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The distinct stage-specific effects of 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid on the activation of MAP kinase and Cdc2 kinase in *Xenopus* oocyte maturation

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Abstract

2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (PACA), pharmacological inhibitor of phospholipase A_2 (PLA_2), inhibits epinephrine-stimulated thromboxane production in human platelets. In this study, we investigated the effect of PACA on meiotic maturation individually in stages V and VI oocytes. PACA prevented the maturation in stage V but merely delayed the process in stage VI oocytes. This was associated with the strong inhibition of Mos synthesis at both stages. Besides, PACA-induced inhibition of MAPK activation was evident in stage V but not in stage VI oocytes. PACA also inhibited the activation of Cdc2 kinase (Cdc2) in stage V but merely delayed the process in stage VI oocytes. Furthermore, 5 μM and higher concentrations of PACA completely inhibited the activation of MAPK and Cdc2 only in stage V, not in stage VI, oocytes. Moreover, we propose PACA as a new tool for the study of *Xenopus* oocyte maturation, which can also play a unique role for the studies of the stage-specific activation of MAPK and Cdc2.

Keywords: 2-(*p*-Amylcinnamoyl)amino-4-chlorobenzoic acid; MAP kinase; Cdc2 kinase; *Xenopus*; Oocyte; Meiotic maturation; GVBD

1. Introduction

In the ovaries of *Xenopus laevis* oocytes are naturally arrested in prophase of the first meiotic division (prophase I) at the G_2 prophase boundary. During this stage oocytes synthesize and store several mRNAs, such as those for Mos, cyclins, etc., that will be used later in development. Specific signals, often hormones, break this arrest and oocytes complete the first meiotic division and then become arrested again in metaphase of the second meiotic cell division (metaphase II), a process known as meiotic maturation. Progesterone, the natural mitogen of *Xenopus* oocytes, triggers various signal transduction pathways in the oocytes which lead to the post-transcriptional activation of maturation or M-phase promoting factor (MPF), also known as the Cdc2-cyclin B complex. In *Xenopus* prophase I-arrested oocytes, MPF is maintained in an inactive form by the phosphorylation of Cdc2 kinase on Thr 14 and Tyr 15. In response to

Abbreviations: MAPK, mitogen-activated protein kinase; Cdc, cell division cycle; MPF, maturation or M-phase promoting factor; MEK, MAPK kinase; PACA, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid; GVBD, germinal vesicle breakdown; PLA_2, phospholipase A_2; MBS, modified barth solution.

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progesterone, Cdc2 kinase is activated in a protein synthesis-dependent manner (with a drop of cAMP leading to the inhibition of PKA) by the cdc25 phosphatase, which removes the inhibitory phosphates of Cdc2 kinase. MPF activity falls after metaphase I, then rises again and remains high during metaphase II arrest until fertilization [1]. It is still a major challenge to identify the proteins and their functions that are critical at the beginning steps of meiotic maturation.

Unlike mammalian cells, which express both p42 and p44 MAPKs, *Xenopus* oocytes express only p42 [2,3]. MAPKs are unique in that they are active only when tyrosine and threonine/serine residues are phosphorylated [4]. Progesterone-stimulated MAPK activation is biphasic in *Xenopus* oocytes [5]. Inappropriate activation of $p42^{MAPK}$ in *Xenopus* egg extracts prior to entry into M-phase induces arrest of the cell cycle in G2 [6–8]. On the other hand, reports from different laboratories have shown that $p42^{MAPK}$ is not essential for entry into meiosis I [9,10]. Mos-MEK-MAPK is thought to constitute a positive-feedback loop [11,12]. Mos accumulates at the time of germinal vesicle breakdown (GVBD), leading indirectly to $p42^{MAPK}$ activation, and ultimately to phosphorylation of RSK (Ribosomal S6 kinase) [13]. Polyadenylation of Mos mRNA is dependent on MAPK [12]. Thus, MAPK activation and Mos synthesis are considered interdependent. Overexpression of either Mos, constitutively active MEK $p42^{MAPK}$ or RSK in *Xenopus* prophase oocyte induces Cdc2 kinase activation and maturation in the absence of progesterone [14–16]. Inhibition of Mos synthesis by antisense oligodeoxyribonucleotide has been shown to prevent progesterone-induced Cdc2 kinase activation [17] indicating that Mos is both necessary and sufficient for Cdc2 kinase activation. It has also been reported that Mos is unable to induce Cdc2 kinase activation in the absence of MAPK [10]. $p42^{MAPK}$ activation stabilizes MPF activity (cyclinB/Cdc2) by blocking the degradation of cyclin B [18,19]. MAPK activation is required for efficient Cdc2 kinase activation during *Xenopus* oocyte maturation [9,20,21]. While Cdc2 kinase activation is essential for GVBD in all species, differences in the timing of, and requirement for, MAPK activation occur between different species [22]. In contrast, Mos accumulation and MAPK activation are under the control of Cdc2 kinase [23]. Although it has recently been proposed that Mos synthesis and MAPK activation are not required to induce MPF activation in *Xenopus* oocytes, they most probably facilitate this process [9,10,24]. Moreover, Carnero et al. [25] reported that PLA_2 induced maturation of *Xenopus* oocytes. However, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (PACA), a pharmacological inhibitor of phospholipase A_2 (PLA_2), has not been used yet for the study of *Xenopus* oocyte meiotic maturation. In an attempt to account for these contradictory findings on the role of Mos and MAPK in MPF formation, we studied the molecular basis of the progesterone-induced meiotic maturation process individually in stage V and stage VI oocytes, which are often studied without strict selection, by using PACA. In this study we found the distinct stage-specific effects of PACA on MAP kinase and Cdc2 kinase activation. From these findings, we propose PACA as a new tool for the study of the *Xenopus* oocyte maturation. Thus, PACA can play a unique role for the studies of the distinct stage-specific molecular mechanisms of MAP kinase and Cdc2 kinase activation during progesterone-induced *Xenopus* oocyte maturation.

2. Materials and methods

2.1. Materials

X. laevis adult females (Johoku Seibutsu Kyozai, Shizuoka, Japan) were bred and maintained under laboratory conditions. [γ-^{32}P] ATP (10 mCi/ml) was purchased from Amersham Biosciences and arachidonic acid (AA) [5,6,8,9,11,12,14,15-^{3}H(N)]-(0.1 mCi/ml) was purchased from PerkinElmer Life Sciences. PACA, arachidonyltrifluoromethyl ketone ($AACOCF_3$) and 1,6-bis(cyclohexyloximinocarbamylamino) hexane (RHC-80267) were purchased from Biomol Research Laboratories. Bromoenol lactone (BEL) and mepacrine were purchased from Calbiochem. AA, lysophosphatidylcholine (LPC), lysophosphatidylserine (LPS) and lysophosphatidylinisitol (LPI) were obtained from Doosan Serdary Research Laboratories. The other reagents, unless otherwise specified, were from Sigma.

2.2. Isolation of Xenopus oocytes and induction of meiotic maturation

Ovaries were surgically removed after keeping the *X. laevis* in ice for 20–30 min, and oocyte clusters were washed in 1×MBS containing 10 mM HEPES (pH 7.5), 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ and 50 μg/ml each penicillin and streptomycin, and cut into small pieces (approximately 20 oocytes per piece) using forceps and defolliculated by incubation in MBS containing 1.5 mg/ml collagenase for 2 h with continuous stirring at room temperature. Collagenase was washed out after several washings with MBS and the oocytes were harvested in MBS medium. In experiments involving later induction of meiotic maturation, oocytes were cultured in Leibovitz's L15 medium (GIBCO, Life Technologies) supplemented with 50 μg/ml each penicillin and streptomycin. Stage V and stage VI oocytes were selected [26] and cultured in MBS. For experiments examining the effects of various concentrations of PACA on maturation, pools of 30–50 oocytes, and for examining the time course, series of 200 oocytes, were incubated in 10 ml of MBS in small dishes and treated with 5 μg/ml progesterone. Maturation was monitored at 1-h intervals by microscopic observation of GVBD indicated by a white spot at the animal pole. For the time course, 10 oocytes were collected at 1-h interval until 10 h.