Pharmacology Biochemistry & Behavior, Vol. 19, pp. 549–552, 1983. © Ankho International Inc. Printed in the U.S.A.

Separation of Clonazepam-Induced Head Twitches and Muscle Relaxation in Mice

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Received 7 March 1983

NAKAMURA, M. AND J. M. CARNEY. *Separation of clonazepam-induced head twitches and muscle relaxation in mice.* PHARMACOL BIOCHEM BEHAV 19(3) 549–552, 1983.—Stereotyped head twitches in mice were induced by clonazepam. The number of head twitches produced was directly related to the clonazepam dose. In addition to head twitches, clonazepam produced dose-related muscle relaxation. Methysergide antagonized the action of clonazepam on head twitches. However, methysergide failed to block the muscle relaxant action. In contrast to methysergide, the benzodiazepine receptor antagonists CGS 8216 and Ro 15-1788 blocked the muscle relaxant effects of clonazepam. Neither CGS 8216 nor Ro 15-1788 blocked the clonazepam-induced head twitches. These data suggest that the muscle relaxant effects of clonazepam are mediated by benzodiazepine/GABA receptor systems that can be blocked by CGS 8216 and Ro 15-1788. On the other hand, it is proposed that the benzodiazepine-induced head twitch effect is mediated by a benzodiazepine/serotonin 2 receptor system.

Clonazepam CGS 8216 Ro 15-1788 Methysergide Head twitches Muscle relaxation

THE presence of stereospecific saturable binding sites for benzodiazepines in the central nervous system was first reported by Möhler and Okada [11] and by Squire and Braestrup [18]. Subsequent to those initial studies there have been many reports describing benzodiazepine receptors in brain. There is a good correlation between the affinity of a series of benzodiazepine compounds for putative receptor sites in brain and their relative potencies as muscle relaxants, anxiolytics and anticonvulsants. These relationships suggest that many of the pharmacological effects of benzodiazepine might be mediated through brain benzodiazepine receptors [11,18]. Benzodiazepine receptors have been reported to be functionally coupled to GABA receptors [19,20].

The discovery of benzodiazepine receptors in the brain also has promoted the search for synthetic compounds which are able to bind to benzodiazepine receptors. Recently, CGS 8216, a pyrazoloquinoline derivative, and Ro 15-1788, an imidazodiazepine derivative, have been shown to be potent brain benzodiazepine receptor antagonists both *in vitro* and *in vivo* [5,8]. These compounds also antagonized the action of diazepam in several pharmacological tests and were devoid of benzodiazepine-like activity in these tests [1,8]. Ro 15-1788 appeared to be a selective benzodiazepine antagonist. In contrast, CGS 8216 antagonized the action not only of benzodiazepine but also of phenobarbital and meprobamate.

We previously suggested that the benzodiazepine induced head twitches were produced by increasing the sensitivity of serotonin receptors [12, 13, 15]. The present study was designed to determine if the head twitches induced by benzodiazepines were mediated through benzodiazepine receptor systems in brain. This was accomplished by determining the extent to which the benzodiazepine antagonists, CGS 8216 and Ro 15-1788 were able to block the effects of clonazepam in mice. These results were compared to the effect of methysergide pretreatments.

METHOD

Subjects

Subjects were male CF1 mice (Sasco, Omaha, NB) that weighed 20–25 g at the start of the study. Mice were group housed (6 per cage) in the OUHSC animal facility. Both water and food were continuously available. Mice were kept under a standard 12/12 light-dark cycle. The ambient temperature was 24±1 °C and the humidity was 55±5%. Groups of eight mice each were used for all studies.

Procedure

Methysergide and CGS 8216 were injected intraperitoneally (IP) 30 min before the oral dose of clonazepam. In contrast, Ro 15-1788 was injected IP 40 minutes after the oral clonazepam dose. The number of head twitches in 4 minutes were counted at 45 and 90 minutes after oral dosing with clonazepam. Individual mice were placed in individual plastic cubicles for the head twitch experiment. Student's *t*-test was used to determine the statistical significance of the difference between the means.

Muscle relaxant activity was assayed as previously described [13]. Briefly, mice were required to support their

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TABLE 1

ANTAGONISM OF THE CLONAZEPAM-INDUCED MUSCLE RELAXATION IN MICE*

Drugs	Dose (mg/kg)	Muscle relaxant activity of clonazepam ED_{50} (mg/kg)	(95% confidence limits)
CGS 8216	0	1.1	(0.69–1.8)
	3	1.7	(1.1–2.8)
	10	4.5†	(2.5–8.1)
	30	7.6†	(4.0–14)
Ro 15-1788	0	0.70	(0.39–1.3)
	10	10†	(5.3–19)
	30	18†	(9.4–34)
Methysergide	0	0.85	(0.50–1.4)
	10	0.65	(0.38–1.1)

*CGS 8216 and methysergide were injected IP 30 minutes prior to clonazepam. Ro 15-1788 was injected IP 40 minutes after clonazepam. See the Method section for further details.
†$p<0.05$.

TABLE 2

EFFECT OF CGS 8216, Ro 15-1788 AND METHYSERGIDE ON THE HEAD TWITCHES INDUCED BY CLONAZEPAM IN MICE*

Drugs	Dose (mg/kg)	Number of head twitches (mean ± S.E.) Clonazepam (mg/kg) 0	3	10	30
CGS 8216	0	0.6 ± 0.3	4.5 ± 1.1	11.3 ± 4.0	20.4 ± 6.9
	10	0.4 ± 0.3	5.2 ± 1.6	13.2 ± 2.6	19.6 ± 7.8
	30	0.4 ± 0.3	6.1 ± 1.7	15.3 ± 2.8	25.4 ± 6.4
	100	0.3 ± 0.2	6.4 ± 1.9	17.3 ± 2.9	27.8 ± 6.8
Ro 15-1788	0	0.3 ± 0.2		8.6 ± 1.7	17.8 ± 3.9
	10	0.3 ± 0.2		10.2 ± 2.3	18.3 ± 2.7
	30	0.4 ± 0.3		12.1 ± 1.9	20.1 ± 4.1
Methysergide	0	0.3 ± 0.2			17.0 ± 3.9
	3	0.1 ± 0.1			5.2 ± 1.3†
	10	0			2.6 ± 0.7†

*Same injection conditions as described in Table 1.
†$p<0.01$.

weight by grasping a horizontal wire (1 mm diameter) with their forepaws. The ED_{50} for muscle relaxation was defined as the clonazepam dose that prevented 50% of the mice from touching the wire more than twice with their hind paws within 10 seconds. Three successive trials were conducted for each mouse. All muscle relaxant experiments were conducted 45 minutes after oral clonazepam. The ED_{50} values for muscle relaxant effects and the significance of the potency ratio between ED_{50} values were calculated using the method of Litchfield-Wilcoxon [9].

Drugs

Clonazepam (Hoffmann-La Roche), CGS 8216 (CIBA-GEIGY) and Ro 15-1788 (Hoffmann La Roche) were suspended in 0.5% methylcellulose. Methysergide maleate (Sandoz) was dissolved in sterile saline.

RESULT

Clonazepam produced a dose-related increase in the number of head twitches and in the degree of muscle relaxation (Tables 1 and 2). The ED_{50} for muscle relaxation was 0.7–1.1 mg/kg. Both CGS 8216 and Ro 15-1788 antagonized the muscle relaxant effects. Increasing the pretreatment dose of CGS 8216 and Ro 15-1788 resulted in corresponding increase in the ED_{50} value. For example, CGS 8216 pretreatment doses of 3, 10 and 30 mg/kg resulted in clonazepam ED_{50} values of 1.7, 4.5 and 7.6 mg/kg respectively, compared to a control ED_{50} value of 1.1 mg/kg. Pretreatment with

Ro 15-1788 also produced a substantial increase in the ED_{50} value. In contrast to the benzodiazepine antagonists, methysergide failed to antagonize the muscle relaxant effects of clonazepam.

The clonazepam-induced head twitches were blocked by methysergide doses (3 and 10 mg/kg) that had no effect on the muscle relaxant action of clonazepam (Table 2). Consistent with the apparent pharmacological separation of head twitches and muscle relaxant effect, neither CGS 8216 nor Ro 15-1788 were able to block the clonazepam-induced head twitches.

DISCUSSION

Benzodiazepines are well known to produce sedation, muscle relaxation, anxiolytic effects and to be effective anticonvulsants both in experimental animals and in humans. These effects are thought to be mediated, at least in part, by the interaction of benzodiazepines with high affinity binding sites in the CNS [11,18]. Benzodiazepines have been shown to potentiate GABA mediated inhibitory pathways in the CNS and the neuronal response to exogenously applied GABA [2, 7, 10]. Costa and Guidotti [3] reported that benzodiazepines increase the sensitivity of the GABA receptor by removing an endogenous protein that regulates the affinity of the GABA receptor. The muscle relaxation observed in the present study induced by clonazepam was antagonized by benzodiazepine receptor antagonists, CGS 8216 and Ro 15-1788. This result was in agreement with the work of Bernard *et al.* [1] and Hunkeler *et al.* [8]. The muscle relaxant action of benzodiazepines has been known to show rapid tolerance development after a few doses [6,13]. Moreover, chronic benzodiazepine administration induced a decrease in the number of benzodiazepine binding sites in the brain [4,17]. Thus, the muscle relaxant action of benzodiazepines is likely to be mediated via brain benzodiazepine receptors that are associated with GABA receptors.

On the other hand, both CGS 8216 and Ro 15-1788 failed to block the clonazepam-induced head twitch response at doses that effectively prevented the muscle relaxant effects. In contrast, methysergide prevented the head twitch response, but failed to block the muscle relaxant effect. We previously reported that the head twitches induced by benzodiazepine was not affected by GABA antagonists such as bicuculline and picrotoxin or by aminooxyacetic acid which increased GABA levels, and that the ability of benzodiazepines to induce head twitches was not changed by chronic administration [12,14]. These results strongly suggest that the mechanism of benzodiazepine-induced head twitches is different from muscle relaxation and is not related to benzodiazepine receptors coupled to GABA receptors.

Recently, Peroutka *et al.* [16] reported that there are two distinct serotonin receptors designated serotonin 1 which was labeled by ^{3}H-5-HT and serotonin 2 by ^{3}H-Spiroperidol. Serotonin 1 receptors were related to the serotonin sensitive adenylate cyclase, whereas the head twitches induced by central serotonin stimulation were mediated by serotonin 2 receptors. The head twitches induced by benzodiazepines were completely blocked by serotonin antagonists such as cyproheptadine and methysergide [12,14]. The potencies of many drugs such as serotonin antagonists, neuroleptics and antidepressants in blocking the head twitches induced by clonazepam closely correlate with affinites for serotonin 2 receptors in cerebral cortex labeled by ^{3}H-spiroperidol ($r=.86$, $p<0.01$, unpublished observation). These data suggest that the head twitches induced by benzodiazepines are mediated by serotonin 2 receptors in the brain.

It is conceivable that there might be pharmacologically distinct benzodiazepine receptors which are coupled to serotonin 2 receptors. Benzodiazepines would bind to the benzodiazepine receptors coupled to serotonin 2 receptors as well as to benzodiazepine receptors coupled to GABA receptors, and would increase the sensitivity of serotonin 2 receptors. In support of this hypothesis is the finding that the behavioral actions of serotonin receptor agonists such as serotonin, mescaline and 5-methoxytryptamine were potentiated by pretreatment with benzodiazepines [12, 13, 15]. As proposed above, CGS 8216 and Ro 15-1788 would be antagonists for benzodiazepine receptors coupled to GABA receptors. Thus, the slight increase in clonazepam-induced head twitches produced by CGS 8216 and Ro 15-1788 (see Table 2) was presumably due to an increase in available clonazepam at benzodiazepine receptors coupled to serotonin 2 receptors, since the benzodiazepine receptors coupled to GABA receptors were blocked. Since relatively higher doses of benzodiazepines (3 to 30 mg/kg) were necessary to produce head twitches, compared to muscle relaxation (0.5 to 5 mg/kg), the affinity of benzodiazepine receptors coupled to serotonin 2 receptors might be less than that to GABA receptors.

ACKNOWLEDGEMENT

We thank Mrs. Edith Martin for her excellent typing of the manuscript.

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