FEBS Letters 351 (1994) 19–21

FEBS 14415

Relationship between corticotropin-releasing factor and interleukin-2: evolutionary evidence

Enzo Ottaviani[a,*], Antonella Franchini[a], Eva Caselgrandi[b], Andrea Cossarizza[c], Claudio Franceschi[c]

[a]*Department of Animal Biology, via Berengario 14, 41100 Modena, Italy*
Department of Biomedical Sciences, [b]Section of Hygiene and [c]Section of General Pathology, via Campi 287, 41100 Modena, Italy

Received 6 June 1994; revised version received 16 July 1994

Abstract

The addition of corticotropin-releasing factor (CRF) to molluscan hemocytes induces the release of biogenic amines (norepinephrine, epinephrine, dopamine), a phenomenon we have considered as an ancestral type of stress response [(1992) Gen. Comp. Endocrinol. 87, 354–360]. A similar but less significant response was observed following the addition of interleukin-2 (IL-2). Pre-incubation of hemocytes with IL-2 or anti-IL-2 monoclonal antibody significantly reduced or completely eliminated the CRF-induced release of biogenic amines. Further direct evidence of competition between CRF and IL-2 was revealed by immunocytochemical and cytofluorimetric analysis. The data are compatible with the presence of a unique (ancestral?) receptor on molluscan hemocytes, capable of binding both CRF and IL-2, two key molecules of the neuroendocrine and immune system, respectively.

Key words: Corticotropin-releasing factor; Interleukin-2; Molluscan hemocyte; Biogenic amines; Stress response; Cytokine receptor

1. Introduction

Stress response is an integrated reaction to agents which threaten body homeostasis. Besides activation of the hypothalamic-pituitary-adrenal axis, several studies have suggested that the immune system is also involved in the stress response. Using invertebrate models, we and others have demonstrated that corticotropin-releasing factor (CRF) and adrenocorticotropin hormone (ACTH) are present in invertebrate immune cells (hemocytes) and cell-free hemolymph, and that they mediate immune responses such as cell migration and phagocytosis [1–3]. Moreover, we have demonstrated that molluscan hemocytes are also able to exert an ancestral type of stress response (release of biogenic amines) when exposed to CRF and ACTH [4,5]. An intriguing relationship appears to exist between cytokines and stress response in mammals [6]. Wishing to trace the evolutionary basis of this relationship, and taking into account recent evidence of the presence of cytokine-like molecules in invertebrates [7–11], we investigated the possible role of cytokines in an invertebrate stress response.

Hemocytes from two invertebrates were used, i.e. *Planorbarius corneus* and *Viviparus ater*. In both molluscs, phagocytic hemocytes contain pro-opiomelanocortin (POMC)-derived peptide-like molecules, such as ACTH and β-endorphin, and the enzymes responsible for the biosynthesis of biogenic amines [1,5,12]. The results obtained suggest that IL-2 is capable of inducing the release of biogenic amines from the hemocytes of both molluscs and of interferring with the CRF-induced release of biogenic amines. The results can be explained by assuming the presence on the plasma membrane of the molluscan hemocyte of a common receptor able to bind both IL-2 and CRF.

2. Materials and methods

2.1. Specimens

Adult specimens of *Planorbarius corneus* (L.) and *Viviparus ater* (Cristofori and Jan) maintained in dechlorinated freshwater at room temperature were used.

2.2. Determination of the biogenic amines

Hemolymph of *P. corneus* and *V. ater* was divided into six portions (3 ml each one) and placed in plastic tubes. In the first tube (control sample), snail saline solution (SSS) (300 μl) [13] was added, in the second, human CRF (Sigma Chem. Co., St. Louis, USA) (10^{-8} M final concentration), and in the third, human recombinant interleukin-2 (IL-2) (Boehringer, Mannheim, Germany) (20 U/ml final concentration). The sample in the fourth tube was preincubated with IL-2 (20 U/ml final concentration) for 2 h before adding CRF (10^{-8} M final concentration), while those in the fifth and sixth tubes were incubated with anti-IL-2 monoclonal antibody (mAb) (Serva, Heidelberg, Germany) (1:1) for 2 h, before adding CRF (10^{-8} M final concentration) to the sixth tube only. All the tubes were incubated for 20 min at room temperature and immediately centrifuged ($600 \times g$ for 15 min). After centrifugation, the supernatant (serum) and the pellet (hemocytes) were collected, and the amount of biogenic amines in the serum was immediately determined by HPLC. Serum samples were prepared and analyzed by the addition of activated aluminium oxide to the Clin-Rep-Catecholamine Kit (Pharma Vertriebs GmbH and Co. KG, Munich, Germany), as described elsewhere [14], and subsequently treated and analyzed as previously reported [4]. Each experiment was carried out at least three times.

2.3. Immunocytochemical procedures

Hemolymph of *V. ater* was collected as previously described [15]. The hemocytes were cytocentrifuged (Cytospin 2 Cytocentrifuge, Shandon) on a slide at 1000 rpm for 10 min and air-dried. The unfixed hemocytes were then processed by an immunocytochemical procedure, as described elsewhere [16]. Hemocytes were treated with anti-IL-2 mAb (1:10) and anti-CRF polyclonal antibody (pAb) (1:250) (Accurate

*Corresponding author.

SSDI 0014-5793(94)00802-7

Chemical and Scientific Corp., New York, USA) overnight at 4°C. The reaction was visualized by avidin-biotin-peroxidase complex [17]. Controls were performed either by omitting the primary antibody or by testing the antibody on the hemocytes after pre-absorption in liquid phase with their homologous antigens (in excess) overnight at 4°C. Nuclei were counterstained with hematoxylin. Anti-IL-2 mAb was also tested on the hemocytes after pre-absorption with CRF (10^{-6} M).

2.4. Cytofluorimetric analysis

Hemocytes from *V. ater* were incubated with optimal concentrations of anti-IL-2 mAb for 30 min at 4°C, washed with cold SSS, incubated with a TRITC-conjugated rabbit anti-mouse Ig for 30 min at 4°C, washed with cold SSS and analyzed with a FACSCAN cytofluorimeter, as described elsewhere [2]. In some cases, staining with anti-IL-2 mAb was performed in the presence of 5×10^{-7} M CRF or of 20 U/ml IL-2.

3. Results and discussion

Table 1 shows that, as expected, release of biogenic amines, and particularly of norepinephrine (NA) and epinephrine (A), was observed when *P. corneus* or *V. ater* hemocytes were incubated with CRF. However, when cells were pre-incubated with IL-2, this effect was drastically reduced. IL-2 alone was also able to induce a release of biogenic amines, but the effect was much less potent than that observed with CRF. These data would suggest that CRF and IL-2 bind to the same receptor but with a different affinity, probably much higher for CRF than for IL-2. The addition of anti-IL-2 mAb did not, per se, provoke the release of biogenic amines. However, this antibody was able to inhibit the CRF-induced release of NA and A. We hypothesized that IL-2 and CRF could share at least one epitope, causing competition for a common receptor on molluscan hemocytes, recognized by anti-IL-2 mAb. To further test this hypothesis, immunocytochemical and cytofluorimetric analysis were performed. Hemocytes from *V. ater* were positively stained when treated with antibodies to IL-2 and CRF

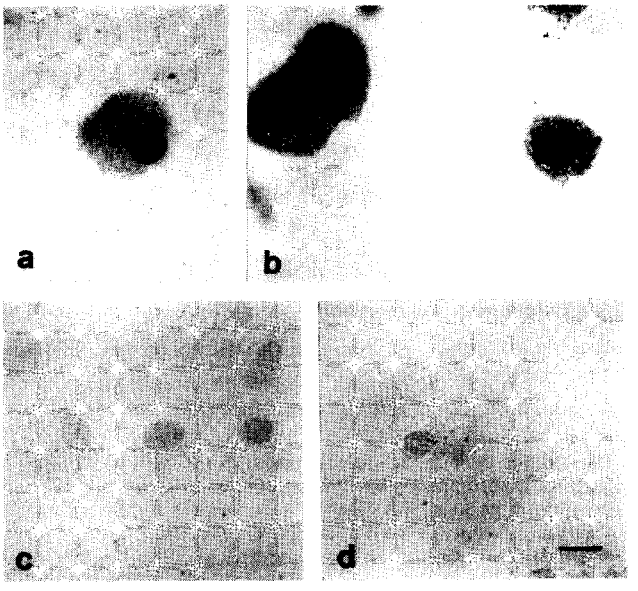

Fig. 1. (a–d) Positive immunoperoxidase staining of molluscan hemocytes to anti-IL-2 mAb (a) and anti-CRF pAb (b). Negative control for anti-IL-2 mAb (c). Negative immunostaining of hemocytes for the anti-IL-2 mAb after pre-absorption with CRF (d). Nuclei were counterstained with hematoxylin. Bar = 10 μm.

(Fig. 1a,b). As expected, when the staining was performed with an anti-IL-2 mAb pre-absorbed with IL-2, a negative result was observed (Fig. 1c), however, the same inhibition was also observed when anti-IL-2 mAb was pre-absorbed with CRF (Fig. 1d). Further proof of the competition between CRF and IL-2 was revealed by cytofluorimetric analysis: anti-IL-2 mAb was able to stain molluscan hemocytes (Fig. 2a), but this reaction was inhibited not only in the presence of IL-2, as expected, but also with CRF (Fig. 2b).

Table 1
Concentrations of biogenic amines determined by HPLC in cell-free hemolymph from *Planorbarius corneus* and *Viviparus ater* after the addition to hemolymph of 10^{-8} M CRF, IL-2 (20 U/ml), CRF after pre-incubation with IL-2 and anti-IL-2 mAb

		Addition of					
		Saline	CRF	IL-2	IL-2 + CRF	anti-IL-2mAb	anti-IL-2mAb + CRF
Na (%)	*P. corneus*	28.0 ± 7.6[a] (100)[b]	293.0 ± 31.6** (1046)	70.0 ± 18.1* (250)	284.0 ± 28.8** (1015)	31.0 ± 10.4 (111)	33.0 ± 7.0 (118)
	V. ater	131.0 ± 16.5 (100)	394.0 ± 36.0** (301)	250.0 ± 28.3** (191)	349.0 ± 39.8** (266)	143.0 ± 24.4 (109)	137.0 ± 30.7 (106)
A (%)	*P. corneus*	87.0 ± 16.9 (100)	187.0 ± 25.0** (215)	145.0 ± 14.3* (167)	152.0 ± 28.8* (175)	96.0 ± 31.4 (110)	103.0 ± 26.0 (118)
	V. ater	92.0 ± 24.5 (100)	299.0 ± 45.2** (325)	219.0 ± 38.0** (238)	312.0 ± 37.0** (339)	93.0 ± 35.2 (101)	109.0 ± 20.5 (118)
DA (%)	*P. corneus*	246.0 ± 24.6 (100)	325.0 ± 22.9* (132)	250.0 ± 17.6 (102)	296.0 ± 21.1 (120)	257.0 ± 22.0 (104)	267.0 ± 23.1 (108)
	V. ater	380.0 ± 22.4 (100)	512.0 ± 50.1* (135)	417.0 ± 41.7 (110)	524.0 ± 25.5** (138)	422.0 ± 38.6 (111)	426.0 ± 51.9 (112)

NA = norepinephrine; A = epinephrine; DA = dopamine. [a]The mean ± standard deviation of three experiments is shown. [b]Control values are taken as 100%. $^{**}P < 0.01$ vs. control (saline); $^{*}P < 0.05$ vs. control (saline). Statistical analysis was performed by Student's *t*-test.

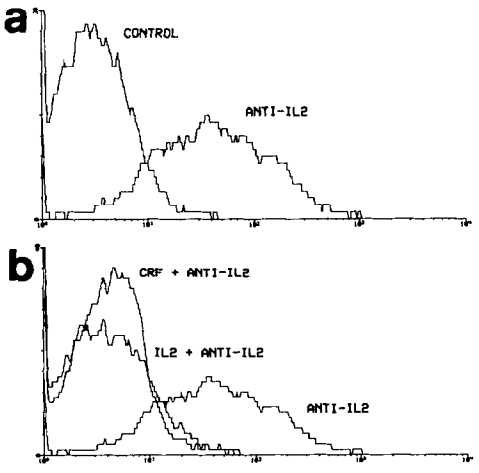

Fig. 2. (a–b) Identification by a FACSCAN cytofluorimeter of IL-2-like molecules on molluscan hemocyte plasma membrane by Anti-IL-2 mAb (a) and competition of this antibody with IL-2 or CRF (b). Ordinate = cell number; abscissa = fluorescence intensity. Data refer to one experiment representative of 4.

In conclusion, our main findings suggest that IL-2 and CRF bind to the same receptor on molluscan hemocyte plasma membrane. The nature of this molluscan receptor is unknown. A possible scenario of this intriguing relationship between CRF, IL-2 and IL-2 receptors on snail hemocytes could be as follows. In basal conditions, one type of receptor able to bind either IL-2- or CRF-like molecules with low or high affinity, respectively, could be present on the plasma membrane of snail hemocytes. This occupancy would be insufficient to give an appreciable release of biogenic amines. The addition of IL-2 or CRF might provoke the release of biogenic amines, and this phenomenon would be greater with CRF owing to its higher affinity for the receptor on hemocyte membranes. Such a situation would explain why the preincubation with both IL-2 and anti-IL-2 mAb significantly reduced the CRF-induced release of biogenic amines. In particular, anti-IL-2 mAb, which can bind to IL-2 and CRF present on the hemocyte membrane, would be unable to provoke amine release, leaving most of the antibody molecules free and able to bind to CRF, so inhibiting the CRF-induced release of the amines.

The fact that the IL-2 receptor can be involved in the binding of molecules other than IL-2 is not unprecedented. It has been recently demonstrated that in mammalian cells the γ chain of the IL-2 receptor is functionally involved in the IL-4 and IL-7 receptor complex [18–20]. Our findings enlarge the number of potential candidate ligands for the IL-2 receptor, suggesting that this phenomenon has a long evolutionary history. These results are probably the first demonstration that an important cytokine, IL-2, a crucial hormone for the stress response, CRF, and their receptors may have co-evolved. In any case, these data represent new evidence in favour of the strict relationship between the immune and neuroendocrine systems, and particularly of our hypothesis of the 'mobile immune-brain' [2,5]. Co-evolution of ligand- receptor pairs has been recently described for LH and FSH receptors [21].

Further work is needed to ascertain whether IL-2 and CRF derive from a primordial molecule and whether their receptors share a common primordial origin.

Acknowledgements: This work was supported by grants from C.N.R. (Italy) to E.O. and C.F.

References

[1] Ottaviani, E., Petraglia, F., Montagnani, G., Cossarizza, A., Monti, D. and Franceschi, C. (1990) Regul. Peptides 27, 1–9.
[2] Ottaviani, E., Cossarizza, A., Ortolani, C., Monti, D. and Franceschi, C. (1991) Proc. R. Soc. Lond. B 245, 215–218.
[3] Genedani, S., Bernardi, M., Ottaviani, E., Franceschi, C., Leung, M.K. and Stefano, G.B. (1994) Peptides 15, 203–206.
[4] Ottaviani, E., Caselgrandi, E., Petraglia, F. and Franceschi, C. (1992) Gen. Comp. Endocrinol. 87, 354–360.
[5] Ottaviani, E., Caselgrandi, E., Franchini, A. and Franceschi, C. (1993) Biochem. Biophys. Res. Commun. 193, 446–452.
[6] Imura, H., Fukuta, J.-I. and Mori, T. (1991) Clin. Endocrinol. 35, 107–115.
[7] Hughes Jr., T.K., Smith, E.M., Chin, R., Cadet, P., Sinisterra, J., Leung, M.K., Shipp, M.A., Scharrer, B. and Stefano, G.B. (1990) Proc. Natl. Acad. Sci. USA 87, 4426–4429.
[8] Ottaviani, E., Franchini, A. and Franceschi, C. (1993) Biochem. Biophys. Res. Commun. 195, 984–988.
[9] Stefano, G.B., Smith, E.M. and Hughes, T.K. (1991) J. Neuroimmunol. 32, 29–34.
[10] Raftos, D.A., Stillman, D.L. and Cooper, E.L. (1991) Immunol. Cell Biol. 69, 225–234.
[11] Raftos, D.A., Cooper, E.L., Habicht, G.S. and Beck, G. (1991) Proc. Natl. Acad. Sci. USA 88, 9518–9522.
[12] Ottaviani, E., Capriglione, T. and Franceschi, E. (1994) Brain Behav. Immun. (in press).
[13] Ottaviani, E. (1983) Dev. Comp. Immunol. 7, 209–216.
[14] Goldstein, D.S., Feuerstein, G., Izzo, J.L. and Keiser, H.R. (1988) Life Sci. 28, 467–475.
[15] Ottaviani, E. (1989) J. Moll. Stud. 55, 379–382.
[16] Ottaviani, E. and Cossarizza, A. (1990) FEBS Lett. 267, 250–252.
[17] Hsu, S.M., Raine, L. and Fanger, H. (1981) J. Histochem. Cytochem. 29, 577–580.
[18] Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K.-I. and Sugamura, K. (1993) Science 262, 1874–1877.
[19] Noguchi, M., Nakamura, Y., Russell, S.M., Ziegler, S.F., Tsang, M., Cao, X. and Leonard, W.J. (1993) Science 262, 1877–1880.
[20] Russell, S.M., Keegan, A.D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M.C., Miyajima, A., Puri, R.K., Paul, W.E. and Leonard, W.J. (1993) Science 262, 1880–1883.
[21] Moyle, W.R., Campbell, R.K., Myers, R.V., Bernard, M.P., Han, Y. and Wang, X. (1994) Nature 368, 251–255.