

Ligation of Toll-Like Receptor 3 Differentially Regulates M2 and M3 Muscarinic Receptor Expression and Function in Human Airway Smooth Muscle Cells

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Key Words

Airway hyperreactivity • Airway smooth muscle cells • Double-stranded RNA • Toll-like receptor 3

Abstract

Background: Viral infection causes asthma exacerbations and airway hyperreactivity. Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA) of viral or synthetic origin in a fashion different from protein kinase R (PKR). The aim of this study was to examine the expression and function of TLR3 in human airway smooth muscle (ASM) cells. **Methods:** Expression of TLR3 and muscarinic receptor (MR), histamine receptor (HR), and cysteinyl leukotriene receptor (CysLTR) subtypes was analyzed by quantitative real-time PCR, flow cytometry, or Western blotting. It was assessed whether ASM cells respond to polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of dsRNA, with alterations in M2R, M3R, H1R, and CysLT1R expression. The function of these subtypes was evaluated by cholinergic regulation of forskolin-stimulated cyclic AMP accumulation or by mobilization of intracellular calcium upon stimulation. **Results:** ASM cells expressed TLR3 and PKR, and intracellular TLR3 expression was demonstrated. Poly I:C caused decreased M2R and increased M3R expression, without affecting H1R and CysLT1R expression. Poly I:C-treated cells showed decreased cholinergic inhibition of forskolin-stimulated cyclic AMP accumulation and enhanced calcium flux in response to ace-

tylcholine, but not to histamine and LTD₄. These modulating effects of poly I:C were reversed by chloroquine, but not by 2-aminopurine. **Conclusions:** The data indicate that poly I:C internalized by ASM cells differentially regulates M2R and M3R expression and function by interacting with TLR3 rather than with PKR, suggesting that these changes may contribute to airway hyperreactivity.

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Introduction

Respiratory viral infections are a major cause of asthma exacerbations and airway hyperreactivity [1]. The recognition of pathogen-associated molecular patterns expressed by infectious agents, including viruses, involves members of the Toll-like receptor (TLR) family that mediate early host defense [2, 3]. Of these known members, TLR3, TLR7, and TLR8 sense viral infection and activate antiviral innate immune responses [4–6]. TLR3 recognizes double-stranded RNA (dsRNA), a product of viral replication, and TLR7 and TLR8 recognize single-stranded RNA viruses. TLR3 also responds to polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of dsRNA. Although TLR3 functions as a sensor for viral dsRNA, the participation of the dsRNA sensing system in the development of airway hyperreactivity remains poorly understood.

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Airway hyperreactivity to different stimuli acting through disparate pathways is one of the characteristic features of asthma. Stimuli act either directly on airway smooth muscle (ASM) or via release of constrictors from inflammatory cells and nerves [7–9]. ASM is constricted not only by mediators such as histamine and cysteinyl leukotrienes, but also by neurotransmitters such as acetylcholine (ACh) and neurokinin A. Receptors for these mediators and neurotransmitters have subtypes that bind specific agonists and antagonists. For example, four histamine receptors (H1R, H2R, H3R, and H4R), two cysteinyl leukotriene receptors (CysLT1R and CysLT2R), and five muscarinic receptors (M1R, M2R, M3R, M4R, and M5R) have been identified based on amino acid structure of different cloned genes [10–12]. Of these subtypes, H1R, CysLT1R, and M3R, which belong to the G protein-coupled receptor family, mediate constriction of ASM, whereas M2R on ASM plays a role for cholinergic inhibition of adenylate cyclase via an inhibitory G protein, resulting in inhibition of cyclic AMP (cAMP) synthesis [7, 8]. In addition, neuronal M2R limits bronchoconstriction by inhibiting release of ACh from parasympathetic nerves. Viruses and poly I:C are known to cause neuronal M2R dysfunction [13, 14].

TLR3, a pattern recognition receptor for dsRNA, is expressed in dendritic cells, fibroblasts, epithelial cells, NK cells, and T cells [15–20]. In addition, recently published studies show that ASM cells express TLR3 and respond to dsRNA with increased cytokine and chemokine production [21–23]. However, no information as to whether TLR3 ligation in ASM cells regulates the expression and function of receptor subtypes that mediate bronchoconstriction or limit bronchodilation has been provided to our knowledge. The present study was therefore undertaken to examine whether poly I:C treatment of ASM cells modulates the expression and function of M2R, M3R, H1R, and CysLT1R subtypes. Since protein kinase R (PKR) is activated upon binding dsRNA to undergo dimerization and autophosphorylation [24, 25], a potential role for PKR in mediating responses to poly I:C in ASM cells was also examined.

Materials and Methods

ASM Cells

Primary cultures of normal human ASM cells from three different donors were obtained from Clonetics (San Diego, Calif., USA). Cells were grown in smooth muscle cell basal medium containing 5% fetal bovine serum, 1 ng/ml epidermal growth factor, 2 ng/ml fibroblast growth factor, 10 µg/ml insulin, 50 µg/ml

gentamycin, and 50 ng/ml amphotericin B (Clonetics), according to the manufacturer's instructions. Confluent cells from passages 3–5 were serum/growth factor-deprived for 24 h before treatment.

Reagents

Poly I:C (<0.125 EU/mg) was obtained from InvivoGen (San Diego, Calif., USA). ACh chloride, histamine dihydrochloride, gallamine triethiodide, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), mepyramine, chloroquine, 2-aminopurine, and forskolin were obtained from Sigma-Aldrich (St. Louis, Mo., USA). LTD₄ was obtained from Cayman Chemical (Ann Arbor, Mich., USA), and MK571 was from Biomol (Plymouth Meeting, Pa., USA). Fluo-3 acetoxymethyl ester was obtained from Molecular Probes (Eugene, Ore., USA). All other chemicals were of analytical grade.

Antibodies

Phycoerythrin (PE)-labeled mouse monoclonal anti-human TLR3 antibody (clone TLR3.7) was obtained from eBioscience (San Diego, Calif., USA). Rabbit polyclonal anti-human H1R antibody directed against the cytoplasmic loop (No. 3) of the receptor was obtained from Chemicon (Temecula, Calif., USA), and rabbit polyclonal anti-human CysLT1R antibody directed against the carboxy-terminal portion of the receptor was from Cayman Chemical. Rabbit polyclonal anti-human M2R and anti-human M3R antibodies and mouse monoclonal anti-human smooth muscle α-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Rabbit polyclonal anti-human PKR and anti-phospho-human PKR (Thr451) antibodies were obtained from Cell Signaling Technology (Danvers, Mass., USA).

RT-PCR

Extraction of total RNA and cDNA synthesis by reverse transcriptase were performed as described previously [26]. Primer sets for TLR3, TLR7, TLR8, H1R, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Roche Molecular Biochemicals (Mannheim, Germany). To analyze the expression profiles of TLR3, TLR7, and TLR8 in ASM cells, semiquantitative PCR was performed on a thermal cycler using a Pyrobest DNA polymerase kit (TaKaRa, Tokyo, Japan). The amplified products were subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. Quantitative real-time PCR was performed on a LightCycler using the LightCycler Fast-Start DNA Master SYBR Green I kit (Roche Molecular Biochemicals) according to the protocol provided in the parameter-specific kits. The PCR was also performed using the following sense and antisense primers optimized for the LightCycler: CysLT1R, 5'-TATTCACGTACCATTCACC-3' and 5'-CCTTCTTTCTGGGTACATAA-3'; M2R, 5'-AGTGCTGTTGCCTCTAATAT-3' and 5'-TCTGCTTTTCATCTCCATTC-3'; M3R, 5'-TTCCCATC-CAGCTAGAG TCA-3' and 5'-GCTTTCTTCTCCTTGACCAG-3'. The thermal cycler conditions were set to 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 5 s (CysLT1R) or 8 s (M2R and M3R). In each PCR, no amplification of unspecific products was observed when a melting curve analysis was carried out to control for specificity of the amplification products. The copy number of TLR3, TLR7, TLR8, H1R, CysLT1R, M2R, and M3R was calculated as the number of tran-

scripts per 1,000 copies of the housekeeping gene GAPDH and was indicated as the expression rate.

Flow Cytometry

Expression and localization of TLR3 were analyzed by flow cytometry. For surface staining, cells were incubated with PE-labeled anti-TLR3 antibody. For intracellular staining, cells were fixed and permeabilized with the intraPrep permeabilization reagent kit (Immunotech, Marseille, France) according to the manufacturer's instructions and then incubated with PE-labeled anti-TLR3 antibody. To analyze H1R and CysLT1R expression, fixed and permeabilized cells were incubated with anti-H1R or anti-CysLT1R antibody directed against the cytoplasmic domain of the receptor and reacted with a PE-labeled secondary antibody (BioSource, Camarillo, Calif., USA). In each assay, isotype control was used for negative staining. Samples were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson, San Jose, Calif., USA). Expression of immunoreactive proteins was expressed as a ratio of the mean fluorescence intensity (MFI) obtained with isotype control antibody.

Western Blot

Expression of M2R, M3R, and total and phosphorylated PKR was analyzed through Western blot analysis. Preparation of cell lysates and measurement of protein concentration were performed as described previously [26]. The lysates were resolved on 10% SDS-PAGE and electroblotted onto PVDF membranes (Bio-Rad, Hercules, Calif., USA). The membranes were blocked with 2% bovine serum albumin in TBS-Tween 20, probed with anti-M2R, anti-M3R, anti-PKR, or anti-phospho-PKR antibodies, and then incubated with a horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories, San Francisco, Calif., USA). Blots were visualized by using enhanced chemiluminescence (Pierce, Rockford, Ill., USA). Smooth muscle α -actin was run in parallel as an internal control. The density of the immunoreactive bands was quantified by using the Multianalyst software system (Bio-Rad) and was expressed as an intensity ratio.

Intracellular Calcium Mobilization

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured as follows. Cells were loaded in Ca^{2+} -free Tyrode's solution with 10 μM fluo-3 for 1 h at room temperature. Loaded cells were washed, suspended in loading buffer, and maintained at 37°C in a Fluoroskan (Labsystems, Helsinki, Finland). The concentration of extracellular Ca^{2+} was brought to 1.8 mM by adding CaCl_2 30 min before recordings. Maximal cell fluorescence and minimal fluorescence were determined by adding 1% NP-40 and 0.1 M EGTA, respectively.

Measurement of Intracellular cAMP

Cells were treated with the indicated agents before the addition of forskolin, a stimulator of adenylate cyclase. After an additional 15-min incubation, the intracellular cAMP level was measured by using the cAMP Biotrack EIA system (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

Statistical Analysis

Statistical significance of differences was determined with paired Student's *t* test. Differences were considered significant at $p < 0.05$.

Results

Analysis of TLR3 Expression

We first examined the expression profiles of TLR3, TLR7, and TLR8, which sense virus infection, in human ASM cells. RT-PCR analysis showed that the cells were positive for TLR3 mRNA and negative for TLR7 and TLR8 mRNAs (fig. 1a). The expression rate of TLR3 mRNA was about 0.4 as determined by quantitative real-time PCR analysis (fig. 1b). Since TLR3 was expressed at the mRNA level, its protein expression was analyzed by flow cytometry. TLR3 was detected by intracellular staining, but not by cell surface staining (fig. 1c, d). Thus, TLR3 was predominantly localized intracellularly and not on the cell surface. Additionally, we observed a marked upregulation of TLR3 mRNA expression after 24-hour treatment with poly I:C (25 $\mu\text{g}/\text{ml}$), a synthetic analog of dsRNA, by quantitative real-time PCR (fig. 2a). This upregulation was manifested at the intracellular expression level of TLR3, as analyzed by flow cytometry (fig. 2b, c).

Analysis of PKR Expression

We next examined whether ASM cells express PKR because this kinase responds to dsRNA in a fashion different from TLR3. Western blotting analysis showed that ASM cells expressed detectable levels of PKR and treatment with poly I:C (25 $\mu\text{g}/\text{ml}$) for 24 h led to an increase in levels of both total and phosphorylated PKR, indicating that PKR is expressed and activated by poly I:C (fig. 3a, b).

Effect of Poly I:C on Expression of MR, HR, and CysLTR Subtypes

Based on the observation that ASM cells expressed TLR3 and PKR, we next examined whether poly I:C treatment modulates the expression of MR, HR, and CysLTR subtypes that mediate bronchoconstriction or limit bronchodilation. Quantitative real-time PCR was performed to determine the expression levels of mRNA for M2R, M3R, H1R, and CysLT1R in cells treated with poly I:C (25 $\mu\text{g}/\text{ml}$) for up to 24 h. The results are summarized in figure 4. Poly I:C caused decreased M2R and increased M3R mRNA expression, but did not modify H1R and CysLT1R mRNA expression. The modulating effects of poly I:C on M2R and M3R mRNA expression were observed after 12 h and were significant by 24 h. The protein expression levels of M2R, M3R, H1R, and CysLT1R in untreated and poly I:C-treated cells were further analyzed by Western blotting or flow cytometry. Consistent with the results of PCR analysis, treatment with poly I:C for 24 h signifi-

Fig. 1. Expression and localization of TLR3 in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence and starved of serum and growth factors before analysis. **a** RT-PCR analysis of TLR3, TLR7, and TLR8 mRNA expression in cells from one representative donor (of three) is shown. **b** Quantitative real-time PCR was performed to determine the expression levels of mRNA for TLR3, TLR7, and TLR8. Results are presented as means $\pm$ SEM of three different donors. ND = Not detected. **c** Localization of TLR3 protein was analyzed by surface and intracellular staining. Intact cells or fixed and permeabilized cells were stained with PE-labeled anti-TLR3 antibody and analyzed by flow cytometry. The dotted tracing in each panel indicates background staining. Data from one representative donor (of three) are shown. **d** The MFI for surface and intracellular expression of TLR3 was determined. Results are presented as means $\pm$ SEM of three different donors. ** $p < 0.01$.

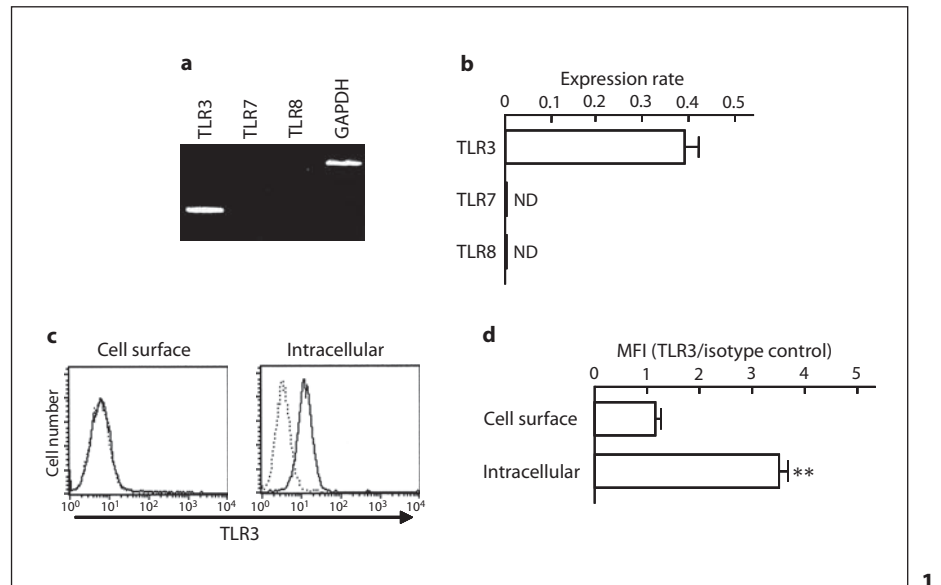
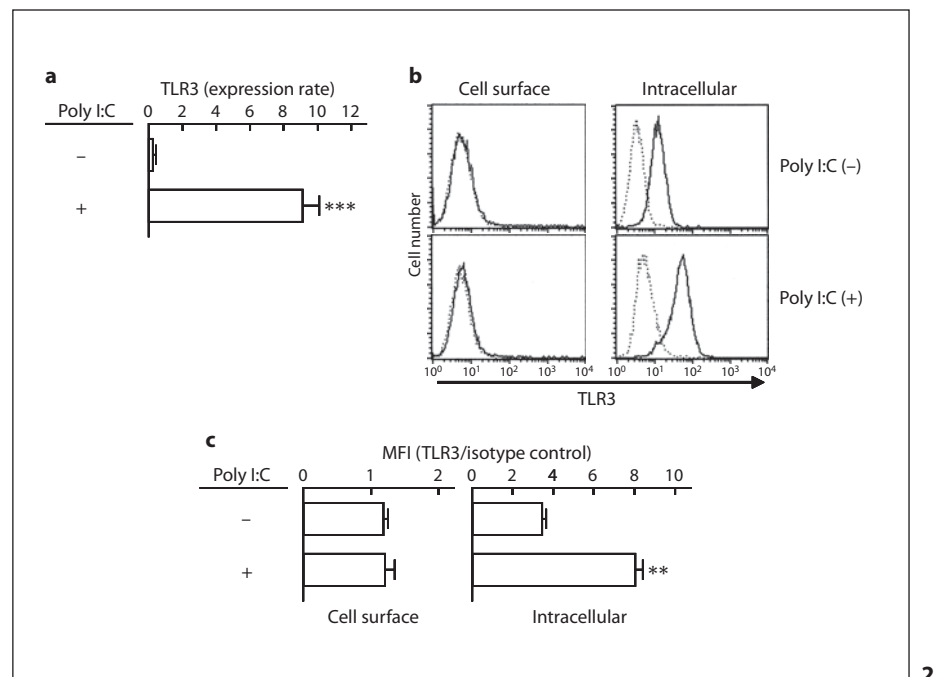


Fig. 2. Upregulation of TLR3 expression by poly I:C in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, treated with or without poly I:C (25 μ g/ml) for 24 h, and analyzed for TLR3 expression. **a** Quantitative real-time PCR analysis of TLR3 mRNA expression is shown. Results are presented as means $\pm$ SEM of three different donors. *** $p < 0.001$. **b** Flow-cytometric analysis of surface and intracellular expression of TLR3 in cells from one representative donor (of three) is shown. The dotted tracing in each panel indicates background staining. **c** The MFI for surface and intracellular expression of TLR3 was determined. Results are presented as means $\pm$ SEM of three different donors. ** $p < 0.01$.



cantly decreased M2R and increased M3R expression (fig. 5a, b), without affecting H1R and CysLT1R expression (fig. 5c, d). A higher concentration of poly I:C (50 μ g/ml) also gave similar results (data not shown).

Analysis of Receptor Function

The function of M3R, H1R, and CysLT1R in ASM cells treated with or without poly I:C (25 μ g/ml) for 24 h was evaluated by mobilization of intracellular calcium upon

stimulation with ACh (1 μ M), histamine (10 μ M), or LTD₄ (1 μ M). As shown in figures 6 and 7, both untreated and poly I:C-treated cells responded to ACh, histamine, and LTD₄ with increased calcium flux. Furthermore, poly I:C-treated cells showed enhanced responsiveness to ACh, but not to histamine and LTD₄. The [Ca²⁺]_i increase in response to ACh was significantly higher in poly I:C-treated cells than in untreated cells. In contrast, no significant differences in the [Ca²⁺]_i increases evoked by his-

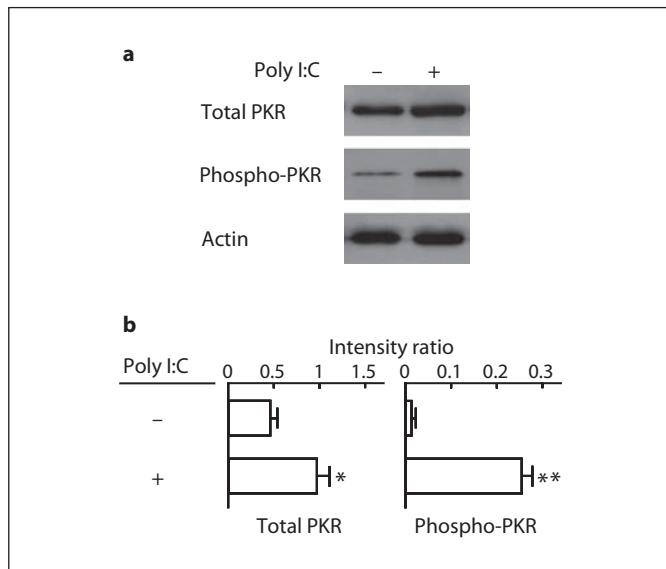


Fig. 3. Expression and activation of PKR in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, treated with or without poly I:C (25 µg/ml) for 24 h, and analyzed for the expression of total and phosphorylated PKR by Western blotting. Smooth muscle α -actin was measured as a control. **a** Data from one representative donor (of three) are shown. **b** The intensity ratio of total and phosphorylated PKR to actin was determined by densitometric analysis. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$, ** $p < 0.01$.

tamine or LTD₄ were seen between untreated and treated cells. Our observation of the modest increase in $[Ca^{2+}]_i$ induced by LTD₄ accorded with a previous report which showed that the response to LTD₄ was very low compared with that to histamine [27]. The responses to ACh, histamine, and LTD₄ in untreated and poly I:C-treated cells were completely blocked by the M3R antagonist 4-DAMP (1 µM), the H1R antagonist mepyramine (10 µM), and the CysLT1R antagonist MK571 (1 µM), respectively. 4-DAMP was applied in a concentration selective for M3R over M2R [28]. Complete inhibition of ACh-mediated calcium responses by 4-DAMP in untreated and poly I:C-treated cells implies that basal and upregulated M3R is functional.

The function of M2R was evaluated in terms of cholinergic regulation of forskolin-stimulated cAMP accumulation. As shown in figure 8, both untreated and poly I:C-treated cells responded to forskolin (5 µM) with intracellular cAMP accumulation. Although poly I:C treatment tended to enhance forskolin-stimulated cAMP accumulation, no significant differences in cAMP level

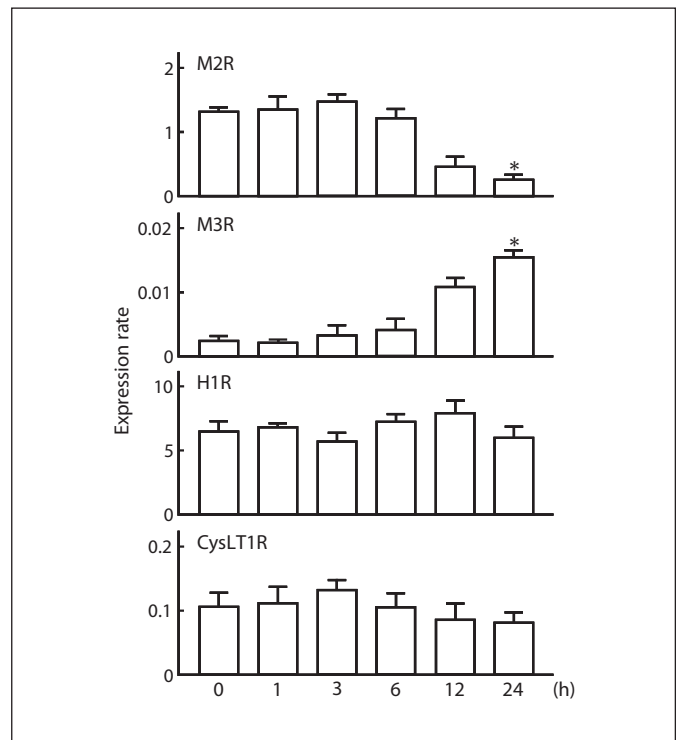


Fig. 4. Effect of poly I:C on M2R, M3R, H1R, and CysLT1R mRNA expression in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, treated with poly I:C (25 µg/ml) for up to 24 h, and analyzed for the expression of mRNA for receptor subtypes by quantitative real-time PCR. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$.

were seen between untreated and treated cells. In untreated cells, cAMP accumulation was significantly reduced by ACh (100 µM), and this reduction was abrogated by the M2R antagonist gallamine (10 µM). The agent was applied in a concentration selective for M2R over M3R [28]. In poly I:C-treated cells, cAMP accumulation was to a lesser extent attenuated by ACh, and this attenuation was also abrogated by gallamine. Our data imply that the M2R downregulation by poly I:C is associated with decreased cholinergic inhibition of adenylate cyclase.

Involvement of TLR3 in Mediating Responses to Poly I:C

As already shown in figure 2, poly I:C upregulated TLR3 expression in ASM cells. Accordingly, we asked whether upregulated TLR3 is involved in mediating responses to poly I:C. To examine this, ASM cells that had been exposed to poly I:C (25 µg/ml) for 24 h were rested

Fig. 5. Effect of poly I:C on M2R, M3R, H1R, and CysLT1R protein expression in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, treated with or without poly I:C (25 μ g/ml) for 24 h, and analyzed for the expression of receptor subtypes. **a** The expression of M2R and M3R was analyzed by Western blotting. Smooth muscle α -actin was measured as a control. Data from one representative donor (of three) are shown. **b** The intensity ratio of M2R and M3R to actin was determined by densitometric analysis. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$. **c** The expression of H1R and CysLT1R was analyzed by flow cytometry. The dotted tracing in each panel indicates background staining. Data from one representative donor (of three) are shown. **d** The MFI for H1R and CysLT1R expression was determined. Results are presented as means $\pm$ SEM of three different donors.

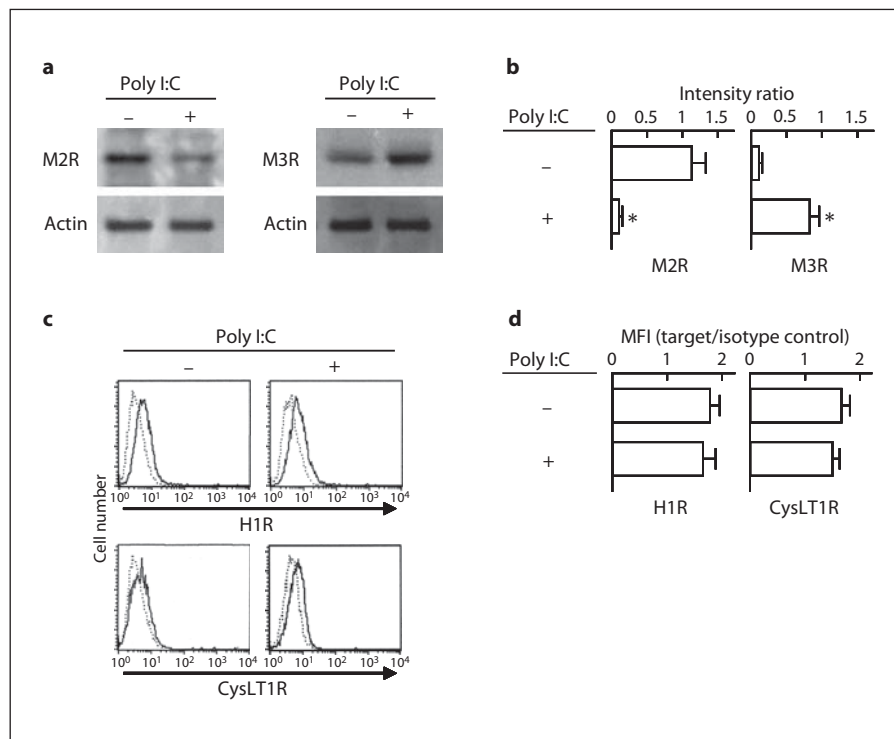
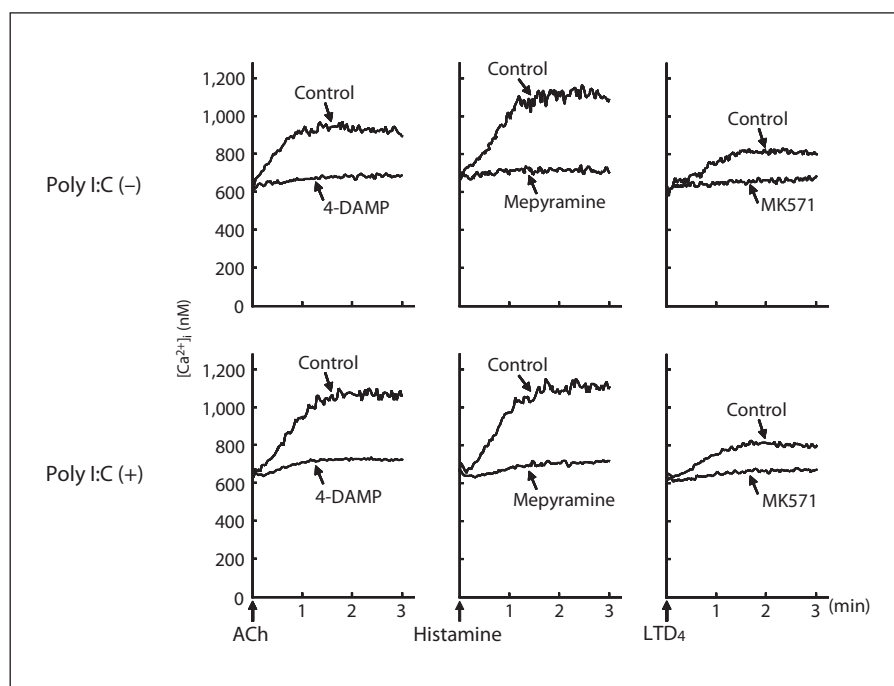


Fig. 6. Intracellular calcium mobilization upon stimulation in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, treated with or without poly I:C (25 μ g/ml) for 24 h, loaded with fluo-3, and stimulated with ACh (1 μ M), histamine (10 μ M), or LTD₄ (1 μ M) in the absence or presence of 4-DAMP (1 μ M), mepyramine (10 μ M), or MK571 (1 μ M). To determine $[Ca^{2+}]_i$, cells were analyzed on a Fluoroskan. Results shown are representative of three different donors.



for 12 h, rechallenged with or without poly I:C for 24 h, and then analyzed for M2R, M3R, and TLR3 mRNA expression by quantitative real-time PCR and for PKR phosphorylation by Western blotting. Rechallenge with

poly I:C caused further decreased M2R and increased M3R and TLR3 mRNA expression (fig. 9a), but did not alter the expression level of phosphorylated PKR (fig. 9b, c). This observation suggests that TLR3 upregulation af-

Fig. 7. Intracellular calcium mobilization upon stimulation in ASM cells. The data obtained with figure 6 were quantified. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

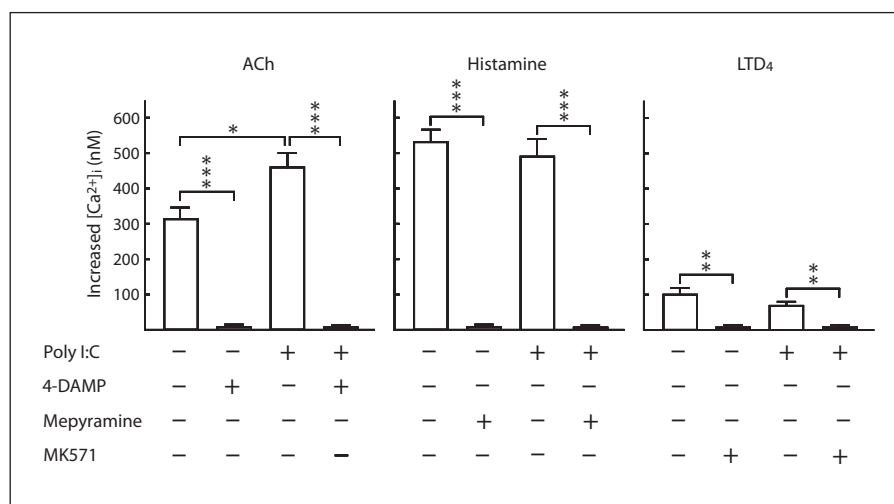
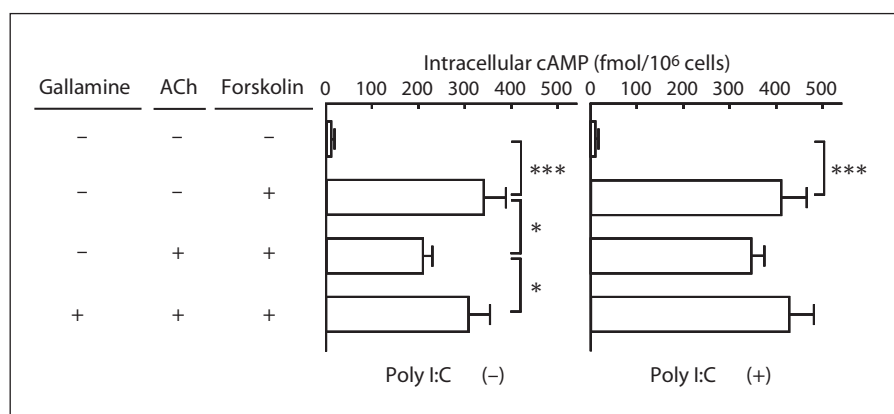


Fig. 8. Cholinergic regulation of forskolin-stimulated cAMP accumulation in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, treated with or without poly I:C (25 μ g/ml) for 24 h, and stimulated in the absence or presence of gallamine (10 μ M) or ACh (100 μ M) with forskolin (5 μ M) for 15 min. The intracellular cAMP levels were determined by EIA. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$, *** $p < 0.001$.



ter the first exposure may account for more pronounced effects of poly I:C following rechallenge.

We further sought to determine whether either TLR3 or PKR is involved in the modulation of M2R and M3R expression by poly I:C. To that end, we used two different inhibitors, chloroquine and 2-aminopurine. It is known that chloroquine is an endosomal acidification inhibitor that blocks intracellular TLR-mediated signaling [18, 29], whereas 2-aminopurine is a PKR inhibitor [30, 31]. Quantitative real-time PCR showed that poly I:C-induced changes in M2R and M3R mRNA expression were significantly reversed by chloroquine (5 μ g/ml), but not by 2-aminopurine (10 mM) (fig. 10a). Consistent with the PCR data, Western blotting analysis gave similar results (fig. 10b, c). Although 2-aminopurine at the concentration used had no significant effect on poly I:C-induced changes in M2R and M3R expression, the agent completely inhibited poly I:C-induced phosphorylation of PKR (fig. 11a, b), without affecting cell viability (fig. 11c).

It has been reported that 1–10 mM of 2-aminopurine is required for the inhibition of PKR phosphorylation and activation [30, 31]. Functional analyses of M2R and M3R showed that in poly I:C-treated cells, chloroquine, but not 2-aminopurine, was effective for further cholinergic inhibition of forskolin-stimulated cAMP accumulation (fig. 12) and for attenuation of ACh-stimulated calcium flux (fig. 13a, b). In untreated cells, neither chloroquine nor 2-aminopurine affected functional responses to ACh. These results demonstrate that TLR3 rather than PKR is actively involved in the differential regulation of M2R and M3R expression and function by poly I:C.

Discussion

This study shows that primary human ASM cells express TLR3 and PKR and that TLR3 is predominantly localized intracellularly and not on the cell surface. We

Fig. 9. Analyses of M2R, M3R, and TLR3 mRNA expression and PKR phosphorylation in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, incubated with or without poly I:C (25 μ g/ml) for 24 h, rested for 12 h, and treated with or without poly I:C for 24 h. **a** M2R, M3R, and TLR3 mRNA expression was analyzed by quantitative real-time PCR. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$, ** $p < 0.01$. **b** PKR phosphorylation was analyzed by Western blotting. Smooth muscle α -actin was measured as a control. Data from one representative donor (of three) are shown. **c** The intensity ratio of phosphorylated PKR to actin was determined by densitometric analysis. Results are presented as means $\pm$ SEM of three different donors. ** $p < 0.01$.

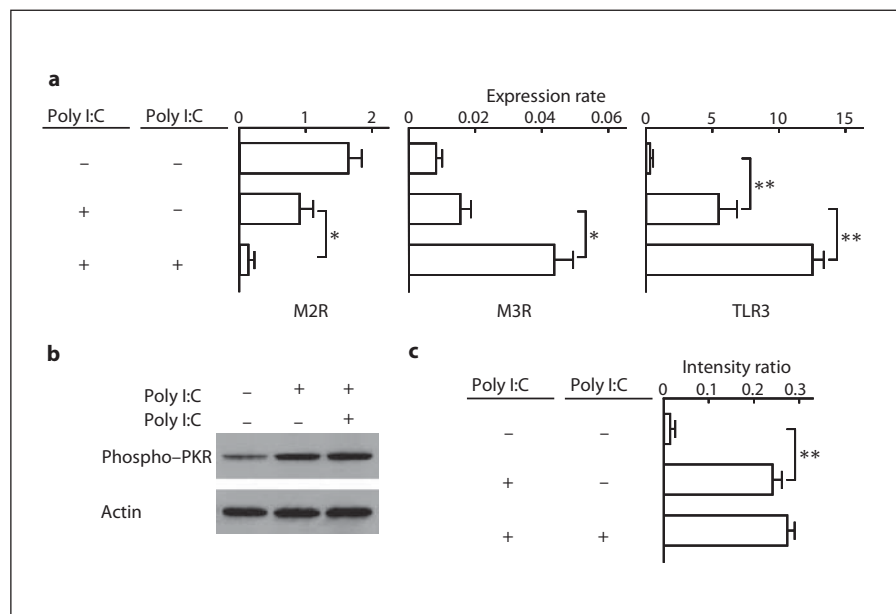
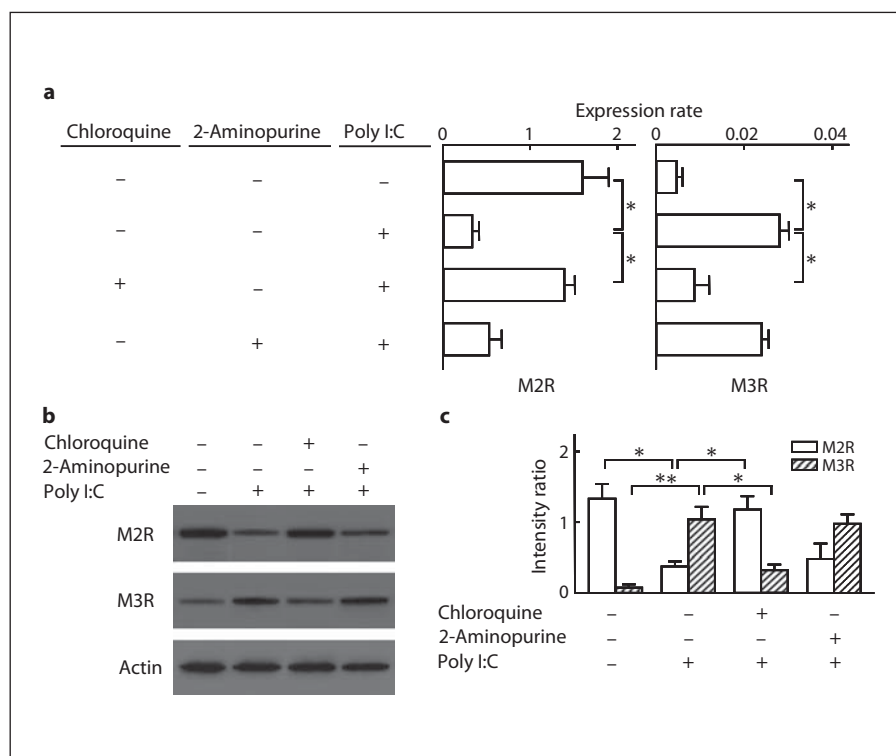


Fig. 10. Effects of chloroquine and 2-aminopurine on poly I:C-induced changes in M2R and M3R expression in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, incubated with or without chloroquine (5 μ g/ml) or 2-aminopurine (10 mM) for 1 h, treated with poly I:C (25 μ g/ml) for 24 h, and analyzed for M2R and M3R expression. **a** The expression levels of mRNA for M2R and M3R were determined by quantitative real-time PCR. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$. **b** The protein expression levels of M2R and M3R were analyzed by Western blotting. Smooth muscle α -actin was measured as a control. Data from one representative donor (of three) are shown. **c** The intensity ratio of M2R and M3R to actin was determined by densitometric analysis. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$, ** $p < 0.01$.



found that treatment with poly I:C, a synthetic analog of dsRNA, causes decreased M2R and increased M3R expression and function. The cellular effects of dsRNA are mediated by TLR3, PKR, retinoic acid-inducible gene-1, or melanoma differentiation-associated gene-5 depending on the cell type/cell response [18–24, 32, 33]. Poly

I:C-induced changes in M2R and M3R expression and function were significantly reversed by chloroquine, an endosomal acidification inhibitor, but not by 2-aminopurine, a PKR inhibitor. Thus, the effects observed in poly I:C-treated ASM cells may be due to TLR3 ligation rather than to PKR activation.

Fig. 11. Effect of 2-aminopurine on poly I:C-induced phosphorylation of PKR in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, incubated with or without 2-aminopurine (10 mM) for 1 h, and treated with or without poly I:C (25 μ g/ml) for 24 h. **a** PKR phosphorylation was analyzed by Western blotting. Smooth muscle α -actin was measured as a control. Data from one representative donor (of three) are shown. **b** The intensity ratio of phosphorylated PKR to actin was determined by densitometric analysis. Results are presented as means $\pm$ SEM of three different donors. ** $p < 0.01$. **c** Cell viability was determined by the trypan blue dye exclusion procedure. Results are presented as means $\pm$ SEM of three different donors.

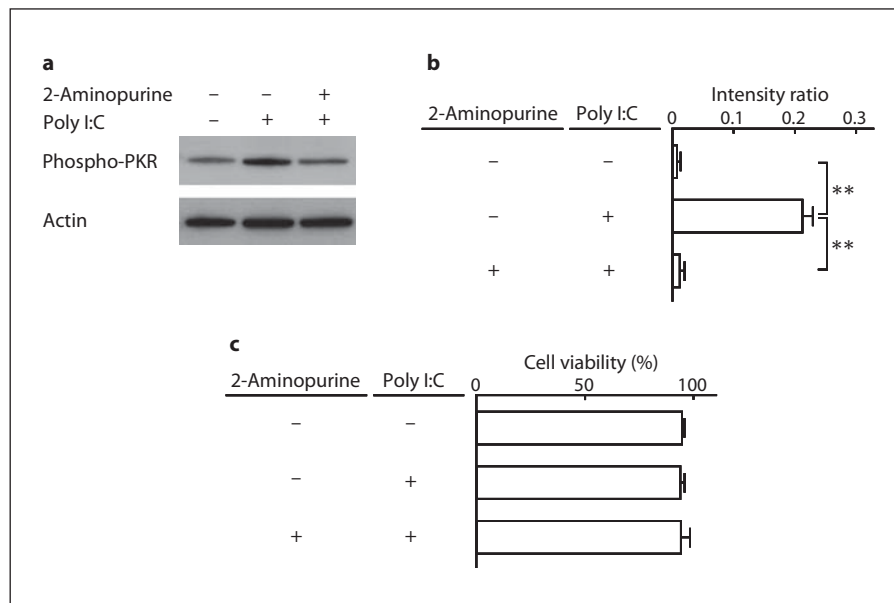
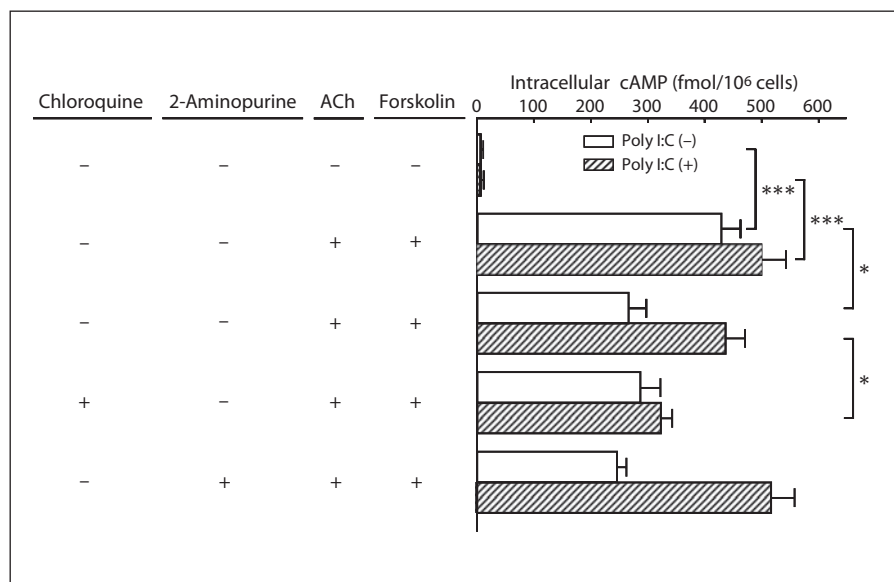


Fig. 12. Effects of chloroquine and 2-aminopurine on cholinergic inhibition of forskolin-stimulated cAMP accumulation in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, incubated with or without chloroquine (5 μ g/ml) or 2-aminopurine (10 mM) for 1 h, treated with or without poly I:C (25 μ g/ml) for 24 h, stimulated in the absence or presence of ACh (100 μ M) with forskolin (5 μ M) for 15 min, and analyzed for the intracellular cAMP levels by EIA. Results are presented as means $\pm$ SEM of three different donors. * $p < 0.05$, *** $p < 0.001$.



Our observation of TLR3 mRNA and protein expression in ASM cells is in keeping with three recently published studies [21–23], but the protein expression pattern is controversial among them, despite the use of the same anti-TLR3 antibody (clone TLR3.7). While one study showed intracellular but not surface expression of TLR3 [21], the other showed surface but not intracellular expression [22]. Further, the third showed that TLR3 immunoreactivity was detectable both intracellularly and on the cell surface [23]. The expression pattern we observed was similar to that obtained in the former study,

fitting the pattern in dendritic cells and T cells in which TLR3 is expressed intracellularly but not on the cell surface [18, 20]. Upregulation of TLR3 protein in poly I:C-stimulated ASM cells is also controversial. While two groups showed no upregulation of TLR3 at the protein level by poly I:C, despite upregulation at the mRNA level [21, 22], we observed TLR3 upregulation both at the mRNA and the protein level after poly I:C stimulation. Although the reason for the discrepancy in these results, including ours, is not clear, it might be possible that differences in selection of ASM cell donors affect a pattern

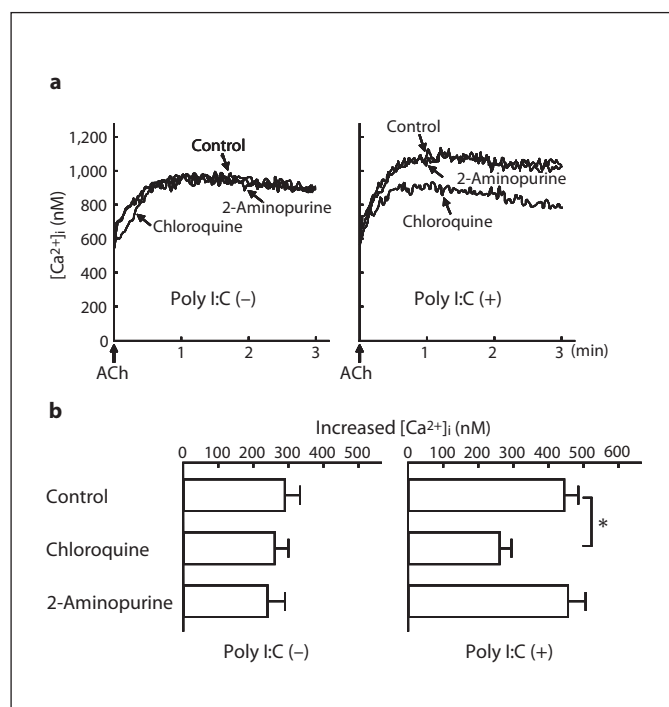


Fig. 13. Effects of chloroquine and 2-aminopurine on ACh-mediated calcium responses in ASM cells. Cells were grown in culture medium containing serum and growth factors to confluence, starved of serum and growth factors, incubated with or without chloroquine (5 μ g/ml) or 2-aminopurine (10 mM) for 1 h, treated with or without poly I:C (25 μ g/ml) for 24 h, loaded with fluo-3, and stimulated with ACh (1 μ M). $[Ca^{2+}]_i$ was determined on a Fluoroskan. **a** Data from one representative donor (of three) are shown. **b** Changes in $[Ca^{2+}]_i$ are presented as means $\pm$ SEM of three different donors. * $p < 0.05$.

of TLR3 expression. However, this possibility, suggesting that there might be a crucial, yet to be determined, difference in the condition of the ASM cells used, remains to be explored.

We observed that poly I:C treatment of ASM cells caused decreased M2R and increased M3R expression, but did not modify H1R and CysLT1R expression. These were all confirmed both at the mRNA and the protein level. Although H1R and CysLT1R were detectable by flow cytometry, M2R and M3R were detectable by Western blotting, but not by flow cytometry using different commercially available polyclonal anti-M2R and anti-M3R antibodies. The presence of M2R and M3R on ASM cells has been demonstrated by radioligand binding [34]. The failure to detect M3R by flow cytometry may be at least partly explained by a minor population of this subtype [7, 8]. The modulating effects of poly I:C on M2R and

M3R expression were significantly reversed by chloroquine, suggesting that intracellular TLR3 mediates the effects. This suggestion is supported by the observation that TLR3 upregulated by poly I:C operated on the modulation of M2R and M3R expression. Although poly I:C also induced an increase in levels of both total and phosphorylated PKR, 2-aminopurine had no significant effect on poly I:C-induced changes in M2R and M3R expression. Thus, PKR does not appear to play a significant role in mediating these responses. Taken together, our results indicate that poly I:C is internalized by ASM cells and differentially regulates M2R and M3R expression by interacting with intracellular TLR3 rather than with PKR. This is in line with previous findings which showed that cellular responses to poly I:C in intracytoplasmic TLR3-expressing cell types were markedly inhibited by endosomal acidification inhibitors such as chloroquine and bafilomycin A1 [18, 20, 21, 23].

Although both untreated and poly I:C-treated ASM cells responded to ACh, histamine, and LTD₄ with increased calcium flux, treated cells showed enhanced responsiveness to ACh, but not to histamine and LTD₄. Furthermore, complete inhibition of ACh-mediated calcium responses by the M3R antagonist 4-DAMP in untreated and poly I:C-treated cells implies that basal and upregulated M3R is functional. In addition, enhanced responsiveness to ACh in poly I:C-treated cells was reversed by chloroquine, but not by 2-aminopurine. However, the function of the M3R subtype with respect to ACh-stimulated calcium mobilization is controversial [7–9, 34]. The reason for this discrepancy is not clear, but might be related to the difference between culture conditions. Although ASM cells grown in the culture medium with or without growth factors have been used in different experiments [21–23, 27, 35–39], we used cells grown in growth factor-supplemented culture medium in the present study. The M3R-mediated enhancement of calcium flux following stimulation with ACh may involve potentiation of ASM contraction, since the receptor is coupled to the Ca²⁺-dependent contractile apparatus containing inositol triphosphate [7, 8, 34]. However, it appears unlikely that poly I:C would affect release of inositol phospholipids because the $[Ca^{2+}]_i$ increases caused by histamine or LTD₄ do not differ between untreated and poly I:C-treated cells.

On the other hand, alterations in expression levels of M2R on ASM have been shown to be associated with the degree of cholinergic inhibition of adenylate cyclase [8, 34, 40]. In line with such associations, the decrease in M2R caused by poly I:C was accompanied by the reduc-

tion of cholinergic inhibition of forskolin-stimulated cAMP accumulation. Also, poly I:C-induced changes in M2R function was reversed by chloroquine, but not by 2-aminopurine. These findings suggest that ligation of TLR3 by viral dsRNA may attenuate cholinergic inhibition of β -agonist-induced relaxation of ASM by down-regulating M2R expression and function. However, the functional significance of M2R in relation to broncho-protection is not completely understood, because stimulation of M2R on ASM does not play a direct role in bronchoconstriction [7, 8] and because β -adrenergic relaxant responses are impaired in asthma [9, 41].

The expression and function of MR and CysLTR subtypes in the lung are modulated by some cytokines. For example, IFN- γ decreases M2R expression in airway parasympathetic neurons, leading to increased vagal ACh release [13]. In addition, IFN- γ increases CysLT1R expression and responsiveness to LTD₄ in ASM cells [36]. TGF- β ₁ and IL-13 also increase CysLT1R expression and LTD₄ responses in ASM cells [37]. However, these three cytokines were not detectable in the supernatants from poly I:C-treated ASM cells (data not shown). This observation may at least explain that poly I:C does not have the ability to modulate CysLT1R expression and function. ASM cells have been shown to produce several cytokines and chemokines, including IL-6 and IL-8, in response to poly I:C [21–23]. However, the addition of IL-6 and IL-8 to cultures of ASM cells did not cause decreased M2R and increased M3R mRNA expression (data not shown). Thus, it appears unlikely that the modulating effects of poly I:C on M2R and M3R expression are mediated through the autocrine production of cytokines and chemokines.

The ability of poly I:C, via an effect on TLR3 expressed in ASM cells, to cause decreased M2R and increased M3R expression may have important implications for the development of airway hyperreactivity. The decrease in M2R would attenuate cholinergic inhibition of adenylate cyclase-dependent bronchodilation. This attenuation also predicts an indirect effect of bronchoconstriction. The increase in M3R would enhance sensitivity to ACh and promote bronchoconstriction. Collectively, viral dsRNA may directly modulate cholinergic responses of ASM via the TLR3 pathway. Retinoic acid-inducible gene-1 and melanoma differentiation-associated gene-5 have been found to serve as another sensor for cytoplasmic dsRNA [32, 33]. Accordingly, the participation of these sensors in some of the effects of poly I:C cannot be ruled out. Further studies are needed to elucidate the signal transduction pathways involving poly I:C-mediated M2R downregulation and M3R upregulation.

In summary, we have shown that human ASM cells express TLR3 and PKR and respond to poly I:C with decreased M2R and increased M3R expression and function. The observed effects of poly I:C, however, appear to be largely dependent on TLR3 rather than on PKR. Our results suggest a possible mechanism by which viral dsRNA recognition by TLR3 expressed in ASM cells may contribute to airway hyperreactivity by differentially regulating M2R and M3R expression and function.

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