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# Deltorphins: A family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites

**(amphibian skin peptides/mouse vas deferens assay/receptor binding assay)**

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ABSTRACT **Deltorphins are endogenous linear heptapeptides, isolated from skin extracts of frogs belonging to the genus *Phyllomedusa*, that have a higher affinity and selectivity for δ opioid binding sites than any other natural compound known. Two deltorphins with the sequence Tyr-Ala-Phe-Asp(or Glu)-Val-Val-Gly-$NH_2$ have been isolated from skin extracts of *Phyllomedusa bicolor*. The alanine in position 2 is in the D configuration. These peptides, [D-Ala²]deltorphins I and II, show an even higher affinity for δ receptors than the previously characterized deltorphin, which contains D-methionine as the second amino acid. These peptides show some similarity to another constituent of *Phyllomedusa* skin, dermorphin, which is highly selective for μ-opioid receptors. These peptides all have the N-terminal sequence Tyr-D-Xaa-Phe, where D-Xaa is either D-alanine or D-methionine. While this structure seems to be capable of activating both μ and δ opioid receptors, differences in the C-terminal regions of these peptides are probably responsible for the observed high receptor selectivity of dermorphin and deltorphin.**

The endogenous opioid ligands isolated from vertebrate brain show little selectivity toward the different types of opioid receptors. Peptides isolated from amphibian skin appear to be more selective. In 1981 Montecucchi *et al.* (1) extracted from the skin of the Argentinian frog *Phyllomedusa sauvagei* a heptapeptide named dermorphin, which preferentially binds to μ-type opioid receptors (2). By recombinant DNA techniques, it was demonstrated that dermorphin, like numerous other peptides, is derived in multiple copies from larger precursors. In addition, from inspection of the sequence of one of the cloned cDNAs for these precursors, the existence of another heptapeptide with an N-terminal region similar to that of dermorphin was predicted (3). We recently succeeded in isolating small quantities of this peptide from the skin of *P. sauvagei* and named it deltorphin, because of its high affinity and selectivity for the δ opioid binding site (4). Both dermorphin and deltorphin contain a D amino acid (D-alanine and D-methionine, respectively) as the second amino acid. In the cloned cDNAs, codons for the corresponding L amino acids—i.e., GCG for alanine and ATG for methionine—were found at these positions. This characteristic suggested that the processing of these peptides includes a reaction whereby an L amino acid residue is converted to its D isomer within peptide linkage (3). Here we describe the isolation of two other heptapeptides from the skin of *Phyllomedusa bicolor*, which show an affinity and selectivity for δ opioid receptors several times higher than that of deltorphin and the cyclic enkephalin derivative [D-Pen²,D-Pen⁵]enkephalin (DPDPE, where D-Pen is D-penicillamine) (5). Once again, these peptides contain a D-alanine residue in the second position and share with dermorphin and deltorphin the N-terminal sequence Tyr-D-Xaa-Phe. We refer to these peptides, which differ by the presence of an aspartic or glutamic residue in position 4, as [D-Ala²]deltorphins I and II, respectively, to distinguish them from the peptide already isolated (4). This study presents the isolation, characterization, synthesis, and pharmacological profiles of [D-Ala²]deltorphins. The biological and receptor binding properties of these peptides are compared with those of the respective L isomers, deltorphin, dermorphin, and other synthetic opioid peptides.

## MATERIALS AND METHODS

**Isolation of [D-Ala²]Deltorphins.** Fresh skins (92 g) obtained from eight frogs (*P. bicolor*) captured near Oberà (Paranà, Brazil) were minced with scissors and extracted with 500 ml of methanol for 1 week at room temperature. The liquid was decanted and filtered, and the skin was extracted again with 5 parts (vol/wt) of 80% methanol. The filtrates were pooled and stored in a dark bottle at 2°C until processed further. A volume of filtered extracts equivalent to 85 g of skin was concentrated under reduced pressure (40°C) and lyophilized from water to yield 2.9 g of yellow-brown syrupy residue, which was resuspended under stirring in 10 ml of water and dissolved by adding 190 ml of ethanol to give a clear yellow solution. The liquid was loaded on two chromatographic columns filled with 200 g of alkaline alumina (Merck, Darmstadt, F.R.G.) and eluted with a stepwise gradient of aqueous ethanol (nine steps from 95% to 10% ethanol; each step, two or three fractions of 200 ml). Fractions eluted with 70% and 60% ethanol, which contained the opioid activity, were collected and lyophilized to yield 318 mg of crude material. To ascertain that no epimerization occurred during alumina chromatography, a sample (100 μg) of synthetic [L-Ala²]-deltorphin I was passed through the alumina column and processed exactly as above. No opioid activity was recovered in eluates, demonstrating that conversion of the inactive L isomer into the active D isomer did not occur during chromatography.

An aliquot (19 mg) of the lyophilized active material was purified by preparative HPLC on a PLC-18 Supelcosil column (2.12 × 25 cm, 20–30 μm; Supelco) with a linear gradient of solvent B (5 mM trifluoroacetic acid in acetonitrile) in solvent A (5 mM trifluoroacetic acid in water) (Fig. 1A). Active fractions were pooled and chromatographed on a LC-18-DB column (4.6 × 250 mm, 3 μm; Supelco) with the gradient described above. Two partially resolved active peaks were collected separately and passed again through the

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Abbreviations: DADLE, [D-Ala², D-Leu⁵]enkephalin; DAGO, [D-Ala²,Phe(Me)⁴,Gly-ol⁵]enkephalin; DPDPE, [D-Pen²,D-Pen⁵]enkephalin (where D-Pen is D-penicillamine).

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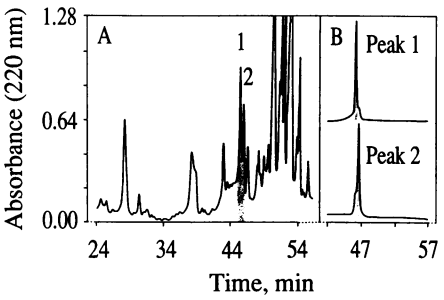


FIG. 1. Purification of [D-Ala$^2$]deltorphins from skin extracts of *P. bicolor*. (*A*) HPLC (PLC-18 Supelcosil reversed-phase column) of bioactive fractions from alumina chromatography. Stippled area shows opioid activity. The two active peaks, 1 and 2, were separately collected and further purified by HPLC. (*B*) Final chromatography of the two bioactive peaks on a Supelcosil LC-18-DB column. Stippled areas show fractions used for amino acid analysis and sequencing.

same column until complete separation was achieved. The final yields were 76 μg of [D-Ala$^2$]deltorphin I and 98 μg of [D-Ala$^2$]deltorphin II, with purities higher than 85%. The remaining amount of crude material was purified with the same procedure to yield 1.080 mg of [D-Ala$^2$]deltorphin I and 1.650 mg of [D-Ala$^2$]deltorphin II.

**Structural Analysis.** Amino acid analyses were performed with an LKB (Biochrom) 4400 amino acid analyzer after hydrolysis of the peptides (0.2–0.5 nmol) in 6 M HCl at 110°C for 24 hr *in vacuo*. Automated Edman degradation was performed with an Applied Biosystems 470A protein sequencer equipped with a 120A phenylthiohydantoin (PTH) analyzer. Manual dansyl Edman degradation was performed according to the method of Hartley (6). Reaction with D-amino acid oxidase (Sigma) was carried out by incubating the peptide hydrolysate (0.5 nmol) with the enzyme for 3 hr at room temperature in 20 mM Hepes (pH 7.9) and was followed by derivatization of the mixture with dansyl chloride and amino acid analysis under conditions described above.

**Synthesis of [Ala$^2$]Deltorphins.** [D-Ala$^2$,Glu$^4$]- and [D-Ala, Asp$^4$]deltorphinamides, their L isomers, and 1-(3,5-diiodotyrosyl) [D-Ala$^2$]deltorphins {[Tyr(I$_2$)$^1$,D-Ala$^2$]deltorphins} were prepared by solid-phase synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) polyamide active ester chemistry (7) on a Biolynx automated peptide synthesizer (Pharmacia Biochrom), and the products were purified by HPLC as described above. Amino acid analysis after 6 M HCl hydrolysis (24 hr, 110°C, *in vacuo*) gave ratios in accord with the desired structures, and expected amino acid sequences were confirmed by automated Edman degradation.

**Preparation of Tritiated Deltorphins.** For catalytic tritiation, 2.5 mg of [Tyr(I$_2$)$^1$, D-Ala$^2$]deltorphin I was dissolved in 1 ml of methanol with 10 μl of triethylamine and the solution was frozen. Palladium oxide (30 mg; Fluka) was dispersed on the surface of the frozen solution. Tritium exchange was carried out as described by Amiche *et al.* (8) and the labeled peptide was purified by analytical HPLC. The specific radioactivity of [$^3$H][D-Ala$^2$]deltorphin I was calculated as 47 Ci/mmol (1739 GBq/mmol).

**Pharmacological Assays in Isolated Tissues.** Preparations of the myenteric plexus longitudinal muscle obtained from the small intestine of male guinea pigs (400–500 g) and preparations of vasa deferentia of mouse, rat, and rabbit were used for field stimulation with bipolar rectangular pulses of supramaximal voltage as described (9). Synthetic reference peptides were dermorphin (Farmitalia Carlo Erba, Milano, Italy), [D-Ala$^2$,D-Leu$^5$]enkephalin (DADLE) (Cambridge Research Biochemicals, Cambridge, UK), and DPDPE (Bachem). Assays were performed in the presence or absence of naloxone and the δ opioid receptor antagonist naltrindole (10). The equilibrium constant $K_{eq}$ of the opioid antagonists was calculated by using the formula $K_{eq} = Q/(DR - 1)$, where $Q$ is the antagonist concentration (nM) and $DR$ is the dose ratio in antagonist-treated versus antagonist-free preparations.

**Binding Assays.** Binding of the peptides was assayed in crude membrane preparations (11) from rat brain (μ and δ sites) or guinea pig cerebellum (κ sites) at pH 7.4 in 50 mM Tris/HCl buffer. Each assay mixture contained, in a final volume of 2 ml, the membrane preparation (0.6–0.8 mg of protein, equivalent to 20–27 mg of brain wet tissue) and the tritiated ligand at the desired concentration with or without unlabeled ligand. The μ binding site was selectively labeled with [$^3$H][D-Ala$^2$,Phe(Me)$^4$,Gly-ol$^5$]enkephalin ([$^3$H]DAGO, 1 nM; Amersham). The δ binding site was labeled with tritiated, S—S cyclized [$^3$H]DPDPE (Amersham). Nonspecific binding was determined in the presence of 1 μM DAGO and 1 μM DPDPE in μ and δ binding assays, respectively. For the κ binding site a paired-tube paradigm as suggested by Mosberg *et al.* (12) was used, in which specific binding was defined as bound (−)-[$^3$H]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 methanesulfonate (U50,488; 500 nM; Upjohn). When [$^3$H][D-Ala$^2$]deltorphin I was used as primary radioligand (0.2 nM), nonspecific binding was determined in the presence of 100 nM [D-Ala$^2$]deltorphin I or 10 μM naloxone. After a 45-min incubation at 35°C the samples were cooled at 4°C and the free ligand was separated from membrane-bound ligand by filtration under reduced pressure over Whatman GF/B paper (soaked for 1 hr in incubation buffer with 0.1% bovine serum albumin), followed by three washes with 5 ml of ice-cold buffer. Separation time never exceeded 20 sec. Radioactivity was extracted in 10 ml of Filter-Count

Table 1. Inhibitory potency ($IC_{50}$) of dermorphin, DADLE, DPDPE, deltorphin, [D-Ala$^2$]deltorphins, and their L isomers on electrically evoked contractions of mouse vas deferens (MVD) and guinea pig ileum (GPI) preparations

| Peptide | $IC_{50}$, nM [mean ± SEM (*n*)] MVD | $IC_{50}$, nM [mean ± SEM (*n*)] GPI | MVD/GPI $IC_{50}$ ratio |
|---|---|---|---|
| Dermorphin | 17.80 ± 2.10 (29) | 1.06 ± 0.06 (14) | 16.7 |
| DADLE | 0.75 ± 0.09 (10) | 9.10 ± 0.53 (7) | $8 \times 10^{-2}$ |
| DPDPE | 6.05 ± 0.68 (13) | ≥3,000 (15)* | $\leq 2 \times 10^{-3}$ |
| Deltorphin | 0.97 ± 0.05 (75) | ≥3,000 (11)* | $\leq 3 \times 10^{-4}$ |
| [L-Met$^2$]Deltorphin | 1186 ± 282 (5) | ≥10,000 (3)* | $\leq 1 \times 10^{-1}$ |
| [D-Ala$^2$]Deltorphin I, natural | 0.21 ± 0.01 (20) | ≥1,500 (7)* | $\leq 1 \times 10^{-4}$ |
| [D-Ala$^2$]Deltorphin I, synthetic | 0.16 ± 0.01 (27) | ≥1,500 (11)* | $\leq 1 \times 10^{-4}$ |
| [D-Ala$^2$]Deltorphin II, natural | 0.32 ± 0.05 (5) | ≥3,000 (7)* | $\leq 1 \times 10^{-4}$ |
| [D-Ala$^2$]Deltorphin II, synthetic | 0.37 ± 0.04 (44) | ≥3,000 (9)* | $\leq 1 \times 10^{-4}$ |
| [L-Ala$^2$]Deltorphin I | 1320 ± 190 (4) | ≥10,000 (4)* | $\leq 1 \times 10^{-1}$ |

Numbers in parentheses are the numbers of experiments.
*In these experiments $IC_{50}$ values varied widely and were probably greater than could be measured.

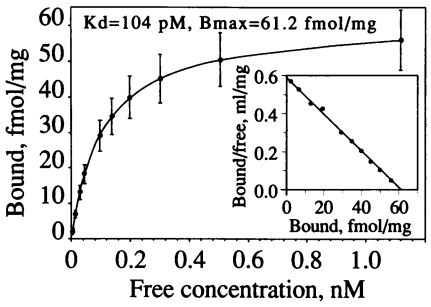


FIG. 2. Binding isotherm of [$^3$H][D-Ala$^2$]deltorphin I with rat brain membranes. (*Inset*) Scatchard transformation.

scintillation fluid (Packard Instrument) and measured in a liquid scintillation counter (LS 100, Beckman). Soaking the filters in 0.1% bovine albumin decreased radioactivity binding to filters to <50 dpm. Competition curves were determined in triplicate and affinities of each ligand in the $\mu$, $\delta$, and $\kappa$ systems are expressed as log $K_i$ and graphically represented as a binding selectivity profile (13) by plotting the three affinity values of each peptide on a horizontal logarithmic scale. The standard deviation of calculated log $K_i$ was within 0.1 decimal logarithmic unit. Kinetic experiments with [$^3$H][D-Ala$^2$]deltorphin I were performed by addition of the radioligand to a flask containing the other reagents as above. The mixture was incubated at 35°C and continuously agitated. To determine the time course of binding, the reaction was stopped at various intervals by immediate filtration of a 1-ml aliquot. After the steady state was reached, dissociation was initiated by dilution of the mixture with the same volume of a levorphanol solution (final concentration 10 $\mu$M). The residual binding was measured at intervals for 2 hr.

**Data Analysis.** The inhibitory constant ($K_i$) of the various nonradioactive peptides was calculated from $IC_{50}$ by means of the equation $K_i = IC_{50}/[2L/L_0) + (L/K_d) - 1]$, where $L$ is free radioligand concentration in equilibrium with $IC_{50}$ of unlabeled ligand and $L_0$ is free radioligand concentration in the absence of competing ligand (14). The maximal number of binding sites ($B_{max}$) and the dissociation constant ($K_d$) for the radioligands were determined from least-squares regression analysis of Scatchard transformation of the saturation isotherms. Kinetics of [$^3$H][D-Ala$^2$]deltorphin I binding was assumed to follow a bimolecular association model (second-order kinetics) and a unimolecular dissociation model (first-order kinetics). The dissociation curve was graphically represented by plotting $RL_{\Delta t}/RL_{eq}$ vs. time, with $RL_{\Delta t}$ and $RL_{eq}$ equal to the occupied receptor concentrations at a given time and at equilibrium, respectively. The slope of the plot $\ln(RL_{\Delta t}/RL_{eq})$ vs. time is related to $k_{-1}$. The apparent on-rate constant ($k_{+1(app)}$) was calculated as the slope of the plot $\ln[RL_{eq}/(RL_{eq} - RL_{\Delta t})]$ vs. time, according to the equation $\ln[RL_{eq}/(RL_{eq} - RL_{\Delta t})] = k_{+1(app)}t$, where $k_{+1(app)} = (k_{+1}L) + k_{-1}$. The accuracy in the determination of binding parameters was evaluated from the standard deviation of the error of the raw data, $SD(E_{rad})$ (15). Calculations were performed on a Macintosh II computer using the regression-analysis program StatView II (Abacus Concepts, Berkeley, CA).

**Enzymatic Degradation of Deltorphins by Brain Membranes.** Crude rat brain membranes (2 mg of protein per ml) in 50 mM Tris/HCl (pH 7.4) were incubated with 0.1 mM deltorphin, [D-Ala$^2$]deltorphin I or II, or L isomers for various times at 37°C. The reaction (in 200 $\mu$l) was stopped by addition of 20 $\mu$l of 0.5 M HCl and heating at 80°C for 5 min. After centrifugation at 100,000 × $g$ for 5 min the supernatant was directly injected into a reversed-phase HPLC column for peptide quantification.

## RESULTS

Fig. 1 shows the two final stages of chromatographic separation of [D-Ala$^2$]deltorphins from extracts of skin of *P. bicolor*. Eluates were tested for opioid activity on the mouse vas deferens preparation. The active fractions (Fig. 1*B*) were used for sequence analysis. Automated Edman degradation of these two peptides gave the sequences Tyr-Ala-Phe-Glu-Val-Val-Gly (peak 1, 45.6 min) and Tyr-Ala-Phe-Asp-Val-Val-Gly (peak 2, 46.1 min), which were consistent with the corresponding amino acid compositions $Glu_{1.0}Gly_{1.0}$-$Ala_{0.9}Val_{1.8}Tyr_{0.8}Phe_{1.0}$ (peak 1) and $Asp_{0.8}Gly_{1.0}Ala_{0.9}Val_{1.7}$-$Tyr_{0.7}Phe_{1.0}$ (peak 2). To test whether these peptides terminate with a free carboxyl group or an amide, 10 nmol of each peptide was sequenced by manual dansyl Edman degradation. After the sixth cycle of degradation (i.e., after removal of the penultimate valine), the sample was reacted with dansyl chloride and an aliquot was directly analyzed without previous acid hydrolysis. The presence of a fluorescent compound with chromatographic characteristics identical to those of a sample of authentic dansylglycinamide was detected by both thin-layer chromatography on polyamide sheets and reversed-phase HPLC. A second aliquot of the sample was similarly analyzed after acid hydrolysis and showed the presence of dansylglycine.

The L or D configuration of the alanine residue in these peptides was demonstrated by incubating aliquots of the acid hydrolysates with D-amino acid oxidase. Amino acid analysis revealed the disappearance of alanine in the samples subjected to the enzymatic treatment. Thus, both peptides contain D-alanine in position 2 and a C-terminal glycinamide. The peptide containing Asp-4 was named [D-Ala$^2$]deltorphin I, and the peptide with Glu-4, [D-Ala$^2$]deltorphin II. The correctness of the proposed structures was confirmed by synthesis followed by comparison of the chromatographic and pharmacological properties of the natural and synthetic molecules. Samples of synthetic [D-Ala$^2$]deltorphins had chromatographic properties identical to those of the corresponding natural peptides, and synthetic L isomers were devoid of any biological activity.

The results obtained with [Ala$^2$]deltorphins, deltorphin, dermorphin, DADLE, and DPDPE in the bioassays are summarized in Table 1. These tests demonstrated the high $\delta$-receptor selectivity and potency of deltorphin and [D-Ala$^2$]deltorphins. On the same preparation, $K_{eq}$ values (nM, mean ± SEM) of naloxone were 135 ± 22 for [D-Ala$^2$]-deltorphin I, 118 ± 15 for [D-Ala$^2$]deltorphin II, 76.9 ± 4.1 for deltorphin, and 43.2 ± 3.4 for DPDPE. This confirms that naloxone is a weak antagonist of $\delta$-receptor-selective opioids. In contrast, naltrindole very potently inhibited the depression produced by deltorphins on electrically evoked contraction of mouse vas deferens. $K_{eq}$ values (nM, mean ± SEM) of naltrindole were 1.2 ± 0.4 for [D-Ala$^2$]deltorphin I, 0.64 ± 0.12 for [D-Ala$^2$]deltorphin II, and 0.38 ± 0.08 for deltorphin. Deltorphins are almost inactive on guinea pig ileum and rat vas deferens ($\mu$ site), as well as on rabbit vas deferens ($\kappa$ site). On the other hand, on guinea pig ileum, dermorphin showed an $IC_{50}$ value in the nanomolar range, which was 17 times lower than that on mouse vas deferens, confirming its preferential binding to the $\mu$ opioid receptor sites.

The binding isotherm for [D-Ala$^2$]deltorphin I with rat brain membranes (Fig. 2) was obtained at 35°C to avoid possible discrepancies between the results of binding and pharmacological experiments. The Scatchard transformation is consistent with the binding to a single type of site with an affinity ($K_d$ = 0.104 ± 0.009 nM) about 2 orders of magnitude higher than that obtained in the same membrane preparations with DPDPE ($K_d$ = 8.8 ± 1.2 nM). The estimate of $B_{max}$ (61.2 ± 5.9 fmol/mg of protein, or 2.02 ± 0.02 pmol/g of brain) with this peptide is in the range of that obtained for $\delta$ sites with

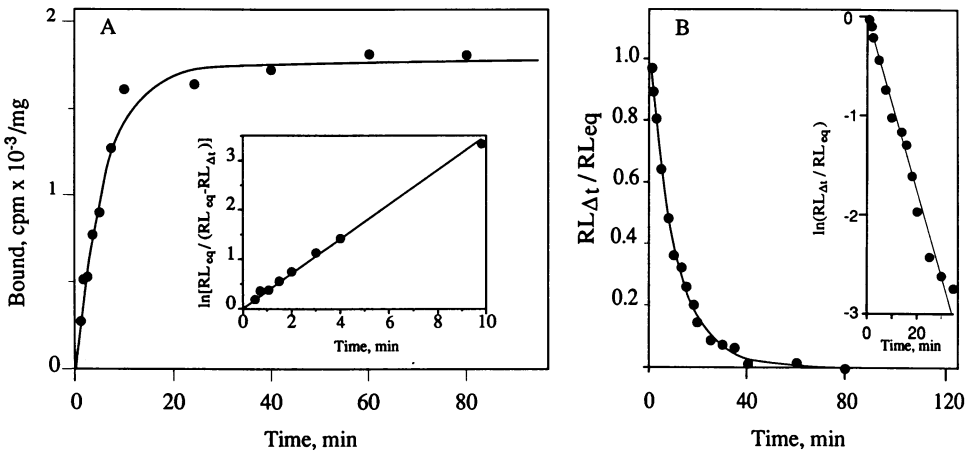


FIG. 3. Kinetics of the specific binding of 0.2 nM [$^3$H][D-Ala$^2$]deltorphin I to rat brain membranes. (*A*) Association curve of the labeled peptide. Results are from one experiment. (*Inset*) Plot of the association according to the second-order kinetics equation. (*B*) Dissociation curve of the peptide after addition of 10 $\mu$M levorphanol. (*Inset*) Plot of the ligand dissociation from the receptor according to the first-order kinetics equation.

DPDPE (78.8 ± 3.6 fmol/mg of protein, or 2.60 ± 0.01 pmol/g of brain) and close to values previously determined with other $\delta$ ligands (16, 17). The $K_d$ and $B_{max}$ values of both peptides did not change when $\mu$ binding suppression was obtained with a fixed ratio of unlabeled DAGO (30 nM) to the tritiated ligand ($1 \times K_d$) (results not shown).

The on-rate constant ($k_{+1}$) for [D-Ala$^2$]deltorphin I was calculated to be $2.36 \times 10^7$ $M^{-1} \cdot sec^{-1}$, the steady state being reached in about 25 min at 35°C (Fig. 3*A*). Nonspecific binding was very low, <7% of total bound radioactivity. The dissociation curve of [D-Ala$^2$]deltorphin I was monophasic with a half-life of 8.3 min and $k_{-1} = 1.49 \times 10^{-3}$ $sec^{-1}$ (Fig. 3*B*). The kinetically derived dissociation constant ($K_d = k_{-1}/k_{+1}$) was 0.063 nM.

The rate of degradation of deltorphins in contact with a crude preparation of rat brain membranes was very slow, since <10% hydrolysis occurred after 50 min of incubation. In contrast, L isomers were completely degraded in 10–25 min (results not shown).

To further characterize the selectivity pattern of [D-Ala$^2$]deltorphin I, several opioid compounds were assayed for their relative potency to inhibit the specific binding of [$^3$H][D-Ala$^2$]deltorphin I and [$^3$H]DAGO to rat brain membrane preparations (Table 2). The Hill coefficients ($n_H$) accounted for the homogeneity of the competition curves observed. DPDPE selectively displaced [$^3$H][D-Ala$^2$]deltorphin I and its $K_i$ value was close to the $K_d$ value calculated from saturation experiments (16). As expected, the highly selective $\mu$ opioid peptide DAGO displaced [$^3$H][D-Ala$^2$]deltorphin I with very low potency. The self-competition of unlabeled [D-Ala$^2$]deltorphin I gave a $K_i$ value close to the $K_d$ calculated from the Scatchard plot and 2 orders of magnitude higher than the $K_i$ estimate for DPDPE. Deltorphin and [D-Ala$^2$]deltorphin II were also more effective than DPDPE in displacing the radioligand. These results are represented in Fig. 4 as binding selectivity profiles.

## DISCUSSION

We have described the isolation of two high-affinity ligands from amphibian skin that are selective for $\delta$ opioid receptors. These peptides are referred to as [D-Ala$^2$]deltorphin I and II to distinguish them from deltorphin (D-methionine at position 2), an amphibian peptide isolated previously (4). These results demonstrate that the skin of frogs belonging to the genus *Phyllomedusa* contains two families of opioid peptides, dermorphins and deltorphins. These peptides have in common the N-terminal sequence Tyr-D-Xaa-Phe, where D-Xaa is D-alanine or D-methionine. Since this initial sequence is conserved, we presume that it is necessary for binding to both $\mu$ and $\delta$ sites of opioid receptors. Unlike dermorphin, the deltorphins have a charged amino acid in position 4, namely histidine in the case of deltorphin and aspartic or glutamic acid in the case of [D-Ala$^2$]deltorphins. Obviously, the presence of positively or negatively charged amino acids in this position has little influence on the binding characteristics. It thus seems likely that other features in the C-terminal regions of deltorphins are essential for the observed receptor selectivity.

Deltorphins are more flexible linear peptides than the conformationally restricted cyclic peptide DPDPE. Nevertheless, they showed an affinity for $\delta$ sites from 10 to 200 times higher than that of the synthetic enkephalin analogue. Thus, the suggestion that the highest level of selectivity toward a given opioid binding site may be achieved only by imposing conformational restrictions on peptide chains is not supported by these observations. The high affinity and potency of the deltorphins could allow the precise biochemical and pharmacological characterization of the $\delta$ opioid receptor. As shown in this communication, [$^3$H][D-Ala$^2$]deltorphin I is a valuable probe for binding studies, since its affinity and selectivity are the highest of all the $\delta$-selective ligands known

Table 2. Inhibitory potencies of enkephalin analogues, deltorphin, and [D-Ala$^2$]deltorphins on the specific binding of 0.2 nM [$^3$H][D-Ala$^2$]deltorphin I at $\delta$ site and of 1 nM [$^3$H]DAGO at $\mu$ site in rat brain membranes at 35°C

| Peptide | [$^3$H]DAGO | | [3H][D-Ala$^2$]deltorphin I | |
|---|---|---|---|---|
| | $K_i$, nM | $n_H$ | $K_i$, nM | $n_H$ |
| DAGO | 3.36 ± 0.89 | 1.02 ± 0.18 | 820 ± 33 | 0.82 ± 0.06 |
| DPDPE | 1990 ± 98 | 0.80 ± 0.04 | 8.87 ± 0.64 | 0.96 ± 0.08 |
| Deltorphin | 1630 ± 114 | 0.85 ± 0.03 | 2.41 ± 0.03 | 0.98 ± 0.02 |
| [D-Ala$^2$]Deltorphin I | 3150 ± 202 | 0.92 ± 0.08 | 0.15 ± 0.01 | 0.96 ± 0.03 |
| [D-Ala$^2$]Deltorphin II | 2450 ± 185 | 0.84 ± 0.07 | 0.71 ± 0.01 | 0.88 ± 0.04 |

Values are means ± SEM; $n_H$ is the Hill coefficient.

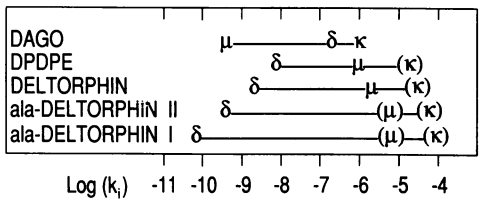


FIG. 4. Binding selectivity profiles of DAGO, DPDPE, deltorphin, and [D-Ala$^2$]deltorphins. Greek letters represent the three types of opioid binding sites under study; each is positioned according to log $K_i$ of the ligand at that site (for details see ref. 13). Parentheses indicate that the value of log $K_i$ was greater than could be measured.

to date. Since amphibian skin peptides are often similar or even identical to mammalian hormones and neurotransmitters (18), the question whether relatives of the dermorphin/deltorphin peptides also occur in mammals clearly deserves further attention.

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