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Provinol Inhibits Catecholamine Secretion from the Rat Adrenal Medulla

Jung-Hee Lee[1], **Yu-Seung Seo**[2], **and Dong-Yoon Lim**[2,*]

[1]*DNA Repair Research Center, Chosun University,* [2]*Department of Pharmacology, College of Medicine, Chosun University, Gwangju 501-759, Korea*

The aim of the present study was to examine the effect of provinol, which is a mixture of polyphenolic compounds from red wine, on the secretion of catecholamines (CA) from 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. 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) plus 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 recovered to the considerable extent of the corresponding control secretion in comparison with the 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 in comparison to its basal release. Taken together, these data 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 perfused rat adrenal medulla. This inhibitory effect of provinol seems to be exerted by inhibiting the influx of both calcium and sodium into the rat adrenal medullary 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.

Key Words: Provinol, Catecholamine secretion, Adrenal medulla, Cholinergic receptors, Nitric oxide

INTRODUCTION

Provinol represents the polyphenolic compounds extracted from French 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 has been 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., 2006). 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 has been 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 (Bernátová 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

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Corresponding to: Dong-Yoon Lim, Department of Pharmacology, College of Medicine, Chosun University, 375, Seosuk-dong, Dong-gu, Gwangju 501-759, Korea. (Tel) 82-62-230-6335, (Fax) 82-62-227-4693, (E-mail) dylim@chosun.ac.kr

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ABBREVIATIONS: CA, catecholamines; NO, nitric oxide; DMPP, 1.1-dimethyl-4-phenyl piperazinium iodide, methyl-1,4-dihydro-2; ACh, acetylcholine; McN-A-343, 3-(m-chlloro-phenyl-carbamoyl-oxy-2-butynyl-trimethyl ammonium chloride; BAY-K8644, 6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate.

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). 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 suppression of 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 the 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 perfused model of normotensive rats, and to establish its mechanism of action.

METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 200 to 300 grams, were anesthetized with thiopental sodium (40 mg/kg) intraperitoneally. The adrenal gland was isolated using some modification of the methods described previously (Wakade, 1981). The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by placing 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 the entrance of the adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid was released only from the slit made in the adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula were 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°C.

Perfusion of adrenal gland

The adrenal glands were perfused by means of an ISCO pump (WIZ Co.) at a rate of 0.33 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of the 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 perfusion of DMPP (10^{-4} M) and McN-A-343 (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 the perfusion stream via a three-way stopcock, respectively. 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 returned 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 had 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 quinine for 20 min, then the perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the stimulating agent alone or with provinol, and the perfusates were collected for the same period of time as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was measured directly using the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using a 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 the secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of the control samples (unstimulated). The sample blanks were also lowest for perfusates of the 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 de-

tected 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 quantified as the current detected at the electrode 30 min after the presence of provinol at room temperature. NO release was calculated in picomoles. NO production was also measured indirectly by measuring the 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 the means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made using the computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: Provinol [(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 their molar base.

RESULTS

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

After the perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, the basal CA release from the 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., 2006), there was an initial attempt 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- and 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 increased the CA secretion (1,192±58 ng for 0～4 min), as shown in Fig. 1 (left). However, the pretreatment with provinol in the range of 0.3～3 μg/ml for 90 min relatively inhibited ACh-stimulated CA secretion in a concentration- and time- dependent manner. In the presence of provinol as shown in Fig. 1 (left),

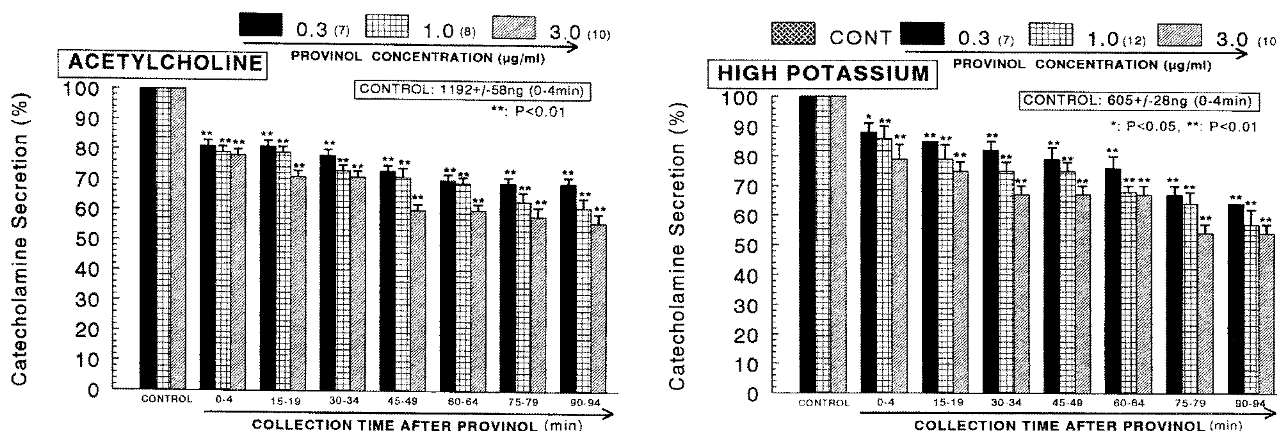


Fig. 1. Dose-dependent effects of provinol on the secretory responses of catecholamines (CA) evoked by acetylcholine (left) and high potassium (right) from the perfused rat adrenal medulla. The CA secretion by a single injection of ACh (5.32×10^{-3} M) and K^+ (5.6×10^{-2} M) in a volume of 0.05 ml was evoked at 15 min intervals during loading with 0.3, 1.0 and 3.0 μg/ml of provinol for 90 min as indicated by the arrow marks, respectively. The numbers in parentheses indicate the number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (% of control). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-treated group of provinol. ACh- and high K^+-induced perfusates were collected for 4 minutes, respectively. **p<0.01.

CA releasing responses were inhibited to 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, markedly stimulates 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 (Fig. 1- right). 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 (1,160±62 ng for 0~8 min). However, as shown in Fig. 2 (left), DMPP-stimulated CA secretion after pretreatment with provinol was maximally reduced to 53% of the control release in the last period (80~88 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. 2 (right).

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

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. 3 (left).

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

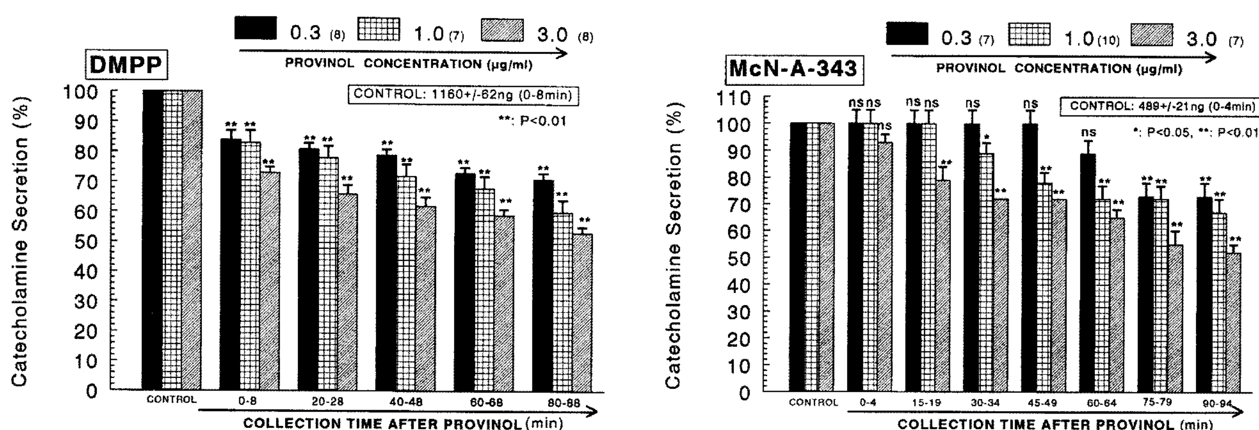


Fig. 2. Dose-dependent effects of provinol on the CA secretory responses evoked by DMPP (left) and McN-A-343 (right) from the perfused rat adrenal medulla. The CA secretion by perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min was induced at 15 and 20 min intervals during loading with 0.3, 1.0 and 3.0 μg/ml of provinol for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of provinol. DMPP- and McN-A-343-induced perfusates were collected for 8 and 4 minutes, respectively. Other legends are the same as in Fig. 1. *p<0.05, **p<0.01. ns: Not statistically significant.

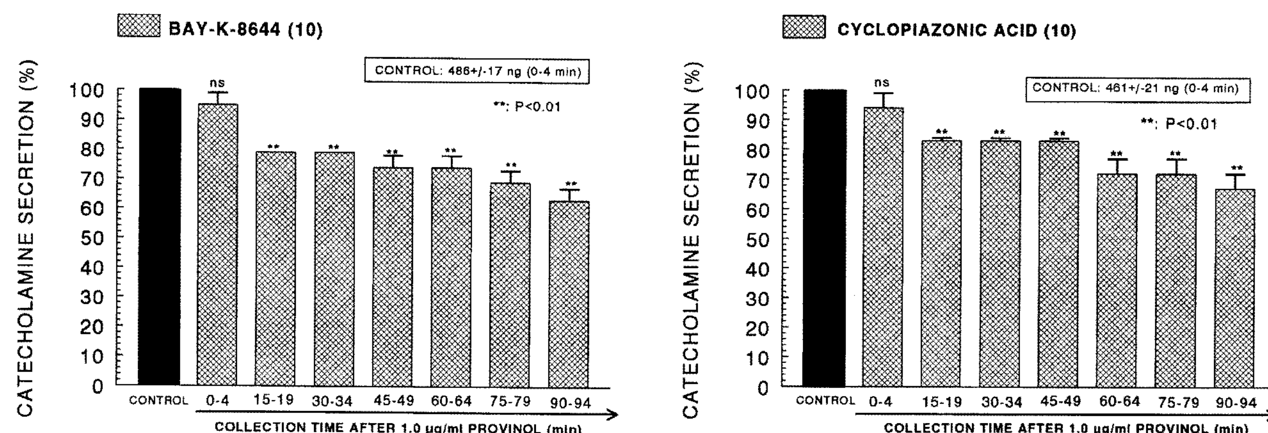


Fig. 3. Time-course effects of provinol on the CA secretion evoked by Bay-K-8644 (left) and cyclopiazonic acid (right) from the perfused rat adrenal medulla. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals during loading with provinol (1.0 μM) for 90 min. Other legends are the same as in Fig. 1. **p<0.01. ns: Not statistically significant.

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. 3 (right), 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 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. 4, veratridine greatly produced CA secretion (1,184±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.

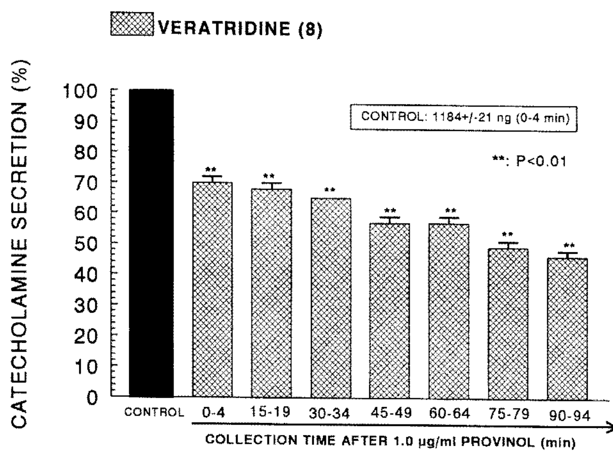
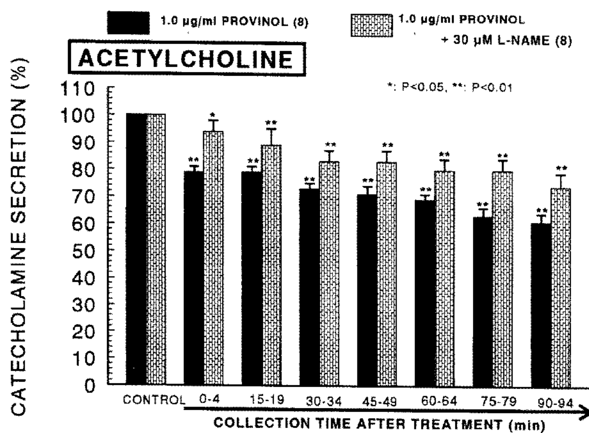


Fig. 4. Time-course effects of provinol on the CA secretion evoked by veratridine from the perfused rat adrenal medulla. Veratridine (10^{-4} M) was perfused into an adrenal vein for 4 min at 15 min intervals during loading with provinol (1.0 μM) for 90 min. Other legends are the same as in Fig. 1. **p<0.01.

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

It has also been found that, as shown in Fig. 1~4, 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 recovered to 74~94% of the corresponding control release compared to results after loading of provinol alone as illustrated in Fig. 5 (left). High K^+ (56 mM)-evoked CA release in the simultaneous presence of provinol (1 μM) and L-NAME (30 μM) for 90 min also recovered to 75~100% of the corresponding control release during all periods in comparison to the data of treatment with provinol alone (Fig. 5-right).

As shown in Fig. 6 (left), the simultaneous perfusion of provinol and L-NAME for 90 min overcame 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



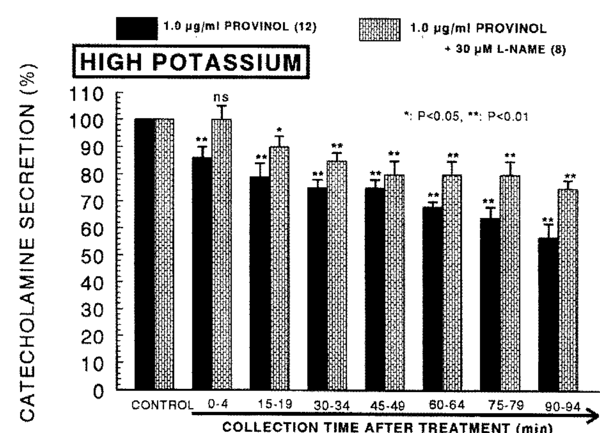


Fig. 5. Effects of provinol plus L-NAME on the CA secretory responses evoked by acetylcholine (left) and high potassium (right) from the perfused rat adrenal medulla. The CA secretion by a single injection of ACh (5.32×10^{-3} M) and K^+ (5.6×10^{-2} M) in a volume of 0.05 ml was evoked at 15 min intervals during simultaneous loading with provinol (1.0 μM) plus L-NAME (30 μM) for 90 min. Statistical difference was obtained by comparing the corresponding control (CONTROL) with provinol-treated group or group treated with provinol+L-NAME. Other legends are the same as in Fig. 1. *p<0.05, **p<0.01. ns: Not statistically significant.

secretory response evoked by McN-A-343 (10^{-4} M for 4 min) recovered to 75~100% of the corresponding control release compared to results of the provinol-treatment alone, as shown in Fig. 6 (right).

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

As shown in Fig. 7 (left), 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 also recovered to 73~100% of the control release in comparison to the results following the treatment with provinol alone (Fig. 7-right).

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

As shown in Fig. 5~7, 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 greatly recovered to a considerable extent of the corresponding control secretion from 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. 8, 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

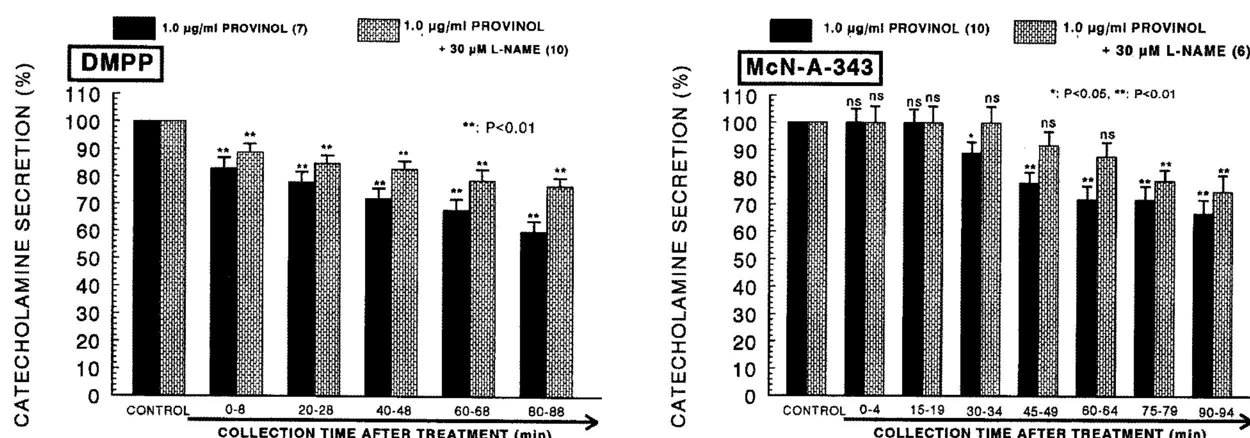


Fig. 6. Effects of provinol plus L-NAME on the CA secretory responses evoked by DMPP (left) and McN-A-343 (right) from the perfused rat adrenal medulla. The CA secretion by perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min was induced at 15 and 20 min intervals after preloading with provinol (1.0 μM) plus L-NAME (30 μM) for 90 min, respectively. Other legends are the same as in Fig. 1 and 5. *p<0.05, **p<0.01. ns: Not statistically significant.

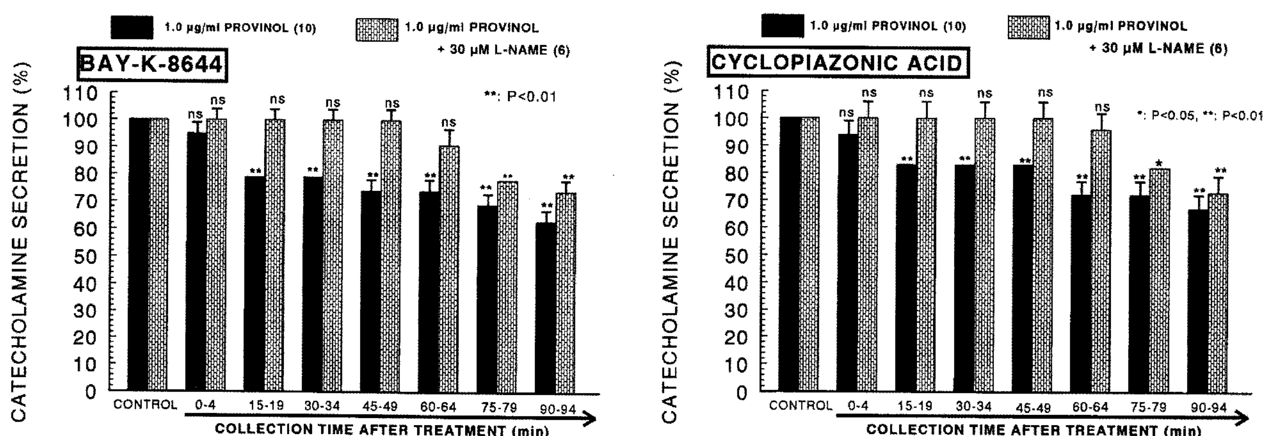


Fig. 7. Effects of provinol plus L-NAME on the CA secretory responses evoked by Bay-K-8644 (left) and cyclopiazonic acid (right) from the perfused rat adrenal medulla. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals during simultaneous loading with provinol (1.0 μM) for 90 min. Other legends are the same as in Fig. 1 and 5. *p<0.05, **p<0.01. ns: Not statistically significant.

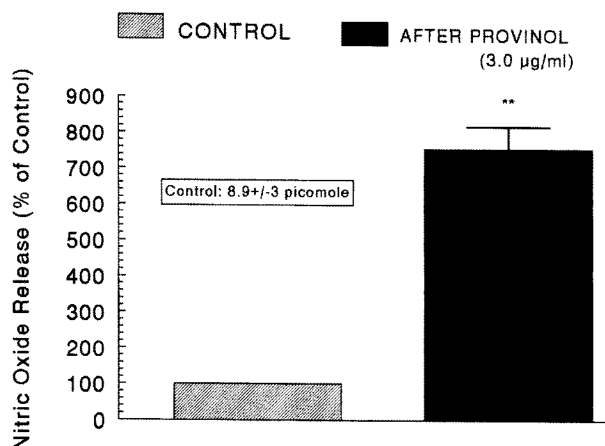


Fig. 8. Effects of provinol on nitric oxide (NO) production in the perfused rat adrenal medulla. Perfusate sample was taken for 8 min after loading the perfusion of provinol (3.0 μM) at a rate of 0.31 ml/min. Ordinate: the amounts of NO released from the adrenal medulla (% of control). Abscissa: Treatment (before and after provinol). Statistical difference was made by comparing the control with provinol-treated group. $^{**}p<0.01$.

650% of the control release. Consequently, it was confirmed that provinol practically increase the level of NO released from the rat adrenal medulla.

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 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 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 PCRW 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., 2004), there may be some assumtions 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 significantly recovered to a considerable level of the corresponding control secretion in comparison to inhibition of treatment with provinol alone. This result is well consistent with report that PCRC 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 the different anthocyanins identified in wine, only delphinidin caused endothelium-dependent relaxation, although it was slightly less potent than provinol (Andriambeloson et al., 1998). Moreover, it has been reported that the NO synthase 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). 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.

PCRW is also found to right 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 BHR. Data suggest that reduction of BP in BHR as well as the improvement of vasorelaxation in provinol-treated WKY rats were associated with other rather than NO-dependent mechanisms (Bernátová 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.

Polyphenolic compounds have been documented to relax pre-contracted 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 grapes 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 intra-

cellular 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 (Schramm et al., 1983; Garcia et al., 1984). 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 (Yanagihara et al., 1979; Fisher et al., 1981). It has also been 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 and Wakade, 1983; Lim and Hwang, 1991) and isolated bovine adrenal chromaffin cells (Kilpatrick et al., 1981 & 1982; Knight and 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 and 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 (Oka et al., 1979; Burgoyne, 1984). 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., 1985b). In the present study, provinol suppressed the veratridine-evoked CA secretory response. This result suggests that the inhibitory effect 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 and 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 cyclopiazonic 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; Frankel et al., 1993a; Demrow and Slane, 1995; Andriambeloson et al., 1997; Flesch et al., 1998; Leikert et al., 2002). The polyphenolic compound resveratrol presented in red wine is thought to be responsible for the 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 (Frankel et al., 1993b; Pace-Asciak et al., 1995; Chen and Pace-Asciak, 1996; Rotondo et al., 1998; Wallerath et al., 2002).

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 can attenuate the CA secretion induced by stress or emotional excitation, thus causing the stimulation of sympathetic nerves and the adrenal medulla. Although the CA plays a pivotal role in the regulation of normal functions in cardiovascular systems, stress-induced overexpression 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; Freedman and Lefkowitz, 2004; Westfall and Westfall, 2005; Lymperopoulos et al., 2007).

The results of the present study conclusively demonstrate that provinol inhibits the CA secretion by stimulation of cholinergic nicotinic receptors as well as by membrane depolarization from the 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 cells and consequent reduction of the CA level in the circulation.

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