HYDRODYNAMIC PROPERTIES OF A [^{3}H]ETORPHINE MACROMOLECULAR COMPLEX FROM THE RAT BRAIN

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1. Introduction

In the central nervous system, opiates produce their various pharmacological effects primarily by interacting with stereospecific binding sites [1–3]. These sites are presumably carried by membrane-bound opiate receptors which, as such, have not yet been unambigously identified and, a fortiori, purified.

A first step toward these major goals is to prove that macromolecular (?) species which bind opiates with high affinity and stereospecifically do exhibit well defined hydrodynamic properties in solution. A classical approach [4] makes use of highly selective, nearly irreversible radioactive ligands to label the receptor molecule(s). The resulting membrane-bound, radioactive complex is then solubilized with the appropriate chaotropic agent after which its hydrodynamic properties are analyzed by conventional techniques, namely gel filtration and ultracentrifugation [5].

Here, following [6], we have selected [^{3}H]etorphine as the specific radioactive probe but a solubilizing agent different from Brij 36 T:sodium cholate, an anionic detergent. We show that a crude membrane fraction from the rat brain contains a component of high M_r which, in solution, exhibits well-defined although unusual hydrodynamic properties. Given our experimental protocol, this component may represent an opiate receptor. However, the possibility that there exist, in addition to this particular entity, other lower M_r species with similar binding characteristics, is discussed.

2. Materials and methods

2.1. *Preparation of the crude membrane fraction (CMF) from rat brain*

Adult Wistar rats were killed by decapitation. The brains (minus the cerebellum) were rapidly dissected out and homogenized at 4°C in a total volume (v_o) of 12 ml/g wet tissue of 0.32 M sucrose in Tris–HCl (1 mM, pH 7.4). Homogenization was completed in a Potter-Elvehjem tissue grinder by 10 strokes of the teflon pestle, motor driven at 800–1000 rev./min. The resulting suspension was incubated for 30 min at 35°C and centrifuged (0–2°C) in a Beckman rotor type 30 for 30 min at 30 000 rev./min. The pellet was dispersed (Polytron) in a large excess of ice-cold Tris–HCl (50 mM, pH 7.4) (from now on referred to as 'buffer') and centrifuged as before. The washed pellet was homogenized (Polytron) in $v_o/2$ of buffer to yield the CMF (12–14 mg protein/ml).

2.2. *Labelling of the CMF with [^{3}H]etorphine*

This was carried out routinely by incubating for 20 min at 35°C the following mixture: 4.0 ml CMF, 1.0 ml buffer (with and without 20 μM levorphanol) and 5.0 ml 4 nM [^{3}H]etorphine (41.4 Ci/mmol, the Radiochemical Center, Amersham), in buffer. After incubation, the reaction mixture was chilled down to 0°C, centrifuged and washed as above. The final pellet was dispersed (Polytron) in 5.0 ml ice-cold buffer to give the [^{3}H]CMF (10–11 mg protein/ml).

2.3. *Solubilization of the [^{3}H]CMF with sodium cholate*

To 2.4 ml [^{3}H]CMF were added 0.3 ml of ice-cold buffer and 0.3 ml of Na-cholate at the desired concentration (usually 10%, w/v, in buffer). After 15 min

at 0°C, the mixture was centrifuged in the cold, in a Beckman rotor type 30 for 30 min at 30 000 rev./min. The supernatant (2 mg protein/ml) constituted the radioactive cholate extract.

2.4. *Gel filtration*

Sepharose 6B (Pharmacia, 83 ml) was packed into a glass column of 1.5 by 45 cm and fully equilibrated at cold-room temperature with Na-cholate (1%, w/v) in Tris–HCl (50 mM, pH 7.4). The column was calibrated with a Pharmacia HMW kit (blue dextran 2000, thyroglobulin, ferritin, catalase, aldolase), cytochrome *c* (Merck) and [^{3}H]etorphine. An aliquot (0.5 ml) of the radioactive detergent extract was applied and eluted at a constant flow rate (Mariotte flask) of ~13 ml/h. Fractions of 1.0 ml were collected and 0.4 ml each fraction was mixed with 6 ml home-made scintillation liquid (toluene 0.7 l:Triton N-101 0.3 l:butyl-PBD 7 g) and counted in an Intertechnique model SL-30 counter.

2.5. *Density gradient ultracentrifugation*

Density gradients in Na-cholate (1%, w/v)–Tris–HCl (50 mM, pH 7.4) were prepared in the appropriate centrifuge tubes with a conventional gradient maker. They consisted of 5 ml sucrose (Merck) 5–20% (w/v). An aliquot (0.15 ml) of the radioactive detergent extract was layered on top of the gradient and spun at 0–2°C in a Beckman rotor type SW 65 for 4 h at 64 000 rev./min. The gradients were 'calibrated' with a mixture of aldolase and catalase from the Pharmacia HMW kit. Fractions of 5 drops were collected automatically (peristaltic pump) by a LKB piercing unit connected to an Isco fraction collector. The radioactivity of each fraction was counted as above.

2.6. *Protein assay*

Protein concentrations were estimated as in [7] with bovine serum albumin as standard.

3. Results

3.1. *Labelling of the CMF with [^{3}H]etorphine and solubilization of the [^{3}H]etorphine labelled CMF by sodium cholate*

Under the labelling conditions in section 2, 80–90% of the bound radioactivity was levorphanol-'displaceable'; i.e., non-specific binding amounted to only 10–20% of total binding. The optimal solubilizing concentration of Na-cholate was determined by incubating (15 min, 0°C) the [^{3}H]CMF with increasing amounts of detergent. Fig.1 demonstrates that Na-cholate is a very efficient solubilizing agent over 0.5–1% (w/v). It is especially important to note that at 1% (w/v) of Na-cholate, solubilization of the membrane-bound radioactivity was nearly quantitative (80–90%). Therefore 1% Na-cholate was routinely used to get the high speed supernatant referred to as the soluble detergent extract.

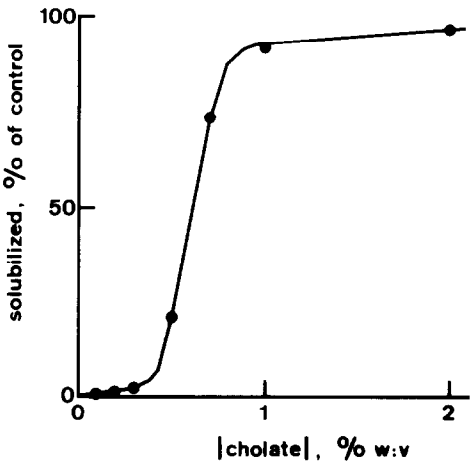

Fig.1. Solubilization of the radioactivity specifically bound to a rat brain CMF by increasing concentrations of Na-cholate.

3.2. *Filtration of the soluble [^{3}H]CMF cholate extract through Sepharose 6B*

First, the applied radioactivity was totally recovered upon agarose chromatography. Second, only trace amounts of labelled material were eluted in the void volume of the column, indicating that the detergent extracted radioactivity was entirely soluble.

Fig.2 shows that Sepharose 6B filtration resolved the levorphanol sensitive radioactivity into 2 major well-separated components: Component X (25% of the input radioactivity) was eluted as a fairly sharp and almost perfectly symmetrical peak between ferritin (M_r 440 000; a = 61 Å) and thyroglobulin (M_r 669 000; a = 85 Å). Its apparent Stokes radius (r_S) was estimated to be 67 Å (fig.2, insert), corresponding to M_r ~ 500 000. This value is substantially higher than that in [6,8]. Peak X was levorphanol-sensitive, so that component X is likely to represent a specific opiate binding macromolecule; i.e., *a* opiate receptor. The second component almost completely included in the

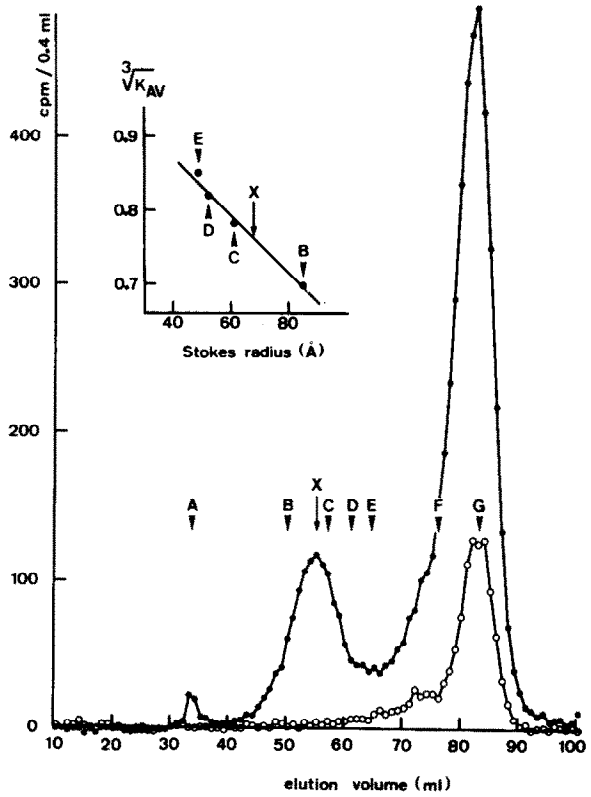

Fig. 2. Filtration through Sepharose 6B of the soluble cholate extract. The CMF had been labelled with [^{3}H]etorphine in the absence (•) and in the presence (○) of 2 μM levorphanol. (A) Blue dextran 2000; (B) thyroglobulin; (C) ferritin; (D) catalase; (E) aldolase; (F) cytochrome *c*; (G) [^{3}H]etorphine. Insert: calibration curve according to [10].

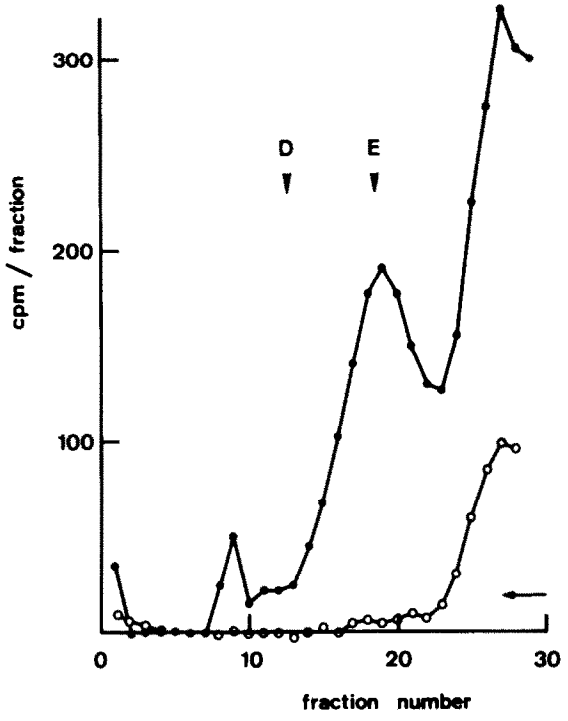

Fig.3. Centrifugation in sucrose density gradient of the soluble cholate extract. The CMF had been labelled with [^{3}H]-etorphine in the absence (•) and in the presence (○) of 2 μM levorphanol. (D) Catalase; (E) aldolase. The arrow indicates the gravity field.

gel was hardly separated from free [^{3}H]etorphine. It consisted possibly of 'low' M_r species being eluted slightly ahead of cytochrome *c* (M_r 13 000).

3.3. *Centrifugation of the soluble [^{3}H]CMF cholate extract in sucrose density gradients*

In sucrose density gradients, the levorphanol-sensitive radioactivity was resolved into two major components, in agreement with the agarose elution profile (fig.3). No radioactivity was recovered in the pellet. Greater than 60% of the applied counts did not migrate whereas the other 30–40% co-sedimented with aldolase, a globular, water-soluble enzyme of M_r 158 000. This sedimentable [^{3}H]etorphine macromolecular complex, likely to be identical to component X (in preparation), thus does not behave in sucrose gradients as one would have expected from its app. r_S estimated by agarose chromatography.

4. Discussion

These results demonstrate clearly that a crude membrane fraction from the rat brain contains a macromolecular species with well defined hydrodynamic parameters and whose binding characteristics are those of an opiate receptor. Upon filtration through agarose (Sepharose 6B), this component elutes as would a globular, hydrosoluble protein of M_r ~500 000 (app. r_S 67 Å). However, in sucrose gradients, it sediments along with aldolase, a water-soluble enzyme of M_r 158 000! This unusual hydrodynamic behavior is reminiscent of that of the cholinergic receptor protein solubilized from *Electrophorus electricus* electroplax [4,5] and may be accounted for by either an elongated shape or a low density (presence of lipids) or both. In this respect, the answer rests on the measurement of the frictional ratio and apparent specific volume of the [^{3}H]etorphine macromolecular complex in crude cholate extracts [5].

In [6,8] it was claimed that an opiate macromolec-

ular complex of M_r ~380 000 had been solubilized with Brij 36T. Unfortunately, this important advance was not, in either study, substantiated by actual experimental results (agarose elution profiles not shown).

However, in a similar approach and using identical techniques [9] no defined peak of radioactivity of either 380 000 or 500 000 M_r could be shown. In [9], in addition to 'a broad peak of 100 000–500 000 molecular weight, ... several other species of less than 20 000 molecular weight', was shown suggesting that 'a large number of distinct membrane components are capable of stereospecifically binding opiates in vivo'.

We have been confronted, in the course of similar studies, with the following discrepancy: filtered on short columns (PD-10, Pharmacia) of Sephadex G-25, the cholate-extracted radioactivity appears to be mainly 'macromolecular' (75% in v_0) whereas peak X (fig.2) accounts for a mere 25% of the total input!

A trivial explanation would reside in a rapid dissociation of the macromolecular complex during chromatography at cold room temperature. In fact, it is easy to demonstrate (in preparation) that there is no detectable dissociation of the complex in question (in the presence of a large excess of unlabelled etorphine) for as long as 18 h after solubilization. More likely then, G-25 excludes labelled components that are almost completely included in Sepharose 6B. Indeed, fig.2 reveals a levorphanol-sensitive, etorphine binding material slightly ahead of cytochrome *c* (M_r 13 000, marker F).

Therefore, in the mammalian brain there may be several distinct membrane components that bind opiates specifically. These components, which need not be as numerous as suggested in [9], may differ in their hydrodynamic properties, chemical nature and pharmacological specificity. Were this true it would open a new avenue of research in the field.

References

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