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Naloxone potentiates anxiolytic-like actions of diazepam, pentobarbital and meprobamate but not those of Ro19-8022 in the rat

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Abstract

The elevated plus-maze test was used to determine if the opiate antagonist naloxone could potentiate the anxiolytic-like effects of the benzodiazepine diazepam, the barbiturate pentobarbital, the propanediol carbamate meprobamate and the partial benzodiazepine receptor agonist [*R*]-1-[(10-chloro-4-oxo-3-phenyl-4*H*-benzo[*a*]quinolizin-1-yl) carbonyl]-2-pyrrolidine-methanol (Ro19-8022) in the rat. A subeffective dose of each of these compounds was combined with naloxone, 10 mg/kg. Naloxone had no effect by itself, but potentiated all drugs except Ro19-8022. The proportion of entries on the open arm increased while the total number of arms entries was not modified. These results coincide with and extend data previously obtained in the mouse. One possible explanation for naloxone's effect could be that it blocks opioid inhibition of GABAergic (γ-aminobutyric acid) neurons thereby enhancing the effects of benzodiazepines. Another possibility is that naloxone blocks opioid effects on adenosinergic systems.

Keywords: Anxiety; Plus-maze; Opiate; Benzodiazepine; Naloxone

1. Introduction

We have previously reported that naloxone potentiates the anxiolytic-like actions of subeffective doses of the benzodiazepines chlordiazepoxide and diazepam, the propanediol carbamate meprobamate and the 5-HT_{1A} receptor agonist buspirone in the elevated plus-maze and in the light/dark choice procedure in mice (Belzung and Ågmo, 1997; Belzung et al., 2000). A recent study showed that naloxone also potentiates the anxiolytic-like effects of a tachykinin NK_3 receptor agonist, senktide, in the elevated plus-maze test in mice (Ribeiro and De Lima, 1998). The potentiation seems to be specific to anxiolytic-like effects since motor actions of the drugs were not modified by naloxone. It has also been reported that naloxone fails to potentiate the amnesic action of chlordiazepoxide in the radial maze or in a passive avoidance task (Belzung and Dubreuil, 1998).

All studies reporting naloxone-induced potentiation of anxiolytic-like effects have been performed on mice. It seems essential to determine if this potentiation is a peculiarity for mice, or if it also occurs in another commonly used species, the rat. This was the main purpose of the present study. In order to obtain some additional information concerning the mechanism of action, we included a partial benzodiazepine receptor agonist, the quinolizinone [*R*]-1-[(10-chloro-4-oxo-3-phenyl-4*H*-benzo[*a*]quinolizin-1-yl) carbonyl]-2-pyrrolidine-methanol (Ro19-8022) (Jenck et al., 1992), and a barbiturate, pentobarbital, in addition to drugs already used in mice, viz. diazepam and meprobamate. Thus, the present studies not only extend previous research from the mouse to the rat but also to drugs not previously used in this kind of studies.

2. Materials and methods

2.1. Subjects

Male Wistar rats were purchased from Centre d'Elevage Janvier (Le Genest St. Ile, France) and were housed in pairs in standard rat cages. Commercial rodent pellets and tap water were available ad libitum. The room temperature was about 22°C and humidity was not controlled. Lights

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were on between 2000 and 0800 h. Experiments started about 3 weeks after the animals' arrival to the laboratory. The subjects were treated in accordance with the European Community Council directive 86/609/EEC.

2.2. Apparatus and procedure

The plus-mazes were made of plywood and the arms' surface was covered by a kind of gray rubber carpet. The arms measured 50 * 10 cm, and they were united by a central platform measuring 10 × 10 cm. Two opposing arms had 40-cm-high walls on three sides. The mazes were elevated to a height of 50 cm above floor level. A video camera was installed about 1.5 m above the center of the maze. The light intensity during experimental sessions was 5.7 lx on the surface of the open arms and 1.2 lx on the closed arms. At the beginning of a test, the rat was placed on the central platform with its head facing an open arm. The experimental sessions were recorded on tape and analyzed later. The frequency of visits to the arms was registered with a hand-held computer (Psion organizer). A rat was considered to be on the central platform when at least two paws were on it (i.e. either both front paws or both hind paws or all four paws) and on an arm whenever the four paws were on it. All tests were performed between the 3rd and the 7th hour of the dark period. The procedure has been described in detail previously (Ågmo et al., 1995).

2.3. Drugs

Diazepam and Ro19-8022 were both obtained from Hoffman-La Roche, Basel, Switzerland and suspended in physiological saline to which a drop of Tween® 80 was added to each 5 ml. The suspension was sonicated for about 5 min and shaken immediately before use. Pentobarbital and naloxone HCl (both from Sigma, St. Louis, MO) were dissolved in physiological saline whereas meprobamate was obtained as a commercial solution (Equanil®, Parke-Davis). The solution was diluted to the appropriate concentration with distilled water. The interval between drug injection and test was 30 min for diazepam, Ro19-8022 and meprobamate and 15 min for pentobarbital and naloxone. All drugs were injected intraperitoneally in a volume of 1 ml/kg body weight. Saline was used as control. All animals in all experiments were given a total of two injections so as to keep stress associated with the injection procedure constant.

The intervals between drug administration and test employed here are standard intervals used in many behavioral studies in rats (e.g. Ågmo et al., 1995, naloxone and diazepam in the plus-maze test, pentobarbital in the Vogel conflict test; Jenck et al., 1992, Ro19-8022 in the Geller-Seifter conflict test and in the pentetrazole anticonvulsant activity test; Vogel et al., 1971, meprobamate in the Vogel conflict test) and coincide with maximal plasma and/or brain concentrations (e.g. Tepperman et al., 1983; Guthrie et al., 1987; Sato et al., 1995).

2.4. Design

A parallel group design was used in such a way that all treatments in a particular experiment were run at every session. The order of treatments within the session was counterbalanced between animals. Since it was not practically possible to run all animals in a given experiment at a single session, a small number of subjects (3–4) per treatment were run at each session. This was then repeated until there was at least 10 animals per treatment. Due to a technical failure, there is one group with data from only eight animals.

2.5. Statistics

The total number of entries onto the arms as well as the proportion of entries upon the open arms, expressed as percent, ((number of entries on the open arms/total number of entries) × 100) were evaluated with one-factor analyses of variance or the *t*-test when only two groups were used. A posteriori tests were performed with the Neuman–Keul procedure.

It has repeatedly been reported that the proportion of open arm entries is a sensitive indicator of anxiolytic-like effects while the total number of entries represents ambulatory activity (Pellow et al., 1985; Cruz et al., 1994). Thus, these two parameters contain all the useful information that can be obtained on the plus-maze.

3. Results

As can be seen in Fig. 1, diazepam increased the proportion of open arm entries at a dose of 1 mg/kg,

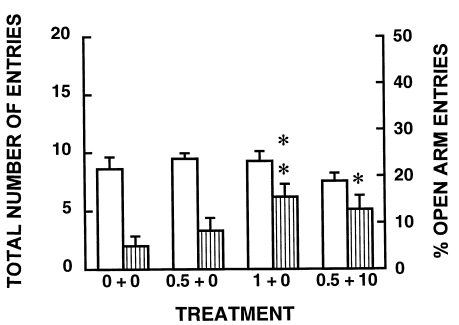

Fig. 1. Total number of arms entries (open bars, left axis) and proportion of open arm entries (striped bars, right axis) in male rats treated with diazepam alone or in combination with naloxone. The diazepam dose is given to the left of the plus sign and the naloxone dose (both in mg/kg) to the right. Data are mean + S.E.M. ANOVA revealed differences between groups for proportion of open arm entries, $F(3,56) = 3.09$, $P = 0.03$, but not for total number of arms entries, $F(3,56) = 1.23$, NS. $N = 15$ in all groups. *, **, Different from control, $P < 0.05$ and $P < 0.01$.

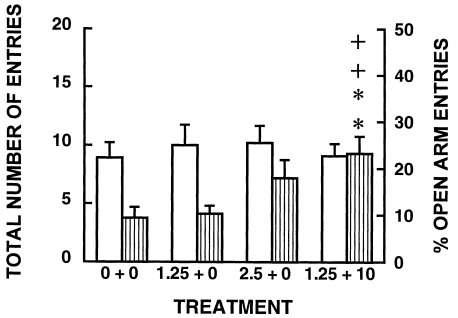

Fig. 2. Total number of arms entries (open bars, left axis) and proportion of open arm entries (striped bars, right axis) in male rats treated with pentobarbital alone or in combination with naloxone. ANOVA showed that there was a significant difference between groups for proportion of open arm entries, $F(3,40) = 5.08$, $P = 0.004$, but not for total number of entries, $F(3,40) = 0.25$, NS. Number of animals per group varied from 10 to 12.**, Different from control, $P < 0.01$; ++, different from pentobarbital 1.25 + saline, $P < 0.01$.

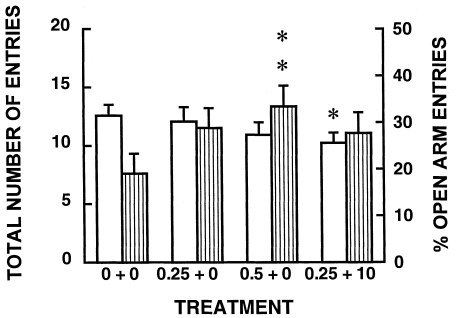

Fig. 4. Total number of arms entries (open bars, left axis) and proportion of open arm entries (striped bars, right axis) in male rats treated with the partial benzodiazepine agonist Ro19-8022 alone or in combination with naloxone. ANOVA revealed a significant difference for the proportion of open arm entries, $F(4,47) = 44.42$, $P < 0.001$, as well as for the total number of arm entries, $F(4,47) = 130.26$, $P < 0.001$). $N = 13$ in all groups except in controls where $N = 12$. *, Different from saline + saline, $P < 0.05$.

whereas 0.5 mg/kg was ineffective. The drug did not modify the total number of arms entries. When the 0.5 mg/kg dose was combined with naloxone, 10 mg/kg, the proportion of open arm entries was significantly increased while no effect was observed on total number of entries.

Two doses of pentobarbital, 1.25 and 2.5 mg/kg, were used. None of them had any significant effect on plus-maze parameters. However, the proportion of open arm entries appeared to be increased by the 2.5 mg/kg dose, although statistical significance was not obtained. Nevertheless, we decided to combine the totally ineffective dose of 1.25 mg/kg with naloxone, 10 mg/kg. The proportion of open arm entries was now not only different from that of controls but also from that of animals given the same dose of pentobarbital + saline. Thus, a clear-cut potentiation of the anxiolytic-like action of pentobarbital was obtained. No effect was obtained on the total number of arms entries, showing that locomotor activity was unaffected. Data are shown in Fig. 2.

Meprobamate, 120 mg/kg, increased the proportion of open arm entries while 60 mg/kg was ineffective. Neither dose affected the total number of arm entries. When the ineffective dose (60 mg/kg) was combined with naloxone, 10 mg/kg, it significantly increased the proportion of open arm entries without modifying total number of entries. Results are illustrated in Fig. 3.

The partial benzodiazepine receptor agonist Ro19-8022 showed an anxiolytic-like effect at a dose of 0.5 mg/kg, increasing the proportion of open arm entries. A lower dose was ineffective. When this lower dose was combined with naloxone, 10 mg/kg, the only effect obtained was a reduction of the total number of arm entries. It appears, then, that the combined treatment reduced locomotor activity without showing any sign of anxiolytic-like effects. Data are displayed in Fig. 4.

Naloxone was by itself ineffective (Fig. 5).

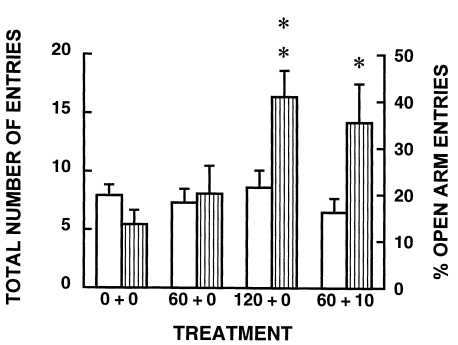

Fig. 3. Total number of arms entries (open bars, left axis) and proportion of open arm entries (striped bars, right axis) in male rats treated with meprobamate alone or in combination with naloxone. Groups differed significantly with regard to the proportion of open arm entries, $F(3,37) = 4.95$, $P = 0.005$, but not with regard to the total number of entries, $F(3,37) = 0.55$, NS. Number of subjects in each group varied from 8 to 13. *, ** Different from control, $P < 0.05$ and $P < 0.01$, respectively.

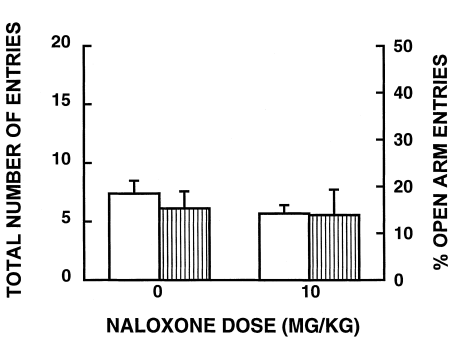

Fig. 5. Total number of arms entries (open bars, left axis) and proportion of open arm entries (striped bars, right axis) in male rats treated with naloxone. All subjects were given a saline injection 15 min before naloxone. *t*-tests showed no difference between controls and naloxone-treated rats ($t(18) = 0.24$, NS for the proportion of open arm entries and $t(18) = 1.36$, NS, for the total number of entries). $N = 10$ in both groups.

4. Discussion

Subeffective doses of diazepam, pentobarbital and meprobamate turned out to have anxiolytic like effects in rats when combined with a dose of naloxone that did not affect plus-maze behavior by itself. It can, then, be maintained that naloxone has the capacity to potentiate anxiolytic-like effects of these drugs. It cannot be argued that naloxone is potentiated because this drug lacks anxiolytic-like effects even in very large doses (Ågmo et al., 1995). These results show that the previously reported potentiation of anxiolytic-like effects of diazepam, chlordiazepoxide and meprobamate by naloxone in mice is not specific for that species. Furthermore, they demonstrate that another drug, pentobarbital, is also potentiated. Thus, all full agonists at the benzodiazepine/barbiturate/$GABA_A$ receptor hitherto tested (and meprobamate) have their anxiolytic-like activity potentiated by the opiate antagonist. However, the effects of the partial benzodiazepine receptor agonist Ro19-8022 were not modified by naloxone. In order to explain this observation, some speculations about the mechanisms responsible for the potentiation are needed.

The first issue here is whether naloxone is specific to the opioid receptors or if its effects could be attributed to actions at some other receptor. This does not seem likely, however. Naloxone is unable to block the motor effects of benzodiazepines, even when extremely large doses of the antagonist are administered (Ågmo et al., 1995). Since such effects are easily blocked by flumazenil and picrotoxin these observations suggest that naloxone does not have any significant antagonistic action neither on benzodiazepine nor on $GABA_A$ receptors. This conclusion is reinforced by data showing that the deleterious effects of diazepam on learning in the Morris water maze are not reduced by naloxone at a dose that completely blocks the effects of morphine (McNamara and Skelton, 1992). Furthermore, receptor binding studies have shown that naloxone has no functionally relevant affinity for $GABA_A$ (Goldinger et al., 1981), $serotonin_{1A}$ (Martin et al., 1991) or dopamine (Carlsson and Seeger, 1982) receptors. These are the main receptor sites that have been related to anxiolytic/anxiogenic effects. Thus, it is most probable that the effects of naloxone observed in the present studies are a consequence of opioid receptor blockade.

One obvious way to explain naloxone's effect is to assume that the drug removes inhibitory opioidergic influences on GABA neurons. It has repeatedly been shown that activation of opioid receptors in several brain areas inhibits GABAergic activity (Cohen et al., 1992; Stanford and Cooper, 1999). Under the condition that opioid systems are active during the plus-maze test, blockade of opioid receptors would remove inhibitory influences on GABA neurons. Because benzodiazepines and barbiturates are supposed to exert their anxiolytic effects through enhanced GABAergic transmission, their effects should then be reinforced by opioid receptor antagonists. Opioids are released in response to stress (Boone Jr. and McMillen, 1994; Larsen and Mau, 1994; Yamada and Nabeshima 1995), and the plus-maze experience is as stressful as mild electric foot-shock, provided that corticosteroid release is accepted as an indicator of stress intensity. Plasma concentrations are in fact elevated to a similar degree after short foot-shock and after exposure to the plus-maze (Friedman et.al., 1967; Pellow et.al., 1985; Weinstock et. al., 1998a,b). Thus, it is conceivable that opioid receptor blockade enhances the effects of drugs with agonist properties at the $GABA_A$ receptor, exactly as occurred in the present experiments.

The lack of effect of Ro19-8022 can be a consequence of it being a partial agonist. Such agonists display anxiolytic-like activity when receptors are close to saturation, at difference from full agonists which are active at only moderate receptor occupancy (Facklam et al., 1992a). Furthermore, while the curves relating GABA potentiation to fractional benzodiazepine receptor occupancy are hyperbolic for full agonists, they are parabolic for partial agonists (Facklam et al., 1992b). This means that the latter kind of drugs have a very small effect until receptors are almost saturated, while the former display gradually increasing effects from very low receptor occupancy and on. Thus, a leftward shift of the dose–effect curve may have large effects for full agonists but slight or no effect for partial agonists. Since there is no reason to believe that naloxone neither modifies receptor affinity nor intrinsic efficacy, it is improbable that the partial agonist would attain the receptor occupancy required for an anxiolytic effect even after the combined treatment.

Another possible explanation for the absence of naloxone-induced potentiation of Ro19-8022 could be that the combined treatment had a sedative effect, masking possible anxiolytic-like actions. This proposal is reinforced by the fact that the combination indeed reduced the total number of arms entries. However, the reduction was of small magnitude and it seems unlikely that such a weak inhibitory effect on locomotion could account for the lack of potentiation.

While the above explanation holds for the benzodiazepines and pentobarbital, it is not evident that it can be applied to meprobamate. This drug affects the $GABA_A$ receptor only at high concentrations in vitro (Macksay and Ticku, 1988; Rho et.al., 1997), and it is unlikely that the concentrations obtained in vivo have any significant effect on that receptor complex. In fact, the concentrations of meprobamate required for a direct action on the $GABA_A$ receptor complex are within the range producing coma in the human (Bailey, 1981).

We (Belzung et al., 2000) have previously speculated about a possible role for adenosine receptors in naloxone-induced potentiation of anxiolytic effects. Adenosine A_1 receptor agonists have anxiolytic-like properties in mice (Jain et.al., 1995; Florio et.al., 1998) and both benzodiazepines and meprobamate inhibit adenosine reuptake at

therapeutic doses (Bruns et.al., 1983; Metha and Kulkarni,1984; Phillis, 1984; Phillis and O'regan, 1988). The adenosine A_1 receptor inhibits adenylylcyclase, whereas opioids have been reported to inhibit or stimulate this enzyme. Inhibition is mediated by G_i GTP binding or G_0 proteins (Childers, 1991) while stimulation depends on G_s proteins (Sarne et. al., 1996, 1998; Crain and Shen, 1998; Wu et. al., 1998). If opioids stimulate adenylylcyclase in brain regions important for anxiolysis, they would have actions on adenosinergic neurons opposite to those of benzodiazepines and meprobamate. Therefore, opioid receptor blockade should enhance the effects of these latter drugs. 5-HT_{1A} receptor agonists also interact with adenosinergic systems (Harrington et al., 1988), making it possible to include the potentiation of buspirone in this explanation. There are also data suggesting that tachykinins acting at the tachykinin NK_3 receptor, another class of compounds displaying anxiolytic-like activity (Ribeiro et al., 1999) potentiated by naloxone (Ribeiro and De Lima, 1998), stimulate adenosine release (Cahill et al., 1997). However, it is not known whether this latter effect is mediated through the tachykinin NK_3 receptor. The hypothesis outlined here is very speculative, but may be subjected to experimental test. Before performing such tests, it seemed essential to determine if naloxone-induced potentiation of anxiolytic effects is a robust phenomenon, existing in several species.

Although great expectancies were associated with the partial benzodiazepine receptor agonists, they have had limited success in the clinical treatment of anxiety. Extensive trials with abecarnil have shown that the drug has, at best, a short-term effect inferior to that of classical benzodiazepines (Pollack et. al., 1997; Aufdembrinke, 1998). Strangely enough, a low dose was more efficient than a high dose in one study (Small and Bystritsky, 1997). This is in sharp contrast to drugs such as barbiturates, meprobamate, buspirone and the benzodiazepines, whose clinical efficacy is established beyond any doubt. Interestingly, all the anxiolytic drugs with firmly established clinical efficacy are potentiated by naloxone while a drug with doubtful clinical utility (Ro19-8022) is not. It is possible that naloxone-potentiation could be a good predictor of the clinical usefulness of anxiolytic drugs.

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