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European Journal of Pharmacology 319 (1997) 85–90

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Modulation of the inflammatory response by corticotropin-releasing factor

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Received 22 February 1996; revised 12 August 1996; accepted 15 October 1996

Abstract

Peptides of the corticotropin-releasing factor (CRF) family have been shown to have either pro- or anti-inflammatory activities. CRF (10–30 μg/kg) administered subcutaneously or intravenously could inhibit edema and dye leakage in the rat paw produced by several injuries. These findings are opposed to some results suggesting a predominantly pro-inflammatory effect of CRF mainly in arthritic processes. The purpose of this work was to identify in vivo and in vitro the conditions for the pro- or anti-inflammatory actions of CRF in order to clarify its physiological and pharmacological function. Using the rat paw edema test we observed that only the highest doses of CRF employed (5 μg) induced a moderate and sustained swelling. Pre-treatment with low doses of CRF (0.5–5 ng) was able to inhibit the edema induced by *Naja naja naja* phospholipase A_2, carrageenin or histamine. Higher doses (50 ng–5 μg) had no anti-inflammatory activity. When co-injected with *Naja naja naja* phospholipase A_2 or histamine the peptide did not modify the swelling at doses up to 500 ng, showing at 5 μg an additive edema with *Naja naja naja* phospholipase A_2. In vitro, CRF did not modify the release of histamine but slightly increased the release of arachidonic acid to the medium. Our findings show a clear dose dependence on the local effects of CRF in inflammatory responses. These results suggest that the mechanisms of the two dose-related phenomena may be distinct.

Keywords: CRF (corticotropin-releasing factor); Inflammation; Histamine; Arachidonic acid

1. Introduction

Edema and leakage of plasma from the vascular compartment follow after injury of skin by external agents such as heat, noxious chemicals or trauma (Wei et al., 1993). Peptides of the corticotropin-releasing factor (CRF) family have been shown to have either pro- or anti-inflammatory activities (Wei and Kiang, 1989; Karalis et al., 1991). Administration of CRF after heat or cold injury reduced the swelling, when given within minutes after injury, before inflammation had progressed to its maximum (Wei et al., 1993). CRF (10–30 μg/kg) administered subcutaneously or intravenously could inhibit edema and dye leakage in the paw produced by immersion in hot (48–58°C) or cold (−20°C) solutions, by exposure to concentrated inorganic acids, or by local intradermal injections of inflammatory mediators such as histamine, serotonin or substance P (Wei and Kiang, 1987). In this context, displaceable binding sites for iodinated CRF were found on blood vessels and on epithelial cells in proximity to sites of vascular leakage (Dashwood et al., 1987). The mechanism by which CRF reduces the edema and protein extravasation is not known. However, the anti-inflammatory activities would be independent of pituitary, steroid release or hypotensive effects (Tsagarakis and Grossman, 1994).

These findings are opposed to the results of Karalis et al. (1991), who showed that CRF is secreted locally in acute carrageenin-induced inflammation in rats and that the immunoneutralization of CRF resulted in a significant decrease of both the volume and cellularity of the inflammatory exudate. These later results suggested a predominantly pro-inflammatory effect of CRF in the setting of acute carrageenin-induced inflammation in rats. Moreover,

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PII S0014-2999(96)00824-2

immunoreactive CRF was found in synovium from patients with rheumatoid arthritis and osteoarthritis (Crofford et al., 1993). The peptide is also locally expressed in acute and chronic streptococcal cell wall-induced and adjuvant-induced arthritis (Crofford et al., 1992).

There is ample evidence relating CRF to the inflammatory response. However, after the experimental evidence obtained so far the potential activity of CRF in inflammatory processes in vivo remains controversial. The purpose of this work was to identify in vivo and in vitro the conditions for the pro- or anti-inflammatory actions of CRF with the hope that the results would clarify its physiological and pharmacological function. Our results suggest a bimodal modulatory activity of CRF in inflammation that depends on the doses and administration time.

2. Materials and methods

2.1. Drugs and animals

Carrageenin, dextran (mean molecular weight 180 000), Hanks' balanced salt solution (HBSS), RPMI-1640 medium, *o*-phthaldialdehyde and histamine were from Sigma. Human/rat CRF was from Peninsula Laboratories. Phospholipase A_2 from *Naja naja naja* venom was purified as described (Bianco et al., 1995). Thioglycolate medium was from Difco. [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid ([^{3}H]arachidonic acid), 100 Ci/mmol, 1 Ci = 37 Gbq) was from New England Nuclear. All other reagents and solvents were of the highest grade commercially available.

Wistar rats weighing 200–300 g were used.

2.2. Hind paw edema

Hind paw edema was induced by the injection (Hamilton syringe) of 10 μl of either CRF, *Naja naja naja* phospholipase A_2 (0.3–0.6 mg/ml), 1% carrageenin, or histamine (1–2 mg/ml) into the foot pad. Swelling measured with a micrometer at the times indicated was calculated by subtracting the value at time zero from the readings taken after the injection (Correa et al., 1991).

2.3. Cell preparation

Resting or thioglycolate-elicited peritoneal exudate cells were harvested in HBSS, washed twice and resuspended in RPMI-1640 medium to obtain the suitable concentration for additional assays (Mosier, 1984). When adherent cells were required, peritoneal exudate cells were incubated in 24-well plastic tissue culture plates for 2 h at 37°C (2×10^6 peritoneal exudate cells/ml, 0.5 ml/well). The non-adherent cells were removed by three washes with warm RPMI-1640 medium and discarded.

2.4. Measurement of [^{3}H]arachidonic acid release

The adherent cells (1×10^6 cells/well) were radiolabelled including [^{3}H]arachidonic acid (0.1 μCi/ml) in the medium overnight (Balsinde et al., 1994). At the end of the 16-h labelling period adherent cells were washed and placed in serum-free medium for 30–60 min before addition of the indicated amount of CRF in serum-free medium containing 2 mg/ml bovine serum albumin (fatty acid-free) as a trap for liberated [^{3}H]arachidonic acid. After 30 min, the supernatants were removed, cleared of detached cells by centrifugation and assayed for radioactivity by liquid scintillation counting. Extracellular [^{3}H]arachidonic acid release is expressed as the percentage of radioactivity released to the incubation medium compared with the total cellular radioactivity (Balsinde et al., 1994). Viability assessed by Trypan blue exclusion test was always better than 90%.

2.5. Histamine release

Peritoneal cells were harvested with Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 2 mM EGTA. Cells were washed twice, adjusted to 2×10^6 cells/ml and kept in chilled HBSS until used. The release of histamine was induced by the indicated amounts of *Naja naja naja* phospholipase A_2 at 37°C for 30 min. Released histamine was measured by a fluorescence method using *o*-phthaldialdehyde (May et al., 1970) and expressed as μg of histamine per 10^6 cells.

2.6. Statistical analysis

Each point of in vivo experiments represents the mean and S.E. of at least 6 determinations. In vitro assays were performed in triplicate. The significance of differences was determined by analysis of variance and Bonferroni test.

3. Results

3.1. Role of CRF in the in vivo inflammatory response

Table 1 shows the effect of different doses of CRF on the rat paw edema. CRF (0.5 ng to 5 μg) was dissolved in HBSS, and 10 μl of each solution was injected into the rat hind paws. The swelling was measured at 15, 30 or 60 min. As shown in Table 1, only 5 μg of CRF induced a significant swelling. At this dose, the edema was moderate and sustained more than 1 h and typically associated to redness.

In the next step, animals were pre-treated with CRF in order to check whether CRF could be modulating the inflammatory response induced by other agents by some kind of cell priming. CRF (0.5 ng to 5 μg) was dissolved

Table 1
Effect of CRF on the rat paw edema

CRF (ng)	Mean paw swelling (mm)		
	15 min	30 min	60 min
0	0.24 ± 0.05	0.12 ± 0.05	0.17 ± 0.06
0.5	0.24 ± 0.09	0.10 ± 0.05	0.13 ± 0.04
5	ND	0.20 ± 0.20	ND
50	0.10 ± 0.09	0.13 ± 0.06	ND
500	ND	0.13 ± 0.08	ND
5×10^3	0.28 ± 0.16	0.38 ± 0.05 [a]	0.40 ± 0.10 [a]

CRF was dissolved in HBSS, 10 μl of each solution was injected in the hind paws of rats. [a] $P < 0.01$ vs. HBSS. Data represent means ± S.E. of two independent determinations performed in triplicates. ND: not determined.

in HBSS and 10 μl of each solution was injected in the rat hind paws 30 min before the injection of several agents: 2–6 μg of *Naja naja naja* phospholipase A_2, 1% carrageenin, 10 μg histamine, all in 10 μl HBSS. As shown in Fig. 1, low doses of CRF, up to 5 ng, were able to inhibit the swelling induced by 2.5 μg of *Naja naja naja* phospholipase A_2 (20–50% inhibition). Pre-treatment with intermediate and high doses of the peptide did not modify the *Naja naja naja* phospholipase A_2-induced edema (data not shown). Up to 6 μg of *Naja naja naja* phospholipase A_2 was tested with identical results. Similar effects were observed for carrageenin- or histamine- induced edema: pre-treatment with CRF was able to inhibit the swelling at

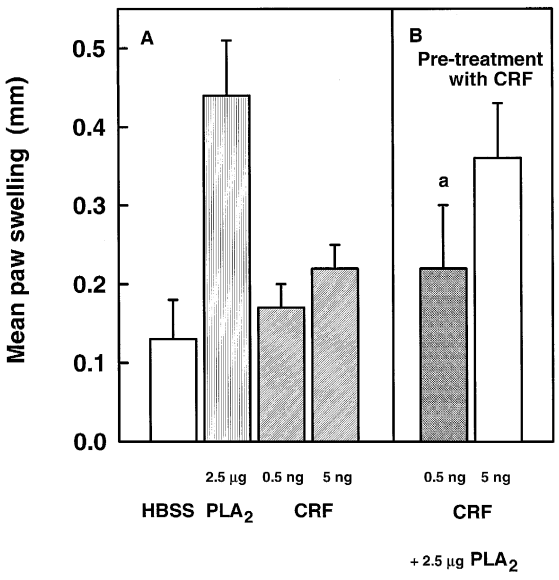

Fig. 1. Effect of CRF pre-treatment on the edema induced by phospholipase A_2. (A) HBSS, 2.5 μg phospholipase A_2 (PLA_2) or CRF (0.5 or 5 ng) in a final volume of 10 μl was injected in the rat hind paws. The swelling was measured 30 min after the injection. (B) Rat hind paws were pre-injected with CRF (0.5 or 5 ng) in a final volume of 10 μl. After 30 min, the paws were challenged with 2.5 μg phospholipase A_2 (PLA_2) and the swelling was measured 30 min later. [a] $P < 0.01$ vs. phospholipase A_2.

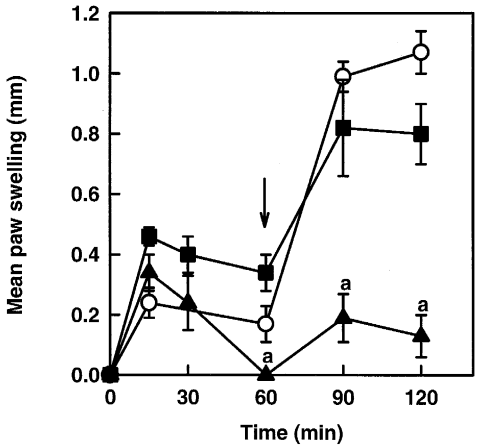

Fig. 2. Time course for the inhibition of the carrageenin-induced edema by low doses of CRF. Rat hind paws were injected with HBSS (○), 0.5 ng (▲) or 5 ng (■) CRF; subplantar injection of 10 μl of 1% carrageenin was, in all groups, conducted after 60 min (arrow). The swelling was measured every 30 min. [a] $P < 0.01$ vs. HBSS.

doses up to 5 ng, with no activity at intermediate or higher doses (Figs. 2 and 3, respectively).

In order to explore the time dependency of the observed pro- and anti-inflammatory effects, different doses of CRF were co-injected with *Naja naja naja* phospholipase A_2 or histamine and the swelling was measured at 30 or 60 min (Table 2 and Fig. 3, respectively). At 5 ng, CRF did not modify the swelling induced by *Naja naja naja* phospholipase A_2 or histamine, indicating that some time is required for CRF to exert its anti-inflammatory activity. On the

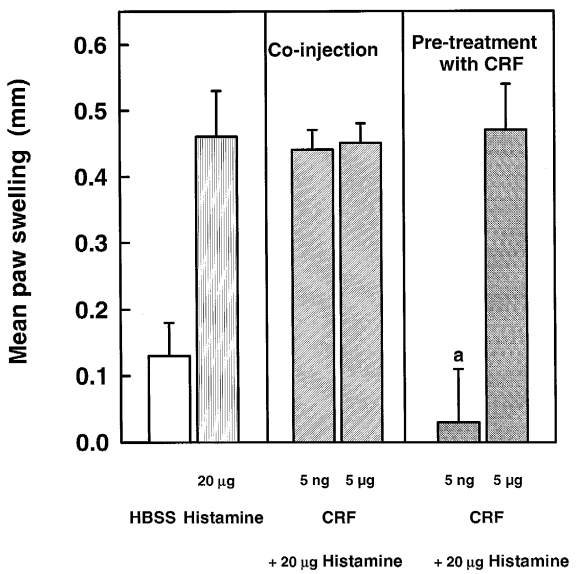

Fig. 3. Effect of CRF on the histamine-induced edema. Rat hind paws were injected with HBSS or histamine (left), co-injected with histamine (20 μg) and 5 ng or 5 μg CRF (middle) or pre-injected with 5 ng or 5 μg CRF 30 min before challenge with 20 μg histamine (right). In all cases the volume injected was 10 μl. The swelling was measured after 30 min. [a] $P < 0.001$ vs. histamine.

Table 2
Effect of CRF and *Naja naja naja* phospholipase A_2 co-injection on the rat paw edema

CRF (ng)	Mean paw swelling (mm)	
	30 min	60 min
0	0.70 ± 0.06	0.57 ± 0.06
5	0.80 ± 0.12	0.55 ± 0.07
50	0.72 ± 0.07	ND
500	0.83 ± 0.24	ND
5×10^3	1.00 ± 0.10 [a]	0.77 ± 0.07 [b]

CRF was dissolved in HBSS, 10 μl of each solution was injected in the hind paws of rats 1 h before the injection of 6 μg of *Naja naja naja* phospholipase A_2 in 10 μl of PBS. [a] $P < 0.05$ vs. HBSS, [b] $P < 0.01$ vs. HBSS. Data represent means ± S.E. of two independent determinations performed in triplicates. ND: not determined.

other hand, the co-injection of 5 μg of the peptide with *Naja naja naja* phospholipase A_2 induced an edema that was almost additive but did not modify the edema induced by histamine. These results suggest that the pro-inflammatory effects of CRF could share the pathway with histamine.

3.2. Histamine release in CRF-stimulated peritoneal exudate cells

Given the results suggesting a possible relationship between CRF and histamine-induced edema, it was of interest to explore whether CRF could modulate the in vitro release of histamine either directly or induced by 5 μg *Naja naja naja* phospholipase A_2. Up to 4 μg CRF/10^6 peritoneal exudate cells did not modify either the basal or the *Naja naja naja* phospholipase A_2-induced release of histamine (Fig. 4).

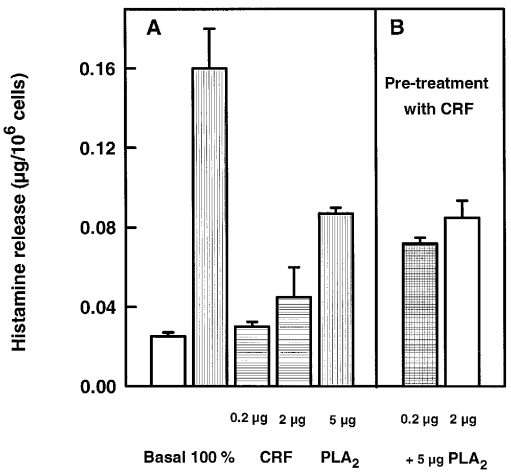

Fig. 4. Effect of CRF on the histamine release from peritoneal cells. (A) Cells (1×10^6/tube) were incubated with medium (basal), perchloric acid (100%), CRF (0.2 or 2 μg) and 5 μg phospholipase A_2 (PLA_2) for 30 min or (B) pre-treated with CRF (0.2 ng or 2 μg) for 30 min before stimulation with 5 μg phospholipase A_2. Authentic histamine was used as standard to quantify the released histamine as described in Section 2.

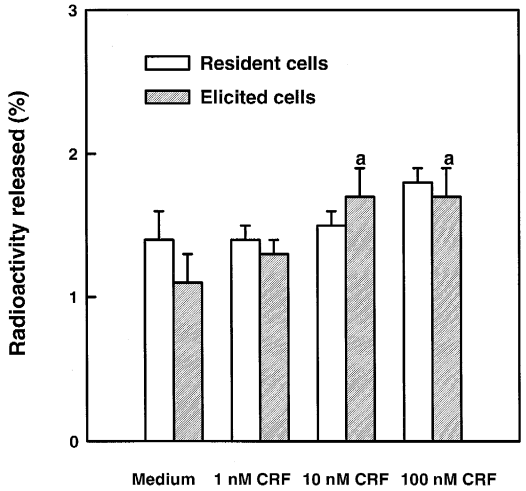

Fig. 5. Effect of CRF on the [^{3}H]arachidonic acid release from peritoneal cells. Adherent resident or elicited peritoneal cells plated to 1×10^6 cells/well were radiolabelled with [^{3}H]arachidonic acid overnight and stimulated with the indicated amounts of CRF for 30 min. Released [^{3}H]arachidonic acid is expressed as a percentage of total radioactivity. [a] $P < 0.05$ vs. medium.

3.3. Arachidonic acid mobilization in CRF-stimulated macrophages

The release of arachidonic acid from membrane phospholipids is one of the earliest events that follow stimulation of phagocyte cells with a variety of agonists (Irvine, 1982; Balsinde et al., 1994). In an attempt to gain insight into the molecular aspects involved in CRF modulation of the inflammatory response we investigated the mobilization of arachidonic acid by CRF-treated adherent peritoneal cells. Fig. 5 shows the CRF-induced arachidonic acid release from pre-labelled cells. As expected from the in vivo results, low doses of CRF did not induce any significant increment in the basal arachidonic acid release. However, a slight but reproducible increment in the arachidonic acid release to the extracellular medium was observed with the higher doses employed, which is more evident when elicited macrophages are exposed to CRF (Fig. 5).

4. Discussion

It has been shown that communication between neuroendocrine and immune systems exists (Blalock, 1994) and that immunity and inflammation are not solely peripheral but also under control of neural influences (Goetzl and Sreedharan, 1992). Earlier data indicate that in certain cases neuropeptides would enhance inflammatory processes (Stanisz, 1994). CRF is found in inflamed synovial tissues at the same concentrations detected in hypophyseal portal venous blood (Crofford et al., 1993). Moreover, CRF produced in high amounts in inflammatory sites

would promote inflammation by potentiating the pro-inflammatory activities of cytokines and other mediators of inflammation (Vamvakopoulos and Chrousos, 1994). Interestingly, other authors have shown that the exposure of tissues to exogenous CRF produces a reduction in the detrimental effects of several injuries (Wei et al., 1993). It has been suggested that pharmacologic doses of CRF administered i.v. or s.c. may act in a different manner than CRF expressed locally at an inflammatory site, where there are infiltrating inflammatory cells and a vast array of inflammatory mediators (Crofford et al., 1993).

The present study was carried out to address the effects of CRF in acute inflammation. Our first observation illustrated that CRF per se could exhibit moderated inflammatory activity only when locally injected at high doses. Moreover, CRF could not enhance the swelling when co-injected with inflammatory agents, except at high doses. Interestingly, in that case, CRF did not show synergistic but additive effect. In rats, peptides of the CRF superfamily enhance blood flow in the hind limb by relaxing arterial smooth muscle (Bolt et al., 1989). It has been suggested that enhanced blood flow after CRF treatment would potentiate the edema (Wei et al., 1993). The fact that CRF does not affect the release of histamine rules out a role for this mediator in the CRF inflammatory response. In this connection eicosanoids generated from the slight amount of arachidonic acid released could be, at least in part, responsible for the edema observed.

In contrast, in animals pre-treated with the peptide before the inflammatory challenge, we observed an anti-inflammatory effect at doses up to 5 ng. The anti-inflammatory effect following a single injection of an exogenous peptide does not exclude the possibility that endogenous peptides, which may be continuously released in vivo, have effects of longer duration. How the peptide reduces the edema is not known, but several possibilities have been documented. CRF may act directly as an agonist on cellular elements in or near the microcirculation to reduce vascular leakage after injury (Wei et al., 1993). Changes in cytosolic free calcium probably play an essential role in CRF protection because other CRF actions have been shown to involve Ca^{2+} influx (Kiang, 1994). Hypotension does not seem to account for the anti-inflammatory effects of CRF, because at subcutaneous doses which did not produce hypotension CRF still inhibited vascular leakage in rat skin, mucosa, muscle and lung (Serda and Wei, 1992). Anti-inflammatory effects of CRF observed in vivo cannot be explained in terms of substance P antagonism (Wei et al., 1993).

It has been suggested that the discrepancies between CRF effects may be related to examination times. The inhibition of plasma extravasation by CRF is measured very early after stimulus (60 min), whereas the decrease in volume and cellularity by immunoneutralization of CRF was measured at 5 h after stimulus (Karalis et al., 1991). In our studies, we employed an identical time course for all in vivo experiments. The present data show that CRF possesses different effects depending on the dose employed, i.e. low doses are anti-inflammatory and large doses are pro-inflammatory. High and low affinity CRF binding sites have been characterized in human peripheral blood leukocytes and monocytes as well as on resident macrophages of mouse spleen (Singh and Fudenberg, 1988; Webster et al., 1990; Grigoriadis et al., 1993). In this connection, a membrane-bound receptor for human CRF has been recently cloned (Chen et al., 1993). Moreover, displaceable binding sites for iodinated CRF were found on blood vessels and on epithelial cells in proximity to sites of vascular leakage (Dashwood et al., 1987). Our findings suggest that the mechanisms of the two dose-related phenomena may be distinct. It remains to be established whether CRF exerts its modulatory activity either acting directly on epithelial cells or via effects on inflammatory mediator release from immune cells.

In summary this paper shows a bell-shaped dose dependence of the local effects of CRF in inflammatory responses. Although the mechanisms still remain to be elucidated the results of this study clearly indicate that any additional work should be performed taking into account an extended range of concentrations.

Acknowledgements

This work was supported by SECyT UNC, CONICET, CONICOR and Fundación Antorchas Argentina. C.M.R. and I.D.B. are career members of CONICET.

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