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〈NOTE〉

Calcium in the Endoplasmic Reticulum, but not in Mitochondria, is Involved in VTG Production in the Hepatocyte Cultures of Rainbow Trout, *Oncorhynchus mykiss*

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Vitellogenin (VTG) is the egg-yolk precursor protein that is synthesized in the liver in response to circulating estrogen and released into the blood. VTG is a macromolecule formed by a calcium-binding protein-phospholipid-glycolipoprotein complex and contains about 0.7% calcium in rainbow trout *Oncorhynchus mykiss* (Fremont and Riazi, 1988).

Calcium is required for protein synthesis in a variety of cells (Brostrom and Brostrom, 1990). It was reported that blocking sequestered calcium led to the inhibition of protein production in the rat liver (Altin and Bygrave, 1985), which inhibits the calcium-dependent translational initiation of protein production through reduced of activated eIF-2 and the phosphorylation of the α-subunit of eIF-2 (Brostrom *et al.*, 1989; Kimball and Jefferson, 1992). Chin *et al.* (1988) showed that sequestered calcium, presumably stored in the endoplasmic reticulum (ER), is required for optimal rates of protein synthesis in rat hepatocytes. Yeo and Mugiya (1997) found that in primary cultures of rainbow trout hepatocytes, VTG production required extracellular calcium. Moreover, recently they suggested that calcium stored in intracellular sequestrators plays a regulatory role in VTG production by trout hepatocytes (Yeo and Mugiya, 1998). However, little information is available about the relation of various intracellular sequestrators in the synthesis of VTG. In the present study, the effects of intracellular sequestered calcium in ER and mitochodria in VTG production were examined using calcium agonists.

Rainbow trout weighing 250~330 g were obtained from the Nanae Fish Culture Experimental Station, Hokkaido University, and maintained at about 14℃ in outdoor ponds at our laboratory. Hepatocytes were prepared following Hayashi and Ooshiro (1975) as described by Kwon *et al.* (1993). Isolated cells were precultured for 2 days, and then estradiol-17β (E_2, 2×10^{-6}M in 3 $\mu\ell$ of 95% ethanol) and calcium agonists were simultaneously added to the dishes. Calcium agonists added were thapsigargin (10^{-7}~10^{-6}M) and oligomycin (1~10 μg/mℓ). These concentrations were effectively used to change calcium states in various cells (Jones and Sharpe, 1994; Verbost *et al.*, 1996). Thapsigargin specifically inhibits Ca-ATPase activity in ER and depletes calcium in ER (Thastrup *et al.*, 1990). Oligomycine inhibits Ca-ATPase activity and depletes calcium in mitochondria (Verbost *et al.*, 1996). The effects of these agonists on VTG synthesis were examined 7 days after addition, during which time media were changed daily. The survival rate of hepatocytes was no differences between the control (E_2 only) and calcium agonists-treated cultures on day 7.

Synthesized proteins were analyzed by 5~20% gradient SDS-PAGE according to the method of Laemmli (1970). Identification of the VTG band was based on the results of a previous study (Kwon *et al.*, 1993) in which isolated rainbow trout hepatocytes that were incubated with E_2 synthesized a protein of the same molecular weight (175kDa) as in the present study. After SDS-PAGE, the integrated optical density (IOD) of the main VTG band was measured by a Bio Image (Millipore)

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and expressed as a percentage of the experimental to control (E_2 only). This type of expression has the benefit of excluding effects of variations in the number of cultured cells and in the amount of proteins applied to the lanes of electrophoresis.

Thapsigargin reduced the rate of VTG production in a concentration-dependent way and a significant difference was obtained at concentrations of 5×10^{-7} M and 10^{-6}M ($P<0.01$, Fig. 1). The rate of inhibition was about 26% and 32% of the control at these concentrations, respectively. However, the rate of VTG production at all concentrations of oligomycin did not differ from the control level with E_2 alone (Fig. 2).

Thapsigargin is unique in depleting sequestered calcium specifically in ER (Ghosh *et al.*, 1991). The fact that thapsigargin reduced the production of VTG in a concentration-dependent way suggests that calcium in ER is involved in the regulation of VTG production by hepatocytes in rainbow trout. The necessity of sequestered calcium in ER for protein production was reported in rat hepatocytes (Brostrom and Brostrom, 1990; Kimball and Jefferson, 1992; Tinton *et al.*, 1995).

Oligomycin is also unique in depleting sequestered calcium specifically in mitochondria (Verbost *et al.*, 1996). Because oligomycin was not effective in reducing VTG production, calcium in the mitochondria may not be essential for the regulation of VTG production.

In conclusion, the present study suggests that calcium stored in ER, not in mitochodria, is involved in VTG production by trout hepatocytes.

However, the intracellular mechanisms whereby calcium depletion specifically inhibits VTG production are not well understood. In addition, because VTG is a highly phosphorylated protein in nature, it is possible that calcium depletion induces anomalous phosphorylation by inactivating calcium-dependent protein kinase C (Cohen, 1985), resulting in the specific inhibition of VTG production. 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. Moreover, experiments with the focus of the phosphorylation and dephosphorylation of VTG molecules would provide a more detailed model for VTG production.

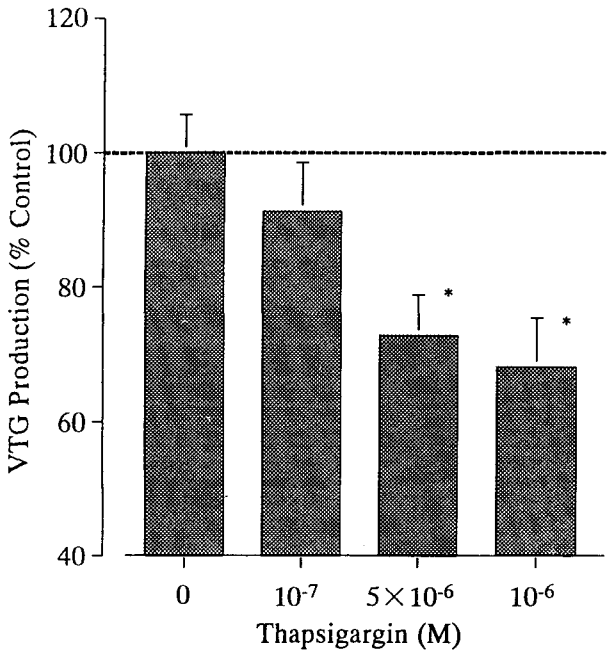

Fig. 1. Effects of thapsigargin on the E_2-induced production of VTG. Hepatocytes were cultured in media containing E_2(2×10^{-6}M) and various concentrations of thapsigargin for 7 days. Vertical bars represent the average (mean ± SE) percentage of nine experiments. *$P<0.01$ for E_2 alone.

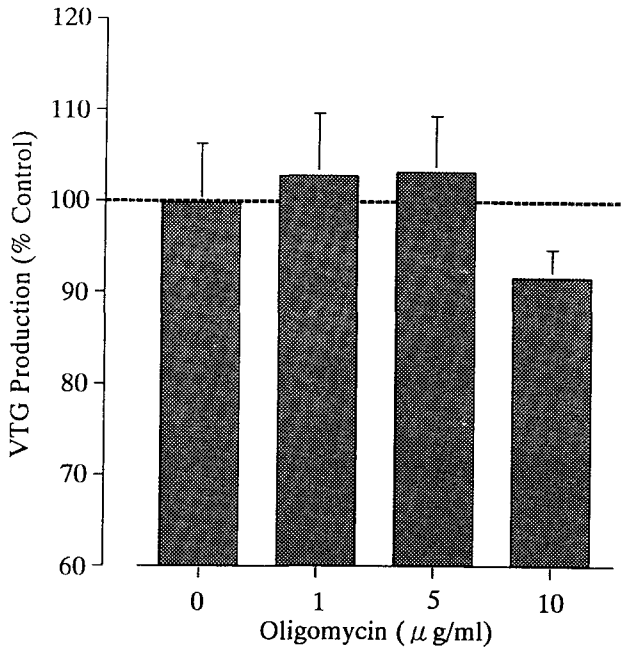

Fig. 2. Effects of oligomycin on the E_2-induced production of VTG. Hepatocytes were cultured in media containing E_2 (2×10^{-6}M) and various concentrations of oligomycin for 7 days. Vertical bars represent the average (mean ± SE) percentage of four experiments.

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