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Influence of PD 123319 (AT_2-Receptor Antagonist) on Catecholamine Secretion in the Perfused Rat Adrenal Medulla

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ABSTRACT

Background: The aim of this study was to examine whether PD 123319 (an angiotensin II type 2 [AT_2] receptor antagonist) can influence the release of catecholamines (CA) from the perfused model of the rat adrenal medulla. **Methods:** The adrenal gland was isolated by the modification of Wakade method, and perfused with normal Krebs-bicarbonate solution. The content of CA was measured using the fluorospectrophotometer. **Results:** During perfusion of PD 123319 (range, 5 to 50 nM) into an adrenal vein for 90 minutes the CA secretory responses evoked by acetylcholine (ACh), high K^+, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), and McN-A-343 was dose- and time-dependently inhibited. Furthermore, loading with PD 123319 for 90 minutes also markedly inhibited the CA secretory responses evoked by 4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl -phenyl)-pyridine-5-carboxylate (Bay-K-8644), cyclopiazonic acid, veratridine, and angiotensin II (Ang II). PD 123319 did not affect basal CA output. Simultaneous perfusion of PD 123319 and CGP 42112 perfused into an adrenal vein for 90 minutes rather more potently inhibited the CA seretory responses evoked by Ach, high K+, DMPP, Bay-K-8644, veratridine, and Ang II compared to the inhibitory effect by PD123319-treated alone. **Conclusions:** Taken together, these results show that PD 123319 inhibits the CA secretion evoked by both cholinergic and Ang II receptor stimulation from the perfused rat adrenal medulla. This inhibitory effect of PD 123319 seems to be exerted by blocking the influx of both Na^+ and Ca^{2+} through their voltage-dependent channels into the rat adrenomedullary chromaffin cells as well as by reducing the Ca^{2+} release from its cytoplasmic calcium store, which may be relevant to AT_2 receptor blockade. Based on these present data, it is thought that PD 123319 has different activity from previously known AT_2 antagonist activity in the perfused adrenal medulla, and that AT_2 receptors may be involved in the rat adrenomedullary CA secretion.

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Key Words: PD 123319; Angiotensin II type 2 receptor blockers; Catecholamine secretion; Adrenal medulla

Introduction

It has been found that angiotensin II (Ang II) is the principal effector hormone of the renin-angiotensin system, a system that plays a major role in the control of peripheral vascular resistance, blood pressure, and fluid and electrolyte homeostasis. Ang II is known to exert its actions through at least two major subtypes of receptors, Ang II

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type 1 (AT_1) and type 2 (AT_2) receptors, which are localized on the surface of plasma membrane of various tissues throughout the body.[1-5] Ang II is a secretogogue for secretion of catecholamines (CA) that is believed to be mediated through inositol-1,4,5-triphosphate (IP_3) production by AT_1.[6,7] Indeed, it has been shown that Ang II-induced CA release is mediated by AT_1 in the rat adrenal medulla.[6] AT_1-mediated phospholipase C activation and subsequent IP_3 formation may increase cytosolic Ca^{2+} levels by releasing Ca^{2+} from intracellular storage, with subsequent activation of CA release.[7] Moreover, it has been found that addition of IP_3 to permeabilized bovine chromaffin cells releases intracellular Ca^{2+}.[8] Also, addition of Ca^{2+} to permeabilized bovine chromaffin cells was reported to cause CA secretion.[9] In the adrenal medulla, Ang II increases the basal CA output through the activation of AT_1 receptors either *in vitro*[1,10,11] or *in vivo*.[12] However, a number of previous studies have shown that the selective blockade of AT_1 receptors did not abolish the increase of Ang II-evoked CA secretion in adrenal medulla.[6,12-14] These observations suggest the existence of additional mechanisms other than those mediated through AT_1 receptors. Generally, both AT_1 and AT_2 Ang II receptors are expressed in the adrenal medulla. In the rat, AT_2 receptors predominate, AT_1 receptors representing only 5% to 10% of the total number of Ang II receptors.[15,16] In fact, AT_2 is also abundantly expressed in the adrenal medulla of adult rats.[17,18] Previous studies have demonstrated that the CA-releasing effect of Ang II is mediated primarily via the activation of AT_2 receptors in adrenal medullary fragments isolated from the rat adrenal gland,[19,20] and also in cultured porcine adrenal medullary chromaffin cells.[21] In contrast, it has been suggested that the AT_2 agonist, CGP 42112, inhibits CA biosynthesis through a decrease in cyclic guanosine monophosphate (cGMP) production in cultured porcine adrenal medullary cells.[22] AT_2 receptors are known to play a role in mediating CA secretion by the adrenal medulla of anesthetized dogs in response to Ang II receptor agonist administration *in vivo*.[23] PD 123319 (AT_2 antagonist) and CGP 42112 (AT_2 agonist) inhibited the increase in adrenal CA secretion induced by local administration of Ang II without any agonist actions.[23] Notwithstanding, such a functional implication of AT_2 receptors in the isolated perfused adrenal medulla has yet to be confirmed in other species. Therefore, the present study was designed to evaluate whether AT_2 receptors are functionally involved in the CA secretion in the perfused model of the isolated rat adrenal medulla. The specific purpose of the present study was to analyze modulations of the CA secretion from the perfused rat adrenal gland in response to locally administered several secretagogues including AngII in the presence of either PD 123319 or CGP 42112, both of which are highly specific and selective ligands to angiotensin AT_2 receptor in various tissues.[24]

Subjects and methods

1. Experimental procedure

All procedures involving animal experiment were approved by the committee of experimental animals, Chosun University School of Medicine. Male Sprague-Dawley rats, weighing 250 to 350 g, were anesthetized with thiopental sodium (50 mg/kg) intraperitoneally. The adrenal gland was isolated by the methods described previously.[25] 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°C ± 1°C.

2. Perfusion of adrenal gland

The adrenal glands were perfused by means of peristaltic pump (ISCO pump; WIZ Co., Lincoln, NE, USA) at a rate of 0.33 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, and 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 to 7.5. The solution contained disodium ethylenediamine tetraacetate (10 μg/mL) and ascorbic acid (100 μg/mL) to prevent oxidation of catecholamines.

3. Drug administration

The perfusions of Ang II (10^{-7} M) and 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, 10^{-4} M) for 1 minute and/or a single injection of acetylcholine (Ach, 5.32×10^{-3} M) and KCl (5.6×10^{-2} M) in a volume of 0.05 mL were made into perfusion stream via a three-way stopcock, respectively. 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride (McN-A-343, 10^{-4} M), veratridine (10^{-4} M), 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate (Bay-K-8644, 10^{-5} M) and cyclopiazonic acid (10-5 M) were also perfused for 4 minutes, respectively. In the preliminary experiments, it was found that upon administration of the above drugs, the secretory responses to Ang II, ACh, KCl, McN-A-343, veratridine, Bay-K-8644, and cyclopiazonic acid returned to preinjection level in about 4 minutes, but the responses to DMPP in 8 minutes.

4. Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 minutes 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 minutes. 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 PD 123319 on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing PD 123319 for 90 minutes, 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 PD 123319, 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.

5. Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre[26] without the intermediate purification alumina for the reasons de-

scribed earlier[25] 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 (NE, base) equivalents.

6. Statistical analysis

The statistical difference between the control and pretreated groups was determined by the Student's t-test and analysis of variance test. 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. The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray.[27]

7. Drugs and their sources

The following drugs were used: PD 123319, CGP 42112, cyclopiazonic acid, acetylcholine chloride, DMPP, norepinephrine bitartrate, methyl-1, BAY-K-8644, veratridine hydrochloride, Ang II acetate salt (Sigma Chemical Co., St. Louis, MO, USA), and McN-A-343 (RBI, Natick, MA, USA). 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 are expressed in terms of molar base.

Results

1. Influence of PD 123319 on the CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the perfused rat adrenal glands

We determined the effects of PD 123319 on the CA secretion from the isolated perfused model of rat adrenal

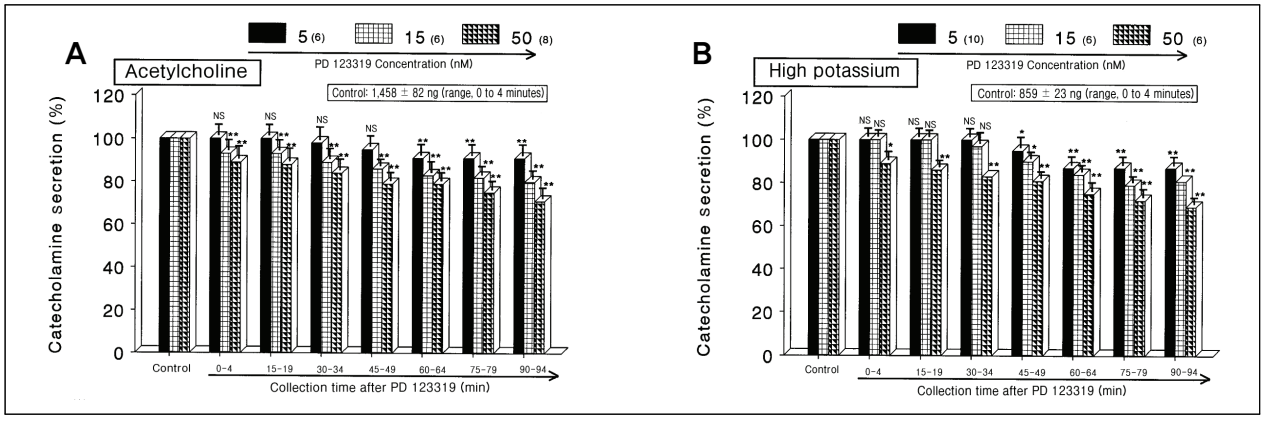

Fig. 1. Dose-dependent effects of PD 123319 on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh) (A) and high potassium (B) from the perfused rat adrenal medullas. The CA secretion by a single injection of ACh (5.32 mM) or high K^+ (56 mM) in a volume of 0.05 mL was evoked at 15 intervals during loading with 5, 15, and 50 nM of PD 123319 for 90 minutes as indicated at an arrow mark, respectively. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean. 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 PD 123319. ACh- and high K^+-induced perfusates were collected for 4 minutes, respectively. NS, statistically not significant. *$p < 0.05$, **$p < 0.01$.

medulla stimulated by some choligergic agonists and high potassium. When the rat adrenal medullae were perfused with PD 123319 for 90 minutes, the CA secretion evoked by these secretagogues was significantly diminished. After the perfusion with oxygenated Krebs-bicarbonate solution for 1 hour, basal CA release from the perfused rat adrenal medulla was 20 ± 3 ng for 2 minutes (n = 12).

When ACh (5.32×10^{-3} M) in a volume of 0.05 mL was injected into the perfusion stream, the amount of CA secreted was 1,458 ± 82 ng for 4 minutes. However, in the presence of PD 123319 in the range of 5 to 50 nM for 90 minutes, ACh-evoked CA secretion was concentration- and time-dependently reduced by PD 123319. As shown in Fig. 1A, the treatment of PD 123319 inhibited ACh-evoked CA releasing responses by maximally 71% of the corresponding control release. Also, high K^+ directly depolarizes the cell membranes without Na+ influx and results in Ca^{2+} influx through voltage-dependent Ca^{2+} channels and, consequently, catecholamine secretion.[28] High potassium markedly evoked the CA secretion from the rat adrenal medulla (859 ± 23 ng for 0 to 4 minutes). However, following the pretreatment with PD 123319 (range, 5 to 50 nM), high K^+ (5.6×10^{-2} M)-stimulated CA secretion was significantly inhibited by about 69% of the control at last period (range, 90 to 94 minutes) as shown in Fig. 1B. To further confirm the actions of PD 123319 on nicotinic acetylcholine receptors, using the perfused rat adrenal medulla, we examined the effects of PD 123319 on the DMPP-induced CA secretion. PD 123319 (15 μM) greatly reduced the DMPP-induced secretion from the adrenal medulla. 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 (1,358 ± 32 ng for 0 to 8 minutes). However, as shown in Fig. 2A, DMPP-evoked CA secretion during loading with PD 123319 was also maximally reduced to 71% of the control release at last period. It has been found that in voltage-clamped guinea-pig chromaffin cells, muscarine produced a transient intracellular Ca^{2+} concentration increase and CA release.[29] McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-receptor agonist, [30] perfused into an adrenal gland for 4 minutes also caused an increased CA secretion (569 ± 26 ng for 0 to 4 minutes). However, in the presence of PD 123319,

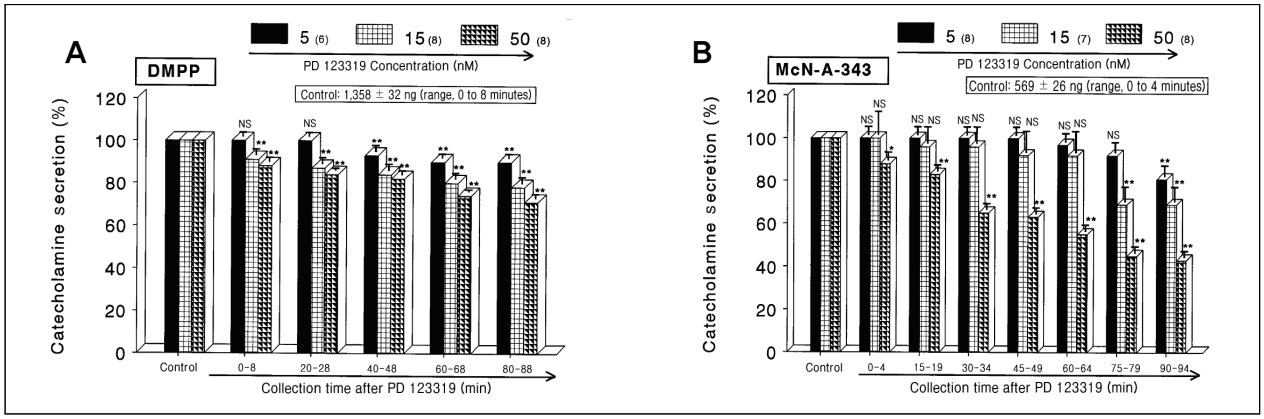

Fig. 2. DDose-dependent effects of PD 123319 on the catecholamines (CA) secretory responses evoked by 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (A) and McN-A-343 (B) from the perfused rat adrenal medullas. The CA secretion by perfusion of DMPP (100 μM) for 1 minute and 3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride (McN-A-343, 100 μM) for 4 minutes was induced at 20 and 15 intervals during loading with 5, 15, and 50 nM of PD 123319 for 90 minutes, respectively. DMPP- and McN-A-343-induced perfusates were collected for 8 and 4 minutes, respectively. NS, statistically not significant. *p < 0.05, **p < 0.01.

McN-A-343-evoked CA secretion was markedly depressed to about 43% of the corresponding control secretion (Fig. 2B).

2. Influence of PD 123319 on the CA secretion evoked by Bay-K-8644, cyclopiazonic acid, veratridine and Ang II from the perfused rat adrenal glands

It is well-known that Na^+ and Ca^{2+} influxes into chromaffin cells through the nicotinic ACh receptor-operated cation and voltage-sensitive Ca^{2+} channels, respectively, are essential for the ACh-evoked CA secretion from the adrenomedullary cells. Since Bay-K-8644 is known to be a calcium channel activator, which enhances basal Ca^{2+} uptake[31] and CA release,[32] it was of interest to examine the effect of PD 123319 on Bay-K-8644-evoked CA secretion from the perfused rat adrenal glands. Bay-K-8644 (10^{-5} M)-evoked CA secretion in the presence of PD 123319 (15 nM) was reduced to maximally 63% of the control at 75 to 94 minutes period as compared to the corresponding control release (525 ± 13 ng for 0 to 4 minutes) from 10 adrenal glands as shown in Fig. 3A.

It has been described that cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum.[33,34] The inhibitory action of PD 123319 on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 3B. In the presence of PD 123319 (15 μM) from 10 adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was also inhibited to 70% of the control response (512 ± 10 ng for 0 to 4 minutes) at 45 to 94 minutes period.

It has also been found 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.[35] To characterize the pharmacological action of PD 123319 on voltage-dependent Na^+ channels, the effect of PD 123319 on veratridine-evoked CA secretion was examined here. As shown in Fig. 4A, veratridine (10^{-4} M) greatly produced the CA secretion (1,395 ± 30 ng for 0 to 4 minutes). However, in the presence of PD 123319 (15 μM), veratridine-evoked CA secretion was greatly inhibited to 66%

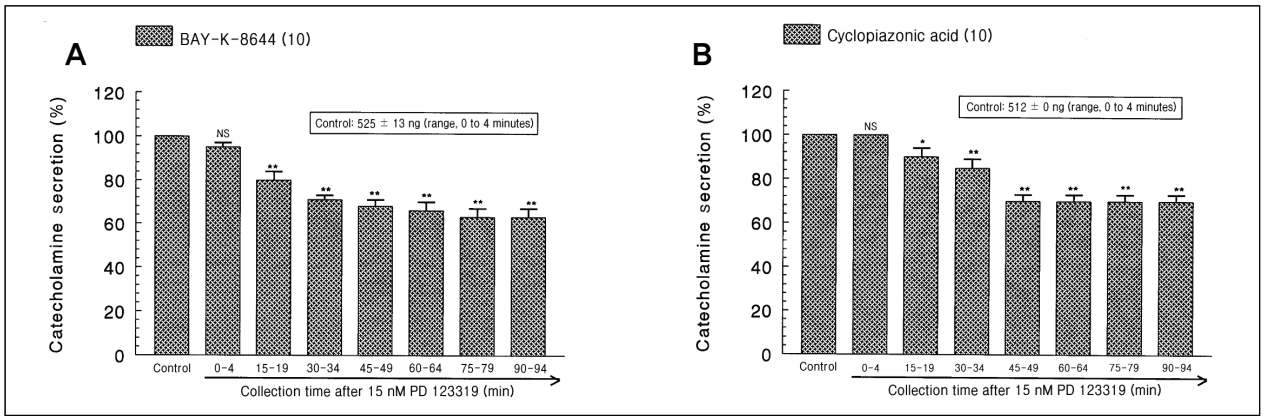

Fig. 3. Time-course effects of PD 123319 on the catecholamines (CA) release evoked by 4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate (Bay-K-8644) (A) and cyclopiazonic acid (B) from the perfused rat adrenal medullas. Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) were perfused into an adrenal vein for 4 minutes at 15-minute intervals during loading with PD 123319 (15 nM) for 90 minutes, respectively. NS, statistically not significant. *$p < 0.05$, **$p < 0.01$.

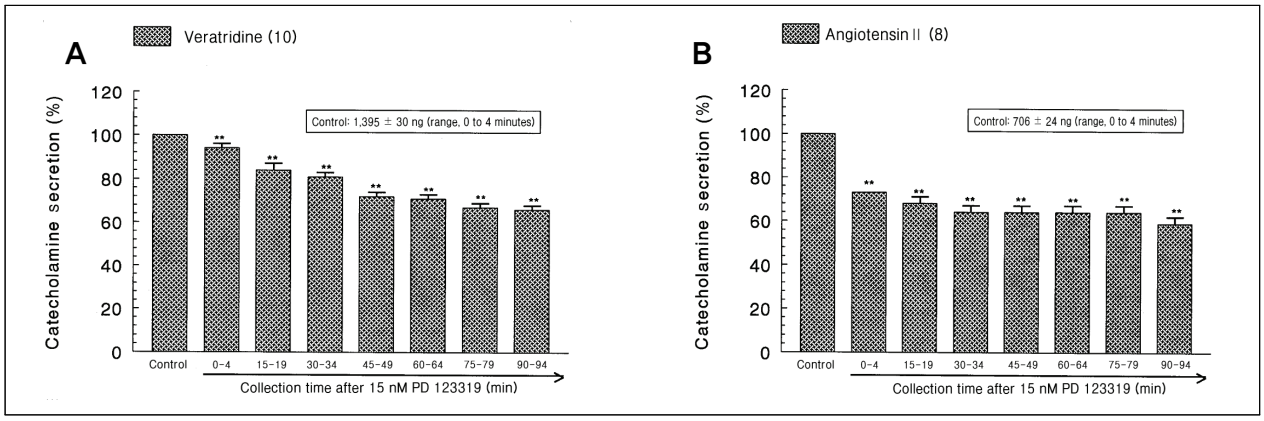

Fig. 4. Time-course effects of PD 123319 on the catecholamines (CA) release evoked by veratridine (A) and angiotensin II (B) from the perfused rat adrenal medullas. Veratridine (100 μM) for 4 minutes and angiotensin II (100 nM) for 1 minute were perfused into an adrenal vein and at 15-minute intervals during loading with PD 123319 (15 nM) for 90 minutes, respectively. **p < 0.01.

of the corresponding control release.

Since it has been demonstrated that AT_2 stimulation induces CA secretion in cultured porcine chromaffin cells,[21] but Hano et al.[11] have suggested that Ang II increase epinephrine release from the adrenal medulla via the AT_1 receptors, it was likely interesting to examine the effect of Ang II on the CA rease. Ang II (10^{-7} M) significantly evoked the CA secretory response (706 ± 24 ng for 0 to 4 minutes) whereas, in the presence of PD 123319 (15 μM), Ang II (100 nM)-evoked CA secretion was greatly inhibited to 59% of the corresponding control release (Fig. 4B).

3. Influence of PD 123319 plus CGP 42112 on the CA secretion evoked by ACh, excess K^+, DMPP and Ang II from the perfused rat adrenal glands

As shown in Figs. 1, 2, PD 123319 inhibited the CA secretory response evoked by cholinergic stimulation as ell as by direct membrane depolarization in the perfused rat adrenal gland. It has also been demonstrated that AT_2 receptpr agonist (CGP 42112) reduces both tyrosine hydroxylase (TH)-enzyme activity and TH-synthesis in cultured porcine adrenal medullary cells and that these inhibitory effects could be mediated by decrease of cGMP production. Therefore, in order to investigate the influence of CGP 42112 on PD 123319-induced inhibitory effects, it was likely of interest to examine effect of PD 123319 plus CGP 42112 on the CA secretion evoked by ACh, high K^+, and DMPP from the perfused rat adrenal glands. In the present study here, CGP 42112 (15 nM) itself did not affect basal CA output from perfused rat adrenal glands (data not shown). In subsequent experiments, PD 123319 (15 nM) plus CGP 42112 (15 nM) were simultaneously loaded into the rat adrenal medulla for 90 minutes immediately after establishment of control responses to cholinergic receptor-stimulation as well as direct membrane-depolarization. ACh (5.32 mM)-evoked CA release before perfusion with PD 123319 plus CGP 42112 was 1,372 ± 46 ng (range, 0 to 4 minutes). However, in the simultaneous presence of PD 123319 (15 nM) plus CGP 42112 (15 nM) for 90 minutes, it was more attenuated to 69% of the control release (Fig. 5A). High K^+ (56 mM)-evoked CA release under the coexistence of PD 123319 (15 nM) plus CGP 42112 (15 nM) was also reduced to 71% of the corresponding control secretion (854 ± 22 ng for 0 to 4 minutes), as shown in Fig. 5B. DMPP (10^{-4} M) perfused into the adrenal gland produced great CA secretion (1,088 ± 32 ng for 0 to 8

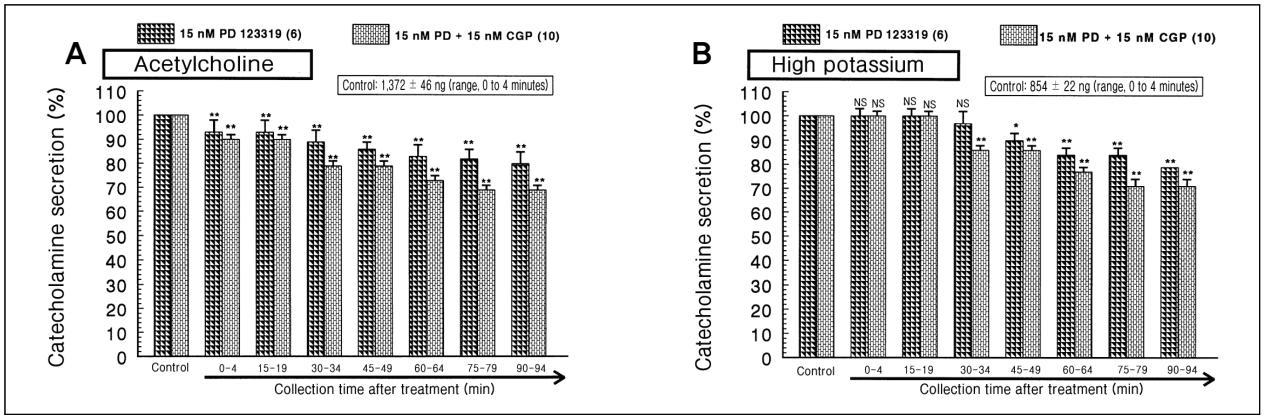

Fig. 5. Influence of PD 123319 (or PD) plus CGP 42112 (CGP) on the catecholamines (CA) secretory responses evoked by acetylcholine (A) and high potassium (B) from the perfused rat adrenal medulla. The CA secretion by a single injection of acetylcholine (Ach) (5.32 mM) or high K^+ (56 mM) in a volume of 0.05 mL was evoked at 15 intervals during simultaneous loading with PD (15 nM) plus CGP (15 nM) for 90 minutes. Statistical difference was obtained by comparing the corresponding control (control) with group of PD 123319-treated alone or group treated with PD + CGP. NS, statistically not significant. *p < 0.05, **p < 0.01.

minutes) prior to loading with PD 123319 plus CGP 42112, but following perfusion with PD 123319 plus CGP 42112 it was markedly diminished to 51% of the corresponding control release (Fig. 6A). Moreover, in the presence of PD 123319 plus CGP 42112, Ang II-evoked CA secretory responses was also more potently inhibited by 42% of the control secretion (660 ± 21 ng for 0 to 4 minutes), as shown in Fig. 6B.

4. Influence of PD 123319 plus CGP 42112 on the CA secretion evoked by Bay-K-8644 and veratridine from the perfused rat adrenal glands

In the simultaneous presence of PD 123319 plus CGP 42112, the secretory responses evoked by Bay-K-8644 (10^{-5} M) given into the adrenal gland for 4 minutes were greatly depressed to 42% of the corresponding control responses (596 ± 15 ng for 4 minutes) (Fig. 7A). The CA secretion

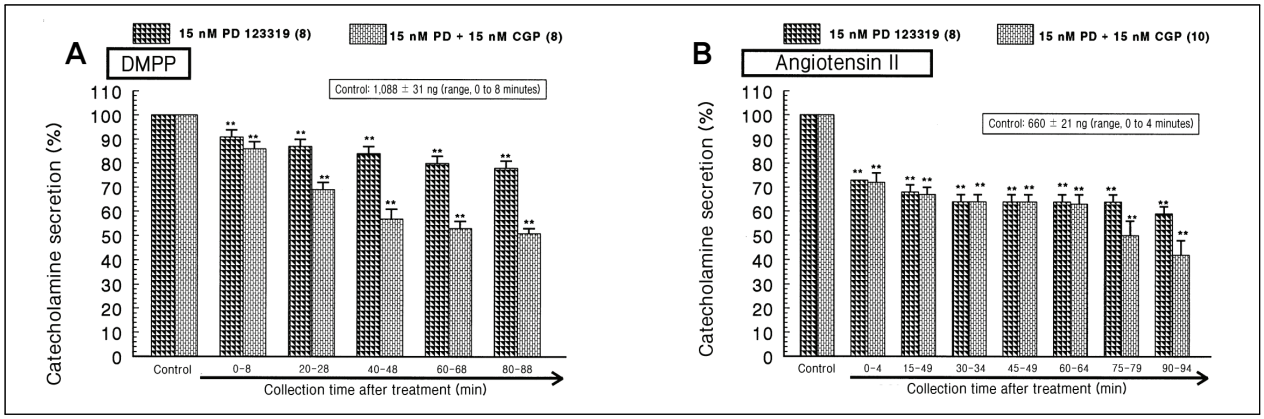

Fig. 6. Effects of PD 123319 plus CGP 42112 on the catecholamines (CA) secretory responses evoked by 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (A) and angiotensin II (B) from the perfused rat adrenal medulla. The CA secretion by perfusion of DMPP (100 μM) and angiotensin II for 1 minute was induced at 20- and 15-minute intervals during simultaneous loading with PD (15 nM) plus CGP (15 nM) for 90 minutes, respectively. **p < 0.01.

evoked by veratridine (10^{-4} M), an activator of Na^+ channels, was greatly elevated to 1,351 ± 24 ng for 4 minutes before loading of PD 123319 plus CGP 42112. However, in the simultaneous presence of PD 123319 (15 nM) plus CGP 42112 (15 nM), it was significantly inhibited to 59% of the corresponding control secretion, as shown in Fig. 7B.

Discussion

In this study, PD 123319 inhibited the CA secretory responses evoked by ACh, DMPP and McN-A-343 as well as by high K^+ and Ang II from the perfused rat adrenal medulla in a concentration-dependent manner (Figs. 1, 2, 4). It also did a time-dependently reduce the CA secretion evoked by Bay-K-8644, veratridine, and cyclopiazonic acid (Figs. 3, 4). These results strongly suggest that PD 123319 can inhibit the CA secretion evoked by cholinergic (both nicotininc and muscarinic) receptor as well as by Ang II receptor stimulation from the rat adrenal medulla. This inhibitory effect of PD 123319 seems to be mediated by blocking both receptor-operated cation channels and the voltage-sensitive Ca^{2+} or Na^+ channels as well as by inhibiting the release of Ca^{2+} from cytoplasmic store through the blockade of Ang II receptors located on the presynatic membrane of the rat adrenomedullary chromaffin cells, which are also relevant to adrenal nicotinic receptor blockade.

We found that perfusion of PD 123319 into the adrenal gland resulted in a significant decrease in the CA secretory responses evoked by cholinergic stimulation as well as by direct membrane-depolarization in time- and dose-dependent fashion while PD 123319 itself did not affect the basal CA secretion. We also demonstrated that PD 123319, a selective antagonist to AT_2 receptors, a time-dependently diminished Ang II-evoked CA release in the perfused rat adrenal gland. In support of this idea, it has been well established that PD 123319 expresses highly selective and specific affinity to AT_2 receptors in various tissues, including the adrenal medulla.[1,36] Furthermore, PD 123319 is widely accepted to be a selective nonpeptide AT_2 receptor antagonist.[37] For example, in the rat portal vein, which contains both AT_1 and AT_2 receptors, Ang II-induced contractions were partially inhibited by selective AT_2 receptor blockade with PD 123319.[38] In the adrenal gland, AT_2 re-

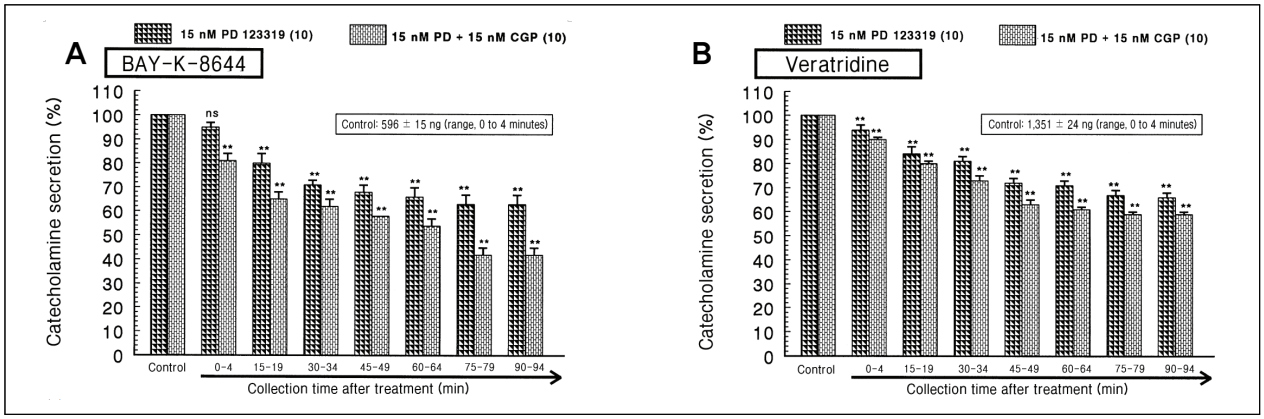

Fig. 7. Effects of PD 123319 plus CGP 42112 on the catecholamines (CA) secretory responses evoked by 4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate (Bay-K-8644) (A) and veratridine (B) from the perfused rat adrenal medulla. Bay-K-8644 (10 μM) and veratridine (100 μM) were perfused into an adrenal vein for 4 minutes at 15-minute intervals during simultaneous loading with PD (15 nM) plus CGP (15 nM) for 90 minutes. NS, not statistically significant. **p < 0.01.

ceptors have been implicated in cortisol secretion in response to Ang II in bovine adrenal fasciculata cells *in vitro* as PD 123319 significantly diminished the secretion of cortisol induced by Ang II.[39] Therefore, it suggests that the PD 123319-induced inhibitory effect on the CA release may be mediated with AT_2 receptor blockade. This observation is compatible with previous result indicating that PD 123319 inhibits Ang II-evoked CA release from rat adrenal medullary fragments[19,20] as well as from the adrenal gland of the anesthetized dogs.[23] These *in vitro* and *in vivo* results are in accordance with this hypothesis that the CA secretory effect evoked by Ang II as well as cholinergic receptors stimulation involves, at least in part, activation of AT_2 receptors localized on the rat adrenal medullary chromaffin cells.

It has also been found that CGP 42112 affects AT_2 receptors in tissues with a highly heterogeneous distribution of receptor subtypes by virtue of its high affinity, high selectivity, low nonspecific binding, and high stability.[40,41] CGP 42112 is considered to be a selective AT_2 agonist.[37] However, unlike PD 123319, the pharmacological activity of CGP 42112 is likely to vary depending on tissues and species studied. For example, CGP 42112 has been claimed to act as a full AT_2 agonist in intact PC12W cells[42] and in the rat adrenal medullary fragments *in vitro*.[19] However, in this work, the findings that simultaneous perfusion of PD 123319 plus CGP 42112 more strongly inhibited the CA release evoked by ACh, high K+, DMPP, veratridine and Ang II are not consistent with the results acting as a full AT_2 agonist. By contrast, CGP 42112 was found to behave as a selective AT_2 antagonist in cultured rat cardiac myocytes[43] as well as in the duck adrenal gland and subfornical organ *in vitro*.[44] In this study, CGP 42112, by itself, did not affect basal CA output at a dose used in the experiment. It has been previously shown that Ang II markedly increased the basal CA secretion in a dose-dependent manner within the dose range similar to that used for CGP 42112.[12] The increase in adrenal CA output in response to Ang II was inhibited by about 80% following the largest dose of PD 123319, and CGP 42112 significantly attenuated the CA response to Ang II by about 70%.[23] Furthermore, both PD 123319 and CGP 42112 inhibited the increase in adrenal CA secretion induced by local administration of Ang II.[23] In isolated rat hearts, CGP 42112A has been shown to suppresse nerve stimulation-induced NE overflow in the same way as the combination of Ang II and losartan, and this suppression was abolished by PD 123319.[45] Moreover, it has been shown that the binding potency of CGP 42112 at AT_2 receptors is significantly greater than that of Ang II.[41] Therefore, the absence of any positive effect of CGP 42112 on the basal CA secretion is compatible with the view that CGP 42112 is devoid of any agonist activity in the canine adrenal medulla *in vivo*.[23] However, the absence of any agonist activity on AT_2 receptors is sharply contrasted with the recent observation in rat adrenal medullary fragments in which basal CA release increased significantly in response to CGP 42112 in a concentration-dependent manner within the range from 0.001 to 1.0 mM.[19] The reason for this discrepancy is at present unclear. It may be accounted for by the different experimental conditions (*in vivo* vs. *in vitro* [perfused model]) or the different animal species (dog vs. rat) employed in those studies. Indeed, chromaffin cells in adrenal medullary fragments *in vitro* may differ from those in adrenal medulla *in vivo* in their reactivity in response to Ang II. For example, chromaffin cells PD 123319, had to be incubated with Ang II for 90 minutes to obtain an appropriate CA response,[19] while adrenal medulla tissues studied *in vivo* are affected when exposed to Ang II for only 1 minute at an infusion rate

of 0.5 mL/min[12,23] and also the perfused rat adrenal gland increased the CA release when exposed to Ang II for only 1 minute at an perfusion rate of 0.31 mL/min.[46] Furthermore, the distinctive reactivity of such tissues may differ among species. In the present stud, it has been demonstrated that adrenal CA release evoked by Ang II as well as cholinergic stimulation and direct membrane depolarization is rather potently inhibited in the coexistance of PD 123319 and CGP 42112. Takekoshi et al.[22] have shown that CGP 42112 (AT_2 agonist) reduces both TH-enzyme activity and TH-synthesis biosynthesis in cultured porcine adrenal medullary cells and that these inhibitory effects could be mediated by decrease of cGMP production. Because CGP 42112 is highly selective to AT_2 receptors with low nonspecific binding,[40,41] it is plausible that the CGP 42112-induced inhibition of the CA release is primarily attributable to specific blockade of AT_2 receptors in the adrenal medulla. Based on the results obtained from studying the nonpeptidic AT_2 antagonist PD 123319, these results support the hypothesis that AT_2 receptors are functionally involved in local regulation of CA secretion in the perfused rat adrenal medulla.

In contrast, it has been reported that, in cultured porcine chromaffin cells, AT_2 stimulation induces CA secretion by mobilizing Ca^2 through voltage-dependent Ca^{2+} channels without affecting intracellular pools and that these effects could be mediated by a decrease in cGMP production.[21] Worck et al.[47] have also speculated that Ang II through binding to both receptor subtypes (both AT_1 and AT_2) facilitates the sympathoadrenal reflex response by actions at several anatomical levels of the neural pathways involved in the sympathoadrenal reflex response elicited during insulin-induced hypoglycemia in conscious chronically instrumented rats. In light of these results, the present findings seem to be disagreement with those results that adrenal CA secretion is mediated through AT_2 receptors. On the other hand, Armando et al.[48] have demonstrated that both adrenomedullary AT_1 and AT_2 receptor types maintain and promote the adrenomedullary CA synthesis and the transcriptional regulation of TH in rats. Instead of opposing effects, these results indicate a complex synergistic regulation between the AT_1 and AT_2 receptor types.

The nicotinic receptor is a neurotransmitter-gated cation-conducting ion channel that is opened by binding of agonists such as ACh and DMPP.[49] The opening of this channel triggers Ca^{2+} uptake and secretion of CA from chromaffin cells.[35] To determine if the inhibition of DMPP-stimulated secretion by AT_2 antagonist was due to an effect on the activity of the nicotinic receptor, the effect of PD 123319, on DMPP-stimulated CA secretion was examined. As shown in Fig. 2A, treatment with PD 123319 greatly inhibited DMPP-evoked CA secretion, reducing by 71% of the corresponding control release. It seems to be plausible that PD 123319 can activate a signal transduction pathway that changes the activity of both nicotinic receptors and voltage-sensitive Na^+ channels.

In the present study, PD 123319 inhibited the CA secretory responses evoked by high potassium, a direct membrane depolarizer, as well as by Bay-K-8644, an activator of voltage-dependent L-type Ca^{2+} channels, which facilitates the influx of Ca^{2+} into the chromaffin cells. This findings that AT_1-selective antagonist inhibited the CA secretion evoked by Bay-K-8644 was surprising, as Takekoshi et al.[21] have reported that removal of external Ca^2 significantly suppressed either Ang II plus CV-11974 (AT_1 antagonist, 100 nM; which simulates specific AT_2 stimulation) or CGP 42112 (AT_2 agonist)-induced CA secretion in cultured porcine adrenomedullary chromaffin cells. It is unclear how the blockade of AT_2 receptors results in the inhibition of secretion seen in these cells. The

simplest explanation is that the decrease in Ca^{2+} uptake by PD 123319 is responsible for the inhibition of the CA secretion. However, such an explanation is complicated because of the complex relationship between the CA secretion and intraceIlular free Ca^{2+} levels. Both the intracellular location of the Ca^{2+} level increase[50,51] and the magnitude of the Ca^{2+} level increase[52] can affect the relationship between intracellular free Ca^{2+} levels and secretion. Holz et al.[52] have reported that when Ca^{2+} uptake is large, changes in Ca^{2+} uptake resulted in less than proportional changes in CA secretion. Consequently, although the decrease in Ca^{2+} uptake (influx) into the adrenal chromaffin cells may explain the decrease by PD 123319 in CA secretion, it is still unclear whether this is only or even most important factor contributing to the inhibition of CA secretion by the AT_2 antagonist. However, in view of these results, it seems that the voltage-sensitive Ca^{2+} channels located on the rat chromaffin cell membrane could be the target site for PD 123319 mediated inhibition of CA secretion.

In this study, PD 123319 also inhibited the CA secretory responses evoked by cyclopiazonic acid, which is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum.[33,34] Therefore, it is thought that PD 123319-induced inhibitory effect on the CA secretion evoked by ACh or McN-A-343 may be associated with the mobilization of intracellular Ca^{2+} in the rat chromaffin cells. This result suggests that the blockade of AT_2 receptors can inhibit the Ca^{2+} release from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the CA secretion. In the present work, PD 123319 also time- and concentration-dependently inhibited the CA secretion evoked by McN-A-343, a selective muscarinic M_1-agonist. This finding suggests new other concept that AT_2 receptors are involved in regulating the CA secretory process induced by activation of muscarinic M_1-receptors in the rat adrenal medulla. In supporting this concept, it has been reported that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increased the subsequent Ca^{2+} release from those storage sites and thereby increase of Ca^{2+}-dependent K^+-current.[53] 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.[50,54] However, in the present study, it is not clear whether PD 123319-induced inhibitory effect on Ca^{2+} movement from intracellular pools is mediated to their direct effect on the PI response or an indirect effect as a result of AT_2 receptor blockade by PD 123319. Based on previous results, the finding of the present study suggests that AT_2 receptor blockade-induced inhibition by PD 123319 may be involved in regulating CA secretion evoked by muscarinic M_1-receptor stimulation in the rat adrenal medullary chromaffin cells.

Collectively, these results show that PD 123319 inhibits the CA secretion evoked by cholinergic receptor as well as by Ang II receptor stimulation from the perfused rat adrenal medulla. PD 123319 seems to possess antagonist activity at nicotinic receptors of the rat adrenal medulla. It is also thought that PD 123319-induced inhibitory effect is exerted by blocking the influx of both Na^+ and Ca^{2+} through their channels into the rat adrenomedullary chromaffin cells as well as by inhibiting the Ca^{2+} release from its cytoplasmic calcium store, which is likely to be relevant to AT_2 receptor blockade. Based on these findings, it is thought that PD 123319 has different activity from previously known AT_2 antagonist activity in the perfused

adrenal medulla, and that AT_2 receptors may be involved in regulating the rat adrenomedullary CA secretion.

Summary

연구배경: 본 연구의 목적은 흰쥐 부신의 적출관류모델에서 선택성 AT_2수용체 차단제인 PD 123319가 카테콜아민 유리에 영향을 미치는지를 검색하는데 있다.

방법: Wakade방법을 약간 변형하여 흰쥐에서 부신을 적출하여 leucite chamber에 고정한 후 Krebs-bicarbonate 용액으로 관류하면서 약물을 처치한 후 관류액을 채집하여 형광분도기를 이용하여 카테콜아민 함량을 측정하였다.

결과: PD 123319 (범위, 5-50 μM)을 부신정맥 내로 90분간 관류 시 acetylcholine (ACh), 고칼륨, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), 및 McN-A-343에 의한 catecholamines (CA) 분비반응을 비교적 용량 및 시간 의존적으로 유의하게 억제하였다. 또한, 90분 동안 PD 123319 (15 μM) 존재하에서, L형 칼슘통로 활성화제인 Bay-K-8644, 세포질의 내형질세망막에서 Ca^{2+}-ATPase 억제제인 cyclopiazonic acid, 선택성 나트륨통로 활성화제인 veratridine 및 angiotensin II (Ang II)에 의한 CA 분비반응이 뚜렷이 억제되었다. PD 123319 자체는 기초 CA 분비량에 영향을 미치지 않았다. 그러나 PD 123319와 CGP 42112 공존하에서, ACh, 고칼륨, DMPP, Bay-K-8644, veratridine 및 Ang II에 의한 CA 분비반응은 PD 123319 단독처치 시에 비해 오히려 더욱 억제되었다.

결론: 이와 같은 연구결과를 종합하여 보면, 흰쥐 적출관류 부신수질에서 PD 123319는 Ang II뿐만 아니라 콜린성 수용체 흥분에 의한 CA 분비작용을 유의하게 억제함을 제시하였다. 이들 두 약물은 흰쥐 부신수질의 니코틴 수용체에서 길항작용을 나타내는 것으로 여겨진다. 또한 이러한 PD 123319의 CA 분비 억제작용은 흰쥐 적출 부신수질의 크롬친화세포 내로 전압의존성 Na^+ 및 Ca^{2+} 이온통로를 통한 세포 내로 이들 이온의 유입을 차단하고 세포질 내 칼슘저장고로부터 칼슘유리를 억제함으로써 나타나며, 이는 AT_2수용체 차단작용과 관련이 있는 것으로 생각된다. 이와 같은 연구결과로 보아, PD 123319는 흰쥐 관류 부신수질에서 이전에 알려진 AT_2수용체 길항제 활성과 다른 작용을 가지며, 또한 AT_2수용체가 흰쥐 부신수질의 CA 분비에 관여하는 것으로 생각된다.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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