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Characterization and distribution of μ opioid receptors in rabbit cerebellum

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ABSTRACT The binding characteristics and distributions of [^{3}H]ohmefentanyl ([^{3}H]OMF), [^{3}H]etorphine, [^{3}H] *N*-methyl-*N*-[7-(I-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl] benzeneacetamide [^{3}H]U-69593), and [^{3}H] (*D*-penicillamine^2, *D*-penicillamine^5) enkephalin ([^{3}H]DPDPE) in rabbit cerebellum were studied. In saturation experiment, [^{3}H]OMF bound to a single population of sites with $K_d = 2.2 \pm 1.3$ nmol · L^{-1} and $B_{max} = 69 \pm 13$ fmol/mg protein. The results of [^{3}H]etorphine ($K_d = 1.0 \pm 0.4$ nmol · L^{-1} and $B_{max} = 16 \pm 6$ fmol/mg protein) were similar to those of [^{3}H]OMF. There were no specific bindings of [^{3}H]U-69593 and [^{3}H]DPDPE in rabbit cerebellar membranes. In autoradiographic study, the gray scales of specific binding for [^{3}H]OMF, [^{3}H]etorphine, [^{3}H]U-69593, and [^{3}H]DPDPE were 20.2 ± 4.6, 8.5 ± 2.7, 2.0 ± 0.7, and 3.2 ± 4.3, respectively. The gray matter of rabbit cerebellum was intensely labeled by [^{3}H]OMF and [^{3}H]etorphine, but not by [^{3}H]U-69593 or [^{3}H]DPDPE. These results suggested that the rabbit cerebellum contained μ opioid receptors.

KEY WORDS ohmefentanyl; etorphine; enkephalins; endorphin receptors; cerebellum; radioligand assay; autoradiography

Evidenced by radioligand assay and autoradiography, [^{3}H]etorphine binding sites were present in rabbit cerebellum[1]. Competitive experiments demonstrated that the specific binding sites of [^{3}H]etorphine in rabbit cerebellum were mainly of the μ opioid receptor subtype[2,3]. Because etorphine interacts equally well with μ, δ, and κ opioid receptor subtypes in nervous tissue, the subclassification of opioid receptor sites in rabbit cerebellum awaiting to be determined using highly selective ligand directly to μ, δ, and κ opioid receptor subtypes.

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Ohmefentanyl (OMF), designed and synthesized by our laboratory, is a highly selective μ opioid receptor subtype agonist[4,5]. In Goldstein's laboratory found that the affinity of OMF was the highest among 47 opioid ligands and the selectivity of OMF for μ opioid receptor was parallel to that of [*D*-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAGO) and was higher than that of sulfentanyl[6]. *N*-methyl-*N*-[7-(I-pyrrolidinyl)-1-oxaspiro(4,5) dec-8-yl]benzeneacetamide (U-69593) and [*D*-penicillamine^2, *D*-penicillamine^5] enkephalin (DPDPE) are typical ligands for κ and δ opioid receptor subtypes, respectively[7,8]. In the present work, using ligands of [^{3}H]OMF, [^{3}H]U-69593, and [^{3}H]DPDPE, we went further into the subclassification of opioid receptor sites in the rabbit cerebellum.

MATERIALS AND METHODS

New Zealand white adult rabbits of either sex weighing $2.6 \pm s$ 0.5 kg ($n = 16$) were used. OMF and [^{3}H]OMF (2.07 PBq · mol^{-1}) were produced by Shanghai Institute of Materia Medica and Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, respectively. [^{3}H]Etorphine (1.11 PBq · mol^{-1}, produced by Shanghai Medical University) was a gift from Professor XU Shao-Fen, Department of Neurobiology, Shanghai Medical University. Etorphine was produced by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. [^{3}H]U-69593 (1.77 PBq · mol^{-1}), New England Nuclear Corp, USA. [^{3}H]DPDPE (1.78 PBq · mol^{-1}), Amersham Corp. U-69593 and DPDPE, Sigma Co.

Preparation of cerebellar membrane protein Cerebellum was homogenized in 30 vol of ice-cold sucrose 0.32 mol · L^{-1}. The homogenate was cen-

trifuged at 1000×g for 10 min. The supernatant was recentrifuged at 39 000×g for 30 min, and the resulting pellet was resuspended in 30 vol of Tris-HCl buffer a 50 mmol · L^{-1} (pH 7.4). The homogenization and centrifugation procedures were repeated once. The final pellet was resuspended in Tris buffer at 10 mg · ml^{-1} concentration and stored at −30℃. Protein concentrations were determined by the colorimetric method.

Binding study Bindings of ligands ([^{3}H]OMF, [^{3}H]etorphine, [^{3}H]U-69593, and [^{3}H]DPDPE) were measured by incubating 300 μl of the prepared cerebellar membranes (1 mg of protein for [^{3}H]OMF binding study and 2 mg protein for [^{3}H]U-69593, [^{3}H]DPDPE, and [^{3}H]etorphine binding studied) with 100 μl of buffer containing displacer (unlabeled ligands 1 μmol · L^{-1} for non-specific binding) or of buffer alone (total binding); 100 μl of the tritiated ligand at different concentrations for 45 min at 25℃. Incubation was terminated by rapid filtration through Whatman GF/C filters, which were washed immediately with 3—5 ml portions of ice-cold Tris buffer. Radioactivity on the filters was determined by liquid scintillation counting.

Sectioning of cerebellum The rabbits were decapitated. The cerebella were mounted on cryotome chucks and frozen under powdered dry ice. The mounted cerebella were warmed to −20℃ and 10—15 μm coronal sections were cut and thaw-mounted onto gelatin coated slides, which were stored at −30℃.

Autoradiography Cerebellar sections were incubated in 100 μl of Tris-HCl buffer 50 mmol · L^{-1}, pH 7.4, at 25℃ for 45 min, with [^{3}H]OMF or other tritiated ligands 2 nmol · L^{-1} for total binding. Adjacent sections were incubated under the same conditions but in the presence of unlabeled ligands 1 μmol · L^{-1} for determination of non-specific binding. The slides were washed sequentially through 6 rinses of Tris buffer and 3 rinses of distilled water at 4℃ and dried in air. The dry tissue sections were tightly juxtaposed against tritium-sensitive film (produced by Beijing Institute of Photochemistry) at 4℃ for 12 wk. The films were developed in Kodak D-19 and fixed in F-5.

The gray scales of sections of film were measured by a computer-assisted densitometer. Gray scales from at least 3 different sections in each rabbit were averaged and the average values obtained from 4 rabbits were expressed as $\bar{x}\pm s$.

RESULTS

Binding studies of tritiated ligands Saturation experiments were carried out in rabbit cerebellar membranes. Specific binding of [^{3}H]OMF was proved to be saturated with respect to increasing radioligand concentrations from 0.5 to 10.0 nmol · L^{-1} (Fig 1B). Scatchard analysis yielded a linear plot indicating an apparently single type of binding sites with K_d values of 2.2±1.3 nmol · L^{-1} and B_{max} values of 69±13 fmol/mg protein (n=5, $\bar{x}\pm s$, Fig 1A).

Specific binding of [^{3}H]etorphine was also found with K_d=1.0±0.4 nmol · L^{-1} and B_{max} =16±6 fmol/mg protein (n=4, $\bar{x}\pm s$). The characterization of specific binding of [^{3}H]etorphine was similar to that of [^{3}H]OMF, although [^{3}H]etorphine bound less sites than [^{3}H]OMF did: 16 *vs* 69 fmol/mg protein (Fig 1).

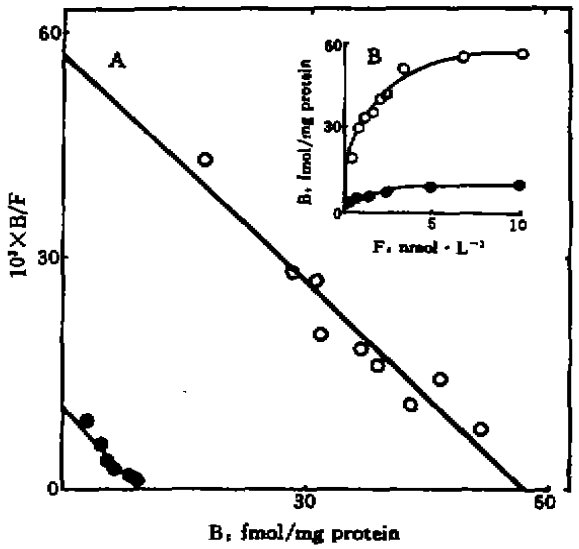

Fig 1. Specific binding of [^{3}H]OMF (○) and [^{3}H]etorphine (●) to rabbit cerebellar membranes. A: Scatchard plot; B: Saturation curve.

Specific binding sites of [^{3}H]U-69593 and [^{3}H]DPDPE were not found in saturation ex-

periment under the same conditions. Ratios of total binding to non-specific binding for [^{3}H]OMF, [^{3}H]etorphine, [^{3}H]U-69593, and [^{3}H]DPDPE were 4.5, 2.6, 1.1, and 1.1, respectively.

Autoradiography For [^{3}H]OMF and [^{3}H]etorphine, the gray scales of total binding were higher than those of non-specific binding (Tab 1). For [^{3}H]U-69593 and [^{3}H]DPDPE, there were no significant differences between total binding and non-specific binding. The gray scales of specific binding (total binding substracted non-specific binding) for [^{3}H]OMF, [^{3}H]etorphine, [^{3}H]U-69593, and [^{3}H]DPDPE were 20.2±4.6, 8.5±2.7, 2.0±0.7, and 3.2±4.3, respectively.

Tab 1. Gray scale of autoradiography of [^{3}H]OMF, [^{3}H]etorphine, [^{3}H]DPDPE, and [^{3}H]U-69593 in rabbit cerebellum. $n=4$, $\bar{x}\pm s$. $^*P>0.05$, $^*P<0.01$ *vs* non-specific binding group.

Groups	Gray scale	
	TB	NSB
[^{3}H]OMF	34.4±4.4*	14.1±2.3
[^{3}H]Etorphine	23.2±3.4*	14.1±1.8
[^{3}H]DPDPE	16.0±3.4*	14.2±2.9
[^{3}H]U-69593	14.2±2.6*	14.2±3.9

The autoradiogram for [^{3}H]OMF and [^{3}H]etorphine showed clear cerebellar structures: the gray matter was intensely labeled, while the white matter was devoid of labeling. The gray matter was more heavily labeled in [^{3}H]OMF group than that in [^{3}H]etorphine group (Fig 2, Plate 1).

DISCUSSION

Our saturation experiment and autoradiographic studies showed that the rabbit cerebellum contained only μ opioid receptors. Thus the rabbit cerebellum may be used for μ opioid receptor preparation to investigate the effect of opioids on adenylate cyclase and for tissue supply to isolate and purify the μ opioid receptor binding protein.

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兔小脑 μ 阿片受体的特性及其分布

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摘要 应用放射配体结合试验和放射自显影研究了[^{3}H]羟甲芬太尼([^{3}H]OMF), [^{3}H]埃托啡([^{3}H]Eto), [^{3}H]U 69593和[^{3}H]DPDPE 与兔小脑的结合特性. [^{3}H]OMF 和[^{3}H]Eto 与兔小脑有一呈饱和性的单位点的结合, 它们的 K_d 分别为2.2±1.3和1.0±0.4 nmol·L^{-1}, B_{max}分别为69±13和16±6 fmol/mg 蛋白. [^{3}H]U 69593和[^{3}H]DPDPE 与兔小脑无结合. 放射自显影显示兔小脑上也仅有[^{3}H]OMF 和[^{3}H]Eto 的结合位点分布. 表明兔小脑阿片受体是μ受体亚型.

关键词 羟甲芬太尼; 埃托啡; 脑啡肽; 内啡肽受体; 小脑; 放射配位体测定; 放射自显影