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β-ENDORPHIN RECEPTORS ON CULTURED AND FRESHLY ISOLATED LYMPHOCYTES FROM NORMAL SUBJECTS

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[125I-(D-Ala^2)-βEndorphin (βEP*) binding to Epstein-Barr Virus (EBV) transformed B Lymphocytes or to freshly isolated lymphocytes was characterized. The binding was time-, temperature- and pH-dependent; furthermore, it was reversible and cell concentration dependent. Maximum binding appeared at 15C in Tris buffer (pH 7.6) containing both BSA 0.5% and Bacitracin 0.1 mg/ml. βEP inhibited βEP* binding to transformed lymphocytes and to freshly isolated lymphocytes by 50% at approximately $4x10^{-8}$ M and $8x10^{-9}$ M, respectively. Peptides representing amino-acid sequences 1-5, 1-16 and 1-17 of βEP did not inhibit βEP binding, neither did the opiates compounds Naloxone, Morphine, Bremazocine and Ethylketocyclazocine. On the contrary, βEP (6-31) inhibited βEP* binding as effectively as βEP, thus indicating that βEP* binding to lymphocytes does not involve the N-terminal region of βEP. $2x10^7$/ml freshly isolated lymphocytes bound 1.53±0.75%, whereas $2x10^6$/ml transformed lymphocytes bound 1.64±0.75% (mean±SD) βEP* at tracer concentration. EBV transformed lymphocytes and freshly isolated lymphocytes may be useful to study βEP receptors in humans.

It has been suggested that β-Endorphin (βEP) has various receptor-mediated actions on lymphocytes such as modulation of both the Phytohaemoagglutinin- and Concanavalin A-induced proliferative responses (1, 2), and stimulation of both Natural Killer activity (3-5) and Interferon production (6). Furthermore, it has been suggested that βEP receptors may play a role in certain clinical states, such as Non-Insulin Dependent Diabetes Mellitus (7), Polycystic Ovarian Disease (8) and eating disorders (9). Therefore, experiments have been carried out in order to characterize βEP receptors in lymphocytes since they are easily obtainable from human beings and they should be a suitable model to study: i) the immunomodulatory role of βEP; ii) the presence of βEP receptor defects in humans; and, iii) the role of receptor defects in the pathogenesis of disease states.

MATERIALS AND METHODS

RPMI 1640, Fetal Calf Serum (FCS), Phosphate Buffer Saline Solution (PBS), Penicillin-Streptomycin and Glutamine were from Flow Laboratories (Irvin, Scotland); Lymphoprep was from Niegard and Co. A/S (Oslo, Norway); Di-n-buthyl-phtalate was from Merck (Darmstadt, FRG), Bovine Serum Albumin (fraction V), Bacitracine,

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α, β and γ Endorphin, Met- and Leu-Enkephalin, ACTH 1-39, α, β and γMSH, Naloxone were from SIGMA (St.Louis, USA); Bremazocine was kindly provided by Sandoz (Basilea, Switzerland).
Blood samples, drawn from the antecubital vein, were obtained from volunteers. They were normal as far as body weight (Geigy Table, 7th edition), hematochemical parameters and physical examination. Furthermore, volunteers had no family history of diabetes mellitus, obesity, hypertension, headache, neurological or psychiatric disorders. None had taken any drug during the last two months.

Cell preparation procedures: blood was collected in a heparinized syringe, diluted 1:2 in PBS, peripheral blood mononuclear cells isolated on a gradient of Lymphoprep according to Boyum (10) and finally washed three times in PBS. In the case of transformed lymphocytes, cells were suspended in RPMI 1640 medium supplemented with 20% FCS, 50 I.U./ml Penicillin, 50 ug/ml Streptomycin and 2 mM Glutamine, and subsequently B Lymphocytes were transformed with Epstein-Barr Virus (EBV) obtained from the supernatant of the Marmoset lymphoblastoid line B95-8, as previously described (11). EBV exposed cells were incubated at 37C in presence of an atmosphere of 5% CO -95%O and began to proliferate after 5 weeks of incubation. In the case of freshly isolated lymphocytes, cells were suspended in RPMI 1640 medium, incubated for 1 hr at 37C in a plastic Petri dish, non adherent cells harvested, washed repeatedly and finally suspended in the binding buffer. In the final sample over 98% of the cells were lymphocytes.

[125I]-[D-Ala^2]-βEP binding studies: human [D-Ala^2]-βEP was labeled by means of Chloramine T according to the method described by Hazum et al. (12). Specific activity was approximately 200 uCi/ug. Transformed lymphocytes ($2x10^6$/ml), or freshly isolated lymphocytes ($2x10^7$/ml), were suspended, unless otherwise indicated, in TRIS 25mM, KCl 5mM, NaCl 120mM, EDTA 1.1mM, MgSO 1.4mM, Glucose 10mM buffer (pH 7.6) (TRIS buffer) (13) containing both BSA 0.5% and Bacitracine 0.1 mg/ml. Cells were incubated with [125I]-[D-Ala^2]-βEP ($2x10^{-10}$M) (βEP*) in the presence or absence of increasing amouns of βEP, various opioid peptides or opiate compounds. At the end of the incubation period 200 ul aliquots were layered over 200 ul Di-n-buthyl-phtalate (d=1.05) (13) and centrifuged in Beckman Microfuge E. Non specific binding was estimated as the binding of βEP* in the presence of 10^{-5}M βEP. Cell viability, measured at the beginning and at the end of each incubation period by the Tripan Blue exclusion test, was always over 97%. βEP* degradation was measured by both the Trichloroacetic acid precipitation technique and rebinding studies.

RESULTS AND DISCUSSION

Initially, time-course binding experiments have been performed on transformed lymphocytes in TRIS buffer (pH 7.6) at 15C. Under these experimental conditions non specific binding was about 40% of total binding due to tracer degradation (data not shown). In order to overcome this problem, the binding buffer was supplemented with 0.1 mg/ml Bacitracine plus BSA at concentrations ranging from 0.1 to 1%. Tracer degradation was completely abolished in the presence of both 0.5% BSA and 0.1 mg/ml Bacitracine. Therefore, subsequent experiments were carried out adding these compouns to the binding buffer.

Figure 1 shows that βEP* binding to transformed lymphocytes was time- and temperature-dependent, the maximum specific binding appearing at 15C. At this temperature steady state was achieved in 1hr and lasted until 3hrs without any considerable tracer degradation; non specific binding was 20% of total binding. Conversely, at 24C the steady state was achieved in 30min and lasted 1hr whereas at 37C the steady state was achieved in 20 min (Fig.1). It is noteworthy that after 1hr of incubation tracer degradation was 14% at 24C and 18% at 37C. At these temperatures degradation disappeared in the presence of 1 mg/ml Bacitracine but the specific binding decreased consistently. βEP* binding was pH-dependent; maximum specific binding was obtained in a pH range from 7.4 to 7.8. βEP* binding was reversible, in fact at the steady state

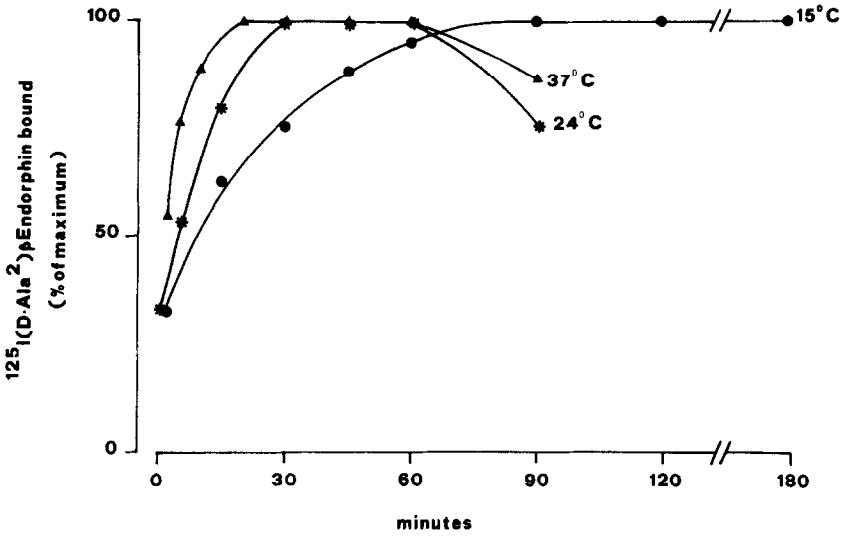

Figure 1 The effect of incubation time and temperature on specific [125I]-[D-Ala^2]-βEndorphin (βEP*) binding to transformed lymphocytes. 2x10^{6}cells/ml were incubated with βEP* at tracer concentration in TRIS buffer (pH 7.6) at 15, 24 and 37C for varying times in the presence or absence of 10^{-5}M βEP. Values represent means of three experiments, each done in triplicate.

(90 min at 15C), the addition of 10^{-5}M βEP to the incubation medium induced a progressive dissociation of the bound radioactivity, so that after 1hr about 80% of the radioactivity was dissociated (data not shown). The binding reaction was carried out in the presence of a variety of buffers and media (Hank's, Krebs-Ringer Bicarbonate, Hepes-TRIS) but the highest specific binding was obtained in the presence of the aforementioned TRIS buffer (data not shown). Experiments carried out on circulating lymphocytes gave superimposable results. The binding increased as function of transformed lymphocyte concentration in the range from 0.5x10^6 to 4x10^6 cells/ml (fig.2a). Furthermore, binding also increased as function of freshly isolated lymphocyte concentration in the range from 0.5x10^7 to 3x10^7/ml.

2x10^6 /ml transformed lymphocytes from 5 normal subjects bound specifically 1.64±0.75% (mean±SD), whereas 2x10^7/ml freshly isolated lymphocytes from 9 normal subjects bound specifically 1.53±0.75% (mean±SD) βEP* at tracer concentration.

Competition-inhibition studies showed that βEP and βEP(6-31) have similar inhibitory properties for βEP* binding, 50% inhibition occurring at about 4x10^{-8}M in the case of transformated lymphocytes (Fig.3a) and at about 8x10^{-9}M in the case of freshly isolated lymphocytes (Fig.3b). The former value is in close agreement with data reported by Hazum et al. (14) on βEP receptors in RPMI 6237 cultured lymphocytes. Therefore, circulating lymphocytes seem to bind βEP with higher affinity in comparison to transformed lymphocytes. Scatchard analysis of data showed curvilinear plots suggesting the presence of two different receptor

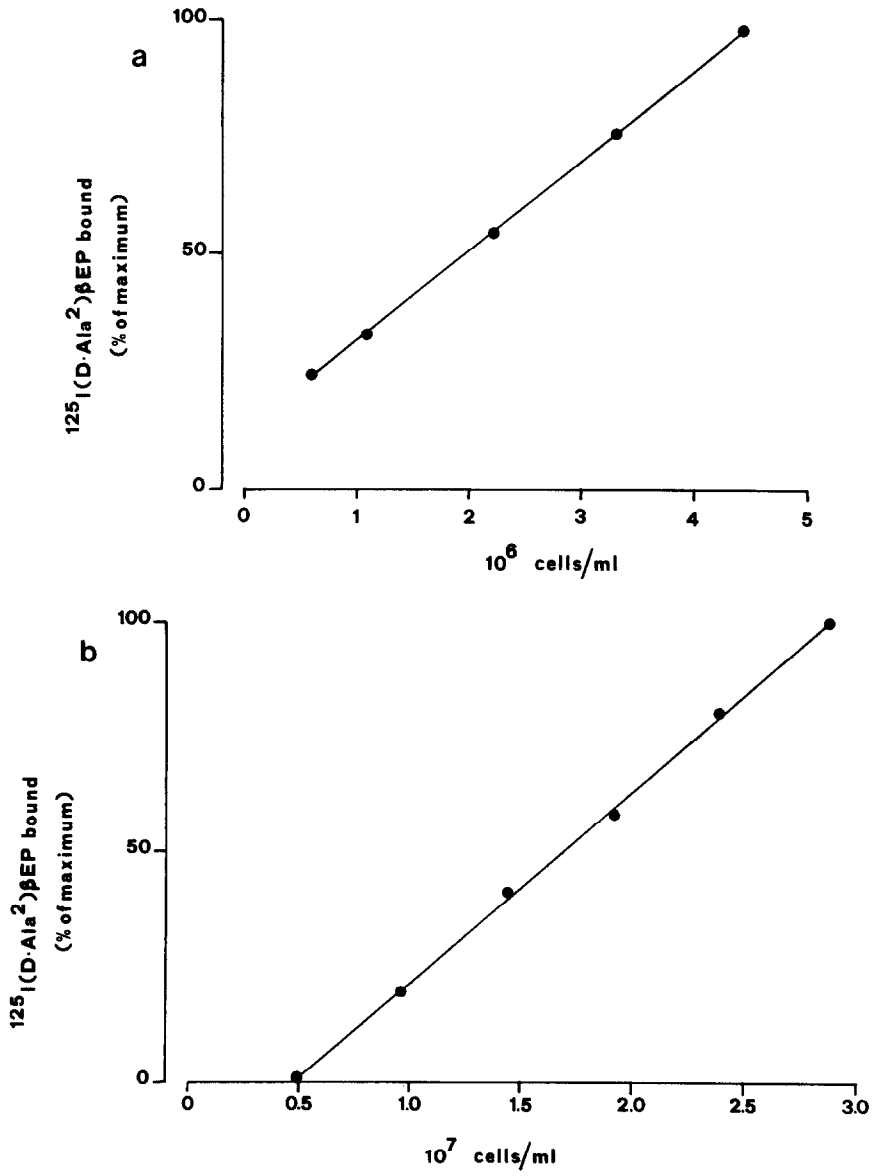

Figure 2 The effect of cell number on specific [125I]-[D-Ala^2]-βEndorphin (βEP*) binding to transformed lymphocytes (a) or freshly isolated lymphocytes (b). Cells were incubated at 15C and at varying concentrations in TRIS buffer (pH 7.6) with βEP* at tracer concentration for 90 min in the presence or absence of 10^{-5}M βEndorphin. Values represent means of three experiments, each done in duplicate.

populations or negative cooperativity (fig.3b; inset). Therefore, it is not possible to calculate the affinity constant (15).

It has been reported that βEP influences lymphocyte proliferative (1) or blastogenic (2) response through its non-opioid C-terminal segment rather than its opioid active moiety (i.e. the N-terminal segment of the molecule). N-terminus is not involved in the binding to both freshly isolated lymphocytes and transformed lymphocytes. In fact Leu-Enkephalin, Met-Enkephalin (corresponding to the N-terminal aminoacid 1-5), αEP and

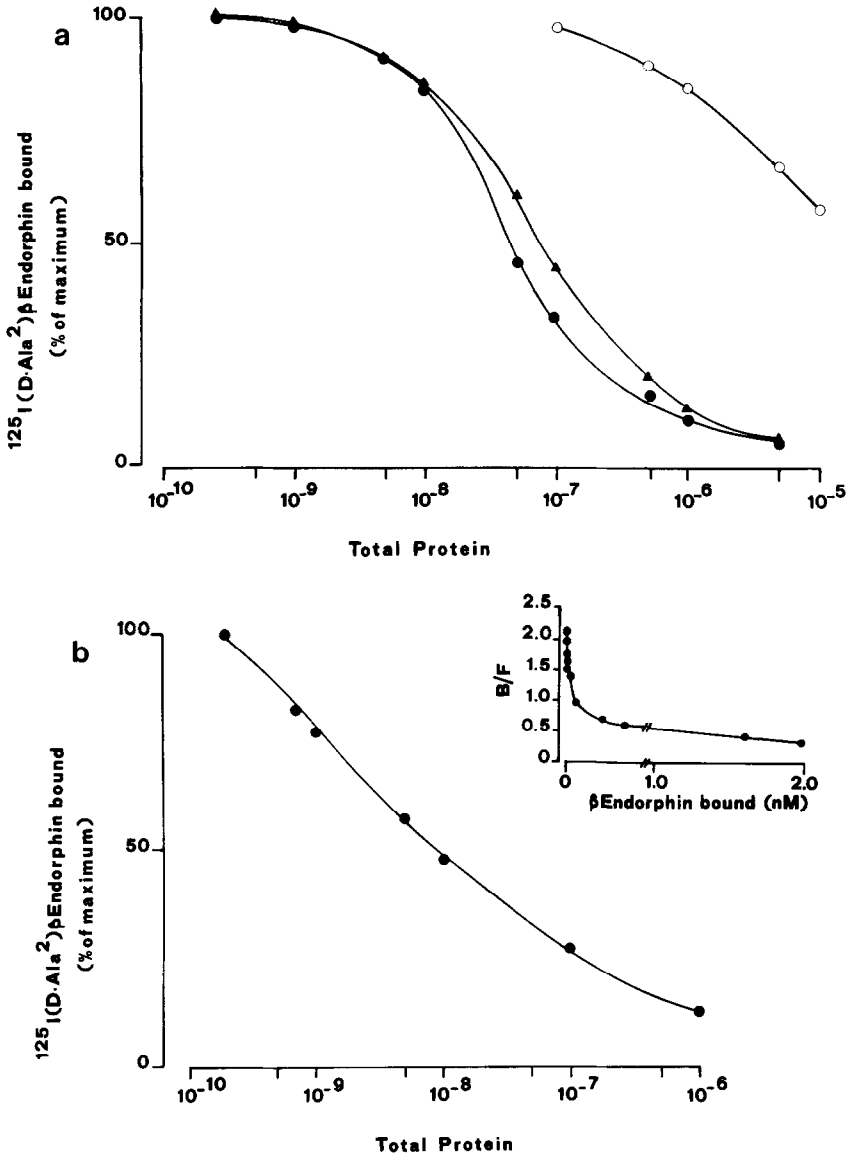

Figure 3 The competition-inhibition effect of unlabeled βEndorphin (●——●), βEndorphin(6-31) (▲——▲) and ACTH (○——○) on specific [125I]-[D-Ala^2]-βEndorphin (βEP*) binding to transformed lymphocytes (2x10^6/ml) (a) or unlabeled βEndorphin on specific βEP* binding to freshly isolated lymphocytes (2x10^7/ml) (b). Cells were incubated at 15C in the presence or absence of increasing amounts of the aforementioned compounds. Data were plotted as percentage of the specific binding obtained in the presence of βEP* at tracer concentration alone. The binding obtained in the presence of 10^{-6}M βEndorphin was considered non-specific and subtracted. Values represent means of data obtained with cells from 5 normal subjects, each point done in duplicate. Inset: Example of Scatchard plot of the βEndorphin competitive binding data.

γEP (corresponding to the N-terminal sequence 1-16 and 1-17, respectively) were not able to inhibit βEP* binding at 10^{-6}M (data not shown). On the other hand, βEP(6-31) lacking the N-terminal sequence 1-5, inhibited βEP* binding as effectively as βEP, indicating that βEP interacts with lymphocyte receptors through the central-, C-terminal fragment. These data are in agreement with results reported in RPMI 6237 cultured

lymphocytes (14), EL4 mouse thymoma cells (16), human neuroblastoma cell lines (17) and human SF 126 cells (18), and in various rat tissues (19). Moreover Naloxone, Bremazocine, Morphine and Ehtylketocyclazocine did not inhibit βEP* binding at 10^{-5}M (data not shown) confirming that both transformed lymphocytes and freshly isolated lymphocytes possess non-opioid βEP receptors.

αMSH, βMSH and γMSH at 10^{-5}M did not influence βEP* binding (data not shown). Conversely, ACTH inhibited βEP* binding at high concentration (fig.3a). We do not know the biological relevance of this result; it is, however, noteworthy that βEP and ACTH originate from the same prohormone, and that opioid and corticotrophic systems have several opposite or overlapping effects (20, 21).

In conclusion, this report describes the presence of non-opioid βEP receptors on both EBV transformed B lymphocytes and freshly isolated lymphocytes. These two models might be useful to study βEP receptors in human beings; in particular, transformed lymphocytes have been proven useful to study receptors for other hormones (22, 23) since it is possible to obtain large quantities of homogenous cells that continue to proliferate indefinitely bearing the genetic receptor pattern of the donor.

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