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α_2-Adrenoceptor Agonists and Stress-Induced Analgesia in Rats: Influence of Stressors and Methods of Analysis

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DE KOCK, M. AND T. F MEERT. *α_2-Adrenoceptor agonists and stress-induced analgesia in rats: Influence of stressors and methods of analysis.* PHARMACOL BIOCHEM BEHAV **58**(1) 109–117, 1997.—The present experiments were designed to investigate the role of housing and handling conditions during testing, as well as data analysis, on the outcome of antinociceptive testing of α_2-adrenoceptor agonists, fentanyl, and a high dose of chlordiazepoxide in the tail withdrawal reaction test (TWR test) in rats. Dose–response curve data were obtained with fentanyl, clonidine, xylazine, dexmedetomidine, and 40.00 mg/kg chlordiazepoxide and were compared under normal TWR test conditions and during immobilization or immobilization with continuous painful stimulation. Data were analyzed in terms of all-or-none criteria as well as percentage maximum possible effect (%MPE) analysis over the total measurement period or at any specific time point during testing. The results indicate that stress, induced by immobilization and immobilization with long-term-applied paw pressure, unmasked possible antinociceptive properties of the various α_2-adrenoceptor agonists and potentiated the effects of fentanyl. Stress also unmasked the positive effects of benzodiazepines. The manner of data analysis was shown to significantly affect the outcome measured in stress and nonstress conditions. The MPE analysis, particularly at one time point, appeared much more sensitive than the all-or-none criteria. The data indicate that the housing and handling conditions of animals during testing, together with data analysis, may affect the outcome of different classes of compounds in the TWR test, and this knowledge may help control for false positive results. © 1997 Elsevier Science Inc.

α_2-Adrenoceptor agonists Benzodiazepine Data analysis Opioid Stress Tail withdrawal reaction test

THE results of analgesia testing with various classes of pharmacological agents in animals may depend not only on the animal models used but also on several external factors, such as handling and housing conditions. The manner in which the results are analyzed can also be a source of variability in outcome (35). Particularly with regard to the efficacy of α_2-adrenoceptor agonists, controversial results have been reported in rodents. Antinociceptive activity has been reported in several pain models, including the formalin test, the hot plate test, and the writhing test (3,7,16,17,20,24,31). In the tail withdrawal reaction test (TWR test), a spinal reflex model particularly sensitive to opioids (11), an antinociceptive effect was not always present with α_2-adrenoceptor agonists. Whereas positive results were reported by several authors (10,23,26, 30), other groups were unable to demonstrate any antinociceptive activity (5,19,24). When these studies are compared, differences in the definition of functional activity (such as a doubling of baseline latencies or a percentage of maximum possible effect between 50 and 80% on one hand vs. all-or-none criteria on the other) and differences in the test conditions (such as single manipulations, repeated handling, anesthetized animals, immobilization, etc.) become apparent.

To evaluate the influence of handling and housing conditions during testing, as well as of different methods of data analysis, on the functional performance of α_2-adrenoceptor agonists in the TWR test, we performed a series of experiments with clonidine, xylazine, and dexmedetomidine in rats subjected to normal handling conditions, immobilization, and immobilization with pain. The results were analyzed by use of all-or-none criteria and maximal possible effect analysis. As

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pharmacological references, the opioid fentanyl and the benzodiazepine chlordiazepoxide were included in the study.

MATERIALS AND METHODS

Animals

Approval from the Institutional Animal Care and Use Committee was obtained to perform the experiments described.

In all tests, male Wistar rats weighing 200–300 g were used. During testing, the animals were housed individually in standard observation cages equipped with a grid floor (normal TWR conditions) or they were placed in bolman cages. The bolman cage consists of a cylinder of metal bars restraining the body of the rat but allowing completely free movement of the tail.

All experimental housing as well as group housing 24 h before testing, was in a laboratory that was air conditioned (temperature 21 ± 1°C, relative humidity 65 ± 5%) and continuously illuminated. All testing took place during the morning between 0800 and 1200 h.

Tail Withdrawal Reaction Procedure

The TWR procedure used has been described in detail by Janssen et al. (11). Briefly, for the normal TWR test, the rat was placed in a cylindrical rat holder with its tail hanging freely outside the cage. The distal 5 cm of the tail was immersed in a hot water bath (55 ± 1°C) and the time for tail withdrawal was measured to the nearest 0.1 s. To minimize tissue damage from repeated testing, a cutoff time of 10.0 s was adopted. With these criteria, control experiments demonstrated that animals could be repeatedly tested over time, for a whole day, if needed, without introducing any shifts in baseline response or any damage to the tail tissue of the animals. For animals in the bolman cages, a similar procedure was used.

Experimental Design

Three groups of testing conditions were used: the TWR was recorded on animals in normal conditions, on animals placed in bolman cages, and on animals placed in bolman cages and with a crocodile clip on their right hind paw. Placement of such a clip did not result in a traumatic tissue reaction over the time period tested. In all conditions, a TWR latency determination was made in advance of any test housing manipulation (referred to as no-stress baseline). For the bolman cage and the bolman cage plus clip groups, a second control TWR latency (referred to as stress baseline) was measured 10 min after placement in the final test condition. For all three conditions, subsequent measurements were taken at 5, 10, 15, 20, 30, 45, and 60 min after treatment. Dose–response curves were collected for fentanyl, clonidine, xylazine, and dexmedetomidine. For each condition, a saline control and a 40.00-mg/kg chlordiazepoxide group were also included. Under each drug and test condition, results for five different animals were collected.

To evaluate whether the effects on TWR latency observed with 0.16 mg/kg clonidine and dexmedetomidine in the bolman cages were naloxone-reversible, additional groups of five animals were tested using combined treatments of subcutaneous injections of 0.16 mg/kg clonidine or dexmedetomidine with an intravenous injection (lateral tail vein) of 2.50 mg/kg naloxone.

Drugs

Clonidine HCl, chlordiazepoxide HCl, dexmedetomidine HCl, fentanyl citrate, xylazine HCl, and naloxone were freshly prepared as aqueous solutions. The drugs were given subcutaneously in a total volume of 1 ml/100 g body weight. Test doses were selected either from the geometrical series 0.00063, 0.0025, . . . , 2.50, 10.00 mg/kg or from the series 0.01, 0.02, . . . , 5.00, 10.00 mg/kg. Naloxone was injected in a volume of 0.2 ml/100 g body weight.

Data Analysis

The TWR latency was evaluated in three different ways. First of all, comparable to the method frequently described, the TWR latency was analyzed in terms of the number of animals reaching the all-or-none criterion of TWR latencies >6.0 and $\geqslant 10.0$ s as indices for significant and profound antinociceptive activity, respectively (5,11,18,19).

Secondly, the data were analyzed in terms of the percentage maximal possible effect (%MPE) (20), according to the formula

$$\%\text{MPE} = \frac{\text{postdrug latency} - \text{predrug latency}}{\text{cutoff time} - \text{predrug latency}} \times 100\,,$$

with the cutoff time being 10.0 s and the predrug latency being the no-stress and the stress baseline latencies in two different series of analyses. Based on these MPE values for each measurement period, mean values for each rat over the entire 60-min observation period were calculated. This analysis is comparable to the maximum area under the curve and is referred to as the maximal possible effect over the entire measurement period.

Thirdly, the highest MPEs obtained in a particular measurement period were also used for calculation of the maximum possible %MPE, which is called the maximal possible effect at any given time point during testing. Reference is made to both the no-stress and stress baselines.

Data on %MPE are presented as mean (±1 SEM) values for five rats per treatment condition.

To evaluate possible statistical differences between experimental conditions evaluated with the all-or-none criterion, the Fisher exact probability test (two-tailed) (28) was used. For the testing of differences in terms of %MPE within groups (at different measurements) or between different groups, the Wilcoxon matched-pairs signed-ranks test and the Mann–Whitney *U*-test (two-tailed) (28) were used, respectively. Because data analysis in terms of analysis of variance with post hoc testing revealed comparable results, these methods will not be reported here.

RESULTS

Saline Controls

With regard to the all-or-none criteria, none of the saline-pretreated animals revealed a TWR latency > 6.0 s in the normal TWR test conditions over the 60-min period (Table 1). After placement of the animals in the bolman cages, the TWR latency increased somewhat, but only one out of five rats ever reached a TWR latency > 6.0 s. At no time did a TWR latency $\geqslant 10.0$ s occur. In rats exposed to both the bolman cage and the clip, three out of five animals reached TWR latencies > 6.0 s, and for one animal even a TWR latency $\geqslant 10.0$ s was noted.

With %MPE analysis over the entire 60-min measurement period, the saline-treated animals under normal testing conditions reached an average %MPE of 4.77 ± 1.73% (Fig. 1, upper panel). In the bolman cages and the bolman cages plus clip, increases in %MPE were observed due to the increases

TABLE 1

TWR LATENCY IN TERMS OF ALL-OR-NONE ANALYSIS

		Normal TWR		Bolman Cage		Bolman Cage + Clip	
Compound	Dose (mg/kg)	TWR > 6.0 s	TWR ⩾ 10.0 s	TWR > 6.0 s	TWR ⩾ 10.0 s	TWR > 6.0 s	TWR ⩾ 10.0 s
Saline		0/5	0/5	1/5	0/5	3/5	1/5
Fentanyl	0.0025			0/5	0/5	0/5	0/5
	0.01	0/5	0/5	5/5*	2/5	4/5	2/5
	0.02			5/5*	5/5**	4/5	3/5
	0.04	2/5	1/5	5/5*	5/5**	5/5	5/5*
	0.16	5/5**	5/5**	5/5*	5/5**	5/5	5/5*
Clonidine	0.00063			2/5	0.5		
	0.0025			3/5	0/5		
	0.01			3/5	0/5		
	0.04			3/5	2/5	2/5	0/5
	0.16	0/5	0/5	5/5*	1/5	0/5	0/5
	0.63	0/5	0/5	3/5	0/5	0/5	0/5
	2.50	0/5	0/5	0/5	0/5	1/5	0/5
	10.00	1/5	0/5				
Xylazine	0.63	0/5	0/5	1/5	0/5	3/5	0/5
	2.50	0/5	0/5	1/5	0/5	1/5	1/5
	10.00	0/5	0/5	4/5	1/5	2/5	2/5
	40.00	1/5	0/5	0/5	0/5	0/5	0/5
Dexmedetomidine	0.0025	0/5	0/5				
	0.01	0/5	0/5	0/5	0/5	4/5	0/5
	0.04	0/5	0/5	2/5	2/5	4/5	1/5
	0.16	2/5	2/5	5/5*	5/5**	4/5	3/5
	0.63	2/5	0/5	5/5*	2/5	3/5	3/5
	2.50	0/5	0/5	5/5*	3/5	5/5	5/5*
Chlordiazepoxide	40.00	0/5	0/5	1/5	0/5	5/5	5/5*

Given for the different test conditions are the numbers of animals reaching a TWR latency > 6.0 s and ⩾ 10.0 s over the number of animals tested. Differences from the saline-treated controls were evaluated using the Fisher exact probability test (two-tailed): *$p < 0.05$, **$p < 0.01$.

in TWR latencies after immobilization and immobilization plus clip before the injection of saline. With the baseline under normal conditions (no-stress baseline) taken into account as a starting reference, the mean %MPE value for the saline-treated animals increased from 4.77 ± 1.73% to 16.67 ± 3.60% ($p > 0.05$) and 35.64 ± 7.13% ($p < 0.05$) in the bolman cages and the bolman cages plus clip, respectively (Fig. 1, middle and lower panels). With the stress baselines before the injections taken into account, saline did not result in a detectable effect. Thus, although the TWR latencies became longer in the saline-treated animals in the bolman cages with and without clip, the average %MPE values clearly depend on the control latencies used as a reference. For the saline-treated group in the bolman cage with clip, for instance, the mean %MPE was 35.64 ± 7.13% with reference to the no-stress baseline latency and 6.60 ± 3.60% with reference to the stress baseline latency ($p < 0.05$). Therefore, all drug data in the bolman cages and the bolman cages plus clip were evaluated using both the no-stress and the stress baseline latencies.

A similar effect was observed when the mean %MPE at any given time point during testing was used, a very sensitive way to detect a change in the results. With this method, the mean %MPE values were mostly higher than those when the whole measurement period was taken into account. For the saline controls under normal TWR test conditions, however, there was no difference ($p > 0.05$) between the mean %MPE obtained over the entire treatment period (4.77 ± 1.73%) and at one moment (4.65 ± 1.43%). In the bolman cages, the mean %MPE of the saline controls reached 29.60 ± 6.62% and 15.39 ± 4.67%, respectively, for the no-stress and stress baseline latencies (Fig. 2, middle panel). In the bolman cages plus clip, the average %MPE of the saline controls became higher, and a significant difference ($p < 0.05$) was present between the mean values calculated on the basis of the no-stress (61.67 ± 12.53%) and the stress (26.76 ± 13.35%) baseline latencies (Fig. 2, lower panel).

Fentanyl

Fentanyl produced a dose-dependent antinociceptive effect in all three test conditions, and this was independent of the method of data analysis. In terms of all-or-none criteria (Table 1), the doses of fentanyl resulting in TWR latencies of > 6.0 and ⩾ 10.0 s in all animals tested dropped from 0.16 mg/kg in the normal TWR condition to 0.01 mg/kg (TWR latency > 6.0 s) and 0.02 mg/kg (TWR latency ⩾ 10.0 s) after placement of the rats in bolman cages. For the bolman cage plus clip condition, a complete antinociceptive activity (TWR latency ⩾ 10.0 s) was observed with 0.04 mg/kg fentanyl in all animals tested. Under all three test conditions and at all the doses of fentanyl tested, there were usually significant differences from saline-treated controls (Table 1).

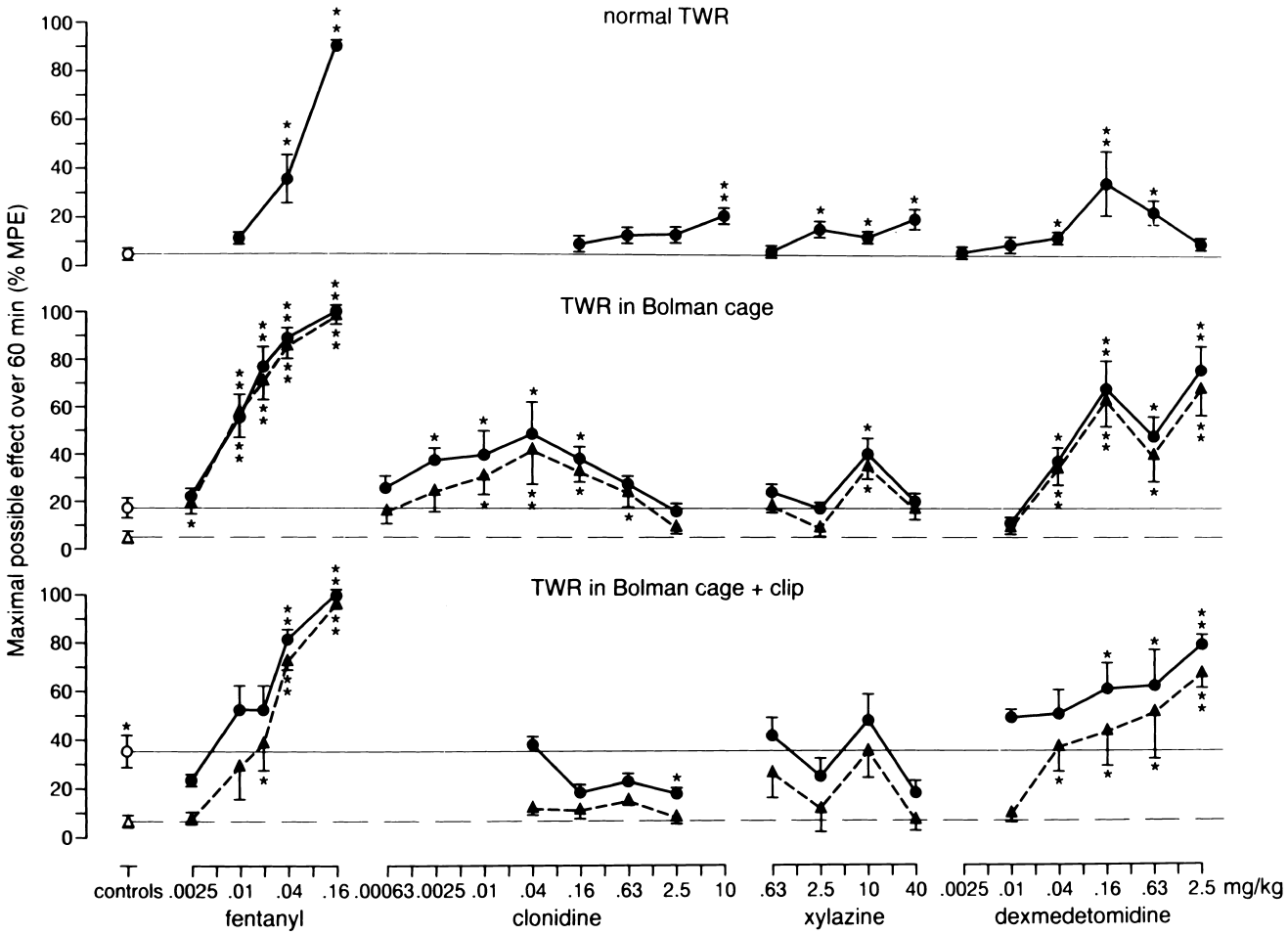

FIG. 1. Antinociceptive activity of various agents in the TWR test as a function of test conditions. Given are dose–response curves of the compounds tested in terms of the maximum possible effect over the entire 60-min measurement period, an analysis comparable to area under the curve. The animals were tested under normal housing conditions (upper panel), after being placed in bolman cages (middle panel), and after being placed in bolman cages with a clip on one hind paw. The data were plotted as a function of the nonstressed (circles with solid lines) and stressed (triangles with dashed lines) baseline latencies. Statistical differences from the corresponding saline controls were evaluated with the Mann–Whitney *U*-test (28): $*p < 0.05$, $**p < 0.01$.

In terms of the %MPE over 60 min, the first significant difference from saline was present: a) with 0.04 mg/kg fentanyl in normal test conditions (Fig. 1, upper panel), b) with doses $\geq$0.0025 or 0.01 mg/kg fentanyl in the bolman cages (Fig. 1, middle panel), and c) with 0.02 or 0.04 mg/kg fentanyl in bolman cage plus clip (Fig. 1, lower panel), depending on the no-stress or stress baseline latencies for the two last conditions. With the analysis in terms of %MPE at one time point (Fig. 2), the lowest effective dose of fentanyl was: a) 0.01 mg/kg in normal test conditions, b) 0.01 mg/kg in the bolman cage, and c) 0.04 mg/kg in the bolman cage plus clip; this was independent of the use of stress or no-stress baseline latencies as starting points.

Clonidine

With clonidine, using all-or-none criterion analysis, no antinociceptive activity (TWR latency > 6.0 s) was detected under normal test conditions at doses ranging from 0.00063 to 10.00 mg/kg (Table 1). In the bolman cages, all animals revealed a TWR latency > 6.0 s after 0.16 mg/kg clonidine, reaching a significant difference from saline controls. Higher doses of clonidine resulted in fewer animals reaching the TWR > 6.0 s criterion, producing a biphasic dose–response curve. Only three out of all animals tested reached a TWR latency $\geq$ 10.0 s. Also, in the bolman cages with clip, very limited effects were observed, without any difference from saline controls. In terms of %MPE over 60 min, clonidine reached a significant difference from saline only at 10.00 mg/kg under normal conditions (Fig. 1, upper panel). In the bolman cages, activity started at 0.0025 or 0.01 mg/kg clonidine, depending on the baseline used (Fig. 1, middle panel). In the bolman cages with clip, no differences from saline were present (Fig. 1, lower panel). At 2.50 mg/kg clonidine, values lower ($p <$ 0.05) than the saline controls were observed. With the analysis in terms of %MPE at one time point (Fig. 2), a more sensitive outcome in terms of lowest active dose was detected than during normal testing. Here, a lowest active dose of 0.63 mg/kg clonidine was obtained (Table 2).

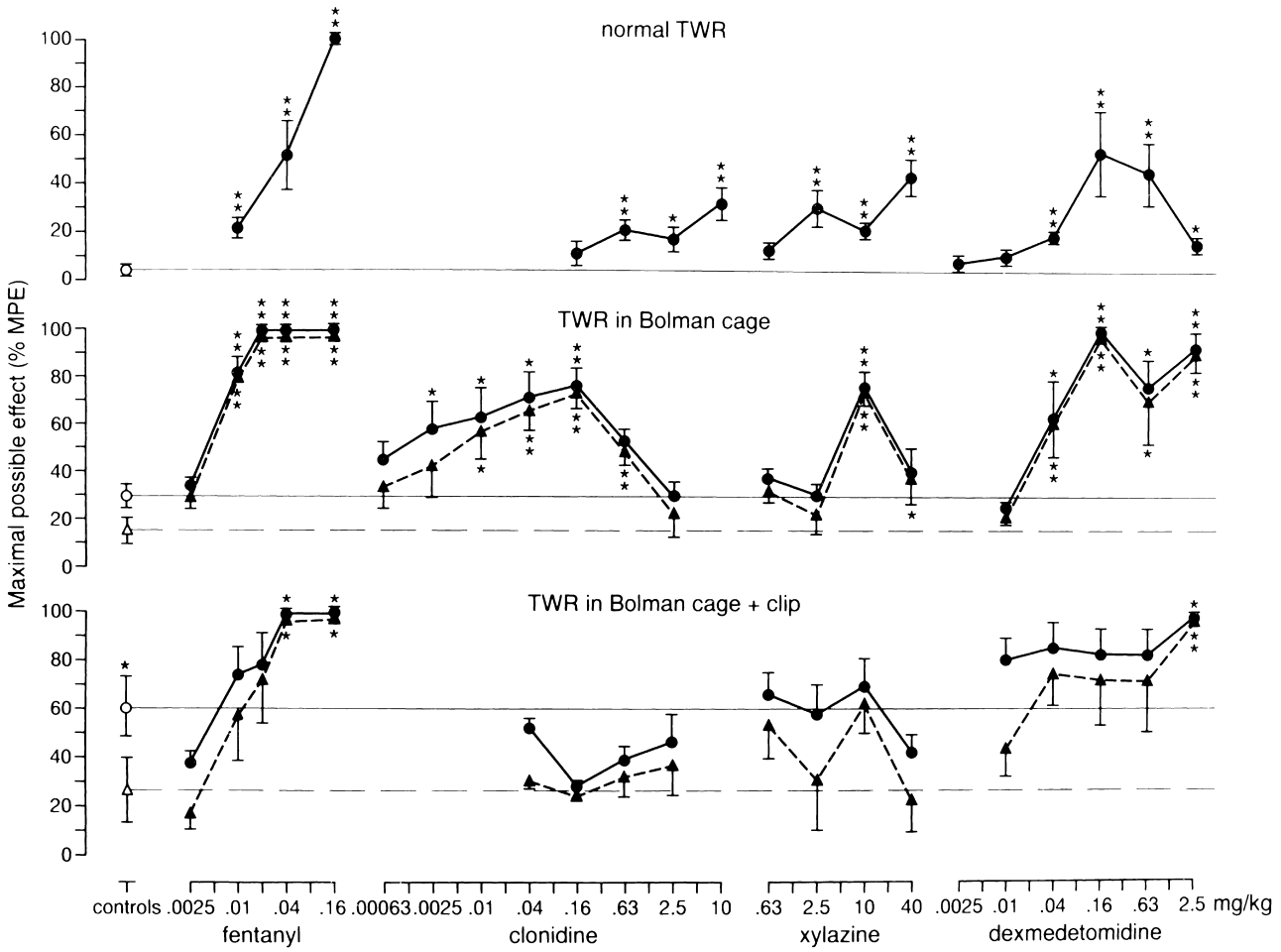

FIG. 2. Antinociceptive activity of various agents in the TWR test as a function of test conditions. Given are dose–response curves of the compounds tested in terms of the maximum possible effect at any one time point during testing. The animals were tested under normal housing conditions (upper panel), after being placed in bolman cages (middle panel), and after being placed in bolman cages with a clip on one hind paw. The data were plotted as a function of the nonstressed (circles with solid lines) and stressed (triangles with dashed lines) baseline latencies. Statistical differences from the corresponding saline controls were evaluated with the Mann–Whitney *U*-test (28): $*p < 0.05$, $**p < 0.01$.

Xylazine

In terms of all-or-none criteria, xylazine was without any effect at the doses used in the various test conditions (Table 1). Both methods of %MPE analysis revealed activity from 2.50 to 40.00 mg/kg xylazine in normal TWR testing (Figs. 1, 2). In the bolman cage, activity was consistently present with both analyses at 10.00 mg/kg xylazine. In the bolman cage with clip, no functional differences from saline were present.

Dexmedetomidine

Dexmedetomidine did not show any significant antinociceptive activity during normal TWR testing with the all-or-none criterion (Table 1). However, in contrast to the other α_2-adrenoceptor agonists and the saline-treated animals, some rats (two out of five) reached the antinociceptive criterion of TWR latency $\geq$ 10.0 s. In the bolman cages, some dose-dependent antinociceptive activity was measured. For TWR latency $>$ 6.0 s, all animals reached the criterion from doses $\geq$ 0.16 mg/kg, showing statistical differences from saline controls. For the TWR latency $\geq$ 10.0 s criterion, complete antinociceptive activity was measured at 0.16 mg/kg dexmedetomidine. However, higher doses resulted in less activity. Adding a clip to the animals in the bolman cages resulted in gradually more animals reaching the required criteria for antinociception. Only with 2.50 mg/kg dexmedetomidine was a significant difference from saline controls noted. With dexmedetomidine, both %MPE analyses resulted in inverted U-shaped curves under normal TWR testing conditions (Figs. 1, 2, upper panels). The first active dose in both cases was 0.04 mg/kg dexmedetomidine, and the maximum effect was present at 0.16 mg/kg, with maximum percentages lower than the 50% MPE level. In the bolman cages, the dose–response curves of dexmedetomidine were comparable to those of fentanyl (Figs. 1, 2, middle panels). Activity started at 0.04 mg/kg dexmedetomidine, and a nearly maximum efficacy was measured with 0.16 mg/kg dexmedetomidine. Higher doses resulted in comparable outcomes, revealing a ceiling effect. In the bolman cages plus clip, maximum activity was present with both %MPE analyses at 2.50 mg/kg dexmedetomidine.

TABLE 2

SUMMARY OF THE ANTINOCICEPTIVE ACTIVITY OF THE AGENTS TESTED IN THE THREE TEST CONDITIONS

Compound	Test Condition	All-or-None		% MPE over 60 min	% MPE at One Time Point
		TWR > 6.0 s	TWR ⩾ 10.0 s		
Fentanyl	Normal TWR	0.16	0.16	0.04	0.01
	Bolman cage	0.01	0.02	0.01	0.01
	Bolman cage + clip	No	0.04	0.02	0.04
Clonidine	Normal TWR	No	No	10.00	0.63
	Bolman cage	0.16	No	0.0025	0.0025
	Bolman cage + clip	No	No	No	No
Xylazine	Normal TWR	No	No	2.50	2.50
	Bolman cage	No	No	10.00	10.00
	Bolman cage + clip	No	No	No	No
Dexmedetomidine	Normal TWR	No	No	0.04	0.04
	Bolman cage	0.16	0.16	0.04	0.04
	Bolman cage + clip	No	2.50	0.04	2.50
Chlordiazepoxide	Normal TWR	No	No	40.00	40.00
	Bolman cage	No	No	40.00	40.00
	Bolman cage + clip	No	40.00	40.00	40.00

Given, compared with the corresponding saline controls, are the lowest active doses (in mg/kg) as a function of data analysis. Data were evaluated in terms of reaching the all-or-none criterion of a TWR latency > 6.0 s and ⩾ 10.0 s, the percent maximal possible effect (%MPE) over the 60-min measurement period and the %MPE at one measurement period. "No" refers to no statistical difference from the saline control. For chlordiazepoxide, only 40.00 mg/kg was tested.

Chlordiazepoxide

Using the all-or-none criterion, chlordiazepoxide tested at a dose of 40.00 mg/kg resulted in antinociceptive activity in animals placed in bolman cages with clip. Under these conditions, all animals reached TWR latency ⩾ 10.0 s (Table 1).

Independent of the %MPE analysis, 40.00 mg/kg chlordiazepoxide had a limited effect (but significantly different from saline activity) during normal TWR testing (Fig. 3, left section). The %MPE was limited to nearly 20%. For both methods of analysis, the maximum %MPE observed with 40.00 mg/kg chlordiazepoxide increased to about 40% in the bolman cages (Fig. 3; middle section) and to 100% in the bolman cages plus clip (Fig. 3, right section).

To summarize all of the different test results in a more systematic way, a review table is presented (Table 2). Given are the lowest active doses showing significant differences from saline under the different test conditions and using the various methods of data handling. In the conditions of the bolman cages and the bolman cages plus clip, a significant difference from saline had to be present using both the stress and no-stress baseline latencies to overcome possible bias in the statistical analysis.

Naloxone Reversibility

In the bolman cages, a dose of 2.50 mg/kg naloxone given intravenously together with either 0.16 mg/kg clonidine or 0.16 mg/kg dexmedetomidine prevented increases in TWR latencies induced by the α_2-adrenoceptor agonists. Table 3 represents for each individual animal the prestress baseline latency in the TWR test, the stress-baseline latency after the placement of the animal in the bolman cage, and the maximal observed TWR latency at any moment during the 60-min testing period after treatment with the α_2-adrenoceptor agonist in combination with naloxone. At no time, and independent of the different ways of analyzing the results, were differences ($p > 0.05$) from the control latencies observed.

DISCUSSION

The results of the present study clearly indicate that handling and housing conditions affect the activity of various α_2-adrenoceptor agonists, of fentanyl, and of chlordiazepoxide in the TWR test in rats. The measured antinociceptive activity of clonidine and dexmedetomidine, in particular, increased in animals subjected to immobilization produced by placing the animals in bolman cages. The efficacy was observed in terms of more animals reaching higher levels of functional antinociception (increased TWR latencies) and in terms of decreases in the doses showing significant differences from saline controls. Immobilization thus seems to unmask the antinociceptive properties of clonidine and dexmedetomidine in the TWR testing. In saline-treated animals, immobilization in bolman cages also resulted in higher antinociception values. Because immobilization is an important stressor in rodents (13), the above-mentioned results may be ascribed to stress-induced analgesia. As a consequence, the increased functional activity of the α_2-adrenoceptor agonists during immobilization may be the result of a positive interaction between these substances and the endogenous mechanism underlying stress-induced analgesia. The functional interaction between α_2-adrenergic mechanisms and stress-induced analgesia has been reported using various stressors and animal pain models (2,4,22,29). Stress-induced antinociception is a complex phenomenon that probably involves several endogenous pain-modulating systems.

Immobilization stress-induced analgesia is recognized as an endogenous opiate-mediated phenomenon (9,15). This is confirmed in our experiments by the reversal of antinociceptive properties of 0.16 mg/kg clonidine or 0.16 mg/kg dexme-

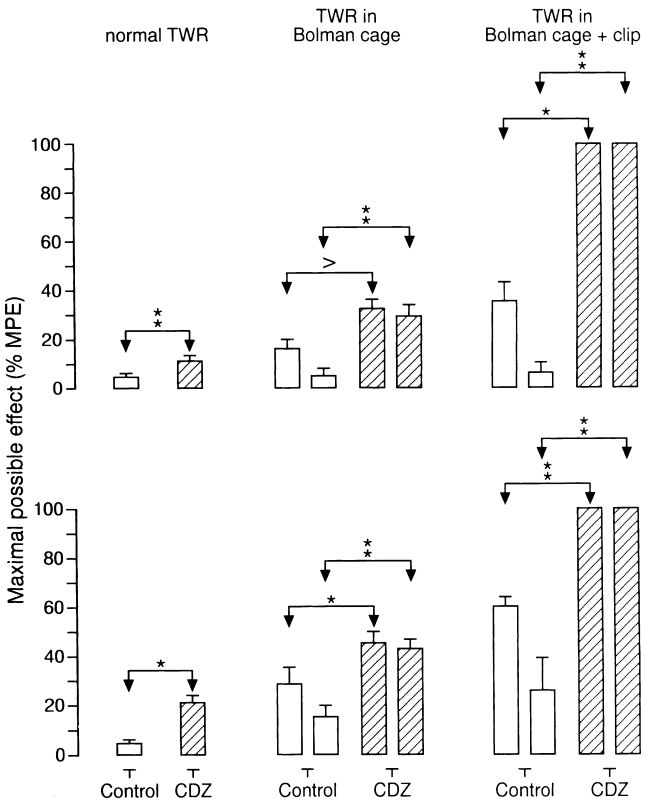

FIG. 3. Antinociceptive activity of 40.00 mg/kg chlordiazepoxide in the TWR test as a function of test conditions. Data are presented in terms of the maximum possible effect over the entire 60-min measurement period (upper panel) and in terms of the maximum possible effect measured at one time point during testing (lower panel). The data were plotted as a function of the nonstressed (left bars) and stressed (right bars) baseline latencies in the bolman cages and the bolman cages plus clip. Statistical differences from the corresponding saline controls were evaluated with the Mann–Whitney *U*-test (28): $*p < 0.05$, $**p < 0.01$.

detomidine by naloxone in animals placed in the bolman cages. Because the endogenous opiate system appears to be activated during immobilization stress, it is understandable that the minimal effective dose of the opioid fentanyl is reduced during TWR testing in bolman cages. Potentiation of opioid antinociception with endogenous endorphins or enkephalins, as well as exogenously administered endorphins and enkephalins, has been demonstrated repeatedly (36). The potentiation of the endogenous opiate system with α_2-adrenoceptor agonists may also account for the functional antinociceptive activity observed here with the various α_2-adrenoceptor agonists in the bolman cages. Such a potentiation between the α_2-adrenergic and the opiate systems is regularly demonstrated in animal and human studies by the co-administration of α_2-adrenoceptor agonists and opioids (1,5,6,18,19,33).

The biphasic dose–response curves obtained with clonidine (and somewhat for xylazine), as well as the plateau reached with dexmedetomidine at the doses tested here, fit with the idea of an interaction between the α_2-adrenoceptor and the opiate systems. The reduced activity with clonidine at the higher dose range may be explained by an increasing presence of α_1-adrenergic activity, which can overrule the antinociceptive effects due to an interaction between the α_2-adrenoceptor and

TABLE 3

REVERSAL OF THE ANTINOCICEPTIVE PROPERTIES OF CLONIDINE AND DEXMEDETOMIDINE WITH NALOXONE IN THE TWR TEST DURING STRESS

	Animal				
Condition/Treatment	#1	#2	#3	#4	#5
Clonidine					
Prestress baseline	1.4	2.0	1.4	1.4	1.8
Stress baseline	3.1	2.4	1.6	4.2	3.2
Maximal TWR latency	3.0	3.6	3.4	3.4	4.6
Dexmedetomidine					
Prestress baseline	1.8	1.6	2.0	3.2	2.0
Stress baseline	3.0	2.0	4.0	2.6	3.2
Maximal TWR latency	2.9	4.0	3.1	4.4	4.1

Given for each animal tested are the prestress baseline latencies, the stress baseline latencies after placement in the bolman cages, and the maximal observed latencies obtained at any moment during the 60-min testing period. Drug treatment consisted of a combination of a subcutaneous injection with 0.16 mg/kg of either clonidine or dexmedetomidine with an intravenous injection of 2.5 mg/kg of naloxone. The TWR latencies are given in seconds.

the opiate systems. A similar effect was described after systemic application of α_2-adrenoceptor agonists with opioids (19). The ceiling effects observed with the dose range of dexmedetomidine tested can be explained by an important antistress effect of selective α_2-agonism (32), which may limit the output of the stress-induced release of endogenous opiates. A similar antistress effect of the α_2-adrenoceptor agonists may also account for the biphasic dose–response curves observed with the other α_2-adrenoceptor agonists. Whether higher doses of dexmedetomidine would result in a biphasic dose–response curve, as observed with the other α_2-adrenoceptor agonists, could not be determined from our data.

When a pain stimulus was added to immobilization stress, by a clip being put onto the animal's hind paw during testing, smaller differences from saline controls were observed with the α_2-adrenoceptor agonists and fentanyl as compared with the results in the bolman cages alone. The reduced activity was mainly due to an increased baseline observed in the saline controls. If for fentanyl and dexmedetomidine, for instance, the data from doses in the bolman cages are compared with those obtained in the bolman cages plus clip, no statistical differences were found. For clonidine and xylazine, some reduced antinociceptive properties were seen after the additional placement of the clip. The functional differences in agonistic activity of these two agents for α_2-adrenoceptors at higher doses, or the insufficient antinociceptive activity on the additional introduction of pain stimuli, may account for the drop in activity observed. Furthermore, our behavioral observations during normal TWR testing indicated an increased reactivity to stimulation at the higher doses of xylazine and clonidine, which could interfere with the responding to the TWR stimulation.

The benzodiazepine chlordiazepoxide resulted in functional activity during various handling conditions at a dose of 40.00 mg/kg. Chlordiazepoxide or benzodiazepines in general, as the α_2-adrenoceptor agonists, have been reported to potentiate opioid analgesia (12,34). An intrinsic antinociceptive activity of benzodiazepine alone, as, for instance, based on interaction with spinal GABA receptors (8), may not account for the functional effects observed here due to the lack of effi-

cacy in normal TWR testing conditions. Maximal antinociceptive activity was detected in animals placed in bolman cages with clip. This effect was comparable with the results obtained with 0.04 mg/kg fentanyl in a similar test condition. Compared with normal TWR testing, it is still a fourfold decrease in the dose of fentanyl, pointing to the functional role of the test environment on the outcome. The increased activity of chlordiazepoxide, especially compared with the α_2-adrenoceptor agonists, might be explained in terms of a broader potentiating effect of the benzodiazepine. Pain plus stress, as presented by placement of the animals in bolman cages with clip, may induce the release of important amounts of endogenous opiates and norepinephrine (NE). Benzodiazepines not only potentiate the effects of the opiate system (12,35) but they can also potentiate the α_2-adrenergic activity of endogenous norepinephrine, as they do for exogenous α_2-adrenoceptor agonists (27).

The α_2-adrenoceptor agonists have some limiting effects on the functional role of NE because of a presynaptic inhibitory control on the release of NE in the central nervous system (14). As a net result, benzodiazepines might have broader potentiating activity than α_2-adrenoceptor agonists.

Although benzodiazepines possess potentiating effects on the analgesia provided by various classes of analgesics, they are not recognized as analgesic compounds on their own in clinical settings. It should be noted that the results obtained here with chlordiazepoxide might be explained in terms of a change in the awareness of the painful stimuli because of their well-known hypnotic, sedative, and anxiolytic properties (21,25).

The second variable considered in this study was the influence of the method of data analysis on the functional performance of α_2-adrenoceptor agonists in the TWR test. Globally it appears that the %MPE analysis, and more specifically the %MPE at one time point, is more sensitive than all-or-none criteria for objectively assessing the antinociceptive properties of various compounds in the TWR test. The increased sensitivity for detecting antinociceptive properties was present in terms of either a reduction in the lowest active doses or, in some cases, compounds even becoming active. For example, whereas the α_2-adrenergic agonists and chlordiazepoxide were without any effect in the TWR testing under normal conditions using the all-or-none criteria (TWR latency > 6.0 s or $\geqslant 10.0$ s), antinociceptive activity was present using the %MPE analysis (Table 2). Furthermore, the %MPE analysis appeared to be sensitive to the baseline values used. The use of baseline latencies before or after stress application may considerably affect the results (Figs. 1–3).

In conclusion, the present series of experiments indicates that housing and handling conditions may affect the performance of different classes of compounds in the TWR test in rats. The use of highly sensitive methods of data analysis may also have an important effect on the outcome of antinociceptive drug testing. When clinical reality is taken into account, we may advocate the use of the somewhat less sensitive but more robust methods of data analysis (such as the all-or-none criteria) because benzodiazepines and α_2-adrenoceptor agonists, as used alone for acute pain treatment, do not appear to possess major analgesic properties.

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