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조선대학교 대학원

의학과

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흰쥐 관류부신에서 카테콜아민 분비작용에 대한 Provinol 의 억제효과

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이 논문을 의학박사 학위신청논문으로 제출함.

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의학과

서유승

서 유 승의 박사학위논문을 인준함

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서 유 승

(지도교수 : 임 동 윤)

조선대학교 대학원 의학과

본 연구의 목적은 Provinol (적포도주에서 분리한 폴리페놀 혼합물)이 정상혈압 흰쥐로부터 분리 적출한 부신의 관류모델에서 카테콜아민 (catecholamines, CA) 분비작용에 미치는 영향을 검색하고 그 작용기전을 규명하는데 있으며, 본 연구를 시행하여 다음과 같은 결과를 얻었다.

Provinol (0.3~3 μg/ml)을 부신정맥 내로 90 분간 관류 시 비교적 용량 및 시간 의존적으로 ACh (5.32 mM), 고칼륨 (56 mM, 막탈분극제), DMPP (100 μM, 선택성 니코틴 N_N 수용체 작동제), 및 McN-A-343 (100 μM, 선택성 무스카린 M1-수용체 작동제)에 의한 CA 분비를 억제하였다. 그러나, Provinol 자체는 기초 CA 분비량에 영향을 미치지 않았다. 또한, Provinol (1 μg/ml) 존재 하에서, 전압-의존성 나트륨통로 활성화제인 veratridine (100 μM), L 형 칼슘통로 활성화제인 Bay-K-8644 (10 μM) 및 세포질내 내형질세망막에서 Ca_{2+}-ATPase 억제제인 cyclopiazonic acid (10 μM)에 의한 CA 분비가 유의하게 억제되었다. 흥미롭게도, Provinol (1 μg/ml)과 L-NAME (NO Synthase 억제제, 30 μM)을

90 분간 동시 처치하였을 때 ACh, 고칼륨, DMPP, Bay-K-8644 및 cyclopiazonic acid 의 CA 분비효과가 Provinol 단독처치 시 나타나는 억제효과가 억제되어 거의 대조치 수준까지 회복되었다. 또한 실제로 Provinol 처치 후에 NO 유리량이 기초 유리량에 비해 현저하게 증가하였다.

이와 같은 연구결과를 종합하여 보면, 흰쥐 적출 관류 부신수질에서 Provinol 은 콜린성(니코틴 및 무스카린) 수용체 흥분 및 막탈분극에 의한 CA 분비에 대하여 억제효과를 나타내었다. 이러한 Provinol 의 억제효과는 흰쥐 부신수질에서 NO Synthase 의 활성화에 의한 NO 생성증가로 인하여 부신크롬친화세포 내로 전압의존성 나트륨 및 칼슘통로를 통한 Na^+ 및 Ca^{2+} 유입억제와 세포 내 칼슘저장고로부터 칼슘유리의 억제작용에 기인되는 것으로 생각된다. 이와 같은 연구결과로 보아, Provinol 이 심혈관계 질환, 즉 고혈압 및 협심증의 예방 및 치료에 유익할 것으로 사료된다

I. INTRODUCTION

Provinol is a mixture of different polyphenolic compounds isolated from French red wine. Provinol represents the polyphenolic compounds isolated from red wine and it involves (in mg/g of dry powder) 480 proanthocyanidins, 370 polymeric tannins, 61 total anthocyanins, 19 free anthocyanins, 38 catechin, 18 hydroxycinnamic acids and 14 flavonols. Various epidemiological reports have shown that regular intake of natural polyphenols in grape juice, red wine and in some other beverages is associated with reduced risk of cardiovascular diseases (Fuster et al. 1992; Middleton et al. 2000). The French Paradox is defined as a low incidence of coronary heart disease while consuming a diet rich in saturated fat. The Mediterranean diet, rich in fruits and red wine, was shown to protect against the development of cardiovascular diseases (Hertog et al 1995; De Lorgeril et al. 1996).

Provinol at the concentration producing the maximal endothelium-dependent relaxation, restored the relaxation of the femoral artery to acetylcholine abolished by superoxides and enhanced partially the relaxant responses of sodium nitroprusside suggesting the ability of Provinol to preserve NO from degradation (Zenebe et al. 2003; Pecháňová et al. 2006a). Provinol partially prevents L-NAME induced hypertension via the different mechanisms depending on the duration of treatment in male Wistar rats. Prevention of oxidative damage in the brain with modulating effect on NO synthase activity is suggested (Jendeková et al, 2006). Provinol reduced blood pressure (BP) only in borderline hypertensive rats (BHR). Data suggest that reduction of BP in BHR as well as the improvement

of vasorelaxation in Provinol-treated Wistar-Kyoto (WKY) rats were associated rather with other than NO-dependent mechanisms (Bernatova et al, 2007). Similarly, red wine polyphenolic compounds (PCRW) caused a dose-dependent relaxation in rabbit aorta with intact endothelium (Cishek et al. 1997). In healthy volunteers, the coronary flow-velocity reserve was increased after drinking red wine, but not after drinking the same quantity of alcohol in white wine or vodka (Shimada et al., 1999). The endothelium-dependent vasodilation was also improved after acute intake of 500 ml of red wine or red wine without alcohol in men, as determined by ultrasonography of the brachial artery (Hashimoto et al., 2001). Although Huang et al. (1999) demonstrated epicatechin-induced endothelium-dependent vasorelaxation in rat mesenteric arteries, it seems that polymeric rather than monomeric phenols were responsible for NO-dependent relaxation. Resveratrol, a natural phenolic trihydroxystilbene present in red wine, produced mainly endothelium-dependent and nitric oxide-mediated vasodilation in human internal mammary artery but partially in saphenous vein rings and improved their endothelial reactivity. This may have a therapeutic potential in cardiovascular diseases (Rakici et al, 2005). It has been suggested that the mechanisms of vasorelaxation induced by resveratrol are heterogeneous, two mechanisms participating partially in the relaxation of the isolated porcine coronary artery were detected in the study, one being the nitric oxide released from the endothelium, the other causing inhibition of Ca^{2+} influx, but estrogen receptors were not involved in resveratrol-induced relaxation (Liu et al, 2006). Lim (2008) has shown that resveratrol inhibits cholinergic stimulation-evoked secretion of catecholamines (CA) through suppressing ion influx into the rat adrenomedullary cells due to the increased NO production.

There is, however, little evidence regarding the effects of Provinol on the CA secretion from adrenal medulla. Therefore, the aim of the present study was to investigate whether Provinol can modify the CA secretion evoked by stimulation of cholinergic receptors and direct membrane-depolarization in the isolated perfused model of normotensive rats, and to establish its mechanism of action.

II. MATERIALS AND METHODS

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. 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 CA.

Drug administration

The perfusions of DMPP (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32 x 10^{-3} M) and KCl (5.6 x 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), veratridine (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, veratridine, 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 Provinol on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing Provinol 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 Provinol, 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 Provinol 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: Provinols [(purchased product, mixture of polyphenols developed by INRA (Institut National de Recherche Agronomique, Montpellier-France) in partnership with the Société Française de Distilleries Co. in France)], 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 acid, veratridine hydrochloride (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 Provinol used are expressed in terms of molar base.

III. RESULTS

Effects of Provinol 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, the basal CA release from the isolated perfused rat adrenal glands amounted to 22±3 ng for 2 min (n=9). Since Provinol at the concentration producing the maximal endothelium-dependent relaxation, restored the relaxation of the femoral artery to acetylcholine abolished by superoxides and enhanced partially the relaxant responses of sodium nitroprusside suggesting the ability of Provinol to preserve NO from degradation (Zenebe et al. 2003; Pecháňová et al. 2006a), it was attempted initially to examine the effects of Provinol itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, Provinol (0.3 ~ 3 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 Provinol on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion. Secretagogues were given at 15 to 20 min-intervals. Provinol 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 (1192±58 ng for 0-4 min), as shown in Fig. 2. However, the pretreatment with Provinol in the range of 0.3 ~ 3 μg/ml for 90 min relatively concentration- and

time-dependently inhibited ACh-stimulated CA secretion. In the presence of Provinol as shown in Fig. 3, CA releasing responses were inhibited by 56% 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 (605±28 ng for 0-4 min). However, following the pretreatment with Provinol (0.3 ~ 3 μg/ml), high K^+ (5.6×10^{-2} M)-stimulated CA secretion was significantly inhibited to 54% of the control after 75 min period. DMPP (10^{-4} M), which is a selective nicotinic N_N-receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (1160±62 ng for 0-8 min). However, as shown in Fig. 4, DMPP-stimulated CA secretion after pretreatment with Provinol was maximally reduced to 53% of the control release at last period (90-94 min). 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 (489±21 ng for 0-4 min). However, McN-A-343-stimulated CA secretion in the presence of Provinol was markedly depressed to 52% of the corresponding control secretion (100%) as depicted in Fig. 5.

Effects of Provinol 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 Provinol 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 Provinol (1 μg/ml) was greatly blocked to 63% of the control release except for the initial 0-4 min as compared to the corresponding control release (486±17 ng for 0-4 min) from 10 rat adrenal glands as shown in Fig. 6.

In order to investigate the effect of Provinol on the mobilization of intracellular Ca^{2+}, the effect of Provinol 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. 7, in the presence of Provinol in 10 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was also inhibited to 67% of the control response (461±21 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 Provinol on voltage-dependent Na^+ channels, the effect of Provinol on the CA secretion induced by veratridine was examined here. As shown in Fig. 8, veratridine greatly

produced CA secretion (1184±21 ng for 0-4 min). However, in the presence of Provinol (1 μg/ml), veratridine (100 μM)-evoked CA secretion from 8 glands was greatly inhibited to 46% of the corresponding control release in a time-dependent manner.

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

It has also been found that, as shown in Fig. 2~8, Provinol inhibits the CA secretory response evoked by cholinergic stimulation in the perfused rat adrenal gland. Therefore, to study the relationship between NO and Provinol-induced inhibitory effects on the CA release from the rat adrenal glands, Provinol-induced inhibitory responses of CA secretion evoked by cholinergic receptor-stimulation as well as membrane depolarization was examined in the presence of L-NAME. In the simultaneous presence of Provinol (1 μM) and L-NAME (30 μM) for 90 min, ACh-evoked CA release was recovered by 74~94% of the corresponding control release compared to results after loading of Provinol alone as illustrated in Fig. 9. High K^+ (56 mM)-evoked CA release in the simultaneous presence of Provinol (1 μM) and L-NAME (30 μM) for 90 min was also recovered by 75~100% of the corresponding control release during all periods in comparison to data of treatment with Provinol alone (Fig. 10).

As shown in Fig. 11, the simultaneous perfusion of Provinol and L-NAME for 90 min got over the DMPP-evoked CA release to 77~89% of the control response compared to the corresponding control response in comparison to that of the Provinol-treatment alone. Moreover, in the presence of Provinol (1 μM) and

L-NAME (30 μM), the CA secretory response evoked by McN-A-343 (10^{-4} M for 4 min) was recovered to 75~100% of the corresponding control release compared to results of the Provinol-treatment alone, as shown in Fig. 12.

Effects of Provinol plus L-NAME on CA release evoked by BAY-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

As shown in Fig. 13, the simultaneous perfusion of Provinol (1 μM) and L-NAME (30 μM) for 90 min made the CA release evoked by Bay-K-644 (10 μM, an activator of voltage-dependent L-type calcium channel) to 74~100% of the corresponding control response compared to the results of Provinol-treatment alone. After the simultaneous perfusion with Provinol and L-NAME, cyclopiazonic acid (10 μM, an inhibitor of Ca^{2+}-ATPase of endoplasmic reticulum)-evoked CA release was also recovered by 73~100% of the control release in comparison to the results following the treatment with Provinol alone (Fig. 14).

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

As shown in Fig. 9~14, it has been shown that Provinol-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 recovered to the considerable extent of the corresponding control secretion by simultaneous treatment with L-NAME, an inhibitor of NO synthase,

compared to the inhibitory effects of Provinol-treatment alone. Therefore, it was of interest to determine directly the level of nitric oxide released from adrenal medulla following the perfusion of Provinol-containing Krebs-bicarbonate solution. As shown in Fig. 15, the basal level of NO before loading of Provinol was 8.9±3 picomole. However, 30 min after the presence of Provinol (3 μg/ml), it was greatly enhanced to 650% of the control release. Consequently, it was confirmed that Provinol practically increase the level of NO released from the rat adrenal medulla.

IV. DISCUSSION

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

In support of this idea, it was documented that Provinol elicited endothelium-dependent relaxation of rat femoral artery by the Ca^{2+}-induced increase of NO synthase activity and by protecting NO from degradation (Zenebe et al. 2003). Because the action of red wine polyphenolic compounds has been associated with the improvement of endothelium-dependent relaxation and elevation of NO synthase activity and/or expression in several in vitro and in vivo experiments (Andriambeloson et al. 1998, Pecháňová et al. 2004a), it may be assumed about possible therapeutic effect of Provinol in diseases associated with reduced NO bioavailability such as endothelial dysfunction or atherosclerosis. Furthermore, in the simultaneous presence of L-NAME (an inhibitor of nitric oxide synthase) and Provinol, the CA secretory responses evoked by cholinergic stimulation and direct membrane-depolarization was significantly recovered to considerable level of the corresponding control secretion in comparison to

inhibition of treatment with Provinol 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 Provinol, anthocyanins and oligomeric condensed tannins had the same pharmacological profile as Provinol (Andriambeloson et al., 1998). Of different anthocyanins identified in wine, only delphinidin caused endothelium-dependent relaxation, although it was slightly less potent than Provinol (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). This result strongly

indicates that Provinol-induced inhibitory effect of the CA secretion is mediated by the increased NO production due to activation of NO synthase in adrenomedullary cells.

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 (iNOS). 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. 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. On the contrary, it has been reported that L-NAME inhibits ACh-induced CA secretion in bovine chromaffin cells (Uchiyama, et al., 1994) and that the NO donor 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). Anyway, in the light of above findings, the present studies suggest that Provinol can activate nNOS in the rat adrenal medullary chromaffin cells, in addition to the direct inhibitory effects on the CA secretion.

Polyphenolic compounds of red wine (PCRW) are also found to lower blood pressure in normotensive and hypertensive rats (Mizutani et al., 1999; Diebolt et al., 2001). It has been shown that in endothelium-dependent fashion, red wines and grapes exhibit vasorelaxation 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; Andriambeloson et al. 1997; Fitzpatrick et al., 2000; Zenebe et al., 2003). Recently, Provinol reduced blood pressure only in borderline hypertensive rats (BHR). Data suggest that reduction of BP in BHR as well as the improvement of vasorelaxation in provinol-treated Wistar-Kyoto (WKY) rats were associated with other rather than NO-dependent mechanisms (Bernatova et al, 2007). Moreover, Provinol partially prevents L-NAME induced hypertension via the different mechanisms depending on the duration of treatment in male Wistar rats. Prevention of oxidative damage in the brain with modulating effect on NO synthase activity is suggested (Jendeková et al, 2006). Based on these findings, the present experimental results indicate that Provinol-induced inhibitory activity of CA secretory response evoked by stimulation of nicotinic receptors might contribute at least partly to its hypotensive mechanism.

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). Based on this result, the present finding that Provinol significantly inhibited the CA secretory responses evoked by ACh, high K^+, DMPP and McN-A-343 suggests that Provinol can produce the similar effect with that of PCRC in adrenal medulla of the normotensive rats.

Polyphenolic compounds have been documented to relax precontracted smooth muscle of the arteries with intact endothelium. Moreover, some of them were also shown to relax endothelium-denuded arteries (Fuster et al. 1992; Andriambeloson et al. 1997). Several authors have reported that extracts from grape and wine induce endothelium-dependent relaxation via enhanced generation and/or increased biological activity of NO which leads to the elevation of cGMP level (Fitzpatrick et al. 1993; Andriambeloson et al. 1997). The increase in the intracellular Ca^{2+} level proceeds via a redox-sensitive pathway the activation of NO synthase, the production of NO and thus endothelium-dependent vasodilatation in different types of arteries from different species (Andriambeloson et al. 1999, Zenebe et al. 2003, Duarte et al. 2004). Another therapeutic effect of flavonoids may be their ability to interact with the generation of NO from vascular endothelium, which leads not only to vasodilatation, but also to the expression of genes that protect the cardiovascular system (Middleton et al. 2000; Zenebe and Pecháňová 2002; Curin and Andriantsitohaina 2005). In terms of these findings, the results of the present study seem likely that Provinol can cause the depressor effect by the inhibition of CA secretion from the adrenal medulla.

In the present study, Provinol 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 Provinol 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 Provinol indicates that this inhibitory effect of Provinol 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 Provinol inhibited CA secretion evoked by Bay-K-8644 as well as by high K^+ suggest that Provinol 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 Provinol inhibits the DMPP-evoked CA secretion by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

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, Provinol suppressed the veratridine-evoked CA secretory response. This result suggests that the inhibition of Provinol 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 Provinol 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 Provinol 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 Provinol 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 Provinol 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 Provinol 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 Provinol-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 Provinol on Ca^{2+} movement from intracellular pools is due to its direct effect on the response of phosphoinositides or the indirect effects.

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 Provinol promotes the endothelium-dependent relaxation, activates NO synthase, inhibits platelet aggregation, and prevents 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).

In addition to these pharmacological effects of Provinol, in the present study, it

was shown that Provinol inhibits the CA induced by cholinergic (both nicotinic and muscarinic) receptor stimulation, suggesting that Provinol 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 failure, 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. 16, conclusively, the results of the present study demonstrate that Provinol inhibits the CA secretion by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization from the isolated perfused adrenal glands of the normotensive rats. It seems that this inhibitory effect of Provinol is mediated by blocking the influx of Na^{+} and Ca^{2+} ions through calcium and sodium channels into the rat adrenal medullary chromaffin cells as well as by inhibiting the release of Ca^{2+} from the cytoplasmic calcium store, which are exerted at least partly by the increased NO production due to the activation of nitric oxide synthase. These experimental results may greatly contribute to the hypotensive effect of Provinol 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 examine the effect of Provinol, which is a mixture of polyphenolic compounds isolated from red wine, on secretion of catecholamines (CA) from the isolated perfused rat adrenal medulla, and to elucidate its mechanism of action.

Provinol (0.3~3 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). Provinol itself did not affect basal CA secretion (data not shown). Also, in the presence of Provinol (1 μg/mL), the secretory responses of CA evoked by Bay-K-8644 (a voltage-dependent 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. Interestingly, in the simultaneous presence of Provinol (1 μg/mL) and L-NAME (a selective inhibitor of NO synthase, 30 μM), the CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclpiazonic acid were recovered to the considerable extent of the corresponding control secretion in comparison with inhibition of Provinol-treatment alone. Under the same condition, the level of NO released from adrenal medulla after the treatment of Provinol (3 μg/mL) was greatly elevated compared to the corresponding basal release.

Taken together, the present results demonstrate that Provinol 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 Provinol seems to be exerted by inhibiting the influx of both calcium and sodium into the rat adrenal medullary chromaffin cells along with the blockade of Ca^{2+} release 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, Provinol may be beneficial to prevent or alleviate the cardiovascular diseases, including hypertension and angina pectoris.

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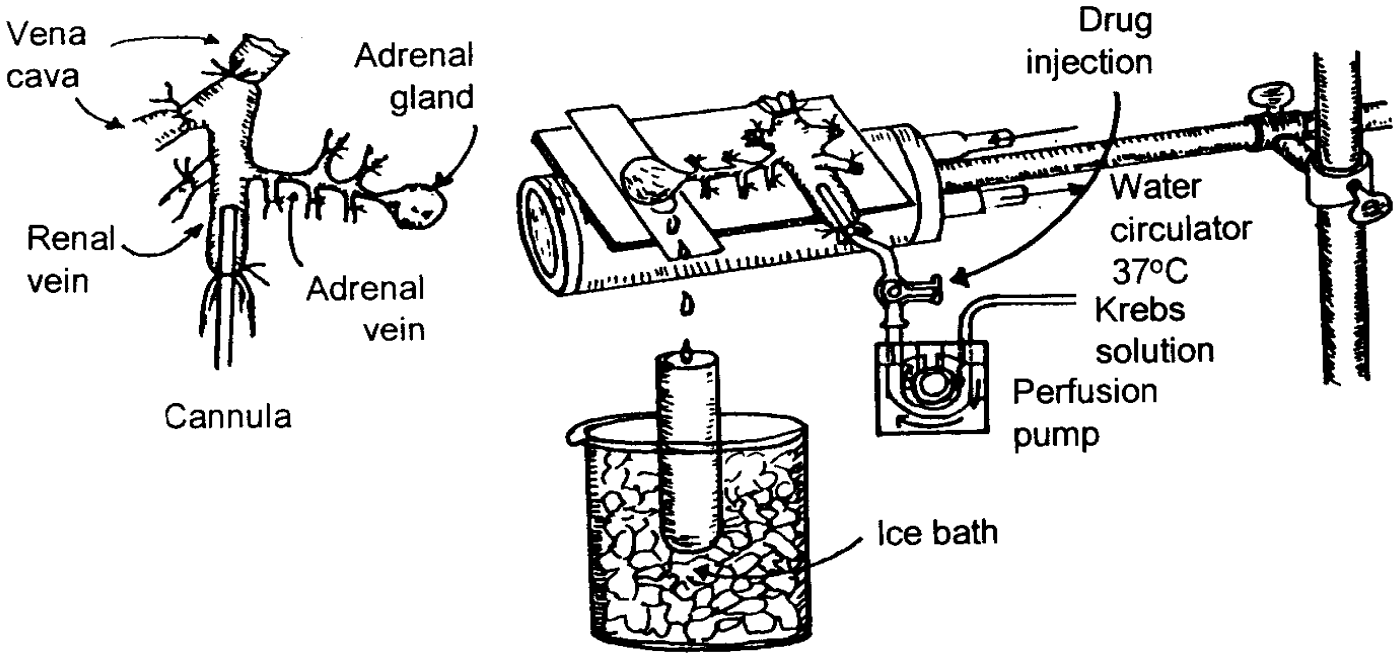

Fig. 1. Schematic drawing of the preparation used to study secretion of catecholamines in the isolated perfused rat adrenal gland.

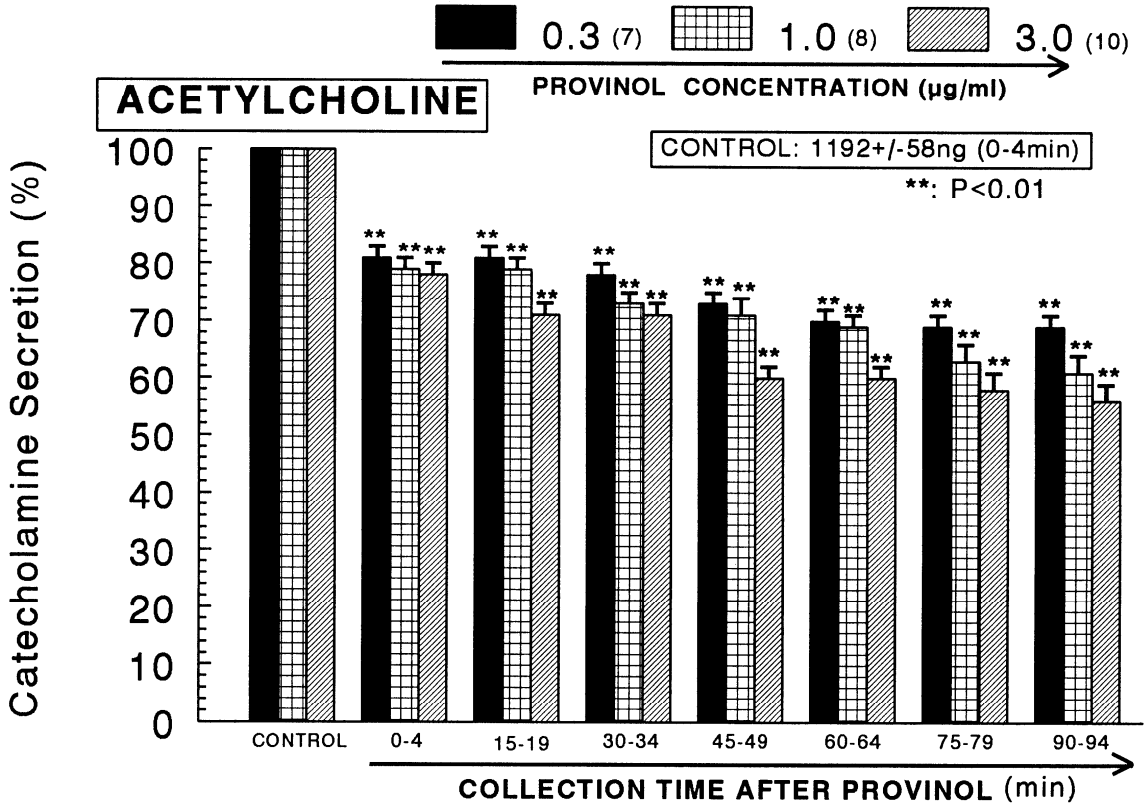

Fig. 2. Effects of Provinol on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh) in the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was evoked at 15 min intervals during loading with 0.3, 1.0 or 3.0 μg/mLof Provinol 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 the control). Abscissa: collection time of perfusate (min). Statistical significance was tested by comparing the corresponding control with each concentration-pretreated group of Provinol. ACh-induced perfusate was collected for 4 minutes. **: $P < 0.01$.

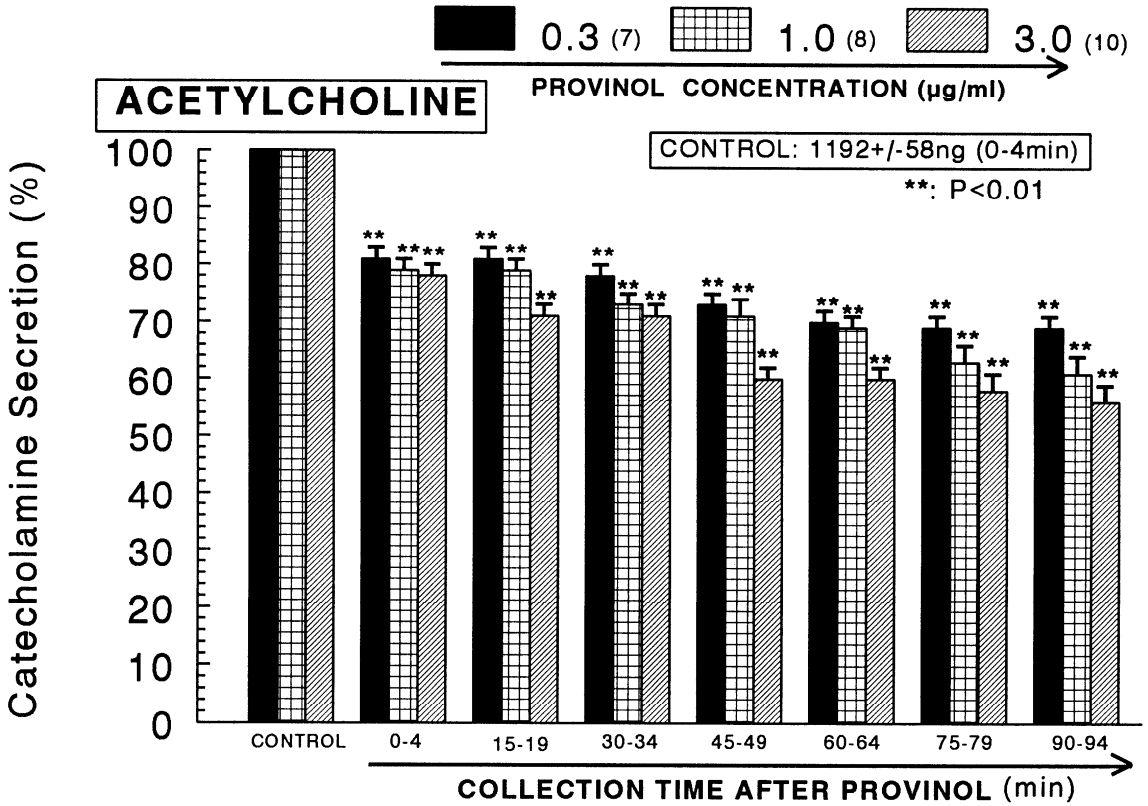

Fig. 3. Effects of Provinol on the secretory responses of catecholamines (CA) evoked by high K^+ in the isolated perfused rat adrenal glands. CA secretion by a single injection of K^+ (56 mM) was evoked at 15 min intervals during loading with 0.3, 1.0 or 3.0 μg/mLof Provinol for 90 min. Statistical significance was tested by comparing the corresponding control with each concentration-pretreated group of Provinol. K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. *: $P < 0.05$, **: $P < 0.01$.

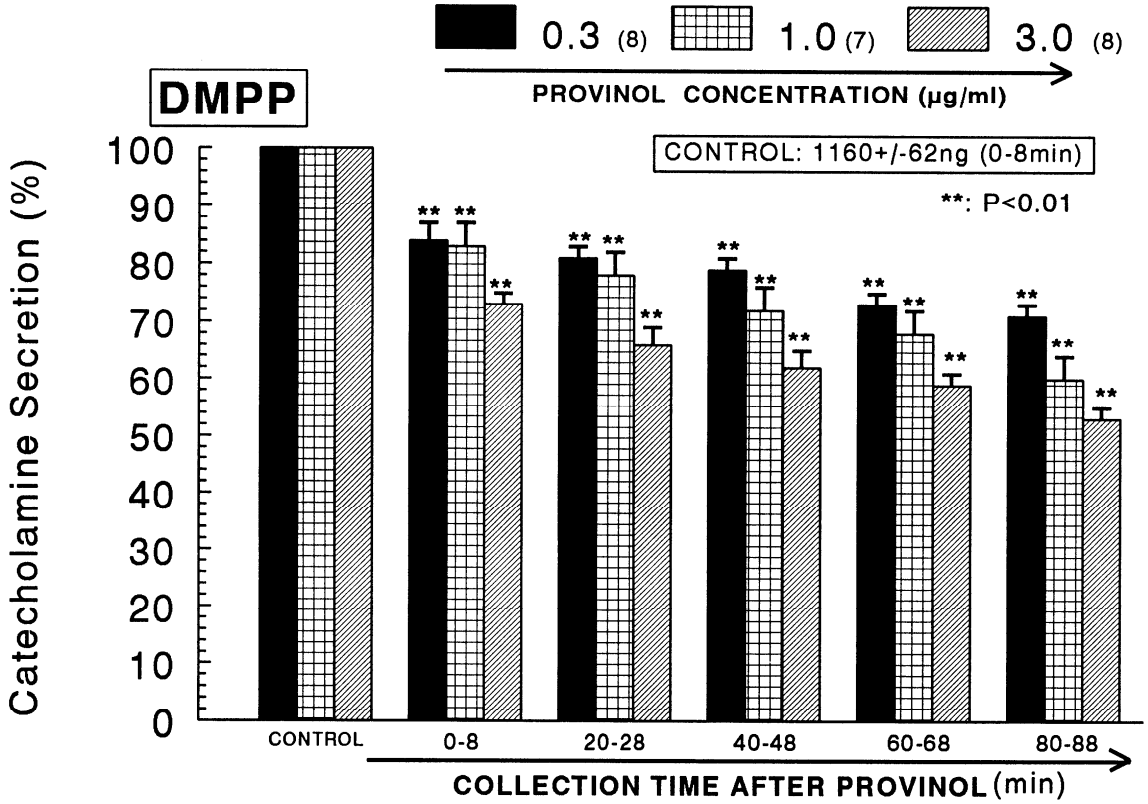

Fig. 4.Effects of Provinol on the secretory responses of catecholamines (CA) evoked by DMPP in the isolated perfused rat adrenal glands. CA secretion by the perfusion of DPPP (10^{-4} M) was evoked for 2 min at 20 min intervals during loading with 0.3, 1.0 or 3.0 μg/mLof Provinol for 90 min. Statistical significance was tested by comparing the corresponding control (1160±62 ng for 8 min) with each concentration-pretreated group of Provinol. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 2. **: P < 0.01.

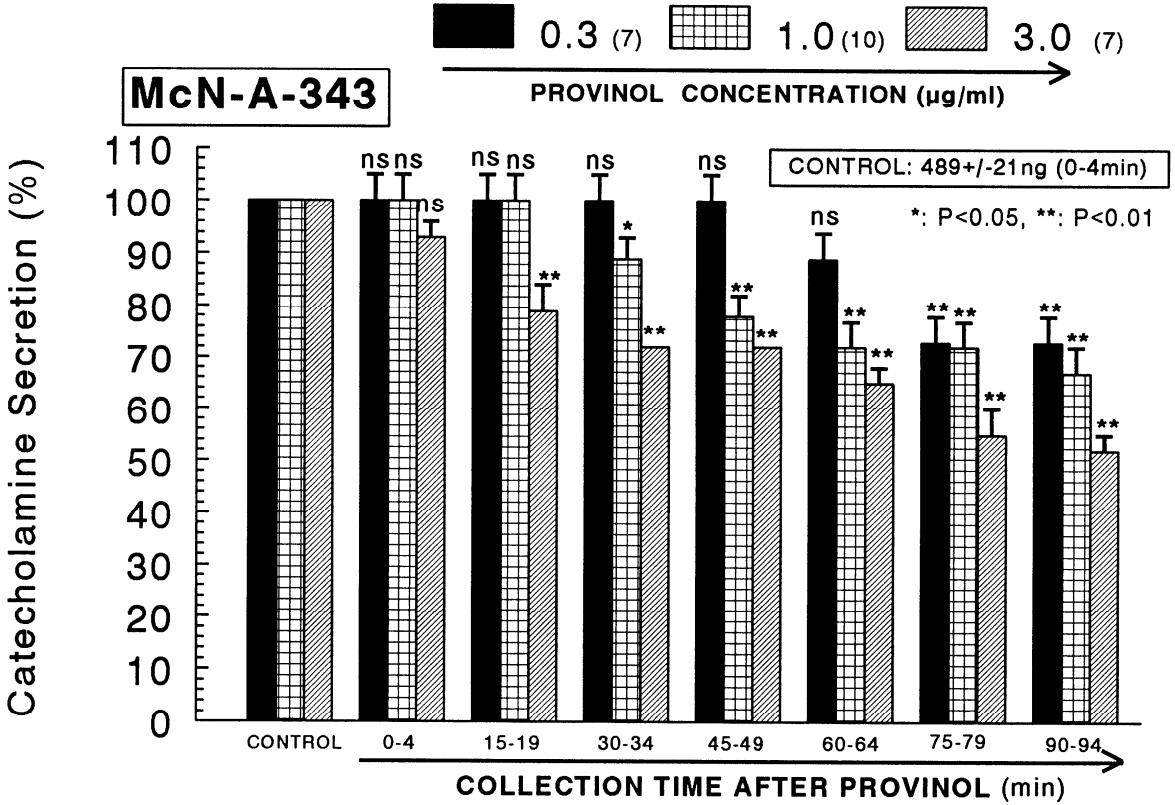

Fig. 5. Effects of Provinol on the secretory responses of catecholamines (CA) evoked by McN-A-343 in the isolated perfused rat adrenal glands. CA secretion by the perfusion of McN-A-343 (10^{-4} M) was evoked for 4 min at 15 min intervals after preloading with 0.3, 1.0 or 3.0 μg/mLof Provinol for 90 min. Statistical significance was tested by comparing the corresponding control with each concentration-pretreated group of Provinol. McN-A-343-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 2. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

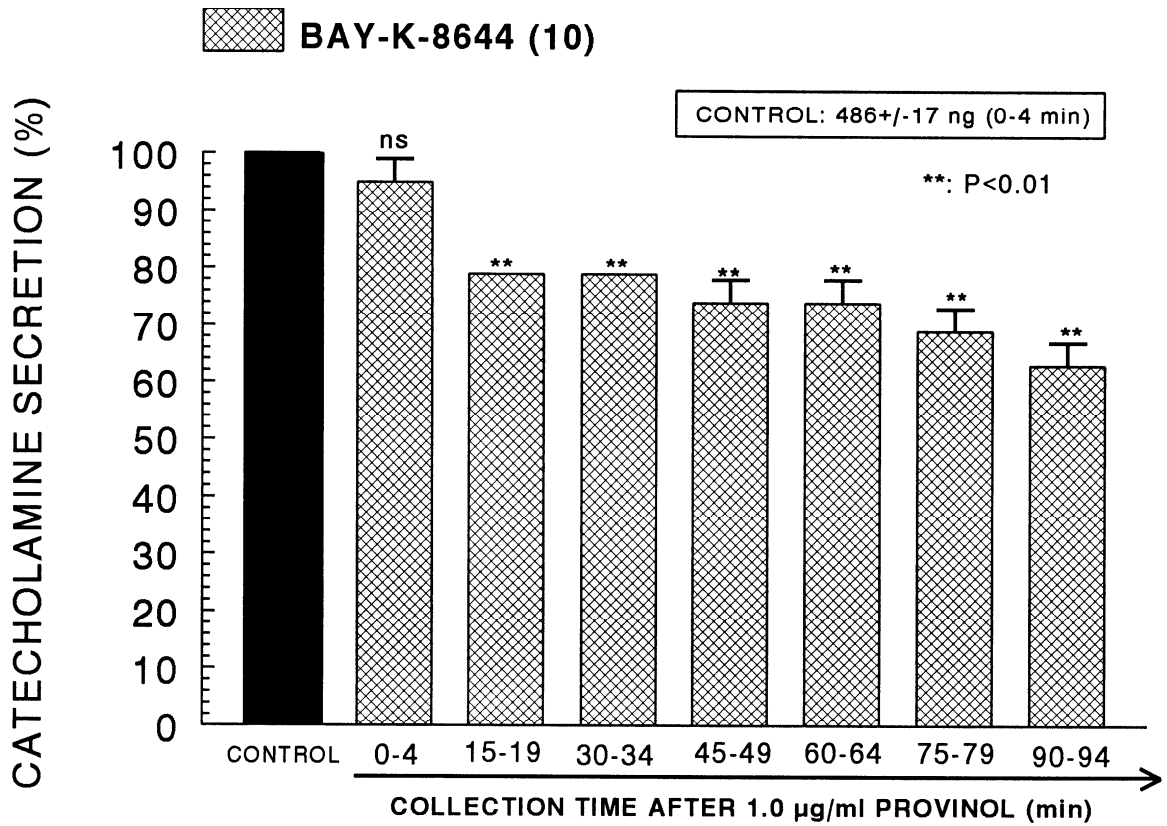

Fig. 6. Time-course effect of Provinol on CA release evoked by Bay-K-8644 from the rat adrenal glands. Bay-K-8644 (10^{-5} M) was perfused into an adrenal vein for 4 min at 15 min intervals during loading with Provinol (1.0 µg/mL) for 90 min. Statistical significance was tested by comparing the corresponding control with each period after treatment with Provinol. Other legends are the same as in Fig. 2. **: $P < 0.01$. ns: Statistically not significant.

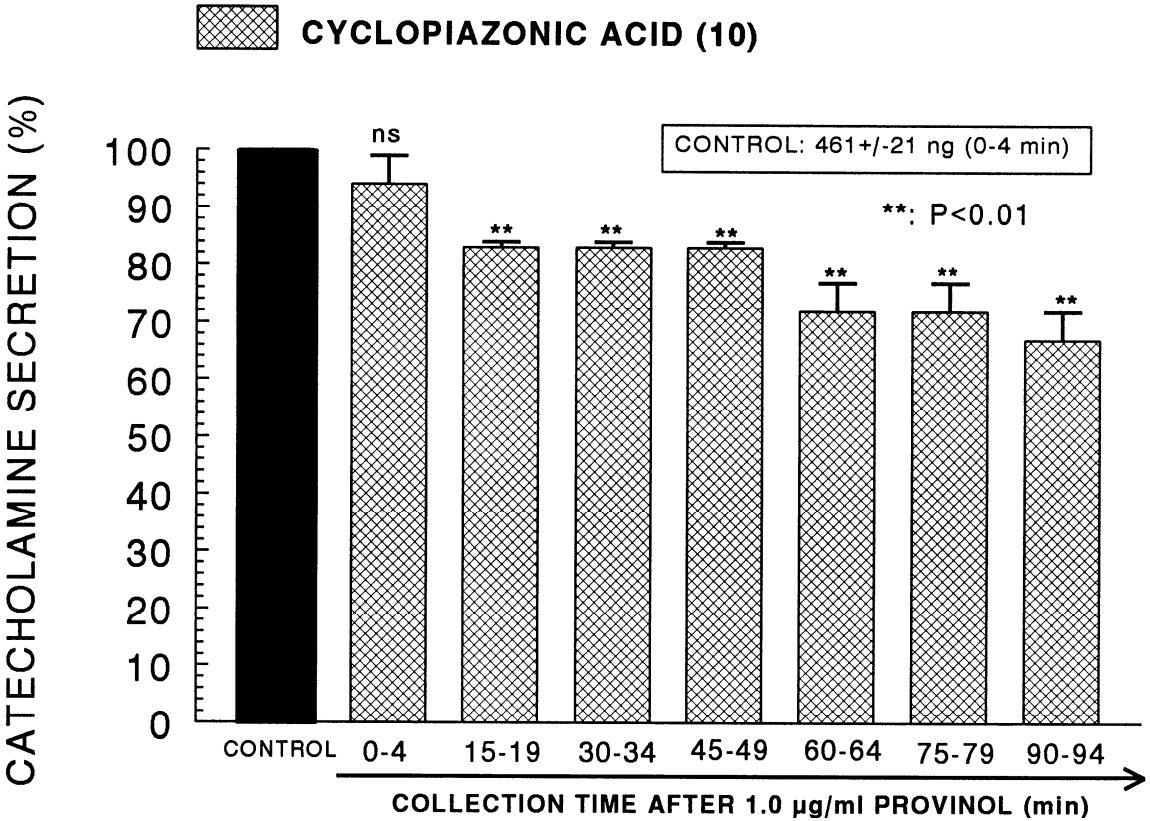

Fig. 7. Time-course effect of Provinol on CA release evoked by cyclopiazonic acid from the rat adrenal glands. Cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals during loading with Provinol (1.0 µg/mL) for 90 min. Statistical significance was tested by comparing the corresponding control with each period after pretreatment with Provinol. Other legends are the same as in Fig. 2. **: P < 0.01. ns: Statistically not significant.

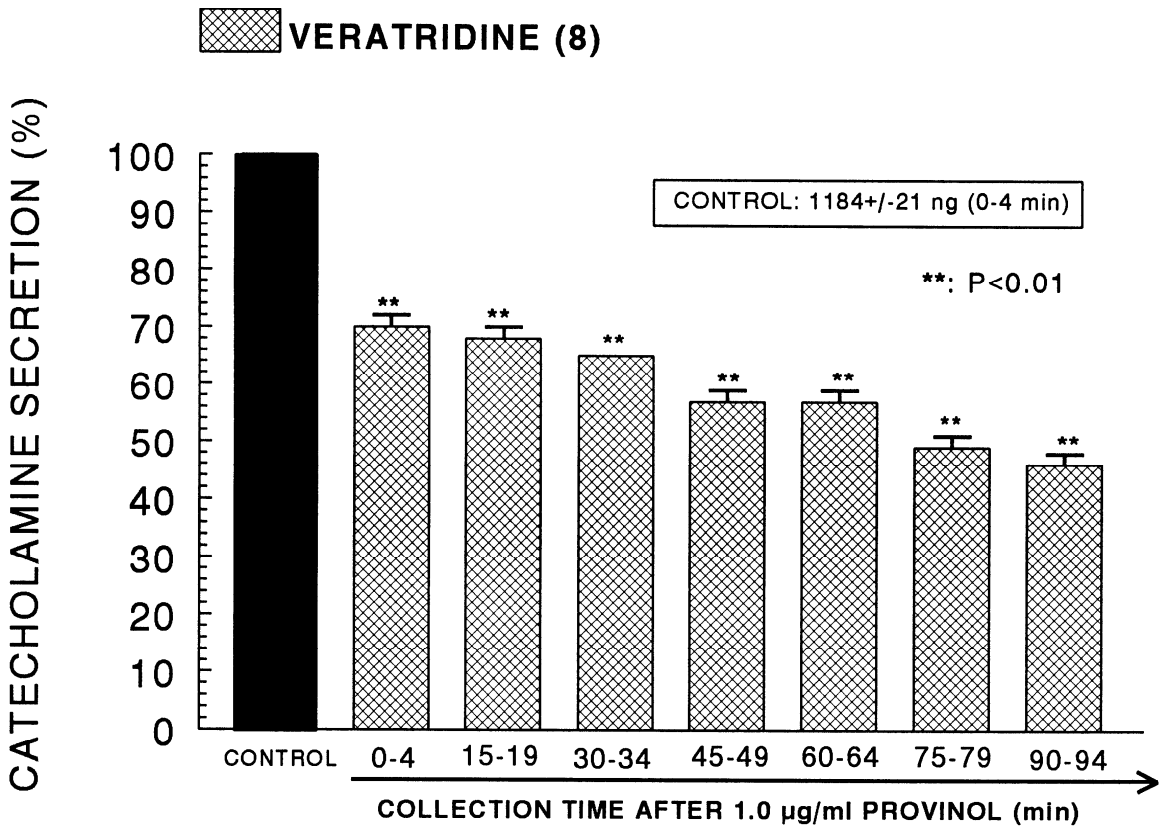

Fig. 8. Time-course effect of Provinol on the CA release evoked by veratridine from the rat adrenal glands. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals during loading with Provinol (1.0 μg/ml) for 90 min. Other legends are the same as in Fig. 2. **: $P < 0.01$.

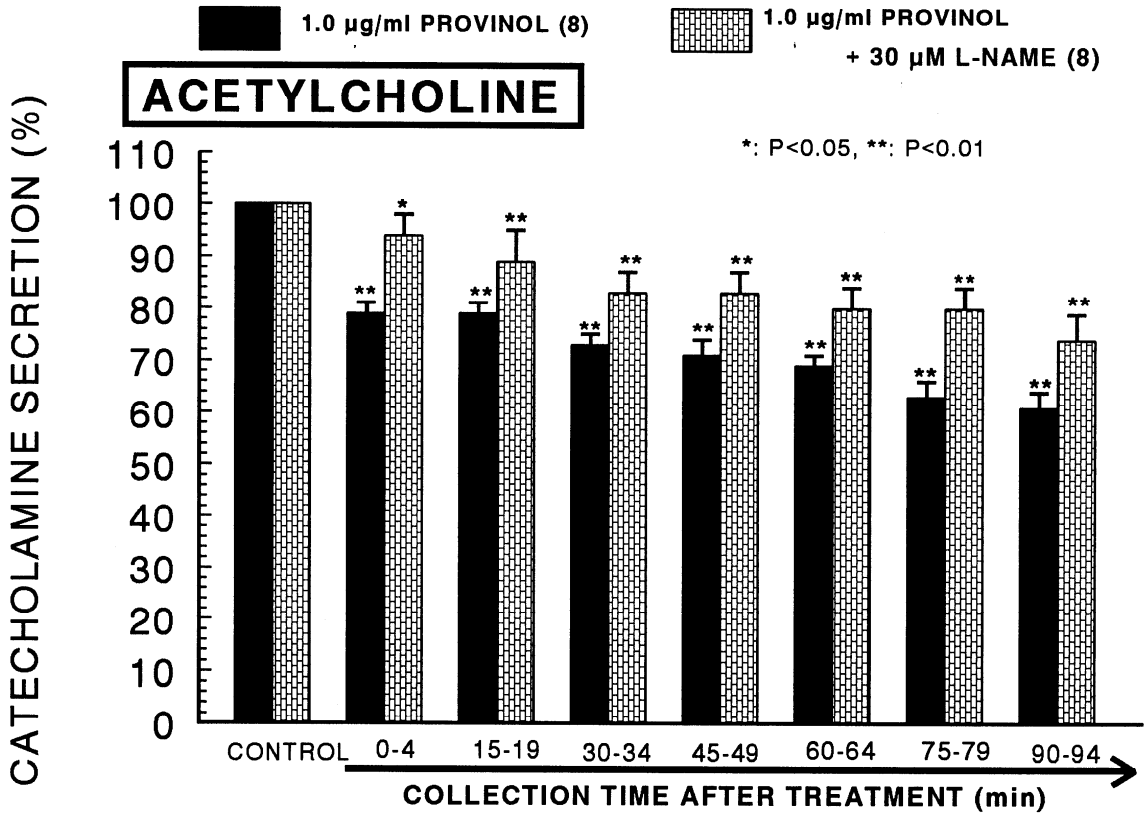

Fig. 9. Effects of Provinol plus L-NAME on the CA secretory responses evoked by ACh in the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml was induced before (CONTROL) and after loading with Provinol (1.0 µg/ml) plus L-NAME (30 µM) for 90 min. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 2. *: $P < 0.05$, **: $P < 0.01$.

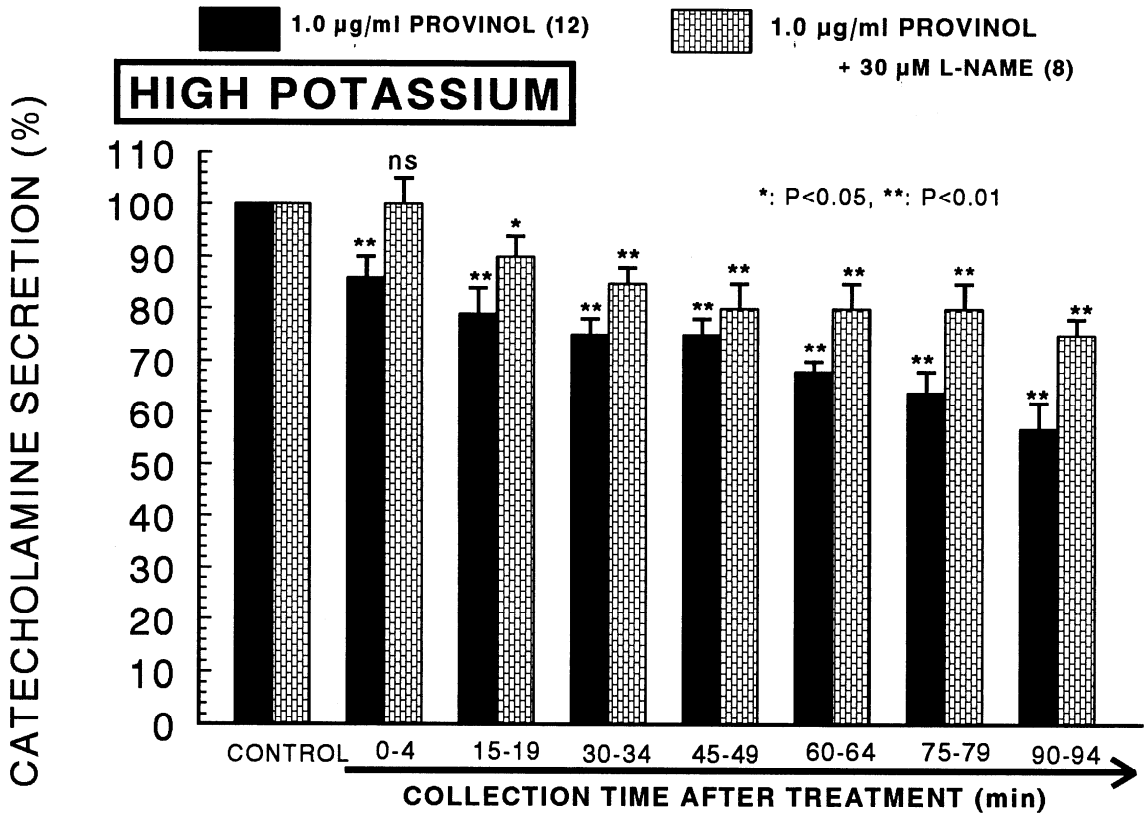

Fig. 10. Effects of Provinol plus L-NAME on the CA secretory responses evoked by high potassium fin the isolated perfused rat adrenal glands. The CA secretion by a single injection of high K^+ (5.6×10^{-2} M) in a volume of 0.05 ml was induced before (CONTROL) and after loading with Provinol (1.0 µg/ml) 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. 2. **: $P < 0.01$. ns: Statistically not significant.

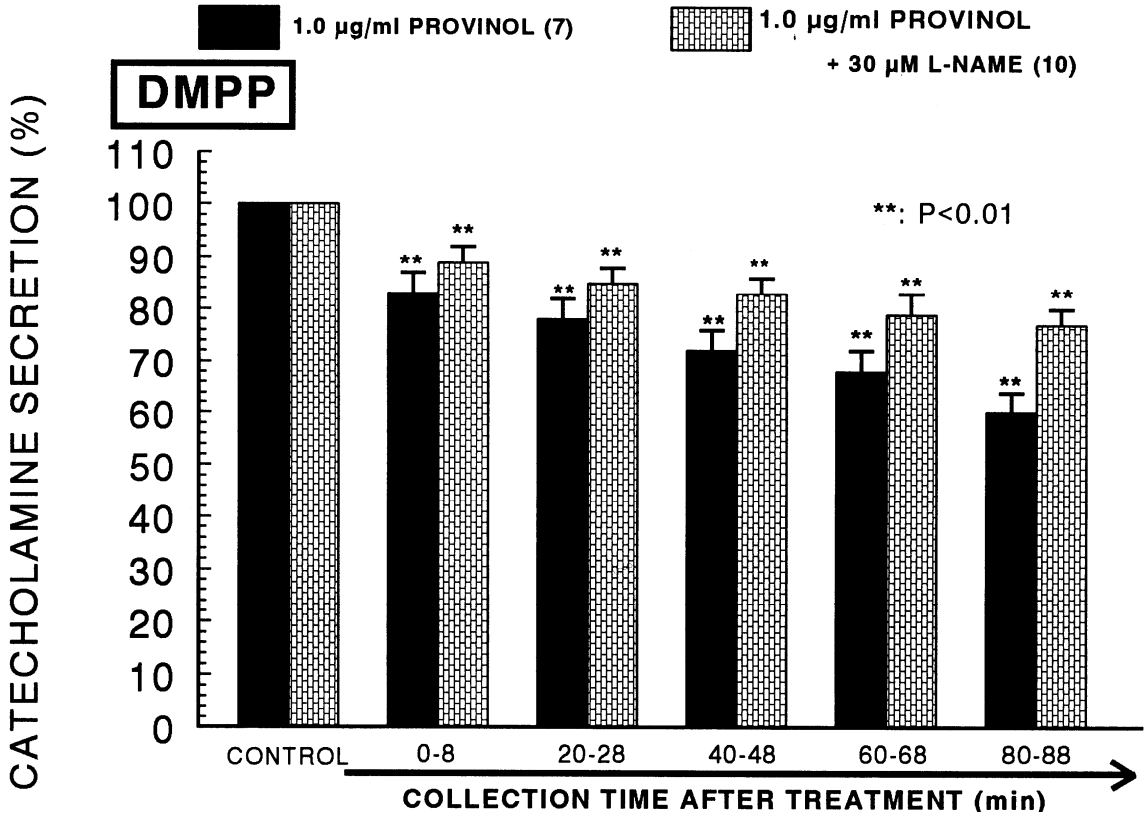

Fig. 11. Effects of Provinol plus L-NAME on the CA secretory responses evoked by DMPP in the isolated perfused rat adrenal glands. The CA secretion by perfusion of DMPP (10^{-4} M) for 2 min was induced before (CONTROL) and after preloading with Provinol (1.0 µg/ml) plus L-NAME (30 µM) for 90 min. Perfusates were collected for 8 minutes at 20 min-intervals. Other legends are the same as in Fig. 2. **: P < 0.01.

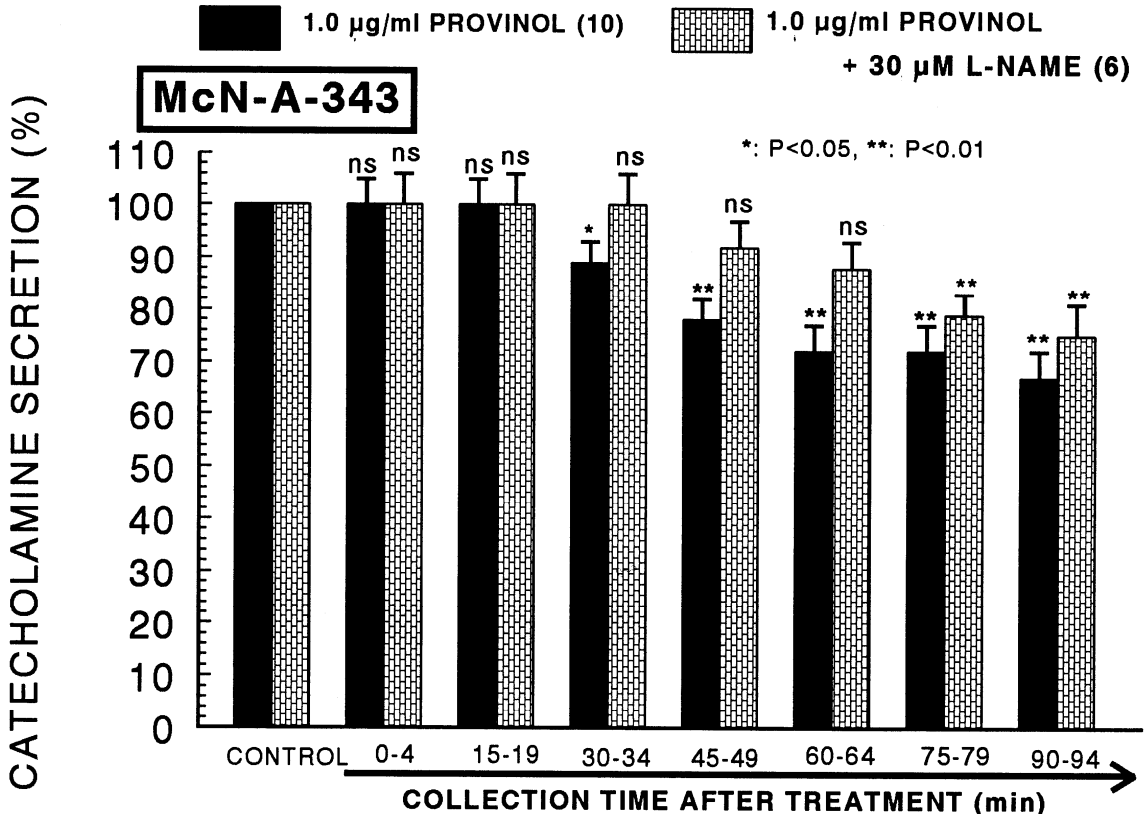

Fig. 12. Effects of Provinol plus L-NAME on the CA secretory responses evoked by McN-A-343 in the isolated perfused rat adrenal glands. The CA secretion by perfusion of McN-A-343 (10^{-4} M) for 4 min was induced before (CONTROL) and after loading with Provinol (1.0 µg/ml) plus L-NAME (30 µM) for 90 min. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 2. **: P < 0.01, **: P < 0.01. ns: Statistically not significant.

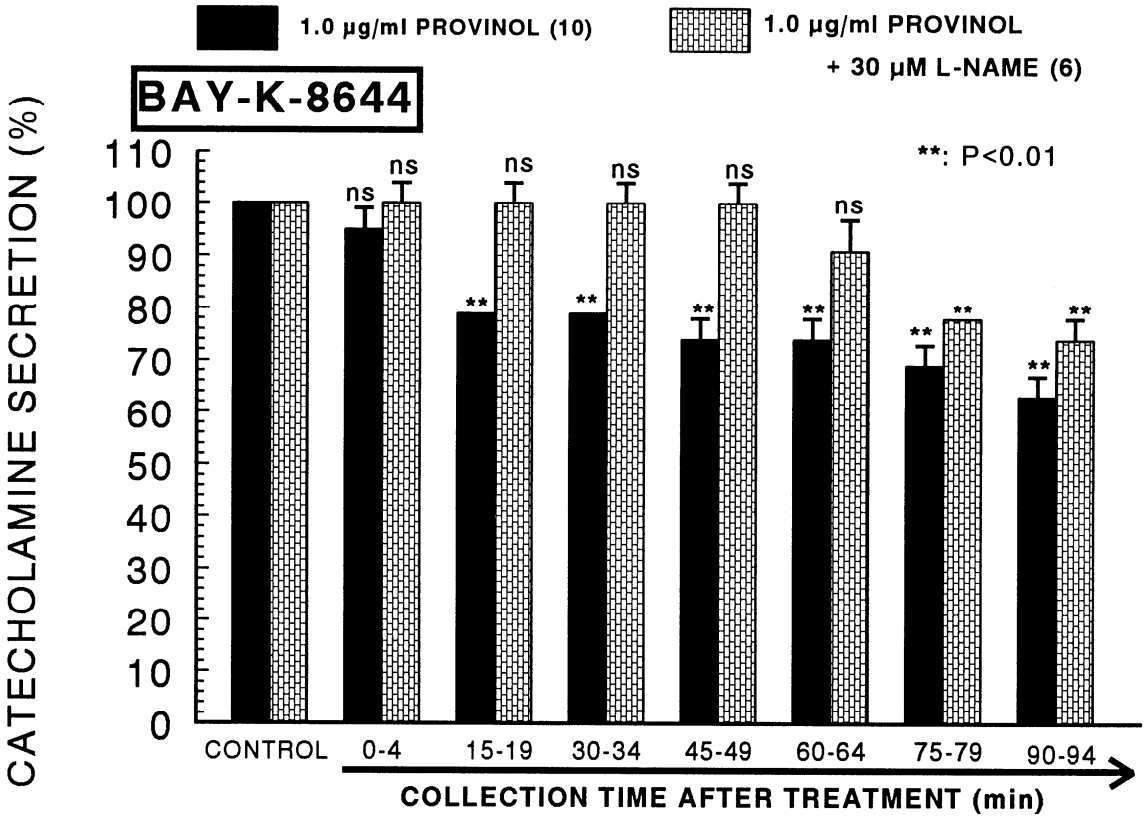

Fig. 13. Effects of Provinol plus L-NAME on the CA secretory responses evoked by Bay-k-8644 in the isolated perfused rat adrenal glands. The CA secretion by perfusion of Bay-k-8644 (10^{-5} M) for 4 min was induced before (CONTROL) and after loading with Provinol (1.0 µg/ml) plus L-NAME (30 µM) for 90 min. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 2. **: P < 0.01. ns: Statistically not significant.

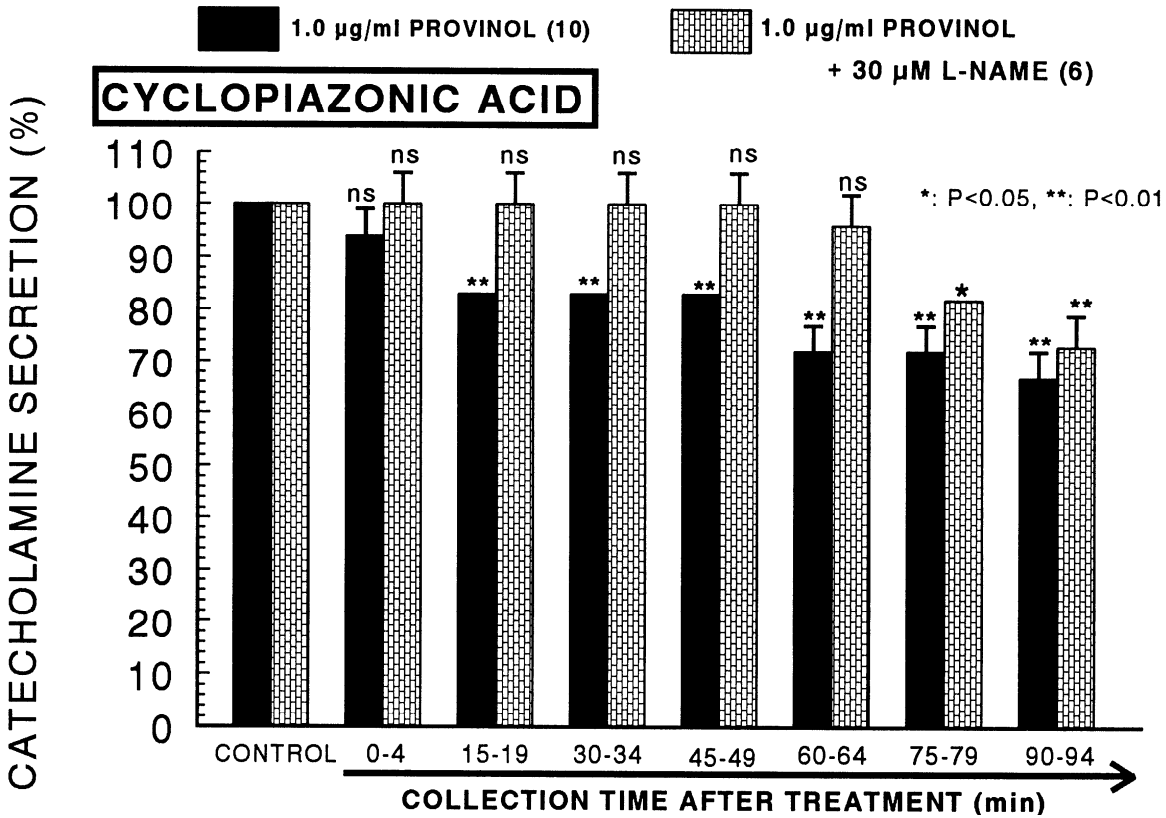

Fig. 14. Effects of Provinol plus L-NAME on the CA secretory responses evoked by cyclopiazonic acid from the isolated perfused rat adrenal glands. The CA secretion by perfusion of cyclopiazonic acid (10^{-5} M) for 4 min was induced before (CONTROL) and after loading with Provinol (1.0 µg/ml) plus L-NAME (30 µM) for 90 min. Perfusates were collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 2. **: P < 0.01. ns: Statistically not significant.

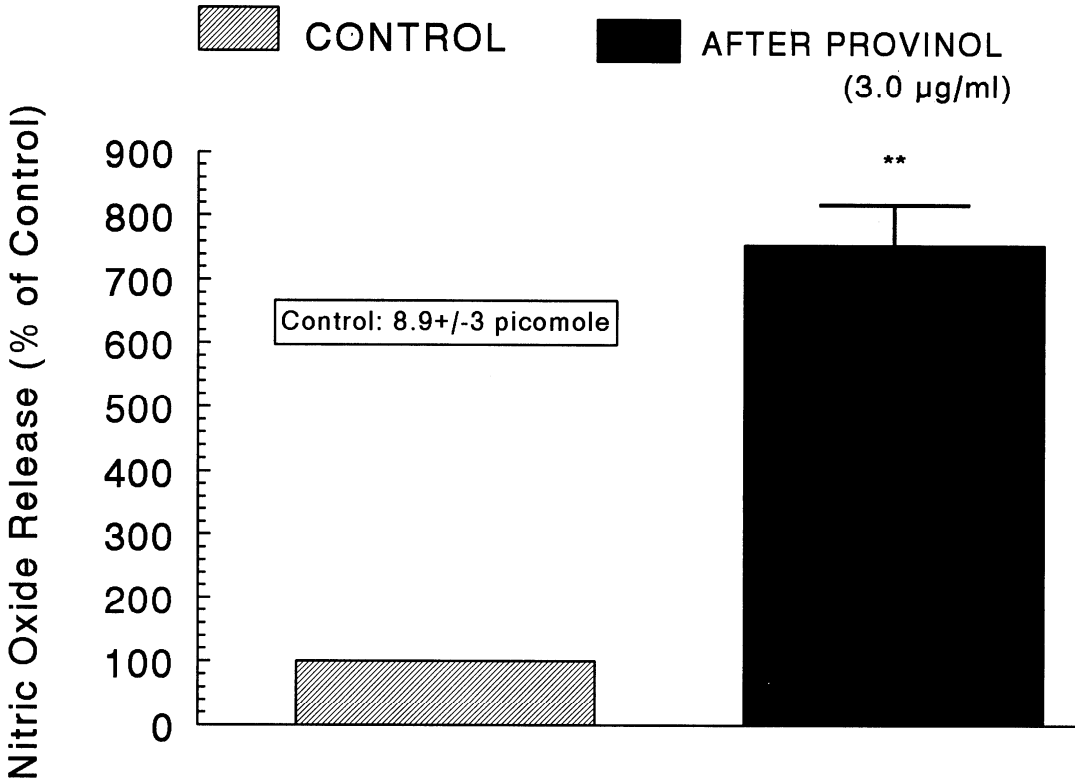

Fig. 15. Effect of Provinol on nitric oxide (NO) production in the isolated perfused rat adrenal medulla. Perfusate sample was taken for 10 min after perfusion of Provinol (3.0 ug/ml) for 90 min. Ordinate: the amounts of NO released from the adrenal medulla (% of control). Abscissa: Treatment (before and after Provinol). Statistical difference was made by comparing the control (8.9±3 picomoles) with Provinol-treated group. **: P< 0.01.

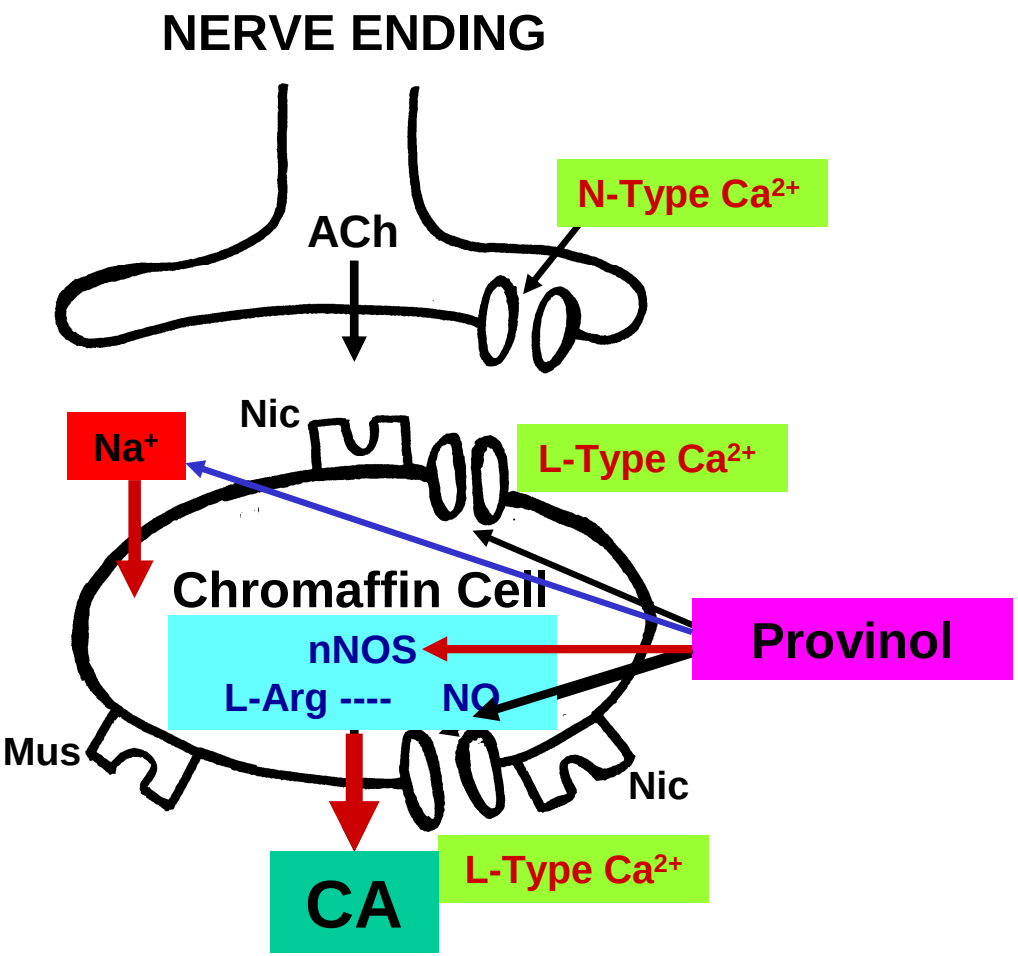

Fig. 16. Schematic diagram of possible action site of Provinol in the rat adrenal gland. This diagram demonstrates possible localizations of voltage-dependent Na^+ and Ca^{2+} channels and cholinergic receptors mediating secretion of adrenal catecholamines (CA). CA-containing cells possess synaptic nicotinic receptors, extrasynaptic nicotinic and muscarinic receptors, and L-type voltage-dependent Ca^{2+} channels close to the extrasynaptic nicotinic receptors.

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논문제목	한글 : 흰쥐 관류부신에서 카테콜아민 분비작용에 대한 Provinol의 억제효과 영어 :Inhibitory Effect of Provinol on Catecholamine Secretion in the Perfused Rat Adrenal Gland				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2008년 6월 일

저작자: 서 유 승 (서명 또는 인)

조선대학교 총장 귀하