COMPARISON OF THE RELAXANT EFFECTS OF DIAZEPAM, FLUNITRAZEPAM AND MIDAZOLAM ON AIRWAY SMOOTH MUSCLE

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SUMMARY

The mechanisms by which benzodiazepines produce muscle relaxation and respiratory depression are not know, but they may include actions on peripheral benzodiazepine receptors or central GABA receptors, or a direct action on airway smooth muscle may also be involved. We have compared, therefore, the effects of diazepam, flunitrazepam and midazolam on airway tone by measuring isometric tension of guineapig trachealis muscle. Cumulative concentrations of diazepam, flunitrazepam and midazolam caused concentration-dependent relaxation of resting tone in the tracheal smooth muscle with no significant differences in pD₂ values (−log EC₅₀—an index of potency) or intrinsic activities (% of maximum response) for relaxations for the three compounds. Pretreatment with propranolol 10⁻⁶ mol litre⁻¹, flumazenil 10⁻⁷ and 10⁻⁶ mol litre⁻¹ or PK11195 10⁻⁶ mol litre⁻¹ had no effect on diazepam- or midazolam-induced relaxation. Diazepam 3 x 10⁻⁶ mol litre⁻¹ pretreatment shifted the concentration-response curves for acetylcholine, histamine and serotonin (5-HT) to the right by a factor of approximately 2. Flunitrazepam 3 x 10⁻⁶ mol litre⁻¹ pretreatment also shifted the curves for histamine and 5-HT similarly to the right, whereas midazolam pretreatment did not inhibit any agonist-induced contractions. These results suggest that benzodiazepines relax airway smooth muscle, not via neural pathways or central and peripheral benzodiazepine receptors, but by a direct action on airway smooth muscle.

KEY WORDS


Benzodiazepines have been used widely as adjuvants in both regional and general anaesthesia and in high risk patients in the intensive care unit. However, the effects of benzodiazepines on airway tone and reactivity have not been studied well, and it is also not known if the benzodiazepine antagonist flumazenil may be used safely for the asthmatic patient treated with benzodiazepines. Although some studies on airway relaxant effects of benzodiazepines have been published [1, 2], the relative potencies of benzodiazepines and their mechanisms of action on airways are still in question. We have therefore evaluated the direct effects of diazepam, flunitrazepam and midazolam on tracheal smooth muscle isolated from guineapigs.

The effects of flumazenil (a specific central-type antagonist of benzodiazepines), PK11195 (a specific peripheral-type antagonist of benzodiazepines), atropine (a muscarinic antagonist) and propranolol (a beta adrenergic antagonist) were examined also to elucidate any mechanisms involved. In addition, the inhibitory effects of the three agents were investigated against contractions induced by contractile agonists: potassium chloride, acetylcholine (ACh), bethanechol (BCh), histamine and serotonin (5-HT).

MATERIALS AND METHODS

Male guineapigs weighing 300–700 g were killed under enflurane anaesthesia vaporized into a glass jar, and the tracheas, from the larynx to the carina, removed. Single tracheal ring strips were prepared by modifying a technique reported previously (paired strips) [3]. Six preparations were obtained from each animal. Every preparation was mounted in an organ bath filled with 20 ml of a Krebs-Ringer type solution maintained at 37 °C and aerated with 5% carbon dioxide in oxygen. The solution contained the following (mmol litre⁻¹): sodium chloride 120.7, potassium chloride 5.9, calcium chloride 2.5, magnesium chloride 1.2, sodium bicarbonate 15.5, sodium dihydrogen phosphate 1.2 and glucose 11.5.

The isometric tension of each sample was measured continuously with a strain gauge transducer (Minebea Co., Ltd, Japan) and displayed on a pen recorder (San-ei Instrument Co., Ltd, Japan). Each preparation was set at approximately 1.5 g of resting tension and was allowed to equilibrate for at least 40 min before addition of drug, while being
washed repeatedly with fresh Krebs solution. The spontaneous tension change within 30 min was less than 3% of the maximal relaxation produced by dyphylline $6 \times 10^{-8}$ mol litre$^{-1}$. Benzodiazepines (diazepam, flunitrazepam and midazolam) were administered cumulatively to the tracheal smooth muscle. In the presence of flumazenil $10^{-7}$ and $10^{-6}$ mol litre$^{-1}$, propranolol $10^{-4}$ mol litre$^{-1}$ or PK11195 $10^{-4}$ mol litre$^{-1}$, diazepam and midazolam were tested also in the same fashion as above. In order to examine the inhibitory effects of benzodiazepines, contractile agonists (KCl, BCh, ACh, histamine and 5-HT) were administered cumulatively in the presence of each benzodiazepine ($3 \times 10^{-6}$ mol litre$^{-1}$) which had been administered at least 20 min before adding the cumulative concentration of the contractile agonists. To elucidate the parasympathetic inhibitory effect of benzodiazepines, the effect of atropine $10^{-6}$ mol litre$^{-1}$ on relaxations induced by diazepam or midazolam was studied also on preparations partially precontracted with KCl $30$ mmol litre$^{-1}$ or histamine $10^{-8}$ mol litre$^{-1}$.

For each contractile agonist, responses were plotted as a percentage of their own maximum contraction. Relaxant responses were compared with the maximal relaxation achieved by dyphylline $6 \times 10^{-3}$ mol litre$^{-1}$ added at the end of each experiment and the response was represented as a relative percentage of the relaxation induced by dyphylline as 100%. Dyphylline $6 \times 10^{-3}$ mol litre$^{-1}$ was used as it is as potent as isoproterenol $10^{-4}$ mol litre$^{-1}$ in producing maximal airway relaxation [4] and is not affected by beta adrenergic receptor antagonists. Values of $pD_2$, which represents the potency of agonists, were calculated as the negative logarithm of $EC_{50}$ (concentration at 50% of the maximum response) in each drug response. Intrinsic activities indicate the maximum relaxation of each benzodiazepine corresponding to % of the maximal relaxation obtained with dyphylline $6 \times 10^{-3}$ mol litre$^{-1}$.

Atropine sulphate, acetylcholine chloride, bethanechol hydrochloride, histamine dihydrochloride, 5-hydroxytryptamine, potassium hydrochloride and $D_1$-propranolol hydrochloride were obtained from Sigma Chemical (U.S.A.), diazepam, flumazenil and midazolam from Yamanouchi Pharmaceutical Co. (Japan) and dyphylline and flunitrazepam from Eisai Co. Ltd (Japan). PK11195 was provided by Rhône-Poulenc Rorer Inc. (U.S.A.). Differences in $pD_2$ values and intrinsic activities were compared by Student's paired $t$ test for two groups or by analysis of variance for more than two groups. $P < 0.05$ was considered significant.

**RESULTS**

*Effects on resting tone of tracheal smooth muscle*

At concentrations greater than $3 \times 10^{-7}$ mol litre$^{-1}$, cumulative administration of diazepam, flunitrazepam and midazolam caused concentration-dependent relaxation of the untreated tracheal smooth muscle (fig. 1). Relative potencies of the three benzodiazepines and maximum responses were similar (fig. 1). No significant differences were found in $pD_2$ values or in intrinsic activities (table I). There were seven guineapigs in each benzodiazepine study.
7. No significant shift in each curve compared with the $n = 1$ (A) on the concentration-response curves to bethanechol litre$^{-1}$ (D) or flunitrazepam 3 x 10$^{-6}$ mol litre$^{-1}$ (O). Effects of midazolam 3 x 10$^{-6}$ mol litre$^{-1}$ had no effect on the concentration–response curves to diazepam (fig. 2). Flumazenil 10$^{-6}$ mol litre$^{-1}$ also had no effect on concentration–response curves to diazepam (fig. 2). The $pD_2$ values for two benzodiazepines in the presence and absence of either flumazenil 10$^{-6}$ and 10$^{-7}$ mol litre$^{-1}$ or propranolol 10$^{-6}$ mol litre$^{-1}$ were not different (table II). Five experiments were performed in each study. PK11195 10$^{-6}$ mol litre$^{-1}$ pretreatment did not affect concentration–response curves to diazepam or midazolam (data not shown, $n = 3$).

### Effects of propranolol, flumazenil and PK11195

Beta adrenergic block with propranolol 10$^{-8}$ mol litre$^{-1}$ had no effect on diazepam-induced concentration–response curves (fig. 2). Flumazenil 10$^{-6}$ mol litre$^{-1}$ also had no effect on concentration–response curves to diazepam (fig. 2). The $pD_2$ values for two benzodiazepines in the presence and absence of either flumazenil 10$^{-7}$ and 10$^{-8}$ mol litre$^{-1}$ or propranolol 10$^{-8}$ mol litre$^{-1}$ were not different (table II). Five experiments were performed in each study. PK11195 10$^{-6}$ mol litre$^{-1}$ pretreatment did not change KCl response curves (table III). Although the curves for ACh were shifted slightly to the right by pretreatment with benzodiazepines (fig. 3), significant shifts were observed only in preparations pretreated with diazepam 3 x 10$^{-6}$ mol litre$^{-1}$ (table III). No significant shift, however, was found in the concentration–response curves to BCh with any benzodiazepine pretreatment (fig. 4, table III). The curves for histamine (fig. 5) and 5-HT (fig. 6) were shifted significantly (each by a factor of approximately 2) to the right by pretreatment with either diazepam 3 x 10$^{-6}$ mol litre$^{-1}$ or flunitrazepam 3 x 10$^{-6}$ mol litre$^{-1}$ (table III). In contrast, pretreatment with midazolam 3 x 10$^{-6}$ mol litre$^{-1}$ had no effect on the concentration–response curves to any contractile agonists (figs 3–6, table III). The number of preparations for each agonist study was seven.

### Effect of atropine on benzodiazepine-induced relaxation

Diazepam and midazolam also caused concentration-related relaxation of the muscle partially precontracted with KCl 30 mmol litre$^{-1}$ or histamine 10$^{-6}$ mol litre$^{-1}$. Pretreatment with atropine 10$^{-8}$ mol litre$^{-1}$ of KCl 30 mmol litre$^{-1}$ or hist-
Thus the mechanism of airway relaxation seems to differ from the central depressant effect of benzodiazepines. This suggests that diazepam may possess some stimulant effect on cholinesterase activity, as ACh is more susceptible than BCh to hydrolysis by cholinesterase. It is conceivable that the inhibitory mechanism of benzodiazepines in airway smooth muscle is not mediated by peripheral muscarinic mechanism of benzodiazepines in airway smooth muscle, because pretreatment with atropine did not alter the relaxant response to diazepam and midazolam.

Fig. 5. Mean (SEM) effects of midazolam 3 x 10^{-4} mol litre^{-1} (C), diazepam 3 x 10^{-4} mol litre^{-1} (D) or flunitrazepam 3 x 10^{-6} mol litre^{-1} (A) on the concentration-response curves to histamine (B). n = 7. Significant shifts are observed in the curves after diazepam and flunitrazepam pretreatments compared with the control curve (P < 0.05 in pD2 values).

Fig. 6. Mean (SEM) effects of midazolam 3 x 10^{-4} mol litre^{-1} (C), diazepam 3 x 10^{-4} mol litre^{-1} (D) or flunitrazepam 3 x 10^{-6} mol litre^{-1} (A) on the concentration-response curves to 5-HT (B). n = 7. Significant shifts are observed in the curves after diazepam and flunitrazepam pretreatments compared with the control curve (P < 0.05 in pD2 values).

DISCUSSION

The main finding of the present study was that diazepam, flunitrazepam and midazolam concentration-dependently relaxed uncontracted guineapig trachealis muscle at similar potencies (3 x 10^{-4} - 3 x 10^{-6} mol litre^{-1}). Moreover, this effect was not blocked by flumazenil or PK11195. In terms of sedative and hypnotic potencies in clinical dose ranges, flunitrazepam is said to be about five times as potent as midazolam and about 10 times as potent as diazepam [5, 6], whereas relaxant potencies for the three agents evaluated in this study were similar. Thus the mechanism of airway relaxation seems to differ from the central depressant effect of benzodiazepines. Although the concentrations of benzodiazepines required for relaxation of trachealis muscle were similar to the plasma concentrations achieved clinically (3 x 10^{-4} - 10^{-3} mol litre^{-1}) [5], EC50 for relaxations are in excess of the plasma concentration ranges, because the concentration-response curves to each benzodiazepine were steep for concentrations greater than 10^{-4} mol litre^{-1} (fig. 1).

As there are cholinergic and adrenergic nerves or nerve terminals in isolated trachealis muscle, we examined the effect of atropine or propranolol. We could not obtain any evidence to suggest involvement of cholinergic or adrenergic mechanisms in the relaxant effects of benzodiazepines, because pretreatment with atropine 10^{-3} mol litre^{-1} or propranolol 10^{-4} mol litre^{-1} (concentrations known to block muscarinic or beta adrenergic receptors [4]) did not alter the relaxant response to diazepam and midazolam.

There are two types of benzodiazepine receptor antagonist available currently. In agreement with our findings, PK11195 (a peripheral-type antagonist) has been reported to have no effect on midazolam-induced relaxation [1]. However, central-type benzodiazepine antagonists, such as flumazenil, seem to be more useful clinically than peripheral-type antagonists. Concentrations of flumazenil 10^{-2} mol litre^{-1} and 10^{-4} mol litre^{-1} used in the present study are likely to correspond to estimated plasma concentrations in clinical use (0.004 - 0.04 mg kg^{-1}) [7-9]. Benzodiazepines probably do not relax airways by activating central benzodiazepine receptors, as flumazenil did not block the concentration-related relaxation of diazepam and midazolam.

If benzodiazepines block Ca^{2+} channels in airway smooth muscle, specific inhibition should have been found in KCl-contracted tissues [3] after benzodiazepine administration. Our results strongly suggest that the relaxation was not caused by block of voltage-dependent Ca^{2+} channels, as the pD2 value for KCl contraction was not significantly decreased by pretreatment with each benzodiazepine (table III).

Although a weak but significant inhibitory effect of diazepam was observed in ACh-induced contraction, BCh-induced contractions were not affected by pretreatment with any of the three benzodiazepines. This suggests that diazepam may possess some stimulant effect on cholinesterase activity, as ACh is more susceptible than BCh to hydrolysis by cholinesterase. It is conceivable that the inhibitory mechanism of benzodiazepines in airway smooth muscle is not mediated by peripheral muscarinic receptors. Our observations that atropine had no influence on the relaxant effects of diazepam and midazolam in muscle precontracted with histamine 10^{-6} mol litre^{-1} or KCl 30 mmol litre^{-1} also support this view. We chose KCl and histamine as precontractile drugs to investigate the mechanism of benzodiazepines because the actions of these agonists are largely unaltered by atropine. Our results with atropine do not conflict with a previous study showing that relaxation of the airway by benzodiazepines was caused by inhibition of parasympathetic activity [2]. That study was undertaken in cats to which benzodiazepines were administered centrally.
We have no explanation for the different effects of midazolam and the other two benzodiazepine pretreatments seen during histamine and 5-HT induced contractions. Significant differences were observed only in pD₂ values, while the maximum responses to contractile agonists were not influenced by pretreatment with diazepam or flunitrazepam, indicating possible antagonism at the receptor sites. This finding suggests that usual doses of diazepam and flunitrazepam are useful in preventing bronchoospasm induced by ACh, histamine or 5-HT, because the equipotent doses of benzodiazepines (3 x 10^-4 mol litre^-1) used in the present study are similar to those used clinically, and they have also been shown to cause significant relaxation by themselves (fig. 1). In addition, our study suggests that flumazenil may be used safely for asthmatic patients who have been treated with benzodiazepines, as flumazenil did not antagonize airway relaxation elicited by benzodiazepines. However, it should be remembered that the benzodiazepines may depress the ventilatory response to carbon dioxide [10] and influence breathing pattern and chest wall mechanics [11].

In summary, we observed that the benzodiazepines (diazepam, flunitrazepam and midazolam) relaxed uncontracted airway smooth muscle in a concentration-dependent manner. Relaxant potencies and maximum responses were similar for all three agents. The mechanism of airway relaxation was probably a direct effect on smooth muscle.

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REFERENCES