Int. J. Dev. Biol. 39: 485-491 (1995)

Original Article

4-aminopyridine acts as a weak base and a Ca^{2+} mobilizing agent in triggering oocyte meiosis reinitiation and activation in the Japanese clam *Ruditapes philippinarum*

ISABELLE GOBET[1], MONICA LIPPAI[1] MARTINE TOMKOWIAK[1], YVES DUROCHER[1], CATHERINE LECLERC[2], MARC MOREAU[2] and PIERRE GUERRIER[1*]

[1]*Laboratoire de Biologie moléculaire et cellulaire, UMR 49, Ecole Normale Supérieure de Lyon, Lyon,*
[2]*Centre de Biologie du développement, UMR 9926, Université Paul Sabatier, Toulouse*
and [1,2]*Laboratoire de Biologie marine du Collège de France, Concarneau and Station biologique, Roscoff, France*

ABSTRACT Ovarian oocytes of the prosobranch mollusc *Patella vulgata* and the pelecypod *Ruditapes philippinarum* are arrested during prophase of the first maturation division. Release from this blockade, which is revealed by germinal vesicle breakdown, drives these oocytes to a second arrest in metaphase I, at which time the oocytes become fertilizable. The respective roles of Ca^{2+} and H^+ ion movements during this early step in meiosis reinitiation has not been fully established yet. In this work we reveal the presence of acidic vesicles and report that bafilomycin A_1 and N,N'-dicyclohexylcarbodiimide, two inhibitors of the vacuolar-type H^+-ATPase, applied to *Ruditapes* oocytes, produce a significant inhibition of their response to the natural neurohormone serotonin. Since sodium deprivation did not affect this response, this suggests that a v-type ATPase pump, possibly located in the membrane of these acidic vesicles, may play a subtle role in the cascade of events that releases oocytes from their prophase block. We then describe how 4-aminopyridine, a drug reputed to be a K^+ channel antagonist, triggers both meiosis reinitiation and activation of *Patella* and *Ruditapes* oocytes. This agent acts as a weak base, its effect depending on external pH. Moreover, using the fluorescent probes BCECF and Fluo-3/AM, we observe that this drug both alkalinizes the endoplasm and promotes an intracellular Ca^{2+} surge. This dual effect may explain why *Ruditapes* oocytes no longer stop in metaphase under these conditions and behave like other bivalve species which are directly fertilizable at the germinal vesicle stage.

KEY WORDS: *4-aminopyridine, cytoplasmic alkalinization, intracellular calcium surge, meiosis reinitiation and activation, mollusc oocyte*

Introduction

The control of cell division cycle (and essentially that of re-entry in M-phase) is preferably studied in female gametes of both vertebrates and invertebrates. These constitute an excellent model system since they offer a considerable population of large, quite synchronized cells which are blocked during late prophase of the first meiotic division, after DNA replication.

Meiosis reinitiation depends on various external stimuli such as sperm or a hormonal signal. The cytological marker for this maturation process is germinal vesicle breakdown (GVBD), i.e., disruption of the nuclear envelope. Then, in certain species (as the bivalves *Spisula*, *Pholas* or *Barnea*), maturation goes to completion, while in others the resumption of meiosis is a two-step process with a second block occurring later in metaphase I. This is the case for the prosobranch gastropod *Patella vulgata* and for some bivalves such as *Mytilus* or *Ruditapes*.

Abbrevations used in this paper: 4-AP, 4-aminopyridine; 5HT, 5-hydroxytryptamine or serotonin; 9-AA, 9-aminoacridine; ASW, artificial seawater; $BafA_1$, bafilomycin A_1; BAPTA/AM, 1,2,-bis (o-aminophenoxy)ethane-N,N,N'N'-tetraacetic acid, acetomethyl ester; BCECF/AM, 2',7'-bis-(carboxymethyl)-5(6)-carboxyfluorescein; $[Ca^{2+}]_i$, intracellular free Ca^{2+}; CaFSW, calcium-free seawater; CaNaFSW, calcium- and sodium-free seawater; DCCD, N,N'-dicyclohexylcarbodiimide; DMSO, dimethylsulfoxide; EGTA, [ethylenebis-(oxyethilenenitrilono)] tetraacetic acid; Fluo-3/AM, Fluo-3-pentaacetomethyl ester; GVBD, germinal vesicle breakdown; IP_3, inositol trisphosphate; MPF, M-phase promoting factor; NaFSW, sodium-free seawater; pH_0, extracellular pH; pH_i, intracellular pH; TEA, tetraethylammonium; V-ATPase, H^+-ATPase of the vacuolar type.

***Address for reprints:** Laboratoire de Biologie moléculaire et cellulaire, UMR 49, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cédex 07, Fance. FAX: 33.72728686.

0214-6282/95/$03.00

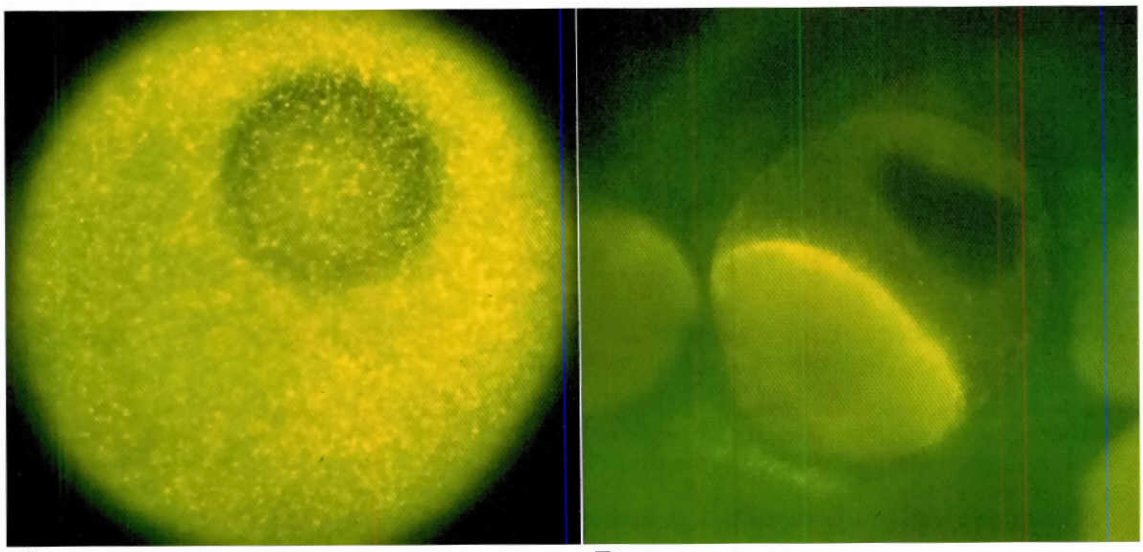

Fig. 1. Acidic vesicles revealed by 9-aminoacridine in prophase-arrested oocytes of *Patella vulgata* (A) and their sedimentation by *in vivo* centrifugation on a sucrose cushion (B).

In *Patella*, release from the prophase I block has been shown to depend on an intracellular pH rise (Guerrier *et al.*, 1986a,b). In the bivalves *Spisula* and *Barnea*, it has been established that release from the prophase I block is accompanied by an efflux of H^+ ions and an alkalinization of intracellular pH (pH_i). However, the role of this alkalinization is not yet clear since: (a) D-600 (methoxyverapamil), an inhibitor of Ca^{2+} movements in the cell, inhibits meiosis resumption without preventing proton extrusion (Dubé and Guerrier, 1982); (b) removal of external Na^+ does not inhibit release from the prophase I block in *Patella* (Guerrier *et al.*, 1986a) and only delays this process in *Spisula* (Dubé and Coutu, 1990). These observations seem to exclude the possibility that the plasma membrane Na^+/H^+ exchanger may play a major role in meiosis resumption. Here, we demonstrate the presence of acidic vesicles in *Patella* and *Ruditapes* oocytes that accumulate fluorescent weak bases such as 9-aminoacridine and test the effects of H^+ v-ATPase inhibitors on the meiotic process.

The second maturation step in *Patella*, i.e., release from the metaphase I block, seems to depend on a transient rise in intracellular free Ca^{2+} ($[Ca^{2+}]i$, as reported in a variety of animal species. This classical event has often been presented as the principal, and probably the prerequisite ionic signal responsible for oocyte activation (Guerrier *et al.*, 1978; Berger, 1992 and Berridge, 1993, for review).

In contrast to the situation observed in *Patella*, a $[Ca^{2+}]_i$ increase seemed sufficient to release *Ruditapes* oocytes from their two successive blocks in prophase and metaphase I (Abdelmajid *et al.*, 1993a,b; Guerrier *et al.*, 1993). In particular, the natural neurohormone serotonin (5 hydroxytryptamine or 5HT) triggered GVBD via a specific G protein-coupled receptor which induced an early inositol 1,4,5-trisphosphate (IP_3) surge and was responsible for the necessary increase in $[Ca^{2+}]_i$ that precedes the phosphorylation cascade leading to M-phase promoting factor (MPF) activation (Gobet *et al.*, 1994). In this report, we will describe the effects of two inhibitors of the v-H^+ ATPase which inhibit GVBD in *Ruditapes* and reveal that 4-aminopyridine (4-AP), a drug previously known as a K^+-channel antagonist, could trigger both a cytoplasmic alkalinization and an intracellular Ca^{2+} surge. Under these conditions, oocytes complete maturation, skipping the usual arrest they normally exhibit in metaphase I, stage at which they are normally fertilized.

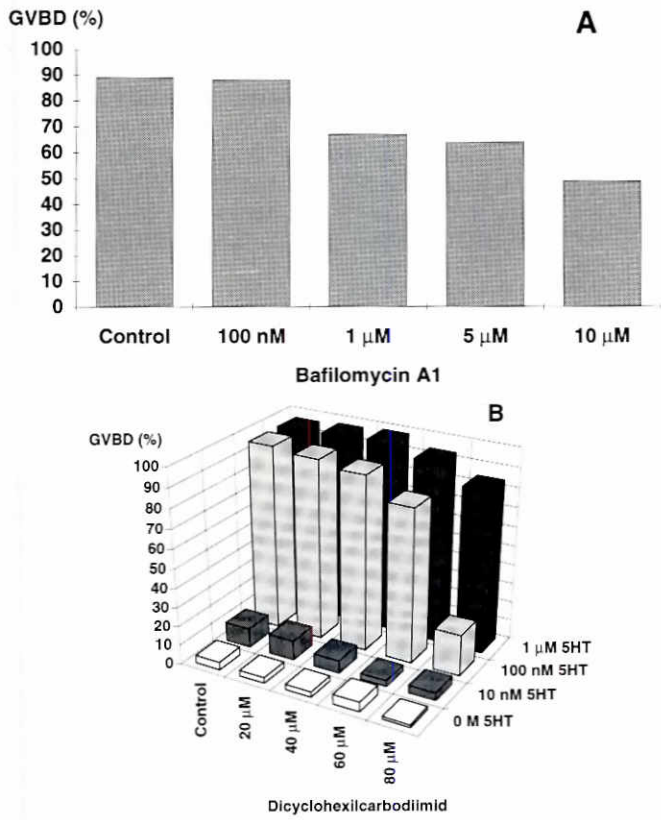

Fig. 2. Effects of two inhibitors of H^+ v-type ATPase on 5HT-induced GVBD in *Ruditapes philippinarum* prophase-arrested oocytes. (A) *Inhibitory effect of bafilomycin A_1 in the presence of 100 nM 5HT;* **(B)** *effect of dicyclohexylcarbodiimide (DCCD) observed at different 5HT concentrations.*

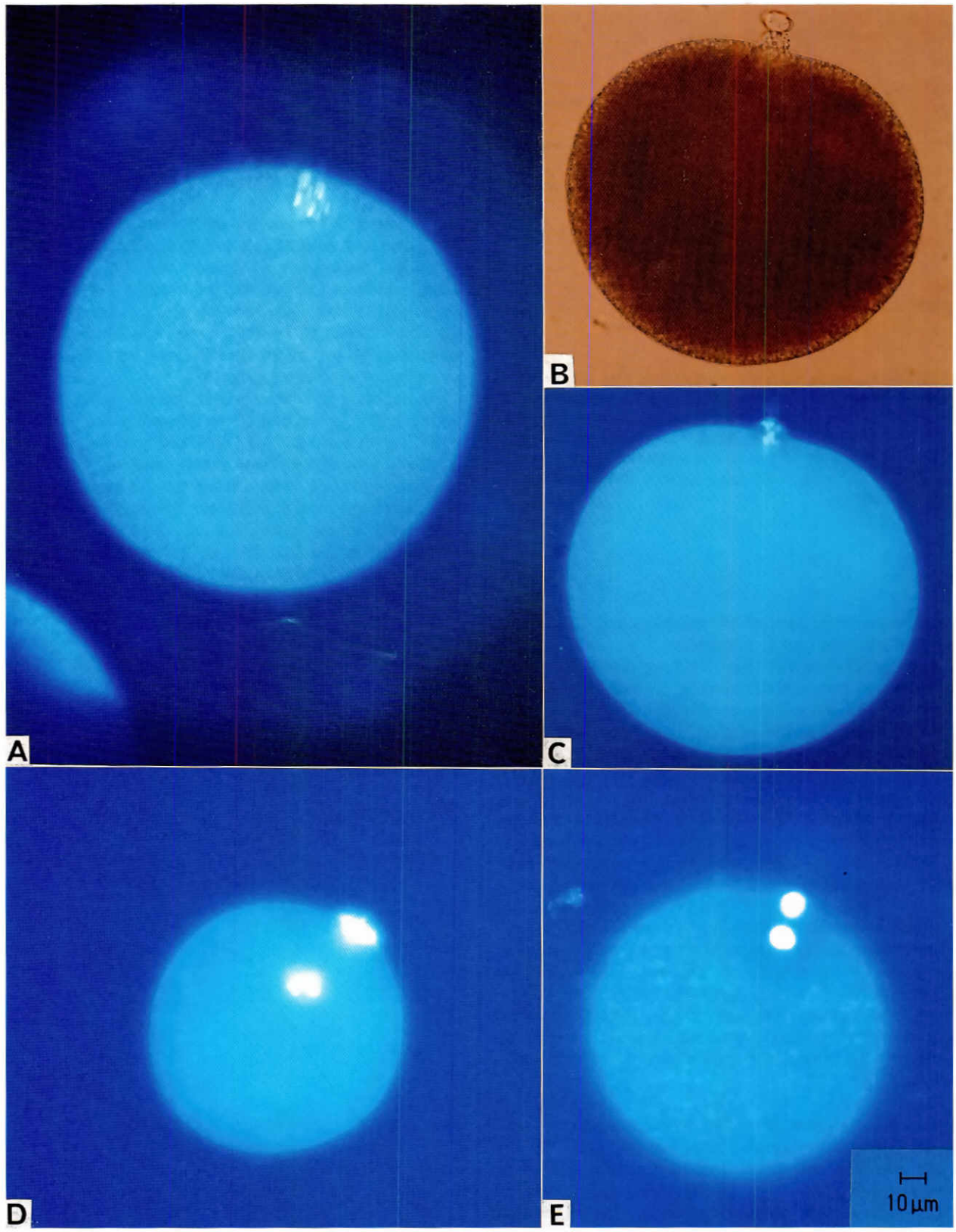

Fig. 3. Cytological consequences of the application of 2 mM 4-aminopyridine (4-AP), pH 8.5 to follicle-free, prophase-arrested oocytes of *Patella vulgata* (A to C) or to prophase-arrested oocytes of *Ruditapes philippinarum* (D,E). *In both cases, this treatment triggers activation, i.e., GCBD followed by anaphase and first polar body extrusion. Activation also occurs when 4-AP is delivered to metaphase-arrested oocytes of both species.*

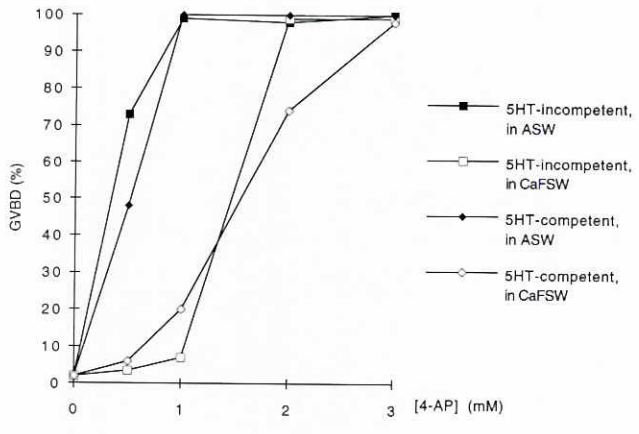

Fig. 4. Dose-response curves illustrating the effect of 4-aminopyridine (4-AP) in triggering GVBD in ASW or CaFSW. *The response of two batches of oocytes are presented. When stimulated with 10 μM 5HT, the batch symbolized by squares attained only 20% GVBD in ASW and was defined as 5HT-incompetent, while the other batch appeared highly competent, reaching 90% GVBD in ASW and 80% GVBD in CaFSW.*

Results

Na^+/H^+ exchange-dependent alkalinization is presumably not required for Ruditapes oocyte maturation

It has been established that sperm, KCl and ammonia-induced meiosis reinitiation was accompanied, in bivalve oocytes, by a slight increase in the intracellular pH (Dubé and Guerrier, 1982; Moreau *et al.*, 1985; Guerrier *et al.*, 1986a; Abdelmajid *et al.*, 1993a). Our preliminary experiments, using BCECF, strongly suggest that this is also the case after effective 5HT stimulation of *Ruditapes* prophase-arrested oocytes. To check for the physiological significance of this feature, we resolved to block any possible Na^+/H^+ exchange process. *Ruditapes* oocytes were thus repeatedly washed and resuspended for 2 h in NaFSW. Final external Na^+ concentration was in the nM range (Dubé and Coutu, 1990). Under these conditions, where the oocytes cannot alkalinize their cytoplasm by extruding H^+, the kinetics of 5HT-induced maturation was not affected. In particular, GVBD occurred normally within 13-15 min, as observed in control oocytes suspended in ASW. This establishes that Na^+/H^+ exchange-dependent alkalinization is unlikely to play a key role in the process of meiosis reinitiation. We thus focused our attention on the potential role that intracellular acidic vesicles may have to play in this process.

Evidence for the existence of acidic cortical vesicles

To demonstrate the presence of acidic vesicles in *Patella* and *Ruditapes* oocytes, we used the fluorescent probe 9-aminoacridine (9-AA), which, as a weak base, accumulated in these organelles. *Patella* oocytes, devoid of follicle cells and treated or not with NH_4Cl, were incubated with 200 μM 9-AA in ASW for 20-25 min, protected from light and washed with ASW. The use of 10 μM 9-AA for only 10 min proved to be sufficient for loading *Ruditapes* or *Mytilus* oocytes.

After fluorescent microscopical observation using blue light through an OLYMPUS DM 500 filter, we could observe the presence of 2 μm yellow vesicles within the cortex of all three species. These granules, which were still present after maturation and gathered around mitotic figures after fertilization, could be easily displaced by centrifuging oocytes on a sucrose cushion. They occupied a thin layer between the light centripetal yolk plug and the central hyaline cytoplasm (Fig. 1A,B).

Inhibition of the response of Ruditapes oocytes to 5HT by bafilomycin A_1 and dicyclohexylcarbodiimide

To test our hypothesis concerning a possible role of acidic vesicles in *Ruditapes*, we decided to test the effect of bafilomycin A_1 on 5HT-dependent maturation. $BafA_1$ is known to be a highly specific inhibitor of vacuolar-type H^+-ATPases (V-ATPases), which sequester protons into different intracellular particles such as endosomes, coated vesicles, etc.

The oocyte response to 5HT (10 nM to 1 μM) was tested after a 15 min preincubation period with different concentrations of $BafA_1$ in ASW (100 nM to 10 μM), GVBD being scored 30 min after 5HT addition. Some inhibition was already observed at 1 μM 5HT when at least 1 μM $BafA_1$ was applied; but the inhibitory effect of this compound was more significant in the presence of the threshold 100 nM 5HT concentration (Fig. 2A).

We also tested the effect of N,N'-dicyclohexylcarbodiimide (DCCD) which binds covalently to v-ATPase and is a potent blocker of ATP-dependent proton translocation (Arai *et al.*, 1987; Sun *et al.*, 1987). Oocytes were preincubated with DCCD (1 to 80 μM) in ASW for 15 min before they were treated with 5HT (10 nM to 1 μM), GVBD being scored 30 min later. As found previously for $BafA_1$, the stimulating effect of 1μM 5HT was barely affected by concentrations of DCCD below 60-80 μM, while inhibition was again more effective at the threshold concentration of 100 nM 5HT (Fig. 2B). In the absence of 5HT, $BafA_1$ or DCCD had no visible effect on the oocytes.

Cytological effects of 4-aminopyridine (4-AP)

2 mM 4-AP (pH 8.5) was assayed on follicle-free prophase-arrested *Patella* oocytes and on prophase-blocked *Ruditapes*

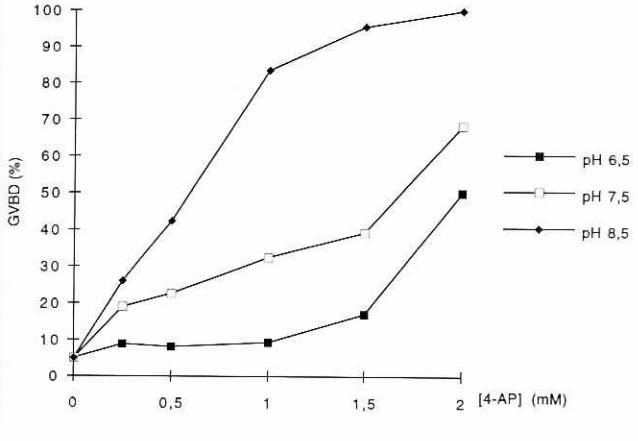

Fig. 5. Effects of increasing external pH on the efficiency of different 4-AP concentrations to trigger GVBD in *Ruditapes philippinarum* prophase-arrested oocytes. *The percentage of GVBD was measured 40 min after 4-AP addition.*

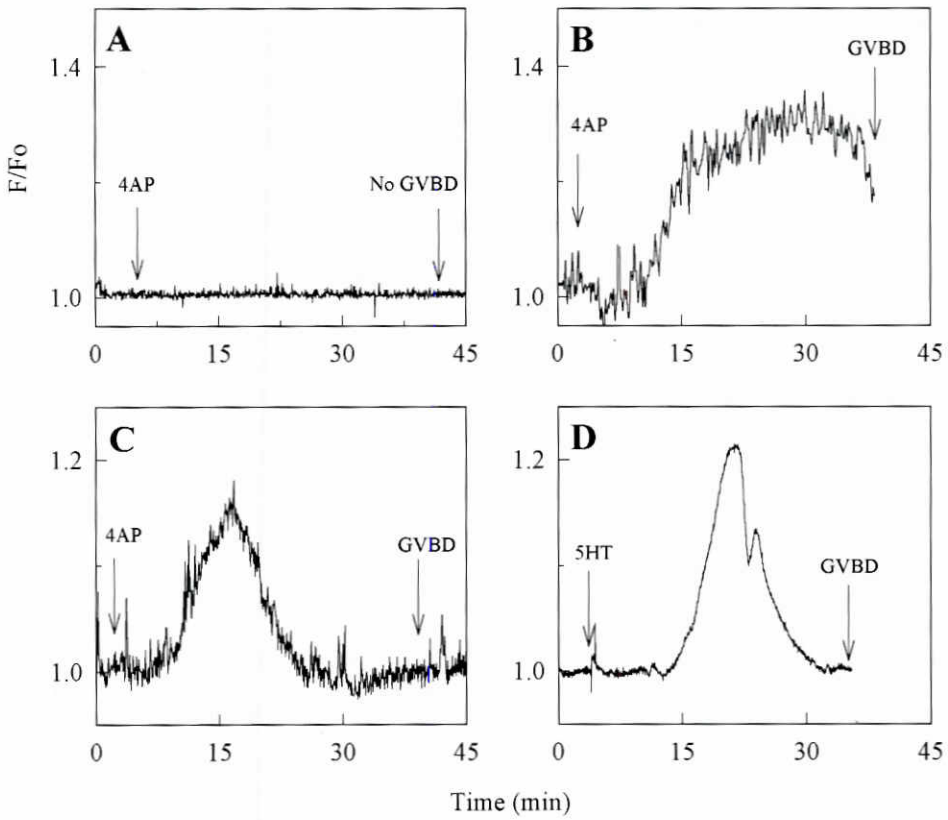

Fig. 6 . Relative transient pH and Ca^{2+} surges observed after the addition of 4-aminopyridine (4-AP), to BCECF/AM (A,B) and Fluo-3/AM-loaded (C,D) prophase-arrested oocytes of *Ruditapes philippinarum* suspended in ASW. *Abscissa, time in minutes; ordinates, relative changes in fluorescence (F/F_0). Excitation was 490 nm for Fluo3 and 500 nm for BCECF; emission was recovered at 530 nm. Each record has been obtained from one individual oocyte and is representative of at least four similar independent experiments.* **(A)** *In this experiment, 0.5 mM 4-AP, pH 8 could not trigger GVBD nor a change in the intracellular pH.* **(B)** *2 mM 4-AP, pH 8 produces a significant intracellular pH change and GVBD.* **(C)** *Transient Ca^{2+} response and GVBD as observed after the addition of 2 mM 4-AP, pH 8.* **(D)** *A similar transient surge is observed after the addition of 2 mM 5HT.*

oocytes. To follow meiotic events, treated oocytes were stained *in vivo* for 10 min with Hoechst 33342 before microscopical observation. In both species, meiosis reinitiation did not arrest at first metaphase but proceeded at least up to first polar body extrusion (Fig. 3). The application of 4-AP to metaphase I-blocked oocytes also resulted in their activation. In contrast, the classical K^+ channel antagonists tetraethylammonium chloride or bromide (TEA, 50 to 150 mM) were found unable to trigger GVBD or to inhibit 5HT-dependent GVBD.

Effects of external calcium removal on 4-AP-dependent meiosis reinitiation

To check whether the absence of Ca^{2+} in the external medium could modify or not the action of 4-AP, *Patella* and *Ruditapes* oocytes were treated with different concentrations (0.5-3 mM) of 4-AP applied either in ASW or in CaFSW + 2 mM EGTA. In both cases, the absence of external Ca^{2+} resulted in a decrease in the biological response at low 4-AP concentrations. However, 2-3 mM 4-AP produced the same percentage of GVBD, whether this drug was added in ASW or in CaFSW. Moreover, in *Ruditapes*, we found that 4-AP was also effective on batches of incompetent oocytes which barely responded to 5HT (Fig. 4).

Mode of action of 4-AP

The effect of 4-AP depends on external pH and leads to cytoplasmic alkalinization

Using three ASW media with different pH-values (6.5, 7.5, 8.5), we could study the effect of external pH at increasing 4-AP concentrations (0.25-2 µM), working on the same oocyte population of *Ruditapes* oocytes (Fig. 5). In every case, the biological response to 4-AP was significantly stronger at higher pH levels, particularly at pH 8.5. Identical results were obtained using whole or follicle-free *Patella* oocytes. Within 30 min, these oocytes reached 100% GVBD at pH 9 in the presence of 0.5 mM 4-AP, while 1 mM and 2 mM of this agent were respectively required at pH 8 and pH 7 to get the same score. To control that 4-AP actually affected intracellular pH, we used BCECF-loaded oocytes of *Ruditapes*. Under these conditions, we found that only effective treatments resulting in GVBD triggered a slow progressive increase in pH, which peaked some 20 min after drug addition (Fig. 6A and B). Its amplitude was twice that observed after 5HT stimulation.

Fluorometric evidence for the existence of an internal Ca^{2+} surge triggered by 4-AP

It has already been demonstrated that, during meiosis reinitiation of prophase-arrested oocytes of *Ruditapes*, transduction of the 5HT signal involved an intracellular Ca^{2+} surge and that BAPTA/AM inhibited GVBD (Guerrier *et al.*, 1993; Gobet *et al.*, 1994). After loading the oocytes with Fluo3/AM we observed that a transient Ca^{2+} surge actually occurred within 10 min of 4-AP addition, a response similar to that obtained following 5 HT stimulation (Fig. 6C and D).

Discussion

Possible role of pH and acidic vesicles

The significance of pH_i alterations observed during oocyte maturation or fertilization (Dubé *et al.*, 1985; Moreau *et al.*, 1985; Stith and Maller, 1985; Busa, 1986) remained questionable despite intensive research. The incontestable existence of acidic vesicles in the cortex of some invertebrate oocytes (Christen, 1983; Lee and Epel, 1983; Schroeder, 1985) and the fact that fertilization acidifies a cortical compartment in sea urchin eggs (Allemand *et al.*, 1987) incited us to examine the possible role of these elements in pH_i changes and oocyte maturation. Actually, we succeeded in visualizing them in *Patella*, *Mytilus* and *Ruditapes* oocytes.

Working on *Ruditapes* oocytes we found that two specific blockers of the vacuolar-type H^+-ATPase, $BafA_1$ (1 to 10 μM) and DCCD (20 to 80 μM), actually exerted an inhibitory effect on 5HT-induced oocyte maturation, as observed in ASW. Since the Na^+/H^+ exchanger is unlikely to play an important role in maturation (cf. our experiments in NaFSW), this suggests that these drugs really act on some v-ATPases involved in this process. These pumps are likely to be located in the membrane of the acidic vesicles, which would sequester protons, contributing to the acidification of their lumen and alkalinization of the cytoplasm. By blocking their function, $BafA_1$ or DCCD may affect the normal regulation of pH_i required for maturation to occur. However, this has not been demonstrated in our experiments and it remains possible that these drugs may alternatively affect plasma membrane v-ATPases, as described to occur and function in murine macrophages (Swallow *et al.*, 1993). Whatever may be the case, our data, at least indicate that negative results concerning the Na^+/H^+ exchanger did not definitively close the question of the biological significance of pH_i changes during meiosis reinitiation.

Possible role of Ca^{2+}

4-AP, a classical plasma membrane K^+ channel antagonist has been recently shown to inhibit the ATP-driven Ca^{2+} uptake in guinea pig sarcoplasmic reticulum (Ishida and Honda, 1993). In mollusc oocytes, a transient increase in the intracellular Ca^{2+} level has already been shown to play an important role in the release from both prophase and metaphase blocks (Guerrier *et al.*, 1986a; Abdelmajid *et al.*, 1993a,b). Moreover, previous results obtained with 5HT, ammonia, procaine and thapsigargin already demonstrated that both external and internal Ca^{2+} stores were involved in mediating maturation of *Ruditapes* oocytes (Guerrier *et al.*, 1993; Gobet *et al.*, 1994). This prompted us to test the effects of 4-AP on female gametes of these species.

In fact, our first results showed that 2 mM 4-AP triggered GVBD both in *Patella* and in *Ruditapes* oocytes, while TEA, another potent K^+ channel blocker, had no similar effect when applied either to *Patella* (Moreau *et al.*, 1990) or *Ruditapes* oocytes (this study). Moreover, it appeared that 4-AP-dependent meiosis proceeded at least up to the appearance of the first polar body, which contrasts with 5HT-induced maturation that stops at metaphase I. This suggested that 4-AP might act in a way different from that used by 5HT. Actually, we found that 4-AP was effective both on 5HT-competent and -incompetent oocytes, whether these were suspended in ASW or CaFSW. This result supported the assumption that internal Ca^{2+} stores were mainly involved in the action of this agent and we could actually detect an important transient Ca^{2+} surge within 10 min of 4-AP addition. Taken together, these data are consistent with the hypothesis that 4-AP may act via an inhibition of the intracellular Ca^{2+} ATPase pumps which sequester Ca^{2+} in the endoplasmic reticulum, as already reported for mammalian cells (Ishida and Honda, 1993).

Another interesting observation was that the effect of 4-AP depended on the external pH. This suggested that 4-AP, similarly to the free membrane permeating forms of the weak bases NH_4Cl or procaine, was susceptible to catch protons from the cytosol, thus increasing pH_i. This assumption has been supported by our recordings using BCECF-loaded oocytes of *Ruditapes*, which revealed that a slow increase in pH_i occurred after 4-AP addition. As already observed following ammonia stimulation (Guerrier *et al.*, 1993), it is possible that this higher internal pH level, coupled to the Ca^{2+} response, would contribute to facilitate some specific processes leading the oocytes to skip their stage of arrest in metaphase I. This hypothesis will be further tested experimentally by measuring simultaneously the pH and Ca^{2+} surges which occur during maturation of *Patella* and of different species of bivalves which arrest *(Ruditapes)* or not *(Barnea, Pholas* and *Spisula)* in metaphase I.

Materials and Methods

Solutions

Artificial seawater (ASW) and calcium-free seawater (CaFSW) were prepared according to the formulae of Shapiro (1941), to which 2 mM Tris were added, pH being adjusted to 8.2 with HCl. CaFSW contained 2 mM EGTA. Sodium-free seawater (NaFSW) and calcium- and sodium-free seawater (CaNaFSW) were prepared by substituting Tris base for NaCl.

NH_4Cl was prepared from a 2 M stock solution containing 10 mM Tris, pH 8.5. Stock solutions were prepared daily, in ASW for 5HT (10 mM), tetraethylammoniumchloride or bromide (TEA, 1 M) and $MnCl_2$ (1 M), in ethanol for 4-AP (1 M). We used frozen stock solutions made in ethanol for ionomycine (1 mM), 9-aminoacridine (9-AA, 50 mM), bafilomycin A_1 ($BafA_1$, 1 mM) and N,N'-dicyclohexylcarbodiimide (DCCD, 20 mM) and in DMSO for Hoechst 33342 and 33258 (100 μg/ml), Fluo-3-pentaacetomethyl ester (Fluo-3/AM, 1 mM) or 2',7'-bis-(carboxymethyl)-5(6)-carboxyfluorescein (BCECF/AM, 1 mM).

All these chemicals were obtained from Sigma (St Louis, MO, USA) except Fluo-3 and BCECF which were purchased from Molecular Probes (Eugene, OR, USA).

Handling of oocytes

The limpets *Patella vulgata* were collected from September to January in the vicinity of Roscoff and were maintained in Lyon at 14°C under a shallow flow of running sea water. Oocytes were obtained by mincing the gonads with scissors in ASW. They were filtered through cheese cloth and washed repeatedly by short centrifugation and elimination of the supernatant. To remove follicle cells, the oocytes were washed 3 times in CaNaFSW (Guerrier *et al.*, 1986a). The vitelline envelope was removed by a 20 min treatment with 0.02% pronase in the same medium followed by washing in CaFSW and resuspension in ASW.

Ruditapes philippinarum clams were obtained from commercial sources in Golfe du Morbihan or provided by IFREMER (La Tremblade). They were kept in running sea water tanks until used. Their gonads and oocytes were treated as described for *Patella*. To remove the vitelline envelope, they were incubated in CaFSW containing 1 mM EGTA and 0.02% trypsin (Sigma, type III), washed 3 times in CaFSW and maintained in ASW (Osanai and Kuraishi, 1988).

Triggering meiosis and quantification

Meiosis reinitiation of prophase-blocked oocytes of *Patella* was triggered by adding to the oocyte suspensions 10 mM NH_4Cl or 0.5 to 3 mM 4-AP. *Ruditapes* oocytes were induced to reinitiate meiosis by incubating 1 ml aliquots of the cell suspension (0.5 to 1%, v:v) with various concentrations of 5HT or 4-AP at room temperature for a 30-90 min period. For quantification of maturation, eggs were mounted and flattened by removing excess fluid at the edge of the coverslip. Under these conditions, GVBD was easily observed *in vivo* and scored by random counting of 100-200 oocytes per treatment. For cytological observations, chromosomes were either stained *in vivo* using the fluorescent dye Hoechst 33342 (0.5 µg/ml) or *in vitro,* after fixation in glucamine acetate buffer (GA) containing Hoechst 33258 (Dufresne *et al.,* 1988). All experiments were performed at least 3 times.

Intracellular free Ca^{2+} and pH measurements

Oocytes, suspended in ASW, were incubated for 30 min in the presence of 5 µM Fluo-3/AM or BCECF/AM for $[Ca^{2+}]_i$ or pH measurements, respectively. Excess dye was removed by washing the oocytes in ASW. Then, oocytes were attached to a coverslip at the bottom of the observation chamber using a 1% solution of protamine sulfate in ASW. Measurements were performed with the Argus 50 system from Hamamatsu (Japan) coupled to an OLYMPUS IMT2 inverted microscope and a 486 HP computer fitted to a printer. Data were saved on floppy discs and corrected for photobleaching attenuation using a specific program. Excitation wavelengths were 490 nm for Fluo-3 and 500 nm for BCECF. Emission was recovered at 530-535 nm. No calibration was performed for pH measurements and only relative changes of fluorescence emitted by BCECF were followed. Instead, the concentration of cytosolic free Ca^{2+} could be estimated according to the formula given by Kao *et al.* (1989): $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, with Kd= 400 nm, where F is the fluorescence value; F_{max} the maximal value obtained by calibration with 5 µM ionomycine; F_{min} the minimal value observed after quenching Fluo-3 fluorescence using 50 mM $MnCl_2$. Taking into account autofluorescence of the oocytes, resting free intracellular Ca^{2+} concentration varied from 160 to 600 nM, as previously reported (Guerrier *et al.,* 1993). Relative changes in fluorescence (F/F_0), which are linearly proportional to the real Ca^{2+} concentrations, were chosen to present our recordings, selected from a number of independent experiments.

Acknowledgments

We are especially grateful to Dr. Michel Charbonneau who drove our interest towards acidic vesicles and to Dr. André Gérard and his colleagues (IFREMER, La Tremblade) for preparing and sending us conditioned Ruditapes clams. This work has been supported by grants from the Association pour la Recherche sur le Cancer (ARC 6145), IFREMER (922521145) and a fellowship from the French government to M.L. (Hungary).

References

ABDELMAJID, H., GUERRIER, P., COLAS, P., DUROCHER, Y., GOBET, I., KRANTIC, S., LECLERC-DAVID, C., MOREAU, M., NEANT, I., RIVAILLER, P. and TOMKOWIAK, M. (1993a). Role of calcium during release of mollusc oocytes from their blocks in meiotic prophase and metaphase. *Biol. Cell 78:* 137-143.

ABDELMAJID, H., LECLERC-DAVID, C., MOREAU, M., GUERRIER, P. and RYAZANOV, A. (1993b). Release from the metaphase I block in invertebrate oocytes: possible involvement of Ca^{2+}/calmodulin-dependent kinase III. *Int. J. Dev. Biol. 37*: 279-290.

ALLEMAND, D., RENZIS, G., GIRARD, J-P. and PAYAN, P. (1987) Activation of amino acid uptake at fertilization in the sea urchin egg. Requirement for proton compartmentalization during cytosolic alkalosis. *Exp. Cell Res. 169*: 169-177.

ARAI, H., BERNE, M., TERRES, H., PUOPOLO, K. and FORGAC, M. (1987). Subunit composition and ATP site labelling of the coated vesicle (H+)-ATPase. *Biochemistry 26:* 6632-6638.

BERGER, F. (1992). Mechanisms of initiation and propagation of the calcium wave during fertilization in deuterostomes. *Int. J. Dev. Biol. 36:* 245-262.

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature 361:* 315-325.

BUSA, W.B. (1986). Mechanisms and consequences of pH-mediated cell regulation. *Annu. Rev. Physiol. 48:* 389-402.

CHRISTEN, R. (1983). Acidic vesicles and the uptake of amines by sea urchin eggs. *Exp. Cell Res. 143*: 319-325.

DUBÉ F. and COUTU, L. (1990). The sodium requirement for activation of surf clam oocytes. *Cell Biol. Int. Rep. 14:* 463-471.

DUBÉ, F. and GUERRIER, P. (1982). Activation of *Barnea candida* (Mollusca pelecypoda) oocytes by sperm or KCl, but not by NH4Cl, requires a calcium influx. *Dev. Biol. 92:* 408-417.

DUBÉ, F., SCHMIDT, T., JOHNSON C.H., and EPEL, D. (1985). The hierarchy of requirements for an elevated intracellular pH during early development of sea urchin embryos. *Cell. 40:* 657-666.

DUFRESNE, L., DESROCHES, M., BOURGAULT, C., GICQUAUD, C. and DUBE, F. (1988). Relationships between intracellular pH, protein synthesis and actin assembly during parthenogenetic activation of sea urchin eggs. *Biochem. Cell Biol. 66:* 780-791.

GOBET, I., DUROCHER, Y., LECLERC, C., MOREAU, M. and GUERRIER, P. (1994). Reception and transduction of the serotonin signal responsible for meiosis reinitiation in oocytes of the Japanese clam *Ruditapes philippinarum. Dev. Biol. 164:* 540-549.

GUERRIER, P., BRASSART, M., DAVID, C. and MOREAU, M. (1986a). Sequential control of meiosis reinitiation by pH and Ca^{2+} in oocytes of the prosobranch mollusk *Patella vulgata. Dev. Biol. 114*: 315-324.

GUERRIER, P., GUERRIER, C., NEANT, I. and MOREAU, M. (1986b). Germinal vesicle nucleoplasm and intracellular pH requirements for cytoplasmic maturity in oocytes of the prosobranch mollusk *Patella vulgata. Dev. Biol. 116:* 92-99.

GUERRIER, P., LECLERC-DAVID, C. and MOREAU, M. (1993). Evidence for the involvement of internal calcium stores during serotonin-induced meiosis reinitiation in oocytes of the bivalve mollusc *Ruditapes philippinarum Dev. Biol. 159:* 474-484.

GUERRIER, P., MOREAU, M. and DOREE, M. (1978). Control of meiosis reinitiation in starfish: calcium ion as the primary effective trigger. *Ann. Biol. Anim. Bioch. Biophys. 18:* 441-452.

ISHIDA Y. and HONDA, H. (1993). Inhibitory action of 4-Aminopyridine on Ca^{2+}-ATPase of the mammalian sarcoplasmic reticulum. *J. Biol. Chem. 268*: 4021-4024.

KAO, J.P.Y., HAROOTUNIAN, A.T. and TSIEN, R.Y. (1989). Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. *J. Biol. Chem. 264:* 8179-8184.

LEE, H.C. and EPEL, D. (1983). Changes in intracellular acidic compartments in sea urchin eggs after activation. *Dev. Biol. 98:* 446-454.

MOREAU, M., DAVID, C. and AZZI, L. (1990). Changes in membrane properties during *in vitro* meiotic maturation of the limpet *Patella vulgata. Int. J. Dev. Biol. 34*: 441-451.

MOREAU, M., GUERRIER, P. and VILAIN, J.P. (1985). Ionic regulation of oocyte maturation. In *Biology of Fertilization*, Vol. 1 (Eds. C.B. Metz and A. Monroy). Academic Press, New York, pp. 299-345.

OSANAI, K. and KURAISHI, R. (1988). Response of oocytes to meiosis-inducing agents in pelecypods. *Bull. Mar. Biol. Stn. Asamushi (Tohoku Univ.) 18:* 45-56.

SCHROEDER, T.E. (1985). Cortical expressions of polarity in the starfish oocyte. *Dev. Growth Differ. 27:* 311-321.

SHAPIRO, H. (1941). Centrifugal elongation of cells and some conditions governing the return to sphericity and cleavage time. *J. Cell Comp. Physiol. 18:* 61-78.

STITH, B.J. and MALLER, J.L. (1985). Increased intracellular pH is not necessary for ribosomal protein S6 phosphorylation, increased protein synthesis, or germinal vesicle breakdown in *Xenopus* oocytes. *Dev. Biol. 107:* 460-469.

SUN, S.Z. XIE, X.S. and STONE, K. (1987). Isolation and reconstitution of the DCCD sensitive proton pore of the clathrin-coated vesicle proton translocating system. *J. Biol. Chem .258:* 14790-14794.

SWALLOW, C.J., GRINSTEIN, S., SUDSBURY, R.A., and ROTSTEIN, O.D. (1993). Relative roles of Na^+H^+ exchange and vacuolar-type H^+-ATPases in regulating cytoplasmic pH and function in murine peritoneal macrophages. *J. Cell. Physiol. 157:* 453-460

Accepted for publication: March 1995