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Photoaffinity Labeling of Peripheral-Type Benzodiazepine-Binding Sites

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SUMMARY

The use of a novel photoaffinity label for the peripheral-type benzodiazepine-binding site is described. This compound, PK 14105, has high affinity (4 nM) and selectivity for cardiac benzodiazepine-binding sites. Under ultraviolet light, PK 14105 couples covalently to an 18,000-Da membrane protein which apparently corresponds to the (or a part of the) cardiac benzodiazepine-binding site. Since covalent attachment of PK 14105 totally precludes the binding of other ligands to this binding site, it is suggested that, during ultraviolet irradiation, this compound inserts covalently into the binding domain of the peripheral-type benzodiazepine-binding site.

The use of photoaffinity ligands for hormone and neurotransmitter receptors has been invaluable in the unraveling of receptor structure and has also been of a certain utility in studying receptor turnover and the pharmacological sequelae of receptor activation. The wide range of receptors to which this approach has been applied has been reviewed recently (1).

Of the two types of benzodiazepine-binding site, the central-type receptor (coupled to GABA-activated chloride channels) can be photolabeled by a range of 7-nitrobenzodiazepines, including flunitrazepam (2, 3). The peripheral-type benzodiazepine-binding site, however, although it does recognize flunitrazepam, cannot be photolabeled by this compound (3). The reason for this differential sensitivity of the two subtypes of binding site to flunitrazepam is not clear, but presumably reflects the lack of an appropriately positioned "receptive" group in the binding site of the peripheral-type binding site.

The current study examines the use of PK 14105, a nitrophenyl derivative of the selective peripheral-type ligand, PK 11195, as a potential photoaffinity label for these sites.

Materials and Methods

Synthesis of PK 14105. 1-(2-Fluoro-5-nitrophenyl)-3-isoquinolinecarboxylic acid was synthesized from 2-(2-fluoro-5-nitrophenyl)-4-phenylmethylene-5-(4-H)oxazolone (4). This acid was reacted with ethylchloroformate in toluene containing triethylamine and the mixed anhydride so formed reacted with *N*-methyl-2-butanamide to yield 1-(2-fluoro-5-nitrophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 14105). The tritiated product was synthesized by methylation of the *N*-desmethyl precursor with [^{3}H]-methyl iodide in dimethoxyethane in the presence of sodium hydroxide at ambient temperature.

Preparation of cardiac membranes. Rats (Sprague-Dawley males, 200 g) were killed by cervical dislocation and their hearts dissected. The atria were discarded and the ventricles homogenized in 4 volumes of 5 mM Tris-HCl containing 1 mM $MgCl_2$ and 0.25 M sucrose. A crude membrane preparation was prepared therefrom as described previously (5). These membranes were stored frozen until required, without any noticeable loss of binding activity.

Radioligand binding assays. The binding of [^{3}H]PK 11195 and [^{3}H]PK 14105 to cardiac membranes was performed at ambient temperature using the assay conditions described previously for [^{3}H]PK 11195 (5). The concentrations of radioligand used routinely in the binding assays were 1 nM ([^{3}H]PK 11195) and 2 nM ([^{3}H]PK 14105). The binding of [^{3}H]QNB to heart membranes was performed as described previously (5) and that of [^{3}H]flunitrazepam to cortical membranes was performed as described by Speth *et al.* (6). The binding of [^{3}H]spiroperidol to D_2-dopamine receptors and S_2-serotonin receptors in frontal cortex was performed using the incubation conditions described by Hamblin *et al.* (7), using cinanserin (10^{-6} M) and sulpiride (10^{-6} M) to define the nonspecific binding.

Photolabeling of binding sites with PK 14105. A Blak-Ray ultraviolet lamp (Ultraviolet products, San Gabriel, CA) was used to irradiate the membranes; this lamp emits a broad spectrum ultraviolet radiation, with a maximum at 366 nm. Aliquots (1 ml) of these were placed in 1.7-cm^2 cell culture dishes and irradiated at 22°. The intensity of the radiation received in each dish was estimated by ferrioxalate actinometry to be 4.24 nE/min using the method of Parker (8). Membranes (50 μg of protein/ml) were preincubated for 5 min with PK 14105 (10^{-7} M) in the dark, before being irradiated further for 1 hr. They were then harvested by centrifugation and washed twice by resuspension and centrifugation in 50 mM Tris-HCl buffer, pH 7.4,

ABBREVIATIONS: QNB, quinuclidinyl benzilate; DEP, diethylpyrocarbamate.

containing $MgCl_2$ (10 mM) to remove any PK 14105 not bound covalently to the membranes. Control samples were prepared which had undergone either irradiation in the absence of PK 14105 or incubation for 1 hr with PK 14105 with no irradiation. The values presented in the Results are expressed as a percentage of these latter controls. To estimate the covalent attachment of [^{3}H]PK 14105, membrane samples were preincubated for 5 min with [^{3}H]PK 14105 (2 nM) in 50 mM Tris-HCl buffer at pH 8.5 containing 10 mM $MgCl_2$ and then irradiated for 1 hr. The samples then were removed from under the lamp and unlabeled PK 11211 (10^{-5} M) was added to them in order to displace from the binding sites any remaining [^{3}H]PK 14105 not covalently bound. Pilot experiments showed that 15 min of incubation was sufficient to displace any noncovalent specific binding, and this incubation time was used routinely thereafter. The membranes were then filtered through Whatman GF/C filters and washed twice with 50 mM Tris-HCl, 5 ml, pH 7.4. The radioactivity retained by the filters was estimated by liquid scintillometry in the usual way.

Gel electrophoresis. The molecular weights of the protein labeled covalently by [^{3}H]PK 14105 were measured by gel electrophoresis using the method of Laemmli (9). The membranes were solubilized by boiling for 10 min in 0.1 M Tris-HCl, pH 6.8, containing dithiothreitol (5%), glycerol (10%), and bromophenol blue (0.02%). Aliquots corresponding to 50–250 μg of protein were applied to the top of polyacrylamide gels through which they migrated under a voltage of 60 V overnight. Standard proteins labeled with ^{14}C were applied to a parallel lane. After electrophoresis, the gels were sliced into 50 bands (2 mm) and each band digested in hydrogen peroxide (1 ml) overnight at 60° in scintillation vials. The next morning, scintillation fluid (Insta-Gel, 10 ml) was added and the radioactivity therein was estimated by liquid scintillometry. Other gels were processed by autoradiography: these were dessiccated under vacuum and apposed to tritium-sensitive film (LKB Ultrofilm) for 2 months at −80°.

Data handling. Saturation, displacement, and association curves were fitted by nonlinear regression analysis using an iterative curve-fitting procedure on an IBM-PC microcomputer to the following models:

$$\text{Saturation curves: } [B] = [B_{max}][L]^n/(K_D + L^n)$$

$$\text{Inhibition curves: } [B] = [B]_O\ IC_{50}/(IC_{50} + [I])$$

$$\text{Association curves: } [B]_t = [B]_{eq}\ (1 - e^{-(k_1 L\ +\ k_{-1})})$$

$$\text{Dissociation curves: } [B]_t = [B]_{eq}\ e^{-k_{-1}t}$$

where B_{max} represents the maximal binding capacity, K_D the dissociation affinity constant of the radioligand, L the concentration of radioligand, I the concentration of inhibitor, k_1 the association rate constant, k_{-1} the dissociation rate constant, and n the Hill coefficient. K_i, the dissociation affinity constant for an inhibitor, was calculated according to the equation $K_i = IC_{50}/(1 + K_D\ L)$.

Materials. [N-*methyl*-^{3}H]RO5-4864 (76 Ci/mmol), [^{3}H]QNB (30.2 Ci/mmol), and [^{3}H]spiperone (27.6 Ci/mmol) were obtained from New England Nuclear. [*N-methyl*-^{3}H]Flunitrazepam (78 Ci/mmol) was from Amersham, and [^{3}H]-PK 11195 (40 Ci/mmol) and [^{3}H]14105 (87 Ci/mmol) were from CEA. Benzodiazepines were generously donated by Hoffmann-La Roche (Nutley, NJ). Cinanserin was a kind gift of E. R. Squibb Ltd. and sulpiride was from Delagrange. Atropine was obtained from Prolabo.

RO5-4864 is 4′-chlorodiazepam. PK 11195 is 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide, PK 11211 its 2-fluorophenyl analogue, and PK 14105 the 2-fluoro-5-nitrophenyl analogue.

Results

Photoreactivity of PK 14105. Exposure of PK 14105 in aqueous solution to ultraviolet light does not lead to an irreversible change in the mass or ultraviolet absorption spectrum (Fig. 1A). Similar results were obtained for flunitrazepam. In diethylamine, however, PK 14105 was susceptible to nucleophilic substitution of its activated fluorine atom by the amine group of the solvent (Fig. 1B). The formation of the (2-diethylamino-5-nitrophenyl) reaction product was identified by nuclear magnetic resonance spectroscopy.

Displacement by PK 14105 of the binding of [^{3}H]PK 11195. In competition experiments, PK 14105 potently displaced the specific binding of [^{3}H]PK 11195 with a K_i of 1.79×10^{-8} M (Table 1). This compound thus has an affinity for the peripheral-type benzodiazepine-binding site some 5-fold lower than the K_D of PK 11195 itself. The Hill coefficient of the displacement curve was 1.16 (±0.05), compatible with simple competition for a single binding site. PK 14105 had no detectable affinity for central benzodiazepine receptors or for serotonin, muscarinic, or dopamine receptors ($IC_{50} > 10^{-5}$ M).

Binding of [^{3}H]PK 14105 to cardiac membranes. [^{3}H]PK 14105 bound to a single saturable population of binding sites on rat cardiac membranes. The pooled data from 12 independent saturation curves, one of which is illustrated in Fig. 2, yielded K_D and B_{max} values of 4.0 (2.2–5.9) $\times 10^{-9}$ M and 5.8 (4.8–6.8) pmol/mg of protein. The Hill coefficient was 1.03. The capacity of these sites is thus comparable to their capacity for [^{3}H]RO5-4864 (5.7 pmol/mg of protein; Ref. 5). At the concentration of radioligand routinely used (1 nM), specific binding represented >95% of the total binding of [^{3}H]PK 14105.

The specific binding of [^{3}H]PK 14105 could be displaced by a number of compounds active on peripheral-type benzodiazepine-binding sites (Table 1). The affinities of these compounds are in good agreement with their affinities for the site labeled by [^{3}H]PK 11195. The displacement curve had Hill coefficients not significantly different from unity.

Association and dissociation of [^{3}H]PK 14105 to/from its binding sites followed monoexponential processes and were extremely rapid at 25° (Fig. 3). The rate constants of association (k_1) and dissociation (k_{-1}) were 1.12×10^{10} M^{-1} sec^{-1} and 1.71 sec^{-1}, respectively at 4°. The equilibrium dissociation constant ($K_D = k_{-1}/k_1$) derived from these data was 0.15 nM.

The binding of [^{3}H]PK 14105 was unaltered on membranes that had been irradiated prior to the binding experiment. Irradiation of solutions of the ligand led to a small decrease in the binding (Table 2, parameter 1).

Covalent modification by PK 14105 of peripheral-type benzodiazepine-binding site. Irradiation of cardiac membranes with PK 14105, followed by repeated (four) washings, led to a decrease in the ability of these membranes to bind [^{3}H]PK 11195 or [^{3}H]RO5-4864 (Figs. 4–6). No effect on [^{3}H]PK 11195 binding was observed following either irradiation of the membranes in the absence of PK 14105, exposure of the membranes to PK 14105 without irradiation, or exposure to previously irradiated PK 14105 (Table 2, parameter 3). The inhibition of the binding of [^{3}H]PK 11195 observed depended on the concentration of PK 14105 used and on the duration of the irradiation; a half-maximal effect was observed at 1.5 (0.6–2.4) $\times 10^{-7}$ M (Fig. 4). Saturation analysis of the binding of [^{3}H]PK 11195 or [^{3}H]RO5-4864 to membranes exposed to PK 14105 under ultraviolet light revealed that the inhibition of binding observed was due to a decrease in the number of the specific binding sites. In these experiments, the B_{max} value for both [^{3}H]PK 11195 and [^{3}H]RO5-4864 was some 70% higher

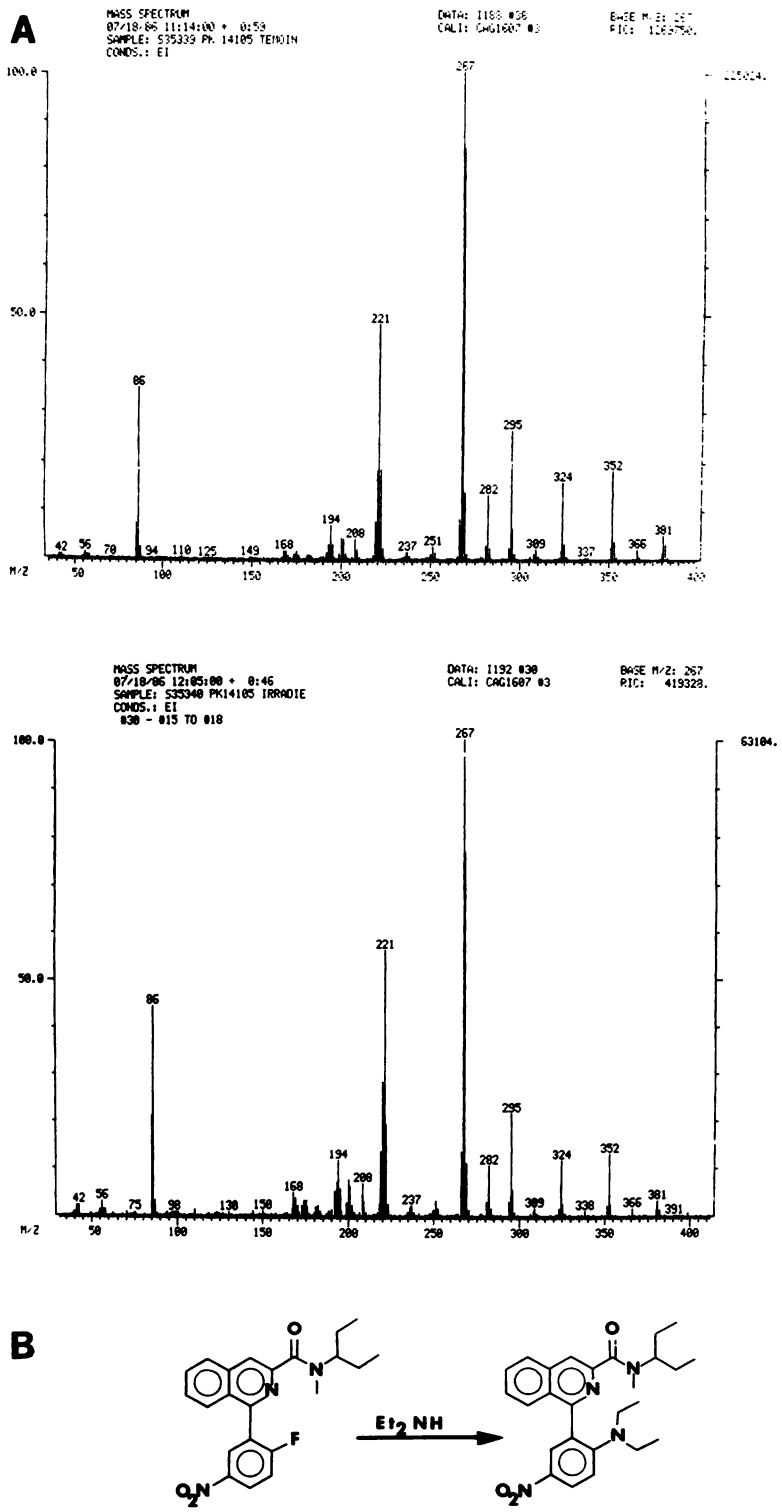

Fig. 1. A. Mass spectra of PK 14105 (10^{-5} M) recorded before (*top*) and after (*bottom*) irradiation for 1 hr with ultraviolet light at 366 nm. B. Structure of PK 14105 and its reaction product with diethylamine.

than that found in previous experiments (e.g., Refs. 5 and 10). To verify that there was, in fact, no significant difference in the binding capacity of the sites for [^{3}H]PK 14105 compared to the other two ligands, the binding of [^{3}H]PK 14105 was studied in these same three membrane preparations; again, the B_{max} value for this ligand was 80% higher than the mean value. The affinity of the remaining sites for [^{3}H]PK 11195 was not changed by the treatment (Fig. 7), but there was a slight *increase* in their affinity for [^{3}H]RO5-4864. The Hill coefficients remained at unity. The time course of the photo-occlusion of [^{3}H]PK 11195 binding is illustrated in Fig. 6.

Neither muscarinic receptors in the same membranes labeled by [^{3}H]QNB, central-type benzodiazepine receptors on cortical membranes labeled by [^{3}H]flunitrazepam, D_2-dopamine receptors, nor S_2-serotonin receptors were modified by exposure to PK 14105 (10^{-7} M) under ultraviolet light.

Photolabeling of cardiac membranes by [^{3}H]PK 14105. If membranes were exposed to ultraviolet light in the

TABLE 1

Pharmacological specificity of the binding sites for [^{3}H]PK 11195 and [^{3}H]PK 14105 in rat cardiac membranes

IC_{50} values for various compounds as inhibitors of the specific binding of 1 nM [^{3}H]PK 11195 or [^{3}H]PK 14105 to rat cardiac membranes are given. Each value represents the IC_{50} value and its 95% confidence limits obtained by nonlinear regression analysis of the data from three independent 11-point displacement curves. In column 4 (Protection), the data were obtained from experiments in which the compounds were incubated with [^{3}H]PK 14105 under ultraviolet illumination, and the extent of irreversible incorporation of [^{3}H]PK 14105 was determined. The values for [^{3}H]PK 11195 binding have been culled from Ref. 10. No appreciable inhibition of either the reversible binding or the covalent incorporation was seen with the following compounds (all tested at 10^{-4} M): atropine, picrotoxin, morphine, glutamic acid, cinanserin, GABA, verapamil, and fluphenazine; quinacrine and propranolol were inactive at 10^{-5} M.

	[^{3}H]PK 11195	[^{3}H]PK 14105	
		Competition	Protection
PK 14105	$2.71\ (1.58–3.87) \times 10^{-8}$	$1.12\ (0.82–1.42) \times 10^{-8}$	$2.88\ (1.09–4.67) \times 10^{-8}$
PK 11195	$8.27\ (6.82–10.1) \times 10^{-9}$	$3.8\ (2.70–4.90) \times 10^{-9}$	$5.5\ (1.8–9.2) \times 10^{-9}$
RO5-4864	$4.83\ (3.74–6.23) \times 10^{-8}$	$3.47\ (2.45–4.5) \times 10^{-8}$	$8.00\ (3.10–12.9) \times 10^{-8}$
Diazepam	$5.17\ (2.86–9.32) \times 10^{-7}$	$5.79\ (4.1–7.48) \times 10^{-7}$	$7.87\ (2.16–1.36) \times 10^{-7}$
Clonazepam	$7.43\ (1.70–32.3) \times 10^{-5}$	$2.19\ (1.45–2.94) \times 10^{-5}$	$1.19\ (0.44–1.95) \times 10^{-5}$

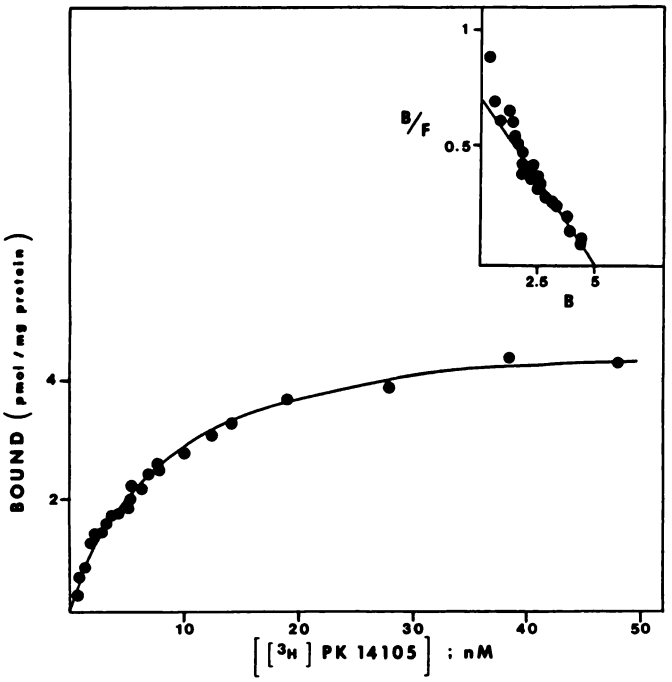

Fig. 2. Saturation of peripheral-type benzodiazepine-binding sites by [^{3}H] PK 14105. The data represent specific binding from a single 24-point saturation curve. The *line* represents the best fit to a single hyperbola determined by iterative nonlinear regression analysis. The *inset* represents a Scatchard transformation of the same data.

presence of [^{3}H]PK 14105, the radioligand became attached to the membranes in such a way that it could no longer be removed by repeated (four) washings. This irreversible incorporation could be blocked completely by incubating the membranes with high concentrations of PK 11195 or RO5-4864 but not by clonazepam. For a variety of ligands, the concentrations necessary to prevent the photolabeling of the membranes by [^{3}H] PK 11195 were in good agreement with those necessary to occupy the peripheral-type benzodiazepine-binding site in this tissue (Table 1). These data suggest that PK 14105 is a specific photoaffinity label for the peripheral-type benzodiazepine-binding site.

If either the membranes or the ligand were irradiated separately and the binding examined subsequently, no irreversible incorporation was observed. The reversible binding, however, remained unchanged (Table 2, parameters 1 and 2). This implies that the photoactivation of PK 14105 is reversible.

The time course of incorporation of [^{3}H]PK 14105 was slow compared to the rate at which it bound reversibly to the receptor (Fig. 7; cf. Fig. 3). This suggests that attainment of equilibrium is not rate-limiting in the formation of the covalent complex, i.e., $k_1 \gg k_2$ in the mechanism:

$$R + L \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} R{:}L \xrightarrow[k_2]{} R - L$$

where $R{:}L$ represents the reversible and $R - L$ the covalent complex. The formation of covalent complex is thus zero order with $k_2 = 5 \times 10^{-9}$ M sec^{-1} under the experimental conditions used. This represents a quantum efficiency of 7.2×10^{-9}. This low quantum efficiency may reflect the possibility that the irradiation might not have been performed at the optimal wavelength.

Further evidence for a zero order mechanism is provided by the finding that the rate of fixation of a low concentration (2 nM) of [^{3}H]PK 14105 in this experiment was comparable to the rate of inactivation of the peripheral-type benzodiazepine receptor induced by a 100-fold higher concentration of PK 14105 in the experiment shown in Fig. 6.

Decreasing the temperature to 4° led to a doubling of the rate of incorporation ($k_2 = 1.3 \times 10^{-18}$ M sec^{-1}), but this was less than might have been expected from the markedly decreased dissociation rate (cf. Fig. 3).

At concentrations of [^{3}H]PK 14105 up to and around its K_D, the amount of radioligand covalently incorporated corresponded to the number of binding sites occupied reversibly by that concentration of ligand at equilibrium (Fig. 8). At higher concentrations, the amount of incorporation was more variable, but averaged around 80%.

Unlike the reversible binding of [^{3}H]PK 14105 to its binding site, which shows a broad pH tolerance, the efficiency of covalent incorporation was highly pH dependent, with a maximum at pH 9.0 (Fig. 9).

The photolabeled site could be solubilized in sodium dodecyl sulfate and migrated as a single band on polyacrylamide gel electrophoresis (Fig. 10). By reference to marker proteins separated on the same gels, the apparent molecular weight of the labeled protein was calculated to be 18,000. The labeling of this protein band could be blocked completely by co-incubating the membranes with compounds active at peripheral-type benzodiazepine-binding sites during the photoreaction (Fig. 11).

Discussion

PK 14105, like flunitrazepam, does not undergo irreversible modification on exposure to ultraviolet light, even though it is prone to photocatalyzed incorporation into membrane proteins. This implies that this compound is a reversibly photoactivated ligand forming a short-lived photoactive intermediate which will only form covalent bonds when juxtaposed to a suitably reactive group. The reaction of PK 14105 with diethylamine shows that it is susceptible to substitution of its 2′-fluorine by a nucleophilic group. Similar photocatalyzed nucleophilic substitutions have been reported for other *p*-fluoronitrophenyl compounds (11). Possible nucleophilic groups in the membrane protein labeled by PK 14105 may be the terminal amino group of a ω-amino amino acid or the thiol group of a cysteine. Reversibly photoactivated ligands have several advantages over irreversibly photoactivated ligands, such as azido compounds, one of which being that it is easier to prevent nonspecific photolabeling outside the binding domain, since it is only when the ligand is bound to the receptor that the attachment site is suitably positioned for reaction.

PK 14105 had a high affinity for the sites labeled by [^{3}H]PK

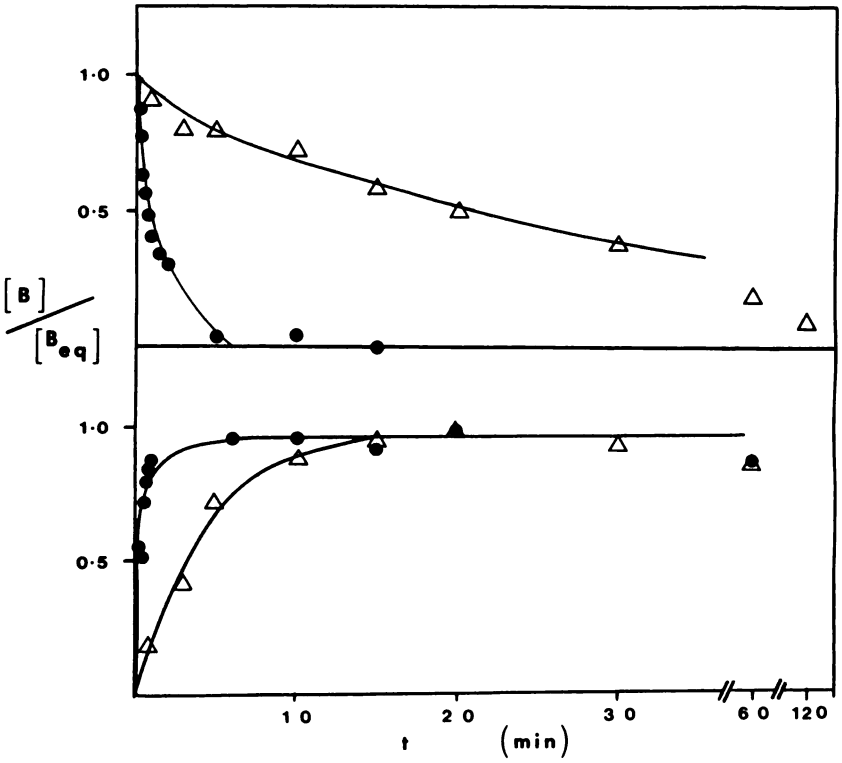

Fig. 3. Time course of the binding of [^{3}H]PK 14105 to peripheral-type benzodiazepine-binding sites in heart membranes. Determinations were made at 4° (△) and at 25° (●). Each point represents the mean of three independent determinations; the standard errors are not shown for clarity but were less than 15% of the mean. The lines are the best fits to single exponentials determined by nonlinear regression analysis. *Top:* Dissociation of [^{3}H]PK 14105 initiated by addition of PK 11211 (10^{-6} M) 60 min after the radioligand. *Bottom:* Association of [^{3}H]PK 14105 to cardiac membranes.

TABLE 2

Effect of ultraviolet irradiation on the binding properties of peripheral-type benzodiazepine-binding sites

The binding of [^{3}H]PK 14105 (2 nM) and [^{3}H]PK 11195 (0.75 nM) was examined under four conditions: A, control; B, to membranes irradiated in the absence of PK 14105; C, to non-irradiated membranes using previously irradiated PK 14105 (tritiated or nontritiated); and D, to irradiated membranes with irradiated PK 14105. Four parameters were investigated: 1) the reversible binding of [^{3}H]PK 14105, 2) the covalent incorporation of [^{3}H]PK 14105, 3) the ability of PK 14105 to irreversibly modify the subsequent binding of [^{3}H]PK 11195, and 4) the ability of PK 14105 to displace the binding of [^{3}H]PK 11195. Irradiations were performed for 1 hr, as were the non-irradiated control incubations in 2A, 2B, 2C, 3A, 3B, and 3C. Data were obtained from a single experiment whose component parts were repeated on separate occasions with similar results. The data are expressed as the percentage of the binding seen in non-irradiated controls (A), except for those for parameter 2 which are expressed as a percentage of the incorporation seen in condition D, and those for parameter 4 which are expressed as the percentage of the binding of [^{3}H]PK 11195 to the control membranes (A) in the absence of PK 14105 obtained in the presence of the indicated concentration of PK 14105. The binding of [^{3}H]PK 11195 to irradiated membranes represented 98% of that seen with non-irradiated membranes.

Parameter	A Neither membranes nor PK 14105 irradiated	B Membranes only irradiated	C PK 14105 only irradiated		D Both membranes and PK 11195 irradiated	
			[^{3}H]	[^{1}H]	Together	Separately
	%	%	%		%	
1. Reversible binding of [^{3}H]PK 14105	100	94.2	77.5			79.7
2. Covalent incorporation of [^{3}H]PK 14105	0	0	0		100	
3. Covalent modification of [^{3}H]PK 11195 binding by PK 14105 (10^{-6} M)	100	120		113	19.6	
4. Displacement of [^{3}H]PK 11195 binding by [^{3}H] PK 14105						
10^{-8}	75	83		87		80
3×10^{-8} M	41	56.9				51

11195, with a K_i value of 17.9 mM, close to that of PK 11195 itself (K_i = 7.27 nM) (10). PK 14105 seemed specific for this site, as no displacement of radioligand binding was observed for a variety of other receptors.

The binding of PK 14105 to the peripheral-type benzodiazepine-binding site was characterized directly using a tritiated derivative. [^{3}H]PK 14105 bound reversibly to a population of binding sites with the same capacity as that of those for [^{3}H]RO5-4864. The binding interaction followed classical mass action kinetics and had a pharmacological specificity appropriate for the peripheral-type benzodiazepine-binding site.

When exposed to ultraviolet light, PK 14105 reduced the ability of membranes to bind subsequently [^{3}H]PK 11195 and [^{3}H]RO5-4864, and this inhibition persisted after washing sufficiently to remove all traces of free PK 14105. The loss of binding ability was related to a decrease in the number of binding sites, and could be total if the concentration of PK 14105 was sufficiently high. This suggests irreversible photocatalyzed inactivation of the binding site by PK 14105.

Irreversible inactivation of the peripheral-type benzodiazepine-binding site had been demonstrated previously (12) with the histidine-modifying reagent, DEP. Two important differences were observed between the inactivation of the peripheral-

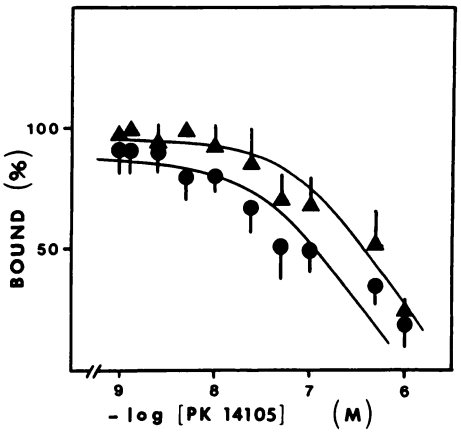

Fig. 4. Inactivation of the radioligand binding capacity of peripheral-type benzodiazepine-binding sites by PK 14105. Membranes were irradiated for 60 min with the indicated concentrations of PK 14105 under ultraviolet light as described in Materials and Methods. After washing, the binding of [^{3}H]PK 11195 (●) and [^{3}H]RO5-4864 (▲) was measured. The data are expressed as the percentage of the binding seen in membranes irradiated without PK 14105 and represents the mean ± standard error of three ([^{3}H]PK 11195) or two ([^{3}H]RO5-4864) independent determinations.

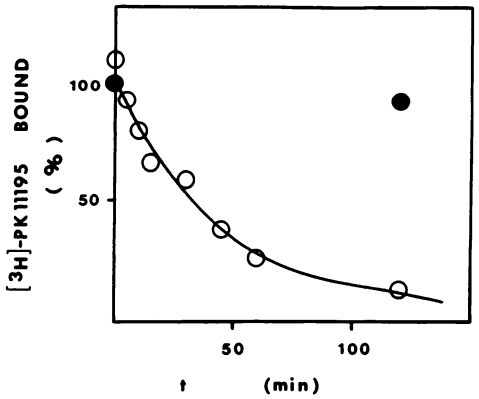

Fig. 6. Time course of the inactivation of peripheral-type benzodiazepine-binding sites by PK 14105 (10^{-7} M). The data are expressed as the percentage of the binding of [^{3}H]PK 11195 observed in non-irradiated membranes exposed to 10^{-7} M PK 14105. Each point represents the mean of two independent determinations, which differed from one another by less than 10%. ●, determinations made on non-irradiated control samples.

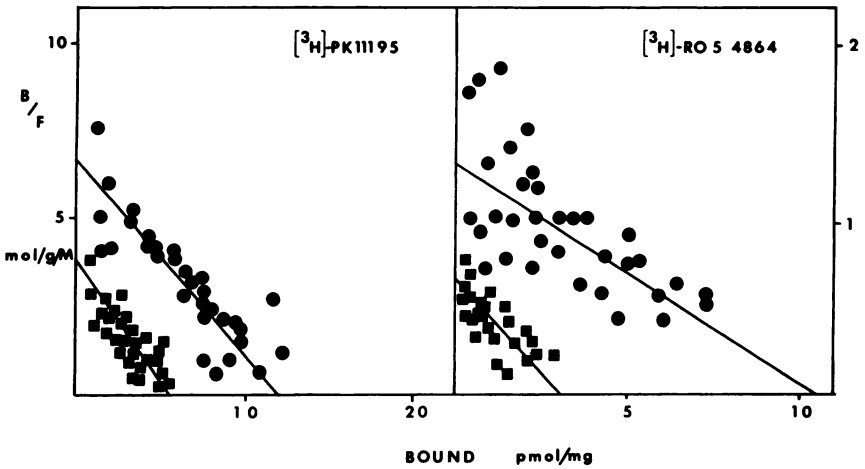

Fig. 5. Scatchard transformations of the saturation of peripheral-type benzodiazepine-binding sites by [^{3}H]PK 11195 and [^{3}H]RO5-4864 in irradiated (■) and nonirradiated (●) membranes incubated with 10^{-7} M PK 14105. The data are pooled from three independent 12-point saturation curves and the *lines* represent the best fits to single hyperbolae obtained by nonlinear regression analyses of the nontransformed data. The K_D and B_{max} values (with their 95% confidence limits) obtained are indicated below, as are the Hill coefficients (n_H):

	[^{3}H]PK 11195		[^{3}H]RO5-4864	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
K_D (nM)	1.83 (1.22–2.44)	1.42 (0.97–1.37)	8.03 (5.37–10.7)	4.86 (2.60–7.12)
B_{max} (pmol/mg prot.)	12.1 (10.6–13.5)	5.47 (4.90–6.04)	10.5 (8.7–12.3)	3.09 (2.40–3.78)
n_H	1.0	1.1	1.0	1.0

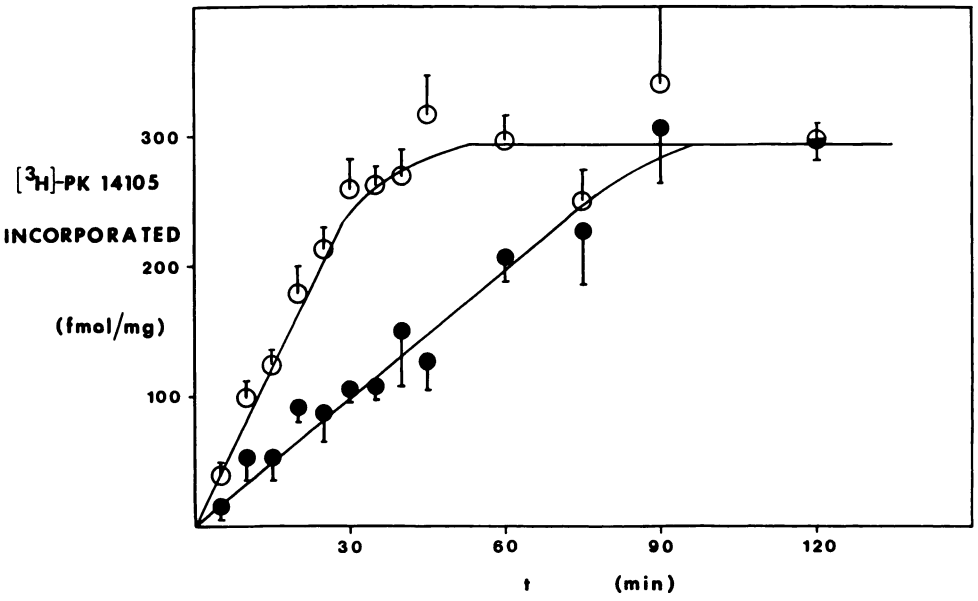

Fig. 7. Time course of the photolabeling of peripheral-type benzodiazepine-binding sites by [^{3}H]PK 14105 (2×10^{-9} M). The data represent the mean and standard error of three independent determinations. The time course was examined at 22° (●) and at 4° (○).

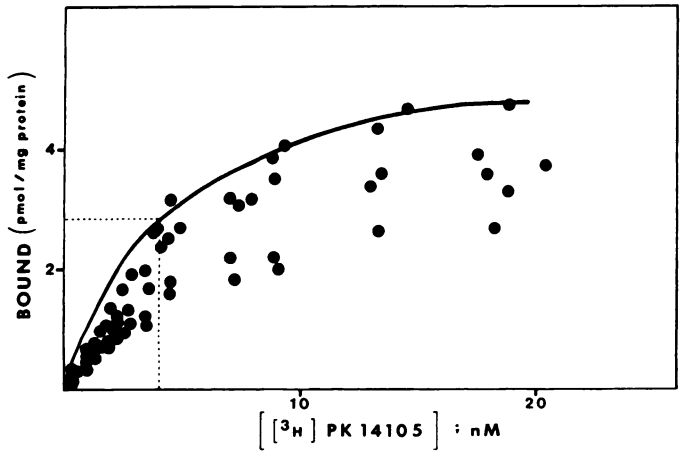

Fig. 8. Photolabeling of peripheral-type benzodiazepine-binding sites by [^{3}H]PK 14105. Membranes were irradiated with the indicated concentrations of [^{3}H]PK 14105 for 60 min. The data are pooled from six independent determinations. The *solid line* represents the saturation curve for the *reversible* binding of [^{3}H]PK 14105 and the *dashed line* indicates half-maximal saturation of the sites at the K_D.

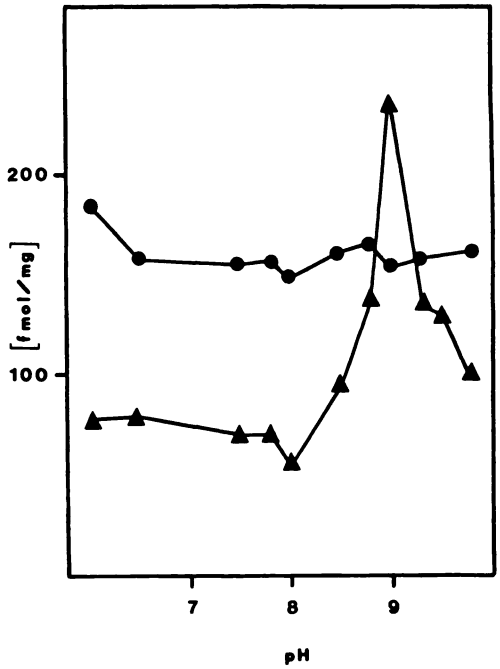

Fig. 9. Effect of pH on the reversible binding (●) and covalent attachment (▲) of [^{3}H]PK 14105 (1 nM) to peripheral-type benzodiazepine-binding sites. The data were obtained from a single experiment.

type benzodiazepine-binding site induced by DEP and that induced by PK 14105. First, PK 14105 was able to inactivate all of the binding sites, whereas only half of them were sensitive to DEP. Second, with DEP, only the binding of [^{3}H]PK 11195 was affected, the membranes conserving their ability to bind [^{3}H]RO5-4864; with PK 14105, however, the binding of both ligands was affected equally.

The availability of radiolabeled [^{3}H]PK 14105 made it possible to determine whether or not this molecule actually became incorporated into the receptor site during photocatalyzed inactivation. This indeed does seem to be the case, and nearly all of the sites available for the reversible binding of [^{3}H]PK 14105 could be labeled covalently by [^{3}H]PK 14105. This contrasts with the photolabeling of the central benzodiazepine receptor by flunitrazepam, where only a quarter of the binding sites can be photolabeled (3, 13). This may imply that the peripheral-type benzodiazepine-binding site is monomeric (unlike the tetrameric central-type receptor) or that PK 14105 is an antagonist that does not induce any allosteric changes in coupled binding sites [as has been suggested (14) for the central-type irreversible ligand RO15-4513]. The latter possibility may explain the increase in affinity of [^{3}H]RO5-4864 (which may be an agonist) for the residual sites after irradiation.

The specificity of PK 14105 for the peripheral-type benzodiazepine-binding site is indicated first by the finding that neither central-type benzodiazepine receptors nor muscarinic, dopaminergic, or serotoninergic receptors are modified by exposure to PK 14105 under ultraviolet light, and, second, by the presence of only a single band of radiolabeled protein after gel

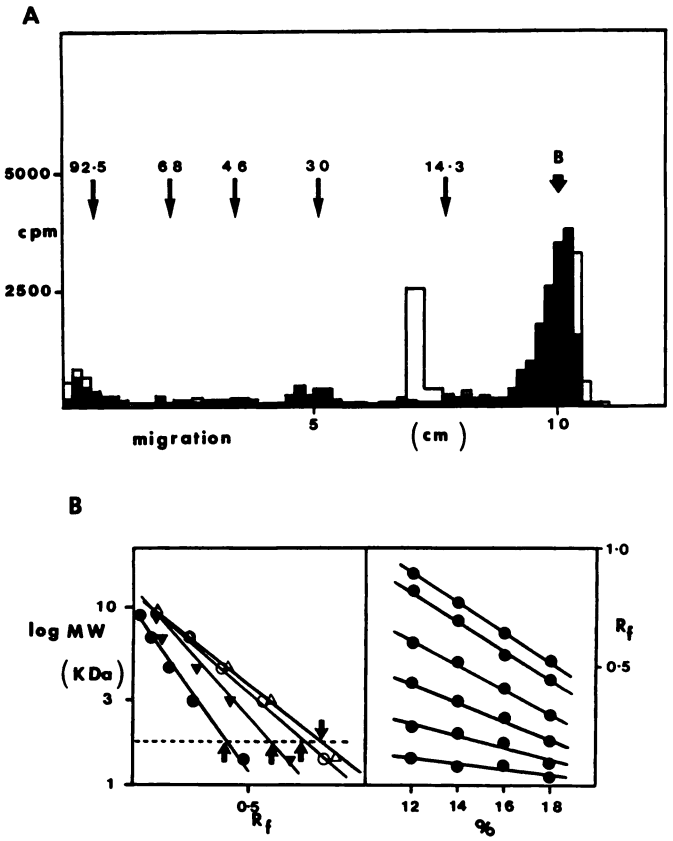

Fig. 10. A. Gel electrophoresis of membrane proteins labeled covalently by [^{3}H]PK 14105. Fifty μg of protein solubilized from labeled heart membranes were separated on a 14% polyacrylamide gel. The tritium was localized on slices of the gel. The *solid columns* represent a parallel lane on the same gel containing membranes photolabeled in the presence of 10^{-6} M PK 11211. The migration of the bromophenol blue dye front (*B*) and of standard proteins of known molecular weight (noted in kDa) are indicated by *arrows*. B. *Left:* Migration of the standard proteins as a function of molecular weight on gels containing 12% (△), 14% (○), 16% (▼), and 18% (●) polyacrylamide. The *arrows* indicate the migration of the peripheral-type benzodiazepine receptor, corresponding to a molecular weight of 18,000 (*dashed line*). *Right:* The same data represented as a function of gel concentration. The *lines* correspond to (in descending order): lysozyme (14.3 kDa), the protein labeled with [^{3}H]PK 14105, carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (68 kDa), and phosphorylase B (92.5 kDa).

Fig. 11. Gel electrophoresis of membrane proteins labeled covalently by [^{3}H]PK 14105. Autoradiogram of a 10% polyacrylamide gel after the migration of 60 μg of protein solubilized from photolabeled heart membranes. The *lanes* represent samples labeled in the presence of the following compounds (10^{-6} M): *A*, control; *B*, PK 11211; *C*, clonazepam; *D*, PK 14105; *E*, diazepam; *F*, RO5-4864. The *numbers* on the *right* indicate the migration of standard proteins a molecular weight markers.

electrophoresis of membranes photolabeled with [^{3}H]PK 14105. This specificity is probably due to the fact that nitrophenyl compounds, unlike azides, are photoactived reversibly and weakly, only reacting in the presence of an appropriate acceptor group. The finding that the photolabeling of the peripheral-type benzodiazepine-binding site has a pH maximum close to the pK value for thiol groups may implicate a cysteine residue as the site of incorporation.

The solubilized, photoaffinity-labeled peripheral-type benzodiazepine-binding site migrated on gel electrophoresis as a single protein band of molecular weight 18,000. This value corresponds well with that (23,000) obtained previously for this binding site using the technique of irradiation inactivation (5) and probably reflects the monomer size of the binding site. This protein is thus considerably smaller than most receptor proteins studied to date.

In conclusion, this study demonstrates the feasibility of irreversibly labeling the peripheral-type benzodiazepine-binding site with PK 14105 and opens the horizon to the purification and molecular characterization of this protein.

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