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Affinity Labeling of μ and κ Receptors with Naloxone Benzoylhydrazone

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SUMMARY

Naloxone benzoylhydrazone (NalBzoH) labels both μ and κ receptors in standard homogenate binding assays. We now report that [^{3}H]NalBzoH can effectively photoaffinity label opioid receptors. By modifying [^{3}H]NalBzoH binding conditions, we can selectively label either μ or κ_3 receptors in calf striatal membranes or classical U50,488-sensitive κ_1 receptors in guinea pig cerebellar membranes. After removal of unbound radioligand, the [^{3}H]NalBzoH-labeled membranes were irradiated with UV light to couple the bound radioligand to its binding site. No specific μ, κ_1, or κ_3 binding remained after a 20-hr dissociation at 25° without UV irradiation. In contrast, approximately 45% of μ and 40% of κ_1 and κ_3 binding remained after 2 min of UV exposure. In time course studies, increasing the UV exposure from 30 sec to 3 min produced a progressive increase in radioligand incorporation, which did not increase further with UV exposure times up to 5 min. A portion of the binding in irradiated membranes appeared to be covalently coupled to proteins. Following solubilization of irradiated membranes with sodium dodecyl sulfate, approximately 30–40% of the specifically bound radioactivity precipitated with trichloroacetic acid (TCA). These levels of TCA-precipitable binding corresponded to the amount of dissociation-resistant binding described above. No specific binding could be TCA precipitated in samples that were not exposed to UV. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{3}H]NalBzoH-labeled membranes revealed a number of labeled peaks with levels of radioactivity that did not correspond to the intensity of the protein bands, suggesting that this technique was not randomly labeling proteins. This approach may be a useful method to affinity label, characterize, and purify μ and κ opiate receptor subtypes.

Since the discovery of opiate binding sites and the identification of receptor subtypes, numerous investigators have tried to isolate and characterize them. Early attempts to solubilize receptors proved difficult, possibly due to the reversible inhibition of binding by low concentrations of detergents and the very high sensitivity of the binding site to proteases and phospholipases (1, 2). Several groups have labeled solubilized sites and purified them (3–8), and one has even sequenced a protein believed to be related to the μ receptor (9). However, all these approaches suffer from the drawback that affinities and selectivities of these receptors may change following their removal from the membrane, making the identification of specific subtypes difficult.

An alternative approach is to label the receptor while it is still in the membrane and retains its well documented binding characteristics. Initially, investigators prebound the receptor with slowly dissociating ligands and then solubilized the membranes (10–12). Although this proved more successful, subsequent purification steps were greatly limited by the continued dissociation of the ligand. This drawback can be overcome by affinity labeling of the receptor. This technique requires chemically reactive radioligands that selectively and covalently label the receptor, with high ratios of specific to nonspecific binding. A number of chemically reactive opiate derivatives with high receptor affinities have been synthesized (13–16), but they suffer from high levels of nonspecific binding (17). More recently, studies using [^{3}H]*cis*-(+)-3-methylfentanylisothiocyanate (18, 19), [^{3}H]-β-funaltrexamine (20–25), and alkylating derivatives of enkephalin (26–31) have been reported, but they are limited to μ and δ receptors. Other techniques involved the attachment of nonreactive compounds to the receptor, through either cross-linking methods, such as those reported with β-

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ABBREVIATIONS: NalBzoH, (−)-6-desoxy-6-benzoylhydrazido-*N*-allyl-14-hydroxydihydronormorphinone; Gpp(NH)p, 5′-guanylylimidodiphosphate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr^6; DADL, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; U50,488, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide.

^{125}I-endorphin (32) and the enkephalins (33, 34), or UV exposure (35–37).

Recently we reported an unusual opiate ligand, NalBzoH (38–40). With modifications of binding conditions and tissues, [^{3}H]NalBzoH can selectively label μ, κ_1, or κ_3 sites (41). We now show that UV exposure of prebound [^{3}H]NalBzoH activates the ligand to covalently attach to proteins and, by choosing the appropriate binding conditions, we can affinity label μ, κ_1, and κ_3 receptors.

Materials and Methods

All radioligands, Formula 963 and Econofluor scintillation fluors, and Protosol were purchased from New England Nuclear Corporation (Boston, MA). [^{3}H]NalBzoH was synthesized as previously reported (38–40). Peptides were purchased from Peninsula Laboratories (Belmont, CA). Rainbow molecular weight markers were obtained from Amersham (Arlington Heights, IL). Fresh calf brains were obtained locally, dissected, prepared as previously reported (42), and frozen. Tissue prepared in this manner and maintained at −70° retained its binding for at least 3–4 weeks. Frozen guinea pig brains were obtained from Charles River (Wilmington, MA). Each brain was thawed and the cerebellar tissue was prepared and frozen as described above.

The standard μ and κ assays were all performed as previously reported (40–42). [^{3}H]NalBzoH binding was performed as described below.

μ assays. Bovine striatal membrane homogenates (2 ml of 10 mg of wet weight/ml) were preincubated in the presence of magnesium sulfate (5 mM) and potassium phophate buffer (50 mM, pH 7.2), in the presence or absence of levallorphan (1 μM), for 30 min at 25°. At the end of that time, [^{3}H]NalBzoH (1 nM) was added for an additional 60 min at 25°, at which time the κ binding component was dissociated by addition of levallorphan (1 μM) and continuation of the incubation for an additional 60 min (42). The ratio of total to nonspecific binding in membranes was approximately 3:1 for μ binding.

κ assays. Guinea pig cerebellar (κ_1) or bovine striatal (κ_3) membrane homogenates (10 mg of wet weight/ml) were incubated in potassium phosphate buffer (50 mM, pH 7.2) containing K_2EDTA (5 mM) and [^{3}H]NalBzoH (1.0 and 1.2 nM, respectively), for 60 min at 25° (41). To determine membrane binding, all assays were filtered over Whatman glass fiber filters, the filters were washed with two 4-ml aliquots of cold buffer, and the radioactivity was determined. All binding was performed in triplicate, and all assays were replicated at least three times unless otherwise noted. Nonspecific binding was determined in the presence of levallorphan (1 μM). Only specific binding is reported. The ratio of total to nonspecific binding in membranes was approximately 3:1 for κ_1 and 4:1 for κ_3 binding.

Affinity labeling studies. Tissues were prelabeled as described above, and the membranes were centrifuged (49,000 × g, 20 min). Tissues were resuspended (40 mg of wet weight/ml) in potassium phosphate buffer (50 mM, pH 7.2) containing $MgSO_4$ (5 mM) and levallorphan (1 μM) for μ assays, K_2EDTA (5 mM) for κ_3 assays, or no addition for κ_1 assays. The following protease inhibitors were also added to all resuspensions: bestatin (0.5 μg/ml), chymostatin (5 μg/ml), antipain (5 μg/ml), aprotinin (5 μg/ml), soybean trysin inhibitor (25 μg/ml), pepstatin (5 μg/ml), N-carboxymethyl-Phe-Leu (2 μg/ml), leupeptin (5 μg/ml), and bacitracin (10 μg/ml). These concentrations of protease inhibitors did not significantly affect binding. The homogenates were then exposed to UV radiation (254 nm) from a Mineralight lamp (model R-52-G), at 0–4° for 0–5 min. Under these conditions, the temperature of the solution never rose above 10°.

For dissociation studies, we added either Gpp(NH)p (100 μM) or levallorphan (1 μM) or we lowered the pH to 5 with acetic acid. All values are presented as the means ± standard errors. Statistical evaluations were determined using either Student's t test or analysis of variance, depending upon the comparisons.

SDS-PAGE. Gel electrophoresis was performed according to the method of Laemmli (43), using slab gels (1.5 × 120 mm) consisting of 5–15% polyacrylamide gradients for the separating gel and 3% stacking gels. The samples were prepared for electorphoresis by resuspension of concentrated tissue pellets in 50 mM Tris·HCl (pH 7.7) containing 2% SDS. Samples were boiled for 5 min and precipitated with TCA (10%), and the pellet was extracted with ethanol/ether (1:1) for 10 min. After centrifugation, pellets were resuspended in SDS sample buffer (50 mM Tris, pH 8.0, containing 5% β-mercaptoethanol, 10% glycerol, 1% SDS, 200 mM Na_2HPO_4, and 0.001% bromophenol blue) and boiled for 5 min, and separate gels were run to localize protein bands and to determine the distribution of radiolabel. To visualize proteins, gels were fixed for 60 min in 10% acetic acid/50% methanol, stained for 60 min in the same solution containing 0.1% Coomassie blue, and diffusion destained overnight. To determine the location of radiolabel, gels were immediately sliced into 250-μm sections and incubated with 15 ml of Econofluor with 5% Protosol, for 36 hr at 36°, and the radioactivity was determined. Apparent molecular weights were calculated from a graph of mobility versus logarithm of molecular weight. Molecular weight standards used were myosin (M_r 200,000), phosphorylase b (M_r 97,400), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,300).

Results

First we examined whether UV exposure of prebound [^{3}H]NalBzoH irreversibly labeled receptors (Fig. 1). We selectively labeled μ, κ_1, or κ_3 sites and washed away free [^{3}H]NalBzoH. After resuspending the membrane pellets, we exposed them to UV for 2 min and looked at reversibility of binding by lowering the pH to 5, which rapidly dissociates reversible opioid binding (41). μ binding in nonirradiated control membranes decreased over 90% after 1 hr and was undectectable after 20 hr. In contrast, approximately 45% of specific binding in irradiated membranes remained stable for 20 hr. Irradiation of bound [^{3}H]NalBzoH in guinea pig cerebellar membranes, a measure of κ_1 binding, yielded similar results. Although κ_1 binding in unexposed membranes dissociated more slowly than μ binding, no detectable binding remained by 20 hr, compared with approximately 40% of binding in UV irradiated samples. As with the other two assays, UV irradiation also appeared to couple about 40% of κ_3 binding.

We next examined whether this persistent labeling following UV exposure was resistent to denaturation with SDS (Table 1). After irradiation, membranes were solubilized overnight with SDS (2%). Under these conditions, free [^{3}H]NalBzoH alone does not precipitate. Furthermore, if [^{3}H]NalBzoH is added to SDS-solubilized membranes without UV exposure and then treated with TCA, no radioactivity is precipitated. Thus, precipitation of radiolabel with TCA may provide an indication of covalency. Following exposure to UV of membranes prebound with [^{3}H]NalBzoH, between 30 and 40% of the radiolabel precipitated with the protein, compared with less than 10% in similarly treated nonirradiated membranes. Furthermore, if [^{3}H]NalBzoH was added to membranes immediately before UV irradiation, with no incubation, there was little or no specific labeling of protein.

We next examined the time course of UV exposure and irreversible incorporation of [^{3}H]NalBzoH (Fig. 2). We dissociated reversible μ binding over 20 hr at 25°, by lowering the pH to 5 or by adding the GTP analog Gpp(NH)p, which rapidly dissociates reversibly bound [^{3}H]NalBzoH from μ receptors (41). Simply adding levallorphan to dissociate μ binding was not adequate, because dissociation was so slow under these

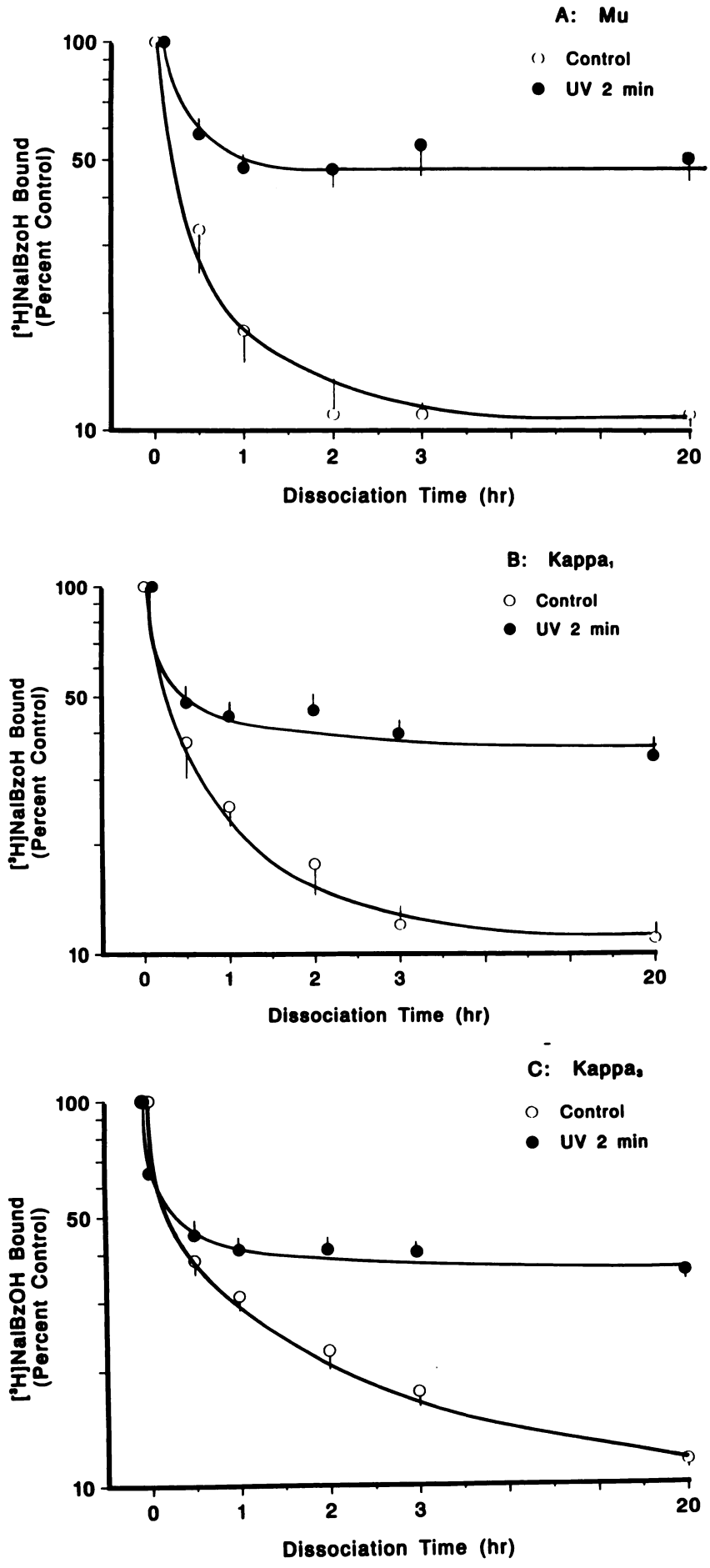


Fig. 1. Dissociation of [^{3}H]NalBzoH binding in control and UV-exposed membranes. Binding assays labeling μ (A), κ_1 (B), and κ_3 (C) receptors, using [^{3}H]NalBzoH, were performed as described in Materials and Methods. Dissociation studies were then performed in control (nonirradiated) membranes and in homogenates that were UV irradiated for 2 min. The pH was lowered to 5 with acetic acid, and residual binding was determined at the stated times after the addition. Binding before the dissociation for the control and UV-exposed membranes, respectively, was: μ, 3200 ± 165 and 2298 ± 108 cpm; κ_1, 3217 ± 393 and 2273 ± 269 cpm; and κ_3, 8729 ± 670 and 5646 ± 352 cpm. Results are the means ± standard errors of three separate experiments.

TABLE 1

UV irradiation of [^{3}H]NalBzoH results in irreversible binding to calf striatal membranes

Membrane homogenates were incubated with [^{3}H]NalBzoH to selectively label μ, κ_1, or κ_3 receptors, as described in Materials and Methods, and the membranes were washed. Half the homogenates were irradiated for 3 min and the other half served as the unradiated control. Binding was determined using a filtration assay at this point in both groups for all the assays and was used to determine the percentage of TCA-precipitable radioactivity. All homogenates were then centrifuged, and the pellets were resuspended in 0.5 ml of phosphate buffer containing SDS (2%) and EDTA (1 mM) and were boiled 5 min. The samples were then diluted with water, precipitated with TCA (12.5%), and allowed to sit overnight at ambient temperature, and the pellet was washed with 1 ml of ethanol/ether (1:1, v/v) and counted (38). Values are expressed as the mean ± standard error, for three experiments, of the percentage of radioactivity precipitable with TCA, based upon the binding for each group determined before the solubilization step, as described above.

Binding assay	TCA-precipitable radioactivity		
	No irradiation	UV irradiation	
	% of membrane binding		
μ	5.2 ± 0.9	41.3 ± 4.2	$p < 0.001$
κ_1	5.7 ± 0.8	37.0 ± 6.1	$p < 0.01$
κ_3	6.7 ± 0.4	31.3 ± 6.3	$p < 0.05$
$\mu + \kappa_3$	4.2 ± 0.2	26.4 ± 2.6	$p < 0.001$

binding conditions ($\tau_{1/2} \sim 24$ hr). Extending the UV exposure beyond 2 min increased irreversible incorporation of [^{3}H]NalBzoH. Both methods of dissociation yielded comparable results, with a maximal level of incorporation seen by 3 min that was not appreciably increased by 5 min.

Similar incorporation rates were observed with κ_1 and κ_3 binding. [^{3}H]NalBzoH binding to these sites was readily reversible ($\tau_{1/2} < 15$ min), permitting us to measure dissociation by adding a high concentration of levallorphan (1 μM). We also examined the effects of lowering the pH to 5. Under both conditions, approximately 30–40% of specific κ binding remained after a 20-hr dissociation at 25° with UV exposure. In all cases, no significant specific binding remained in the control tissue that was not irradiated.

Finally, we examined the effects of the UV radiation on [^{3}H]NalBzoH binding when the membranes were irradiated before the addition of the radiolabel (Fig. 3). Preexposure with UV greatly reduced binding to all the receptor subtypes. The binding of [^{3}H]NalBzoH to μ receptors declined at the same rate as the selective binding to both the μ_1 and μ_2 receptor subtypes. Similarly, the UV exposure lowered [^{3}H]NalBzoH binding to κ_1 sites with a time course similar to that of [^{3}H]U69,593. The reduction in binding to all the subtypes showed a similar rate of decrease. Preincubation of membranes with opioid peptides did not protect the binding sites from inactivation by UV irradiation (data not shown).

To help distinguish between random protein labeling and selective incorporation into opioid binding sites, we examined the distribution of radioactivity using SDS-PAGE. [^{3}H]NalBzoH binding and irradiation were performed in calf striatal membranes under conditions that labeled μ and κ_3 sites (Fig. 4a). A number of bands of radioactivity were observed. The largest peak migrated with an apparent molecular weight greater than 200,000, with another at approximately M_r 90,000. Delineation of the other potential bands was difficult, due to the low resolution achieved by counting of gel slices. However, we consistently saw smaller peaks at approximately M_r 83,000 and 67,000. Clearly, these peaks did not correspond to the two major protein bands, which had apparent molecular weights of approximately 55,000 and 45,000.

To further investigate whether we were randomly labeling membrane proteins, we prepared rat liver membranes and exposed them to UV with [^{3}H]NalBzoH at 70,000 cpm/ml. Liver membranes contain no specific binding sites and, therefore, serve as a reasonable control. Despite a concentration of [^{3}H]NalBzoH in the liver membranes during UV irradiation that was over 5-fold higher than the concentration of [^{3}H]NalBzoH present in brain membranes during UV treatment, we observed less than half the incorporation into membranes and no identifiable peaks of radioactivity on SDS-PAGE. Indeed, we observed less than 5% incorporation of radiolabel into the liver membranes under these UV irradiation conditions.

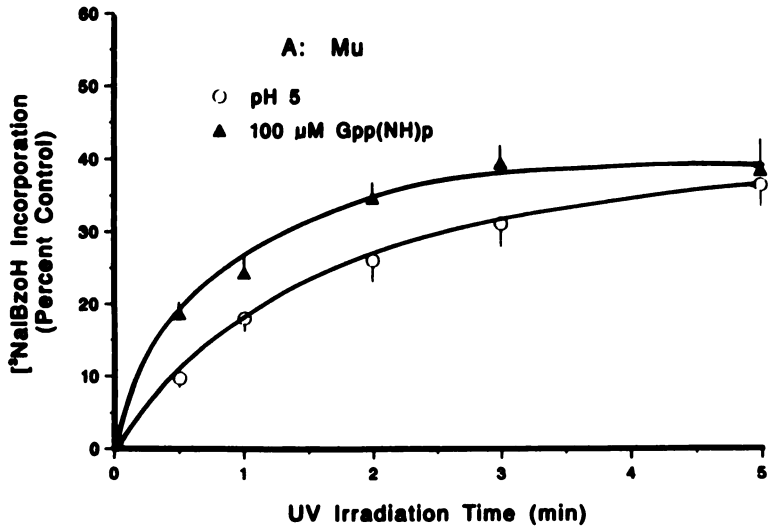
We also examined the SDS-PAGE running pattern of [^{3}H]NalBzoH in the absence of tissue. Greater than 95% of the radioligand ran with the dye front, but a significant proportion ran with an apparent molecular weight of approximately 30,000, presumably due to its association with SDS micelles. Thus, the peaks in this range seen with affinity-labeled tissue may correspond to free radioligand. This conclusion is supported by the reduction of this band in affinity-labeled brain membranes caused by activated charcoal, which adsorbs free [^{3}H]NalBzoH (data not shown).

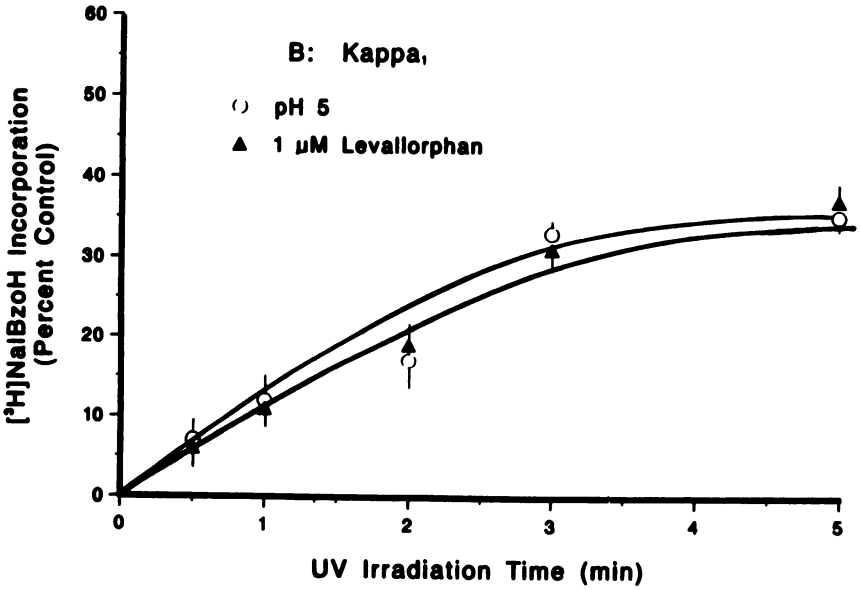
Discussion

With appropriate binding conditions and tissue, [^{3}H]NalBzoH can label either μ (μ_1 and μ_2), κ_1, or κ_3 receptors (40, 41). We now show that UV exposure of [^{3}H]NalBzoH prebound to membranes will irreversibly label membrane proteins. Although the resistance of a significant proportion of irradiated binding to dissociation over 20 hr suggested covalent incorporation, alternative explanations still had to be considered. However, the ability of TCA to precipitate radiolabel from SDS-solubilized membranes argues strongly in favor of a covalent linkage. The similar levels of radiolabel resistant to dissociation and to TCA precipitation suggest that they probably represent the same binding.

We routinely observed a 30–40% level of incorporation, which reached maximal values between 3 and 5 min of UV exposure. The failure of extended UV exposures, beyond 3 min, to further increase incorporation probably results from two competing actions. Although UV exposure of prebound [^{3}H]NalBzoH leads to protein incorporation, UV also markedly lowers receptor binding. Receptor inactivation presumably leads to a rapid dissociation of the ligand, eliminating its ability to selectively label receptors. Irradiation of unlabeled membranes for a similar period destroys approximately 80–90% of the binding sites. Thus, after a 3-min UV exposure of prebound [^{3}H]NalBzoH, very few sites capable of binding ligands remain, and continued radiation does not increase the incorporation of [^{3}H]NalBzoH into specific sites. The mechanism through which [^{3}H]NalBzoH covalently labels proteins remains unknown. Other opiate alkaloids have been covalently coupled to proteins following UV irradiation.

The question of labeling selectivity cannot yet be answered definitively. SDS-PAGE of affinity-labeled striatal membranes yielded a number of peaks of radioactivity, with large differences between total and nonspecific samples. Although these differences were determined in the presence of levallorphan (1 μM), morphine at concentrations as low as 1 nM gives similar results (data not shown). The difference between total and nonspecific labeling is consistent with specific labeling of bind-





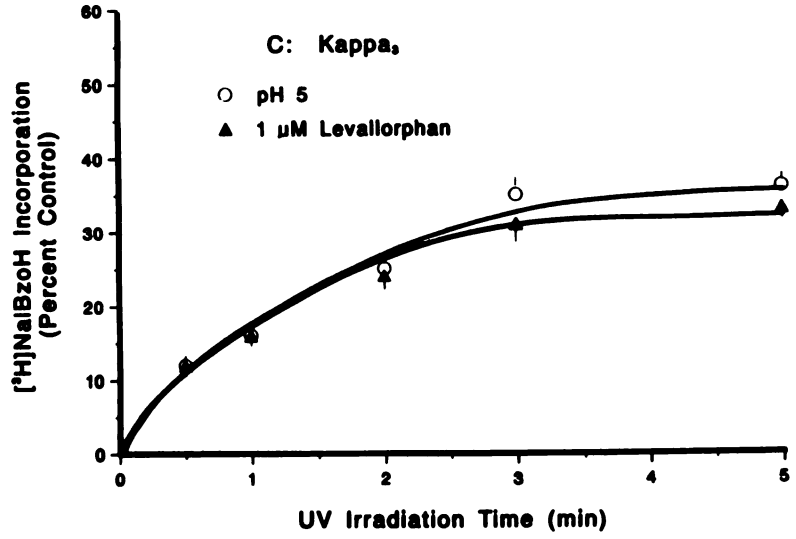
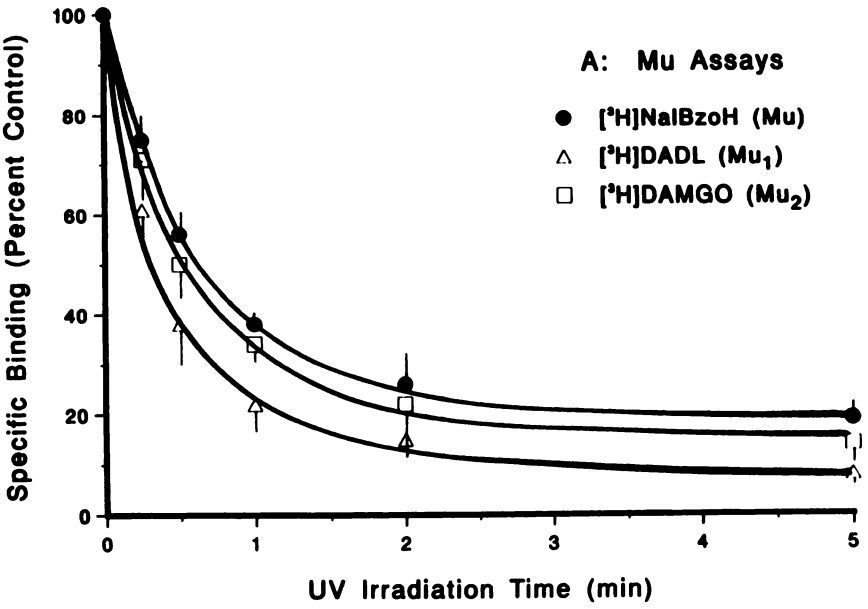
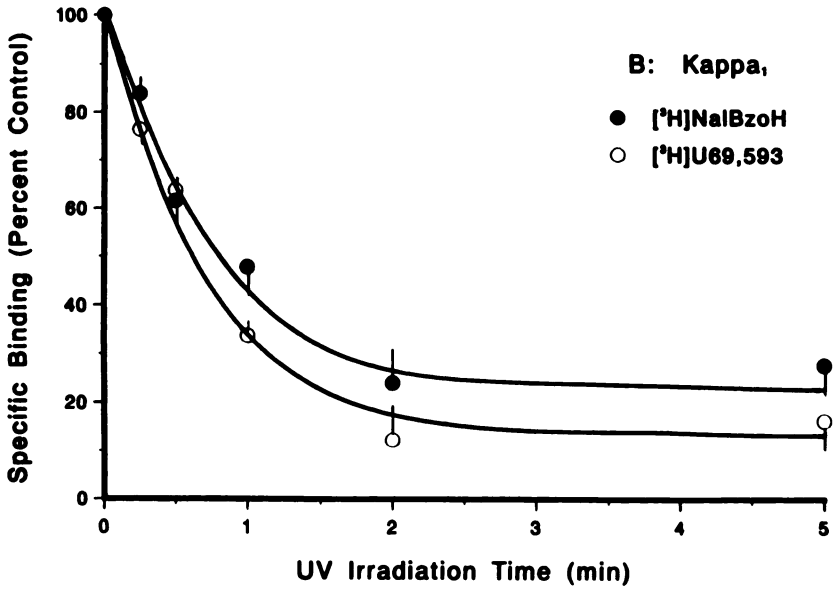


Fig. 2. Time course of irreversible incorporation of [^{3}H]NalBzoH into membranes. Binding assays labeling μ (A), κ_1 (B), and κ_3 (C) receptors, using [^{3}H] NalBzoH, were performed as described in Materials and Methods. After free radiolabel was removed, homogenates were UV irradiated for the stated time, after which reversible labeling was dissociated over 20 hr at 25°. No significant specific binding remained in control samples that were not exposed to UV radiation. A, For μ binding, dissociations were initiated by lowering the pH to 5 with acetic acid or by adding Gpp(NH)p (100 μM). For κ_1 (B) and κ_3 (C) binding, reversible binding was dissociated by lowering the pH to 5 with acetic acid or adding levallorphan (1 μM). Binding before the dissociation was: μ, 3219 ± 104 cpm; κ_1, 3217 ± 393 cpm; and κ_3, 8720 ± 670 cpm. Results are the means ± standard errors of three separate experiments.





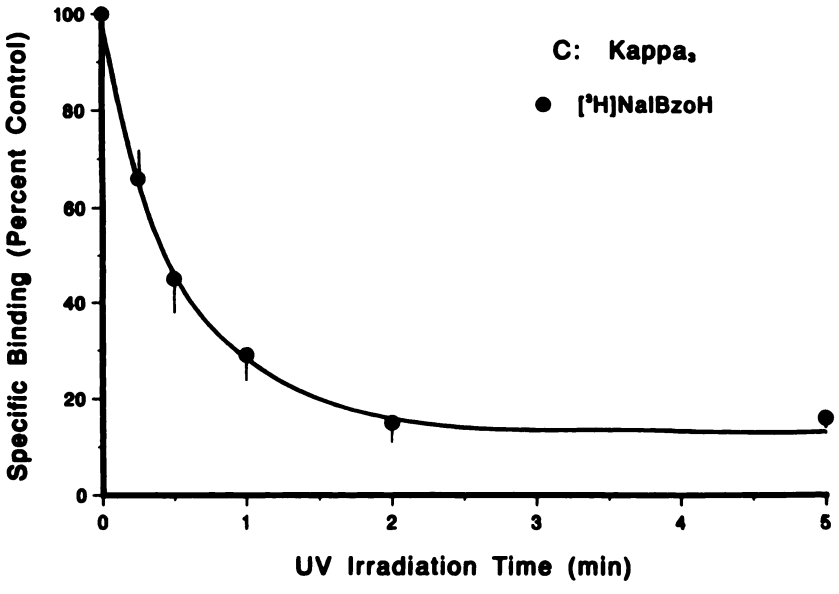


Fig. 3. Effect on μ and κ binding of UV exposure to membranes. Binding assays labeling μ (A), κ_1 (B), and κ_3 (C) receptors, using [^{3}H]NalBzoH, were performed as described in Materials and Methods. Calf striatal (μ and κ_3) and guinea pig cerebellar (κ_1) homogenates were exposed to UV for the stated time, after which selective binding assays were performed. A, Control μ binding with [^{3}H]NalBzoH (44.1 Ci/mmol) was 8684 ± 1488 cpm. Control μ_1 binding with [^{3}H]DADL (0.7 nM, 39.3 Ci/mmol) in the presence of DPDPE (1 nM) was 3348 ± 1386 cpm. Control μ_2 binding with [^{3}H]DAGO (1 nM, 40.8 Ci/mmol) ([^{3}H]DAMGO) in the presence of DSLET (5 nM) was 3348 ± 1386 cpm. B, Control κ_1 binding with [^{3}H]NalBzoH and with [^{3}H]U69,593 (1 nM, 75 Ci/mmol) was 3475 ± 60 and 1767 ± 83 cpm, respectively. C, Control κ_3 binding with [^{3}H]NalBzoH was 10,001 ± 582 cpm. Results are the means ± standard errors of three separate experiments.

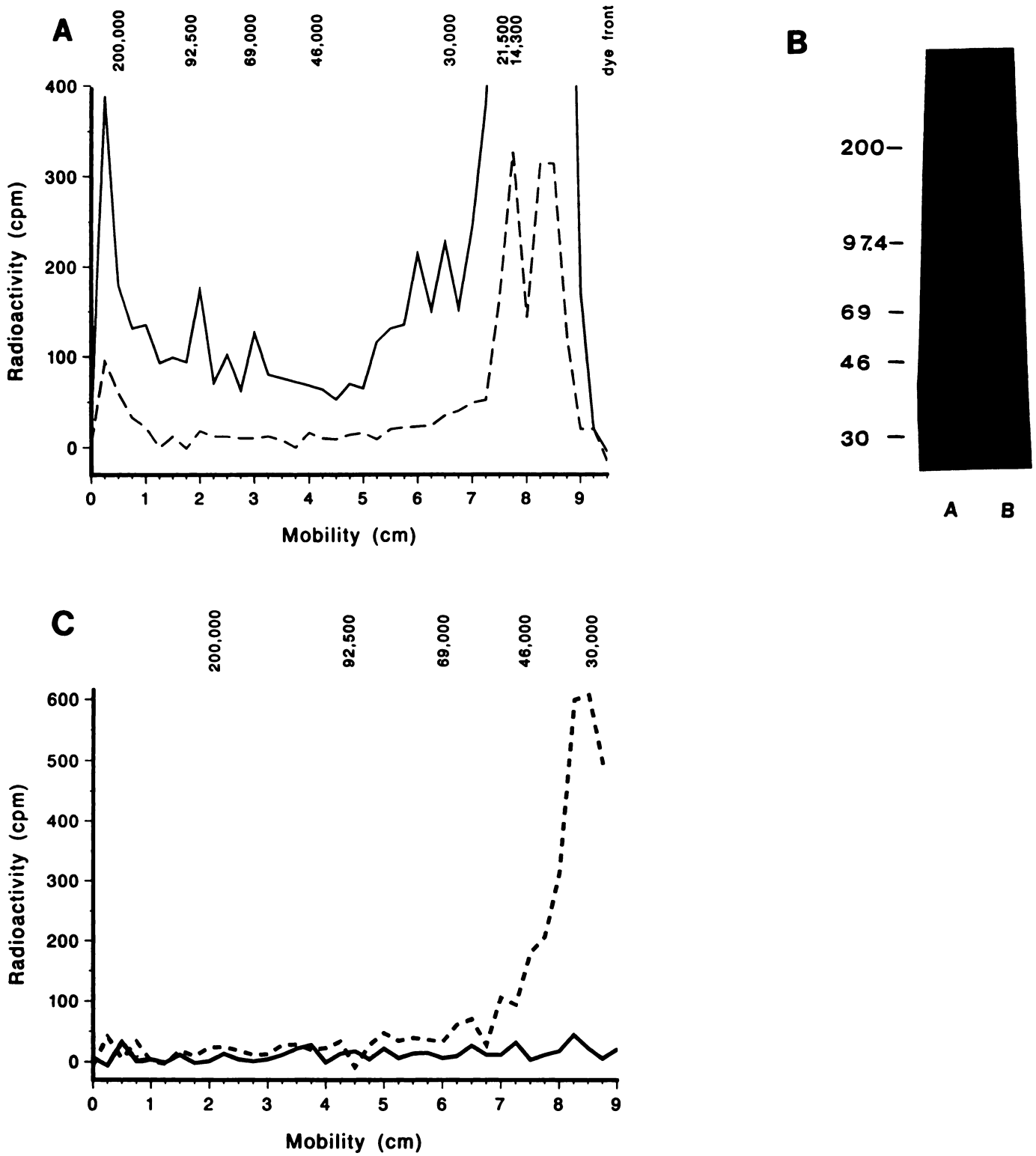


Fig. 4. Protein and [^{3}H]NalBzoH distribution in SDS-PAGE. A, Calf striatal membranes were labeled with [^{3}H]NalBzoH (1 nM) in the presence of $MgSO_4$ (5 mM) for 60 min, in the absence (total binding) (——) or presence of levallorphan (1 μM; nonspecific binding) (– – –). Under these conditions, [^{3}H]NalBzoH labels both κ and μ sites. Free [^{3}H]NalBzoH was removed by centrifugation and resuspension, after which the membranes were irradiated and prepared for SDS-PAGE as described previously. Each lane was loaded with approximately 1.6 mg of protein, corresponding to 8130 cpm for total and 2385 cpm for nonspecific binding. B, Calf striatal membranes (approximately 1.6 and 3.2 mg of protein for *lanes A* and *B*, respectively) were subjected to incubation times and temperatures described previously, prepared for SDS-PAGE, and stained. The two major protein bands had molecular weights of approximately 55,000 and 45,000. C, Liver membranes (——). Liver membranes were irradiated with [^{3}H] NalBzoH (70,000 cpm/ml), centrifuged, solubilized with SDS sample buffer, and run on SDS-PAGE, using approximately 1.6 mg of protein, corresponding to 3500 cpm. Free [^{3}H]NalBzoH (– – –). [^{3}H]NalBzoH was boiled with sample buffer and run on SDS-PAGE. The peak of radioactivity seen with a mobility of 8.5 cm represents approximately 1–2% of the added radioactivity. The remainder migrated with the dye front (9.5 cm) and is not included on this graph.

ing sites, but other possibilities still remain. If [^{3}H]NalBzoH were randomly labeling proteins, the total sample would have greater incorporation than the nonspecific binding, simply due to the greater amount of radiolabel present at the time of irradiation. If this were the case, however, we might expect the pattern of radioactivity on SDS-PAGE to correspond to protein bands. In fact, we observed large differences between the protein banding pattern and the distribution of radioactivity. The major peaks of radioactivity ran with apparent molecular weights far greater than that of either of the two major protein bands.

The results of the liver membrane labeling study also argue against a random incorporation of label. Because this tissue contains no specific binding sites, the incorporation of [^{3}H]NalBzoH is random. Under UV irradiation conditions identical to those used in the brain membranes, less than 5% of [^{3}H]NalBzoH present during the irradiation was actually incorporated into membranes, indicating that [^{3}H]NalBzoH is not very reactive under these conditions. Even though the concentration of [^{3}H]NalBzoH present during UV exposure was greater than 5-fold higher than that present during the affinity labeling of brain membranes, we observed less than half the incorporation. Together, these results are consistent with a selective incorporation of radiolabel.

Despite these controls, we still cannot be certain that the labeled proteins actually represent specific opioid binding sites. Even if the labeling is specific, correlation of specific peaks with binding sites remains difficult. First, total [^{3}H]NalBzoH binding comprises a mixture of μ_1, μ_2, and κ_3 sites. Although we observed labeling in the range of M_r 65,000, a number of higher molecular weight peaks also were present. Although these additional peaks might represent sites previously not reported (i.e., μ_1 or κ_3), they might also correspond to proteins in close proximity to the receptor rather than the binding site itself. Clearly, this is an issue that requires additional investigation.

The major strength of this technique, using [^{3}H]NalBzoH, is its potential to selectively affinity label μ, κ_1, and κ_3 receptors. Both μ_1 and μ_2 sites are labeled together, but κ_1 and κ_3 receptors can be labeled separately. Although some affinity labels have been reported for μ receptors, affinity labels for κ_1 receptors are very limited, and [^{3}H]NalBzoH remains the only κ_3 affinity ligand.

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