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Report

[Biocytin^{13}]Dynorphin A 1-13 Amide: A Potential Probe for the κ-Opioid Receptor

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A dynorphin A 1-13 amide (DYN) derivative biotinylated in position lysine 13 (B-DYN) has been prepared by automated solid-phase peptide synthesis. The derivative retained its ability to bind to avidin, and B-DYN–avidin complex showed a dissociated half-life of 10 hr at 37°C. Opioid receptor binding was measured in membrane preparations of rat brain (μ), NG-108-15 neuroblastoma–glioma hybrid cells (δ), and guinea pig cerebellum (κ). Biotinyl substitution of DYN either did not affect receptor binding (δ) or slightly reduced binding affinity (μ and κ). Binding of B-DYN to the κ receptor was very tight, with an IC_{50} value in the low picomolar range, while binding to μ and δ sites was over two orders of magnitude lower. Preassociation of B-DYN with avidin resulted in a reduction of the affinities to the investigated opioid receptors by 100- to 1000-fold. However, the apparent affinity of B-DYN–avidin for the κ-opioid receptor is sufficient to suggest that B-DYN may be a useful tool for κ-opioid receptor assay, localization, and purification.

KEY WORDS: biotinylated receptor ligand; biocytin; avidin; dynorphin; κ-opioid receptor; receptor–avidin cross-linking.

INTRODUCTION

The biotin–avidin system has emerged as a powerful and versatile tool in the analysis of proteins and peptides, because of the extremely high affinity of the egg white protein avidin for the small vitamin molecule, biotin. Biotin can be covalently linked to marker molecules without losing its avidin affinity, while avidin can be coupled with a variety of sensitive detection systems, e.g., fluorescein or enzymes. Biotinylated receptor ligands in conjunction with enzyme-labeled or immobilized avidin have been successfully employed for the detection or purification of hormone and neurotransmitter receptors (1–3), because of the ability of biotinylated ligands to cross-link receptor with avidin. We have recently described the preparation of biotinylated β_h-endorphin derivatives by biotinylation of the preformed peptide and their use as molecular probes for the μ- and δ-opioid receptor (4). Further, a biotinylated β-endorphin probe was used as a tracer in a biotin–avidin-linked ELISA[4] of β-endorphin, affording a sensitivity of 0.5 fmol per sample (5).

Highly selective ligands for the isolation of κ-opioid receptors by affinity chromatography and their subsequent detection are currently not available. Dynorphin A 1-13 amide interacts with a high affinity and some selectivity at the κ-opioid receptor (6). It is resistant to proteolytic degradation by amino- and carboxypeptidases (7). In the present report, we describe the automated solid-phase synthesis of a dynorphin 1-13 amide derivative biotinylated in position 13 [(biocytin^{13}) dynorphin A 1-13 amide] and its properties as a potential κ-opioid receptor–avidin cross-linking agent.

MATERIALS AND METHODS

Materials

[^{3}H]DADL (sp act, 50 Ci/mmol), [^{3}H]DAGO (sp act, 50 Ci/mmol), and [^{3}H]diprenorphine (sp act, 41 Ci/mmol) were purchased from Amersham (Arlington Heights, Ill.). The following compounds were obtained from the indicated sources: bovine serum albumin (BSA), bacitracin, casein, *p*-nitrophenyl phosphate, L-leucyl-L-leucine, and biotin, 2′-(4′ hydroxyazobenzene)-benzoid acid (HABA) from Sigma (St. Louis, Mo.); *N*-carboxymethyl-L-Phe-L-Leu from Serva (Heidelberg, F.R.G.); thiorphan, dynorphin A 1-13

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[4] Abbreviations used: AP, alkaline phosphatase; DADL, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,MePhe^4, gly-ol^5]enkephalin; BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; HABA, 2′-(4′-hydroxyazobenzene)-benzoic acid; DYN, dynorphin A 1-13 amide; B-DYN, [biocytin^{13}]dynorphin A 1-13 amide (biocytin is ε-biotinyl lysine); DCM, dichloromethane; DMF, *N,N*-dimethyl formamide; DCC, *N,N′*-dicyclohexylcarbodiimide; Boc, t-butyloxycarbonyl; Boc-Bct, N_α-Boc-biocytin; DIPCDI, diisopropylcarbodiimide; HOBT, hydroxybenztriazole.

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amide, [D-Ala[2], D-Leu[5]]enkephalin (DADL), and [D-Ala[2],MePhe[4],Gly-ol[5]]enkephalin (DAGO) from Peninsula (Belmont, Calif.); captopril from Squibb; diprenorphine • HCl from NIDA (Rockville, Md.); 96-well EIA plates from Costar (Cambridge, Mass.); avidin–alkaline phosphatase conjugate from Vector (Burlingame, Calif.); Boc derivatives of amino acids and *p*-methylbenzhydroxylamine resin from Applied Biosystems (Foster City, Calif.); polypropylene net (74 μm) from MacMaster-Carr (Los Angeles, Calif.); and *N*-hydroxysuccinimide and diisopropylcarbodiimide from Aldrich (Milwaukee, Wis.). All other reagents were analytical grade.

Biotin-N-hydroxy Succinimide Ester. This was prepared by a slight modification of reported procedures (8). A mixture of 5.0 g biotin (20.5 mmol), 3 g *N*-hydroxysuccinimide (26 mmol), and 4.0 g (19.4 mmol) *N,N*-dicyclohexylcarbodiimide in 60 ml DMF was stirred overnight at ambient temperature. The precipitate was removed by filtration and the filtrate was evaporated under reduced pressure. The residue was recrystallized from 450 ml acetonitrile. Yield, 4.5 g (64%); m.p., 207–209°C; lit m.p., 208–210°C (8).

N_α-*Boc-L-biocytin.* A mixture of 2.5 g N_α-Boc-L-lysine (10 mmol) and 4.2 g biotin-*N*-hydroxysuccinimide ester (12 mmol) in 50 ml DMF were stirred at ambient temperature until a clear solution was obtained. After the addition of 200 μl triethylamine, the mixture was stirred at 20°C for 48 hr. The solvent was removed under reduced pressure, and the residue was extracted with 140 ml of cold, acqueous 0.1 *M* $NaHCO_3$. The cold solution was acidified to pH ~4 with 1 *M* HCl and chilled in the refrigerator overnight. The white precipitate was filtered and dried *in vacuo* with P_2O_5. The product (3.4 g) was twice recrystallized from 140 ml ethanol and washed twice with 2 × 75 ml ether. Yield, 3.0 g, 63.5%; m.p., 139–141°C. The product gave a single peak on C18 RP-HPLC using a 0 to 50% MeOH linear gradient. LSI-MS (Kratos MS-50RF) yielded major ions at *m/z* 472, 471 (M^{+1}), and 397 (M^+ Boc).

[Biocytin[13]]Dynorphin A 1-13 Amide. Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-Leu-(biotinyl)Lys-$CONH_2$ was synthesized using a combination of the manual "teabag" (9) and automated solid-phase peptide synthesis methods. A 2.5-cm^2 polypropylene bag (74-μm mesh) was used to contain 0.42 g (0.25 mmol) of *p*-methylbenzhydrylamine resin (0.6 mmol/g;150–200 mesh). The packet was heat-sealed, placed in a 25-ml Nalgene wide-mouth bottle, and washed with 2 × 10 ml DCM for 30 sec, 2 × 10 ml 5% (v/v) diisopropylethylamine in DCM for 2 min, and 2 × 10 ml DCM for 30 sec. Boc-biocytin (472 mg, 1 mmol) was dissolved in 5 ml DMF, and 0.135 g HOBT (1 mmol) in 2 ml DMF and 126 mg (0.157 ml, 1 mmol) DIPCDI were added. The bottle cap was tightened and the mixture was agitated for 1 hr in a mechanical shaker. The bag was washed with 10 ml DMF for 30 sec and 2 × 10 ml DCM for 30 sec. After drying in a desiccator, a corner of the bag was cut and the resin transferred into the reaction vessel of an ABI peptide synthesizer. The remainder of the synthesis was performed using the ABI protocols and reagents. After completion of the synthesis, the peptide-resin (0.98 g) was cleaved with 10 ml HF containing 1 g *p*-cresol. The HF was removed using a stream of N_2 gas, and the cleaved peptide-resin was washed with 3 × 10 ml ethyl acetate. The peptide was extracted using 2 × 20 ml 50% acetic acid. The extract was diluted with water to bring the concentration to 20% acetic acid and lyophilyzed. The peptide was redissolved in 100 ml water and lyophilyzed again. Yield, 280 mg (75%) crude peptide. Analytical HPLC was performed on a Vydac C18 column (0.46 × 25 cm) using a 50-min linear gradient of 15 to 35% acetonitrile in 0.1% trifluoroacetic acid in water and monitoring at 215 nm. The product eluted at 28% acetonitrile and represented approximately 80% of the UV absorbing material. A portion of the crude product (5 mg) was purified by reversed-phase HPLC using the same system. The fraction containing the main product, shown to be pure on analytical HPLC, was lyophilyzed and stored at −20°C. The structure and purity of the product were verified by amino acid analysis, sequencing the first 12 amino acids, and by liquid secondary ion mass spectrometry, performed on a Kratos MS 50 RF mass spectrometry using the method described earlier (4). The measured MH^+ ion at *m/z* 1828 is consistent with the molecular weight (MH^+) of B-DYN.

Avidin Binding

The binding of B-DYN to avidin was assessed according to the method of Green (10); 1.0 ml of avidin solution (0.2 mg/ml in PBS) was mixed with 24 μl of 10 m*M* HABA, which changes its absorption maximum upon binding to avidin, and the decrease in optical density at 500 nm was measured after successive addition of biotin or biotinylated peptide.

Dissociation from Avidin

Solutions of B-DYN (100 μl of 10 μg/ml) in PBS were added to wells of microtiter plates, then incubated overnight at 4°C, and the wells were washed four times with 250 μl washing buffer (PBS, 0.1% Tween 20) to remove unbound B-DYN. Then 250 μl of a mixture of 1% casein, 10% ethanolamine in 1 *M* $NaHCO_3$ was added to each well to block remaining free plastic-surface binding sites. After 4 hr at 20°C, the wells were washed four times with 250 μl of washing buffer, and 100 μl of a solution of avidin–alkaline phosphatase conjugate (5 μg/10 ml) in PBS was added and incubated for 45 min at 37°C. To block free avidin binding sites, 10 μl of a 100 m*M* aqueous solution of biotin (adjusted to pH 8.5) was added to individual wells at different time intervals (0–5 hr). After 5 hr, the plates were rinsed five times with 250 μl washing buffer, and the B-DYN bound enzyme activity was determined using a modification of a published procedure (11). Briefly, 100 μl of 2 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine-HCl, pH 9.8, and 0.3 *M* $MgCl_2$ were added, and the mixture was incubated at 20°C for 15 min. The reaction was quenched with 50 μl 3 *N* NaOH, and the content of the wells was transferred to 1-ml cuvettes. After the addition of 500 μ*M* water, the absorbance was measured at 405 nm. Control wells not coated with B-DYN, treated in the same fashion, were used to determine the nonspecific absorbance, which was subtracted. All determinations were performed in duplicate with less than 5% variability.

Receptor Binding

Competitive receptor binding experiments were per-

formed with washed membranes of rat brain for μ-opioid receptor analysis, guinea pig cerebellum for κ-opioid receptor, and NG-108-15 cells for δ-opioid receptor. For the preparation of rat membranes, the whole brain without cerebellum of male, Sprague–Dawley rats, weighing 120–140 g, was homogenized in 60 vol of ice-cold 50 m*M* Tris–HCl buffer (pH 7.4) containing 100 m*M* sodium chloride. After 1 hr at 20°C, the preparation was centrifuged at 4°C and 20,000 rpm for 20 min. The resuspended pellet was washed twice with 50 m*M* Tris buffer and stored at −70°C in 10% sucrose. Washed membranes of guinea pig cerebellum (Hartley, 300–500 g) were prepared and stored as described for rat membranes. For the preparation of NG108-45 cell membranes, cells were grown, harvested, and homogenized as described earlier (12), and the membranes were stored in 10% sucrose at −70°C. All binding studies were performed in 50 m*M* Tris buffer substituted with 0.1% BSA and a mixture of protease inhibitors (0.01% bacitracin, 30 μ*M* bestatin, 1 m*M* L-leucyl-L-leucine, 0.3 μ*M* thiorphan, 10 μ*M* captopril, and 50 μ*M* *N*-carboxymethyl-L-Phe-L-Leu). Affinity to the μ-opioid receptor was determined in rat membranes with [^{3}H]DAGO tracer (1 n*M*), while NG-108-15 membranes were used with [^{3}H]DADL (1 n*M*) to determine the interaction at the δ-opioid receptor. The κ-opioid receptor sites were assayed in guinea pig cerebellum with [^{3}H]diprenorphine (1 n*M*) in the presence of 10 μ*M* morphiceptin and 0.5 μ*M* DADL to block μ and δ sites (12). Membranes were incubated in polyethylene tubes with radioactive ligand and different concentrations of competing nonradioactive ligand for 1 hr at 20°C. Nonspecific binding was determined for all preparations in the presence of 10 μ*M* unlabeled diprenorphine. Binding studies in the presence of avidin were performed with preformed avidin–B-DYN complexes which were obtained by mixing increasing amounts of B-DYN with large excesses of avidin (1 μ*M*). Bound and unbound radioactivities were separated by filtration through Whatman GF/B filters as described (12), and radioactivity on the filters was measured. IC_{50} values were determined by nonlinear least-squares regression analysis of competition curves (12).

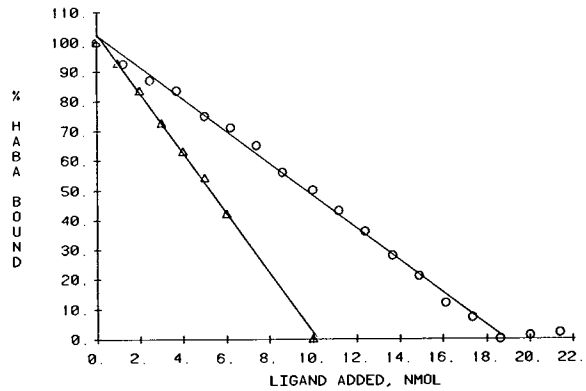

Fig. 1. Dissociation of preformed avidin–HABA complex by subsequent addition of biotin (△) and B-DYN (○). Percentage HABA bound was calculated from absorbance at 500 nm corrected for dilution and background adsorbance.

RESULTS AND DISCUSSION

Chemistry

Several methods have been reported for the synthesis of biotinylated peptides, mostly involving biotinylation of Lys residues of performed peptides (4). The shortcoming of this approach is the difficulty of controlling the reaction when more than one free amino group is present in the peptide reactant. The direct solid-phase synthesis of biotin-containing peptides has not been extensively used. The methods described here are modifications and improvements of procedures which are adaptable for preparation of peptides containing biocytin in specific positions using current methods and instrumentation in peptide synthesis.

The preparation of biotin *N*-hydroxysuccinimide followed standard methods (8). The major improvement was the finding that the product could readily be crystallized from small volumes of acetonitrile with a good recovery, rather than the large volumes of other solvents previously used, which provide the product in a low yield. Similarly, the major improvement in the preparation of N_α-Boc–biocytin was the method of recovery from the reaction mixture. Dissolution of the crude product in $NaHCO_3$ followed by slight acidification resulted in the slow crystallization of an almost pure product, which could readily by recrystallized from ethanol.

The synthesis of B-DYN was accomplished using a combination of a manual method for coupling of Boc to the resin (9), followed by automated solid-phase peptide synthesis methods. We found that with DMF as solvent, N_α-Boc–biocytin could readily be coupled to the benzhydrylamine resin using DIPCDI and HOBT. Although coupling of N_α-Boc–biocytin was not used in the automated cycles, there is no reason why it would not be directly applicable. At the outset of this work, we also did not know whether the biotin heterocycle would withstand HF cleavage of the peptide-resin. As shown here, the HF cleavage reaction proceeds without difficulty. Adaptations of the methods described here should be suitable for placing biocytin any-

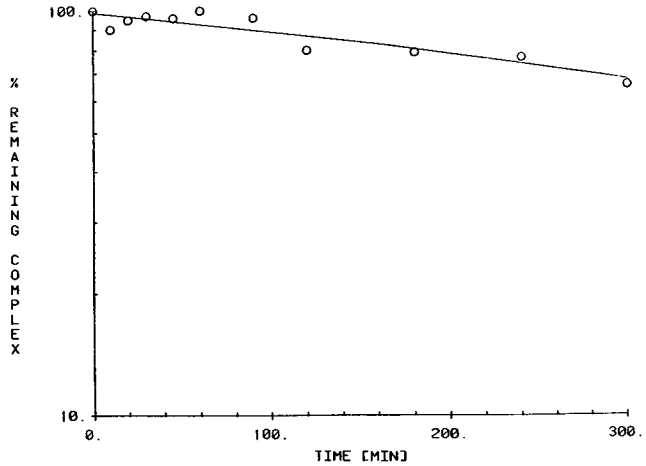

Fig. 2. Dissociation of the preformed avidin–B-DYN complex. B-DYN was immobilized to wells of microtiter plates, and dissociation of the formed B-DYN–avidin–alkaline phosphatase complex was monitored upon addition of excess biotin by determining the plate-bound enzyme activity.

Table I. Binding of B-DYN to the μ-, δ-, and κ-Opioid Receptors[a]

Receptor subtype	IC_{50} value (n*M*)		
	DYN	B-DYN	B-DYN–Avidin
μ	0.1 (0.08–0.12)	0.8 (0.6 –0.9)	63 ± 15
δ	7 ± 2	8.3 ± 3.4	350 ± 96
κ	0.002 ± 0.001	0.005 (0.002–0.008)	7 (6–8)

[a] Competitive binding experiments were performed with guinea pig (κ), NG-108-15 (δ), and rat (μ) membranes as described under Materials and Methods. The range of values in parentheses was obtained from two independent experiments. Otherwise, the standard error of the nonlinear regression is given.

where within a synthetic peptide and for introducing biotin analogues with different spacer arms.

Binding of B-DYN to Avidin

The ability of B-DYN to bind to avidin was assessed by two methods. A sharp end point in the avidin–biotin titration assay (Fig. 1) indicated that B-DYN displayed a high affinity to avidin. However, the different slopes of the regression lines for B-DYN and biotin suggested that the amount of B-DYN present in solution was overestimated. The lower activity of B-DYN could be caused by strong nonspecific binding of dynorphin derivatives to glass and plastic, which has previously resulted in an underestimation of their receptor binding affinities (13). BSA, used in the receptor binding studies to reduce nonspecific binding, could not be employed here, since it interferes with the HABA–avidin assay system (12). While the shape of the HABA displacement curve indicates a high affinity of B-DYN to avidin, the binding affinity cannot be quantitated from the experiment because of the much lower HABA affinity which is displayed by B-DYN in a nearly stoichiometric fashion.

We also measured the rate of dissociation of the B-DYN–avidin complex by monitoring the release of avidin-AP from an immobilized B-DYN–avidin-AP complex. Here, B-DYN was first adsorbed to plastic surfaces of microtiter wells and conjugated to avidin-AP. Excess biotin was then added as a competitor, and the retention of B-DYN-bound avidin-AP was monitored with time as a measure of dissociation of the B-DYN–avidin complex. Using this method, the dissociation half-life was determined to be 10 hr at 37°C (Fig. 2). For comparison, the biotin–avidin complex shows $t_{1/2}$ = 200 days, biotin–succinoavidin shows $t_{1/2}$ = 127 days (15), and $N^{\alpha,B1}$-biotinyl–insulin–avidin shows $t_{1/2}$ = 4.8 hr (16). Although these dissociation rates are not directly comparable to our immobilized system, it is clear that the B-DYN–avidin is sufficiently stable for many of the intended applications in analysis and purification. As previously described (16), the affinity of the analogue for avidin could, in principle, be modified as needed by increasing the spacer arm or modifying the biotin moiety.

Binding of B-DYN to Opioid Receptors

Binding affinities of DYN and B-DYN to the μ-, δ-, and κ-opioid receptors were determined by competitive binding

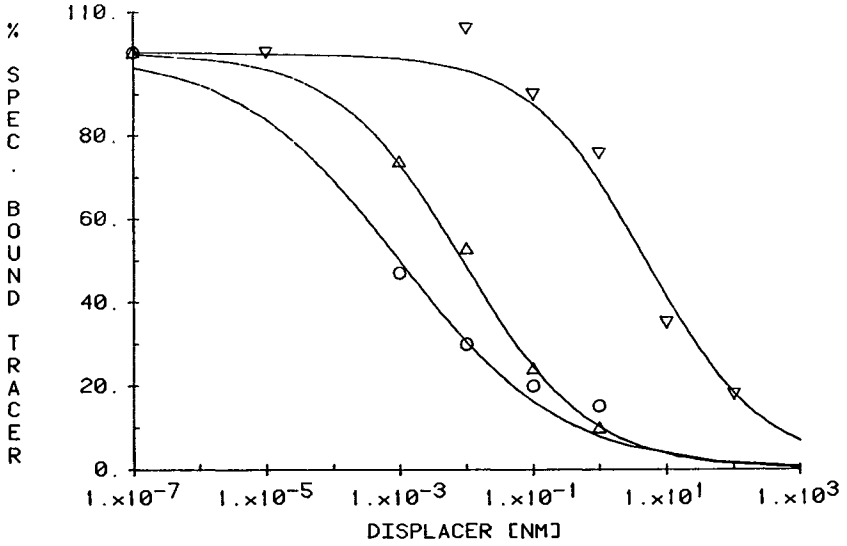

Fig. 3. Competitive inhibition of [^{3}H]diprenorphine to guinea pig cerebellum membranes in the presence of 50 μ*M* morphiceptin and 0.5 μ*M* DADL to block the μ- and δ-opioid receptor. (○) Dynorphin A1-13 amide; (△) B-DYN; (▽); B-DYN in the presence of 1 μ*M* avidin.

assays using specific binding conditions for each of the receptors (Table I). The affinities measured for dynorphin A 1-13 amide were roughly comparable to those described in the literature. James and Goldstein (6) reported IC_{50} values for DYN of 0.6 n*M* (μ), 1.6 n*M* (δ), and 0.02 n*M* (κ), with the use of 3H-EKC and guinea pig brain membranes pretreated with β-chlornaltrexamine. The higher κ affinity in our study could result from the additional use of BSA to block nonspecific binding and the different labeling conditions in guinea pig cerebellum. Less specific labeling conditions employed in previous studies (17) are not comparable. With dissociation constants for the κ opioid receptor in the low picomolar range, it is difficult to obtain precise estimates, because at these low concentrations of DYN and B-DYN, it is difficult to control for nonspecific binding. The results clearly show, however, that both DYN and B-DYN displayed extremely high affinities in the κ receptor assay with guinea pig cerebellum. Hill slopes of the binding curves were less than 1 (0.4–0.8), suggesting some form of binding-site heterogeneity. As shown in Table I, the relative order of binding of DYN to the three receptors (κ> μ> δ) is maintained with B-DYN. Thus, the specificity of receptor binding is not altered by the biotin moiety. Moreover, biotinylation of DYN only slightly decreased its affinity to μ and κ sites, with little change in δ receptor binding. Hence, the positively charged ε-amino group of Lys-13 of DYN is not essential for binding to the receptor. This is in accord with the retained κ receptor affinity of dynorphin derivatives truncated on the C terminal (17,18).

Binding of Opiod Receptors to Avidin–B-DYN Complexes

The primary purpose of incorporating biotin into peptide ligands is to exploit its tight binding to avidin for assays and purification. To test the ability of B-DYN to cross-link the receptor with avidin, binding studies were performed with preformed B-DYN–avidin complexes. Compared to B-DYN, binding affinities of B-DYN–avidin complexes to all three opioid receptor subtypes (μ, δ, and κ) decreased considerably (Table I, Fig. 3), showing that the avidin compromises receptor binding. We previously found a small (two- to threefold) decrease in μ and δ receptor binding affinities for β_h-endorphin derivatives that were biotinylated in position 9 of the molecule, while biotinylation at position 19, 24, 27, or 28 had little effect on binding (4). The rather large decrease in opioid receptor affinity of B-DYN upon binding to avidin is thus surprising, as the avidin moiety is further removed from the enkephalin binding site in position 13 of B-DYN than in position 9 of the biotinylated β-endorphin probe. Possibly, avidin interferes more strongly with the folded structure of dynorphin than with that of the longer β-endorphin. Nevertheless, considerable affinity for the κ receptor is retained with the B-DYN–avidin complex with selectivity over μ and δ receptors, suggesting that B-DYN is able to cross-link efficiently the κ-opioid receptor with avidin. We cannot eliminate the possibility that the apparent binding observed is due to free B-DYN in equilibrium with the avidin–B-DYN complex. We do not think this is the case since the avidin concentration was kept at a very large excess (1 μ*M*) over the presumed K_d of the avidin–B-DYN complex. Given the extremely tight binding of B-DYN to avidin (dissociation $t_{1/2}$ = 10 hr), the fraction of free B-DYN would be expected to be far below the K_d of B-DYN at the κ receptor at the measured IC_{50_κ} of the B-DYN–avidin complex. Further, the ratios of affinities of the avidin–B-DYN complex versus B-DYN to the three receptors are quite different; one might expect that if free B-DYN were the ligand, the same concentrations would be present in all experiments and similar structure–binding relationships would be observed.

The ultimate proof that B-DYN can cross-link the κ receptor to avidin awaits experiments in which direct binding of avidin to the B-DYN–receptor complex is demonstrated, either by direct assay of the complex with avidin conjugates or by purification of the receptor with avidin-affinity methods. On the other hand, B-DYN could be covalently cross-linked to the κ receptor, followed by receptor solubilization, avidin affinity chromatography, and avidin-ELISA detection. The latter application is independent of the ability of B-DYN to cross-link noncovalently opioid receptors and avidin.

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