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Natriuresis Induced by Intracerebroventricular Diazepam in Rabbits

Jeong-Tae Koh and Young-Johng Kook[1]

Departments of Pharmacology, Chonnam University Dental and [1]Medical Schools, Kwangju 501–190, Korea

The renal function is under regulatory influence of central nervous system (CNS), in which various neurotransmitter and neuromodulator systems take part. However, a possible role of central GABA-benzodiazepine system on the central regulation of renal function has not been explored. This study was undertaken to delineate the renal effects of diazepam. Diazepam, a benzodiazepine agonist, administered into a lateral ventricle (*icv*) of the rabbit brain in doses ranging from 10 to 100 μg/kg, elicited dose-related diuresis and natriuresis along with improved renal hemodynamics. However, when given intravenously, 100 μg/kg diazepam did not produce any significant changes in all parameters of renal function and systemic blood pressure. Diazepam, 100 μg/kg *icv*, transiently decreased the renal nerve activity (RNA), which recovered after 3 min. The plasma level of atrial natriuretic peptide (ANP) increased 7-fold, the peak coinciding with the natriuresis and diuresis. Muscimol, a GABAergic agonist, 1.0 μg/kg given *icv*, elicited marked antidiuresis and antinatriuresis, accompanied by decreases in systemic blood pressure and renal hemodynamics. When *icv* 0.3 μg/kg muscimol was given 3 min prior to 30 μg/kg of diazepam *icv*, urinary flow and Na excretion rates did not change significantly, while systemic hypotension was produced. These results indicate that *icv* diazepam may bring about natriuresis and diuresis by influencing the central regulation of renal function, and that the renal effects are related to the increased plasma ANP levels, not to the decreased renal nerve activity, and suggest that the effects may not be mediated by the activation of central GABAergic system.

Key Words: Diazepam, Renal function, Renal nerve activity, Atrial natriuretic peptide

INTRODUCTION

The central nervous system exerts integrative regulatory influence on the excretory function of kidney to maintain homeostasis of the body fluids, either through humoral agents or nerve pathways (DeWardener, 1973; Gottschalk, 1979; Rauch et al, 1990). Numerous neurotransmitter and neuromodulator systems in the brain have been shown to participate in central regulation of renal function, including adrenergic (Kook et al, 1985), dopaminergic (Kook et al, 1986, 1988a), tryptaminergic (Kook et al, 1988b; Kook et al, 1990), and histaminergic systems (Kook et al, 1987).

Diazepam, the prototype of benzodiazepine agents, has been known to possess potent pharmacodynamic actions on the CNS function, including antianxiety, anticonvulsion, sedation, muscle relaxation, and central modulation of cardiovascular function (William et al, 1989). It is also well established that its action mechanism is related to modulating the activation of GABAergic receptor-chloride channel complex. In the absence of GABA the benzodiazepine drugs do not open the channel, while they can increase the frequency of channel opening only in the presence of GABA (Twyman et al, 1989). In the brain, radioactive benzodiazepines were bound in cerebral cortex, amygdala, hippocampus, hypothalamus, cerebellum, etc, coinciding with the GABA-binding sites (Richards et al, 1987). Besides brain tissue, some benzodiazepines including diazepam were also bound to peripheral tissues such as kidney and heart, although the functions of the binding sites are not clearly elucidated yet (Braestrup & Nielsen, 1985; Kurumaji &

Corresponding to: Jeong-Tae Koh, Department of Pharmacology, Chonnam University Dental School, Hak-Dong 5, Kwangju 501-190, Korea. (Tel) 062-220-4230 (Fax) 062-232-6974

Toru, 1996). In the rat kidney, benzodiazepine receptors were concentrated in the thick ascending limb of the loop of Henle and in early distal tubule (Beaumont et al, 1984). Diazepam given intravenously elicited marked increases in fractional excretions of water, sodium and potassium in rats due to impairment of tubular reabsorption and to modification of the response of the collecting tubules to desmopressin (Monasterolo & Elias, 1993), with no hemodynamic changes, suggesting a direct effect on tubular transport mechanisms (Monasterolo et al, 1995).

In receptor-binding studies, [^{3}H]diazepam also binds to circumventricular organs including choroid flexus and ependyma, which are related to the central regulatory area of body fluid (Braestrup & Squires, 1977a, b). However, the involvement of benzodiazepine system on the central regulation of renal function has not been clearly established. This study was undertaken to observe the renal effect of *icv* diazepam and to elucidate its mechanism.

METHODS

Animal preparation and analyses of urine and plasma samples

Adult white rabbits of either sex, weighing 1.8~2.4 kg, were anesthetized with 1 g/kg urethane s.c., and the airway was kept free with a T-tube inserted in the trachea. 0.3% NaCl and 3% glucose solution containing 45 mg% of para-aminohippuric acid (PAH) and 250 mg% of creatinine (cr) was infused into an ear vein at a rate of 0.5 ml/min. Through a small midline incision close to the symphysis, both ureters were cannulated with polyethylene (PE) tubings for the collection of urine samples, and for the blood sampling a femoral artery was cannulated with a PE tubing, which was then kept patent with heparin-saline (400 U/ml). For intracerebroventricular (*icv*) administration of the agents, a lateral ventricle of the cerebrum was cannulated. A hole was drilled on the skull at a point 1.5 cm rostral to the occiput tubercle and 0.5 cm lateral to the midline, and a PE cannula of 1.5 mm O.D. was introduced obliquely until cerebrospinal fluid appeared in the cannula, and then it was plugged and kept in place by cementing to the bone. The volume administered did not exceed 0.15 ml. At the end of each experiment the location of the cannula tip was checked by dissection. When the urine flow rate had stabilized several hours after the infusion began, clearance experiment started. After two 10-min control clearance samples were collected, the agent was administered *icv*, and then two 10-min and three 20-min urine samples were collected. The blood samples were obtained at midpoint of each clearance period via the femoral artery cannula and immediately centrifuged to separate the plasma.

Quantitative analyses of creatinine were done by the method of Phillips (1944) and PAH by that of Smith et al (1945). Na and K concentrations were determined by flamephotometry, and the osmolality with osmometer.

Measurement of renal nerve activity

To measure renal nerve activity (RNA), the left kidney was exposed retroperitoneally, and a branch of left renal nerve was carefully isolated from the surrounding connective tissue, and suspended on a bipolar electrode, which fixed to a micromanipulator, in a pool of mineral oil. Multiunit renal nerve activity was monitored on an oscilloscope and an ink-writing recorder at the same time. In order to ascertain the validity of the nerve preparation, reflex decrease of nerve activity was confirmed by intravenous injection of 30 μg/kg phenylephrine (Peterson et al, 1983). RNA was quantified with an integrator amplifier coupled to a level window discriminator.

Measurement of plasma atrial natriuretic peptide

Plasma concentration of atrial natriuretic peptide was measured by radioimmunoassay technique as described by Kim et al (1989). The blood-sampling tubes contain a mixture of 2.7 mM ethylenediamine tetraacetate, 600 μM phenylmethylsulfonylfluoride, 0.2 KIU soybean trypsin inhibitor, and 0.54 TIU aprotinin per 1 ml of blood. 1.5 ml blood samples were placed in the tubes on ice and then were immediately centrifuged at 4°C, 3000 rpm for 10 min. The separated plasma were passed through Sep-Pak C18 cartridges (Waters) for extracting ANP, and then they were eluted with 4 ml of 0.1% trifluoroacetic acid (TFA) and 2 ml of 60% acetonitrile in 0.1% TFA. The eluent was dried with Speed-Vac® evaporator and was kept at −70°C until analyzed for ANP by radioimmunoassay.

Drugs

Diazepam and other drugs for assay were obtained from Sigma Chemicals Co. Muscimol was purchased from Research Biochemical Inc. Diazepam was dissolved in 100% ethanol as a stock solution, and then diluted with distilled water to contain 40% ethanol immediately before use. Other drugs administerd were diluted with saline.

Statistical analysis

Statistical significance was assessed either with Student's t-test or with ANOVA. If significant difference was detected with ANOVA, further analyses as required were performed to determine which of the groups differed from the appropriate controls. For multiple group comparison Bonferroni's modified t-test was applied.

RESULTS

Renal effects of icv diazepam

Diazepam given *icv* in doses less than 10 μg/kg did not produce any significant changes in renal function. With 30 μg (=0.1 moles) per kg, urine flow rate (Vol)

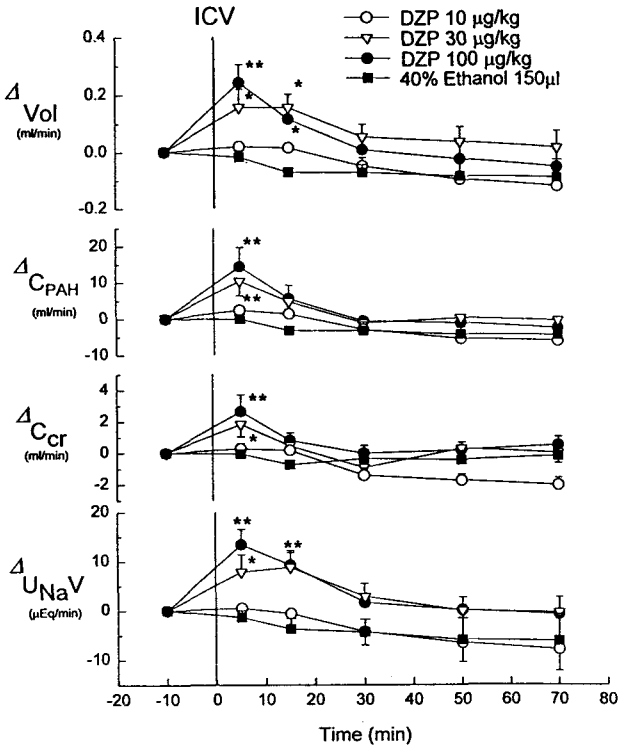

Fig. 1. Effects of *icv* diazepam (DZP) and 40% ethanol, the vehicle, on rabbit renal function. Mean changes from the control values with one S.E. are shown. Significant differences from the control values are marked with asterisks, *=p<0.05, **=p<0.01. Other legends are as in Table 1.

Table 1. Effects of *icv* diazepam, 100 μg/kg, on the rabbit renal function

	Control	0'~10'	10'~20'	20'~40'	40'~60'	60'~80'
Vol (ml/min)	0.269±0.057	0.509±0.085**@	0.382±0.052*@@	0.275±0.044	0.243±0.038	0.214±0.028
C_{PAH} (ml/min)	17.29±2.46	31.61±6.69**	22.84±4.31	16.62±2.62@	16.11±2.58	14.61±2.01
C_{cr} (ml/min)	7.50±0.86	10.12±1.75**	8.30±1.05@	7.47±1.00	7.67±0.88	7.97±1.22
FF (%)	44.58±3.72	33.54±2.31	38.28±2.77	45.68±3.30	49.57±4.24	54.21±4.80*
$U_{Na}V$ (μEq/min)	6.06±2.03	19.41±4.67**@	15.29±2.74**@@	7.66±1.24	6.10±1.72	5.24±1.44
FE_{Na} (%)	0.641±0.200	1.624±0.399**@	1.452±0.256**@@	0.816±0.090	0.681±0.182	0.607±0.167
U_KV (μEq/min)	3.24±0.53	5.00±0.67**@	4.30±0.50**@	3.55±0.51	3.32±0.57	3.55±0.58
C_{osm} (ml/min)	0.408±0.065	0.678±0.086**@	0.582±0.074**@@	0.467±0.065**@	0.439±0.060	0.423±0.056
T^CH_2O(ml/min)	0.140±0.020	0.168±0.052	0.199±0.044	0.193±0.031	0.197±0.034	0.210±0.035
MAP (mmHg)	98.7±6.2	95.3±4.4@	95.7±6.2@	95.7±6.6	93.0±8.2@	91.7±8.0*

Abbreviations: Vol, rate of urinary flow; C_{PAH} and Ccr, clearances of PAH and creatinine, resp.; FF, filtration, as calculated from $Ccr/C_{PAH}\times 100$; $U_{Na}V$ and U_KV, excretory rates of sodium and potassium, resp.: FE_{Na}, fractional excretory rate of filtered sodium, as $U_{Na}V/(P_{Na}\times Ccr)\times 100$; Cosm, clearance of osmotically active substances; T^CH_2O, reabsorption of osmotically free water; MAP, mean arterial pressure. The agent was given at 0 time. Significant differences from control values are marked with asterisks. *=p<0.05; **=p<0.01. Mean±S.E. from 6 experiments. Significant differences from administration of vehicle (40% ethanol *icv*, 150 μl) group are marked with @ and @@, @=p<0.05 and @@=p<0.01.

increased 1.5-fold over the control value for 20 min and returned to the control level. Na excretional rate ($U_{Na}V$) doubled with increasing renal plasma flow (C_{PAH}; RPF) and glomerular filtration rate (C_{cr}; GFR). Fractional excretory rate of filtered sodium (FE_{Na}) also doubled in the second 10-min period (Fig. 1). However, free water clearance (T^cH_2O) and systemic blood pressure (MAP) did not change significantly. With the dose tripled to 100 μg/kg *icv*, marked diuresis, natriuresis, and kaliuresis were observed. Urine flow rate doubled in the first 10-min period, and the diuresis lasted for 20 min. Na and K excretory rates also increased by 220% and 50% respectively, with increased RPF and GFR. Osmolar clearance increased, reflecting the increase in the excretion of sodium, but free water clearance did not change (Table 1). 40% ethanol, the vehicle for diazepam, given *icv*, did not produce any significant changes, as shown in Fig. 1.

Renal effects of intravenous diazepam

To ascertain that the renal effects of diazepam given into lateral ventricle are elicited through central mechanism, not by direct renal action of the agent possibly leaked into the systemic circulation from the *icv* site, the response to intravenous administration of diazepam was examined. Diazepam, 100 μg/kg given intravenously, did not elicit diuresis and natriuresis, and it decreased RPF and GFR by 30% and 24%, respectively, with decreasing the systemic blood pressure (Fig. 2). It is thus clear that the *icv* diazepam acts through central mechanism, not through direct action on kidney.

Changes of renal nerve activity

As shown in Fig. 3-A, 100 μg/kg diazepam transiently decreased the RNA for 1-2 min, and then the activity recovered (upper tracing). The lower tracing shows integrated RNA by full scale to reset mode. Fig. 3-B summarizes the results of 6 experiments each of 10, 30, and 100 μg/kg *icv* diazepam. The RNA dipped slightly immediately following the administration; however, no significant changes of RNA were noted.

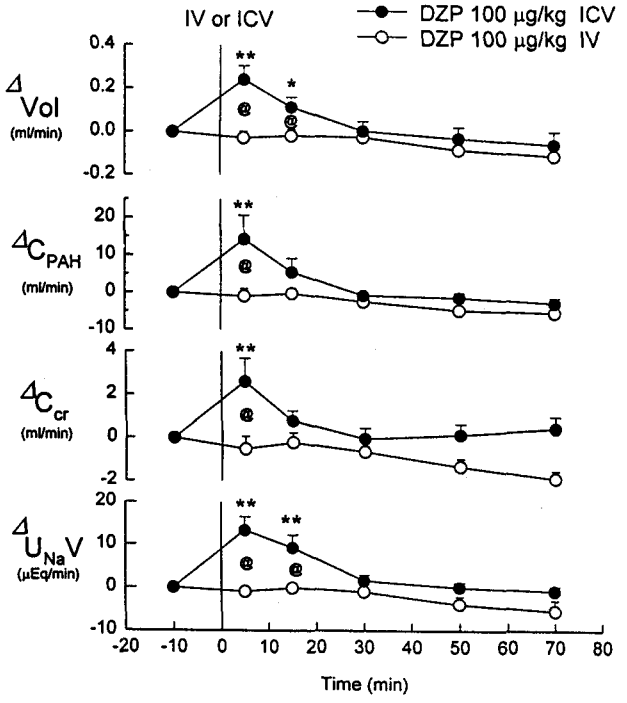

Fig. 2. Effects of 100 μg/kg diazepam, *icv* and *iv*, on rabbit renal function. Significant differences between both groups are marked with @, @=$p<0.05$. Other legends are as in Fig. 1.

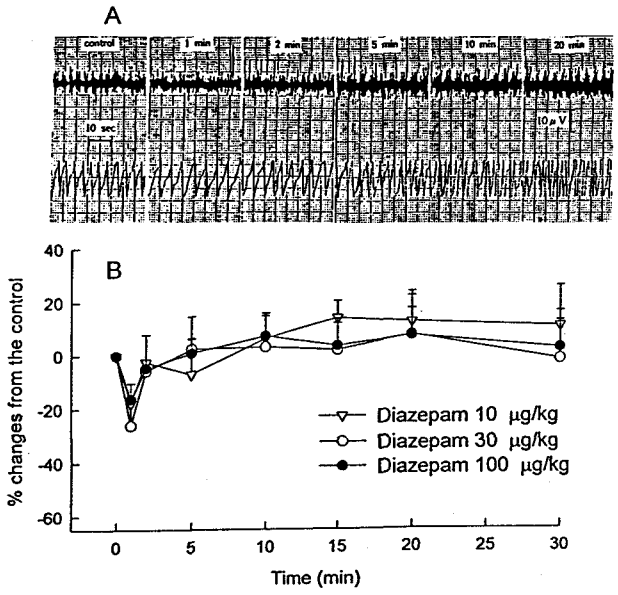

Fig. 3. Influence of 100 μg/kg diazepam *icv* on renal nerve activity. A, the upper tracing shows a renal nerve activity before and after administration, and the lower a integrated renal nerve activity by "full scale to reset" integration mode. B, changes of renal nerve activities by *icv* diazepam. Percent changes from the control level are plotted against time. Mean±S.E. from 6 experiments each are shown.

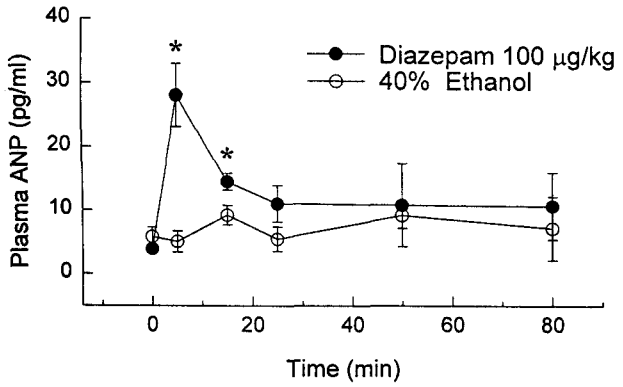

Fig. 4. Changes of plasma concentration of atrial natriuretic peptide (ANP) by *icv* diazepam 100 μg/kg and 40% ethanol, the vehicle. Significant differences from the control values are marked with asterisks, *=p<0.05.

Changes of plasma atrial natriuretic peptide

When 100 μg/kg diazepam administered *icv*, the plasma ANP level markedly increased from the control value of 3.94±0.70 pg/ml to 28.07±8.93 at 5 min, and then gradually returned to the pre-administration level. The ANP peak coincided with the diuresis and natriuresis (Fig. 4).

Renal effects of icv muscimol

To examine the involvement of GABA receptor in the action, renal response of *icv* muscimol was observed. With 0.1 and 0.3 μg/kg muscimol *icv*, no significant changes in renal function were noted. With 1.0 μg/kg muscimol *icv*, systemic blood pressure decreased by 57 mmHg within 10 min and then gradually recovered. Both RPF and GFR were markedly

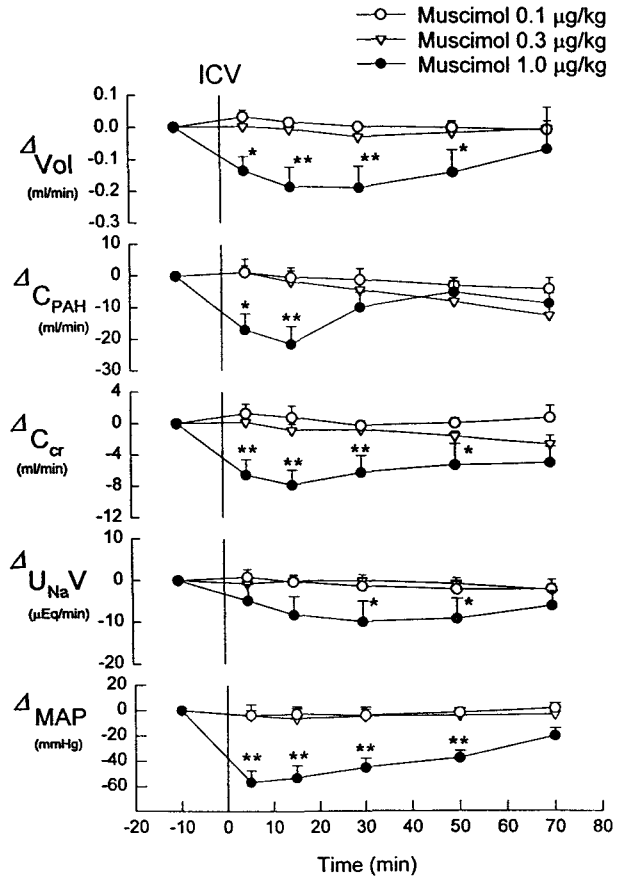

Fig. 5. Effects of *icv* muscimol on rabbit renal function. Significant differences from the control values are marked with asterisks, *=p<0.05, **=p<0.01.

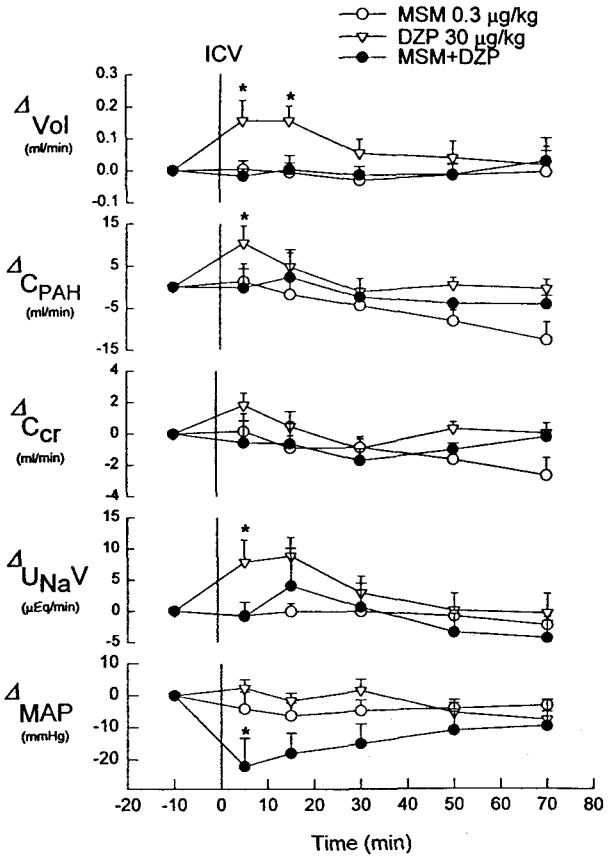

Fig. 6. Influence of *icv* muscimol, 0.3 μg/kg, on the renal effects of *icv* diazepam, 30 μg/kg. In the MSM+DZP group, muscimol was administered 3 min prior to diazepam. Significant differences from the control values are marked with asterisks, *=p<0.05.

depressed to one-fourth of control rates at 10~20 min. Urine flow rate also decreased by 78% of control rate in 20~40 min, with Na and K excretion rates also decreasing correspondingly (Fig. 5).

Renal effects of icv diazepam in muscimol-pretreated rabbits

When 30 μg/kg diazepam was administered *icv* 3 min after 0.3 μg/kg muscimol *icv*, which elicits no renal effects and severe changes of blood pressure by itself, the diuretic and natriuretic effects of 30 μg/kg diazepam *icv* disappeared with marked decreases in systemic blood pressure (Fig. 6).

DISCUSSION

In the present study, diazepam given intracerebroventricularly elicited diuretic and natriuretic responses in doses ranging from 10~100 μg/kg. Diazepam also increased RPF and GFR, suggesting that natriuretic and diuretic responses may be related to the improved renal hemodynamics. The increased FE_{Na} suggests that tubular function is also affected. As for the renal effects of *iv* diazepam, Monasterolo and Elias (1993) reported that diazepam induced diuresis and natriuresis without detectable changes in GFR or p-aminohippuric acid clearance in conscious rats. Also, in an isolated and perfused rat kidney, diazepam decreased the reabsorption rates of sodium and water without promoting hemodynamic changes, indicating direct effect of diazepam on tubular transport mechanisms (Monasterolo et al, 1995). We found, however, in our present study that in the rabbit 100 μg/kg diazepam given *iv* did not produce diuretic and natriuretic responses, as seen with *icv* diazepam. The discrepancy in renal effect of *iv* diazepam between our result and previous observations may have been caused by the difference in species. Anyway, this result further indicates that the natriuretic and diuretic responses of *icv* diazepam are brought about through the central mechanism.

Central regulatory influence on the renal function has been ascribed either to the alteration of renal nerve activity or to the mediation of humoral factors. When the renal nerve was electrically stimulated, antidiuretic and antinatriuretic responses were produced in a frequency-dependent manner, accompanied with decreased RPF, GFR and FE_{Na} (Bello-Reuss et al, 1976). On the contrary, severing the renal nerve elicits marked diuresis and natriuresis (Bonjour et al, 1969; Christy et al, 1994), and decreased renal sympathetic tone also causes decrease in the renal tubular sodium reabsorption, producing diuresis and natriuresis (Prosnit & DiBona, 1978). Thus, renal function is controlled through the alteration of sympathetic activity, which is ultimately under the control of CNS. In the present study the renal nerve activities were not changed significantly by *icv* diazepam up to 100 μg/kg, which produced natriuretic and diuretic responses. This finding indicates that the responses of *icv* diazepam are not directly related to the alteration of renal sympathetic nerve activities.

As the alternative, the involvement of certain humoral factor was explored. It has been well known that ANP, among many known agents, has an important role in the regulation of renal function. ANP exerts a prominent influence on the renal vasculature, mesangium, renin secretion, and sodium excretion. The most striking feature of these actions is the increase in GFR followed by natriuresis. The increase in GFR results from increased glomerular capillary hydrostatic pressure, ensuing from the afferent arteriolar vasodilation and the efferent arteriolar constriction (Fried et al, 1986). When administered *iv*, ANP also produces diuresis and natriuresis by decreasing renal tubular reabsorption of sodium and water, resulting in severe hypotension (DeBold et al, 1981; Brenner et al, 1990). Other natriuretic factors, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are also found (Sudoh et al, 1990), but their blood levels are too low to be ascribed a role comparable to ANP (Aburaya et al, 1991). In this study, the plasma ANP level increased about 7-fold following the *icv* diazepam, which lasted more than 25 min, coinciding with the peak diuresis and natriuresis. This suggests that the renal effects of *icv* diazepam are most likely related to the increased plasma ANP.

To obtain clues on whether the renal effects of *icv* diazepam are related to central GABAergic system, the influence of *icv* muscimol, a GABA agonist, was investigated. With less than 0.3 μg/kg muscimol, which did not appreciably affect the systemic blood pressure, the renal function did not change; however, 1.0 μg/kg muscimol produced severe antinatriuresis and antidiuresis along with a marked decrease in blood pressure. Renal hemodynamics and GFR were also decreased. The hypotension evoked by *icv* mus-

cimol results from decreased sympathetic discharge from CNS to periphery, as reflected by the decreased renal nerve activity (Choi, 1994). In the present study, diazepam under muscimol-pretreatment potentiated the hypotensive effect of muscimol; on the contrary, however, *icv* diazepam effects on the renal function disappeared. It appears that the loss of renal effects may have resulted from severe hypotension caused by a combination of muscimol and diazepam. Bicuculline, an antagonist of GABA receptor, elicited natriuresis and diuresis, and diazepam under bicuculline-pretreatment tended to produce additive, not depressing, effects in renal function (unpublished data). These findings suggest that benzodiazepine system on the central regulation of renal function may not be directly associated to GABA system.

Recently, the GABA receptors have been subdivided into three groups ($GABA_A$ $GABA_B$, $GABA_C$) based upon pharmacological evidence. $GABA_A$ receptor is the most prevalent among the known GABA receptors in CNS. This subtype is known to be directly associated with chloride channel, which is allosterically modulated by benzodiazepines and barbiturates. When this receptor is stimulated by muscimol or isoguvacine, chloride influx into the neuronal cell increases. This change results in hyperpolarization of the neuron, which attenuates the release of transmitters, and it is selectively blocked by bicuculline (Bernard et al, 1989; Cooper et al, 1991). $GABA_B$ receptor is present at a lower level in the CNS than the $GABA_A$ receptor and is not linked to a chloride channel. $GABA_B$ receptor plays a role in attenuating the release of amines, excitatory amino acid, neuropeptide, and hormones, and it is antagonized by phaclofen. This $GABA_B$ receptor is coupled to ion channels via secondary messenger systems, and it is linked through GTP-sensitive proteins to a calcium channel. It is also distinguished pharmacologically from the $GABA_A$ receptor by its selective affinity for the agonist baclofen and its lack of affinity for muscimol and bicuculline. Activation of the $GABA_B$ receptor by baclofen decreases calcium conductance and transmitter release. Unlike the $GABA_A$ receptor, the $GABA_B$ receptor is not modulated by benzodiazepines and barbiturates (Bowery et al, 1989; Cooper et al, 1991). $GABA_C$ receptor has been characterized as another subtype by *cis*-4-aminocrotonic acid, although its pharmacological properties have not been established (Kebabian & Neumeyer, 1994). These reports and our present findings suggest that GABA system may not be directly involved in the renal responses of central diazepam.

Receptors for benzodiazepines were identified initially by the binding of [^{3}H]diazepam to membrane homogenates from rat brain (Braestrup & Squires, 1977a, b; Möhler & Okada, 1977), but they were also found in several peripheral tissues, such as the kidney (Braestrup & Squires, 1977b). The properties of the binding sites for [^{3}H]diazepam in the kidney differ considerably from those in the brain. For instance, clonazepam, a potent anticonvulsant, binds with nanomolar affinity to benzodiazepine receptors in the brain (central-type benzodiazepine receptors), but with less than micromolar affinity to the receptors in the kidney (peripheral-type benzodiazepine receptors). In contrast, Ro5-4864, a benzodiazepine without anxiolytic effects, is 1000 times more potent in displacing [^{3}H] diazepam binding in the kidney than in the brain (Braestrup & Squires, 1977a, b). Unlike the one to the central-type receptor, the binding ligand to the peripheral sites is not sensitive to chloride or GABA (Patel & Marangos 1982; Shoemaker et al, 1983). When diazepam was administered with *icv* muscimol, systemic blood pressure decreased synergistically, supporting that benzodiazepine receptors are modulating the activities of central GABAergic receptors and chloride channels on central regulation of cardiovascular system (Gillis et al, 1989; Barron et al, 1997); however, renal responses of diazepam were not potentiated by muscimol, indicating that the benzodiazepine system involved in the central regulation of renal function may not be directly linked to GABA ergic system. It is also supported by the result that β-CCM, an inverse agonist which binds competitively with benzodiazepines for central-type benzodiazepine receptor, did not affect the diuretic and natriuretic effects of *icv* diazepam (unpublished data). On the other hand, peripheral benzodiazepine receptors, which are not related to chloride-channel sensitive GABAergic system, are also discovered in olfactory bulb, choroid flexus, and ependymal lining of the third ventricle of the brain (Anholt et al, 1985; Anholt, 1986; Gehlert et al, 1985). These areas are also known as the regions of central regulation of renal function (Lichardus et al, 1987). Thus, the renal effects of *icv* diazepam may have occurred through the peripheral-type benzodiazepine receptors in the brain, although further studies are required.

In summary, *icv* diazepam may bring about natriuresis and diuresis by influencing the central regu-

lation of renal function, and the renal effects are related to the increased plasma ANP levels, but not to the decreased renal nerve activity. These effects of diazepam may not be mediated by the activation of central GABA system.

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