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Inhibition of GABA Metabolism in Rat Brain Synaptosomes by Midazolam (RO-21-3981)

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Benzodiazepines are known to potentiate GABA (γ-aminobutyric acid) action in the brain. The effects of midazolam, a water-soluble benzodiazepine, on GABA disposal ($^{14}CO_2$ from [1-^{14}C]GABA) and on the individual processes of GABA uptake, GABA release, and GABA-transaminase in the rat brain synaptosomal model system were studied. A 10 per cent inhibition of action was defined as ID_{10}. Midazolam inhibited overall GABA disposal at ID_{10} = 13 μM. The ID_{10} values for the three contributing processes in the overall GABA disposal process are 580 μM for GABA-transaminase activity, 96 μM for GABA release, and 13 μM for GABA uptake. The value for GABA release is probably not valid since it fell outside of the linear part of the regression line which was used for calculation. Therefore, GABA uptake inhibition appears to be responsible for the overall inhibition of GABA disposal. This value is consistent with the proposed hypothesis that anesthesia involves excess GABA in the synaptic area. (Key words: Brain: gamma-aminobutyric acid; synapses. Hypnotics: benzodiazepines, midazolam. Theories of anesthesia.)

IT IS GENERALLY ACCEPTED that benzodiazepines act by potentiation or facilitation of GABA (γ-aminobutyric acid) action.[1,2] Diazepam has been used for clinical anesthesia but has undesirable side effects because of the vehicle in which it is dissolved. Midazolam (RO-21-3981; 8-chloro-6(2-flurophenyl)-1-methyl-4H-imidazo-(1,5-a)(1,4)benzodiazepine maleate; WSB) has been developed as a water-soluble benzodiazepine anesthetic agent and these side effects are eliminated.[3–5] We studied the effects of midazolam on GABA metabolism using rat brain synaptosomes as a model.

Materials and Methods

Synaptosomes were prepared from rat forebrain by ultracentrifugation at 0.9–1.2 M sucrose.[6] They were incubated in Ringer's solution (Krebs-phosphate in the presence of 10 μM [1-^{14}C]GABA, 0.5 μCi/ml) for 1 h at 30° C, with or without midazolam. After incubation, the synaptosomes were inactivated with 1 M H_2SO_4, and the liberated $^{14}CO_2$ was trapped in 2 N NaOH in a suspended plastic cup. The cup was dropped into scintillation fluid and its radioactivity determined as a measure of "GABA-disposal."

GABA-transaminase (4-aminobutyrate:2-oxoglutarate aminotransferase; EC 2.6.1.19) activity was determined with or without midazolam by coupling to excess aldehyde dehydrogenase.[7] The reduction of NAD^+ was followed spectrophotometrically at 340 nm. Absorption change between 10 min and 40 min after initiation of the reaction was used as a measure of GABA-transaminase activity.

GABA release was studied using synaptosomal beds in which synaptosomes (from striatum only) were packed by centrifugation into a thin layer 8 mm in diameter. The centrifuge tube contained a glass sleeve (8 mm, ID) which sat on top of a piece of polyester fabric (14 mm diameter). The fabric sat on top of a flat rubber washer which formed a cushion between the glass sleeve and epoxy block below. Ringer's solution (Krebs-phosphate) was added to the tube followed by synaptosomes in 0.32 M sucrose containing 0.5 mg protein. After the suspended synaptosomes were stirred gently with a thin glass rod, the centrifuge speed was increased slowly to 250 × *g* to insure uniform sedimentation. After a few minutes, the centrifugal force was increased to 1000 × *g* to pack the synaptosomes. After centrifugation, the fabric containing the synaptosomal bed was carefully recovered so as not to disturb the bed. It was covered with another piece of fabric and clamped between silver frames to lend rigidity. The assembly was temperature equilibrated at 30° C for 10 min in Ringer's solution, then removed, drained for several seconds, and transferred into Ringer's solution containing [1-^{14}C]-GABA (10 μM, 0.5 μCi/ml) for 5 min for GABA loading. The assembly was then transferred serially through twenty 2-ml Ringer's solution washes at 2.5-min intervals. These washes are indicated as Fraction Numbers. In some experiments, the presence of KCl or midazolam in fractions 9 through 12 is indicated by dashed vertical lines after the eighth and twelfth fractions. Subsequently, the washed synaptosomal bed was dissolved in 1 N NaOH on a steam bath. Aliquots from all washes, incubation solutions and the NaOH extract were taken for radioactivity determination. Data are reported as per cent of total radioactivity, that is, the sum of radioactivity from wash number five through the NaOH extract. The first four washes

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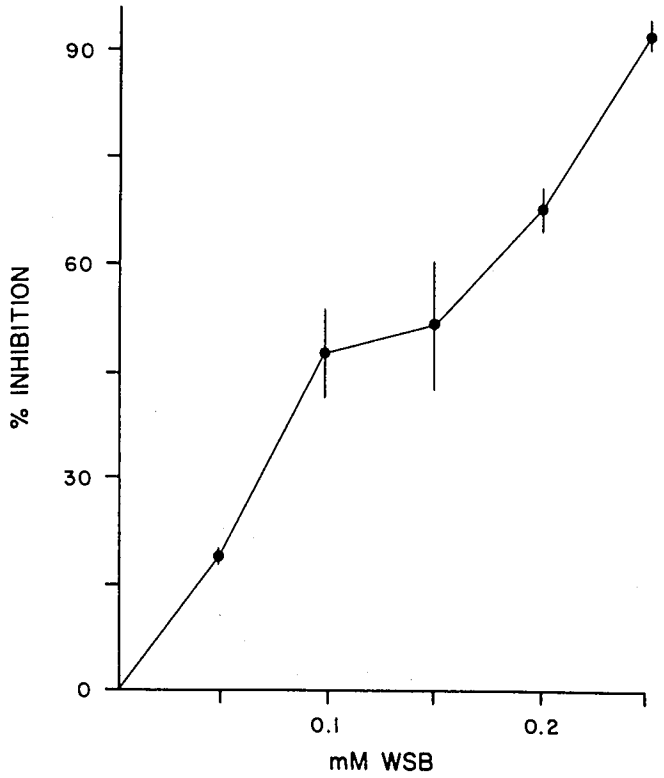

FIG. 1. Inhibition by midazolam (WSB) of GABA disposal by rat brain synaptosomes. GABA disposal is measured by $^{14}CO_2$ liberated from [1-^{14}C]GABA. The regression line, Y = 329.1X + 5.8 (r = 0.89) with N = 6 or 7 for each point, has P values of <0.001 for regression and correlation coefficients, and 0.19 for the Y-intercept.

were neglected since their higher radioactivity represented mostly surface radioactivity. The radioactivity in the NaOH extract represented radioactivity remaining in the synaptosomes at the end of the washing process.

GABA uptake was measured by incubation of synaptosomes (0.6–0.7 mg protein) in Ringer's solution containing tritiated GABA (50 μM, 2 μCi/ml) for 10 min at 30°C. At the beginning and end of incubation, aliquots were diluted tenfold in ice-cold Ringer's solution containing 50 mM GABA, and mixed immediately. This represented a 1000-fold dilution of GABA radioactivity so that even if further GABA uptake occurred, it did not significantly change the amount of radioactive GABA in the synaptosomes. They were centrifuged at 10,000 × g. The space in the pellet represented 0.2 per cent of the total radioactivity in the sample and was neglected. The difference in radioactivity in pellets between 0 and 10 min represented uptake of GABA into the synaptosomes.

Protein was determined with phenol reagent.[8] Radioactivity was determined using a Packard TRI-CARB® Liquid Scintillation Spectrometer (Model 3385). Its efficiency was continuously monitored and was approximately 70 per cent efficient for ^{14}C, and 27 per cent efficient for ^{3}H.

Data were analyzed using Student's t tests for unpaired data. Values were reported as means ± SEM. The number of experiments (N) is given in the Results section. Least square regression lines (Y = bX + a) were calculated where X was midazolam concentration in mM, and Y was percentage inhibition. The P value of the regression coefficient (b), which is the same for the correlation coefficient (r), and the P value of the Y-intercept (a) were calculated with conventional statistical methods.[9] The doses giving 10 per cent inhibition (ID_{10}) and 50 per cent inhibition (ID_{50}) were obtained from the regression lines. In release experiments, effective dose (ED) was used instead of inhibitory dose (ID).

Results

The control rate of overall "GABA disposal" was 3.56 ± 0.10 nmol/h × mg protein at N = 226. It was inhibited by midazolam in a dose-related manner (fig. 1). The calculated ID_{10} was 13 μM, and the ID_{50} was 134 μM, which is equivalent to 0.56 and 5.9 mg/100 ml, respectively.

GABA-transaminase activity for controls was 0.44 ± 0.01 μmol/h × mg protein (N = 331). It was inhibited by midazolam in a dose-related manner, but

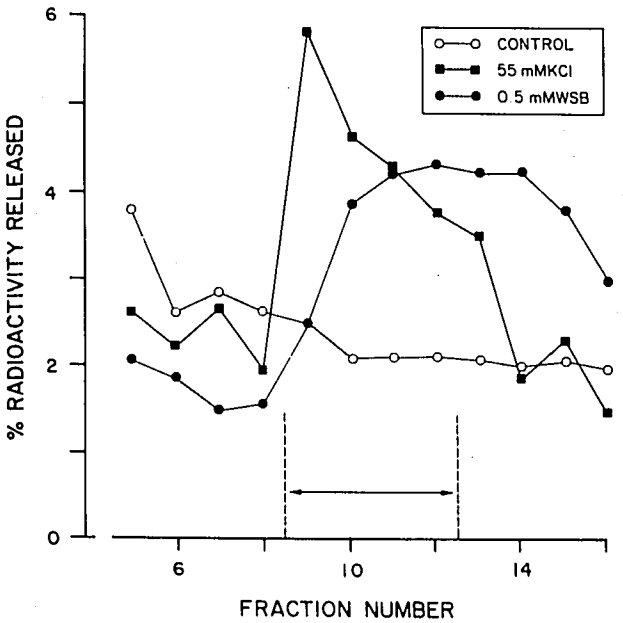

FIG. 2. Effect of midazolam (WSB) and KCl on release of radioactivity from synaptosomes preloaded with [1-^{14}C]GABA. Washes are indicated by Fraction Numbers. Dashed vertical lines indicate that midazolam or KCl was added from the ninth through twelfth washes. Each point represents a single determination of parallel consecutive observations for each condition using three synaptosomal beds from one preparation of synaptosomes.

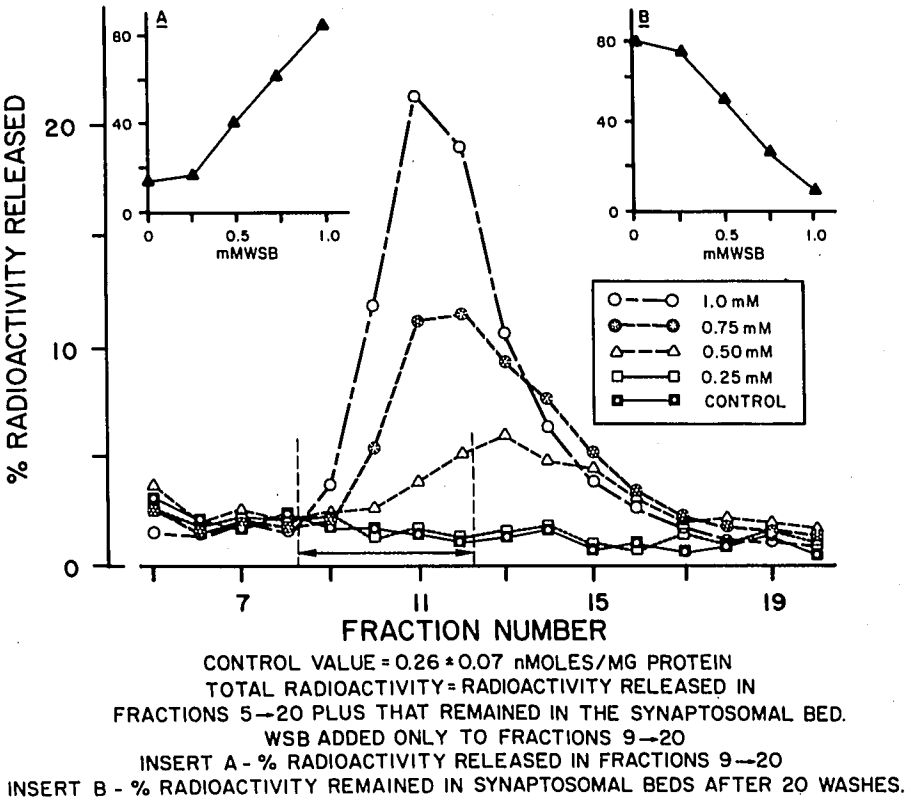

FIG. 3. Dose-related release of radioactivity by midazolam (WSB) from [1-^{14}C]-GABA preloaded synaptosomes. In the main graph, each line represents response to a single dose with 20 successive washes indicated by Fraction Numbers with N = 5 for each data point. SEM values are deleted for clarity. Dashed vertical lines indicate that midazolam may or may not be added from the ninth through twelfth washes. Inserts A and B represent cumulative release or retention respectively at each dose level, expressed as per cent of radioactivity originally present in the synaptosomal bed. Control values (*i.e.*, without midazolam) do not fall on the regression lines in both inserts. Based on the four concentrations studied, the regression for release is represented by Y = 89.2X − 3.9 (r = 0.90) and for retention by Y = 86.0X + 93.1 (r = −0.87). The *P* values for GABA release are <0.001 for regression and correlation coefficients, and 0.58 for Y-intercept. The *P* values for GABA retention are <0.001 for regression and correlation coefficients, and 0.39 for Y-intercept.

the concentration required was relatively high. The regression line with N = 21 of Y = 13.5X + 2.1 (r = 0.92) has *P* values of <0.001 for regression and correlation coefficients, and 0.37 for Y-intercept. The calculated ID_{10} is 580 μM and ID_{50} is 3,534 μM.

GABA was released in the absence of drug at a rate of 0.26 ± 0.07 nmol/mg protein for washes 9–20 (*i.e.*, 0.5 h) with N = 5. This slow rate of release probably represents both control release of GABA and discharge of GABA metabolites (possibly CO_2) into the medium. For the 12 washes (9–20) in the five control experiments, mean release was 1.28 per cent of initial radioactivity loaded into the synaptosomes with an average SEM of 0.36. Induced release can be seen with both 55 mM KCl and various concentrations of midazolam (figs. 2 and 3). The release by KCl was immediate. Midazolam caused a delayed but prolonged release of GABA. The dose-related release of GABA is shown in the two inserts in figure 3. The calculated ED_{50} values are 604 μM and 501 μM, respectively, for GABA release and retention with an average of 552 μM and the calculated ED_{10} values are 156 μM and 36 μM, respectively (average 96 μM).

Normal GABA uptake occurred at a rate of 7.95 ± 0.67 nmol/h × mg protein (N = 10). Its inhibition by midazolam was also dose-related but the relationship was not linear (fig. 4). The ID_{50} from figure 4 is 110 μM and ID_{10} is 13 μM.

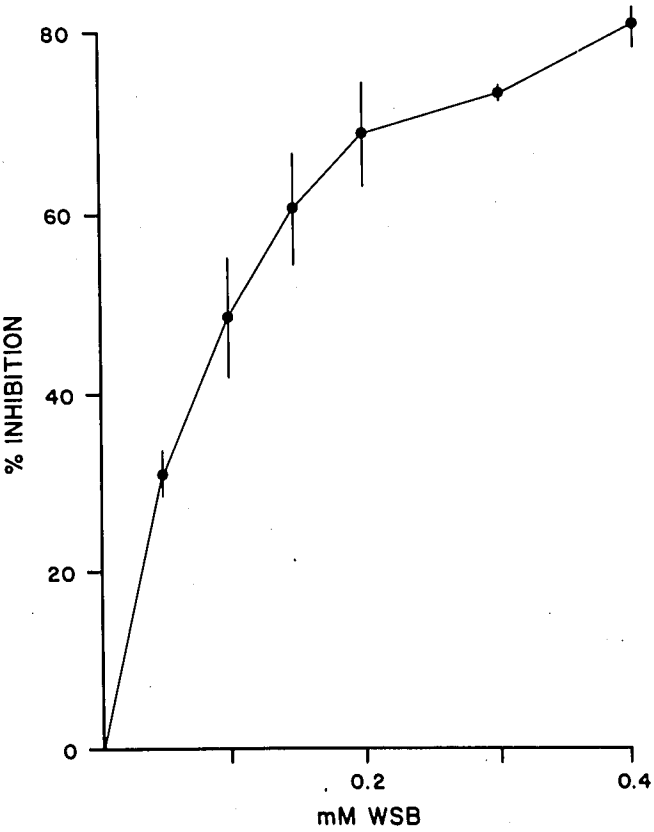

FIG. 4. Inhibition by midazolam (WSB) of ^{3}H-GABA uptake by synaptosomes (N = 5–7).

TABLE 1. Effects of Midazolam on Different Steps of Synaptosomal GABA Metabolism

	ID_{10} (μM)	ID_{50} (μM)
GABA disposal	13	134
GABA-T	580	3,534
GABA release	96	552
GABA uptake	13	110

The ID_{10} and ID_{50} values are calculated from regression lines except in the case of GABA uptake where the values are estimated from the curve in figure 4. In the case of GABA release, the average ED values calculated from GABA release and GABA retention regression lines are given here.

Discussion

We previously postulated that GABA may play an important role in causing anesthesia.[7,10–15] Using rat brain synaptomoses as a model, we were able to demonstrate a dose-related inhibition by anesthetic agents of GABA disposal measured as $^{14}CO_2$ liberation from [1-^{14}C]GABA. Furthermore, the ID_{10} values of this inhibition roughly correspond to the ED_{50} values for the clinical actions of the anesthetic drugs studied.[10,13,15]

The concentration of midazolam causing 10 per cent inhibition of GABA disposal, ID_{10}, was found to be 13 μM (ID_{50} = 134 μM) (table 1). Pharmacologically, no data are available on midazolam concentration in the brain at the time when anesthesia is attained.[4] However, the antimetrazol action in mice by midazolam peaks at the same time when the brain concentration of midazolam is highest (approximately 8.2 μM).‡

The ID_{10} value of GABA disposal found in this study (13 μM, table 1) appears to originate from an inhibition of GABA uptake (ID_{10} = 13 μM) rather than by any other mechanism. The inhibition of GABA-transaminase activity (ID_{10} = 580 μM) requires much higher concentrations. Induced GABA release also requires higher drug concentrations (ED_{10} = 90 μM and ED_{50} = 552 μM). The accuracy of the ED_{10} values is questionable since they fall into the region where the regression curve is nonlinear, but the ED_{50} values support the validity of the ED_{10} observations since it is also much larger than the ID_{50} (134 μM) for GABA disposal. Midazolam action can therefore be attributed to inhibition of GABA uptake. Excess GABA in a GABA synapse would inhibit impulse transmission. Midazolam appears to act by a potentiation of synaptic inhibition via reduced GABA reuptake from the synaptic clefts.

This deduction is in agreement with our hypothesis[10–15] that excess GABA in the synaptic area may contribute to the state of anesthesia. We have shown that thiopental in clinically relevant concentrations, inhibits GABA-transaminase.[7] Others have shown earlier that volatile anesthetic agents inhibit complex I in the electron transport chain.[16] This inhibition should cause an accumulation of NADH followed by an accumulation of succinic semialdehyde and of GABA. Although NADH accumulation has not been demonstrated in the brain, it has been shown in the kidney[17] and calculated to occur in the liver.[18] The failure in demonstrating NADH accumulation in the brain may lie in the phenomenon of metabolic compartmentation.[19] NADH accumulation occurring in one brain area may become diluted out in chemical analysis of the whole brain. Although NADH accumulation in the brain has not yet been demonstrated, an increase in GABA content in brain cortex slices under the influence of halothane does occur.[11,20]

The logical conclusions from these observations support the hypothesis that various anesthetic agents cause increases in GABA levels in the synaptic area by various means. The increase in GABA content may be compartmentalized in the brain. Anesthesia, therefore, is not the expected result of all drugs which cause an increase in brain GABA content. For example, aminooxyacetic acid, which causes greatly elevated brain GABA content, does not cause anesthesia, implying a localization of GABA accumulation in areas other than the synapses. It is also known that the metabolic effect of thiopental in the mouse brain is compartmented.[21]

The stability and low level of radioactivity release from [1-^{14}C]GABA preloaded synaptosomal beds in the absence of drugs and their immediate response to KCl depolarization indicate the suitability of this preparation for GABA release studies. Studies not reported here indicate that 55 mM KCl liberated nearly all the releasable radioactivity, and the response is reproducible. The dose-related response to midazolam and its delayed nature is also reproducible in magnitude and in time, indicating that fractional responses are measurable. Reduced release by 25 mM KCl depolarization (not reported here) also confirms these observations.

The induced release of GABA by midazolam is different than that caused by KCl, suggesting that the action of midazolam is not a simple depolarization. A plot of radioactivity released by midazolam appears to take the form of a S-shaped curve, since release above 1.0 mM approaches 100 per cent. (These data cannot be obtained because of drug solubility problems.) This suggests a cooperative, allosteric type of reaction. Both the induction of release and return to resting values after removal of the drug is slow. Some sort of receptor binding action by midazolam is sug-

‡ Sepinwall J: personal communication.

gested. Recent work has not proven a direct correspondence of brain GABA-receptors and benzodiazepine receptors but suggests that benzodiazepine binding may affect GABA binding.[22]

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