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Expression of Vasopressin V2 Receptor in Xenopus laevis Oocytes by Porcine Kidney Cell Line (LLC-PK1) Messenger RNA

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Vasopressin V2 receptor was expressed in Xenopus laevis oocytes which were injected with poly(A)$^+$RNA from porcine kidney cell line LLC-PK1. Pharmacological antagonism of the expressed V2 receptor was observed between arginine vasopressin and two potent and selective vasopressin antagonists: [d(CH_2)$_5$,D-Phe^2,Ile^4,Ala^9-NH_2]arginine vasopressin and [d(CH_2)$_5$,D-Ile^2,Ile^4]arginine vasopressin. Activation constant for arginine vasopressin concentration was 1.32×10^{-10}M. The nucleotide length of the mRNA encoding for vasopressin V2 receptor was deduced to be approximately 2 kilobases.

Vasopressin, a posterior pituitary octapeptide, is a hormone which regulates blood pressure and body fluid maintenance. Vasopressin receptors are grouped in two types (V1 and V2); V1 receptors on vascular smooth muscle (1) and on hepatocytes (2) which modulate vasopressor and glycogenolytic responses to vasopressin, are linked to phosphatidylinositol turnover and alteration of intracellular calcium concentration (3). V2 receptors on renal tubules which modulate antidiuretic responses to vasopressin, are linked to activation of adenylate cyclase and cyclic AMP production (4). Meyerhof et al showed expression of V1 receptor by electrophysiological method in Xenopus laevis oocytes injected with rat liver poly(A)$^+$RNA (5). However, they were unable to detect expression of V2 receptor in oocytes injected with poly(A)$^+$RNA from rat kidney. In this report vasopressin V2 receptor was shown to be expressed in Xenopus

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laevis oocytes when they were injected with poly(A)$^+$RNA from V2 receptor-containing porcine kidney cell line LLC-PK1, through direct measurement of vasopressin-dependent adenylate cyclase activity in the oocyte membranes. The nucleotide length of the mRNA encoding for V2 receptor was deduced.

MATERIALS AND METHODS

[d$(CH_2)_5$,D-Phe^2,Ile^4,Ala^9-NH_2]arginine vasopressin and [d$(CH_2)_5$,D-Ile^2,Ile^4]arginine vasopressin were from Peninsula Laboratories. Arginine vasopressin was from Peptide Institute. Xenopus laevis was from a local animal supplier, Hamamatsu Seibutsu Kyozai Co.

Cell culture---LLC-PK1 cell line was a gift from Japanese Cancer Research Resources Cell Bank. The cells were grown in medium 199 supplemented with 3% fetal bovine serum.

RNA extraction and poly(A)$^+$RNA purification---RNA was extracted from the LLC-PK1 cells by guanidine thiocyanate/CsCl centrifugation method (6). Poly(A)$^+$RNA was purified from the total RNA by passing through oligo dT cellulose column twice. Resulting poly(A)$^+$RNA was heated at 70° C for 4 min and cooled immediately on ice, then it was layered on top of 10-30% sucrose linear gradient solution containing 50 mM Tris-HCl,pH 7.5, 0.2 M LiCl, 1 mM EDTA, 0.2% SDS, and centrifuged at 25,000 rpm for 20 hours at 4° C. At the end of centrifugation the tube contents were fractionated and ethanol precipitated at -20° C. The precipitated poly(A)$^+$RNA was washed with ethanol three times, and dissolved in H_2O, and used for microinjection.

Microinjection of Xenopus laevis oocytes---Mature female Xenopus laevis was induced to ovulate by HCG treatment 2 to 5 weeks prior to sacrifice. The frog was killed by decapitation and the ovary was dissected into small pieces. The pieces of the ovary were digested with 2% collagenase (Sigma type 2) in Barth's medium (7) at 20° C for 3 hours with occasional shaking. The oocytes were defolliculated by forceps and incubated at 20° C overnight. Intact oocytes of the stage V (Dumont) were injected with poly(A)$^+$RNA (3 mg/ml, 20 nl). The injected oocytes were incubated in Barth's medium at 20° C for 4 days.

Adenylate cyclase measurement---The oocytes were pinched in a flat forceps to remove the contents off. The oocyte membranes were washed in 5 mM Hepes-NaOH,pH7.5, 3 mM $MgCl_2$, 1 mM EDTA twice and homogenized. Vasopressin-dependent cyclic AMP production of the membrane was performed by the method described previously (8), but in a much smaller reaction volume (8 µl). The amount of cyclic AMP produced was determined by radioimmunoassay (YAMASA cyclic AMP kit).

RESULTS

Xenopus laevis oocytes do not have vasopressin V2 receptor in nature, but they have guanine nucleotide binding regulatory

protein of the stimulative type, Gs and catalytic unit of adenylate cyclase. When V2 receptor is newly synthesized and incorporated into oocyte membranes in the same way as reported in many other membrane receptors (9,10,11), the V2 receptor should couple to the Gs and the catalytic unit, which would result in emergence of vasopressin-dependent adenylate cyclase activity in the oocyte membrane.

Vasopressin-dependent increase of the adenylate cyclase activity of the oocytes which had been injected with total poly(A)$^+$RNA from LLC-PK1 cells, was very small. Therefore, the poly(A)$^+$RNA was further fractionated by sucrose-density gradient centrifugation and these fractions were injected into oocytes (Fig 1). Fractions number 24, 25 and 26 showed vasopressin-dependent increase of adenylate cyclase activity. From absorbance profile at 260 nm, the size of the mRNA encoding for vasopressin V2 receptor was 18s.

An aliquot of the fraction of poly(A)$^+$RNA, which gave the highest vasopressin-dependent cyclic AMP production, was injected into oocytes, and using the injected oocyte membranes pharmacological antagonism of the expressed V2 receptor between arginine vasopressin and two potent and selective vasopressin V2 antagonists was observed (Fig 2). Potent and selective vasopressin V2 antagonist:[d$(CH_2)_5$,D-Ile^2,Ile^4]arginine vasopressin decreased vasopressin-dependent adenylate cyclase activity significantly ($p<0.05$). More potent vasopressin V2 antagonist:[d$(CH_2)_5$,D-Phe^2,Ile^4,Ala^9-NH_2]arginine vasopressin decreased vasopressin-dependent adenylate cyclase activity more significantly ($p<0.01$).

The activation constant for arginine vasopressin concentration was calculated from the data in Fig 2 inset and it was 1.32×10^{-10} M, which was in good agreement with the activation constant of

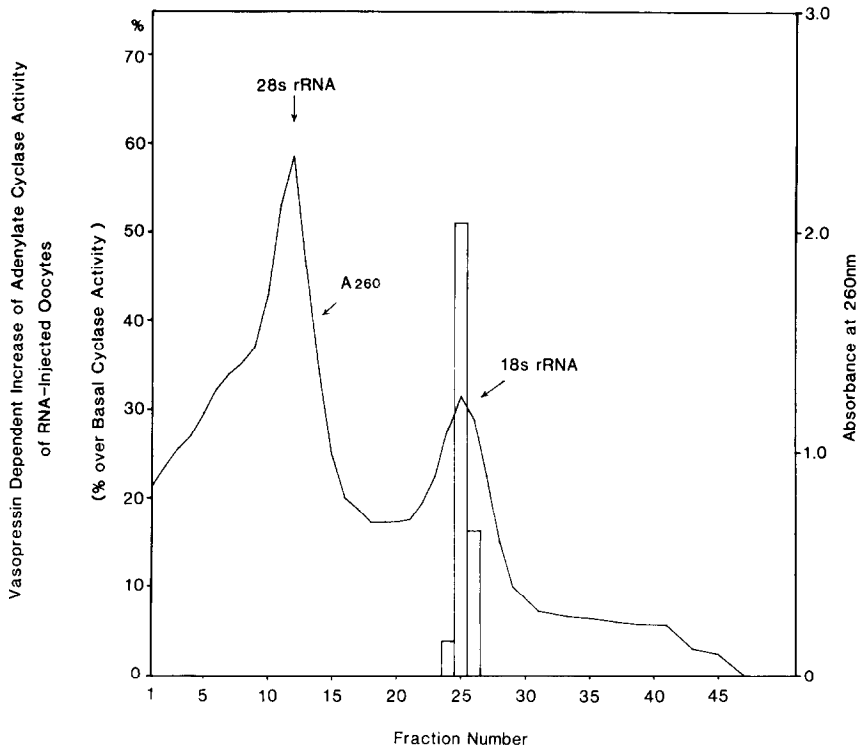

Fig. 1. Vasopressin-dependent adenylate cyclase activity of the oocytes injected with LLC-PK1 poly(A)$^+$RNA fractions separated through sucrose density gradient centrifugation. LLC-PK1 poly(A)$^+$ RNA (1.2 mg) was fractionated on a 37 ml 10-30% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 0.2 M LiCl, 1 mM EDTA, 0.2% SDS at 25,000 rpm for 20 hours at 4° C. Forty-seven fractions were collected; RNA in each fraction was precipitated with ethanol, washed with ethanol tree times, and dissolved in 5 µl H_2O. An aliquot (approx. 30 ng per oocyte) of each fraction was injected into Xenopus oocytes. Adenylate cyclase activity of the membranes of the injected oocytes were determined in the presence and absence of 10^{-7} M arginine vasopressin. Open columns stand for vasopressin-dependent cyclic AMP production of the injected oocyte membranes.

native vasopressin-dependent adenylate cyclase of LLC-PK1 cells. Since the oocytes, which had been injected with H_2O or the oocytes which had not been injected at all, did not show any vasopressin-dependent increase of adenylate cyclase activity, it was concluded that vasopressin V2 receptor was expressed in the oocytes injected with the poly(A)$^+$RNA from LLC-PK1 cells, and that nucleotide length of the mRNA encoding for vasopressin V2 receptor was approximately 2 kilobases.

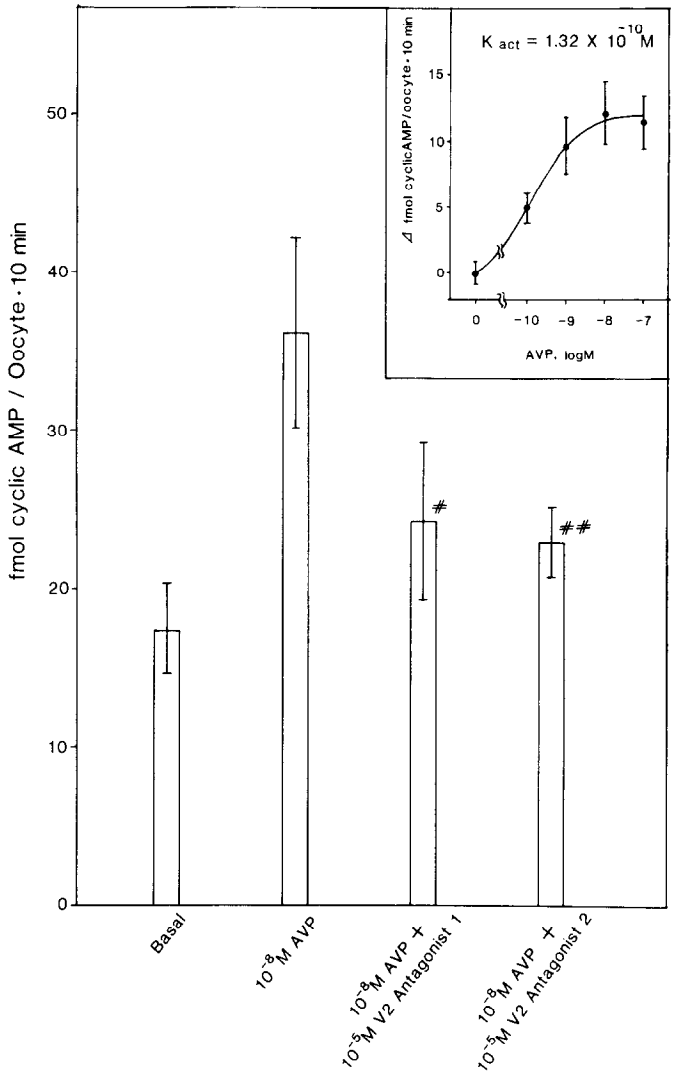

Fig. 2. Adenylate cyclase activity of the oocytes injected with poly(A)$^+$ RNA fraction of the highest activity in Fig 1 in the presence of arginine vasopressin and two vasopressin V2 antagonists. Sixty ng poly(A)$^+$ RNA of the fraction number 25 in Fig 1 was injected in each oocyte. Adenylate cyclase activity of the injected oocyte membranes was determined in the presence of arginine vasopressin (AVP) and two vasopressin V2 antagonists: V2 antagonist-1:[d(CH_2)$_5$,D-Ile^2,Ile^4]arginine vasopressin, V2 antagonist-2:[d(CH_2)$_5$,D-Phe^2,Ile^4,Ala^9-NH_2]arginine vasopressin. # and ##:statistically significant decrease from 10^{-8} M arginine vasopressin in t-test, #:$p<0.05$, ##:$p<0.01$. Inset, Arginine vasopressin dose-dependence of adenylate cyclase activity of the injected oocyte membranes. Activation constant of 1.32 x 10^{-10} M was calculated from a double reciprocal plot.

DISCUSSION

The data presented here indicate that vasopressin V2 receptor was expressed in Xenopus laevis oocytes when they were injected with

poly(A)$^+$RNA from V2 receptor-containing porcine kidney cell line LLC-PK1. Meyerhof et al showed that vasopressin V1 receptor was expressed in Xenopus laevis oocytes which were injected with rat liver poly(A)$^+$RNA. However, they could not ascertain expression of V2 receptor by electrophysiological method in the oocytes which were injected with rat kidney poly(A)$^+$RNA (5). Then, they proposed two possibilities. One is that only those receptors which are linked to phosphatidylinositol turnover and alteration of intracellular calcium concentration, may elicit electrophysiological responses in the injected oocytes. The other is that V2 receptor would have a complex assembly composed of more than one subunit. The data presented here indicate the former possibility. Because the LLC-PK1 poly(A)$^+$RNA of a discrete size was able to induce expression of vasopressin V2 receptor in oocytes, possibility of a complex assembly of V2 receptor was unlikely.

Understanding of molecular nature of the receptors necessitates their molecular cloning and analysis of the molecular basis of their function. But many receptors exist in biological membrane in a very small amount and purification of them is usually very difficult. Recently, a novel technique utilizing Xenopus laevis oocytes was devised to clone receptor genes without receptor protein purification step (12). However, this technique is based on receptor-evoked intracellular events, i.e. phosphatidyl-inositol turnover and alteration of intracellular calcium concentration, which induce opening of Cl^- or K^+ channels of Xenopus oocytes. Adenylate cyclase-coupled receptors which are expressed in oocytes are not known to induce opening of these channels, or they might be inducing to open the channels at too low a level to be detected electrophysiologically. The method presented in this paper may indicate possibility of cloning

adenylate cyclase-coupled membrane receptors including vasopressin V2 receptor.

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