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Electrophysiological effects of erythromycin, but lack of mechanical effects, in airway smooth muscle

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Abstract

The antibiotic erythromycin has been shown to modulate a variety of electrophysiological and mechanical responses in many cell types. We investigated whether it did so in airway smooth muscle using standard patch clamp, fura-2 fluorimetric and organ bath techniques. Erythromycin (10^{-4} M) evoked a small transient inward current with reversal potential and time-course similar to that of the Ca^{2+}-dependent Cl^- currents seen in these cells. Unlike its effects in other cell types, however, it did not alter basal $[Ca^{2+}]_i$, voltage-dependent Ca^{2+} currents, nor mechanical tone at rest, nor the corresponding responses to cholinergic stimulation (membrane currents; release of internally sequestered Ca^{2+}, nor contractions evoked by neural stimulation or exogenously added cholinergic agonist). In conclusion, erythromycin does exert interesting electrophysiological actions in airway smooth muscle, but does not alter mechanical activity as it has been shown to do elsewhere.

Keywords: Airway smooth muscle contraction; Airway hyperresponsiveness; Erythromycin; Macrolide antibiotic; Ca^{2+}-dependent Cl^- current

1. Introduction

The primary innervation to the airways is cholinergic in nature (Taylor et al., 1984). We have shown previously that acetylcholine acts at the postjunctional receptors on the airway smooth muscle to cause release of internally sequestered Ca^{2+} (Janssen and Sims, 1993a; Janssen et al., 1999), activation of Ca^{2+}-dependent Cl^- currents (Janssen, 1996; Janssen and Sims, 1992), and the resultant membrane depolarization (Janssen and Daniel, 1990; Janssen and Sims, 1992); depending on the degree of membrane depolarization, this could then lead to opening of voltage-dependent Ca^{2+} channels (Janssen, 1997) and Ca^{2+} influx.

In addition to their therapeutic uses as antibacterial agents, macrolide antibiotics such as erythromycin have been found to augment voltage-dependent Ca^{2+} currents in jejunal circular smooth muscle cells (Farrugia et al., 1995), inhibit acetylcholine-activated Cl^- currents in guinea-pig nasal gland cells (Ikeda et al., 1995), and reduce cholinergic responses in isolated human bronchus (Tamaoki et al., 1995) and guinea-pig small intestine (Minocha and Galligan, 1991). The concentrations required to achieve these effects exceed the conventional therapeutic doses.

In light of the influence that these commonly used clinical agents may have on ion channels and Ca^{2+}-homeostasis, and the ramifications this has on airway smooth muscle excitability, we sought to examine the effects of erythromycin on membrane currents, Ca^{2+}-handling and mechanical tone.

2. Methods

2.1. Preparation of isolated tissues and single cells

Whole lobes of lung and tracheae were obtained from dogs which had been euthanized using pentobarbital sodium (100 mg/kg). Tracheal smooth muscle was isolated by removing connective tissue, vasculature and epithelium, then cut into strips parallel to the muscle fibers (≈ 1 mm wide). Lobes of lung were pinned out, the overlying parenchyma and pulmonary vasculature were removed, and ring segments (≈ 4–5 mm long) of fifth to sixth order (outer diameter 2–6 mm) were excised. For single cell studies, tracheal smooth muscle strips (0.5–1.0 g wet weight) were transferred to dissociation

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buffer (composition given below) containing collagenase (type IV; 2.7 U ml^{-1}), elastase (type IV; 12.5 U ml^{-1}), and bovine serum albumin (1 mg ml^{-1}), then were either dissociated immediately or stored at 4 °C for dissociation at a later time (less than 48 h later); we have previously found that cells used immediately and those used after 48 h of refrigeration exhibit similar functional responses (i.e., contraction and activation of Ca^{2+}-dependent ion conductances). In order to dissociate into single tracheal smooth muscle cells, tissues in enzyme-containing solution were incubated at 37 °C for 60–120 min, then gently triturated. Tissues were either used immediately or stored at 4 °C for use the next day; we found no functional differences in tissues, which were studied immediately compared to those used after 24-h refrigeration.

2.2. Patch clamp electrophysiology

Single tracheal smooth muscle cells were allowed to settle and adhere to the bottom of a recording chamber (1 ml bath volume perfused at 2–3 ml min^{-1}) and were studied within 6 h after dissociation. Membrane currents were recorded using the nystatin perforated-patch method, which we have described in detail previously (Janssen, 1997; Janssen et al., 1997, 1999). Briefly, cells were held under voltage clamp at − 70 mV using an Axopatch-1D patch clamp amplifier (Axon Instruments, Foster City, CA). Electrodes had a tip resistance of 1–3 MΩ and were filled with an electrode solution containing the following (mM): CsCl, 140; $CaCl_2$, 0.4; $MgCl_2$, 1; EGTA, 1; HEPES, 20; pH 7.2; nystatin (200 μg ml^{-1}). Access resistance ranged from 10 to 40 MΩ and 60–80% series resistance compensation was employed. Data were filtered at 1 kHz, sampled at 2 kHz using pClamp6 software (Axon Instruments), and stored on the computer hard-drive for later analysis using pClamp6 and SigmaPlot software. Corrections were not made for liquid junction potentials (previously found to be only ≈ 2 mV (Janssen and Sims, 1992)). Agonists were applied by pressure ejection from a puffer pipette (Picospritzer II; General Valve, Fairfield, NJ).

2.3. Fura-2 fluorimetry

Freshly dissociated cells were studied using a filter-based photometric system (DeltaScan; Photon Technology International, South Brunswick, NJ). After settling onto a glass coverslip mounted onto a Nikon TMD inverted microscope, cells were loaded with the membrane-permeant form of fura-2 (fura-2/AM, 2 μM for 30 min at 37 °C), then superfused continuously with Ringers buffer (2–3 ml min^{-1}) at 37 °C. Cells were illuminated alternately (0.5 Hz) at the excitation wavelengths and the emitted fluorescence (measured at 510 nm) induced by 340 nm excitation (F_{340}) and that induced by 380 nm excitation (F_{380}) was measured using a photomultiplier tube assembly. Agonists were applied by pressure ejection from a puffer pipette (Picospritzer II; General Valve).

2.4. Muscle bath technique

Ring segments were mounted into 3 ml muscle baths using stainless steel hooks inserted into the lumen. One hook was fastened to a Grass FT.03 force transducer using silk thread (Ethicon 4-0); the other was attached to a plexiglass rod, which served as an anchor. Tissues were bathed in Krebs–Ringer's buffer (see below for composition) containing indomethacin (10 μM), bubbled with 95% O_2/5% CO_2, and maintained at 37 °C; tissues were passively stretched to impose a preload tension of ≈ 1 g (determined to allow maximal responses). Isometric changes in tension were amplified and plotted using a chart recorder. Tissues were equilibrated for 2 h before commencing the experiments, during which time the tissues were challenged with 60 mM KCl at least once to assess the functional state of each tissue. Electrical field stimulation was delivered via two platinum rods (4 mm apart) on either side of the tissue. Electrical pulses (0.5 ms duration; 50–70 V) were delivered in pulse trains with frequencies of 1 or 10 pulses per second (pps).

2.5. Solutions and chemicals

Dissociation buffer contained (in mM): NaCl, 125; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; HEPES, 10; EDTA, 0.25; D-glucose, 10; L-taurine, 10; pH 7.0. Single cells were studied in Ringer's buffer containing (in mM): NaCl, 130; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; HEPES, 20; D-glucose, 10; pH 7.4. Intact tissues were studied using Krebs–Ringer's buffer containing (in mM): NaCl, 116; KCl, 4.2; $CaCl_2$, 2.5; NaH_2PO_4, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 22; D-glucose, 11; bubbled to maintain pH at 7.4. Indomethacin (10 μM) was also added to the latter to prevent generation of cyclooxygenase metabolites of arachidonic acid.

Chemicals were obtained from Sigma, with the exception of fura-2 acetoxymethyl ester (Calbiochem, La Jolla, CA). ACh was prepared as an aqueous solution; erythromycin was prepared in methanol; the final concentration of methanol to which the cells/tissues were exposed ranged from 0.01% to 1%.

2.6. Data analysis

Responses are reported as mean ± S.E.M.; *n* refers to the number of animals. Statistical comparisons were made using paired Student's *t*-test; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Electrophysiological effects of erythromycin

In airway smooth muscle cells held under voltage clamp at a holding potential of − 70 mV and perfused internally with a Cs^+-containing electrode solution (to block outward

K^+ currents), erythromycin (10^{-4} M) evoked a transient inward current in 7 of 10 cells tested, an example of which is given in Fig. 1; the 3 non-responding cells nonetheless exhibited the typical electrophysiological response to caffeine which we have described in the past (Janssen, 1997; Janssen et al., 1997, 1999). Such inward currents were not evoked by the diluent alone (i.e., methanol; $n=8$). The mean magnitude of this inward current in the responding cells was 167 ± 45 pA. The current–voltage relationship of these currents was investigated using ramp depolarizing commands (from -100 to $+50$ mV, at a rate of 150 mV/s, from a holding potential of -70 mV): these currents were linear at negative potentials, reversed at $+2 \pm 1$ mV ($n=3$; see Fig. 1 inset), and somewhat outwardly rectifying at positive potentials.

The time-course and current–voltage relationship of these currents are similar to the currents which are evoked in these cells by agonists such as acetylcholine and histamine (Janssen and Sims, 1992, 1993b), and we have previously been able to show "cross-talk" between the signaling pathways activated by these agonists in that application of one agonist occluded the subsequent response to the other agonist (Janssen and Sims, 1993b). We used the same approach to examine whether erythromycin interacted with the cholinergic signaling pathway. Cells were challenged with acetylcholine (10^{-4} in application pipette), then exposed for 5–10 min to either erythromycin (10^{-4} M) or to Ringers buffer followed by a second test response to acetylcholine. Responses were expressed as a per cent of control, and comparisons made between responses obtained during application of Ringers versus erythromycin. We found no significant effect of erythromycin on the magnitude of the cholinergically evoked membrane current responses: these were $70.1 \pm 20.0\%$ ($n=4$) of control in Ringers-treated tissues and $85.5 \pm 17.3\%$ ($n=5$) of control after erythromycin.

Finally, we examined the effects of erythromycin on the Ca^{2+} currents in these cells: we have characterized these in detail previously (Janssen, 1997) and shown them to be primarily of the "L-type" variety in canine tracheal myocytes. In this study, we used depolarizing pulses to $+10$ mV (100 ms duration; from the holding potential of -70 mV) at 15 s intervals, to evoke a series of Ca^{2+} currents of reproducible amplitude (see Fig. 2). These currents were unaffected in one cell, enhanced in two cells and reduced partially in four others. Fig. 2 shows the Ca^{2+} currents evoked in one of the responder cells, and how these were first augmented slightly, but then suppressed in a reversible fashion. On the whole, however, erythromycin had no

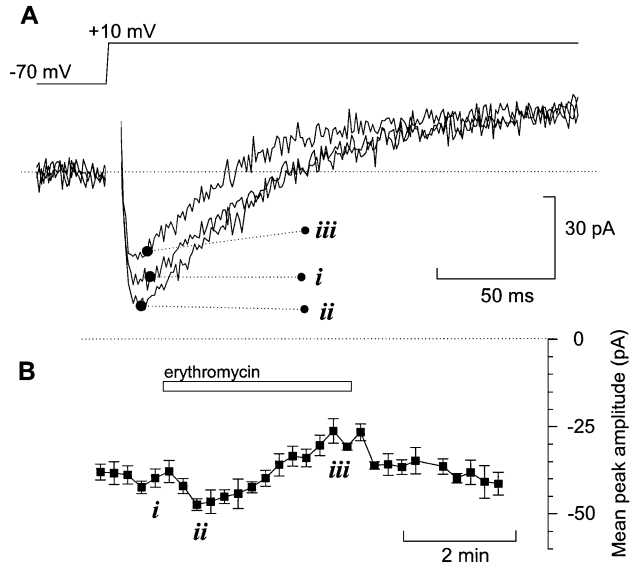

Fig. 2. Effect of erythromycin on voltage-dependent Ca^{2+} currents. Voltage-dependent Ca^{2+}-currents were recorded from a cell using a Cs^+-containing electrode solution and depolarizing pulses (to $+10$ mV from a holding potential of -70 mV; 250 ms duration; delivered at 15 s intervals). (A) Representative tracings of the currents evoked in this way before (i) application of erythromycin (10^{-4} M in the application pipette), as well as 30 s (ii) and 3 min after (iii), are superimposed. (B) Mean peak amplitudes of the Ca^{2+} currents evoked throughout the experiment represented in panel A: i, ii and iii indicate the specific current responses shown in panel A.

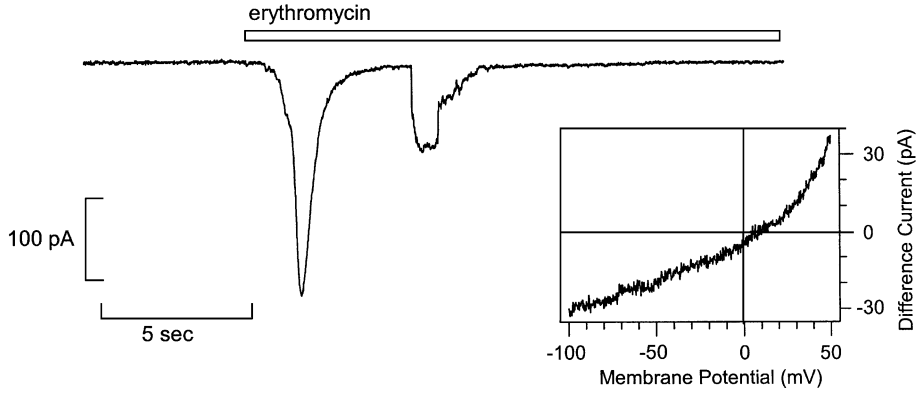

Fig. 1. Membrane current evoked by erythromycin. In a single canine tracheal myocyte perfused internally with a Cs^+-containing electrode solution and held under voltage clamp at -70 mV, erythromycin (10^{-4} M in the application pipette) evoked a transient inward current. Inset: current–voltage relationship of the erythromycin-evoked membrane current, obtained using ramp depolarizations (from -100 to $+50$ mV over the course of 1 s); reversal of the current occurred at $+6$ mV.

significant effect on the Ca^{2+} currents: mean values of peak current were 70.7 ± 13.1 pA in its absence and 53.5 ± 14.1 pA in its presence ($n = 6$). The time-courses of activation and of inactivation were also not altered by erythromycin.

3.2. Fluorimetric responses to erythromycin

The time-course and current–voltage relationship of these currents are similar to the currents which are evoked in these cells by agonists such as acetylcholine and histamine via release of internally sequestered Ca^{2+}. It may be, then, that erythromycin somehow causes a similar release of Ca^{2+}. We used fura-2 fluorimetric techniques to test this hypothesis, and found no discernable changes in $[Ca^{2+}]$ upon application of erythromycin, and cholinergically evoked Ca^{2+}-transients were not significantly reduced by erythromycin: these were 78.9 ± 13.2% ($n = 15$) and 74.4 ± 4.1% ($n = 16$) of control.

3.3. Effects of erythromycin on mechanical responses

Finally, we examined the effects of erythromycin on the contractions evoked by stimulation of the cholinergic nerve endings as well as direct activation of the postjunctional cholinergic receptors. Tracheal and bronchial tissues were first challenged with 60 mM KCl (in order to standardize all subsequent responses), after which the tissues were treated with either 10^{-4} M erythromycin or vehicle, then electrically stimulated (100 pulses delivered at either 1 or 10 pps), followed by addition of carbachol added in 10-fold increments in cumulative fashion. Erythromycin did not evoke any measurable change in mechanical tone, nor did it have any significant effect on the magnitudes of electrically evoked responses, nor those evoked by addition of carbachol, in either tracheal or bronchial tissues (Fig. 3).

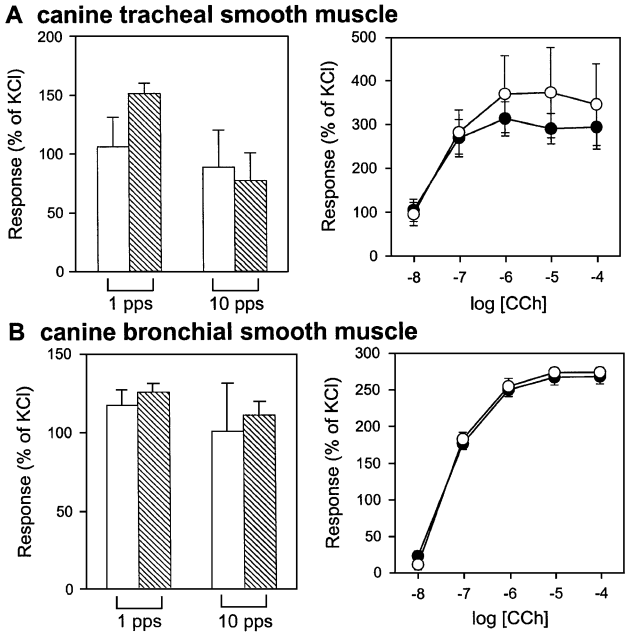

Fig. 3. Lack of effect of erythromycin on cholinergically evoked contractions. Mean peak contractions in isolated canine tracheal (A) and bronchial (B) tissues, evoked by either neural stimulation (100 pulses delivered at 1 or 10 pps; left-hand panels) or by exogenously added carbachol (right-hand panels) in the absence or presence of erythromycin (10^{-4} M; open or hatched bars/symbols, respectively).

4. Discussion

Macrolide antibiotics are in common and widespread use. However, as with any drug, there is always the possibility that these agents have biological effects other than those originally intended. For example, in airway tissues, macrolide antibiotics (albeit at concentrations far in excess of their therapeutic doses) have been found to reduce cholinergic responses (Tamaoki et al., 1995). Although the mechanism(s) underlying this reduction is quite unclear, macrolides have been shown elsewhere to augment voltage-dependent Ca^{2+} currents (Farrugia et al., 1995) and inhibit acetylcholine-activated Cl^- currents (Ikeda et al., 1995), both of which are believed to be activated during airway smooth muscle contraction.

In this study, we found erythromycin activated a small inward current with many properties which are hallmark features of the Ca^{2+}-dependent Cl^- currents present in these cells (Janssen and Sims, 1992, 1993b, 1995): (1) it is transient, rising to a peak and then resolving back to baseline within only a few seconds, even though the agent continues to be applied; and (2) its current–voltage relationship is linear at negative potentials and reverses at +2 mV. While the cholinergically evoked current is accompanied by (in fact, triggered by) a substantial elevation of $[Ca^{2+}]_i$, we were unable to detect any change in $[Ca^{2+}]_i$ upon application of erythromycin. However, it should be pointed out that the cholinergic membrane current response is considerably larger than the erythromycin-evoked current-typically on the order of several nanoamperes (Janssen and Sims, 1992, 1993b), compared to a few hundred picoamperes (Fig. 1), respectively—and thus it may be that the corresponding fluorimetric response is also present but exceedingly small.

On the other hand, while erythromycin seems to inhibit Cl^- currents in guinea-pig nasal gland cells (Ikeda et al., 1995), it did not do so in canine tracheal myocytes in this study. Likewise, despite its salutory effect on voltage-dependent Ca^{2+} currents in jejunal circular smooth muscle cells (Farrugia et al., 1995), and inhibitory effects on Ca^{2+} channels in mast cells (Franzius et al., 1994), erythromycin did not have a systematic (nor statistically significant) effect on voltage-dependent Ca^{2+} currents in canine tracheal myocytes.

Finally, and perhaps most importantly, we did not find that erythromycin had any significant effect whatsoever on neurogenic cholinergic contractions, nor those evoked by exogenously added cholinergic agonist, even when used at

concentrations which far exceed its clinically relevant doses. This finding contradicts those of others who show a reduction of cholinergic responses in isolated human bronchus (Tamaoki et al., 1995) and guinea-pig small intestine (Minocha and Galligan, 1991). The reason(s) for this discrepancy is unclear, but may include species- and tissue-related differences.

In conclusion, while erythromycin did evoke a biological response in canine tracheal myocytes—in the form of a membrane current with properties of a Ca^{2+}-dependent current—it had no significant effect on: voltage-dependent Ca^{2+} currents, Ca^{2+}-homeostasis, or tone directly, nor did it alter in any way the membrane currents, change in $[Ca^{2+}]_i$, nor contractions evoked by cholinergic stimulation.

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