Journal of Cellular Biochemistry 98:287–300 (2006)

Intracellular Acidification Delays Hormonal G2/M Transition and Inhibits G2/M Transition Triggered by Thiophosphorylated MAPK in *Xenopus* Oocytes

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Abstract *Xenopus* oocyte maturation is analogous to G2/M transition and characterized by germinal vesicle breakdown (GVBD), spindle formation, activation of MPF and Mos-Xp42^{Mpk1} pathways. It is accompanied prior to GVBD by a transient increase in intracellular pH. We determined that a well known acidifying compound, NH_4Cl, delayed progesterone-induced GVBD in a dose-dependent manner. $GVBD_{50}$ was delayed up to 2.3-fold by 10 mM NH_4Cl. Cyclin B2 phosphorylation, Cdk1 Tyr15 dephosphorylation as well as p39^{Mos} accumulation, Xp42^{Mpk1} and p90^{Rsk} phosphorylation induced by progesterone were also delayed by incubation of oocyte in NH_4Cl. The delay induced by NH_4Cl was prevented by injection of MOPS buffer pH 7.7. In contrast to acidifying medium, alkalyzing treatment such as Tris buffer pH 9 injections, accelerated GVBD, MPF and Xp42^{Mpk1} activation, indicating that pHi changes control early steps of G2/M dynamics. When injected in an immature recipient oocyte, egg cytoplasm triggers GVBD through MPF auto-amplification, independently of protein synthesis. In these conditions, GVBD and Xp42^{Mpk1} activation were delayed by high concentration of NH_4Cl, which never prevented or delayed MPF activation. Strickingly, NH_4Cl strongly inhibited thiophosphorylated active MAPK-induced GVBD and MPF activation. Nevertheless, Tris pH 9 did not have any effects on egg cytoplasm- or active MAPK-induced GVBD. Taken together, our results suggest that dynamic of early events driving Xp42^{Mpk1} and MPF activation induced by progesterone may be negatively or positively regulated by pH_i changes. However Xp42^{Mpk1} pathway was inhibited by acidification alone. Finally, MPF auto-amplification loop was not sensitive to pH_i changes. J. Cell. Biochem. 98: 287–300, 2006.

Key words: intracellular pH; MPF; MAPK; cell cycle control; oocyte; meiosis

Intracellular pH (pH_i) plays crucial functions in many aspect of cellular regulation, including cell growth, growth factor activity, proliferation, cell cycle, oncogenesis, oncogene expression, and malignant transformation [Harguindey et al., 1995; Putney et al., 2002]. A crucial pH_i-regulating mechanism involves the ion exchanger Na^+/H^+ antiport (NHE). Many studies, such as those of Hesketh et al. [1985], have shown that a pH_i increase up to 0.2 pH unit follows cell stimulation by appropriate mitogens. Permissive effects of pH_i elevation and NHE activation on cell proliferation have been clearly established [Pouyssegur et al., 1984; Kapus et al., 1994]. When injected, activated p21Ras induces a sustained alkalization that is necessary for oncogene-induced mitosis [Hagag et al., 1987]. Conversely, proto-oncogenic form of Ha-Ras that does not induce intracellular alkalization presents a weak mitogenic effect [Doppler et al., 1987]. Transfection of fibroblasts with a yeast H^+ pumping membrane ATPase, which induced a

Grant sponsor: French "Ministère de l'Education Nationale"; Grant number: EA 1033.

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Received 4 November 2005; Accepted 8 November 2005
DOI 10.1002/jcb.20764

permanent increase of pH_i, is associated with the acquisition of a transformed phenotype [Perona and Serrano, 1988]. Similarly, NHE activation and subsequent-induced pH_i elevation has been suggested to be necessary for oncogenic transformation and maintenance of the transformed phenotype [Reshkin et al., 2000]. Nevertheless, mechanisms by which pH_i changes are related to cell cycle control and proliferation remains poorly understood.

Progression through cell cycle M-phase is regulated by the G2/M chekpoint and the metaphase-anaphase transition. M-phase entry is promoted by a cytoplasmic factor, the M-phase promoting factor (MPF). The latter has been demonstrated to be universal regulator of entry into mitosis and meiosis: MPF is made up of a catalytic subunit, Cdk1 and a regulatory subunit, the Cyclin B. The activation of this Cyclin-Cdk (cyclin dependant kinase) complex is controlled by phosphorylation or proteolysis of its subunits [for review see Nurse and Hartwell, 1998; Masui, 2001]. Association between regulatory subunit and Cdk1 requires phosphorylation of Cdk1 on residue Thr161 by Cdk activating kinase [Lorca et al., 1992; Solomon, 1993]. Then, to be catalytically active, Cdk1 is dephosphorylated on Thr 14 and Tyr15 by Cdc25 dual specificity phosphatase [Gautier et al., 1988; Kumagai and Dunphy, 1991]. Simultaneously to Cdk1 dephosphorylation, Cyclin B becomes phosphorylated [Gautier and Maller, 1991]. *Xenopus* immature oocytes, which are synchronized at diplotene stage of first meiosis, contain large amount of inactive MPF, or pre-MPF [Nurse and Hartwell, 1998; Masui, 2001]. Pre-MPF is thought to consist of a complex of Cyclin B and Cdk1. Inhibition of pre-MPF is provided by Myt1 that phosphorylates Cdk1 and then inactivates the complex. Pre-MPF can be directly activated by Cdc25 injection [Rime et al., 1994] or by an auto-amplification loop. The auto-amplification loop is triggered by MPF contained in cytoplasm from eggs or metaphase II arrested oocytes [Masui and Markert, 1971]. This loop does not depend upon protein synthesis [Wasserman and Masui, 1975] and involves MPF ability to phosphorylate and activate Cdc25 [Hoffmann et al., 1993; Strausfeld et al., 1994].

Simultaneously to MPF activation, the Erk-like $Xp42^{Mpk1}$ (extracellular regulated kinase) is phosphorylated and activated [Ferrell et al., 1991]. $Xp42^{Mpk1}$ belongs to the mitogen activated protein kinase (MAPK) pathway that is turned on by the synthesis of Mos in response to progesterone stimulation [Sagata et al., 1988]. Once activated, $Xp42^{Mpk1}$ phosphorylates and activates ribosomal S6 kinase ($p90^{Rsk}$), which negatively regulates Myt1 [Palmer et al., 1998]. Activation of Mos-$Xp42^{Mpk1}$ pathway has been shown not to be required for MPF activation but for timely M phase entry to occur when oocytes are stimulated either by progesterone or insulin [Fisher et al., 1999; Dupre et al., 2002; Baert et al., 2003]. Moreover, this pathway has been shown to be responsible for S-phase suppression between MI and MII, and for arrest at metaphase II [Abrieu et al., 2001; Bodart et al., 2002].

Among the early events that precede germinal vesicle break down (GVBD) and spindle morphogenesis [Hausen and Riebesell, 1991], a transient increase in intracellular pH by 0.2–0.4 units has been described in *Xenopus* oocytes [Lee and Steinhardt, 1981; Cicirelli et al., 1983] as well as in urodeles oocytes [Rodeau and Vilain, 1987]. Elevation of pH_i is thought to result from NHE activity, whose up-regulation has been proposed to involve phosphorylation by MAPK family members: Mos [Rezai et al., 1994], Raf [Kang et al., 1998], and c-Jun kinase [Goss et al., 2001]. Observations have carried out controversial data on the function of pH_i changes on the molecular mechanisms regulating cell cycle progression during meiosis. Lowering pH_i by sodium acetate containing medium or by incubation in low extracellular pH (pH_e) media were shown to induce a decrease in the rate of protein synthesis that occurs in response to progesterone [Houle and Wasserman, 1983]. Though there was a good correlation between S6 phosphorylation and alkalization during meiosis resumption, lowering pH_e never prevented S6 phosphorylation [Wasserman and Houle, 1984; Stith and Maller, 1985]. Nevertheless, S6 phosphorylation appeared not to be sufficient to trigger GVBD because S6 phosphorylation levels were not affected in oocytes exhibiting GVBD [Stith and Maller, 1985]. Traditionally, weak bases like trimethylamine (TMA) or procaine, a local anesthetic, have been used in progesterone-free medium to artificially increase pH_i [Houle and Wasserman, 1983]. The interpretations of the results obtained using these compounds to alkalize cytoplasm have to be shaded. Two independent studies have pointed out that the short initial alkalization induced by TMA is promptly followed by a

subsequent acidification [Burckhardt and Thelen, 1995] and that a significant acidification occurred following procaine exposure [Rodeau et al., 1998]. Besides, though procaine triggers a transient activation of MPF in *Xenopus* oocytes [Flament et al., 1997], alkalization using pH9-buffered solutions never triggered GVBD [Flament et al., 1996] suggesting that procaine could promote GVBD and activate MPF independently of pH. Similarly, Houle and Wasserman reported that GVBD could occur following TMA addition in absence of hormonal stimulation [Houle and Wasserman, 1983].

Correlations between pH_i oscillations and MPF activity have been studied during cell cycle of *Xenopus laevis* early embryos [Grandin et al., 1991]. pH_i values were oscillated together with the cycling of MPF activity and suggested that pH_i oscillation could be an integral component of the cell cycle oscillator. So far, no exhaustive studies have been carried out to characterize the relationship between pH homeostasis, MPF and MAPK during oocyte maturation. In order to study, the effects of pH_i changes on cell cycle progression, we analyzed the effects of NH_4Cl, Tris and MOPS buffers on the molecular events driving G2/M transition in *Xenopus laevis* oocytes. The results reported in the present study provide a frame work to explain the role of pHi in meiosis and outline that early mechanisms leading to Mos-$Xp42^{Mpk1}$ and MPF activations can either be accelerated by alkalization or delayed by cytoplasm acidification. In contrast to MPF autoamplification loop, which was not sensitive to pH_i changes, thiophosphorylated active MAPK-induced GVBD was inhibited by pH_i decrease.

MATERIALS AND METHODS

Handling, Treatment, and Micro-Injection of Oocytes

Adult *Xenopus laevis* females were purchased from University of Rennes (France). After anaesthesia with 1 mg/ml MS222 (Sandoz), 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, pH 7.5/NaOH). Full-grown stage VI oocytes [Dumont, 1972] were obtained by manual defolliculation after 40 min incubation in 1 mg/ml collagenase A (Roche Applied Science). Oocytes were kept at 14°C in ND96 medium until use.

Acidification of the oocyte cytoplasm was obtained by incubation in NH_4Cl (Sigma)-containing medium (from 1 to 10 mM), which induces fast decrease in pH_i [Burckhardt and Fromter, 1992; Flament et al., 1996]. Modified ND was used for incubation in NH_4Cl 10 mM to preserve the osmotic balance. pH of external solutions was adjusted to 7.5. In order to buffer pH_i variations, 50 nl of MOPS buffer (morpholino propane sulfonic acid, 400 mM, pH 6.9) were microinjected in immature oocytes using a positive displacement digital micropipette [Nichiryo; Flament et al., 1997]. Distilled water was used as control for microinjection experiments. Injected oocytes were allowed to heal for 1 h in ND96 medium.

Oocyte cytoplasm alkalization was induced by micro-injection of 50 nl of Tris pH 8.5 or 9 into immature oocytes 1 h before hormonal stimulation.

Resumption of meiosis was stimulated by incubating oocytes in ND96 medium containing 10 μM progesterone (Sigma). Progesterone-treated oocytes used as cytoplasm donor for microinjection experiments, were rinsed several times before cytoplasm withdrawn. Fifty nanoliters of cytoplasm were transferred into each immature recipient oocyte.

GVBD was also triggered by recombinant thiophosphorylated active MAPK (Calbiochem, 454855). Twenty nanoliters equal to 1.3 μ specific activity were injected per oocyte.

Maturation Analysis

Time course experiments were performed and maturation was assessed by the occurrence of the white spot (WS), due to displacement of the germinal vesicle toward the apex of the cell. GVBD was confirmed by dissection of heat-fixed oocytes (100°C, 5 min). Batches of oocytes, in which the proportion of immature (absence of WS) versus matured oocytes (presence of WS) was in agreement with the total population, were placed at 4°C every hour, unless otherwise specified. All experiments were performed at 20°C and at least in triplicate.

For cytological analysis, oocytes were fixed overnight in Smith's fixative, dehydrated, and embedded in paraffin. Sections (7 μm thickness) were stained with nuclear red for detecting nuclei and chromosomes whereas picro indigo carmine was used to reveal cytoplasmic structures [Flament et al., 1996].

Electrophoresis and Western Blotting

Frozen oocytes were homogenized in a homogenization buffer and centrifuged for 10 min at 13,000*g* (4°C) to eliminate yolk platelets. After brief centrifugation, one volume of 2× Laemmli sample buffer was added to one volume of supernatant. Proteins were denatured by heating the samples (100°C, 5 min) and then separated by 10% ($p39^{Mos}$) or 17.5% (proteins except $p39^{Mos}$) SDS-polyacrylamide gel electrophoresis (SDS–PAGE). Half oocyte was loaded per well. Separated proteins were subjected to Western blot analysis as previously reported. Cyclin B2 and Cdk1 tyrosine 15 phosphorylation were detected using respectively a polyclonal antibody JG103 [Goodger et al., 1996] or a phospho-tyr 15 Cdk1 (Cell Signaling). Both were used at 1/1,000 in TBS. Protein kinases Mos and $p90^{Rsk}$ were detected using antiserum raised against the carboxy-terminal part of the enzyme, diluted at 1/1,000 in TBS (C-238 and C-21 respectively, Santa Cruz biotechnology, Heidelberg, Germany). $Xp42^{Mpk1}$ was detected using the anti-Erk2 monoclonal antibody D-2 (Santa Cruz biotechnology, Heidelberg, Germany) (1/2,500 in TBS). Signals were detected using ECL chemoluminescence system (Amersham Biosciences).

Statistical Analysis

Values were compared using the chi-square analysis of contingency tables or the Fisher exact test if one or more values were equal to zero. Each experiment was performed at least in triplicate on oocytes obtained from different females.

RESULTS

Progesterone-Induced G2/M Transition Is Delayed by Cytoplasm Acidification Induced by NH_4Cl

When applied in the external medium at a concentration of 10 mM, NH_4Cl triggers an intracellular acidification of 0.3–0.4 pH units within 4 min [Burckhardt and Fromter, 1992; Flament et al., 1996; Rodeau et al., 1998]. To analyze the effects of intracellular acidification on progesterone-induced maturation, oocytes were treated with increasing concentration. In these conditions, we observed that the G2/M transition was delayed in a dose-dependent manner (Fig. 1A, n = 8). G2/M transition was assessed through the appearance of a white spot at the apex of the oocytes and GVBD was confirmed by heat-fixation followed by hemi-dissection of the oocytes. When the oocytes were treated with 1 mM NH_4Cl, $GVBD_{50}$ (time required for 50% of treated oocytes to undergo GVBD) was delayed by 1.7 ± 0.1 fold in six females in comparison to $GVBD_{50}$ of control oocytes treated with progesterone alone. $GVBD_{50}$ was never reached in batches of oocytes from 2 other females (Table I). Similarly, $GVBD_{50}$ was not reached in 50% of 10 mM NH_4Cl-treated oocytes stimulated by progesterone (n = 4 females) while $GVBD_{50}$ was delayed by at least 2.3 fold in the other cases (Fig. 1A). To confirm that delay of GVBD was due to pH_i variation and not to NH_4Cl itself, injections of MOPS buffer at pH 6.9 were also performed. The latter has been previously shown to buffer oocyte cytoplasm and to prevent the pH_i increase that accompanies maturation [Flament et al., 1996]. In such conditions, $GVBD_{50}$ was dramatically delayed ($3.2 \times GVBD_{50}$ of H_2O-injected oocytes, data not shown).

TABLE I. Effects of NH_4Cl on Progesterone-Induced GVBD

Number of female	NH_4Cl 1 mM + progesterone	NH_4Cl 10 mM + progesterone
1	× 1.49	nd
2	× 1.28	× 1.50
3	× 1.79	(++)
4	× 1.59	nd
5	(++)	(++)
6	× 1.93	× 2.24
7	(++)	nd
8	× 2.27	nd

Delays are expressed by comparison of $GVBD_{50}$ of NH_4Cl-treated oocytes to $GVBD_{50}$ of control oocytes solely treated with progesterone; (++), $GVBD_{50}$ was not reached in these oocytes; nd, not determined.

Progesterone-Induced MPF and $Xp42^{Mpk1}$ Activations Are Delayed by NH_4Cl

Due to low level of GVBD in 10 mM NH_4Cl-treated *Xenopus* oocytes, we chose to use a 1 mM concentration to study the effects of NH_4Cl on the molecular events that underlie G2/M transition because in most cases, 100% of the oocytes exhibited GVBD 15 h post-progesterone addition. In immature oocytes, Cyclin B2, $Xp42^{Mpk1}$ and $p90^{Rsk}$ are not phosphorylated. Phosphorylated forms of $Xp42^{Mpk1}$ and $p90^{Rsk}$ were detected 5–9 h post-progesterone addition (Fig. 1B, P). Cyclin B2 phosphorylation was correlated to dephosphorylation of Cdk1 Tyr15. As well,

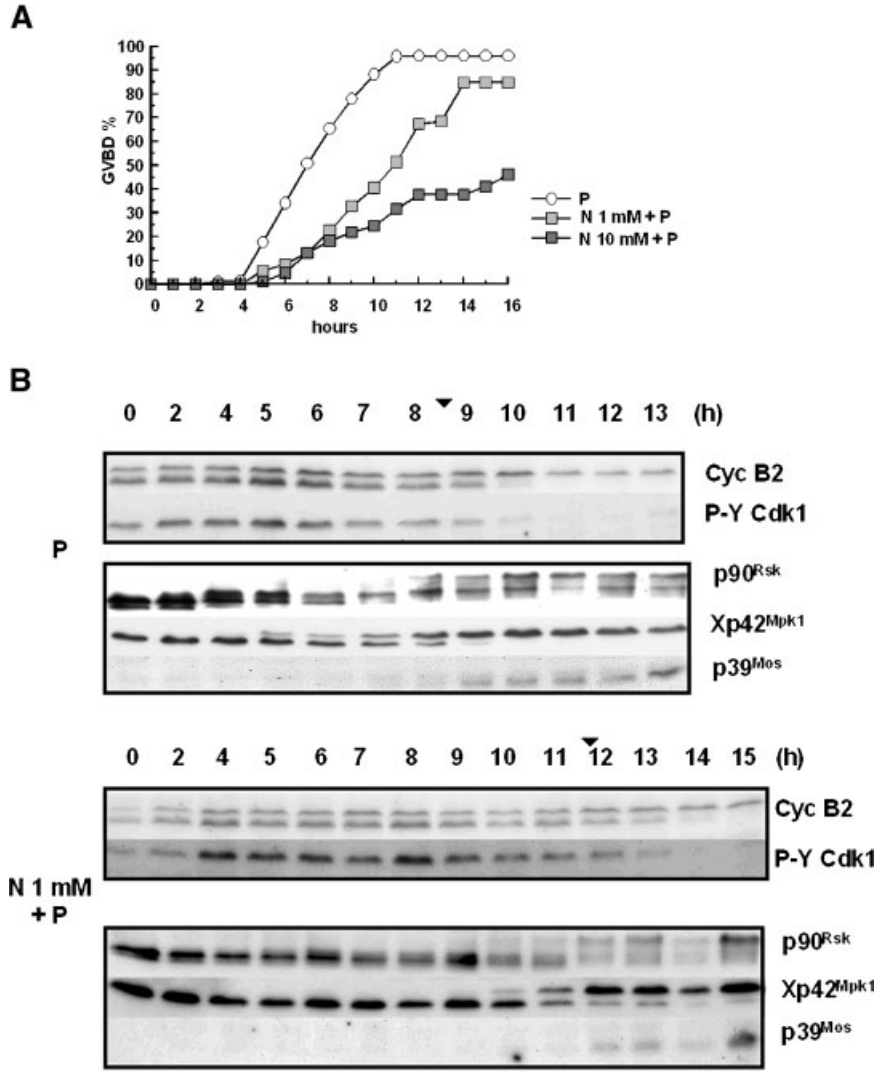

Fig. 1. Dose-dependent effects of NH_4Cl on progesterone-induced G2/M transition. **A**: Immature oocytes were incubated in progesterone without (open circle) or with NH_4Cl (1 mM, gray square; 10 mM, dark gray square). NH_4Cl was added 1 h prior to hormonal stimulation with progesterone. Results expressed are means of eight experiments on eight different females. **B**: Effects of NH_4Cl 1 mM on progesterone-induced MPF and Mos-$Xp42^{Mpk1}$ pathway activation. $GVBD_{50}$ was observed 8 h 20 after progesterone addition in control oocytes whereas it occurred 11 h 50 post-progesterone in 1 mM NH_4Cl (arrows). At the time points indicated, lysates of the same batch of oocytes treated with progesterone (P) or NH_4Cl 1 mM + Progesterone (P + N 1 mM) were subjected to lysates to Western blot analysis using polyclonal antibody against Cyclin B2 (1/1,000), P-Tyr15 Cdk1 (1/1,000), Mos (1/1,000) and $p90^{Rsk}$ (1/1,000) and monoclonal antibody against $Xp42^{Mpk1}$ (1/2,500). This result is representative of three experiments.

phosphorylations of $Xp42^{Mpk1}$ and $p90^{Rsk}$ were correlated to the accumulation of $p39^{Mos}$, which appeared to be complete after 10 h in control oocytes. Typical pattern of phosphorylation, which characterizes mature oocytes, was observed after 10–11 h in progesterone-stimulated oocytes. In the presence of NH_4Cl 1 mM (Fig. 1B, N 1 mM + P), such a pattern of phosphorylation was observed only after 14–15 h. Cyclin B2 phosphorylation and Cdk1 Tyr 15 dephosphorylation were delayed by 3–4 h. $p39^{Mos}$ accumulation was also slowed down and was detected only 14–15 h after the beginning of the treatment. Consequently, $Xp42^{Mpk1}$ and $p90^{Rsk}$ phosphorylations were delayed and appeared complete only after 14 h. Similarly, injection of MOPS (pH 6.9) or incubation in physiological medium at $pH_e = 6$ prior to progesterone stimulation induced a delay of both Mos-$Xp42^{Mpk1}$ and MPF activation (data not shown). Then the delay

in G2/M transition induced by acidification was correlated with delay in the activation of both $Xp42^{Mpk1}$ and MPF pathways.

MOPS pH 7.7 Injection Prevents NH_4Cl-Induced Delay of G2/M Transition

In order to prevent NH_4Cl-induced delay of G2/M transition, MOPS buffers at different pH were used. In our hands, MOPS pH 7.7 was the only one that did not affect the GVBD kinetics of progesterone-stimulated oocytes. Indeed, $GVBD_{50}$ of oocytes injected with 50 nl of MOPS pH 7.7 was not different from those of control (0.91 ± 0.03 vs. 1, Fig. 2A). If $GVBD_{50}$ was delayed by 1.17 ± 0.05 fold ($n = 3$) when oocytes were treated with NH_4Cl 1 mM, this delay was abolished in MOPS pH 7.7 injected oocytes (0.92 ± 0.04 vs. 1.17 ± 0.05, $P > 0.05$, Fig. 2A). In the latter case, $GVBD_{50}$ was not different from those of MOPS pH 7.7-injected oocytes (0.92 ± 0.04 vs. 0.91 ± 0.03, $P < 0.01$, Fig. 2). At the end of the treatment (10 h in this set of experiment), we observed that neither MOPS pH 7.7 nor NH_4Cl prevented the activation of

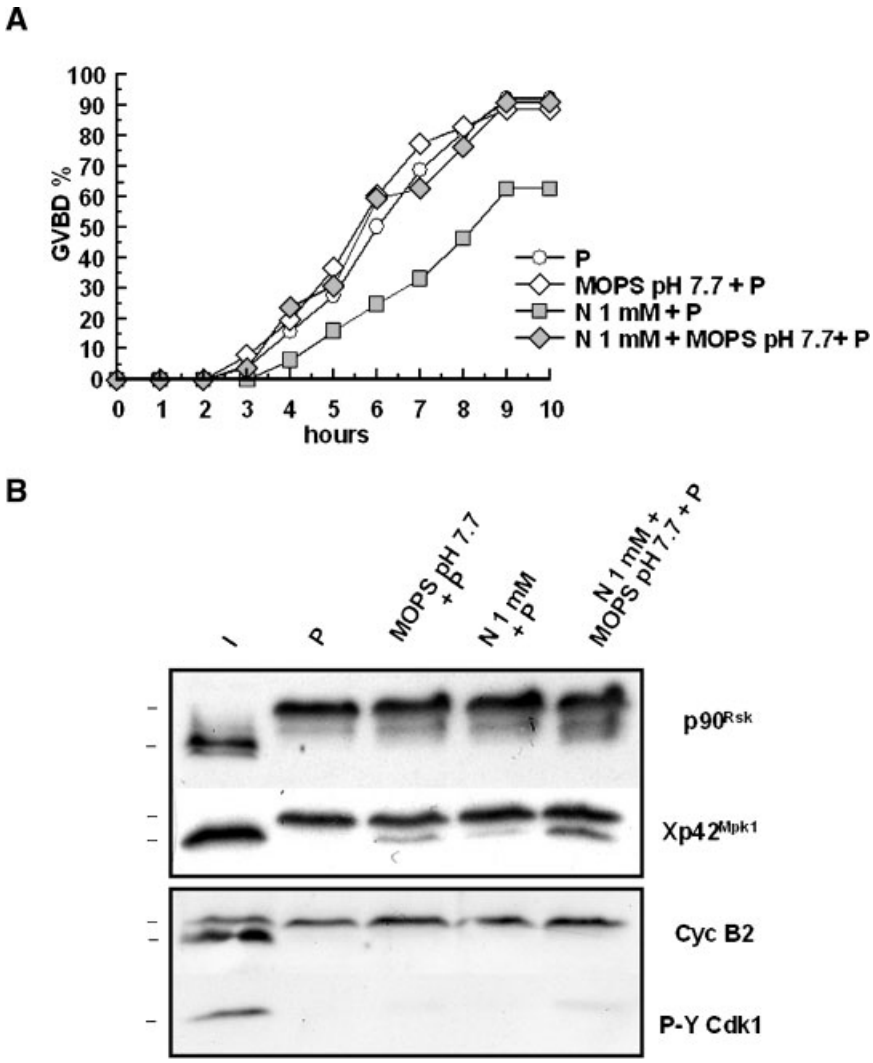

Fig. 2. MOPS pH 7.7 injection prevents NH_4Cl-induced delay of G2/M transition. Oocytes were microinjected with MOPS pH 7.7 prior treatment with NH_4Cl 1 mM (1 h) and then stimulated by progesterone. GVBD kinetics (**A**) shown that GVBD in MOPS pH 7.7 injected oocytes was not delayed by NH_4Cl 1 mM treatment (P + 1 mM + MOPS 7.7). These batches exhibited a significative difference toward NH_4Cl-treated oocytes (P + N 1 mM + MOPS 7.7 vs. P + N 1 mM, $P < 0.01$). **B**: Western blot analyses from oocytes after 10 h of progesterone treatment: phosphorylation of $Xp42^{Mpk1}$, $p90^{Rsk}$ and Cyclin B2 and Cdk1 Tyr 15 dephosphorylation was observed in progesterone-treated oocytes (P), progesterone-treated oocytes injected by MOPS pH 7.7 (P + MOPS 7.7), progesterone-treated oocytes incubated in NH_4Cl (P + N 1 mM) and progesterone-treated oocytes incubated in NH_4Cl and injected by MOPS pH 7.7 (P + N 1 mM + MOPS 7.7). Immature oocyte (I) exhibited phosphorylated Cdk1 whereas $Xp42^{Mpk1}$, $p90^{Rsk}$, and Cyclin B2 remained under their non-phosphorylated forms.

MPF and phosphorylation of $Xp42^{Mpk1}$ and $p90^{Rsk}$ (Fig. 2B).

Tris Buffer pH 9 Injection Accelerates Progesterone-Induced G2/M Transition, MPF, and $Xp42^{Mpk1}$ Activation

Previous works performed in our lab have shown that Tris pH 8-microinjection does not induce significant pHi changes while Tris pH 9-microinjection triggers a pHi increase of 0.4 pH unit [Flament et al., 1996]. We have then tested the effects of Tris buffer pH 8.5 and Tris buffer pH 9-microinjection on the progesterone-induced G2/M transition. Tris pH 8.5-microinjected oocytes exhibited the same kinetic of maturation that those that were either not injected or injected with H_2O. No significant differences between the $GVBD_{50}$ of these batches were observed (Fig. 3A,B, $n = 3$ females). However, $GVBD_{50}$ was observed 1 h earlier in Tris pH 9-microinjected oocytes than in the other conditions (Fig. 3B). Relative time to $GVBD_{50}$ for Tris pH 9-microinjected oocytes was 0.78 ± 0.03 those of control oocytes (Fig. 3A).

Acceleration of GVBD induced by Tris pH 9 injection was correlated to earlier activation of MPF and MAPK (Fig. 3C). In absence of progesterone stimulation, Tris pH 9 injection did not trigger MPF nor $Xp42^{Mpk1}$ activation (without P, Fig. 3C). In control oocytes, dephosphorylation of Cdk1 and phosphorylation of $Xp42^{Mpk1}$ were observed 7–8 h post-progesterone addition. Activation of MPF, as attested by Cdk1 Tyr 15 dephosphorylation and Cyclin B2 phosphorylation was complete after 11 h. In contrast to control oocytes, complete activation of MPF was observed after 7 h in Tris pH 9-injected oocytes (Tris pH 9 + P). Similarly, $Xp42^{Mpk1}$ and $p90^{Rsk}$ were fully phosphorylated after 7 h in Tris pH 9-injected oocytes (Tris pH 9 + P) whereas they were activated after 9–10 h in control (Fig. 3C). $p39^{Mos}$ accumulation was

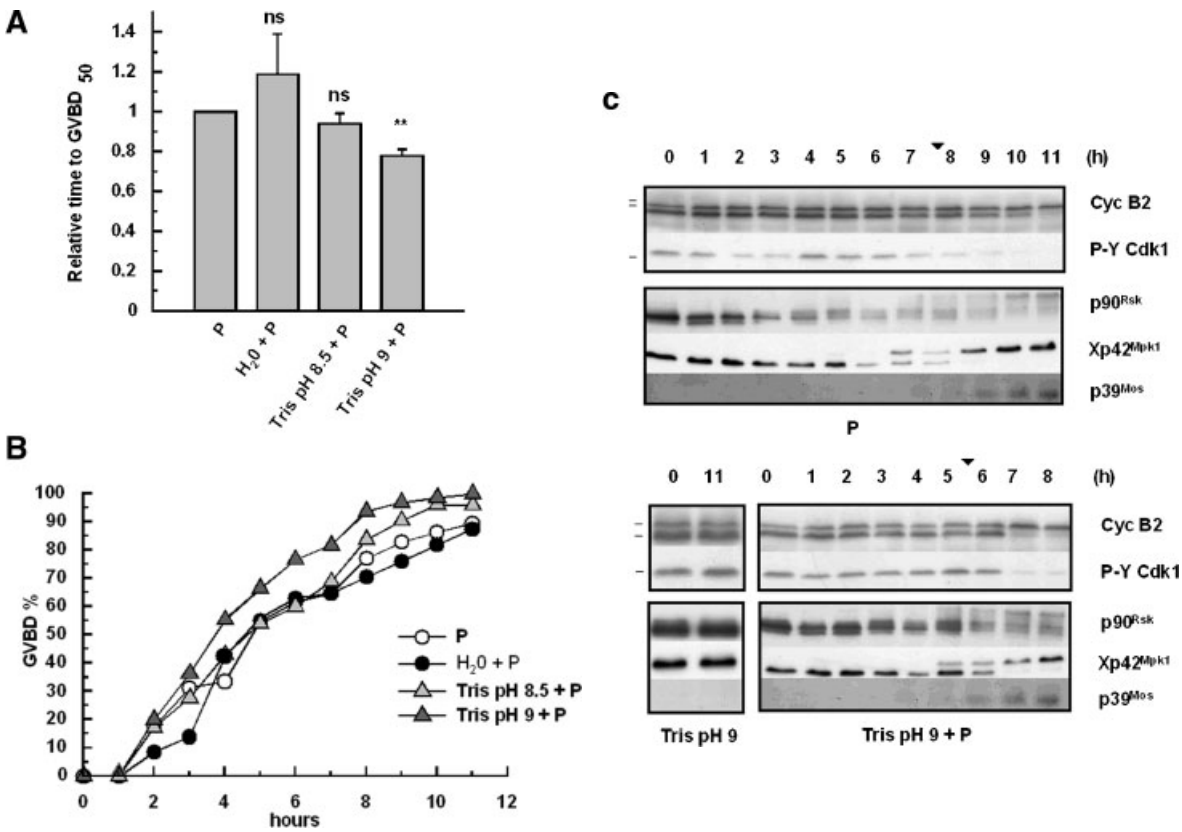

Fig. 3. Effects of Tris pH 9 buffer injection on G2/M transition, MPF and $Xp42^{Mpk1}$ activation. Oocytes were microinjected with Tris pH 9 buffer 1 h before stimulation by progesterone. Distilled water-injected oocytes (H_2O) were used as control. Relative time to $GVBD_{50}$ (**A**) and GVBD kinetics (**B**) revealed no significant diffences between the $GVBD_{50}$ of control oocytes (P), H_2O-injected oocytes ($P + H_2O$) and Tris pH 8.5 (P + Tris pH 8.5) stimulated by progesterone (respectively 1, 1.19 ± 0.20, and 0.94 ± 0.05, $P < 0.05$); ns, no significant difference between control and treated oocytes; **significant difference between control and treated oocytes ($P < 0.05$). G2/M acceleration in Tris pH 9-injected oocytes was assessed by Western blot analysis using Cyclin B2, Cdk1 P-Tyr 15, $Xp42^{Mpk1}$, $p90^{Rsk}$, and p39Mos antibodies (**C**). Arrows represent $GVBD_{50}$.

also accelerated in Tris pH 9 injected oocytes (Tris pH 9 + P).

MPF Auto-Amplification Loop Induced by Egg Cytoplasm Injection Is not Sensitive to pH_i Changes

Because GVBD and activation of MPF induced by egg cytoplasm injection are independent of the $Xp42^{Mpk1}$ pathway [Wasserman and Masui, 1975; Sellier and Bodart, personal observations], we used this cellular context to analyze the effects of an intracellular acidification on GVBD and MPF activation induced by egg cytoplasm injection.

First, GVBD was not delayed by incubation in NH_4Cl 1 mM (Fig. 4A and B, n = 7 females), in contrast to hormonal stimulation. However, oocytes treated by NH_4Cl 10 mM underwent maturation but GVBD kinetic was dramatically delayed: $GVBD_{50}$ was reached more than 1 h later in comparison to control oocytes ($GVBD_{50} = 1.75 \pm 0.19 \times GVBD_{50}$ of control oocytes (three females)). Though GVBD was delayed by high concentration of NH_4Cl (Table II), Western blot analysis revealed that neither the dephosphorylation of Cdk1 Tyr 15 nor the phosphorylation of Cyclin B2 were impaired or delayed by NH_4Cl even at 10 mM (Fig. 4C). In all treatment, activation of MPF was observed 1 h after egg cytoplasm injection, as attested by Cyclin B2 phosphorylation and Cdk1 Tyr 15 dephosphorylation.

Second, Tris injection did not accelerate egg cytoplasm-induced GVBD (Fig. 5A). Tris pH 9-egg cytoplasm co-injected oocytes $GVBD_{50}$ was comparable to control oocytes: $GVBD_{50} = 1.08 \pm 0.02 \times GVBD_{50}$ of control oocytes (H_2O + Cy). MPF activation was not accelerated (Fig. 5B): Cdk1 Tyr 15 dephosphorylation and Cyclin B2 phosphorylation occurred at the same

TABLE II. Effects of NH_4Cl on GVBD Induced by Egg Cytoplasm Injection

Number of female	NH_4Cl 1 mM + Cy	NH_4Cl 10 mM + Cy
1	× 1.00	× 2.34
2	× 0.90	× 2.09
3	× 1.13	× 1.38
4	× 1.55	× 2.43
5	× 1.08	× 1.54
6	× 1.03	× 1.15
7	× 1.10	× 1.32

Delays are expressed by comparison of $GVBD_{50}$ of NH_4Cl-treated oocytes to $GVBD_{50}$ of control oocytes injected with egg cytoplasm

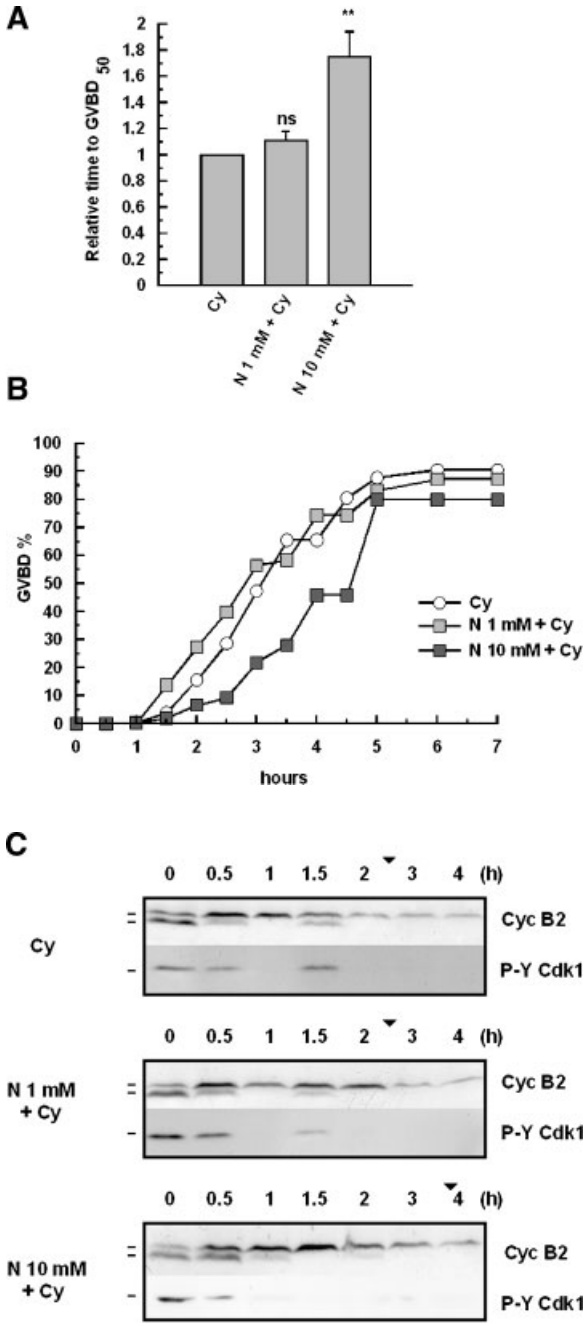

Fig. 4. Effects of NH_4Cl on egg cytoplasm-induced GVBD and MPF activation. Relative time to $GVBD_{50}$ (**A**) and GVBD kinetics (**B**). NH_4Cl was added 1 h prior to injection of metaphase-II arrested eggs (50 nl by oocytes); ns, no significant difference between control and treated oocytes; **significant difference between control and treated oocytes ($P < 0.05$). **C**: NH_4Cl was added 1 h prior to injection of metaphase-II arrested eggs (50 nl by oocytes). Oocytes were taken every ½ h during 2 h after egg cytoplasm injection. MPF activation was analysed using Cyclin B2 (1/1,000) and Cdk1 P-Tyr 15.

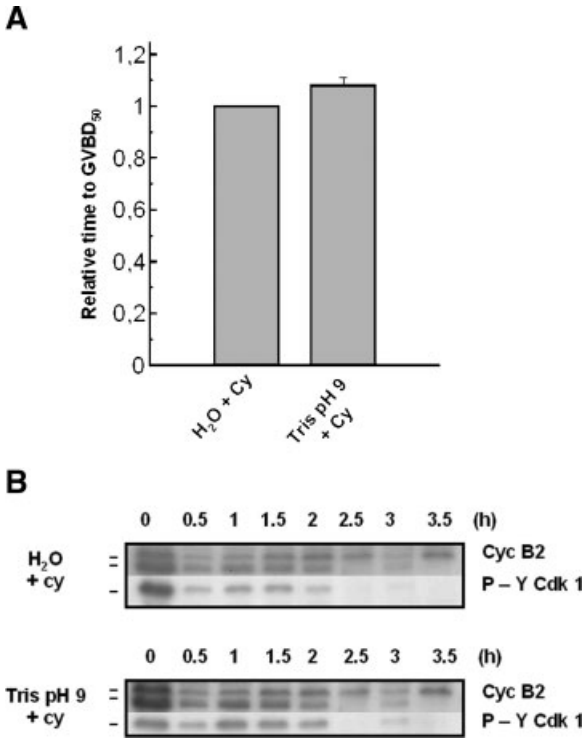

Fig. 5. Effects of Tris pH 9 injection on egg cytoplasm-induced GVBD and MPF activation. **A**: Relative time to $GVBD_{50}$, (**B**) MPF activation kinetic was detected using Cyclin B2 and Cdk1 P-Tyr 15 antibodies.

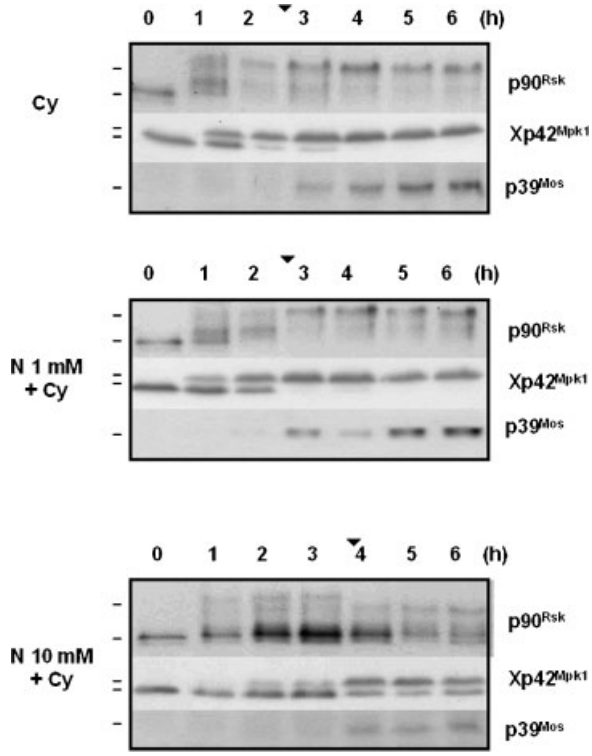

Fig. 6. Effects of NH_4Cl on egg cytoplasm-induced $Xp42^{Mpk1}$ phosphorylation and $p39^{Mos}$ accumulation. Oocytes were treated with NH_4Cl 1 h prior to injection of metaphase-II arrested eggs (50 nl by oocytes) and they were taken every ½ h for 2 h after egg cytoplasm injection. Activation of the MAPK pathway was analyzed through p39Mos accumulation, $Xp42^{Mpk1}$ and $p90^{Rsk}$ phosphorylation.

time in control oocyte ($H_2O + Cy$) and Tris-injected oocytes (Tris pH 9 + Cy).

MPF-Induced $Xp42^{Mpk1}$ Activation Is Delayed by Cytoplasm Acidification

Egg cytoplasm injection also induced $p39^{Mos}$ accumulation and subsequent $Xp42^{Mpk1}$ and $p90^{Rsk}$ phosphorylation in absence of hormonal stimulation (Fig. 6). We observed in control oocytes and in NH_4Cl 1 mM-treated oocytes that $p39^{Mos}$ accumulation, $Xp42^{Mpk1}$ and $p90^{Rsk}$ phosphorylation occured at the same time, 3 h and 2–3 h post-injection respectively, while $p39^{Mos}$ accumulation was delayed in NH_4Cl 10 mM-treated oocytes. In these oocytes, $Xp42^{Mpk1}$ and $p90^{Rsk}$ were not fully activated: both active and inactive isoforms were detected. Thus, at a concentration of 1 mM, NH_4Cl did not prevent MPF and $Xp42^{Mpk1}$ activation whereas a 10 mM concentration delayed $Xp42^{Mpk1}$ activation without impairing MPF activation (Fig. 6).

Thiophosphorylated MAPK-Induced GVBD Is Inhibited by Cytoplasm Acidification

When injected into immature oocytes, thiophosphorylated active MAPK triggers GVBD (Fig. 7A, n = 3) whereas control oocytes injected with H_2O or boiled inactivated MAPK (data not shown) did not exhibited any GVBD. As reported earlier by Haccard et al. [1995], we observed that protein synthesis was required for thiophosphorylated active MAPK to induce GVBD (Fig. 7A) and MPF activation: Cdk1 remained phosphorylated on Tyr 15 and Cyclin B2 did not exhibited any phosphorylation when oocytes were incubated in cycloheximide (CHX, 10 μg/ml) prior to MAPK injection. Tris pH 9 injection had no effects on thiophosphorylated active MAPK-induced GVBD. Neither GVBD kinetic nor GVBD rates were enhanced by cytoplasm alkalization (Fig. 7A). Though Tris pH 9 injection did not accelerated thiophosphorylated active MAPK-induced GBVD, incubation in increasing concentration of NH_4Cl had dramatic effects on the GVBD rate; control oocytes exhibited $87.3 \pm 6.7\%$ GVBD whereas oocytes incubated in NH_4Cl 1 and 10 mM exhibited respectively 35.0 ± 17.3 and $0 \pm 0\%$ GVBD (Fig. 7A). We failed to detect Cyclin B and

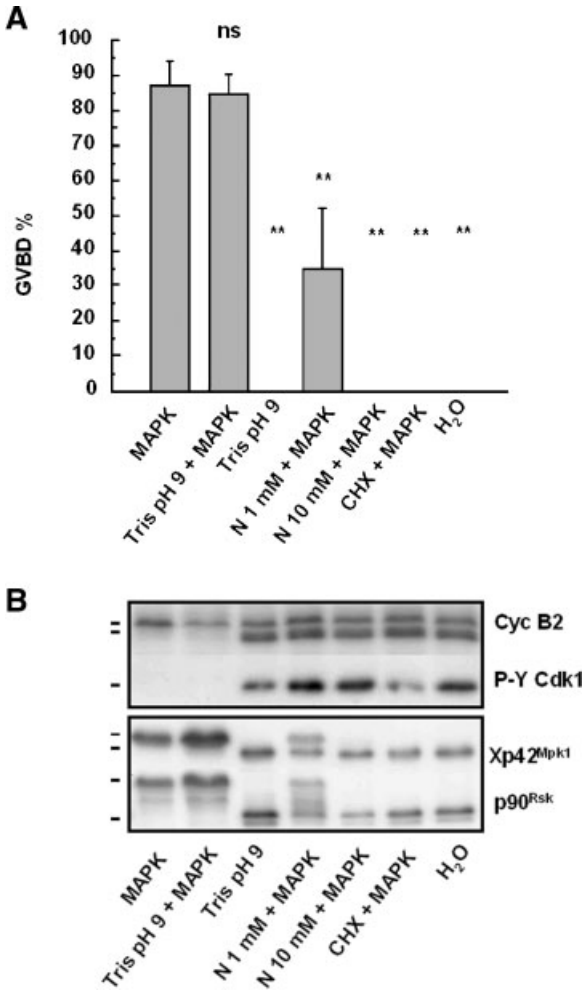

Fig. 7. Acidification inhibits thiophosphorylated MAPK-induced GVBD. Recipient immature oocytes were injected with thiophosphorylated active MAPK (1.3 μ/oocyte) to induce GVBD. **A**: Percentages of maturation 33 h post MAPK injection. Oocytes were treated with NH_4Cl 1 mM (N 1 mM) or 10 mM (N 10 mM) or microinjected with Tris pH 9 or incubated with CHX (10 μg/ml) 1 h prior to injection with active MAPK. Control oocytes were injected with Tris pH 9 or water. MPF and $Xp42^{Mpk1}$ activations were analyzed by Western blot at the end of the experiment (**B**).

Cdk1 under their active forms in both 1 mM and 10 mM NH_4Cl incubated oocytes (Fig. 7B). The decrease of GVBD induced by thiophosphorylated active MAPK-injected was also correlated with partial activation of endogenous $Xp42^{Mpk1}$ and $p90^{Rsk}$ in 1 mM NH_4Cl and lack of activation of endogenous $Xp42^{Mpk1}$ and $p90^{Rsk}$ in 10 mM NH_4Cl (Fig. 7B).

DISCUSSION

Intracellular pH (pH_i) regulates many key functions in cellular life. The transient alkalization that occurs during meiosis resumption (G2/M transition) has been well described in *Xenopus laevis* oocytes. However, its role during G2/M transition, as well as the identity of its molecular targets, remains poorly elucidated.

Conclusions drawn from experiments perfomed with compounds such as trimethylamine and procaine may be questioned and shaded because such compounds do not alkalize but rather acidify cytoplasm [Burckhardt and Thelen, 1995; Rodeau et al., 1998]. Procaine induces MPF activation after longtime exposure in absence of hormonal stimulation [Flament et al., 1997] and trimethylamine has also been reported to induce spontaneous GVBD [Houle and Wasserman, 1983], suggesting that increase of protein synthesis observed with these compounds might be due to the weak bases themselves and not to their pHi-related effects. In the present study, we focused on another compound, NH_4Cl. Due to the unusual high permeability of *Xenopus* oocyte membrane to NH_4^+ ions [Burckhardt and Fromter, 1992], NH_4^+ dissociation into NH_3 and H^+ in cytosol results in pHi decrease. We observed that incubation in increasing concentration of NH_4Cl delays in a dose-dependent manner G2/M transition induced by hormonal stimulation in prophase-arrested oocytes. Delays of entry into meiosis induced by NH_4Cl did not appear to be a specific effect of ammonium chloride. Indeed, other compounds that are able to inhibit pHi changes such as MOPS buffer pH 6.9 injection [Flament et al., 1996] or incubation in pH 6.0 buffered medium, that acidifies, delayed *Xenopus* oocytes G2/M transition (data not shown). Because MOPS pH 7.7 prevented the effects of NH_4Cl, one shall suggest that the GVBD delay observed is due to pHi acidification and not to unrelated effects of NH_4Cl. These results are in agreement with Houle and Wasserman observation [Houle and Wasserman, 1983]: prior to GVBD, an acidifying medium such as sodium acetate, slows down the kinetic of oocytes maturation induced by progesterone. In contrast to NH_4Cl or MOPS pH 6.9 injections, we notices that injections of Tris pH 9, which alkalizes cytoplasm [Flament et al., 1996], accelerated progesterone-induced GVBD but never triggered GVBD on their own.

Following progesterone stimulation, if GVBD occurs in absence of intracellular alkalization in NH_4Cl-treated or in MOPS-injected oocytes, alkalization facilitates both MPF and $p39^{Mos}$

to $Xp42^{Mpk1}$ pathway activation induced by progesterone. 1 mM NH_4Cl, as well as 10 mM NH_4Cl [Sellier and Bodart, personal observations], delayed MPF activation (Cdk1 Tyr dephosphorylation and Cyclin B2 phosphorylation), $p39^{Mos}$ accumulation and the subsequent phosphorylation of $Xp42^{Mpk1}$ and $p90^{Rsk}$. Both activation of $p39^{Mos}$ to $Xp42^{Mpk1}$ pathway and MPF, which are delayed by NH_4Cl and accelerated by Tris pH 9 injections, occurred at the same time relatively to $GVBD_{50}$, suggesting that both pathways are sensitive to pH_i changes. Because these pathways are interconnected, several hypotheses might explain these simultaneous delay or acceleration. There have been considerable discussions about the role of this alkalization in the control of protein synthesis during meiosis [Lee and Steinhardt, 1981; Cicirelli et al., 1983; Stith and Maller, 1985]. By affecting global protein synthesis, pHi variations might modulate timely-progesterone-induced MPF and $Xp42^{Mpk1}$ activations because both factors are dependent upon protein synthesis for their activation. On one hand, $p39^{Mos}$ accumulation has been proposed to be regulated through MPF-dependent mechanisms [Castro et al., 2001; Frank-Vaillant et al., 2001]. Then, delay in MPF activation, by impairing Cdc25 activation or the autoamplification loop of MPF [Gautier and Maller, 1991; Hoffmann et al., 1993; Strausfeld et al., 1994], will result in a delayed $p39^{Mos}$ accumulation. On another hand, $p39^{Mos}$ synthesis and $Xp42^{Mpk1}$ activation have been shown to be required for timely GVBD to occur when oocytes are stimulated by progesterone [Gross et al., 2000; Dupre et al., 2002] or by insuline [Baert et al., 2003]. If absence of $p39^{Mos}$ accumulation or MAPK activation delay M phase entry, injection of members of the $p42^{Mpk1}$ cascade accelerate hormonal-induced GVBD [Baert et al., 2003; Bodart et al., 2005] then acceleration or delay in $p39^{M\circ s}$ accumulation may respectively accelerate or delay activation of MPF induced by hormonal stimulation.

Egg cytoplasm contains sufficient amount of active MPF to trigger the activation of inactive pre-MPF, which is stored in immature recipient oocytes [Masui and Markert, 1971; Gautier and Maller, 1991]. The activation of this auto-amplification loop is achieved through the activation of Cdc25 and is independent upon protein synthesis [Wasserman and Masui, 1975; Hoffmann et al., 1993; Rime et al., 1994; Strausfeld et al., 1994]. Though it has been pointed out that egg cytoplasm triggers GVBD in absence of protein synthesis, for example, $p39^{Mos}$, GVBD is noticeably delayed in these conditions [Wasserman and Masui, 1975; Sellier and Bodart, personal observation]. These conditions offer possibilities to explore directly the effects of pH_i variation on MPF activity and on the auto-amplification activation loop induced by egg cytoplasm injection. NH_4Cl (1–10 mM) never delayed Cyclin B2 phosphorylation or Cdk1 Tyr 15 phosphorylation induced by egg-cytoplasm injection leading to the conclusion that MPF auto-amplification loop was not affected by acidification. Similarly, Tris injection had no effect on MPF activation or GVBD kinetic when induced by egg cytoplasm injection. Taken together, these observations lead us to propose that neither Cdc25 nor Plx1 activities, involved in the autocatalytic loop of MPF [Rime et al., 1994; Strausfeld et al., 1994; Karaiskou et al., 2004], are impaired by acidification.

Best candidate for proton-sensitive mechanisms is the $p39^{Mos}$ to $Xp42^{Mpk1}$ pathway. NH_4Cl at 10 mM concentration but not 1 mM delayed $p39^{Mos}$ synthesis and subsequent $Xp42^{Mpk1}$ and $p90^{Rsk}$ phosphorylations induced by MPF in egg cytoplasm injected oocytes. The GVBD delay observed at this concentration is similar to the delay induced by the lack of $p39^{Mos}$ accumulation in p39Mos antisense-egg cytoplasm co-injected oocytes (data not shown). Moreover, delay observed in NH_4Cl-treated oocytes following hormonal stimulation is in a range close to the one observed in absence of $p39^{Mos}$ synthesis, when GVBD and MPF activation are induced by progesterone [Gross et al., 2000; Dupre et al., 2002] or insulin [Baert et al., 2003]. Acidification might impair the positive feed back between $Xp42^{Mpk1}$ and its upstream activator $p39^{Mos}$, through $p39^{Mos}$ Ser 3 phosphorylation [Matten et al., 1996] or mRNA recruitment by polyadenylation [Howard et al., 1999].

Erk proteins have been shown in other models to be potentially regulated by intracellular pH. Evidence have been accumulated that antagonist of NHE-1 could partially inhibit stretch-induced Erk activation in cardiomyocytes [Takewaki et al., 1995]. Similarly, Erk can be regulated by NHE in RASM cells [Mukhin et al., 2004]. In *Xenopus laevis* oocytes, thiophosphorylated active MAPK triggers M-phase entry in a protein synthesis dependent manner

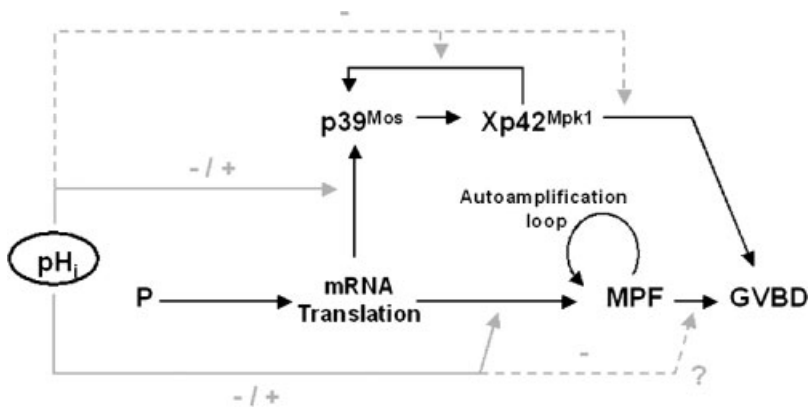

Fig. 8. Working model for the influence of pHi on signaling pathways of meiosis. Precocious steps leading to MPF and MAPK activation stimulated by progesterone are sensitive to pHi changes: their activation is delayed by acidification or accelerated by alkalization (gray). GDVB induced by active MAPK is inhibited by acidification but not affected by alkalization (gray, broken line). MPF auto-amplification loop is not affected by pHi variation but MPF induced GVBD is delayed by high concentration of NH_4Cl (10 mM). Nevertheless, this delay might account for the delay in p39Mos accumulation.

[Haccard et al., 1995]. In our hands, GVBD and MPF activation, induced by thiosphosphorylated active MAPK, were not impaired by alkalization (Tris pH 9 injections) but were dramatically inhibited by pHi decrease induced by NH_4Cl. MAPK-induced GVBD might be inhibited at two different levels: (1) Though no study has been carried out to determine the influence of pHi on thiophosphorylated active MAPK, the latter might be sensitive to proton, like reported for MAPK; indeed, protonation of functional groups of the activation segment of MAPK prevent in vitro the activation of the enzyme by promoting a stable closed conformation at pH 5.5 [Tokmakov et al., 2000]. (2) Protein synthesis is required for thiosphosphorylated active MAPK to induce the activation of the endogenous pool of $Xp42^{Mpk1}$ and the M-phase entry. In one hand, acidification could prevent the establishment of a positive feed back loop between $Xp42^{Mpk1}$ and its upstream activator $p39^{Mos}$. In another hand, acidification could inhibit the synthesis of another protein, which remain to be determined and would be necessary for thiosphosphorylated active MAPK to trigger GVBD.

These observations provide a new framework to decipher the influence of pHi changes on signaling pathways in amphibian meiosis. Because MPF autocatalytic loop is not delayed and because there is no delay between $p39^{Mos}$ accumulation and $Xp42^{Mpk1}$ phosphorylation, it can be hypothesized that early mechanisms leading to $p39^{Mos}$ synthesis and MPF activation are proton-sensitive: they can be either accelerated by alkalization or delayed by acidification (Fig. 8). Moreover, our results provide in vivo evidence that MAPK pathway activity is severely inhibited by acidification and does not promote M-phase entry in these conditions. Since these signaling pathways are highly conserved in eukaryotic cells, this model might be extended to other species or cell type.

ACKNOWLEDGMENTS

We thank Arlette Lescuyer-Rousseau for technical assistance. We also thank Drs Duesbery, Antoine-Paul, and Standart for helpful suggestions and comments, as well as Mrs Dehennaut.

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