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"DAKLI": A multipurpose ligand with high affinity and selectivity for dynorphin (κ opioid) binding sites

(opioid receptor/radioreceptor binding assay/peptide ligand)

AVRAM GOLDSTEIN†, JOHN J. NESTOR, JR.,‡, ASHA NAIDU†, AND SHERRY R. NEWMAN‡

†Department of Pharmacology, Stanford University, Stanford, CA 94305; and ‡Institute of Bio-Organic Chemistry, Syntex Research, Palo Alto, CA 94303

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ABSTRACT **We describe a synthetic ligand, "DAKLI" (Dynorphin A-analogue Kappa LIgand), related to the opioid peptide dynorphin A. A single reactive amino group at the extended carboxyl terminus permits various reporter groups to be attached, such as ^{125}I-labeled Bolton–Hunter reagent, fluorescein isothiocyanate, or biotin. These derivatives have high affinity and selectivity for the dynorphin (κ opioid) receptor. An incidental finding is that untreated guinea pig brain membranes have saturable avidin binding sites.**

To label one type of receptor binding site in the presence of related types, a radioligand should (*i*) have very high affinity for the site to be labeled, (*ii*) be highly selective for that site, (*iii*) have high enough specific radioactivity to be usable at a low concentration to maximize its binding at the preferred site relative to that at the next-preferred site, (*iv*) for convenience incorporate a radioisotope such as ^{125}I with a high-energy mode of decay.

Dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln) is a 17-residue endogenous opioid peptide with selectivity for the κ opioid receptor (1–3). Dynorphin A-(1–13) is as potent and as selective for the κ binding site as is the natural full-length peptide (4), so we surmised that this fragment could be modified safely by extension at the C terminus. For flexibility of functionalization with various reporter groups, we used a primary amino function at the C terminus as a handle. Lysine-11 and lysine-13 (especially the former) play essential roles in determining κ affinity and selectivity (5). To abolish the reactive ε-amino groups, yet retain the charges at these two positions, we replaced lysine with arginine. Our C-terminal extension consisted of glycine-14, followed by 1,5-diaminopentane. The resulting peptide is called "DAKLI" (Dynorphin A-analogue Kappa LIgand): [Arg^{11,13}]dynorphin A-(1–13)-Gly-NH$(CH_2)_5NH_2$.

After synthesis of the peptide by the solid-phase method and its removal from the resin by aminolysis with 1,5-diaminopentane, the free α-amino group of tyrosine-1 remains blocked by the *t*-butoxycarbonyl (Boc) group and the only reactive amine is at the extended C terminus. This feature allows the facile production, from [Boc]DAKLI, of several potentially useful ligands. The Boc Group is subsequently removed to provide the free ligand. By coupling ^{125}I-labeled diiodinated Bolton–Hunter reagent (BH) (4000 Ci/mmol; 1 Ci = 37 GBq), we could obtain a radioligand, which we call DAKLII** (each asterisk denotes one atom of ^{125}I). With fluorescein isothiocyanate we produced a fluorescent derivative, DAKLIF. Biotinylation yielded DAKLIB, for labeling with fluorescein-coupled avidin or phycoerythrin-coupled streptavidin (PE-streptavidin). Finally, DAKLI is suitable for attachment to an activated solid matrix to yield an affinity ligand for receptor purification studies. These ligands are proving useful in detecting dynorphin receptors on transfected cells in expression cloning experiments.

MATERIALS AND METHODS

Synthesis of [Boc]DAKLI. [Boc]Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Arg-Leu-Arg-Gly was prepared as a benzylic ester on standard Merrifield resin (polystyrene/1% divinylbenzene crosslinked) using carbodiimide couplings and N^{α}-*t*-butoxycarbonyl protection but no side-chain protection. The corresponding amide was formed by aminolysis of 7.1 g of peptide resin (≈0.3 mmol of peptide) with 3.5 ml of 1,5-diaminopentane in 35 ml of MeOH (60 hr, 25°C). The resin was filtered, washed well with MeOH, and the filtrate was concentrated *in vacuo*. The residue was triturated three times with Et_2O, dissolved in 50% AcOH, and filtered. The filtrate was concentrated *in vacuo* (<40°C) and lyophilized from H_2O to yield the crude peptide amide as a foam. The product was purified by preparative HPLC in two portions on a column (2.5 × 100 cm) of C_{18} Vydac 218 TP54 (The Separations Group, Hesperia, CA) (20–30 μm) using 28% CH_3CN (0.3 M in NH_4OAc, pH 4.5) as the eluant. Product fractions were pooled and concentrated *in vacuo*, and the residue was lyophilized from H_2O to yield 50 mg of white powder, $[\alpha]_D^{-25}$ −22° (*c* 0.4, AcOH). Reversed-phase analytical HPLC (as above but 4 × 250 mm; particle size, 5 μm; gradient, 10–50% CH_3CN; 0.03 M in NH_4OAc, pH 4.5; *A* = 280 nm) showed the product to be 90% pure, and the amino acid analysis after 6 M HCl hydrolysis (18 hr, 110°C) gave ratios in accord with the desired structure: Pro, 1.1 (1); Gly, 2.9 (3); Ile, 0.9 (1); Leu, 2.0 (2); Tyr, 1.3 (1); Phe, 1.1 (1); Arg, 4.9 (5). The expected amino acid sequence was obtained on an Applied Biosystems (Foster City, CA) 470A microsequencer (K. Jarnagin, Syntex Research).

Preparation of Iodinated Peptides DAKLII and DAKLII.** BH (Sigma) was iodinated by the chloramine-T method as follows: to 5 mg of dry BH was added 50 mg of NaI in 40 μl of 50 mM sodium phosphate buffer, followed immediately by 0.5 ml of chloramine T (30 mg/ml in 250 mM sodium phosphate buffer). The reaction was terminated after 30 sec by 0.1 ml of sodium metabisulfite (150 mg/ml in 50 mM sodium phosphate buffer). All buffers were at pH 7.4. The reaction mixture was extracted twice with benzene, and the organic phases were pooled and dried under N_2. The dried extract was taken up in 100 μl of benzene and run on HPLC [Altex (Berkeley, CA) Lichrosorb Si60 column, 4.6 mm × 25 cm] in isocratic 70% toluene with 30% ethyl acetate. A small amount of tracer diiodinated ^{125}I-labeled BH was added as a marker for the elution position of the product, which was collected, dried, and coupled to [Boc]DAKLI (235 nmol in 50

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Abbreviation: PE-streptavidin, phycoerythrin-coupled streptavidin; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; DPDPE, [D-penicillamine^2,D-penicillamine^5]enkephalin.

μl of 0.1 M $NaHCO_3$, pH 8.5) on ice for 1 hr, and then overnight at 4°C. The reaction mixture and the washes of the reaction tube with MeOH/HCl (MeOH/0.1 M HCl, 1:1, vol/vol) were injected onto reversed-phase HPLC (μBondapak C_{18}, 3.9 × 300 mm, Waters Associates) and eluted by a linear gradient of CH_3CN in 5 mM trifluoroacetic acid (20–60%, 30 min). The coupled peptide was collected and lyophilized, and the Boc group was removed in 66% trifluoroacetic acid (30 min, 23°C) and purified by HPLC as described above. Overall yield of DAKLII was 3–5%.

Essentially the same procedure was followed for DAKLII**, using radioactive diiodinated BH (4000 Ci/mmol; Amersham). The benzene solution, 500 μCi as furnished, was dried under N_2 on ice, then reacted with 25 μl of [Boc]DAKLI (2 mM in 0.1 M $NaHCO_3$, pH 8.5) on ice for 1 hr, and then overnight at 4°C. Removal of the Boc group and purification of the product were as described above with appropriate volume reductions. Overall yield of DAKLII** was 20–30%.

Preparation of Biotinylated Peptide DAKLIB. To 120 μl of 0.1 M $NaHCO_3$ (pH 8.5) was added 120 μl of 25 mM sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce) in $NaHCO_3$ and 25 μl of 10 mM [Boc]DAKLI in water. The mixture was incubated at 23°C for 2 hr, and then on ice for 1 hr and overnight at 4°C. The coupled peptide was purified on reversed-phase HPLC as described above, lyophilized, dissolved in 150 μl of MeOH/HCl, and the Boc group was removed as described above. If necessary, this step was repeated on HPLC fractions containing the peptide, from which the Boc group had not been removed. Overall yield of DAKLIB was 14–18%. Avidin and fluorescein-labeled avidin were from Sigma, PE-streptavidin was from Biomeda (Foster City, CA).

Preparation of Fluorescein-Coupled Peptide DAKLIF. To 500 μl of sodium phosphate (pH 9.5) was added 25 μl of freshly prepared 10 mM fluorescein isothiocyanate (isomer I; Sigma) in sodium phosphate, and 25 μl of 1 mM [Boc]DAKLI in water, and incubated 30 min at 23°C. A blank reaction was carried out identically without peptide. The incubation mixtures were passed through Sep-Pak C_{18} cartridges (Waters Associates) that had been prewashed with MeOH, CH_3CN, and water. The cartridges were washed with 3 ml of water and then eluted with 2 ml of 90% CH_3CN in 5 mM trifluoroacetic acid. The eluates were lyophilized, dissolved in MeOH/HCl, and purified on HPLC as described above. Since many UV-absorbing peaks were observed, it was necessary to compare the elution profile of the blank reaction mixture to identify the position of the coupled peptide accurately. After elution and lyophilization, the coupled peptide was dissolved again, deblocked as described above, and purified on HPLC. Overall yield of DAKLIF was 20–35%.

Binding. Standard binding assays for three types of opioid receptors were carried out with guinea pig brain membranes at pH 7.4, in Tris buffer as described (6), and also in Krebs Hepes buffer (KH; 118 mM NaCl/4.8 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/25 mM Hepes). Briefly, tritiated [D-Ala^2,$MePhe^4$,$Gly\text{-}ol^5$]enkephalin (DAGO; New England Nuclear) (7) was used to label μ sites. ^3H-labeled S–S cyclized [D-$penicillamine^2$,D-$penicillamine^5$]enkephalin (DPDPE; Amersham) (8) was used to label δ sites. In both cases, high concentrations of the nonradioactive ligands defined nonsaturable binding. For κ sites, a paired-tube paradigm was used (6), in which specific binding was defined as bound [^3H]bremazocine (New England Nuclear) that was displaced by the highly κ-selective ligand *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methane sulfonate (500 nM) (U50,488; Upjohn) (9). The

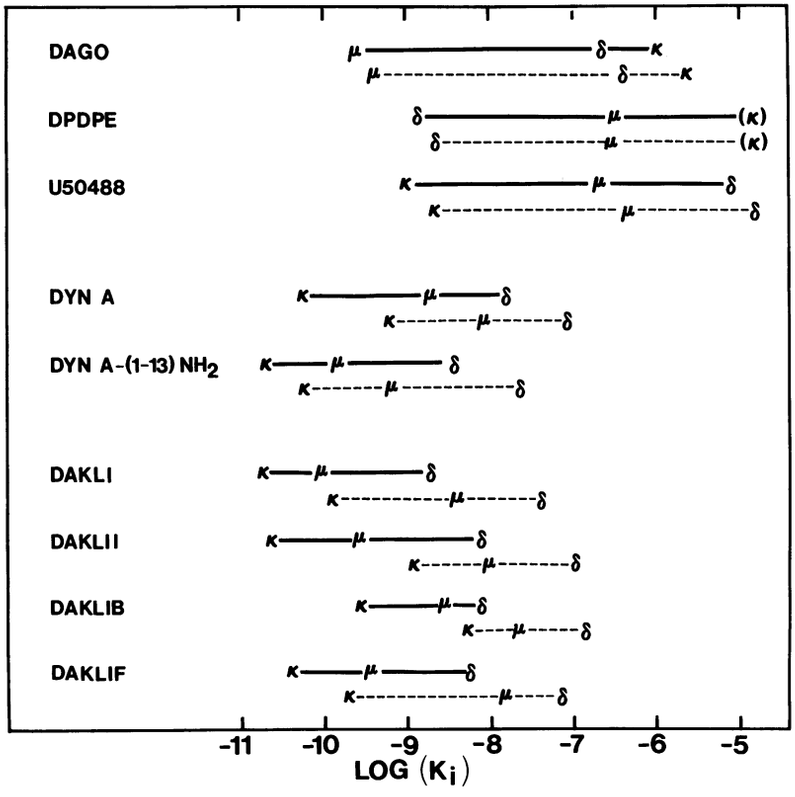

FIG. 1. Binding selectivity profiles of the key ligands, two dynorphins, and several DAKLI peptides. For interpretation of profiles see text description. Solid lines are in Tris, broken lines are in KH. Greek letters represent the three types of opioid binding sites under study; each is positioned according to log(K_i) of the ligand at that site. Parentheses indicate that the value of log(K_i) was greater than could be measured. The nonradioactive forms of the key ligands DAGO, DPDPE, and U50,488 are at the top, showing the selectivity of each for its corresponding site. DAKLII is nonradioactive diiodinated DAKLI, the nonradioactive form of DAKLII**.

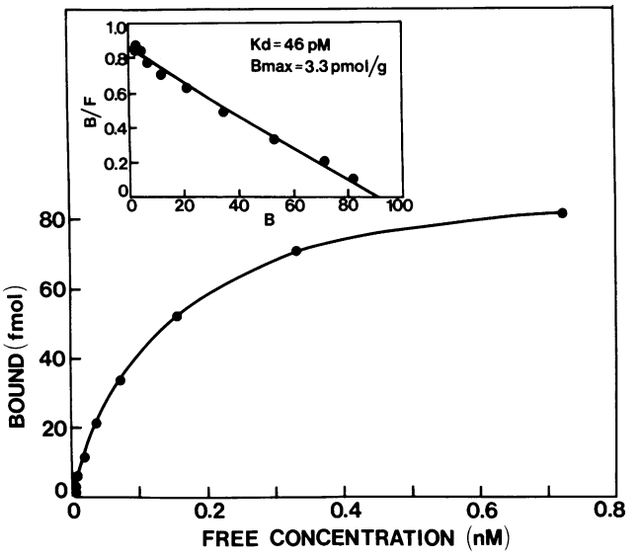

FIG. 2. Binding isotherm of DAKLII** with guinea pig brain membranes. (*Inset*) Scatchard transformation. These data were obtained in KH (pH 7.4).

membranes were suspended in 2 ml of buffer, incubated (23°C, 2 hr), harvested on glass fiber filters, and washed repeatedly with ice-cold buffer. Free radioligand concentrations were measured by determining radioactivity in supernatants, and radioligand integrity was verified by reversed-phase HPLC at the end of the incubation.

Competition curves were determined in duplicate, and IC_{50} values were converted to K_i by means of a full equation (10) rather than the Cheng–Prusoff approximation (11). The standard deviation of IC_{50} determinations (and therefore of K_i) in our hands was 0.1 decimal logarithmic unit (i.e., ≈25%).

Results from competition experiments with each ligand in the μ, δ, and κ systems are expressed as a *binding selectivity profile* (12), which is a graphic representation of the logarithms of the three K_i values plotted on a horizontal logarithmic scale. This presentation shows at a glance both the absolute affinities at each site and—as a horizontal distance—the selectivity of the ligand for the preferred over the nonpreferred sites.

In some binding assays DAKLII** was used as primary radioligand. Because of high background binding to the filters, membranes were centrifuged (3500 × *g*, 10 min). Free concentrations were determined by measuring the radioactivity of 200 μl of supernatant. Pellets were taken up in 2 ml of KH, transferred to new test tubes, and centrifuged again. Supernatants were removed, and tubes containing the pellets were transferred to a γ-counter. Nonspecific binding was determined in the presence of 500 nM U50,488. As we intended to use DAKLII** to detect dynorphin binding sites on whole viable cells, we conducted these binding assays only in KH.

For binding experiments with avidin, membranes were preincubated (30 min, 23°C) with DAKLIB (0.5 nM) or an equivalent volume of MeOH/HCl in KH and then centrifuged (3500 × *g*, 10 min). The pellets were taken up in 2 ml of KH, incubated (90 min, 23°C) with ^{125}I-labeled avidin (New England Nuclear), centrifuged, and washed as described above. In competition experiments with PE-streptavidin, the same procedure was followed except that the second incubation was for 30 min with both ^{125}I-labeled avidin and added PE-streptavidin.

RESULTS

Fig. 1 shows the binding selectivity profiles of the DAKLI peptides in comparison with dynorphin A, dynorphin A-(1–13) amide, and the three standard type-selective ligands (DAGO, DPDPE, and U50,488) for the μ, δ, and κ sites, respectively. The DAKLI peptides are all selective for dynorphin (κ) sites, with dissociation constants in the subnanomolar range.

The binding isotherm for DAKLII** with guinea pig brain membranes is shown in Fig. 2. The Scatchard transformation is consistent with binding to a single type of site with very high affinity (K_d = 46 pM). The estimate of B_{max} is comparable to that obtained for κ sites in guinea pig brain membranes with [^{3}H]bremazocine and U50,488 as described above.

DAKLII** has a very fast on-rate (Fig. 3). The off-rate was also measured by incubating DAKLII** with membranes for 1 hr and then adding U50,488 (500 nM) and determining the loss of radioactivity from the membranes at intervals for 4 hr. The decay curve (not shown) was log linear with a half-time of 1.0 hr.

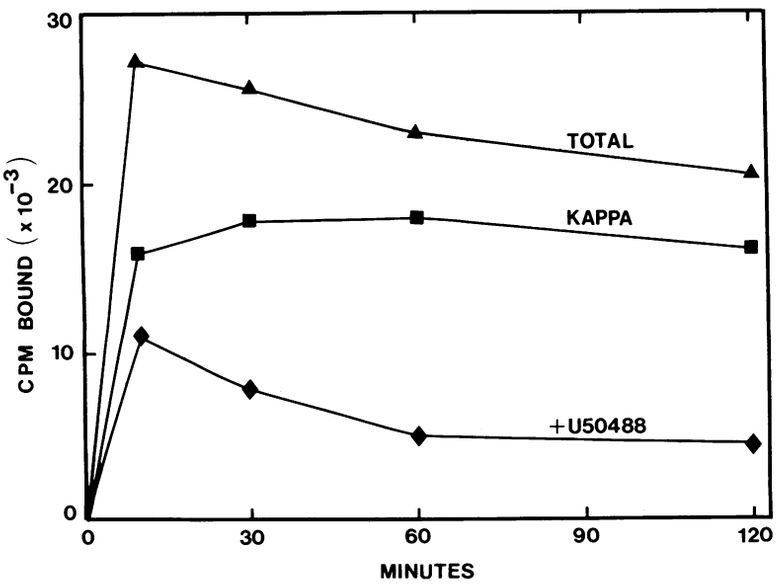

FIG. 3. Kinetics of association of DAKLII**. Upper curve, total bound radioactivity. Lower curve, residual bound radioactivity in the presence of U50,488 added at time zero. Middle curve, specific κ binding as the difference between upper and lower curves.

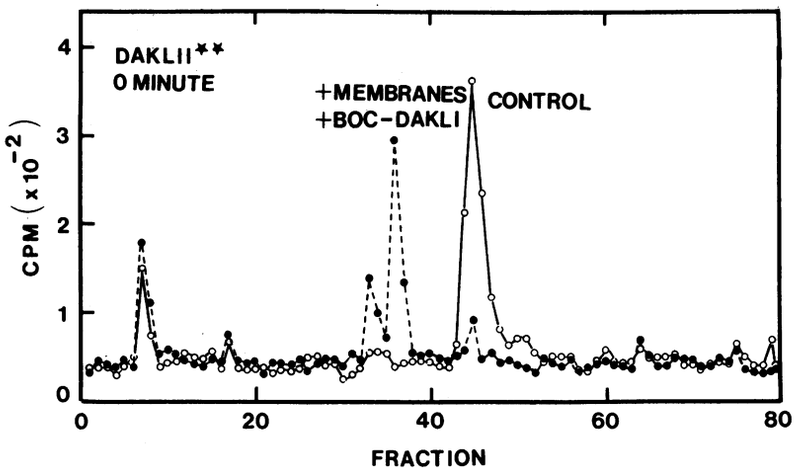

FIG. 4. Degradation of DAKLII** in contact with membranes. Reversed-phase HPLC, CH_3CN linear gradient (20–40% in 20 min) in 5 mM trifluoroacetic acid. Solid line (control) is DAKLII** before contact with membranes. Dotted line is immediately after contact with membranes as in the binding assay; incubation mixture was centrifuged, and supernatant was applied directly to HPLC. In the experiment shown here, 50 nM [Boc]DAKLI was added in an attempt to protect DAKLII**; it was ineffective, as was 500 nM [Boc]DAKLI (data not shown).

Immediately upon addition of DAKLII** to the usual membrane suspension (10 mg/ml based on initial wet weight of brain) followed by mixing (Vortex) and placing on ice ("0 time"), all the radioligand was degraded (Fig. 4). Two labeled fragments (as yet unidentified) were eluted from the HPLC column earlier than the parent peptide. [Boc]DAKLII** was not degraded under these conditions. The degradation of DAKLII** could not be prevented by [Boc]DAKLI (0.5 μM), nonradioactive DAKLII (10 μM), or DAKLI (1.0 μM). Boiling the membrane preparation prevented degradation. Thus, a membrane-bound aminopeptidase of low affinity and high capacity appears to be responsible.

Table 1 compares affinities of several ligands for the dynorphin (κ) sites as determined in our standard system (6) and using DAKLII** as radioligand. That the same sites are labeled by the two different radioligands is suggested by the similar quantitative rank orders of dissociation constants of the competing ligands. With one exception, DAKLII** was more easily competed against than [^{3}H]bremazocine (i.e., K_i values of the competing ligands were lower), presumably because degradation reduced the effective free radioligand concentration. B_{max} values determined from binding isotherms using the [^{3}H]bremazocine/U50,488 and DAKLII**/U50,488 systems were approximately the same (3–4 pmol per g wet weight of brain).

Table 1. Comparisons of ligand dissociation constants determined by competition against two radioligands used to label dynorphin (κ opioid) binding sites

Competing ligand	Radioligand: [^{3}H]Bremazocine/ U50,488	Radioligand: DAKLII**/ U50,488
DAGO	−5.66	−6.60
DPDPE	(−4.84)	−4.72
U50,488	−8.66	−8.95
Dyn A	−9.29	−9.54
Dyn A-(1–13)amide	−10.32	−10.06
DAKLI	−9.91	−10.54
DAKLII	−9.70	−10.33
DAKLIB	−8.95	−10.13
DAKLIF	−8.29	−9.23

[^{3}H]Bremazocine with and without U50,488 is the standard paired-tube system described elsewhere (6); DAKLII** was used with U50,488 (500 nM) to define nonspecific binding. KH, pH 7.4. Data are mean values of log (K_i) computed from duplicate or triplicate IC_{50} estimates in independent experiments. Parenthetic value for DPDPE is a limit; high enough concentration to obtain 50% inhibition could not be attained. Dyn, dynorphin.

Several ways of using fluorescence to detect dynorphin binding sites were considered. Direct coupling of fluorescein (DAKLIF) produced a peptide with suitable binding properties (cf. Fig. 1) but with substantial quenching of fluorescence. Measured under the same conditions [KH, pH 7.4, Aminco-Bowman (American Instrument) spectrophotometer], the relative fluorescence intensities, based on molar concentrations of peptide or protein, were as follows: fluorescein, 1.00; DAKLIF, 0.10; fluorescein-avidin, 0.42; PE-streptavidin, 1.77. Optimal excitation and emission wavelengths 488 and 518 nm were used (but 495 and 576 for PE-streptavidin).

To ascertain whether avidin would recognize DAKLIB in the binding sites, we incubated membranes with and without DAKLIB at a concentration 10 times K_d (91% site occupancy). Then after removing free DAKLIB by washing, we measured the binding of increasing concentrations of ^{125}I-labeled avidin. As Fig. 5*A* shows, there was considerable binding of avidin in the absence of DAKLIB. There was also a DAKLIB-dependent binding, with apparent K_d of 130 pM and B_{max} of 4.3 pmol per g wet weight of original brain, in good agreement with the value found using the [^{3}H]bremazocine/U50,488 system to label κ sites, as noted above.

PE-streptavidin has significant advantages over fluorescein-avidin (13). To determine whether PE-streptavidin would recognize DAKLIB in the κ binding sites, we carried out a competition experiment using ^{125}I-labeled avidin as radioligand at 2.2 pM in the absence and presence of DAKLIB. As Fig. 5*B* shows, this compound recognized the same DAKLIB-occupied sites as did labeled avidin, with apparent K_i of 28 pM computed from the IC_{50}.

It is noteworthy that most of the binding of ^{125}I-labeled avidin (Fig. 5*A*, Control) and of PE-streptavidin (Fig. 5*B*) in the absence of DAKLIB is saturable, as though there were high-affinity avidin binding sites on the well-washed brain membranes, with K_d of ≈200 pM for avidin (B_{max}, 25 pmol/g) and 140 pM for PE-streptavidin.

DISCUSSION

We have described the properties of a high-affinity ligand, DAKLI, which is selective for dynorphin (κ opioid) binding sites. When the primary amino group of tyrosine-1 is blocked by Boc, only a single reactive NH_2 remains, at the extended C terminus. [Boc]DAKLI is a multipurpose ligand. It can be coupled to ^{125}I-labeled BH to yield a radioligand, DAKLII**.

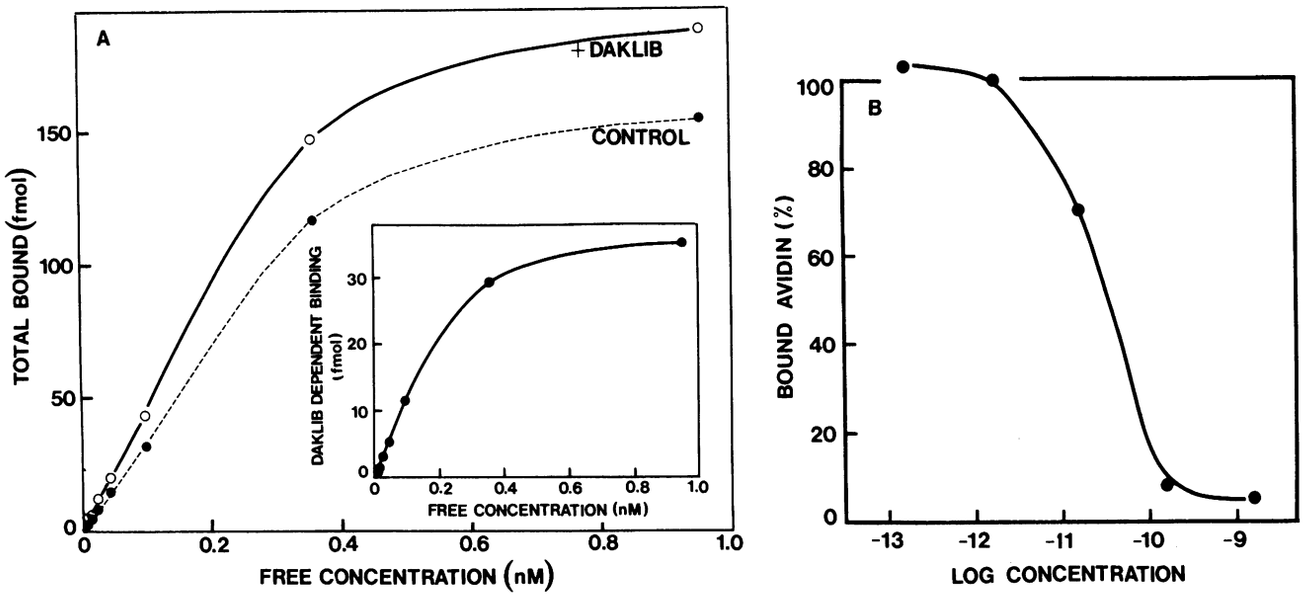

FIG. 5. DAKLIB-dependent binding of avidin or PE-streptavidin to guinea pig brain membranes. (*A*) Binding of ^{125}I-labeled avidin in the presence and absence of DAKLIB. Membranes were incubated first with or without 0.5 nM DAKLIB (30 min, 23°C) and then, after removing excess free DAKLIB by washing and centrifugation, with radiolabeled avidin for 90 min at various concentrations. Measured free concentration of ^{125}I-labeled avidin is on the *x* axis. (*Inset*) Difference plot, representing that portion of total avidin binding that is due to DAKLIB. (*B*) Competition by PE-streptavidin for DAKLIB-dependent avidin binding sites. Conditions are the same as for *A*, but concentration of ^{125}I-labeled avidin was fixed at 2.2 pM, incubation was for 60 min, and PE-streptavidin concentration was varied. After the preincubation with or without DAKLIB and the removal of DAKLIB by washing, PE-streptavidin was added, followed immediately by ^{125}I-labeled avidin.

DAKLII**, with apparent K_d of ≈50 pM in a physiological salts buffer, selectively labels the κ binding sites on guinea pig brain membranes. The ^{125}I label offers the practical advantages of γ-counting over scintillation counting in assays with membrane preparations or whole cells. The high specific radioactivity (4000 Ci/mmol) makes it feasible to use extremely low concentrations (for optimum selectivity) and to lower the detection limit. We have used DAKLII** to find rare clones of transfected mouse fibroblasts that express binding sites by simply flooding confluent cell layers with DAKLII**, washing, and placing the culture flasks on x-ray film for a few days (unpublished observations).

DAKLII** is degraded by guinea pig brain membranes at the very start of an incubation, evidently by a membrane-bound aminopeptidase. Removal of tyrosine-1 is known to destroy the ability of the peptide to recognize the binding sites, but site-bound DAKLII** has a very slow off-rate ($t_{1/2}$, 1 hr). Thus, the site-bound radioligand is protected, while the free radioligand is converted to products that may be less "sticky," reducing nonspecific binding to membranes and adsorption to tube surfaces. Since no reversible equilibrium is achieved, estimated dissociation constants for the DAKLI peptides must be regarded only as approximations; the true affinity of DAKLII**, in particular, may be substantially greater. Although not much discussed in the literature, it is essential in binding assays with any peptide ligand not only to measure free concentration but also to determine whether the peptide remains intact during the incubation.

We have converted DAKLI to a fluorescent ligand by coupling to fluorescein isothiocyanate or indirectly by coupling first to a biotin derivative. As with the addition of BH at the C terminus, these adducts retain high affinity and selectivity for the binding sites. When DAKLIB occupies the κ sites, its biotin can be recognized by avidin or PE-streptavidin. Thus, it may now be possible to study dynorphin receptors in tissues by fluorescence cytochemistry, as well as to detect cells expressing such receptors in the fluorescence-activated cell sorter.

An interesting incidental observation was the specific saturable high-affinity binding of avidin or PE-streptavidin to untreated guinea pig brain membranes. This suggests the presence of membrane-bound biotin-containing enzymes or other proteins, as has been noted in HeLa cells (14).

The DAKLI peptides should find numerous useful applications in the study of dynorphin (κ opioid) receptors. Until it becomes available commercially, [Boc]DAKLI will be available in small amounts from Dr. John J. Nestor, Jr. (Institute of Bio-organic Chemistry, Syntex Research, P.O. Box 10850, Palo Alto, CA 94303). We thank Dr. Guo-xi Xie for carrying out the experiment of Fig. 4, and Dr. K. Jarnagin for sequencing DAKLI. This work was supported, in part, by Grants DA-1199 from the National Institute on Drug Abuse and BNS-8416617 from the National Science Foundation to A.G.

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