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c-AMP-dependent protein kinases and the control of progesterone-induced maturation in amphibian oocytes

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Summary. 1. Methylxanthines (IBMX, caffeine, theophylline), which are known inhibitors of c-AMP phosphodiesterase inhibited reversibly progesterone-induced maturation of *Xenopus* oocyte.

2. The time course of protein phosphorylation during maturation stimulated by progesterone was analysed in *Xenopus* oocyte : protein phosphorylation increased around germinal vesicle breakdown (GVBD). No quantitative changes were observed before this period.

3. Injection of the heat-stable inhibitor (I) of c-AMP-dependent protein kinases into *Xenopus* and *Discoglossus* oocytes triggered GVBD with 30-50 p. 100 efficiency. When (I) was injected into *Pleurodeles* oocytes, maturation did not occur, but a rapid and reversible condensation of lampbrush chromosomes was observed.

Introduction.

The vertebrate oocyte is blocked in meiotic prophase. It has been shown *in vivo* and *in vitro* in amphibian that gonadotropic hormone stimulates the follicular envelopes to produce a steroid hormone, progesterone, which acts directly on the oocyte to induce maturation (Fortune *et al.*, 1975 ; Thibier-Fouchet *et al.*, 1976). The molecular connections between the initial progesterone oocyte interaction and the completion of oocyte maturation remain largely unknown (Smith, 1975). The possibility that c-AMP-dependent protein kinases are involved in the initiation of maturation (early steps of progesterone action) is suggested by very recent experiments reported by Maller and Krebs (1977). They demonstrated that microinjection of highly purified preparation of regulatory subunit (R) of c-AMP-dependent protein kinase induces meiotic maturation of *Xenopus laevis* oocyte and that catalytic subunit (C) blocks progesterone-induced maturation ; the inhibitory action of (C) upon maturation occurs only if (C) is present during the first hours of progesterone exposure. It was suggested from these results that precocious decrease in c-AMP concentration may temporarily inhibit the phosphorylation of specific proteins ; these proteins, in turn cause oocyte maturation.

To directly test this hypothesis, we have analysed the time course of protein phosphorylation during oocyte maturation stimulated by progesterone in *Xenopus*. The biological effects of the heat-stable c-AMP-dependent protein kinase inhibitor (I) was investigated with regard to protein phosphorylation and oocyte maturation. We also report that methylxanthines, which are known inhibitors of c-AMP phosphodiesterase, inhibit progesterone-induced maturation.

Material and methods.

Xenopus laevis (De Rover, Holland), *Discoglossus pictus* (from Tunisia) and *Pleurodeles waltlii* females were bred and maintained under laboratory conditions. Full-grown *Xenopus* oocytes (stage 6, Dumont, 1972) were isolated by collagenase treatment and selected under stereotaxic microscope as already described (Bellé *et al.*, 1976). Denuded oocytes were equilibrated overnight in medium A containing NaCl, 88mM ; KCl, 1mM ; $Ca(NO_3)_2$, 0.41mM ; $MgSO_4$, 0.82mM ; Tris 2mM ; pH 7.4. Penicillin (100 IU/ml) and Streptomycin (0.1 mg/ml) were added to incubation flasks. The rate of oocyte maturation in the presence of progesterone 1 μM was determined. The criterion for muturation was breakdown of the germinal vesicle (GVBD).

For ^{32}P-labelling of proteins, incubation was made at room temperature (22 °C) under O_2/CO_2 (95 p. 100/5 p. 100) atmosphere, continuously shaking the flasks, in Medium A (25 oocytes/ml) and in the presence of 100 μCi/ml of $H_3\,^{32}PO_4$ (Radiochemical Center, Amersham) and 10μM phosphate in standard conditions. At the indicated time (2 hrs in ^{32}P experiments) oocytes were washed four times in cold Medium A and homogeneized at 4 °C in 0.4 ml of Medium B containing Tris, 0.05M ; KCl, 0.075M ; sucrose, 0.25M ; NaF, 0.3M and H_3PO_4, 10mM ; pH 7.4. The homogenate was centrifuged at 2 000 × g ; the supernatant was mixed with one equal volume of buffer C (final concentration Tris-KCl, 0.0625M ; sodium dodecyl sulfate (SDS), 2 p. 100 ; glycerol 10 p. 100 and mercaptoethanol 5 p. 100). Samples were heated at 100 °C for 30 min. Aliquots (0.1 ml) were put onto paper discs (Whatman 31 ET) and precipitated in 10 p. 100 trichloroacetic acid (TCA) 2 to 12 hrs. After washing in 5 p. 100 TCA at 4 °C for 15 min., filters were treated in 5 p. 100 TCA at 90 °C for 30 min. rinsed in 5 p. 100 TCA at 20 °C for 15 min., extracted in ethanol-ether (1/1) at 37 °C for 30 min., then in ethanol-chloroform (1/2) at 20 °C for 15 min. and finally rinsed twice in ether at 20 °C for 15 min., then dried. The radioactivity was measured by Cerenkov counting in 10 ml of water (40 p. 100 efficiency). The heat-stable protein kinase inhibitor (I) was gift of Dr. Nunez (INSERM, Bicêtre). Inhibitor was prepared from beef brain and purified up to the Sephadex G-75 filtration step (Majumder, 1974). Inhibitor was tested in the incubation conditions described by Reimann *et al* (1971) using histone Sigma IIA as substrate and endogenous protein kinases of the 190 000 × g supernatant of Rat liver (1 g liver homogeneized in 2,5 ml). Maximum inhibition of 10 μl supernatant was obtained with 1,5 μl of the inhibitor preparation.

Results.

Inhibition of oocyte maturation by methylxanthines. — When *Xenopus* oocytes are treated continuously with either 3-isobutyl-1-methylxanthine (IBMX), theophylline or caf-

feine (1mM) following progesterone exposure, maturation is completely inhibited. IBMX is the most efficient inhibitor. Lower concentrations of methylxanthines (between 5.10^{-5}M and 10^{-4}M) lead to a significant delay in maturation (fig. 1).

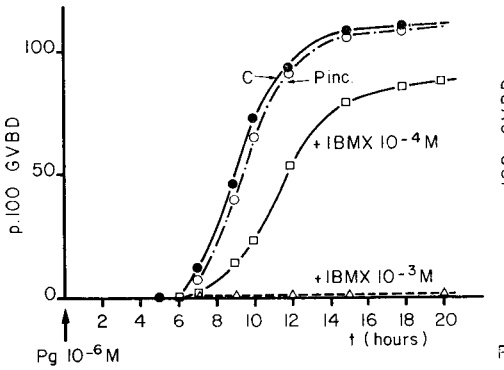

FIG. 1.

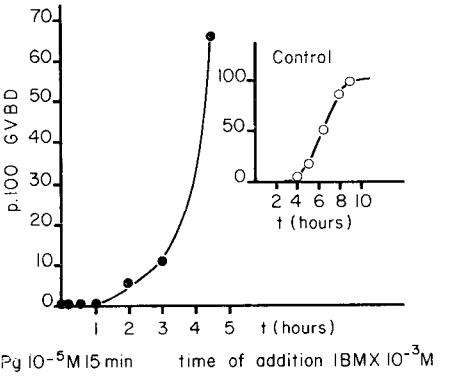

FIG. 2.

FIG. 1. — *IBMX dose effect on oocyte maturation.*

●—● C : Control oocytes incubated with progesterone 10^{-6}M.
□—□ Oocytes incubated with both progesterone 10^{-6}M and IBMX 10^{-4}M.
△--△ Oocytes incubated with both progesterone 10^{-6}M and IBMX 10^{-3}M.
o--o Pinc : oocytes preincubated with IBMX 10^{-3} (12 hrs) then stimulated by progesterone 10^{-6}M.

FIG. 2. — *Oocytes were exposed to progesterone* 10^{-5}*M for* 15 *min ; at various times they were transferred in IBMX solution* (10^{-3}M).

Oocytes were preincubated in the presence of 1mM IBMX alone ; 12 hrs later they were removed and the drug washed out. Then, oocytes were stimulated by progesterone ; maturation normally occurred (fig. 1). IBMX (50 nl, 5mM) or theophylline (50 nl, 13mM) were microinjected into oocyte ; progesterone-stimulated maturation again normally occured. These results demonstrate that IBMX blocks progesterone action only when it is present in the extracellular medium ; they further show that effect of extracellular methylxanthines is perfectly reversible.

Results presented in figure 2 reveal that IBMX effectively inhibits maturation during the first hours after progesterone exposure. Thus, only the early steps of maturation were sensitive to inhibition by IBMX. When oocytes are treated continuously for 12 hrs, with both progesterone (10^{-6}M) and IBMX (5.10^{-4}M), maturation is blocked. After removal of the drug, the preincubated oocytes were subjected to a second progesterone stimulation ; maturation always occurs but surprisingly the lag period between first progesterone exposure and 50 p. 100 GVBD is shortened, indicating that IBMX does not block all the effects of progesterone.

Phosphorylated proteins during progesterone-stimulated meiotic division.

— *Endogenous protein phosphorylation.* — In order to generate intracellular radioactive ATP needed for labelling of proteins, denuded full-grown *Xenopus* oocytes were incubated in medium A in the presence of ^{32}P (100 μCi/ml) and various

H_3PO_4 concentrations (10^{-8}M to 10^{-2}M). The uptake of exogenous phosphate increases linearly with phosphate concentration until 5×10^{-5}M. All experiments were further performed at a phosphate concentration of 10^{-5}M.

In these conditions, uptake was found to be linear with incubation time during the first two hours. Protein phosphorylation, estimated in the 2 000 × g supernatant in different females, is relatively constant when estimated as a percent of ^{32}P uptake (table 1). As shown in fig. 3 numerous proteins are phosphorylated ; five major bands were invariably found (MW : 18 000 ; 20 000 ; 30 000 ; 38 000 ; 110 000.)

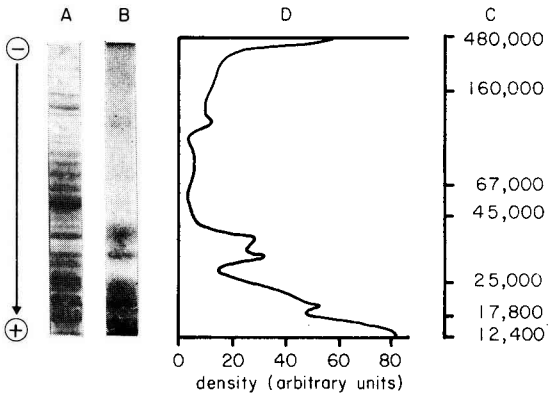

FIG. 3. — *Electrophoretic patterns of the* 2 000 × *g supernatant of isolated* Xenopus *oocytes.* Oocytes were incubated with ^{32}P and the 2 000 × g supernatant was submitted to SDS polyacrylamide gel electrophoresis (10 p. 100).

(A) protein staining (Coomassie blue) pattern of the dried gel.
(B) autoradiograph of phosphorylated proteins.
(C) molecular weight scale determined with standard proteins.
(D) densitometric scanning of the autoradiograph, obtained with a ISCO spectrometer equipped with a linear transport accessory.

TABLE 1

[^{32}P] *uptake and* [^{32}P] *protein incorporation in the* 2 000 × *g supernatant of defolliculated oocytes isolated from different* Xenopus laevis *females*

Oocytes isolated from different females were incubated in the presence of ^{32}P orthophosphate (100 μCi/ml) and 10^{-5}M phosphate for 2 hrs at room temperature

Experiment	^{32}P uptake (dpm/oocyte/2 hrs)	^{32}P proteins (p. 100 of uptake)
1	127 000 ± 17 000	0.33 ± 0.44
2	31 000 ± 1 500	0.44 ± 0.09
3	104 000 ± 15 000	0.58 ± 0.02
4	284 000 ± 24 000	0.28 ± 0.01
5	49 000 ± 3 000	0.39 ± 0.04
6	35 600 ± 200	0.63 ± 0.07
7	32 000 ± 7 000	0.63 ± 0.06
8	166 000 ± 14 000	0.45 ± 0.04
mean ± SD	104 000 ± 89 000	0.46 ± 0.13

— *Effects of progesterone.* — ^{32}P uptakes into progesterone-treated oocytes or into control oocytes are comparable until GVBD ; around this period a pronounced uptake decrease is observed (fig. 4). Protein phosphorylation was concomitantly analysed ; when expressed as a percent of ^{32}P in protein *versus* ^{32}P uptake, phosphorylation increases around GVBD (fig. 5). Preliminary experiments were conducted to test early effects of progesterone. Oocytes were incubated simultaneously with progesterone and ^{32}P for 2 hrs. The 2 000 × g supernatant was analysed by SDS polyacrylamide gel electrophoresis. Neither quantitative nor qualitative changes were observed during the whole period. It must be remarked that 2 000 × g pellet was not analysed and also that experimental conditions may not be optimum for the detection of early changes in phosphorylated proteins. Experiments were performed in the presence of IBMX or theophylline ; the drugs do not modify total protein phosphorylation following 2-hr incubation.

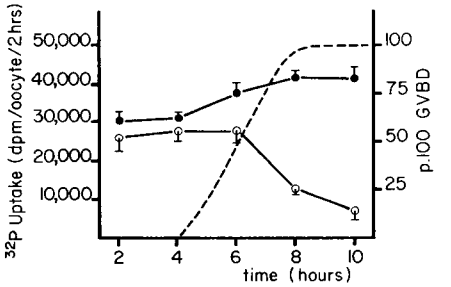

FIG. 4.

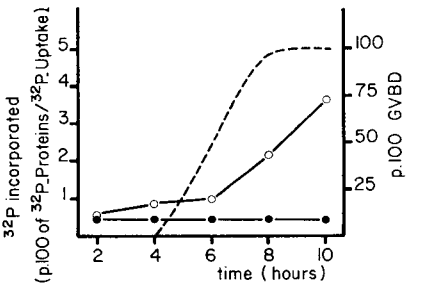

FIG. 5.

FIG. 4. — ^{32}P *phosphate uptake during the course of maturation.*

Groups of 50 oocytes were preincubated in 2 ml Medium A (0—0), or Medium A containing progesterone 10^{-6}M (0—0).

After 0h, 2h, 4h, 6h, 8h of preincubation, oocytes were transferred for 2-hr incubation in the same media containing in addition ^{32}P phosphate. (---) percent of germinal vesicle breakdown. Each point is the mean ± SD of two independent determinations.

FIG. 5. — ^{32}P *incorporation into proteins during the course of maturation.*

●—● ^{32}P incorporation into control oocytes.

o—o ^{32}P incorporation into progesterone-stimulated oocytes (10^{-6}M).

- - - - p. 100 of germinal vesicle breakdown. Each point is the mean ± SD of two independent determinations.

Injection of a heat-stable inhibitor protein of c-AMP-dependent protein kinases. — The inhibitor (I) is effective in blocking the activity of catalytic subunit (C) of the holoenzyme (Walsh and Ashby, 1973). Injection of (I) into *Xenopus* oocyte (50 nl) induces germinal vesicle breakdown directly in the absence of hormone. Similar experiments performed in *Discoglossus pictus* lead to the same result. In both species efficiency of (I) injection in inducing GVBD was between 30 p. 100 to 50 p. 100 but in all injected oocytes we observed a migration of the germinal vesicle toward the animal pole even when GVBD was not obtained. Following injection into *Xenopus* oocyte of (I) a 30 p.100 decrease in the amount of phosphorylated proteins (tested after a 2-hr pulse in ^{32}P) was observed.

The heat-stable inhibitor (I) (50 nl) was also injected into full-grown *Pleurodeles waltlii* oocytes (urodele amphibian) in May and June 1977 ; maturation did not occur. This negative result may depend on the physiological state of tested females (oocytes isolated from *Pleurodeles* at this period of the year did not mature in the presence of progesterone) or also on the doses of injected inhibitor. The effects of (I) injection on lampbrush chromosomes was investigated. Figure 6 shows that it induces a rapid regression of the chromosome loops and also a condensation of the chromosome axis. The phenomenon was observed in every experiment. The same action of (I) was also found in small vitellogenic oocytes (diameter 0.8 mm). When lampbrush chromosomes were observed 24 hrs after (I) injection, in every preparation chromosomes had again become normally developed. This indicates that, although (I) does not induce GVBD in our conditions, it provokes a rapid and total condensation of chromosomes which is reversible 24 hrs later (experiments in collaboration with Lacroix and Loones). Parallel experiments were performed in the presence of progesterone ; in no *Pleurodeles* females tested, did progesterone induce GVBD or chromosome condensation in full-grown or small oocytes.

Discussion.

Our experimental results suggest that early events following progesterone stimulation of amphibian oocyte involve an initial shift in c-AMP concentration, which results in change in the activity of c-AMP-dependent protein kinases.

Inhibitors of c-AMP phosphodiesterase block progesterone-induced maturation only if present in the extracellular medium. This finding has to be correlated with the demonstration of a phosphodiesterase activity associated with oocyte membrane (about 50 p. 100 of the total phosphodiesterase activity measured in *Xenopus* oocytes homogenate is membrane-bound). The enzymatic activity is not active on extracellular c-AMP, since exogenous ^{3}H-c-AMP is not metabolized when incubated with denuded oocytes (unpublished results in collaboration with Fontaine). Therefore c-AMP phosphodiesterase is probably localized in the inner membrane face, where it may regulate c-AMP concentration in an intracellular compartment near or at the membrabrane level. c-AMP concentration in *Xenopus* oocytes is high (10^{-6}M) (O'Connor and Smith, 1976 ; Pays-de Schutter *et al.*, 1975). Extracellular theophylline does not change total intracellular c-AMP level (O'Connor and Smith, 1976) ; in this presentation we report that extracellular IBMX does not modify quantitatively protein phosphorylation. These results suggest that the inhibition of membrane-associated c-AMP phosphodiesterase does not regulate the whole c-AMP pool, but probably a very

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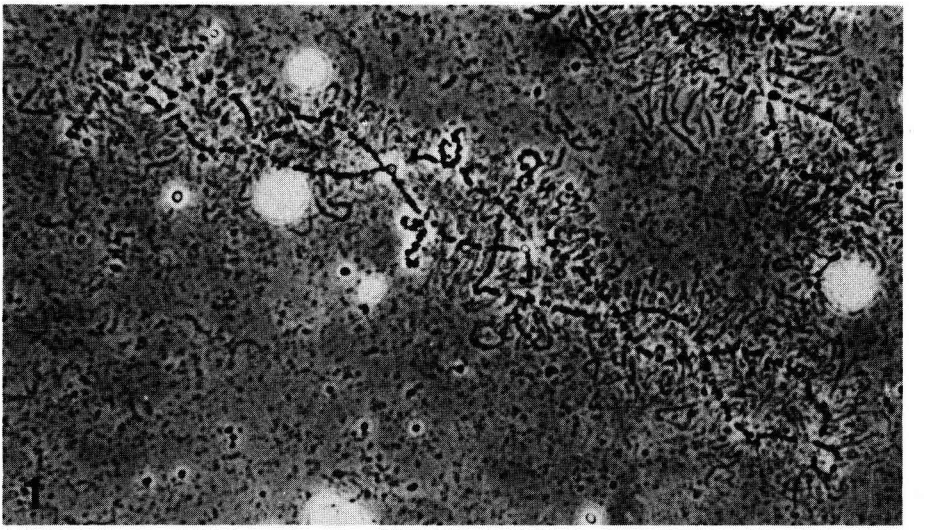
FIG. 6. — *Preparation of lampbrush chromosomes isolated from full-grown* Pleurodeles *oocytes.*

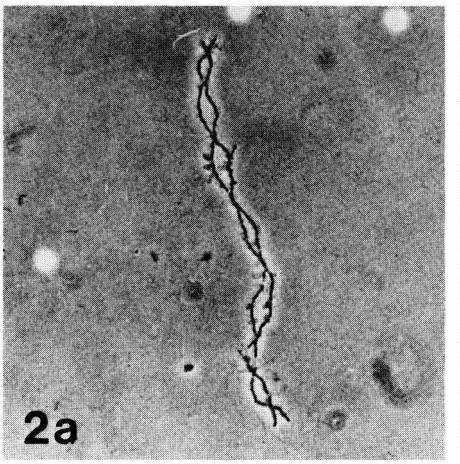
1 Control lampbrush chromosomes.

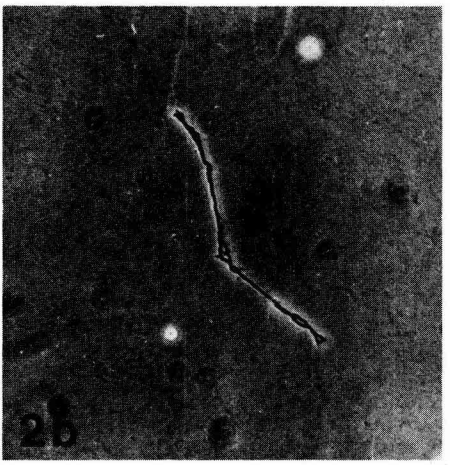
2a Chromosomes isolated 60 min. after (I) injection.

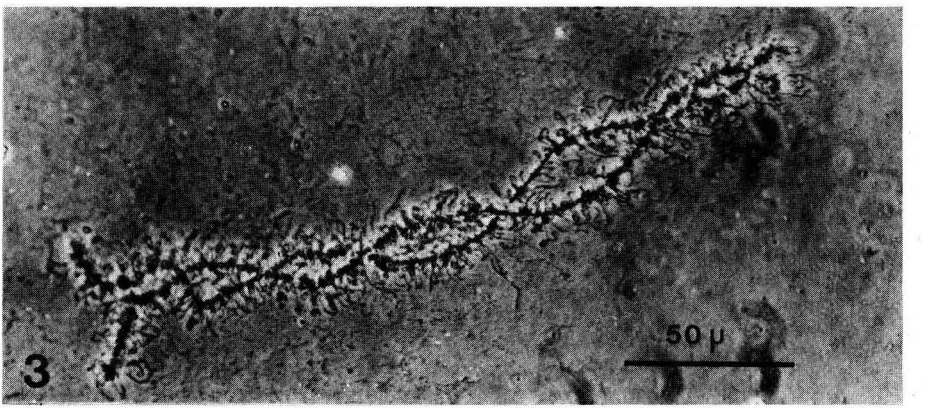
2b Chromosomes isolated 180 min. after (I) injection.

3 Lampbrush chromosomes isolated 24 hrs after (I) injection.





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localized pool in a cell compartment near the membrane. The most probable consequence of a local lowering in c-AMP concentration would result in a modification of phosphorylation-dephosphorylation equilibrium permitting maturation to proceed.

Protein phosphorylations are increased during progesterone-induced maturation (fig. 5). This increase was not observed before GVBD or several hours after progesterone exposure. Early transient changes induced by progesterone in phosphorylated proteins were not detected in our experimental conditions. It is worth while to notice that only proteins in the 2 000 × g supernatant were analysed by SDS polyacrylamide gel electrophoresis. New experiments are needed to investigate this problem more accurately.

As formerly demonstrated by Maller and Krebs (1977), injection of the heat-stable inhibitor (I) into *Xenopus* oocyte triggers germinal vesicle breakdown. Whether (I)-induced maturation may allow normal development remains to be determined. Injection of (I) does not lead to oocyte maturation in all cases especially in *Pleurodeles*. In this species, lampbrush chromosomes are well developed (Lacroix, 1968) and it is known that progesterone stimulates oocyte maturation during the breeding season (Ozon *et al.*, 1975). After progesterone stimulation chromosome regression does not occur before six to ten hours, but precedes germinal vesicle breakdown (12-20 hrs) (M. T. Loones, personnal communication). We have shown that (I) injection into full-grown or small vitellogenic oocytes during the non-breeding season does not induce GVBD but a rapid reversible regression of chromosomes. This important observation indicates that :

— condensation of chromosomes involves the inhibition of subunit (C) of c-AMP-dependent protein kinases ;

— condensation of chromosomes can be induced independently of GVBD, suggesting that the mechanism of action of (I) may involve several distinct c-AMP-dependent protein kinases.

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Résumé. 1. Les méthylxanthines : caféine, théophylline et IBMX inhibent une c-AMP phosphodiestérase membranaire et bloquent la maturation provoquée par la progestétone, de l'ovocyte de Xénope. Cet effet est réversible.

2. Pendant la maturation, induite par la progestérone sur l'ovocyte isolé, on observe une augmentation des phosphorylations des protéines ovocytaires. Cette action n'est observable qu'au moment de la rupture de la membrane nucléaire.

3. L'inhibiteur (I) de la sous-unité catalytique (C) des protéines kinases c-AMP-dépendantes a été injecté dans les ovocytes de plusieurs Amphibiens. Chez *Xenopus* et chez *Discoglossus*, (I) provoque la rupture de la membrane nucléaire dans 30 à 50 p. 100 des cas. Chez *Pleurodeles*, (I) n'a pas provoqué cette rupture mais une condensation réversible des chromosomes en écouvillon.

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