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Influence of Polyphenol Compounds Isolated from Red Wine on Catecholamine Release in the Perfused Rat Adrenal Medulla

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의학과

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흰쥐 관류 부신수질에서 적포도주에서 분리한 폴리페놀 화합물이 카테콜아민 유리작용에 미치는 영향

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흰쥐 관류 부신수질에서 적포도주에서 분리한 폴리페놀 화합물이 카테콜아민 유리작용에 미치는 영향

고 우 석

(지도교수 : 임 경 준)

조선대학교 대학원 의학과

본 연구의 목적은 적포도주에서 분리한 폴리페놀 화합물(PCRW)이 정상혈압 흰쥐로부터 분리 적출한 부신의 관류모델에서 카테콜아민 (catecholamines, CA) 분비작용에 미치는 영향을 검색하여 그 작용기전을 규명코자 본 연구를 시행하여 다음과 같은 결과를 얻었다.

PCRW (20~180 μg/ml)을 부신정맥내로 90분간 관류시 비교적 용량 및 시간 의존적으로 ACh (5.32 mM), 고칼륨 (56 mM, 막탈분극제), DMPP (100 μM, 선택성 니코틴수용체 작동제), 및 McN-A-343 (100 μM, 선택성 무스카린 M_1-수용체 작동제)에 의한 CA 분비반응을 억제하였다. 그러나, PCRW 자체는 기초 CA 분비량에 영향을 미치지 않았다. 또한, PCRW (60 μg/ml) 존재 하에서, 전압-의존성 나트륨통로 활성화제인 veratridine (100 μM), L형 칼슘통로 활성화제인 Bay-K-8644 (10 μM) 및 세포질내 내형질세망막에서 Ca^{2+}-ATPase 억제제인 cyclopiazonic acid (10 μM)에 의한 CA 분비반응이 억제되었다. 흥미롭게도, PCRW (60 μg/ml)과 L-NAME (NO

Synthase 억제제, 30 μM)을 90분간 동시 처치하였을 때 ACh, 고농도의 K^+, DMPP, Bay-K-8644 및 cyclopiazonic acid의 CA 분비효과가 PCRW 단독처치 시 나타나는 억제효과에 비교하여 상응하는 대조치의 수준까지 회복되었다. 또한 실제로 PCRW처치 후에 NO 유리량이 기초 유리량에 비해 현저하게 증가하였다.

이와 같은 연구결과를 종합하여 보면, 흰쥐 적출 관류 부신수질에서 PCRW는 콜린성(니코틴 및 무스카린 수용체) 흥분작용 및 막탈분극에 의한 CA 분비작용에 대하여 억제작용을 나타내었다. 이러한 PCRW의 억제작용은 흰쥐 부신수질에서 NO Synthase의 활성화에 의한 NO 생성증가로 인하여 부신크롬친화세포 내로 나트륨 및 칼슘유입억제와 세포 내 칼슘저장고로부터 칼슘유리의 억제작용에 기인되는 것으로 생각된다.

이와 같은 PCRW의 작용으로 보아 PCRW가 심혈관계 질환의 예방 및 치료에 유익할 것으로 사료된다.

I. INTRODUCTION

Epidemiological evidence in support of a cardioprotective role of red wine comes from the observation that the mortality rate from coronary heart disease in France, where red wine is commonly taken with meals, is approximately half that of other Western countries, despite the presence of similar cardiovascular risk factors (Renaud and de Lorgeril, 1992; Tunstall-Pedoe et al., 1999). This was named as the 'French Paradox'. Cardiovascular disease remains a leading cause of morbidity and mortality in developed countries. Previously, the possible cardiovascular benefit of low to moderate consumption of alcoholic beverages, particularly of red wine, has received increasing attention (Rimm et al., 1996, Mukamal et al., 2003 and Wollin and Jones, 2001). There is some evidence of the desirable effects of a moderate wine intake, such as: an increase in the production of high density lipoproteins (HDL) (Gaziano et al., 1993), a reduction of oxidized low density lipoproteins (LDL), a vasodilatory effect of some wine components as quercetin (Perez-Vizcaino et al., 2002) and tannic acid (Flesch et al., 1998) and also an antioxidative effect due to the wine's content of polyphenols (Frankel et al., 1993a; 1993b).

Red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Fitzpatrick et al., 1995; Fitzpatrick et al., 2000; Zenebe et al., 2003). *In vivo* polyphenolic compounds of red wine (PCRW) were shown to reduce blood pressure in normo and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001; Bernátová et al.,

2002). The administration of purple grape juice improved the endothelium dependent, flow-mediated vasodilation in coronary artery disease patients with impaired endothelial function (Stein et al., 1999). Resveratrol, one of the polyphenolic compounds presented in red wine is thought to be responsible factor for its beneficial cardiovascular effects. Since resveratrol has similar effects to PCRW such as promotion of vasodilation, activation of nitric oxide synthase, inhibition of platelet aggregation and leukocyte activation, prevention of oxidation of LDL-cholesterol and reduction of cholesterol synthesis (Chen and Pace-Asciak, 1996; Rakici et al., 2005).

Evidence has accumulated that aberrant acute or chronic activation of the sympathoadrenal system is involved causally in the development of various cardiovascular diseases such as heart failures (Kaye, et al., 1995). Furthermore, a recent study reported that upregulation of adrenal medullary G protein-coupled receptor kinase 2 mediates sympathetic hyperactivity in heart failure through an increase in secretion of catecholamines (CA) in two different models of heart diseases (Lymperopoulos, et al., 2007). These evidences suggest that the CA play an important role in the pathological events of cardiovascular diseases. There is, however, little evidence regarding the effects of PCRW on ion channel-mediated CA secretion. Therefore, the purpose of the present study was to examine the effects of PCRW on the CA secretion evoked by stimulation of cholinergic (muscarinic and nicotinic) receptors and direct membrane-depolarization in the isolated perfused model of normotensive rats, and to establish its mechanism of action.

Ⅱ. MATERIALS AND METHODS

Isolation of polyphenolic compounds

Polyphenolic compounds were prepared as described by Caderni et al (2000), using adsorption chromatography from a 2-year-old red a cabernet sauvignon red wine made from Cabernet Sauvignon grapes by standard red wine making procedures at the Arzens Cooperative winery (Arzens, Aude, France), as follows (Fig. 1): alcohol was eliminated by distillation of batches and the remaining solution was deposited on a Diaion HP-20 column (Mitsubish Chemical Industries, Japan). After rinsing with water to remove sugars and organic acids, the phenolic pool of chemicals present in wine was eluted with 100% ethanol in water, concentrated by vacuum, evaporation and atomized, lyophilized by Coldvac–80 (Hanil R & D, Korea). 2.1 g PCRW from 1 L red grape wine was obtained. The working solution of this PCRW was prepared by dissolving in 0.9% NaCl solution on the day of each experiment and filtered before administration.

Experimental procedure

Mature male Sprague-dowley rats, weighing 200 to 350 grams, were used in the experiment. The animals were housed individually in separate cages, and food (Cheil Animal Chow) and tap water were allowed *ad libitum* for at least a week to adapt to experimental circumstances. On the day of experiment, a rat was anesthetized with thiopental sodium (50 mg/kg) intraperitoneally, and tied in supine position on fixing panel.

Isolation of adrenal glands: The adrenal gland was isolated by the

modification of previous method (Wakade, 1981). The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by the placement of three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations. A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulations. A small slit was made into the adrenal cortex just opposite entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at 37 ±1 ^{o}C (Fig. 2).

Perfusion of adrenal gland

The adrenal glands were perfused by means of peristaltic pump (Isco, St. Lincoln, NE, U.S,A.) at a rate of 0.31 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solution was constantly bubbled with 95 % O_2 + 5 % CO_2 and the final pH of the solution was maintained at 7.4 ~ 7.5. The solution contained disodium EDTA (10 μg/ml)

and ascorbic acid (100 μg/ml) to prevent oxidation of catecholamines.

Drug administration

The perfusions of DMPP (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32×10^{-3} M) and KCl (5.6×10^{-2} M) in a volume of 0.05 ml were made into perfusion stream via a three-way stopcock, respectively. McN-A-343 (10^{-4} M), Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were also perfused for 4 min, respectively.

In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated sample's was collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effect of PCRW on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing PCRW for 90 min, and

then the perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the stimulating agent or along with PCRW, and the perfusates were collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

The content of perfusate was measured directly by the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Milano, Italy).

A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Measurement of NO release

NO release was measured using a NO-selective microelectrode (amiNO-700, innovative Instruments Inc) and an amplifier (inNO meter, Innovative Instruments). Platelet NO production was quantified as the integrated signal detected by the microelectrode after platelet activation, as previously described (Freedman et al., 2000). The electrode was calibrated by producing standardized concentrations of NO in 0.5% (wt/vol) KI in 0.1 mol/L H_2SO_4 from $NaNO_2$

standards. NO release was quantitated as the current detected at the electrode 30 min after the presence of PCRW at room teperature. NO release was calculated as picomole. NO production was also measured indirectly by measuring nitrite content in the supernatant.

Statistical analysis

The statistical difference between the control and pretreated groups was determined by the Student's *t* and ANOVA tests. A P-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: PCRW (manufactured in pharmacology Lab), 1.1-dimethyl-4 -phenyl piperazinium iodide (DMPP), acetylcholine chloride, norepinephrine bitartrate, potassium chloride (KCl), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate (BAY-K-8644), cyclopiazonic acd, (Sigma Chemical Co., U.S.A.), and (3-(m-cholro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was dissolved in 99.5 % ethanol and diluted appropriately with Krebs-bicarbonate solution (final concentration of alcohol was

less than 0.1 %). Concentrations of all drugs except PCRW used are expressed in terms of molar base.

III. RESULTS

Effects of PCRW on CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the perfused rat adrenal glands

After the perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 21±2 ng for 2 min (n=9). Since *in vivo* PCRW was shown to reduce blood pressure in normotemsive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001; Bernátová et al., 2002), it was attempted initially to examine the effects of PCRW itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, PCRW (20 ~ 180 μg/ml) itself did not produce any effect on basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of PCRW on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion. Secretagogues were given at 15 to 20 min-intervals. PCRW was present for 90 minutes after the establishment of the control release.

In the perfused rat adrenal medulla, stimulation of nicotinic acetylcholine receptor-ion channels with acetylcholine, a physiological secretagogue, injected into the perfusion stream in a volume of 0.05 ml greatly caused the CA secretion (1279±48 ng for 0-4 min), as shown in Fig. 3. However, the pretreatment with PCRW in the range of 20 ~ 180 μg/ml for 90 min relatively concentration- and time-dependently inhibited ACh-stimulated CA secretion. As shown in Fig. 3, in the presence of PCRW, CA releasing responses were inhibited by 70% of the corresponding control release (100%). Also, it has been found that depolarizing

agent like KCl, an activator of voltage-dependent Ca^{2+} channels, stimulates markedly CA secretion (631±32 ng for 0-4 min). High K^+ (5.6×10^{-2} M)-stimulated CA secretion after the pretreatment with 20 μg/ml PCRW was not affected for 0-64 min period as compared with its corresponding control secretion (100%) (Fig. 4). However, following the pretreatment with higher concentrations of PCRW (60 ~ 180 μg/ml), high K^+ (5.6×10^{-2} M)-stimulated CA secretion was significantly inhibited to 75% of the control after 75 min period, although it was not initially affected by PCRW. DMPP (10^{-4} M), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (1172±32 ng for 0-8 min). However, as shown in Fig. 5, DMPP-stimulated CA secretion after pretreatment with PCRW was greatly reduced to 81% of the control release. McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 4 min caused an increased CA secretion (560±24 ng for 0-4 min). However, McN-A-343-stimulated CA secretion in the presence of PCRW was markedly depressed to 73% of the corresponding control secretion (100%) as depicted in Fig. 6.

Effects of PCRW on CA secretion evoked by Bay-K-8644, cyclopiazonic acid and veratridine from the perfused rat adrenal glands

Since Bay-K-8644 is known to be a calcium channel activator, which enhances basal Ca^{2+} uptake (Garcia et al., 1984) and CA release (Lim et al., 1992), it was of interest to determine the effects of PCRW on Bay-K-8644-stimulated CA

secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10^{-5} M)-stimulated CA secretion in the presence of PCRW (60 μg/ml) was greatly blocked to 73% of the control except for the early 0-34 min as compared to the corresponding control release (480±21 ng for 0-4 min) from 8 rat adrenal glands as shown in Fig. 7.

In order to investigate the effect of PCRW on the mobilization of intracellular Ca^{2+}, the effect of PCRW on the CA secretion evoked by cyclopiazonic acid, as a secretagogue, was examined. Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler et al., 1989). As shown in Fig. 8, in the presence of PCRW in 8 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was also inhibited to 71% of the control response (448±24 ng for 0-4 min).

The voltage-dependent Na^+ channels consist of the principal α-subunit, which is associated with a noncovalently attached β_1-subunits, and a disulfide-linked β_2-subunit (Catterall, 2000). The α-subunits issued from a large multigene contain the ion-pore and the toxin binding sites, i.e., site 1 for tetrodotoxin, site 2 for veratridine, site 3 for α-Scorpion toxin (α-ScTx), site 4 for β-Scorpion toxin (β-ScTx), and site 5 for *P. brevis* toxin-3 (PbTx-3) (Catterall, 2000). It has also been known that veratridine-induced Na^+ influx mediated through Na^+ channels increased Ca^{2+} influx via activation of voltage-dependent Ca^{2+} channels and produced the exocytotic secretion of CA in cultured bovine adrenal medullary cells (Wada et al., 1985a). To characterize the pharmacological action of PCRW on voltage-dependent Na^+ channels, the effect of PCRW on the CA secretion induced by veratridine was examined here. As shown in Fig. 9, veratridine greatly

produced CA secretion (1115±35 ng for 0-4 min). However, in the presence of PCRW (60 µg/ml), veratridine (100 µM)-evoked CA secretion was greatly inhibited to 70% % of the corresponding control release in a time-dependent manner.

Effects of PCRW plus L-NAME on CA release evoked by ACh, high K^+, DMPP, McN-A-343, BAY-K-8644 and cyclopiazonioc acid from the perfused rat adrenal glands

It has also been found that, in this study, PCRW inhibits the CA secretory response evoked by cholinergic stimulation in the perfused rat adrenal gland. Therefore, to study the relationship between NO and PCRW-induced inhibitory effects on the CA release from the rat adrenal glands, the effect of L-NAME on PCRW-induced inhibitory responses of CA secretion evoked by cholinergic receptor-stimulation as well as membrane depolarization was examined. In the present study, ACh (5.32 mM)-evoked CA release before perfusion with PCRW plus L-NAME was 1178±32 ng (0-4 min) from 10 rat adrenal glands. In the simultaneous presence of PCRW (60 µg/ml) and L-NAME (30 µM) for 90 min, it was initially not affected at 0-34 min, but later inhibited by 87% of the corresponding control release only at the period of 90-94 min in comparison with that of PCRW-treated group only as illustrated in Fig. 10. High K^+ (56 mM)-evoked CA release in the presence of PCRW (60 µg/ml) and L-NAME (30 µM) for 90 min was also not changed for 0-64 min, but later inhibited to 83% of the corresponding control release only at the last period of 90-94 min period in comparison to the control secretion (725±29 ng, 0-4 min) from 12 glands (Fig. 11).

As shown in Fig. 12, DMPP-evoked CA release prior to the perfusion with PCRW and L-NAME was 1280±34 ng (0-8 min). The simultaneous perfusion of PCRW and L-NAME for 90 min no longer inhibited DMPP-evoked CA release for the period of 0-48 min from 8 experiments while rather depressed to 90% of the control release only at the period of 80-88 min. Moreover, in the simultaneous presence of PCRW and L-NAME for 90 min, McN-A-343-evoked CA secretory responses was also time-dependently inhibited by 78% of the control secretion (512±27 ng, 0-4 min) at the period of 90-94 min from 10 glands, as shown in Fig. 13.

As shown in Fig. 14, the simultaneous perfusion of PCRW (60 μg/ml) and L-NAME (30 μM) for 90 min no longer inhibited the CA release evoked by Bay-K-8644 for the period of 0-64 min from 10 experiments, but later depressed to 76% of the control release only at the last period of 90-94 min in comparison to their corresponding control responses (448±24 ng, 0-4 min). The cyclopiazonic acid (10^{-5} M)-evoked CA secretory response prior to treatment with PCRW and L-NAME was 458±19 ng for 0-4 min. As shown in Fig. 15, however, in the presence of PCRW (60 μg/ml) and L-NAME (30 μM) for 90 min in 12 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was depressed to 75-84% of the control response (100%) only at the period of 75-94 min.

Effect of PCRW on the level of nitric oxide released from the perfused rat adrenal medulla

As shown in Fig. 10~15, it has been shown that PCRW-induced inhibitory effects

on the CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, BAY-K-8644 and cyclopiazonioc acid from the perfused rat adrenal glands were greatly attenuated by simultaneous treatment with L-NAME, an inhibitor of NO synthase, compared to the inhibitory effects of PCRW-treatment alone. Therefore, it was of interest to determine directly the level of nitric oxide released from adrenal medulla following the perfusion of PCRW-containing Krebs-bicarbonate solution. As shown in Fig. 16, the basal level of NO before loading of PCRW was 66.1±29 picomole. However, 30 min after the presence of PCRW (180 μg/ml), it was greatly enhanced to 185% of the control release. Consequently, it was confirmed that PCRW practically increase the level of NO released from the rat adrenal medulla.

IV. DISCUSSION

The present study provides the first evidence that PCRW significantly inhibits the CA secretory responses evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization from the isolated perfused adrenal gland of the normotensive rats. This inhibitory effect of PCRW seems to be exerted by inhibiting the influx of both calcium and sodium into the rat adrenal medullary chromaffin cells as well as the release of Ca^{2+} into the cytoplasmic calcium store at least partly through the increased NO production due to the activation of nitric oxide synthase.

In general, the adrenal medulla has been employed as a model system to study numerous cellular functions involving not only noradrenergic nerve cells but also neurons. Usually, two mechanisms are involved in the secretion of adrenal medullary hormones. During neurogenic stimulation of the adrenal medulla, ACh is released from splanchnic nerve endings and activates cholinergic receptors on the chromaffin cell membrane (Viveros, 1975). This activation initiates a series of events known as stimulus-secretion coupling, culminating in the exocytotic release of CA and other components of the secretory vesicles into the extracellular space. Based on this fact, the present findings that PCRW inhibited the CA secretory responses evoked by nicotinic receptor stimulation as well as by membrane depolarization in the rat adrenal medulla seem to be able to support the fact that, in *in vivo* studies, PCRW lowers blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001). It has been reported that red wines and grapes exhibit endothelium-dependent relaxation of

blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Fitzpatrick et al., 1995; Fitzpatrick et al., 2000; Zenebe et al., 2003).

These experimental results indicate that PCRW-induced inhibitory activity of CA secretory response evoked by stimulation of nicotinic receptors might contribute at least partly to its hypotensive mechanism. ACh, the physiological presynaptic transmitter at the adrenal medulla, which is released by depolarizing splanchnic nerve terminals and then activates nicotinic receptors, releases CA, and induces dopamine β-hydroxylase by calcium dependent secretory process (Dixon et al, 1975; Viveros et al, 1968). In terms of this fact, the present results suggest that PCRW may inhibit CA secretion evoked by nicotinic stimulation from the splanchnic nerve ending through the blockade of nicotinic receptors. The release of epinephrine from the adrenal medulla in response to splanchnic nerve stimulation or nicotinic agonist is mediated by activation of nicotinic receptors located on the chromaffin cells. The exocytotic CA release from the chromaffin cells appears to be essentially similar to that occuring in noradrenergic axons (Douglas, 1968; Sorimachi & Yoshida, 1979). ACh-evoked CA secretion has shown to be caused through stimulation of both nicotinic and muscarinic receptors in guinea-pig adrenal gland (Nakazato et al, 1988) as well as in the perfused rat adrenal glands (Lim & Hwang, 1991).

In support of this idea, more recently, it has been shown that polyphenolic compounds isolated from *Rubus coreanum* (PCRC) inhibits the CA secretory responses evoked by cholinergic stimulation and membrane depolarization in the adrenal medulla isolated from the normotensive rat (Kee and Lim, 2007). In this study, PCRW inhibited the CA secretory responses evoked by ACh, high K^+,

DMPP and McN-A-343. It suggests that PCRW can produce the similar effect in adrenal medulla of the normotensive rats with that of PCRC in adrenal medulla of the normotensive rats.

Tannins contained in green tea are also found to induce the depressor effect in rat with renal hypertension (Yokozawa et al., 1994). Extracts of tea (Fitzpatrick et al., 1995) and flavonoids found in tea (Fitzpatrick et al., 1993) have been shown to give vasodilator effects. In a cohort of Norwegian men and women, higher consumption of black tea was associated with lower systemic blood pressure (Stensvold et al., 1992). In terms of these findings, the results of the present study seem likely that PCRW can cause the depressor effect by the inhibition of CA secretion from the adrenal medulla. The present findings appeared to be similar at least partly to the facts that extracts of tea (Fitzpatrick et al., 1995) and flavonoids found in tea (Fitzpatrick et al., 1993) produced vasodilator effects, but not to the fact that tea ingestion in the normotensive men caused larger acute increases in blood pressure than caffeine alone (Hodgson et al., 1999). Furthermore, it has also been found that green tea extract inhibits the CA secretory responses evoked by cholinergic stimulation and membrane depolarization in the adrenal medulla isolated from the rat (Lim et al., 2003) and the rabbit (Lim, 2005).

In the present study, PCRW also time-dependently depressed the CA secretory response evoked by Bay-K-8644, which is known to activate L-type voltage-dependent Ca^{2+} channels (Garcia et al, 1984; Schram et al, 1983). This result indicates that PCRW may inhibit Ca^{2+} influx to the rat adrenomedullary cells. In support of this idea, in cultured bovine adrenal medullary cells, nicotinic (but not muscarinic) receptors mediate the Ca^{2+}-dependent secretion of CA

(Fisher et al., 1981; Yanagihara et al, 1979). It has been also known that the activation of nicotinic receptors stimulates CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal glands (Wakade & Wakade, 1983; Lim & Hwang, 1991) and isolated bovine adrenal chromaffin cells (Kilpatrick et al, 1981; 1982; Knight & Kesteven, 1983). Wada and his coworkers (1985b) have found that the adrenomedullary chromaffin cells have (i) nicotinic receptor-associated ionic channels, responsible for carbachol-induced Na^{+} influx, (ii) voltage-dependent Na^{+} channels, responsible for veratridine-induced Na^{+} influx and (iii) voltage-dependent Ca^{2+} channels, suggesting that the influx of Na^{+} caused either by carbachol or by veratridine leads to activate voltage-dependent Ca^{2+} channels by altering membrane potentials, whereas high K^{+} directly activates voltage-dependent Ca^{2+} channels without increasing Na^{+} influx. In the present study, the finding that high K^{+}-induced CA secretory response was depressed by pretreatment with PCRW indicates that this inhibitory effect of PCRW is exerted through the direct inhibition of calcium influx into the rat adrenal chromaffin cells. Furthermore, slight elevation in the extracellular potassium concentration increases both the frequency of spontaneous action potentials and the secretion of CA (Kidokoro & Ritchie, 1980), suggesting that the influx of calcium that occurs during action potentials is directly linked to the rate of secretion. These findings that PCRW inhibited CA secretion evoked by Bay-K-8644 as well as by high K^{+} suggest that PCRW inhibits directly the voltage-dependent Ca^{2+} channels. In the bovine chromaffin cells, stimulation of nicotinic, but not muscarinic ACh receptors is known to cause CA secretion by increasing Ca^{2+} influx largely through voltage-dependent Ca^{2+} channels (Burgoyne, 1984; Oka et al., 1979). Therefore,

it seems that PCRW inhibits the DMPP-evoked CA secretion by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors with DMPP.

The mechanism by which the stimulation of ACh receptors activates voltage-dependent Ca^{2+} channels in adrenal medullary cells is well understood. It has also been shown that ACh depolarizes chromaffin cell membranes and that this is dependent on the inward movement of Na^{+} into the cells (Douglas et al., 1968). Kidokoro and Ritchie (1980) demonstrated that ACh generates Na^{+}-dependent action potentials and that these are mediated by nicotinic (but not muscarinic) ACh receptors. Taking these previous observations into account, it has been suggested that the influx of Na^{+} via nicotine receptor-associated ionic channels leads to the activation of voltage-dependent Ca^{2+} channels by altering the membrane potentials (Wada et al., I985b). In the present study, PCRW suppressed the veratridine-evoked CA secretory response. This result suggests that the inhibition of PCRW on the CA secretion evoked by veratridine as well as by ACh and DMPP is responsible for the inhibition of Ca^{2+} influx, resulting in reduced CA secretion. Therefore, it seems likely that the predominant site of action of PCRW is nicotinic receptor-gated ionic channels in the rat adrenomedullary chromaffin cells.

Veratridine-induced influx of Na^{+} is a requisite for triggering Ca^{2+} influx and the CA secretion (Wada et al., 1985a; 1985b). Therefore, the inhibition by PCRW of voltage-dependent Na^{+} channels is responsible for the inhibition of Ca^{2+} influx and the CA secretion. Voltage-dependent Na^{+} channels are indispensable for axonal conduction in central and peripheral neurons.

The present study has also shown that PCRW inhibits the CA secretion

evoked by cyclopiazonic acid. Cyclopiazonic acid is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger & Riley, 1989; Siedler et al., 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki et al., 1992). Therefore, it is felt that the inhibitory effect of PCRW on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that the PCRW has an inhibitory effect on the release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the secretion of CA. It has been shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceeding Ca^{2+} load (Suzuki et al., 1992). This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cylopiazonic acid (Uyama et al., 1992). Suzuki and his coworkers (1992) have shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase in the subsequent Ca^{2+} release from those storage sites. Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide metabolism, resulting in the formation of inositol 1,4,5-trisphosphate, which induces the mobilization of Ca^{2+} from the intracellular pools (Cheek et al., 1989; Challis et al., 1991). The present results suggest that PCRW-induced depression of the CA secretion evoked by McN-A-343 and

cyclopiazonic acid may be due to the inhibition of Ca^{2+} release from the intracellular pools induced by stimulation of muscarinic ACh receptors. However, in the present study, it is uncertain whether the inhibitory effect of PCRW on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

In the present study, in the simultaneous presence of PCRW and L-NAME (NOS inhibitor), the CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were considerably recovered to the extent of the corresponding control secretion compared to those of PCRW treatment alone. This result is well consistent with report that polyphenolic compounds isolated from red wine produced the endothelium-NO-dependent relaxation through an extracellular Ca^{2+}-dependent mechanism (Andriambeloson et al., 1999). Amongst the different classes of polyphenolic compounds present in PCRW, anthocyanins and oligomeric condensed tannins had the same pharmacological profile as PCRW (Andriambeloson et al., 1998). Of different anthocyanins identified in wine, only delphinidin caused endothelium-dependent relaxation, although it was slightly less potent than PCRW (Andriambeloson et al., 1998).

It has also been shown that (-) epicatechin, one of polyphenolic components of green tea, concentration-dependently relaxed U46619-contracted arteries without the functional endothelium. It is unlikely that (-) epicatechin acts as an antagonist at prostaglandin receptors to cause relaxation since it reduced arterial contraction induced by other vasoconstrictors, such as phenylephrine and endothelin-1 (Huang et al., 1998). The endothelium-independent relaxation induced by (-) epicatechin may be partly mediated through inhibition of Ca^{2+} influx through

voltage-sensitive Ca^{2+} channels in vascular smooth muscle cells because (-) epicatechin significantly reduced the high K^+-induced contraction in the same preparation (Huang et al., 1998). It was also found that (-) epicatechin could act on endothelium to increase intracellular Ca^{2+} and nitric oxide release, which may account for the endothelium-dependent relaxation (Huang et al., 1999). In addition, (-) epicatechin-induced relaxation in endothelium-intact tissues may be also mediated by nitric oxide-dependent activation of iberiotoxin-sensitive K^+ channels. These mechanisms may be associated with a beneficial effect of green tea epicatechins on vascular system (Huang et al., 1999).

Some epidemiological studies indicate an association between moderate consumption of red wine and reduced risk of coronary heart disease (Renaud and de Lorgeril, 1992; German and Walzem, 2000). It has been shown that PCRW promote the endothelium-dependent relaxation, activate NO synthase, inhibit platelet aggregation, and prevent oxidation of LDL-cholesterol (Fitzpatrick, et al, 1993; Andriambeloson, et al., 1997; Flesh, et al., 1998; Leikert, et al., 2002; Demrow and Slane, 1995; Frankel, et al., 1993a). The polyphenolic compound resveratrol presented in red wine is thought to be responsible factor for its beneficial cardiovascular effects. Since resveratrol has similar effects to RWPC such as promotion of vasodilation, activation of nitric oxide synthase, inhibition of platelet aggregation and leukocyte activation, prevention of oxidation of LDL-cholesterol and reduction of cholesterol synthesis (Chen and Pace-Asciak, 1996; Wallerath, et al., 2002; Pace-Asciak, et al., 1995; Rotondo, et al., 1998; Frankel, et al., 1993b).

Furthermore, these effects of resveratrol are agreement with the present result that PCRW can inhibit the CA secretory responses evoked by cholinergic

stimulation and membrane depolarization at least partly by activation of nitric oxide synthase in the isolated perfused rat adrenal medulla, since this inhibitory effect of PCRW on the CA secretory responses was significantly attenuated in the presence of L-NAME, an inhibitor of nitric oxide synthase.

In support of this idea, generally, NO is produced enzymatically from the terminal guanidino nitrogen of L-arginine by the action of NO synthase (NOS) (Palmer, et al., 1988; Sakuma, et al., 1988). There are at least three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS. The adrenal medulla possesses characteristic postganglionic sympathetic neurons, and the presence of nNOS has been demonstrated (Marley, et al., 1995; Oset-Gasque, et al., 1994; Palacios, et al., 1989; Schwarz, et al., 1998). In vitro studies using NOS inhibitors and NO donors were performed to examine the role of NO in modulating CA secretion from the adrenal medulla but the results remain controversial. In the present work, in presence of L-NAME, the inhibitory responses of PCRW on the CA secretion were recovered to the considerable extent of the control secretion compared with the inhibitory effects of PCRW alone. This result demonstrates that PCRW can inhibit the CA release at least partly through the activation of nNOS in the rat adrenal medulla. In the support of this finding, it has been reported that the NOS inhibitor, L-NAME enhances K^+-stimulated CA secretion in cultured bovine chromaffin cells (Torres, et al., 1994) and that sodium nitroprusside (SNP) inhibits ACh-induced CA secretion in bovine chromaffin cells (Rodriguez-Pascual, et al., 1996). These studies suggest that NO may play an inhibitory role in the control of CA secretion. Moreover, the presence of endothelial cells has been reported to inhibit the K^+-induced or the nicotinic receptor agonist DMPP-induced CA secretion in cultured bovine

chromaffin cells (Torres, et al., 1994), suggesting that not only nNOS but also eNOS may play roles in modulating adrenal CA secretion. In contrast, it has been reported that L-NAME inhibits acetylcholine (ACh)-induced CA secretion in bovine chromaffin cells (Uchiyama, et al., 1994) and that the NO donor sodium nitroprusside (SNP) enhances nicotine-induced CA secretion in cultured bovine chromaffin cells (O'Sullivan and Burgoyne, 1990). These findings suggest that NO may facilitate cholinergic agonist-induced CA secretion. On the other hand, a few in vivo studies have suggested that NO does not play a role in regulation of adrenal CA secretion (Breslow, et al., 1992; Breslow, et al., 1993). Based on these reports, the present studies suggest that PCRW possesses the ability partly to activate nNOS in the rat adrenal medullary chromaffin cells, in addition to the direct inhibitory effects on the CA secretion.

In addition to these pharmacological effects of PCRW, in the present study, it was shown that PCRW inhibits the CA induced by cholinergic (both nicotinic and muscarinic) receptor stimulation, suggesting that PCRW attenuates the CA secretion induced by stress or emotional excitation, thus causing the stimulation of sympathetic nerves and the adrenal medulla. Although the CA play a pivotal role in the regulation of normal functions in cardiovascular systems, stress-induced over expression of the CA would contribute to the involvement and augmentation of cardiovascular diseases such as heart failures, atherosclerosis, coronary heart disease and hypertension. Indeed, chronic heart failure is associated with activation of the sympathetic nervous system as manifested by increased circulating level of norepinephrine and increased regional activity of the sympathetic nervous system (Kaye et al., 1995; Lymperopoulos et al., 2007; Freedman and Lefkowitz, 2004; Westfall and

Westfall, 2005).

As shown in Fig, 17, conclusively, the results of the present study demonstrate the first evidence that PCRW inhibits the CA secretion by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization in the isolated perfused adrenal glands of the normotensive rats. It seems that this inhibitory effect of PCRW is exerted by blocking the influx of calcium and sodium into the rat adrenal medullary chromaffin cells as well as the release of Ca^{2+} into the cytoplasmic calcium store at least partly via the increased NO production due to the activation of nitric oxide synthase, which are relevant to the direct interaction with the nicotinic receptor itself. These experimental results may greatly contribute to the hypotensive effect of PCRW components, through inhibition of the CA secretion from adrenomedullary chromaffin cells and consequent reduction of the CA level in the circulation.

V. SUMMARY

The aim of the present study was to investigate whether polyphenolic compounds (PCRW) isolated from red wine may affect release catecholamine from the isolated perfused rat adrenal medulla, and to elucidate its mechanism of action.

PCRW (20~180 μg/mL) perfused into an adrenal vein for 90 min dose- and time-dependently inhibited the CA secretory responses evoked by ACh (5.32 mM), high K^+ (a direct membrane-depolarizer, 56 mM), DMPP (a selective neuronal nicotinic N_N receptor agonist, 100 μM) and McN-A-343 (a selective muscarinic M_1 receptor agonist, 100 μM). PCRW itself did not affect basal CA secretion (data not shown). Also, in the presence of PCRW (60 μg/mL), the secretory responses of CA evoked by Bay-K-8644 (a L-type dihydropyridine Ca^{2+} channel activator, 10 μM), cyclopiazonic acid (a cytoplasmic Ca^{2+}-ATPase inhibitor, 10 μM) and veratridine (an activator of voltage-dependent Na^+ channels, 10 μM) were significantly reduced, respectively. Interestingly, in the simultaneous presence of PCRW (60 μg/mL) and L-NAME (a selective inhibitor of NO synthase, 30 μM), the inhibitory responses of PCRW on the CA secretion evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclpiazonic acid were considerably recovered to the extent of the corresponding control secretion compared with the inhibitory effect of PCRW-treatment alone. Practically, the level of NO released from adrenal medulla after the treatment of PCRW (60 μg/mL) was greatly elevated compared to the corresponding basal released level.

Taken together, these results obtained from the present study demonstrate

that PCRW inhibits the CA secretory responses from the isolated perfused adrenal gland of the normotensive rats evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. This inhibitory effect of PCRW seems to be exerted by inhibiting the influx of both calcium and sodium into the rat adrenal medullary chromaffin cells together with the release of Ca^{2+} from the cytoplasmic calcium store at least partly through the increased NO production due to the activation of nitric oxide synthase. Based on these effects, it is also thought that PCRW may be beneficial to prevent or treat the cardiovascular diseases.

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Influence of Polyphenol Compounds Isolated from Red Wine on Catecholamine Release in the Perfused Rat Adrenal Medulla

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BACKGROUND

Red wines and grapes exhibit endothelium-dependent relaxation of blood vessels via enhanced generation and/or increased biological activity of NO, leading to the elevation of cGMP levels (Fitzpatrick et al., 1993; Fitzpatrick et al., 1995; Fitzpatrick et al., 2000; Zenebe et al., 2003). In vivo red wine polyphenols (PCRW) were shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001). The administration of purple grape juice improved the endothelium dependent, flow-mediated vasodilation in coronary artery disease patients with impaired endothelial function (Stein et al., 1999). PCRW enhanced NO synthesis and cGMP accumulation only in the presence of functional endothelium. In denuded aortic rings, PCRW concentration 10^3-fold higher was necessary to induce relaxation (Ndiaye et al., 2003; Corder et al., 2001). However, recently, we have reported that EGCG, one of potent catechins isolated from green tea, did not affect the secretion if release of catecholamines (CA) evoked by ACh, high K^+ and Bay-K-8644 from the isolated perfused rat adrenal gland (Lim et al, 2002). Despite of these effects, there has been no report on the effect of PCRW on the CA release. Therefore, the purpose of the present study was to examine the effects of PCRW on CA release in the perfused model of the rat adrenal gland.

MATERIALS AND METHODS

Experimental Animals

Mature male Sprague-Dawley rats, weighing 180-250g were anesthetized with intraperitoneal thiopental-Na (40 mg/kg). The adrenal gland was isolated by the methods described previously.

Perfusion of Adrenal Gland

Perfused by means of a peristaltic pump at a rate of 0.31 ml/min with Krebs-bicarbonate solution constantly bubbled with 95% O2 ± 5% CO2. The final pH of the solution was maintained at 7.4 - 7.5.

Drug Administration and Collection of Perfusate

The drug administration was made by perfusion or a single injection into perfusion stream via a three-way stopcock. Secretory responses to ACh, high K^{+}, McN-A-343, Bay-K-8644 and cyclopiazonic acid were collected for 4 min, but the responses to DMPP for 8 min.

Measurement of Catecholamines

Measured directly by the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina (Wakade, 1981), using Fluorospectrophotometer (Kontron, Italy) or HPLC with electrochemical detector

Statistical Analysis

All data were presented as means with their standard errors, and analyzed by Student's paired t-test using the computer system as previously described (Tallarida and Murray, 1987) or by ANOVA-test.

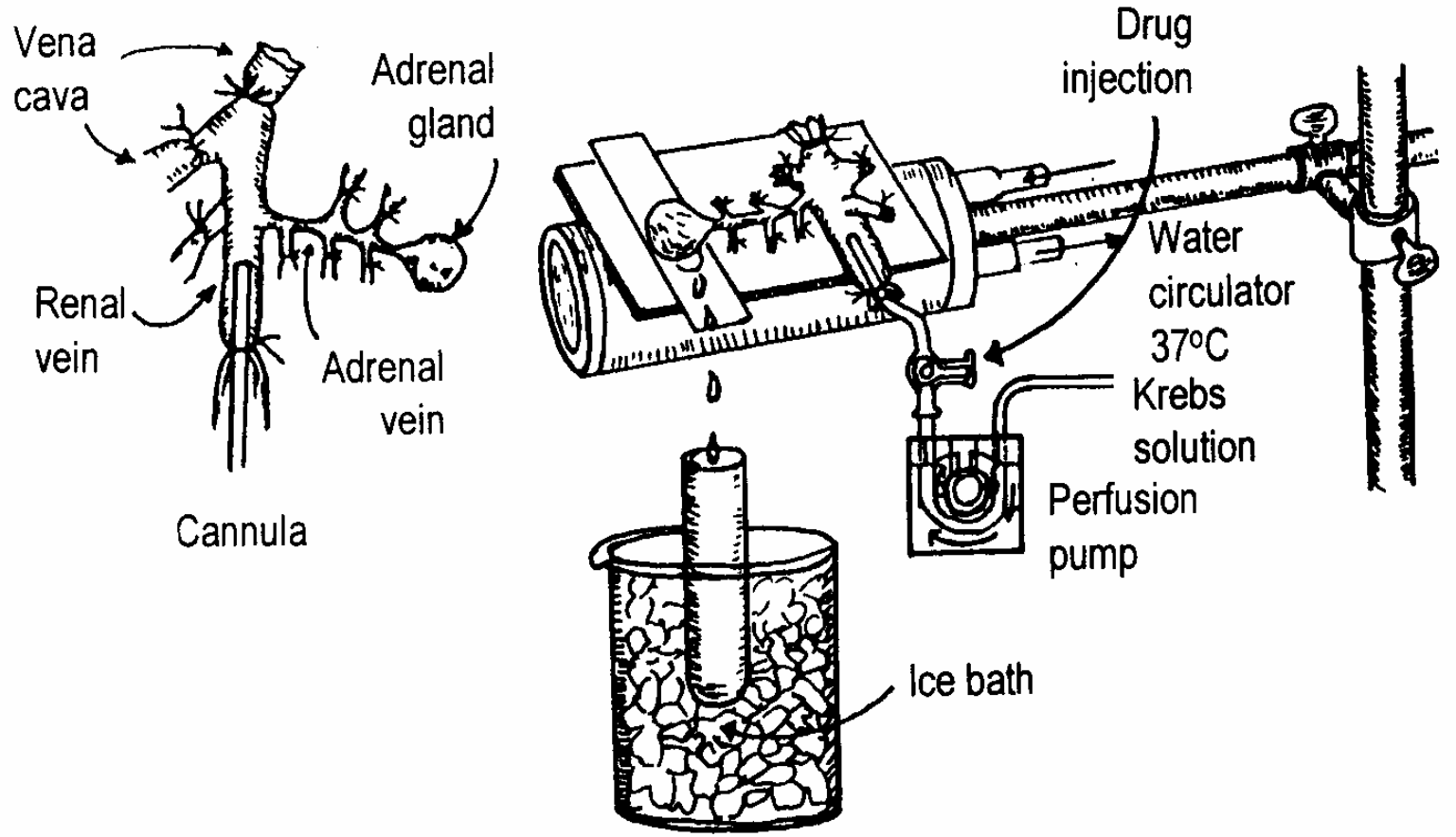

Fig. 2. Schematic drawing of the preparation used to study secretion of catecholamines in the isolated perfused adrenal gland of the SHRs.

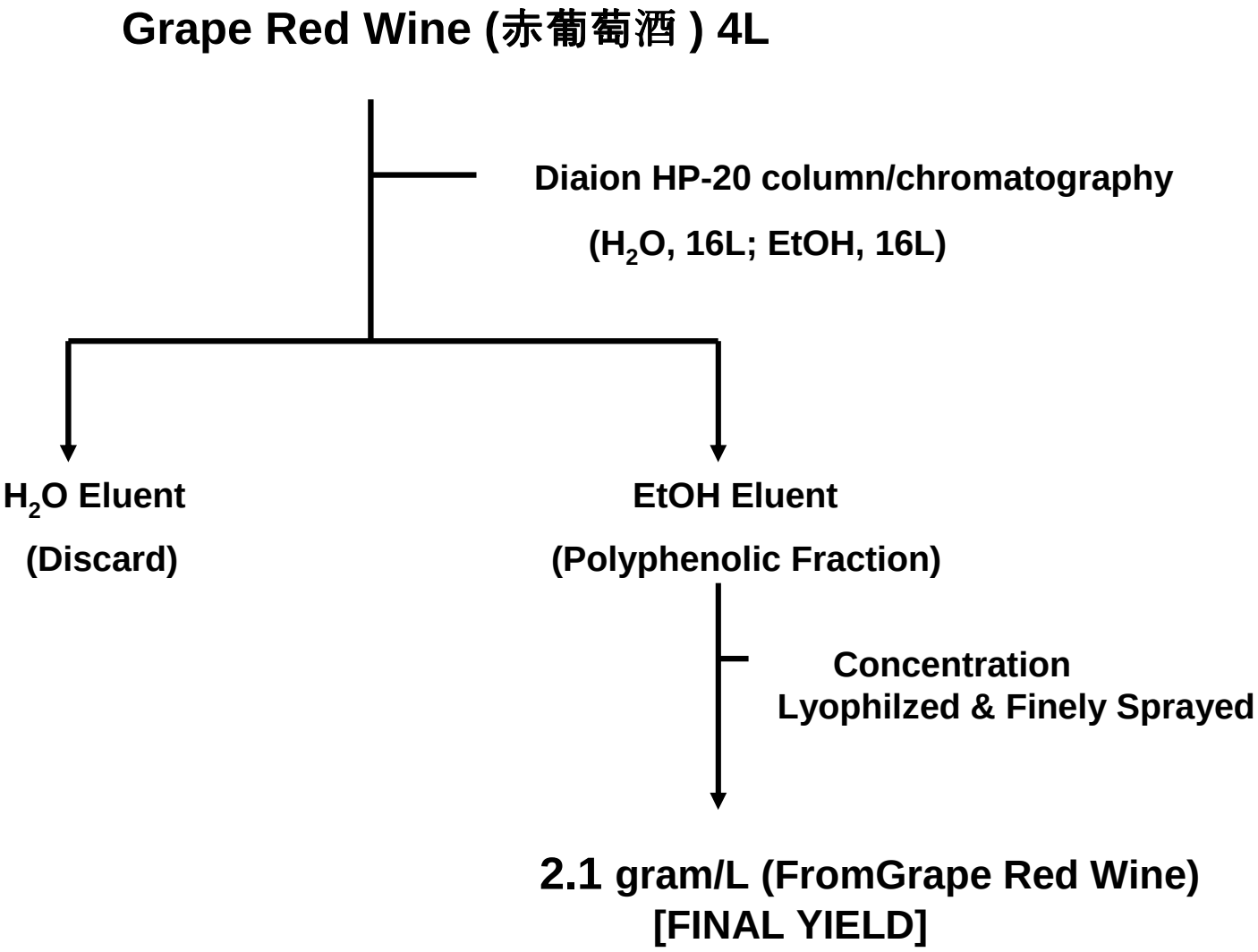

Fig. 1. Preparation of polyphenolic compounds from grape red wine (赤葡萄酒).

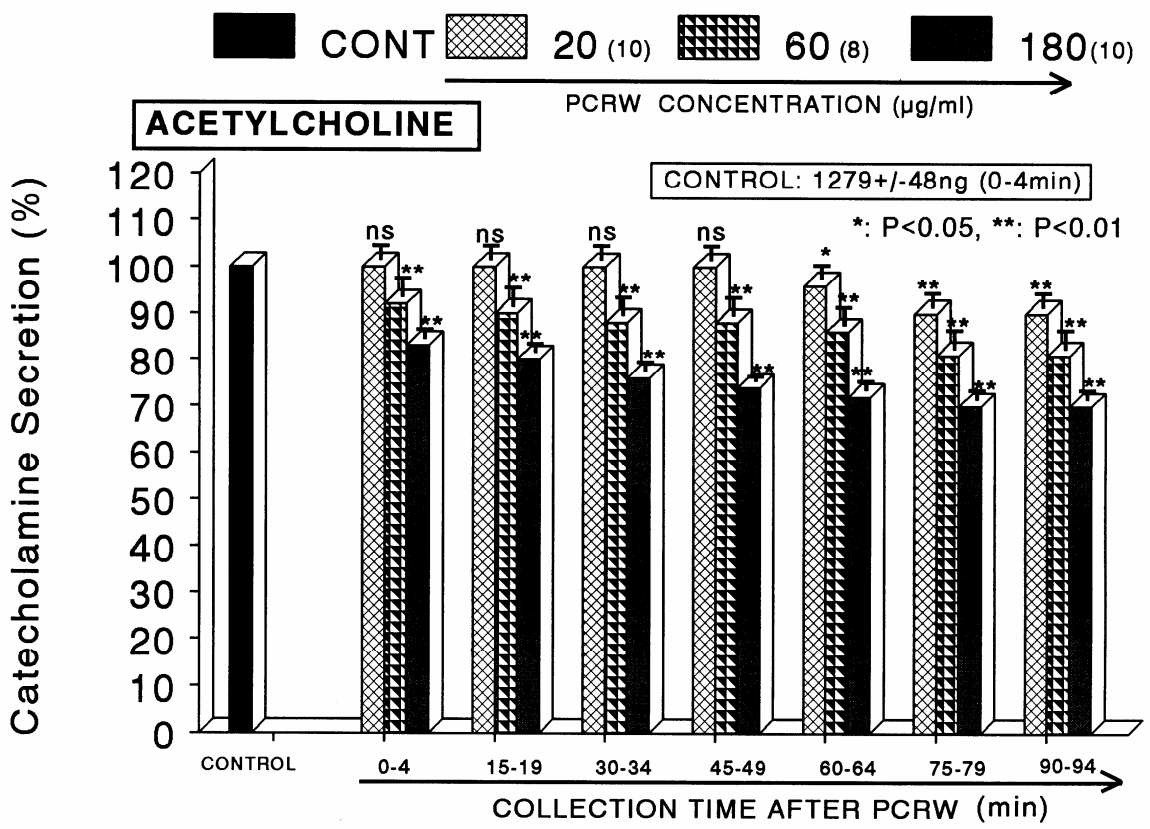

Fig. 3. Dose-dependent effect of polyphenolic compounds extracted from red wine (PCRW) on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 20, 60, 180 μg/mLof PCRW for 90 min as indicated at an arrow mark. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (% of control for 4 min). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of PCRW. ACh-induced perfusate was collected for 4 minutes. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

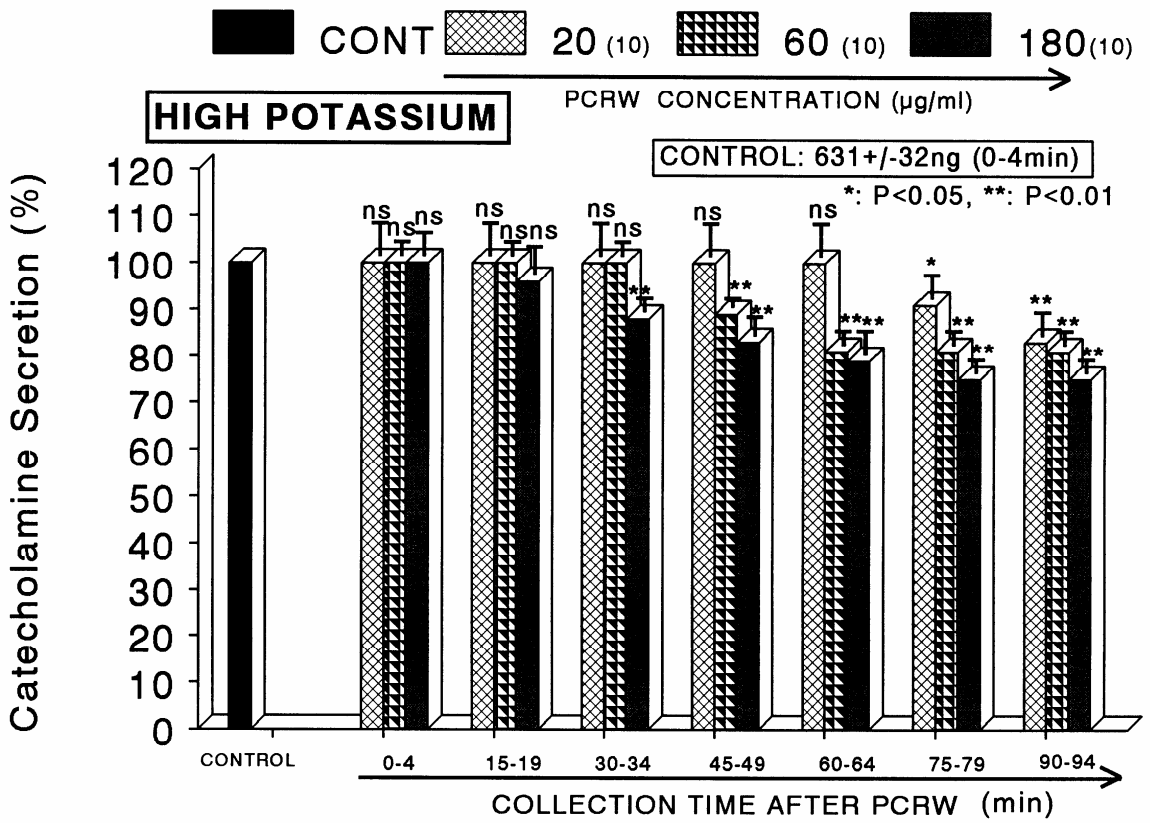

Fig. 4. Dose-dependent effect of PCRW on the secretory responses of catecholamines (CA) evoked by high K^+ from the isolated perfused rat adrenal glands. CA secretion by a single injection of K^+ (56 mM) was injected in a volume of 0.1 ml at 15 min intervals after preloading with 20, 60, 180 μg/mLof PCRW for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of PCRW. K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

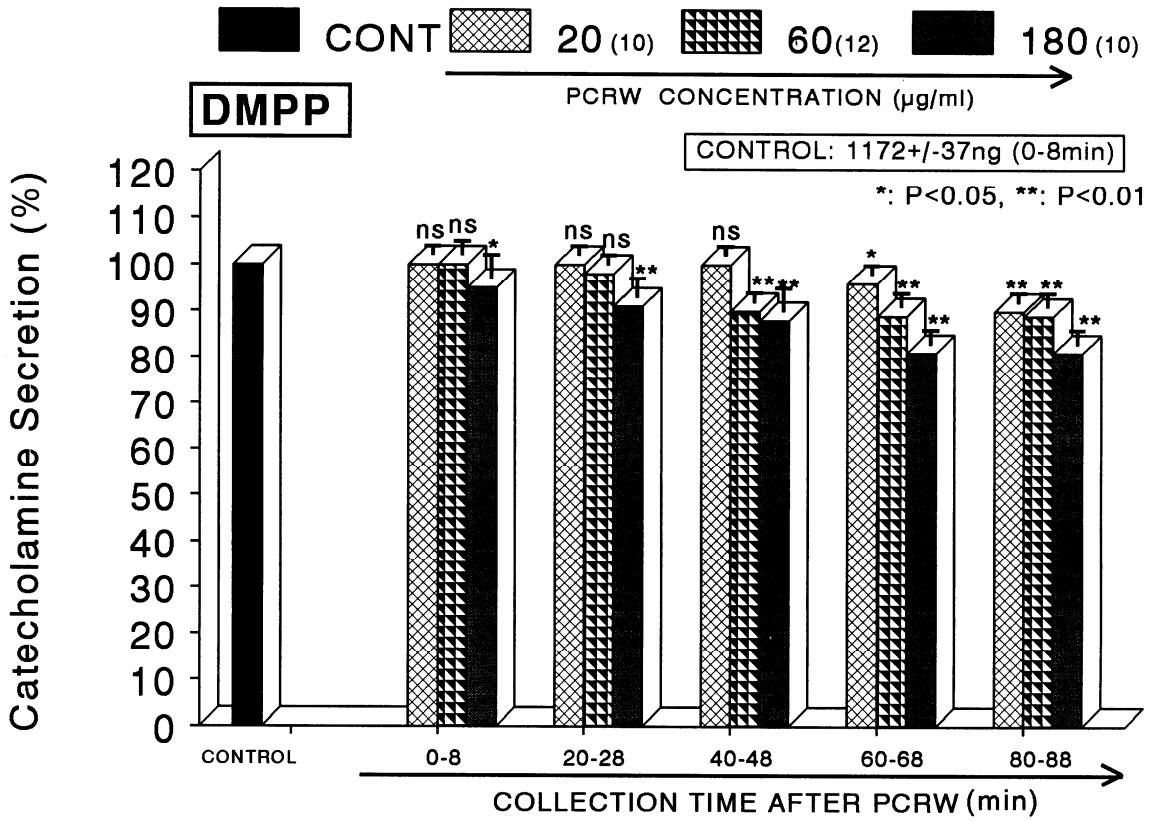

Fig. 5. Dose-dependent effect of PCRW on the secretory responses of catecholamines (CA) evoked by DMPP from the isolated perfused rat adrenal glands. CA secretion by the perfusion of DPPP (10^{-4} M) was infused for 2 min at 20 min intervals after preloading with 20, 60, 180 μg/mLof PCRW for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 1172±37 ng for 8 min) with each concentration-pretreated group of PCRW. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

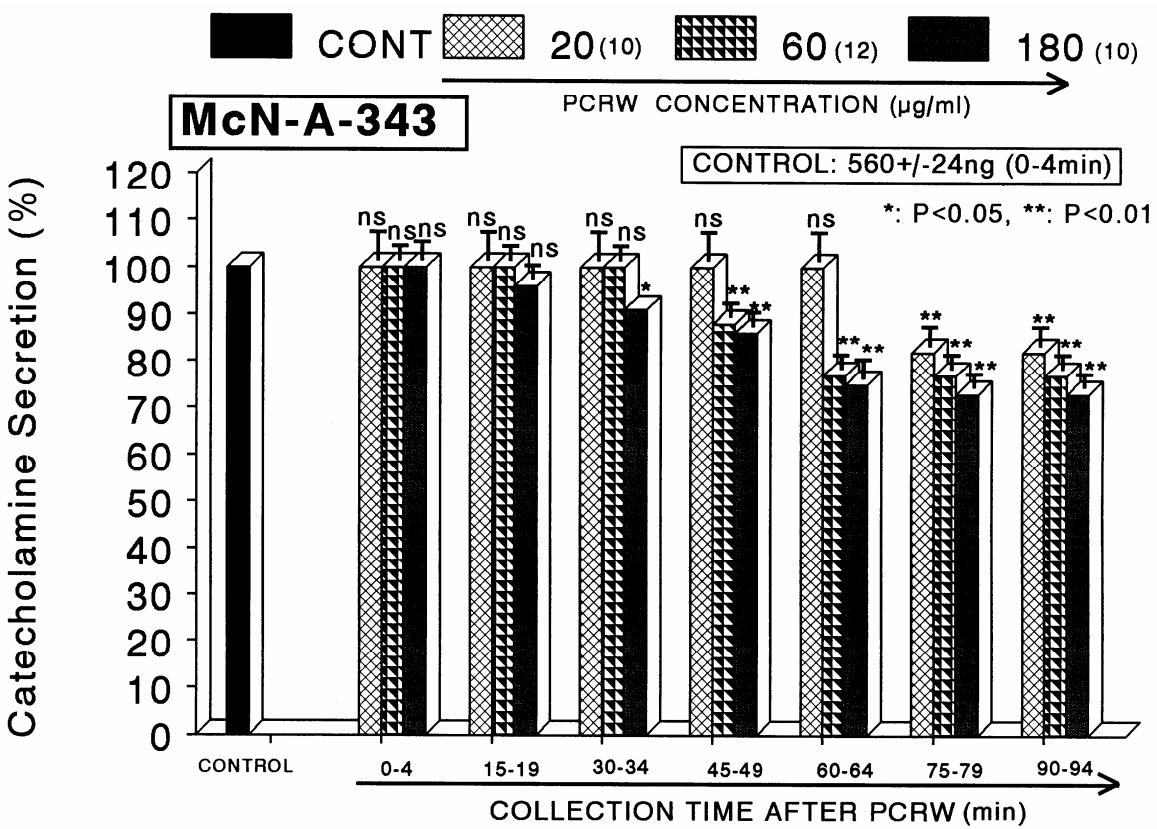

Fig. 6. Dose-dependent effect of PCRW on the secretory responses of catecholamines (CA) evoked by McN-A-343 from the isolated perfused rat adrenal glands. CA secretion by the perfusion of McN-A-343 (10^{-4} M) was infused for 4 min at 15 min intervals after preloading with 20, 60, 180 μg/mLof PCRW for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of PCRW. McN-A-343-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

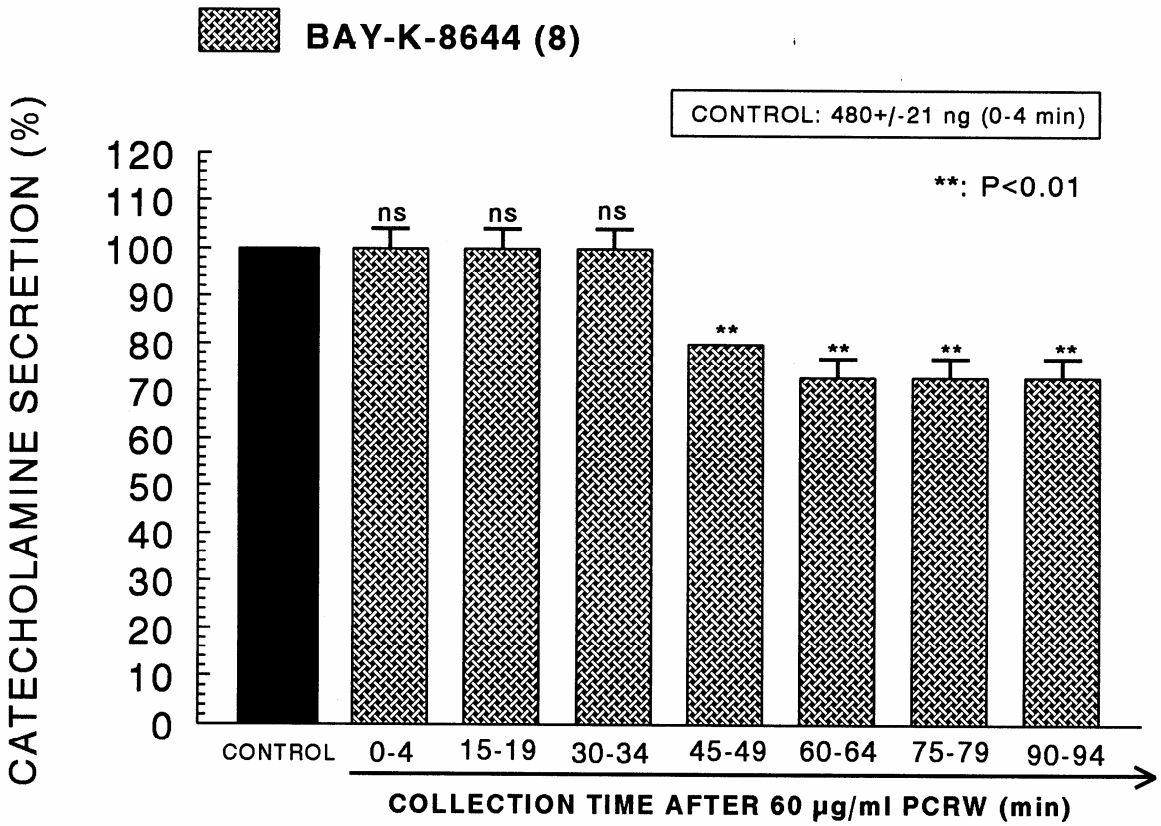

Fig. 7. Time-course effects of PCRW on CA release evoked by Bay-K-8644 from the rat adrenal glands. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of PCRW (60 µg/mL) for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each period after pretreatment with PCRW. Other legends are the same as in Fig. 3. **: $P < 0.01$. ns: Statistically not significant.

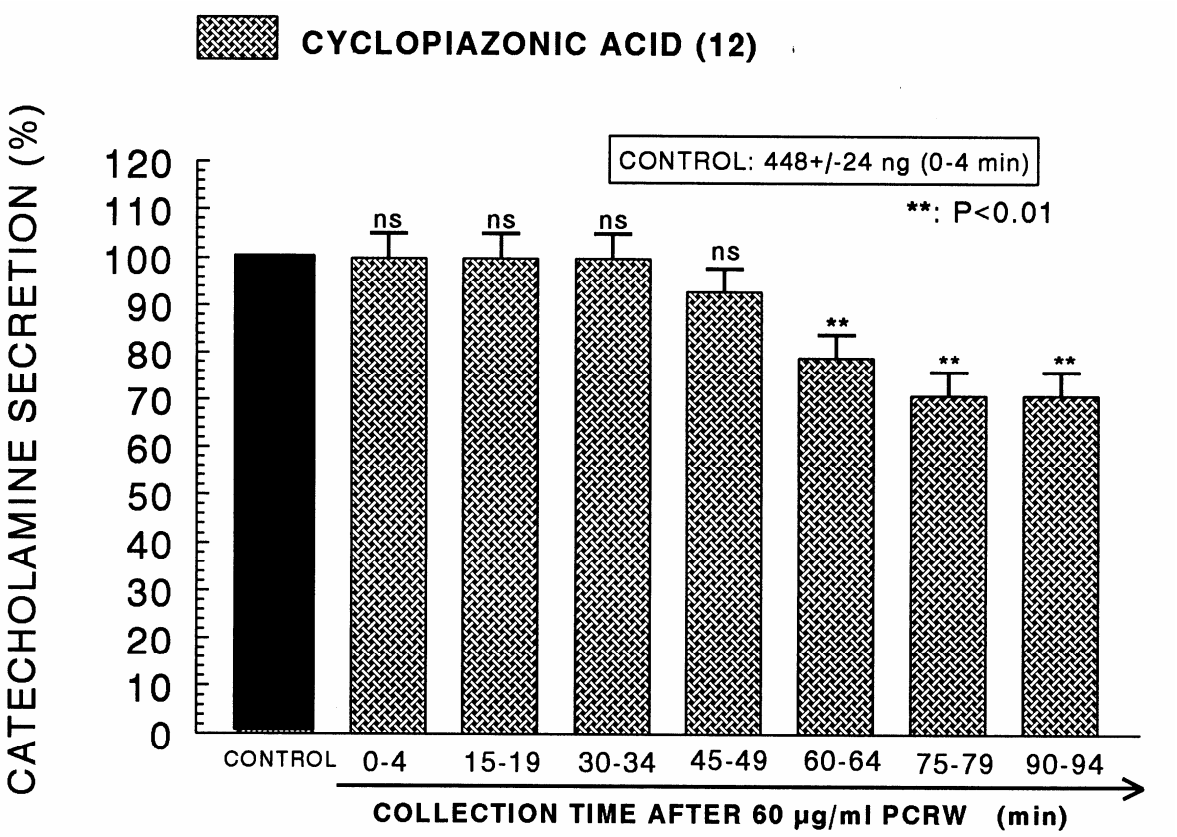

Fig. 8. Time-course effects of PCRW on CA release evoked by cyclopiazonic acid from the rat adrenal glands. Cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of PCRW (60 µg/mL) for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each period after pretreatment with PCRW. Other legends are the same as in Fig. 3. **: $P < 0.01$. ns: Statistically not significant.

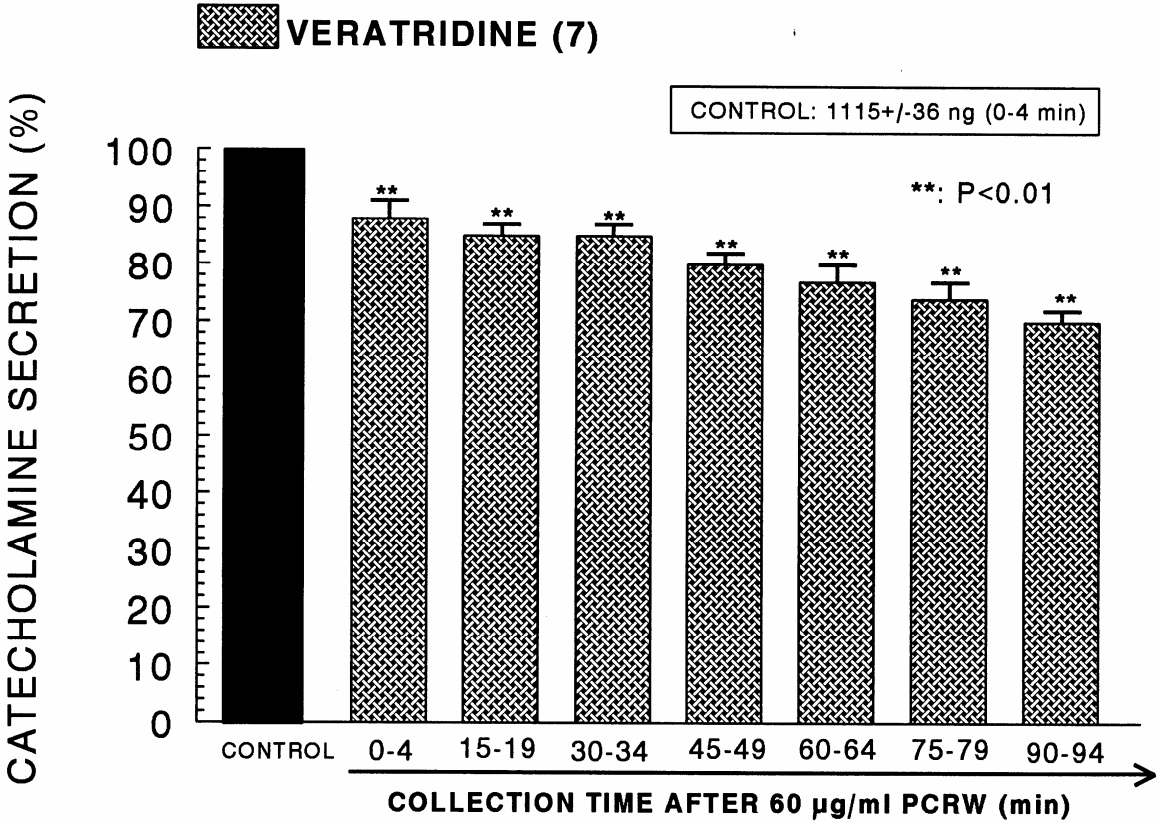

Fig. 9. Time-course effects of PCRC on the CA release evoked by veratridine from the rat adrenal glands. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with PCRC (60 µg/ml) for 90 min, respectively. Other legends are the same as in Fig. 3. **: $P < 0.01$.

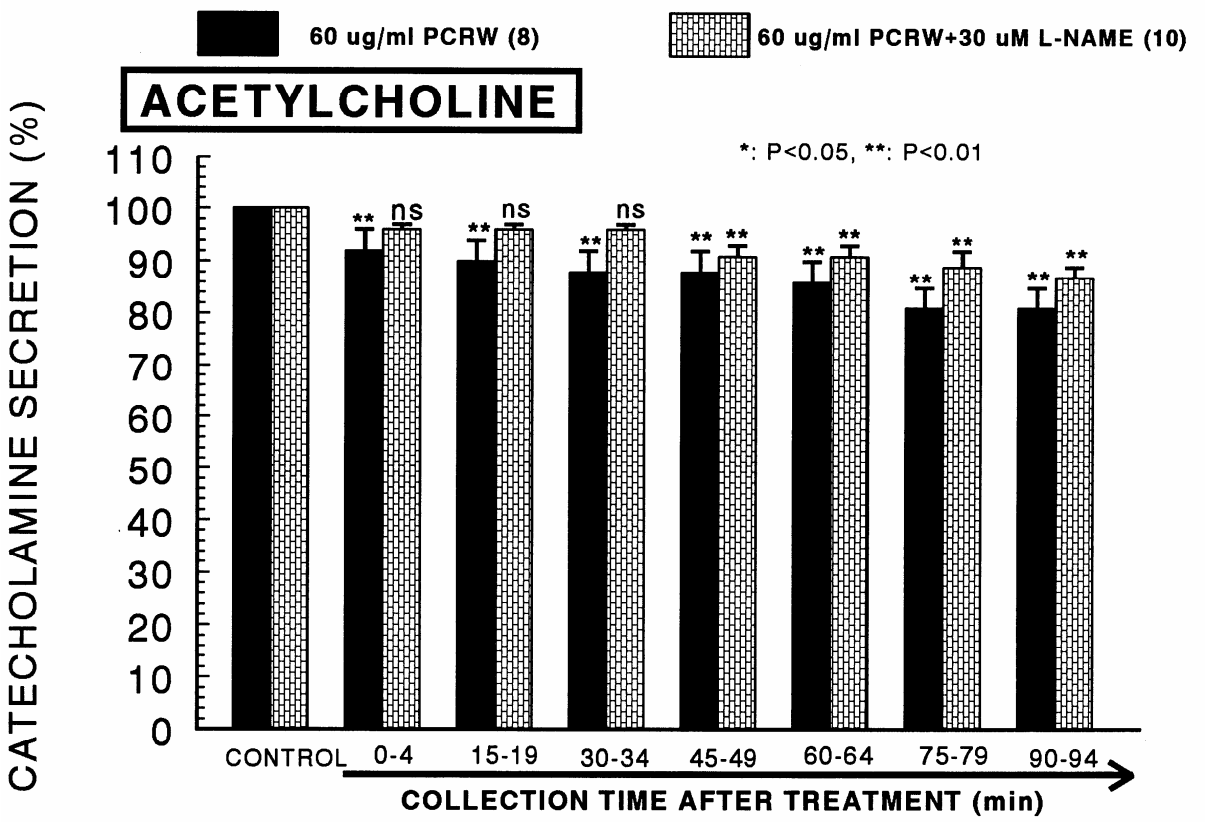

Fig. 10. Effects of PCRW plus L-NAME on the CA secretory responses evoked by ACh from the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was induced before (CONTROL), after preloading with PCRW (60 μg/ml) alone, and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$.

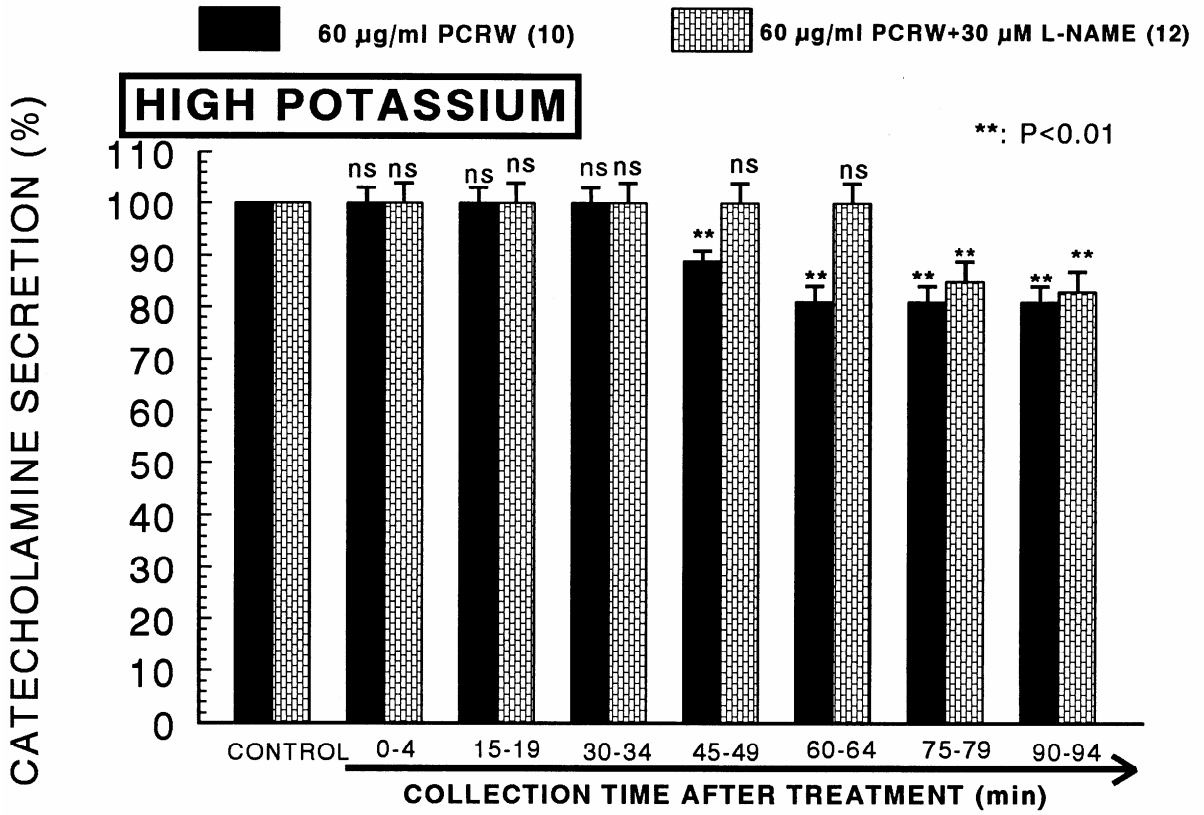

Fig. 11. Effects of PCRW plus L-NAME on the CA secretory responses evoked by high potassium from the isolated perfused rat adrenal glands. The CA secretion by a single injection of high K^+ (5.32×10^{-3} M) in a volume of 0.05 ml was induced before (CONTROL), after preloading with PCRW (60 μg/ml) alone, and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 3. **: $P < 0.01$. ns: Statistically not significant.

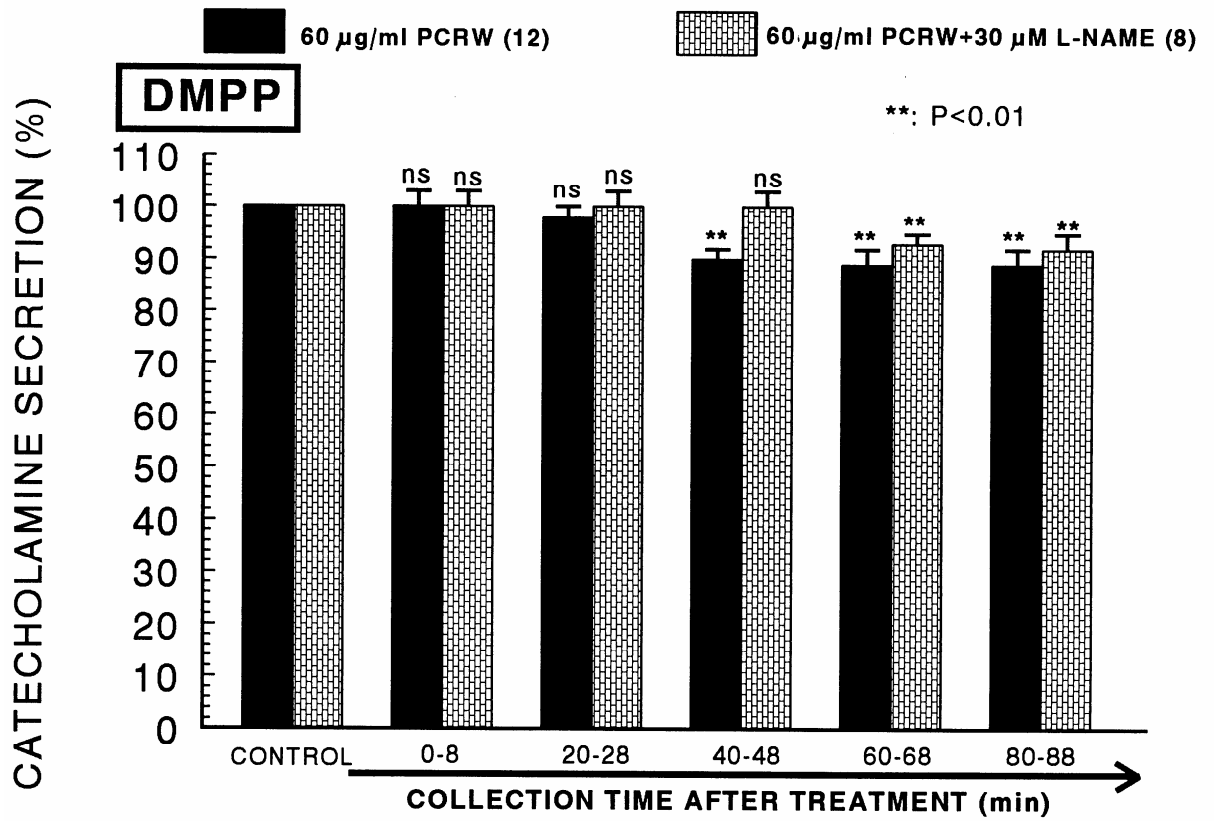

Fig. 12. Effects of PCRW plus L-NAME on the CA secretory responses evoked by DMPP from the isolated perfused rat adrenal glands. The CA secretion by perfusion of DMPP (10^{-4} M) for 2 min was induced before (CONTROL), after preloading with PCRW (60 μg/ml) alone, and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min, respectively. Perfusates were collected for 8 minutes at 20 min-intervals. Other legends are the same as in Fig. 3. **: $P < 0.01$. ns: Statistically not significant.

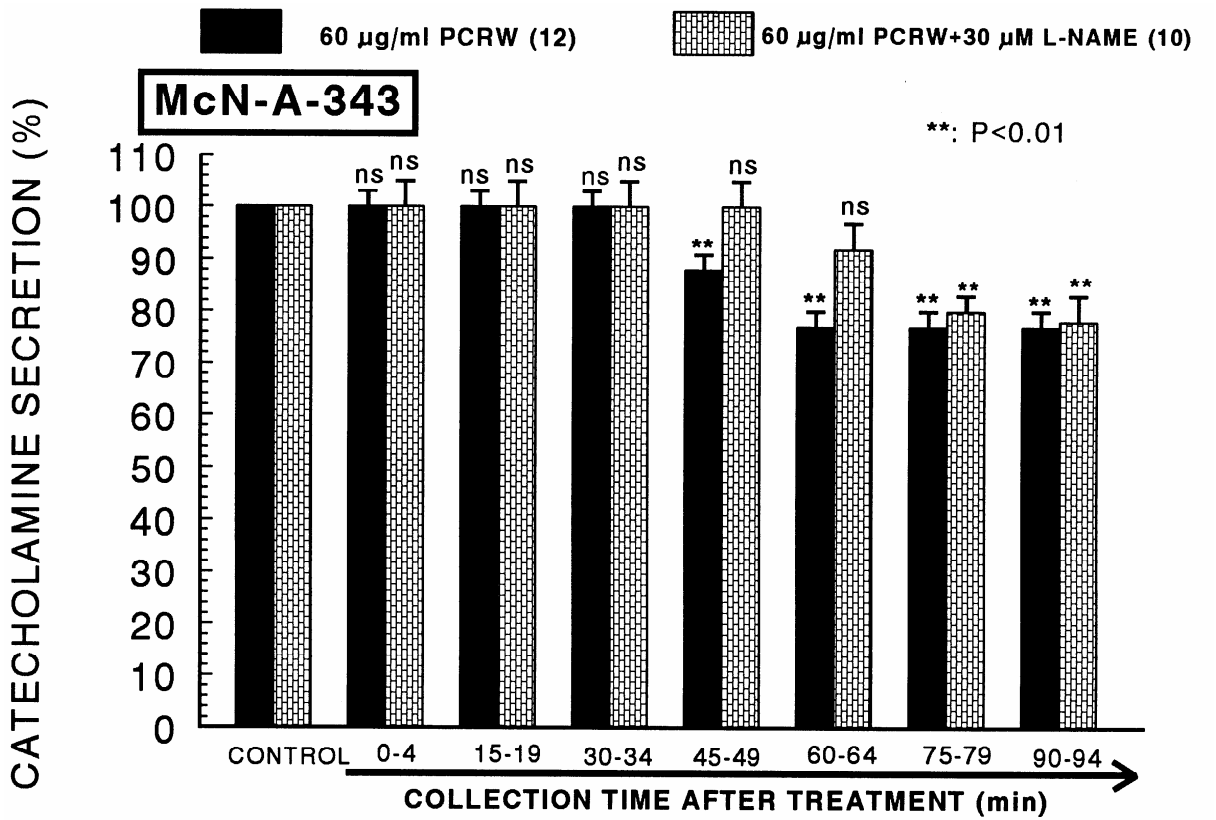

Fig. 13. Effects of PCRW plus L-NAME on the CA secretory responses evoked by McN-A-343 from the isolated perfused rat adrenal glands. The CA secretion by perfusion of McN-A-343 (10^{-4} M) for 4 min was induced before (CONTROL), after preloading with PCRW (60 μg/ml) alone, and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 3. **: $P < 0.01$. ns: Statistically not significant.

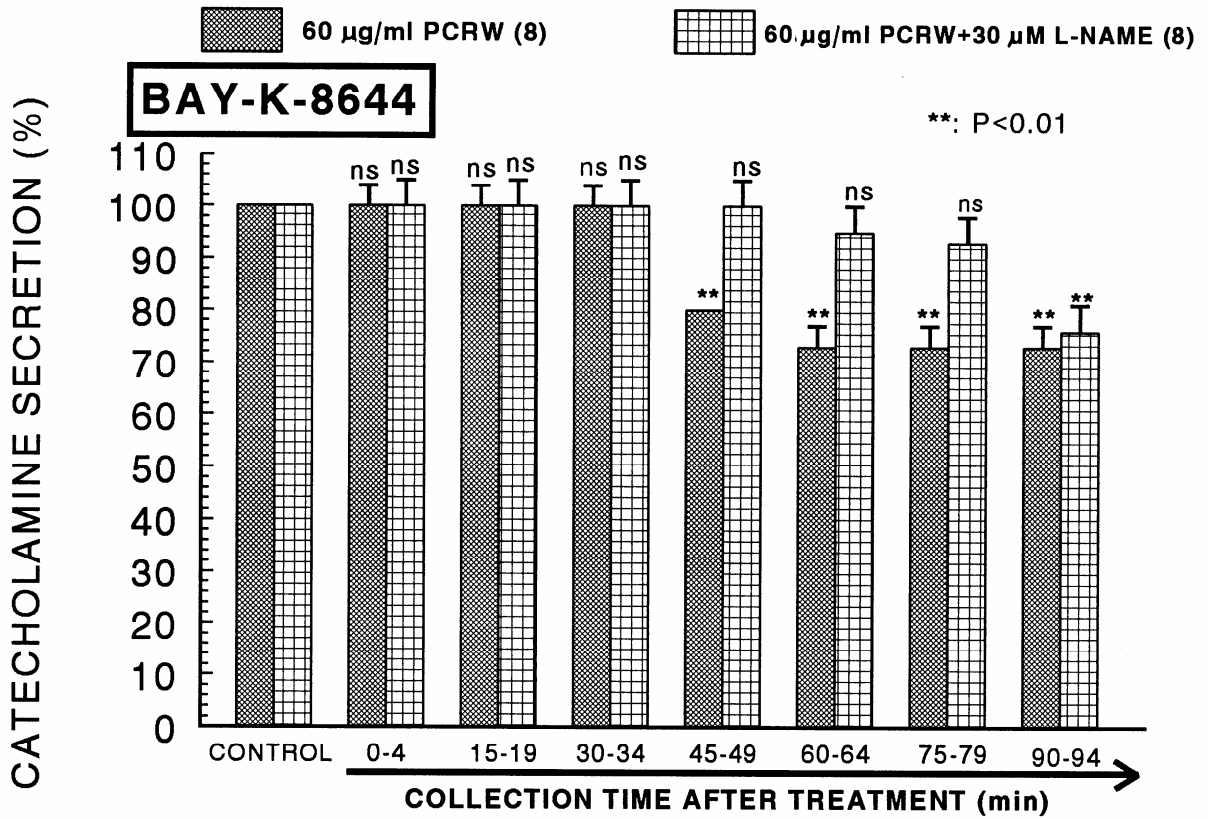

Fig. 14. Effects of PCRW plus L-NAME on the CA secretory responses evoked by Bay-k-8644 from the isolated perfused rat adrenal glands. The CA secretion by perfusion of Bay-k-8644 (10^{-5} M) for 4 min was induced before (CONTROL), after preloading with PCRW (60 μg/ml) alone, and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 3. **: P < 0.01. ns: Statistically not significant.

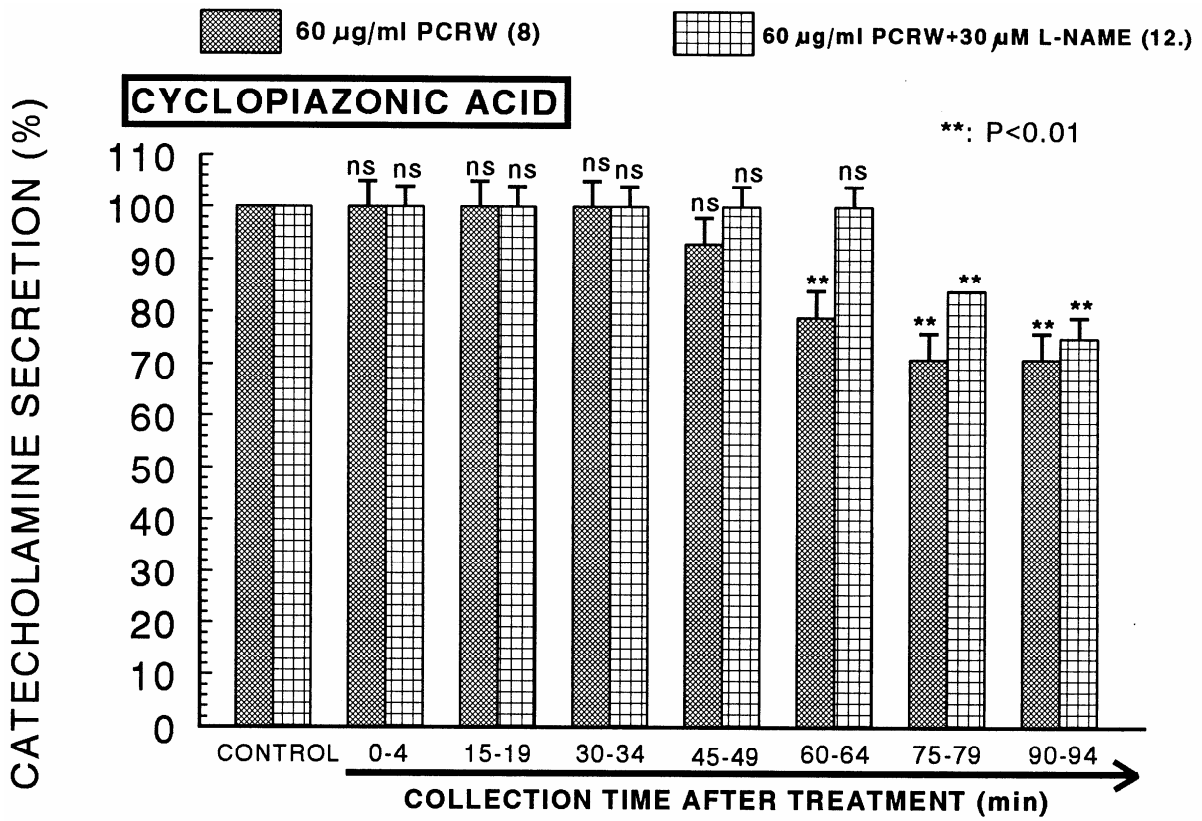

Fig. 15. Effects of PCRW plus L-NAME on the CA secretory responses evoked by cyclopiazonic acid from the isolated perfused rat adrenal glands. The CA secretion by perfusion of cyclopiazonic acid (10^{-5} M) for 4 min was induced before (CONTROL), after preloading with PCRW (60 μg/ml) alone, and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min, respectively. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 3. **: $P < 0.01$. ns: Statistically not significant.

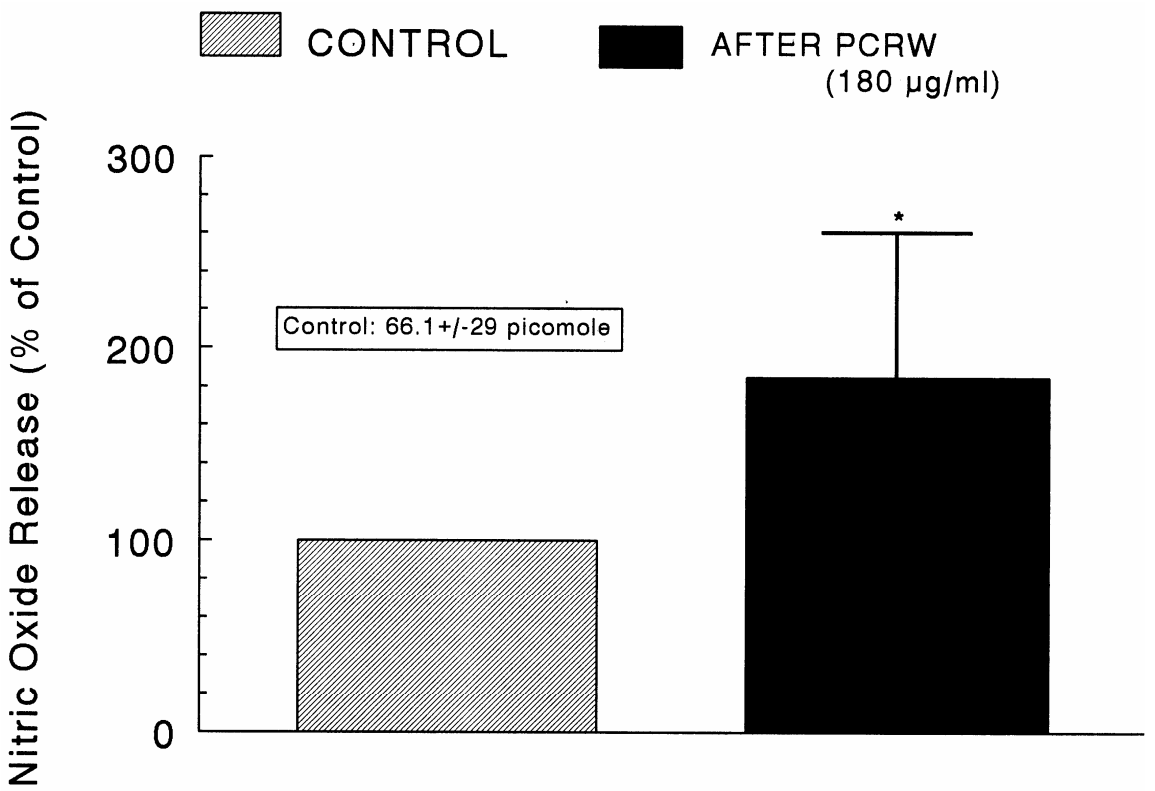

Fig. 16. Comparison of nitric oxide (NO) production before (CONTROL) and after administration of PCRW in the isolated perfused rat adrenal medulla. Perfusate sample was taken for 8 min after loading the perfusion of PCRW (180 ug/ml) at a rate of 0.31 ml/min. Ordinate: the amounts of NO released from the adrenal medulla (% of control). Abscissa: Treatment (before and after PCRW). Statistical difference was made by comparing the control (66.1±29 picomoles) with PCRW-treated group. **: P< 0.01.

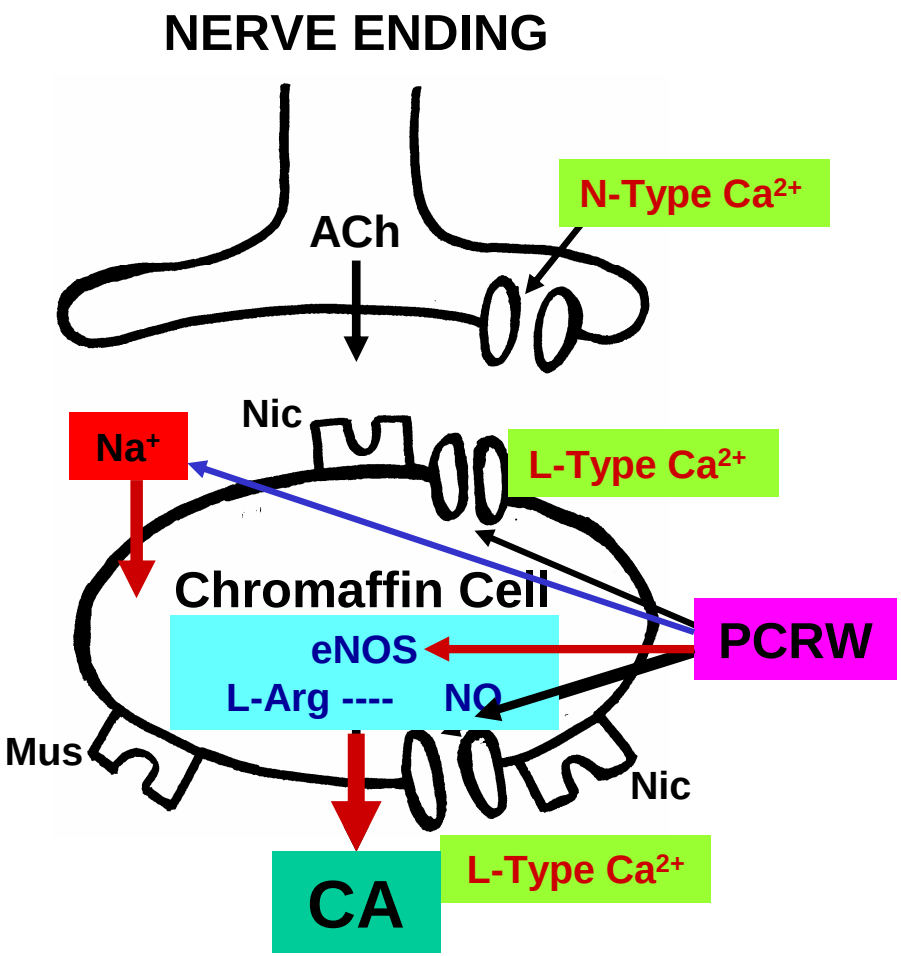

Fig. 17. Schematic diagram of possible action site of PCRW in the rat adrenal galnd. This diagram demonstrates possible localizations of voltage-dependent Ca^{2+} channels and cholinergic receptors mediating secretion of adrenal catecholamines (CA). CA-containing cells possess synaptic nicotinic receptors, extrasynaptic nicotinic and muscarinic receptors, and L-type voltage-dependent Ca^{2+} channels close to the extrasynaptic nicotinic receptors.

CONCLUSIONS

Taken together, these results obtained from the present study demonstrate that PCRW inhibits the CA secretory responses from the isolated perfused adrenal gland of the normotensive rats evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. It seems that this inhibitory effect of PCRW is exerted by inhibiting both the calcium influx into the adrenal medullary chromaffin cells of the normotensive rats and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the increased NO production due to the activation of nitric oxide synthase, which are at least relevant to the direct interaction with the nicotinic receptor itself.