Cholinomimetic action of macrolide antibiotics on airway gland electrolyte secretion

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Irokawa, Toshiya, Tsukasa Sasaki, Sanae Shimura, Kan Sasamori, Takako Oshiro, Masayuki Nara, Tsutomu Tamada, and Kunio Shirato. Cholinomimetic action of macrolide antibiotics on airway gland electrolyte secretion. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L951–L957, 1999.—We investigated the acute effects of erythromycin (EM) and its derivatives on ionic currents in airway glands from feline tracheae. Therapeutic concentrations of EM or clarithromycin (CAM) attenuated the whole cell currents evoked by ACh in a competitive manner. The maximally stimulated inward Cl⁻ currents were reduced to 54 and 83% and the outward K⁺ currents to 55 and 84% of control values by EM and CAM, respectively, whereas the responses induced by phenylephrine, norepinephrine, caffeine, or ionomycin were unaffected by EM, CAM, or EM523, a synthetic derivative of EM. K⁺ channels in excised outside-out patches were not influenced by macrolides. Although therapeutic concentrations of macrolides showed no effect on the baseline currents, high concentrations of macrolides alone evoked currents mimicking the ACh response, which were abolished completely by atropine. We concluded that macrolides act as a partial agonist on cholinergic receptors, resulting in a reduction of Cl⁻ secretion at pharmacological doses of the agents, which may exhibit a pronounced effectiveness on hypertrophied and/or cholinergically sensitized submucosal glands in pathological airways.

submucosal gland; chloride secretion; patch clamp; erythromycin; partial agonist

ERYTHROMYCIN (EM) and its derivatives are known to relieve the symptoms related to airway hypersecretion in such lung diseases as chronic bronchitis and diffuse panbronchiolitis (13, 23, 32). A variety of mechanisms have been reported to explain the basis of the clinical benefit of macrolides in the airways. For instance, EM attenuates the production of cytokines including interleukin (IL)-8 from Pseudomonas aeruginosa-stimulated human neutrophils (18) and from cultivated human bronchial epithelial cells induced by Haemophilus influenzae endotoxin (12). Neutrophil elastase is a strong secretagogue of the airway submucosal gland (21). It is therefore of interest to clarify whether macrolides affect water secretion as well as mucus secretion from the gland.

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mg/ml) for 30 min at 37°C. After dispersion and a wash with centrifugation at 180 g, the cells were resuspended in a standard extracellular solution (see Electrical recordings) until used.

Electrical recordings. Ionic currents were measured with a patch-clamp amplifier (EPC9, HEKA Electronic), low-pass filtered at 1.0 kHz, and monitored on both a built-in software oscilloscope and a pen recorder (RECTI-HORIZ-8K, Nippon-Denki Sato, Tokyo, Japan). The solutions employed were of the following compositions (in mM): extracellular (bath) solution, 120 KCl, 1.13 MgCl2, 0.5 EGTA, 1 Na2ATP, 10 glucose, and 10 HEPES; and intracellular (pipette) solution, 130 KCl, 1 Na2ATP, 0.5 EGTA, 10 glucose, and 10 HEPES. The fluids were superfused over the cell(s) by withdrawing of high concentration EM (Fig. 1A). Airway gland cells still responded to ACh after the withdrawal of high concentration EM (Fig. 1A), indicating the physiological relevance of the EM-induced response. This agonistic action was found also with EM523 (>10^-4 M; 220 ± 44 pQ/s; n = 5), a synthetic derivative of the macrolide (17). The stimulatory effect of high concentrations of macrolides was significantly inhibited in the presence of the muscarinic antagonist atropine. As shown in Fig. 3, the responses to 1–5 mM EM were reduced to 32 ± 10% of control value in the presence of 10^-6 to 10^-4 M atropine (P < 0.01; n = 10).

As exemplified in Fig. 1B, higher concentrations (10^-8 to 10^-7 M) of ACh induced oscillatory responses imposed on sustained currents, which were also reduced in the presence of EM (10^-6 M; 25 ± 7% of preceding control value; P < 0.01; n = 12) and CAM (10^-6 to 10^-5 M; 76 ± 5%; P < 0.05; n = 11), one of the derivatives of EM (Fig. 1B). The dose-dependent inhibitory effect of EM on the ACh (20 nM)-induced response is summarized in Fig. 4. The maximal inhibitory effect was observed at 10^-5 M EM. This suppressive effect of low-dose macrolides on the ACh-induced response was rapidly reversible (Fig. 1).

A maximal concentration of ACh (10^-6 M) stimulated large and sustained currents without oscillation. As shown in Fig. 1C, both EM and CAM attenuated the maximal stimulation of the currents. To quantify the effects of macrolides on maximally stimulated inward and outward currents, we compared the electric charge responses to three successive ACh applications of short duration (~30 s), with 3-min intervals. Namely, to avoid contamination from desensitization to maximal ACh stimuli, mean ionic currents of the first and third responses without macrolide were compared with the second response, which was in the presence of macrolide (see Fig. 1C). The inward Cl^- current was reduced to 54 ± 9% of the nontreatment control values by 10^-5 M EM (P < 0.05; n = 7). The outward current was also attenuated significantly to 55 ± 7% of the control value.
P, 0.05; n = 7). CAM (10^{-5} M) also suppressed the ACh-induced currents significantly; i.e., the inward and outward currents were reduced to 83 ± 6% (P, 0.05; n = 5) and 84 ± 3% (P < 0.05; n = 5) of control values, respectively.

Effect of macrolides on K^+_1-channel activity in the excised membrane patch. To examine whether macrolides act directly on ion channels in the plasma membranes of acinar cells, excised outside-out patch experiments were performed. As previously reported (27), a large-conductance K^+_1 channel was the most frequent channel at the basolateral aspect of this cell type. The channel conductance was ~165 pS under K^+-rich saline on both sides of the patch membrane, and the activity was eradicated by outside TEA, a maxi-K^-channel inhibitor. This channel was Ca^{2+} dependent because 1) in the inside-out excised-patch experiment, the channel activity was totally abolished when Ca^{2+} was removed from the solution, with 1 mM EGTA added at the cytosolic aspect of the membrane, even at positive membrane potentials, and 2) the outward current at a 0-mV HP in the whole cell configuration was abolished in the presence of either TEA or 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid, a membrane-permeable Ca^{2+} chelator. Thus the outward current at a 0-mV HP in the whole cell configuration was carried by K^+_1 released through the maxi-K^-channel (BK channel). In the present study, the activity of the maxi-K^-channel was unaffected by either EM (10^{-5} and 3 × 10^{-3} M; n = 5 each), CAM (10^{-5} M; n = 3), or EM523 (10^{-4} M; n = 3; Fig. 5). The open-state probability of the K^- channel was 0.133 ± 0.020 for control activities and 0.132 ± 0.044 after 10^{-5} M EM (not significant; n = 5).

Effects of macrolides on currents induced by various stimulants. Macrolides suppressed the ACh-induced K^+ and Cl^- currents concurrently, and the activity of the BK channel itself on the excised membrane was...
insensitive to macrolides. Therefore, we considered that the point of action of macrolides might be upstream from the channel activation. To elucidate further the mechanism involved in this acutely suppressive effect of low-dose macrolides, experiments were carried out with various Ca\textsuperscript{2+}-mobilizing agents because both currents are Ca\textsuperscript{2+}-dependent (27). There are two sources of cellular Ca\textsuperscript{2+}, extracellular and intracellular, and there are at least two mechanisms in cellular Ca\textsuperscript{2+} release, one mediated by inositol 1,4,5-trisphosphate, a product of phospholipase (PL) C coupled to receptor activation, and the other Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. The latter mechanism has been known to be facilitated by caffeine (31). In previous studies, Sasaki et al. (27) and Shimura et al. (28) confirmed that both mechanisms can operate in human and feline submucosal gland acinar cells. The extracellular Ca\textsuperscript{2+} appears to have a direct connection to the occurrence and cessation of cellular Ca\textsuperscript{2+} oscillations in cells lacking Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release Ca\textsuperscript{2+} storage, such as the avian salt gland (14). However, in the tracheal gland, the agonist-induced oscillations in ionic currents were attenuated gradually by the removal of extracellular Ca\textsuperscript{2+} and took several minutes to be abolished completely (27). In the present study, macrolides interrupted the ACh-induced oscillations abruptly (Fig. 1), in clear contrast to the effect of Ca\textsuperscript{2+} removal. We therefore considered that the inhibitory effect of macrolides was not derived from its action on the Ca\textsuperscript{2+}-influx pathway but from its action on the cellular release mechanisms. Hence we tested the possibility of macrolides influencing these intracellular Ca\textsuperscript{2+}-mobilizing mechanisms. Currents evoked by the α-adrenergic agonist phenylephrine (10\textsuperscript{-4} M; n = 4), the sympathetic neurotransmitter norepinephrine (10\textsuperscript{-4} M; n = 4), or caffeine (2×10\textsuperscript{-2} M; n = 6) were unaffected by EM (Fig. 6, A and B). Furthermore, currents induced by ionomycin (5×10\textsuperscript{-7} M), a Ca\textsuperscript{2+} ionophore, were also insensitive to macrolides (CAM, n = 5; EM523, n = 4; 10\textsuperscript{-5} M each). These results suggested that the inhibitory action of macrolides was derived neither by influencing the mechanisms of cellular Ca\textsuperscript{2+} mobilization downstream from PLC nor by decreasing the Ca\textsuperscript{2+} sensitivity of the effector ion channels.

**DISCUSSION**

In some exocrine glands including those from airways, ACh (via the muscarinic receptor), phenylephrine, and norepinephrine (via the α-adrenergic receptor) activate K\textsuperscript{+} and Cl\textsuperscript{−} currents through Ca\textsuperscript{2+} as a common second messenger (20, 27). These secretagogues activate, via a guanine nucleotide-binding protein (G protein)-dependent mechanism, the plasma membrane PLC that generates inositol 1,4,5-trisphosphate and diacylglycerol, the former releasing Ca\textsuperscript{2+} from intracellular pools and initiating the Ca\textsuperscript{2+} signal. In the present study, we showed that EM and its derivatives reversibly attenuated the ACh-evoked but not the phenylephrine- and norepinephrine-induced Ca\textsuperscript{2+}-dependent ionic currents in freshly isolated feline tracheal glands. This ACh-specific inhibitory effect of therapeutic levels of macrolides (16) indicates that the point of action of the agents is upstream from the G protein linked to the muscarinic receptor. Furthermore, high doses of macrolides per se stimulated the ionic currents in a manner similar to that of ACh, and these were abolished in the presence of atropine. This finding indicates that macrolides have a certain affinity to muscarinic receptors. The term “partial agonist” is defined as a drug that produces submaximal tissue responses and competitively blocks the effects of those with higher intrinsic efficacies (11). Although descriptive pharmacological analyses were not carried out, EM may act as a partial agonist with a certain threshold on airway gland electrolyte secretion.

The inhibitory action of macrolides on ACh-evoked currents has also been reported in the guinea pig nasal gland (9), although secretagogues other than ACh were not examined. These investigators found that the ACh-evoked K\textsuperscript{+} and Cl\textsuperscript{−} currents were attenuated concurrently in the presence of macrolides as in the present study. However, the authors attributed the effect of macrolides to their direct inhibitory action on Cl\textsuperscript{−} conductance. If that were true also in tracheal glands,
it would be necessary for macrolides to block the $K^+$ channel directly as well as the $Cl^-$ channel. However, this possibility is remote in tracheal gland acinar cells because the $Ca^{2+}$-dependent $K^+$ channel was not sensitive to macrolides in the present excised-patch experiments (Fig. 5). In vivo, it might be possible that a membrane hyperpolarization due to a $Cl^-$-channel blockade could induce an inhibition of $K^+$ channels secondarily because the dominant $K^+$ channels on the tracheal gland plasma membrane are activated by membrane depolarization as well as by cellular $Ca^{2+}$ (27). But this would not be pertinent to the voltage-clamp experiments. Finally, the possibility of a direct $Cl^-$-channel inhibition was excluded in the tracheal gland preparation because macrolides were without effect on the inward $Cl^-$ currents stimulated by adrenergic agonists and ionomycin (Fig. 6).

A direct effect of EM on respiratory mucus secretion has been investigated in an in vitro preparation of human airways (7). In contrast to the present results in electrolyte secretion, EM was found to reduce both spontaneous (baseline) and stimulated glycoconjugate secretion (by either histamine or the cholinergic agonist methacholine) from airways in culture. It is known that the mucus and fluid secretions from the submucosal gland are regulated individually. That is, the mucus secretion is regulated largely by $\beta$-adrenergic stimuli via cAMP (5), whereas the fluid secretion is regulated mainly by $\alpha$-adrenergic stimuli and cholinergic stimuli energize both secretions (1). Additionally, it has recently been shown in porcine bronchi that the ACh-induced liquid secretion could be uncoupled from mucus secretion by pretreatment with blockers of both $Cl^-$ and $HCO_3^-$ transporters (10), which clearly demonstrated that different mechanisms underlie the two types of secretions. Expectedly, methacholine in combination with EM yielded almost the same mucus production compared with that in control cells (7), and the inhibitory effect of EM on mucus secretion was more specific to histamine. Their findings are consistent with ours in regard to the anticholinergic effect. However, with respect to the effect on histamine-induced mucus secretion, probably mediated by cAMP through the activation of histamine $H_2$ receptors, a distinctive pathway other than anticholinergic may exist, that is, a blockade of PLA$_2$, as they proposed (7).

In the present study, we could not find a common mechanism responsible for the reported clinical and experimental observations as a whole. This was probably because the present investigation focused on the acute effects of the agents, at most several tens of minutes. Macrolides exhibit not only a secretory inhibitory effect but also anti-inflammatory actions by inhibiting the release of cytokines and/or the migration of inflammatory cells (12, 18) to the extent that it results in a structural restoration of pathological lungs (8). It is notable, in this respect, that the clinical efficacy of
Macrolides on copious airway secretion has been shown to be attained very rapidly, even within a few days (13), whereas it takes months of treatment to achieve the anti-inflammatory effects (8, 18). Macrolides have at least two sites of action. One is a cell surface receptor, which is known as "motilide action," resulting from an interaction with motilin receptors. In addition, macrolides have been shown to penetrate into the cell interior (3). One of the EM derivatives, FK506, clinically used as an immunosuppressant, has been shown to inhibit cytokine production, including IL-2 and IL-8, at the gene transcription level in activated T lymphocytes (19). This anti-inflammatory action may not be detected within an order of minutes. Taken together, in the present investigation, we have isolated a cholinomimetic action as a novel acute extracellular-phase behavior out of a broad spectrum of actions of macrolide antibiotics.

The airway submucosal gland has been demonstrated to exhibit a marked hypertrophy and hyperplasia.

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Fig. 5. Continuous recording showing effect of CAM on K⁺-channel activity in excised outside-out patch. Outside-out patch was obtained after establishment of whole cell configuration, and pipette was then raised to air-water interface. Seal resistance was >1 GΩ, and maxi-K⁺ channel (BK channel) was validated by its sensitivity to tetraethylammonium (TEA; 1 mM). Membrane potential was held at +20 mV throughout. No discernible effect on BK-channel activity was found.

Fig. 6. Effects of macrolides on tracheal gland responses to stimulants other than ACh. Macrolides were without effects on norepinephrine (NE; A), caffeine (B), and ionomycin (C)-activated currents. In A and C, membrane currents were monitored at 3 different HPs (nos. on right), which was accomplished by applying 100-ms-duration +40- or −40-mV pulses to −40-mV HP. Apparent double traces at −40-mV HP are due to fluctuations in capacitance current.
zia in chronic inflammatory lung diseases (22). Furthermore, an upregulated secretory response specific to the cholinergic muscarinic pathway has also been reported in an experimental bronchitic airway (15). The anticholinergic effect of EM and its derivatives may exhibit a pronounced effectiveness in such pathological airways.

We thank Brent Bell for reading the manuscript.

This work was supported by Grant-in-Aid no. 0770650 for Scientific Research from The Ministry of Education, Science, Sports and Culture, J apan (to T. Sasaki).

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Received 20 August 1998; accepted in final form 26 February 1999.

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