Pharmacokinetic-Pharmacodynamic Analysis of the EEG Effect of Alfentanil in Rats Following β-Funaltrexamine-Induced μ-Opioid Receptor "Knockdown" *In Vivo*

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Purpose. The objective of this investigation was to determine the influence of pre-treatment with the irreversible μ-opioid receptor antagonist β-funaltrexamine (β-FNA) on the pharmacokinetic-pharmacodynamic (PK/PD) relationship of alfentanil in rats.

Methods. The PK/PD correlation of alfentanil (2 $mg.kg^{-1}$ intravenously in 20 min) was determined in chronically instrumented rats using amplitudes in the 0.5–4.5 Hz frequency band of the EEG as pharmacodynamic endpoint. β-FNA was administered intravenously (10 $mg.kg^{-1}$) either 35 min or 24 h prior to the PK/PD experiments.

Results. Pre-treatment with β-FNA had no influence on the pharmacokinetics of alfentanil. The *in vivo* concentration-EEG effect relationships, however, were steeper and shifted towards higher concentrations with no difference between the 35-min and the 24-h pre-treatment groups. Analysis of the data on basis of the operational model agonism revealed that the observed changes could be explained by a 70–80% reduction in alfentanil efficacy in β-FNA pre-treated rats. This is consistent with results from an *in vitro* receptor bioassay showing a 40–60% reduction in the number of specific μ-opioid binding sites in the brain.

Conclusions. This investigation confirms the validity of a previously postulated mechanism-based PK/PD model for the effect of synthetic opiates in rats.

KEY WORDS: alfentanil; β-funaltrexamine; μ opioid receptors; operational model of agonism; pharmacokinetic-pharmacodynamic modeling.

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ABBREVIATIONS: ANOVA, analysis of variance; β-FNA, β-funaltrexamine; BSA, bovine serum albumin; 95%CI, 95% confidence interval; Cl, clearance; CV, coefficient of variation; E/[A], concentration-effect; EEG, electroencephalogram; MRT, mean residence time; PK/PD, pharmacokinetic/pharmacodynamic; RIA, radioimmunoassay; $t_{1/2,\beta}$, elimination half life; V_{SS}, volume of distribution at steady-state.

INTRODUCTION

Recently, a number of integrated pharmacokinetic/pharmacodynamic (PK/PD) models have been proposed for characterisation of functional adaptation to the effects of opiates. Some of these models describe tolerance development on basis of distribution to a hypothetical tolerance compartment (1). Others postulate a physiological counter-regulatory mechanism to characterise functional adaptation (2). A limitation of these models is that they are in empiric nature and that they are not based on receptor regulation as the mechanism of functional adaptation. It is generally believed that changes in the expression and function of μ-opioid receptors play an important role in the development of functional tolerance both *in vitro* (4) and *in vivo* (3). Recently, the functional role of the μ-opioid receptor in the pharmacological actions of morphine *in vivo* has been convincingly demonstrated in knockout mice (5).

In a previous investigation we have proposed a mechanism-based pharmacokinetic-pharmacodynamic (PK/PD) model for the effects of synthetic opiates on the brain (6). By a combination of *in vivo* and *in vitro* technologies it was shown that the *in vivo* concentration-EEG effect relationships could be explained by the operational model of agonism according to Black and Leff (7), on the assumption of a considerable receptor reserve. The mechanism-based PK/PD model has subsequently been applied to examine functional adaptation to the EEG effects of synthetic opiates in a quantitative manner. This study showed that the observed loss of potency of alfentanil upon repeated administration could be explained by a 50% loss of functional μ-opioid receptors (8).

The objective of the present investigation was to obtain experimental validation of the proposed mechanism-based PK/PD model for synthetic opiates. To this end the role of receptor reserve as a determinant of the pharmacodynamics of alfentanil *in vivo* was examined by pre-treatment with the irreversible μ-opioid receptor antagonist β-funaltrexamine (β-FNA) (9). Radioligand binding studies were performed in parallel to allow for an integration of *in vitro* receptor binding characteristics with the *in vivo* pharmacology.

METHODS

Animals and Surgery

Male Wistar rats with a body weight between 250–350 g were used in the experiments. Rats were housed individually in plastic cages at 21°C, normal light-dark cycle (8:00 a.m.–8:00 p.m.) with food (Standard Laboratory Rat Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. One week before the experiment, seven cortical EEG electrodes were implanted under fentanyl/droperidol anaesthesia (Hypnorm® Janssen Pharmaceutica BV, Beerse, Belgium) as described before (10). Two days before the experiment, four permanent cannulas were implanted, two in the left jugular vein and one in the femoral vein for administration of alfentanil and β-FNA, midazolam and vecuronium, respectively, and one in the femoral artery for blood sample collection. The protocol of the study was approved by the Committee on Animal Experimentation of Leiden University.

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Pharmacokinetic-Pharmacodynamic Experiments

Rats were pre-treated with an intravenous infusion of 10 $mg.kg^{-1}$ of β-FNA or saline for 2 min at a constant rate of 200 $\mu l.min^{-1}$. Following a washout period of 35 min or 24 h the pharmacokinetic-pharmacodynamic correlation of alfentanil was determined. Briefly, alfentanil (2 $mg.kg^{-1}$) was administered via an intravenous infusion over 20 min at a constant rate of 20 $\mu l.min^{-1}$. During periods of alfentanil-induced muscle rigidity and respiratory depression the animals received repeated intravenous bolus injections of vecuronium bromide (0.15 mg) and were artificially ventilated with air. Adequacy of artificial ventilation was ascertained by monitoring of the arterial pO_2, pCO_2 and pH. During and after the infusion 18 arterial blood samples (20–50 μl) were collected for determination of the concentration-time profile of alfentanil. In addition three blood samples (100 μl) were collected for determination of the midazolam concentration. From 20 min prior to until 140 min after the administration of alfentanil the EEG was continuously recorded and subjected to off-line fast Fourier analysis for quantification. Amplitude in the 0.5–4.5 Hz frequency band of the EEG was selected as pharmacodynamic endpoint. During the entire experiment a continuous infusion of midazolam (5.5 $mg.kg^{-1}.h^{-1}$) was given to prevent alfentanil-induced seizure activity.

Pharmacokinetics of β-Funaltrexamine

The pharmacokinetics of β-FNA were determined in a separate experiment. Two days before the experiment, animals were implanted with a cannula in the femoral artery for blood sampling and another one in the jugular vein for drug administration. β-FNA was administered by an intravenous infusion of 10 mg kg^{-1} in 2 min and twelve blood samples (100–200 μl) were collected at regular intervals. Each blood sample was haemolysed immediately with 400 μl of deionized water and stored at −20°C until HPLC assay (see below).

Radioligand Binding

The effect of β-FNA pre-treatment radioligand receptor binding was also determined. Rats were pre-treated with 10 mg kg^{-1} β-FNA or saline and brains were removed after a 35 min or 24 h washout period and stored in nitrogen at −80°C. Brains (minus cerebellum) were homogenised in 10 volumes 50 mM Tris-HCl buffer (pH = 7.4) at 25°C and stored at −80°C. The suspension was centrifuged at 48.000 g for 20 min and the pellet was washed three times with buffer. Aliquots of 100 μl were incubated in duplicate for 30 min with 0.3–15 nM [^{3}H]naloxone (New England Nuclear-719, specific activity 57.7 Ci $mmol^{-1}$, New England Nuclear, Boston MA, U.S.A.). Samples were then filtered through a pre-soaked Whatman GF/B glass fibre filter and eluted six times with 3 ml Tris-HCl buffer of 4°C under reduced pressure. The filters were submerged in 3.5 ml of Packard Ultima Gold scintillation fluid (Packard, Merden, CT, U.S.A.) and radioactivity was measured using a Packard Tri-Carb 1500 liquid scintillation analyser (Packard, Downers Grove, IL, U.S.A). Non-specific binding of [^{3}H]naloxone was determined in the presence of 100 μM fentanyl. Displacement of a fixed concentration (2.5 nM, $\sim K_D$) of [^{3}H]naloxone by alfentanil was determined in the presence and absence of 100 mM NaCl. Protein concentration in the homogenates was determined using the Pierce Micro BCA assay (Pierce, Rockford, IL, U.S.A.).

Drug Assay Alfentanil

The concentrations of alfentanil in blood were determined by the radioimmonoasay (RIA) method described by Cox *et al.* (6). Under the actual experimental conditions the binding ability (B_0) was ∼30% and the limit of detection was 10 ng ml^{-1}. The intra- and interassay variability were less than 5%.

Drug Assay β-Funaltrexamine

The β-FNA concentrations were determined by HPLC with electrochemical detection at 800 mV. Haemolysed blood samples were mixed with the internal standard, naloxone, and 200 μl borate buffer (1M, pH = 9) and subsequently extracted with 3 ml ethylacetate. After centrifugation the organic layer was evaporated at 30°C and the residue was dissolved in 100 μl mobile phase (mixture of phosphate buffer (0. 05M, pH = 5.5) and acetonitrile 78:22 v/v) of which 50 μl was injected into the chromatographic system consisting of an Applied Biosystems pump, an Antech electrochemical detector and a Shimadzu C-R3A Chromatopac integrator. Chromatography was performed on a C18 ODS ultrasphere column (25 cm × 4.6 mm i.d., and 5 μm) and a precolumn Alltech Guard column C18, flow rate 1ml min^{-1}). The retention times for β-FNA and naloxone were 6.1 and 4.5 min, respectively. The limit of detection for β-FNA was 20 ng ml^{-1} and a linear calibration curve was obtained in the concentration range of 20–500 ng ml^{-1}. The intra- and inter-assay coefficients of variation were 5.9 and 3.3%, respectively, and the extraction efficiency was 78%.

Drug Assay Midazolam

The blood concentrations of midazolam were determined by HPLC with ultraviolet detection as described before (10). The intra-and interassay variation was less than 6%.

Data Analysis

Standard pharmacokinetic compartmental analysis was performed by fitting the blood concentration-time profiles to a biexponential equation (6) by use of the WinNonlin Pro software package (version 1.5, Pharsight, Mountain View, CA, U.S.A.). From the fit, the pharmacokinetic descriptors, clearance (Cl), volume of distribution at steady-state (V_{SS}), mean residence time (MRT) and elimination half life ($t_{1/2,\beta}$) were calculated. From each individual time-EEG effect profile (n = 5–6 per treatment group), 50 data points were sampled at regular intervals between the start of the infusion and the time of the last blood-concentration measurement. The individual estimates of the pharmacokinetic model parameters were then used to calculate alfentanil blood concentrations at the times of the EEG sampling. Individual alfentanil E/[A] curves were then fitted simultaneously to the Hill equation:

$$E = E_0 + \frac{(\alpha - E_0) \cdot [A]^{nH}}{EC_{50}^{nH} + [A]^{nH}} \quad (1)$$

to obtain estimates of the lower asymptote (E_0), upper asymptote

(α), midpoint location (EC_{50}) and midpoint slope parameter (Hill slope, n_H). The fits were performed by use of the non-linear mixed effect modeling software package, NONMEM (11) utilising a method which takes into account interindividual variability in the model parameters (12). An IBM-compatible personal computer (Pentium^R 133 MHz) running under Windows 95 and Visual-NM 2.2.2 (RDPP, Montpellier, France) was used with the Microsoft FORTRAN PowerStation 4.0 compiler and NONMEM version IV, level 2.0 (double precision; NONMEM project group, University of California, San Francisco, U.S.A.). Parameters and associated S.E.'s were estimated using the so-called first-order method and additive intraindividual and multiplicative interindividual residual error models were assumed (11–12). Estimates of the interindividual variability were expressed as coefficient of variation (CV). Individual parameter estimates for each subject were calculated using the first-order Bayesian estimation method implemented in the NONMEM software.

The E/[A] data were also analysed on the basis of the operational model of agonism (7):

$$E = E_0 + \frac{(E_m - E_0) \cdot \tau^n \cdot [A]^n}{(K_A + [A])^n + \tau^n \cdot [A]^n} \quad (2)$$

where E_m is the maximum effect achievable in the system, K_A is the agonist dissociation equilibrium constant, n is the slope index for the occupancy-effect relationship and τ is the efficacy parameter, which is defined by the ratio of total receptor concentration and the concentration of agonist-receptor complex required to produce half-maximal effect. The potency parameter in the Hill-equation (EC_{50}) can be expressed in terms of the operational model of agonism as follows (7):

$$EC_{50} = \frac{K_A}{(2 + \tau^n)^{1/n} - 1} \quad (3)$$

Inspection of Equation (3) shows that $EC_{50}/K_A \rightarrow 1/(2^{1/n}-1)$ when $\tau \rightarrow 0$ (Fig. 1). Furthermore, with high efficacy values (reflected in high n's) Equation (3) approximates to a simple linear relationship, $EC_{50}/K_A = 1/\tau$, regardless of the value of n (Fig. 1) (13).

The receptor binding characteristics of the radioligand (L), [^{3}H]naloxone, were determined by fitting the following equation to the specific-binding data from the saturation experiments using the GraphPad Prism software package version 2.0 (GraphPad, San Diego, CA, U.S.A.):

$$B = \frac{B_{max} \cdot [L]}{K_d + [L]} \quad (4)$$

where B is the concentration of bound ligand, B_{max} is the total number of specific binding sites and K_d is the ligand dissociation equilibrium constant. Individual alfentanil (A) competition curves were fitted to the following equation:

$$B = \frac{B_0 \cdot [A]}{IC_{50} + [A]} \quad (5)$$

where B_0 is the total number of specific binding sites occupied by [^{3}H]naloxone in the absence of alfentanil and IC_{50} the concentration of alfentanil that inhibits 50% of the specific binding. Subsequently, the dissociation equilibrium constant (K_i) for alfentanil was calculated using the Cheng and Prusoff (14) equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}} \quad (6)$$

The effects of β-FNA pre-treatment on pharmacokinetic and radioligand-binding parameter estimates were assessed using InStat (version 3.00, GraphPad, San Diego, CA, U.S.A.) by one-way analysis of variance (ANOVA) and, in case of a significant effect, further evaluated with the Bonferroni-corrected t-test. Values of $P < 0.05$ were considered to be significant. Because the simultaneous E/[A] curve fitting analysis with NONMEM employed in this study yields population parameters rather than individual estimates, ANOVA could not be used for statistical comparison of the *in vivo* pharmacodynamic parameters of the different treatment groups. Instead, the effect of β-FNA treatment was assessed by estimating the change in the pharmacodynamic parameters compared to the control. When the 95% confidence interval (95%CI) of this estimate did not include 0, the change was considered to be significant. In the case of EC_{50}, K_d and K_i the individual estimates were transformed to logarithms before statistical analysis, since these parameters are assumed to be log-normally distributed (15).

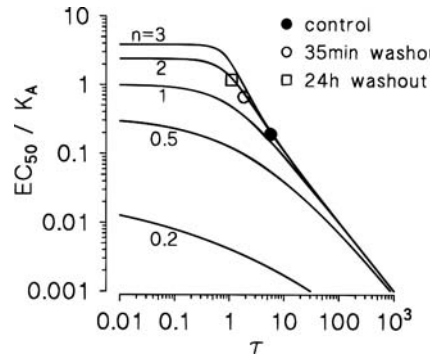

Fig. 1. Simulations of Equation (3) showing the relationship between the ratio EC_{50}/K_A and the efficacy parameter, τ, for different values of the slope parameter of the transducer function (n). The symbols represent the average estimates for alfentanil in control and β-FNA-treated rats (see text).

Compounds

Compounds were obtained from the following sources: alfentanil hydrochloride (Janssen Pharmaceutica BV, Belgium); β-funaltrexamine hydrochloride (β-FNA, Tocris Cookson, U.K.); midazolam (Roche, The Netherlands); naloxone hydrochloride (Sigma, The Netherlands); vecuronium bromide (Organon Technika BV, The Netherlands).

RESULTS

Effect of β-FNA Pre-treatment on the Pharmacokinetics and Pharmacodynamics of Alfentanil *In Vivo*

In vehicle-treated rats, alfentanil (2 mg kg^{-1} i.v. in 20 min) produced a rapid increase in the EEG effect (Fig. 2). After the infusion was stopped, the EEG effect returned to baseline values within 60–90 min. The pharmacokinetic behaviour of

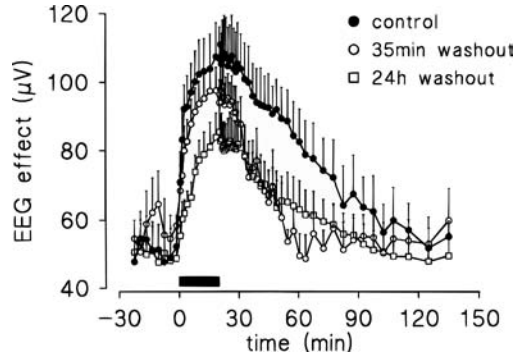

Fig. 2. Time-EEG effect profiles after intravenous administration of 2 mg kg^{-1} alfentanil in 20 min in rats pre-treated with a 2 min intravenous infusion of vehicle, 10 mg kg^{-1} β-FNA followed by 35 min washout and 10 mg kg^{-1} β-FNA followed by 24 h washout. The symbols and error bars represent EEG effects as mean ± S.E., respectively (n = 5–6). The solid bar indicates the duration of the alfentanil infusion.

alfentanil could be described adequately by a two-compartment model (Table 1) and the alfentanil concentration in blood could be related directly to the EEG effect by the Hill equation to obtain estimates of the upper asymptote, midpoint location and midpoint slope parameter (α, pEC_{50} and n_H, respectively) of the concentration-effect relationship (Table 2; Fig. 3).

Administration of β-FNA (10 mg kg^{-1} i.v. in 2 min) followed by washout had no significant effect on the average baseline EEG effect measured during 15 min before the start of the alfentanil infusion (50.4 ± 4.4, 54.2 ± 5.3 and 47.4 ± 7.2 μV for the vehicle-treated, 35 min and 24 h washout groups, respectively; $P > 0.5$). Blood concentrations of β-FNA declined very rapidly and it was not possible to obtain a complete time-concentration profile and pharmacokinetic parameter estimates in each rat. The concentration of β-FNA was always below the limit of detection (20 ng ml^{-1}) 30 min after administration (Fig. 4). Alfentanil produced a rapid increase in the EEG activity in β-FNA pre-treated rats, but the effect was markedly reduced compared to the control group (Fig. 2). The loss of *in vivo* efficacy appeared not to be due to changes in the disposition of alfentanil, since pharmacokinetic parameters were not significantly different following β-FNA treatment (Table 1). However, simultaneous fitting of the blood concentration-effect data to the Hill equation revealed that alfentanil E/[A] curves were significantly shifted to the right and steeper in β-FNA pre-treated animals compared to the control group (Table 2 and Fig. 3). The upper (α) and lower (E_0) asymptotes of alfentanil E/[A] curves following β-FNA pre-treatment appeared to be lower and higher, respectively, but these effects were not significant as tested (Table 2 and Fig. 3). However, when the maximum response was expressed as (α − E_0), a significant reduction was found following β-FNA treatment (α − E_0 = 70.6 ± 5.3, 43.5 ± 3.0 and 29.7 ± 6.4 μV for vehicle-treated, 35 min. and 24 h. washout groups, respectively).

Alfentanil produced marked respiratory depression and muscle rigidity in the control and 24 h washout groups, necessitating artificial ventilation and the administration of vecuronium, respectively. Interestingly, however, in the 35 min washout group alfentanil had no visible effect on respiration and muscle tone and blood gas values remained within physiological ranges without administration of air and vecuronium (data not shown).

Analysis of blood samples taken 10 min before and 10

Table 1. Pharmacokinetic Parameter Estimates for Alfentanil (2 mg kg^{-1} i.v. in 20 min) in Vehicle-and β-FNA-Pre-treated (10 mg kg^{-1} i.v. in 2 min) Rats

Pre-treatment	Cl (ml min^{-1} kg^{-1})	V_{SS} (l kg^{-1})	MRT (min)	$t_{1/2,\beta}$ (min)
Control	39.3 ± 2.2	0.89 ± 0.05	29.4 ± 6.6	20.3 ± 1.4
β-FNA, 35 min washout	34.8 ± 5.7	0.91 ± 0.10	26.1 ± 7.5	22.0 ± 5.4
β-FNA, 24 h washout	24.7 ± 3.8	0.81 ± 0.07	37.2 ± 6.2	30.1 ± 6.2

Note: Parameter estimates (mean ± S.E., n = 5–6) of Cl (clearance), V_{SS} (volume of distribution at steady-state), MRT (mean residence time) and $t_{1/2,\beta}$ (elimination half life) were obtained by fitting the individual time-concentration data to a standard two-compartment model as described under "Methods."

Table 2. Pharmacodynamic Parameter Estimates for the EEG Effect of Alfentanil (2 mg kg^{-1} i.v. in 20 min) in Vehicle- and β-FNA-Pre-treated (10 mg kg^{-1} i.v. in 2 min) Rats Obtained by PK/PD Analysis with the Hill Equation

Pre-treatment	E_0 (μV)	α(μV)	n_H	pEC_{50} (M)
Control	39.5 ± 6.6 (33.8%)	112.0 ± 9.3 (16.4%)	1.23 ± 0.26 (28.0%)	6.33 ± 0.18 (2.9%)
β-FNA, 35 min washout	57.0 ± 4.7 (18.4%)	101.0 ± 4.4 (9.4%)	2.75 ± 0.32* (64.1%)	5.86 ± 0.02* (3.9%)
β-FNA, 24 h washout	48.3 ± 6.8 (27.6%)	83.6 ± 9.5 (24.1%)	2.73 ± 0.19* (168.2%)	6.05 ± 0.02* (2.8%)

Note: Parameter estimates (mean ± S.E., n = 5–6) of E_0 (lower asymptote), α (upper asymptote), n_H (Hill slope parameter) and pEC_{50} (potency) were obtained by fitting the concentration EEG effect data Equation 1 with NONMEM as described under "Methods." Estimates of interindividual variability are shown as CV in parentheses.
* Significantly different from control ($P < 0.05$).

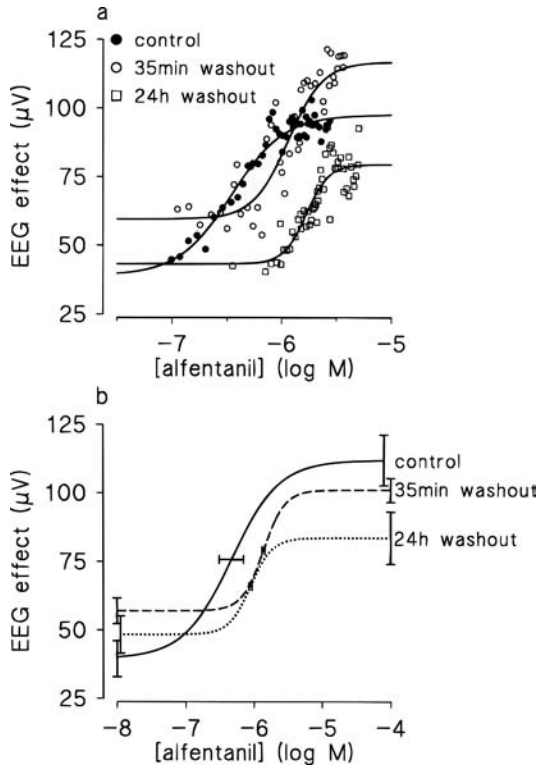

Fig. 3. *In vivo* concentration-effect relationships for the EEG effect of 2 mg kg^{-1} alfentanil administered in a 20 min intravenous infusion in vehicle- and β-FNA-treated rats. (a) Example of individual curve fits obtained in three typical rats. The lines shown superimposed on the experimental data points represent the individual fits with the Hill equation with the following NONMEM Bayes parameter estimates: E_0 = 39.1, 59.5, and 43.3 μV; α = 97.7, 116.8, and 79.5 μV; n_H = 1.81, 2.72, and 4.22; pEC_{50} = 6.46, 5.94, and 5.77 for control, 35 min and 24 h washout groups, respectively. (b) Average *in vivo* concentration-effect relationships. The curves were simulated with the Hill equation using the mean NONMEM population parameter estimates (Table 2). Vertical error bars represent S.E.'s of E_0, α and horizontal error bars represent S.E.'s of pEC_{50} (Table 2).

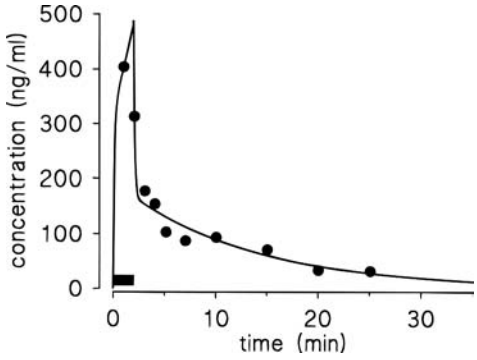

Fig. 4. Blood concentration versus time profile of β-FNA in an individual rat following intravenous administration of 10 mg kg^{-1} in 2 min. The line shown superimposed on the experimental data points represents the best fit of the data to a standard two-compartment pharmacokinetic model (Cl = 3.4 l min^{-1} kg^{-1}, V_{SS} = 34.4 l kg^{-1}, MRT = 10.1 min and $t_{1/2,\beta}$ = 8.9 min). The solid bar indicates the duration of the β-FNA infusion.

and 120 min after the start of the alfentanil infusion confirmed that midazolam concentrations were at steady-state during the time course of the experiment and no significant differences ($P > 0.05$ in all cases) were found between the three treatment groups (t = −10: 1059 ± 170, 863 ± 91 and 1161 ± 220 ng ml^{-1}; t = 10: 1332 ± 286, 870 ± 124 and 1141 ± 109 ng ml^{-1}; t = 120: 1194 ± 204, 1228 ± 263 and 1211 ± 68 ng ml^{-1} for the vehicle-treated, 35 min and 24 h washout groups, respectively).

Effect of β-FNA Pre-treatment on μ-Opioid Receptor Binding *In Vitro*

Pre-treatment with β-FNA (10 mg kg^{-1} i.v. in 2 min) produced an average reduction of ~40 and 61% in the number of specific binding sites for [^{3}H]naloxone following 35 min and 24 h washout, respectively (B_{max} = 157.8 ± 20.3, 95.1 ± 17.7 and 61.4 ± 24.8 fmol mg^{-1} protein for the vehicle-treated, 35 min and 24 h washout groups, respectively, n = 4, $P < 0.05$). β-FNA pre-treatment had no significant effect on the affinity of [^{3}H]naloxone (pK_D = 8.36 ± 0.07, 8.41 ± 0.04 and 8.49 ± 0.11 for the vehicle-treated, 35 min and 24 h washout groups, respectively, n = 4, $P > 0.50$).

The potency of alfentanil for displacing [^{3}H]]naloxone was practically identical in the three groups (pK_i = 6.80 ± 0.21, 7.01 ± 0.35 and 6.46 ± 0.05 for the vehicle-treated, 35 min and 24 h washout groups, respectively, n = 3, $P > 0.30$). Addition of 100 mM NaCl, which is believed to prevent coupling of the agonist-occupied receptor complex to G proteins (6), produced a 4-20 rightward shift of the alfentanil inhibition curve and pK_i values estimated in β-FNA pre-treated rats under these conditions were again not significantly different from the control group (pK_i = 5.60 ± 0.05, 5.69 ± 0.05 and 5.88 ± 0.09 for the vehicle-treated, 35 min and 24 h washout groups, respectively, n = 3, $P > 0.05$).

Integration of *In Vivo* and *In Vitro* Data

In theory E/[A] curves obtained before and after receptor inactivation by an irreversible antagonist can be fitted directly to the operational model of agonism (Eq. 2) to obtain estimates of agonist affinity and efficacy *in vitro* (16). Initially, we attempted to use this approach to analyse the *in vivo* alfentanil E/[A] curves but the model fitting did not converge into a unique solution. An explanation for this is that direct curve fitting can only be employed when pre-treatment with the irreversible antagonist produces a significant depression of the maximal response and eliminates the receptor reserve of the system. In the present study, upper asymptotes of the alfentanil E/[A] curves tended to be lower after β-FNA treatment, but this effect was not significant as tested and apparently was overshadowed by the between-animal variability in the model fitting. In contrast, a significant rightward shift was observed, indicative for the existence of a receptor reserve. A second reason why individual E/[A] curves could not be fitted directly to the operational model of agonism is that the significant steepening of the alfentanil curves following β-FNA treatment cannot be accounted for by Eq. (2) under the assumption of a common transducer slope (n). However, in the case of a high-efficacy system, the relationship between agonist potency and efficacy simplifies to $EC_{50}/K_A = 1/\tau$, irrespective of the value of n (Fig. 1). Therefore, for each rat the ratio of individual *in vivo*

EC_{50} estimates and average in *vitro* K_i values in the presence of NaCl (which can be assumed to represent agonist dissociation equilibrium constants uncontaminated by the expression of efficacy; (6)) was calculated (EC_{50}/K_i = 0.19 ± 0.03, 0.66 ± 0.12 and 1.18 ± 0.27 for the vehicle-treated, 35 min and 24 h washout groups, respectively, $P < 0.005$). From these ratios, it was calculated that the efficacy of alfentanil was reduced by ~68 and 81% in β-FNA treated animals (τ = 5.71 ± 0.68, 1.84 ± 0.35 and 1.10 ± 0.29 for the vehicle-treated, 35 min and 24 h washout groups, respectively; Fig. 1).

DISCUSSION

In this investigation a new approach is presented for the examination of mechanism-based pharmacokinetic-pharmacodynamic (PK/PD) models *in vivo*, which is based on an analysis of the effect of gradual "knockdown" of functional receptors by pre-treatment with an irreversible antagonist. The method was applied to a previously proposed mechanism-based model for the CNS effect of synthetic opiates in rats (6). β-Funaltrexamine (β-FNA) was used as the irreversible antagonist. Several studies have shown that this compound acts as a selective irreversible antagonist at the μ-opioid receptor both *in vivo* and *in vitro* (9,17–18). Three important lines of evidence suggest that the changes in the *in vivo* responses of alfentanil following β-FNA pre-treatment observed in the present study were indeed due to a specific, irreversible knockdown of functional μ-opioid receptors. First, it was demonstrated that the clearance of β-FNA is extremely high in rats and levels in blood were always below the detection limit after 30 min washout (Fig. 4). Therefore, it is very unlikely that the loss of effect of alfentanil was due to the presence of significant concentrations of β-FNA in the circulation during the time of the experiment. Second, it was shown that β-FNA pre-treatment had no effect on the whole blood pharmacokinetics of alfentanil (Table 1), indicating that the reduced response to alfentanil *in vivo* was solely due to a pharmacodynamic change. Finally, the radioligand binding studies revealed significant reduction in B_{max} values in β-FNA-treated animals while affinities for [^{3}H]naloxone and alfentanil, both in the absence and presence of NaCl, were unchanged, consistent with expectations for the effects of an irreversible receptor antagonist.

The analysis of the change in the E/[A] curve with β-FNA-pretreated rats with the operational model of agonism showed that the *in vivo* data can be accounted for by a ~75% reduction of the efficacy parameter, τ, which includes total receptor concentration. In the light of the rather large interindividual variability, this modeling-derived estimate of the degree of *in vivo* receptor knockdown is in good agreement with the ~55% reduction of B_{max} observed in the *in vitro* radioligand binding study.

Although the upper asymptotes of alfentanil E/[A] curves tended to be lower in β-FNA treated rats, this effect was not significant as tested (Table 2). This is in line with our hypothesis that there exists a μ-opioid receptor reserve in rat brain *in vivo*, although the slight decrease in intrinsic activity suggests that the reserve is smaller than proposed previously (6,8). Furthermore, the present observation that a 50–75% loss of μ-opioid receptors produces a ~2-3-fold reduction in potency of alfentanil is in agreement with our previous prediction that the 2-fold increase in EC_{50} observed following repeated administration of alfentanil in the same *in vivo* model can be explained by a 40% decrease in the efficacy parameter, τ(8).

Although the nature of the slope parameter remains subject of debate (19), the operational model of agonism predicts that E/[A] curves with Hill slopes greater than unity become flatter with decreasing values of τ(20). In the present study, however, β-FNA pre-treatment produced the opposite effect and alfentanil E/[A] curves became steeper (Table 2 and Fig. 3). Although further research is required to illuminate this observation, antagonist-induced curve-steepening could be indicative for receptor heterogeneity (21) and it could be speculated that the EEG effect of alfentanil is mediated via multiple receptor types which differ in their sensitivity to β-FNA. In this respect, it is of interest to note that other synthetic opioids tested previously in the EEG model (fentanyl, sufentanil, remifentanil and GR90291) yielded E/[A] curves with Hill slopes between 2–3 in control animals (8,10), similar to the values obtained for alfentanil in β-FNA-treated rats in the present study (Table 2). Very recently, three new alternatively spliced μ-opioid receptor isoforms have been identified, which might be involved with different aspects of the pharmacology of alfentanil (22).

Interestingly, in the present study (indirect) information on the rate of turn-over of μ- opioid receptors can also be inferred. The data seem to indicate that the turn-over is slow, since the 35 min. and 24h washout groups yielded similar *in vivo* and *in vitro* estimates of the degree of receptor knockdown. These data seem to be in agreement with observations by Zernig *et al.* (23,24) who investigated *in vivo* μ receptor turnover after irreversible blockade with clocinnamox and found that the half life of receptor reappearance after receptor inactivation was 6.3 and 3.2 days in rhesus monkey and mice, respectively.

Interestingly, in the present study evidence for differential effects of receptor knockdown on different pharmacodynamic endpoints was obtained. The effects on ventilation and muscle tone appeared to be identical in the control and 24 h washout groups, but were never observed in the 35 min washout group. This could suggest that the turnover of the receptors mediating respiratory depression and muscle rigidity is faster than that of those mediating EEG effects. Although the mechanisms behind this observation remain to be elucidated, it is of interest that the rat μ opioid receptor is alternatively spliced into two isoforms, which resensitize with different rates following agonist-induced desensitization (25–26). Furthermore, the μ receptors mediating discriminative stimulus effects of opioids in a pigeon model have also been reported to recover fully within one day following β-FNA pre-treatment (27).

In conclusion, we have demonstrated that integrated PK/PD analysis of the effects of an irreversible antagonist can be used to study the relationship between receptor concentration and pharmacological effect *in vivo* in a novel manner and that this may serve as a basis for the validation of mechanism-based PK/PD models. By using an irreversible antagonist, it is possible to knockdown receptors *in vivo* in a gradual and controlled manner and to monitor receptor recovery under various experimental conditions, which can provide insights in the mechanisms of receptor turnover. These two features (i.e., gradual, controlled knockdown and receptor recovery) distinguish our proposed model from models of genetically-engineered, knockout animals (5) and make it particularly useful to study phenomena that are related to dynamic changes in receptor expression, such as tolerance development

REFERENCES

1. M. Ekblom, M. Hammeralund-Udenaes, and L. K. Paalzow. Modelling of tolerance development and rebound effect during different intravenous administrations of morphine to rats. *J. Pharmacol. Exp. Ther.* **266**:244–252 (1993).
2. J. W. Mandema and D. R. Wada. Pharmacodynamic model for acute tolerance development to the electroencephalographic effects of alfentanil in the rat. *J. Pharmacol. Exp. Ther.* **275**: 1185–1194 (1995).
3. D. E. Keith, B. Anton, S. R. Murray, P. A. Zaki, P. C. Chu, D. V. Lissin, G. Monteillet-Agius, P. L. Stewart, C. J. Evans, and M. Von Zastrow. μ-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vitro* and in the mammalian brain. *Mol. Pharmacol.* **53**:377–384 (1998).
4. F. Sehba, A. Duttaroy, S. Shah, B. Chen, J. Carroll, and B. C. Yoburn. In vivo homologous regulation of μ-opioid receptor gene expression in the mouse. *Eur. J. Pharmacol.* **339**:33–41 (1997).
5. B. L. Kieffer. Opioids: first lessons from knockout mice. *Trends Pharmacol. Sci.* **20**:19–26 (1999).
6. E. H. Cox, T. Kerbusch, P. H. Van der Graaf, and M. Danhof. Pharmacokinetic-pharmacodynamic modeling of the electroencephalogram effect of synthetic opioids in the rat: correlation with the interaction at the *mu*-opioid receptor. *J. Pharmacol. Exp. Ther.* **284**:1095–1103 (1998a).
7. J. W. Black, and P. Leff. Operational models of pharmacological agonism. *Proc. R. Soc. Lond. B.* **220**:141–162 (1983).
8. E. H. Cox, J. A. Kuipers, and M. Danhof. Pharmacokinetic-pharmacodynamic modeling of the EEG effect of alfentanil in rats: assessment of rapid functional adaptation. *Br. J. Pharmacol.* **124**:1534–1540 (1998b).
9. P. S. Portoghese, D. L. Larson, L. M. Sayre, D. S. Fries, and A. E. Takemori. A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. *J. Med. Chem.* **23**:233–234 (1980).
10. E. H. Cox, J. G. N. van Hemert, E. J. Tukker, and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the EEG effect of alfentanil in rats. *J. Pharmacol. Toxicol. Meth.* **38**:99–108 (1997).
11. R. C. Schoemaker and A. F. Cohen. Estimating impossible curves using NONMEM. *Br. J. Clin. Pharmacol.* **42**:283–290 (1996).
12. P. H. Van der Graaf, E. A. Van Schaick, R. A. A. Mathot, A. P. IJzerman, and M. Danhof. Mechanism-based pharmacokinetic-pharmacodynamic modeling of the effects of N^6-cyclopentyladenosine analogs on heart rate in rat: estimation of *in vivo* operational affinity and efficacy at adenosine A_1 receptors. *J. Pharmacol. Exp. Ther.* **283**:809–816 (1997).
13. P. H. Van der Graaf, E. A. Van Schaick, S. A. G. Visser, H. J. M. M. De Greef, A. P. IJzerman, and M. Danhof. Mechanism-based pharmacokinetic-pharmacodynamic modeling of anti-lipolytic effects of adenosine A_1 receptor agonists in rats: prediction of tissue-dependent efficacy *in vivo*. *J. Pharmacol. Exp. Ther.* **290**:702–709 (1999).
14. Y-C. Cheng and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
15. A. Christopoulos. Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends Pharmacol. Sci.* **19**:351–357 (1998).
16. P. H. Van der Graaf and M. Danhof. On the reliability of affinity and efficacy estimates obtained by direct operational model fitting of agonist concentration-effect curves following irreversible receptor inactivation. *J. Pharmacol. Toxicol. Meth.* **38**:81–85 (1997).
17. R. B. Rothman, J. B. Long, V. Bykov, A. E. Jacobson, K. C. Rice and H. W. Holaday. β-FNA binds irreversible to the opiate receptor complex: *in vivo* and *in vitro* evidence. *J. Pharmacol. Exp. Ther.* **247**:405–416 (1988).
18. C. Chen, J-C. Xue, J. Zhu, Y-W. Chen, S. Kunapuli, J. K. De Riel, and L-Y. Liu-Chen. Characterization of irreversible binding of β-funaltrexanine to the cloned rat μ-opioid receptor. *J. Biol. Chem.* **270**:17866–17870 (1995).
19. J. Giraldo. The slope parameter and the receptor reserve. *Trends Pharmacol. Sci.* **19**:445 (1998)
20. J. W. Black, P. Leff, and N. P. Shankley, with an appendix by J. Wood. An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br. J. Pharmacol.* **84**:561–571 (1985).
21. P. H. Van der Graaf, N. P. Shankley, and J. W. Black. Analysis of the activity of α_1-adrenoceptor antagonists in rat aorta. *Br. J. Pharmacol.* **118**:299–310 (1996).
22. G. Zernig, E. R. Butelman, J. W. Lewis, E. A. Walker, and J. H. Woods. *In vivo* determination of the μ-opioid receptor turnover in monkeys after irreversible blockade with clocinnamex. *J. Pharmacol. Exp. Ther.* **269**:57–65 (1994).
23. Y-X. Pan, J. Xu, E. Bolau, C. Abbadie, A. Chang, A. Zuckerman, G. Rossi, and G. W. Pasternak. Identification and characterization of three new alternatively spliced μ-opioid receptor isoforms. *Mol. Pharmacol.* **56**:396–403 (1999).
24. G. Zernig, T. Burke, J. W. Lewis and J. H. Woods. Mechanism of clocinnamoxblockade of opioid receptors: evidence from *in vitro* and *ex vivo* binding and behavioral assays. *J. Pharmacol. Exp. Ther.* **279**:23–31 (1996).
25. T. Koch, S. Schulz, H. Schroder, R. Wolf, E. Raulf, and V. Holt. Carboxyl-terminal splicing of the rat μ opioid receptor modulates agonist-mediated internalization and receptor resensitization. *J. Biol. Chem.* **273**:13652–13657 (1998).
26. R. Wolf, T. Koch, S. Schulz, M. Klutzny, H. Schroder, E. Raulf, F. Buhling, and V. Hollt. Replacement of threonine 394 by alanine facilitates internalization and resensitization of the rat μ opioid receptor. *Mol. Pharmacol.* **55**:263–268 (1999).
27. D. Morgan and M. J. Picker. The μ opioid irreversible antagonist beta-funaltrexamine differentiates the discriminative stimulus effects of opioids with high and low efficacy at the μ opioid receptor. *Psychopharmacol.* **140**:20–28 (1998).