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Characterization of [^{3}H]thiocolchicoside binding sites in rat spinal cord and cerebral cortex

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Abstract

Thiocolchicoside, a semi-synthetic derivative of the naturally occurring compound colchicoside with a relaxant effect on skeletal muscle, has been found to displace both [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) and [^{3}H]strychnine binding, suggesting an interaction with both GABA and strychnine-sensitive glycine receptors. In order to gain further insight into the interaction of thiocolchicoside with these receptors, the binding of [^{3}H]thiocolchicoside in rat spinal cord–brainstem and cortical synaptic membranes was characterized. [^{3}H]Thiocolchicoside binding was saturable in both tissues examined. In spinal cord–brainstem membranes, we found a K_D of 254 ± 47 nM and a B_{max} of 2.39 ± 0.36 pmol/mg protein, whereas in cortical membranes, a K_D of 176 nM and a B_{max} of 4.20 pmol/mg protein was observed. A similar K_D value was found in kinetic experiments performed in spinal cord–brainstem membranes. Heterologous displacement experiments showed that GABA and strychnine displaced the binding in a dose-dependent manner, whereas glycine was ineffective. [^{3}H]Thiocolchicoside binding was also displaced by several $GABA_A$ receptor agonists and antagonists, but not by baclofen, flunitrazepam, guvacine, picrotoxin or by other drugs unrelated to GABA transmission. In spinal cord–brainstem, and to a lower extent, in cortical membranes, GABA and its analogs were not able to completely displace [^{3}H]thiocolchicoside specific binding indicating that, besides $GABA_A$ receptors, thiocolchicoside can bind to another unidentified site. Unlabelled thiocolchicoside, however, completely displaced [^{3}H]muscimol binding both in cortical and in spinal cord–brainstem synaptic membranes with an IC_{50} in the low μM range. Neurosteroids were found to modulate the binding in cortical but not in spinal cord–brainstem synaptic membranes. We conclude that [^{3}H]thiocolchicoside binding shows a pharmacological profile indicating an interaction with the $GABA_A$ receptor. The different affinities for the $GABA_A$ receptor agonists and antagonists and sensitivity to neurosteroids obtained in the cerebral cortex and in the spinal cord may indicate a preferential interaction of the compound with a subtype of the $GABA_A$ receptor. The data also indicate that [^{3}H]thiocolchicoside binds to another site(s), whose nature remains to be elucidated.

Keywords: Thiocolchicoside; GABA receptor; Strychnine; Glycine receptor; Radioreceptor binding; (Rat)

1. Introduction

Thiocolchicoside, a semi-synthetic derivative of the naturally occurring compound colchicoside, has a relaxant effect on skeletal muscle, which is also accompanied by anti-inflammatory and analgesic activity (Janbroers, 1987). By virtue of these effects, this drug has long been used in a number of orthopaedic, traumatic and rheumatological conditions.

In rats, thiocolchicoside has been reported to significantly reduce the polysynaptic reflex elicited in biceps femoris muscle in normal animals and to markedly decrease the stretch reflex elicited in triceps surae muscle in spastic animals after repeated administration (Françon et al., 1998). The actions of thiocolchicoside have been attributed, at least in part, to the activation of GABA receptors. Indeed, Biziere et al. (1981a,b) reported that thiocolchicoside can interact with GABA receptors, since it was able to inhibit both the binding of [^{3}H]GABA on rat cortical and cerebellar membranes (with an IC_{50} in the micromolar range) and the tonic seizures induced by picrotoxin. The effect of thiocolchicoside on GABA receptors does not seem to be caused by an interaction with the

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benzodiazepine modulatory binding site, since the compound was not able to inhibit [^{3}H]flunitrazepam binding (Biziere et al., 1981a).

Besides its effects on GABA receptors, thiocolchicoside also seems to interact with strychnine-sensitive glycine receptors. The drug was found to delay the appearance of strychnine-induced seizures (Biziere et al., 1981a) and to displace [^{3}H]strychnine binding (Hunt and Raynaud, 1977; Biziere et al., 1981a). Recently, Cimino et al. (1996) studied in more detail the interaction of thiocolchicoside with strychnine-sensitive glycine receptors in rat brainstem–spinal cord synaptosomal membranes. They found that thiocolchicoside displaces [^{3}H]strychnine binding with similar kinetic parameters of glycine and suggested that thiocolchicoside could directly interact with the glycine binding site of the receptor.

In the present study, the nature of the receptor(s) interacting with thiocolchicoside was evaluated using [^{3}H]thiocolchicoside as radioligand. The conditions under which maximal specific binding occurs were established, and its displacement by various compounds acting on both $GABA_A$, $GABA_B$ and strychnine-sensitive glycine receptors, as well as other receptor types was investigated. Because strychnine-sensitive glycine receptors have not been found in the cerebral cortex, a brain area particularly rich of $GABA_A$ receptors, experiments were performed using membrane preparations from both cerebral cortex and spinal cord–brainstem. Furthermore, the interaction of thiocolchicoside with $GABA_A$ receptors was also studied by assessing the ability of the compound to displace [^{3}H]muscimol binding in rat brainstem–spinal cord synaptic membranes.

2. Methods

2.1. Materials

GABA, isoguvacine, bicuculline, piperidine-4-sulphate, guvacine, picrotoxin, alphaxalone, allotetrahydrodeoxycorticosterone, pregnenolone, baclofen and flunitrazepam were obtained from RBI, Natick, MA, USA. Strychnine was obtained from Sigma-Aldrich, Milano, Italy. [^{3}H]Muscimol was purchased from Amersham Italia, Milano. [^{3}H]Thiocolchicoside was customer-labelled by Amersham, and kindly provided by Dr. J. Allen from Synthlabo Recherche, France.

2.2. [^{3}H]Thiocolchicoside binding assay

Sprague–Dawley rats (200–300 g) were decapitated and the spinal cord–brainstem and cerebral cortex rapidly removed on ice. The tissues were homogenized in 15 vol of ice-cold 0.32 M sucrose with 10 strokes of a glass/Teflon Potter homogenizer. The homogenate was centrifuged for 10 min at $1000 \times g$, the pellet discarded, and the resulting supernatant fluid centrifuged for 20 min at $20{,}000 \times g$. The crude synaptosomal pellet was resuspended in 15 vol of ice-cold distilled H_2O and homogenized for 30 s with an Ultra-Turrax homogenizer. The suspension was centrifuged for 20 min at $8000 \times g$. The supernatant was collected, and the pellet, a bilayer with a soft buffy uppercoat, was rinsed carefully with the supernatant fluid to collect the upper layer. The supernatant fluid was then centrifuged at $48{,}000 \times g$ for 20 min. The final pellet was stored at -20°C for at least 18 h. On the day of the experiment, membranes were resuspended in 15 vol of ice-cold 50 mM Tris–citric acid buffer (pH 7.1), and maintained for 30 min at 37°C. Membranes were then centrifuged at $48{,}000 \times g$ for 10 min. The resulting pellets were resuspended and centrifuged twice as above. To measure specific binding of [^{3}H]thiocolchicoside (40 Ci/mmol, Amersham) to spinal cord–brainstem or cortical membranes, aliquots of crude synaptic membranes (0.14–0.35 mg of protein) were incubated in Eppendorf tubes for 30 min at 4°C in a final volume of 250 μl of 50 mM Tris–citric acid buffer (pH 7.1) containing 5–800 nM [^{3}H]thiocolchicoside (saturation experiments) or 40 nM [^{3}H]thiocolchicoside (displacement experiments) alone or in the presence of 1 mM cold thiocolchicoside or various concentrations of other drugs. Since preliminary experiments indicated that the affinity of [^{3}H]thiocolchicoside for its binding site was in the high nanomolar range, to avoid rapid dissociation of the receptor-ligand complex the reaction was terminated by centrifugation at $15{,}000 \times g$ for 5 min at 4°C. The supernatant fluid was then aspirated and the pellet surface was washed once with 0.5 ml of cold buffer. The tips of the Eppendorf tubes were cut off and placed in scintillation mini-vials; 4 ml of scintillation fluid (Optiphase ''Hisafe'' 3; EG&G, Wallach) were added and the vials were shaken overnight to extract the radioactivity associated with each pellet. [^{3}H]Thiocolchicoside does not bind to Eppendorf tubes. Radioactivity was determined with a Wallach 1409 Liquid Scintillator counter with 50% efficiency. Specific [^{3}H]thiocolchicoside binding was obtained by subtracting from the total bound radioactivity the amount not displaced by cold 1 mM thiocolchicoside.

2.3. Association and dissociation experiments

The time necessary for [^{3}H]thiocolchicoside to reach the steady-state, and the affinity (K_D) for its binding sites were also assessed in association and dissociation experiments. In these assays, which require rapid binding determination, incubation was terminated by rapid filtration under pressure (Young and Snyder, 1974). In association experiments, [^{3}H]thiocolchicoside was incubated with aliquots of synaptic membranes for different times and rapidly filtered under pressure through Whathman GF/B filters which were washed once with 3 ml of cold buffer. The filters were placed in scintillation mini-vials, 4 ml of scintillation fluid (Optiphase ''Hisafe'' 3; EG&G) were added, and the vials were shaken overnight to extract the radioactivity associated with each filter. The binding of

[^{3}H]thiocolchicoside to filters was negligible. In dissociation experiments, samples were incubated for 30 min with [^{3}H]thiocolchicoside, then 1 mM cold thiocolchicoside was added and incubation stopped at different times by filtration through GF/B filters, as described above. Association and dissociation parameters were calculated by non-linear least-squares regression and K_D values for [^{3}H]thiocolchicoside were determined as the ratio of K_{off}/K_{on}.

2.4. [^{3}H]Muscimol binding

The interaction of thiocolchicoside with $GABA_A$ receptors was tested in a [^{3}H]muscimol binding assay, which was performed according to the procedure of Beaumont et al. (1978) as described by Banfi et al. (1984). Membranes were obtained as described for the [^{3}H]thiocolchicoside binding assay with the exception that, after thawing, the pellet was incubated at 37°C for 30 min with 0.05% Triton X-100. The membrane suspension was then centrifuged at $48,000 \times g$ for 10 min and the pellet washed three times with Triton-free buffer. The binding was determined by incubating membrane aliquots with 5 nM [^{3}H]muscimol and increasing concentrations of thiocolchicoside in 50 mM tris–citrate buffer, pH 7.1, in a final volume of 1 ml. After 30 min incubation at 4°C, the samples were rapidly filtered through Watman GF/B glass fiber filters which were then washed three times with 5 ml of ice-cold buffer. Non-specific binding was determined in the presence of 200 μM unlabeled GABA.

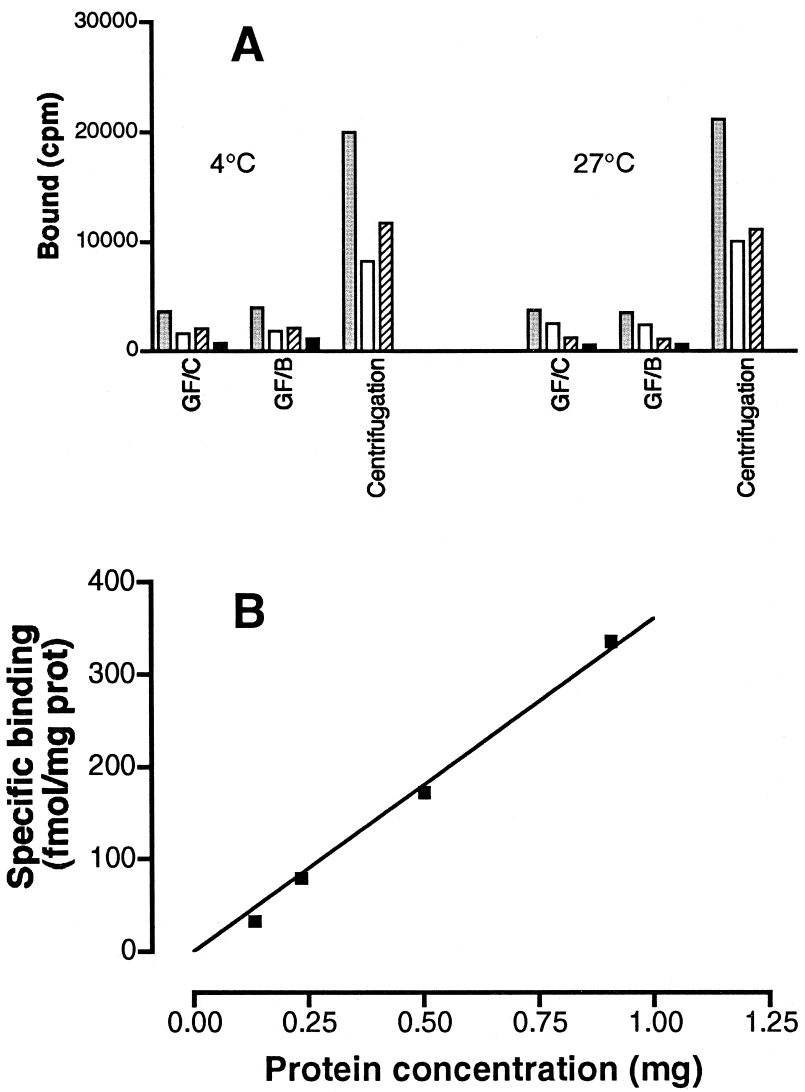

Fig. 1. (A) Effect of temperature, filtration through GF/C or GF/B filters previously soaked for 60 min in 0.05% poly(ethylenimine), and centrifugation on specific [^{3}H]thiocolchicoside binding. Membranes were incubated at 4 or 27°C for 30 min with 40 nM [^{3}H]thiocolchicoside and then filtered or centrifuged as described in Section 2. Gray bar, total binding; open bar, non-specific binding; hatched bar, specific binding; dark bar, filter binding. (B) Effect of different protein concentrations on specific [^{3}H]thiocolchicoside binding. Various amount of synaptic proteins obtained from rat spinal cord–brainstem were incubated with 40 nM [^{3}H]thiocolchicoside for 30 min at 4°C. Reaction was determined by centrifugation. Non-specific binding was determined in the presence of 1 mM cold thiocolchicoside. Values are from one experiment performed in triplicate.

2.5. Protein determination

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.6. Data analysis

Results were analyzed by non-linear fitting using the computer program Prism[Tm] (GrapPad Software, USA). The K_i values were calculated from the IC_{50} values using the Cheng–Prusoff equation. The *F*-test was used to assess whether the fitting using a two-site model equation was significantly better ($P < 0.05$) than that obtained with a one-site model.

3. Results

3.1. Binding conditions

Preincubation of synaptic membranes in buffer at 37°C for 30 min significantly decreased non-specific binding. Addition of 0.05% Triton X-100 during this preincubation period decreased total binding by about 50% without significant effects on non-specific binding (data not shown). Accordingly, binding experiments were routinely performed on membranes preincubated in buffer in the absence of Triton X-100.

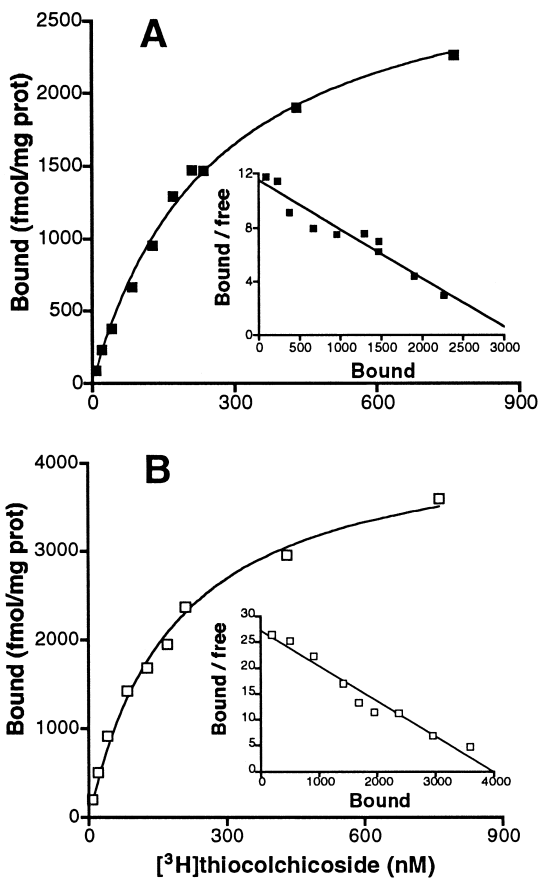

Fig. 2. Saturation of specific [^{3}H]thiocolchicoside binding in synaptic membranes isolated from rat spinal cord–brainstem (A) and cerebral cortex (B). Experiments were performed as described in Section 2. Results are from one representative experiment performed in triplicate. The experiment was replicated four times in the spinal cord and once in the cerebral cortex. Inset: Scatchard transformation of the saturation data.

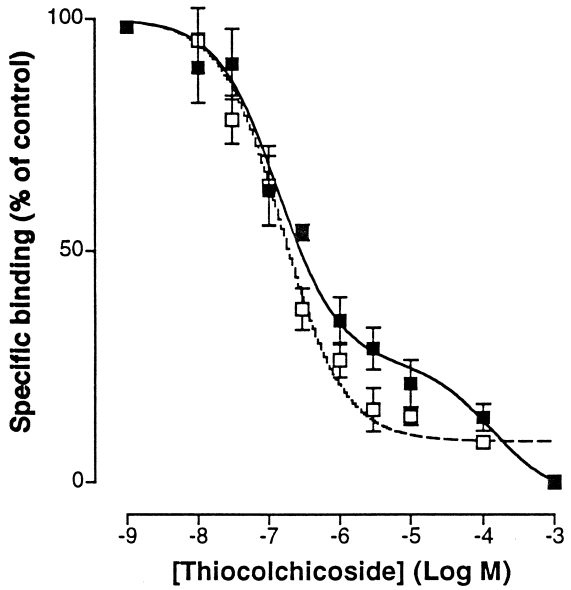

Fig. 3. Homologous displacement curves of [^{3}H]thiocolchicoside binding in synaptic membranes isolated from rat spinal cord–brainstem (■) and cerebral cortex (□). Results represent the mean ± SEM of four (spinal cord–brainstem) or three (cerebral cortex) experiments performed in triplicate.

To study the influence of temperature on binding, spinal cord–brainstem synaptic membranes were incubated with a single concentration of [^{3}H]thiocolchicoside for 30 min in the presence or absence of cold 1 mM thiocolchicoside at 4°C or 27°C. Incubation was interrupted by filtration under pressure through Watman GF/C or GF/B filters previously soaked for 60 min in 0.05% poly(ethylenimine) or by centrifugation at 15,000 rpm for 5 min. Specific binding was about 10 times higher when centrifugation was used, whereas the increase in the incubation temperature from 4°C to 27°C had only a negligible effect on specific binding (Fig. 1A).

Under standard assay conditions (40 nM [^{3}H]thiocolchicoside), 1.5–2.5% of the total ligand available was bound at equilibrium and specific binding represented approximately 32 and 54% of the total binding in spinal cord–brainstem and cerebral cortex membranes, respectively.

As shown in Fig. 1B, specific [^{3}H]thiocolchicoside binding to spinal cord–brainstem membranes was linear between 0.13 and 0.9 mg of protein. Binding studies were therefore performed within this range.

3.2. Saturation and homologous displacement experiments

Saturation curves from [^{3}H]thiocolchicoside binding studies to membrane preparations from spinal cord–

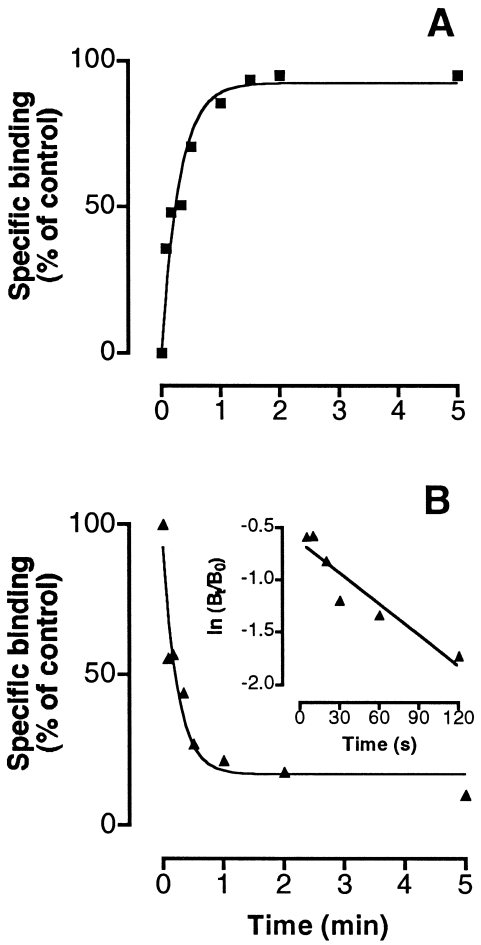

Fig. 4. Time course of association (A) and dissociation (B) of [^{3}H]thiocolchicoside specific binding to rat spinal cord–brainstem synaptic membranes. The experiment was performed as described in Section 2. Values are shown as percent of control specific binding obtained after 30 min incubation with the radioligand. Non-specific binding was determined in the presence of 1 mM cold thiocolchicoside. Points represent the mean of triplicate determinations from a single experiment. The experiment was repeated twice with similar results. Inset: plot of the dissociation data expressed as ln of the ratio between the amount of specific binding at time t (B_t) and the amount of specific binding at steady-state level (B_0).

brainstem and cortical membranes are reported in Fig. 2. In both tissues, specific [^{3}H]thiocolchicoside binding was saturable with increasing concentrations of the radiolabelled compound (5–800 nM) whereas non-specific binding, detected in the presence of 1 mM cold thiocolchicoside, increased linearly (not shown). Non-linear least-squares regression analyses indicated the presence of a single binding site: in spinal cord–brainstem membranes binding experiments ($n = 4$) gave a K_D of 254 ± 47 nM and a B_{max} of 2.39 ± 0.36 pmol/mg protein, whereas in cortical membranes ($n = 1$) the binding parameters were $K_D = 176$ nM and $B_{max} = 4.20$ pmol/mg protein.

Homologous displacement experiments using a single [^{3}H]thiocolchicoside concentration (40 nM) and increasing concentrations of cold thiocolchicoside were also performed (Fig. 3). In spinal cord–brainstem synaptic membranes, computer-assisted data analysis gave the best fit using a two-site model ($P < 0.01$): 72% of the binding sites showed a relatively high affinity ($K_i = 0.135$ μM) and 28% a low affinity ($K_i = 127$ μM). The latter binding site was not detected in saturation experiments since the highest concentration of [^{3}H]thiocolchicoside used was 800 nM. In cortical membranes, on the other hand, displacement binding data fit to a single component curve with a K_i of 0.127 μM. The Hill coefficients calculated from spinal cord–brainstem and cortical synaptosomal membranes were 0.5 and 0.75, respectively.

3.3. Kinetic experiments

As shown in Fig. 4, in rat spinal cord–brainstem membranes [^{3}H]thiocolchicoside binding reached equilibrium in about 1 min at 4°C ($T_{1/2} = 0.28$ min). A similar rapid dissociation rate was also observed ($T_{1/2} = 0.34$ min). K_{on} and K_{off} values were 0.0091 M^{-1} min^{-1} and 2.02 min^{-1}, respectively, and the K_D, calculated as K_{off}/K_{on} ratio was 221.9 nM, which was similar to the value obtained in saturation experiments.

3.4. Amino acid and drug competition

The effect on [^{3}H]thiocolchicoside binding of various aminoacids and of several drugs which interact with different receptor systems was evaluated in spinal cord–brainstem synaptic membranes using a single concentration of each compound. Among the aminoacids tested, only GABA, β-alanine and, to a lesser extent, taurine were able to displace the binding. The activity of the latter two amino acids is not surprising since it is known that they

Table 1
Aminoacids and other compounds effective in displacing [^{3}H]thiocolchicoside specific binding in spinal cord–brainstem membranes
Inhibition of specific [^{3}H]thiocolchicoside binding was determined as described in Section 2. Values are expressed as percent displacement of specific [^{3}H]thiocolchicoside binding determined in the presence of 1 mM cold thiocolchicoside and are the mean ± SEM of three separated experiments. The following compounds were found to be ineffective at 1 mM concentration: glycine, alanine, L-proline, L-serine, L-glutamate, L-cysteine, valine, histidine, leucine, pargiline, sulpiride, atropine, SCH23390, phenilephrine, isoproterenol, 5,7-dichlorokynurenic acid (DCKA), 2-amino-5-phosphonopentanoic acid (AP5), nicotine, guvacine, picrotoxin, pentylentetrazole, flunitrazepam (100 μM), baclofen.
All values were significantly different from control, $P < 0.01$ (Student's t-test).

Compound (1 mM)	Specific binding (percent of control)
GABA	50.8 ± 9.9
β-alanine	53.6 ± 2.8
Taurine	71.8 ± 7.6
Bicuculline	36.9 ± 5.5
Isoguvacine	39.0 ± 0.5
Piperidine-4-sulphate	20.2 ± 1.0
Strychnine	26.9 ± 4.3

interact with both GABA (Horikoshi et al., 1988; Maddison et al., 1990; Bureau and Olsen, 1991) and glycine receptors (Young and Snyder, 1974; Kishimoto et al., 1981). Of the different drugs tested, only GABA, the $GABA_A$ receptor antagonist bicuculline, and strychnine displaced the binding of [^{3}H]thiocolchicoside, whereas all the other drugs evaluated, most of which are unrelated to GABAergic transmission, were ineffective (see legend to Table 1). A lack of effect, however, was also observed with the GABA uptake inhibitor guvacine as well as picrotoxin. Baclofen, a $GABA_B$ receptor agonist, and flunitrazepam were also ineffective in displacing the binding.

To further characterize the pharmacological profile of [^{3}H]thiocolchicoside binding, we performed dose–response curves using unlabeled GABA, strychnine, glycine and several $GABA_A$ receptor interacting compounds as displacers. In spinal cord–brainstem membranes, both GABA and strychnine inhibited [^{3}H]thiocolchicoside binding in a dose-dependent manner (Fig. 5A). It should be noted that in this area, strychnine ($IC_{50} = 3.9 \pm 2.9$ μM; $n = 3$) was more potent that GABA ($IC_{50} > 1$ mM), and was able to displace a higher amount of specific binding (residual specific binding: strychnine 27.5 ± 1.5%, GABA 58.4 ± 9.7%). In contrast, in the cerebral cortex (Fig. 5C), both the specific binding displaced and the IC_{50} of the two compounds were comparable (residual specific binding: strychnine 24.3 ± 4.5%, GABA 25.6 ± 8.8%; IC_{50}: GABA $= 5.5 \pm 3.7$ μM, strychnine $= 10.6 \pm 3.4$ μM). Glycine, on the other hand, did not displace the binding in both tissues. The displacement profile of other $GABA_A$ receptor agonists and antagonists was also rather different in the two areas evaluated. In spinal cord–brainstem membranes, bicuculline, isoguvacine and piperidine-4-sulphate displaced the binding with similar potency (IC_{50} of 12.8 ± 3.9 μM, 30.1 ± 13.0 μM and 22.7 ± 7.6 μM, respectively). Isoguvacine and bicuculline had the same potency

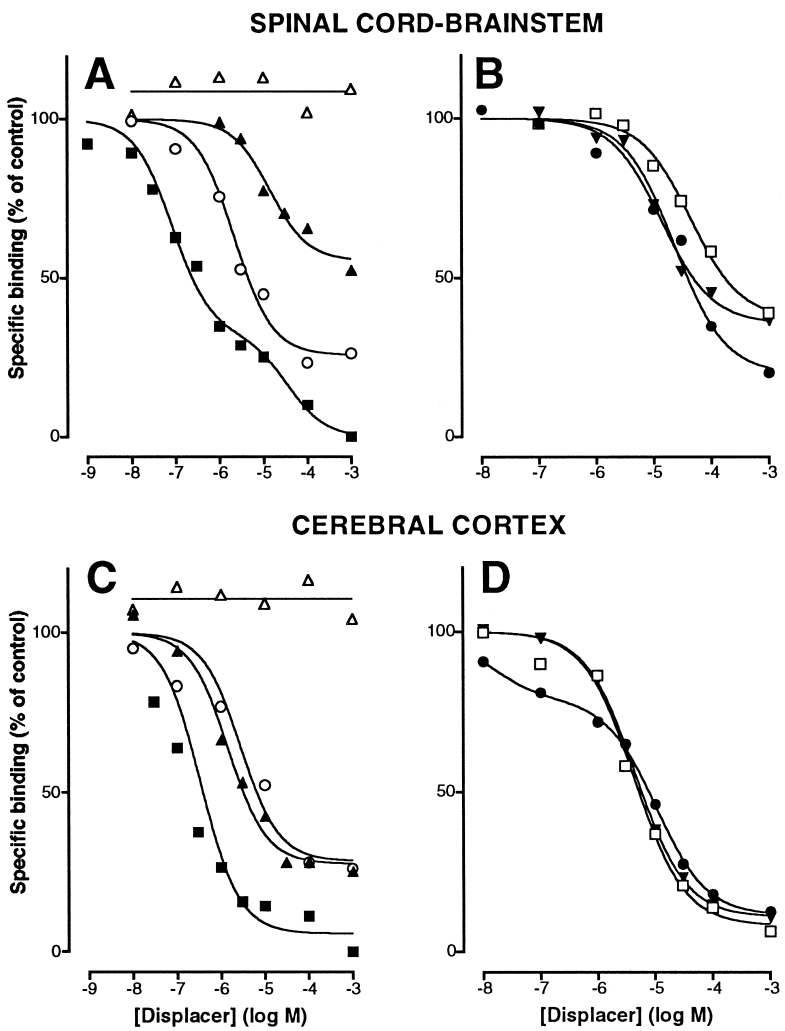

Fig. 5. Displacement curves of [^{3}H]thiocolchicoside binding in synaptic membranes from rat spinal cord–brainstem and cerebral cortex. Results represent the mean of three to four experiments performed in triplicate. Standard errors have not been reported in the figure and varied less than 10%. Data for cold thiocolchicoside are the same reported in Fig. 3. (■) Thiocolchicoside; (▲) GABA; (△) glycine; (○) strychnine; (▼) isoguvacine; (□) bicuculline; (●) piperidine-4-sulphate.

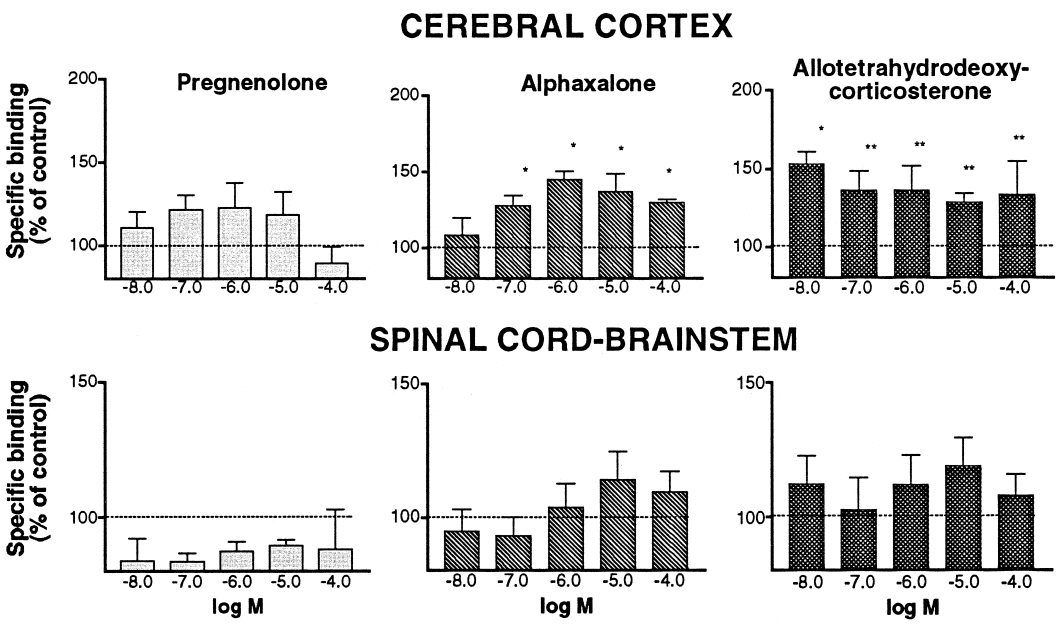

Fig. 6. Effect of several neurosteroids on [^{3}H]thiocolchicoside binding in synaptic membranes from rat cerebral cortex and spinal cord–brainstem. Results represent the mean ± SEM of three to four experiments performed in triplicate. $^*P < 0.01$; $^{**}P > 0.05$ (Student's *t*-test).

in displacing the binding also in cortical membranes (IC_{50} 3.9 ± 0.7 μM and 4.7 ± 0.5 μM, respectively). Piperidine-4-sulphate, on the other hand, showed a better fit to a two-site model as compared to a single site model, with an IC_{50H} of 0.06 ± 0.05 μM and an IC_{50L} of 11.9 ± 5.5 μM (Fig. 5D). It should be noted that in spinal cord–brainstem membranes, the IC_{50} of all GABAergic compounds were higher than in the cerebral cortex and, furthermore, all compounds displaced only 60–70% of the specific binding.

An unexpected observation of the present study was the displacement of [^{3}H]thiocolchicoside binding by strychnine in the cerebral cortex, a brain area devoid of strychnine-sensitive glycine receptors (Bristow et al., 1986). We therefore tested whether strychnine may also interact with $GABA_A$ receptors. Strychnine, indeed, was able to completely displace [^{3}H]muscimol binding with an IC_{50} value of 30 μM (data not shown), which is comparable to that found for the displacement of [^{3}H]thiocolchicoside binding (10.68 μM).

3.5. Modulation of [^{3}H]thiocolchicoside binding by neurosteroids

$GABA_A$ receptors can be modulated by various compounds, including neurosteroids. It has been reported that several neurosteroids, including the anesthetic alphaxalone, significantly increase [^{3}H]muscimol binding (Turner et al., 1989; Lambert et al., 1995). Since the experiments described above suggest that [^{3}H]thiocolchicoside interacts with the GABA receptor, we decided to evaluate whether [^{3}H]thiocolchicoside binding could also be modulated by neurosteroids, both in cortical and in spinal cord–brainstem synaptic membranes. Fig. 6 shows that in cortical membranes, both alphaxalone and allotetrahydrodeoxycorticosterone significantly increased the binding, whereas pregnenolone was ineffective. In contrast, the same compounds, when tested in spinal cord–brainstem membranes, were unable to modulate the binding.

3.6. Effect of cold thiocolchicoside on [^{3}H]muscimol binding

The evaluation of the interaction of thiocolchicoside with $GABA_A$ receptors was also carried out on brain-

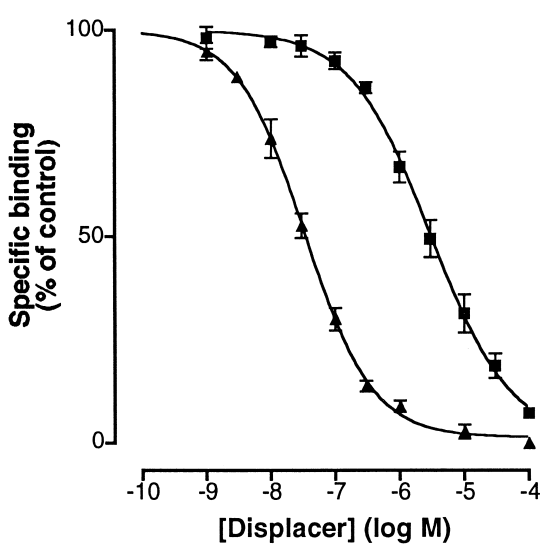

Fig. 7. Displacement of [^{3}H]muscimol binding by different concentrations of GABA, or thiocolchicoside in spinal cord–brainstem synaptic membranes. Results are the mean of three different experiments performed in triplicate. (▲) GABA; (■) thiocolchicoside.

stem–spinal cord membranes using [^{3}H]muscimol as radioligand (Fig. 7). It was found that thiocolchicoside ($IC_{50} = 2.013 \pm 0.359$ μM; nH = 0.71) completely inhibits [^{3}H]muscimol binding, although the IC_{50} value is much higher than that of GABA ($IC_{50} = 0.030 \pm 0.005$ μM; nH = 0.84). A complete inhibition of [^{3}H]muscimol binding was also observed in the cerebral cortex (data not shown).

4. Discussion

In the present study, we demonstrate the presence of [^{3}H]thiocolchicoside specific binding in rat spinal cord and cerebral cortex. The binding is displaced, in a dose-dependent manner, by GABA and several $GABA_A$ receptor agonists and antagonists which directly interact with the GABA binding site (isoguvacine, piperidine-4-sulphate and bicuculline), and by strychnine, but not by compounds acting on different sites on the $GABA_A$ receptor (picrotoxin, flunitrazepam) or other receptor systems. The observation that baclofen, a $GABA_B$ receptor agonist, is not able to displace [^{3}H]thiocolchicoside binding excludes an interaction of the drug with this receptor subtype.

In the cerebral cortex, it appears that the main target for thiocolchicoside are $GABA_A$ receptors. This is suggested by the displacement profile of the different $GABA_A$ receptor agonists and antagonists and also by the finding that [^{3}H]thiocolchicoside binding is enhanced in the presence of neurosteroids (Fig. 6), a kind of effect previously observed on [^{3}H]muscimol binding (Turner et al., 1989). Furthermore, cold thiocolchicoside completely displaces [^{3}H]muscimol binding (data not shown). Since both agonists and antagonists showed an IC_{50} in the μM range, it may be hypothesized that thiocolchicoside selectively interact with the low affinity site of the $GABA_A$ receptor which seems to be responsible for the physiological activity of GABA (Sieghart, 1992, 1995).

In spinal cord–brainstem membranes, instead, the binding profile was more complex. Homologous displacement experiments identified two binding sites and strychnine was the most potent compound in displacing the binding. GABA showed a low potency and, even at 1 mM concentration, was unable to displace more than 50% of the binding. Also, the other $GABA_A$ receptor-interacting compounds, however, although more potent than GABA, did not displace more than 60–70% of the specific binding. In the spinal cord–brainstem, therefore, only a fraction of the [^{3}H]thiocolchicoside binding sites are sensitive to GABA and its analogs, indicating that, besides $GABA_A$ receptors, thiocolchicoside binds to other unidentified site(s).

The observation that glycine was not able to displace the binding of [^{3}H]thiocolchicoside was unexpected since we recently reported that in spinal cord membranes cold thiocolchicoside behaves like glycine in displacing [^{3}H]strychnine binding. These results were interpreted as if thiocolchicoside could directly interact with the glycine recognition site of the strychnine-sensitive glycine receptor, and that this interaction could be responsible for the allosteric modulation of [^{3}H]strychnine binding (Cimino et al., 1996). The results obtained with [^{3}H]thiocolchicoside, however, do not support a direct interaction of the drug with the glycine binding site of the receptor.

Another unexpected finding also concerns the potency of strychnine in inhibiting [^{3}H]thiocolchicoside binding, particularly in the cerebral cortex where strychnine-sensitive glycine receptors are not present. Strychnine-sensitive glycine receptors, $GABA_A$ receptors, as well as nicotinic receptors belong to the transmitter-gated receptor superfamily and show with other members of this family several structural and functional similarities. The cDNAs for all the subunits for the various members are evolutionarily related, sharing a common sequence homology and predicted topological structure for the polypeptides. Vanderberg et al. (1992) performed in vitro mutagenesis and functional analysis of the α1-subunit of the glycine receptor to study the domains that form the strychnine binding site, and found that several residues involved in the binding of strychnine are conserved among subunits of all the members of the ligand-gated ion channel receptor superfamily. Indeed, there is evidence indicating that strychnine can also interact with $GABA_A$ and nicotinic receptors. Goldinger and Muller (1980), for example, reported that the GABA receptor antagonist bicuculline inhibits specific [^{3}H]strychnine binding in spinal cord synaptosomal membranes. In agreement with the results of these authors, we found that in cortical membranes strychnine is able to displace [^{3}H]muscimol binding (data not shown). Furthermore, it has recently been reported that strychnine acts as a competitive antagonist of α-bungarotoxin-sensitive nicotinic receptors (Matsubayashi et al., 1998). Thiocolchicoside, therefore, might interact with the same binding sites of strychnine but, differently from strychnine, it has higher affinity for the $GABA_A$ receptor. In fact, we found that in cortical membranes, strychnine displaces [^{3}H]muscimol binding with an IC_{50} of 30 μM, whereas the IC_{50} for thiocolchicoside was 2.01 μM. In addition, thiocolchicoside is not able to allosterically interact with the glycine site of the strychnine-sensitive glycine receptors, since glycine does not displace the binding of [^{3}H]thiocolchicoside binding but displace in a dose-dependent manner [^{3}H]strychnine binding (Cimino et al., 1996).

There is evidence to support the possibility of a multiplicity of $GABA_A$ receptors. Regional variations in mRNAs for the different $GABA_A$ receptor subunits have been demonstrated within the central nervous system (Bureau and Olsen, 1991; Sieghart, 1995), which may result in receptors with different function and regulation. For example, regional differences have been shown in neurosteroid modulation of $GABA_A$ receptor complexes obtained from the frontal cortex and spinal cord (Gee and Lan, 1991), or in modulation by benzodiazepines (Costa, 1998). The

different sensitivity of [^{3}H]thiocolchicoside binding to $GABA_A$ receptor agonists and antagonists and to strychnine in the spinal cord compared to the cerebral cortex suggests that the compound may recognize different subtypes of $GABA_A$ receptors differentially expressed in the two areas of the CNS considered. This hypothesis is also supported by the effect of neurosteroids that increase the binding of [^{3}H]thiocolchicoside in cortical but not in spinal cord–brainstem synaptic membranes (Fig. 6). Furthermore, autoradiographic studies show an uneven distribution of [^{3}H]thiocolchicoside binding sites in the gray matter of the spinal cord (manuscript in preparation). Binding and functional experiments with recombinant $GABA_A$ receptors may help to elucidate the subunit composition which preferentially bind thiocolchicoside and whether the compound acts as an agonist or an antagonist on these receptors.

In conclusion, the results reported in this study demonstrate the presence of [^{3}H]thiocolchicoside binding sites in the rat cerebral cortex and in the spinal cord. The different affinities for the $GABA_A$ receptor agonists and antagonists and sensitivity to neurosteroids obtained in the cerebral cortex and in the spinal cord may indicate a preferential interaction of the compound with a subtype of the $GABA_A$ receptor. Furthermore, in the spinal cord, [^{3}H]thiocolchicoside binds also to another site(s), the nature of which remains unknown and deserves further investigation to clarify the mechanism responsible for the myorelaxant action of thiocolchicoside observed in humans.

References

Banfi, S., Dorigotti, L., Abbracchio, M.P., Balduini, W., Coen, E., Ragusa, C., Cattabeni, F., 1984. Methylazoxymethanol microencephaly as a model for studying nootropic drugs: neurochemical characterization and behavioral studies with oxiracetam. Pharmacol. Res. Commun. 16, 67–84.

Beaumont, K., Chilton, S.W., Yamamura, H.I., Enna, S.J., 1978. Muscimol binding in rat brain: association with synaptic GABA receptors. Brain Res. 148, 153–162.

Biziere, K., Huguet, F., Narcisse, G., Breteau, M., 1981a. Le thiocolchicoside, un agoniste des récepteurs GABAergiques. Thérapie 36, 95–96.

Biziere, K., Huguet, F., Narcisse, G., Breteau, M., 1981b. Affinity of thiocolchicoside and thiocolchicoside analogues for the postsynaptic GABA receptor site. Eur. J. Pharmacol. 75, 167–168.

Bristow, D.R., Bowrey, N.G., Woodruff, G.N., 1986. Light microscopic autoradiographic localization of [^{3}H]Glycine and [^{3}H]Strychnine binding sites in rat brain. Eur. J. Pharmacol. 126, 303–307.

Bureau, M.H., Olsen, R.W., 1991. Taurine acts on a subclass of $GABA_A$ receptors in mammalian brain in vitro. Eur. J. Pharmacol. 207, 9–16.

Cimino, M., Marini, P., Cattabeni, F., 1996. Interaction of thiocolchicoside with [^{3}H]strychnine binding sites in rat spinal cord and brainstem. Eur. J. Pharmacol. 318, 201–204.

Costa, E., 1998. From $GABA_A$ receptor diversity emerges a unified vision of the GABAergic inhibition. Annu. Rev. Pharmacol. Toxicol. 38, 321–350.

Françon, D., Adam, R., Decobert, M., Depoortere, H., 1998. Thiocolchicoside: a muscle relaxant devoid of sedative effect 358. Arch. Pharmacol. 358 (N.1), R39, Suppl. 1.

Gee, K.W., Lan, N.C., 1991. Gamma-amminobutyric $acid_A$ receptor complexes in rat frontal cortex and spinal cord show differential responses to steroid modulation. Mol. Pharmacol. 40, 995–999.

Goldinger, A., Muller, W.E., 1980. Stereospecific interaction of bicuculline with specific [^{3}H]strychnine binding to rat spinal cord synaptosomal membranes. Neurosci. Lett. 16, 91–95.

Horikoshi, T., Asanuma, A., Yanagisawa, K., Anzai, K., Goto, S., 1988. Taurine and beta-alanine act on both GABA and glycine receptors in *Xenopus* oocyte injected with mouse brain messenger RNA. Brain Res. 464, 97–105.

Hunt, P., Raynaud, J.P., 1977. Benzodiazepine activity: is interaction with the glycine receptor, as evidenced by displacement of strychnine binding, a useful criterion?. J. Pharm. Pharmacol. 29, 442–444.

Janbroers, J.M., 1987. Review of the toxicology, pharmacodynamics and pharmacokinetics of thiocolchicoside, a GABA-agonist muscle relaxant with anti-inflammatory and analgesic actions. Acta Therapeutica 13, 221–250.

Kishimoto, H., Simon, J.R., Aprison, M.H., 1981. Determination of the equilibrium dissociation constants and number of glycine binding sites in several areas of the rat central nervous system, using a sodium-independent system. J. Neurochem. 37, 1015–1024.

Lambert, J.J., Belelli, D., Hill-Venning, C., Peters, J.A., 1995. Neurosteroids and $GABA_A$ receptor function. Trends Pharmacol. Sci. 16, 295–303.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maddison, J.E., Leong, D.K., Dodd, P.R., Johnston, G.A., 1990. Plasma GABA-like activity in rats with hepatic encephalopathy is due to GABA and taurine. Hepatology 11, 105–110.

Matsubayashi, H., Alkondon, M., Pereira, E.F.R., Swanson, K.L., Albuquerque, E.X., 1998. Strychnine: a potent competitive antagonist of the α-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal neurons. J. Pharmacol. Exp. Ther. 284, 904–913.

Sieghart, W., 1992. $GABA_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug-binding sites. Trends Pharmacol. Sci. 13, 446–450.

Sieghart, W., 1995. Structure and pharmacology of the γ-aminobutyric $acid_A$ receptor subtypes. Pharmacol. Rev. 47, 181–234.

Turner, D.M., Ransom, R.W., Yang, J.S.J., Olsen, R.W., 1989. Steroid anesthetics and naturally occurring analogs modulates the GABA receptor complex at a site distinct from barbiturates. J. Pharmacol. Exp. Ther. 248, 960–966.

Vanderberg, R.J., French, C.R., Barry, P.H., Shine, J., Schofield, P.R., 1992. Antagonism of ligand-gated ion channel receptors: two domains of the glycine receptor α subunit from the strychnine-binding site. Proc. Natl. Acad. Sci. U.S.A. 89, 1765–1769.

Young, A.B., Snyder, S.H., 1974. Strychnine binding in rat spinal cord membranes associated with the synaptic glycine receptor: cooperativity of glycine interactions. Mol. Pharmacol. 10, 790–809.