Specific Binding of β-Endorphin to the Isolated Renal Basolateral Membranes *in Vitro*

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Binding of human β-endorphin (β-EP) to rat renal basolateral membranes was characterized using [^{125}I]Tyr^{27}-β-EP ([^{125}I]β-EP) as a primary ligand. Ten millimolar of ethylenediaminetetra acetic acid (EDTA) completely inhibited the degradation of [^{125}I]β-EP in the incubation mixture at 4 °C, thus making it possible to quantitatively examine the [^{125}I]β-EP binding. The specific binding of [^{125}I]β-EP to the basolateral membranes was reversible and saturable, and a nonlinear least-squares regression analysis of a saturation isotherm revealed two different classes of specific binding sites. One class had an apparent dissociation constant (*Kd*) of 0.68 nM and a lower number of binding sites (33 fmol/mg protein), whereas the other class had a lower affinity (apparent *Kd* of 210 nM) and a higher number of binding sites (7.3 pmol/mg protein). Inhibition of the [^{125}I]β-EP binding by naloxone (10 μM) was approximately only 20%, and that by D-Ala^2–D-Leu^5–enkephalin (10 μM) was null, suggesting the major role of a non-opioid binding component in specific [^{125}I]β-EP binding to basolateral membranes. Moreover, a 50% inhibition by 10 μM of dynorphin(1—13) suggests that a certain region of the primary structure of β-EP, excluding at least the NH_2-terminal enkephalin sequence, is of particular importance for the [^{125}I]β-EP binding. These lines of evidence suggest the existence of two different classes of specific binding sites for β-EP on the renal basolateral membranes, and the high- and low-affinity bindings may be attributed to opioid and non-opioid receptors, respectively, as judged by known characteristics of opioid and non-opioid receptors in other peripheral tissues.

Keywords β-endorphin; renal basolateral membrane; opioid receptor; non-opioid receptor

Introduction

β-Endorphin (β-EP) is a 31-residue endogenous peptide with potent opioid properties.[1] Since the specific binding sites of β-EP have been found not only in the brain but also in several peripheral tissues,[2-6] increasing attention has been paid to peripheral endorphin actions *via* direct interactions. Recent studies have revealed that peripheral specific binding and actions of β-EP have been characterized as non-opioid in nature.[3,4,7-10] Dave *et al.*[4] suggested the existence of two different classes of binding sites of ^{125}I-labeled acetyl-human β-EP on crude membranes prepared from homogenates of several peripheral tissues, such as the kidney.

In the kidney, specific receptors for various polypeptide hormones have been identified on the basolateral membranes,[11-14] and their physiological roles have been implicated in relation to hormone levels in systemic circulation.[15,16] However, whether there are specific binding sites of β-EP on the basolateral side of renal tubule cells remains to be known at present, although β-EP was reported as a kidney trophic hormone.[17] Our recent study[2] using an *in vivo* labeling technique demonstrated the specific binding of intravenously administered [^{125}I]β-EP to the lung and liver of the rat under physiological conditions, but failed to significantly show specific [^{125}I]β-EP binding to the kidney, possibly due to a high degree of nonspecific binding and/or reabsorption of the peptide *via* the filtration-luminal uptake route.[18] Therefore, in this study, we examined whether specific binding sites of β-EP exist on the isolated basolateral membranes by *in vitro* binding experiments.

Experimental

Chemicals (3-[^{125}I]Iodotyrosyl^{27})human-β-endorphin (*ca.* 2000 Ci/mmol, designated as [^{125}I]β-EP) and (3-[^{125}I]iodotyrosyl^{A14})human-insulin (*ca.* 2000 Ci/mmol, designated as [^{125}I]insulin) were purchased from Amersham International Ltd. (Buckinghamshire, UK) and trichloroacetic acid (TCA), *N*-ethylmaleimide (NEM) and ethylenediaminetetraacetic acid (EDTA) dipotassium salt from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Unlabeled β-EP, naloxone, D-Ala^2–D-Leu^5–enkephalin (DADLE), bovine serum albumin (BSA, fraction V), bestatin and bacitracin were obtained from Sigma Chemical Co. (St. Louis, MO), dynorphin(1—13) from Peptide Institute Inc. (Osaka, Japan), and polyethyleneimine (molecular weight 60000—80000) from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents were those commercially available and of an analytical grade. The peptidase inhibitors (EDTA, bestatin, bacitracin and NEM) and unlabeled opioid ligands (β-EP, naloxone, dynorphin(1—13) and DADLE) were dissolved in a 25 mM Tris–HCl buffer (pH 7.4) containing 0.1% BSA. The monoiodinated β-EP was dissolved in phosphate buffered saline (PBS) containing 0.1% BSA and stored at −20 °C until the time of the study. The labeled β-EP used was at least 98% pure as assayed by the TCA-precipitation method.[19] Distilled, deionized water was used throughout the experiments.

Preparation of Basolateral Membranes Basolateral membranes were isolated from the renal cortex of male Wistar rats (200—300 g, Sankyo Laboratory Co., Toyama, Japan) by the method of Percoll density gradient centrifugation.[20] The obtained membranes were finally suspended in a 25 mM Tris–HCl buffer (pH 7.4) containing 300 mM mannitol, and stored in a small volume at −70 °C until the time of use.

The purity of the basolateral membranes was assessed by determining the activities of marker enzymes. Moreover, in order to confirm whether hormone receptors remained active on the basolateral membrane preparations obtained, the specific binding of [^{125}I]insulin in the basolateral membrane suspension was examined in the presence of a peptidase inhibitor, NEM (1 mM), as previously described.[11]

β-Endorphin Binding Assays For the binding assays of [^{125}I]β-EP, basolateral membrane fractions were thawed and diluted in a 25 mM Tris–HCl buffer (pH 7.4) containing 300 mM mannitol to give a protein concentration of approximately 5 mg/ml. Forty microliters of the membrane suspension were mixed with 80 μl of 25 mM Tris–HCl buffer (pH 7.4) containing 0.125% BSA and 12.5 mM EDTA (designated as incubation buffer; EDTA was not included in case the degradation of [^{125}I]β-EP was assessed) with and without an unlabeled opioid ligand. Then 80 μl of [^{125}I]β-EP (0.04 μCi, 0.1 nM in a final concentration) dissolved in the incubation buffer was added, and allowed to incubate at 4 °C or 25 °C in a final volume of 200 μl for 90 min (except when equilibration time of [^{125}I]β-EP binding was examined). The incubation was terminated by filtrating the assay mixture under low vacuum through Whatman GF/C filters pre-soaked with ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% BSA and 0.1% polyethyleneimine (designated as presoaking buffer). The filters were then washed twice with an additional 5 ml of ice-cold 25 mM Tris–HCl buffer (pH 7.4) containing 0.1% BSA. The ^{125}I-radioactivity on the filters was determined in a γ-counter (model ARC-605, Aloka Co., Tokyo, Japan).

In order to assess the degradation of [^{125}I]β-EP during the binding

assay experiments, incubation was performed at 4 °C or 25 °C in the presence and absence of peptidase inhibitors (*i.e.*, EDTA, bestatin, bacitracin and NEM). At designated times, the incubation mixtures (200 μl) were added to 200 μl of a 15% TCA aqueous solution containing 1% BSA (as a precipitation carrier), mixed well, and the total radioactivity was counted in a γ-counter. The mixtures were then rapidly filtered under low vacuum through a GF/C filter, and the radioactivity on the filters was measured as TCA-insoluble radioactivity. Thus, the percentage of unchanged [^{125}I]β-EP in the incubation medium was estimated from TCA-insolubility calculated as follows:

$$\text{TCA-insolubility (\%)} = (\text{TCA insoluble cpm})/(\text{total cpm}) \times 100 \quad (1)$$

To demonstrate reversibility of the [^{125}I]β-EP binding, basolateral membranes were incubated with 0.1 nM [^{125}I]β-EP at 4 °C for 30 min; thereafter, a high concentration (10 μM final) of unlabeled β-EP was added to the incubation mixture and total binding was determined at serial time intervals after β-EP addition.

In saturation experiments, nonspecific binding of [^{125}I]β-EP was defined as the amount (fmol per mg protein) bound to basolateral membranes which was not inhibited by 10 μM of unlabeled β-EP. Specific binding was, therefore, defined as the amount of total binding minus nonspecific binding.

Analytical Methods Enzyme activities for Na^+, K^+-ATPase, alkaline phosphatase, γ-glutamyltransferase, acid phosphatase, succinate dehydrogenase and lactate dehydrogenase were measured by the methods of Scharschmidt *et al.*,[21] Walter and Schutt,[22] Orlowski and Meister,[23] Rothstein and Blum,[24] King[25] and Scalera *et al.*,[26] respectively. Protein concentration was determined by the method of Bradford,[27] using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) with BSA as a standard.

Data Analysis Data from saturation experiments were analyzed using the following equation:

$$C_b/P = B_{max,1} \cdot C_f/(K_{d,1} + C_f) + B_{max,2} \cdot C_f/(K_{d,2} + C_f) \quad (2)$$

where P represents the protein concentration (mg/ml); C_b and C_f are the specifically bound and unbound concentrations (nM) of β-EP in the assay mixtures, respectively; $B_{max,1}$ and $B_{max,2}$ are the binding capacities (pmol/mg protein) on basolateral membranes at sites 1 and 2, respectively; and $K_{d,1}$ and $K_{d,2}$ are the affinity constants (nM) at sites 1 and 2, respectively. The left-side hand represents the amount of bound β-EP (pmol) per mg protein of the incubation mixture, which was measured in the above-mentioned binding experiments. Thus, the C_f *vs.* C_b/P curve was fitted to Eq. 2 to estimate the values of $B_{max,1}$, $K_{d,1}$, $B_{max,2}$ and $K_{d,2}$, using an iterative nonlinear least-squares regression curve-fitting program, MULTI.[28] Moreover, data from the saturation experiments were expressed in the Scatchard plots using the following linear equation:

$$C_b/P/C_f = -(C_b/P - B_{max,i})/K_{d,i} \quad (3)$$

where $B_{max,i}$ and $K_{d,i}$ denote the binding kinetic parameters for the i-th binding site.

Results and Discussion

Previous *in vitro* studies have identified opioid receptors in the kidney,[4,29,30] but have not yet provided evidence for the presence of opioid receptors on the basolateral membranes. Therefore, the present study was designed to demonstrate the specific binding of β-EP to the basolateral membranes prepared from the rat renal cortex. The degree of purification of basolateral membranes was determined by measurement of the specific activities of several marker enzymes: Na^+, K^+-ATPase, the marker enzyme for basolateral membranes, was enriched 19.3±0.7 folds (mean±S.E.M.; $n=12$) with respect to the initial homogenate. On the other hand, alkaline phosphatase and γ-glutamyltransferase, the marker enzymes for the brush-border membranes, were enriched only 1.82±0.16 (mean±S.E.M.; $n=12$) and 1.59±0.12 folds ($n=4$), respectively. Contamination of acid phosphatase derived from lysosomes, succinate dehydrogenase from mitochondria and lactate dehydrogenase from cytoplasm was small, as judged by their enrichment factors of 2.22±0.16, 0.318±0.074 and 0.067±0.005 (mean±S.E.M.; $n=3$), respectively. These enrichment factors of marker enzymes indicate that the isolated membrane fraction was properly purified and sufficiently rich in the basolateral membrane. Moreover, the isolated basolateral membrane fraction exhibited a significant specific binding activity for [^{125}I]insulin (result not shown), and the presence of insulin receptors on the membranes was confirmed. Therefore, the obtained membrane fraction was considered to be adequate for the investigation of opioid receptors at the basolateral side of renal tubule cells *in vitro*.

In preliminary experiments of β-EP binding assays, we attempted to minimize the adsorption of [^{125}I]β-EP to GF/C filters without basolateral membranes in assay tubes. In the absence of BSA and polyethyleneimine in the presoaking buffer, the percentage of adsorbed [^{125}I]β-EP to the filters was as high as 71.1±1.3% (mean±S.E.M.; $n=3$) of the total amount of β-EP applied. By contrast, when 0.1% BSA and 0.1% polyethyleneimine were added to the presoaking buffer, the percentages of adsorbed [^{125}I]β-EP were only 1.6±0.3% and 7.2±2.8% (mean±S.E.M.; $n=3$), respectively. Therefore, both 0.1% BSA and 0.1% polyethyleneimine were routinely included in the presoaking buffer thereafter.

In degradation experiments, we determined the optimal conditions required to preserve the stability of [^{125}I]β-EP during the incubation necessary for binding studies. Figure 1 presents the degradation of [^{125}I]β-EP in the incubation mixtures under different conditions. Upon exposure to renal basolateral membranes (1 mg protein/ml) at 4 °C, [^{125}I]β-EP rapidly degraded in the absence of peptidase inhibitors. However, in the presence of either EDTA (10 mM) or other peptidase inhibitors (bestatin 0.1 mM, bacitracin 50 μM and NEM 1 mM), the degradation was markedly reduced. In particular, 10 mM EDTA could completely inhibit the degradation of [^{125}I]β-EP at 4 °C, whereas its inhibitory effect was somewhat diminished at 25 °C (Fig. 1). Therefore, we routinely added 10 mM EDTA in the assay mixture to avoid the degradation of [^{125}I]β-EP during the binding assays at 4 °C. These results suggest that metallopeptidases are involved, at least in part, in the [^{125}I]β-EP degradation in the basolateral membrane suspensions. In the brain, the inactivation of the enkephalins has been suggested

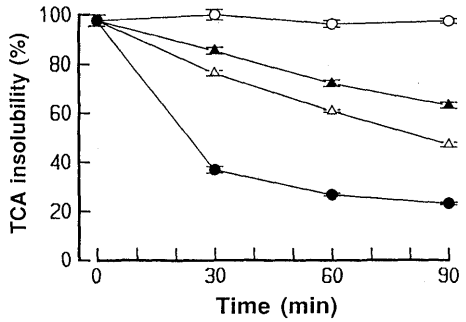

Fig. 1. Time Courses of TCA-Insolubility (%) of [^{125}I]β-EP during Incubation with Rat Renal Basolateral Membranes

[^{125}I]β-EP (0.1 nM) incubated with basolateral membranes (1 mg/ml) for designated time intervals was mixed with 15% TCA, filtered through GF/C filters, and the TCA-insoluble radioactivity on the filter was counted. Each point and vertical bar represent the mean±S.E.M. ($n=4$). ●, at 4 °C without peptidase inhibitors; Δ, at 4 °C with bestatin 0.1 mM, bacitracin 50 μM and NEM 1 mM; ▲, at 25 °C with EDTA 10 mM; ○, at 4 °C with EDTA 10 mM.

to proceed *via* the action of two membrane-bound peptidases[31a,b]; one of them is an aminopeptidase characterized by its sensitivity to inhibition by bestatin. The second peptidase, originally given the name "enkephalinase," has been shown to be a neutral metallopeptidase with specificity directed toward cleavage on the amino side of hydrophobic amino acids. The latter enzyme has been shown to be abundantly distributed in the kidney and lung, which is consistent with the present results of rapid [^{125}I]β-EP degradation at a neutral pH at 4 °C.

As illustrated in Fig. 2, the amount of [^{125}I]β-EP that binds to basolateral membranes varied as a function of the protein concentration. With a protein concentration between 0.2 and 1.5 mg/ml, a linear increase in total and nonspecific binding of [^{125}I]β-EP (0.1 nM) was noted ($r=0.99$). Therefore, the protein concentration of 1.0 mg/ml was routinely used in the subsequent binding assays.

The binding of [^{125}I]β-EP was also dependent on the time of incubation (Fig. 3A), although its association process was relatively rapid even at 4 °C. In contrast, at 25 °C, the [^{125}I]β-EP binding reached a maximum at 5 min and gradually decreased with time, due to the degradation of [^{125}I]β-EP during incubation. As shown in Fig. 3B, an excess of unlabeled β-EP rapidly displaced [^{125}I]β-EP from the putative binding sites, while no change was observed after the addition of a vehicle only (without unlabeled β-EP). Since there appeared to be two binding compartments from which [^{125}I]β-EP was dissociable at different rates, the data presented in Fig. 3B were analyzed by a nonlinear least-squares regression analysis[28] using the following equation:

$$(\%\text{ of bound }[^{125}\text{I}]\beta\text{-EP}) = a_1 \cdot \exp(-k_1 t) + a_2 \cdot \exp(-k_2 t) \quad (4)$$

where k_1 and k_2 denote first-order dissociation rate constants, and a_1 and a_2 represent the relative percentages of the two compartments such that $a_1 + a_2 = 100$. Dissociation after 30 min binding leads to k_1 and k_2 values of 1.48 min^{-1} and 0.0165 min^{-1}, respectively, with a_1 and a_2 being 30.4% and 69.6%, respectively. Obviously, the simulation curve (Fig. 3B) was in good agreement with the observed data, suggesting the feasibility of a two-compartment model of ligand dissociation.

Figure 4A shows the specific binding of [^{125}I]β-EP (0.1 nM) to the renal basolateral membranes, as well as the inhibition by various concentrations of unlabeled β-EP in a series of ten saturation experiments. Since the inhibition curve was biphasic, there appeared to be two binding sites on basolateral membranes, which show high and low

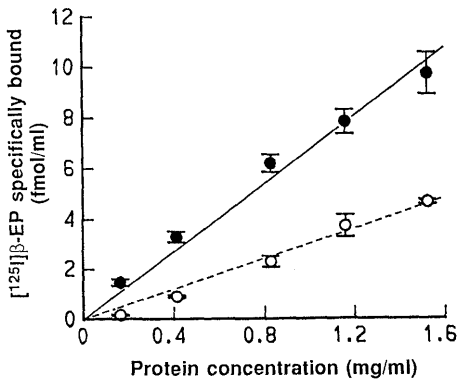

Fig. 2. Total and Nonspecific Bindings of [^{125}I]β-EP as a Function of Rat Renal Basolateral Membrane Concentration

Protein concentrations between 0.18 and 1.5 mg/ml were incubated with [^{125}I]β-EP (0.1 nM) for 90 min at 4 °C in the presence of 10 mM EDTA. Nonspecific binding was determined in the presence of 10 μM unlabeled β-EP. Each point and vertical bar represent the mean ± S.E.M. ($n=3$—4). —●—, total binding; – –○– –, nonspecific binding.

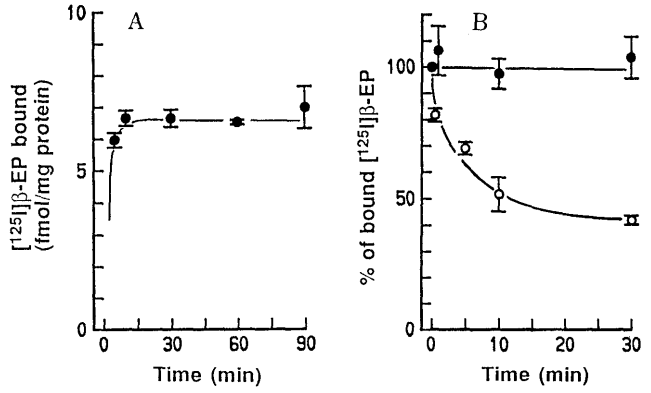

Fig. 3. Time Courses of Association (A) and Dissociation (B) of [^{125}I]β-EP to Rat Renal Basolateral Membranes

In panel B, dissociation of [^{125}I]β-EP (0.1 nM), which was bound to basolateral membranes (1 mg/ml) for 30 min in the presence of 10 mM EDTA, was examined after dilution with (○) and without (●) unlabeled β-EP (10 μM), and the dissociation curve for the displacement of [^{125}I]β-EP by unlabeled β-EP was drawn by a nonlinear least-squares regression analysis[28] according to Eq. 3.

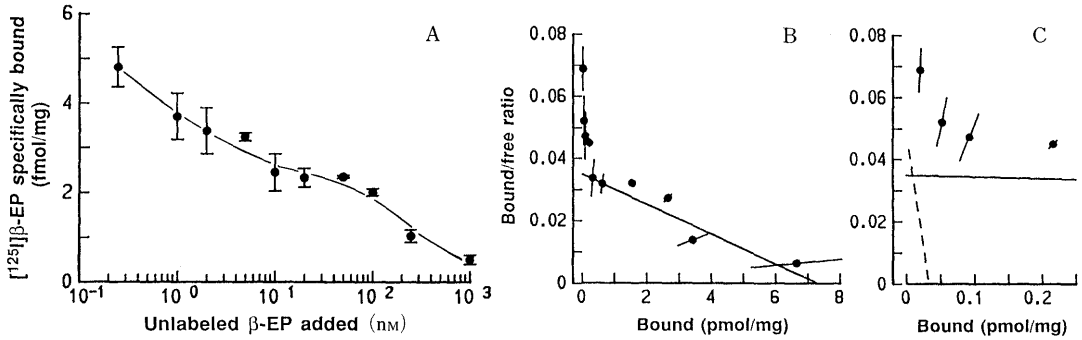

Fig. 4. Inhibition of Specific Binding of [^{125}I]β-EP to Rat Renal Basolateral Membranes by Various Concentrations of Unlabeled β-EP (A) and Scatchard Plots for [^{125}I]β-EP Binding (B, C)

[^{125}I]β-EP (0.1 nM) was incubated with basolateral membranes (1 mg/ml) at 4 °C for 90 min in the presence of 10 mM EDTA. In panel A, the inhibition curve was drawn by eye fitting. In panels B and C, the ratio of bound [^{125}I]β-EP (pmol/mg protein) to free [^{125}I]β-EP (nM) is plotted against bound [^{125}I]β-EP in both wide (0—7 pmol/mg protein) and narrow (0—0.25 pmol/mg protein) concentration ranges, respectively. The solid lines in panels B and C were drawn by Eq. 3 using the kinetic parameters for the low-affinity binding site, and the dotted line in panel C and was plotted for the high-affinity binding site. Each point and bar represent the mean ± S.E.M. ($n=3$—5).

TABLE I. Kinetic Parameters for the Specific Binding of [^{125}I]β-Endorphin to Rat Renal Basolateral Membranes

Parameter	Estimated value[a)]
$B_{max,1}$ (pmol/mg protein)	0.0331±0.0237
$B_{max,2}$ (pmol/mg protein)	7.34±0.974
$K_{d,1}$ (nM)	0.678±0.736
$K_{d,2}$ (nM)	210±43

a) Determined by fitting the C_f *vs.* C_b/P curve using an iterative nonlinear least-squares regression analysis,[28)] and expressed as the mean±S.D. of the estimated parameter.

affinities to [^{125}I]β-EP. These saturation data were replotted as C_f *vs.* C_b/P (figure not shown), and the kinetic parameters were calculated by a nonlinear least-squares regression analysis[28)] based on a two-binding-site model expressed as Eq. 2. Thus, obtained K_d and B_{max} values are listed in Table I. By substituting the obtained parameters to Eq. 2 and considering the rank order of K_m > the tracer concentration of [^{125}I]β-EP (0.1 nM) > C_f, the binding ratios ($C_b/P/C_f$) of [^{125}I]β-EP at high- and low-affinity binding sites can be approximately expressed as $B_{max,1}/K_{m,1}$ (=0.049) and $B_{max,2}/K_{m,2}$ (=0.035), respectively. This indicates that [^{125}I]β-EP binds to high- and low-affinity binding sites to similar extents at the tracer concentration, consistent with the above observation that [^{125}I]β-EP concentration, consistent with the above observation that [^{125}I]β-EP was dissociated from two distinguished binding pools.

Figures 4B and 4C illustrate the Scatchard plots for β-EP binding at the different concentration ranges of bound β-EP, *i.e.*, 0—7 and 0—0.2 pmol/mg protein, respectively. It can be also noted in Fig. 4B that the β-EP binding to the renal basolateral membranes consists of two components. The straight lines calculated by Eq. 2 using the obtained kinetic parameters (Table I) were in good agreement with the observed data points, although the presence of a high-affinity component (depicted in Fig. 4C) is not comprehensively shown owing to its much smaller capacity (0.0331 pmol/mg protein) than the large capacity (7.34 pmol/mg protein) of the low-affinity component. While there is a degradation mechanism of polypeptides called "receptor-mediated degradation"[32,33)] by which peptides can only be degraded after binding with their receptors, there is another type of metabolism of neuropeptides[34)] by which the peptides can only be degraded after dissociation from their receptors and diffusion from the vicinity of the receptors to inactivating peptidases. Taking into consideration that enkephaline are degraded *via* the latter mechanism,[35)] it is a likely assumption that [^{125}I]β-EP is also rapidly degraded upon exposure to isolated renal basolateral membranes after dissociation from its receptors, in the absence of peptidase inhibitors.

In terms of Akaike's Information Criteria (AIC),[36)] the binding of β-EP to the basolateral membranes was better predicted by assuming two binding sites (AIC = −17.3) rather than assuming only one binding site (AIC = −7.2). The high-affinity, low-capacity binding site may well be a population of opioid receptors, as judged by the small K_d value similar to that (2.5 nM) reported previously for tritiated β-EP binding to brain synaptic membranes at 5 °C.[37)]

The effects of several opioid ligands on the total binding of [^{125}I]β-EP to the renal basolateral membranes are

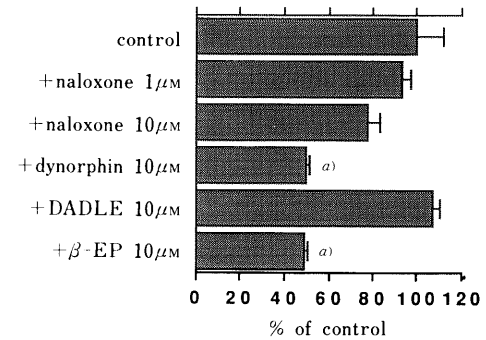

Fig. 5. Effects of Several Opioid Ligands on the [^{125}I]β-EP Binding to Rat Renal Basolateral Membranes

[^{125}I]β-EP (0.1 nM) was incubated with basolateral membranes (1 mg/ml) at 4 °C for 90 min in the absence and presence of an unlabeled opioid ligand, and the total [^{125}I]β-EP binding was determined. Each column and bar represent the mean ±S.E.M. (n=5). The significance of differences between the binding experiments in the absence and presence of an unlabeled opioid ligand was assessed by means of Student's t-test; *a*) $p<0.01$.

illustrated in Fig. 5. Since the nonspecific binding was as large as 50% of the total [^{125}I]β-EP binding and might affect the accuracy of the inhibition data with other opioid ligands, these inhibitory effects were not expressed as the specific binding (%), but as the total binding (%) of [^{125}I]β-EP. Of special note is the observation that naloxone (both 1 μM and 10 μM) and DADLE (10 μM) did not exhibit significant inhibitory effects on the [^{125}I]β-EP binding, while 10 μM of dynorphin(1—13) showed a significant inhibition to the same extent (approximately 50%) as that by 10 μM of β-EP. One might suppose from these results that a significant population of kappa sites exist on the renal basolateral membranes, and that β-EP and dynorphin(1—13) compete with each other at these sites. If that was the case, however, binding of [^{125}I]β-EP should have been displaced by naloxone 1 μM, since naloxone inhibited kappa sites with a K_1 value of 17.1 nM.[38)] In addition, it was previously shown that 1 μM naloxone and DADLE inhibited no more than 5% of the "non-opioid" binding of β-EP to lymphocytes.[9)] Thus, the inability of naloxone and DADLE to inhibit the [^{125}I]β-EP binding suggests that a non-opioid component, which is defined as the specific binding of opioid peptides insensitive to naloxone, is involved in the [^{125}I]β-EP binding to basolateral membranes. Moreover, taking into considering that the non-opioid bindings of β-EP have been characterized as having low affinities to target cells or proteins such as 34 nM in adrenal medullary membranes,[3)] 61 nM in heparin-treated human plasma[7)] and 100 nM in cultured human lymphocytes,[9)] the observed low-affinity, high-capacity binding site (K_d, 210 nM; B_{max}, 7.34 pmol/mg protein) on the renal basolateral membranes could be considered to be non-opioid in nature. Since no opioid-insensitive component of [^{125}I]β-EP binding was seen in the rat brain,[3)] it is unlikely that the opioid-insensitive component found in the basolateral membranes resulted artificially from the iodination of β-EP. This is also evidenced by the finding that binding characteristics of β-EP monoiodinated at Tyr^{27} are indistinguishable from that of β-EP,[39)] while β-EP iodinated at both Tyr^{27} and the biologically active N-terminal tyrosine (Tyr^1) retains little specific binding activity to the brain synaptic membranes.[2)]

Figure 5 also indicates that 10 μM of dynorphin(1—13)

significantly inhibited the [^{125}I]β-EP binding to basolateral membranes by 50%, suggesting that a certain region of the β-EP molecule, excluding at least the NH_2-terminal enkephalin sequence, is of particular importance for the [^{125}I]β-EP binding. Considering that the COOH-terminal sequence of the β-EP molecule is essential for the non-opioid component of [^{125}I]β-EP binding to several types of cells, membranes and proteins,[3,7-9] a COOH-terminal fragment may also be important for the non-opioid β-EP binding to renal basolateral membranes. The presence of β-EP in serum and the existence of non-opioid specific receptors on the renal basolateral membranes suggest that β-EP may mediate some peripheral physiological functions in the kidney by mechanisms distinct from those associated with traditional opioid receptors. However, it is uncertain whether or not dynorphin(1—13) binds specifically to the basolateral membranes, because the exact mechanism (*e.g.*, competitive, noncompetitive, *etc.*) of its inhibitory effect on [^{125}I]β-EP binding is not known at present, and is beyond the scope of the present study. In this respect, this study must be viewed as a first step in determining interactions of β-EP with putative non-opioid binding sites in the renal basolateral membrane *in vitro*.

In conclusion, we have shown for the first time the existence of two different classes of specific binding sites for β-EP on the renal basolateral membranes, and the high- and low-affinity bindings may well be attributed to opioid and non-opioid receptors, respectively. These results are consistent with a possible physiological role of β-EP in peritubular circulation, which requires further investigation.

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