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Effects of activation of protein kinase C on the regulation of atrial natriuretic peptide(ANP) by isolated perfused left atria

Chang-won Kang, Hyung-sub Kang, Ho-il Lee

College of Veterinary Medicine, Chonbuk National University
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백서의 심방관류모델에서 protein kinase C의 활성화가 atrial natriuretic peptide(ANP) 조절에 미치는 영향

강창원 · 강형섭 · 이호일

전북대학교 수의과대학
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초 록 : 심방근 세포는 심방이뇨호르몬을 합성, 저장 그리고 분비하며, 세포내외 이온의 농도, 수분균형 및 혈압 등을 조절하는 것으로 알려져 있다. 또한 심방근의 인장자극에는 Atrial Natriuretic Peptide(ANP)를 2단계(분비, 유리)의 과정으로 이루어져 있으며, 이에 따른 심방이뇨호르몬의 분비 조절기전에 대하여서는 명확히 알려져 있지 않다. 따라서 본 연구는 백서의 심방근 적출관류 모델을 이용하여 protein kinase C와 ANP 조절의 상관관계를 밝히고 분비와 유리의 과정중 어떠한 과정을 이용하여 분비자극에 영향을 주는지를 관찰하기 위하여 본 실험을 실시하였다. PKC 활성제인 PMA(phorbol 12-mystrate 13-acetate)는 ANP의 유리를 현저하게 증가시켰으며, PKC 억제제인 H-7(1-(5-isoquinoline sulfonyl)-2-methyl piperazine dihydrochlo-ride)에 의해 유리를 억제시켰다. PMA와 H-7을 동시에 처리한 경우 PMA에 의하여 증가된 ANP의 유리가 H-7에 의하여 차단됨을 관찰할 수 있었다.

따라서 백서의 관류 심방에서의 ANP 분비 증가는 PKC 활성화에 의하여 이루어지며, ANP분비의 2단계중 ANP 유리에 영향을 줌을 알 수 있었다.

Key words : atrial natriuretic peptide, protein kinase C, heart.

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Address reprint request to Dr. Chang-won Kang, College of Veterinary Medicine, Chonbuk, National University.

Introduction

Atrial myocytes synthesize, store and release atrial natriuretic peptide (ANP). ANP is involved in the regulation of extracellular fluid volume and blood pressure[1] such as natriuresis, diuresis, relaxation of vascular smooth muscle and suppression of renin-angiotensin-aldosterone system. Among the factors related to the regulation of ANP secretion, atrial stretch and an increase of atrial rate have been considered major stimuli[2,3,4,5,6]. Stretch of atrial myocytes changes in the rate of contraction and multiple neurohumoral and pharmacological agents stimulate ANP secretion[7]. Other group have demonstrated that the increase of ANP secretion in response to atrial stretch is caused by the reduction in atrial distension rather than by the distension[8,9,10]. In addition, ANP secretion is regulated by a sequential mechanism. Extracellular fluid with ANP is released into the intercellular space and then translocated by atrial contraction into the atrial lumen[11]. It has also been proposed that the atrial secretion of ANP in the nonbeating atria is regulated by a two-step sequential mechanism[11,12]. First, ANP is released from myocytes into the surrounding paracellular space. Second, translocation of the ANP-containing extracellular fluid (ECF) into the bloodstream is induced by atrial contraction.

The stimulation of phosphoinositide hydrolysis by agonists is an important signal transduction mechanism in many tissues[12,13]. When a ligand binding receptors on the cell surface, inositol phospholipids are hydrolyzed and then produced diacylglycerol and inositol triphosphates are produced. The primary effect of diacylglycerol is activated protein kinase C(PKC) and phosphorylate many proteins. The phosphodiester cleavage of phosphatidylinositol-4,5-bisphosphate by phospholipase C produces both inositol triphosphate(IP_3) and diacylglycerol (DAG). IP_3 induces mobilization of intercellular stored Ca^{2+}, whereas DAG activates PKC[14]. The cellular processes involved in the mediation of ANP release by PKC are not clear yet.

In this study, we investigated the effect of PKC on the release and the secretion of ANP in rat left atria during the two step sequential mechanism and regulation of ANP related to the via the PKC pathway in isolated nonbeating perfused atrial preparations.

Materials and methods

Isolated perfused atrial preparation : Male Sprague-Dowley rats (0.2~0.3kg) were sacrificed by decapitation and chests were opened. Isolated perfused atrial preparation were prepared by the modificated method from described previously[3]. Briefly, the hearts were rapidly dissected and placed in oxygenated warm saline (37℃). Left atria were dissected from hearts and were separately perfused. An isolated tygon[4] made from three small catheters was inserted into the left atria and the atria were dissected. One catheter in the apex, was for inflow; one catheter, adjacent to the atrium, was for the outflow. New isolated probe-tip was used to prevent air-generating in atria. The isolated probe-tip was secured by ligature around the atrioventricular sulcus. The other catheter was used to record pressure changes in the atrium. The cannulated atrium was then transferred to an oxygen chamber (36.5℃). Atria were immediately perfused with Krebs-Henseleit bicarbonate buffer solution using a peristaltic pump (0.45ml/2min) for each tube. The perfusate was prewarmed (36.5℃) by passage through a water bath and equilibrated with 95% O_2 - 5% CO_2 mixture by passage through silicone tubing in gas mixing chamber. Perfused gases and pH of the buffer was monitored via pericardiac sampling and measured with a Corning 175 automatic pH-blood gas system to ensure that pH was maintained at 7.4. The organ chamber was sealed air tight with one outlet connected to a calibrated microcapillary tube. Atrial volume changes were monitored by measuring the length of the column of buffer solution within organ chamber. One of the catheter was connected to physiograph (MK-IV, Narco Biosystems, Houston, TX, USA).

Experimental protocol : Atria were perfused with ^{3}H-inulin for 30 min. to stabilize and equilibrate the extracellular space(ECS) with ^{3}H-inulin. Pericardial medium was introduced by ^{3}H-inulin at the start of atrial perfusion. The atria were perfused for 30 min to stabilize ANP secretion rate and equilibrate the ECS with a marker in baseline, disten-

sion, during distension and reduction were repeated several times. The perfusate was collected in siliconized tubes at 4℃. ECF translocation radio-labelled inulin was added to the pericardial space of the organ chamber with cold inulin as a carrier. In one series of experiments, after four collection periods at the baseline distension, atrial distension was induced by changing the elevation of the out-flow catheter tip by 1, 2, 4 and 6 cmH_2O above the atrium. Every 2 min. of induced atrial distension was followed by an induced atrial volume reduction for 8 min. Atrial volume was reduced by lowering the elevation of the out-flow catheter tip. Coincidentally, rapid transmural atrial transports of ^{3}H-inulin from pericardial space to atrial lumen were measured.

These experiments were carried out using 3 protocols. In protocol 1, the groups of atria were perfused, with regular buffer (n=13), and buffer containing phorbol 12-myristate 13-acetate (PMA, $5x10^{-8}$M, 10^{-7}M, $5x10^{-7}$M), a protein kinase C activator. The pericardial solution was replaced with the ^{3}H-inulin perfusate. After four collection periods with the same solution at the basal rates, atria were distended by elevating the outflow catheter tip 1, 2, 3 and 6 cmH_2O above the atria. In protocol 2,the groups of atria were perfused with regular buffer or 1-(5-isoquinolinesulfonyl) 2-methylpiperazine-dihydrochloride (H-7, 10^{-7}M) buffer, a PKC blocker. The pericardial solution was replaced as in protocol 1. In protocol 3, we had two groups. In one group, 10^{-7}M PMA was perfused alone. In the other group, both 10^{-7}M H-7 and 10^{-7}M PMA buffer were perfused simultaneously. The pericardial solution was replaced as in protocol 1 and 2.

Measurement of ANP by radioimmunoassay : Concentration of ANP in the perfusate, was directly measured by radioimmunoassay as described previously[3]. The radioimmunoassay was performed in tri-acetate buffer (0.1M, pH 7.4), containing 0.2% neomycin, 1.0mM EDTA, 50 benzoyl arginine ethyl ester units/ml soy bean trypsin inhibitor, 200KIU/ml aprotinin, 0.4% phyenyle-methyl sulfamyl fluoride, 0.02% sodium azide and 1% bovine serum albumin. Standard and perfusate sample (100μl) were incubated with antibody for 24hr at 4℃. Samples were incubated with ^{125}I-ANP(specific activity) for 24hr at 4℃. Separation of free tracer(%) from antibody-bound tracer was achieved by adding 1ml of dextran-charcoal suspension. Anti-ANP antibody equally reacted with atropeptides III and rat-ANP(1-28). Several dilutions of the perfusate inhibited the binding of ^{125}I-ANP to the antibody parallel with the standard curve. Radioimmunoassay for irANP was done on the day of experiments. The secreted amount of ANP (ng/ANP/2min) was calculated by multiplying the perfusate volume (2min) with the irANP concentration (ml).

Measurement of the clearance of ECF marker : The ECF translocated was measured as described previously[11,12]. The radioactivities of atria perfusate and pericardial buffer solution were measured with a liquid scintillation system (Tri-Carb 300C, Parkard, Downers Grove, IL) and the amount of ECF translocated through the atrial wall was calculated as follows:

ECF translocated in μl/s/g atrial wet wt(μl/2min/g) =

$$\frac{\text{total radioactivity in perfusate in cpm/s(cpm/2min)}}{\text{radioactivity in the pericardial reservoir in cpm/}\mu\text{l}}$$

divided by atrial wet wt.(mg) x 1,000.

Radioimmunoassay of immunoreactive ANP : Immunoreactive (ir) ANP in the perfusate was measured by radioimmunoassay as described previously[3]. A single radioimmunoassay was performed on the same day of experiments. Nonspecific binding was < 3%. The 50% intercept was at 26.6±2.9pg/tube. The secreted amount of ANP was expressed as ng of ANP per 2 min per g of tissue. The molar concentration of irANP released was calculated as follows;

$$\text{ANP released } (\mu M) = \frac{\text{ANP in pg/s/g}}{\text{the ECF translocated in } \mu\text{l/s/g}} / 3060$$

The denominator 3,060 refers to the molecular mass of ANP1-28 (Da), since the ANP secreted was found to be mainly processed ANP[8,12].

Statistical analysis : Statistical significance of difference of means were tested using Student,s *t* -test and was defined as a p-value of less than 0.05. Linear regressions were calculated by the least-squares method. The results were given as mean±SEM.

Results

PMA effect on stretch-induced ANP secretion : As shown in Fig 1, changes of ECF translocation increased the amount of ANP secretion. ANP secretion was linear by ECF translocation. ANP secretion depended on the concentration of PMA ($5x10^{-8}$M, 10^{-7}M, $5x10^{-7}$M). ANP secretion was increased by PMA concentration in a dose-dependent manner but the amount of ANP secretion was not influenced by $5x10^{-8}$M PMA compared to control groups. As a result, Fig 2 showed that 10^{-7}M PMA increased the basal rate of ANP secretion more than control groups. The basal rate of ANP secretion was 26.20±6.98ng/2min/g in the perfused left atria of rat with regular buffer solution. Atrial stretch-and-release of control groups by 1, 2, 4 and 6 cmH_2O resulted in increased ANP secretion, with peak volumes of 27.84±5.54, 51.39±8.89, 68.94±9.23 and 108.13±28.90ng/2min/g, respectively. All peaks were significantly different from the basal rate. 10^{-7}M PMA buffer solution accentuated ($p<0.05$) the basal rate of ANP secretion 46.4±8.41ng/2min/g compared to control group (26.20±6.98ng/2min/g). Atrial stretch-and-release by 1, 2, 4 and 6

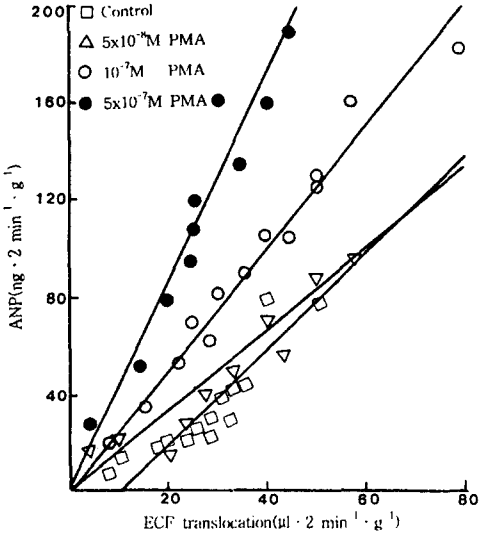

Fig 1. A dose response curve of PMA on ANP secretion. ECF, extracellular fluid; ANP, atrial natriuretic peptide.

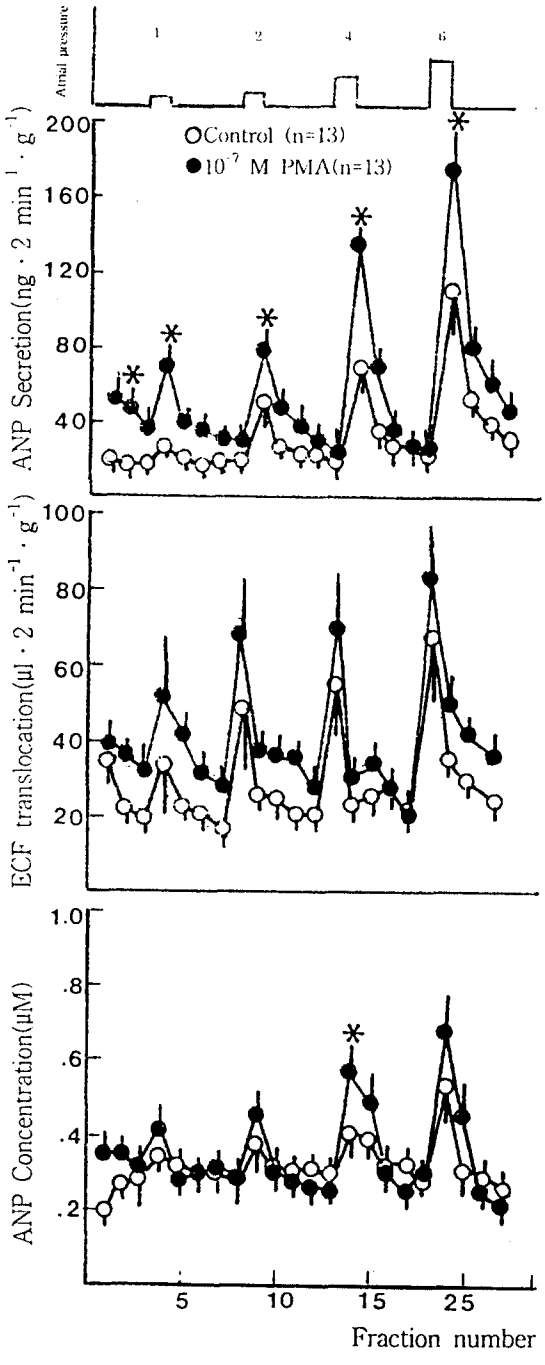

Fig 2. Effect of 10^{-7}M PMA, on ANP secretion, ECF translocation and ANP concentration in the isolated perfused rat atria. Atrial natriuretic peptide was induced by changing the elevation of the outflow catheter tip by 1,2,4 and 6cmH_2O above the atrium. ANP, atial natriuretic peptide; ECF, extracellular fluid. Statistical comparisons were done by student's paired *t*-test. This results are mean±SE. *$p < 0.05$.

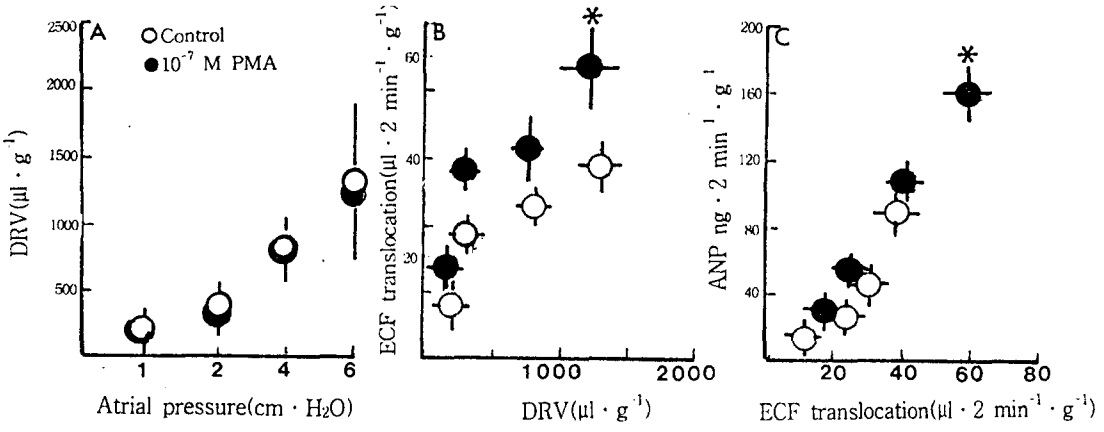

Fig 3. (A) Effect of 10^{-7}M PMA, on the DRV in pressure changes. (B) Effect of 10^{-7}M PMA, response in ECF translocation to increasing DRV. (C) ANP secretion to increasing ECF translocation in left rat atria. ANP, atial natriuretic peptide ; ECF, extracellular fluid ; DRV, distension reduction volume. *p < 0.05.

cmH_2O increased ANP secretion by 10^{-7}M PMA treatment, 58.22±6.98, 78.24±8.21, 135.04±5.41 and 176.05±16. 12ng/2min/g, respectively. ECF translocation tended to increase, but was not different between treatments. ANP concentration in response to pressure elevations were significantly different from 4 cmH_2O, but 1, 2 and 6cmH_2O pressure elevations did not change of ANP concentration (Fig 2). The distension-reduction volume (DRV) was not af-

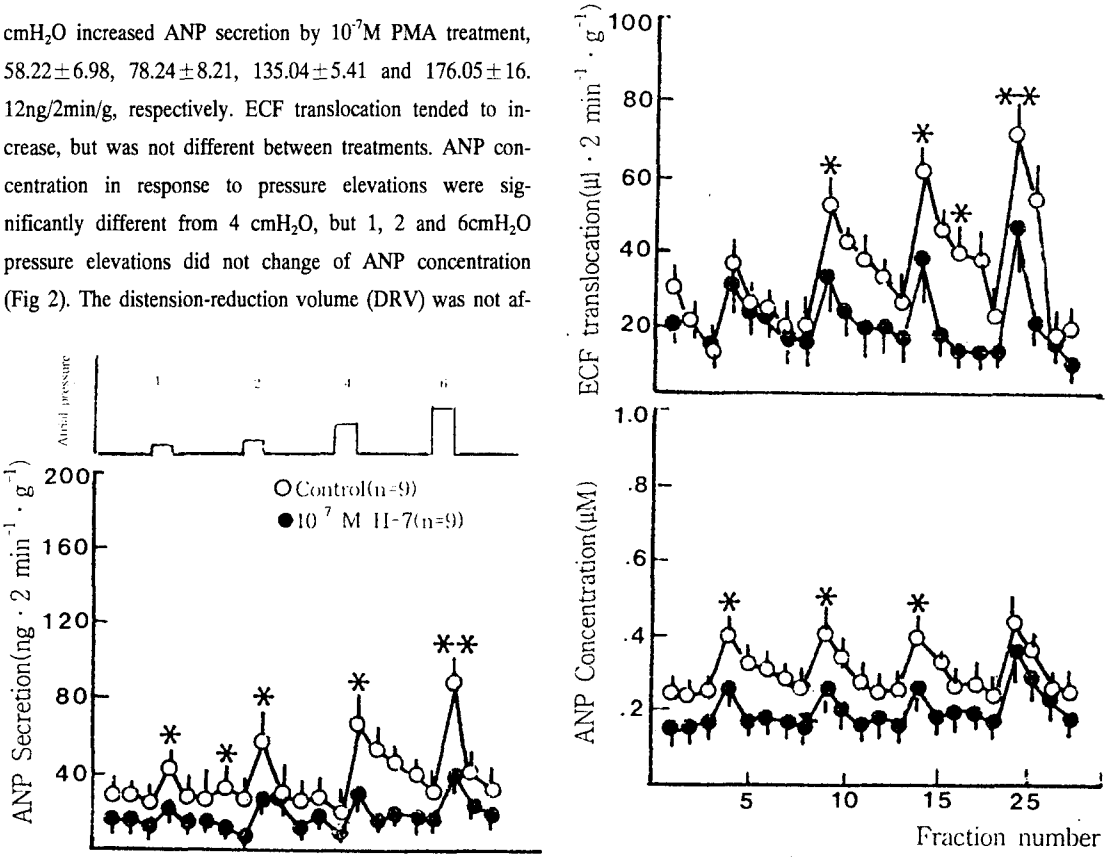

Fig 4. Effect of 10^{-7}M H-7 on ANP secretion, ECF translocation and ANP concentration in the isolated perfused rat atria. Atrial natriuretic peptide was induced by changing the elevation of the outflow catheter tip by 1, 2, 4 and 6cmH_2O above the atrium. ANP, atrial natriuretic peptide; ECF, extracellular fluid, Statistical comparisons were done by student's paired *t*-test. This results are mean± SE. *p < 0.05, **p < 0.01.

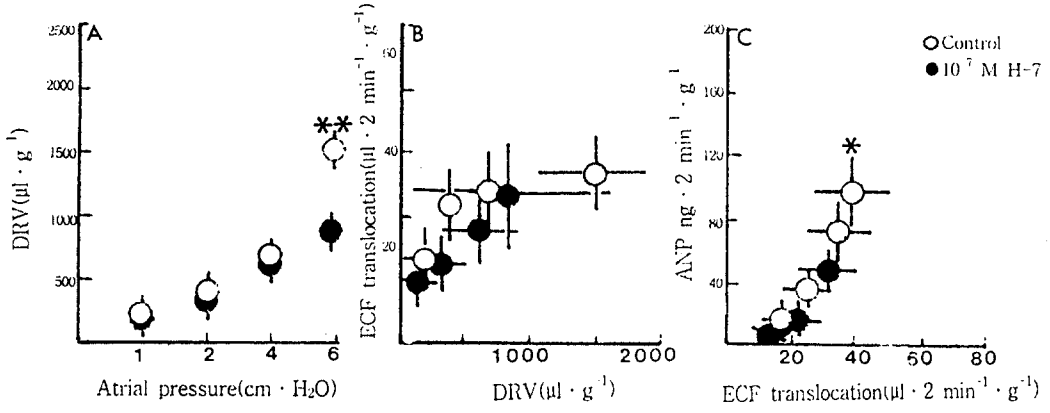

Fig 5. (A) Effect of 10^{-7}M H-7 on the DRV in pressure changes. (B) Effect of 10^{-7}M H-7, response in ECF translocation to increasing DRV. (C) ANP secretion to increasing ECF translocation in left rat atria. ANP, atrial natriuretic peptide ; ECF, extracellular fluid ; DRV, distension reduction volume, $^{*}p < 0.05$, $^{**}p < 0.01$.

fected by the 1, 2, 4 and 6 cmH_2O atrial pressure compared to control groups (Fig 3A). ECF translocation was significantly different between 10^{-7}M PMA and control groups with change of DRV (Fig 3B), 10^{-7}M PMA increased the amount of ANP secretion compared to control groups in ECF translocation (Fig 3C). The results of this experiments suggest that 10^{-7}M PMA increase ANP release from the atrial myocytes.

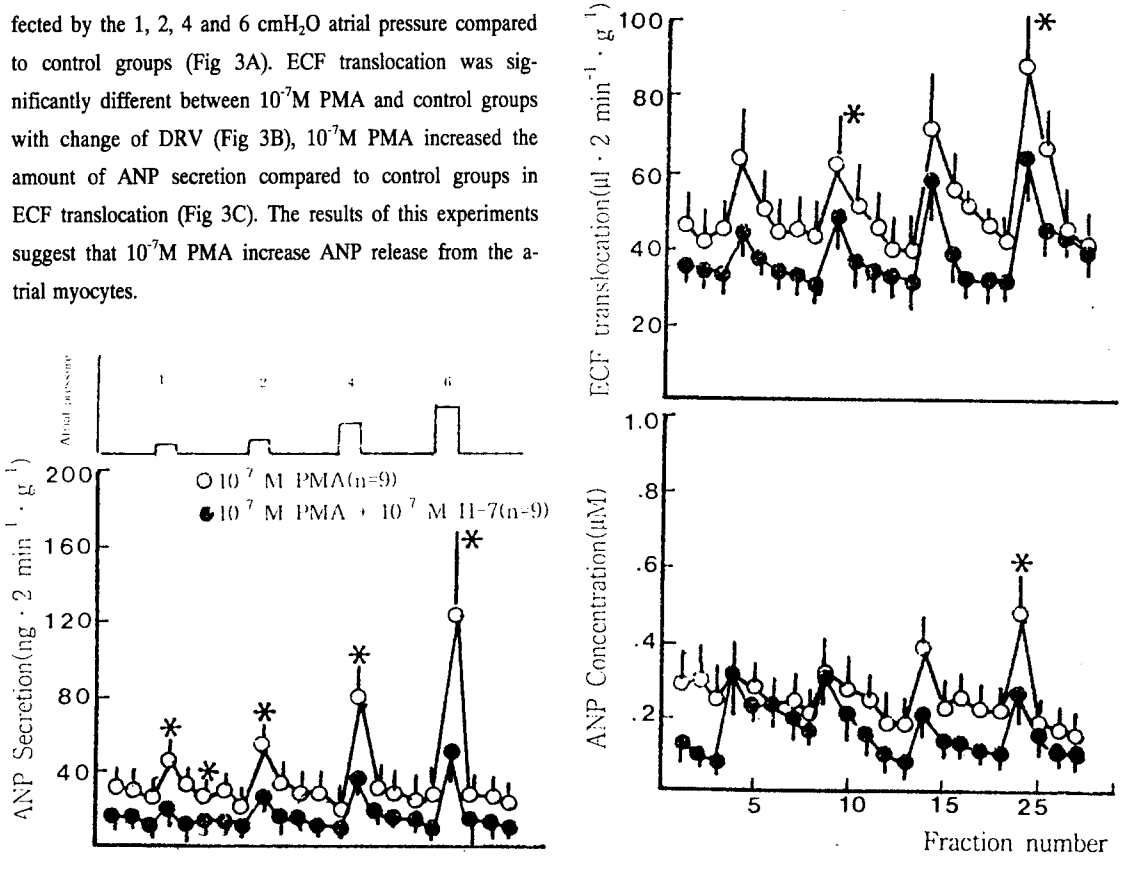

Fig 6. Effect of the combination 10^{-7}M H-7 and 10^{-7}M PMA on ANP secretion, ECF translocation and ANP concentration in the isolated perfused rat atria. Atrial natriuretic peptide was induced by changing the elevation of the outflow catheter tip by 1, 2, 4 and 6cmH_2O above the atrium. ANP, atrial natriuretic peptide; ECF, extracellular fluid. Statistical comparisons were done by student's paired *t*-test. This reslts are mean±SE. $^{*}p < 0.05$.

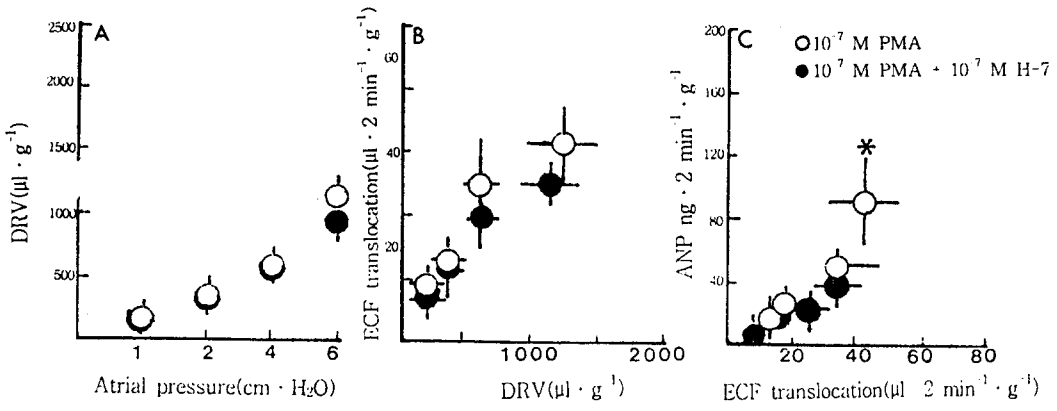

Fig 7. (A) Effect of the combination 10^{-7}M H-7 and 10^{-7}M PMA on the DRV in pressure changes. (B) Effect of both, response in ECF translocation to increasing DRV. (C) ANP secretion to increasing ECF translocation in left rat atria. ANP, atrial natriuretic peptide ; ECF, extracellular fluid ; DRV, distension reduction volume. *p < 0.05.

H-7 effect on stretch-induced ANP secretion. : 10^{-7}M PMA may regulate ANP secretion by mechanical activation. The PKC blocker, 10^{-7}M H-7 was used to confirm the influence of PKC on the secretion of ANP. 10^{-7}M H-7 suppressed ANP secretion 20.42±3.21, 24.51±5.41, 30.54±8.50 and 40.61±8.10ng/2min/g at 1, 2, 4 and 6 cmH_2O, respectively, compared to control groups (Fig 4). H-7 also suppressed 31.54±6.21, 33.50±8.10, 39.15±10.40 and 48.50±11.47μl/2min/g, ECF translocation at 1, 2, 4 and 6 cmH_2O, respectively, compared to control groups. 10^{-7}M H-7 suppressed ANP concentration compared to control groups (Fig 4). ECF translocation and DRV were not influenced at 10^{-7}M H-7 compared to control groups but 6 cm H_2O was affected (Fig 5A, B). The amount of ANP secretion with change of ECF translocation was significantly increased compared to control groups (Fig 5C). The amount of ANP secretion from the left atria of rat is suppressed by H-7.

PMA and H-7 combination effect on stretch-induced ANP secretion : To test the blocking of 10^{-7}M H-7, we increased ANP secretion by PMA and atria were perfused with 10^{-7}M PMA buffer containing 10^{-7}M H-7. As shown in Fig 6, effect of increase by PMA-induced ANP secretion was significantly suppressed by H-7. Various pressure elevations, between 4 and 6 cmH_2O, affected ECF translocation and concentration of ANP differently. 10^{-7}M H-7 suppressed the effect of 10^{-7}M PMA-induced ANP secretion of basal rate and PMA-induced ECF translocation. Neither ECF translocation nor DRV were influenced compared to control (Fig 7A, B). 10^{-7}M H-7 suppressed PMA-induced ANP concentration (Fig 7C). Thus, Prorein kinase C activation increases ANP release and it is suggest that regulation of ANP involved to the via the PKC pathway from atria myocytes.

Discussion

Atrial natriuretic peptide is a hormone that regulates salt, water balance and blood pressure. It is synthesized, stored and secreted from mammalian myocytes[1]. Although stretching atrial myocytes, changing the rate of contraction, and multiple neurohumoral and pharmacological agents stimulate ANP secretion[7], the specific mechanism for the regulation of ANP secretion has not been elucidated. Cho *et al* [11,12] proposed a two-step sequential mechanism for ANP secretion. Cho *et al* [8,9,10] studies consistently confirmed that atrial stretch increases ECF translocation and two distinct steps are involved in the secretory process of ANP from the atrial myocytes.

First, in this study, we used a modified method of the iso-

lated perfused rabbit atrial mode[13]. The improved isolated perfused rat atrial model was developed to investigate the regulation of ANP mechanism at low pressures. This model improved than isolated perfused model of Cho *et al*[11] at low pressure. Accordly, we were available to experiment of physiological pressure by isolated perfused rat atrial model. The present results indicated that the amount of ANP released into the perfusate augmented a linear correlation as extracellular fluid changed by atrial pressure. This was agreed with data from other groups[15~17] and suggested that ANP secretion was influenced by change of mechanical induced atria.

To investigate the 10^{-7}M ANP release via the PKC pathway, we used by PMA and H-7. Our results show that changes of ECF translocation increased the amount of ANP secretion. ANP secretion was increased by PMA concentration in a dose-dependent manner. As a PKC activator, PMA was so accentuate stretch-induced ANP secretion, basal ANP secretion and ECF translocation. PMA increased ANP concentration of the left atria at low pressure in the rat. PMA regulates positively stretch-induced ANP secretion. Therefore, PMA may increase release of ANP from atrial myocytes into the surrounding ECF. An important mechanism for regulating cellular physiology involves activation of phosphoinositide hydrolysis with subsequent production of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of sequestered Ca^{2+} into the cytoplasmic compartment[18], while DAG causes the association of protein kinase C with plasma membrane and the conversion of PKC to an active, Ca^{2+}-sensitive protein kinase C[14]. Sustained cellular responses that are mediated by the phosphoinositol system and increases the DAG content of the plasma membrane, activates the membrane-associated PKC. The combined function of these second messengers, IP_3 and DAG, synergistically regulate many endocrine organs[14,17,18]. The binding of phorbol esters to the same sites normally occupied by DAG may activate PKC[14]. Protein kinase C is located in both membrane and cytosolic fractions in the heart[19, 20]. Its activity appears to be higher in the atria than in the ventricles[21]. ANP secretion is stimulated by 10^{-7}M PMA in both isolated perfused rat hearts[15~17,22] and isolated cultured myocytes[24~26]. TPA (15-160nM) produced a dose-dependent increase in ANP secretion[15,16,22] in perfused or spontaneous beating rat hearts. In ventricular myocytes of the adult rat, activated PKC[32] decreased contractile force, whereas some researchers reported an increase in contractile force[28]. Other group reported that PKC activity increased myocardial contractility and coronary perfusion pressure, but decreased heart frequency and coronary atrial contraction[29]. Administration of TPA in isolated perfused rat hearts stimulates PKC, activates Na^+/H^+ exchanges and subsequently elevates intracellular Ca^{2+} [28,29]. TPA produced dose-dependent negative changes in inotropy and chronotrophy[20]. Change of PMA concentration produced a positive or negative inotropic effect on electrically stimulated left guinea pig atria and increased the frequency of spontaneous beating atria[30]. In the experiments, 10^{-7}M PMA increased secretion of ANP, however, 10^{-7}M H-7 inhibited basal and stretch induced ANP secretion, extracellular fluid translocation, and the amount of released ANP in relation to the ECF translocation. It is suggests that H-7 suppresses the release of ANP. According to a previous study[30], H-7 inhibited PMA-induced protein phosphorylation in the HL-60 cell. H-7 blocks cGMP and cAMP in the cardiac cell and suppresses the activation of PKC. Other group have suggested that H-7 also suppresses the secretion of ANP[31]. Thus, our results of these experiments agreed with other previous groups[32,33].

To investigate the ANP release via the protein kinase C pathway specifically, 10^{-7}M PMA buffer containing 10^{-7}M H-7 were tested in this experiment. H-7 suppressed PMA-induced ANP release and ANP secretion. This indicated that PKC regulated basal and stretch-induced ANP release.

Therefore, we suggest that PKC increase ANP secretion to the via the PKC pathway from the left atrial myocytes and is related with ANP release in the two-step sequential mechanism.

Conclusion

To investigate the role of PKC in the ANP secretion, a perfused rat atrial perfused model was desired. We found that stretch-induced ANP secretion take place in two-steps

sequential mechanism. The secretory response, PMA and H-7, alone or in combination, were studies. ANP secretion was increase by PMA concentration in a dose-dependent manner, whereas H-7 suppressed on ANP secretion. PMA induced ANP secretion was suppressed by H-7.

Therefore, we suggest that PKC increases ANP secretion to the via the PKC pathway from the left atria myocytes and is related with ANP release in the two-step sequential mechanism.

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