

# Activated M-phase-promoting factor (MPF) is exported from the nucleus of starfish oocytes to increase the sensitivity of the Ins(1,4,5)P<sub>3</sub> receptors

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## Abstract

Starfish oocytes that are extracted from the ovaries are arrested at the prophase of the first meiotic division. At this stage of maturation, they are characterized by a large nucleus called the germinal vesicle. Meiosis resumption (maturation) can be induced *in vitro* by adding the hormone 1-methyladenine (1-MA) to the seawater in which the oocytes are suspended. Earlier work in our laboratory had detected Ca<sup>2+</sup> increases in both the cytoplasm and the nucleus of the oocytes approx. 2 min after the 1-MA challenge. The nuclear Ca<sup>2+</sup> increase was found to be essential for the continuation of the meiotic cycle, since the injection of bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) into the nuclear compartment completely blocked the re-initiation of the cell cycle. We have recently confirmed, using confocal microscopy, that the cytoplasmic and nuclear Ca<sup>2+</sup> pools are regulated independently and that the nuclear envelope in starfish oocytes is not freely permeated by the Ca<sup>2+</sup> wave that sweeps across the nuclear region. Studies by others have shown that the sensitivity of the Ins(1,4,5)P<sub>3</sub> (IP<sub>3</sub>) receptors (IP<sub>3</sub>Rs) to IP<sub>3</sub> increases during oocyte maturation, so that they release progressively more calcium in response to the injection of IP<sub>3</sub>, as maturation proceeds. We have now shown that the increased sensitivity of the IP<sub>3</sub>Rs may depend on the activation of the cyclin-dependent kinase, MPF (M-phase-promoting factor) that occurs in the nucleus. MPF does not directly phosphorylate IP<sub>3</sub>Rs but phosphorylates instead the actin-binding protein actin depolymerization factor (ADF)/cofilin.

## Nuclear calcium signaling

Evident Ca<sup>2+</sup> swings have been described in oocytes of the starfish *Asterina pectinifera* during the resumption of meiosis (maturation process) [1], which culminates in the breakdown of the nuclear envelope of the large nucleus, called the germinal vesicle (GVBD), approx. 30 min after the initiation of exposure to 1-methyladenine (1-MA). The maturation process is induced by the hormone 1-MA, and is characterized by the gradual increase of the sensitivity of the cytoplasmic Ins(1,4,5)P<sub>3</sub> (IP<sub>3</sub>) receptors (IP<sub>3</sub>Rs) to their own ligand, IP<sub>3</sub> [2]. A linkage may exist between the sensitization of the receptors and the Ca<sup>2+</sup> swings, which occur both in the cytoplasm and in the nucleus of the oocytes [1]. The matter of the Ca<sup>2+</sup> waves in the cytoplasm and in the nucleus in preparation for meiosis is the subject of a vigorous debate, essentially centred on whether cytosolic Ca<sup>2+</sup> signals are attenuated at the level of the nuclear envelope or immediately and passively transmitted to the nucleoplasm [3,4]. A number of cell types have provided compelling

evidence in favour of persistent gradients of Ca<sup>2+</sup> between the cytoplasm and the nucleus [5], as well as evidence that Ca<sup>2+</sup> can be released into the nucleoplasm from nuclear tubular structures [6]. However, equally convincing experiments on other cells have failed to detect significant attenuation in the transmission of the cytosolic Ca<sup>2+</sup> waves to the nucleus in stimulated cells, and have shown that the kinetics of the Ca<sup>2+</sup> increase in the cytoplasm and in the nucleus are similar [7,8]. Recent work using a new generation of fluorescent proteins, termed 'pericams', and dual-excitation ratiometric dyes that minimize the effects of artefacts that are unrelated to changes in the concentration of free Ca<sup>2+</sup>, have shown a delay in the transmission of the Ca<sup>2+</sup> wave at the border between the cytosol and the nucleus, suggesting a restricted permeability of the nuclear envelope to Ca<sup>2+</sup> ions [9]. Oocytes, particularly those of starfish, have been the source of the most convincing experiments in favour of the idea that the nucleus is insulated from cytosolic Ca<sup>2+</sup> transients [1]. However, other cells, such as polarized pancreatic acinar cells, have also shown that the nucleus is protected from the relatively large Ca<sup>2+</sup> transients that are generated in the secretory pole [10]. In this case, the mechanism of attenuation has not been attributed to the nuclear envelope itself, but to a sieving mechanism performed by perinuclear mitochondria, which protect the nuclear area against invasion by the Ca<sup>2+</sup> waves. It has also been suggested

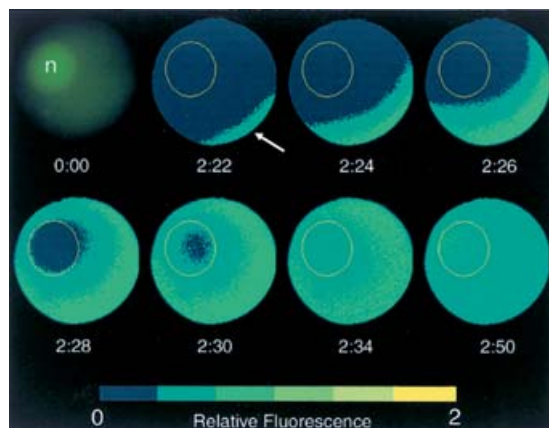
**Key words:** cytoskeleton, nucleus.

**Abbreviations used:** ADF, actin depolymerization factor; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; CCD, charge-coupled device; GVBD, germinal vesicle breakdown; IP<sub>3</sub>, Ins(1,4,5)P<sub>3</sub>; IP<sub>3</sub>R, IP<sub>3</sub> receptor; 1-MA, 1-methyladenine; MPF, M-phase-promoting factor; OGBD, Oregon Green 488 BAPTA-1.

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### Figure 1 | $\text{Ca}^{2+}$ wave elicited by the addition of 1-MA to an immature oocyte

The immature starfish oocyte was pre-injected with the fluorescent dye (5 mg/ml) OGBD coupled to a 10 kDa dextran. 1-MA ( $1\ \mu\text{M}$ ) induced a  $\text{Ca}^{2+}$  wave, which was measured with a CCD camera, shown by the relative fluorescence images. The wave initiated at a localized point beneath the plasma membrane (arrow). The relative fluorescence images show that the  $\text{Ca}^{2+}$  wave embraced the nucleus (n) without spreading into the nucleoplasm.



that perinuclear mitochondria play an important role in confining  $\text{Ca}^{2+}$  signals that are generated inside the nucleus to this organelle [11]. To sum up the most important recent evidence, a consensus of opinion is now emerging, according to which the  $\text{Ca}^{2+}$  barrier at the nuclear envelope may be a cell-specific property, particularly evident in oocytes.

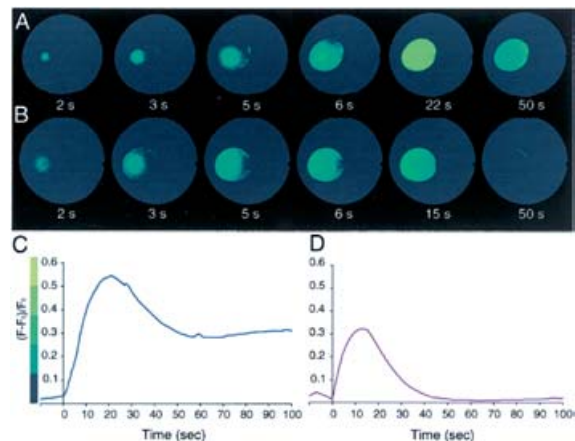
Our laboratory has been working on oocytes since 1994 [1] and has produced a number of results using either confocal microscopy or a sensitive CCD (charge-coupled device) camera, which have analysed the traffic of  $\text{Ca}^{2+}$  between the cytoplasm and the nucleus in detail [12,13].

A particularly significant experiment is described in Figure 1, which shows an immature oocyte injected in the cytoplasm with Oregon Green 488 bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA)-1 (OGBD) coupled to 10 kDa dextran. After approx. 15 min, the dye can be assumed to have diffused to the entire cytoplasm and to the nucleus as well (first fluorescence image). At this point, 1-MA was added to the medium. In this particular oocyte, which was observed with a sensitive CCD camera, a  $\text{Ca}^{2+}$  wave was initiated 2 min and 20 s later. Strikingly, the wave initiated at a circumscribed point with a concave front beneath the plasma membrane, expanded to a cortical half moon, and eventually invaded all of the cytoplasm. The relative fluorescence images taken 2 min and 28 s after the application of 1-MA show that the  $\text{Ca}^{2+}$  wave eventually engulfed the nucleus but its penetration into it was delayed by about 2–4 s. Evidently, the nucleus was not passively permeated by the  $\text{Ca}^{2+}$  that had increased in the perinuclear area following 1-MA stimulation. The mechanism by which the  $\text{Ca}^{2+}$  wave becomes globalized before meiosis

is under debate, and recent work has surprisingly denied the involvement of  $\text{IP}_3\text{Rs}$  in the maturation process of starfish oocytes [14]. Since it is now documented that most enzymes of the phosphoinositide machinery are also present in both the nuclear envelope and the nuclear matrix [15,16], it is plausible to assume that  $\text{IP}_3$  that is liberated within the nucleus in response to plasma membrane signals may induce the increase of  $\text{Ca}^{2+}$  in the nucleoplasm. This is supported by the experiment shown in Figure 2(A) in which  $\text{IP}_3$  was injected directly into the nucleus of an oocyte that had also been previously injected with OGBD conjugated to 70 kDa dextran. The size of the dye obviously prevented its diffusion to the cytoplasm. The relative fluorescence images of the  $\text{Ca}^{2+}$  increase detected with a CCD camera, together with graph of Figure 2(C), show that the injection of  $\text{IP}_3$  elicited a very evident  $\text{Ca}^{2+}$  increase that started at the point of the  $\text{IP}_3$  delivery and propagated to the entire nucleoplasm. The same results were obtained using a confocal microscope (not shown). The observation that the increase of nuclear  $\text{Ca}^{2+}$  was not confined to the inner membrane of the nuclear envelope is in line with the recent finding that antibodies against the subtype 2 of the  $\text{IP}_3\text{R}$ , which is abundantly expressed in different mammalian cells, interact with intranuclear structures [17]. The nuclear  $\text{Ca}^{2+}$  increase declined after approx. 20 s, but failed to reach the baseline, remaining instead very elevated for the entire duration of the experiment. The criticism that the injection of  $\text{IP}_3$  may have brought contaminating  $\text{Ca}^{2+}$  into the nucleus is proven invalid by the lack of any  $\text{Ca}^{2+}$  increase following the delivery of the injection buffer without  $\text{IP}_3$ . In addition, the injection

### Figure 2 | $\text{IP}_3$ and $\text{Ca}^{2+}$ injection into the nucleus of starfish oocytes

(A) The injection of  $50\ \mu\text{M}$   $\text{IP}_3$  into the nucleus of an immature oocyte pre-loaded with OGBD coupled to 70 kDa dextran induced a  $\text{Ca}^{2+}$  increase, measured with a CCD camera, which started at the point of the  $\text{IP}_3$  delivery and propagated throughout the nucleoplasm. The increase started to decline after 20 s, but  $\text{Ca}^{2+}$  remained elevated. (B) The injection of 1 mM  $\text{Ca}^{2+}$  elicited a  $\text{Ca}^{2+}$  increase, which decayed to the baseline in approx. 40 s. The graphs in (C) and (D) show the differences in the kinetics of the  $\text{Ca}^{2+}$  responses to  $\text{IP}_3$  and  $\text{Ca}^{2+}$  respectively.



of 1 mM  $\text{Ca}^{2+}$  into the nucleus raised very significantly the  $\text{Ca}^{2+}$  level there (Figure 2B). This was naturally expected; however, at variance with  $\text{IP}_3$  after 15–20 s, the signal declined and disappeared completely in approx. 40 s. Evidently, then,  $\text{IP}_3$ , which is known to act on cytosolic endoplasmic reticulum receptors, also liberates  $\text{Ca}^{2+}$  in the nucleoplasm acting from within the nucleus itself. Logically, one would have to assume that the nuclear targets of  $\text{IP}_3$  are  $\text{IP}_3$ Rs of the nuclear envelope. However, the alternative unorthodox suggestion could be cautiously advanced that  $\text{IP}_3$  may have other targets within the nucleus, which are unrelated to the nuclear envelope membranes. In this connection, the recent finding that antibodies against the  $\text{IP}_3$ Rs of starfish oocytes stained the nucleoplasm, even after the nuclear envelope had disappeared, is certainly interesting [14]. An interesting point is the complete disappearance of the  $\text{Ca}^{2+}$  that was injected into the nucleus in approx. 40 s (Figure 2B). Three possibilities could account for the observation. (i)  $\text{Ca}^{2+}$  could be taken up into the lumen of the nuclear envelope [18]. This is a distinct possibility, but it has been claimed that the ATPase that should mediate the uptake of  $\text{Ca}^{2+}$  is only located on the cytosolic side of the envelope [19]. (ii)  $\text{Ca}^{2+}$  could exit from the nucleoplasm via the envelope pores [20]. This is certainly a realistic possibility; however, we have recently performed experiments on starfish oocytes showing that the diffusion of injected uncaged  $\text{Ca}^{2+}$  in the cytoplasm is severely restricted (Figure 3).  $\text{Ca}^{2+}$  was monitored with a confocal microscope in both the cytoplasm and the nucleus, since the cell was injected with OGBD conjugated to 10 kDa dextran. The uncaging of 100  $\mu\text{M}$   $\text{Ca}^{2+}$  in a zone beneath the plasma membrane produced an immediate strong elevation of  $\text{Ca}^{2+}$  in the region of irradiation, which declined to the baseline approx. 40 s later without diffusing to either neighbouring domains of the cell or the nucleus. The mechanisms by which the diffusion of  $\text{Ca}^{2+}$  was so severely restricted are reminiscent of classical observations on squid axons [21], but may be more than one, beginning with the rapid uptake into the matrix of neighbouring mitochondria [11]. Therefore, if  $\text{Ca}^{2+}$  had exited from the nucleus through the pores, it would, in all

likelihood, have remained confined to the perinuclear space. (iii) Finally,  $\text{Ca}^{2+}$  in the nucleoplasm could become buffered by non-membranous structures, e.g. specific proteins. At this point, it may also be added that CD38, the enzyme that synthesizes the second messenger cADP-ribose, hitherto considered to be an ectoenzyme, has been recently identified in the inner nuclear membrane [22].

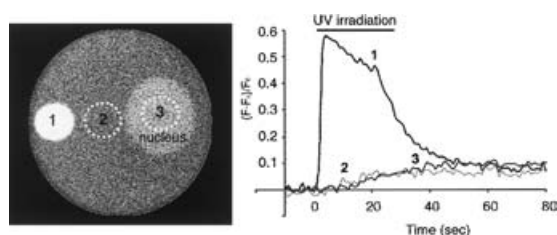
### Cytoskeleton and the increased sensitivity of the $\text{IP}_3$ Rs

To return to the central theme of the present paper, i.e. the increased sensitivity of the  $\text{IP}_3$ Rs in oocytes during the maturation process, the main point is that more  $\text{Ca}^{2+}$  is released at the end of maturation compared with that released in an immature oocyte that is injected with the same amount of  $\text{IP}_3$  [2]. The sensitization process has been recently shown not to be due to the increased expression of  $\text{IP}_3$ Rs [14]. We have recently confirmed these results using the caged variant of  $\text{IP}_3$ , and have investigated the pattern of development of the increased sensitivity of the  $\text{IP}_3$ Rs during the maturation process in some detail. We have found that it starts at the animal hemisphere of the oocytes 12 min after the addition of 1-MA and then propagates along the animal/vegetal axis of the cell. The increased sensitivity coincides with a previously undetected  $\text{Ca}^{2+}$  increase around the nucleus which precedes GVBD (D. Lim, E. Ercolano, K. Kyojuka, K. Lange and L. Santella, unpublished work) and which is assumed to stimulate the  $\text{IP}_3$ Rs. The molecular details of the process by which  $\text{Ca}^{2+}$  leaves the lumen of the endoplasmic reticulum through the open channels in the receptors have not been clarified, but the process is expected to be complex. Various means of regulating (gating) the channels have been described, ranging from the binding of  $\text{Ca}^{2+}$  itself [23,24], and calcium binding proteins [25], through phosphorylation reactions mediated by protein kinases A and C, and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II [26,27], to the interaction with  $\text{Ca}^{2+}$  and calmodulin [28].

Recent experiments in our laboratory have now identified a possible involvement of the actin cytoskeleton in the function of the receptors based on a massive liberation of  $\text{Ca}^{2+}$  in the cytoplasm of mature starfish oocytes induced by the actin depolymerizing drug latrunculin-A [29]. Although latrunculin-A could, in principle, liberate  $\text{Ca}^{2+}$  directly from actin, it seemed worth exploring the possibility that its depolymerization acted instead by activating the  $\text{IP}_3$ R channels. Thus it was decided to search for a natural mechanism that would induce the same changes in the actin cytoskeleton as those that were initiated by latrunculin-A. One plausible possibility seemed to be a kinase-mediated phosphorylation process. The working hypothesis was entertained that such a kinase could be the M-phase-promoting factor (MPF), a key regulator of the cell-cycle progression, which consists of the regulatory cyclin B and the Cdc2 protein kinase. The hypothesis was prompted by previous work that had shown that MPF regulated the

#### Figure 3 | Local uncaging of $\text{Ca}^{2+}$ generates a $\text{Ca}^{2+}$ spike, which does not propagate across the oocyte

An immature oocyte pre-injected with OGBD coupled to 10 kDa and caged  $\text{Ca}^{2+}$  (nitrophenyl-EGTA) was exposed to UV confocal laser light. A small region of the oocyte beneath the plasma membrane (region 1) was selectively irradiated to liberate  $\text{Ca}^{2+}$ . The local uncaging of  $\text{Ca}^{2+}$  generated a  $\text{Ca}^{2+}$  spike, which did not propagate across the cytoplasm (region 2) and to the nucleus (region 3), as indicated by the graph.



IP<sub>3</sub>-dependent Ca<sup>2+</sup> oscillatory signals in ascidian oocytes [30] and the Ca<sup>2+</sup> signal at fertilization in sea urchin eggs [31]. At this point, it was logical to involve the nucleus in the process because, during the maturation process of starfish oocytes, the final process that transforms MPF into an active kinase occurs in the nucleus, and we had previously shown that the removal of the nucleus from the oocytes prior to the application of 1-MA to induce the resumption of the meiotic cycle greatly affected the Ca<sup>2+</sup> elevation that was induced either by the photoliberation of injected caged IP<sub>3</sub>, or by the direct challenge with the sperm [32]. Thus it was decided to measure the activity of MPF in starfish oocytes from which the nucleus was removed, and in which the propagation of the Ca<sup>2+</sup> wave following the injection of IP<sub>3</sub> was inhibited [30]. The experiments have recently shown that the activity of MPF in the lysates of enucleated oocytes was decreased by about 50%, i.e. there was a parallel between the decreased effect of IP<sub>3</sub> and the decreased activity of MPF (D. Lim, E. Ercolano, K. Kyozyuka, K. Lange and L. Santella, unpublished work). The parallel could, of course, be purely coincidental and it was therefore decided to investigate whether active MPF could phosphorylate the actin cytoskeleton. This would have provided more direct support for the suggestion of MPF involvement in the altered function of the IP<sub>3</sub>Rs. The experiments have indeed revealed that active MPF phosphorylated a partially purified actin preparation. Interestingly, IP<sub>3</sub>Rs and actin itself did not become phosphorylated. The target of MPF phosphorylation was instead a low-molecular-mass protein that bound actin in the partially purified preparation. Work is now in progress to identify this protein: preliminary experiments indicate that it may be actin depolymerization factor (ADF)/cofilin.

In conclusion, the work detailed in the present paper has shown that the nucleus, or, more precisely, one of its components, participates in the regulation of the sensitivity of the IP<sub>3</sub>Rs to IP<sub>3</sub>. It appears likely that the increased sensitivity may be linked to a change in the interaction of components of the actin cytoskeleton, i.e. ADF/cofilin with the IP<sub>3</sub>Rs, as previously suggested for ankyrin [33]. The fine mechanism by which this interaction brings about the regulation of the receptor channels will be the subject of future investigations.

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