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SELECTIVE ANTAGONISM BY BENZODIAZEPINES OF NEURONAL RESPONSES TO EXCITATORY AMINO ACIDS IN THE CEREBRAL CORTEX

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1 The recently discovered benzodiazepine receptor exists in high concentration in the cerebral cortex. We have, therefore, examined the effects of diazepam and chlordiazepoxide on cortical neurone responses to excitatory and inhibitory amino acids and acetylcholine, in the cortex of rats anaesthetized with urethane.

2 Chlordiazepoxide applied by microiontophoresis reduced the responses to glutamate and aspartate but acetylcholine responses were unaffected on most cells even by much higher doses of benzodiazepine. γ-Aminobutyric acid (GABA) and taurine responses were unaffected on most cells, but were reduced on 4 of 25 units. After intravenous diazepam, responses to GABA and taurine were reduced on 3 cells and unchanged on 11.

3 On Purkinje cells in the cerebellum a number of cells (5 of 16) exhibited a substantial increase in responses to GABA and taurine following intravenous or iontophoretic application of benzodiazepines.

4 It is suggested that the highly selective reduction of excitatory amino acid responses in the cerebral cortex may be of particular relevance to the behavioural effects of benzodiazepines.

Introduction

Much has been written recently on the mechanism of action of benzodiazepines. Of the opinions expressed, the most popular view seems to be that these compounds facilitate the neuronal depressant effects of γ-aminobutyric acid (GABA), probably by a postsynaptic mechanism (Costa & Greengard, 1975; Costa, Guidotti, Mao & Suria, 1975; Keller, Schaffner & Haefely, 1976; Polc & Haefely, 1976; Haefely, 1977; Choi, Farb & Fischbach, 1977; Gallagher, 1978; Tsuchiya & Fukushima, 1978). However, the opposing view, that benzodiazepines reduce the efficacy of GABA also has its support (Gähwiler, 1976; Steiner & Felix, 1976), whilst two recent studies have claimed that benzodiazepines reduce the effects of excitatory amino acids and acetylcholine (Evans, Francis & Watkins, 1977; Davies & Polc, 1978). Part of this disagreement may relate to the doses of benzodiazepines used. Macdonald & Barker (1978), for example, have found that in cell culture, benzodiazepines can potentiate GABA responses at very low doses but may antagonize them at higher doses.

Another possible reason for this disagreement is that a variety of sites in the central nervous system have been studied, including spinal cord (Choi *et al.*, 1977; Evans *et al.*, 1977; Davies & Polc, 1978) medulla (Dray & Straughan, 1976; Gallagher, 1978) and cerebellum (Curtis, Lodge, Johnston & Brand, 1976; Gähwiler, 1976; Steiner & Felix, 1976; Geller, Taylor & Hoffer, 1978).

A new dimension has now been added to this debate by the demonstration of a specific benzodiazepine binding site, and the tentative demonstration of an endogenous ligand for this site (Squires & Braestrup, 1977; Marangos, Paul, Greenlaw, Goodwin & Skolnick, 1978; Mohler, Okada, Heitz & Ulrich, 1978). Of special relevance is the finding that the benzodiazepine receptor exists in greatest concentration in the cerebral cortex with, generally speaking, a declining concentration as one descends through the nervous system (Squires & Braestrup, 1977; Mohler *et al.*, 1978; Williamson, Paul & Skolnick, 1978). We have therefore investigated the interactions between chlordiazepoxide (a soluble benzodiazepine) excitatory and inhibitory amino acids and acetylcholine on single neurones in the cerebral cortex.

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Methods

Male Porton Wistar rats weighing 250 to 350 g were anaesthetized with urethane (1.25 g./kg. i.p.). The animal was placed in a stereotaxic frame and the body temperature maintained at 37 to 38°C by means of an automatically regulated heating blanket and a rectal probe. An area approximately 4 mm square of cerebral cortex was exposed immediately anterior to the bregma suture, to allow access to the motorsensory cortex. The dura was removed and, after positioning the electrodes, the exposed cortex and muscles were covered with a layer of 5% agar in saline (0.9% w/v NaCl solution) to prevent cooling and drying, and to reduce respiratory and vascular movements of the brain. The agar layer was changed after each microelectrode penetration, though usually only one or two penetrations were made per experiment.

Purkinje cells in the cerebellum were identified by their characteristic discharge pattern, including climbing fibre bursts and subsequent inactivation pauses (Eccles, Ito & Szentagothaí, 1967; Stone, 1979).

For the microiontophoretic application of compounds 7-barrelled micropipettes were used. The barrels were filled immediately before use to approximately 1 cm from the top of the pipette. The centre barrel was always left unfilled. The pipettes were broken under microscopic control so that overall tip diameter was about 10 μm. Iontophoretic ejection was effected by a Digitimer Neurophore Unit.

Intravenous injections were made through a cannula inserted into the contralateral jugular vein. Diazepam was injected as the commercial preparation of Valium for injection, diluted with saline as required. Control injections of saline were always performed, and all solutions were warmed to 37°C before injection.

For iontophoresis, the following solutions were used: L-glutamate sodium, 0.2 M, pH 7.0; L-aspartate potassium, 0.2 M, pH 7.0; γ-aminobutyric acid, 0.2 M, pH 3.5; chlordiazepoxide hydrochloride, 50 mM, pH 4.5; taurine hydrochloride, 0.2 M, pH 4.0; glycine hydrochloride, 0.2 M, pH 5.0; acetylcholine chloride, 0.5 M, pH 4.5. When not being ejected, compounds were subjected to a retaining current of 10 to 15 nA.

Extracellular recordings of unit activity were made with single glass microelectrodes containing 1 M potassium acetate or chloride, having d.c. resistances of 2 to 8 MΩ. The tips of these electrodes were bent to an angle of 10 to 20° during the pulling process to facilitate fixing alongside the multibarrel pipette (Stone, 1973). The tips of the single and multibarrel pipettes were initially approximated by eye, and then under microscopic control. The electrodes were held together by Plasticine. Tip approximation was always confirmed microscopically before and after each experimental penetration. The recording electrode was arranged to project 10 to 25 μm beyond the multibarrel tip.

Unit activity was amplified by a Grass P511 amplifier, and the spikes were passed through a window discriminator, the output pulses of which were counted and displayed as a record of spikes per s on a Grass polygraph. Spikes were simultaneously displayed on oscilloscopes and were also available for recording on magnetic tape and for on-line generation of poststimulus time histograms by an Ortec time histogram analyser.

Results

Effects of chlordiazepoxide on cortical neurones

It was noted that when applied by microiontophoresis chlordiazepoxide (Cdpx) itself had a mild excitatory action on most of the neurones tested (48 of 76 cells). This was seen usually as an approximate doubling of the spontaneous firing frequency even when applying low doses of about 20 nA of Cdpx. A depression of firing was seen, unaccompanied by excitation, on 12 cells. Cdpx appeared to have little effect on spike height.

Following intravenous injection of Cdpx (5 mg/kg) or diazepam (0.1 to 3 mg/kg), changes of spontaneous firing were more variable. Combining the results with both drugs, 4 of 12 cells (4 animals) showed a fall of firing rate, and 6 showed an increase.

There seemed to be no relationship between the depth at which neurones were encountered in the cortex and their responses to Cdpx alone, or in conjunction with amino acids, as in the following paragraphs. Cells were studied at all cortical depths between 0.54 mm and 1.88 mm.

Interactions with agonists

Cdpx could be applied iontophoretically at a wide range of doses from 20 nA for 10 s to 120 nA for 6 min, with no apparent changes of neuronal inhibitory responses to GABA, taurine or glycine on most cells (21 of 25). The amino acid doses were always adjusted to ensure submaximal responses. On 4 units, GABA and taurine responses showed some reduction in size, but on 2 of these glycine was also applied and was unaffected by Cdpx. On none of these 25 cells was a potentiation of the inhibitory amino acids seen.

After intravenous Cdpx (5 mg/kg) or diazepam (0.1 to 3 mg/kg) a similar ratio of effects was noted. GABA and taurine were unaffected on most cells (11 of 14 cells), whilst on 3 cells a reduction of response size was seen. Responses to GABA and taurine were reduced roughly in parallel in these cases.

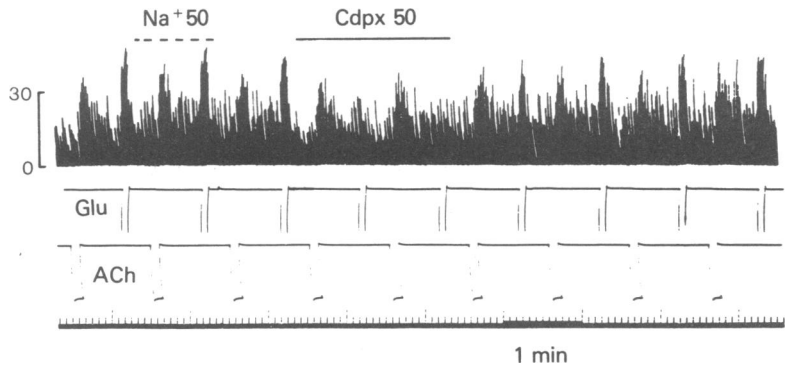

Figure 1 Record of the firing rate (ordinate scale, spikes per s) of a neurone in the cerebral cortex, excited by alternate applications of glutamate, 80 nA (Glu) and acetylcholine, 95 nA (ACh) by microiontophoresis. An application of chlordiazepoxide, 50 nA (Cdpx) blocks completely the response to glutamate, without affecting the response to acetylcholine. A control current passed through the NaCl barrel (Na^+) had no effect on the response.

When using acetylcholine and the amino acids glutamate and asparatate as excitatory agonists, a clear and highly reproducible pattern of interaction emerged. On 38 of 43 neurones, Cdpx induced a reversible reduction of the excitatory amino acid responses (Figure 1). Small doses of Cdpx were required, usually less than 50 nA, and in order to confirm that the outward ('positive') drug current was not interfering with the inward ('negative') amino acid current, separate current controls were performed (Figure 1). The possibility that Cdpx might discriminate between glutamate and aspartate was specifically examined on 13 neurones, on which the amino acid ejecting currents were adjusted to produce responses of approximately equal peak amplitude. Care was taken to maintain the responses at a submaximal level. Cdpx was then applied at a variety of doses, but at all doses which produced reduction of the amino acids, both aspartate and glutamate responses were reduced in parallel.

In the case of 31 of 38 neurones showing amino acid antagonism, excitatory responses to acetylcholine were also obtained. As illustrated in Figure 1, the acetylcholine responses were unaffected by doses of Cdpx causing amino acid blockade. Indeed, on 18 of these cells, the Cdpx dose was increased to try and achieve a block of acetylcholine and compare the relative sensitivities to Cdpx of acetylcholine and the amino acids. But no reasonable dose of Cdpx (up to 300 nA for 5 min) could be found which would produce such a blockade.

It would thus appear from this selection of cells, recorded from 8 animals, that Cdpx exhibits a powerful and specific antagonistic action towards excitatory amino acids but not acetylcholine.

However, it should be mentioned that 3 cells were encountered in one animal on which a low dose of Cdpx (20 nA) produced a rapid and reversible reduction of acetylcholine excitation (Figure 2). None of these 3 cells could be induced to respond to glutamate

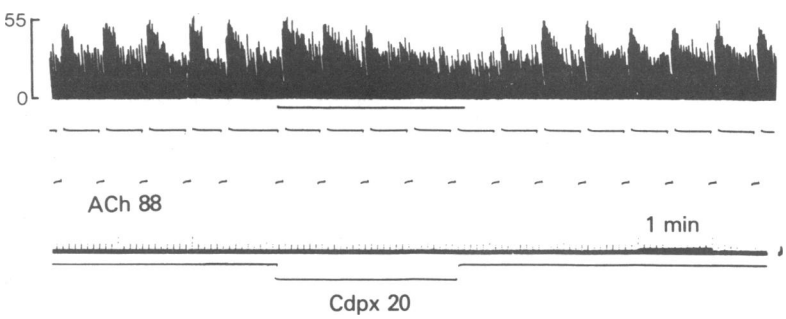

Figure 2 Record of the firing rate of one of three neurones encountered in the cerebral cortex on which excitatory responses to acetylcholine, 88 nA (ACh) were abolished by chlordiazepoxide, 20 nA (Cdpx and also shown by the bar immediately below the recording). These cells could not be excited by glutamate, and are discussed in the text. Ordinate scale in spikes per s.

with ejecting currents of up to 250 nA for 60 s. Nine of 15 other cells studied in the same animal would not respond to glutamate, however, and the possibility must be considered that either the cells in this animal were extremely sensitive to small amounts of Cdpx leaking spontaneously from the micropipette, or that a relatively large amount of Cdpx was diffusing from the barrel. From the preceding sections, either explanation would be expected to preclude responses to glutamate and, presumably, reduce the threshold of acetylcholine responses to Cdpx blockade.

The fact that this problem only arose on 3 units is a partial indication that Cdpx was not normally leaking from the pipettes to a large extent. An additional finding is that when micropipettes of the size used in these experiments were filled with only glutamate or GABA, and retaining currents of 15 nA applied, cells were found not to change substantially their firing rate for periods of up to 1 h, supporting the view that little leakage was occurring.

Studies on cerebellar Purkinje cells

As the above results stand in marked contrast to the results obtained in most previous studies (see Introduction) we have also examined the pharmacology of a small number of Purkinje cells in the cerebellum. These were identified by the usual characteristics of firing rate, climbing fibre bursts and inactivation pauses (Eccles *et al.*, 1967; Stone, 1979).

It has been found that when applied iontophoretically Cdpx had no effect on the responses of 6 of 8 of these cells to GABA or taurine. Injected intravenously, Cdpx (5 mg/kg) or diazepam (0.2 and 1 mg/kg) had no effect on responses to GABA of 2 cells and 3 cells respectively. However, on the other 2 cells studied iontophoretically, and on 1 cell with intravenous Cdpx and 2 cells with intravenous diazepam, a substantial increase in the size of GABA and taurine responses was noted. An example is illustrated in Figure 3. It may be of relevance to the existing controversy on benzodiazepines that the potentiation developed over a period of 5 to 10 min after intravenous injection, and was often preceded, in the period 1 to 2 min after injection, by a *decrease* of response size. This could conceivably reflect a concentration effect, initial high local concentrations causing GABA blockade, giving way to a potentiation as local concentrations fall due to drug redistribution. A dose-effect relationship of this type has been proposed by MacDonald & Barker (1978).

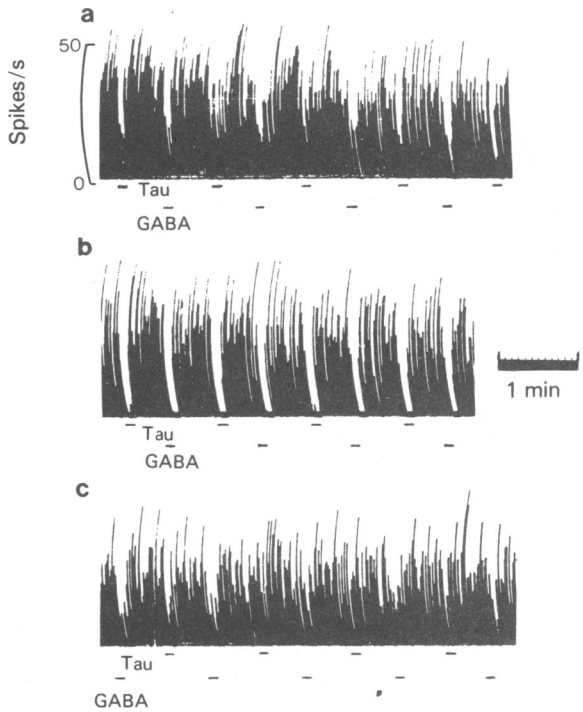

Figure 3 Record of the firing rate of a Purkinje cell in the cerebellum, responding with a weak inhibition of firing rate to small doses of GABA, 15 nA, and taurine, 20 nA (Tau): (a), (b) and (c) were recorded respectively, before, 15 min after, and 80 min after an intravenous injection of diazepam, 1 mg/kg.

Discussion

Part of the reason for the existing controversy concerning the action of benzodiazepines is probably the diversity of central structures that have been investigated in previous studies (Curtis *et al.*, 1976; Gähwiler, 1976; Dray & Straughan, 1976; Steiner & Felix, 1976; Choi *et al.*, 1977; Evans *et al.*, 1977; Davies & Polc, 1978; Gallagher, 1978; Geller *et al.*, 1978). This suggestion is supported by our observation that in the cerebellum, benzodiazepines can indeed potentiate Purkinje cell depression produced by GABA, though as we have noted above, this potentiation was usually preceded by a reduction of response size. In the cerebral cortex on the other hand we have seen no marked or consistent changes of inhibitory amino acid responses after iontophoretic or systemic Cdpx or diazepam. As the recently discovered high affinity benzodiazepine binding site exists in greatest concentration in the cerebral cortex (Squires & Braestrup, 1977; Mohler *et al.*, 1978; Williamson *et al.*, 1978), we would suggest that our main finding, that in the cortex chlordiazepoxide can reversibly and selectively reduce neuronal responses to excitatory amino acids, may be more relevant to the behavioural effects of benzodiazepines.

Dray & Straughan (1976) have in fact observed blockade of excitatory agonists by benzodiazepines, but in their study, in the rat medulla, Cdpx and flurazepam reduced the effects of both glutamate and acetylcholine together, and both roughly in parallel with a depression of background firing rate.

Two subsequent studies on spinal cord neurones have also led to the conclusion that excitatory agonists can be blocked by benzodiazepines, though in one study no non-amino acid control was used (Evans *et al.*, 1977) while in the other acetylcholine and several amino acids were all reduced by a water-soluble benzodiazepine (Davies & Polc, 1978).

We would tentatively attribute the apparent specificity of amino acid antagonism in our experiments to the use of cerebral cortex, with its high concentration of benzodiazepine receptors. Elsewhere, particularly in medulla and spinal cord, the observation of benzodiazepine effects may require high doses which produce a relatively non-specific effect. This is supported by the observation of Curtis *et al.* (1976) that the chief effect of benzodiazepines in the spinal cord was to reduce spike height.

While we feel that the preceding discussion is valid, and may help to resolve some of the arguments currently surrounding benzodiazepines, it is important to recognise that the discussion is limited to conventional views of synaptic transmission. Burnstock (1976) has already emphasized the short-sightedness of attempts to explain divergent results by an explanation devised within the constraints of classical concepts, and we suggest that some thought be given to the following speculation.

It has been pointed out that transmitter precursors, such as glutamate in GABA-containing neurones, may be 'co-transmitters' or 'neuromodulators'. If glutamate and GABA were released simultaneously from some neurones then any reduction of the effect of glutamate might appear as a potentiation of GABA. Such an explanation, which would satisfy at once many of the studies in this field, would imply that benzodiazepines would potentiate synaptic inhibition more than inhibition by exogenous GABA. It is therefore of great interest that just such a distinction was reported by Curtis *et al.* (1976).

Finally, it should be noted that in cases where changes in responses to GABA were seen in this study, responses to taurine were usually changed in parallel. As it has been found by other groups that GABA antagonists such as bicuculline and picrotoxin are also effective antagonists of taurine (Curtis & Tebecis, 1972; Curtis & Johnston, 1974) it would seem that the pharmacology of these amino acids is sufficiently similar that none of the pharmacological tools available, including convulsant antagonists and benzodiazepines should be relied upon to discriminate between GABA- and taurine-mediated synaptic inhibition.

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