Separation of κ-opioid receptor subtype from frog brain

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Complete separation of the [^{3}H]ethylketocyclazocine ([^{3}H]EKC) specific binding (κ subtype) from tritiated Tyr-D-Ala^2-Me-Phe^4-Gly-ol^5 enkephalin (DAGO) and Tyr-D-Ala^2-L-Leu^5-enkephalin (DALA) binding (μ- and δ-subtypes, respectively) was achieved by Sepharose-6B chromatography and sucrose density gradient centrifugation of digitonin solubilized frog brain membranes. The apparent sedimentation coefficient ($s_{20,w}$) for the κ receptor-detergent complex was 13.1 S and the corresponding Stokes radius 64 Å. The isolated fractions exhibited high affinity for EKC and bremazocine, whereas μ- and δ-specific ligands were unable to compete for the [^{3}H]EKC binding sites, indicating that the κ subtype represents a separate molecular entity from the μ and δ receptor sites.

Opioid receptor subtype *Solubilization* *Separation* *Sucrose density gradient centrifugation* *Gel filtration* *Hydrodynamical parameters*

1. INTRODUCTION

It is well known from pharmacological and biochemical studies that the opioid receptor system consists of multiple subtypes, i.e., μ, δ, κ, σ [1–3]. Although it has recently been found by us and other laboratories that opioid receptors may display different molecular forms [4–7], there is controversy over their function and relationship to the different subtypes.

We had described earlier solubilization of active opioid receptors with good yield from frog brain using digitonin [8]. Two distinct receptor populations were found by sucrose density gradient ultracentrifugation and Sepharose-6B gel filtration, using an opioid antagonist, [^{3}H]naloxone (labelling μ-, δ- and κ-sites equally) in receptor binding assays [4]. In the present work selective, tritiated ligands were used for labelling the subtypes. One population of receptors with κ specificity was identified and completely separated from the partially comigrating μ and δ sites.

Abbreviations: EKC, ethylketocyclazocine; DAGO, Tyr-D-Ala^2-Me-Phe^4-Gly-ol^5-enkephalin; DALA, Tyr-D-Ala^2-L-Leu^5-enkephalin; DADLE, Tyr-D-Ala^2-D-Leu^5-enkephalin; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

2. MATERIALS AND METHODS

(Tyr-3.5-[^{3}H])^1-D-Ala^2-L-Leu^{5}enkephalin ([^{3}H]-DALA: 1.37 TBq/mmol; 37.3 Ci/mmol) was synthesized by Dr G. Tóth [9]. [9-^{3}H]Ethylketocyclazocine ([^{3}H]EKC: 0.74 TBq/mmol; 19.9 Ci/mmol) was purchased from New England Nuclear. (Tyr-3.5-[^{3}H])^1-D-Ala^2-MePhe^4-Gly-ol^5 enkephalin ([^{3}H]DAGO): 1.67 TBq/mmol; 45 Ci/mmol, was from Amersham. Levorphanol was kindly donated by Hoffmann La Roche. EKC was provided by Sterling Winthrop Research Institute. DAGO was a generous gift of Dr D. Römer, Sandoz Ltd. DALA was synthesized by Dr K. Medzihradszky et al., Central Res. Inst. for Chemistry, Budapest, Hungary. Sepharose-6B beads and HMW calibration kit were obtained from Phar-

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macia. All other chemicals were of analytical grade.

Particulate membrane fraction of frog (*Rana esculenta*) brain was prepared in buffer A (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 40 KIU/ml trasylol, 20 μg/ml bacitracin, 1 mM PMSF) and solubilized in 1% digitonin as described earlier [8].

Gel filtration of the solubilized supernatant was carried out on a Sepharose-6B column, which was equilibrated with buffer B (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA and 0.05% digitonin) and calibrated with Pharmacia HMW kit.

Sucrose density gradients were prepared in buffer A containing 0.05% digitonin. Samples were centrifuged at 200000 × g for 16 h at 4°C in a Beckman SW-41 Ti rotor.

Aliquots of the sucrose density gradient and Sepharose-6B fractions were incubated in buffer A with [^{3}H]DAGO for 40 min at 35°C [10], [^{3}H]DALA for 90 min at 0°C [9] and [^{3}H]EKC for 40 min at 25°C [3] in a final volume of 1 ml. Following incubation, the samples were directly filtered on polyethyleneimine-treated Whatman GF/C or GF/B filters [11], and washed twice with 10 ml ice-cold 50 mM Tris-HCl (pH 7.4) buffer. Radioactivity was counted in a toluene-based scintillation cocktail. Specific binding was defined as the difference between total binding and binding in the presence of 10 μM unlabelled ligands. Measuring [^{3}H]DAGO binding 10 μM levorphanol was used for demonstration of nonspecific binding. For determining δ binding sites the cross-reactivity of [^{3}H]DALA to the μ binding sites was reduced by the addition of 10 nM unlabelled DAGO [3]. For the demonstration of $\varkappa$ binding sites, the cross-reactivity of [^{3}H]EKC to both μ and δ binding sites was decreased by addition of 100 nM unlabelled DALA and DAGO.

Protein concentrations were determined by the method of Bradford [12].

3. RESULTS AND DISCUSSION

To establish the specificity of the previously observed distinct molecular forms of frog brain opioid receptors [4,8], subtype specific ligands – [^{3}H]DAGO, [^{3}H]DALA and [^{3}H]EKC (with μ, δ and $\varkappa$ specificity) – were used for labelling the fractions of gel filtration and sucrose density gradient centrifugation. All labelled ligands were used above saturating concentration. As shown in fig.1, a single activity peak appeared with [^{3}H]EKC specific binding on a Sepharose-6B column. These fractions (40–45) are completely separated from the μ and δ sites (measured by [^{3}H]DAGO and [^{3}H]DALA specific binding). The Stokes radius of the $\varkappa$ receptor-detergent complex was 64 Å. The μ and δ subtypes comigrated and showed two distinct activity peaks with a higher and a lower Stokes radius. There was an earlier report on partial separation of $\varkappa$ and μ receptor subtypes from CHAPS extract of rat brain on a Sepharose CL-6B column [6]. They detected two populations of $\varkappa$ receptors: one of them coincided with the μ subtype, whereas the other one partially separated from that. Because they were not able to recover the δ subtype after solubilization, there is no information on the position and size of the δ receptor.

We have also estimated the molecular masses of the different subtypes by sucrose density gradient centrifugation. Similar to gel filtration, low digitonin concentration (0.05%) was used because the detergent interferes in the binding assay. The

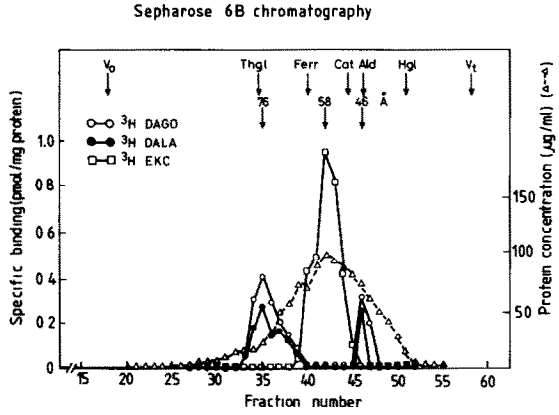

Fig.1. Sepharose-6B gel filtration of digitonin extract of frog brain membranes. Solubilized supernatants (3 ml) were applied to a Sepharose-6B column (1.2 × 100 cm) and eluted with buffer B at a constant flow rate of 10 ml/h. 0.6-ml aliquots of the fractions were measured in duplicate for 10 nM [^{3}H]DAGO, 10 nM [^{3}H]DALA and 7 nM [^{3}H]EKC specific binding as described in section 2. Protein markers and their Stokes radii were as follows: thyroglobulin 85 Å, ferritin 61 Å, catalase 52.2 Å, aldolase 48.1 Å and hemoglobin 24 Å. This experiment was repeated 4 times.

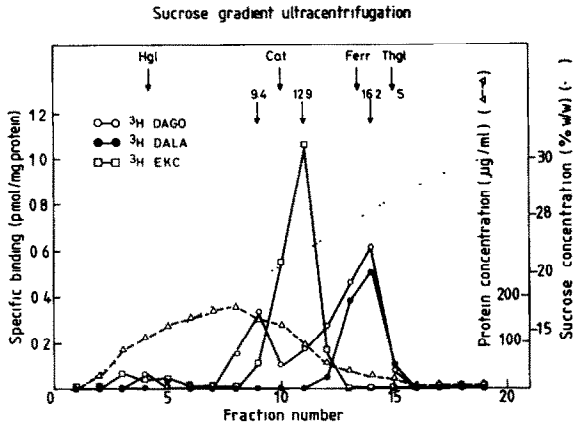

Fig.2. Sucrose density gradient (10–30%, w/w) ultracentrifugation of digitonin solubilized opioid binding sites from frog brain. 1 ml solubilized supernatant or mixture of standard proteins were layered on top of the gradient. After centrifugation, 0.5-ml aliquots were assayed for 10 nm [^{3}H]DAGO, 10 nM [^{3}H]DALA and 7 nM [^{3}H]EKC specific binding as described in section 2. Protein markers for the estimation of sedimentation coefficient were as follows: thyroglobulin (19.3 S), ferritin (15.4 S), catalase (11.3 S), hemoglobin (4.2 S). This experiment was replicated 4 times.

separation of the $\varkappa$ subtype by this method was also successful (fig.2) and the apparent sedimentation coefficient ($s_{20,w}$) for the $\varkappa$ subtype was found to be 13.1 S. [^{3}H]EKC binding was observed in fraction 9–12, where the presence of μ and δ receptors was low. When measuring δ receptors with a more specific ligand ([^{3}H]DADLE) identical results were obtained. The separation of μ and δ receptors could not be solved by this method, either. The recovery of the different subtypes in the solubilized preparation was about the same (basically no loss in activity). The distribution of the μ, δ and $\varkappa$ receptors did not significantly change on the Sepharose column and sucrose density gradient either.

Itzhak et al. [5] recently published the separation of the $\varkappa$ subtype from digitonin solubilized extract of guinea-pig brain and cerebellum. Because of the instability of their preparation, the gradient was run only for 2.5 h, which was insufficient for complete separation. Expressing their data in cpm/mg protein shows less separation.

The specificity of our $\varkappa$ receptor subtype was further proved by displacement experiments. 2 nM [^{3}H]EKC was used for labelling the pooled fractions of sucrose density gradient (fractions 9–12) and Sepharose-6B column (fractions 40–45). Unlabelled EKC and bremazocine showed high affinity (IC_{50} 4 and 30 nM, respectively). μ- and δ-selective ligands up to 10 μM concentration did not decrease the [^{3}H]EKC binding considerably, indicating that the separated fractions contain the $\varkappa$ subtype exclusively.

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