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Inhibitory Mechanism of Polyphenols Isolated from *Rubus coreanum* on Catecholamine Release in the Perfused Adrenal Medulla of SHRs

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자연발증고혈압쥐의 관류 부신수질에서 카테콜아민 유리작용에 대한 복분자 폴리페놀의 억제기전

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이 논문을 의학박사 학위신청논문으로 제출함.

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나 덕 미의 박사학위논문을 인준함

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나 덕 미

(지도교수 : 임 동 윤)

조선대학교 대학원 의학과

본 연구의 목적은 복분자 *(Rubus coreanum* MIQUEL, 覆盆子)로 양조한 복분자주에서 분리한 폴리페놀 화합물(PCRC)이 자연발증고혈압쥐 (SHRs)로부터 분리 적출한 부신의 관류모델에서 카테콜아민 (catecholamines, CA) 유리작용에 미치는 영향을 검색하여 그 작용기전을 규명코자 본 연구를 시행하여 다음과 같은 결과를 얻었다. PCRC (20~180 μg/ml)을 부신정맥 내로 90분간 관류시 비교적 용량 및 시간 의존적으로 ACh (5.32 mM), 고칼륨 (56 mM, 막탈분극제), DMPP (100 μM, 선택성 니코틴수용체 작용제), 및 McN-A-343 (100 μM, 선택성 무스카린 M_1-수용체 작용제)에 의한 CA 분비반응을 억제하였다. 그러나, PCRC 자체는 기초 CA 분비량에 영향을 미치지 않았다. 또한, PCRC (60 μg/ml) 존재 하에서, 선택성 나트륨통로 억제제인 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, PCRC (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 PCRC 단독처치 시 나타나는 억제효과에 비교하여 상응하는 대조치의 수준까지 회복되었다. 또한 실제로 PCRC처치 후에 NO 유리량이 기초 유리량에 비해 현저하게 증가하였다.

이와 같은 연구결과를 종합하여 보면, 자연발증 고혈압쥐의 적출 관류 부신 수질에서 PCRC는 콜린성(니코틴 및 무스카린 수용체) 흥분작용 및 막탈분극에 의한 CA 분비작용에 대하여 억제작용을 나타내었다. 이러한 PCRC의 억제작용은 SHRs의 부신수질에서 NO Synthase의 활성화에 의한 NO 생성증가로 인하여 부신크롬친화세포 내로 나트륨 및 칼슘유입과 세포 내 칼슘저장고로부터 칼슘유리의 억제작용에 기인되는 것으로 생각된다.

이와 같은 PCRC의 작용으로 보아 PCRC가 심혈관계 질환의 예방 및 치료에 유익할 것으로 사료된다.

I. INTRODUCTION

Bokboonja wine is also the principal products of Gochang county, Chonbuk province, Korea, where is famous for wine brewed from *Rubus coreanum* MIQUEL (覆盆子酒). *Rubus coreanum* MIQUEL (覆盆子) has been presently used in treating the disease of the aged, spermatorrhea and impotence in oriental medicine. *Rubus coreanum* has been found to possess several polyphenolic compounds, such as (-)-epicatechin, (+)-catechin, proanthocyanidin, etc. There are no reports about its cardiovascular effects so far, while there are many reports about those of polyphenolic compound isolated from red wine.

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 *in vivo* studies, red wine polyphenolic compounds (PCRWs) 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). PCRWs enhanced NO synthesis and cGMP accumulation only in the presence of functional endothelium. In denuded aortic rings, 103-fold higher concentration of PCRWs was necessary to induce relaxation (Ndiaye et al., 2003; Corder et al., 2001). 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).

Recently, it has been shown that green tea extract inhibits the secretory responses of catecholamines (CA) evoked by cholinergic (nicotinic and muscarinic) stimulation and direct membrane-depolarization in the perfused adrenal medulla isolated from the rat (Lim et al., 2003) and the rabbit (Lim, 2005). However, epigallocatechin-3-gallate, one of potent catechins isolated from green tea, did not affect the secretion of catecholamines (CA) release evoked by the above serectagogues. As aforementioned, there are so far many reports about the effects of red wine on cardiovascular system. Despite of these studies, there are no reports on in vitro functional effects of polyphenolic compounds (PCRC) from wine, which is brewed from *Rubus coreanum* MIQUEL, on the cardiovascular system. Therefore, the aim of the present study was to investigate the ability of PCRC on secretion of CA in the perfused adrenal gland of SHRs, and to establish its mechanism of action.

Ⅱ. MATERIALS AND METHODS

Experimental procedure

Mature male spontaneously hypertensive rats (purchased from DAMOOL SCIENCE, International Customer Service, Seoul, Korea), weighing 200 to 300 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. 1).

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.

Isolation of polyphenolic compounds

Polyphenolic compounds were prepared as described by Caderni et al (2000), using adsorption chromatography from a 1-year old wine brewed from *Rubus coreanum* Miquel (覆盆子酒) at the Research Institute of Bokboonja, Gochang County, Cheollabukdo Province, Korea or 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. 2): alcohol was eliminated by distillation, 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 freezing dryer (Coldvac -80, Hanil R & D, Korea). About 2.9 g PCRC was obtained from 1 L Bokboonja wine, and 2.1 g PCRW from 1 L red grape wine. The working solution of this PCRC was prepared by dissolving in 0.9% NaCl solution on the day of each experiment and filtered before administration.

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

perfusate 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 PCRC on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing PCRC 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 PCRC, 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 (Anton & 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 CA content in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Measurement of NO release

NO release was measured using a NO-selective microelectrode (ami700, Innovative Instruments Inc) and an amplifier (inNo meter, Innovative Instruments Inc). Adrenomedullary NO production was quantified as the integrated signal detected by the microelectrode after perfusion of PCRC into adrenal medulla of SHRs, as previously described (McVeigh et al., 2002). The electrode was calibrated by producing standardized concentrations of NO in 0.5% (wt/vol) KI in 0.1 mol/LH2SO4 from $NaNO_2$ standards. NO release was quantitated as the current detected at the electrode after loading PCRC into adrenal medulla. NO release was calculated as picomoles.

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: PCRC, 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-car

boxylate (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 PCRC used were expressed in terms of molar base.

III. RESULTS

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

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=12). Since, in *in vivo* study, PCRW was shown to reduce blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001), it was attempted initially to examine the effects of PCRC itself on CA secretion from the perfused model of the adrenal glands of the SHRs. However, in the present study, PCRC (20 ~ 180 μg/ml) itself did not produce any effect on basal CA output from the perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of PCRC on cholinergic receptor stimulation- as well as membrane depolarization-evoked CA secretion. Secretagogues were given at 15 to 20 min-intervals. PCRC was present for 90 minutes after the establishment of the control release.

When ACh (5.32×10^{-3} M) in a volume of 0.05 ml was injected into the perfusion stream, the amount of CA secreted was 1589±77 ng for 4 min. However, the pretreatment with PCRC 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 PCRC, the CA releasing responses were inhibited by 69% of the corresponding control release (100%). Also, it has been found that depolarizing agent like KCl stimulates markedly CA secretion (718±38 ng for 0-4 min). High K^+ (5.6×10^{-2} M)-stimulated CA secretion

after the pretreatment with 20 μg/ml PCRC was not affected for the first 45 min period as compared with its corresponding control secretion (Fig. 4). However, following the pretreatment with higher concentrations of PCRC (60 ~ 180 μg/ml), high K^+ ($5.6x10^{-2}$ M)-stimulated CA secretion was maximally inhibited to 68% of the control after 75 min period, although it was not initially affected at 60 μg/ml of PCRC. DMPP (10^{-4} M), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (1449±60 ng for 0-8 min). However, as shown in Fig. 5, DMPP-stimulated CA secretion after the pretreatment with PCRC was greatly reduced to 75% 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 (565±23 ng for 0-4 min). However, McN-A-343-stimulated CA secretion in the presence of PCRC was markedly depressed to 73% of the corresponding control secretion (100%).

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

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 PCRC 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 PCRC was greatly blocked to 75% of the control except for the early 15 min period as compared to the

corresponding control release (512±28 ng for 0-4 min) from 7 adrenal glands of SHRs, as shown in Fig. 7.

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). The inhibitory action of PCRC on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 8. However, in the presence of PCRC in 8 adrenal glands of SHRs, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was also inhibited to 73% of the control response (480±21 ng for 0-4 min).

It has 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 CA secretion in cultured bovine adrenal medullary cells (Wada et al., 1985a). As shown in Fig. 9, veratridine greatly produced CA secretion (1216±54 ng for 0-4 min). PCRC (60 μg/ml) also attenuated veratridine-induced CA secretion by 68% of the corresponding control release in a time-dependent manner.

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

It has also been found that, in this study, PCRC inhibited the CA secretory response evoked by cholinergic stimulation in the perfused adrenal glands of SHRs. Therefore, to study the relationship between NO and PCRC-induced inhibitory effects on the CA release from the adrenal glands of SHRs, the effect of

L-NAME on PCRC-induced inhibitory responses of CA secretion evoked by cholinergic receptor-stimulation as well as membrane depolarization was examined. In the present study, in the simultaneous presence of PCRC (60 μg/ml) and L-NAME (30 μM) for 90 min from 8 adrenal glands of SHRs, ACh (5.32 mM)-evoked CA release was initially not affected at first 4 min, but later rather recovered to 90% of the corresponding control release at the period of 90-94 min compared to that of PCRC (60 μg/ml)-treated group only, as illustrated in Fig. 10. High K^+ (56 mM)-evoked CA release in the presence of PCRC (60 μg/ml) and L-NAME (30 μM) for 90 min was also not changed for 0-64 min, and then recovered to 84% of the corresponding control release at the last period of 90-94 min period in comparison to that of PCRC (60 μg/ml)-treated group only from 7 glands (Fig. 11). As shown in Fig. 12, the simultaneous perfusion of PCRC and L-NAME for 90 min no longer inhibited DMPP-evoked CA release for the period of 0-68 min from 10 adrenal glands while later rather recovered to 92% of the control release at the period of 80-88 min. Moreover, in the simultaneous presence of PCRC and L-NAME for 90 min, McN-A-343-evoked CA secretory responses was also time-dependently recovered to 81% of the control secretion compared to that of PCRC (60 μg/ml)-treated group only from 8 glands as shown in Fig. 13, although they were not affected at period of 0-64 min.

As shown in Fig. 14, the simultaneous perfusion of PCRC (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 glands, and then also recovered to 80% of the control release at the last period of 90-94 min in comparison to that of PCRC (60 μg/ml)-treated group only. As shown in Fig. 15, in the presence of PCRC (60 μg/ml) and L-NAME (30 μM) for 90 min in 10 rat adrenal glands,

cyclopiazonic acid (10^{-5} M)-evoked CA secretion was recovered to 78% of the control response (100%) at the period of 90-94 min in comparison to that of PCRC (60 μg/ml)-treated group only.

Effect of PCRC on the level of nitric oxide released from the perfused adrenal medulla of the SHRs

As shown in Fig. 10~15, the inhibitory effects of PCRC on cholinergic stimulation- and direct membrane depolarization-evoked CA secretory responses were significantly reduced in the presence of l-NAME. Therefore, it was decided directly to determine the level of NO released from adrenal medulla of SHRs after the treatment of PCRC. Moreover, it is found 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). In 10 adrenal glands, the basal amount of NO released from medulla prior to administration of PCRC was 15.5±2.3 picomoles. However, after loading with PCRC it was greatly elevated to 49.3±8.2 picomoles, which was 328% of the basal release, as shown in Fig. 16.

IV. DISCUSSION

The present experimental results have suggested that PCRC inhibits the CA secretory responses from the isolated perfused adrenal gland of the SHRs evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. It seems that this inhibitory effect of PCRC is exerted by inhibiting both the calcium influx into the adrenal medullary chromaffin cells of the SHRs and the uptake of Ca^{2+} into the cytoplasmic calcium store partly through the activation of NO production.

In the present study, in the simultaneous presence of PCRC and L-NAME (NO Synthase inhibitor), the CA secretory responses evoked by ACh, DMPP, high K^+ and Bay-K-8644 were considerably recovered to the extent of the corresponding control secretion compared to those of PCRC treatment alone. This result is well consistent with the 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). Moreover, In this study, following treatment of PCRC into adrenal medulla of SHRs NO production was greatly elevated as shown in Fig. 16. Taking into account these findings, in the present study, it is likely that PCRC inhibits the CA secretory response evoked by

various secretagogues through increasing NO production in adrenal chromaffin cells since PCRC-induced inhibitory responses of CA secretion were significantly reduced in the presence of L-NAME, an inhibitor of NO synthase, and PCRC practically enhanced NO release from adrenal medulla of SHRs.

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, wt al., 1993). 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., 1993).

Furthermore, these effects of resveratrol and PCRW are agreement with the present result that PCRC 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 adrenal medulla of SHRs, since this inhibitory effect of PCRC 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 PCRC on the CA secretion were recovered to the considerable extent of the control secretion compared with the inhibitory effects of PCRC-treatment alone. This result demonstrates that PCRC can inhibit the CA release at least partly through the activation of nNOS in the adrenal medulla of SHRs. 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 the 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 PCRC possesses the ability partly to activate nNOS in the adrenomedullary chromaffin cells of SHRs, in addition to the direct inhibitory effects on the CA secretion.

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. 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. Usually, two mechanisms are involved in the secretion of adrenal medullary hormones. Upon excitation of splanchnic nerves, ACh is released from the nerve terminals, and then activates nicotinic the CA secretion. Based on this fact, the present findings that PCRC inhibited the CA secretory responses evoked by nicotinic receptor stimulation as well as by membrane depolarization in the adrenal medulla of SHRs 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 PCRC-induced inhibitory activity of the 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 the 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 PCRC 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 occurring 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 and Hwang, 1991). In support of this idea, it has 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 this study, PCRC inhibited the secretory responses of CAs evoked by ACh, DMPP, McN-A-343 and high K^+. It suggests that PCRC can produce the similar effect in adrenal medulla of the normotensive rats with that of green tea extract in adrenal medulla of the normotensive rats and rabbits.

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 PCRC can cause the depressor effect by the inhibition of CA secretion from the adrenal medulla. The present findings appeared to contribute at least partly to the facts that extracts of tea (Fitzpatrick

et al., 1992) 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).

In the present study, PCRC 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; Schramin et al, 1983). This result indicates that PCRC may inhibit Ca^{2+} influx to the adrenomedullary cells of SHRs. In support of this idea, in cultured bovine adrenal medullary cells, nicotinic (but not muscarinic) receptors mediate the Ca^{2+}-dependent CA secretion (Fisher et al., 1981; Yanagihara et al, 1979). It has also been known that the activation of nicotinic receptors stimulates the 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 PCRC indicates that this inhibitory effect of PCRC is exerted through the direct inhibition

of calcium influx into the adrenal chromaffin cells of SHRs. Furthermore, slight elevation in the extracellular potassium concentration increases both the frequency of spontaneous action potentials and the CA secretion (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 PCRC inhibited the CA secretion evoked by Bay-K-8644 as well as by high K^+ suggest that PCRC 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 these inhibitory effects of PCRC on the CA secretion evoked by DMPP and veratridine may be mediated by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels due to activation of nicotinic receptor-associated ionic channels, responsible for carbachol-induced Na^+ influx, as well as of voltage-dependent Na^+ channels, responsible for veratridine-induced Na^+ influx, respectively.

The present study has also shown that PCRC 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; Seidler 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 PCRC on the 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 PCRC 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 CA secretion. 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 of 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 an 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 PCRC-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 PCRC on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

In conclusion, as shown in Fig. 17, the results of the present study have suggest that PCRC inhibits the CA secretion by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization in the isolated perfused adrenal

glands of SHRs. It seems that this inhibitory effect of PCRC is exerted by blocking influx of sodium and calcium into the adrenal medullary chromaffin cells of SHRs as well as the uptake of Ca^{2+} into the cytoplasmic calcium store at least partly via the increased NO production due to the activation of nitric oxide synthase. Based on these experimental results, the ingestion of PCRC may be helpful to prevent or alleviate the cardiovascular diseases, through inhibition of CA secretion from adrenomedullary chromaffin cells and consequent reduction of the CA level in the circulation.

V. SUMMARY

The present study was attempted to investigate whether polyphenolic compounds isolated from wine, which is brewed from *Rubus coreanum* MIQUEL (覆盆子酒), may affect the release of catecholamines (CA) from the isolated perfused adrenal medulla of the spontaneously hypertensive rats (SHRs), and to establish its mechanism of action.

Polyphenolic compounds (PCRC, 20~180 μg/mL) perfused into an adrenal vein for 90 min relatively 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). PCRC itself did not affect basal CA secretion (data not shown). Also, in the presence of PCRC (60 μg/mL), the secretory responses of CA evoked by veratridine (a selective Na^+ channel activator (10 μM), Bay-K-8644 (a L-type dihydropyridine Ca^{2+} channel activator, 10 μM), and cyclopiazonic acid (a cytoplasmic Ca^{2+}-ATPase inhibitor, 10 μM) were significantly reduced, respectively. Interestingly, in the simultaneous presence of PCRC (60 μg/mL) and L-NAME (an inhibitor of NO synthase, 30 μM), the inhibitory responses of PCRC on the CA secretion evoked by ACh, high K^+, DMPP, and Bay-K-8644 were considerably recovered to the extent of the corresponding control secretion compared with the inhibitory effect of PCRC-treatment alone. Practically, the level of NO released from adrenal medulla after the treatment of PCRC (60 μg/mL) was greatly elevated compared to the corresponding basal released level.

Taken together, these results obtained from the present study demonstrate that PCRC inhibits the CA secretory responses from the isolated perfused adrenal medulla of the SHRs evoked by stimulation of cholinergic (both muscarinic and nicotinic) receptors as well as by direct membrane-depolarization. It seems that this inhibitory effect of PCRC is mediated by inhibiting both the influx of calcium and sodium into the adrenal medullary chromaffin cells of the SHRs and the uptake of Ca^{2+} into 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 PCRC may be beneficial to prevent or treat the cardiovascular diseases.

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Wine of *Rubus coreanum* MIQUEL (覆盆子酒)
or Grape Red Wine (赤葡萄酒)

Diaion HP-20 column/chromatography

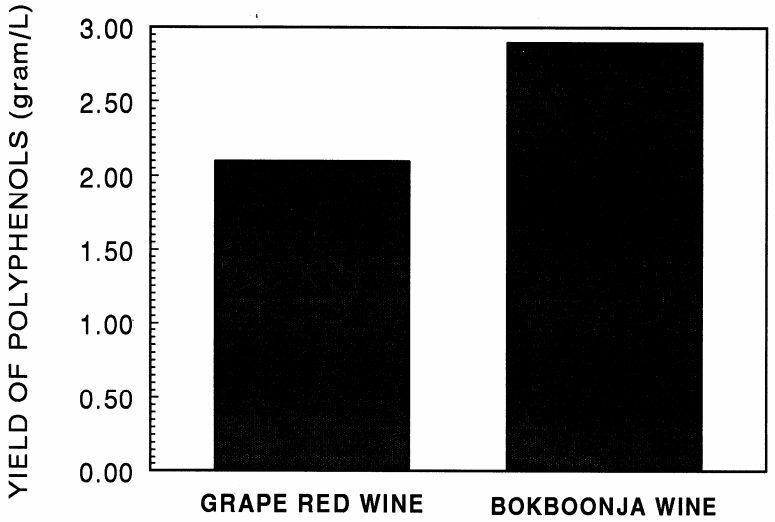
(H_2O, 16L; EtOH, 16L)

H_2O Eluent

(Discard)

EtOH Eluent

(Polyphenolic Fraction)



Concentration
Lyophilzed & Finely Sprayed

2.9 gram/L (From Bokboonja Wine)
2.1 gram/L (From Grape Red Wine)
[FINAL YIELD]

Fig. 1. Preparation of polyphenolic compounds from Bokboonja wine (*Rubus coreanum* MIQUEL, 覆盆子酒) and grape red wine (赤葡萄酒).

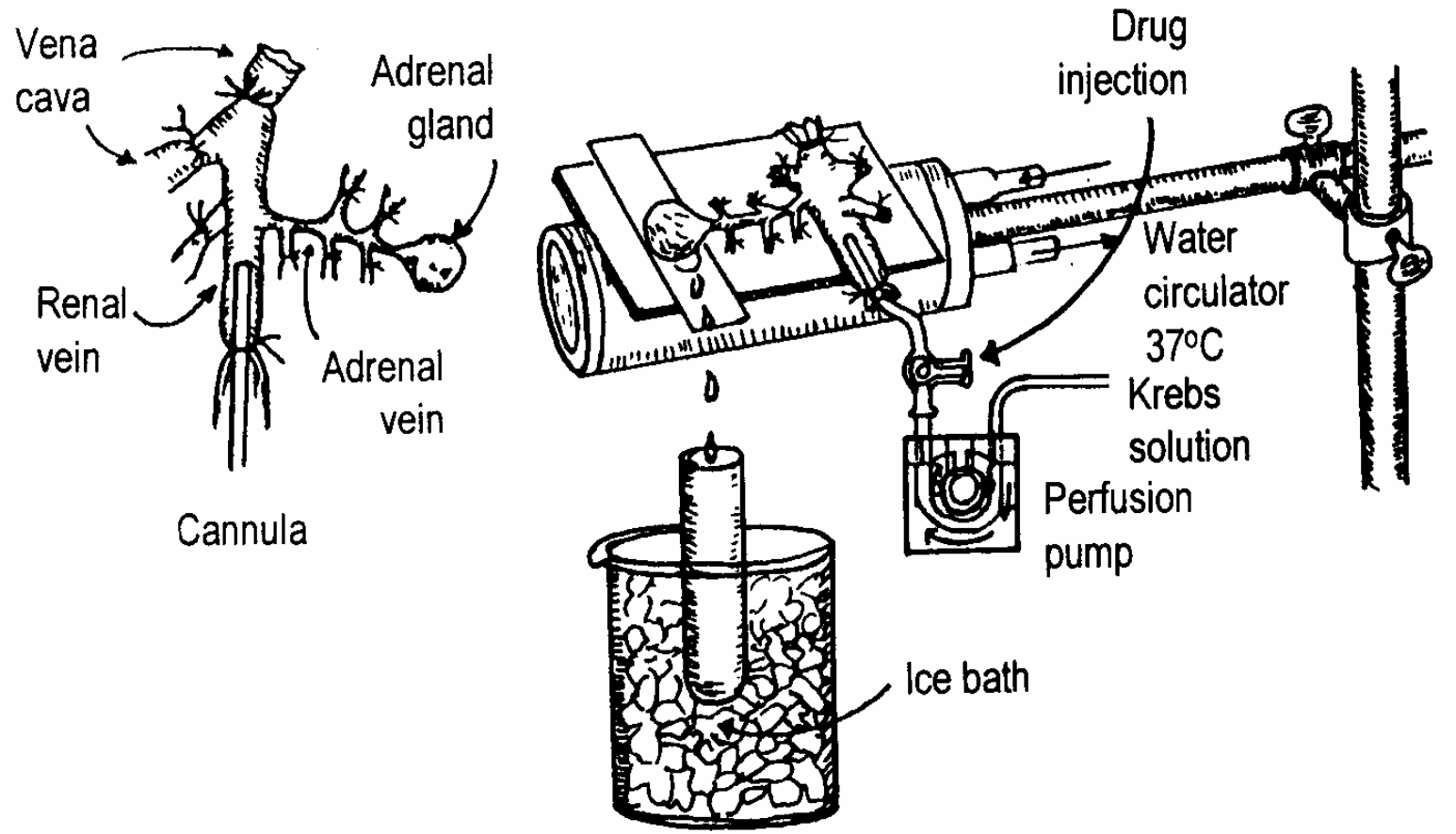


Fig. 1. Schematic drawing of the preparation used to study secretion of catecholamines in the isolated perfused adrenal gland of the SHRs.

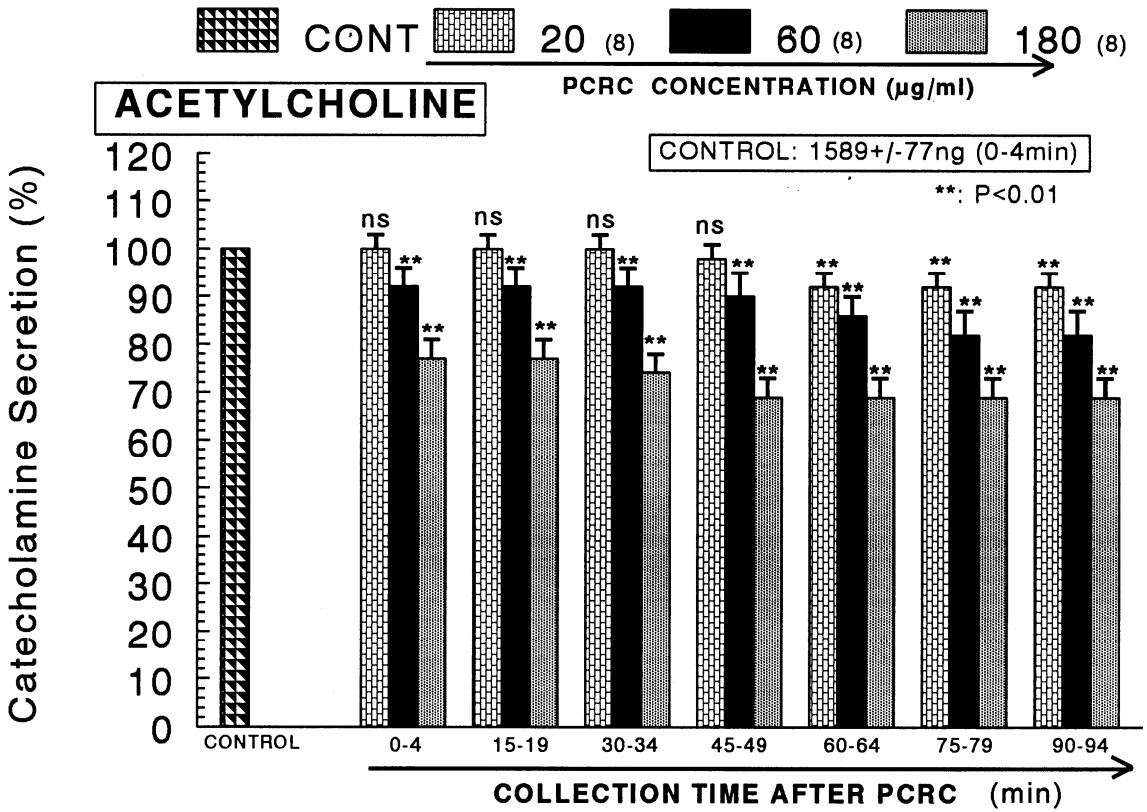


Fig. 3. Dose-dependent effect of PCRC (polyphenolic compounds from *Rubus coreanum*) on the CA secretory responses evoked by acetylcholine from the isolated perfused adrenal glands of spontaneouly hypertensive rats (SHRs). The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 20, 60, 180 μg/ml of PCRC for 90 min as indicated at an arrow mark. Numbers in the parenthesis indicate number of 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). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of PCRC. ACh-induced perfusates were collected for 4 minutes. **: $P < 0.01$. ns: Statistically not significant.

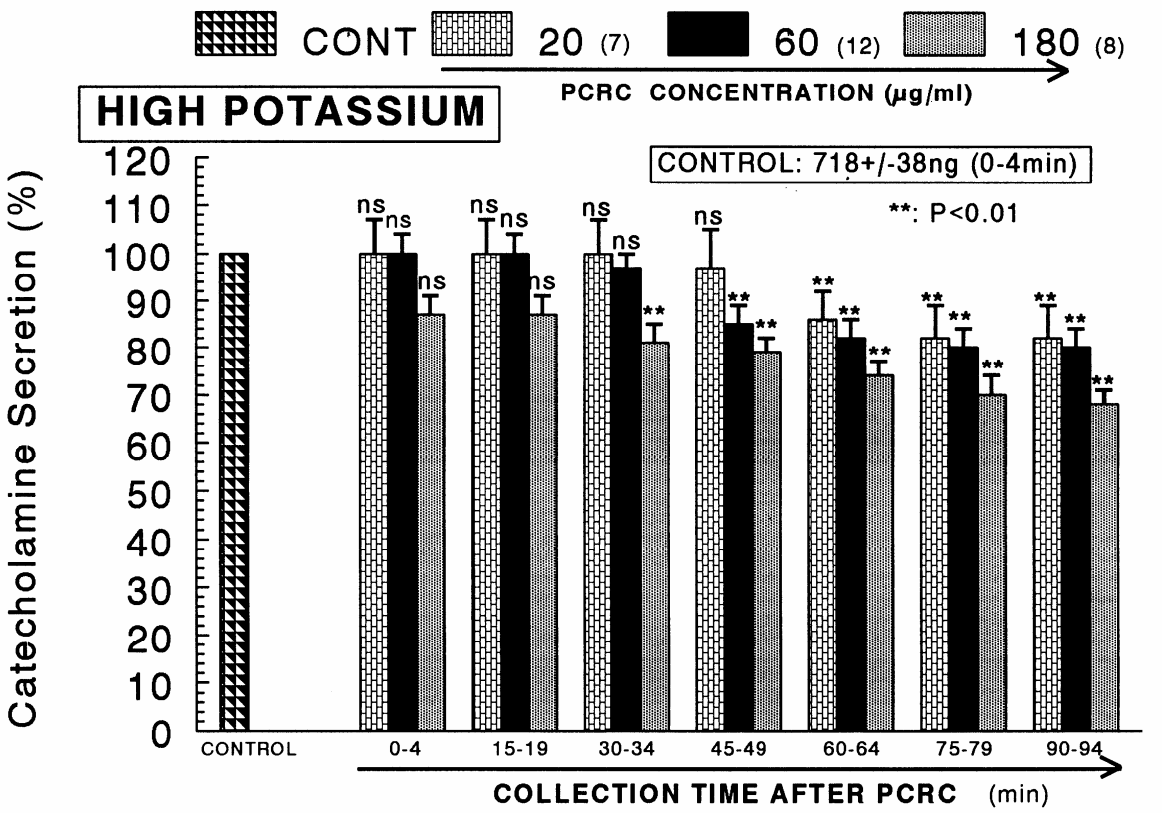


Fig. 4. Dose-dependent effect of PCRC on the CA secretory responses evoked by high K^+ from the isolated perfused adrenal glands of SHRs. The CA secretion by a single injection of K^+ (56 mM) was injected in a volume of 0.1 ml at 15 min intervals after preloading with 20, 60, 180 μg/mLof PCRC for 90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

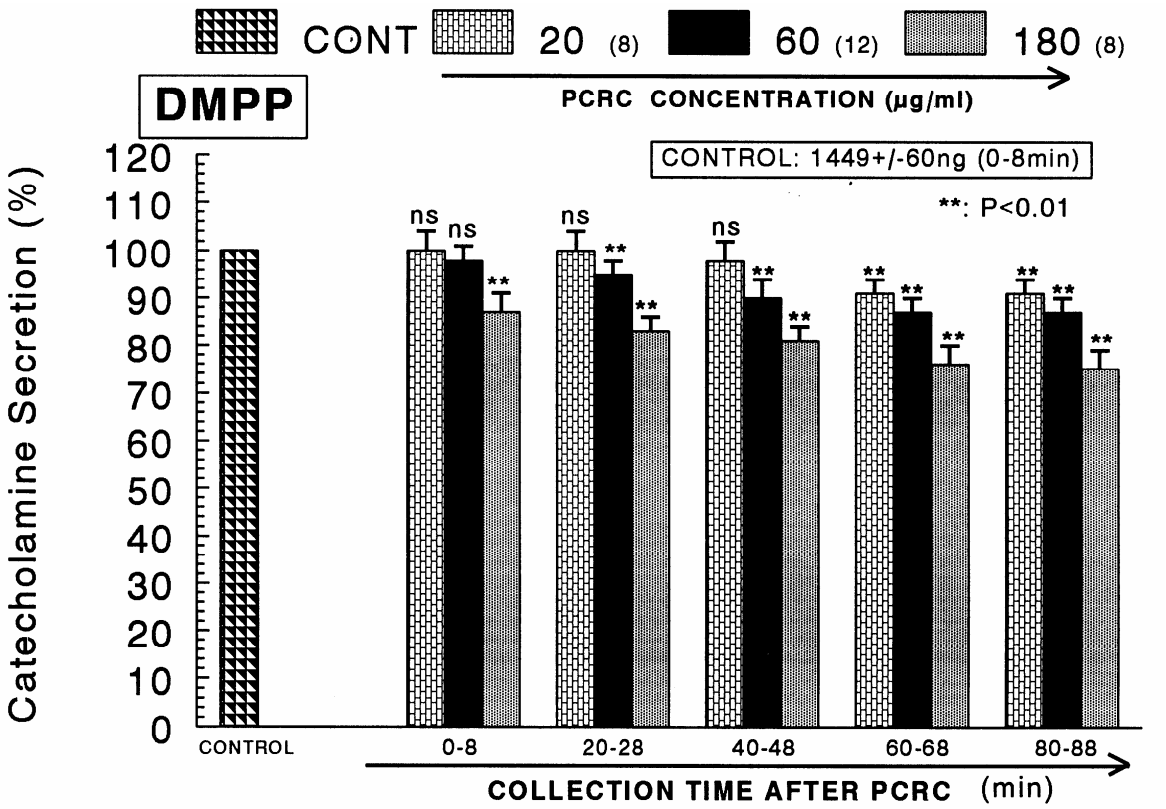


Fig. 5. Dose-dependent effect of PCRC on the CA secretory responses evoked by DMPP from the isolated perfused adrenal glands of SHRs. The CA secretion by the perfusion of DPPP (10^{-4} M) was infused for 2 min at 20 min intervals after preloading with 20, 60, 180 μg/mLof PCRC for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 1449±60 ng for 8 min) with each concentration-pretreated group of PCRC. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 3. **: P < 0.01. ns: Statistically not significant.

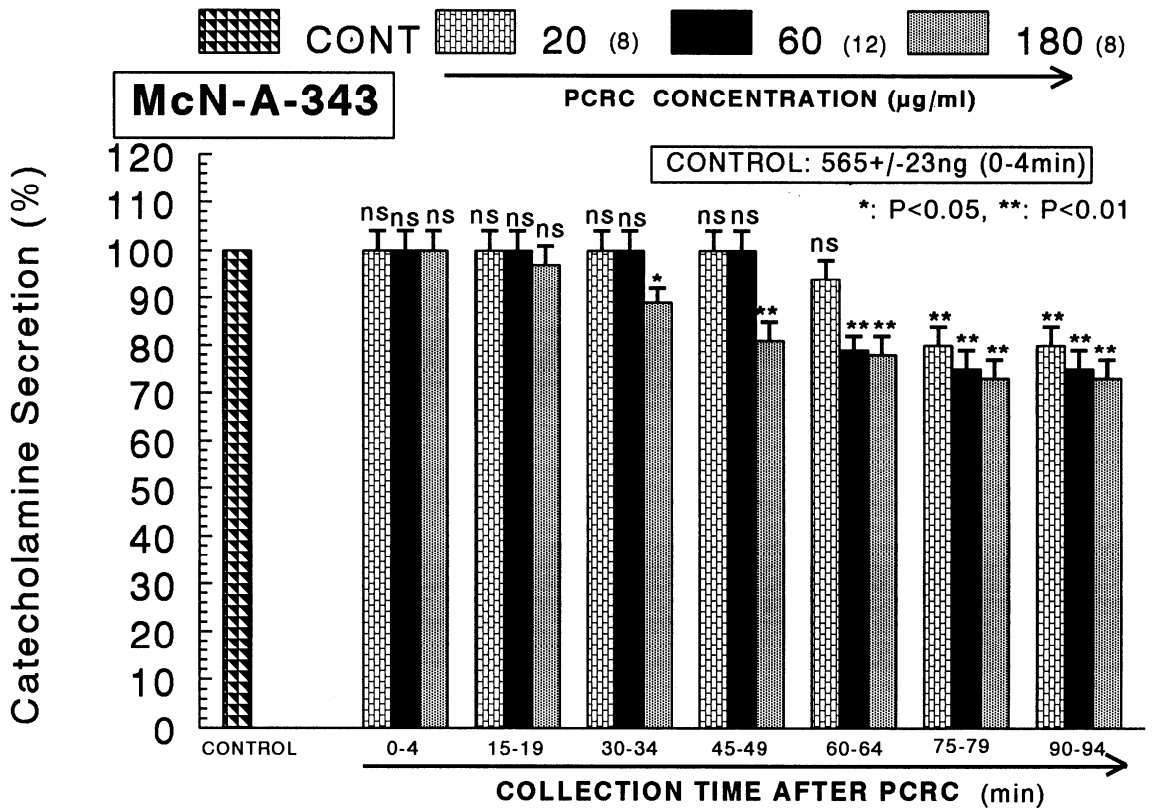


Fig. 6. Dose-dependent effect of PCRC on the CA secretory responses evoked by McN-A-343 from the isolated perfused adrenal glands of SHRs. The CA secretion by the perfusion of McN-A-343 (10^{-4} M) was infused for 4 min at 15 min intervals after preloading with 20, 60, 180 μg/mLof PCRC for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of PCRC. 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.

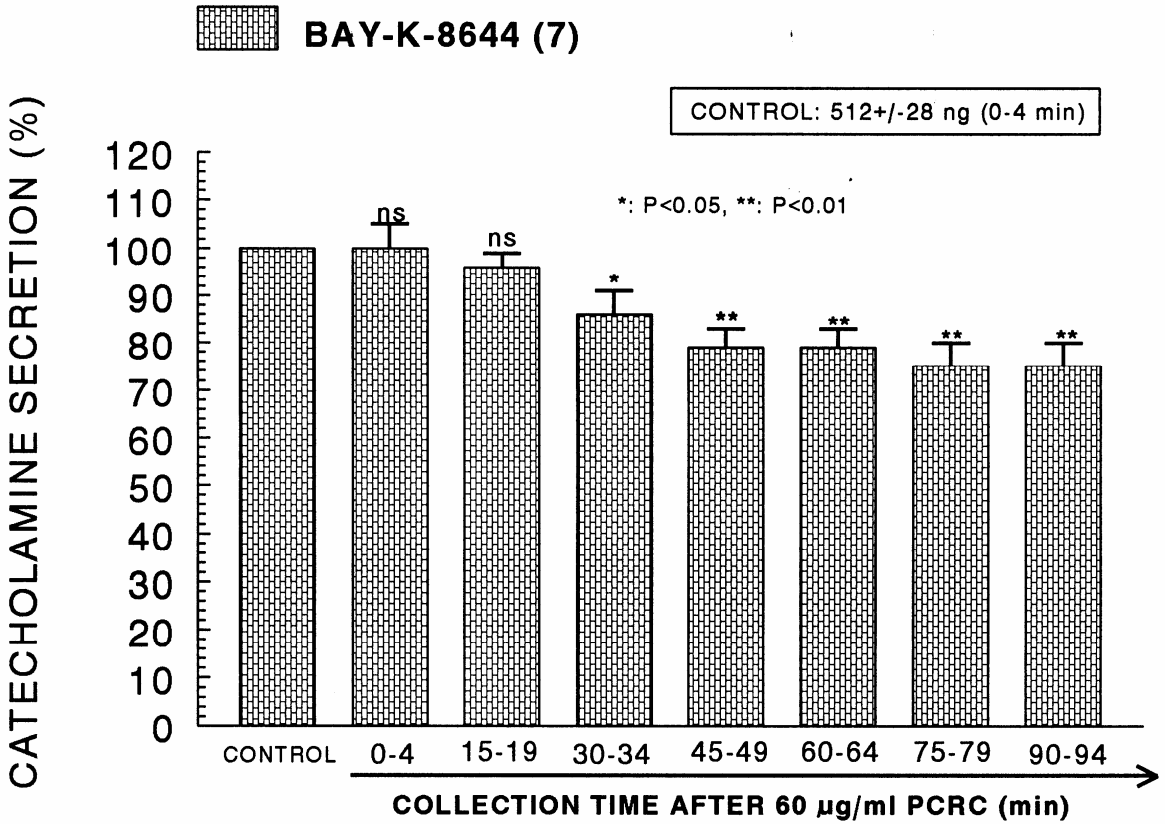


Fig. 7. Time-course effects of PCRC on the CA release evoked by Bay-K-8644 from the adrenal glands of SHRs. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of PCRC (60 µg/mL) for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT) with each period after pretreatment with PCRC. Other legends are the same as in Fig. 3. *: $P < 0.05$. **: $P < 0.01$. ns: Statistically not significant.

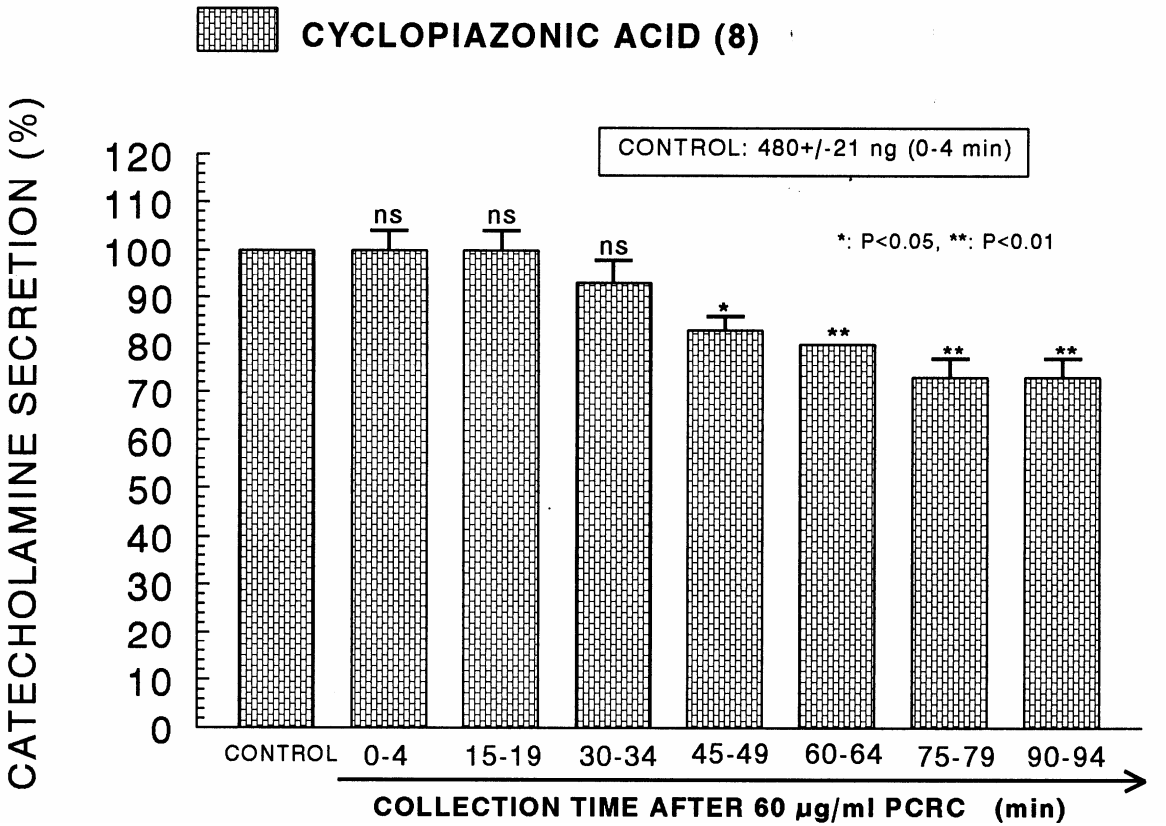


Fig. 8. Time-course effects of PCRC on the CA release evoked by cyclopiazonic acid from the adrenal glands of SHRs. Cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of PCRC (60 µg/mL) for 90 min. Statistical difference was obtained by comparing the corresponding control (CONT) with each period after pretreatment with PCRC. Other legends are the same as in Fig. 3. **: $P < 0.01$. *: $P < 0.05$. ns: Statistically not significant.

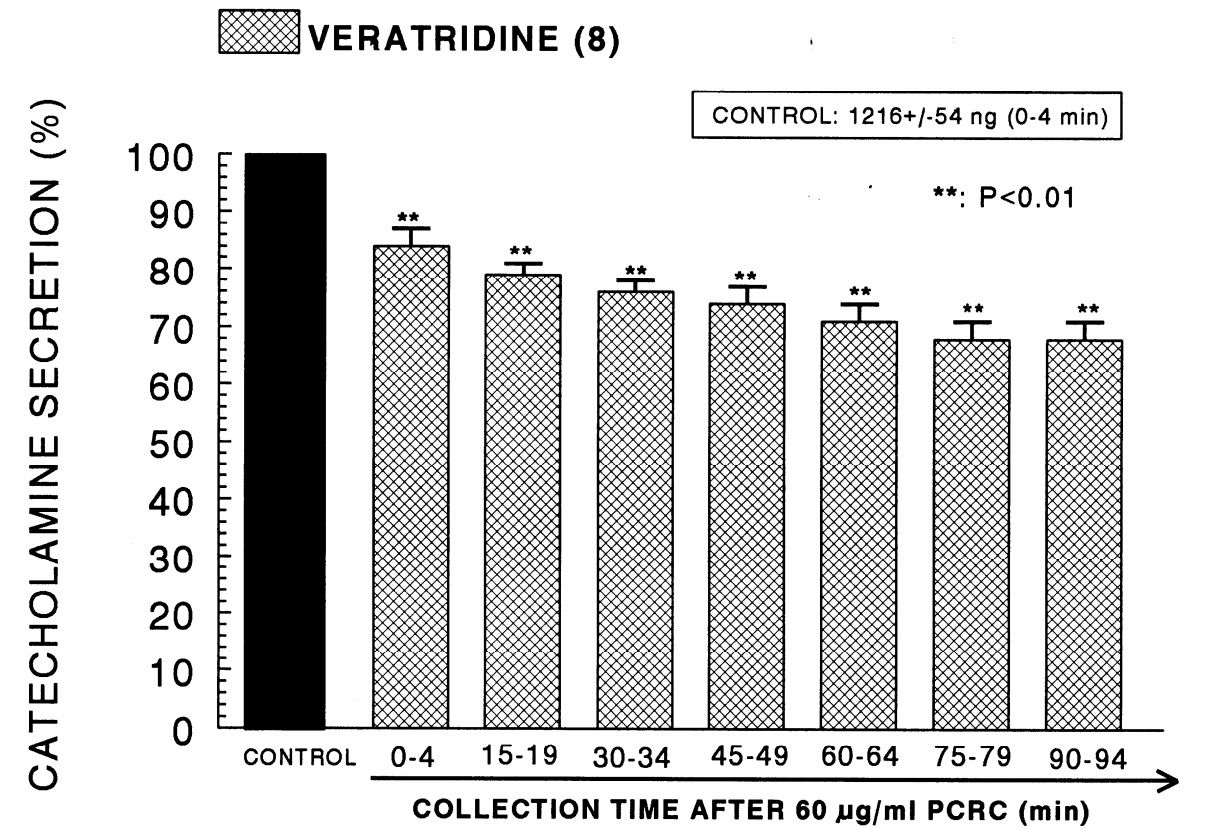


Fig. 9. Time-course effects of PCRC on the CA release evoked by veratridine from the adrenal glands of SHRs. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with PCRC (60 µg/ml) for 90 min, respectively. Other legends are the same as in Fig. 3.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

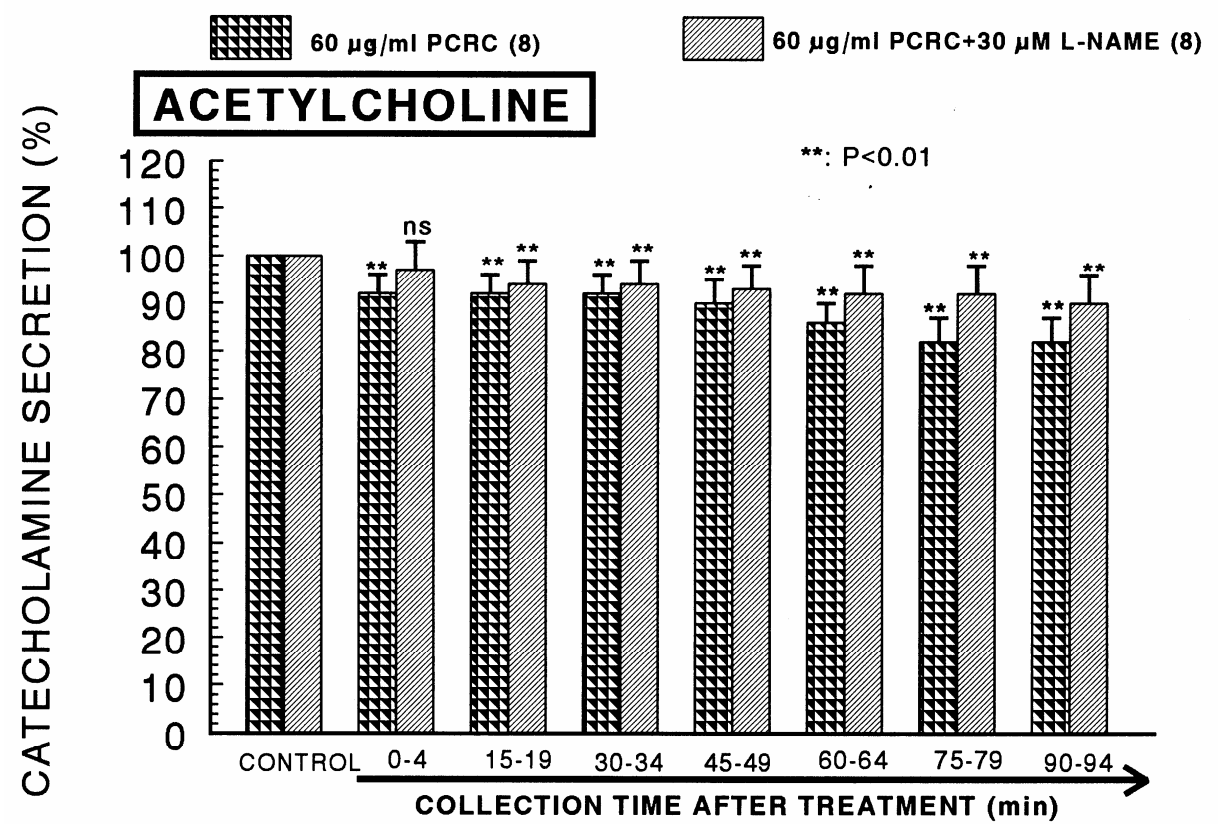


Fig. 10. Effects of PCRC plus L-NAME on the CA secretory responses evoked by ACh from the isolated perfused adrenal glands of SHRs The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was induced before (CONTROL) and after preloading with PCRC (60 μg/ml) plus L-NAME (30 μM) for 90 min. 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.

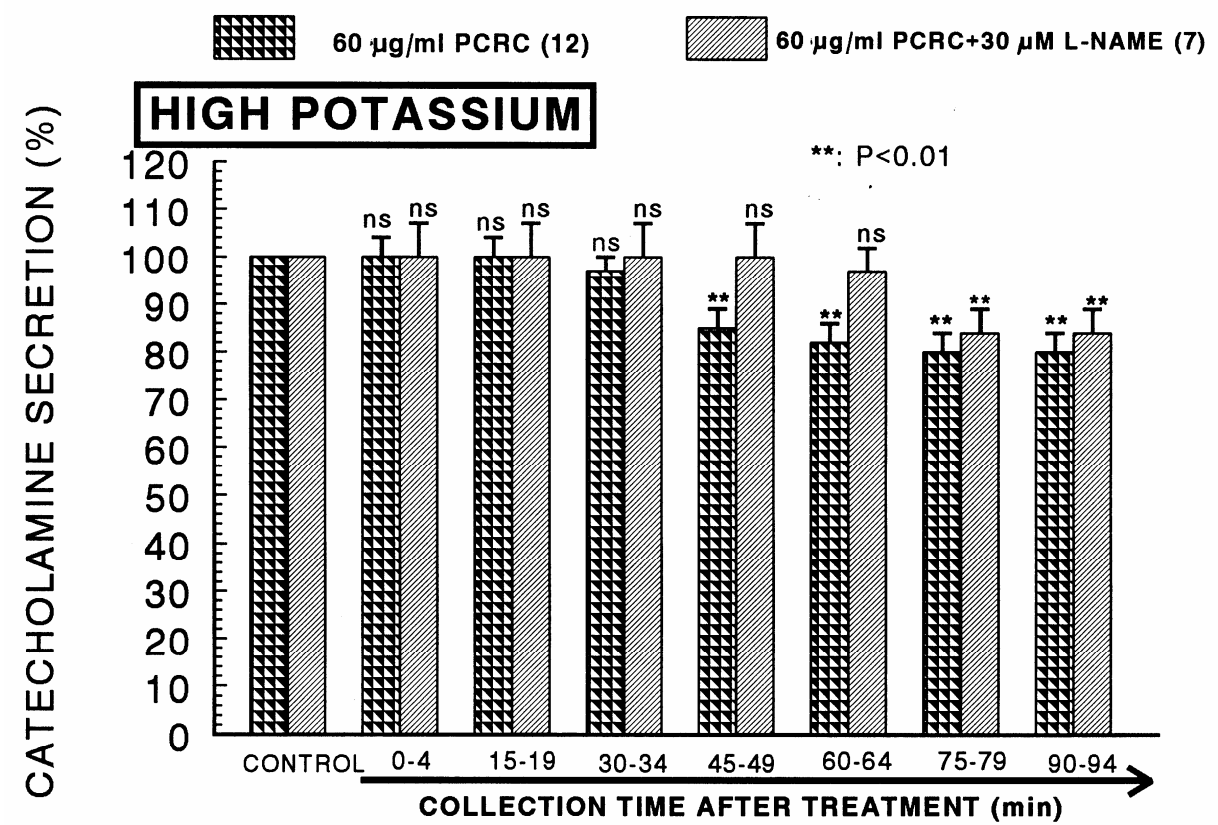


Fig. 11. Effects of PCRC plus L-NAME on the CA secretory responses evoked by high potassium from the isolated perfused adrenal glands of SHRs. The CA secretion by a single injection of high K^+ (5.32×10^{-3} M) in a volume of 0.05 ml was induced before (CONTROL) and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min. 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.

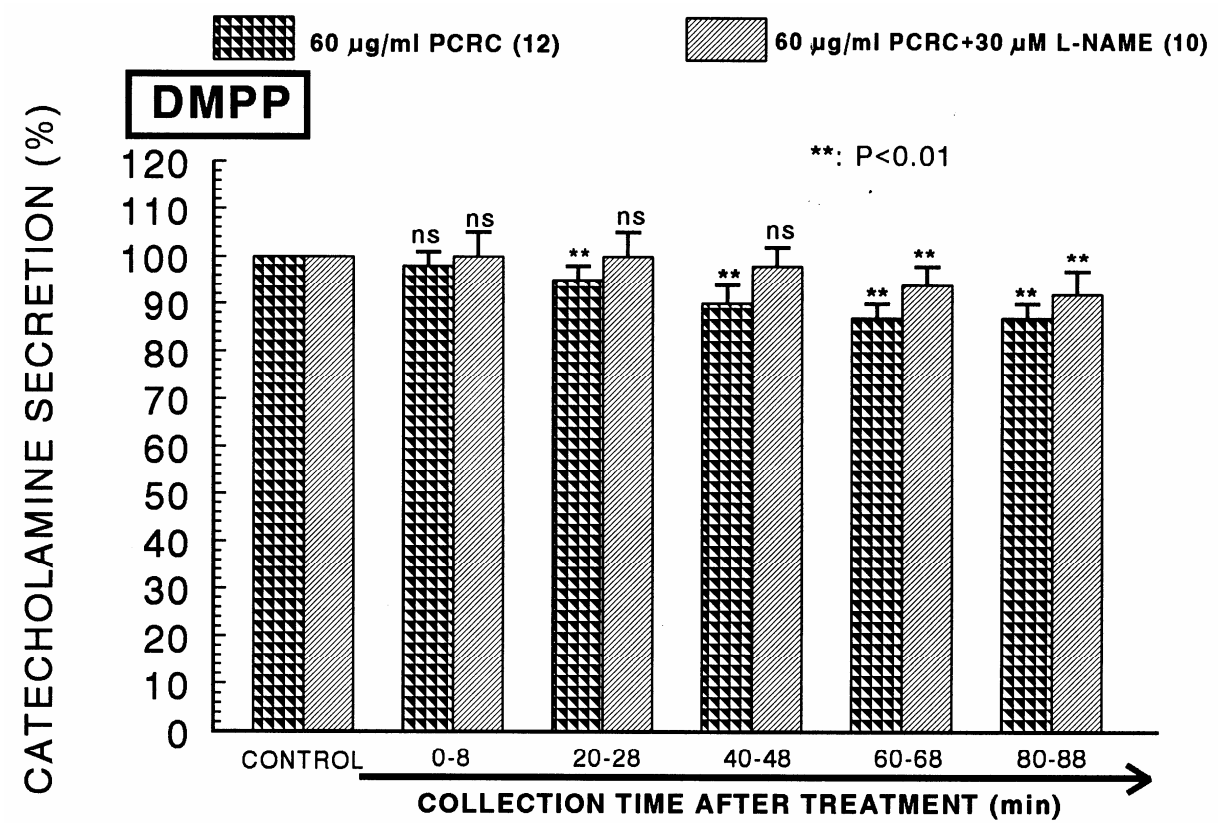


Fig. 12. Effects of PCRC plus L-NAME on the CA secretory responses evoked by DMPP from the isolated perfused adrenal glands of SHRs. The CA secretion by perfusion of DMPP (10^{-4} M) for 2 min was induced before (CONTROL) and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min. Perfusates were collected for 8 minutes at 20 min-intervals. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

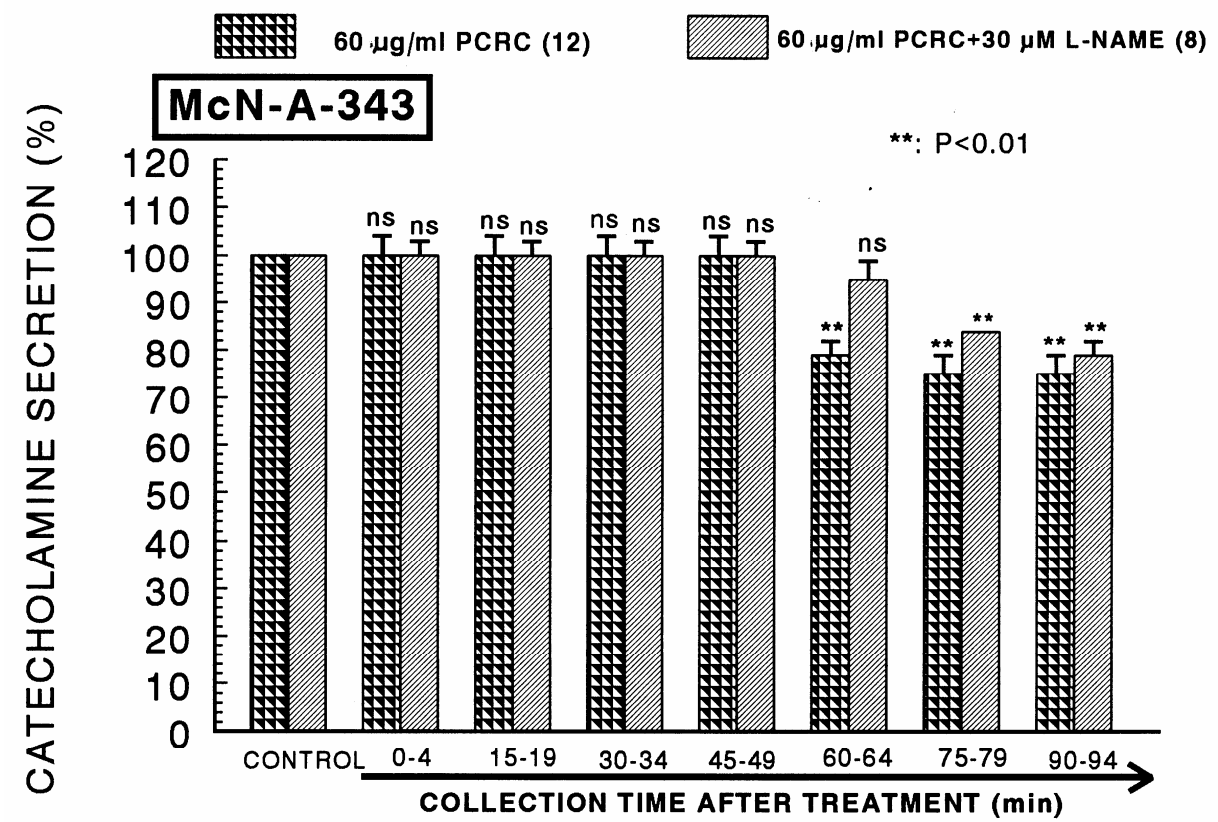


Fig. 13. Effects of PCRC plus L-NAME on the CA secretory responses evoked by DMPP from the isolated perfused adrenal glands of SHRs. The CA secretion by perfusion of DMPP (10^{-4} M) for 2 min was induced before (CONTROL) and after preloading with PCRW (60 μg/ml) plus L-NAME (30 μM) for 90 min. Perfusates were collected for 8 minutes at 20 min-intervals. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

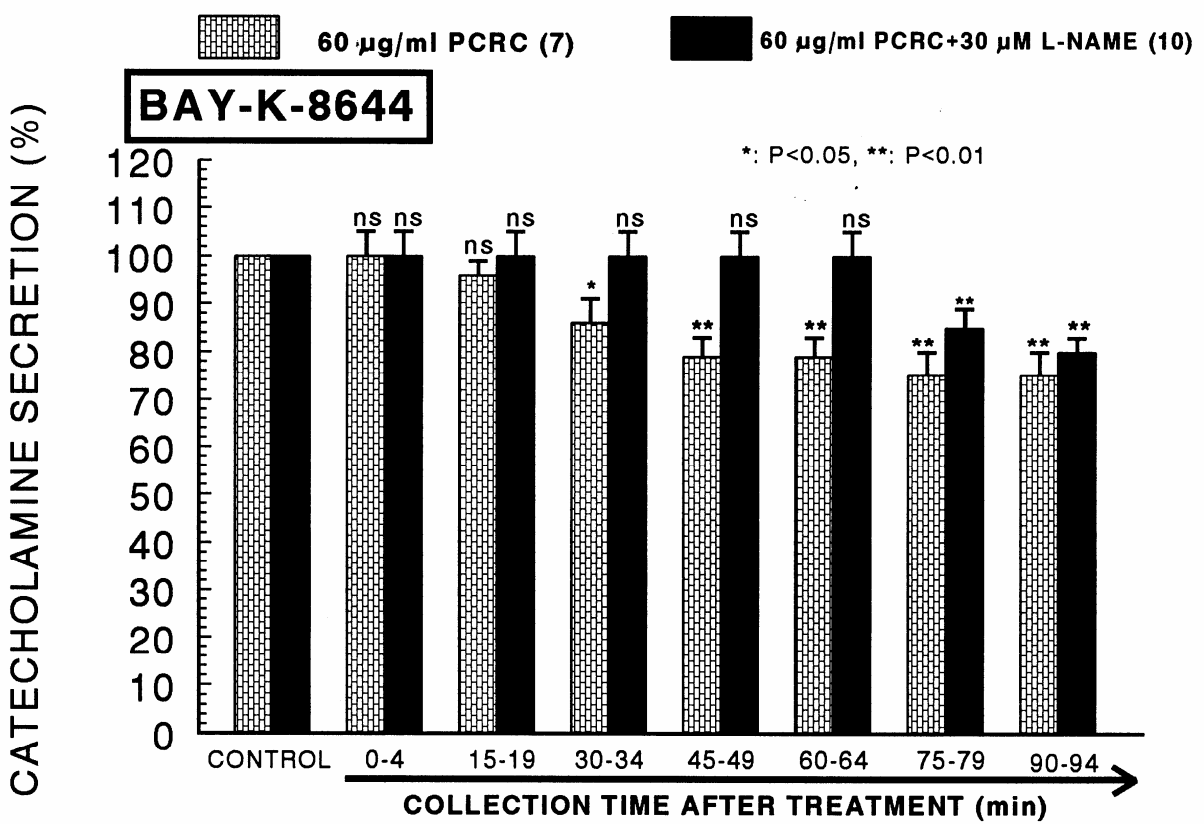


Fig. 14. Effects of PCRC plus L-NAME on the CA secretory responses evoked by Bay-K-8644 from the adrenal glands of SHRs. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with PCRC (60 μg/ml) for 90 min. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

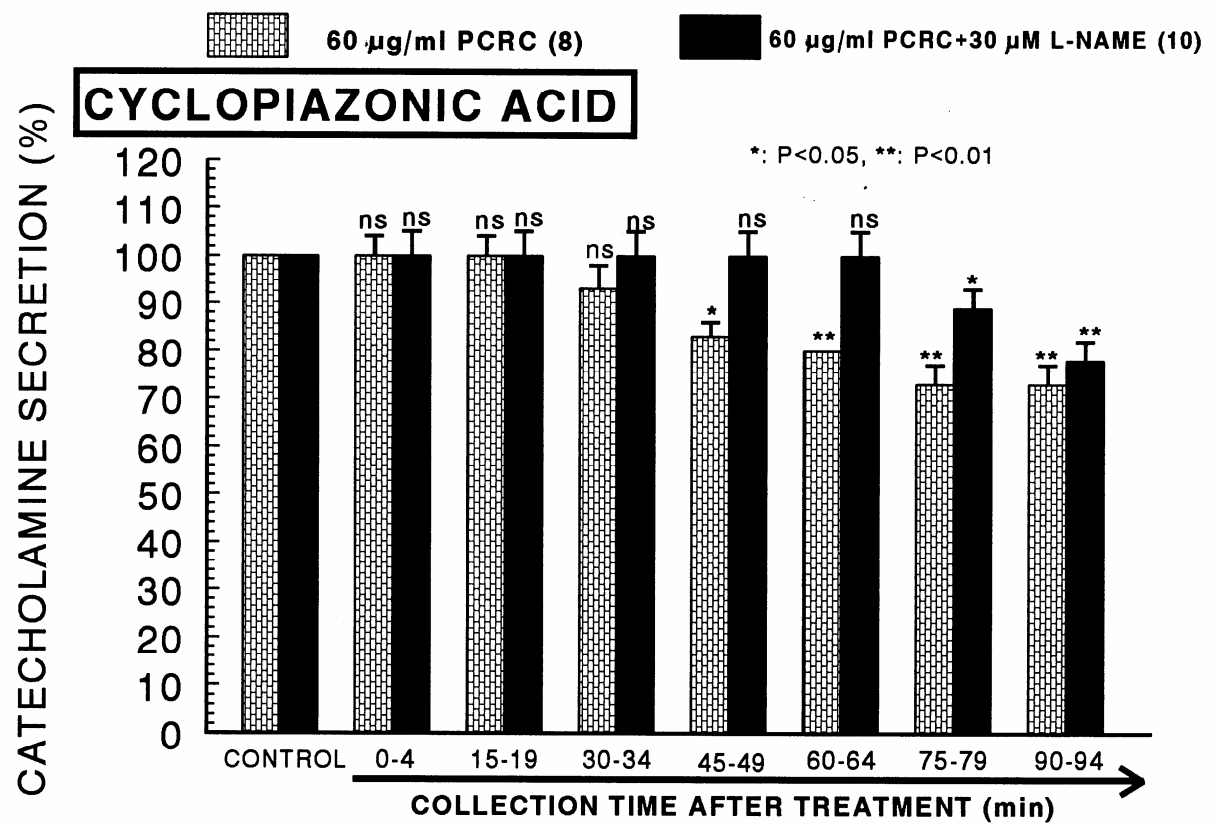


Fig. 15. Effects of PCRC plus L-NAME on the CA secretory responses evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower) from the adrenal glands of SHRs. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with PCRC (60 μg/ml) for 90 min, respectively. Other legends are the same as in Fig. 3. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

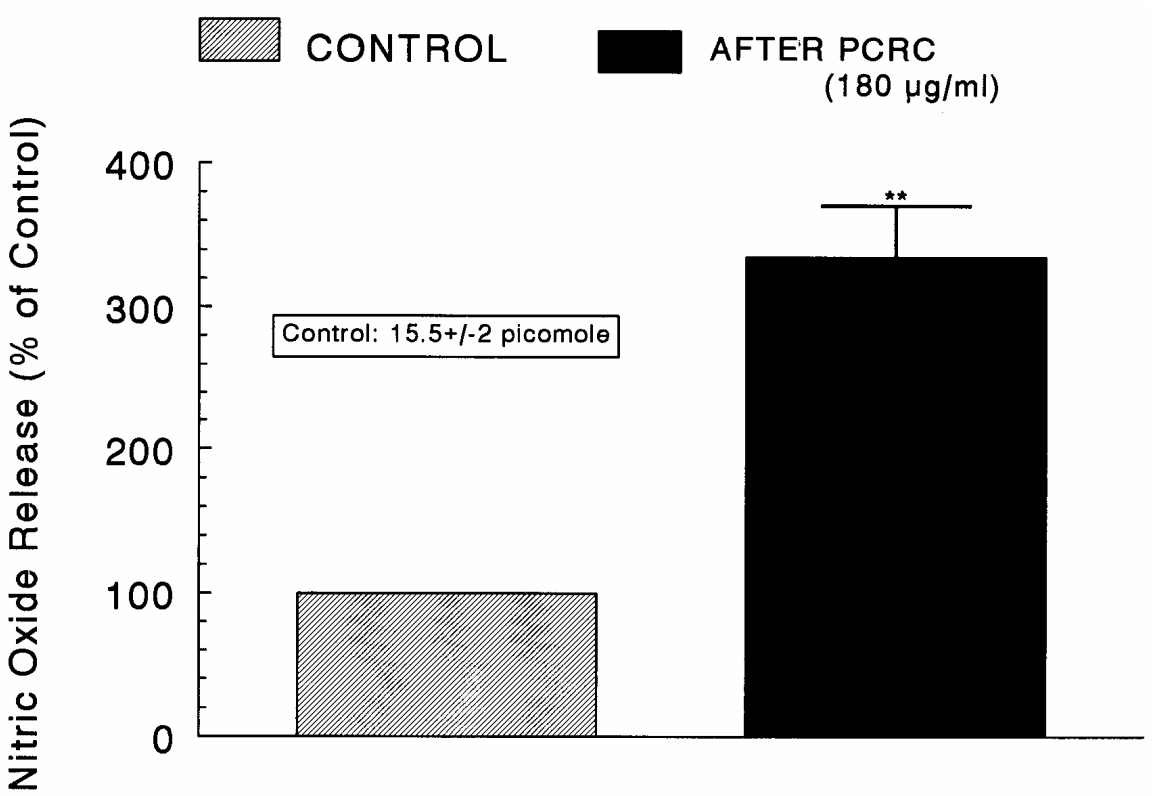


Fig. 16. Comparison of nitric oxide (NO) production before (CONTROL) and after administration of PCRC in the isolated perfused adrenal medulla of the SHRs. Perfusate sample was taken for 8 min after loading the perfusion of PCRC (180 ug/ml) at a rate of 0.31 ml/min. Ordinate: the amounts of NO released from the adrenal medulla (% of control). Abscissa: Treatment (before and after PCRC). Statistical difference was made by comparing the control (15.5±2 picomoles) with PCRC-treated group. **: P< 0.01.

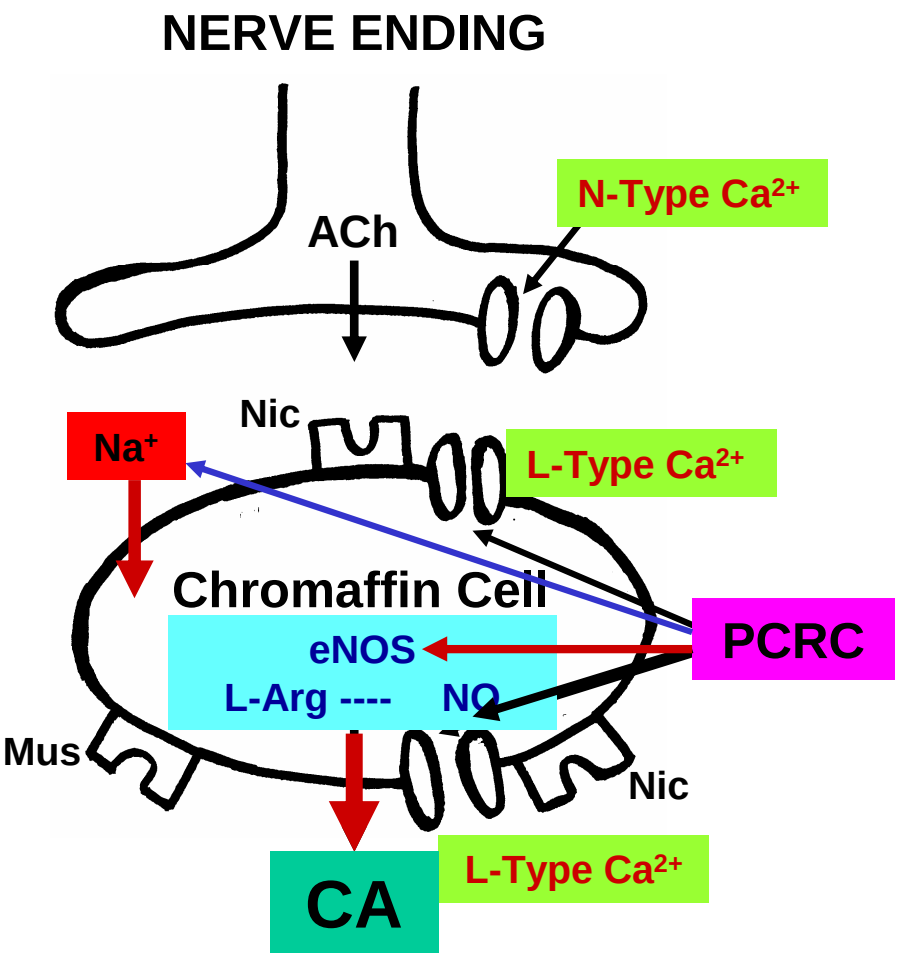


Fig. 17. Schematic diagram of possible action site of PCRC in the adrenal galnd of the SHRs. This diagram demonstrates possible localizations of voltage-dependent Ca^{2+} channels and cholinergic receptors mediating secretion of adrenal catecholamines (CA). CA-containing cells possess synaptic nicotinic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