Modulatory Effects of Rat Endothelin on Central Cardiovascular Control in Rats

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Abstract To characterize the modulatory action of rat endothelin (endothelin-3 or ET-3) on the cardiovascular control by the central nervous system (CNS), ET-3 was injected into the cisterna magna of urethane-anesthetized and immobilized rats. An injection of 100 pmol of ET-3 caused immediate rises in arterial pressure (AP), renal nerve activity (RNA), and heart rate (HR). These variables subsequently decreased and, in 5-20 min, fell below the pre-injection level. Simultaneously, the arterial baroreceptor reflex was almost totally suppressed. Although RNA and HR subsequently returned to, or often exceeded, pre-injection levels in 20 to 60 min and reflex activity recovered, AP sometimes remained below control for at least 2 h. A similar pattern of changes was elicited in unanesthetized precollicular decerebrated rats. The responses to ET-3 were abolished by hexamethonium chloride, but were not conspicuously altered by arginine vasopressin antagonist or angiotensin II antagonist. The CNS sites responsible for ET-induced changes were subsequently searched. Topical application of ET-3 to the ventral surface of the medulla (VSM) caused the pattern of changes in AP, RNA, and HR similar to that following intracisternal injection. Microinjection of ET-3 into the nucl. tractus solitarius (NTS) increased AP and RNA, whereas intrathecal administration of it decreased them. We conclude that intracisternally administered ET-3 centrally modulates both tonic and reflex control of AP by the sympathetic nervous system and that the VSM appears to be primarily responsible for the modulation, although NTS and spinal cord may also be involved in it.

Key words: rat endothelin, arterial pressure, renal nerve activity, ventral surface of medulla, nucleus tractus solitarius.

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Endothelin (ET) is a potent vasoconstrictor peptide originally isolated from the supernatant of cultured porcine vascular endothelial cells (YANAGISAWA *et al.*, 1988b). Soon after discovery of this substance in the pig, rat genomic DNA library was screened and a gene predicting a 21-residue peptide remarkably similar to, but distinct from, porcine ET was cloned and sequenced. The peptide synthesized accordingly was termed rat ET, since an intravenous injection of this agent in rats caused potent and long-lasting vasoconstrictor and pressor responses similar to those elicited by porcine ET (YANAGISAWA *et al.*, 1988a). It was later found, however, that human, porcine, and rat genomes ubiquitously possessed chromosomal loci predicting porcine and rat ET (INOUE *et al.*, 1989). The two substances, originally termed porcine and rat ETs, were also called ET-1 and ET-3, respectively. Throughout this paper, rat ET and ET-3 are used interchangeably.

A peculiar feature of ET-3 is that it is expressed in the central nervous system (CNS) but not in the vascular endothelium (SHINMI *et al.*, 1989; YANAGISAWA and MASAKI, 1989). With respect to the expression of ET in the CNS, Shinmi and colleagues (SHINMI *et al.*, 1989) detected both ET-1 and ET-3 immunoreactivity in a homogenate of porcine spinal cord. They further confirmed that ET-3-like immunoreactivity was coeluted with synthetic ET-3 on a reverse-phase high-performance liquid chromatography. A more recent study by MATSUMOTO *et al.* (1989) in rats based on enzyme immunoassay demonstrated abundant presence of ET-3 in the brain. Furthermore, MACCUMBER *et al.* (1989) employed the Northern blot analysis and *in situ* hybridization in rat tissues. They maintain that the brain expresses ET-3 predominantly, whereas the lung expresses both ET-1 and ET-3. These and other results have led to a hypothesis that ET-3 is the type of ET family members specific to the nervous system (INOUE *et al.*, 1989; VANE, 1989).

In the present study in anesthetized and immobilized rats, we first investigated whether or not ET-3 modulated the central neural control of arterial pressure (AP), renal nerve activity (RNA), and heart rate (HR). Both tonic and reflex CNS controls were examined for modulatory action of ET-3. We then sought to identify the CNS sites responsible for such modulation. Special attention was paid on CNS sites in the lower brainstem and the spinal cord. We eventually confirmed modulatory action of ET-3 on tonic and phasic cardiovascular controls and identified CNS sites that possibly participated in this modulation.

MATERIALS AND METHODS

Preparation of animals. Experiments were performed on male Sprague-Dawley rats weighing between 330 and 460 g. They were anesthetized, except in eight rats noted in RESULTS, with an intraperitoneal injection of urethane (initially 1 g/kg). Supplementary doses of urethane were intravenously given when required, as judged from recordings of AP and RNA. For the purpose of comparing the effect of different anesthetics, five rats were anesthetized by an intraperitoneal injection of α -chloralose (100 mg/kg, initial dose) or sodium pentobarbital

(60 mg/kg, initial dose). After insertion of tracheal, arterial, and venous cannulas, the animal was paralyzed with gallamine triethiodide (initially 10 mg/rat i.v., thereafter 6–8 mg/h, i.v.) and artificially ventilated with oxygen-enriched room air. End-tidal P_{CO_2} was continuously monitored (Sanei-NEC, Respina IH 26) and maintained between 3 and 4%. Body temperature was maintained between $37 \pm 0.5^\circ\text{C}$ by a heating pad controlled by a thermistor probe inserted into the rectum.

Measurement of cardiovascular variables and renal and phrenic nerve discharges. Instantaneous and mean APs and HR were monitored continuously in all experiments. AP was recorded from the abdominal aorta by a polyethylene catheter inserted through the femoral artery and connected to a transducer (Nihon Kohden, TP-200T). HR was computed from the AP pulse by a tachometer (Nihon Kohden, AT-600G).

The left renal nerve was approached retroperitoneally through a left flank incision, and prepared for recording from near the renal artery. To record efferent discharges of the renal nerve, the central cut end of the nerve was placed on bipolar Ag hook electrodes connected to an amplifier (Nihon Kohden, AVB-8) and displayed on an oscilloscope (Tektronix, 5113). The lower and higher cut-off frequencies of the recording system were 100 and 3,000 Hz, respectively.

Renal nerve discharges were quantitated by one of the following two methods. First, discharges were full-wave rectified, integrated over a 10-s interval and stored in a tape recorder (TEAC, SR-31DF) together with original renal nerve discharges, AP, HR, ECG (lead II), and timing pulses of drug injection or other event signals. At the end of the experiment, the renal nerve was cut proximally to the recording electrode and the level of the instrumentation noise was determined. The noise level thus determined was integrated over 10-s intervals for several minutes and the average value of the integrated noise level was calculated. When the recorded signals were reproduced and processed, the rectified and integrated renal nerve discharges were subtracted by this average value. The second method was to convert renal nerve discharges into standard pulses by a window discriminator (Nihon Kohden, EN-601J) at the time of reproducing the recorded signals, and to determine the discharge frequency by a frequency counter (Nihon Kohden, ET-612J). To obtain standard pulses from nerve discharges, the threshold was set slightly above the noise level. The first method was used in the majority of experiments, especially in all that dealt with the response magnitude. Throughout this paper, renal nerve discharges quantitated in either method are called renal nerve activity (RNA).

In six experiments, the left phrenic nerve was approached dorsally by separating the neck muscles. The recorded multifiber phrenic discharges were processed as with renal nerve discharges to obtain phrenic nerve activity (PNA).

Intracisternal and topical applications of ET-3. After isolation of the renal nerve, rats were placed prone in a stereotaxic frame with the bit bar set at -12 mm. Through an occipital incision the atlanto-occipital membrane was exposed. When necessary, part of the occipital bone was removed to expose the obex region of the medulla oblongata. Under a dissection microscope, the needle of a microsyringe

(Terumo, MS-N10) carried in a stereotaxic micromanipulator (Narishige, BE-8) was advanced through the membrane and the needle tip was placed within the cisterna magna. Either vehicle (artificial cerebrospinal fluid, pH=7.4; LINDVALL *et al.*, 1978) or ET-3 (Peptide Institute, Osaka) dissolved in 10 μ l of artificial cerebrospinal fluid was injected into the cisterna magna. The injected solution contained 1.0% of Evans Blue to check, after the experiment, the extent of the brain area where the drug solution reached. Doses of ET-3 employed for the i.c. injection were either 10 or 100 pmol. Injections were made by hand in less than 1 min.

ET-3 was microinjected into several medullary structures such as the nucl. tractus solitarius (NTS) through micropipettes fabricated from glass capillary tubing with tips (ca. 50 μ m) shaped by a standard laboratory micropipette puller. Pipette shanks were long enough to produce minimal distortion of the medulla. The pipettes were carried in a stereotaxic micromanipulator (Narishige, SM-11) and connected by polyvinyl tubing to the pressure source. While observing the meniscus in the micropipette (Drummond Microcaps, i.d.=0.28 mm), through a dissection microscope (Nagashima, MD-2) equipped with an ocular micrometer, the desired volume was injected by adjusting manually the opening time of a solenoid valve (CKD Corp., AG31021), placed in the pneumatic circuit, while maintaining the source pressure about 1.5 atm (LIPSKI *et al.*, 1988). The volume of the injected fluid ranged from 0.1 to 0.2 μ l and the injection time was 5–10 s.

For intrathecal administration of ET-3, a polyethylene catheter (o.d.=0.5 mm) was passed into the subarachnoid space via an incision in the atlanto-occipital membrane and advanced caudally so that its tip lay at Th₅-L₁ (YAKSH and RUDY, 1976).

In six rats, the ventral surface of the medulla (VSM) was exposed for topical application of ET-3. The animal's head was fixed to a stereotaxic frame in a supine position. After the tracheal cannula was inserted, the trachea and esophagus were cut high in the neck between the two ligatures. The longus capitus muscle was removed on both sides at its attachment to the occipital bone. By carefully removing the basilar part of the occipital bone under the dissecting microscope, the VSM was exposed by incising the dura and the arachnoid. The exposed VSM was chemically stimulated by topical application of ET-3 dissolved in artificial cerebrospinal fluid and soaked into a small piece of filter paper. The piece, about 2 $\times$ 2 mm in size, was applied unilaterally to the area overriding the entry root of the XIIth cranial nerve.

Precollicular transection of the brainstem. In three rats, unanesthetized animal preparations were made by transecting the brainstem at the precollicular level. Under halothane anesthesia, a temporal craniotomy was performed by perforating a hole with a dental drill at a point approximately 1 mm rostral to the lambdoid suture and 1 mm left to the midline. Air emboli were protected by dropping saline during drilling, and with bone wax. The hole was then enlarged laterally and rostrally. Part of an occipital lobe was aspirated so that a spatula was inserted into

the midbrain to perform a precollicular transection. After the brain tissue rostral to the brain section was removed by aspiration, the anesthesia was discontinued. A 2-h intermission was interposed to eliminate the effect of halothane.

Examination of the arterial baroreceptor reflex. In 15 experiments the arterial baroreceptor reflex was elicited and the evoked response was compared before and after the i.c. injection of ET-3. The reflex response compared was (i) the change in RNA in response to a bolus i.v. injection of phenylephrine (1–10 μg) or nitroglycerin (5–50 μg), or (ii) the amplitude of the cardiac-related changes in RNA. In the latter type of experiments, the post R-wave time histogram of RNA was obtained by processing 64–128 successive sweeps (Nihon Kohden, ATAC 350).

Analysis of data. While reproducing mean AP (MAP), HR, and renal nerve discharges processed as explained above, they were sampled at the rate of 1 Hz by an analog-to-digital converter (Canopus Electronics, ADX-98H). The mean values of these variables over the period of successive 1- or 5-min intervals were then calculated by a computer (NEC, PC-9801RX). The time course of these variables before and after the drug administration was both numerically and graphically printed out. The baseline level was defined as the average of each variable over the 10-min interval immediately before an i.c. or local injection of ET-3. Variables were expressed as percent of the baseline values.

Statistical analysis of the results was carried out using Student's *t*-test for paired and unpaired data. Differences in data were considered to be significant when $p < 0.05$. Results were expressed as mean $\pm$ S.E.

Histological examination. At the end of each experiment, the animal was perfused through the heart with saline followed by 10% formalin. The brain and spinal cord were cut at 50 μm on a vibrating microtome (Dohsaka EM, DTK-1000). The sites of dye were identified and related to histological structures before and after staining the section with cresyl violet or neutral red.

RESULTS

Effects of intracisternal injection of ET-3 on AP, RNA, and HR

An injection of 100 pmol of ET-3 into the cisterna magna in 10 rats invariably resulted in a distinct pattern of changes in AP, RNA, and HR. The time course of these drug-induced changes consisted, in most animals, of three phases, i.e., phases I, II, and III, although there were individual variations in the duration and magnitude of responses in each phase (Figs. 1A and 2B). Baseline values of MAP, RNA, and HR of this group of rats are listed, along with corresponding values in other experimental groups, in Table 1.

Phase I is characterized by increases in AP, RNA, and HR. The response occurred within 3 min after the drug administration and reached maximal values by 3–15 min. Peak values in MAP, RNA, and HR, expressed as percent changes from the baseline values, were 19 ± 4 , 26 ± 6 , and $6 \pm 2\%$, respectively. They were all statistically significant. The three variables then gradually declined and, at

Fig. 1. Polygraph records illustrating cardiovascular, sympathetic, and respiratory responses to intracisternal (i.c.) administration of 100 pmol of ET-3 observed in two rats (A and B). Arterial pressure (AP) and heart rate (HR) were recorded in both cases, whereas renal nerve activity (RNA) was recorded in A, and phrenic nerve activity (PNA) and the number of bursts of PNA per min (burst rate) were recorded in B only.

5–30 min after the injection, reached baseline levels again.

During phase II, the three variables remained below the baseline levels for the next 20–60 min. The lowest values in MAP, RNA, and HR attained during this

Table 1. Baseline values of MAP and HR in three experimental groups in which ACSF or ET-3 (10 or 100 pmol) was administered.

Group	<i>n</i>	MAP (mmHg)	HR (beats/min)
ACSF	5	92.6 ± 6.4	438 ± 32
ET-3			
10 pmol	8	93.8 ± 3.2	462 ± 13
100 pmol	10	90.9 ± 1.2	465 ± 16

Values are mean ± S.E. *n*, number of experiments; ACSF, artificial cerebrospinal fluid; ET-3, endothelin-3; MAP, mean arterial pressure; HR, heart rate.

phase, expressed as percent changes from the pre-injection values, were -51 ± 4 , -41 ± 8 , and $-13 \pm 3\%$, respectively. They were all statistically significant. During phase III, AP, RNA, and HR increased, often quite rapidly, and in most instances exceeded the baseline levels. In four instances, however, AP remained below control until the end of the 2-h observation period and phase III was absent.

In six experiments, PNA was simultaneously recorded. The time course of the change in PNA was remarkably similar to those in AP, RNA, and HR (Fig. 1B). Thus, intracisternal ET-3 affects the central neural control of circulation and respiration simultaneously. The effects of ET-3 on respiration and its CNS sites of action shall be described systematically in a separate paper.

When 10 pmol of ET-3 was injected intracisternally in eight rats, the time course of changes in AP, RNA, and HR was somewhat different. As with an injection of 100 pmol, initial increases in the three variables characterizing phase I were always elicited (Fig. 2A). The response lasted for 5–20 min. Peak increases in MAP, RNA, and HR, expressed as above, were 14 ± 3 , 20 ± 5 , and $6 \pm 2\%$, respectively. They were all statistically significant. However, AP subsequently returned to the baseline level, and remained below that level during the 2-h observation period. On the other hand, RNA and HR decreased after the peak level was attained but in most animals remained elevated above the pre-injection levels. These sustained elevations of RNA and HR were ascribable, at least partly, to reflex responses to a decrease in AP. Actually, similar changes were observed even after injection of artificial cerebrospinal fluid (see below). In only two animals out of eight, the pattern of changes characterized as phase II was present. In no instances, however, was phase III discerned. Thus, the direct response to intracisternal administration of 10 pmol of ET-3 appeared to be initial transient increases in AP, RNA, and HR.

When artificial cerebrospinal fluid was injected as vehicle in five rats, no consistent initial changes corresponding to phase I were observed. However, there was a tendency that AP decreased slightly during the 2-h observation period, while RNA and HR drifted toward the other direction. Two hours after injection of the vehicle, changes in AP, RNA, and HR from the baseline levels were $-6 \pm 5\%$,

Fig. 2. Effects, on mean arterial pressure (MAP), RNA, and HR, of i.c. administration of 10 pmol (A, 8 rats) or 100 pmol (B, 10 rats) of ET-3. Responses observed in individual animals are represented by dotted lines, whereas the average values at each moment are represented by heavy lines. In this and succeeding figures, all the variables are expressed as percent of the baseline values determined as the average over 10 min immediately before the drug administration.

$19 \pm 8\%$, and $6 \pm 4\%$, respectively. These changes were statistically insignificant.

In order to examine whether intracisternally administered ET-3 might have reached the peripheral circulation and acted directly on the cardiovascular system, 100 pmol of ET-3 was intravenously injected as a bolus. The injection resulted, as observed in conscious rats (YANAGISAWA *et al.*, 1988a), in an immediate fall in AP, lasting for less than 1 min, by about 10–20 mmHg below control, followed by a pressor response, lasting for 15–20 min, by about 20–30 mmHg above the pre-injection level. At a dose of 10 pmol, a bolus intravenous injection did not produce any appreciable cardiovascular changes. Thus, intracisternally administered ET-3 caused responses quite different, with respect to their temporal pattern, magnitude, and the threshold dose, from its direct cardiovascular effects.

These results may be summarized as follows. (1) Intracisternal ET-3 could elicit both a short-latency pressor response with sympathoexcitation and tachycardia and a subsequent depressor response with sympathoinhibition and a bradycardia. (2) The pressor response with sympathoexcitation and a tachycardia was evoked

at doses smaller than that required for the depressor response with sympathoinhibition and a bradycardia. (3) These changes were centrally mediated. We thus concluded that centrally administered ET-3 modulated tonic control of AP, RNA, and HR by the central nervous system.

The extent of the brain surface stained with Evans Blue simultaneously injected with ET-3 was wide. It usually encompassed the dorsal, lateral, and ventral aspects of the brainstem exposed to the subarachnoid space. Furthermore, the ventral aspect of the cerebrum and the upper segments of the spinal cord were stained in most instances. However, the surface of the cerebroventricular space including the floor of the fourth ventricle was not visibly stained.

Effect of precollicular decerebration and various general anesthetics on the response to ET-3

In three rats, decerebration was performed at the precollicular level as described in MATERIALS AND METHODS. About 2 h after halothane was discontinued, intracisternal ET-3 (100 pmol) resulted in a pattern of changes in AP, RNA, and HR, which consisted of three phases as observed in the urethane-anesthetized rats with the intact neuraxis. The magnitude and duration characterizing each phase were comparable to those under urethane anesthesia (Fig. 3A). A similar pattern of responses was elicited under chloralose anesthesia (100 mg/kg, i.p., initial dose; Fig. 3B). When the rat was anesthetized by pentobarbital (60 mg/kg, i.p., initial dose), however, the dose of intracisternal ET-3 required to elicit comparable cardiovascular responses had to be 5–10 times that under urethane or chloralose anesthesia, or after precollicular decerebration (Fig. 3C).

It appeared that a similar pattern of cardiovascular and sympathetic responses was expected to an intracisternal administration of ET-3 under the three types of anesthesia or under unanesthetized and decerebrated condition. For this reason, and for its relatively stable and long-lasting anesthetic effect, urethane was used as general anesthesia in the experiments described below.

Effects of ganglion blockade, vasopressin antagonist, and angiotensin II antagonist on ET-induced cardiovascular changes

The changes in AP, RNA, and HR induced by intracisternal ET-3 were totally eliminated by a continuous intravenous infusion of hexamethonium chloride at the rate of 30 mg/(kg · h) after an initial dose of 30 mg/kg (Fig. 4). Thus, the ET-induced response of AP was mediated by the sympathetic nervous system. Furthermore, since the vagal control of HR was already suppressed as a result of vagolytic action of gallamine triethiodide continuously infused throughout the experiment (SON and WAUD, 1978), absence of the HR change after ganglion blockade in the present experiments implied that it was also mediated by the sympathetic nerve.

On the other hand, pretreatment with 10 µg/kg (i.v.) of arginine vasopressin antagonist, (1-(β-mercapto-β,β-cyclopentamethylene-propionic acid), 2-(O-methyl)-tyrosine)arginine-vasopressin (Manning compound), in four rats did not

Fig. 3. Responses to intracisternally administered ET-3 on MAP, RNA, and HR in rats unanesthetized and precollicular decerebrated (A), and anesthetized by intraperitoneal injection of α -chloralose (100 mg/kg, B) or sodium pentobarbital (60 mg/kg, C). Each of A–C represents results obtained from one animal. Doses of intracisternal ET-3 were 100 pmol in A and B, and 1,000 pmol in C.

conspicuously alter cardiovascular responses to intracisternal ET-3. In these experiments, the antagonistic effect of this compound was confirmed as follows. After the antagonist was given to the animal, arginine vasopressin was injected, before and after ET-treatment, intravenously as a bolus up to 0.1 μ g/kg. The lack of a pressor response was the criterion for the antagonistic effect in question.

Likewise, the ET-induced responses were not affected by salarasin (12 μ g/(kg·min), i.v., in one rat), an angiotensin II antagonist. The antagonistic effect was confirmed as above by examining the lack of a pressor response to a bolus injection of angiotensin II by up to 0.5 μ g/kg. On the bases of these results, we concluded that the efferent mechanism responsible, either primarily or exclusively, for the effect of intracisternally administered ET-3 on AP and HR was the sympathetic nervous system.

Fig. 4. Effects of ganglion blockade on cardiovascular and sympathetic responses to ET-3 (100 pmol, i.c.). Hexamethonium chloride was injected at a dose of 30 mg/kg (i.v.) initially at the moment marked by the first arrowhead and continuously given at the rate of 30 mg/(kg·h) thereafter. Note that responses to administration of ET-3, marked by the second arrowhead, were abolished by pre-treatment with ganglion blockade.

Effects of intracisternal ET-3 on the arterial baroreceptor reflex

Two types of experiments disclosed that intracisternally administered ET-3 (100 pmol) modulated the cardiovascular control by the arterial baroreceptor reflex. In the first group of experiments in 10 rats, the cardiac-related fluctuations of RNA, reflecting baroreceptor-initiated sympathoinhibition (GEBBER and BARMAN, 1980), were compared before and after administration of ET-3 (100 pmol, i.c.). ET-3 reduced the amplitude of the cardiac-related changes in RNA during the latter period of phase I (Fig. 5A-b). Throughout this period, MAP remained above the threshold pressure of the arterial baroreceptor reflex (78 ± 9 mmHg in normotensive rats according to BROWN *et al.*, 1976). During phase II, RNA did not exhibit the cardiac-related changes because MAP decreased below the threshold level (Fig. 5A-c). The arterial baroreceptor reflex was restored during phase III (Fig. 5A-d).

In the second group of experiments in five rats, the reflex change in RNA was correlated to opposite changes in MAP induced by bolus injection of phenylephrine (1–10 μ g, i.v.) or nitroglycerin (5–50 μ g, i.v.) before and after administration of ET-3 (100 pmol, i.c.). As illustrated in Fig. 6, the slope of the RNA-MAP relationship, reflecting the gain of the arterial baroreceptor reflex, was markedly reduced during phase II but recovered during phase III.

Based on the two types of experiments, we concluded that intracisternally administered ET-3 inhibited the reflex cardiovascular control by the arterial

Fig. 5. Effect of intracisternal ET-3 on arterial baroreceptor reflex. In A, cardiac-related fluctuations of RNA, determined as the post R-wave time histogram over 64 successive sweeps, were obtained at 4 points, indicated by a–d in B, before and after injection of ET-3 (100 pmol, i.c.). In a, b, and d, MAP remained within the operating range of the arterial baroreceptor reflex. Then, the amplitude of fluctuations represented effectiveness of the arterial baroreceptor reflex. Note that the reflex was already inhibited at the latter part of phase I (b) but fully recovered at phase III (d).

baroreceptor reflex during the latter period of phase I and during phase II.

Effects of local CNS application of ET-3

We sought to determine the CNS site(s) mediating responses of AP, RNA, and HR to an intracisternal administration of ET-3. As explained above, ET-induced changes were preserved after precollicular decerebration. Furthermore, the arterial

Fig. 6. Relationship between responses of MAP (Δ MAP) and RNA (Δ RNA), both expressed as the change from pre-injection level, to bolus injection of phenylephrine (1–10 μ g, i.v.) or nitroglycerin (5–50 μ g, i.v.). These tests were performed before (open circles), 20–90 min after (phase II, filled circles), and 120–180 min after (phase III, crosses) the administration of ET-3 (100 pmol, i.c.). Note that the slope of the regression line, representing the gain of the arterial baroreceptor reflex, was markedly reduced during phase II, but mostly recovered during phase III. Data points were collected from five experiments.

baroreceptor reflex was inhibited by intracisternal ET-3. Therefore, at least part of CNS sites responsive to ET-3 must be located below the midbrain and along the reflex pathway subserving the arterial baroreceptor reflex.

A unilateral injection of ET-3 (2–10 pmol in 0.2 μ l) into the NTS near the obex in two rats resulted in increases in AP, RNA, and HR. The response occurred within 1 min and lasted for more than 30 min (Fig. 7). There were no subsequent decreases in these variables corresponding to phase II. When the drug was microinjected within the medulla 1 mm ventral or lateral to the NTS or into the area postrema in another five rats, no changes were elicited.

To examine the role of the VSM, ET-3 was delivered to the VSM by a micropipette penetrated through the medulla from the dorsal surface (five rats) or through a direct application to the VSM after exposure of the ventral surface of the lower brainstem (six rats). In the former type of experiments, the micropipette was gently advanced ventrally, passed through the medulla, and its tip was positioned within the subarachnoid space near the VSM. A unilateral injection of ET-3 by the amount as small as 20 pmol elicited the pattern of responses in AP, RNA, and HR that was remarkably similar to that following the intracisternal injection (Fig. 8A). The area reached by the solution, as estimated by simultaneously injected Evans Blue, varied from animal to animal. Whenever the response similar to that following intracisternal ET-3 was elicited, the stained surface area covered the portion of the brainstem medial and rostral to the root of the XIIth cranial nerve (Fig. 8B). This part of the medullary surface overlapped with the central chemosensitive area in the VSM (BRUCE and CHERNIACK, 1987). That ET-3 acted directly on the VSM was demonstrated by a topical application to this area. Following application of a piece of filter paper soaked with ET-3 to the VSM, AP, RNA, and HR all exhibited

Fig. 7. Effects of local microinjection of ET-3 (2 pmol in 0.2 μ l of artificial cerebrospinal fluid) into nucl. tractus solitarius (NTS) at the level 0.5 mm rostral to calamus scriptorius. The injection caused rises in MAP, RNA, and HR as shown in A. The injection site, corresponding to the dorsal and dorsolateral portions of tractus solitarius within NTS, is represented by the shaded area in B. Abbreviations in A and B are as follows: ACSF, artificial cerebrospinal fluid; AP, area postrema; DMN, dorsal motor nucleus of vagus nerve; ION, inferior olivary nucleus; LRN, lateral reticular nucleus; NA, nucleus ambiguus; NTS, nucleus of solitary tract; NV, spinal tract; TS, solitary tract.

responses characterizing phases I through III. On the other hand, microinjection or topical application of artificial cerebrospinal fluid to the region of the VSM in three rats did not produce any appreciable change in AP, RNA, or HR.

Finally, an intrathecal injection of ET-3 in three animals caused transient increases in AP and RNA followed by decreases in them which lasted about 1 h (Fig. 9). These changes, however, were much smaller than those following intracisternal injection or VSM application of ET-3. Simultaneously injected Evans Blue spread over 5–10 segments of both the dorsal and ventral surfaces of the spinal cord that encompassed thoracic and lumbar segments. Specific segments of the spinal cord or the exact location within a spinal segment for the drug action was not determined so far.

DISCUSSION

Major findings of the present study are summarized as follows. In the

Fig. 8. Typical example illustrating effects of topical application of ET-3 to the ventral surface of medulla (VSM). A micropipette filled with ET-3 in artificial cerebrospinal fluid was penetrated at the dorsal surface of the medulla, advanced ventrally, passed through the brainstem, and placed in the subarachnoid space below the area of VSM. Responses to 20 pmol of ET-3, pressure-injected in a volume of 2 μ l, are illustrated by A. The extent of the brain surface directly reached by ET-3 was assessed by simultaneously injected Evans Blue (B). Note that the area reached by dye overlapped with the central chemosensitive area. Abbreviations in A and B are as follows: ACSF, artificial cerebrospinal fluid; Basilar a., basilar artery; Pyr, pyramidal tract; V, trigeminal nerve; X, vagus nerve; XII, hypoglossal nerve.

urethane-anesthetized and paralyzed rats, an intracisternal injection of 10 pmol of ET-3 caused an increase in AP accompanied by sympathoexcitation and tachycardia. At a dose of 100 pmol, a subsequent depressor response with sympathoinhibition and bradycardia appeared. The same pattern of responses was produced in unanesthetized precollicular decerebrated rats. In addition to modulation of the tonic control of cardiovascular and sympathetic activity by the CNS, intracisternal ET-3 modulated the reflex control as evidenced by suppression of the arterial baroreceptor reflex. Although certain brain sites were found to cause cardiovascular and sympathetic responses on topical application of ET-3, the VSM was the only site identified so far which produced the same pattern of changes as that following an intracisternal injection. Since a bolus intravenous injection of 100 pmol of ET-3

Fig. 9. Effect of intrathecal administration of ET-3 (100 pmol) observed in three rats. Responses in individual animals and their means at each moment are represented by dotted and thick lines, respectively.

in the same rat did not cause the pattern of cardiovascular changes comparable to those following intracisternal administration, the observed cardiovascular effect of intracisternally administered ET-3 was centrally mediated but not due to the leakage of the agent to the peripheral circulation.

Because ET is a newly discovered endogenous vasoactive substance, there are, to our knowledge, only two systematic studies on its effects on the central neural control of circulation (OUCHI *et al.*, 1989; MINAMIZAWA *et al.*, 1989). In these studies, ET-1 was administered to the lateral (OUCHI *et al.*, 1989) or the third ventricular (MINAMIZAWA *et al.*, 1989) of unanesthetized rats. In the study by Ouchi and co-workers, an injection of ET-1 (8.25–66 pmol) caused gradual increases in AP and HR, which reached the peak levels in 5 to 20 min. At the same time, plasma epinephrine and norepinephrine concentrations were markedly elevated. At high doses of ET-1 such as 66 pmol, the animal sometimes died of circulatory shock after AP reached the peak value. The drug-induced pressor response was suppressed by pretreatment with phenoxybenzamine, indicating active participation of the sympatho-adrenal system. On the other hand, Minamizawa and colleagues observed a complicated response characterized by a gradually developing pressor response intermingled with episodes of a transient fall in AP below the preinjection level. Soon after AP reached the maximal level in about 20 min after the drug injection (78 pmol), three out of nine of their rats suddenly died.

Results of these two studies cannot be readily compared to ours, since there are certain fundamental differences in the experimental conditions such as the drug examined (ET-1 vs. ET-3), the site of drug injection (third or lateral ventricle vs. cisterna magna), and the conscious state of the animal (unanesthetized vs. anesthetized). Nevertheless, a close look at the three studies reveals common features. First, the pressor response in their experiments corresponds, by and large, to that during phase I in our study. Furthermore, at the dose of 100 pmol of ET-3 in our study, a hypotension during phase II in some instances lasted for the 2-h observation period. In view of the fact that ET-3 markedly suppressed respiration as evidenced by diminished phrenic discharges, these animals might have died of hypoxia as in conscious rats, had they not been supported by artificial ventilation.

In those two studies in conscious rats, the lack of a long-lasting depressor response corresponding to phase II following an intracisternal injection in our study may be most likely attributed to the following two reasons. First and most important was that the third or lateral ventricle, as opposed to the cisterna magna, was the injection site in those two studies. The intracerebroventricular administration was expected to influence predominantly periventricular CNS structures such as the anteroventral third ventricle (AV3V). On the other hand, intracisternal injection of ET-3 in our study elicited the cardiovascular responses comparable to those following direct topical application of ET-3 to the VSM. Actually, intracisternal Evans Blue simultaneously injected with ET-3 always reached the VSM.

Second, in the studies in conscious rats, the animal breathed spontaneously and the drug-induced respiratory changes, which possibly underlied circulatory shocks in some of their animals, might have affected AP. As explained in RESULTS, intracisternal ET-3 (100 pmol) first augmented and then markedly reduced PNA. Intracerebroventricular ET-1 might have inhibited respiration, and if the animal had withstood it, the ensuing hypoxia might have offset the depressor response corresponding to phase II in our study.

Although urethane anesthesia is said to distort cardiovascular responses to certain drugs such as clonidine (ARMSTRONG *et al.*, 1982), it is unlikely that the hypotension during phase II in our study was due to this anesthetic. Whether the animal was decerebrated and unanesthetized or anesthetized by urethane, chloralose, or pentobarbital, responses to ET-3 were very similar. An extraordinarily high dose of ET-3 required to produce comparable cardiovascular and sympathetic changes under pentobarbital anesthesia may be ascribable to its inhibitory effect on the VSM (FELDBERG and GUERTZENSTEIN, 1976).

It should be noted that the hypotension was not specific to ET-3. Our preliminary results indicated that the same pattern of changes in AP, RNA, and HR were observed following intracisternal administration of ET-1. Although the precise dose-response relationship was hard to obtain because of complexity of surgical procedures in a small animal and requirement of a long-lasting stability of the experimental preparation, the dose required to elicit comparable centrally mediated cardiovascular responses was about 10 to 100 times smaller with ET-1

than those with ET-3. Furthermore, similarity of the drug-induced responses suggests that both ET-1 and ET-3 act on the same CNS sites, although specific binding sites for the two isopeptides may differ, as demonstrated in the rat myocytes (WATANABE *et al.*, 1989). In this connection, there is an abundance of binding sites for ET-1 throughout the brain, including those areas such as the NTS that participate in the central cardiovascular control (KOSEKI *et al.*, 1989; JONES *et al.*, 1989). The number of the ET-1 binding sites in the brainstem as assessed by $B_{\max}$ (280 fmol/mg protein) and K_d (0.5 nM) is comparable to that of dopamine binding sites in the corpus striatum ($B_{\max}$ = 300–500 fmol/mg protein and K_d = 0.1–1 nM; SEEMAN, 1980).

Distribution of binding sites of ET-3 in the brain has yet to be determined. Nevertheless, recent studies demonstrated definitively that ET-3 was present in the rat's brain (MACCUMBER *et al.*, 1989; MATSUMOTO *et al.*, 1989) and in the pig's spinal cord (SHINMI *et al.*, 1989; YOSHIZAWA *et al.*, 1989). In the rat's medulla oblongata, where ET-3 is expected to exert centrally mediated cardiovascular effects, the content of ET-3 was about 18% of ET-1 (MATSUMOTO *et al.*, 1989). Since part of ET-1 in the assay is derived from the vascular endothelium which does not contain ET-3, the amount of ET-3 relative to ET-1 in neuronal elements should be even greater.

Admittedly, there has been no direct evidence at the moment that ET-3, even if it is there, plays a physiological role, e.g., as a neurotransmitter or neuromodulator, in the central neural control of the circulation. However, abundance of ET-3 in the rat's brain in addition to the intestine and pituitary gland makes it quite plausible that the ET-3, perhaps as a brain-gut peptide, bears some roles in the integrative neurohumoral control of the circulation under physiological or patho-physiological settings.

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