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# Opioid Binding Sites in the Guinea Pig and Rat Kidney: Radioligand Homogenate Binding and Autoradiography

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**SUMMARY**

The specific binding of the selective μ-, δ-, and κ-opioid ligands [$^3$H][D-Ala$^2$,MePhe$^4$,Gly-ol$^5$]enkephalin ([$^3$H] DAGOL), [$^3$H][D-Pen$^2$,D-Pen$^5$]enkephalin ([$^3$H]DPDPE), and [$^3$H]U69593, respectively, to crude membranes of the guinea pig and rat whole kidney, kidney cortex, and kidney medulla was investigated. In addition, the distribution of specific $^3$H-opioid binding sites in the guinea pig and rat kidney was visualized by autoradiography. Homogenate binding and autoradiography demonstrated the absence of μ- and κ-opioid binding sites in the guinea pig kidney. No opioid binding sites were demonstrable in the rat kidney. In the guinea pig whole kidney, cortex, and medulla, saturation studies demonstrated that [$^3$H]DPDPE bound with high affinity ($K_D$ = 2.6–3.5 nM) to an apparently homogeneous population of binding sites ($B_{max}$ = 8.4–30 fmol/mg of protein). Competition studies using several opioid compounds confirmed the nature of the δ-opioid binding site. Autoradiography experiments demonstrated that specific [$^3$H]DPDPE binding sites were distributed radially in regions of the inner and outer medulla and at the corticomedullary junction of the guinea pig kidney. Computer-assisted image analysis of saturation data yielded $K_D$ values (4.5–5.0 nM) that were in good agreement with those obtained from the homogenate binding studies. Further investigation of the δ-opioid binding site in medulla homogenates, using agonist ([$^3$H]DPDPE) and antagonist ([$^3$H]diprenorphine) binding in the presence of $Na^+$, $Mg^{2+}$, and nucleotides, suggested that the δ-opioid site is linked to a second messenger system via a GTP-binding protein. Further studies are required to establish the precise localization of the δ binding site in the guinea pig kidney and to determine the nature of the second messenger linked to the GTP-binding protein in the medulla.

The concept of multiple opioid receptors, originally suggested by Martin (1), has been followed by extensive characterization of μ, δ, and κ receptors in mammalian brain tissue. However, pharmacological (2–4), electrophysiological (5, 6), biochemical (7, 8), and autoradiographical (9, 10) lines of evidence indicate that opioid receptors are not confined to the central nervous system. For example, there is evidence that increased micturition in rats after treatment with κ-opioid agonists is at least partially due to renal κ-opioid receptors (11, 12). Furthermore, it has been suggested that morphine (μ-opioid)-induced antidiuresis in rats may also have a peripheral component (13), in addition to its considered main central action (14).

Despite this pharmacological evidence and except for one report on the existence of κ binding sites (15), there has been no detailed biochemical investigation on the distribution of μ, δ, and κ binding sites in the kidney. This study has, therefore, investigated the presence of opioid binding sites in the guinea pig and rat kidney, using radioligand binding to tissue homogenates and to slide-mounted sections of the kidney. Although an absence of μ ([$^3$H]DAGOL) and κ ([$^3$H]U69593) binding sites was observed in the guinea pig and rat kidney in the present investigation, δ ([$^3$H]DPDPE) binding sites were found to be present in the guinea pig kidney.

The properties of the δ receptor have been studied most extensively in several cultured cell lines, including the neuroblastoma × glioma hybridoma NG 108-15 (16–19), the neuroblastoma N 18TG2 (20), and the neuroblastoma SH-SY5Y (21). In these cell lines, there is clear evidence of the δ receptor being associated with second messenger amplification signals. Agonist occupation of the receptor has been found to lead to inhibition of adenylate cyclase (20–23), with the signal transduction being modulated by the inhibitory G protein $G_i$ (24, 25). The present study, therefore, examined the probable interaction of the renal δ binding site with membrane G proteins by investigating the effects of ions and nucleotides on radiolabeled agonist ([$^3$H]DPDPE) and antagonist ([$^3$H]diprenorphine) binding.

## Materials and Methods

### Homogenate Binding Studies

**Preparation of membranes.** Guinea pig kidney membranes were prepared fresh each day. Guinea pigs were killed by cervical dislocation,

**ABBREVIATIONS:** DAGOL, [D-Ala$^2$,MePhe,Gly-ol$^5$]enkephalin; DPDPE, [D-Pen$^2$,D-Pen$^5$]enkephalin; DADLE, [D-Ala$^2$,D-Leu$^5$]enkephalin; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; App(NH)p, adenyl-5′-yl-imidodiphosphate; EKC, ethylketacyclazocine; G protein, GTP-binding protein; U69593, 5α, 7α, 8β-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-8-yl]-benzeneacetamide.

and the kidneys were removed immediately and placed in ice-cold 50 mM Tris·HCl buffer, pH 7.4. The kidney cortex was separated from the medulla when necessary; otherwise, the whole kidney was utilized. The tissue was initially minced with scissors and then homogenized for 10 sec, using a Polytron PT 10 at setting 6.0, followed by three strokes of a Teflon pestle in a glass homogenizer. This homogenate was filtered through a fine nylon mesh to remove any residual large particles. The filtrate was centrifuged for 20 min at 39,000 × *g* on a Beckman J21 centrifuge at 4°, the pellet was resuspended in approximately 10 volumes of 50 mM Tris·HCl buffer (pH 7.4), and the suspension was incubated for 45 min at 37°, in order to degrade endogenous ligands. After the period of incubation, the homogenate was recentrifuged at 39,000 × *g* for 20 min at 4°. The resulting pellet was resuspended in ice-cold Tris·HCl and centrifuged as before. The final pellet was resuspended in Tris·HCl (pH 7.4) and made up to a final concentration of 1.0 mg/ml whole kidney or cortex or 0.5 mg/ml medulla.

**Receptor binding assay conditions.** To start the reaction, 50-μl aliquots of membrane were added to 450 μl of 50 mM Tris·HCl assay buffer containing the appropriate concentration of radioligand, competing ligands, nucleotides, or cations (NaCl or $MgCl_2$). The samples were incubated for 90 min at 25°, and the reaction was terminated by the addition of 3.0 ml of ice-cold Tris·HCl, followed by rapid filtration through Whatman GF/B filters. The filters were then washed twice with 3.0 ml of ice-cold 50 mM Tris·HCl and transferred to vials containing 4.0 ml of scintillation cocktail, and the radioactivity counted by liquid scintillation spectrometry.

The specific binding of the radioligand was defined as that binding remaining in the presence of 10 μM naloxone. In saturation experiments, 8–12 concentrations of radioligand (0.5–30 nM) were investigated and, in competition experiments, 6–8 concentrations of unlabeled ligand.

The time dependence of [$^3$H]DPDPE binding to guinea pig kidney tissue was investigated by determination of the specific binding of the radioligand at various time points between 0.5 and 120 min. The reaction was started by addition of 50 μl of membranes to 450 μl of 50 mM Tris·HCl assay buffer containing radioligand, in the presence or absence of 10 μM naloxone, to determine nonspecific and total binding, respectively. The reaction was terminated at each time point by the addition of 3.0 ml of ice-cold buffer, followed by rapid filtration through individual Whatman GF/B filters mounted on a Millipore filter block. Each filter was then washed twice with ice-cold buffer, and the radioactivity was counted as described above.

Protein content of membrane preparations was determined using the method of Lowry *et al.* (26), with bovine serum albumin used as standard.

## Autoradiography Studies

Guinea pig kidneys were frozen in dry ice on aluminium chucks, using plastic embedding material. After equilibration at −18° for 1 hr, the frozen kidneys were sectioned (10 μm) longitudinally through the tip of the papilla, on a Bright Cryostat at −15°, and the sections were thaw-mounted on precleaned, dust-free, gelatin-coated, glass microscope slides. These slides were stored at −20° for a maximum of 3 days.

For receptor binding assays, the slide-mounted sections were gradually brought up to room temperature and then preincubated at 37° for 45 min in 50 mM Tris·HCl buffer (pH 7.4, without added salts), in order to degrade endogenous opioids. The sections were rinsed in 50 mM Tris·HCl (25°), laid flat on glass rods, and then incubated with 500 μl/section of the appropriate tritiated opioid ligand, in 50 mM Tris·HCl buffer, at room temperature for 90 min.

In saturation studies, a minimum of six concentrations of each radioligand, namely [$^3$H]DPDPE (0.2–40 nM), [$^3$H]DAGOL (0.1–10 nM), and [$^3$H]U69593 (0.2–40 nM), were used. Nonspecific binding was determined by treatment of a parallel set of slides, consisting of adjacent sections, with the same concentration of $^3$H-ligand and 10 μM unlabeled naloxone.

After the 90-min incubation period, the assays were terminated by draining the assay mixture off the slides and rinsing the slides with three consecutive 250-ml 50 mM Tris·HCl washes (pH 7.4, 4°). Slides incubated with [$^3$H]DPDPE and [$^3$H]U69593 were given three 10-sec washes, whereas those incubated with [$^3$H]DAGOL were given three 1-min washes. After the Tris washes, all slides were rinsed (2 sec) in a 250-ml wash of distilled water (4°) to remove buffer salts. The slides were then quickly dried in a stream of cool air and dessicated overnight.

The slides were next apposed to tritium-sensitive LKB Ultrofilm in X-ray cassettes in the dark, together with brain paste standards containing known amounts of radioactivity. Films were incubated at room temperature for 3 months, after which they were developed as follows: Kodak D-19 developer (4 min, 18°), deionized water wash (30 sec, 25°), Unifix fixer (3 min, 25°), and a final tap water wash for at least 10 min, before drying in a warm cabinet.

The film (i.e., autoradiogram) was then used directly for analysis, using the Quantimet 920 computer-assisted image analysis system (27).

Briefly, adjacent nonspecific binding sections were realigned on the outlines of the total binding sections, and the specific binding was determined by automatic digital subtraction of the former from the latter images. Quantification of the data was by reference to a series of tritiated brain paste standards, using a natural logarithmic plot of absorbance versus radioactivity. Ideally, tritiated kidney paste standards should have been used as reference. However, because the measurements made were absolute, without comparison to other work, brain paste standards were used as the arbitrary source of measurement for these experiments.

In saturation experiments, the specific binding, expressed as amol/mm$^2$, was determined at each radioligand concentration, and these data were analyzed using LIGAND, as described in Data Analysis.

## Data Analysis

The receptor binding saturation data were analyzed by using a version of the nonlinear, least squares, curve-fitting program LIGAND (28). The source of the LIGAND program was ELSEVIER-BIOSOFT; the program is part of a collection of radioligand binding analysis programs originally produced by McPherson (29). The competitive inhibition data were analyzed by using ALLFIT (30). $K_i$ values were then calculated from the Cheng and Prusoff (31) equation. Competition data for [$^3$H]diprenorphine binding were analyzed by using LIGAND, in order to ascertain changes in affinities and slope values under different conditions.

## Materials

[$^3$H]DPDPE (27–37 Ci/mmol) and [$^3$H]DAGOL (60 Ci/mmol) were obtained from Amersham, and [$^3$H]U69593 (40.5 Ci/mmol) was from New England Nuclear. The following unlabeled drugs, peptides, and nucleotides were used: bremazocine (Sandoz), U69593 (Upjohn Co.), naloxone-HCl (Sigma), DAGOL (Cambridge Research Biochemicals), DADLE (Cambridge Research Biochemicals), DPDPE (Bachem), Gpp(NH)p (Boehringer Mannheim), App(NH)p (Boehringer Mannheim), ATP (Sigma), and GTP (Sigma).

# Results

**Characteristics of specific [$^3$H]DAGOL and [$^3$H]U69593 binding to guinea pig and rat kidney homogenates and of specific [$^3$H]DPDPE binding to rat kidney homogenates.** Equilibrium binding saturation studies performed with [$^3$H]DAGOL and [$^3$H]U69593 in guinea pig and rat whole kidney, cortex, and medulla showed an absence of specific binding. Saturation binding studies performed with [$^3$H]DPDPE in rat whole kidney, cortex, and medulla also indicated an absence of specific binding.

**General characteristics of specific [$^3$H]DPDPE binding to guinea pig kidney homogenates.** Specific binding of

[$^3$H]DPDPE increased linearly with protein concentration, up to a concentration of approximately 4.0 mg/ml for homogenates of whole kidney and cortex and 1.0 mg/ml for medulla homogenates. A protein concentration of approximately 3.0 mg/ml (100 mg of tissue/ml) for whole kidney and cortex homogenates and 0.6 mg/ml (50 mg/ml) for medulla homogenates was used routinely in each binding assay. At these tissue concentrations, <10% of the radioligand available in the incubation volume was bound at equilibrium.

Specific binding of [$^3$H]DPDPE at concentrations used in competition experiments (1.5–2.0 nM) was 40–50% of total binding in the whole kidney and cortex and 50–60% of that in the medulla.

The time dependence of specific [$^3$H]DPDPE binding to medulla homogenates is shown in Fig. 1. Specific binding of [$^3$H]DPDPE achieved equilibrium after 80 min and was stable for at least an additional 40 min. The time course of [$^3$H]DPDPE binding to either the whole kidney or cortex followed a pattern identical to that of the medulla. Membranes were, therefore, incubated with the radioligand for 90 min at 25° in subsequent saturation and competition studies.

**Equilibrium binding saturation and competitive inhibition studies of [$^3$H]DPDPE in guinea pig kidney homogenates.** Specific binding of [$^3$H]DPDPE to whole kidney, cortex, and medulla homogenates was saturable, whereas, in contrast, nonspecific binding was linear with increasing concentration. The saturation isotherm of [$^3$H]DPDPE in the medulla is shown in Fig. 2. The [$^3$H]DPDPE saturation isotherms in the whole kidney and cortex followed a similar pattern. The radioligand bound with high affinity to an apparently homogeneous population of noninteracting sites in the membrane preparations. The $K_D$ and $B_{max}$ values of [$^3$H]DPDPE in the kidney are presented in Table 1. The $K_D$ values (2.6–3.5 nM) were not significantly different in the three preparations. In contrast, the $B_{max}$ value in the whole kidney (8.4 fmol/mg of protein) was significantly lower ($p < 0.05$, Mann Whitney $U$ test) than that observed in the medulla (33 fmol/mg of protein), but it was not significantly different from the $B_{max}$ obtained in the cortex (7.2 fmol/mg of protein). The most likely explanation for this apparent inconsistency is simply that the whole kidney is >90% cortex by weight and, therefore, the increase observed when the medulla is used in isolation is a result of a concentration effect, due to the major localization of binding sites to this area of the kidney.

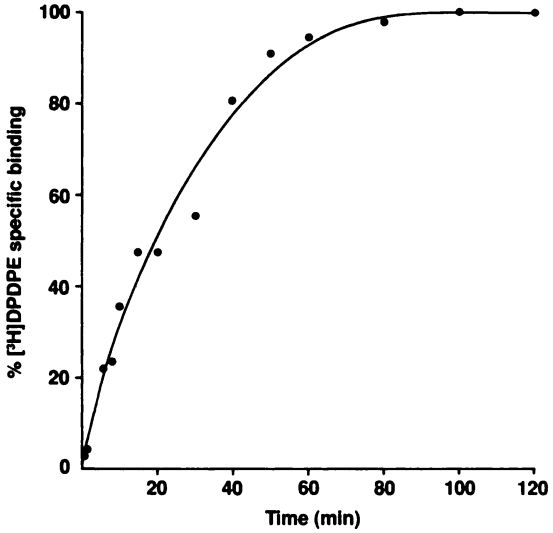


**Fig. 1.** Time dependence of [$^3$H]DPDPE (3.6 nM) binding to guinea pig kidney medulla homogenates at 25°. Equilibrium was reached after 80 min. Data are presented as a percentage of specific binding of [$^3$H]DPDPE at equilibrium. Nonspecific binding of the radioligand was defined with 10 μM naloxone. *Points* shown are from a representative experiment performed in triplicate, as described in Materials and Methods. Similar results were obtained in two additional experiments. The time course of [$^3$H]DPDPE binding in the whole kidney and cortex was similar to that in the medulla.

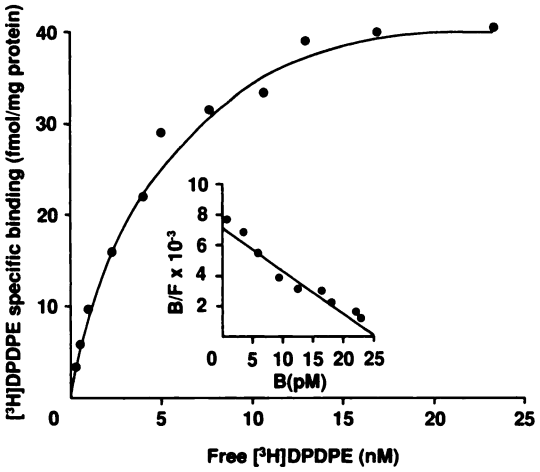


**Fig. 2.** Saturation isotherm for [$^3$H]DPDPE specific binding to guinea pig kidney medulla homogenates. *Inset*, Scatchard transformation of specific binding data. *B*, bound; *B/F*, bound/free. Nonspecific binding of [$^3$H]DPDPE, defined with 10 μM naloxone, was linear over the selected concentration range. *Points* shown are from a representative experiment performed in triplicate. Similar results were obtained in four additional experiments. Corresponding equilibrium binding parameters for [$^3$H]DPDPE are detailed in Table 1.

TABLE 1

**Equilibrium binding parameters of [$^3$H]DPDPE specific binding to kidney tissue homogenates**

The $K_D$ and $B_{max}$ values were determined by LIGAND analysis, as described in Materials and Methods. Each value represents the mean ± standard error from between three and five experiments, each performed in triplicate.

| Tissue | $K_D$ | Slope | $B_{max}$ |
|---|---|---|---|
| | *nM* | | *fmol/mg of protein* |
| Whole kidney | 2.7 ± 1.2 | 1.01 ± 0.02 | 8.4 ± 0.9[a] |
| Cortex | 2.6 ± 0.3 | 0.96 ± 0.02 | 7.2 ± 0.9 |
| Medulla | 3.5 ± 0.4 | 0.98 ± 0.02 | 33 ± 4.3 |

[a] $p < 0.05$ (Mann Whitney $U$ test), compared with $B_{max}$ in medulla.

In competition studies, all unlabeled compounds produced a concentration-dependent competitive inhibition of specific [$^3$H]DPDPE binding in the whole kidney, cortex, and medulla. The $K_i$ values of naloxone, bremazocine, DPDPE, DADLE, DAGOL, and U69593, measured against [$^3$H]DPDPE, are presented in Table 2. The slopes of the competition curves were not significantly different from unity, and the curves fitted a one-site model. The inhibition curves for the unlabeled compounds in the medulla are shown in Fig. 3.

In all three membrane preparations, the relative potencies of the unlabeled opioids showed a typical δ profile, i.e., the δ-selective agents had high affinity at the [$^3$H]DPDPE binding site, whereas μ- and κ-selective agents had very low affinity. The $K_i$ values for DAGOL were >600 nM, whereas those of U69593 were >900 nM. Such affinities for μ- and κ-specific ligands at the δ sites in the kidney correlate closely with those reported in guinea pig brain (32).

TABLE 2

**Inhibition constants of selected opioids against [$^3$H]DPDPE specific binding to kidney tissue homogenates**

The $IC_{50}$ and slope values were determined by ALLFIT analysis, as described in Materials and Methods. $K_i$ values were then derived from the Cheng and Prusoff (31) equation. All slopes were not significantly different from unity. Each value represents the mean ± standard error from between three and six experiments, each performed in triplicate.

| Unlabeled ligand | $K_i$ | | |
|---|---|---|---|
| | Whole kidney | Cortex | Medulla |
| | nM | nM | nM |
| DPDPE | 0.6 ± 0.1 | 0.5 ± 0.1 | 1.4 ± 0.1 |
| DADLE | 1.0 ± 1.0 | 0.3 ± 0.2 | 0.3 ± 0.1 |
| Bremazocine | 0.5 ± 0.2 | 0.7 ± 0.4 | 0.7 ± 0.1 |
| Naloxone | 44 ± 2.8 | 58 ± 14 | 43 ± 11 |
| U69593 | 925 ± 176 | 1141 ± 254 | 1321 ± 586 |
| DAGOL | 903 ± 521 | 1119 ± 680 | 669 ± 385 |

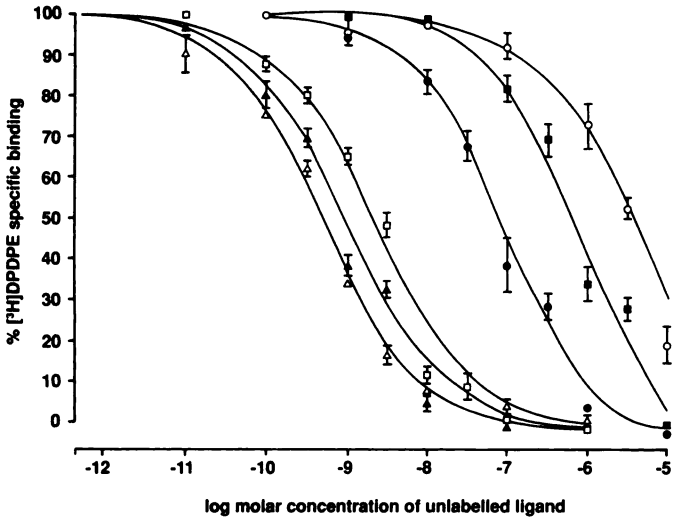


**Fig. 3.** Competitive inhibition curves for unlabeled opioids versus [$^3$H] DPDPE (1.5 nM) specific binding to guinea pig kidney medulla homogenates. Nonspecific binding of [$^3$H]DPDPE was defined with 10 μM naloxone. Each *point* represents the mean of three independent determinations, each done in triplicate; *vertical lines* represent the standard error, shown where they can be clearly distinguished from the *symbol* at that point. Corresponding inhibition constants are listed in Table 2. △, DADLE; ▲, bremazocine; □, DPDPE; ●, naloxone; ■, DAGOL; ○, U69593.

**Effects of cations and nucleotides on specific [$^3$H] DPDPE binding to medulla homogenates.** Because the characteristics of the δ binding site were closely correlated in the medulla and cortex and because, in addition, the [$^3$H] DPDPE $B_{max}$ in the medulla was much higher than that in the cortex, the effects of ions and nucleotides on the equilibrium binding parameters of [$^3$H]DPDPE in the medulla alone were further examined.

The $Mg^{2+}$ concentration curve was bell-shaped (Fig. 4A). $Mg^{2+}$ at concentrations of 1–6 mM produced a maximal (121%) increase in specific [$^3$H]DPDPE (3.6 nM) binding, with the concentration of $Mg^{2+}$ producing half-maximal increase being 0.2 mM. The nature of the $Mg^{2+}$-induced increase in agonist binding was examined by saturation analysis of [$^3$H]DPDPE binding. The presence of 3 mM $Mg^{2+}$ increased the affinity of [$^3$H]DPDPE to 1.6 ± 0.2 nM (compared with 3.5 ± 0.44 nM in the absence of $Mg^{2+}$), although the $B_{max}$ (35 ± 3.5 fmol/mg) remained unchanged (three experiments) (Table 3).

$Na^+$ produced a concentration-dependent inhibition of specific [$^3$H]DPDPE binding, with the maximal effect (30%) being observed at 200 mM $Na^+$ (Fig. 4A). The concentration of $Na^+$ giving half-maximal inhibition was 10 mM.

The guanine nucleotide GTP and its nonhydrolyzable analogue Gpp(NH)p both inhibited specific [$^3$H]DPDPE binding in a dose-related manner, causing a maximum inhibition of 30–40% at 100 μM nucleotide (Fig. 4B). The effect was minimal when $Mg^{2+}$ was absent from the buffer; therefore, the results reported were obtained when 3 mM $Mg^{2+}$ was included in the buffer. The adenine nucleotides ATP and App(NH)p had no effect on specific [$^3$H]DPDPE binding, in either the presence or the absence of 3 mM $Mg^{2+}$ (Fig. 4B).

The saturation binding parameters for [$^3$H]DPDPE were assessed in the presence of Gpp(NH)p (100 μM), when $Mg^{2+}$ (3 mM) was present in the buffer (Fig. 5). Under these conditions, the affinity of [$^3$H]DPDPE was 3.7 ± 0.2 nM, whereas the $B_{max}$ was 29 ± 0.5 fmol/mg of protein (three experiments) (Table 3). In the presence of App(NH)p (100 μM) and $Mg^{2+}$ (3 mM), the $K_D$ and $B_{max}$ values for [$^3$H]DPDPE were 1.3 ± 0.2 nM and 31 ± 1.5 fmol/mg of protein, respectively (four experiments) (Fig. 5, Table 3), values that were not significantly different ($p > 0.05$, Mann Whitney $U$ test) from the parameters observed in the presence of $Mg^{2+}$ alone. However, the $K_D$, but not the $B_{max}$ value, for [$^3$H]DPDPE in the presence of Gpp(NH)p was significantly different ($p < 0.05$, Mann Whitney $U$ test), compared with the corresponding values obtained in the presence of App(NH)p.

**Characteristics of specific [$^3$H]diprenorphine binding to medulla homogenates.** Equilibrium binding saturation studies revealed that [$^3$H]diprenorphine bound with high affinity ($K_D$ = 0.40 ± 0.06 nM, $B_{max}$ = 36.5 ± 3.1 fmol/mg of protein; three experiments) to an apparently homogeneous population of noninteracting sites (Fig. 6A).

$Mg^{2+}$ (3 mM), $Na^+$ (130 mM), and Gpp(NH)p (100 μM) plus $Mg^{2+}$ had no significant effect on specific [$^3$H]diprenorphine (1.5 nM) binding.

Competition curves for unlabeled DPDPE versus specific [$^3$H]diprenorphine binding in $Mg^{2+}$-containing buffer were monophasic (Fig. 6B). Binding data were best fitted to a single-site model, using LIGAND analysis, with a $K_i$ for DPDPE of 1.8 ± 0.2 nM (four experiments) (Fig. 6B). When competition studies were then carried out in the presence of Gpp(NH)p plus $Mg^{2+}$, LIGAND analysis again revealed a single binding site for DPDPE, but with a $K_i$ of 5.6 ± 1.6 nM (three experiments), which was significantly ($p < 0.01$, Mann Whitney $U$ test) different from the control in the absence of Gpp(NH)p.

In order to confirm that guanine nucleotides had no effect on antagonist affinity for the δ binding site, competition curves for naloxone versus specific [$^3$H]diprenorphine binding were constructed in the absence and presence of Gpp(NH)p. Under either condition, the competition curves were best fitted to a single site, and there was no change in the affinity of naloxone for the radioligand binding site. The $K_i$ values of naloxone in the absence and presence of Gpp(NH)p were 62 nM (two experiments) and 66 nM (two experiments), respectively.

**Quantitative *in vitro* autoradiography studies.** The regional distribution of total [$^3$H]DPDPE binding sites in the guinea pig kidney is shown in Fig. 7. Adjacent sections to measure nonspecific binding were treated with [$^3$H]DPDPE (7.5 nM) in the presence of 10 μM naloxone. However, the level of binding under these conditions was too low to allow for

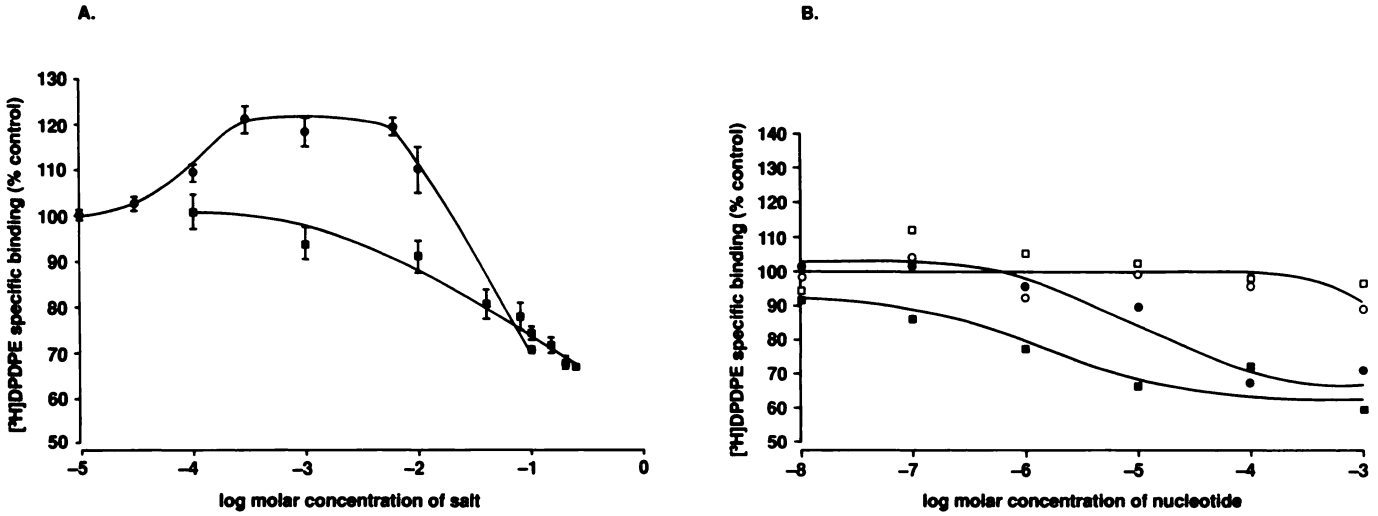


**Fig. 4.** A, Effect of increasing concentrations of $Na^+$ and $Mg^{2+}$ on the specific binding of [$^3$H]DPDPE (3.6 nM) in guinea pig kidney medulla homogenates. Specific binding of [$^3$H]DPDPE, defined with 10 $\mu$M naloxone, was determined at each salt concentration for each set of conditions. Control specific binding was that observed in 50 mM Tris·HCl buffer, in the absence of added salts. Each *point* represents the mean of three independent determinations, each performed in triplicate; *vertical lines* represent the standard error of the mean. ●, $Mg^{2+}$; ■, $Na^+$. B, Effect of increasing concentrations of nucleotides on the specific binding of [$^3$H]DPDPE (1.7 nM) in guinea pig kidney medulla homogenates in $Mg^{2+}$-containing buffer. Specific binding of [$^3$H]DPDPE, defined with 10 $\mu$M naloxone, was determined at each nucleotide concentration for each set of conditions. Control specific binding was that observed in 50 mM Tris·HCl buffer, in the absence of added nucleotides and in the presence of $Mg^{2+}$. Each *point* represents the mean of two independent determinations performed in triplicate. ○, App(NH)p; □, ATP; ●, Gpp(NH)p; ■, GTP.

TABLE 3

**Effect of $Mg^{2+}$ and nucleotides on the equilibrium binding parameters of [$^3$H]DPDPE specific binding to kidney medulla homogenates**

The $K_D$ and $B_{max}$ values were determined by LIGAND analysis, as described in Materials and Methods. Each value represents the mean ± standard error from separate analysis of between three and five individual experiments, each performed in triplicate.

| $Mg^{2+}$/ nucleotide | $K_D$ | Slope | $B_{max}$ |
|---|---|---|---|
| | *nM* | | *fmol/mg of protein* |
| None | 3.5 ± 0.4 | 0.98 ± 0.02 | 33 ± 4.3 |
| $Mg^{2+}$ | 1.6 ± 0.2 | 1.00 ± 0.03 | 33 ± 3.5 |
| $Mg^{2+}$ + App(NH)p | 1.3 ± 0.2 | 0.98 ± 0.02 | 31 ± 1.5 |
| $Mg^{2+}$ + Gpp(NH)p | 3.7 ± 0.2 | 0.99 ± 0.02 | 29 ± 0.5 |

suitable photographic reproduction. Radial distribution of discrete specific binding sites was apparent in both the outer and inner medulla. The $K_D$ value for [$^3$H]DPDPE was determined separately in the outer and inner medullary regions, by using quantitative autoradiography. In both regions, specific binding of [$^3$H]DPDPE was saturable, whereas nonspecific binding was linear with increasing concentrations of the radioligand. The $K_D$ values for the radioligand in the inner and outer regions of the medulla were 4.53 ± 0.79 nM ($n_H$ = 0.87 ± 0.02) and 5.01 ± 0.82 nM ($n_H$ = 0.82 ± 0.04), respectively, whereas the $B_{max}$ values were 221 ± 38 amol/mm$^2$ and 190 ± 45 amol/mm$^2$, respectively (three experiments).

In the cortex of the guinea pig kidney, specific [$^3$H]DPDPE binding sites were restricted to a narrow band along the corticomedullary junction, while being absent in the rest of the cortical region. The saturation binding parameters for [$^3$H]DPDPE in the corticomedullary region were similar to those found in the medulla ($K_D$ = 4.8 ± 0.8 nM, $B_{max}$ = 180 ± 30 amol/mm$^2$).

Autoradiography performed on slide-mounted sections of the

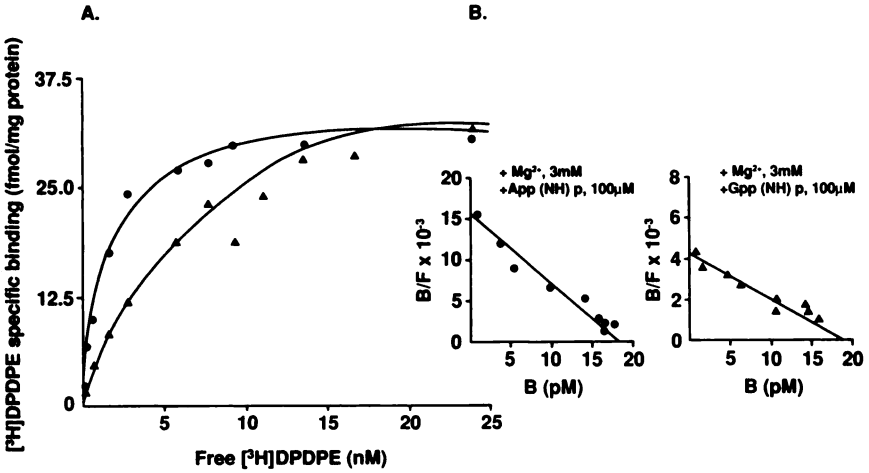


**Fig. 5.** A, Saturation isotherms of [$^3$H]DPDPE specific binding to guinea pig kidney medulla homogenates in the presence of 100 $\mu$M App(NH)p (●) or Gpp(NH)p (▲), with $Mg^{2+}$ present in the buffer. B, Scatchard transformation of the corresponding specific binding data. *B*, bound; *B/F*, bound/free. Nonspecific binding of [$^3$H]DPDPE, defined with 10 $\mu$M naloxone, was linear over the selected concentration range. *Points* shown are from single representative experiments performed in triplicate. Similar results were obtained in two or three additional experiments. Corresponding binding parameters for [$^3$H]DPDPE are listed in the text.

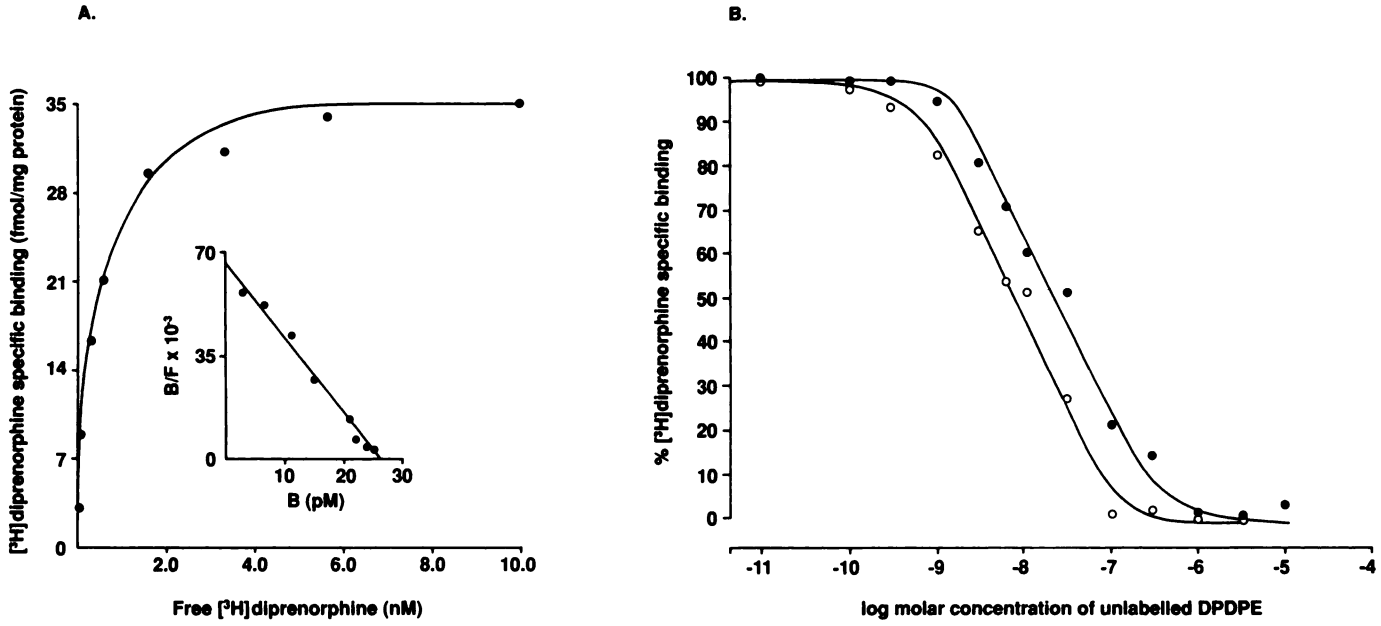


**Fig. 6.** A, Saturation isotherm for [$^3$H]diprenorphine specific binding to guinea pig kidney medulla homogenates. *Inset*, Scatchard transformation of specific binding data. *B*, bound; *B/F*, bound/free. Nonspecific binding of [$^3$H]diprenorphine, defined with 10 $\mu$M naloxone, was linear over the selected concentration range. *Points* shown are from a single representative experiment performed in triplicate. Similar results were obtained in two additional experiments. Corresponding equilibrium binding parameters for [$^3$H]diprenorphine are detailed in the text. B, Competition with [$^3$H]diprenorphine (1.4 nM) specific binding to kidney medulla homogenates by unlabeled DPDPE, in $Mg^{2+}$-containing buffer, in the absence (○) or presence (●) of 100 $\mu$M Gpp(NH)p. Nonspecific binding of [$^3$H]diprenorphine was defined with 10 $\mu$M naloxone. *Points* shown are from single representative experiments performed in triplicate. Similar results were obtained in two or three additional experiments. Data for both curves were best fitted to a single-site model. Corresponding inhibition constants are listed in the text.

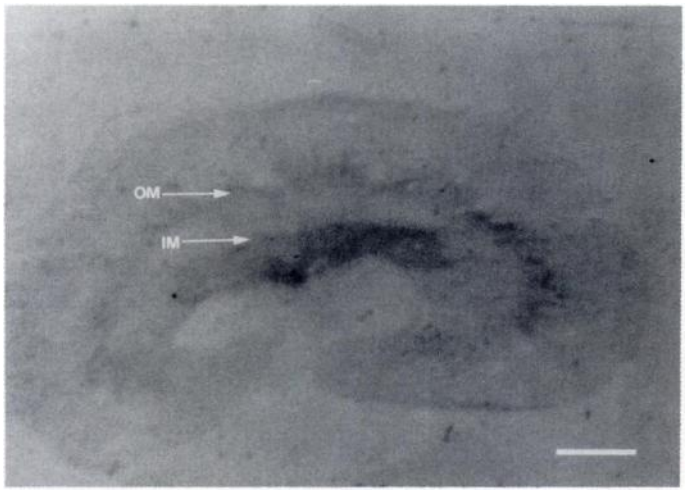


**Fig. 7.** Computer-assisted image enhancement of the autoradiographical localization of total [$^3$H]DPDPE (7.5 nM) binding sites in a longitudinal section of the guinea pig kidney. [$^3$H]DPDPE specific binding was observed in two areas, i.e., in the inner medulla (*IM*) and outer medulla (*OM*) of the kidney. Autoradiography experiments were performed as detailed in Materials and Methods. *Scale bar*, 2.5 mm.

guinea pig kidney, using [$^3$H]DAGOL and [$^3$H]U69593 saturation studies, demonstrated an absence of specific binding at all radioligand concentrations, thereby confirming the results of the homogenate study showing that $\mu$ and $\kappa$ binding sites were not present in the guinea pig kidney.

A lack of specific radioligand binding was revealed when [$^3$H]U69593, [$^3$H]DAGOL, and [$^3$H]DPDPE were used to investigate $\kappa$-, $\mu$-, and $\delta$-opioid binding sites, respectively, in slide-mounted sections of the rat kidney.

## Discussion

Radioligand binding studies performed with highly selective $\mu$ ([$^3$H]DAGOL), $\delta$ ([$^3$H]DPDPE), and $\kappa$ ([$^3$H]U69593) opioid ligands in rat kidney homogenates clearly indicated a lack of detectable opioid binding sites in either the cortex or medulla. Autoradiographical studies, used to detect opioid sites that may be discretely localized in the kidney and that may not be detected by homogenate assays, further confirmed the findings described above.

A previous radioligand binding study performed on rat kidney sections described high affinity specific binding sites for the nonselective $\kappa$ agonists [$^3$H]EKC and [$^3$H]etorphine, under conditions that suppressed binding to $\mu$ and $\delta$ sites (15). It could be argued that Quirion *et al.* (15) detected a subtype of the $\kappa$ receptor, because nonselective benzomorphans such as EKC, but not arylacetamides such as U69593, U50488, or PD117302, have been shown to recognize these sites ($\kappa_2$ sites) with high nanomolar affinity in central nervous tissue (33). In addition, it has also been shown that the $\kappa_2$ site is relatively insensitive to dynorphin (33–35). In the rat kidney, however, the affinity of dynorphin for the [$^3$H]EKC sites appears to be of the same order of magnitude as the affinities of the oripavines (etorphine) and benzomorphans (EKC and bremazocine) (15). In this respect, the [$^3$H]EKC binding sites are more likely to be $\kappa_1$ sites, which should have been detected by [$^3$H]U69593 in these experiments. A reasonable explanation cannot, therefore, be given at present to explain the differences observed in the two studies. However, an absence of $\kappa$ sites in the rat kidney is consistent with functional studies that have recently shown that the diuretic effect of $\kappa$ agonists can be completely abolished through adrenal demedullation (36).

In addition to studies in the rat, binding studies performed

on both homogenates and slide-mounted sections of the guinea pig kidney revealed an absence of $\mu$ and $\kappa$ sites. Conversely, both binding techniques established the presence of specific [$^3$H]DPDPE binding sites in the guinea pig kidney.

On separation of the guinea pig kidney into its cortical and medullary components, specific high affinity binding sites for [$^3$H]DPDPE were demonstrated in homogenates of both regions. The characteristics of these sites were consistent with those of the $\delta$ receptor, in that DPDPE, bremazocine, and DADLE, all $\delta$-active compounds, displayed high affinity, whereas the $\mu$- and $\kappa$-selective ligands DAGOL and U69593, respectively, showed low affinity for specific [$^3$H]DPDPE binding sites. In addition, the potency of naloxone against [$^3$H]DPDPE was similar to values reported for the naloxone affinity ($K_e$, 40–80 nM) for the $\delta$ receptor in pharmacological bioassay studies (37–39).

Autoradiography studies confirmed the presence of specific [$^3$H]DPDPE binding sites in the guinea pig kidney. Two distinct regions of radially arranged binding sites were visualized in the inner and outer medulla. In the cortex, the [$^3$H]DPDPE binding sites were localized in a narrow region near the corticomedullary junction, in continuity with the sites observed in the outer medulla. These sites may represent $\delta$ receptors on collecting ducts, renal tubules, or vascular tissue; however, further studies are required to localize them to discrete structures in the kidney. The $K_D$ values for [$^3$H]DPDPE determined by Quantimet image analysis were in good agreement with those obtained from homogenate studies in Tris·HCl buffer in the absence of added salts.

It would appear that the small population of $\delta$-opioid binding sites characterized in cortical homogenates corresponds to the sites visualized in the corticomedullary region. Although autoradiography studies suggest that the receptor densities in the medulla and cortex are approximately equal, the lower $B_{max}$ observed in cortical homogenates may be due to a dilution effect as a result of localization of binding sites in a discrete area of the cortex.

In guinea pig kidney medulla homogenates, the observed increase in specific binding of [$^3$H]DPDPE in the presence of $Mg^{2+}$ (up to a concentration of 10 mM $Mg^{2+}$) and the reduction in the presence of $Na^+$ (up to a concentration of 200 mM) are consistent with effects of cations on opioid binding and, furthermore, with results reported previously for [$^3$H][Leu$^5$]enkephalin binding to $\delta$ receptors in NG 108-15 cells (16). Both these effects were agonist specific because $Mg^{2+}$ (3 mM) and $Na^+$ (130 nM) had no effect on specific [$^3$H]diprenorphine binding.

Direct $^3$H-agonist binding studies showed that the presence of 3 mM $Mg^{2+}$ caused a 2-fold increase in the affinity of [$^3$H]DPDPE, without affecting the maximum binding capacity. Such sensitivity to $Mg^{2+}$ may be related to the receptor being linked to a second messenger through a G protein or to the presence of multiple affinity states of the receptor, with the presence of $Mg^{2+}$ stabilizing the agonist-bound receptor-G protein high affinity complex (40, 41).

The observation that the guanine nucleotides GTP and Gpp(NH)p reduced specific [$^3$H]DPDPE binding, whereas the adenine nucleotides had no such effect, and, furthermore, the observation that Gpp(NH)p had no influence on specific [$^3$H]diprenorphine binding demonstrated the selectivity of guanine nucleotides for modulating $\delta$ agonist binding in the medulla.

When a comparison was made, by saturation analysis, of the effect of App(NH)p and Gpp(NH)p on specific [$^3$H]DPDPE binding, with $Mg^{2+}$ present in the buffer, the 3-fold shift from a single high affinity state to another single state of lower affinity supported the idea that a G protein was involved in the receptor activation process. Activation of the G protein by guanine nucleotide analogues causes a dissociation of the receptor-G protein complex, resulting in a decrease in receptor affinity for the agonist. On the basis of these results, it appears that the effects of Gpp(NH)p on $^3$H-agonist affinity would not be apparent if $Mg^{2+}$ were excluded from the incubation medium, because $Mg^{2+}$ is required to promote the formation of the high affinity receptor-G protein complex.

The 3-fold shift to lower affinity of DPDPE, but not of naloxone, observed at the [$^3$H]diprenorphine (antagonist) binding site when Gpp(NH)p was present in the incubation medium again confirmed the agonist-specific guanine nucleotide effect. Because antagonists do not participate in postreceptor mechanisms, changes in antagonist affinity at the binding site were not observed in the presence of Gpp(NH)p.

In conclusion, radioligand binding studies performed with homogenates and autoradiographic studies both have revealed the presence of a homogeneous $\delta$-opioid receptor population in the guinea pig kidney. The effects of $Mg^{2+}$, $Na^+$, and guanine nucleotides on specific [$^3$H]DPDPE and [$^3$H]diprenorphine binding in the medulla suggest the involvement of a G protein in the action of $\delta$ agonists in the medulla. However, the nature of the second messenger system associated with the receptor-G protein complex needs to be determined.

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