

Corticotropin-Releasing Hormone Induces Skin Mast Cell Degranulation and Increased Vascular Permeability, A Possible Explanation for Its Proinflammatory Effects*

THEOHARIS C. THEOHARIDES, LEENA K. SINGH, WILLIAM BOUCHER, XINZHU PANG, RICHARD LETOURNEAU, ELIZABETH WEBSTER, AND GEORGE CHROUSOS

Department of Pharmacology and Experimental Therapeutics (L.K.S., W.B., X.P., R.L., T.C.T.), Tufts University School of Medicine, Boston, Massachusetts 02111; Section of Pediatric Endocrinology (E.W., G.C.), National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT

Mast cells are involved in atopic disorders, often exacerbated by stress, and are located perivascularly close to sympathetic and sensory nerve endings. Mast cells are activated by electrical nerve stimulation and millimolar concentrations of neuropeptides, such as substance P (SP). Moreover, acute psychological stress induces CRH-dependent mast cell degranulation. Intradermal administration of rat/human CRH (0.1–10 μ M) in the rat induced mast cell degranulation and increased capillary permeability in a dose-dependent fashion. The effect of CRH on Evans blue extravasation was stronger than equimolar concentrations of the mast cell secretagogue compound 48/80 or SP. The free acid analog of CRH, which does not interact with its receptors (CRHR), had no biological activity. Moreover, systemic administration of antalarmin, a nonpeptide CRHR₁ antagonist, prevented vascular permeability only by CRH and not by compound 48/80 or SP. CRHR₁ was also identified in cultured leukemic human mast cells using RT-PCR. The stimulatory effect of CRH, like that of compound 48/80 on skin vasodilation, could not be elicited in the mast cell deficient W/W^v mice but was present in their +/+ controls, as well as in C57BL/6J mice; histamine could still induce vasodilation in the W/W^v mice. Treatment of rats neonatally with capsaicin had no effect

on either Evans blue extravasation or mast cell degranulation, indicating that the effect of exogenous CRH in the skin was not secondary to or dependent on the release of neuropeptides from sensory nerve endings. The effect of CRH on Evans blue extravasation and mast cell degranulation was inhibited by the mast cell stabilizer disodium cromoglycate (cromolyn), but not by the antisecretory molecule somatostatin. To investigate which vasodilatory molecules might be involved in the increase in vascular permeability, the CRH injection site was pretreated with the H₁-receptor antagonist diphenhydramine, which largely inhibited the CRH effect, suggesting that histamine was involved in the CRH-induced vasodilation. The possibility that nitric oxide might also be involved was tested using pretreatment with a nitric oxide synthase inhibitor that, however, increased the effect of CRH. These findings indicate that CRH activates skin mast cells at least via a CRHR₁-dependent mechanism leading to vasodilation and increased vascular permeability. The present results have implications for the pathophysiology and possible therapy of skin disorders, such as atopic dermatitis, eczema, psoriasis, and urticaria, which are exacerbated or precipitated by stress. (*Endocrinology* **139**: 403–413, 1998)

CRH PRODUCED in the hypothalamus and the brain stem is a principal coordinator of the stress response, through activation of the limbic system, the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (1). CRH is also secreted peripherally and has proinflammatory and other actions (1). For instance, systemic administration of rabbit anti-CRH serum suppressed both the amount of exudate and inflammatory cell accumulation in carrageenin-induced sc inflammation and ameliorated the severity of experimental autoimmune uveitis in rodents (2, 3). Immunocytochemistry verified the presence of CRH also in human tissues undergoing inflammatory processes, including joints of patients with rheumatoid arthritis and osteoarthritis, thyroids of patients with Hashimoto thyroiditis, and gut lesions of patients with ulcerative colitis (4–6).

CRH may be synthesized locally at inflammatory sites, as evidenced by the presence of CRH messenger RNA (mRNA) in chronically inflamed synovia in rats (7). Both immunoreactive CRH (iCRH) and CRH mRNA have also been demonstrated in various components of the immune system, whereas mitogenic stimulation of human T lymphocytes results in synthesis of CRH (for review, see Ref. 8). However, there is a discrepancy between the abundance of iCRH and the paucity of its mRNA at inflammatory sites in the early, acute phase of inflammation. The demonstration of CRH-like immunoreactivity in the dorsal horn of the spinal cord and dorsal root ganglia (9, 10), as well as in sympathetic nerve cell bodies in sympathetic ganglia (9, 11), support the hypothesis that the majority of iCRH in early inflammation is of neuronal rather than immune cell origin.

CRH administration to humans or animals *iv* causes significant dose-dependent peripheral vasodilation, manifested as flushing and hypotension (12). These effects may derive from activation of mast cells, which are located perivascularly, close to nerves (for review see Ref. 13). In fact, acute psychological stress in rats resulted in dura mast cell activation and rat mast cell protease I secretion, which were

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Address all correspondence and requests for reprints to: T. C. Theoharides, Ph.D., M.D., Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111. E-mail: ttheoharides@infonet.tufts.edu.

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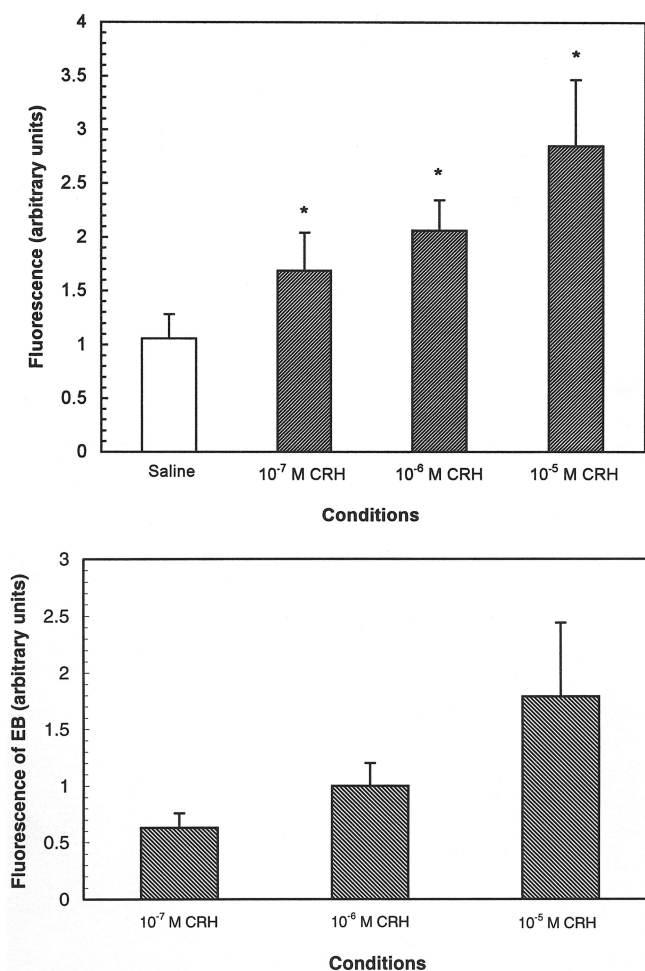
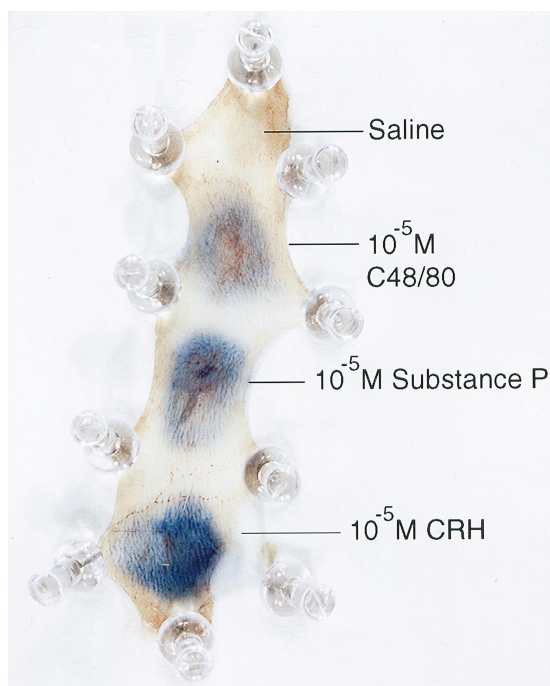


FIG. 1. Photographs of rat skin and graphic representations showing the vasodilatory effect of CRH ($n = 8$). A, Comparison of equimolar concentrations (10^{-5} M) of CRH to that of the known mast cell secre-

CRH-dependent (14). Mast cells can be activated by nerve stimulation (15) or sensory neuropeptides such as SP (16), and they secrete potent vasoactive and proinflammatory mediators that include histamine, cytokines, prostanoids, and proteases (17).

The ability of CRH to activate mast cells may explain its proinflammatory actions and the pathophysiology of certain skin conditions, which are precipitated or exacerbated by stress, such as atopic dermatitis, eczema, psoriasis, and urticaria. Here we studied the ability of CRH to activate skin mast cells and increase vascular permeability in a receptor-specific, dose-dependent fashion.

Materials and Methods

Materials

The drugs used were obtained from the following sources: the mast cell secretagogue, compound 48/80 (C48/80), SP, cromolyn, diphenhydramine, and the nitric oxide (NO) inhibitor N^G -nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. (St. Louis, MO). CRH was obtained from Peninsula (Belmont, CA); the inactive free acid form of rat/human CRH (r/h CRH-OH) and the CRH-receptor peptide antagonist [D-phe¹², Nie^{21,38}, Ala³²]rCRH(12-41) were kindly provided by Neurocrine Biosciences (San Diego, CA). The nonpeptide CRH receptor antagonist antalarmin was prepared at the NIH (Bethesda, MD). Somatostatin (somatotropin release inhibitory factor, SRIF) was purchased from Bachem (Torrance, CA). The anti-SP serum was obtained from Zymed (South San Francisco, CA). All solutions, except antalarmin (see below), were prepared fresh in normal saline the morning of the experiment.

Evans blue extravasation

Male Sprague Dawley rats, each weighing approximately 350 g (Charles River, NY), were anesthetized with a single ip injection of 0.2 ml ketamine and 0.05 ml xylazine (100 mg/ml each) and injected iv via the tail vein with 0.6 ml of 1% Evan's blue, 10 min before treatment. The same procedure was repeated with 0.01 ml of 10 mg/kg xylazine and 80 mg/kg ketamine in 8-week-old male C57B/6J, as well as W/W^v mast cell deficient mice [WBBGF₁ (WB - $W/+ \times C57BL/6-W^v$) lineage] and their $+/+$ normal counterparts (Jackson Laboratories, Bar Harbor, ME), which were kept in virus-free sections. The W geno-type was inferred from the white coat (depletion of hair pigment) of the animals. All animals were housed in a modern animal facility and were allowed *ad libitum* access to food and water. Drugs were tested by intradermal injection in 0.05 ml normal saline using a tuberculin syringe. The CRH was drawn in one syringe while, when appropriate, the pretreatment solutions were prepared in different syringes to avoid any mixing between the two solutions. The pretreatment solution was injected first and was allowed to remain in the skin for 5 min before CRH. Antalarmin was dissolved as 20 mg/0.05 ml in absolute ethanol and the appropriate amount (to correspond to the desired mg/kg BW) was then dissolved in normal saline; unfortunately, it quickly came out of solution and clearly resulted in variable bioavailability. The final amount injected was 0.5 ml iv in the tail vein 6 h before CRH injection. The animal was killed 15 min later by asphyxiation over CO_2 vapor and decapitated; the skin was removed, turned over, and photographed. Animals were handled one at a time in an isolated procedure room inside the animal facility to minimize any effect of the stress of handling, change of environment, or presence of other injected animals.

tagogue C48/80 and SP; each solution used is shown on the right and is connected to the corresponding injection site by a solid line. B, Dose-response curve showing the effect of CRH on Evans blue extravasation extracted by formamide ($n = 4$). C, The same results shown in (B) but recalculated after subtracting the control value due to saline from all experimental values obtained in the same rat. *, $P < 0.05$ using ANOVA.

Evans blue extraction

Evans blue extravasated in the skin was extracted by incubating the skin samples in 99% *N,N*-dimethyl-formamide (Sigma) for 24 h at 55 C and was quantitated fluorometrically at an excitation wavelength of 620 nm and an emission wavelength of 680 nm (18).

Microscopy

In rats or mice not injected with Evans blue, the skin from the site where drugs were introduced was rapidly removed at the end of the injection period and was fixed in 4% paraformaldehyde for light microscopy (19). The tissue was then frozen and thin sections (7 μ m) were cut using a cryostat (Jung CM 3000, Leica, Deerfield, IL). The sections were stained with acidified (pH < 2.5) toluidine blue (Sigma), and all mast cells were counted by two different researchers, blinded to the experimental conditions, at 200 \times magnification using a Diaphot inverted Nikon microscope (Don Santo Corp., Natick, MA). For electron microscopy, samples were fixed in modified Kanovsky's fixative containing 0.2% paraformaldehyde, 3% glutaraldehyde, and 0.5% tannic acid in 0.1 mM Na-cacodylate buffer prepared as before (20) and photographed using a Philips-300 transmission electron microscope.

TABLE 1. Skin mast cell activation by CRH

Conditions	Dye extravasation (color intensity)	Mast cell activation ^a		
		Total ^b	Activated	% total
Normal saline	– ^d	2840	850	20 \pm 3
C48/80 (1.0 μ g/ml)	+++	841	375	45 \pm 15 ^c
CRH-OH (10 ^{–4} M)	–	710	110	14 \pm 9
CRH (10 ^{–4} M)	++++	902	426	49 \pm 15 ^{c§}
CRH (10 ^{–5} M)	+++	1886	696	38 \pm 3 ^c
CRH (10 ^{–6} M)	++	810	331	42 \pm 7 ^c
CRH (10 ^{–7} M)	++	1008	329	37 \pm 5 ^c
CRH (10 ^{–8} M)	++	790	254	32 \pm 2 ^c
CRH (10 ^{–9} M)	+	725	202	28 \pm 6
CRH (10 ^{–10} M)	–	880	239	26 \pm 5

^a Three sections were examined from each of four blocks from each rat; n = 3 except for normal saline (n = 8) and 10^{–5} M CRH (n = 3); the higher number of mast cells listed for saline and CRH reflect the greater number of animals studied.

^b Means all mast cells counted in all of the rats studied.

^c P < 0.05 compared with saline.

^d Signifies no color, whereas ++++ signifies maximal color.

Capsaicin treatment

For sensory nerve neuropeptide depletion, one entire litter of rats was injected with capsaicin within 2 days of birth, and the male rats were used 5 weeks later as described before (15). The effectiveness of this procedure was confirmed by immunocytochemistry (21), which showed that there were no SP-positive cells or nerve processes in the skin of capsaicin-treated rats.

Immunohistochemistry

Frozen sections were cut at 7 μ m by cryostat (Jung CM 3000, Leica) and thaw-mounted. All specimens were treated with 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase. After briefly rinsing in PBS, the sections were incubated in 5% normal goat serum in PBS for 30 min, then exposed to rabbit anti-SP polyclonal antibody in PBS containing 5% normal goat serum at 1:4000 dilution for 48 h at 4 C. The sections were incubated for 2 h at 4 C in rhodamine-conjugated goat antirabbit immunoglobulin (Cappel Laboratories, Cochranville, PA), diluted 1:500 with 1% normal goat serum and 0.02% Triton X-100 in 0.1 M PBS. Final observation and photography were performed with Nikon Diaphot inverted microscope (Don Santo).

RT-PCR amplification of CRH-receptor mRNA in HMC-1 cells

Human leukemic mast cells (HMC-1) were obtained from Dr. But-terfield (Mayo Clinic, MN) and were grown as described previously (22). To determine whether mast cells express CRH receptors, mRNA was extracted from 1 \times 10⁶ HMC-1 cells or RBL cells using Tri-Reagent (Molecular Resources; Cincinnati, OH). For RT-PCR analysis, complementary DNA (cDNA) was synthesized using Superscript Preamplification System (GIBCO-BRL, Gaithersburg, MD). Two successive amplifications were conducted using overlapping, nested primers. Primer selection was based on the published sequence of CRHR₁ (23). In the first amplification, PCR was carried out for 30 cycles using the 5' outer primer CCGAATTCGGGCATTCAGGACGGTAGCCGAGCGAGC and the 3' outer primer CCGGATCCCCATGACCTGCCAGCTCAGACTGCTGTG. The second amplification was performed for 20 cycles using 1 μ l of the first reaction product and the 5' inner primer CCGAATTC AATGGGAGGGCACCCGACGCTCCGTCTCGTC and the 3' inner primer CCGGATCCCCATGACCTGCCAGCTCAGAC. An *Eco*R1, CCGAA-TTC and a *Bam*H1, CCGGATCC, restriction enzyme site was included on the 5' end of the 5' and 3' primers, respectively. A product of identical size was also simultaneously amplified by RT-PCR, using pituitary polyA RNA (Clontech, Palo Alto, CA) under identical conditions.

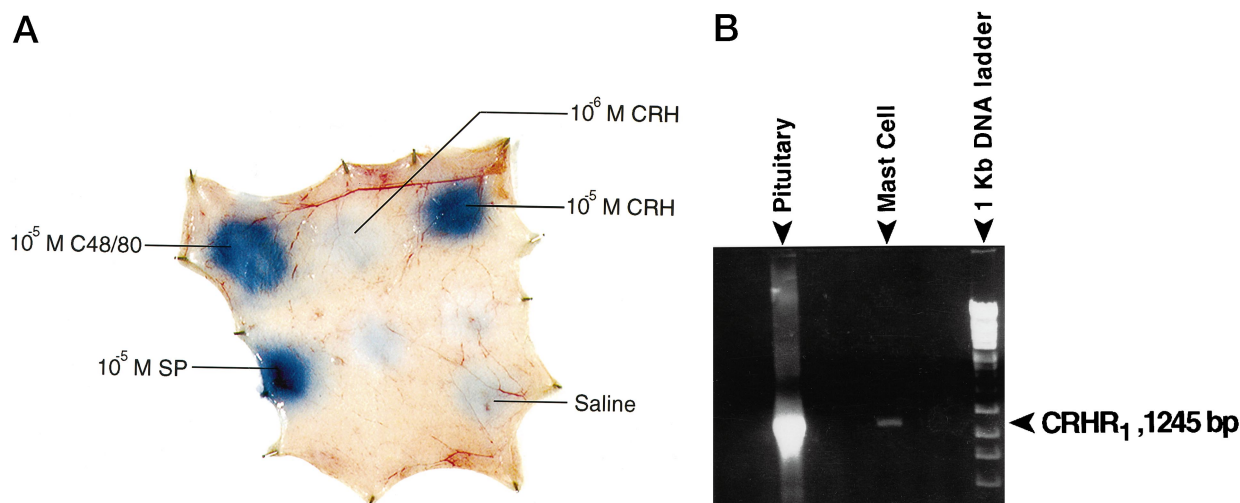


FIG. 2. A, Photographs of rat skin showing the inhibitory effect of antalarmin (n = 4). Rats were pretreated iv with 10 mg/kg BW of antalarmin 6 h before intradermal injection of CRH (10^{–5} M), SP (10^{–5} M), C48/80 (1 μ g/ml), or saline as shown (far left); note the absence of an inhibitory effect of antalarmin on the effect of SP or C48/80. B, Agarose gel (1.2%) run as described before (5), showing the presence of CRHR₁ mRNA by RT-PCR in HMC-1 cells.

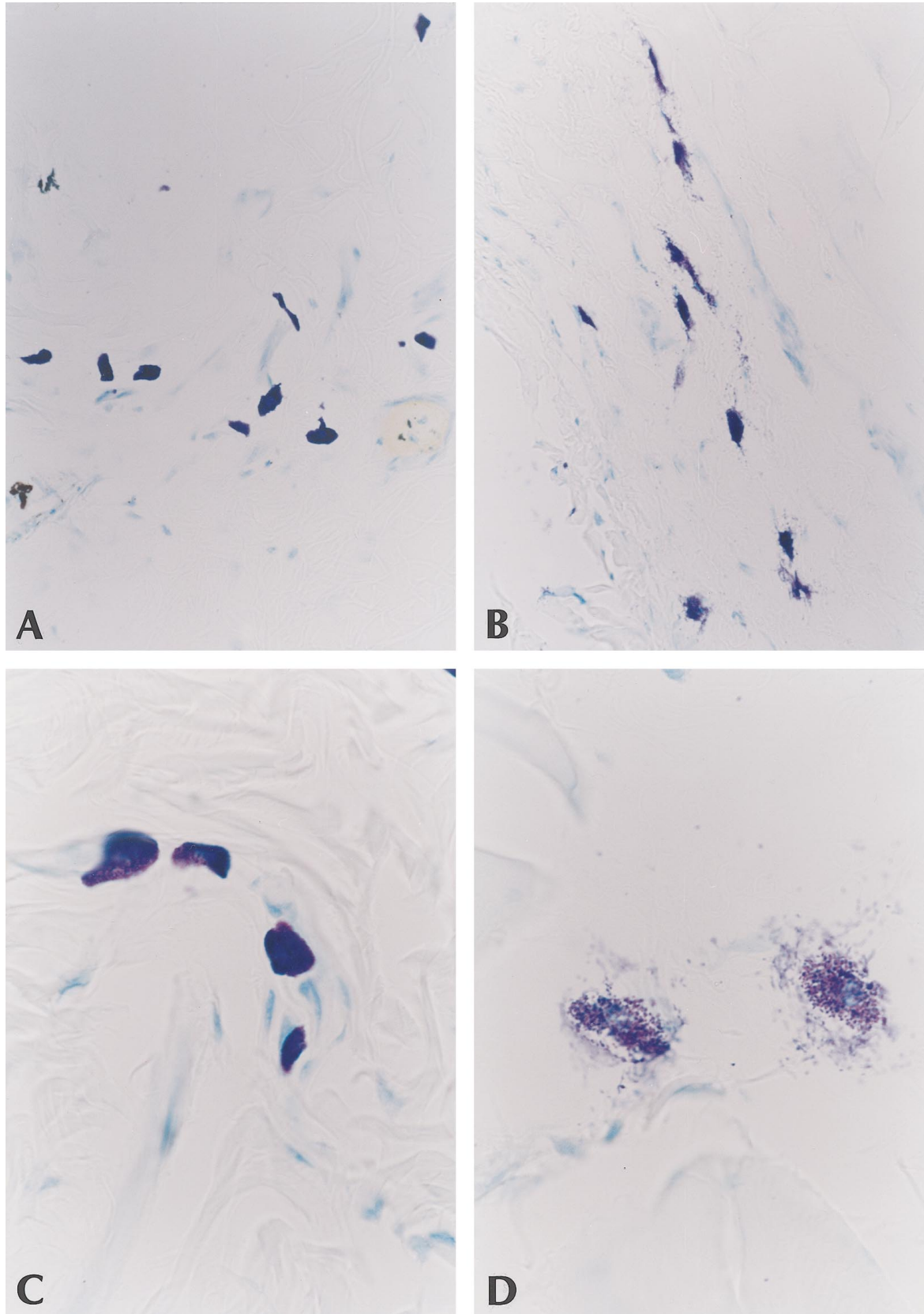


FIG. 3. Photomicrographs of rat skin mast cells stained with toluidine blue. A, Control site with intact mast cells; B, experimental site injected with CRH (10^{-6} M). Magnification, $\times 400$. Higher magnifications of mast cells from (C) a control site and (D) a CRH site; note activated mast cells in (D) showing secretory granule content outside the cell periphery. Magnification, $\times 1000$. (Actual number of rats examined is listed on Table 1).

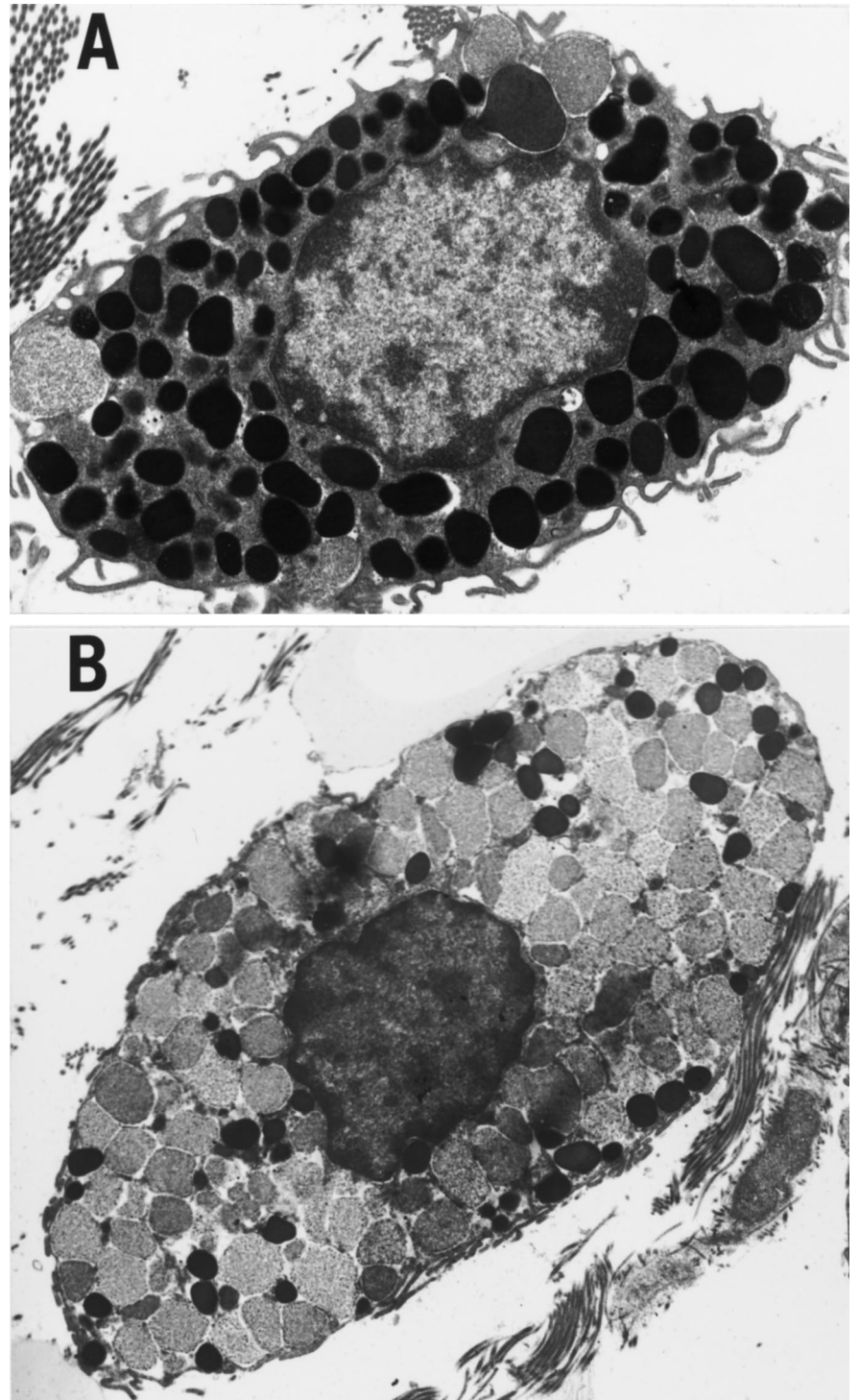


FIG. 4. Electron photomicrographs of skin rat mast cells from (A) control site injected with normal saline and (B) site injected with CRH (10^{-6} M) showing ultrastructural changes of the granule content with loss of electron density indicating secretory activity. Magnification, $\times 6,600$.

Presentation of results

Results are presented as the mean \pm SD values of Evans blue extravasation under different experimental conditions. The number of animals tested *in vivo* or *in vitro* is denoted by (n). Evans blue extravasation is evaluated by ANOVA and Student-Newman-Keul's tests, whereas the mast cell degranulation results are compared using nonparametric analysis with Mann Whitney *U* test. Significance is denoted by $P < 0.05$.

Results

CRH effects on vascular permeability and mast cell degranulation

CRH induced marked skin vasodilation and vascular permeability, as evidenced by Evans blue extravasation, an effect that was more pronounced than that obtained by an equimolar concentration (10^{-5} M) of the mast cell secreta-

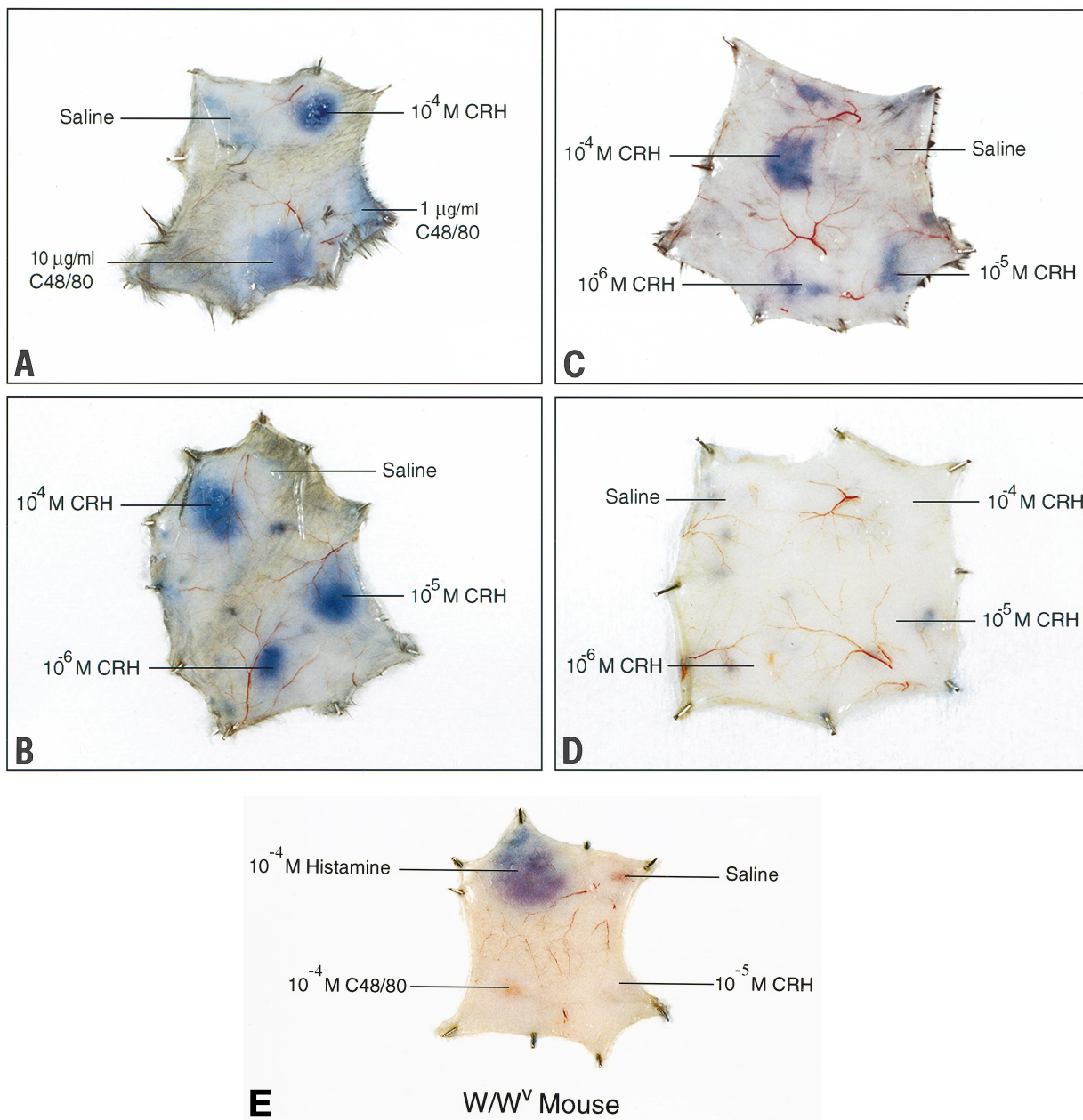


FIG. 5. Photographs of mouse skin showing the effect of CRH ($n = 5$). A and B, C57 Black; C, $+/+$ controls for W/W^v ; D) W/W^v mast cell-deficient mouse; E, W/W^v mast cell deficient mouse showing that the effect of C48/80 is also absent, whereas that of histamine is present, indicating that the mouse can still mount a mast cell-independent, direct vasodilatory response.

gogue C48/80 or SP (Fig. 1A). The effect of CRH on both vasodilation and mast cell degranulation was dose dependent (Table 1). Evans blue extraction confirmed these results and showed that the increased vascular permeability induced by CRH was statistically significant ($P < 0.05$) from 10^{-5} to 10^{-7} M (Fig. 1B). Little intrarater variability was observed during these experiments ($3.08 \pm 0.12\%$, $n = 3$ for CRH and 0.55 ± 0.22 , $n = 4$ for normal saline), but there were considerable interrater differences. To examine the possible confounding effect of such variability, the results were recalculated after subtracting the control value obtained from the saline injection in each rat. The results showed that all

concentrations (10^{-5} M to 10^{-7} M) were again statistically significant (Fig. 1C).

The inactive free acid CRH-OH produced no vasodilation (Table 1), suggesting that these peripheral actions of CRH are mediated by specific receptors. Pretreatment iv with the non-peptide CRH receptor antagonist antalarmin (10 mg/kg BW), which is specific for the CRH-receptor type 1 (CRHR₁), inhibited Evans blue extravasation (Fig. 2A), in response to 10^{-5} M CRH by $33.0 \pm 10.6\%$ ($n = 3$) and to 10^{-6} M CRH by 51.0 ± 21.8 ($n = 3$), both of which were significant ($P < 0.05$), but it did not inhibit the effect of SP or C48/80 (Fig. 2A). The inhibitory effect of antalarmin required 6 h to become evident

(results not shown), most likely because it was extremely insoluble in aqueous medium. Surprisingly, the peptide CRH-receptor antagonist [D-phe¹², Nie^{21,38}, Ala³²]rCRH(12–41) did not block the effect of CRH at any concentration; instead, it mimicked the effect of CRH at high concentrations (10^{-4} M), although it was less potent both as measured by plasma extravasation and mast cell degranulation (results not shown).

RT-PCR using RNA from HMC-1 and specific primers for the human CRH-R₁ receptor yielded a specific band of approximately 1245 bp corresponding to the full sequence of CRHR₁ (Fig. 2B). A product of identical size was also simultaneously amplified by RT-PCR, using pituitary polyA RNA under identical conditions as positive control. No such product was identified in human cultured synoviocytes used under the same conditions (results not shown).

The CRH effect on mast cell degranulation was investigated morphologically and was judged by granule content extrusion and/or more than 50% loss of cellular staining (Fig. 3). Degranulation was observed in $49 \pm 15\%$ of the mast cells at skin sites treated with 10^{-4} M CRH ($n = 5$ rats, 902 mast cells counted), which was statistically higher ($P < 0.05$) when compared with $20 \pm 3\%$ ($n = 8$ rats, 2840 mast cells counted) of the mast cells from control sites (Table 1). The inactive acid form of CRH had no effect (Table 1) on mast cell degranulation ($14 \pm 9\%$, $n = 3$, 710 mast cells counted). Ultrastructural observations of mast cells from CRH-injected sites had obvious signs of degranulation evidenced by loss of the electron dense content of their secretory granules (Fig. 4).

Mast cell dependence of CRH effects on the skin

To ascertain whether the effect of CRH on skin vasodilation was mast cell-dependent, the W/W^v mast cell deficient mice and their +/+ controls, as well as C57B/6J mice, were used. C48/80 and CRH caused Evans blue extravasation between 10^{-4} to 10^{-6} M (Fig. 5A), which was weaker than that seen in the rat C57B/6J mice (Fig. 5B). This effect was also present in the +/+ controls of the W/W^v mast cell-deficient mice (Fig. 5C) but was entirely absent in W/W^v mice (Fig. 5D). C48/80 (10^{-4} M) used as negative control was also unable to induce dye extravasation in W/W^v mice (Fig. 5E), whereas histamine (10^{-5} M) used as a positive control did induce a strong vasodilatory effect (Fig. 5E). The increased vascular permeability induced by CRH at 10^{-5} M in the +/+ controls was also accompanied by mast cell degranulation of $37 \pm 7.0\%$ ($n = 3$, 697 mast cells counted), as compared with $20 \pm 2\%$ ($n = 3$, 1014 mast cells counted) in the sites injected with normal saline alone ($P < 0.05$).

In animals that had been treated neonatally with capsaicin to destroy neuropeptide containing sensory nerve termini, immunohistochemistry for SP showed no fibers positive for this peptide (Fig. 6, A and B). In these animals, the vasodilatory effect of CRH or of C48/80 was not affected (Fig. 6C), indicating that the increased vascular permeability was not dependent on the presence of neuropeptides such as SP.

We also investigated whether the effect of CRH could be inhibited by agents known to either stabilize the mast cell surface or inhibit neuronal secretion. Pretreatment of the injection site with the mast cell stabilizer cromolyn (10^{-4} M)

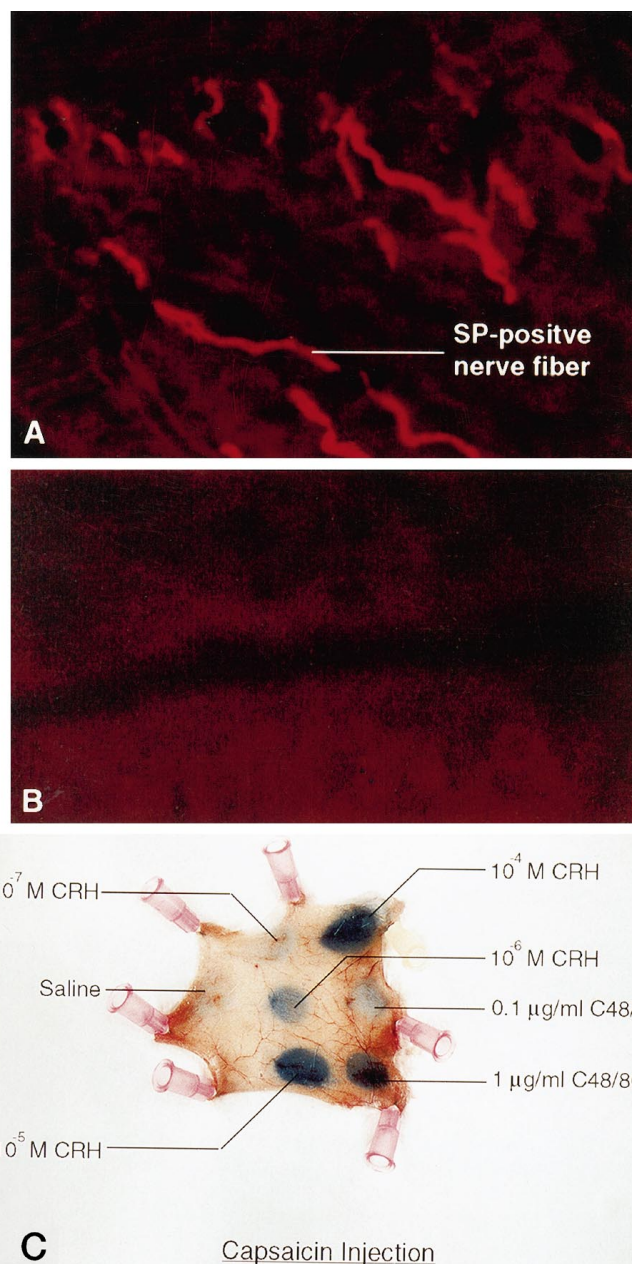


FIG. 6. Photomicrographs of skin from (A) control rats showing the presence of SP-positive fibers visualized by indirect immunofluorescence using rhodamine-conjugated anti-SP serum; B, after treatment neonatally with capsaicin showing the absence of SP-positive nerve fibers; C, photograph of rat skin showing the lack of any inhibition after treatment with capsaicin neonatally on the effect of CRH and C48/80, on dye extravasation ($n = 5$).

for 5 min before the injection of CRH (10^{-5} M) blocked the effect of CRH (Fig. 7A). Evans blue extraction showed that cromolyn inhibited the effect of CRH (10^{-5} M) by $87 \pm 12.7\%$ ($n = 4$) at 10^{-5} M and $74.2 \pm 22\%$ ($n = 4$) at 10^{-6} M, both of which were significant ($P < 0.05$). Pretreatment with somatostatin before CRH (10^{-5} M) resulted in more dye extravasation than 10^{-5} M CRH alone (Fig. 7B); in fact, somatostatin alone caused significant fluid extravasation, which at millimolar concentrations was as strong as that seen with CRH (Fig. 7B).

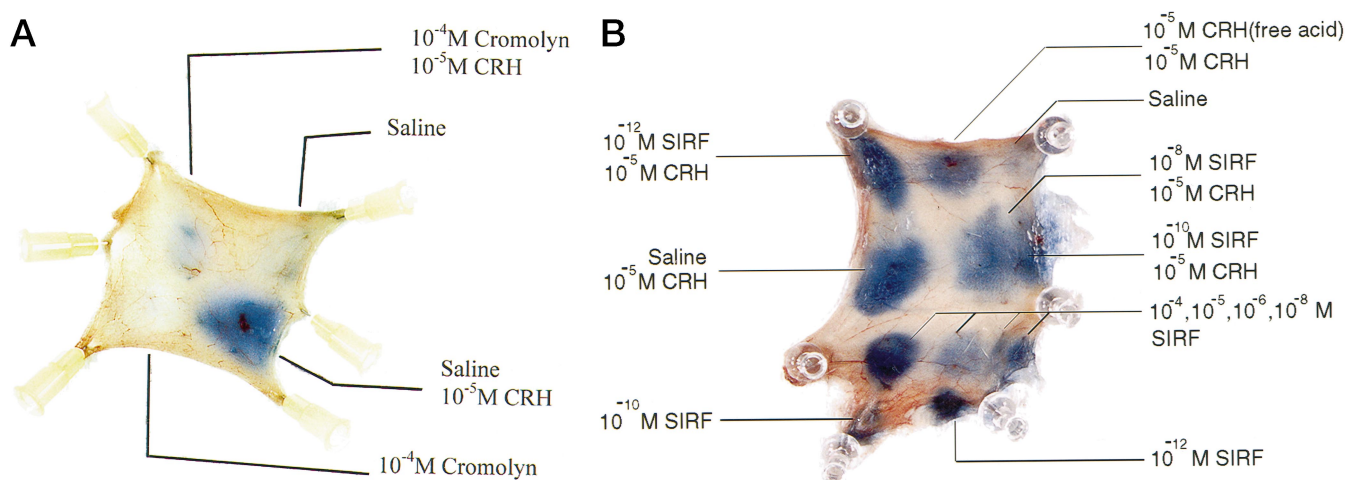


FIG. 7. Photographs of rat skin showing the effect of pretreatment with (A) cromolyn (10^{-4} M) or (B) SRIF (as indicated) before injection of CRH, on dye extravasation ($n = 5$ each). The concentrations and/or combinations of drugs used are indicated by a solid line emanating from the site of injection.

To investigate which mast cell-derived vasodilatory molecule mediated the vasodilatory effect of CRH, the injection sites were pretreated with the histamine-1 receptor antagonist diphenhydramine for 5 min (Fig. 8A). Fluid extravasation in response to CRH (10^{-6} M) was reduced by $86.9 \pm 10.6\%$ ($n = 5$) with 10^{-4} M and by $77.7 \pm 9.9\%$ ($n = 5$) with 10^{-5} M diphenhydramine. However, this drug only partially inhibited extravasation at higher ($>10^{-6}$ M) CRH concentrations (Fig. 8A). These results suggest that, whereas histamine is the main molecule through which CRH induces fluid extravasation, some of this effect may be mediated by other vasodilatory molecules. We, therefore, investigated whether NO might also be involved because it has been shown to be secreted from mast cells. Pretreatment of rats ip with the 10^{-9} M NO-synthase inhibitor, L-NAME, surprisingly augmented the effect of 10^{-5} M CRH (Fig. 8B). In fact, L-NAME by itself resulted in vasodilation as evidenced by increased vascular permeability over a concentration range from 10^{-4} M to 10^{-8} M.

Discussion

The present findings show that CRH triggers rat skin mast cell degranulation, which results in vasodilation and increased vascular permeability documented by Evans blue extravasation. This effect shows little intrarat but considerable interrat variability. The smallest CRH concentration ($0.1 \mu\text{M}$) consistently capable of eliciting a statistically significant response was about two orders of magnitude less than those reported for other neuropeptides. For instance, 0.03 mM SP, vasoactive intestinal peptide (VIP), or somatostatin were required for about 15% release of histamine from dispersed human skin mast cells (24). It should also be noted that these peptides carry a net positive charge thought to be important for triggering mast cell secretion, whereas CRH has one net negative charge.

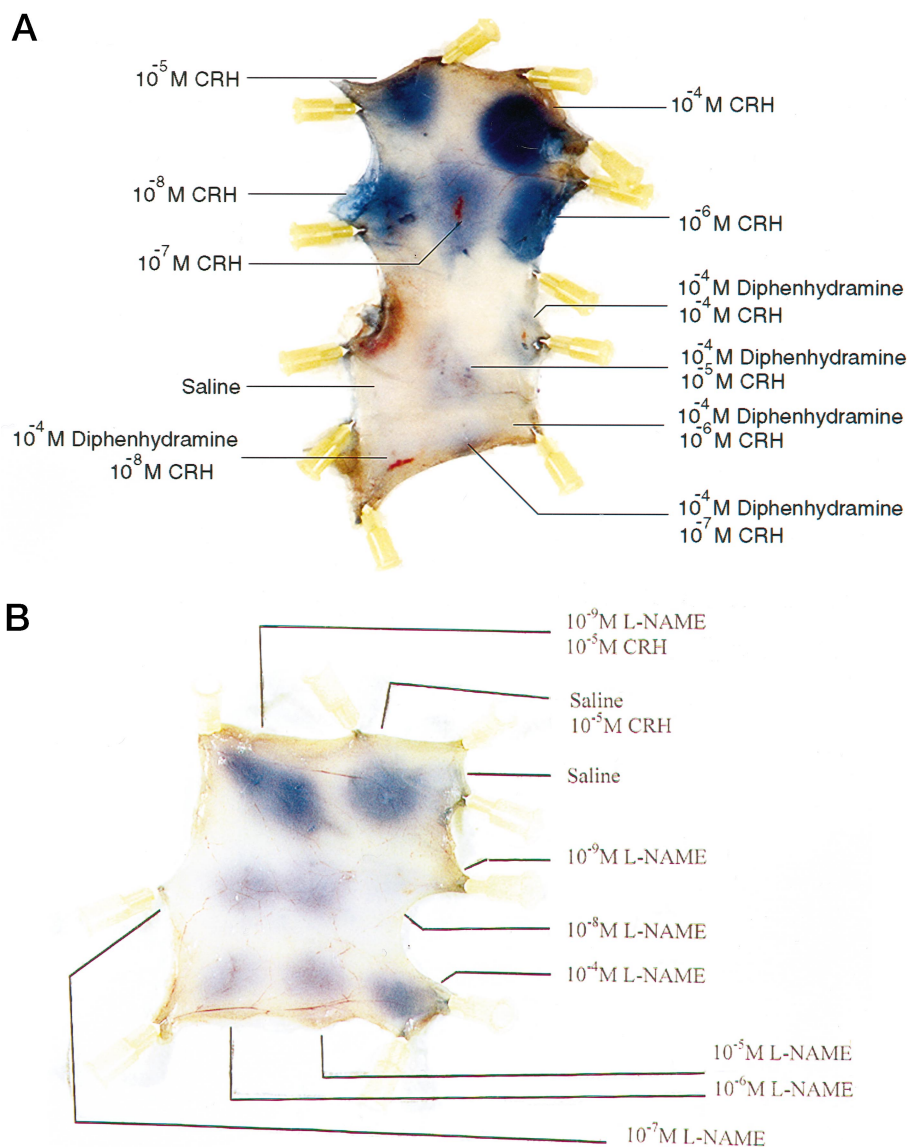
The vasodilatory effect of CRH was blocked by the anti-allergic drug cromolyn, which can inhibit mast cell secretion from connective tissue mast cells (25), providing indirect evidence that mast cells are necessary. The morphological

evidence clearly indicated that CRH results in mast cell degranulation. The strongest evidence for the dependence of the vasodilatory effect of CRH on mast cells, however, came from the absence of this action in W/W^v mast cell deficient mice. Other studies using W/W^v mice concluded that SP-induced increase in vascular permeability and granulocyte infiltration was also mast cell dependent (26, 27). Somatostatin did not block the effect of CRH on fluid extravasation, but increased vascular permeability itself. This is not surprising because somatostatin had previously been shown to stimulate rat (28) and human (29) mast cell secretion.

The increase in vascular permeability induced by CRH appears to be receptor mediated because only the amidated form of CRH, which interacts with CRH receptors (30), was active. Moreover, the nonpeptide CRHR₁ antagonist antalarmin reduced the effect of intradermal CRH, suggesting that CRH-induced skin mast cell activation and increased vascular permeability involved CRHR₁ at least partially. Antalarmin, an analog of Pfizer's CP-154,526 which was shown to block the effect of exogenously administered CRH on ACTH levels (31), also inhibited carrageenin-induced sc inflammation (32). The fact that antalarmin did not inhibit the CRH-induced response entirely is most likely due to its insolubility in aqueous media, which results in poor bioavailability. Alternatively, CRHR₂ may also be involved. In this context, we recently showed that urocortin, which has 45% sequence identity with CRH and is more potent agonist of the CRHR₂ (33), is more potent than CRH in increasing skin vascular permeability (34). *In vivo*, some direct action of CRH on blood vessels cannot be precluded because a CRHR₂ subtype was recently identified on arterioles (35). Our finding that the peptide CRH antagonist [D-phe¹², Nie^{21,38}, Ala³²]rCRH(12–41) acted as a partial agonist for mast cell degranulation was unexpected. However, similar effects have also been reported for SP antagonists (36) and LHRH antagonists (37).

The presence of mRNA for CRHR₁ in HMC-1 cells supports the findings with antalarmin, suggesting that CRHR₁ is also expressed in rat skin mast cells. We believe that the

FIG. 8. Photographs of rat skin showing the effect on dye extravasation induced by CRH ($n = 5$ each) of pretreatment with (A) the histamine-1 receptor antagonist diphenhydramine or (B) L-NAME. The concentration of CRH used and/or combinations of drugs used are indicated by a solid line connecting them to the corresponding site of injection.



band seen with RT-PCR in HMC-1 cells does signify CRHR₁ receptor expression in this cell line because it was reliably obtained, whereas no such band was seen (when simultaneously run using identical solutions) in cultured human synoviocytes obtained from patients undergoing joint replacement. Admittedly, it is hard to extrapolate from leukemic mast cells to normal rat skin mast cells and confirmation will have to await *in situ* hybridization of rat skin samples. Nevertheless, our results are supported by other evidence showing that human skin expresses genes for CRH receptors and for CRH (38).

At this time, the relative contribution of CRH in inflammatory processes, compared with other neuropeptides colocalized in postganglionic sympathetic and/or afferent sensory endings is not known. The lack of an inhibitory effect of capsaicin treatment on the effect of injected CRH indicates that, at least when given exogenously, the action of CRH does not depend on the presence of sensory afferent fiber terminals or SP release. The identification of CRH in primary

sensory afferent fibers (10), however, suggests that antidromic release of CRH from unmyelinated C fibers innervating the skin may participate *in vivo*. For instance, skin mast cells degranulated in response to electrical stimulation (ES) of sensory nerves (39), whereas dura mast cells degranulated after ES of the trigeminal (15) or cervical (40) ganglion. CRH is also present in neurons of sympathetic chain ganglia (41) from the terminals of which it may be also secreted during stress. In general, mast cell-neuron interactions appear to be important in hypersensitivity reactions (16) and in neuroinflammatory syndromes (42).

CRH binding sites have been found on human peripheral blood leukocytes (for review see Ref. 8) and inflamed synovia from arthritic rats (7). Consequently, CRH may either initiate or potentiate the inflammatory process mediated by mast cells via the release of cytokines. This possibility is not excluded by our present results since CRH stimulates secretion of IL-1 from monocytes (43), whereas IL-1 (44) and stem cell factor (45) have been reported to induce mast cell secretion.

However, inflammatory cells are unlikely to be present under normal conditions in the skin.

The fact that the histamine-1 receptor antagonist diphenhydramine could not block the CRH effect entirely indicates that vasodilatory molecules other than histamine may be involved. For instance, vasodilation in rodents is equally dependent on histamine and serotonin (46), whereas cytokines present in mast cells (17) may also be involved. For instance, tumor necrosis factor (TNF) secreted from skin mast cells in response to morphine sulfate was shown to result in skin vasodilation and expression of endothelial adhesion molecule-1 (47). Another vasoactive candidate is NO, which can also be released from mast cells (48). Surprisingly, inhibition of the inducible pathway of NO synthesis, using L-NAME (49), augmented the effect of CRH. A similar increase in vasodilation following inhibition of NO synthesis by L-NAME (documented as increased intestinal permeability and secretion) was attributed to release of histamine from mast cells and not to a direct effect of NO (49). In fact, the inducible isozyme of NO synthase is known to be up-regulated in mast cells and macrophages in intestinal inflammation (50). The relation between CRH and NO, however, is far from clear at the moment. For instance, fetoplacental vessels are dilated in response to CRH via a NO-mediated mechanism (51), whereas NO inhibits placental CRH secretion (52). In the hypothalamus, NO has been reported to both stimulate (53) and inhibit (54) CRH secretion.

Our hypothesis is that, during stress *in vivo*, CRH is released from postganglionic sympathetic nerves and/or peripheral sensory afferents and acts on local mast cells to induce vascular permeability. The inflammatory mediators released could recruit and activate immune cells, as well as act on nerve endings to release more peptides, thus further stimulating mast cells. Preliminary results indicate that rat skin mast cells degranulate during 30 min of acute stress by immobilization (55). This same process was previously shown to induce degranulation of dura mast cells through CRH release (14). In keeping with this hypothesis, a random poll of systemic mastocytosis patients indicated that cutaneous flushing was exacerbated by emotional or physical stress in 40/45 patients (Theoharides, unpublished information). Novel nonpeptide CRH-receptor antagonists, or other molecules that could interfere with CRH-induced skin mast cell activation, might be useful in the management of skin disorders, which are triggered or exacerbated by stress, such as atopic dermatitis, eczema, psoriasis or urticaria.

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