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SPECIFIC BINDING FOR OPIATE-LIKE DRUGS IN THE PLACENTA

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Abstract—The properties of human placental opiate binding sites were analysed using [^{3}H]etorphine in terms of kinetic parameters, specificity, subcellular distribution and effect of ionic environment. *In vitro* these sites display a pharmacological profile similar to that of the pituitary opiate receptor site. In particular, they exhibit lower affinities for opioid peptides than do brain opiate receptors. They were detected exclusively in human placenta, in which they appeared during the first half of pregnancy.

β Endorphin was originally isolated from the pituitary gland [1] and was later found to exist in brain [2]. Recently Nakai *et al.* [3], Genazzani *et al.* [4] and Odagiri *et al.* [5] showed that β endorphin could be extracted from human placenta. Liotta and Krieger [6] reported that this organ was able to synthesize β endorphin *in vitro*. In brain, the distribution of endogenous opioid peptides is related to that of the opiate receptor [7]. Simantov *et al.* [8] reported the existence of a stereospecific opiate binding site in the pituitary gland and the relationship between opioid peptides and hypophyseal endocrine processes is now well documented [9]. Previously we gave some evidence for the presence of such specific opiate binding sites in human placenta [10]; the aim of the present work is to characterize this placental opiate binding site.

MATERIALS AND METHODS

Preparation of tissues fraction

Placenta. Human full term placentas were obtained immediately after vaginal delivery or Caesarian section. Placentas of earlier gestation were obtained after Brindeau Caesarian section or legal abortion at 9 gestational weeks. Pregnant rats, mice, rabbits and hamsters were obtained after mixing males with females in a ratio of 1/5. Gravid females were killed when close to term and placental tissue was excised.

Placentas were collected on ice, minced with scissors and washed several times with cold Tris–HCl buffer (0.05 M, pH 7.4, 25°). All further operations were carried out at 4°. The tissue was freed from blood vessels and connective tissue, and homogenized in 5 vol. Tris–HCl buffer (0.05 M, pH 7.4) in a Potter–Elvehjem homogenizer (five strokes at maximal speed). The homogenate was centrifuged (1000 *g* for 10 min) and the supernatant fluid was spun at 100,000 *g* for 30 min; the resulting pellet was washed once and spun again for an additional period of 30 min at 100,000 *g*. The final pellet was homogenized and then diluted with Tris buffer (0.05 M, pH 7.4, 25°) to give a final protein concentration of about 1 mg/ml.

Brain. Wistar rats were decapitated and the brain minus cerebellum was homogenized in a Potter–Elvehjem homogenizer. The homogenate was centrifuged (100,000 *g*, 30 min) and brain membranes were then obtained as described for placenta.

Binding assay

The reaction mixtures (1 ml) contained 0.8 mg of placental or brain protein, 1 pmole of [^{3}H]etorphine with and without 1 nmole of levorphanol. They were incubated always in triplicate, at 37° for 30 min. Immediately after incubation, they were filtered under reduced pressure through Whatman glass fiber disks (GF/B) and washed with 10 ml of ice-cold Tris buffer (0.05 M, pH 7.4, 25°). The filters were dried and counted in 10 ml of toluene scintillation cocktail. In some experiments free and membrane-bound etorphine were separated by centrifugation at 100,000 *g* for 30 min. The pellet was washed with 2 ml of cold Tris buffer (0.05 M, pH 7.4, 25°) and counted in 10 ml of toluene scintillation cocktail.

Protein concentration were estimated by the method of Lowry *et al.* [12]. Specific [^{3}H]etorphine binding was defined as the difference between the radioactivity bound to membranes in the absence and in the presence of 1 μM of levorphanol.

Chemicals

Chemicals were obtained from the following sources: [15.16(*n*)-^{3}H]etorphine, 35.4 Ci/mmole (Radiochemical Centre, Amersham, U.K., dextrorphan and levorphanol (Hoffman-La Roche, Basel, Switzerland), naloxone (Endo, Brussels, Belgium), morphine and codeine (Francopia, Paris, France), cyclazocine (Winthrop, U.K.), diprenorphine (Reckitt & Colman, London, U.K.), haloperidol (Janssen, Brussels, Belgium), D-Ala$_2$-leucine and methionine enkephalinamide (Dr. Mazarguil, Toulouse), β endorphin (Beckman).

RESULTS

Specific [^{3}H]*etorphine binding to subcellular fractions of human term placenta.* Table 1 summarizes the results of [^{3}H]etorphine binding determination

Table 1. Distribution of [^{3}H]etorphine binding site in subcellular fractions of the human placenta*

Fraction	Specific binding sites [^{3}H]etorphine (1 nM) (fmoles/mg protein)	Number of binding sites expressed as per cent of homogenate
Homogenate	14 ± 4.5	100
Nuclear (1000 *g*)	8.5 ± 3.3	38 ± 9.5
Mitochondrial (20,000 *g*)	42 ± 12.5	44 ± 9.5
Microsomal (100,000 *g*)	34 ± 5	11 ± 1

* The tissue fractions were prepared by homogenization and differential centrifugation in 0.32 M sucrose as described in Material and Methods. Each fraction was homogenized in Tris buffer (0.05 M, pH 7.4) and diluted with the same buffer to give a final protein concentration of 1 mg/ml. The results represents mean ± S.E.M. of four independent experiments.

carried out on different subcellular fractions. The data indicated a 3-fold increase in the specific activity of the 20,000 and 100,000 *g* pellets compared to that of the homogenate. Electron microscopic examination of these fractions indicate that the pellet obtained at 20,000 *g* contained mitochondria and large unidentified fragments, whereas microsome-like vesicles were located in the 100,000 *g* pellet. Using marker enzyme analysis, Whitsett and Lessard [11] reported a similar constitution of the placenta subcellular fraction. The etorphine binding sites (93 per cent) cosedimented with the particulate fractions (nuclear, mitochondrial and microsomal), indicating that these sites were membrane-bound. Since the 20,000 and 100,000 *g* pellets have almost identical site densities, a crude membrane fraction was used in all subsequent experiments. We checked that specific [^{3}H]etorphine binding was similar whether the [^{3}H]etorphine–membrane complex was isolated by filtration through Whatman glass fiber filter or centrifugation at 100,000 *g* for 30 min as described in Materials and Methods.

Kinetics of binding. Binding of [^{3}H]etorphine to human term placenta membrane was time and temperature dependent. Figure 1 shows the time course of binding of 1 nM [^{3}H]etorphine at 4° and 37°. At 37° the equilibrium was reached within 10 min and we have previously found an association constant K_1 of 0.2×10^9 $M^{-1}.min^{-1}$ [10]. When the incubation was carried out at 4°, maximal binding was observed after 90 min and represented 73 ± 2.5 per cent (mean ± S.E.M. of six experiments) of maximal binding measured at 37°. We have shown previously [10] that at 37° [^{3}H]etorphine binding is reversible with a dissociation rate constant K_{-1} of 0.075 min^{-1}.

The concentration dependence of [^{3}H]etorphine binding was studied over concentrations ranging between 1×10^{-10} and 4×10^{-9} M. According to the Scatchard plot (Fig. 2) placental membrane exhibited a single class of noninteracting binding site for [^{3}H]etorphine. These binding sites were characterized by a dissociation constant of 0.43 ± 0.06 nM and a binding capacity of 64 ± 4.8 fmoles (mean ± S.E.M. of 4 independent experiments). The K_D value derived from the Scatchard plot agrees very well with that (0.39 nM) calculated from the ratio K_{-1}/K_1.

Specificity. The specificity of the opiate binding site in the human term placenta was assessed by the

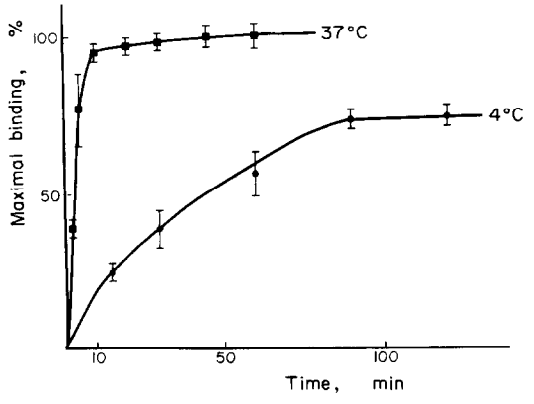

Fig. 1. Time course of [^{3}H]etorphine specific binding to human term placental membranes. The binding of [^{3}H]etorphine (1nM) was followed at 4° and 37° as indicated and expressed (mean ± S.E.M. of four experiments) as per cent of the maximal binding.

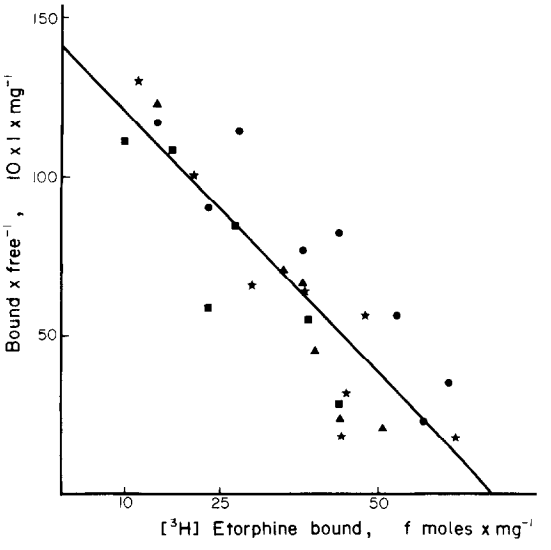

Fig. 2. Scatchard plot of specific [^{3}H]etorphine binding to human term placental membranes. The ratio of bound to free [^{3}H]etorphine is represented as a function of [^{3}H]etorphine concentration added in the incubation medium. Results of four independent experiments are indicated by the different symbols.

Table 2. Relative potencies of drugs in reducing [^{3}H]etorphine binding to human term placental membrane and opiate brain receptor*

	IC_{50} (nM)	
	Placenta (N = 4)	Brain (N = 2–3)
Cyclazocine	2 ± 0.5	4
Levorphanol	2.4 ± 0.3	12.5
Diprenorphine	3.9 ± 0.5	3.3
Naloxone	8.8 ± 2.8	14
Morphine	350 ± 140	63
β Endorphin	309 ± 65	24
D-Ala_2-Leu-enkephalinamide	866 ± 88	11
D-Ala_2-Met-enkephalinamide	1000 ± 260	14
Haloperidol	7500 ± 2400	
Codeine	25,000 ± 500	
Dextrorphan	> 25,000	

* [^{3}H]Etorphine (1 nM) was used. Incubations were carried out at 4° for 2 hr. IC_{50} values from independent experiments were determined from log-probit regression analysis.

ability of various drugs to inhibit binding of [^{3}H]etorphine at 4° (Table 2). In this test, levorphanol was 1000-fold more potent than dextrorphan, indicating stereospecific opiate–site interaction in placental membrane. Opiates and opioid peptides competed with [^{3}H]etorphine for binding. The apparent affinities of opioid-like drugs for the placental site were compared with those measured in the same condition for opiate brain receptor (Table 2). The opiate antagonists diprenorphine and naloxone, and the opiate agonist–antagonist, cyclazocine, were nearly as effective in inhibiting binding of the radioactive ligand in placenta as in brain.

Morphine and opioid peptides were equipotent in antagonizing the binding of [^{3}H]etorphine to placental site; however, these compounds are weaker inhibitors in placenta than in brain. Codeine and haloperidol, which did not interact with opiate brain receptor, displayed no significant affinities for the [^{3}H]etorphine placental binding site.

Etorphine binding on human placenta obtained at various ages of gestation. The [^{3}H]etorphine binding capacity was measured at 2, 5 and 9 months of pregnancy. The results are reported in Fig. 3. Specific [^{3}H]etorphine binding increased 10-fold between 2 and 5 months of gestation after which it remained constant until term. Kinetics study of [^{3}H]etorphine binding on 2-, 5- and 9-months-old placenta indicated that [^{3}H]etorphine affinity did not change, so that the increase observed reflected an increase of the number of binding sites.

Etorphine binding on placenta obtained from various species. The specific binding of [^{3}H]etorphine was measured on membrane prepared from the placentas of different mammals. Rabbit, hamster and mouse placentas showed no detectable specific binding whereas those of rats had low levels of specific binding: 13 per cent of those measured under the same conditions in human placental membranes (Table 3).

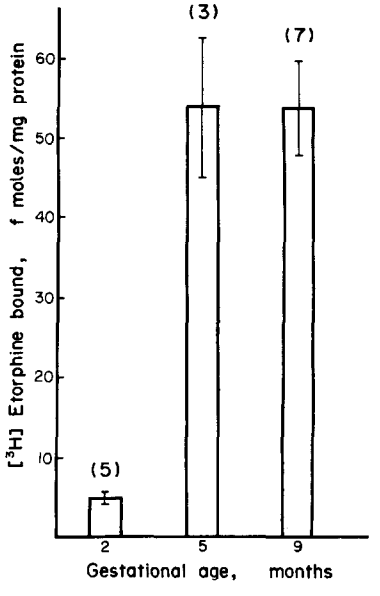

Fig. 3. Specific [^{3}H]etorphine binding to human placental membrane at various ages. The binding capacity of 5-months-old and term placenta was measured after incubation of various concentrations with placental membrane; the number of binding sites was determined from a Scatchard plot. The results reported on this graph represented mean ± S.E.M. of the binding capacity of three to seven placentas (number in parentheses). The maximal binding of [^{3}H]etorphine to membrane of 2-months-old placenta was studied with 2 nM [^{3}H]etorphine.

Table 3. Specific binding of [^{3}H]etorphine to placental membrane of various species*

Species	[^{3}H]Etorphine bound (fmoles/mg protein)	Number of animals
Mouse	Not detectable	5
Rat	7.5 ± 1.4	4
Hamster	Not detectable	5
Rabbit	Not detectable	4
Sheep	Not detectable	1
Human	53.5 ± 9	7

* Membranes were prepared from term placental of various species and incubated with [^{3}H]etorphine (2 nM) for 30 min at 37°.

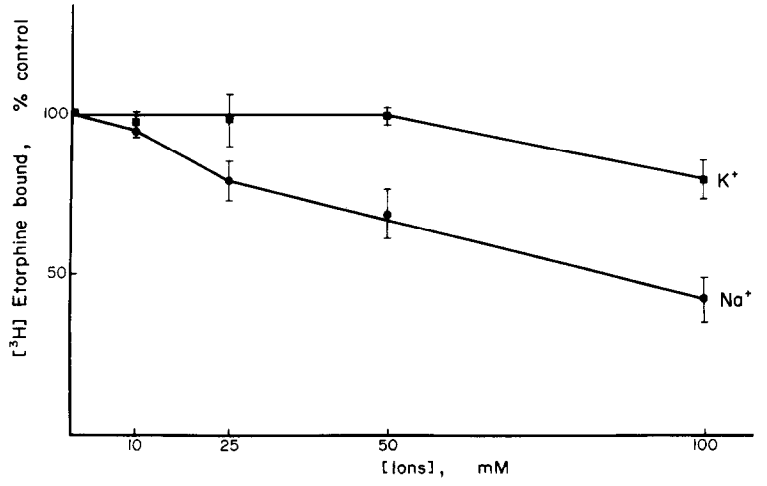

Fig. 4. Effect of sodium and potassium on the stereospecific binding of [^{3}H]etorphine. Human term placental membranes, suspended in Tris–HCl buffer with or without sodium or potassium at various concentrations were incubated at 37° for 30 min with 1 nM [^{3}H]etorphine. Specific binding is expressed as per cent of control measured in Tris–HCl.

Effect of Na^+ *and* K^+ *on the specific* [^{3}H]*etorphine binding on human term placenta.* Opiate brain receptor binding is markedly modified by the ionic environment: low concentrations of Na^+ selectively enhance the binding of opiate antagonists and decrease the binding of opiate agonists [13, 14]. In the case of term placental opiate binding sites, Fig. 4 indicates that a 50 per cent inhibition of 1 nM[^{3}H]etorphine binding was observed in the presence of 80 mM Na^+; K^+ was less effective with a 15 per cent inhibition at the same concentration.

DISCUSSION

The results of the present work confirms our earlier observations [10]: a stereospecific binding site for opiate-like drugs is present in human placenta.

The opiate binding site must be located on membranes because the specific activity is maximal in the mitochondrial and microsomal fractions.

The [^{3}H]etorphine binding site in placental membranes satisfies all the major criteria for a specific opiate binding site. Binding is rapid and reversible [10], saturable and stereospecific. At equilibrium maximal binding is greater at 37° than at 4°; a similar observation was reported by Simantov for opiate brain receptor [15]. Etorphine binds with a high affinity (K_D = 0.43 nM) to one single class of non-interacting binding sites. The kinetics parameters and the affinity determined by the Scatchard plot are in good agreement.

The placenta binds opiate-like drugs with stereospecific selectivity. The results of the binding studies suggests that opiate agonists and antagonists have similar potencies in placenta and in brain. However, morphine displays a very low potency in placenta; these results confirm our previous observation [10]. Furthermore, opioid peptides are weaker inhibitors in placenta than in brain. Two possible hypotheses can be formulated to explain such a phenomenom: the influence of a metabolic process, at least for opioid peptides, or the presence of a particular binding site. Indeed, placenta contains large amount of proteolytic enzymes. We have performed the incubation at 4° and checked the potency of opioid peptides using metabolically stable derivatives. Accordingly, β endorphin is still degraded by placental membrane at 4° because we have observed a loss of 25 per cent in the binding activity, corresponding to a decrease of 50 per cent in the concentration compared to incubation carried out with boiled placental membranes. Under the same conditions such a result is also found for the two enkephaline derivatives but to a lesser extent (30 per cent decrease in the concentration), whereas levorphanol exhibit no measurable loss of activity. Metabolism of opioid peptides in placenta differs markedly from that reported in brain [16, 17]; its characterization is in progress. A metabolic process cannot, however, explain the difference in the potency of opioid for placenta binding and brain receptor. Simantov *et al.* [7] reported that hypophyseal opiate receptor displays an affinity lower for morphine and enkephaline derivatives than opiate brain receptor: it seems likely that pituitary and placenta opiate binding site present similar pharmacological specificity which is quite different from that of opiate brain receptor.

One of the most characteristic feature for brain opiate receptor is its selective alteration by sodium ions [13, 14]. A similar property is found for placental opiate binding site; the [^{3}H]etorphine binding decreased with the sodium concentration, 50 mM sodium induced a 35 per cent reduction, whereas 10–50 mM potassium had no effect.

Etorphine binding to human placenta can be demonstrated early in pregnancy; the maximal number of binding sites increases 10-fold during the first part of gestation and remains constant until term. The ontogenetic evolution of etorphine binding sites is parallel to placental maturation, as this process take place until 4 months of gestation; thereafter only placental weight increases. Phylogenic studies indi-

cated that this site seems specific for human placental type.

The present study demonstrates specific binding sites for opioid-like drugs in the human placenta and raises the question of their physiological significance. Protein hormones similar to those produced by the anterior pituitary gland have been isolated from placental tissue: chorionic gonadotropin [18], somatomammotropin [19], corticotropin [20] and β endorphin [3]. In addition, we have found that placental opiate binding site specificity is quite similar to those of pituitary. In the placenta little is known about whether or not the release of these peptidic hormones is under the control of some local factor.

It will be assumed that as in the hypothalamo-hypophyseal axis, β endorphin participates in the regulation of placental endocrine function during gestation. The specific opiate binding site demonstrated in this study could be implicated in such a physiological role.

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