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THE ROLE OF MEMBRANE LIPIDS IN RECEPTOR MECHANISMS

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INTRODUCTION

In spite of the abundance of data on the interaction of ligands with their membrane receptors, the molecular role of lipids in the subsequent vectorial transfer of information across the membrane bilayer has not been fully elucidated. The amphiphatic properties of the lipids are believed to provide both a suitable environment for the anchoring of the hydrophobic regions of the proteins as well as a barrier for the intra- and extracellular ions and solutes. These static roles of lipid have been suggested in a paper by Day & Levy (1), in which they proposed that the primary role of the phospholipids was to provide a molecular "cement" for the construction of membranes and other cellular organelles. However, numerous lines of evidence suggest that lipids also have a dynamic role in the function of membrane-bound protein, as illustrated by their effect on the mitochondrial enzymatic activites (2). In the membrane enzymes that constitute a vectorial system, such as Na^+-K^+-ATPase, the enzymatic activities depend greatly on the physical state of the endogenous lipids (3, 4). Moreover, in addition to the effect on catalytic rate constants, lipids can modulate enzymatic activity by affecting the ligand-binding properties of the membrane proteins, e.g. thiamine pyrophosphate binding to pyruvate oxidase (5).

It is therefore tempting to visualize similar dynamic roles of lipids in receptor mechanisms, perhaps also including the ability to act directly as binding sites themselves. Because most neurotransmitters and drug molecules are cationic at physiological pH, acidic lipids are the most likely candidates for the latter role. Hence, in this review we summarize existing data on the role of the lipid, and in particular acidic lipids, in the series of

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molecular events which start with the binding of a unique ligand to the membrane receptor. Three possible roles of lipids are discussed: (*a*) their direct involvement in the ligand binding, (*b*) their role as cofactor for the functional receptor, and (*c*) their serving as regulators for the coupling between the receptor and the effector system. However, we do not discuss the metabolism of lipids as a consequence of ligand-receptor interaction, such as the phosphorylation and dephosphorylation of the lipid phosphatidylinositol in response to various neurotransmitters. For a review of these topics the reader is referred to an excellent article by R. H. Michell (6).

THE FLUID MOSAIC MODEL AND STRUCTURE OF THE MEMBRANE

A brief discussion of the current model for the structure of biological membranes follows.

In 1972, Singer & Nicholson (7) combined the existing models and proposed the fluid mosaic model in which the structural determinants in the biological membrane were composed of asymmetric phospholipid bilayers. In this model, the polar head groups of the lipids are exposed to the external aqueous phase and their hydrophobic regions form an apolar environment in the backbone of the membrane. Complex carbohydrate oligosaccharides and glycoproteins are located at the outside of the bilayer. The membrane proteins are also asymmetrically disposed. They may be located at the surface of the bilayer (peripheral proteins) or be partially immersed in the bilayer, sometimes penetrating the membrane completely (integral proteins). The latter are surrounded by a shell of immobilized lipid known as the boundary lipid or lipid annulus, and usually these phospholipids remain with the protein after solubilization with organic solvents or detergents (7, 8). In contrast, the peripheral proteins can be extracted from the bilayer with gentle techniques such as varying ionic strength or sonication. It is believed that the peripheral proteins bind to the integral proteins rather than to the lipid bilayer (7). There is a tendency for the lipid in the membrane to organize into domains (9) and the fluidity of these domains is believed to regulate the enzymatic activity (3).

The essential feature of this mosaic model is that the membrane components, both phospholipids and proteins, are capable of lateral mobility in the membrane plane (10). The lipid undergoes a lateral phase separation during thermal-induced phase transition (10, 11) and this separation has a great effect on protein distribution in the membrane. Freeze fracture electron microscopy for the reconstituted complexes of lecithins and rhodopsin (12–14) reveals a random arrangement of protein molecules in the bilayer at temperatures above the onset of phase transition and clustering of parti-

cles below the transition temperature. Hence, the physical state of the lipid must greatly influence the activity of the membrane proteins. Transmembrane rotation of the phospholipids ("flip-flop") is observed to occur at a slower rate in the artificial membrane (15, 16) than in native membrane such as erythrocytes (17). The oligosaccharides and glycoproteins at the outside of the bilayer would not undergo such transmembrane rotational movement. Thus, the fluidity of the bilayer halves can be regulated not only by the existing phospholipid asymmetry but also by the lateral mobility and the rotational movement of the lipid. The lateral mobility of the membrane proteins, in turn, will be regulated by the alteration in the physical state of the lipid [for a detailed discussion on the control of protein lateral mobility, the reader is referred to the review article by Nicholson (18)].

This fluid mosaic model is based largely on studies of the erythrocyte membrane (7) and other simple systems (193). But studies of nerve membranes, which contain most of the receptor systems discussed in this review, have shown that they too possess most or all of the features of this model (194–198). In particular, it is clear that at least some proteins are mobile in nerve membranes and that their mobility is restricted in certain regions (199).

Within the proposed fluid mosaic model for the structure of the biological membrane, the role of lipid in the receptor mechanism can be summarized as follows: (*a*) the lipid per se or in combination with a membrane protein serves as the recognition sites for the ligands; (*b*) the lipids that surround the receptor molecules, the lipid annulus of the receptor, modulate the receptor's three-dimensional structure and hence regulate the ligand affinity for the receptor, and (*c*) the physical state of the lipid domain in which the receptor is situated regulates the lateral mobility of the receptor and, in turn, the interaction of the ligand-receptor complex with the effector molecule, such as adenylate cyclase or an ion channel. With regard to *a,* if a specific lipid has a role in the recognition of the ligand, the purified and synthetic lipid should have high affinity for the ligand and should compete with the biological membrane containing the receptor for the specific ligand. Since the glass fiber membrane commonly used in receptor binding assay also exhibited high affinity for the radioactive insulin (19) and opiates (20), the lipid affinity for the ligand should, therefore, also demonstrate ligand specificity; that is, the relative affinity of various ligands for the lipid should correspond to those observed with the receptor in the membrane. With regard to *c,* if the lipid has a role in the subsequent molecular event after the recognition of the ligand, removal or addition of the particular lipid involved would modulate the cellular response to the presence of the ligand without significant alteration of the ligand-receptor interaction. A classic example for illustrating both these possible roles of lipid is the data on the cholera toxic-ganglioside interaction.

GANGLIOSIDE AS THE RECEPTOR FOR CHOLERA TOXIN

The role of ganglioside in the action of cholera toxin has been recently summarized by Fishman & Brady (21) and Gill (22). The role of ganglioside as the receptor for the toxin was initially recognized by the experiments of Van Heyningen et al (23), who found that the lipid extract from the homogenates of epithelia scraped from the small intestine of rabbit was a potent deactivator of cholera toxin. The effective component in this lipid extract was subsequently discovered to be the sialidase-resistant monosialoganglioside G_{M1} [galactosyl-*N*-acetylgalactosaminyl-(sialyl)-galactosylglucosyl ceramide] (24–29). G_{M1} being the recognition site for cholera toxin was further illustrated by the positive correlation between the amount of toxin bound to various cell types (22, 30) or intestine of various species (22, 31) and the density of G_{M1} in these tissues. Most convincingly, Cuatrecasas (32) illustrated the structural requirement of several gangliosides to compete for ^{125}I-toxin binding to liver plasma membrane with G_{M1} being the most potent ganglioside. Removal of the lipid from liver membrane by solvent extraction or increase of the G_{M1} level in the membrane with neuramidase treatment subsequently decreased or increased the ^{125}I-cholera toxin binding to the liver plasma membrane (32). The affinity of toxin for G_{M1}, as measured by the dissociation constant, K_d, has been determined to be $\sim 10^{-9}$ M (22, 31). Hence, the lipid G_{M1} fulfills two of the criteria for being the cellular recognition sites of cholera toxin—toxin binds to the lipid with high affinity and the lipid competes with liver plasma membrane for toxin. Furthermore, it appears that G_{M1} has a functional role in the expression of cholera toxin action. Addition and incorporation of the exogenous G_{M1} to the membrane increases the cellular response to cholera toxin (28, 31, 33). Increase in the lipolysis of fat cells (28) and increase in the adenylate cyclase activity in the erythrocyte membrane (33) or small intestine (31) in the presence of cholera toxin was observed after pretreatment with G_{M1}. Such observations could be attributed to the removal of some inhibitory ligand by G_{M1} which blocked the endogenous "functional" glycoprotein receptor for cholera toxin in the cellular membrane. This possibility is supported by the observations that various glycoproteins, e.g. fetulin and thyroglobulin (32), are able to bind cholera toxin and that additional G_{M1} enhanced the subsequent rate but not the extent of toxin-induced lipolysis of the fat cells (34). However, further evidence for a functional role of G_{M1} is provided by the work of Moss et al (35) and Fishman et al (36) with a clone of transformed mouse fibroblasts containing nondetectable quantities of G_{M1}. These fibroblasts, NCTC 2071 cells, lack two biosynthetic enzymes for ganglioside biosynthesis—CMP-sialic acid : lactosylceramide sialyltransferase and UDP-galactose : G_{M2} : galactosyltransferase—and were shown to be unre-

sponsive to cholera toxin (35, 36). However, after the incorporation of the nonmetabolized G_{M1} in the growth medium into the cellular membrane, the fibroblasts were observed to respond to cholera toxin (35, 36). There was a correlation between the amount of G_{M1} incorporated and the increase in the amount of cAMP accumulated by cells induced by the presence of toxin (35, 36). Incorporation of other gangliosides by the cells did not exhibit such activity. Hence, these experiments clearly demonstrated that ganglioside G_{M1} is the functional receptor for the cholera toxin. The action of cholera toxin at the target tissue most likely is initiated by binding of the toxin to the carbohydrate moiety of the G_{M1} molecule (29, 37) and/or other surface glycoproteins. Though the exact mechanism is unknown, the G_{M1} participates in the regulation of cellular response-activation of the enzyme adenylate cyclase by cholera toxin. The enhancement of lipolysis rates in fat cells (34) by G_{M1} suggests a further role for this lipid other than as recognition sites in the cholera toxin mechanism.

THE ROLE OF LIPID IN ADRENERGIC RECEPTOR MECHANISM

The possible involvement of lipid in adrenergic receptor action was implicated initially by the studies of Dikstein (38) and Yong et al (39) with radioactive dibenamine, an α-adrenergic blocker. By labeling the α-adrenergic receptor in the aortic tissue with ^{14}C-dibenamine, Yong et al (39) observed that one third of total radioactivity could be extracted with organic solvent and that there was a difference in radioactivity between the lipid extract of the tissue previously protected from the nonradioactive dibenamine with α-agonists and the extract not protected. Dikstein (38) reported that boiling chloroform:methanol (2:1) removed 80% of the ^{3}H-dibenamine bound to the aortic segments. These studies inferred that the binding component of dibenamine in the aortic tissue was lipid in nature. However, both studies had used low specific radioactivity of dibenamine. Dibenamine not only could react with the adrenergic receptor, but also could interact with serotonin and acetylcholine receptor (39). Without comparing the amount of radioactivity extracted when the tissue was labeled with dibenamine in the presence or absence of excess nonradioactive α-agonists, we might conclude that the dibenamine associated with the lipid extract is a reflection of the nonspecific binding of the ligand to the lipid. This could explain the contradictory results reported by Lewis & Miller (40) which suggested that ^{3}H-phenoxybenzamine bound to rat seminal tissue was not extractable by chloroform:methanol (2:1).

The nonspecific interaction of α-agonists with lipid was further demonstrated by the norepinephrine-lecithin interaction. Cuthbert et al (41) reported an uptake of ^{3}H-norepinephrine from the aqueous phase of sodium

phosphate at pH 6.5 into the ether phase containing lecithin [phosphatidylcholine (PC)]. However, this interaction between the α-agonist and the lipid:lecithin appeared to be nonspecific, because the amount of ^{3}H-norepinephrine "bound" to lecithin was identical in the presence of (+) or (–) norepinephrine and in the absence of any nonradioactive norepinephrine. The high capacity of the lipid for interaction with norepinephrine might reflect the nonspecific low affinity unsaturable binding sites observed in every receptor binding assay. But there appears to be some lipid specificity in this high capacity interaction. Formby (42, 43) reported the interaction of ^{3}H-norepinephrine with phosphatidylserine (PS) and phosphatidylinositol (PI) by a two-phase partitioning system. By partitioning the α-agonists between the aqueous phase (25 mM Tris pH 7.2) and the organic phase [chloroform:methanol (2:1)], and after correcting for partitioning coefficient of the agonists, Formby demonstrated the binding of norepinephrine to PS and PI and no binding to the lipids sphingomyelin, lecithin, phosphatidylethanolamine (PE), cerebroside- and sialic acid–free glycolipids. But the physiological significance of this observation was greatly diminished by Formby's report of maximal norepinephrine binding to PS at pH 10.1. Hence, binding occurred at the nonpolar state of norepinephrine. Again, these observations do not reflect the specific interaction between the α-agonist and the receptor, but they might represent the initial partitioning of the ligand into the membrane bilayer prior to the ligand interaction with the receptor.

Other data that might implicate the involvement of lipid in the cellular recognition of adrenergic ligands are the extraction and partial purification of a proteolipid that binds ^{3}H-norepinephrine from the bovine spleen [DeRobertis and his co-workers (44–47)]. By partitioning the lyophilized spleen homogenate containing bound ^{3}H-norepinephrine between aqueous phase and organic phase of chloroform:methanol (2:1), De Plazas & DeRobertis (44, 45) isolated a proteolipid constituting 0.055% of total protein in spleen that bound the α-agonists. By increasing the polarity of the eluting solvent of a Sephadex LH-20 column, the proteolipid that bound ^{3}H-norepinephrine was separated from other proteolipids (45). Affinity constants of the α-agonist for this purified proteolipid were determined to be 0.33 and 18 μM and the density of the proteolipid receptor was calculated to be 1.5×10^{11} receptors/mg dried spleen tissue. All these values appear to be within the reasonable range of a specific receptor on the target organ. However, when the lipid content of this proteolipid was analyzed, it was observed to contain PE, PI, and PS. As discussed previously, Formby (42, 43) observed high capacity ^{3}H-norepinephrine binding to the phospholipid PS and PI. Hence, DeRobertis's proteolipid receptor for α-agonist could simply be the binding of ^{3}H-norepinephrine by the lipid in the extract. However, when this proteolipid was added to the artificial lipid mem-

brane (black lipid membrane–containing cholesterol alone or cholesterol + PI/PE/PC), Ochoa et al (46, 47) could demonstrate a sudden increase in conductance which lasted for 20–40 sec with the addition of *d*-norepinephrine and not with *l*-norepinephrine. This response was blocked by phentolamine and only observable in the presence of the proteolipid. PE, PI, or PC (low concentrations) did not give a response. Thus, there appeared to be receptor specificity. *l*-Isoproterenol, a β-agonist, produced a lower response than norepinephrine, and cholinergic agonists did not alter the conductance of the artificial membrane containing the proteolipid. On the other hand, unusually high concentrations of α-agonist were needed to produce such an effect (0.5–50 mM) and conductance change (20–40 sec) was of relatively long duration, indicating that more studies are necessary before conclusions can be drawn. With the K_{diss} values of norepinephrine for this proteolipid reported to be 0.33 and 18 μM (45), the relatively high concentration of norepinephrine required to produce an increase in conductance suggested the interaction of the α-agonist with some low affinity binding sites, possibly the artificial lipid membrane. Nevertheless, the ability of this preparation to distinguish the *d*- and *l*-isomers of norepinephrine offers a tantalizing possibility for the involvement of lipid or a proteolipid as the cellular recognition site for α-agonist.

Another set of data that suggests the participation of lipid in the recognition of adrenergic ligands is the hydrolysis of frog erythrocyte membrane phospholipids by phospholipases and the effect on ^{3}H-dihydroalprenolol binding [Limbird & Lefkowitz (48)]. It is commonly known that various phospholipases hydrolyze the triglyceride ester linkage of the lipid differently. Phospholipase A hydrolyzes the ester linkage at β-position to yield a fatty acid and phosphatide. Phospholipase C and D catalyze the hydrolysis of phosphodiester linkage between L-α-phosphatidic acid and nitrogen base, and between α,β-diglyceride and phosphorated base, respectively. In general, treatment with phospholipase A will affect the hydrophobicity of the bilayer, and treatment with phospholipase C will alter the polar head groups of the lipids. When Limbird & Lefkowitz (48) treated the frog erythrocyte membrane with phospholipases A, C, and D, they reported a decline in the ^{3}H-dihydroalprenolol binding without altering the K_{diss} values. Furthermore, addition of PC, PS, or PI did not restore the phospholipases' inhibitory effect. Such results could be explained by the overall destabilization of the membrane conformation after the phospholipase treatment. This could be the case with the phospholipase A inhibition. Since the lysophospholipid, a product of phospholipase A, is not easily released from the membrane (49) and the product has surface-active properties, serving as solubilizing agent (50), the inhibitory effect of phospholipase A on the β-antagonist binding could be due to the hydrolysis products. The hydrolysis products could be sequestered with the defatted bovine serum

albumin (BSA). Without such an experiment, the interpretation of phospholipase A effect observed by Limbird & Lefkowitz (48) is rather difficult. On the other hand, the inhibitory effects of phospholipase C and D on ^{3}H-dihydroalprenolol binding suggest the involvement of the polar head groups of the phospholipid in the binding of the β-antagonist. Circular dichroism measurement of the erythrocyte membrane after phospholipase C treatment indicated, even after removal of 60–75% of the polar head groups of the phospholipids, the gross physical state of the membrane, and the proteins were not affected (51). With no apparent decrease in the K_{diss} value but a decrease in number of binding sites (48), the polar head groups of the lipid removed by phospholipase C possibly are the recognition sites for β-antagonist. However, the possibility remains that the receptor protein was released into the incubation medium or that protease contaminants were present in the phospholipase C preparation. Therefore, the role of lipid in adrenergic receptor binding is not at all conclusive.

The involvement of lipid in the coupling of the adrenergic-receptor complex to the effector is more clear-cut. The best-studied system is the activation of adenylate cyclase by β-agonists. It has been proposed by De Häen (52) from the existing data that the basic mechanism for the hormone receptor action is the floating receptor model; that is, one can picture the hormone receptor complex floating in a sea of lipid and an adenylate cyclase molecule interacting with more than one type of hormone receptor complex (53, 54). Hence, one possible role of lipid in the hormone-activating adenylate cyclase system is regulation of the lateral mobility of the hormone receptor complex. This was demonstrated by experiments with polyene antibiotics (48, 55). Demel et al (56) and Sessa & Weissman (57) showed that the polyene antibiotics filipin, amphotericin B, and nystatin penetrate the membrane preferentially at the region containing cholesterol. The formation of such antibiotic:cholesterol complexes was shown not to involve lysis and disintegration of the membrane (55). When the pigeon erythrocyte membrane was treated with filipin (55), the isoproterenol- and guanylyl nucleotide–stimulated adenylate cyclase was inhibited with no apparent alteration of the β-agonist binding to the filipin-treated membrane. Moreover, the NaF-stimulated adenylate cyclase was not affected by filipin, indicating that the antibiotic's action was not in the inactivation of the catalytic subunit of the enzyme. The filipin effect could be reversed by removing the antibiotics with cholesterol (55). Similar results were observed by Limbird & Lefkowitz (48) with the filipin and amphotericin B effect on the frog erythrocyte membrane. Studies with a fluorescent dye (55), perylene, suggested that the structural order of the lipid matrix predominates over the microviscosity in determining the signal transfer of the receptor to the adenylate cyclase.

On the other hand, studies involving alteration of the membrane fluidity also suggest the importance of the environmental factor in β-adrenergic agonist action (58–62). Orly & Schramm (58), by the in vitro addition of various fatty acids to the turkey erythrocyte membrane, enhanced the isoproterenol activation of the adenylate cyclase at 20°C up to 25-fold. Arrhenius plots suggested that the activation energy of the isoproterenol-adenylate cyclase reaction after the addition of fatty acid was decreased (58). This observation of Orly & Schramm was carried on further by the experiments reported by Levitzki and co-workers (59, 60). By incubating the turkey erythrocyte membrane with *cis*-vaccinic acid, Levitzki and co-workers reported an increase in adenylate cyclase sensitivity to *l*-epinephrine (59, 60), with no apparent modification of the β-receptor–binding properties. Furthermore, by measuring the microviscosity of the membrane using the fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH), which has been demonstrated to dissolve in the hydrocarbon region of the membrane (61, 62), Hanski, et al (60) observed a direct correlation between increased microviscosity and the amount of *cis*-vaccinic acid added to the membrane. Thus, Hanski et al (60) suggested a direct correlation between epinephrine activation of the adenylate cyclase and the microviscosity of the membrane.

Activation of the cyclase by β-receptor is believed to be a diffusion-controlled process; by altering the membrane lipid composition and hence the membrane fluidity, the vectorial transferring of information can be regulated. However, Shattil & Cooper (63) and Insel et al (64) observed that the epinephrine-induced human platelet aggregation was dependent on the fluidity of the membrane. By incubating the platelet with cholesterol vesicles containing various levels of phospholipid, the cholesterol/phospholipid ratio (C/PL) in the platelet membrane was altered. It has been shown with PDH fluorescent measurements that cholesterol increases the order in the bilayer and decreases random rotational motion of the lipid and, hence, the membrane fluidity (63, 65). By increasing the C/PL ratio, the sensitivity to epinephrine-induced platelet fusion increases (63, 65, 66). By incubating the platelets with cholesterol-free phospholipid and thus decreasing the cholesterol content in the platelet membrane (64), an 18-fold decrease in the epinephrine potency to induced aggregation was observed. Such a decrease in epinephrine potency is not accompanied by a decrease in the affinity of epinephrine to the α-receptor (64). Furthermore, in the cholesterol-rich platelet the potency of epinephrine to inhibit prostaglandin E_1–stimulated (PGE_1-stimulated) adenylate cyclase was significantly increased over the control platelet. Thus, these findings tend not to support the idea that *cis*-vaccinic acid increases epinephrine potency in turkey erythrocyte by increasing membrane fluidity (58–60).

It is true that in these two sets of findings two different types of receptor were involved, α- and β-adrenergic. However, one has to be cautious in interpreting the fluorescence measurement of microviscosity. As discussed by Shinitzky & Cooper (63), the measurement of microviscosity with DPH reflects only the average microviscosity of the membrane. A small, local variation of fluidity would be masked. Especially in cases when 85% of the total fatty acid added was being incorporated by the membrane (58, 60), the change in microviscosity might reflect the fluidity of the lipid domain rich in added fatty acid. Without available data on the kinetics of DPH partitioning among lipid domain, the actual alteration in the microviscosity surrounding the receptor is difficult to evaluate. Though DPH interacts with the hydrophobic region of the bilayer (61), the measured microviscosity could be that of the inner lipid bilayer which does not contain the receptor.

Besides being affected by the order of the lipid matrix or the microviscosity of the receptor environment, the adrenergic receptor mechanism appeared to be modulated by specific membrane lipid. In a series of experiments with the myocardium adenylate cyclase, Levey (67) and Rethy et al (68) reported restoration of the non-ionic detergent Lubrol-PX–solubilized adenylate cyclase sensitivity to norepinephrine by the lipid PI. Furthermore, there was lipid specificity effect. PI restored norepinephrine and PS restored glucagon and histamine sensitivity of the Lubrol-solubilized enzyme (67). Such lipid specificity is not observed with the adenylate cyclase on rat liver plasma membrane (68). When the plasma membrane was extracted with organic solvent, Rethy et al (68) reported a loss of NaF-, norepinephrine- and glucagon-stimulated activity. This loss of enzymatic activity was restored by the addition of PS but not with PI, PE, or PC. These findings could be explained by the indirect effect due to the stabilization of the enzyme receptor complex by lipid. Levey (67) and Birnbaumer (53) suggested that the phospholipid was directly involved through the interaction with specific sites on the cyclase system and hence the lipid specificity. However, this explanation could remain feasible only if the Lubrol-solubilized adenylate cyclase contained the receptor. Later studies by Lefkowitz (69) and Gilman (70) demonstrated that β-receptor could be resolved from adenylate cyclase after Lubrol PX extraction of the macromolecules from the membrane. Therefore, most likely the Lubrol-solubilized enzymes (67, 68) did not contain the receptor molecule. The reason the added lipid can restore the adenylate cyclase sensitivity to norepinephrine is still unclear.

The involvement of specific phospholipids in the coupling between β-adrenergic receptor and adenylate cyclase has been suggested by the recent works of Axelrod and co-workers (202–205). Using rat reticulocytes as a model, Axelrod and co-workers proposed that activation of the adenylate cyclase in the reticulocytes by β-adrenergic agonists is closely associated

with the methylation of the membrane PE to PC. Axelrod and co-workers (202, 203) demonstrated the presence of two *S*-adenosylmethionine-dependent (SAM-dependent) methyltransferases in the erythrocytes' membrane. These two enzymes are spatially oriented with the methyltransferase I, which converts PE to phosphatidylmonomethylethanolamine (PME), at the cytoplasmic side and with methyltransferase II, which converts PME to PC at the outer bilayer. Interestingly, Hirata et al (204) observed that the active isomers of β-agonists stimulated the methyltransferases in the intact reticulocytes. The magnitude of stimulation was dependent on the amount of β-receptor available. Direct activation of adenylate cyclase by agents such as guanine nucleotide (GTP, G_{ppNHP}) or NaF did not enhance the transferases' activity. Ability of phentolamine to inhibit the L-isoproterenol stimulation of the methyltransferases suggested that the conversion of PE to PC might be involved in the β-receptor activation of the adenylate cyclase. Potentiation of the β-agonist stimulation of adenylate cyclase by the addition of SAM implied that stimulation of the methyltransferases was required. Since the microviscosity of the membrane after the methylation of PE was decreased significantly (204), Hirata et al (204) suggested that the possible result of such methyltransferase stimulation by β-agonists was the fluidization of the microenvironment and thus facilitation of the lateral mobility of the β-receptor. Hence, the coupling between receptor and adenylate cyclase is enhanced.

The conversion of PE to PC stimulated β-agonist activity, and prolonged methylation of the reticulocytes' membrane produced an increase in the β-agonist binding (205). Strittmatter et al (205) reported an increase in the number of ^{3}H-dihydroalprenolol binding sites due to the unmasking of the cryptic β-adrenergic receptor by the methylation of PE to PC. There was no apparent alteration in the affinity of the receptor. Such an observation is in accord with the sensitivity of β-adrenergic binding to phospholipase C and D as reported by Limbird & Lefkowitz (48). Because under the reported experimental conditions de novo synthesis of new receptors is not possible, the appearance of new sites could be due to alteration in the membrane charges or microenvironment of the membrane surrounding the receptor. In either case, the involvement of specific phospholipids PC and PE in the β-adrenergic receptor action is implicated.

LIPID IN THE CHOLINERGIC RECEPTOR ACTION

The proteinaceous nature of the nicotinic receptor has been unequivocally demonstrated by the purification of the α-bungarotoxin-binding sites from the electroplax membrane of *Electrophorous electricus, Torpedo californica,* and *Torpedo mamorata* with affinity chromatography of the detergent-extracted membrane (71–75). These high affinity binding sites were shown

to be the acetylcholine receptor by successful reconstitution of a chemical excitable membrane by Changeux (76) and Raftery (77) as measured by $^{22}Na^+$ flux. Chemical analysis of this purified receptor revealed only protein and traces of carbohydrate, with no lipid phosphorous. Thus, on the basis of these data it appears that lipid has no role in the recognition of the nicotinic ligand. Sensitivity of the crude preparation toward phospholipases (78–80) might reflect proteolytic enzyme contamination in the phospholipase preparation or the detergent effect of the lipid hydrolysis products, as discussed in the previous section.

However, when the nicotinic binding site was labeled with ^{14}C-dimethyl-*d*-tubocurarine and the radioactivity was extracted from *Electrophorous electricus* or rat cerebral cortex with organic solvent (chloroform : methanol, 2 : 1) and chromatographed on a Sephadex LH-20 column, using either the radioactive ligand (81–83) or a cholinergic fluorescent probe (84), De Robertis and co-workers demonstrated high affinity binding of cholinergic agonists to a proteolipid fraction [K_d for acetylcholine, 10^{-7} and 10^{-5} M (81)]. This proteolipid was later extracted from the rat cerebral cortex at –60°C by Isumi & Freed (85, 86), indicating that it is loosely bound to the membrane. However, this proteolipid receptor for the nicotinic ligands appeared to have immunological properties different from those extracted with affinity chromatography (87). Antisera raised against the detergent-extracted receptor would not precipitate the organic solvent–extracted proteolipid receptor or vice versa (87). De Robertis and his co-workers (88–90) reported conductance change in an artificial membrane induced by nicotinic agonists and blocked by antagonists after the introduction of the proteolipid into the membrane. But, as in the case of their work with the adrenergic proteolipid (44, 45), the response to added agonists was long (~120 sec) and high concentrations of agonists were required to elicit a response (~10^{-3} M). Thus, whether this extracted proteolipid is similar to the nicotinic binding site as isolated with the detergent-affinity chromatography method is unclear.

Barrantes et al (87) reported the loss of α-bungarotoxin binding activity when the proteolipid was emulsified in 1% Triton X-100 solution or water, making it appear that the nicotinic binding activity is not associated with the protein moiety of the proteolipid extract. This suggests that the binding activity was associated with one or all of the lipid found in this fraction: PC, PE, PS, and PI (81). Wu et al (91) found that the proteolipid extracted by De Robertis and his co-workers contained a large quantity of triphosphoinositol (TPI) and the binding behavior of the proteolipid on the LH-20 column could be reproduced using the lipid TPI. Further evidence for interaction between TPI and nicotinic agonists was presented by Cho et al (92), who reported that cholinergic ligands modulated TPI partitioning between aqueous phase (2 mM Tris buffer, pH 7.4) and organic phase

(heptane). The ability of various cholinergic ligands to induce ^{3}H-TPI transfer from aqueous phase to the organic phase suggested an interaction between the ligand and the lipid and subsequent alteration of the physical properties of the lipid. More important, there was an excellent correlation between the ED_{50} of the various cholinergic ligands to induce the TPI transfer, the in vivo potency of these ligands to produce neuromuscular blockade, and the in vitro ligand affinity for the nicotinic receptor (92). Thus, these studies suggest that even though lipid is not the recognition site as isolated with the detergent extraction method (71–75), the lipid TPI is able to bind cholinergic ligands in receptor-like fashion.

The involvement of lipid in the cellular recognition of muscarinic agonist is ambiguous. The proteinaceous nature of the receptor was implicated by the sensitivity of the ^{3}H-3-quinuclidinyl benzilate (QNB), a muscarinic antagonist, binding to sulfhydryl reagent (93, 94). However, treatment of the rat brain membrane with phospholipase A or C inhibited the ^{3}H-QNB binding (95). Addition of PS or dipalmitoyl phosphatidic acid (PA) partially restored the ^{3}H-QNB binding (95). But when the receptor was solubilized with high salt, the inhibitory action of phospholipase C disappeared (96). Hence, it appears that the inhibitory effect of phospholipase A could be due to the surface action of the degradation product (50). This is supported by the observation that both fatty acid and lysophosphatides inhibited ^{3}H-QNB binding to rat brain membrane (95).

Does lipid have a role in the action of cholinergic receptor as in the case of adrenergic receptor? The existing data can only suggest a possible involvement. Schiebler & Hucho (97) suggested that the reproducibility of experiments involving the reconstituted excitable membrane with the *Torpedo californica* nicotinic receptor could be greatly enhanced if the tightly bound lipids were allowed to remain associated with the receptor. This could result from the reported stabilization effect of phospholipids on the ion channel associated with the receptor (98). Stabilization of the ion channels could also explain the restoration of acetylcholine-induced contractility in the rectus abdominus muscle of *Bufo viridis* by PE or PS after 25% acetone extraction (99). However, in this study (99), with no data on cholinergic receptor binding, the lipid effect on the receptor-ligand interaction could not be ruled out.

Unlike the lipid effect on the mobility of the adrenergic receptor, the studies by Axelrod et al (100) did not indicate a dependency of the cholinergic receptor mobility on membrane fluidity. By using a fluorescent lipid membrane probe, dioctadecylindocarbocyanine (DiI), and a fluorescent α-bungarotoxin, tetramethylrodamine-α-bungarotoxin (TMR-α-Bgt), and the fluorescent photobleaching recovery method, Axelrod et al (100) were able to measure the lateral mobility of the lipid, DiI, and the cholinergic receptor, TMR-α-Bgt, in the cultured chick embryo myotube membrane at

different temperatures and at various alterations of the membrane fatty acid composition. They observed no effect on the rate of lateral mobility of either DiI or TMR-α-Bgt after substantial alteration of membrane fatty acid. Since the fluorescent probe DiI has been observed to label uniformly planar membrane without segregation from any lipid domain (101, 102), the constancy of lipid and cholinergic receptor mobility upon changes in the lipid acyl side chains suggests that the mobility of the receptor is regulated by a mechanism other than changes in the fluidity of the surrounding lipids (100).

LIPID INVOLVEMENT IN HORMONE RECEPTOR ACTION

The involvement of lipid in the cellular recognition of peptide hormones is best illustrated by the role of ganglioside in the binding of thyrotropin (TSH), human chorionic gonadotropin (hCG), and luteinizing hormone (LH) to their respective target organ plasma membrane. Since there are sequence analogies between these peptide hormones and the B subunit of the cholera toxin (103, 104), it is tempting to believe that these hormones might have affinities for the ganglioside G_{M1}—the putative cellular receptor for cholera toxin, as discussed in a previous section—and this has been demonstrated to be the case. TSH binding to the thyroid membrane was shown to be inhibited by the ganglioside added to the incubation mixture (105). There was a structural relationship in the TSH inhibition by ganglioside with G_{D1b} (galactosyl-*N*-acetylgalactosaminyl-*N*-acetyl-neuraminyl-*N*-acetylneuraminyl-galactosylglucosyl ceramide) > G_{T1} (*N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-*N*-acetylneuraminyl-galactosylglucosyl ceramide) > G_{M1} > $G_{M2} = G_{M3}$ (*N*-acetylneuraminylgalactosylglucosyl ceramide) > G_{D1a} (*N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-gactosylglucosyl ceramide). Moreover, the inhibition was due to TSH-ganglioside interaction rather than the interaction of ganglioside with the membrane TSH receptor since pretreatment of the thyroid membrane did not produce inhibition (105). Ganglioside inhibition of hCG and LH binding to testis was also observed (106, 107). The potencies of various gangliosides to compete for the hCG and LH were somewhat different from their potencies to compete for the TSH binding. For hCG, the order was $GI_{T1} > G_{D1a} > G_{D1b} > G_{M2} > G_{M1}$ (106) and for LH it was $G_{T1} > G_{D1b} > G_{D1a} > G_{M1} > G_{M2}$ (107). Nevertheless, in all three cases, acid residue gangliosides are more potent in their interaction with the peptide hormone than the monosialic acid ganglioside such as G_{M1}.

The peptide interaction with ganglioside was further demonstrated by measuring peptide fluorescence intensity alteration after binding to ganglio-

side (105–107). Similar to the observations with cholera toxin (23, 30), there was a positive correlation between the concentration of ganglioside in the membrane and TSH binding (108). Rat thyroid tumor (1-8R) membrane, because of its lack of *N*-acetylgalactosaminyl transferase and hence its inability to synthesize complex gangliosides, binds only 20% of the amount of the TSH bound to normal thyroid membrane (108).

Apparently, however, ganglioside is not the sole component of the receptor for these peptide hormones, as it is with cholera toxin. When the TSH receptor from thyroid membrane was solubilized with lithium dicodosalicylate (109–111), the binding components, after trypsinization, had a molecular weight of 15,000 to 30,000, which is considerably larger than GT_1 = 2057. But this solubilized TSH receptor can be labeled with ^{14}C-glucosamine (111) and its binding is still sensitive toward neuramidase (109). Thus, the TSH receptor could be a glycoprotein. Preliminary measurements indicated that the receptor consists of 30% carbohydrate and 10% sialic acid (110). Glycoprotein properties of the receptor were further substantiated by no apparent enrichment of a specific ganglioside in the supernatant of the solubilized receptor, though the supernatant contained 80% of the total TSH binding (111). Possibly the lipid could regulate the physical properties of the TSH receptor, for a decrease in the Na^+ inhibitory effect on TSH binding was observed after the glycoprotein was removed from the membrane environment (111).

The effect of the membrane lipid environment on hormone receptor binding was further demonstrated by experiments in which the membrane lipids were altered by phospholipases (112–114) or in which the glycoprotein receptor was incorporated into defined artificial membrane (115–118). Phospholipase C inhibited the ^{3}H-TSH binding by decreasing the affinity of the TSH receptor (112). Phospholipase A_1 and A_2 and C, but not phospholipase D, inhibited the gonadotropin binding to bovine corpus luteum (113, 114). Though there was no correlation between the amount of phospholipid hydrolyzed and the degree in the inhibition of gonadotropin binding (114), the phospholipase effect resulted from the decrease in number of binding sites rather than from a change in affinity of the receptor (113). Since the putative receptor has been identified as a glycoprotein (109–111) and the phospholipases have no effect on the solubilized receptor (113), the observed decrease in number of sites after phospholipase treatment could be due to either (*a*) the extraction of receptor by the hydrolysis products or (*b*) the detergent-like effect of the lysophosphatide, and not to the phospholipid's being a component of the receptor. Experiments with lysophosphatide did not indicate extraction of the receptor (113). Restoration of gonadotropin binding after phospholipase A treatment was accomplished by washing the membrane with defatted BSA so as to remove the lysophosphatides (114). Thus, these experiments indicated that the

lipid surrounding the receptor has a vital role in the ligand receptor interaction.

The importance of the lipid environment on the glycoprotein receptor interaction with TSH, hCG, and LH is again illustrated by the properties of TSH binding during altered membrane fluidity (115) and in artificial liposomes (116–118). TSH binding to human thyroid membrane was shown to depend on temperature with a sharp break at 30°C (115). Membrane fluidity measurement with fluorescent probe or the alteration of fluidity with Ca^{+2} or Mg^{+2} indicated that the increase in number of binding sites for TSH in the thyroid membrane correlated with the increase in membrane fluidity (115). Thus, though the TSH receptor is a glycoprotein, the phospholipids in the membrane have a role in the expression of the receptor action. This role of phospholipid is better exemplified by the properties of the TSH receptor in liposomes (116–118). It was demonstrated that the phospholipids and cholesterol liposomes containing gangliosides have a K_{diss} for TSH [5×20^{-9} M (118)] that is similar to that of thyroid membrane [2.5×10^{-8} M (109, 110)]. Thus, the TSH receptor in liposomes will retain its binding properties. When the TSH receptor was extracted with Triton X-100 and incorporated into vesicles of either dipalmitoyl lecithin (DPL) or dismyristoyl lecithin (DML), the temperature-dependent TSH binding reflected the transition temperature of the lipids, 24°C for DPL vesicles and 42°C for DML vesicles (117). However, when the TSH receptor was incorporated into mixed DML and DPL vesicles, the transition temperature for TSH binding was 24°C, the melting temperature of the mixed vesicles, rather than 28°C (117). This is due to separation of the DPL and DML into lipid domains (9) and segregation of TSH into the fluid lipid state—DPL. Furthermore, the TSH glycoprotein receptor in dioleoyl lecithin has better binding capacity than the TSH receptor in the dipalmitoyl lecithin vesicles (118). Thus, these data clearly indicate that there is a direct interaction between the glycoprotein receptor for TSH and the membrane phospholipids.

Modification of receptor binding by the membrane lipids can be observed with the insulin receptor also. Dietary changes could induce a decrease in insulin binding (119–123). Rats fed with a high fat diet have a lower number of insulin binding sites in the liver plasma membrane than rats fed with a glucose diet (123). Treatment of the fat cells with either phospholipase A or C increased the number of insulin binding sites with no change in the affinity in the membrane (124). Polyene antibiotics (nystatin, amphotericin B) that do not disrupt phospholipid structure did not enhance insulin binding whereas filipin, which does disrupt phospholipid structure, did enhance binding (124). Thus, the alteration of phospholipid content in membrane either by dietary method (119–123) or biochemical treatment (124) can result in the modification of insulin binding. In view of the

interaction of the insulin receptor with a nonreceptor glycoprotein molecule in the membrane (125), the degree of the fluidity of the lipid might affect insulin binding by regulating interaction of insulin receptors with other membrane proteins.

The involvement of lipids in other hormone receptor binding is implicated by the sensitivity of binding toward phospholipase treatment. Thyrotropin releasing hormone (TRH) binding to the plasma membrane of the bovine anteria pituitary gland was inhibited by phospholipase A, C, and digitoxin (126). This inhibition was due to a decrease in ^{3}H-TRH receptor affinity and not in the number of binding sites (126). Surprisingly, instead of increasing binding, the phospholipids, PC, PE, PI, and PS, with a concentration of 1 mg/ml in the assay mixtures, all decreased the number of ^{3}H-TRH binding sites without any effect on K_d (126). It appears, then, as in the case of TSH, hCG, LH, and insulin, that the phospholipids can regulate the receptor ligand interaction. Binding of the hormone glucagon to the liver plasma membrane can also be inhibited with phospholipid removal (127, 128). Treatment of the liver plasma membrane with phospholipase A (127, 128) or phospholipase C (128) inhibited the ^{125}I-glucagon binding to the membrane. The phospholipase A effect was partially reversible by the addition of phospholipids, with PS having the greatest effect (127). Since the phospholipase A hydrolysis product, lysophosphatide, is not easily released from the membrane (49), replacement of the hydrolyzed phospholipid with exogenous lipid is rather difficult (49). Without any determination of the amount of lipid incorporated, the actual role of lipid in glucagon binding cannot be surmised. But it is clear that phospholipase C does decrease the affinity of the glucagon receptor and not the number of binding sites (128). Furthermore, the phospholipase C–treated receptor has the same apparent affinity as the nontreated receptor for des-His-glucagon, a competitive antagonist (128), and phospholipase C has no effect on des-His-glucagon binding (128). Hence, the phospholipid polar head groups may interact with the histidine moiety of the glucagon (128).

As discussed in the adrenergic receptor section of this article, the present working hypothesis for the hormone receptor–adenylate cyclase interaction is the floating receptor mechanism (52). Mobility of the hormone receptor is illustrated by capping of the uniformly distributed receptor after the addition of ligand as measured by fluorescent hormones (129) or the fluorescent photobleaching recovery technique (130). In either case, the lateral diffusion rate of the hormone receptor complexes is greatly dependent on the temperature of incubation which is a reflection of the membrane order. Hence, the alteration of the lipid environment should have a pronounced effect on the hormone-stimulated adenylate cyclase. Hydrolysis of the phospholipids in the thyroid and liver plasma membrane with phospholipase A or C inhibited the TSH- (131) and glucagon- (127, 128) stimulated adeny-

late cyclase activity, respectively, with no apparent effect on the basal or fluoride-stimulated enzymatic activity. These phospholipase effects could result from destruction of the hormone binding sites. However, the TSH receptor, being a glycoprotein, is not sensitive to phospholipases. Glucagon affinity for the binding sites was attenuated by phospholipase A or C (127, 128). Hence, in these two hormones' receptors the binding sites remained after phospholipase treatment. These data suggest the lipid might have a part in coupling of the receptor hormone-complex with adenylate cyclase. There appears to be lipid specificity. When liver plasma membrane was treated with phospholipase C isolated from *Bacillus cereus,* which preferentially removes the polar head groups of PS and PI but not sphingomyelin, the glucagon-stimulated effect on the cyclase was abolished (128). Whereas, when phospholipase C from *Clostridium perfringens* was used, which preferentially hydrolyzed neutral phospholipids PC, PE, and sphingomyelin, only partial loss of glucagon activity was observed even after removal of 60% of the total phospholipids (128). Furthermore, restoration of the glucagon sensitivity in the phospholipase A–treated (127) or Lubrol-solubilized (67, 132, 133) adenylate cyclase could be accomplished by addition of PS. Addition of PC to phospholipase A–treated or Lubrol PX–treated enzyme could also restore adenylate cyclase response to TSH (131). However, as in the case of adrenergic receptor action, hormone receptor and adenylate cyclase being two distinct molecules (69, 70), the exact role of the phospholipid in restoring hormone activation in the solubilized adenylate cyclase is unclear.

Some data suggest that the lipid environment adjacent to the receptor, the microviscosity of the lipid annulus, might influence the hormone receptor action. By fusing the liver membrane with synthetic PC vesicles, Houslay et al (134) altered the lipid composition of membrane and observed a change in glucagon-stimulated adenylate cyclase activity. Arrhenius plots of the NaF or the guanylyl nucleotide–stimulated (G_{ppNHP}-stimulated) activity were not modulated by lipid substitution (134). In contrast, the break at 28.5°C in the Arrhenius plot of the glucagon-stimulated cyclase in the control membrane was shifted upward by dipalmitoyl PC, downward by dimyristoyl PC, and abolished by dioleoyl PC (134). The authors attributed these observations to lipid phase separation and concluded that the hormone-stimulated enzyme was sensitive to the lipid environment or the fluidity of the membrane. Houslay further substantiated this point by altering the fluidity of the annulus lipid with benzyl alcohol (135) which increased both fluoride- and glucagon-stimulated adenylate cyclase activity (135). Lowering of the breakpoint of the Arrhenius plot of the glucagon-stimulated cyclase was also observed (135). The benzyl alcohol effect was abolished in the Lubrol PX–solubilized enzyme (135). Hence, membrane fluidity as the explanation for these observations is a logical one. However,

when a lipid undergoes a phase transition, in addition to fluidity other physical parameters also change, such as lipid packing density and degree or order, and degree of hydration at the lipid interface, with the fluid-like phase more hydrated than the solid-like phase (136–138). Hence, modification of the breakpoints in the Arrhenius plot by exogenous lipid (134) or benzyl alcohol (135) might involve factors in addition to membrane fluidity.

This point was illustrated by the work of Engelhard et al (139, 140). By supplementing the growth medium of the mouse LM cells with polar head groups and/or fatty acids with different degrees of unsaturation, Engelhard et al (139, 140) were able to increase the levels of basal, NaF-, and PGE-stimulated adenylate cyclase as well as to alter the temperature-dependent properties of the enzyme. The modification of the enzymatic activity was not observed with detergent-solubilized enzyme (139), thus suggesting adenylate cyclase sensitivity to its lipid environment. Although there is a correlation between the increase in the PGE_1-stimulated cyclase activity and the average degree of unsaturation of the phospholipid fatty acids, or with the degree of alkylation in the choline polar head group (140), there is no correlation between the altered enzymatic activity and the microviscosities of the membrane as measured with fluorescent probe (139, 140). Supplementation with polar head groups and fatty acids simultaneously produced the maximal increase (139, 140). Hence, in the mouse LM cells, the PGE_1-stimulated adenylate cyclase depends on both the lipid polar head groups and the fluidity. Therefore, not only the lipid environment (fluidity) but also the degree of the lipid order (affected by polar head groups) is important in the hormone receptor mechanism.

The important role that membrane fluidity plays in the hormonal regulation of adenylate cyclase activity could be discounted by the above studies. The PGE_1-stimulated adenylate cyclase in cholesterol-rich platelet was attenuated when compared with normal platelet (66). Since cholesterol increased the order in the membrane bilayer and thus reduced the membrane fluidity (63, 65), the decrease in the enzymatic response to the hormone PGE_1 could result from attenuation in the lateral diffusion of PGE_1-receptor complex. However, the attenuation of the NaF-stimulated adenylate cyclase in the cholesterol-rich platelet implied a possible alteration in the enzyme conformation. Thus, other possible roles in the alteration of the membrane fluidity and hormone receptor action are suggested.

ACIDIC LIPIDS AND OPIATE RECEPTOR MECHANISM

The involvement of phospholipid in the stereospecific binding of opiate ligands to the brain membrane has been implicated by receptor sensitivity to phospholipases (141–144). In general, opiate binding to the membrane

can be diminished by prior treatment of the membrane with phospholipase A_2 and not at all or only slightly with phospholipase C or D (141, 142). It has been surmised that the inhibitory effect of phospholipase A_2 is the detergent-like effect of the lysophosphatide products on the membrane, since the addition of 1% defatted BSA to the binding mixture can restore binding capacity (143). However, the addition of exogenous lysophosphatides does not inhibit binding (143) and not all defatted albumin used demonstrates binding reversibility (143). Thus, it is possible that the reversal effect of BSA in this case is the restoration of membrane lipid order.

Very recent work in our laboratory has provided direct evidence for a role of lipids in opiate binding. When brain membranes were incubated with one of several different radioactive opiates and then extracted with the non-ionic detergent Brij 36J (191), a number of binding components were observed (192). Several of these components were of very low molecular weight, and analysis of the latter by thin-layer chromatography revealed a large assortment of lipids, including CS and PS (A. Smith, unpublished data). Since bound ligand was removed during thin-layer chromatography, it could not be directly shown that opiate was associated with any of these lipids, but since protein could not be detected in any of these low molecular weight fractions, direct interaction of opiate ligands with one or more lipids is inferred.

To determine the binding of drugs to lipids, there appears to be some interaction between opiate and the phospholipids. Abood and co-workers (144–146) demonstrated stereospecific interaction between opiates and phosphatidylserine (PS). Exposing the brain membrane to PS resulted in an enhancement of opiate binding to the brain membrane (136) and an interaction with the lipid in an organic-aqueous phase partitioning system (145). There appears to be a correlation between the fatty acids of PS and PE released and inhibition of opiate binding by phospholipase A_2 (144). Furthermore, restoration of opiate binding by phospholipids after phospholipase A treatment was also reported (144). Thus, these data suggest a specific requirement of PS in the receptor vicinity for opiate stereospecific binding to the brain membrane. However, in their studies, Abood and co-workers did not report data on the quantity of PS incorporated into the membrane (144, 146). With only a slight increase in binding (30%) (144), there is a possibility that the phospholipids could have removed some endogenous inhibitory ligands. Though phospholipase D did not inhibit opiate binding, PS decarboxylase, which converts PS to PE, inhibited binding (146). Moreover, with no data on the correlation of the relative affinity of various opiate ligands to PS in the partitioning studies (145) and the in vivo potency of the ligands, the physiological significance of the opiate-PS interaction remains to be determined.

Opiate was observed to interact with another type of acidic lipid, cerebroside sulfate (CS). In a structure-activity analysis, Loh et al (148, 149) found that among the membrane acidic lipids examined, a postulated conformation of CS not only fulfilled the requirements of the opiate receptor proposed by Beckett & Casy (147), but also possessed the essential key binding determinants predicted from structure-activity relationships (149). Moreover, this conformation of CS served to explain most of the structure-activity relationships in various structurally unrelated opiates such as the derivatives of oripavine, morphine, oxymorphone, morphinan, benzomorphan, 4-phenylpiperidine, methadone, and fentanyl.

We then determined CS-opiate interaction experimentally by demonstrating that opiates bind saturably and stereospecifically to CS with high affinity in an organic-aqueous solvent partitioning system (148–150). The relative affinities of various opiates for CS correlated remarkably well with their in vivo potencies (149), distinguishing the CS-opiate interaction from the nonspecific high affinity interaction of opiate ligands with the glass fiber membrane (20). Furthermore, the CS-opiate interaction could also distinguish the opiate agonist and antagonist (150, 151); the ability of various agonists to induce ^{3}H-CS transfer from aqueous to organic phase was distinctly different from that of the antagonist (150) in that the former were much more effective than the latter. The ^{3}H-CS transfer induced by agonists was inhibited by antagonists at concentrations that do not induce ^{3}H-CS transfer by itself. This indicated that CS-agonist and CS-antagonist complexes have different physical properties. More recent studies have shown that the former complex is more hydrophobic, while the latter is more hydrophilic in nature. Similar opiate interaction with CS could be demonstrated in an aqueous environment (to 50 mM Tris buffer pH 7.4) and the interaction of agonists with CS in aqueous buffer was demonstrated to be Na^+ dependent (151), as in the case of opiate agonist binding to brain membrane (152, 153). Thus, the acidic lipid CS opiate interaction possesses many properties of the opiate-receptor interaction.

Because of this remarkable resemblance of CS-opiate interaction to opiate binding to the brain receptor, the possibility of CS being involved as the cellular recognition site for opiate was investigated in our laboratory. When the chemical nature of a partially purified opiate receptor reported by Lowney et al (154) was analyzed, the major component in this proteolipid was found to be CS (148). The Sephadex LH-20 elution profile of the proteolipid was reproduced by chromatographing the acidic lipid CS (148).[1] This again suggested that some CS molecules in the brain could participate

[1]Our published data have proven conclusively that CS is virtually identical with this partially purified opiate receptor.

in the stereospecific interaction of the opiate. Inhibition of opiate binding to the synaptic membrane by the cationic dye, Azure A (155), which has high affinity for CS (156), and by treatment of the membrane with the sulfatide degradative enzyme, cerebroside sulfatase (E.C.3.1.6.8) (157), also support a possible involvement of CS in the opiate receptor binding.

Since there is no correlation between the CS content and opiate receptor distribution in the brain, the involvement of every molecule of CS in opiate receptor binding is unlikely. In the cerebroside sulfatase studies (157), 50% of the specific binding of ^{3}H-naloxone was inhibited while only 2% of the CS present was hydrolyzed. In at least some tissues, such as myelin, where the highest concentration of CS in the brain is located and opiate receptor binding is absent, the CS molecule is known to bind very tightly to A_1 basic protein in myelin. These observations could probably explain why one fails to observe a correlation between CS levels in various cell lines (158) or the developing chick (159) and opiate receptor density. In fact, opiate can bind to myelin CS after the basic proteins are removed, and myelin A_1 basic proteins are also known to inhibit ^{3}H-levorphanol binding to CS (T. M. Cho, personal communication).

The increasing evidence for multiple opiate receptors, as well as the known heterogeneity in chemical nature of opiate receptors, also argues against a strict correlation between CS levels and opiate receptor distribution. In light of the work of Law et al (190), who showed that the membrane binding sites of ^{3}H-dihydromorphine, ^{3}H-enkephalin, and ^{3}H-naloxone are not identical, Cho has observed that the binding of ^{3}H-dihydromorphine to CS can be inhibited by morphine and β-endorphin but not by enkephalin. These data indicate that the CS cannot be involved in the binding of all opioid ligands. Whether or not this acidic lipid CS is indeed an opiate recognition site in biological membrane cannot be fully established at present, since in the studies with both Azure A (155) and cerebroside sulfatase (157) the possibility of destruction or extraction of membrane receptor protein could not be ruled out. Furthermore, our preliminary experiments indicate that incorporation of CS by a neuroblastoma cell line, N18TG2, which possesses opiate binding sites (160) did not increase the number of receptor sites but did alter the affinity of the receptor. This seems to contradict our previous findings that treatment with both Azure A and cerebroside sulfatase reduces the number of opiate binding sites in synaptic plasma membrane. One explanation of this discrepancy lies in the use of the Langmuir absorption isotherm which, in fact, does not fit with the opiate binding to the membrane. Binding of a charged molecule-like opiate to the negatively charged membrane obeys Stern type of isotherm (200) rather than Langmuir adsorption isotherm, and binding of the Stern type exhibits a negative cooperativity as a result of the decrease of the negative surface

potential with increasing concentration of the ligand (200, 201). If this is the case, the number of binding sites cannot be determined by Scatchard plot. Incorporation of CS into the membrane should increase the affinity of opiate because the negatively charged CS should increase the negative potential of the membrane. Thus, Scatchard plots may not be a useful tool to analyze the binding of opiate.

Some preliminary studies in our laboratory have suggested that sulfatide could also be involved in the expression of opiate action other than being a possible component of the recognition sites. In both Azure A–treated mice and Jimpy mice in which the sulfatide level is significantly lower than in littermate control mice (161, 162), the analgesic potency of morphine was decreased relative to that of control (155). Injection of an antiserum specific for sulfatide in the periaqueductal gray area, the center for opiate analgetic action, antagonized morphine actions (163). These data suggest that the subsequent cellular event after the opiate-receptor interaction as measured by this action could be modulated by the availability of the CS molecule in the vicinity of the opiate receptor.

Since the interpretation of studies carried out with intact animals is rather difficult, the effect of CS on opiate action in in vitro cell cultures is also being studied in our laboratory. The presence of opiate receptors in the neuroblastoma X glioma NG108–15 cell lines and the inhibition by opiates of basal and PGE_1-stimulated adenylate cyclase are well documented (164–167). But in the parent cell line, neuroblastoma N18TG2, which contains opiate binding sites (160), opiate agonists, and opiate peptides, have little or no effect on the adenylate cyclase activity (160, 166). After the incorporation of the acidic lipid CS by N18TG2, the opiate ligands inhibited the adenylate cyclase activity in the parent cells with a potency similar to that found in the daughter cells, NG108–15 (168, 169). This lipid potentiation effect could not be observed in cell lines which do not possess opiate receptor (169). These results suggest that the addition of the lipid to the parent plasma membrane promoted coupling between receptors and adenylate cyclase by changing either the lipid composition of the membrane and thus the packing order of the membrane lipid or the membrane fluidity. Further experiments are presently being undertaken in an attempt to discriminate between these possibilities.

LIPIDS IN OTHER MEMBRANE RECEPTOR ACTION

The involvement of membrane ganglioside in serotonin receptor action was initially implicated by the work of Woolley & Gommi (170, 171). Rat stomach tissue treated with neuramidase and EDTA was rendered insensitive to serotonin (5-HT), and sensitivity was restored by exposing the

treated tissue to ganglioside (170). Moreover, rats reared from infancy on a high galactose diet which inhibited synthesis of galactoprotein (172) had a reduced concentration of 5-HT receptor in the stomach and also exhibited attenuated response to 5-HT (171). These data suggest the possible role of ganglioside in the cellular recognition of 5-HT. Although Fiszer & De Robertis (173) and Goodwin & Sneddon (174) were successful in extracting a proteolipid which bound 5-HT, whether the proteolipid is the glycoprotein for 5-HT receptor is unclear. Moreover, the chemical analysis of the Sephadex LH-20 fractions of Goodwin & Sneddon (174) revealed CS, PI, and di-phosphoinositol (DPI) (175). The chromatographic pattern of the CS, PI, ^{3}H-5-HT mixtures resembled that of putative "proteolipid" 5-HT receptor (175), and saturable high affinity binding of 5-HT to the lipids PS ($K_d = 0.4$ μM), PA ($K_d = 1.9$ μM), and TPI ($K_d = 10$ μM) was observed in isobutanol (176). Thus, it is possible that acidic lipids rather than ganglioside could be involved in 5-HT binding. However, the binding affinities of 5-HT to these acidic lipids in aqueous medium are significantly lower than they are in organic solvent, with K_d's in the former ranging from 9.4 μM for DPI to 1250 μM for CS (177).

Interaction of 5-HT with purified ganglioside could be demonstrated with equilibrium dialysis (178, 179) in the broadening of the nuclear magnetic resonance (NMR) signal of 5-HT by ganglioside (180). Though these observations were specific for ganglioside and showed no broadening of the NMR signals with other membrane lipids (180), the concentration of 5-HT used (10^{-3} M) was too high to evaluate the physiological significance of the studies. Ochoa & Bangham (181) reported 5-HT affinities for ganglioside to be 3 to 5 mM. Recent studies with the antipsychotic drugs, chlorpromazine and chlorimipramine, which have high affinities for the dopamine receptor in the brain (182), likewise indicated an interaction with the ganglioside (183–185). However, the low K_d values obtained with equilibrium dialysis (185), K_d 10^{-4} M, or the use of a high concentration of drugs to produce a change in fluorescence (2×10^{-5} M) (184) suggested that the observed interaction between drugs and ganglioside could be a nonspecific interaction.

Data on the interaction between other ligands and lipids are limited. A study by Lembeck et al (186) suggested that Substance P, a putative peptide neurotransmitter, could interact with the phospholipid PS. Probably the lipid binding properties of Substance P is not related to the specific receptor for the peptide since the capacity of the total brain lipid extract (TLE) to bind Substance P is extraordinarily high (3 μg/ml of TLE) (186). Lembeck et al (186) suggest that this observation could reflect the brain storage mechanism for Substance P.

Lipid involvement in other receptors has been implicated by the phospholipase studies. Phospholipase A_2 decreased dopamine receptor binding

(J. Wu, personal communication) and inhibited the γ-aminobutyric acid (GABA) binding to rat cerebellum (187, 188). Because treatment with lysolecithin and the ionic detergent, sodium dodecyl sulfate, also inhibited GABA binding (187, 188), the phospholipase A_2 effect could be due to the surface detergent action of the hydrolysis product. However, it is of interest to note that GABA binding is enhanced by phospholipase C treatment (187, 188). With 70% of the polar head group hydrolyzed, the GABA binding was increased 260% (187, 188). Thus, the polar head groups of lipid may be competing with GABA for receptor as postulated by Watkins (189). PE was also shown to possess an inhibitory effect (188). Thus, besides affecting the fluidity or lipid order of the membrane, the phospholipid also appears to interact competitively with the cationic sites of the receptor.

CONCLUSION

This article reviews the existing data on the role of membrane lipids in receptor mechanisms. Our assumption that lipids serve as the *recognition sites* for various ligands was based on experiments with direct drug-lipid interaction or with alterations in membrane lipid content. However, because of the lack of correlation between ligand affinity for the lipid and the in vivo potency of the ligand, low ligand affinity for the lipid, or other reasons, the lipid molecule has not been established as the recognition site. Even in the case with cholera toxin, TSH, LH, hCG, where gangliosides exhibit high affinity for these ligands and compete with membrane-bound receptor for the ligands, the lipid per se could not be unequivocally demonstrated to be the binding sites. As in the case of opiate receptors, many indirect studies indicate the involvement of CS in opiate receptor mechanisms. Furthermore, direct binding studies indicate that CS fulfills many criteria as opiate receptor associated with ligand interactions. However, because of the lack of correlation between the distribution of CS and opiate receptor density, the exact role of CS in opiate receptor mechanism remains an enigma.

Since lipid-protein in combination could constitute the receptor (or the recognition site), as in the case of the glycoprotein receptor for TSH, it is possible that the lipid environment surrounding the receptor could *regulate receptor binding activity.* This is clearly demonstrated by the modification of TSH binding with alterations in membrane fluidity and by alterations in membrane hydrophobicity with phospholipase A. Also, modifying polar head groups with phospholipase C can usually result in receptor binding attenuation. However, in all the studies, because of the inconsistency in the mode of binding attenuation—either by decrease in affinity or in the number of binding sites—a unified mechanism for the lipid modification of receptor could not be proposed. Probably, as with the lipid effect on mitochondrial

enzymes, the membrane lipids affect the conformation of the receptor protein molecule and thus the ligand affinity for the receptor.

Besides being the possible regulator for ligand affinity for the receptor, lipid can also *modulate receptor function* at the subsequent molecular event after receptor-ligand interaction. In the floating hormone receptor model, one can easily surmise that the possible role of lipid in this receptor model might be at the coupling of the receptor with the effector molecule, such as adenylate cyclase. Thus, it is not surprising to observe correlations between membrane fluidity, diffusion coefficient of the receptor, and the adrenergic or various hormonal receptor potency on the adenylate cyclase activity. However, as discussed earlier, caution is needed in interpreting these data. Values obtained by measurements of microviscosity with fluorescent probe represents only the average fluidity of the membrane. The physiological significance of the receptor clustering phenomenon, which is a reflection of receptor mobility, is not well understood. Hence, the hypothesis that receptor mobility predetermines the cellular response to the ligand can only be inferred from existing data. In some cases, such as the acetylcholine receptor, mobility of the receptor was independent of the microviscosity. Therefore, the lipid bilayer fluidity cannot be the only determinant in regulating receptor action. Lipid could be the specific coupler for specific receptor and adenylate cyclase, as suggested by Levey and Birnbaumer. Lipid could induce the receptor-ligand complex and the effector molecule such as adenylate cyclase into favorable conformation for coupling. Thus, other than fluidity, physical parameters such as hydration or packing order of the lipids require future consideration in the action of receptor.

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