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IN VIVO EVIDENCE FOR THE SPECIFIC BINDING OF HUMAN β-ENDORPHIN TO THE LUNG AND LIVER OF THE RAT

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Abstract—Specific binding of human β-endorphin (β-EP) was demonstrated in the lung and liver of the rat *in vivo* by the following lines of evidence, using [^{125}I-Tyr^{27}]β-EP as a radiolabeled tracer. First, the tissue-to-serum concentration ratios of the intact labeled peptide 15 min after intravenous administration were decreased significantly in the lung and liver by a simultaneous injection of unlabeled β-EP (48.5 nmol/kg), whereas in the other tissues such a decrease was not observed. Second, serum concentrations of the preadministered labeled peptide were increased rapidly after an additional intravenous injection of unlabeled β-EP via the femoral vein, but not via the carotid artery into the heart. Third, the immunoreactive labeled β-EP (^{125}I-β-EP), which was purified on a Sephadex G-50 column and did not specifically bind to the rat brain membranes, did not accumulate in the lung and liver and was not displaced by unlabeled β-EP *in vivo*, in contrast to [^{125}I-Tyr^{27}]β-EP, the commercially available HPLC-purified labeled peptide. Fourth, an additional injection of dynorphin (1–13) or ethylketocyclazocine (kappa agonist) also increased the serum concentrations of preadministered [^{125}I-Tyr^{27}]β-EP but injection of Ala^2-D-Leu^5-enkephalin (delta agonist) or naloxone (mu antagonist) did not, suggesting kappa-type binding sites in the lung.

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 some peripheral tissues [2–5], increasing attention has been paid to the peripheral direct endorphin actions. On the other hand, in the field of pharmacokinetics, the important roles of the peripheral receptor binding in the distribution and metabolism of peptide hormones, such as insulin [6–8] and arginine vasopressin [9], have been demonstrated. Recently, Whitcomb *et al.* [10] described a unique *in vivo* radioreceptor assay for the identification of tissue insulin receptors. In the present study, therefore, we used a similar method to examine the peripheral specific binding of [^{125}I-Tyr^{27}]β-EP, which is commercially available, purified by high-performance liquid chromatography, and specifically binds to the opioid receptors in the brain. We also used an *in vivo* displacement technique to certify the specific binding of the labeled peptide to the lung. Moreover, we performed the above experiments using an unfractionated labeled β-EP (^{125}I-β-EP) which lacks the opioid receptor binding activity, in order to examine the effect of receptor binding activity on *in vivo* distribution of β-EP, by comparing the distribution characteristics of the two radioiodinated peptides.

MATERIALS AND METHODS

Bindings of labeled β-endorphins to rat brain membranes. The rat brain membrane (RBM) fraction was prepared as previously described [11] and used for the receptor binding assay. The binding values of [^{125}I-Tyr^{27}]β-EP and of ^{125}I-β-EP to RBM were determined as follows. The membrane pellet was resuspended in ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% bovine serum albumin, 3 mM $MgSO_4$, 500 KIU/ml Trasylol, 0.02% bacitracin, 0.001% leupeptin, and 0.001% pepstatin to a concentration of 2.0 mg protein/ml (designated as the membrane solution). Labeled β-EP (10,000 cpm) and various concentrations of unlabeled human β-EP (0, 1, 4, 10, 100, and 1000 nM) were added to 1.5 ml of the membrane solution and incubated at 0° for 2 hr. Binding was terminated by centrifugation for 1 min in a Beckman tabletop microcentrifuge at 4°, and residual unbound ligand was removed rapidly by aspiration. The radioactivity was counted in the supernatant fraction and in the precipitate, respectively, and the percent bound was defined as (cpm in precipitate)/[(cpm in supernatant) + (cpm in precipitate)] × 100. Each binding assay was performed in triplicate.

Tissue distribution of the labeled peptides after intravenous injection. Adult male Wistar rats (Nihon Seibutsu Zairyo, Tokyo, Japan) weighing 240–280 g were used throughout the experiments. Under light ether anesthesia, the femoral vein and artery were cannulated with polyethylene tubing (PE-50) for drug administration and blood sampling,

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respectively. After recovery from anesthesia, [^{125}I-Tyr^{27}]β-EP (4 μCi/kg) with or without unlabeled β-EP (48.5 nmol/kg) or only ^{125}I-β-EP (4 μCi/kg) was injected through the femoral vein. The rats were killed by exsanguination by cutting the carotid artery at 15 min; the liver, kidney, lung, muscle, adipose (retroperitoneal fat), gut (first third of the small intestine), heart, brain, and skin were quickly excised, rinsed with ice-cold saline, and blotted dry. A portion of each tissue was weighed and counted for total radioactivity in a gamma-counter (model ARC-300, Aloka Co. Ltd., Tokyo, Japan) with a counting efficiency of approximately 80%. Another portion (0.5 g) was quickly added to 5 ml of ice-cold 20% acetic acid solution containing ammonium acetate (designated as AcOH solution) and stored at −40°. Determination of the percent of trichloroacetic acid (TCA)-precipitable radioactivity in each tissue was performed as previously described [12]. Briefly, each tissue was homogenized in the acetic acid solution in a motor-driven Potter homogenizer; 1 ml of the homogenate was added to 1 ml of 30% TCA, mixed well, and centrifuged; and TCA-precipitable radioactivity was determined.

Additional injection of unlabeled β-EP or selective opioid ligands after labeled tracer injection. [^{125}I-Tyr^{27}]β-EP or ^{125}I-β-EP (4 μCi/kg) plus saline up to 200 μl was injected through the femoral vein in conscious rats, followed by an additional injection of unlabeled β-EP (48.5 nmol/kg) at 15 min. Unlabeled β-EP was also injected through the cannulated carotid artery into the heart, followed by an equal injection of saline, when [^{125}I-Tyr^{27}]β-EP was preadministered. Moreover, 48.5 nmol/kg of dynorphin(1-13) (DYN) or ethylketocyclazocine (EKC), 3.1 μmol/kg of naloxone, or 240 nmol/kg of D-Ala^2-D-Leu^5-enkephalin (DADLE) was injected intravenously 15 min after i.v. injection of [^{125}I-Tyr^{27}]β-EP. In each of the above experiments, blood was withdrawn through the femoral artery at designated times, serum (50–200 μl) was added to 2 ml of 15% TCA, and the subsequent procedure was the same as described above for tissue samples. Some samples were gel chromatographed on a Sephadex G-50 column (1 × 45 cm) eluted with the acetic acid solution.

Data calculations. TCA-precipitable radioactivity (cpm) was determined in serum or tissues as described above, and concentrations of the labeled β-EPs were expressed as the percent of the dose injected per ml serum or g tissue. For the tissues, the tissue-to-arterial serum concentration ratios (designated as $K_{p_{app}}$) 15 min after the tracer injection were calculated as (cpm/g tissue)/(cpm/ml serum).

Materials. [^{125}I-Tyr^{27}]β-EP was obtained from the Radiochemical Center (Amersham, Arlington Heights, IL) and was reportedly prepared by iodination of synthetic human β-EP using Na^{125}I and chloramine-T and fractionated by HPLC. The unfractionated labeled human β-EP (^{125}I-β-EP) with a specific activity of 83 μCi/μg was prepared by the chloramine-T method and gel chromatography on Sephadex G-50 as previously described [13]. The labeled peptides (fractionated and unfractionated) were stored at −40° until studied. As assayed by chromatography on Sephadex G-50, these labeled tracers were at least 95% pure for a month. Following N-terminal cleavage of these tracers by leucine aminopeptidase (0.2 mg/ml) at 37° for 1 hr and thin-layer chromatography of the digestion products eluted with butanol-1-ol/acetic acid/water (75:13:12, by vol.) as previously described [14], approximately 30% of the radioactivity appeared as monoiodotyrosine from the unfractionated tracer, while there was no radioactivity from the fractionated tracer. Therefore, the unfractionated tracer was found to be iodinated at Tyr^1 as well as Tyr^{27}, whereas the fractionated tracer was labeled only at Tyr^{27}. DYN and EKC were supplied by the Eizai Co. (Tokyo, Japan). Naloxone and DADLE were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), human β-EP and TCA were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Sephadex G-50 was from the Pharmacia Fine Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade and were commercially available without the need for further purification.

RESULTS

Binding of labeled β-EPS to RBM. Figure 1 shows the bindings of [^{125}I-Tyr^{27}]β-EP (fractionated by HPLC) and ^{125}I-β-EP (unfractionated) to RBM and the inhibition by unlabeled β-EP. The values of specific and nonspecific binding of [^{125}I-Tyr^{27}]β-EP were 16.5 and 8.7% of the labeled tracer added, respectively, whereas those of ^{125}I-β-EP were 1.1 and 13.4%, respectively, indicating that the unfractionated tracer retained little opioid receptor binding activity. The IC_{50} for [^{125}I-Tyr^{27}]β-EP binding was 4.9 nM which is close to the previously reported value (3.5 nM) obtained by inhibition of [^{3}H]-β-EP binding to RBM by unlabeled β-EP at 0° [15]. Therefore, it follows that the fractionated labeled tracer was close to native human β-EP in the binding property, whereas the unfractionated tracer was not.

Tissue distribution of the labeled peptides after intravenous injection. Table 1 shows the tissue-to-arterial serum concentration ratios ($K_{p_{app}}$) of [^{125}I-Tyr^{27}]β-EP with and without unlabeled β-EP and of ^{125}I-β-EP 15 min after the tracer injection via the femoral vein. The volume ratios of the interstitial

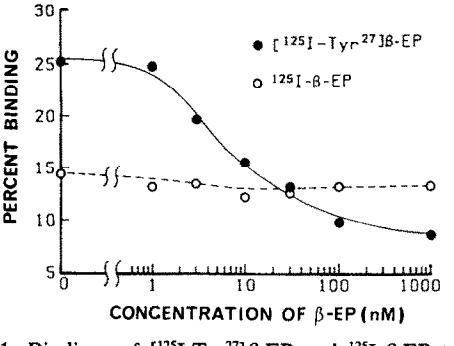

Fig. 1. Bindings of [^{125}I-Tyr^{27}]β-EP and ^{125}I-β-EP to rat brain membranes, and inhibition of it by unlabeled human β-EP. Each point is the mean of triplicate determinations.

Table 1. Tissue-to-serum concentration ratios ($K_{P_{app}}$) of [^{125}I-Tyr^{27}]β-EP and ^{125}I-β-EP in the rat

	$K_{P_{app}}$ of [^{125}I-Tyr^{27}]β-Ep*		$K_{P_{app}}$ of ^{125}I-β-EP‡	Fractional IFV§
	Tracer only	Tracer + β-EP†		
Liver	1.37 ± 0.39	0.35 ± 0.04‖	0.37 ± 0.03	0.16
Kidney	4.03 ± 0.68	3.50 ± 0.93	2.2 ± 0.22	0.20
Lung	4.46 ± 0.80	1.14 ± 0.29¶	0.79 ± 0.18	0.19
Muscle	0.21 ± 0.06	0.12 ± 0.01	0.10 ± 0.02	0.12
Adipose	0.14 ± 0.02	0.10 ± 0.02	0.095 ± 0.019	0.14
Gut	0.76 ± 0.18	0.43 ± 0.06	0.23 ± 0.10	0.094
Heart	0.42 ± 0.15	0.17 ± 0.05	0.18 ± 0.01	0.10
Brain	0.068 ± 0.027	0.036 ± 0.015	0.038 ± 0.005	0.004
Skin	0.40 ± 0.07	0.38 ± 0.08	0.65 ± 0.17	0.30

* Values are means ± SE (N = 4).
† Unlabeled β-EP (48.5 nmol/kg) was injected simultaneously with [^{125}I-Tyr^{27}]β-EP.
‡ Values are means ± SE (N = 3). Taken from Ref. 12.
§ Volume ratio of the interstitial fluid in the tissue; taken from Refs. 16 and 17.
‖ Significantly different ($P < 0.05$) from tracer only injection.
¶ Significantly different ($P < 0.02$) from tracer only injection.

fluid to the whole tissue (designated as IFV, the fractional interstitial fluid volume) in several tissues examined are cited from Refs. 16 and 17 and are included in Table 1 for comparison with $K_{P_{app}}$ of the labeled β-EPs. The tissues showing $K_{P_{app}}$ of more than 1 for the fractionated and unfractionated tracers were liver, kidney, and lung, and only kidney, respectively, indicating that these tissues accumulated more of the respective peptide than the serum concentration at pseudoequilibrium (terminal phase after i.v. injection). A significant decrease in the $K_{P_{app}}$ values of the fractionated tracer by the simultaneous injection of unlabeled β-EP was observed for the lung ($P < 0.02$) and the liver ($P < 0.05$). The $K_{P_{app}}$ values of the unfractionated tracer and the fractionated tracer with unlabeled β-EP were similar to the fractional IFV in each tissue except kidney.

Additional injection of unlabeled β-EP or selective opioid ligands after labeled tracer injection. Figure 2A presents the effect of an additional injection of unlabeled β-EP via the femoral vein or carotid artery on serum disappearance of [^{125}I-Tyr^{27}]β-EP. There was an immediate increase in serum concentration of [^{125}I-Tyr^{27}]β-EP by 153 ± 69% (mean ± SE, N = 3) 1 min after i.v. injection of unlabeled β-EP, whereas such an increase was not observed after arterial injection. Figure 2B presents the effect of an additional injection of unlabeled β-EP via the

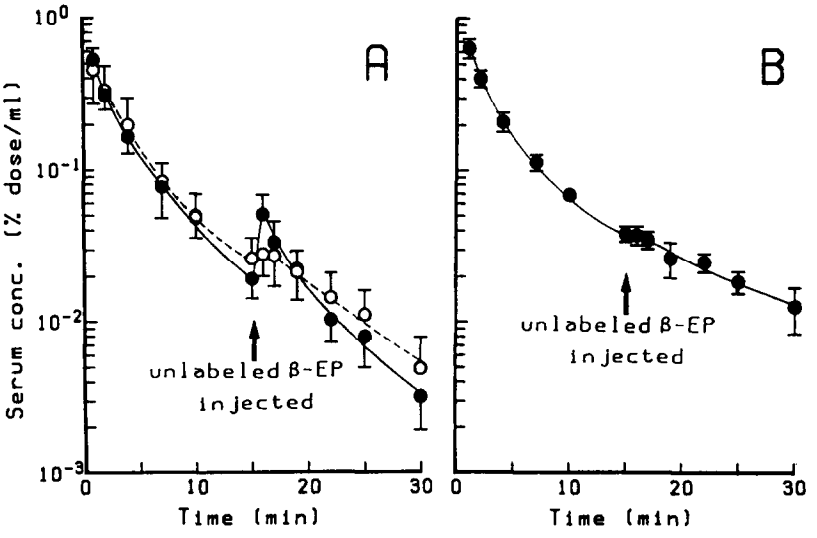

Fig. 2. (A) Effect of an additional injection of unlabeled β-EP (48.5 nmol/kg) through the femoral vein (●) or carotid artery (○) on the serum concentrations of preadministered [^{125}I-Tyr^{27}]β-EP in the rat. (B) Effect of an additional injection of unlabeled β-EP (48.5 nmol/kg) through the femoral vein on serum concentrations of preadministered ^{125}I-β-EP in the rat. Each point and vertical bar represent the mean ± SE of three rats in panels A and B.

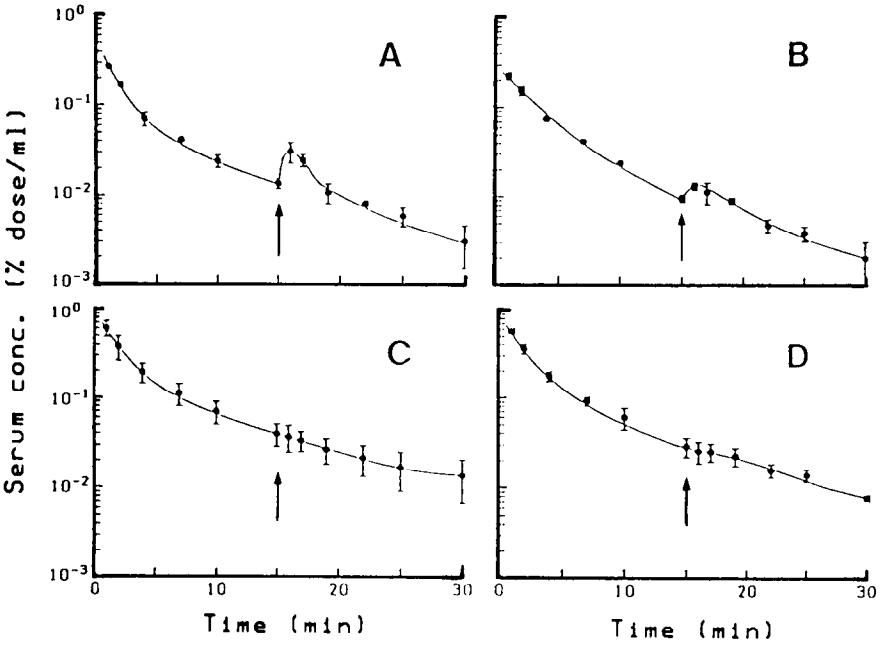

Fig. 3. Effect of an additional injection of 48.5 nmol/kg of DYN (A) or EKC (B), 3.1 μmol/kg of naloxone (C), or 240 nmol/kg of DADLE (D) through the femoral vein on the serum concentrations of preadministered [^{125}I-Tyr^{27}]β-EP in the rat. An arrow indicates the time of the additional injection. Each point and vertical bar represent the mean ± SE of three rats.

femoral vein on the serum disappearance of ^{125}I-β-EP. An increase in serum concentration of ^{125}I-β-EP was not observed, in contrast to [^{125}I-Tyr^{27}]β-EP. As shown in Fig. 3, A–D, *in vivo* displacement of [^{125}I-Tyr^{27}]β-EP was demonstrated by an additional injection of DYN or EKC, but not by that of a much larger amount of naloxone or DADLE.

DISCUSSION

There are two tyrosine residues in the β-EP molecule that can be iodinated; the importance of the N-terminal tyrosine for its biological activity has been reported [18]. Moreover, Houghten *et al.* [19] reported that the presence of diiodotyrosine in position 1 and in positions 1 and 27 dramatically reduces the opiate activity (< 1% of native β-EP), whereas diiodination of β-EP in position 27 still leaves 37% of the opiate activity. TLC after leucine aminopeptidase digestion showed that ^{125}I-β-EP was iodinated at Tyr^{1} and Tyr^{27}. It is likely, therefore, that nearly complete reduction of the receptor binding activity of ^{125}I-β-EP resulted from the iodination of β-EP at the biologically active N-terminal tyrosine, while monoiodination of β-EP at Tyr^{27} did not change the receptor binding activity of β-EP. In the present study, therefore, the unfractionated tracer (^{125}I-β-EP) was used to examine the effect of receptor binding activity on the *in vivo* distribution of β-EP, by comparing the distribution characteristics of the two radioiodinated peptides.

We demonstrated in this study that [^{125}I-Tyr^{27}]β-EP specifically binds to lung and liver of the rat, using an *in vivo* radioreceptor assay technique [10] with the HPLC-fractionated and unfractionated labeled peptides as tracers. The saturable binding of [^{125}I-Tyr^{27}]β-EP to the lung and liver appears to be related to the receptor binding activity, from the observations that the $K_{p_{app}}$ values of the unfractionated tracer were similar to those of the fractionated tracer with unlabeled β-EP in each tissue except for the kidney and that ^{125}I-β-EP was not displaced by i.v. injection of unlabeled β-EP, in contrast to [^{125}I-Tyr^{27}]β-EP. The unfractionated tracer possessed nonspecific binding activity similar to that of the fractionated tracer but lacked specific binding activity in peripheral tissues as in the brain synaptosomal membranes. We cannot, in a strict sense, attribute the observed *in vivo* displacement of the HPLC-purified tracer by unlabeled β-EP simply to the dissociation of the radiolabeled ligand from the peripheral opioid receptors, because there is the possibility of saturable binding of β-EP to some peripheral binding sites (distinct from receptors in the brain) or a capacity-limited uptake of β-EP by macrophages. The present use of the Sephadex-purified iodinated β-EP would support the involvement of receptors in the lung and liver.

When [^{125}I-Tyr^{27}]β-EP was injected into the carotid artery, serum concentrations of tracer measured at the femoral artery were much higher than the concentrations of the intravenously injected tracer (results not shown), thus excluding the possibility that the arterially injected unlabeled β-EP was not efficiently mixed with circulating blood due to the arterial blood pressure. The tissue-to-serum partition coefficient (K_p), which represents the real con-

centration ratio of tissue to serum at equilibrium, can be calculated from the $K_{p_{app}}$ values based on a physiological model [20]. According to this model, the tissue-to-arterial serum concentration ratio ($K_{p_{app}}$) obtained at pseudoequilibrium should be equal to or larger than K_p for noneliminating organs with large distribution volumes and less than K_p for eliminating organs such as liver and kidney. Such a correction factor is the same in all experiments with and without a simultaneous injection of unlabeled β-EP, and therefore the $K_{p_{app}}$ values (not corrected to the K_p values) deserve an appropriate interpretation for the saturability of the distribution of labeled peptides in tissues, which is the purpose intended.

An intravenously (not arterially) administered substance is subject to a first pass through the lung before it reaches the systemic circulation. It follows from *in vivo* displacement experiments (Fig. 2A) that the lung is responsible for the rapid dissociation of [^{125}I-Tyr^{27}]β-EP after intravenously injecting unlabeled β-EP, suggesting that the binding site in the lung is located close to the pulmonary capillaries. Sapru *et al.* [21] showed that an enkephalin-analog stimulates the pulmonary J receptors (juxta-pulmonary capillary receptors) located at the alveolar level close to the pulmonary capillaries. Moreover, these responses are abolished by naloxone [22], indicating the presence of mu-receptors which would activate pulmonary J receptors in the lung. The present study provides unique *in vivo* evidence for the existence of a peripheral specific binding site(s) of β-EP, for the interaction of β-EP with the lung has not been described elsewhere. Moreover, from *in vivo* displacement (washout) experiments using selective opioid ligands, it was suggested that the specific binding sites in the lung have a significant kappa-type property, although it is accepted that the affinity of β-EP to kappa sites is not as high as to mu or delta sites in the central nervous system. On the other hand, it is known that naloxone is not a specific antagonist for mu-receptors, and its K_i values for mu-, delta- and kappa-receptors in the brain are reported to be 1.78, 27.0 and 17.2 nM respectively [23]. From previous pharmacokinetic studies of naloxone [24], the serum concentration of naloxone at the present i.v. dose of 1 mg/kg (3.1 μmol/kg) is expected to be 0.29 μg/ml (0.89 mM) at 5 min. Consequently, inability of concomitantly injected naloxone to exhibit *in vivo* displacement of the fractionated tracer (Fig. 3C) was inconsistent with the fact that the K_i values of naloxone for kappa-receptors were much lower than the expected serum concentrations of concomitantly injected naloxone, at least within 5 min, suggesting that the contribution of mu-receptors in the specific binding of β-EP was relatively small compared with that of the putative kappa-type receptors in the lung in the *in vivo* conditions. This is supported by Werling *et al.* [25, 26] who discriminated opioid receptor binding sites in several types of neural membranes under physiological conditions of ionic strength and temperature and reported that sodium and guanine nucleotide (GTP) inhibited the binding of agonists to mu- and delta-receptors but were less effective at kappa-receptors.

The lung was shown to play an important role in the distribution of β-EP as a depot when the serum concentrations were monitored in the artery (except for the pulmonary artery) after the additional i.v. injection of unlabeled β-EP, because the blood in the artery (emerging from the lung) was in equilibrium with the pulmonary tissue. Similarly, in order to examine the dissociation of the labeled peptide from the liver by unlabeled peptide, we should measure the concentrations of the labeled peptide in hepatic vein (emerging from the liver) after the additional i.v. injection of unlabeled peptide.

That [^{125}I-Tyr^{27}]β-EP showed specific binding to the rat liver corresponds to the recent observation by Dave *et al.* [2] in which ^{125}I-labeled acteyl-human β-endorphin (Ac-hBE) was used as a tracer. Dave *et al.* observed specific binding of Ac-hBE to kidney, adrenal, spleen, and testis of the rat using isolated membrane preparations. Although we observed the decrease in $K_{p_{app}}$ in the kidney (Table 1), our *in vivo* radioreceptor assay did not detect significantly the specific binding of [^{125}I-Tyr^{27}]β-EP, mainly due to a relatively large nonspecific binding of the peptide in the kidney ($K_p = 3.50$, determined at the i.v. dose of 48.5 nmol/kg) in contrast to the liver and lung. Such a large nonspecific retention in the kidney may be related to the physiological functions of the kidneys (i.e. glomerular filtration and retention in the tubules, followed by peptidase digestion) maintained in the intact organism.

It has been reported that *in vitro* opioid receptor binding can be affected by various experimental conditions, i.e. the composition of cations and guanine nucleotide of the medium [26, 27], disruption of membranes and artificial buffers [28]. The present observations on specific binding of β-EP in the lung can be supported by *in vitro* binding assays using homogenates, isolated membranes or slices of the lung. It is important to realize that, however, in *in vitro* assays it is impossible to reproduce the exact environmental conditions that apply in the intact organism, to which the major attention of the present study has been directed. Accordingly, our present study must be viewed as a first step in determining interactions of β-EP (or β-EP like peptides) with putative kappa-type opioid binding sites in the lung, which should be validated in the future by independent *in vitro* approaches.

In conclusion, peripheral specific binding of human β-EP was demonstrated in the lung and liver of the rat by an *in vivo* radioreceptor assay using [^{125}I-Tyr^{27}]β-EP as a radiolabeled tracer. *In vivo* displacement of the tracer by unlabeled β-EP, DYN, and EKC (but not by naloxone and DADLE) suggests that the binding sites in the lung have a significant kappa-type property. It was also shown that the lung plays an important role in the distribution of the peptide as a depot after i.v. administration in the rat.

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