Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 263-268

DISTRIBUTION OF STEREOSPECIFIC OPIATE RECEPTOR BINDING ACTIVITY BETWEEN SUBCELLULAR FRACTIONS FROM OVINE CORPUS STRIATUM

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Received May 15,1980

SUMMARY: The thermodynamic binding constants of the opiate ligand etorphine to subcellular fractions prepared from ovine central nervous system tissue are reported. Those examined were synaptosomal, microsomal and myelin fractions. The dissociation constant is smallest and the binding capacity greatest in the microsomal fraction. The results have implications concerning future work on opiate receptor heterogeneity and isolation.

One of the objectives of the initial investigations of the opiate receptor(s) was to determine the approximate localization of the receptor binding activity in subcellular fractions from central nervous tissue. Initially, the majority of stereospecific binding activity (defined as picomoles of opiate ligand stereospecifically bound per milligram of protein) was reported to be in the microsomal fraction from rat brain (1). On further analysis it was reported, however, that it is the synaptosomal fraction which exhibits the greatest binding (2). This latter localization has since been generally taken to be definitive.

However, examination of these reports shows that, in fact, the extent to which specific opiate binding activity occurs in the microsomal fraction was not necessarily established since in this fraction the particulate material was not separated from soluble protein for the binding and protein analyses. The resolution of this problem is important: (a) in view of recent work on the ultrastructural localization of the biological opiate/opioid peptide receptor(s) (3); (b) because of the implications concerning

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0006-291X/80/130263-06$01.00/0

the proposed heterogeneity of opiate receptors (4); (c) as a starting-point for procedures aimed at the isolation and molecular characterization of opiate receptors from central nervous system tissue (5,6,7).

In view of this we have reinvestigated the question. Our general procedure was to prepare subcellular fractions from a region of brain known to have high opiate binding activity (8). We then characterized these fractions by ultrastructural and biochemical analysis. Finally, using a ligand (etorphine) which is known (9) to bind well to all putative opiate receptors we determined binding isotherms for each of the subcellular fractions.

EXPERIMENTAL: The work reported here was performed on microsomal, synaptosomal and myelin fractions prepared from corpus striata dissected from thawed ovine whole brains which had been frozen to -70°C within one hour of slaughter. A typical weight of dissected corpus striatum was 3 grams per brain. The subcellular fractions were prepared as pellets, following precisely the methods described by Bradford et al (10), using discontinuous sucrose density gradient centrifugation to separate the synaptosomal from the myelin fraction. The microsomal fraction was collected by centrifuging the S_2 supernatant at 150,000 g for 1 hour. Detailed electron microscopic examination was made of each subcellular fraction from corpus striata, prepared as above from fresh ovine brains or from freshly thawed frozen ovine brains, or from each of these when the final treatment of the pellets, before fixing, was in isotonic medium which avoided the lysis of the subcellular organelles which otherwise occurred. The levels of the cytoplasmic marker enzyme, lactic dehydrogenase, which served as an indicator of the intactness of the membrane envelopes in the final pellets (11), were also determined in each of these cases. The results of all these studies showed that there were no apparent differences between organelles prepared from fresh or frozen striata, whether lysed or unlysed. Opiate binding studies are usually performed on lysed samples and we therefore continued this practice in the work described below. The ultrastructural morphologies of our preparations were not distinguishable from those contained in detailed studies of the subcellular fractionation of brain tissue (10,12,13). The number density of intact synaptosomes visible in our fields were comparable to that obtained for this fraction by other workers (Dr.J. de Belleroche, private communication). Preliminary binding studies showed no difference in the pattern of binding levels between fresh and frozen material. Thus, we carried out the analyses described below with frozen brains.

The binding assays were made in triplicate, as follows. [^{3}H]-etorphine; specific activity 36 Ci/mmol; Amersham) in tris-HCl buffer (50 mM, pH 7.4) was pipetted into Eppendorf reaction tubes in final concentrations of 0.1 to 50 nM (from stock solutions of 10 and 100 nM). Tris-HCl buffer was added to 0.9 ml total volume, the tubes being protected from light. Incubations were begun by addition of 0.1 ml of resuspended tissue pellets (5-20 mg pellet per tube) in tris-HCl buffer. The tubes were incubated in a shaking water bath at 25°C for 50 minutes in the dark. The suspensions were then filtered by suction on Whatman glass fiber filters (GF/C, 2.4 cm), washed rapidly with two 5 ml portions of ice cold tris-HCl buffer and dried. Non-stereospecific binding was determined by performing the incubation in the presence of 1.5 micromolar non-radioactive etorphine (gift from Reckitt and Colman, Ltd., U.K.) added as 20 microliters of 0.78 mM solution. The radio-

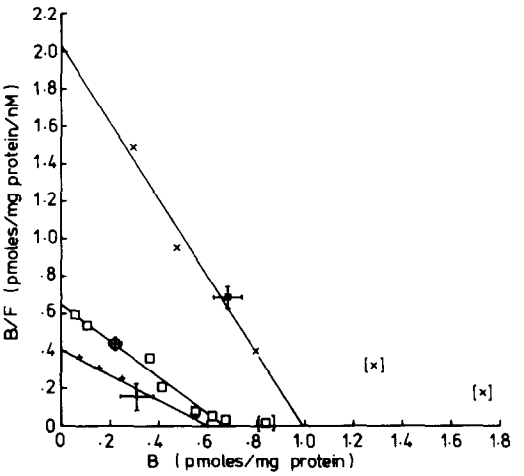

Figure 1 Scatchard plot of binding isotherms (25°C) of etorphine to subcellular fractions from ovine corpus striatum: × microsomes, □ synaptosomes, + myelin. Error bars are worst case counting standard deviations propagated through the calculation for each fraction. Bracketed points are those not used in determining the thermodynamic constants presented in this paper. Abscissa are stereospecific binding in pmoles/mg of protein; ordinate is stereospecific binding divided by free ligand concentration expressed in nM.

activity remaining on each filter was determined by scintillation counting after solubilization in a standard tissue solubilizing scintillation fluid. The vials were allowed to stand in the dark at 4°C for several hours before counting.

Protein analyses of the resuspended pellets were performed by the Folin-Lowry method following digestion of the suspension in 0.25 M NaOH for one hour at 37°C. The analyses were based upon duplicate samples in a range of at least two concentrations falling on the standard curve (which was determined by 5 points in duplicate using crystalline bovine serum albumin).

RESULTS AND DISCUSSION: Figure 1 summarizes our results in the form of binding isotherms, presented as Scatchard plots, from the 3 fractions studied. The derived constants, evaluated from a least squares analysis of the approximately linear portions of these plots is given in Table I.

It is apparent from Figure 1 that the binding isotherm for at least the microsomal fraction is quite non-linear and therefore the constants derived, while doubtless in the correct order, represent approximations to the actual cases. In an experiment of the type we have carried out the dissociation constant (K_D) should be equal to the inhibition constant (K_I) determined by competition, in the binding assay, for etorphine sites by a dissimilar

TABLE I

Dissociation constants (K_D) and binding capacities (B_o) for etorphine to opiate receptors in subcellular fractions from ovine corpus striatum.

Fraction	K_D (nM)	B_o (pmoles/mg protein)
microsomal	0.49	0.99
synaptosomal	1.04	0.67
myelin	1.50	0.61

molecule such as levorphanol (14). Using whole brain homogenates from guinea pig, Gillan et al (9) have determined the following constants: K_D = 0.5 nM([^{3}H]-etorphine, etorphine), K_I = 0.29 nM ([^{3}H]-etorphine, levorphanol), B_o = 15 pmoles/gm fresh brain tissue. With respect to the latter we find that the average protein content of our fractions is approximately 3% of the pellet weight.

We have chosen not to carry out further numerical analyses of the binding isotherms such as decomposition of the curves into "high affinity" and/or "low affinity" sites. As work of Glasel et al (15) has shown in another binding system, and as has been recently forcefully re-emphasized (16) such interpretations are usually misleading. An interpretation of the Scatchard plot in terms of a model for binding (such as the Scatchard model, stepwise equilibrium model, cooperative binding models, etc.) would require data to be taken over a vastly greater range of bound ligand values than we have attempted here, or has previously been attempted in these systems. We do, however, note that the exclusion of the non-linearity in, particularly, the microsomal fraction isotherm will cause our reported K_D to be a considerable over-estimate. That is, we have given a very conservative value for K_D in this fraction and inclusion of the above effects would give values much less than 0.5 nM. Within the errors inherent in this method it is conceivable that a self-consistent interpretation of the data is that the three subcellular fractions have at least one set of receptors each with similar binding capacities, but dissimilar binding constants.

Our results may be summarized as follows. The microsomal subcellular fraction from ovine corpus striatum has highest binding affinity and binding capacity for etorphine. Our results are roughly in accord, taking into account the remarks of the previous paragraph, with recent studies of Gillan et al (9), insofar as the weighted average of our inhibition constants and binding capacities (with change of units) are in qualitative agreement with their results.

The term "microsomal" is perhaps somewhat of a misnomer for this fraction from central nervous system tissue. Under ultrastructural examination its appearance is very different from the microsomal fraction from liver, for example. In particular, the rough endoplasmic reticula form only a small fraction of the stainable features. Other reports have questioned whether or not glial cross-examination is an important feature of the three subcellular fractions studied in this work (17). On the other hand, there is no evidence that junctional complex debris is present in our microsomal fraction. We have, in fact, prepared post-synaptic density fractions from our synaptosomal pellets indicating that post-synaptic elements are concentrated there.

We conclude that there is not a concentrated localization of opiate receptors specifically in the synaptosomes; rather non-synaptosomal structures present in striatum microsomes contain more of the receptors. Moreover, it appears that the thermodynamic quantities describing etorphine binding to these receptors differ in various subcellular fractions.

ACKNOWLEDGEMENTS

J.A.G. wishes to acknowledge the support of a National Institutes of Health Fogarty Senior International Fellowship and the use of funds from grant NIH/NS 15700-01. R.F.V. is a Medical Research Council Postdoctoral Fellow. We thank Mr. John Cavanagh for preparation of the electron micrographs. Prof. H.F. Bradford gave expert advice in the comparative analysis of these photographs.

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