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Aluminium Inhibits Protein Kinase C Activity in the Liver without Any Effect on Vitellogenin Production in Salmon *Oncorhynchus masou*

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Abstract

Protein kinase C (PKC) in the liver was studied in relation to Al-induced inhibition of vitellogenin (VTG) production in immature and male salmon, *Oncorhynchus masou*. The brain was also studied as a positive control. Addition of Al ($\geqq 10^{-7}$ or 10^{-8} M) to the assay cocktail significantly reduced PKC activity by about 25% in the liver and the brain. Estradiol-17β (E_2) induced marked vitellogenesis on Days 7 and 10 after a single administration and also on Day 3 after the second administration. E_2 doubled PKC activity in the brain, while it had no effect on the activity in the liver. PKC activity was reduced by Al ($\geqq 10^{-8}$ M) to about half of the respective control values in both the brain and liver of the E_2-treated fish. However, no correlation ($r^2=0.061$) between PKC activity in the liver and plasma VTG levels was obtained in the estrogenized fish. These results suggest that Al inhibits VTG production through processes that are unrelated to PKC.

Key words: Aluminium, Brain, Liver, Plasma, Protein kinase C, Salmon, Vitellogenin

Introduction

Aluminium (Al) is a metal that is toxic to fish and reaches high concentrations of 3.7-34.8 μM in acidic rivers and lakes (Brumbaugh and Kane, 1985; Borg, 1986). Such environments have been shown to impair oogenesis due to the poor accumulation of egg yolk in perch *Perca fluviatilis* (Runn et al., 1977) and a reduction in egg number in pupfish *Cyprinodon nevadensis* (Lee and Gerking, 1980). Mugiya and Tanahashi (1998) showed the more direct evidence that Al inhibited the induction of vitellogenin (VTG) synthesis in hepatocyte culture of rainbow trout *Oncorhynchus mykiss*. However, the inhibitory mechanisms remain to be studied.

VTG is an egg yolk precursor protein and is synthesized in the liver in response to estrogen. This protein is transported to the developing ovary and is accumulated in oocytes after splitting into lipovitellin, phosvitin and β components (Ng and Idler, 1983). Phosvitin is a highly phosphorylated molecule containing as much as 50% phosphoserine (Wiley and Wallace, 1981) and contains about 10% phosphorus as esterified orthophosphate in barfin flounder *Verasper moseri* (Matsubara and Sawano, 1995). Protein phosphorylation and dephosphorylation are under control of protein kinases including protein kinase C (PKC), a Ca^{2+}-phospholipid-dependent enzyme. Since an Al concentration of 2×10^{-8} M was reported to inhibit PKC by 90% in the brain of rats (Cochran et al., 1990), there is a possibility that Al inhibits VTG production by inhibiting PKC activity in the liver.

The present study was undertaken to evaluate whether Al inhibits PKC activity in the liver and thereby impairs VTG production in salmon *Oncorhynchus masou*. The brain was also assayed as a positive control, because it is known to have a high activity of PKC (Minakuchi et al., 1981).

Materials and Methods

Salmon *Oncorhynchus masou* weighing about 150 g were obtained from the Hokkaido Fish Hatchery at Mori and kept in outdoor ponds with running water at about 14°C. The salmon were fed trout food pellets once a day except the day of sampling. Maturing females were not included.

Blood sampling and enzyme extraction

Fish were anesthetized with 2-phenoxyethanol and then blood was collected by cutting the tail of fish and by draining the blood into heparinized capillary tubes. Plasma was separated by centrifugation ($350\times g$, 15 min) and used for protein determination and electrophoresis to ascertain vitellogenesis in the fish treated with estradiol.

The liver and the brain were dissected after bleeding, weighed, and homogenized on ice with a Potter homogenizer (50 strokes at 1,000 rpm, GTR-1000; Eyela, Tokyo) in 5 volumes of the solution (0.25 M

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sucrose, 5 mM ethylenediaminetetraacetic acid, 10 mM mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 25 μl/mL aprotinin, pH 7.5). After ultrasonic treatment for 1 min, the samples were centrifuged at 100,000$\times g$ for 60 min (4°C) and the supernatants (cytosol fractions) were used for a PKC assay.

PKC assay

PKC activity was determined using the PKC assay kit (Upstate Biotech., NY) containing 20 mM (*N*-morpholino)propanesulfonic acid (pH 7.2), 25 mM β glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.5 mg/mL phosphatidyl serine, 0.05 mg/mL diacylglycerol, 75 mM $MgCl_2$, inhibitor cocktail (2 μM protein kinase A inhibitor peptide and 20 μM compound R24571), 500 μM PKC substrate peptide, and 500 μM adenosine triphosphate (ATP). Each sample (10 μL) was added to the assay cocktail (40 μL) supplemented with [γ-^{32}P] ATP (74 kBq/nmol ATP, Dupont/NEN, Boston) and incubated at 30°C for 3 min. The reaction was stopped by adsorbing the sample (20 μL) to a p81 phosphocellulose paper (Whatman, Maidstone). The paper was washed three times in 0.75% phosphate solution and once in acetone, dried, and added to 5 ml scintillation cocktail (Scintisol EX-H, Dojindo, Kumamoto) for radioactive counting (LSC 5000; Aloka, Tokyo). This assay kit is suitable not only for column-purified fractions but also for crude cell lysates according to the assay kit instructions.

PKC activity was expressed in terms of the amount (μmol) of inorganic phosphate (Pi) incorporated from [γ-^{32}P] ATP into the substrate/μL of the assay cocktail. The protein concentration in the cocktail was not considered in expressing the results because estrogen also increases liver proteins by synthesizing VTG.

Protein determination and electrophoresis

Protein concentrations of the plasma and PKC assay samples were determined according to the method of Bradford (1976) using Coommassie brilliant blue (CBB) G-250.

Plasma proteins were analyzed by 9.8% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (1970). The gels were run at 30 mA and stained with CBB R-250. Standard proteins used for molecular mass determinations were carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97 kDa), β-galactosidase (116 kDa), and myosin heavy chain (205 kDa).

Experiment 1. Effect of Al on PKC activity

Al ($AlCl_3 \cdot 6H_2O$; Wako, Osaka) was dissolved in distilled water and added to the PKC reaction cocktail at final concentrations of 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. The volume of solvent added was 2 μL/60 μL of cocktail. The control received an equivalent amount of the solvent only.

Experiment 2. Effect of estrogen on PKC activity

If PKC is involved in VTG production, estrogen should increase PKC activity in the liver. To test this idea, fish were intraperitoneally administered with estradiol-17β (E_2; Sigma, St. Louis) at a dose of 1 mg E_2/0.1 mL dimethyl sulfoxide/100 g body weight and sampled after 7 and 10 days. Other fish received E_2 twice on Days 0 and 5 at a dose of 0.5 mg E_2/0.1 mL 90% ethanol/100 g body weight each and were sampled on Day 8. The control received the respective solvents only. The liver and the brain were assayed to examine the effect of E_2 on PKC activity.

The relative amount of plasma VTG was expressed by 60 μg (the amount of total proteins applied to each lane)$\times$ V/T, where V is the integrated optical density (IOD) of the main VTG band with 175 kDa, and T is the IOD of total proteins. The protein band with 175 kDa was identified as the main VTG band by Western blotting and immunoelectrophoresis (Kwon et al., 1993). The IOD was measured by a Bio Image System (Millipore, Bedford).

Experiment 3. Effect of Al on PKC activity in E_2-treated fish

Fish received a single administration of E_2 and were sampled after 7 and 10 days. The dose was the same as described in Exp. 2. Liver and brain PKC were assayed in cocktail containing Al at the same concentrations as described in Exp. 1.

Statistical analysis

Statistical evaluation of differences between mean values was tested by ANOVA (Scheffe's *F*-test). Significance was accepted at $P<0.05$. Ratio data were statistically analyzed after being arcsine transformed.

Results

PKC activity in the extract was about 0.19 Pi pmol/μL for the liver and 0.43 Pi pmol/μL for the brain, being 2.3 times higher the brain than the liver. When Al was added to the assay cocktail at concentrations of $\geqq 10^{-8}$ M for the liver and 10^{-7} M for the brain, the activity decreased significantly to about 75% of the respective control values ($P<0.01$) (Fig. 1).

SDS-PAGE showed that the administration of E_2 induced an intense VTG band with 175 kDa on Days 7

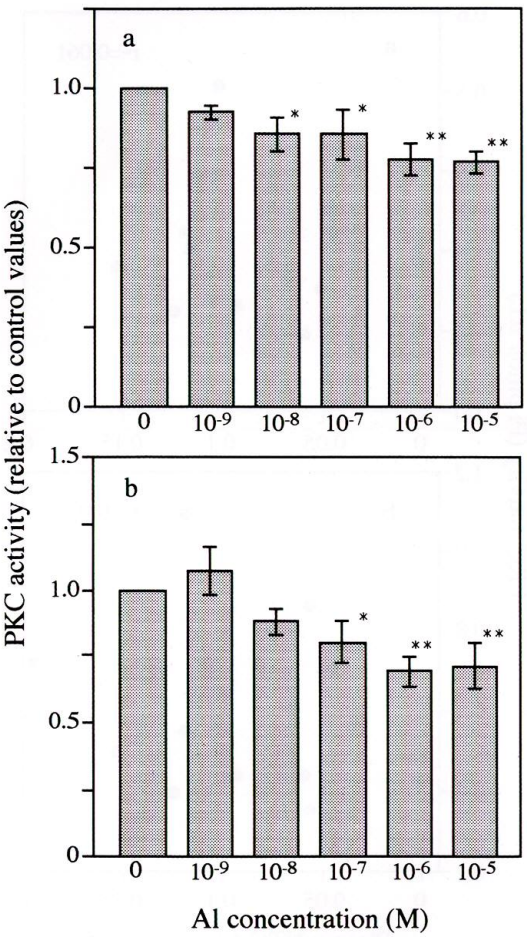

Fig. 1. Inhibition of PKC activity by Al in the liver (a) and the brain (b) of salmon. Vertical bars represent the SE of the mean for five fish. $*P<0.05$, $**P<0.01$ for each control (Al=0 M).

and 10 after a single administration and also on Day 3 after the second administration (Fig. 2). The control fish had no equivalent band.

The liver and the brain obtained from these fully estrogenized fish were subjected to PKC assays. E_2 had no effect of the enzyme activity at any examination times in the liver (Fig. 3), while E_2 characteristically increased the activity in the brain ($P<0.01$) and doubled it on Day 3 after the second administration.

PKC activity was individually plotted against the amount of plasma VTG calculated from SDS-PAGE. No correlation was obtained between PKC activity in the liver ($r^2=0.061$) or in the brain and plasma VTG levels ($r^2=0.077$) (Fig. 4).

The liver and the brain obtained from the estrogenized fish on Days 7 and 10 were assayed for PKC activity after addition of Al. Al ($\geqq 10^{-8}$ M) reduced the activity in both tissues to 82～52% of the respective control values on Day 7 (Fig. 5), showing results similar to those obtained with the E_2-untreated fish as shown in Fig. 1. On Day 10, Al reduced the activity in a concentration-dependent way in the liver and to 75% of the control value in the brain (Fig. 6).

Discussion

PKC is known to play a significant role in the transduction of extracellular signals into intracellular events in many tissues and organs (Nishizuka, 1992). The molecular basis of PKC actions depends on the phosphorylation of serine/threonine residues. Since VTG contains a large amount of phosphoserine, this enzyme

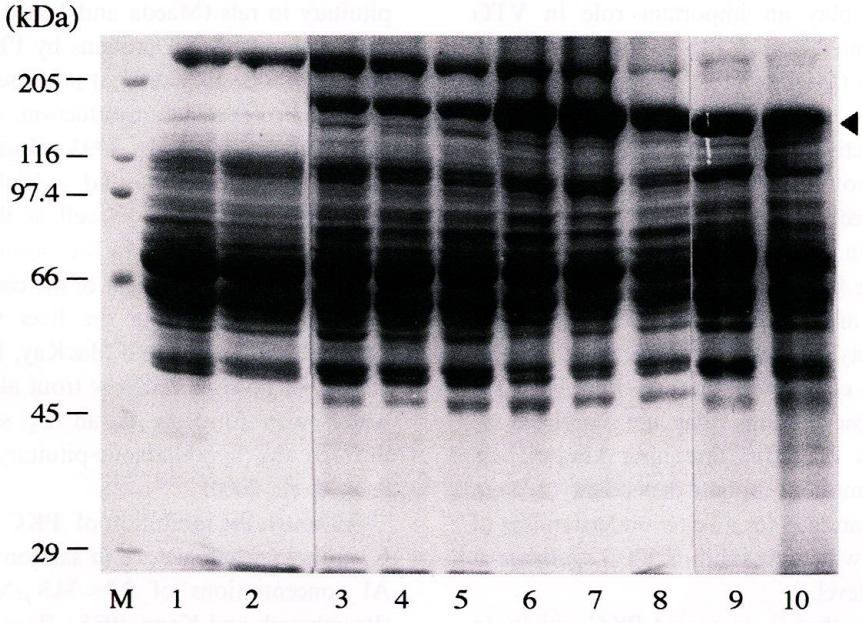

Fig. 2. SDS-PAGE of plasma in E_2-administered salmon, showing the expression of VTG bands (arrowhead). Lanes 1 and 2: control; Lanes 3-5: 7 days after administration; Lanes 6-8: 10 days after administration; Lanes 9 and 10: twice administered, 8 days after the first administration; M: molecular mass markers.

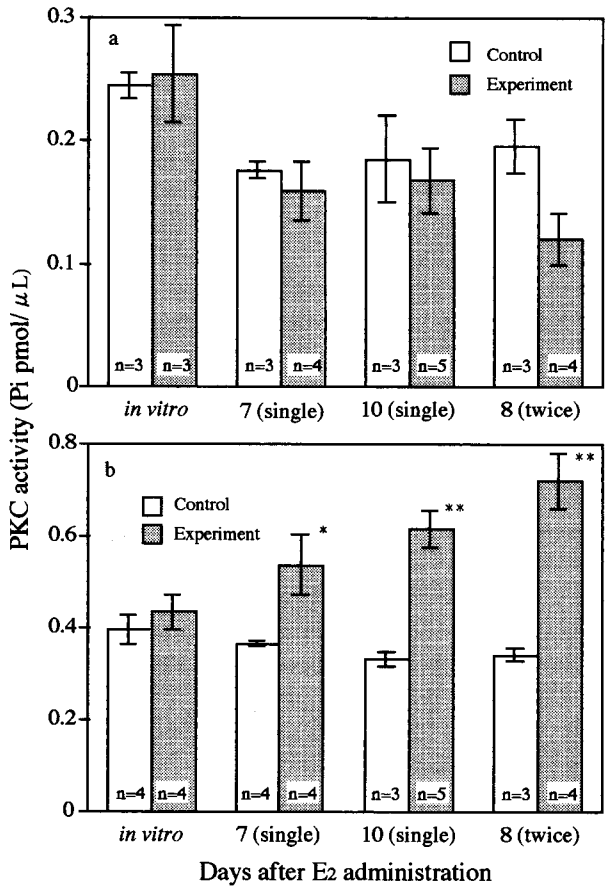

Fig. 3. Effects of E_2 on PKC activity in the liver (a) and the brain (b) in samon. Vertical bars represent the SE of the mean. *In vitro*: E_2 was added to the assay coktail of non-estrogenized fish. $*P<0.05$, $**P<0.01$ for respective controls.

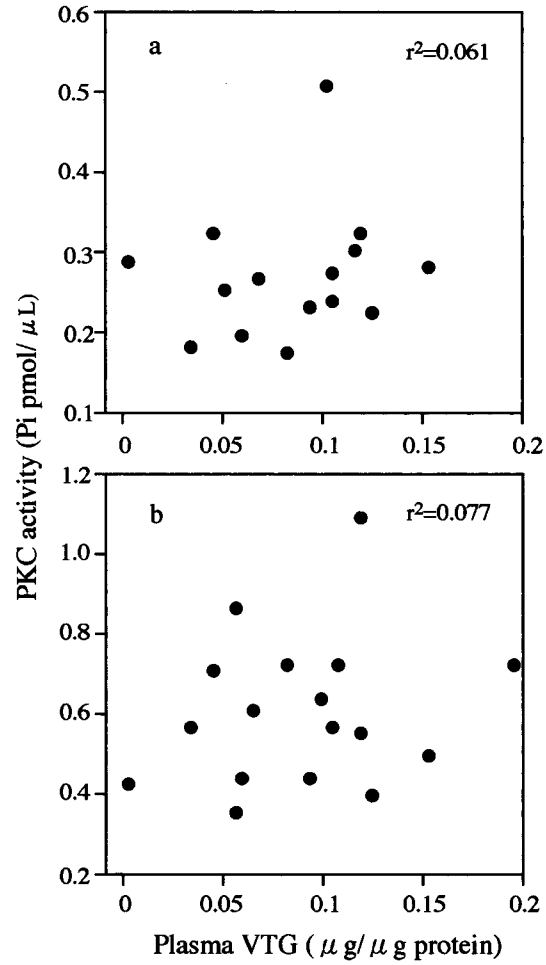

Fig. 4. Relationship between the PKC activity of the liver (a) or the brain (b) and the amount of plasma VTG in E_2-treated salmon.

was considered to play an important role in VTG synthesis. Al inhibited both VTG production in rainbow trout *O. mykiss* (Mugiya and Tanahashi, 1998) and PKC activity in the rat brain (Cochran et al., 1990). Therefore, if PKC activity is inhibited by Al in the liver, VTG production would be impaired. In the present study, Al actually reduced PKC activity in the liver as well as in the brain. However, E_2 did not increase PKC activity in the liver, in which VTG was actively being synthesized, and no correlation was obtained between PKC activity and plasma VTG levels. These facts led us to the conclusion that Al inhibits VTG synthesis through mechanisms that are unrelated to PKC. Other types of serine/threonine kinases, e.g. adenosine cyclic monophosphate-dependent protein kinase, should be examined for a better understanding of the mechanisms by which Al inhibits VTG synthesis at a posttranslational level.

It is not surprising that E_2 increased PKC activity in the brain. This result coincides with the report that estrogen enhanced the level of PKC activity in the pituitary in rats (Maeda and Lloyd, 1993). The phosphorylation of brain proteins by PKC plays an important role in several neuronal processes such as neurotransmitter release, signal transduction, and synaptic plasticity (Murakami et al., 1993; Rajanna et al., 1995). Rogers et al. (2000) found a high expression of E_2 receptors in the brain as well as the liver in Atlantic salmon *Salmo salar*. In the brain these receptors are related to the development of the central nervous system (Callard, 1983) and in the liver they are related to vitellogenesis (Lazier and MacKay, 1993). The hypothalamus of juvenile rainbow trout also has E_2 receptors, which may function as an E_2 secretion modulator through the hypothalamic-pituitary-gonadal axis (Allison et al., 2000).

Although the inhibition of PKC by Al did not affect E_2-induced vitellogenesis in salmon *O. masou*, elevated Al concentrations of 3.7~34.8 μM in acidic waters (Brumbaugh and Kane, 1985; Borg, 1986) may result in dysfunction of physiological processes through depressing phosphorylation in some tissues, because the present

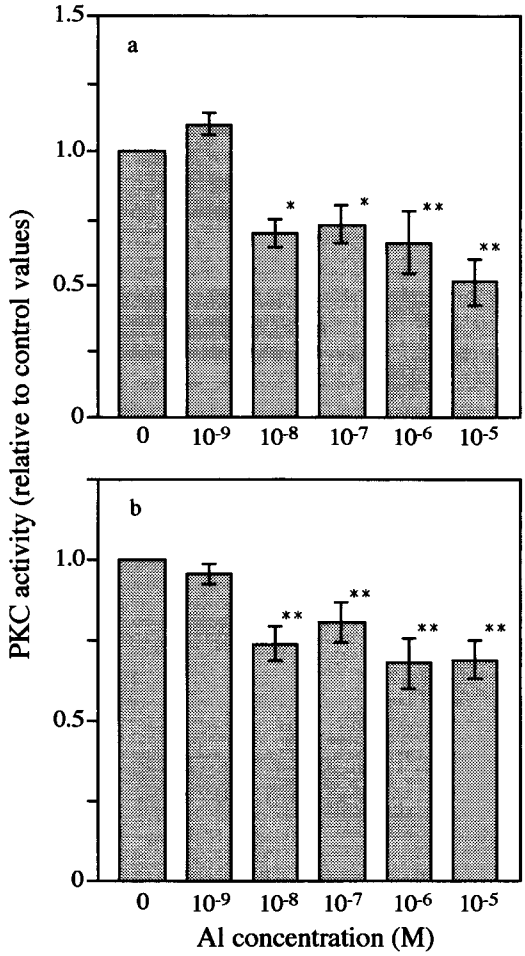

Fig. 5. Inhibition of PKC activity by Al in the liver (a) and the brain (b) 7 days after E_2 administration in salmon. Vertical bars represent the SE of the mean for five fish. $*P<0.05$, $**P<0.01$ for each control (Al= 0 M).

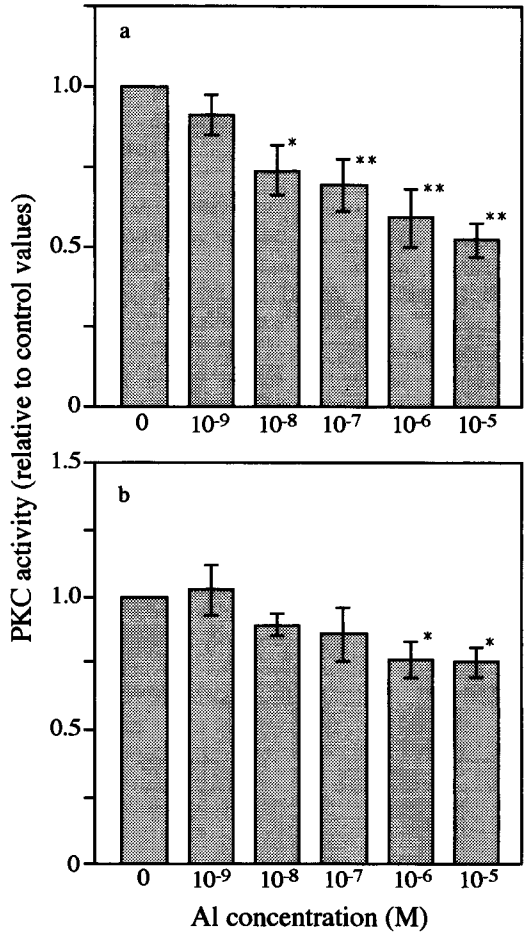

Fig. 6. Inhibition of PKC activity by Al in the liver (a) and the brain (b) 10 days after E_2 administration in salmon. Vertical bars represent the SE of the mean for five fish. $*P<0.05$, $**P<0.01$ for each control (Al= 0 M).

study showed that low concentrations (10^{-7} M$\sim$$10^{-8}$ M) of Al inhibited PKC activity in the brain and the liver.

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