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Modification of the GABA/benzodiazepine receptor with the arginine reagent, 2,3-butanedione

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Treatment of either crude or purified preparations of the γ-aminobutyrate (GABA)/benzodiazepine receptor complex with arginine-specific reagents resulted in a time- and concentration-dependent loss of [^{3}H]muscimol binding activity. Following exposure to either 2,3-butanedione or phenylglyoxal ($\leq$20 mM), [^{3}H]muscimol binding was inhibited by up to 80%. [^{3}H]Flunitrazepam binding was much less sensitive to the effects of the reagents. Scatchard analysis of the binding data indicated that treatment with butanedione resulted in a loss of [^{3}H]muscimol binding sites with little effect on binding affinity. Considerable protection against inactivation was provided by arginine and by the endogenous receptor ligand, GABA. These results indicate that arginine residues play a critical role in maintaining the GABA receptor in a conformation capable of ligand binding, possibly by participating in the binding site through interaction with the carboxylate moiety of GABA.

GABA receptor; Benzodiazepine receptor; Arginine residue; Chemical modification

1. INTRODUCTION

γ-Aminobutyrate (GABA) is the principal inhibitory neurotransmitter in the mammalian central nervous system. A major class of GABA receptors ($GABA_A$ receptors) are associated with anion channels which in many cases constitute part of a protein complex which also possesses binding sites for benzodiazepines and other centrally active drugs [1,2]. Highly purified preparations of this receptor complex have been found, in most cases, to be comprised of at least two distinct polypeptides [3–5], and two recent reports indicate that GABA- and benzodiazepine-receptor ligands photolabel different subunits of the receptor complex purified from bovine cerebral cortex [6,7]. However, little is known of the nature of the amino acids which participate directly in the interaction between ligands and their binding sites. In view of a recent report, describing the primary amino acid sequence of $GABA_A$ receptor subunits [8], studies aimed at defining the amino acid residues of the polypeptides involved with ligand binding may help to elucidate the precise mechanism through which the receptor complex operates. A critical role for histidine residues in the binding of benzodiazepines has been reported [9]. The same report also demonstrated the ability of diazotized sulphanilate to eliminate low-affinity GABA binding sites. However, the reactivity of this compound is only moderately selective and the identity of the modified amino acids remains unclear. In the present study we report on the ability of arginine-specific reagents to inactivate [^{3}H]muscimol binding to the receptor complex. The data indicate that arginine residues may be intimately involved with the binding of GABA to the $GABA_A$ receptor.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

[*methylamine*-^{3}H]Muscimol (13 Ci/mmol) and [*N-methyl*-^{3}H]flunitrazepam (85 Ci/mmol) were from Amersham (Bucks, England). Phenylglyoxal and 2,3-butanedione were from Sigma (Dorset, England). The sources of materials used for receptor purification were as noted previously [5]. All other chemicals were obtained from BDH (Dorset, England).

2.2. *Preparation of crude synaptic membranes and isolation of the receptor complex*

A crude preparation of synaptic membranes was prepared from pig cerebral cortex as in [10] except that all media contained the protease inhibitors EDTA (1 mM), benzamidine (1 mM), phenylmethanesulphonyl fluoride (0.3 mM), trypsin inhibitors type IIS and II-O (each 10 mg/l). Membranes were stored frozen at −20°C for up to 2 months. Before chemical modification and assay of receptor binding, the membranes were thawed, washed once with 20 mM potassium phosphate buffer (pH 7.5), 100 mM KCl, 0.1 mM EDTA (referred to as 'buffer A') and dialysed for 20 h against 500 vol. buffer A.

The GABA/benzodiazepine receptor complex was purified from a Triton X-100-extract of pig brain membranes by a combination of affinity chromatography, on Ro1986/1-agarose, and ion-exchange chromatography as described in [5]. The concentration of [^{3}H]muscimol binding sites in the isolated preparation was typically in the range of 50–100 pmol/ml. The purified receptor complex was stored frozen at −20°C for up to 1 month before use.

2.3. *Chemical modification with arginine reagents*

For modification with butanedione [11], an aliquot (~50 μl) of purified receptor was diluted 4-fold with 50 mM sodium borate (pH 8.2), 0.1% Triton X-100 containing butanedione at indicated concentrations. After incubation at 25°C for up to 2 h, receptor binding activities were assayed either directly or, where indicated, after chromatography on a column of Sephadex G-25 in 50 mM sodium borate, 0.1% Triton X-100. Aliquots (400 μl) of the membrane preparations (10 mg/ml) were treated similarly except that Triton X-100 was omitted from the incubation buffer. Modification of the receptor complex with phenylglyoxal was performed as for butanedione but in 0.1 M sodium carbonate buffer (pH 8.0) instead of sodium borate.

2.4. *Binding assays*

Except where stated otherwise, ligand binding to the purified receptor complex was measured by a polyethylenimine/filtration assay modified from that described in [5,12]. The purified receptor complex was incubated with 40 nM [^{3}H]muscimol or 10 nM [^{3}H]flunitrazepam at 0°C for 60 min. Other conditions were as stated [5]. Following incubation, the assay mixture was filtered on polyethylenimine-soaked filters [12]. The filters were washed twice with 4 ml of 10 mM potassium phosphate (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, dried and counted for radioactivity in 4 ml of scintillation fluid. All assays were performed in duplicate. Membranes (~0.5 mg protein) were incubated with 8 nM [^{3}H]muscimol or 2 nM [^{3}H]flunitrazepam at 0°C for 60 min in a total volume of 500 μl buffer A. Following incubation, the assay mixtures were filtered and counted as described above.

2.5. *Sephadex G-25 chromatography*

Desalting, subsequent to the butanedione treatment, was performed by chromatography on a column (1.3 × 8.5 cm) of Sephadex G-25 pre-equilibrated with 50 mM sodium borate (pH 8.2), 0.1% Triton X-100. The receptor preparation (200 μl) was applied to the column and chromatographed at 0.15 ml/min in equilibration buffer. The initial 1.12 ml of eluate was discarded and the following 2.25 ml was retained for determination of ligand binding activities.

3. RESULTS AND DISCUSSION

The binding of [^{3}H]muscimol to crude membranes, or to purified preparations of the GABA/benzodiazepine receptor complex, was reduced substantially after treatment with the arginine-modifying reagent, 2,3-butanedione (fig.1A,B). In contrast, the binding of [^{3}H]flunitrazepam was much less sensitive to the effects of the reagent (fig.1A,B). [^{3}H]Muscimol binding to the purified receptor complex was also

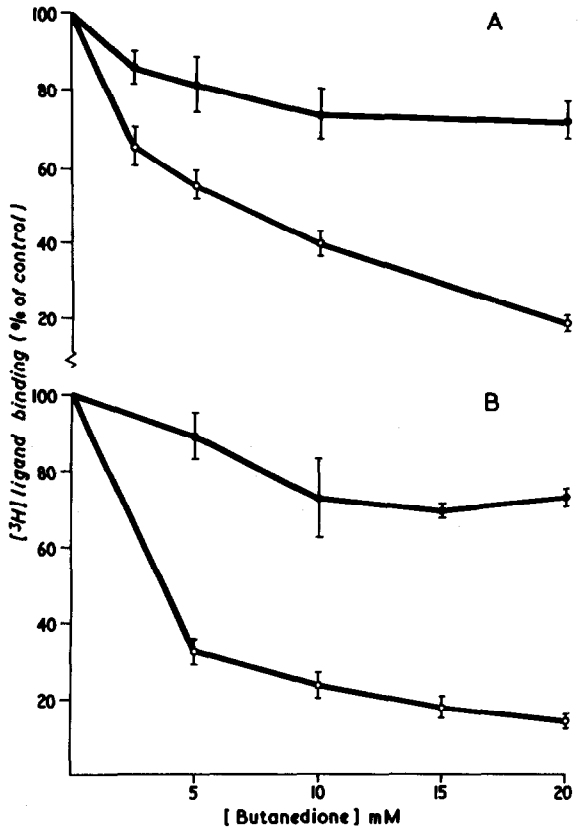

Fig.1. Effect of butanedione-treatment on ligand binding to the GABA/benzodiazepine receptor complex. Aliquots of membranes (A) or purified receptor complex (B) were incubated for 2 h in the absence or presence of butanedione as described in section 2. Following incubation, samples were assayed for [^{3}H]muscimol (○) or [^{3}H]flunitrazepam (●) binding activity. Results represent the mean and standard error of four independent experiments. Representative preparations of membranes or purified receptor complex for which specific [^{3}H]muscimol binding activities were determined, indicated values of approx. 1.5 pmol/mg and 3.5 nmol/mg, respectively. Typically, the concentration of [^{3}H]muscimol binding sites in the modification reaction mixture was ~4 pmol/ml (membranes) or ~15 pmol/ml (purified receptor complex).

inactivated (>80% inhibition at 10 mM) selectively, after treatment with another arginine-modifying reagent, phenylglyoxal (not shown). Inactivation of [^{3}H]muscimol binding by butanedione was both concentration- and time-dependent (fig.2). Incubation for 2 h in the presence of

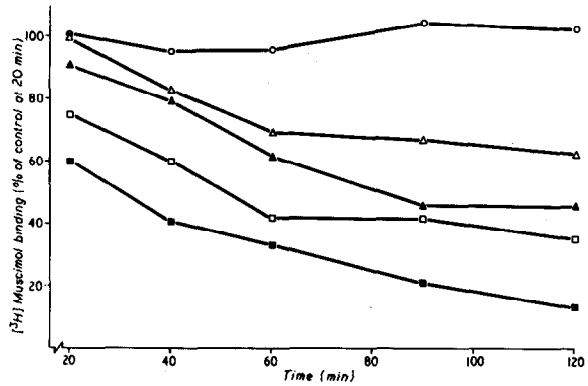

Fig.2. Time- and concentration-dependent inactivation of [^{3}H]muscimol binding activity. Binding of [^{3}H]muscimol was measured after incubation of the purified receptor complex in the absence (○) or presence of 2.5 mM (△), 5 mM (▲), 10 mM (□) or 20 mM (■) butanedione. Each point represents the mean of three individual experiments. The standard errors for each point were less than 9%.

20 mM butanedione reduced [^{3}H]muscimol binding activity by up to 80%.

The inactivation of [^{3}H]muscimol binding was not reversible and binding activity could not be restored after removal of the reagent by gel filtration (tables 1,2). Scatchard analysis of [^{3}H]muscimol binding activity after treatment of

Table 1

Scatchard analysis of [^{3}H]muscimol binding following butanedione treatment

Pretreatment	K_d (nM)	B_{max} (%)
Control	11.2 ± 1.1	100
Butanedione	8.4 ± 1.6	43 ± 8

The purified receptor complex was incubated in the absence or presence of 20 mM butanedione for 2 h. Prior to the binding assays, both treated and untreated samples were chromatographed on Sephadex G-25 as described in section 2. In order to detect the maximum amount of bound ligand, [^{3}H]muscimol binding was measured using a polyethyleneglycol-precipitation/centrifugation assay [5], over a concentration range of 1 to 100 nM. The butanedione-induced inactivation of binding activity determined by this procedure did not differ significantly from that measured by the filtration method. Results are the mean and standard error of four independent experiments

Table 2

Protection of [^{3}H]muscimol binding activity from inactivation by butanedione

Pretreatment	[^{3}H]Muscimol binding (% of unmodified control)
Butanedione[a]	11.6 ± 2.8
+ 5 mM arginine	11.5 ± 1.7
+ 10 mM arginine	17.8 ± 3.9
+ 20 mM arginine	35.6 ± 5.5
+ 30 mM arginine	69.1 ± 8.9
+ 40 mM arginine	81.8 ± 1.9
+ 40 mM lysine	18.5 ± 5.2
Butanedione[b]	18.2 ± 1.7
+ 1 μM GABA	80.4 ± 1.3

[a] [^{3}H]Muscimol binding activity was determined after incubation of the purified receptor for 2 h at 25°C with arginine or lysine in the presence or absence of 20 mM butanedione

[b] Aliquots of purified receptor were preincubated at 25°C for 30 min in the absence or presence of 1 μM GABA. Samples from each preincubation mixture were then incubated for 90 min at 25°C in the absence or presence of 20 mM butanedione. Prior to determination of [^{3}H]muscimol binding activity, all samples were chromatographed on Sephadex G-25 as described in section 2. Preincubation with GABA did not inhibit the binding of [^{3}H]muscimol to unmodified samples; 116 (110–125)% of control. All results represent the mean and standard error of three independent experiments

the purified receptor with butanedione revealed that the loss of activity resulted principally from a reduction in the apparent number of binding sites, whereas the binding affinity, as represented by the K_d values, was not altered significantly (table 1). These data indicate that the arginine residues modified by butanedione play a critical role in maintaining the GABA receptor in a conformation capable of ligand binding, possibly by comprising part of the binding site itself.

Butanedione has the potential for side reactions with α-amino groups and with the ϵ-amino group of lysine although, under the conditions employed in these studies, its reactivity is reported to be directed at arginine residues specifically [11]. The ability of arginine (10–40 mM) but not lysine (40 mM) to protect up to 70% of [^{3}H]muscimol binding activity from inactivation (table 2) suggests that it is a modification of arginine residues which results in loss of activity. [^{3}H]Muscimol binding activity was also protected from inactivation by preincubating the receptor complex in the presence of GABA prior to treatment with butanedione. At a concentration of 1 μM, GABA protected approx. 60% of the binding activity from inactivation (table 2). The ability of GABA to protect [^{3}H]muscimol binding activity from inactivation is consistent with the view that arginine residues, essential for GABA receptor binding, are components of the ligand binding sites.

Radiolabelled arginine-reactive compounds may therefore supplement covalent labelling with [^{3}H]muscimol [13] as tools for exploring the molecular structure of GABA-binding sites.

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