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Rat Interleukin-1β Binding Sites in Rat Hypothalamus and Pituitary Gland

Christel Marquette[a,b]
Anne-Marie Van Dam[c]
Elisabeth Ban[a]
Philippe Lanièce[d]
Michèle Crumeyrolle-Arias[a]
Gilles Fillion[a]
Frank Berkenbosch[c,†]
France Haour[a]

[a] Unit of Pharmacology Neuro-Immuno-Endocrinienne, and
[b] Rabies Unit, Pasteur Institute, Paris, France;
[c] Research Institute Neurosciences, Free University, Medical Faculty, Department of Pharmacology, Amsterdam, The Netherlands;
[d] Institut de Physique Nucléaire, Paris-Sud University, Orsay, France

In this study, radiolabeled recombinant rat interleukin-1β (r^{125}I-IL-1β) was used to localize and characterize IL-1β binding in rat hypothalamus and pituitary gland by quantitative autoradiography. The ability of this ligand to bind to type I IL-1 receptor was first tested on murine lymphoma cells (EL-4). In the rat-tissue sections, high densities of specific r^{125}I-IL-1β binding sites were localized in the anterior as well as the posterior pituitary and in the choroid plexus. A fine labeling was observed in meninges and third ventricle walls while no binding was detected in the hypothalamic nuclei. Saturation experiments, in the anterior and posterior pituitary, revealed one specific binding site with an affinity constant (K_d) of 0.5 n*M*. Competition experiments were achieved using either rat IL-1β (rIL-1β) or human IL-1s (hIL-1α, hIL-1β and IL-1 receptor antagonist: hIL-1a). Affinity constants (K_i) were drastically different according to the ligand used, while K_i values were found similar in anterior and posterior pituitary. Competition with rIL-1β revealed one binding affinity (K_i of 0.1 n*M* range). In contrast, competition with hIL-1β revealed two binding affinities: a high (K_i: 0.1 p*M* range) and a low one (K_i: 1 n*M* range). Competition with hIL-1ra was obtained for high concentrations only (K_i: 10–100 n*M* range), whereas human IL-1α (hIL-1α) was unable to compete at 1–100 n*M*. Therefore, two types of IL-1 binding affinities have been identified in rat pituitary tissues: a very high affinity binding, detected with hIL-1β only and a lower affinity binding. Human IL-1β can therefore be a better ligand for rat receptors than the rat ligand itself while hIL-1α is not. This is suggestive for the existence of several IL-1 receptor subtypes and is in accordance with the biological activity of hIL-1β in the rat. Moreover, the high density of binding sites observed in both anterior and posterior pituitary indicates that pituitary tissues are direct targets for IL-1 stimulation. It is worth noting that IL-1 binding sites cannot be detected in the rat hypothalamus. This raises the question of the mechanism of the hypothalamic IL-1-mediated effects and suggests a role of the third ventricular walls in this mediation.

Introduction

Interleukin 1 (IL-1) is a pleiotropic cytokine, widely involved in interaction between the immune, nervous and endocrine systems [1]. The effects of IL-1 on neuroendocrine and brain functions are actually well documented [2–4]. Peripheral or central administrations of IL-1 in rodents has potent neuroendocrine actions on the hypothalamus-pituitary-adrenal (HPA) axis through an increase in corticotropin-releasing factor (CRF) secretion from the hypothalamus followed by an increase in adrenocorticotropic hormone (ACTH) [5–9].

At the pituitary gland level, IL-1 is also directly involved in the regulation of anterior pituitary hormones such as ACTH, GH, LH, TSH and PRL [8, 10–15] and in the liberation of the posterior pituitary hormones vasopressin (AVP) and oxytocin (OXY) [16, 17]. In addition to these neuroendocrine actions, brain-mediated effects are observed following human IL-1 (hIL-1) administration (i.v. or i.c.v.) in rat and mouse brain such as fever [18], analgesia [19], modulation of food intake [20], increase in slow wave sleep [21], behavioral changes [3, 22], as well as immunosuppressive effects [23]. These effects are thought to be mediated by the action of IL-1 at specific sites in the hypothalamus.

IL-1 receptors (IL-1R) have been localized by autoradiography in the mouse anterior pituitary using hIL-1α and hIL-1β as a ligand [24–26] and characterized as type I receptor [27–29], but no binding was detected in any hypothalamic nucleus. In contrast, in the rat brain and pituitary we did not visualize any binding site using the same ligand (personal results) while a widespread distribution of IL-1 binding sites has been reported using murine IL-1α (mIL-1α) [30]. In rat hypothalamic membrane preparations, low-affinity high-capacity binding has been detected using hIL-1β, while no binding was detectable in the pituitary gland [31].

We consider that these discrepancies in IL-1 binding in the rat brain are related to the use of species heterologous ligands. This hypothesis has been tested with the use of recombinant rat ^{125}I-IL-1β (r^{125}I-IL-1β) by a quantitative autoradiographic study of IL-1 binding to rat hypothalamus and pituitary gland.

Material and Methods

Animals

Male Wistar rats (180–200 g, IFFA CREDO) and male C_3H/He/OU mice (7–8 weeks old, Pasteur) were kept under standard laboratory conditions in which the light period was from 07:00 until 19:00 h. Sterilized laboratory food and water were available ad libitum. Animals were killed by decapitation. Brain and pituitary gland were dissected rapidly, frozen in dry ice powder and kept at −80°C until use.

Radioligand

Recombinant rat IL-1β (rIL-1β) was iodinated (100 μCi/μg) according to the lodogen method [32, 33]. Briefly, lodogen (1,3,4,6-tetra-chloro-3-alpha-6-alpha-diphenylglycouracil, Pierce Chemicals, Rockford, Ill., USA)-coated polyethylene conical tubes (Greiner, The Netherlands) containing 2.5–5 μg recombinant rIL-1β dissolved in 10 μl of 0.1 *M* Tris-HCl buffer (pH 7.3) were incubated for 15 min at room temperature with 1 n*M* of Na-^{125}I (10 μl of a NaOH solution, pH 7–11, Amersham, UK). To separate the labeled IL-1β protein from aggregates and free iodine, the labeling mixture was loaded on a Sephadex G25 medium column and eluated with a buffer consisting of 0.1 *M* Tris-HCl containing 0.02% gelatin. The preparation was further purified by gel filtration using a Sephadex G50 fine column with 0.1 Tris-HCl containing 0.02% BSA as elution buffer. The binding characteristics of the radiolabeled ligand were tested using membranes of a murine T-cell lymphoma cell line (EL-4 cells) and mouse pituitary.

Unlabeled Reagents

Recombinant cytokines: human IL-1α and human IL-1β were a gift of Dr. S. Gillis (Immunex Corporation, Seattle, Wash., USA), human IL-1 receptor antagonist (hIL-1ra) was from Synergen (Boulder, Colo., USA), human IL-2 was from Roussel Uclaf (Romainville, France). Rat IL-1β was kindly provided by Dr. E. Kawashima from Glaxo IMB (Geneva, Switzerland).

EL-4 Membrane-Binding Assay

Murine EL-4 cell membranes were used to test the binding of the radiolabeled rIL-1β ligand. Saturation experiments were performed on 32 μg of EL-4 cell membranes incubated for 1 h at room temperature with increasing concentrations (0.1 p*M* to 4 n*M*) of r^{125}I-IL-1β in a final volume of 50 μl per well of a 96-well filter plate. Nonspecific binding was defined as binding in the presence of 10 μ*M* hIL-1β. Competition experiments were performed with 5.7 p*M* to 10 n*M* rIL-1β and 0.87 p*M* to 28.6 n*M* hIL-1β in the presence of 45 p*M* r^{125}I-IL-1β. Following incubation, the filters were washed and dried. Each filter was punched out of the filter plate and the radioactivity was counted.

Autoradiography

Mapping of r^{125}I-IL-1β binding was carried out on serial coronal sections of rat and mouse pituitary gland and through the rat brain from bregma −2.3 mm to −5.3 mm according to the rat brain atlas coordinates [34]. Frozen sections (20 μm) were obtained using a Reichert-Jung cryostat (Frigocut 2800) and stored at −20°C until use. The slide-mounted sections were dried and preincubated for 15 min at room temperature in PBS (0.1 *M*) pH 7.4 followed by incubation in RPMI 1640 containing 1% BSA and 0.2 n*M* r^{125}I-IL-1β at room temperature for 2 h. Nonspecific binding was performed in the pres-

ence of 100 n*M* rIL-1β. Saturation experiments were performed on pituitary gland slices with increasing concentrations of r^{125}I-IL-1β (60 p*M* to 2.7 n*M*) and the nonspecific binding was determined in the presence of 100 n*M* rIL-1β. Competition experiments in rat and mouse pituitary glands were performed in the presence of 0.2 n*M* r^{125}I-IL-1β and a range of 0.01 p*M*-1 μ*M* in concentrations of unlabeled rIL-1β, hIL-1β, hIL-1α and hIL-1ra. Following incubation, the slices were washed 5 times 5 min in PBS (0.1 *M*) buffer at 4°C and dried.

Quantitative Image Analysis

High-Resolution Radio-Imager. Saturation experiment was quantified with a prototype of a high-resolution radioimage (HRRI) (prototype IPN, Orsay, France) [35–37], since the radioimager provides a linear dose response over a large range of radioactivity. On the contrary, radiosensitive films exhibit a saturation for high concentrations of radioactive ligand. The radiolabeled slices were overlaid with a thin scintillator sheet, and signals were detected generating a 100 times higher efficiency than conventional image analyzing systems. HRRI permits, in real-time acquisition, a sensitive quantitative analysis of specific radioactivity bound to the tissue in a linear dose-response scale. The results are expressed in moles per pixel ($\times 10^{-21}$).

Autoradiography. Slices from mapping and competition experiments were exposed to Amersham ^{3}H-Hyperfilm for 14–21 days. Autoradiograms were quantified by computerized densitometry of the relative grain density using an image analyzer (RAG 200, Biocom, Les Ulis, France), as previously described [24]. Standardization was achieved using polymer [^{125}I]-microscale (batch No. 5, Amersham). The results are expressed in femtomoles of radiolabeled ligand bound per milligram of protein.

Data Analysis

Dissociation constant (K_d), inhibition constant (K_i), inhibitory concentration 50% (IC_{50}) and maximal binding (B_{max}) were calculated using a curve fitting program (GraphPad Program, Harvey Motulsky, San Diego, Calif., USA).

Results

Rat ^{125}I-IL-1β Binding Characterization on EL-4 Membranes

Competition with various concentrations of unlabeled rIL-1β or hIL-1β (fig. 1) displayed K_i values of 0.57 and 0.25 n*M*, respectively. Analysis of the saturation characteristics of IL-1-binding sites on EL-4 membranes revealed a K_d of 2.5 n*M* (data not shown).

Mapping of r^{125}I-IL-1β Binding in Rat Hypothalamus and Pituitary Gland

Autoradiograms of rat brain sections revealed specific binding sites for r^{125}I-IL-1β localized on walls of the third ventricle (fig. 2A), on the choroid plexus (fig. 2B), and on the meninges (fig. 2C). High densities of binding sites were observed in the anterior and posterior pituitary (fig. 3A), but no binding was detected in the pars intermedia or in the hypothalamic area.

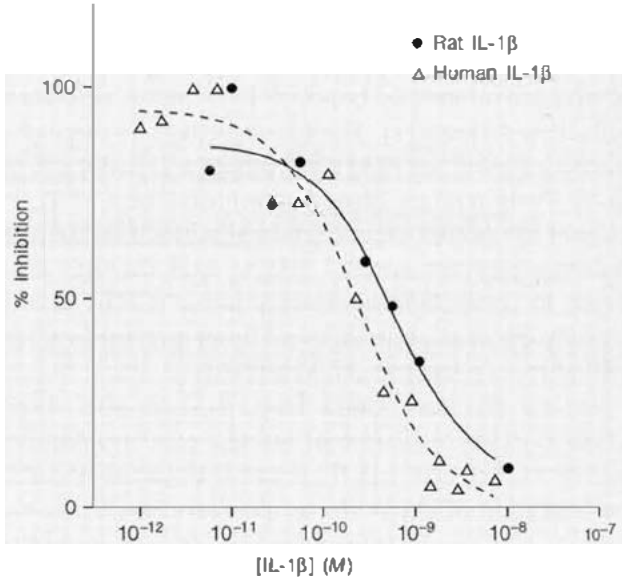

Fig. 1. Competition of human and rat IL-1β with rat ^{125}I-IL-1β binding to EL-4 cell membranes.

This binding can be totally inhibited by unlabeled rIL-1β (100 n*M*) (fig. 2A′, B′, C′, 3A′), but not by recombinant human IL-2 (data not shown).

Pharmacological Characterization of r^{125}I-IL-1β Binding Sites in the Rat Anterior and Posterior Pituitary Gland

Saturation experiments (fig. 4) revealed similar affinities K_d in anterior and posterior pituitary (1 and 0.9 n*M*, respectively). When calculated for the entire pituitary tissue, maximal binding could be approximated around 5.6 fmol/anterior pituitary ($72 \cdot 10^{-21}$ mol/pixel × 1,301.5 pixels × 60 sections/pituitary gland) and 0.25 fmol/posterior pituitary ($52 \cdot 10^{-21}$ mol/pixel × 188.5 pixels × 25 sections/pituitary gland).

Competition experiments were performed using unlabeled rIL-1β, hIL-1α, hIL-1β and hIL-1ra. The K_i values are summarized in table 1. Competition of rIL-1β (fig. 5A) displayed a single high-affinity binding site in the anterior and posterior pituitary with similar K_i values (0.08 and 0.22 n*M*, respectively). In contrast, competition with hIL-1β (fig. 6A) displayed two binding affinities in both the anterior and posterior pituitary, one with a high-affinity (K_i = 0.23 and 0.37 p*M*, respectively) and one

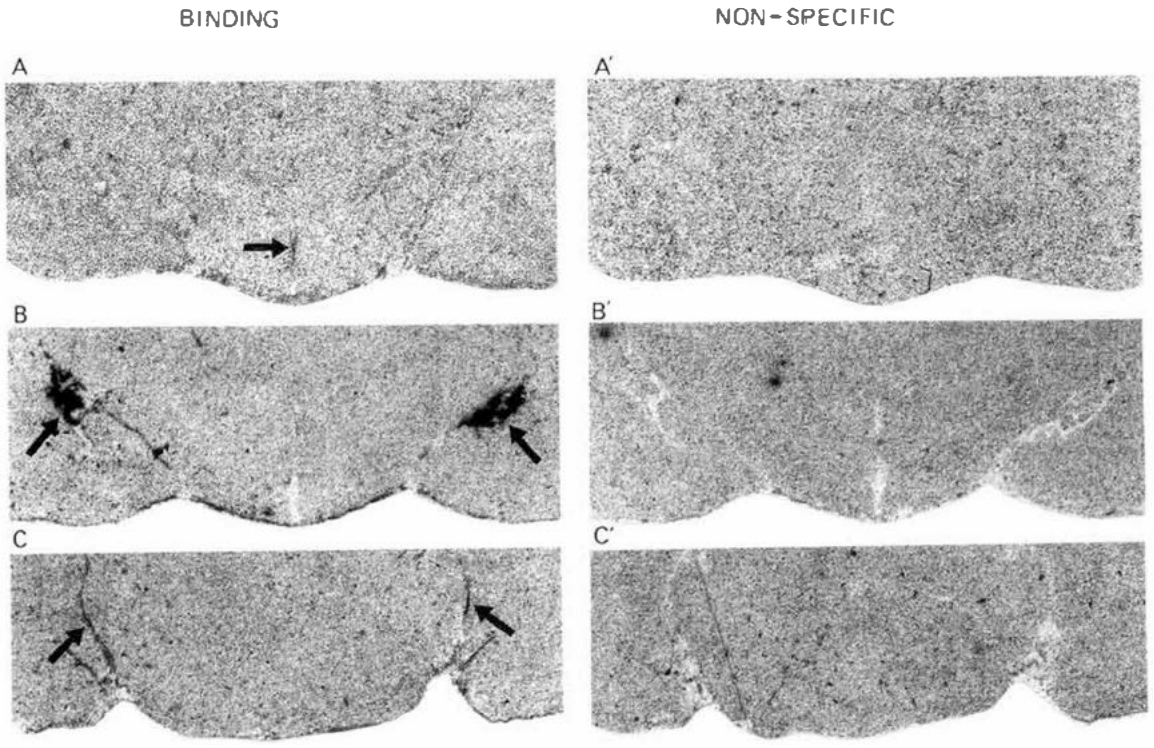

Fig. 2. Autoradiograms of rat ^{125}I-IL-1β binding in the rat hypothalamus. (× 6.6). **A, B, C** Total binding obtained by incubation with 0.2 n*M* rat ^{125}I-IL-1β showing binding sites in (**A**: bregma: −2.3 mm) on the walls of the third ventricle (arrow) (**B**: bregma: −4.3 mm) on the choroid plexus (arrows) (note: on this slice the epithelial wall of the third ventricle has been tirred during slicing), and (**C**: bregma: −5.3 mm) on the meninges (arrows). **A', B', C'** Nonspecific binding was obtained by addition of an excess of rat IL-1β (100 n*M*).

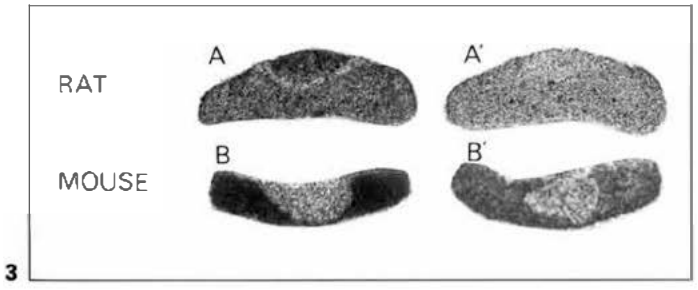

Fig. 3. Autoradiograms of rat ^{125}I-IL-1β binding in the rat (× 6.6) and mouse (× 10.3) pituitary gland. **A, B** Total binding obtained by incubation with 0.2 n*M* r^{125}I-IL-1β. **A', B'** Nonspecific binding was obtained by addition of excess rIL-1β (100 n*M*).

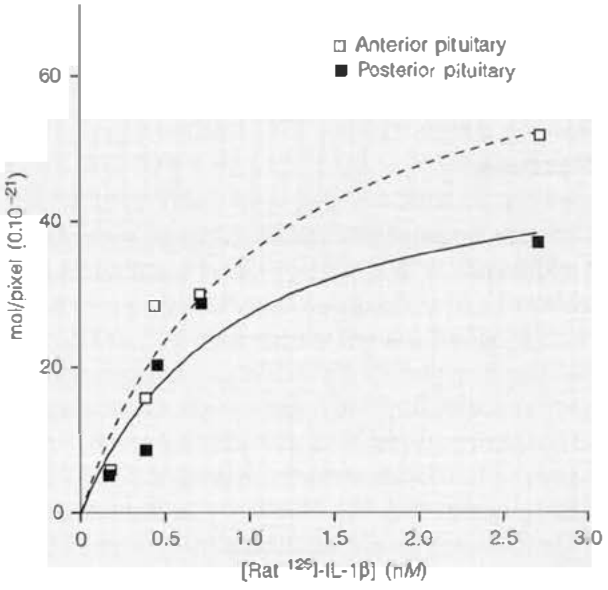

Fig. 4. Saturation curves of specific r^{125}I-IL-1β binding to rat anterior and posterior pituitary. Specific radioactive binding is given in mol/pixel (× 10^{-21}) and was obtained by computerized image analysis obtained with HRR1. Each point is the mean of global radioactivity measured on 2 sections (the mean area per section was 1,301.5 and 188.5 pixels for anterior and posterior pituitary, respectively).

Table 1. K_i obtained by competition of rat ^{125}I-IL-1β binding with rat IL-1β, human IL-1β, human IL-1α and human IL-1 receptor antagonist in the rat anterior and posterior pituitary

	Anterior pituitary	Posterior pituitary
Rat IL-1β	0.8 n*M*	0.22 n*M*
Human IL-1β	0.23 p*M*	0.37 p*M*
	0.7 n*M*	0.87 n*M*
Human IL-1ra	98 n*M*	12 n*M*
Human IL-1α	<100 n*M*	<100 n*M*

with a low-affinity site (K_i = 0.70 and 0.87 n*M*, respectively).

Competition with hIL-1α was not detectable (fig. 6A) in spite of the high concentration of IL-1α used (1–100 n*M*), whereas hIL-1ra compete with r^{125}I-IL-1β binding (fig. 6B) with low affinities (K_i = 98 and 12 n*M* in anterior and posterior pituitary).

Mapping and Speci of r^{125}I-IL-1β Binding in Mouse Pituitary Gland

Under the same experimental conditions, r^{125}I-IL-1β binding was achieved on mouse pituitary gland sections (fig. 3B). The presence of specific binding is evident in the anterior pituitary but undetectable in the posterior pituitary. As previously described [24–26], no binding was detected in the hypothalamus (data not shown) under these experimental conditions.

Competition of rIL-1β showed a single IL-1 binding site in the mouse anterior pituitary with an apparent affinity of 12.5 p*M* (IC_{50} value) (fig. 5B).

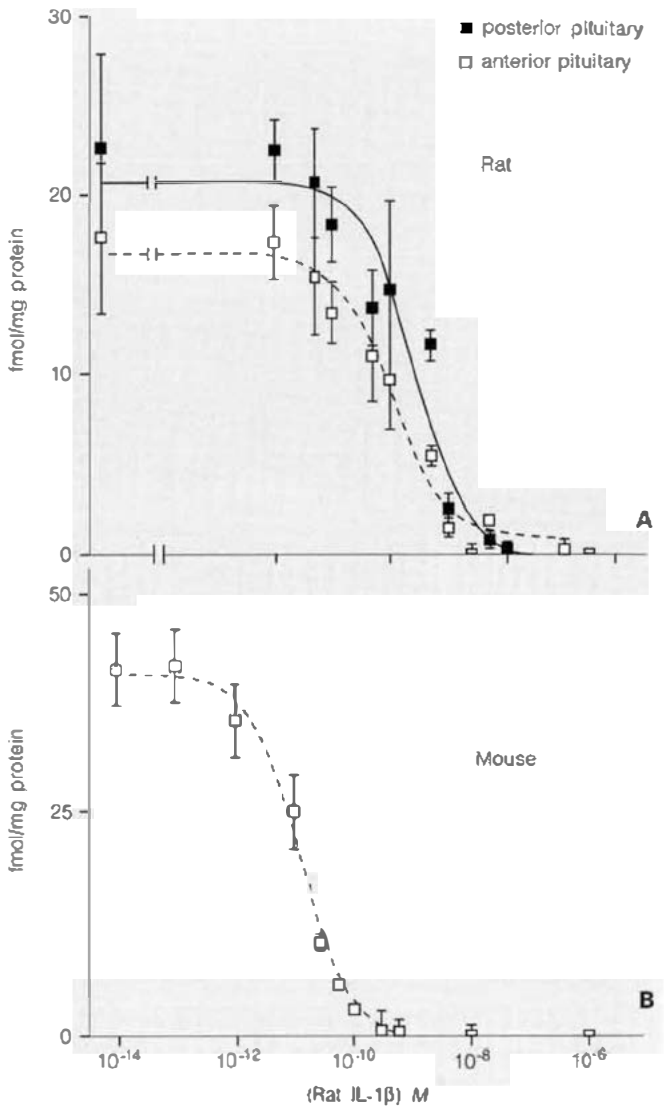

Fig. 5. Competition of 0.2 n*M* r^{125}I-IL-1β by rIL-1β in rat anterior and posterior pituitary (**A**) and in mouse anterior pituitary (**B**). Specific radioactive binding is given in fmol/mg protein and was obtained by computerized image analysis of the autoradiographic images. Data represent the mean ± SD of 6 or 8 determinations in the anterior and posterior pituitary, respectively. Error bars not shown are contained within the symbol.

Discussion

The detection and pharmacological characterization of IL-1 binding sites in the rat pituitary and brain has been obtained using rat IL-1β radiolabeled ligand. This ligand binds to murine EL-4 cell membranes, known to possess a single class of high-affinity binding sites characterized as type I IL-1 receptors [38]. Saturation and competition experiments on this cell line provided K_d and K_i values in the same order of magnitude as those described for type I hIL-1β receptors [38–40]. Similarly, in mouse pituitary, r^{125}I-IL-1β binding revealed localization and binding characteristics identical to those reported for h^{125}I-IL-1α and h^{125}I-IL-1β [26, 41]. Hence, these results indicate that r^{125}I-IL-1β is a useful ligand for the detection of IL-1 binding sites.

In rat hypothalamus no binding sites can be detected in the nervous tissue under our experimental conditions. However, direct effects of IL-1 have been described in the hypothalamus, such as secretions of catecholamines [42]

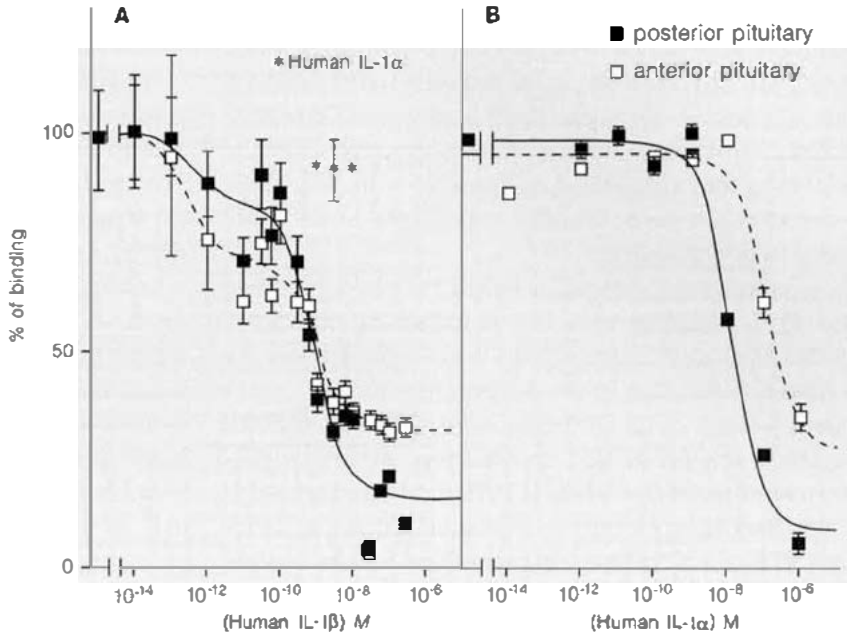

Fig. 6. Competition of r^{125}I-IL-1β binding to the rat anterior and posterior pituitary. **A** By human IL-1β and human IL-1α. **B** By human IL-1 receptor antagonist. Specific bound radioactivity is given in fmol/mg protein and is obtained by computerized image analysis of the autoradioradiographic images. Data represent the mean ± SD of 6 or 8 determinations in the anterior and posterior pituitary, respectively.

and CRH [9, 43, 44]. It is worth noting that this discrepancy has also been found by others using either the same method [41], or in situ hybridization detection of IL-1 receptor type I mRNA [45]. Using IL-1 receptor type I mRNA probes, Yabuuchi et al. [45] did not visualize any specific signal in the hypothalamic paraventricular nuclei. However, endothelial cells provide a signal in this region [45]. In mouse brain similar results had been also described [24–27]. This lack of IL-1 receptor detection is in discordance with IL-1 biological activity on hypopthalamic neurohormone release. Several hypotheses could explain this lack of coherence: (1) hypothalamic sites of IL-1 action differ from the common IL-1 receptors type I already described [3] involving a new type of receptor not detected by this ligand; (2) only a low level of IL-1 receptors with a very high affinity might be necessary for inducing CRF response [44], and then not detected by our current technqiue; (3) the presence of IL-1 binding sites in the epithelial cells of the walls of the third ventricle and choroid plexus might be a possible primary target for IL-1 action which could be followed by secondary signals thereafter (TNF, IL-6, PGE_2). This last hypothesis is supported by the detection of IL-1 receptor mRNA in the epithelial cells as well as in the endothelial cells in these structures [45], and the characterization of a functional IL-1 receptor type I on these cells in the rat brain [Van Dam, pers. commun.]. Thus, a general idea emerges that IL-1 would bind to receptors in epithelial and endothelial cells, inducing secondary mediators and activation of CRH neurons and HPA axis.

The rat anterior and posterior pituitary displayed a specific high-affinity binding site using r^{125}I-IL-1β as ligand, while binding sites are detectable in the mouse anterior pituitary only, using rat or human IL-1 [24, 26]. These results support the existence of species-specific differences in IL-1R types [41]. The affinity of the binding site for rIL-1β in the rat pituitary tissues is in accordance with those reported for type I IL-1 receptors expressed on pituitary AtT-20 cell line [46], on mouse anterior pituitary [24–26], on fibroblasts [47], and on immunocompetent cells [48, 49]. However, hIL-1α do not compete with r^{125}I-IL-1β binding. Thus, these binding sites do not seem to meet the requirement for a classification as type I or type II as defined by hIL-1 binding [48]. This can be compared to the IL-1 binding to soluble IL-1R (IL-1Rs): the protein binds IL-1β but not IL-1α, and IL-1ra [50] fails to inhibit IL-1β binding to IL-1Rs [51]. It is worth noting that in our study hIL-1β reveals two affinities sites in the rat pituitary: one high affinity (subpicomolar) and one low affinity (nanomolar). Such a heterogeneity for IL-1 binding has been also previously described in cell lines [47–49]. Therefore, several hypotheses are feasible: (1) the binding sites for hIL-1β concern one receptor with two states of affinity; (2) two distinct receptors bind hIL-1β

with different affinities and rIL-1β with one class of affinity; (3) one receptor displays two affinities according to its association in a homo- or heterocomplex with another protein [52]. Alternatively, in the mouse pituitary, mRNA coding for type I and type II IL-1 receptors has been detected [53] whereas binding studies detected only one type of receptors using hIL-1 as ligand [24–26, 53]. Thus, the nature of the high- and the low-affinity binding site remains to be elucidated.

It is interesting to note that, in spite of hIL-1β binding to rat IL-1 receptors and the induction of biological response, the radiolabeled hIL-1β is not a good ligand for receptors visualization in the rat [pers. results].

The presence of IL-1 binding sites in the rat anterior pituitary is related to IL-1 modulation of various hormone release (ACTH, GH, LH, TSH and PRL)[8, 10–15] and pituitary activity during infection and stress in rodents [7]. In vivo characterization of the cellular type of IL-1 binding strictly depends on the animal species and the typeof IL-1 (α or β) used. Up to now, only one result in mice indicates that this binding site is likely to be localized on mouse somatotroph cells [54]. In rat posterior pituitary, IL-1 receptors could be related to the direct effect of IL-1β on AVP and OXY release during immune or inflammatory reactions [16, 17, 55]. Since the concentration of IL-1 for half-maximal release of vasopressin is in the range of K_i for the low affinity site (in 0.1–1 n*M* range), this binding site is probably involved in the control of vasopressin release [10].

In conclusion, in the rat, IL-1 binding sites have been demonstrated in the anterior and posterior pituitary, the epithelium of choroid plexus and the walls of the third ventricle and in the meninges. The lack of receptors in the rat hypothalamus is in discordance with the biological activity of IL-1 but is in agreement with previous results in rat and in mouse studies. Therefore, the initial target for IL-1 stimulation of the hypothalamo-pituitary axis remains to be clarified in the rat as well as in the mouse. Results obtained in the rat pituitary indicate a direct IL-1 control on pituitary functions. Demonstration of two IL-1 binding sites which cannot be strictly identified with type I and II IL-1 receptors suggests the existence of several IL-1 receptor subtypes.

Acknowledgments

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