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Original Article

Thimerosal triggers meiosis reinitiation in oocytes of the Japanese clam *Ruditapes philippinarum* by eliciting an intracellular Ca^{2+} surge

MONICA LIPPAI[1], ISABELLE GOBET[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 bivalve mollusc Ruditapes philippinarum are arrested during first meiotic prophase. Release from this blockade is triggered by the neurohormone serotonin (5HT or 5-hydroxytryptamine), which promotes germinal vesicle breakdown and drives these oocytes to a second arrest in metaphase I. 5HT action involves binding to a specific G protein-coupled receptor which results in a transient rise in IP_3 and in the intracellular free Ca^{2+} concentration. Here we analyze the cytological effects and mode of action of the sulphydryl reagent thimerosal which could also trigger meiosis reinitiation in *Ruditapes*. No metaphase I spindle formed under these conditions since thimerosal was found to be able to preclude or reverse tubulin polymerization when applied to prophase- or to metaphase-arrested oocytes, respectively. Our results strongly suggest that the common final target for 5HT and thimerosal actions consists in a transient rise in internal free Ca^{2+} level that we could follow using Fluo3/AM as a probe. The effect of thimerosal in promoting oocyte maturation and increasing intracellular free Ca^{2+} concentration was improved by excess KCl. In addition, thimerosal, but not KCl, was found to facilitate 5HT-induced maturation at subthreshold hormone concentrations which, by themselves, did not produce an intracellular Ca^{2+} surge. These data suggest that thimerosal may inhibit Ca^{2+} pumps of the endoplasmic reticulum and unmask the plasma membrane voltage-sensitive Ca^{2+} channels which also appear after 5HT-induced GVBD.

KEY WORDS: *bivalve oocytes, intracellular calcium stores, meiosis reinitiation, microtubules, sulphydryl reagent*

Introduction

Vertebrate and invertebrate oocytes constitute an excellent model to study control of the cell division cycle (and essentially that of M-phase) since they offer a considerable population of large and physiologically synchronized cells. These cells have already replicated their DNA and are blocked at the germinal vesicle stage during late prophase of the first meiotic division.

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), an event which attests for their re-entry in M-phase. Then, in certain species such as the bivalves *Spisula*, *Pholas* and *Barnea*, maturation proceeds to completion while in others such as *Mytilus* and *Ruditapes*, during metaphase I, there occurs a second block which is only released upon fertilization or artificial activation.

We already described that release of *Ruditapes philippinarum* oocytes from their two successive blocks in prophase and metaphase depended on an intracellular-free calcium surge (Abdelmajid *et al.*, 1993a,b; Guerrier *et al.*, 1993). In this species, the natural hormone serotonin (5-hydroxytryptamine, 5HT) was found to trigger GVBD via a specific G protein-coupled receptor which induced an early inositol 1,4,5-trisphosphate (IP3) surge (Gobet *et al.*, 1994). Moreover, GVBD could not occur following incubation of the oocytes with the permeant Ca^{2+} chelator BAPTA/AM but was elicited via alternative treatments by ionophores, weak bases and thapsigargin, which also trigger an intracellular Ca^{2+} surge (Guerrier *et al.*, 1993).

Abbreviations used in this paper: ASW, artificial sea water; $(Ca^{2+})_i$, intracellular free (Ca^{2+}); BAPTA/AM, 1,2,-bis(o-aminophenoxy) ethane-N,N,N'N'-tetraacetic acid, acetomethyl ester; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylene-glycol-bis(b aminoethyl ether) N,N,N',N'-tetraacetic acid; Fluo 3/AM, fluo-3 pentaacetomethyl ester; GVBD, germinal vesicle breakdown; 5HT, 5-hydroxytryptamine; IP3, inositol trisphosphate; TMS, thimerosal.

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

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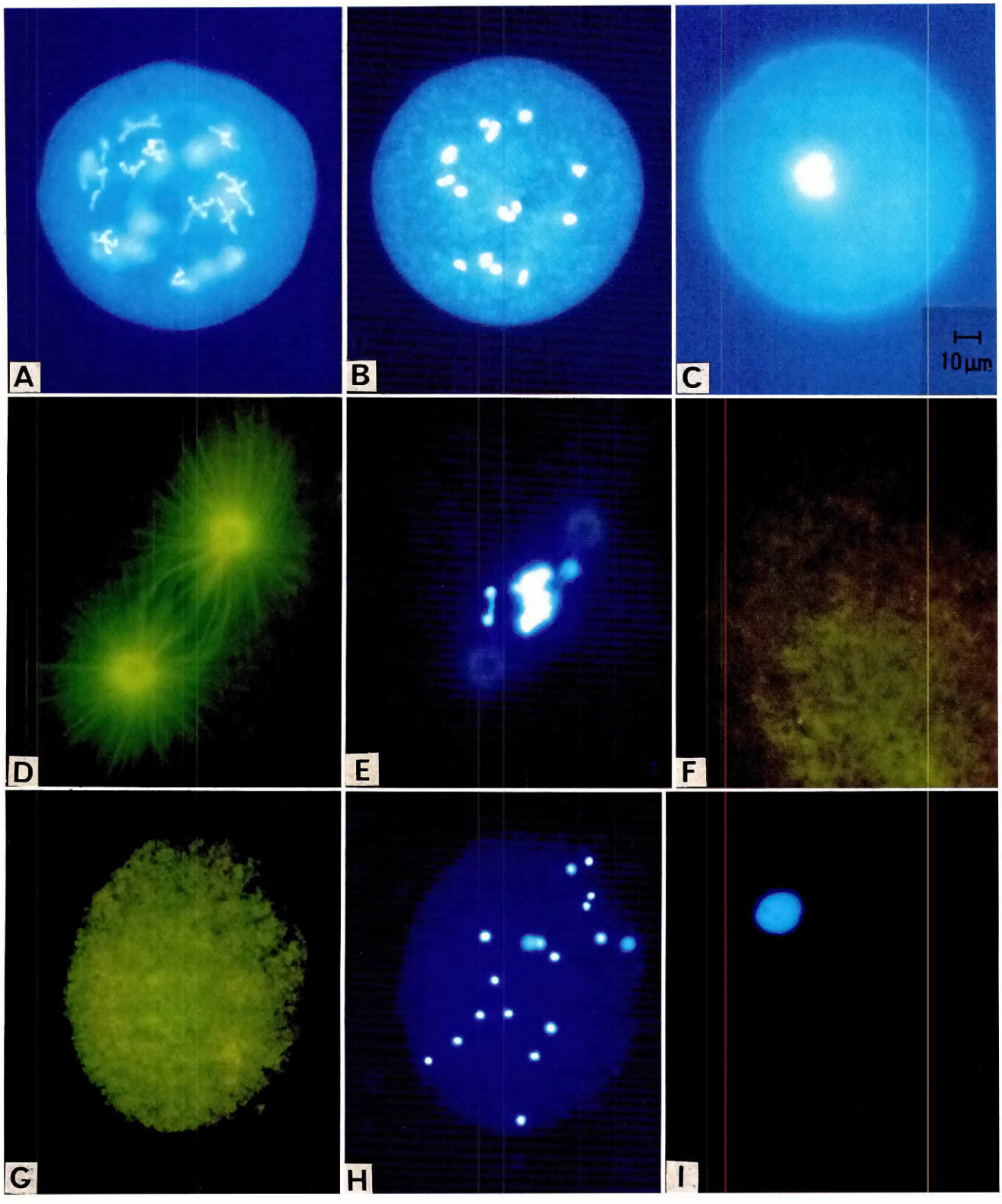

Fig. 1. Cytological consequences of the application of 100 µM thimerosal (TMS) to prophase- or metaphase-arrested oocytes of *Ruditapes philippinarum*. **(A-C)** *Vital staining with Hoechst 33342.* **(A)** *Germinal vesicle stage with tetrades;* **(B)** *GVBD has occurred but condensed chromosomes remain scattered;* **(C)** *metaphase chromosomes remain grouped.* **(D-I)** *Oocytes fixed and processed to reveal tubulin and chromosomes (Hoechst 33258).* **(D,E)** *Control metaphase-arrested oocyte;* **(F,I)** *after treatment with TMS, spindle is no longer present and a nucleus has formed;* **(G,H)** *prophase-treated oocyte with no spindle and dispersed chromosomes. Pictures taken with immersion oil objectives x40 for A,B,C,G,H and x100 for D,E,F,I.*

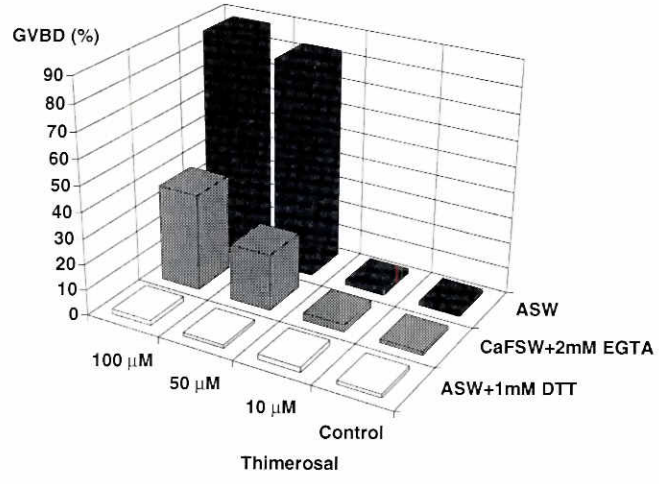

Fig. 2. Histogram *illustrating the effect of TMS in triggering GVBD of prophase-arrested oocytes of Ruditapes philippinarum in ASW or CaFSW, and inhibition of this response in the presence of 1 mM dithiothreitol (DTT). % GVBD was scored 50 min after TMS addition.*

In contrast to the situation found in some bivalves such as *Spisula*, *Pholas* and *Barnea*, which are readily fertilizable at the germinal vesicle stage, the addition of excess KCl could neither mobilize Ca^{2+} nor trigger GVBD in prophase-arrested *Ruditapes* oocytes. These are only able to respond to this agent after they have undergone GVBD and reached metaphase I, presumably depending on the recruitment or unmasking of K+ sensitive voltage-gated Ca^{2+} channels (Abdelmajid *et al.*, 1993a,b; Guerrier *et al.*, 1993).

In this report, we show that thimerosal (TMS), an oxidant of sulfhydryl groups, can also trigger GVBD in *R. philippinarum* oocytes by mobilizing intracellular calcium, even though this treatment does not allow formation of a normal first metaphase spindle.

Results

Cytological effects of thimerosal (TMS)

Vital staining with Hoechst 33342 revealed that 100 μM TMS usually triggered GVBD while preventing formation of the metaphase I spindle: condensed chromosomes remained dispersed in the cytoplasm (Fig. 1A,B). The same treatment, applied to metaphase-arrested oocytes results in the clumping and decondensation of the metaphase plate chromosomes which do not move to the poles and will eventually reform a closed nucleus (Fig. 1C).

To better visualize the effects of TMS on the mitotic apparatus, we used oocytes presenting a high percentage (50-60%) of spontaneous maturation, which allowed us to study simultaneously the effects of TMS both on prophase- and metaphase-blocked oocytes. After vitelline membrane removal, 100 μM TMS was added for 30 min. Then oocytes were stuck to coverslips, extracted, fixed and treated with monoclonal β tubulin antibody and Hoechst 33258, as described in Materials and Methods.

In control metaphase-arrested oocytes, a normal mitotic spindle was present with condensed chromosomes on the metaphase plate (Fig. 1D,E). In prophase-treated oocytes, spindle microtubules did not form and chromosomes remained scattered after GVBD (Fig. 1G,H). Similarly, when oocytes had already reached first metaphase before TMS-treatment, the previously formed mitotic spindle disappears, while chromosomes remain together and decondense to produce a resting nucleus (Fig. 1F,I). Oocytes simultaneously treated with 100 mM TMS and effective concentrations of 5 HT did not form a spindle and behaved as illustrated in Fig. 1B or G,H.

Effects of external calcium removal on TMS responses of Ruditapes oocytes

We checked whether the absence of Ca^{2+} in the external medium could modify or not the action of TMS. GVBD was scored after a 50-60 min treatment with different TMS concentrations (10-100 μM) applied either in ASW or in CaFSW+2 mM EGTA. We repeatedly observed that TMS was effective in both cases, even if oocytes suspended in CaFSW did not often respond as efficiently as those maintained in ASW (Fig. 2). Moreover, we noticed that, even in ASW, the response to TMS greatly varied (from 0 to 100% GVBD) from one batch of oocytes to another.

Mode of action of TMS in Ruditapes oocytes

The effect of TMS depends on its oxidative properties

We found that 1 mM dithiothreitol (DTT), which is known to reduce disulfide bonds, fully inhibited TMS-induced maturation (Fig. 2). We also established that this inhibition was effective even when DTT was delivered up to 5-10 min after TMS addition (Fig. 3). DTT alone had no activating or visible harmful effect on oocytes when used alone and its inhibitory effect was found to be reversible even at high concentrations (5 mM). We also observed that a 15 min preincubation with 1 mM DTT, which was sufficient to block TMS-dependent GVBD, had no effect on the maturations induced by 0.1 or 1 μM 5HT (Fig. 4). Only higher DTT concentrations (3-5 mM) proved able to inhibit this physiological response. Moreover, this inhibitory effect was perfectly reversible since oocytes from the same batch, washed after a longer preincubation period with DTT (45 min), did not exhibit any significant alteration in their response to 5HT.

The response to TMS is facilitated by KCl

Excess KCl is known to trigger GVBD in prophase-arrested oocytes of several bivalves by depolarizing the cell and creating an

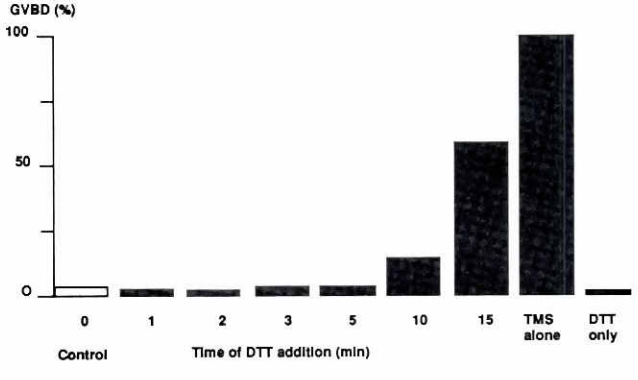

Fig. 3. Effects of adding 1 mM DTT at different times *after treating Ruditapes philippinarum prophase-arrested oocytes with 100 mM TMS. % GVBD was scored 90 min after TMS addition.*

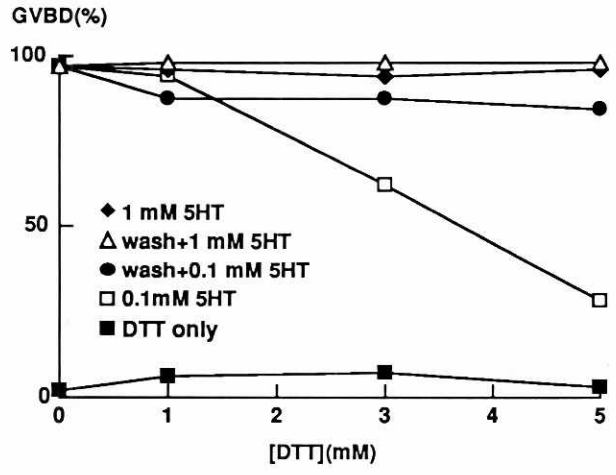

Fig. 4. Dose-response curves illustrating the effect of DTT on the efficiency of the 5HT-triggered *Ruditapes* oocyte maturation. *Oocytes were incubated in the presence of different concentrations of DTT for 15 min or 45 min and then washed, before adding 0.1 or 1 mM 5HT. % GVBD was scored 30 min after 5HT addition.*

influx of external calcium (Dubé and Guerrier, 1982; Abdelmajid *et al.*, 1993b). Interestingly, this agent failed to promote GVBD in prophase-arrested oocytes of *Ruditapes* (Guerrier *et al.*, 1993). Thus, we decided to study the effect of excess KCl on TMS-dependent maturation in this species. To our surprise, we found that excess KCl (+53 mM) was able to increase the GVBD-triggering effect of TMS (Fig. 5). We observed this facilitating effect even when KCl was applied 20 min after TMS addition, while KCl alone, as expected, did not promote GVBD.

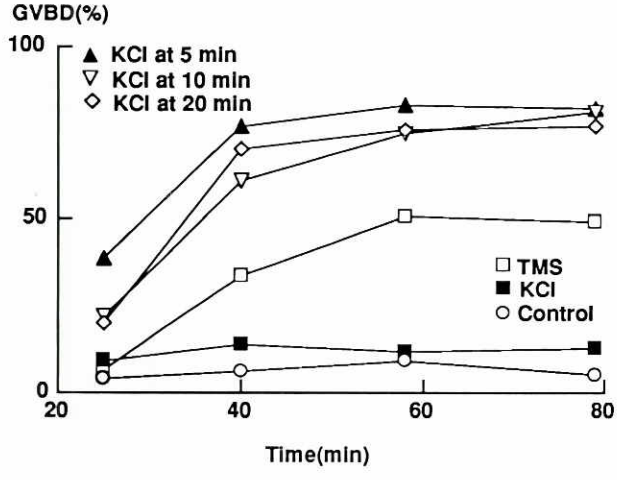

Fig. 5. Cooperative effect of KCl (+53 mM) and TMS (100 mM) on the amplitude and kinetics of the biological response (GVBD) of *Ruditapes philippinarum* prophase-arrested oocytes. *In this particular experiment, KCl was added to ASW at different times (5, 10, 20 min) after addition of 100 mM TMS. Similar results were obtained upon simultaneous addition of both agents.*

The response to 5HT is facilitated by TMS but not by KCl

We also studied the influence of TMS and KCl on 5HT-induced oocyte maturation. Interestingly, we found that, while 100 μM TMS significantly increased the response to 5HT at any (even subthreshold) 5HT concentrations, the addition of 53 mM KCl had no significant effect on this hormonal response (Fig. 6A and B).

Besides, we found that agonists of the ryanodine-gated intracellular Ca^{2+} stores such as caffeine (10 mM) or ryanodine (up to 200 μM) neither improved TMS or 5HT-induced maturation, nor did they produce any effect by themselves (data not shown).

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

In *Ruditapes* oocytes, it has been already shown that transduction of the 5HT signal involved an intracellular Ca^{2+} surge (Guerrier *et al.*, 1993). Therefore, we were interested to check whether TMS might act by mobilizing the same intracellular second messenger, using Fluo-3/AM loaded oocytes. Actually, we observed that 100 μM TMS triggered a transient Ca^{2+} surge within 20 min of its application as performed either in ASW or CaFSW (Fig. 7A and B). Under the conditions of this assay, where DTT may be partly photolyzed and diffusion of the drugs is limited, we found that an

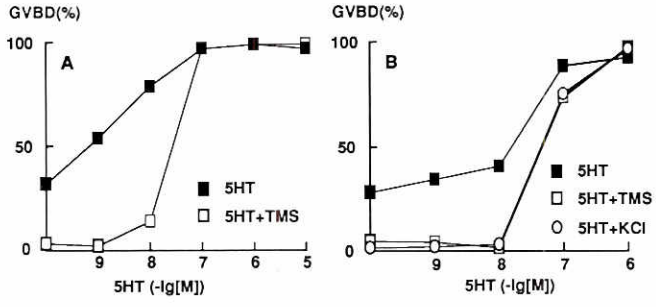

Fig. 6. Dose-response curves illustrating the cooperative effect of 5HT and 100 μM TMS in promoting GVBD of *Ruditapes philippinarum* prophase-arrested oocytes *(two typical independent experiments* **A** *and* **B**). *Instead, no facilitation is observed when excess KCl (+53 mM) is delivered simultaneously with 5HT* **(B)**. *GVBD was scored 30 min after drug addition.*

early addition of 5 mM, but not of 2 mM DTT, dramatically reduced the resulting Ca2+ surge and precluded GVBD (Fig. 7C and D). Instead, a late application of DTT (20 min after TMS treatment) had no significant effect on the already triggered $[Ca2+]_i$ increase and did not supress GVBD. These results agree with our observations concerning time-dependence of the inhibition of TMS-induced GVBD by DTT (Fig. 3). Specificity of such a DTT effect was evidenced by the fact that 5 mM DTT did not block ionomycin-induced Ca^{2+} surge even after a 20 min preincubation period in the presence of this agent (data not shown).

Discussion

TMS is a thiol reagent that probably acts by oxidizing sulfhydryl groups. This drug has been shown to cause Ca2+ release from platelets and leukocytes (Hecker *et al.*, 1989; Hatzelman *et al.*,

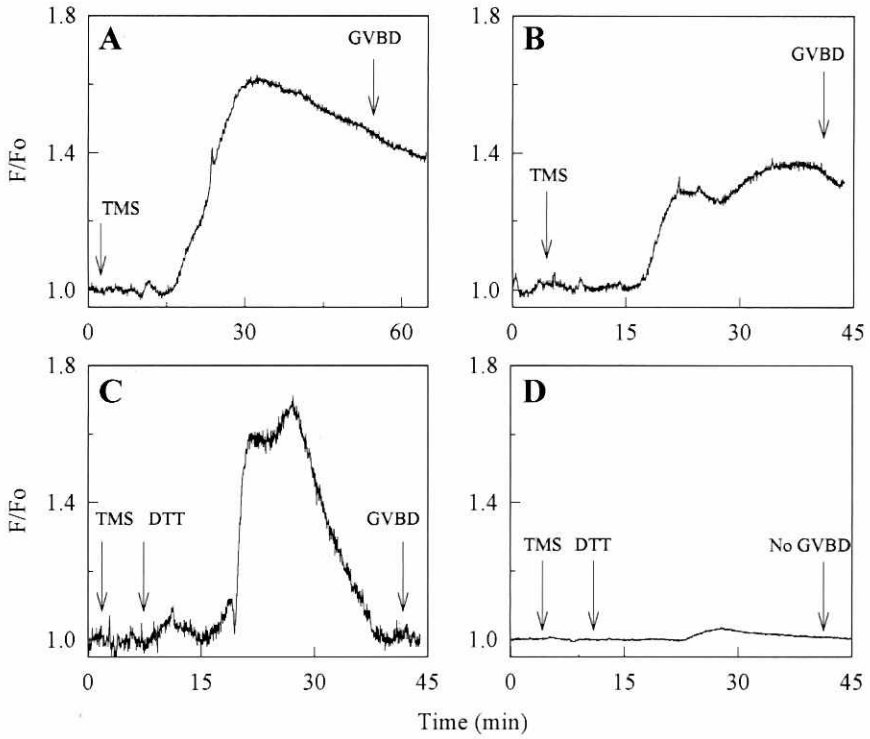

Fig. 7. Transient Ca^{2+} surges observed after the addition of TMS to Fluo-3/AM-loaded prophase-arrested oocytes of *Ruditapes philippinarum*. *Abscissa, time in minutes; ordinates, relative changes in fluorescence (F/F_0). Excitation, 490 nm; emission, 530 nm. Each record refers to an individual oocyte and is representative of at least 4 similar independent experiments.* **(A)** *Transient Ca^{2+} surge observed after the addition of 100 mM TMS in ASW. GVBD occurred within 35 min in 8 out of 8 companion oocytes from the same preparation.* **(B)** *Similar response recorded following the addition of 100 mM TMS in CaFSW. GVBD occurred within 40 min in 5 out of 8 companion oocytes.* **(C)** *The addition of 2 mM DTT after TMS stimulation did not block the Ca^{2+} surge and GVBD occurred at 25 min.* **(D)** *The addition of 5 mM DTT after TMS dramatically reduces the Ca^{2+} response and precludes GVBD.*

1990), Ca^{2+} oscillations in unfertilized hamster (Swann, 1991), mouse (Miyazaki *et al.*, 1992a,b; Swann, 1992; Cheek *et al.*, 1993) and sea urchin oocytes (Galione *et al.*, 1993; McDougall *et al.*, 1993; Tanaka and Tashjian, 1994), as well as in HeLa cells (Bootman *et al.*, 1992) and other permeabilized cells (Missiaen *et al.*, 1991; Renard *et al.*, 1992; Hilly *et al.*, 1993). All these effects were reversed by DTT.

TMS may act in different but not exclusive ways: (1) by increasing the affinity of the IP3 receptor (Finch *et al.*, 1991; Missiaen *et al.*, 1992; Renard *et al.*, 1992; Hilly *et al.*, 1993); (2) by reducing the effectiveness of the ATPase driven Ca2+ pump which sequesters Ca^{2+} in the endoplasmic reticulum as first suggested by Dikstein (1971) and Rebhun (1976) and later observed in some systems (Jones *et al.*, 1983; Bootman *et al.*, 1992); (3) by favoring simultaneously or alternatively other Ca^{2+}-induced Ca^{2+} release (CICR) processes which may involve ryanodine-dependent Ca^{2+} stores when present in the cell (Islam *et al.*, 1992; Salama *et al.*, 1992a,b; McDougall *et al.*, 1993) or, (4) finally, by affecting intravesicular Ca^{2+} handling as proposed by Tanaka and Tashjian (1994).

In this study, we have shown that TMS could release *Ruditapes* oocytes from their prophase block and promote GVBD. This effect, as reported in other cases, may arise from an oxidation of relevant thiol groups since DTT completely inhibits TMS-triggered GVBD. We actually demonstrated that this reagent did promote an important intracellular Ca^{2+} surge that could be blocked by DTT. The first effect of TMS is presumably to mobilize internal Ca^{2+} stores, as shown by the fact that this compound was able to trigger maturation, whether oocytes were suspended in ASW or in CaFSW. This response is likely to stimulate an additional Ca^{2+} influx since the efficiency of TMS in triggering GVBD is usually better in the presence of external calcium. TMS was also found to facilitate the oocyte response to 5HT even at subthreshold concentrations, where 5HT does not produce any measurable Ca^{2+} surge (Guerrier *et al.*, 1993). Such an observation can be accounted for according to 3 different hypothesis. A first possibility would be that TMS increases the extent of the 5HT-dependent IP3 peak response but this is unlikely since TMS inhibits, rather than stimulates, IP_3 production (Bootman *et al.*, 1992). Alternatively, TMS may increase the sensitivity of the IP_3 receptors themselves, as reported to occur in another system (Hilly *et al.*, 1993). However, even though TMS increases the extent of GVBD, it does not significantly affect EC_{50}s which remain similar in both conditions. Finally, the most likely explanation for this TMS effect is that $[Ca^{2+}]_i$ has increased due to the inhibition of Ca^{2+} ATPase pumps. This agrees with our previous observations showing that the efficiency of 5HT in triggering GVBD depends on the resting intracellular Ca^{2+} level and that thapsigargin also facilitates 5HT-dependent maturation (Guerrier *et al.*, 1993).

In addition, our finding that KCl facilitates TMS-dependent maturation suggests that this thiol reagent may exert an alternative action on the voltage-operated Ca^{2+} channels which normally appear after GVBD, following 5HT stimulation. One may assume that these channels, which are perhaps present but inactive at the GV-stage, might be unmasked and activated by TMS and thus would become sensitive to excess extracellular KCl, producing an additional Ca^{2+} influx. This effect of TMS may be due to its oxidative properties since no such facilitating effect of KCl was observed following 5HT stimulation. Finally, it seems rather unlikely that ryanodine-gated channels are involved in these responses since we found that caffeine or ryanodine did not improve either TMS or 5HT-induced maturation.

Another effect of TMS was to preclude the formation of the mitotic apparatus when applied to *Ruditapes* prophase-arrested oocytes or to depolymerize spindle microtubules when delivered at metaphase. A similar effect has already been described to occur after TMS treatment of mouse oocytes (Cheek *et al.*, 1993). Such

behavior is not surprising since the existence of an equilibrium between S=S and SH-HS groups has often been considered to control stability of the mitotic apparatus (Rapkine, 1931; Sakai, 1978). Moreover, Kuriyama and Sakai (1974) have shown that oxidation of tubulin thiol groups interfered with tubulin polymerization and could be reversed by DTT. Alternatively, it has been shown that an increased intracellular free Ca^{2+} concentration did affect microtubule assembly (Heilbrunn, 1921; Weisenberg, 1972) both *in vivo* (Kiehart, 1981) and *in vitro* (Salmon and Segall, 1980; Suprenant, 1986). However, this last possibility must be rejected since we have recently found that a giant first polar body was extruded following a dual treatment by 5HT and low concentrations of staurosporine which produced a larger intracellular Ca^{2+} surge than observed after TMS treatment. Further experiments will be designed to examine whether TMS mainly exerts its action by increasing sensitivity of the IP_3 receptors or by inhibiting Ca^{2+} ATPase pumps of the endoplasmic reticulum.

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.

Stock solutions were prepared daily in ASW for 5HT (10 mM), caffeine (10 mM), and MnCl2 (1M), in 20% DMSO for ryanodine (20 mM). We used frozen stock solutions made in distilled water for TMS (10 mM) and dithiothreitol (DTT, 1M), in ethanol for ionomycine (1 mM), and in DMSO for Fluo-3-pentaacetomethyl ester (Fluo-3/AM, 1 mM) and Hoechst fluochromes 33342 or 33258 (100 µg/ml).

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

Handling of oocytes

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. 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 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). All experiments were performed on oocyte populations which did not show a percentage of spontaneous maturation higher than 5%.

Triggering meiosis and quantification

5HT-induced maturation was triggered by incubating 1 ml aliquots of the cell suspension (0.5%) with various concentrations of 5HT at room temperature for 30 min. TMS was used at various concentrations in 0.5 ml aliquots of a 1% oocyte suspension, within a 30-90 min period.

For quantification of maturation, eggs were mounted and flattened by removing excess fluid at the edge of the coverslip. GVBD was easily observed *in vivo* due to high transparency of the cytoplasm and was 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 (Dufresne *et al.*, 1988), containing Hoechst 33258.

All experiments were performed at least 3 times. Results from only one typical experiment were selected for figure presentation.

Immunolocalization of tubulin

After vitelline membrane digestion, oocytes were allowed to stick to clean coverslips coated with 1% poly-L-lysine. To stabilize mitotic spindles, we used the KGE ISOL medium of Paweletz *et al.* (1984) containing 0.8% paraformaldehyde from a freshly prepared 4% stock solution. This was obtained by warming to 60°C, 8 g of dry powder in 100 ml distilled water supplemented with some drops of NaOH to facilitate full dissolution. 100 ml of 2x Sorensen phosphate buffer (0.02 M , pH 7.2) was added after the solution came back to room temperature. After 1 h incubation in KGE-paraformaldehyde mixture, coverslips were rinsed with Sorensen buffer to rehydrate them. Permeabilization was performed for 45 min in Sorensen pH 7, containing 1% bovine serum albumin (BSA) and 0.03% saponin. Mouse monoclonal antitubulin antibody (Sigma) was diluted 1/1000 in the same buffer containing 1 µg/ml Hoechst 33258 and applied overnight at 4°C. Slides were washed in Sorensen-saponin and incubated for 1 h at room temperature with FITC-conjugated, Fab specific goat anti-mouse IgG (Sigma, dilution 1/150) in Sorensen-saponin BSA. Slides were washed 4-5 times in Sorensen buffer containing Evans blue (50 ml of a 0.2% stock solution in 100 ml) and mounted in Sorensen:glycerol (1:1) Evans blue. Controls incubated with the second antibody alone gave no fluorescence. Preparations were observed via an Olympus microscope fitted for epifluorescence and photographs taken using Ektachrome 160 T films.

Intracellular free Ca^{2+} measurements

Oocytes, suspended in ASW, were incubated for 30 min in the presence of 5 µM Fluo-3/AM for $[Ca^{2+}]_i$ measurements. 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 sulphate 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 wavelength was 490 nm and emission was recovered at 530-535 nm.

The concentration of cytosolic free Ca^{2+} could be estimated according to the formula given by Kao *et al.* (1989):

$$[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F), \text{ with } K_d = 400 \text{ nM}.$$

F is the fluorescence value; F_{max} is the maximal value obtained by calibration with 5 µM ionomycine; F_{min} is 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+} concentrations varied from 160 to 600 nM. 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 (n>6).

Acknowledgments

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