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Isobolographic analysis of non-depolarising muscle relaxant interactions at their receptor site

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Abstract

Administration of certain combinations of non-depolarising muscle relaxants produces greater than expected neuromuscular blockade. Synergistic effects may be explained by drug interactions with the postsynaptic muscle nicotinic acetylcholine receptor. To investigate this hypothesis, the adult mouse muscle nicotinic acetylcholine receptor ($\alpha_2\beta\delta\varepsilon$) was heterologously expressed in *Xenopus laevis* oocytes and activated by the application of acetylcholine (10 μM). The effects of five individually applied muscle relaxants and six combinations of structurally similar and dissimilar compounds were studied. Drug combinations containing equipotent concentrations of two agents were tested and dose–response curves were determined. All compounds tested alone and in combination produced rapid and readily reversible, concentration-dependent inhibition. Isobolographic and fractional analyses indicated additive interactions for all six tested combinations. These findings suggest that synergistic neuromuscular blocking effects, observed for the administration of certain combinations of muscle relaxants, do not result from purely postsynaptic binding events at the muscle nicotinic acetylcholine receptor, but rather from differential actions on pre- and postsynaptic sites.

Keywords: Acetylcholine receptor; Muscle relaxant; (Antagonist); Interaction; Isobologram; Potency; *Xenopus* oocyte

1. Introduction

Non-depolarising muscle relaxants block neuromuscular transmission by binding competitively to the muscle nicotinic acetylcholine receptor in the neuromuscular junction. When two non-depolarising muscle relaxants are given simultaneously, greater than additive, i.e. synergistic, neuromuscular blockade has been observed for some drug combinations, both in clinical and in vitro studies (Lebowitz et al., 1981; Waud and Waud, 1985). This synergy appears pronounced when the two combined muscle relaxants are members of different structural classes (Jalkanen et al., 1994; Rautoma et al., 1995a; Erkola et al., 1996), as for example, shown for the combination of the aminosteroid pancuronium with the monoquaternary benzylisoquinolinium d-tubocurarine (Pollard and Jones, 1983). However, a recent clinical study also described a synergistic interaction between two benzylisoquinolinium compounds, cis-atracurium and mivacurium (Kim et al., 1998).

The underlying mechanism(s) by which synergistic interactions occur is not known and could involve both pre- and postsynaptic actions (Lebowitz et al., 1980). However, other authors have considered synergy to arise entirely from postsynaptic actions (Waud and Waud, 1984; Jalkanen et al., 1994). It is well known that the nicotinic acetylcholine receptor is a ligand-gated ion channel that is composed of four homologous subunits associated pseudosymmetrically in a pentameric structure to create a central ion-conducting pathway (Changeux et al., 1990; Unwin, 1993). The adult or junctional form of the nicotinic acetylcholine receptor is composed of α, β, δ, and ε subunits (Mishina et al., 1986). Two extracellular binding sites, with different affinities for agonists and antagonists, are formed at the α–ε and α–δ subunit interfaces (Pedersen and Cohen, 1990; Sine and Claudio, 1991). To open the channel pore, both binding sites need to be occupied by an agonist molecule (acetylcholine).

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Therefore, one hypothesis to support a purely postsynaptic mechanism for synergy is that two muscle relaxants applied in combination influence each others affinity for the two binding sites within the postsynaptic receptor (Waud and Waud, 1985). If the acetylcholine receptor possesses a high affinity binding site and a low affinity binding site for antagonists A and B, synergistic effects could occur from the binding of antagonist A to the high affinity site, which produces a conformational change in the low affinity binding site for antagonist B increasing affinity for antagonist B. This mechanism of cooperative binding of antagonists would be similar to the well established effect for the agonist acetylcholine.

Previous work with in vitro nerve–muscle preparations has been able to exclude the influence of drug uptake and distribution, which might impact on observations in clinical studies, to explain synergy. However, the neuromuscular junction is left intact, which makes a precise discrimination between pre- and postsynaptic effects impossible. Therefore, the goal of the present study was to determine the interactions of various combinations of two muscle relaxants on the pure postsynaptic receptor, using a postsynaptic receptor model in which the adult muscle nicotinic acetylcholine receptor was heterologously expressed in *Xenopus laevis* oocytes. Our aim was to study the role of combinations of non-depolarising muscle relaxants with similar and dissimilar molecular structures in producing synergistic effects.

2. Materials and methods

2.1. Harvesting and microinjection of Xenopus oocytes

All experimental procedures involving the South African clawed frog (*Xenopus laevis*) were approved by the Committee on Animal Research of the University of California, San Francisco, and are similar to those previously described (Kindler et al., 2000). Briefly, unfertilised oocytes were removed from anaesthetised adult frogs (Nasco, Fort Atkinson, WI) and washed twice in a Ca^{2+}-free, high Mg^{2+}-containing Ringer's solution (composition in mM: 82 NaCl, 2 KCl, 5 HEPES, 20 $MgCl_2$, pH 7.4), followed by collagenase (type A, Boehringer Mannheim, Indianapolis, IN) treatment (2 mg/ml) for 1–2 h with constant agitation to remove the follicular cell layer. Oocytes were washed again and transferred into modified Barth's solution with HEPES (MBSH, composition in mM: 88 NaCl, 1 KCl, 10 HEPES, 7 $NaHCO_3$, 1 $CaCl_2$, 1 $Ca(NO_3)_2$, pH 7.0).

Within 16 h after removal, mature oocytes (stages V and VI) were injected with diluted mixtures of complementary RNA (cRNA) for the subunits of the adult nicotinic acetylcholine receptor ($\alpha\beta\delta\varepsilon$), using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). Aliquots of each cRNA, synthesised from plasmids encoding the α, β, and δ subunits, were diluted 1:1000 and the ε-subunit 1:20 in ribonuclease-free water and mixed in the ratio of 2:1:1:1.

Expression plasmids pSPα1, pGEMβ, and pSPδ encoding complementary DNA coding sequences for mouse muscle nicotinic acetylcholine receptor subunits α, β, δ, respectively, were provided by Drs. John Forsayeth and Zach Hall (Department of Physiology, University of California, San Francisco, CA), and expression plasmid pSPε by Dr. Paul Gardner (Department of Biochemistry, Dartmouth Medical School, New Hampshire, MA). These plasmids contain an SP6 promoter 5′ to the translation start codon that allows in vitro synthesis of RNA that directs the translation of each subunit. After cytoplasmic injections of cRNA, oocytes were cultured for 3 to 5 days at 18 °C in modified Barth's solution to which 50 mg/ml gentamycin, 2.5 mM sodium pyruvate, 5% heat-inactivated horse serum, and 5 mM theophylline were added.

2.2. Electrophysiological recording and compounds

Electrophysiological experiments were performed at room temperature (20–22 °C). A single defolliculated oocyte was placed in a continuous-flow recording chamber (25 μl volume) and superfused with 3–5 ml/min MBSH containing 0.5 μM atropine sulfate. The oocyte was impaled with two glass electrodes filled with 3 M KCl (0.4–2.5 MΩ) and voltage clamped with the holding potential set at −60 mV (Axoclamp 2A; Axon Instruments, Foster City, CA). Signals were filtered using an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) set at a 40-Hz cutoff before sampling at 100 Hz. Resulting signals were digitised and stored on a Power Macintosh 7100 (Apple Computer, Cupertino, CA) using data acquisition software (MacLab; ADInstruments, Milford, MA).

Acetylcholine, atropine and gallamine, a trisquaternary phenolic ether, were purchased from Sigma (St. Louis, MO). Other muscle relaxants were obtained in preparations for clinical use: pancuronium, a bisquaternary steroidal compound, (Elkins-Sinn, Cherry Hill, NJ), vecuronium, the 2-desmethyl monoquaternary analogue of pancuronium, (Baxter Healthcare, Deefield, IL), mivacurium, a benzylisoquinolinium diester, and d-tubocurarine, a monoquaternary benzylisoquinolinium compound (Abbott Laboratories, Chicago, IL). All drugs were dissolved in MBSH. Solutions and their dilutions to the experimental concentrations were prepared immediately before the experiments.

2.3. Experimental design

Test solutions containing either 10 μM acetylcholine alone or in combination with various concentrations of one muscle relaxant were typically applied for 20 s and the peak current was determined. The control response to acetylcholine alone was repeated after each application of antagonist and the mean value of these two acetylcholine applications was taken as the "average control current", to which the

antagonist response was compared (percent inhibition of average control current), using the following equation:

$$\%\ \text{inhibition} = 100 \times [1 - (\text{current in presence of antagonist} / \text{average control current})].$$

After each drug application, a washout period of at least 60 s was applied to minimise the amount of desensitisation during the course of the experiment. For each data point, measurements from 5 to 8 oocytes were used, and for each experiment, oocytes from at least two different batches were used. Concentration–response relationships for muscle relaxant inhibited membrane currents were fit to the four parameter logistic equation to obtain estimates of the Hill coefficients and IC_{50} values.

To test the hypothesis that synergistic effects of two muscle relaxants may be a purely postsynaptic receptor effect, we co-applied combinations of two muscle relaxants. According to the procedure of Tallarida et al. (1989), drugs were combined in a fixed equipotent ratio, based on their IC_{50} values determined during the first part of the study. This mixture was then diluted and a concentration–response curve was determined. We first tested two combinations of two muscle relaxants of the same structural category, pancuronium/vecuronium with steroidal structures and mivacurium/d-tubocurarine with a benzylisoquinolinium structure. Then two combinations of a steroidal with a benzylisoquinolinium compound were tested, pancuronium/d-tubocurarine and vecuronium/mivacurium. Finally, combinations of gallamine with either pancuronium or d-tubocurarine were studied.

2.4. Data analysis

The concentration–response relations for each muscle relaxant were fitted by non-linear regression analysis using GraphPad Prism 3.0, a software for Macintosh (GraphPad Software, San Diego, CA) from which the inhibitor concentrations for half-maximal response (IC_{50}) and Hill slopes were determined. Individual drug potencies (IC_{50} value) were tested for significant differences by one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered significant. Unless otherwise specified, results are expressed as means ± standard deviation (S.D.) or 95% confidence intervals (CI).

Isobolographic analysis was used to define the interaction between muscle relaxants (Berenbaum, 1989; Tallarida et al., 1989). This method characterises the effect resulting from the administration of two compounds, mixed at their equieffective concentration ratios. For example, the IC_{50} for gallamine divided by the IC_{50} for pancuronium, both determined in the first part of this study, was 203; thus, for this combination, a "new drug" containing gallamine and pancuronium mixed in the ratio of 203:1 was generated. A concentration–response curve was established testing dilutions of each mixture. The IC_{50} (and 95% CI) for each drug mixture was estimated by fitting the concentration–response curve to the data using the Prism software package, and it was then used to calculate the experimentally determined IC_{50} for each drug of the combination, based on its ratio in the mixture.

Isobolograms were constructed by plotting single drug IC_{50} values on the *x*-and *y*-axes. A straight line connecting these single drug IC_{50} points constitutes the "theoretical additivity line" or isobole (*isos*, equal, *bole*, effect) and defines an infinite number of drug combinations that are to be predicted to produce 50% inhibition. If the experimentally derived IC_{50} of a combination falls on the theoretical additivity line, the effect of the drug mixture is additive. Points below the 95% confidence interval of the isobolar plot suggest a synergistic interaction, whereas points above the 95% confidence interval line suggest a subadditive or antagonistic interaction. Confidence intervals for each point were calculated from the variances of each component alone.

Fractional analysis was also used to evaluate muscle relaxant interactions. The fractional value describes the actual IC_{50} value as a fraction of the additive IC_{50}. The analysis is made by calculating the component effect of each agent in combination as the fraction of the dose that produces the same effect when given separately. The sum of fractional doses, as expressed by the following equation, indicates the type of interaction:

$$cA/(IC_{50})_A + cB/(IC_{50})_B$$

where $(IC_{50})_A$ and $(IC_{50})_B$ = the IC_{50} concentrations of drugs A and B, respectively, given alone, and cA and cB = the concentrations of drugs A and B, respectively, that when combined are equipotent with $(IC_{50})_A$ or $(IC_{50})_B$.

Values near 1 indicate additive interaction, values greater than 1 imply an antagonistic effect and values less than 1 indicate a synergistic interaction.

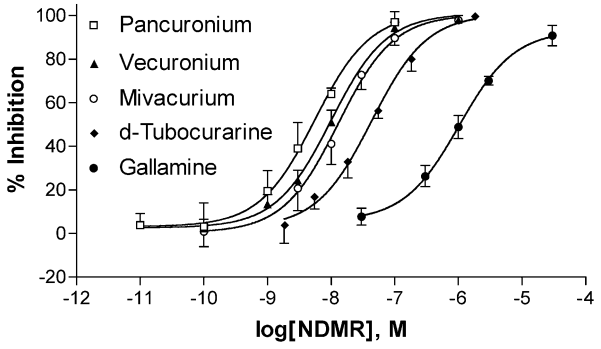

Fig. 1. Concentration–response effects of pancuronium, vecuronium, mivacurium, d-tubocurarine and gallamine for inhibition of acetylcholine-induced (10 μM) currents in oocytes expressing adult-type nicotinic acetylcholine receptors. Data points are mean ± S.D. (error bars) of 5–8 oocytes. Error bars not visible are smaller than symbols. Continuous lines through the data points are the best fits obtained with the Hill equation.

Table 1
Equi-effective concentrations (IC_{50}) for pancuronium, vecuronium, mivacurium, d-tubocurarine, and gallamine administered alone and in combination in a fixed-concentration ratio

Group	Pancuronium		Vecuronium		Mivacurium		d-Tubocurarine		Gallamine		Sum of IC_{50} fractions
	Fraction of IC_{50}	Concentration (nM)	Fraction of IC_{50}	Concentration (nM)	Fraction of IC_{50}	Concentration (nM)	Fraction of IC_{50}	Concentration (nM)	Fraction of IC_{50}	Concentration (nM)	
Single-drug study											
Pancuronium	1.00	5.5 (4.2–6.8)	–	–	–	–	–	–	–	–	1.00
Vecuronium	–	–	1.00	9.9 (8.4–11.4)	–	–	–	–	–	–	1.00
Mivacurium	–	–	–	–	1.00	12.9 (9.9–15.9)	–	–	–	–	1.00
d-Tubocurarine	–	–	–	–	–	–	1.00	43.4 (33.6–56.2)	–	–	1.00
Gallamine	–	–	–	–	–	–	–	–	1.00	995 (846.1–1144)	1.00
Interaction studies											
Pancuronium + Vecuronium	0.55	3.0 (2.5–3.6)	0.55	5.5 (4.5–6.5)	–	–	–	–	–	–	1.1
Mivacurium + d-Tubocurarine	–	–	–	–	0.38	5.0 (3.7–6.3)	0.38	16.7 (12.3–21.1)	–	–	0.76
Pancuronium + d-Tubocurarine	0.53	2.9 (2.5–3.3)	–	–	–	–	0.53	23.1 (20.1–26.0)	–	–	1.06
Vecuronium + Mivacurium	–	–	0.52	5.2 (4.2–6.2)	0.52	6.8 (5.5–8.1)	–	–	–	–	1.02
d-Tubocurarine + Gallamine	–	–	–	–	–	–	0.48	20.8 (17.3–24.4)	0.48	476.8 (395.1–558.4)	0.96
Pancuronium + Gallamine	0.64	3.5 (2.9–4.2)	–	–	–	–	–	–	0.64	641 (522.9–759.2)	1.28

Values are means (95% CI) of 5–8 oocytes.

3. Results

3.1. Potencies of individually applied non-depolarising muscle relaxants

Oocytes injected with cRNA coding for subunits of the junctional nicotinic acetylcholine receptor responded to the application of various concentrations of acetylcholine under two-electrode voltage clamping with inward currents described previously for this model (Kindler et al., 2000). Pancuronium, vecuronium, mivacurium, d-tubocurarine, and gallamine reversibly inhibited the inward currents elicited by co-application of 10 μM acetylcholine in a concentration-dependent fashion (Fig. 1). The concentrations producing a 50% inhibition (IC_{50}) of acetylcholine-induced currents were 5.5 nM (nHill = 0.95), 9.9 nM (1.06), 12.9 nM (1.07), 43.4 nM (0.78), and 995 nM (0.91) for pancuronium, vecuronium, mivacurium, d-tubocurarine, and gallamine, respectively (Table 1). The rank order of potency according to the derived IC_{50} values was pancuronium > vecuronium ~ mivacurium > d-tubocurarine > gallamine.

3.2. Interactions of combined drug applications

As with muscle relaxants applied individually, all tested muscle relaxant combinations also produced readily reversible, concentration-dependent inhibition of currents elicited by the co-application of acetylcholine. An example of the raw data obtained for one drug combination (pancuronium/d-tubocurarine) is shown in Fig. 2.

3.2.1. Interactions of muscle relaxant combinations from the same structural class

Fig. 3A shows the isobolographic analysis of the steroidal compound combination pancuronium/vecuronium. The experimentally determined IC_{50} values for the combination were 3.0 nM (2.5–3.6, 95% confidence intervals) for pancuronium and 5.5 nM (4.5–6.5) for vecuronium. The theoretical additive IC_{50} values were calculated as 2.8 nM (2.1–3.4) for pancuronium and 5.0 nM (4.2–5.7) for vecuronium, which overlap closely with the experimentally derived IC_{50}, indicating an additive interaction.

For the mivacurium/d-tubocurarine combination (Fig. 3B), the experimentally determined IC_{50} values for the combination were 5.0 nM (3.7–6.3) for mivacurium and 16.7 nM (12.3–21.1) for d-tubocurarine. The theoretical additive IC_{50} was calculated as 6.5 nM (5.0–8.0) for mivacurium and 21.7 nM (16.8–26.6) for d-tubocurarine. Therefore, the experimental IC_{50} values for the individual drugs in combination fell at the synergistic edge of the 95% confidence interval.

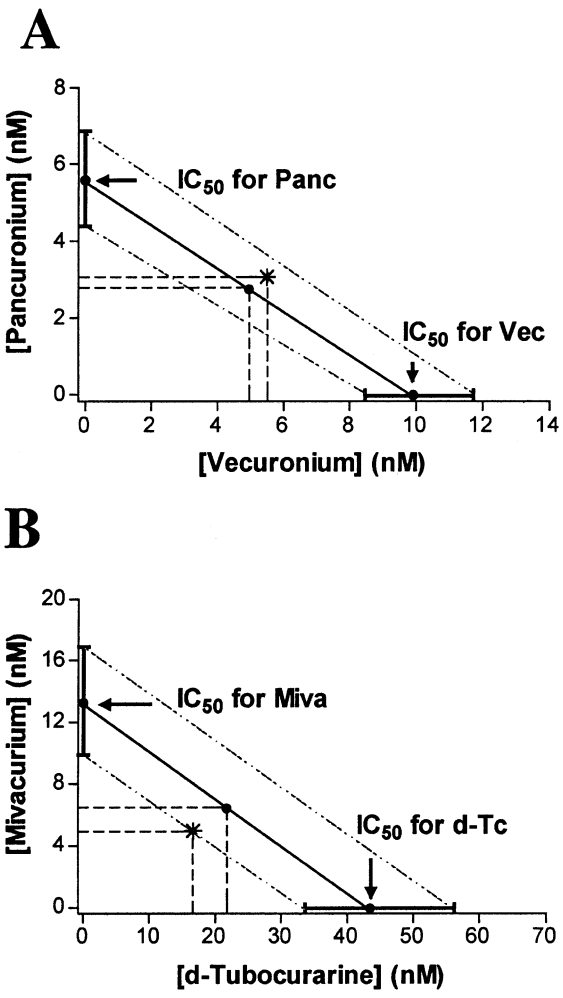

Fig. 3. IC_{50} isobolograms for the interaction of combinations of either two steroidal non-depolarising muscle relaxants (pancuronium [Panc] and vecuronium [Vec], panel (A)) or two benzylisoquinolinium compounds (mivacurium [Miva] and d-tubocurarine [d-Tc], panel (B)). The solid line connecting the single drug IC_{50} concentrations of the single drug IC_{50} points is the theoretical additive line; points on this line are the theoretical additive points (dashed lines = 95% CI). The theoretical IC_{50} is indicated by the filled circle (●) and the experimentally derived IC_{50} concentration is indicated by the asterisk (*).

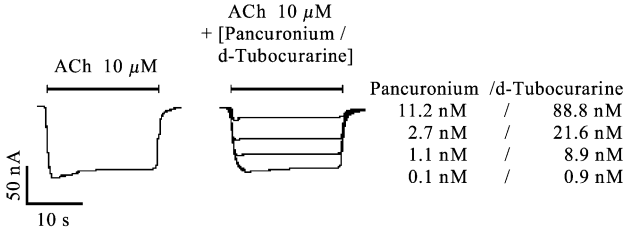

Fig. 2. Concentration-dependent effects of a pancuronium/d-tubocurarine combination on the adult-type nicotinic acetylcholine receptors (ε-nAChR) expressed in *Xenopus* oocytes. Tracings represent raw currents observed during the application of acetylcholine (10 μM) for 20 s, either alone (as control) or in combination with various concentrations of pancuronium/d-tubocurarine as indicated. Current tracings for each application of the drug combination from a single oocyte are superimposed.

3.2.2. Interactions of muscle relaxant combinations from different structural classes

Both combinations of a steroidal compound with a benzylisoquinolinium compound (pancuronium/d-tubocurarine and vecuronium/mivacurium) showed additive interactions. For the pancuronium/d-tubocurarine interaction (Fig. 4A), the experimentally determined IC_{50} values for the combination was 2.9 nM (2.5–3.3) for pancuronium and 23.1 nM (20.1–26.0) for d-tubocurarine. The theoretical additive IC_{50} was calculated as 2.8 nM (2.1–3.4) for pancuronium and 21.7 nM (16.8–26.6) for d-tubocurarine. The confidence intervals of these points overlapped with the experimentally derived values. Likewise, the experimentally determined IC_{50} values for the vecuronium/mivacurium combination (Fig. 4B) were 5.2 nM (4.2–6.2) for vecuronium and 6.8 nM (5.5–81) for mivacurium. The theoretical additive IC_{50} was calculated as 5.0 nM (4.2–5.7) for vecuronium and 6.5 nM (5.0–8.0) for mivacurium. The confidence intervals of these points also overlapped with the experimentally determined values.

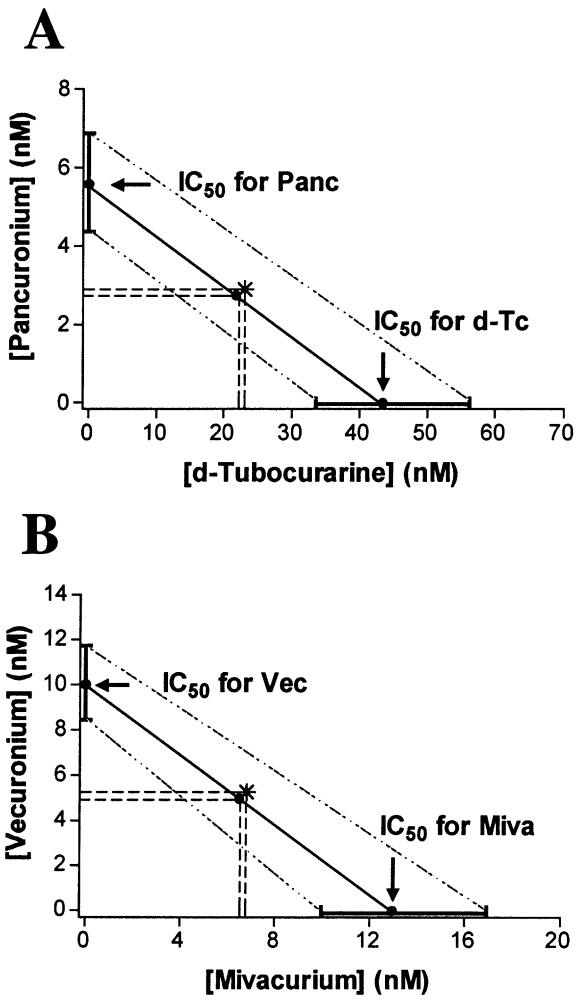

Fig. 4. IC_{50} isobolograms for the interaction of two combinations of a steroidal non-depolarising muscle relaxant with a benzylisoquinolinium compound (vecuronium [Vec] and mivacurium [Miva], panel (A); pancuronium [Panc] and d-tubocurarine [d-Tc], panel (B)). The solid line connecting the single drug IC_{50} concentrations of the single drug IC_{50} points is the theoretical additive line; points on this line are the theoretical additive points (dashed lines = 95% CI). The theoretical IC_{50} is indicated by the filled circle (●) and the experimentally derived IC_{50} concentration is indicated by the asterisk (*).

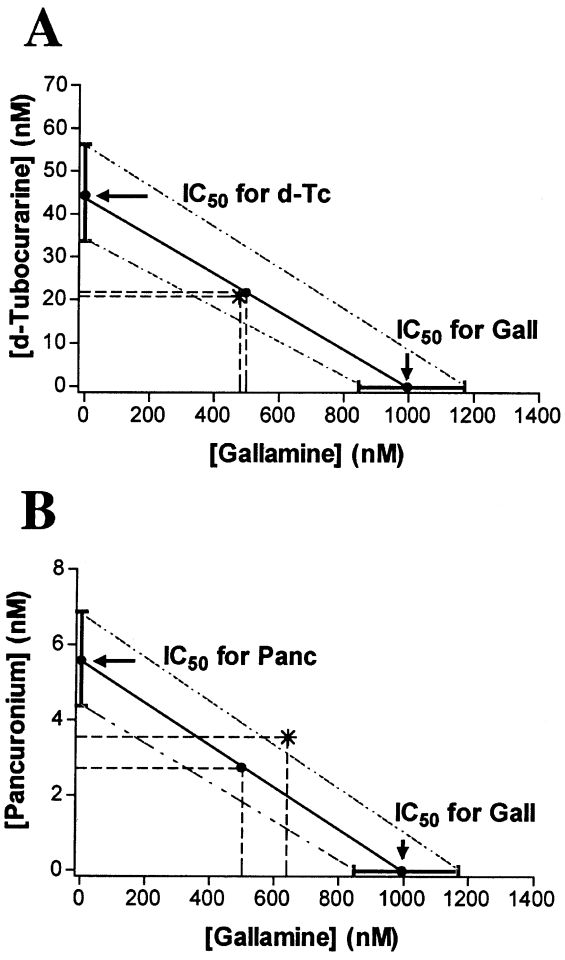

Fig. 5. IC_{50} isobolograms for the interaction of d-tubocurarine [d-Tc] and gallamine [Gall] (panel A) and pancuronium [Panc] and gallamine [Gall] (panel B). The solid line connecting the single drug IC_{50} concentrations of the single drug IC_{50} points is the theoretical additive line; points on this line are the theoretical additive points (dashed lines = 95% CI). The theoretical IC_{50} is indicated by the filled circle (●) and the experimentally derived IC_{50} concentration is indicated by the asterisk (*).

Similar observations were noted for the d-tubocurarine/gallamine interaction (Fig. 5A). The experimentally determined IC_{50} values were 20.8 nM (17.3–24.4) for d-tubocurarine and 476.8 nM (395.1–558.4) for gallamine. The theoretical additive IC_{50} was calculated as 21.7 nM (16.8–26.6) for d-tubocurarine and 497.5 nM (423.0–571.9) for gallamine. Again, for this combination, the confidence intervals of the experimentally derived and theoretical points overlapped. For the pancuronium/gallamine interaction (Fig. 5B), the experimentally determined IC_{50} value for the combination was 3.5 nM (2.9–4.2) for pancuronium and

641.0 nM (522.9–759.2) for gallamine. The theoretical additive IC_{50} was calculated as 2.8 nM (2.1–3.4) for pancuronium and 497.5 nM (423.0–571.9) for gallamine. The experimentally determined IC_{50} values therefore fall outside the 95% confidence interval to the antagonistic side of the isobologram. However, the 95% confidence interval for the experimentally determined IC_{50} overlapped largely with that of the theoretical additive IC_{50}.

Results of fractional analyses of these interactions are summarised in Table 1. The calculated values range from 0.78 to 1.28 with four of the six combinations being almost exactly 1.0. The low value (0.78) identified the mivacurium/d-tubocurarine combination as possibly synergistic whereas the high value (1.28) was associated with the pancuronium/gallamine combination.

4. Discussion

The results of our study indicated no synergistic interactions for various combinations of two non-depolarising muscle relaxants on heterologously expressed adult muscle nicotinic acetylcholine receptors; this result was found for drug combinations with similar as well as dissimilar molecular structures. Hence, it appeared unlikely that the clinically observed synergism for some muscle relaxant combinations is a result of purely postsynaptic interactions with the nicotinic acetylcholine receptor. The interactions of the two tested combinations deviated somewhat from the theoretical line of additive action, one combination deviated in the synergistic direction and one combination in the antagonistic direction. Even though the IC_{50} values we derived experimentally for these combinations suggested non-additivity, we conclude that these combinations are also additive in nature, since the errors associated with the experimentally determined values (listed in Table 1) overlapped strongly with the 95% confidence limits calculated for an additive interaction. These results have important implications for understanding the mechanism by which combinations of muscle relaxants interact at the neuromuscular junction.

The administration of two muscle relaxants in combination was introduced during the 1980s. The intent of this practice was to reduce the autonomic and cardiovascular side effects of individual compounds by giving smaller doses of each drug as a combination (Lebowitz et al., 1981). The clinical impression of practitioners suggested the existence of synergy with some muscle relaxant combinations. In subsequent clinical studies, the combined administration of various pairs of aminosteriodal compounds, including the pancuronium/vecuronium combination, produced additive muscle blocking effects (Pandit et al., 1986; Naguib and Abdulatif, 1993; Naguib et al., 1995), whereas studies on combinations of benzylisoquinolinium compounds reported additive (Naguib et al., 1994) or synergistic effects (Kim et al., 1998). Moreover, combinations of an aminosteroidal with a benzylisoquinolinium compound exhibited synergistic effects in clinical and in in vitro studies using nerve-muscle preparations (Lebowitz et al., 1980; Pollard and Jones, 1983; Waud and Waud, 1985; Jalkanen et al., 1994).

In the present study, we found only additive effects for muscle relaxant combinations from the same structural class and for two aminosteroid/ benzylisoquinolinium combinations. We also explored interactions with the obsolete muscle relaxant gallamine. We chose to study this agent because its structure, a trisquarternary phenolic ether, represented a third type of muscle relaxant distinct from the aminosteroid and benzylisoquinolinium families. Combining gallamine with either d-tubocurarine or pancuronium had an additive effect, which is in agreement with some in vitro studies using a nerve-muscle preparation (Riker and Wescoe, 1951; Waud and Waud, 1984). However, other investigators have reported synergy for these combinations (Wong and Jones, 1971; Ghoneim et al., 1972; Schuh, 1981).

Our results exclude mechanisms for synergy primarily based on postsynaptic receptors and suggest that other causes, including presynaptic drug effects, contribute to muscle relaxant synergy. Presynaptic effects have been reported for d-tubocurarine and pancuronium (Riker, 1975). Presynaptic effects may lead to a decrease in the mobilisation and storage of acetylcholine in the nerve terminal (Kurihara and Brooks, 1975). Bowman and Webb (1976) suggested a positive feedback mechanism of acetylcholine binding to presynaptic receptors allowing nerve endings to "keep pace with transmitter release during high frequency stimulation". They concluded that fade during tetanic or train-of-four stimulation presumably results from prejunctional receptor blockade.

Some investigators have attributed greater presynaptic effects to d-tubocurarine and greater postsynaptic effects to pancuronium (Bowman and Webb, 1976; Su et al., 1979). The overall effect of a combination of two non-depolarising muscle relaxants could be synergistic, if the two drugs differ greatly in their presynaptic and postsynaptic affinities. The presynaptic effect of the first drug may lead to a decreased release of acetylcholine, making the postsynaptic membrane more sensitive to the second muscle relaxant. This mechanism would reduce the margin of safety for neuromuscular transmission, especially so if the second muscle relaxant binds predominantly to postsynaptic acetylcholine receptors. Results of several studies suggested that drug combinations with dissimilar molecular structures were more synergistic than combinations with similar structures (Lebowitz et al., 1980; Pollard and Jones, 1983; Meretoja et al., 1993; Rautoma et al., 1995b).

Other underlying mechanisms may contribute to synergistic effects seen in clinical studies for certain muscle relaxant combinations. For example, pancuronium is known to inhibit plasma cholinesterase activity, which could result in a reduction of the degradation of mivacurium if both are administered in combination (Savarese et al., 1988). Early reports of synergistic actions for some combinations of muscle relaxants could been related to the use of volatile anaesthetics in many clinical interaction studies (Ghoneim et

al., 1972; Lebowitz et al., 1980), because they enhance the effect of muscle relaxants (Waud, 1979; Wright et al., 1995). However, the possibility that combined administration of two muscle relaxants influences their protein binding in plasma to such a degree that a greater than expected proportion of the free, unbound drug reaches its neuromuscular site of activity (Lebowitz et al., 1980) was disproven for the combination of pancuronium and d-tubocurarine (Martyn et al., 1983). Different binding affinities of non-depolarizing muscle relaxants to muscle proteins may also be considered as a mechanism for clinically observed synergy. A low potency muscle relaxant may have a high affinity to muscle protein sites, potentially allowing a high potency muscle relaxant an unobstructed diffusion passage to the neuromuscular junction.

With the oocyte expression system, large numbers of functional receptors are assembled and inserted into the oocyte membrane, which mimics the postsynaptic junctional environment. This receptor population can be exposed rapidly to various concentrations of specific agonists or antagonists as it occurs in vivo with terminal synaptic agonist release. However, some potential limitations of our model need to be considered. The concentration of agonist used here (acetylcholine 10 μM) may be lower than the transient peak concentrations that occur within the neuromuscular junction. In order to study pharmacodynamic aspects of interactions between two muscle relaxants, we used this agonist concentration as the standard test concentration to ensure robust baseline responses and to minimise receptor desensitisation due to repetitive acetylcholine applications. We maintained a holding voltage of -60 mV because the antagonistic effects of muscle relaxants have been reported to be independent of holding voltages ranging from -100 to -40 mV (Fletcher and Steinbach, 1996; Garland et al., 1998). Our experiments were performed at room temperature, whereas normally the receptor is expressed in a homeothermic animal (mouse). However, we have previously reported that the open probability and single channel conductance of adult mouse nicotinic acetylcholine receptors expressed in *Xenopus* oocytes are the same as those reported for native receptors at physiological temperature (Yost and Winegar, 1997). Finally, the use of mouse receptors is justified because of the close sequence homology and functional similarity with human nicotinic acetylcholine receptors (Boulter et al., 1985).

In conclusion, our data suggest that synergistic neuromusclar blocking effects, observed for the administration of certain combinations of non-depolarising muscle relaxants, do not result from purely postsynaptic binding events at the muscle nicotinic acetylcholine receptor, but rather from differential actions on pre- and postsynaptic sites.

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