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RESPONSES OF NEURONS OF THE CANINE AREA POSTREMA TO NEUROTRANSMITTERS AND PEPTIDES

David O. Carpenter, M.D.[1,2]
Dean B. Briggs, M.S.[1]
Norman Strominger, Ph.D.[2]

[1]New York State Department of Health
Albany, New York 12201

[2]Albany Medical College
Albany, New York 12208

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USAF SCHOOL OF AEROSPACE MEDICINE
Aerospace Medical Division (AFSC)
Brooks Air Force Base, Texas 78235

Southeastern Center for Electrical Engineering Education
1101 Massachusetts
St. Cloud, Florida 32769

NOTICES

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The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

Thomas E. Dayton

THOMAS E. DAYTON, Captain, USAF
Project Scientist

Donald N. Farrer

DONALD N. FARRER, Ph.D.
Supervisor

Royce Moser, Jr.

ROYCE MOSER, Jr.
Colonel, USAF, MC
Commander

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The responses of 122 neurons in the area postrema of anesthetized dogs to 17 common transmitters and peptides were determined. Recordings were made from one barrel of a 7-barrel ionophoretic electrode.

All neurons were silent at rest, but most could be detected and excited by application of glutamate. The glutamate response was a brief, high-frequency response of less than 1-sec duration.

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20. ABSTRACT (Continued)

Excitatory responses were also found to histamine, norepinephrine, serotonin, dopamine, apomorphine, angiotensin II, neurotensin, leucine-enkephalin, vasoactive intestinal polypeptide (VIP), thyrotropin-releasing hormone (TRH), gastrin, vasopressin, and substance P. While most neurons were excited by dopamine and apomorphine, approximately half of those studied were excited by the other substances. Inhibitory responses were found to norepinephrine (6 of 15 cells) and histamine (3 of 45 cells). No responses were found to acetylcholine, somatostatin, or cholecystokinin (CCK).

The responses to all 13 excitatory substances other than glutamate were similar. Typically these responses had a latency of 2-20 sec and lasted for 30 sec to 5 min on their first application. The frequency of discharge was usually low (~ 0.5 Hz). Multiple applications of these agents often induced a maintained spontaneous discharge of low frequency, although each application induced a transient incremental discharge but with a frequency that rarely exceeded 2 Hz.

The area postrema has been proposed to be the "chemosensory trigger zone" for emesis. All of the agents which excite area postrema neurons, with the exception of serotonin and norepinephrine, have been shown to be emetic, while none of the three agents without excitatory effects are emetic. Thus these results provide strong support for the central role of the area postrema in emesis.

The similarity of response to so many substances on small neurons suggests a common ionic and/or metabolic mechanism underlying the response. The prolonged nature of the response to brief administration of these agents would seem to be an appropriate one for neurons which subserve a sensation and behavior such as nausea and vomiting.

RESPONSES OF NEURONS OF THE CANINE AREA POSTREMA TO NEUROTRANSMITTERS AND PEPTIDES

INTRODUCTION

The area postrema, one of the circumventricular organs, lies outside of the blood-brain barrier (41) on the wall of the fourth ventricle at the level of the obex. It is composed of large vascular spaces (30) with abundant small neurons (25, 27), mast cells (10) and neuroglia, including a transitional type referred to as "glialoid" (32). The area postrema is known to be a major neural component in the emetic reflex, and it has been named the "chemosensory trigger zone" (CTZ) for emesis by Borison and Wang (6) on the basis of the fact that emesis due to drugs (such as apomorphine), radiation, and motion sickness is abolished after ablation of the area postrema. It has been suggested that the area postrema is involved in osmoreception (14), regulation of the choroid plexus (34), regulation of EEG synchronization sleep cycles (8, 9), and blood pressure regulation (1, 42), especially in mediating the cardiovascular response to angiotensin II (21). However, the role of the area postrema in some of these functions has been questioned, (4, 43). The neurons of the area postrema have been reported to project to many brain stem structures including the solitary nucleus (28), dorsal motor nucleus of the vagus, hypoglossal nucleus, mesencephalic trigeminal nucleus, locus ceruleus and superior and inferior colliculi (35). Afferents to the area postrema have been described from the vagus (23) and aortic nerves (24), hypothalamus (19), and adjacent bulbar nuclei such as ambiguuns, solitarius, dorsal motor vagus, intercalatus, and hypoglossal (15).

In spite of a considerable knowledge of the connections and possible functions of the area postrema, information is lacking concerning the electrophysiological and chemosensory properties of its neurons. Two attempts have been made to record neurons in this area. Borison et al. (5) recorded in cats and applied drugs systemically. They found it very difficult to hold neurons for any period of time due to their small size and the pulsation of the tissue. Nevertheless they recorded a few units that were probably within the area postrema and a number of units from surrounding structures. They found excitation to cardiac glycosides and ATP. Brooks et al. (7) attempted recordings in an isolated brain tissue preparation and found neurons unresponsive to glutamate, serotonin, angiotensin II, dopamine, and osmotic changes. We have attempted to record from units in the area postrema of the dog with multiple barrel microelectrodes in order to characterize their responsiveness to common transmitters and peptides.

METHODS

Conditioned random-source dogs (10 to 15 kg) were obtained from commercial sources. Dogs were anesthetized with IV Surital and then were either maintained on a Surital drip or anesthetized with α chloralose (65 mg/kg) or

Nembutal (25 mg/kg). No significant differences in the nature of electrical activity of area postrema neurons were detected as a function of anesthetic. A Bird Mark 7 respirator was used as needed.

Following placement of femoral venous and arterial catheters and insertion of a tracheal cannula, animals were mounted in a Kopf stereotaxic frame and positioned with pronounced ventral bending at the neck. The floor of the fourth ventricle was exposed by removal of the section of skull over that area and the cerebellum retracted forward with a specially made device resembling a bent spatula. When the neck was thus angled, it was possible to expose all of the area postrema without removing the cerebellum. In early experiments we found that attempts to remove the uvula gave bleeding which obscured visualization of the surface of the area postrema.

Recordings were made using 7-barreled electrodes (R & D Scientific Glass, Spencerville, Md.). One barrel was filled with 1 M NaCl for recording while the others were used for ionophoresis. One barrel always contained 1 M Na glutamate (Aldrich, pH 7-8). Others contained various combinations of the transmitters and peptides listed in Table 1.

The electronic equipment utilized included a Dagan Model 2400 preamplifier, a Tektronix Model 5111 oscilloscope with 5A26 differential amplifiers, WP Instruments pulse generators, a Honeywell Model 1858 Visicorder and an ionophoretic control module as described by Willis et al. (40) which is a constant voltage source, varying time rather than current. Electrodes were advanced using a Kopf digital hydraulic microdrive.

The electrode was placed in the transverse center of the area postrema under direct visualization. All placements were in the rostral two-thirds of the structure, with the majority from the mid-region where the area postrema is widest. The digital microdrive was set to zero at the point of contact of the electrode with the tissue, and units were only accepted for study within the first 700 μm of the surface. Brief pulses of glutamate were used to detect units. Once a unit was found, responses to all drugs in the ionophoretic electrode were tested as often as possible.

At the conclusion of electrophysiological tests, dogs were perfused transcardially with 0.9% saline followed by 10% neutral formalin. Brainstems were removed and blocked in the transverse plane. Following postfixation in neutral formalin, material either was embedded in paraffin and sectioned at 7 μm or was cut at 20 μm on a freezing microtome. Sections through the area postrema were stained with hematoxylin and eosin or cresyl violet.

Two normal dogs were anesthetized with Nembutal and perfused as above. These cases were used to study the cytology and spatial relationships of the area postrema. Frozen sections (20 μm) were prepared to minimize shrinkage. One case was cut sagittally and the other in the transverse plane. Sections were stained with cresyl violet-luxol blue or neutral red.

TABLE 1. SUBSTANCES IONOPHORETICALLY APPLIED TO AREA POSTREMA NEURONS

Drug	pH	Molarity	Medium*	Company
L-Glutamic Acid (sodium salt)	7-8	1 M	DW	Aldrich
5-Hydroxytryptamine (5HT)	3-4	1 M	DW	Sigma
Acetylcholine chloride (ACh)	3-4	1 M	DW	Sigma
Histamine	3-4	1 M	DW	Sigma
3-Hydroxytryptamine (Dopamine)	3-4	1 M	S	Sigma
Apomorphine hydrochloride	6-7	3×10^{-3} M	S	Sigma
Norepinephrine	3-4	1 M	DW	Sigma
Angiotensin II	6-7	10^{-3} M	S	Sigma
Neurotensin	6-7	10^{-3} M	S	Sigma
Thyrotropin-releasing hormone (TRH)	6-7	3×10^{-3} M	S	Sigma
Vasoactive intestinal polypeptide (VIP)	6-7	3×10^{-4} M	S	Sigma
Gastrin	6-7	10^{-3} M	S	Sigma
Substance P	6-7	10^{-3} M	S	Sigma
Vasopressin	6-7	10^{-3} M	S	Calbiochem
Leucine enkephalin	6-7	2×10^{-3} M	S	Sigma
Somatostatin	6-7	10^{-3} M	S	Sigma
Cholecystokinin (CCK) 26-33 octapeptide amide sulfate	6-7	10^{-3} M	S	Sigma

*DW = Distilled H_2O
S = Saline

RESULTS

The area postrema in living preparations is visible as a translucent, slightly elevated piece of tissue lining the rim of the fourth ventricle and clearly delineated from the surrounding ependyma. Figure 1 shows the canine area postrema in the sagittal and transverse planes. The maximum depth of the structure is approximately 1 mm, perhaps slightly more if shrinkage is taken into consideration. The rostro-caudal and transverse dimensions are about 1.5 mm and ..0 mm respectively, at their largest extents. The nucleus is much narrower near the ventral border. As indicated in the photomicrographs the area postrema is bordered dorsolaterally and laterally by the rostral division of the gracile nucleus and the medial nucleus of the solitary tract, and ventrally by the dorsal motor nucleus of the vagus.

To ensure that recordings were being made from the area postrema, it was required that units be within 700 μm of the surface. This requirement allowed for a considerable margin before the electrode would enter adjacent nuclei. It was often clear when the recording electrode passed from area postrema into dorsal motor vagus or medial solitary nucleus since the size of action potentials suddenly increased. This increase never occurred at depths of less than 700 μm. Thus, we are confident that the neurons included in this study are truly in the area postrema. In spite of persistent attempts with fast green or $FeCl_3$ ionophoresis, we were unsuccessful in marking the recording site in electrophysiological preparations. Because of the great vascularity of this area we believe that the markers were rapidly cleared.

The neurons of the area postrema were silent at rest but could be easily detected by short ionophoretic applications of glutamate, which excited the cells. Most units had action potentials of small amplitude, often more easily heard on the audio monitor than seen on the oscilloscope. However, there were no obvious differences in the patterns of response of small units as compared to larger units suitable for illustration. Because of the circulatory and respiratory movements of the brain stem it often was not possible to hold units for protracted periods, but positive responses to transmitter ionophoresis were accepted only if obtained on at least two applications.

Figure 2 illustrates the responses of a neuron recorded at 543 μm depth. This cell was excited by glutamate and gastrin but not by VIP or angiotensin II. The response to glutamate was of short latency, brief duration, and relatively high frequency. In contrast, the response to gastrin had a latency of greater than 10 sec. The discharge frequency to gastrin was very slow (0.5 Hz) and the discharge lasted about 30 sec. Figure 3 shows a similar response to VIP from another neuron.

Figure 4 shows the responses of a cell to glutamate, norepinephrine, and thyrotropin-releasing hormone (TRH). This cell was excited by glutamate and TRH, but inhibited by norepinephrine. The response to TRH, like that to gastrin in Figure 3, was of long latency (3 sec), reached its maximal frequency only at about 20 sec, and lasted for about 4 min. The response

illustrated was that to the first application of TRH. A second application 2 min after the first resulted in an excitatory reponse which did not turn off, leaving the cell with a maintained slow spontaneous activity. The second response to glutamate and the inhibition due to norepinephrine were obtained during this spontaneous activity. When the cell was spontaneously active, application of glutamate produced a brief excitation followed by a pause; this pause was apparent in almost all neurons that were made spontaneously active.

Figure 5 shows another neuron which was excited by angiotensin II, neurotensin, and glutamate. As for gastrin and TRH, the neurotensin and angiotensin II responses were at a relatively low frequency and long duration. The glutamate response illustrated was obtained after both neurotensin and angiotensin had been applied twice, so that the cell was spontaneously active.

Table 2 shows the responsiveness of 122 area postrema units. All but one unit were excited by glutamate, but this may reflect only the fact that glutamate was used to search for units. Inhibitory responses were found only to norepinephrine and histamine, but these could be detected only after prolonged peptide excitation. Excitatory responses were found for 7 peptides, apomorphine, serotonin, norepinephrine, and histamine on some but not all cells. All of these substances appeared to elicit the same type of response, characterized by a long latency (2-20 sec), low discharge frequency ($\sim$0.5 Hz), and long duration (30 sec - 5 min). Often all substances, including the amines, stimulated a spontaneous discharge which lasted for many minutes, sometimes for as long as the cell could be held.

Neurons which were stimulated into prolonged spontaneous discharge by application of an excitatory amine or peptide were still capable of incremental excitation. Figure 6 shows a neuron which was caused to discharge by application of serotonin. Application of both serotonin and TRH induced clear increases in the discharge frequency. These increases were also very long lasting.

DISCUSSION

These studies are the first to record from a population of neurons in which one has reasonable assurance that they are located in the area postrema, and to test the responsiveness of these neurons to ionophoretic application of peptides and transmitters. Our results augment those of Borison et al. (5) who attempted recordings in this region and injected substances intra-arterially. Most of the units which they studied were at the junction between the area postrema and the solitary nucleus or dorsal motor vagus. They found that the neurons recorded responded to injections of ouabain and ATP into the vertebral artery, but not consistently to apomorphine. The only other report of a study in this region is by Brooks et al. (7), who in an isolated rat brain stem preparation found no responses to glutamate, serotonin, angiotensin II, and dopamine perfusion. These results are inconsistent with our observations, perhaps due to species (rats vs. dog) or preparation (isolated vs. intact).

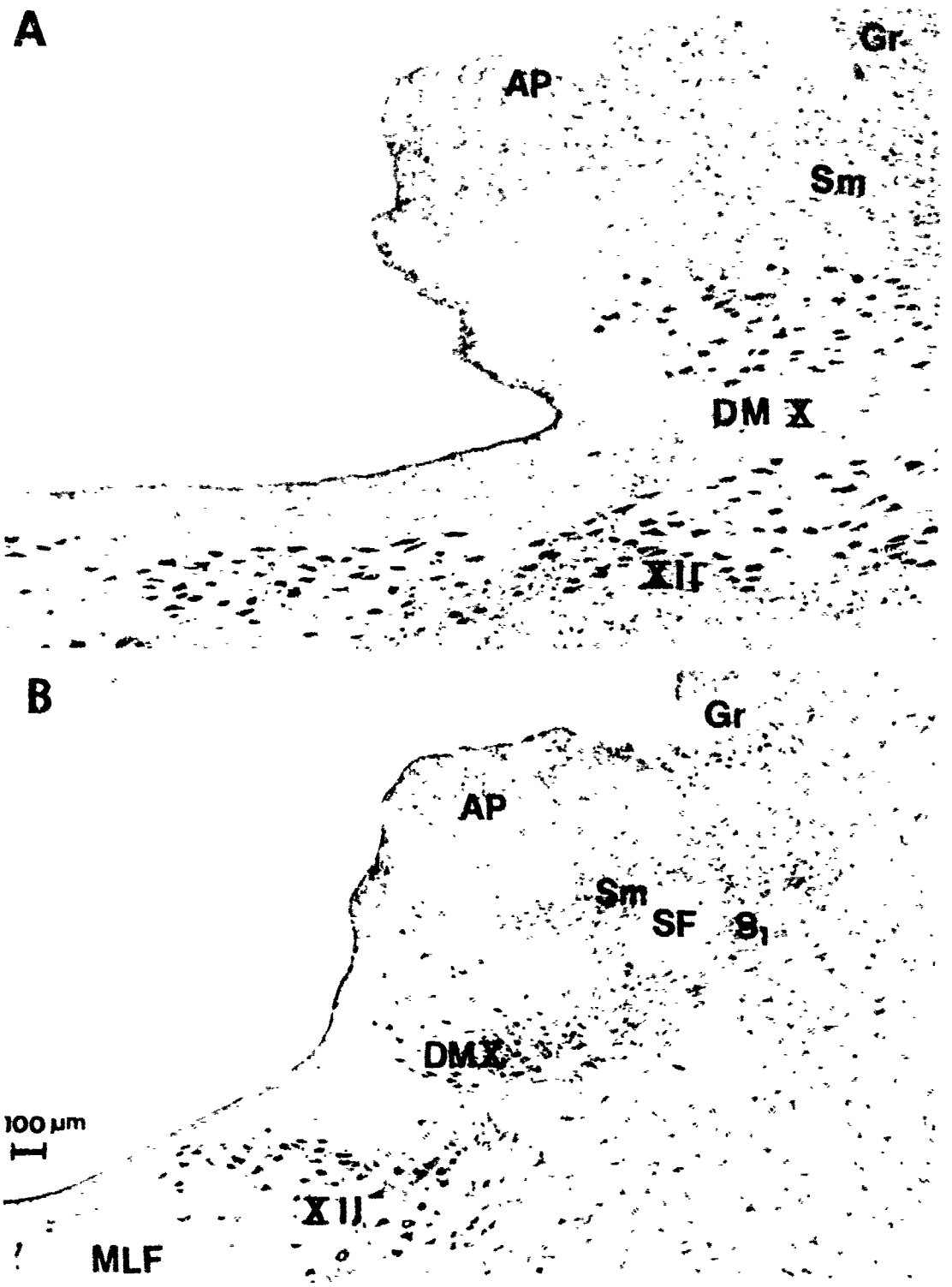

Figure 1. Photomicrographs of the dorsomedial aspect of the medulla show the area postrema (AP) and surrounding nuclei in the (A) sagittal and (B) transverse planes. In the sagittal photomicrograph, anterior is to the left. The transverse photomicrograph is of the right side. Gr, rostral division of the gracile nucleus; DM $\underline{X}$, dorsal motor nucleus of the vagus; MLF, medial longitudinal fasciculus; SF, solitary fasciculus; Sl, lateral solitary nucleus; Sm, medial solitary nucleus; XII, hypoglossal nucleus; 20-µm frozen sections, neutral red.

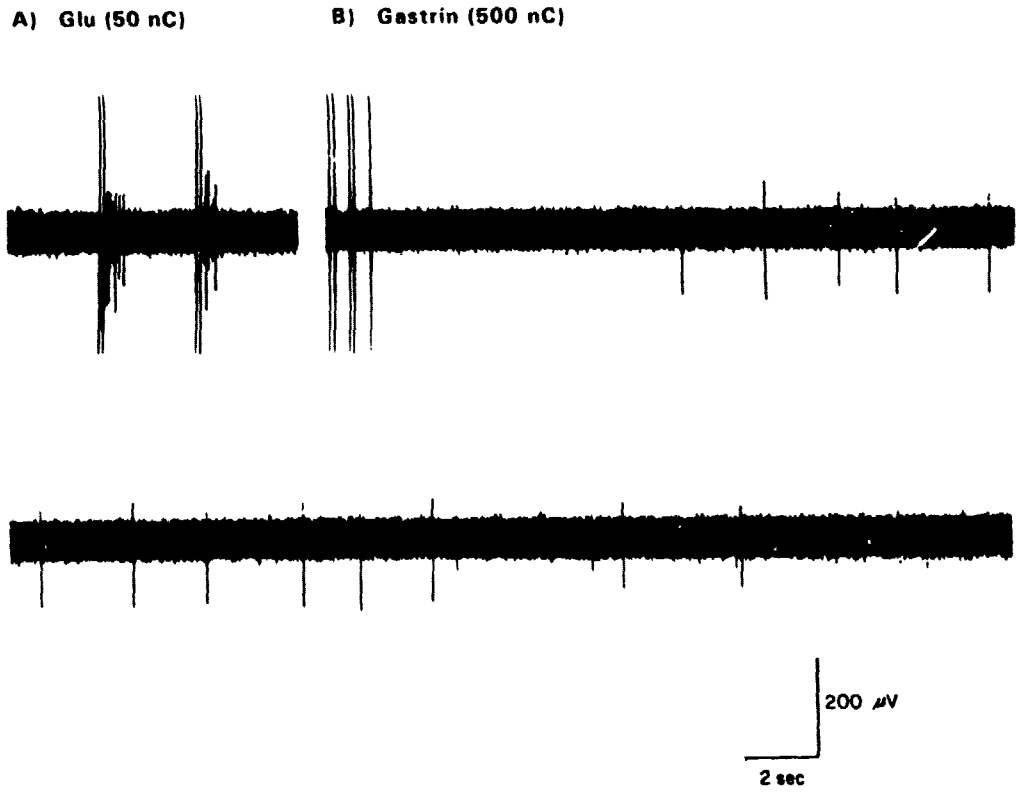

Figure 2. Responses of an area postrema neuron to glutamate (Glu) and gastrin. The neuron was at 543 μm and did not respond to either VIP or angiotensin II. The large deflections are ionophoretic artifacts. Glutamate was applied as a single pulse of 50 nC. Gastrin was applied as 5 pulses, each 100 nC, of which only the last two are shown.

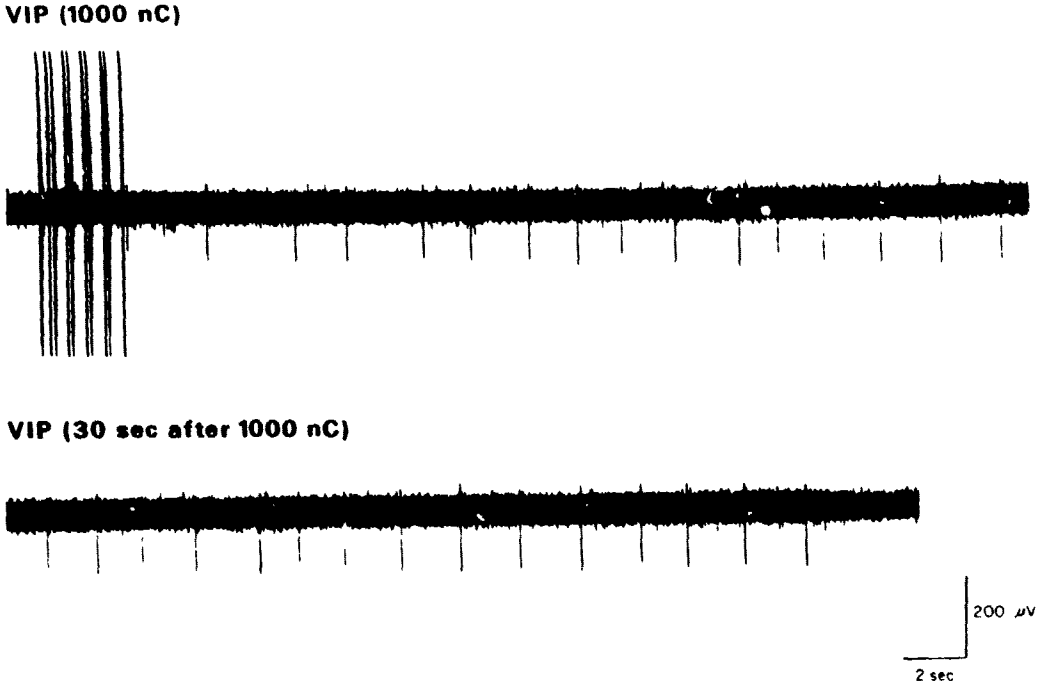

Figure 3. Response of an area postrema neuron 680 μm from the surface to application of vasoactive intestinal polypeptide (VIP). The large deflections at the beginning of the trace are the artifacts resulting from application of 5 pulses, each 200 nC, through the ionophoretic barrel. The VIP response began about 6 sec after the beginning of ionophoresis and lasted somewhat more than 1 min. This neuron was also excited by glutamate, TRH, and serotonin, was inhibited by histamine, and was unaffected by substance P.

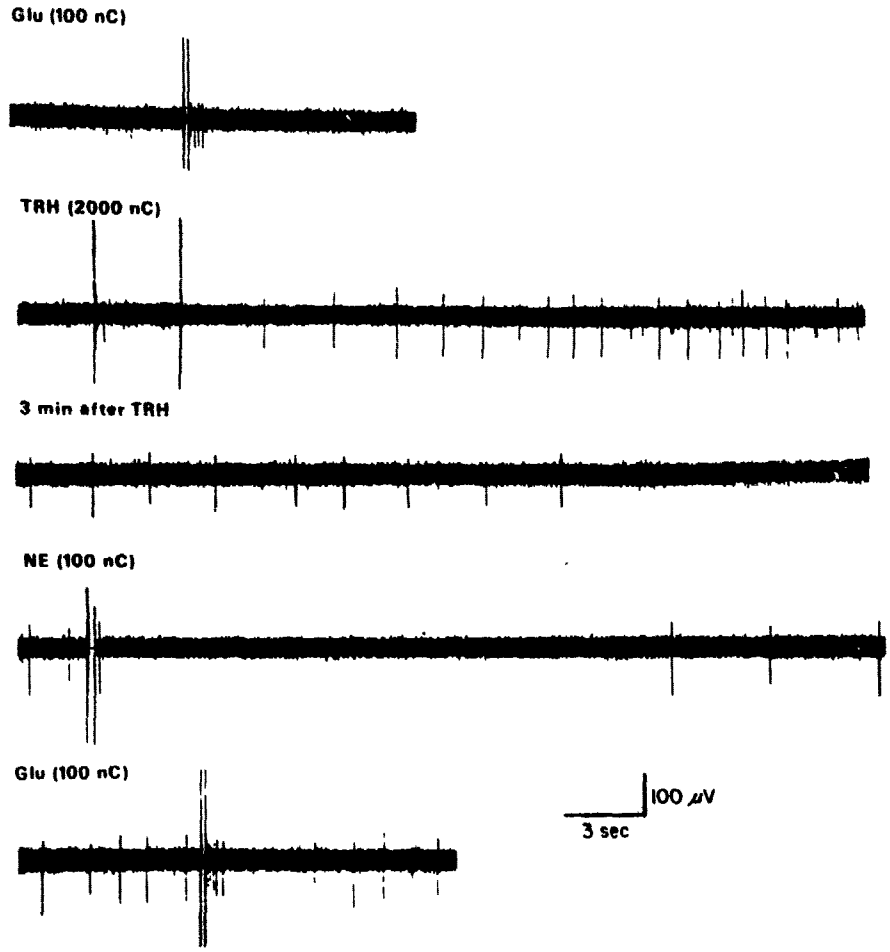

Figure 4. Responses of an area postrema neuron 490 μm from the surface to glutamate (Glu), thyrotropin-releasing hormone (TRH), and norepinephrine (NE). This record is from the same neuron shown in Figure 3. The response to glutamate was over within 1 sec and was of relatively high frequency. TRH, applied between the large artifacts at 2000 nC, induced a discharge with 6-sec latency, reached a maximal frequency of less than 0.5 Hz only after about 20 sec and lasted for 3.5 min. After two applications each of TRH and VIP the neuron remained spontaneously active. Under these circumstances it was possible to observe a very prolonged inhibition to norepinephrine at 100 nC. When the neuron was spontaneous, the glutamate response was unchanged, but was followed by a pause in the spontaneous activity.

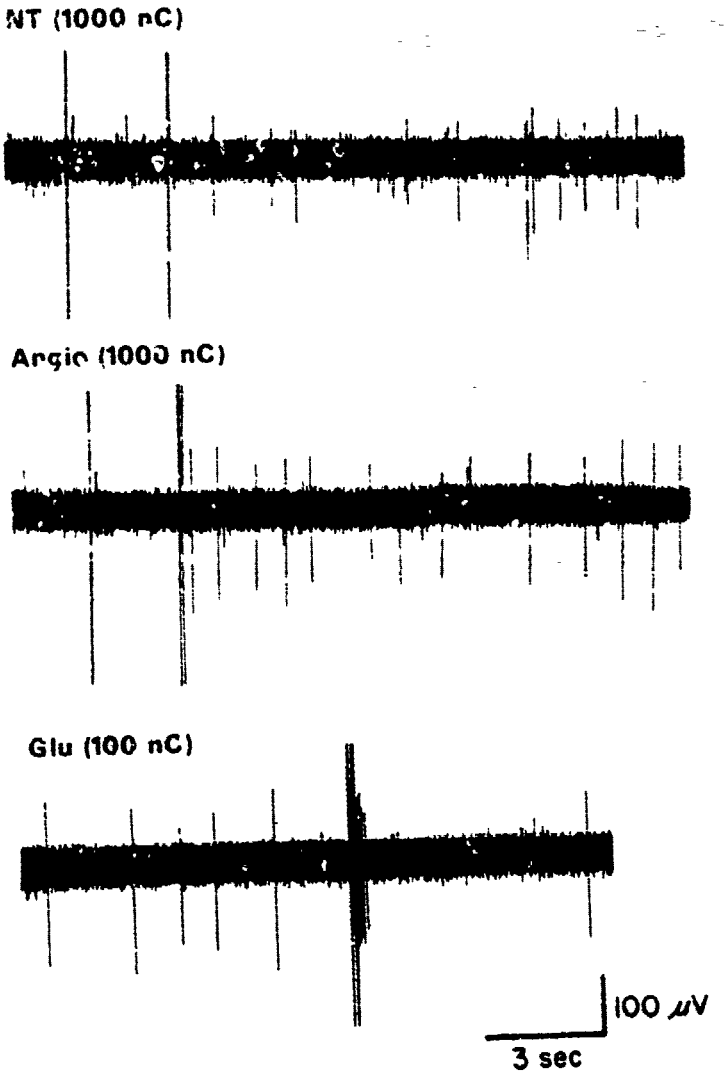

Figure 5. Neurotensin (NT) and angiotensin II (Angio) excitation of an area postrema neuron at 480 μm. As in other figures the large lines are shock artifacts indicating the ionophoretic applications. Both substances were excitatory on this neuron at 1000 nC. The responses to each lasted over 30 sec on the initial application. After a repeat application of each the neuron remained spontaneously active. The lowest record shows the response to glutamate (Glu) during this spontaneous activity.

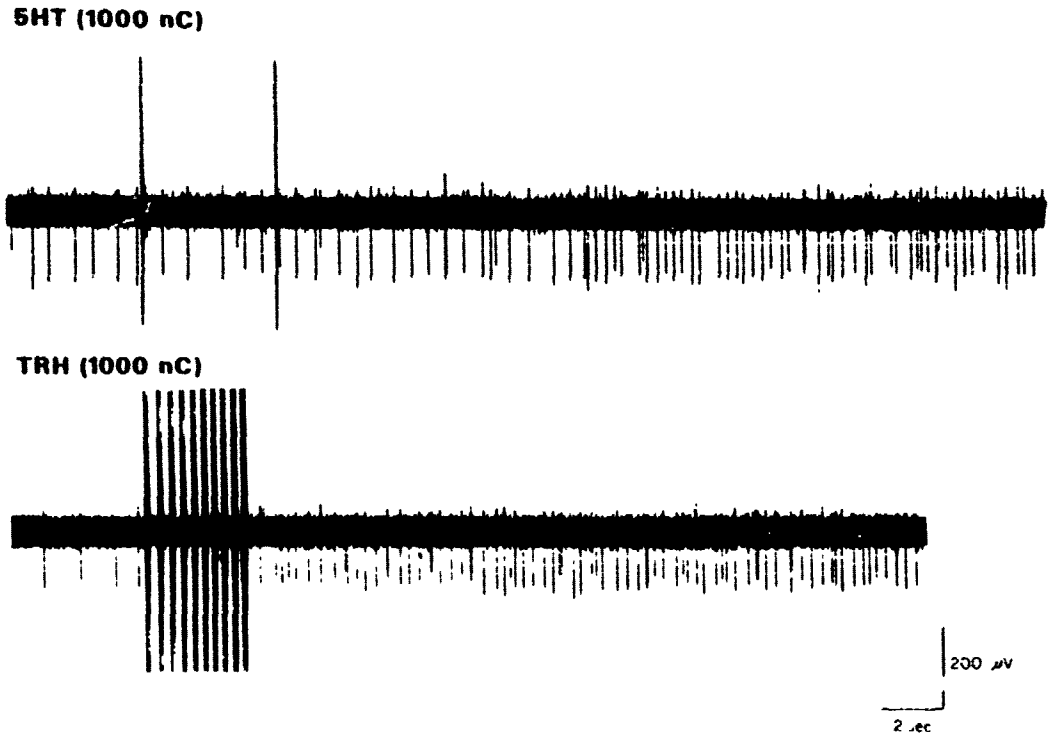

Figure 6. Responses of the neuron illustrated in Figures 3 and 4 to serotonin (5HT) and thyrotropin-releasing hormone (TRH). These records were obtained subsequent to those in the other figures, at which time the cell had received multiple applications of glutamate, two applications each of TRH and VIP, and several tests of the inhibitory response to norepinephrine. The cell was stable in a state of spontaneous discharge. However, application of both a single pulse of 1000 nC serotonin and then 100 nC pulses of TRH caused an incremental excitation, with the maximal discharge frequency reaching about 2 Hz. The discharge returned toward control over 3-5 min after each application.

TABLE 2. RESPONSES OF AREA POSTREMA NEURONS

Substance	Excitation	Inhibition	No Change
Glutamate	121/122	0/122	1/122
5HT	21/32	0/32	11/32
Norepinephrine	6/15	6/15	3/15
ACh	0/9	0/9	9/9
Histamine	29/45	3/45	13/45
Dopamine	8/11	0/11	3/11
Apomorphine	12/14	0/14	2/14
Angiotensin II	21/68	0/68	47/68
Neurotensin	4/18	0/18	14/18
TRH	34/52	0/52	18/52
VIP	18/39	0/39	21/39
Gastrin	5/11	0/11	6/11
Substance P	11/23	0/23	12/23
Vasopressin	1/2	0/2	1/2
Leucine enkephalin	7/16	0/16	9/16
CCK	0/11	0/11	11/11
Somatostatin	0/10	0/10	10/10

Several generalizations can be made concerning the patterns of response of area postrema neurons. All units were silent when first encountered but could be readily identified by their brief excitatory responses to iontophoretic application of glutamate. This is in apparent contrast to the conclusions of Borison et al. (5), who concluded that these neurons had a slow regular spontaneous activity. We found that a slow, regular spontaneous discharge could be induced in almost all area postrema neurons by application of appropriate substances, and that once initiated the slow, spontaneous discharge was very long lasting. Since Borison et al. (5) applied substances by intraarterial injection, it seems likely that they turned on many units with their first injection, thus giving the appearance of spontaneous activity.

The responses to all other transmitters are remarkable in several regards. Of the 17 substances tested, excitatory responses were obtained to all but three (acetylcholine, somatostatin, and CCK). For such small neurons to have presumably specific receptors for so many substances is surprising, although not inconsistent with the proposed role of these neurons in emesis. While dopamine and apomorphine excited most neurons, the other substances all excited approximately half of the neurons tested. Analysis of the patterns of response did not show an indication that there were multiple populations of neurons, as defined by receptor profiles. The high frequency of excitatory responses and the lack of a clear pattern of association among the responses is most consistent with the conclusions that these neurons comprise a single population. The failure to obtain an excitatory response to any one of these substances on every neuron is possibly a result of the tip of the ionophoretic electrode being at a site distant to the receptors, not because of a lack of receptors on every neuron. The presence of both excitatory and inhibitory responses to norepinephrine and histamine may reflect localization of receptors mediating the two polarity responses on different portions of the cell body or dendritic tree, although it is not clear what the functional meaning of such an organization would be. Such biphasic responses have been found in many neurons, including other brain stem neurons, to a variety of transmitters (cf. Greene and Carpenter (17)).

Another remarkable feature of the chemosensitivity of area postrema neurons is the nature of their response to excitatory substances other than glutamate. While there was variability in the sensitivities of individual neurons to particular substances, the characteristics of the responses to these excitatory substances were identical. The responses were relatively slow in onset, very long in duration, and of a relatively low maximal frequency. This is a situation unlike any previously reported. While so many substances have rarely been tested on a defined population of neurons, there is no precedent for finding so similar a response to so many substances on identified neurons. The similarity of response is suggestive of a common ionic and/or metabolic mechanism, although unfortunately no information is available on what this common mechanism might be.

Although the majority of neuronsstudied were recorded from the central and widest region of the area postrema, no obvious differences in sensitivity or pattern of response were apparent as a function of location throughout the rostral two-thirds of the area postrema. The lower one-

third was not studied because of the fact that the structure is thin there, making it difficult to be certain of the boundary. Chernicky et al. (13) have described three layers within the canine area postrema, based on Golgi preparations. They are a mantle region on the surface, containing glialoid cells, a central region containing small stellate (5-15 μm) neurons which do not project out of the area postrema, and a junctional region, deep to the others, containing small stellate (5-8 μm) neurons which project to other brain stem areas. It is likely that most or all of our recordings are from the central region, based both on the depth of recordings and the fact that these are the largest cells, and, therefore, the most easily recorded. The fact that these neurons appear to be of a single population is consistent with Borison's (4) conclusion that the area postrema has a single function, that being to function as the chemosensory trigger for emesis.

If the physiologic role of these neurons is to detect circulating emetic agents, then all substances which excite these neurons should be emetic, and all substances which do not excite the neurons should not be emetic. This pattern does appear to be the case. Glutamic acid infusion in humans causes nausea and vomiting (26), as well as a kainic-acid-type toxicity of a mouse area postrema neurons (29). Apomorphine is well known to induce emesis (38), presumably by its agonist action at dopamine receptors, known to exist in the area postrema (33). Dopamine infusion also induces emesis (20). Morphine (39), enkephalin (2), and histamine (3) all are emetic and their actions are dependent upon integrity of the area postrema. We have found that angiotensin II, neurotensin, leu-enkephalin, TRH, VIP, gastrin, substance P, and vasopressin are emetic on IV administration while CCK and somatostatin are not (11, 12). Together these observations provide strong support for the proposed function of the area postrema as being the chemosensory trigger zone for emesis.

While the other proposed functions of the area postrema are less certain, these results have relevance to one issue. While some have argued that the area postrema is the origin of an angiotensin II stimulated, sympathetically mediated increase in blood pressure (1, 16, 21, 22, 42), others have suggested that all angiotensin receptors are in the vagal nuclei rather than the area postrema (18). Our results indicate that there are angiotensin II receptors in the area postrema, although not providing positive evidence for their direct involvement in cardiovascular reflexes.

For neurons whose primary function may be to respond to humoral emetic agents, the response characteristics of area postrema neurons are probably appropriate. Nausea, which almost always precedes vomiting and which also is dependent upon the integrity of the area postrema (37), is a relatively prolonged event often with a long, undefined latency. Not only do these neurons show a low threshold for a maintained discharge, but also they are capable of incremental increases in discharge frequency when exposed to additional excitants. It seems quite reasonable to assume that nausea results from moderate spontaneous discharge impinging at the level of the motor emetic center, and that with a suitable increment of activity a threshold is reached which activates the full emetic reflex.

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