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# Regulation of the Transport of Vitellogenin by Heterotrimeric G-Proteins during Oogenesis of a Polychaete, *Pseudopotamilla occelata*

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**Coelomoic fluid protein (CP), a vitellogenin contained in the coelomic fluid of polychaetes, is transported by receptor-mediated endocytosis that is controlled by GTP-binding proteins. Transport of $^{125}$I-CP was markedly inhibited by $AlF_4$ and toxins such as cholera toxin and pertussis toxin. These effects appear to be mediated by cAMP, since $^{125}$I-CP transport was also greatly inhibited by dibutyryl cAMP. The results strongly suggest that hetero trimeric G-protein is involved in the regulation of $^{125}$I-CP transport through the activation of adenylyl cyclase. Immunoblotting tests with antibodies against $Gs_\alpha$ and $Gi_\alpha$ subunits showed a $Gs_\alpha$ subunit of 45 kDa in the membrane of oocytes of intermediate and large size classes and a $Gi_\alpha$ subunit of 41 kDa only in the oocytes of the intermediate size class.**

Transport of vitellogenin into the oocytes of the polychaete, *Pseudopotamilla occelata,* is carried out in specific stages by receptor-mediated endocytosis (Lee and Kim, 1993; Lee et al., 1997). GTP-binding proteins were found to be involved not only in the vesicular protein transport (Helms, 1995) and in the endocytosis of proteins (Brunskill et al., 1996) but also in the transport of vitellogenin during oogenesis of *Xenopus* and the polychaete (Schmalzing, 1994; Nam et al., 1996; Mukhopadhyay et al., 1997). Heterotrimeric G-proteins have been known to accelerate maturation of oocytes in sea urchin and starfish (Chiba et al., 1993; Jaffe et al., 1993) and to be involved in controlling fertilization in *Xenopus* (Downs et al., 1992; Tang, 1992) and early developmental stages (Allworth et al., 1991).

Toxins such as cholera toxin (CTX) and pertussis toxin (PTX) and aluminum fluoride ($AlF_4$) have been used to explore functional aspects of G-proteins, since these toxins and $AlF_4$ show different effects on G-proteins. In general, PTX acts on inhibitory G-protein (Gi), whereas CTX acts on the α-subunit of stimulatory G-protein (Gs), affecting the sensitivity of G-protein in GTP (Allworth et al., 1991). For instance, the association of Gs protein with endosome fusion was found using CTX, and the association of G-protein with oocyte maturation in starfish was shown using PTX (Shilling, 1989; Colombo et al., 1994). The effects of toxins have been known to be dependent on the level of cAMP in some physiological phenomena such as exocytosis in sea urchin eggs (Turner et al., 1987), endosome internalization in rat liver cells (Janicot et al., 1991) and oocyte maturation in *Xenopus* (Gallo et al., 1995). Aluminum fluoride has been known to activate heterotrimeric G-protein (Katada et al., 1984; Blackmore et al., 1985; Kanaho et al., 1985; Strnad and Wong, 1985; Gilman,1987).

In the present paper we studied the effects of cholera toxin and pertussis toxin on the receptor-mediated transport of vitellogenin specifically into the oocytes of the intermediate size class so that we could characterize the G-protein involved in the transport and explore an association of the regulation of vitellogenin transport with the elevation of cAMP.

## Materials and Methods

### *Materials*

Oocytes of *Pseudopotamilla occelata* Moore were collected on the east coast of Korea and kept in sea water at 10℃. Separation of oocytes into three size classes of small (30-80 μm in diameter), intermediate (80-140 μm in diameter) and large (180-200 μm in diameter) was as described by Nam et al. (1996).

### *Iodination of coelomic fluid proteins*

Coelomic fluid was collected by rinsing the dissecting area of the female worms when dissected for the purpose of collection of oocytes. The proteins were extracted and iodinated as described by Lee and Kim (1993).

### *Extraction and electrophoresis of membrane proteins*

Membrane proteins were extracted from the membranes

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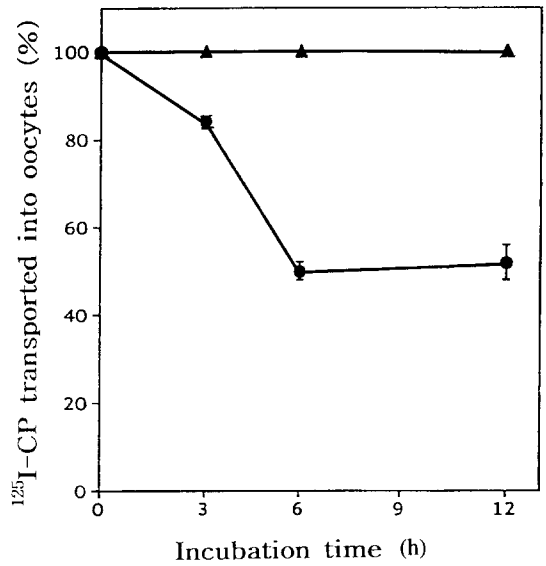


**Fig. 1.** Effects of $AlF_4$ on $^{125}$I-CP transport into the oocytes of the intermediate size class. The oocytes were incubated with (●) or without (▲) $AlF_4$. $AlF_4$ was prepared with 20 mM NaF and 10 μM $AlCl_3$.

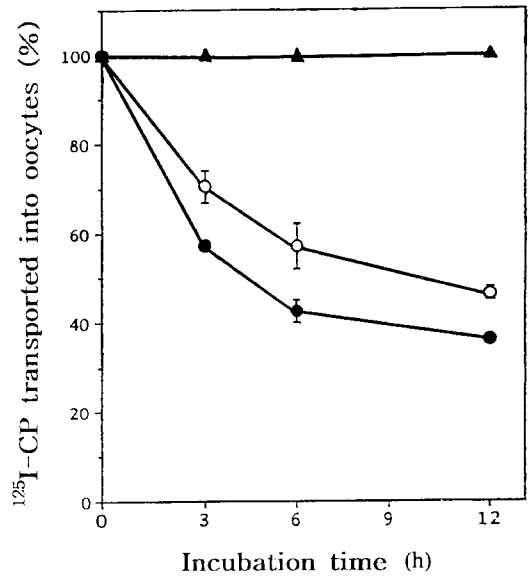


**Fig. 2.** Effects of cholera toxin on $^{125}$I-CP transport into the oocytes of the intermediate size class. The oocytes were incubated with cholera toxin at the concentration of 2 μg/ml (○) and 4 μg/ml (●) or without (▲) cholera toxin.

of the oocytes of the intermediate size class. Isolation of membranes, extraction of membrane proteins and gel electrophoresis were carried out as described by Lee (1988).

*Immunoblotting*

Membrane proteins of the oocytes were electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose (NC) membrane from the gel and blocked with 5% non-fat dry milk at 4℃ for 24 hours. NC membrane containing membrane proteins was washed three times with washing buffer (TBS + 0.1% Tween 20), each time for 10 minutes, and primarily incubated with rabbit polyclonal IgG in binding buffer (20 mM Tris-Hcl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1% Triton X-100, 0.5% gelatin) and secondarily with peroxidase-labeled antibody. After incubation, the NC membrane was immersed in peroxidase-ECL solution (Amersham) for one minute and then exposed to X-ray film (XR O-mat, Kodak) for 3-5 minutes (Serafini, 1991).

## Results

*Effect of Aluminum fluoride on the transport of vitellogenin*

Aluminum fluoride, a selective inhibitor of heterotrimeric G-protein, was introduced into the medium in which the oocytes of the intermediate size class were incubated with a labeled vitellogenin, $^{125}$I-labeled coelomic fluid proteins ($^{125}$I-CP), which were previously characterized by gel elctrophoresis (Lee et al., 1997). Oocytes were incubated with 20 mM NaF and 10 μM $AlCl_3$ for 3 hours at 12℃ with gentle rotatory shaking, and then $^{125}$I-CP of 4054 CPM/μl was introduced into the medium. The effect of $AlF_4$ on the transport of $^{125}$I-CP into oocytes was remarkable: the transport was greatly reduced down to 50% of the control by the end of 6 hours of incubation without further reduction during subsequent incubation up to 12 hours (Fig. 1).

*Effects of CTX and PTX on the transport of vitellogenin*

CTX and PTX, both known to be bacterial toxins controlling different functions of G-proteins, were used to demonstrate the kind of G-protein associated with the transport of vitellogenin. The toxins were activated by incubating in 100 mM dithiothreitol at 37℃ for 10 minutes.

The effect of CTX was assayed at two different concentrations of 2 μg/ml and 4 μg/ml. Fig. 2 shows that transport of $^{125}$I-CP into the oocytes of intermediate size class drastically decreased at both concentrations with greater inhibition effect at the higher concentration. During 3 hours of incubation the transport decreased almost linearly with 43% reduction; it reached a plateau of 64% reduction by 12 hours.

The effect of PTX was similar to that of CTX but the reduction effect was not as great as that of CTX. The transport decreased with 40% reduction at 4 μg/ml for 12 hours (Fig. 3).

*Effect of dbcAMP on the transport of vitellogenin*

The effect of dibutyryl cAMP(dbcAMP) on the transport was also assayed in order to see whether the toxin

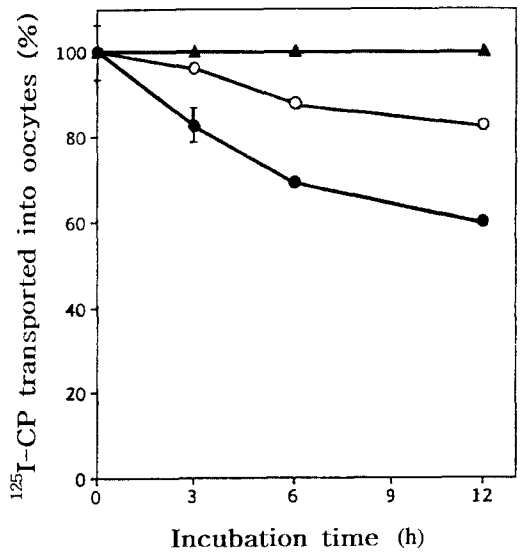


**Fig. 3.** Effects of pertussis toxin on $^{125}$I-CP transport into the oocytes of the intermediate size class. The oocytes were incubated with pertussis toxin at the concentration of 2 μg/ml (○) and 4 μg/ml (●) or without (▲) pertussis toxin.

effects may result simply from the elevation of cAMP by activation of adenylate cyclase affected by G-protein. As shown in Fig. 4, transport of $^{125}$I-CP was not affected at 50 mM dbcAMP but was greatly inhibited at 100 mM dbcAMP with more than 50% reduction.

*Identification of G-proteins*

From the studies on the effects of the toxins and $AlF_4$

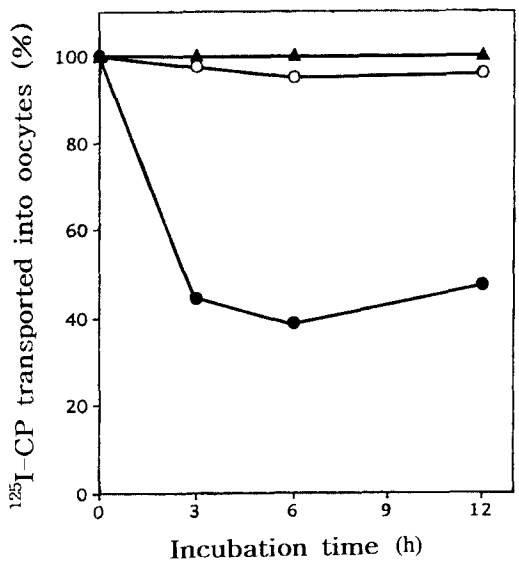


**Fig. 4.** Effects of dbcAMP on $^{125}$I-CP transport into the oocytes of intermediate size class. The oocytes were incubated with dbcAMP at the concentration of 50 mM (○) and 100 mM (●) or without (▲) dbcAMP.

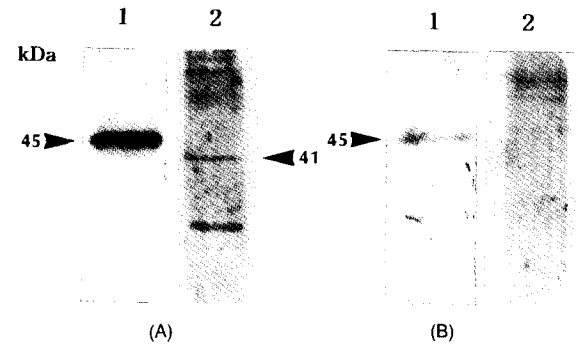


**Fig. 5.** Immunoblots of membrane proteins of the intermediate (A) and the large size classes (B) oocyte with antibodies raised against α subunits of G-protein. Blotting results of immunoblotting with antibodies against $Gs_\alpha$ and $Gi_\alpha$ are displayed in lane 1 and lane 2, respectively. The amount of protein used for gel electrophoresis was 200 μg for the intermediate and 150 μg for the large size class. $Gs_\alpha$ of 45 KDa was detected in both size classes but $Gi_\alpha$ of 41 KDa was detected only in the oocytes of the intermediate size class.

on the transport of $^{125}$I-CP, the G-protein involved in the vitellogenin transport into the oocytes was expected to be heterotrimeric G-protein. In order to characterize G-proteins mediating vitellogenin transport, the membrane proteins of the intermediate and large oocytes were electrophoresed after treatment with β-mercaptoethanol and heat and immunoblotted with rabbit polyclonal IgG against $Gs_\alpha$ and $Gi_\alpha$, which bind specifically to the C-terminal of the G subunit. A protein of 45 kDa, reacting positively to the antibody against the $Gs_\alpha$ subunit, and three proteins of 60 kDa, 41 kDa and 29 kDa, reacting positively to the antibody against the $Gi_\alpha$ subunit, were detected from the membrane proteins of the intermediate size class (Fig. 5). However, from the large size class only the proteins of 45 kDa and 60 kDa were detected and not the 41 kDa protein, which is assumed to be the $Gi_\alpha$ subunit.

## Discussion

The present study on the effects of $AlF_4$ and toxins on the transport of $^{125}$I-CP strongly suggest that heterotrimeric G-protein is involved in the receptor-mediated endocytosis of vitellogenin specifically into the oocytes of intermediate size, in which transport is the most active compared to the small and large size classes (Lee and Kim, 1993). $AlF_4$, a selective inhibitor of heterotrimeric G-protein (Chabre, 1990; Kahn, 1991; Helms, 1995), inhibits budding of clathrin-coated vesicles during endocytosis, in which multiple GTP-binding proteins, including heterotrimeric G-proteins, are involved (Carter et al., 1993). Endocytosis was also inhibited in the oocytes of *Xenopus* when incubated with $AlF_4$ (Mukhopadhyay et al., 1997). The inhibition by $AlF_4$ appears to be closely associated with a serious reduction in the transport of $^{125}$I-CP into the oocytes of *Pseudopotamilla* by CTX in the present study. These results could be due to activation of adenylate cyclase by altering the conformation of the $Gs_\alpha$ subunit of

heterotrimeric G-protein by treating the oocyte membrane with CTX (Janicot et al., 1991). Such interpretation is strongly supported by an observation that the transport of $^{125}$I-CP was greatly reduced by dbcAMP, leading to a speculation that the reduction in the transport of vitellogenin may arise as a result of increased concentration of cAMP. The CTX effect appears to be closely associated with the function of $Gs_\alpha$, since oocyte maturation was inhibited when the oocytes were treated with CTX or G-proteins of the oocyte membrane were bound with the antibody against $Gs_\alpha$ subunit (Maller et al., 1979; Godeau et al., 1980; Gallo, 1995). This inhibition also resulted from the elevation of cAMP by the activation of Gs subunit with CTX (Schultz et al., 1983; Cork et al., 1990).

A significant association between the concentration of cAMP and the transport activity of vitellogenin appears in the immunoblotting studies, in which the $Gs_\alpha$ subunit of 45 kDa was detected in intermediate and large size classes, whereas $Gi_\alpha$ of 41 kDa was found only in the intermediate size class. Two proteins of 60 kDa and 29 kDa which reacted positively to the antibody against the $Gi_\alpha$ subunit were disregarded, as the molecular weight of the $Gi_\alpha$ subunit generally 38-42 kDa. In the oocytes of *Xenopus,* the $Gs_\alpha$ and $Gi_\alpha$ subunits were confirmed to be 43 kDa and 39 kDa, respectively (Gallo et al., 1995). Presence of the $Gi_\alpha$ subunit of 38 kDa was also reported in the oocytes and cell-stage embryos of mouse (Jones and Schultz, 1990). In the oocytes of Chinese hamsters, $Gi_{2\text{-}3}$ subunits of 42 kDa and 45 kDa were detected by immunoblotting tests (Xu, 1996). Detection of the $Gi_\alpha$ subunit specifically in the oocytes of the intermediate size class is very significant in connection with the transport of vitellogenin. Activation of the $Gi_\alpha$ subunit may result in a decrease of cAMP concentration, leading to an enhancement of vitellogenin transport, though the mechanism for this is not clear. However, a previous result that GTP enhanced the transport of vitellogenin, strongly suggested that the transport can be regulated by GTP-binding proteins other than heterotrimeric G-protein (Nam et al., 1996). This result was demonstrated in *Xenopus* oocytes, in which endocytosis is increased by injection of Rab 5 into the oocytes of a mutant with decreased GTPase activity (Mukhopadhyay et al., 1997). It may, therefore, be tentatively concluded that multiple GTP-binding proteins, including small G-proteins as well as heterotrimeric G-proteins, are all involved in the regulation of the vitellogenin transport in the oocytes of *Pseudopotamilla occelata* through the activation of adenylyl cyclase.

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