Vol. 189, No. 1, 1992
November 30, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 79-84

THAPSIGARGIN INDUCES MEIOTIC MATURATION IN SURF CLAM OOCYTES

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Received August 28, 1992

I report here that thapsigargin, an inhibitor of Ca^{2+}-ATPase activities in internal Ca^{2+} stores, induces meiotic maturation in prophase I-arrested surf clam (*Spisula solidissima*) oocytes. The half-maximal dose for triggering germinal vesicle breakdown (GVBD) is 120 nM. Thapsigargin-induced GVBD is followed by all normal subsequent steps of meiotic maturation including extrusions of first and second polar bodies, with almost normal timing as compared with K^+-induced activation. Thapsigargin-induced GVBD requires the presence of external Ca^{2+} at a half-maximal concentration of 0.6 mM. In normal sea water, thapsigargin-induced activation is accompanied by a slightly increased $^{45}Ca^{2+}$ uptake by the oocytes and by an intracellular pH rise of 0.3 U. These results show that thapsigargin-sensitive Ca^{2+} pools regulating Ca^{2+} fluxes exist in surf clam oocytes, and they also further establish that Ca^{2+} ions are the major initial trigger for meiosis resumption in this species.

Meiosis resumption in prophase I-arrested mollusc oocytes is normally triggered by fertilization. In surf clam (*Spisula solidissima*) oocytes, germinal vesicle breakdown (GVBD) and later steps of meiotic maturation may be induced by sperm or by a number of artificial agents such as increased K^+ ions, ionophore A23187 or serotonin which all require the presence of external Ca^{2+} to be efficient (1-4). Increased $^{45}Ca^{2+}$ uptake by the oocytes accompanies activation induced by sperm, K^+ ions or serotonin (2,4). The general view arising from these studies is that the initial trigger leading to meiosis resumption in prohase I-arrested oocytes is an increased Ca^{2+} influx from the surrounding medium (5-7). However, it has been shown that injection of inositol trisphosphate may also induce GVBD in surf clam oocytes, thus suggesting that internal Ca^{2+} stores may also be involved in the activation process (8). Recently, the tumor

ABBREVIATIONS: DMO, dimethyloxazolidinedione; DMSO, dimethylsulfoxide; GVBD, germinal vesicle breakdown; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IP3, inositol 1,4,5-trisphosphate; MPF, Meiosis/mitosis promoting factor; TG, thapsigargin; TPA, 12-O-tetradecanoyl-13-phorbol acetate.

0006-291X/92 $4.00

promoter thapsigargin has been shown, in various mammalian cell types, to specifically inhibit Ca^{2+}-ATPases of various Ca^{2+} internal stores and to cause Ca^{2+} release resulting in elevated intracellular free Ca^{2+}, without formation of inositol phosphates or inhibition of plasma membrane Ca^{2+}-ATPases. (9-13). Moreover, thapsigargin has been observed to promote increased Ca^{2+} influx in numerous cell types as a consequence of emptying internal Ca^{2+} stores which increases the plasma membrane Ca^{2+} permeability (10). According to a new model currently developed, regulation of Ca^{2+} entry would be coordinated with the extent of depletion of intracellular Ca^{2+} stores (9,11). Given that increased Ca^{2+} influx appears an almost universal consequence of addition of thapsigargin to mammalian cells (11,13), that IP3-sensitive Ca^{2+} stores are especially thapsigargin-sensitive (12,13), that such stores appear to exist in surf clam oocytes (8), and that it is thought that increased Ca^{2+} influx is the primary trigger for meiosis resumption in this species (2,7), I decided to verify the possible activating properties of thapsigargin in these oocytes. I report here that thapsigargin efficiently induces meiotic maturation of surf clam oocytes. This activation is dependent upon the presence of external Ca^{2+} and is accompanied by slightly increased $^{45}Ca^{2+}$ uptake by the oocytes and by a normal rise of intracellular pH. These results confirm the major role of elevated Ca^{2+} in initiating the early cell cycles in surf clam oocytes.

MATERIALS AND METHODS

General procedures and reagents.

Surf clam specimens and gametes were obtained and handled as previously described (14). The experiments were performed in artificial sea water (normal or Ca^{2+}-free) prepared according to the MBL formulae (15) with the addition of 2 mM HEPES, pH 8.0. K^+ activation was performed by adding 10% (v/v) isotonic KCl (0.52 M) to the oocyte suspensions. Thapsigargin (Calbiochem) was dissolved in DMSO as stock solutions and kept in frozen aliquots until use. The final DMSO concentration was always kept below 0.5% (v/v) which had no effect on the processes observed when added alone. All experiments on kinetics or using radioactive isotopes were performed at 18-20°C in a temperature-controlled water bath while other experiments were performed at room temperature (20-22°C). GVBD was assessed under a light microscope by random counting of 100-200 oocytes per sample. Meiotic stages were determined by observations of fixed and Hoechst-stained oocytes under an epifluorescence microscope as previously described (14).

$^{45}Ca^{2+}$ uptake.

$^{45}Ca^{2+}$ uptake determinations were performed as previously described (2,16). $^{45}Ca^{2+}$ (as an aqueous solution, Amersham Canada Ltd.) was used at a final concentration of 8-10 µCi/ml. The pellets of washed samples (0.5 ml of 1.5-2% v/v oocyte suspensions) were dissolved in 0.5 N NaOH and counted with a Beckmann LS5801 scintillation counter. The data are expressed as CPM corrected for protein content determined by the method of Bradford (17).

Measurements of pHi.

Measurements of pHi were performed by the DMO method exactly as previously described (18) with proper control experiments to validate the use of [^{14}C]DMO method with surf clam

oocytes, as will be fully reported elsewhere (Dubé, in prep.). Determinations of average pHi were done with 4 samples per condition (1 ml of 1-2% oocyte suspensions), using artificial sea water prepared with 10 mM HEPES, pH 7.6.

RESULTS AND DISCUSSION

Thapsigargin was found to induce GVBD, in a dose-dependent manner, when added to surf clam oocytes, with 50% GVBD at a concentration of 120 nM (Fig. 1). Thapsigargin not only induced GVBD, the first visible sign of meiosis resumption, but also all subsequent steps of meiotic maturation, including extrusions of first and second polar bodies (Fig. 2). Unlike another tumor promoter, TPA, which activates directly protein kinase C and was found to activate only part of the meiotic process with a great delay (16,19), the kinetics of meiotic maturation induced by thapsigargin closely resembled that of K^+-induced maturation (Fig. 2) known to best mimic the normal process (20). As seen, the delay in thapsigargin-activated oocytes is less than 5 min for GVBD and less than 10 min for extrusion of the second polar body (Fig. 2).

In order to test whether external calcium was required for thapsigargin-induced meiotic maturation, oocyte suspensions were prepared at various external Ca^{2+} concentrations before addition of thapsigargin (Fig. 3). As seen, thapsigargin-induced GVBD could not occur in Ca^{2+}-free or low Ca^{2+}-containing sea water (Fig. 3), as is the case for K^+- or serotonin-induced

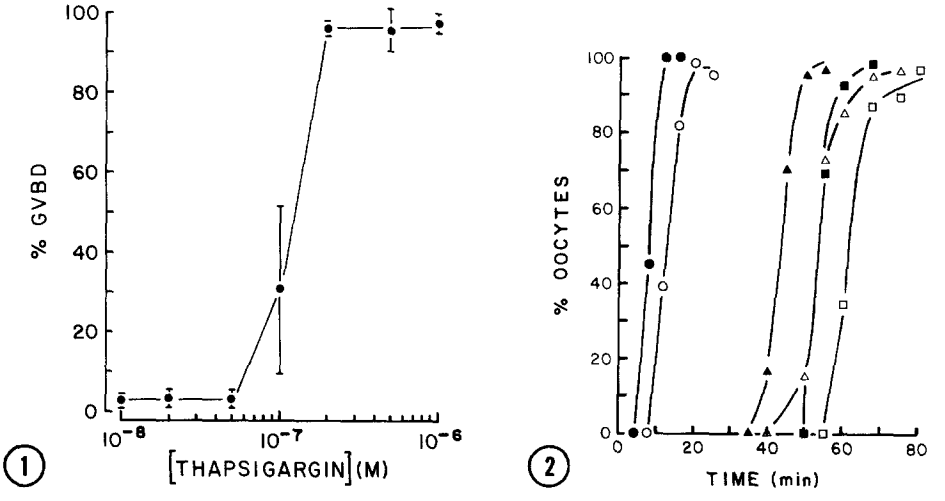

Figure 1. Dose-response curve of thapsigargin-induced GVBD. Oocyte suspensions (0.5%, v/v) were incubated in presence of indicated concentrations of thapsigargin for 30 min and the percentages of oocytes having undergone GVBD were determined. Mean results (± S.E.M.) of three experiments with two batches of oocytes.

Figure 2. Kinetics of meiotic maturation induced by thapsigargin. Two oocyte suspensions were activated with the addition of either 52 mM K^+ ions (closed symbols) or 1 µM thapsigargin (open symbols). At the different indicated times, samples of oocytes were fixed, stained with Hoechst and the percentages of oocytes having completed GVBD (●,○), first polar body extrusion (▲,△), or second polar body extrusion (■,□) were determined.

activations (4). The half-maximal concentration of external calcium was found to be 0.6 mM (Fig. 3), thus showing the absolute requirement of external Ca^{2+} ions for the activation process.

This result suggested that, like the addition of excess K^+ ions or serotonin (4,16), the addition of thapsigargin might result in an increased Ca^{2+} influx as the primary trigger for meiosis resumption. This was tested by measuring $^{45}Ca^{2+}$ uptake by the oocytes after the addition of thapsigargin as compared to untreated or K^+-activated oocytes (Fig. 4). As seen, the addition of K^+ ions induces a major biphasic increase of $^{45}Ca^{2+}$ uptake by the oocytes (Fig. 4), as previously reported (16). Thapsigargin does not induce such a large burst in $^{45}Ca^{2+}$ uptake but promotes a slightly increased rate of $^{45}Ca^{2+}$ uptake over basal level of unactivated oocytes (Fig. 4). This thapsigargin-induced increase in $^{45}Ca^{2+}$ uptake, though slight, is reproducible from one experiment to another and differs from the effect of TPA which was shown not to promote any differential $^{45}Ca^{2+}$ uptake by the oocytes, over the same time period, as compared to untreated oocytes (16). Presumably, this increased $^{45}Ca^{2+}$ uptake induced by thapsigargin is related to emptying of some intracellular Ca^{2+} stores as seen in other cells (11,13). It appears unlikely that this increased $^{45}Ca^{2+}$ uptake is sufficient for activation since similarly small or even higher $^{45}Ca^{2+}$ induced by subthreshold K^+ concentrations do not promote GVBD (2). Based on known effects of thapsigargin, I consider, however, this increased Ca^{2+} permeability as indicative of an earlier intracellular Ca^{2+} release which could be directly involved in the activation process.

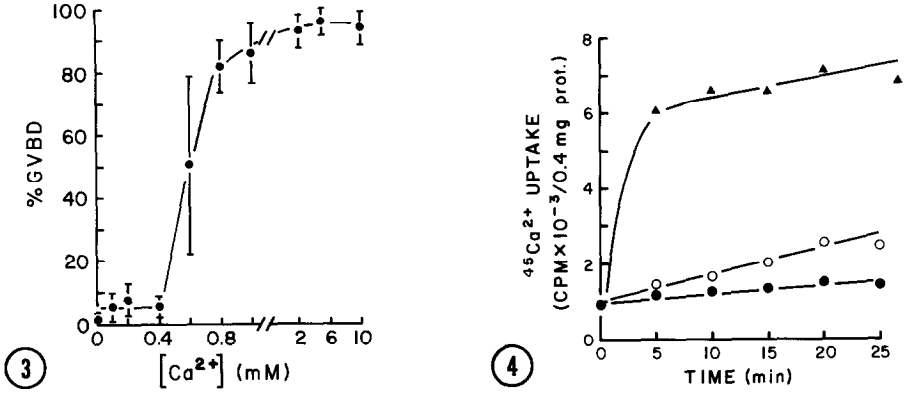

Figure 3. External Ca^{2+} requirement for thapsigargin-induced GVBD. An oocyte suspension in Ca^{2+}-free sea water was divided in several lots to which were added various known amounts of isotonic $CaCl_2$ (0.35 M) for the final indicated Ca^{2+} concentrations. Thapsigargin (1 µM) was then added and the oocytes were fixed after 30 min. and scored for GVBD. Mean results (± S.E.M.) of three experiments using two batches of oocytes.

Figure 4. $^{45}Ca^{2+}$ uptake by oocytes after addition of thapsigargin. An oocyte suspension (1.8%, v/v) was preequilibrated for 10 min in presence of 10 µCi/ml $^{45}CaCl_2$ and divided in three lots which were either activated by the addition of 52 mM K^+ ions (▲), 10 µM thapsigargin (○) or were left untreated (●). At different indicated times, the oocytes were sampled and rapidly washed and processed as described in Materials and Methods. The results are expressed as CPM corrected for protein content. Percentages of GVBD at 30 min were: untreated, 1%; K^+, 100%; TG, 100%. Two other experiments gave similar results.

Finally, we tested whether thapsigargin-induced meiosis resumption would be accompanied by another normally associated ionic response, the pHi rise. Figure 5 shows that the pHi of unactivated oocytes rises, after 30 minutes, from 7.22 (± .02) to 7.56 (± .02) in K^+-activated oocytes and to 7.53 (± .01) in thapsigargin-activated oocytes. The 0.3 U pHi rise triggered by thapsigargin is therefore absolutely comparable to that induced by K^+ ions, despite the very different $^{45}Ca^{2+}$ uptake promoted by these two agents. Even though such a pHi rise does not appear required for GVBD (21, and Dubé, in prep.), this shows that thapsigargin-induced activation shares similarities as well as differences with other activating agents such as K^+ ions.

Taken altogether, our results clearly show that thapsigargin efficiently induces meiotic maturation through a pathway involving external Ca^{2+}. Thapsigargin does not induce such a large increase in $^{45}Ca^{2+}$ uptake as seen with sperm, K^+ ions or serotonin (2,4) but nevertheless causes an higher $^{45}Ca^{2+}$ uptake than seen in untreated oocytes, unlike TPA (16). Since thapsigargin may also cause internal Ca^{2+} release, it is possible that this contributes to activate the oocytes but since external Ca^{2+} is required, this suggests that such an intracellular Ca^{2+} release, if necessary, might not be sufficient alone for triggering meiosis resumption. Further investigations will be required to precisely establish the possible respective contributions of both internal and external Ca^{2+} in the turning on of the cell cycle by thapsigargin. Interestingly, our observation that a normal pHi rise is promoted by thapsigargin supports the hypothesis that elevated Ca^{2+} may directly regulate pHi independently of protein kinase C and the inositol phospholipid hydrolysis pathway (22). It also shows that this pHi rise can be partly uncoupled from the normal, larger, $^{45}Ca^{2+}$ uptake seen with other activating agents. This is but one of many examples illustrating that thapsigargin is a powerful new tool to further analyze the interrelationships between various intracellular events that could not be previously uncoupled. In the surf clam oocytes, thapsigargin should

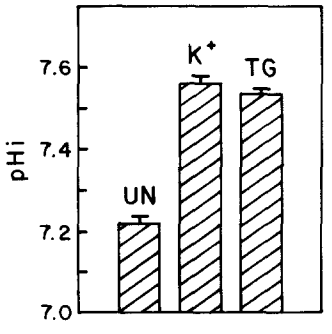

Figure 5. pHi of thapsigargin-activated oocytes. An oocyte suspension (1.7%, v/v) was divided in three lots which were either activated with 52 mM K^+ ions (K^+), 10 µM thapsigargin (TG) or left untreated (UN). The oocytes were sampled after 30 min in presence of [^{14}C]DMO and their pHi was determined as described in Materials and Methods. The results are expressed as average pHi (± S.D.) calculated for four identical samples. Percentages of GVBD at 30 min were: UN, 0%; K^+, 100%; TG, 97%.

prove very useful to precisely establish the respective contributions of intracellular Ca^{2+} release and increased Ca^{2+} influx in the early steps leading to MPF (Meiosis/Mitosis-promoting factor) activation, and further progress through the cell cycles as well where Ca^{2+} is thought to regulate specific transitions.

ACKNOWLEDGMENTS: Renaud Gonthier, Marie-Pascale Morin and Sandra Morissette are gratefully acknowledged for technical assistance, and Louise Dufresne for critical reading of the manuscript. This work was supported by NSERC-Canada and FCAR-Québec grants.

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