BIOCHEMICAL CHARACTERIZATION OF OPIATE RECEPTORS IN THE HUMAN PLACENTAL MEMBRANE FRACTIONS

Ph.D.THESIS

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II. ABBREVIATIONS

B_{max}	: Maximum number of binding site
Beta-EP	: Beta-endorphin
CNS	: Central nervous system
Cam-20	: Cambridge-20 (PD-117302, no name available)
DADLE	: D-Ala^2,D-Leu^5-enkephalin
DAGO	: D-Ala^2,N-Me-Phe^4,Gly-ol^5-enkephalin
DALAMID	: D-Ala^2-enkephalin 5-L-Methionine amide
DALE	: Tyr-D-Ala^2-Leu^5-enkephalin
DAMGE	: Tyr-D-Ala^2-D-Leu^5-enkephalin
DPDPE	: D-Pen^2-D-Pen^5-enkephalin
EDTA	: Ethylenediaminotetra acetic acid
EKC	: Ethylketocyclazocine
hPL	: Human placental lactogen
IC_{50}	: The observed value for 50 % inhibition of radiolabelled ligand binding in displacement assays
K_D	: Equilibrium dissociation constant
NEM	: N-ethylmaleimide
PCP	: Phencyclidine
PMSF	: Phenylmethyl sulphonylfluoride

PNS	:	Peripheral nervous system
RVD	:	Rat vas deferens
SE	:	Standart error
STI	:	Soybean trypsin inhibitory
TCP	:	N-(1-/2-thienyl/cyclopiperidine)
Tris	:	-(hydroxymethyl)aminometan
U-50,488H	:	Trans-(+/-)-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamide methane sulfonate

III.ABSTRACT

The human placental membrane-bound opioid receptors were studied. In our procedure concerning microvillous membrane preparation contained of about 90 % number of total naloxone binding sites.

In kinetical assays, a rapid and a slow phase of dissociation were obtained using ^{3}H-naloxone and ^{3}H-EKC. However, in the case of equilibrium analysis Scatchard curve showed a single binding site with ^{3}H-naloxone (K_D= 7.4 nM; B_{max}= 114.8 fmol/mg protein), although two binding sites appeared in the case of ^{3}H-EKC (K_D= 0.9 nm ; B_{max}= 5 fmol/mg protein for high affinity site and K_D= 25 nM ; B_{max}= 85 fmol/mg protein for low affinity site).

A series of kappa-selective ligands (PD-117302, Dynorphin(1-13), EKC, and U-50,488H) were tested in displacement experiments, and found to be potent inhibitors of agonist and antagonist binding.

The stereospecific property of the opiate receptors from human placenta was proved by using the active enantiomer levorphanol and inactive dextrorphan.

We also observed the decreasing effect of Na^{+} on the ^{3}H-EKC binding.

Finally we could confirm that human placental microvillous membrane contains mainly (89 %) the kappa-opiate binding sites.

1.INTRODUCTION

1.1.RECEPTORS

Ever since Langley proposed the term "receptor" almost 100 years ago, biochemists have been interested to know more about the structures of these elusive macromolecular conglomerates, but the detailed chemical structures are still just partly known.

The word "receptor" is used differently in different scientific fields. In physiology, it is used for sensory devices for olfaction, vision, blood pressure regulation, mechanical contact etc., and is not always defined at the molecular level. In pharmacology and biochemistry, the concept of receptor is a defined macromolecular structure, that specifically combines with a ligand to convey a signal into a biological compartment (Koman, 1985). However one should be aware that the term receptor usually implies both a binding site and a transducing element that permits the binding of suitable ligands to trigger the events that lead to the physiological response (Simon, 1981). A ligand is a low molecular weight substance that binds to the receptor specifically.

One has to deal with several kinds of receptors, for instance neurotransmitter receptors, hormone receptors, and

finally drug receptors. It is obvious that not all receptors are classified once and for all, since the classification depends on the biological role of the ligand, which can be either endogenous or synthetic (Braestrup, 1980).

Many receptor systems consist of seperate molecular units for recognition, transduction, and catalysis. Affinity of a receptor is that recognition units of a receptor bind ligands very specifically, and affinity for a receptor does not necessarily implicate activation.

The ligand may or may not have the properties that will cause the convenient change in the recognition site resulting in further transfer of the stimulus. This property of the ligand is defined as efficacy, and reflects the relative agonist and antagonist characteristics of the ligand. A pure antagonist ligand may bind to the recognition unit strongly, i.e. have high affinity, but not induce a signal, i.e. have zero efficacy. Many ligands behave as partial agonists and depending on the assay system, they may show predominantly agonist or antagonist characteristic (Koman, 1985).

Most receptors, however , are integral membrane proteins and are means of communication between the two compartments physically seperated by the membrane which is a permeability barrier consisting of a "fluid mosaic" lipid bilayer (Singer, 1972). Molecular pharmacology of receptors started in the seventies when receptor binding assays were developed to measure the binding of radio-labeled ligands to the receptor.

Beside equilibrium and nonequilibrium measurements to determine binding constants the other goal of molecular "receptorology" was to establish the primary, secondary, and tertiary structure of receptors. The first procedural step in such studies consists of solubilizing the receptor out of membranes and its purification (generally by affinity chromatography), followed by partial determination of the amino acid sequence of the pure protein and/or nucleotide sequence of the receptor gene. Finally, in order to convincingly demonstrate that the receptor structure determined is correct and functional, it is necessary to reincorporate the isolated protein into membranes, to demonstrate that binding of the ligand is accompanied by the anticipated ionic permeability changes or by other biological parameters of receptor activation. Therefore, reconstitution of the purified receptor into the membrane is necessary in order to establish the receptor function of the binding protein (Wollemann, 1987).

In higher organisms, receptor may be classified into those for neurotransmitters, for neuromodulators and for hormones, and exist in the CNS, PNS or various non-neural cells.

1.2.OPIOID RECEPTORS

The extract of the opium poppy is among the oldest

materials used for medicinal purposes, and the study of the major alkaloid in this extract, morphine is one of the oldest areas of biological research. Human beings have been using opiates for centuries to releive pain. The opiates produce a large variety of pharmacological responses, the most important of which are analgesia, euphoria, and addiction. The latter includes the development of tolerance and physiological dependence. The receptors are tightly bound to cell membranes and appear to be concentrated in the synaptic region. Opioid receptors were first postulated by Beckett and Casey in 1953 and were confirmed to exist in 1973 by Terenius, Simon et al. and Pert and Snyder. Since 1973, there is considerable evidence that opioid drugs interact stereospecifically with opioid receptors on the neuronal cell membrane. Martin and co-workers (1976) postulated the existence of heterogenous opiate receptors. The discovery of opiate receptors in the central nervous system of animals, man, and more recently in some invertebrates (Stefano et al. 1980), and the resulting discovery of endogenous opiate like ligands for these receptor have given rise to considerable activity and excitement in the field of opiate research (Simon et al. 1973, 1978; Pert and Snyder, 1973; Terenius, 1973). Binding of opiates is stereospecific, saturable and of high affinity (K_D's range from 10^{-10}M to 10^{-7}M). The pH optimum for binding is in the physiological range (pH 7-8). Binding is very sensitive to proteolytic enzymes and to a

large number of protein reagents, suggesting the involvement of protein in opiate binding (Simon, 1987). The hypothesis that opiates must bind to specific sites located on the surface of or inside nerve cells in order to exert their effects has been put forth by investigators for several decades (Simon et al. 1978; Snyder, 1986).

The finding that these receptors exist in a wide variety of species and have survived throughout evolution suggested that they must serve an important function for the organism that carries them. The search for endogenous ligands was begun among the many known brain chemicals, but none of the classical transmitters and neurohormones exhibited significant affinity for opiate binding sites (Simon, 1987).

The molecular properties of the opioid receptors are under investigation, but the relationship between the data from biochemical and pharmacological experiments of the multiple opioid system are not well understood. The visualization of opiate receptor complexes and models can also be found in the literature on the basis of experimental data.

1.3.TERMINOLOGY

Terms such as "narcotics", "opiates", and "opioids" are often used to describe compounds that have a pharmacological profile similar to morphine. The word "narcotic" was at one

time used to describe any substance that induced sleep. Although in sufficient doses, all morphine-like drugs will induce sleep or even anasthesia, the term narcotic is not specific for this class of drugs and can be equally applied to other classes, e.g. barbiturates or benzodiazepines. Opiates are, strickly speaking, drugs that are either directly or indirectly derived from one of the opium alkaloids. However, many of the newer compounds, although having pharmacological properties similar to the opium alkaloids, are fully synthetic substances that have chemical structures that bear little similarity to them. While morphine is an opiate, pethidine (meperidine) and fentanyl are not. The prefered generic name for the class of drugs with morphine-like activity is "opioid". An opioid is an exogenous substance, natural or synthetic, that binds specifically to any of the several sub-species of opioid receptors and produces some agonist effect (Bovill, 1987). In discussing the multiple opioid receptors a consistent terminology is helpful. At the 1983 International Narcotic Research Conference, a committee on nomenclature (A.Goldstein and H.W.Kosterlitz) endorsed the term "opioid receptor" in place of "opiate receptor". They agreed with the current tendency to drop "endorphin" as a generic term for endogenous ligands, and to use it only for opioid products of the pro-opiomelanocortin gene (Goldstein et al. 1984).

1.4.CLASSIFICATION OF OPIOID RECEPTORS

Since the discovery of opiate receptors in the CNS (Pert and Snyder, 1973; Simon et al. 1973; Terenius, 1973) a number of lines of evidence have suggested that several subtypes of the receptor may exist. Martin et al. (1976) and subsequently others (Lord et al. 1977; Simantov et al. 1978; Smith et al. 1980) demonstrated pharmacological and biochemical evidence for the existence of heterogenous opiate receptor populations in several different tissues. Frenk et al. (1978) reported different pharmacological responses on administration of enkephalin and opiates into different brain regions. Lord et al. (1979) and Beddell et al. (1977) observed different potencies of opioid peptides and opiates in the mouse vas deferens and guinea pig ileum. The brain contains at least three sepeŕate opoid receptor subcategories, mu, delta, and kappa. Current evidence suggest that each of the three large protein precursors, pro-opiomelanocortin, pro-enkephalin, and pro-dynorphin, produces opioid peptides that interact differentially with mu-, delta-, and kappa-receptors ,respectively. However, this is in no way exclusive. For instance, enkephalins that appear to interact predominantly with delta-receptors can also interact with mu-receptors that mediate analgesia. Also it seems that mu- and delta-receptors have an equal affinity for beta-endorphin, which has a strong analgesic action. In fact, beta-endorphin could well exert

its actions through its own specific category or receptor ,epsilon-receptor, (Schulz et al. 1979; Wuster et al. 1979, 1980; Garzon et al. 1985; Bradford, 1986). The several forms of endogenous opioids not only are biologically active, but almost all of them can interact with several kinds of opioid receptors (Holaday, 1985). Pharmacological studies of morphine congeners and of opioid peptides enabled in turn a more detailed classification of various opioid receptor types (Table-1). The main types are classified as MU for morphine-type ligands, DELTA for enkephalins, the shortest opioid peptides, and KAPPA for dynorphin and some benzomorphans (Paterson et al. 1983; Koman, 1985; Lynch and Snyder, 1986; Bovill, 1987); each of them binds its specific ligand with maximal affinity, thus giving rise to a physiological effect. Whether SIGMA receptors should physiologically be classified as opioid receptors is controversial.

Multiple classes of opiate receptors have been studied by autoradiographic techniques in vitro. Mu-receptors are localized mostly in areas associated with pain perception, such as the medial thalamus and periaqueductal grey, suggesting their involvement in the analgesic effects of opiates (Goodman et al. 1980). Delta-receptors are localized in limbic areas, and may mediate euphoric effects (Goodman et al. 1980). Kappa-receptors are more concentrated in deep layers of the cerebral cortex (Goodman and Snyder, 1982;

TABLE-1. SUMMARY OF GENERALLY ACCEPTED RECEPTOR CLASSES.

Receptor subtype	Endogenous ligand	Prototype ligand	Invitro bioassay
MU	Beta-endorphin	Morphine	Guinea-pig ileum
DELTA	Leu-(met-) enkephalin	D-Ala^2-D-Leu^5 enkephalin	Mouse vas deferens
KAPPA	Dynorphin	Ethylketo-cyclazocine	Rabbit vas deferens
EPSILON	Beta-endorphin	?	Rat vas deferens
SIGMA	?	SKF-10,047	?

Abbreviations : ?=Not known; SKF-10,047= N-allylnorcyclazocine.

Maurer et al. 1983; Slater et al. 1986) and may cause sedative effects or analgesic effects. Sigma-receptors are highly concentrated in a series of motor nuclei, in large neuronal cells, as well as in the sensory nuclei and in the pyramidal cells of the hippocampus (Largent et al. 1984) and may cause psychotomimetic effects. Tempel and Zukin (1987) have been shown that their finding of distinct localizations for those receptor classes is consistent with the hypothesis that these receptors are synthesized through independent biosynthetic pathways and serve distinct functions. In addition, Zukin and Zukin (1984) have reviewed that these four receptors (mu, delta, kappa, and sigma) are thought to mediate distinct behavioral syndroms. Particularly striking are:

1.- The kappa syndrome which includes ataxia and pronounced sedation.

2.- The sigma syndrome which includes hallucinations and dysphoria.

Most of the known opiate drugs and opioid peptides interact with more than one of these receptor sites. Thus, the complex neuropharmacological actions of a given opioid would appear to reflect its interaction with varying potencies at a combination of these sites. Different potencies between enkephalins and opiates in their ability to compete for the binding of ^{3}H-labelled narcotics and ^{3}H- or ^{125}-I labelled

enkephalins or their substituted analogues have been reported by Lord et al. (1977) and confirmed by many other laboratories (Simantov et al. 1978; Chang et al. 1978; Law et al. 1978). Differential effects of cations (Law et al. 1978; Chang et al. 1979), temperature, N-ethylmaleimide and 3,5-diiodo-4-diazosulfonilic acid on the binding of ^{3}H-enkephalin or ^{125}I(D-Ala,D-Leu)-enkephalin and ^{3}H-dihydromorphine have been observed.

Goldstein (1984) mentioned that an ideal criteria for differentiating between types of opioid receptor have not yet been realized. For differentiation of receptor types and measurement of selectivity in pharmacologic assays we still need highly selective antagonists. Meanwhile, selective alkylation (and protection from alkylation) and selective induction of tolerance in bioassays are yielding useful information about the pharmacologic selectivity of agonist for the multiple types of opioid receptor.

1.5.CHARACTERIZATION OF MU-AND DELTA-RECEPTORS (SIMILARITIES AND DIFFERENCES)

Comparison of the results of binding studies in the brain with those of bioassays in preparations innervated by cholinergic or adrenergic nerves suggested that opiate receptors populations in both the central and peripheral

systems are heterogenous (Zukin and Zukin, 1981a). The characteristics of the receptor in the guinea-pig ileum resembled Martin's mu-receptor and was termed the mu-receptor while the receptor in the mouse vas deferens was called delta-receptor. Guinea-pig brain appeared to parallel the mouse vas deferens most nearly in its receptor sub-class distribution. By contrast, many neuroblastoma cell lines have been shown to bear only enkephalin or delta receptors (Chang and Cuatrecasas, 1979). More recently, studies involving the competiton of ligands for radiolabelled opiate binding sites in specific brain regions (Chang and Cuatrecasas, 1979; Simantov et al. 1978; Leslie et al. 1980) have provided further biochemical evidence for mu- and delta-receptors, and indicate that these have somewhat different distributions throughout the brain. Thus, for instance, the thalamus and hypothalamus were shown to be relatively enriched in mu-receptors. In contrast, the frontal cortex and striatum appeared to have equivalent densities of these receptor types. Moreover, Pasternak and his coworkers have shown that high affinity morphine binding sites, identified as a class of mu-receptors mediating analgesia and low affinity sites, possibly receptors, appear at different times in the developing rat brain (Zhang and Pasternak, 1980). The multiplicity of opioid receptor subtypes on the basis of pharmacological and biochemical studies was investigated by Wolozin et al. (1981, 1982) and Nishimura et al. (1984).

They demonstrated that the biphasic displacement curves of ^{3}H-labelled D-Ala^{2}-Met^{5}-enkephalinamide, D-Ala^{2}-D-Leu^{5}-enkephalin, and dihydromorphine by all opiates and enkephalins tested becomes monophasic carrying the high affinity displacement after treatment of the tissue with irreversibly labeling ligand, naloxazone or oxymorphazone (Varga et al.1987). They termed this high affinity binding site the mu_1-site. This site is involved in the mediation of the analgesic effects of opiates. Other laboratories also proved the existence of mu_1-binding sites (Pasternak, 1980, 1982; Pasternak et al. 1980, 1981; Hahn, 1982; Zhang and Pasternak, 1981). Together these studies provide considerable substatiation for mu- and delta-receptors.

The identification of distinct mu- and delta-receptor types has been facilitated by the recent synthesis of highly sub-type selective ligands. Newman and Barnard (1984) using highly selective ligands, DAMGE (approx.150-fold more selective for mu-than for delta-sites) and DPDPE (approx.200-fold more selective for delta-than for mu-sites), provided strong evidence that the major ligand binding proteins of mu-and delta-receptors are indeed distinct entities of different molecular sizes. In Simon's laboratory (1975) the sulfhydryl reagent N-ethylmaleimide ,NEM, was used for the irreversible inactivation of opiate receptors in rat brain. ^{3}H-naltrexone binding was protected effectively by morphine and naloxone and less effectively by the enkephalins.

Tritiated (D-Ala^2-D-Leu^5)-enkephalin ,DADLE, binding was protected 80 times more with unlabelled enkephalin than with opiate alkaloids. Robson and Kosterlitz (1979) irreversibly inactivated the opiate receptors using phenoxybenzamine. The delta-binding sites showed a high for (D-Ala^2,D-Leu^5)-enkephalin and they could be protected more effectively by enkephalin than by the mu-selective dihydromorphine. On the other hand, unlabelled dihydromorphine exhibited a higher degree of protection of the ^{3}H-dihydromorphin binding sites than of the ^{3}H-(D-Ala^2,D-Leu^5-enkephalin binding sites. The relatively non-selective peptide (D-Ala^2,D-Leu^5)enkephalinamide protected both sites equally well. Those results which were mentioned above suggested the existence of mu- and delta-receptor subtypes. In addition, mu-and delta-receptors increase membrane potassium conductance leading to an inhibition of transmitter release and cell firing (North, 1986).

1.6.CHARACTERIZATION OF KAPPA- AND SIGMA-RECEPTORS

To establish the presence of kappa- and sigma-receptors has been more difficult. Thus, the first in vitro receptor binding studies involving ^{3}H-ethylketocyclazocine (EKC),putative mu and kappa ligand (Hiller et al. 1979; Pasternak et al. 1980; Chang et al. 1980), concluded that there were no distinct kappa-receptors in brain. These investigations involved competitive displacement analysis and regional distribution studies of the 3H-EKC high affinity

binding component only (Zukin and Zukin, 1981a). A number of investigators were unable to find evidence for the existence of distinct kappa-receptors in the brain due to the fact that EKC bound almost equally well to the mu- and delta-binding site (Hiller et al 1980; Harris, 1980; Pasternak, 1980). Furthermore, the kappa-binding site in the rat brain was found to be about 10-15 % of the total opiate binding sites. Kosterlitz and coworkers (1978, 1980a and 1980b; 1981a and 1981b) demonstrated a relatively higher concentration of kappa-binding sites in the guinea pig brain in the presence of blocking agent for the delta-(100 nM D-Ala^2-D-Leu^5-enkephalin) and the mu-sites (100 nM D-Ala,-(Me)Phe,Gly-ol-enkephalin). Goodman et al. (1982) and Cross et al. (1987) have been shown that the highest density and most selective localization of putative kappa-receptors occurs in layers V and VI of the cerebral cortex. These deep cortical kappa receptors may account for the unique sedative and analgesic actions of kappa opiates. So far kappa-receptors have never been reported to be paired with an adenylate cyclase and are known to inhibit the voltage-sensitive calcium (Ca^{+2}) channel (North, 1986).

Vincent et al. (1979), and Zukin and Zukin (1979) first reported the existence of PCP-binding sites in brain membranes. Zukin and Zukin (1981b) suggested that Phencyclidine (PCP) binding sites and sigma receptor are the same receptor protein in rat brain. This idea was further

supported by the behavioral experiments of Holtzman (1980) and Harris (1980). Gundlach et al. (1985) labelled brain membranes with ^{3}H-N-allylnormetazocin and designated the high affinity site to the PCP-receptors. Zukin et al. (1983) demonstrated the existence of PCP binding sites in rat brain using ligands with different specificity. Using a PCP-derivative, ^{3}H-TCP (^{3}H-N-(1-/2-thienyl/cyclohexyl) 3,4-piperidine) in the receptor binding studies, Vignon et al. (1983) found 10 fold higher affinity to the receptor than using PCP.

1.7.CHARACTERIZATION OF EPSILON-RECEPTORS

The epsilon-opioid receptor subtype has been postulated to be specific for beta-endorphin on the basis of structure activity studies in the isolated rat vas deferens (RVD) (Miranda et al. 1979; Schulz et al. 1979). The fact that only beta-endorphin (beta-EP) was a potent agonist in RVD led to the proposal of a novel type of opioid receptor, the epsilon-receptor (Schulz et al. 1979; Wuster et al. 1979, 1980). In subsequent studies Schulz et al. (1981) concluded that in the RVD beta-EP acts through an opioid receptor type different from mu, delta, and kappa. Beta-EP binds with similar affinities to some of these receptors complicates the issue of characterizing the epsilon-receptor. Therefore, it is likely that cross-protection techniques as well as the

specific blockade of the different opioid receptors binding beta-EP will help in ascertaining the presence of this epsilon-receptor in the central nervous system (Garzon et al. 1985).

1.8.AIM OF THESIS

The placenta is a highly complex, metabolically very active organ which has multiple enzymes, protein and steroid hormones, and neurotransmitters (e.g. acetylcholine) (Sastry et al. 1979). Peripheral changes in endogenous opioids and the identified binding sites could directly, or by interactions with these other placental transmitters, affect placental or fetal cardiovascular or neuroendocrine function, maternal blood pressure, cardiac output, or pulse rate, resulting in acute or chronic effects on the fetus or placenta (Ahmed et al. 1986).

The placenta is a readily available tissue for studies of human receptors. It has been shown to contain receptors for hormones such as insulin and catecholamines (Ahmed et al. 1981). Valetta et al. (1979) identified and demonstrated stereospecific binding sites for etorphine and other opiates in human placenta. Placenta has also been reported to contain neurotransmitters such as acetylcholine (Sastry and Sadavongvivad, 1979), beta-endorphin (by radioimmunoassay) (Nakai et al. 1978), and methionine enkephalin and beta-

endorphin (Sastry et al. 1980). Thus, in spite of the fact that placenta is not innervated (Fujiyama et al. 1971), it displays many of the nervous tissue, however, the natural ligands for placental receptors might be different from those of nervous system opioid receptors (Ahmed et al. 1981).

The human placental microvillous tissue contains membrane bound etorphine binding sites where they appear during the first half of pregnancy (Porthe et al. 1982; Valetta et al. 1980). The opiate binding site must be located on membranes because the specific activity shows maximum activity in the mitochondrial and microsomal fraction (Valette et al. 1980). Morphine and enkephalins display a very low affinity for placental opiate binding sites suggesting that these binding sites do not correspond either to mu- or to delta-opiate receptors (Valetta et al. 1980). It was suggested that the human placenta contains a single class of opiate binding sites whose pharmacological characteristics correspond to those of kappa opiate binding site (Porthe et al. 1981; Ahmed et al. 1987; Valette et al. 1987). Similar binding sites are not found in the placenta of several mammals (e.g. hamster, sheep, mouse, and rabbit) (Porthe et al. 1982).

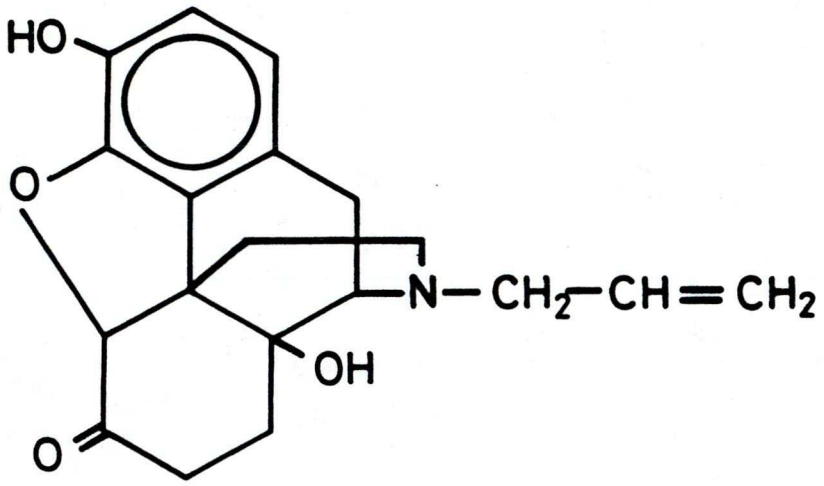
The aim of the studies presented in this thesis was to describe:

1- the optimal conditions for opioid receptor binding assays in the placental microvillous membrane fraction

(method of preparation of membrane fraction, incubation time, temperature and protein curve) for radioligand binding).

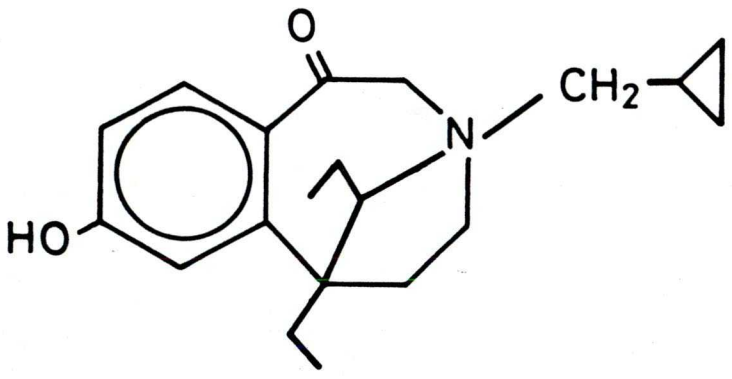
2- the distribution of opioid receptors in the placental microvillous membrane fraction.

3- the biochemical properties of human placental opioid receptors using ^{3}H-EKC,agonist kappa-site preferring ligand. This agonist binding is compared to the universal opioid antagonist, naloxone.



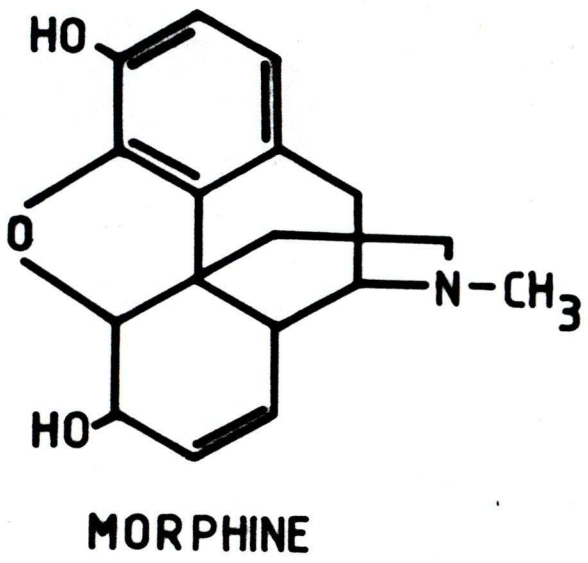
NALOXONE

Fig.-1. The chemical structure of naloxone.



Ethylketocyclazocine (EKC)

Fig.-2. The chemical structure of ethzlketoczclayocine.



MORPHINE

Fig.-3. The chemical structure of morphine.

2.MATERIALS AND METHODS

2.1.CHEMICALS:

^{3}H-Ethylketocyclazocine (27Ci/mmol) was purchased from New England Nuclear, ^{3}H-Naloxone (57Ci/mmol) was prepared at the Isotope Laboratory of Biological Research Center. Naloxone hydrochloride was kindly donated by Endo Laboratories, Garden City, NY. Levorphanol and dextrorphan tartarate were kindly provided by Hoffmann-La Roche, Inc., Nutley, NJ. PD-117302 (Cambridge-20) was kindly donated by Warner-Lambert/Parke-Davis Pharmaceut. Res. Div. MI,(USA) Dynorphin(1-13) was supplied by BACHEM (CH-4416 Bubendorf/Switzerland). U-50,488H was provided by Upjohn Co.Kalamazoo,MI,(USA). Unlabelled EKC was provided by Sterling Winthrop Research Institute and Tyr-D-Ala-Leu-(Me)Phe-Gly-ol (DAGO) was a generous gift of Dr Roemer, Sandoz Ltd. D-Ala^{2}-Leu^{5} enkephalin (DALE) and D-Ala^{2}-D-Leu^{5} enkephalin were synthesized by Dr. K. Medzihradszky et al. Central Research Inst. for Chemistry, Budapest, Hungary. Bacitracin, phenylmethyl-sulphonylfluoride (PMSF) EDTA.2H_2O, Benzamidine.HCl were obtained from Sigma Chem. Co., St. Louis. All other chemicals were of analytical grade.

2.2.METHODS:

2.2.1.MEMBRANE PREPARATION:

Human placentae were obtained from Department of Obstetrics and Gynecology of A.Szent-Györgyi University,Szeged, just after delivery of normal term pregnancies and were brought to the laboratory immersed in ice and processed within 1h after obtention. Patient consent and medical history were obtained. The umbilical cord and amniotic membranes were removed. All further operations were carried out at $4^{o}C$. The whole placenta was rinsed several times with 0.05 M Tris-HCl buffer, pH 7.4. Cotyledons were trimmed and washed in the same buffer until the color turned pink. They were then homogenized using Ultra-Turrax from Janke and Kunkel GmbH Co. for 30 sec intervals in high speed in 5 volumes of buffer-A containing 50 mM Tris-HCl, pH 7.4, 1mM EDTA, 40 KIU Trasylol, 20 ug/ml Bacitracin, 1mM PMSF, 0.1uM Thiorphan, 10 uM Captopril 1mM Benzamidine and 0.002 % Soybean Tripsin Inhibitor. The homogenate was centrifuged at 800xg for 10 min using a Sorvall RC5C preparative centrifuge (GSA 10 fixed angle rotor) to remove fragments of tissue and red blood cells. The pellet was discarded and the supernatant was centrifuged at 10,000xg for 10 min to remove intracellular debris. The resultant supernatant was subjected to ultracentrifugation at 100,000xg for 1h using a Sorvall OTD65B ultracentrifuge (A-641 fixed angle rotor).

The jelly like pellet was resuspended in 10 volume of 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM sucrose. The homogenate was stirred for 30 min in the presence of 10 mM $MgCl_2$ to give a final concentration of 1 mM $MgCl_2$ to aggregate the nonmicrovillous membranes which were removed by centrifugation at 3,000xg (5,000rpm) for 10 min. The supernatant was spin at 45,000xg (20,000 rpm) for 45 min to yield a pellet enriched in microvilli. Then the pellet was resuspended by homogenization in 10 mM Tris-HCl,pH 7.4, containing 0.32 M sucrose and frozen in liquid nitrogen and stored at $-70^{o}C$. Under this conditions the binding activity of the membranes remained stable for several weeks. Protease inhibitors were used to protect the opiate receptors from degradation.This membrane preparation was used for assaying the opioid receptors using a radio-receptor assay. Alternatively, the whole placenta after washing with 0.05 M Tris-HCl buffer was firstly frozen in liquid nitrogen and then after stored at $-70^{o}C$. Figure-4 shows the schematic diagram of membrane preparation.

2.2.2.RECEPTOR BINDING ASSAYS:

Specific binding in the microvillous membrane (0.05-0.6 mg protein/ml) was measured by incubation with ^{3}H-Naloxone for 50 min at $0^{o}C$ and ^{3}H-EKC for 60 min at $0^{o}C$ and 20 min at room temperature ($25^{o}C$) under red light (^{3}H-EKC is sensitive

to normal light), respectively, in the absence and presence of 10 uM unlabelled ligand. In the case of ^{3}H-EKC binding assay 100 nM DAGO and 100 nM DADLE were present in the incubation mixture as suggested by Kosterlitz et al. (1981a) (in order to block mu-and delta-sites). Following incubation, the samples were filtered directly on vacuum through Whatman GF/B glass fiber filters. After filtration, the filters were washed twice with 10 ml ice-cold 50 mM Tris-HCl ,pH 7.4, buffer and dried overnight at room temperature (25^{o}C) or at 37^{o}C for 3 hours. Then the radioactivity was measured in a toluene based scintillation coctail in a LKB 1211 Minibeta liquid scintillation counter. Efficiency of counting was 35-40 % (see appendix-A) Specific binding was defined as the difference between total binding and the binding in the presence of 10 uM unlabelled ligand (non-specific binding). Routinely specific binding represented 40-50 % of total binding. All experiments have been replicated 3-5 times with membrane preparations derived from different patients. The ratio of the receptor concentration to the free ligand was about 1-2 % in the incubation medium. Values are the mean of 3-5 experiments which varied by 10 %. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

2.2.3.DATA ANALYSIS

The experimental data were fitted to derive the equilibrium binding constants by KINETFIT computer programme provided by Dr.C.Bagyinka (Institute of Biophysics, Biological Research Center of Hungarian Academy of Sciences, Szeged/Hungary) using a Commodore-64 computer.

The $k_{obs.}$ was calculated from the slope of the line where ln (B_e/B_e-B_t) is plotted vs time (t). The pseudo first order rate constant (k_{+1}) was calculated where

$$k_{+1} = (k_{obs.}-k_{-1})/ L \quad *$$

where L = free ligand concentration, k_{-1} = first order rate constant for dissociation.

For evaluation of data KINETFIT computer program was used.

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* for derivation see appendix-B.

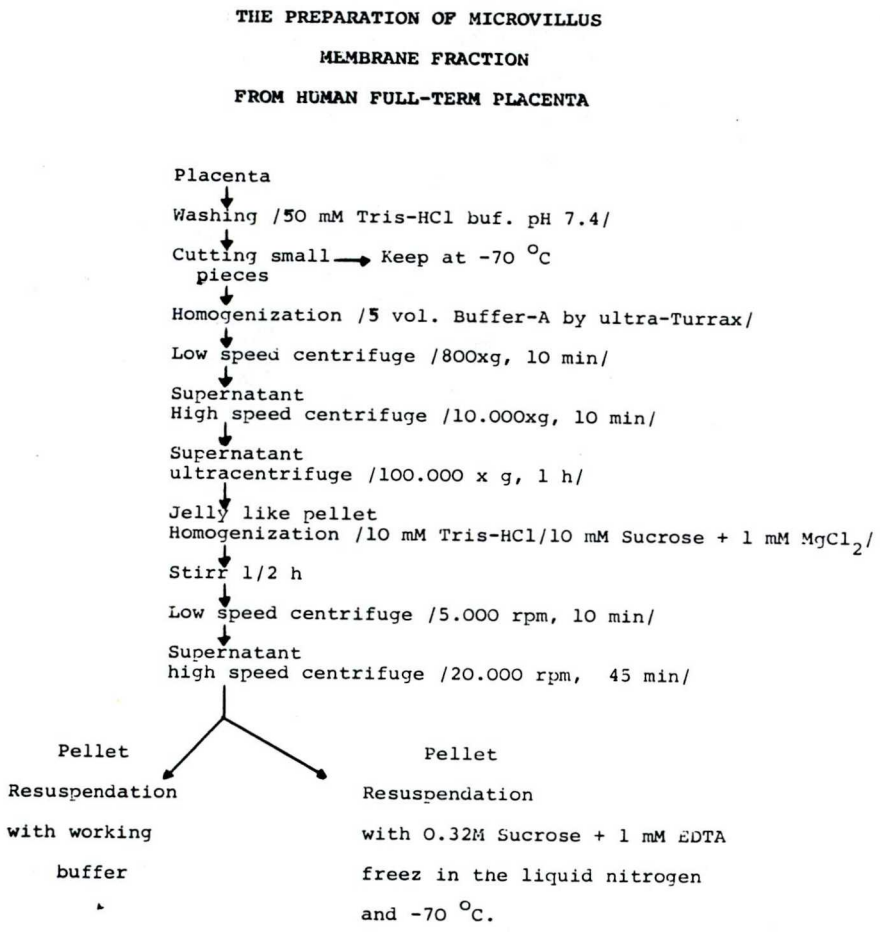

Fig.-4. Schematic diagram of the membrane preparation from human placental tissue.

3.RESULTS

3.1. KINETIC PARAMETERS OF ^{3}H-NALOXONE AND ^{3}H-EKC BINDING TO HUMAN PLACENTA MICROVILLOUS MEMBRANE.

When ^{3}H-naloxone binding was studied, the binding was proportional to the protein concentration up to 0.6 mg protein/ml (Fig.5).

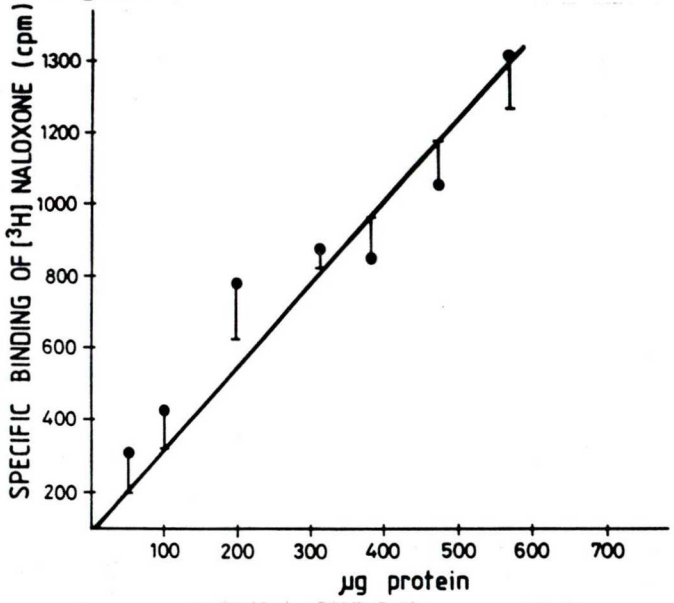

Fig-5. Binding of ^{3}H-naloxone to placental membranes as a function of protein concentration.

Binding of ^{3}H-naloxone to term placental microvillous membranes was studied under equilibrium conditions using tissue protein concentrations ranging from 0.05-0.6 mg/ml. Tissue

fractions were prepared as described in Material and Methods, and incubations were carried out at $0^{o}C$ for 50 min in the presence and absence of a ^{3}H-naloxone. Each point represents the mean (±SE) of specific binding and results from triplicate incubations with three to five seperate experiments.

The time course of ^{3}H-naloxone binding to placental membrane is shown in Fig.6.

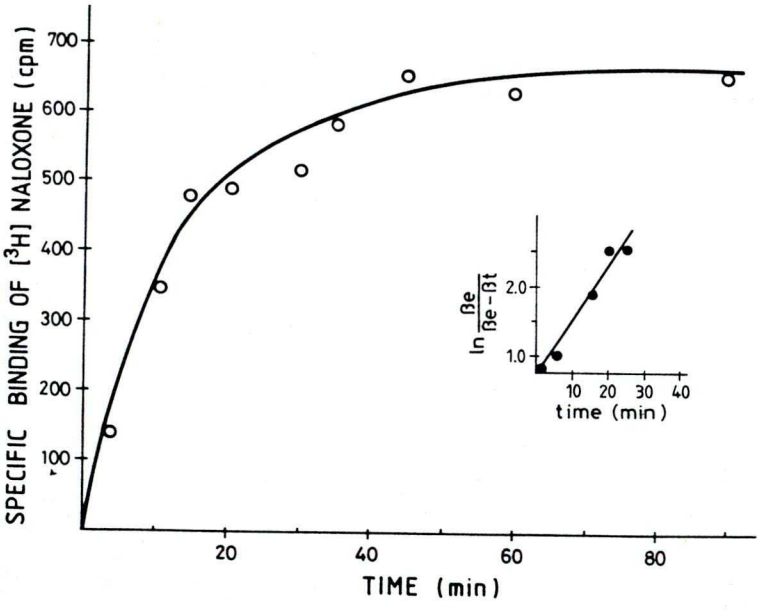

Fig-6. Association curve of ^{3}H-naloxone binding to human placental membranes.

The ^{3}H-naloxone (10 nM) was incubated with membranes (0.05-0.6 mg protein/ml) in the presence or absence of 10 µM unlabelled naloxone at $0^{o}C$ for the times indicated. The specific binding is demonstrated. The

inset shows the graphic estimation of the observed association rate constant according to equation $B_t = B_e(1-e^{-k_{obs}xt})$, where B_t is the bound radioactive ligand at the time, t , B_e is the bound ligand in equilibrium, and k_{obs} is the observed rate constant.

At 0^oC the specific binding of the tritiated ligand (10 nM) reached equilibrium within 50 min and remained constant at least up to 90 min. The apparent association rate constant was calculated on the basis of pseudo first order kinetics (see Fig.6, insert). The value of k_{obs} was found to be 0.069 min^{-1}.

Fig.7 shows the dissociation of ^{3}H-naloxone from its specific binding sites at 0^oC. In the assays, the membranes were incubated with ^{3}H-naloxone for 50 min. After reaching the equilibrium a great excess of unlabelled naloxone (10 μM) was added to induce dissociation. The amount of the bound radioactive ligand was determined at various time intervals. Kinetic data were obtained using the first order rate equation. The dissociation rate constants for the rapid and slow phase of dissociation were 0.13 min^{-1} ($t_{1/2}$= 5.3 min) and 0.0038 min^{-1} ($t_{1/2}$= 182.4 min), respectively.

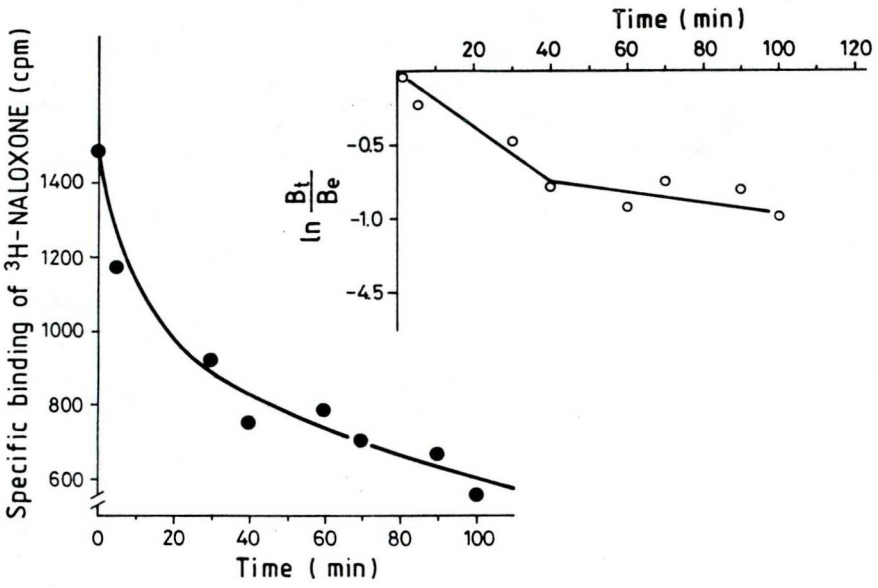

Fig.-7. Dissociation of ^{3}H-naloxone from human placental membranes. ^{3}H-naloxone was incubated with the membrane preparation at 0^oC with or without 10 µM unlabelled naloxone. After 50 min incubation time 10 µM unlabelled naloxone was added and aliquots were taken at the times indicated (Inset shows the graphic estimation of the dissociation rate constant).

When ^{3}H-EKC binding was studied, the binding was directly proportional to protein concentration up to 0.6 mg protein/ml (Fig.8).

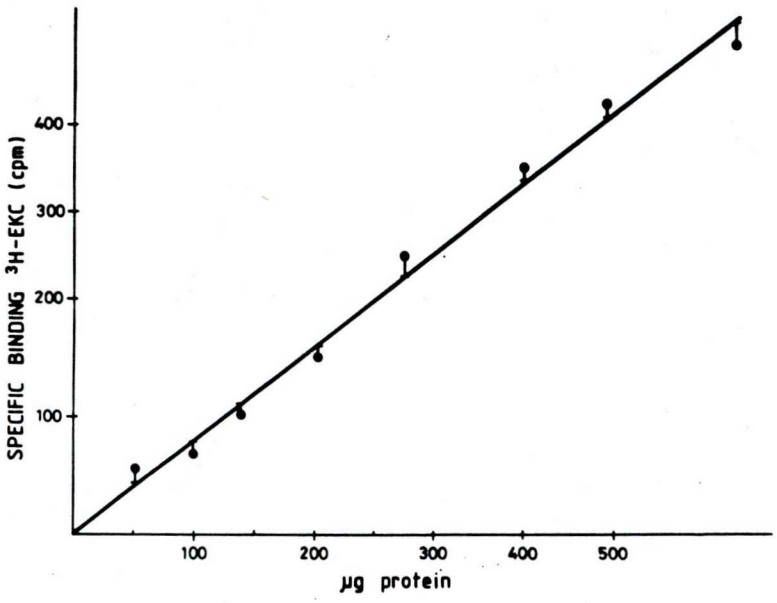

Fig.-8. Binding of ^{3}H-EKC to placental membranes as a function of protein concentration. Binding of ^{3}H-EKC to placental microvillous membranes was studied. Protein concentrations ranging 0.05-0.6 mg/ml. The incubations were carried out at 25^{o}C for 20 min in the presence and absence of a saturating dose of ^{3}H-EKC. Each point represents the mean (± SE) of specific binding and results from triplicate incubations with three to five separate experiments.

In the case ^{3}H-EKC which is kappa preffering ligand , the equilibrium was reached within 20 min at 25^{o}C and 60 min at 0^{o}C (Figure.9). Dissociation of ^{3}H-EKC binding was

carried out at 25°C. Membranes were incubated with the radioligand for 20 min and then 10 μM of unlabelled ligand was added. The amount of the bound radioactivty was determined at various time intervals. The dissociation rate constant for the rapid and slow phase of dissociation were 0.097 min^{-1} ($t_{1/2}$= 7.1 min) and 0.0049 min^{-1} ($t_{1/2}$= 141.2 min), respectively (Fig.10).

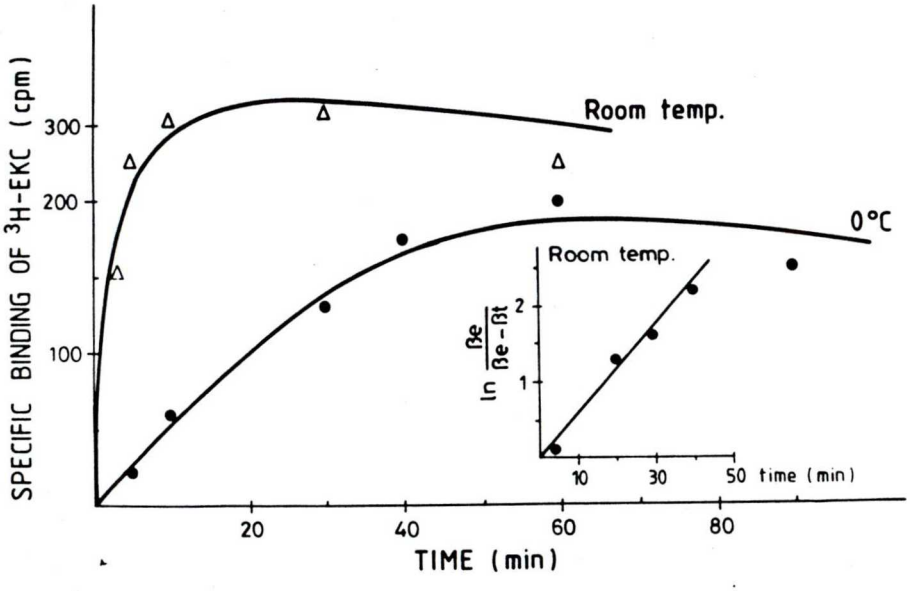

Fig.-9. ^{3}H-EKC binding to human placental membranes as a function of incubation time. ^{3}H-EKC (10 nM) was incubated with placental membranes for the indicated periods of time at 0°C and at 25°C (Inset shows the graphic estimation of the observed association rate constant at 25°C).

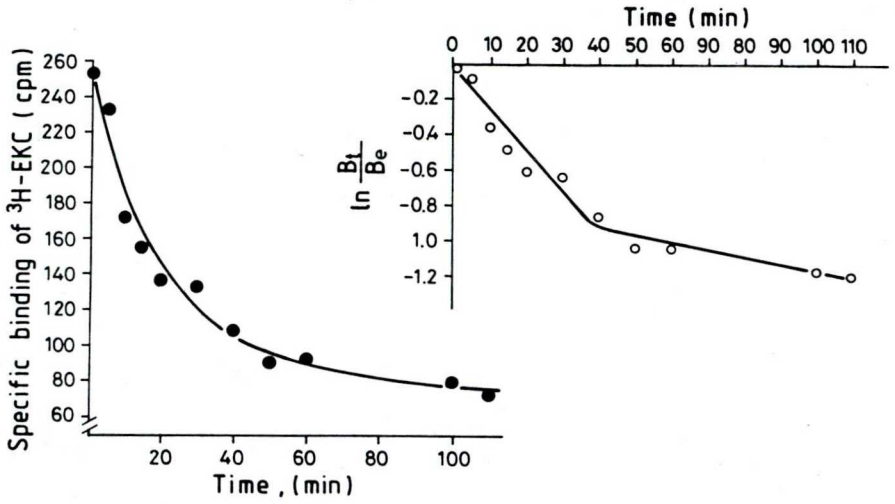

Fig.-10. Dissociation of ^{3}H-EKC from human placental microvillous membrane. ^{3}H-EKC was incubated with the membrane preparation at 25^{o}C with or without 10 µM unlabelled EKC. After 20 min incubation time 10 µM unlabelled EKC was added and aliquots were taken at the times indicated (Inset shows the graphic estimations of the dissociation rate constant).

3.2.EQUILIBRIUM STUDIES

Equilibrium binding assays were carried out at 0^{o}C for 50 min in the case of ^{3}H-naloxone (radioligand concentrations

were increasing from 0.2 to 15 nM), and at 25°C for 20 min in the case of ^{3}H-EKC (radioligand concentrations are increasing from 0.1 to 9 nM). As it can be seen on Fig.11 and Fig.12 the specific binding was saturable in both case.

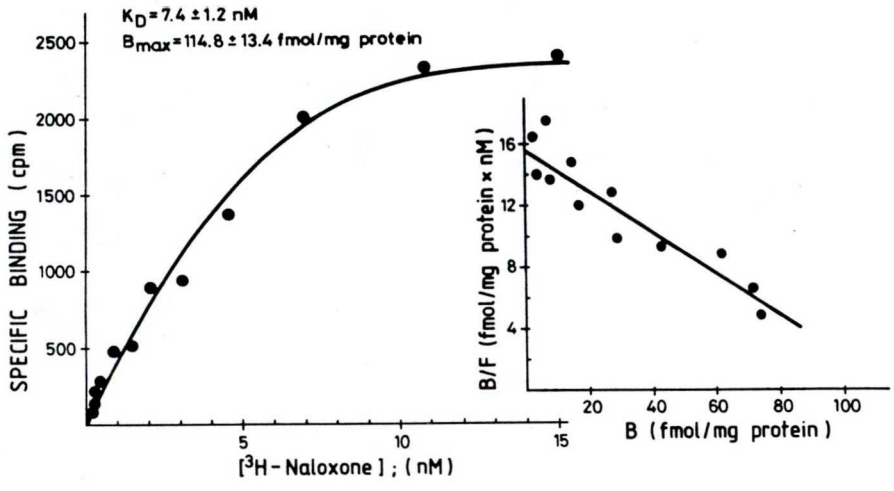

Fig.-11. Saturation curve for the binding of ^{3}H-naloxone to human placental microvillous membrane.
The membrane preparations were incubated with increasing concentrations of ^{3}H-naloxone in the presence or absence 10 μM unlabelled naloxone (Inset shows the data plotted according to computer-assisted Scatchard analysis).

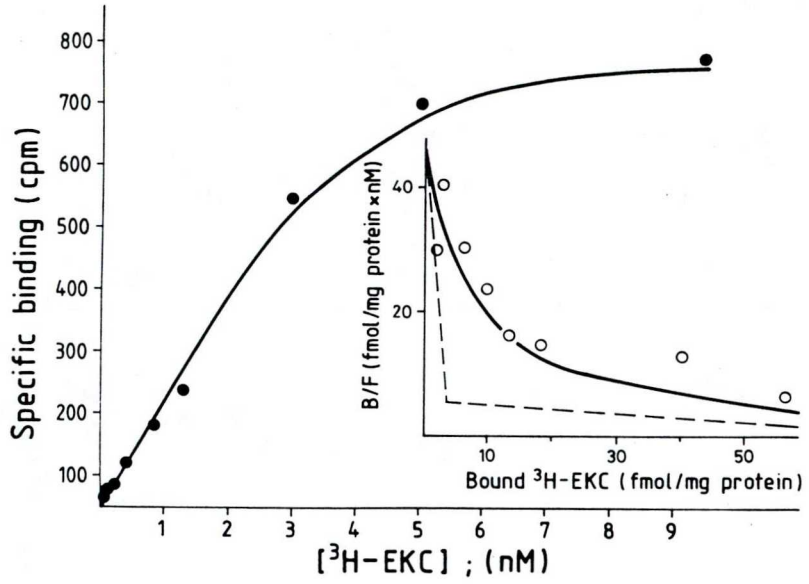

Fig.-12. Saturation curve for the binding of ^{3}H-EKC to human placental microvillous membrane.
The membrane preparations were incubated with increasing concentrations of ^{3}H-EKC in the presence or absence 10 µM unlabelled naloxone (Inset shows the data plotted according to computer-assisted Scatchard analysis).

The ratios of specific binding to nonspecific binding are shown in Fig. 13 and Fig. 14 , respectively.

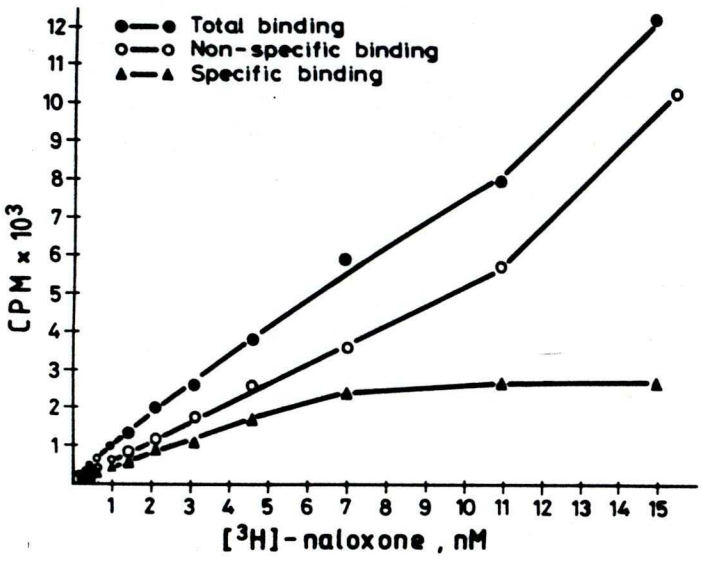

Fig.-13. Ratio of specific binding to nonspecific binding for ^{3}H-naloxone.

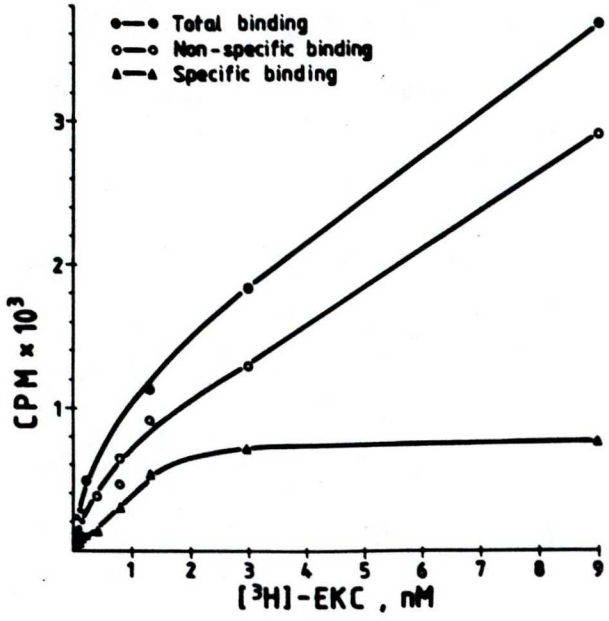

Fig.-14. Ratio of specific binding to nonspecific binding for ^{3}H-EKC.

On the basis of Scatchard transformation of data two binding sites were obtained for ^{3}H-EKC, and a single binding site for ^{3}H-naloxone. The binding of the ^{3}H-naloxone and ^{3}-EKC were found to be saturable and stereospecific. ^{3}H-naloxone Scatchard analysis showed a linear curve with single binding site (K_D= 7.4 nM ; B_{max}= 114 fmol/mg protein). Whereas, ^{3}H-EKC Scatchard analysis showed a biphasic curve with two classes of binding sites (for high affinity site K_D= 0.9 nM ; B_{max}= 5.0 fmol/mg protein and for low affinity site K_D= 25 nM ; B_{max}= 85 fmol/mg protein).

3.3.BINDING SPECIFICITY

The effects of various opioid ligands on ^{3}H-naloxone and ^{3}H-EKC binding were tested. The placental microvillous membrane fractions were incubated with radioligands (1 nM ^{3}H-naloxone or 1.5 nM ^{3}H-EKC) in the presence of various opioid ligands in wide concentration range (10^{-11}M - 10^{-5} M). Displacement curves were constructed (Fig.15 and Fig.16) and IC_{50} values were calculated (See Table-2)

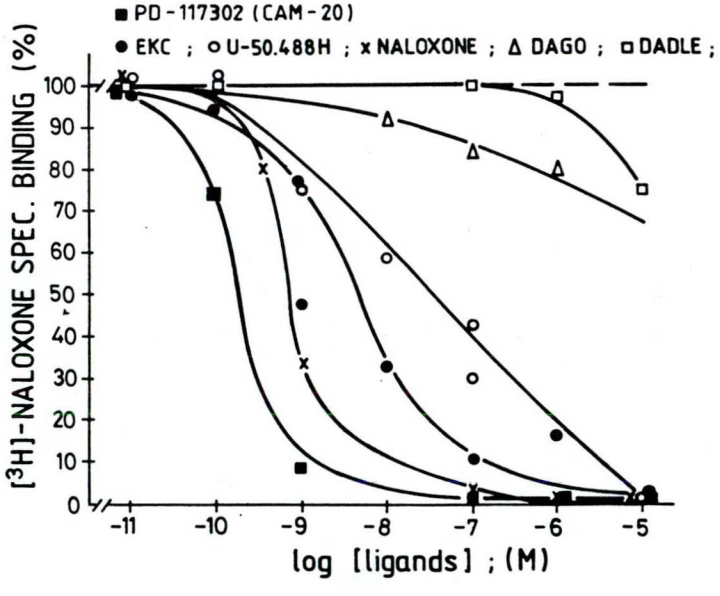

Figure-15. Displacement of ^{3}H-naloxone (1 nM) binding from the human placental microvillous membrane by different opioid ligands.

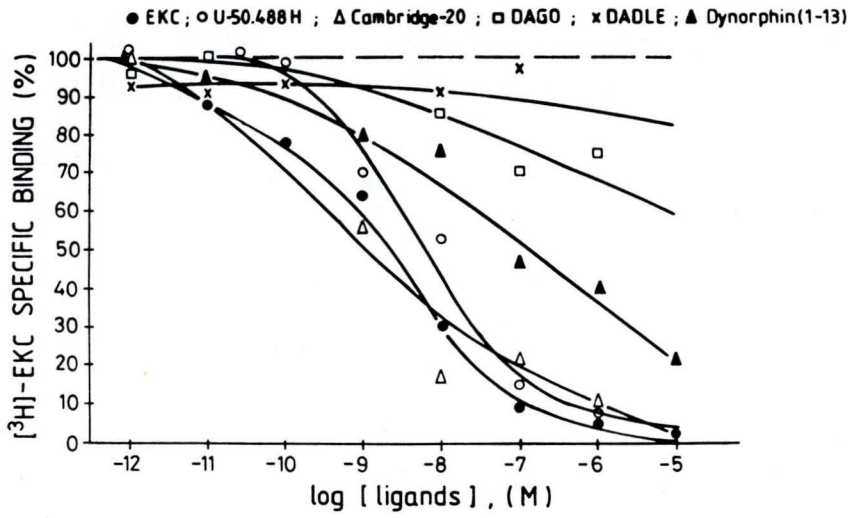

Fig.-16. Displacement of ^{3}H-EKC (1.5 nM) binding from the human placental microvillous membrane by different opioid ligands.

TABLE-2. POTENCIES OF OPIATE LIGANDS IN REDUCING OF ^{3}H-EKC (1.5 nM) AND ^{3}H-NALOXONE (1 nM) BINDING TO HUMAN PLACENTAL MICROVILLOUS MEMBRANE PREPARATION.

Unlabelled ligands	Specificity	IC_{50} (nM)	
		^{3}H-EKC	^{3}H-naloxone
Ethylketocyclazocine	Kappa	3.5	7.0
U-50,488H	Kappa	8.0	60.0
PD-117302 (Cam-20)	Kappa	0.5	0.3
Dynorphin(1-13)	Kappa	100.0	80.0
DAGO	Mu	>100,000.0	>100,000.0
DADLE	Delta	>100,000.0	>100,000.0
Naloxone	Universal	N.T.	0.9

Abbreviation: NT= Not tested.

3.4.STEREOSPECIFICITY

The stereospecific nature of ^{3}H-naloxone binding to placental membranes is shown in Fig.17. The pharmacologically active enantiomer opioid ligand levorphanol caused 50 percent inhibition of ^{3}H-naloxone specific binding at approximately $5x10^{-7}$ M concentration.On the other hand the inactive enantiomer dextrorphan was much less effective.

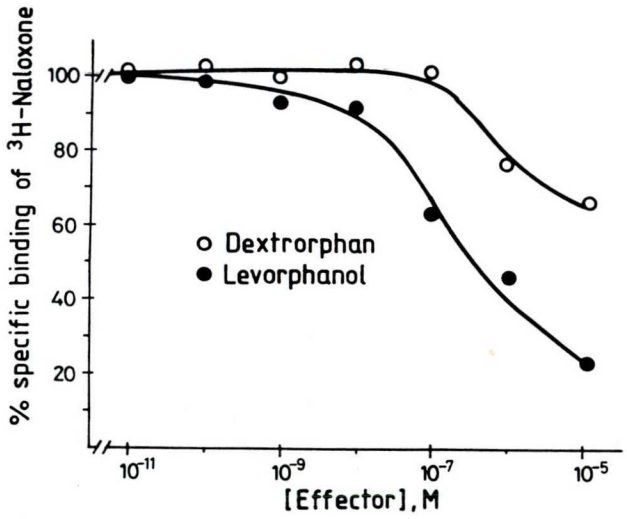

Fig.-17. Displacement of specifically bound ^{3}H-naloxone by different concentrations of dextrorphan and levorphanol. Remaining ^{3}H-naloxone bound is expressed as % of radioligand specifically bound in the absence of the enantiomers.

levorphanol. Remaining ^{3}H-naloxone bound is expressed as % of radioligand specifically bound in the absence of the enantiomers.

3.5. THE DISTRIBUTION OF OPIOID RECEPTOR SUBTYPES IN HUMAN PLACENTAL MEMBRANE.

The distribution of opioid receptor sub-types was carried out using by sub-type specific radiolabelled ligands (the concentration range is 2-5 nM) in the presence of sub-type specific unlabelled ligands as blocking agents. As it can be seen in Table-3, it was found that human placental membrane contains mainly kappa sub-type opioid receptor.

TABLE-3: THE DISTRIBUTION (%) OF OPIOID RECEPTOR SUBTYPES IN HUMAN PLACENTAL MEMBRANE.

Radioligand	Sub-type	B_{max} (fmol/mg protein)	%
^{3}H-Naloxone	Universal	71.5 ± 26.9	100.0
^{3}H-DAGO	Mu	N.D.	-
^{3}H-DALE	Delta	N.D.	-
^{3}H-EKC	Kappa	64.2 ± 25.7	88.8

Abbreviation: N.D.= Non detectable. Results are the mean from 3-4 experiments. Each was replicated three times.

3.6. EFFECT OF Na^+ ON THE SPECIFIC ^{3}H-EKC BINDING ON PLACENTAL MEMBRANE.

Effect of sodium on the specific binding on placental membrane was studied. Fig.18 shows that Na^+ (100 mM) has a decreasing effect on the ^{3}H-EKC binding from the human placental microvillous membrane.

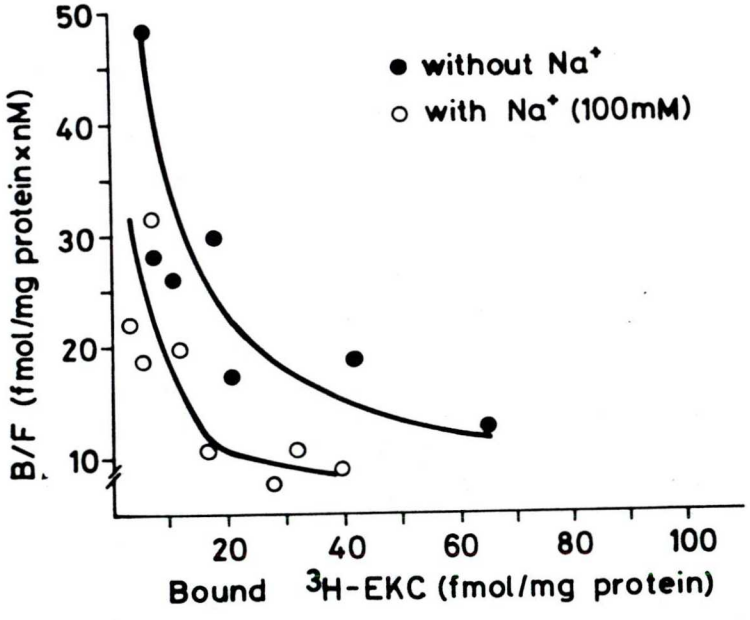

Fig.-18. The effect of Na^+ (100 mM) on the binding of ^{3}H-EKC in the human placental microvillous membrane fraction.

4.DISCUSSION

The human placental tissue has been reported to contain acetylcholine (Sastry et al. 1977; Olubadewo et al. 1978), opioid peptides (Sastry et al. 1981), and opiate binding sites(Porthe et al. 1982). It was proposed that these sites have binding properties identical to those of the kappa-opioid receptors in their membrane- bound (Porthe et al. 1982; Ahmed, 1983) and solubilized (Ahmed et al. 1981) forms. The physiological role of opiate receptors is currently unclear in human placenta which is a metabolically very active organ.

We have measured opiate binding sites in different placental membrane fractions of microvillous tissue where their location had been suggested earlier by Valetta et al. (1979, 1980) and Porthe et al. (1981, 1982). Valetta et al.(1980) have indicated that 93 % of the total etorphine binding sites is located in the human placenta. In our procedure of the isolation of microvillous membrane fraction, as described in Material and Methods, we could recover of about 90 % number of naloxone binding sites (data not shown).

Porthe et al.(1982) have pointed out that the opiate binding sites found on microvillous membrane fraction have similar pharmacological characteristics to the kappa-

opiate binding site. They suggested that human placental microvillous membranes could be considered as a tissue containing mainly kappa-opioid binding sites.

Therefore, microvillous membrane fractions were prepared (see Figure-4). Following our preparation, the binding activity was remained for several weeks (at least 3-4 weeks) (data not shown). Placenta contains large amount of proteolytic enzymes. Hence, it was required to use several protease inhibitors (PMSF for serin proteases, captopril and triorphan for enkephalinase, benzamidine for general proteases, STI for trypsin-like proteases, and trasylol for kallikrein and trypsin proteases) in the buffer system during the membrane isolation.

Large protein concentrations may effect the ratio of bound to free (B/F) ligand especially at low ligand concentration. Therefore, the protein concentration was kept under 0.6 mg protein/ml in each assay, because the binding was proportional to the protein concentration up to 0.6 mg protein/ml as it can be seen in Figure-5 and Figure-8.

Kinetic data of both ^{3}H-naloxone and ^{3}-EKC show a rapid and a slow phase of dissociation (see Fig.7 and Fig.10). However, in the case of equilibrium analysis Scatchard curve shows a single binding site for ^{3}H-naloxone, although two binding sites (high and low affinity) appeared in the case of ^{3}-EKC. This discrepancy might be due to the EKC itself which is a partial agonist for the mu-receptor (Hiller et al. 1979;

Pasternak et al. 1980; Chang et al. 1980) while it is a kappa ligand or the different properties of the two ligands. Usually antagonists exhibit higher affinities than agonists. Naloxone might have a high affinity site associated with too low number of this binding sites in this case. This means that the present experimental conditions do not allow us to detect this site in a reproducible way, because this might be in the range of the experimental error.

Ahmed et al.(1986) have demonstrated lower opioid receptor numbers in term placentas from generally healthy mothers and babies delivered by vaginal versus abdominal routes (for vaginal route B_{max}= 72 fmol/mg protein and for abdominal route B_{max}= 187 fmol/mg protein using ^{3}H-etorphine). Our results concerning with opioid receptor numbers of placenta which obtained by vaginal routes are in agreement with their data (B_{max}= 114 fmol/mg protein and B_{max}= 85 fmol/mg protein using ^{3}H-naloxone and ^{3}H-EKC, respectively. Moreover, we were not able to compare the opioid receptor numbers between vaginal and abdominal routes because of insufficient amount of placentas obtained from abdominal route. It is possible to explain the apparent lower number of opioid receptors in vaginal deliveries compared with the abdominal route by suggesting that the rise in endogenous opioid peptides during vaginal delivery may result in receptor desensitization or down regulation.

Consequently, Ahmed et al.(1986) have investigated whether chronic abuse of opioids during pregnancy could also result in placental opioid receptor desensitization. Among mothers who used opioids during their pregnancies, they found a decrease in the number of opioid receptors in full-term placentas, irrespective of the route of delivery. In addition, Valetta et al.(1980) reported that ^{3}H-etorphine-binding rised up 10-fold between 2. and 5. months of gestation after which it remained constant until term. Kinetic study of ^{3}H-etorphine binding on 2-, 5-, and 9-months-old placentas show that ^{3}H-etorphine affinity does not change, so that the increase observed reflected an increase of the number of binding sites.Recently, Belisle et al. (1988) observed more than one class of binding sites using ^{3}H-EKC on the term placental cells. Belisle et al.(1988) have been shown that the receptor concentrations increased up to the fifth month of gestation, thereafter remaining stable at 60-70 fmol/mg placental protein. In addition, they also found that placental cells from term gestations also contained kappa-specific opioid binding sites. However, in contrast to the syncytiotropho-blastic plasma membranes,term placental cells contained more than one population of kappa-opioid binding sites; the first population of sites had the affinity, specificity, and concentration of those in term plasma membranes, while the second population of sites had lower affinity but much higher capacity for opioid ligands. Moreover, a number of

laboratories have been shown that human placenta contains a single class of opiate binding sites whose pharmacological characteristics correspond to those of kappa-opiate binding site (Porthe et al.,1981; Ahmed et al., 1987; Valetta et al., 1987).

We have observed large variations in the opiate receptor content of different placentae (data not shown). It is not yet clear whether this variability is a reflection of the physiological or pharmacological state of the donor, or simply represents biological individuality. A related question that remains to be answered is that of the nature of the natural ligand for these receptors (Ahmed et al.1981). Both our and recent results of others have been indicated the placenta represents a unique situation in which only one specific type of opioid receptor has been demonstrated.

The competetion ability of various opioid ligands for ^{3}H-naloxone and ^{3}H-EKC binding sites were tested. The kappa selective opioid ligands, U-50,488H, PD-117302 (Cam-20), and the kappa preferring ligand EKC were able to compete with high affinity (IC_{50} values are in the nanomolar range) for naloxone binding sites using microvillous membrane preparation. Dynorphine (1-13) (Goldstein et al. 1979), but even this peptide binds with a relatively low affinity. We also have found that Dynorphin(1-13) has low affinity against to naloxone binding site in the human placental membrane. However, this low affinity of dynorphin

might be due to its degradation during the incubation. In fact, the placenta of many species including man is known to exhibit drug metabolizing activity (Juchau et al.1968). Therefore, low affinity of dynorphin might be result from metabolic inactivation of the drug.

The solubilized opioid receptors from human placenta show the same specificity for binding to ligands of different subclasses of the opiate receptors as that of the membrane-bound receptor (Ahmed, 1983). However, affinity appears to be either changed slightly in case of etorphin, naloxone, and cyclazocine, or levorphanol. This could be due to either the different assay conditions for the solubilized receptor which are not identical to the membrane bound (i.e., presence and absence of a detergent) or the possible existence of a receptor conformation in a detergent-vesicle complex which may result in a change of affinity but not in binding specifities (Ahmed, 1983). The placental receptor, both in the membrane-bound and soluble state, appears to have an unusual range of binding affinities and may therefore represent a new category of opiate receptor. It shares with mu-receptors a high affinity for some alkaloids, such as etorphine and levorphanol, but displays only a low affinity for the opioid peptides beta-endorphin and DALAMID, as well as for the classical mu-ligand, morphine.

Evidence for the stereospecific nature of binding sites of naloxone to placental membranes is shown in Fig.17. We

observed that levorphanol (biologically active enantiomer of opioid ligand) at approximately $5x10^{-7}$M caused 50 per cent inhibition of the specific naloxone binding. Meanwhile inactive enantiomer dextrorphane was much less effective. Ahmed et al. (1981) have found the same result for etorphine binding site on the human placenta. However, the affinity of levorphanol to the placental receptor is about ten times less than to brain.

The binding characteristics of ^{3}H-naloxone (an universal opioid antagonist) and ^{3}H-EKC (a kappa agonist) were studied. We could confirm that human placental microvillous membrane contains mainly the kappa-opioid binding sites (see Table-3). We could not observe a meaningful specific winding activity on the placental microvillous membrane fraction using by mu-specific radiolabelled ligand, DAGO or the fairly delta-selective radiolabelled DALE.

One of the most characteristic feature for brain opiate receptor is its selective alteration by sodium ions. A similar property is found for placental opiate binding site. Valetta et al.(1980) have found that the ^{3}H-etorphine binding decreased with the sodium concentration. 50 mM sodium induced a 35 percent reduction, whereas 10-50 mM potassium had no effect. Na^{+} selectively enhances the binding of opiate antagonist and decreases the binding of opiate agonist (Pert et al.1973; Simon et al. 1973). In our result, also the decreasing effect of Na^{+} (100 mM) on the ^{3}H-EKC binding in

the placental microvillous membrane was observed. 100 mM sodium resulted in a 34 and 19 percent reduction on the B_{max} for the high and low affinity site, respectively.

The localization of opiate binding sites on the highly specialized syncitial brush border membrane at the maternal-placenta interface may suggest a possible involvement of these opiate binding sites in the regulation of placental endocrine function (Porthe et al.1982).

It is known that the opioid receptors in placental tissue have physiological significance. Specifically, kappa-opiates stimulated placental cell hPL production in a dose-dependent manner at concentrations of peptides in the range of their affinities for the receptor. Furthermore, kappa-opiate stimulation of hPL production was inhibited by a bioactive opiate antagonist, naloxone (Belisle et al.1988).

Although many questions remain to be answered regarding the function of the opoiate receptor in the placenta, a nonneural tissue (Fujiyama et al. 1971), perhaps it is appropriate to know that beta-lipoprotein, and methionine enkephalin have been demonstrated in human placenta (Sastry et al. 1980, 1981). It has been postulated that these peptides play the role of a negative feedback modulator of AcCh release by the placenta (Olubadewo et al.1978). A similar role has been shown in rat brain, suggesting that mu- and kappa-opiate receptors regulate cholinergic functions (Wood et al.1981). Another possible role for the opiate

receptor in human placenta is modulation of hormonal release in a manner analogous to their modulation of hormones such as luteinizing hormone-releasing hormone in rat mediobasal hypothalamic neurons (Drouva et al.1980).

Speculations on other functions for the opiate receptors in human placenta need experimental data for support. One such function could be the involvement of the receptor as a first step in a process to protect the fetus from opiate peptides circulating in the maternal serum. Since it has been reported that increasing levels of beta-endorphin in maternal serum occur prior to partition (Fletcher et al.1980), such a speculation may correlate with the reported observation. Another speculation for receptor function would be through a central mechanism for pain regulation prior to placental detachment.

Some properties of human placenta opioid receptors were recently compared to that of other tissues (e.g. guinea-pig cerebellum, rat thalamus, and rabbit cerebellum) by interaction with lectins (Valetta et al.1987) and differences were found in the carbohydrate moiety.Gioiannini et al.(1982) and Valetta et al.(1983) have been demonstrated the kappa subtype seems to contain less carbohydrates than the mu-receptor. Further molecular studies are needed to establish the structural homologies or differences related to the sub-types and tissue source.

All these results show that human placental membranes seem to be considered as a tissue containing mainly kappa-opioid binding sites.

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APPENDIX

APPENDIX-A: DERIVATION OF INTEGRATED EQUATION FOR ASSOCIATION RATE CONSTANT

Abbreviations : L= unbound ligand; R= unoccupied receptor; RL= receptor-ligand complex.

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \qquad [1]$$

defines the general second order association and first order dissociation binding processes. The association rate equation is

$$\frac{d[RL]}{dt} = k_1[L][R] - k_{-1}[RL] \qquad [2]$$

If the binding experiment is performed in zone A, [L] remains essentially constant and a new association rate constant k_1' can be defined as $k_1' = k_1[L]$. Substituting into Eq.(2) yields

$$\frac{d[RL]}{dt} = k_1'[R] - k_{-1}[RL] \qquad [3]$$

At any given time t the concentration of unoccupied receptor ([R]) is equal to

$$[R] = [R]_o - [RL]_t \qquad [4]$$

Appendix

At equilibrium, the net formation of RL is zero. Substituting Eq.(4) into Eq.(3) at equilibrium yields

$$k_1' ([R]_o - [RL]_{eq}) = k_{-1} [RL]_{eq} \qquad [5]$$

$$k_{-1} = \frac{k_1' [R]_o}{[RL]_{eq}} - k_1' \qquad [6]$$

Substituting Eq.(6) into Eq.(3)

$$\frac{d[RL]}{dt} = k_1' ([R]_o - [RL]) - \left(\frac{k_1' [R]_o}{[RL]_{eq}} - k_1' \right) [RL] \qquad [7]$$

Eq.(7) reduces to

$$\frac{d[RL]}{dt} = k_1 \frac{[R]_o ([RL]_{eq} - [RL])}{[RL]_{eq}} \qquad [8]$$

which rearranges to

$$\frac{d[RL]}{[RL]_{eq} - [RL]} = \frac{k_1 [R]_o}{[RL]_{eq}} dt \qquad [9]$$

Integrating Eq.(9) from t_1 to t_2 yields

$$\ln \frac{[RL]_{eq} - [RL]_{t_1}}{[RL]_{eq} - [RL]_{t_2}} = \frac{k_1' [R]_o}{[RL]_{eq}} (t_2 - t_1) \qquad [10]$$

Appendix

Eq.(10) is a general equation that can be used for any two time points on the association curve for an equilibrium process. Eq.(10) can be further reduced by setting $t_1 = 0$; therefore $[RL]_{t_1} = 0$.

$$\ln \frac{[RL]_{eq} - 0}{[RL]_{eq} - [RL]_{t_2}} = \frac{k_1' [R]_o t_2}{[RL]_{eq}} \qquad [11]$$

From Eq.(6)

$$k_{-1} + k_1' = \frac{k_1' [R]_o}{[RL]_{eq}} \qquad [12]$$

Substituting Eq.(12) and $k_1' = k_1[L]$ into Eq.(11) yields

$$\ln \frac{[RL]_{eq}}{[RL]_{eq} - [RL]_t} = (k_{-1} + k_1 [L])t \qquad [13]$$

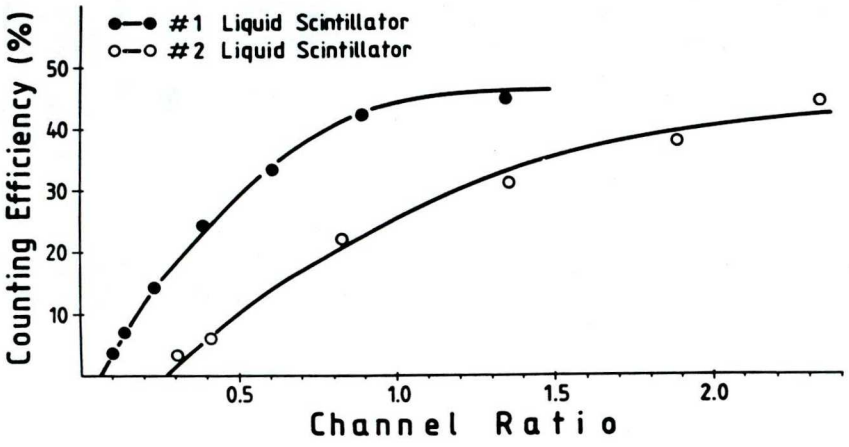
A plot of $\ln \frac{[RL]_{eq}}{[RL]_{eq} - [RL]_t}$ versus time yields a line with a slope

$$k_{obs} = k_{-1} + k_1 [L]$$

Therefore

$$k_1 = \frac{k_{obs} - k_{-1}}{[L]}$$

APPENDIX-B:



Quench curve