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MOUSE BRAIN OPIOID RECEPTOR IDENTIFICATION BY DIRECT ULTRAVIOLET PHOTOAFFINITY LABELING

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Abstract—[^{3}H]Morphine, PL-017[prolyl-3,4-^{3}H,D-prolyl,3,4-^{3}H] ([^{3}H]PL-017) and enkephalin-(2-D-penicillamine,5-D-penicillamine)[tyrosyl-2,6-^{3}H] ([^{3}H]DPDPE) were directly cross-linked to mouse brain opiate receptors by an ultraviolet (254 nm) irradiation procedure. [^{3}H]Morphine preferentially and specifically labeled a 58 kDa protein. The labeling of this protein was suppressed by the addition of excess naloxone. Dithiothreitol reduced the irreversible binding of [^{3}H]morphine and [^{3}H]PL-017 to the receptor protein. In the acid hydrolysate of [^{3}H]DPDPE-labeled opiate receptors, dityrosine, was detected. These results suggest that the [^{3}H]tyrosine residue of [^{3}H]DPDPE covalently bound the tyrosine residue of δ-opioid receptors. The direct UV-photoaffinity labeling method using commercially available radioactive opiates described here should be a useful tool for characterization and purification of the opiate receptors.

The existence of various types of opiate receptors, e.g. μ, κ and δ, has been demonstrated by pharmacological and ligand-binding studies. However, the molecular nature of these receptors is controversial and a necessary first step in studying the structure and function of the opiate receptors is the identification of the receptors by appropriate techniques. The photoaffinity labeling technique has been widely used to identify opiate receptors [1, 2]. However, this technique generally requires modification of the parent ligands to introduce photoreactive moieties such as azido or nitrophenylazido groups into the molecules. These selectively and covalently label the receptors, and have high ratios of specific to nonspecific binding. On the other hand, a simple photoaffinity labeling technique has been used for various receptors, and some ^{125}I-labeled peptide ligands, without the introduction of any artificial photoreactive group, were covalently cross-linked to their receptors by simple ultraviolet (UV) irradiation [3–5].

In this paper, we report the direct covalent linking of [^{3}H]morphine and other commercially available radiolabeled opiate ligands with opiate receptors in the mouse brain by UV irradiation. Furthermore, we also report that opiate receptors have a tyrosine residue at the ligand binding site in opiate receptors.

MATERIALS AND METHODS

Materials. Male ddY mice were purchased from SLC (Shizuoka, Japan). [^{3}H]Morphine[*N-methyl*-^{3}H] (50.6 Ci/mmol) and PL-017-[prolyl-3,4-^{3}H,D-prolyl,3,4-^{3}H] ([^{3}H]PL-017) (110.2 Ci/mmol), enkephalin-(2-D-penicillamine,5-D-penicillamine)-[tyrosyl-2,6-^{3}H] ([^{3}H]DPDPE) (51.5 Ci/mmol, and enkephalin-(2-D-ala,5-D-leu)[tyrosyl-3,5-^{3}H] ([^{3}H]-DADLE) (34.4 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Wheat germagglutin (WGA†)–agarose gel was from Hohnen Co. Ltd (Tokyo, Japan). X-ray film (XAR-5) was from Kodak, and the fluorographic reagent Enlightning was from New England Nuclear. Naloxone, electrophoresis chemicals and premixed standards were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The dityrosine standard was synthesized by the procedure of Amado *et al.* [6]. All other solvents and reagents were of the highest grade available.

Direct UV photoaffinity labeling of opiate receptors. The procedures for photoaffinity labeling were essentially the same regardless of the ^{3}H-labeled ligand. The typical procedure for [^{3}H]morphine was as follows. Mouse brain P_2-membranes were prepared as reported previously [7]. P_2-Membranes (2–3 mg/mL) were incubated with 10 nM [^{3}H]-morphine for 15 min at 30° in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After preincubation, the mixture was irradiated on ice with a UV mercury lamp (8 W) at a distance of about 10 cm (254 nm, 1200 μW/cm^2). Unless otherwise indicated, the irradiation time was 30 min. At the end of the irradiation period, 5-fold excess 50 mM Tris–HCl buffer, pH 7.4, containing 10 μM of naloxone was added, the mixture was left to stand for 15 min at 30°, and the P_2 membranes were washed. Photoaffinity bound [^{3}H]-morphine was separated from free [^{3}H]morphine by rapid filtration through GF/B glass fiber filters. The filters were washed with 20 mL of 50 mM Tris–HCl buffer, pH 7.4, containing 10 μM naloxone. Nonspecific binding was determined in the presence of 10 μM naloxone. The filters were then transferred to vials containing 10 mL of Aquasol-2, and radioactivity was determined in a liquid scintillation spectrometer (Packard 2000CA TRI-CARB). For the analysis of ^{3}H-ligand-labeled proteins by sodium

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† Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

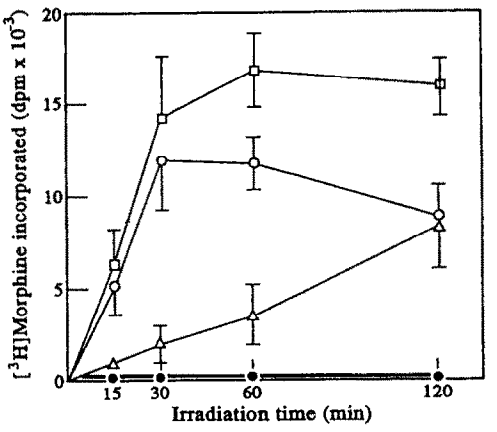

Fig. 1. Effect of UV irradiation time on cross-linking of [^{3}H]morphine in P_2-membranes. Mouse brain membranes were incubated with 2 nM [^{3}H]morphine in the presence or absence of 10 μM naloxone as described in Materials and Methods. Labeled membranes were placed on ice and treated as follows: (●) membranes were dark treated (exposed to room light only) before filtration; (□, △) membranes were UV irradiated before filtration. Non-specific and total cross-linking were determined in the presence (△) and absence (□) of 10 μM unlabeled naloxone, respectively. Specific cross-linking (○) was the difference between the total and non-specific cross-linking values. Each experiment was performed in triplicate. Data represent the means ± SE of three experiments.

dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the membranes were collected by centrifugation at 20,000 *g* for 15 min at 4°. The pellet was washed once with the same buffer and processed for SDS–PAGE according to the method of Laemmli [8].

Effect of dithiothreitol (DTT) on the photoaffinity labeling of [^{3}H]*morphine or* [^{3}H]*PL-017 to its receptors.* The P_2-membranes were preincubated with 10 nM [^{3}H]morphine or [^{3}H]PL-017 in 50 mM Tris–HCl buffer, pH 7.4, containing 0–1.0 mM DTT for 15 min at 30°. After preincubation, the P_2-membrane preparation was irradiated for 30 min on ice with the UV mercury lamp at a distance of about 10 cm. The membranes were then collected by centrifugation at 20,000 *g* for 15 min at 4°. The pellet was washed once with the same buffer. The membrane preparation was then subjected to SDS–PAGE.

Chemical characterization of dityrosine photolabeled [^{3}H]*DPDPE-opiate receptors.* Photoaffinity labeling of opiate receptors using [^{3}H]DPDPE was performed as described above. The P_2-membranes photoaffinity labeled with [^{3}H]DPDPE were partially purified using the WGA–agarose column. WGA–agarose column-purified labeled receptors were placed in a glass tube containing 2 mL of 6 N HCl, sealed under N_2, and hydrolysed overnight at 115°. The hydrolysate was then diluted 8-fold with water, and evaporated under reduced pressure. [^{3}H]-Dityrosine was analysed by the method described by Zaitsu *et al.* [9]. In brief, after the dityrosine standard was added, the sample was applied to a Dowex 1 × 8 column (CH_3COO^- form, 1 × 10 cm) and eluted with 0.1 N acetic acid (pH 3.2). The fluorescence fraction was pooled and then applied to HPLC. The dityrosine was analysed by ion exchange chromatography using a Radialpac SAX column (8 mm × 10 cm, Waters, Milford, MA, U.S.A.) and 0.1 M acetic acid buffer, pH 3.2 at a flow rate of 0.4 mL/min. The dityrosine was monitored with a spectrofluorometer at an emission wavelength of 404 nm and an excitation wavelength of 287 nm, and 0.2-mL fractions were collected. The fractions were pooled and a 0.1 mL aliquot of each fraction was counted in a scintillation spectrometer.

SDS–slab gel electrophoresis. SDS–PAGE was performed according to the method of Laemmli [8] using 9% polyacrylamide gel (1 × 120 × 135 mm). Upon completion of the electrophoresis, the gel was stained with Coomassie brilliant blue R-250, dried and autoradiagraphed at −80° for 1–2 weeks using Enlightning.

RESULTS

Photoaffinity labeling of P_2-membrane with ^{3}H-labeled opiate ligands

Figure 1 shows the effect of irradiation time on

Table 1. Photoaffinity labeling of ^{3}H-opiates in mouse brain P_2-membranes

	[^{3}H]Morphine	[^{3}H]PL-017	[^{3}H]DADLE	[^{3}H]DPDPE
	Incorporated ^{3}H-radioactivity (dpm/mg protein)			
Treatment				
UV irradiated	14,265 ± 4301	5742 ± 765	1371 ± 326	5386 ± 288
with naloxone	2127 ± 1472	2274 ± 139	1240 ± 167	2876 ± 361
Non-irradiated	1212 ± 467	701 ± 146	1214 ± 79	1182 ± 49

Mouse brain membranes were labeled using 2 nM ^{3}H-ligand in the presence or absence of 10 μM naloxone under the conditions described in Materials and Methods. Membranes were then UV irradiated for 30 min on ice. UV irradiated or non-irradiated membranes were washed as described in Materials and Methods. The membranes labeled with ^{3}H-ligand were collected on a glass fiber filter (GF/B), and the radioactivity was measured.

Data represent the means ± SE of three experiments. Each experiment was carried out in triplicate.

Table 2. Effect of UV wavelength on photoaffinity labeling of opioid receptors with [^{3}H]PL-017

Wavelength	Incorporated radioactivity (dpm/mg protein) Without naloxone	With naloxone
Non-irradiated	756 ± 197	701 ± 146
254 nm	5742 ± 765	2274 ± 139
312 nm	1947 ± 299	1565 ± 237
360 nm	886 ± 87	755 ± 33

Mouse brain membranes were UV irradiated for 30 min on ice using 2 nM [^{3}H]-PL-017 in the presence or absence of 10 μM naloxone under the conditions described in Materials and Methods. The membranes labeled with [^{3}H]PL-017 were collected on a glass fiber filter (GF/B), and the radioactivity was measured.

Data represent the means ± SE of three experiments. Each experiment was carried out in triplicate.

the photoaffinity labeling of [^{3}H]morphine, a μ-receptor ligand, in mouse P_2-membranes. Photolabeled ^{3}H-radioactivity increased with the duration of irradiation, and the incorporation of radioactivity reached a plateau at 60 min. In contrast, samples exposed only to room light showed no irreversible incorporation over a 120 min period. Non-specific labeling measured in the presence of a large excess of naloxone also increased gradually with time. Thus, maximal specific labeling was observed after 30–60 min of irradiation. Longer irradiation times decreased the incorporation of radioactivity, possibly a result of slow damage to receptors.

When photoaffinity labeling of opiate receptors by other ^{3}H-opiate ligands was carried out, [^{3}H]-morphine, [^{3}H]PL-017 and [^{3}H]DPDPE photolabeled the P_2-membranes irreversibly using UV irradiation of 254 nm. In contrast, irreversible labeling was not observed with [^{3}H]DADLE (Table 1). None of these ligands showed irreversible labeling with UV irradiation of 356 nm (Table 2).

To confirm these results, we analysed the photolabeled molecules by SDS–PAGE. After irradiation, the membranes were solubilized, and submitted to SDS–PAGE under reducing conditions. Figure 2 shows the fluorography of these experiments. [^{3}H]-Morphine was incorporated into one major peak with an M_r of 58,000 (Fig. 2, lane c). The addition of naloxone completely suppressed the labeling of the 58 kDa protein (Fig. 2, lane b).

Effect of DTT on the photoaffinity labeling of [^{3}H]-*morphine or* [^{3}H]*PL*-017 *to its receptors*

We assumed that photoaffinity labeling occurred as a result of radicals from the ^{3}H-labeled ligands being activated by UV irradiation and that the presence of a radical scavenger would suppress the labeling. To confirm this, we studied the effect of DTT on the reaction of [^{3}H]morphine or [^{3}H]PL-017 on the P_2-membranes. Treatment of membranes with DTT alone has no effect on specific binding of receptors [10, 11]. The effect of DTT on the photoaffinity labeling of [^{3}H]morphine to the P_2-membrane preparation is shown in Fig. 3. The presence of DTT (0.1–0.5 mM) in the irradiating medium resulted in diminution of the labeling of

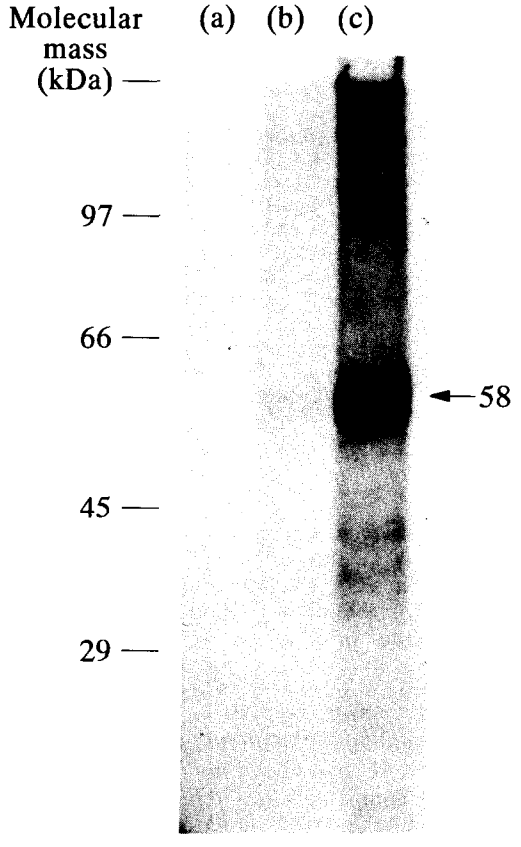

Fig. 2. Autoradiograms of photoaffinity labeled [^{3}H]-morphine in opiate receptors. P_2-membranes were incubated with [^{3}H]morphine in the presence (lane b) or absence (lane c) of 10 μM naloxone for 15 min at 30°, then UV irradiated for 30 min on ice and analysed by 10% SDS–PAGE. The non-irradiated reference sample was allowed to stand on ice in room light for 30 min (lane a). Gels were stained, destained, impregnated with Enlightning (New England Nuclear), and dried.

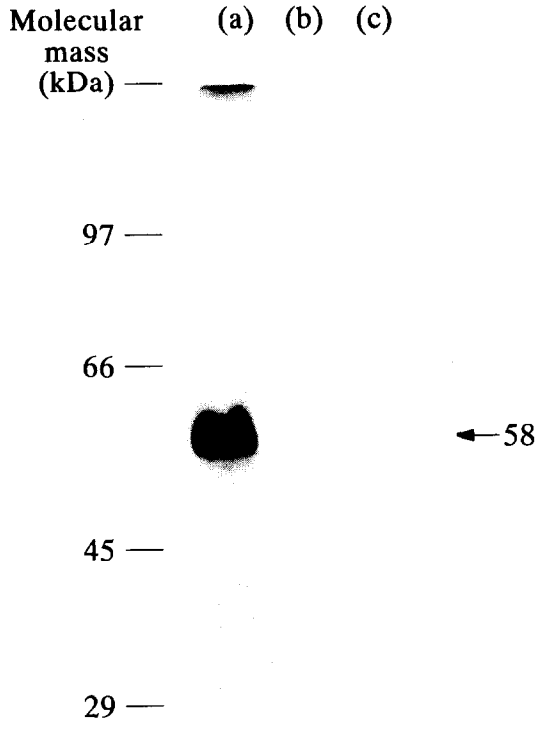

Fig. 3. Effect of DTT on the photoaffinity labeling of [^{3}H]-morphine to its receptor. The P_2-membrane preparation was preincubated for 15 min at 30° with 10 nM [^{3}H]-morphine in a 50 mM Tris–HCl buffer, pH 7.4, containing 0–1.0 mM DTT. After preincubation, the P_2-membrane preparation was irradiated with a UV mercury lamp as described in Materials and Methods. Membranes were then collected by centrifugation at 20,000 *g* for 15 min at 4°, and the supernatant containing free [^{3}H]morphine was discarded. The membrane preparation was subjected SDS–PAGE. Lane a: without DTT, lane b: with 0.1 mM DTT, lane c: with 0.5 mM DTT.

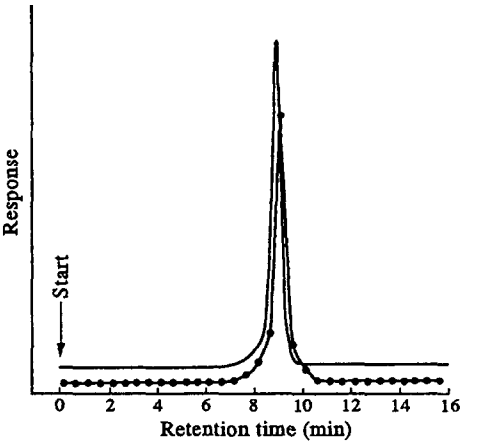

Fig. 4. Elution profile of acid hydrolysed [^{3}H]DPDPE photolabeled opiate receptors. Partially purified [^{3}H]-DPDPE photolabeled opiate receptors were hydrolysed by 6 N HCl. The interfering compound in the acid hydrolysate was removed by a Dowex 1 × 8 column. The fluorescence fraction was analysed by HPLC using a Radialpac SAX column, 8 mm × 10 cm (Waters, Milford, MA, U.S.A.) and 0.1 M acetic acid buffer, pH 3.2, at a flow rate of 0.4 mL/min. The dityrosine was monitored by a spectrofluorometer operating at an emission wavelength of 404 nm and an excitation wavelength of 287 nm, and 0.2-mL fractions were collected. The fractions were pooled and a 0.1-mL aliquot of each fraction was counted in a scintillation spectrometer. (●) Radioactivity; (—) fluorescence activity.

[^{3}H]morphine, and the presence of 0.5 mM of DTT photoaffinity labeling of [^{3}H]PL-017 was 30% of control. Thus, DTT markedly attenuates the photoaffinity labeling of [^{3}H]morphine and [^{3}H]PL-017 to receptor proteins.

Chemical characterization of dityrosine photolabeled [^{3}H]DPDPE-opiate receptors

Partially purified [^{3}H]DPDPE photolabeled opiate receptors were hydrolysed by 6 N HCl, and the interfering compounds was removed by a Dowex 1 × 8 column. Figure 4 shows a chromatogram of the hydrolysate of [^{3}H]DPDPE labeled receptors. The major ^{3}H-radioactivity was coeluated with a dityrosine standard.

DISCUSSION

The preparation of suitable photoreactive ligands requires sophisticated chemical techniques and may generate fragile and unstable compounds. Following synthesis, these compounds must be tested for the maintenance of affinity binding and receptor selectivity in competition binding assays. These ligands must be synthesized in radioactive form. The advantage of direct cross-linking by UV irradiation is its simplicity as it eliminates the need to synthesize chemically modified ligands. For this reason, cross-linking of a hormone to its receptors by the direct UV irradiation method has been used for various receptors [3–5].

It has been reported that some commercially available ^{3}H-opiates are intrinsically photo-labile, and irradiation of short-wave length UV light causes photochemical decomposition [12]. In addition, opiate receptors are very sensitive to UV light, which causes rapid destruction of opiate binding activity [12]. This study showed that some commercially available radioactive opiate ligands possess the intrinsic property of photoaffinity probes for direct UV irradiation. [^{3}H]Morphine and [^{3}H]PL-017 were irreversibly incorporated into receptors upon irradiation with UV light of 254 nm. On the other hand, UV irradiation failed to cause irreversible incorporation of [^{3}H]DADLE (Table 1). These results agree well with those observed previously [13].

Kooper *et al.* [13] reported that [^{3}H]etrophine and other ^{3}H-labeled opiate ligands were labeled irreversibly to opiate receptors by UV irradiation, but they did not determine the amino acid residues of the ligand binding site. We attempted to clarify the chemical mechanism for photoaffinity labeling

of tyrosine residue containing ^{3}H-labeled ligand. Photoaffinity cross-linking of a ligand to its receptor can be achieved when both molecules have reactive groups in suitable configurations such as phenolic residues. [^{3}H]Morphine and [^{3}H]PL-017 possess this residue, which may be involved in the reaction. In addition, this residue is not labeled by tritium. In the case of [^{3}H]DADLE, the phenolic residue is labeled by tritium at the 3 and 5 positions. Therefore, it is possible that tritium may be eliminated by UV irradiation. This speculation was supported by the results that [^{3}H]morphine and [^{3}H]PL-017 specifically and irreversibly labeled receptors, whereas [^{3}H]-DADLE failed to cause irreversible labeling (Table 1).

We have used UV irradiation to cross-link [^{3}H]-morphine to its receptor sites on mouse brain P_2-membranes. In this study, we estimated the apparent molecular weight of the protein that incorporated [^{3}H]morphine to be 58 kDa. This protein probably represents the opiate receptor, since labeling was inhibited by the presence of naloxone. The M_r 58,000 agrees well with that reported for the μ-opiate receptor identified by affinity labeling methods [14–16].

Tyrosyl radicals have been generated by UV irradiation of aqueous tyrosine solution [17], and antioxidants are capable of scavenging the tyrosyl radical [18]. The generation of dityrosine during ultraviolet irradiation of poly(L-tyrosine), copolymers, and the dipeptide Try–Try was demonstrated by Lehrer and Fasman [17]. Moreover, the presence of Ca^{2+} dityrosine was detected by UV irradiated calmodulin [19].

In this study dityrosine was detected in the acid hydrolysate of opiate receptors photoaffinity labeled by [^{3}H]DPDPE. Receptors bound the ligand at the binding site, which affected the reactivity, accessibility, and/or proximity of the tyrosine residue of receptors and the [^{3}H]tyrosine residue of [^{3}H]-DPDPE suggesting that the binding site of δ-opiate receptors has a tyrosine residue.

Photo-oxidation destroys cysteine, methionine and tyrosine residues [20]. DTT in the UV irradiation medium diminished the photoaffinity labeling of opiate receptors (Fig. 3). This result indicates that DTT acts as a scavenger and protects against the photo-oxidation of [^{3}H]morphine and [^{3}H]PL-017, and reduces the covalent binding of ^{3}H-radioactivity to opiate receptors.

The advantage of direct cross-linking by UV irradiation is its simplicity; with this procedure, it is not necessary to produce selective affinity labels for each receptor type. These radiolabeled ligands are commercially available, eliminate the need for the addition of cross-linking arms and have been extensively characterized for binding affinity and receptor selectivity. Direct cross-linking by UV irradiation may prove to be a powerful analytical tool in identifying and characterizing opiate receptors.

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