*, 1987 for mouse) is not only required but is sufficient to trigger further meiotic progression.

The current view is thus that cAMP-dependent phosphorylation by protein kinase A (PKA) regulates the activity of one or more proteins still to be identified, one of them possibly 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 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, respectively, mammalian and amphibian oocytes (Schultz *et al.*, 1983 ; Urner *et al.*, 1983 ; Sato and Koide, 1984 for mouse ; Schorderet-Slatkine and Baulieu, 1982 for *Xenopus laevis*).

This general mechanism of release from prophase I seems to apply not only to amphibian and mammalian oocytes, but seems to extend to other vertebrates as well, such as brook trout (*Salvelinus fontinalis*) and Catfish (*Clarias batrachus*) oocytes (DeManno and Goetz, 1987 ; Haider and Chaube, 1996) , as well as bovine and rabbit oocytes (Rose-Hellekant and Bavister, 1996 ; Rchard *et al.*, 1997 ; Yoshimura *et al.*, 1992) , although the detailed mechanism of oocyte maturation further needs to be elucidated. 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 since the natural, presumably hormonal, trigger for meiosis reinitiation in this species is unknown and whether the forskolin treatment mimicks the normal physiological process remains to be established. However, in other echinoderms, it has been shown that 1-methyl-adenine-induced meiotic maturation of starfish oocytes is also accompanied by a decrease in cAMP which, if altered, does not prevent but at least retards meiotic progression (Meijer and Zarutskie, 1987). Thus, there appears to be an almost universal decrease in oocyte cAMP, which is sufficient for triggering the release from prophase I arrest, or is at least positively affecting it. However, echinoderms and vertebrates belong to deuterostome animals and, as such, have a quite different developmental regulation than 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. Little is known, however, about the possible involvement of cAMP in the regulation of meiotic maturation, especially in those protostome species which are normally fertilized at the prophase I stage.

The oocytes of surf clam, a bivalve mollusc, are released at the prophase I stage and fertilization normally reinitiates the meiotic maturation process. Artificial activation may be induced through the use of compounds raising intracellular Ca^{2+}, such as ionophore or high K^+ seawater (Allen, 1953 ; Schuetz, 1975), and also by the neurohormone serotonin (Hirai *et al.*, 1988), 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 presence of forskolin or IBMX, would 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 likewise relying on decreased intracellular cAMP to achieve meiotic maturation.

The aim of the present work was to reexamine 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. 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 relying 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 methodes

Handling of Gametes

Specimens of surf clams (*Spisula solidissima*) were collected at Iles-de-la-Madeleine (Quebec, Canada) from mid-June to late July. Large stocks of animals were kept at the Station Aquicole de Pointe-au-Père (Université du Québec à Rimouski) in running seawater tanks. Small numbers (20 -30) of clams were shipped, when required, to Montreal where they were kept in a 200 L. closed system aquarium set at 7°C. Gametes were obtained and handled as described by Allen (1953).

Chemicals and solutions

IBMX, an inhibitor of phosphodiesterase, and dbcAMP (N^6,2'-0-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) solution, and was mixed 1 : 1 with the oocytes to be fixed.

Fertilization or Artificial Activation of Oocytes

Oocytes were washed three to five times with ASW by unit gravity and resuspended in 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 a proper amount of a 1 M stock solution in ASW, adjusted to pH 8.0 just prior to its use, to oocyte suspensions. Fertilization was achieved by adding to oocyte suspension 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 were processed as described in the legend of each figures. Briefly, tubes containing oocytes were rapidly centrifuged to pellet oocytes and discard ASW. These tubes were rapidly transfered 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 min in the presence of 0.5 ml 90% ethanol. Near the end of 30 min, oocytes suspended in ethanol were transferred to a Kimble and Kontes tissue homogenizer on ice and were homogenized for 1 to 2 min. A volume equivalent to 1/200 of the whole homogenate was taken out to check the state of homogenization under light microscopy. After transfering 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 min at -20°C, tubes were centrifuged (12 000 g) for 15 min at 4°C. The supernatant was separated from the pellet and either evaporated in a SpeedVac concentrator right away 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 was measured by radioimmunoassay (Brooker *et al*, 1979). Samples and standards were incubated with 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 6 hours at 4°C. Radioactivity in the pellets was measured in a Gamma counter. The results are

reported as picomoles cAMP per mg protein. The sensitivity of the assay was 5 pmoles/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 levels 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 ($P < 0.05$) are depicted by asterisks (*).

Result

Cyclic AMP-increasing treatments do not inhibit serotonin- or KCl- induced GVBD

It has been reported that cAMP raising treatments, more specifically the incubation of oocytes in presence of forskolin and IBMX at 5 μg/ml (12 μM) and higher concentrations, dramatically inhibit 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 described in the previous report by Sato *et al.* Surprisingly, when forskolin was tested up to 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 what has been reported (data not shown). We therefore examined whether the combined addition of both chemicals would affect GVBD. Figure 1 shows the result of such experiments which, once again, did not reveal any significant inhibitory effect of various combination 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 to 1 hr, measured the oocyte cAMP levels and examined their effect on subsequent 5-HT-induced GVBD. As shown in Figure 2A, the cAMP concentration (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 (untreated) oocytes (Fig. 2A, Dunnet's method, $P<0.05$), but this nevertheless resulted in apparently normal GVBD upon the subsequent addition of 5-HT (Fig. 2B). It was also noted that prolonged incubations of oocytes with IBMX and forskolin, without other usual activating agents never resulted in GVBD.

In many systems, membrane permeable cAMP analogs such as dbcAMP, 8-Bromo-cAMP and Sp-cAMPs have been demonstrated to be a potent activators of cAMP-dependent protein kinases. In figure 3, we tested their possible inhibitory effect on 5-HT- or KCl-induced GVBD of *Spisula* oocytes. Dibutyryl cAMP was unable to inhibit GVBD when concentrations up to 5 mM were tested (Fig. 3A and B), 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 as 8-Bromo-cAMP at concentrations up to 5 mM (Fig. 3C and D), or Sp-cAMPs at concentrations up to 100 μM (Fig. 3E and F) showed no inhibitory effect on GVBD triggered by either 5-HT or KCl.

Interestingly, there was a dramatic inhibition of GVBD (Fig. 4), as previously reported (Sato *et al.*, 1985). The concentration of the two chemicals were shown to be ineffective in inhibiting GVBD induced by 5-HT or KCl (Fig.1). This inhibition was further characterized by determining the percent incorporation of sperm pronuclei into the oocytes observed under the fluorescence microscope after staining oocyte DNA with Hoechst 33258. After a normal insemination, most oocytes showed condensed maternal chromosomes and a decondensed male pronucleus (Fig. 5A, after 30 min of insemination), and polar bodies were visible by 60 minutes (Fig. 5B). However, most

oocytes showed intact GV following insemination in the presence of IBMX and forskolin, even 60 minutes after insemination (Fig. 5C and D). Though there were visible sperm heads bound to the oocytes, they remained at the periphery of the oocytes and were not incorporated into them (Fig. 5C and D). Table 1 shows the state of sperm incorporation in the oocytes at different times after the insemination in the presence or the absence of IBMX and forskolin. At 30 minutes after the addition of sperm suspension, 96% of control oocytes have undergone GVBD, with at least one sperm pronucleus incorporated. However, in the case of oocytes inseminated in presence of IBMX and forskolin, almost none of the oocytes underwent GVBD (<10 %, Table 1). Upon treatment with IBMX and forskolin, the GV-intact oocytes were generally devoid of an incorporated male pronucleus (Table 1). This indicates that fertilization and incorporation of sperm pronuclei are inhibited by IBMX and forskolin, but not GVBD as such.

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, for an overall average value of 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. 6). In control (untreated) oocytes, the cAMP rise starts within 2 min, reaches a plateau between 5-10 min, and then the cAMP slowly declines, thus establishing that the increase in cAMP occurs prior to GVBD (at 10 min) and remains higher than in unactivated oocytes throughout this period (Fig. 6A). 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. 6A). When the same experiment is performed with oocytes pre-incubated in 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.) is seen within the same period after adding 5-HT,

but the subsequent decrease after GVBD is less obvious while the kinetics of GVBD is unaffected (Fig. 6B).

The increase of cAMPi 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 opening up voltage-gated Ca^{2+} channels (Dubé, 1988). To test whether this increase in cAMP concentration was restricted to 5-HT-activated oocytes, we have compared the effect of KCl activation to that of 5-HT activation on the cAMP concentration (Fig. 7A). Interestingly, a similar transient increase in the cAMP concentration was also observed in KCl-activated oocytes (Fig. 7A), 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 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 presence of NH_4Cl on their cAMP level and observed no significant changes (Fig. 7A). 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. With both 5-HT or excess K^+ added to oocytes under conditions that do not result in GVBD, no significant changes in cAMP levels could be detected (Fig. 7B). This indicates that the rise in cAMP is not immediately linked to any early events such as ligand binding to its receptor or initial membrane depolarization, but might be linked to the early Ca^{2+} influx or some other required step(s) for oocyte activation. Finally, we further examined this latter possibility by testing whether the oocyte cAMP levels would be sensitive to a graded series of Ca^{2+} influx, below or above the required threshold for activation, as can be induced by a graded series of added K^+ (Dubé, 1988). Figure 8 illustrates that no increase of oocyte cAMP could be detected with any concentration of added 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 highly 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 almost perfectly sperm-induced activation, we wanted to verify whether a normal fertilization would similarly 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 9 shows that fertilization indeed results in an early and steady increase of cAMP before GVBD. Despite the slower kinetics of GVBD due to imperfect synchrony of oocytes upon fertilization (20 ± 11 at 20 min, 86.5 ± 3.5 at 30 min, %GVBD ± S.E.M., Fig. 9), this rise of cAMP seems to eventually surpass that seen after the addition of 5-HT (Fig. 6), and this might be related to the absence of a detectable secondary decrease over the monitored period (Fig. 9). The measured cAMP cannot originate from spermatozoa since most of them were washed out prior to oocyte sampling and 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 detectable changes 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 to the cascade of events leading to GVBD, possibly through the cAMP-dependent phosphorylations. 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 have treated oocytes with SQ 22,536, a potent adenylyl cyclase inhibitor. A pre-incubation of oocytes with SQ 22,536 (1 mM), for 1 hr prior to the addition of 5-HT or excess K^+, resulted in GVBD,

but with a much slower kinetics than that seen with control untreated oocytes, with a 50% GVBD of 16.5 min vs 9.5 min (Fig. 10). This suggests that inhibition of adenylyl cyclase, and possibly interfering with the normal increase of cAMP, alter the normal program of oocyte activation. This is further supported by the results presented in Fig. 11 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 suggests that this lower rise in cAMP is related to the observed delayed GVBD (Fig. 11). This indicates that the rise of cAMP might indeed 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 (see Faure *et al.*, 1998 ; Ferrell, 1999 ; Heikinheimo and Gibbons, 1998 ; Dekel, 1996 and references therein). Artificially elevating cAMPi by treatments which activates adenylyl cyclase or inhibits PDEs (phosphodiesterases), or 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 (see Meijer and Zarutskie, 1987, and references therein). It has been reported that increasing cAMP by forskolin and/or IBMX, or incubations in 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 an universal required step for the release from prophase block.

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, also at concentrations ten times higher than those reported inhibitory (Sato *et al.*, 1985). The reason for this discrepancy between our results and previous ones is unclear, but may be due to some contaminants in the chemicals used, many years ago, in previous studies. Sperm-induced GVBD is blocked in presence of IBMX and forskolin, but we have shown that this inhibition is at the level of sperm-oocyte fusion/incorporation. We thus interpret this inhibition as an effect on the sperm itself which becomes unable to fertilize when raising its cAMP content. One likely possibility is that the acrosome reaction is somewhat affected by this treatment, perhaps prematurely triggered, since this process is generally associated with increased cAMP (reviewed in Breitbart and Spungin, 1997), but further investigations would be required to confirm this hypothesis.

Surprisingly, in addition, direct measurements of oocyte cAMP showed that it rises 20% to 40% very early 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 which failed to detect any significant changes in oocyte cAMP levels after fertilization (Adeyemo *et al.*, 1987). This could be due to their 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, since 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, 1993) is of a G_s protein-coupled type since 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 by induced membrane depolarization (Colas and Dubé, 1998). Taken altogether, these results reveal an unsuspected rise of occyte cAMP at the onset of release from prophase I arrest which is in strong contrast with what was known, to our knowledge, in all other animal species so far studied.

Is the rise in oocyte cAMP required for GVBD ?

As seen, incubations of oocytes in presence of forskolin and IBMX are not inhibitory but, instead, mimick the normal rise in cAMP seen after activation by KCl or serotonin. However, even prolonged incubations in presence of these chemicals (> 3 hours) never resulted in GVBD unless another activating agent was subsequently added to the oocytes. This indicates that the rise of cAMP is not sufficient alone 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. 11). 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 cAMP, GVBD might have been completely prevented. Notwithstanding this limitation in possible interpretations, 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 remains unknown 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 and which is absolutely required for GVBD to occur (Dubé, 1988 ; Colas and Dubé, 1998), followed by 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 any 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 is known to promote Ca^{2+} influxes of increasing amplitudes beyond and above a threshold level that results in GVBD (Dubé, 1988). We observed no changes in cAMP levels in oocytes which 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, e.g. 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 (reviewed by 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 what is 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 a rise in cAMP may 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. While this process is most likely 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.

For example, MAP kinase (mitosis-associated protein kinase) activation is concomitant or follows MPF activation, in *Xenopus* or starfish oocytes, respectively (Nebreda and Hunt, 1993, Picard *et al.*, 1996). While MAP kinase activation is required in the former species, it is obviously not in the latter. In *Spisula* oocytes, MAP kinase activation seems required for and precedes MPF activation (Shibuya *et al*, 1992), whereas in the annelid *Chaetopterus* oocytes, early MAP kinase activation occurs but is not required for MPF activation (Eckberg, 1997). These observations illustrate that the pathways leading to active MPF appear diverse from one species to another.

A drop in oocyte cAMP is associated with release from prophase arrest by its effect in reducing PKA activity which, in turn, is thought to maintain the arrest by phosphorylation of a regulatory susbtrate, remaining to be identified. In starfish oocyte maturation, cAMP seems to negatively affect the activation of MPF through mik1, wee1, and cdc25 (Meijer and Aion, 1991), while a decrease in cAMP alone is insufficient itself to trigger oocyte activation (see Meijer *et al.*, 1989). In best-known *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) making difficult the identification of causal effects. 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 tightly 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 there is no evidence for the presence of c-mos in invertebrates (Eckberg, 1997). In light of current knowledge, it is thus difficult to speculate which process could be positively affected by increased cAMP and enhanced PKA activity to promote cell cycle reentry 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 identify any specific substrate phosphorylated by PKA that may contribute to oocyte activation. It will be interesting to verify whether increased cAMP is a common step in the activation of oocytes from protostome animals, as opposed to deuterostomes.

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Table 1. Effect of IBMX and forskolin on the percent incorporation of sperm pronucleus into the oocytes.

Condition/time after fertilization (min.)	Oocytes with intact GV [3], no male pronucleus	Sperm incorporated with GV intact	GVBD [4] without sperm pronucleus	GVBD with sperm pronucleus
C[1], 1	98	2	0	0
C , 15	0	73	3	24
C , 30	0	2	2	96
Ibmx+forsk[2] , 1	94	6	0	0
Ibmx+forsk , 15	99	0	1	0
Ibmx+forsk , 30	98	0	2	0
Ibmx+forsk , 60	91	0	1	8

[1]C. Fertilization using approximately 1 : 50 000 sperm suspension with 0.25 % v/v oocytes.

[2]Ibmx+forsk.. Fertilization in presence of 17.5 µg/ml IBMX and 25 µg/ml forskolin.

[3]GV. Germinal vesicle.

[4]GVBD. Germinal vesicle breakdown.

Fig. 1. Effect of forskolin and IBMX pretreatments on 5-HT- or KCl-induced GVBD of *Spisula* oocytes. Oocytes were pretreated with various combinations of forskolin and IBMX, at indicated concentrations, for 15 min. before adding 5-HT (5 μM, A) or KCl (45 mM, B). DMSO: DMSO vehicle alone; Forsk/IBMX: Forskolin and IBMX alone (65/60 μM) without any activating agent. GVBD was scored after 20 min. Mean results (± S.E.D.) of 4 experiments.

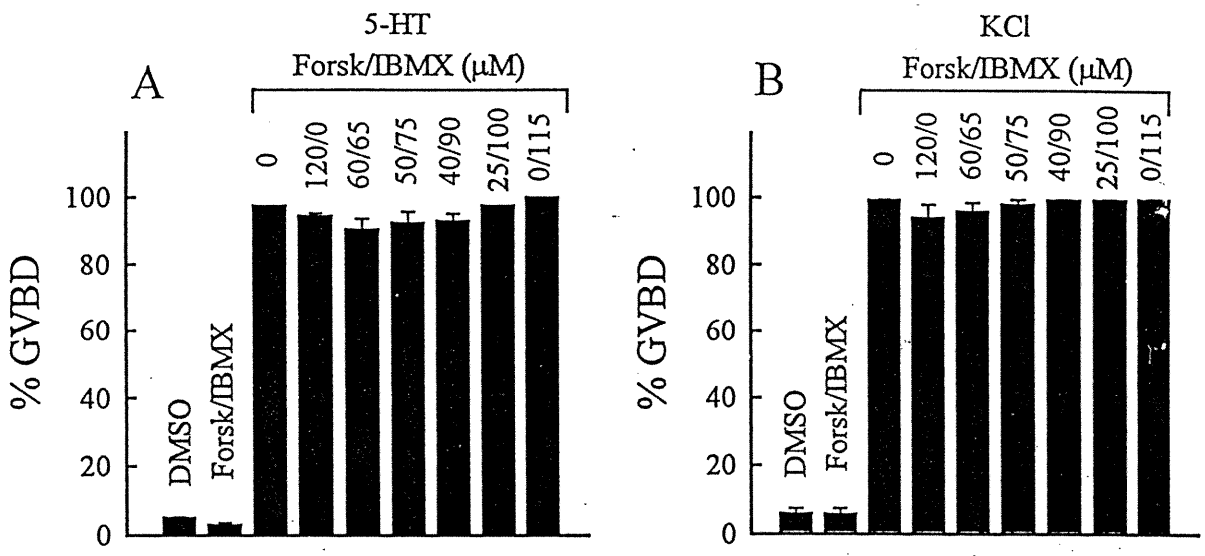

A
5-HT
Forsk/IBMX (μM)
% GVBD
100
80
60
40
20
0
DMSO
Forsk/IBMX
0
120/0
60/65
50/75
40/90
25/100
0/115
B
KCl
Forsk/IBMX (μM)
% GVBD
100
80
60
40
20
0
DMSO
Forsk/IBMX
0
120/0
60/65
50/75
40/90
25/100
0/115

Fig. 2. Effects of forskolin and/or IBMX pretreatments on the concentration of oocyte cAMP and on 5-HT-induced GVBD. A) Effect of incubating oocytes for one hour in presence of forskolin (120 μM), IBMX (115 μM), or a combination of both (60/65 μM) on oocyte cAMP concentration determined as described in Materials and Methods (Panel A) and on subsequent 5-HT-induced GVBD (Panel B). Unt.: oocytes left untreated. DMSO: oocytes treated with the DMSO vehicle alone (Panel A) and to which 5-HT was added (Panel B). Asterisk (*) : condition that resulted in an oocyte cAMP concentration significantly different from that of untreated oocytes. Mean results (± S.E.M.) of three experiments.

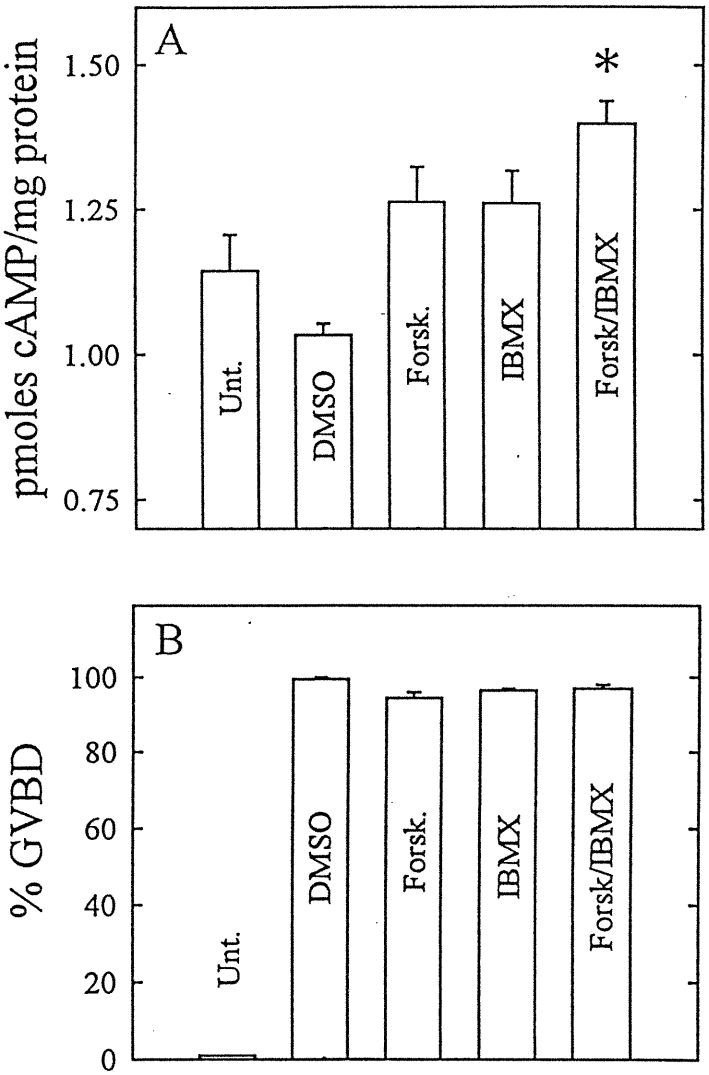

A
pmoles cAMP/mg protein
1.50
1.25
1.00
0.75
*
Unt.
DMSO
Forsk.
IBMX
Forsk/IBMX
B
% GVBD
100
80
60
40
20
0
Unt.
DMSO
Forsk.
IBMX
Forsk/IBMX

Fig. 3. Effect of dbcAMP, 8-bromo-cAMP, or Sp-cAMPs pretreatment on 5-HT- or KCl-induced GVBD. Oocytes were pretreated using various indicated concentrations of dbcAMP (A, B), 8-bromo-cAMP (C, D), or Sp-cAMPs (E, F) for 15 min. prior to the addition of 5-HT (5 μM, A, C, E) or KCl (45 mM, B, D, F). dbcAMP: dbcAMP alone; 8-Br-cAMP: 8-bromo-cAMP alone; Sp-cAMPs; Sp-cAMPs alone. Mean results (± S.E.M.) of four experiments.

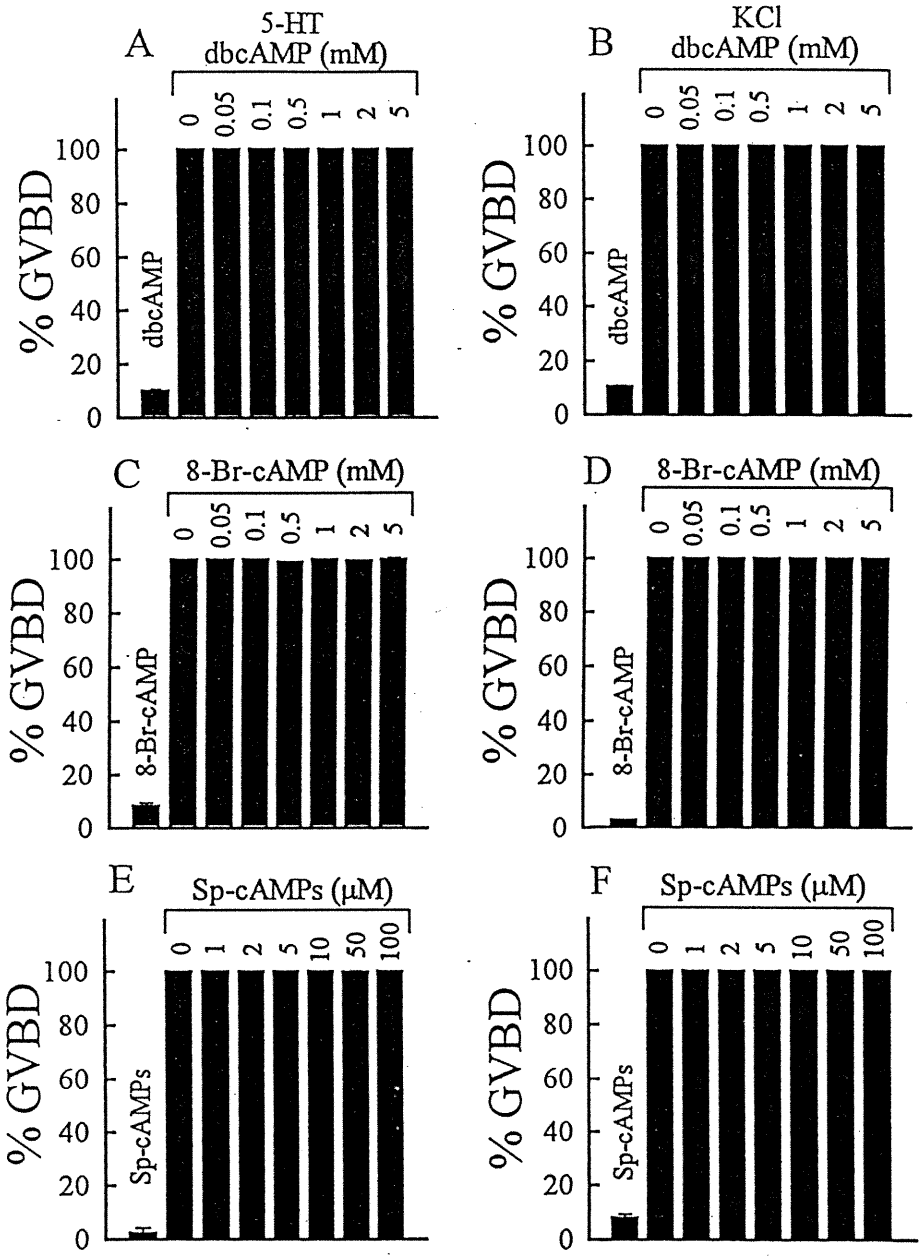

A
5-HT
dbcAMP (mM)
0
0.05
0.1
0.5
1
2
5
% GVBD
100
80
60
40
20
0
dbcAMP
B
KCl
dbcAMP (mM)
C
8-Br-cAMP (mM)
8-Br-cAMP
D
E
Sp-cAMPs (μM)
0
1
2
5
10
50
100
Sp-cAMPs
F

Fig. 4. 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 (O, DMSO), for 15 min prior to insemination at time t = 0. Aliquots were sampled at indicated times for determinations of percentage GVBD. Mean results (± S.E.M.) of two separate experiments.

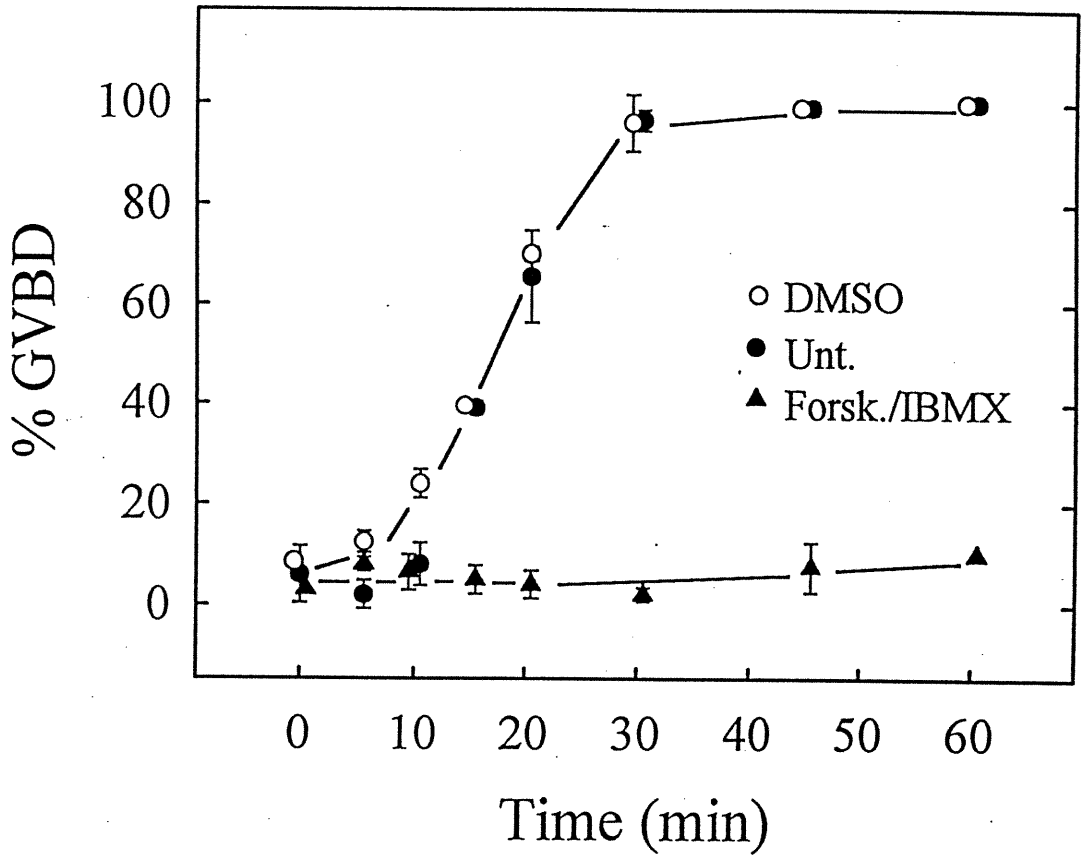

% GVBD
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)
DMSO
Unt.
Forsk./IBMX

Fig. 5. 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 (bar represents 20 μm).

A
mc
mp
B
mp
mc
pb
C
sh
gv
sh
sh
gv
D
gv
gv

Fig. 6. Effect of 5-HT on cAMP concentration of *Spisula* oocytes. A) Oocytes were either treated with 5-HT (5 μM, ●) or left untreated (○). At different times, aliquots were removed and processed for cAMP determinations as described in Materials and Methods, and the results are expressed as % of initial cAMP concentration of untreated oocytes. B) A similar experiment using oocytes pre-incubated in presence of forskolin and IBMX (60/65 μM) for 15 min. 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.

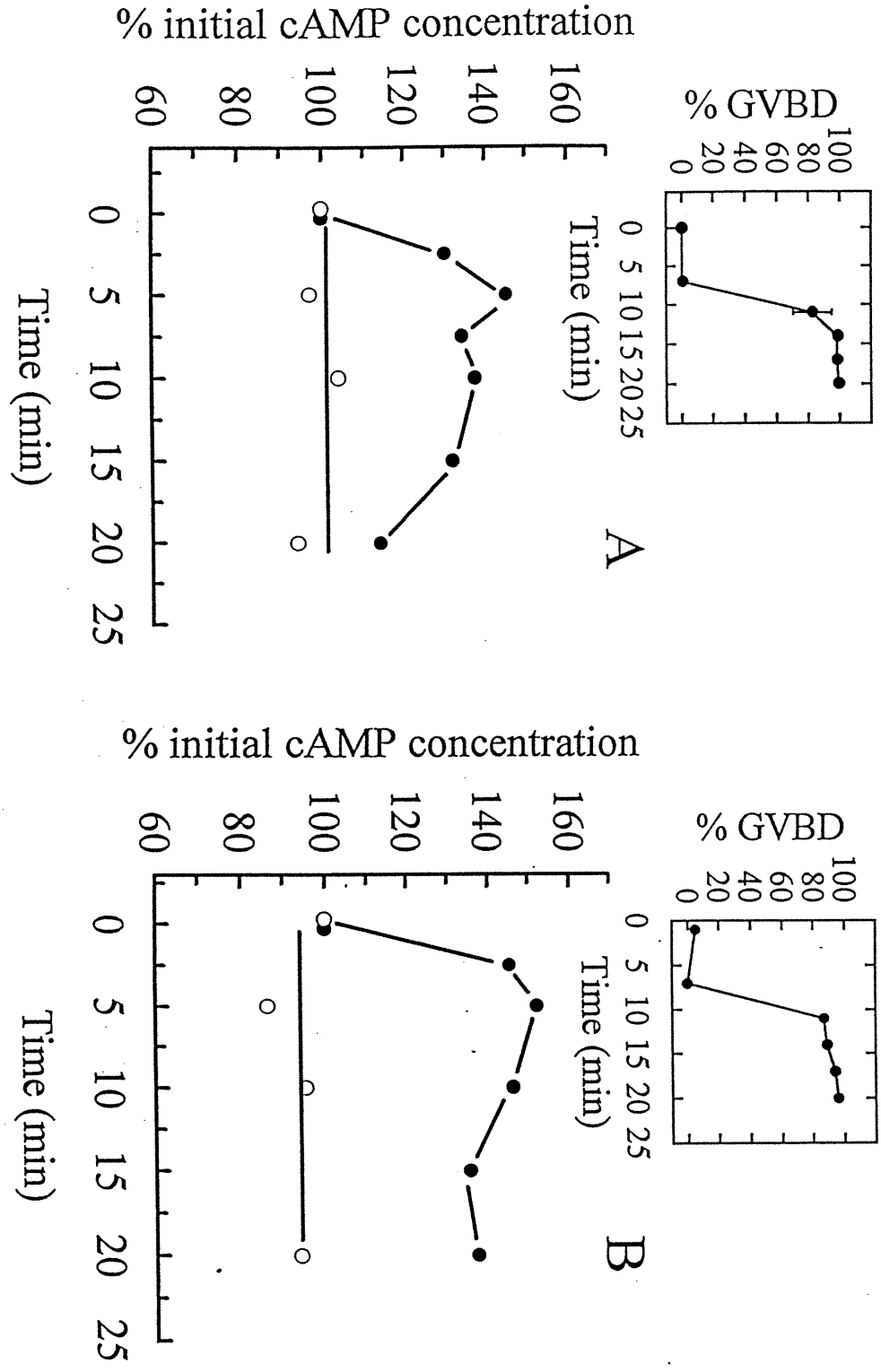
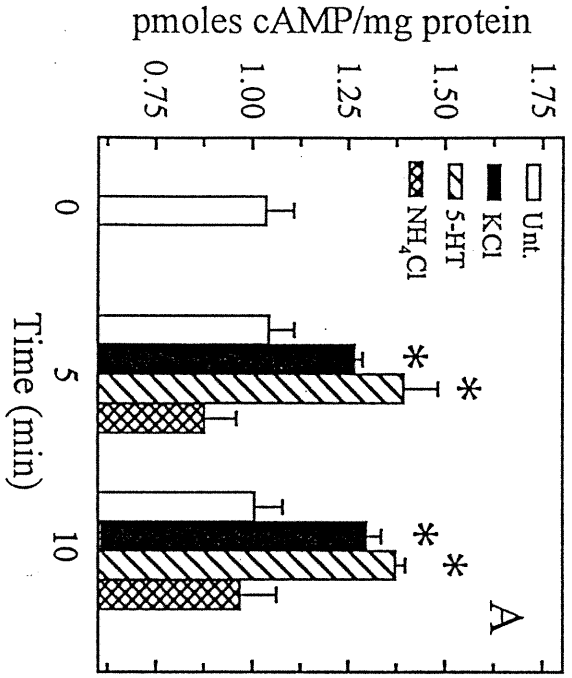

% initial cAMP concentration
160
140
120
100
80
60
0 5 10 15 20 25
Time (min)
% GVBD
100
80
60
40
20
0
0 5 10 15 20 25
Time (min)
A
B

Fig. 7. Effects of KCl, 5-HT, or NH_4Cl on the cAMP concentration of *Spisula* oocytes. Panel A 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 to untreated oocytes. Percentages of GVBD scored 15 min. after adding 5-HT, KCl, or NH_4Cl were 100%, 100%, and 0% percent, respectively. Panel B shows a similar experiment in which KCl or 5-HT were added to oocytes kept in artificial Ca^{2+}-free seawater with no resulting GVBD, as determined after 15 min. Mean results (± S.E.M.) of three experiments.


pmoles cAMP/mg protein
0.75
1.00
1.25
1.50
1.75
Unt.
KCl
5-HT
NH4Cl
0
5
10
Time (min)
*
*
*
*
A

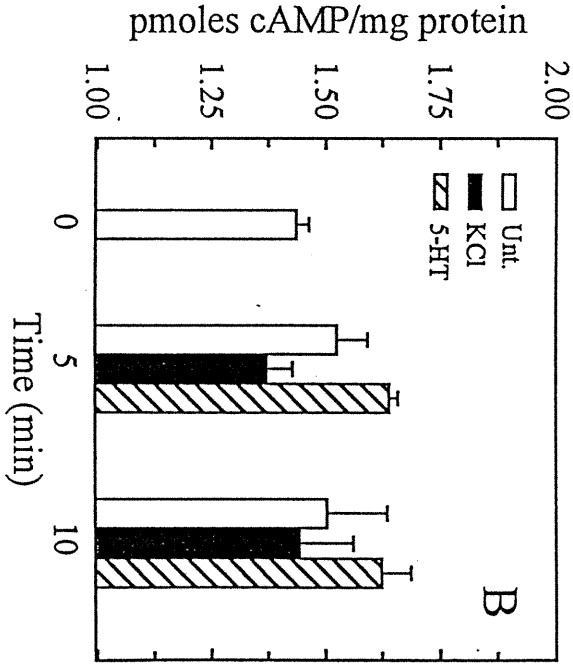

pmoles cAMP/mg protein
1.00
1.25
1.50
1.75
2.00
Unt.
KCl
5-HT
0
5
10
Time (min)
B

Fig. 8. Effect of various added K^+ on oocyte cAMP concentration. Various amounts of isotonic KCl (0.52 M) were added to oocytes for the indicated final concentrations (% v/v). Oocytes were sampled 10 min later for determination of cAMP concentration, as described in Materials and Methods. GVBD, as scored after 15 min, 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.

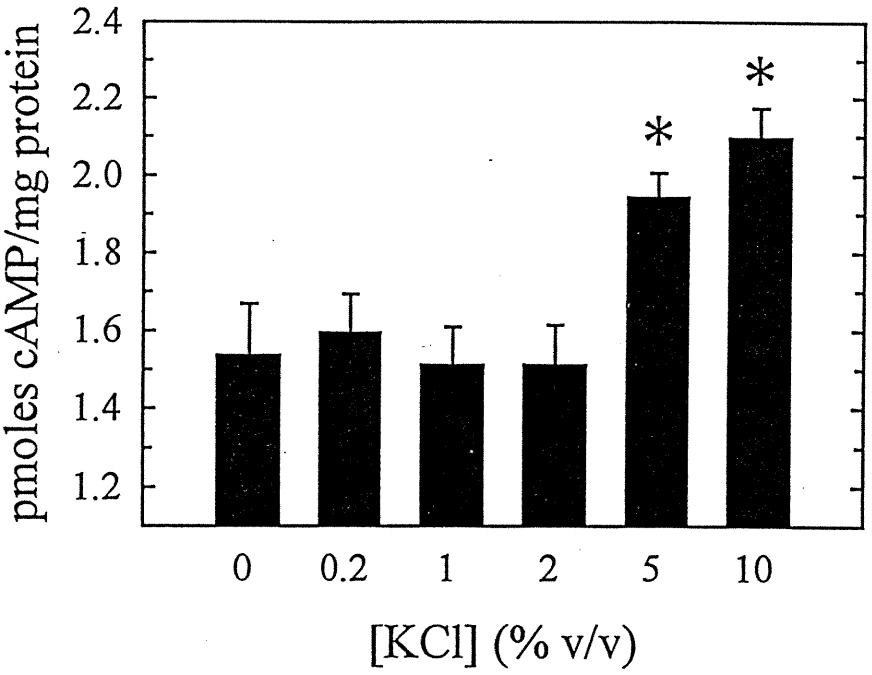

pmoles cAMP/mg protein
2.4
2.2
2.0
1.8
1.6
1.4
1.2
*
*
0
0.2
1
2
5
10
[KCl] (% v/v)

Fig. 9. 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.

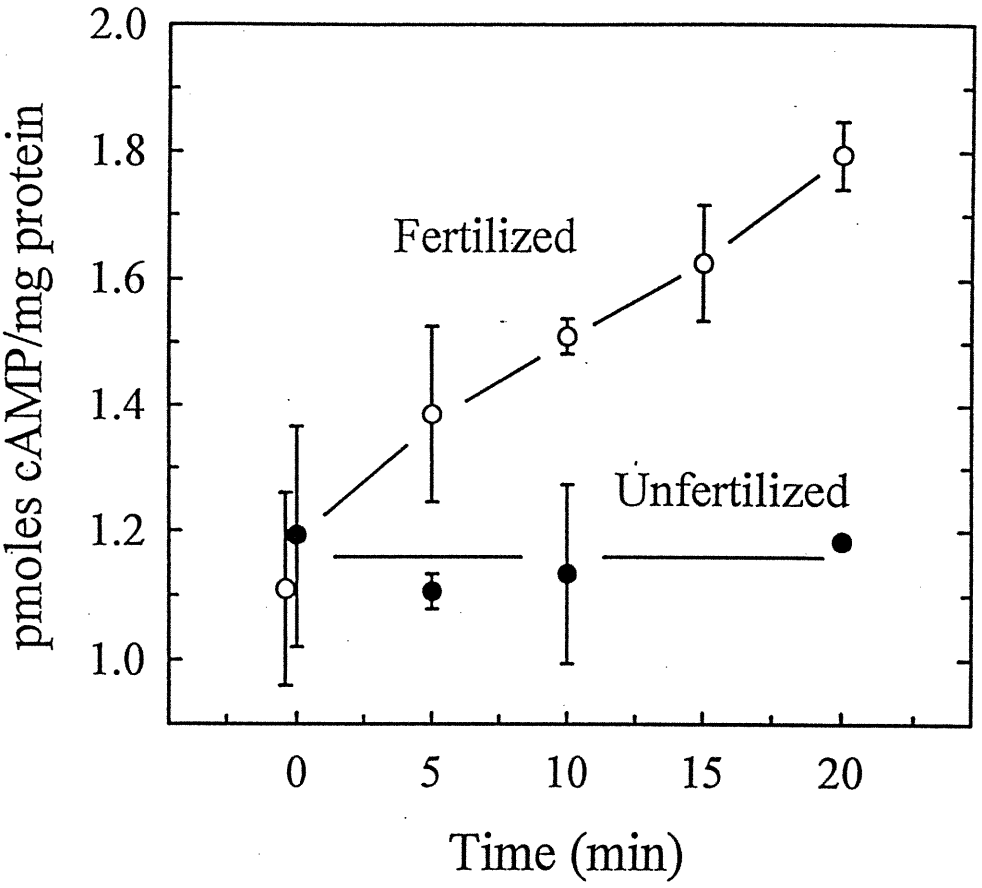

2.0
1.8
1.6
1.4
1.2
1.0
pmoles cAMP/mg protein
Fertilized
Unfertilized
0
5
10
15
20
Time (min)

Fig. 10. Effect of pretreatment of oocytes with SQ 22,536 on the time course of 5-HT- or KCl-induced GVBD. Oocytes were either pretreated with 1 mM SQ 22,536 (open symbols, O,) or left untreated (filled symbols, ●, ▲) for 1 hr prior to the addition of 5-HT (5 μM, ● , O) or KCl (45 mM, ▲ , Δ), at time 0. At indicated times, oocytes were fixed for determinations of percentage GVBD. Mean results (± S.E.M.) of two experiments.

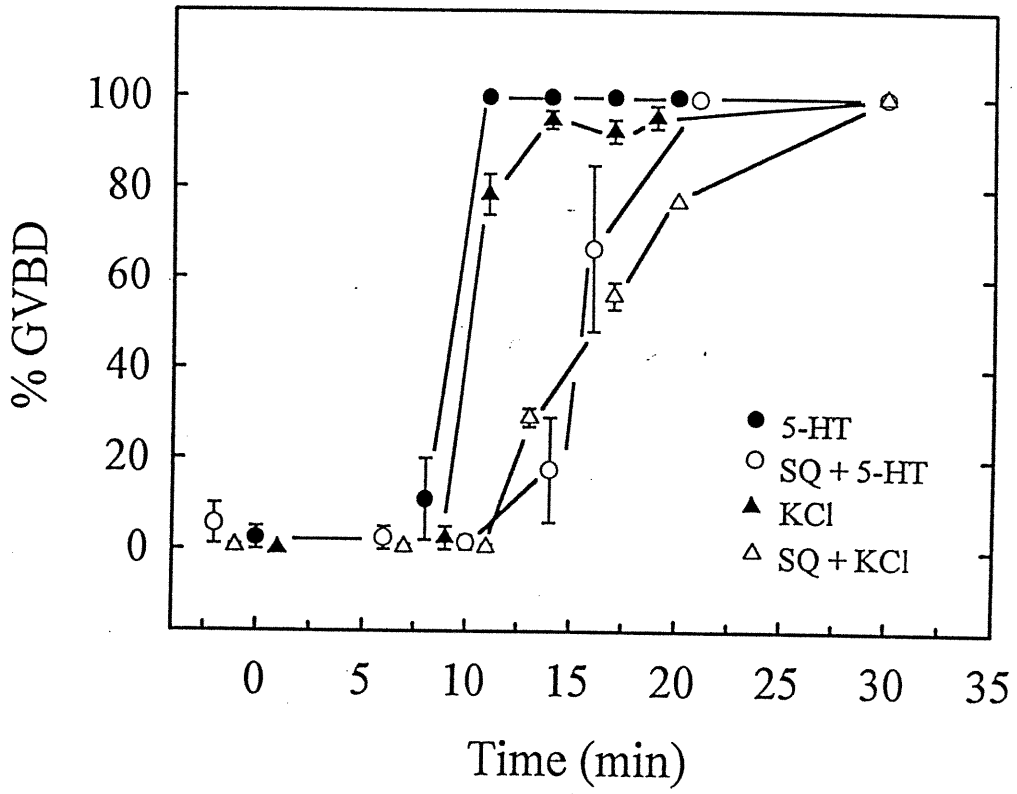
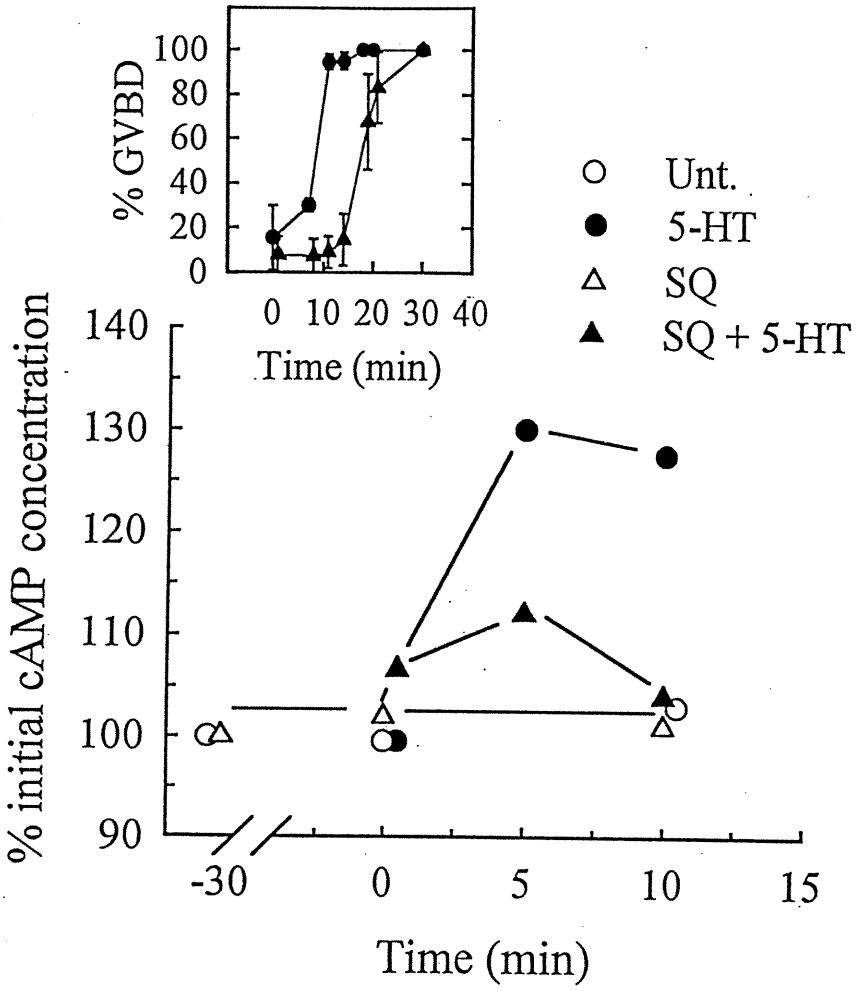

% GVBD
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (min)
5-HT
SQ + 5-HT
KCl
SQ + KCl

Fig. 11. Effect of pretreatment of oocytes with SQ 22,536 on cAMP concentration before and after addition of 5-HT. Oocytes were divided in 2 lots, one to which SQ 22,536 (1 mM) was added (Δ) while the other was left untreated (○). One hour later, 5-HT (5 μM) was added to an aliquot of both SQ22,536-treated (▲) and untreated oocytes (●). At indicated times, samples of oocytes were frozen and later processed for determinations of cAMP concentration expressed as percent initial cAMP concentration of oocytes at the beginning of 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.


% GVBD
100
80
60
40
20
0
0 10 20 30 40
Time (min)
Unt.
5-HT
SQ
SQ + 5-HT
% initial cAMP concentration
140
130
120
110
100
90
-30
0
5
10
15
Time (min)

3.0 DISCUSSION AND CONCLUDING REMARKS

The resumption of meiosis is known to be triggered by a universal intracellular M-phase-promoting factor, which is a complex of at least 2 subunits; Cdc2-kinase and cyclin B (Labbé *et al.*, 1989). This MPF, usually present as an inactive form in many prophase-arrested oocytes, requires for its activity the dephosphorylation of tyrosine and threonine residues of the Cdc2-kinase subunit, as well as the phosphorylation of the cyclin B subunit. In *Xenopus*, clam and starfish oocytes, PKA seems to be responsible for the dephosphorylation of certain proteins that are either directly or indirectly linked to the activity of MPF (Meijer and Arion, 1991; Rime *et al.*, 1992). This is supported by several lines of evidence (well reviewed in Meijer and Zarutskie, 1987). For example, compounds that increase cyclic AMP levels (such as forskolin and cholera toxin) inhibit oocyte maturation just as the microinjection of C-subunit of PKA does. In addition, cyclic AMP levels drop during oocyte maturation, both in mammals (Shultz *et al.*, 1983; Vivarelli *et al.*, 1983) and amphibians (Cicirelli and Smith, 1985; Maller, 1985), as well as in starfish (Meijer and Zarutskie, 1987). Until now, this hypothesis seemed to apply to *Spisula* oocytes as well since it has been reported that cyclic AMP-raising treatments were inhibitory on the oocyte activation, as reflected by GVBD induced with sperm or 5-HT (Sato *et al.*, 1985). However, we present here evidence that cyclic AMP-raising treatments do not inhibit 5-HT/KCl-induced GVBD in *Spisula* oocytes, contrary to the previous report. Forskolin, an adenylyl cyclase activator proven in many systems to greatly increase intra-oocyte cyclic AMP levels, was without effect on *Spisula* oocytes. IBMX, a non-specific PDE inhibitor, failed to show inhibitory effects upon 5-HT/KCl-induced activation. Other cyclic AMP analogs, dbcAMP, 8-bromo-cAMP, and Sp-cAMPs, were not inhibitory as well. These results contradict the previously reported (Sato *et al.*, 1985) and currently accepted hypothesis that *Spisula* oocyte activation, similar to mouse/*Xenopus*/starfish oocyte maturations, necessitates a decrease in cyclic AMP level, and that cyclic AMP-raising treatments inhibit GVBD. This suggests that the re-initiation of meiosis in prophase-arrested *Spisula* oocytes may be regulated differently from that of mammalian, *Xenopus* or starfish oocytes where a decrease in cyclic AMP

levels, which leads to protein(s) dephosphorylation necessary for functional MPF, precedes GVBD.

Our data show that oocyte cyclic AMP concentrations increased significantly by treating cells with IBMX and forskolin combined, whereas neither chemical was able to significantly increase cyclic AMP concentrations when used alone (Fig. 2). This was rather unexpected since, under similar condition, forskolin has been shown to increase cyclic AMP level over 35-fold over its original level in *Evasterias troschelii* oocytes (Meijer and Zarutskie, 1987) and up to 4-fold in *Xenopus laevis* oocytes (Schorderet-Slatkine and Baulieu, 1982). This might be an indication that *Spisula* oocytes possess an efficient compensatory mechanism that rapidly lowers cyclic AMP level, such as the activation of phosphodiesterases or stimulation of cyclic AMP efflux (Harden, 1983). Alternatively, adenylyl cyclase may be less sensitive to forskolin for yet undetermined reasons. In fact, forskolin did not activate adenylyl cyclase in bull or boar sperm (Forte *et al.*, 1983) suggesting that the action of forskolin is not universal. It is thus reasonable to assume that, in *Spisula* oocytes, adenylyl cyclase might also be insensitive to forskolin treatment, but further analysis of the activity of adenylyl cyclase, as well as the activity of PDEs involved in the regulation of cyclic AMP, are required to test this hypothesis. Nonetheless, the presumed maintenance of cyclic AMP levels by the phosphodiesterase inhibitor IBMX, which blocked 1-MA-induced activation in starfish oocytes (Dorée et al., 1976), seems neither to inhibit nor to delay the 5-HT-induced activation of *Spisula* oocytes. This leads us to conclude that the maintenance of oocyte cyclic AMP concentration at the same level as in unactivated oocytes, as well as a slight increase in cyclic AMP levels caused by application of both chemicals, has no inhibitory effect on the activation of *Spisula* oocytes.

Our results on the measurements of cyclic AMP levels by RIA reveal a transient increase of cyclic AMP level in 5-HT-activated oocytes (Fig. 6A) and a similar increase in KCl-activated oocytes (Fig. 7A). This increase is detected as early as 2.5min post activation, and the cyclic AMP decreases to its original level after GVBD (around 15 min) in serotonin-activated oocytes (Fig. 6A). When external calcium is

absent, this increase does not occur (fig. 7B), indicating that the Ca^{2+} influx is necessary for the increase in cAMP levels by both 5-HT and KCl, as well as for the activation itself. In *Spisula* oocytes, a radioligand binding study has shown the existence of a new type of receptor (Krantic *et al.*, 1993) that involves an increase in the uptake of $^{45}Ca^{2+}$. The putative receptor was later postulated to mobilize a phospholipase C pathway resulting in the production of IP-3 and DAG through hydrolysis of phosphatidylinositol-bisphosphate (PIP-2) (Colas and Dubé, 1998). The increase in oocyte cyclic AMP levels by 5-HT does not necessarily involve a G-protein coupled to the receptor that directly activates the adenylyl cyclase, since a similar increase is detected in KCl-activated oocytes. This is further supported by the fact that the absence of Ca^{2+} in the external medium, which abolishes GVBD by both 5-HT or KCl, also inhibits 5-HT- or KCl-induced increase in cyclic AMP level (Fig. 7B). The absence of GVBD by 5-HT in oocytes treated with BAPTA/AM also demonstrates the absolute involvement of Ca^{2+} in the activation cascade triggered by 5-HT (personal observation). The similarity in the progression of meiosis in serotonin- or KCl- stimulated oocytes, as well as the reported influxes of $^{45}Ca^{+2}$ under both conditions, strongly suggest the possibility that the cascade utilized by both artificial activating agents eventually merge into a single cascade, involving the Ca^{2+} influx.

We have also performed several experimental manipulations known to alter the Ca^{2+} influx, by adding 5 to 52 mM K^+ ions to the oocytes, and examined the dependency of the increase in cyclic AMP concentration upon the Ca^{2+} influx. As previously shown, adding 5 to 15 mM K^+ ions may stimulate up to half the normal increase of Ca^{2+} influx, but it does not allow any GVBD nor the protein phosphorylation to occur (Dubé, 1988, Dubé *et al.*, 1991). Under similar conditions (Fig. 8), we were unable to detect any significant increase in cyclic AMP levels for those oocytes treated with K^+ that would trigger a Ca^{2+} influx below the threshold level for GVBD. The increase in cAMP level therefore seems not to be directly linked to the Ca^{2+} influx, but rather, closely linked to GVBD and probably accounts for the behaviour of the increase in protein phosphorylation reported earlier (Dubé *et al.*, 1991). It is probable that the event(s) responsible for this increase in cAMP levels lies

1991). It is probable that the event(s) responsible for this increase in cAMP levels lies downstream of the initial Ca^{2+} influx (hence the crucial concentration of Ca^{2+}) leading to the triggering of the activation of MPF and the GVBD. What then, could be the cause of such an increase in cyclic AMP levels occurring post-activation?. In a recent study on cell-free extracts of *Xenopus laevis* oocytes, the cyclic AMP-PKA cascade was activated by the kinase activity of MPF after its appearance (Grieco *et al.*, 1996). Perhaps the increase in cyclic AMP, apparently requiring the activation as a prerequisite, might be due to the kinase activity of the MPF. However, this hypothesis could be ruled out, since the increase in cyclic AMP precedes the appearance of histone H1 kinase activity (Walker *et al.*, 1999). Other cell factors (e.g., Ca^{2+}) that may stimulate the production of cyclic AMP independently of the receptor (De Vivo and Maayani, 1988), could also contribute to the increase observed in activated *Spisula* oocytes. Further analysis of the activity of adenylyl cyclase, as well as the activity of PDEs, seems necessary to address this question.

When oocytes were incubated with the adenylyl cyclase inhibitor SQ 22,536, the basal levels of cyclic AMP did not decrease significantly from that of control oocytes. However, the increase in cyclic AMP was significantly blocked and the GVBD was delayed in 5-HT-activated oocytes (Fig. 11), suggesting that the activation of the adenylyl cyclase is indeed responsible for the increase in cyclic AMP. The inhibition by SQ 22,536 of the 5-HT-induced increase in cyclic AMP seems to be responsible for the delay in the kinetics of the GVBD (Fig. 11). The GVBD was likewise delayed in KCl-activated oocytes as well, probably through the inhibition of the increase in cyclic AMP concentration. Thus the 5-HT- or KCl-induced increase in cyclic AMP appears to be due to the activation of adenylyl cyclase turned on by the same event(s) that requires the full oocyte activation as a prerequisite. However, the cyclic AMP rise seems to be dispensable since oocytes pretreated with SQ 22,536 nevertheless reaches full GVBD after 5-HT or KCl addition. The increase in cyclic AMP alone is not causal for the GVBD, since a similar increase, observed in the oocytes treated with forskolin and IBMX combined (Fig. 2A), was not sufficient to induce activation of oocytes. Taken together, these results suggest that, although an

the increase in cyclic AMP that results from activation itself is required for the normal kinetics of GVBD. Blocking the increase in cyclic AMP by inhibiting adenylyl cyclase presumably inhibits the phosphorylation of protein(s) involved in the normal kinetics of GVBD, thus resulting in a delay.

Fertilization also involves a steady increase in cyclic AMP levels (Fig. 9), thus suggesting that the increase in cyclic AMP is indeed a physiological event that is required for the normal process of activation in *Spisula* oocytes. This agrees with the previously reported general increase in protein phosphorylation during the period after fertilization and prior to GVBD (Adeyemo *et al.*, 1987; Eckberg *et al.*, 1987; Dubé *et al.*, 1991) and the transient increase in cyclic AMP by 5-HT/KCl shown in this paper. Adeyemo *et al* (1987) have previously reported that cyclic AMP changes upon fertilization in clam oocytes were not significant. However, we believe that the conditions for the fertilization experiment described in their paper were not adequate to give a high percentage of fertilized, synchronous eggs, according to the optimized fertilization parameters reported previously in this species (Clotteau and Dubé, 1993).

Fertilization is inhibited in the presence of IBMX and forskolin. However, our results indicate that this effect is likely to be on the sperm itself since no incorporation of male pronucleus occurs. Thus, the inhibition of GVBD cannot be considered as an inhibition of oocyte activation but is rather an inhibition of some critical step(s) by which the spermatozoon becomes able to fuse with the oocyte. One such likely step, possibly affected by increased cyclic AMP, might be the acrosome reaction accompanied by changes in cyclic AMP levels. Indeed, the progesterone-induced acrosome reaction is inhibited by PKI in human sperm (Harrison *et al.*, 2000), and the addition of dbcAMP alone or together with IBMX induced acrosome reaction in hamster spermatozoa incubated in $NaHCO_3$-free medium (Visconti *et al.*, 1999). In addition, in hamster sperm, a pre-incubation for 3 hours under capacitating conditions with compounds such as caffeine, theophylline and IBMX effectively inhibits the acrosome reaction (Rogers and Garcia, 1979). Whether the presence of forskolin and IBMX in the medium prematurely caused the acrosome reaction of *Spisula*

acrosome reaction (Rogers and Garcia, 1979). Whether the presence of forskolin and IBMX in the medium prematurely caused the acrosomal reaction of *Spisula* spermatozoa well before the contact of two gametes took place or inhibited acrosomal reaction to occur, awaits further studies. At this point, it seems clear that the inhibition of fertilization by forskolin and IBMX reported earlier (Sato *et al.*, 1985) resulted from the inability of spermatozoa to fuse with the oocyte (this study).

The present report raises several questions as to the implication of cyclic AMP in the resumption of meiosis in *Spisula* oocytes relative to the other species reported, first being the immediate downstream effect of the increase in cyclic AMP. Interesting is the rapid, one time activation of MAPK which precedes the dephosphorylation of tyrosine residue in $p34^{cdc2}$ reported earlier (Shibuya *et al.*, 1992). A complete activation of MPF does not occur until after the GVBD in clam oocytes (Shibuya *et al.*, 1992), although histone H1 kinase activity can be detected as early as 6 min postactivation (Walker *et al.*, 1999). The timing of *Spisula* oocyte's MAPK activation relative to that of tyrosine dephosphorylation differs from *Xenopus* oocytes where the activation of MAPK and MPF occurs simultaneously (Nebreda and Hunt, 1993), and also to the starfish oocytes where the MAPK activation occurs after the activation of MPF (Picard *et al.*, 1996). Previous protein phosphorylation studies revealed three major phosphorylated bands occuring immediately following the activation, of 41, 48, and 56 kDa (Clotteau and Dubé, 1996), possibly being $p42^{MAPK}$ (Shibuya *et al.*, 1992), p49 nucleoplasmine (Herlands and Maul, 1994), and cyclin B (Dubé *et al.*, 1991), respectively. The cyclic AMP increase might have a role in phosphorylating one of these proteins. For example, although cyclic AMP inhibits MAPK activation in mouse oocytes (Sun *et al.*, 1999), cyclic AMP might activate MAPK in clam oocytes, judging from the time course of both increase in cyclic AMP levels and the tyrosine phosphorylation of MAPK (Shibuya *et al.*, 1992). In fact, in neonatal rat cardiomyocytes, PKA activators significantly activate Raf-1 and MAP kinases, and the elevation of intracellular cyclic AMP was also shown to activate MAPK in PC12 cells (Yamazaki *et al.*, 1997; Frodin *et al.*, 1994; Vossler *et al.*, 1997; Yao *et al.*, 1998, see Sun *et al.*, 1999). Without further results on the possible effect of cyclic AMP on

interesting possibility to be considered is the connection between cyclic AMP and the rise in pH_i. The delay (but not an inhibition) in kinetics of GVBD occuring during the activation of oocytes in the presence of amiloride derivatives (Dubé and Eckberg, 1997) resembles the delay oberved in SQ 22,536-treated oocytes (this study). Considering the fact that the artificial rise in pH_i did not increase cyclic AMP levels (fig. 7A), it is possible that the cyclic AMP rise is required for the rise in pH_i, which is reported to allow the progression of GVBD at optimal rate (Dubé and Eckberg, 1997). Further analysis involving microinjection of catalytic/regulatory subunits of PKA seems necessary to determine the exact role of cAMP on the resumption of meiosis, since neither SQ 22,536 nor forskolin alone could totally affect cyclic AMP levels, hence the activity of PKA in this study.

How then does the increase in cyclic AMP and PKA activity fit into the current knowledge on the regulation of MPF activity? With protein synthesis being unecessary for at least the completion of meiosis I (Hunt *et al.*, 1992), the regulation of MPF seems to rely on the post-translational modifications of cyclin/cdc2 complexes. However, PKA inhibits the phosphorylation cascade leading to the activation of MPF in both mouse oocytes (Rime and Ozon, 1991) and *Xenopus* oocytes (Matten *et al.*, 1994). The regulation of MPF in *Spisula* oocytes thus seems to differ from mouse and *Xenopus* oocytes and further studies are required to fill the missing link between the activation-induced cyclic AMP increase and the cascade of events leading to the activation of MPF in *Spisula* oocytes.

To summarize, compounds known to increase the intracellular cyclic AMP do not have any effect on the meiosis resumption in *Spisula* oocytes, as evidenced by normal GVBD. Further measurements of cyclic AMP levels after artificial activation or fertilization show an increase in cyclic AMP content, further demonstrating that, unlike vertebrate or starfish oocyte maturation, the activation does not involve any decrease, but rather, an increase in cyclic AMP levels in *Spisula* oocytes. This increase in the intracellular cyclic AMP seems to be independent of Ca^{2+} and pH rise, and results from an activation of adenylyl cyclase by downstream events and absolutely

requires the activation of the oocyte. GVBD is delayed, both in 5-HT- or KCl-induced activation, in the presence of the adenylyl cyclase inhibitor SQ 22,536 that successfully inhibits the post-activation increase in cyclic AMP level of oocytes. The fact that sperm incorporation was blocked when insemination takes place in the presence of IBMX and forskolin suggest that fluctuations in cyclic AMP may be important for the sperm physiology, if not for the oocytes. Taken together, our results establish the emergence of a new original model where the oocyte maturation does not involve a decrease, but rather an increase in cyclic AMP levels. Further studies on the changes in the activity of PDEs, PKAc/r microinjection experiments, and the activity of adenylyl cyclase should shed more light on this new cascade of events occurring during the resumption of meiosis.

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