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In-vitro Smooth Muscle Relaxant Activity of a Series of Vecuronium Analogues in the Rat Aorta

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Abstract—The ability of a series of 17-ester analogues of vecuronium to elicit a direct relaxant effect on vascular smooth muscle has been studied using rat isolated aortic rings contracted with 40 mM KCl. The IC50 for inhibition of KCl-induced contractions increased with increasing size of the 17-ester substituent, such that vecuronium (17β-acetate) was the least potent with an IC50 of around 500 μM and Org-9827 (17α-pivalate) was the most potent with an IC50 of around 5 μM. In addition, for the weaker-acting compounds, the 17α-esters were more potent than their corresponding 17β-esters, although this difference was lost as the size of the 17-ester substituent increased. From the results obtained here, it is concluded that the hypotensive activity of some of the newer neuromuscular blocking steroids seen in cats, pigs and dogs in-vivo is probably, at least in part, a consequence of a direct relaxant effect of the compound on vascular smooth muscle through inhibition of voltage-activated, L-type, calcium channels. This may have both advantageous and disadvantageous clinical consequences when using large doses of one of the newer vecuronium analogues with a low relative neuromuscular-blocking potency.

One of the complications of using tubocurarine clinically as a muscle relaxant is its hypotensive activity. Ganglion block may contribute to this effect, although in man it is mainly due to histamine release since the effect is prevented by pretreatment with the antihistamine drug, promethazine (Stoelting & Longenecker 1972; Moss et al 1981). Clinically-effective doses of the steroidal neuromuscular blocking agent, vecuronium bromide (Fig. 1), on the other hand, cause no reduction in blood pressure even at very high doses (Lienhart et al 1983; Morris et al 1983). However, several of the newer variants of vecuronium, particularly at high concentrations in-vivo, have been shown to reduce blood pressure in a variety of anaesthetized animal models. Thus, Org-9616 (the 17α-butyrate analogue of vecuronium) and Org-7617 (the 17β-butyrate, 16*N*-allyl analogue of vecuronium) both produced a marked fall in blood pressure with no accompanying change in heart rate at a concentration of three times their ED90 neuromuscular blocking dose in anaesthetized cats and pigs (Muir et al 1989). In the anesthetized dog, both Org-9616 and Org-9991, the 16-homopiperidino, 17β-butyrate analogue of vecuronium, at concentrations three times their ED90 concentration for neuromuscular block, have been shown to produce significant decreases in mean arterial blood pressure (Cason et al 1990). Org-9382 (the 17β-butyrate, 2-morpholino analogue of vecuronium) also reduces blood pressure in anaesthetized cats and pigs (R. J. Marshall, personal observations).

In the anaesthetized cat, Muir et al (1991) observed no ganglion block, as assessed by effects on contractions of the preganglionically-stimulated nictitating membrane, with Org-9991 at ten times its ED90 neuromuscular-blocking concentration. Also, Cason et al (1990) reported that the hypotension produced by Org-9616 and Org-9991 was accompanied by a decrease in systemic vascular resistance.

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Therefore, the possibility must be considered that the newer vecuronium analogues may exert a direct relaxant effect on vascular smooth muscle.

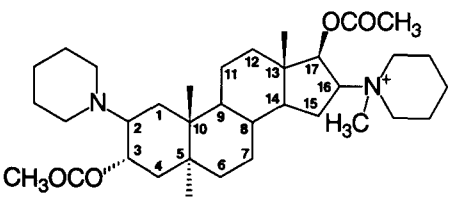

FIG. 1. Chemical structure of vecuronium bromide (1-((2β, 3α, 5α, 16β, 17β)-3,17-bis(acetyloxy)-2-(1-piperidinyl)-androstan-16-yl)-1-methyl-piperidinium bromide). The numbering indicates the standard carbon atom labelling of the androstane nucleus of the molecule.

One common feature of all the vecuronium analogues showing a hypotensive effect in anaesthetized animal models is the presence of a relatively bulky butyryl ester group at position 17. Hence, Org-9273 (the 2-morpholino, 3-hydroxy analogue of vecuronium) is, like vecuronium, a 17β-acetate and, again like vecuronium, it does not produce hypotension in anaesthetized patients even at 3–4 times its ED90 neuromuscular blocking concentration (van den Broek et al 1991). Therefore, in an attempt to identify the mechanism of the hypotensive effect of the newer vecuronium analogues, and to determine the structural requirements for the effect, we have examined the ability of a series of 17-ester analogues of vecuronium to inhibit smooth muscle contractile responses in the rat isolated aorta. Vecuronium analogues with 17-ester substituents ranging in size from a two-carbon acetate to a five-carbon pivalate have been studied. In addition to size, the importance of the orientation of the 17-ester substituent was also studied, both the 17α- and the 17β-ester being examined for each different substituent. Our results indicate that the compounds have a direct relaxant effect on vascular smooth

muscle, probably due to a block of the muscle membrane voltage-activated L-type calcium channels. We also confirm that their potency in this effect is related to the size and orientation of the 17-ester substituent.

Materials and Methods

Aortic ring preparation

An approximate 20-mm length of thoracic aorta was removed from male Sprague-Dawley rats, 150–250 g, that had been killed by excess anaesthesia with CO_2 followed by immediate exsanguination. Several 3-mm rings were cut from the vessel and each ring was suspended on wire supports in a 10-mL tissue bath under 1 g of resting tension. All tissues were bathed in Krebs–Henseleit solution (at 32°C) of the following composition (mM); NaCl 159, $NaHCO_3$ 30, KCl 5, $CaCl_2$ 2·5, $MgSO_4$ 1, KH_2PO_4 1, glucose 11 gassed with 95% O_2–5% CO_2 to a pH of 7·2–7·4. Atropine (0·1 μM) and propranolol (1·7 μM) were added to all solutions to eliminate any muscarinic and β-adrenoceptor activities, respectively. In the calcium re-admission studies, no $CaCl_2$ was added to the initial physiological solution. In addition, 1 mM EGTA was added to the bathing solution to buffer any residual calcium, present as an impurity of other salts, in the calcium-free solution. Isometric tension was measured using Grass FT03C force displacement transducers linked to a Grass model 79D polygraph recorder.

Inhibition of KCl-induced contractions

Aortic rings were contracted with 40 mM KCl, a concentration which was shown in initial experiments to produce about 75% maximum force in the tissue. A regular protocol was devised where aortic rings were exposed to 40 mM KCl for 15 min every 25 min. Once repeatable contractions to KCl had been established, a series of KCl-induced contractions was elicited in the presence of a range of concentrations of one of the compounds under examination. Each concentration of the test compound was added to the tissue bath 5 min after the wash-out of a dose of KCl, i.e. 5 min before the subsequent KCl exposure. At least five different concentrations of the test compound were used in each experiment, including a concentration having no effect on the KCl-induced contraction and one which completely abolished the contraction. Contractile force was measured at the end of each KCl exposure, just before wash-out, and all results were expressed as a percentage of the value seen following exposure to 40 mM KCl alone.

For each set of contractions, an IC50 value was obtained by using a nonlinear iterative curve-fitting procedure to find the best fit for the following equation:

$$\text{Response (as \% control maximum)} = 100/(1 + ([\text{antagonist}]/\text{IC50})^{P}) \quad (1)$$

In addition to providing an estimate of the IC50 of the compound, the fit also provides a value for the slope of the concentration-response relationship (P). For each compound under examination between two and seven individual estimates of the IC50 were made in different tissues and these were averaged to give the presented mean and standard error of the mean (s.e.m.) values.

A small number of experiments was also performed to determine the ability of some of the steroidal muscle relaxants to prevent contractions of aortic rings elicited by 1 μM phenylephrine instead of 40 mM KCl. For these phenylephrine experiments, all other experimental conditions were identical to those for the KCl experiments and IC50 values were calculated as described above.

Inhibition of calcium-dependent contractions

Cumulative concentration-effect curves to exogenously added calcium were obtained from aortic rings continually exposed to 40 mM KCl in the presence and absence of one of the test compounds. A minimum of four calcium concentrations, up to a maximum of 5 mM, was used to define each concentration-effect curve and for each set of contractions an ED50 value was obtained using a nonlinear iterative curve-fitting procedure to find the best fit for equation 1. The maximum contractile response was taken as that seen with 3 mM calcium in the absence of any test compound and this maximum was assumed to be the same for the calcium concentration-effect curve in the presence of the test compounds. Unfortunately, it was not always possible to reach the maximal contractile responses in the presence of high concentrations of the test compounds, since concentrations of $CaCl_2$ above 3 mM tended to depress contractions probably due to an osmotic effect on the tissue. Therefore, to avoid complications associated with incomplete concentration-effect curves, all the compounds were tested for their ability to inhibit calcium-induced contraction of the aorta only at their minimally effective concentration. It should be stressed that the assumption that there was no change in the maximal response in the presence of the test compound means that the calculated ED50 values have to be regarded as apparent values, since we have no evidence that the maximal response could genuinely be reached in the presence of any of the test compounds. For each compound under examination between three and eight individual estimates of the ED50 were made in different tissues and these were averaged to give the presented mean and s.e.m. values.

Drugs

All of the neuromuscular blocking steroids under investigation (Table 1) are simple analogues of vecuronium, the only structural change being the size and orientation of the 17-ester substituent, with the exception of Org-9382 which is the 2-morpholino derivative of Org-9453. All the

Table 1. Designation of 17-ester-substituted vecuronium analogues under investigation.

Name	17-Ester substituent
Vecuronium (Org-NC45)	β-Acetate
Org-9535	α-Acetate
Org-9489	β-Proprionate
Org-9784	α-Proprionate
Org-9453	β-Butyrate
Org-9616	α-Butyrate
Org-9643	β-Pivalate
Org-9827	α-Pivalate
Org-9382	β-Butyrate*

*Org-9382 is a 2-morpholino compound, all others are 2-piperidino.

vecuronium analogues were the generous gift of Organon Laboratories Ltd (Newhouse, Lanarkshire). Stock solutions of 10 mM of the neuromuscular-blocking steroids were made in 10 mM citric acid and these were diluted using physiological saline, on the day of use, to give the required concentrations. In addition, and for comparison, all experiments were also performed using the classical voltage-activated calcium-channel blocker, verapamil hydrochloride (Sigma Chemical Co., Poole).

Statistics

All data are presented as mean and s.e.m. of results from two to eight individual experiments. The small number of results obtained with some of the lower potency compounds was due to the extremely limited supply of these analogues. Where appropriate, statistical testing was performed using either a one-sample or an unpaired Student's t-test with statistical significance set at $P < 0.05$.

Results

Inhibition of KCl-induced contractions

All of the steroidal compounds studied produced a concentration-dependent inhibition of the contraction of the aortic rings elicited by 40 mM KCl. Averaged concentration-effect curves for the most and least potent steroidal compound, along with verapamil as a reference, are shown in Fig. 2. There were wide variations in potency for the steroidal compounds. IC50 values ranged from around 500 μM for vecuronium (17β-acetate) to around 5 μM for Org-9827 (17α-pivalate). A bar graph of the IC50 values for all eight 17-ester substituted vecuronium analogues studied is shown in Fig. 3. For both the 17α- and the 17β- ester substituted series of analogues of vecuronium the potency increased as the size of the 17-ester substituent increased. In addition, for each substituted moeity except the most potent pivalates, the 17α-enantiomer was significantly more potent than the 17β-enantiomer ($P < 0.05$, unpaired Student's t-test). As with overall potency, the enantiomer selectivity appeared to be dependent on the size of the 17-ester substituent, the greatest selectivity for the 17α-enantiomer being seen with the smallest 17-ester substituent. For all eight compounds, similar slope values for the concentration-effect curve were

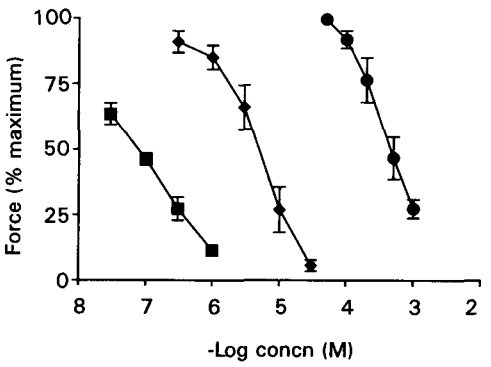

FIG. 2. Averaged concentration-effect curves showing the inhibition of KCl-induced contraction of aortic rings by verapamil (■), Org-9827 (◆, 17α-pivalate) and vecuronium (●, 17β-acetate). Each plotted point is the mean and s.e.m. of data obtained from between three and seven individual preparations.

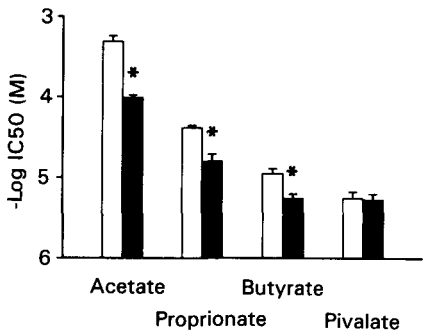

FIG. 3. Bar graph showing mean and s.e.m. IC50 values for inhibition of KCl-induced contractions of aortic rings for the eight 17-ester vecuronium analogues. Data are shown for four 17β-esters (□) and their corresponding 17α-esters (■). Data are the average of between two and seven individually obtained results for each compound. *$P < 0.05$ compared with the corresponding 17β-ester.

calculated suggesting that all compounds inhibited the KCl-induced contractions by a similar mechanism of action.

One common modification of the structure of steroidal neuromuscular blocking drugs, such as in the clinically evaluated compounds, Org-9273 and Org-9426 (rocuronium; the 2-morpholino, 3-hydroxy, 16*N*-allyl-pyrrolidino derivative of vecuronium), is the replacement of the 2-piperidino moeity with a 2-morpholino moeity. With respect to the effects of the compounds on KCl-induced contractions of the rat aorta, this substitution had no effect on potency. Hence, Org-9382 (2-morpholino, 17β-butyrate analogue of vecuronium) had an IC50 of 9·3 ± 1·8 μM (n = 4) which was not significantly different from the value of 12·2 ± 1·8 μM (n = 4, $P > 0.5$, unpaired Student's t-test) seen for Org-9453 (2-piperidino, 17β-butyrate analogue of vecuronium).

Antagonism of calcium concentration-effect relationship

To test whether the effect of the steroidal compounds on KCl-induced contractions of the aorta was indeed due to an inhibition of muscle-membrane voltage-activated calcium channels, concentration-effect curves to calcium were performed on aortic rings continuously exposed to 40 mM KCl in the presence and absence of one of verapamil (3 nM), vecuronium (25 μM), Org-9616 (0·25, 0·5 or 1 μM), Org-9453 (1 μM) or Org-9643 (0·5 μM). In the absence of any test compound, calcium ions produced a concentration-dependent contraction of the aortic ring which was maximal at around 5 mM. The average ED50 calcium concentration for all experiments was 481 ± 43 μM (n = 31). All the test compounds, at the concentrations studied, shifted the calcium ED50 values (Table 2). As examples, averaged concentration-effect curves for the most (verapamil, 3 nM) and least (vecuronium, 25 μM) potent compounds studied are shown in Fig. 4. The concentrations of test compounds used were chosen to produce a rightward shift in the calcium concentration-effect curve of approximately threefold. Even with this relatively small dose-ratio, at the maximum calcium ion concentration studied (5 mM) only about 75% of the maximal control response was observed in the presence of the test compounds.

There was a good correlation between the concentration of the compound required to produce an approximate threefold rightward shift in the calcium concentration-

Table 2. Calcium ED50 values for contractions of aortic rings exposed to 40 mM KCl in the presence and absence of verapamil and four neuromuscular blocking steroids.

Compound	Concn (μM)	n	Control ED50 (μM)	Test ED50 (μM)	Ratio
Verapamil	0·003	3	0·22 ± 0·06	0·48 ± 0·14	2·20 ± 0·21*
Vecuronium	25	8	0·42 ± 0·06	1·50 ± 0·49	3·55 ± 0·74*
Org-9453	1	5	0·56 ± 0·09	1·24 ± 0·29	2·11 ± 0·44*
Org-9643	0·5	4	0·77 ± 0·19	1·71 ± 0·39	2·47 ± 0·68*
Org-9616	1	4	0·42 ± 0·03	1·83 ± 0·47	4·33 ± 0·98*

*$P < 0·05$ compared with 1·0.

effect curve (Table 2) and the IC50 values determined from the concentration-dependent inhibition on KCl-induced contractions (Fig. 2) for the five compounds for which both measures were obtained. This supports the suggestion that the IC50 for inhibition of KCl-induced contractions of the tissue is a measure of the activity of the compounds at the muscle-membrane voltage-activated calcium channels.

Inhibition of phenylephrine-induced contractions

Five compounds were tested for their ability to inhibit phenylephrine-induced contractions of the isolated aortic rings: three of the simple 17-ester vecuronium analogues (Org-9784, Org-9453 and Org-9643), rocuronium (Org-9426) and verapamil. All five compounds inhibited the contractions and average IC50 values are shown in Table 3. The IC50 value for verapamil was similar to the value obtained for this compound against KCl-induced contractions. However, all three of the 17-ester vecuronium analogues were more potent against phenylephrine than against KCl. The increase in potency was variable ranging from approximately threefold for Org-9784 and Org-9453 to approximately ninefold for Org-9643. Org-9426, which was not tested against KCl-induced contraction, was potent against phenylephrine-induced contractions despite its lack of an in-vivo hypotensive effect (Guill et al 1991).

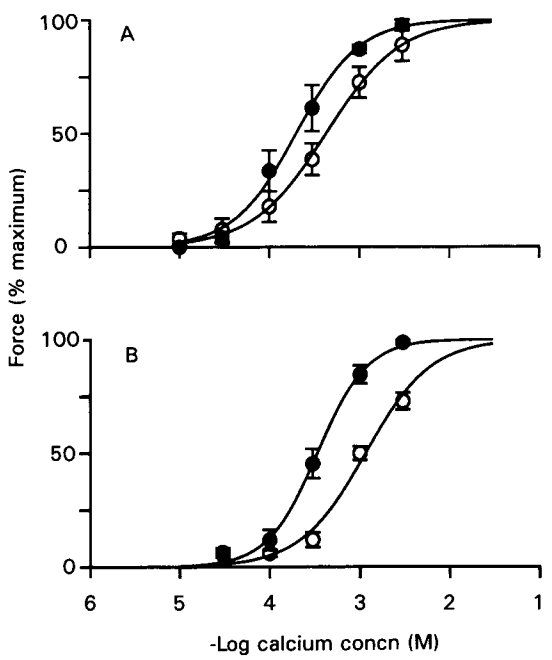

FIG. 4. Representative averaged calcium concentration-effect curves measured in the absence (●) and presence (○) of 3 nM verapamil (A) and 25 μM vecuronium (B). Each plotted point is the mean and s.e.m. of three (verapamil experiments) or eight (vecuronium experiments) individually obtained results. Full lines are the best-fit concentration-effect curves using the plotted average data.

Discussion

Structure-activity relationship for calcium-channel block

We confirm the earlier suggestion (Cason et al 1990) that certain analogues of vecuronium have a direct vascular smooth muscle relaxant activity. This appears to be a consequence of an inhibition of voltage-activated verapamil-sensitive (presumably L-type) calcium channels. Further, we have elucidated one of the structural requirements for this effect of the compounds. For smaller 17-ester substituents, both the size and orientation of the substituent is critical in determining calcium-channel activity. However, these factors seem less critical for the larger 17-ester substituents. The five-carbon pivalate may represent an upper limit to the allowable size of the 17-ester substituent. Increasing this substituent further, such as in a 17-cyclohexanoate, may in fact decrease the binding of the compound to the calcium channel, i.e. result in a decrease in vascular smooth-muscle-relaxant potency. Due to the non-availability of vecuronium analogues with 17-ester substituents larger than the pivalates, we were not able to test this possibility.

Hypotensive activity of muscle relaxants in-vivo

There is only a fivefold difference in neuromuscular-blocking potency between the most (vecuronium) and least (Org-9453) active of the compounds studied here, as assessed in the anaesthetized cat (R. J. Marshall, personal communication). This is considerably less than the 100-fold potency range we have determined for vascular smooth-muscle-relaxant activity. This suggests that the principal factor governing the appearance of hypotension at equi-effective neuromuscular-blocking concentrations is the potency of

Table 3. IC50 values for the inhibition of calcium-dependent contractions of aortic rings exposed to 1 μM phenylephrine.

Compound	n	IC50 (μM)
Verapamil	4	0·0458 ± 0·0067
Org-9784	4	6·1 ± 1·8
Org-9453	4	4·2 ± 2·0
Org-9643	7	0·60 ± 0·28
Org-9426	5	1·13 ± 0·13

the compound at calcium channels rather than its potency at the neuromuscular junction. Thus, one would expect to associate the greatest hypotensive activity with the analogues with the largest 17α-ester substituents. Indeed, the high vascular smooth-muscle-relaxant potency for Org-9616 and Org-9382 agrees well with the observations that these compounds produce decreases in blood pressure in the anaesthetized cat.

A significant degree of hypotension associated with a clinically-used muscle relaxant could be regarded as both advantageous and disadvantageous. Anaesthetists and surgeons frequently favour a degree of hypotension during surgery since it is thought to minimize bleeding from cut skin and fat. Also, it has been suggested that the increased blood flow to skeletal muscle associated with the lowering of blood pressure could markedly influence the time course of muscle relaxation, considerably speeding up the onset of neuromuscular block (Wierda et al 1993). A disadvantage of a hypotensive action of muscle relaxants is the risk of an uncontrolled hypotensive crisis, particularly if the anaesthetic also produces hypotension.

The similarity of the IC50 values for verapamil against phenylephrine- and KCl-induced contractions suggest that external calcium entering through voltage-operated calcium channels plays an important part in the contractions elicited by α-adrenoceptor agonists. However, the increased potency of the neuromuscular steroids against phenylephrine-induced contractions compared with those produced by KCl suggests that these compounds may have some slight degree of α-adrenoceptor-blocking activity. As yet, no systematic study has been made of such a putative action of these compounds and the consequences of such an action on the in-vivo hypotensive and muscle relaxant effects of the neuromuscular blocking steroids are unknown.

Neuromuscular consequences of calcium-channel block

With any muscle relaxant exhibiting calcium-channel-blocking activity there exists the possibility of a synergistic interaction between the calcium-channel blocking and acetylcholine-receptor blocking properties of the compounds at the neuromuscular junction. As with hypotension, this synergism could have either desirable or deleterious consequences. Wierda et al (1993) argued that any potentiation of neuromuscular block through calcium-channel block would be advantageous if it decreased the onset-time of neuromuscular block without severely compromising the duration of block or its reversibility. However, such a synergism would be deleterious if it resulted in a neuromuscular block which was either markedly prolonged or not easily reversed by classical pharmacological means.

It has been shown both in-vivo (Durant et al 1984) and in-vitro (Bikhazi et al 1982; Kraynack et al 1983) that verapamil potentiates the neuromuscular-blocking effects of muscle relaxants such as pancuronium. Also, Jones et al (1985) observed marked residual neuromuscular block which was resistant to neostigmine treatment following pancuronium administration to a patient on long-term therapy with verapamil. The ability of verapamil to potentiate neuromuscular block may reflect an action of the compound at a site other than at calcium channels, such as a direct effect on the postjunctional nicotinic acetylcholine receptor/ion channel complex (Chang et al 1990), and, as such, would not necessarily be a common feature of all compounds that inhibit L-type calcium channels. Alternatively, it could be a consequence of an action at verapamil-sensitive calcium channels either prejunctionally on the nerve terminal membrane or postjunctionally on the muscle-fibre membrane. Verapamil-sensitive calcium channels exist on mouse motor nerve terminals (Penner & Dreyer 1986; Anderson & Harvey 1987). Unfortunately, a precise role for these calcium channels is at present unknown. They are clearly distinct from the ω-conotoxin-sensitive voltage-activated calcium channel associated with the normal process of evoked transmitter release. However, nerve terminal verapamil-sensitive calcium channels may possibly have a regulatory role in neuromuscular transmission. Consequently, any secondary action of an acetylcholine-receptor antagonist to block these channels might conceivably have an additive effect on its neuromuscular-blocking activity. Verapamil-sensitive calcium channels also exist on the skeletal muscle membrane (Glossmann et al 1983). As with the nerve-terminal, verapamil-sensitive calcium channels, a precise role for these muscle membrane calcium channels is unknown. Certainly, they are not directly involved in excitation-contraction coupling since the activation of skeletal muscle does not require external calcium (Armstrong et al 1972; Luttgau & Spiecker 1979). However, based on the combined effects of verapamil and vecuronium on nerve-evoked and acetylcholine-induced contractions of the cat tibialis muscle in-vivo, Anderson & Marshall (1985) concluded that the muscle-membrane, verapamil-sensitive calcium channels do play an important role in the synergistic effects of verapamil on the neuromuscular block produced by vecuronium. The usefulness of a secondary action of a muscle relaxant at either the nerve terminal or muscle-membrane verapamil-sensitive calcium channels is hard to predict and would depend largely on the nature of any ensuing synergism as described above.

Finally, it is possible that in addition to their activity on verapamil-sensitive calcium channels, the vecuronium analogues studied here might, unlike verapamil, also block the motor nerve-terminal, ω-conotoxin-sensitive, voltage-activated calcium channels involved in mediating evoked acetylcholine release. This would produce a marked reduction in the amount of acetylcholine released upon nerve stimulation, much like that seen with magnesium ions (for review see Silinsky 1985). As yet, an analysis of acetylcholine release has not been performed with any of the compounds studied here so we are unable, at present, to say whether their neuromuscular block contains any appreciable presynaptic component due to a block of the nerve-terminal calcium channels associated with evoked acetylcholine release.

Conclusion

The hypotensive effects of muscle relaxants has traditionally been associated with ganglion block and histamine release (Bowman 1990). However, the ability of certain analogues of the steroidal muscle relaxant vecuronium to directly elicit relaxation of KCl-contracted isolated aortic rings of the rat points to the potential for some muscle relaxants to produce hypotension, in-vivo, by a direct action on vascular smooth muscle to inhibit calcium entry through L-type calcium

channels. The advantageous and disadvantageous consequence of such an action should be carefully considered in the search for new and better muscle relaxants.

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