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Electrophysiological studies in cultured mouse CNS neurones of the actions of an agonist and an inverse agonist at the benzodiazepine receptor

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1 The action of agents which bind with the benzodiazepine (BZ) receptor has been investigated by use of intracellular recordings from dissociated mouse neurones grown in tissue culture.

2 The agents tested were midazolam (an agonist at the BZ receptor) and methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM – an inverse agonist at the BZ receptor). These were applied to the neurone under study by one of the following methods: iontophoresis; pressure application of known concentrations from blunt pipettes; directly in the perfusing medium.

3 On only very few occasions did midazolam or DMCM have a direct effect on the membrane potential (E_M) or conductance (G_M) of the impaled neurone. For the neurones where direct effects were present, there was no consistent pattern of response. Neither substance affected the threshold for action potential generation.

4 The effect of midazolam and DMCM on responses evoked by iontophoretic application of γ-aminobutyric acid (GABA) was also investigated. Three parameters were used to quantify GABA responses: the depolarization (V_{GABA}); the increase in G_M (g_{GABA}) measured with constant current pulses; using voltage clamp, the GABA current (I_{GABA}).

5 The GABA response should be quantified by a parameter which is linearly related to the number of GABA-operated channels which are conducting at any instant. V_{GABA} does not fulfil this criterion. g_{GABA} is an appropriate parameter, but is difficult to determine for large responses where the membrane is nearly short circuited. I_{GABA} measured during voltage clamp fulfils this criterion.

6 Midazolam ($> 10^{-6}$ M) reliably potentiated GABA responses with a parallel shift to the left of the dose-response curve. This is in agreement with biochemical studies where BZs increase the affinity of the GABA receptor for its ligand.

7 The effect of DMCM on GABA responses was more variable. In the majority of cases GABA responses were reduced by DMCM. The threshold dose for this depression was usually around 10^{-6} M, but was sometimes as low as 10^{-8} M. Dose-response curves of I_{GABA} or g_{GABA} showed the inhibition to be of a non-competitive nature. The maximum inhibition achieved was around 70%.

8 For a given neurone, and at doses which did not necessarily cause a reduction of the response to GABA, DMCM could antagonize the potentiating action of midazolam on GABA responses. A possible interpretation is that more than one BZ site per receptor complex must be occupied by a BZ agonist (or inverse agonist) before the functional changes for the complex as a whole can occur.

9 Desensitization to GABA was increased by midazolam.

Introduction

Benzodiazepines (BZs) were first introduced into clinical practice in the early 1960s (Lader, 1978) for their anxiolytic, sedative, anti-convulsive and muscle relaxant properties. Specific binding sites for BZs in the brain were, however, first demonstrated in 1977 (Squires & Bræstrup, 1977; Möhler & Okada, 1977). At about the same time, what is probably the major pharmacological mechanism of action was discovered – that BZs enhance the electrophysiological response to the inhibitory transmitter γ-aminobutyric acid (GABA) and potentiate inhibitory postsynaptic potentials (i.p.s.ps) at synapses where GABA is

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thought to be the natural transmitter (Polc & Haefely, 1976; Choi *et al.*, 1977).

BZ and GABA receptors interact at the molecular level and probably co-exist as a functional unit in the neuronal membrane (Olsen, 1982). The GABA receptor is of the low affinity variety (Skerritt *et al.*, 1982; Krogsgaard-Larsen *et al.*, 1984). The affinity of the BZ receptor for its ligand is increased in the presence of GABA (see Bræstrup & Nielsen, 1983). The converse, i.e. that BZs cause an increase in the affinity of the GABA receptor, probably occurs, but is less well documented (Olsen, 1982; but see Skerritt *et al.*, 1982; Bræstrup & Nielsen, 1983; Skerritt & Johnston, 1983).

In view of the very high clinical potency of BZs and the existence of specific receptors, attempts have been made to identify an endogenous ligand. The result of such a search led Bræstrup and his colleagues to the discovery that ethyl-β-carboline-3-carboxylate (β-CCE) binds with high affinity to BZ receptors (Bræstrup *et al.*, 1980). The behavioural profile of this and other β-carboline derivatives (convulsant/proconvulsant, anxiogenic) was broadly opposite to that of the classical BZs. β-Carbolines therefore became known as inverse agonists at the BZ receptor (Polc *et al.*, 1982). This was the first demonstration that different agents acting at the *same* receptor could produce opposite responses. In contrast to BZ agonists, GABA decreases the binding of inverse agonists to the BZ receptor (Bræstrup & Nielsen, 1981), while β-CCE and the methyl ester, β-CCM, have no effect on GABA binding (Skerritt *et al.*, 1983). Subsequently, β-carbolines with partial agonist (e.g. ZK 91296, Meldrum *et al.*, 1983; Petersen *et al.*, 1984) and antagonist (e.g. ZK 93426, Jensen *et al.*, 1984) properties have been produced.

In the present electrophysiological study on mouse neurones in tissue culture, we have attempted to elucidate the mechanism of action of the convulsant β-carboline, methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), which is probably the most potent inverse agonist at the BZ receptor (Petersen, 1983; Petersen *et al.*, 1983). The following data concerning the GABA-BZ system in cultured neurones have already been established: the BZ receptor present in dissociated cultures closely resembles receptors isolated from the intact nervous system (Huang *et al.*, 1980; White *et al.*, 1981) though the BZ receptors may take a month or more to develop fully, when two distinct BZ binding sites become evident (Sher, 1983). Further, for cultures from cortex, low affinity BZ binding sites ($K_d = 240$ nM) may be preferentially located on neurones (Sher & Machen, 1984). GABA responses are mediated predominantly by an increase in g_{Cl} (Barker & Ransom, 1978; Barker & Mathers, 1981) which is voltage-dependent (Study & Barker, 1983). GABA responses are potentiated by BZs (Choi *et al.*, 1981; MacDonald & Barker, 1978) via an increase in frequency of opening of the GABA-activated channels (Study & Barker, 1981). In general, convulsants act by reducing the frequency of activation ion channels operated by neutral amino acids (Barker *et al.*, 1983).

It was previously thought that certain GABA mimetics were either without influence (e.g. THIP) or even had a negative effect (e.g. piperidine-4-sulphonic acid, (P4S)) on BZ binding (Bræstrup *et al.*, 1979). These original binding studies were performed in Cl^--free media at 0°C. In an earlier study, we compared the modulation of neuronal responses to THIP and P4S by DMCM and midazolam to that of responses to GABA. Under the physiological conditions of our electrophysiological experiments (Cl^--containing media at 35°C) responses to all three agonists were, in fact, modulated to approximately the same extent by DMCM and midazolam (Jensen & Lambert, 1984a, b).

Here, we have compared the action of DMCM with the water soluble BZ, midazolam, on the basis that the consequence for neuronal behaviour of results obtained from binding studies should be demonstrable in an electrophysiological system. We have shown that, while midazolam potentiates GABA responses with a leftward shift of the dose-response curve, low doses of DMCM depress GABA responses in a non-competitive manner. DMCM was able to reduce markedly the potentiation of GABA responses by midazolam at doses where DMCM itself had little effect.

Part of this work has appeared in abstract form (Jensen & Lambert, 1983a, b).

Methods

Neurones from the brains and spinal cords of 12–14 day old mouse embryos were grown in tissue culture. The method of culturing and the media are those according to Ransom *et al.* (1977), except that trituration was performed mechanically by drawing the tissue up into and expelling it from a syringe. This operation was performed 10 times with each of the following needles used sequentially: 1.1, 0.9, 0.8, 0.7 and 0.6 mm diameter. Initially we used disposable polypropylene syringes (10 ml, Monoeject). A later batch of these syringes proved to be highly toxic (traces of a chemical sterilizing agent, ethylene oxide, were possibly still present). Little or no tissue survived the trituration procedure. Thereafter, we used well rinsed, heat sterilized glass syringes. The dissociated tissue was sown in 35 mm dishes containing poly-L-lysine coated cover slips (Banker & Cowan, 1977).

The methods used for electrophysiological recording and drug application have been published previously (Jensen & Lambert, 1984b). The following

will be confined to those aspects considered necessary to facilitate interpretation of the results presented here.

Electrophysiology

Cultures used for electrophysiology were 1–3 months old. The culture was perfused with oxygenated Hanks Balanced Salt Solution (BSS) at 34–36°C and a flow of 30 ml h^{-1}. Hanks BSS contained (mM): NaCl 136.9, $CaCl_2$ 1.3, $MgSO_4$ 0.4, $MgCl_2$ 0.5, K_2HPO_4 0.4, Na_2HPO_4 0.3, $NaHCO_3$ 4.2, HEPES 20 and glucose 11; pH 7.35. Tetrodotoxin (TTX, 2.5×10^{-7} M) or $MgCl_2$ (9.1 mM) were usually added to the BSS. Where pertinent, this is noted in the text and legends to the figures.

Electrodes for intracellular recording were pulled from 1.2 mm glass (Clark Electromedical, GC 120F-15) and filled with KCl (1 or 3 M) or K_2SO_4 (0.6 M). Resistances were 25–50 MΩ). The recording amplifier was custom built following a design of S. Lærke (Copenhagen) and had facilities for bridge-balance and single-electrode-voltage-clamp (SEVC) (see Finkel & Redman, 1984).

Voltage clamp experiments were confined to larger neurones (> 30 μm, 3 M KCl electrodes). With smaller cells (< 20 μm) it was difficult to obtain stable penetrations which laster for more than a few minutes. Following impalement of small cells by a low resistance microelectrode, a number of morphological changes became apparent. The cell lost its phase-bright appearance and became granulated; larger vacuoles appeared in the soma; the soma and dendrites became distended. Electrophysiological changes were a decrease in resting E_M, an increase in resting membrane conductance (G_M), an inability to generate action potentials and, ultimately, loss of penetration. These changes did not occur so readily with high resistance electrodes, where the tip oriface is presumably finer. It therefore seems likely that the small cells were unable to cope with the osmotic load of hypertonic KCl from the larger tipped electrodes.

With the SEVC, values for 'waiting time', 'injecting time' and loop gain were selected so that minor electrical disturbances (e.g. capacitively coupled transients from the iontophoretic system and changes in the electrode capacity caused by small changes in the depth of the recording medium) did not cause the system to oscillate. This meant that the clamp was occasionally of poorer quality than it would otherwise have been for a short duration experiment, with consequent small changes in the voltage (E_M) recording (e.g. see Figure 6).

Drug application

(a) Iontophoresis Double-barrelled iontophoretic electrodes were pulled from 1.5 mm glass (Clark Electromedical, GC 150F-15). The barrels were filled with GABA (0.1–0.5 M, pH 3.5) and glycine (0.1–0.5 M, pH 2.5). Electrode tips were not broken back (total tip diameter < 1 μm), but it was still necessary to use a retaining voltage. Because the retaining voltage causes depletion of the agonist at the tip of the electrode, reproducibility was achieved by applications at constant intervals following a period of 'warming-up' the electrode by a few trial ejections. The agonists were always applied to the neuronal soma.

Midazolam (10^{-2} M, pH 4) and DMCM 10^{-2} M, pH 2–3) were applied from separate iontophoretic electrodes (tip diameter 1–1.5 μm). Each electrode was moved up in turn close to the soma. Retaining voltages were not used since they usually caused irreversible electrode blockage.

(b) Pressure application Two, three or four barrelled electrodes were pulled from 1.5 mm glass without fibres (Class Electromedical GC 150-15) and the tips broken back so that the internal diameter was 3–5 μm per barrel. The barrels were filled by back-filling with DMCM (10^{-12}–10^{-5} M) and midazolam (10^{-12}–10^{-4} M) which were dissolved in Hanks BSS perfusion medium. The pressure electrode was positioned downstream from the impaled neurone to avoid unwanted leakage effects and moved up to a measured distance (usually about 50 μm) from the neurone during application. Square pressure pulses (5–10 psi) were applied to the individual barrels. Flow through the barrels was usually checked before and after application by observing the movement of poorly attached cellular matter during the application of a pressure pulse. This test was performed downstream at a long distance from the impaled neurone. Experiments with GABA (10 μM) in the pressure electrodes showed that the response was relatively independent of the pressure applied and the duration of the pulse, but was very dependent on the distance from the impaled neurone. The concentrations of the applied agents reaching the cell membrane were therefore probably less than those in the pressure electrodes. Pressure and iontophoretic applications were usually not made simultaneously because flow from the pressure pipette often disturbed the iontophoretic application. Rather, the modulator was applied by a pressure pulse for a few seconds followed by a pause of 1–2 s, then the agonist was ejected iontophoretically.

(c) Bath perfusion A few experiments were performed where DMCM and midazolam were applied directly in the perfusing medium. However, reversibility of the drug effects, especially DMCM, was poor. This may be because the agents bind to glass (i.e. the coverslip and base of the recording chamber) to some extent (C.

Bræstrup, personal communication) and this drug depot is only depleted slowly.

Quantification of the GABA responses

Four methods were used, the relative merits and shortcomings of which are discussed in the text: (a) Measurement of the GABA-induced change in E_M (V_{GABA}, always a depolarization with KCl electrodes). (b) Measurement of the GABA-induced increase in G_M. This was achieved by injecting constant current pulses into the neurone. The size of these pulses was adjusted to give a voltage deflection of 10–15 mV. Sometimes individual pulses were measured (e.g. Figure 3). Otherwise, pulses were averaged (using a Datalab model DL 4000 B) before and at a defined time after the start of GABA application. The conductance associated with the GABA activated channels (g_{GABA}) was obtained by subtracting the resting G_M from that measured during the response to GABA. (c) Using the SEVC, the peak membrane current during a GABA application was measured. An accurate value for I_{GABA} is achieved only when there is no drift in the system, no iontophoretic 'coupling' artifact and E_{GABA} does not change significantly during the course of the experiment. (d) The neuronal transmembrane potential was clamped at a pre-determined level with the SEVC (sometimes E_{GABA}) and stepped to another value before and during the application of GABA. The currents required to produce the voltage steps were measured.

Results

The results presented here are based on intracellular recordings from 100 neurones (see Table 1) of which 13 were from brain and 87 from spinal cord cultures. No systematic difference was seen between the results obtained from brain and spinal cord, so these have been grouped together. DMCM and/or midazolam were applied by iontophoresis to 28 of these neurones and by bath perfusion and/or pressure to 72.

Method of modulator application

In an earlier study (Jensen & Lambert, 1983c) midazolam and DMCM were applied routinely by iontophoresis. There were some features of these experiments which could not be repeated when DMCM was applied from pressure electrodes or by direct bath perfusion. These features were: (a) DMCM caused a direct decrease in G_M (with little change in E_M) in about 40% of the neurones tested. (b) Respon-

Table 1 Interaction of methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and midazolam with γ-aminobutyric acid (GABA) responses on cultured brain and spinal cord neurones

A. *Iontophoretic application of DMCM and midazolam*

DMCM ($n = 28$)			*Midazolam* ($n = 19$)		
GABA ↑	*GABA* =	*GABA* ↓	*GABA* ↑	*GABA* =	*GABA* ↓
2	2	24	16	2	1

B. *Pressure or perfusion application of DMCM and midazolam*

Modulator dose	*DMCM* ($n = 74$) GABA ↓/*Neurones tested*	*Midazolam* ($n = 51$) *GABA* ↑/*Neurones tested*
$1–2 \times 10^{-9}$ M	0/8	0/3
$1–2 \times 10^{-8}$ M	2/19	0/6
$1–2 \times 10^{-7}$ M	11/16	2/7
$1–2 \times 10^{-6}$ M	21/26	20/21
$1–2 \times 10^{-5}$ M	5/5	12/12
1×10^{-4} M	—	2/2

The alteration of the GABA response in the presence of a modulator is represented thus: increase ↑; no change =, decrease ↓. (**A**) Summary of the changes of the response to GABA which were seen during the iontophoretic application of the modulators. (**B**) All neurones shown were tested with pressure application of the modulators. For about 20% of the neurones, the results were subsequently confirmed by bath perfusion of the modulator(s) at the same concentration(s) as those present in the pressure electrode. The response to GABA was considered to be depressed by DMCM or enhanced by midazolam when this change persisted for at least 10 s following termination of the pressure pulse. The number of neurones fulfilling this criterion is represented as a fraction of the total number of neurones tested at each dose range of modulator.

ses to glycine were reduced on a significant number of occasions. (c) Depression of the response to GABA was often greater after the DMCM ejecting current was turned off.

Application of a positive current to an electrode containing DMCM 10^{-2} M at pH 2–3 will probably result in the ejection of a significant quantity of H^+ ions. Moreover, there will also be considerable variation between pipettes (Kelly, 1971). When applied to neurones, protons are known to cause complex and multiple effects, including depression of amino-acid evoked responses (Gruol *et al.*, 1980; Jensen & Lambert, unpublished observations). It is likely that some, if not all, of the above features are functions of proton effects. In addition to these interpretive problems, there were also the following technical considerations of the iontophoretic technique: the effective concentration of the modulators is unknown; when the solubility product for DMCM was exceeded on ejection into BSS at pH 7.5, crystals of DMCM formed on the tip of the electrode; retaining currents frequently caused crystal formation which led to pipette blockage. Iontophoretic application of the modulators was therefore abandoned at an early stage of this study.

Direct effects of midazolam and DMCM on the membrane potential and conductance

Most of the experiments described here were performed with pressure application of the modulators followed by applications of the agonist(s) in rapid succession. This precluded a rigorous study of the direct effects of midazolam and DMCM. Our general impression was, however, that neither agent had any consistent effect on either E_M or G_M.

There were, however, two characteristic artefacts which were readily recognizable: (a) A pressure artefact where a small change in E_M was seen during and shortly after the pressure pulse (e.g. Figure 2). (b)

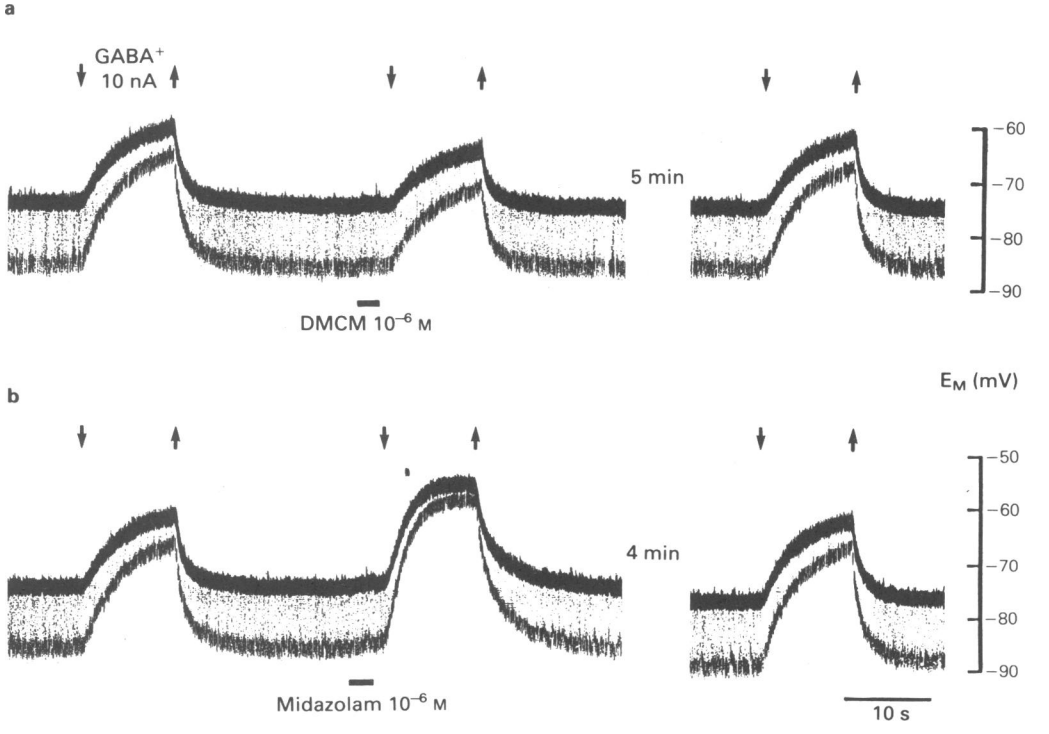

Figure 1 γ-Aminobutyric acid (GABA) response is potentiated by midazolam and depressed by methyl 6,7-dimethoxy-4-ethyl β-carboline-3-carboxylate (DMCM). Spinal cord neurone bathed with BSS containing tetrodotoxin 2.5×10^{-7} M and impaled with 3 M KCl electrode. Traces are modulated by negative-going voltage deflections caused by the injection of −0.6 nA pulses, 20 ms duration, 6 s^{-1}. (a) Pressure application of DMCM (10^{-6} M; 15 psi, 2 s) caused a reduction in the GABA response from 13 to 10 mV. (b) Following pressure application of midazolam (10^{-6} M; 15 psi, 2 s) the GABA response increased from 12 to 18 mV. In both cases, recovery was complete as shown by the responses to the right which were evoked after the indicated times had elapsed.

Pressure application of midazolam sometimes resulted in a weak GABA-mimetic response. This was caused by leakage of GABA from the iontophoretic pipette. The retaining voltage on the GABA barrel was set in normal BSS, where any GABA leakage was subthreshold, but was sometimes disclosed in the presence of midazolam. Although the tips of the iontophoretic pipettes were unbroken, this leakage could not always be contained by further increasing the retaining voltage. Indeed, this sometimes resulted in an even larger 'leakage' of GABA, presumably because significant amounts of GABA were carried in the bulk flow.

On occasions, however, direct effects were seen with midazolam and DMCM which could not be dismissed as artefacts. Both substances have been seen to evoke hyperpolarizations and depolarizations. The occurrence of such events was not frequent enough to warrant detailed investigation.

There have been a few reports in the literature where it has been suggested that direct effects exerted by low concentrations of BZ may contribute to the overall pharmacological action of these drugs. Thus, MacDonald & Barker (1982) report that flurazepam in the concentration range 10^{-12}–10^{-8} M increased P_{Cl} and/or elevated the spike threshold in a significant number of cultured mouse spinal cord neurones. Carlen *et al.* (1983) have shown that nanomolar concentrations of midazolam potentiated a Ca^{2+}-activated K^+ conductance in hippocampal CA1 neurones. In view of these results, we have tested the effect of low doses of midazolam and DMCM applied by pressure onto 15 neurones. We found that neither agent in the concentration range 10^{-12}–10^{-9} M had direct effects on E_M or G_M. There was also no effect on the voltage threshold (E_{th}) or the rheobase current (I_{rheo}) for spike generation (6 neurones). E_{th} was measured by injecting a 30 ms duration current pulse, the strength of which (I_{rheo}) was just sufficient to evoke an AP.

Interaction of midazolam and DMCM with GABA responses

(a) Specificity In an earlier study (Jensen & Lambert, 1983c), in which glycine application was used as a control, we demonstrated that GABA responses were specifically enhanced by midazolam and depressed by DMCM. Here, we have applied glycine to eight neurones and found the response to be unaltered when the modulators were applied by pressure pulses.

Figure 1 shows a GABA response which is decreased following a pressure application of 10^{-6} M DMCM (Figure 1a) and potentiated following a pressure application of 10^{-6} M midazolam (Figure 1b). The action of both modulators was fully reversible.

(b) Dose-dependency of midazolam and DMCM modulation of GABA responses We have not rigorously investigated which doses of DMCM and midazolam were just sufficient to cause a depression and an enhancement of the GABA response respectively. A definitive statement about such threshold doses is also precluded by the aforementioned uncertainty about the concentration at the neuronal membrane of agents applied by pressure. Bearing these reservations in mind, it can be seen from Table 1 that doses with which GABA responses were appropriately altered in about half the neurones tested would be in the range 10^{-7} M for DMCM and between 2×10^{-7} M and 1×10^{-6} M for midazolam. The threshold dose for DMCM effect was more variable than that for midazolam. Thus, on 2 (out of 17) occasions, 10^{-8} M DMCM caused a detectable reduction in the GABA response, while in 20% of the neurones tested, $1-2 \times 10^{-6}$ M DMCM was without effect on the GABA response.

Following short pressure applications of midazolam and DMCM, the time courses of recovery from the action of both agents were very similar. $t_{\frac{1}{2}}$ was usually 20–30 s with full recovery within 2 min (see also Jensen & Lambert, 1984b).

(c) Depression of GABA responses by DMCM: dose-response relationships Figure 2 shows responses to GABA in normal Ringer (a_1) and following pressure ejection of 10^{-5} M DMCM (a_2). The dose-response curve (Figure 2b) has been constructed by measuring the size of the GABA-evoked depolarizations at the point where the GABA ejecting current was terminated. DMCM depresses the GABA response with an apparent rightward shift of the curve. The ED_{50} for GABA has apparently increased from 22 to 36 nA in the presence of DMCM. The depolarization to GABA corresponding to the control ED_{50} (22 nA GABA) was reduced from 18.75 mV to 8.5 mV in the presence of DMCM.

When investigating the action of the modulators with GABA responses, it is important that the appropriate parameter is chosen to quantify the response. GABA operates ionophores, each of whose contribution to the total G_M is a function of the channel conductance and its rate and duration of opening. As g_{GABA} increases, so E_M moves towards E_{GABA} by an amount, V_{GABA}. The relationship between V_{GABA} and g_{GABA} is hyperbolic. As E_M approaches E_{GABA}, the driving force on Cl^- decreases and a greater number of GABA-operated channels must be recruited to cause a given incremental change in V_{GABA} (see equation 3 of Choi & Fischbach, 1981). Furthermore, with large applications of GABA, the Cl^- gradient across the membrane is disturbed and E_{GABA} does not remain constant, but (with KCl electrodes) moves negatively. V_{GABA} is, therefore, an inappropriate parameter for quantifying large GABA responses. An accurate reflection of the number of ionophores successfully operated during GABA ac-

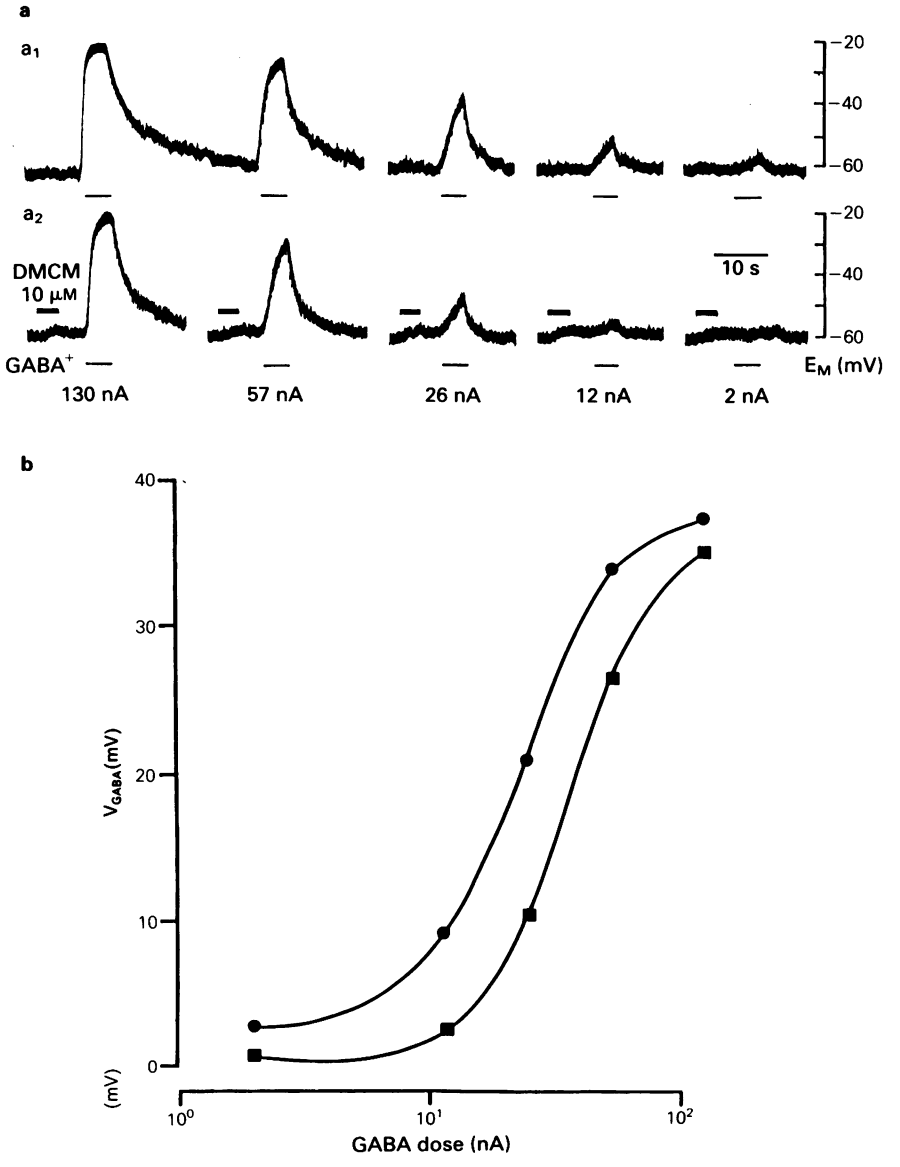

Figure 2 Depression of GABA evoked responses by DMCM. Spinal cord neurone impaled with 1 M KCl electrode. (a) Intracellular recordings: (a_1) control responses to five iontophoretic doses of GABA, (a_2) same series of GABA applications as in (a_1) except that 9 s before the start of the iontophoretic application a 4 s pressure pulse of DMCM (10^{-5} M, a supramaximal dose) was applied to the neurone. (b) Dose-response curve for the results shown in (a). The GABA ejecting current is plotted on a logarithmic scale, while the response is assessed as the maximum size of the GABA-evoked depolarization (V_{GABA}). With this method of quantification, there is an apparent shift to the right of the dose-response curve in the presence of DMCM (■) when compared with the control (●).

tion can be obtained either by measuring g_{GABA}, or by voltage clamping the membrane so that the gradient for Cl^- ions is kept constant and then measuring I_{GABA}. With these methods of quantification each recruited ionophore will contribute an equal increment to g_{GABA} or I_{GABA} respectively. There are, nevertheless, limitations for both of these methods, as outlined below.

(i) Measurement of g_{GABA} Subtracting the value of G_M for the resting membrane from the conductance measured during GABA action gives a value for g_{GABA}, i.e. the total conductance of the GABA operated channels. Strictly speaking, allowance should be made for membrane rectification whereby G_M changes as a result of the passive depolarization *per se*. We have not done this routinely since changes in G_M on passive depolarization were minor compared with the increase in G_M in the presence of GABA. Furthermore, it is not known whether the ionic mechanisms responsible for rectification will still be operating in the presence of GABA (see Jensen & Lambert, 1984b).

Figure 3 is a high speed, high fidelity recording of a GABA response before (a) and immediately following (b) an application of DMCM (10^{-5} M). The resting G_M was 5.74 nS. At the end of the control application, G_M in the presence of GABA was 22.4 nS, i.e. g_{GABA} was (22.4 − 5.74) = 16.6 nS. DMCM application did not alter the resting G_M, but g_{GABA} in the presence of DMCM was 5.46 nS, i.e. only 33% of that in the control situation. DMCM had, therefore, reduced the GABA response by 67%. Similar calculations applied to measurements of g_{GABA} for the responses in Figure 1 show that midazolam potentiates the GABA response by 125% and DMCM depresses it by 47%. (From measurements of V_{GABA}, the values are +50% and −23% for midazolam and DMCM, respectively).

Accurate measurements of G_M cannot be made during large GABA responses where the increase in G_M is so large that the membrane is effectively short-circuited. Current injection then gives very little, if any, voltage deflection. The inherent electrode properties such as rectification and polarization (due to charge movement at the electrode tip, see Finkel & Redman, 1984) become dominant, and measurements of g_{GABA} are very unreliable. Correction for the electrode's non-linear properties cannot always be

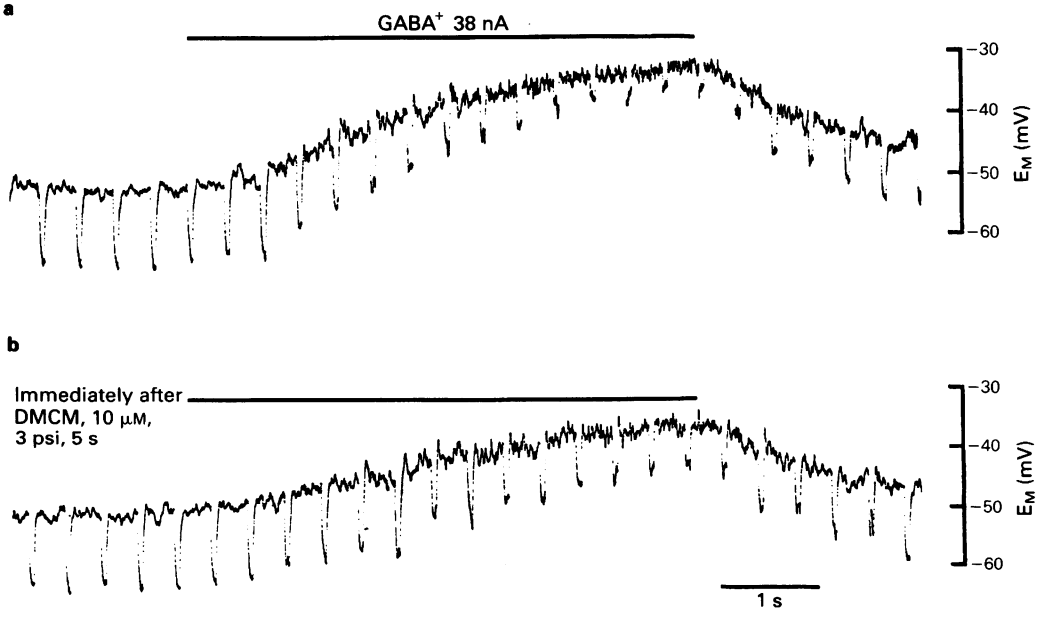

Figure 3 GABA evoked depolarization and conductance increase are depressed by DMCM. Spinal cord neurone impaled with a 1 M KCl electrode. The trace is interrupted by G_M measuring pulses evoked by the injection of − 0.07 nA constant current pulses. (a) An iontophoretic application of GABA evoked a depolarization of 18 mV which was accompanied by an increase in G_M of 230%. (b) Following a pressure application of DMCM (10^{-5} M), the depolarization was decreased to 15 mV and accompanied by an increase in G_M of only 80% (i.e. in the presence of DMCM, g_{GABA} was only 35% of that before DMCM).

achieved by subtracting transients recorded with the electrode extracellular from those recorded intracellularly. This is because the electrical properties of the electrode are not necessarily the same in the two situations (unpublished observations).

Figure 4 shows a dose-response curve where g_{GABA} has been measured by averaging conductance measuring pulses during the responses evoked by a range of GABA doses. For the above-mentioned reasons, g_{GABA} cannot be measured for large GABA responses. In the presence of DMCM 10^{-5} M, however, the maximum g_{GABA} is markedly reduced. DMCM thus has the properties of a non-competitive inhibitor. In similar experiments (not shown), midazolam caused a parallel leftward shift of the dose-response curve (see also Figure 3, Jensen & Lambert, 1984b and Choi *et al.*, 1981).

(ii) Measurement of I_{GABA} Maximum GABA responses may be quantified by measuring I_{GABA} under voltage clamp conditions. A dose-response curve for such an experiment is shown in Figure 5. During increasing doses of GABA, I_{GABA} was computed from the current flowing during 20 mV potential steps as described in the legend in Figure 5. The maximum I_{GABA} was found by interpolation to be 3.7 nA, for which the ED_{50} for GABA was 15 nA. The experiment was then repeated after 10^{-5} M DMCM had been added to the perfusion medium. DMCM caused a reduction in the response to all doses of GABA (mean $35.1 \pm 2.34\%$ (s.e.mean, $n = 7$)), while ED_{50} was unchanged (15 nA GABA). This again is characteristic of non-competitive inhibition. From a total of 14 dose-response curves it was shown that the maximum inhibition that could be achieved with DMCM was around 70%.

Desensitization of responses to GABA

Responses to GABA evoked by either large applications of the agonist or in the presence of midazolam often showed a decay in the plateau phase (e.g. see Figure 6). Three factors contribute to the decay: (i) A change in E_{GABA}. Using KCl recording electrodes and under steady state conditions, E_{GABA} (and, presumably, E_{Cl}) was around -25 mV and Cl^- diffused out of the neurone at the same rate as it was injected (both by diffusion and, sometimes, by current injection) from the electrode. During large GABA responses, however, the marked increase in g_{Cl} meant that the neurone lost Cl^- faster than it was being supplied and E_{Cl} became progressively more negative. As the driving force [$E_M - E_{Cl}$] decreased, so I_{GABA} decreased. (ii) Following a pulse application of midazolam, its effect will decline progressively ($t_{\frac{1}{2}}$ for recovery following midazolam was usually 20–30 s). (iii) Desensitization to GABA. This could be studied in isolation by

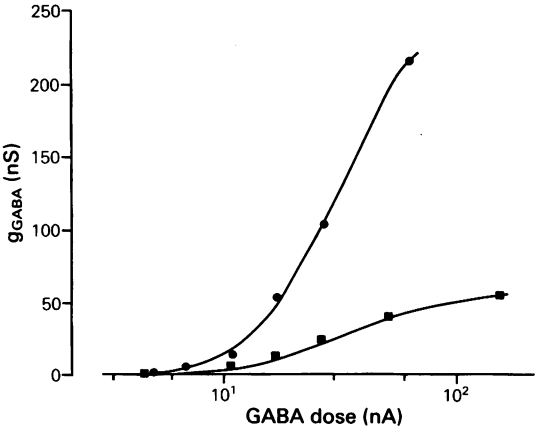

Figure 4 Effect of DMCM on dose-response curve to GABA. Spinal cord neurone bathed with BSS containing tetrodotoxin 2.5×10^{-7} M and impaled with a 3 M KCl electrode. DMCM (10^{-5} M) was applied by pressure ejection using a similar experimental protocol to that in Figure 3. The GABA dose is expressed as the iontophoretic ejecting current on a logarithmic scale. The response is expressed as g_{GABA}, and no allowance has been made for membrane rectification. Although the maximum g_{GABA} was too large to measure in the control situation (●), it was clearly reduced in the presence of DMCM (■).

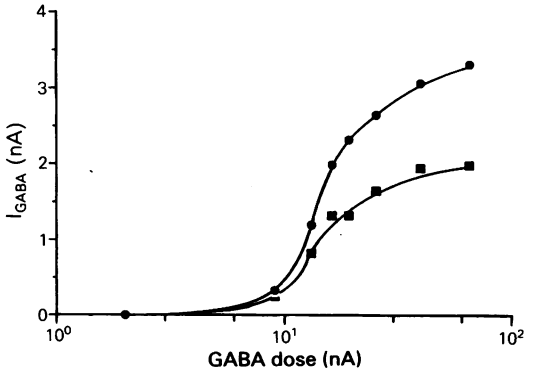

Figure 5 Effect of DMCM on dose-response curve to GABA. Spinal cord neurone bathed with BSS containing tetrodotoxin 2.5×10^{-7} M and impaled with 3 M KCl electrode. The neurone was clamped at -70 mV using the SEVC. E_M was transiently shifted to -50 mV using short duration current pulses. Increasing iontophoretic doses of GABA were applied to the neurone. I_{GABA} was computed by subtracting the pulse current in the control situation from the maximum pulse current during GABA application (●). The experiment was repeated in the presence of 10^{-5} M DMCM applied in the perfusion medium (■). DMCM caused a reduction in the GABA responses of about 40%, while ED_{50} was unchanged at 15 nA, i.e. non-competitive inhibition.

clamping E_M at E_{GABA} (using small voltage steps to determine I_{GABA}) and using bath applications of the modulators. Under these conditions, desensitization to GABA was found to be enhanced by midazolam. This enhancement was even more pronounced when the GABA-mimetic, P4S, was used.

Interaction of DMCM and midazolam

Since GABA responses are enhanced by midazolam and depressed by DMCM, it would be expected that each agent would mutually antagonize the effects of the other, irrespective of whether the modulators were

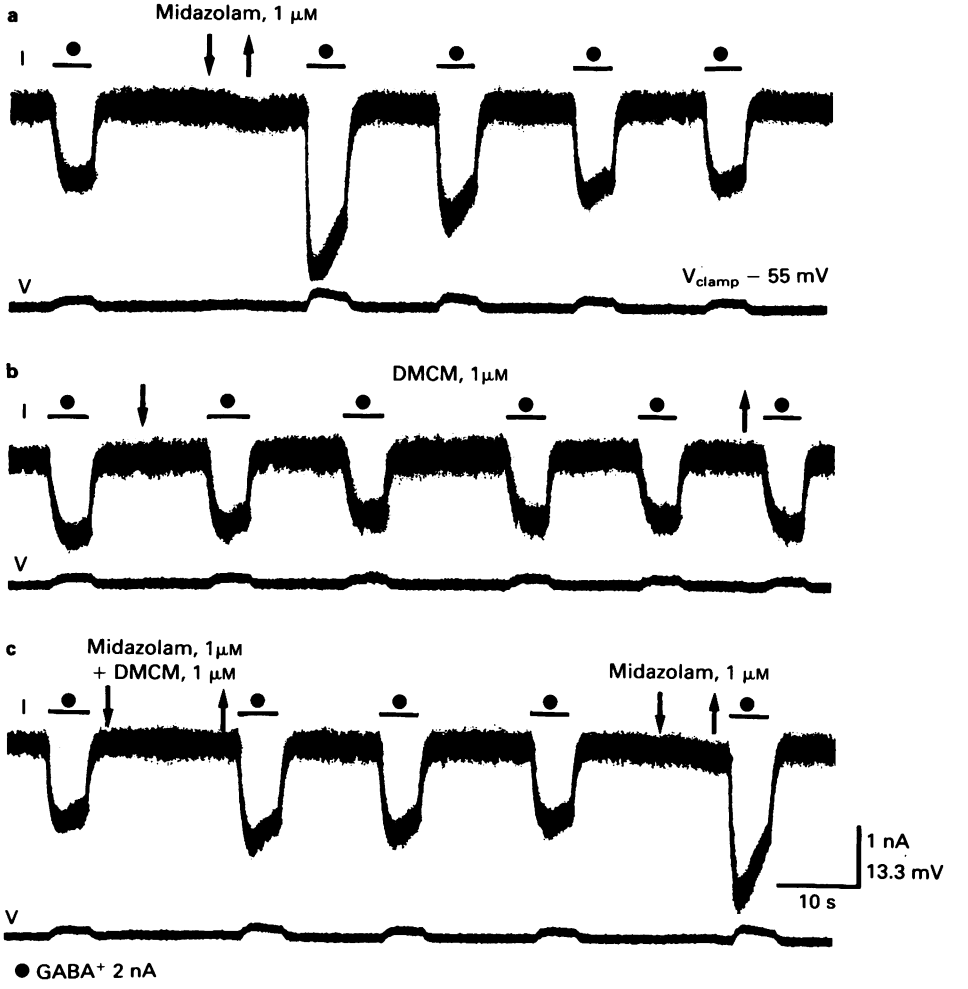

Figure 6 DMCM antagonizes the midazolam-induced potentiation of a GABA response. Spinal cord neurone bathed with BSS containing 10 mM Mg^{2+} and impaled with 3 M KCl electrode. The SEVC was used to clamp E_M at − 55 mV throughout. In order to avoid instability and oscillations, the clamp was not absolute (as can be seen from the small deflections in the potential recording (V) during GABA applications (●)). (a) A pressure application of midazolam (10^{-6} M) caused a marked potentiation of the GABA-evoked current, the size and time course of which can be assessed from the traces (see also text). (b) A pressure application of DMCM (10^{-6} M) had little effect on the GABA-evoked current. (N.B. The small reduction in the GABA responses *during* DMCM application is probably an artefact since flow from the DMCM pipette probably disturbed the iontophoretic application of GABA. A true assessment of the attenuation by DMCM is seen by comparing the last GABA response with that before DMCM.) (c) Following an ejection of a mixture of midazolam and DMCM, potentiation of the GABA response is much less than that seen in (a). A subsequent dose of midazolam resulted in a much larger potentiation of the GABA response.

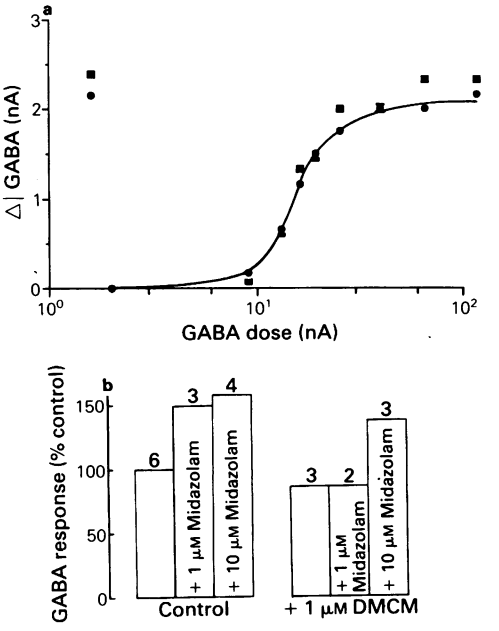

Figure 7 Midazolam-potentiated GABA responses are reduced by a subthreshold dose of DMCM. Same neurone as in Figure 5, where an explanation of the dose-response curve is given, except that 10 mV voltage steps were used here. (a) DMCM 10^{-6} M (■) applied in the perfusing medium had no significant effect on the GABA response. The line has been drawn through the control (●) responses to GABA. (b) The histogram shows the effect of midazolam in the same neurone in the absence and presence of DMCM (10^{-6} M) on the response to a single dose of GABA (13 nA). The GABA response was normalized to 100% in the absence of the modulators (column furthest to the left). The number of observations is given above each column. In the absence of DMCM, the GABA response was potentiated by 50% in the presence of 10^{-6} M midazolam and by 58% with 10^{-5} M midazolam. DMCM 10^{-6} M caused a reduction of the GABA response by 15% but this is probably not significant (see dose-response curve). Midazolam 10^{-6} M was then without effect, while 10^{-5} M midazolam potentiated the GABA response by only 40%.

competing for the same receptor. This expectation was fulfilled in the seven neurones in which the interactions of midazolam and DMCM were tested. More interestingly, it could be demonstrated that a dose of DMCM, which had little or no effect on GABA responses itself, could strongly antagonize the midazolam potentiation. This observation is important, since it infers that DMCM and midazolam are acting on the same receptor and, at the doses used, DMCM is predominantly acting as an antagonist. Such an experiment is shown in Figure 6. A pressure ejection of 10^{-6} M midazolam caused a potentiation of the GABA response by 120% and the effect of midazolam could still be detected after 60 s ($t_{\frac{1}{2}}$ = 12 s, Figure 6a). A 70 s application of DMCM (Figure 6b) caused a depression of only 7% (comparing the response just *after* DMCM ejection with the control, see legend). Following an ejection of a solution containing DMCM and midazolam (both at 10^{-6} M), the GABA response was potentiated by only 25%. A subsequent dose of midazolam alone showed that the neurone could still respond to midazolam. The potentiation in this case (75%) was less than the control potentiation (120%) because traces of DMCM were still present. Later applications of midazolam demonstrated full recovery.

Another example where DMCM acts as an apparent antagonist is shown in Figure 7. These results were obtained with the same neurone as in Figure 5, where 10^{-5} M DMCM had previously been shown to cause a depression of the GABA response by 40%. In Figure 7 it can be seen that 10^{-6} M DMCM was without any marked effect on the dose-response curve, if anything there was a small potentiation of the GABA response. The histogram (Figure 7b) shows the interaction of 10^{-6} M DMCM with the potentiation of the response to a single dose of GABA (+ 13 nA) by midazolam in the same neurone. GABA was tested a number of times (n) in each situation. The control I_{GABA} was set as 100% (n = 6). This was potentiated by 50% (n = 3) with 10^{-6} M midazolam and by 58% (n = 4) with 10^{-5} M midazolam. DMCM 10^{-6} M caused an apparent reduction of the GABA response by 15% (n = 3) (but this is probably not significant, see dose-response curve). Midazolam 10^{-6} M was now without effect (n = 2), while midazolam 10^{-5} M potentiated the GABA resonse by only 40% (n = 3). Thus, in this neurone, DMCM (10^{-6} M) acted as a relatively pure antagonist, while 10^{-5} M DMCM had a direct inverse agonist action (see Figure 5).

Discussion

Quantification of the GABA response

If V_{GABA} is chosen as the parameter to quantify the GABA response (Figure 2), two erroneous conclusions may be drawn: (i) That there are 'spare receptors', i.e. there are more GABA receptors present than are needed to evoke the maximum response of which the neurone is capable. At the dose where V_{GABA} has reached a maximum (within the resolution of the recording system) not all the GABA-operated ionophores will, in fact, be conducting maximally. (ii) That, in the presence of DMCM, there is a parallel shift to the right of the dose-response curve (Figure 2). This could lead to the conclusion that DMCM is

acting as a competitive antagonist.

g_{GABA} and, under voltage clamp conditions, I_{GABA} are directly related to the number of conducting ionophores. Measurements of g_{GABA} are limited by electrode performance, and it is not possible to obtain values for maximum GABA responses in the control situation (Figure 4) or in the presence of midazolam. For I_{GABA} it is technically difficult to obtain a perfect space clamp of the soma in a longer experiment (e.g. Figure 6).

Interaction of midazolam with responses to GABA

Midazolam reliably potentiated responses to GABA (Figures 1, 6 and 7). The threshold dose for midazolam was usually between 10^{-7} and 10^{-6} M (Table 1), which would be expected from the results of Sher & Machen (1984) where a low affinity BZ binding site (K_d 2.4×10^{-7} M) is primarily associated with neurones in culture. Our results are thus in agreement with the established mechanisms for BZ action. BZs increase the affinity of the GABA receptor (Bræstrup & Nielson, 1983) with a potentiation of the postsynaptic response resulting from an increase in frequency of channel openings (Study & Barker, 1981) and a parallel shift to the left of the GABA dose-response curve (Choi *et al.*, 1981; Jensen & Lambert, 1984b).

Interaction of DMCM with responses to GABA

DMCM reduced the GABA-evoked change in both E_M and G_M (Figures 1–5). This is not surprising since, from behavioural and binding studies (Bræstrup *et al.*, 1982a; Petersen, 1983), DMCM is known to be an inverse agonist at the BZ receptor. The threshold dose for DMCM intrinsic action (when present) varied between 10^{-8}–10^{-6} M (Table 1).

An explanation for the variability in responses to DMCM would be that the BZ receptor can exist in more than one conformational state. One conformation would favour an agonist while another would favour an inverse agonist (Bræstrup *et al.*, 1983c). If the former state were the most frequently encountered, this would explain why, in terms of threshold dose and reproducibility, midazolam's effects were more reliably evoked than DMCM's. On the other hand, more than one BZ receptor may have been involved in our study. The existence of at least two BZ receptors is now reasonably well established (Petersen *et al.*, 1983; Sieghart *et al.*, 1983). These receptors can be recognized by their different affinities for β-carboline derivatives (Nielsen & Bræstrup, 1980), the BZ_2 receptor having approximately 10 times higher affinity for DMCM than the BZ_1 receptor. The relative occurrence and distribution of these receptors on the neurones studied here is unknown. It is unlikely that an endogenous BZ ligand was present in our cultures. The cultures were incubated in a defined medium (Hanks BSS) for at least 1 h before experiments began. Furthermore, applications of the modulators would be expected to displace an endogenous ligand. The possibility cannot be excluded, however, that a regulating factor involving the intracellular medium (e.g. receptor turnover) is involved.

The manner by which DMCM depresses GABA responses is characteristic of non-competitive inhibition (Figures 4 and 5). Responses to GABA were reduced by a maximum of about 70%, while the ED_{50} was unaltered. This rules out the possibility that DMCM is acting as a competitive antagonist at the GABA receptor in the same manner as bicuculline. The results are, however, reminiscent of the action of pictrotoxin (Simmonds, 1980), which interferes with the operation of the Cl^- ionophore.

That about 30% of the response to GABA is resistant to DMCM cannot be explained at present. This might represent the maximum compliance of the GABA receptor-ionophore complex in the negative direction. Alternatively, it is possible that the response is mediated by GABA receptors which are not coupled to BZ receptors. It is known, for example, that BZ receptors are coupled preferentially to low affinity GABA receptors (Skerritt *et al.*, 1983; Krogsgaard-Larsen *et al.*, 1984).

Interaction of midazolam and DMCM

The potentiation of GABA responses by midazolam was attenuated by DMCM. This would be expected from the interaction of modulators with opposing actions. DMCM could also attenuate the action of midazolam when DMCM itself had little or no intrinsic action (Figures 6 and 7). On these occasions DMCM seems to be acting as a relatively pure antagonist.

Evidence has accumulated which suggests that receptors for BZs and DMCM are identical. Thus, DMCM completely inhibits specific flunitrazepam (FNM) binding at several sites in the brain (Bræstrup *et al.*, 1982b) and BZ 'antagonists' inhibit the effect of DMCM (Bræstrup *et al.*, 1982a). Furthermore, the number of [^{3}H]-DMCM binding sites is approximately equal to the number of [^{3}H]-FNM binding sites (Bræstrup & Nielsen, 1981; Bræstrup *et al.*, 1983b) and radiation inactivation experiments show that FNM and DMCM binding sites are of approximately equal size (Nielsen *et al.*, 1983).

The BZ receptor is a tetramer (Bræstrup *et al.*, 1983). The subunits appear to be identical, each being able to bind one molecule of ligand. There is apparently no co-operativity between the subunits (E.N. Petersen, personal communication). However, although there is clearly an overlapping of the recognition of DMCM and BZs, there are discrete differences in the

actual binding sites. Thus, in the presence of FNM, 25% of the BZ binding sites are irreversibly inactivated following exposure to u.v. light (Möhler, 1982), and the binding of BZ agonist ligands is greatly decreased, while DMCM binding is actually increased (Chan *et al.*, 1982; Bræstrup *et al.*, 1983c).

The GABA-BZ receptor and chloride ionophore complex is composed of highly integrated subunits (probably four) each of which is able to bind one molecule of GABA and one molecule of a BZ type ligand. This tetramer forms a functional unit in the membrane (see model of Nielsen *et al.*, 1985). The operation of the ionophore requires binding of two GABA molecules (Barker & Ransom, 1978). Presumably then, for a given complex where the ionophore is conducting, at least two GABA receptors are occupied while the remainder are vacant. It is not known at present how many GABA and BZ receptors must be occupied simultaneously for the BZ to increase GABA efficacy. It is possible that a low concentration of BZ-receptor ligand (which is insufficient to occupy enough sites to influence operation of the ionophore) can nevertheless interfere with the access to the receptors of another BZ ligand. This might explain why DMCM acts as an apparent antagonist at lower doses (Figure 7) and an inverse agonist at higher doses (Figure 5). Alternatively, a particular, but undefined, BZ receptor subtype may have been involved in these experiments.

Desensitization of the GABA response

The observation that desensitization to GABA is enhanced by midazolam may be of importance for the interpretation of results from binding studies. It is reasonable to assume that desensitization will also occur when membrane fragments are incubated with GABA. Thus, GABA stimulates BZ binding under conditions where the ionophore is presumably not operating. This may be of relevance, since specific binding of DMCM and diazepam is Cl^- dependent (Bræstrup *et al.*, 1983a), and the binding sites for both agents are thought to be coupled to the Cl^- ionophore (Bræstrup *et al.*, 1983c).

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