

Effects of macrolide antibiotics on neurally mediated contraction of human isolated bronchus

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Background: Long-term administration of macrolide antibiotic substances is an alternative therapy used in the treatment of asthma and airway hyperresponsiveness, but neither its mechanism of action nor whether this substance exerts an immediate action in the airways is known.

Methods: Contractile responses of human isolated bronchial strips to electrical field stimulation (EFS) and acetylcholine were assessed under isometric conditions in the absence and presence of erythromycin, roxithromycin, or clarithromycin.

Results: Incubation of tissues with erythromycin (3×10^{-5} mol/L) attenuated the contractile responses to EFS so that the stimulus frequency required to produce 50% of the maximal contraction increased from 4.1 ± 0.5 to 10.1 ± 0.7 Hz (mean $\pm$ SE; $p < 0.001$). In contrast, contractile responses to acetylcholine were not changed. Erythromycin reduced the EFS-induced contraction in a concentration-dependent fashion; the maximal decrease from the baseline response was $92.8\% \pm 3.6\%$ ($p < 0.001$). This inhibitory effect was not altered by propranolol, indomethacin, ouabain, charybdotoxin, or mechanical removal of the epithelium. Roxithromycin and clarithromycin likewise inhibited neurally mediated contraction.

Conclusions: These results suggest that macrolides may inhibit cholinergic neuroeffector transmission in the human airway smooth muscle, probably by reducing exocytotic release of acetylcholine from the nerve terminals. (*J ALLERGY CLIN IMMUNOL* 1995;95:853-9.)

Key words: Airway smooth muscle, bronchial hyperreactivity, erythromycin, acetylcholine, vagal nerve, asthma

Several reports have shown that macrolide antibiotics including erythromycin and troleandomycin favorably affect the clinical status of patients with severe steroid-dependent asthma.¹⁻⁴ Although the precise mechanism remains uncertain, macrolides' steroid-sparing effect^{5,6} and inhibition of theophylline clearance, which can result in higher theophylline concentrations,⁷ have been postulated to contribute to their beneficial actions in the treatment of asthma.

Miyatake et al.⁸ showed that long-term administration of low-dose erythromycin reduced airway

Abbreviations used

EFS: Electrical field stimulation
L-NAME: L-N^G-arginine methylester

hyperresponsiveness, a characteristic of asthma,⁹ probably through its antiinflammatory actions. However, it is unknown whether macrolides exert immediate effects on airway functions. Because the increased parasympathetic tone regulating airway caliber may be involved in the pathogenesis of asthma^{10,11} and because erythromycin has been shown to depress nerve-mediated contraction in guinea pig intestinal smooth muscle by inhibiting the release of acetylcholine from cholinergic nerve endings,¹² we hypothesized that macrolide antibiotics could similarly affect neural component of contractile responses of airway smooth muscle preparations. To test this hypothesis, we examined the effects of erythromycin and other newly developed macrolides, roxithromycin and clarithromycin

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cin, on cholinergic neuroeffector transmission in human isolated bronchial strips under isometric conditions in vitro.

METHODS

Preparation of tissues

Human lung tissues were obtained from 31 patients during thoracotomies performed because of carcinoma. After surgical removal, pieces of macroscopically normal lung tissues were rapidly immersed in Krebs-Henseleit solution consisting of the following (in millimoles per liter): NaCl, 118; KCl, 5.9; CaCl_2 , 2.5; MgSO_4 , 1.2; NaH_2PO_4 , 1.2; NaHCO_3 , 25.5; and D-glucose, 5.6. The solution was gassed with a mixture of 95% O_2 and 5% CO_2 . Cartilaginous bronchi, 2 to 4 mm in internal diameter, were then dissected free of parenchyma, fat, and surrounding connective tissues and cut helically at a 45° pitch to obtain bronchial strips 2 to 3 mm in width and approximately 20 mm in length. Between three and eight strips were dissected from each specimen and mounted in organ chambers filled with 14 ml of Krebs-Henseleit solution maintained at 37° C and continuously aerated with 95% O_2 plus 5% CO_2 to obtain a pH of 7.4, a carbon dioxide pressure of 38 mm Hg, and an oxygen pressure of greater than 500 mm Hg.¹³ The lower end of the tissue was attached to a glass hook at the base of the organ chamber by a loop of silk thread, and the upper end was attached to a force-displacement transducer (model TB-652T, Nihon Kohden, Tokyo, Japan) for continuous recording of isometric tension by a pen recorder (model WT-685G, Nihon Kohden). Each organ chamber was fitted with two rectangular platinum electrodes (6 × 25 mm) placed beside the tissue for transmural electrical field stimulation (EFS) (biphasic square wave, 1-millisecond pulse duration, supramaximal voltage of 40 V for 15 seconds). In our preliminary experiments the contractile responses of human bronchial strips to EFS (1 to 50 Hz) were abolished by atropine (10^{-6} mol/L) and were therefore cholinergic in nature, and the maximal contraction induced by EFS was approximately 73% of the maximal response to acetylcholine (71 ± 5 vs 97 ± 7 gm per gram of tissue, $n = 8$ for each). The tissues were allowed to equilibrate for 60 minutes while they were washed with Krebs-Henseleit solution every 15 minutes, and the resting tension was adjusted to a value that gave the maximal contractile response to EFS. Separate studies on the relationship between resting tension and active tension showed maximal responses with 2 gm of resting tension. A contractile response was determined as the difference between the peak tension developed and the resting tension.

Effect of macrolides on contractile responses

To assess whether macrolides affect neurally mediated smooth muscle contraction, contractile responses to EFS were determined before and after the addition of erythromycin, roxithromycin, or clarithromycin (each at 3×10^{-5} mol/L). We first obtained the baseline responses to

EFS at increasing frequencies of stimulation (1 to 50 Hz); we then added each macrolide to the chamber, and after 15 minutes we repeated the measurements. To test the effects of other antibiotics, we similarly constructed the frequency-response curves before and after the addition of penicillin or cephazolin (3×10^{-5} mol/L). In control experiments we examined the effect of the solvent (Krebs-Henseleit solution) alone on the responses to EFS in a similar manner. In evaluating the macrolide effect on EFS-induced contraction, we determined the stimulus frequency required to produce 50% of the maximal contraction by linear regression analysis.

We then assessed concentration-dependent effect of erythromycin. After baseline contractile responses to EFS at 5 Hz were obtained, erythromycin at concentrations ranging from 10^{-5} to 10^{-4} mol/L was added to the chamber in a cumulative manner, during which intervals the responses to EFS at the same frequency of stimulation were determined 15 minutes after each addition.

To identify whether the site of action of macrolide is prejunctional or postjunctional in the cholinergic motor pathway, we examined the effect of erythromycin, roxithromycin, and clarithromycin (3×10^{-5} mol/L) on the contractile responses to exogenously applied acetylcholine. After we established the first concentration-response curves, tissues were washed with Krebs-Henseleit solution until the tension returned to the resting levels and 60 minutes later each macrolide was added. Fifteen minutes later the second concentration-response curves for acetylcholine were generated. We also examined the effects of ampicillin and cephazolin (3×10^{-5} mol/L) on the acetylcholine-induced contractions in a similar manner. To characterize the concentration-response curves, the negative logarithm of molar concentrations of acetylcholine required to produce a half-maximal contraction were determined by linear regression analysis.

To assess possible involvement of β -adrenergic receptor activation,¹⁴ synthesis and release of inhibitory prostaglandins,¹⁵ activation of Na^+ - K^+ -ATPase,¹⁶ opening of Ca^{2+} -activated K potassium channels,¹⁷ and the release of nitric oxide from nonadrenergic noncholinergic inhibitory nerves¹⁸ in the action of erythromycin, we determined erythromycin-induced (3×10^{-5} mol/L) inhibition of the contractile response to EFS (5 Hz) in the absence and presence of the following pharmacologic blocking agents: propranolol (10^{-5} mol/L), a β -adrenergic receptor antagonist; indomethacin (3×10^{-6} mol/L), a cyclooxygenase inhibitor; ouabain (10^{-7} mol/L), an Na^+ - K^+ -ATPase inhibitor; charybdotoxin (10^{-7} mol/L), a Ca^{2+} -activated K potassium channel blocker¹⁹; and L-N^G-arginine methylester (L-NAME; 10^{-3} mol/L), an inhibitor of nitric oxide synthase.²⁰ In addition, to test whether the release of epithelium-derived relaxing factor is involved,²¹ we compared the effects of erythromycin on EFS-induced contractions between intact tissues and the tissues with their epithelial layer removed. Bronchial strips were gently rubbed off their luminal surface with a moist cotton swab, and confirmation of

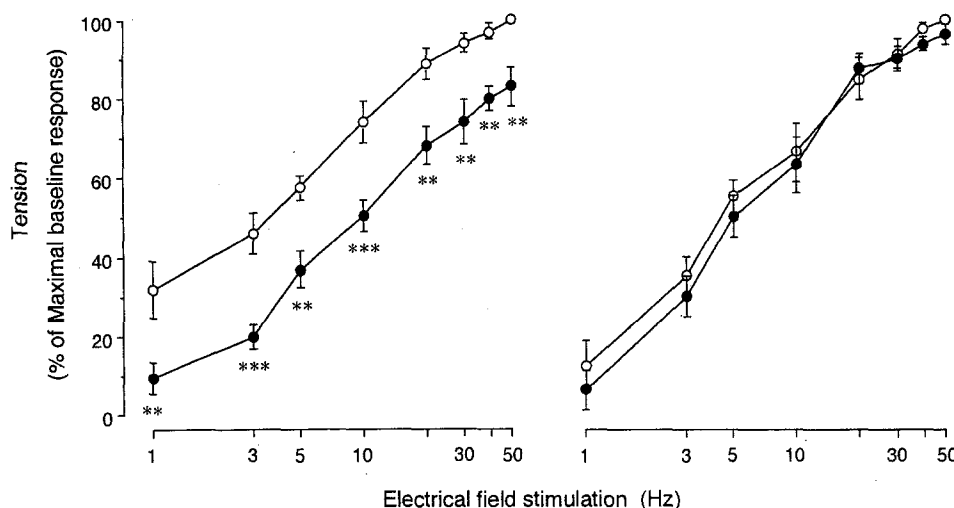


FIG. 1. Effect of erythromycin (*left*) and its solvent (Krebs-Henseleit solution) alone (*right*) on contractile responses of human bronchial strips to EFS. After baseline responses (○) were obtained, erythromycin (3×10^{-5} mol/L) or Krebs-Henseleit solution was added and 15 minutes later measurements were repeated (●). Responses are expressed as percent of maximal baseline response. Data expressed as mean $\pm$ SE; $n = 12$ for each curve. Significant difference from corresponding baseline responses at $p < 0.01$ (**) or at $p < 0.001$ (***).

the successful removal of the epithelium was histologically performed at the end of the experiment.

Drugs

The following drugs were used: erythromycin base, acetylcholine chloride, DL-propranolol hydrochloride, indomethacin, ouabain, L-NAME (Sigma Chemical Co., St. Louis, Mo.); charybdotoxin (Peptide Institute Ltd., Osaka, Japan); roxithromycin (Eisai Co., Tokyo, Japan); clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan); ampicillin (Takeda Pharmaceutical Co., Osaka, Japan); and cephazolin (Fujisawa Pharmaceutical Co., Osaka, Japan). Indomethacin was dissolved in absolute ethanol at 10^{-2} mol/L and subsequently diluted in Krebs-Henseleit solution, and other drugs were dissolved in Krebs-Henseleit solution. Working solutions of all drugs were kept on ice and added to the organ chamber in 140 μ l aliquots.

Statistical analysis

All values are expressed as means $\pm$ SE. Statistical analysis was performed by analysis of variance or the Newman-Keuls multiple comparison test, and a p value of less than 0.05 was considered significant.

RESULTS

Addition of any antimicrobial agent to the chamber at concentrations used in this study did not alter the resting tension of human bronchial strips. As demonstrated in Fig. 1, erythromycin (3×10^{-5} mol/L) substantially decreased the contractile responses to EFS at all stimulus frequen-

cies, causing an increase in the stimulus frequency required to produce 50% of the maximal contraction from 4.1 ± 0.5 to 10.1 ± 0.7 Hz ($p < 0.001$, $n = 12$), whereas the solvent alone had no effect. The concentration-response relationship of the inhibitory effect of erythromycin is shown in Fig. 2. Erythromycin inhibited the EFS-induced (5 Hz) contraction in a concentration-dependent fashion; the maximal decrease from the baseline response was $92.8\% \pm 3.6\%$ ($p < 0.001$, $n = 10$), observed at a concentration of 10^{-4} mol/L. The data can be linearized in the form of a Woolf-Augustinsson-Hofstee plot²²; the apparent Michaelis-Menten constant K_m characterizing the inhibition of contractile responses to EFS at 5 Hz by erythromycin was estimated to be 2.2×10^{-5} mol/L.

To determine the site of erythromycin action in the vagal motor pathway, we examined the effect of this drug on the contractile responses to exogenous acetylcholine. In contrast to the effect on EFS-induced contraction, erythromycin did not affect the concentration-response curves for acetylcholine (Fig. 3). Other macrolides including roxithromycin and clarithromycin likewise attenuated the contractile responses to EFS without affecting those to acetylcholine (Table I). Neither ampicillin nor cephazolin significantly altered EFS- and acetylcholine-induced contractions.

Addition of propranolol, indomethacin, charybdotoxin, and L-NAME increased the contractile

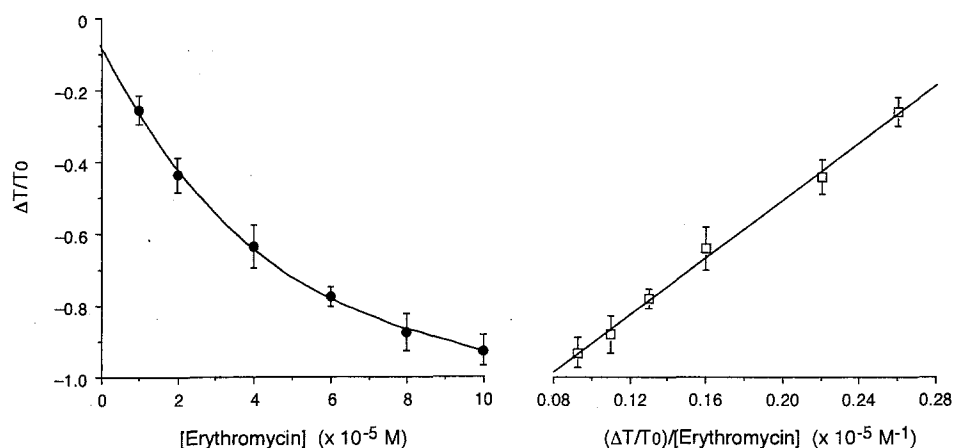


FIG. 2. Concentration-dependent effect of erythromycin on contractile responses of human bronchial strips to EFS at 5 Hz (*left*) and its Woolf-Augustinsson-Hofstee plot (*right*). Values are expressed as change of tension (ΔT) from baseline response obtained before addition of erythromycin (T_0). Data expressed as mean $\pm$ SE; $n = 10$ for each point.

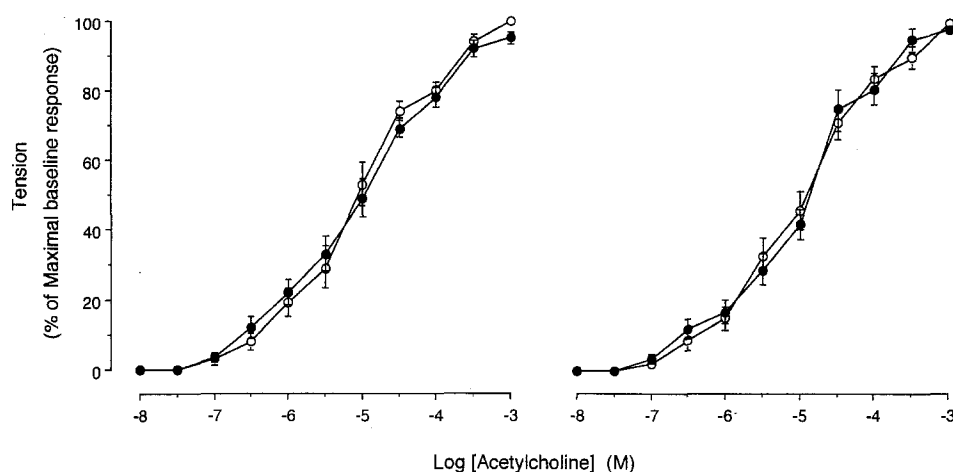


FIG. 3. Effect of erythromycin (*left*) and its solvent (Krebs-Henseleit solution) alone (*right*) on contractile responses to acetylcholine. After baseline responses ($\circ$) were obtained, erythromycin (3×10^{-5} mol/L) or Krebs-Henseleit solution was added and second measurements were made ($\bullet$). Responses are expressed as percent of maximal baseline response. Data expressed as mean $\pm$ SE; $n = 12$ for each curve.

responses to EFS (5 Hz) by $4.6\% \pm 1.3\%$, $8.9\% \pm 1.7\%$ ($p < 0.05$), $12.0\% \pm 2.1\%$ ($p < 0.05$), and $16.0\% \pm 2.4\%$ ($p < 0.01$), respectively, by themselves ($n = 8$ in each case). Under these conditions erythromycin at 3×10^{-5} mol/L still attenuated the EFS-induced contraction; the magnitude of the decrease was not significantly different from the value obtained in the absence of a blocker (Fig. 4). Similarly, mechanical removal of the epithelial cells per se increased the contractile responses to EFS by $19.7\% \pm 2.6\%$ ($p < 0.01$, $n = 8$) but did not alter the inhibitory effect of erythromycin.

DISCUSSION

Our in vitro studies demonstrate that erythromycin does not change the resting tone of human isolated bronchial strips but attenuates neurally mediated contraction, presumably by inhibiting cholinergic neuroeffector transmission in airway smooth muscle. This effect was likewise observed with other macrolide antibiotics such as roxithromycin and clarithromycin, whereas ampicillin and cephazolin had no effect. Thus the inhibitory action appears to be specific for macrolides.

It has been known that transmural electrical

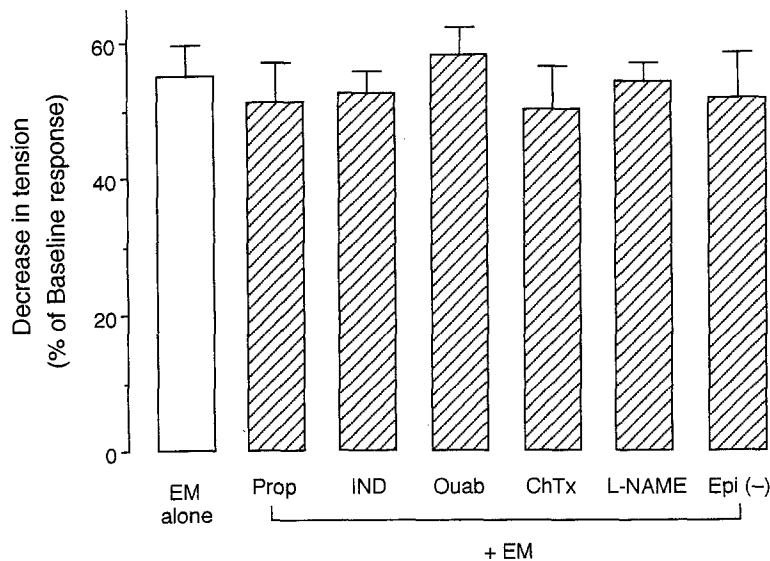


FIG. 4. Effects of propranolol (*Prop*, 10^{-5} mol/L), indomethacin (*IND*, 3×10^{-6} mol/L), ouabain (*Ouab*, 10^{-7} mol/L), charybdotoxin (*ChTx*, 10^{-7} mol/L), L-NAME (10^{-3} mol/L), and mechanical removal of epithelium (*Epi(-)*) on erythromycin-induced (*EM*, 3×10^{-5} mol/L) decrease in contractile responses to EFS at 5 Hz. Data expressed as percent decrease in tension from baseline responses obtained before addition of EM. Values expressed as mean $\pm$ SE; $n = 8$ for each column.

TABLE I. Effects of antibiotic substances on contractile responses to EFS and acetylcholine

Antibiotic drug	EFS (ES_{50} , Hz)		Acetylcholine (pD_2)	
	Before	After	Before	After
Erythromycin	4.1 ± 0.5	$10.1 \pm 0.7^*$	5.2 ± 0.2	5.0 ± 0.3
Roxithromycin	4.3 ± 0.2	$8.0 \pm 0.4^*$	4.9 ± 0.4	5.1 ± 0.4
Clarithromycin	4.1 ± 0.3	$7.7 \pm 0.5^\dagger$	4.8 ± 0.2	5.0 ± 0.5
Ampicillin	4.4 ± 0.3	4.2 ± 0.1	4.9 ± 0.4	4.9 ± 0.2
Cephazolin	3.9 ± 0.4	4.1 ± 0.3	5.2 ± 0.2	4.8 ± 0.4

Data are means $\pm$ SE; $n = 12$ for erythromycin, $n = 10$ for other drugs.

ES_{50} , Stimulus frequency (in hertz) required to produce 50% of maximal baseline response; pD_2 , negative logarithm of acetylcholine concentration required to produce half-maximal baseline response.

*Significantly different from value before drug addition ($p < 0.01$).

†Significantly different from value before drug addition ($p < 0.05$).

stimulation of the isolated human airway smooth muscle, as performed in the present study, stimulates postganglionic nerve fibers and produces the excitatory and inhibitory responses through activation of parasympathetic and nonadrenergic noncholinergic mechanisms, respectively.¹⁸ Thus the attenuation of EFS-induced contraction produced by macrolides can result from a decrease in parasympathetic contraction or an increase in nonadrenergic noncholinergic relaxation. However, the latter mechanism is not likely because the inhibition of nitric oxide synthase activity by L-NAME²⁰ and the concomitant blockade of the release of nitric oxide, a proposed neurotransmit-

ter of nonadrenergic noncholinergic nerves in the human airway,¹⁸ did not alter the inhibitory effect of erythromycin on the contractile responses to EFS. In addition, attenuation of EFS-induced contraction could be associated with the stimulation of β -adrenergic receptors on the vagal nerve endings,¹⁴ the production of inhibitory prostanoids such as prostaglandin E_2 that potentially reduce acetylcholine release,¹⁵ the stimulation of Na^+ - K^+ -ATPase activity and the subsequent repolarization or hyperpolarization of nerve cells,¹⁶ or the release of epithelium-derived relaxing factor.²¹ However, these possibilities seem unlikely because the effect of erythromycin was not influenced by

mechanical removal of the epithelial cells of the bronchial strips or pretreatment of tissues with the following pharmacologic blocking agents: propranolol, a β -adrenergic receptor antagonist; indomethacin, a cyclooxygenase inhibitor; and ouabain, an inhibitor of Na^+ - K^+ -ATPase.

To determine the site of macrolide action on the cholinergic motor pathway in our smooth muscle preparations, we compared the effects of macrolides on airway contractions induced by EFS with those induced by exogenously administered acetylcholine and found that erythromycin, roxithromycin, and clarithromycin did not alter the contractile responses to acetylcholine. This finding implies that the effects of macrolides were not associated with concomitant alterations in smooth muscle function such as decreased muscarinic receptor binding or increased degradation of endogenous acetylcholine. Therefore macrolide antibiotics are probably acting at a postganglionic prejunctional site in the cholinergic efferent neural pathway and the depression of neurally induced contraction might thus be attributable to a decrease in the exocytotic release of acetylcholine.

Our data do not provide evidence for a specific neuronal mechanism of macrolide action on transmitter release, but previous studies have shown that some antibiotics inhibit Ca^{2+} entry into nerve terminals. For example, aminoglycosides inhibit depolarization-induced Ca^{2+} uptake into rat brain synaptosomes,²³ and erythromycin has been reported to induce paralysis of a mouse nerve-diaphragm preparation by way of an action on the cholinergic nerve terminals.²⁴ Additionally, Lees and Percy²⁵ showed that some aminoglycosides inhibit nerve-mediated contractions of the guinea pig ileum without affecting responses to exogenous acetylcholine and that the inhibitory effects of the antibiotics are overcome by raising the extracellular Ca^{2+} concentration. The results of the present study may be consistent with but may not prove the hypothesis that inhibitory effects of macrolide antibiotics could be due in part to an inhibition of acetylcholine release as a result of decreased Ca^{2+} entry into airway cholinergic nerve terminals.

Increasing evidence suggests an efficacy of macrolide antibiotics in the treatment of asthma¹⁻⁴ and airway hyperresponsiveness,⁸ for which several mechanisms are proposed such as steroid-sparing effects,^{5,6} inhibition of theophylline clearance,⁷ and antiinflammatory actions including inhibition of chemotaxis²⁶ and generation of reactive oxygen species by polymorphonuclear leukocytes.²⁷ Our results on human airway smooth muscle indicate

that inhibition of cholinergic neuroeffector transmission might also be involved in the favorable effects of macrolides on asthma. It has been shown that the mean serum concentration after the ingestion of 500 mg erythromycin by the adult volunteers is 1.6×10^{-6} mol/L,²⁸ whereas in our in vitro studies the decrease in EFS-induced contraction was observed at erythromycin concentrations of 10^{-5} mol/L and higher. However, these findings may not necessarily negate its clinical significance, because with long-term administration this drug can concentrate intracellularly more than 10-fold.²⁹

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