EFFECT OF LIPIDS ON BINDING CHARACTERISTICS OF OPIOID RECEPTORS

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ABSTRACT

EFFECT OF LIPIDS ON BINDING CHARACTERISTICS OF OPIOID RECEPTORS

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Effect of lipids on binding characteristics of opioid receptors in membranes prepared from rat brain were studied. Lipid concentrations causing changes in specific binding of [^{3}H]Endomorphin-1 ($ProE_1$), an opioid agonist highly specific to mu-type opioid, [^{3}H]Ile^{5,6}deltorphin II (DIDI), an agonist ligand highly specific to delta type receptor and [^{3}H]Naloxone (Nlx), a universal opioid receptor antagonist were determined. Inhibition of [^{3}H]$ProE_1$, [^{3}H]DIDI and [^{3}H]Nlx specific binding was also examined by homologous displacement experiments in the presence and absence of lipids. In order to understand whether the changes occurring in the specific binding is due to changes in equilibrium dissociation constant (K_D) or maximum number of binding sites (B_{max}), the equilibrium binding experiments were performed.

Arachidonic acid (AA) inhibited binding of both agonist and antagonist ligand in a dose dependent manner with IC_{50} values of 0.15, 0.1, and 0.6 mM for [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx, respectively. K_d values were not affected while B_{max} values decreased 38 % and 76 % for mu, and delta receptor subtypes, respectively. For [^{3}H]Nlx, B_{max} values decreased 20 and 56 % in the absence and presence of 100 mM NaCl, respectively.

Cholesteryl hemisuccinate (CHS) enhances (100 % of control) ligand binding at mu-sites however no effect was encountered at delta sites. Furthermore, CHS also enhances (50 % of control) the binding of antagonist ligand in the absence of NaCl. B_{max} values were increased by 70 % for mu sites and 40% for antagonist ligand binding site. Under similar conditions Kd values were not affected.

Phosphatidic acid (PA) and phosphatidylcholine (PC) exhibited negligible effect on ligand binding. PA decreased specific binding of ProE$_1$ and DIDI by 16 and 10 %, respectively. Specific binding of antagonist ligand Nlx decreased 11 % in the presence of NaCl whereas in the absence of NaCl specific binding is very close to control. In the presence of PC specific binding of both agonist and antagonist ligands were around control values.

In this study modulatory effect of lysophospholipids, lysophosphatidic acid and lysophosphatidylcholine on opioid binding sites were evaluated for the first time. Both lysophospholipids exhibited similar effects: decreasing specific binding in receptor subtype independent manner between 0.1 to 1 mM range. K_d values were not significantly affected, while remarkable decrease (45-75 %) in B_{max} values were observed.

Keywords: Arachidonic acid, Cholesteryl hemisuccinate, Lysophospholipid, Opioid receptors, Phospholipid, Rat brain membranes.

ÖZ

LİPİTLERİN OPİOİD RESEPTÖRLERİNİN BAĞLANMA ÖZELLİKLERİ ÜZERİNE ETKİSİ

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Lipitlerin sıçan beyinlerinden elde edilen hücre zarlarındaki opiat reseptörlerine bağlanma özellikleri çalışılmıştır. Mu tipine özel agonist ligand [^{3}H]Endomorphin-1 ($ProE_1$), delta tipine özel [^{3}H]Ile^{5,6}deltorphin II (DIDI) ve genel opiat reseptör antagonisti [^{3}H]Naloxone (Nlx) ligandlarının bağlanmasında değişikliğe yol açan lipit konsantrasyonları tespit edilmiştir. [^{3}H]$ProE_1$, [^{3}H]DIDI ve [^{3}H]Nlx ligandlarının bağlanmasındaki azalmalar ortamda lipit yokken ve varken homolog yer değiştirme deneyleriyle tespit edilmiştir. Özgün bağlanmada oluşan değişimin bağlanmanın afinitesi (K_D) ya da bağlanma alanlarında (B_{max}) oluşan değişiklikten kaynaklandığını anlamak için denge bağlanma deneyleri gerçekleştirilmiştir.

Arakidonik asit (AA) agonist ve antagonist ligandların bağlanmalarını doza bağlı şekilde [^{3}H]$ProE_1$, [^{3}H]DIDI and [^{3}H]Nlx için sırasıyla 0.15, 0.1 and 0.6 mM IC_{50} değerleri ile azaltmıştır. K_d değerleri etkilenmezken, B_{max} değeri mu ve delta reseptör alt tipleri için sırasıyla % 38 ve % 76 azalmıştır. [^{3}H]Nlx

ligandı için B_{max} değerleri ortamda 100 mM NaCl varken ve yokken sırasıyla % 20 ve % 56 azalmıştır..

Kolesterol hemisaksinat (CHS) ligand bağlanmasını mu tiplerinde artırmış (kontrole göre % 100) fakat delta tiplerinde herhangi bir etki göstermemiştir. CHS, antagonist ligandın bağlanmasını ortamda NaCl yokken artırmıştır (kontrole göre % 50). B_{max} değerleri mu tipleri için % 70 ve antagonist ligand bağlanma alanları için % 40 artmıştır. Benzer koşullar altında K_d değerleri etkilenmemiştir.

Fosfatidik asit (PA) ve fosfatidilkolin (PC), ligand bağlanması üzerine az bir etkiye sahiptir. PA, [^{3}H]ProE$_1$ ve [^{3}H]DIDI özgün bağlanmasını sırasıyla % 16 ve % 10 azaltmıştır. Antagonist ligand Nlx'un özgün bağlanması ortamda NaCl varken % 11 azalmıştır. Ortamda NaCl yokken özgün bağlanma kontrol seviyelerine çok yakındır. Agonist ve antagonist ligandların özgün bağlanması PC ortamda iken kontrol değerleri civarında gerçekleşmiştir.

Bu çalışmada lizofosfolipitlerin (lizofosfatidikasit ve lizofosfatidilkolin) opiate bağlanma alanları üzerine yaptıkları etkiler ilk kez çalışılmıştır. Her iki lizofosfolipitde benzer etkiler göstererek özgün bağlanmayı alt tiplere bağımlı olarak 0.1 ile 1 mM arasında azaltmıştır. K_d değerleri önemli şekilde etkilenmezken, B_{max} değerlerinde önemli bir azalma (% 45-75) tespit edilmiştir.

Anahtar Kelimeler:, Arakidonik asit, Kolesteril hemisaksinat, Fosfolipit, Lizofosfolipit, Opiat reseptörleri, Sıçan beyin zarları.

To My Elder Brother

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AA	arachidonic acid
AC	adenylate cyclase
B_{max}	maximum number of binding site
BSA	bovine serum albumin
$ProE_1$	endomorphine I
CHS	cholesteryl hemisuccinate
DIDI	$Ile^{5,6}$ Deltorphine II
GPCR	G-protein coupled receptors
K_d	equilibrium dissociation constant
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
Nlx	naloxone
PA	phosphatidic acid
PC	phosphatidylcholine
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
Trizma base	Tris[hydroxymethyl]aminomethane

CHAPTER 1

INTRODUCTION

1.1. History of Opioids

The ability of poppy (*Papaverum somniferum*) to produce mood-altering and sleep-inducing effects has been known for thousands of years. Poppy is mentioned in Sumerian records (5000 to 4000 BC) and Assyrian tablets refer to medicinal properties of opium (name coming from "*opos*", Greek word for juice), substance obtained from the unripe seed pods of the poppy plant. There is an opinion that mysterious drug nepenthe ("against sorrow") which, according to Homer, "drowning cares and angers, did decline / All thought of ill. Who drunk her cup could shed / All that day not a tear…" (Odyssey IV, 295-297, translated by George Chapman) was also nothing else but opium.

Famous physician, writer, and philosopher Galen (AD 129-ca.216) was of very high opinion of the virtues of opium, and his writings greatly influenced European medical theory and practise. Later Paracelcus (1493-1541) introduced the alcoholic tincture of opium called *laudanum* as an analgesic and sedative. *Paregoric*, another opium-based medicine widely used for treating of diarrhoea, was made by combining camphor with tincture

of opium. Since there were few alternative therapeutics or painkillers until the 19th century, opium was somewhat of a medicinal panacea. Opium and opium-based products were easily available and widely prescribed by physicians.

The habit of opium smoking began after importing into Europe the practise of North American Indians to smoke tobacco in pipes. Gradually opium smoking became a preferred method of taking the drug.

Morphine (named after the Greek god of dreams Morpheus), the first of 20 or more alkaloids found in opium, was isolated in the very beginning of the 19th century by the young German pharmacist Serturner. The isolation of other alkaloids, such as codeine and papaverine, followed soon. These pure products earned popularity in the medical world. In 1853 a French physician Charles Pravaz invented the hypodermic syringe, and it made possible the administration of morphine by injection. This way the effect of the drug is much stronger than when the same amount is taken orally.

The fact that the use of opium leads to addiction is known since antiquity. However, it seems that this did not create serious social problems until comparatively recent times. The situation changed with the use of hypodermic needle for intravenous injections of opioids. During the Civil War in the U.S.A. about 400,000 soldiers treated with opiates became addicted. With the synthesis of more powerful drugs the problem further escalated. Thus, the search for better cough, chest and lung medicine led the Bayer Company to the synthesis of heroin (the name derived from the adjective “heroisch”) in 1898. There was considerable interest in the highly effective new drug, which was initially declared to be non-addictive. Unfortunately, heroin, though being a more potent and faster acting painkiller than morphine, soon was found to be even more addictive.

Opioid addiction soon became a serious political concern, leading to the Opium Convention that was signed in Hague in 1912 to control opium trade. Its conditions were added to the Treaty of Versailles after the end of World War I. In the U.S. all domestic manufacture of heroin was banned in 1924. In Europe the League of Nations basically drove heroin manufacture underground by the early 1930s.

Despite all efforts the situation with opiate and other narcotic abuse remains a serious problem worldwide. According to the U.S. National Household Survey on Drug Abuse, in 1999 an estimated 208,000 Americans were current users of heroin, more than tripling the number since 1993, with rapidly dropping the average age of heroin users. Heroin-related deaths are rising as a result of the increasing purity and decreasing price of the drug. UN World Drug Report 2000 says that “the UN estimates that some 180 million people worldwide - 4.2 per cent of people aged 15 years and above – were consuming during the late 90s; this figure includes 13 million people abusing opiates, 9 million of whom were addicted to heroin”.

1.2. OPIOID RECEPTORS

1.2.1. Discovery of opioid receptors

At the end of the 19th century, Fisher proposed the lock-and-key model for the enzyme-glycozide system. This idea was developed further and applied for receptors as well. The term “receptor” was introduced by P. Ehrlich. In general, receptors are protein molecules, components of a cell or organism, which interact with a drug and initiate a stream of biochemical reactions. Receptor-mediated drug effects involve two processes: binding (i.e. ligand-receptor complex formation) and receptor activation, which mediates the effect. All ligands, which upon binding to a receptor produce certain response, are called agonists; compounds, which bind to the receptor

but produce no effect, are called antagonists. Term “opiate” is usually used for the ligand of non-peptide nature, and “opioid” is a broader term describing both peptide and non-peptide compounds.

Different pharmacological evidence led to the idea of the existence of specific receptors for opioid ligands in central nervous system (CNS). First demonstration of these receptors was done by three independent research groups in 1973 (Simon, 1973; Terenius, 1973; Pert, 1973). In 1976 experiments on spinalized dogs performed by Martin and co-workers clearly showed that opioids are not a homogenous group, as it was believed before, but consist of several different types. According to the drugs used in the experiments, three types of receptors discovered were named mu (μ, for morphine), kappa (κ, for ketocyclazocine) and sigma (σ, for SKF (10,047(+)-N-allylnormetazocine)). Next year Lord et al. (1977) described one more opioid receptor type found in the mouse *vas deferens* preparation, and this type was named delta (δ) receptor. Later sigma receptor was found to be non opioid, and by now only mu, delta and kappa receptor types are widely accepted, though several other receptor types were proposed as opioid receptors (epsilon (ε, Wuster et al., 1979), lambda (λ, Grevel et al., 1985) and zeta (ζ, Zagon et al., 1991). For each type of opioid receptor several subtypes are described (Wolozin and Pasternak, 1981; Jiang et al., 1991; Sofuoglu et al., 1993; Wollemann et al., 1993). Following the isolation of DNAs encoding the delta-, kappa- and mu-opioid receptors, low stringency homology based screening of cDNA libraries identified a receptor that appeared to be a fourth member of opioid receptor family, nociceptin receptors (NOP-R initially called LC132, ORL1, or nociceptin/orphanin FQ, Bunzow et al., 1994; Mollereau et al., 1994; Meunier et al., 1995). According to the nomenclature suggested by the International Union of Pharmacology (IUPHAR), the opioid receptors are referred to as MOP, DOP, KOP, and NOP receptors for the mu, delta, kappa, and nociceptin receptors, respectively. Nomenclature of opioid receptors are given in Table 1.1.

Table 1.1. Nomenclature of opioid receptors

Opioid Receptors		
Pharmacology Nomenclature	**IUPHAR Recommendation**	**Molecular Biology Nomenclature**
Delta	OP1	DOR, OPRD
Kappa	OP2	KOR, OPRK
Mu	OP3	MOR, OPRM
Nociceptin	OP4	NOP-R, N/OFQ

1.2.2. Heterogeneity of opioid receptors: pharmacological, biochemical and anatomical evidence

Pharmacological evidence for the multiplicity of opioid receptors has come from many areas including in *vivo* bioassays, binding experiments with selective radioligands, and selective protein and inactivation experiments with alkylating and acylating agents. The pharmacological properties of the three opioid receptor classes are distinct and can be clearly differentiated (Wood and Iyengar, 1988) (Table 1.2.). The most important pharmacological effect of activating opioid receptors is analgesia, the decrease of pain perception. This effect can be evoked through all the three types of opioid receptors, though different forms of pain might be regulated by different types of receptors. Mu and delta sites are considered to mediate analgesia at supraspinal, while kappa and delta sites at spinal sites. A number of autonomic responses are also mediated by opioid receptors. The respiratory depression (Florez and Mediavilla, 1977) is mediated by mu receptors, and this is the main undesirable side effect of narcotic analgesics.

Nausea, hypothermia, bradycardia and antidiuretic side effects are also mediated through mu sites. The delta sites are responsible for mediating hyperthermia and hypotension, while kappa sites mediate miosis and diuresis.

The targets of the endocrine effects of opioids are mainly the pituitary hormones (Holaday and Loh, 1979). They increase the release of adrenocorticotropic hormone (ACTH), (mu, kappa), melanocyte-stimulating hormone (MSH) (mu), prolactin (mu), growth hormone (delta), while inhibit the release of antidiuretic hormone (ADH) (kappa), luteinizing hormone (LH) (mu) and oxytocin (mu, kappa). Opioids cause behavioral and mood changes: mu receptors mediate euphoria, while the opposite effect, dysphoria is mediated by kappa sites. Agonists acting at kappa sites cause sedation and appetite suppression as well. The elevated loco motor activity is mediated by kappa sites. The role of opioid receptors in mental illness, memory and learning behaviour has also been reported (Olson, 1995).

In addition, there is evidence that opioid receptors also participate in the control of immune system (Roy and Loh, 1996). More than two decades ago, existence of opioid receptors on cells involved in host defense and immunity has been shown (Wybran et al.," 1979). The search for a deeper understanding of the pathogenesis of human acquired immunodeficiency syndrome has stimulated sustained interest in the immunomodulatory actions of opioids. Although HIV-contaminated needles may account in part for the increased prevalence of AIDS in drug abusing populations that administer drugs intravenously, several laboratories have demonstrated that opioids directly modify the immune response to HIV.

Genetic approaches are available to study in *vivo* the role of mammalian opioid systems in regulating many physiological functions, including pain perception and analgesia, responses to stress, aggression and dominance (Matthes et al., 1996; König et al., 1996).

Investigation of the behavioral effects of morphine in mice revealed that the lack of mu-receptors abolish the analgesic effects of morphine, place-preference activity and physical dependence (Matthes et al., 1996). It has been suggested that delta and kappa receptors do not mediate, even partially, any of the major biological actions of morphine in the absence of the mu-receptor. This raises the important issue of cooperativity between the opioid receptors (Traynor and Elliot, 1993) which might take place at the molecular level through allosteric interactions or second messenger systems, or occur at a functional level on separate neurons.

1.2.3. Structure of the opioid receptors

Opioid receptors are members of the membrane-bound neuroreceptor family that are functionally coupled to G-proteins. Thus, opioid receptors share G-protein coupled receptors (GPCR) classical structure: seven hydrophobic transmembrane helices connected by intracellular and extracellular loops (Figure 1.1). Cloned mu, delta and kappa opioid receptors consist of 398, 372 and 380 amino acids, respectively in rat and mouse (Benyhe et al., 1994; Kieffer et al., 1995).

Opioid receptors were shown to have an extracellular N-terminal with multiple glycosylation sites, third intracellular loop with multiple amphiphatic α-helices and forth intracellular loop with putative palmitoylation sites. Two conserved cysteine residues, which are thought to be involved in disulfide bonding, are found in the first and second extracellular loops of these three receptors.

Table 1.2. Summary of generally accepted opioid receptor classes

Receptor	Mu	Delta	Kappa
Prototype Ligand	Morphine	enkephalins	Ethylketocyclazocine (EKC)
Endogenous Ligand	β-endorphin endomorphins dermorphins	met-enkephalin leu-enkephalin deltorphins	Dynorphin A
Selective agonists	DAMGO Morphiceptin Sulfentanyl PLO17	DADLE DPDPE DSLET D-Ala^{2}deltorphins	U50488 U69593 ICI197067
Antagonists	Naloxone	Naloxone	Naloxone
Selective antagonists	CTAP Cyprodime	ICI-174864 Naltrindole TIPP	Nor-binaltorphimine (nor-BNI)
Effects	Analgesia Euphoria Respiratory depression Release of Prolactin	Analgesia Hypotension	Analgesia Dysphoria Diuresis Release of ADH
Location	Rat brain rabbit cerebellum Guinea-pig ileum	Rat brain NG-108-15 cell line Mouse vas deferens	Rat brain Rabbit cerebrum Frog brain Guinea-pig cerebellum

A cysteine residue in the C-terminal domain, which is a potential site for palmitoylation, is conserved and the amino acid sequences from the start of the C-terminal intracellular domain to this cysteine residue is highly conserved across these receptors. This suggests that this region constructs a fourth intracellular loop that plays an important role in coupling to second messenger systems. (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Fukuda et al., 1993).

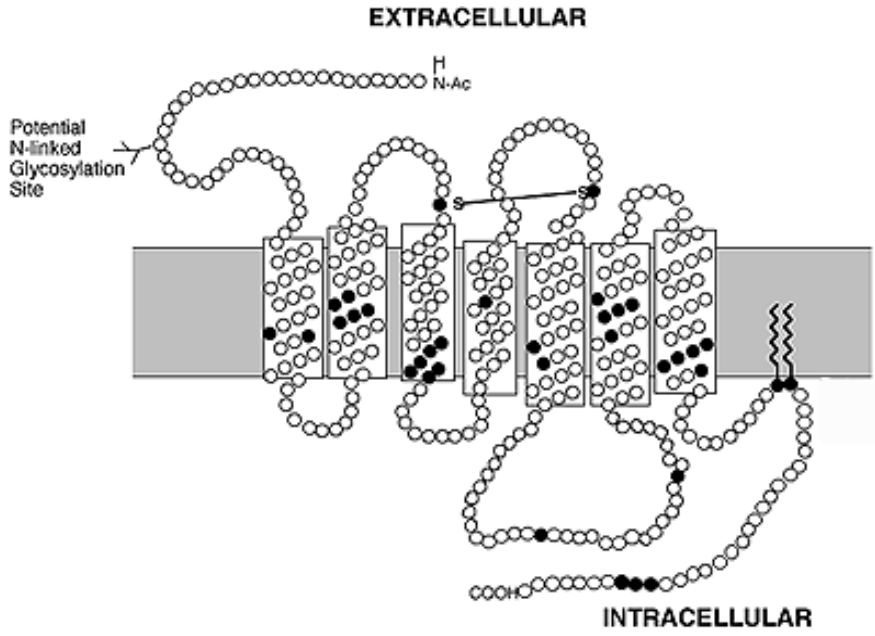


Figure 1.1. Schematic model of an opioid receptor

Among the other G-protein coupled receptors, somatostatin receptors are the most similar to these opioid receptors, having 34-42 % identity in amino acid sequences overall. Furthermore, AT_{1A} and AT_{1B} angiotensin II receptors, formylmethionylleucylphenylalanine receptor, NPY_1 and NPY_3 neuropeptide Y receptors and interleukin-8 receptor have 20-30% identity.

The amino acid sequences of mu, delta and kappa receptors are approximately 60 % identical to each another, with the highest homology found in transmembrane domains (73-76% of those identified) and intracellular regions (63-66 % of those identified) and third extracellular loops as well as the N- and C-terminal tail which are extracellular and intracellular, respectively (Table 1.3). The third intracellular loop is implicated in binding G

proteins and has consensus sequences for phosphorylation, which might be involved in regulatory processes as G protein docking. High homology of this region suggests that various opioid receptors may interact with similar G-protein complexes. The greatest divergences in amino acid sequence occur mostly in the N-terminus, second and third extracellular loops and the C-terminus.

1.2.4. Distribution of Opioid Receptors

Opioid receptors are widely distributed throughout the brain and peripheral tissues of all animals, although pattern of distribution of mu-, delta- and kappa- receptors, and the quantity of receptors varies between species and various major anatomical regions.

The relative abundance of opioid binding sites from the species varies dramatically. The ontogeny of different types of receptors is quite distinct. In rat, mu-, and kappa- receptors appear earlier in development than delta-receptor does (Kornblume et al., 1987).

Opioid receptor distribution varies between anatomical regions. In general, the laminar patterns distribution is distinctive (Mansour et al., 1988; Hiller and Fan, 1996). In the CNS, the mu- opioid receptors have been localized by autoradiographical studies in the caudate putamen, neocortex, thalamus, nucleus accumbens, hippocampus, amygdala, hypothalamus, periaquenductal gray, raphe nuclei, globus pallidus and spinal cord. The delta- opioid receptors have a more restricted distribution in the CNS than other opioid receptors. They are denser in olfactory bulb, caudate putamen, nucleus accumbens and neocortex. The kappa- receptors are particularly enriched in the cerebral cortex, striatum, substantia nigra and hypothalamus (Mansour et al., 1988).

In the periphery, opioid receptors are found in myenteric plexus and in certain smooth muscles, such as guinea pig ileum, mouse vas deferens, hamster vas deferens and rat vas deferens. Peripheral tissues are extremely useful as in vitro bioassay systems for opioids and their receptors (Leslie, 1987).

Table 1.3. Amino acid identity of different types of receptors

	Amino acid identity		
	μ/δ %	**μ/κ %**	**δ/κ %**
Extracellular regions	34	36	40
N-terminal	25	33	31
First loop	72	67	72
Second loop	42	35	54
Third loop	18	18	11
Transmembrane (TM) regions	76	73	74
TM1	69	62	62
TM2	100	84	84
TM3	82	91	91
TM4	45	32	55
TM5	79	79	75
TM6	77	73	68
TM7	86	95	90
Intracellular regions	63	66	63
First loop	90	100	90
Second loop	91	91	95
Third loop	87	87	83
C-terminal (before palm.)	82	91	82
C-terminal (after palm.)	27	31	21

1.2.5. Molecular Biology of Opioid Receptors

A lot of effort has been made in order to elucidate the primary structure of the opioid receptors by several investigators since eukaryotic gene cloning became generally practicable. The first trials used receptor purification and peptide sequencing to lead on to oligonucleotide probes for hybridisation screening. Only in one case this approach led to a protein sequence (Schofield et al., 1989), but the cDNA obtained gives no expression of opioid binding and lacks any putative transmembrane domains. Cloning opioid receptor cDNA by homology screening has also failed. Finally, receptor expression cloning led to success, though the first cDNA obtained from human placenta using this method (Xie et al., 1992) displays low affinity towards both peptide and alkaloid opioid ligands and is expressed in tissues where opioid receptors were not known to be present (skeletal muscle, kidney). In an attempt to isolate a cDNA encoding an opioid receptor Cabon et al. (1993) also used the expression cloning approach, but the sequence found, though coding a protein having high affinity for opiate alkaloids, turned out to be the part of the *E.coli* genome.

The first cloning of real opioid receptor gene identified was the one of the delta opioid receptor type described simultaneously by two research groups (Kieffer et al., 1992; Evans et al., 1992). Both of these groups used cDNA libraries derived from NG-108 neuroblastomaglioama cell line. The clones encode a 372 amino acid-residue protein which refers to the mouse delta opioid receptor. The first opioid receptor to be cloned was the mouse DOR in 1992 from a NG108-15 glioma hybridoma cell line, followed by the discovery of the rat DOR, the human DOR in 1994, and an amphibian DOR. Although only one DOR gene has been cloned, on the basis of in vitro and in vivo pharmacological characteristics, a variety of subtypes of DOR have been proposed. Specifically, the opioid ligands [D-Pen^{2}, D-Pen^{5}]enkephalin

(DPDPE), [D-Ala^2, D-Leu^5]enkephalin (DADLE), [D-Ala^2, D-Leu^5]enkephalyl-Cys (DALCE), and 7-benzylidenenaltrexone (BTNX) have been shown to bind with high affinity to DOR sites designated as δ1, while [D-Ser^2,Leu^5]enkephalyl-Thr (DSLET), [D-Ala^2]deltorphin II, naltrindole 5-isothiocyanate (5-NTI), and naltribene bind to δ2-sites.

Soon after successful cloning of delta receptors, the cloning of kappa and mu opioid receptors was reported (Yasuda et al. 1993; Chen et al. 1993a,b; Wang et al., 1993). To date, MOR genes have been cloned from rat, mouse, human, rhesus and crab-eating monkey The MOR-1 gene, encoding for one form of the μ-receptor, shows approximately 50-70% of homology to the genes encoding for the δ- (DOR-1), κ- (KOR-1) and orphan (ORL_1) receptors. Two splice variants of the MOR-1 gene have been cloned, differing only in the presence or absence of 8 amino acids in the C-terminal tail. The splice variants exhibit differences in their rate of onset and recovery from agonist-induced internalization but their pharmacology does not appear to differ in ligand binding assays. The μ_1/μ_2 subdivision was proposed by Pasternak and colleagues to explain their observations, made in radioligand binding studies, that tritium labelled mu-, delta- and kappa- ligands displayed biphasic binding characteristics.

The cloned μ- opioid receptor has high affinity to morphine, naloxone and DADLE. Furthermore, the μ- selective ligand DAMGO but not the δ-selective ligand DPDPE or the κ- selective ligands (U50,488H) binds to the receptor with a high affinity, confirming that the cloned receptor is of the μ-type. The mu opioid receptor is widely distributed throughout the central nervous system, with higher levels found in the periaqueductal gray region and in the superficial dorsal horn of the spinal cord.

KOR have been cloned from rat, mouse, human, guinea pig, amphibian, and zebra fish. The cloned KOR has high affinity for the endogenous peptide dynorphin A (1-17), and a variety of selective agonists and antagonists have been developed for the KOR. As for the other opioid receptor classes, a number of subtypes have been described for the kappa type receptors pharmacologically; κ1 sites bind to dynorphin 1-17 but not to DADLE (representing the cloned KOR profile), κ2 sites bind (Arg6, Phe7)-Met-enkephalin and DADLE, and the so-called κ3 site is sensitive to naloxone benzoylhydrazone.

Further attempts to clone additional opioid receptor types and/or subtypes led several laboratories to isolate a cDNA encoding a homologous protein with a high degree of sequence similarity to the opioid receptors but did not bind opiates and opioid receptor antagonists with high affinity. This receptor remained ‘’orphan’’ until late 1995, when two groups independently reported the isolation of the endogenous peptide ligand from brain tissue of rat and porcin. The new ligand was a hectadecapeptide closely related to the KOR-selective peptide dynorphin A and was termed nociceptin or orphanin FQ. Main features of the cloned opioid receptors are given in Table 1.4.

1.3. Endogenous Opioid Peptides

1.3.1. Discovery of Opioid Peptides

When the opioid receptors were discovered the endogenous ligands acting upon these receptors were unknown. The finding of opiate binding sites and the fact that opiate antagonists exerted some intrinsic activity in opiate naive subjects and could diminish non drug induced analgesia, stimulated thoughts about endogenous compounds with opiate like action (Lasagna, 1965; Jacob et al., 1974; Akil et al., 1976). The first indication for endogenous opioids came from studies showing that brain extracts contain

opioid-like activity (Terenius and Wahlström, 1974; Kosterlitz and Waterfield, 1975). Further investigations led to the isolation and characterization of the endogenous morphine-like substance which named Enkephalin for ″in the head″ (Hughes et al., 1975) and of the C-fragment of the pituitary hormone β-lipotropin, later termed β-endorphin (Bradbury et al., 1976) (Table 1.3.1). The enkephalins and β-endorphin were shown to induce similar actions as morphine in a number of *in vitro* and *in vivo* test procedures. Among others, they were self-administered by laboratory animals, indicating the rewarding properties and addictive potential of these substances. Another class of endogenous opioids, the dynorphins (Goldstein et al., 1979; Goldstein et al., 1981), and nociceptin/orphanin FQ as the endogenous ligand of the orphan receptor has a clear resemblance.

Table 1.4. Characteristics of the cloned opioid receptors

	Mu	**Delta**	**kappa**
Gene family	GPCR	GPCR	GPCR
Number of introns	3	2	2
Size of mRNA	10-16 kb	4.5-11.0 kb	5.2 kb
Amino acid length of the gene product	400 aa [human]	372 aa [human]	380 aa [human]
	398 aa [mouse]	372 aa [mouse]	380 aa [mouse]
	398 aa [rat]	372 aa [rat]	380 aa [rat]
Number of glycosylation sites	5	2	2
Number of phosphorylation sites	3-4	4-7	5-7
Chromosomal localisation [human]	6 q24-25	1 p34-36	8 q11-12

1.3.2. Distribution of Opioid Peptides

Enkephalins the first endogenous opioids extracted more widely distributed in the brain than beta-endorphins, being present in several areas including hypothalamic nuclei, limbic structures, caudate-putamen, the brain stem, several layers of the dorsal horn, peripheral nerves, and the adrenal medulla. The most powerful of the opioids, dynorphins, are found throughout the central and peripheral nervous systems. Some research supports the theory that they regulate pain at the spinal cord level, influence feeding behaviour at the hypothalamic level, and function with other endogenous opioids to regulate the cardiovascular system. Dynorphins also may be involved in inhibiting intestinal motility, a phenomena that occurs when the body perceives pain. The presence of a large precursor to this opioid in the anterior pituitary suggests that it has many peripheral targets. Another opioid called neo-endorphin also is classified in the dynorphin group.

Opioid peptides have also been isolated in invertebrates. Enkephalin peptides were found in molluscs (Leung and Stefano, 1984) and in annelids (Salzet et al., 1995).

1.3.3. Properties of Opioid Peptides

Endogenous opioid peptides are mainly derived from four precursors, pro-opiomelanocortin, proenkephalin, prodynorphin, and pronociceptin/orphanin FQ. Except for pronociceptin/orphanin FQ, all derived from the other precursors consists of a pentapeptide sequence TyrGlyGlyPheMet/Leu (YGGFM/L). Nociceptin/orphanin FQ, however, contains a phenylalanine (F) instead of the N-terminal tyrosine, a residue necessary for high-affinity binding to the classic opioid receptors.

Proopiomelanocortin gives rise to endorphins, as well as non-opioid adrenocorticotrop hormone and melanocyte stimulating hormone (Mains et al., 1977). Proenkephalin contains one copy of Leu-enkephalin, four copies of Met-enkephalin and two extended Met-enkephalins (Noda et al., 1982). Prodynorphins gives rise to dynorphin A and B, and α-, β-neoendorphins (Goldstein et al., 1979, 1981). Some of the endogenous opioid peptides and their precursors are shown in Table 1.5.

The amino acid sequence of nociceptin/orphanin FQ has homology with other opioid peptides especially the prodynorphin fragment dynorphin A, suggesting a close evolutionary relationship between the precursors. Nociceptin/orphanin FQ, however, has a C-terminal phenylalanine (F) whereas peptides derived from the other precursors all have the pentapeptide sequence TyrGlyGlyPheMet/Leu (YGGFM/L) at their N-termini. These peptides vary in their affinity for mu-, delta- and kappa-receptors, and have negligible affinity for ORL1-receptors, but none binds exclusively to one opioid receptor type.

All pro-endorphins are synthesized in the nucleus and transported to the nerve terminal by microtubule transport. At the nerve terminal they are cleaved by specific proteases. These protease recognize the double basic amino acid sequences positioned just before and after the opioid peptide. The peptides are released when the nerve fires and bind to post-synaptic receptors, stimulating second messenger systems. The action of opioid peptides is terminated by membrane-bound proteases which cleave the terminal Gly-3-Tyr-4 bonds. These data indicates that endogenous opioids act as neurotransmitter. Opioids were also shown to have influence on long-term potentiation process.

Table 1.5. Opioid peptides and their structures

Opioid peptide	Structure
β -endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
Met-enkephalin-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met-Arg-Phe
dynorphin $A_{(1-8)}$	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile
dynorphin $A_{(1-13)}$	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
dynorphin $A_{(1-17)}$	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gly
α-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
β-neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Dermorphins	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-OH
	Tyr-D-Ala-Phe-Trp-Tyr-Pro-Lys-OH
	Tyr-D-Ala-Phe-Trp-Asn-OH
	Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Glu-Ala-OH
	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Glu-Ala-Lys-Lys-Ile-OH
Deltorphins	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2
	Tyr-D-Ala-Phe-Asp-Val-Val-Gly- NH_2
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly- NH_2
	Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH_2
endomorphin 1	Tyr-Pro-Trp-Phe-NH_2
endomorphin 2	Tyr-Pro-Phe-Phe-NH_2
Nociceptin	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln

Although endogenous peptides usually have some binding preferences to a certain receptor type, they are able to bind to all three types of receptors. [Met]- and [Leu]enkephalin have high affinities for delta-receptors, ten-fold lower affinities for mu-receptors and negligible affinity for kappa-receptors. Other products of processing of pro-enkephalin, which are N-terminal extensions of [Met]enkephalin, have a decreased preference for the delta-receptor with some products, e.g. metorphamide displaying highest affinity for the mu-receptor. The opioid fragments of pro-dynorphin, particularly dynorphin A and dynorphin B, have high affinity for kappa-receptors but also have significant affinity for mu- and delta-receptors. Beta-endorphin is equiactive at mu- and delta- receptors with much lower affinity for kappa-receptors; the post-translational product, N-acetyl-beta-endorphin, has very low affinity for any of the opioid receptors. Dermorphins and deltorphins, another group of endogenous opioid peptides that were found in the skin of amphibia Phyllomedusa, are mostly mu- and delta-selective ligands, respectively (Mignona et al., 1992; Erspamer et al., 1989). These two classes of amphibian peptides share a common N-terminal amino acid sequence, Tyr-D-Xaa-Phe, where Xaa is either D-Ala, D-Met or D-Leu.

Opioid peptides identified in mammalian brain are considered to be endogenous agonists for the delta- (enkephalins) and kappa- (dynorphins) receptors, but none of the identified endogenous opioids has absolute pharmacological specificity for a given receptor type (Mansour et al., 1995b). In 1997, Zadina and colleagues isolated two new tetrapeptides from bovine brain, both of which had high affinity and selectivity for the mu- receptor. These endogenous peptides were named endomorphin 1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (Tyr-Pro-Phe-Phe-NH_2). Later endomorphins were found in human brain and spinal cord (Hackler et al., 1997) and immune tissues (Jessop et al., 2000). These peptides exhibited the highest specifity and affinity for the mu-receptor among all endogenous opioids so far discovered in the mammalian systems.

1.3.4. Pharmacology of Opioid Peptides

Enkephalins have been associated with addiction to and withdrawal from morphine. Morphine intensely stimulates the enkephalin receptors which causes a negative feedback machanism suppressing the activity of the enkephalin neurons. This decrease in enkephalin neural activity necessitates an increase in the amount of morphine in order to maintain the same level of analgesia. This biological phenomenon is responsible for the drug tolerance and addiction associated with morphine.

Unlike opioids, the body's endorphins are not addicting. When endorphins are not activating receptors, no withdrawal symptoms are felt. Enzymes break down endorphins as soon as they act at receptors, so they are never in contact with receptors long enough to form tolerance and dependency.

The most popular action of endogenous opioids is their creation of a feeling of euphoria during exercise, which endurance athletes frequently refer to as runner's high. Numerous investigators have observed an increase in blood plasma levels of beta-endorphin following physical activity. However, plasma levels of this substance have not been found to increase proportionally with exercise intensity.

In addition to analgesia, endogenous opioids have also been found to maintain feeding behavior. This finding is supported by research indicating enhanced feeding responses following beta-endorphin injections and work nothing that naloxone inhibited these responses. Endogenous opioids also have been linked to moderation of drinking behavior and cough suppression.

Endogenous opioids have a great variety of functions throughout the body. Their distribution and the distribution of the opioid receptos ensure a highly effective pain management system. Gastrointestinally in stomach they decrease gastric motility, thus prolonging gastric emptying time. This may lead to esophageal reflux. In small intestine they decrease biliary, pancreatic, and intestinal secretions and delays digestion of food in the small intestine. Resting tone increases and periodic spasms occur. In large intestine propulsive peristaltic waves in the colon are diminished and tone increases until it spasms. This, along with the inattention to the normal stimuli for defecation reflex, contribute to constipation. They increase smooth muscle tone in the urinary tract and can induce spasms. Urinary urgency and difficulty with urination may result (Kastin et al., 1997).

1.4. Opioid Receptors and Signal Transduction

1.4.1. The role of the G-proteins

The transmissions of extracellular information that are carried by opioid ligands are mediated by opioid receptors interacting with heterotrimeric guanine nucleotide binding proteins (G-proteins). These heterotrimeric G-proteins are members of a guanine nucleotide-binding protein superfamily as are cytoskeletal proteins and low molecular weight GTP-binding proteins (ras p21 protooncogenes and ras related proteins) (Spiegel, 1987; Gilman, 1987). The features of regulatory G-proteins include: 1) association with the cytoplasmic surface of the plasma membrane; 2) function as receptor-effector couplers; 3) heterotrimeric structure with subunits designated α; β and γ . These subunits are distinct gene products. To date 23 α-subunits (encoded by 17 different genes), 5 β-subunits and 12 γ-subunits have been cloned at this time (Simon, 1991; Kehlenbach et al., 1994; Nurnberg et al., 1995). The γ-subunits, in contrast to the β-subunits, show a high degree of diversity (Ray et al., 1995; Morishita et al., 1995).

The α-subunits are subdivided into 4 main families: G_s, G_i, G_q, G_{12}. α-subunits are required for the specificity in receptor-effector coupling, bind guanine nucleotides with high affinity and specificity, possess intrinsic GTP-ase activity, serve as substrates for ADP-ribosylation by bacterial toxins and directly regulate effector activity. βγ-subunits, coupled tightly but noncovalently to the complex, are responsible for receptor G-protein coupling, directly modulate effector activity and can inhibit G-protein activation by blocking α-subunit dissociation (Spiegel A.M., 1990). The G_s-protein, the stimulatory of G-protein of adenylyl cyclase (AC) was discovered in 1977 by Pfeuffer and is responsible for mediating AC activation.

The effect of opioid ligands are mediated by pertussis toxin sensitive G_i and G_o proteins. G_i-proteins were initially purified from rabbit liver (Bokoch et al., 1983) and human erythrocytes and can regulate a great variety of effector systems such as AC, phospholipase C (PLC), phospholipase A_2 (PLA_2), phospholipase D (PLD), K^+ channels, Ca^{2+} channels. G_o proteins activate also ion channels (Spiegel A.M. 1990).

When an agonist interacts with its receptor, the receptor associates with a specific heterotrimeric G-protein that is in the inactive, GDP bound form. The activated receptor induces the release of GDP from the α subunit. The nucleotide free α-subunit binds GTP and induces conformational changes leading to dissociation of the G-protein from the receptor and dissociation of the heterotrimeter into GTP-liganded α subunit and βγ dimer. Both of them can regulate effector molecules including AC and phospholipases as well as certain ion channels. Due to its GTP-ase activity the α-subunit restores the inactive form of the Gα-subunit and the termination of G-protein activation is achieved by the reassociation of the α-subunit with the βγ dimer (Birnbaumer et al. 1990; Birnbaumer, 1992). Agonist induced G-protein activation is demonstrated in Figure 1.2.

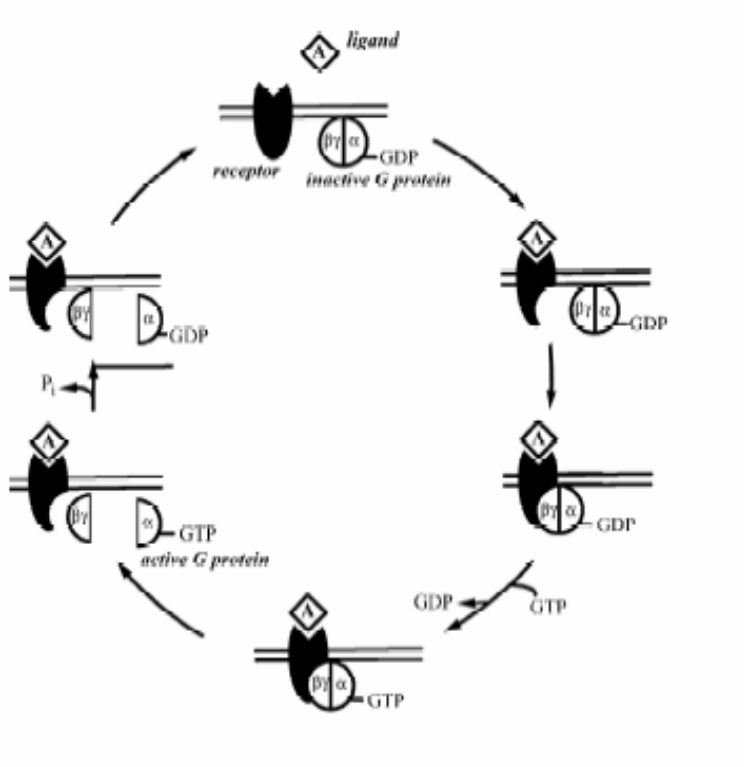


Figure 1.2. G protein activation

Agonists differ in their maximal ability to cause biological responses. The term efficacy has been introduced first by Stephenson (1997). In the case of G-protein coupled receptors it is determined by two signal transduction components: 1) the ability to induce receptor stimulated guanine nucleotide exchange on the G-protein α-subunit 2) the number of G-proteins activated by occupied receptors (Selley et al., 1997). Intrinsic efficacy is characteristic of a certain drug and in contrast to efficacy, is independent of receptor density. It represents the amount or response induced per unit of receptor occupation (Johnson and Fleming, 1989). Full agonists are able to produce maximal response without occupying all the available receptors. In the case of classical partial agonists the activation of the whole receptor pool is required to produce the maximal effect. Drugs with modest intrinsic efficacy can be partial agonists in a tissue with relatively low receptor concentration and full agonists in another tissue with higher density of the same receptors.

This type of ligands are called mixed full/partial agonists (Johnson and Fleming, 1989; Selley et al., 1997).

Divalent and monovalent cations and guanine nucleotide, guanosine 5'-triphosphate (GTP) and thiol reagents affect differentially the binding of opioid agonists and antagonists. For example, Na^+ ions reduce the affinity of opioid receptor for agonists but not for antagonists. Different mechanisms have been proposed to explain this phenomenon. It has ben hypothesized that Na^+ ions allosterically transforms opioid receptor sites from conformations which bind agonists more readily to conformations which bind antagonists more readily (Pert and Snyder, 1974). To investigate the mechanisms through which Na^+ modulates opioid receptor properties Kong et al. (1993) mutated the Asp95 residue, in the second transmembrane region of the protein molecule, to Asn in the cloned delta receptor and found that Na^+ regulation of agonist binding was lost. This residue seems to play a role in the Na^+ regulation of agonist binding rather than being directly involved in agonist ligand recognition. Sulfhydryl reagents, such as iodoacetamide, N-ethylmaleimide and p-hydroxymercuri-benzoate inhibited opioid receptor specific binding. N-ethylmaleimide affected opioid agonist binding by at least two mechanisms: direct inhibition and indirect inhibition due to uncoupling of receptors from G proteins.

1.4.2. Effector mechanisms

It seems that most type of cloned receptors belong to the G_i/G_o-coupled superfamily of receptors and share several common properties in their ability to interact with second messenger systems. The direct G-protein ($\beta\gamma$ or α subunit) mediated effects are: 1) inhibition of AC, 2) inhibition of voltage operated Ca^{2+} channels; 3) activation of an inwardly rectifying K^+ channels; 4) regulation of PLC activation; 5) activation of mitogen-activated protein kinases (MAPK) pathways.

1.4.2.1. Adenylate cyclase

Opiates may, like many other drugs and neurotransmitters, modulate the activity of AC in brain. Coupling of the opioid receptors to the inhibitory system of AC has been studied in transformed cell lines and in brain membranes. The mechanisms of the receptor coupling to AC have been studied mostly in NG108-15 cell line which contain high levels of opioid receptor binding sites (Hamprecht, 1977). Opioid receptors in this cell line were identified as being of the delta-type (Chang and Cuatrecasas, 1979; Gilbert et al., 1982) and they inhibit both basal and prostaglandin E1-stimulated AC activity (Traber et al., 1975a; Brandt et al., 1976 and 1977). Pertussis toxin abolishes the inhibition of AC by opioids NG108-15 cells (Hsia et al., 1984), suggesting that G_i- (or G_o-) protein is coupled to these opioid receptors to exert their inhibitory effects.

Interestingly, there are reports suggesting that the complexity of the AC system allows different routes for opioids not only to inhibit, but also to stimulate cAMP production (Sarne et al., 1998). Thus, $G_{\beta\gamma}$ complex was demonstrated to stimulate AC of type 2, 4 and 7 (Federman et al., 1992; Tsu et al., 1995). Since AC type 2 and 4 are expressed in the brain, it is quite possible that some of the central actions of the opioids are mediated by these enzymes. So, the regulation of intracellular cAMP by opioids seems to be rather complicated (Law et al., 2000).

1.4.2.2. Calcium Channels

One cellular event that underlies the effects of opioids to reduce cellular excitability and neurotransmitter release is the inhibition of voltage-dependent Ca^{2+} channels. The activation of all three types of opioid receptors are able to inhibit different types of Ca^{2+} channels (North, R.A., 1992; Rhin et al., 1994; Connor et al., 1999). This reduction of Ca^{2+} currents through opioid receptors is blocked by pertussin toxin, indicating the involvement of G_i-

and/or G_o-proteins. Presently it is realized that $G_{\beta\gamma}$ subunits inhibit Ca^{2+} channels rather than $G_{\alpha 0}$ subunit (Ikeda, 1996: Herlitze et al., 1996).

Cloned rat mu opioid receptor is functionally coupled to N-type Ca^{2+} channels (Morikawa et al., 1995). Cloned mu and delta opioid receptors expressed in GH3 pituitary cells inhibited voltage-activated L-type Ca^{2+} channels (Piros et al., 1996). Kappa opioid receptors also are able to modulate Ca^{2+} channels activity (Kaneko et al., 1994).

1.4.2.3. Potassium Channels

Membrane hyperpolarization caused by an increase in K^+ conductance is another cellular event which is thought to be important for opioids to reduce cellular excitability and inhibit neurotransmitter release. The activation of mu- and delta-opioid receptors increases an inwardly rectifying K^+ conductance in various preparations, including the locus coeruleus, hippocampus, and submucosal plexus. The kappa-opioid receptor also increases K^+ conductance in guinea pig substantia gelatinosa neurons. These increases in K^+ conductance caused by the activation of opioid receptors are sensitive to pertussis toxin, indicating mediation through G_i- and/or G_o-proteins. The increase in K^+ conductance by the stimulation of opioid receptors has been confirmed using the Xenopus oocyte system, in which the cloned mu- or kappa-opioid receptor and G-protein-activated K^+ channel are co-expressed (Chen and Yu, 1994). These increases of K^+ conductance were also blocked by pertussis toxin.

1.4.2.4. Phospholipase C

PLC is another effector molecule for opioid receptors. Opioid stimulation of PLC was shown in SH-SY5Y human neuroblastoma cells (Smart et al., 1994). Mechanism of PLC activation may involve $G\beta\gamma$ subunits (Chan et al., 1995) or activation of L-type Ca^{2+} channels (Smart et al., 1995).

Opioids mobilize Ca^{2+} from the inositol 1,4,5-trisphosphate-sensitive store in NG108-15 cells. This mobilization of Ca^{2+} from internal stores was mediated by G_i- and/or G_o- protein and depended upon the cell growth conditions. The cloned opioid receptors expressed on Xenopus oocytes can mediate the oscillatory Cl^- current response. This type of oocyte response is mediated by mobilizing Ca^{2+} from internal stores via inositol phosphate formation. G_i- and/or G_o- proteins are probably involved in this response in Xenopus oocytes expressing opioid receptors, since pertussis toxin blocked it.

1.4.2.5. MAPK Pathways

MAPKs are a family of serine/threonine protein kinases that are activated in response to various extracellular stimuli, including growth factors and hormones. MAPKs, has been shown to be activated by G protein-growth factors and hormones. Recently, G protein-coupled receptors have been shown to activate MAPKs through the action of the heterotrimeric G protein by two distinct mechanisms. The 85-kDa cytosolic PLA_2, which selectively cleaves arachidonate from phospholipid, is a substrate for MAPK and has been shown to be activated by phosphorylation by MAPK at a specific serine residue. All types of opioid receptors functionally couple with the MAPK cascade and PLA_2 through the action of PTX-sensitive G proteins (G_i or G_o) (Fukuda et al., 1996).

1.5. Oligomerization of Opioid Receptors

Oligomerization of GPCR is a burgeoning field of research that is significantly advancing and remodeling understanding of GPCR regulation and function.

A general structural basis for GPCR oigomerization has yet to be established. It is commonly thought that multiple factors, such as transmembrane domain hydrophobicity, extracellular disulfide bonds and intracellular coiled-coil interactions, cooperatively mediate oligomerization. Other factors could additionally account for receptor-receptor interactions, such as cross-linking through accessory proteins or leucine zipper interactions. Sensitivity to reducing agents implicate disulfide bonds in KOR dimerization, and in DOR-KOR and MOR-DOR hetero-dimerization (Samuel et al., 2003).

Oligomerization may be necessary for signal amplification, such that agonist activation of a limited number of receptors triggers activation of other associated receptors, resulting in increased effector coupling, receptor phosphorylation, G-protein uncoupling and internalization of an entire receptor complex.

More recently, several groups have reported dimerization of several GPCRs, including opioid receptors (Angers et al., 2001 and Lee et al., 2001). In fact, heterodimerization of opioid receptors has been shown to alter opioid ligand properties and affect receptor trafficking in cell culture model systems and *in vivo*. There are also reports of heterodimerization of the opioid receptors with other classes of GPCR; these include the NK1 receptor and the β2-adrenergic receptor (Jordan et al., 2001).

Opioid receptor oligomerization has significant potential implications in therapeutic drug design because ligands that interact with multiple receptors simultaneously may be more efficacious. The concept of bivalent ligands has previously been used in the design of numerous opioid ligands of increased potency and specificity.

1.6. Opioid tolerance and dependence

1.6.1. Tolerance, dependence and withdrawal

Tolerance develops after chronic (sustained or repeated) opioid drug treatment and refers to the decrease of the sensitivity of the biological system to an opiate (Cox B.M., 1993; Trujillo and Akil, 1991). Prolonged administration of opioid drugs also lead to psychial and physical dependence, a persistent relapsing disorder. The former reflects the special behavior and craving, an incentive to satisfy the appetite for drus (Terenius, 1997). The latter refers to the latered function of the organisms influenced by the drug whereby further administration is necessary to avoid physical disturbance. Termination of drug-treatment leads to ‘withdrawal’ syndromes. This hyper excitable state shows typically opposite signs to the acute actions of the drug, including restlessness, irritability, tremor, diarrhoea, goose flesh and muscle cramps (Trujillo and Akil, 1991).

Koob and Bloom (1988) described two possible mechanisms of drug tolerance: a within system and a between-systems adaptation. Opioid receptors uncoupling from G-proteins and receptor down-regulation, in particular the receptor’s high affinity sites, are well-known mechanisms (the within-system) of opioid tolerance. A series of recent studies have proposed that a between-systems, particularly the pain facilitatory systems (opiate-activated opponent systems), may also involve in the development of opioid tolerance.

Several lines of evidence suggest that N-methyl-D-asparate (NMDA) receptors activation and the subsequent nitric oxide (NO) production probably play a between-systems mechanism of opioid tolerance. Recently, it has been found that cyclooxygenase (COX) inhibitors could attenuate the opioid tolerance without enhancing morphine's anti-nociceptive effect.

In summary, except the opioid receptor uncoupling and opioid receptor down-regulation, chronic morphine treatment may also activate pain facilitatory systems (NMDA receptor activation, NO production, and COX activation) during opioid tolerance development. It implies that some complicated interactions may happen among the opioid receptor, NMDA-receptor, NO, and COX systems and are worth further investigations.

1.6.2. Desensitization, internalization and down-regulation

Prolonged exposure of cells to agonists results in a loss of responsiveness to further agonist stimulation through a process termed desensitization. The mechanism underlying homologous desensitization involves phosphorylation of the agonist-activated GPCRs by membrane of the GPCR kinase family, which promotes binding of arrestin proteins, effectively uncoupling the productive interaction of activated GPCRs and heterotrimeric G proteins. The β-adrenergic receptors undergo phosphorylation during the desensitization process and it leads to functional uncoupling of the receptor from the effector system in turkey erythrocytes (Sibley et al., 1984). Receptor-G-protein uncoupling requires minutes to occur from the exposure to ligands.

Internalization or sequestration is generally envisioned as movement of the receptor into a cell compartment distinct from the plasma membrane (Harden, 1983; Mahan et al., 1987). This membrane compartment is essentially defined by two criteria; it is not accessible to hydrophilic ligands, and it has a lower density than plasma membranes, allowing separation of the two compartments by sucrose density gradient centrifugation (Stadel et al., 1983; Strader et al., 1984). Internalized receptors can be either stored or destroyed in intracellular membrane compartments (coated vesicles, endosomes and lysosomes) or recycled to the cell surface. It has been shown that delta and mu opioid receptors are phosphorylated in an agonist-

dependent manner by G-protein coupled receptor kinases, and are endocytosed in a dynamin-dependent process via clathrin-coated pits. Overexpression of arrestin or GRK leads to enhanced agonist-induced internalization. Delta opioid receptors do not apparently recycle to the plasma membrane efficiently. Following chronic treatment of cells for several hours with agonist, delta opioid receptors are apparently associated with lysosomes.

After chronic agonist exposure receptor down-regulation might also occurs, which entails a decrease in the total pool (surface+intracellular) of receptors. This process develops more gradually and becomes significant after hours of agonist treatment (Strader et al., 1984; Sibley and Lefkowitz, 1985; Koenig and Edwardson, 1997). Down-regulation usually entails receptor internalization by endocytosis and subsequent degradation.

1.7. Lipids

1.7.1. Lipids as Bioeffectors

The term ″lipids″ encompasses a great number of slightly polar natural compounds with various chemical structures, such as free fatty acids, neutral glycerides, waxes, phospholipids, sphingolipids, oxylipins, sterols, etc. For an appreciable length of time it was thought that lipids play a relatively conservative role in cell activity. They were considered mainly as metabolic fuel stores. Later, it was established that lipids also play another vital function: they are the main structural component of cell membranes.

Studies about the role of lipids in vital systems was initiated at the beginning of the 1960s, after establishing the structure, biosynthetic pathways, and physiological role of prostaglandins. During the two last decades, especially in the 1990s, many studies have shown that other lipids also act as important biological effectors, regulators, and mediators which

participate in virtually all biological processes in an organism as a whole such as immune response, transmission of neuronal information, regulation of vessel and muscle tonus, homeostasis, inflammation, etc. and in biochemical reactions of animal and human cells. As second messengers they provide transduction of various external signals into the cell, and they simultaneously act as intercellular mediators.

1.7.2. Phosphatidic Acid

Phosphatidic acid (PA) is an essential substrate for enzymes involved in the synthesis of glycerophospholipids and triacylglycerols. It can be synthesized via two different acylation pathways named after their respective precursor, glycerol 3-phosphate (Gro3P) pathway, and the dihydroxyacetone (GrnP) pathway. In the Gro3P pathway, the first step of acylation catalyzed by Gro3P acyltransferase leads to the formation of lysophosphatidic acid (LPA). In the GrnP pathway, the first intermediate formed by GrnP acyltransferase is 1-acyl-dihydroxyacetone phosphate. This compound is converted to LPA in an NADPH-dependent reaction. LPA either formed through the Gro3P pathway or the GrnP pathway, respectively, is further acylated to PA by 1-acylGro3P acyltransferase (Athenstaedt and Daum, 1999).

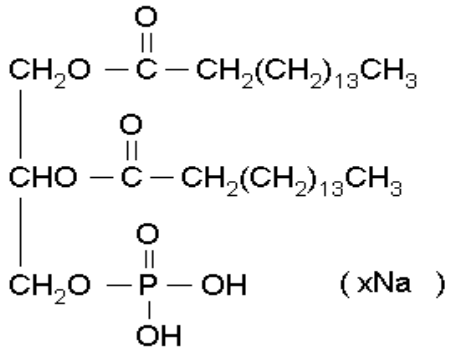


Figure 1.3. Phosphatidic acid

Besides *de novo* synthesis of PA via the two acylation pathways, this lipid can be formed by alternative biosynthetic routes. Diacylglycerol derived from deacylation of triacylglycerol by triacylglycerol lipase, or by hydrolysis of glycerophospholipids through the action of PLC can be phosphorylated by diacylglycerol kinase. Hydrolysis of glycerophospholipids catalysed PLD results directly in the formation of PA (Athenstaedt and Daum, 1999).

PA plays a major role in the biosynthesis of membrane phospholipids and triacylglycerols. Conversion of PA to diacylglycerol, however, is also important for regulatory aspects because diacylglycerol can serve as a lipid second messenger and as a mediator of vesicle formation. The enzyme which catalyses hydrolysis of PA and generates 1,2-diacylglycerol and Pi is phosphatidate phosphatase. In mammalian cells diacylglycerol is not only a precursor for glycerolipid synthesis, but it can also act as a second messenger. During classical signal transduction the production of diacylglycerol occurs by PLC-catalysed breakdown of phosphatidylinositol-4,5-bisphosphate. An alternative pathway of diacylglycerol formation comprises the enzymatic activity of phosphatidate phosphatases (Vance, 1998).

It has also been proposed that PA may be a mediator of protein secretion. It has been shown that PA derived from PLD-catalysed turnover of phosphatidylcholine (PC), which is under control of Sec14p, promotes protein secretion in yeast. PA generated upon a stimulatory signal by PLD catalysed breakdown of phospholipids can also act as a potent growth factor molecule, stimulate PLC, protein and lipid kinases, mobilise Ca^{2+} flux, activate NADPH oxidase (Rizzo and Romero, 2002), induce hormone release (Dunlop and Larkins, 1989; Metz and Dunlop, 1990), platelet aggregation (Kroll et al., 1989), muscle contraction (Ohata and Momose, 1991; Ohata et al., 1991) and gene transcription (Knauss et al., 1990; Cano et al., 1992; Mollinedo, 1994).

When applied to cells exogenously, PA exerts potent mitogenic effects (Siegmann, 1987; Pearce et al., 1994; Krabak and Hui, 1991; Knauss et al., 1990; Wood et al., 1993). Some of the observed mitogenic effects may be attributed to contamination with LPA. However, endogenous PA has been implicated in proliferative responses to certain receptor-dependent agonists, including epidermal growth factor, platelet derived growth factor, interleukin 2, interleukin 1 and interleukin 11. In addition, PA apparently mediates the mitogenic effects of sphingosine and sphingosine 1-phosphate.

1.7.3. Lysophosphatidic Acid

LPA is the simplest phospholipid and consists of a glycerol backbone with a hydroxyl group at the sn-2 (or sn-1) position, a phosphate group at the sn-3 position, and a fatty acid chain at the sn-1 (or sn-2) position. Although it is present at very low levels only in animal tissues, it is extremely important biologically, influencing many biochemical processes.

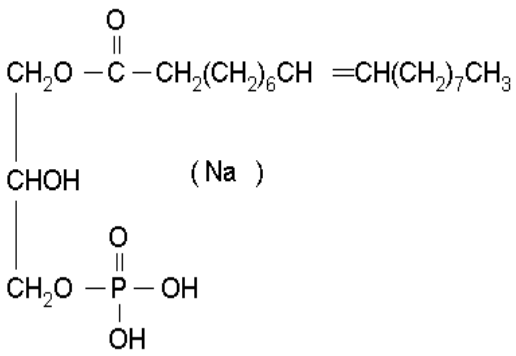


Figure 1.4. Figure of lysophosphatidic acid

Synthesis and release of LPA into the extracellular space is initiated by enzymatic degradation of phospholipids. Platelets are the major source of LPA in the blood. As a product of the blood-clotting process and the activity of a specific LPD, LPA is abundant in serum (not plasma) in an albumin

-bound form where it accounts for much of the biological activity. However; further studies have established that it is produced by a wide variety of cell types, and that most mammalian cells express receptors for LPA. It may initiate signalling in the cells in which it is produced, as well as affecting neighbouring cells.

The glycerophosphate acyltransferase (GPAT), located in both endoplasmic reticulum and mitochondria catalyzes the formation of LPA by acylation of glycerol-3-phosphate (Tigyi and Parrill, 2003). The reduction of acyl dihydroxy acetone phosphate in peroxysomes is also an alternative pathway to LPA production (Dunlop and Larkins, 1985). LPA may also be synthesized by the action of a monoacylglycerol kinase on monoacylglycerol, as an important precursor of phosphatidylinositol (PI) synthesis. (Simpson et al., 1991). Another lysophospholipid, lysophosphatidylcholine (LPC) located in the outer plasma membrane leaflet or present in biological fluids constitute potential sources of extracellular LPA (van Dijk et al., 1998).

In the last few years, the characterization of cloned LPA receptors in combination with strategies of molecular genetics has allowed determination of both signalling and biological effects that are dependent on receptor mechanisms. Experimental activation of this receptor has shown that a range of downstream signalling cascades mediate LPA signalling. These include activation of protein kinases, AC and PLC, release of arachidonic acid (AA), and much more (Moolenaar, 1995 and Ishii, 1995).

LPA signalling may result in changes in cell morphology. Contractile changes induced by LPA are signalled mainly by the small G proteins ras and rho, as well as kinase pathways such as MAP. RhoA stimulates the appearance of stress fibers, focal adhesions, and tyrosine phosphorylation in quiescent cells, resulting in myosin light chain phosphorylation and subsequent contractile changes in smooth muscle and other cells (Ridley and Hall, 1992; Kranenburg and Moolenaar, 2001).

Platelet derived LPA function in normal wound-healing processes. Thus in addition to activating platelets in an autocrine fashion, LPA can promote the proliferation of fibroblasts and vascular smooth muscle cells, as well as induce cellular tension and cell-surface fibronectin assembly, which are important events in wound repair (Zhang et al., 1994; Mosher, 1995; Nietgen and Durieux, 1998).

LPA functions as a mitogen and induces Ca^{2+} release from intracellular stores. It may also be a key regulator of coagulation and wound healing. Recently, breast and ovarian cancer cell lines have been shown to proliferate in the presence of ovarian cancer activating factor which was determined to be LPA. LPA is significantly elevated in patients with various gynecologic malignancies, including ovarian (early and late stage), cervical, and endometrial carcinomas, suggesting LPA functions in a positive feedback loop and represents a potential biomarker for gynecologic cancers (Xu et al., 1998).

1.7.4. Phosphatidylcholine

PC is the most abundant phospholipid in eukaryotic membranes. Besides having a structural role in membranes and lipoproteins, PC plays an important role in signal transduction as it is a major source of lipid secondary messengers in eukaryotes.

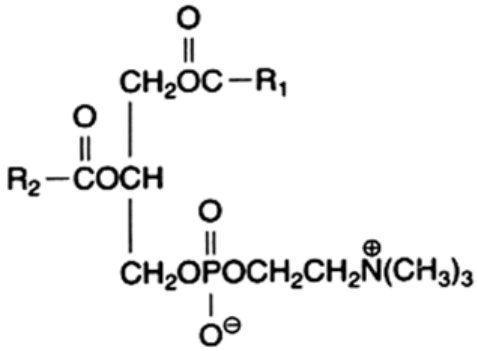


Figure 1.5. Structure of PC

For PC two major biosynthesis pathways exist in eukaryotes, the phospholipid N-methylation pathway and the CDP-choline pathway. The N-methylation pathway is a de novo pathway for PC biosynthesis, whereas the CDP-choline pathway is a scavenging pathway (re)incorporating choline and choline phosphate that have been released during the turnover of PC by phospholipases (Sohlenkamp, 2003).

In eukaryotes two additional pathyways can lead to PC formation. PC can be formed via head group exchange, that is if choline exchanges the head group of phosphatidylserine (PS), PC can be formed and free serine be released or vice versa (Kanfer, 1980). Finally, PC can be synthesized by the acylation of LPC (Choy, 1989). In mammals, the major route for PC biosynthesis is the CDP-choline pathway, whereas the phospholipid N-methylation pathway only seems to have an auxiliary function.

PC is an essential component of membranes of most eukaryotes and probably plays an important role in some prokaryotic organisms. In its most prominent role, PC is a structurally component of lipid bilayers in membranes in both, eukaryotes and prokaryotes. Within the major membrane lipids, PC has a special status because of its bilayer-forming properties and its zwitterionic state at physiological pH. Most other bilayer-forming lipids like PI or phosphatidylglycerol (PG) are anionic at physiological pH. The zwitterionic phospholipids, mainly PC and/or phosphatidylethanolamine (PE) together comprise the majority of the membrane phospholipids of eukaryotic cells.

In addition to its structural role, PC is involved in a set of diverse cellular processes. PC is together with other phospholipids like phosphatidylinositol and its phosphorylated derivatives a substrate for phospholipases during receptor-mediated signal transduction in eukaryotes, thereby being a source of secondary messengers like diacylglycerol or PA acid. In mammals PC appears to be required for very low density lipoprotein secretion.

1.7.5. Lysophosphatidylcholine

LPC, 1-acyl-sn-glycero-3-phosphocholine, is well known as an intermediate of metabolism of PC, the main phospholipid component in all eukaryotic and many prokaryotic cells.

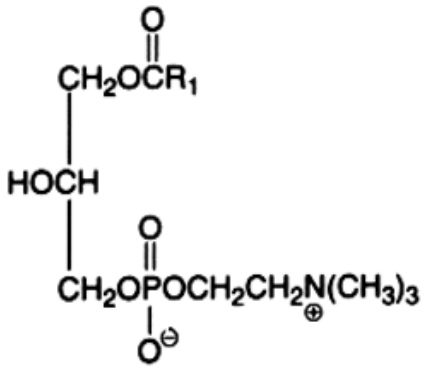


Figure 1.6. Structure of lysophosphatidylcholine

LPC is produced as a result of hydrolysis of PC with various isoforms of PLA_2 or in reactions catalyzed by lecithin-cholesterol acyltransferase, which transfers a fatty acid residue from PC to cholesterol and is the key enzyme in lipoprotein metabolism in animal and human blood plasma.

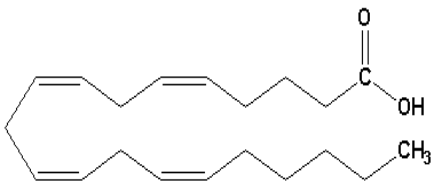
Although signalling properties of LPC have been studied in a variety of cellular systems, specific cell membrane receptors for LPC had not been identified previously. Four G protein-coupled receptors, ovarian cancer G protein-coupled receptor 1 (OGR1, also known as GPR68), G protein-coupled receptor 4 (GPR4), G2A, and T cell death-associated gene 8 (TDAG8), belong to a new subfamily of GPCRs which is known as OGR1 subfamily. (Xu,1996; Choi et al., 1996; Heiber et al., 1995 and Weng et al., 1998). Recently the ligands for G2A and GPR4 was identified as LPC (Xu et al., 2000 and Xu et al., 2001).

The biological effects and the signaling properties of LPC have been most extensively studied *in vitro* in cells related to atherosclerosis, including endothelial cells, smooth muscle cells, monocytes, and lymphocytes (Prokazova et al., 1998 and Hara et al., 1997). LPC activates PLC (Okajima et al., 1998) and PKC (Prokazova et al., 1998) induces increases in intracellular calcium (Okajima et al., 1998; Okita et al., 1997), and activates or inhibits MAP kinase (Bassa et al., 1998 and Rikitake et al., 2000). LPC has been demonstrated to be atherogenic. However, antiatherogenic actions of LPC have also been reported (Hara et al., 1997). It will be important to determine whether this dual function of LPC is related to receptor-mediated vs. nonreceptor-mediated effect, LPC and receptor compertmentalization, and/or LPC effects mediated via different types of proteins/receptors. The endogenous expression of G2A and GPR4 in these cell types remains to be determined.

The levels of LPC are elevated in many diseases related to inflammation (Prokazova et al., 1998 and Murphy et al., 1998). More recently, elevated LPC levels (in particular, the ratios of palmitoyl-LPC to linoleoyl-LPC) have been reported in ovarian cancer (Okita et al., 1997) and multiple myeloma (Sasagawa et al., 1999).

1.7.6. Arachidonic Acid

AA is a polyunsaturated fatty acid (all-cis-5,8,11,14-Eicosatetraenoic acid) which is released from phospholipids in cells stimulated by many first messengers, including neurotransmitters, neuromodulators, and neurohormones.



Arachidonic Acid
(all-cis-5,8,11,14-eicosatetraenoic acid)

Figure 1.7. Structure of AA

AA has a short lifespan, during which it may interact with and affect the activity of ion channels and protein kinases within the cell. Alternatively, it may be transformed to a family of metabolites, the eicosanoids, which may also produce important effects on intracellular targets. In both cases, the AA cascade affects neuronal excitability by fulfilling the primary criteria defining a second messenger system that is, receptor-dependent formation and intracellular site of action. Where the eicosanoids differ from "classical" second messengers is in their ability to cross the cell membrane, diffuse through the extracellular space, and interact with high-affinity receptors located on neighboring neurons. Eicosanoid receptors have been characterized in the brain and have been shown to be linked to second messengers, such as cyclic AMP, very much like the receptors recognized by dopamine, noradrenaline, and so on

In resting cells, AA is stored within the cell membrane, esterified to glycerol in phospholipids. A receptor-dependent event, requiring a transducing G protein, initiates phospholipid hydrolysis and releases the fatty acid into the intracellular medium. Three enzymes may mediate this deacylation reaction: PLA_2, PLC, and PLD. PLA_2 catalyzes the hydrolysis of phospholipids at the sn-2 position in a single-step reaction. By contrast, PLC and PLD do not release free AA directly. Rather, they generate lipid products

containing arachidonate which are diacylglycerol and PA, respectively, which can be released subsequently by diacylglycerol- and monoacylglycerol-lipases (Daniela Piomelli, 2000).

Once released, free arachidonate has three possible fates: reincorporation into phospholipids, diffusion outside the cell, and metabolism. Metabolism is carried out by three distinct enzyme pathways expressed in neural cells: cyclooxygenase, lipoxygenases, and cytochrome P450. Several products of these pathways act within neurons to modulate the activities of ion channels, protein kinases, ion pumps, and neurotransmitter uptake systems. The newly formed eicosanoids may also exit the cell of origin and act at a distance, by binding to G-protein-coupled receptors present on nearby neurons or glial cells. Finally, the actions of the eicosanoids may be terminated by diffusion, uptake into phospholipids, or enzymatic degradation (Daniela Piomelli, 2000).

AA has been implicated to have modulatory functions in both physiological and pathophysiological processes. For example, it effects the function of many receptors including $GABA_A$ receptors (Nielsen et al., 1987; Koenig and Martin, 1992; Witt et al., 1996) and NMDA receptors (Casado and Ascher, 1998). It modulates ion channels including both K^+ (Ordway et al., 1989) and the L-type Ca^{2+} channels (Urushidani et al., 1996) and regulates activity of many enzymes, such as protein kinase A (Farooqui et al., 1997) and protein kinase C (McPhail et al., 1984).

1.7.7. Cholesteryl hemisuccinate

Cholesteryl hemisuccinate (CHS) is a biologically active lipid that demonstrates antiproliferative activity in cancer cells (Fariss et al., 1994 and Djuric et al., 1997), protects cells from drug induced toxicity (Fariss et al., 1997), and can regulate cellular enzymatic activities (Chelliah et al., 1994).

Studies using non-hydrolyzable ether analogs of these compounds have demonstrated that the intact molecule is needed for these biological activities (Fariss et al., 1994 and Djuric et al., 1997). The acidic nature of this compound is also needed for biological activity (Ray et al., 1996). It has been postulated that the antiproliferative action of these compounds may be related to their physicochemical properties that allow incorporation into cell membranes (Djuric et al., 1997). CHS has been demonstrated to alter membrane acyl chain motion or fluidity in model and cell membranes (Dumas et al., 1997; Knudsen et al., 1997; Lai et al., 1997).

1.7.8. Membrane Lipids as Regulators of Receptors

Although lipid metabolites, free fatty acids and lysophospholipids are present in the cell in small quantities, they play important roles in biological systems. They modify the function of membrane proteins including receptors and ion channels. Such alterations can occur through signal transduction pathways, but may also result from direct effects of the metabolite on the protein.

Lundbaek and Anderson showed that lysophospholipids including LPC, lysophosphatidylinositol (LPI), lysophosphatidylethanolamine (LPE) and lysophosphatidylserine (LPS) modify the function of gramicidin channels in planar bilayers. Their effects are qualitatively similar: the channel appearance rate and average duration increase, and the standard free energy for channel formation are decreased. The relative potency of the lysophospholipid is correlated with the size of their polar head groups. The magnitude of the increase in channel duration varies as a function of the channel length (Lundbaek and Anderson, 1994).

Pulkkinen and Hamalainen characterized the phospholipid inhibition of estradiol and progesterone binding to guinea-pig and human myometrial receptors. They studied several compounds and found that phosphatidyIinositol, LPA and LPC were the most active inhibitors and they concluded that certain phospholipids may inhibit the binding of sex steroids to their receptors, through their detergent properties, thus changing the functional environment of the receptors (Pulkkinen and Hamalainen, 1995).

Casado and Ascher showed that effects of lysophospholipids are indeed opposite to those of arachidonic acid. Lysophospholipids inhibited the NMDA currents in a fully reversible and voltage independent manner (Casado and Ascher, 1998).

1.7.9. Effect of Lipids on Opioid Receptor

In the literature there are few studies concerning different types of lipids and opiate receptors. In an early study, McGee studied the effects of alterations in membrane fatty acid composition on receptors for opiates in clonal NG108-15 following exposure to unsaturated fatty acids including AA. Decreases in binding of [^{3}H]etorphine was observed in cells exposed to AA which were primarily associated with changes in the number of binding sites (McGee, 1982).

Hasegawa et al. showed that the lower binding affinity of isolated receptors can be restored by the addition of lipids containing an acidic head group and a fatty acid with two or more double bonds, such as AA (Hasegawa et al., 1987).

Medzihradsky et al. demonstrated that oleic acid (cis), elaidic acid (trans) and the cis and trans isomers of vaccenic acid can influence both agonist and antagonist opioid ligand-receptor interactions (Medzihradsky,

1990). More recently, the same group describes the modulator role of membrane microviscosity on mu-opioid receptors (Emmerson,.2000)

Previously our group also demonstrated modulator role of AA on binding of an antagonist ligand (Nlx) at opioid receptor subtype level (Apaydın, 1997 and 2000). Furthermore in a preliminary study inhibitory action of LPA on naloxone binding was also documented (Apaydın, 1998).

1.8. Aim of the Study

It is well documented that lipids can modulate the functioning of membrane bound receptors. As outlined above, there is rather limited information on lipid modulation of opioid receptor binding activity. Furthermore, to our knowledge there is no available literature data on modulatory role of lysophospholipids on opioid receptors. For these reasons, in this thesis we aim to study the effect of phospholipids and lysophospholipids on binding properties of opioid receptors and tried to characterize the influence at receptor subtype level.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

[^{3}H]Endomorphin I ($ProE_1$) with specific activity of 63.7 Ci/mmol, [^{3}H]Ile 5,6 Deltorphin II (DIDI) 49.5 Ci/mmol and [^{3}H]Naloxone (Nlx) with specific activity equals to 35 Ci/mmol were synthesized in the Isotope Laboratory of Biological Research Center, Szeged. Naloxone hydrochloride, $ProE_1$, DIDI were kindly donated by Dr. Anna Borsodi from Biological Research Center of Hungarian Academy of Sciences. AA, CHS, PA, LPA, PC and LPC were obtained from Sigma. All other reagents were obtained from Sigma or Merck.

Wistar rats were from the Hıfzıssaha and Animal House of the Biological Research Center (Szeged, Hungary).

2.2. Methods

2.2.1. Preparation of Brain Membranes

A crude membrane fraction of Wistar rat brains were prepared according to the procedure of Pasternak (Pasternak, 1974). After decapitation of animals, brains were removed without cerebella, and placed in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose.

Brains were washed several times to remove blood and unwanted tissue particles. They were sliced into small pieces and homogenized at 4 °C in 60 volumes of 50 mM Tris-HCl buffer, pH 7.4 with a teflon-glass homogenizer. The homogenate was filtered through several layers of cheese-cloth and the filtrate was centrifuged at 40,000 g for 20 min at 0-4 °C in Sorvall RC5C centrifuge. Resulting pellets were suspended in 60 volumes of 50 mM Tris-HCl buffer, pH 7.4 by vortexing and by several passes through a syringe and incubated at 37 °C for 30 min to remove endogenous opioid peptides. This suspension was centrifuged under the same conditions and final pellets were suspended in 5 volumes of 50 mM Tris-HCl buffer, pH 7.4 containing 0.32 M sucrose to give a final protein concentration of 3-4 mg/ml. The membranes were stored at – 70 °C until use.

2.2.2. Lipid Modification of Membranes

CHS and AA were added as a solution in ethanol to membranes suspended in 50 mM Tris buffer, pH 7.4 at 25 °C with vigorous mixing. Control membranes were incubated with same amounts of ethanol. After incubation for 30 min at 25 °C, the mixture was centrifuged for 15 min at 20,000 g at 4 °C, and the pellet was re-suspended in 50 mM Tris buffer, pH 7.4. Suspension of membranes were used during the binding assay.

Lysophospholipids and phospholipids were added to membranes in the form of small unilamellar vesicles. For preparation of vesicles lipids were dissolved in Chloroform: Methanol 2:1 mixtures. Organic solvents were removed under a gentle stream of nitrogen in the fume hood. When dry to remove any residual organic solvent an additional vacuum was performed for an additional 60 minutes. Dried lipids were dissolved with vigorous vortexing in 50 mM Tris-HCl buffer, pH 7.4 at 25 °C containing 0.1 % BSA (essentially fatty acid free) to obtain vesicles at the desired concentrations. Finally vesicles were subjected to 10 times freezing-thawing cycle and prepared unilamellar vesicles were stored at –20 °C. During the day of experiment

vesicles were sonicated with probe-type sonicator until water clear suspensions obtained in order to get small unilamellar vesicles. Suspension of small unilamellar vesicles were used within 2 hours since it is not possible to keep the vesicles in this form more than 2 hours.

2.2.3. Radioligand Binding Assays

Prior to perform the binding assays, frozen rat brain membranes were thawed and separated from the Tris-HCl/sucrose medium by centrifugation at 40,000 g for 20 min at 4 °C. Experiments were carried out in plastic test tubes for the tritium labeled peptides or glass test tubes for the tritium labeled alkaloid ligands. Binding experiments were performed in 50 mM Tris-HCl buffer, pH 7.4.

The final volume of the reaction mixture was 1 ml consisting of:

800 μl of membrane suspension (0.3-0.4 mg protein)

100 μl of radioligand

100 μl of Tris-HCl buffer (to determine total binding) /100 μl of 10 μM cold ligand (to determine non-specific binding) / 100 μl of lipids at various concentrations (for competition experiments)

Reaction was started with the addition of membrane suspension. Assay tubes were kept in water-bath shaker at proper temperature for a period of time. Incubation conditions for the used radioligands are listed in Table 2.1. Incubations were terminated by rapid filtration under vacuum through Whatman glass fiber filters (GF/B or GF/C), followed by washing three times with 5 ml of ice-cold 50 mM Tris-HCl, pH 7.4 using Brandel Cell Harvester. Filters were dried and the bound radioactivity was left to dry and then filters were immersed in a Toluene based scintillation cocktail for a few second and then counted in a scintillation counter having a counting efficiency of % 50 for tritium.

All assays were performed in duplicate tubes, and repeated at least three times. The given values represent the means (± SEM). Protein concentration was measured according to the method of Bradford (1976) using BSA as a standard.

TABLE 2.1. Binding Assay Conditions Used in The Study

Radioligand	Concentration (nM)	Incubation Time	Temperature	Filter	Test tube
[^{3}H]ProE$_1$	1.0	45 min	25°C	GF/C	glass
[^{3}H]DIDI	0.5	45 min	35°C	GF/C	plastic
[^{3}H]Nlx	1.0	60 min	0°C	GF/B	glass

2.2.3.1. Equilibrium Binding Experiments

The effect of lipids on opioid receptor binding was studied at 10^{-3} M through 10^{-7} M lipid concentrations. Binding experiments were carried out by protein incubation with radioligands in the presence of different concentrations of lipids, in the absence or presence of 10 μM unlabeled ligands to define total and non-specific binding, respectively. Conditions used for different radioligands are given in Table 2.1. The specific binding was calculated by subtraction of the non-specific binding from the total binding. The molar concentration of the lipids causing 50% inhibition (IC_{50}) or 50% increase (EC_{50}) in specific binding of radioligands were recorded.

2.2.3.2. Displacement (Competition binding experiment)

Displacement binding experiments were carried out by incubation of membranes with radioligands in the presence of different concentrations of unlabelled competing ligand concentration ranging between 10^{-5} to 10^{-11} M.

Conditions used for different radioligands are listed in Table 2.1. Effect of different lipids on binding was studied by including IC_{50} value or EC_{50} value of the lipids. Reaction was terminated as described before.

2.2.3.3. Saturation binding experiments

Saturation binding experiments were performed with increasing concentrations of radioligands. At each concentration of radioligand two tubes being used for total binding and two for non-specific binding. Six to eight different radioligand concentrations were used where the highest concentration was 200 nM. Then, serial dilutions of the radioligands were made in order to obtain radioligand concentrations between 5 to 200 nM. Effect of different lipids on binding parameters of opioid receptors were studied by including IC_{50} value or EC_{50} value of the lipids. Reaction was terminated as explained previously.

2.2.4. Analysis of Binding Data

A radioligand is a radioactively labelled compound that can associate with a receptor, transporter, enzyme, or any protein of interest. Measuring the rate and extent of binding provides information on the number of binding sites, and their affinity and accessibility for various drugs. Radioligand binding experiments are easy to perform, and provide useful data in many fields.

There are two major types of radioligand binding experiments: direct and indirect. Direct binding assays measure the direct interaction of a radioligand with a receptor and indirect binding assays measure the inhibition of the binding of a radioligand by an unlabelled ligand to deduce the affinity of the receptors for the unlabelled ligand. Radioligand binding assays can be used in a variety of preparations. This method allows to measure the affinity

of the drug towards its receptor rapidly and allows to quantify the number of receptor in the tissue.

The simplest model describing the interaction of a receptor with a ligand to form a complex, RL, is the bimolecular reaction:

$$\text{Ligand+Receptor} \leftrightarrow \text{Ligand-Receptor}$$

According to the Principle of Mass Action, at equilibrium, ligand-receptor complexes form at the same rate that they dissociate:

$$[\text{Ligand}][\text{Receptor}]K_{+1}=[\text{Ligand-Receptor}]K_{-1},$$

where K_{+1} is the association rate constant in units of $M^{-1}min_{-1}$

K_{-1} is the dissociation rate constant in units of min_{-1}

[Ligand-Receptor] is the concentration of ligand-receptor complex, also referred to as the amount bound, [B].

Equilibrium dissociation constant (K_d) is defined as

$$K_d = K_{-1}/K_{+1}=[\text{Ligand}][\text{Receptor}]/[\text{Ligand-Receptor}]$$

Thus, the equilibrium dissociation constant, K_d expressed in units of moles/liter or molar, is the concentration of a ligand, which, in case of a bimolecular, one-step reaction, occupies half of the receptors at equilibrium.

The concentration of unlabelled drug that results in radioligand binding halfway between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%).

Two principal parameters characterize the ligand-receptor interaction.

(1) Affinity is a measure of the ability of a ligand to bind to a specific receptor, and is represented by equilibrium dissociation constant (K_d). The K_d of a radioligand may be determined by: (a) kinetic experiments or (b) saturation experiments.

(2) Selectivity can be defined as the ratio of the dissociation constants at primary and secondary sites, obtained in competition binding experiments.

2.2.4.1. Displacement (Competition) Studies

If kinetic and saturation binding studies are used to measure the direct interaction of a radioligand with a receptor, the competitive inhibition of the binding of a radioligand by unlabeled compounds can be used to indirectly characterize this interaction. Indirect binding assays are essential to characterize completely a population of receptors.

In displacement binding experiments the binding of the unlabelled compound to a receptor is measured by its ability to displace the specific binding of a low fixed concentration of a radioligand. As the concentration of unlabeled ligand increases, the amount of radioligand bound to the receptor decreases. The binding parameter obtained from this experiment is the concentration of unlabeled ligand that inhibits the binding of the radioactive ligand by 50%. The dissociation constant for the unlabelled ligand for the receptor is referred as the K_i (equilibrium inhibition constant).

2.2.4.2. Saturation Studies

Saturation binding experiments measure specific binding at equilibrium at various concentrations (often 6-12) of the radioligand to determine receptor number and affinity. Specific binding is plotted against the concentration of the radioligand. As the concentration of radioactive ligand increases, a point reached where the amount of ligand bound no longer increases. The linear transformation of the saturation curve called as Scatchard plot is used the calculate B_{max} (maximal number of binding sites) and K_d (equilibrium dissociation constant) values.

A linear Scatchard plot reflects intercation of a ligand in a simple bimolecular manner with a single class of binding sites, or with multiple classes of binding sites with equal affinity. Non-linear Scatchard plots may reflect more complex models, including cooperative interactions between binding sites or the presence of multiple classes of binding sites for which the radioligand has different affinities.

2.2.4.2. Statistical Analysis

Experimental data from competitive radioligand binding experiments were analysed to determine binding parameters for unlabelled compounds. In the saturation experiments the K_d and B_{max} values of the binding were determined. Obtained data were analysed by GraphPad Prism (Stannard, 1995).

CHAPTER 3

RESULTS

3.1. Effect of AA on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

Effect of AA on opioid receptor binding was studied with serial experiments. In these experiments first concentration of AA that cause 50% inhibition in specific binding was determined. Then, inhibition of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx specific binding was studied by homologous displacement experiments in the absence and presence of AA and EC_{50} values were determined. The effect of AA on ligand binding was evaluated further by equilibrium binding experiments and the binding parameters, K_D and B_{max} were obtained.

AA was dissolved in ethanol first and then desired concentrations were obtained by dilutions made with Tris-HCl buffer. Control experiments show that at the used concentrations ethanol did not effect the binding result.

3.1.1. Determination of IC_{50} values for AA

IC_{50} value of AA was determined with equilibrium binding experiments by including different concentrations of AA into the reaction mixture. It was observed that AA caused a decrease in specific binding of [^{3}H]ProE$_1$,

[^{3}H]DIDI and [^{3}H]Nlx to rat brain opioid receptor in a concentration-dependent manner (Figure 3.1, Figure 3.2). The IC_{50} values for AA was $1.5x10^{-4}$ and $1x10^{-4}$ M for [^{3}H]ProE_1, [^{3}H]DIDI, respectively and for [^{3}H]Nlx $7.3x10^{-5}$ and $6x10^{-5}$ M in the absence and presence of NaCl (Table 3.1).

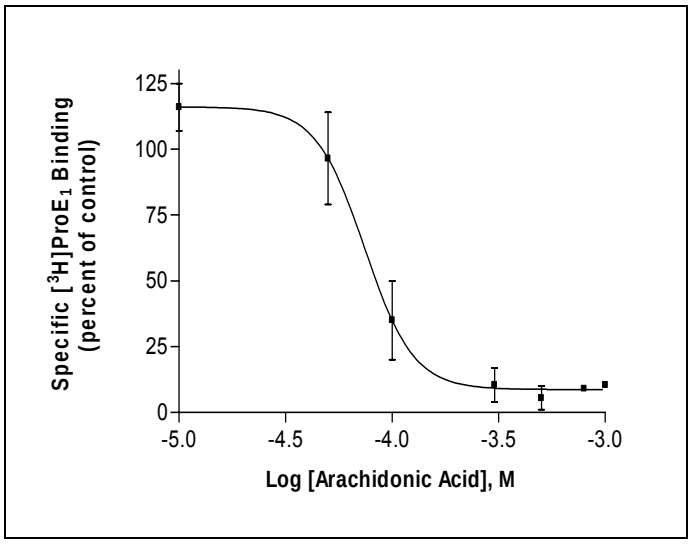


Figure 3.1. Effect of AA on specific binding of [^{3}H]ProE_1.in rat brain membranes. Membranes (0.4 mg protein/ml in the assay) were incubated with indicated concentrations of AA with 1 nM [^{3}H]ProE_1 for 45 minute at 25 ºC. Result is expressed as percent of control.

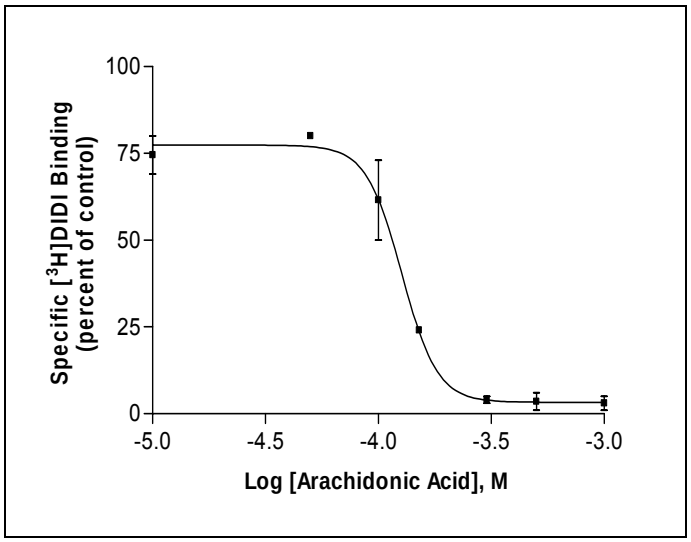


Figure 3.2. Effect of AA on specific binding of [^{3}H]DIDI in rat brain membranes. Membranes (0.4 mg protein/ml in the assay) were incubated with indicated concentrations of AA with 1 nM [^{3}H]DIDI for 45 minute at 35 ºC. Result is expressed as percent of control.

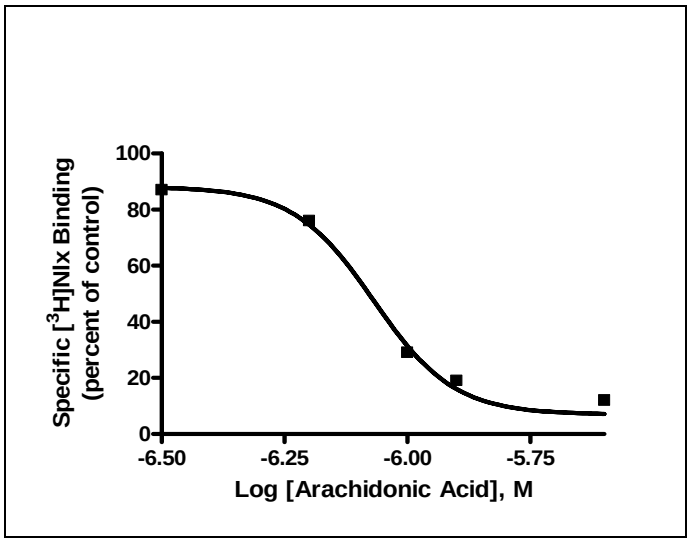


Figure 3.3. Effect of AA on specific binding of [^{3}H]Nlx in rat brain membranes. Membranes (0.4 mg protein/ml in the assay) were incubated with indicated concentrations of AA with 1 nM [^{3}H]Nlx for 60 minute at 0 ºC. Result is expressed as percent of control.

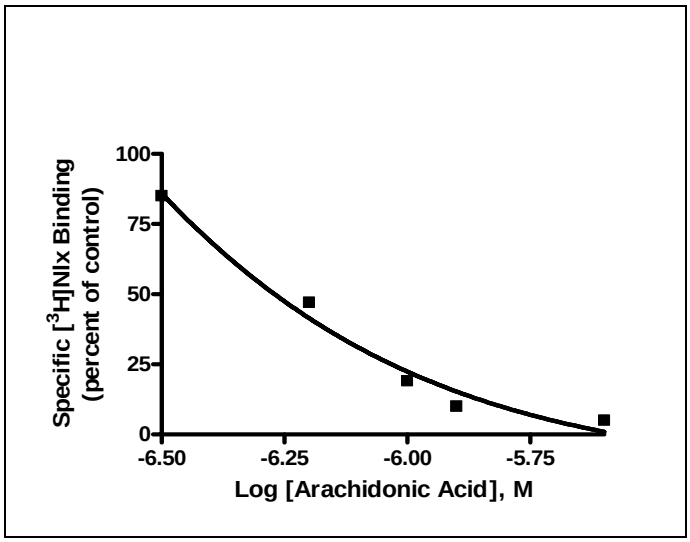


Figure 3.4. Effect of AA on specific binding of [^{3}H]Nlx in the presence of NaCl. Membranes were incubated with the indicated concentrations of AA in the presence of 100 mM NaCl with 1 nM [^{3}H]Nlx for 60 minute at 0 ºC. Result is expressed as percent of control.

Table 3.1. IC_{50} values for inhibition of [^{3}H]Pro-E1, [^{3}H]DIDI and [^{3}H]Nlx binding by AA.

IC_{50} (M)			
[^{3}H]ProE_1	[^{3}H]DIDI	[^{3}H]Nlx	
		+ NaCl	-NaCl
$1.5x10^{-4}$	$1x10^{-4}$	$6x10^{-4}$	$7.3x10^{-4}$

3.1.2. Displacement Experiments

In displacement experiments, non-specific component of binding is measured with increasing concentrations of cold ligands of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]NIx in the absence and presence of AA (Figure 3.3, Figure 3.4). To see effect of AA on displacement of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]NIx, AA was added to the reaction mixture at the concentration of IC_{50} values determined in the equilibrium binding experiments (Table 3.1). There was no significant difference in IC_{50} values obtained from displacement experiments.

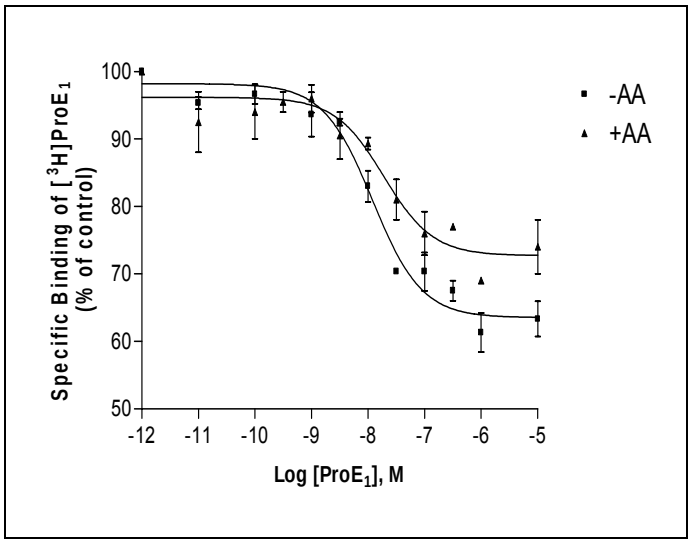


Figure 3.5. Inhibition of [^{3}H]ProE$_1$ specific binding to rat brain membranes by ProE$_1$. Membranes were treated with [^{3}H]ProE$_1$ in the presence of various concentrations of cold ProE$_1$ in the absence and presence of 1.5×10^{-4} M AA, under equilibrium conditions. Result is expressed as percent of control.

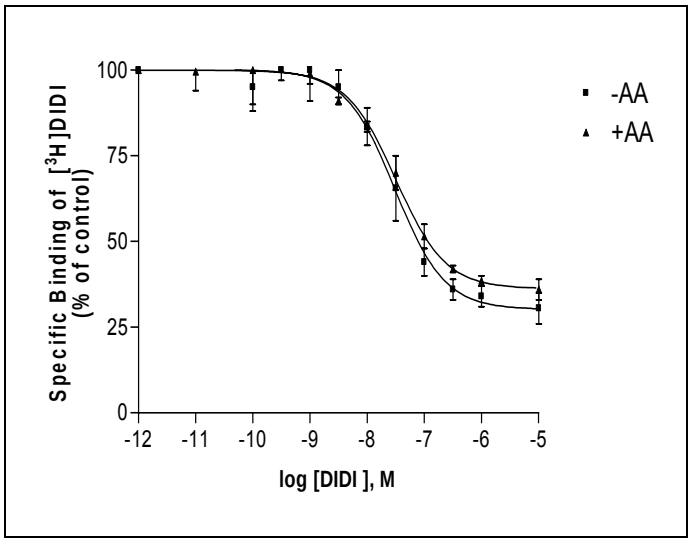


Figure 3.6. Inhibition of [^{3}H]DIDI specific binding to rat brain membranes by DIDI. Membranes were treated with [^{3}H]DIDI in the presence of various concentrations of cold DIDI in the absence and presence of $1x10^{-4}$ M AA, under equilibrium conditions. Result is expressed as percent of control.

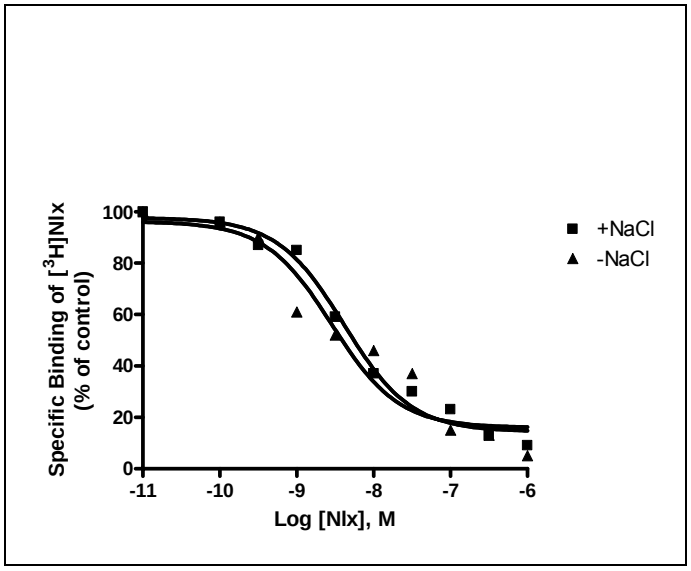


Figure 3.7. Inhibition of [^{3}H]NIx specific binding to rat brain membranes by NIx. Membranes were treated with [^{3}H]NIx in the presence of various concentrations of cold NIx in the absence of AA or NaCl in the medium and in the presence of NaCl, under equilibrium conditions. Result is expressed as percent of control.

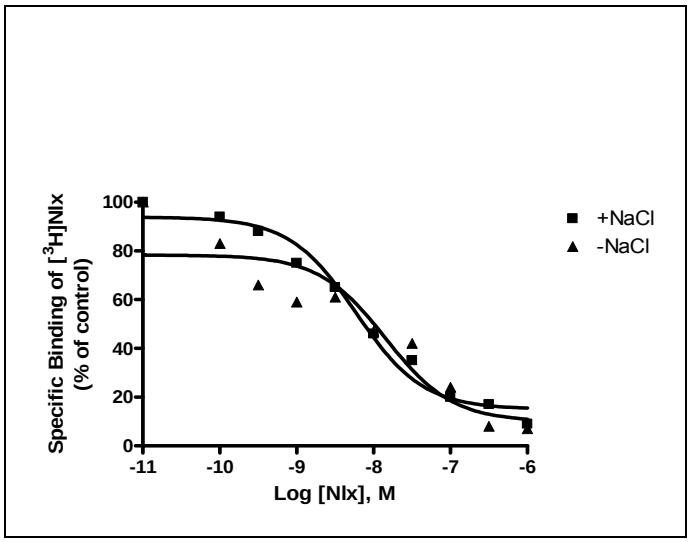


Figure 3.8. Inhibition of [^{3}H]Nlx specific binding to rat brain membranes by Nlx in the presence of AA. Membranes were treated with [^{3}H]Nlx in the presence of various concentrations of cold Nlx when $6x10^{-4}$ M AA was present in the medium and when both 100 mM NaCl and $7.3\ x10^{-4}$ M AA was present in the medium under equilibrium conditions. Result is expressed as percent of control.

Table 3.2. IC_{50} values obtained from displacement experiments

Ligand	AA	NaCl	IC_{50} (M)
[^{3}H]ProE$_1$	-	-	$1.051x10^{-8}$
	+	-	$7.878x10^{-9}$
[^{3}H]DIDI	-	-	$3.764x10^{-8}$
	+	-	$2.226x10^{-8}$
[^{3}H]Nlx	-	-	$1.47x10^{-8}$
	+	-	$4.56x10^{-8}$
	-	+	$2.72x10^{-8}$
	+	+	$2.93x10^{-8}$

NaCl was added at 100 mM concentration.

3.1.3. Scatchard Experiments

Equilibrium saturation binding of ligands in AA modified membranes was performed to determine if the changes in binding were due to alterations in ligand binding affinity or capacity. The binding of [^{3}H]ProE$_1$ in unmodified membranes revealed one population of saturable binding sites with a B_{max} 85 fmol/mg of protein and K_D of 0.4 nM. Addition of AA decreased B_{max} value to 53 fmol/mg of protein while K_D value was not affected significantly. The binding parameters K_D and B_{max} of [^{3}H]DIDI were affected in the presence of AA (Table 3.3). With antagonist ligand Nlx binding parameters were affected both in the presence and absence of NaCl in the medium and in the presence of NaCl binding sites are more susceptible to inhibition (Table 3.3).

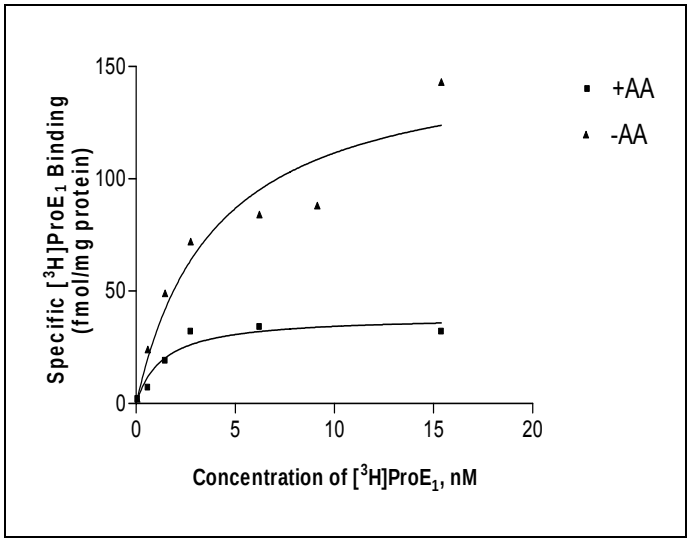


Figure 3.9. Equilibrium saturation binding of [^{3}H]ProE$_1$. Membranes (0.4 mg/ml assay medium) were incubated with varying concentrations of [^{3}H]ProE$_1$ in the absence and presence of 1.5×10^{-4} M AA. Result is expressed as fmol/mg protein.

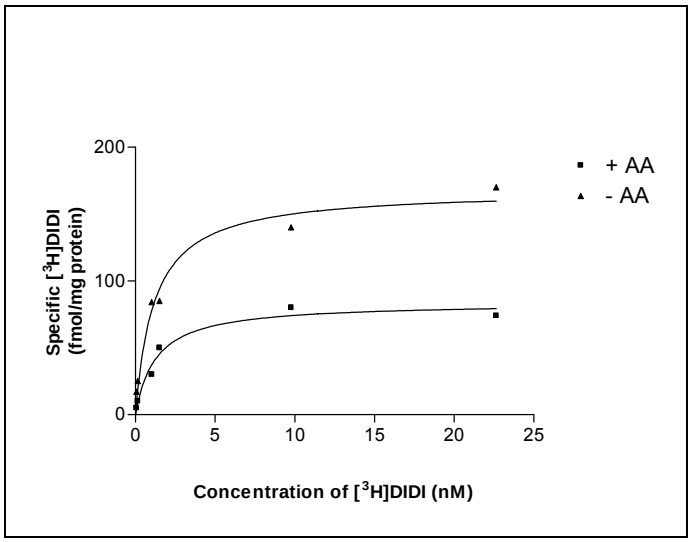


Figure 3.10. Equilibrium saturation binding of [^{3}H]DIDI. Membranes (0.4 mg/ml assay medium) were incubated with varying concentrations of [^{3}H]DIDI in the absence and presence of $1x10^{-4}$ M AA. Result is expressed as fmol/mg protein.

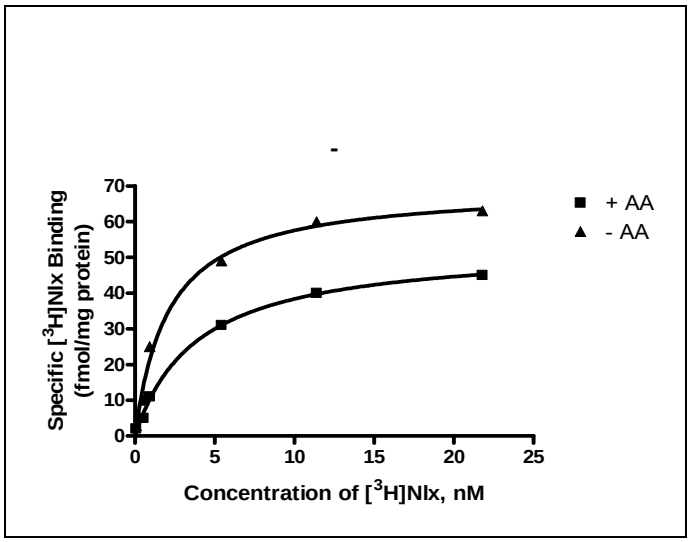


Figure 3.11. Equilibrium saturation binding of [^{3}H]Nlx. Membranes (0.4 mg/ml assay medium) were incubated with varying concentrations of [^{3}H]Nlx in the absence and when $6x10^{-4}$ M AA was present in the medium. Result is expressed as fmol/mg protein.

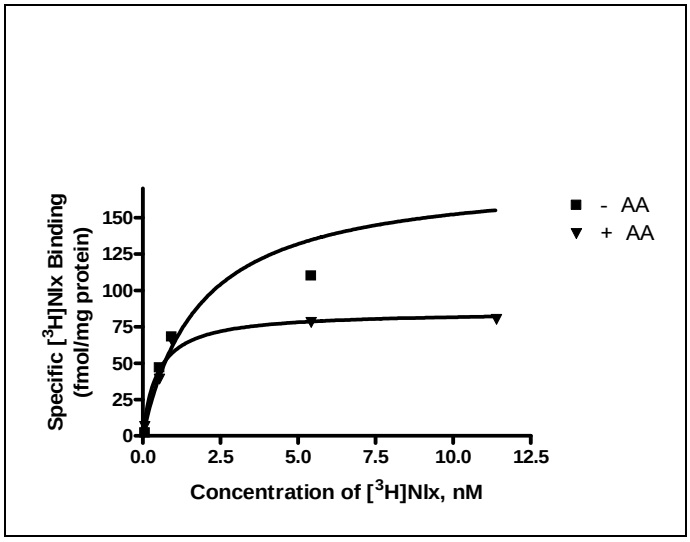


Figure 3.12. Equilibrium saturation binding of [^{3}H]Nlx. Membranes (0.4 mg/ml assay medium) were incubated with varying concentrations of [^{3}H]Nlx in the absence and when 7.3×10^{-4} M AA and 100 mM NaCl was present in the medium. Result is expressed as fmol/mg protein.

Table 3.3. Parameters of ligand binding

Ligand	AA	NaCl	K_D (nM)	B_{max} (fmol/mg of protein)
[^{3}H]ProE$_1$	-	-	0.4	85
	+	-	0.48	53
[^{3}H]DIDI	-	-	2.05	157
	+	-	1.52	53[a]
[^{3}H]Nlx	-	-	5.7	67
	+	-	13.1[a]	53
	-	+	5.4	187
	+	+	11.7[a]	83

[a] $p<0.05$, significant difference between K_D and B_{max} values from control and AA-treated membranes.

3.2. Effect of Cholesteryl Hemisuccinate on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

Effect of CHS on opioid receptor binding was studied with serial experiments. First concentration of CHS causing 50% increase in specific binding was determined by including different concentrations of CHS to the incubation medium. Then, inhibition of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx specific binding was studied by homologous displacement experiments in the absence and presence of CHS. Finally, the effect of CHS on ligand binding was evaluated by equilibrium binding experiments in the absence and presence of CHS and K_D and B_{max} were obtained.

CHS was prepared by dissolving in ethanol and then desired concentrations were obtained by dilutions made with TrisHCl buffer. As controls experiments were performed in the presence of ethanol.

3.2.1. Determination of EC_{50} values

EC_{50} value of CHS was determined with equilibrium binding experiments by using different concentrations of CHS. It was observed that CHS caused an increase in specific binding of [^{3}H]ProE$_1$ in a concentration-dependent manner. CHS increased [^{3}H]Nlx specific binding to opioid receptor in the absence of NaCl. [^{3}H]DIDI and [^{3}H]Nlx specific binding in the presence of NaCl was reduced in the CHS treated membranes. (Figure 3.13, Figure 3.14, Table 3.4.).

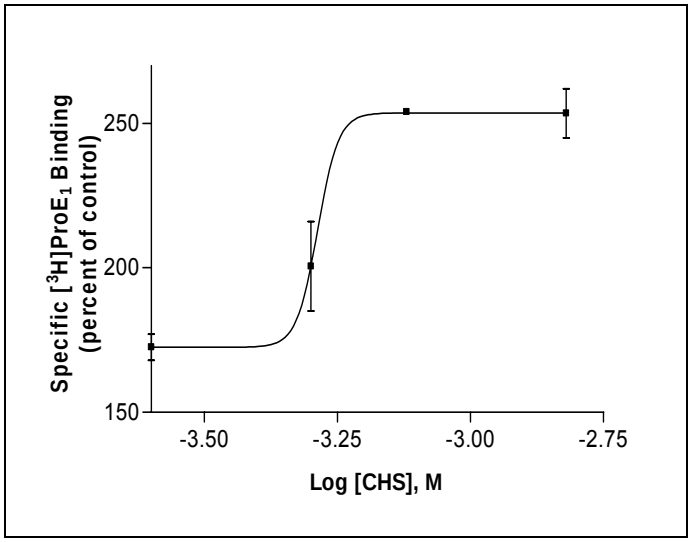


Figure 3.13. Effect of CHS on specific binding of [^{3}H]ProE$_1$ in rat brain membranes. [^{3}H]ProE$_1$.binding assay was performed on membranes in the presence of various concentrations of CHS under equilibrium conditions with 1 nM final [^{3}H]ProE$_1$.concentrations. ,Result is expressed as % of control.

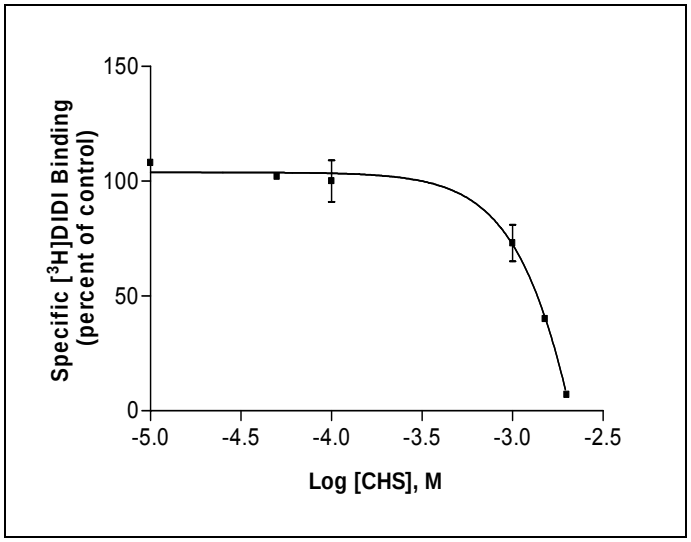


Figure 3.14. Effect of CHS on specific binding of [^{3}H]DIDI.in rat brain membranes. [^{3}H]DIDI binding assay was performed on membranes in the presence of various concentrations of CHS under equilibrium conditions with 1 nM final [^{3}H]DIDI concentrations. ,Result is expressed as % of control.

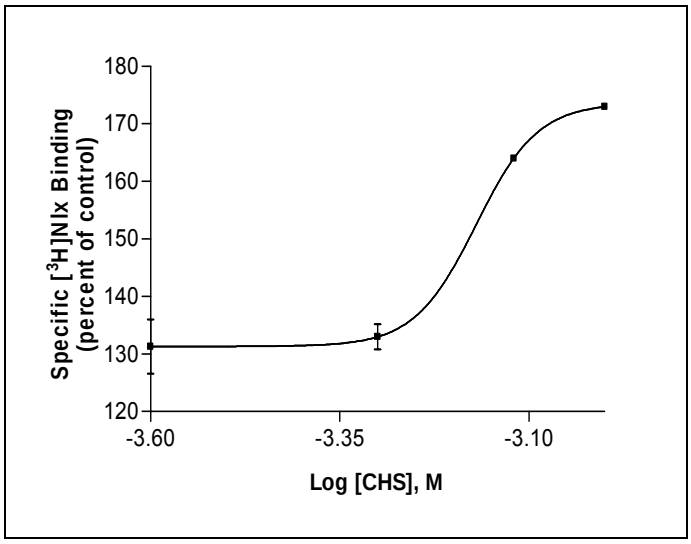


Figure 3.15. Effect of CHS on specific binding of [^{3}H]Nlx in rat brain membranes. [^{3}H]Nlx binding assay was performed on membranes in the presence of various concentrations of CHS under equilibrium conditions with 1 nM final [^{3}H]Nlx concentrations. ,Result is expressed as % of control.

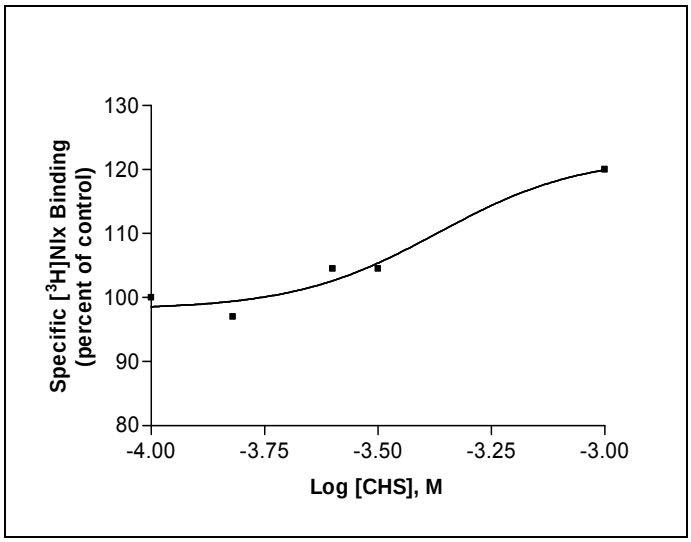


Figure 3.16. Effect of CHS on specific binding of [^{3}H]Nlx in rat brain membranes. [^{3}H]Nlx binding assay was performed on membranes in the presence of various concentrations of CHS under equilibrium conditions with 1 nM final [^{3}H]Nlx when 100 mM NaCl was present in the medium. Result is expressed as % of control.

Table 3.4. EC_{50} values for [^{3}H]ProE_1 and [^{3}H]Nlx specific binding by CHS

EC_{50} (mM)	
[^{3}H]ProE_1	[^{3}H]Nlx (- NaCl)
1	0.2

3.2.2. Displacement Experiments

In displacement experiments, non-specific component of binding is measured with increasing concentrations of cold ligands of [^{3}H]ProE_1 and [^{3}H]Nlx (Figure 3.17, Figure 3.18). IC_{50} values obtained from displacement experiments are given in Table 3.4.

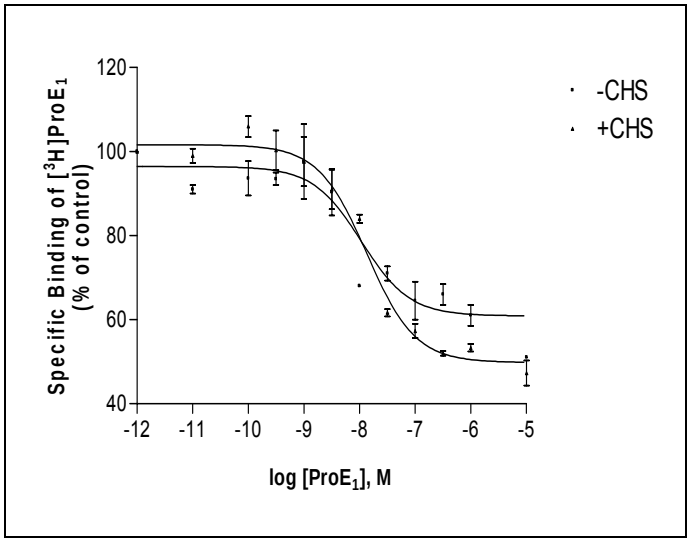


Figure 3.17. Inhibition of [^{3}H]ProE_1 specific binding to rat brain membranes by ProE_1. Membranes were treated with [^{3}H]ProE_1 in the presence of various concentrations of cold ProE_1 in the absence and presence of 1 mM CHS, under equilibrium conditions. Result is expressed as percent of control.

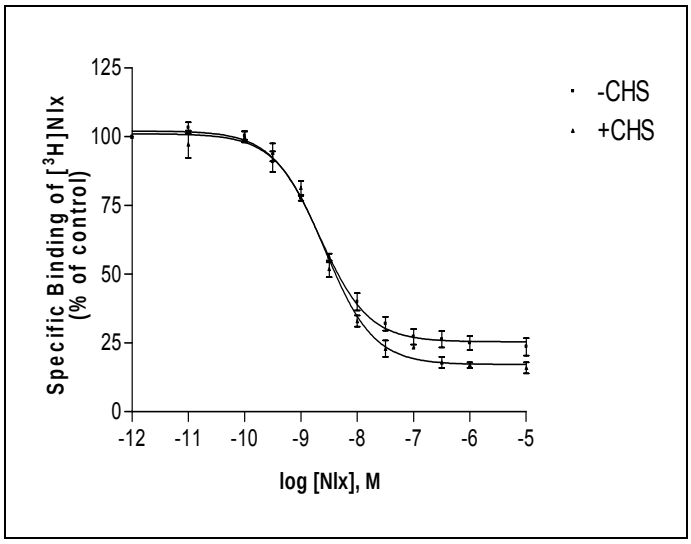


Figure 3.18. Inhibition of [^{3}H]Nlx specific binding to rat brain membranes by Nlx. Membranes were treated with [^{3}H]Nlx in the presence of various concentrations of cold Nlx in the absence and presence of 0.2 mM CHS, under equilibrium conditions. Result is expressed as percent of control.

Table 3.5. IC_{50} values obtained from displacement experiments

Ligand	CHS	NaCl	IC_{50} (M)
[^{3}H]ProE$_1$	-	-	1.051×10^{-8}
	+	-	7.878×10^{-9}
[^{3}H]Nlx	-	-	2.177×10^{-8}
	+	-	1.976×10^{-8}

3.3.3. Saturation Experiments

The effect of CHS on ligand binding parameters were evaluated further by saturation experiments (Figure 3.19, 3.20). The binding parameters, K_D and B_{max} were obtained in the absence and presence of CHS. In [^{3}H]ProE$_1$ specific binding, in the unmodified membranes B_{max} value was obtained as 120 fmol/mg of protein and in the CHS treated membranes this value increased to 207 fmol/mg of protein while K_D was not affected.

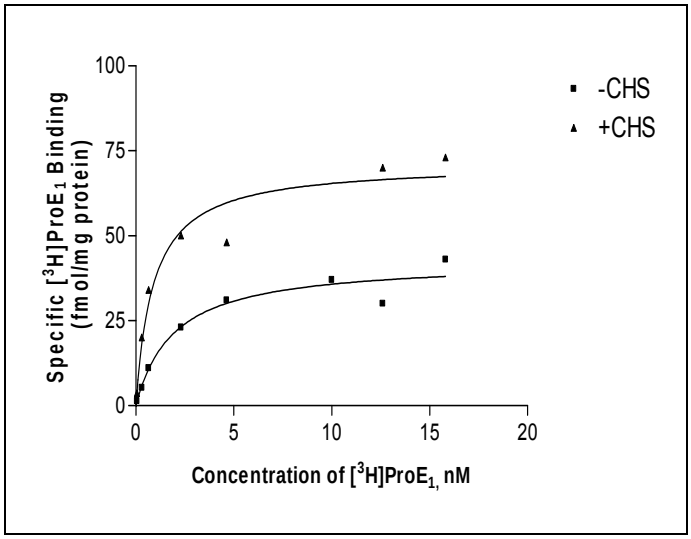


Figure 3.19. Equilibrium saturation binding of [^{3}H]ProE$_1$. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]ProE$_1$ in the absence and presence of 1 mM CHS. Result is expressed as fmol/mg protein.

In [^{3}H]Nlx specific binding in the unmodified membranes B_{max} value was obtained as 43 fmol/mg of protein and K_D as 1.9 nM and in the CHS treated membranes B_{max} increased to 72 fmol/mg of protein while K_D reduced to 0.9 nM (Figure 3.20, Table 3.6).

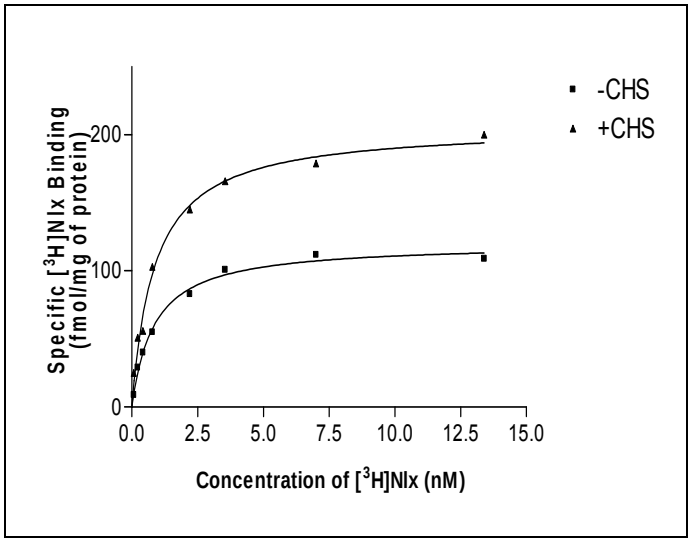


Figure 3.20. Equilibrium saturation binding of [^{3}H]Nlx. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]Nlx in the absence and presence of 0.2 mM CHS. Result is expressed as fmol/mg protein.

Table 3.6. Parameters of ligand binding

Ligand	**CHS**	**K_D (nM)**	**B_{max} (fmol/mg protein)**
[^{3}H]ProE_1	-	0.85	120
	+	0.87	207[a]
[^{3}H]Nlx	-	1.9	42.7
	+	0.9	71

[a] p<0.05, significant difference between B_{max} values from control and CHS-treated membranes.

3.3. Effect of Lysophosphatidylcholine on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

3.3.1. Equilibrium Binding Experiments

Effect of LPC on specific binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx were determined by using different concentrations of LPC. LPC decreased specific binding of [^{3}H]ProE$_1$ and [^{3}H]DIDI with IC_{50} values of $2x10^{-4}$ M and $1.4x10^{-4}$ M, respectively (Figure 3.21 and 3.22). Specific binding of [^{3}H]Nlx decreased with IC_{50} values of $1.6x10^{-4}$ M and $4.9x10^{-4}$ M in the absence and presence of NaCl, respectively (Figure 3.23 and 3.24).

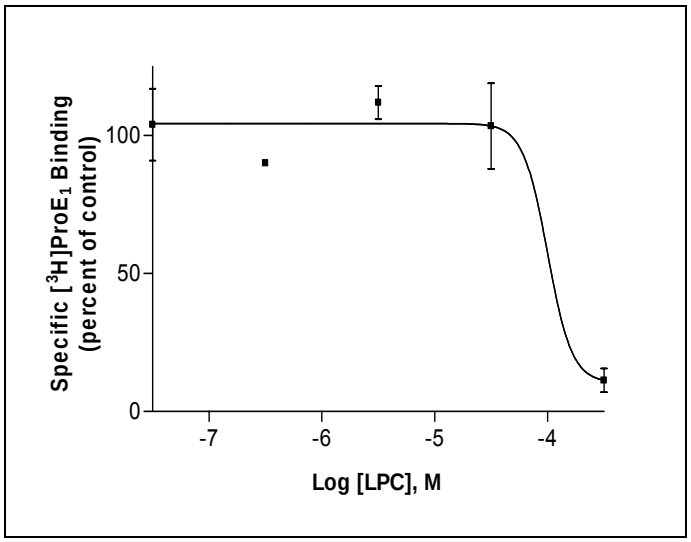


Figure 3.21. Effect of LPC on specific binding of [^{3}H]ProE$_1$ in rat brain membranes. Binding assay was performed on membranes in the presence of various concentrations of LPC under equilibrium conditions. Result is expressed as % of control.

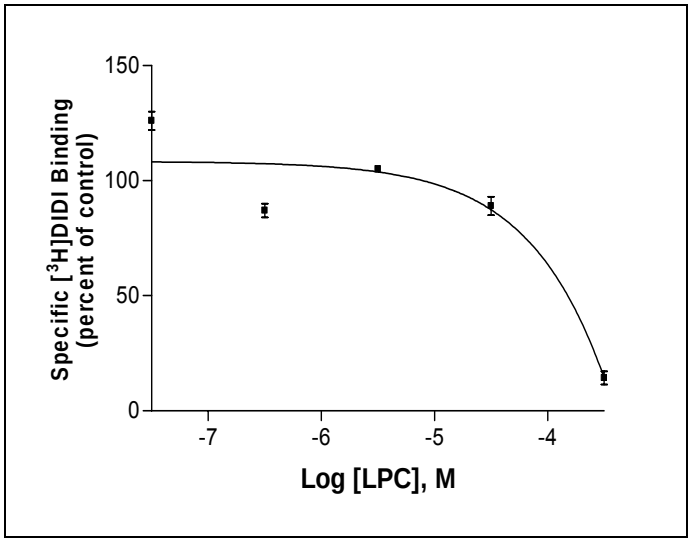


Figure 3.22. Effect of LPC on specific binding of [^{3}H]DIDI in rat brain membranes. Binding assay was performed on membranes in the presence of various concentrations of LPC under equilibrium conditions. Result is expressed as % of control.

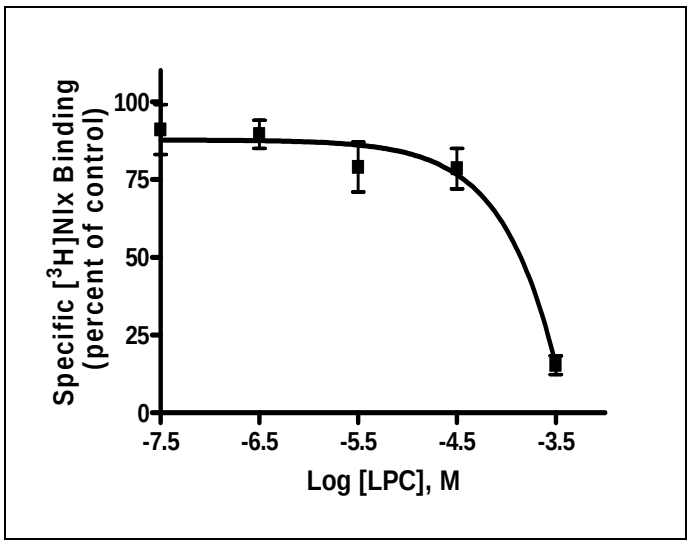


Figure 3.23. Effect of LPC on specific binding of [^{3}H]Nlx in rat brain membranes. Binding assay was performed on membranes in the presence of various concentrations of LPC under equilibrium conditions in the absence of 100 mM NaCl. Result is expressed as % of control.

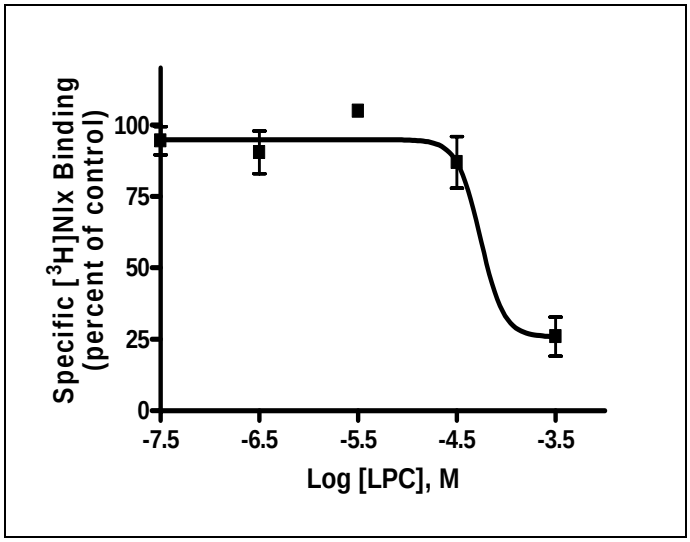


Figure 3.24. Effect of LPC on specific binding of [^{3}H]Nlx in rat brain membranes. Binding assay was performed on membranes in the presence of various concentrations of LPC under equilibrium conditions in the presence of 100 mM NaCl. Result is expressed as % of control.

Table 3.7. IC_{50} values for inhibition of [^{3}H]ProE_1, [^{3}H]DIDI and [^{3}H]Nlx binding by LPC

IC_{50} (M)			
[^{3}H]ProE_1	[^{3}H]DIDI	[^{3}H]Nlx	
		+ NaCl	-NaCl
$2x10^{-4}$	$1.4x10^{-4}$	$1.6x10^{-4}$	$4.9x10^{-5}$

3.3.2. Displacement Experiments

In displacement experiments, non-specific component of binding is measured with increasing concentrations of cold ligands of [^{3}H]ProE_1, [^{3}H]DIDI and [^{3}H]Nlx (Figure 3.25, 3.26, 3.27, 3.28). IC_{50} values determined from displacement experiments are given in Table 3.8.

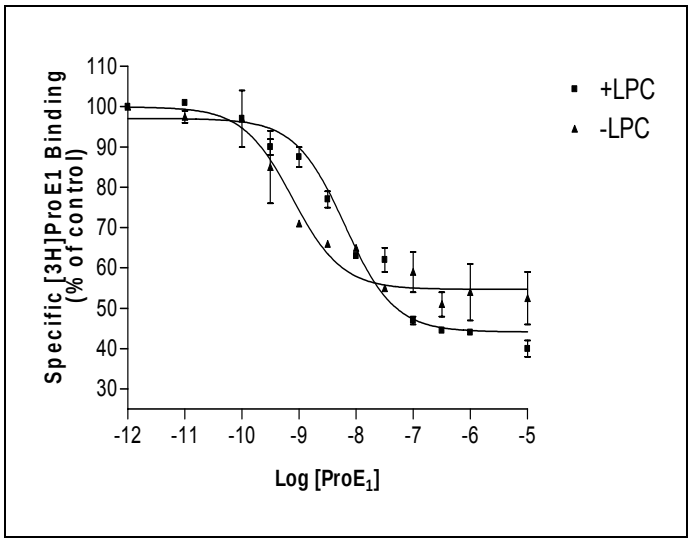


Figure 3.25. Inhibition of [^{3}H]ProE$_1$ specific binding to rat brain membranes by ProE$_1$. Membranes were treated with [^{3}H]ProE$_1$ in the presence of various concentrations of ProE$_1$ in the absence and presence of 0.2 mM LPC, under equilibrium conditions. Result is expressed as percent of control.

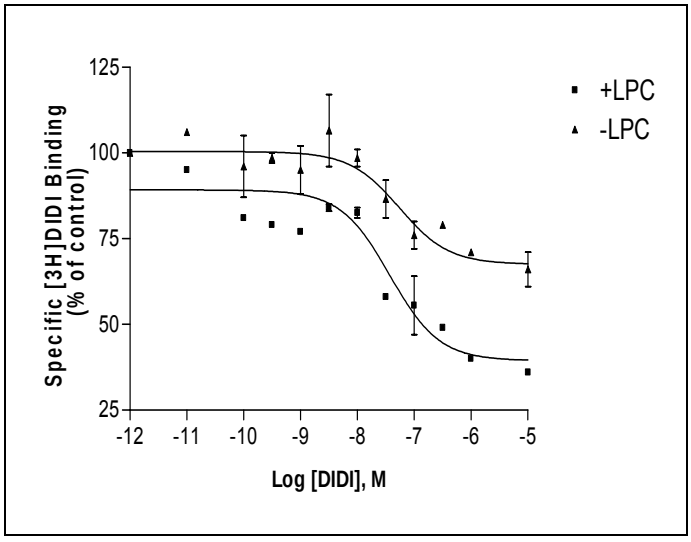


Figure 3.26. Inhibition of [^{3}H]DIDI specific binding to rat brain membranes by DIDI. Membranes were treated with [^{3}H]DIDI in the presence of various concentrations of DIDI in the absence and presence of 0.14 mM LPC, under equilibrium conditions. Result is expressed as percent of control.

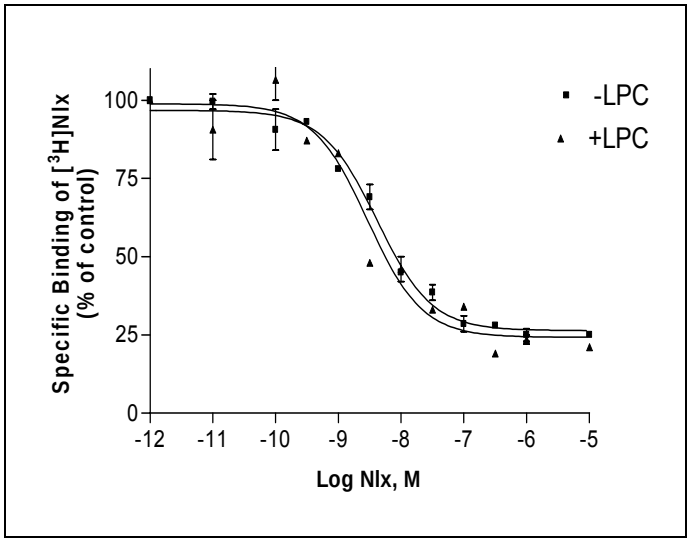


Figure 3.27. Inhibition of [^{3}H]Nlx specific binding to rat brain membranes by Nlx. Membranes were treated with [^{3}H]Nlx in the presence of various concentrations of Nlx in the absence and presence of 0.16 mM LPC, under equilibrium conditions. Result is expressed as percent of control.

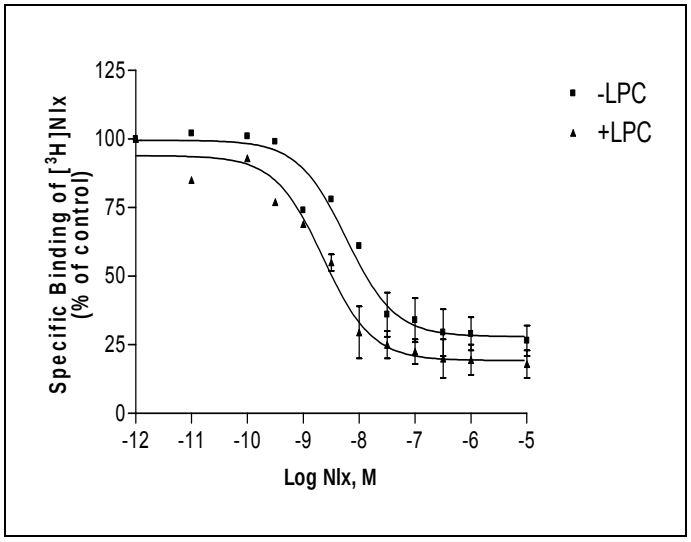


Figure 3.28. Inhibition of [^{3}H]Nlx specific binding to rat brain membranes by Nlx. Membranes were treated with [^{3}H]Nlx in the presence of various concentrations of Nlx in the absence and presence of 0.49 mM LPC and in the presence of 100 mM NaCl under equilibrium conditions. Result is expressed as percent of control.

Table 3.8. IC_{50} values obtained from displacement experiments

Ligand	LPC	NaCl	IC_{50} (M)
[^{3}H]ProE$_1$	-	-	$7.676x10^{-10}$
	+	-	$6.349x10^{-9}$
[^{3}H]DIDI	-	-	$5.492x10^{-8}$
	+	-	$3.728x10^{-8}$
[^{3}H]Nlx	-	-	$4.148x10^{-9}$
	+	-	$2.937x10^{-9}$
	-	+	$5.869x10^{-9}$
	+	+	$2.279x10^{-9}$

3.3.3. Saturation Experiments

Equilibrium saturation binding of ligands in LPC modified membranes showed that LPC is affecting binding capacity of ligands (Figure 3.29, 3.30, 3.31, 3.32). The binding of [^{3}H]ProE$_1$ in unmodified membranes one binding sites obtained with a B_{max} value of 93.5 fmol/mg of protein and K_D of 1.435 nM. Addition of LPC decreased B_{max} value to 77.20 fmol/mg of protein while K_D value determined as 2.29 nM. The binding parameters K_D and B_{max} of [^{3}H]DIDI determined as 1.69 nM and 100.9 fmol/mg protein in the LPC unmodified membrane and when LPC disincluded into the medium B_{max} reduced to 75 fmol/mg protein while K_D was not affected. With antagonist ligand Nlx binding parameters were affected both in the presence and absence of NaCl in the medium and in the absence of NaCl binding sites are more susceptible to inhibition (Table 3.9).

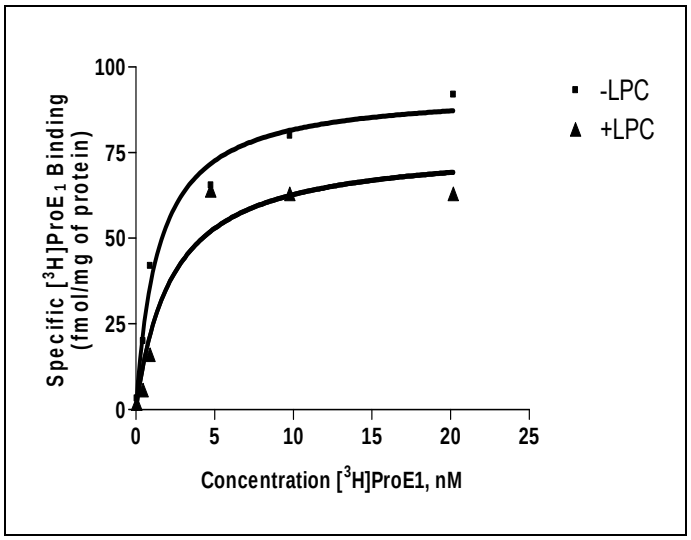


Figure 3.29. Equilibrium saturation binding of [^{3}H]ProE$_1$.in rat brain membranes. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]ProE$_1$ in the absence and presence of LPC. Result is expressed as fmol/mg protein.

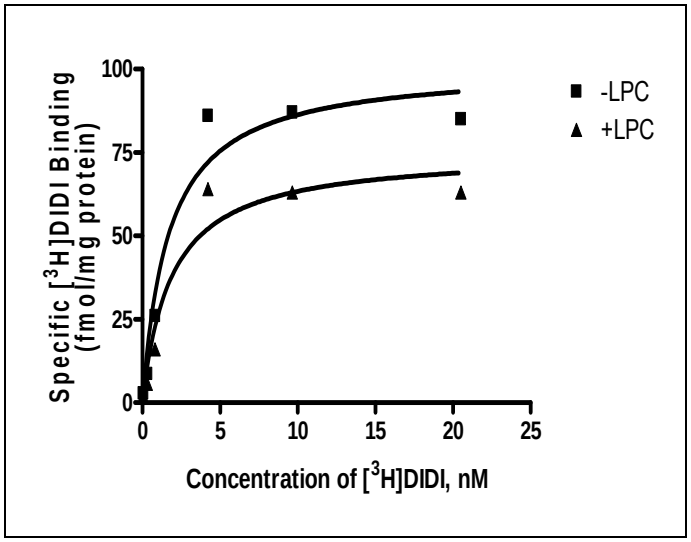


Figure 3.30. Equilibrium saturation binding of [^{3}H]DIDI in rat brain membranes. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]DIDI in the absence and presence of LPC. Result is expressed as fmol/mg protein.

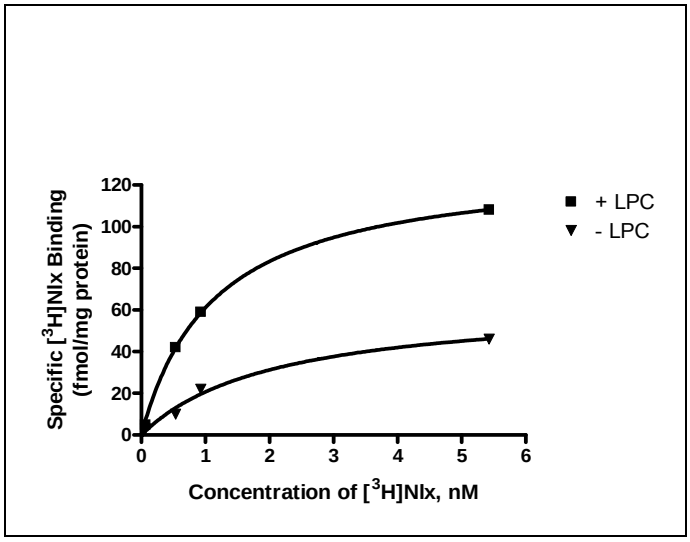


Figure 3.31. Equilibrium saturation binding of [^{3}H]Nlx. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]Nlx in the absence and presence of LPC when NaCl is not present in the medium. Result is expressed as fmol/mg protein.

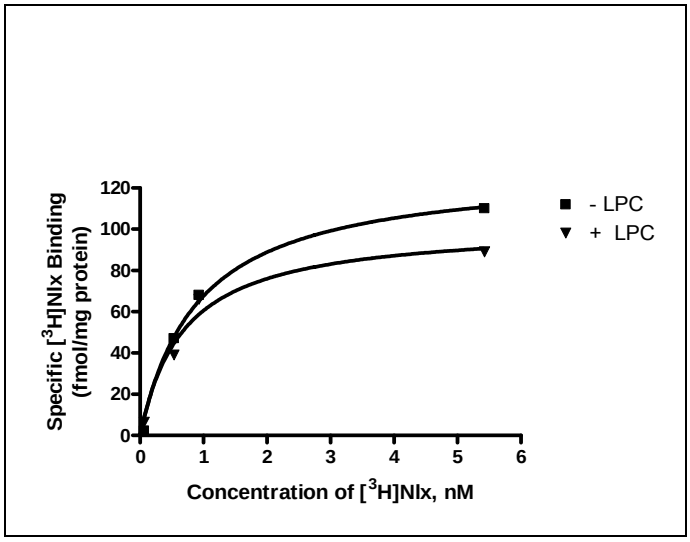


Figure 3.32. Equilibrium saturation binding of [^{3}H]Nlx in rat brain membranes. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]Nlx in the absence and presence of LPC when 100 mM NaCl is present in the medium. Result is expressed as fmol/mg protein.

Table 3.9. Parameters of ligand binding

Ligand	LPC	NaCl	K_D (nM)	B_{max} (fmol/mg of protein)
[^{3}H]ProE$_1$	-	-	1.44	93.5
	+	-	2.29	77.20
[^{3}H]DIDI	-	-	1.69	100.9
	+	-	1.86	75
[^{3}H]Nlx	-	-	1.14	130.9
	+	-	2.1	64
	-	+	0.91	129.4
	+	+	0.66	102.1

3.4. Effect of Lysophosphatidic acid on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

3.4.1. Equilibrium Binding Experiments

Effect of LPA on specific binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx were determined by using concentrations of LPA ranging from 10^{-4} to 10^{-8} M concentrations. LPA decreased specific binding of [^{3}H]ProE$_1$ and [^{3}H]DIDI with IC_{50} values of $1.5x10^{-4}$ M and $1x10^{-4}$ M, respectively (Figure 3.33 and 3.34) while specific binding of [^{3}H]Nlx changed marginally in the absence and presence of NaCl, respectively (Figure 3.35 and 3.36).

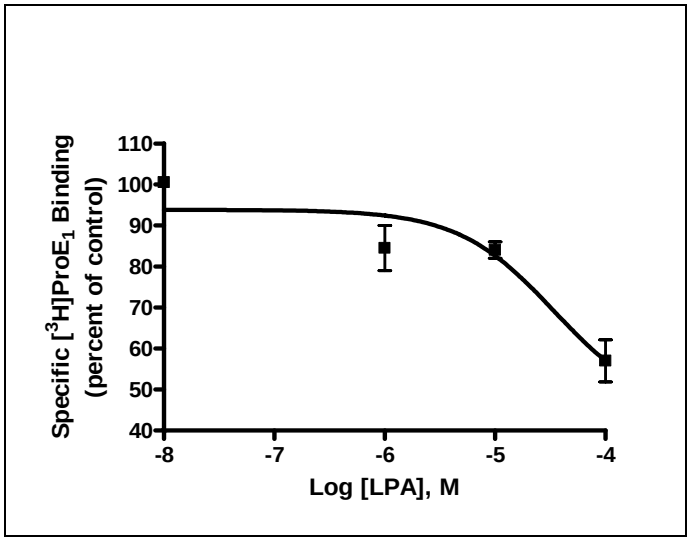


Figure 3.33. Effect of LPA on specific binding of [^{3}H]ProE$_1$.in rat brain membranes. Membranes were incubated with 1 nM [^{3}H]ProE$_1$ in the presence of various concentrations of LPA under equilibrium conditions. Result is expressed as % of control.

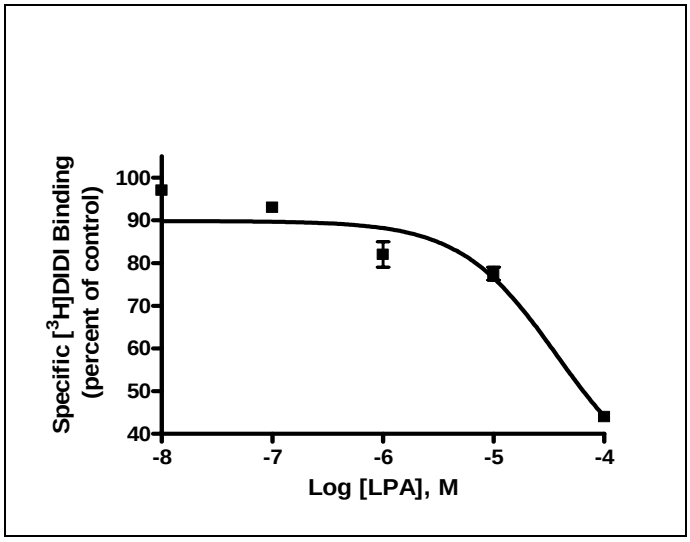


Figure 3.34. Effect of LPA on specific binding of [^{3}H]DIDI in rat brain membranes. Membranes were incubated with 1 nM [^{3}H]DIDI in the presence of various concentrations of LPA under equilibrium conditions. Result is expressed as % of control.

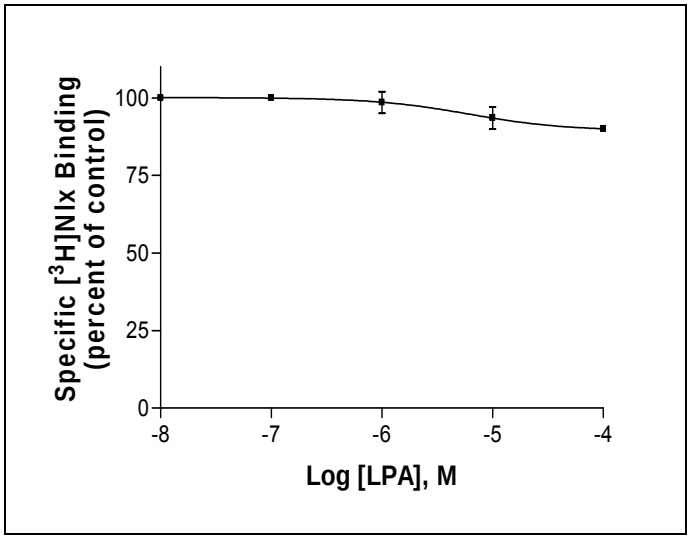


Figure 3.35. Effect of LPA on specific binding of [^{3}H]Nlx in rat brain membranes. Membranes were incubated with 1 nM [^{3}H]Nlx in the presence of various concentrations of LPA under equilibrium conditions. Result is expressed as % of control.

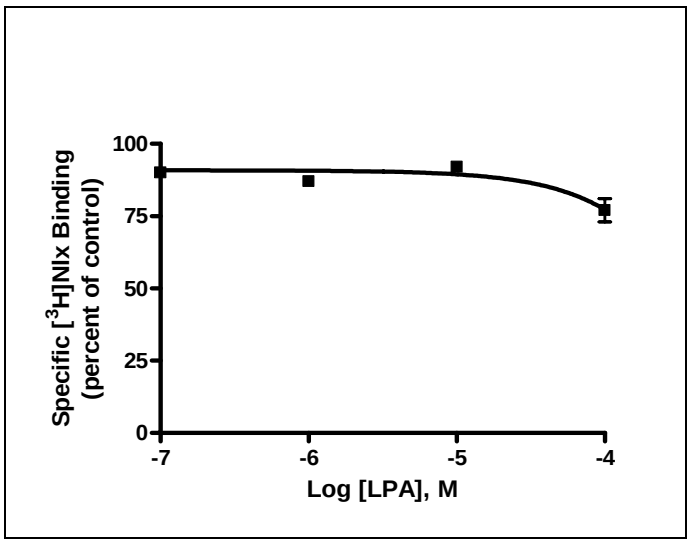


Figure 3.36. Effect of LPA on specific binding of [^{3}H]Nlx in rat brain membranes. Membranes were incubated with 1 nM [^{3}H]Nlx in the presence of various concentrations of LPA in the presence of 100 mM NaCl. Result is expressed as % of control.

Table 3.10. IC_{50} values for inhibition of [^{3}H]ProE_1 and [^{3}H]DIDI specific binding by LPA

IC_{50} (M)	
[^{3}H]ProE_1	[^{3}H]DIDI
$1.5x10^{-4}$	$1.0x10^{-4}$

3.4.2. Displacement Experiments

In displacement experiments, non-specific component of binding is measured with increasing concentrations of cold ligands of [^{3}H]ProE_1,and [^{3}H]DIDI (Figure 3.37, 3.38) in the absence and presence of LPC. IC_{50} values determined from displacement experiments are given in Table 3.11.

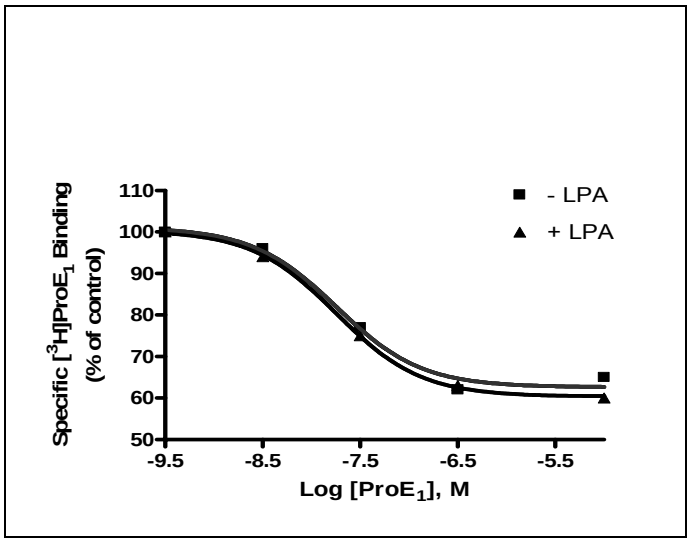


Figure 3.37. Inhibition of [^{3}H]ProE_1 specific binding to rat brain membranes by ProE_1. Membranes were treated with 1 nM [^{3}H]ProE_1 in the presence of various concentrations of ProE_1 in the absence and presence of 0.15 mM LPA under equilibrium conditions. Result is expressed as percent of control.

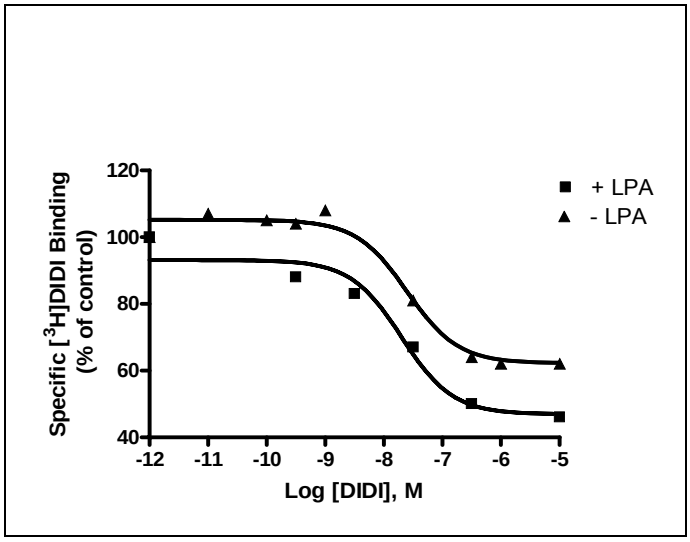


Figure 3.38. Inhibition of [^{3}H]DIDI specific binding to rat brain membranes by DIDI. Membranes were treated with 1 nM [^{3}H]DIDI in the presence of various concentrations of DIDI in the absence and presence of 0.1 mM LPA under equilibrium conditions. Result is expressed as percent of control.

Table 3.11. IC_{50} values obtained from displacement experiments

Ligand	LPC	IC_{50} (M)
[^{3}H]ProE$_1$	-	1.85×10^{-8}
	+	1.82×10^{-8}
[^{3}H]DIDI	-	2×10^{-8}
	+	2.48×10^{-8}

3.4.3. Saturation Experiments

Equilibrium saturation binding of [^{3}H]ProE$_1$ in LPA modified membranes showed that LPA is affecting both binding capacity and affinity of ligand of ligands (Figure 3.39). In unmodified membranes B_{max} value of 218 fmol/mg of protein and K_D value of 5.37 were determined. Treatment of membranes with LPA decreased B_{max} value to 117 fmol/mg of protein and K_D value to 10.32 nM (Table 3.12). Analysis of equilibrium saturation binding data for [^{3}H]DIDI revealed that in the presence of LPA B_{max} value decreased 49 % while K_D value did not changed.

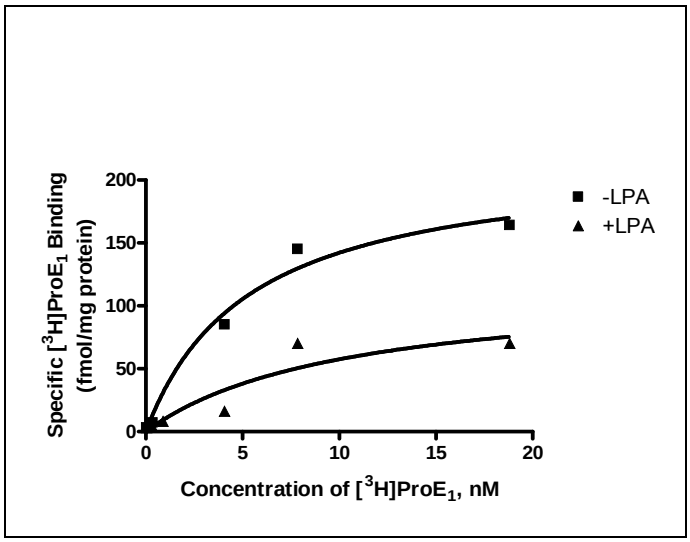


Figure 3.39. Equilibrium saturation binding of [^{3}H]ProE$_1$.in rat brain membranes. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]ProE$_1$ in the absence and presence of 0.15 mM LPA. Result is expressed as fmol/mg protein.

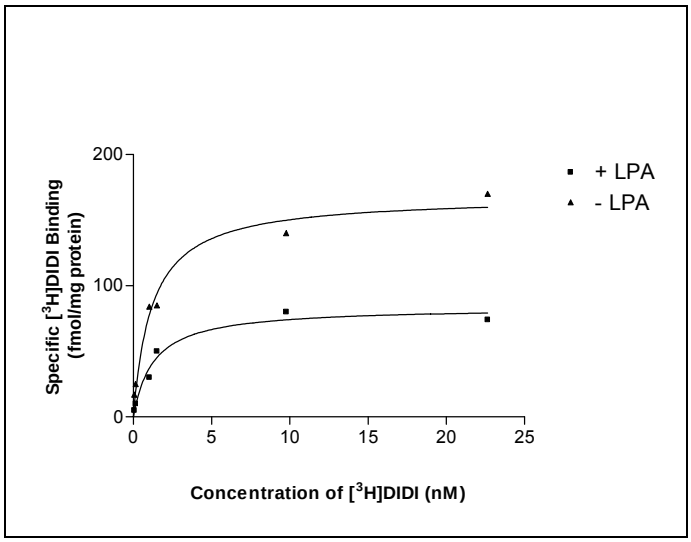


Figure 3.40. Equilibrium saturation binding of [^{3}H]DIDI in rat brain membranes. Membranes (0.4 mg/ml assay medium) were incubated with increasing concentrations of [^{3}H]DIDI in the absence and presence of 0.1 mM LPA. Result is expressed as fmol/mg protein.

Table 3.12. Parameters of ligand binding

Ligand	LPA	K_D (nM)	B_{max} (fmol/mg of protein)
[^{3}H]ProE$_1$	-	5.37	218.5
	+	10.2	116.8
[^{3}H]DIDI	-	1.19	168
	+	1.28	83

3.5. Effect of Phosphatidic acid on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

Effect of PA was studied at concentrations ranging between 10^{-4} M to 10^{-8} M concentrations. Equilibrium binding experiments showed that PA has negligible effect even at the highest concentration studied. It decreased specific binding value of [^{3}H]ProE$_1$,and [^{3}H]DIDI by 16 and 10 %, respectively. In the presence of NaCl specific binding of antagonist ligand [^{3}H]Nlx decreased 11 % whereas in the absence of NaCl specific binding is very close to control value.

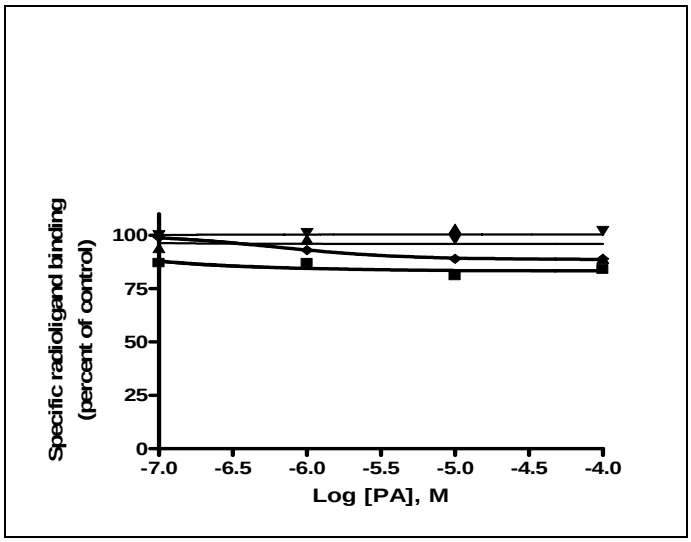


Figure 3.41. Equilibrium binding of [^{3}H]ProE$_1$ (■), [^{3}H]DIDI (▲), [^{3}H]Nlx (+ NaCl) (♦) and [^{3}H]Nlx ▼ (-NaCl)in the absence and presence of different concentrations of PA. Result is expressed as percent of control.

3.6. Effect of Phosphatidylcholine on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

Effect of PC on specific binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx was studied at concentrations varying between 10^{-4} M to 10^{-8} M. Equilibrium binding experiments showed that PC has negligible effect even at the highest concentration studied which is 10^{-3} M.

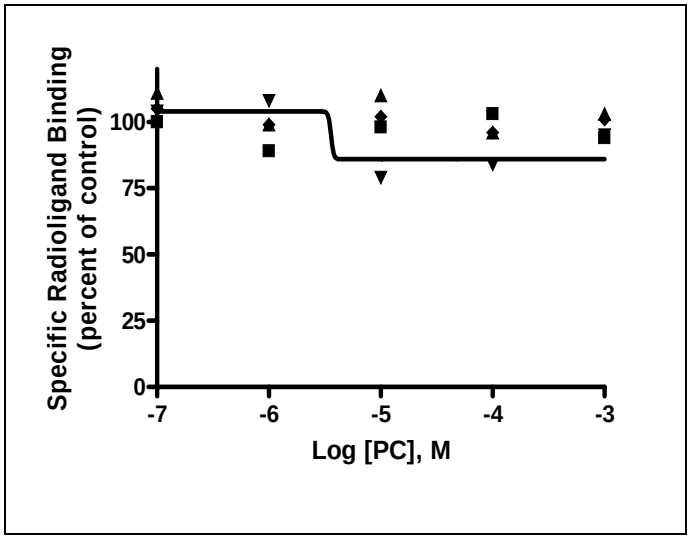


Figure 3.42. Equilibrium binding of [^{3}H]ProE$_1$ (■), [^{3}H]DIDI (▲), [^{3}H]Nlx (+ NaCl) (♦) and [^{3}H]Nlx ▼ (-NaCl) in the absence and presence of different concentrations of PC. Result is expressed as percent of control.

CHAPTER 4

DISCUSSION

In this study, the effect of lipids AA, CHS, LPC, LPA, PA and PC on binding characteristics of opioid receptors in membranes prepared from rat brain were determined. In these experiments concentrations of lipids causing changes in specific binding of [^{3}H]ProE$_1$ an opioid agonist highly specific to mu-type, [^{3}H]DIDI an agonist ligand highly specific to delta type and [^{3}H]Nlx a universal opioid receptor antagonist were determined. Inhibition of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx specific binding was studied also by homologous displacement experiments in the presence and absence of lipids. In order to understand whether the changes occurring in the specific binding is due to changes in K_D or B_{max} equilibium binding experiments were performed.

4.1. Effect of AA on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

Fatty acids have been reported to exert a modulatory effect on a great variety of cellular processes, ranging from synaptic transmission to eliciting the inhibition of Na^+ and Ca^{2+} currents, the capping phenomenon in lymphocytes, enzyme activation and regulation of cell surface receptors, transporters and ionic channels. AC, guanylate cyclase, protein kinase C or 5'-nucleotidase are among the enzymes amenable to modulation by fatty acids. Similarly, Na^+ and Ca^{2+} currents and Na^+/D-glucose transporters are inhibited by fatty acids. Some cis-unsaturated fatty acids have been reported to affect the function of several voltage- and ligand-gated ion channels.

Fatty acid effects on membrane proteins can be direct or indirect. Indirect effects involves alterations of the bulk lipid properties of the membrane, for instance, by acting as detergents to perturb the lipid membrane, by altering membrane fluidity, bilayer stiffness and/or membrane curvature, or by changing the ‘’protein-lipid interface’’. Direct effects results from the interaction of fatty acids with the ion channel proper, without intermediate steps or metabolites, whereas indirect effects are mediated by fatty acid metabolic products through, e.g. the lipoxygenase or the cyclooxygenase cycles.

In our study we used AA (20:4) which is a cis-unsaturated fatty acid and released from membrane phospholipids by a different number of neurotransmitters. Our results showed that AA caused a concentration-dependent inhibition both with agonist and antagonist ligands. Concentration of AA that causing 50 % inhibition in specific binding of radioligands were $1.5x10^{-4}$ M, $1x10^{-4}$ M for [^{3}H]ProE_1 and [^{3}H]DIDI, respectively. For an antagonist ligand [^{3}H]Nlx the concentration of $7.3x10^{-4}$ M and $6x10^{-4}$ M in the absence and presence of NaCl were determined respectively.

In the literature there are studies showing effects of AA in several different systems. In an earlier study, Mc Gee et al. (1982) studied effects of opioid receptors, prostaglandin receptors and AC activity by using NG108-15 cells where AA caused a decrease in binding of agonist ligand [^{3}H]etorphine. Hasegawa et al. (1987) in a later study found that lipids containing an acidic head group and a fatty acid with two or more double bonds including AA are necessary for obtaining binding activity in partially purified opioid receptors.

In displacement experiments, inhibition of binding of a radiolabelled ligand by unlabelled compounds was studied which can be used to indirectly characterize the interaction of the receptor with competing unlabelled compounds. EC_{50} values obtained from displacement experiments in the ab-

sence and presence of IC_{50} values of AA showed that these values are similar to each other. This shows us that changes in specific binding were due to mainly changes in binding capacity.

Analysis of saturation binding experiments which is done to see whether effect of lipids are mainly due to changes in affinity or binding capacity, revealed a one site model for the interaction of both agonist and antagonist radioligands with the mebrane bound total receptor sites. Decrease in the B_{max} value in the presence of AA, strongly suggest that AA exerts its affect by causing a decrease in the membrane fluidity.

AA has been shown to be an important cellular messenger in a wide variety of cell types. From a physiological point of view, a number of transmitters and hormones produce AA as second messengers and this could be the source of inhibitory effects on opioid receptor.

4.2. Effect of CholesterylHemisuccinate on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]NIx to Opioid Receptors

The effect of cholesterol on lipid bilayer structure is quite well understood, and the structural requirements for action are quite strict. On the other hand, the modulator role of cholesterol on membrane proteins has not been explored in detail, and the mechanisms involved remain obscure; yet, it is becoming evident that cholesterol plays an important modulator role in disease processes and in drug action. For example, it modulates proteolytic cleavage of the amyloid precursor protein and may be involved in etiology of Alzheimer's disease; it modulates the action of general and local anesthetics on acetycholine receptors, and of neurosteroids on the $GABA_A$ receptor.

There are three regions of the membrane with which cholesterol might modulate membrane proteins: in the bilayer, the lipid-protein interface, or on the protein itself. In the first, the protein's function depends on some physical property of the bulk lipid bilayer such as fluidity. In the second, lipid properties at the lipid-protein interface, such acycl chain length or lateral pressure, might be important. In the third, allosteric binding sites, specific for cholesterol would exist on the protein, either in the lipid-protein interface or within the protein itself (Addona et al., 1998).

In our study we used CHS which is a negatively charged cholesterol analog in order to modify the membrane. CHS increased specific binding of $ProE_1$ which is a mu-type agonist whereas decreased binding of DIDI an agonist specific to delta-type opioid receptor. Specific binding of Nlx is also increased when membranes were treated with CHS while in the presence of NaCl it did not effect the specific binding of antagonist ligand Nlx.

In a study conducted by Emmerson (2000) effect of CHS on mu-opioid agonist and antagonist binding was investigated in membranes prepared from SH-SY5Y cells and CG glioma cell lines. In agonist ligands, sulfentanyl and DAMGO, addition of CHS was shown to increase ligand binding upto 1 µmole/mg protein concentration. On the other hand, binding of antagonist ligands (naltrexone and diprenorpine) was unaffective at the same CHS concentrations. Like wise, in our experiments in the presence of Na^+ ions (antagonist receptor configuration) CHS has no effect on receptor binding which can be explained by protective effect of sodium ions. Modulation of opioid receptor binding by sodium has been well described. It increases the number of binding sites of antagonists and decreases the number of binding sites of agonists (Medzihradsky, 1997). The differential effect of sodium on binding of agonists and antagonists were interpreted in terms of conformational changes of the receptor from an agonist-selective state to a

state with higher affinity for antagonist. On the other hand, CHS increased binding of mu-receptor specific agonist ligand (proendomorphine) whereas no effect was observed on binding of delta-receptor specific ligands (DIDI). Previously, mu-opioid binding sites were shown to localized in an anionic environment, whereas delta sites were found in cationic region of membranes (Sargent, 1988). Therefore, due to the negative charge CHS would exhibit easier access to cationic microenvironment of the lipid bilayer containing the delta binding sites thus decreasing the bindig activity of delta specific agonist ligand DIDI.

4.3. Effect of Lysophosphatidylcholine on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

Effect of LPC on various cellular processes and molecules has been investigated. In platelets LPC was shown to induce an increase in cAMP levels, resulting in the inhibition of aggregation (Yuan, 1996). LPC also increases cytoplasmic free Ca^{2+} concentration in several types of cells including endothelial, smooth muscle cells, leukocytes and macrophages although the lipid has been reported to inhibit agonist induced Ca^{2+} increase (Inoue, 1992; Tokumura, 1994; Chen, 1995).

More recently effect LPC on NMDA responses was studied in nucleated patches taken from cultured cortical and diencephalic neurons of mice together with cells expressing recombinant NMDA receptors (Casado and Ascher, 1988). It was shown that LPC at 2 μM inhibits NMDA receptor currents in a fully reversible and voltage independent manner. It was proposed that this action of LPC seemed to required insertion in to the lipid bilayer. LPC was also shown to activate various protein kinases including protein kinase-C, p38 MAP kinase, p42 MAP kinase and the jun kinase (Jing et al., 2000).

To our knowledge effect of LPC on opiate receptors has not been evaluated yet. In our experiments, LPC was shown to inhibit specific binding of both agonist and antagonist ligands at around an IC_{50} value of 100 μM. Although the mechanism of action of this inhibitory action of LPC remains unclear, it is known that at high concentrations lysophospholipids can decrease the acyl chain order parameter of lipid membranes. This indicates that fluidity of the membrane interior is increased so the effects of lysophospholipids on membrane protein function have been ascribed to their effect on membrane fluidity. The results obtained in our study can be explained in terms of fluidity changes since analysis of saturation binding data showed that LPC decreased B_{max} value of $ProE_1$, DIDI and Nlx in the absence of NaCl by 20, 26 and 50 %, respectively. In the presence of NaCl B_{max} value of naloxone decreased 20%. On the other hand, K_D values were not affected. This observation is an indirect evidence that effect of LPC is due to a change in membrane fluidity. Effects of LPC might be important from a pathophysiological view of inflammatory disorders and vascular artherosclerosis; the normal LPC concentration in serum or plasma is reported to be approximately 100 μM and this value would increase in these disorders. Our findings might be important for the actions of LPC in the cells involved in the inflammatory disorders and vascular atherosclerosis.

4.4. Effect of Phosphatidic acid and Lysophosphatidic acid on Specific Binding of [^{3}H]$ProE_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

PA and LPA are critical intermediates in de *novo* lipid biosynthesis but also are produced rapidly in significant quantities upon cell activation, suggesting a possible role of PA and LPA as second messengers. In addition, PA and LPA may function as agonists in that they exert their own biological effects when added extracellularly to appropriate target cells. For example, both PA and LPA have been reported to induce contraction of smooth muscle cells (Tokumura et al., 1980). Both compounds have also

been reported to cause Ca^{2+} mobilization in various cell types (Moolenaar et al., 1986a; van Corven et al., 1989), to inhibit adenylate cyclase (Murayama and Ui, 1987; van Corven et al., 1989), and to stimulate DNA synthesis and cell division in fibroblasts (Moolenaar et al., 1986a; van Corven et al., 1989).

In our study we studied effect of PA at concentrations ranging between 10^{-4} M to 10^{-8} M concentrations. At the highest concentration studied PA decreased specific binding value of $ProE_1$,and DIDI by 16 and 10 %, respectively. Specific binding of antagonist ligand Nlx decreased 11 % in the presence of NaCl whereas in the absence of NaCl specific binding is very close to control value. Since IC_{50} value did not obtained further experiments were not carried out with PA.

In our study effect of LPA determined by using concentrations of LPA ranging from 10^{-4} to 10^{-8} M and found that LPA decreased specific binding of [^{3}H]$ProE_1$ and [^{3}H]DIDI while specific binding of [^{3}H]Nlx decreased 10 and 19 % in the absence and presence of NaCl in the medium. Displacement and saturation experiments carried out by using [^{3}H]$ProE_1$ and [^{3}H]DIDI showed that mainly B_{max} values are subjected to inhibition.

In the literature there are very few studies showing effect of LPA on receptors. In one of the study, effect of several lysophospholipids including LPA was studied in cortical and diencephalic neurons obtained from mice and it was found that LPA had no effect on NMDA responses at 10 μM concentration (Casado and Ascher, 1998).

4.5. Effect of Phosphatidylcholine on Specific Binding of [^{3}H]ProE$_1$, [^{3}H]DIDI and [^{3}H]Nlx to Opioid Receptors

PC is an essential component of membranes of most eukaryotes and probably plays an important roles in some prokaryotic organisms. Most bilayer-forming lipids like PI or PG are anionic at physiological pH while PC has a special status since its zwitterionic state at physiological pH.

In a study made to investigate modulation of 1,25-dihydroxyvitamin D_3 receptor by phospholipids and fatty acids it was found that at a concentration of 30 μM, PC with one or two C-18 unsaturated fatty acids were shown to be more effective than those with two saturated fatty acids of C-14, C-16, C-18, or C-20 chain length. This suggest that the fatty acid moiety of the PC molecule plays very important role in modulation (Chen, 1984). Aloj et al. found that acidic phospholipids PI, PS, and neutral phospholipids PE although less potent interact with ^{125}I-thyrotropin and inhibits its binding to thyroid plasma membranes but other neutral phospholipids PC is not similarly effective (Aloj et al., 1979). In an earlier study performed on crude membrane preparations from rat brain it was found that synthetic dipalmitoyl phosphatidylcholine and whole brain PC were without effect on opiate binding (Abood et al., 1977).

In our study, we found that PC from bovine brain has negligible effect even at the highest concentration 10^{-3} M on both agonist and antagonist ligands of opioid receptor which can be explained due to neutral charges of the PC.

CHAPTER 5

CONCLUSION

Growing evidence suggest that lipids including fatty acids, lysophospholipids and phospholipids in addition to their roles as a structural components of membrane lipids, act as a second messengers or regulators of signal-transducing molecules.

Effect of various lipids on binding properties of rat brain opioid receptors at subtype level was evaluated.

AA was found to decrease specific binding of all receptor subtypes in a concentration dependent manner by causing a decrease in the maximum number of binding sites. At delta sites AA eliminated majority of binding sites with compared to mu sites and at antagonist configuration of receptor in the presence of NaCl binding sites were more susceptible to AA inhibition.

The role of cholesterol in specific binding of opioid ligands was studied. Specific binding of agonist ligand $ProE_1$ specific to mu sites enhanced whereas specific binding of other agonist ligand DIDI specific to delta sites was not effected. CHS showed its effect on antagonist ligand naloxone when NaCl is not present in the medium. Effect of CHS on specific binding was mainly on number of binding sites while affinity was not effected significantly.

Modulatory effect of LPA and LPC on opioid binding sites were determined. LPC decrease specific binding in receptor subtype independent manner while LPA showed its effect mainly on mu and delta sites. Both lysophospholipids exhibited similar effects, K_d values were not significantly affected, while B_{max} values decreased. Phospholipids PA and PC has no significant effect on opioid receptor specific binding.

These effects indicate that lipids are regulator of receptor binding. Changes in lipid composition may also regulate protein function and protein-protein interactions through effects on membrane biophysical properties such as microviscosity, thus potentially affecting protein lateral diffusion or protein conformational changes. The changes in membrane composition and biophysical properties resulting from diet, age, and pathophysical states suggest a regulatory role for the membrane bilayer in cellular signal transduction.

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APPENDIX A

Specific Binding of Radioligands in the presence of Different Concentrations of Lipids

[CHS], M	[^{3}H]ProE$_1$ S.B. (cpm)	[^{3}H]DIDI S.B. (cpm)	[^{3}H]Nlx S.B.(cpm)	[^{3}H]Nlx S.B. (+ NaCl) (cpm)
Control	1197	1818	1944	5460
$2.5x10^{-4}$	1712	1940	2479	4774
$5x10^{-4}$	1972	1922	2987	3062
10^{-3}	2952	1326	2785	2654
$1.5x10^{-3}$	2516	751	2133	2798
$2x10^{-3}$	1809	126	2107	690

[LPC], M	[^{3}H]ProE$_1$ S.B. (cpm)	[^{3}H]DIDI S.B. (cpm)	[^{3}H]Nlx S.B.(cpm)	[^{3}H]Nlx S.B. (+ NaCl) (cpm)
Control	1523	1074	1070	1936
$3x10^{-4}$	169	123	172	621
$3x10^{-5}$	1085	947	959	1921
$3x10^{-6}$	1368	1123	926	2213
$3x10^{-7}$	1226	889	992	2036
$3x10^{-8}$	1162	1105	1049	1921

[LPA], M	[^{3}H]ProE$_1$ S.B. (cpm)	[^{3}H]DIDI S.B. (cpm)	[^{3}H]Nlx S.B.(cpm)	[^{3}H]Nlx S.B. (+ NaCl) (cpm)
Control	1868	1431	1475	2492
10^{-4}	1127	1013	1197	2355
10^{-5}	1608	1395	1358	2333
10^{-6}	1345	1450	1278	
10^{-7}		935		
10^{-8}		974		

[PC], M	[^{3}H]ProE$_1$ S.B. (cpm)	[^{3}H]DIDI S.B. (cpm)	[^{3}H]Nlx S.B.(cpm)	[^{3}H]Nlx S.B. (+ NaCl) (cpm)
Control	2835	1087	1081	1830
10^{-3}	2671	1118	1028	1850
10^{-4}	2927	1045	910	1755
10^{-5}	2774	1197	854	1821
10^{-6}	2516	1078	1170	1825
10^{-7}	2846	1215	1123	1928

[PA], M	[^{3}H]ProE$_1$ S.B. (cpm)	[^{3}H]DIDI S.B. (cpm)	[^{3}H]Nlx S.B.(cpm)	[^{3}H]Nlx S.B. (+ NaCl) (cpm)
Control	1789	1444	1876	1968
10^{-4}	1771	1376	1886	2336
10^{-5}	1616	1498	1756	2162
10^{-6}	1744	1397	1780	2140
10^{-7}	1754	1301	1237	2128

[AA], M	[^{3}H]ProE$_1$ S.B. (cpm)	[^{3}H]DIDI S.B. (cpm)
Control	610	1818
10^{-5}	737	1309
$5x10^{-5}$	750	1444
10^{-4}	332	878
$2.5x10^{-4}$	118	692
$5x10^{-4}$	84	0

[AA], M	[^{3}H]Nlx S.B.(cpm)	[^{3}H]Nlx S.B. (+ NaCl) (cpm)
Control	3768	2741
$3x10^{-7}$	3278	2330
$6x10^{-7}$	2864	1309
$1x10^{-6}$	1092	520
$2.5x10^{-5}$	716	274
10^{-5}	452	137

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MSc. Thesis, Middle East Technical University, Biochemistry Ankara Turkey
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Papers and Abstracts

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4. S. Apaydin[1], Ö. Gündüz[2], S. Benyhe[2], H.A. Öktem[1]. Modulation of Opioid Receptor Binding by Arachidonic Acid. European Opioid Conference, Poster of Abstract, April 4-7, 2004, Visegrad.

5. Apaydin S. and Öktem H.A. "Opioid Receptor Modulation by Lysophosphatidic Acid and Arachidonic Acid: A Possible Cross Talking between Lysophosphatidic Acid and Opioid Signal Transduction Systems". Turkish Biochemical Society XIV. National Congress of Biochemistry (with International participation), Book of Abstract, October 28-31, 1997, İzmir

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