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Photoaffinity Labeling of Opiate Receptors Using Intrinsically Photoactive ^{3}H-Opiates

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SUMMARY

Opiate receptors in rat and cow brain membranes have been labeled irreversibly using the intrinsic photolability of ^{3}H-opiates. Membranes were incubated with ^{3}H-ligand and then irradiated with UV light of 254 nm. Nonspecific binding was determined in the presence of 10 μM unlabeled levallorphan. Irreversible binding was defined as binding which survived heat or acid denaturation of membranes. Specific incorporation of label into denatured samples was observed only when unbound or loosely bound ^{3}H-ligand was washed free from the membranes prior to irradiation. There was a general correlation between photosensitivity of the ^{3}H-ligand and its ability to photolabel receptors. Hence, photolabeling presumably results by covalent attachment of highly reactive species generated during photochemical decomposition of ligand. With ^{3}H-etorphine, optimal irradiation time was 5 min. In addition to ^{3}H-etorphine, receptors could be labeled irreversibly with ^{3}H-oxymorphone, ^{3}H-dihydromorphine, and ^{3}H-ethylketocyclazocine. Of the specific binding present in irradiated, nondenatured samples, 45–60% remained attached to receptors upon denaturation. ^{3}H-Ethylketocyclazocine exhibited an 86% yield of incorporation. Signal-to-noise levels of 50–80% could be achieved in denatured samples. Therefore, this method provides a means of covalently labeling opiate receptors in high yield and with high signal-to-noise ratios. The opioid peptides, ^{3}H-D-Ala^2,D-Leu^5-enkephalin, ^{3}H-D-Ser^2,Leu^5,Thr^6-enkephalin, ^{3}H-D-Ala^2,Met^5-enkephalin amide, and ^{3}H-D-Ala^2,N-MePhe^4,Gly-ol^5-enkephalin, as well as the benzomorphan, ^{3}H-bremazocine, apparently lack the structural characteristics which allow photolabeling. ^{3}H-Etorphine was incorporated at high specific activity into a glycoprotein fraction of bovine brain membranes which was retained on wheat germ agglutinin affinity columns. Unretained proteins were essentially unlabeled. ^{3}H-Etorphine was also incorporated at high specific activity into proteins of 17,000 and <13,700 Da and, at lower specific activity, into a protein of 2.4 × 10^6 Da in guinea pig brain membranes when κ receptors were selectively labeled. This method therefore allows labeling of specific membrane proteins and will prove useful in molecular characterization of opiate receptors.

Receptors for opiate alkaloids and opioid peptides have been divided into five major classes based on biochemical and pharmacological evidence (1, 2). These classes are μ, δ, κ, σ, and ϵ. In spite of abundant pharmacological and ligand binding evidence for this classification scheme, the molecular basis of this heterogeneity has eluded clarification. In addition, various structural models for the receptors have been proposed (3–5). The initial step in elucidating the molecular structure of the receptor is identification of the proteins which specifically bind opiates. One approach is affinity labeling of the binding site. Several opiate ligands have been derivatized with electrophilic groups which can be attacked by receptor nucleophiles (6–10). Opiate ligands have also been derivatized with photoactive groups which form covalent bonds to receptor upon photodecomposition with light of the appropriate wavelength (11–18).

These ligands in radiolabeled form have met with varying degrees of success at identification of opiate receptor proteins. Using ^{3}H-fentanyl isothiocyanate, an isothiocyanate derivative of fentanyl, Klee and co-workers (19, 20) reported labeling of an M_r 58,000 protein, from NG108-15 hybrid cell membranes, which is a component of the δ receptor. Using ^{3}H-DALECK, a chloromethyl ketone derivative of leucine enkephalin, Newman and Barnard (21) reported labeling of an M_r 58,000 protein in rat brain membranes which is a component of the μ receptor. However, production of these affinity probes often involves time-consuming synthesis of several analogs which then must be characterized for binding affinity, receptor selectivity, and ability to bind covalently to the receptor. Many of these analogs are found to lack the ability to bind with high affinity or, when radiolabeled, exhibit high amounts of nonspecific binding, mak-

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ABBREVIATIONS: ETOR, etorphine; EKC, ethylketocyclazocine; BREM, bremazocine; OXY, oxymorphone; DHM, dihydromorphine; DADLE, D-Ala^2,D-Leu^5-enkephalin; DSTLE, D-Ser^2,Leu^5,Thr^6-enkephalin; DAMA, D-Ala^2,Met^5-enkephalin amide; DAGO, D-Ala^2,*N*-MePhe^4,Gly-ol^5-enkephalin; Mn(OAc)$_2$, manganese acetate; WGA, wheat germ agglutinin; NAG, *N*-acetyl glucosamine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetate.

ing them unsuitable as affinity probes. In addition, some ligands with electrophilic centers have been shown to bind with high affinity and selectivity but not to form covalent bonds to receptor, presumably due to lack of nearby receptor nucleophiles or unfavorable orientation in the binding site (10).

Photoaffinity labeling offers some advantages over the use of ligands with electrophilic centers (22–24). Since covalent attachment does not occur until the sample is irradiated, more control is exerted over the reaction. Ligand bound to nonreceptor sites can be removed before covalent attachment occurs, leading to lower levels of nonspecific labeling. In addition, the reactive species generated in photoaffinity labeling is of a free radical nature and forms several types of covalent bonds. This eliminates the requirement for the presence of specific nucleophiles in a favorable orientation to the probe. In spite of these important advantages and the many compounds which have been synthesized, photoaffinity labeling of membrane preparations has, to date, been unsuccessful at producing covalent attachment of a radiolabeled opiate ligand in high yield and with high levels of specific binding.

Glasel and Venn (25) reported that some commercially available ^{3}H-opiates are intrinsically photolabile. When irradiated with short-wave UV light, ^{3}H-ETOR undergoes photochemical decomposition. If irradiation was carried out in the presence of membranes, ^{3}H-ETOR (or a decomposition product) became tightly bound to the protein. However, these investigators could demonstrate little or no specificity in the labeling, as assessed by incubation and irradiation in the presence and absence of excess unlabeled opiate. Dramatic increases in nonspecific binding were observed. In addition, although a reduced rate of label washout was demonstrated for ^{3}H-ETOR after UV irradiation, irreversible (covalent) labeling was not clearly demonstrated.

Using a modification of the procedure of Glasel and Venn (25) we have been able to demonstrate both specific and irreversible labeling of rat brain membranes with several ^{3}H-opiates upon irradiation with UV light. Photolabeling occurs in high yield and with high levels of specific binding, characteristics which are requisite for the success of the photoaffinity labeling approach. Labeled membrane preparations can be generated which are suitable for solubilization and analysis under denaturing conditions. Using this technique, we report specific labeling of a glycoprotein fraction of bovine brain membranes and high and low molecular weight proteins in guinea pig brain membranes. Therefore, this method of using the intrinsic photolability of commercially available radiolabeled opiate ligands which bind with known affinity, known selectivity, and high signal-to-noise ratios could be a viable alternative for irreversible labeling of receptors when synthesis of suitable affinity probes is impossible.

Materials and Methods

Preparation of membranes. The crude mitochondrial (P_2) membrane fraction of rat brain was used for these studies. Male Sprague-Dawley rats (Taconic Farms), 180–250 g, were sacrificed by decapitation. Whole brains minus cerebellum were placed in ice-cold 10 mM Tris-HCl, pH 7.4, containing 320 mM sucrose (Tris/sucrose buffer) and homogenized in a Potter-Elvehjem homogenizer by 10 strokes of a motor-driven Teflon pestle in a volume of 10 ml/g of tissue wet weight. The homogenate was centrifuged at 1,000 × *g* for 10 min and the supernatants saved. The pellets were resuspended by vortexing in 2 ml/g ice-cold Tris/sucrose and were centrifuged again at 1,000 × *g* for 10 min. The combined 1,000 × *g* supernatants were centrifuged at 31,000 × *g* for 15 min. The pellets were resuspended by vortexing in 3 ml/g 10 mM Tris-HCl, pH 7.4, and the suspension was allowed to incubate at 25° for 15 min. Following centrifugation at 31,000 × *g* for 15 min, the pellets were resuspended by gentle Potter-Elvehjem homogenization to a final volume of 1.53 ml/g in 10 mM Tris-HCl, pH 7.4. Aliquots were stored at −80° until used. Protein was determined by the method of Lowry *et al.* (26).

Preparation of reversibly labeled membranes. P_2 membrane fraction (6.5 mg of protein) was incubated with 2 nM ^{3}H-ligand in the presence or absence of 10 μM levallorphan in 7 ml of incubation buffer under the following conditions: ^{3}H-ETOR, 10 mM Tris-HCl, pH 7.4, at 25° for 90 min; ^{3}H-EKC, 10 mM Tris-HCl, pH 7.4 + 100 mM NaCl at 4° for 90 min; ^{3}H-BREM, 50 mM K_2HPO_4, pH 7.4 + 400 mM NaCl at 4° for 4 hr; ^{3}H-OXY, ^{3}H-DHM, ^{3}H-DAGO, 10 mM Tris-HCl, pH 7.4 + 3 mM $Mn(OAc)_2$ at 25° for 2 hr; ^{3}H-DADLE, ^{3}H-DSTLE, and ^{3}H-DAMA, 10 mM Tris-HCl, pH 7.4 + 100 mM NaCl + 3 mM $Mn(OAc)_2$ + 2 μM GTP at 25° for 90 min.

Irradiation of labeled membrane suspensions and assessment of irreversible binding. Following incubation, the labeled membranes were washed as follows. The incubation mixture was centrifuged for 5 min at 37,000 × *g* and the supernatant was carefully removed and discarded. The pellet was resuspended in 6 ml of ice-cold 10 mM Tris-HCl, pH 7.4, and centrifuged at 37,000 × *g* for 5 min. Membranes labeled with μ ligands (OXY, DHM, and DAGO) were then given a final resuspension in 6 ml of ice-cold incubation buffer. Membranes labeled with all other ligands were washed a second time. Immediately after the final resuspension in incubation buffer, aliquots of 500 μl were filtered through Whatman GF/B or Schleicher and Schuell glass fiber filters under reduced pressure and washed three times with 5.5 ml of ice-cold 10 mM Tris-HCl, pH 7.4. These samples were used to determine the amount of specific binding present after washing and before any subsequent manipulations (zero time value). The remaining membrane suspension was transferred to polystyrene weighing boats on ice. The square weighing boats provided a large surface area for irradiation. A shortwave UV lamp of 254 nm (Mineralight R52G, Ultra-Violet Products, Inc., San Gabriel, CA) was positioned over the samples approximately 1 cm above the liquid surface. Irradiation at 254 nm was carried out for the designated time period. Nonirradiated controls were treated in the same manner except that samples remained on ice in room light for the designated time period. Following these treatments, 500-μl aliquots were filtered and washed as described above. Other 500-μl aliquots were placed in a 90° water bath for 10 min before filtration and washing. This treatment denatures the membranes and, in most experiments, released >90% of specifically bound counts from nonirradiated controls. Thus, label remaining associated with membranes after heat denaturation was assumed to be irreversibly and covalently attached to membrane components. Specific binding values were obtained by subtracting binding observed in the presence of 10 μM levallorphan from that observed in its absence. All samples were counted in 4.5 ml of Ultrofluor or Ecoscint (National Diagnostics, Somerville, NJ) in minivials using a Packard model 4450 scintillation spectrometer.

Irradiation of labeled filter-trapped membranes and assessment of irreversible binding. Following incubation with ^{3}H-ligand as described above, membranes were filtered through Whatman GF/B or Schleicher and Schuell glass fiber filters and washed three times with 5.5 ml of ice-cold Tris-HCl, pH 7.4, under reduced pressure to rapidly remove loosely associated ligand. The membrane-laden filters were removed from the filter manifold and laid with the side containing the membranes face up on a glass plate which was resting on ice. The filters were either exposed to room light for 10 min or irradiated for 10 min at a distance of 0.5 cm using the UV lamp described above. They were then immediately transferred back to the filter manifold and washed under reduced pressure with either 16.5 ml of 10 mM Tris-HCl (nondenatured samples) or 15 ml of 30% acetic acid (denatured sam-

ples). Acid denaturation was shown to release >90% of specifically bound ligand from nonirradiated samples. Label remaining associated with membranes after acid treatment was assumed to be irreversibly and covalently bound. Specific binding values were obtained by subtracting binding observed in the presence of 10 μM levallorphan from that observed in its absence.

Photolabeling of cow striatal membranes and WGA chromatography of photolabeled proteins. Fresh cow brains were obtained from a local slaughterhouse and the striata were removed. Striatal P_2 membrane fraction was prepared by the method described above. Membranes were suspended in 140 ml of 10 mM Tris-HCl (2 mg of protein/ml) and incubated with 5 nM ^{3}H-ETOR as described above. Incubation was carried out in the presence or absence of 10 μM levallorphan. Membranes were then washed twice as described above, and irradiated for 10 min on ice with stirring to ensure uniform exposure of membranes to light. After irradiation, membranes were washed twice to remove unbound ^{3}H-ETOR. After the final wash, photolabeled membranes were resuspended in a final volume of 10 ml of 10 mM Tris-HCl, pH 7.4. Aliquots could be stored at −80° until further use. Uniformly (nonspecifically) labeled membranes were prepared in the same manner, but with the omission of the wash step prior to irradiation.

Photolabeled membranes were solubilized by homogenization in the presence of 13 mM CHAPS and 1% Lubrol. The homogenate was incubated on ice for 15–30 min and then centrifuged at 105,000 × *g* for 75 min at 4°. The clear supernatant was removed and dialyzed overnight against 10 mM Tris-HCl, pH 7.4, at 4° to remove free ^{3}H-ETOR.

Chromatography on wheat germ lectin-Sepharose 6MB was carried out on a 3-ml column as follows. Two ml of extract were applied to the column, 1 ml at a time. Each aliquot was applied to the bed and allowed to incubate for 15 min. The column was washed with 10 mM Tris-HCl, pH 7.4, containing 10 mM CHAPS and 1 mM EDTA. Retained proteins were eluted with the same buffer containing 0.25 M *N*-acetyl D-glucosamine. Protein was estimated by absorbance at 280 nm, and a 50-μl aliquot of each 1-ml fraction was counted in a scintillation spectrometer to detect tritium.

Photolabeling of guinea pig brain membranes and chromatography of labeled proteins on Sepharose 6B-CL. Frozen guinea pig brains were obtained from Pel-Freeze (Rogers, AR). Cerebella were removed and the P_2 membrane fraction was prepared as described above. Membranes were photolabeled with 5 nM ^{3}H-ETOR as described above for bovine striata, except that incubation was carried out in the presence of 100 nM unlabeled DAGO and 100 nM unlabeled DSTLE to mask μ and δ receptors, respectively. Labeled membranes were solubilized with 13 mM CHAPS/1% Lubrol as described above. Free ^{3}H-ETOR was removed by passage of extract over Sephadex G-25 equilibrated and eluted with a buffer consisting of 100 mM Tris-HCl, pH 7.4, 10 mM dipotassium EDTA, and 0.5% sodium cholate. The resulting void volume was concentrated to a final protein concentration of 10.8 mg/ml (1570 cpm/mg of protein). The protein (13 mg) was applied to a 167-ml Sepharose 6B-CL column equilibrated with a buffer consisting of 75 mM Tris-HCl, pH 7.4, 10 mM dipotassium EDTA, and 0.5% sodium cholate. Elution was carried out in the same buffer at a flow rate of 14 ml/hr at 4°. The absorbance of each 2-ml fraction was determined at 280 nm and the fractions were counted in an equal volume of Hydrofluor (National Diagnostics) in a Packard scintillation spectrometer.

The column was calibrated as follows. To determine void and included volumes, a mixture of blue dextran (2 mg/ml) and ^{3}H-ETOR (277,000 cpm/ml) was loaded onto the column and eluted with a buffer consisting of 75 mM Tris-HCl, pH 7.4, 10 mM dipotassium EDTA, and 290 mM NaCl. Eight molecular weight standards (Pharmacia, Piscataway, NJ) were then loaded onto the column two at a time and eluted as above, giving rise to a linear calibration curve.

Materials. ^{3}H-EKC, ^{3}H-BREM, ^{3}H-DADLE, ^{3}H-DSTLE, ^{3}H-DAMA, and ^{3}DAGO were obtained from New England Nuclear (Boston, MA). ^{3}H-ETOR, ^{3}H-OXY, and ^{3}H-DHM were obtained from Amersham Radio Chemicals (Arlington Heights, IL). DSTLE and DAGO were obtained from Bachem (Torrance, CA). Tris-HCl, GTP, trypsin, and NAG were obtained from Sigma Chemical Co. (St. Louis, MO). Ultrofluor, Ecoscint, and Hydrofluor were obtained from National Diagnostics. CHAPS was obtained from Boehringer-Mannheim (Indianapolis, IN). Wheat germ lectin-Sepharose 6MB, Sephadex G-25, and Sepharose 6B-CL were obtained from Pharmacia. All other chemicals were reagent grade.

Results

Table 1 shows the effect of 10 min irradiation of 2 nM ^{3}H-ETOR in unwashed (Section A) and washed (Section B) membranes. In both cases, irradiation with UV light gave irreversible labeling of the membranes. However, only when membranes were washed free of unbound ^{3}H-ligand prior to irradiation was *specific* irreversible (covalent) labeling obtained. In unwashed membranes (Table 1, Section A), irradiation caused a 13-fold and 1.6-fold increase in nonspecific and total binding values, respectively, leading to an overall decrease in specific binding in irradiated samples compared to nonirradiated samples. In nonirradiated samples, denaturation by heating for 10 min in a 90° water bath caused an increase in nonspecific binding and a decrease in total binding, leading to an overall decrease in specific binding. Heat denaturation of irradiated samples gave similar results, leading to a total loss of specific binding. Therefore, although irradiation caused a large amount of irreversible incorporation of label into membranes, all specific signal was lost.

We surmised that these results were most likely due to unbound and loosely bound ^{3}H-ligand in the binding milieu becoming covalently attached to membranes upon irradiation and, to some extent, by heating. When membranes were washed by twice repeated centrifugation and resuspension *prior* to irradiation, specific covalent labeling was observed (Table 1, Section B). Unlike unwashed samples, there was no significant change in nonspecific binding upon irradiation, and there was a decrease in the total binding value. This lead to a 29% decrease in specific binding in the irradiated sample compared to nonirradiated samples. In addition, heat denaturation of nonirradiated samples lead to little change in nonspecific binding and a large decrease in total binding, resulting in a >90% loss of specific binding. By contrast, upon irradiation and denaturation, only 54% of the specific binding was lost. Therefore, 46% of the specific binding present in irradiated samples survived heat denaturation. This value was defined as the per cent yield of covalent photoincorporation. Importantly, the covalent binding is 71% specific. In subsequent experiments with other ligands (see below), specific covalent labeling has been observed as high as 79% with yields as high as 86%.

Fig. 1 shows the effect of irradiation time on specific irreversible incorporation of ^{3}H-ETOR into brain membranes. Samples exposed only to room light (Fig. 1, *DARK*) showed no significant change in binding over the 25-min period tested. Increased time of UV irradiation lead to a progressive decrease in specific binding (Fig. 1, *IRRAD*). In the dark-treated samples, denaturation practically eliminated all specific binding at each time point. However, in irradiated samples, heat denaturation revealed a time-dependent, irreversible incorporation of ^{3}H-ligand into membranes. Maximal incorporation was observed after 5 min of irradiation. Longer irradiation times served only to decrease the number of counts incorporated, possibly by excessive UV damage to protein.

TABLE 1

Effect of washing on photoincorporation of ^{3}H-ETOR into membranes

^{3}H-ETOR (2 nM) was incubated with rat P_2 membranes and the labeled membranes were either treated immediately (Unwashed) or washed twice by centrifugation (Washed) prior to any of the specified treatments. "Zero time" refers to aliquots (500 μl) filtered immediately after centrifugal washing and before any other treatments. "Non-irrad" refers to samples allowed to stand on ice in room light for 10 min. "Irrad" refers to samples irradiated on ice for 10 min with 254 nm of UV light as described under Materials and Methods. "Denat" refers to samples which were heated in a 90° bath for 10 min after either room light exposure (Non-irrad/Denat) or irradiation (Irrad/Denat). "NSB" and "Total" were incubated in the presence and absence of 10 μM unlabeled levallorphan, respectively. SB = specific binding and is the difference between the total and NSB values; % SB = percentage of total binding which is specific. "Yield" refers to that percentage of *specific* binding in irradiated samples which survives denaturation. This was taken to be covalent binding. Values are average cpm from duplicate 500-μl aliquots which were filtered and washed as described under Materials and Methods. The experiment was repeated twice with less than 5% variation between experiments. Actual standard errors are omitted for table clarity.

Treatment	A Unwashed				B Washed			
	NSB	Total	SB	% SB	NSB	Total	SB	% SB
	cpm				*cpm*			
Zero time	458	5429	4971	92%	355	2635	2280	87%
Non-irrad	392	4877	4485	92%	315	2419	2104	87%
Non-irrad/Denat	910	921	11	1%	318	435	117	27%
Irrad	5995	8736	2741	31%	371	1867	1496	80%
Irrad/Denat	5889	5605	0	0%	276	965	689	71%
			(Yield 0%)				(Yield 46%)	

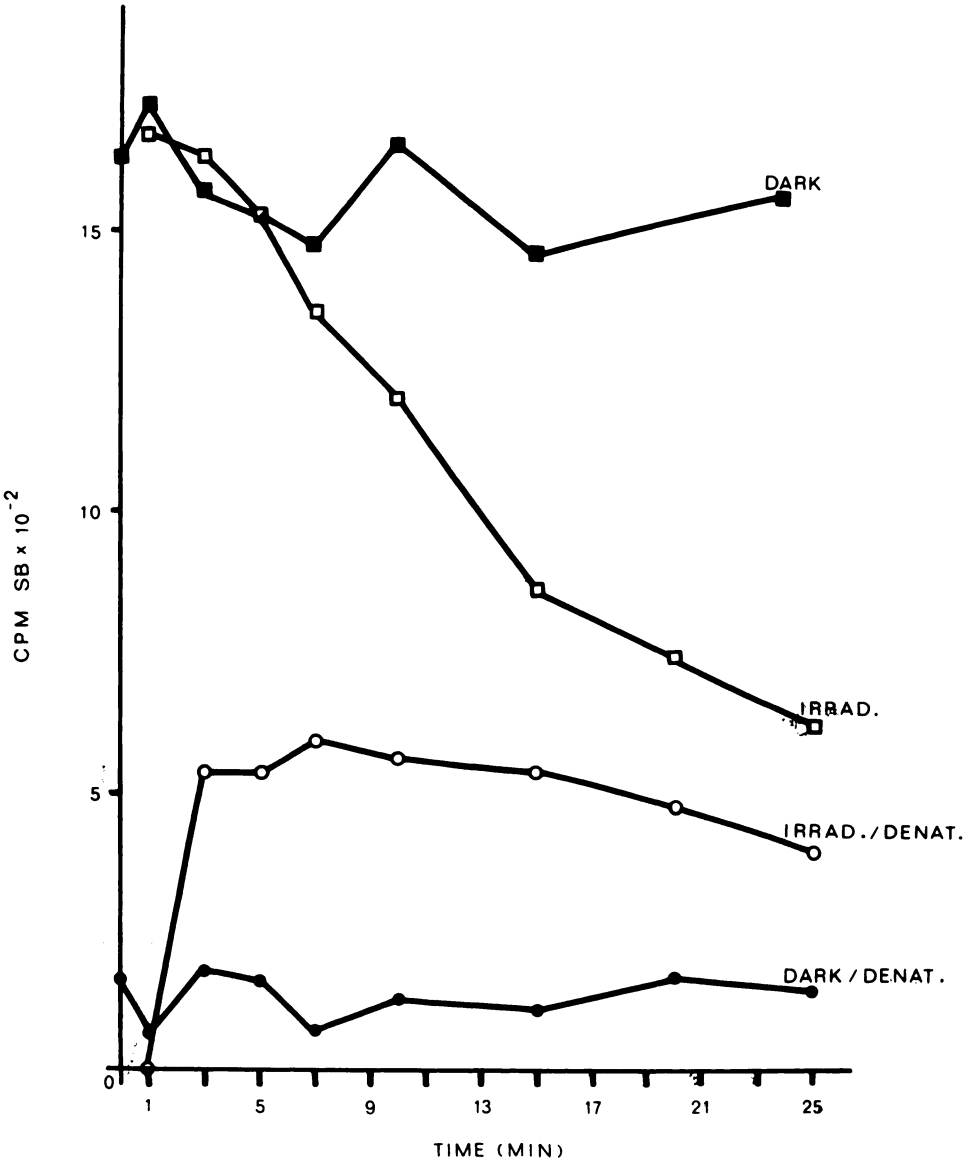

Fig. 1. Effect of time of irradiation on irreversible incorporation of ^{3}H-ETOR into membranes. Rat brain membranes were labeled with 2 nM ^{3}H-ETOR in the presence or absence of 10 μM levallorphan and washed as described under Materials and Methods. Labeled membranes were placed on ice and treated as follows: ■, membranes which were dark treated (exposed to room light only) prior to filtration; ●, membranes which were dark treated and heat denatured prior to filtration; □, membranes which where irradiated prior to filtration; ○, membranes which were irradiated and heat denatured prior to filtration. Times refer to time of irradiation or dark treatment. Heat denaturation was for 10 min at 90° Values given are cpm of specific binding. The experiment was repeated twice with duplicate determinations. There was less than 10% variation between experiments.

To determine whether protein components of the membrane were being photolabeled, we determined the effect of trypsin on covalently bound counts. Table 2 shows the results of these experiments. Trypsin caused a marked loss of counts from reversibly labeled (control) and photolabeled membranes, indicating that ^{3}H-ETOR is attached to a protein moiety in the membrane preparation. Loss of counts from boiled photolabeled membranes confirms loss from covalently labeled sites.

Table 3 shows that, in addition to ^{3}H-ETOR, ^{3}H-EKC, ^{3}H-OXY, and ^{3}H-DHM also became irreversibly and specifically bound to opiate receptors upon irradiation with UV light. Specific binding levels in irreversibly labeled membranes ranged from 47% to 79%, and yields of incorporation of bound ligand ranged from 46% to 86%. It is noteworthy that ^{3}H-EKC incorporated with such a high yield, whereas the related compound, ^{3}H-BREM, did not become irreversibly incorporated. Four opioid peptides were tested for their ability to photolabel membranes: DADLE, DSTLE, DAMA, and DAGO. All of these peptides failed to incorporate irreversibly upon irradiation with UV light.

In the case of DADLE, DSTLE, and DAMA, low cpm values were observed at zero time. These low values resulted from dissociation of ligand during centrifugal washing of membranes. In order to circumvent this problem, the experiments whose results are shown in Table 4 were carried out. In these experiments, membranes were incubated with ^{3}H-ligand and bound ligand was separated from free by rapid filtration through glass fiber filters. The membranes were then washed by filtration of ice-cold buffer. The faster wash time reduced dissociation of all ligands during the wash step, as exemplified by comparison of values for specific binding at zero time in Tables 3 and 4. The trapped, washed membranes were then either irradiated with UV light or subjected to room light, followed by a subsequent buffer wash or acetic acid wash by filtration. As with membrane suspensions, ^{3}H-ETOR, ^{3}H-OXY, and ^{3}H-DHM specifically and irreversibly labeled receptors using this method. With the exception of ^{3}H-DHM, the yields were somewhat lower than those with membrane suspensions, presumably due to shielding of those membrane fragments deeply imbedded in the filter fibers. By contrast, UV irradiation failed to cause irreversible incorporation of ^{3}H-DADLE, ^{3}H-DSTLE, or ^{3}H-DAMA.

As pointed out above, ^{3}H-BREM was the only opiate alkaloid which did not photolabel receptors. In addition, it is the only ligand not incubated in Tris-HCl buffer; a phosphate buffer was used. Since irradiation is carried out in incubation buffer, we tested whether buffer conditions had any effect on photolabeling. Membranes were incubated with ^{3}H-ETOR or ^{3}H-BREM in their normal buffers as described under Materials and Methods. After washing, a buffer switch was effected prior to irradiation: membranes labeled with ^{3}H-ETOR were resuspended in the phosphate buffer and membranes labeled with ^{3}H-BREM were resuspended in Tris-HCl buffer. Despite the different irradiation buffers, ^{3}H-ETOR photolabeled membranes as usual, while ^{3}H-BREM still failed to photoincorporate (data not shown). Therefore, the ability to photolabel membranes is indeed a property of the ligand and not of buffer conditions.

In Fig. 2, we investigated the photosensitivity of various opiate ligands in an attempt to correlate photosensitivity with their ability to photolabel receptors. For all four ligands shown, pre-irradiation with UV light decreased their ability to bind to native receptors in a time-dependent manner. However, whereas ^{3}H-ETOR and ^{3}H-DHM (also ^{3}H-OXY, not shown) were destroyed very rapidly, ^{3}H-DADLE and ^{3}H-BREM were much more resistant to destruction by UV light. As shown in

TABLE 2

Effect of trypsin on photolabeled membranes

Membranes photolabeled with ^{3}H-ETOR in the presence or absence of 10 μM levallorphan were diluted with 10 mM Tris-HCl, pH 7.4, to a final concentration of 1 mg/ml. Membranes were then incubated in the presence or absence of 100 μg/ml trypsin for 90 min at 25° with constant shaking. Following incubation, some 500-μl aliquots were filtered through glass fiber filters under reduced pressure and washed three times with 5.5 ml of ice-cold 10 mM Tris-HCl, pH 7.4. Other aliquots were denatured by boiling for 10 min prior to filtration. As a control for trypsin activity, naive membranes were incubated with 2 nM ^{3}H-etorphine in the presence or absence of 10 μM levallorphan in 10 mM Tris-HCl, pH 7.4, for 60 min at 25°. Either trypsin (100 μg/ml) or buffer was then added and incubation was carried out for an additional 90 min before filtration and washing as described above. Values are cpm of specific binding ± standard error of three experiments. Each experiment was carried out in triplicate.

Treatment	Specific binding	Effect of trypsin
Control	3775 ± 89	
Control/Trypsin	242 ± 102	−94 ± 2.3
Photolabeled	1025 ± 37	
Photolabeled/Trypsin	169 ± 56	−83 ± 5.7
Photolabeled/Denat	737 ± 36	
Photolabeled/Trypsin/Denat	262 ± 5.3	−65 ± 2.2

TABLE 3

Photolabeling of membrane suspensions

Rat brain membranes were labeled using 2 nM ^{3}H-ligand in the presence or absence of 10 μM levallorphan under the conditions described under Materials and Methods. Membranes were then washed in ice-cold incubation buffer and either irradiated or exposed to room light as described under Materials and Methods. "% SB Irrev" refers to the percentage of binding in irradiated/denatured samples which is specific. All other treatments and terms are as defined in the legend of Table 1. Values are cpm of specific binding ± standard error for the number of experiments (*n*) shown at the bottom of each column. Each experiment was carried out in duplicate.

	Specific binding by ^{3}H-ligand								
	ETOR	DHM	OXY	EKC	BREM	DAGO	DADLE	DSTLE	DAMA
					cpm				
Zero time	2874 ± 757	2317 ± 400	1380	903 ± 161	2718 ± 230	1313 ± 218	563 ± 35	340	468
Non-Irrad	2817 ± 760	2016 ± 309	1490	425 ± 62	2649 ± 634	881 ± 177	339 ± 86	242	455
Non-Irrad/Denat	145 ± 44	129 ± 69	18	6 ± 6	99 ± 64	3 ± 2	6 ± 6	17	0
Irrad	1954 ± 589	1737 ± 439	1280	211 ± 20	1293 ± 276	534 ± 167	283 ± 39	159	237
Irrad/Denat	688 ± 61	1070 ± 285	587	182 ± 25	138 ± 11	9 ± 5	22 ± 11	11	17
% SB Irrev	72 ± 3	68 ± 2	79	47 ± 5	77 ± 5				
% Yield	47 ± 4	61 ± 4	46	86 ± 4	11 ± 2	3 ± 2	7 ± 4	7	7
	n = 7	*n* = 5	*n* = 1	*n* = 3	*n* = 3	*n* = 3	*n* = 3	*n* = 1	*n* = 1

TABLE 4

Photolabeling of filtered membranes

Rat brain membranes were incubated with 2 nM ^{3}H-ligand in the presence and absence of 10 μM levallorphan under the conditions described under Materials and Methods. Labeled membranes were then filtered through glass fiber filters. The filters containing the membranes were then immediately irradiated or exposed to room light, followed by the treatments described in the appropriate section of Materials and Methods. Denaturation in these experiments was achieved by treatment with acetic acid. Values are cpm of specific binding ± standard error for the number of experiments (*n*) shown at the bottom of each column. Each experiment was carried out in duplicate.

	Specific binding by ^{3}H-ligand					
	ETOR	DHM	OXY	DADLE	DSTLE	DAMA
Zero time	5746 ± 719	3156 ± 13	2260	1937 ± 403	1390	2680
Non-Irrad	2821 ± 248	714 ± 248	2010	1032 ± 459	1140	2180
Non-Irrad/Denat	38 ± 19	98 ± 69	12	108 ± 54	0	336
Irrad	2928 ± 554	1050 ± 62	1350	916 ± 153	551	951
Irrad/Denat	786 ± 14	768 ± 103	390	43 ± 27	23	54
% SB Irrev	79 ± 5	64 ± 3	67			
% Yield	27 ± 4	74 ± 14	29	4 ± 2	4	6
	n = 3	*n* = 2	*n* = 1	*n* = 3	*n* = 1	*n* = 1

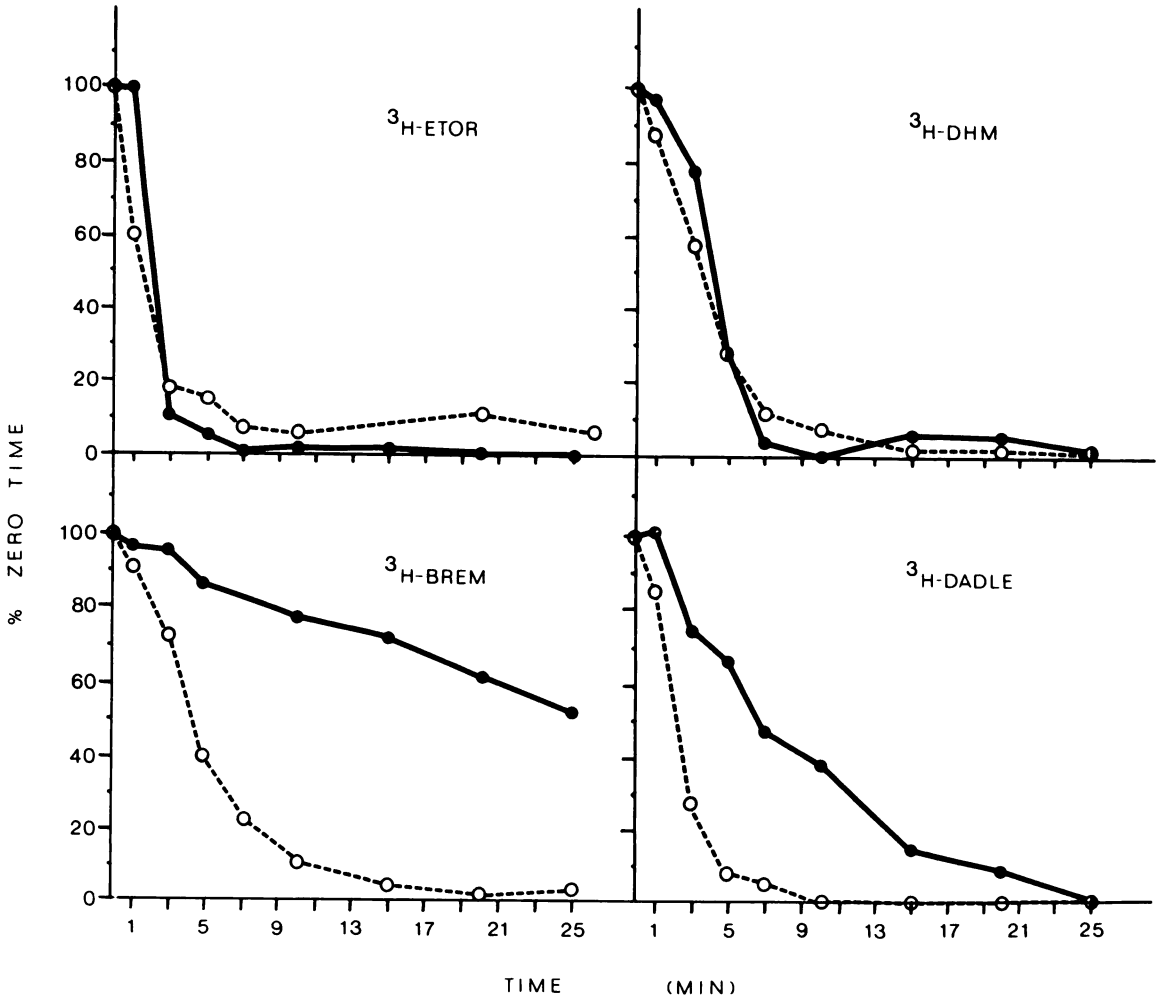

Fig. 2. Kinetics of ligand destruction and binding site destruction by UV irradiation. ●, ^{3}H-ligand was irradiated on ice at a concentration of 20 nM in 10 mM Tris-HCl, pH 7.4, for the designated periods of time. Irradiated ligand (2 nM) was then incubated in duplicate with native membranes under the conditions described under Materials and Methods. ○, rat brain membranes (1 mg of protein/ml) were irradiated on ice for the designated periods of time in 10 mM Tris-HCl, pH 7.4. Irradiated membranes were then concentrated by centrifugation and incubated in duplicate with untreated ^{3}H-ligand (2 nM) under the conditions described under Materials and Methods. The incubation conditions described under Materials and Methods were used to carry out standard binding assays using 600 μg of membrane protein in a total volume of 0.5 ml. Nonspecific binding for each ligand was determined in the presence of 10 μM levallorphan. After the designated incubation time, the mixture was filtered through glass fiber filters (Whatman GF/B or Schleicher & Schuell) under reduced pressure, followed by three washes with 5.5 ml of ice-cold 10 mM Tris-HCl, pH 7.4. The four panels represent results obtained with the following ligands: ^{3}H-ETOR, ^{3}H-DHM, ^{3}H-BREM, and ^{3}H-DADLE. Results similar to those obtained with ^{3}H-DHM were also obtained with ^{3}H-OXY (data not shown).

Tables 3 and 4, ^{3}H-ETOR, ^{3}H-DHM, and ^{3}H-OXY photolabeled receptors, whereas no irreversible labeling was observed with ^{3}H-DADLE or ^{3}H-BREM. Therefore, those ligands that exhibit a high degree of photosensitivity can irreversibly label receptors by this technique, whereas those that are relatively stable to irradiation with 254 nm UV light do not have the ability to photolabel receptors. Kinetics of ^{3}H-ligand destruction by UV light could therefore be used to predict which ligands would photolabel receptors. Also shown in Fig. 2 is the photosensitivity of membrane-binding sites for various ligands. When membranes were preirradiated for various times, binding for all four ligands was rapidly decreased, presumably by denaturation of receptor protein or destruction of key amino acid residues.

It is noteworthy that, for those ligands which photolabel receptors, the kinetics of ligand destruction follow very closely the kinetics of binding site loss. This presents the possibility that receptors are being destroyed, ligand is dissociating, and irreversible binding is occurring to sites other than receptors. To address this, the experiment described in Fig. 3 was carried out. Ligand was allowed to bind to steady state, membranes were washed, and then ligand was induced to dissociate from receptors into the milieu before irradiation or exposure to room light only. The pattern of ligand incorporation was then compared to that in membranes where no prior dissociation occurred. In the dissociated group, the pattern of labeling upon irradiation was markedly different from that in the undissociated group. In the dissociated group, comparison of nonirradiated samples to irradiated samples revealed that irradiation alone caused a marked increase in incorporation of ^{3}H-DHM. The net result was that the irradiated/denatured samples contained more counts than the non-irradiated/native samples, due to nonspecific incorporation of counts. Irradiation of undissociated membranes caused a slight decrease in binding compared to nonirradiated samples. Furthermore, irradiated/denatured samples contained fewer counts than nonirradiated/native samples in the undissociated group. Similar results were obtained with ^{3}H-ETOR, where simply boiling nonirradiated samples caused an incorporation of counts in the dissociated group. This was not observed in the undissociated group, where a decrease in binding was always observed upon boiling (not shown). These observations make it unlikely that the photolabeling observed when bound ligand is irradiated results from incorporation of dissociated ligand to non-receptor sites.

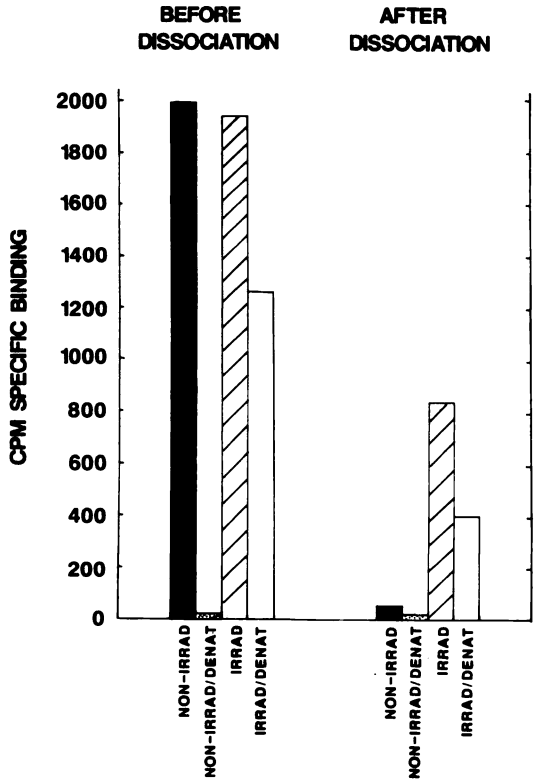

Fig. 3. Irradiation before and after dissociation of ^{3}H-DHM. Rat brain membranes were incubated with 2 nM ^{3}H-DHM in the presence or absence of 10 μM levallorphan and washed as described under Materials and Methods. The washed membranes were then resuspended in 10 mM Tris-HCl, pH 7.4, containing 250 mM NaCl. Half the suspension was immediately irradiated or exposed to room light only (*BEFORE DISSOCIATION*). The other half was allowed to incubate at 37° for 5 hr to allow dissociation of ligand into the milieu before irradiation or exposure to room light (*AFTER DISSOCIATION*). Similar results were obtained for ^{3}H-ETOR (not shown).

Fig. 4 shows results obtained when CHAPS/Lubrol extracts from cow striatal membranes photolabeled with ^{3}H-ETOR were chromatographed on a WGA column. Fig. 4A shows results from membranes which were washed prior to irradiation. Approximately 85% of the protein flowed through the column unretained, while 15% was retained and could be eluted with NAG. By estimating the ratio of cpm to protein in the two peaks, it was observed that the NAG-eluted fraction was labeled to a much higher specific activity than the flowthrough (specific activity of NAG-elute/specific activity of flowthrough = 13.3:1). Fig. 4B shows results from membranes which were not washed prior to irradiation. Compared to washed membranes, the number of counts incorporated was much higher in both fractions. In addition, there was no difference in the specific activity in the flowthrough and the NAG-eluted fraction (specific activity of NAG elute/specific activity of flowthrough = 0.96:1). This indicates random labeling of proteins and reiterates the need for removal of unbound ligand before the irradiation step.

Others have reported that complexes of ^{3}H-ETOR and receptor formed in intact membranes are relatively stable when solubilized with some detergents (27–29). These complexes could be subjected to dialysis and to gel filtration. We have observed that when membranes were bound with ^{3}H-ETOR, washed, and solubilized with CHAPS, the ^{3}H-ETOR did not readily dissociate from solubilized receptors (as assessed by gel filtration on Sephadex G-25). This would seem to indicate that covalent attachment of ^{3}H-ETOR to receptors is not necessary for carrying out chromatographic analyses of the complexes. However, heating extracts from nonirradiated membranes at 60° for 30 min caused almost complete dissociation due to denaturation. By contrast, when photolabeled membranes were used, heating of CHAPS extracts in this manner caused little dissociation (data not shown). This supports covalent attachment of ^{3}H-ETOR upon irradiation and demonstrates the utility of this technique if analysis of denatured receptors is required.

Fig. 5 shows results obtained when ^{3}H-ETOR was used to photolabel κ receptors in guinea pig brain membranes and detergent extracts chromatographed on Sepharose 6B-CL. κ receptors were selectively labeled in the presence of 100 nM unlabeled DSTLE and 100 nM unlabeled DAGO to mask δ and μ receptors, respectively. Membranes were then washed, irradiated, and extracted with CHAPS/Lubrol. After removal of

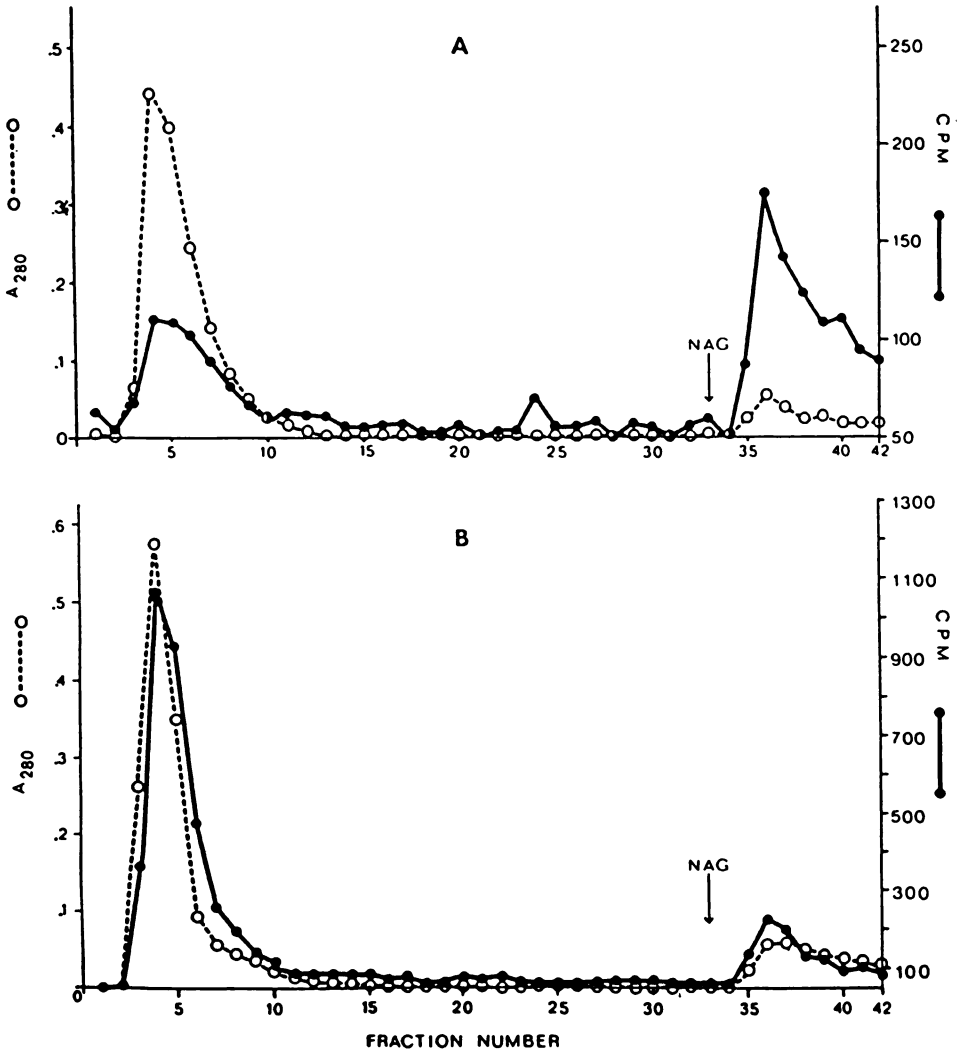

Fig. 4. WGA chromatography of detergent extract of cow brain membranes photolabeled with ^{3}H-ETOR. Bovine striatal P_2 membranes were incubated with 2 nM ^{3}H-ETOR, irradiated with or without prior washing, washed after irradiation, and extracted with CHAPS/Lubrol, all as described under Materials and Methods. After removal of free ^{3}H-ETOR by overnight dialysis, the solubilized proteins were subjected to lectin affinity chromatography on wheat germ lectin-Sepharose 6MB. Retained proteins were eluted with 0.25 M NAG as described under Materials and Methods. A. Membranes were washed three times by repeated centrifugation and resuspended in ice-cold buffer prior to irradiation. B. Membranes were irradiated without any prior washing. ↓, the start of NAG elution. The cpm are from 50-μl aliquots of each 1-ml fraction. Protein concentration was determined by absorbance at 280 nm (A_{280}). Results shown are from membranes incubated in the absence of 10 μM levallorphan. When membranes were incubated in the presence of 10 μM levallorphan, nonspecific binding was only 10–20% of total.

free ^{3}H-ETOR by chromatography on Sephadex G-25, the void volume was concentrated and chromatographed on Sepharose 6B-CL. The major peak of protein eluted with an apparent molecular weight of 2.8×10^6 Da (Fig. 5, peak *I*). Lower molecular weight proteins eluted at 17,000 Da (Fig. 5 peak *II*) and <13,700 Da (Fig. 5, peak *III*). Measurement of radioactivity associated with each peak revealed a differential distribution among these proteins. A major peak of radioactivity migrated at 2.4×10^6 Da, slightly shifted from the bulk of protein in peak I. Peaks II and III were also labeled. By estimating the ratio of cpm to protein in each peak, it was observed that the two low molecular weight peaks were labeled to much higher specific activity than the high molecular weight peak. The specific activity of peak II was 5 times higher than that of peak I, whereas the specific activity of peak III was 8 times higher than that of peak I. Neither of these peaks coeluted with free ^{3}H-ETOR. It is noteworthy, however, that peak III eluted after the included volume. This indicates a labeled protein which interacts with the column matrix, making it impossible to estimate its molecular weight under these elution conditions. Thus, ^{3}H-ETOR was not randomly incorporated but was selectively incorporated into proteins at high specific activity. These proteins may represent components of the κ receptor.

Discussion

Synthesis of photoaffinity labels requires the chemical modification of parent ligands to incorporate photoactive moieties such as azido and nitrophenyl azido groups into the molecule (22–24). Following synthesis, these compounds must be tested for maintenance of high affinity binding and receptor selectivity in competition binding assays. These ligands must then be synthesized in radiolabeled form and tested for specificity of incorporation of label into the membranes. This study shows that some commercially available radiolabeled opiate ligands possess the intrinsic property of being photoaffinity probes, in that they can be incorporated specifically and irreversibly into membrane proteins upon irradiation with UV light of 254 nm.

Labeling with high signal-to-noise ratio was only observed when unbound ligand was removed from the membranes prior to irradiation. This presumably prevents the photoincorporation of ^{3}H-ligand at non-receptor sites. Comparison of samples incubated in the presence of 10 μM unlabeled levallorphan with those incubated in the absence of unlabeled ligand revealed that specific binding levels of 50–80% could be achieved in denatured samples. This is a suitable signal-to-noise ratio for analysis of ligand-receptor complexes. In addition to surviving boiling in 10 mM Tris buffer as described, we have observed that photoincorporated counts also survive boiling in Laemmli

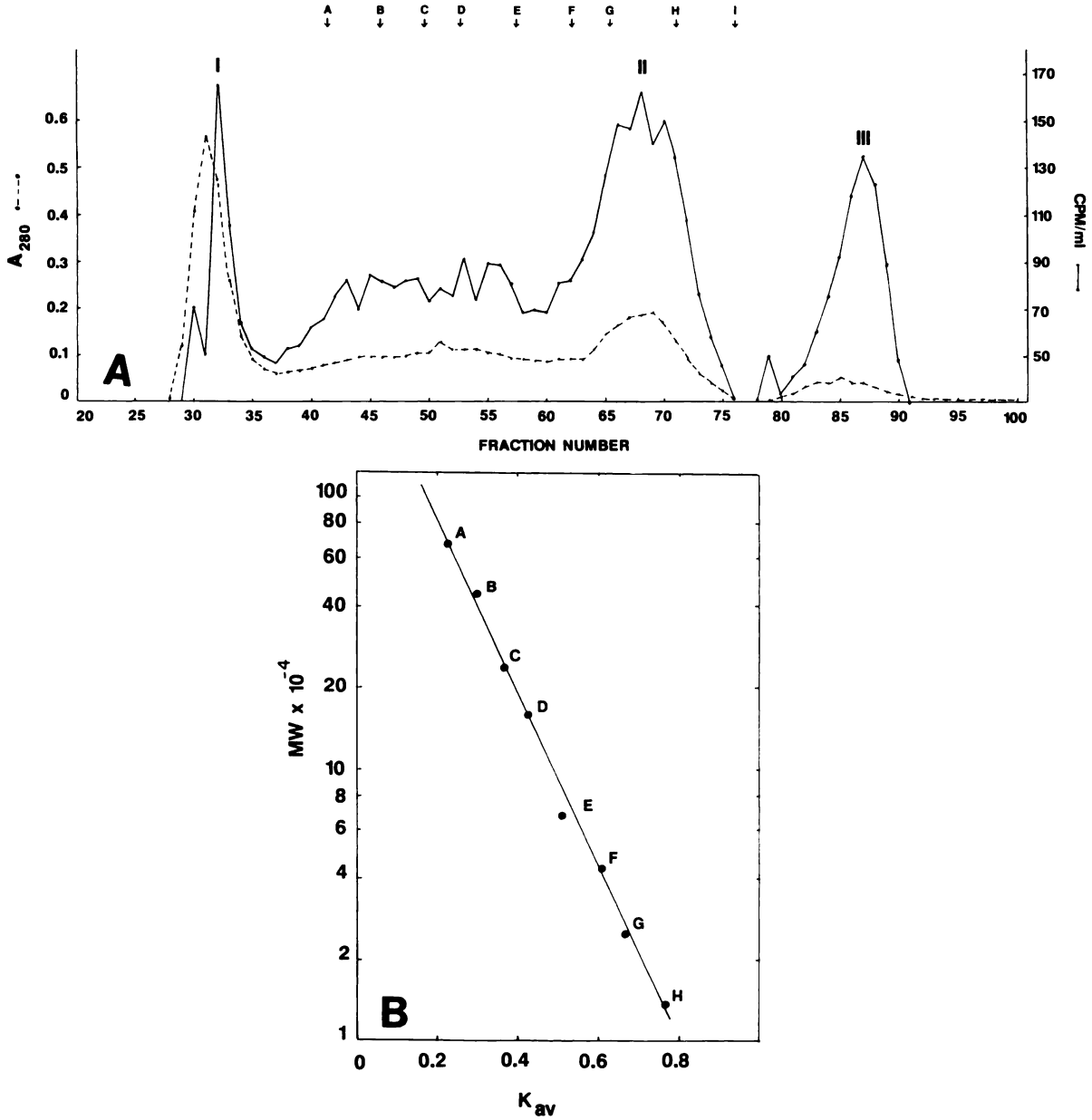

Fig. 5. Sepharose 6B-CL chromatography of detergent extract of guinea pig brain membranes photolabeled with ^{3}H-ETOR. A. Guinea pig brain membranes were incubated with 5 nM ^{3}H-ETOR as described under Materials and Methods except that 100 nM unlabeled DAGO and 100 nM unlabeled DSTLE were included. These conditions result in selective labeling of κ opiate receptors. Membranes were then washed, irradiated, and extracted with 13 mM CHAPS/1% Lubrol as described under Materials and Methods. Free ^{3}H-ligand was removed by chromatography on Sephadex G-25. The concentrated void volume was applied to Sepharose 6B-CL and eluted as described under Materials and Methods. Fractions of 2 ml were collected. Protein concentration was determined by absorbance at 280 nm (A_{280}) and tritium was monitored by scintillation spectrometry. Positions of molecular weight standards are indicated by *arrows*: A, thyroglobulin, 669 kDa; B, ferritin, 440 kDa; C, catalase, 232 kDa; D, aldolase, 158 kDa; E, bovine serum albumin, 67 kDa; F, ovalbumin, 43 kDa; G, chymotrypsinogen A, 25 kDa; H, ribonuclease A, 13.7 kDa; I, ^{3}H-ETOR. The column calibration curve is shown in B.

sample buffer (30). This further confirms that the incorporation of label is of a covalent nature.

Opiate receptors have been shown to be sensitive to trypsin, indicating that at least a major component of the receptor is proteinaceous and is exposed from the lipid bilayer (31). Trypsin treatment of membranes photolabeled with ^{3}H-ETOR caused marked loss of counts from membranes. This loss is presumably due to cleavage and release of fragments of the receptor which bear the covalently attached ^{3}H-ligand. This indicates that a protein component in the membrane is photolabeled. A lower per cent loss was observed in the nonspecific binding samples, indicating that nonspecific binding may be largely to non-proteinaceous sites (not shown).

There was a high degree of correlation between photolability of ligand and its ability to photolabel receptors. Those ligands which were highly sensitive to UV irradiation also photolabeled receptors. Ligands resistant to photodecomposition or those which were slowly photodecomposed lacked the ability to photolabel receptors under these conditions. The labeled products which bind covalently to the receptors are unknown. Presumably, the active species is of a free radical nature resulting from photodecomposition of the ^{3}H-ligand. Unlike ligands with elec-

trophilic moieties, this highly reactive species may not require specific nucleophiles at or near the ligand-binding site in order to form a covalent bond with the receptor.

In addition, the binding sites for these ligands were all highly sensitive to UV irradiation. Binding site photosensitivity has been observed by others (25) and could be due to UV-induced denaturation of protein or selective destruction of UV-absorbing amino acid residues. For ligands which photolabeled receptors, the rate of ligand destruction closely paralleled the rate of binding site loss. This may account for the average 50% yield of photoincorporation observed with most ligands. As ligand is photolyzed, receptor is denatured, preventing greater incorporation of ligand. It is interesting to note that ^{3}H-EKC was unusual in that it gave a 90% yield of photoincorporation. We have observed that, unlike binding sites for other opiate ligands, including ^{3}H-BREM, the binding sites for ^{3}H-EKC were relatively resistant to destruction by UV light (not shown). This could account for the high yield of incorporation. The reason for the difference in photosensitivity of binding sites for ^{3}H-EKC and ^{3}H-BREM, which have similar binding characteristics, is not known and is presently under study.

It is interesting to note that photosensitivity of binding sites was apparently condition dependent. For example, Roques and co-workers (18) observed that irradiation of membranes with a 50-W mercury lamp at 20 cm destroyed 15% of δ sites (7 min irradiation) and 25% of μ sites (14 min irradiation). The differences in receptor loss which they observed and the results reported here are most likely due to differences in irradiation intensity. We have not investigated greater irradiation distances. However, we have observed that irradiation with a lower intensity lamp resulted in decreased loss of binding sites but at the expense of a concomitant decrease in photoincorporation of ^{3}H-ligand. How photodamage to receptors might affect the validity of physicochemical information obtained by analysis of these labeled complexes is a question which must be kept in mind.

The loss of binding sites upon irradiation opens the possibility that, during irradiation, ligand could dissociate and become covalently attached to non-receptor sites. Experiments were performed to ascertain the pattern of labeling when bound ligand was released from receptors into the milieu and membranes irradiated. When ligand was dissociated from receptors, the labeling obtained after the various manipulations was markedly different from that observed when ligand was bound. Most notable was that irradiation itself caused a marked incorporation of counts into membranes, the net result being that irradiated/denatured membranes incorporated more counts than nonirradiated/native samples. Since the pattern of label incorporation was different in dissociated membranes, it is unlikely that label incorporated in the standard procedure is due to ligand which has dissociated from receptors and become attached to other proteins.

Gioannini *et al.* (32) reported that opiate binding activity, present in detergent extracts of brain membranes from several species, could be retained on WGA affinity columns. This activity could be eluted by competition with NAG. With extract from cow striatal membranes, these investigators showed that 11% of the total ^{3}H-diprenorphine-binding activity was present in the flowthrough whereas 47% was retained and eluted with NAG. These data demonstrate that the opiate receptor(s) contain(s) a carbohydrate moiety. In the present study, retention of CHAPS/Lubrol-solubilized receptors photolabeled with ^{3}H-ETOR on WGA is consistent with the glycoprotein nature of the receptor. The specific activity of the NAG eluate was 13-fold higher than the flowthrough, indicating specific incorporation of label into receptors and not random incorporation into various proteins. Random incorporation was obtained, however, when membranes were not washed prior to irradiation. Further purification of receptors can be obtained upon resolution of the glycoproteins in the NAG-eluate.

Guinea pig brain membranes have been shown to contain a high concentration of κ opiate receptors (33). κ receptors can be labeled with nonselective ^{3}H-ligands when binding to μ and δ receptors is blocked by simultaneous incubation in the presence of unlabeled μ and δ ligands (34). Proteins from guinea pig brain membranes photolabeled under these conditions were subjected to molecular sizing on Sepharose 6B-CL. Proteins of 17,000 Da and <13,700 Da were labeled to high specific activity. A peak in the high molecular weight fraction was labeled with lower specific activity. Low molecular weight proteins which bind ^{3}H-ETOR have been observed by others (29). Also, Itzhak *et al.* (35) resolved a 4×10^5 Da protein from guinea pig brain on sucrose density gradients. This fraction exhibited binding of ^{3}H-BREM in the presence of μ and δ blockers. The proteins photolabeled by ^{3}H-ETOR may represent κ receptors or fragments thereof. This result again shows that random proteins are not being labeled and shows the potential of this photolabeling method for characterization of receptor components.

In conclusion, this method produced irreversible binding of several selective and nonselective ^{3}H-ligands, in high yield and with high signal-to-noise ratios. Incubation conditions could be manipulated (3), unlabeled blocking ligands could be used, or membranes could be pretreated with receptor acylating or alkylating agents (36, 37) so as to target even nonselective photolabile ^{3}H-ligands to specific receptor types. Therefore, it is not necessary to produce selective affinity labels for each receptor type. These radiolabeled ligands are commercially available and have been extensively characterized for binding affinity and receptor selectivity. Use of their intrinsic photolability may circumvent the costly synthesis and characterization of new photoaffinity probes and will greatly facilitate molecular studies of opiate receptors.

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