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β-endorphin binding in cultured adrenal cortical cells

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The polypeptide β-endorphin binds to cultured bovine adrenal cortical cells in a naloxone insensitive manner. β-endorphin and N-Acetyl-β-endorphin are equipotent in inhibiting binding. The amino terminal 27 amino acid fragment referred to as β-endorphin[1-27] shows no ability to inhibit binding, whereas the carboxy-terminal tetrapeptide Lys-Lys-Gly-Glu partially inhibits binding. ACTH, angiotensin II and met-enkephalin show little or no ability to inhibit β-endorphin binding. Competition binding reveals an apparently single affinity class with Kd of 33 nM. Molecular cross linking experiments reveal putative receptor subunits of 85 kD, 64 kD, 54 kD and 44 kD. The lower molecular weight bands are preferentially cross-linked by a hydrophobic cross linking reagent, in contrast to the two higher molecular weight bands, which are cross linked equally by hydrophobic and water soluble cross linking reagents. The β-endorphin binding characteristics of adrenal cortical cells revealed here are quite similar to those of a class of non-opioid β-endorphin receptors previously shown to exist in cells of the immune system.

Keywords: β-endorphin; adrenal; receptor

Introduction

β-endorphin, first characterized as an endogenous opioid of the central nervous system, is now considered to play a role in the functioning of the immune system as well. A variety of experimental data support this view: β-endorphin has been implicated in the stimulation of natural killer cell cytotoxicity (Mathews *et al.*, 1983; Mandler *et al.*, 1986; Fiatarone *et al.*, 1988) and in a variety of effects on mononuclear cells, including stimulation of chemotaxis (Van Epps & Saland, 1984; Ruff *et al.*, 1985), stimulation of interferon production (Brown & Van Epps, 1986), and inhibition of interleukin 1β release (Brummitt *et al.*, 1988). Injection of β-endorphin into rat cerebral ventricles induced migration of macrophage-like cells into the area (Saland *et al.*, 1983). β-endorphin stimulated the generation of cytotoxic T lymphocytes (Carr & Klimpel 1986), and enhanced the lymphocyte proliferative response (Gilman *et al.*, 1982; Kusnecov *et al.*, 1987, 1989) as well as stimulated production of IL2 (Kusnecov *et al.*, 1987; Gilmore & Weiner, 1988). β-endorphin appears to affect lymphocytes in a complex manner which may involve interactions of β-endorphin and IL-2 receptors (Ray *et al.*, 1990) and which may take various forms depending on the concentration of ligand and time of exposure (Van Den Bergh *et al.*, 1991). The role of endogenous opioids in the immune system has been reviewed by Carr (1991).

β-endorphin has been shown to bind to mu and delta type opioid receptors in the periphery (Shook *et al.*, 1988) and in the central nervous system (McLean *et al.*, 1989). The existence of a receptor for β-endorphin in cells of the immune system was first shown by Hazum *et al.* (1979) in experiments involving specific binding of radiolabeled ligand to a transformed lymphocyte cell line. Schweigerer *et al.* (1985) found specific binding of β-endorphin to EL4 thymoma cells. More recently, Shahabi *et al.* (1990a) have characterized a putative β-endorphin receptor on murine splenocytes using competition binding and molecular cross-linking studies. In another study by the same group (Shahabi *et al.*, 1990b), it was shown that β-endorphin binds specifically to a human mononuclear cell line, and that this binding shows cation and guanosine nucleotide effects.

The extensive variety of physiological effects on cells of the immune system appear to involve interactions both with classical opioid type cellular receptors, defined by the ability of antagonists such as naloxone to block β-endorphin action, as well as non-opioid type interactions, defined by the inability of antagonists such as naloxone to block activity. Interestingly, studies which have demonstrated specific binding of β-endorphin to cells of the immune system often involve the non-opioid type of interaction (Hazum *et al.*, 1979; Schweigerer *et al.*, 1985; Shahabi *et al.*, 1990a,b).

β-endorphin has been shown to bind to isolated rat liver membranes and to stimulate the adenyl cyclase-cAMP system (Dave *et al.*, 1985). In the same study, a variety of whole tissue preparations were screened for the ability to bind β-endorphin. Binding was observed in spleen, kidney, testis and adrenal, but not in ventral prostate or pancreas. This study did not attempt to distinguish whether adrenal β-endorphin binding was to medullary or cortical components.

Although β-endorphin effects are well established in the CNS and the immune system, little is known about effects in the endocrine system. There is, however, reason to suspect that such a function exists. β-endorphin infusion into human volunteers significantly suppressed the stimulation of serum cortisol by ACTH (Beyer *et al.*, 1986), without significantly affecting the stimulation of secretion of other adrenal steroids by the same ACTH infusion, thus implying the possibility that β-endorphin may act directly on the adrenal cortex. Baird *et al.* (1983) found that natural ovine β-endorphin affected steroid production in cultured human fetal adrenal cells, although this result was not

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observed to occur in the presence of synthetic porcine β-endorphin. Shanker & Sharma (1979) found that β-endorphin stimulated corticosterone production in isolated rat adrenal cells, implying a direct effect of the peptide on the adrenal cortex. Recently, Clarke *et al.* (1994) have shown that β-endorphin stimulates the secretion of the adrenal androgens dehydroepiandrosterone and androstenedione in a suspension of adult human adrenal cortical cells. Moreover, when β-endorphin and ACTH were added simultaneously, stimulation of cortisol secretion was significantly inhibited by β-endorphin, whereas androstenedione secretion was stimulated to a higher level than was induced by ACTH alone. Thus, according to these data, β-endorphin is capable of functioning as an adrenal androgen stimulating substance. Parker *et al.* (1993) have recently identified another putative adrenal androgen stimulating substance.

Although the data presented here suggest non-opioid receptor binding, the possibility that opioid type β-endorphin receptors may also exist in the adrenal cortex is suggested by the finding that met-enkephalin stimulates growth of the rat adrenal zona fasciculata (Robba *et al.*, 1986), and that naloxone stimulates steroidogenesis in isolated cells of the adrenal cortex (Lymangrover *et al.*, 1981).

In the current study, we investigated the ability of β-endorphin to bind to cultured adrenal cortex cells. We show that β-endorphin binds specifically in a naloxone insensitive manner and identify by molecular cross linking a set of putative adrenal cortical β-endorphin receptors or receptor subunits.

Results

Cultured bovine adrenal cortex cells were incubated in the presence of ^{125}I labeled β-endorphin for increasing lengths of time either in the presence (triangles) or absence (closed circles) of 2 μM unlabeled β-endorphin (Figure 1). Binding of label is suppressed by excess unlabeled peptide at each time point. Taking the binding in the presence of the unlabeled peptide to be non-specific binding, it can be seen that specific binding (open circles), reaches a plateau by about 90 min, and this plateau is maintained at least until 3 hr. Further binding experiments were therefore carried out using incubation times of 90–120 min, as indicated.

Preliminary experiments showed no significant pH effect over a range of pH 6.6 to pH 7.8, nor was there any significant calcium ion effect over the range of 1 to 10 mM, except that specific binding was approximately 10% higher at 5 mM than at 1 mM (data not shown). Binding experiments using several preparations of radioactively labeled peptide demonstrated binding which was 77–91% displaceable by 2 μM unlabeled peptide, resulting in a range of specific binding of 11.9% to 20.1% in five separate preparations (Table 1).

Since β-endorphin has been shown not only to bind to opioid type receptors but also to act on some cells of the immune system in a naloxone insensitive manner, we tested whether the binding observed here could be inhibited by naloxone. Under conditions in which β-endorphin inhibited binding by 82%, binding in the presence of 5 μM naloxone averaged 98.8 ± 2.97% SD of the binding in the absence of any inhibitor. ACTH also showed no significant inhibitory effect on β-endorphin binding (average binding 101.8 ± 0.5% of control).

Since it is generally considered that the N-terminal regions of the endorphin family confer opioid specificity and that the C-terminal extensions are involved in other types of interactions, various naturally occurring N and C-terminal derivatives of β-endorphin[1–31] were tested for the ability to inhibit binding of [^{125}I]β-endorphin[1–31] to cultured adrenal cortical cells. β-endorphin which is missing the last four carboxy terminal amino acids, β-endorphin[1–27] was unable to inhibit binding (Figure 2). This experiment was repeated using a second batch of peptide prepared by a different manufacturer, which also showed no inhibitory activity (data not shown). β-endorphin[1–27] showed no apparent inhibitory effect even at 20 μM (Figure 3). The N-terminal acetylated form of β-endorphin[1–31] was equipotent with the non-acetylated form in inhibition of binding (Figure 2). No inhibition was seen in the presence of angiotensin II or met-enkephalin (Figure 2).

The inability of the C-terminal truncated peptide to compete for binding with the full length peptide suggests that the carboxy terminus of β-endorphin must be important in the interaction with the putative receptor. To test whether the carboxy terminal region was sufficient by itself to inhibit binding of labeled β-endorphin, binding was carried out in the presence of a fragment which consists only of the carboxy-terminal tetrapeptide Lys-Lys-Gly-Glu (Figure 3). Binding was moder-

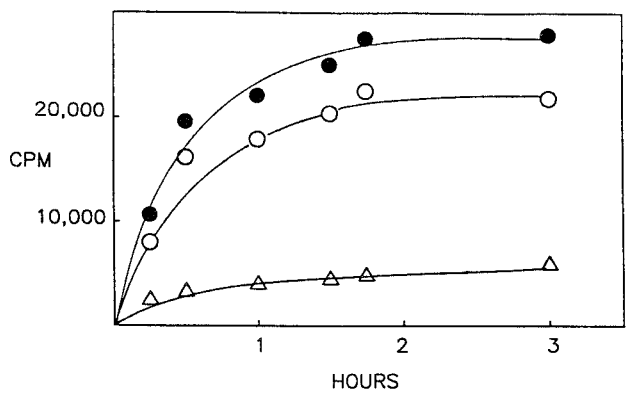

Figure 1 Bovine adrenal cortical cells in tissue culture were exposed to labeled β-endorphin for various times as described under Materials and methods. Non specific binding was determined as label bound in the presence (△) of 2 μM unlabeled peptide. Total binding (●); specific binding (○)

Table 1

Experiment	*Applied CPM*	*Bound CPM*	*% Bound*	*% Displaced*	*% Specific*
1	74 190	10 722	14.5	82.0	11.9
2	275 600	71 271	25.9	77.6	20.1
3	50 000	7 080	14.2	87.9	12.4
4	156 900	33 670	21.5	91.2	19.6
5	294 550	64 418	21.9	79.7	17.4
Average			19.6 ± 5.1	83.7 ± 5.7	16.3 ± 3.9

Five different bovine adrenal cortex cell cultures were grown up. β-endorphin was freshly radioiodinated and applied to cells as described under Materials and methods. Averages are presented ± standard deviation

ately inhibited at 2 μM and significantly inhibited at 20 μM. Thus, binding of labeled β-endorphin to cultured bovine adrenal cortical cells appears to depend critically on the C-terminal region of the molecule.

Binding of labeled β-endorphin in the presence of increasing concentrations of unlabeled peptide results in the competition curve shown in Figure 4. A major component with a Kd of approximately 33 nM is observed. Binding at 4°C for 16 h yielded a similar pattern.

In an attempt to characterize the target molecule(s) for β-endorphin binding, radioactively labeled β-endorphin was cross-linked using two varieties of homobifunctional cross linking reagent. DSS is hydrophobic and was added to the incubation medium dissolved in DMSO. BS^3, the sulfated derivative of DSS, is water soluble and was applied dissolved in aqueous buffer.

The results are shown in Figure 5. Cross-linking with BS^3 (Lane 1) or with DSS (Lane 4) yields a pattern of labeled bands which are abolished when labeling is carried out in the presence of 2 μM unlabeled peptide (Lanes 2 and 5, respectively). Binding in the presence of naloxone yields patterns which are essentially identical to those obtained in its absence (Lane 3: BS^3; Lane 6: DSS). Both BS^3 and DSS cross-link labeled β-endorphin to bands running at 85 KD and 64 KD apparent molecular weights.

Since β-endorphin is capable of binding to serum components including complement (Schweigerer *et al.*, 1983), control experiments were carried out to rule out the possibility that binding was to something other than the cultured cells. Addition of bovine serum albumen at 50 μg/ml had no detectable effect on binding efficiency (data not shown). Control bindings to medium pretreated culture wells (lanes 7–10) showed no cross-linking of significance, except for a trace band running at about 69 kD in the BS^3 cross-linked sample (Lane 9) which is not present in the patterns obtained when cells were present, and which may represent a low level of binding to a serum protein. Finally, label could be recovered from tissue scraped from culture dishes: cells were incubated in the presence of [^{125}I]β-endorphin, washed, and removed from the culture well using a teflon cell scraper. The amount of label recovered by this method ranged from 75–90% of the label which was recovered using NaOH solubilization rather than scraping (data not shown). We conclude that the binding observed in these experiments was to cells rather than to serum components bound to the culture well.

Interestingly, DSS preferentially cross-links labeled β-endorphin to bands running at 54 kD (arrow) and 44 kD. DSS lacks the sulfate groups present on BS^3 and is presumably capable of dissolving into and migrating through the lipid bilayer of the cell membrane.

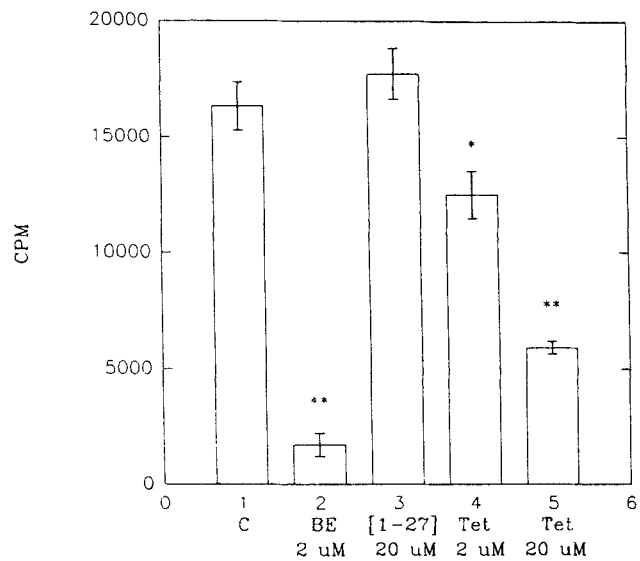

Figure 3 Binding of labeled β-endorphin was carried out in the presence of the tetrapeptide Lys-Lys-Gly-Glu, which is the C-terminal tetrapeptide of human β-endorphin. C: control. BE: β-endorphin[1–31] at 2 μM. [1–27]: β-endorphin[1–27] at 20 μM. Tet: tetrapeptide Lys-Lys-Gly-Glu at 2 μM and 20 μM shown in bars 4 and 5, respectively. $*P<0.02$; $**P<0.001$

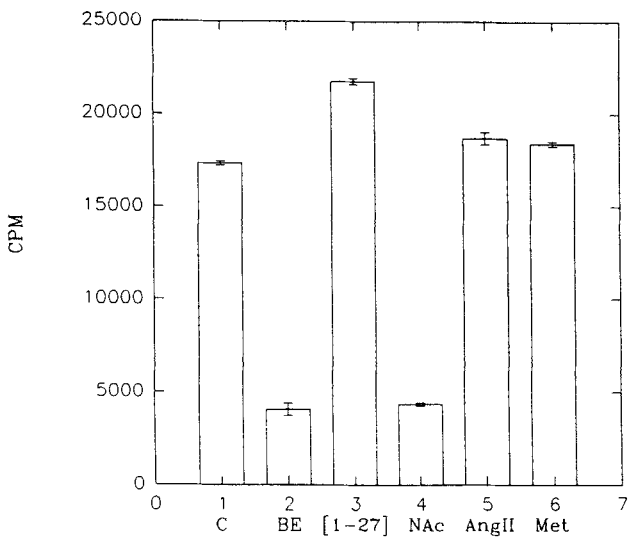

Figure 2 Binding of labeled β-endorphin was carried out in the presence of 1 μM concentrations of β-endorphin[1–31], β-endorphin[1–27], N-acetyl-β-endorphin[1–31], angiotensin II, and met-enkephalin and in the absence of added peptide, as control. C: control. BE: β-endorphin[1–31]. [1–27]: β-endorphin[1–27]. NAc: N-acetyl-β-endorphin[1–31]. AngII: angiotensin II. Met: methionine-enkephalin

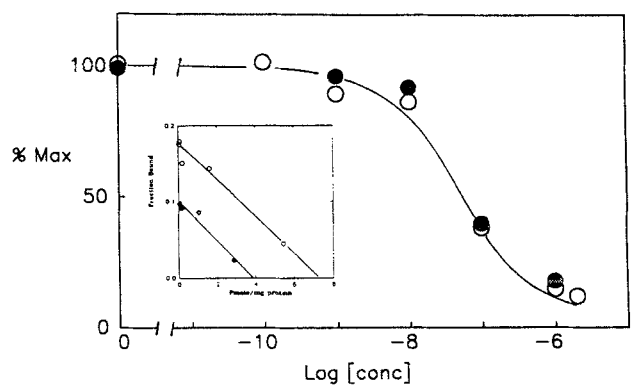

Figure 4 Competition binding of labeled β-endorphin in the presence of increasing concentrations of unlabeled peptide. Data from two separate experiments are plotted, normalized to % maximum binding. Scatchard analysis yields Kd = 33 ± 1.9 SE nM, Bmax = 5.7 ± 1.8 SE pmole/mg protein. Experiment 1: open circles: total binding 17.3% of applied label. Experiment 2: closed circles: total binding 14.5% of applied label. Inset: Scatchard plot

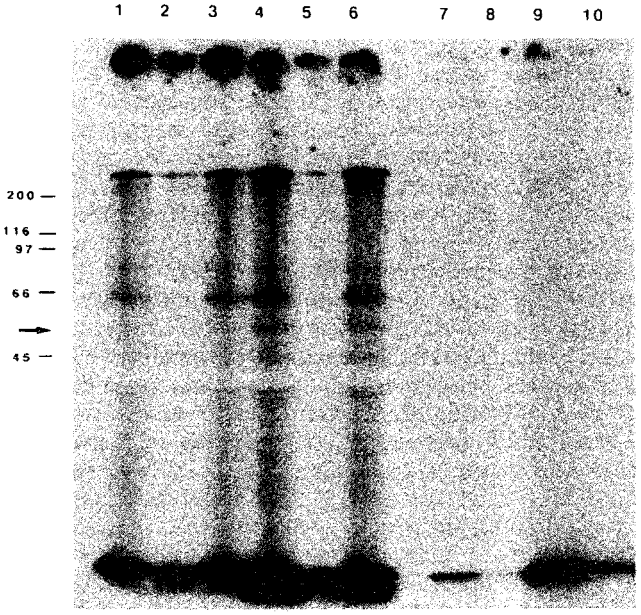

Figure 5 Cross linking of labeled β-endorphin to cultured adrenal cortical cells followed by gel electrophoresis and autoradiography for 6 weeks. BS^3 cross-linking is shown in lanes 1–3 in the presence of unlabeled β-endorphin (lane 2) or naloxone (lane 3). DSS cross-linking is shown in lanes 4–6 with unlabeled β-endorphin (lane 5) or naloxone (lane 6). Lanes 7–10: binding of labeled β-endorphin to culture plate pretreated with culture medium – Lanes 7 and 8 are DSS cross-linked in the absence or presence of unlabeled β-endorphin, respectively; Lanes 9 and 10 are the analogous experiments using BS^3 cross-linking

Thus, the preferential cross-linking of the 54 and 44 kD peptides may represent an intramembrane location for the cross-link targets on these molecules.

Discussion

We have identified a set of molecules which bind β-endorphin in a naloxone insensitive manner. Gel electrophoresis of β-endorphin target molecules cross-linked to radioactively labeled ligand reveals a set of bands which presumably represent the polypeptide subunits of one or more of these targets or receptors. The use of water soluble and insoluble cross-linking reagents reveals some target molecules which are recovered at about equal levels (85 kD and 64 kD) and some which are more strongly labeled in the presence of the insoluble cross linker DSS (54 kD and 44 kD). Since DSS is thought to function by dissolving into the cell membrane lipid bilayer, it may be that these bands represent polypeptides which contain membrane domains.

Binding of β-endorphin to adrenal cortical cells was not substantially affected by N-terminal acetylation, but was abolished by removal of the C-terminal tetrapeptide. Thus, the properties of β-endorphin binding shown for this system differ substantially from those of central nervous system opioid type β-endorphin receptors (Akil *et al.*, 1981), in which N-terminal acetylation abolishes activity, but C-terminal truncation is only partially inhibitory. These data, taken together with the observed insensitivity of binding to naloxone, suggest the existence of non-opioid β-endorphin receptors on cells of the adrenal cortex. The ability of the C-terminal tetrapeptide Lys-Lys-Gly-Glu to inhibit binding also suggests the non-opioid nature of adrenal β-endorphin binding. This same tetrapeptide activity has been shown previously in some cases of immune cell β-endorphin binding (Shahabi *et al.*, 1990a,b). It is of interest that the C-terminal tetrapeptide of β-endorphin has previously been shown to be the active factor in melanotropin potentiating activity in a lizard skin assay (Carter *et al.*, 1979), reinforcing the view that the amino and carboxy regions of the β-endorphin molecule can elicit different physiological responses depending on the target tissue.

β-endorphin is highly conserved, showing 94% amino acid sequence homology, between human and mouse, and 81% homology between human and Xenopus (Martens, 1986). In addition to its well known role as an endogenous opioid in the central nervous system, it is also produced along with ACTH in the pituitary in response to stress as part of the metabolism of the precursor peptide pro-opiomelanocortin. More recent data also demonstrate β-endorphin production by lymphocytes at sites of inflammation (Przewlocki *et al.*, 1992).

As described above, Dave *et al.* (1985) have shown that β-endorphin can bind to liver membrane, eliciting stimulation of the adenyl cyclase system. In that same study, it was shown that β-endorphin can bind to extracts of whole adrenal. However, little or no published data currently exist regarding specific binding of β-endorphin to adrenal cortical cells. In this study, we have shown that β-endorphin binds specifically to adrenal cortex cells in culture and that radioactively labeled β-endorphin can be cross-linked to a distinct set of putative target molecules on these cells. Using similar techniques to those described in the current study, we have recently completed studies showing that β-endorphin binds specifically to cultured mouse macrophages and that this binding shows many of the same characteristics described here for adrenal cortical cells, and by several other groups for other cells of the immune system (Gelfand *et al.*, in press).

In studies on thymocytes, splenocytes, and monocytes (Schweiger *et al.*, 1985; Shahabi *et al.*, 1990a,b), β-endorphin binding in the immune system was characterized. The results of those studies show remarkable similarities when compared with the results reported here for cells of the adrenal cortex. Immune cell receptors or receptor subunits were reported to exist in 66 kD, 59 kD and 44 kD forms, which are similar to the sizes reported here (85 kD, 64 kD, 54 kD, 44 kD). Likewise, binding was found to be insensitive to naloxone and to the carboxy truncated peptide β-endorphin[1–27] (Schweigerer *et al.*, 1985; Shahabi *et al.*, 1990a,b), but was partially sensitive to the carboxy terminus tetrapeptide (Shahabi *et al.*, 1990a,b). Finally, competition binding yielded Kd values ranging from 4 to 65 mM, the range in which β-endorphin binding to adrenal cortical cells was observed in these studies. The data described here indicate the existence of β-endorphin receptors which therefore may be related to the corresponding immune system receptors.

Little is known regarding the physiological role of β-endorphin in adrenal cortical functioning. One pre-

vious study from this laboratory demonstrated that β-endorphin can partially inhibit the steroidogenic effect of ACTH (Beyer *et al.*, 1986), and Shanker & Sharma (1979) reported a stimulatory effect of β-endorphin on steroidogenesis in cultured adrenal cortical cells. In the current study, we found no effect of ACTH on β-endorphin binding, implying that the physiological role played by β-endorphin in steroidogenesis, if any, is not mediated by competition of ACTH and β-endorphin for common receptors. This is in contrast to the speculation by Shanker & Sharma (1979) for a common receptor, based on lack of additivity when ACTH and β-endorphin were added together to cultured cells. Eggens *et al* (1989) found that high levels of β-endorphin stimulated the secretion of cortisol, but not aldosterone, in human adrenocortical cells obtained from cadaver kidney donors.

Recently, Clarke *et al.* (1994) have shown that β-endorphin is capable of stimulating the secretion of the adrenal androgens androstenedione and dehydroepiandrosterone in human adrenal cells. Moreover, β-endorphin partially inhibited the ACTH-stimulated increase in cortisol secretion. Taken together, these studies suggest that β-endorphin may play a role in the regulation of adrenocortical steroid synthesis.

Recent findings suggest that β-endorphin is made in a number of different cell types including those of the immune system and adrenal medulla, in addition to the well known pituitary and central nervous system sources. If the binding affinity described here reflects the true magnitude *in vivo*, then it seems unlikely that the adrenal cortex could be much affected by circulating β-endorphin of pituitary origin, since serum levels are less than nanomolar (Facchinetti *et al.*, 1983; Genazzini *et al.*, 1983). Moreover, the circulatory anatomy of the adrenal gland in humans does not favor the direct transport of β-endorphin from the medulla to the cortex, but, rather, the reverse. Nevertheless, indirect evidence exists for medulla-to-cortex transport of material. Hinson (1990) has suggested that such a pathway exists, and hypothesizes that the pathway involves neural innervation of the adrenal cortex. If indeed such a pathway exists, it could result in the accumulation of β-endorphin in the adrenal cortex at concentrations much higher than found in the circulation. Another possible mechanism for accumulation of β-endorphin in the cortex may involve the observation that chromaffin cells, typical of the adrenal medulla, have been found in the adrenal cortex (Hinson 1990). Finally, macrophages have been shown to exist in the adrenal cortex by immunohistochemical techniques (Hume *et al.*, 1984) and the ability of macrophages as well as other cells of the immune system to produce β-endorphin has been suggested (Harbour *et al.*, 1987; Przewlocki *et al.*, 1992). Alternatively, Stolz *et al.* (1992) have suggested that under physiological conditions, positively charged peptides such as ACTH and β-endorphin are concentrated in the region next to the adrenal cell membrane by electrostatic interactions, both as a function of the net positive charge on the peptide and of the strength of the negative charge at the cell surface. Although β-endorphin is not as strongly charged as ACTH[1–24] used in that study (Stolz *et al.*, 1990), it contains 5 positively charged Lys residues, compared with three other negatively charged Glu residues, for an expected net positive charge. By this same argument, the ability of the tetrapeptide Lys-Lys-Gly-Glu to inhibit β-endorphin binding might be potentiated by the net positive charge of this fragment at physiological pH. In any case, it is of interest that the steroidogenic activity of β-endorphin in cultured adrenal cells shown by Shanker & Sharma (1979) occurred over a range of concentrations similar to that observed in the binding experiments described here: the half-maximal stimulation was observed in those experiments at approximately 40 nM.

It should be pointed out that the argument regarding circulating levels of pituitary derived β-endorphin also applies to cells of the immune system. Nevertheless, these cells demonstrate a multitude of physiological responses to β-endorphin mediated by opioid and non-opioid receptor types. It is therefore possible that β-endorphin effects in immune cells are due to β-endorphin of non-pituitary origin, and we may speculate that the same holds for adrenal cortical cells.

In summary, the present study is consistent with an intriguing hypothesis. β-endorphin may be considered to be an informational molecule used by several organ systems, including the adrenal cortex. Blalock (1989) has suggested that the immune, endocrine and central nervous systems communicate with each other at the molecular level using a shared set of informational molecules which include amoung others the enkephalins, endorphins and ACTH. β-endorphin is known to bind to opiate type receptors in the central nervous system and has more recently been found to interact with cells of the immune system by both opioid and non-opioid mechanisms. The earlier paradigm for the stress response involving the hypothalamo-pituitary-adrenal cortical axis secreting CRH, ACTH and finally cortisol has therefore given way to a model revealing more complex interactions which include cells of the immune system in addition to the endocrine system.

Materials and methods

β-Endorphin [1–31], the C-terminal truncated form β-endorphin [1–27], and the N-terminal acetylated form N-Ac-β-endorphin [1–31] were obtained from Sigma Chemical Company. The lactoperoxidase iodinating reagent Enzymobeads were obtained from BioRad (Burlingame, Ca). The cross linking reagents disuccinimidyl suberate (DSS) and bis-(sulfosuccinimidyl) suberate (BS^3) were obtained from Pierce (Rockford, Il). Na[^{125}I] was from Dupont-NEN. Tissue culture medium was obtained from Gibco: Dulbecco's Modified Eagle's Medium, mixed 1:1 with Ham's F12 (DMEM:F12). Fetal bovine serum was obtained from Gemini Biotech (Calabasas, Ca), collagenase P was obtained from Boehringer (Indianapolis, In), and DNase and bovine serum albumen (BSA) were purchased from Sigma. Gel electrophoresis reagents were obtained from BioRad and used according to the manufacturer's recommendations.

β-Endorphin was radioiodinated using Enzymobeads according to the manufacturer's instructions. Label was purified by reverse phase HPLC through a Beckman C18 column, using an 8–80% acetonitrile gradient in 0.1% trifluoroacetic acid. A strongly labeled peak was observed at approximately 38% ACN and was used in binding experiments.

Bovine adrenals from animals less than 2 years of age were obtained from a local slaughterhouse and transported to the

laboratory at 0°C. Two adrenal glands were trimmed of adherent fat, soaked in 10% aqueous iodine for 1–2 min to destroy contaminating microorganisms, blotted dry, and sectioned lengthwise. Medullary tissue was removed using curved scissors, and cortical tissue was gently scraped off using a number 22 scalpel blade. The tissue was disaggregated by incubation for 90 min at 37°C in 1:1 DMEM:F12 medium (Gibco) containing 0.01 volume Gibco antibiotic-antimycotic solution, 1 mg/ml collagenase, 2 mg/ml BSA, and 0.05 mg/ml DNAse. The enzyme treatment was carried out in a volume of 50 ml in a 250 ml erlenmeyer flask on a rotary shaking water bath at 110 RPM. Following this treatment, the cell suspension was transferred to a 50 ml plastic conical tube and large undigested fragments were allowed to settle for 1 min. The remainder was centrifuged for 10 min at 1000 RPM. Cell pellets were resuspended in complete medium (DMEM:F12, 0.01 volume of Gibco antibiotic/antimycotic solution, 0.1 volume fetal bovine serum) and plated on 100 mm Falcon Primeria tissue culture plates (six plates total) which had been precoated for 30 to 60 min with 1 μg/cm^2 human plasma fibronectin dissolved in DMEM:F12 medium. Cells were routinely passaged for three to four passages onto uncoated Primeria plates or to Falcon polystyrene six or 12 well tissue culture plates. Cells in culture showed homogeneous morphology similar to that of the adrenal cortical cells described by Hornsby and Gill (1978), and continued to produce the adrenal steroids dehydroepiandrosterone and androstenedione for at least four passages, as demonstrated by radioimmunoassay. The oil-red-O stain indicative of lipid containing cells was used to rule out any substantial contamination by other cell types. Cells showed numerous strongly staining bodies which were similar in appearance but on the average smaller than stained bodies in similarly prepared human adrenal cells. No cells lacking oil-red-O staining bodies were observed in the bovine tissue cultures used in these studies.

Binding studies were carried out as follows. Tissue culture 12 well plates containing confluent cell cultures in third or fourth passage were used. Except where indicated, all steps were carried out at 20°C. Each well was washed twice with HEPES-Saline (20 mM HEPES, pH 7.4, 0.9% NaCl, 5 mM $CaCl_2$) by sequentially aspirating the contents of the well, gently pipeting the wash buffer, and aspirating the wash buffer. To each well, 485 μl of incubation buffer (HEPES-Saline containing 1 μg/ml aprotinin and leupeptin and 1 mM PMSF) was added, followed by 5–10 μl of non-radioactive competitive inhibitor or buffer, followed by 10 μl of radioactively labeled hormone. Following incubation, the incubation solution was aspirated off the cells, and each well was washed three times with 1 ml HEPES-Saline. Radioactivity was released by incubating the contents of each well with 0.5 ml 1 N NaOH at 37°C for 20–30 min. The contents of each well was removed, combined with an additional 0.5 ml wash of the respective well, and counted in an Isodata 10 well gamma counter. Competition binding results were analysed by the computer program Ligand (Munson & Rodbard, 1980) using a one site model.

For chemical cross linking experiments, cells were labeled and washed by the same procedure, then subjected to 30 min incubation in either 1 μM DSS or 0.1 μM BS^3 in HEPES-Saline followed by two washes with HEPES-Saline. Cells were dissolved in gel application buffer (10 mM Tris pH 6.8, 5 mM dithiothreitol, 0.1% SDS, 10% glycerol, 0.05% bromophenol blue), heated at 95–100°C for 5 min, cooled to room temp, and electrophoresed by the method of Laemmli (1970) through a 10% polyacrylamide gel containing a 4% stacking gel. BioRad high molecular weight protein markers were run in parallel with the sample lanes. Gels were fixed and stained for 1 h with 40% methanol, 10% acetic acid containing 0.1% coomassie Blue, followed by extensive destaining in the same buffer absent the dye. Gels were reswelled for 30 min in 10% ethanol and dried on a BioRad gel dryer at 80°C for 2 h. Radioactively labeled bands were revealed by autoradiography for 6 weeks at −70°C using Kodak Xomat film and a Dupont Cronex intensification screen.

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