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Photoaffinity Labeling of Opioid δ Receptors with an Iodinated Azido-Ligand: [^{125}I][D-Thr^2,pN$_3$Phe^4,Leu^5]enkephalyl-Thr^6

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SUMMARY

The photoaffinity ligand of the δ opioid receptor Tyr-D-Thr-Gly-pN$_3$Phe-Leu-Thr (azido-DTLET) was iodinated and purified by high performance liquid chromatography. Monoiodo-azido-DTLET displayed a high affinity (K_D = 15 nM) and is selective ($K_{I\mu}/K_{I\delta}$ = 9.8) for rat brain δ opioid receptors (for comparison, the corresponding values for tritiated azido-DTLET are K_D = 1.66 nM and $K_{I\mu}/K_{I\delta}$ = 27). On rat brain sections, the anatomical distribution of [^{125}I]azido-DTLET binding sites revealed by autoradiography corresponds to that of δ receptors. On rat brain membrane homogenates and NG108-15 hybrid cells, UV irradiation of the receptor-ligand complex results in the irreversible binding to membrane proteins of 14% of the bound radioactivity. Gel electrophoresis of [^{125}I]azido-DTLET-labeled proteins followed by autoradiography shows a different pattern in rat brain and NG108-15 cells. In rat brain, labeling of two of these proteins, with molecular weights of 44,000 and 34,000, was inhibited by 30 nmol/liter of nonradioactive DTLET, a δ-selective ligand but not by the same concentration of [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin, a μ-selective ligand. In NG108-15 cells, this 44-kDa protein was not visualized; the main band was at 33 kDa and disappeared in the presence of levorphanol.

The existence of several types of opioid receptors in mammalian nervous system is now well established. At least three types of receptors, μ, δ, and κ have been identified on the basis of their different binding properties and pharmacological profiles (for review see Ref. 1). In addition, opioid receptors have also been detected in culture of cells derived from tumors and, particularly, in the NG108-15 hybrid cells. In these cells, which result from the fusion of two cell lines, a rat glioma and a mouse neuroblastoma, opioid receptors of the δ type only are found (2–4). However, the exact relationship between these types of receptors is still unclear. It is not known, for instance, whether the three different types of binding site correspond to different protein sequences, to oligomeric complexes with some common subunits, or even to proteins sharing the same amino acid sequence but undergoing different post-translational modifications. It is also unknown whether these different receptors represent truly distinct biochemical entities or the interconvertible forms of a single opioid receptor. The biochemical characterization of opioid receptors should help to clarify these points and to elucidate the mechanisms of action of endogenous and exogenous opioid compounds. Many attempts have been made to solubilize active opioid receptors but their binding sites have proved to be very sensitive to detergents and only recently have several groups succeeded in obtaining soluble receptors still able to bind ligands (5–7). To avoid the difficulties associated with solubilization, several *in situ* methods have been tested to achieve covalent labeling of the receptor in the membranes. They include the use of alkylating agents (8, 9), bifunctional agents to cross-link radioactive ligands (10), and photoaffinity ligands, which can be activated by light to react with the receptor (11–17). This article describes the use of the radioactive iodinated form of the photoaffinity ligand azido-DTLET derived from the δ-selective opioid agonist DTLET (18), to label the δ opioid receptors of rat brain and of the neuroblastoma-glioma hybrid cells NG108-15.

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Materials and Methods

Chemicals

[^{3}H]DTLET (2.1 TBq/mmol, 57 Ci/mmol) was synthesized by CEA, Service des molècules marquèes, Saclay, France. Na^{125}I (carrier-free) and [^{3}H]DAGO (1.48 TBq/mmol, 40 Ci/mmol) were obtained from

ABBREVIATIONS: azido-DTLET, Tyr-D-Thr-Gly-pN$_3$Phe-Leu-Thr; DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr; I-azido-DTLET, iodinated azido-DTLET; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TCA, trichloroacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin.

New England Nuclear (Boston, MA). Levorphanol tartrate was purchased from Hoffmann-La Roche (Basel, Switzerland) and porcine skin gelatin (175 Bloom grade) from Sigma Chemical Co. (St Louis, MO). Other chemicals were of analytical grade.

Cell Lines

The initial stocks of the hybrid cell lines NG108-15 and NG108-5 and their parents, the mouse neuroblastoma N18TG2 and the rat glioma C6BU-1, were gifts from Drs. Reiser and Hamprecht (University of Tübingen, Federal Republic of Germany). Cells were used between passages 20 and 25.

Ligand Preparation

The synthesis of azido-DTLET has already been described elsewhere (13). For this study (I-azido-DTLET) has been prepared by the chloramine T method (19); briefly, 8 μg of azido-DTLET in 2 μl of water, 15 μl of 0.5 M pH 7.4 sodium phosphate buffer, and 37 MBq (1 mCi) of $Na^{125}I$ or an equivalent amount of nonradioactive $Na^{127}I$ were added successively in a 1.5-ml Eppendorf tube. The reaction was initiated by the addition of 5 μl of 7 mM chloramine T and stopped after 30 sec at room temperature with 10 μl of 26 mM sodium metabisulfite, both in phosphate buffer. The reaction products were separated by HPLC on an Ultrasphere Octyl column (0.45 × 25 cm; Altex, San Ramon, CA) eluted with a 30-min linear gradient of 0% to 50% acetonitrile in triethylamine formate, 0.25 M, pH 3, at a flow rate of 1 ml/min. The radioactivity of the eluant from the column was monitored continuously with the γ probe (SMIG 724) of a Nardeux MIP 10 radioactivity detector and the corresponding signal was recorded. The same method was used for the preparation of iodinated DTLET. When nonradioactive ligand was prepared, the UV absorbance was measured at 280 nm with a Waters model 441 UV detector. The molecular weight of nonradioactive Iazido-DTLET was verified using a Varian mass spectrometer with the fast atom bombardment ionization technique. The iodinated peptides were stored in the elution buffer at −20° and were used with similar results up to 6 weeks after the iodination.

Equilibrium Binding to Rat Brain Membranes

Membrane preparation. Rats (Sprague Dawley, male, 250 g) were sacrificed by decapitation and the brains were quickly removed. The brain minus cerebellum was homogenized (10 strokes) in a Thomas glass-Teflon homogenizer in 12 volumes of ice-cold Tris·HCl buffer, 1 mM, pH 7.4, containing 0.32 M sucrose. The homogenate was then incubated 30 min at 37° to remove any endogenous ligand and centrifuged 30 min at 160,000 × *g* in a Beckman Ti60 rotor. The resulting pellet was resuspended in Tris-HCl, 50 mM, pH 7.4, centrifuged a second time under the same conditions, and finally resuspended in the initial volume of Tris·HCl, 50 mM, pH 7.4. This preparation constituted the crude membrane fraction. In several experiments the membranes were prepared using only the neostriatum.

Ligand binding. Binding experiments were performed at 37° with continuous shaking in polypropylene tubes protected from the light by stainless steel jackets. The incubation was carried out in 50 mM Tris·HCl, pH 7.4, in a final volume of 1 ml; radioactive ligand in Tris·HCl (containing 0.01% BSA when an iodinated ligand was used), increasing quantities of nonradioactive ligand and, for nonspecific binding determination, levorphanol to a final concentration of 10 μM, were each added in 100 μl of Tris·HCl to the appropriate volume of buffer. The reaction was initiated by the addition of 100 μl of crude membrane fraction [approximately 400 μg of protein as determined by the method of Lowry *et al.* (20) with BSA as a standard]. After incubation, the reaction was stopped by addition of 4 ml of ice-cold Tris·HCl with 0.01% BSA (TBSA) to each tube. The mixture was immediately filtered under reduced pressure on Whatman GF/B filters soaked in TBSA and the filters were rinsed twice with 4 ml of ice-cold TBSA. The radioactivity retained on the filters was counted in a multi-well γ-counter (LKB Wallace, Turku, Finland) for iodide or with 10 ml of Aquasol 2 scintillation fluid (New England Nuclear) in a Rackbeta scintillation counter (LKB) for tritium. In kinetic experiments the initial incubation volume was 50 ml and aliquots were taken at the required intervals and filtered.

UV Irradiation of Membranes

For photoaffinity labeling of the receptors, the procedure was identical to that used in equilibrium binding experiments but the membranes were filtered at the end of the incubation (in this case BSA was omitted from the incubation and from the soaking and rinsing solutions). The filters were rapidly laid on an ice-cold surface and irradiated 8 min with a Spectroline UV lamp (model ENF-24/F; Spectronic Corp., Westbury, NY) placed 7 cm above the filters. The proteins were solubilized from the filters overnight in 1 ml of 50 mM Tris·HCl, pH 8, containing 2% SDS and 1 mM EDTA. Samples were then diluted to 9 ml with water, precipitated with 12.5% TCA for 30 min and washed with 1 ml of ethanol/ether, 1:1 (v/v). The pellets were dissolved in 100 μl of electrophoresis sample buffer and kept frozen (−20°).

In another set of experiments, the tubes were centrifuged at the end of the incubation in a Beckmann Ja21 rotor at 50,000 × *g* for 30 min at 4°. The pellets were then resuspended in 1 ml of ice-cold Tris·HCl buffer and irradiated on ice for 8 min with the UV lamp placed 7 cm above the bottom of the tubes. After a second centrifugation under the same conditions, the pellets were dissolved in 100 μl of electrophoresis sample buffer and kept frozen (−20°).

The output of the lamp was measured with a compensated CA 1 thermopile (Kipp and Zonen, Delft, Netherland) and was 77 $\mu W/cm^2$, at a wavelength of 254 nm and a distance of 7 cm.

Binding to Rat Brain Sections

The brains were frozen immediately after removal by immersion in isopentane at −50°. Cryostat sections (20 μm thick) taken across the neostriatum were thaw-mounted on gelatin-coated slides and stored at −80° until used. Incubation was carried out in the dark for 45 min at room temperature by covering each brain section with 200 μl of the ligand solution in TBSA. At the end of the incubation, the sections were rinsed sequentially in five or six histology dishes containing ice-cold Tris·HCl buffer. They were then processed in one of three ways. A first series was immediately air-dried at room temperature, in a dust-free atmosphere. A second series was first fixed by immersion for 30 min at 4° in 4% paraformaldehyde, defatted in increasing concentrations of ethanol (70%, 90%, 100%, v/v) and pure xylene, rehydrated through an inverse series of ethanol, and then air-dried as were the others. Finally, a third series was left in TBSA on ice and irradiated for 10 min under conditions comparable to those used for membrane preparations (wavelength, 254 nm; distance, 10 cm). All sections from this third series were then fixed with paraformaldehyde and defatted as described above, except those destined for electrophoresis, for which the fixation step was omitted. Sections from both series were then either scraped off the slides with a razor blade for radioactivity measurements and/or electrophoresis or autoradiographed by apposition to tritium-sensitive film (Ultrofilm; LKB) inside x-ray cassettes, at 4°. These autoradiograms were developed after 1 week of exposure, in a Kodak GBX developer.

Studies on Cells

Cell culture conditions. The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 25 mM glucose, supplemented with 44 mM sodium bicarbonate, 2 mM L-glutamine, 10% Myo-clone fetal calf serum (GIBCO), and HAT (hypoxanthine, 0.1 mM; aminopterin, 10 μM; and thymidine, 16 μM). Cultures were maintained without antibiotics, in an atmosphere of 5% CO_2 with 90% relative humidity, and subcultivated in 75-cm^2 Corning flasks at a 1:2 split ratio. To avoid the loss of opioid receptors sometimes resulting from trypsin treatment (2), the cells were detached from their support by simple shaking.

Binding to cells and photolabeling. Cells at confluency were harvested in the growth medium and centrifuged at 400 × *g* for 8 min.

The pellets were washed twice with 50 ml of a solution containing 137 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 5.5 mM glucose, and 0.22 mM KH_2PO_4 and were resuspended in a Tris-sucrose solution (0.3 M sucrose, 0.01 M Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 0.75 M NaCl) containing protease inhibitors (50 mM EGTA, 5 μM leupeptin, 50 μM soybean trypsin inhibitor, and 0.1 mM phenylmethanesulfonyl fluoride, as described by Diekmann-Gerber *et al.* (4). The cell suspensions were kept on ice before the binding assay.

The cell suspension (5×10^5 cells) in 400 μl of Tris-sucrose solution was incubated in the dark with 200 pmol/liter [^{125}I]azido-DTLET for 20 min at 37° in a shaking water bath. After the cells were washed twice in the Tris-sucrose buffer by centrifugation at $100 \times g$, they were irradiated on ice for 10 min at 254 nm with the Spectroline UV lamp.

Cell membrane preparation. After the photoaffinity labeling, the intact cells were incubated 30 min on ice in a hypotonic solution (10 mM Tris·HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$), then homogenized in a Dounce-Potter glass homogenizer (about 20 strokes) and centrifuged 10 min at $700 \times g$ at 4°. The supernatant was centrifuged 1 hr at $160,000 \times g$ at 4°. The pellets were kept frozen at −20° before SDS-PAGE. Protein concentrations were determined by amido black staining of nitrocellulose dot blot (21).

SDS-PAGE

SDS-PAGE was performed as described by O'Farrel (22) for the second dimension except that urea was included in both stacking and resolving gel at a final concentration of 5 M, the stock solution of acrylamide contained 28.4% acrylamide and 1.6% bisacrylamide (w/v), and the acrylamide concentration of the stacking gel was 4%. Samples were applied onto the gel in a sample buffer consisting of 5% SDS, 5% β-mercaptoethanol, and 5 M urea in 62.5 mM Tris·HCl, pH 6.8. Resolving gels were 12% acrylamide. When gradient gels (5 to 12% acrylamide in a linear gradient) were used, the high concentration solution contained 15% (w/v) sucrose. After electrophoresis, gels were fixed 30 min in cold TCA (10%), stained with Coomassie blue R, 0.25% (w/v) in 45% methanol/10% acetic acid, and destained in 30% ethanol/10% acetic acid. Gels were then soaked for 20 min in 60% methanol to prevent cracking and dried in a Bio-Rad slab gel dryer. The gels were subjected to autoradiography using Kodak X'OMAT AR x-ray film and Du Pont Cronex Lightning-plus intensifying screen at −80°.

Results

Ligand preparation. The retention time of azido-DTLET on reverse phase HPLC eluted by a 30-min acetonitrile gradient under the conditions described above was 24.6 min, as determined from the UV absorbance. Iodination of azido-DTLET yielded a product with a retention time of 27.2 min (Fig. 1). When a trace amount of radioactive iodide was incorporated in the reaction mixture, the radioactivity eluted from the column with the 27.20 min peak. In addition, mass spectrometry of this product gave a peak corresponding to a molecular weight of 868. These results indicate that the new species was indeed monoiodinated azido-DTLET. Iodination of DTLET also resulted in the appearance of a new radioactive species with a retention time on HPLC longer than that of DTLET. As the iodinated and noniodinated peptides were separated on HPLC, the specific activity of [^{125}I]azido-DTLET and [^{125}I]DTLET was assumed to be identical to that of Na^{125}I (74 TBq/mmol, 2000 Ci/mmol).

Ligand binding. Time-course experiments on rat brain membranes showed that [^{125}I]azido-DTLET binding reaches equilibrium after 30 min of incubation at 37°, using a ligand concentration of 22 pmol/liter (100,000 cpm/ml). An incubation time of 45 min was, therefore, chosen for equilibrium studies. At this time, 1700 cpm of iodide radioactivity were retained on the filters (total binding). Nonspecific binding, as determined in the presence of 10 μM levorphanol, usually accounted for 50% to 70% of the total.

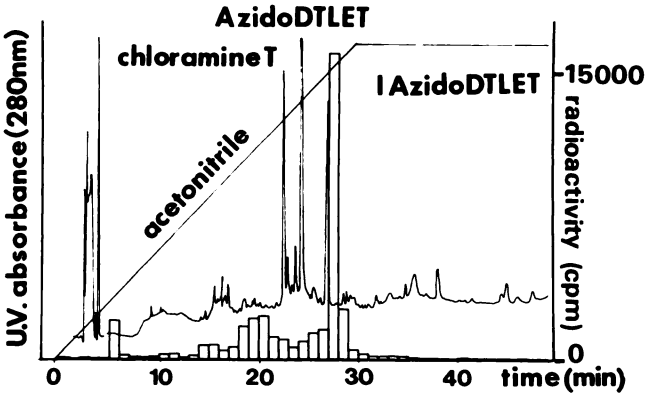

Fig. 1. Preparation of iodinated azido-DTLET by HPLC. Sodium iodide and azido-DTLET were allowed to react in phosphate buffer in the presence of chloramine T. The reaction was stopped with sodium metabisulfite and the resulting products were injected on a C8 reverse phase HPLC column. Elution was with a 30-min 0 to 50% acetonitrile gradient in 0.25 M triethyl ammonium formate, pH 3. The histogram shows the radioactivity in the 1-min, 1-ml fractions collected.

TABLE 1

Affinity of DAGO, DTLET, and I-azido-DTLET for opioid receptors in rat brain

Binding was performed on the rat brain crude membrane preparation as described in Materials and Methods. Radioactive ligand, nonradioactive displacer, and membranes were incubated 45 min at 37° in triplicate. Incubation was terminated by rapid filtration under vacuum on Whatman GF/B filters, which were then counted. Owing to the low concentrations of ligand used, the values expressed for the displacement of [^{125}I]azido-DTLET are IC_{50}. Other values are K_i values derived from the Cheng and Prussof equation: $K_i = IC_{50}/(1 + L/K_D)$.

Competing ligand	Radioactive ligand			K_i DAGO/ K_i DTLET
	[^{3}H]DAGO (1 nmol/liter)	[^{3}H]DTLET (1 nmol/liter)	[^{125}I]Azido-DTLET (22 pmol/liter)	
	nM			
DAGO	3.9[a]	700[a]	668	0.0056
DTLET	25[a]	1.4[a]	2.5	17.8
[^{127}I]Azido-DTLET	136	13.8	14.9	9.8

[a] Values from Ref. 12 and K_D from Ref. 18.

The δ-selective ligand DTLET and the μ-selective ligand DAGO both abolished specific [^{125}I]azido-DTLET binding. DTLET was found to be 267 times more potent ($K_i = 2.5$ nM) than DAGO ($K_i = 668$ nM) in this regard (Table 1). Conversely, nonradioactive Iazido-DTLET inhibited the binding of tritiated DAGO, tritiated DTLET, and iodinated azido-DTLET with K_i values of 136, 13.8, and 14.9 nM, respectively (Table 1; Fig. 2). These results indicate that Iazido-DTLET binds to δ opiate receptors, although its affinity and selectivity for this receptor are lower than those reported for noniodinated azido-DTLET (16).

Covalent binding. Irradiation of membrane bound [^{125}I]-azido-DTLET resulted in covalent binding of 14% of the radioactivity (Table 2). This was measured by filtering the membranes at the end of the incubation, irradiating the filters, solubilizing their content, and precipitating the solubilizate with TCA.

The mechanism of this reaction was further investigated in a similar experiment (Table 3); when the irradiation step was omitted or when nonphotoactivable [^{125}I]DTLET was used, no radioactivity was precipitated. This shows that the covalent

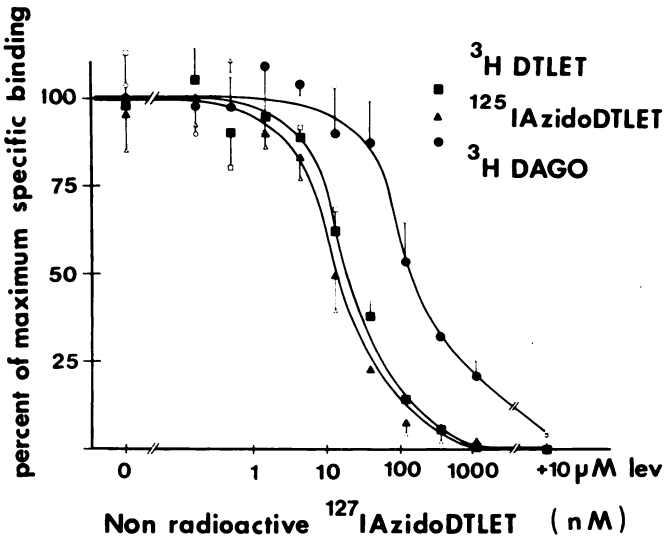

Fig. 2. Displacement of selective opioid ligands by nonradioactive [^{127}I]-azido-DTLET. [^{3}H]DTLET (1 nmol/liter), [^{3}H]DAGO (1 nmol/liter), and [^{125}I]azido-DTLET (22 pmol/liter) were incubated with a crude rat brain membrane preparation in the presence of increasing concentrations of nonradioactive [^{127}I]azido-DTLET. Incubation was stopped by rapid filtration. *Points* are mean of triplicates and *bars* represent standard deviations for each point. Values obtained for the affinities are listed in Table 1.

TABLE 2

UV irradiation of [^{125}I]azido-DTLET results in irreversible specific binding to rat brain proteins

Rat brain membranes were incubated with 227 pmol/liter [^{125}I]azido-DTLET as described in Materials and Methods, in the presence or absence of 10 μM levorphanol, and filtered. After irradiation, the filters were solubilized overnight in 1 ml of 50 mM Tris·HCl, pH 8, containing 2% SDS and 1 mM EDTA. Samples were diluted to 9 ml with water, precipitated with 12.5% TCA, and washed with 1 ml of ethanol/ether, 1:1 (v/v). Values are mean of quadruplicate determinations ± standard error. *p*, difference between with and without levorphanol (Student *t* test).

	Binding after filtration	Binding after irradiation	
		After TCA	After TCA and ethanol/ether wash
	cpm		
Without levorphanol	8,124 ± 245	1,609±39	1,149 ± 122
With 10 μM levorphanol	5,097 ± 195	997 ± 42	736 ± 73
	$p = 0.015\%$	$p = 0.011\%$	$p = 2.7\%$

linkage of [^{125}I]azido-DTLET to proteins is indeed due to the reaction of the azide moiety and not to the incorporation into the proteins of free iodide, which would have been dissociated from the ligand upon irradiation.

SDS-PAGE followed by autoradiography allowed a more detailed analysis of the labeled proteins; the distribution of the radioactive proteins consists of four major bands with molecular weights of 52,000, 44,000, 38,000, and 34,000. When incubation was carried out in the presence of levorphanol, the 44-kDa band was no longer labeled and the labeling of the 34-kDa band was strongly reduced (Fig. 3). The distribution of the radioactivity was estimated from the density scan of the film; the 44-kDa band represented 3.5% and the 34-kDa band 5% of the total radioactivity present in the gel after electrophoresis.

In another experiment, binding was carried out in the presence of DTLET or DAGO. As shown in Fig. 4, the intensity of the radioactive bands at 44 and 34 kDa was reduced in the presence of 30 nmol/liter DTLET but not in the presence of the same concentration of DAGO. It is also noteworthy that the addition of protease inhibitors (6 mM EDTA, 5 μM leupeptin, 50 μM soybean trypsin inhibitor, 1 mM phenylmethanesulfonyl fluoride, and 0.01% bacitracin), in this experiment, did not change the repartition of the radioactivity on the gel.

Binding on cells. In order to compare these results with data from another source of opioid receptors, similar labeling experiments were carried out with the hybrid cells NG108-15 and NG108-5 as well as with their two parental lines, the rat glioma C6BU-1 and the mouse neuroblastoma N18TG2. The presence of opioid receptors on these cells was investigated using the tritiated ligand [^{3}H]DTLET in saturation experiments on whole cells. No specific binding of [^{3}H]DTLET was seen on the rat glioma cells. In contrast, on the mouse neuroblastoma and the hybrid cells, opioid binding sites were present with the following binding parameters: $K_D = 1.40$ nM and $B_{max} = 545$ fmol/mg of protein for the N18TG2 cells, $K_D = 1.04$ nM and $B_{max} = 492$ fmol/mg of protein for the hybrid cells NG108-15 and $K_D = 0.54$ nM and $B_{max} = 103$ fmol/mg of protein for the hybrid cells NG108-5, a cell line resulting from the same fusion as the NG108-15. Cells were irreversibly labeled with [^{125}I]azido-DTLET, the membranes were prepared, and the distribution of the radioactivity was studied by SDS-PAGE and autoradiography. Fig. 5 shows an axonometrical representation of the optical density of the resulting film. In all cells except the C6BU-1 glioma, the general features of repartition of the radioactivity were similar. A strong band, with a molecular weight of 38,000 was particularly visible. An equally strong band, with a molecular weight of 33,000 was present in N18TG2 (Fig. 5, *lane D*) and NG108-15 (Fig. 5, *lane C*), for which opioid binding B_{max} was approximately 500 fmol/mg of protein. In NG108-5, for which B_{max} was 5 times lower, this latter 33-kDa band was much less intense (Fig. 5, *lane E*). In the presence of 10 μM levorphanol, this 33-kDa band disapeared (Fig. 5, *lane B*). In addition, this 33-kDa band is not detectable in the C6BU-1 line (Fig. 5, *lane A*).

Binding on rat brain slices. Binding of [^{125}I]azido-DTLET to sections of rat neostriatum was almost entirely displaceable

TABLE 3

UV irradiation of [^{125}I]azido-DTLET but not of [^{125}I]DTLET results in irreversible binding to rat brain proteins

Rat brain membranes were prepared using only the neostriatum and incubated as described in Materials and Methods with [^{125}I]azido-DTLET or [^{125}I]DTLET in the presence or absence of 10 μM levorphanol and centrifuged. After irradiation, the pellets were solubilized overnight in 300 μl of 50 mM Tris·HCl, pH 8, containing 2% SDS and 1 mM EDTA. Samples were diluted to 3 ml with water, precipitated with 12.5% TCA, and washed with 1 ml of ethanol/ether, 1:1 (v/v). Values are mean ± standard error for the number of replicate determinations indicated.

Ligand	Binding after centrifugation	Binding after TCA precipitation and ethanol/ether wash	
		No irradiation	UV irradiation
	cpm		
[^{125}I]Azido-DTLET (120 pmol/liter)	22,346 ± 839 ($n = 6$)	552 ± 90 ($n = 3$)	2,550 ± 549 ($n = 3$)
[^{125}I]DTLET (220 pmol/liter)	30,485 ± 1980 ($n = 6$)	449 ± 155 ($n = 3$)	363 ± 38 ($n = 3$)

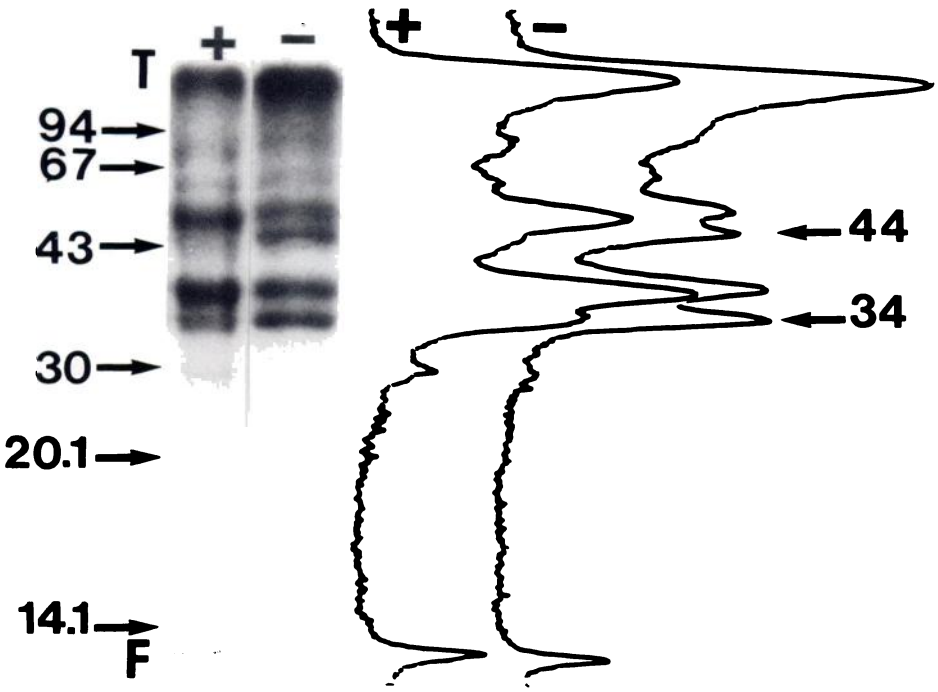

Fig. 3. Autoradiography of rat striatal membrane proteins labeled with [^{125}I]azido-DTLET. Rat striatal membranes were prepared as described in Materials and Methods and incubated with 162 pmol/liter [^{125}I]azido-DTLET in the presence (+) or absence (−) of 10 μmol/liter levorphanol, centrifuged, and resuspended in Tris buffer for UV irradiation on ice. Samples were run on a 12% acrylamide gel, which was subjected to autoradiography (2-month exposure). Both lanes were loaded with an equal amount of radioactivity (3500 cpm). The *right panel* of the figure shows densitometer scan of the bands shown in the *left panel*. Molecular masses of the standards are in kDa. *F*, front; *T*, top. This experiment was repeated more than 10 times with similar results.

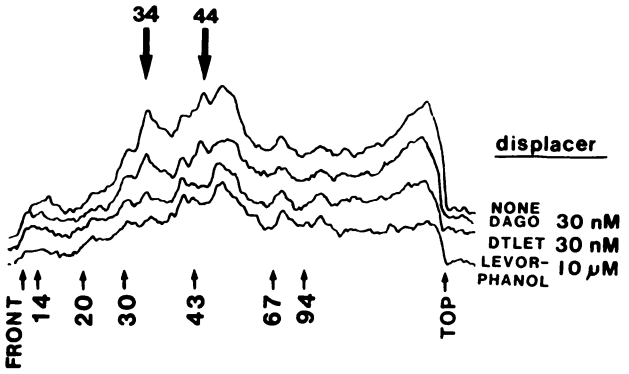

Fig. 4. Selective inhibition of [^{125}I]azido-DTLET photoaffinity labeling. Rat striatal membranes were incubated with 184 pmol/liter [^{125}I]azido-DTLET in the presence of various nonradioactive ligands and filtered as described in Materials and Methods. Samples were run on a gradient gel, and the autoradiography of the dried gel was scanned. *Arrows* above the scans indicate the position of the radioactive bands that disappear in the presence of displacer.

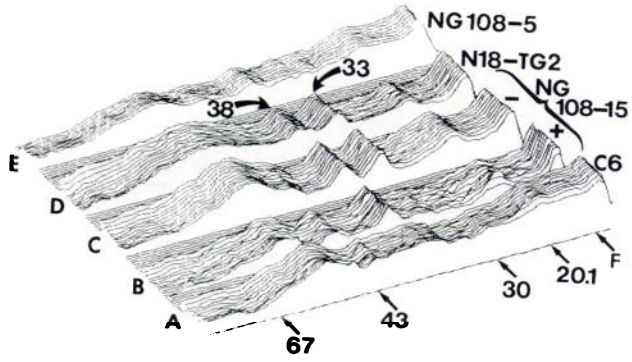

Fig. 5. Covalent labeling of different cell lines by [^{125}I]-azido-DTLET. Whole cells were incubated with 193 pmol/liter [^{125}I]azido-DTLET and irradiated and the membranes were prepared as described in Materials and Methods. Samples were run on a gradient gel, which was then exposed for autoradiography. The picture is a three-dimensional representation of the optical density of the resulting film. *A*, C6BU-1; *B*, NG108-15 plus levorphanol; *C*, NG108-15; *D*, N18-TG2; and *E*, NG108-5. The *curved arrows* indicate the position of the band labeled specifically (33 kDa) and nonspecifically (38 kDa). Molecular weights of standards are in kDa. *Arrow labeled F*, front. Labeling of NG108-15 was repeated seven times with similar results.

(90%) with nonradioactive levorphanol (Table 4). The autoradiographic distribution of bound radioligand molecules was characterized by a dense and relatively homogeneous labeling of the neostriatum and the olfactory tubercle and by a bilaminar pattern of moderate labeling densities in the supragranular and the deep layers of the cerebral cortex (Fig. 6A). This distribution pattern conformed to that previously reported for [^{3}H] DTLET (23, 24) or other δ-selective ligands (25, 26). Approximately 25% of the bound radioactivity was still present in sections after UV irradiation, fixation in 4% paraformaldehyde, and defatting (Table 4). The autoradiographic distribution of these retained radiolabeled molecules was similar to that observed at the end of the incubation (Fig. 6B). In contrast, the radioactivity was almost entirely washed out from sections that had not been photoirradiated but were otherwise similarly fixed and defatted (Table 4). Analysis by gel electrophoresis of photoirradiated sections recovered from the slides revealed an

TABLE 4

Irreversible binding of [^{125}I]azido-DTLET to rat brain sections after UV irradiation, fixation, and delipidation

Sections from rat neostriatum (20 μm thick) were incubated 45 min at room temperature under 200 μl of 50 mM Tris·HCl containing 0.2 nmol/liter [^{125}I]azido-DTLET (200,000 cpm). Sections were then rinsed in several baths of ice-cold Tris·HCl, UV irradiated, fixed in 4% paraformaldehyde, and defatted as described in Materials and Methods. Values are mean ± standard error (five determinations).

	No irradiation No fixation No delipidation	10 min UV irradiation Fixation Delipidation	No irradiation Fixation Delipidation
Total binding (cpm)	10,875 ± 777	2,488 ± 516	264 ± 105
Nonspecific binding (cpm)	663 ± 285	255 ± 134	253 ± 129

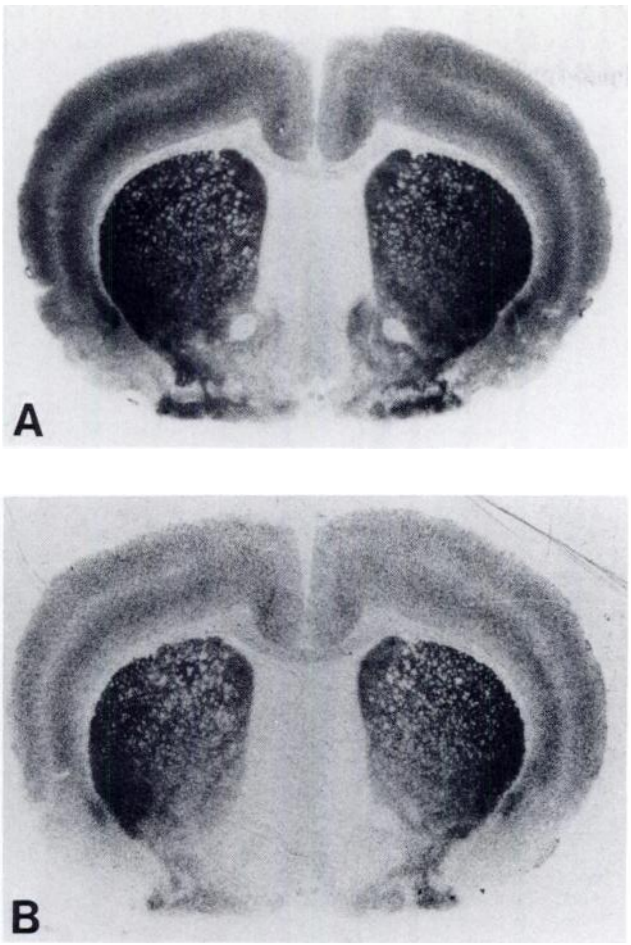

Fig. 6. Autoradiographic localization of [^{125}I]azido-DTLET binding. Mid-striatal brain sections (20 μm thick) were incubated under 200 μl of Tris buffer containing 0.2 nmol/liter [^{125}I]azido-DTLET and rinsed in several baths of ice-cold Tris. Section A was air-dried and processed for autoradiography immediately after incubation. Note the intense and diffuse labeling of the neostriatum and olfactory tubercle and the bilaminar distribution of the label in cerebral cortex. Section B was photoirradiated, fixed with paraformaldehyde, and defatted before being autoradiographed. The distribution of the covalently bound molecules is comparable to that seen in A.

autoradiographic pattern of labeled radioactive proteins identical to that seen with the membrane preparation. Varying the duration of the irradiation only changed the amount of radioactivity bound, without altering the migration pattern (data not shown). The proportion of receptors labeled was estimated on this preparation; after binding and irradiation at a concentration of [^{125}I]azido-DTLET of 2×10^7 cpm/ml (4.5 nmol/liter) the specific 44-kDa band on the gel was counted and found to correspond to 700 cpm (0.16 fmol of [^{125}I]azido-DTLET). This represents 1/60 of the 10 fmol of receptor expected in the 100 μg of rat brain protein loaded on the gel. At the 20 times lower concentration of [^{125}I]azido-DTLET usually used (10^6 cpm/ml, i.e., 0.2 nmol/liter) the specific band could not be counted but one can estimate that 1/1200 of the receptors are labeled.

Laser irradiation. To study more precisely the influence of the UV light wavelength and in an attempt to reduce ligand dissociation during the 8-min irradiation, a light source different from the hand-held UV lamp was used. The rat sections were irradiated with two different UV-pulsed excimer lasers. These machines gave a 20-nsec flash of 12 mJ at 308 nm and a 15-nsec flash of 200 mJ at 249 nm, respectively. The use of lasers offered the advantage of an irradiation at a single known wavelength, together with a very short irradiation time. In addition, the total energy received by the sample was similar to the one given by the small UV lamp. Binding and rinsing were carried out as described above and the beam of the laser was then directed onto the sample for irradiation. The sections were then dried and scraped from the slides for counting and dissolved in SDS-PAGE sample buffer for further analysis. Irreversible binding to proteins was measured after TCA precipitation and ethanol/ether wash as described for membranes in Materials and Methods. Interestingly, irradiation with up to 10 flashes at a wavelength of 308 nm did not result in any incorporation of radioactivity into the proteins (1.48 ± 0.6%, four determinations of the radioactivity present in the slides after rinsing was precipitated). On the other hand, one single flash of the laser at 249 nm caused a strong labeling of the brain proteins (30.6 ± 0.9%, four determinations, of the radioactivity was precipitated). However, the SDS-PAGE pattern obtained with these samples was similar to the one obtained with the less powerful UV lamp. Again, in this preparation virtually no incorporation of radioactivity followed the irradiation of nonazidated [^{125}I]DTLET with the 249 nm laser (2.4% of the radioactivity precipitated).

Discussion

As is commonly seen with other peptides (27), iodination of azido-DTLET induces an increase in hydrophobicity, which, in turn, results in an increase of the retention time on reverse phase HPLC. This hydrophobicity probably explains the relatively low value of the specific to total binding ratio on rat brain membranes. This ration (between 30% and 50%) is indeed lower than the one obtained using the corresponding tritiated peptides [^{3}H]DTLET or [^{3}H]azido-DTLET (16). Iodination also induces a reduction of the affinity of azido-DTLET. This effect is consistent with results obtained after iodination of the enkephalin analog D-Ala^2-Leu^5-enkephalin (28) and may presumably be accounted for by the fact that iodide is a bulky subtituent on the N-terminal tyrosine, which is known to play a crucial role in opioid peptide binding (29). However, the reduction in affinity is counterbalanced by the very high specific activity obtained with ^{125}I. This allows the use of low concentrations of radioactive ligand in binding experiments, thus minimizing nonspecific binding.

Experiments on membrane preparations, tissue sections, and cells demonstrate that photoactivation of bound [^{125}I]azido-DTLET results in its covalent binding to proteins. The mechanism of this reaction clearly involves the aryl azide moiety inasmuch as covalent binding does not occur when either the nonazidated, iodinated peptide [^{125}I]DTLET or a wavelength of 308 nm, distinct from the aryl azide absorbance maximum at 254 nm, is used.

These properties of [^{125}I]azido-DTLET have allowed us to label components of the δ opioid receptors from two different sources, rat brain and cells in culture. In both preparations, [^{125}I]azido-DTLET specifically labeled a protein with molecular weight of 34,000 in rat brain and 33,000 in cells. However, another protein, with a molecular weight of 44,000, was labeled only in rat brain and not in the cells.

The molecular weight of this latter protein is comparable to

the value of 46,000 obtained in the rat by Yeung (15) using [^{125}I]-[D-Ala^2,pN$_3$Phe^4,Met^5]enkephalin as a photoaffinity probe. More recently, Szücs *et al.* (30) have described the irreversible labeling of opiate receptors from several sources with a chloromethyl ketone derivative of [D-Ala^2,Leu^5]enkephalin. In rat brain, five proteins of, respectively, 74, 65, 56, 43, and 34 kDa were labeled. Although this derivative is described as a rather μ-selective ligand, it is possible that the labeling of the 43-kDa and the 33-kDa bands are in fact due to a small cross-reactivity with the δ receptor and thus correspond to the 44-kDa and 34-kDa proteins described here.

Simon's group (10) has used a chemical cross-linking agent to label opioid receptors from various sources with iodinated human β-endorphin. On the basis of the differences in the relative abundance of μ and δ binding sites in several tissues, these authors suggested that μ receptors have a molecular weight of 65,000 in rat brain whereas δ receptors have a molecular weight of only 53,000. Using our ligand we were not able to detect this protein.

The opioid receptor labeled and purified by Klee's group (8, 31) from NG108-15 cells has a molecular weight of 58,000. Similarly, Nakayama *et al.* (32) purified the receptor from the same source and found three polypeptides with molecular weights of 58,000, 65,000, and 71,000, all of which are above the value of 33,000 we obtain in the same cells (this report and Ref. 17). However, due to the high background of radioactivity in the zone of the gel between 67 and 44 kDa (Fig. 5), the presence in our autoradiography of a minor, levorphanol-displaceable, 58-kDa band could not be ruled out. To exclude the possibility that the 33-kDa band seen here is in fact a degradation product of the 58-kDa protein detected by others using different ligands, we carried out the experiments in the presence of a cocktail of peptidase inhibitors but this did not cause any change in the pattern of the autoradiography.

The simpler assumption regarding the mechanism of irreversible labeling of a receptor with a specific ligand is that the ligand will react with the subunit of the receptor bearing the binding site. The fact that we found two major specific bands in rat brain, and only one in NG108-15 cells, with molecular weights different from those generally reported in the literature is puzzling. We do not think that protein degradation is responsible for this discrepancy. One explanation could be that opioid receptors are composed of several subunits and that different reports are in fact dealing with different subunits bearing different binding sites. This would be an argument in favor of a hetero-oligomerical structure of δ opioid receptor. However, this is not in accordance with the fact that δ opioid receptors are coupled to a G protein (33) and that many of the G protein-coupled receptors characterized so far, including the receptors for the putative neuropeptide substance K, seem to be monomers (34–36).

In view of our results, we could speculate that the above assumption, that irreversible labeling is restricted to the binding site of the receptor, is not justified and, indeed, we have found that part of the irreversibly bound radioactivity could be copurified with glycolipids (data not shown). A more likely explanation for this multiplication of labeled proteins is that not all the membrane proteins labeled by our ligand and other affinity ligands are subunits of the receptor carrying the binding site. They could be other subunits or neighboring proteins involved, for instance, in signal transduction or receptor anchoring. If this is the case, caution must be exercised in the use of such an irreversible labeling to monitor the purification of the receptor. However, this does not hamper the use of these ligands for studies of the anatomical localization of receptors at photonic or electronic microscopic resolution.

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