JCI The Journal of Clinical Investigation

Virus- and interferon-induced loss of inhibitory M2 muscarinic receptor function and gene expression in cultured airway parasympathetic neurons.

D B Jacoby, … , Y Chan-Li, A D Fryer

J Clin Invest. 1998;102(1):242-248. https://doi.org/10.1172/JCI1114.

Research Article

Viral infections increase vagally mediated reflex bronchoconstriction. Decreased function of inhibitory M2 muscarinic receptors on the parasympathetic nerve endings is likely to contribute to increased acetylcholine release. In this study, we used cultured airway parasympathetic neurons to determine the effects of parainfluenza virus and of interferon (IFN)-gamma on acetylcholine release, inhibitory M2 receptor function, and M2 receptor gene expression. In control cultures, electrically stimulated acetylcholine release increased when the inhibitory M2 receptors were blocked using atropine (10(-)5 M) and decreased when these receptors were stimulated using methacholine (10(-)5 M). Acetylcholine release was increased by viral infection and by treatment with IFN-gamma (300 U/ml). In these cells, atropine did not further potentiate, nor did methacholine inhibit, acetylcholine release, suggesting decreased inhibitory M2 receptor function and/or expression. Using a competitive reverse transcription-polymerase chain reaction method, we demonstrated that M2 receptor gene expression was decreased by more that an order of magnitude both by virus infection and by treatment with IFN. Thus, viral infections may increase vagally mediated bronchoconstriction both by directly inhibiting M2 receptor gene expression and by causing release of IFN-gamma which inhibits M2 receptor gene expression.

Virus- and Interferon-induced Loss of Inhibitory M_2 Muscarinic Receptor Function and Gene Expression in Cultured Airway Parasympathetic Neurons

David B. Jacoby,* Hui-Qing Xiao,* Norman H. Lee,§ Yee Chan-Li,* and Allison D. Fryer‡

**Division of Pulmonary and Critical Care Medicine, Department of Medicine, ‡Department of Environmental Health Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21209; and §The Institute for Genomic Research, Gaithersburg, Maryland 21205*

Abstract

Viral infections increase vagally mediated reflex bronchoconstriction. Decreased function of inhibitory M_2 muscarinic receptors on the parasympathetic nerve endings is likely to contribute to increased acetylcholine release. In this study, we used cultured airway parasympathetic neurons to determine the effects of parainfluenza virus and of interferon (IFN)-γ on acetylcholine release, inhibitory M_2 receptor function, and M_2 receptor gene expression. In control cultures, electrically stimulated acetylcholine release increased when the inhibitory M_2 receptors were blocked using atropine (10^{-5} M) and decreased when these receptors were stimulated using methacholine (10^{-5} M). Acetylcholine release was increased by viral infection and by treatment with IFN-γ (300 U/ml). In these cells, atropine did not further potentiate, nor did methacholine inhibit, acetylcholine release, suggesting decreased inhibitory M_2 receptor function and/or expression. Using a competitive reverse transcription-polymerase chain reaction method, we demonstrated that M_2 receptor gene expression was decreased by more that an order of magnitude both by virus infection and by treatment with IFN. Thus, viral infections may increase vagally mediated bronchoconstriction both by directly inhibiting M_2 receptor gene expression and by causing release of IFN-γ which inhibits M_2 receptor gene expression. (*J. Clin. Invest*. 1998. 102:242–248.) Key words: asthma • parainfluenza • bronchoconstriction • hyperresponsiveness • acetylcholine

Introduction

Viral infections are associated with exacerbations of asthma (1) and cause vagally mediated hyperresponsiveness in normal subjects (2, 3). Studies in virus-infected guinea pigs demonstrate that the efferent arm of vagally mediated reflex bronchoconstriction is abnormal, as release of acetylcholine in response to electrical stimulation of the vagus is increased (4).

Under normal circumstances, the release of acetylcholine from airway parasympathetic nerves is limited by inhibitory muscarinic M_2 receptors on the nerve endings. These receptors, which have been demonstrated in guinea pigs (5), rats (6), cats (7), dogs (8), and humans (9, 10), are markedly dysfunctional in parainfluenza virus–infected guinea pigs (11) and rats (12). Previously, we have suggested several mechanisms by which viral infections may impair the function of airway neuronal M_2 receptors. Studies in which guinea pigs were depleted of leukocytes before parainfluenza virus infection demonstrated that both leukocyte-dependent and leukocyte-independent mechanisms may be involved (13).

Recently, we have described a method for growing primary cultures of guinea pig airway parasympathetic neurons (14). These cells synthesize acetylcholine and release it in response to electrical stimulation. They express M_2 receptors that can be demonstrated at the mRNA level using reverse transcription (RT)[1]-PCR, at the protein level by immunocytochemistry, and functionally. Stimulated acetylcholine release from these cells is potentiated when the M_2 receptors are blocked by atropine, and inhibited when the M_2 receptors are stimulated using methacholine (14).

In these studies, we investigated the effects of infection of the nerve cells with parainfluenza virus and of treatment with IFN-γ (a cytokine that is produced by lymphocytes in response to viral infections) on the expression and function of M_2 receptors.

Methods

Animals

Female Dunkin-Hartley guinea pigs (180–200 g; supplied by Hilltop Animal Farms, Scottsdale, PA) were handled in accordance with the standards established by the USA Animal Welfare Acts set forth in National Institutes of Health guidelines and the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee.

Cell culture

Parasympathetic nerve cells were isolated and cultured using a method modified from Burnstock et al. as described previously (14). In brief, guinea pig trachealis muscle, containing parasympathetic ganglia, was minced and disaggregated using collagenase (0.2%). The resultant cell suspension was plated in 35 × 10 mm polystyrene culture dishes and incubated at 37°C in 5% CO_2. After 90 min, all nonadherent cells were gently collected and replated on Matrigel-coated (Collaborative Biomedical Products, Bedford, MA) culture dishes. Cells were grown in serum-free medium that contained 50% Ham's

These data were presented at the American Thoracic Society annual meeting in San Francisco, 1997, and were published in abstract form (Jacoby, D.B., N.H. Lee, H.Q. Xiao, and A.D. Fryer. 1997. *Am. J. Respir. Crit. Care Med.* 155:A208).

Address correspondence to Allison D. Fryer, Department of Environmental Health Sciences, School of Hygiene and Public Health, 615 N. Wolfe Street, Johns Hopkins University, Baltimore, MD 21209. Phone: 410-614-5449; FAX: 410-955-0299; E-mail: afryer@jhsph. edu

Received for publication 7 July 1997 and accepted in revised form 16 March 1998.

J. Clin. Invest.

0021-9738/98/07/0242/10 $2.00
Volume 102, Number 1, July 1998, 242–248
http://www.jci.org

1. *Abbreviation used in this paper:* RT-PCR, reverse transcription-PCR.

F12 and 50% DME supplemented with 20 $\mu g \cdot ml^{-1}$ L-glutamine (BioWhittaker, Walkersville, MD), 500 $\mu g \cdot ml^{-1}$ fatty acid–free BSA (Calbiochem Corp., San Diego, CA), 20 $\mu g \cdot ml^{-1}$ rat transferrin, 10 $\mu g \cdot ml^{-1}$ bovine insulin, 100 $ng \cdot ml^{-1}$ β-nerve growth factor (Sigma Chemical Co., St. Louis, MO), and 125 $U \cdot ml^{-1}$ penicillin (GIBCO BRL, Gaithersburg, MD). Cultures were incubated at 37°C in 5% CO_2 and were fed every 48 h. 24 h after plating, cytosine arabinoside (1.0 μM) was added to the medium and remained thereafter to inhibit growth of any dividing cells remaining in the culture. Cells were generally used in experiments 7–10 d after plating.

Viral infection and IFN-γ treatment of cultured nerve cells

Parainfluenza type 1 (Sendai virus; ATCC VR-105) was grown in embryonated chicken eggs for 2 d at 34°C. Infected fluid was cleared by low-speed centrifugation and stored in aliquots at −70°C.

Viral content was determined by exposing Rhesus monkey kidney cell monolayers (Viromed, Minnetonka, MN) to serial 10-fold dilutions of the virus stock solution. After incubation for 1 wk at 34°C, infection was determined using a hemadsorption assay (15). The monolayers were washed and the medium replaced with a 0.5% suspension of guinea pig erythrocytes. After 1 h, the erythrocytes were washed off, and the monolayers were examined under an inverted phase-contrast microscope (Olympus) for evidence of hemadsorption. Viral content was determined as the multiple of the amount of stock solution required to produce infection in 50% of the Rhesus monkey kidney cell monolayers, the $TCID_{50}$ (TCID = tissue culture infectious dose).

Cultured nerve cells were infected by adding 2×10^4 $TCID_{50}$ to each well. Virus was allowed to adhere to the neurons for 1 h at 34°C. The infecting medium was then removed, the cells were washed, and fresh medium was added. Infection of these cells was demonstrated both by recovering virus from the culture medium 24 h later and by a hemadsorption assay done directly on the infected nerve cells.

To treat cells with IFN-γ, mouse recombinant IFN-γ (Sigma Chemical Co.) was added to the culture medium at a final concentration of 300 U/ml. This was left in until functional experiments were done or RNA was harvested. All studies of acetylcholine release, M_2 receptor function, and M_2 receptor gene expression were done 24 h after either viral infection or treatment with IFN-γ.

Measurement of acetylcholine using chemiluminescence

Acetylcholine was measured as described previously (14) using a modification of a method described by Israel and Lesbats (16). This method is based on the following reactions: (*1*) Acetylcholine→choline + acetate; (*2*) Choline→betain + H_2O_2; where (*1*) is catalyzed by cholinesterase and (*2*) is catalyzed by choline oxidase. When horseradish peroxidase and luminol are exposed to this product, it leads to a chemiluminescent reaction of the luminol with the H_2O_2 that is proportional to the original concentration of acetylcholine. This was read in a chemiluminometer (Wallac, Gaithersburg, MD). As little as 0.4 nM acetylcholine can be measured using this technique.

96-well plates of neurons were grown for 7–10 d. Cells were washed with Krebs-Henseleit solution of the following composition (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.5, NaH_2PO_4 1.28, $NaHCO_3$ 25.0, and dextrose 5.55, bubbled with 5% CO_2 and 95% O_2; pH 7.4. Cells were then washed twice more for 1 min/wash with 100 μl of physiological solution, and 10-μl samples of each wash were taken for measurement of acetylcholine. Cells were stimulated by field stimulation using electrodes placed into the wells, and a 10-μl sample of the physiological solution was taken for measurement of acetylcholine release at the end of 1 min. The physiological solution was then replaced with physiological solution containing 80 mM KCl, and acetylcholine was measured 5 min later.

Each 10-μl sample was mixed with 0.5 ml of chemiluminescent reaction mixture made from 7.5 ml Krebs-Henseleit solution, 80 μl of 1,000 U/ml cholinesterase, 100 μl of 250U/ml choline oxidase, 50 μl of 2 mg/ml horseradish peroxidase, and 100 μl of 1 mM luminol. Acetylcholine was measured in relative light units over 1 min. The increase in acetylcholine with electrical field stimulation was calculated as a percentage of the baseline. The total amount of acetylcholine in each well was calculated as the concentration of acetylcholine from two 1-min washes, added to the acetylcholine in the field-stimulated sample, and acetylcholine released by KCl. Because of the possibility of variability in the number of nerve cells from culture to culture, each data point in the acetylcholine release studies represents the mean of five wells.

Testing the function of neuronal muscarinic receptors

To test the function of the M_2 muscarinic receptors on these nerve cells, the receptors were either blocked using atropine (10 μM) or stimulated using methacholine (0.1 μM). Because methacholine is not susceptible to degradation by cholinesterase, it does not interfere with the acetylcholine assay described above (as we have demonstrated previously [14]).

For experiments in which the M_2 receptors were blocked using atropine, the stimulus parameters were frequency = 5 Hz, pulse duration = 0.1 ms, stimulus intensity = 1 V, 30 pulses/train. For experiments in which the M_2 receptors were stimulated using methacholine, the stimulus parameters were frequency = 2 Hz, pulse duration = 0.1 ms, stimulus intensity = 1 V, 30 pulses/train. The reason for the different stimulus parameters in these two sets of experiments is that the receptor's response to endogenous agonist is greater at higher stimulus frequency (5), making it easier to demonstrate using atropine. Conversely, it is easier to demonstrate the effect of stimulating the receptor with an exogenous agonist (methacholine) when the receptor is not being stimulated by endogenous agonist, i.e., at lower stimulus frequency. A frequency–response curve was also generated using stimulus parameters of frequency = 0.2–10 Hz, pulse duration = 0.1 ms, stimulus intensity = 1 V, 6 s/train.

Competitive RT-PCR assay for M_2 receptor gene expression

Generating internal standard M_2 receptor cRNA. We extracted RNA from guinea pig heart by homogenizing tissues in RNAzol lysis buffer and extracting with chloroform and phenol/chloroform. RNA samples were treated for 30 min at 37°C with 2 U RNase-free DNase (Bethesda Research Laboratories, Gaithersburg, MD) to eliminate contaminating genomic DNA. 1 μg of RNA was reverse-transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (2.5 U/μl), dATP, dCTP, dGTP, and cTTP (1 mM each) RNase inhibitor (1 U/μl), and $oligo(dT)_{16}$ (2.5 μM). Reverse transcription was carried out at 42°C for 20 min, after which the reverse transcriptase was inactivated by heating to 99°C for 5 min. An aliquot of the reverse transcription reaction was used for PCR to amplify a portion of the guinea pig M_2 cDNA. As the guinea pig M_2 receptor sequence has not been determined previously, we used oligonucleotide primers based on the rat sequence (17): 5′-AAA AAG AAT TCC ACG AAA CCT CTG ACC TAC CC-3′ and 5′-AAA AAG GAT CCT CTG ACC CGA CGA CCC AAC TA-3′ (underlined sequences are EcoRI and XhoI adapter sites for subcloning). Agarose gel electrophoresis demonstrated a 0.7-kb product, as would be predicted from the rat sequence. We subcloned the PCR product from guinea pig heart into pGEM11. Sequencing reactions were performed on both strands using dye-terminator reactions on a thermocycler (model 9600; Perkin-Elmer Cetus Corp., Norwalk, CT) with the Prism Ready Reaction Dye Terminator Cycle sequencing kits (Applied Biosystems, Inc., Foster City, CA).

To generate an internal standard for the competitive RT-PCR assay, we excised a 43-base segment of the guinea pig M_2 receptor cDNA in pGEM3 using the restriction endonucleases XhoI and MscI. The resulting digest was reacted with Klenow to fill in the 3′ recessed ends and ligated. This yielded a template that was cut with BamHI, and transcribed in vitro using T7 polymerase to synthesize cRNA that was 43 bases smaller than the original M_2 receptor RNA.

Nerve cell RNA extraction and RT-PCR. To carry out the competitive RT-PCR assay, we used RNA extracted from control nerve cells, nerve cells that were infected with parainfluenza virus for 24 h,

and nerve cells that were exposed to IFN-γ (300 U/ml) for 24 h. Cells were grown for 7–10 d on 12-well Matrigel-coated plates. RNA was extracted using the RNAzol B method, and the RNA was precipitated in isopropanol overnight. These samples were treated for 30 min at 37°C with 2 U RNase-free DNase to eliminate contaminating genomic DNA. 500 ng of RNA was added to internal standard cRNA (0.01–10 pg/sample) in the presence of Moloney murine leukemia virus reverse transcriptase (2.5 U/μl), dATP, dCTP, cGTP, and dTTP (1 mM each), RNase inhibitor (1 U/μl), and random hexamer primers (2.5 μM). Reverse transcription was carried out at 42°C for 20 min, after which the reverse transcriptase was inactivated by heating to 99°C for 5 min. The absence of contaminating genomic DNA was confirmed by carrying out control reactions in which the reverse transcriptase was omitted.

Samples of cDNA obtained in the above reactions were amplified using AmpliTaq polymerase (1.5 U/50 μl) in the presence of 2 mM $MgCl_2$, dNTPs (0.4 mM), and paired primers based on the guinea pig M_2 receptor sequence. We designed new primers based on the guinea pig sequence. The upstream primer was 5′-TCC TCT CTT TCA TCC TCT GG-3′ and the downstream primer was GTG CCT GAG TCA CCT TTT TG-3′. These primers yield a PCR product of 568 bases using guinea pig M_2 mRNA, and 525 bases using the truncated internal standard cRNA. PCR was conducted for 40 cycles at 95°C for 1 min, 62°C for 1 min, using a Perkin Elmer-Cetus thermocycler. Final extension was at 72°C for 10 min. Aliquots of DNA products (10 μl) were run on agarose gels (4%), stained with ethidium bromide (0.5 μg/ml), and visualized with ultraviolet illumination. Amounts of PCR product were determined by densitometry. The input amount of M_2 receptor mRNA was then determined by interpolating to determine the amount of internal standard cRNA that yielded a ratio of M_2 mRNA product/cRNA product of 1.

Analysis of data

The frequency–response relationship for stimulated acetylcholine release was analyzed using repeated-measures ANOVA. The effects of atropine and methacholine on acetylcholine release in control, virus-infected and IFN-treated cells were compared using a multiway ANOVA. The levels of M_2 receptor mRNA in control, virus-infected, and IFN-treated cells were compared using a Friedman test.

Results

Infection of nerve cells. Cultured neurons became infected with parainfluenza virus as demonstrated both by hemadsorption (Fig. 1) and by recovering virus from the culture medium 24 h after infection.

Effects of viral infection and IFN-γ on acetylcholine release and M_2 receptor function. Neither viral infection nor treatment with IFN-γ changed the acetylcholine content of the cells (control, 134.7±12.2 pmol/well, virus-infected, 127.5±11.3 pmol/well, and IFN-treated, 125.0±11.0 pmol/well).

Cultured neurons released acetylcholine in frequency-dependent fashion in response to electrical field stimulation (Fig. 2). Pair-wise comparison revealed that the release of ace-

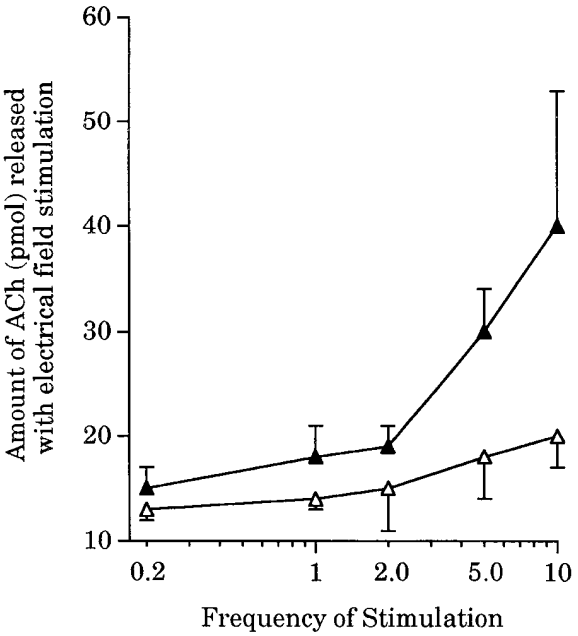

Figure 2. Electrically stimulated acetylcholine release from cultured airway neurons. Cells were stimulated for 6 s at 1 V, 0.1-ms pulse duration, at frequencies of 0.2–10 Hz. A frequency-dependent release of acetylcholine was observed in both control (*open triangles*) and virus-infected cells (*closed triangles*). This was potentiated at high (≥ 5 Hz) but not at low, frequencies in virus-infected cells.

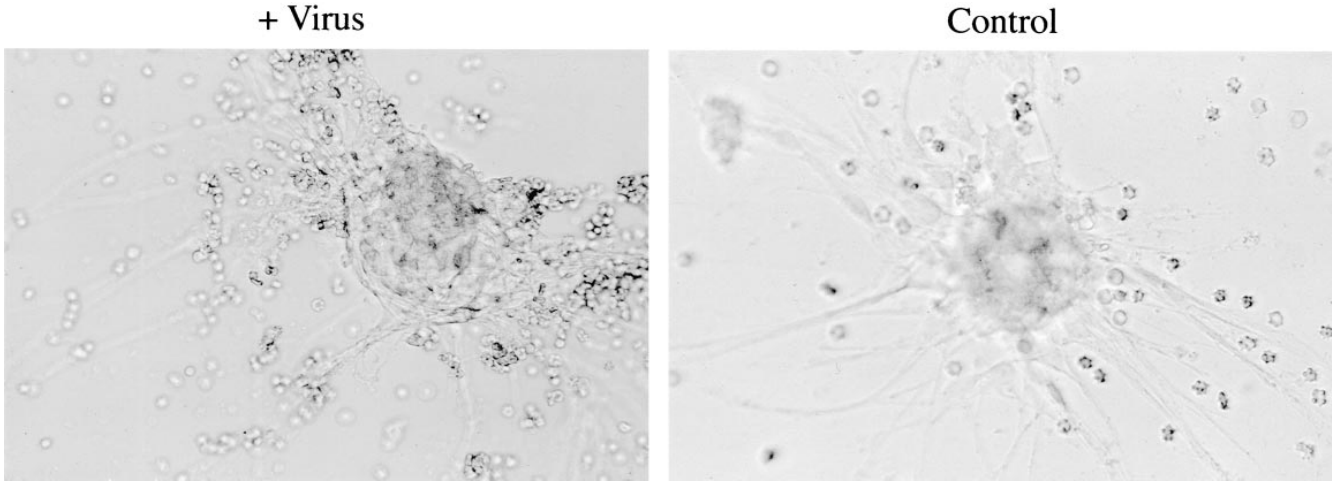

Figure 1. Neurons that have been exposed to parainfluenza virus become infected, as demonstrated by hemadsorption. Infected neurons bind guinea pig erythrocytes via viral hemagglutinin expressed on the neuron cell membrane (*left*). In contrast, uninfected neurons do not bind erythrocytes (*right*).

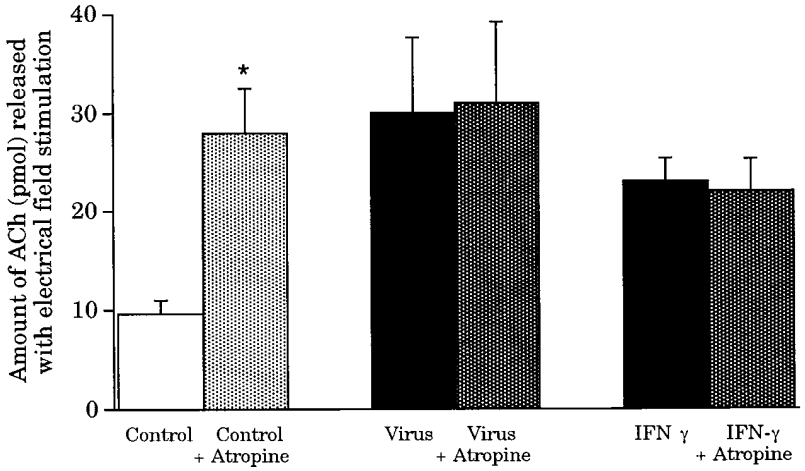

Figure 3. Electrically stimulated acetylcholine release from cultured airway neurons. In control neurons, acetylcholine release was potentiated when the M_2 receptors were blocked by atropine (10^{-5} M). Acetylcholine release was increased to about the same level by infection with parainfluenza virus and by treatment with IFN-γ (300 U/ml). Addition of atropine to virus-infected and IFN-treated cultures did not potentiate acetylcholine release further. Cells were stimulated at 1 V, 0.1-ms pulse duration, at a frequency of 5 Hz, for a train of 30 pulses. Data are mean of five experiments carried out in triplicate with SE.

tylcholine was significantly potentiated by viral infection at high (≥ 5 Hz) but not low stimulus frequencies.

In control neurons, acetylcholine release was potentiated threefold when the M_2 receptors on the cells were blocked using atropine (10^{-5} M; Fig. 3). Acetylcholine release was inhibited when cells were treated with methacholine (10^{-7} M; Fig. 4).

Both viral infection and treatment with IFN-γ increased the release of acetylcholine when the cells were stimulated at 5 Hz (Fig. 3) but not at 2 Hz (Fig. 4). Treatment of these cells with atropine did not further increase acetylcholine release (Fig. 3). Treatment of these cells with methacholine did not decrease acetylcholine release (Fig. 4).

Sequencing of guinea pig M_2 receptor segment. RT-PCR using mRNA from guinea pig heart and oligonucleotide primers based on the rat M_2 receptor sequence yielded a 683-base product, as would be predicted from the known rat sequence. Analysis of the subcloned M_2 receptor segment from the guinea pig heart revealed 85% sequence identity with the rat receptor sequence (Fig. 5). After excising the 43-base segment shown, RT-PCR using the new oligonucleotide primers based on the guinea pig sequence yielded a 525-base product starting with the truncated internal standard and a 568-base product starting with guinea pig heart or nerve cell mRNA.

Effects of viral infection and IFN-γ on M_2 receptor gene expression. Internal standard cRNA added in various amounts to the mRNA extracted from nerve cells competed with nerve cell M_2 receptor mRNA for primers and bases, yielding two bands distinguishable by agarose gel electrophoresis (Fig. 6 *A*). The log ratio of nerve cell product to internal standard product varied inversely and linearly with the log of the amount of internal standard added (Fig. 6 *B*). This allowed calculation of the amount of input M_2 receptor mRNA as equal to the amount of input internal standard cRNA added when the ratio of nerve cell product to internal standard product was 1. Competitive RT-PCR measurement revealed that both viral infection and treatment with IFN-γ decreased M_2 receptor mRNA substantially (Fig. 7).

Discussion

In these studies, we investigated the effects of IFN-γ, and of infection with parainfluenza virus, on M_2 receptor expression and function in primary cultures of airway parasympathetic neurons. As the predominant lymphocyte response to viral infection of the airways is a $CD8^+$ T lymphocyte response (18), producing IFN-γ, this cytokine is likely to be relevant to the leukocyte-dependent effects of viral infections on the M_2 receptor.

In uninfected nerve cell cultures, treatment with atropine blocked the inhibitory M_2 receptors and increased the release of acetylcholine. This confirms our previously reported studies

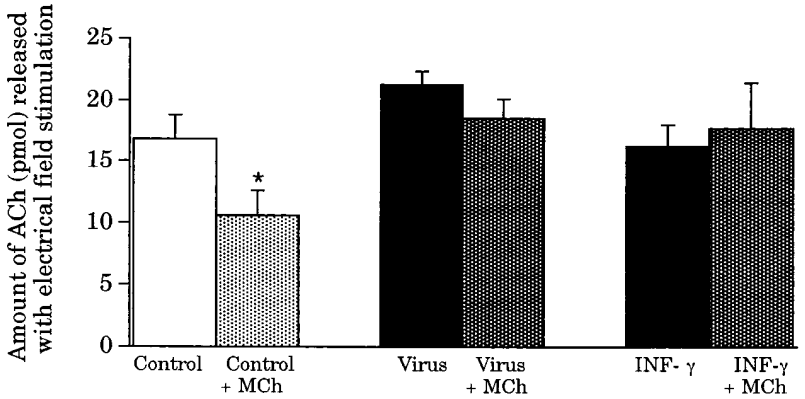

Figure 4. Electrically stimulated acetylcholine release from cultured airway neurons. In control neurons, acetylcholine release was inhibited when the M_2 receptors were stimulated by methacholine (10^{-7} M). Addition of methacholine to virus-infected and IFN-treated cultures did not inhibit acetylcholine release. Cells were stimulated at 1 V, 0.1-ms pulse duration, at a frequency of 2 Hz, for a train of 30 pulses. Data are mean of five experiments carried out in triplicate with SE.

```
AGTTAAGCGG  ACCACAAAAA  TGGCAGGAAT  GATGATTGCA  GCTGCCTGGG    50
←---------primer--------→
TCCTCTCTTT  CATCCTCTGG  GCTCCAGCCA  TCCTCTTCTG  GCAGTTCATA   100

GTAGGGGTGA  GAACTGTGGA  GGATGGGGAA  TGCTACATTC  AATTTTTTTC   150

CAATGCTGCT  GTCACCTTTG  GCACTGCCAT  TGCAGCCTTC  TACTTGCCAG   200
                                              ←-----------
TAATCATCAT  GACTGTGCTG  TATTGGCATA  TATCTCGAGC  CAGCAAGAGC   250
---------excised segment----------------→
AGGATAAAGA  AGGAGAAAAA  AGAGCCCGTG  GCCAACCAAG  ATCCTGTGTC   300

TCCAAGTCTG  GTGCAAGGAA  GAATAGTGAA  GCCAAACAAC  AACAACATGC   350

CCGGCAGTGA  CAGTGCCCTA  GAGCACAACA  AAATCCAGAA  TGGCAAGACC   400

CCCAGAGGTG  TTGTGACAGA  AAACTGTGTC  CAGGGGGAGG  AGAAAGAGAG   450

CTCCAATGAC  TCCACCTCAG  TCAGTGCCGT  TGCCTCTAAT  ATGAGAGATG   500

ATGAAATAAC  CCAGGATGAG  AACACAATAT  CCACTTCCCT  GGGCCAGACT   550
                                              ←--
AAAGATGAGA  ACTCTAAGCA  AACATGCATC  AAAATTGTCA  CCAAGACTCA   600
-------primer---------→
AAAAGGTGAC  TCAGGCACCC  CGAGTCACAC  CACTGTGGAG  T
```

Figure 5. Partial sequence of guinea pig muscarinic M_2 receptor cDNA. The product sequenced was generated by RT-PCR of mRNA from guinea pig heart using primers designed based on the sequence for the rat M_2 receptor gene (see text). Primers for competitive PCR were designed based on this sequence as shown. A 43-base segment was excised from this product to make the template for generation of the cRNA internal standard for competitive RT-PCR.

(14). Furthermore, as we have shown previously, methacholine, by stimulating the M_2 receptors, inhibited the release of acetylcholine.

Both treatment with IFN-γ and infection with parainfluenza virus markedly increased the release of acetylcholine from the nerve cell cultures. This effect was evident in the experiments reported in Fig. 3, where a higher frequency of stimulation (5 Hz) was used. This maximizes the effect of endogenous acetylcholine in stimulating the M_2 receptors (5, 14). Thus, it is easier to demonstrate blockade of the M_2 receptor with atropine at higher frequencies and similarly to demonstrate decreased receptor function at higher frequencies.

In the experiments reported in Fig. 4, where a lower frequency of stimulation (2 Hz) was used, a small increase in acetylcholine release was seen in virus-infected, but not in IFN-treated, cultures. The lower frequency of stimulation decreases the effect of endogenous agonist on the M_2 receptor, making it possible to demonstrate the function of the M_2 receptors by stimulating them with methacholine. At the same time, this makes it more difficult to demonstrate an increased release of acetylcholine due to M_2 receptor dysfunction.

In virus-infected and IFN-treated cultures, neither atropine nor methacholine altered the release of acetylcholine, demonstrating loss of M_2 receptor function. Furthermore, the observation that the release of acetylcholine in virus-infected and IFN-treated cells was the same as in control cells treated with atropine suggests that loss of M_2 receptor function is responsible for the increased acetylcholine release.

We have shown previously that viral infections impair the function of M_2 receptors in vivo via mechanisms that are both leukocyte-dependent and leukocyte-independent (13, 19). Among the effects of leukocytes on M_2 receptor function, we have shown that several positively charged proteins of the

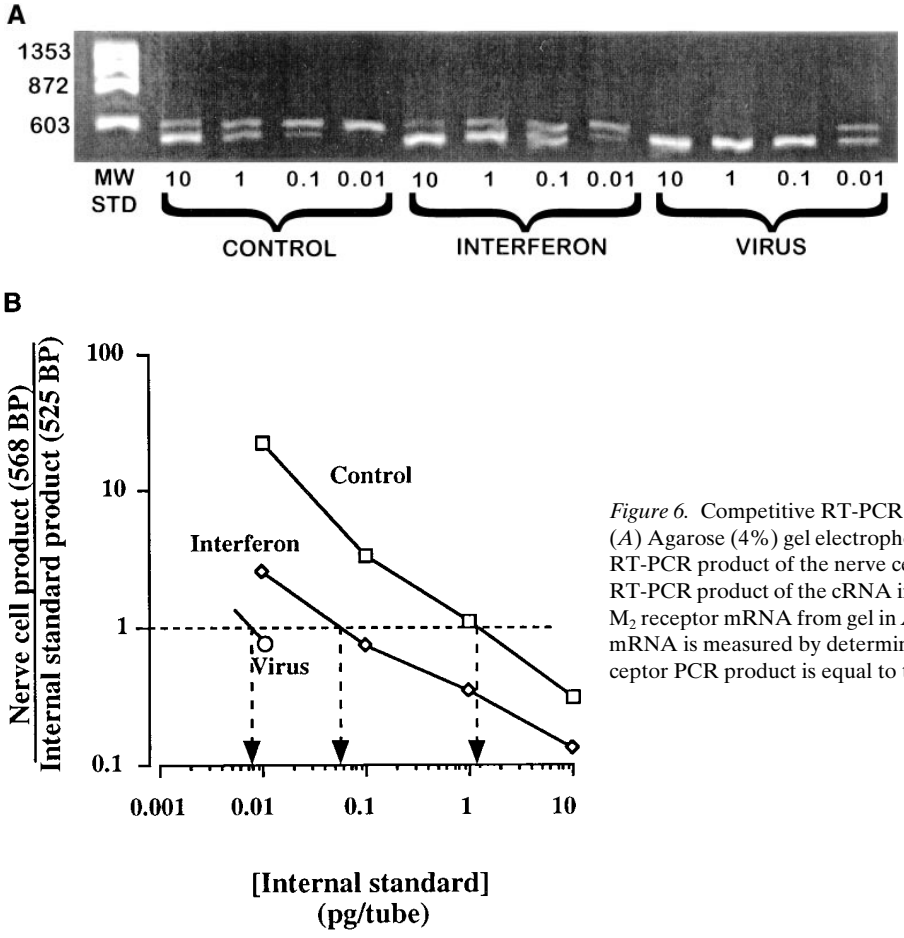

Figure 6. Competitive RT-PCR analysis of nerve cell M_2 receptor expression. (*A*) Agarose (4%) gel electrophoresis of PCR products. The upper band is the RT-PCR product of the nerve cell M_2 receptor mRNA. The lower band is the RT-PCR product of the cRNA internal standard. (*B*) Calculation of nerve cell M_2 receptor mRNA from gel in *A*. The amount of input nerve cell M_2 receptor mRNA is measured by determining the point at which the amount of M_2 receptor PCR product is equal to the amount of internal standard PCR product.

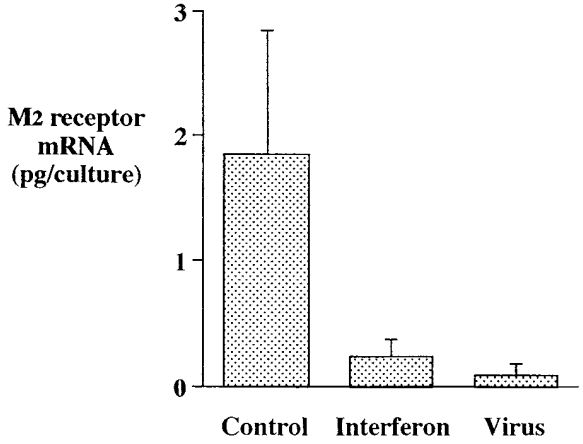

Figure 7. Effect of viral infection and IFN treatment on nerve cell M_2 receptor mRNA expression, determined by competitive RT-PCR. Each point represents the mean±SE of four experiments.

eosinophil act as allosteric antagonists at the M_2 receptors (20). This appears to be an important mechanism of M_2 receptor dysfunction in antigen-challenged guinea pigs, as neutralizing the positively charged proteins using heparin reverses the loss of receptor function (21), while blocking the effect of eosinophil major basic protein using an antibody prevents antigen-induced loss of receptor function (22). However, the failure of heparin to reverse the effects of viral infections in virus-infected guinea pigs (13) suggests that the leukocyte-dependent mechanisms of M_2 receptor dysfunction after viral infection are not related to eosinophil proteins. Among the possible leukocyte-independent mechanisms, we showed that parainfluenza virus itself causes a 10-fold loss of agonist affinity for the M_2 receptor when incubated with the receptor in vitro (19). This was due to cleavage of sialic acid residues by neuraminidase, an enzyme expressed by influenza and parainfluenza viruses.

The decreased expression of the M_2 receptor gene in the current studies may be an additional mechanism of M_2 receptor dysfunction. IFN-γ is produced by both $CD8^+$ and $CD4^+$ T lymphocytes in response to viral infections (23). The ability of this cytokine to potentiate the release of acetylcholine from neurons and to make this release unresponsive to atropine and methacholine strongly suggests the loss of a functional M_2 receptor on the cells. The decreased M_2 receptor mRNA in IFN-γ–treated neurons suggests that the mechanism of the effects on acetylcholine release and M_2 receptor function may be at the level of gene expression.

Likewise, viral infection of the neurons also decreased M_2 receptor function and decreased M_2 receptor mRNA. Although the primary site of viral infection is the airway epithelial cell, it is possible that infection of the airway nerves also takes place. Mori and colleagues (24) showed that exposure to parainfluenza virus leads to infection of the olfactory nerves. In the absence of direct infection of the airway parasympathetic nerves, production of IFN-γ in response to infection of other airway tissues could still decrease the expression of the M_2 receptor gene.

This is the first report of the regulation of M_2 receptor gene expression in cultured neurons. However, expression of the M_2 receptor gene has been shown to be regulated in chick heart, cerebellar granular cells, and human lung fibroblasts. Exposure to the muscarinic agonist carbachol decreases M_2 receptor mRNA in chick heart (25) and in cerebellar granular cells (26). Muscarinic agonists may also decrease the M_2 receptor content of cells by mechanisms other than decreased gene expression (27). In the HEL 299 cell line (human embryonic lung fibroblasts), M_2 receptor expression is decreased by activation of protein kinase C (28), and by the cytokines IL-1β, TNF-α (29), TGF-β (27), and platelet-derived growth factor (30).

The effects of viral infection and IFN-γ on M_2 receptor gene expression, as well as on acetylcholine release and M_2 receptor function, may be important in explaining the effects of viral infection on airway function. It has long been recognized that viral infections are associated with exacerbations of asthma (1) and that they can lead to increased vagally mediated reflex bronchoconstriction (2, 3). The function of the M_2 muscarinic receptor on the airway nerves is lost after viral infections in both guinea pigs (11) and rats (12), leading to increased vagally mediated bronchoconstriction (4). Loss of M_2 receptor gene expression as a result either of direct infection of the nerve with virus or of production of IFN-γ in virus-infected airways may be responsible for loss of the inhibitory feedback that normally limits the release of acetylcholine in the airways. This may explain the increased reflex bronchoconstriction and exacerbation of asthma symptoms seen during viral airway infections.

Acknowledgments

We thank Bethany Yost for technical assistance.

This work was supported by National Institutes of Health grants HL-54659 (Dr. Jacoby), HL-44727 and HL-55543 (Dr. Fryer), and by grants from the Center for Indoor Air Research (Dr. Jacoby) and from the American Heart Association (Dr. Jacoby and Dr. Fryer).

References

1. Johnston, S.L., P.K. Pattemore, G. Sanderson, S. Smith, F. Lampe, L. Josephs, P. Symington, S. O'Toole, S.H. Myint, D. Tyrrell, and S.T. Holgate. 1995. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *Br. Med. J.* 310:1225–1229.

2. Empey, D.W., L.A. Laitinen, L. Jacobs, W.M. Gold, and J.A. Nadel. 1976. Mechanisms of bronchial hyperreactivity in normal subjects following upper respiratory tract infection. *Am. Rev. Respir. Dis.* 113:523–527.

3. Aquilina, A.T., W.J. Hall, R.G. Douglas, and M.J. Utell. 1980. Airway reactivity in subjects with viral upper respiratory tract infections: the effects of exercise and cold air. *Am. Rev. Respir. Dis.* 122:3–10.

4. Buckner, C.K., V. Songsiridej, E.C. Dick, and W.W. Busse. 1985. In vivo and in vitro studies of the use of the guinea pig as a model for virus-provoked airway hyperreactivity. *Am. Rev. Respir. Dis.* 132:305–310.

5. Fryer, A.D., and J. Maclagan. 1984. Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.* 83:973–978.

6. Aas, P., and J. Maclagan. 1990. Evidence for prejunctional M_2 muscarinic receptors in pulmonary cholinergic nerves of the rat. *Br. J. Pharmacol.* 101:73–76.

7. Blaber, L.C., and A.D. Fryer. 1985. The response of the cat airways to histamine in vivo and in vitro. *Br. J. Pharmacol.* 84:309–316.

8. Ito, Y., and T. Yoshitomi. 1988. Autoregulation of acetylcholine release from vagus nerve terminals through activation of muscarinic receptors in the dog trachea. *Br. J. Pharmacol.* 93:636–646.

9. Ayala, L.E., and T. Ahmed. 1989. Is there loss of a protective muscarinic receptor in asthma? *Chest.* 96:1285–1291.

10. Minette, P., and P.J. Barnes. 1988. Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea-pig airways. *J. Appl. Physiol.* 64:2532–2537.

11. Fryer, A.D., and D.B. Jacoby. 1991. Parainfluenza virus infection damages inhibitory M_2 muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig. *Br. J. Pharmacol.* 102:267–271.

12. Sorkness, R., J.J. Clough, W.L. Castleman, and R.F. Lemanske. 1994. Virus-induced airway obstruction and parasympathetic hyperresponsiveness in adult rats. *Am. J. Respir. Crit. Care Med.* 150:28–34.

13. Fryer, A.D., K.A. Yarkony, and D.B. Jacoby. 1994. The effect of leukocyte depletion on pulmonary M_2 muscarinic receptor function in parainfluenza virus-infected guinea-pigs. *Br. J. Pharmacol.* 112:588–594.

14. Fryer, A.D., C.L. Elbon, A.L. Kim, H.-Q. Xiao, A.I. Levey, and D.B. Jacoby. 1996. Cultures of airway parasympathetic nerves express functional M_2 muscarinic receptors. *Am. J. Respir. Cell Mol. Biol.* 15:716–725.

15. Shelokov, A., J.E. Vogel, and L. Chi. 1958. Hemadsorption (adsorption-hemagglutination) test for viral agents in tissue culture with special reference to influenza. *Proc. Soc. Exp. Biol. Med.* 97:802–809.

16. Israel, M., and B. Lesbats. 1987. The use of bioluminescence techniques in neurobiology, with emphasis on the cholinergic system. *In* Neurochemistry: A Practical Approach. A.J. Turner and H.S. Bachelard, editors. IRL Press, Washington, DC. 113–125.

17. Wei, J., E.A. Walton, A. Milici, and J.J. Buccafusco. 1994. m1-m5 muscarinic receptor distribution in rat CNS by RT-PCR and HPLC. *J. Neurochem.* 63:815–821.

18. Graham, M.B., D.K. Dalton, D. Giltinan, V.L. Braciale, T.A. Stewart, and T.J. Braciale. 1993. Response to influenza infection in mice with a targeted disruption in the interferon gamma gene. *J. Exp. Med.* 178:1725–1732.

19. Fryer, A.D., E.E. El-Fakahany, and D.B. Jacoby. 1990. Parainfluenza virus type 1 reduces the affinity of agonists for muscarinic receptors in guinea-pig heart and lung. *Eur. J. Pharmacol.* 181:51–58.

20. Jacoby, D.B., G.J. Gleich, and A.D. Fryer. 1993. Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M_2 receptor. *J. Clin. Invest.* 91:1314–1318.

21. Fryer, A.D., and D.B. Jacoby. 1992. Function of pulmonary M_2 muscarinic receptors in antigen challenged guinea pigs is restored by heparin and poly-L-glutamate. *J. Clin. Invest.* 90:2292–2298.

22. Evans, C.M., D.B. Jacoby, G.J. Gleich, A.D. Fryer, and R.W. Costello. 1997. Antibody to eosinophil major basic protein protects M_2 receptor function of antigen challenged guinea pigs in vivo. *Am. J. Respir. Crit. Care Med.* 155: A574.

23. Graham, M.B., V.L. Braciale, and T.J. Braciale. 1994. Influenza virus–specific $CD4^+$ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J. Exp. Med.* 180:1273–1282.

24. Mori, I., T. Komatsu, K. Takeuchi, K. Nakakuki, M. Sudo, and Y. Kimura. 1995. Parainfluenza virus type 1 infects olfactory neurons and establishes long-term persistence in the nerve tissue. *J. Gen. Virol.* 76:1251–1254.

25. McKinnon, L.A., M. Rosoff, S.E. Hamilton, M.L. Schlador, S.L. Thomas, and N.M. Nathanson. 1997. Regulation of muscarinic receptor expression and function in cultured cells and in knock-out mice. *Life Sci.* 60:1101–1104.

26. Longone, P., I. Mocchetti, M.A. Riva, and W.J. Wojcik. 1993. Characterization of a decrease in muscarinic m2 mRNA in cerebellar granule cells by carbachol. *J. Pharmacol. Exp. Ther.* 265:441–446.

27. Haddad, E.B., J. Rousell, J.C. Mak, and P.J. Barnes. 1996. Transforming growth factor-beta 1 induces transcriptional down-regulation of m2 muscarinic receptor gene expression. *Mol. Pharmacol.* 49:781–787.

28. Rousell, J., E.B. Haddad, J.C.W. Mak, and P.J. Barnes. 1995. Transcriptional down-regulation of m2 muscarinic receptor gene expression in human embryonic lung (hel 299) cells by protein kinase c. *J. Biol. Chem.* 270:7213–7218.

29. Haddad, E.B., J. Rousell, M.A. Lindsay, and P.J. Barnes. 1996. Synergy between tumor necrosis factor alpha and interleukin 1beta in inducing transcriptional down-regulation of muscarinic M_2 receptor gene expression. Involvement of protein kinase A and ceramide pathways. *J. Biol. Chem.* 271: 32586–32592.

30. Barnes, P.J., E.B. Haddad, and J. Rousell. 1997. Regulation of muscarinic M_2 receptors. *Life Sci.* 60:1015–1021.