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# Brain phospholipase C–diacylglycerol lipase pathway is involved in vasopressin-induced release of noradrenaline and adrenaline from adrenal medulla in rats

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**Abstract**

Recently, we reported that intracerebroventricularly (i.c.v.) administered arginine–vasopressin evokes the release of noradrenaline and adrenaline from adrenal medulla by brain thromboxane $A_2$-mediated mechanisms in rats. These results suggest the involvement of brain arachidonic acid in the vasopressin-induced activation of the central adrenomedullary outflow. Arachidonic acid is released mainly by two pathways: phospholipase $A_2$ ($PLA_2$)-dependent pathway; phospholipase C (PLC)- and diacylglycerol lipase-dependent pathway. In the present study, therefore, we attempted to identify which pathway is involved in the vasopressin-induced release of both catecholamines from adrenal medulla using urethane-anesthetized rats. Vasopressin (0.2 nmol/animal, i.c.v.)-induced elevation of plasma noradrenaline and adrenaline was dose-dependently reduced by neomycin [0.28 and 0.55 μmol (250 and 500 μg)/animal, i.c.v.] and 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U-73122) [5 and 10 nmol (2.3 and 4.6 μg)/animal, i.c.v.] (inhibitors of PLC), and also by 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC-80267) [1.3 and 2.6 μmol (500 and 1000 μg)/animal, i.c.v.] (an inhibitor of diacylglycerol lipase). On the other hand, mepacrine [1.1 and 2.2 μmol (500 and 1000 μg)/animal, i.c.v.] (an inhibitor of $PLA_2$) was largely ineffective on the vasopressin-induced elevation of plasma catecholamines. These results suggest that vasopressin evokes the release of noradrenaline and adrenaline from adrenal medulla by the brain PLC- and diacylglycerol lipase-dependent mechanisms in rats.


*Keywords:* Adrenal medulla; Brain; Diacylglycerol lipase; Vasopressin; Phospholipase C

## 1. Introduction

Vasopressin is commonly recognized as an important neuropeptide involved in water conservation (Acher, 1993) and pituitary adrenocorticotropic hormone (ACTH) secretion (Gillies et al., 1982; Rivier et al., 1984; Antoni, 1993). Increases in pituitary ACTH secretion during activation of the hypothalamic-pituitary adrenal axis are accompanied by elevated synthesis of vasopressin in perikarya located in magno- and parvocellular parts of the paraventricular nucleus of the hypothalamus (De Goeij et al., 1992; Antoni, 1993). Vasopressin has also been recognized as a neurotransmitter or neuromodulator to modulate diverse brain functions such as memory and behavior (Ferris et al., 1984; De Wied et al., 1991), fever (Wilkinson and Kasting, 1987) and central cardiovascular regulation.

Intracerebroventricularly (i.c.v.) administered vasopressin has been shown to have central pressor and tachycardiac effects in rats (Pittman et al., 1982; Zerbe et al., 1983). However, the central mechanisms are largely undefined. Recently, we reported that the centrally administered vasopressin evokes the release of noradrenaline and adrenaline from adrenal medulla by the brain thromboxane $A_2$-mediated mechanisms in rats (Okada et al., 2002, 2003a). In addition, centrally administered

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arachidonic acid elevated plasma levels of both catecholamines and these elevations were abolished by central pretreatment with indomethacin, an inhibitor of cyclooxygenase (Yokotani et al., 2000). These results suggest that centrally administered vasopressin evokes the release of arachidonic acid in the brain, thereby activating central adrenomedullary outflow in rats.

Arachidonic acid has been shown to be released mainly by two different pathways: (1) phospholipase $A_2$ ($PLA_2$) hydrolyzes the *sn*-2 ester bond of membrane phospholipids, thereby releasing arachidonic acid (Flower and Blackwell, 1976; Irvine, 1982; Axelrod, 1990; Soloff et al., 2000); (2) diacylglycerol lipase hydrolyzed diacylglycerol to yield arachidonic acid (Bell et al., 1979; Irvine, 1982; Axelrod, 1990; Broad et al., 1999). Diacylglycerol is formed in different ways. While agonist-induced activation of phosphoinositide-specific phospholipase C (PLC) may produce rapid, transient increases in diacylglycerol along with inositol 1,4,5-trisphosphate, more sustained elevation of diacylglycerol is believed to result largely from phosphatidylcholine breakdown. Diacylglycerol generation from phosphatidylcholine may involve phospholipase D (PLD), forming phosphatidic acid, which can then be converted to diacylglycerol by phosphatidate phosphohydrolase (Hammond et al., 1995; Exton, 1997). Vasopressin has also been shown to activate PLD in addition to PLC in rat hepatocytes, however, the PLD activating effect was rapidly desensitized (Pittner and Spitzer, 1993; Dajani et al., 1999).

Recently, we reported that the brain $PLA_2$ and PLC–diacylglycerol lipase are, respectively, involved in the centrally administered melittin (a $PLA_2$ activator)- and corticotropin-releasing hormone-induced activation of the central sympatho-adrenomedullary outflow in rats (Yokotani et al., 2000; Okada et al., 2003b). Hence, the present study was designed to clarify which phospholipase is involved in the vasopressin-induced activation of the central adrenomedullary outflow using anesthetized rats.

## 2. Materials and methods

### 2.1. Experimental procedures

Male Wister rats weighing about 350 g were maintained in an air-conditioned room at 22–24 °C under a constant day–night rhythm for more than 2 weeks and given food (laboratory chow, CE-2; Clea Japan, Hamamatsu, Japan) and water ad libitum. Under urethane anesthesia (1.2 g/kg, i.p.), the femoral vein was cannulated for infusion of saline (1.2 ml/h), and the femoral artery was cannulated for collecting blood samples, as shown in our previous papers (Yokotani et al., 1995; Okada et al., 2003b). After the animal was placed in a stereotaxic apparatus, the skull was drilled for intracerebroventricular administration of test substances using stainless-steel cannula (0.3 mm outer diameter). The stereotaxic coordinates of the tip of cannula were as follows (in mm): AP −0.8, L 1.5, V 4.0 (AP, anterior from the bregma; L, lateral from the midline; V, below the surface of the brain), according to the rat brain atlas (Paxinos and Watson, 1986). Then, 3 h were allowed to elapse before the application of blocking reagents [mepacrine, neomycin, U-73122 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) and RHC-80267 (1,6-bis(cyclohexyloximinocarbonylamino)hexane)].

Mepacrine and neomycin dissolved in sterile saline were slowly injected into the right lateral ventricle in a volume of 5 μl/animal, while U-73122 and RHC-80267 dissolved in 2.5 μl of 100% *N*,*N*-dimethylformamide (DMF)/animal were i.c.v. administered using a 10-μl Hamilton syringe. Vasopressin dissolved in sterile saline was i.c.v. administered in a volume of 10 μl/animal using a 25-μl Hamilton syringe 30 min after application of U-73122 and RHC-80267, and 180 min after application of mepacrine and neomycin due to their slightly elevating effects on the basal plasma levels of catecholamines. Correct placement of the cannula was confirmed at the end of each experiment by verifying that Cresyl Violet, injected through the cannula, had spread throughout the entire ventricular system.

All experiments were conducted in compliance with the guiding principles for the care and use of laboratory animals approved by the Kochi Medical School.

### 2.2. Measurement of plasma catecholamines

Blood samples (400 μl) were collected through an arterial catheter and preserved on ice during experiments. Plasma was prepared immediately after the final sampling. Catecholamines in the plasma were extracted with a slight modification and assayed electrochemically with high performance liquid chromatography (HPLC) (Anton and Sayre, 1962, Okada et al., 2003b). Briefly, after centrifugation, the plasma (100 μl) was transferred to a centrifuge tube containing 30 mg of activated alumina, 2 ml of double deionized water, 1 ml of 1.5 M Tris Buffer (pH 8.6) containing 0.1 M disodium EDTA and 1 ng of 3,4-dihydroxybenzylamine as an internal standard. The tube was shaken for 10 min and the alumina was washed three times with 4 ml of ice-cold double deionized water. Then, catecholamines adsorbed onto the alumina were eluted with 300 μl of 4% acetic acid containing 0.1 mM disodium EDTA. A pump (EP-300: Eicom, Kyoto, Japan), a sample injector (Model-231XL; Gilson, Villiers-le-Bel, France) and an electrochemical detector (ECD-300: Eicom) equipped with a graphite electrode were used with HPLC. Analytical conditions were as follows: detector, +450 mV potential against an Ag/AgCl reference electrode; column, Eicompack CA-50DS, 2.1×150 mm (Eicom); mobile phase, 0.1 M $NaH_2PO_4$–$Na_2HPO_4$ buffer (pH 6.0) containing 50 mg/l EDTA dihydrate, 0.75 g/l sodium 1-octanesulfonate and

15% methanol at a flow of 0.18 ml/min; injection volume, 40 μl. The amount of catecholamines in each sample was calculated using the peak height ratio relative to that of 3,4-dihydroxybenzylamine, as internal standard. By this assay, coefficients of variation (CV) for intra- and inter-assay were 3.0% and 3.7%, respectively, and 0.5 pg of noradrenaline and adrenaline were accurately determined.

### 2.3. Treatment of data and statistics

All values are expressed as the means±S.E.M. The data were analyzed by repeated-measure analysis of variance (ANOVA), followed by post-hoc analysis with the Bonferroni method for comparing a control to all other means. *P* values less than 0.05 were taken to indicate statistical significance.

### 2.4. Compounds

The following drugs were used: mepacrine (quinacrine) dihydrochloride (Research Biochemicals, Natik, MA, USA); neomycin sulfate (Sigma, St. Louis, MO, USA); RHC-80267, U-73122 (Biomol Research Laboratory, Plymouth Meeting, PA, USA); synthetic arginine–vasopressin (vasopressin) (Peptide Institute, Osaka, Japan). All other reagents were the highest grade available (Nacalai Tesque, Kyoto, Japan).

## 3. Results

### 3.1. Effect of mepacrine, an inhibitor of $PLA_2$, on the vasopressin-induced elevation of plasma catecholamines

Mepacrine [1.1 and 2.2 μmol (500 and 1000 μg)/animal, i.c.v.] had no effect on the basal plasma levels of catecholamines. Since we previously reported that vasopressin (0.1, 0.2, and 0.5 nmol/animal, i.c.v.) dose-dependently elevated plasma levels of both catecholamines (Okada et al., 2002), we used the dose of 0.2 nmol/animal in the present experiment. Administration of vasopressin (0.2 nmol/animal) rapidly increased plasma levels of noradrenaline and adrenaline. These responses reached a maximum 5 min after administration of the peptide and then declined toward their basal levels (Fig. 1). The vasopressin-induced elevation of plasma catecholamines was not attenuated by mepacrine [1.1 μmol (500 μg)/animal, i.c.v.], while the elevation of plasma noradrenaline, but not adrenaline, was attenuated by a higher dose of mepacrine [2.2 μmol (1000 μg)/animal, i.c.v.] (Fig. 1).

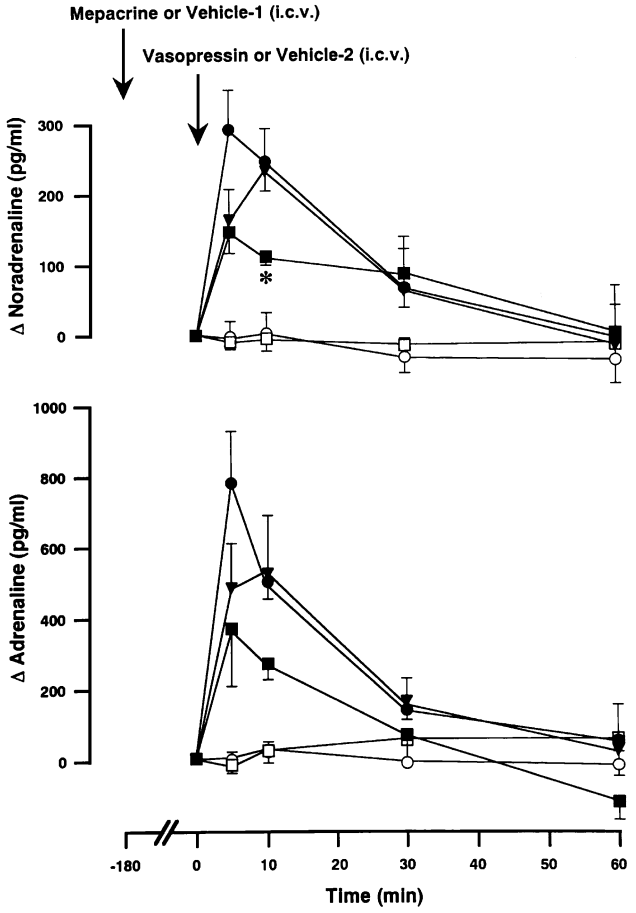


Fig. 1. Effect of mepacrine on the vasopressin-induced elevation of plasma catecholamines. ΔNoradrenaline and ΔAdrenaline: increments of noradrenaline and adrenaline above the basal. Mepacrine [1.1 and 2.2 μmol (500 and 1000 μg)/animal] or vehicle-1 (5 μl saline/animal) was intracerebroventricularly (i.c.v.) administered 180 min before the administration of vasopressin (0.2 nmol/animal, i.c.v.) or vehicle-2 (10 μl saline/animal, i.c.v.). Arrows indicate the i.c.v. administrations of mepacrine/vehicle-1 and vasopressin/vehicle-2. ○, vehicle-1 plus vehicle-2 (*n*=5); ●, vehicle-1 plus vasopressin (*n*=6); □, mepacrine (1.1 μmol/animal) plus vehicle-2 (*n*=5); ▼, mepacrine (1.1 μmol/animal) plus vasopressin (*n*=6); ■, mepacrine (2.2 μmol/animal) plus vasopressin (*n*=5). Each point represents the mean ±S.E.M. *Significantly different (*P*<0.05) from vehicle-1- and vasopressin-treated group. The actual values for noradrenaline and adrenaline at 0 min were 371.1±46.9 and 399.6±75.4 pg/ml in the vehicle-1 (saline)-pretreated group (*n*=11); 270.0±50.4 and 444.8±105.5 pg/ml in the mepacrine (1.1 μmol/animal)-pretreated group (*n*=11); 331.5±90.0 and 584.1±44.3 pg/ml in the mepacrine (2.2 μmol/animal)-pretreated group (*n*=5), respectively.

### 3.2. Effect of neomycin, a nonselective inhibitor of PLC, on the vasopressin-induced elevation of plasma catecholamines

Pretreatment with neomycin [0.28 and 0.55 μmol (250 and 500 μg)/animal, i.c.v.] had no effect on the basal plasma levels of catecholamines. Neomycin reduced the vasopressin-induced elevation of both catecholamines in a dose-dependent manner (Fig. 2). The increments of plasma noradrenaline and adrenaline at 5 min were 106.9±53.1 and 264.7±91.0 pg/ml in the neomycin [0.55 μmol (500 μg)/animal]- and vasopressin-treated group (*n*=5). These values were significantly different from those in the vehicle-1 (saline)- and vasopressin-treated group (289.1±58.3 and 777.0±146.4 pg/ml, *n*=6) (Fig. 2).

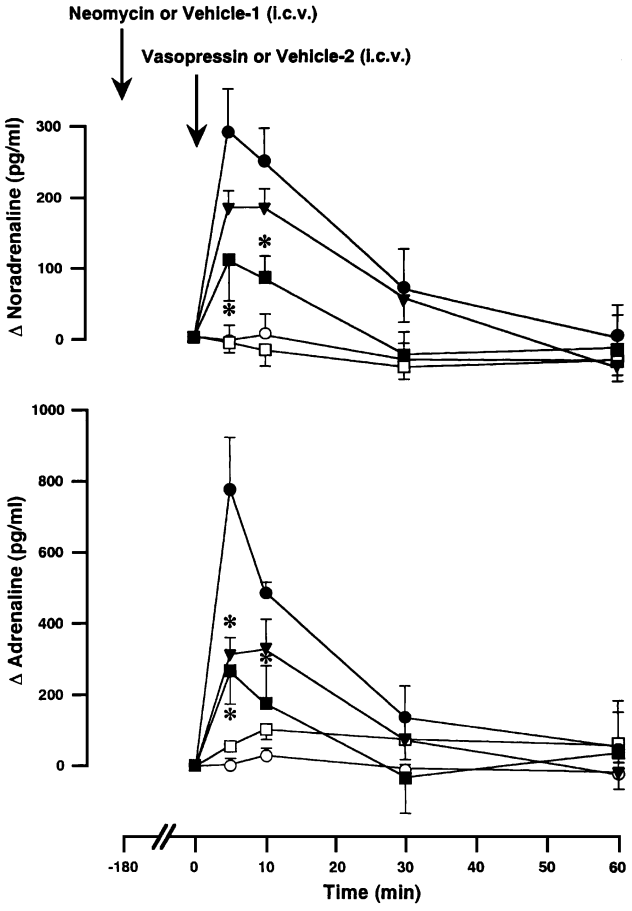


Fig. 2. Effect of neomycin on the vasopressin-induced elevation of plasma noradrenaline and adrenaline. Neomycin [0.28 and 0.55 μmol (250 and 500 μg)/animal, i.c.v.] or vehicle-1 (5 μl saline/animal, i.c.v.) was administered 180 min before the administration of vasopressin (0.2 nmol/animal, i.c.v.) or vehicle-2 (10 μl saline/animal, i.c.v.). ○, vehicle-1 plus vehicle-2 (cited from Fig. 1); ●, vehicle-1 plus vasopressin (cited from Fig. 1); □, neomycin (0.55 μmol/animal) plus vehicle-2 ($n$=5); ▼, neomycin (0.28 μmol/animal) plus vasopressin ($n$=5); ■, neomycin (0.55 μmol/animal) plus vasopressin ($n$=5). *Significantly different ($P$<0.05) from vehicle-1- and vasopressin-treated group. Other conditions were the same as those in Fig. 1. The actual values for noradrenaline and adrenaline at 0 min were 272.0±16.7 and 264.7±59.5 pg/ml in the neomycin (0.28 μmol/animal)-pretreated group ($n$=5); 446.3±73.1 and 583.1±91.2 pg/ml in the neomycin (0.55 μmol/animal)-pretreated group ($n$=10), respectively.

### 3.3. Effect of U-73122, a selective inhibitor of PLC, on the vasopressin-induced elevation of plasma catecholamines

Pretreatment with U-73122 [5 and 10 nmol (2.3 and 4.6 μg)/animal, i.c.v.] or vehicle-1 (2.5 μl of 100% DMF, i.c.v.) had no effect on the basal plasma levels of noradrenaline and adrenaline. U-73122 reduced the vasopressin-induced elevation of noradrenaline and adrenaline in a dose-dependent manner (Fig. 3). The increments of plasma noradrenaline and adrenaline at 5 min were 28.8±23.9 and 214.7±44.3 pg/ml in the U-73122 [10 nmol (4.6 μg)/animal]- and vasopressin-treated group ($n$=5). These values were significantly different from those in the vehicle-1 (DMF)- and vasopressin-treated group (186.7±38.1 and 716.0±102.7 pg/ml, $n$=8) (Fig. 3).

### 3.4. Effect of RHC-80267, an inhibitor of diacylglycerol lipase, on the vasopressin-induced elevation of plasma catecholamines

Pretreatment with RHC-80267 [1.3 and 2.6 μmol (500 and 1000 μg)/animal, i.c.v.] had no effect on the basal plasma levels of noradrenaline and adrenaline. RHC-80267 reduced the elevation of plasma noradrenaline and adrenaline evoked by vasopressin in a dose-dependent manner (Fig. 4). The increments of plasma noradrenaline and adrenaline at 5 min were 9.3±46.4 and 279.0±67.6 pg/ml in the RHC-80267 [2.6 μmol (1000 μg)/animal]- and vasopressin-treated group

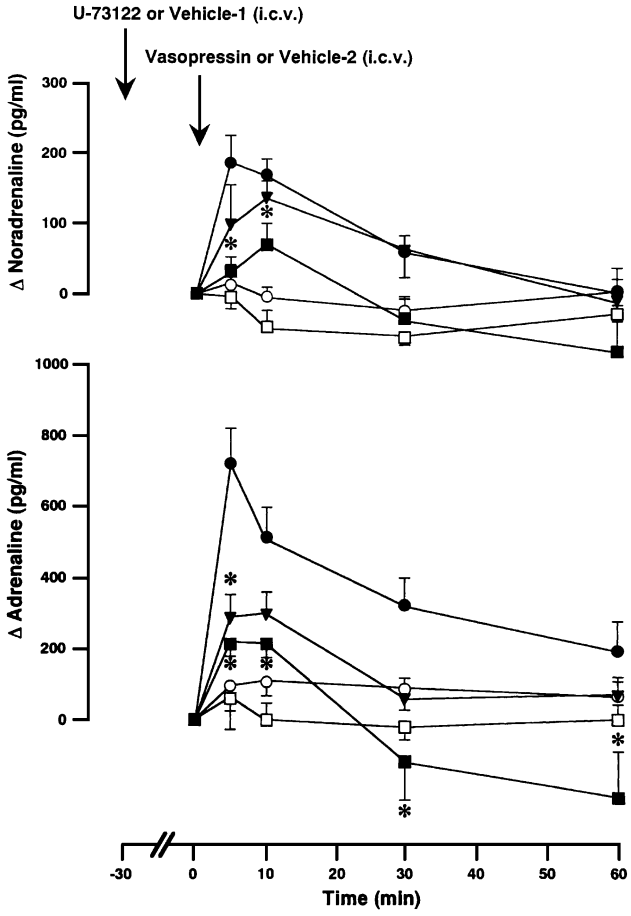


Fig. 3. Effect of U-73122 on the vasopressin-induced elevation of plasma noradrenaline and adrenaline. U-73122 [5 and 10 nmol (2.3 and 4.6 μg)/animal, i.c.v.] or vehicle-1 (2.5 μl of 100% DMF/animal, i.c.v.) was administered 30 min before the administration of vasopressin (0.2 nmol/animal, i.c.v.) or vehicle-2 (10 μl saline/animal, i.c.v.). ○, vehicle-1 plus vehicle-2 ($n$=4); ●, vehicle-1 plus vasopressin ($n$=8); □, U-73122 (10 nmol/animal) plus vehicle-2 ($n$=5); ▼, U-73122 (5 nmol/animal) plus vasopressin ($n$=4); ■, U-73122 (10 nmol/animal) plus vasopressin ($n$=5). *Significantly different ($P$<0.05) from vehicle-1- and vasopressin-treated group. Other conditions were the same as those in Figs. 1 and 2. The actual values for noradrenaline and adrenaline at 0 min were 312.5±40.8 and 532.6±135.2 pg/ml in the vehicle-1 (DMF)-pretreated group ($n$=12); 272.5±7.8 and 110.2±6.3 pg/ml in the U-73122 (5 nmol/animal)-pretreated group ($n$=4); 310.9±61.1 and 562.5±149.9 pg/ml in the U-73122 (10 nmol/animal)-pretreated group ($n$=10), respectively.

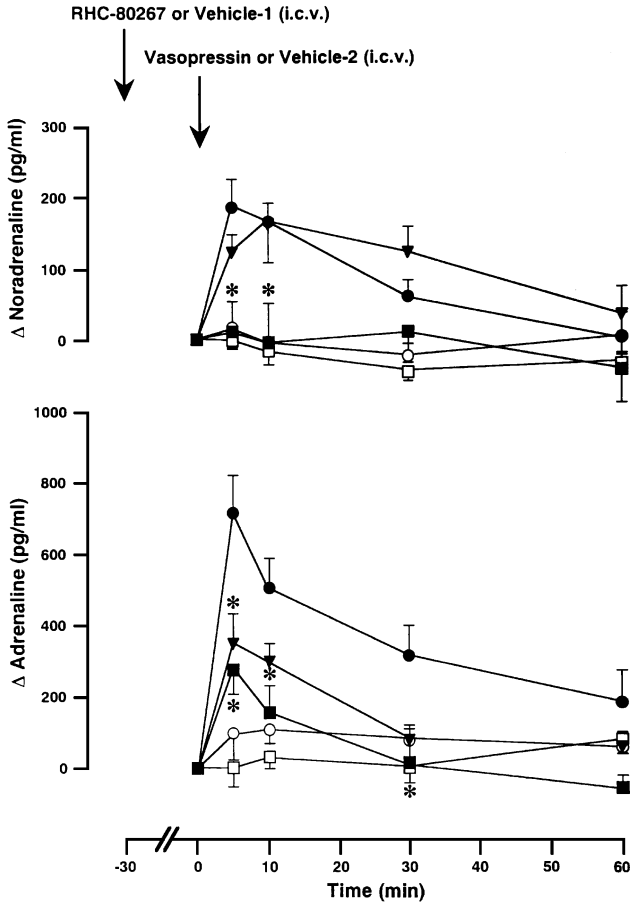


Fig. 4. Effect of RHC-80267 on the vasopressin-induced elevation of plasma noradrenaline and adrenaline. RHC-80267 [1.3 and 2.6 μmol (500 and 1000 μg)/animal, i.c.v.], or vehicle-1 (2.5 μl of 100% DMF/animal, i.c.v.) was administered 30 min before the administration of vasopressin (0.2 nmol/animal, i.c.v.) or vehicle-2 (10 μl saline/animal, i.c.v.). ○, vehicle-1 plus vehicle-2 (cited from Fig. 3); ●, vehicle-1 plus vasopressin (cited from Fig. 3); □, RHC-80267 (2.6 μmol/animal) plus vehicle-2 ($n$=5); ▼, RHC-80267 (1.3 μmol/animal) plus vasopressin ($n$=5); ■, RHC-80267 (2.6 μmol/animal) plus vasopressin ($n$=5). *Significantly different ($P$<0.05) from vehicle-1- and vasopressin-treated group. Other conditions were the same as those in Figs. 1–3. The actual values for noradrenaline and adrenaline at 0 min were 267.1±27.4 and 235.4±40.5 pg/ml in the RHC-80267 (1.3 μmol/animal)-pretreated group ($n$=5); 246.0±42.8 and 468.8±154.6 pg/ml in the RHC-80267 (2.6 μmol/animal)-pretreated group ($n$=10), respectively.

($n$=5). These values were significantly different from those in the vehicle-1 (DMF)- and vasopressin-treated group (186.7±38.1 and 716.0±102.7 pg/ml, $n$=8) (Fig. 4).

## 4. Discussion

It has been shown that mepacrine inhibits the release of arachidonic acid induced by *N*-methyl-D-aspartate at concentrations that inhibit $PLA_2$ activity, but not affect PLC activity, in primary cultures of cerebellar granule cells (Lazarewicz et al., 1990). Mepacrine also blocks melittin (a $PLA_2$ activator)-stimulated prostaglandin $E_2$ release from renal cortex slices (Churchill et al., 1990). Recently we also reported that central pretreatment with mepacrine (500 μg/animal, for 60 min) abolished the centrally administered melittin-induced elevation of plasma noradrenaline and adrenaline in rats (Yokotani et al., 2000). In the present experiment, however, central pretreatment with mepacrine (500 μg/animal, for 180 min) was ineffective on the vasopressin-induced elevation of plasma catecholamines, while the pretreatment with a large dose of this reagent (1000 μg/animal, for 180 min) had a weak reducing effect on the vasopressin-induced elevation of plasma noradrenaline alone. Mepacrine has been used to distinguish the activities of $PLA_2$ and PLC (Lapetina et al., 1981), however, the reagent can also inhibit PLC at high concentrations (Hofmann et al., 1982). The evidence suggests the possibility that mepacrine inhibits brain PLC rather than $PLA_2$, thereby attenuating the vasopressin-induced elevation of plasma noradrenaline in rats.

In the next experiment, we examined the effect of neomycin on the vasopressin-induced elevation of plasma catecholamines. Neomycin (250 and 500 μg/animal, i.c.v.) dose-dependently reduced the vasopressin-induced elevation of both catecholamines. Neomycin has been shown to be one of the inhibitors of PLC due to its binding to phosphatidylinositol 4,5-bisphosphate (Negishi et al., 1990). However, this reagent also inhibits some types of ion channels, including volume-sensitive $Cl^-$ channels (Mitchell et al., 1997), voltage-sensitive $Na^+$ channels (Charpentier et al., 1995) and voltage-sensitive $Ca^{2+}$ channels (Pichler et al., 1996). On the other hand, U-73122 has been shown to be a selective inhibitor of PLC in human platelets and polymorphonuclear neutrophils (Bleasdale et al., 1990; Smith et al., 1990). Therefore, we also examined the effect of this reagent on the vasopressin-induced elevation of plasma catecholamines. U-73122 (2.3 and 4.6 μg/animal, i.c.v.) dose-dependently reduced the vasopressin-induced elevation of plasma catecholamines. Hence, these results suggest the involvement of the brain PLC in the vasopressin-induced elevation of plasma catecholamines in rats. Vasopressin has already been shown to activate PLC in rat hepatocytes (Pittner and Spitzer, 1993; Dajani et al., 1999).

PLC catalyzes the breakdown of phosphatidylinositol, which results in the generation of two lipid molecules, diacylglycerol and inositol triphosphate, which function as second messengers. However, diacylglycerol is an important cellular source of arachidonic acid which may be released by diacylglycerol lipase (Grillone et al., 1988; Balsinde et al., 1991; Hou et al., 1996). RHC-80267 has been shown to selectively inhibit diacylglycerol lipase activity in canine platelets (Sutherland and Amin, 1982), human adrenal glomerulosa cells (Natarajan et al., 1988) and rat thyroid lobes (Levasseur et al., 1984). In the present experiment, RHC-80267 (500 and 1000 μg/animal, i.c.v.) dose-dependently reduced the vasopressin-induced elevation of plasma catecholamines. The result suggests the involvement of the brain diacylglycerol lipase in the vasopressin-induced

elevation of plasma catecholamines, in addition to the brain PLC, in rats. The PLC- and diacylglycerol lipase-dependent mechanisms have also been shown to be involved in the vasopressin-induced release of arachidonic acid in rat smooth muscle cells (Broad et al., 1999).

The biological effects of vasopressin are mediated by three receptor subtypes (vasopressin $V_{1a}$, $V_{1b}$ and $V_2$ receptors). Vasopressin $V_{1a}$ receptors (distributed in liver, vascular smooth muscle, platelets and the central nervous system) and vasopressin $V_{1b}$ receptors (widely distributed in the brain) are coupled to PLC, while kidney vasopressin $V_2$ receptors are linked to adenylyl cyclase (Burbach et al., 1995; Lolait et al., 1995; Vaccari et al., 1998; Hernando et al., 2001). Recently, we reported that the central vasopressin $V_1$ receptors are involved in the vasopressin-induced elevation of plasma catecholamines in rats (Okada et al., 2002). Vasopressin $V_1$ receptors- and PLC-mediated release of arachidonic acid has already been shown in the smooth muscle cells (Grillone et al., 1988). These results further confirm the involvement of the brain vasopressin $V_1$ receptor-PLC signaling pathway in the vasopressin-induced elevation of plasma catecholamines in rats.

In summary, we demonstrated here that the brain arachidonic acid generated by brain PLC- and diacylglycerol lipase-dependent mechanisms is involved in the vasopressin-induced release of noradrenaline and adrenaline from adrenal medulla in rats.

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