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Interaction of Diazosulfanilic Acid and the Opiate Receptor

Inhibition of Specific Binding to Synaptic Membranes and Labeling of a Membrane Lipid Stereospecifically Protected by Opioids

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SUMMARY

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3,5-Diiodo-4-diazosulfanilic acid (DIDSA) inhibited the opiate specific binding to the rat brain synaptic membrane. DIDSA inhibited [^{3}H]dihydromorphine binding more rapidly and to a greater extent than [^{3}H]naloxone binding. That the inhibition of the opiate binding resulted from the derivatization of the opiate receptor sites by DIDSA was demonstrated by Scatchard analysis of the binding data and by the ability of 0.1 μM concentrations of various opiate ligands and not 0.1 μM dextrorphan to attenuate the DIDSA inhibition. DIDSA was probably reacting with a population of sites distinct from those which reacted with *N*-ethylmaleimide (NEM). This was demonstrated by the ability of 100 mM Na^+ to decrease NEM inhibition of [^{3}H]naloxone binding and not that of DIDSA inhibition. The difference in NEM and DIDSA reactive sites was further demonstrated by the ability of hypoosmotic lysis of synaptosomes to enhance DIDSA but not NEM inhibition of naloxone binding. When synaptic membranes were derivatized with a combination of 0.4 mM DIDSA and 0.1 mM NEM in the presence of levorphanol and subsequently labeled with [^{125}I]diazosulfanilic acid (DISA), there was a levorphanol concentration-dependent increase in radioactivity incorporated into the chloroform:methanol (2:1) extractable membrane lipids previously protected from the reagents with levorphanol but not those lipids protected with dextrorphan. There was a parallel increase in the amount of opiate receptor binding activity protected by the levorphanol. Moreover, the 2-fold increase in the radiolabeling of the protected membrane polar lipids could be eliminated by the addition of 0.1 μM levorphanol but not by the addition of dextrorphan in the reaction mixtures containing [^{125}I]DISA. The identity of the polar lipid selectively protected by levorphanol was determined by thin-layer chromatography in silica gel G plates in three different solvent mixtures. The chromatographic patterns of the membrane polar lipids extract in these solvent mixtures compared favorably with those of the standard phosphatidylethanolamine. Separation of membranous protein with Sephadex column chromatography and polyacrylamide gel isoelectric focusing did not resolve any proteins which were significantly protected by levorphanol.

INTRODUCTION

Although the existence of binding sites in the neural tissues stereospecific for opiates has been demonstrated (1–3), the exact chemical identity of the putative receptor has not been fully elucidated. Its proteinaceous nature is inferred from the reduction in stereospecific opiate binding that occurs following reaction with proteolytic enzymes (1, 4) and alkylating agents, especially sulfhydryl reagents (5). However, there is also strong circumstantial evidence involving membrane acidic lipids in the specific interaction between opiates and their receptor. For example, opiate binding to the synaptic membranes can be inhibited by prior treatment of the membranes with

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phospholipase A_2 (4, 6). Second, it has been demonstrated in this laboratory (7, 8) and by others (9, 10) that CS[2] and PS, respectively, can bind opiates stereospecifically. This involvement of lipids in opiate-receptor interaction is further supported by experiments demonstrating that maneuvers designed to decrease available CS in the central nervous system resulted in a decrease in analgetic response to morphine. Studies with injections of azure A, a cationic dye which has high affinity for CS (11), and with Jimpy mice, a genetic mutant strain with drastically reduced CS levels, indicated that such animals had lowered analgetic responses to morphine (increased morphine AD_{50}) and a concomitant decrease in the amount of [^{3}H]morphine specifically bound to synaptic membranes *in vitro* (12). These and other data have prompted Lee and Smith (13) to propose a protein-lipid model for the opiate binding site. In their model, alkaloids, such as morphine, will interact with the lipid portion of the receptor, while the enkephalin peptides will interact with the protein portion of the receptor complex.

In view of the apparent involvement of membrane lipids in opiate-receptor interaction, an attempt was made to label the binding sites with an agent which would react with membrane lipids. The agent DIDSA, was chosen because it does not penetrate membranes and because the opiate receptor has been postulated to be located at the surface of the synaptic membranes. Moreover, the ^{125}I-labeled analogue of this reagent is available with high specific radioactivity. In the present communication, we wish to report on the effect of DIDSA on opiate receptor binding and on the attempt to identify a membranous component in the chloroform:methanol (2:1) extract which is stereospecifically protected by opiates from this reagent.

METHODS

Preparation of synaptic membranes. Two different preparations of synaptic membranes were used in the present studies. For the effect of DIDSA on opiate binding, synaptic membrane was isolated from the P_2 fraction of crude rat brain homogenates (pellet obtained after centrifugation at 22,000 × *g* for 20 min) with a discontinuous sucrose gradient (0.6 M:1.0 M sucrose buffered with 5 mM Hepes, pH 7.6) according to the method of Gray and Whittaker (14) as modified by Terenius (15). As suggested by Terenius (15), such a preparation yielded synaptic membranes with a purity of about 60%. The opiate receptor binding activity was determined to be stable for at least 3 months when membranes were stored at −70°. In the experiments in which the opiate receptor in the synaptic membrane was labeled with [^{125}I]DISA, the membrane used was prepared from the P_2 fraction by the method of Cotman (16). Synaptosomes were initially separated from myelin and extrasynaptosomal mitochondria in the P_2 fraction by a discontinuous Ficoll gradient (8.5%:13% Ficoll buffered with 5 mM Hepes, pH 7.6). Synaptic membrane was obtained by isolating the membrane from the hypoosmotically lysed synaptosomes with a discontinuous sucrose gradient (0.6:1.0 M sucrose buffered with 5 mM Hepes, pH 7.6). This procedure yielded membranes with a greater purity (65–75%) than did the single-gradient method (16).

Synthesis of DIDSA. DIDSA was prepared by the conversion of 3,5-diiodosulfanilic acid with sodium nitrite in acidic pH according to the method of Helmkamp and Sears (17). The salmon-color crystals of the 3,5-diiodosulfanilic acid (1 g), recrystallized from acidic water, were dissolved in 25 ml of 0.1 N HCl. After the solution was cooled to 0°, 3 ml of cold sodium nitrite solution (2 g/25 ml) was added, whereupon yellow crystals of DIDSA precipitated from solution. After stirring at 0° for 15 min, the DIDSA crystals were separated from the sulfanilic acid by centrifuging at 10,000 × *g* for 10 min. After the residual pellet was washed twice with 10-ml portions of ice-cold water, it was dried and stored under vacuum in a desiccator containing Drilite.

[^{125}I]DISA was prepared from [^{125}I]sulfanilic acid as suggested by the supplier, New England Nuclear Corporation, Boston, Mass.). The solvent (propanol:water, 1:1) from aliquots of [^{125}I]sulfanilic acid, 50μCi to 500 μCi, was removed by evaporation under a stream of N_2 and the residue was redissolved in 50 μl to 0.5 ml of 0.1 N HCl, respectively. After cooling the solutions to 0°, 50 μl to 0.5 ml of 0.05 N sodium nitrite was added and the mixture was Vortexed. After standing in the dark for 15 min at 0°, the reaction was terminated by the addition of 100 μl to 1.0 ml of 0.1 M phosphate buffer (K^+), pH 7.6. Aliquots of the resulting solution containing 50 μCi of [^{125}I]DISA was used for labeling synaptic membranes. The final concentration of DISA used in each of the labeling experiments was between 10^{-7} M and 100^{-6} M.

Interaction of synaptic membranes with DIDSA or NEM. Sucrose was removed from the synaptic membrane preparation by diluting the membrane (3.4–4.6 mg of protein) with 25 ml of 25 mM Hepes buffer, pH 7.6 (Buffer B), and centrifuging the suspension of 23,5000 × *g* for 20 min. The pellet was resuspended in 1 ml of 50 mM phosphate (K^+) buffer, pH 7.6 (Buffer A); preincubated at 0°, 24°, or 37° for 5 min; and 1 ml of a freshly prepared solution of DIDSA or NEM (twice the final concentration) in Buffer A was added. Incubations were carried out for 30 min at the desired temperature. For incubations with DIDSA, the mixtures were kept in the dark because DIDSA was observed to decompose in the light. The alkylations with DIDSA and NEM were terminated, respectively, by the addition of 5 ml of Buffer A containing 20 mM histidine and by 5 ml of Buffer A containing 5 mM dithiothreitol. The supernatants were removed after centrifuging the mixtures at 23,500 × *g* for 20 min. The pellets were washed twice with 20 ml of phosphate buffer and then resuspended in 2.4 ml of Buffer B. Aliquots (0.2 ml) of the resulting membrane suspensions were used in each opiate receptor binding assay. Control membranes were treated under identical conditions in the absence of the reagents.

Effect of lysis on the inhibition of specific opiate binding by DIDSA and NEM. Crude synaptosomal pellets, P_2, were isolated freshly from excised rat brain minus cerebellum by differential centrifugation. To the final pellet, equivalent to P_2 obtained from one rat brain, 20 ml of 5 mM phosphate (K^+) lysing buffer, pH 7.6, were

[2] The abbreviations used are: CS, cerebroside sulfate; PS, phosphatidylserine; DIDSA, 3,5-diiodo-4-diazosulfanilic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DISA, diazosulfanilic acid; NEM, *N*-ethylmaleimide; C:M, chloroform:methanol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.

added. To another pellet, 20 ml of 100 mM phosphate (K^+) control buffer, pH 7.6 were added. After resuspending the pellets by homogenization, the suspensions were incubated at 0° for 30 min and the lysed and the nonlysed crude synaptosomes were collected by centrifuging at 23,500 × *g* for 20 min. After the pellets were resuspended with 8 ml of 100 mM phosphate buffer, 1.0 ml of the lysed or nonlysed P_2 was reacted with the alkylating agents DIDSA or NEM (final alkylating agents concentration, 10 μM to 2.5 mM) in a final volume of 2 ml. After incubating at room temperature for 30 min, the reaction was terminated by centrifuging at 23,500 × *g* for 20 min. The pellets thus obtained were resuspended in Buffer B, and [^{3}H]naloxone binding to these treated membranes was determined as described below.

Opiate receptor binding assays. Specific binding assays of [^{3}H]dihydromorphine, [^{3}H]naloxone, or [^{3}H]etorphine to treated or control synaptic membranes were performed in Buffer B. The incubation of the membranes with tritiated drugs was carried out at 24° for 15 min. In a typical assay, synaptic membrane (200–300 μg of protein) was incubated in triplicate for 5 min with or without 5 μM levorphanol followed by the addition of a tritiated ligand. After 15 min, the membranes were collected on Whatman glass-fiber circles (GF-C) and washed four times with 4 ml of Buffer B at 0°. The filters were allowed to stand overnight at room temperature in 9 ml of Scintiverse (Fisher Scientific Company, Pittsburgh, Pa.) before measurement of radioactivity in Packard Tri-Carb scintillation spectrometer with preset efficiency. Specific binding was defined as the difference in binding in the absence and presence of levorphanol.

Labeling of phospholipids and sulfatides with [^{125}I]DISA. Selected phospholipids and sulfatides were labeled with ^{125}I-DISA in Buffer A. In each case, 1 μmole of lipids, dissolved in 2 ml of chloroform:methanol (2:1), was evenly coated at the bottom of 13 × 100 mm tubes by evaporating the solvent with a stream of nitrogen. The lipids were resuspended into 0.9 ml of Buffer A by sonicating the mixtures for 2 min at full power with a Bronsen sonicator fitted with a microtip. For lipids which cannot form stable sonicated vesicles, e.g., PE (18), 1 mole of β-palmitoyl lysolecithin was added to 4 moles of phospholipids before sonication. After the lipids were uniformly suspended, 50 μCi of [^{125}I]DISA (final concentration 1 mM) in 100 μl of buffer were added. The reaction was carried out at room temperature in the dark for 15 min and then terminated by the addition of 3 ml of C:M (2:1). After the addition of 1 ml of methanol, the lipids were extracted by Vortexing for 30 sec. The lower chloroform phase was then washed twice with 2 ml of 0.1 N KCl in 50% methanol. Free [^{125}I]DISA was separated from labeled lipids by thin-layer chromatography in silica gel G plates developed in a solvent mixture of methanol:water:ammonia (50:50:1). The origins of the chromatograms, where the lipids were located, were extracted twice with 5 ml of C:M (2:1). The specific activity of each lipid was determined by measuring the inorganic phosphate produced by acid hydrolysis of the phospholipids (19). In the case of sulfatides, the lipid concentration was determined by the method of Kean (11), utilizing the dye azure A.

Labeling of membranous components associated with opiate binding. In order to label the membranous components involved in the specific binding of opiates with [^{125}I]DISA, other membranous constituents which were not involved with opiate binding were alkylated beforehand with nonradioactive reagents. In a typical reaction, synaptic membranes (200–500 μg of protein) were freed of sucrose by washing with Buffer A. The pellets obtained from centrifuging at 23,500 × *g* for 20 min were resuspended with 1.6 ml of Buffer A after a 0.2-ml aliquot of varying concentration of levorphanol or 1 μM of dextrorphan was added to the membrane suspensions. The mixture was incubated at room temperature for 10 min, then 0.1 ml of 2 mM NEM and 0.1 ml of 8 nM DIDSA were added. The reaction was carried out at room temperature for 30 min in the dark, in order to minimize the decomposition of DIDSA. The reaction was terminated by the addition of 25 ml of cold Buffer A and the membranes were collected by centrifugation at 23,500 × *g* for 20 min. Excess alkylating agents and opiates which remained bound to the receptor were removed by washing the membranes first with 25 ml of Buffer A containing 100 mM NaCl and then twice with 25 ml of Buffer A alone. After the third wash, the synaptic membranes were resuspended in 0.7 ml of Buffer A, and 0.1 ml of 1 μM dextrorphan was added. The membranes were labeled by the addition of 50 μCi of [^{125}I]DISA converted from [^{125}I]iodosulfanilic acid without the addition of nonradioactive DIDSA (in 200 μl). After incubating at room temperature for 30 min, the labeling reaction was terminated by centrifugation. The pelleted membranes were washed three times with 1 ml of Buffer A, and the pellets were resuspended in Buffer A by sonication at full power for 10 sec with a Bronsen sonicator equipped with a microtip.

Materials. [^{3}H]Dihydromorphine (46 Ci/mmole), [^{3}H]naloxone (20 Ci/mmole), and [^{125}I]iodosulfanilic acid (>1000 Ci/mmole) were purchased from New England Nuclear Corporation. [^{3}H]Etorphine (42 Ci/mmole) was supplied by Amersham/Searle Corporation, Arlington Heights, Ill. Sulfanilic acid and NEM were obtained from Sigma Chemical Company, St. Louis, Mo. Supelco, Inc., Bellefonte, Pa., supplied all lipids used in the present studies with the exception of *N*-methyldipalmitoylphosphatidylethanolamine and *N,N*-dimethyldipalmitoylphosphatidylethanolamine, which were obtained from Vegas Chemical Company, Las Vegas, Nev., and Sigma Chemical Company, respectively. Other chemicals were reagent grade or spectral quality. Precoated thin-layer silica gel G plates were obtained from Analabs. Simonsen supplied male Sprague-Dawley rats (180–200 g).

RESULTS

Inhibition of opiate binding to synaptic membrane by DIDSA. The interaction of opiate ligands with the brain receptor has been reported to be extremely sensitive to the alkylation of the sulfhydryl groups on the synaptic membrane (5, 20). Since DIDSA has reactivity toward amino groups and sulfhydryl groups (21), reduction of the opiate binding activity should be observed when the synaptic membrane is derivatized with this reagent. As shown in Fig. 1a, inhibition of [^{3}H]dihydromorphine and [^{3}H]naloxone specific binding was observed when the synaptic membrane was treated with 2 mM DIDSA at 24°. Under the present opiate binding assay conditions,

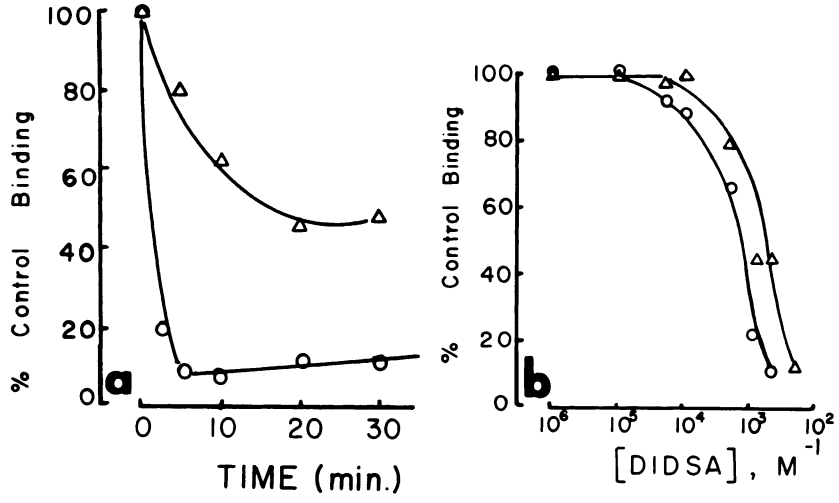

FIG. 1. *Time-dependent (a) and concentration-dependent (b) DIDSA inhibition of the opiate binding to the synaptic membrane*

a. Inhibition of [^{3}H]dihydromorphine (○——○) and [^{3}H]naloxone (△——△) binding to the synaptic membrane treated with 2 mM DIDSA at 24° for various periods of time was carried out as described under Methods.

b. Inhibition of the opiate binding by various concentrations of DIDSA was carried out at 24° for 20 min as described under Methods.

The final concentrations of [^{3}H]dihydromorphine and [^{3}H]naloxone in the binding mixtures were 2 nM and 5 nM, respectively. Values for specific binding to the control membranes were 7500 dpm/mg of protein for [^{3}H]dihydromorphine and 7600 dpm/mg of protein for [^{3}H]naloxone.

i.e., in the absence of 100 mM NaCl, 2 mM DIDSA inhibited [^{3}H]dihydromorphine specific binding to a greater extent than that of [^{3}H]naloxone. Furthermore, the rate at which DIDSA inhibited the dihydromorphine binding was significantly greater than that of the naloxone binding (Fig. 1a). [^{3}H]Dihydromorphine specific binding was inhibited maximally (90%) after 5 min of exposure to the reagent, while [^{3}H]naloxone specific binding was not maximally inhibited (55%) until 20 min after the initiation of the DIDSA incubation. Increase in the DIDSA concentration in the incubation mixtures or the elevation of the reaction temperature to 37° did not eliminate the difference in the rate of inhibition of opiate agonist and antagonist binding. Apparently, the naloxone binding site was less accessible to DIDSA than the dihydromorphine binding site. This apparent difference could be demonstrated also by the determination of the DIDSA IC_{50} values to inhibit [^{3}H]dihydromorphine and [^{3}H]naloxone specific binding. As shown in Fig. 1b, at 24° 0.5 mM and 1.5 mM DIDSA were required to inhibit by 50% the [^{3}H]dihydromorphine and [^{3}H]naloxone specific binding to the synaptic membrane, respectively. Analogous differences in the IC_{50} values were obtained when the DIDSA reaction was carried out at 37°.

The observed DIDSA inhibition of the opiate binding could be attenuated by the presence of various opioid ligands. As summarized in Table 1, the ability of 0.75 mM DIDSA to inhibit the [^{3}H]dihydromorphine binding was modulated by the presence of 0.1 μM of various opiates. A 10-min preincubation with the opiate ligands was required for the observed protection. However, incomplete protection of the opiate binding was observed with all of the opiates tested. Nevertheless, this protection was determined to be stereospecific. The ability of 0.1 μM levorphanol but not 0.1 μM dextrorphan to protect the opiate binding sites from DIDSA was observed in the experiments summarized in Table 1 and Fig. 2. The time-dependent decrease in the [^{3}H]dihydromorphine binding capacity of the synaptic membrane derivatized with 0.5 mM DIDSA in the presence of 0.1 μM dextrorphan was not observed when the reaction was carried out in the presence of 0.1 μM levorphanol (Fig. 2). When the incubation of the synaptic membrane with DIDSA was carried out longer than 20 min, a gradual decrease of the opiate binding activity was observed even in the presence of 0.1 μM levorphanol.

A direct interaction between the opiate receptor binding sites and DIDSA was demonstrated further by a Scatchard analysis (22) of the [^{3}H]dihydromorphine binding data. The specific binding of [^{3}H]dihydromorphine could be resolved into two components, with K_{diss} = 0.6 nM and 31 nM (Fig. 3). When the synaptic membrane was pretreated with 0.75 mM DIDSA at 24°, there

TABLE 1

Inhibition by DIDSA of the specific binding of [^{3}H]dihydromorphine to synaptic membrane: protection by various opiates

Incubations of synaptic membrane with 0.75 mM DIDSA in the presence or absence of various opioid compounds were carried out in Buffer A as described under Methods. After the removal of the alkylating agents and opioid compounds by repeated washings, [^{3}H]dihydromorphine binding to the DIDSA-treated and control membranes was carried out at 24° for 15 min. The final [^{3}H]dihydromorphine concentration was 2 nM. Specific binding to the control membranes was 5800 ± 400 dpm/mg of protein.

Opiate	[^{3}H]Dihydromorphine specific binding[a]
	% of control
None	35.8 ± 4.9
Dextrorphan	38.1 ± 10.2
Levorphanol	61.1 ± 3.9
Morphine	52.7 ± 5.2
Naloxone	52.0 ± 2.3
Methadone	43.0 ± 7.8
Leucine^5-enkephalin	53.9 ± 9.6

[a] Mean ± standard error of the mean of five separate incubations of each opiate.

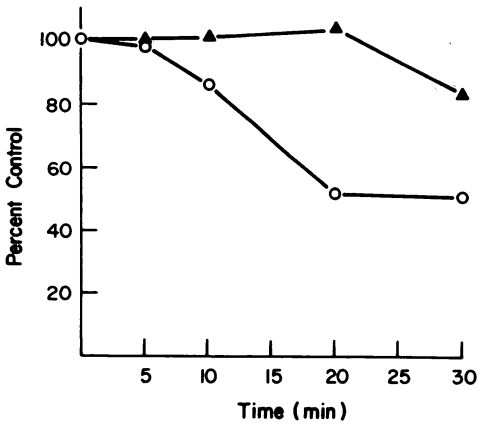

FIG. 2. *Kinetics of DIDSA inhibition of [^{3}H]dihydromorphine binding in the presence of opiates*

Synaptic membranes (1.2 mg) in 2 m of Buffer A were incubated with 0.5 mM DIDSA in the presence of 0.1 μM dextrorphan (○——○) or 0.1 μM levorphanol (▲——▲). At the designated time, 5 ml of 50 mM phosphate plus 20 mM histidine, pH 7.6, were added and the membranes were pelleted by centrifuging at 22,000 × *g* for 20 min. [^{3}H]Dihydromorphine (final concentration 2 nM) binding was then carried out with the resuspended membranes. Specific binding of 5300 dpm/mg of protein was observed with control membranes.

was a significant decrease in the number of binding sites from 0.66 to 0.26 pmole/mg of protein and from 2.22 to 0.56 pmole/mg of protein for the high-affinity and low-affinity sites, respectively. When the DIDSA concentration in the incubation mixtures was increased, the number of binding sites was observed to decrease further with no apparent decrease in the binding affinities.

Differences in DIDSA and NEM inhibition. Although DIDSA shows preferential reactivity toward the phenolic hydroxy group of tyrosine and the imidazole of histidine in protein molecules, the reagent also demonstrates reactivity toward sulfhydryl groups (21). It is probable, therefore, that the observed inhibition of opiate binding to synaptic membranes by DIDSA results in addition from blockade of sulfhydryl groups at the active sites, as is the case with NEM. This probability can be established by the two reagents' activity in the presence of Na^+. If DIDSA is reacting in a manner similar to that of NEM, then the inhibitory action of the reagent should also be attenuated by Na^+ (20). In confirmation of findings by others (5, 20), when the synaptic membranes were treated with 0.5 mM NEM, significant inhibition of [^{3}H]naloxone binding was observed (Fig. 4), with a maximal effect occurring after 15 min of incubation of 37°. In the presence of 100 mM Na^+, the same concentration of NEM did not inhibit the naloxone binding significantly after 30 min of incubation. Similar observations were made with higher concentrations of NEM. However, when synaptic membranes were incubated with 2 mM DIDSA, the inhibitory rate of [^{3}H]naloxone binding was significantly faster than that of NEM, and, in contrast to NEM, the magnitude and the rate of the DIDSA inhibition were identical in the absence or presence of 100 mM Na^+ (Fig. 4). Hence, DIDSA and NEM appeared to be interacting with nonidentical groups in the opiate binding sites in the synaptic membranes.

By exploiting the dissimilarity in the permeability of the two reagents, the difference between NEM and DIDSA inhibition of the opiate binding was illustrated further. Since DIDSA has been demonstrated to be nonpenetrating (23), and NEM penetrates the membrane freely, the two reagents should react differently with intact and lysed synaptosomes. As shown in Fig. 5, the logit-log plots of the inhibition data, lysis did not materially affect the inhibitory action of NEM on a crude synaptosomal preparation (P_2, pellet obtained after centrifugation at 23,500 × *g* for 20 min). In both the lysed and nonlysed preparations of P_2, the IC_{50} values of NEM to inhibit the [^{3}H]naloxone binding were similar, being 1.4 mM and 1.3 mM for lysed and nonlysed synaptosomes, respectively. In striking contrast, when the experiments analogous to those of NEM were carried out with DIDSA, the amount of DIDSA required to inhibit the [^{3}H]naloxone binding to the nonlysed synaptosomes was determined to be 2.0-fold higher than that required for the lysed synaptosomes. The IC_{50} values for the lysed and nonlysed synaptosomes were 0.88 mM and 1.8 mM, respectively.

Labeling of membrane lipids stereospecifically protected by opiates: dependence on levorphanol concentration of [^{125}I]DISA incorporation by synaptic membranes.

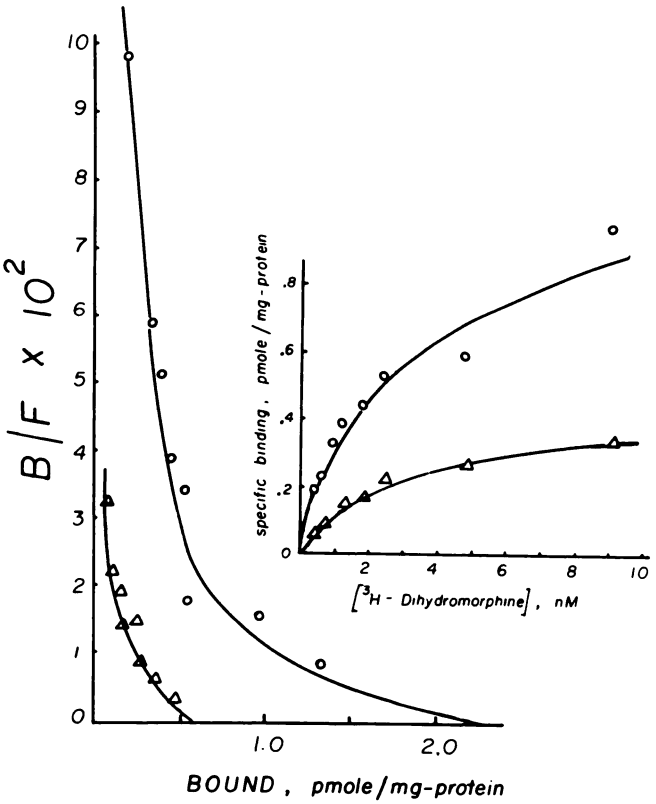

FIG. 3. *Scatchard analysis of DIDSA inhibition*

Synaptic membranes (12 mg), alkylated with 1 mM DIDSA in 20 m of Buffer A for 30 min at room temperature, were suspended in Buffer B, and [^{3}H]dihydromorphine binding to these treated membranes was determined (△——△). Control membranes (○——○) were from the same synaptic membrane preparation and were carried through similar manipulations in the absence of DIDSA. The *inset* represents the amount of [^{3}H]dihydromorphine specifically bound to control (○——○) and treated (△——△) membranes.

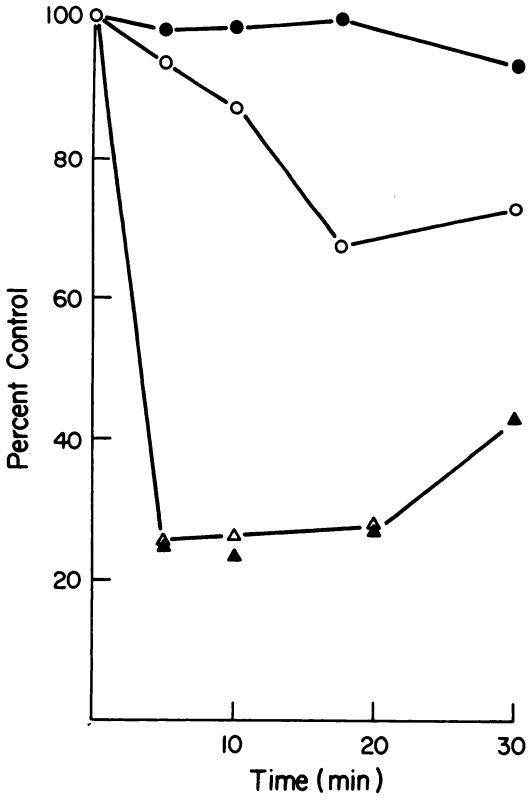

FIG. 4. *Effect of Na^+ on DIDSA and NEM inhibition of opiate binding to synaptic membranes*

Synaptic membranes (1.5 mg) in 1.0 ml of Buffer A were reacted with 0.5 mM NEM (○——○) or 2 mM DIDSA (△——△) at room temperature. At various time intervals, the reactions were terminated with the addition of 5 ml of Buffer A containing 5 mM dithiothriotol or 20 mM histidine for the incubations with NEM and DIDSA, respectively. In parallel experiments, 100 mM Na^+ was added to the reactions with NEM (●——●) and DIDSA (▲——▲). After the removal of the alkylating agents, [^{3}H]naloxone (5 nM) binding was performed with the resuspended membranes. Specific binding of naloxone to the control membranes was 6800 dpm/mg of protein.

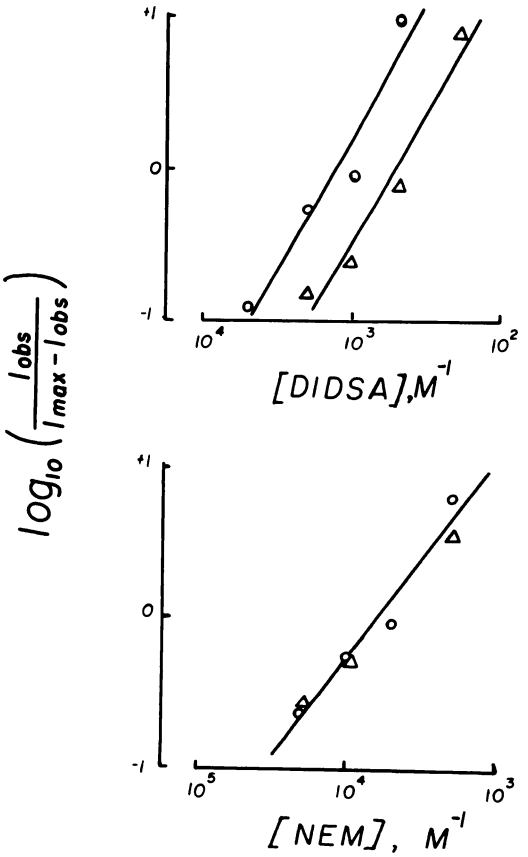

FIG. 5. *Logit-log plots of the DIDSA and NEM inhibition of [^{3}H] naloxone binding: effect of lysis*

Incubations of lysed and nonlysed crude synaptosomal preparations with various concentrations of (a) DIDSA (*upper*) and (b) NEM (*lower*) at 24° for 30 min were carried out as described under Methods. Values for maximal inhibition of [^{3}H]naloxone binding by DIDSA and NEM were determined to be 90% and 50%, respectively. Specific binding of [^{3}H]naloxone to the lysed (○——○) and nonlysed (△——△) synaptosomes was determined as described under Methods to be 5700 dpm/mg of protein and 5200 dpm/mg of protein, respectively.

From the studies on the inhibition of opiate binding by the derivatizing agents, it appears that DIDSA and NEM may be reacting with overlapping sets of binding sites. In an attempt to identify the chemical nature of the opiate binding sites, labeling of opiate receptors was carried out with high specific activity [^{125}I]DISA. Membranous sites which are not involved in opiate binding and which react with the reagents were initially derivatized as described under Methods. In order to attain maximal derivatization of the nonopiate binding sites and a complete protection of opiate receptors, a mixture of 0.4 mM DIDSA and 0.1 mM NEM was used. Opiate receptor binding sites were protected from the reagent by the prior addition of varying concentrations of levorphanol.

As summarized in Table 2, when the membranes were treated with NEM and DIDSA, specific binding of [^{3}H] naloxone to the membrane was completely abolished, whereas treatment of synaptic membrane with the same concentration of the reagents separately did not elicit complete inhibition (Figs. 1 and 5). The inhibition of naloxone binding could be averted by preincubation of the membrane with 0.2 μM levorphanol, whereas dextrorphan failed to produce such an effect (Table 2). Thus, the chemical nature of the receptor could be characterized by labeling the membrane with [^{125}I]DISA after derivatization/alkylation and the removal of excess nonradioactive reagents and opiates. Subsequently, by comparing the radioactivity incorporated by the membranes previously protected by levorphanol or dextrorphan, the amount of [^{125}I]DISA incorporated by the opiate-specific binding sites could be determined.

When the opiate receptor was labeled in this fashion, the amount of [^{125}I]DISA incorporated into the membrane was observed to the dependent on the concentration of levorphanol previously used to protect the receptor from the nonradioactive DIDSA and NEM. The final concentration of 0.5 μM [^{125}I]DISA in the incubation mixture was used because further dilution of the radioactive reagent would diminish the difference between the radioactivity incorporated into membranes protected by levorphanol and dextrorphan. When the amount of [^{125}I] DISA incorporated by the membranes was standardized with respect to the protein content, a 25% increase in radioactivity was observed only in membranes protected

TABLE 2

Inhibition of [^{3}H]naloxone binding to synaptic membranes alkylated with N-ethylmaleimide and DIDSA

Synaptic membranes (2 mg in 5 ml of Buffer A) were derivatized with 0.4 mM DIDSA and 0.1 mM NEM at 37° for 30 min in the presence of (A) 0.1 μM dextrorphan and (B and C) 0.1 μM levorphanol. In all three cases the opiates were allowed to equilibrate with the membranes for 10 min before the addition of the alkylating agents. Excess agents and opiates were removed by twice washing the membranes with 20 ml of phosphate buffer after the incubations. [^{3}H]Naloxone (final concentration 5 nM) was then added to the control and treated membranes at 4° for 2 hr. In treatment C, after the removal of excess opiates and alkylating agents by washings, the membranes were then treated with 10 μM DIDSA at 37° for 30 min. [^{3}H]Naloxone binding was then determined after removal of the DIDSA.

Treatment	[^{3}H]Naloxone binding	% of control
	fmoles	
Control	21.6	100
A. + dextrorphan + reagents	1.4	6.5
B. + levorphanol + reagents	18.2	84.2
C. + levorphanol + reagents, wash, + 10 μM DIDSA	19.2	88.9

by levorphanol at a concentration of 10^{-7} M or greater. However, when the amount of radioactivity associated with the lipids was determined, a greater difference was observed with the C:M (2:1) extract from the membrane previously protected with levorphanol when compared with the dextrorphan-protected extract. Nonreacted [^{125}I]DISA was separated from labeled lipids by thin-layer chromatography with silica gel G plates developed in methanol:water:ammonia (50:50:1) (Fig. 6). With this solvent mixture, 50% of the total counts in the C:M (2:1) membrane extract applied to the plate remained stationary at the origin. All of the derivatized polar lipids tested, i.e., CS, PS, PI, PC, and PE remained at the origin. Free [^{125}I]DISA migrated with the solvent front as shown by the fact that, when nonreacted PS and [^{125}I]DISA were applied to the plate sequentially, greater than 90% of the total radioactivity was at the solvent front (Fig. 6, *upper* panel) whereas organic phosphate remained at the origin. Thus, free [^{125}I]DISA and labeled polar lipids can easily be separated under these conditions.

When ^{125}I-labeled synaptic membrane C:M (2:1) extracts were devoid of free [^{125}I]DISA, a levorphanol dose-dependent protection of membranous polar lipids from the derivatizing reagents was observed (Fig. 7). At a levorphanol concentration of 10^{-7} M or greater, the increase in the amount of [^{125}I]DISA incorporated into the membrane was nearly twice that observed with dextrorphan. This increase was parallel to the increase in the protection of etorphine binding activity in the synaptic membranes derivatized with the reagents in the presence of various concentrations of levorphanol (Fig. 7).

If the [^{125}I]DISA is labeling the opiate receptor, then the increased labeling seen by the prior addition of levorphanol in the derivatizing mixture should be eliminated when the same concentration of drug is added concomitantly with [^{125}I]DISA to the final incubation mixture, but dextrorphan should have no effect. As shown in Fig. 7, after the synaptic membranes previously protected from the derivatizing reagents with 10^{-6} M

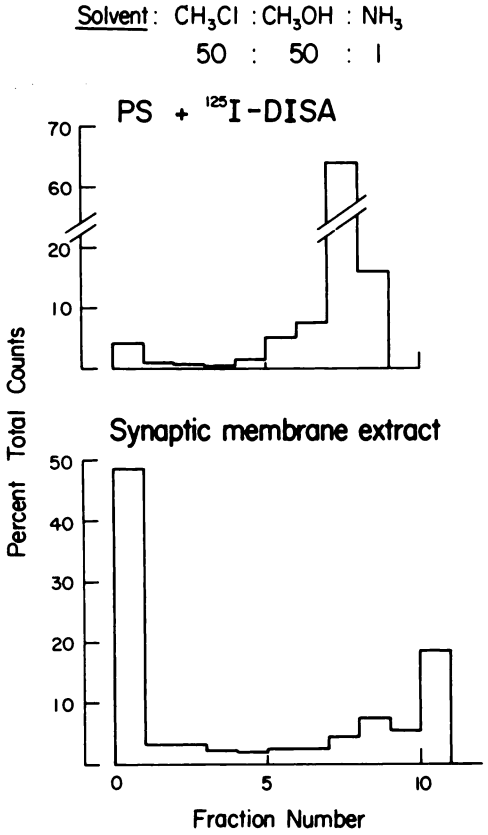

FIG. 6. *Thin-layer chromatographic pattern of a C:M (2:1) extract of the lyophilized reaction mixtures containing 2 mg of PS and 1 m M [^{125}I]DISA (upper panel) and the synaptic membranes labeled with 50 μCi of [^{125}I]DISA (lower panel)*

Reaction conditions for both cases were as described under Methods. The thin-layer silica gel G plates were developed in a solvent mixture of methanol:water:ammonia (50:50:1). One-centimeter fractions were removed and radioactivity was determined.

levorphanol were labeled with [^{125}I]DISA in the presence of 10^{-6} M levorphanol, the increase in radioactivity was abolished. This implies that when the opiate receptor is occupied with the ligand levorphanol, the membrane polar lipids are no longer available for the reaction with the radioactive reagent.

Identification of the membranous lipids labeled by [^{125}I]DISA. The ability of levorphanol to protect membranous constituents extractable by C:M (2:1) suggested that the polar lipids might play an important role in the interaction of the opiates with the receptor. In order to identify the lipids involved, the C:M extract was separated by thin-layer chromatography on silica gel G plates and developed with solvent mixtures as outlined by Rouser (24). When standard lipids were reacted with [^{125}I]DISA and chromatographed on silica plates under identical conditions, they exhibited different reactivity toward the [^{125}I]DISA. As shown in Table 3, PE and *N*-methyldipalmitoylphosphatidylethanolamine had the greatest reactivity. The specific activity of the labeled PE was 3.9-fold higher than that of labeled PI. Since ethanolamine contains a primary amine which is a nucleophile, condensation with the electrophilic diazonium salt should be rapid. However, if the primary amine is

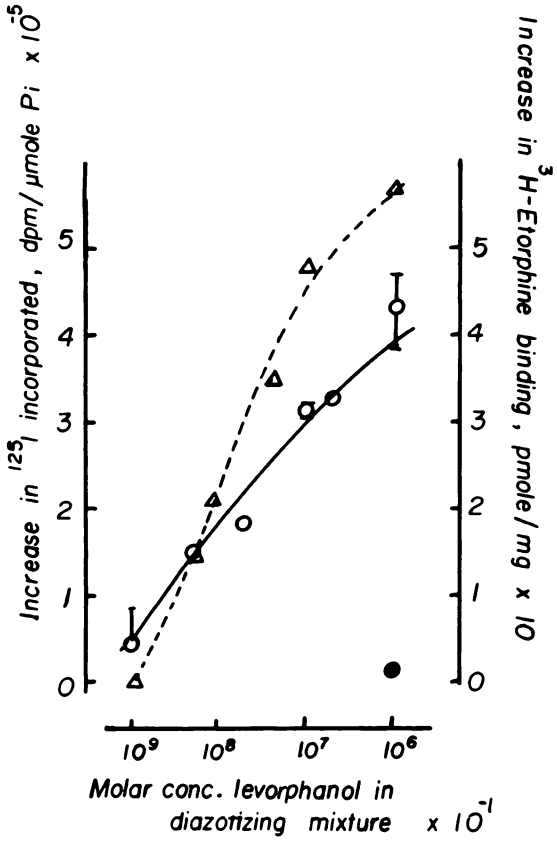

FIG. 7. *Effect of levorphanol on [^{125}I]DISA labeling of the membrane polar lipids*

Membrane polar lipids were labeled and extracted as described under Methods. The *X* axis represents the amount of levorphanol in the incubation mixtures when the synaptic membrane was derivatized with 0.1 mM NEM and 0.4 mM DIDSA. The *left Y* axis (○——○) represents the difference between the amount of radioactivity incorporated by the membrane lipid extract previously protected with 0.1 μM of dextrorphan. The background radioactivity, less than 5%, contributed by [^{125}I]sulfanilic acid, was subtracted from each value. The amount of [^{125}I]DISA incorporated by the membrane lipids previously protected with dextrorphan was determined to be 4.0 ± 0.2 × 10^{5} cpm/μmole of Pi. ●, The difference in the radioactivity incorporated by the membrane previously protected with 0.1 μM dextrorphan and those protected with 1 μM levorphanol and subsequently labeled with [^{125}]DISA in the presence of 0.1 μM dextrorphan and 1 μM levorphanol, respectively. The other *Y* axis (Δ- - -Δ) represents the amount of [^{3}H]etorphine (2 nM) specifically bound after the removal of excess opiates to the synaptic membrane treated with NEM and DIDSA in the presence of various concentrations of levorphanol. [^{3}H]Etorphine specific binding in the control membrane was determined to be 0.72 pmole/mg of protein.

TABLE 3

Specific activity of standard lipids labeled with [^{125}I]DISA

Standard lipids were sonicated in Buffer A with a Bronsen sonicator fitted with a microtip for 2 min at full power in a lipid concentration of 2 mg/ml. In the cases of PE and *N*-methylated PE, 1 mmole of β-palmitoyllysophosphatidylcholine was added to 4 mmoles of the phosphatides to stabilize the sonicated vesicles. Then 1 μmole of the sonicated lipid vesicles was aliquoted to 13 × 100 mm test tubes containing Buffer A. Labeling reactions were initiated by the addition of 1 mmole of [^{125}I]DISA (15 μCi). After incubating at 24° for 15 min in the dark, the reactions were terminated by the addition of 3.0 ml of C:M (2:1). Lipids were extracted after the addition of 1.0 ml of methanol by Vortexing the mixture for 30 sec. The lower chloroform phase was washed twice with 2.0 ml of 0.1 M KCl in 50% methanol. Free radioactive DISA was removed from the labeled lipids by thin-layer chromatography as described in the text. The amount of phospholipid was determined by measuring the inorganic phosphate liberated by the acid hydrolysis of the lipds located at the origin of the chromatograms (21). The amount of sulfatide was measured by the method described by Kean (11).

Lipid	Specific activity
	dpm/μmole × 10^{-4}
CS (bovine brain)	10.5 ± 0.1
PS (bovine brain)	14.4 ± 1.2
PI (plant)	19.9 ± 3.5
PE (bovine brain)	76.7 ± 4.2
N-Methyldipalmitoylphosphatidylethanolamine	75.1 ± 3.7
N,N-Dimethyldipalmitoylphosphatidylethanolamine	2.8 ± 0.5
PC (bovine brain)	9.2 ± 0.1
β-Palmitoyllysophosphatidylcholine	6.4 ± 0.8

methylated, condensation with DISA should be diminished. This is consistent with the difference in the specific radioactivities of the lipids with varying degrees of methylation in the ethanolamine head group. As summarized in Table 3, the presence of one methyl group in the ethanolamine moiety did not alter the specific activity of the labeled lipid (*N*-methyldipalmitoylphosphatidylethanolamine) significantly from that of PE. However, the presence of two methyl groups on the polar head group of the lipid (*N,N*-dimethyldipalmitoylphosphatidylethanolamine) reduced the specific activity of the labeled lipid to 3.6% of that of PE. The dipalmitoyl analogue of the synthetic PC was not labeled by [^{125}-I]-DISA. Instead of the nonreactivity observed toward the synthetic dipalmitoyl analogue, PC from bovine brain reacted with [^{125}I]DISA, and the specific activity of this labeled lipid compared favorably with that of labeled CS, PS, and PI. Apparently, this diazotizing agent can also react with the hydrophobic region of the lipids, the unsaturated fatty acids.

When the C:M (2:1) extract of the synaptic membrane labeled with [^{125}I]DISA was chromatographed with three different solvent mixtures, complex labeling patterns were observed (Fig. 8). The chromatographic patterns of the extract from membrane previously protected from the reagents DIDSA and NEM with levorphanol were identical with those protected with dextrorphan. These labeling patterns were distinctly different from those of the free label in the three solvent mixtures used (Fig. 9). In all three solvent mixtures used, the free label chromatographic pattern consisted of two major peaks, one radioactive peak remaining at the origin, the other having an R_F value ≤0.5. The materials which can be moved by the organic solvents are most likely the condensation products of [^{125}I]DISA with the solvents and do not represent the dimerization of the label itself or the impurities in the reagent. This was demonstrated by the alteration of the R_F values of the more hydrophobic component of the free label in these three solvent mixtures when the free label was mixed with excess of serine immediately before the application of the [^{125}I]DISA

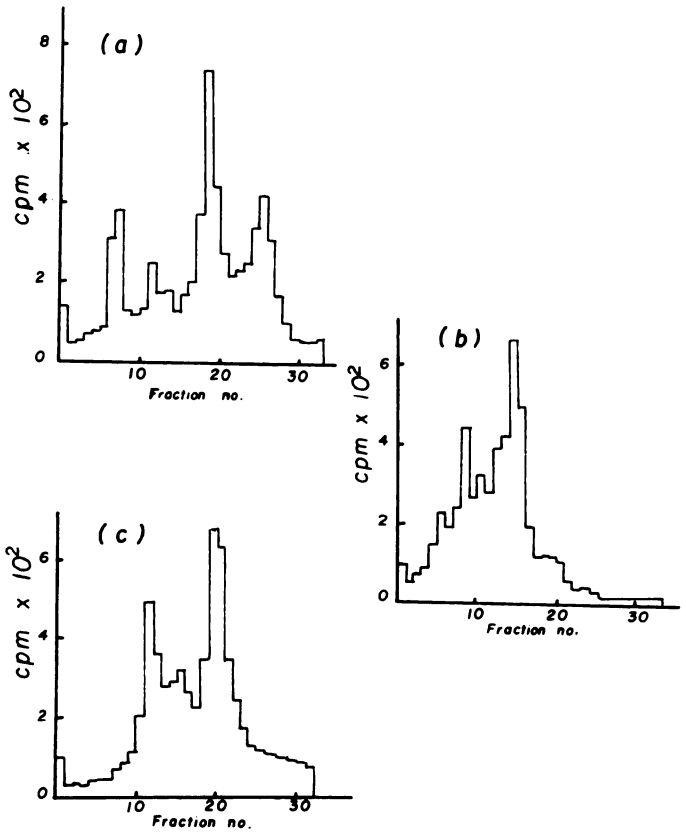

FIG. 8. *Thin-layer chromatographic pattern of the C:M (2:1) extract of the ^{125}I-labeled synaptic membrane previously protected by opiates*

Synaptic membranes (0.2–0.5 mg) were extracted and free [^{125}I]DISA was separated from labeled lipids as described under Methods. The extracts were applied onto precoated silica gel G plates and the plates were developed in the solvent mixtures of chloroform:methanol:ammonia (62:35:5) (a) chloroform:methanol:water (65:25:4) (b), and 1-propanol:propionic acid:chloroform:water (3:2:2:1) (c). Subsequently the chromatograms were subdivided into 0.5-cm fractions and the radioactivity of each fraction was determined with a liquid scintillation spectrometer.

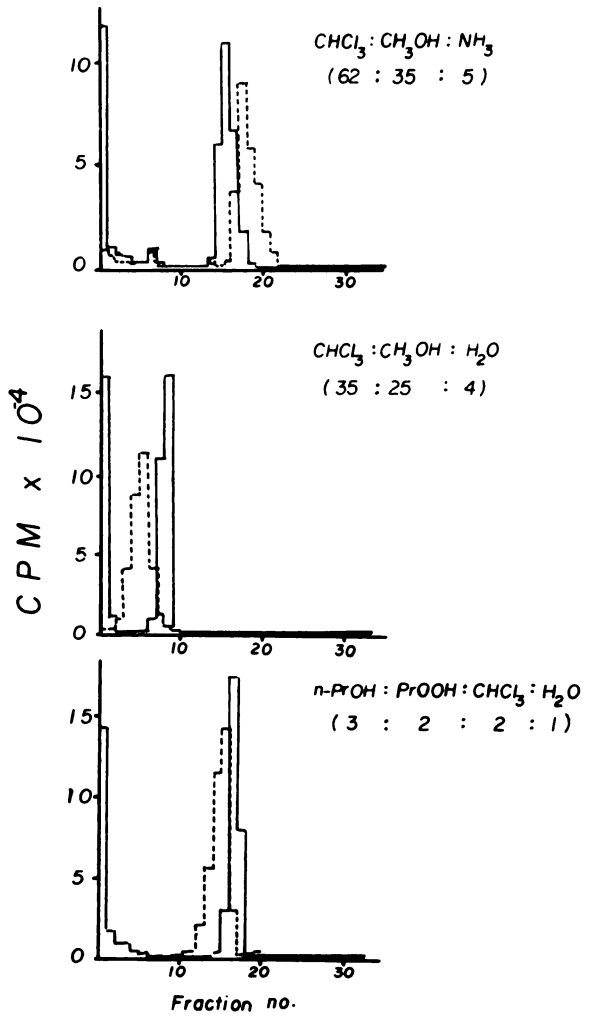

FIG. 9. *Thin-layer chromatographic pattern of [^{125}I]DISA*

The nonreacted free label [^{125}I]DISA was applied to silica gel G plates and the plates were developed in the solvent mixtures as described in Fig. 8. The radioactivity of each 0.5-cm fraction of the chromatograms was determined. – – –, The chromatographic patterns of the free label in the presence of excess serine. Serine (1 mmole) was added to 0.1 ml of a 1 mM solution of [^{125}I]DISA immediately before the application of the radioactivity onto the silica gel G plates.

onto the silica gel G plates. Not only was the amount of radioactivity observed at the origins of the chromatograms significantly reduced, but the R_F values of the [^{125}I]DISA serine adduct were also different from those of the free label alone (Fig. 9).

The identity of the labeled lipids was determined by comparing the chromatographic patterns of standard lipids labeled by [^{125}I]DISA and those of the C:M (2:1) extract of the labeled membrane in the three different solvent mixtures. As shown in Fig. 10, all of the standard lipids labeled with this reagent, with the exception of PE and PC from bovine brain, have simple chromatographic patterns. The mobilities of all of the major radioactive peaks of CS, PS, and PI correspond to the R_F values of the unlabeled standard lipids as determined by iodine vapor reactivity (Fig. 10). Although the mobility of the labeled lyso-PC, monomethylated PE, and dimethylated PE did not correspond to the R_F values of the standard lipids, all three labeled lipids migrated as a single peak on the silica gel G plates developed in the three solvent mixtures used. Only bovine brain PE and PC labeled with [^{125}I]DISA had multiple peaks in their thin-layer chromatograms. The chromatograms of the C:M (2:1) extracts resembled those of the labeled PE. Analogous to the chromatograms of labeled PE, the C:M (2:1) extracts had the distinct three major peak chromatographic patterns when the plates were developed in the solvent mixture $CHCl_3$:CH_3OH:NH_3 (62:35:5) and the double major peak pattern when the plates were developed in $CHCl_3$:CH_3OH:H_2O (65:25:4). The chromatograms of the extract were distinctly different from those of the labeled PC in particular when the silica gel G plates were developed in 1-propanol:propanoic acid:chloroform:water (3:2:2:1). Instead of a major radioactive peak at R_F = 0.36, as in the case of labeled PC, the chromatograms of the membrane extract had radioactive peaks at R_F = 0.36, 0.48, and 0.64, which was analogous to the pattern of labeled PE when the plates were developed in this solvent mixture (Figs. 8 and 10).

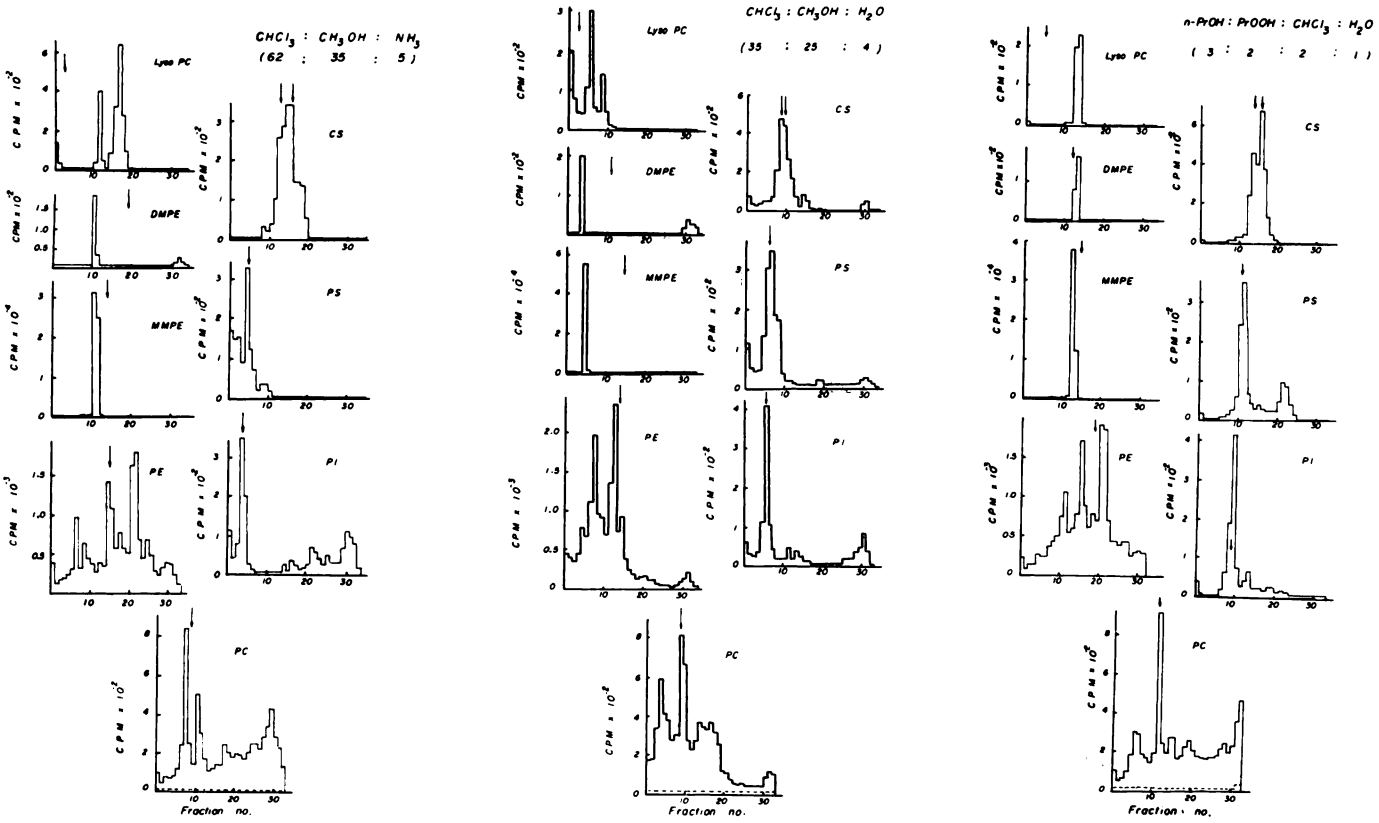

FIG. 10. *Thin-layer chromatographic patterns of various standard lipids labeled with [^{125}I]DISA*

Phospholipids and sulfatides were labeled with 1 mM [^{125}I]DISA as described under Methods and in Table 3. Free label was removed from labeled lipids as described in the text. The solvent mixtures used were identical with those described in Fig. 8. The chromatograms were subdivided into 0.5-cm fractions and the radioactivity of each fraction was determined. The *arrows* represent the location of the standard lipids in the chromatograms as determined by positive iodine reactivity.

DISCUSSION

The ability of a nonspecific membrane-modifying reagent, DIDSA, to react with opiate binding sites was demonstrated clearly in the present studies by the fact that the inhibitory action of this reagent on [^{3}H]dihydromorphine binding could be blocked by the presence of morphine, levorphanol, naloxone, and leucine^5-ekephalin but not by dextrorphan. Moreover, Scatchard analysis of the data indicated a direct interaction of opiate receptor sites with DIDSA with the resulting loss in binding sites. DIDSA, similar to alkylating agents such as NEM, inhibited [^{3}H]dihydromorphine binding to a greater extent than [^{3}H]naloxone binding at all temperatures tested. As a consequence, it may appear that DIDSA has a mode of action similar to that of NEM; however, there are some distinct differences, for example, the ability of Na^+ to block the inhibitory effect of NEM on opiate binding but not that of DIDSA (Fig. 4). This suggests that DIDSA and NEM could not be interacting with the identical membrane constituents within or near the opiate ligand binding sites.

It is interesting that better results were obtained in the present study when NEM and DIDSA were used in combination to derivatize the sites on the membrane which were not involved in the stereospecific binding of the opiates. When the two reagents were utilized separately, at concentrations of 0.1 mM NEM and 0.4 mM DIDSA, incomplete inhibition of opiate binding was observed. However, when combined, almost 100% inhibition of opiate binding was achieved at the same concentrations (Table 2, treatment A). In contrast, more than 1 mM DIDSA alone was needed to reach 100% inhibition (Fig. 1). However, at this concentration of DIDSA, complete protection of the opiate receptor with levorphanol could not be achieved (Table 1). On the other hand, with the lower concentration of DIDSA in combination with NEM, levorphanol could completely protect the receptor from the reagents (Table 2). Hence, by the use of the combination of NEM and DIDSA, virtually 100% of the receptor was accessible for derivatization by [^{125}I]DISA.

The problem presented by the relatively low concentration of opiate receptor was not resolved completely by the availability of a highly radioactive reagent, [^{125}I]DISA. In order to label all of the receptor sites protected beforehand by levorphanol, 1 mM [^{125}I]DISA in the final incubation mixtures was required (Fig. 1). Rather than adding large quantities of nonradioactive reagent to the incubation mixtures, [^{125}I]DISA was used at concentrations of 0.1–1 μM, at which concentrations the opiate receptors protected by levorphanol were not inhibited by the reagent (Table 2). If it is assumed that only one molecule of DISA reacts with one lipid molecule and that there is only one lipid molecule per receptor site, with a specific radioactivity of [^{125}I]DISA = 1000 Ci/mmole the calculated maximal amount of opiate receptor which can

be labeled is 3 fmoles/mg of protein [obtained by subtracting the amount of ^{125}I incorporated after protection by 10^{-6} M dextrorphan from that after 10^{-6} M levorphanol and determining the amount of organic phosphate per milligram of protein (Fig. 7)]. The maximal number of low-affinity (K_{diss} = 20–30 nM) binding sites in synaptic membrane, prepared by the present method, was previously reported to be 2.4 pmoles/mg of protein (12). Thus, only 0.1% of the total available receptors was labeled by the present technique. Whether such a population is a true representation of all of the opiate receptors in the synaptic membrane remains an open question.

In a previous report (25) using a similar chromatographic technique, we concluded that [^{125}I]DISA had labeled CS, previously protected stereospecifically by the opiates. However, we have now established that the above finding may be due to the incomplete removal of the reagent, [^{125}I]DISA. In the original study, the mobility of the diazonium compound in all three of the solvent mixtures used in developing the thin-layer chromatograms was very similar to that of standard sulfatides (Figs. 9 and 10). In the present study, however, after the removal of the [^{125}I]DISA which was not covalently reacted with a membrane component, complex chromatographic patterns were observed with the labeled membranous lipids instead of a single radioactive peak in each chromatogram (Fig. 8). In all three solvent mixtures tested these patterns resembled those of the standard PE labeled with the reagent.

The basis for concluding that a polar lipid such as PE could be involved in the opiate ligand-receptor interaction is 2-fold: (a) the degree of protection of the lipid from the reagents DIDSA and NEM was dependent on the concentration of the opiate, and (b) when 1 μM levorphanol was present in the incubation mixtures containing [^{125}I]DISA, there was no difference in the amount of radioactivity incorporated into the lipid fractions between those previously protected from DIDSA and NEM derivatization with levorphanol and those with dextrorphan (Fig. 7). This observation suggests that PE is in the proximity of the opiate ligand binding sites. Granted, without detailed analysis of the labeled products, one cannot conclude that the polar lipid in proximity with the opiate binding sites is PE. However, because of the complexity of the chromatographic patterns of the membrane extract, lipids extractable with C:M (2:1) other than PE or PC can be eliminated from consideration. The identity of the labeled membrane extract is more likely to be PE rather than PC because of (a) similarity in the chromatographic patterns and (b) the greater reactivity of PE toward the reagent [^{125}I]DISA (Table 3). PE itself does not appear to be the opiate receptor since both Loh *et al.* (7, 8) and Abood and Hoss (9) did not find opiate binding with PE. Furthermore, these observations do not necessarily preclude that CS or PS is essential to the opiate receptor-ligand interaction. The amount of CS in the synpatic membrane is relatively low and is still debatable. One-third of the total phospholipids in the synaptic membrane have been determined to be PE (26). Since [^{125}I]DISA has greater reactivity toward PE (Table 3), it is likely that the reagent may have reacted with a small percentage of the PE molecules which were protected by levorphanol rather than with the CS or PS, which might have been protected also.

The ability of levorphanol and not dextrorphan to protect a certain population of phospholipids in the synaptic membrane suggests an important role for the phospholipid in stereospecific interactions of opiates and their receptor. The function of the phospholipid, possibly PE, in the vicinity of the opiate binding site could be related to the cellular response to opiate other than stabilization of the receptor conformation. It has been suggested by Hirata and his co-workers (27) that there is a close correlation between the activation of adenylate cyclase by *beta*-adrenergic agonists and the transmethylation of the PE molecules to PC in the erythrocyte membrane. Moreover, stimulation of PE methylation by transmethylase has been reported to enhance ligand-receptor interaction for *beta*-adrenergic agonists (27, 28), benzodiazepine (28), and human growth hormone (29). Hence, if a similar situation exists with respect to opiate interaction with its receptor, the presence of a PE molecule in the proximity of the binding site would be prerequisite. Our preliminary studies with the neuroblastoma × glioma NG108-15 hybrid cells and the striatal membrane have indicated an apparent opiate receptor regulation of the transmethylase activity in these membrane preparations. Hence, methylation of these lipid molecules could be involved in the molecular basis for the opiate action.

Unfortunately, in the present studies a stereospecific protection of the membrane protein was not observed. Although vigorous analytical procedures such as polyacrylamide disc gel electrofocusing and Sephadex exclusion gel column chromatography were used to separate individual membrane protein components according to charge and size, a significant increase was not observed in the amount of [^{125}I]DISA incorporated by the protein previously protected from the reagents with levorphanol when compared with those previously protected with dextrorphan. This could be due to the concentration of the reagents used in derivatizing the opiate binding sites. As illustrated by Table 2 and Fig. 1, at concentrations of 10^{-7}–10^{-5} M, DIDSA had no effect on the opiate receptor binding activity. Most likely, under the present incubation conditions, the radioactive reagents did not interact with the protein portion of the opiate binding sites.

In conclusion, it has been possible to label a membrane polar lipid selectively protected by levorphanol and not by dextrorphan. This polar lipid has been tentatively identified to be PE. Nevertheless, the demonstration of a polar lipid to be protected by opiate ligands suggests a possible role of membrane lipids in opiate receptor-ligand interaction and the subsequent cellular response. The identification of the polar lipid as PE could be utilized to stabilize the solubilized receptor and facilitate the isolation of the opiate binding site.

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