

FIRST SESSION

Stress-depression

THE ACTION OF STRESS AND ANXIOLYTIC AND ANXIOGENIC BENZODIAZEPINE RECEPTORS LIGANDS ON [35 S] T-BUTYLBICYCLOPHOSPHOROTHIONATE BINDING IN THE RAT CEREBRAL CORTEX

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Summary. - *The effect of foot shock stress on [35 S] t-butylbicyclophosphorothionate (TBPS) binding to fresh unwashed membrane preparations from rat cerebral cortex was studied and was compared to those of positive and negative modulators of the GABAergic transmission. 35 S-TBPS binding was increased (30%) in cerebral cortex of rats exposed to foot shock compared to the non stressed rats. In contrast, the in vitro addition and the in vivo administration of anxiolytic and positive modulators of the GABAergic transmission inhibited the specific binding of 35 S-TBPS. On the other hand, the anxiogenic β -carbolines DMCM, β CCM, FG 7142 and β CCE mimicked in vivo and in vitro the effect of stress. The demonstration that stress, similar to anxiogenic β -carbolines and opposite to benzodiazepines and anxiolytic β -carbolines, increases 35 S-TBPS binding in the rat cerebral cortex, suggests that some emotional state related to stress and anxiety may result from a diminished GABAergic transmission at the level of the GABA/benzodiazepine receptor/chloride ionophore complex.*

Riassunto (Effetto dello stress e dei ligandi ansiolitici e ansiogenici per i recettori alle benzodiazepine sul binding del 35 S-TBPS nella corteccia cerebrale del ratto). - *L'effetto dello stress e dei farmaci ansiogenici e ansiolitici sulla funzione del canale allo ione cloro GABA-dipendente è stato studiato nella corteccia cerebrale di ratto utilizzando il binding del [35 S] t-butilbicyclofosforotionato (TBPS), un ligando selettivo per i recettori associati al canale allo ione cloro. Il foot-shock stress aumenta il legame del 35 S-TBPS nella corteccia cerebrale di ratti non stressati; questo effetto è simile a quello indotto dalla somministrazione in vivo o dalla aggiunta in vitro di beta-carbolina ansiogene ed è opposto a quello indotto dalle benzodiazepine e dalla β -carbolina ansiolitica ZK 93423. Nel loro insieme, i nostri dati in vitro ed in vivo forniscono l'evidenza sperimentale che, misurando le variazioni del legame del 35 S-TBPS al canale allo ione cloro, è*

possibile studiare la modulazione fisiologica e farmacologica delle sinapsi GABAergiche. Inoltre questi risultati dimostrano che il complesso recettoriale GABA_A/benzodiazepine/canale al cloro è un sensibile target all'azione degli stimoli stressanti ed ha un ruolo cruciale nella modulazione degli stati emozionali e nella farmacologia dei farmaci ansiolitici e ansiogenici.

Introduction

It is well established that several anxiolytic and anxiogenic drugs exert most of their pharmacological effects by acting at the level of the GABA/ionophore receptor complex [1, 2]. Thus, the anxiolytic benzodiazepines and barbiturates and the anxiogenic β -carbolines, picrotoxin, tetrazol, and the cage convulsant organophosphoric derivative t-butylbicyclophosphorothionate (TBPS) exert their pharmacological effects via an opposite allosteric modulation of the GABA_A-gated chloride ion channel. Indeed, β -carbolines, picrotoxin, and tetrazol reduce the function of the GABA-coupled Cl⁻ channel [3-6] and cause experimental anxiety in animals [7-10] and generalized anxiety and panic attack in man [11, 12]. On the other hand, benzodiazepines and barbiturates, which facilitate the GABAergic transmission, [13-16] have anticonflict effect in rats [17-19] and an anxiolytic action in humans [20].

The discovery that the activation of benzodiazepine receptors may produce both anxiolytic and anxiogenic effects depending on the shifts in the conformation of the GABA/benzodiazepine/Cl⁻ ionophore receptor complex elicited by anxiolytic benzodiazepines and anxiogenic β -carbolines respectively [21], has allowed to suggest that the GABA_A receptor complex plays a major role not only in the pharmacology of the above drugs but also in the physiopathology of stress and anxiety.

On the basis of these considerations a very important problem is to understand the molecular events which mediate the effects of anxiolytic and anxiogenic drugs and the action exerted by stress and anxiety on the function of the GABA_A-gated chloride ion channel in the mammalian central nervous system.

In an attempt to elucidate this problem we used the binding of ³⁵S-TBPS to rat cerebral cortex as a tool to study the changes in the function of the GABA_A/benzodiazepine receptor complex elicited by different anxiolytic and anxiogenic drugs and by foot shock stress.

TBPS is a compound which reduces the central GABAergic transmission by binding to recognition sites at the level of the chloride ionophore-associated to the GABA_A/benzodiazepine receptor complex [22]. The specific binding of ³⁵S-TBPS to brain tissue is modulated by different classes of compounds which alter the GABA dependent chloride ionophore function. Thus, the binding of this ligand is competitively displaced by picrotoxin and a variety of cage convulsants and allosterically modulated by GABA, GABA mimetics, barbiturates and benzodiazepine recognition site ligands such as benzodiazepines, β -carbolines and pyrazolopyridine derivatives [22-25]. Thus, by measuring ³⁵S-TBPS binding seems to be possible to evaluate biochemically the pharmacological and physiological changes related to the function of the chloride ionophore coupled to the GABA_A/benzodiazepine receptor complex.

The results of the present study demonstrate that foot-shock increases ³⁵S-TBPS binding sites in the cerebral cortex of unstressed rats. This effect is mimicked by the *in vivo* administration or the *in vitro* addition of anxiogenic β -carbolines and opposite to those elicited by benzodiazepines and anxiolytic β -carbolines.

Materials and methods

Male Sprague Dawley rats (Charles River, Como, Italy) weighing 180-200 g were used. They were divided into two groups: naive and handling-habituated rats. As previously reported [26] handling-habituated animals were rats habituated to the manipulations that precede killing. Naive animals were left in their home cage for 6-8 days and were then sacrificed as were the handling-habituated rats. After this training we considered handling-habituated rats not-stressed animals and naive rats the stressed ones.

The foot shock stress consisted of a series of electrical foot shocks delivered in individual boxes, with floors made of brass rods, 2 cm apart. Shocks were provided by a stimulator which delivered a shock of 0.2 mA every 500 ms with 500 ms duration. Rats were foot shocked continuously for 20 min.

After sacrifice the brains were rapidly removed and the cerebral cortex was dissected out. The fresh brain tissue was homogenized with a Polytron PT 10 (setting 5, for 20 s) in 50 vol of ice-cold 50 mM Tris-citrate buffer (pH 7.4 at 25 °C) containing 100 mM NaCl, centrifuged at 20,000 \times g for 20 min and reconstituted in 50 vol of Tris-citrate buffer. ³⁵S-TBPS binding was measured as previously described [23].

Anxiolytic benzodiazepine receptor ligands and ³⁵S-TBPS binding

The effect of the *in vitro* addition and the *in vivo* administration of different anxiolytic ligands for benzodiazepine recognition sites was studied on ³⁵S-TBPS binding in unwashed membrane preparation of rat cerebral cortex.

As shown in Table 1, the *in vitro* addition of different benzodiazepines to membrane preparation from rat cerebral cortex decreased, like GABA and GABA agonists [22, 23, 25], ³⁵S-TBPS binding. This effect was shared by the anticonvulsant and anxioly-

Table 1. - Effect of benzodiazepine receptor ligands on ³⁵S-TBPS binding to rat unwashed membrane preparations from rat cerebral cortex

Drugs	Concentration	³⁵ S-TBPS binding (% of solvent)
Solvent		100 \pm 5.2
Quazepam	10 μ M	21 \pm 3.2 *
ZK 93423	10 μ M	40 \pm 2.9 *
Flunitrazepam	10 μ M	41 \pm 2.9 *
Diazepam	10 μ M	45 \pm 2.7 *
Lorazepam	10 μ M	48 \pm 4.0 *
Triazolam	10 μ M	61 \pm 4.5 **
Clonazepam	10 μ M	68 \pm 5.4 **
Alprazolam	10 μ M	69 \pm 5.5 **
Flurazepam	10 μ M	76 \pm 3.5 **
DMCM	0.01 μ M	185 \pm 11.5 *
β CCM	0.01 μ M	134 \pm 5.6 **
FG 7142	1 μ M	125 \pm 7 **
β CCE	0.01 μ M	123 \pm 6 **

Drugs were dissolved in dimethyl sulfoxide (stock solutions 0.1 mM) and dilutions were made up in 50 mM Tris-citrate buffer pH 7.4. The control groups were incubated with an equivalent amount of solvent. Incubation of 2 nM ³⁵S-TBPS were maintained at 25 °C for 90 min. Each value is the mean \pm SEM of 3 to 8 experiments performed in triplicate. * p < 0.01 vs solvent; ** p < 0.05 vs solvent.

tic β -carboline derivative ZK 93423 which, by acting at the benzodiazepine recognition sites exhibits pharmacological properties similar to those of anxiolytic and anticonvulsant benzodiazepines [27]. However, the degree of inhibition produced by the various benzodiazepine receptor ligands examined at the concentration of 10 μ M was different. In fact, quazepam, flunitrazepam, diazepam, lorazepam and ZK 93423 inhibited 35 S-TBPS binding more than 50%. Among these compounds quazepam was the most effective producing 80% of inhibition. On the other hand other benzodiazepines (triazolam, clonazepam, alprazolam and flurazepam) showed a lower efficacy respect to the previous drugs. Thus, using unwashed membranes, it seems possible to differentiate the intrinsic activity of the various benzodiazepines on the basis of their efficacy in decreasing 35 S-TBPS binding.

The effect of ZK 93423 like that of benzodiazepines, was reversed by the addition of the benzodiazepine receptor antagonists RO 15-1788 and ZK 93426 indicating that these effects were mediated by specific receptor interaction (data not shown).

All together these results indicate that 35 S-TBPS binding is a sensitive index of the changes in the function of the GABAergic synapses. Since TBPS inhibits the GABAergic neurotransmission by a direct blockade of the GABA-gated Cl^- channels [28], the inhibition of 35 S-TBPS binding to the chloride channel by these anxiolytic drugs implies an increased ability to generate chloride current and this results in an enhanced function of the GABAergic synapses.

This conclusion is further supported by the demonstration that the *in vivo* administration of diazepam (3 mg/kg i.p.) clonazepam (3 mg/kg i.p.) and alprazolam (0.5 mg/kg i.p.), as well as their *in vitro* addition, reduce 35 S-TBPS binding in the rat cerebral cortex (Table 2).

This finding suggests that the pharmacological profile of different benzodiazepine recognition site ligands may be revealed by measuring *ex vivo* 35 S-TBPS binding to rat brain.

Anxiogenic benzodiazepine receptor ligands and 35 S-TBPS binding

To further investigate the sensitivity of 35 S-TBPS binding to the changes in the function of the GABA coupled chloride channel we studied whether the anxiogenic ligands for benzodiazepine receptors have an opposite effect on this parameter respect to the anxiolytic drugs.

As shown in Table 1 the *in vitro* addition of DMCM, β CCM, FG 7142 and β CCE, the anxiogenic, convulsant and proconvulsant β -carboline derivatives which decrease the GABAergic trans-

Table 2. - *Opposite effect of the in vivo administration of anxiolytic and anxiogenic benzodiazepine receptor ligands on 35 S-TBPS binding to rat cerebral cortex*

Drugs	Doses	35 S-TBPS binding (% of vehicle)
Vehicle		100 \pm 7
Diazepam	3 mg/kg i.p.	70 \pm 6 *
Clonazepam	3 mg/kg i.p.	75 \pm 5 *
Alprazolam	0.5 mg/kg i.p.	75 \pm 7 *
DMCM	0.12 mg/kg i.v.	160 \pm 15 *
β CCM	0.4 mg/kg i.v.	155 \pm 14 *
β CCE	0.4 mg/kg i.v.	145 \pm 11 *
FG 7142	0.8 mg/kg i.v.	136 \pm 11 *

Benzodiazepines and β -carbolines were given 20 and 15 min before sacrifice, respectively. Drugs were dissolved in saline with one drop of Tween 80 per 5 ml. Control rats received an equivalent volume of vehicle (2 ml/kg). Incubations of 2 nM 35 S-TBPS were maintained at 25 $^{\circ}$ C for 90 min. Each value is the mean \pm SEM of 4 to 6 separate experiments, each done in triplicate. *p < 0.05 vs vehicle.

mission [4, 6, 21, 29], increased 35 S-TBPS binding in the rat cerebral cortex.

The effects of β -carbolines indicate that a decrease in the function of the GABA_A/benzodiazepine receptor complex results in a parallel increase of 35 S-TBPS binding to the GABA-dependent chloride channel. This conclusion is further supported by the data showing that the *in vivo* administration of these anxiogenic β -carboline derivatives increases 35 S-TBPS binding measured *ex vivo* in the rat cerebral cortex (Table 2).

These *in vivo* and *in vitro* results indicate that the anxiogenic actions elicited by negative modulators of the GABAergic transmission can be revealed biochemically measuring 35 S-TBPS binding.

Stress and 35 S-TBPS binding

The finding that 35 S-TBPS binding can be modulated in opposite direction *in vitro* and *in vivo* by drugs which by acting at the level of the GABA/benzodiazepine receptor complex induce anxiolytic or anxiogenic effects prompted us to study whether changes in the emotional state of the animals modified 35 S-TBPS binding in the rat cerebral cortex.

We investigated the effect of stress on the chloride channel-associated binding sites by measuring 35 S-TBPS binding in the cerebral cortex of handling-habituated (unstressed) and handling-habituated foot-shocked (stressed) rats.

Table 3. - Foot shock stress increases ^{35}S -TBPS binding in the rat cerebral cortex of handling-habituated rats

	^{35}S -TBPS binding (% of control)
Control	100 \pm 8.4
Foot shock	130 \pm 7.0 *

Rats were handling-habituated as described under «Materials and methods». Unwashed membrane preparations from cerebral cortex were incubated in the presence of 2 nM ^{35}S -TBPS for 90 min at 25 °C. Data are the mean \pm SEM of 3 separate experiments performed in triplicate. *p < 0.05 vs control.

As shown in Table 3, ^{35}S -TBPS binding was increased (30%) in the cerebral cortex of rats exposed to foot shock stress compared to that of non stressed animals. On the contrary, foot shock stress failed to modify ^{35}S -TBPS binding in cortical membranes of naive (not handling-habituated) rats (data not shown).

The finding that foot shock stress, like anxiogenic ligands for benzodiazepine receptors, increases ^{35}S -TBPS binding to rat cerebral cortex suggests that this treatment decreases the function of the GABA-benzodiazepine receptor complex and its associated chloride ionophore. Thus a decrease in the function of the GABA-dependent chloride channel may be a crucial molecular event involved in some anxious states.

Discussion

The results here reported indicate that the GABA-dependent chloride channel plays a major role in the neurochemistry and pharmacology of anxiolytic and anxiogenic ligands for benzodiazepine receptors as well as in the physiopathology of stress and anxiety.

This conclusion is strongly suggested by our finding showing that foot-shock stress induces a significant enhancement in the density of ^{35}S -TBPS binding

sites in the cerebral cortex of handling-habituated (unstressed) rats.

Since TBPS has specific recognition sites at the level of the GABA-gated Cl^- channel, the stress-induced enhancement in the density of ^{35}S -TBPS binding sites indicates that the GABA/benzodiazepine/chloride ionophore receptor complex is a sensitive target for the action of environmental stress. Thus, our data also suggest that a decrease in the GABA-dependent chloride permeability might be an important molecular event involved in some anxious states.

Accordingly, the negative allosteric modulators of the GABAergic transmission mimic the effect of stress on ^{35}S -TBPS binding to fresh unwashed cortical membrane preparations of rat brain. In fact, in agreement with their pharmacological profile, anxiogenic and convulsant β -carboline derivatives DMCM, βCCM , FG 7142 and βCCE shares with foot shock the capability to increase ^{35}S -TBPS binding in the rat brain.

Accordingly, these specific benzodiazepine recognition site ligands, which induce proconflict effect in rats and mice [7, 30] experimental anxiety in cats [10] and monkeys [9], anxiety and panic attack in man [11], decrease the GABAergic transmission through a negative modulation of GABA ionophore receptor complex [4, 6, 21, 29, 31, 32].

On the contrary, benzodiazepines and the positive modulator of the GABAergic transmission ZK 93423 modulate *in vivo* and *in vitro* ^{35}S -TBPS binding in a manner opposite to that of stress and anxiogenic β -carbolines. In fact, these compounds, similarly to GABA [22, 23, 25] decrease ^{35}S -TBPS binding to fresh unwashed cortical membrane preparations.

In conclusion, the present *in vitro* and *in vivo* biochemical data give a functional evidence that by measuring the changes of ^{35}S -TBPS binding to the GABA-dependent chloride ionophore it is possible to study the physiological and pharmacological modulation of the GABAergic synapses.

Accordingly, our results have shown that the GABA_A/benzodiazepine/ionophore receptor complex is a sensitive target for the action of stressful stimuli and plays a major role in the modulation of some emotional states.

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