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Original Article

Xenopus oocyte maturation: cytoplasm alkalization is involved in germinal vesicle migration

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ABSTRACT **In *Xenopus laevis* oocytes a transient increase in intracellular pH has been reported to occur during progesterone-induced maturation. Using a cytological approach, we have systematically analyzed germinal vesicle breakdown and meiotic spindle formation in various experimental conditions either preventing or promoting pH_i changes. Injection of a neutral buffer (MOPS pH 6.9) induced a cytosolic acidification of 0.3 pH unit and inhibited by 30% the formation of the maturation white spot after progesterone exposure; in oocytes displaying a white spot, only half showed a spindle, often located far from the plasma membrane. Similar results were observed with a Na-free medium which prevents oocyte alkalization. Injection of an alkaline buffer (Tris pH 9) was able to induce migration of the germinal vesicle in 25% of the oocytes in the absence of progesterone, but failed to induce GVBD. Taken together, these results suggest that the increase in pH_i observed during maturation may be involved in the migration of the germinal vesicle towards the plasma membrane. We also incubated oocytes in the presence of procaine, a weak base often used to artificially alkalize the oocyte cytoplasm. The changes induced by exposure to procaine were different from those resulting from alkaline buffer injection. Indeed procaine promoted GVBD, as well as spindle formation and chromosome condensation. However these events appeared without migration of the germinal vesicle, suggesting that the expected alkalization did not occur.**

KEY WORDS: *Xenopus oocyte, maturation, intracellular pH, procaine*

Introduction

Full grown *Xenopus laevis* oocytes are physiologically arrested in prophase of the first meiotic division. In response to progesterone, such oocytes undergo maturation. The germinal vesicle (GV) first moves towards the animal pole thus creating the classical white spot (WS). This is followed by germinal vesicle breakdown (GVBD), spindle formation and chromosomes condensation. The first meiotic division occurs and is completed by the formation of the first polar body, while the second division is initiated but arrested at metaphase (Hausen and Riebesell, 1991).

One of the changes induced by progesterone stimulation is the activation of the M-phase promoting factor (MPF). MPF has been extensively studied (King *et al.*, 1994). However, MPF activation is a late event in the time course of oocyte maturation. Indeed, it occurs at 0.4 $GVBD_{50}$ (40% of the time required for half the responding oocytes to undergo GVBD; Stith and Maller, 1984). Among early events, a transient increase in intracellular pH (pH_i) has been described (Lee and Steinhardt, 1981). Maturing oocytes alkalize by an average 0.2-0.4 pH unit at 0.2 $GVBD_{50}$, then pH_i decreases close to its original value just before GVBD. This is probably the result of an upregulation of the Na^+/H^+ antiporter in which the $p39^{mos}$ kinase seems to be involved (Rezai *et al.*, 1994).

Several strategies have been used in order to determine the role of this pH_i increase. On the one hand, some authors attempted to keep the cytoplasm from alkalizing during progesterone-induced maturation, but GVBD still occurred (Lee and Steinhardt, 1981; Stith and Maller, 1985). On the other hand, weak bases as trimethylamine (TMA) or procaine were used to artificially increase pH_i. Weak bases with no added progesterone induced GVBD in a time course approximately three times longer than control cells treated with progesterone (Lee and Steinhardt, 1981; Houle and Wasserman, 1983), but chromosomes and spindle did not appear (Houle and Wasserman,

Abbreviations used in this paper: GV, germinal vesicle; GVBD, germinal vesicle breakdown; MPF, maturation promoting factor; TMA, trimethylamine; MOPS, (3-[*N*-morpholino]propanesulfonic acid); WS, white spot.

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1983). Taken together, the results of both strategies suggested that the increase in pH_i is not a prime necessity in maturation.

Recent pH_i measurements have shown that a short initial alkalization is followed by a subsequent acidification during TMA application (Burckhardt and Thelen, 1995). The modulation by pH_i of the cytological events accompanying maturation in *Xenopus laevis* oocytes may thus have been underestimated.

Therefore, we have investigated the effects of weak bases, as well as those of buffer injections, by correlating the presence or absence of the WS with the results of a systematic examination of cytological sections. Our study reveals that a sufficiently alkaline pH_i seems to be particularly required for GV migration towards the oocyte surface.

Results

Effects of neutral buffer injection on progesterone-induced maturation

In order to keep the cytoplasm from alkalizing during maturation, 50 nl of 400 mM MOPS pH 6.9 were injected per oocyte. To check whether such treatment was able to prevent any pH_i variation we used transient NH_4Cl applications which induce fast changes in pH_i (Burckhardt and Frömter, 1992). The remaining pH_i of MOPS-injected oocytes was 7.16±0.08 (n= 9) while those of control oocytes was 7.46±0.10 (n= 10). When MOPS-injected oocytes were superfused with NH_4Cl, pH_i did not change (Fig. 1B).

Since such MOPS-injected oocytes appeared to be strongly buffered, their response to progesterone stimulation was studied (Table 1). The number of oocytes showing a WS was significantly lower in MOPS-injected ones (60.0% of total) than in water-injected controls (97.5%); GVBD was confirmed by cytological examination for these oocytes. Besides, the cytological analysis revealed a dramatic effect of MOPS pH 6.9 injection on spindle formation: spindle and chromosomes were observed in only 50% of the MOPS-injected oocytes having reached GVBD, as compared to 100% in water-injected controls. Moreover 66.6% of these spindles were ectopic in MOPS-injected oocytes (vs 2.5% in water-injected ones): they were oriented either parallel to the plasma membrane or far from the animal pole (Fig. 2A). The spindle position was normal in control oocytes that received water before their exposure to progesterone (Fig. 2B). MOPS- or water-injected oocytes that were not incubated with progesterone were still in the prophase I stage at the end of the experiment (not shown).

Effects of Na-free medium on progesterone-induced maturation

After a control overnight incubation in ND96 medium in the presence of progesterone, 90% of the oocytes (27 out of 30) showed a classical WS. When oocytes were incubated in Na-free medium (which is another way to keep the cytoplasm from alkalizing by inhibiting the Na^+/H^+ antiporter) containing the same amount of progesterone, only 40% of them showed a WS (12 out of 30). The histological analysis revealed the presence of ectopic spindles in 60% of the oocytes that had reached GVBD in Na-free medium, whereas spindles were always located near the plasma membrane in the presence of sodium. So these results were similar to those obtained with MOPS buffer injections.

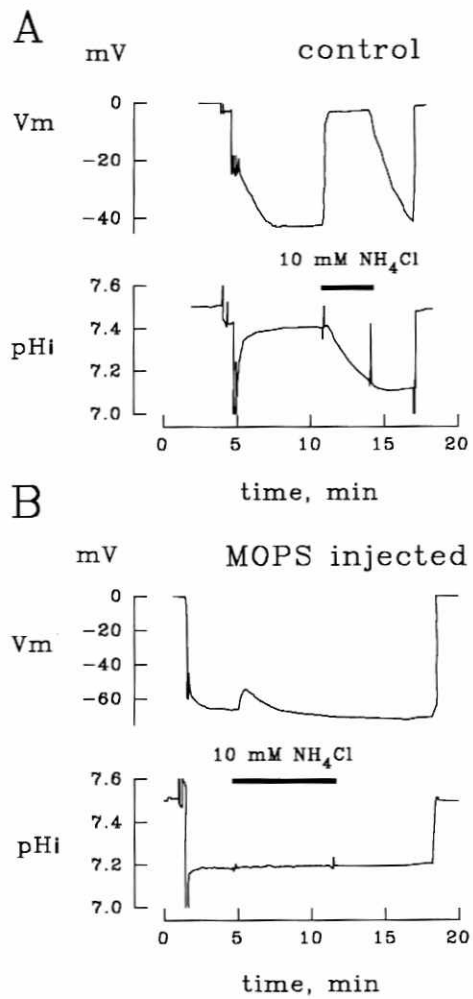

Fig. 1. Changes in membrane potential (Vm) and intracellular pH (pH_i) during application of 10 mM NH_4Cl (pH 7.5; horizontal bars). **(A)** *Control oocyte: addition of ammonium to the external medium induced membrane depolarization (Vm), as well as pH_i decrease from 7.41 to 7.13 within 3 min.* **(B)** *MOPS pH 6.9 injected oocyte (20 mM final): except for a small transient depolarization, no changes in Vm or pH_i occurred after ammonium addition. The initial and final deflections in traces correspond to penetration or withdrawal of the microelectrodes.*

Effects of alkaline buffer injection

In a second set of experiments, we determined the effects of alkaline buffer injection in the absence of progesterone. Several assays were performed with 580 mM Tris, pH 8. None of the injected oocytes showed any sign of maturation. We failed to observe WS or GVBD. Four hours after the injection, there was no significant difference between pH_i values in buffer-injected oocytes (7.51±0.08, n= 3) and control oocytes (7.61±0.01, n= 3).

We therefore used 650 mM Tris pH 9. pH_i values in these buffer-injected oocytes were higher than Tris pH 8 injected ones (8.08±0.14, n= 4). The results from 3 batches of 30 oocytes from 3 animals were not significantly different from one another and were pooled together. Migration of the GV occurred in 25.6% of the oocytes injected with this buffer (23 out of 90). This could be observed 2 to 5 h after the injection, and the number of affected oocytes did not change afterwards. Cytological analysis con-

TABLE 1

EFFECT OF MOPS INJECTION ON THE OOCYTE MATURATION INDUCED BY PROGESTERONE

Injection medium	Total	White spot	GVBD	Spindle formation	Normal spindle	Ectopic spindle
Water	40	39	39	39	38	1
MOPS pH 6.9	40	24*	24*	12*	4*	8*

Pooled numbers of oocytes from two animals, each batch containing 20 oocytes; there was no significant difference between the results of the two experiments. *Shows a significant difference ($p<0.001$, Chi square test or Fisher's exact test) between MOPS-injected and water-injected oocytes.

firmed that, although the GV had really moved, GVBD did not occur (Fig. 3A). Half of the GV migrations observed did not occur towards the animal pole, but obliquely with respect to the "animal pole/vegetal pole" axis.

Effects of procaine

Since procaine is able to alkalize the oocyte (Houle and Wasserman, 1983), we compared its action with that of alkaline buffer injection (Table 2). Oocytes incubated in procaine never showed a WS but their pigmentation always looked like a hair-net after overnight incubation. After 16 h incubation in a medium containing 10 mM procaine at pH 7.5, we failed to observe GVBD on histological sections. However, after 24 h at pH 7.5, 59% of the oocytes showed GVBD. When the incubation was performed at pH 8.5, 44% of GVBD was observed after 16 h, and 91% after 24 h. The percentage of GVBD observed after 24 h incubation is significantly higher at pH 8.5 than at pH 7.5.

At both pH values, GVBD could be followed by spindle formation and chromosomes condensation after 24 h incubation, but never after 16 h. The proportion of oocytes having reached GVBD and where spindle and chromosomes were observed, was 26% at pH 7.5 (14 out of 53) and 28% at pH 8.5 (23 out of 82); these values did not differ significantly. Most of these spindles were ectopic: 86% and 91% of spindles formed at pH 7.5 and pH 8.5 respectively (not significantly different). They were never found in the vicinity of the plasma membrane (Fig. 3B).

Discussion

Up to date, the intracellular alkalization that occurs during *Xenopus laevis* oocyte maturation was not held to be a prime necessity. The results of our study suggest that the increase in pH_i that occurs during progesterone-induced maturation of *Xenopus* oocyte is necessary for the migration of the GV towards the animal pole.

First, when alkalization was prevented by neutral buffer injection, GVBD still occurred following progesterone addition but meiotic spindles were not located close to the plasma membrane. The same result was obtained with Na-free medium that

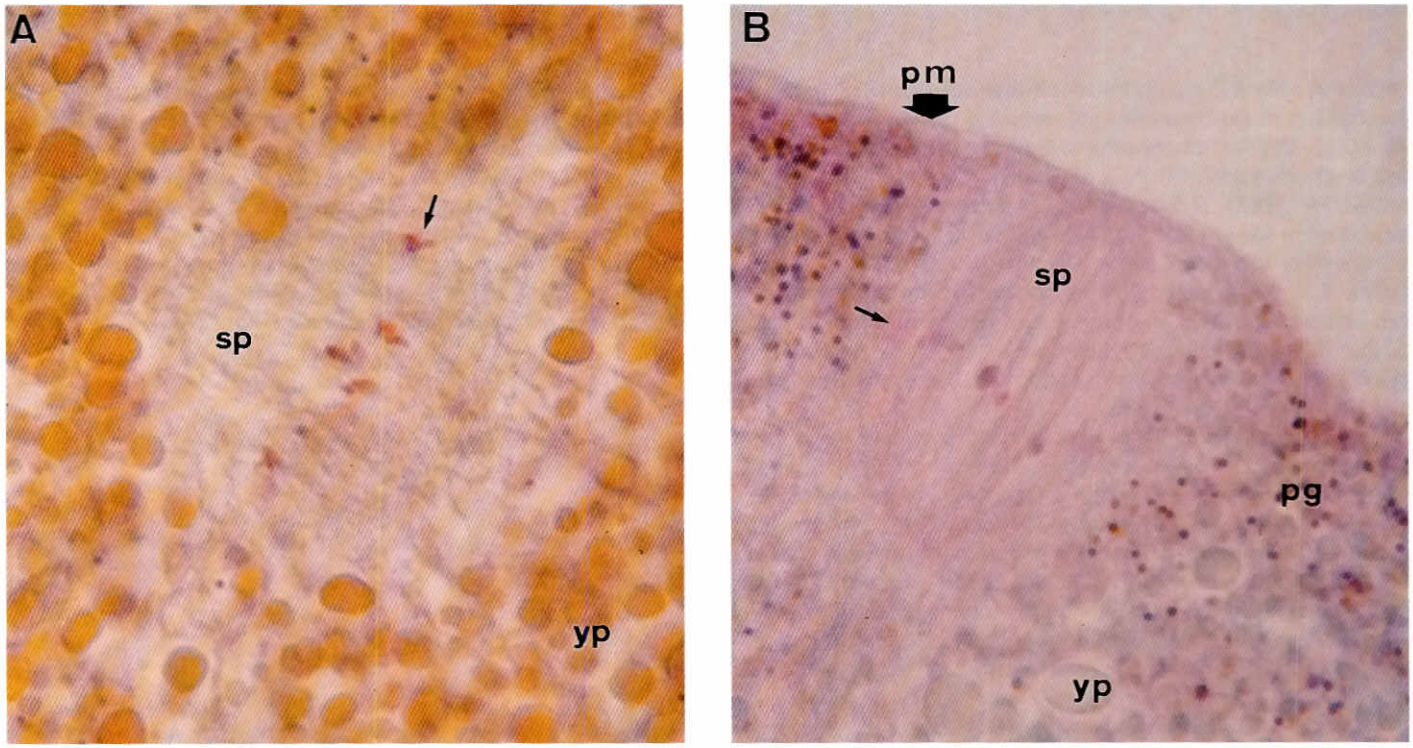

Fig. 2. Injection of MOPS pH 6.9 promotes ectopic spindles in oocytes stimulated by progesterone. (A) *After injection of MOPS pH 6.9, progesterone induced the appearance of WS in only 60% of the oocytes. Observation of cytological sections of such oocytes revealed that, although spindle (sp) and chromosomes (arrow) were present, they were not located near the plasma membrane but encircled by yolk platelets (yp). (x4500).* **(B)** *A control oocyte injected with water. Following progesterone exposure, spindle and chromosomes are located close to the plasma membrane (pm) among the pigment granules (pg). This section was stained only with nuclear red. (x5000).*

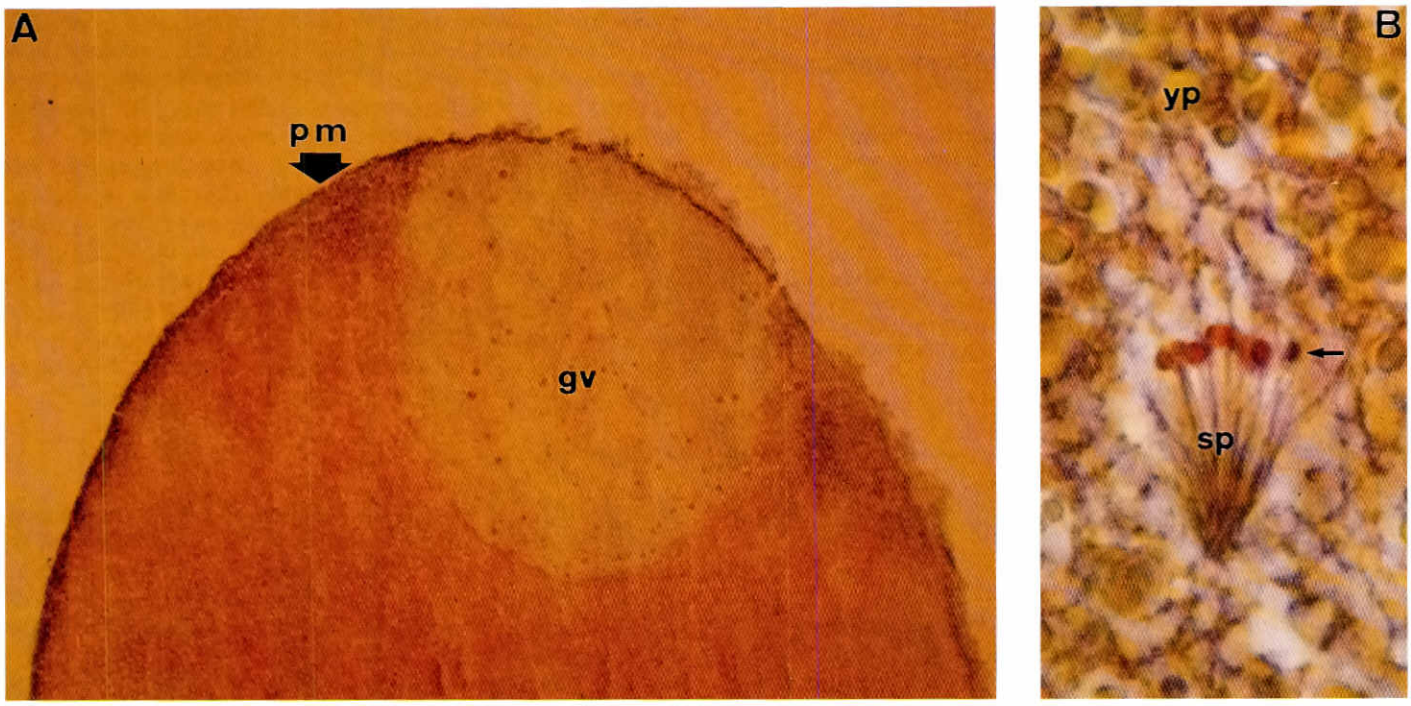

Fig. 3. Comparison of procaine exposure versus Tris pH 9 injection. (A) *When Tris pH 9 was injected in stage VI Xenopus oocytes, the germinal vesicle (gv) appeared in the animal hemisphere within 2 to 4 h after injection. Although the GV had moved towards the animal pole close to the plasma membrane (pm), its breakdown had not occurred. (x400).* **(B)** *A cytological section of an oocyte after a 24 h exposure to 10 mM procaine pH 7.5. This treatment induced not only GVBD, but also chromosomes condensation (arrow) and spindle formation (sp). However, this structure was not located near the plasma membrane but in the center of yolk platelets (yp). (x4500).*

inhibits the Na^+/H^+ antiporter which is the principal H^+ extrusion mechanism in the membrane of *Xenopus* oocyte (Burckhardt *et al.*, 1992). We did not use amiloride because it is well known that this drug inhibits GVBD by a pH independent mechanism (Stith and Maller, 1985). The percentage of ectopic spindles in oocytes incubated in Na-free medium was similar to that in MOPS-injected oocytes; this does not agree with a toxic effect of MOPS buffer. The presence of ectopic spindles following inhibition of pH_i variation in stage VI oocytes has not been reported previously. This is probably because, in most cases, a systematic cytological analysis was not performed (Lee and Steinhardt, 1981; Stith and Maller, 1985).

The second approach we used consisted in alkalizing oocyte cytoplasm by buffer injection. The use of Tris pH 8 was not efficient. This was not the result of a toxic effect of the buffer since such injected oocytes still undergo GVBD following progesterone exposure (not shown). The pH_i measurement revealed that pH_i does not reach 8, suggesting that the buffering power of the cell is high enough to counterbalance the buffer injection. The use of Tris pH 9 induced a real increase in pH_i and enhanced the migration of the GV. The nucleus moved either towards the animal pole or equatorially. This strange displacement may be due to too strong a pH change or to a toxicity of Tris buffer. The time course of the migration following Tris pH 9 injection ranged from 2 to 5 h, and this is consistent with the situation observed following progesterone addition. Indeed, GV migration is finished after approximately 4 h, at which time pH_i begins to decrease, just before GVBD (Lee and Steinhardt, 1981).

As opposed to Tris buffer injection, the incubation of oocytes in procaine-containing medium never induced the appearance of WS. However, on histological sections, we observed that procaine induced GVBD. This result is consistent with those of Houle and Wasserman (1983). However, these authors reported that procaine and some other weak bases failed to induce visible spindles and chromosomes anywhere in the oocyte. In our hands, procaine enhanced not only GVBD but also spindle formation and chromosomes condensation in some cases. These

TABLE 2

EFFECT OF PROCAINE ON THE MATURATION OF STAGE VI *XENOPUS* OOCYTES

10 mM procaine incubation medium	Total	White spot	GVBD	Spindle formation	Normal spindle	Ectopic spindle
pH 7.5, 16 h	90	0	0	0	0	0
pH 7.5, 24 h	90	0	53	14	2	12
pH 8.5, 16 h	90	0	40	0	0	0
pH 8.5, 24 h	90	0	82*	23	2	21

Pooled numbers of oocytes from three animals, each batch containing 30 oocytes; there was no significant difference between the results of the three experiments, except in the case of the 24 h incubation in the pH 7.5 medium, where some variability was observed. Results for GVBD or spindles at 24 h were all significantly different from counts at 16h, either at pH 7.5 or at pH 8.5 ($p<0.001$, Chi square test or Fisher's exact test). When comparing 24 h incubations at pH 7.5 and at pH 8.5, only GVBD counts were significantly different (*, $p<0.001$).

structures appeared within the oocyte far from the plasma membrane, as if GVBD was induced without prior migration. The fact that spindles and chromosomes have never been observed previously might be explained by the lack of a systematic cytological study of oocytes exposed to weak bases.

So the results of procaine exposure are different from those of alkaline buffer injection. This is astonishing since procaine is expected to induce an increase in pH_i too (Houle and Wasserman, 1983). This difference might be explained by an intricate action of procaine: this local anesthetic possesses many other properties in various cell types (Ritchie and Greene, 1990). Another hypothesis is that procaine does not alkalize the cytoplasm of the oocyte as expected up to here. Indeed, recent experiments showed that TMA, previously used as a weak base, induced an intracellular acidification of the *Xenopus* oocyte as long as TMA was present in the perfusion bath (Burckhardt and Thelen, 1995). A similar decrease in pH_i seems to occur following procaine exposure (our unpublished results). This is in agreement with the effects reported here. Other studies are currently being performed to determine the way of procaine action. It might be interesting to determine whether the appearance of spindles and chromosomes following procaine exposure involves MPF activation or a still unknown pathway.

In conclusion, our study shows that the role of the cytoplasm alkalization that occurs during progesterone-induced maturation of *Xenopus* stage VI oocytes is more important than expected previously: it appears necessary for the migration of the GV towards the animal pole. It is tempting to hypothesize that this pH variation stimulates microtubule assembly. Indeed, a fibrillar network was always observed under the GV after its migration. Microtubules are involved in GV migration in *Xenopus* (Huchon *et al.*, 1981; Gard, 1992). In *Spisula solidissima* it has been shown that plus-end microtubule elongation is favored in alkaline pH (Suprenant, 1991). Interestingly, our results agree with those previously obtained with stage IV *Xenopus* oocytes (Wasserman *et al.*, 1984). Indeed, when injected in these oocytes, MPF (cytosol) induced GVBD but the GV did not migrate to the animal pole. The pH_i of these stage IV recipient oocytes increased by 0.3 pH units (from 6.93 to only 7.24) but did not reach the 7.5-7.6 level measured in maturing stage VI oocytes. Therefore, in these stage IV oocytes, the lack of a sufficient pH_i increase does not allow the migration of the GV, which corroborates the findings reported in this paper.

Materials and Methods

Handling of oocytes

Adult *Xenopus laevis* females were purchased from C.R.B.M. (CNSR, Montpellier France). They were not primed with any gonadotropins. After anaesthesia with 1 g/l MS222, ovarian lobes were surgically removed and placed in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes/NaOH, pH 7.5). Full grown stage VI oocytes (Dumont, 1972) were obtained by defolliculation using a 2 h treatment in 2 mg/ml collagenase A. Oocytes were stored at 12°C in ND96 medium.

Experimental conditions

Batches of 20 to 30 oocytes were incubated at 20°C in test or in control media. Test solutions containing 10 mM procaine-HCl were obtained by substituting the corresponding amount of NaCl in ND96 medium (no progesterone was added during procaine incubations). The pH of procaine solutions was either 7.5 or 8.5 since its effect is expected to be greater with higher external pH (Wasserman and Houle, 1984). In the Na-free solution, all NaCl was replaced by choline chloride and pH was adjusted to 7.5 with KOH. Progesterone was used at 10 µM.

Injections of 50 nl per oocyte of either MOPS buffer (400 mM, pH 6.9 KOH), Tris buffer (tris(hydroxymethyl)aminomethane; 580 mM, pH 8; or 650 mM, pH 9 HCl), or distilled water (control), were performed using a pressure apparatus (Inject+Matic, Geneva). MOPS is well tolerated by the oocyte (Lee and Steinhardt, 1981).

Cytological analysis

Oocytes were fixed overnight in Smith's fixative, dehydrated and embedded in paraffin. 7 µm thick sections were stained with nuclear red which reveals nucleus or chromosomes, and with picroindigo carmine which reveals cytoplasmic structures.

pH_i determination

pH_i was measured as previously described (Rodeau and Vilain, 1987). Briefly, pH_i was measured with a H^+-selective liquid exchanger microelectrode (hydrogen ionophore II, cocktail A, Fluka) with respect to the Vm electrode. The pH-sensitive electrodes were calibrated in the experimental chamber before and after each experiment, and had an average slope of -50 mV/pH unit.

Chemicals

MOPS, procaine-HCl, and progesterone were purchased from Sigma. Collagenase A was from Boehringer Mannheim. All other reagents were obtained from Aldrich.

Statistics

The results are expressed as the mean±SD. The significance of the difference between means of groups was tested according to the Student's *t* test. Observed counts were compared using the chi square analysis of contingency tables or the Fisher exact test.

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