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# Studies on Kinetics of [$^3$H]$\beta$-Funaltrexamine Binding to $\mu$ Opioid Receptor

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**SUMMARY**

$\beta$-Funaltrexamine ($\beta$-FNA) was shown to be a reversible $\kappa$ agonist and an irreversible $\mu$ antagonist. [$^3$H]$\beta$-FNA at low concentrations (<10 nM) covalently binds to $\mu$ but not $\delta$ or $\kappa$ opioid receptors in brain membranes. The interaction between $\beta$-FNA and $\mu$ opioid receptors was thought to involve two steps; a reversible ligand-receptor complex is formed before the formation of an irreversible complex, based on observations in bioassays *in vitro*. In this study, we investigated whether such a two-step process occurred in binding using bovine striatal membranes and determined the kinetic parameters by examining the time courses of both reversible and irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors. Specific binding was defined as the difference between binding in the presence of levorphanol and dextrorphan (1 $\mu$M). Reversible binding was determined as the difference between membrane (reversible and irreversible) binding and irreversible binding. At 25°, the rate of formation of irreversible [$^3$H]$\beta$-FNA-receptor complex increased as the concentration increased and reached a plateau at 2 nM; further increase in [$^3$H] $\beta$-FNA concentration did not enhance the rate of formation, indicating that the rate saturation effect exists for irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors. At 10° and low concentrations (<1 nM) of [$^3$H]$\beta$-FNA, appreciable reversible binding to opioid receptors occurred before any irreversible [$^3$H]$\beta$-FNA-receptor complex could be detected. These observations support the notion that reversible binding occurs before alkylation of the receptor. The binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors was thus modeled to allow for such a two-step process:

$$D + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} DR \xrightarrow{k_2} DR^*$$

A mathematical analysis method was derived to allow determination of all kinetic parameters ($k_{+1}$, $k_{-1}$, $k_2$, and $K_d$) of such a two-step reaction. Values of $k_2$, $k_{+1}$, $k_{-1}$, and $K_d$ were determined at 10° for 0.5, 0.25, and 0.125 nM [$^3$H]$\beta$-FNA and were found to be very similar among these three concentrations. Raising the incubation temperature from 10° to 37° greatly enhanced the values of $k_{+1}$, $k_{-1}$, and $k_2$ without affecting $K_d$. At 37° incubation without 200 mM NaCl significantly decreased the values of $k_{+1}$, $k_{-1}$, and $k_2$ without affecting $K_d$. NaCl increased the irreversible binding, probably by shifting the equilibrium towards a conformation that binds more easily with $\beta$-FNA. Under all conditions examined, the value of $k_{-1}$ was found to be at least 5-fold greater than $k_2$, indicating that the majority of the reversible complex dissociates and only a small portion proceeds to form irreversible complex. This finding is consistent with published observations that only a portion of $\beta$-FNA binding to $\mu$ opioid receptors is irreversible. In conclusion, [$^3$H]$\beta$-FNA binds reversibly to $\mu$ opioid receptors before forming covalent bonding. The kinetic parameters were determined from time course experiments, which may be useful in detecting subtle changes in receptors. This mathematical analysis method should be generally applicable to any irreversible ligand that undergoes such a two-step reaction, provided that it interacts with a homogeneous population of receptors.

$\beta$-FNA, the fumaramate methyl ester of naltrexamine, was synthesized by Portoghese *et al.* (1) and found to have reversible $\kappa$ agonist and irreversible $\mu$ antagonist activities in *in vitro* preparations of the guinea pig ileum (1–4) and the mouse vas deferens (3–5) and in *in vivo* antinociceptive tests (6). Binding of $\beta$-FNA to opioid receptors in brain membranes has been characterized. Pretreatment of membranes with high concentrations of $\beta$-FNA (>0.1 $\mu$M) reduced $\mu$ binding with or without changing $\delta$ binding (3, 7–11). Pretreatment of guinea pig brain membranes with low concentrations (1–5 nM) of $\beta$-FNA followed by extensive washing reduced $\mu$ opioid receptor binding without affecting $\delta$ or $\kappa$ receptor binding (12). The reduction of $\mu$ binding by $\beta$-FNA pretreatment appeared to be due to a reduction in $B_{max}$ without a change in the $K_d$ of [$^3$H]DAGO and this effect was more pronounced in the presence than in the absence of 100 mM NaCl(12). It was also shown (13) that [$^3$H]

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**ABBREVIATIONS:** FNA, funaltrexamine; DAGO, [D-Ala$^2$, *N*-Me-Phe$^4$, Gly$^5$-ol]-enkephalin; EGTA, ethylene glycol-bis($\beta$-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TCA, trichloroacetic acid; DADLE, [D-Ala$^2$-D-Leu$^5$]-enkephalin; TEL buffer, Tris·HCL·buffer containing 1 mM EGTA and 10 $\mu$M leupeptin, concentration refers to that of Tris·HCL buffer.

$\beta$-FNA (5 nM), in the presence of 100 mM NaCl, covalently labeled the binding site subunit of the $\mu$ opioid receptor with high specificity in bovine striatal membranes. The high selectivity and irreversible nature of its action make $\beta$-FNA a very useful pharmacological tool both *in vivo* and *in vitro* (for a review, see Ref. 14).

When a chemical affinity ligand covalently binds to a receptor, there are two most probable mechanisms; (a) a bimolecular reaction occurs between the ligand and the receptor or (b) the ligand first binds to the receptor to form a reversible ligand-receptor complex followed by the formation of a covalent bond. It was thought that the second mechanism was involved in the interaction between the $\mu$ opioid receptor and $\beta$-FNA based on some observations (14–16). The 6$\alpha$-isomer of $\beta$-FNA ($\alpha$-FNA) acts reversibly at the $\mu$ opioid receptor and can protect the receptor against irreversible blockade by $\beta$-FNA (15). Whereas the *trans* form (in the double bond of fumaramate) can irreversibly block the $\mu$ opioid receptor, the *cis* form acts only as a reversible ligand (15). When X-ray crystal structures of $\alpha$-FNA and $\beta$-FNA were superimposed, it was found that the fumaramate double bond of $\alpha$-FNA was more than 2 Å away from that in $\beta$-FNA and in the wrong orientation for nucleophilic attack to take place (16). Thus, proper alignment of the electrophilic group in $\beta$-FNA (fumaramate) with the nucleophile in the receptor (sulfhydryl group) is essential in the covalent bond formation, indicating that reversible binding has to occur before the formation of an irreversible complex. Some evidence from binding studies also supports this notion. $\beta$-FNA irreversibly binds to $\mu$ receptors at concentrations close to its $K_i$ (12, 13). Opioid compounds added before [$^3$H]$\beta$-FNA can completely inhibit specific binding of [$^3$H]$\beta$-FNA, whereas those added 1 hr after [$^3$H]$\beta$-FNA can displace only 65–75% of its specific binding (12). All these are results obtained in *in vitro* bioassay and binding studies. In order to gain more insights into the mechanism of the reaction between $\beta$-FNA and the $\mu$ opioid receptor, kinetic studies of [$^3$H]$\beta$-FNA binding to the $\mu$ opioid receptor are needed. The two mechanisms can be distinguished kinetically because in the first case the rate of the irreversible reaction is linearly proportional to the concentration of the ligand, whereas in the second case, where an initial receptor-ligand complex is formed, the linear relationship between the rate of the reaction and the ligand concentration exists only when the ligand concentration is below or equal to the concentration that saturates the receptor. Once the ligand saturates the receptor, further increase in its concentration would not increase the rate of irreversible binding. This phenomenon has been described in enzymology as a "rate saturation effect" (17). At 37°, the irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors follows apparent first-order kinetics (13). The observation of apparent first-order kinetics does not readily differentiate these two mechanisms, because in most experiments the concentration of the ligand greatly exceeds that of the receptor. To differentiate these two mechanisms, experiments were performed to examine whether this rate saturation effect was present. The data we obtained indicated that such a two-step process did occur and we, therefore, modeled the reaction as follows.

$$D + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} DR \xrightarrow{k_2} DR^*$$

A mathematical analysis method was derived according to this model, and experiments were performed to determine all three kinetic parameters ($k_{+1}$, $k_{-1}$, and $k_2$). The equilibrium dissociation constant ($K_d$) of the reversible receptor-ligand interaction, which cannot be determined by Scatchard analysis due to its partially irreversible nature, can thus be derived ($k_{-1}/k_{+1}$).

## Experimental Procedures

**Materials.** [$^3$H]$\beta$-FNA (specific activity, 21.8 Ci/mmol) was supplied by the National Institute on Drug Abuse. Levorphanol and dextrorphan were obtained from Hoffman-La Roche Co. (Nutley, NJ). [$^3$H]DAGO (specific activity, 30.3 Ci/mmol) was purchased from DuPont-NEN Research Products (Boston, MA); naloxone from Research Biochemicals, Inc. (Wayland, MA); leupeptin and EGTA from Sigma Chemical Co.; TCA and GF/B filters from Fisher Co.; and Liquiscint from National Diagnostics Co. (Manville, NJ).

**Bovine striatal membrane preparation.** The procedures for membrane preparation were carried out at 0–4°. Fresh bovine brains were obtained from a slaughterhouse shortly after animals were sacrificed. Striata were dissected out, cleaned of surrounding tissues and white matter, and frozen on dry ice immediately. Membranes were prepared according to the method described previously (13, 18). Briefly, tissues were homogenized in 10 volumes of 0.32 M sucrose in 10 mM Tris·HCl buffer (pH 7.5) containing 10 mM glucose, 1 mM EGTA, and 10 $\mu$M leupeptin and were centrifuged at 920 × $g$ for 10 min. The pellet was homogenized and centrifuged again. The combined supernatant was centrifuged at 40,000 × $g$ for 20 min to yield the crude membrane fraction ($P_2$). $P_2$ membrane was swollen twice in 5 mM Tris·HCl buffer containing 1 mM EGTA and 10 $\mu$M leupeptin (pH 7.5), homogenized to disrupt synaptosomes, and centrifuged at 40,000 × $g$ for 20 min. The top white $P_2$ membrane pellet was brought up in 5 volumes (of the original weight) of 50 mM Tris·HCl buffer containing 1 mM EGTA and 10 $\mu$M leupeptin (TEL buffer, pH 7.5) and aliquots of membrane were stored at −70° until use.

**Time course of binding of [$^3$H]$\beta$-FNA to opioid receptors: membrane, irreversible, and reversible binding.** Bovine striatal membranes, at approximately 1 mg of protein/ml of 50 mM TEL buffer containing 200 mM NaCl, were incubated with levorphanol or dextrorphan (final concentration, 1 $\mu$M) for 30 min at 10° or 10 min at 25° or 37°. Membranes were then incubated with [$^3$H]$\beta$-FNA in 50 mM TEL buffer, in the presence of 200 mM NaCl, at 10°, 25°, or 37°. In some specified experiments, NaCl was not included in the incubation buffer. At various intervals, samples were taken for determination of membrane, irreversible, and reversible binding.

For irreversible binding, two 1-ml samples were taken into microfuge tubes containing 250 $\mu$l of 50% TCA (final 10%). Irreversible binding was determined according to a modification of the method of Liu-Chen and Phillips (13). TCA-precipitated membranes were centrifuged at 2000 × $g$ for 20 min at 4°, and the supernatant was aspirated. The precipitate was washed with 0.3 ml of water by sonication, precipitated again with 1.0 ml of 15% TCA, allowed to remain on ice for at least 10 min, filtered with GF/B filters under reduced pressure. The filters were washed once with 5 ml of ice-cold 10% TCA and then twice with 5 ml of water. Filters are then placed in 7-ml scintillation vials and 5 ml of Liquiscint were added and shaken for at least 2 hr before counting.

For membrane binding, two 1-ml samples were filtered immediately over GF/B filters under vacuum, followed by three washes each with 5 ml of ice-cold 50 mM Tris·HCl buffer (pH 7.5). Radioactivity on the filters was determined by liquid scintillation counting.

Specific reversible binding was determined as the difference between specific membrane binding and specific irreversible binding.

For each condition, the experiment was performed at least three times. Variations of duplicates from the mean were less than 10%. Data presented in Tables 1, 2, and 3 represented mean ± standard error of at least three independent experiments, whereas those in Figs. 1, 2,

and 3 were results from one experiment. Counting efficiency of the $\beta$-counter was approximately 55%.

**Dissociation of [$^3$H]$\beta$-FNA-opioid receptors complex.** Bovine striatal membranes, at approximately 1 mg of protein/ml in 50 mM TEL buffer containing 200 mM NaCl, were incubated with [$^3$H]$\beta$-FNA at 10° for 45 min or 37° for 10 min. Naloxone was then added to a final concentration of 10 $\mu$M. At various intervals after the addition of naloxone, samples were taken for the determination of membrane, irreversible, and reversible binding, as described above.

**Protein determination.** Protein contents of membrane preparations were determined by the method of Lowry *et al.* (19), using bovine serum albumin as the standard.

**Mathematical analysis.** The binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors is modeled to allow for an initially reversible process followed by passage to an irreversible complex according to the scheme:

$$D + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} DR \xrightarrow{k_2} DR^* \quad (1)$$

where $D$ = drug, $R$ = receptor, $DR$ = reversible complex of drug and receptor, $DR^*$ = irreversible complex of drug and receptor, $k_{+1}$ = association rate constant, $k_{-1}$ = dissociation rate constant, and $k_2$ = rate constant of formation of irreversible ligand-receptor complex.

The reversible component, $DR$ in amount $X$, is at steady state

$$X_{ss} = \frac{D \cdot R_T}{D + K_m} \quad (2)$$

where

$$K_m = \frac{k_{-1} + k_2}{k_{+1}} \quad (3)$$

The irreversible complex, $DR^*$ in amount $Y$, increases linearly with time, given by

$$Y = \frac{k_2 \cdot D \cdot R_T}{D + K_m} \cdot t \quad (4)$$

Thus,

$$Y = k_2 \cdot X_{ss} \cdot t \quad (5)$$

and $k_2$ can, thus, be obtained from the slope of the time-dependent plot from

$$k_2 = \frac{\text{slope}}{K_{ss}} \quad (6)$$

Therefore, $k_2$ and $K_m$ can be determined from a single set of time courses of irreversible binding and the plateau of the reversible binding. From the time course of reversible binding, the apparent association constant ($K_{app}$) can be obtained according to the method of Williams and Lefkowitz (20), in which

$$K_{app} = k_{+1} \cdot [D] + k_{-1} \quad (7)$$

By solving Eqs. 3 and 7, both $k_{+1}$ and $k_{-1}$ can be determined. The equilibrium dissociation constant ($K_d$) can be calculated according to

$$K_d = k_{-1}/k_{+1} \quad (8)$$

Alternatively, for a reaction that proceeds as outlined in Eq. 1, a kinetic method was developed for the evaluation of the dissociation constant ($K_d$) of the reversible receptor-ligand complex and of the first-order rate constant ($k_2$) for the irreversible interaction between the receptor and the irreversible ligand (21–23). The equation to describe such a process is shown below, where [$D$] is the ligand concentration and $K_{obs}$ is the observed first-order rate constant for irreversible binding.

$$\frac{1}{K_{obs}} = \frac{K_d}{k_2} \frac{1}{[D]} + \frac{1}{k_2}$$

Thus, if the time courses of irreversible binding of the receptor are performed at various ligand concentrations, a plot of $1/K_{obs}$ versus $1/[D]$ should give a straight line, of which the intercept is $1/k_2$ and the slope is $K_d/k_2$.

## Results

**Time course of membrane binding, irreversible binding, and reversible binding of [$^3$H]$\beta$-FNA to opioid receptors.** Figs. 1, 2, and 3 represent time courses of membrane, irreversible, and specific binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors. These three figures were derived from the same experiment. At 10° and 0.5 nM [$^3$H]$\beta$-FNA, the specific membrane binding to opioid receptor increased quickly (Figs. 1 and 3), whereas the irreversible binding occurred much more slowly (Figs. 2 and 3). Reversible specific binding reached a plateau at 40 min (Fig. 3). When the amount of irreversible binding was plotted against time, the slope of the regression line is the rate of complex formation, as shown in Fig. 2, *inset*. Using the data on reversible specific binding, one can calculate the apparent association rate constant ($K_{app}$) according to the method of Williams and Lefkowitz (20) (Fig. 3, *inset*). Qualitatively, the higher the temperature, the sooner the reversible specific binding reached the plateau and the higher the rate of formation of irreversible complex. Increasing [$^3$H]$\beta$-FNA concentration had the same effect. At 10°, appreciable reversible binding to opioid receptor occurred before any irreversible [$^3$H]$\beta$-FNA-receptor complex could be detected. The length of this lag period depended on the concentration of [$^3$H]$\beta$-FNA and temperature. The higher the concentration, the shorter the lag period. At 25° and 37°, no lag period was observed for as low as 0.25 nM [$^3$H]$\beta$-FNA. The observation that such a lag period exists supports the notion that reversible binding occurs before the formation of irreversible complex.

**Effect of [$^3$H]$\beta$-FNA concentration on the rate of formation of irreversible [$^3$H]$\beta$-FNA-receptor complex.** The relationship between [$^3$H]$\beta$-FNA concentration and the rate of formation of irreversible [$^3$H]$\beta$-FNA-receptor complex at 25° is shown in Fig. 4. At 0.5–2 nM, the rate increased with [$^3$H]$\beta$-FNA concentration; however, at 4 nM, the rate of formation was the same as that at 2 nM. This finding indicates that the rate saturation effect does exist for irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors.

**Determination of the number of $\mu$ receptors in bovine striatal membranes.** Scatchard analysis of saturation experiments using [$^3$H]DAGO showed that there appeared to be a homogeneous population of binding sites with $K_d$ of 1.36 ± 0.06 nM (three experiments) and $B_{max}$ of 141.1 ± 8.2 fmol/mg of protein (three experiments). Generally, we used 0.8–0.9 mg of protein/assay tube in a volume of 1 ml, which represents the opioid receptor concentration of approximately 120 pM.

**Determination of $k_{+1}$, $k_{-1}$, $k_2$, and $K_d$ at 10° at three concentrations.** All these parameters were calculated from data obtained experimentally, as outlined in Mathematical Analysis. Results are summarized in Table 1. These results indicate that $k_2$ values do not differ significantly among different concentrations at the same temperature. Similarly, $k_{+1}$, $k_{-1}$, and, thus, $K_d$ are not different among these three concentrations.

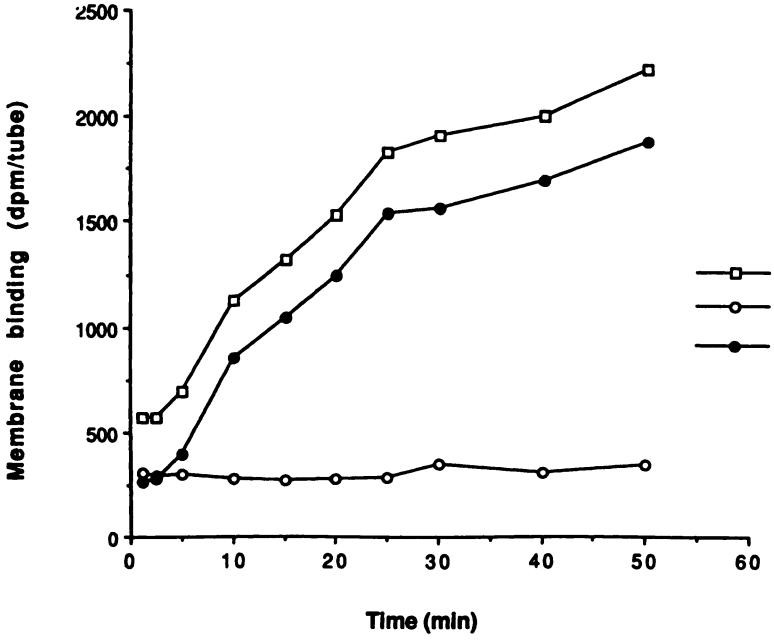


**Fig. 1.** Time course of membrane binding of 0.5 nM [$^3$H] $\beta$-FNA to $\mu$ opioid receptors in bovine striatal membranes at 10°. *TB*, *NSB*, and *SB*, total, nonspecific, and specific binding, respectively. Protein contents in each tube were determined to be 0.87 mg. Data presented in Figs. 1, 2, and 3 were derived from one single incubation mixture. This experiment was performed three times.

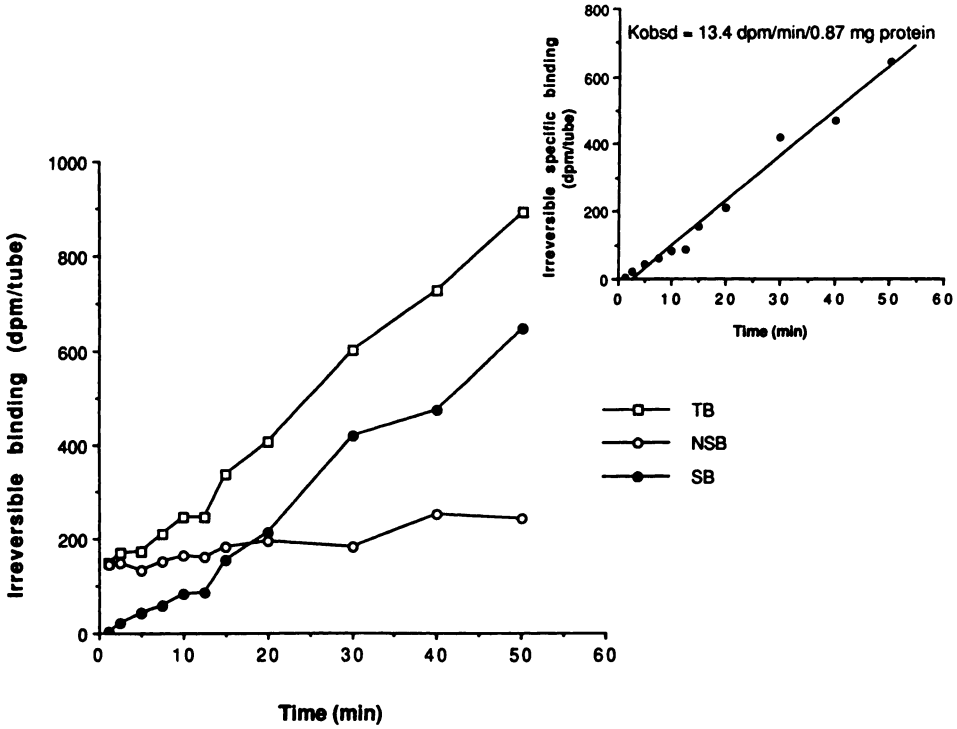


**Fig. 2.** Time course of irreversible binding of 0.5 nM [$^3$H]$\beta$-FNA to $\mu$ opioid receptors in bovine striatal membranes at 10°. *TB*, *NSB*, and *SB*, total, nonspecific, and specific binding, respectively. When the specific irreversible binding was plotted against time, the slope of the regression line represented the rate of irreversible ligand-receptor complex formation (*inset*). This experiment was performed three times.

**Effect of temperature and NaCl on $k_{+1}$, $k_{-1}$, $k_2$, and $K_d$.** When the binding was carried out with 0.5 nM [$^3$H]$\beta$-FNA at 37°, the values of $k_{+1}$, $k_{-1}$, and $k_2$ increased significantly from those at 10° ($p < 0.01$, when compared with 0.5 nM at 10°, by Student's $t$ test) (Table 2). $K_d$ remained unchanged when the temperature was raised from 10° to 37° (Table 2). When, at 37°, NaCl was eliminated from incubation buffer, the values of $k_{+1}$, $k_{-1}$, and $k_2$ were significantly less than those obtained with NaCl included in the incubation mixture ($p < 0.01$ by Student's $t$ test) (Table 3). Yet, $K_d$ remained unchanged under this incubation condition (Table 3).

**Calculation of $K_d$ and $k_{+2}$ based on the method of Kitz and Wilson (21).** The transformation, according to the method of Kitz and Wilson (21), of data obtained at 10° for four different concentrations is shown in Fig. 5. $K_d$ was calculated to be 1.72 nM and $k_{+2}$ was 0.0102/min.

**Determination of $k_{-1}$ from dissociation experiments.** An example of the dissociation experiments is shown in Fig. 6. Dissociation rate constants for the reversible [$^3$H]$\beta$-FNA-receptor complex were calculated to be 0.079 ± 0.016 min$^{-1}$ (three experiments) for 10° and 0.711 ± 0.080 min$^{-1}$ (three experiments) for 37°.

## Discussion

In this report, we demonstrated that the rate saturation effect existed for irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid recep-

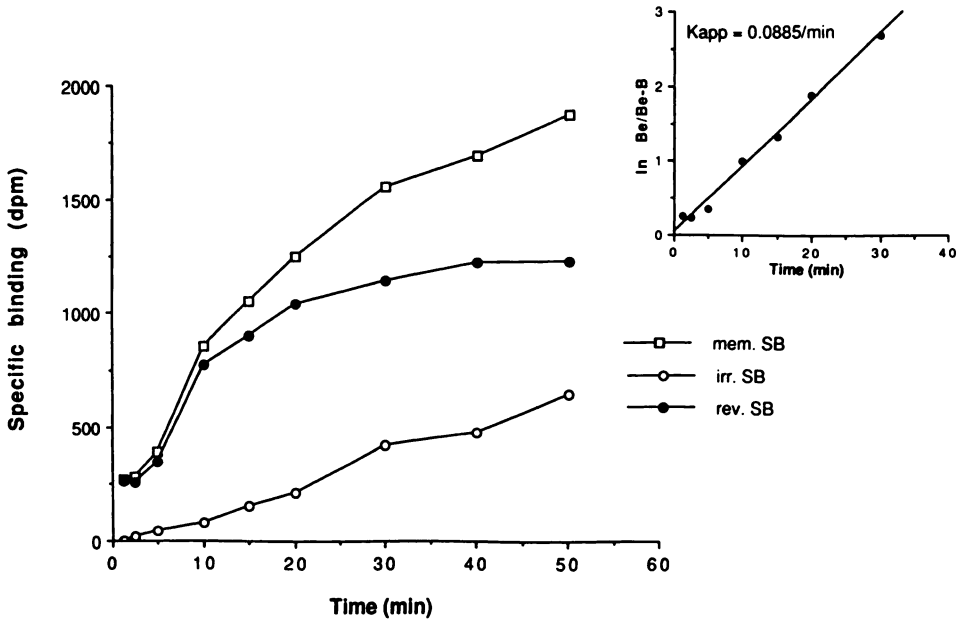


**Fig. 3.** Time course of membrane (*mem.*), reversible (*rev.*), and irreversible (*irr.*) specific binding of 0.5 nM [$^3$H]$\beta$-FNA to $\mu$ opioid receptors in bovine striatal membranes at 10°. Reversible specific binding was calculated as the difference between membrane and irreversible specific binding. When the data on specific reversible binding were transformed according to the method of Williams and Lefkowitz (20) and plotted against time, the slope of the regression line represented the apparent association rate constant ($K_{app}$) (*inset*). This experiment was performed three times.

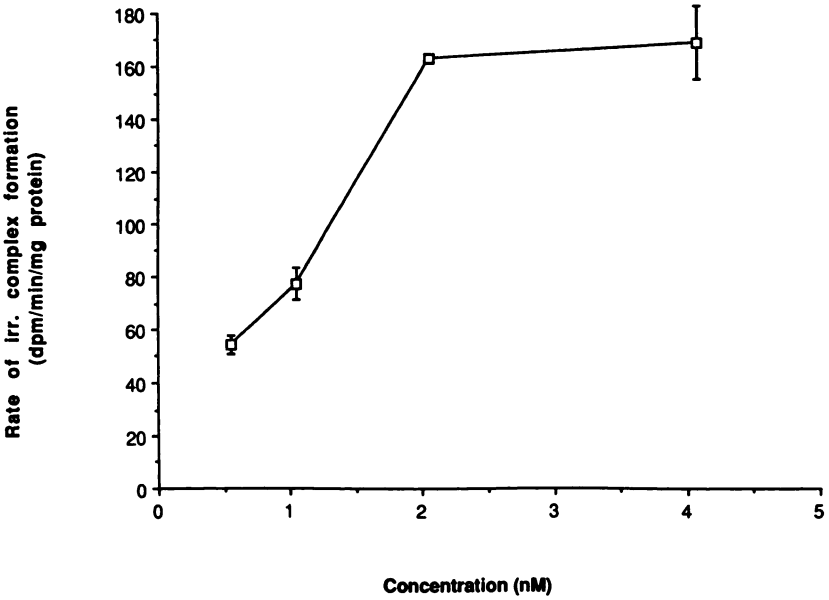


**Fig. 4.** The relationship between the observed rate of formation of irreversible complex and [$^3$H]$\beta$-FNA concentration at 25°. Note that the observed rate reached a plateau at 2 nM. Each *point* represented the mean ± standard error of three (2 and 4 nM) of four (0.5 and 1 nM) determinations.

TABLE 1

**Values of $k_{+1}$, $k_{-1}$, $k_2$, and $K_d$ at three concentrations of [$^3$H]$\beta$-FNA at 10°**

Data are expressed as mean ± standard error. Numbers in parentheses are numbers of determinations.

| | 0.125 nM | 0.25 nM | 0.5 nM |
|---|---|---|---|
| $k_{+1}$ (nM$^{-1}$·min$^{-1}$) | 0.047 ± 0.005 (3) | 0.052 ± 0.004 (3) | 0.043 ± 0.004 (4) |
| $k_{-1}$ (min$^{-1}$) | 0.082 ± 0.012 (3) | 0.080 ± 0.009 (3) | 0.064 ± 0.006 (4) |
| $k_2$ (min$^{-1}$) | 0.012 ± 0.001 (3) | 0.013 ± 0.001 (3) | 0.010 ± 0.001 (4) |
| $K_d$ (nM) | 1.76 ± 0.09 (3) | 1.54 ± 0.08 (3) | 1.50 ± 0.12 (4) |

tors and that at 10° and concentrations less than 1 nM there was a lag period between the onset of reversible binding and the onset of irreversible binding. This is the first kinetic evidence from a binding study to support the concept that reversible ligand-receptor binding occurs before alkylation of the receptor takes place. This finding is in agreement with those of Sayre *et al.* (15) and Griffin *et al.* (16). The binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors was modeled as a two-step process (Eq. 1). A mathematical analysis method was developed to determine experimentally the kinetic parameters ($k_{+1}$, $k_{-1}$, and $k_2$) of both reversible and irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors. These parameters are found to be independent of ligand concentration to a certain extent, whereas raising the temperature or including NaCl in incubation buffer significantly increased not only the rate constants of reversible association and dissociation but also the rate constant of irreversible complex formation. Under all conditions examined, $k_{-1}$ is always greater than $k_2$ by a factor of at least 5, indicating that the majority of the reversible complex dissociates and only a small portion proceeds to form irreversible complex. This

TABLE 2

**Values of $k_{+1}$, $k_{-1}$, $k_2$, and $K_d$ at 0.5 nM [$^3$H]$\beta$-FNA at 10° and 37°**

Data are expressed as mean ± standard error. Numbers in parentheses are numbers of determinations.

| | 10° | 37° |
|---|---|---|
| $k_{+1}$ (nM$^{-1}$·min$^{-1}$) | 0.043 ± 0.004 (4)* | 0.427 ± 0.058 (3)* |
| $k_{-1}$ (min$^{-1}$) | 0.064 ± 0.006 (4)* | 0.686 ± 0.096 (3)* |
| $k_2$ (min$^{-1}$) | 0.010 ± 0.001 (4)* | 0.041 ± 0.001 (3)* |
| $K_d$ (nM) | 1.50 ± 0.12 (4) | 1.63 ± 0.17 (3) |

* $P < 0.01$ by Student's $t$ test for differences between 10° and 37°.

TABLE 3

**Values of $k_{+1}$, $k_{-1}$, $k_2$, and $K_d$ at 0.5 nM [$^3$H]$\beta$-FNA at 37°: effect of NaCl**

Data are expressed as mean ± standard error. Numbers in parentheses are numbers of determinations.

| | TEL buffer only | TEL buffer + 200 mM NaCl |
|---|---|---|
| $k_{+1}$ (nM$^{-1}$·min$^{-1}$) | 0.088 ± 0.010 (4)* | 0.427 ± 0.058 (3)* |
| $k_{-1}$ (min$^{-1}$) | 0.170 ± 0.017 (4)* | 0.686 ± 0.096 (3)* |
| $k_2$ (min$^{-1}$) | 0.008 ± 0.001 (4)* | 0.041 ± 0.001 (3)* |
| $K_d$ (nM) | 1.96 ± 0.17 (4) | 1.63 ± 0.17 (3) |

* $P < 0.01$ by Student's $t$ test for differences between incubations with and without NaCl.

study represents the first attempt to delineate kinetically the interaction of both reversible and irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors.

$K_d$ and $k_2$ obtained based on the method of Kitz and Wilson (21) and Schaeffer *et al.* (22) are in good agreement with the values obtained using the method presented in this report. Their method allows one to determine $K_d$ and $k_2$ from the time course of irreversible binding of at least three different concentrations of the irreversible ligand (inhibitor). The present method allows one to determine association and dissociation rate constants of the reversible reaction, in addition to $K_d$ and $k_2$, from time courses of both reversible and irreversible binding of a single concentration of the irreversible ligand. In the case of [$^3$H]$\beta$-FNA, it is more informative to know all four parameters than just $K_d$ and $k_2$. To our knowledge, this is the first method to calculate $k_{+1}$, $k_{-1}$, $k_2$, and $K_d$ of an irreversible ligand undergoing such a two-step reaction to bind irreversibly to the receptor. The underlying assumption is that it binds to a homogeneous population of receptors. We, therefore, chose to use [$^3$H]$\beta$-FNA at concentrations of no more than 0.5 nM for reversible binding and no more than 4 nM for irreversible binding to ensure that binding only occurred with $\mu$ opioid receptors, and not with $\delta$ or $\kappa$ receptor (see below).

The values of $k_{-1}$ calculated as outlined in Mathematical Analysis were 0.064 ± 0.006 min$^{-1}$ (four experiments) for 10° and 0.686 ± 0.096 min$^{-1}$ (three experiments) for 37°. The values determined experimentally were 0.079 ± 0.016 min$^{-1}$ (three experiments) for 10° and 0.711 ± 0.080 min$^{-1}$ (three experiments) for 37°. At both temperatures, the values were very similar, thus further validating this analysis method.

There is a good agreement in the literature that $\beta$-FNA binds reversibly, but not irreversibly, to $\kappa$ opioid receptors; yet, whether it binds irreversibly to $\mu$ and/or $\delta$ opioid receptors has been controversial (3, 7–13, 24, 25). At the concentration of levorphanol and dextrorphan used (1 $\mu$M), levorphanol completely inhibits specific irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptors, whereas dextrorphan has no effect (13). We believe that, under the incubation conditions used in this study, we examined the irreversible binding of [$^3$H]$\beta$-FNA to only $\mu$ opioid receptors for the following reasons. First, previously it was reported that pretreatment of brain membranes with $\beta$-FNA at concentrations less than 10 nM followed by extensive washing decreased $\mu$ opioid receptor binding but not $\delta$ binding. This reduction in $\mu$ binding appeared to be due to a reduction in $B_{max}$ without a change in $K_d$ when binding was performed with [$^3$H]DAGO (12). Second, the concentrations of [$^3$H]$\beta$-FNA used in irreversible binding were so low (the highest being 4 nM) that its occupancy at $\delta$ opioid receptors was negligible. The $K_i$ values of $\beta$-FNA have been determined previously to be 2.2, 14, and 78 nM for $\mu$, $\kappa$, and $\delta$ opioid receptors, respectively (12). At 4 nM, the receptor occupancy at $\delta$ opioid receptors is only approximately 5%; thus, the irreversible binding to $\delta$

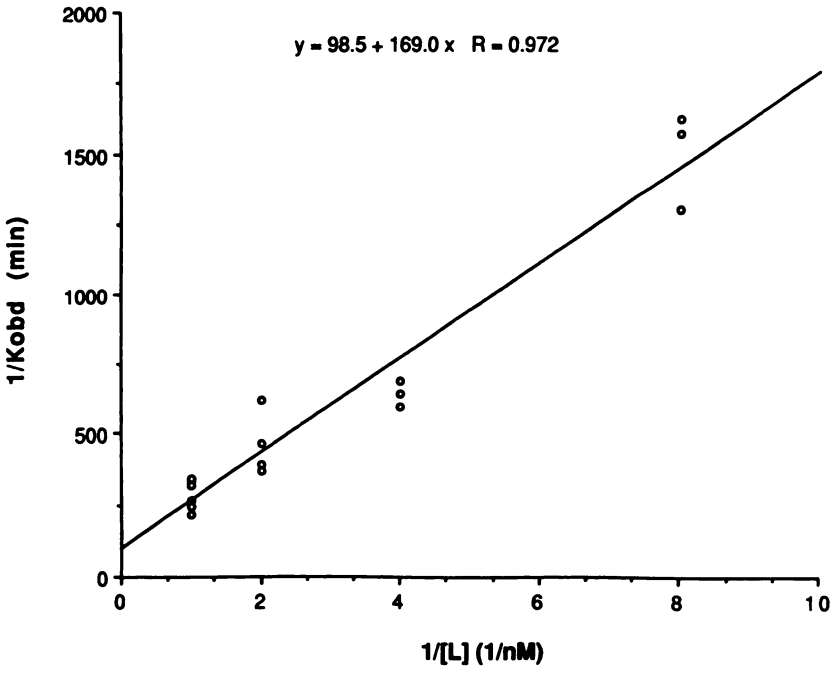


**Fig. 5.** Calculation of $k_2$ and $K_d$ at 10° according to the method of Kitz and Wilson (21). Data on irreversible specific binding of four concentrations of [$^3$H]$\beta$-FNA (0.125, 0.25, 0.5, and 1 nM) to opioid receptors were transformed (see Mathematical Analysis) and plotted. Values of $k_2$ and $K_d$ were determined to be 0.0102/min and 1.72 nM, respectively.

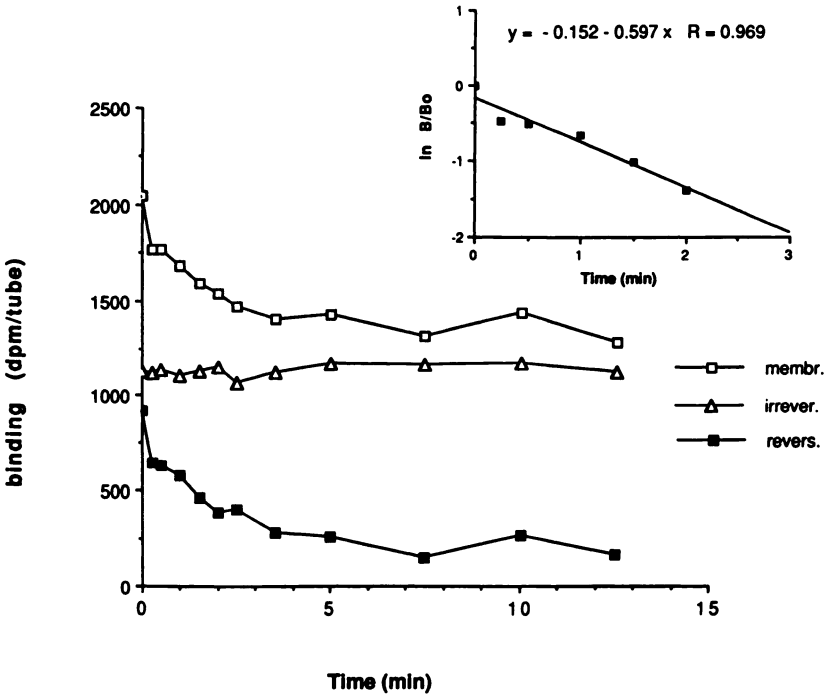


**Fig. 6.** Time course of dissociation of reversible [³H]β-FNA-receptor complex. Membranes were incubated with 0.5 nM [³H]β-FNA at 37° for 10 min and then 10 μM naloxone was added (time 0). Membrane binding, irreversible binding, and reversible binding were determined at various time points as described in Experimental Procedures. The dissociation rate constant was determined to be 0.597/min (*inset*). Each tube contained 0.85 mg of protein. This experiment was performed three times.

receptors, if any, is minimal. Third, specific irreversible binding of 5 nM [³H]β-FNA to opioid receptors was inhibited potently by sufentanil, a μ-selective ligand, yet not at all by ICI174,864, a selective δ antagonist (13). Last, autoradiography of irreversible binding of [³H]β-FNA to opioid receptors was recently examined in rat brain sections. It was found that [³H]β-FNA (5 or 10 nM) labeled a population of opioid receptors of which the distribution was very similar to that of [³H]DAGO (26). We conclude, therefore, that in this study we examined the irreversible binding of β-FNA to μ receptors. However, we are aware of the findings of Rothman *et al.* (9), who demonstrated that β-FNA (1 μM) treatment followed by extensive washing resulted in a 60% decrease in $B_{max}$ and no change in $K_d$ of the lower affinity [³H]DADLE binding site (termed $\delta_{cx}$ site), whereas it induced no change in $B_{max}$ and a 2-fold increase in $K_d$ of the [³H]DAGO binding site (μ receptor). Similar results were obtained, following a 20-nmol β-FNA intracerebroventricular injection, for the lower affinity [³H]DADLE binding site, yet this treatment produced no change in either $K_d$ or $B_{max}$ for the [³H]DAGO binding site (9). Along the same line, intracerebroventricular injection of 2.5 μg of β-FNA produced a small reduction in $B_{max}$ of [³H]DAGO binding only in thalamus but no change in caudate and central gray (25). The reason for this discrepancy is not quite clear. For the *in vitro* membrane binding study, perhaps differences in both incubation condition and source of tissues may account for it.

As to reversible binding, the data were obtained from concentrations of [³H]β-FNA no more than 0.5 nM, to minimize the interaction with δ and κ types of opioid receptors. Previously it was reported that, at 1.5 nM or less, β-FNA inhibited only μ opioid receptor binding and not that of δ or κ receptors. At 2 nM, β-FNA started to have an inhibitory effect on κ binding, yet it did not inhibit δ receptor binding until the concentration was equal to or more than 10 nM (12). In the study mentioned above (12), κ receptor binding was carried out in guinea pig brain membranes with 1 nM [³H]ethylketocyclazocine, in the presence of 20 nM sufentanil and 500 nM DADLE to block μ and δ binding, respectively. Recently, we performed the same experiment in bovine striatal membranes (the tissue that was used in this study) and found that β-FNA at 1.0 nM or less did not inhibit κ binding. We, thus, conclude that at 0.5 nM or less [³H]β-FNA binds to μ opioid receptors only and there is no significant binding to δ or κ receptors.

It was demonstrated that including NaCl in the incubation buffer greatly enhanced irreversible binding of [³H]β-FNA to μ opioid receptors (10, 12, 13). In this study, we found that NaCl enhanced formation of the reversible receptor-ligand complex as well as the irreversible complex. This finding further supports the notion that NaCl shifts the equilibrium toward a certain conformation that is more conducive to the binding of an antagonist (in this case [³H]β-FNA) and also to the formation of a covalent bond between the receptor and [³H]β-FNA.

It was shown previously that specific irreversible binding of [³H]β-FNA to opioid receptors at 37° reached a plateau at 5 nM after a 90-min incubation and further increases in [³H]β-FNA concentration or incubation time did not increase specific binding. At the plateau level, only approximately 50% of the μ opioid receptor population were labeled (13). It was puzzling at the time why 20 nM [³H]β-FNA, a concentration 10 times its $K_i$, labeled only 50% of the μ receptor population. The determination of $k_{-1}$ and $k_2$ allows an explanation of such an observation. Because $k_{-1}$ is at least 5-fold greater than $k_2$ under all conditions examined, the majority of reversible receptor-ligand complex formed dissociates and only a small percentage proceeds to form an irreversible complex, presumably due to energy required for formation of a covalent bond. This finding can also account for the finding that at 37°, while the reversible binding of 0.5 nM [³H]β-FNA reached an apparent plateau in 10 min or less, the irreversible binding did not reach a plateau at the end of a 50-min incubation. The findings in this report are in accordance with the report of Recht and Pasternak (11), who found that, under their incubation condition (25°, 30-min

incubation, no NaCl), a 10-fold higher concentration of [$^3$H]$\beta$-FNA was needed for irreversible binding than that used in competitive binding assays. Because the value of $k_{+1}$ is very small in the absence of NaCl, the formation of reversible complex is very slow. Within 30 min, only a small amount of reversible complex is formed. Most of reversible complex formed dissociates, and only a small portion proceeds to form a covalent bond. In the absence of NaCl, the value of $k_2$ is very small and thus, a higher concentration of $\beta$-FNA is required to make the reaction proceed to irreversible complex.

In this study, [$^3$H]$\beta$-FNA bound irreversibly to $\mu$ opioid receptors at a concentration as low as 0.125 nM. This observation is contrary to that of Recht and Pasternak (11). The discrepancy is mainly due to differences in incubation conditions, as discussed above. In some studies (7–11), 1 $\mu$M $\beta$-FNA was used. At this concentration, $\beta$-FNA would bind irreversibly not only to $\mu$, but also to $\delta$ receptors (3, 10, 12, 27). Recent autoradiography results of [$^3$H]$\beta$-FNA irreversible binding to opioid receptors indicated that its specificity for $\mu$ opioid receptor only occurred at concentrations below 10 nM (in the presence of NaCl); at concentrations above 10 nM, the specific binding to $\mu$ receptors was obscured by its extremely high nonspecific binding (26). Thus the specificity of $\beta$-FNA for $\mu$ opioid receptor is concentration dependent.

Recently, Takahashi *et al.* (28) demonstrated that in slices of striatum and midbrain of morphine-tolerant and -dependent rats there was a significantly higher rate of irreversible binding of [$^3$H]$\beta$-FNA to $\mu$ opioid receptor, when compared with control rats. In calculating the rate constant of [$^3$H]$\beta$-FNA-receptor irreversible complex formation, they did not take into account the existence of the reversible receptor-ligand complex, although they did propose such a two-step reaction. The higher rate of irreversible complex formation is most likely due to an increase in $k_{+1}$ and/or a decrease in $k_{-1}$, as they have discussed. An increase in $k_2$ cannot be excluded, however. The term association rate constant in their paper (they termed it $k_{+1}$) is a function of $k_{+1}$, $k_{-1}$, and $k_2$.

In conclusion, in this report, we provided evidence from binding studies to support the notion that reversible $\beta$-FNA-$\mu$ opioid receptor binding occurs before the formation of a covalent bond. A mathematical analysis method was derived to determine kinetic parameters of both reversible and irreversible binding of $\beta$-FNA to the opioid receptor. This method should be generally applicable to any irreversible ligand that undergoes such a two-step reaction to bind covalently to the receptor. $k_{-1}$ was at least 5-fold greater than $k_2$ under all conditions examined and this may account for the finding that only a fraction of $\mu$ opioid receptors were labeled even when a concentration 10 times its $K_i$ was used. Raising the temperature or including NaCl in the incubation buffer enhanced the irreversible binding mainly by increasing $k_2$.

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