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BRIEF COMMUNICATION

Quantitative Analysis of Naloxone Antagonism of the Discriminative Stimulus Properties of Morphine in the Pigeon

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WESSINGER, W. D. AND D. E. McMILLAN. *Quantitative analysis of naloxone antagonism of the discriminative stimulus properties of morphine in the pigeon.* PHARMACOL BIOCHEM BEHAV **25**(1) 209–214, 1986.—Pigeons were trained to discriminate morphine (5.0 mg/kg) from saline under a second-order fixed ratio 10 (fixed-ratio 5) color-tracking schedule for food reinforcement. After reliable stimulus control was established, cumulative graded doses of morphine (0.3–30.0 mg/kg) were tested and resulted in dose-dependent increases in morphine-appropriate key pecking and decreases in response rate. Cumulative doses of naloxone (0.1–10.0 mg/kg) or consecutive injections of saline did not elicit morphine-appropriate responding or affect response rate. Pre-treatment with naloxone (0.1–1.0 mg/kg) before determination of cumulative dose-effect curves for morphine caused the morphine generalization curves to be shifted, in a parallel manner, rightward. Dose-ratio analysis of naloxone antagonism of morphine generalization, using a Schild plot with the slope constrained to −1, gave an apparent pA_2 value (95% confidence limits) of 6.53 (6.18–6.89).

Morphine Naloxone Drug discrimination pA_2 Schild plots Pigeons

THERE is considerable evidence that the discriminative stimulus properties of morphine are mediated by the same types of receptors that mediate other well-known actions of morphine (e.g., analgesia, respiratory depression, subjective effects). One observation which supports this hypothesis, is that the discriminative stimulus effects of morphine can be completely blocked by low doses of pure antagonists, such as naloxone and naltrexone. The antagonist blockade of the stimulus effects can be surmounted by increasing the dose of agonist [2]. The purpose of the present study was to characterize naloxone antagonism of morphine discriminative-stimulus effects in pigeons trained to discriminate 5 mg/kg morphine from saline using the second-order color-tracking schedule of reinforcement developed in our laboratory [6,7]. Because of the competitive nature of the antagonism, it was possible to determine the apparent pA_2 value for the morphine-naloxone pair using dose-ratio analysis [1, 9, 10]. The apparent pA_2 is the negative logarithm of the molar dose of an antagonist which reduces the effect of a double dose of an agonist to that of a single dose. The pA_2 of a competitive antagonist is a reflection of the affinity of the antagonist for the receptor mediating the agonist effect and is thus useful for classifying drugs and receptors [12,13]. The results of dose-ratio analysis have been reported for the antagonism of morphine's discriminative stimulus effects in the rat [2, 5, 11] and squirrel monkey [15]; however, to our knowledge, this has not been previously reported for the pigeon.

METHOD

Subjects

Four male White Carneaux pigeons, 9 to 11 years old and weighing 540 to 580 g at the beginning of these experiments, were used. These birds had previously been used for other drug discrimination experiments and had been tested with a variety of narcotic agonists, antagonists, and other psychoactive drugs [8]. Throughout this experiment they were maintained at 80% of their free feeding weight by restricted post-session feeding of mixed grain. They were individually housed in a room maintained under a 12-hour normal phase

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lighting cycle. Tap water and oyster grit were freely available in the home cages.

Apparatus

A standard operant test chamber (Model G-7410, Gerbrands Corp., Arlington, MA), housed inside a light and sound-attenuating enclosure (G-7211, Gerbrands Corp.) equipped with a fan for air circulation was used. Three translucent pigeon keys (G-6315, Gerbrands Corp.) which could be transilluminated with colored lights were arranged horizontally in the test chamber 20 cm above the grid floor. Centered below was an opening through which mixed grain could be presented by a grain magazine (G-5610, Gerbrands Corp.) when schedule contingencies were met. A small relay mounted on the chamber produced auditory feedback with each effective key peck during sessions. The test chamber was illuminated by a single houselight (28 V-DC) which remained lit during experimental sessions, except during feed cycles when only the grain hopper was illuminated. White noise was supplied continuously to the room housing the behavioral environments. Schedule contingencies and data collection were programmed by a microcomputer (TRS-80, Model III, Radio Shack) through an interface (Microcomputer Interface II, MED Associates, Inc., East Fairfield, VT) and behavior was recorded by a cumulative recorder (Model C-3, Gerbrands Corp.) located in an adjacent room.

Procedure

Discrimination training. The pigeons were trained to discriminate 5.0 mg/kg morphine from saline as described by Paule and Wenger [8]. In order to obtain food reinforcement, the pigeons were required to track the location of a red or green transilluminated side key under a second-order schedule depending on whether morphine (red key correct) or saline (green key correct) had been administered. At the start of the session, the houselight and center observing key (white) were illuminated. A single peck to the white center key extinguished it and lighted the two side keys, one red and the other green. Completion of five responses on either side key (fixed-ratio 5, FR5) extinguished the side keys and relighted the center key to return the original condition. The position of the red and green side keys was varied randomly after each center-key response. During training sessions, mixed grain (8-sec access) was presented only after ten FR5 units had been completed on the appropriate side key for the training stimulus conditions. Thus, this schedule required that the pigeon select a side key (right or left) which was appropriate for the stimulus conditions (red after morphine, green after saline) and respond under an FR5 on the side key, ten times before mixed-grain was presented. This schedule is referred to as FR10 (FR5) according to the terminology of Kelleher [3] for second-order schedules. Training sessions terminated after 6 mixed-grain presentations or 40 min, whichever occurred first.

Sessions were usually conducted Monday through Friday, with pigeons receiving morphine or saline according to a double alternation schedule (i.e., M,M,S,S,M. . .). With repeated pairing of the drug stimulus conditions and reinforcement contingencies, responding came under stimulus control, thus the pigeons responded on the appropriate key color with a high degree of accuracy. Before testing was begun each bird was required to complete at least 14 consecutive training sessions in which no less than 80% of the side-key responses prior to the first reinforcement were distributed on the appropriate key. Under this criteria, no more than 12 incorrect responses, prior to the first reinforcement, were permitted.

Discrimination testing. After stimulus control was established training continued, but tests were conducted on Thursdays or Fridays, if the subjects met criteria (80% correct key responding prior to the first reinforcement) on the two preceeding training days. The two training sessions preceeding test days were always in a saline-morphine or morphine-saline sequence and each drug or drug combination was tested twice, once after each sequence, in the course of the experiments.

Cumulative-dosing procedures were used which involved administration of increasing doses of drug (in one-half log unit increments) before each test trial (a total of 5 test trials/test session) so that dose-effect curves for an individual subject could be determined within a single session. Each test trial began with an IM injection, followed by a 15-min pretreatment period and a 5-min response period. During the response period, the completion of ten FR5's on either key color was reinforced. Each test trial ended after mixed-grain presentation or 5 min, whichever occurred first. The interjection interval was always 20 min. In addition to drug testing, 5 consecutive saline injections (1 ml/kg) were tested in an analogous fashion in order to insure that successive trial drug-key responding did not develop as a result of the multiple-trial testing procedure. Cumulative dose-effect curves were determined for morphine (0.3–30.0 mg/kg) and naloxone (0.1–10.0 mg/kg) alone. The antagonism of morphine discrimination by naloxone was tested by administering a single dose of 0.1, 0.3, or 1.0 mg/kg naloxone 5 min prior to beginning cumulative morphine dose-effect determinations (0.3–30.0 mg/kg morphine). During the course of the experiment, the sequence of testing drugs, drug combinations and saline was mixed, but all pigeons were tested in the same order. Each dose-effect curve was determined twice in each subject. Data obtained prior to reinforcement on the two training days immediately preceding test days served as controls.

Data analysis. Data from test sessions were analyzed in terms of drug effects on the percent of total responses occurring on the morphine-appropriate key and the side-key response rate (responses/sec). Side-key response rate refers only to side-key (both red and green) responding with the observing-key response latencies and reinforcement time omitted. Data were calculated for individual subjects and are presented as the mean and standard error of the mean calculated for the group. When responding during a trial did not result in reinforcement, the percent morphine-appropriate responses, were not included in the average, however, all response rate values were used. For morphine and morphine-naloxone dose-effect curves, the doses of morphine estimated to produce 50% morphine-appropriate responding (ED50) and 95% confidence limits (95% C.L.) were obtained by least squares linear regression of the linear portions of the dose-effect curves [14]. The resultant best fit lines were tested for parallelism using the t-test [14].

The apparent pA_2 for naloxone was determined using a Schild plot [1, 9, 10]. The ED50 values for morphine in the presence of three different doses of naloxone were divided by the ED50 for morphine alone to obtain dose ratios. The log of the dose ratio minus one (log DR−1) was plotted on the ordinate and the negative log of the dose of naloxone in moles/kg was plotted on the abscissa. A regression line through these points was determined to not differ signifi-

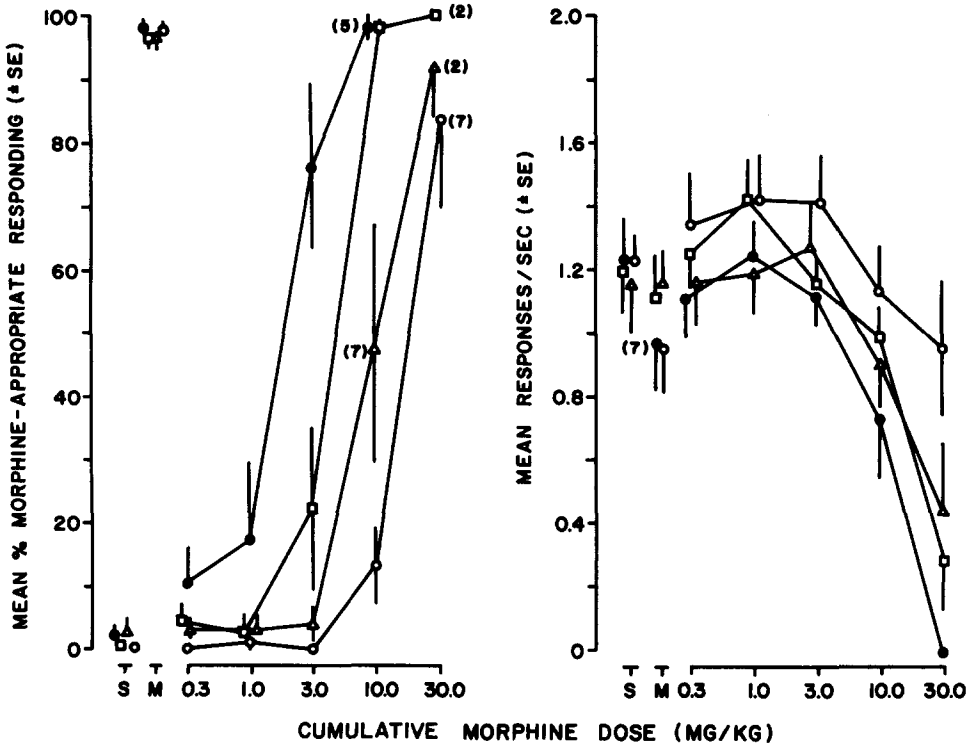

FIG. 1. Dose-effect curves for cumulative doses of morphine alone (●), or morphine preceded by 0.1 (□), 0.3 (△) or 1.0 (○) mg/kg naloxone in pigeons. The mean percent morphine-appropriate responding (left panel) and mean side-key response rate (right panel) are on the ordinates with the cumulative morphine dose in mg/kg (log scale) on the abscissa. The vertical bars indicate the standard errors. The points over S and M are control data for saline or the training dose of morphine (5 mg/kg), respectively, from training days which immediately preceeded test days. Each point represents the mean (±SE) of 8 values (2 determinations in each of 4 pigeons) except where indicated in the figure. When responding during the test session did not result in reinforcement delivery, the percent morphine-appropriate responding data was excluded from the mean calculation.

cantly from −1 (actual value±SE = −0.88 + 0.26). When Schild plots are made for pure competitive antagonists (i.e., morphine and naloxone), the underlying theory requires the regression line to be constrained to −1, for only then does $pA_2 = \log K_B$ (K_B being the affinity of the antagonist for the receptor) [12]. Therefore, the Schild plot analysis for determination of the apparent pA_2 for naloxone antagonism of morphine-discrimination was done by constraining the slope to −1 as described by Tallarida and Murray [14]. The intercept of this best fit line on the abscissa is the apparent pA_2 value.

Drugs. Morphine sulfate (Mallinckrodt, Inc., St. Louis, MO) and naloxone hydrochloride (ENDO Laboratories, Garden City, NJ) were dissolved in 0.9% physiological saline (used also for saline controls) to an injection volume of 1 ml/kg and administered IM into a breast muscle. For multiple injections, each successive injection was into muscle on alternate sides of the breast. Doses are expressed as mg/kg and refer to the salt, except for the dose-ratio analysis where the naloxone dose was converted to moles/kg. Doses shown in figures are the total dose administered (e.g., where 1.0 mg/kg for morphine is indicated, this represents the first trial dose of 0.3 mg/kg plus the subsequent second trial dose of 0.7 mg/kg for a total dose of 1.0 mg/kg, etc.).

RESULTS

The effects of cumulative graded doses of morphine alone on the distribution of responses and the rate of side-key responding (responses/sec) are shown in Fig. 1. Also shown are the training control data obtained prior to the determination of dose-effect curves which demonstrate that the subjects responded to saline (over S) or the 5 mg/kg morphine training dose (over M) with a high degree of stimulus control (>95% accuracy). Low doses of morphine (0.3 and 1.0 mg/kg) engendered primarily saline-appropriate responding, while doses of 3.0 and 10.0 mg/kg morphine resulted in increasing morphine-appropriate responding (filled cirlces, left panel, Fig. 1). Response rate decreased in a dose-dependent manner at cumulative doses greater than 3.0 mg/kg (filled circles, right panel, Fig. 1). None of the subjects emitted any responses following a cumulative dose of 30.0 mg/kg morphine.

When cumulative doses of morphine were tested in the presence of three different doses of naloxone (0.1–1.0 mg/kg) the morphine generalization curve was shifted in a parallel manner (*t*-test, [14]) progressively to the right along the abscissa (left panel, Fig. 1). The ED50's (95% confidence limits) were 3.90 (3.01–5.05), 10.24 (6.18–16.95), and 16.17

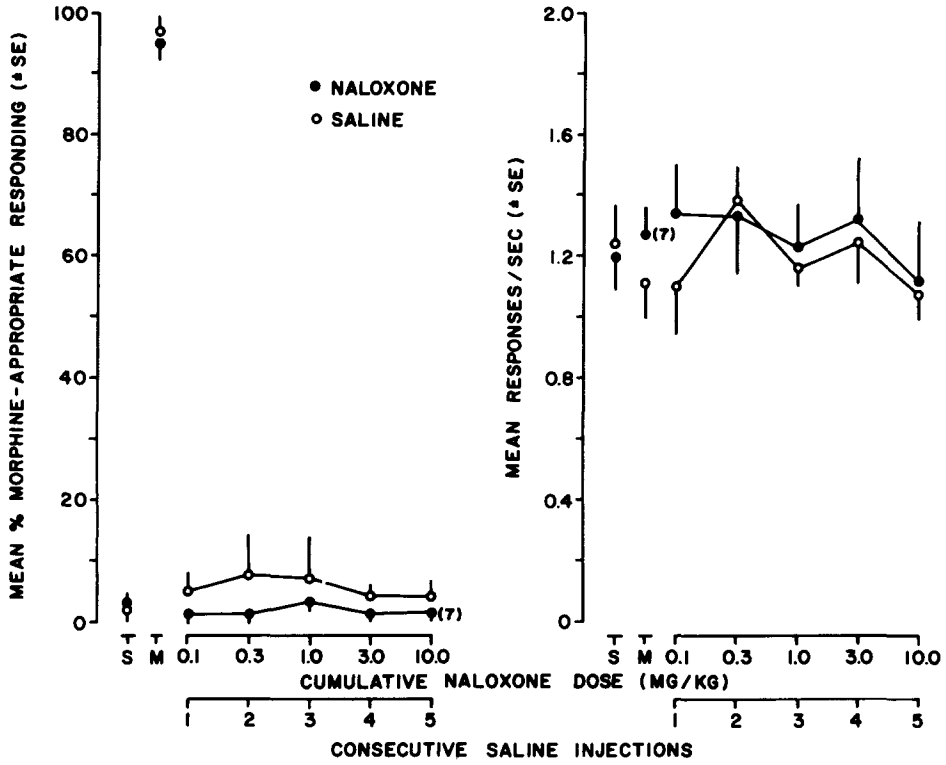

FIG. 2. Dose-effect curves for cumulative doses of naloxone (●) or consecutive saline (○) injections in pigeons. The mean percent morphine-appropriate responding (left panel) and mean side-key response rate (right panel) are on the ordinates and the cumulative naloxone dose in mg/kg (log scale) or consecutive saline injection numbers are on the abscissa. Other details are as described for Fig. 1.

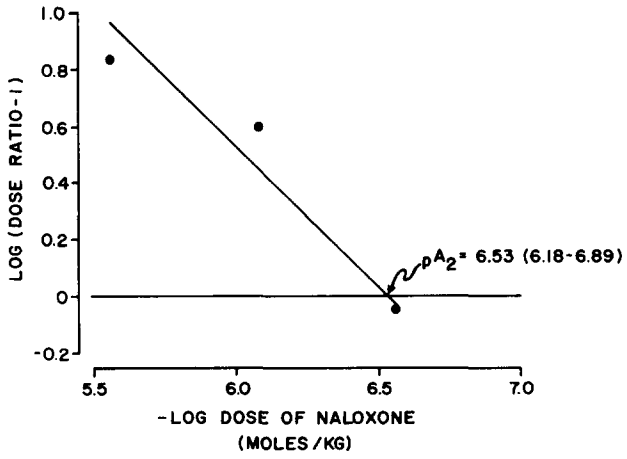

FIG. 3. Schild plot of antagonism of the morphine-like discriminative stimulus effects of morphine by naloxone in pigeons. The log of the dose ratio minus 1 is plotted on the ordinate and the negative log of the dose of naloxone in moles/kg is plotted on the abscissa. Points were calculated from the ED50 values of the dose-effect curves in the left panel of Fig. 1. The slope of the regression line was constrained to −1 as required by theory for competative antagonism as described by Tallarida and Murray [14].

(11.00–23.76) for morphine in combination with 0.1, 0.3 and 1.0 mg/kg naloxone, respectively. The calculated ED50 (95% confidence limits) for morphine alone was 2.05 (1.35–3.12). The response rate-decreasing portions of the dose-response curves (right panel, Fig. 1) were similarly shifted rightward in a dose-dependent manner by pretreatment with naloxone; however, these shifts were not as great, nor as orderly, as the shifts in the generalization curves. Moderate response rate-increasing effects (above saline controls) were observed at 0.1 and 1.0 mg/kg naloxone plus 1.0 mg/kg morphine and 1.0 mg/kg naloxone plus 3.0 mg/kg morphine. While response rate was totally suppressed following a cumulative dose of 30 mg/kg or morphine alone, the response rates following this dose of morphine were 0.28, 0.44 and 0.95 responses/sec with 0.1, 0.3 and 1.0 mg/kg naloxone pretreatment, respectively.

The effects of cumulative doses of naloxone alone (0.1–10.0 mg/kg) and consecutive injections of saline (1–5) on stimulus control in birds trained to discriminate morphine from saline and on response rate are shown in Fig. 2. Neither naloxone (filled circles) nor saline (open circles) produced appreciable morphine-appropriate responding (left panel, Fig. 2). Likewise, neither naloxone up to 10 mg/kg, nor saline resulted in any change in the mean side-key rate of responding (right panel, Fig. 2).

The ED50 values for morphine alone and morphine with naloxone were used to construct a Schild plot to determine the apparent pA_2 for naloxone (Fig. 3). The negative log dose of naloxone in moles/kg was plotted against the log of the

dose ratio minus 1. The linear regression line calculated for these points, constrained to a slope of −1 as dictated by competitive theory, intercepted the abscissa (dose ratio=2) at 6.53 (6.18–6.89) which is the pA_2 (95% confidence limits).

DISCUSSION

The results of this study further support the hypothesis that the discriminative stimulus properties of morphine are mediated by opiate receptors [2]. This study is unique in that this evidence was obtained in the pigeon using a relatively new procedure, the second-order color-tracking schedule of reinforcement. To our knowledge, apparent pA_2 values for naloxone antagonism of morphine discriminative stimulus properties have not been reported in this species, nor have pA_2 values for other antagonists or naloxone antagonism of other agonists been reported in the pigeon. Because of the potential value dose-ratio analysis has for classifying receptor types [12,13] such an approach could prove useful in identifying the neuronal substrates mediating the discriminative stimulus effects of other agonists and agonist-antagonists in the pigeon.

In the present study, increasing doses of naloxone given 5 min prior to the determination of the cumulative morphine dose-effect curves, caused the curves to be shifted progressively, and in a parallel manner, to the right. This presumptive evidence of competative antagonism suggested the use of dose-ratio analytical procedures to quantify the naloxone antagonism. Schild [9] introduced the term pA_2 as a quantitative measure of the strength of a competitive antagonist. Developed originally for antagonism experiments in *in vitro* preparations, investigators have more recently determined the "apparent" pA_2 values from *in vivo* experiments as well, using a variety of endpoints. Many of these studies and the use of pA_2 analysis for *in vivo* experiments are reviewed by Tallarida *et al.* [13].

Dose-ratio analysis has had limited application in drug discrimination pharmacology. Krimmer and Barry [5] reviewed earlier studies of naloxone antagonism of morphine in three groups of rats trained to discriminate three different doses of morphine [4]; the pA_2 values were in close agreement. In rats trained to discriminate fentanyl from saline, the pA_2 values for naloxone antagonism of fentanyl and methadone were higher than those for naloxone antagonism of morphine and heroin, suggesting that the synthetic opioids act on a different population of receptors [5]. The slopes of the linear regression lines used to determine the pA_2 values in these drug discrimination studies were not reported.

Teal and Holtzman [15] and Holtzman [2] have reported pA_2 values for antagonism of the discriminative effects of morphine by naltrexone in squirrel monkeys and rats. In squirrel monkeys, the apparent pA_2 (±SE) was 8.25+0.2 and in rats, 7.69+0.07. In both cases, however, the slope of the regression lines differed from the predicted −1 value dictated by competative theory [12,13]. Shannon *et al.* [11] reported an apparent pA_2 value for naloxone antagonism of morphine discriminative stimulus effects in rats. In this case, the slope was not different from −1 and the pA_2 value (±95% confidence limits) for a Schild plot (constrained to −1) was 7.85+0.36. Dose-ratio analysis is based on a number of assumptions which are difficult to verify *in vivo:* (1) the concentrations of the drugs at the receptors must be proportional to the administered dose; (2) they must be acting in a competative manner according to the Langmuir equation; and (3) the agonist and antagonist must be in equilibrium with the receptor [2, 11, 15]. Failure to meet the first two assumptions can result in Schild plots which vary from the theoretical slope of −1. Failure to meet the third assumption can result in a pA_2 value which underestimates the antagonist equilibrium dissociation constant [11].

In the present study in pigeons, the slope of the Schild plot regression line was not significantly different from −1 suggesting that the first two assumptions were reasonably met and the constrained Schild plot (slope=−1) yielded a pA_2 value (±95% confidence limits) of 6.53±0.36. This value is more than an order of magnitude less than that reported by Shannon *et al.* [11] for the rat and suggests that naloxone is less potent as an antagonist of the discriminative stimulus effects of morphine in the pigeon than in the rat. Indeed, 0.1 mg/kg naloxone (base) was sufficient to block the stimulus effects of 3.0 mg/kg morphine in the rat [11], while in the present study 1.0 mg/kg naloxone (salt) reduced the mean morphine-appropriate responding following 10.0 mg/kg morphine from 98% to 13%. Caution must be exercised in comparing pA_2 values in different species, however, since kinetics are species-dependent [13]. Dose-ratio analysis has proven to be of value in elucidating the nature of receptors mediating the discriminative stimulus properties of opioids in other species. This study demonstrates the feasibility of determining pA_2 values for the discriminative stimulus properties of opiates in the pigeon. Such a quantitative approach should prove useful in elucidating the nature of receptors mediating discriminative stimulus properties of opioids for future studies in pigeons.

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