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学位論文題目　Identification of the α Subunits of Inhibitory G-Proteins Coupled to 17α, 20β-Dihydroxy-4-pregnen-3-one Receptors in Oocytes of Medaka (*Oryzias latipes*)

論文審査委員　主査　教授　野田昌晴
教授　鈴木義昭
教授　長濱嘉孝
教授　山森哲雄

論文内容の要旨

Fully grown oocytes of vertebrates are arrested at prophase of the first meiotic division and must progress to the second meiotic metaphase before fertilization is possible. The resumption of meiosis is brought about the action of maturation-inducing hormone (MIH) which is secreted from ovarian follicles under the influence of a pituitary gonadotropin (luteinizing hormone, GTH II in fishes). Among vertebrates, MIHs have been most extensively investigated in several species of fishes and amphibians. In these animals, the MIH is a steroidal substance, and its major function is known to induce resumption of meiosis (final meiotic maturation). 17α, 20β-Dihydroxy-4-pregnen-3-one (17α, 20β-DP) was identified, for the first time in any vertebrate, as MIH of amago salmon (*Oncorhynchus rhodurus*). In the most widely investigated group, the anuran amphibians, MIH is generally considered to be progesterone.

The mosut fundamental insight into hormone action that has emerged from the study of oocyte maturation is the identification of a new type of steroid hormone action that involves a plasma membrane receptor. Unfortunately, efforts to purify and characterize MIH receptors have been difficult and unsuccessful, largely due to relatively low ligand affinities and specificity of 17α, 20β-DP and progesterone. A specific 17α, 20β-DP binding to plasma membranes prepared from defolliculated oocytes of rainbow trout was identified and characterized. Recently, it has been reported that pertussis toxin (PT)-sensitive inhibitory G-proteins (Gi) is involved in the signal transduction of 17α, 20β-DP during meiotic maturation of rainbow trout oocytes. However, the details of the receptors and their signaling pathway are poorly understood. The refore, the present study was designed to further investigate the involvement of G-proteins in the signal transduction pathway during steroid hormone-induced oocyte maturation. To this end, he choses the medaka (*Oryzias latipes*) as an experimental animal. Medaka, under a photoperiod of 14 hr light 10 hr dark at 26℃, usually spawns daily within 1 hr of the onset of light for a number of consecutive days. By this method, various phases of oocyte maturation can be timed accurately. This orderly spawning allows them to collect oocytes at predictable stages, which makes medaka an ideal model for the study of hormonal regulation of oocyte growth and maturation. 17α, 20β-DP was shown to be a major naturally occurring MIH in medaka. Rainbow trout (*Oncorhynchus mykiss*) was also used for comparison.

In chapter I, he examined changes in Giα protein contents and 17α, 20β-DP binding activity during naturally occurring oocyte maturation in both rainbow trout and medaka. The antibody AS/7 specific for Giα1,2 subunits

immunodetected Giα proteins (40kDa) in medaka and rainbow trout plasma membranes from postvitellogenic oocytes. The α subunit of G-proteins detected in medaka oocyte membranes has properties characteristic of a member of the Gi family. This protein was shown to be a substrate for PT. Time course changes in the amount of the 40 kDa band were examined using medaka membrane preparations obtained during various periods (every 6 hr from 41 hr prior to spawning to ovulation) of oocyte growth and maturation. Gi protein was maximal in immature oocytes (23 hr prior to spawning), decreased in oocytes undergoing GVBD and was not detected in ovulated eggs. A similar decrease in Gi protein contents also occurs in membrane fractions of oocytes undergoing GVBD and ovulated eggs in rainbow trout.

Changes in 17α, 20β-DP binding activity in oocyte plasma membranes were examined using medaka oocytes collected in the same time course as that used for Gi protein content measurements. 17α, 20β-DP binding activity was barely detectable at 35 hr prior to spawning. The binding activity increased significantly to reach a maximal level at 23 hr prior spawning, followed by a rapid drop during oocyte maturation and ovulation. The maturational competence was also determined by counting percentage GVBD. The percentage of GVBD was only 5% at 38 hr prior to spawning, but increased to 48% at 29 hr prior to spawning, and reached 100% at 23 hr prior to spawning. At 17 hr prior to spawning, oocytes underwent spontaneous maturation in the absence of 17α, 20β-DP. The increases in the percentage of GVBD and 17α, 20β-DP binding activity occurred concomitantly.

In chapter Ⅱ, he focuses on the characterization of Giα subunits expressed in medaka postvitellogenic oocytes as candidates of signal transducer of the 17α, 20β-DP receptor. Cloning and sequencing of full-length cDNAs encoding medaka Giα subunits were also carried out. In addition, he also investigated the expression of Gs in medaka oocytes. He first used reverse transcription-polymerase chain reaction (RT-PCR) to amplify α subunits of G-proteins using total RNA from intact ovarian follicles as a template. Five different PCR products were obtained. Sequence analyses indicate that these clones include three subtypes of Giα (Giαa, Giαb and Giαc) and two subtypes of Gsα (Gsαd and Gsαe). Full length cDNA clones for Giαa and Giαc were isolated from a medaka ovarian follicle cDNA library. Predicted amino acid sequences of Giαa and Giαc exhibited significant homology with Giα1 and Giα2 of other species, respectively. Both Giαa and Giαc possessed a specific Cys residue in the C-terminal region which was the site for ADP-ribosylation by pertussis toxin. A method was then developed to extract medaka oocyte RNA without contamination of follicle cell RNA. Using this method, he demonstrated that oocytes expressed both Giαa and Giαc, but not Giαb. Goα, another G-

protein which is ADP-ribosylated by PT, was not expressed in oocytes, although this expressed in brain tissue. The results presented here suggest that the 17α, 20β-DP-dependent stimulation of oocyte maturation is mediated through the G-protein α subunits, Giαa and/or Giαc.

In chapter Ⅲ, the coupling of 17α, 20β-DP receptors and G-protein α subunits in medaka oocyte plasma membranes was investigated. Three kinds of antisera (A2, C4, and M1) against peptides corresponding to the unique internal sequences of medaka Giα subtypes, Giαa and Giαc, were produced. Antisera A2 and C4 recognized Giαa and Giαc, respectively, while antiserum M1 recognized both subtypes of Giα. Immunoblotting using these antisera showed that medaka oocytes express two Giα, Giαa and Giαc concomitantly. The results also indicated that Giαa behaved a little larger than Giαc on SDS-PAGE.

Giα in medaka oocyte membrane preparations could be ADP-ribosylated with PT after solubilization with CHAPS. This indicates that the solubilization condition used in this study was so mild that Gi retains the heterotrimeric form even after solubilization. As expected, antiserum M1 successfully precipitated solubilized two Giα, Giαa and Giαc. Furthermore, the β subunit of G-protein as also found in the immunoprecipitates. These results indicate that Gi still retains the heterotrimeric form in the immunoprecipitate, becouse the β and γ subunits are known to tightly associate each other.

He found significant amounts of the specific 17α, 20β-DP binding in the immunoprecipitates, indicating that the 17α, 20β-DP receptor is directly coupled with Gi. This is the first demonstration of direct coupling of the MIH receptor and heterotrimeric G-proteins. He has not yet attempted to quantify yield of 17α, 20β-DP binding and Giα in the immunoprecipitates. Several technical problems need to be overcome: these include the difficulty of assaying 17α, 20β-DP binding in solubilized membrane preparations.

It should be emphasized that evidence for plasma membrane steroid hormone receptors is not limited to the amphibian and fish oocyte system. For example, it is becoming increasingly apparent that some steroid hormones may effect rapid alterations in brain function independently of classical intracellular receptors. By producing three polyclonal anitibodies which recognize Giα, he was able to demonstrate, by immunoprecipitation and ligand binding assay, that the 17α, 20β-DP receptor is coupled directly with Gi, and its α subunit is Giαa and/or Giαc. Further studies will focus on the identification of the 17α, 20β-DP receptor using this new coimmunoprecipitation method.

審査結果の要旨

卵成熟は卵を受精可能にする過程であり、卵濾胞細胞で生成される卵成熟誘起ホルモン(MIH)の働きで起こる。MIHは脊椎動物では魚類でのみ単離され、17α、20β-ジヒドロキシ-4-プレグネン-3-オン(17α、20β-DP)と同定された。17α、20β-DPはステロイドでありながら卵表の膜受容体／情報伝達系を介して卵成熟を起こす。本研究では、17α、20β-DPに連結する抑制性G-蛋白質の構造と機能を明らかにする目的で行った。実験材料としては光周期、水温の調整により卵成熟過程を同調できるメダカを主に使用したが、比較にニジマスも用いた。

第一章では、卵成熟時における卵細胞膜中の抑制性G-蛋白質(Gi)と17α、20β-DP受容体の蛋白量の変動を調べた。メダカとニジマスの卵細胞質には百日咳毒素(PT)感受性のGiαサブユニット(40 kDa)が存在し、その蛋白量は卵黄形成を完了した未成熟卵（メダカでは産卵２３時間前）で最大で、卵成熟の開始後に減少し、排卵後では検出されなかった。一方、17α、20β-DP膜受容体量は、卵の17α、20β-DPに対する感受性の増加に伴い上昇し、Giα蛋白量と同様に卵成熟、排卵時に急減した。

第二章では、卵成熟時のメダカ卵に発現するGαサブユニットの同定を行った。RT-PCR法を用いてメダカ卵胞（卵とそれを取り囲む濾胞細胞）より５種のG-蛋白質クローンを得て、それらの塩基配列の解析より３種のGiαサブユニット(Giαa, Giαb, Giαc)と２種のGsαサブユニット(Gsαd, Gsαe)に分類した。次に、独自に考案した卵細胞RNA抽出法を駆使することにより、卵細胞に特異的に発現するG-蛋白質がGiαaとGiαcであることを示すとともに、メダカ卵胞のcDNAライブラリーよりこれらの全長cDNAクローンを得て、全塩基配列を決定した。

第三章では、17α、20β-DP受容体とGiαサブユニットとのカップリングについて解析した。この目的のために、Giα(A2)とGiαc(C4)のそれぞれに特異的な抗体およびそれら両方を認識する抗体(M1)の三種を作成した。まず、メダカの卵細胞膜からCHAPSを用いて可溶化したGiαaはPTによりADP-リボシル化することを確認した。次に、この可溶化Giαを M1抗体を用いてGiβとともに免疫沈降させることに成功した。さらに、これらGiα／Giβ免疫沈降物中には17α、20β-DP受容体が多量に含まれていることを結合実験により示した。

以上、本研究では、卵成熟誘起ホルモン17α、20β-DPの作用発現に17α、20β-DP膜受容体／抑制性G-蛋白質Giが重要な役割を果たすことを明確に示すとともに、ステロイドホルモン受容体にGi蛋白質が連結することをはじめて直接的に示した。したがって、審査委員は全員一致で博士論文として十分なものと判断した。

試験結果においては、提出論文の内容について口述発表させた後、その内容と学問的背景に関して申請者に対する面接試験を行った。論文内容、関連分野の基礎知識、今後の課題等に関する質問が出たが、申請者の応答はいずれも的確であった。また英語力についても提出論文は英文で書かれており、学位授与に際して必要な水準に達していると判断した。以上、総合的に判断して合格とした。