Possible Involvement of Calcium-Calmodulin Pathways in the Positive Chronotropic Response to Angiotensin II on the Canine Cardiac Sympathetic Ganglia

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ABSTRACT—We investigated the ganglionic effects of angiotensin II (Ang II) and the signal transduction involved in the cardiac sympathetic ganglia by the direct administration of agents to the ganglia through the right subclavian artery and monitoring the heart rate as an indicator of the ganglionic function in pithed dogs. Ang II given i.a. caused increases in the heart rate, which was inhibited by the treatment with the AT_1-receptor antagonist forasartan, but not by the AT_2-receptor antagonist PD-123319. The stimulation by Ang II, but not by acetylcholine, was inhibited after treatment with an inhibitor of phospholipase C, U-73122; a cell-permeant modulator of the Ins(1,4,5)P_3 receptors, 2-aminoethoxydiphenyl borate; an intracellular calcium and calcium-associated protein kinase inhibitor, HA-1077; calmodulin (CaM) inhibitor, W-7; Ca^{2+}/CaM-dependent protein kinase II inhibitor, KN-93; a selective protein kinase C inhibitor, calphostin C; and Na^+-H^+ exchange inhibitor, dimethylamiloride. These results suggest that Ang II stimulates the ganglionic transmission at postsynaptic sites via the activation of AT_1 receptor coupled to either activation of phospholipase C, phosphoinositide hydrolysis and subsequent increase in intracellular Ca^{2+} and activation of protein kinase C and Ca^{2+}/CaM kinase II, although this ganglionic stimulation seems to involve, at least in part, the protein kinases-dependent increase of amiloride-sensitive Na^+ inflow.

Keywords: Angiotensin II, Sympathetic ganglion, Ca^{2+}/calmodulin-dependent protein kinase, Protein kinase C, Amiloride-sensitive Na^+ channel

A variety of peptides, angiotensin II (Ang II) and other neuropeptides, have been identified in the ganglia by immunofluorescence. They appear to be localized to particular cell bodies, nerve fibers or SIF cells; and they are released upon nerve stimulation (1). It has been proposed that angiotensinogen (Ao) mRNA is located in both sensory neural tissue as well as the sympathetic ganglia in the rat (2). Recently, we also demonstrated Ao mRNA to be located in the dog stellate and inferior cervical ganglia, while preganglionic stimulation to the isolated ganglia released endogenous Ang II to the incubation medium (3). Based on these findings, the angiotensin system may thus play an independently important role in ganglionic transmission in the sympathetic ganglia.

The octapeptide Ang II is well known to exert a wide range of physiological effects on the cardiovascular, renal and endocrine systems, as well as on the peripheral and central nervous systems (4). The peptide is a potent stimulant of the sympathetic ganglia by exerting its action directly on the postganglionic cells in the superior cervical (5) and the stellate ganglia (6, 7). Specific binding sites for Ang II have been shown to exist at multiple sites in the sympathetic nervous system, including the sympathetic stellate and superior cervical ganglia (8), the brain (9) and the adrenal medulla (10).

Recent studies with non-peptide Ang II antagonists have provided more definitive evidence for the existence of Ang II receptor subtypes. Ang II receptors have thus been divided into two subtypes, which are referred to as AT_1 and AT_2 (11).

As for the possible involvement of the signal transduction in Ang II responses, biochemical investigations have also been done. Based on the findings of such biochemical studies, Ang II has been proposed to stimulate phospholipase C through a G-protein and accelerate the production of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) as well as diacylglycerol and the mobilization of intracellular Ca^{2+} in the vascular smooth muscle cells (12). Ang II has also been reported to inhibit the adenylate cyclase-cAMP-dependent
protein kinase cascade through activation of the receptor-coupled G-protein G_i in cultured rat heart (13). The rat superior cervical ganglia also contain AT_1-type angiotensin receptors, which are probably G-protein linked, and their stimulation results in an increased inositol phospholipid metabolism (14). However, there is little information on the signal transduction pathways of the peptide in the sympathetic ganglia provided by functional changes in the innervated peripheral organ. Such in vivo experiments to elucidate the participation in the signal transduction pathways involved are clinically significant.

In the present study, the effect of Ang II on the ganglionic transmission and the possible involvement of signal transduction in this effect were investigated by utilizing changes of heart rate as indicator of in vivo canine sympathetic ganglionic function.

MATERIALS AND METHODS

Animal care

Mongrel adult dogs of either sex, provided by the Fukuoka City Animal Control Center, were kept for about 1 week in the Facilities for Experimental Animals of Fukuoka University and during that time they underwent a general medical examination. Each dog was housed in an individual cage located in a temperature-controlled room (22°C) that was humidified (50–60%) and maintained on a 12 h/12 h light/dark cycle (8 AM/8 PM). All animals had free access to water and were fed standard solid laboratory food (ED-1; Sanwa Chemicals, Inc., Tokyo), 300 g/animal per day. The experimental procedures were carried out under protocols approved by the Experimental Animal Care and Use Committee of Fukuoka University and The Japanese Pharmacological Society. The total number of animals used in this experiment was 68 dogs.

Surgical preparation

The dogs (6–12 kg) were anesthetized with 30 mg/kg of pentobarbital sodium administered intravenously (i.v.). The trachea was cannulated and the animals put on a Harvard respirator (24 strokes/min, 300 ml of tidal volume). To eliminate reflex changes in the autonomic activity, both vagus nerves were cut at the cervical region and both carotid arteries were ligated. The spinal cord was severed and then the brain was pithed with a steel rod through the atlantooccipital foramen, while the foramen was thereafter rapidly sealed with cork. The surgical procedures were performed principally according to the methods described by Fleisch et al. (15). The chest was opened by severing the upper three ribs, and the right internal mammary and vertebral arteries were ligated. The right brachial artery was cannulated with a polyethylene catheter connected to a stopcock. The catheter was advanced proximally until the tip was approximately at the junction of the right internal mammary and subclavian artery and was then secured tightly in place. The temperature in the area of the ganglia was maintained at between 37°C and 38°C by a heat lamp and a heating pad placed under the animal. The blood pressure was measured with a pressure transducer (MPU-0.5-290-11; Nihon Kohden Co., Tokyo) connected to a cannula placed in the right femoral artery. The heart rate as an indicator of the ganglionic function was recorded continuously by a cardiographograph (RT-45, Nihon Kohden) triggered by the femoral arterial pulse wave. The output signals from the pressure transducer and the cardiographograph were both recorded on a polygraph (RBL-45, Nihon Kohden). Because the blood pressure decreased after the spinal cord was severed, the pressure was thus maintained at about 80 mmHg during the experiment by infusing dextran (6% in saline containing 5% glucose) into the femoral vein, as previously described (15). All agents were rapidly injected intraarterially (i.a.) at a volume of 0.1 to 0.2 ml through a catheter inserted in the subclavian artery. The agents administered via the subclavian artery reached the cardiac sympathetic ganglia and affected the peripheral functions through the sympathetic system (15–17).

Drug administration

The drugs to be systemically administered were injected through a catheter inserted in the right femoral vein. As postganglionic stimulants, acetylcholine (ACh) and Ang II were used in the present study. For dose-response studies with ACh and Ang II, the drugs were given in increasing doses after a complete recovery from the preceding effect in the respective groups of animals. To prevent tachyphylaxis from developing with the successive administration of Ang II, a 10-min interval was thus allowed between each administration of Ang II, according to our previously reported data (18, 19). The interval between two successive dose-response runs for Ang II and ACh were 60 min. As for the drug pretreatment, forasartan, PD-123319, U-73122, 2-APB, W-7, KN-93, calphostin C, HA-1077 and dimethylamiloride were administered i.a. in order to directly reach the ganglia. According to our previous report (20) in which 3 min after the administration of the membrane permeable antagonists were allowed to elapse before another dose-response to Ang II, 3 min after the administration of these antagonists in the present experiment, either Ang II or ACh were administered i.a.

Drugs

The drugs used in this study were acetylcholine chloride (Daichi Pharmaceutical, Tokyo); angiotensin II (Protein Research Foundation, Osaka); nifedipine, 5-(N,N-dimethyl)-amiloride (dimethylamiloride) and 2-aminoethoxydiphenyl borate (2-APB) (Sigma Chemical Co., St. Louis, MO, USA);
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N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7 hydrochloride) and N-[2-[[3-(4'-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxy-benzensulfonamide phosphate (KN-93) (Seikagaku Kogyo Co., Tokyo); calphostin C (LC Laboratories, New Boston St. Wobum, MA, USA); 9-(tetrahydro-2-furyl)-9H-purin-6-amine (SQ-22536) and 1-[6-[[17β-3-methoxyestradiol-1,3,5(10)-tri-en-17-yl]amino]hexyl]-1H-pyrrrole-2,5-dione (U-73122) (Research Biochemicals International, Natick, MA, USA). 5-[(3,5-Dibutyl-1H-1,2,4-triazol-1-yl)methyl]-2-[2-(1H-tetrazol-5-ylphenyl)]pyridine (forasartan), 1-[[4-(dimethylamino)-3-methylphenyl]-methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]-pyridine-6-carboxylic acid, ditri-fluoroacetate, monohydrate (PD-123319) and 1-5-(isoquinolinesulfonyl)-homo-piperazine (HA-1077) were generous gifts from Searle (Skokie, IL, USA), Parke-Davis (Ann Arbor, MI, USA) and Asahi Chemical Industry (Tokyo), respectively.

Because of the poor solubility of calphostin C and nifedipine in water, both 0.02 and 10 mg/ml stock solutions of the drugs were prepared with absolute ethanol, stored in a freezer and at the time of administration, an aliquot of the stock solutions was thus diluted with distilled water. The maximum concentration of ethanol used (10%) at a volume of 0.2 ml had no effect on the positive chronotropic effect of the ganglionic stimulants in the cardiac sympathetic ganglia. Forasartan was dissolved in 0.05 N NaOH and then adjusted to within a range of pH 7–8 with 0.5 N HCl. All other agents were dissolved in distilled water.

**Statistical analyses**

The values are the mean ± S.E.M. The values between more than two mean values of the dose-response run in the same animals were evaluated by the Dunnett test. Comparisons of the values before and after antagonists in the same animals were assessed statistically by an analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. Values of P<0.05 were considered to be significantly different.

**RESULTS**

**Effects of forasartan and PD-123319 on the positive chronotropic responses to Ang II**

Thirteen groups consisting of 4–7 dogs per group were used in this study. The values of the mean basal blood pressure and heart rate in all pithed animals before the treatments were 87 ± 6.3 mmHg and 147 ± 16 beats/min, respectively, (n = 120).

As shown in Fig. 1, Ang II (0.1, 0.2, 0.4 μg) given to the cardiac ganglia through the subclavian artery produced a dose-dependent increase in the heart rate with a latency of about 2 s. The peak effect was reached within 20 s and the response lasted for 2–4 min, depending on the dose. The dose-response run to Ang II was performed first. At 1, 2 and 3 h later, the dose-dependent effects of the peptide showed no alteration, thus indicating that the effects were quite reproducible (data not shown).

The dose-response to Ang II was unaffected by successive i.v. treatment with hexamethonium (10 mg/kg) and atropine (0.1 mg/kg), the rate increase induced by a maximal dose of Ang II, 0.4 μg, being unaltered from the control of 42 ± 8.3 to 46 ± 7.6 beats/min after the treatment.

Intraarterial administration of forasartan, a nonpeptide selective AT₁-receptor antagonist, 50, 100 and 200 μg, and PD-123319, a selective AT₂-receptor antagonist, 5 and 10 mg, did not cause any changes in the basal heart rate levels. As shown in Fig. 1, the i.a. administration of forasar-
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tan produced a dose-dependent marked inhibition of the postganglionic stimulatory response to Ang II. On the other hand, PD-123319 failed to affect the ganglionic stimulation by Ang II.

**Effects of N-ethylmaleimide, U-73122 and 2-APB on the positive chronotropic responses to Ang II and ACh**

N-Ethylmaleimide, a G-protein inhibitor, at doses of 2.5 – 20 μg dose-dependently inhibited the postganglionic stimulatory effects of Ang II. The effects of ACh were also similarly reduced by this agent (data not shown).

As shown in Fig. 2, U-73122, a phospholipase C inhibitor, at 0.1, 0.2 and 0.4 mg reduced the postganglionic stimulatory effect of Ang II but did not influence that of ACh.

As shown in Fig. 3, 2-APB, a cell-permeant modulator of the Ins(1,4,5)P₃ receptors, at 0.5, 1 and 2 mg inhibited the stimulatory effects of Ang II, but failed to reduce that of ACh.

**Effects of nifedipine and HA-1077 on the positive chronotropic responses to Ang II and ACh**

As shown in Fig. 4, nifedipine (50 and 100 μg, i.a.), voltage-gated Ca²⁺-channel antagonist, inhibited the ganglionic stimulatory response to Ang II and ACh.

As shown in Fig. 5, HA-1077, an intracellular Ca²⁺ and protein kinase inhibitor, at 10 – 80 μg dose-dependently inhibited the ganglionic stimulatory response to Ang II but failed to reduce the response to ACh.

**Effects of SQ-22536 and calphostin C on the positive chronotropic responses to Ang II and ACh**

SQ-22536, an adenylyl cyclase inhibitor, at 5 – 20 μg, failed to inhibit the rate increase elicited by Ang II and ACh (data not shown).

As shown in Fig. 6, calphostin C, a selective protein kinase C inhibitor, at doses of 0.16, 0.32 and 0.64 μg, i.a. reduced the postganglionic responses to Ang II, but did not influence those to ACh.
Effects of Ang II and KN-93 on the positive chronotropic responses to Ang II and ACh

As shown in Fig. 7, W-7 (0.5 and 1 mg, i.a.), a CaM antagonist, reduced the postganglionic stimulatory effect of Ang II in a dose-dependent fashion, without affecting the ganglionic stimulation induced by ACh.

As demonstrated in Fig. 8, KN-93 (25 and 50 μg, i.a.), a Ca²⁺/CaM-dependent protein kinase II inhibitor, reduced
the postganglionic stimulatory effect of Ang II at a high dose of 50 μg, but had no similar effect on the ACh-induced response.

Effect of dimethylamiloride on the positive chronotropic responses to Ang II and ACh

As seen in Fig. 9, dimethylamiloride (0.1, 0.2 and 0.4 mg, i.a.), a Na⁺-channel antagonist, reduced the postganglionic stimulatory effect of Ang II in a dose-dependent manner but had no similar effect on ACh.

DISCUSSION

In the present in vivo experiments, the agents administered through the catheter inserted into the artery are able to reach the cardiac sympathetic ganglia and affect the cardiac functions via the sympathetic system (15, 19, 20). In the same in vivo experiment as that employed in the
present study, exogenous ACh injected i.a. was able to stimulate the cardiac sympathetic ganglion by either nicotinic or muscarinic receptor activation (15, 16). Nicotinic receptors are ligand-gated ion channels, and their activation always causes a rapid increase in the cellular permeability to Na⁺ and Ca²⁺, depolarization and excitation (21). In contrast, muscarinic receptors belong to the class of so-called G-protein-coupled receptors and the responses to muscarinic agonists are not necessarily linked to changes in ion permeability (21). In the present investigation, the ganglionic stimulatory response to Ang II was not inhibited by the combined treatment with hexamethonium and atropine that strongly diminished both the frequency-dependent response to preganglionic stimulation and the dose-dependent response to ACh in spinal dogs (15, 16). This finding is fully compatible with the findings of previous reports in which the positive chronotropic response to Ang II injected i.a. toward the cardiac sympathetic ganglia in spinal cats and dogs was unaltered when the ganglionic transmission was blocked with hexamethonium and atropine (6, 18). Consequently, the ganglionic stimulatory effect of Ang II does not involve the cholinergic system in the ganglionic transmission.

In the pithed rat, losartan, an AT₁-receptor antagonist, but not PD-123177, an AT₂-receptor antagonist, inhibited the tachycardiac response to Ang II that is due to the stimulation of the cardiac sympathetic ganglia (22), thus suggesting that the Ang II receptor in the sympathetic ganglia most likely has an AT₁ subtype (23). This finding is also consistent with the findings of an in vitro functional study showing that the Ang II-induced depolarization of the rat superior cervical ganglion was blocked by losartan, but not by PD-123177 (24). In the present in vivo experiment, the ganglionic stimulatory response to Ang II in the dog cardiac sympathetic ganglia was also inhibited by forasartan, which is described as a non-peptide competitive AT₁-receptor antagonist (25), but not by PD-123319, an AT₂-receptor antagonist. Therefore, the Ang II-induced stimulation of the dog sympathetic ganglia is also mediated by the AT₁ subtype of the Ang II receptor.

A number of biochemical studies have demonstrated that Ang II receptors are coupled via a number of different G-proteins to a variety of signal transduction pathways, which include the stimulation of phospholipase C and the mobilization of intracellular calcium (26). In this study, ACh as well as Ang II-induced ganglionic stimulation was inhibited by N-ethylmaleimide, which causes the uncoupling of GTP-binding proteins from receptors by the same mechanism as IAP, islet-activating protein (27). However, U-73122 identified as the inhibitor of phospholipase C in neuronal cells (28), inhibited the ganglionic stimulatory response to Ang II, but not to ACh. Furthermore, the ganglionic stimulatory response to Ang II but not ACh was also reduced by the administration with 2-APB, a recently identified and selective cell-permeant modulator of the Ins(1,4,5)P₃ receptors, which inhibits the contraction of thoracic aorta isolated from rabbits induced by Ang II, but showed no effect on the contraction of potassium-depolarized muscle (29). Furthermore, ACh-induced muscarinic ganglionic stimulation is mediated by M₁-ACh receptors coupled to G-protein associated with guanylate cyclase and cyclic GMP pathways in the superior cervical ganglia of rabbits (30). The fact supports the present results that in contrast with the response to Ang II, ACh-induced ganglionic stimulation was not due to the activation of the signal transduction of phospholipase C – phosphoinositide breakdown pathways. Accordingly, the ACh and Ang II-induced ganglionic stimulation is mediated via the activating receptors coupled with G-protein, and the effect of Ang II thus involves phospholipase C activation and a stimulation of phosphoinositide hydrolysis.

In the present in vivo experiment, the treatment of the cardiac sympathetic ganglia with SQ-22536, adenosine cyclase inhibitor, did not affect the ganglionic stimulation induced by Ang II. In our previous report (20), the application of dibutyryl cyclic AMP, a membrane permeable cyclic nucleotides, to the dog cardiac sympathetic ganglia also did not affect the positive chronotropic response to Ang II. These findings thus indicate that the activation of cyclic nucleotides is not involved in the ganglionic response to Ang II. Intraarterial administration of nifedipine, a voltage-dependent Ca²⁺-channel antagonist, also inhibited the ganglionic stimulatory effect of Ang II, but the inhibitions by the antagonist at a subsequent high dose of 100 μg were limited to 50% and 41% inhibition from the controls. It has recently been demonstrated that HA-1077, an intracellular calcium-associated protein kinase inhibitor, potently inhibits protein kinases, such as cyclic nucleotide-depend-ent protein kinase and Ca²⁺/CaM-dependent myosin light chain kinase (31). Furthermore, the antagonistic effects of HA-1077 on protein kinase are also likely related to the blocking action of intracellular calcium (32). In this study, the ganglionic stimulatory responses to Ang II were inhibited in a dose-dependent manner by HA-1077, whereas those to ACh were unaffected. The above described findings thus imply that the ganglionic stimulatory response to Ang II involves not only the intracellular stored Ca²⁺ mobilization but also the extracellular Ca²⁺ influx. Besides, the ganglionic stimulation elicited by Ang II but not ACh was blocked by calphostin C, described as a potent and specific protein kinase C inhibitor (33). The fact is consistent with the finding that the AT₁-receptor-mediated reduction of neuronal delayed rectifier current by Ang II in cultured neurons which contributes to neuronal membrane depolarization is partially reduced by treatment with calphostin C (34).
W-7, a CaM inactivator (35), inhibited the ganglionic stimulating response to Ang II in a dose-dependent fashion, but not that to ACh, in this study. The ganglionic stimulatory effect of Ang II and the enhancement in the effect after repetitive preganglionic stimulation were also prevented in a dose-dependent manner by W-7 in our previous study (20). Furthermore, it has been demonstrated that based on a biochemical study, stimulation of AT₁ receptors on cultured neurons stimulates PI hydrolysis, increases in intracellular Ca²⁺ and increases activation of CaM kinase II (36). In the present study, a selective Ca²⁺/CaM-dependent protein kinase II inhibitor, KN-93 (37), also inhibited the ganglionic effects of Ang II, without affecting those of ACh. In line with current results, the AT₁-receptor-mediated reduction of neuronal delayed rectifier current by Ang II is partially blocked by either the CaM antagonist W-7 or the specific CaM kinase II inhibitor KN-93 (38). The findings indicate that, the ganglionic stimulatory response to Ang II may involve an intracellular second messenger, the Ca²⁺/CaM-dependent protein kinase II pathway, although the activities of Ca²⁺/CaM-dependent protein kinase II in the cardiac sympathetic ganglia after Ang II still remain to be determined.

Furthermore, Ca²⁺/CaM kinase II is a known modulator of ion channels and is activated by Ang II via AT₁-receptors in vascular smooth muscle cells (39). It has been demonstrated that Ang II, via AT₁ receptor, opens a non-selective Na⁺/Ca²⁺ channel and elicits neuronal depolarization (40). Based on electrophysiological studies, it has thus been proposed that the ganglionic stimulating effect of Ang II is due to an increase of the permeability of the postsynaptic membrane to Na⁺ (5). Ang II stimulates an amiloride-sensitive Na⁺/H⁺ exchange system in cultured vascular smooth muscle cells which is mediated in part by protein kinase C-dependent mechanism (41). In the present in vivo study, the ganglionic stimulant effect of Ang II, but not ACh, was clearly inhibited by dimethylamiloride. According to these facts, it is presumed that the signal transduction to the ganglionic stimulatory response to Ang II involves, in part, a Ca²⁺/CaM kinase II-operated Na⁺ influx.

Based on our findings, we therefore propose that the signal transduction mechanisms of the postganglionic stimulatory response to Ang II probably involve, in part, the activation of AT₁-receptor coupled to G-protein-associated phospholipase C with generation of IP₃ and subsequent intracellular Ca²⁺ mobilization and activation of protein kinase C and Ca²⁺/CaM kinase II to mediate the rapid actions of Ang II on neuronal membrane amiloride-sensitive Na⁺ influx.

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