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Calcium and neostigmine antagonize gentamicin, but augment clindamycin-induced tetanic fade in rat phrenic nerve-hemidiaphragm preparations

Soo-il Lee, Jong-Hwan Lee, Seung Cheol Lee, Jung Moo Lee, and Ji Hyeon Lee

Department of Anesthesiology and Pain Medicine, Dong-A University Medical Center and Medicine College, 1,3 Ka, Dongdaeshin-Dong, Seo-Gu, Busan 602-715, Korea

Abstract

Purpose. A reduction in acetylcholine release induced by gentamicin may limit neostigmine-induced increases in acetylcholine concentration in the neuromuscular junction. An increase in acetylcholine concentration caused by neostigmine and calcium may enhance the use-dependent ion channel block of the nicotinic acetylcholine receptor caused by clindamycin. The purpose of this study was to determine whether calcium and neostigmine antagonize the neuromuscular blockade caused by gentamicin and augment the blockade caused by clindamycin during both single-twitch (0.1 Hz) and tetanic stimulation (50 Hz for 1.9 s).

Methods. Left phrenic nerve-hemidiaphragm preparations (Male Sprague-Dawley rats, 150–250 g) were mounted in Krebs solution. The concentration-response curves of gentamicin and clindamycin were obtained. The reversal effects of treatment with 5 mM calcium or 250 nM neostigmine on the effects of 1.5 mM gentamicin, which caused 72% reduction of single twitch, were studied. The effects of calcium or neostigmine on the effects of clindamycin were studied by examining the shift of the concentration-response curve of clindamycin with pretreatments with these agents. The effective concentrations were determined by a probit model.

Results. Calcium antagonized the single-twitch depression and tetanic fade caused by gentamicin more effectively than neostigmine. The effective concentration of 50% maximal effect (EC_{50}) values of clindamycin for tetanic fade in the presence of 5 mM calcium or 250 nM neostigmine were reduced by approximately 52%.

Conclusion. Clindamycin and gentamicin interfere with neuromuscular transmission and cause tetanic fade. Neostigmine and calcium antagonized the neuromuscular blockade caused by gentamicin, but augmented that caused by clindamycin.

Key words Calcium · Clindamycin · Gentamicin · Neostigmine · Neuromuscular blockade

Address correspondence to: S. Lee

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Introduction

Gentamicin blocks calcium channels at the motor nerve terminal and decreases the release of acetylcholine [1,2]. Gentamicin has neuromuscular blocking effects [3–5], augments the effect of nondepolarizing neuromuscular blocking agents [6,7], and aggravates symptoms of myasthenia gravis [8].

Clindamycin produces an open ion channel block on the end-plate [9,10] and decreases acetylcholine release at the motor nerve terminal [11,12]. Clindamycin alone induces profound, long-lasting neuromuscular blockade [13,14] and prolongs the blockade of nondepolarizing muscle relaxants [15–18].

Neostigmine and increased calcium concentrations reverse the neuromuscular blockade caused by gentamicin, but do not reverse the blockade caused by clindamycin [19]. Treatment with clindamycin alone resulted in twitch depression of 15%–20% compared to that of the control; however, treatment with neostigmine (20 $ng \cdot ml^{-1}$) or calcium (81 $\mu g \cdot ml^{-1}$) resulted in a 5% and 11% increase in twitch tension, respectively [17]. The train-of-four ratio produced by an overdose of clindamycin improved slightly after treatment with calcium chloride (1.5 $mg \cdot kg^{-1}$ i.v.), edrophonium (20 mg), and neostigmine (2 mg) [14].

Most of the aforementioned studies considered only single-twitch stimulation. The mechanisms underlying the reduction in single-twitch tension are different from those underlying an increase in the tetanic fade [20,21]. Therefore, tetanic stimulation may demonstrate a response that is different from the response to single-twitch stimulation.

A reduction in acetylcholine release induced by gentamicin [1,2] may limit neostigmine-induced increases in acetylcholine concentration in the neuromuscular junction. An increase in acetylcholine concentration caused by neostigmine and calcium may enhance the use-dependent ion channel block of the

nicotinic acetylcholine receptor (nAChR) caused by clindamycin [9,10] and further impair neuromuscular transmission.

The purpose of this study was to determine whether calcium and neostigmine antagonize the neuromuscular blockade caused by gentamicin and augment the blockade caused by clindamycin, when measured by both single-twitch and tetanic stimulation.

Materials and methods

Male Sprague-Dawley rats weighing between 150 and 250 g were used in all experiments. Our Institutional Animal Care and Use Committee approved the experimental protocol. Thirty-three rats were anesthetized by perivertebral injection of ketamine ($150\ mg \cdot kg^{-1}$) at the lumbar level, and then killed. The left phrenic nerve was separated from the middle portion of the thymus to the point of branching near the hemidiaphragm surface. The phrenic nerve and diaphragm were excised en bloc and immersed in a 50-ml bowl which contained oxygenated (95% O_2, 5% CO_2) Krebs solution (118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 30 mM $NaHCO_3$, 1 mM KH_2PO_4, 1 mM $MgCl_2$, and 11 mM glucose). Excess thoracic and abdominal wall bones and muscles were cut down until only an 8- to 10-mm-high costal wall remained. The left hemidiaphragm was separated from the rest of the diaphragm, preserving the middle diaphragmatic ligament from the vertebral bodies to the xyphoid process of the sternum. Left phrenic nerve-hemidiaphragm preparations were then mounted in a 20-ml organ bath filled with Krebs solution. The bath solution was maintained at 32°C by circulating heated water in the space between the double walls, and it was continuously aerated with a gas mixture of 95% oxygen and 5% carbon dioxide. The pH of the bath was maintained at 7.38 to 7.42. Spent Krebs solution was exchanged for fresh solution 10 min after the preparation was mounted. The preparations were attached to a force transducer (Model 1030; UFI, Morro Bay, CA, USA) with a stainless steel wire and allowed to stabilize for 20 min in the bath. The phrenic nerve was connected to a stimulating electrode and stimulated with supramaximal square wave impulses of 0.2-ms duration, using a stimulator (Model ML112; AD Instruments, Bella Vista, NSW, Australia).

The preparation was stretched until the maximum output tension was recorded after stimulation; then another 10 min elapsed for stabilization before the experiment began. Three consecutive single-twitch tensions at 0.1 Hz, and a 1.9-s tetanic tension of 50 Hz were digitized and stored on a Power Macintosh 7100 (Apple Computer, Cupertino, CA, USA), using data acquisition software (MacLab; AD Instruments).

After the baseline tension was measured, the concentrations of gentamicin sulfate ($n = 6$) and clindamycin phosphate ($n = 6$) in the bath were cumulatively increased until an 80%–90% reduction of a single twitch was attained. At least 20 min was allowed to establish a pseudo-steady-state condition of the drug concentration between the bath and the preparation, and measurement of tensions was made during the pseudo-steady state and after each experiment in a drug-free solution. Data were analyzed only if single-twitch tension returned to greater than 90% of the baseline recording in a drug-free solution.

To assess the reversibility of muscle paralysis produced by gentamicin, a 72 ± 7% reduction of single twitch (accompanied by a 61 ± 14% increase of tetanic fade) was established by 1.5 mM gentamicin, and either 5 mM calcium chloride ($n = 5$) or 250 nM neostigmine ($n = 5$) was added. The extent of reversal was measured during the pseudo-steady state, and the recovery value was expressed as antagonism (%) [22], as defined below.

A 5-mM concentration of calcium and a 250-nM concentration of neostigmine augmented the degree of neuromuscular blocking (70 ± 10%) of clindamycin, and the reversibility of muscle paralysis caused by clindamycin could not be accurately assessed. Instead of evaluating antagonism (%) for clindamycin, the concentration of clindamycin in Krebs solution pretreated with 5 mM calcium ($n = 5$) or 250 nM neostigmine ($n = 6$) was increased cumulatively until an 80%–90% reduction of single twitch was attained. Tension was measured during the pseudo-steady state.

Single tension was determined based on the mean of three consecutive single-tension measurements. Single-twitch and peak tetanic tensions of agents at each concentration step were compared with the control tension (percent reduction of control), using the following equation:

$$\text{Percent reduction} = [1 - (\text{tension in presence of agent}/\text{control tension})] \times 100$$

The response of agents with regard to tetanic fade at each concentration step was calculated (as percent increase of tetanic fade compared to peak tetanic tension in a tetanic tension), using the following equation:

$$\text{Percent increase} = [1 - (\text{end tetanic tension}/\text{peak tetanic tension})] \times 100$$

The effects of antagonists on the neuromuscular action of gentamicin were calculated as antagonism (%), using the following equation:

$$\text{Antagonism (\%)} = [1 - \{\text{\% reduction (or increase) after antagonist}/\text{\% reduction (or increase) before antagonist}\}] \times 100$$

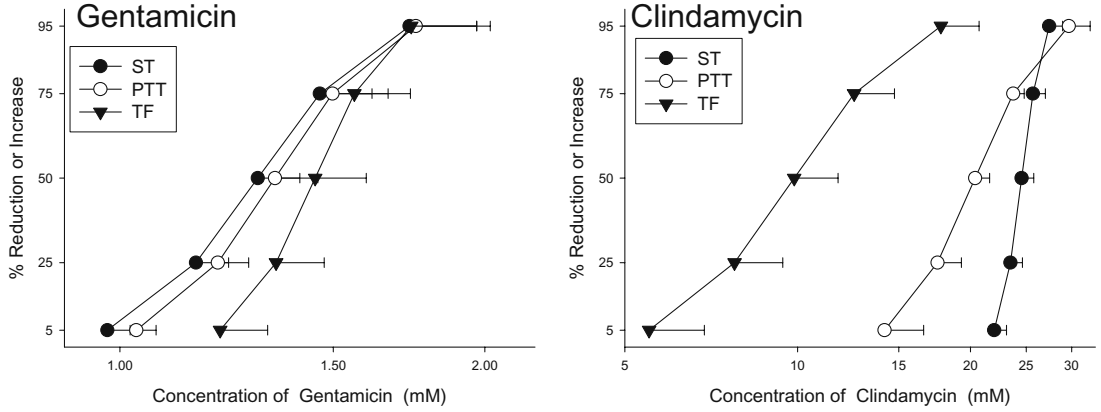

Fig. 1. Cumulative concentration-effect curves of gentamicin and clindamycin on single-twitch tension (*ST*) at 0.1 Hz, and peak tetanic tension (*PTT*) and tetanic fade (*TF*) at 50 Hz for 1.9 s. The effects on single-twitch and peak tetanic tensions were calculated as percent inhibition of control, and the effects on tetanic fade were calculated, as percent increase of fade. All contractile responses were nerve-evoked. In all cases, preparations were exposed to gentamicin and clindamycin for 20 min before the measuring of contractions. Six preparations were studied for each antibiotic. The indicated concentrations of gentamicin and clindamycin are shown as means and SD

Table 1. EC_{50} Values for ST, PTT, and TF

	EC_{50} (mM)		
Antibiotics	ST	PTT	TF
Gentamicin ($n = 6$)	1.30 ± 0.11	1.34 ± 0.11	1.45 ± 0.15
Clindamycin			
Alone ($n = 6$)	24.6 ± 1.2	20.4 ± 1.2	9.9 ± 1.9
In NS, 250 nM ($n = 6$)	19.6 ± 0.9*	13.2 ± 1.5*	4.8 ± 0.6*
In Ca^{2+}, 5 mM ($n = 5$)	21.2 ± 2.1*	15.0 ± 2.5*	4.8 ± 0.7*

* $P < 0.05$, by *t*-test with Bonferroni's correction, compared to clindamycin alone
Values are means ± SD; *n* indicates the number of experiments
EC_{50}, Effective concentration of 50% maximal effect; ST, single twitch; PTT, peak tetanic tension; TF, tetanic fade; NS, neostigmine

For each experiment, a linear regression was generated by the probit model, and effective concentrations (EC) were determined.

The effective concentration of 50% maximal effect (EC_{50}) values of clindamycin alone, or of clindamycin together with 5 mg calcium or 250 nM neostigmine, were compared using Student's *t*-test with Bonferroni's correction. Differences were considered significant at $P < 0.05$.

Results

In all experiments, tetanic tension was well maintained during repetitive stimulation when the antibiotics were not present. Tetanic fade occurred at higher concentrations of gentamicin, but at lower concentrations of clindamycin, than single-twitch or peak tetanic tension did (Fig. 1 and Table 1).

Calcium (antagonism [%] of single twitch, 84%; that of tetanic fade, 99%) reversed the neuromuscular blockade caused by gentamicin more effectively than neostigmine (antagonism [%] of single twitch, 12%; that of tetanic fade, 31%) in the indirectly elicited responses of five rat phrenic hemidiaphragm preparations (Fig. 2).

Calcium (5 mM) and neostigmine (250 nM) shifted the concentration-response curves of clindamycin to the left (Fig. 3). The EC_{50} values of clindamycin in the presence of 5 mM calcium or 250 nM neostigmine were reduced by 14% and 20% for single twitch, and by approximately 52% for tetanic fade (Table 1).

Discussion

The results of our study show that gentamicin and clindamycin have neuromuscular blocking effects. After gentamicin treatment, the EC_{50} values for reduction in single-twitch and peak tetanic tensions and increase in tetanic fade were 1.30, 1.34, and 1.45 mM, respectively. The concentration-response curve of gentamicin for

tetanic fade was shifted to the right from those for the depression of single-twitch and peak tetanic tensions.

Gentamicin blocks calcium channels at the motor nerve terminal and decreases the release of acetylcholine [1,2]. We found that gentamicin produced tetanic fade, which was almost completely reversed by an increased calcium concentration. Mg^{2+} causes increasing tetanic force ("tetanic ascent"), which spares the depression of tetanic tension [23]. Therefore, gentamicin probably causes tetanic fade due to decreased calcium entry into the presynaptic nerve terminal. A possible mechanism of action may be suggested as follows. Tetanic fade could be the functional correlate of depressed acetylcholine mobilization in the nerve terminal secondary to the blockade of presynaptic nAChR [20,21]. Because of the almost complete reversal of tetanic fade by calcium, which increases the release of acetylcholine at the nerve terminal, it could be postulated that gentamicin competitively blocks presynaptic nAChR. On the other hand, we found that single-twitch tension was reduced by more than 60% when tetanic fade occurred, which indicates that gentamicin may reduce more than 90% of the acetylcholine release [24]. Because of this, the acetylcholine concentration in the synaptic cleft may be too low to stimulate neuronal nAChR and increase calcium entry in the nerve terminal.

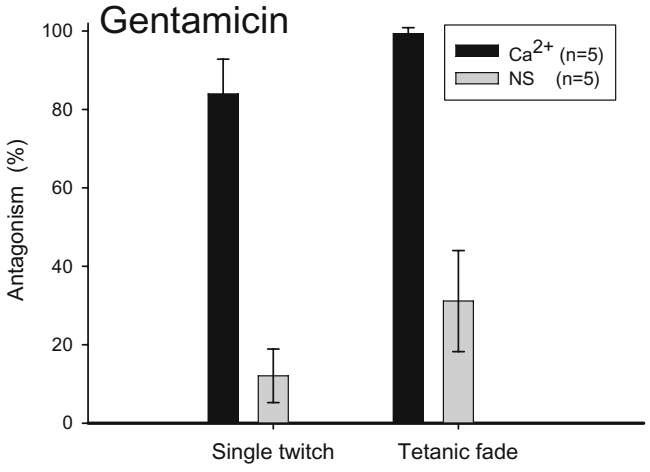

Fig. 2. Neuromuscular blockade caused by gentamicin was reversed by calcium (Ca^{2+}; single-twitch, 84%; tetanic fade, 99%) and neostigmine (*NS*; single-twitch, 12%; tetanic fade, 31%) in the indirectly elicited responses of five rat phrenic nerve-hemidiaphragm preparations. The concentrations of calcium and neostigmine were 5.0 mM and 250 nM, respectively. The results are expressed as antagonism (%). Values are expressed as means and SD

Schlesinger et al. [25] demonstrated that gentamicin could cause competitive inhibition and open ion channel block of the nAChR channel complex at the end-plate. In their study [25], open channel block was observed at a high concentration of gentamicin (10 mM) and calcium almost completely restored the tetanic tension; therefore, it seems that open channel block caused by gentamicin would probably not have contributed to the increasing tetanic fade seen in our study. An increased calcium concentration enhances acetylcholine release at the nerve terminal and results in increased acetylcholine concentration in the synaptic cleft. This indicates that an increased acetylcholine concentration could possibly offset the competitive inhibition of nAChR at the end-plate and maybe at the nerve terminal, or that

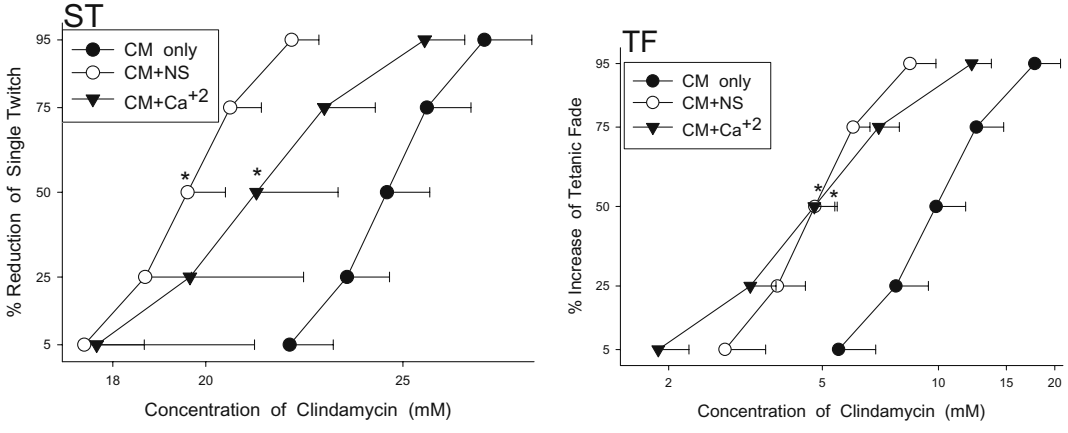

Fig 3. Cumulative concentration-response curves of clindamycin (*CM*) in Krebs solution pretreated with 250 nM neostigmine (*NS*) or 5 mM calcium (Ca^{2+}) for effect on single-twitch (*ST*) tension at 0.1 Hz, and tetanic fade (*TF*) at 50 Hz for 1.9 s. The effects on single-twitch tension were calculated as percent inhibition of control, and the effects on tetanic fade, as percent increase of fade. All contractile responses were nerve-evoked. In all cases, the preparations were exposed to neostigmine and calcium for 20 min before clindamycin was added cumulatively. The indicated concentrations of clindamycin represent the means and SD of five or six preparations. $^*P < 0.05$ by *t*-test with Bonferroni's correction, compared to the effective concentration of 50% maximal effect value for clindamycin alone

increased acetylcholine concentration could stimulate presynaptic nAChR, resulting in the reduction of tetanic fade.

Neostigmine increases acetylcholine concentration in the synaptic space. In our study, neostigmine reduced tetanic fade by 31%. Gentamicin may reduce more than 90% of the acetylcholine release, because it has been shown that single-twitch tension was reduced by more than 60% after gentamicin treatment [24]. Therefore, the amount of acetylcholine increased because of neostigmine treatment may be limited, and this may cause only a small reversal in the reduction of single-twitch tension and the increase of tetanic fade. A partial reversal in tetanic fade by neostigmine also suggests that gentamicin causes muscle relaxation presynaptically [26].

Decreased acetylcholine release at the nerve terminal [1,2] and the competitive inhibition of nAChR at the end-plate [25], or maybe at the nerve terminal, may be important mechanisms underlying the impairment of neuromuscular transmission caused by gentamicin.

In our study, the EC_{50} values of clindamycin for reductions in single-twitch and peak tetanic tensions, and the increase in tetanic fade were 24.6, 20.4, and 9.9 mM, respectively. Clindamycin caused the concentration-response curve for tetanic fade to shift to the left from those for the other two parameters.

Tetanic fade can be produced by the use-dependent blockade of postsynaptic nAChR-operated ion channels [21,27]. Clindamycin produces an open ion channel block on the end-plate [9,10] and decreases acetylcholine release at the motor nerve terminal [11,12]. Because calcium reduced the effective concentrations of clindamycin for tetanic fade, it appears that clindamycin may not cause tetanic fade presynaptically. This indicates that clindamycin blocks the nAChR operated-ion channel and produces tetanic fade.

We found that calcium and neostigmine shifted the concentration-response curve of clindamycin to the left. These results suggest that an increase in acetylcholine concentration in the neuromuscular synapse enhances the open ion channel block. Repetitive stimulations may cause more acetylcholine to accumulate in the synapse than single stimulation, which could explain why the EC_{50} values for tetanic fade were decreased by 52% compared to those for single-twitch tension, which were decreased by 14%–20%. These results suggest that calcium and neostigmine enhance and prolong the muscle relaxation produced by clindamycin.

In contrast to our results, some investigators have reported that calcium or neostigmine partially reversed the muscle relaxation caused by clindamycin [14,17]. The reasons for these differences are not known, but may reflect differences in species, study methods, the degree of block, or the frequency of nerve stimulation.

The maximal therapeutic levels of gentamicin and clindamycin are 12 $ng \cdot ml^{-1}$ ($\fallingdotseq$0.021 mM) and 17 $ng \cdot ml^{-1}$ ($\fallingdotseq$0.034 mM), respectively [5]. The concentrations we studied were 50 times or more these levels. Although the concentrations necessary to cause muscle relaxation are much higher than the therapeutic concentrations, it is likely that the neuromuscular effects of antibiotics would be accentuated when they are used in combination with agents that have a neuromuscular blocking effect [28].

Further investigation is needed to confirm that the effects of antibiotics on muscle fibers may contribute to the change in muscle tension, that neostigmine may antagonize the paralytic effect of a combination of clindamycin and nondepolarizing muscle relaxant, and that a combination of clindamycin and neostigmine may worsen the muscle weakness in myasthenia gravis.

To conclude, we found that clindamycin and gentamicin interfered with neuromuscular transmission and caused tetanic fade. Neostigmine and calcium antagonized the neuromuscular blockade caused by gentamicin and accentuated the blockade caused by clindamycin. It remains speculative whether neostigmine may augment the neuromuscular blockade induced by muscle relaxants in the presence of clindamycin.

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