Effects of Calcium Agonists on Vitellogenin Induction by Estradiol-17 β in Primary Hepatocyte Culture in the Rainbow Trout *Oncorhynchus mykiss*

In-Kyu Yeo and Yasuo Mugiya

Laboratory of Comparative Physiology, Faculty of Fisheries,
Hokkaido University, Minato, Hakodate 041-8611, Japan

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Effects of calcium agonists (A23187, caffeine, ryanodine, and thapsigargin) on estradiol-induced vitellogenin (VTG) induction were examined in primary hepatocyte culture in the rainbow trout *Oncorhynchus mykiss*. These agonists are effective in depleting calcium in various calcium stores. Hepatocytes were precultured for 2 days and then estradiol-17 β (E₂, 2×10^{-6} M) and the agonists were simultaneously added to the incubation medium. The hepatocytes were cultured for 7 more days and then spent medium was analyzed by SDS-PAGE. The relative rate of VTG production was evaluated by measuring the integrated optical density of the main VTG band and was expressed as the percentage of VTG to total proteins including the VTG. The addition of A23187 ($10^{-7} \sim 10^{-6}$ M), caffeine ($10^{-4} \sim 10^{-2}$ M) or thapsigargin ($10^{-7} \sim 10^{-6}$ M) to the incubation medium specifically reduced VTG production by hepatocytes in a concentration-dependent way. Ryanodine also reduced the production to about 60% of the control at a concentration of 10^{-4} M. These results suggest that the synthesis of VTG is more susceptible to intracellular sequestered calcium, at least to calcium in the endoplasmic reticulum, than are other hepatocyte-derived proteins in rainbow trout.

Key words: hepatocyte culture, vitellogenin production, calcium agonists, intracellular calcium store, endoplasmic reticulum, rainbow trout

Vitellogenin (VTG), a calcium-binding and highly phosphorylated protein, is a precursor molecule of egg yolk in oviparous vertebrates. VTG is synthesized in the liver under the stimulation of estrogens, transported to the developing ovary, and accumulated as yolk proteins in eggs after splitting into lipovitellin, phosvitin, and β components.¹⁾ VTG contains about 0.7% calcium in the rainbow trout *Oncorhynchus mykiss*²⁾ and about 2.0% calcium in the bass *Opsariichthys uncirostris*.³⁾ Phosvitin contains calcium⁴⁾ as well as about 10% phosphorus as esterified orthophosphate in barfin flounder *Verasper moseri*.⁵⁾

Calcium is required for protein synthesis in a variety of cells. Chin *et al.*⁶⁾ reported that GH₃ pituitary cells exposed to low extracellular calcium concentrations resulted in a concomitant reduction in the rate of amino acid incorporation into proteins. Similar results were reported in hepatocytes,^{7,8)} tumor cells,⁹⁾ and pituitary cells¹⁰⁾ following calcium depletion with EGTA. Recently, Yeo and Mugiya¹¹⁾ found that in primary cultures of rainbow trout hepatocytes, VTG production is extracellular calcium-dependent. They showed that VTG production is more susceptible to calcium than are other hepatocyte-derived proteins.

Brostrom *et al.*¹²⁾ suggested that A23187 inhibits calcium-dependent translational initiation of protein synthesis through facilitating the mobilization of intracellular sequestered calcium in mammalian eukaryotic cells. Fawell *et al.*¹³⁾ demonstrated that the inhibition of protein synthesis by A23187 occurred possibly through dephosphorylation in GH₃ pituitary tumor cells. Chin *et al.*⁸⁾ showed that sequestered calcium, presumably stored in the endoplas-

mic reticulum, is required for optimal rates of protein synthesis in rat hepatocytes. Protein phosphorylation and dephosphorylation are controlled by various protein kinases including protein kinase C and by calmodulin-dependent protein phosphatase.^{14,15)} In turn these enzymes are activated by an elevated level of cytosolic calcium through an increase in the influx of extracellular calcium and/or in the release of intracellular sequestered calcium. Therefore, VTG synthesis may be highly dependent on an intracellular calcium status because of its highly phosphorylated nature. It was also reported that protein synthesis was inhibited by depletion of intracellular sequestered calcium in mammalian eukaryotic cells, regardless of cytosolic calcium status.^{12,16)} However, little information is available about the role of intracellular sequestered calcium in the synthesis and integration of VTG molecules in fish.

The present study was undertaken to examine the direct role of intracellular sequestered calcium in VTG production in primary hepatocyte culture of rainbow trout. Calcium agonists (A23187, caffeine, ryanodine, and thapsigargin) were used to change calcium status in sequestrators.

Materials and Methods

Fish

Rainbow trout *Oncorhynchus mykiss* weighing 250–330 g were obtained from a trout farm and maintained at about 14°C in outdoor ponds at our laboratory. The fish

were fed trout food pellets once a day but were starved during the last 3 days before the experiments to avoid the induction of bile, which weakens the attachment of hepatocytes to a culture dish.

Hepatocyte isolation and incubation

Hepatocytes were prepared following Hayashi and Ooshiro¹⁷⁾ as described by Kwon *et al.*¹⁸⁾ Cell yield and viability were determined by the Trypan Blue exclusion test.

Isolated hepatocytes were plated into a 60-mm plastic petri dish with positive-charge (Falcon) at a density of 3×10^5 cells per dish. The culture medium was Williams' medium E (Life Technol. Inc.) containing 0.2 μ M bovine insulin (Sigma), streptomycin (100 μ g/ml), penicillin (70 μ g/ml), and NaHCO₃ (23 mM). All incubations were carried out in 3 ml of the medium at 15°C under 5% CO₂ and air with saturated humidity. The culture medium contained 1.8 mM calcium, which was lowered to a level of 0.5 mM by adding 1.3 mM EGTA, if necessary, according to the method described by Yeo and Mugiya.¹¹⁾

In vitro hormone and calcium agonist treatments

Isolated cells were precultured for 2 days and then estradiol-17 β (E₂, 2×10^{-6} M in 3 μ l of 95% ethanol) and calcium agonists (in 3 μ l of 95% ethanol) were simultaneously added to the dishes. Calcium agonists added were A23187 (10^{-7} M $\sim$ 10^{-5} M), caffeine (10^{-4} M $\sim$ 10^{-2} M), ryanodine (10^{-5} M $\sim$ 10^{-4} M), and thapsigargin (10^{-7} M $\sim$ 10^{-6} M). These concentrations were effectively used to change calcium status in various cells.^{12,16,19,20)} The effects of these agonists on VTG synthesis were examined 7 days after addition, during which time media were changed daily. Control cultures received an equivalent amount of the solvent only.

A23187 rapidly increases cytosolic free calcium by facilitating the entry of extracellular calcium and by releasing sequestered calcium.²¹⁾ Caffeine induces depletion of stored calcium by opening calcium-release channels in intracellular calcium stores.²²⁾ Thapsigargin inhibits the Ca ATPase activity of the endoplasmic reticulum (ER) specifically and depletes calcium in the ER.²³⁾ Ryanodine also mobilizes sequestered calcium into the cytoplasm. Therefore, these agonists commonly reduce the level of calcium in sequestrators.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the presence of 2-mercaptoethanol on a precast linear gradient slab gel with a 5 to 20% acrylamide gradient.²⁴⁾ Spent medium (3 ml) was collected and centrifuged to remove any debris in the sample. Total proteins were precipitated by adding cold 50% trichloroacetic acid (TCA) to a final concentration of 10%. The whole precipitate was washed three times with 5 ml of 5% TCA, dissolved in 30 μ l of sample buffer (0.175 M Tris-HCl, 8 M urea, 1% SDS, 0.5% mercaptoethanol, pH 7.4), and applied to each lane of the gel. It was difficult to apply a fixed amount of protein to each lane, because the present experiment was intended to inhibit VTG production. The gels were stained with 0.25% Coomassie brilliant blue R-250 for 30 min. Standard proteins used for molecular weight (MW) determinations were carbonic anhydrase

(MW 29,000), ovalbumin (45,000), bovine serum albumin (66,000), phosphorylase *b* (97,400), β -galactosidase (116,000), and myosin (205,000).

Qualitative and Quantitative analyses of VTG

The identification of VTG bands was based on the results of a previous study¹⁸⁾ in which isolated rainbow trout hepatocytes incubated with E₂ produced a protein of the same electrophoretic mobility (175 kDa) as in the present study. Kwon *et al.*¹⁸⁾ identified this band (the main band) as VTG by immunoblot analysis and immunoelectrophoresis.

After SDS-PAGE, the integrated optical density (IOD) of the main VTG band was measured by a Bio Image (Millipore) and expressed as a percentage of the IOD of the total proteins including VTG. Minor subunits of VTG were not considered as VTG, because the subunits constituted only a fairly small part of VTG and overlapped with other proteins.¹⁸⁾ An excellent correlation ($r=0.99$, $P<0.01$) was reported between the amount of VTG applied to each lane of SDS-PAGE and the IOD over a wide range of VTG concentrations.¹¹⁾

Statistical analysis

A one-way ANOVA by Fisher's protected least significant difference (PLSD) was used for statistical evaluation of mean values. Significance was accepted at $P<0.05$. Percent data were statistically analyzed after being arcsine transformed.

Results

Trout hepatocytes were cultured in the serum-free medium with E₂ for 7 days and the spent media were analyzed by SDS-PAGE. A new protein band in the spent media was electrophoretically detected at a molecular weight position of 175 kDa (Fig. 1). This band was identified as VTG by Kwon *et al.*¹⁸⁾ on the basis of immunoblotting and immunoelectrophoresis. The control culture without E₂ did not induce an equivalent protein.

The addition of A23187 to the incubation medium was not effective in reducing VTG production at concentrations of 10^{-7} M and 10^{-6} M (Fig. 1). However, a higher concentration of 10^{-5} M markedly reduced VTG synthesis. These results were essentially the same despite different calcium concentrations in the medium.

The rate of VTG to total protein concentrations was 9.1% in the control culture (Ca 1.8 mM) without A23187 (Fig. 2). It decreased with increased concentrations of A23187 and reached about 35% of the control ($P<0.01$) at 10^{-5} M A23187. A low calcium concentration of 0.5 mM reduced VTG synthesis by about 28%, compared with 1.8 mM calcium (Fig. 2). However, the effects of A23187 were similar to those in 1.8 mM calcium. A significant reduction occurred at an A23187 concentration of 10^{-5} M.

The addition of caffeine to the incubation medium reduced VTG production. The rate of VTG to total protein concentrations decreased with increasing concentrations of caffeine and a significant difference was obtained at concentrations more than 10^{-3} M caffeine ($P<0.01$, Fig. 3). A caffeine concentration of 10^{-2} M completely inhibited VTG production and its level did not differ from the back-

Fig. 1. Gradient SDS-PAGE showing the effects of A23187 on the E₂-induced production of VTG (arrowhead) in hepatocyte cultures with different calcium concentrations of 0.5 and 1.8 mM.

Spent media were analyzed on day 7 in culture. The amount of sample buffer applied was 30 μ l/lane (see Materials and Methods for details). M: molecular weight (MW) marker. CBB stain.

Fig. 2. Effects of A23187 on the E₂-induced production of VTG.

The activity of VTG production was estimated for the relative optical density of VTG to total proteins after SDS-PAGE on day 7 in cultures with A23187. Vertical bars represent the SE of mean for 8–13 experiments. * $P < 0.01$ for E₂ alone.

ground level without E₂.

Ryanodine was not effective in reducing VTG production at concentrations of 10^{-5} M and 5×10^{-5} M. However, it inhibited the production by about 60% at a concentration of 10^{-4} M (Fig. 4). Therefore, concentration-dependent effects were not observed for ryanodine.

Thapsigargin also reduced VTG production in a concentration dependent way and a significant difference was ob-

Fig. 3. Effects of caffeine on the E₂-induced production of VTG.

The activity of VTG production was estimated for the relative optical density of VTG to total proteins after SDS-PAGE on day 7 in cultures with caffeine. Vertical bars represent the SE of mean for four experiments. * $P < 0.01$ for E₂ alone.

tained at concentrations of 5×10^{-7} M and 10^{-6} M ($P < 0.01$, Fig. 5). The rate of inhibition was about 26% and 32% of the control at these concentrations, respectively.

Discussion

It was suggested that calcium entry into hepatocytes via a receptor-operated calcium channel was an important step for VTG synthesis and secretion in rainbow trout.¹¹⁾ It

Fig. 4. Effects of ryanodine on the E₂-induced production of VTG.

The activity of VTG production was estimated for the relative optical density of VTG to total proteins after SDS-PAGE on day 7 in cultures with ryanodine. Vertical bars represent the SE of mean for four experiments. * $P < 0.01$ for E₂ alone.

Fig. 5. Effects of thapsigargin on the E₂-induced production of VTG.

The activity of VTG production was estimated for the relative optical density of VTG to total proteins after SDS-PAGE on day 7 in cultures with thapsigargin. Vertical bars represent the SE of mean for nine experiments. * $P < 0.01$ for E₂ alone.

was also reported that protein synthesis by hepatocytes required sequestered calcium, presumably stored in the endoplasmic reticulum, in rat.^{7,8} Therefore, we examined the effects of intracellular sequestered calcium on the induction of VTG production using calcium agonists in a hepatocyte culture in rainbow trout. The calcium agonists used are known to change calcium status in various cells in fish.²⁵⁻²⁷

The addition of A23187 to the incubation medium effectively inhibited VTG production. Similar results were reported for protein synthesis in liver cells in rat.^{7,8} The intracellular mechanisms of inhibition are not well under-

stood. Fawell *et al.*¹³ demonstrated that the dephosphorylation of proteins and the inhibition of protein synthesis occurred simultaneously after addition of the ionophore. Phosphorylation is involved in protein synthesis at various sites such as ribosomal protein S6,²⁸ initiation factors eIF-2,²⁹ and the cap-binding protein eIF-4E.³⁰ Therefore, the effect of A23187 on the phosphorylation of the VTG molecule should be examined to understand the intracellular mechanisms of inhibition.

Caffeine, like A23187, is known to act on intracellular calcium dynamics. In addition, it can release calcium from a ryanodine-sensitive calcium store, if any such stores are present.³¹ Although caffeine effectively inhibited VTG production in this study, we could not determine whether the calcium sequestrators that were affected by caffeine were sensitive or nonsensitive to ryanodine. To answer this question, ryanodine was tested.

Ryanodine reduced the production of VTG at a concentration of 10⁻⁴M in this study. This agonist is known to release calcium from a calcium store with ryanodine receptors in various cells including hepatocytes.^{19,32} Therefore, the present results show that trout hepatocytes may have ryanodine-sensitive receptors for mobilizing sequestered calcium.

The common action of the agonists used is that they deplete calcium in various kinds of calcium sequestrators. Among these agonists, thapsigargin is unique in specifically depleting sequestered calcium in ER.³³ Our finding that thapsigargin reduced the production of VTG in a concentration-dependent way suggested that calcium in ER is involved in the regulation of VTG production by hepatocytes in rainbow trout. However, the intracellular mechanisms whereby calcium depletion specifically inhibited VTG production are not well understood. Chin *et al.*⁸ suggested that the depletion of calcium in ER by pharmacologic procedures inhibited protein production at a translational level in rat liver. In addition, because VTG is a highly phosphorylated protein in nature, it is possible that calcium depletion induced anomalous phosphorylation by inactivating calcium-dependent protein kinase C,¹⁴ resulting in the specific inhibition of VTG production. E₂-primed experiments and VTG mRNA analyses would be useful for determining whether intracellular calcium acts at a receptor, translational, or posttranslational level in the pathway of VTG synthesis.

In conclusion, we suggest that calcium stored in intracellular sequestrators, at least in ER, plays a regulatory role in VTG production by trout hepatocytes.

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