rophages, monocytes, endothelial progenitor cells, and so on, resulting in gene expression within these phagocytes.[23,24] These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer.

Currently, a highly efficient and safe gene delivery system is needed for gene therapy in humans. The present study demonstrated that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhanced the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases. However, the initial success of gelatin-mediated AM gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

Study Limitation

First, histological capillary density, calf blood pressure ratio, and laser Doppler flow were significantly higher in the AM-gelatin group than in the naked AM group. However, the angiographic score did not significantly differ between the two. This discrepancy raises the possibility that conventional angiography may have insufficient resolution to fully visualize the angiogenic microvessels. Second, human AM level was slightly elevated in the control group. This implies that the anti-human AM antibody used in this radioimmunoassay had some cross-reactivity with endogenous rabbit AM. Nevertheless, human AM level in the muscles was highest in the AM-gelatin group within 2 weeks after gene transfer. These results suggest that AM DNA-gelatin complexes induces potent and long-lasting AM production.

Conclusions

Intramuscular administration of AM DNA induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases.

Acknowledgments

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Simultaneous monitoring of acetylcholine and catecholamine release in the in vivo rat adrenal medulla

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Abstract

To simultaneously monitor acetylcholine release from pre-ganglionic adrenal sympathetic nerve endings and catecholamine release from post-ganglionic adrenal chromaffin cells in the in vivo state, we applied microdialysis technique to anesthetized rats. Dialysis probe was implanted in the left adrenal medulla and perfused with Ringer's solution containing neostigmine (a cholinesterase inhibitor). After transection of splanchnic nerves, we electrically stimulated splanchnic nerves or locally administered acetylcholine through dialysis probes for 2 min and investigated dialysate acetylcholine, choline, norepinephrine and epinephrine responses. Acetylcholine was not detected in dialysate before nerve stimulation, but substantial acetylcholine was detected by nerve stimulation. In contrast, choline was detected in dialysate before stimulation, and dialysate choline concentration did not change with repetitive nerve stimulation. The estimated interstitial acetylcholine levels and dialysate catecholamine responses were almost identical between exogenous acetylcholine (10 μM) and nerve stimulation (2 Hz). Dialysate acetylcholine, norepinephrine and epinephrine responses were correlated with the frequencies of electrical nerve stimulation, and dialysate norepinephrine and epinephrine responses were quantitatively correlated with dialysate acetylcholine responses. Neither hexamethonium (a nicotinic receptor antagonist) nor atropine (a muscarinic receptor antagonist) affected the dialysate acetylcholine response to nerve stimulation. Microdialysis technique made it possible to simultaneously assess activities of pre-ganglionic adrenal sympathetic nerves and post-ganglionic adrenal chromaffin cells in the in vivo state and provided quantitative information about input–output relationship in the adrenal medulla.

Keywords: Anesthetized rats; Microdialysis; Choline; Norepinephrine; Epinephrine

1. Introduction

Although acetylcholine is one of major neurotransmitters in the peripheral autonomic nervous system as well as central nervous system (Collier, 1977; Fibiger, 1991; Calabresi et al., 2000), it has been difficult to measure endogenous acetylcholine in the in vivo state since acetylcholine released from nerve endings is rapidly degraded by tissue acetylcholinesterase (Taylor and Brown, 1998). Recently, microdialysis technique with improved measurement has made it possible to monitor low levels of acetylcholine in the in vivo central nervous system. In the peripheral autonomic nervous system, we have measured acetylcholine release from post-ganglionic parasympathetic nerve endings using microdialysis technique (Akiyama et al., 1994; Akiyama and Yamazaki, 2000, 2001; Kawada et al., 2001). Little information is, however, available on acetylcholine release from pre-ganglionic autonomic nerve endings in the in vivo state. The assessment of pre-ganglionic autonomic nerve activities is important for understanding the autonomic ganglionic transmission under physiological and pathophysiological conditions.

Adrenal medulla is one candidate suitable for investigating acetylcholine release from pre-ganglionic autonomic nerve endings (Holman et al., 1994). Compared to autonomic ganglia, adrenal gland is solid and suited to microdialysis probe implantation. Furthermore, microdialysis technique in the adrenal medulla provides a distinct advantage to monitor catecholamine release from adrenal medulla following acetylcholine release. Thus, we consider it possible to simultaneously assess pre- and post-ganglionic sympathetic nerve activities by monitoring acetylcholine and catecholamine release in the adrenal medulla.

In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and tested the suitability of microdialysis technique to simultaneously

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monitor acetylcholine and catecholamine release from adrenal medulla.

2. Materials and methods

2.1. Animal preparation

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Wistar rats weighing 390–460 g were anesthetized with pentobarbital sodium (50–55 mg/kg i.p.). The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. The left femoral artery and vein were cannulated for monitoring arterial blood pressure and administration of anesthetic, respectively. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 mg/(kg h) i.v.). Electrocardiogram was monitored for recording heart rate. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38 °C. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a subcostal flank incision, and the left splanchnic nerve was transected. In protocols requiring nerve stimulation, shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden, Japan) with a rectangular pulse (10 V and 1 ms in duration).

2.2. Dialysis technique

The materials of the dialysis probe were the same as those used in our previous dialysis experiments (Akiyama et al., 2003). Briefly, each end of the dialysis fiber (0.31 mm o.d., and 0.20 mm i.d.; PAN-1200 50,000 mol. wt. cutoff, Asahi Chemical, Japan) was inserted into the polyethylene tube (25 cm length, 0.50 mm o.d., and 0.20 mm i.d.; SP-8) and glued. The length of the dialysis fiber exposed was 3 mm. At perfusion speed of 10 μl/min, in vitro recovery rates of acetylcholine, choline, norepinephrine, and epinephrine were (%): 3.08 ± 0.04, 2.93 ± 0.10, 2.09 ± 0.03, and 2.16 ± 0.03, respectively (number of dialysis probes: 3).

The dialysis probe was implanted in the medulla of the left adrenal gland and perfused with Ringer's solution containing the cholinesterase inhibitor, neostigmine (10 μM) at a speed of 10 μl/min using a microinjection pump (CMA/100, Carnegie Medicin, Sweden). Ringer's solution consisted of (in mM) 147.0 NaCl, 4.0 KCl, 2.25 $CaCl_2$. All pharmacological agents tested were locally administered by perfusion through the dialysis probe after being dissolved in Ringer's solution. One sampling period was 2 min (one sample volume = 20 μl). We started the protocols followed by a stabilization period of 3–4 h. Catecholamine release was evoked by 2 min-local administration of acetylcholine or 2 min-electrical stimulation of left splanchnic nerves. In protocols requiring repeated nerve stimulation, electrical stimulation was performed at 30 min-intervals. Taking the dead space volume into account, we continuously collected three dialysate samples per pharmacological or electrical stimulation: one before, one during, and one after stimulation. We subtracted the dialysate acetylcholine, norepinephrine, or epinephrine contents in control from those during stimulation, and expressed these values as indices of dialysate acetylcholine, norepinephrine or epinephrine response to stimulation.

Half of the dialysate sample was injected into high-performance liquid chromatography for the measurement of acetylcholine and choline (Akiyama et al., 1994), and the remaining half was injected into another high-performance liquid chromatography for the measurement of norepinephrine and epinephrine (Akiyama et al., 1991).

2.3. Experimental protocols

2.3.1. Protocol 1

We repeated stimulations of splanchnic nerves at 2 and 4 Hz twice and examined dialysate acetylcholine, choline and catecholamine responses to nerve stimulation and their reproducibility in five rats.

2.3.2. Protocol 2

To compare the estimated interstitial acetylcholine levels between administration of acetylcholine and nerve stimulation, we locally administered acetylcholine (10 μM) in five rats and stimulated splanchnic nerves at 2 Hz in five other rats. The concentration of exogenous acetylcholine was determined to obtain a similar dialysate catecholamine response to nerve stimulation at 2 Hz.

2.3.3. Protocol 3

We raised stepwise the frequency of nerve stimulation from 2 to 4, 10, 20 Hz and examined dialysate acetylcholine and catecholamine responses in five rats. In addition, to examine the input–output relationship in the adrenal medulla, we analyzed the relationship between dialysate acetylcholine and catecholamine responses of five rats.

2.3.4. Protocol 4

We examined the effects of cholinergic receptor antagonists on dialysate acetylcholine and catecholamine responses. Nerve stimulations at 2 and 4 Hz were performed before and after 30 min-local administration of cholinergic receptor antagonists. We tested the nicotinic receptor antagonist, hexamethonium bromide (1 mM) in five rats or the muscarinic receptor antagonist, atropine sulfate (10 μM) in five other rats.

2.4. Statistical methods

To examine the effect of nerve stimulation and pharmacological agents, we analyzed heart rate and mean

arterial pressure, and dialysate acetylcholine, choline, norepinephrine and epinephrine responses, using one- or two-way analysis of variance with repeated measures. When statistical significance was detected, the Newman–Keuls test was applied (Winer, 1971). Statistical significance was defined as $P < 0.05$. Values are presented as mean ± S.E.

3. Results

The experiments were carried in anesthetized rats and had been performed in the presence of neostigmine. Local administration of pharmacological agents did not influence heart rate or mean arterial pressure in any of the protocols. In protocol 3 ($n = 5$), nerve stimulation at 2 Hz decreased heart rate from 420 ± 8 to 397 ± 8 beats/min ($P < 0.05$) and increased mean arterial pressure from 125 ± 4 to 136 ± 3 mmHg ($P < 0.05$). Heart rate and mean arterial pressure recovered after cessation of stimulation. Nerve stimulation at 4, 10 and 20 Hz decreased heart rate to 396±9, 393±7 and 392 ± 9 beats/min, respectively, and increased mean arterial pressure to 134±3, 141±3, and 142±3 mmHg, respectively. In the other protocols, nerve stimulation at 2 or 4 Hz evoked the same responses of heart rate and mean arterial pressure.

3.1. Dialysate acetylcholine and catecholamine

3.1.1. Protocol 1

As shown in the *upper panel* of Fig. 1 ($n = 5$), acetylcholine was not detected in dialysate before nerve stimulation, but substantial acetylcholine was detected in dialysate by nerve stimulation. In contrast, choline, norepinephrine, and epinephrine were detected in dialysate before stimulation. Dialysate choline concentration did not change with repetitive nerve stimulation. Dialysate norepinephrine and epinephrine concentrations increased with nerve stimulation. Stimulation at the same frequency elicited almost identical responses on repetition.

3.1.2. Protocol 2

Using in vitro recovery rate of acetylcholine (3.08%), the estimated interstitial acetylcholine levels were 308 nM in acetylcholine infusion (10 μM, $n = 5$) and 276 ± 15 nM in nerve stimulation (2 Hz, $n = 5$; Fig. 1, *lower panel*). There was no statistical difference in the estimated interstitial acetylcholine levels and dialysate catecholamine responses between the two groups.

3.1.3. Protocol 3

When the frequency of nerve stimulation was increased from 2 to 20 Hz, dialysate acetylcholine, norepinephrine and epinephrine responses were enhanced ($n = 5$; Fig. 2, *upper panel*). We plotted the relationship between dialysate catecholamine response (ordinate) and dialysate acetylcholine response (abscissa) of five rats (Fig. 2, *lower panel*). Dialysate norepinephrine and epinephrine responses correlated with dialysate acetylcholine responses.

3.1.4. Protocol 4

At both 2 and 4 Hz of nerve stimulation, hexamethonium suppressed dialysate norepinephrine and epinephrine responses, but did not affect acetylcholine response ($n = 5$; Fig. 3, *upper panel*). Atropine suppressed epinephrine response at both 2 and 4 Hz of nerve stimulation, but did not affect norepinephrine and acetylcholine responses ($n = 5$; Fig. 3, *lower panel*).

4. Discussion

By now, simultaneous monitoring of adrenal acetylcholine and catecholamine release has been limited to only a few studies using perfused adrenal gland. Collier et al. (1984) measured endogenous acetylcholine and catecholamine effluxes from perfused cat adrenal gland. O'Farrell et al. (1997) preloaded bovine adrenal glands with [^{3}H]-choline and measured the subsequent efflux of [^{3}H]-labelled compound as an index of acetylcholine release and catecholamine efflux. In the present in vivo study, dialysate acetylcholine and catecholamine responses served as indices of acetylcholine release from splanchnic nerve endings and catecholamine release from adrenal medulla, respectively. This simultaneous monitoring implies quantitative measurement of pre- and post-ganglionic neurotransmitter release at the adrenal medulla.

4.1. Source of dialysate acetylcholine

The stimulation of splanchnic nerve induced acetylcholine release from pre-ganglionic nerve endings and increased dialysate acetylcholine concentration. It has been demonstrated that adrenal gland receives parasympathetic efferent and afferent innervation (Coupland et al., 1989; Niijima, 1992; Parker et al., 1993). Branches of parasympathetic efferent nerves conduct through celiac nerves, celiac ganglion and splanchnic nerves to adrenal nerves (Niijima, 1992). In the present study, we electrically stimulated the portion just distal to the sympathetic chain and proximal to the celiac ganglion. This portion does not contain branches of parasympathetic efferent nerves. After transection of splanchnic nerves, basal dialysate acetylcholine was less than the detection limit of high performance liquid chromatography (10 fmol), and substantial acetylcholine was detected in dialysate during the stimulation of this portion. Thus, most of the detected acetylcholine in dialysate derives from pre-ganglionic sympathetic nerve endings.

4.2. Interstitial choline levels in the adrenal medulla

Under physiological conditions, there is enough acetylcholinesterase activity in splanchnic nerve endings, chromaffin cells, and interstitial cells (Coupland, 1965; Palkama, 1967; Lewis and Shute, 1969; Somogyi et al., 1975). Released acetylcholine is degraded to choline and acetate by

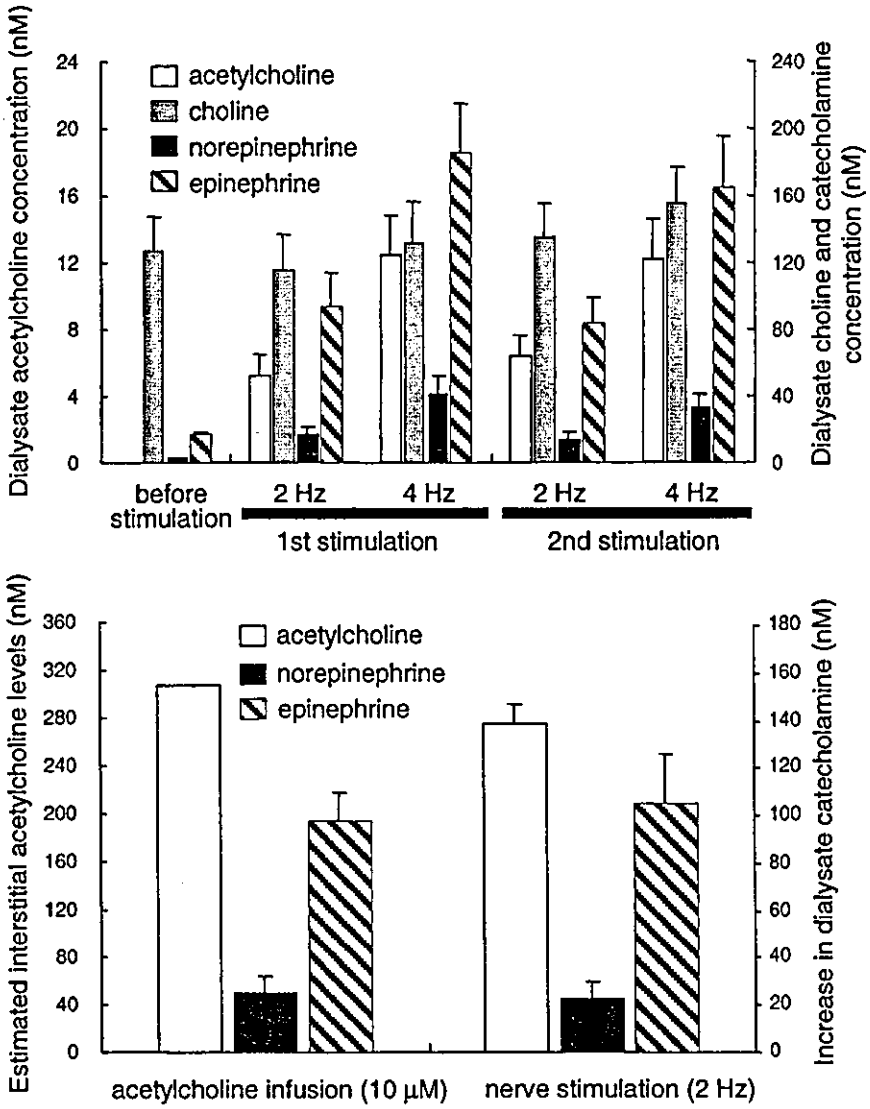

Fig. 1. (*Upper panel*) Acetylcholine was not detected in dialysate before nerve stimulation, but substantial acetylcholine was detected in dialysate by nerve stimulation (5 ± 1 nM at 2 Hz and 12 ± 2 nM at 4 Hz). Dialysate choline concentration did not change with nerve stimulation. Dialysate norepinephrine and epinephrine concentrations increased with nerve stimulation (17 ± 5 and 94 ± 20 nM at 2 Hz, respectively, and 41 ± 12 and 185 ± 29 nM at 4 Hz, respectively). Stimulation at the same frequency elicited almost identical responses on repetition, $n = 5$. Values are mean ± S.E. (*Lower panel*) There was no statistical difference in the estimated interstitial acetylcholine levels and dialysate catecholamine responses between acetylcholine infusion (10 μM, $n = 5$) and nerve stimulation (2 Hz, $n = 5$). Values are mean ± S.E.

acetylcholinesterase. Interstitial choline is carried into the nerve endings through neuronal transporters and used as a precursor for synthesis of acetylcholine (Taylor and Brown, 1998). In in vitro perfused experiments, continuous administration of choline sustains the synthesis and release of acetylcholine from nerve endings. In the present study, the concentration of dialysate choline was more than 10 times that of dialysate acetylcholine during nerve stimulation, and repetitive acetylcholine release did not induce a decrease in dialysate choline concentration. Moreover, nerve stimulation elicited almost identical responses of dialysate acetylcholine on repetition. These results indicate that repetitive acetylcholine release did not decrease interstitial choline levels and did not affect release of acetylcholine. Thus, under in vivo conditions, adrenal interstitial choline levels may be sufficiently high to sustain acetylcholine synthesis in the pre-ganglionic nerve endings.

4.3. Catecholamine release induced by endogenous and exogenous acetylcholine

Either exogenous or endogenous acetylcholine evokes catecholamine release by activating cholinergic receptors on the surface of chromaffin cells (Douglas, 1975). The interstitial acetylcholine levels serves as an index of input into chromaffin cells. We examined whether the estimated interstitial acetylcholine levels were identical between exogenous acetylcholine and nerve stimulation when

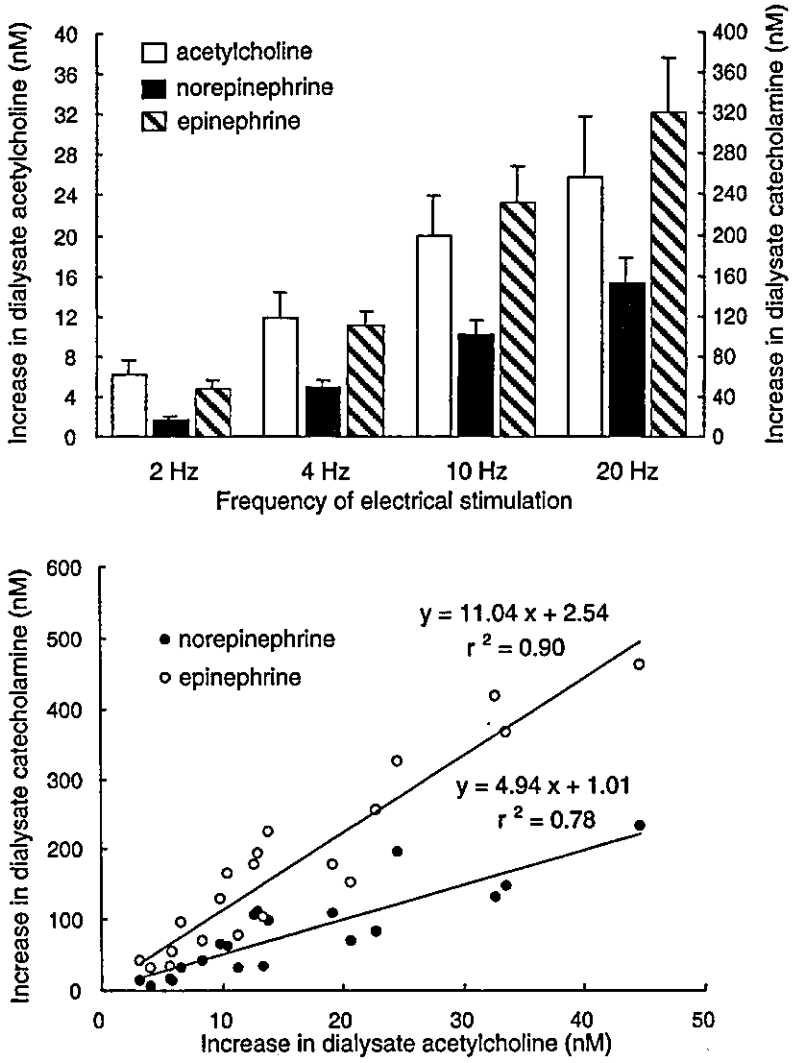

Fig. 2. (*Upper panel*) Dialysate acetylcholine response was enhanced from 6 ± 1 to 26 ± 6 nM when the frequency of nerve stimulation was increased from 2 to 20 Hz. Similarly, dialysate norepinephrine and epinephrine responses were enhanced from 16 ± 4 to 152 ± 26 nM and 48 ± 9 to 321 ± 54 nM, respectively, $n = 5$. Values are mean ± S.E. (*Lower panel*) The relationship between dialysate catecholamine response (*ordinate*) and dialysate acetylcholine response (*abscissa*) of five rats. Dialysate norepinephrine and epinephrine responses correlated with dialysate acetylcholine responses. These relations were expressed by regression equations with correlation coefficients of $y = 4.94x + 1.01$, $r^2 = 0.78$, and $y = 11.04x + 2.54$, $r^2 = 0.90$, respectively.

dialysate catecholamine responses were equal. Actually the estimated interstitial acetylcholine levels during nerve stimulation (2 Hz) were identical with those during acetylcholine infusion (10 μM). These data indicate that inputs into chromaffin cells were almost identical between the two stimulations. It could be inferred from this finding that dialysate acetylcholine concentration reflects acetylcholine levels at the surface of chromaffin cells and serves as an index of cholinergic transmission in the adrenal medulla.

4.4. Relationship of acetylcholine and catecholamine release

Dialysate norepinephrine and epinephrine responses were correlated with the frequency of splanchnic nerve stimulation. This norepinephrine and epinephrine release occurred as a consequence of acetylcholine release by splanchnic nerve stimulation. We found a linear relation between dialysate acetylcholine response and dialysate catecholamine responses. This indicates that the input–output relationship in the adrenal medulla is linear over the range of frequency from 2 to 20 Hz. Dialysate acetylcholine response of 1 nM evoked dialysate norepinephrine response of about 5 nM and dialysate epinephrine response of about 11 nM. This relation between dialysate acetylcholine and catecholamine responses could provide quantitative information about the input–output relationship in the adrenal medulla.

4.5. Effects of cholinergic receptor antagonists

It has been suggested that acetylcholine release from pre-ganglionic nerve endings is modulated by pre-synaptic

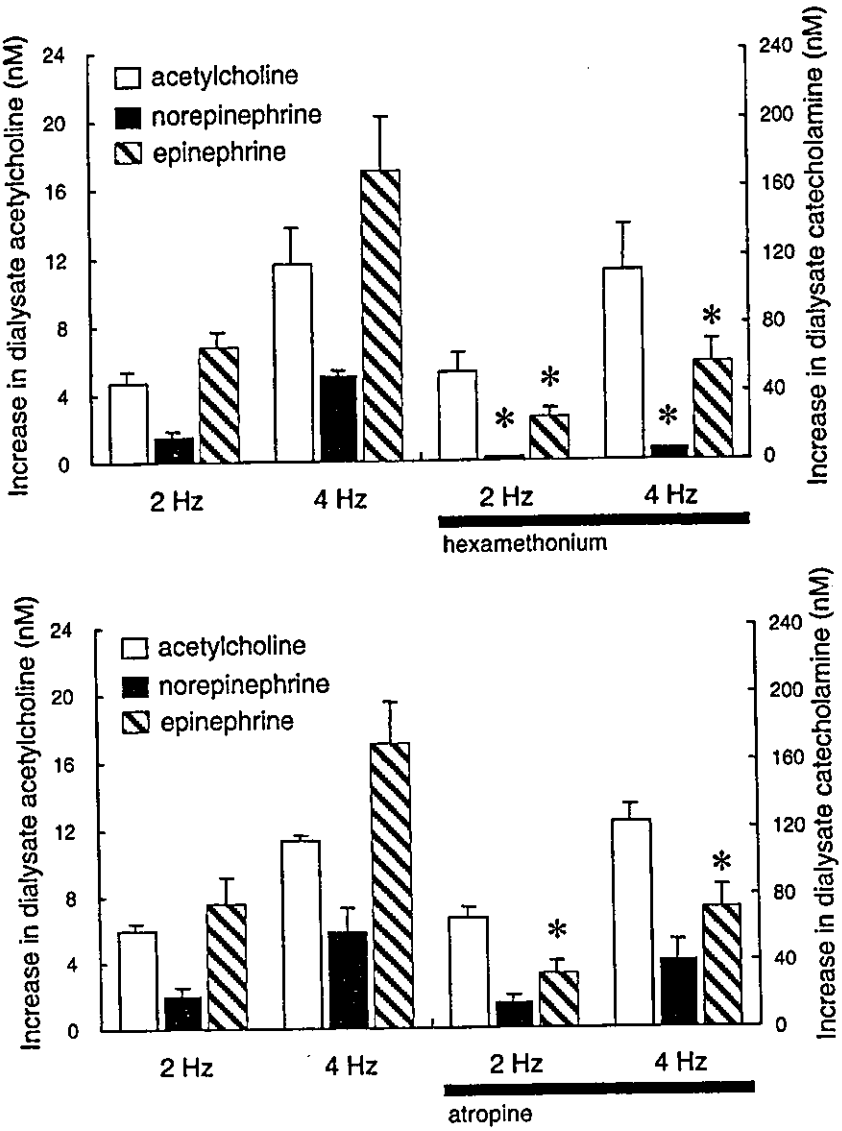

Fig. 3. (*Upper panel*) At both 2 and 4 Hz of nerve stimulation, hexamethonium suppressed dialysate norepinephrine and epinephrine responses, but did not affect acetylcholine response, $n = 5$. Values are mean ± S.E. $^*P < 0.05$ vs. concurrent dialysate norepinephrine or epinephrine response before administration of hexamethonium. (*Lower panel*) At both 2 and 4 Hz of nerve stimulation, atropine suppressed epinephrine response, but did not affect norepinephrine and acetylcholine responses, $n = 5$. Values are mean ± S.E. $^*P < 0.05$ vs. concurrent dialysate norepinephrine or epinephrine response before administration of atropine.

cholinergic autoreceptors (Dujic et al., 1990; Myers and Undem, 1996; Barbara et al., 1998). Neostigmine might induce the activation of pre-synaptic cholinergic receptors by increasing the acetylcholine levels in synaptic regions (Brehm et al., 1992) and suppress acetylcholine release by activating pre-synaptic autoreceptors. In the present study, neither hexamethonium nor atropine affected dialysate acetylcholine response to nerve stimulation at either 2 or 4 Hz. Thus, autoinhibition of acetylcholine release can be considered insignificant in our experimental condition, and dialysate acetylcholine response reflects pre-ganglionic nerve activities. In contrast, hexamethonium suppressed norepinephrine and epinephrine releases by nerve stimulation whereas atropine suppressed only epinephrine release. The muscarinic agonist, muscarine or pilocarpine preferentially enhanced epinephrine release (Douglas and Poisner, 1965; Wakade and Wakade, 1983). These results suggest that both nicotinic and muscarinic receptors exist on the surface of epinephrine-storing cells, while, on the surface of norepinephrine-storing cells, nicotinic receptors are primarily present.

4.6. Methodological limitations

We locally administered neostigmine to adrenal medulla through dialysis probe. Cholinesterase inhibitor was necessary to detect acetylcholine even during splanchnic nerve stimulation because released acetylcholine is rapidly

degraded by acetylcholinesterase before reaching the dialysis fiber. In the same preparation, local administration of neostigmine enhanced the dialysate catecholamine response to nerve stimulation by about three-fold, but did not influence the responses of heart rate and mean arterial pressure (Akiyama et al., 2003). Total catecholamine release from adrenal gland might not change by the local administration of neostigmine. In the present study, dialysate catecholamine response was correlated with the frequency of splanchnic nerve stimulation. Thus, in the presence of neostigmine, absolute value of dialysate catecholamine response is exaggerated, but could reflect relative changes in catecholamine release from adrenal gland.

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Short communication

In vivo assessment of catechol *O*-methyltransferase activity in rabbit skeletal muscle

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Abstract

With the use of microdialysis technique in the anesthetized rabbit, we examined the catechol *O*-methyltransferase (COMT) activity at the skeletal muscle interstitium. We implanted a dialysis probe into the adductor muscle, and monitored dialysate catecholamines and their metabolites with chromatogram-electrochemical detection. Administration of COMT inhibitor (entacapone) decreased dialysate 3-methoxy 4-hydroxyphenylglycol (MHPG) levels. Local administration of dihydroxyphenylglycol induced increases in dialysate MHPG levels. These increases in dialysate MHPG levels were suppressed by the addition of entacapone. The concentration of MHPG in the skeletal muscle dialysate corresponded to the COMT activity in the skeletal muscle. Furthermore, local administration of norepinephrine or epinephrine increased normetanephrine or metanephrine levels in dialysate but not MHPG levels. Skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.

Keywords: Catecholamine; Catechol *O*-methyltransferase; Entacapone; Microdialysis; Skeletal muscle

Catechol *O*-methyltransferase (COMT) exerts a critical action on the inactivation of catecholamrines and catecholestrogens (Boulton and Eisenhofer, 1998). COMT enzyme exists in almost all mammalian tissues and organs (Karhunen et al., 1994; Männistö and Kaakkola, 1999). The wide distribution of COMT in different tissues suggests an important physiological role for COMT activity. In vitro COMT activity has been widely assessed in various tissues (Männistö and Kaakkola, 1999; Tsunoda et al., 2002), while in vivo COMT activity has been assessed only in erythrocyte (Toumainen et al., 1996). To determine whether COMT activity is involved in cardiovascular regulation, we need information about in vivo COMT activity in organs and tissues.

A sophisticated technique using radiotracers has been employed for spillover of organ specific metabolite formed by COMT activity (3-methoxy 4-hydroxyphenylglycol, MHPG) (Lambert et al., 1995). This study suggested that majority of MHPG in plasma was derived from skeletal muscle, with the exception of central nervous system. Dispersed organs, such as skeletal muscle, have a thin and diffuse sympathetic innervation, but skeletal muscle is one candidate suitable for investigating regional MHPG production (Tokunaga et al., 2003a,b). This organ is suited to microdialysis probe implantation. Recently we have developed the skeletal muscle microdialysis for the monitoring of catecholamines and their metabolites. At the skeletal muscle, the small amounts of dialysate norepinephrine and its metabolites could be determined by microdialysis with electrochemical detection.

In the present study, we examined whether COMT blocker affected regional norepinephrine kinetics at the skeletal muscle interstitial spaces. With the use of dialysis technique, the dialysate was sampled from the skeletal muscle, and dialysate catecholamines and their metabolites levels were measured with liquid chromatography. Further, the study was designed to examine regional *O*-methylation products evoked by local administration of catecholamine and determine whether these data provide information about in vivo regional COMT activity.

Male Japanese white rabbits weighing 2.6–3.1 kg each were anesthetized with pentobarbital sodium (30–35 mg/kg,

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i.v.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). After tracheotomy, the animals were ventilated with room air mixed with oxygen. Body temperature was maintained with a heated pad and lamp. All protocols were performed in accordance with the American Physiological Society guidelines for the use of animals. After a longitudinal skin incision was made in the left groin, the dialysis probes were implanted in the left adductor muscle with a fine guiding needle.

For skeletal muscle dialysis, we designed a transverse dialysis probe. The dialysis fiber (13 mm length, 0.31 mm O.D. and 0.2 mm I.D.; PAN-1200, 50,000 molecular mass cutoff, Asahi Chemical, Tokyo, Japan) was glued at both ends into a polyethylene tube (25 cm length, 0.5 mm O.D. and 0.2 mm I.D.) (Akiyama et al., 1991; Tokunaga et al., 2003a,b). The dialysis probe was perfused with Ringer solution at a speed of 10 μl/min using a microinjection pump (CMA 102, Carnegie Medicin, Stockholm, Sweden). Dialysate catecholamines and their metabolite concentrations were measured by high-performance liquid chromatography with electrochemical detection (Takauchi et al., 1997; Tokunaga et al., 2003a,b; Yamazaki et al., 1995).

Basal dialysate norepinephrine, dihydroxyphenylglycol (DHPG) and MHPG levels were presented in Table 1. Entacapone (COMT blocker) was intraperitoneally administered (10 mg/kg) (Illi et al., 1995; Scheinin et al., 1998). Administration of entacapone decreased the MHPG level of dialysate but increased the DHPG levels of dialysate. The dialysate norepinephrine levels were not affected by entacapone. These changes were preserved 2 h after administration of entacapone.

To examine regional COMT activity, we measured the formation of MHPG evoked by local administration of exogenous DHPG via dialysis probe. We determined doses of DHPG based on the dialysate DHPG concentration in the previous experiments (Akiyama and Yamazaki, 2001). Local administration of DHPG (25, 250 ng/ml) dose-dependently increased the MHPG levels of dialysate (Fig. 1). These increases in the MHPG levels were prevented by pretreatment with entacapone.

In this study, exogenous DHPG dose-dependently increased the MHPG levels of dialysate. Exogenous DHPG via the dialysis probe easily traversed the cell membrane and reached skeletal muscle (Goldstein et al., 1998). In contrast, entacapone significantly decreased the MHPG levels of dialysate. These data suggest that regional COMT activity corresponds to the production of dialysate MHPG levels. Furthermore, entacapone induced a decrease in the dialysate MHPG level accompanied by an increase in the dialysate DHPG but not norepinephrine level. Therefore we consider that regional DHPG is one possible substrate for MHPG production, and that the concentration of MHPG or MHPG/DHPG ratio in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle.

Table 1
Basal dialysate NE, DHPG, and MHPG levels in rabbit skeletal muscle

	Before entacapone	After entacapone
NE (pg/ml)	8±1	10±1
DHPG (pg/ml)	27±4	53±11*
MHPG (pg/ml)	198±12	147±18*

NE, norepinephrine; DHPG, dihydroxyphenylglycol; MHPG, 3-methoxy 4-hydroxyphenylglycol. Values are means±SE. n=5.
* P<0.05 vs. values before entacapone.

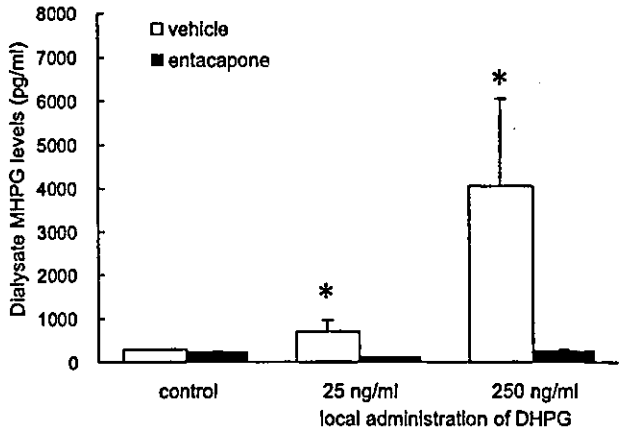

Fig. 1. Effects of exogenous dihydroxyphenylglycol (DHPG) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of DHPG dose-dependently increased the MHPG levels of dialysate. These increases in the MHPG levels were prevented by pretreatment with entacapone. Values are means±SE (n=5). *P<0.05 vs. control.

Earlier studies suggested species and organ differences in extraneuronal uptake and COMT activity (Scheinin et al., 1998; Tsunoda et al., 2002). Extraneuronal norepinephrine uptake and COMT activity were well examined in rabbit heart with the findings suggesting that rabbit heart hardly metabolizes isoprenaline to methoxyprenaline (Lindmar and Löffelholz, 1974). Thus rabbit heart seems to have a very poorly developed extraneuronal system, including weak COMT activity, for the uptake and metabolism of catecholamines (Trendelenburg, 1978). On the other hand, rabbit aortic strips have a high capacity for COMT activity (Levin, 1974). From these and previous data (Tokunaga et al., 2003a,b), the ratio of MHPG/DHPG in myocardium and skeletal muscle were 1.0±0.2 and 7.9±1.3, respectively. Rabbit skeletal muscle seems to have a well-developed COMT activity. In the skeletal muscle sympathetic innervation was not dense, and the DHPG levels were less than that of heart (Tokunaga et al., 2003a,b). Therefore, other compounds or plasma DHPG might be involved in the regional formation of MHPG in the skeletal muscle.

MHPG is produced by extraneuronal *O*-methylation of DHPG formed intraneuronally from norepinephrine or by the extraneuronal combination of COMT and monoamine oxidase (MAO) on norepinephrine and epinephrine (Akiyama and Yamazaki, 2001; Eisenhofer et al., 1988). Therefore, MHPG is mainly yielded from DHPG, norepinephrine or epinephrine at the skeletal muscle. Furthermore,

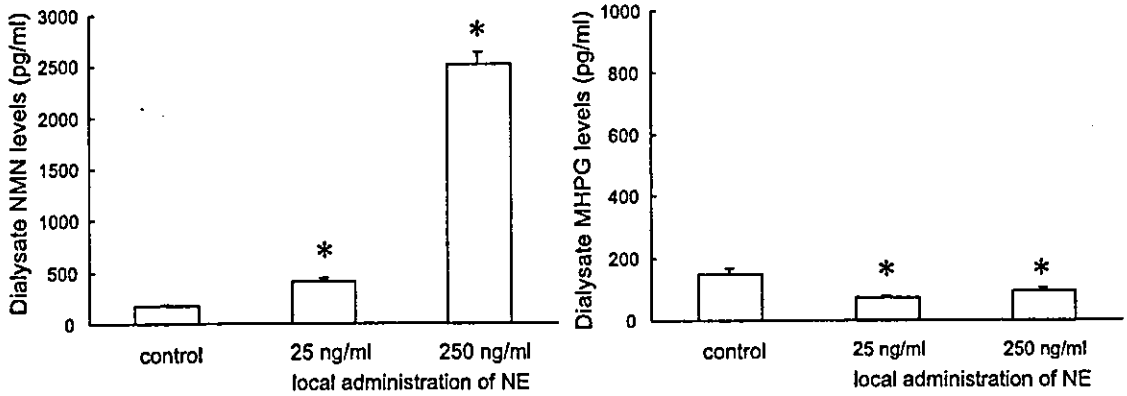

Fig. 2. Effects of exogenous norepinephrine (NE) infusion on the 3-methoxy 4-hydroxyphenylglycol (MHPG) and normetanephrine (NMN) production. Local administration of NE dose-dependently increased the NMN levels of dialysate but not MHPG levels. Values are means±SE (n=5). *P<0.05 vs. control.

O-methylation of catechol compounds includes MHPG, normetanephrine and metanephrine. We examined the relation between catecholamines and their metabolites. To compare norepinephrine and epinephrine with DHPG infusion, norepinephrine or epinephrine infusion with similar doses of DHPG was administered. Local administration of norepinephrine increased the normetanephrine levels of dialysate but not the MHPG levels (Fig. 2). Local administration of epinephrine increased the metanephrine levels of dialysate but not the MHPG levels (Fig. 3). Our data suggest that only DHPG is a possible substrate for MHPG production. Local administration of norepinephrine or epinephrine produced normetanephrine or metanephrine but not MHPG. Or rather, norepinephrine or epinephrine caused a decrease in the dialysate MHPG level. These data are consistent with data on the origins of plasma MHPG in rats, which indicated that most MHPG arises from *O*-methylation of the DHPG by intraneuronal deamination of norepinephrine (Eisenhofer et al., 1994).

Our data indicate that COMT exerts an important role on the degradation of cathecholamines in the skeletal muscular interstitium. Muscular catecholamines derive from circulating blood and surrounding sympathetic nerve systems (Tokunaga et al., 2003a,b). Therefore, COMT activity in the skeletal muscle may be related to regional or systemic sympathetic nerve activity. The relationship between regional COMT activity and sympathetic nerve activity remains to be further examined. Muscle sympathetic nerve activity is involved in the regulation of vascular tone and glucose metabolism in the skeletal muscle (Lundvall and Edfeldt, 1994; Spraul et al., 1994). Further studies concerning the physiological role of regional COMT activity on vascular or metabolic control are warranted.

To our knowledge, this is the first report on the in vivo assessment of COMT activity by direct measurement of dialysate MHPG, normetanephrine, and metanephrine obtained from skeletal muscle. Local administration of DHPG increased the MHPG levels of dialysate. These increases in MHPG were prevented by pretreatment with a COMT inhibitor. Therefore we consider that the concentration of MHPG in the skeletal muscle dialysate might correspond to the COMT activity in the skeletal muscle. Measurement of MHPG/DHPG ratio or MHPG formation evoked by DHPG infusion in skeletal muscle may be particularly appropriate for providing information about regional COMT activity. Thus skeletal muscle microdialysis with local administration of catecholamine offers a new method for in vivo assessment of regional COMT activity.

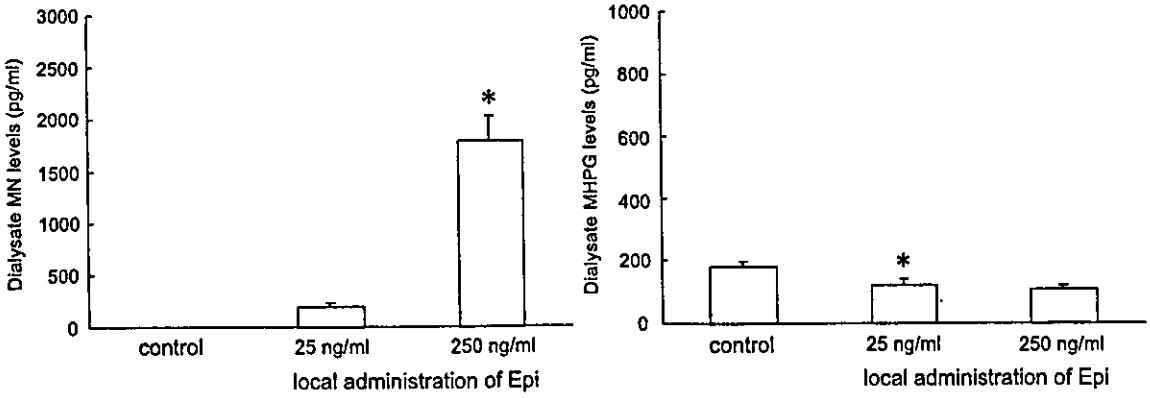

Fig. 3. Effects of exogenous epinephrine (Epi) infusion on the metanephrine (MN) and 3-methoxy 4-hydroxyphenylglycol (MHPG) production. Local administration of Epi increased the MN levels of dialysate but not MHPG levels. Values are means±SE (n=5). *P<0.05 vs. control.

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Effects of Ca^{2+} channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla

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Akiyama, Tsuyoshi, Toji Yamazaki, Hidezo Mori, and Kenji Sunagawa. Effects of Ca^{2+} channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla. *Am J Physiol Regul Integr Comp Physiol* 287: R161–R166, 2004. First published March 18, 2004; 10.1152/ajpregu.00609.2003.—To elucidate the types of voltage-dependent Ca^{2+} channels controlling ACh and catecholamine releases in the in vivo adrenal medulla, we implanted microdialysis probes in the left adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on ACh, norepinephrine, and epinephrine releases induced by nerve stimulation. The dialysis probes were perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine. The left splanchnic nerves were electrically stimulated at 2 and 4 Hz before and after intravenous administration of Ca^{2+} channel antagonists. ω-Conotoxin GVIA (an N-type Ca^{2+} channel antagonist, 10 μg/kg) inhibited ACh release at 2 and 4 Hz by ~40%, norepinephrine release at 4 Hz by ~50%, and epinephrine release at 2 and 4 Hz by ~45%. A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit these releases. ω-Conotoxin MVIIC (a P/Q-type Ca^{2+} channel antagonist, 50 μg/kg) inhibited ACh and epinephrine releases at 4 Hz by ~30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh release at 2 and 4 Hz by ~70% and norepinephrine and epinephrine releases at 2 and 4 Hz by ~80%. Nifedipine (an L-type Ca^{2+} channel antagonist, 300 and 900 μg/kg) did not change ACh release at 2 and 4 Hz; however, nifedipine (300 μg/kg) inhibited epinephrine release at 4 Hz by 20%, and nifedipine (900 μg/kg) inhibited norepinephrine and epinephrine releases at 4 Hz by 30%. In conclusion, both N- and P/Q-type Ca^{2+} channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca^{2+} channels do not. L-type Ca^{2+} channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

anesthetized rats; microdialysis; norepinephrine; epinephrine; preganglionic autonomic nerve endings

Ca^{2+} influx through the voltage-dependent Ca^{2+} channels induces the release of transmitters from neuronal or secretory cells by initiating exocytosis from vesicles. Voltage-dependent Ca^{2+} channels have been classified into L-, N-, P-, Q-, R-, and T-types (12, 25, 30). To better understand the mechanism controlling the release of transmitters, it is important to determine the type of Ca^{2+} channels involved in the release of the transmitters on neuronal or secretory cells.

In the in vivo adrenal medulla, catecholamine release is controlled by central sympathetic neurons through preganglionic splanchnic nerves. Splanchnic nerve endings make synaptic-like contacts with chromaffin cells (9). ACh released from splanchnic nerve endings consequently evokes catecholamine release from chromaffin cells by activation of cholinergic receptors. Thus, in vivo catecholamine release requires Ca^{2+} influx through the voltage-dependent Ca^{2+} channels at two different sites in the adrenal medulla: splanchnic nerve endings and chromaffin cells. Numerous studies have investigated the nature of Ca^{2+} channels controlling transmitter release from postganglionic autonomic nerve endings (8, 11, 32, 33, 36, 37). Little information is, however, available on the type of Ca^{2+} channels controlling the ACh release from preganglionic autonomic nerve endings including splanchnic nerve endings. Moreover, although the types of Ca^{2+} channels controlling catecholamine release have been investigated using isolated chromaffin cells in various species (5, 6, 13, 16, 21, 23, 24), it remains unknown whether endogenous ACh induces Ca^{2+} influx through the same types of Ca^{2+} channels on chromaffin cells.

We have recently developed a dialysis technique to simultaneously monitor ACh and catecholamine releases in the in vivo adrenal medulla (2). This method makes it possible to characterize Ca^{2+} channels controlling ACh release from splanchnic nerve endings and catecholamine release from adrenal medulla in the in vivo state. In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on dialysate ACh and catecholamine responses induced by the electrical stimulation of splanchnic nerves.

MATERIALS AND METHODS

Animal preparation. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996). Adult male Wistar rats weighing 380–450 g were anesthetized with pentobarbital sodium (50–55 mg/kg ip). A cervical midline incision was made to expose the trachea, which was then cannulated. The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. The left femoral artery and vein were cannulated for monitoring arterial blood pressure and administration of anesthetic, respectively. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 $mg \cdot kg^{-1} \cdot h^{-1}$ iv). Electrocardiogram was monitored for recording heart rate. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38°C. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a subcostal flank incision, and the left splanchnic nerve was transected. Shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden) with a rectangular pulse (10 V and 1 ms in duration).

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Dialysis technique. The materials of the dialysis probe were the same as those used in our previous dialysis experiments (1, 2). Briefly, each end of the dialysis fiber (0.31 mm OD, and 0.20 mm ID; PAN-1200 50,000 mol wt cutoff, Asahi Chemical) was inserted into the polyethylene tube (25-cm length, 0.5 mm OD, and 0.2 mm ID; SP-8) and glued. The length of the dialysis fiber exposed was 3 mm.

The left adrenal gland was gently lifted, and the dialysis probe was implanted in the medulla of the left adrenal gland along the long axis by using a fine guiding needle. The dialysis probe was perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine (10 μM), at a speed of 10 μl/min using a microinjection pump (CMA/100, Carnegie Medicin). Ringer solution with no buffer consisted of (in mM) 147.0 NaCl, 4.0 KCl, and 2.25 $CaCl_2$. One sampling period was 2 min (1 sample volume = 20 μl). We started the protocols followed by a stabilization period of 3–4 h and sampled dialysate taking the dead space volume into account.

Dialysate ACh, norepinephrine (NE), and epinephrine (Epi) concentrations were measured as indexes of ACh and catecholamine releases in the adrenal medulla. Half of the dialysate sample was used for the measurement of ACh, and the remaining half for the measurement of NE and Epi. ACh and catecholamine assays were separately conducted using each high-performance liquid chromatography with electrochemical detection as previously described (3, 4).

Experimental design. The experiment was performed based on the previous experiment showing that dialysate ACh and catecholamine responses were reproducible on repetition of stimulation (2). The left splanchnic nerves were electrically stimulated for 2 min at 30-min intervals. Three dialysate samples were continuously collected per electrical stimulation: one before, one during, and one after stimulation. Stimulations at two different frequencies (2 and 4 Hz) were performed before and after intravenous administration of Ca^{2+} channel antagonists.

We tested three types of Ca^{2+} channel antagonists (25): the N-type Ca^{2+} channel antagonist ω-conotoxin GVIA, the P/Q-type Ca^{2+} channel antagonist ω-conotoxin MVIIC, and the L-type Ca^{2+} channel antagonist nifedipine. We determined the first doses of Ca^{2+} channel antagonists based on the dose used in the earlier experiments (7, 14, 26, 29, 37) and tested ω-conotoxin GVIA (10 μg/kg) in six rats, ω-conotoxin MVIIC (50 μg/kg) in six rats, and nifedipine (300 μg/kg) in six rats. Second, we tested a fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) in six rats, a combination of fivefold higher doses of ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) in six rats, and a threefold higher dose of nifedipine (900 μg/kg) in six rats. We did not test a higher dose of ω-conotoxin MVIIC singly because a high dose of ω-conotoxin MVIIC loses its selectivity for P/Q-type and inhibits N-type Ca^{2+} channels (18).

Nifedipine was administered twice before 2- and 4-Hz stimulation, but ω-conotoxin GVIA and MVIIC were administered once before 2-Hz stimulation because the ω-conotoxin family has long-lasting blocking actions (8, 18, 36). We assessed the responses to nerve stimulation 30, 20, and 10 min after administration of ω-conotoxin GVIA, ω-conotoxin MVIIC, and nifedipine, respectively, when heart rate and arterial pressure had already been stabilized.

At the end of the experiment the rats were killed with pentobarbital sodium, and the implant sites were examined. The dialysis probes were confirmed to have been implanted in the adrenal medulla, and no bleeding or necrosis was found macroscopically.

Drugs. Drugs were mixed fresh for each experiment. Neostigmine methylsulfate (Shionogi), ω-conotoxin GVIA (Peptide Institute), and ω-conotoxin MVIIC (Peptide Institute) were dissolved and diluted in Ringer solution. Nifedipine (Sigma Chemical) was dissolved in ethanol and diluted in Ringer solution.

Statistical methods. To examine the effects of nerve stimulation and Ca^{2+} channel antagonists, we analyzed heart rate and mean arterial pressure and dialysate ACh, NE, and Epi responses by using one-way ANOVA with repeated measures. When statistical significance was detected, the Newman-Keuls test was applied (35). Statistical significance was defined as $P < 0.05$. Values are presented as means ± SE.

RESULTS

Effects of Ca^{2+} channel antagonists on heart rate and mean arterial pressure. ω-Conotoxin GVIA (10 μg/kg) decreased heart rate from 418 ± 9 to 328 ± 13 beats/min ($P < 0.05$) and mean arterial pressure from 115 ± 2 to 74 ± 2 mmHg ($P < 0.05$). ω-Conotoxin GVIA (50 μg/kg) did not further decrease heart rate and mean arterial pressure. ω-Conotoxin MVIIC decreased heart rate from 408 ± 3 to 390 ± 5 beats/min ($P < 0.05$) but did not change mean arterial pressure. Combined ω-conotoxin GVIA and MVIIC decreased heart rate from 415 ± 10 to 327 ± 4 beats/min ($P < 0.05$) and mean arterial pressure from 124 ± 2 to 57 ± 2 mmHg ($P < 0.05$). Nifedipine (300 μg/kg) decreased mean arterial pressure from 113 ± 4 to 86 ± 4 mmHg ($P < 0.05$) but did not change heart rate. Nifedipine (900 μg/kg) decreased mean arterial pressure from 124 ± 3 to 73 ± 2 mmHg ($P < 0.05$).

Effects of Ca^{2+} channel antagonists on ACh and catecholamine releases. ACh could not be detected in dialysate before or after stimulation. Thus we expressed dialysate ACh concentration during stimulation as an index of ACh release induced by stimulation. In contrast, substantial amounts of NE and Epi were observed in dialysate before stimulation. Intravenous administration of Ca^{2+} channel antagonists did not affect these basal NE and Epi releases (Table 1). Dialysate NE and Epi concentrations increased by nerve stimulation and rapidly declined after the stimulation. Thus we subtracted the dialysate NE and Epi contents before stimulation from those during stimulation and expressed these values as indexes of NE and Epi releases induced by stimulation.

Effects of ω-conotoxin GVIA. ω-Conotoxin GVIA (10 μg/kg) significantly inhibited ACh release at 2 Hz from 6.2 ± 0.9 to 3.6 ± 0.5 nM, ACh release at 4 Hz from 12.2 ± 1.7 to 7.9 ± 1.2 nM, NE release at 4 Hz from 34 ± 6 to 17 ± 3 nM, Epi release at 2 Hz from 81 ± 13 to 42 ± 3 nM, and Epi release at 4 Hz from 180 ± 21 to 94 ± 7 nM. However, inhibition of NE release at 2 Hz was not statistically significant (Fig. 1*A*). A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not

Table 1. *Basal dialysate NE and Epi concentrations before and after administration of Ca^{2+} channel antagonists*

	NE, nM	Epi, nM
ω-Conotoxin GVIA (10 and 50 μg/kg) (n = 12)		
Before administration	4.9±0.9	20.6±2.9
After administration	3.8±0.6	21.0±2.6
ω-Conotoxin MVIIC (50 μg/kg) (n = 6)		
Before administration	4.1±1.0	20.2±2.6
After administration	4.6±0.9	24.0±3.5
ω-Conotoxin GVIA (50 μg/kg) + MVIIC (250 μg/kg) (n = 6)		
Before administration	4.4±1.3	17.5±3.8
After administration	3.1±0.7	20.8±4.4
Nifedipine (100 and 300 μg/kg) (n = 12)		
Before administration	4.0±0.6	17.4±2.2
After administration	3.3±0.9	17.7±2.9

Values are means ± SE; *n*, no. of rats. NE, norepinephrine; Epi, epinephrine.

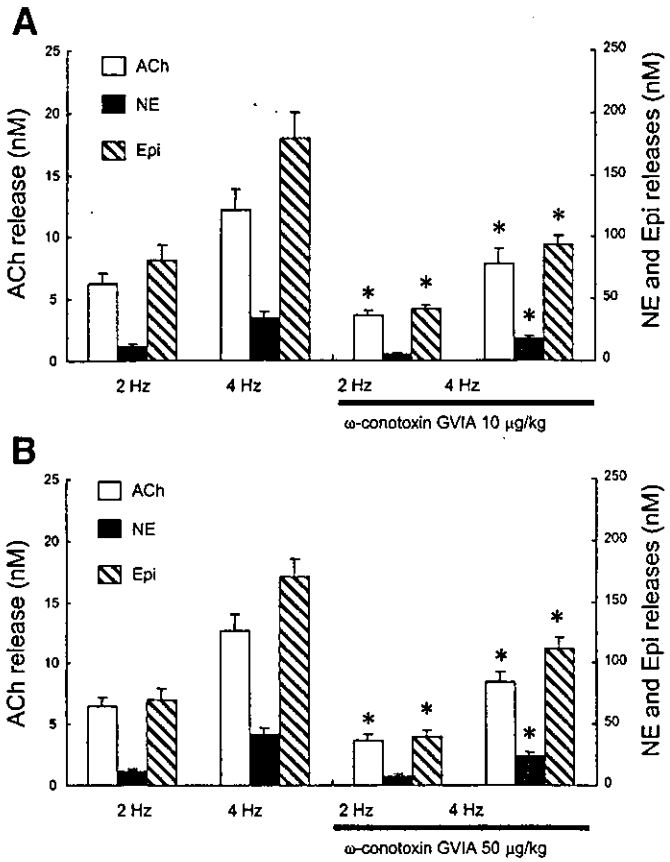

Fig. 1. Effects of ω-conotoxin GVIA on ACh, norepinephrine (NE), and epinephrine (Epi) releases. ω-Conotoxin GVIA (10 μg/kg) inhibited ACh release at 2 and 4 Hz, NE release at 4 Hz, and Epi release at 2 and 4 Hz (*A*). A 5-fold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit these releases (*B*). Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

further inhibit release. ω-Conotoxin GVIA (50 μg/kg) significantly inhibited ACh release at 2 Hz from 6.5 ± 0.7 to 3.7 ± 0.5 nM, ACh release at 4 Hz from 12.6 ± 1.4 to 8.5 ± 0.8 nM, NE release at 4 Hz from 41 ± 6 to 24 ± 4 nM, Epi release at 2 Hz from 70 ± 10 to 40 ± 6 nM, and Epi release at 4 Hz from 170 ± 15 to 112 ± 10 nM (Fig. 1*B*).

Effects of ω-conotoxin MVIIC. ω-Conotoxin MVIIC (50 μg/kg) significantly inhibited ACh release at 4 Hz from 11.7 ± 2.5 to 8.5 ± 2.1 nM and Epi release at 4 Hz from 170 ± 38 to 129 ± 35 nM. Inhibitions of ACh and Epi releases at 2 Hz and NE release at either frequency were not statistically significant (Fig. 2).

Effects of combined ω-conotoxin GVIA and MVIIC. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) significantly inhibited ACh release at 2 Hz from 6.7 ± 0.6 to 1.9 ± 0.3 nM, ACh release at 4 Hz from 12.1 ± 1.3 to 3.8 ± 0.6 nM, NE release at 2 Hz from 11.1 ± 1.1 to 1.2 ± 0.3 nM, NE release at 4 Hz from 36 ± 5 to 8 ± 2 nM, Epi release at 2 Hz from 88 ± 9 to 13 ± 3 nM, and Epi release at 4 Hz from 187 ± 20 to 49 ± 9 nM (Fig. 3).

Effects of nifedipine. Nifedipine (300 μg/kg) did not change ACh release at either frequency but significantly inhibited Epi release at 4 Hz from 172 ± 31 to 135 ± 23 nM. Inhibitions of Epi release at 2 Hz and NE release at either frequency were not statistically significant (Fig. 4*A*). A threefold higher dose of

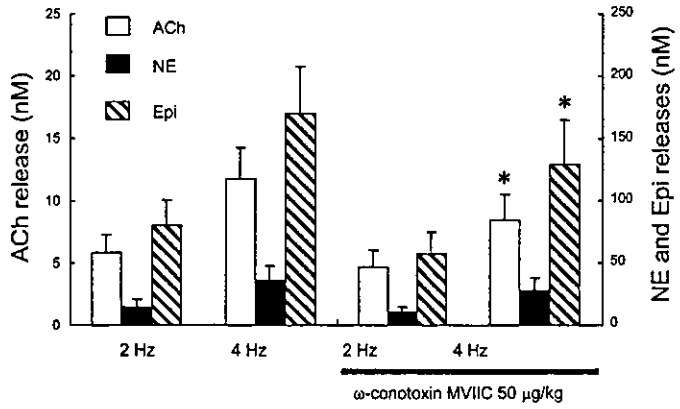

Fig. 2. Effects of ω-conotoxin MVIIC on ACh, NE, and Epi releases. ω-Conotoxin MVIIC (50 μg/kg) inhibited ACh and Epi releases at 4 Hz. Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

nifedipine (900 μg/kg) did not change ACh release but significantly inhibited Epi release at 4 Hz from 188 ± 24 to 128 ± 15 nM and NE release at 4 Hz from 33 ± 5 to 24 ± 4 nM. Inhibitions of NE and Epi releases at 2 Hz were not statistically significant (Fig. 4*B*).

DISCUSSION

Effects of Ca^{2+} channel antagonists on ACh release from splanchnic nerve endings. In the present study, ω-conotoxin GVIA (10 μg/kg) inhibited ACh release at both 2 and 4 Hz by approximately 35–40%. A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit ACh release. ω-Conotoxin MVIIC (50 μg/kg) inhibited ACh release at 4 Hz by ~30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh release at both 2 and 4 Hz by ~70%. N- and P/Q-type Ca^{2+} channels could be present on the splanchnic nerve endings and be involved in ACh release. P/Q-type Ca^{2+} channels may play a role in ACh release at a high frequency of stimulation. ACh release response was resistant to nifedipine (300 and 900 μg/kg) at both 2 and 4 Hz. L-type Ca^{2+} channels could not be present on splanchnic nerve

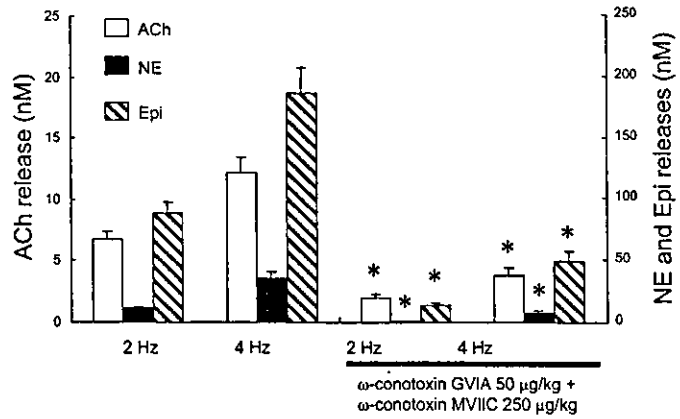

Fig. 3. Effects of combined ω-conotoxin GVIA and MVIIC on ACh, NE, and Epi releases. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh, NE, and Epi releases at 2 and 4 Hz. Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

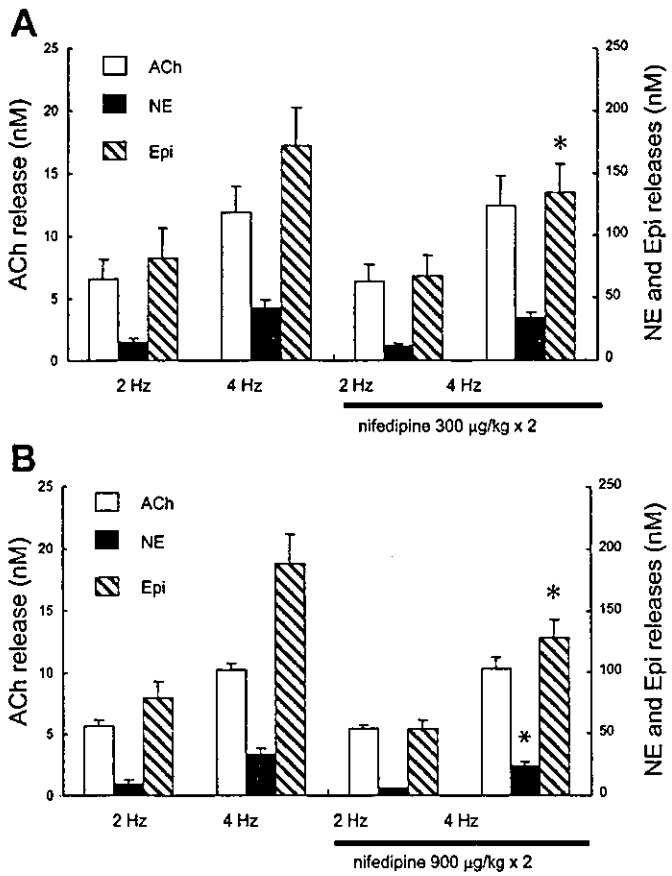

Fig. 4. Effects of nifedipine on ACh, NE, and Epi releases. Nifedipine (300 μg/kg) did not change ACh release at either frequency but inhibited Epi release at 4 Hz (*A*). Nifedipine (900 μg/kg) did not change ACh release at either frequency but inhibited NE and Epi releases at 4 Hz (*B*). Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

endings or not play a major role in ACh release. This is the first direct study to demonstrate the type of Ca^{2+} channels controlling ACh release from splanchnic nerve endings.

In isolated rat adrenal glands, catecholamine release induced by field stimulation is sensitive to P/Q-type Ca^{2+} channel antagonist, whereas that induced by exogenous ACh is insensitive (27). This indirect study suggested the involvement of P/Q-type Ca^{2+} channels in ACh release but failed to show the involvement of N-type Ca^{2+} channels. In isolated bovine adrenal glands, a direct measurement study showed that a reduction of the extracellular Ca^{2+} concentration inhibits ^{3}H-labeled ACh release induced by field stimulation, but N- and L-type Ca^{2+} channel antagonists do not (28). Thus our findings are in part consistent with these direct and indirect studies but inconsistent as to the involvement of N-type Ca^{2+} channels.

This discrepancy might be ascribed to the experimental method. The contribution of Ca^{2+} channels may vary with the type of method used to evoke ACh release. In these studies, ACh release was evoked by electrical field stimulation of isolated adrenal glands, which is known to induce ACh release but not direct depolarization of chromaffin cells (34). In the present study, ACh release was evoked in the in vivo state by electrical stimulation of splanchnic nerves. The type of Ca^{2+} channels involved in ACh release may vary with the frequency, amplitude, or time period of stimulation. Actually, in the present study, we observed the involvement of P/Q-type Ca^{2+} channels at only high-frequency stimulation, while it has been reported in perfused rat adrenal glands that N-type Ca^{2+} channels are involved in the maintenance of catecholamine release in response to long splanchnic nerve stimulation (31). The time period of 2 min in the present study seems to be longer than those in earlier studies but could be within the physiological range. Moreover, the blocking action of ω-conotoxin GVIA is time dependent as well as dose dependent and irreversible (8, 11, 32, 36). The maximum functional effect of ω-conotoxin GVIA has been observed to be at least 15 min after administration. We evaluated the effect of ω-conotoxin GVIA 30 min after intravenous administration, when heart rate and mean arterial pressure had already been stabilized. The evaluation early after administration might lead to underestimation of the inhibitory effects of ω-conotoxin GVIA.

There are many similarities between synaptic transmission from splanchnic nerves to chromaffin cells and sympathetic ganglionic transmission (17). In isolated guinea pig paravertebral ganglia, an electrophysiological study has shown that both N- and P-type Ca^{2+} channel antagonists reduce cholinergic synaptic conductance, whereas L-type Ca^{2+} channel antagonist does not (19). In isolated rat superior cervical ganglia, both N- and P-type Ca^{2+} channel antagonists inhibit the rise in Ca^{2+} concentration in the terminal boutons (22). Moreover, in isolated rat superior cervical ganglia, ^{3}H-labeled ACh release induced by high K^+ is inhibited by both N- and P-type Ca^{2+} channel antagonists but unaffected by L-type Ca^{2+} channel antagonist (15). Our findings are similar to these findings obtained from isolated sympathetic preganglionic nerves.

The inhibition by ω-conotoxin GVIA (50 μg/kg) was almost the same as that by ω-conotoxin GVIA (10 μg/kg). Moreover, the inhibition by combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) was almost algebraically the sum of the individual inhibition by ω-conotoxin GVIA (10 μg/kg) and MVIIC (50 μg/kg). These results suggest that fivefold higher doses of ω-conotoxin GVIA and MVIIC are sufficient to cause inhibition of Ca^{2+} channels. However, ~30% of ACh release was resistant to combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg). Other types of Ca^{2+} channels except for N- and P/Q-types may be involved in ACh release from splanchnic nerve endings. Further examination could be needed.

Effects of Ca^{2+} channel antagonists on catecholamine release from chromaffin cells. In the present study, nifedipine (300 μg/kg) did not change ACh release at 2 and 4 Hz but inhibited Epi release at 4 Hz by ~20%. A threefold higher dose of nifedipine (900 μg/kg) did not change ACh release at 2 and 4 Hz but inhibited NE and Epi releases at 4 Hz by ~30%. Adrenal chromaffin cells are divided into two populations: NE- and Epi-storing cells (10). L-type Ca^{2+} channels could be present on the surface of both NE- and Epi-storing cells and play a role in NE and Epi releases.

Approximately 70% of catecholamine release was resistant to nifedipine (900 μg/kg). This result suggests that other types of Ca^{2+} channels except for L-type are present on chromaffin cells and involved in NE and Epi releases, although we cannot exclude the possibility of incomplete inhibition of L-type Ca^{2+} channels. Species differences in the types of Ca^{2+} channels controlling Ca^{2+} influx and catecholamine release have been

shown with rat, cat, and bovine chromaffin cells (5, 6, 13, 24). In patch-clamp studies of isolated rat chromaffin cells, Ca^{2+} inward current elicited by depolarization is sensitive to both L- and N-type Ca^{2+} channel antagonists (16, 21). The study measuring Ba^{2+} current by patch-clamp technique has shown the existence of L-, N-, and P/Q-type Ca^{2+} channels on rat chromaffin cells and the following distribution of Ca^{2+} channels in decreasing order: L-type > N-type > P/Q-type (13). In the present study, ω-conotoxin GVIA (10 and 50 μg/kg) inhibited NE release at 4 Hz and Epi release at 2 and 4 Hz by approximately 45–50%. ω-Conotoxin MVIIC (50 μg/kg) inhibited Epi release at 4 Hz by ~30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited NE and Epi releases at 2 and 4 Hz by approximately 75–85%. However, these Ca^{2+} channel antagonists simultaneously inhibited ACh release to almost the same extent. It is difficult to determine how much Ca^{2+} antagonists are acting on chromaffin cells when Ca^{2+} channel antagonists inhibit ACh release. Thus, although much of these inhibitions of catecholamine release may be considered to be consequences of the inhibition of ACh release, we cannot exclude the possibility that N- or P/Q-type Ca^{2+} channels may be involved in the in vivo catecholamine release on chromaffin cells.

The inhibition of NE release at 2 Hz by ω-conotoxin GVIA (10 and 50 μg/kg) and the inhibition of NE release at 4 Hz by ω-conotoxin MVIIC (50 μg/kg) were not statistically significant despite significant inhibitions of ACh and Epi releases. In the same preparation, we have shown that cholinergic antagonists almost inhibited NE and Epi releases induced by nerve stimulation (1, 2). However, the correlation between ACh and NE releases was poorer than that between ACh and Epi releases when stimulation frequency was raised stepwise (2). Insignificant inhibitions of NE release may be ascribed to this poor correlation.

In the present study, Ca^{2+} channel antagonists did not affect basal dialysate NE and Epi levels. In our previous study of the same preparation, these basal levels were not affected by neostigmine, hexamethonium, or atropine (1). We then concluded that these basal dialysate NE and Epi levels reflect noncholinergic catecholamine release. N-, P/Q-, and L-type Ca^{2+} channels may not play a major role in basal noncholinergic catecholamine release from adrenal medulla.

Methodological considerations. We administered neostigmine locally to adrenal medulla through a dialysis probe. Cholinesterase inhibitor was necessary to monitor endogenous ACh even during splanchnic nerve stimulation because released ACh is rapidly degraded by acetylcholinesterase before reaching the dialysis fiber. In the same preparation, local administration of neostigmine enhanced the dialysate catecholamine response to nerve stimulation by approximately threefold, but dialysate ACh and catecholamine responses are correlated with the stimulation frequency of splanchnic nerves in the presence of neostigmine (2). Thus dialysate ACh and catecholamine responses are likely to be correlated with the amount of Ca^{2+} influx from voltage-dependent Ca^{2+} channels even in the presence of neostigmine.

Intravenous administration of Ca^{2+} channel antagonists induced changes in heart rate or mean arterial pressure. These changes might affect ACh and catecholamine releases through a baroreflex mechanism. Moreover, these hemodynamic changes might decrease the spillover of ACh or catecholamine from adrenal medulla and affect the dialysate ACh or catecholamine concentrations (20). In our preparation, however, splanchnic nerves had been transected before control sampling, and basal dialysate catecholamine concentrations did not change before or after administration. Thus effects of these hemodynamic changes could be negligible when we considered the effects of Ca^{2+} channel antagonists on nerve stimulation-induced dialysate responses.

In conclusion, we applied dialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on ACh and catecholamine releases induced by electrical stimulation of splanchnic nerves. Both N- and P/Q-type Ca^{2+} channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca^{2+} channels do not. L-type Ca^{2+} channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

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Portable X-ray generator utilizing a cerium-target radiation tube for angiography

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Abstract

The development of a portable X-ray generator with a cerium-target tube and its application to angiography are described. The portable X-ray generator consists of a main controller, a unit with a Cock–Croft circuit and an X-ray tube, and a personal computer. Negative high voltages are applied to the cathode electrode in the X-ray tube, and the tube voltage and current are regulated by the controller or the computer. The X-ray tube is a glass-enclosed double-focus diode with a cerium target and a 0.5 mm-thick beryllium window. The maximum tube voltage and current were 60 kV and 0.8 mA, respectively. The focal-spot sizes were 4 mm × 4 mm (large) and 1 mm × 1 mm (small), respectively. Angiography was performed with a computed radiography system using iodine-based microspheres. The tube voltage, the current, the distance between the imaging plate and the X-ray source, and the spot size were 60 kV, 0.4 mA, 1.5 m, and small, respectively. In this angiography, we observed coronary arteries and fine blood vessels of about 50 μm or less with high contrasts.

Keywords: Cerium-target X-ray tube; Cerium characteristic X-rays; K-absorption edge; High contrast angiography; Microangiogrphy

1. Introduction

In conjunction with single crystals, synchrotrons generate monochromatic X-rays. These rays play an important role in parallel radiography and have been employed to perform high-contrast micro-angiography [1] and phase imaging [2–4]. However, it is difficult to obtain sufficient machine times for various research projects including medical applications.

So far, several different flash X-ray generators have been developed [5,6], and soft generators [7–12] with photon energies of lower than 150 keV can be employed to perform biomedical radiography. In order to produce monochromatic X-rays, plasma flash X-ray generators [13–16] are useful, since quite intense and sharp characteristic X-rays such as lasers have been produced from weakly ionized linear plasmas of nickel, copper and molybdenum, while bremsstrahlung rays are hardly detected at all. Using these generators, the characteristic X-ray intensity substantially increased with corresponding increases in the charging voltage.

Since K-series characteristic X-rays from cerium target are absorbed effectively by iodine-based contrast mediums, a cerium-target X-ray tube is very useful in order to perform high-contrast angiography. On the other hand, cerium is a rare earth element and has a high reactivity, and it is difficult to design the target. However, the development of a cerium-target tube for high-contrast angiography has long been wished for.

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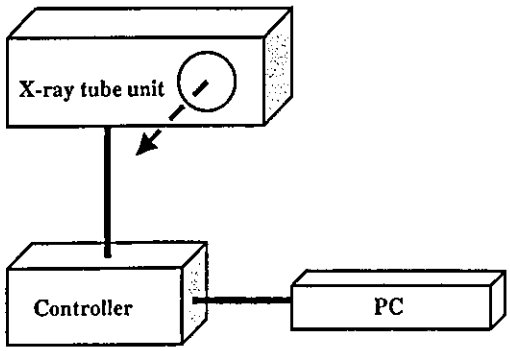

Fig. 1. Block diagram of the portable X-ray generator with a cerium-target radiation tube, which is specially used for angiography using iodine-based contrast mediums. The negative high voltage is applied to the cathode electrode, and the tube current is regulated by the filament temperature. Although the X-ray tube is a double-focus type, we usually employ a small focus in order to measure the radiographic characteristics and to perform angiography.

In the present research, we developed a portable X-ray generator with a cerium-target tube, used to perform preliminary study on angiography achieved with cerium K-series characteristic X-rays.

2. Generator

Fig. 1 shows the block diagram of the X-ray generator, which consists of a main controller, an X-ray tube unit with a Cockcroft circuit and a cerium-target tube, and a personal computer. The negative high-voltage is applied to the cathode electrode, and the anode (target) is connected to the ground potential. In this experiment, the tube voltage was regulated from 40 to 65 kV, and the tube current was regulated within 0.8 mA by the filament temperature. The exposure time is controlled in order to obtain optimum X-ray intensity, and the X-ray tube is a double-focus type with focal-spot dimensions of approximately 4 mm × 4 mm (large spot) and 1 mm × 1 mm (small spot), respectively. The maximum tube current is determined by the spot dimensions, and the currents of small and large spots are 0.4 and 0.8 mA, respectively.

3. Characteristics

3.1. X-ray intensity

X-ray intensity was measured by a Victoreen 660 ionization chamber at 1.0 m from the X-ray source using a small spot with an exposure time of 1.0 s (Fig. 2). At a constant tube current of 40 μA, the X-ray intensity increased when the tube voltage was increased. The intensity was roughly in proportion to the tube current at a constant tube voltage of 60 kV. In this measurement, the intensity with a tube voltage of 60 kV and a current of 90 μA was 2.14 μC/kg at 1.0 m from the source with errors of less than 0.2%.

3.2. X-ray source

In order to measure images of the X-ray source, we employed a pinhole camera with a hole diameter of 50 μm in conjunction with a computed radiography (CR) system (Fig. 3) [17]. When the tube voltage was increased, the spot intensity increased slightly, and spot dimensions seldom varied and had values of approximately 1 mm × 1 mm.

3.3. X-ray spectra

In order to measure X-ray spectra, we employed a cadmium tellurium detector (CDTE2020X, Hamamatsu Photonics Inc.) (Fig. 4). Compared with a germanium detector, this detector has lower energy resolutions. When the tube voltage was increased, both the characteristic X-ray intensity and the maximum photon energy of bremsstrahlung X-rays increased. According to insertion of a monochromatic cerium oxide filter, quasi-monochromatic X-rays were obtained.

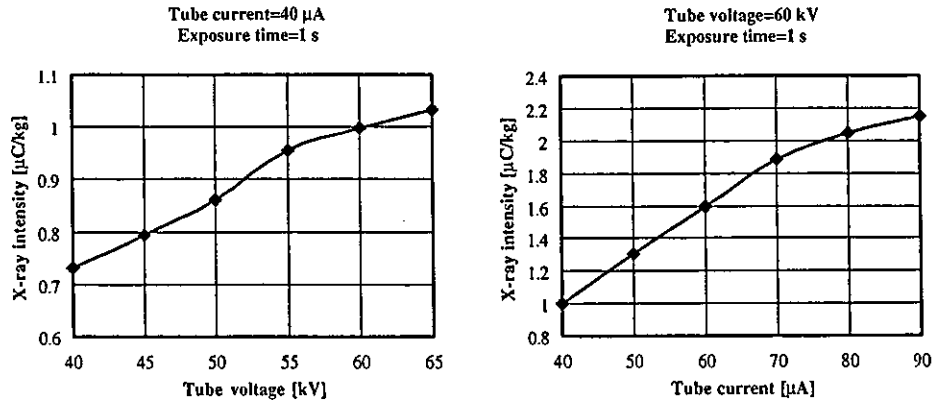

Fig. 2. X-ray intensity measured at 1.0 m from the X-ray source according to changes in the tube voltage and current. In the measurement, we employed an ionization chamber without using a monochromatic filter.