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In vivo apparent affinity and efficacy estimates for μ opiates in a rat tail-withdrawal assay

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Abstract Experiments in a rat tail-withdrawal assay tested the hypothesis that the magnitude and pattern of antagonism of μ opiate agonists by the insurmountable μ opioid antagonist clocinnamox are inversely related to agonist efficacy. In addition, these experiments examined whether this antagonism could be quantified to yield apparent affinity and efficacy estimates for the pharmacological characterization of five opiate agonists. Etonitazene, etorphine, morphine, buprenorphine, and GPA 1657 produced dose-dependent increases in tail-withdrawal latency until 100% maximum possible effect (%MPE) was obtained. Morphine required a higher dose of clocinnamox for a 50% reduction in maximal antinociceptive effect than did buprenorphine or GPA 1657. In contrast, no dose of clocinnamox tested decreased the%MPE for etonitazene or etorphine. These data suggest a rank order of relative efficacy of etonitazene ≥ etorphine > morphine ≥ GPA 1657 ≥ buprenorphine. Similarly, numerical analysis of these data yielded the following apparent affinity and efficacy estimates: etonitazene (0.38 mg/kg, 128); etorphine (0.68 mg/kg, 125); morphine (50 mg/kg, 38), GPA 1657 (6.6, 39); and buprenorphine (0.042 mg/kg, 2.2). These data illustrate that in vivo affinity and efficacy estimates for a number of agonists are remarkably similar across different methods of analysis and are useful for drug classification.

Key words Etonitazene · Morphine · Buprenorphine · Etorphine · GPA 1657 · Affinity · Efficacy · Antinociception · Clocinnamox

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Introduction

An insurmountable antagonist such as the cinnamoylmorphinone clocinnamox prevents receptors from interacting with an agonist by means of either site-directed alkylation or very slow dissociation rates. The functional result of insurmountable antagonism is a progressive rightward shift and eventual flattening of an agonist dose-response curve as the dose of an insurmountable antagonist is increased (for reviews see Furchgott 1966; Kenakin 1993). The notion that clocinnamox produces a long-lasting elimination of μ opioid receptors is supported by radioligand binding studies (Aceto et al. 1989; Burke et al. 1994; Zernig et al. 1995). For example, radioligand binding studies have demonstrated that clocinnamox dose-dependently reduced binding of [^{3}H]DAMGO ([D-Ala^2, *N*-MePhe^4, Gly^5-ol][tyrosyl-3,5-^{3}H]enkephalin) and [^{3}H]-naltrexone without affecting the affinity of either ligand. Furthermore, clocinnamox produced wash-resistant binding to μ but not δ or κ opioid receptors (Zernig et al. 1995, 1996a).

An insurmountable antagonist, such as clocinnamox or β-funaltrexamine (β-FNA), is an indispensable tool for the determination of agonist relative efficacy in behavioral pharmacology (Zernig et al. 1994; Butelman

et al. 1996). Agonists that differ in efficacy or spare receptor population should be differentially sensitive to an insurmountable antagonist, especially with respect to the loss of maximum effect. For example, in the acetic acid writhing test, fentanyl and morphine retain a maximum antinociceptive response after pretreatment with 80 mg/kg β-FNA, whereas buprenorphine and nalorphine do not, suggesting that fentanyl and morphine require fewer receptors to produce the maximum effect than do buprenorphine and nalorphine (Zimmerman et al. 1987). Higher doses of β-FNA and clocinnamox are required to reduce the maximum antinociceptive effect of methadone or fentanyl than are required to reduce the maximum effects of levorphanol or morphine. These data suggest that methadone and fentanyl may require a smaller intact fraction of the μ opioid receptor population for antinociceptive effect than do levorphanol and morphine (Adams et al. 1990; Comer et al. 1992).

In the present study, the relative efficacy of five opioid agonists was characterized in the rat tail-withdrawal assay using the insurmountable antagonist clocinnamox. Previous experiments using the rat tail-withdrawal procedure demonstrated that naltrexone was equipotent as an antagonist of etorphine, morphine, buprenorphine, and GPA 1657, suggesting that these agonists produce their antinociceptive effects through common, presumably μ, opioid receptors (Walker et al. 1994). Prior to beginning studies with clocinnamox, apparent pA_2 analyses with the competitive antagonist naltrexone as well as low efficacy agonist nalbuphine were used to test whether the antinociceptive effects of a fifth agonist, etonitazene, were mediated through similar receptor populations. Under the conditions used, nalbuphine fails to produce antinociceptive effects and serves as an antagonist of higher efficacy opiates (Walker et al. 1994). Inasmuch as pA_2 analyses suggested that the five agonists did not differ in receptor selectivity for antinociceptive effects, experiments with clocinnamox were performed to characterize qualitatively and quantitatively their efficacy as μ agonists.

In order to continue ongoing efforts to compare the analytical quality of different numerical approaches in the determination of in vivo apparent affinity and efficacy estimates, the data were subjected to the following three algorithms: (i) the classical double reciprocal plot of Furchgott (1966); (ii) the Furchgott equation extended by a slope factor introduced to improve curve-fitting (Zernig et al. 1994); and, (iii) an extended version of the operational model of Black and Leff (1983). Because the original Black and Leff model does not express decreases in apparent efficacy as decreases in the receptor pool, it was extended by q, the fraction of available receptors after a partial blockade with an insurmountable antagonist (Furchgott 1966; see Zernig et al. 1996b). The various mathematical models are briefly detailed below.

Materials and methods

Subjects

Male Sprague-Dawley rats were housed individually in a humidity and temperature-controlled room with lights on from 0700 to 2000 hours. Rats were fed a daily ration of 20 to 25 g to maintain body weights of approximately 340 to 360 g and were given continuous access to water. Cumulative-dose tests occurred approximately once a week for an individual rat.

Apparatus

Eight rodent restraint tubes were used. A Precision Model 181 water bath maintained the temperature of the water. The water from the bath was mixed with tap water in a Thermos brand wide-mouth thermos (diameter = 8 cm) to obtain the desired water temperature. Water temperature was measured by a Sensortek Model BAT-12 with Bailey/Sensortek Type T thermocouple, and tail-withdrawal latency was measured with a hand-operated digital stopwatch with time resolution of 1/100 s.

Procedure

Approximately 1 week prior to testing, all rats were habituated to the restraint tubes and the testing room for 25 min on two or three separate occasions. All antinociceptive testing occurred in the morning within a 4-h period of time. Rats were placed into the restraining tubes with their tails hanging freely. The last 5–10 cm of the tail was immersed into a Thermos containing either 40° or 55°C water, and the latency for tail withdrawal was measured. A cut-off time of 15 s was imposed so that if the rat failed to remove its tail within 15 s, the experimenter removed the stimulus.

The first three stimulus presentations during a test were 40°C to control for tail removal independent of water temperature. If the tail was not removed within 15 s on two out of three presentations of 40°C, the rat remained in the experiment. A 2-min interval occurred between each stimulus presentation throughout the experiment. Next, a single control latency value for the 55°C water temperature was obtained, followed by the first injection of test compound SC in the dorsal flank. After a 15-min pretreatment period, latency to withdraw the tail from both 40° and 55°C water was redetermined. On this and all succeeding trials, each temperature was presented once and the order of presentation of 40° and 55°C water was varied randomly. At the conclusion of the 10-min testing period, another injection of test compound was administered so that the total dose was increased 0.5 log unit. After another 15- min pretreatment period, latency values for 40° and 55°C were taken again during a 10-min testing period. As with similar studies (Adams et al. 1990; Paronis and Holtzman 1992; Walker et al. 1994), dosing was stopped when a subject reached maximum effect (15 s) at 55°C water, the solubility limits of a compound were reached, or other behaviors interfered with the measurements (i.e., convulsions).

Antagonism studies

Twelve rats were assigned to one of two groups: one tested after pretreatments of naltrexone ($n = 6$) and one tested after pretreatments of nalbuphine ($n = 6$). Two control dose-response curves were determined for etonitazene in each group. During antagonism studies, pretreatment of naltrexone (0.01, 0.1 or 1.0 mg/kg) or nalbuphine (3.2, 10, and 32 mg/kg) was administered and tested alone in the first trial. Thereafter, a cumulative etonitazene dose-response

curve was determined as described above. Testing occurred once a week for each group.

Clocinnamox studies

Forty rats were assigned to one of eight groups: two tested with etonitazene ($n = 6$) or etorphine ($n = 6$), two with morphine ($n = 5$ and $n = 5$), two with buprenorphine ($n = 4$ and $n = 5$), and two with GPA 1657 ($n = 4$ and $n = 5$). One to three control dose-response curves were determined for the respective agonist in each group. Then a dose of 1.0, 3.2, or 10 mg/kg clocinnamox, SC, was administered 24 h prior to re-determination of the agonist dose-response curve. In preliminary studies, this pretreatment time and route of administration produced the greatest antagonism of morphine following a dose of 10 mg/kg clocinnamox (unpublished observation). Each rat received only one dose of clocinnamox.

Data analysis

Latencies for tail-withdrawal after administration of a drug were converted into percent maximum effect by the formula:

$$\%\ \mathrm{MPE} = \frac{\text{test latency} - \text{control latency}}{(15\ \mathrm{s} - \text{control latency})} \times 100$$

using the control latency measured at the beginning of the experiment. Each rat served as its own control. A value of zero was assigned if the rat withdrew its tail faster than the control latency. For each agonist studied, one to three control dose-response curves were generated in each rat and pooled to determine a control function.

The antagonism data were further analyzed and quantified to determine apparent pA_2 values for naltrexone and nalbuphine. The dose that produced a 50% maximum effect was taken as the ED_{50} for each dose-response curve. ED_{50} values were determined by log-linear interpolation of the linear portion of dose-response curves. Apparent pA_2 values were determined using the ED_{50} values according to the Schild method (Arunlakshana and Schild 1959), with drug doses substituted for drug concentrations (Takemori 1974). Schild plot slopes were considered to be significantly different from unity if the 95% CL of the slope did not include -1.

Dose-response curves for the clocinnamox experiments were fitted using the following semilogarithmic form of the logistic dose-response equation:

$$E = \frac{(\mathrm{Emax}\text{-}\mathrm{Emin}) * 10^{(\log[X]*h)}}{10^{(\log(ED50)*h)} + 10^{(\log[X]*h)}} + \mathrm{Emin}$$

where E is the %MPE, and Emin and Emax are the minimum and maximum of the sigmoid dose-response curve. X is the dose of agonist (in mg/kg). ED_{50} is the agonist dose causing 50% maximum possible effect and h is the slope factor.

The clocinnamox data were further analyzed according to three methods previously described in detail (Furchgott 1966; Black and Leff 1983; Zernig et al. 1994, 1996b). The first analysis used the well-known method developed by Furchgott (1966). Dose-response curves for an agonist alone were compared to dose-response curves in the presence of clocinnamox. Equiactive concentrations at 5% intervals for both dose-response curves (A_1,A_1'), (A_2,A_2') . . . , (A_N,A_N') were determined and the relationship was described by the double reciprocal formula

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{(1-q)}{qK_A}$$

where A and A′ are the equieffective doses of agonist (in mg/kg) before and after clocinnamox administration, respectively, and q is the fraction of receptors remaining after inactivation of the receptors. A plot of the reciprocals $1/[A_1]$ versus $1/[A_1']$ should be a straight line where q, the fraction of receptors remaining after clocinnamox treatment, is determined by the inverse of the slope of the linear regression. K_A, the reciprocal of the in vivo affinity (in mg/kg), was determined by the following:

$$K_A = \frac{(\mathrm{slope} - 1)}{\mathrm{intercept}}$$

(Tallarida and Jacob 1979; Kenakin 1993). Furthermore, an initial estimate of e, the efficacy of the agonist, was obtained from the following equation (Furchgott 1966):

$$e = (K_A/[ED_{50,\ \mathrm{control}}]) - 1.$$

For the second method of analysis, the values K_A, q, and e determined from the double reciprocal plot were used as initial estimates for a fit for both clocinnamox treatment and control functions using the original equation of Furchgott (1966) and modified a slope factor, n, that allowed for better curve-fitting:

$$E = \frac{E_m\ (q \cdot e/(1 + q \cdot e)) + 10^{(\log[A']n)}}{10^{(\log[K_A])n}((1/(1 + q \cdot e)) + 10^{(\log[A']n)}} + c$$

where E_m is the maximum possible response; n is a slope factor; E is the antinociceptive effect of an agonist (% MPE); and c is the baseline latency. This method allows for estimates of K_A, e, q and n from both clocinnamox and control dose-response curves. For an empirical comparison of these two methods, see Zernig et al. (1996b). Mathematical and statistical calculations were performed on an IBM PC with the InPlot and InStat computer packages (GraphPad, San Diego, USA).

The third method of analysis was performed according to the model proposed by Black and Leff (1983) and applied to behavioral data according to methods described extensively by Zernig et al. (1996b). In the previous two methods described above, e was defined as $ED_{50,\ \mathrm{control}} = K_A/(e+1)$ whereas in the present method, tau (an operational definition of efficacy) is defined as $ED_{50,\ \mathrm{control}} = K_A/((2+\mathrm{tau}^n)^{1/n} - 1)$ where n is a slope factor of a transducer function relating receptor occupancy to observed response (Black and Leff 1983). Note that in all methods of mathematical analysis, the efficacy value (e or tau) is the reciprocal of the fraction of receptors necessary to give a half-maximum response. An estimate of the fraction of receptors still available for interaction with an agonist after blockade with a certain dose of an insurmountable antagonist [which corresponds to q in Furchgott's model (1966)] can be obtained by dividing the tau value obtained after inactivation of the receptors by the insurmountable antagonist by the tau value obtained under control conditions (see Zernig et al. 1995, 1996b). For non-hyperbolic E/[A] curves, Black et al. (1985) propose a function of the form:

$$E = \frac{E_m * \mathrm{tau}^n * [A]^n}{(K_A + [A])^n + \mathrm{tau}^n * [A]^n}$$

where E is the effect (% MPE); E_m, the maximum attainable response; [A], the agonist concentration; and n, the slope factor of the transducer function. The semilogarithmic form of the above equation is extended by c, the baseline response, with tau represented as $(q*\mathrm{tau}_{\mathrm{control}})$:

$$E = E_m/(((10^{(\log(K_A) - \log[A])} + 1)/(q*\mathrm{tau}_{\mathrm{control}}))^n + 1) + c.$$

All dose-response curves obtained with a given agonist were simultaneously fitted to the above equation using a non-linear fitting program (Zernig and Issaevitch 1995) and the general mathematical software package Mathematica (Wolfram Research, Champaign, USA; Wolfram 1991). Variance estimates for a given variable were obtained by holding all other fitted curve parameters constant and

allowing the parameter under investigation to vary (constrained 95% confidence intervals; see Zernig et al. 1996b).

The derived values determined by the mathematical analysis of Black and Leff (1983) can be used in the equation by Black et al. 1985:

$$ED_{50,control} = K_A/((2+tau^n)^{1/n}-1)$$

to determine the control ED_{50} value of an agonist. This "back-calculated" ED_{50} value was then compared to the observed ED_{50} value as a measure of internal consistency.

Drugs

The following compounds were used: etonitazene hydrochloride, morphine sulfate, buprenorphine hydrochloride, etorphine hydrochloride, naltrexone hydrochloride (National Institute on Drug Abuse, Rockville, Md., USA), nalbuphine hydrochloride (Research Biochemicals International, Natick, Mass., USA), GPA 1657 [(1)β-2′-hydroxy-2,9-dimethyl-5-phenyl-6,7-benzomorphan] (gift from James H. Woods, University of Michigan, Ann Arbor, Mich., USA), and clocinnamox [14 β-(*p*-chlorocinnamoylamino)-7,8-dihydro-*N*-cyclopropylmethyl-normorphinone mesylate] (gift from John W. Lewis, Reckitt and Colman Pharmaceutical Division, Kingston-Upon-Hull, UK).

Morphine, etonitazene, and etorphine were dissolved in physiological saline. Clocinnamox, GPA 1657 and buprenorphine were dissolved in sterile water. All solutions except clocinnamox were prepared to administer each injection in a volume of 0.1–2.0 ml per 100 g body weight. Due to its limited solubility, clocinnamox was prepared as a suspension to administer each injection in a volume of 3.0 ml per 100 g body weight. Doses are expressed as the forms listed above. Saline was injected in a volume of 1 ml/kg body weight.

Results

Naltrexone and nalbuphine pretreatment experiments

Cumulative doses of etonitazene produced a dose-dependent increase in tail-withdrawal latency until a maximum effect (15 s) was obtained (Fig. 1). Naltrexone pretreatments of 0.01–1.0 mg/kg and nalbuphine pretreatments of 3.2–32 mg/kg produced dose-dependent shifts to the right in the etonitazene dose-response curves. Because all dose-response curves were parallel to initial control curves in the naltrexone and nalbuphine pretreatment experiments, an apparent pA_2 analysis was applicable. The potency of naltrexone and nalbuphine to antagonize the antinociceptive effects of etonitazene was revealed by linear Schild regressions and apparent pA_2 analyses (Table 1). Apparent pA_2 values for naltrexone and nalbuphine were 8.3 (7.2–9.3) and 5.3 (4.1–6.6), respectively. Because the Schild regressions were not significantly different from unity, the slopes were constrained to -1 and the apparent pA_2 values redetermined for naltrexone and nalbuphine as 7.8 (7.2–8.5) and 5.6 (5.1–6.2).

Table 1 Apparent pA_2 values for naltrexone or nalbuphine antagonism of the antinociceptive effects of etonitazene

Antagonist	pA_2 value ± 95% CL	(−)slope ± 95% CL	Constrained pA_2 value[a] ± 95% CL
Naltrexone	8.3 (7.2–9.3)	0.75 (1.2–0.32)	7.8 (7.2–8.5)
Nalbuphine	5.3 (4.1–6.6)	1.4 (3.5–0.71)	5.6 (5.1–6.2)

[a]95% CL of the slopes of all Schild regressions were not different, so slopes were constrained to -1 for this analysis

Clocinnamox pretreatment experiments

Six consecutive saline injections, administered every 25 min, produced less than a 30% maximum effect indicating the experimental procedure contributed little to the antinociception observed (data not shown). Cumulative doses of etonitazene, etorphine, morphine, buprenorphine, or GPA 1657 produced dose-dependent increases in tail-withdrawal latencies until the

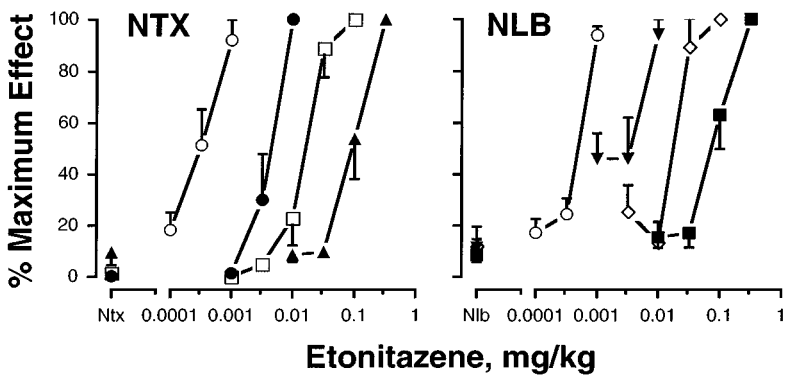

Fig. 1 Naltrexone (*left panel*) or nalbuphine (*right panel*) antagonism of the antinociceptive effects of etonitazene in the rat tail-withdrawal assay. *Ordinate*: the percentage of maximum antinociceptive response (15 s). *Abscissa*: dose of etonitazene, in mg/kg. Control dose-response curves (*open circles*) are the average of two observations in six rats. Other points represent the mean of one observation in six rats. *Left panel*: 0.01 mg/kg naltrexone (*solid circles*); 0.1 mg/kg naltrexone (*open squares*); 1.0 mg/kg (*solid triangles*). *Right panel*: 3.2 mg/kg nalbuphine (*solid inverted triangles*); 10 mg/kg nalbuphine (*open diamonds*); 32 mg/kg nalbuphine (*solid squares*). Naltrexone or nalbuphine was administered 25 min prior to determination of the etonitazene dose-response curve. Points above Ntx and Nlb indicate the antinociceptive effects of naltrexone or nalbuphine alone

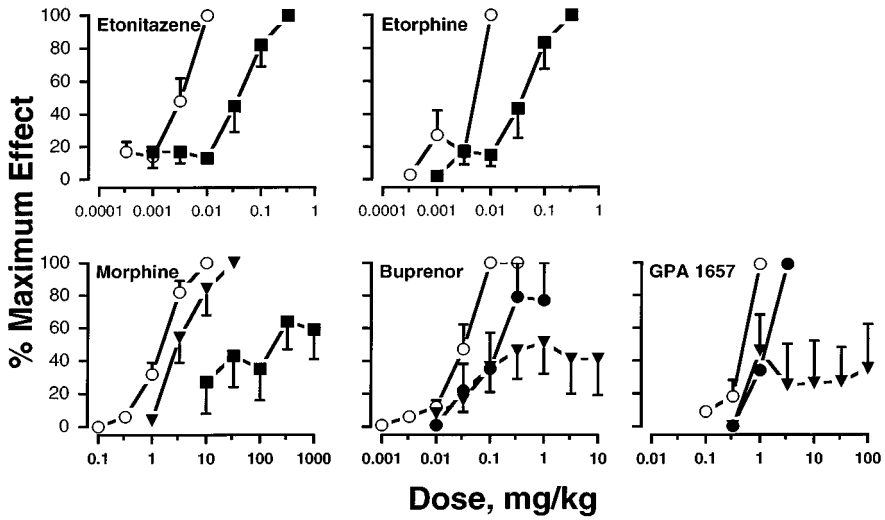

Fig. 2 Effects of clocinnamox (*CCAM*) pretreatments on the antinociceptive effects of etonitazene, etorphine, morphine, buprenorphine, or GPA 1657 in the rat tail-withdrawal assay. Ordinate: latency measures converted into the percent maximum effect. *Abscissa*: dose of agonist, in mg/kg. Control points (*open circles*) are the average of one or two determinations. Each open point represents the mean of four to six rats, with the exception of 100 mg/kg GPA 1657 ($n = 3$), which produced convulsions in one rat. Clocinnamox, SC, was administered 24 h prior to the agonist dose-response curve. Dosing was continued until maximum effect was obtained, flattening of the dose-response curve was evident, or convulsions were observed. ● 1.0, ▼ 3.2, ■ 10 mg/kg CCAM

maximum possible effect (15 s) was achieved (Fig. 2). The observed ED_{50} values (95% CL) for each agonist to produce antinociception are presented in Table 2. The agonists differed in sensitivity to clocinnamox. For buprenorphine and GPA 1657, a dose of 1.0 mg/kg clocinnamox produced a 12- and 2.3-fold increase, respectively, in the ED_{50} values required for antinociception, without decreasing the maximum effect achieved. A higher dose of clocinnamox, 3.2 mg/kg, depressed the dose-response curves for both GPA 1657 and buprenorphine, so that a dose twice or 15 times the initial ED_{50}, respectively, produced only a 50% maximum effect. Further increasing the cumulative dose of buprenorphine by 30-fold, or the GPA 1657 dose by 100-fold, produced no further increase in maximum effect. In contrast, a dose of 3.2 mg/kg clocinnamox did not significantly alter the morphine dose-response curve. A 10 mg/kg dose of clocinnamox flattened the morphine dose-response curve, so that a dose of 320 mg/kg, over 200-times the initial ED_{50}, produced a maximum effect of only 60%, and a further increase to 1800 mg/kg produced no increase in maximum effect. For etonitazene and etorphine, however, the 10 mg/kg dose of clocinnamox significantly increased the ED_{50} by 14- and 4.8-fold, respectively, but did not decrease the maximum antinociceptive effect. Sensitivity to clocinnamox suggests a rank order of relative efficacy of etonitazene ≥ etorphine > morphine > GPA 1657 ≥ buprenorphine. Clocinnamox alone failed to produce antinociceptive effects after 1 or 24 h administration (unpublished observations).

The data presented in Fig. 2 were also analyzed using three mathematical models to obtain estimates for the apparent in vivo dissociation constant, K_A (which is inversely proportional to the agonist affinity), efficacy (e or tau), and the fraction of receptors remaining after clocinnamox treatment (q) (Furchgott 1966; Black and Leff 1983). Generally, etonitazene (0.063–0.31 mg/kg) and etorphine (0.020–0.68 mg/kg) yielded high affinity estimates and the highest efficacy estimates. However, the efficacy estimate for etorphine was quite low determined by the Furchgott (1966) method as compared to the other two methods of estimation (Table 2). Morphine had the lowest affinity estimates (23–50 mg/kg) and the third highest efficacy estimates. Buprenorphine had high affinity estimates (0.013–0.12 mg/kg) but also the lowest efficacy estimates. GPA 1657 had both intermediate affinity estimates (0.90–6.6 mg/kg) and low to intermediate efficacy estimates relative to the other agonists. The derived values determined by mathematical analyses were used to calculate control ED_{50} values for agonists in the absence of clocinnamox in order to assess the internal consistency of the models. Back-calculated ED_{50} values for control dose-response curves were similar to observed ED_{50} values (Table 2).

Values for q, the fraction of receptors remaining available for agonist interaction after insurmountable antagonist treatment, were calculated from each model. Estimates from the Furchgott (1966) equation suggest that 1.0, 3.2 and 10 mg/kg clocinnamox reduced the receptor population by 49%, 28%, and 84%, respectively (Table 3). The lack of a dose-dependent reduction in receptor population with increasing doses of CCAM, suggests that the Furchgott (1966) equation may produce questionable estimates of q under some in vivo conditions. However, estimates from the n-modified Furchgott (1966) equation revealed a

Table 2 Apparent K_A, e or tau, and dervived ED_{50} values determined using three methods of mathematical analyses[a]

	Etonitazene	Etorphine	Morphine	Buprenorphine	GPA 1657
K_A (log mg/kg)					
Furchgott[b]	−0.51	−1.7	1.5 (0.010–2.0)	−1.9 [−1.4–(−2.4)]	−0.045 (−0.045–0.86)
n-Modified[c]	−1.2 [−0.85–(−1.6)]	−0.28 [−0.020–(−0.54)]	1.4 (0.0–1.7)	−0.93 [−0.72–(1.1)]	0.058 (−0.84–1.1)
Black and Leff[d]	−0.41 [−0.33–(−0.50)]	−0.17 [−0.087–(−0.25)]	1.7 (1.7–1.8)	−1.4 [−0.15–(−1.6)]	0.82 (0.81–0.82)
K_A (mg/kg)					
Furchgott	0.31	0.020	31	0.013	0.90
n-Modified	0.063	0.52	23	0.12	1.1
Black and Leff	0.38	0.68	50	0.042	6.6
e or tau					
Furchgott	107	5.7	38 (8.2–68.0)	1.4 (1.1–1.7)	11. (1.2–20.0)
n-Modified	111 (110–112)	231 (174–288)	40 (19.01–61.0)	5.0 (3.0–7.0)	20. (11.01–29.0)
Black and Leff	128 (98–177)	125 (90–160)	38 (37.0–39.0)	2.2 (0.79–3.6)	39. (19.0–59.0)
Calculated ED_{50}, control, [mg/kg][e]					
Furchgott[f]					
n-Modified	0.00056	0.0023	0.59	0.027	0.058
Black and Leff	0.0030	0.0055	1.4	0.035	0.17
Observed ED_{50}, control, (mg/kg)[g]	0.0029 (0.0022–0.0038)	0.0066 (0.0041–0.011)	1.5 (1.1–2.2)	0.021 (0.014–0.032)	0.36 (0.25–0.53)

[a] Dose-response curves used for these analyses are shown in Fig. 2

[b] Data were fitted to the Furchgott (1966) equation by using values determined by double reciprocal analysis of equieffective doses. This analysis could only determine values from the agonist dose-response curves in the presence of clocinnamox. Variance, when it could be determined, is therefore expressed as range of values from tests of two doses of clocinnamox

[c] Data from each separate agonist dose-response curve were fit to the slope-modified Furchgott (1966) equation (Zernig et al. 1994). Values are determined from agonist dose-response curves in the absence and presence of clocinnamox. Variance is expressed as a range for etonitazene and etorphine (control and 10 mg/kg clocinnamox) and SEM for morphine (control, 3.2 and 10 mg/kg clocinnamox), buprenorphine and GPA 1657 (control, 1.0 and 3.2 mg/kg clocinnamox)

[d] Agonist dose-response curves in the absence and presence of clocinnamox were simultaneously analyzed for a given agonist using the Black and Leff (1983) model as applied to behavioral data (Zernig et al. 1995, 1996). Variance is expressed as the 95% CL

[e] ED_{50} values for control dose-response curves were back-calculated using $ED_{50,\ control} = K_A / ((2+tau^n)^{1/n} - 1)$ (Black et al. 1985). See Methods

[f] ED_{50} values cannot be back-calculated in this method of analysis

[g] ED_{50} values determined from Fig. 2

Table 3 Average q values after insurmountable blockade with 1.0, 3.2, or 10 mg/kg clocinnamox as determined by three methods of analyses

	q value[a]	Range or SEM	*n* determinations[b]
Furchgott			
1.0 mg/kg CCAM	0.51	(0.41–0.61)	2
3.2 mg/kg CCAM	0.72	(0.58–0.85)	3
10 mg/kg CCAM	0.16	(0.11–0.22)	3
n-Modified			
1.0 mg/kg CCAM	0.62	(0.55–0.69)	2
3.2 mg/kg CCAM	0.38	(0.30–0.47)	3
10 mg/kg CCAM	0.083	(0.044–0.12)	3
Black and Leff			
1.0 mg/kg CCAM	0.38	(0.17–0.58)	2
3.2 mg/kg CCAM	0.28	(0.15–0.42)	3
10 mg/kg CCAM	0.095	(0.059–0.13)	3

[a] Fraction of receptors remaining for agonist interaction after a given dose of clocinnamox as represented by q

[b] Number of agonists tested with a given dose of clocinnamox (1.0 mg/kg clocinnamox: buprenorphine and GPA 1657; 3.2 mg/kg clocinnamox: morphine, buprenorphine, and GPA 1657; and 10 mg/kg clocinnamox: etonitazene, etorphine and morphine). Variance is expressed as a range or SEM

dose-dependent reduction in receptor population, such that doses of 1.0, 3.2 and 10 mg/kg reduced the receptor population by 38%, 62%, and 92%, respectively. Similarly, the extended Black and Leff (1983) model also revealed a dose-dependent reduction in receptor population such that doses of 1.0, 3.2, and 10 mg/kg clocinnamox reduced the receptor population by 62%, 72%, and 91%, respectively.

Discussion

Prior to studying relative efficacy differences amongst agonists, the agonists must be shown to produce their measured effects through a common receptor population. Apparent in vivo pA_2 analysis, which examines whether the antagonistic potency of an opioid-selective antagonist varies among agonists, provides one useful test of whether a group of agonists exerts behavioral effects through common opioid receptors (Takemori et al. 1972; Dykstra et al. 1988; Woods et al. 1992a). In previous studies, apparent in vivo pA_2 values for the competitive opioid antagonist, naltrexone, as an antagonist of the antinociceptive effects of etorphine, morphine, buprenorphine, and GPA 1657 ranged from 7.3 to 7.7, suggesting that naltrexone does not discriminate among the μ opioid receptors through which these agonists produce antinociceptive effects (Walker et al. 1994). The apparent pA_2 value of 7.8 for naltrexone in the present study suggests that etonitazene exerts its antinociceptive effects through similar μ opioid receptors in rats. Similarly, apparent in vivo pA_2 values for nalbuphine as an antagonist of the antinociceptive effects of etorphine, morphine, buprenorphine, and GPA 1657 ranged from 5.1–5.8, suggesting that nalbuphine also does not discriminate among the μ opioid receptors through which these agonists produce antinociceptive effects (Walker et al. 1994). The apparent pA_2 value of 5.6 for nalbuphine in the present study suggests that etonitazene exerts its antinociceptive effects through μ opioid receptors in rats. In summary, it appears that all the agonists studied in the experiments with clocinnamox produce antinociceptive effects in the rat tail-withdrawal assay through actions at μ opioid receptors.

The naltrexone antagonism studies were important control experiments for additional reasons. Since the high doses of agonists examined in combination with clocinnamox in the present study cannot be examined under control conditions, naltrexone antagonism experiments allow the probing of high doses of agonists without such side effects as convulsions, respiratory suppression, and lethality. For example, doses of 0.32 mg/kg etonitazene, 0.1 mg/kg etorphine, 1000 mg/kg morphine, 3.2 mg/kg buprenorphine, and 100 mg/kg GPA 1657 were all studied in combination with naltrexone in rats without apparent evidence of multiple receptor actions (present study; Walker et al. 1994, 1996). Buprenorphine produces a bell-shaped dose-response curve in some antinociceptive assays so that high doses of buprenorphine produce less antinociception than low doses (Cowan et al. 1977; Woods et al. 1992b; Walker et al. 1995). When buprenorphine was studied in combination with naltrexone, dose-dependent, parallel shifts to the right in the buprenorphine dose-response curves were observed. In these experiments, no evidence of a bell-shaped dose-response curve was observed up to doses of 3.2 mg/kg buprenorphine (Walker et al. 1994).

Once it is assured that similar opioid receptor types are the targets for all agonists tested, alterations in agonist dose-response curves may be attributed to other factors such as agonist relative efficacy. The irreversible antagonist clocinnamox (Lewis et al. 1988; Aceto et al. 1989; Comer et al. 1992) was used qualitatively and quantitatively to examine agonist relative efficacy for five opiate agonists. Clocinnamox pretreatment studies revealed that etonitazene etorphine, morphine, buprenorphine, and GPA 1657 were differentially sensitive to μ receptor inactivation. A low dose of clocinnamox eliminated the ability of buprenorphine and GPA 1657 to produce a maximum effect, but failed to alter the dose-response curve for morphine, suggesting that buprenorphine and GPA 1657 are lower efficacy agonists than is morphine. Furthermore, 10 mg/kg clocinnamox flattened the dose-response curve for morphine, but not those for etonitazene or etorphine, suggesting that morphine may be a higher efficacy agonist than buprenorphine or GPA 1657, but a lower efficacy agonist than etonitazene and etorphine. These changes are probably not due to changes in affinity, since clocinnamox fails to alter the apparent affinity of naltrexone in vivo (Burke et al. 1994; Walker et al. 1996) or [^{3}H]DAMGO and [^{3}H]-naltrexone in vitro and ex vivo (Burke et al. 1994; Zernig et al. 1996). Rather, these patterns of antagonism are most likely due to an agonist's differential sensitivity to the decrease in available μ receptors.

The observation that lower efficacy agonists revealed differential sensitivity to clocinnamox antagonism is in agreement with previous insurmountable antagonism studies in rats with β-FNA (Zimmerman et al. 1987; Adams et al. 1990; Pitts et al. 1996). For example, antinociception studies with β-FNA in rats and squirrel monkeys indicated that etorphine was a higher efficacy agonist than morphine which was a higher efficacy agonist than buprenorphine (Allen et al. 1997; Zimmerman et al. 1987). These data support a hypothesis that the magnitude of clocinnamox antagonism is inversely related to the relative efficacy of the agonists in an antinociception assay.

When an insurmountable antagonist is available, a quantitative estimate of relative efficacy can be determined by the method of partial irreversible blockade (Tallarida and Jacob 1979; Furchgott 1966; for

reviews see Kenakin 1993). In the present experiments, clocinnamox was used to quantify the relative efficacy of etonitazene etorphine, morphine, buprenorphine, and GPA 1657. The qualitative and quantitative analyses performed suggest a rank order of relative efficacy of etonitazene ≥ etorphine > morphine ≥ GPA 1657 ≥ buprenorphine. These quantitative estimates of efficacy are similar to those previously published in the literature. For example, quantitative analyses indicated that etonitazene was higher efficacy than morphine (Walker et al. 1995; Zernig et al. 1995) and etorphine was a higher efficacy agonist than morphine, which was a higher efficacy agonist than buprenorphine (Allen et al. 1997).

In addition to measuring the efficacy estimates for each agonist, the clocinnamox antagonism experiments also allowed calculation of apparent affinity estimates. Buprenorphine, the lowest efficacy agonist, and etorphine and etonitazene, the highest efficacy agonists, had high affinity for their receptors. Similarly, in radioligand binding studies, etonitazene, buprenorphine and etorphine have high affinity for opioid receptor sites (Sadée et al. 1982; Richards and Sadée 1985). GPA 1657 had an intermediate affinity estimate and morphine had the lowest affinity estimate. The values calculated for morphine are similar to in vivo K_A estimates for morphine in antinociceptive assays in rhesus monkeys (13–60 mg/kg) (Zernig et al. 1994; Walker et al. 1995), mice (11–68 mg/kg) (Zernig et al. 1995), and rats (22–25 mg/kg) (Blasig et al. 1979; Porreca et al. 1982; Tallarida and Cowan 1982). The affinity estimates for buprenorphine are in agreement with values obtained in operant experiments with rats (0.11 mg/kg) (E. A. Walker and AM. Young, unpublished observations) and are also in agreement with the affinity estimates for buprenorphine as an antagonist of the tail-withdrawal response in rhesus monkeys (0.12–0.19 mg/kg) (Walker et al. 1995). These experiments suggest that in vivo affinity and efficacy estimates can be used for drug classification, as well as for making hypotheses about the relationship of agonist efficacy to behavioral effects.

Three different methods of mathematical analyses were used to calculate in vivo affinity and efficacy estimates, as well as the percentage of receptors available for agonist interaction after clocinnamox administration. An extensive discussion of the validity, applicability, and limitations of these methods has been presented elsewhere (Zernig et al. 1996b). In brief, Zernig et al. (1996b) concluded that the use of the Black and Leff (1983) equation extended by q, the fraction of available receptors according to Furchgott (1966), has a number of advantages over the traditional double reciprocal Furchgott plot as well as an n-modified Furchgott equation used previously (Zernig et al. 1994; Walker et al. 1995). For example, the derived values from the n-modified Furchgott and the Black and Leff (1983) equations can be used to generate ED_{50} values that match the observed control ED_{50} values calculated by logistic regression, thereby providing information about the internal consistency of these two analyses in vivo. Despite the advantages of one mathematical analysis over another, the estimates determined from the n-modified Furchgott and Black and Leff (1983) models were generally similar to those determined from the more traditional Furchgott (1966) method. The exceptions, however, were the apparent K_A and e values for etorphine, which were much lower when determined using the Furchgott (1966) equation than when determined using the other two methods of analyses. The estimates from the Furchgott (1966) equation were determined from a single dose-response curve in which 10 mg/kg clocinnamox failed to reduce the maximum effect of etorphine. Especially under circumstances without a loss of maximum effect, the use of a double reciprocal plot may result in errors of estimation of K_A and therefore, e (Kenakin 1993).

In vivo affinity and efficacy estimates for a number of agonists are remarkably similar across different laboratories using different species, paradigms, insurmountable antagonists, and different methods of analysis (e.g., Walker et al. 1995). An interesting exception to this rule is the agonist etonitazene. Despite the use of similar antinociception assays, i.e., warm-water tail-withdrawal, etonitazene was shown to be a low efficacy agonist in mice (tau, 7; Zernig et al. 1995) but a high efficacy agonist in rats (tau, 128; present study) and rhesus monkeys (tau 174, Walker et al. 1995). Apparent pA_2 values for opioid antagonists indicate that etonitazene produces its antinociceptive effects through μ opioid receptors in rhesus monkeys (Walker et al. 1993), rats (present study), and mice (Burke et al. 1994). Apart from the obvious species difference, we have no explanation to offer for the considerable difference in relative efficacy for etonitazene. Further studies of etonitazene and clocinnamox in other behavioral assays with mice are required to make any judgements regarding the nature of this discrepancy across species.

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