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| 学位の種類 | 博士（医学） |
| 学位記番号 | 甲第４９３号 |
| 学位授与年月日 | 平成１７年　３月１１日 |
| 学位授与の要件 | 学位規則第４条第１項該当 |
| 学位論文題目 | 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<br>（アフリカツメガエル卵成熟時のMAPキナーゼとCdc2キナーゼの活性化に及ぼす2-(p-amylcinnamoyl)amino-4-chlorobenzoic acidの発育ステージによる異なる効果） |
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## 学位論文の内容の要旨

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). Progesterone-induced synthesis of c-mos proto-oncogene product (Mos) is accompanied by the simultaneous activation of mitogen-activated protein kinase (MAPK) and Cdc2 kinase during *Xenopus* oocyte meiotic maturation. However, it has recently been proposed by many laboratories that Mos synthesis and MAPK activation are not required to induce MPF activation in *Xenopus* oocytes, they most probably facilitate this process. In an attempt to account for these contradictory findings on the role of Mos and MAPK in MPF formation, the molecular basis of the progesterone-induced meiotic maturation process was studied individually in stage-V and stage-VI oocytes, which are often studied without strict selection, by using 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid (PACA), a pharmacological inhibitor of phospholipase $A_2$ ($PLA_2$). In this study the distinct stage-specific effects of PACA on MAPK and Cdc2 kinase activation were observed. Moreover, the distinct stage-specific molecular mechanisms of MAPK and Cdc2 kinase activation were established. From these findings, PACA was proposed 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 MAPK and Cdc2 kinase activation during progesterone-induced *Xenopus* oocyte maturation.

## Experimental procedures

*Xenopus* oocytes isolation and staging were done by Dumont criterion. Oocytes were treated with 10 mM PACA and progesterone (5 mg/ml) was applied after 1 h for induction of meiotic maturation. Groups of oocytes were labeled with 0.1 μM [$^3$H]-labeled arachidonic acid (AA) (1 μCi/ml) and released AA was measured by liquid scintillation counter after individually treated with $PLA_2$ inhibitors and microinjection of four different concentrations of $PLA_2$. Three different concentrations of bacterially expressed and purified recombinant maltose binding protein (MBP)-Mos were injected in stage-V oocytes treated with PACA. Mos mRNA was depleted by antisense oligodeoxynucleotides. The homogenate of oocytes was centrifuged at 15,000 *g* for 10 min at 4°C and the resultant supernatant was used as the sample for Western blotting, kinase assay as well as other analyses. For Western blotting sample (equivalent to 1 oocyte) was electrophoresed using a 12.5% SDS-PAGE Anderson system and transferred proteins were reacted with each antibody. MAPK was immunoprecipitated and its activity was measured using MBP as substrate. Silver staining was done by usual kit. Radioactivity of $^{32}$P was determined by Cerenkov counting using liquid scintillation system.

## Results

PACA can cause marked inhibition of meiotic maturation and $PLA_2$ is not involved in the meiotic maturation process. PACA showed distinct stage-specific effects on meiotic maturation. This drug has similar effects on synthesis of Mos but different effects on the activation of MAPK at stages-V and –VI. The target of PACA-induced inhibition of MAPK activation in stage-V oocytes is at the upstream of MEK and this inhibition is not contributed by any binding protein. Inhibition of $PLA_2$ does not affect the activation of MAPK in both stage-V and stage-VI oocytes. $PLA_2$ and $PLA_2$-generated metabolites can not restore the MAPK activation in PACA-treated stage-V oocytes. Interestingly, recombinant MBP-Mos protein can remove the inhibitory effect of PACA and restore the MAPK activation in stage-V oocytes. Antisense depletion of Mos by conventional oligonucleotide can not prevent the activation of MAPK completely. PACA has no inhibitory effect on MAPK activity in stage-VI oocytes *in vitro* or *in vivo*. PACA has stage-specific effects on the activation of Cdc2 kinase. PACA-induced effects on Mos synthesis and on MAPK and Cdc2 kinase activation are concentration-dependent.

## Discussion

Stage-V and stage-VI oocytes are well documented for the study of *Xenopus* oocyte meiotic maturation. To investigate the involvement of $PLA_2$ the bromoenol lactone, another $PLA_2$ inhibitor was tested which did not inhibit the maturation. Moreover, neither microinjection of $PLA_2$-generated metabolites nor direct microinjection of various concentrations of $PLA_2$, could overcome the PACA-induced inhibition of meiotic maturation. Therefore, $PLA_2$ is not involved in this maturation process. Moreover, it was assumed that the PACA-induced inhibition of meiotic maturation probably resulted from some structural feature since a

structural homologue of PACA, differentiation-inducing factor-1 (DIF-1)-mediated inhibition of maturation is structure-specific.The distinct effects of PACA on MAPK activation in stage-V and –VI in spite of the similar effects on Mos synthesis, clearly indicates the distinct stage-specific molecular mechanisms of MAPK activation. Since microinjection of MBP-Mos can remove the inhibitory effects of PACA on MAPK activation in stage-V oocytes. Therefore the activation of MAPK is clearly dependent on Mos synthesis in both stages. Thus trace amount of Mos can activate the MAPK in stage-VI oocytes but not in stage-V oocytes. Mos-activating system may weaker in PACA-treated stage-V oocytes rather than stage-VI oocytes. This is the basic difference on the Mos-dependent activation of MAPK in stage-V and stage-VI oocytes. The possible mechanism of the delay of Cdc2 kinase activation is the delayed activation of cdc25C in stage-VI and the complete inhibition of Cdc2 kinase activation in stage-V is caused by the inhibition of cdc25C activation by PACA.

### Conclusion

PACA was proposed as a potential new tool for the study of the meiotic maturation of *Xenopus* oocytes. Thus, PACA can play a unique role for the comparative studies of the molecular mechanisms of MAPK and Cdc2 kinase activation in stage-V and stage-VI oocytes during progesterone-induced meiotic maturation of *Xenopus* oocytes.

## 論 文 審 査 の 結 果 の 要 旨

本研究はホスホリパーゼ $A_2$ の阻害剤である 2-(p-amylcinnamoyl)amino-4-chlorobenzoic acid (PACA) が プロゲステロンによるアフリカツメガエル卵母細胞の成熟を抑制するメカニズムを解析したものである。その結果、卵成熟の抑制はホスホリパーゼ $A_2$ を直接阻害によるものではなく、それ以外のシグナル伝達系に作用した結果であり、さらに卵母細胞の発育ステージによってその影響が異なることを明らかにした。即ち、ステージ６の細胞では PACA により卵成熟の開始が遅れるが、ステージ５の細胞では起こらない。これは Cdc2 キナーゼの活性化と対応する。さらに、両ステージの細胞は PACA により Mos キナーゼ蛋白質の合成が阻害されるが、その下流にある MAP キナーゼの活性化はステージ６の細胞では阻害されずステージ５の細胞では強く阻害された。これは Mos の微妙な活性が細胞の発育ステージで異なることを示唆している。このように本論文は PACA が卵母細胞の発育ステージを区別する有用なツールであるとともに卵成熟を誘発するシグナル伝達機構の解明に新しいに手掛かりを与えており、明らかに学術の水準を高めたものと認められる。