

The TLR7 agonist imiquimod induces bronchodilation via a nonneuronal TLR7-independent mechanism: a possible role for quinoline in airway dilation

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Submitted 19 August 2015; accepted in final form 7 April 2016

Larsson OJ, Manson ML, Starkhammar M, Fuchs B, Adner M, Kumlien Georén S, Cardell LO. The TLR7 agonist imiquimod induces bronchodilation via a nonneuronal TLR7-independent mechanism: a possible role for quinoline in airway dilation. *Am J Physiol Lung Cell Mol Physiol* 310: L1121–L1129, 2016. First published April 15, 2016; doi:10.1152/ajplung.00288.2015.—Toll-like receptor (TLR) 7 agonists are known to reduce allergic airway inflammation. Their recently reported ability to rapidly relax airways has further increased their interest in the treatment of pulmonary disease. However, the mechanisms behind this effect are not fully understood. The present study, therefore, aimed to determine whether airway smooth muscle (ASM)-dependent mechanisms could be identified. TLR7 agonists were added to guinea pig airways following precontraction with carbachol in vitro or histamine in vivo. Pharmacological inhibitors were used to dissect conventional pathways of bronchodilation; tetrodotoxin was used or bilateral vagotomy was performed to assess neuronal involvement. Human ASM cells (HASMCS) were employed to determine the effect of TLR7 agonists on intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) mobilization. The well-established TLR7 agonist imiquimod rapidly relaxed precontracted airways in vitro and in vivo. This relaxation was demonstrated to be independent of nitric oxide, carbon monoxide, and cAMP signaling, as well as neuronal activity. A limited role for prostanoids could be detected. Imiquimod induced $[\text{Ca}^{2+}]_i$ release from endoplasmic reticulum stores in HASMCS, inhibiting histamine-induced $[\text{Ca}^{2+}]_i$. The TLR7 antagonist IRS661 failed to inhibit relaxation, and the structurally dissimilar agonist CL264 did not relax airways or inhibit $[\text{Ca}^{2+}]_i$. This study shows that imiquimod acts directly on ASM to induce bronchorelaxation, via a TLR7-independent release of $[\text{Ca}^{2+}]_i$. The effect is paralleled by other bronchorelaxant compounds, like chloroquine, which, like imiquimod, but unlike CL264, contains the chemical structure quinoline. Compounds with quinoline moieties may be of interest in the development of multifunctional drugs to treat pulmonary disease.

asthma; bronchodilation; imiquimod; quinoline; toll-like receptor 7

TOLL-LIKE RECEPTOR (TLR) AGONISTS have recently become of interest as novel therapeutic targets for the treatment of allergy and asthma (2). TLRs, which are a subset of virus- and bacteria-sensing, pathogen-recognition receptors, are pivotal for the early response against infection. Activation results in an immediate production of proinflammatory cytokines (23), and, most commonly, development of a Th1 response.

Treatment with TLR agonists, alone or as adjuvants in allergen-specific immunotherapy (34), has been shown to modify and dampen the allergic inflammatory response (2, 8). In particular, treatment with agonists of TLR7, which recognizes

viral single-stranded RNA, results in a reduction in Th2 cytokine levels, eosinophilia, goblet cell hyperplasia, and total IgE levels (3, 17, 21, 37, 38), as well as airway hyperresponsiveness in vivo (1) and airway reactivity following long-term culture in vitro (12). In the clinic, TLR7 agonists have proved effective in the treatment of seasonal allergic rhinitis (16).

TLR7 agonists are of particular interest as novel studies have demonstrated that the prototypical agonist imiquimod (R-837) also harbors the ability to induce rapid airway relaxations in vitro, as well as in vivo (11, 22). This further highlights a role for the use of these agonists in the treatment of asthma. To date, relaxation has primarily been attributed to a TLR7-dependent release of neuronally derived nitric oxide (NO), but other additional mechanisms must be considered. The present study was designed to characterize this in depth.

MATERIALS AND METHODS

Animals. Male Dunkin-Hartley guinea pigs (250–750 g) were obtained from Harlan (Horst, The Netherlands). They were housed in groups of five in plastic cages with adsorbent bedding in temperature and light-dark cycle (12:12 h) controlled rooms. Food and water were available ad libitum.

Animals were handled in accordance with the Federation for European Laboratory Animal Science Associations guidelines. All animal procedures were approved by the local ethics committee at Karolinska Institutet (Stockholm norra djurförsöksetiska nämnd; ethical permit nos.: N44-12, N41-14, and N143-14). In total, 60 animals were used for this study.

In vitro pharmacology. Organ bath experiments with guinea pig trachea were performed as described previously (32, 33). Animals were killed by CO_2 or an overdose of pentobarbital sodium (Apoteket, Stockholm, Sweden). The trachea was quickly removed and dissected free of surrounding connective tissue in Krebs-Henseleit buffer solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, MgCl_2 1.2, CaCl_2 2.5, NaHCO_3 25, and D-glucose 11.1). Tracheal segments were cut along the cartilage into eight rings of equal length and mounted in 5-ml organ baths [or myographs for electric field stimulation (EFS) experiments] filled with Krebs-Henseleit buffer solution at 37°C, bubbled with carbon gas (5% CO_2 in O_2). Changes in smooth muscle force were detected using an isometric force-displacement transducer linked to a Grass polygraph. For denudation experiments, epithelium was removed by mechanical scraping with a scalpel and confirmed via microscopy.

Segments were equilibrated for 60 min, where the force was adjusted to 30 mN. Tracheal reactivity was assessed through the cumulative addition of histamine (0.1 nM to 0.1 mM), and, after a 30-min wash-out period, further pharmacological studies were conducted. Tracheal rings were precontracted with carbachol (100 nM). Once stable precontractions were obtained, segments were exposed to cumulative concentrations of imiquimod (0.1–100 μM ; Invivogen, San Diego, CA), R-848 (0.1–300 μM ; Invivogen), CL-264 (0.1–300

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μM ; Invivogen), salbutamol (0.1–1,000 nM), or vehicle. At the end of each experiment, maximal relaxations were assessed for each individual segment through the administration of papavarine (100 μM) and sodium nitroprusside (100 μM). For EFS experiments, segments were equilibrated, force was adjusted, and reactivity was assessed as above. Segments were exposed to indomethacin (3 μM) and atropine (1 μM) for 30–40 min and subsequently exposed to histamine (1 μM) until a stable contraction was obtained. Electrodes delivering electrical stimulation were placed on opposite sides of the segments (Current Stimulator model CS200, J.P. Trading, Aarhus, Denmark). Three consecutive 60-s stimulations (16.0 Hz, 55 mA, 1.0-ms pulse duration) were given 150 s apart, to induce reversible tracheal relaxation.

In vivo studies. Guinea pigs were ventilated with a flexiVent animal ventilator (Scireq, Montreal, Quebec, Canada). Following anesthesia with fentanyl (500 $\mu\text{g}/\text{kg}$ ip), midazolam (30 mg/kg ip), and droperidol (5 mg/kg ip) (all Apoteket), animals were placed on a heating pad (37°C), tracheotomized, and connected to the ventilator via a 16-gauge (2.1 mm) cannula. Guinea pigs were given repeated doses of anesthetic, at 50% of the original dose, every 40 min. Pulse and SO_2 levels remained stable throughout the experiment. Airways were precontracted with a continuous infusion of histamine (7.5–12.5 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ iv), to produce an approximate fivefold increase in the basal lung resistance. Once a stable baseline had been reached, aerosolized distilled water (dH_2O) or imiquimod was given. In concentration-response experiments, increasing concentrations of imiquimod were given noncumulatively. A stable baseline of contraction was recovered before treatment with the subsequent concentration of imiquimod. For vagotomy experiments, animals were bilaterally vagotomized before infusion of histamine.

In vitro and in vivo pharmacological interventions. The pharmacological interventions N^G -nitro-L-arginine methyl ester (L-NAME; 100 μM in vitro, 30 mg/kg in vivo) and N^G -monomethyl-L-arginine (L-NMMA; 100 μM) were used to study the involvement of NO; tetrodotoxin (TTX; 1 μM) was used to study the involvement of neurons; KT-5823 (1 μM), Rp-8-Br-PET-cGMPS (0.1 mg/kg iv), H89 (10 μM), and zinc protoporphyrin IX (ZnPP9; 30 μM) were given to study the involvement of protein kinase G (PKG), cyclic guanine monophosphate (cGMP), protein kinase A (PKA), and carbon monoxide (CO); and the TLR7 antagonist IRS661 (100 μM in vitro, 0.2 mg/kg iv in vivo) was used to study the involvement of TLR7. In vitro, L-NAME and L-NMMA were administered on established precontractions, 30 min before imiquimod administration; TTX was administered 30 min before precontraction; KT-5823 and H89 were administered 30 min before the precontractions; ZnPP9 was administered 30 min before precontractions or acutely; and IRS661 was administered 1 h before the induction of precontractions. As the level of precontraction has been shown to affect the response to different bronchorelaxants (e.g., salbutamol), precontractions were adjusted to 50–60% of the reference maximal contractions of the individual rings. Except for studies using indomethacin, all in vitro pharmacological interventions were performed in the presence of EP₁ receptor antagonist ONO-8130 (100 nM), which prevents prostanoid-induced modulation of contractile and relaxing responses (33). In vivo, L-NAME was administered 30 min before imiquimod treatment; Rp-8-Br-PET-cGMPS was administered 0, 15, 30, and 45 min before imiquimod; and IRS661 was administered 60, 90, or 120 min before imiquimod. Stable histamine-induced precontractions were established, and the response to imiquimod was assessed before administration of pharmacological agents. In all experiments using pharmacological interventions in vivo, imiquimod was administered at a concentration of 3 mg/ml.

Measurement of Ca^{2+} flux in human airway smooth muscle cells. Primary bronchial human airway smooth muscle cells (HASMCS) were obtained from Promocell (Heidelberg, Germany). Cells from four separate patients were used, between passages 4 and 6. Cells were cultured in smooth muscle cell growth medium supplemented with 5% fetal calf serum, 0.5 ng/ml epidermal growth factor, 2 ng/ml

basic fibroblast growth factor, 5 $\mu\text{g}/\text{ml}$ insulin, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco, NY), and 0.25 $\mu\text{g}/\text{ml}$ Fungizone (Gibco); they were maintained in a humidified chamber at 37°C with a constant supply of 5% CO_2 . Media and supplements were obtained from Promocell, unless otherwise specified. Before experiments, cells were growth arrested with serum-free media for 24 h. Cells were stained with the calcium indicator fluo 4-AM (3 μM , Molecular Probes, Invitrogen, San Diego, CA), and changes in mean fluorescence intensity over time were detected on an BD Accuri C6 Flow Cytometer (BD, San Jose, CA). Analysis was performed with FlowJo software (Tree Star, Ashland, OR). In *experimental setup 1*, following a 30-s baseline recording of Ca^{2+} fluxes, cells were exposed to imiquimod (100 μM) or histamine (100 μM). In *experimental setup 2*, cells were pretreated with thapsigargin (1 μM) or dH_2O for 30 min. Following a 60-s baseline recording of Ca^{2+} fluxes, cells were exposed to imiquimod (100 μM). In *experimental setup 3*, Ca^{2+} fluxes were continuously monitored. At 60 s, cells were exposed to imiquimod (100 μM) or dH_2O . At 300 s, cells were exposed to thapsigargin (1 μM). In *experimental setup 4*, cells were preincubated with different concentrations of imiquimod, CL264 (10, 30, or 100 μM), or vehicle for 5 min. Following a 30-s baseline recording of Ca^{2+} fluxes, cells were exposed to histamine (100 μM).

Drugs and materials. NaCl, KCl, KH_2PO_4 , MgCl_2 , CaCl_2 , NaHCO_3 , and D-glucose were purchased from VWR (West Chester, PA). Carbachol, histamine, salbutamol, papavarine, sodium nitroprusside, L-NAME, L-NMMA, Rp-8-Br-PET-cGMPS, indomethacin, and IRS661 were purchased from Sigma Aldrich (St. Louis, MO). Imiquimod, R-848, and CL-264 were purchased from Invivogen. KT-5823, H89, thapsigargin, and TTX were purchased from Tocris Bioscience (Bristol, UK). ONO-8130 was a kind gift from ONO Pharmaceuticals (Japan).

Statistics. Data was analyzed using GraphPad Prism 6 Software (San Diego, CA). All results are presented as means $\pm$ SE. For in vitro pharmacology experiments, the concentration-response curve values for pEC_{50} and E_{max} were calculated using nonlinear regression analysis. EC_{50} values were compared using Student's *t*-test. In vivo and Ca^{2+} mobilization experiments were analyzed using a one-way or a two-way ANOVA (vagotomy), followed by Bonferroni post hoc tests.

RESULTS

Imiquimod relaxes precontracted guinea pig airways in vitro and in vivo. Cumulative addition of imiquimod in vitro resulted in concentration-dependent and rapid complete relaxation of tracheal rings precontracted with carbachol (Fig. 1A, $\text{pEC}_{50} = 4.46 \pm 0.05$, $\text{EC}_{50} = 34.8 \mu\text{M}$), verifying previously published results (22).

For in vivo studies, continuous intravenous infusion of histamine resulted in a stable and reproducible contraction 40–50 min after the start of infusion. Challenge with aerosolized imiquimod (0.3–30 mg/ml) resulted in a concentration-dependent relaxation of contraction, as measured by a reduction in Newtonian resistance (R_N ; an approximation of resistance in the conducting airways, Fig. 1B, $P < 0.0001$) and tissue resistance (G ; which reflects energy dissipation in peripheral lung tissue, Fig. 1C, $P < 0.0001$). A similar trend was evident for tissue elastance (H , a reflection of tissue stiffness in peripheral lung tissue), but did not reach significance (data not shown). Relaxation occurred within 10 s of administration; contraction returned to baseline levels after 10–15 min.

Administration of imiquimod alone had no effect on baseline in vitro or in vivo (data not shown).

Imiquimod relaxes precontracted guinea pig airways independently of the epithelium. The tracheal epithelium is known to release mediators involved in bronchodilation. In this light,

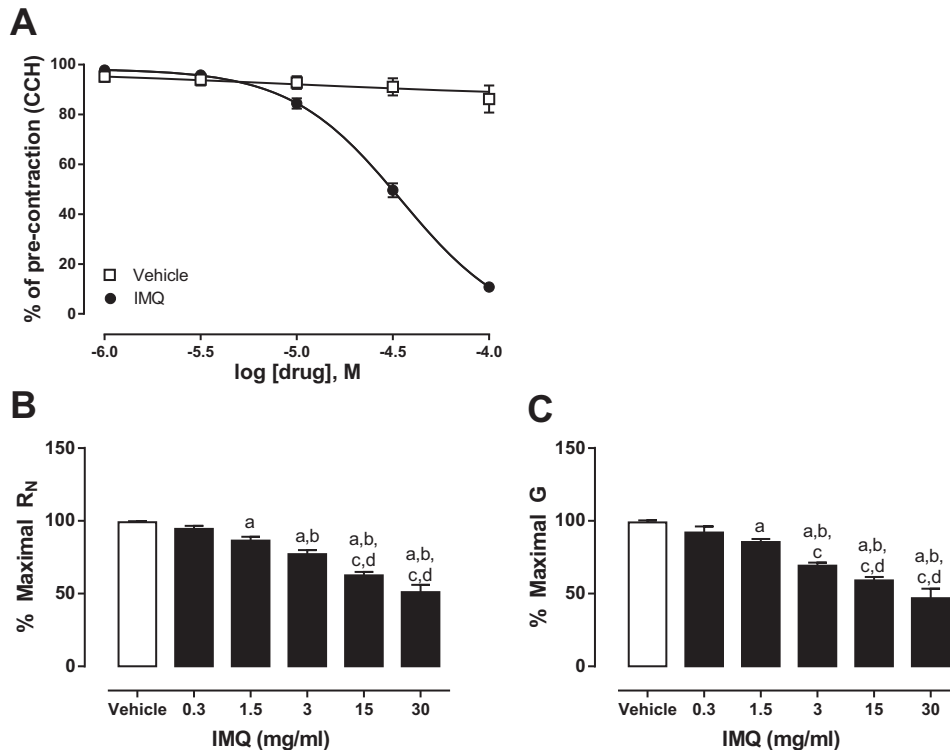


Fig. 1. Imiquimod (IMQ) relaxes precontracted guinea pig airways in vitro and in vivo. **A**: tracheal rings were precontracted with carbachol (CCH) in vitro. Increasing doses of IMQ were added cumulatively, and percent change from maximum contraction was determined ($n = 12-26$). Values are mean percentage of maximal contraction $\pm$ SE. **B** and **C**: guinea pig airways were precontracted with intravenous histamine in vivo. Aerosolized IMQ was added noncumulatively, and changes in maximal Newtonian resistance (R_N ; **B**) and tissue resistance (G ; **C**) were determined. Values are mean percentage of maximal resistance $\pm$ SE. $P < 0.05$ vs. ^avehicle, ^b0.3 mg/ml, ^c1.5 mg/ml, and ^d3 mg/ml using one-way ANOVA, followed by a Bonferroni post hoc test ($n = 7-18$).

we assessed whether the epithelium was necessary for imiquimod-mediated relaxation. However, denudation of the tracheal epithelium had no significant effect on imiquimod-mediated relaxation (Table 1).

Imiquimod relaxes precontracted guinea pig airways independently of conventional pathways of bronchodilation. Using our in vitro and in vivo models, we next examined molecules and associated signaling pathways involved in bronchodilation, including the role of NO and prostanoids, previously implicated in imiquimod-mediated bronchodilation (11, 22), CO, cGMP, PKG, and PKA.

Administration of indomethacin before imiquimod administration in vitro resulted in a small, but significant, decrease in potency (Table 1, $P = 0.021$), compared with administration of vehicle, which is in accordance with previous observations (22). Comparatively, exposure to L-NAME, L-NMMA (NO synthase inhibitors; Fig. 2A, Table 1), ZnPP9 [heme oxygenase 1 (HO-1) inhibitor], and KT-5823 (PKG inhibitor, Fig. 2B,

Table 1) did not significantly alter imiquimod-mediated relaxation. Administration of H89 (PKA inhibitor) significantly reduced the potency of salbutamol-mediated relaxation ($P = 0.014$), but had no effect on imiquimod-mediated relaxation of precontracted guinea pig airways in vitro (Table 1).

Intravenous administration of L-NAME in vivo resulted in a small, nonsignificant trend ($P = 0.08$) toward a reversal of imiquimod (3 mg/ml)-induced reduction in R_N , but had no effect on G (Fig. 3, A and B). Rp-8-Br-PET-cGMPS (cGMP synthase inhibitor) had no effect on imiquimod-mediated reduction in R_N or G , when given before imiquimod (Fig. 3, C and D).

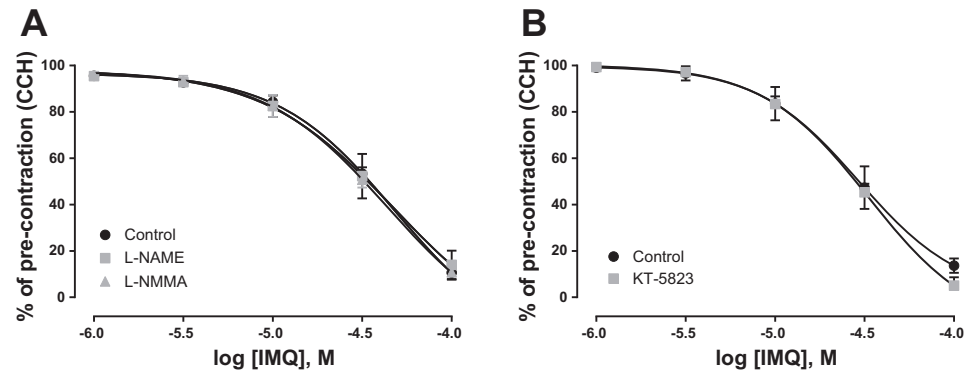
Imiquimod relaxes precontracted guinea pig airways in vivo and in vitro, independently of central and peripheral neurons. Bilateral vagotomy was performed to determine the involvement of central neuronal reflexes on relaxation in vivo. Histamine-induced increases in R_N and G were unaffected by vagotomy (data not shown). There was no significant differ-

Table 1. Relaxation of precontracted guinea pig tracheal rings following incubation with inhibitors of endogenous bronchodilatory mediators

Bronchorelaxant	Signaling Molecule	Intervention	pEC ₅₀ $\pm$ SE (Vehicle)	pEC ₅₀ $\pm$ SE (Inhibitor)	P Value (Vehicle vs. Inhibitor)	n
Imiquimod	Epithelium	Denudation	-4.5 ± 0.06	-4.6 ± 0.03	0.33 (ns)	8
	COX-1/2	Indomethacin	-4.6 ± 0.02	-4.4 ± 0.07	0.02*	5
	NOS	L-NAME	-4.3 ± 0.11	-4.3 ± 0.28	0.51 (ns)	4
		L-NMMA	-4.3 ± 0.11	-4.3 ± 0.11	>0.99 (ns)	4
	HO-1	ZnPP9	-4.5 ± 0.14	-4.5 ± 0.08	0.41 (ns)	4
	PKG	KT-5823	-4.5 ± 0.14	-4.4 ± 0.07	0.59 (ns)	4
	PKA	H89	-4.6 ± 0.15	-4.7 ± 0.11	0.58 (ns)	4
	Neuron	TTX	-4.5 ± 0.06	-4.6 ± 0.06	0.60 (ns)	4

n, No. of animals. Guinea pig tracheal rings were precontracted with carbachol and subsequently exposed to imiquimod or salbutamol. COX-1/2, cyclooxygenase-1/2; NOS, nitric oxide synthase; HO-1, heme oxygenase 1; PKG, protein kinase G; PKA, protein kinase A; TTX, tetrodotoxin; pEC₅₀, log of effective concentration of 50%; ns, nonsignificant. *Significant difference.

Fig. 2. Imiquimod (IMQ) relaxes precontracted guinea pig airways independently of nitric oxide (NO) and protein kinase G (PKG) in vitro. Tracheal rings were precontracted with carbachol (CCH). IMQ was added in the presence or absence of the NO inhibitors L-NAME or L-NMMA (A) or the PKG inhibitor KT-5823 (B). Values are mean percentage of maximal contraction $\pm$ SE ($n = 4$).



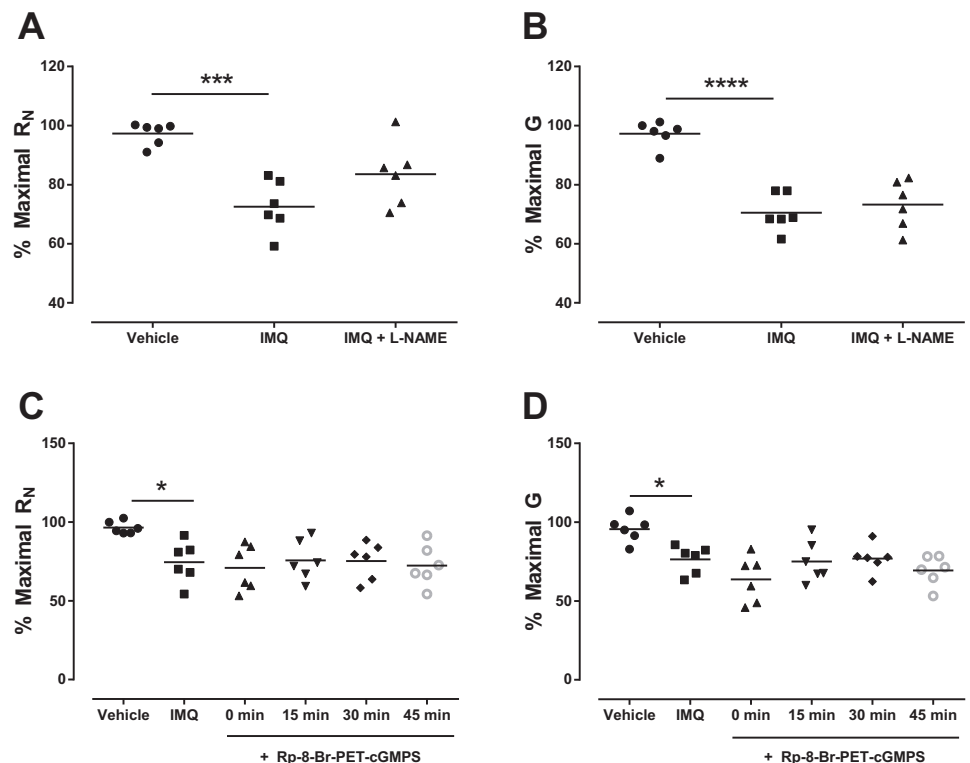
ence in reduction of R_N (Fig. 4A) or G (Fig. 4B) between vagotomized and sham-vagotomized animals following aerosolized imiquimod administration, at any of the concentrations used.

Using our in vitro model, we further assessed if imiquimod-induced relaxation was dependent on bronchodilatory mediators released by inhibitory nonadrenergic, noncholinergic (iNANC) neuronal fibers, using the neurotoxin TTX. Sensitivity of iNANC fibers to TTX was first determined using a model of neuronally mediated bronchodilation. Guinea pig tracheas were precontracted with histamine; EFS was subsequently applied to the tracheas in the presence of atropine, to exclude cholinergic effects. EFS resulted in a rapid and reversible relaxation of tracheas that was completely inhibited by TTX (Fig. 4C). Comparatively, pretreatment with TTX had no effect on imiquimod-induced relaxation (Fig. 4D, Table 1).

Imiquimod induces intracellular Ca^{2+} mobilization and blocks histamine-induced Ca^{2+} mobilization, in isolated HASMCs. The effect of imiquimod on intracellular Ca^{2+} mobilization ($[Ca^{2+}]_i$) a prerequisite of airway smooth muscle

(ASM) cell contraction, was subsequently measured, as the data indicated that imiquimod-mediated relaxation was not mediated by conventional bronchodilatory pathways. Surprisingly, addition of imiquimod to HASMC induced a reproducible increase in mean fluorescence intensity, an arbitrary measure of $[Ca^{2+}]_i$ concentration (Fig. 5A). The increase in $[Ca^{2+}]_i$ was significantly higher than that induced by histamine (Fig. 5, A and B). A similar rise in $[Ca^{2+}]_i$ following imiquimod exposure was evident when extracellular calcium was removed (data not shown). Pretreatment with thapsigargin significantly blunted the imiquimod-induced rise in $[Ca^{2+}]_i$ in HASMC (Fig. 5C); reciprocally, imiquimod inhibited thapsigargin-induced increases in $[Ca^{2+}]_i$ (Fig. 5D), suggesting the endoplasmic reticulum (ER) to be a source of imiquimod-induced $[Ca^{2+}]_i$. To assess if the imiquimod-mediated rise in $[Ca^{2+}]_i$ could impact the rise in $[Ca^{2+}]_i$ following histamine, HASMC were preincubated with imiquimod for 5 min and subsequently exposed to histamine. Imiquimod preincubation resulted in a concentration-dependent reduction in $[Ca^{2+}]_i$ following addition of histamine ($P = 0.004$) (Fig. 5, E and F), with a potency

Fig. 3. Imiquimod (IMQ) relaxes precontracted guinea pig airways independently of nitric oxide (NO) and cGMP in vivo. Guinea pig airways were precontracted with intravenous histamine in vivo. The NO inhibitor L-NAME (30 mg/kg; A and B) or the cGMP inhibitor Rp-8-Br-PET-cGMPS (0.1 mg/kg; C and D) were given before administration of aerosolized IMQ (3 mg/kg). Changes in Newtonian resistance (R_N ; A and C) and tissue resistance (G; B and D) were determined. Values are mean percentage of maximal resistance. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ vs. vehicle using one-way ANOVA, followed by a Bonferroni post-hoc test ($n = 6$).



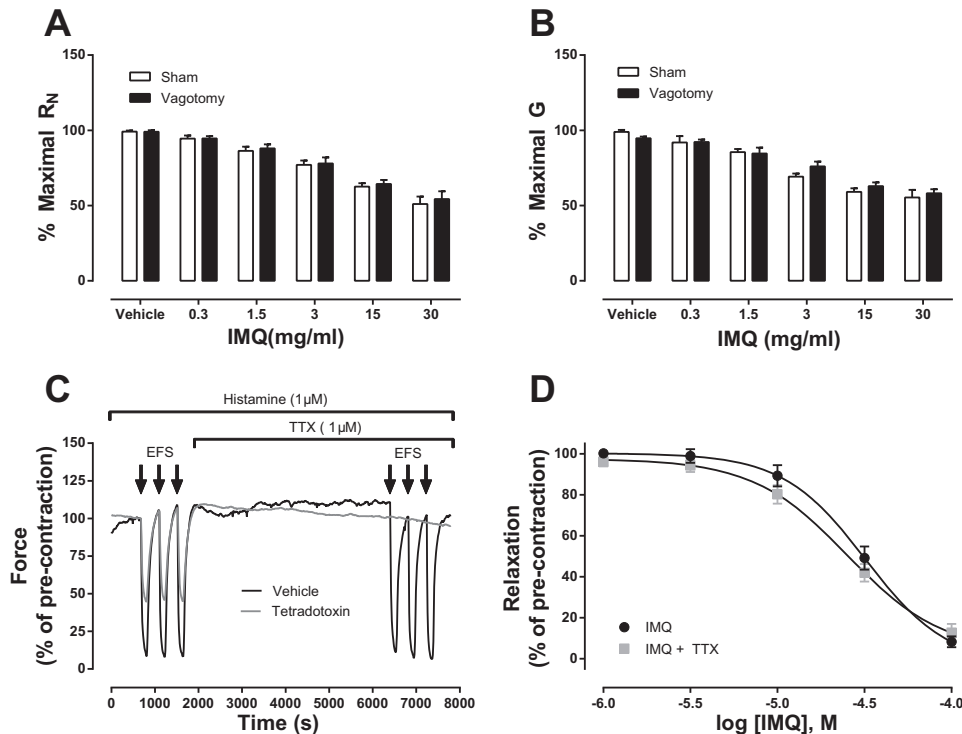


Fig. 4. Imiquimod (IMQ) relaxes precontracted guinea pig airways independently of the neuronal pathways. *A* and *B*: guinea pig airways were precontracted with intravenous histamine in vivo. Before contraction, animals were bilaterally vagotomized or sham equivalent. Aerosolized IMQ was given non-cumulatively, and changes in maximal Newtonian resistance (R_N ; *A*) and tissue resistance (G ; *B*) were determined. Values are mean percentage of maximal resistance $\pm$ SE ($n = 7-18$). Data were analyzed using a two-way ANOVA, followed by a Bonferroni post hoc test. *C*: a representative trace to show inhibition of electric field stimulation (EFS)-induced bronchorelaxation with tetrodotoxin (TTX). *D*: tracheal rings were precontracted with carbachol. IMQ was added in the presence or absence of TTX. Values are mean percentage of maximal contraction $\pm$ SE ($n = 4$).

similar to imiquimod-mediated bronchorelaxation in vitro ($pEC_{50} = 4.68 \pm 0.08$).

Imiquimod-induced relaxation is not dependent on TLR7. The role of TLR7 on imiquimod-induced airway relaxation was subsequently examined, using the TLR7 antagonist IRS661 and other structurally similar and dissimilar TLR7 agonists. Intravenous administration of IRS661 in vivo had no significant effect on imiquimod-mediated reduction in R_N (Fig. 6A) or G (Fig. 6B), when administered before imiquimod. Similarly, no significant difference in imiquimod-induced relaxation in IRS661-treated tracheal rings was found, compared with control (Fig. 6C).

As reported (22), R-848, a TLR7/8 agonist, and imidazoquinoline, with a similar chemical structure to R-837, induced a concentration-dependent relaxation of tracheal rings precontracted with carbachol, but with a lower potency. The structurally dissimilar TLR7 agonist CL264 (19) is a more potent activator of TLR7-specific immune events (10) (data not shown). However, CL264 did not relax precontracted guinea pig trachea (Fig. 6D) and had no effect on histamine-induced increases in $[Ca^{2+}]_i$ (Fig. 6, E and F).

DISCUSSION

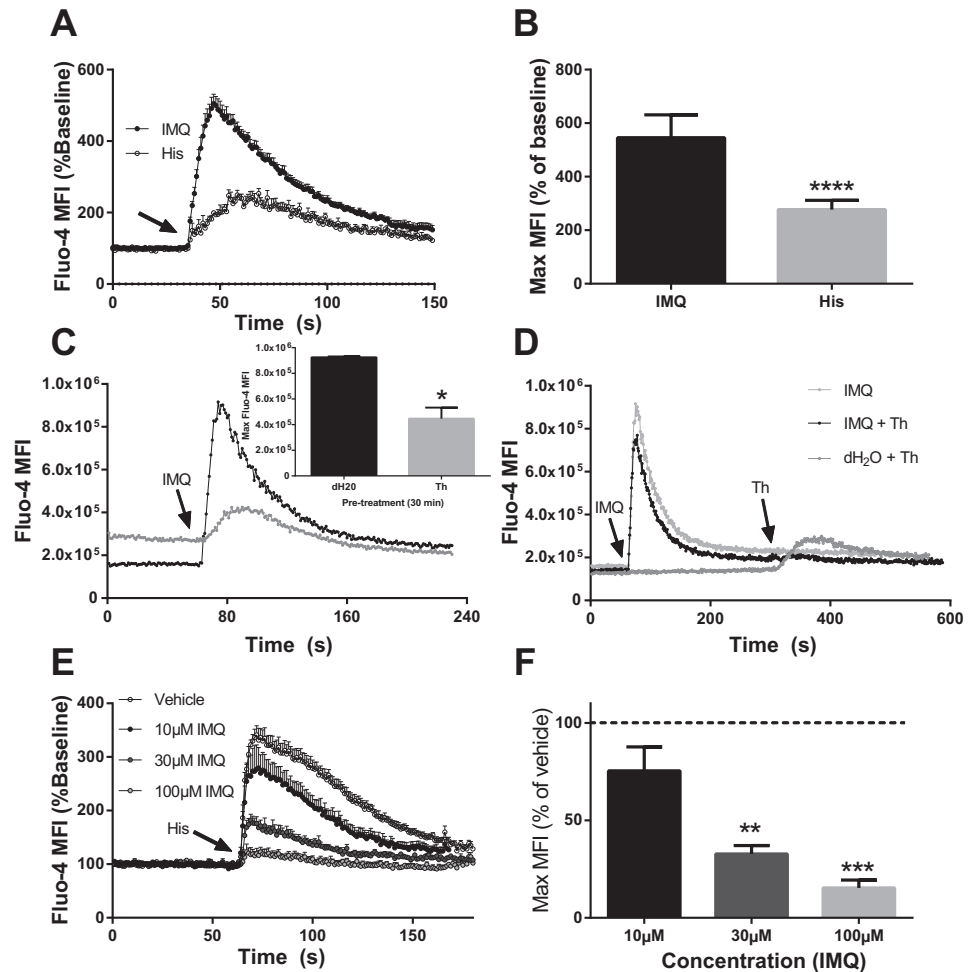
TLR7 ligands have shown preclinical and clinical success in the treatment of allergic disease, due to their ability to modulate and reduce allergic airway inflammation (16, 17, 37, 38). Their recently reported ability to rapidly relax airways (11, 22) has increased their interest in the treatment of pulmonary disease. The present study verified the ability for imiquimod to induce a strong and rapid dilatory effect in isolated guinea pig airways. It also, for the first time, demonstrated that imiquimod could directly relax airways precontracted with histamine in vivo. Epithelial denudation and TTX-mediated blockage of neuronal release ruled out the involvement of epithelium- or

neuron-derived mediators. In addition, various pharmacological inhibitors further dismissed the involvement of traditional airway dilatory mechanisms, including NO, CO, and cAMP signaling. Further investigations in isolated ASM cells demonstrated that imiquimod induced a rise in ER-derived $[Ca^{2+}]_i$, which was associated with an inhibition $[Ca^{2+}]_i$ following histamine exposure. Bronchorelaxatory effects were not affected by TLR7 antagonism, and neither relaxation nor Ca^{2+} inhibition were evident in response to the structurally dissimilar TLR7 agonist CL264, signifying that this effect was independent of TLR7.

To investigate imiquimod-mediated bronchodilation in vivo, we developed a model based on our laboratory's previous studies (5, 6) that allowed extensive evaluation of bronchodilators in guinea pigs in vivo. Animals were anesthetized with a novel triple combination anesthetic, which ensured stable surgical anesthesia for up to 4 h in ventilated animals. A continuous intravenous infusion of histamine resulted in a stable airway contraction, enabling the direct measurement of aerosolized bronchodilatory compounds. Using this in vivo setup, changes in R_N , G , and H could be measured. This approach allowed the dissection of changes in resistance in relation to both central (R_N) and peripheral (G and H) airways (31). Decreases in R_N , G , and H were evident within seconds following imiquimod administration, suggesting that imiquimod concentration-dependently dilated histamine-precontracted airways in both central and peripheral compartments. This study is the first to show that imiquimod can reverse established histamine-induced precontractions in vivo.

To study mechanisms of imiquimod-mediated bronchodilation, we evaluated the effect of epithelial denudation and pharmacological inhibition of conventional pathways of bronchodilation. Removal of the epithelium had no effect on relaxation, suggesting that epithelial derived-mediators were

Fig. 5. Imiquimod (IMQ) induces intracellular calcium mobilization and blocks histamine-induced calcium mobilization. Isolated airway smooth muscle cells were stained with the Ca^{2+} indicator fluo 4 and exposed to various compounds, and changes in mean fluorescence intensity (MFI) were determined using a BD Accuri C6 flow cytometer. **A** and **B**: cells were exposed to IMQ (100 μM) or histamine (His, 100 μM) (arrow). Values are percent change from baseline (**A**) or maximum percent change from baseline (**B**) $\pm$ SE. **** $P < 0.0001$ using Student's *t*-test ($n = 5-15$). **C**: cells were pretreated with thapsigargin (Th; 1 μM) or dH_2O for 30 min and exposed to IMQ (100 μM , arrow). Values are MFI or maximum MFI $\pm$ SE (inset). * $P < 0.05$ using a Student's *t*-test ($n = 3$). **D**: cells were exposed to IMQ (100 μM) or dH_2O (arrow) and subsequently exposed to Th (1 μM , arrow). Values are MFI. **E** and **F**: cells were preincubated with varying concentrations of IMQ for 5 min and subsequently exposed to His (100 μM , arrow). Values are percentage of maximum vehicle MFI $\pm$ SE. ** $P < 0.01$, *** $P < 0.001$ using one-way ANOVA followed by Bonferroni post-hoc test ($n = 4-7$).



not involved. Comparatively, the cyclooxygenase inhibitor indomethacin reduced the potency of imiquimod-mediated relaxations without affecting the maximal dilation. This is in accordance with previous observations (22) and suggests that ASM-derived prostanoids are involved, but are not crucial, for imiquimod-mediated effects. Further analysis revealed that the investigated imiquimod-mediated effects were completely independent of other conventional pathways of bronchodilation. CO, which is endogenously produced via the enzyme HO-1 (13), has been shown to dilate guinea pig airways precontracted with histamine in vivo, in a cGMP-dependent manner (5, 6). In vivo or in vitro inhibition of this pathway, through blockage of HO-1, cGMP synthase, or PKG, had no effect on imiquimod-mediated bronchodilation. Similarly, inhibition of PKA, an important effector molecule for cAMP signaling and β_2 -agonist-mediated bronchorelaxation (4), had no effect.

Vasoactive intestinal peptide and NO, mediators derived from iNANC nerve fibers, are known to potently relax in guinea pig airways (18, 36). However, in vivo and in vitro inhibition of NO had no significant effect on imiquimod-induced relaxation, nor did bilateral vagotomy in vivo, which prevents reflex-induced bronchodilation (28, 29). In addition, neuronal blockade with TTX, which prevented EFS-induced relaxation and, as such, inhibited mediator release from iNANC fibers, had no effect on imiquimod-induced relaxation in vitro. These data indicate that neither neuron- or epithelium-

derived mediators, nor CO or cAMP, are critical for imiquimod-mediated bronchodilation.

The results in this study demonstrate that imiquimod acts directly on the ASM and interferes with Ca^{2+} homeostasis. Exposure of ASM cells to imiquimod resulted in a sharp rise in $[\text{Ca}^{2+}]_i$, likely derived from ER stores. Imiquimod subsequently, significantly, and concentration-dependently inhibited histamine-induced mobilization of $[\text{Ca}^{2+}]_i$. The potencies by which imiquimod induced $[\text{Ca}^{2+}]_i$ release and inhibited histamine-induced $[\text{Ca}^{2+}]_i$ were similar to in vitro relaxation, suggesting that disruption of Ca^{2+} is of importance for bronchorelaxation. However, the mechanism by which a rise in $[\text{Ca}^{2+}]_i$ induces subsequent relaxation is unclear. Previous studies have demonstrated that imiquimod induces rises in $[\text{Ca}^{2+}]_i$ in sensory neurons (24), and PC12 and F11 cell lines (20), with the latter study highlighting a role for the inositol triphosphate (IP_3) receptor activation in this process. Contractions by histamine and carbachol, both of which were reversed by imiquimod, primarily rely on IP_3 signaling and release of ER-derived Ca^{2+} to induce contraction (25). Imiquimod may disrupt this pathway either through binding to and blocking the IP_3 receptor (19), or emptying of ER pools, as demonstrated in this study. Alternatively, it has previously been shown that localized increases in $[\text{Ca}^{2+}]_i$ in ASM can activate Ca^{2+} -dependent potassium channels (e.g., BK_{Ca}), resulting in hyperpolarization and relaxation (9). However, previous studies have

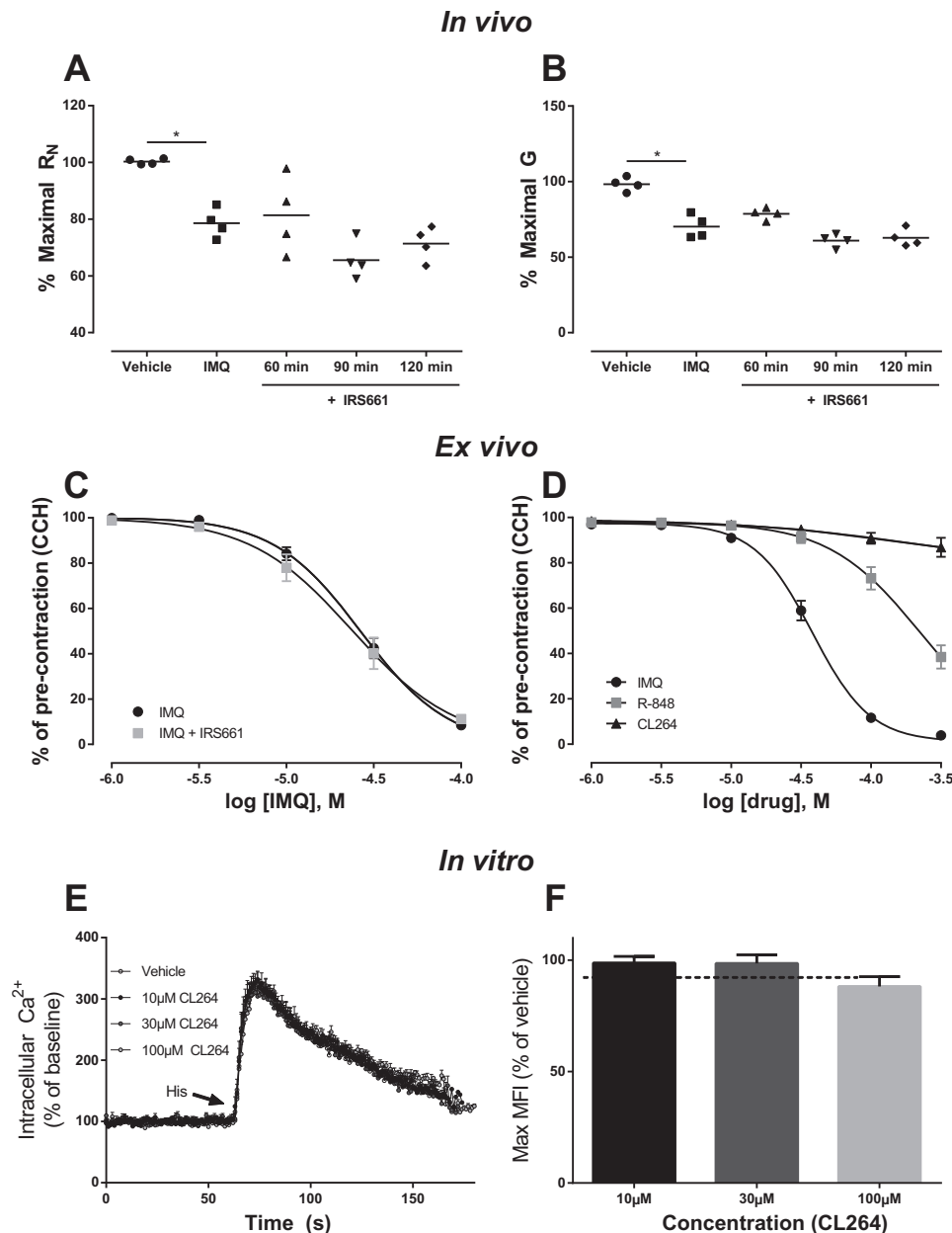


Fig. 6. Imiquimod (IMQ) relaxes precontracted guinea pig airways independently of TLR7 in vitro and in vivo. *A* and *B*: guinea pig airways were precontracted with intravenous histamine in vivo. Aerosolized IMQ (3 mg/kg) was added following intravenous challenge with IRS661 (0.2 mg/kg). Changes in maximal Newtonian resistance (R_N ; *B*) and tissue resistance (G; *C*) were determined. Values are mean percent change from maximum resistance. * $P < 0.05$ vs. vehicle using one-way ANOVA, followed by a Bonferroni post hoc test ($n = 4$). *C*: tracheal rings were precontracted with carbachol (CCH) in vitro. Increasing doses of IMQ were added cumulatively following preincubation with the TLR7 antagonist IRS661 or vehicle. Percent change from maximum contraction was determined. Values are percent change from maximum contraction $\pm$ SE ($n = 5$). *D*: tracheal rings were precontracted with CCH in vitro. Increasing doses of TLR7 agonists IMQ, R-848, or CL264 were added cumulatively. Percent change from maximum contraction was determined. Values are percent change from maximum contraction $\pm$ SE ($n = 8$). *E* and *F*: isolated airway smooth muscle cells were preincubated with varying concentrations of CL264 for 5 min and subsequently exposed to histamine (His; 100 μ M, arrow). Values are percentage of maximum vehicle MFI $\pm$ SE ($n = 4-7$).

reported minimal involvement of such channels in imiquimod-induced relaxation (22), making this less likely. Surprisingly, despite the sharp rise in $[Ca^{2+}]_i$, imiquimod had no effect on basal airway tone; however, the mechanism underpinning this dissociation is beyond the scope of this study. It remains to be determined how imiquimod disrupts Ca^{2+} homeostasis and thus induces relaxation, but the results suggest it exerts its effect by acting directly on the ASM.

Imiquimod has previously been shown to upregulate cytokine and chemokine production and alter cell-surface marker expression on ASM, effects that were evident after 24 h (27). However, the speed at which imiquimod exerts its effect would suggest that the effect on ASM does not occur via traditional TLR-dependent pathways. This is corroborated by the finding that, in our models, imiquimod-mediated bronchodilation of guinea pig airways was not dependent on TLR7, as both in

vitro and in vivo administration of the TLR7 antagonist IRS661 had no effect on imiquimod-mediated bronchodilation. This was true even when the antagonist was used at in vitro concentrations 100-fold higher than previously shown to be effective (27). Additionally, another TLR7 agonist, CL264, that we and others (7, 19) have shown to be more potent than imiquimod in terms of TLR7 agonism had no effect on precontracted airways or on histamine-induced Ca^{2+} mobilization in ASM cells. Indeed, previous studies have demonstrated that the rise in $[Ca^{2+}]_i$ following imiquimod administration is independent of TLR7 (20, 24), substantiating that bronchorelaxation by imiquimod occurs predominantly independently of TLR7.

It is likely that the bronchorelaxatory effects of some, but not all, TLR7 agonists are a consequence of their unique chemical structures. Imiquimod and R-848, but not CL264,

share a chemical structure known as quinoline. This structure, not required for TLR7 agonism (39), is also present in other bronchodilatory compounds with undefined mechanisms of action, including the bitter taste receptor (TAS2R) agonists quinine and chloroquine. Quinine and chloroquine relax precontracted mouse, guinea pig, and human airways, at similar potencies to imiquimod (9, 15, 26, 32) and have been shown to induce $[Ca^{2+}]_i$ mobilization (9), as well as inhibit subsequent calcium release by other agents (14, 30, 35). Indeed, both quinine and chloroquine have been shown to inhibit $[Ca^{2+}]_i$ mobilization primarily by blocking of IP_3 - IP_3 receptor interactions (30, 35), again raising the possibility that imiquimod may disrupt intracellular IP_3 -mediated signaling pathways. Other studies have demonstrated that TLR7 agonists that lack the quinoline-moiety (loxoribine) do not induce a rise in $[Ca^{2+}]_i$ (20, 24), further suggesting that quinoline may be of importance in TLR7-independent mechanisms of imiquimod. It is likely that other bronchodilating TLR7 agonists, such as CL097 and gardiquimod (22), which share similar structural components, dilate airways through similar mechanisms. Quinoline-dependent release and subsequent inhibition of $[Ca^{2+}]_i$ responses represent a novel and potential pathway for imiquimod-mediated bronchodilation, independent of classical bronchodilatory mechanisms.

A subset of the data presented in this study can be perceived as lying in contrast to previously published reports (11, 22). In the cited studies, bronchodilation occurs at similar potencies and time frames, as seen in the present study, but instead occurs via a neuronal, NO-mediated and TLR7-dependent mechanism. The present findings do not dismiss these reports; instead, two mechanisms for imiquimod-mediated bronchorelaxation may conceivably exist, and their relative contribution is highly dependent on the experimental protocol employed. For example, the aforementioned studies induced precontraction mainly through neuronal stimulation, whereas our in vivo models induced ASM contraction through nonneuronal mechanisms. Neuronal NO was likely induced by imiquimod in our models, but could have been masked by the additional ability for imiquimod to inhibit histamine-induced Ca^{2+} mobilization in ASM. The present study supplies an additional and novel TLR7-independent, ASM-dependent mechanism for imiquimod-mediated bronchodilation.

In summary, the present study demonstrates that the prototypical TLR7 agonist imiquimod can directly and rapidly relax precontracted guinea airways in vitro and in vivo. These effects appear to be independent of the epithelium, neuronal activation, and conventional pathways of airway relaxation. Instead, imiquimod acts directly on the ASM and likely causes bronchorelaxation through disruption of Ca^{2+} homeostasis. This effect is independent of TLR7 and may instead be related to the presence of a quinoline structure. This highlights the potential use of drugs that combine the effects of quinoline with the anti-inflammatory action of TLRs in future treatment of inflammatory and hyperactive airway disease.

ACKNOWLEDGMENTS

We thank Marina Leino, Eric Hjalmarsson, and Cecilia Landberg for technical assistance.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

O.J.L., M.L.M., M.S., B.F., M.A., S.K.G., and L.-O.C. conception and design of research; O.J.L., M.L.M., B.F., and S.K.G. performed experiments; O.J.L. and M.L.M. analyzed data; O.J.L., M.L.M., M.S., M.A., S.K.G., and L.-O.C. interpreted results of experiments; O.J.L. prepared figures; O.J.L. and L.-O.C. drafted manuscript; O.J.L., M.L.M., M.S., B.F., M.A., S.K.G., and L.-O.C. edited and revised manuscript; O.J.L., M.L.M., M.S., B.F., M.A., S.K.G., and L.-O.C. approved final version of manuscript.

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