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Abstract

The effect of various non-depolarizing neuromuscular blocking agents (gallamine, pancuronium, vecuronium, d-tubocurarine, metocurine, atracurium and pipecuronium) on [^{3}H] acetylcholine release in the response to field electrical stimulation was investigated in vitro in preparations of the guinea pig right atrium. In this preparation, atropine enhanced and oxotremorine, a muscarinic agonist, reduced the release of [^{3}H] acetylcholine. Atropine reversed the inhibitory effect of oxotremorine in a concentration dependent manner, indicating that there is negative feedback modulation of acetylcholine release from the vagal nerve. While pancuronium, gallamine and atracurium enhanced the release of [^{3}H] acetylcholine, d-tubocurarine, metocurine, vecuronium and pipecuronium did not affect it. Pancuronium and gallamine also reduced the inhibitory effect of oxotremorine and the K_d value of pancuronium for muscarinic receptors located on cholinergic nerve terminals was 2.31 μM. These findings indicate that pancuronium and gallamine enhanced the release of acetylcholine from the atrial parasympathetic nerve, probably by inhibiting presynaptic muscarinic receptors.

KEYWORDS: acetylcholine release, guinea pig atrium, neuromuscular blocking agents, presynaptic inhibition, muscarinic receptors

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The Effect of Non-Depolarizing Neuromuscular Blocking Agents on the Release of Acetylcholine from the Right Atrium of the Guinea Pig

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The effect of various non-depolarizing neuromuscular blocking agents (gallamine, pancuronium, vecuronium, *d*-tubocurarine, metocurine, atracurium and pipecuronium) on [^{3}H]acetylcholine release in the response to field electrical stimulation was investigated *in vitro* in preparations of the guinea pig right atrium. In this preparation, atropine enhanced and oxotremorine, a muscarinic agonist, reduced the release of [^{3}H] acetylcholine. Atropine reversed the inhibitory effect of oxotremorine in a concentration dependent manner, indicating that there is negative feedback modulation of acetylcholine release from the vagal nerve. While pancuronium, gallamine and atracurium enhanced the release of [^{3}H]acetylcholine, *d*-tubocurarine, metocurine, vecuronium and pipecuronium did not affect it. Pancuronium and gallamine also reduced the inhibitory effect of oxotremorine and the K_d value of pancuronium for muscarinic receptors located on cholinergic nerve terminals was 2.31 μM. These findings indicate that pancuronium and gallamine enhanced the release of acetylcholine from the atrial parasympathetic nerve, probably by inhibiting presynaptic muscarinic receptors.

Key words: acetylcholine release, guinea pig atrium, neuromuscular blocking agents, presynaptic inhibition, muscarinic receptors

Certain non-depolarizing neuromuscular blocking agents such as pancuronium and gallamine are known to produce tachycardia in humans (1, 2) and anesthetized animals (3, 4). It has been shown that the antimuscarinic (atropine-like) action of these compounds on presynaptic muscarinic receptors present on the axon terminals of sympathetic neurons in the heart resulted in facilitation of norepinephrine (NE) release (5). Until now, however, there has been no direct neurochemical evidence of whether non-depolarizing neuromuscular blocking agents have any effect on the release of acetylcholine (ACh) in the heart.

In the present study, we investigated the effect of several non-depolarizing neuromuscular blocking agents on the [^{3}H]ACh release from isolated guinea pig atria preloaded with [^{3}H]choline in response to field electrical stimulation. Preliminary data have been presented elsewhere (6).

Materials and Methods

Guinea pig right atrium preparations and loading with [^{3}H]choline. Guinea pigs of either sex, weighting 300-500 g, were sacrificed by a blow on the head and the excised right atria were incubated for 40 min at 37°C in modified Krebs solution (7) containing 0.1 μCi of [methyl-^{3}H]choline chloride (58 mCi/mmol). The Krebs solution was aerated with a mixture of 95% O_2 and 5% CO_2 throughout the experiment. To facilitate the uptake of [^{3}H]choline and the synthesis of [^{3}H]ACh, the preparations were continuously stimulated during incubation at 1 Hz with supramaximal (10 V/cm) square-wave field impulses of 1.0 msec duration, through two platinum electrodes placed above and below the suspended atria, respectively. After the incubation, to remove excess [^{3}H]choline, the atria were transferred to other baths of 3 ml volume and superfused at a rate of 1.0 ml/min for 90 min with Krebs solution containing 10 μM hemicholinium-3 to prevent the reuptake of [^{3}H]choline liberated by the hydrolysis of the released [^{3}H]ACh.

Collection. After the 90 min washout, super-

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fusion was continued at a rate of 1 ml/ min and the 3-min fractions of the superfusate were collected with a fraction collector throughout the experiment. Starting at the beginning of the 4th (S_1), 10th (S_2) and 16th (S_3) fractions, the preparations were stimulated for 2 min with supramaximal square wave impulses of 1.0 msec duration at 2 Hz (240 shocks).

Total ^3H tissue content and [^3H]ACh release at resting and during field electrical stimulation. It was observed that after a 2-min stimulation period the ^3H content of the fractions returned to the resting level within 12 min (*i.e.*, after 4 fractions). Therefore, the fractions which were collected during the stimulation and the three following fractions were used to calculate the stimulation-evoked release (4th, 5th, 6th and 7th fractions for S_1; 10th, 11th, 12th and 13th for S_2; and 16th, 17th, 18th and 19th for S_3). The spontaneous (resting) release of ^3H was determined by measuring the ^3H content of other non-stimulated fractions before and after each stimulation period. The regression line of the resting release was determined with exponential curve fitting. From this regression line, the resting release in fractions collected at specific periods could be estimated. The evoked release of ^3H could be calculated by subtracting the resting release expected to be present during these 4 fractions from the actually measured ^3H content. In previous experiments, it was demonstrated that more than 90 % of the radioactivity released in response to field electrical stimulation (2 Hz, 2 min) was [^3H]ACh, whereas during resting release of [^3H]ACh was only about 40 % of the radioactivity (8–10). Therefore, it was assumed that the radioactivity released by field electrical stimulation represents [^3H]ACh. The absolute amount of radioactivity released was measured (disintegrations per second per gram of tissue: Bq/g) and the percentage of the released radioactivity to total tissue radioactivity in the preparations (fractional release) was calculated. The release expressed as the fractional release was relatively constant from experiment to experiment during successive collection periods.

Measurement of radioactivity. One milliliter of each fraction collected was transferred to a scintillation vial and 7 ml of scintillation fluid was added to each vial. The radioactivity of the samples was measured by liquid scintillation spectrometry (Tri-Carb 4530, Packard, IL, USA). At the end of each experiment, the ^3H content of the atria was also determined. The atria were blotted with filter paper, weighed, homogenized in 1 ml of Soluene (Packard) and kept at room temperature for 24 h. Supernatant (100 μl) was added to 7 ml of scintillation fluid and the radioactivity was measured.

Determination of the influence of compounds on [^3H]ACh release. In control experiments the increase of ^3H caused by field electric stimulation above the resting release of ^3H was determined. It was observed that there was considerable variation in both resting release and the stimulation-induced increase of the release of ^3H from one preparation to another. In contrast, the ratios of the amounts of ^3H released during consecutive stimulation periods, *i.e.*, S_2/S_1 and S_3/S_2 were very similar in different experiments. Therefore, the effect of compounds on evoked release of ^3H was determined by the comparison of the S_3/S_2 ratios in the absence and presence of them. Compounds were added to the perfusing solution 6 min after S_2. Since mainly [^3H]ACh was released in response to field electrical stimulation, the increase or decrease of S_3/S_2 indicated augmentation or inhibition of stimulated [^3H]ACh release, respectively.

Furthermore, to test the possible antimuscarinic activity of non-depolarizing neuromuscular blocking agents and atropine, we investigated the effects of these compounds on the inhibitory effect of oxotremorine on ACh release. At first, the log dose-response curve of oxotremorine on [^3H]ACh release was determined. Then, the effect of oxotremorine 0.5 μM was measured under perfusion with Krebs solution containing one of the 4 non-depolarizing neuromuscular blocking agents (pancuronium, gallamine, vecuronium and pipecuronium) or atropine. From these experiments, dose-ratios of these compounds were calculated, and the dissociation constants (K_d) of these compounds for muscarinic receptors located on cholinergic nerve terminals were determined by the Schild plot.

Statistical analysis. The mean ± SEM of the data are presented. Statistical analysis was carried out with one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered significant.

Results

Fractional release of [^3H]ACh. The resting and stimulated release of ^3H was measured in 4 to 6 atria for each compounds tested. Under the resting condition, 0.20 ± 0.01 % (n = 6) of the total radioactivity was

released in a 3-min period. None of the compounds studied had any effect on resting release of radioactivity. When 2 Hz field electrical stimulation was delivered for 2 min, the release of radioactivity was significantly enhanced above spontaneous release, and 0.22 ± 0.01 % of the total radioactivity was released by the 2-min stimulation (Table 1 and Fig. 1). The fractional release of ^3H in different experiments and the stimulation evoked release of the same experiment were very similar. The ratios of the ^3H released during consecutive stimulation periods, S_2/S_1 and S_3/S_2 in control experiments were 0.81 ± 0.02 and 0.94 ± 0.05, respectively.

Effect of atropine, oxotremorine and non-depolarizing neuromuscular blocking agents on [^{3}H]ACh release. Atropine 1.0 μM enhanced and oxotremorine 0.5 μM reduced the stimulation evoked release of [^{3}H]ACh (Table 2 and Fig. 1), indicating that there is negative feedback modulation of ACh release from the vagal nerve.

Concentrations of the neuromuscular blocking agents were comparable to those in clinical use for neuromuscular blockade (11). *d*-Tubocurarine, metocurine, vecuronium and pipecuronium even at high concentrations had no significant effect on the evoked release of ^3H. Gallamine

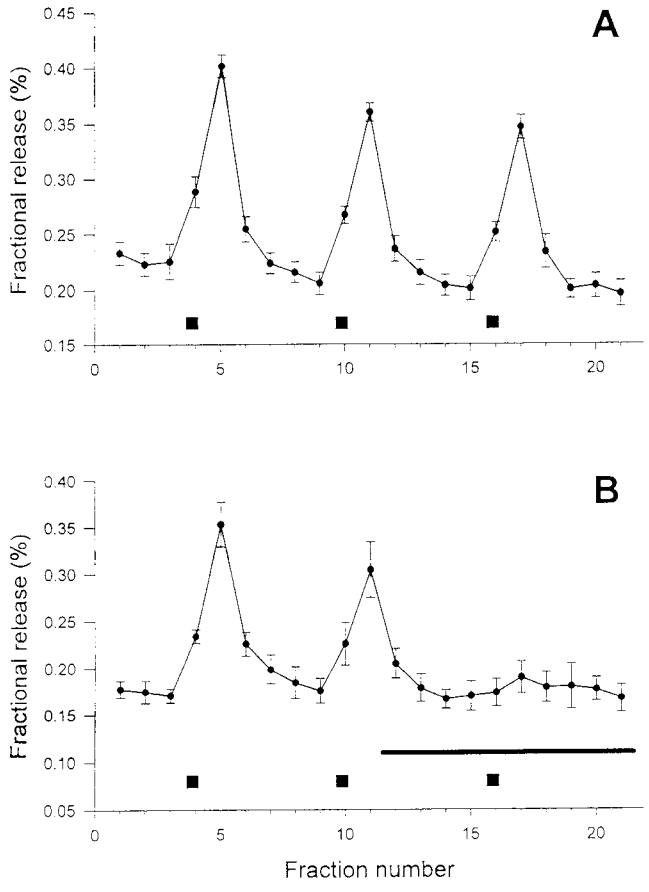

Fig. 1 Effect of field electrical stimulation on the fractional release of [^{3}H]ACh (mean and SEM) from guinea pig right atria. The stimulation, as indicated by closed rectangules, was applied three times during the experiments. **A**: Control experiment, average of 6 experiments. **B**: Oxotremorine 0.5 μM was added to the perfusing solution as indicated with a horizontal bar in the figure, average of four experiments. Oxotremorine reduced the stimulation-evoked release of ACh and S_3 was almost abolished.

Table 1 ^{3}H released from guinea pig atria at rest and during field electrical stimulation

	^{3}H Release	
	Bq/g	Fractional release (%)
Resting (3-min collection)	839 ± 85	0.204 ± 0.011
Stimulation (2 Hz, 240 shocks)	1002 ± 165	0.221 ± 0.013

Mean $\pm$ SEM ($n = 6$)

Table 2 Effect of atropine, oxotremorine and neuromuscular blocking agents on stimulation-evoked release of [^{3}H]ACh from the guinea pig atrium

Compounds		S_2/S_1	S_3/S_2
Control		0.81 ± 0.02	0.94 ± 0.05
Oxotremorine	0.5 μM	0.73 ± 0.06	0.18 ± 0.07*
Atropine	1 μM	0.81 ± 0.06	1.88 ± 0.12*
Pancuronium	2 μM	0.74 ± 0.03	1.11 ± 0.14*
	20 μM	0.87 ± 0.04	1.30 ± 0.11*
Gallamine	70 μM	0.79 ± 0.10	1.11 ± 0.04
	300 μM	0.83 ± 0.02	1.47 ± 0.07*
d-Tubocurarine	1 μM	0.88 ± 0.08	0.87 ± 0.05
	80 μM	0.89 ± 0.06	0.90 ± 0.01
Metocurine	60 μM	0.84 ± 0.02	1.10 ± 0.03
Vecuronium	5 μM	0.77 ± 0.02	0.97 ± 0.04
	20 μM	0.87 ± 0.04	0.95 ± 0.09
Pipecuronium	6 μM	0.88 ± 0.04	1.08 ± 0.09
	20 μM	0.90 ± 0.05	1.10 ± 0.03
Atracurium	45 μM	0.89 ± 0.02	1.19 ± 0.04*

Mean $\pm$ SEM ($n = 4$–6)
In control experiments, no compound was added between S_2 and S_3
* Significant difference ($P < 0.05$) from control values.

significantly increased the stimulation evoked release of [^{3}H]ACh at a concentration of 300 μM. Pancuronium (2 and 20 μM) and atracurium 45 μM significantly increased the S_3/S_2 ratio, indicating that pancuronium and atracurium significantly enhanced the evoked release of [^{3}H]ACh. The effect of pancuronium and gallamine on [^{3}H]ACh release was concentration dependent (Table 2).

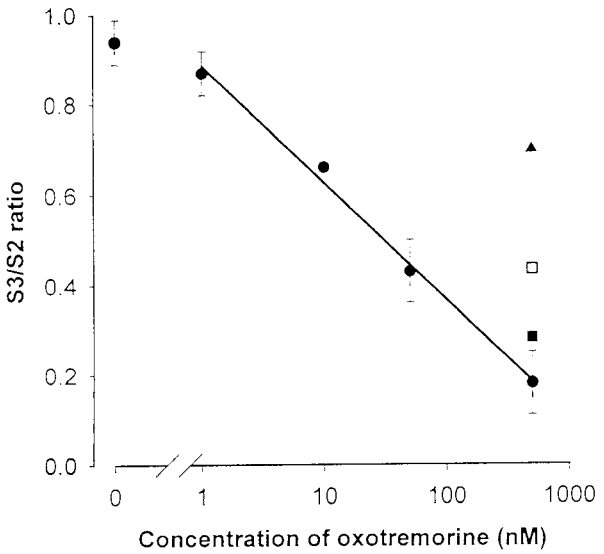

Fig. 2 Log dose-response curve of oxotremorine on [^{3}H]ACh release. Oxotremorine reduced the stimulation evoked release of ACh in a concentration-dependent manner (Closed circles and vertical bars indicate mean and SEM of 4 experiments). Closed rectangle, open rectangle, and closed triangle indicate antagonizing effects of pancuronium (2, 20 and 50 μM, respectively) on the inhibitory effect of oxotremorine on ACh release.

Figure 2 showed the log dose-response curve of oxotremorine on the [^{3}H]ACh release and the antagonism of its inhibitory effect by pancuronium. Figure 3 shows the Schild plots of atropine, pancuronium and gallamine. Data of vecuronium and pipecuronium are also presented. The regression lines for Schild plots had slopes of 0.97 and 1.37 for atropine and pancuronium, respectively. K_d values of atropine and pancuronium for muscarinic receptors located on cholinergic nerve terminals calculated from these curves were 1.38 nM and 2.31 μM, respectively. However, the K_d value of pancuronium may not be accurate because the slope of Schild plot differed from unity.

Discussion

In the present study, we demonstrated that gallamine, pancuronium and atracurium at clinically relevant concentrations increased the stimulation-evoked release of ACh from isolated guinea pig atria, while *d*-tubocurarine, metocurine, vecuronium and pipecuronium showed no significant effects.

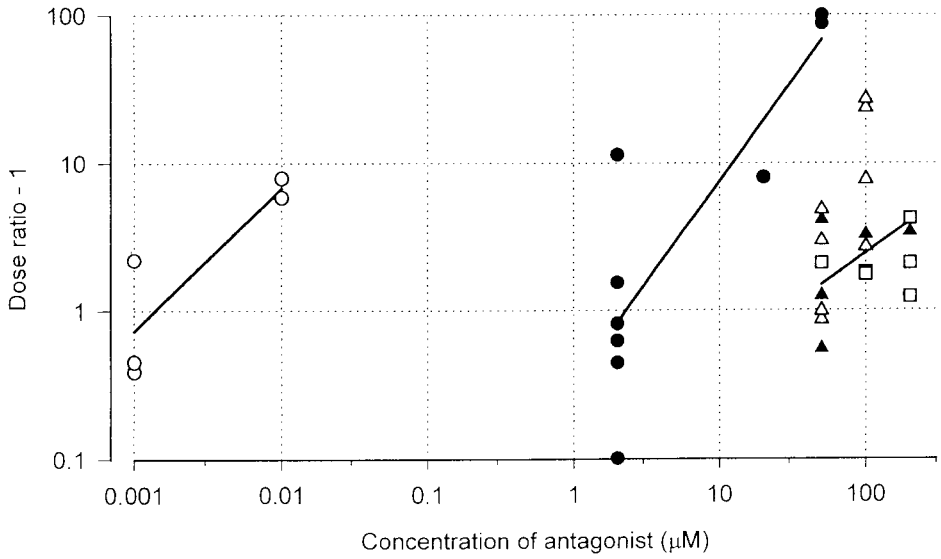

Fig. 3 The Schild plot of atropine and neuromuscular blocking agents. Regression lines of atropine, pancuronium and gallamine are shown in the figure (from left to right). Open circles = atropine; closed circles = pancuronium; closed triangles = gallamine; open triangles = pipecuronium; open rectangles = vecuronium.

Elevation of heart rate (HR) by pancuronium and gallamine has been attributed to the inhibition of the parasympathetic innervation of the cardiac pacemaker (3, 4, 12–14), the facilitation of the release of NE (15–18) and inhibition of the reuptake of released NE from the adrenergic nerve terminals (19–21). Theoretically, the parasympathetic effect on HR could be caused by inhibition of the evoked release of ACh from the cardiac vagus (presynaptic effect) and/or to inhibition of the interaction of ACh with postsynaptic muscarinic receptors (postsynaptic effect) on the cardiac pacemaker. While it has been suggested that the effect of pancuronium and gallamine on HR is due to postsynaptic mechanisms (14, 17), Lee Son and Waud have postulated in a series of experiments on the isolated guinea pig heart (22–24) that the main action of pancuronium and gallamine on cardiac pacemaker is due to their presynaptic effect. However, no direct evidence is available concerning these two hypothetical mechanisms. We demonstrated here that gallamine and pancuronium, instead of inhibiting, increased the stimulation-evoked release of ACh in the isolated guinea pig atria. Therefore, the presynaptic effect of these compounds on the vagus nerve could not be responsible for the elevation of HR by these compounds. We also found that atracurium at high concentrations enhanced the release of ACh. In contrast, *d*-tubocurarine, metocurine, vecuronium and pipecuronium which have no chronotropic effect, using concentrations ranging from clinically equipotent to large, did not affect ACh release.

The release of ACh from the vagus nerve is modulated by negative feedback mechanisms since atropine enhanced and oxotremorine reduced the release of [^{3}H]ACh and atropine prevented the inhibitory effect of oxotremorine in a concentration-dependent manner. ACh reduced its own release via presynaptic muscarinic receptors. In the present study neurochemical evidence has been obtained that at least pancuronium and gallamine were able to antagonize the effect of oxotremorine on ACh release from the vagus nerve and enhance the release of ACh, indicating that these compounds inhibited presynaptic muscarinic receptors on the parasympathetic innervation of the guinea pig atrium and thereby enhanced ACh release.

It has been suggested that pancuronium and gallamine are able to increase the release of NE from sympathetic neurons (17, 18). It has been demonstrated by direct measurement of the evoked release of NE that pancuronium, gallamine (5) and atracurium at high concentrations (25) increased the evoked release of NE from the guinea pig right atrium by inhibiting presynaptic muscarinic receptors located on the sympathetic nerve endings. We previously reported that pancuronium had a higher affinity to muscarinic receptors located on sympathetic nerve terminals than to those located on vagal nerve terminals (10). While the K_d value of pancuronium for muscarinic receptors located on adrenergic nerve terminals was 63.1 nM (10), its K_d value for muscarinic receptors located on cholinergic nerve terminals calculated in this study was 2.31 μM. The difference in these K_d values indicates that the muscarinic receptors located on the sympathetic and parasympathetic axon terminals are pharmacologically different. Vizi *et al.* also reported the heterogeneity among the presynaptic muscarinic receptors (26). Thus, it was assumed that pancuronium given during general anesthesia, inhibited presynaptic muscarinic receptors located on the sympathetic nerve ending and enhanced the release of NE before blocking those located on parasympathetic postganglionic nerve terminals and enhancement of the release of ACh.

In summary, neurochemical evidence obtained in this study indicates that in the heart of the guinea pig, gallamine, pancuronium and atracurium enhance the release of ACh from parasympathetic nerve terminals, and at least pancuronium and gallamine exerted their effects by inhibiting the presynaptic muscarinic receptors on the parasympathetic nerve terminals.

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