

The interrelationship between cholinergic pathway in the magnocellular paraventricular nucleus and natriuresis

Chunyan Wang^{1,2*}, Min Wang^{3*}, Heng-ai Zhang^{1,4}, Xijia Deng⁵, Pengxu Wang⁵, Huizheng Mao¹, Yuan Lin⁶ and Chunling Jiang¹

¹ Department of Physiology, Dalian Medical University, 9 West section, Lvshun south road, Dalian 116044, China

² Renal Division, Inner Mongolia People's Hospital, 20 Zhaowuda Road, Hohhot 010010, China

³ Department of Physiology, School of Medicine, Jiaying University, 118 Jiahang Road, Jiaying 314001, China

⁴ Beijing Key Laboratory of Drug Target Identification and Drug Screening, Chinese Academy of Medical Science and Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, China

⁵ Department of Clinical Medicine, Dalian Medical University, 9 West section, Lvshun south road, Dalian 116044, China

⁶ Department of Pharmacology, Dalian Medical University, 9 West section, Lvshun south road, Dalian 116044, China

Abstract. The central nervous system is known to play important roles in the regulation of renal sodium excretion. The present study was designed to reveal the interrelationship between cholinergic pathway in the magnocellular paraventricular nucleus (PVN) and the natriuresis induced by brain cholinergic stimuli. The results indicated that urinary sodium excretion was significantly increased at 40 min after intracerebroventricular (ICV) injection of carbachol (CBC). Immunohistochemical studies showed that CBC increased choline acetyltransferase-immunoreactivity (ChAT-IR) in the magnocellular PVN and renal proximal convoluted tubule (PCT), respectively. After pretreatment with atropine, urinary sodium excretion was significantly reduced, and carbachol-increased ChAT-IR in the magnocellular PVN and PCT was also significantly decreased. These results suggested that brain cholinergic stimuli induced the natriuresis and increased the activity of cholinergic neurons in the magnocellular PVN and cholinergic system in the PCT. The blockade of muscarinic receptor completely abolished the natriuresis and partially inhibited carbachol-exerted stimulatory effects in the magnocellular PVN and PCT. To summarize, brain cholinergic pathway and peripheral cholinergic system in kidney were found to contribute to the natriuresis following brain cholinergic stimulation. Our findings revealed novel evidence that PVN was involved in the natriuresis *via* humoral mechanisms.

Key words: Carbachol — Natriuresis — Choline acetyltransferase — Magnocellular paraventricular nuclei — Proximal convoluted tubule

Introduction

Recent studies have highlighted the essential role of brain regions such as the hypothalamic paraventricular nucleus (PVN) in the regulation of water intake, urinary volume and

sodium balance (de Arruda Camargo et al. 2010). Microinjections of bicuculline into the PVN were found to produce the antinatriuresis and antidiuresis (Haselton and Vari 1998). Similarly, microinjection of kainic acid in the PVN evoked both diuresis and natriuresis, without any concomitant alteration in glomerular filtration rate (GFR) and renal plasma flow (RPF) (Jin and Rockhold 1989). Specifically, a direct neurons projection was observed to arise from PVN and terminate in the kidney (Schramm et al. 1993). In this light, the neuronal cells in the PVN appeared to act in the kidney directly and indirectly *via* humoral pathway (Badoer 2010),

Correspondence to: Chunling Jiang, Department of Physiology, Dalian Medical University, 9 West section, Lvshun south road, Dalian 116044, China

E-mail: chunling_jiang2006@163.com

* These authors contributed equally to this work.

and the possibility of neuronal mechanisms in the PVN in the regulation of natriuresis induced by central cholinergic stimuli has not been directly examined.

Injection of cholinergic agonists into ventromedial hypothalamus (VMH) and locus coeruleus (LC) exerted the natriuresis and diuresis (De Luca Jr. et al. 1991; Valladao et al. 1992), suggesting that brain cholinergic system played an important role in the regulation of renal sodium and water excretion.

The nephron plays an important role in the maintenance of sodium balance, extracellular fluid volume. Renal infusion of acetylcholine in dogs indicated that the sodium excretion and urinary volume was increased without changing RPF and GFR and the increase was blocked by atropine (Vander 1964), suggesting the inter-relationship between renal cholinergic system and the renal sodium excretion.

Choline acetyltransferase (ChAT)-positive neurons have been detected in the PVN (Yamada et al. 2005). There are M receptors in epithelial cells of renal tubule (Robey et al. 2001). However, it is unknown whether the activity of cholinergic system in the PVN and kidney are changed in the natriuresis induced by intracerebroventricular (ICV) injection of cholinergic agonist.

ChAT catalyzes the biosynthesis of acetylcholine in the cytoplasm, which is the most reliable marker for cholinergic neurons (Kimura et al. 1981). It was widely accepted that the immunoreactivity of ChAT accord with the activity of the enzyme; the activity of cholinergic neurons was demonstrated by ChAT immunohistochemistry (Issa et al. 1999). In the present study, we combined the experiment *in vivo* with immunohistochemistry to examine. (1) The effect of the changes of renal sodium excretion after ICV injection of carbachol, as well as the expression of choline acetyltransferase-immunoreactivity (ChAT-IR) in PVN and kidney, (2) the effect of blocking cholinergic M receptor by atropine on the renal sodium excretion after ICV injection of carbachol, (3) the effect of blocking cholinergic M receptor by atropine on the changes of ChAT-IR in the PVN and kidney induced by ICV injection of carbachol. We attempted to determine the interrelation between brain cholinergic pathway in the PVN and peripheral renal cholinergic system during the natriuresis.

Materials and Methods

Animals

Healthy male Sprague-Dawley rats weighing 250~280 g were kept on a 12 h/12 h light /dark schedule with a free access to standard laboratory food and water at controlled temperature and humidity.

Experimental protocols

Forty-eight rats were anesthetized with aether. A 23-gauge guide cannula was implanted in the right lateral ventricle using a stereotaxic apparatus. Another stylette (31-gauge) was placed in the guide cannula to prevent occlusion. The rats were housed individually and were allowed to recover for one week after the surgery.

ICV injection protocols

All ICV injections were made between 8:00 and 9:00 a.m.. All injected solutions were made in volumes of 5 μ l over a period of 2 min.

Twenty-four rats for the experiment *in vivo* were divided randomly into four groups ($n = 6$ per group): physiological saline + carbachol (NS+CBC) group, Atropine + carbachol (Atro+CBC) group, Atropine + physiological saline (Atro+NS) group and NS+NS group. NS+CBC group received ICV injection of 0.9% NaCl (5 μ l), followed by ICV injection of 0.1 μ g/ μ l carbachol (5 μ l) 20 min later. The treatment of other groups was similar to the NS+CBC group. Another 24 rats for immunohistochemistry were also divided into 4 groups and treated as described in the experiment *in vivo*.

Experiment in vivo

Animals were anaesthetized with urethane (1 ml/100 g b.w., i.p.). The right external jugular vein, the left carotid artery and urinary bladder were cannulated with a polyethylene catheter for intravenous (i.v.) drug administration, mean arterial blood pressure (MAP) recording and urine collection, respectively. After surgery, an infusion of 0.9% NaCl (0.025 ml/min/100 g) was started and maintained for one hour. The first 60 min following stabilization was considered as the reference period and the following 120 min were monitored after the final injection.

In all groups, urine samples were collected every 20 min over the experimental period. MAP was recorded with PcLab polygraph. Urine volume was determined gravimetrically and urinary sodium concentration was measured by Easylyte plus Na/K/Cl Analyzers (Medica Corporation, USA).

Immunohistochemistry

40 min after the final injection, 24 rats were perfused with 1% and 4% paraformaldehyde for the fixation of the brain and kidney tissue under anesthesia. Brain and kidney tissue were removed, post-fixed in 4% paraformaldehyde and immersed into a phosphate buffer saline containing 30% sucrose for three days.

50 μm thick coronal brain sections were sliced on a vibratome. The identification of PVN (Bregma: $-1.80\text{ mm} \sim -2.12\text{ mm}$) was based on the atlas by Paxinos and Watson. Each section of the PVN was used for ChAT-IR using the avidin-biotin-peroxidase technique. The tissue sections were incubated overnight at 4°C in the primary antibody (ChAT-Ab, 1:100, Boster Company, P.R. China). The sections were further incubated in the biotinylated-second antibody (Boster Company, P.R. China) for 2 h and then processed using the the avidin-biotin complex ABC (Boster Company, P.R. China) for 2 h. Diaminobenzidine (DAB; Sigma Company, USA) was used for signal detection. HPIAS series colorful pathology photograph system was used to analyze ChAT-IR positive neurons. The brain sections were observed at magnification $\times 20$. The number and optical density of ChAT-IR positive neurons were calculated *per area* and *per group*.

Kidney tissue was frozen in liquid nitrogen and then mounted in optimum cutting temperature compound (OCT; Sakura Finetek, USA) mounting media. Cryostat sections (8 μm thick) were cut at -12°C and placed onto low iron clear glass slides.

Sections were incubated in the primary antibody (ChAT-Ab, 1:500, Boster Company, P.R. China) overnight at 4°C . The sections were further incubated in the biotinylated-second antibody (Boster Company, P.R. China) for 1 h and then processed using the the avidin-biotin complex ABC (Boster Company, P.R. China) for 1 h. Diaminobenzidine (DAB; Sigma Company, USA) and haematoxylin were used to detect signal. Image Pro Plus image analysis system was used to analyze ChAT-IR positive granules. Kidney sections were observed in a microscope at magnification $\times 20$. The number and optical density of ChAT-IR positive granules were calculated *per area* and *per group*.

Statistical analysis

All data were expressed as mean \pm SEM. Statistical evaluation was done using ANOVA with post hoc test of Least Significant Difference (LSD) in Equar Variances Assumed. In all comparisons, statistical significance was set at $p < 0.05$.

Results

Effect of pretreatment with atropine on the natriuresis induced by ICV injection of carbachol

In NS+CBC group, urinary sodium excretion was immediately increased at 20 min, reached the peak at 40 min, and the enhancement lasted for about 100 min (Fig. 1). However, this effect was significantly attenuated in Atro+CBC group,

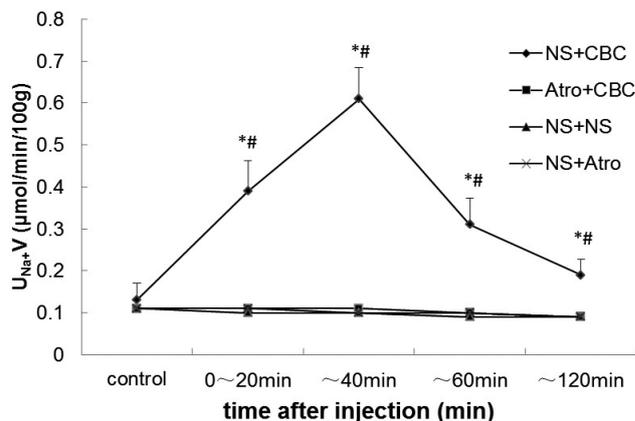


Figure 1. Effect of pretreatment with atropine on the natriuresis induced by ICV injection of carbachol (CBC). * $p < 0.05$ vs. NS+NS group, # $p < 0.05$ vs. Atro+CBC group ($n = 6$). ICV, intracerebroventricular; NS, physiological saline; Atro, atropine; U_{Na^+} , urinary sodium concentration; V, urine volume; U_{Na^+} V, urinary sodium excretion.

compared with NS+CBC group. And there was no significant difference between Atro+CBC group and NS+NS group ($p > 0.05$, Fig. 1). Injection of 30 μg Atropine (Atro+NS group) into the ICV did not induce a significant change in urinary sodium excretion within 120 min observed, compared with that in NS+NS group.

Effect of atropine pretreatment on the change of ChAT-IR in the PVN induced by ICV injection of carbachol

In the hypothalamus of NS+NS group, ChAT-IR positive neurons were distributed predominantly in the PVN. Compared with NS+NS group, NS+CBC group showed an increase of ChAT-IR positive neurons predominantly in the paraventricular hypothalamic nucleus, lateral magnocellular part (PaLM). However, there was a significant decrease in number of ChAT-IR positive neurons in the PaLM in Atro+CBC group (Fig. 2B) when compared with that in NS+CBC group (Fig. 2A), but still higher than that in NS+NS group (Fig. 4A, $p < 0.05$). And there was not marked difference in optical density of ChAT-IR positive neurons in the PaLM in Atro+CBC group, compared with that in NS+CBC group. ChAT-IR in Atro+NS group was similar to that in NS+NS group. The data on ChAT-IR in the PaLM were summarized in Fig. 4A and 4B.

Effect of atropine pretreatment on the changes of ChAT-IR in the PCT induced by ICV injection of carbachol

In the NS+NS group, ChAT-IR positive granules were detected very weakly in the PCT (Fig. 3C). Forty min after

ICV injection of carbachol (0.5 μ g; NS+CBC group), it was evident that ChAT-IR positive granules were densely distributed, strongly stained and detected mainly in PCT (Fig. 3A). The number of ChAT-IR positive granules in the NS+CBC group were significantly increased in renal PCT compared with that in the NS+NS group ($p < 0.05$, Fig. 5). However, there was a significant decrease in the number of ChAT-IR positive granules in renal PCT in the Atro+CBC group (Fig. 3B), compared with that in NS+CBC group, but still higher than that in NS+NS group (Fig. 4, $p < 0.05$). No significant difference was found in optical density of ChAT-IR positive granules in the kidney, when Atro+CBC group was compared with the NS+CBC group. The data above are summarized in Fig. 4C.

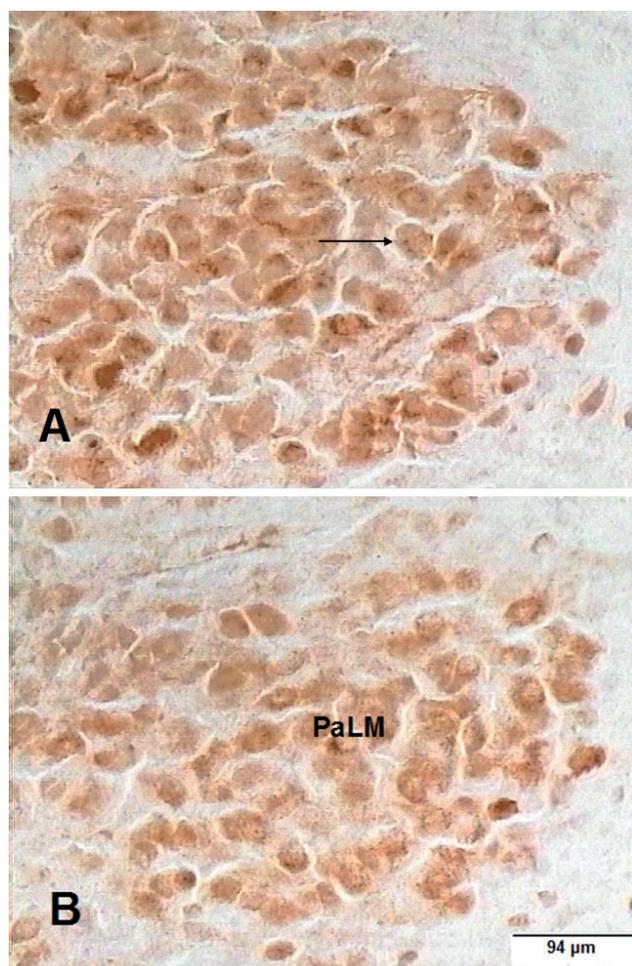


Figure 2. Effect of pretreatment with atropine on the changes of ChAT-IR (choline acetyltransferase-immunoreactivity) in the PaLM (paraventricular hypothalamic nucleus, lateral magnocellular part) induced by central carbachol (CBC). NS+CBC group (A), Atro+CBC group (B). Arrow points to ChAT-IR positive neurons.

Discussion

A number of studies have showed that the central nervous system might play an important role in the control of renal water and electrolyte excretion (De Luca Jr et al. 1991; Valladao et al. 1992; de Arruda Camargo et al. 2010; Badoer 2010).

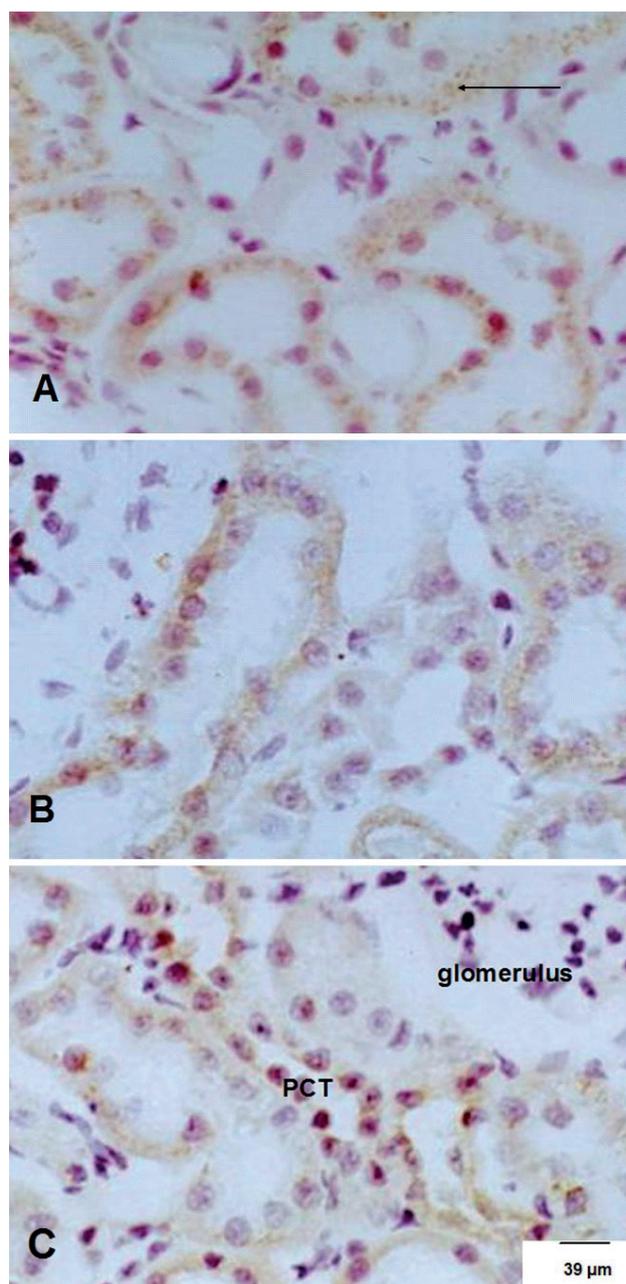


Figure 3. Effect of pretreatment with atropine on the changes of ChAT-IR in the PCT (proximal convoluted tubule) induced by central CBC. NS+CBC group (A), Atro+CBC group (B), NS+NS group (C). Arrow points to ChAT-IR positive granules.

Intraventricular injection of hypertonic saline produced the natriuresis and diuresis (Mathai et al. 1998). Evidence indicated that PVN influenced renal function *via* the renal nerves (Haselton and Vari 1998) and PVN was involved in humoral control in renal sodium excretion.

In the present study, ICV injection of carbachol was found to exert the natriuresis, which could be completely inhibited by M receptor blocker atropine. CNS cholinergic stimulation contributed to the natriuretic effect, indicating the involvement of brain cholinergic system. Similarly, injection of carbachol into lateral ventricle, septal area, and preoptic region exerted the natriuresis and diuresis (Colombari et al. 1992; McCann et al. 1997).

Pretreatment of atropine downregulated carbachol (ICV injection)-increased ChAT-IR especially in the PaLM, indicating that cholinergic neurons in the PVN were activated by central carbachol and suppressed by atropine.

PVN contained abundant cholinergic neurons, mainly in the magnocellular region (Yamada et al. 2005). As for our study, the increase of ChAT-IR positive neurons in the PVN was more prominent in magnocellular region after ICV injection of carbachol, but the changes of ChAT-IR in the PaLM induced by central injection of carbachol were partially reduced by administration of atropine. The activation of cholinergic neuron in the PVN implied the involvement of brain cholinergic pathway in the natriuresis induced by central cholinergic stimuli. Furthermore, muscarinic receptors were strongly involved in these regulatory processes.

Nonetheless, pretreatment with atropine didn't completely block the changes of ChAT-IR in the PVN. This may suggest that the natriuresis induced by ICV injection of carbachol also depended on other humoral/neural pathway in the PVN. Magnocellular region of PVN mainly contained vasopressin and oxytocin neurons as some paper reported (Arima and Aguilera 2000; Honda et al. 2014). Nitric oxide synthase neurons were also located in the same region (Yuan et al. 2006). These evidences implicated that the distribution of those neurons in the PVN were overlapped.

Cholinergic and catecholaminergic nerve terminals were involved in the natriuresis by catecholamine, angiotensin II or atrial natriuretic peptide (Saad et al. 1997), which implied that there were multiple synapses and neurotransmitters responsible for the natriuresis resulted from ICV injection of carbachol. Microinjection of glutamic acid could cause an increase of noradrenaline concentration in the magnocellular PVN as well as in blood, and restrain the cardiovascular reflex (Jin et al. 2001). Similar to these studies, our previous study demonstrated that central angiotensinergic pathway and NO-dependent neural pathway in the locus coeruleus contributed to the natriuresis following brain cholinergic stimulation (Wang et al. 2007). Thus, it is possible that ICV injection of carbachol elicited the release of endogenous acetylcholine, acted on the muscarinic ace-

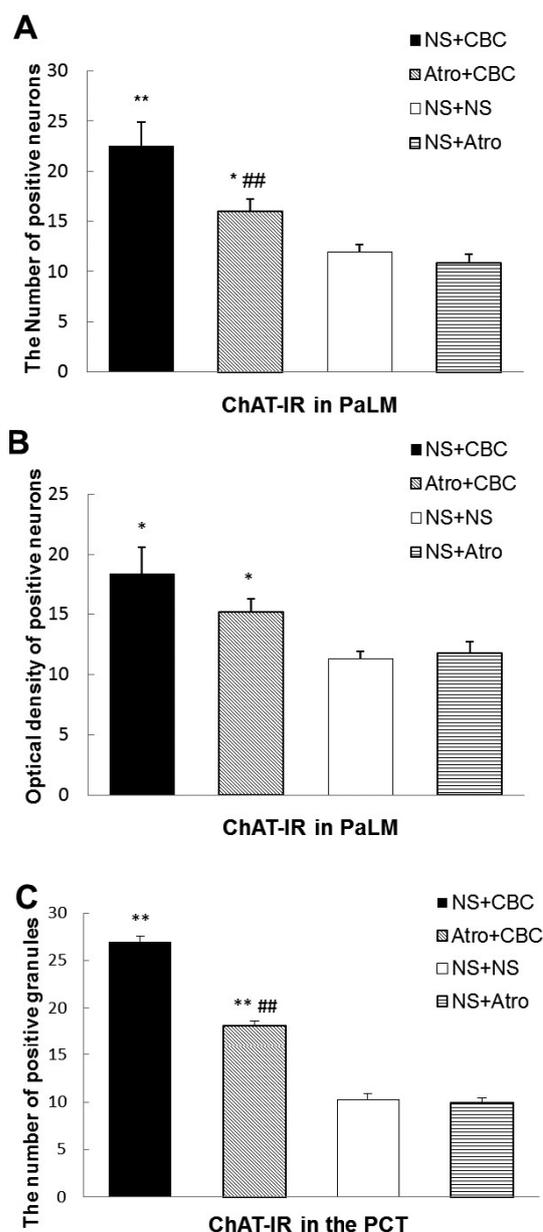


Figure 4. Quantitative analysis of the effect of atropine pretreatment on the change of ChAT-IR in the PaLM and PCT induced by central carbachol (CBC). **A.** The number of ChAT-IR positive neurons in PaLM. **B.** Optical density of ChAT-IR positive neurons in PaLM. **C.** The number of ChAT-IR positive granules in PCT. * $p < 0.05$, ** $p < 0.01$ vs. NS+NS group; ## $p < 0.01$ vs. NS+CBC group ($n = 6$). For more abbreviations see Fig. 2 and 3.

tylcholine receptor (mAChR) in the PVN, resulted in the natriuretic effect through some neurohumoral pathways. In addition, angiotensin II, noradrenaline or other neuronal pathway might participate in the same changes induced by carbachol.

The results presented here revealed strong biochemical connections between brain mAChRs and cholinergic pathway in the PVN involved in the natriuresis induced by central cholinergic stimuli. It has been shown that atropine eliminate the increase of endogenous extracellular signal regulated kinase (ERK) 1/2 activity induced by carbachol to hippocampal slices and cortical cells (Rosenblum et al. 2000), thus, we proposed that ERK signal transduction pathway was probably involved in the natriuresis induced by central cholinergic stimuli.

Kidney is not only the target organ for many other hormones, but also an endocrine organ. It could synthesize and release hormones such as renin, prostaglandins and erythropoietin. Experiments in rats suggested non-neuronal ACh synthesis in the kidney (Maeda et al. 2011). In this study, ChAT-IR was observed in renal epithelial cells, suggesting the existence of intrarenal ACh production. Expression of non-neuronal ACh is increased in pathological conditions such as arthritis, and ulcerative colitis (Forsgren et al. 2009). As ChAT is the limiting factor for the synthesis of ACh, it seems likely that ChAT expression levels might be changed when the rats are in abnormal conditions such as natriuresis and diuresis.

This study further demonstrated that 40 min after ICV administration of carbachol, the ChAT-IR in epithelial cells of the PCT increased, but decreased by atropine pretreatment.

Our results indicated that renal cholinergic system was responsible for the natriuresis induced by ICV injection of carbachol, suggesting the involvement of renal epithelial cells in the renal sodium excretion. As carbachol was an activator of the phosphoinositide hydrolysis pathway in rat inner medullary collecting duct (Chou et al. 1998) the natriuresis induced by central carbachol caused the activation of the phosphoinositide hydrolysis pathway.

Carbachol did not affect renal sodium and water excretion in 120 min *via* injection from peripheral vein or cavum subarachnoidale, suggesting that the natriuresis induced by central carbachol was mediated by activating some brain regions. And also the activity of the renal cholinergic system was modulated by the central cholinergic system, and the final effect was to influence the reabsorption of sodium and water on renal tubule, and resulted in the natriuresis. In line with these findings, a recent report has suggested that there is a close cross-talk between the kidney and CNS. Fos-activity of supraoptic and magnocellular PVN neurons is reduced in the chronic renal failure rats (Palkovits et al. 2013). The involvement of the PVN in control of the kidney through the release of the antidiuretic hormone has been documented (Krowicki and Kapusta 2011).

It is reasonable, therefore, to suggest that PVN affects renal sodium excretion *via* humoral pathway. Cholinergic mechanism in PVN are important in cooperation with the

peripheral cholinergic system in the kidney in the regulation of renal sodium excretion induced by central cholinergic stimuli, though the cellular and molecular mechanism underlying this process remained to be investigated.

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