Degranulation of mast cells located in median eminence in response to compound 48/80 evokes adrenocortical secretion via histamine and CRF in dogs

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Running head: Brain mast cells and CRF-ACTH-cortisol axis
Itsuro Matsumoto, Yasuhisa Inoue, Katsuhiko Tsuchiya, Toshio Shimada, and Tadaomi Aikawa. Degranulation of mast cells located in median eminence in response to compound 48/80 evokes adrenocortical secretion via histamine and CRF in dogs. Am J Physiol Regul Integr Comp Physiol --- The effect of intracerebroventricular infusion of compound 48/80 (C48/80), a mast-cell secretagogue, on adrenal cortisol secretion was investigated in dogs under pentobarbital anesthesia. A marked increase in adrenal cortisol secretion was elicited by C48/80 along with a concomitant increase in the plasma levels of cortisol and immunoreactive-ACTH, but neither arterial blood pressure and heart rate nor the plasma histamine level altered significantly. Pretreatment with either anti-corticotropin releasing factor (CRF) antiserum or pyrilamine maleate (H₁ histamine-receptor antagonist) significantly attenuated the C48/80-evoked increase in cortisol secretion, but pretreatment with metiamide (H₂-receptor antagonist) significantly potentiated it. Significant attenuation of the C48/80-evoked increase in cortisol also occurred in dogs given ketotifen, a mast-cell stabilizing drug, prior to the pharmacologic challenge. In the pars tuberalis (PT) and median eminence (ME), mast cells were highly concentrated in close association with the primary plexus of the hypophyseal portal system. Degranulated mast cells were extensively found in the ME of C48/80-treated animals. These results suggest that mast cells located in these regions liberated histamine within the brain as a result of degranulation induced by C48/80, and that this led to activation of the hypothalamic-pituitary-adrenocortical axis.

Key words: brain mast cells; cortisol; pyrilamine; metiamide; ketotifen
HISTAMINE is widely distributed within the brain, with the highest concentrations being found in the hypothalamus, including ME, in various mammalian species (52). It is well documented that administration of histamine via either the iv or icv route activates the hypothalamic-pituitary-adrenal (HPA) axis (55, 65), indicating that it acts as a neurotransmitter in the brain mediating adrenocortical hormone secretion (52, 60, 65). However, the histamine pool in the brain comprises two compartments: one consists of histamine as a neurotransmitter derived from neuronal cells, the other consists of histamine derived from non-neuronal cells (18, 24, 35, 37, 44). Mast cells are the major source of non-neuronal histamine within the central nervous system (CNS) (52); indeed, high concentrations of mast cells have been found in the hypothalamus (37, 44), especially in the ME (35, 44), and in the circumventricular organs (8, 15). Several lines of evidence -- obtained using α-fluoromethylhistidine (19, 34) or biochemical methods (20, 44), or by experiments on mast cell-deficient animals (34, 67) -- demonstrate that the histamine contents of the two compartments are approximately equal. Furthermore, exposing rats to physical or emotional stress alters not only the concentration and rate of synthesis of histamine (6, 59), but also the number of mast cells (6) in several brain regions. These findings led us to hypothesize that the histamine-induced adrenocortical secretory response might, in part, be mediated by histamine derived from hypothalamic mast cells under some pathophysiological conditions. Our previous investigation demonstrated that an IgE-dependent degranulation of intracranial mast cells does indeed activate the HPA axis in dogs (38), suggesting that brain mast cells may act as an immune sensor serving to detect antigens arriving at the CNS from the peripheral circulation; in other words, as the “gate”
to the HPA system for an antigen from the external world. However, there is little evidence as to the physiological role played by intracranial mast cells within the CNS. The purpose of this study was to determine whether and how degranulation of brain mast cells might activate the HPA axis. For this purpose, we employed C48/80, a specific mast-cell secretagogue (14, 40, 50, 68), to induce degranulation of intracranial mast cells.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Nagasaki University School of Medicine. Furthermore, all experimental protocols used in this study conformed to the Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences (approved by Physiological Society of Japan, revised 2002). Adult male mongrel dogs weighing 9.2 to 19.3 kg (n=128) were used for the experiments on the hypothalamic-pituitary-adrenal response. On the day before the experiment (which started at around 9:00 am) involving a pharmacologic challenge, a 22-gauge stainless-steel tube was implanted as a cerebral guide cannula into the cerebral third ventricle (VIII) of each animal using a stereotaxic instrument under pentobarbital anesthesia (25 mg/kg, iv). The stereotaxic coordinates were: 20 mm anterior and 7 mm dorsal to the external aural meatus line (EAML) and 0 mm from the midline. After the guide cannula had been firmly anchored to the skull using dental cement, a glass cannula for the collection of adrenal venous blood was inserted into the left lumbo-adrenal vein. To lessen the effect of surgery on the experimental results, the retro-peritoneal route was
employed for the cannulation of the lumbo-adrenal vein (i.e. without opening the abdomen) using the method of Satake et al. (51) with some modifications (23, 56). In brief, a straight, longitudinal (rostral-caudal) incision some 6 cm in length was made in the lumbar skin between the tip of the left last rib (twelfth) and the left caudal ventro-iliac spine. Then, the lumbo-dorsal fascia covering the latissimus dorsi muscle was cut. Once the left lumbo-adrenal vein and adrenal gland could be seen, the external and internal abdominal oblique muscles were cut around the boundary of the latissimus dorsi. This made it possible to reach the left adrenal vein, lying along the dorsal wall of the retro-peritoneal cavity, without opening the abdomen (i.e. without a laparotomy) and without any mechanical disturbance of the visceral organs lying within the abdominal cavity. After the accessory side branches of the lumbo-adrenal vein had been doubly ligated and cut, a small glass cannula connected to a rubber tube containing 0.3ml heparin-saline solution (500U/ml) was inserted into the adrenal vein. When required, splanchnicectomy (SPX) (resection of the left greater, lesser, and least splanchnic nerves) was performed around the left adrenal gland before the adrenal cannulation (in 6 dogs). The incision was closed with sutures after administration of penicillin G (30,000 U, intramuscularly) and 2% xylocaine jelly (2 ml, given hypodermatically around the incision; Fujisawa, Japan). The duration of the surgery (implanting cerebral guide cannula and adrenal vein cannula) was approximately 90 min. The animals were placed in a heated recovery room and their rectal temperature maintained above 38 °C. They were allowed to recover for about 24 hours. All animals were awake and could drink normally water as and when they chose by 8 h after the induction of anesthesia. Since they had not undergone a laparotomy operation, lost of blood and
extra-cellar fluids during the surgery was estimated to be less than 20 ml. At 9:00 am on the
day following the above operation, each animal was reanesthetized with pentobarbital
sodium (25 mg/kg, iv). Supplementary pentobarbital was given as required to maintain an
absent palpebral reflex. An indwelling catheter for the withdrawal of peripheral blood
samples to be assayed for adrenocorticotropic hormone (ACTH) and histamine was inserted
into the inferior vena cava via a branch of the femoral vein 4 h before any pharmacologic
challenge. Drugs dissolved in artificial cerebrospinal fluid (ACSF) were administered into
V_{III}, after adjustment to the pH (7.4) and concentration required, at a rate of 15.6 µl/min
through a 27-gauge stainless-steel tube fixed into the cerebral guide cannula.

**Experimental procedure**

Blood sampling was started at 13:00 on the day following the operation. Adrenal or
peripheral venous blood samples were collected in graduated tubes at 10 min before, and at
5, 10, 20, 30, 40, 60, 90, 120, and 150 min after the start of an icv infusion of C48/80 (3.75,
37.5, or 375 µg/kg over a 5-min period; Sigma-Aldrich). Each blood sample (roughly 2.5
ml) was centrifuged immediately after its collection. The resulting plasma samples were
stored at -20 °C until assayed for cortisol etc. The blood cells from each sample were
resuspended in the same volume of saline, and returned to the animal before the next
sampling period. Such replacements ensured that the hematocrit of all blood samples
ranged between 40 and 46 %.

When required, an infusion of pyrilamine maleate (5 µg/kg over 10 min; Sigma-Aldrich,
Japan) or metiamide (~ 500 µg/kg over 10 min; SKF-Japan) was started 5 min before the
onset of the C48/80 infusion and ended 5 min after the start of the pharmacologic challenge.
Lyophilized anti-CRF antiserum (equivalent to 12.5 µl rabbit antiserum against human CRF; Sigma, USA) was infused into VIII over 30 min (ending 5 min before the onset of a pharmacologic challenge). Ketotifen fumarate (Sigma-Aldrich, Japan), a prophylactic anti-asthma drug with a mast cell-stabilizing action (26, 28), was given in one of three ways: (a) simultaneous administration [ketotifen (100~2000 µg/kg) was infused into VIII starting 15 min before the onset of the C48/80 infusion, and infused for 20 min (ending 5 min after the onset of the C48/80-challenge)]; (b) semichronic administration [ketotifen was given twice via the icv route before the C48/80-challenge (the first, starting at 24 h before the onset of the C48/80 infusion, was administered at 1 mg/kg over 4 h, while the second, starting at 4 h before the onset of the C48/80 infusion, was at 0.5 mg/kg over 2 h)]; (c) chronic administration [ketotifen was given via the oral route (per os, po) every morning for seven days, ending on the day before the experiment (2 mg/day; a dose comparable to that given to allergic individuals)]. In dogs given ketotifen either semichronically or chronically, 5 µg/kg histamine was given subsequently as a challenge at 180 min (30 min after the final blood sampling in the experiment on the ketotifen-effect) after the start of the C48/80 infusion via the icv route to establish whether or not ketotifen’s anti-histaminergic effect, one of the side effects of this drug, might be responsible for any change in the C48/80-evoked adrenal cortisol secretory response. Mean arterial blood pressure (MAP) and heart rate (HR) were monitored via the femoral artery through a strain-gauge pressure transducer (Statham P23AC) and a polygraph system (Nihon Kohden AP 620-G) starting before, and continuing after the pharmacologic challenge. To confirm correct placement of the cerebral cannula, cresyl violet dye was infused into VIII (in the same way as the
pharmacologic challenge) after the end of the experiment, and dye distribution within the brain was examined in each animal.

**Determinations**

The cortisol in the adrenal venous plasma was measured by a fluorometric method after purification by thin layer chromatography on silica gel (22, 39). The adrenal cortisol secretion rate (µg/kg/min) was calculated by multiplying adrenal plasma flow (ml/kg body weight/min) by the concentration of cortisol in the adrenal plasma (µg/ml). The integrated adrenal response (area under the curve of the adrenal cortisol secretory response), representing the evoked total cortisol secretion above the basal secretion over a 150-min period, was also calculated for each administration of C48/80, histamine, or vehicle. The concentration of plasma immunoreactive (ir)-ACTH was measured using a commercially available radioimmunoassay kit (CIS Biointernational, France) without an extraction procedure. The limit of determination was 4 pg/tube, and the intra- and inter-assay coefficients of variation were ~ 8.5 and 10.2 %, respectively. Ir-ACTH levels are expressed in pg/ml, with the equivalent synthetic human ACTH (1-39) used as the reference standard. Plasma histamine was assayed by a single-isotope radioenzymatic method, with rat kidney as the source of histamine methyltransferase (53). Briefly, each plasma sample was deproteinized using 1 M HClO₄, and the supernatant was neutralized with NaOH after addition of sodium phosphate buffer (pH 6.5). The sample was applied to a Dowex 50-X-4 column, and eluates were incubated at 37 °C for 90 min with histamine N-methyltransferase (prepared from rat kidney), ³H-S-adenosylmethionine, and sufficient 0.1 M sodium phosphate buffer (pH 7.9). After addition of unlabeled 1-methylhistamine at
the end of the incubation, labeled and unlabeled 1-methylhistamine were extracted using 3 ml chloroform. The radioactivity of the labeled 1-methylhistamine was measured in a liquid scintillation spectrometer (model 3330; Packard, USA) after washing the chloroform-extracts of the incubates with 3 ml distilled water. Plasma catecholamines were measured by the coulometric electrochemical determination method, with the minor modification previously reported (39), using a high-performance liquid chromatographic analytic system (ESA, model 5100A).

**Histology**

For the histological study, we used 13 animals of either sex weighing 4.8 ~ 11.2 kg that had received no experimental treatment. Each animal was given an overdose of pentobarbital sodium. After trimming, the brain that had been perfused transcardially with saline containing 0.1 M phosphate buffer (pH 7.4) was subjected to histological examination under a light microscopy by a previously described method (38). Since a large number of mast cells is found in a circumscribed ventral region of the hypothalamus (38), their distribution in the floor of Viii and in a zone within 2 mm of the midline on either side that contained the ME and the PT was minutely examined in coronal serial sections. The distribution in the rostrocaudal direction was examined by counting the number of mast cells in coronal sections taken every 0.5 mm from 16.8 mm to 22.8 mm rostral to the EAML (i.e., R16.8 - R22.8). The distribution in the horizontal direction was examined at about R19.3, the level at which the mast-cell number was found to be maximal in the rostrocaudal examination. In sections at this level, the PT is seen on both sides and the ME in the central portion of the section. The vertical distribution of mast cells was examined in
the central part (i.e., excluding the PT region) of coronal sections at about R19.3; this central part consists largely of the ME. Degranulation of mast cells was judged by the extrusion of granule content and/or more than 50% loss of cellular staining according to the criteria of Theoharides et al. (58). Degranulated mast cells were surveyed in the PT and ME in dogs treated with (n=4) or without (n=4) C48/80. Although mast cells were also found in the neuro- and adeno-hypophysis (AH) and the leptomeninges, these were not included in the count of intracranial mast cells.

Statistical analysis

All data are presented as means ± SE. Data were analyzed using a one- or two-way analysis of variance (ANOVA) with correction for repeated measures and with an appropriate post hoc test (Fisher’s Protected Least Significant Difference, PLSD) for multiple comparisons.

RESULTS

Effects of an icv infusion of C48/80 on adrenal cortisol secretion and on the plasma levels of cortisol, ir-ACTH, and histamine

A dose-dependent increase in the adrenal cortisol secretion rate was observed in response to icv administrations of 3.75, 37.5, and 375 µg/kg C48/80. When 37.5 µg/kg C48/80 was given, a significant increase in adrenal cortisol secretion was first detected at 5 min after the start of the C48/80 infusion, with the peak at or just after 10 min. The adrenal secretion remained high for up to a further 30 min before gradually declining toward the basal level (Fig. 1A). The peak value seen with this dose of C48/80 was 5.8 times the basal secretion
rate. The integrated adrenal cortisol secretion (area under the curve) showed a
dose-dependent increase (Fig. 1A, inset). The plasma level of cortisol also increased slowly
and reached a peak level at 30 min after an icv administration of 37.5 µg/kg C48/80. Then,
it decreased gradually toward the basal level, but was still raised at 150 min after the
C48/80 challenge (Fig. 1B, top). A significant increase in the plasma ir-ACTH concentration
was first seen at 5 min, and the peak occurred at 10 min after the start of a 37.5 µg/kg
C48/80 infusion (Fig. 1B, middle). The evoked increase in ir-ACTH showed a rapid return
toward the basal level (at 20 min), but the level gradually rose again, with the second peak
at 40 min after the start of the infusion. These peak levels of ir-ACTH were 2.4 times and
1.6 times the basal level (at 10 and 40 min, respectively). Neither the plasma level of
histamine (Fig. 1B, bottom) nor the cardiovascular variables (MAP and HR) (data not
shown) altered significantly as a result of the infusion of 37.5 µg/kg C48/80.

Adrenal cortisol secretion increases in response to histamine

To compare the adrenal response evoked by C48/80 with that induced by histamine, the
latter was given via the icv or iv route. A dose-dependent increase in adrenal cortisol
secretion was evoked when 5, 10, or 25 µg/kg histamine was given via the icv route (see
time-response curves and integrated data in Fig. 2A and inset). When 10 µg/kg histamine
was given via the icv route, cortisol secretion had already increased significantly at 5 min;
it then continued to rise and peaked at about 20 min. Thereafter, it decreased rapidly to the
basal level (Fig. 2A). The integrated response to 25 µg/kg histamine was comparable to that
induced by 37.5 µg/kg C48/80 (Fig. 2A, inset).
However, the levels reached in the early part of the response (5~30 min) to 10 µg/kg histamine were significantly greater than the levels seen at the same time after 37.5 µg/kg C48/80, while in the later part (60~90 min) the levels induced by 10 (or even 25) µg/kg histamine were significantly lower than those induced by C48/80. Neither MAP nor HR altered significantly after infusion of ~ 25 µg/kg histamine via the icv route (Fig. 2B). When 100 µg/kg histamine was administered over 1 min via the iv route, marked increases in adrenal cortisol secretion occurred with a transient decrease in MAP and a transient increase in HR immediately after the histamine injection (Fig. 2B).

**H<sub>1</sub> and H<sub>2</sub> antagonists modulate the C48/80-evoked increase in cortisol secretion**

A significant attenuation of the C48/80 (37.5 µg/kg)-evoked increase in cortisol secretion was observed in the period from 10~60 min after the pharmacologic challenge in dogs previously given 5 µg/kg pyrilamine maleate, an H<sub>1</sub>-histaminergic antagonist, via the icv route (Fig. 3A and inset). This pharmacologic antagonist attenuated the integrated C48/80-evoked cortisol secretory response to about 50 % of that seen in animals without such pretreatment. When the same dose of the H<sub>1</sub>-histaminergic blocker (5 µg/kg) was given via the iv route, no significant change in the C48/80-evoked cortisol secretion was found. However, in dogs treated intravenously with 500 µg/kg pyliramine (100 times that given via the icv route), a significant attenuation was seen in the C48/80-evoked cortisol secretory response. Pretreatment with this H<sub>1</sub>-histaminergic blocker (5µg/kg, icv) had effects on the plasma cortisol and ACTH responses (their peaks were seen at 30 min and 10 min after an infusion of C48/80, respectively) to C48/80 that were similar to its effect on
the adrenal cortisol secretion response to C48/80 (Fig. 3C). In contrast, when 500 µg/kg metiamide, an H2-histaminergic antagonist, was given via the icv route, the C48/80-evoked cortisol secretion was augmented significantly at 10, 20, 30, 40, 120, and 150 min after the start of the infusion of C48/80 (Fig. 3B), but this did not occur when ~ 250 µg/kg metiamide was given by the same route (data not shown). Following pretreatment with 500 µg/kg metiamide (icv), the peak cortisol secretion (at 30 min) was 1.62 times that seen in animals without such pretreatment. The level then gradually declined, but at 150 min (the end of the experimental period) it was still 1.66 times the level seen at the same time in animals not pretreated with metiamide. The integrated cortisol secretory response to C48/80 in metiamide-pretreated animals was 1.44 times that in dogs not given the H2-antagonist. The same dose of metiamide (500 µg/kg), however, failed to change the C48/80-evoked cortisol secretion when it was given via the iv route. Although pretreatment with the H2-antagonist (500 µg/kg, icv) tended to have a similar effect on the C48/80-induced peak levels of plasma cortisol and ACTH as on the increase in the adrenal cortisol secretion rate, no statistically significant effects on the augmented plasma concentrations of those two hormones were observed (Fig. 3C). Neither 5 µg/kg pyrilamine alone nor 500 µg/kg metiamide alone (both icv) caused a significant alteration in the adrenal cortisol secretory response to vehicle (Figs. 3A & B).

**Immunoneutralization with anti-CRF antibody abolishes the C48/80-evoked increases in cortisol secretion**

To explore the mechanism underlying the C48/80-evoked pituitary-adrenal response,
immunoneutralization against the CRF within the brain was employed. When an anti-CRF antibody was delivered into V_{III} before an infusion of C48/80, the C48/80-evoked adrenal secretory response was markedly suppressed in the period 10~90 min after the start of the pharmacologic challenge, with the integrated response showing complete suppression (Fig. 4 and inset). The basal level of cortisol secretion was not altered by such anti-CRF pretreatment. The plasma levels of cortisol (which peaked at 30 min) and ACTH (peaked at 10 min) also showed significant attenuation in dogs pretreated with anti-CRF antiserum (Fig. 4B).

Pretreatment with ketotifen attenuates the C48/80-evoked increase in cortisol secretion

To confirm whether mast cells do or do not participate in the C48/80-evoked adrenal response, ketotifen was employed as a mast-cell stabilizer. Simultaneous administration of ketotifen with the C48/80-challenge (37.5 µg/kg) via the icv route caused no significant change in the C48/80-evoked cortisol secretory response even when a large dose of ketotifen (~ 2000 µg/kg) was given (Fig. 5A). In contrast, in dogs given ketotifen semichronically via the icv route, a significant attenuation of the C48/80-evoked increase was observed at 20 ~ 120 min after the start of the C48/80 infusion (Fig. 5B). Furthermore, in dogs given ketotifen chronically via the oral route, the C48/80-evoked adrenal secretion rates were attenuated significantly at 20 ~ 90 min after the start of the C48/80-infusion (even though no ketotifen was given on the day of the pharmacologic challenge). In each case, the integrated responses were suppressed by some 50 % (inset in Fig. 5B). Although co-administration of ketotifen with C48/80 had no effect on the increases in the plasma
levels of cortisol and ACTH induced by C48/80 administration, pretreatment with ketotifen [given either semichronically (icv) or chronically (po)] attenuated the effect of C48/80 on the adrenal cortisol secretion rate (Fig. 5C). To examine whether the anti-histaminergic action of ketotifen, a side effect of this agent, might be responsible for the above attenuation of the C48/80-evoked adrenal response, 5 µg/kg histamine (minimal effective dose) was given via the icv route at 30 min after the final blood sampling in the experiment on the ketotifen-effect in dogs pretreated either semichronically or chronically with ketotifen. In these animals, the adrenal cortisol secretion rate at 10 min after the start of the histamine infusion was slightly, but not significantly, smaller than that in animals not given ketotifen (Fig. 5D).

SPX abolishes the C48/80-evoked increase in adrenal epinephrine secretion, but not in cortisol

Since administration of C48/80 (icv) also elicits increases in adrenal epinephrine and norepinephrine secretion (I. Matsumoto et al., unpublished data), we examined whether adrenomedullary catecholamines might contribute to the C48/80-evoked increase in adrenal cortisol. In dogs with SPX, 37.5 µg/kg C48/80 given via the icv route evoked no increase in adrenal epinephrine secretion, but the C48/80-evoked increase in adrenocortical secretion was intact (Fig. 6).

Histological study

In the rostrocaudal direction, mast cells were concentrated in a limited region (from
R18.3 to R20.3 rostral to the EAML) (Fig. 7), with the peak population at R19.3 (625 ± 40.3 /100 µm-thick section, n=13).

To analyze the distribution of mast cells in the horizontal direction, each coronal serial section at about R19.3 was divided into five small subregions either side of the midline (I~X, Fig. 8). The mean cell counts in the most lateral subregions on either side (I and X) were about 5-times those in the subregions III ~ VIII, with subregions II and IX (the second most lateral) yielding intermediate counts. The lateral parts of subregions I~II and IX~X, corresponding to the pars tuberalis (PT), consist of enveloping epithelial tissue rising upward from the AH and closely surrounding the neuronal tissue called the median eminence (ME), which extends from the hypothalamus. In fact, subregions III ~ VIII correspond to the ME, which contains ependymal tissue and neuronal axons extending ventrally from the tuber cinereum of the hypothalamus. The population of mast cells in the PT amounted to 63 % (463 ± 53/100 µm-thick section, n=9) of the total population of mast cells in any one coronal section taken close to R19.3 (735 ± 68/100 µm-thick section, n=9). The density of the mast-cell population in this area would be roughly 5000 ~ 8000/mm³.

An analysis of the vertical distribution of mast cells in the ME (corresponding to subregions III ~ VIII in Fig. 8) is shown in Fig. 9, with actual photomicrographs also shown. When the ME, roughly 1 mm in thickness at R19.3, was divided into 5 vertical levels (i~v), each subdivision was about 200 µm thick. The population of mast cells in the most ventral subdivision, the so-called outer palisade layer of the ME (v in Fig. 9), which contacts the basal part of the AH via a thin layer of tissue extending from the PT, was about 10-times that observed either in subdivisions ii ~ iii, the so-called middle fibrous layer of
the ME, or in the floor of V_III (level i in Fig. 9, the inner ependymal layer of the ME). The highest density of mast cells was found in level v as a line along the thin layer of epithelial tissue that seems to form a border between the AH and the outer palisade layer of the ME. This corresponds to the *infundibulum* or *infundibular stalk* (level v). The mast-cell population in level v amounted to 57% (196 ± 15/100 µm, n=9) of the total number of mast cells located in the ME (343± 38 /100 µm, n=9). The mast-cell numbers in the upper levels (i ~ iv in Fig. 9) dwindled gradually in a dorsal direction, with the level just below the floor of the third ventricle (level i in Fig. 9) containing 23.8 ± 5/100 µm (n=9). We noted particularly that numerous mast cells in the ME and the PT resided in close association with the capillaries called the primary plexus of the hypophyseal portal system (arrowheads in Fig. 9A and B).

Histological examination under a light microscope revealed degranulated mast cells, as judged by extrusion of granule contents and/or more than 50% loss of cellular staining (white arrows in Fig. 10A) according to the criteria of Theoharides et al. (52). Such degranulation was detected in 11.2 ± 0.51% of the counted mast cells (total cell number counted was 1662) in PT and ME in 4 dogs without any pretreatment. No significant difference in the ratio of degranulated mast cells to intact cells was found among the areas examined (Fig.10B, left). In three C48/80-treated animals, extensive degranulation of mast cells was seen in and around the ME. Treatment with C48/80 led to significant increases in the ratio of degranulated mast cells to granulated cells (39.8 ± 4.2 % of 1458 mast cells counted). Such degranulation was observed in 21.5 ± 3.2 %, 32.7 ± 5.7 %, 47.9 ± 4.8 %, and 57.0 ± 3.2 % of the counted mast cells located in the PT (cell number: 492, n=4),
ventral part of ME (VM, cell number: 513, n=4), medial part of ME (MM, cell number: 321, n=4), and cranial part of ME (CM, cell number: 132, n=4), respectively (Fig. 10B, right)

DISCUSSION

It is increasingly being recognized that mast cells play diverse roles in homeostatic responses to pathobiologic events (17, 21, 38). We found that an icv administration of C48/80 evoked an increase in adrenal cortisol secretion rate with accompanying elevations in the plasma levels of cortisol and ACTH, but without any significant changes in plasma histamine or cardiovascular variables (MAP & HR). C48/80 is a mast cell-degranulating agent that liberates histamine from mast cells, but not from other histamine-containing cells such as macrophages and lymphocytes (68). Pretreatment with a mast cell-stabilizing drug (ketotifen), whether delivered semichronically or chronically (via the icv or oral route, respectively), significantly attenuated the C48/80-evoked increase in cortisol secretion. These results suggest a) that mast cells play a central role in the C48/80-evoked adrenocortical secretory response, and b) that they would be located in intracranial regions in which the blood-brain barrier (BBB) is lacking.

Furthermore, our previous histological examination revealed that mast cells located in ME and PT can be stained metachromatically with toluidine blue (38). In the present study, the proportion of degranulated mast cells to granulated mast cells in the floor of V_III (inner ependymal layer of the ME) in dogs given C48/80 into V_III was significantly higher than that in the same region in dogs untreated with C48/80. It is well known that mast cells display heterogeneities in their mediator content, in their sensitivity to mast-cell
secretagogues, and in their staining properties, and that such heterogeneities depend on differences in anatomical site and species (2, 13, 14, 16, 24, 40, 45, 47, 50). Two types of mast cells, termed connective tissue mast cells and mucosal mast cells, have been identified in intracranial regions as well as in peripheral tissues. Both of them have IgE receptors and can directly response to IgE-mediated stimuli. Connective tissue mast cells, however, contain highly concentrated histamine, show metachromatic staining with toluidine blue, and degranulate in response to C48/80. In contrast, mucosal mast cells appear to contain low concentrations of histamine, do not display metachromasia, and seem to show a weak responsiveness to activation by C48/80. These data suggest that mast cells located in ME and PT should be classified as connective tissue-type mast cells.

In addition, the present study has demonstrated that pharmacologic antagonists against mast cell-derived mediators (namely, H₁- and H₂-histaminergic blockers) modulated the C48/80-evoked adrenal response when they were given via the icv route. Moreover, the C48/80-evoked adrenocortical response was completely suppressed by immunoneutralization with anti-CRF antiserum. On the basis of the above data, we suggest a) that histamine is liberated from mast cells located in the intracranial region, most probably in the ME and PT, as a result of degranulation, but does not enter the systemic circulation, and b) that the liberated histamine triggers activation of the CRF-ACTH-cortisol axis, which occurs in a cascade-like fashion.

However, we need to discuss some possible limitations in our experimental system and results. It is well known that in dogs, surgery involving a so-called laparotomy potentiates the ACTH or adrenocortical response to subsequent stress such as hypoxia or hemorrhage
However, our animals did not receive a laparotomy or manipulation of visceral organs, and their plasma levels of ACTH and cortisol at 10 min before the pharmacologic challenges were within the physiological resting ranges. Nevertheless, there is a possibility that the pituitary-adrenocortical response to the C48/80- or histamine-administration might have been potentiated because the animals were allowed some 24 hours for recovery from the surgery needed for the cannulations of the cerebral ventricle and adrenal vein. In addition, there is a possibility that some central neuroendocrine factors other than CRF, such as arginine-vasopressin, may play a role in the activation of the pituitary-adrenal axis that occurs in response to C48/80 when it is given into the central third ventricle. If so, C48/80-evoked CRF might act only in a permissive manner to stimulate secretion of ACTH after degranulation of central mast cells. Indeed, in our preliminary examination not only the plasma level of arginine-vasopressin, but also the plasma levels of renin activity and catecholamines increased in dogs given C48/80 centrally (I. Matsumoto et al., unpublished observation). The ketotifen-dependent attenuation of the HPA response evoked by C48/80 is unlikely to be due to any anti-histaminergic action of ketotifen, one of the side effects of this drug (36), because a) simultaneous administration of a large dose of ketotifen (~2 mg/kg, icv) had no effect on the C48/80-evoked HPA response, and b) the histamine-induced increase in cortisol secretion was not impaired significantly in dogs pretreated either semichronically or chronically with ketotifen even though such pretreatments attenuated the C48/80-evoked adrenal increases in the same animals. Furthermore, the C48/80-induced HPA response is unlikely to be due to an evoked release of adrenal catecholamines (or to any other change in adrenal functions evoked via the
adrenal sympathetic outflow) because although the C48/80-evoked secretion of adrenal epinephrine was completely abolished by SPX, the C48/80-evoked increase in cortisol secretion was unaffected.

The C48/80-evoked adrenal response might be thought to be mimetic to the histamine-induced adrenal response. However, we noted some differences between these responses. For instance, although the integrated cortisol secretion evoked by 37.5 µg/kg C48/80 was roughly equal to that induced by 25µg/kg histamine, the peak secretion rate in response to the former (at 30 min) was only 65% of the peak response to the latter (at 20 min). Besides, the C48/80-evoked adrenal response increased more gradually and was longer lasting than the histamine-induced response. To explain these differences, a possible clue may be given by our preliminary observation that when C48/80 was infused into the adrenal glands (where of course the presence of mast cells has been confirmed) in artificial medium, the histamine in the perfusate peaked at 1 min (at 20 times the basal level) and remained high for up to a further 5 min (5 times) after the start of the infusion (T. Aikawa and I. Matsumoto, unpublished data). Therefore, the above-mentioned differences could be due a) to the C48/80 infused into V_III taking several minutes to spread into the areas around V_III, to reach the intracranial mast cells, or to induce a maximal liberation of histamine from these mast cells, and b) to a delay of a few minutes being necessary for the mast-cell histamine to be removed from, or inactivated in the cerebrospinal fluid.

A number of questions arise from the present study. First, where are the degranulated mast cells located, and how do chemical mediators liberated from these mast cells after their degranulation activate the HPA axis? In this study, numerous mast cells were found
in the PT and ME, which lack the BBB, and a lot were observed to be closely apposed to capillaries in the so-called primary plexus of the hypophyseal portal system. Theoharides et al. have reported that mast cells located in the ME are found in close association with CRF-containing neuronal terminals (59). In our previous report, an antigenic challenge delivered via either the icv or iv route triggered a degranulation of mast cells located in the hypothalamus in dogs that had been passively sensitized with IgE, and subsequently evoked an HPA response via histaminergic H1 receptors in the brain (38). In addition, several studies suggest a) that not only histamine (52, 65), but also histaminergic receptors such as H1- or H2-type receptors (4, 33, 43, 52, 63) are highly concentrated in the hypothalamus, including the ME, and b) that H1-histaminergic receptors accelerate (1, 7, 49, 52) while H2-histaminergic receptors retard (49) HPA responses in the brain. In our present investigation, the C48/80-evoked increase in cortisol was significantly attenuated by prior icv administration of 5µg/kg pyrilamine maleate, an H1 antagonist, and significantly augmented by prior icv administration of 500 µg/kg metiamide, an H2 antagonist. Furthermore, there is definite evidence that increases in adrenal cortisol secretions in response to histamine given via the iv route are seriously impaired by lesions in the anterior or posterior ME (23). On the basis of the above evidence, it is most likely that histamine liberated from the mast cells located in the ME and PT that are degranulated in response to C48/80 given into VIII subsequently elicit a release of CRF, and might evoke a release of arginine-vasopressin, via H1-receptors (which may be located within the ME).

The second, and more fundamental, question concerns the physiological significance of the mast cells located in the ME or PT. Brain mast cells have a wide spectrum of actions
leading to a regulation or disruption of the functions of the CNS (17, 21, 25, 54, 57). Although mast cells located in the thalamus, but not in the hypothalamus, activate adrenocortical secretion in response to C48/80 given into the lateral cerebral ventricle in rats (5), the pathophysiological role of the thalamic mast cell-induced adrenocortical reaction remains unclear. It has been shown that histamine release from hypothalamic slices can be induced by C48/80 in vitro (62). However, convincing functional studies on the hypothalamic mast cells, especially on the mast cells located in the ME, are lacking. Many previous studies regarding the organization of the ME and its surrounding area have revealed the following (3, 29, 60, 61, 66). The ME lacks the BBB and has a rich vascularity that constitutes the networks of the primary capillary plexus of the hypothalamic-pituitary portal vessels. In the ME, neurohumoral factors, such as CRF or arginine-vasopressin, are contained within the nerve terminals of neurons projecting from the parvocellular divisions of the paraventricular nuclei, and are subsequently carried into the AH via the capillary networks. Efferent and afferent nerve fibers, which contain neurotransmitters such as catecholamines, acetylcholine, serotonin, and histamine, project from the upper or lower CNS to the ME and innervate the ME. Furthermore, endogenous immune products, such as cytokines or antibodies, and exogenous substances such as antigens, microorganisms etc. would also be carried into the ME via the portal vascular system suggesting that the ME may be one of the most important gates into the CNS for the immune system. The above findings suggest that these humoral factors, neuronal signals, and immune information are integrated in the ME, and that the mast cells located therein take part in the integration. Thus, we should like to propose the speculative notion that the mast cells located in the ME
and PT may participate in the link between the antigens arriving there from the external world and the ensuing adrenocortical response. Indeed, our present histological examination revealed a) that more than 60% of mast cells in the ME (level v in Fig. 9) were crowded along the border between the basal part of the AH and the external part of the ME, the so-called outer palisade layer (29), and b) that in the middle part of the ME, the so-called middle fibrous layer (levels ii ~ iv in Fig. 9)(29), numerous mast cells were scattered along those capillaries that seemed to penetrate upward from the outer palisade layer to the floor of V_{III}, the so-called inner ependymal layer (level i in Fig. 9)(29).

A final question is the significance of the mast cell-dependent activation of the HPA response. IgE-dependent immediate hypersensitivity reactions are frequently associated with dysfunctions of the respiratory or cardiovascular systems, which can have life-threatening consequences (12, 64). It is well known that pharmacological glucocorticoids and physiological adrenal corticosteroids can ameliorate the severity of these dysfunctions and suppress the immune responses and/or subsequent immune-mediated inflammation (11, 30, 41, 42). Furthermore, CRF or proopiomelanocortin-derived peptides, such as α-MSH, provide a counter-regulatory mechanism that modulates inflammatory events (9, 10, 21, 40). One possible answer to the above question is that substances borne by the activated HPA axis after such degranulation of mast cells may evoke down-regulation at multiple levels in the pathobiologic progress of immediate hypersensitivity that is triggered by peripherally located mast cells. Consequently, they may contribute harmoniously as a negative feedback control mechanism not only against immediate hypersensitive reactions, but also against
subsequent progress in inflammation, including so-called late-phase reactions. Interestingly, it has been shown that CRF secreted from sympathetic nerve terminals during stress induces degranulation of mast cells located in the thalamus, leptomeninges, or peritoneal tissue (27, 48, 59). This suggests that brain mast cells located in the ME may cross-talk with CRF-containing neurons. Certainly, the present study has confirmed that degranulation of mast cells located in the ME or PT can elicit adrenal cortisol secretion via CRF released within the brain.
Footnotes

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REFERENCES


42. Munck A, Guyre PM, and Holbrook NJ. Physiological functions of glucocorticoids


45. **Purcell WM, Cohen DL, and Hanahoe THP.** Comparison of histamine and 5-hydroxytryptamine content and secretion in rat mast cells isolated from different anatomical locations. *Int Arch Allergy App Immunol* 90: 382-386, 1989.


50. **Saavedra-Delgado AMP, Turpin S, and Metcalfe DD.** Typical and atypical mast cells of the rat gastrointestinal system: distribution and correlation with tissue histamine.


Figure legends

Fig. 1. Effect of C48/80 on adrenocortical secretion rate and on plasma levels of cortisol, ACTH, and histamine. A: Adrenal cortisol secretion rate before and after icv administration of C48/80 in dogs. C48/80 was dissolved in ACSF. Vehicle (○, n=7) or C48/80 was delivered into V III, the later at a dose of 3.75 µg/kg (○, n=7), 37.5 µg/kg (□, n=7), or 375 µg/kg (◇, n=7). Integrated adrenal cortisol secretion over the 150 min after icv administration of C48/80 (inset). B: Effects of 37.5 µg/kg C48/80 (■, n=6) or vehicle (□, n=5) on plasma cortisol (top), plasma ACTH (middle), and plasma histamine (bottom) concentrations. Values are means ± SE. *, P <0.05; **, P <0.01 versus vehicle control. Numbers in parenthesis indicate number of animals.

Fig. 2. Effect of histamine given via the iv or icv route on adrenocortical secretion rate and cardiovascular function. A: Adrenal cortisol secretion rate before and after an iv administration of 100 µg/kg histamine (□, n=5), or an icv administration of histamine at a dose of 5 µg/kg (◆, n=5), 10 µg/kg (●, n=6), or 25 µg/kg (■, n=7), or an icv administration of 37.5 µg/kg C48/80 (□, n=7). Integrated cortisol secretion in response to the above treatments over the 150 min after their administration (inset). B: Effect of histamine administered via the iv (□, 100 µg/kg, n=4) or icv (■, 25 µg/kg, n=4) route on MAP (top) and HR (bottom). Values are means ± SE. *, P <0.05; **, P <0.01 versus vehicle control (A) or versus animals given 25 µg/kg histamine via the icv route (B). Numbers in parenthesis indicate number of animals.
Fig. 3. Effect of H₁- and H₂-blockers on the C48/80 (37.5 µg/kg)-evoked increase in cortisol secretion (A&B) and plasma levels of cortisol and ACTH (C). A: Adrenal cortisol secretion rate before and after icv administration of vehicle (ACSF) with 5 µg/kg pyrilamine (○, n=5), C48/80 without pyrilamine (□, n=6), or C48/80 with 5 µg/kg pyrilamine (■, n=11), or after icv administration of C48/80 with pyrilamine via the iv route at a dose of 5 µg/kg (◇, n=6), or 500 µg/kg (♦, n=6). Integrated cortisol secretion over the 150 min after the administration (inset). B: Adrenal cortisol secretion rate before and after icv administration of vehicle with 500 µg/kg metiamide (◇, n=5), C48/80 without metiamide (□, n=8), or C48/80 with 500 µg/kg metiamide (■, n=13), or after icv administration of C48/80 with 500 µg/kg metiamide via the iv route (△, n=6). Integrated cortisol secretion over the 150 min after the administration (inset). C: Effects of pretreatment with an H₁- or H₂-blocker on the peak levels of plasma cortisol (at 30 min after the C48/80 challenge) and ACTH (at 10 min). In Fig. 3C, the number of experimental animals was the same as in Fig. 3A (pyrilamine) and Fig. 3B (metiamide). Values are means ± SE. *, P <0.05 versus animals given C48/80 alone.

Fig. 4. Effect of anti-CRF antibody on the C48/80-evoked increases in cortisol secretion (A) and plasma cortisol and ACTH (B). A: Adrenal cortisol secretion rate before and after icv administration of C48/80 with (■, n=9) or without (□, n=5) anti-CRF antibody, or of vehicle (ACSF) without anti-CRF antibody (△, n=5). Integrated cortisol secretion over the 150 min after the administration (inset). B: Effect of anti-CRF antibody on the increases in plasma cortisol (which peaked at 30 min after C48/80-challenge) and plasma ACTH
(peaked at 10 min). Number of animals was the same as in Fig. 4A. Values are means ± SE. *, $P < 0.05$; **, $P < 0.01$ versus C48/80 alone.

Fig. 5. Effect of an anti-allergic agent on the C48/80-evoked pituitary-adrenal response. A: Adrenal cortisol secretion rate before and after icv administration of C48/80. Ketotifen was given simultaneously with 37.5 µg/kg C48/80 via the icv route at a dose of 100 µg/kg (◆, n=4), 500 µg/kg (●, n=4), or 2000 µg/kg (▲, n=4), or alternatively vehicle (ACSF) was delivered with 37.5 µg/kg C48/80 (□, n=4). Integrated cortisol secretion over the 150 min after the administration (inset). B: Adrenal cortisol secretion rate before and after icv administration of C48/80. Ketotifen was delivered in a semichronic (S) way via the icv route (●, n=8) or in a chronic (C) way via the oral route (◆, n=14). Alternatively, 37.5 µg/kg C48/80 without ketotifen (□, n=7), or vehicle plus vehicle (△, n=5) was given via the icv route. Integrated cortisol secretion over the 150 min after the administration (inset). C: Effect of ketotifen-pretreatment on the peak levels of plasma cortisol (at 30 min after the C48/80 challenge) and ACTH (at 10 min). Number of experimental animals was the same as in Fig. 5A (ketotifen co-administration) and Fig. 5B (ketotifen pretreatment). D: Comparison of histamine-induced cortisol secretion among groups given or not given ketotifen-pretreatment in the above ways. Adrenal cortisol secretion rate was at 10 min after the icv administration of either vehicle or 5 µg/kg histamine. Infusion of vehicle or histamine was started at 180 min after the C48/80 administration. Number of animals was the same as in Fig. 2A (vehicle and histamine alone) and Fig. 5B (ketotifen-pretreatment). Values are means ± SE. *, $P <0.05$ versus vehicle control; NS, not significant versus
animals given C48/80 alone.

Fig. 6. Effect of SPX on the adrenocortical and adrenomedullary secretions induced by C48/80. Cortisol secretion rate (left) and epinephrine secretion rate (right) at 10 min after an icv administration of vehicle (n=6) or 37.5 µg/kg C48/80 (n=6) in intact animals and SPX animals (n=6). Values are means ± SE. ** P<0.01 versus C48/80-administered animals without SPX; NS, not significant between intact and SPX animals.

Fig. 7. Rostrocaudal distribution of mast cells in ventral hypothalamic region. Top panel: drawing of midline sagittal section of the area around the median eminence and hypophysis. V₀ indicates the position of EAML in the vertical direction. Shaded area corresponds to PT. Middle panel: drawings of partial coronal sections of the brain at 18, 19, 20, 21 mm rostral to EAML (R₀, not shown). Bottom panel: rostrocaudal distribution of mast cells (cell number in 100 µm-thick serial coronal sections from R16.8 to R22.8 in 13 dogs). Values are means ± SE. AH, adenohypophysis; CM, corpus mammillare; ME, median eminence; OC, optic chiasm, PT, pars tuberalis; Vᵢᵢᵢ, third cerebral ventricle.

Fig. 8. Horizontal distribution of mast cells in ventral hypothalamic region at about R19.3. Top panel: photomicrograph of a partial coronal section of a dog brain at R19.3 showing the 5 subdivisions either side of the midline (subregions I–X). Boxed areas (A and B) are shown at higher magnification in Fig.9. Vᵢᵢᵢ, third cerebral ventricle; PT, pars tuberalis (subregions I–II and IX–X); ME, median eminence (subregions III ~ VIII); AH,
adenohypophysis. Bar = 1000 µm. Bottom panel: horizontal distribution of mast cells (numbers of cells in subdivisions of PT and ME region at about R19.3 in 9 dogs). Values are means ± SE.

Fig. 9. Vertical distribution of mast cells in ME. Left panels: high-magnification photomicrographs of boxed areas shown in Fig. 8 [horizontal subregions III ~ IV (A) and VI~VII (B)]. Bars = 250 µm; White arrows, mast cells stained metachromatically with toluidine blue; Black arrowheads, capillaries in the so-called plexus of the hypophyseal portal system. Right panel: vertical distribution of mast cells in the ME at about R19.3 in 9 dogs (the cell numbers from horizontal subregions III to VIII in Fig. 8 were summed). Values are means ± SE.

Fig. 10. Effect of C48/80 on degranulation of mast cells under histological examination. A: High-magnification photomicrograph of a portion of the cranial part (CM) of ME (level ii in Fig. 9) in a C48/80-treated animal. Black arrow, intact mast cell; White arrow, degranulated mast cell; Black arrowheads, capillaries of the hypophyseal portal system. B: Populations of activated and inactivated mast cells in and around the median eminence in dogs treated with (n=4) or without (n=4) C48/80. CM, cranial part of ME (level i ~ ii in Fig. 9); MM, medial part of ME (level iii ~ iv in Fig. 9); VM, ventral part of ME (level v in Fig. 9); PT, pars tuberalis. Values are means ± SE. *, P <0.05 versus corresponding animals without C48/80.
Fig. 3

A

Cortisol secretion rate vs. Time (min)

- H1(icv) + Vehicle
- H1(iv) + C48/80
- H1(icv) + C48/80
- H1(iv) + C48/80
- Vehicle + C48/80

B

Cortisol secretion rate vs. Time (min)

- H2(icv) + C48/80
- H2(iv) + C48/80
- Vehicle + C48/80
- H2(icv) + Vehicle

C

Peak plasma cortisol and ACTH levels

- Vehicle
- C48/80 alone
- C48/80
- +H2 antago(iv)
- +H2 antago(icv)
- +H2 antago(cv)
**Fig. 4**

**A**

![Graph showing cortisol secretion rate over time](image)

**B**

![Bar graph comparing peak plasma cortisol and ACTH levels](image)
Fig. 5

A

Time (min)

B

Time (min)

C

Vehicle
C48/80 alone
C48/80
+Keto(icv/co-ad)
+Keto(icv-twice)
+Keto(po, 1 w)

D

Vehicle + Vehicle
Vehicle + Hist.(5µg/kg)
Keto.(S)+ Hist.(5µg/kg)
Keto.(C)+ Hist.(5µg/kg)

NS
Fig. 6

10 min after C48/80 infusion

- Intact + Vehicle
- Intact + C48/80
- SPX + C48/80

Cortisol secretion rate (µg/kg/min)

Epinephrine secretion rate (ng/kg/min)

NS
Fig. 7

Rostrocaudal distribution

Fig. 8

Horizontal distribution

Cell Number/100μm section

Subregion at around R19.3
Fig. 9

Vertical distribution in ME

Fig. 10

Cell number/100 µm thickness

Intact cells
Degra.cells

PT VM MM CM Without C48/80
PT VM MM CM With C48/80