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On the Mechanism by Which Midazolam Causes Spinally Mediated Analgesia

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The electrical current thresholds for pain (ECTP) in the skin of the neck and tail were measured in rats with chronically implanted lumbar subarachnoid catheters. The effects of a benzodiazepine antagonist and a γ-aminobutyric acid (GABA) antagonist on the analgesic effects of equivalent doses of midazolam, fentanyl, and ketocyclazocine were studied. These were the minimum doses producing maximal segmental analgesia when given intrathecally (*i.e.*, they all caused a significant and maximum increase in ECTP in the tail, which was similar for all three drugs, but no significant change in the ECTP in the neck). Flumazenil (Ro 15-1788) administration caused a parallel shift to the right of the dose-response curve for midazolam spinal analgesia. Segmental analgesia following midazolam was also significantly attenuated ($P < 0.05$) when the selective GABA antagonist bicuculline was given intrathecally at the same time as midazolam. The highest dose of bicuculline used (50 pmol) caused no significant attenuation of the segmental analgesic effects of either ketocyclazocine or fentanyl. The authors concluded that the segmental analgesia produced by intrathecal midazolam is mediated by the benzodiazepine-GABA receptor complex that is involved in other benzodiazepine actions. (Key words: Analgesia, spinal. Antagonists: bicuculline; flumazenil. Antinociception, intrathecal. γ-aminobutyric acid. Pain: benzodiazepines; midazolam.)

INTRATHECAL INJECTIONS of the water-soluble imidazobenzodiazepine midazolam have produced segmental analgesic effects in both rats[1] and humans.[2] This analgesic effect was not caused by a local anesthetic action of the drug and was not accompanied by sedation. In addition, a dose-response curve constructed for electrical current threshold for pain (ECTP) in the tail of the rat revealed that the analgesia is a dose-dependent phenomenon. Prior administration of the specific benzodiazepine antagonist flumazenil (Ro 15-1788) blocked this response.[1] These experiments suggested that the spinally mediated analgesia following intrathecal midazolam may be the result of a combination of the drug with spinal cord benzodiazepine receptors but pointed to the need for more detailed experiments. It has been shown that such receptors in other areas of the central nervous system are associated with the actions of the inhibitory neurotransmitter γ-aminobutyric acid (GABA).§ The purpose of this study was to prove that the analgesia is the result of a combination with benzodiazepine receptors and to determine whether GABA is involved in the analgesia following intrathecal midazolam. As controls, we used the μ-opioid agonist fentanyl and the κ-opioid agonist ketocyclazocine, both of which produced spinally mediated analgesia in our experimental model.[3,4]

Materials and Methods

This work was carried out with permission from the licensing authorities in Great Britain (Home Office License No. PPL 50-00131), and in all experiments attention was paid to ethical guidelines for investigation of experimental pain in conscious animals.[5]

The ECTPs were measured in the skin of the tail and neck of rats after implanting lumbar intrathecal (it) catheters under halothane anesthesia as described previously.[1] Briefly, a lumbar laminectomy was performed with aseptic precautions at the level of L2/L3. The dura was punctured, and a portex catheter (0.25 mm ID; 0.75 mm OD) passed rostrally for 1.5 cm in the intrathecal space. The rest of the catheter was tunnelled under the skin to an exit wound at the neck where two stainless steel wire electrodes were also implanted 1 cm apart. A number of swellings were located at both ends of the catheter so that it was possible to measure precisely catheter dead space (range, 8–12 μl) as well as volumes of injections. The catheter was fixed to the vertebral bone by means of bone cement, with the last swelling on the catheter situated in the laminectomy crater and embedded in the bone cement. The wound was closed in layers with ethilon (Ethicon) sutures. Correct catheter placement was confirmed by injection of 10 μl of 2% lidocaine into the subarachnoid space 10 min after recovery. The catheter was judged to be intrathecal if paralysis and dragging of the hind limbs occurred within 30 s of this injection.

Male Wistar rats were used for the study (weight, 180–220 g), and only animals with paralysis following lidocaine injection were included. For each series of experiments, all injections and observations on pain thresholds were

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§ Haefely W, Kyburz E, Gerecke M, Mohler H: Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure-activity relationships of their agonists and antagonists. Adv Drug Res 14:156–322, 1985.

performed by the same operator, and all injection volumes were 10 μl, regardless of whether they were solutions of single drugs or drugs in combination. Two stimulating electrodes were moistened with electrode jelly and attached to the tail, the cathode 2 cm from the base of the tail, and the anode 3 cm distal to the cathode. A constant current stimulator was switched to each pair of stimulating electrodes in turn, first the tail and then the neck, for the measurement of ECTP. Rectangular pulses of current (2 ms, 50 Hz, 0–10 mA) were passed through the skin of the tail and neck in turn. The ECTP was defined as the minimum current necessary to produce an obvious aversive movement or strong vocalization. The ECTP was measured in the skin of the tail and the neck every 5 min for 15 min before and 20 min after it injections. A further lidocaine test was performed after each experiment. No more than two experiments were performed on the same animal within a 24-h period, and these two tests were always separated by a period of at least 4 h. To be sure there were no residual effects of previously administered drugs, the control tail pain thresholds were compared with those obtained from the same animal on previous occasions.

The following drugs were used: midazolam (Roche Products), fentanyl (Janssen), ketocyclazocine (Sterling-Winthrop), flumazenil (Roche Products), and bicuculline methiodide (Sigma London).

Experiments with Flumazenil

The ECTP was measured in the skin of the tail and neck in nine rats in response to a range of doses of midazolam (15–138 nmol in 10 μl) it immediately after flumazenil (8.25 μmol/kg in 1 ml intraperitoneally). The analgesic responses were calculated by dividing the mean of three 5-min readings in the tail after intrathecal midazolam by the mean of the three control readings made prior to the intrathecal injection. These were grouped for each midazolam dose and compared with those obtained in previous experiments with intrathecal midazolam alone and published elsewhere.[3]

Experiments with Bicuculline

In initial studies, a group of four rats was given 10-μl it injections of bicuculline ranging up to 100 pmol. Their behavior and responses to electrical stimulation of the tail were observed. At the highest dose of 100 pmol, the rats became agitated with spontaneous scratching of the lower half of the body and hyperexcitability to all forms of stimuli (noxious and non-noxious) applied to the tail. It was not possible to measure ECTP accurately in these animals, and they were killed. It was therefore decided to use only the lower doses of bicuculline for this study.

The rats were divided into three groups of four animals each to receive either it midazolam (46 nmol), ketocyclazocine (a κ-opioid agonist; 40 nmol), or fentanyl (a μ-opioid agonist; 0.74 nmol) dissolved in saline. These were the smallest doses of each agent to produce a maximal spinally mediated analgesic effect and were derived from dose-response curves obtained in previous experiments.[3,4]

Midazolam. The following sequence of 10-μl it injections was given to each rat:

1) it bicuculline alone (50 pmol);
2) it midazolam alone (46 nmol);
3) it midazolam (46 nmol) and bicuculline (50 pmol);
4) it midazolam (46 nmol) and bicuculline (10 pmol);
5) it midazolam (46 nmol) and bicuculline (2 pmol); and
6) it midazolam (46 nmol) alone; this test was performed after 1–6 above.

Responses from 2 and 6 were compared in each animal to investigate if tolerance to the benzodiazepine analgesic effect had occurred during the experiment.

Fentanyl. The ECTPs in the neck and the tail were measured as above in response to the following it injections given on 3 successive days:

1) it bicuculline alone (50 pmol);
2) it fentanyl alone (0.74 nmol); and
3) it fentanyl (0.74 nmol) and bicuculline (50 pmol).

Ketocyclazocine. The same protocol was followed in another four rats, with each animal being given the following it drugs on 3 successive days:

1) it bicuculline alone (50 pmol);
2) it ketocyclazocine alone (40 nmol); and
3) it ketocyclazocine (40 nmol) and bicuculline (50 pmol).

Analysis of Results

The analgesic responses were calculated for each individual rat by dividing the mean of the three readings for ECTPs obtained 10, 15, and 20 min after the it injection by the mean of the three control readings obtained prior to the injection; this was done for both tail and neck electrodes.[3] The response calculated for the tail electrodes (r) was used in subsequent calculations. The results for each group of four rats were combined to produce a mean and SEM.

Wilcoxon sign rank test was used to compare the results of increases in ECTP in the tail following intrathecal agonist alone with those following intrathecal agonist together with 50 pmol bicuculline. In the case of midazolam, the response to agonist alone was the mean of the responses obtained from trials 2 and 6 above. Results were considered to be statistically significant if $P < 0.05$.

The percentage suppression of the response to agonist by bicuculline was calculated as follows: the r values, calculated as above for a particular agonist alone, were

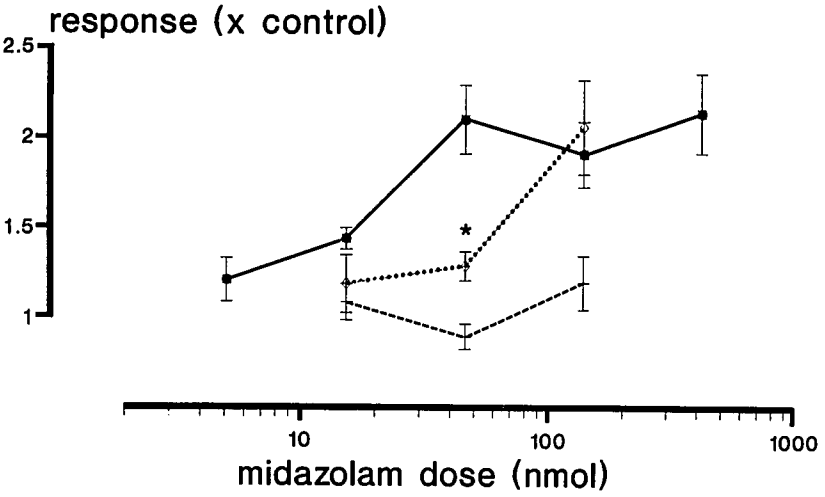

FIG. 1. Midazolam dose-response curves: —● midazolam alone (tail readings only) n = 4; · · · midazolam plus flumazenil (tail readings only) n = 5; - - - midazolam plus flumazenil (neck readings) n = 5. The response to ECTP is expressed as a multiple of control threshold. Points shown are mean ± SEM.

* Significant reduction of the analgesic response to 46 nmol intrathecal midazolam ($P < 0.05$, Wilcoxon's signed rank test).

Values for midazolam alone are from previous studies.[4]

pooled from all animals within the group to produce a mean response for that group (R).

The percentage suppression of the agonist response by each dose of bicuculline was calculated for each animal using the formula:

$$\% \text{ suppression} = \frac{R - r}{R - 1} \times 100$$

The mean (± SEM) percent inhibition by each dose of bicuculline was calculated for each group of animals.

Results

Experiments with Flumazenil

Segmental analgesic effects were observed in experiments in which it midazolam was given immediately after intraperitoneal flumazenil. Flumazenil caused a statistically significant reduction in the analgesic response to 46 nmol of it midazolam ($P < 0.05$, Wilcoxon signed rank test) and a shift to the right of the midazolam dose-response curve (fig. 1).

The tail responses for midazolam alone (15 and 46 nmol) and for midazolam (46 and 138 nmol) in the presence of flumazenil are shown in figure 2 as regression lines ± 90% confidence intervals. This confirms that the response to it midazolam was significantly reduced by intraperitoneal flumazenil (8.25 μmol/kg) and that the dose-response curve for midazolam in the presence of the antagonist is parallel with and significantly to the right of the curve for midazolam alone. The maximum response to it midazolam was 2.23 (mean of all responses to midazolam alone at doses of 46 nmol or more). In figure 2 this represents 100% response, and the ED_{50} for midazolam alone and in the presence of flumazenil are shown.

Experiments with Bicuculline

All three agonists produced spinally mediated analgesia; they caused significant increases in ECTPs in the tail of

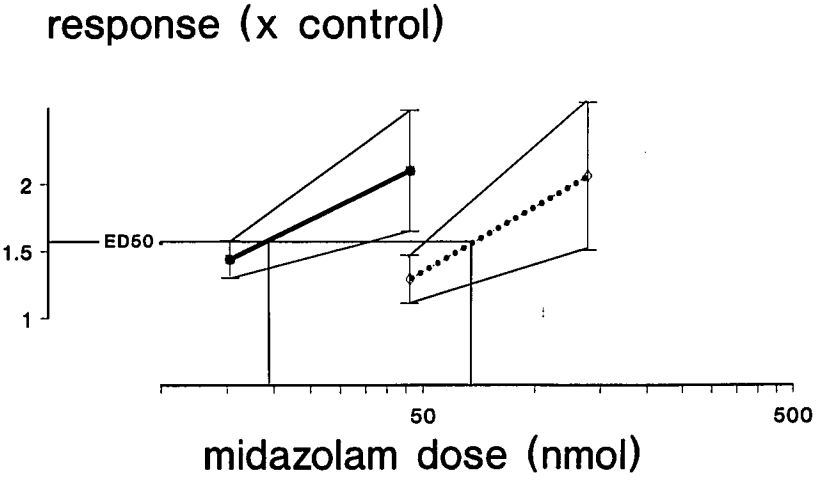

FIG. 2. Midazolam dose-response curves. Lines show mean ± 90% confidence intervals: —●— midazolam alone; · · · midazolam plus flumazenil.

ED_{50} for midazolam = 15.4 nmol alone and 74 nmol after IP flumazenil 8.25 μmol/kg.

TABLE 1. Tail and Neck ECTP Results for Midazolam/Bicuculline Experiments

Tail Readings							Neck Readings						
Rat	(i)	(ii)	(iii)	(iv)	(v)	(vi)	Rat	(i)	(ii)	(iii)	(iv)	(v)	(vi)
822	0.12	0.10	0.22	0.22	0.53	0.35	822	0.70	0.63	0.56	0.83	0.66	0.96
817	0.23	0.13	0.40	0.20	0.22	0.23	817	0.60	0.66	0.60	0.66	0.60	0.60
818	0.36	0.40	0.53	0.65	0.60	0.37	818	0.36	0.66	0.56	0.63	0.43	0.56
823	0.48	0.13	0.45	0.43	0.51	0.46	823	0.43	0.40	0.80	0.63	0.96	0.60
Mean	0.30	0.19	0.40	0.38	0.47	0.35		0.52	0.59	0.63	0.69	0.66	0.68
SEM	0.08	0.07	0.07	0.11	0.08	0.05		0.08	0.06	0.06	0.05	0.11	0.09

Mean control current thresholds for pain (mA) in the tail and neck in the group of rats (n = 4) receiving midazolam on six occasions from (i), the first, to (vi), the last (see text). No more than two tests per day were performed and these were always separated by at least 4 h.

2.2 ± 0.15 for midazolam (mean ± SEM, $P < 0.05$), 1.68 ± 0.07 for fentanyl (mean ± SEM, $P < 0.05$), and 1.71 ± 0.16 for ketocyclazocine (mean ± SEM, $P < 0.05$). There were no significant changes in ECTPs in the neck in any of the rats in these experiments.

There were no significant differences between the analgesic responses produced by the first and the last doses of midazolam ($P > 0.5$), indicating that tolerance to midazolam did not occur during these experiments. Table 1 shows the mean control readings in the neck and tail for four animals prior to it injections of midazolam at testing times (i) to (vi). There was no significant change in the control ECTP at either site and thus no indication of drug accumulation. The administration of bicuculline alone at the highest dose used in these experiments (50 pmol) did not cause any significant change in the ECTP in the tail or any behavioral changes in any group.

Doses of bicuculline (2–50 pmol) administered with 46 nmol midazolam caused a dose-related suppression of the midazolam analgesic response (fig. 3). The highest dose of bicuculline (50 pmol) caused a 51 ± 3.6% (mean ± SEM) suppression of the midazolam response. In contrast this dose of bicuculline produced no significant inhibition of the responses to ketocyclazocine or fentanyl (6 ± 14% and 12.5 ± 6%, respectively).

A lidocaine test performed after every experiment produced anesthesia and paralysis of the rear limbs within 30 s, thus confirming the it position of the catheter.

Discussion

It is commonly accepted that the sedative-hypnotic, anxiolytic, muscle relaxant, and anticonvulsant properties of benzodiazepines are mediated by a receptor complex that includes a $GABA_A$ recognition site, a benzodiazepine binding site, and a chloride channel.§ This view is not universally held, however, and other actions of benzodiazepines have been reported. A role in the regulation of calcium channel activity has been suggested, particularly for ligands at the peripheral benzodiazepine receptor but also in the central nervous system. (For a review see reference 6.) A variety of effects on calcium function have been reported, but these occur mainly at micromolar concentrations. On the other hand, Carlen *et al.*[7] showed that midazolam, at nanomolar concentrations, caused a calcium-mediated increase in potassium conductance in CA1 cells in hippocampal slices. Phillis and his colleagues, among others, have provided powerful evidence for the involvement of adenosine in benzodiazepine actions, sug-

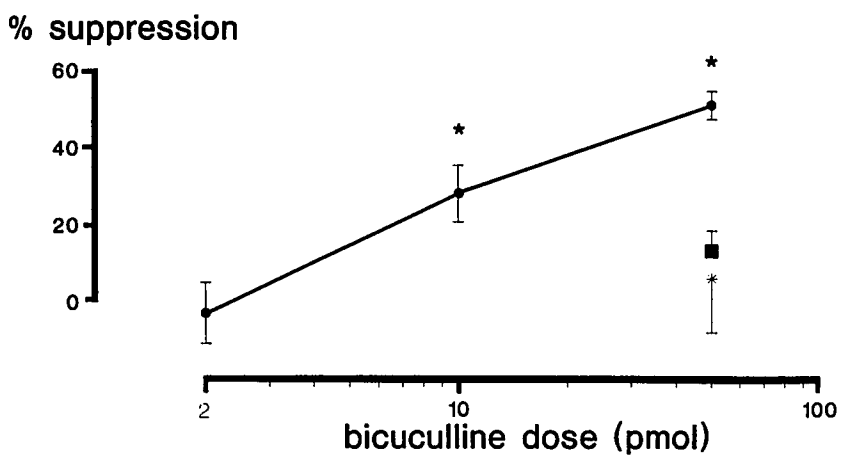

FIG. 3. The percentage suppression by bicuculline of the control analgesic response measured in the tail after intrathecal: —●— midazolam 46 nmol; * ketocyclazocine 40 nmol; ■ fentanyl 0.74 nmol. Points shown are means ± SEM; n = 4.

* $P < 0.05$ statistically significant suppression of the midazolam response (Wilcoxon's signed rank test).

gesting that benzodiazepines may inhibit adenosine uptake or enhance adenosine release.[8] Even the supposed benzodiazepine antagonist flumazenil has been shown to affect adenosine mechanisms, both potentiating and antagonizing adenosine-ergic depressions of cerebral cortical neurons.[9]

The experiments with flumazenil support the suggestion based on previous work that the spinal analgesic effect of midazolam was due to an action on spinal cord benzodiazepine receptors.[1] In previous experiments we demonstrated a complete suppression of the midazolam response by a dose of flumazenil that was greater than that used here (25 mg/kg; 82.5 μmol/kg intraperitoneally). In those experiments, although there were no anti-analgesic effects of flumazenil alone, it is possible that the total suppression of the midazolam response was due to some nonspecific effect of the antagonist. The results from the present experiments show a dose-related suppression of the analgesic response to midazolam and a parallel shift to the right of the dose-response curve. Since all of the animals exhibited segmental blocks (increases in tail but not neck thresholds), we may conclude that the combination of midazolam with spinal cord benzodiazepine receptors is responsible for the analgesia.

To invoke the GABA hypothesis to explain this action of midazolam, we felt it would be necessary to demonstrate either antagonism of the benzodiazepine effect by a GABA antagonist or the ability of a GABA-mimetic to produce similar effects to the benzodiazepine. The latter possibility was not studied as GABA is so widespread in the central nervous system that demonstration of such a selective action was felt to be most unlikely. Our results showed that bicuculline did indeed modify the spinal analgesic action of midazolam. We did find that higher doses of bicuculline (100 pmol) caused a decrease in the pain threshold, the measurements of which were unreliable since they also caused distress even when non-noxious stimuli were applied. However, in the main experiments bicuculline did not cause any changes in pain threshold or behavior when given alone at doses that caused significant attenuation of the midazolam response. Thus, the latter was not a result of a nonspecific action of bicuculline. The obvious conclusion is that GABA is involved in the spinal analgesic effect of midazolam.

The doses of all of the agonists used were taken from equivalent points on their dose-response curves; all three produced a just-maximal spinal analgesic effect.[3,4] Bicuculline had no effect on the analgesic responses to fentanyl or ketocyclazocine. This further argues against a nonspecific effect of bicuculline, but more importantly, it highlights the differences between spinal analgesia evoked by midazolam and that resulting from fentanyl; we previously showed[3] that it fentanyl causes an increase in tail-flick latency but midazolam does not. It is important that a range of tests be used to study spinal analgesics because different mechanisms within the spinal cord may be affected by different drugs. We may conclude that the spinal analgesic effect of it midazolam is mediated by combination with a benzodiazepine receptor that forms part of a typical benzodiazepine-GABA receptor complex within the spinal cord.

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