IN VITRO INTERACTION OF DIAZEPAM AND OXAZEPAM WITH PANCURONIUM AND SUXAMETHONIUM

J. J. DRIESSEN, T. B. VREE, J. VAN EGMOND, L. H. D. J. BOOIJ AND J. F. CRUL

SUMMARY

In vitro studies using the rat phrenic nerve–hemidiaphragm preparation were performed to investigate the effects of diazepam and three of its metabolites on indirectly evoked twitch tension. Diazepam, desmethyldiazepam and temazepam alone caused an increase in twitch tension in lower concentrations, followed by complete depression in higher concentrations. Oxazepam did not cause an initial increase in twitch tension, but showed an immediate and dose-dependent depression. Cumulative concentration–response curves for pancuronium and suxamethonium in the presence of different concentrations of diazepam or oxazepam showed that small concentrations of diazepam, which did not change twitch tension alone, caused antagonism of the action of pancuronium, but not of suxamethonium. With oxazepam no such antagonism was observed. In liminal and supraliminal concentrations, both diazepam and oxazepam potentiated the action of pancuronium and suxamethonium. Possible implications for in vivo interactions are discussed.

The effects of benzodiazepines on muscle contraction are ascribed mainly to an action on spinal and supraspinal mechanisms (Haefely et al., 1981). Recently, however, peripheral effects of benzodiazepines on either neuromuscular transmission or muscle itself have been suggested in skeletal (Wilkinson, Grovestine and Hamilton, 1982; de-Groof, Bianchi and Narayan, 1980), smooth (Clanachan and Marshall, 1980; Hullihan et al., 1983) and heart muscle (Davies and Huston, 1981).

Reported effects of benzodiazepines on the isolated phrenic nerve–hemidiaphragm preparation in the rat, as a model of respiratory skeletal muscle, concern mainly diazepam and chlordiazepoxide and are very conflicting (Haefely et al., 1981). Also, the interactions of benzodiazepines with depolarizing and non-depolarizing myoneural blocking drugs are unclear (Haefely et al., 1981). In the first part of this study we investigated the effect of diazepam and its metabolites on the indirectly evoked twitch tension in the isolated rat phrenic nerve–hemidiaphragm preparation. Desmethyldiazepam, temazepam (3-hydroxydiazepam) and oxazepam are the three metabolites of diazepam which, in vivo, are present each time diazepam is administered although in variable proportion, depending on the species (Zbinden and Randall, 1967). In the second part of the study, and initiated by the results of the first part, we investigated the influence of different concentrations of diazepam and oxazepam on the cumulative concentration–response curves of pancuronium and suxamethonium as the principal representatives of, respectively, non-depolarizing and depolarizing neuromuscular blocking drug.

MATERIALS AND METHODS

Isolated phrenic nerve–hemidiaphragm preparations (Buelbring, 1946) of Wistar rats of 250–300g body weight were suspended in double-walled baths containing 45 ml of mammalian Krebs’ solution (sodium chloride 113.0; potassium chloride 4.7; calcium chloride 2.5; magnesium sulphate 1.2; sodium bicarbonate 25.0; sodium dihydrogen phosphate 2.5; and glucose 11.5 mmol/litre) and aerated with 5% carbon dioxide in oxygen. The temperature was kept constant at 37°C and the pH was adjusted to lie between 7.35 and 7.45 as measured with a Philips digital pH electrode (PW9408). The phrenic nerve was stimulated by a Grass S48 stimulator with supramaximal square wave stimuli of 0.2 ms duration at a rate of 0.1 Hz. The resulting twitch contraction was quantified by an isometric force displacement transducer (Grass FT03) and recorded on a polygraph. The control contractile force was between 20 and 50 gf. For each single experiment six hemidiaphragms from three rats were studied.

Part A

In this group of experiments the effects on the twitch tension of cumulative equimolar concentra-
tions of diazepam, desmethyldiazepam, temazepam and oxazepam were studied, using pure compounds obtained from commercial sources. As these benzodiazepine derivatives are poorly water-soluble and cannot be dissolved in Krebs' solution even after warming to 37°C and automatic stirring, stock solutions of diazepam, desmethyldiazepam, temazepam and oxazepam in 96% ethanol (5 mg ml⁻¹) were made first and further diluted with Krebs' solution. In a control study the effect of ethanol on the twitch tension was tested for amounts of ethanol up to twice those necessary to dissolve the highest concentrations of the four benzodiazepines.

In the main experiments of part A, after the twitch tension was stable for 15 min, cumulative equimolar concentrations (3.5, 35, 87.5, 175 and 262.5 μmol litre⁻¹) of the benzodiazepines were added to the bath at 15-min intervals or after the effect of the previous dose on the twitch tension had equilibrated, and the change in twitch tension was calculated. When 100% depression of the twitch tension was reached, the drugs were washed out with fresh Krebs' solution. The results were expressed as percentage of the control values, that is of the twitch tension recorded before the addition of any compound to the bath. A 50% effective blocking concentration was calculated from the concentration–response curves obtained.

Statistical analysis with Student's t test for unpaired data was performed to compare the changes in twitch response induced by the four drugs. P < 0.05 was considered statistically significant.

Part B
In these experiments cumulative concentration–response curves were determined for pancuronium and suxamethonium, 20 min after the addition of diazepam or oxazepam to the bath. Four main groups of interaction were thus selected: pancuronium–diazepam, pancuronium–oxazepam, suxamethonium–diazepam and suxamethonium–oxazepam. Four different concentrations of diazepam or oxazepam were used in each main group: 0, 3.5, 35 and 70 μmol litre⁻¹, the last three concentrations because they cause respectively subliminal, liminal and supraliminal effects on the twitch tension when applied alone. Each single experiment was conducted with only one concentration of pancuronium (or suxamethonium) and one concentration of benzodiazepine. The results were expressed as percentage of the values of the twitch tension 20 min after the addition of diazepam or oxazepam to the bath.

The concentration–response curves for each subgroup were constructed using the model in which the percentage block is described as a normal probability integral function of the dose of neuromuscular blocking drug in a linear scale (Robertson et al., 1983). For each subgroup the concentration resulting in 50% depression of twitch tension (EC₅₀), the concentration resulting in 90% depression of twitch tension (EC₉₀), and the slope of the concentration–response curve if depicted on a logarithmic scale (α/EC₅₀) were calculated.

The experiments were concentrated in time for each main group to ensure that rats of the same age and comparable environmental circumstances were used. Therefore, the sub-groups of every main group had comparable controls, but controls of the four main groups may not have been comparable.

The results were analysed using Student's t test.
for unpaired data. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

Ethanol did not cause any significant effect on the twitch tension at concentrations considerably greater than those used to dissolve the highest concentrations of benzodiazepines in the following experiments.

**Part A**

The structural formulae of the benzodiazepines investigated in this study are shown in figure 1, which also shows their in vivo metabolic relationships.

The effects of cumulative concentrations of diazepam, desmethyldiazepam, temazepam and oxazepam on twitch tension are shown in table I. Each value represents the mean (±SD) of six experiments. Diazepam, desmethyldiazepam and temazepam initially caused an increase in twitch tension which was followed by depression of the twitch tension at greater concentrations. Oxazepam, however, did not cause an increase in twitch tension, but showed an immediate dose-dependent depression. Typical recordings of the concentration-response curves for diazepam and oxazepam are shown in figures 2 and 3.

All four benzodiazepines caused a complete depression of twitch tension at concentrations ranging from 175 to 262.5 μmol litre⁻¹. After 100% twitch depression was reached, a shift of the baseline was

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**Table I. Change of the twitch tension expressed as percentage of control values at cumulative concentrations of benzodiazepines (μmol litre⁻¹) (mean±SD of six determinations)**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μmol litre⁻¹)</th>
<th>3.5</th>
<th>35</th>
<th>87.5</th>
<th>175</th>
<th>262.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmethyldiazepam</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temazepam</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazepam</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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**Fig. 2.** The effect of cumulative concentrations of diazepam on twitch tension of the in vitro phrenic nerve–hemidiaphragm preparation of the rat.
FIG. 3. The effect of cumulative concentrations of oxazepam on twitch tension of the in vitro phrenic nerve-hemidiaphragm preparation of the rat.

**TABLE II.** Concentrations at which 50% and 90% depression of the twitch tension occurred (EC₅₀ and EC₉₀) and ratio α/EC₅₀ (mean values ± SD) of the concentration-response curves for pancuronium and suxamethonium in the presence of different concentrations of either diazepam or oxazepam. In each subgroup six rat phrenic nerve-hemidiaphragms were studied. *Significant difference (P < 0.05) compared with controls (diazepam or oxazepam concentration of 0.00 μmol litre⁻¹) within the same drug group.

<table>
<thead>
<tr>
<th>Drug groups and concn (μmol litre⁻¹)</th>
<th>EC₅₀ (μmol litre⁻¹)</th>
<th>EC₉₀ (μmol litre⁻¹)</th>
<th>α/EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pancuronium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam 0.00</td>
<td>2.67 (0.36)</td>
<td>3.69 (0.41)</td>
<td>0.30 (0.04)</td>
</tr>
<tr>
<td>Diazepam 3.50</td>
<td>3.58 (0.58)*</td>
<td>5.05 (0.99)*</td>
<td>0.31 (0.07)</td>
</tr>
<tr>
<td>Diazepam 35.00</td>
<td>2.00 (0.14)*</td>
<td>2.88 (0.25)*</td>
<td>0.32 (0.06)</td>
</tr>
<tr>
<td>Diazepam 70.00</td>
<td>2.00 (0.22)*</td>
<td>2.74 (0.31)*</td>
<td>0.29 (0.04)</td>
</tr>
<tr>
<td><strong>Pancuronium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazepam 0.00</td>
<td>3.43 (0.20)</td>
<td>5.1 (0.44)</td>
<td>0.38 (0.06)</td>
</tr>
<tr>
<td>Oxazepam 3.50</td>
<td>3.50 (0.18)</td>
<td>5.5 (0.16)</td>
<td>0.45 (0.03)</td>
</tr>
<tr>
<td>Oxazepam 35.00</td>
<td>3.09 (0.22)</td>
<td>4.6 (0.20)</td>
<td>0.39 (0.04)</td>
</tr>
<tr>
<td>Oxazepam 70.00</td>
<td>2.23 (0.26)</td>
<td>3.7 (0.25)*</td>
<td>0.51 (0.10)*</td>
</tr>
<tr>
<td><strong>Suxamethonium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazepam 0.00</td>
<td>33.09 (1.74)</td>
<td>57.03 (7.80)</td>
<td>0.57 (0.21)</td>
</tr>
<tr>
<td>Diazepam 3.50</td>
<td>31.75 (5.35)</td>
<td>59.10 (11.61)</td>
<td>0.67 (0.14)</td>
</tr>
<tr>
<td>Diazepam 35.00</td>
<td>28.32 (1.70)*</td>
<td>48.07 (5.16)</td>
<td>0.55 (0.20)</td>
</tr>
<tr>
<td>Diazepam 70.00</td>
<td>24.25 (4.96)*</td>
<td>37.75 (6.84)*</td>
<td>0.44 (0.06)</td>
</tr>
<tr>
<td><strong>Suxamethonium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazepam 0.00</td>
<td>28.77 (2.10)</td>
<td>40.45 (2.68)</td>
<td>0.32 (0.04)</td>
</tr>
<tr>
<td>Oxazepam 3.50</td>
<td>28.98 (0.57)</td>
<td>42.32 (4.49)</td>
<td>0.36 (0.10)</td>
</tr>
<tr>
<td>Oxazepam 35.00</td>
<td>17.80 (2.29)*</td>
<td>25.67 (2.64)*</td>
<td>0.35 (0.04)</td>
</tr>
<tr>
<td>Oxazepam 70.00</td>
<td>17.48 (1.18)</td>
<td>24.06 (2.11)*</td>
<td>0.30 (0.07)</td>
</tr>
</tbody>
</table>
observed with diazepam and temazepam, but not with desmethyldiazepam and oxazepam. After the bath had been washed out with fresh Krebs' solution, a recovery of about 75% occurred. The difference in depression of twitch tension between these drugs was statistically significant.

Oxazepam differed significantly from desmethyldiazepam and temazepam in calculated 50% blocking concentration and from diazepam, desmethyldiazepam and temazepam in its effect on twitch increase at lower concentrations.

Part B

Figure 4 (A, B, C, D) shows the cumulative concentration-response curves for pancuronium and suxamethonium in the presence of either diazepam or oxazepam. Each point on the curves represents the mean of six experiments. The concentrations (means ± SD) required to produce 50% and 90% depression of the twitch tension and the values of the σ/EC\textsubscript{50} ratio are listed in table II.

With subliminal concentrations of diazepam (3.5 μmol litre\textsuperscript{-1}) a statistically significant shift of the pancuronium concentration-response curve to the right was seen, while the same subliminal concentration of oxazepam did not cause a significant change. Liminal concentrations (35 μmol litre\textsuperscript{-1}) of both diazepam and oxazepam caused a shift to the left of the concentration-response curve of pancuronium, although this shift was significant only for diazepam. Increasing the concentrations to supraliminal (70 μmol litre\textsuperscript{-1}) caused a significant furth-

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**Fig. 4.** Concentration-response curves for: A, pancuronium in the presence of different concentrations of diazepam; B, pancuronium with oxazepam; C, suxamethonium with diazepam and D, suxamethonium with oxazepam. In vitro rat phrenic nerve–hemidiaphragm preparation. SD of the means are shown only for the control concentration-response curves and, for reasons of clarity of the figure, are omitted for the concentration-response curves in the presence of different concentrations of diazepam or oxazepam.
er shift to the left of the concentration–response curve of pancuronium only in the presence of oxazepam, not of diazepam.

The suxamethonium concentration–response curves were not affected by subliminal concentrations of either diazepam or oxazepam, but the two greater concentrations of both these agents caused a significant shift to the left of the concentration–response curve for suxamethonium. Increasing the concentration of diazepam or oxazepam from 35 to 70 µmol litre⁻¹ did not shift the curve significantly further to the left.

There were no significant differences between the slopes of the concentration–response curves within each main group, except for that of pancuronium with the greatest concentration of oxazepam.

DISCUSSION

Since the discovery of specific and high affinity benzodiazepine receptors in the brain (Moehler and Okada, 1977; Squires and Braestrup, 1977), evidence has been presented of a distinct group of peripheral binding sites for benzodiazepines (Davis and Huston, 1981; Hullihan et al., 1983). Although the correlation of these peripheral binding sites with peripheral pharmacological effects still remains unclear, further studies on peripheral effects are undoubtedly needed. Wilkinson, Grovestine and Hamilton (1982) demonstrated peripheral binding sites for [³H]-flunitrazepam in crude membrane preparations of the rat diaphragm. This discovery has thrown new light on the effects of benzodiazepines on peripheral neuromuscular function and the interaction with myoneural blockers. The effects of diazepam, desmethyldiazepam and temazepam on the rat phrenic nerve–hemidiaphragm preparation show a biphasic effect on the twitch tension: the twitch tension increased in lower concentrations and was depressed in higher concentrations. With oxazepam, however, there was an immediate dose-dependent depression of the twitch tension. Hamilton, studying the effect of diazepam and chlor diazepoxide (Hamilton, 1967) and flurazepam (Hamilton and Stone, 1982) also found this biphasic effect in the in vitro rat diaphragm. A recent study reported that two types of concentration–response curves exist with increasing concentrations of 11 different benzodiazepines—one group with initial twitch increase and subsequent depression and another group with immediate depression of the twitch tension (Driessen et al., 1984).

The effect of different concentrations of diazepam and oxazepam on the cumulative concentration–response curve of pancuronium and suxamethonium was the subject of the second part of the study. Concentration–response curves in the in vitro rat phrenic nerve–hemidiaphragm preparation show considerable variability, especially when some time elapses between experiments. Several factors might be responsible; for example, it has been shown that differences in the age of the rats may be responsible for variations in the margin of safety of the neuromuscular transmission (Kelly and Roberts, 1977). The results of the present in vitro study are interesting because of the small concentrations of benzodiazepines used. Subliminal concentrations of diazepam (3.5 µmol litre⁻¹) antagonized the effect of pancuronium, but not of suxamethonium. Oxazepam antagonized neither pancuronium nor suxamethonium in subliminal concentrations. These results suggest a presynaptic effect on acetylcholine release by diazepam and probably some other benzodiazepines, which may be responsible for the initial increase in twitch tension and for the antagonism of non-depolarizing blocking agents in even lower concentrations. In concentrations of 35 µmol litre⁻¹ and greater, both diazepam and oxazepam potentiate depolarizing and non-depolarizing myoneural blocking drugs. The exact mechanism of this potentiation is not yet clear. One may speculate that the effect is postsynaptic and involves the acetylcholine receptor, because there was no significant difference between the slopes of control concentration–response curves and curves obtained after addition of diazepam or oxazepam. A direct effect on the muscle, however, may also contribute to the effect (deGroof, Bianchi and Narayan, 1980). The incomplete recovery after washing out the preparation in part A of our study affords more evidence for a direct muscle effect.

There have been few, if any, systematic in vivo studies of the interaction of neuromuscular blocking drugs with benzodiazepines, especially those which, like oxazepam, do not cause an increase in twitch tension at lower concentrations. Most previous studies of in vivo interactions involved diazepam. The biphasic action of diazepam might explain some of the controversy in the literature, where no effect, potentiation and antagonism all have been described (Haefely et al., 1981). The in vitro antagonism of non-depolarizing neuromuscular blocking drug by diazepam 3.5 µmol litre⁻¹ may explain why no effect or even slight antagonism of the effect of the relaxant has been found in vivo. Extrapolation of in vitro
concentrations to in vivo concentrations, however, remains impossible.

The different results obtained with oxazepam in this study indicate that diazepam should not be considered to be representative of all benzodiazepines in investigations of the pharmacodynamics of neuromuscular blocking drugs.

ACKNOWLEDGMENT

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REFERENCES


INTERACTION IN VITRO ENTRE LE DIAZEPAM OU L’OXAZEPAM ET LE PANCURONIUM OU LE SUXAMETHONIUM

RESUME

Des études in vitro ont été mises au point pour rechercher les effets du diazepam et de trois ses métabolites sur l’amplitude d’une secousse indirectement provoquée, grâce à une préparation hémidiaphragme-nerf phrénique de rat. Le diazepam, le demethyl Diazepam et le temazepam seuls produisaient une augmentation de l’amplitude de la secousse aux concentrations les plus basses, suivie d’une depression complète aux concentrations les plus élevées. L’oxazepam n’entraînait pas d’augmentation initiale de l’amplitude de la secousse mais provoquait d’emblée une depression dose-dépendante. Les courbes de concentration cumulative-réponse obtenues avec le pancuronium et le suxamethonium en présence de différentes concentrations de diazepam ou d’oxazepam montraient que de faibles concentrations de diazepam, incapables seules de modifier l’amplitude de la secousse, provoquaient un antagonisme de l’action du pancuronium, mais pas du suxamethonium. Avec l’oxazepam, on n’observait rien de tel. A des concentrations liminares ou supraliminares, le diazepam et l’oxazepam potentialisentaient tous les deux l’action du pancuronium et du suxamethonium. Les implications évolutives pour des interactions in vivo sont discutées.
INTERACCIÓN IN VITRO DEL DIAZEPAN Y DEL OXAZEPAN CON EL PANCURONIO Y EL SUXAMETONIO

SUMARIO
Se llevaron a cabo estudios in vitro al usar una preparación de hemidiafragma — nervio frénico de ratón con el objeto de investigar los efectos del diazepan y de tres de sus metabolitos sobre la tensión de contracción indirectamente evocada. El diazepan, el desmetildiazepan y el temazepan solos causaron un aumento de la tensión de contracción en concentraciones más bajas, seguido por una depresión completa en concentraciones más altas. El oxazepan no ocasionó un aumento inicial de la tensión de contracción, pero demostró una depresión inmediata en relación con la dosis. Las curvas cumulativas de respuesta — concentración del pancuronio y del suxametonio en presencia de distintas concentraciones de diazepan o de oxazepan demostraron que las pequeñas concentraciones de diazepan que no producían ningún cambio en la tensión de contracción cuando administrado sólo, provocó un antagonismo de la acción del pancuronio, pero no así del suxametonio. Con el oxazepan, no se observó dicho antagonismo. En concentraciones liminares y supraliminares, tanto el diazepan como el oxazepan potenciaron la acción del pancuronio y del suxametonio. Se discuten las posibles implicaciones para las interacciones in vitro.