Br. J. Pharmac. (1985), **84**, 689–696

Central effects of nicotinamide and inosine which are not mediated through benzodiazepine receptors

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1 The actions of nicotinamide and inosine were investigated on rat cerebellar Purkinje cells using ionophoretic and extracellular recording techniques.

2 Ionophoretic application of nicotinamide or inosine showed that they were potent inhibitors of Purkinje cell firing. This inhibition differed from that induced by benzodiazepines in that it was not reversed by the GABA antagonists bicuculline methiodide and picrotoxin. RO 15-1788, the specific benzodiazepine antagonist, did not reverse the effects of nicotinamide.

3 Chlordiazepoxide has been shown to increase significantly social interaction between pairs of male rats and this increase can be reversed by RO 15-1788, 20 mg kg^{-1} i.p. Nicotinamide also caused a small increase in social interaction but this effect was not reversed by the benzodiazepine antagonist. Inosine did not increase social interaction.

4 [^{3}H]-flunitrazepam binding studies showed that nicotinamide and inosine have only low affinities for the benzodiazepine binding site.

5 These results suggest that while nicotinamide may exert some neuronal depressant and anxiolytic activity, its site of action appears not to be associated with the benzodiazepine receptor site. Similarly, inosine exerts a neuronal depressant effect dissimilar from that of benzodiazepines.

Introduction

The discovery of pharmacologically relevant, high affinity binding sites for the benzodiazepines in the central nervous system has prompted studies on the possible physiological significance of these sites and attempts at isolating endogenous ligands (Squires & Braestrup, 1977). Nicotinamide and inosine are endogenous brain constituents, which have, in the past, been proposed as putative ligands at the benzodiazepine receptor (Mohler *et al.*, 1979; Skolnick *et al.*, 1979). Previous reports by other workers have suggested that both substances have benzodiazepine-like actions. For example, nicotinamide has been shown to protect rats against seizures induced by both 3-mercaptopropionic acid and isoniazid (Balzer *et al.*, 1960), a property shared with the benzodiazepines. Similarly, inosine, like benzodiazepines, antagonizes caffeine-induced seizures (Marangos *et al.*, 1981). In a conflict test nicotinamide restored punishment-suppressed behaviour in rats, an action characteristic of clinically effective anti-anxiety agents (Mohler *et al.*, 1979).

In this study we describe evidence which suggests that although nicotinamide exerts both neuronal depressant and anti-anxiety effects, these actions are mediated by a mechanism(s) other than direct interaction at the benzodiazepine receptors. The electrophysiological and behavioural effects of nicotinamide could not be antagonized by the specific benzodiazepine antagonist RO 15-1788 (Hunkeler *et al.*, 1981). In addition nicotinamide was shown to have only weak affinity for the [^{3}H]-flunitrazepam binding site.

Methods

Electrophysiological studies

Female Wistar rats weighing 150–200 g were anaesthetized initially with chloral hydrate, 350 mg kg^{-1} and maintained with 1–2% halothane in 100% oxygen. The rat was positioned in a Narishige sterotaxic headholder, a small hole trephined in the skull over the dorsal aspect of the cerebellum and the dura incised. Saline (0.9% w/v NaCl solution) was used to protect the exposed cortex from drying during the experiment.

Seven barrelled glass microelectrodes were used with an overall external tip diameter of 5–8 μm. These electrodes were used to record extracellular action potentials from individual Purkinje cells through the central barrel which was filled with 0.5 M sodium chloride. Drugs were administered ionophoretically at the recording site from the solutions in the outer barrels of the electrodes. Retaining currents of 10 nA of appropriate polarity were applied to prevent or minimize spontaneous efflux of compounds from the electrode tip. One of the outer barrels of the electrode was filled with 2 M sodium chloride and used for current balancing (Salmoiraghi & Weight, 1967). Drugs were dissolved in distilled water and the correct pH obtained by the addition of 0.1 M HCl or NaOH. The drug concentration in the electrodes was normally 0.2 M except for RO 15-1788 which was a saturated solution. All the drugs used in this study were ejected as cations. Ejecting currents were in the range of 5–80 nA.

Social interaction test

The method used (Gardner & Guy, 1984) is a modification of that developed by File & Hyde (1978) and is probably due to a more balanced conflict of competing tendencies, and has been shown to be sensitive to the effects of acutely administered benzodiazepines. Male Wistar rats, 180–200 g, were housed in pairs for four days before the experimental test. They were allowed food and water *ad libitum* and were kept on a 12 h light – 12 h dark cycle. Animals were familiarized to the test environment on the two days before the test. Familiarization involved placing cage-mate pairs in the test box for a period of 8 min. The test box (45 × 45 cm with walls 40 cm high) was positioned inside a sound attenuated chamber, lighting was achieved by use of a strip light positioned 75 cm above the floor of the chamber (5 lux). Animals were dosed orally or intraperitoneally with either vehicle (demineralized water, 2 ml kg^{-1}) or test drug 30 min before testing. The antagonist RO 15-1788 was also given intraperitoneally 15 min before testing.

For the behavioural observations, an observation period of 5 min was used, during which the aminal was placed in the test chamber with an animal from another cage. These pairs were allowed to meet only during the observation period. The components of social interaction measured were as follows: sniffing partner; grooming partner; genital investigation; crawling over; crawling under; walking together and following. The duration of occurrence of all the components were added together as a total duration in seconds. Each result is a mean score of seven pairs of rats. Analysis of variance (ANOVA) was used to analyse these data, together with Fisher's least significant difference test and Student's *t* test to indicate specific differences between groups.

[^{3}H]-flunitrazepam binding

For the membrane preparation, male Sprague-Dawley rats, 150–200 g, were killed by cervical dislocation and the brain removed. The cerebellum was rapidly dissected out and subsequently placed in 0.32 M sucrose on ice. Membranes for binding assays were prepared using a modification of the protocol described by Mohler & Okada (1977). The tissue was homogenized in 20 volumes of ice-cold sucrose, the homogenate centrifuged at 1,000 *g* for 10 min. The resulting pellet was discarded and the supernatant recentrifuged at 30,000 *g* for 30 min before resuspension in 100 volumes of Krebs-Tris HCl buffer, pH 7.6 (composition mM: Tris 15, NaCl 118, KCl 4.8, $CaCl_2$ 1.2, $MgCl_2$ 1.2).

Specific flunitrazepam binding was measured by a filtration assay. For routine displacement studies assays contained 0.3 nM [^{3}H]-flunitrazepam (74.2 Ci $mmol^{-1}$). Eight drug concentrations were used for each displacement curve, each being repeated in triplicate. The reproducibility of the triplicates was extremely high, the values differing by less than 10%. Non-specific binding was determined in the presence of 10 μM diazepam. The assay was incubated at 4°C for 90 min and was terminated by filtration through Whatman GF/B glass fibre filters under vacuum. Filters were washed with 2 × 10 ml rinses of ice-cold assay buffer. The filters were dried and bound radioactivity was determined by scintillation spectrometry.

The following compounds were used in this study: γ-aminobutyric acid, glycine (BDH); 5-hydroxytryptamine bimaleate (Koch-Light); bicuculline methiodide, inosine, nicotinamide, noradrenaline bitartrate, picrotoxin (Sigma); flurazepam (Profarmaco Milan); chlordiazepoxide, diazepam, flunitrazepam, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo (1,5a) (1,4) benzodiazepine-3-carboxylate (RO 15-1788, Roche); loprazelam (Roussel).

Results

Electrophysiological studies

Cerebellar Purkinje cells showed a spontaneous firing rate of 25–50 spikes per second. The benzodiazepines, flurazepam, flunitrazepam, chlordiazepoxide and loprazolam all caused a dose-dependent inhibition of firing rate of approximately 60% of the cells tested. The inhibition was often longer lasting but weaker than that of the putative neurotransmitters tested such as γ-aminobutyric acid (GABA) and glycine. The inhibition caused by the benzodiazepines could be readily antagonized by bicuculline methiodide (Figure 1) and picrotoxin, as could the inhibitions due to GABA. The antagonists had no effect on the responses to noradrenaline, glycine, 5-hydroxytryptamine or nicotinamide. RO 15-1788 antagonized the inhibitory

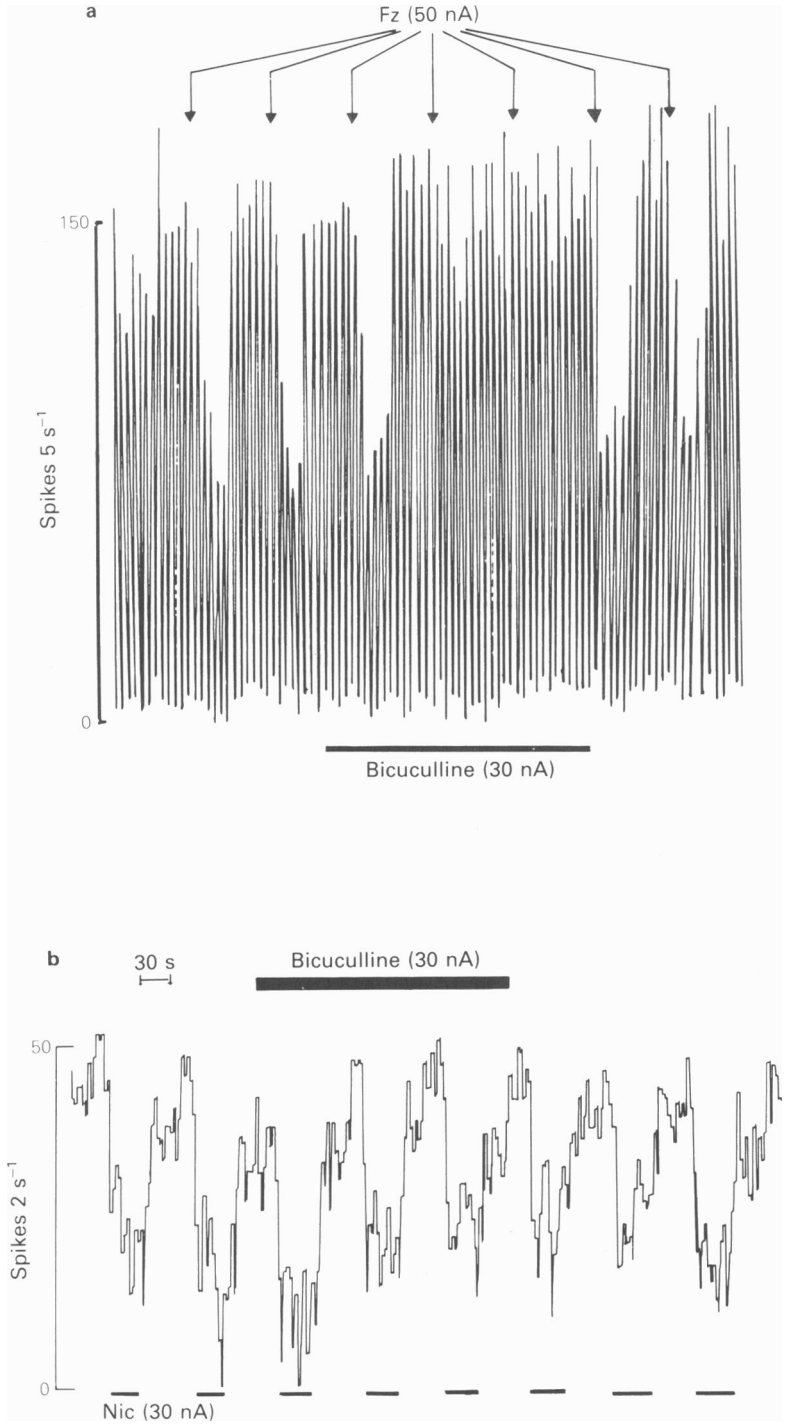

Figure 1 Extracellular ratemeter recordings from two cerebellar Purkinje cells to show the effect of bicuculline methiodide, 30 nA, on the inhibitory effect of (a) flurazepam (Fz), 50 nA, and (b), nicotinamide (Nic), 30 nA. Bicuculline methiodide reversibly reduced the inhibition due to flurazepam but had no effect on the nicotinamide response.

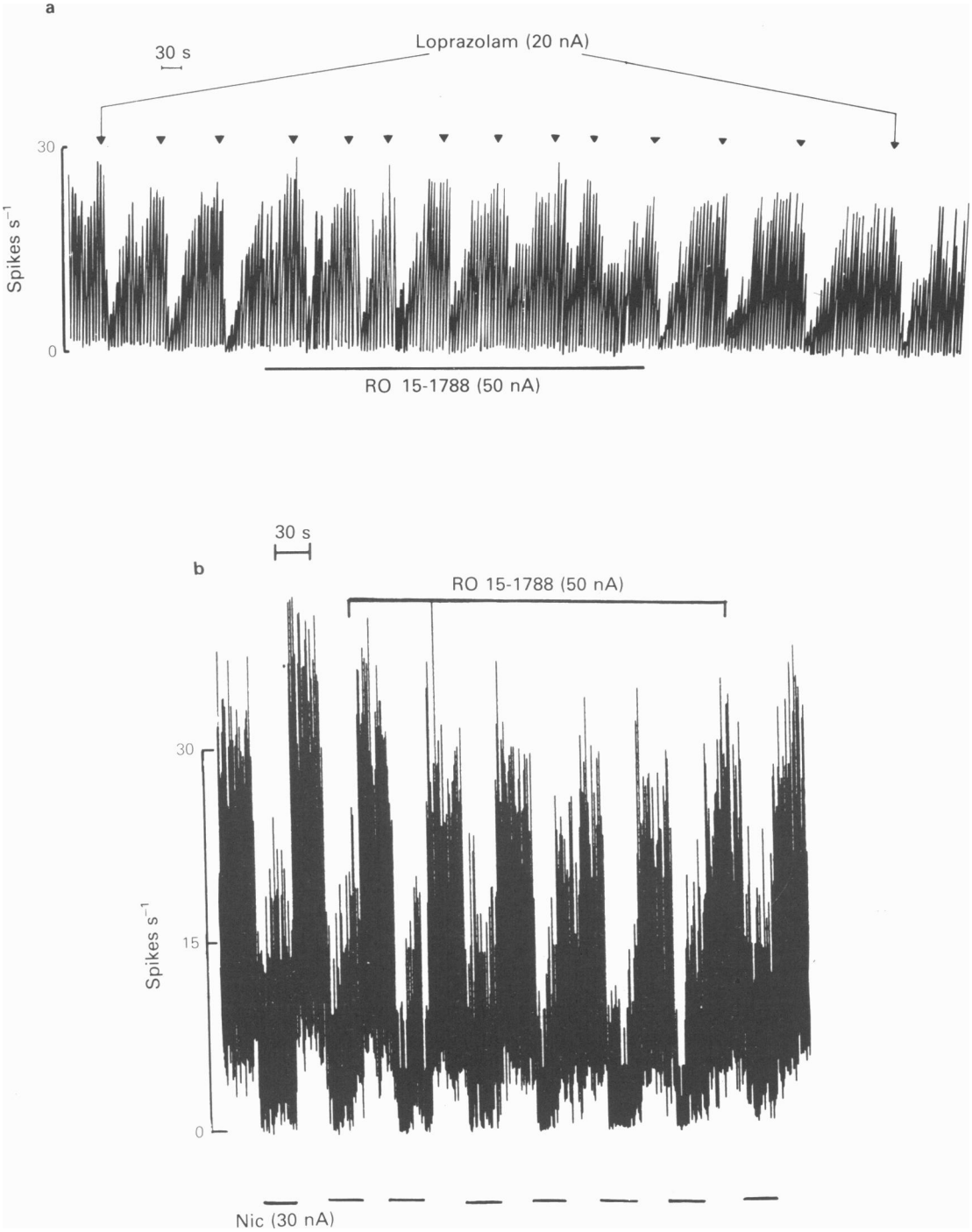

Figure 2 Extracellular ratemeter recordings from two cerebellar Purkinje cells to show the effect of RO 15-1788, 50 nA, on the inhibitory effect of (a) loprazolam, 20 nA, and (b) nicotinamide (Nic), 30 nA. RO 15-1788 reversibly reduced the inhibition due to loprazolam but had no effect on the nicotinamide response.

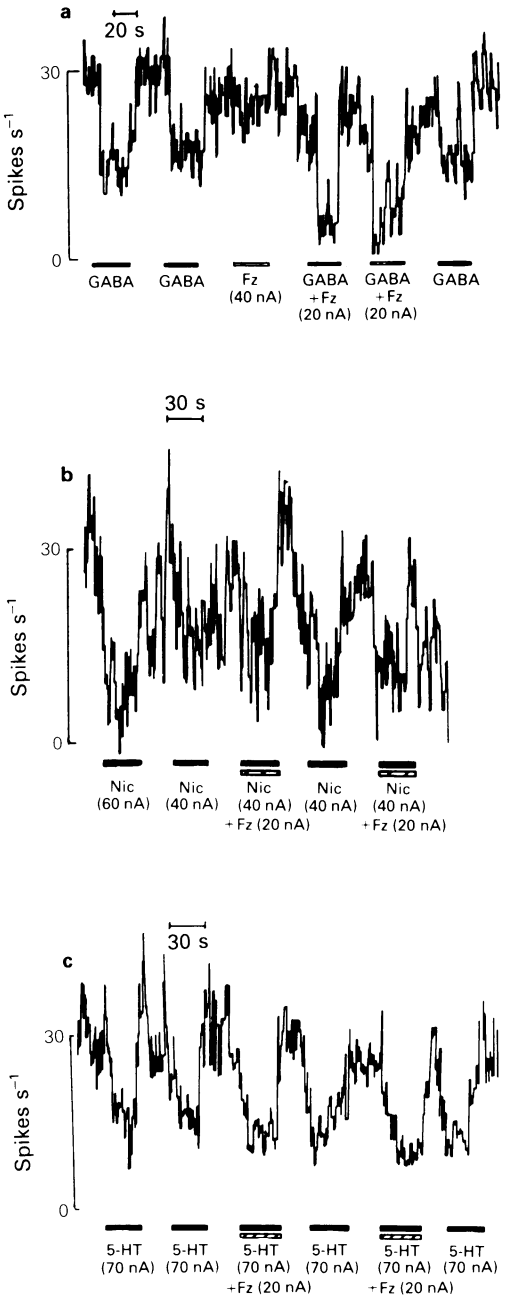

Figure 3 Extracellular ratemeter recordings from a cerebellar Purkinje cell to show the interactions between flurazepam and GABA, nicotinamide and 5-hydroxytryptamine (5-HT); in (a), flurazepam (Fz), 20 nA, potentiated the inhibitory action of GABA, 10 nA; in (b), flurazepam (Fz), 20 nA, had no effect on the response to nicotinamide (Nic), 40 nA; in (c), flurazepam (Fz), 20 nA, had no effect on the response to 5-HT, 70 nA.

responses to the benzodiazepines (Figure 2) and this effect was selective for the benzodiazepines. The responses to GABA, glycine, 5-hydroxytryptamine (5-HT) and noradrenaline were not antagonized by RO 15-1788. The effects of RO 15-1788 were slow in onset and RO 15-1788 itself did not alter the rate of cell firing.

Nicotinamide exerted an extremely potent inhibitory action on all cells tested. It differed from the benzodiazepines in that its action was not sensitive to bicuculline; also picrotoxin and RO 15-1788 failed to antagonize the effect of nicotinamide (Figures 1 and 2, respectively). No interaction was observed between the inhibitory actions of GABA and nicotinamide; in contrast the benzodiazepines markedly enhanced GABA-mediated inhibition (Figure 3). This was tested by finding the ionophoretic current which produced a threshold inhibitory response to GABA and nicotinamide when applied separately and then ionophoresing the two compounds simultaneously. The results from this procedure were always additive and there was no sign of potentiation. There was no synergism between the benzodiazepines and 5-HT, noradrenaline, glycine or nicotinamide. Inosine also induced a bicuculline-insensitive inhibitory effect which did not synergize with the action of GABA.

Social interaction test

Control levels of social interaction were low and stable, ranging from 23 to 30 s in a 5 min observation period. Chlordiazepoxide increased social interaction in a dose-dependent fashion up to 8 mg kg^{-1} (ANOVA, $P < 0.05$, $F = 12.18$, d.f. = 18) (Figure 4). At higher doses the increasing sedative effects suppressed locomotor activity and this was associated with decreased social interaction. Nicotinamide caused statistically significant increases in social interaction at 250 and 500 mg kg^{-1} (ANOVA, $P < 0.05$, $F = 8.28$, d.f. = 18). At 1,000 mg kg^{-1} the rats were highly

Table 1 Inhibition of [^{3}H]-flunitrazepam binding to rat cerebellar membranes

Displacer	IC_{50} (nM)
RO 15-1788	1.7
Loprazolam	3.2
Diazepam	9.0
Flurazepam	28
Bicuculline	80 μM
Inosine	8 mM
Nicotinamide	25 mM

IC_{50} is the concentration required to displace 50% of the specific binding. Each curve was constructed from 8 points. Results represent the mean of at least 3 separate experiments.

sedated. Inosine also induced a marked sedative effect at 500 mg kg^{-1} i.p. Lower doses of inosine (125 and 250 mg kg^{-1} i.p.) still induced some decrease in locomotion but there was no significant change in social interaction. Whilst the increase in social interaction due to chlordiazepoxide could be completely reversed by RO 15-1788 (10 mg kg^{-1}) (ANOVA, $P < 0.05$, $F = 10.23$, d.f. = 18) this specific ben-

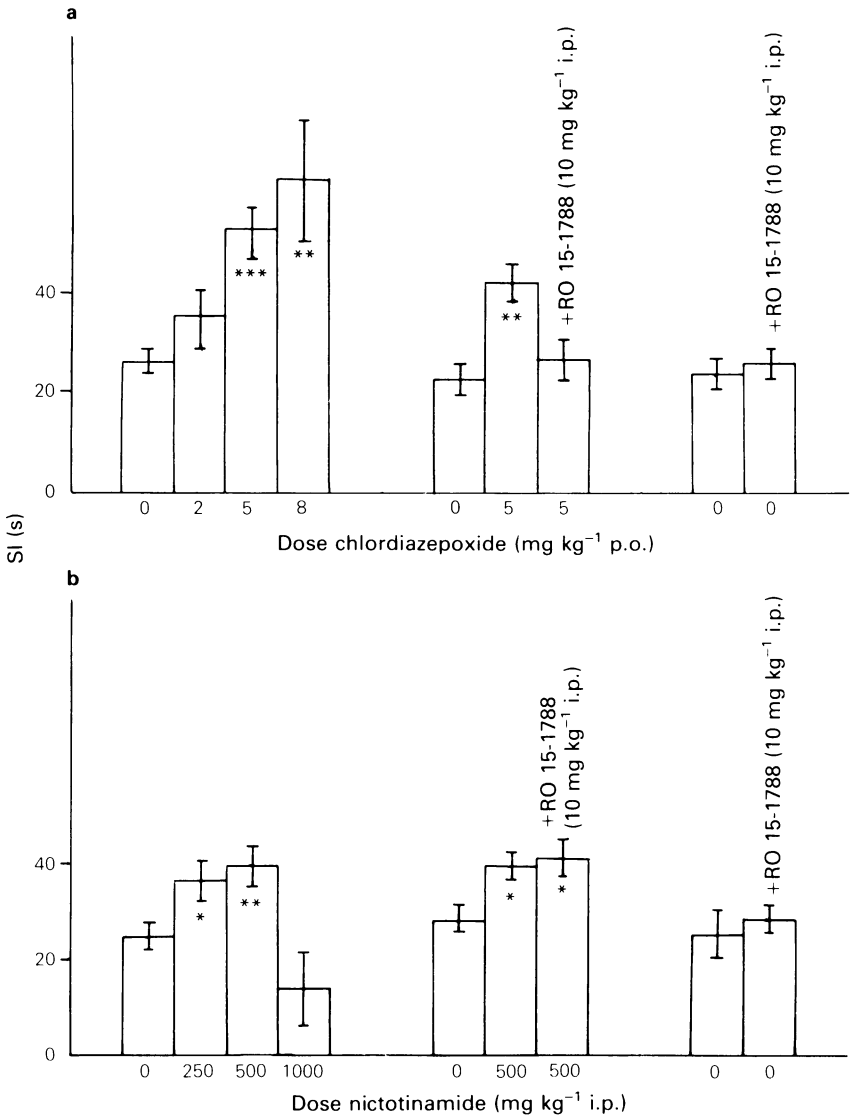

Figure 4 The effect of chlordiazepoxide (Cdzp), 2–8 mg kg^{-1} p.o., and nicotinamide (Nic), 250–1000 mg kg^{-1} i.p., on mean (± s.e.mean) levels of social interaction (SI) in a 5 min observation period. Chlordiazepoxide (a) increased social interaction and this effect was reduced by RO 15-1788, 10 mg kg^{-1} i.p. Nicotinamide (b) also increased social interaction with respect to control, but this effect was not reduced by RO 15-1788. RO 15-1788, 10 mg kg^{-1} i.p., did not alter social interaction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's t test. Analysis of variance and Fisher's least significant difference test indicated significant differences between control and Cdzp 8 mg kg^{-1} and 5 mg kg^{-1} groups, $P < 0.001$. Similarly, nicotinamide at 250 and 500 mg kg^{-1} were significantly different from control, $P < 0.001$. There was no significant difference between the two doses of each drug. The Cdzp plus RO 15-1788 group was significantly different from the group receiving only Cdzp but not from controls. However, the group receiving nicotinamide plus RO 15-1788 was still significantly different from control but not from those receiving nicotinamide alone. All data were analysed at the $P < 0.001$ level.

zodiazepine antagonist did not reverse the effects of nicotinamide. RO 15-1788 itself did not alter the level of social interaction at the dose employed.

[^{3}H]-flunitrazepam binding

The classical benzodiazepines displaced [^{3}H]-flunitrazepam binding in a competitive fashion yielding characteristic sigmoid displacement curves with a linear Scatchard plot to give a Hill coefficient for diazepam of 0.96. RO 15-1788, the specific benzodiazepine antagonist is an extremely potent displacer (Table 1). This observation agrees with other published data (Hunkeler *et al.*, 1981). The putative endogenous ligands nicotinamide and inosine displayed a low affinity for the receptor, having IC_{50} values of 25 mM and 8 mM respectively (Table 1).

Discussion

The electrophysiological data described in this study suggest that while nicotinamide and inosine are potent inhibitors of Purkinje cell activity they have properties which are dissimilar to those of the benzodiazepines. They do not appear to influence GABA-ergic mechanisms and their activities are not reversed by bicuculline or picrotoxin. Other electrophysiological studies using nicotinamide contrast with the present study. For example, the enhancement of presynaptic inhibition in cat spinal cord by local application of nicotinamide has been reversed by (+)-bicuculline (0.5 mg kg^{-1}, i.v.) (Mohler *et al.*, 1979). Our results show that the specific benzodiazepine antagonist, RO 15-1788, failed to antagonize the electrophysiological action of nicotinamide. This presents further evidence for the suggestion that nicotinamide is acting at some site other than the benzodiazepine receptor.

The social interaction test has been proposed as a useful test for the measurement of anxiety (File, 1980). We have been able to show a chlordiazepoxide-induced increase in social interaction upon acute administration. The present results showed that nicotinamide but not inosine increased social interaction in a manner comparable to that of chlordiazepoxide and consistent with its activity in a conflict model of anxiety (Mohler *et al.*, 1979). However, the increase due to nicotinamide was not reversed by RO 15-1788. It would seem that the apparent anxiolytic effect of nicotinamide is induced by some mechanism other than by direct interaction at the benzodiazepine receptor. This idea is supported by the [^{3}H]-flunitrazepam binding studies which showed that, whilst the benzodiazepines displace [^{3}H]-flunitrazepam from cat cerebellar membranes with IC_{50} values in the nM range, the affinities of nicotinamine and inosine are in the mM range. The low pharmacological affinity of nicotinamide coupled with its low concentration in the brain, i.e. 0.1 μmol per gram rat whole brain (Gerber & Deroo, 1970), suggests that nicotinamide is unlikely to be an endogenous ligand for the benzodiazepine binding site. But further studies are required to determine the mechanism responsible for the possible anxiolytic action of this compound.

Although nicotinamide and inosine possess anticonvulsant activity, the profile of antiseizure activity differs from that of the benzodiazepines (Lapin, 1981). Furthermore, recent behavioural studies have led to the suggestion that these two substances may antagonize the actions of benzodiazepines (Slater & Longman, 1979; Crawley *et al.*, 1981). No evidence for antagonism was obtained in these electrophysiological studies although the concentrations of nicotinamide and inosine studied were limited by their bicuculline-insensitive inhibitory action. Indeed, interpretation of the behavioural activities of these agents with respect to benzodiazepine mechanisms may be complicated by this additional, and mechanistically unrelated, neuronal inhibitory action, which may occur at lower brain levels of these drugs (less than 25 mM) than those required to interact with benzodiazepine receptors.

We are grateful to Hoffman-La-Roche for a gift of RO 15-1788. J.M.B. is an SERC (CASE) student.

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(Received August 9, 1984.
Revised October 23, 1984.
Accepted October 29, 1984.)