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Isolation and purification of active opioid receptors from rat brain

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ABSTRACT Active opioid receptor was purified to apparent homogeneity from rat brain membranes. The purification was accomplished in two steps: *Vicia bungei* Ohwi lectin-sepharose affinity chromatography followed by 6-succinylmorphine-$(CH_2)_6$-sepharose affinity chromatography. The purified receptor had a molecular weight of 45 000 as determined by SDS-PAGE and was judged to be homogeneous and genuine opioid receptor by the following criteria: (1) and (2) the $Na^{125}I$ and β-[^{125}I]endorphin labeled protein was analysed by SDS-PAGE separately and a single band was detected in both; (3) a single band was shown in the gel of isoelectric focusing and (4) the purified protein bound with [^{3}H]etorphine in a stereospecific and saturable manner.

KEY WORDS endorphin receptors; lectins; affinity chromatography; autoradiography; electrophoresis; affinity labels

Isolation and purification of opioid receptor had been considered as an important and rather difficult task in neurobiology since the existence of opioid receptor was demonstrated in 1973 by using radio-ligand receptor binding assay. The major problems on this subject were (1) the binding activity of opioid receptor was extremely sensitive to the detergents commonly used in other membrane receptors; (2) the amount of opioid receptor in brain was very small, and scientists have not found any good receptor source like *N*-acetylcholine receptor in torpedo. After arduous attempts about 10 yr, a few research groups claimed that they had purified opioid receptor[1-3]. In this paper, we report that we have got the active, sodium dodecyl sulfate polyacrylamide gel electrophoresis pure opioid receptor from rat brain membranes by using two steps of affinity chromatography, *Vicia bungei* Ohwi lectin (VBL)-sepharose affinity column followed by the 6-succinylmorphine-$(CH_2)_6$-sepharose affinity chromatography.

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MATERIALS

β-[^{125}I] endorphin (human, 67.08 TBq/mmol) was purchased from Amersham. [^{3}H] etorphine (1.48 TBq/mmol) and $Na^{125}I$ (4.07 TBq/ml) were got from Shanghai Medical University and Beijing Institute of Atomic Energy respectively. Bis [2-(succinimidooxycarbonyloxy) ethyl]sulfone (BS-COES) and chloramine T were gifts from Professor H H Loh. VBL was a gift from Professor ZENG Zhong-Kui. SDS-PAGE molecular weight standards were purchased from Sigma. Ampholine (pH 3-10) was got from LKB. Coomassie blue G-250 and α-methyl-D-mannoside were purchased from Fluka. Sepharose 4B was purchased from Pharmacia. Digitonin was got from Hangzhou First Pharmaceutical Factory. 1300-100 Adsorbent Resin was purchased from Shanghai Institute of Pharmaceutical Industry and naloxone was got from Shanghai Medical University.

METHODS AND RESULTS

Preparation of solubilized opioid receptor and [^{3}H]etorphine binding assay By the method previously used in our laboratory[4], the crude rat brain membrane fraction (P_2) was prepared. The final concentration of digitonin 1% and $MgSO_4$ 5 mmol/L were added to P_2 fraction. After stirring for 20 min at 0℃, centrifugation at 100 000 ×g for 60 min was followed. The 1300-100

Adsorbent Resins were added to the supernatant at the ratio of 0.5 g/ml. Stirred again at 0℃ for 60 min so as to adsorb the excess detergent, after filtration, the supernatant was supposed to be the digitonin/$MgSO_4$ solubilized opioid receptor from rat brain which had all characteristics similar to those of P_2 fraction. Opioid receptor binding assay was performed according to the modified polyethylene glycol precipitation method originally described by Cuatrecasas[5]. The protein concentrations were determined by a modified Coomassie Blue assay[8].

Two steps of affinity chromatography VBL had been characterized as a lectin whose suppressive sugar was mannose[7]. We have first found this lectin to have the sugar affinity to opioid receptor (in preparation of publication). VBL was coupled to sepharose by CNBr method[8], and 6-succinylmorphine was prepared and covalently coupled to sepharose 4 B by the method described by Simon[9]. In the typical experiments, 5 ml of soluble opioid receptor was applied to the column at the fluid speed of about 8 ml/h, and was specifically eluted by 5 ml of α-methyl-D-mannoside 0.1 mol/L in buffer A (digitonin 0.02%, $MgSO_4$ 5 mmol/L in Tris-HCl 50 mmol/L, pH 7.5). The sugar elution was directly applied to the second column, 6-succinylmorphine-$(CH_2)_6$-sepharose. Both the VBL and 6-succinylmorphine column were equilibrated and washed with buffer A. Morphine 10 μmol/L was used as the specific elution of the second affinity column. In order to remove the excess of morphine, the morphine elution component was stirred with 1300-100 Resin at the ratio of 0.5 g/ml at 0℃ for 60 min. Thus, the opioid receptor purified by two steps of affinity chromatography was enriched about 1400 folds (Tab 1). The specific binding of the receptor to [^{3}H]etorphine was saturable (Fig 1).

Homogeneity of the purified opioid receptor In order to determine the purity of the opioid receptor, the purified material was iodinated with $Na^{125}I$ in the presence of chloramine T[10]. The affinity labeling of β-[^{125}I]endorphin to opioid receptor was performed according to the method described by Howard *et al*[1]. In our experiments, the concentration of β-[^{125}I]endorphin and naloxone were 2 nmol/L and 3 μmol/L respectively. All samples were boiled for 2 min in SDS-PAGE sample buffer (contain-

Tab 1. Purification of opioid receptors from rat brain

Step	Detergent extract	VBL column eluate	6-Succinyl-morphine column eluate
Protein*(mg)	27.8	0.131	0.014
Specific binding† (fmol/mg)	6.33	1350	9140
Purification fold	—	213	1444‡
Yield(%)	—	0.47	0.05

* Determined by coomassie blue staining using bovine serum albumin as calibration standard.
† The concentration of [^{3}H]etorphine was 3-4 nmol/L.
‡ The final purification folds in 3 other experiments were 1485, 1410 and 1259.

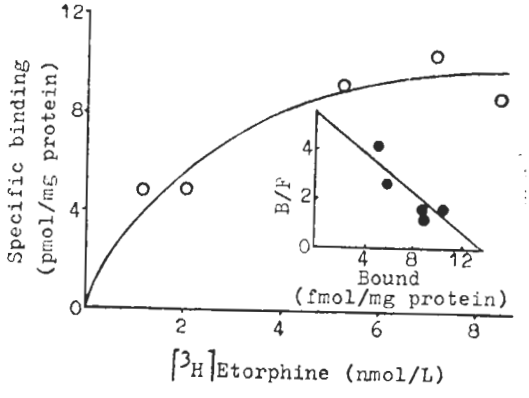

Fig 1. Saturation curve and Scatchard analysis of [^{3}H]etorphine binding to purified opioid receptor(purified by VBL-sepharose and 6-succinyl-morphine-$(CH_2)_6$-sepharose chromatography sequentially). $K_D = 3.3 \pm 0.6$ nmol/L; $B_{max} = 11.3 \pm 2.5$ pmol/mg protein (n = 3).

ing SDS 4%, β-mercaptoethanol 2%, sucrose 15%). SDS-PAGE was carried on for 4-5 h at 15 mA constant current in 0.75 mm slab gels by the method as described by Laemmli[11], using a 9.3% acrylamide separating gel and a 4.2% stacking gel. The gels were fixed in trichloroacetic acid 50% for 15 min and stained in coomassie blue G-250 0.1%. After destained and dried, the gels were exposed to X-ray films for 3-6 wk at -50℃.

As seen in Fig 2, the samples purified by VBL affinity column and 6-succinylmorphine affinity chromatography sequentially showed a single protein band in SDS-PAGE gel, migrating a M_r of 45 000. The samples from VBL and 6-succinylmorphine column separately contained several other protein bands besides the band of M_r 45 000.

The above data indicate that the purified material is homogeneous, but we should verify that the M_r 45 000 protein observed on gels is the material responsible for binding opiates. We cross-linked β-[^{125}I] endorphin to the purified protein by using of BSCOES and analysed by electrophoresis and autoradiography. As shown in Fig 2, a single band migrating at 45 000 is apparent. This labeling is specific since it could be prevented when excess naloxone existed during binding and cross-linking.

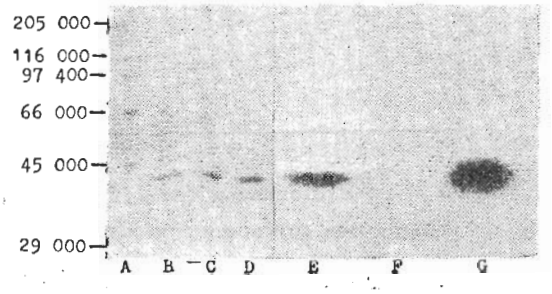

Fig 2. SDS-PAGE of purified and partially purified opioid receptor. Molecular weight standards were shown in lane A (carbonic anhydrase, albumin from egg, albumin from bovine, phosphorylase b, β-galactosidase and myosin). The eluted fractions from 6-succinylmorphine-$(CH_2)_6$-sepharose (lane B), VBL-sepharose (lane C) and VBL column followed by 6-succinylmorphine-$(CH_2)_6$-sepharose column (lane D) were stained by coomassie blue. The eluate from the two steps of chromatography was radioiodinated (lane E). The finally purified opioid receptor was crosslinked to β-[^{125}I]endorphin in the presence (lane F) and the absence (lane G) of naloxone 3 μmol/L.

We have used other method to detect the purity of the purified material. Isoelectric focusing electrophoresis was taken according to the method commonly used[12]. Fig 3 showed that there was only one band in the gel so the receptor is homogeneous in respect to isoelectric point of protein.

DISCUSSION

By utilizing different characteristics of opioid receptor, it is possible to purify the macromolecule using multi-steps of affinity chromatography. Here we report that we have purified opioid receptor from rat brain by using (1) the glycoprotein nature of the receptor; (2) the binding characteristic of the receptor. The facts which supported that the opioid receptor purified by VBL and 6-succinylmorphine affinity chromatography was apparently homogeneous were (1) only one single band was detected when the iodinated protein was analysed by SDS-PAGE and autoradiography and (2) a single band was shown in isoelectric focusing gel. Also, we have used HPLC to characterize the homogeneity of the purified opioid receptor, and the result of this experiment showed that there was only one protein peak in the sample with M_r 45 000 (data not shown).

The M_r 45 000 protein we have got is an active opioid receptor because (1) the purified material exhibits stereospecific and

Fig 3. Analysis of the purified opioid receptor by isoelectric focusing electrophoresis. The gel was 100×2 mm ID. pH 3-10. Protein was stained by coomassie blue.

saturable binding of [^{3}H]etorphine; (2) specific labeling of β-[^{125}I]endorphin to the M_r 45 000 band.

The result of one single band in SDS-PAGE and about 1400 folds in purity could not coincide well, because of, we suppose, (1) the inactivation of opioid receptor during the isolation and purification procedure; (2) a rather large error in protein determination of the purified receptor preparation, which is unavoidable when working with such small amount of protein. Under these circumstances, we think our real purification should be much larger than 1400 folds since the receptor protein is apparently homogeneous.

In summary, we have first purified an active opioid receptor protein with M_r 45 000 that retains its ability to bind opioid ligands *in vitro*.

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大鼠脑内活性阿片受体的分离纯化

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提要 经三齿草藤凝集素-琼脂糖和 6-琥珀酰吗啡-琼脂糖两步亲和层析纯化, 得到保持活性、表观均一的大鼠脑阿片受体, 其 M_r 为 45 000。该纯化蛋白: (1) (2)用 Na^{125}I、β-[^{125}I]内啡肽标记、SDS-PAGE 分析, 均为单一条带; (3) 等电聚焦电泳为单一条带; (4) 与[^{3}H]依托啡的结合是高亲和、可饱和的。

关键词 内啡肽受体; 凝集素; 亲和色谱法; 放射自显影术; 电泳; 亲和力标记