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# Brain phospholipase C and diacylglycerol lipase are involved in corticotropin-releasing hormone-induced sympatho-adrenomedullary outflow in rats

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**Abstract**

Previously, we reported that the elevation of plasma noradrenaline and adrenaline induced by intracerebroventricularly (i.c.v.) administered corticotropin-releasing hormone (CRH) was abolished by i.c.v. administered indomethacin, an inhibitor of cyclooxygenase, in rats [Yokotani et al., Eur. J. Pharmacol. 419, 183-189, 2001]. The result suggests the involvement of active metabolites of brain arachidonic acid in the CRH-induced activation of the central sympatho-adrenomedullary outflow. Arachidonic acid is released mainly by two different pathways: phospholipase $A_2$-dependent pathway; phospholipase C- and diacylglycerol lipase-dependent pathway. In the present study, therefore, we tried to identify which pathway is involved in the CRH-induced elevation of plasma catecholamines in urethane-anesthetized rats. CRH (1.5 nmol/animal, i.c.v.)-induced elevation of plasma noradrenaline and adrenaline was abolished by neomycin [0.55 μmol (500 μg)/animal, i.c.v.] and 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122) [5 nmol (2.3 μg)/animal, i.c.v.] (inhibitors of phospholipase C), and also by 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane (RHC-80267) [1.3 μmol (500 μg)/animal, i.c.v.] (an inhibitor of diacylglycerol lipase). On the other hand, mepacrine [1.1 μmol (500 μg)/animal, i.c.v.] (an inhibitor of phospholipase $A_2$) and 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidinedione (U-73343) [5 nmol (2.3 μg)/animal, i.c.v.] (an inactive analog of U-73122) had no effect. These results suggest that CRH activates the central sympatho-adrenomedullary outflow by the brain phospholipase C- and diacylglycerol lipase-dependent mechanisms in rats.


*Keywords:* Catecholamine; Plasma; Corticotropin-releasing hormone (CRH); Phospholipase C; Diacylglycerol lipase; Brain; Sympatho-adrenomedullary outflow

## 1. Introduction

Corticotropin-releasing hormone (CRH) is a 41 amino acid peptide initially identified as a hypothalamic factor responsible for stimulating corticotropin secretion from the anterior pituitary (Vale et al., 1981). The peptide has been shown to take many roles in the brain functions in addition to its action on the hypothalamic–pituitary adrenal axis (Menzaghi et al., 1993; Chalmers et al., 1996; Dieterich et al., 1997). Intracerebroventricular administration of CRH to laboratory animals produces a wide spectrum of behavioral and autonomic effects. In this regard, centrally administered CRH elicits the activation of sympatho-adrenomedullary system (Brown et al., 1985), the elevation of blood pressure and heart rate (Fisher et al., 1982) and the activation of adrenal sympathetic efferent nerve activity (Kurosawa et al., 1986). Impaired basal and restraint-induced adrenaline secretion has also been shown in the CRH-deficient (knockout) mice (Jeong et al., 2000). However, the central mechanisms that mediate such responses are largely undefined. Recently, we reported that centrally administered CRH elevates plasma levels of noradrenaline and adrenaline and these elevations are abolished by centrally administered indomethacin, an inhibitor of cyclooxygenase, in rats (Yokotani et al., 2001). Centrally administered arachidonic acid also elevates plasma levels of both catecholamines and these elevations were abolished by centrally administered indomethacin (Yokotani et al., 2000). These results suggest the involvement of active metabolites of brain arachidonic acid in the CRH-induced

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activation of the central sympatho-adrenomedullary outflow in rats. Arachidonic acid is released at least by two different pathways: (1) phospholipase $A_2$ hydrolyzes the sn-2 ester bond of membrane phospholipids, thereby releasing arachidonic acid (Flower and Blackwell, 1976; Irvine, 1982; Axelrod, 1990); (2) phospholipase C cleaves the phosphodiester bond, resulting in the formation of diacylglycerol, which can be hydrolyzed by diacylglycerol lipase to yield arachidonic acid (Bell et al., 1979; Irvine, 1982; Axelrod, 1990).

The present study, therefore, was designed to clarify which phospholipase is involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

## 2. Materials and methods

### 2.1. Experimental procedures

Male Wistar 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. After these procedures, the animal was placed in a stereotaxic apparatus, as shown in our previous paper (Yokotani et al., 1995, 2000; Okada et al., 2000).

Three hours after the animal was placed in a stereotaxic apparatus, a stainless-steel cannula (0.35 mm outer diameter) was inserted into the right lateral ventricle according to the rat brain atlas (Paxinos and Watson, 1986). 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). Corticotropin-releasing hormone (CRH) was dissolved in sterile saline and slowly injected into the right lateral ventricle in a volume of 5 μl using a 10-μl Hamilton syringe. Mepacrine dissolved in sterile saline was administered into the right lateral ventricle in a volume of 10 μl/animal, 30 min before application of CRH. Neomycin dissolved in sterile saline was intracerebroventricularly (i.c.v.) administered in a volume of 10 μl/animal, 180 min before application of CRH, since neomycin slightly elevated the basal plasma levels of catecholamines. U-73122 [1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17 yl)amino)hexyl)-1H-pyrrole-2,5-dione] and U-73343 [1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione] dissolved in 100% dimethyl sulfoxide (DMSO) (2.5 μl of 100% DMSO/animal) and RHC-80267 [1,6-bis-(cyclohexyloximinocarbonylamino)-hexane] dissolved in 100% *N*,*N*-dimethylformamide (DMF) (2.5 μl of 100% DMF/animal) were i.c.v. administered, 30 min before application of CRH.

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 (250 μl) were collected through an arterial catheter. Catecholamines in the plasma were extracted by the method of Anton and Sayre (1962) with a slight modification and were assayed electrochemically with high performance liquid chromatography (Okada et al., 2000). 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 5 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 high performance liquid chromatography. Analytical conditions were as follows: detector, +450 mV potential against a 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, 750 mg/l 1-octane sulfate sodium (Nacalai Tesque, Kyoto, Japan) and 15% methanol at a flow of 0.22 ml/min. The amount of catecholamines in each sample was calculated using the peak height ratio relative to that of 3,4-dihydroxybenzylamine, an internal standard. This assay could determine 0.5 pg of noradrenaline and adrenaline accurately.

### 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 (Fig. 3). When only two means were compared, an unpaired Student's *t*-test was used (Figs. 1, 2 and 4). *P* values less than 0.05 were taken to indicate 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, U-73343 (Biomol Research Lab., Plymouth Meeting, PA, USA); synthetic corticotropin-releasing hormone (rat/human) (Peptide Institute, Osaka,

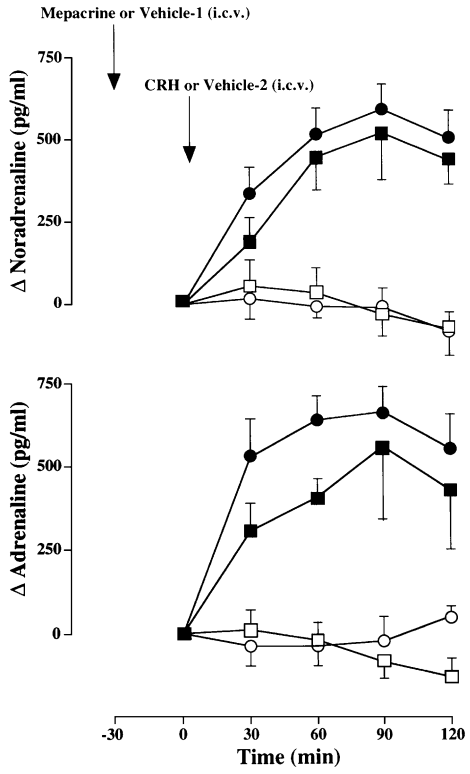


Fig. 1. Effects of mepacrine on the CRH-induced elevation of plasma catecholamines. ΔNoradrenaline and ΔAdrenaline, increase of noradrenaline and adrenaline above the basal. Mepacrine [1.1 μmol (500 μg)/animal] or vehicle-1 (10 μl saline/animal) was intracerebroventricularly (i.c.v.) administered 30 min before the administration of CRH (1.5 nmol/animal, i.c.v.) or vehicle-2 (5 μl saline/animal, i.c.v.). Arrows indicate the i.c.v. administrations of blocker/vehicle-1 and CRH/vehicle-2. ○, vehicle-1 plus vehicle-2 ($n=4$); ●, vehicle-1 plus CRH ($n=6$); ■, mepacrine plus CRH ($n=6$); □, mepacrine plus vehicle-2 ($n=5$). Each point represents the mean ± S.E.M. The actual values for noradrenaline and adrenaline at 0 min were 353.8 ± 60.2 and 208.6 ± 29.8 pg/ml in the vehicle-1-pretreated group ($n=10$) and 350.7 ± 46.2 and 291.8 ± 51.6 pg/ml in the mepacrine-pretreated group ($n=11$), respectively.

Japan). All other reagents were the highest grade available (Nacalai Tesque).

## 3. Results

### *3.1. Effects of mepacrine, an inhibitor of phospholipase $A_2$, on the CRH-induced elevation of plasma catecholamines*

Mepacrine [1.1 μmol (500 μg)/animal, i.c.v.] had no effect on the basal plasma levels of catecholamines. Since we previously reported that corticotropin-releasing hormone (CRH) (0.5, 1.5 and 3.0 nmol/animal, i.c.v.) dose-dependently elevated plasma levels of both catecholamines (Yokotani et al., 2001), we used 1.5 nmol/animal of CRH in the present experiment. CRH (1.5 nmol/animal, i.c.v.) gradually increased plasma levels of noradrenaline and adrenaline and these responses reached a maximum 90 min after administration of the peptide (Fig. 1). The CRH-induced elevation of plasma catecholamines was not influenced by mepacrine [1.1 μmol (500 μg)/animal, i.c.v.] (Fig. 1), while the same dose of this reagent has abolished the elevation of plasma catecholamines induced by i.c.v. administered melittin (an activator of phospholipase $A_2$) in rats (Yokotani et al., 2000).

### *3.2. Effects of neomycin, a nonselective inhibitor of phospholipase C, on the CRH-induced elevation of plasma catecholamines*

Three hours after pretreatment with neomycin [0.55 μmol (500 μg)/animal, i.c.v.], the basal plasma levels of noradrenaline and adrenaline tended to be stable at the preadministered levels. Neomycin abolished the CRH-induced elevation of both catecholamines (Fig. 2). The increments of plasma noradrenaline and adrenaline at 90 min were 78.6 ± 72.3 and 194.6 ± 79.4 pg/ml in the neomycin- and CRH-treated group ($n=6$). These values were significantly

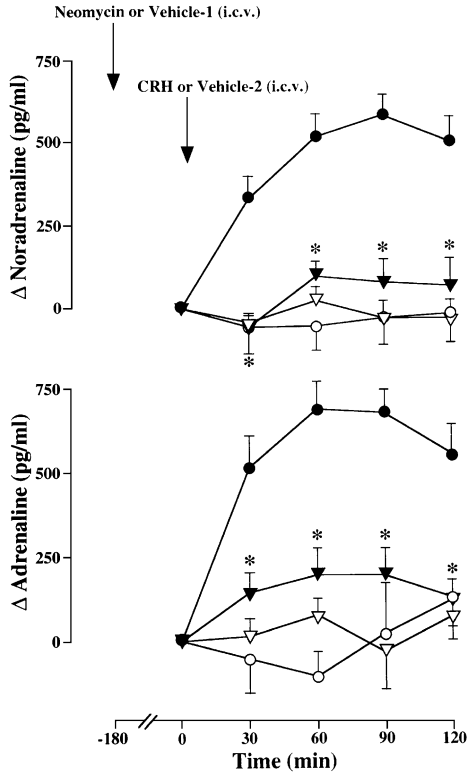


Fig. 2. Effect of neomycin on the CRH-induced elevation of plasma noradrenaline and adrenaline. Neomycin [0.55 μmol (500 μg)/animal, i.c.v.] or vehicle-1 (10 μl saline/animal, i.c.v.) was administered 180 min before the administration of CRH (1.5 nmol/animal, i.c.v.) or vehicle-2 (5 μl saline/animal, i.c.v.). ○, vehicle-1 plus vehicle-2 ($n=3$); ●, vehicle-1 plus CRH ($n=7$); ▽, neomycin plus vehicle-2 ($n=4$); ▼, neomycin plus CRH ($n=6$). *Significantly different ($P<0.05$) from the vehicle-1- and CRH-treated group. Other conditions were the same as those in Fig. 1. The actual values for noradrenaline and adrenaline at 0 min were 353.7 ± 58.0 and 319.2 ± 83.2 pg/ml in the vehicle-1-pretreated group ($n=10$) and 330.9 ± 53.7 and 301.4 ± 56.7 pg/ml in the neomycin-pretreated group ($n=10$), respectively.

different from those in the vehicle-1- and CRH-treated group (583.9 ± 62.0 and 678.9 ± 67.0 pg/ml, $n = 7$) (Fig. 2).

### 3.3. Effects of U-73122, a selective inhibitor of phospholipase C, and U-73343, an inactive analog of U-73122, on the CRH-induced elevation of plasma catecholamines

Pretreatment with U-73122 [5 nmol (2.3 μg)/animal, i.c.v.], U-73343 [5 nmol (2.3 μg)/animal, i.c.v.] or vehicle-1 (2.5 μl of 100% DMSO, i.c.v.) had no effect on the basal plasma levels of noradrenaline and adrenaline. U-73122 effectively reduced the CRH-induced elevation of noradrenaline and adrenaline, while U-73343 had no effect on the CRH-induced elevation of both catecholamines (Fig. 3). The increments of plasma noradrenaline and adrenaline at 90 min were 250.2 ± 59.3 and 413.8 ± 20.2 pg/ml in the U-73122- and CRH-treated group ($n = 6$). These values were

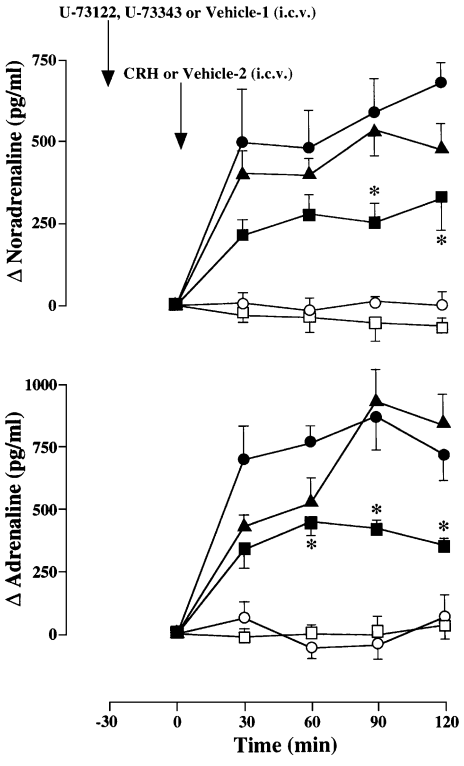


Fig. 3. Effects of U-73122 and U-73343 on the CRH-induced elevation of plasma noradrenaline and adrenaline. U-73122 [5 nmol (2.3 μg)/animal, i.c.v.], U-73343 [5 nmol (2.3 μg)/animal, i.c.v.] or vehicle-1 (2.5 μl of 100% DMSO/animal, i.c.v.) was administered 30 min before the administration of CRH (1.5 nmol/animal, i.c.v.) or vehicle-2 (5 μl saline/animal, i.c.v.). ○, vehicle-1 plus vehicle-2 ($n = 3$); ●, vehicle-1 plus CRH ($n = 6$); □, U-73122 plus vehicle-2 ($n = 4$); ■, U-73122 plus CRH ($n = 6$); ▲, U-73343 plus CRH ($n = 6$). *Significantly different ($P < 0.05$) from the vehicle-1- and CRH-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 354.9 ± 53.8 and 321.1 ± 53.9 pg/ml in the vehicle-1-pretreated group ($n = 9$), 366.2 ± 39.2 and 300.2 ± 84.0 pg/ml in the U-73122-pretreated group ($n = 10$), 227.3 ± 17.2 and 328.1 ± 68.9 pg/ml in the U-73343-pretreated group ($n = 6$), respectively.

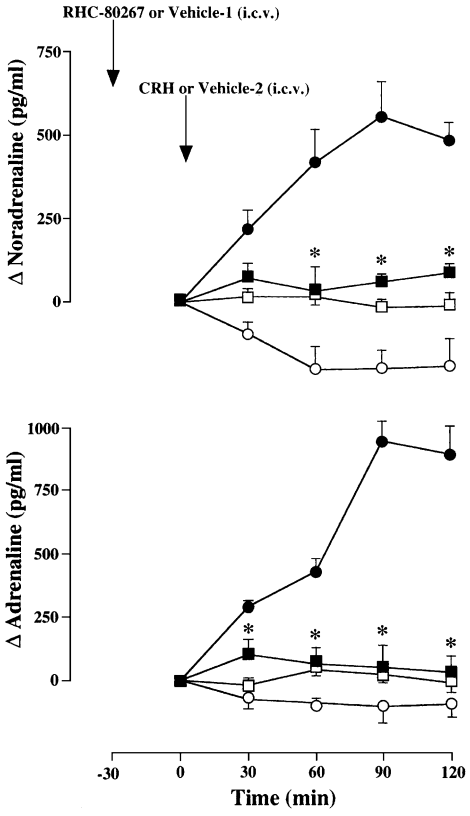


Fig. 4. Effect of RHC-80267 on the CRH-induced elevation of plasma noradrenaline and adrenaline. RHC-80267 [1.3 μmol (500 μg)/animal, i.c.v.] or vehicle-1 (2.5 μl of 100% DMF/animal, i.c.v.) was administered 30 min before the administration of CRH (1.5 nmol/animal, i.c.v.) or vehicle-2 (5 μl of saline/animal, i.c.v.). ○, vehicle-1 plus vehicle-2 ($n = 3$); ●, vehicle-1 plus CRH ($n = 7$); □, RHC-80267 plus vehicle-2 ($n = 4$); ■, RHC-80267 plus CRH ($n = 6$). *Significantly different ($P < 0.05$) from the vehicle-1- and CRH-treated group. Other conditions were the same as those in Figs. 1–3. The actual values for noradrenaline and adrenaline at 0 min were 326.2 ± 51.1 and 379.8 ± 43.7 pg/ml in the vehicle-1-pretreated group ($n = 10$), 295.8 ± 43.7 and 359.9 ± 60.1 pg/ml in the RHC-80267-pretreated group ($n = 10$), respectively.

significantly different from those in the vehicle-1- and CRH-treated group (586.9 ± 102.1 and 861.9 ± 131.9 pg/ml, $n = 6$) (Fig. 3).

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

Pretreatment with vehicle-1 alone (2.5 μl of 100% DMF, i.c.v.) had no effect on the basal plasma levels of catecholamines, while this pretreatment slightly potentiated the CRH-induced elevation of plasma adrenaline (Fig. 4). RHC-80267 [1.3 μmol (500 μg)/animal, i.c.v.] abolished the increases of plasma noradrenaline and adrenaline evoked by CRH (1.5 nmol/animal, i.c.v.) (Fig. 4). The increments of plasma noradrenaline and adrenaline at 90 min were 60.8 ± 25.2 and 50.4 ± 86.2 pg/ml in the RHC-80267- and CRH-treated group ($n = 6$). These values were signifi-

cantly different from those in the vehicle-1- and CRH-treated group (556.9 ± 104.7 and 948.0 ± 77.4 pg/ml, $n = 7$) (Fig. 4).

## 4. Discussion

Previously, we reported that centrally administered melittin elevates plasma levels of noradrenaline and adrenaline in rats and these responses are abolished by mepacrine (500 μg/animal) (Yokotani et al., 2000). Melittin has been shown to activate phospholipase $A_2$ by its interaction with the membrane bound phospholipid side chains (Nishiya, 1991), which results in accelerated lipid hydrolysis of the phospholipase $A_2$–phospholipid complex and thereby increasing arachidonic acid release (Fletcher et al., 1991). Mepacrine inhibits phospholipase $A_2$ activity thereby inhibiting the production of arachidonic acid (Vigo et al., 1980; Hofmann et al., 1982), and also blocks melittin-stimulated prostaglandin $E_2$ release in renal cortex slices (Churchill et al., 1990). In the present experiment, the same dose of mepacrine (500 μg/animal, i.c.v.) had no effect on the CRH-induced elevation of plasma catecholamines. The present result suggests the involvement of the other phospholipase than phospholipase $A_2$ in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

In the next experiment, we examined the effect of neomycin on the CRH-induced elevation of plasma catecholamines. Neomycin abolished the CRH-induced elevation of both catecholamines. Neomycin has been shown to be one of the inhibitors of phospholipase C due to its binding to phosphatidylinositol 4,5-bisphosphate (Orsulakova et al., 1976; Negishi et al., 1990). However, the reagent has been shown to inhibit some types of ion channels, including the volume-sensitive $Cl^-$ channels (Charpentier et al., 1995), the voltage-sensitive $Na^+$ channels (Mitchell et al., 1997) and voltage-sensitive calcium channels (Pichler et al., 1996).

Then, we examined the effect of U-73122 and U-73343 on the CRH-induced elevation of plasma catecholamines. U-73122 effectively reduced the CRH-induced elevation of plasma catecholamines, while U-73343 had no effect on the CRH-induced responses. U-73122 has been shown to be a selective inhibitor of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils (Bleasdale et al., 1990; Smith et al., 1990). U-73343 acts as a weak inhibitor of phospholipase C, thereby used as a negative control (Bleasdale et al., 1990; Smith et al., 1990). The present results, therefore, suggest the involvement of phospholipase C-dependent processes in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

Phospholipase C catalyzes the breakdown of phosphatidylinositol, which results in the generation of two lipid molecules, inositol triphosphate and diacylglycerol, that function as second messengers (Berridge, 1984; Nishizuka, 1984). Diacylglycerol is an important cellular source of arachidonic acid which may be released by diacylglycerol lipase (Moscat et al., 1986; 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 abolished the CRH-induced elevation of plasma catecholamines. The result suggests that the brain arachidonic acid released by diacylglycerol lipase-dependent mechanisms seems to be involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats.

A portion of the released arachidonic acid is metabolized rapidly to oxygenated products by several distinct enzyme systems including cyclooxygenase, and active products may act as intracellular or intramembrane signaling molecules. Feuerstein et al. (1982) have already shown that prostaglandin $E_2$ injected into the rat lateral cerebral ventricle increases plasma levels of catecholamines, especially noradrenaline. We also reported that the centrally administered prostaglandin $E_2$ selectively elevates plasma noradrenaline by activation of the brain prostanoid $EP_3$ receptors in rats (Yokotani et al., 1995). We also reported that the elevation of plasma adrenaline induced by centrally administered CRH is abolished by centrally administered furegrelate, a selective thromboxane $A_2$ synthase inhibitor (Yokotani et al., 2001) and that microinjection of I-BOP, a thromboxane $A_2$ mimetic, into the hypothalamic paraventricular nucleus elevates plasma levels of adrenaline (Murakami et al., 2002). From these results, the brain prostaglandin $E_2$ and thromboxane $A_2$ generated from arachidonic acid seem to be involved, respectively, in the CRH-induced activation of the central sympathetic and adrenomedullary outflow.

In summary, we demonstrated here that the brain arachidonic acid generated by brain phospholipase C- and diacylglycerol lipase-dependent mechanism is involved in the CRH-induced activation of the central sympatho-adrenomedullary outflow in rats: active metabolites of arachidonic acid (prostaglandin $E_2$ and thromboxane $A_2$) seem to be involved the CRH-induced activation of the central sympatho-adrenomedullary outflow.

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