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Specific binding and clearance of [^{3}H]dynorphin (1–13) in the perfused rat lung: an application of the multiple-indicator dilution method

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Abstract—The clearance and binding of a κ-selective opioid peptide, dynorphin (1–13), in the perfused rat lung has been examined, using the multiple indicator dilution method. More than 50% of [^{3}H]dynorphin (1–13) entering the pulmonary circulation was eliminated by the lung during a single passage of a tracer dose. By contrast, when a high dose (100 μM) of dynorphin (1–13) was concomitantly injected, [^{3}H]dynorphin (1–13) behaved like [^{14}C]sucrose, an extracellular marker. The kinetic analyses of the pulmonary venous outflow curves of [^{3}H]dynorphin (1–13) and [^{14}C]sucrose at low and high doses of dynorphin (1–13) indicated that the initial uptake rate constant, extraction ratio and distribution volume of [^{3}H]dynorphin (1–13) decreased significantly in the presence of a high concentration of unlabelled dynorphin (1–13). These results suggest that [^{3}H]dynorphin (1–13) is eliminated by a saturable process and binds to a specific binding site in the perfused lung, which may be the κ-type binding site. The multiple indicator dilution technique, in combination with a moment analysis, was successfully applied to demonstrate the specific binding and clearance of dynorphin (1–13) in the perfused lung.

The lung plays a major role in the regulation of many endogenous substances including polypeptides (Bakhle & Vane 1974; Ryan 1982; Bend et al 1985) and enkephalins (Manwaring & Mullane 1984; Crooks et al 1985; Gillespie et al 1985). We have suggested the existence of a κ-type binding site in the lung (Sato et al 1988), which serves as a saturable depot for exogenously administered β-endorphin in-vivo. While the use of a κ-selective opioid peptide might be adequate to confirm the presence of such a putative binding site, rapid degradation might occur simultaneously with the binding, so a more sophisticated approach is needed.

In the present study, the multiple indicator dilution (MID) technique, which has been shown to be useful for measuring the initial uptake rate constant into liver (Goresky & Bach 1970; Tsao et al 1986), kidney (Silverman et al 1970; Itoh et al 1986), and heart (Ziegler & Goresky 1971; Kuikka et al 1986), together with moment analysis (Yamaoka et al 1978; Kakutani et al 1985), was applied to verify the putative binding site in the lung using the κ-selective opioid peptide, dynorphin (1–13) (Chavkin et al 1982), and to evaluate the pulmonary clearance of the peptide. Little is known about the pharmacokinetic characteristics of dynorphins except for our transport study of a stable analogue of dynorphin using isolated brain capillaries (Terasaki et al 1989).

Materials and methods

Chemicals. Bovine serum albumin (Fraction V; BSA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]Dynorphin (1–13) (spec. act. of 24.4 mCi μmol^{-1}) labelled at Amersham International Ltd (Buckinghamshire, UK), was a gift from Dr Shinro Tachibana (Eisai Co., Ltd, Tokyo, Japan). Unlabelled dynorphin (1–13) was obtained from Peptide Institute Inc. (Osaka, Japan), and trichloracetic acid (TCA) was from Wako Pure Chemical Industries Ltd (Osaka, Japan). [^{14}C]Sucrose was purchased from New England Nuclear Corp. (Boston, MA). Other chemicals were of analytical grade and used without further purification. [^{3}H]Dynorphin (1–13) dissolved in methanol was stored at −20°C, until purified by reverse phase HPLC and lyophilized for use in each experiment.

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Lung perfusion. Adult male Wistar rats (Sankyo Laboratory Co. Ltd, Toyama, Japan), 240–300 g, with free access to food and water, were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (50 mg kg^{-1}). The rats were placed on a heating plate (37°C) in a supine position, the trachea cannulated, and ventilated with the humidified room air at a rate of 60 breaths min^{-1} and a tidal volume of 2–3 mL using an animal respirator (model KN-56, Natsume Seisakusho Co., Tokyo, Japan). The lung was prepared as described by Smith & Bend (1981). Briefly, the chest was opened, the animals were intravenously injected with heparin (1000 units kg^{-1}), the main pulmonary artery and left ventricle (via the pulmonary vein) were cannulated, and single-pass perfusion was started immediately after exsanguination by cutting the aorta. The perfusate was composed of 4·5% (w/v) BSA, 10 mM glucose in Krebs-Ringer bicarbonate (KRB) buffer (mM) NaCl 119, KCl 4·73, $CaCl_2$ $2H_2O$ 2·54, KH_2PO_4 1·18, $MgSO_4$ $7H_2O$ 1·18, $NaHCO_3$ 24·9, pH 7·4), oxygenated with 95% O_2–5% CO_2, and contained in a reservoir in a thermostatic water bath. The lung was maintained at 37°C and perfused at an effective perfusion pressure of 15 cm H_2O at the inlet of the organ and a constant flow rate of 8·6 mL min^{-1} using a peristaltic pump (model MP, Tokyo Rikakikai Co., Tokyo, Japan). To assess the degradation of [^{3}H]dynorphin (1–13) in the reservoir perfusate, a TCA-precipitation method was used as described below.

MID method. The pulmonary uptake rate of [^{3}H]dynorphin (1–13) was measured by the MID method as described for liver (Goresky & Bach 1970). After 15 min stabilization, 100 μL of perfusate containing [^{3}H]dynorphin (1–13) (0·2 μCi mL^{-1}) and [^{14}C]sucrose (0·04 μCi mL^{-1}), as an extracellular reference marker, was rapidly injected with or without 100 μM dynorphin (1–13) into the pulmonary artery. The venous outflow was collected from the pulmonary vein cannula in serial tubes at the rate of one tube per second for 30 s. Two samples from each tube were obtained: one (50 μL) for the determination of the total ^{3}H ([^{3}H–T]) and ^{14}C radioactivity, and the other (50 μL) for determination of unchanged [^{3}H]dynorphin (1–13) by TCA precipitation (see later), using the double-isotope counting method with an appropriate crossover correction in a liquid scintillation counter (model LSC-700, Aloka Co., Tokyo, Japan). In each perfused lung preparation, two bolus injections were given using solutions with and without 100 μM unlabelled dynorphin (1–13), and the second dilution curve was obtained 2 min after the first. In each perfusion experiment, the tracer amount of [^{3}H]dynorphin (1–13) was given first and then the labelled and unlabelled dynorphin (1–13) was given, to avoid a possible effect of unlabelled peptide remaining in the lung on the subsequent injection of labelled peptide alone.

Analytical method. Intact [^{3}H]dynorphin (1–13) in the outflow perfusate was measured as described by Sato et al (1987). Perfusate samples (50 μL) were mixed with an equal volume of

40% (w/v) TCA solution and 20 μL of rat serum as a precipitation carrier. These mixtures were kept standing at 4°C for 30 min and centrifuged at 3000 *g* for 15 min, the supernatants were transferred into separate tubes by aspiration. The ^3H in the supernatants ([^{3}H-S]) was measured in a liquid scintillation counter (model ARC-605, Aloka Co.). The [^{3}H]-radioactivity in the precipitate was calculated as [^{3}H-T] minus [^{3}H-S], and taken as intact [^{3}H]dynorphin (1–13).

Data analysis. The radioactivity in each sample was normalized by dividing it by the injected dose, so that the outflow patterns of [^{3}H]dynorphin (1–13) and [^{14}C]sucrose could be adequately compared. The concentrations in the effluent were thus expressed as the outflow fractions of the dose per mL. During a short time interval when any efflux could be considered to be negligible, the appearance rate constant for pulmonary uptake of dynorphin (1–13) was obtained from MID experiments, using the equation of Goresky & Bach 1970:

$$\ln[FC(t)_{suc}/FC(t)_{dyn}] = k_1\lambda t/(1+\gamma) = k_{1,app}\cdot t \quad (1)$$

where $FC(t)_{suc}$ and $FC(t)_{dyn}$ denote the fractional concentrations of [^{14}C]sucrose and [^{3}H]dynorphin (1–13) per mL of perfusate effluent at time t, respectively; k_1 is the net rate constant for the uptake; λ is the ratio of the cellular space to the vascular space; and γ is the ratio of the interstitial space to the vascular space. Thus, $\lambda/(1+\gamma)$ represents the ratio of cellular space to extracellular space. According to equation 1, the plot of the logarithm of the ratio of the outflow fraction of the test compound to that of an extracellular reference should yield a straight line, and the apparent rate constant for the influx process, $k_{1,app}$, was determined from the slope of this line by a least-squares regression analysis.

Since the injected radioactivity of [^{14}C]sucrose was almost completely recovered in the effluent, the pulmonary extraction (E) of [^{3}H]dynorphin (1–13) was obtained in relation to the recovery ratio of [^{14}C]sucrose as:

$$E = 1 - \Sigma FC(t)_{dyn}/\Sigma FC(t)_{suc} \quad (2)$$

The mean transit time ($\bar{t}$) and the apparent distribution volume (V_d) of each labelled compound were calculated according to model-independent moment analysis (Yamaoka et al 1978; Kakutani et al 1985) as follows:

$$\bar{t} = \Sigma t\cdot FC(t)/\Sigma FC(t) \quad (3)$$

$$V_d = Q\cdot\bar{t}/(1-E) \quad (4)$$

where Q denotes the perfusion flow rate. The significance of differences between the MID experiments in the absence and presence of 100 μM dynorphin (1–13) was assessed by means of the paired Student's *t*-test.

Results and discussion

The present study was designed to verify the κ-type binding site in the lung, which has been suggested to play a role in the distribution of intravenously administered β-endorphin (Sato et al 1988), using the multiple indicator dilution method. For this purpose, a κ-selective opioid peptide, dynorphin (1–13), was used as a tracer ligand.

The lung has been recognized as having a role in regulating the systemic concentration of a variety of vasoactive amines, prostaglandins and polypeptides (Bakhle & Vane 1974; Ryan 1982; Bend et al 1985) by selective removal or metabolism. Moreover, the anatomical location renders a unique significance to the lung, because it is in direct equilibrium with the arterial blood, and intravenously injected substances are subject to first-pass metabolism through it before they reach the systemic circulation. Considering that significant metabolism of enkephalins occurs in the pulmonary circulation (Manwaring & Mullane 1984; Crooks et al 1985; Gillespie et al 1985), it seems important to evaluate quantitatively the pulmonary clearance of dynorphin (1–13) for its in-vivo efficacy.

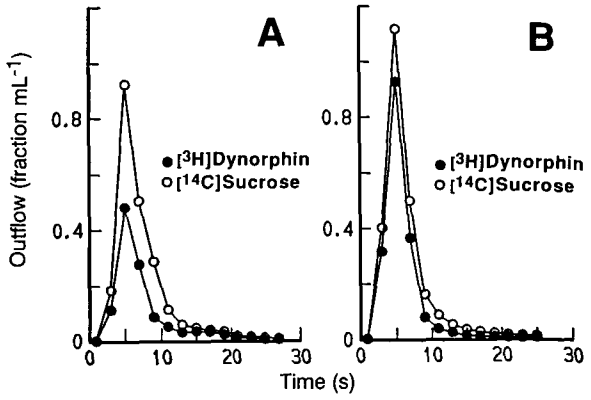

FIG. 1. Typical sets of pulmonary venous outflow curves for [^{3}H]dynorphin (1–13) and [^{14}C]sucrose in the perfused rat lung in the absence (A) and presence (B) of 100 μM unlabelled dynorphin (1–13) in the injection solution.

During the lung perfusion experiment, there was no appearance of oedema, where the perfusion flow rates were constant at 8·55 ± 0·25 mL min^{-1} (mean ± s.e.m.; n = 5), and the variation in perfusion pressure was less than ± 5%. Fig. 1 shows typical sets of pulmonary venous outflow curves for [^{3}H]dynorphin (1–13) and [^{14}C]sucrose in the perfused rat lung in the absence (tracer dose) and presence of 100 μM unlabelled dynorphin (1–13) (high dose) in the injection solution, respectively. After an initial delay (catheter transit time), both materials appeared in the outflow, reached peak concentration in several seconds, and then declined exponentially with time. It is obvious that the outflow patterns of these labelled compounds were similar at the high dose of dynorphin (1–13), while that of [^{3}H]dynorphin (1–13) was much lower than that of [^{14}C]sucrose, indicating a saturable clearance of [^{3}H]dynorphin (1–13) by the lung. There was no significant difference in the outflow pattern of [^{14}C]sucrose between low and high doses of dynorphin (1–13). The ratio plots of $\ln(FC_{suc}/FC_{dyn})$ vs time at the low and high doses of dynorphin (1–13) are shown in Fig. 2. The initial slope of the

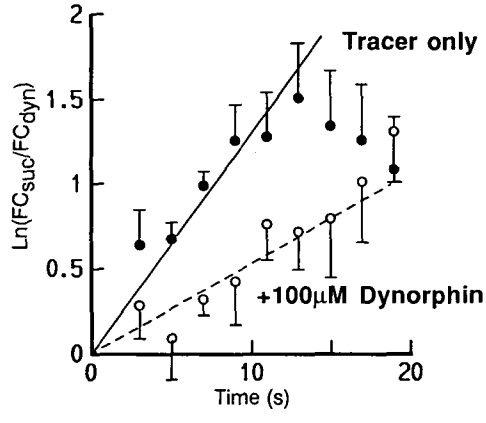

FIG. 2. The ratio of [^{14}C]sucrose to [^{3}H]dynorphin (1–13) fractional concentrations mL^{-1} plotted against time at the low (●) and high (○) doses of dynorphin (1–13) in the perfused rat lungs. Each point and vertical bar represent the mean ± s.e.m. from 5 rats.

Table 1. Apparent initial uptake rate constant, extraction ratio, mean transit time and distribution volume of [^{3}H]dynorphin (1–13) and [^{14}C]sucrose at low and high doses of unlabelled dynorphin (1–13) in the perfused rat lungs.

	[^{3}H]dynorphin (1–13)		[^{14}C]sucrose	
Parameter[a]	Low dose[b]	High dose[c]	Low dose[b]	High dose[c]
$k_{1,app}$ (s^{-1})	0·12 ± 0·017	0·055 ± 0·013*	—	—
E	0·568 ± 0·042	0·228 ± 0·110*	0	0
$\bar{t}$ (s)	5·84 ± 1·09	6·31 ± 1·22	7·43 ± 0·71	7·75 ± 0·76
V_d (mL)	2·08 ± 0·48	1·29 ± 0·29*	1·06 ± 0·10	1·10 ± 0·91

The values are expressed as means ± s.e.m. (n = 5).
[a] The kinetic parameters were calculated by eqns 1–4.
[b] The concentration of [^{3}H]dynorphin (1–13) in the injectate was 8·2 nM.
[c] The concentration of dynorphin (1–13) in the injectate was 100 μM.
* $P < 0·05$, significantly different from the low dose injection of dynorphin (1–13).

ratio plot ($k_{1,app}$) of [^{3}H]dynorphin (1–13) at a high dose was significantly lower than that at a low dose, indicating that the uptake of dynorphin (1–13) is a saturable process.

The concentration of [^{3}H]dynorphin (1–13) in the injectate was 8·2 nM, as calculated from its relatively low specific activity. However, from the injection volume (100 μL) and the calculated V_d of [^{3}H]dynorphin (1–13) (2·08 mL), the average concentration of the tritiated peptide in the lung was estimated to be less than 0·4 nM. Thus, the use of a low concentration of [^{3}H]dynorphin (1–13) could make it possible to investigate the putative κ-type binding site in the lung. The kinetic parameters for [^{3}H]dynorphin (1–13) and [^{14}C]sucrose in the perfused lungs were calculated using equations 1–4, and are listed in Table 1. The catheter transit time ($\bar{t}_{cat}$) was 1·51 ± 0·02s (mean ± s.e.m.; n = 5), as calculated from the catheter volume (V_{cat}) of 0·143 mL and the perfusion flow rate in each perfusion experiment. The mean transit times and distribution volumes of [^{3}H]dynorphin (1–13) and [^{14}C]sucrose obtained (Table 1) reflect the behaviour of these materials in the lung, because $\bar{t}_{cat}$ (1·5 s) and V_{cat} (0·14 mL) are small relative to the corresponding values of $\bar{t}$ and V_d. The V_d value of [^{14}C]sucrose (approximately 1 mL) could be appropriately assigned to the sum of the catheter volume (0·143 mL) and the extracellular fluid volume of the rat lung, i.e. the capillary bed volume (0·55 mL) and interstitial fluid volume (0·3 mL) reported previously (Tsuji et al 1983).

Since the pulmonary extraction ratio of [^{3}H]dynorphin (1–13) was determined to be as high as 0·57 (Table 1), the lung appears to be an important disposing organ for rapidly clearing dynorphin (1–13) from the circulation. As can be seen from inspection of Table 1, the extraction of [^{3}H]dynorphin (1–13) was significantly inhibited by a high dose of dynorphin (1–13). Moreover, the percentage of intact [^{3}H]dynorphin (1–13) in the effluent also significantly increased in the presence of unlabelled dynorphin (1–13) (results not shown). These results indicate that the effective pulmonary clearance of [^{3}H]dynorphin (1–13) is a saturable process, which may be explained by the peptidase enzymes of the pulmonary endothelium (Ryan 1986, 1989). However, it is unlikely that the association of [^{3}H]dynorphin (1–13) with hydrolytic enzymes located on the surface of the pulmonary endothelium would take several seconds (Fig. 2). An alternative explanation is that the polypeptides cannot permeate through the cellular membranes because of their low hydrophobicity and relatively large molecular size, and the observed initial uptake rate is due to the association process of dynorphin (1–13) to a certain binding site, as comprehensively described for the hepatic uptake of epidermal growth factor (Satoh et al 1988).

The mean transit times ($\bar{t}$) of [^{3}H]dynorphin (1–13) and [^{14}C]sucrose were not significantly different, and no significant change was observed between experiments in the absence and presence of unlabelled dynorphin (1–13) in the injection solution (Table 1). This observation indicates that interactions of [^{3}H]dynorphin (1–13) with the plasma membranes of lung alveolar cells and/or the surface of pulmonary endothelium did not significantly alter the transit time of this peptide. However, the diffusibility of dynorphin (1–13) and sucrose within the extracellular cell matrix could be different, and the transit time is not so sensitive to an early association process as to an efflux process, because of its nature as a second-order moment parameter. Consequently, the above observation does not necessarily mean that dynorphin (1–13) lacks any interactions with certain components of the lung tissue.

Table 1 also indicates that the V_d of [^{3}H]dynorphin (1–13) at the high dose (100 μM) is not significantly different from that of [^{14}C]sucrose, suggesting that the peptide behaves like sucrose, an extracellular marker, when its specific binding is effectively blocked. This observation also suggests that the capillary permeability of dynorphin (1–13) is similar to that of sucrose and is not the limiting step for its distribution into the lung. On the other hand, the V_d of [^{3}H]dynorphin (1–13) in the absence of unlabelled dynorphin (1–13) is significantly greater than that in its presence, suggesting a saturable distribution of dynorphin (1–13) into the lung. The 38% decrease of V_d in the presence of excess dynorphin (1–13), together with the above mentioned 54% decrease of $k_{1,app}$ suggests the existence of a specific binding site for dynorphin (1–13) in the lung, in support of the previous in-vivo evidence for the pulmonary κ-type binding capacity (Sato et al 1988). The results presented in the present study might well be explained by a receptor-mediated degradation in which the ligand binding is coupled to subsequent peptidase degradation, as previously suggested for enkephalin degradation in the brain (Knight & Klee 1978). However, the relationship between the enzymatic degradation and the specific binding of dynorphin (1–13) in the lung needs closer study.

In conclusion, the multiple-indicator dilution method was successfully applied to demonstrate the specific binding and clearance of the κ-selective opioid peptide, dynorphin (1–13), in the perfused rat lung.

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Oxidative deamination of aliphatic amines by rat aorta semicarbazide-sensitive amine oxidase

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Abstract—Rat aorta semicarbazide-sensitive amine oxidase (SSAO) exhibits very high affinity in the deamination of an homologous series of aliphatic amines of 1 to 18 straight chain carbon atoms. The K_m value decreases substantially as the chain length of these amines increases. The V_{max} values are higher for the short chain amines. Diamines are poor substrates for SSAO or are not acted upon by the enzyme. The substrate preference for SSAO differs from that for monoamine oxidase.

Blood vessels of various species contain an amine oxidase, namely semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6), which is resistant to inhibition by clorgyline, a type A monoamine oxidase (MAO, EC 1.4.3.4) inhibitor, but quite sensitive to semicarbazide (Clarke et al 1982; Callingham & Barrand 1987) and (E)-2-(3',4'-dimethoxyphenyl)-3-fluoroallylamine (MDL 72145) (Lyles & Fitzpatrick 1985; Elliott et al 1989a). This enzyme can oxidize different endogenous as well as various exogenous amine substrates. It is known that the enzyme is particularly rich in the vascular wall and smooth muscle cells. While MAO is located subcellularly on the outer mitochondrial membrane (Yu 1986), SSAO is found to be heterogenously distributed, i.e. it is present in either the cytoplasm or plasma membrane (Wibo et al 1980). The function of SSAO is not yet well understood (Fuentes & Neff 1977; Lewinsohn et al 1980). Recently is has been found that methylamine (CH_3NH_2) and allylamine ($CH_2=CHCH_2NH_2$) can be deaminated by SSAO, leading to the formation of the toxic compounds formaldehyde (HCHO) and acrolein ($CH_2=CHCHO$), respectively (Boor & Nelson 1982; Nelson & Boor 1982; Precious et al 1988). It has been suggested that SSAO might play a role in certain cardiovascular or smooth muscle disorders. n-Pentylamine can also be deaminated by vascular SSAO (Guffroy et al 1983). Although SSAO is distinctly different from MAO, the substrate spectra of both enzymes appear to be largely overlapping. Recently it has been reported that straight chain saturated aliphatic amines can be oxidized with quite high affinity by MAO-B (Yu 1989). It would be interesting to examine whether vascular SSAO would oxidize other aliphatic amines and to compare its kinetic parameters regarding these substrates with that of MAO.

Materials and methods

SSAO was prepared from the rat aorta as previously described (Clarke et al 1982). Rats (male Wistar, 180–200 g) were killed by cervical dislocation and the aorta dissected out. The aorta was then rinsed in physiological saline to clean away any adhering blood, and freed of surrounding fat. The tissues were then frozen (−20°C) until assayed. Tissues were homogenized by means of a