Eosinophil-Mediated Cholinergic Nerve Remodeling

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Eosinophils are observed to localize to cholinergic nerves in a variety of inflammatory conditions such as asthma, rhinitis, eosinophilic gastroenteritis, and inflammatory bowel disease, where they are also responsible for the induction of cell signaling. We hypothesized that a consequence of eosinophil localization to cholinergic nerves would involve a neural remodeling process. Eosinophil co-culture with cholinergic IMR32 cells led to increased expression of the M_2 muscarinic receptor, with this induction being mediated via an adhesion-dependent release of eosinophil proteins, including major basic protein and nerve growth factor. Studies on the promoter sequence of the M_2 receptor indicated that this induction was initiated at a transcription start site 145 kb upstream of the gene-coding region. This promoter site contains binding sites for a variety of transcription factors including SP1, AP1, and AP2. Eosinophils also induced the expression of several cholinergic genes involved in the synthesis, storage, and metabolism of acetylcholine, including the enzymes choline acetyltransferase, vesicular acetylcholine transferase, and acetylcholinesterase. The observed eosinophil-induced changes in enzyme content were associated with a reduction in intracellular neural acetylcholine but an increase in choline content, suggesting increased acetylcholine turnover and a reduction in acetylcholinesterase activity, in turn suggesting reduced catabolism of acetylcholine. Together these data suggest that eosinophil localization to cholinergic nerves induces neural remodeling, promoting a cholinergic phenotype.

Keywords: cholinergic; eosinophil; muscarinic; asthma

In the airways, parasympathetic nerve stimulation induces acetylcholine release, which leads to mucous secretion and contraction of bronchial smooth muscle. Since mucous production and bronchoconstriction are central features of airway diseases such as asthma and chronic obstructive pulmonary disease (COPD), the parasympathetic nerves may play a pivotal role in the pathogenesis of these conditions. There are several key steps in the synthesis, storage, and release of acetylcholine (ACh). Acetylcholine is synthesized from choline and acetyl CoA under the enzymatic action of choline acetyltransferase (ChAT). ACh is transported into synaptic vesicles by way of the vesicular acetylcholine transporter (VAChT). Once released, ACh stimulates muscarinic receptors on both target organs and the nerves themselves by stimulating M_2 muscarinic receptors. M_2 muscarinic receptors function as autoreceptors to limit further ACh release. ACh is metabolized into choline and acetate by the action of acetylcholinesterase (AChE). Thus, a core group of enzymes and receptors are responsible for controlling the synthesis, activity, and turnover of ACh in cholinergic nerves.

Allergic inflammation is associated with the release of neurotrophins and other factors that have a direct effect on nerves (1). One effect of inflammatory mediators is that they can alter neuronal neurotransmitter content; this effect is termed neural plasticity. Neural plasticity is widely described in afferent nerves, but it is not known if cholinergic nerves are similarly subject to plasticity. Eosinophils are a source of neurotrophins and they accumulate at cholinergic nerves in human and animal models of allergic conditions such as asthma, rhinitis, and eosinophilic gastroenteritis (2–6). Thus, one way in which eosinophils may cause cholinergic nerve cell remodeling is through the release of nerve growth factor (NGF). *In vitro* cell culture studies have also demonstrated that eosinophil contact with nerves activates MAP kinases and other signaling pathways in nerves (9). Thus, eosinophils may influence nerve function via direct contact as well as by their released factors (6–9). The genes involved in the synthesis, control of release, and metabolism of ACh are under the control of a variety of intracellular signaling pathways, including several protein kinases such as the MAP kinase family. Therefore, we hypothesized that eosinophil interactions with nerves may lead to a change in the expression of the genes that control ACh synthesis and metabolism.

To study this hypothesis we employed an established, *in vitro*, co-culture model consisting of eosinophils and the human neuroblastoma cell line IMR32. This nerve cell line displays a cholinergic phenotype when differentiated in culture (6, 7). We studied the effect of purified eosinophils, eosinophil proteins, and surface receptors on cholinergic gene expression in this model system. In addition, we measured the functional activity of the enzymes, the intracellular acetylcholine content and made preliminary observations as to which aspect of the M_2 muscarinic receptor promoter sequence was responding to eosinophils. The results indicate that eosinophils promote a cholinergic phenotype, in particular, through the release of NGF and eosinophil cationic proteins.

MATERIALS AND METHODS

Materials

DMEM Plus Glutamax, FCS, and penicillin/streptomycin solutions were purchased from GIBCO/BRL Life Technologies (Paisley, UK). The IMR32 cell line was obtained from ECACC (Salisbury, UK) and depleted of fibroblasts using immunomagenetic antifibroblast microbeads and LD MACS separation columns (Miltenyi Biotech, Bisley, UK). TRI Reagent, gentamicin, Trypan Blue, diphenyleneiodinium (DPI), CDP-Star chemiluminescent substrate solution, Igepal CA-630, anti-goat IgG alkaline phosphatase (AP) conjugate, phenylmethylsulfonyl fluoride (PMSF), dithithreitol (DTT), and all common buffer constituents were obtained from Sigma (Poole, UK). All primers were obtained from MWG-Biotech AG (Ebersberg, Germany). I-Block for Western blot blocking and Nitro-Block II, chemiluminescent substrate component for AP, were purchased from Tropix (Bedford, MA).

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Protease inhibitors set, 1st strand cDNA synthesis kit for RT-PCR (AMV), and LightCycler-FastStart DNA master SYBR Green 1 were from Roche Molecular Biochemicals (Lewes, East Sussex, UK). Polyclonal rabbit anti-human VAChT antibody (H-160, isotype IgG) and polyclonal rabbit anti-human M_2 antibody (H-170, isotype IgG) were both from Santa Cruz Biotechnology (Santa Cruz, CA,). Polyclonal goat anti-human ChAT antibody was obtained from Chemicon International (Temecula, CA). PCR reaction buffer, *Taq* polymerase, dNTPs, anti-rabbit IgG AP conjugate, Transfectam reagent, Dual Luciferase Reporter assay system, and Wizard PCR preps DNA purification system from Promega (Madison, WI). Image Master VDS-Cl and software Total Lab v1.00 and Ficoll-Paque PLUS were from Amersham Pharmacia Biotech (Little Chalfont, UK). CD16 immunomagnetic beads and VS+ VarioMacs columns were purchased from Miltenyi Biotech. Speedy-Diff was obtained from Clin-Tech Ltd (Clacton-on-Sea, UK). Anti–ICAM-1 and –VCAM-1 antibodies were from Santa Cruz (8). Eosinophil proteins were prepared as previously described (10).

IMR32 Nerve Cell Culture

The human cholinergic neuroblastoma cell line IMR32 was depleted of fibroblasts, as described previously (9). The cells were maintained in culture in proliferation medium (DMEM Plus Glutamax, 5% FCS, 100 U/ml penicillin/streptomycin, 10 μg/ml gentamicin) at 37°C in an atmosphere of 5% CO_2. On achieving confluence, cells were plated at a density of 5×10^5/well in 6-well cell culture dishes and grown in proliferation medium for 48 h. Proliferation media was then replaced by differentiation medium (DMEM Plus Glutamax, 2% FCS, 2 mM sodium butyrate, 100 U/ml penicillin/streptomycin, 10 μg/ml gentamicin) and cells were used for experimentation after a further 6–7 d of differentiation in this medium.

Eosinophil Isolation

Eosinophils were prepared from 45 ml of peripheral blood from healthy human volunteers by a negative immunomagnetic selection technique, essentially as described previously (6). Only populations of eosinophils which were > 98% pure and > 95% viable were used in experimentation. For experimentation purposes 2×10^5 eosinophils/well were added to differentiated IMR32 cells plated as above in 6-well cell culture plates.

Eosinophil Membrane Preparation

Immediately upon isolation, eosinophils were resuspended in cold, sterile dH_2O, incubated on ice for 15 min, then centrifuged at $1{,}500 \times g$ for 10 min at 4°C, as described previously (11). This process was repeated twice, and the resulting lysed cell membranes were resuspended in differentiation medium.

Co-Culture Experiments

Prior studies indicated that 2×10^5/ml of eosinophils was optimal for transcription factor activation in IMR32 cells (9–12); therefore, this number was used for the current studies. IMR32 cells (5×10^5) were differentiated for 6–7 d with sodium butyrate as described above and then incubated with 2×10^5 eosinophils for varying time periods from 1–48 h at 37°C. In some experiments, IMR32 cells were pretreated with inhibitors of eosinophil adhesion for 30 min or an antibody to nerve growth factor for 2 h, and co-culture experiments with eosinophils were performed in the presence or absence of these inhibitors. In other experiments, IMR32 cells were pretreated with eosinophil membranes, which contain the eosinophil adhesion molecules but not eosinophil RNA nor proteins (9). Eosinophil membranes were prepared as described above and were added at a concentration equivalent to 1×10^5 eosinophils.

Nuclear, Cytoplasmic, and Membrane Protein Preparation

Nuclear and cytoplasmic extracts were isolated from IMR32 cells, essentially as described in Ref. 9. For membrane preparations, cells were rinsed with PBS and detached with 0.5 mM EDTA in PBS at 37°C. The detached cells were pelleted at 6,000 rpm for 5 min and suspended in 100 μl buffer A containing 5 mM Tris (pH 6.8), 2 mM EDTA, and freshly added protease inhibitors (5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 5 μg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride) at 4°C. The cells were forced through a 22-gauge needle five to eight times, and the lysate was spun in a Beckman Ultracentrifuge (Beckman, Krefeld, Germany) at 55,000 rpm for 20 min at 4°C to collect the membrane pellet. The pellet was resuspended in buffer B containing 20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, and 1% Triton X-100 with freshly added protease inhibitors (5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 5 μg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and stored at −80°C. Protein concentration was established by the Bradford method (13) and extracts were stored at −80°C.

Western Blotting

Protein extracts (10 μg for M_2 analysis or 20 μg for ChAT and VAChT analysis) were heated to 95°C in sample buffer (100 mM Tris pH 6.8, 2% [wt/vol] SDS, 0.002% [wt/vol] bromophenol blue, 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol) and separated by SDS-PAGE on 10% polyacrylamide separating gel overlaid with 4% stacking gel at 500 V for 1 h. The separated proteins were transferred to a nitrocellulose membrane in transfer buffer (20 mM Tris, 150 mM glycine, 0.01% [wt/vol] SDS, 20% [vol/vol] methanol) at 500 V overnight. For immunodetection with rabbit anti-human M_2 antibody, goat anti-human ChAT antibody, or rabbit anti-human VAChT antibody, membranes were incubated in blocking buffer (Dulbecco's PBS [Invitrogen Ltd, Paisley, UK] containing 0.2% [wt/vol] I-block and 0.1% [vol/vol] Tween-20) for 1 h at room temperature then incubated for 2 h in blocking buffer containing the individual respective antibody (1:200 for each). Following six 5-min washes in washing buffer (PBS [Sigma] pH 7.4, 0.1% [vol/vol] Tween-20) membranes were incubated for 1 h in blocking buffer containing a dilution of the appropriate anti-goat IgG (ChAT) (1:10,000) or anti-rabbit IgG (M_2, VAChT) (1:7,500) AP conjugate. Membranes were then washed six times for 5 min each and exposed to CDP Star chemiluminescent substrate solution plus Nitro-Block II chemiluminescent substrate compound for AP (19:1) for 5 min at room temperature. Blots were then exposed to X-OMAT light-sensitive film (Kodak, Stuttgart, Germany) to obtain an image.

mRNA Analysis

Total RNA was isolated from the cells with TRI reagent, according to the manufacturer's instructions. For both quantitative LightCycler PCRs and semiquantitative RT-PCRs, 1 μg of total RNA was reverse-transcribed into cDNA using an oligo $(dT)_{15}$ primer using the first-strand cDNA synthesis system. Amplification of cDNA was performed by quantitative PCR on the LightCycler using fast start Taq DNA polymerase containing the double-stranded DNA binding dye SYBR Green 1. The samples were continuously monitored during the PCR, and fluorescence was acquired every 0.1°C. PCR mixtures contained 0.5 μM of either β-actin–specific primers (forward, 5′ TCC TGT GGC ATC CAC GAA ACT 3′; reverse, 5′ GAA GCA TTT GCG GTG GAC GAT 3′) M_2-specific primers (forward, 5′ GTG GTC AGC AAT GCC TCA GTT AT 3′; reverse, 5′ TCC CCA TCC TCC ACA GTT CTC 3′) ChAT-specific primers (forward, 5′ TTG TGA GAG CCG TGA CTG AC 3′; reverse, 5′ CAC AGG ACC ATA GCA GCA GA 3′), VAChT-specific primers (forward, 5′ ATA GTG CCC GAC TAC ATC GC 3′; reverse, 5′ TCT TCG CTC TCC GTA GGG TA 3′), MnSOD-specific primers (forward, 5′ AGA TCA TGC AGC TGC ACC ACA 3′; reverse, 5′ GTT CTC CAC CAC CGT TAG GGC 3′), or AChE-specific primers (forward, 5′ CCT CCT TGG ACG TGT ACG AT 3′; reverse, 5′ CTG ATC CAG GAG ACC CAC AT 3′). The samples were denatured at 95°C for 10 min followed by 45 cycles of annealing and extension at 95°C for 12 s, 55°C for 5 s, and 72°C for 8 s (β-Actin, MnSOD, ChAT), 10 s (M_2, VAChT), or 6 s (AChE). Characteristic melting curves were obtained at the end of amplification by cooling the samples to 65°C for 15 s followed by further cooling to 40°C for 30 s. Serial 10-fold dilutions were prepared from known quantities of β-actin, M_2, ChAT, VAChT, AChE, and MnSOD PCR products, which were then used as standards to plot against the unknown samples. Quantification of data was analyzed using the LightCycler analysis software, and values were normalized to the level of β-actin expression for each sample on the same template cDNA. In semiquantitative RT-PCR, the integrity of RNA extraction and cDNA synthesis was verified by PCR by measuring the amounts of β-actin cDNA in each sample using β-actin–specific primers (as above). PCR mixtures contained 10× reaction buffer, 2.5 mM $MgCl_2$, 1.25 units of *Taq* polymerase,

and 0.2 mM of each dNTP. Thermocycling conditions for M_2 cDNA were 95°C for 5 min, 42 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Twenty-nine cycles were used to amplify the more abundant β-actin cDNA. A final extension step of 72°C for 10 min was followed by resolution of the 211–bp M_2 products and the 314–bp β-actin products on a 1.5% Tris borate-EDTA agarose gel containing 0.5 μg/ml ethidium bromide. M_2 PCR products were captured and quantified by densitometry using the Image Master VDS-Cl and software Total Lab v1.00.

ACh Measurements

ACh content was determined by high performance liquid chromatography with an enzymatic reactor containing acetylcholinesterase and choline oxidase and an electro-chemical detector using a commercial kit (Bioanalytical Systems, Inc., Warwickshire, UK) based on the method of Potter (14). Protein concentration was determined by the method of Bradford (13). The cells were pre-incubated for 20 min in a choline-free physiologic salt solution (pH 7.4) consisting of NaCl 13 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 0.75 mM, glucose10 mM, and HEPES 10 mM. The cells were then incubated in the same buffer supplemented with neostigmine (50 μM) with or without PMA100 nM for 45 min. The cells were scraped into 1 ml of methanol, and this mixture transferred to a tube containing formic acid 100 μl 1M, vortex mixed, and centrifuged. The pellet was collected for protein assay while ACh was extracted by mixing the supernatant fluid with chloroform and water (1:2:1 vol/vol). The samples were vortex mixed and centrifuged for 5 min at 3,000 rpm. The aqueous phase (containing ACh) was collected and dried under a vacuum.

Measurement of Acetylcholinesterase Activity

IMR32 cells (1×10^6) were homogenized in 200 μl of a solution of 50 mM Tris pH 8.0/0.2 mM EDTA (buffer A), centrifuged at $12{,}000 \times g$ for 10 min, and supernatant was removed. Fifty microliters of 100ug/ml DTNB (Ellmans Reagent) and 50 μl 20 mM acetyl (β-methyl) thiocholine iodide were added to 50 μl cell supernatant. A blank containing no cells was prepared in tandem. All samples were incubated at 37°C for 5 min. The reaction was followed in a plate reader at 410 nm every 5 min for 20 min. For the initial experiment, supernatants were diluted 1:2, 1:5, 1:10, and 1:20 to determine if the reaction was saturated.

Identification of the Promoter Sequence of the M_2 Muscarinic Receptor in IMR32 Nerve Cells

Preparation of promoter deletion constructs and reporter assay analysis Preparation of RNA, 5′ Rapid Amplification of cDNA ends, cloning, and sequencing from IMR32 cells were performed as described previously in human airway smooth muscle cells (15). Regions upstream of each of the three identified major transcription start sites were investigated for promoter activity based on information obtained from the analysis of the 5′RACE results. Regions upstream of each of the three identified major transcription start sites were investigated. Each region was amplified from human genomic DNA using PCR primers with restriction site consensus sequences for *Mlu*1 and *Xho*1built into the terminal regions of the oligonucleotides. This was necessary to enable subsequent directional cloning into the pGL3E firefly luciferase reporter vector. All PCR reactions were as described in Ref. 15.

Preparation of promoter deletion constructs and transient transfection of IMR32 cells. Transient transfections were performed using Transfectam reagent. Each clone pGL3E firefly luciferase reporter plasmid was co-transfected with pRL-SV40, a plasmid that expresses renilla luciferase under the influence of an SV40 promoter. The latter plasmid was used as a transfection efficiency control. Transfection solutions, containing 0.75 μg cloned pGL3E DNA, 18.75 ng pRL-SV40 DNA (Ratio 50:1), and Transfectam reagent to give a 2:1 ratio, were prepared in serum-free cell culture media. Transfection was performed via the dropwise addition of each transfection solution to a well containing differentiated IMR32 cells. Cells were then incubated at 37°C 5%CO_2 for 48 h. Eight constructs were transfected, as well as an empty pGL3E plasmid and a nontransfected control. Each vector construct was transfected into six wells, with each individual experiment being repeated four times. After the 48-h incubation, three wells of each transfected construct were treated with whole human eosinophils for a further 24 h, while three wells were left untreated. This was also performed for the empty vector and the nontransfected control.

Dual luciferase reporter assay. Luciferase assays were performed using the dual luciferase reporter assay system according to the manufacturer's instructions. The 6-well plates were removed from the incubator, and the growth medium was removed. The wells were rinsed with PBS and aspirated. Passive lysis buffer was then added to each well, and the plates were left at room temperature for 15 min. The lysates were assayed for firefly luciferase activity by the addition of lysate to luciferase assay reagent II, and luminescence was measured in a Wallac Victor^2 mutilabel counter (PerkinElmer, Boston, MA). Renilla luciferase activity was subsequently assayed by adding Stop and Glo reagent and luminometric measurement. Results were normalized for variations in transfection efficiency by using the ratio of firefly:renilla luciferase activity as an index of promoter activity. The promoter activity of each construct was expressed as a fold value over baseline reporter expression activity (transfection with empty pGL3E vector).

Statistical Analyses

Values are expressed as mean ± SD. The statistical significance of differences between treated samples and the appropriate time point control was evaluated by ANOVA; $*P < 0.05$, $**P < 0.005$.

RESULTS

Eosinophils Do Not Express mRNA for the M_2 Muscarinic Receptor nor Cholinergic Genes

In these control experiments (Figure 1A), RNA was extracted from 2×10^6 eosinophils, reverse transcribed, and subjected to real-time RT-PCR using the relevant primers. Results demonstrated that eosinophils did not express mRNA for M_2 muscarinic receptors nor the cholinergic genes VAChT, ChAT, and AChase. However, the β-actin gene product was detected in all cases, demonstrating mRNA integrity. In contrast, all of the above genes were detected in human IMR32 nerve cell RNA (Figures 1B and 1C). Figure 1C shows a representative amplification plot, demonstrating the stability of the β-actin message expression.

Eosinophils Induce both M_2 mRNA and Protein Production in Differentiated IMR32 Cells

Real-time RT-PCR analysis revealed a 225% increase in M_2 mRNA relative to the untreated control after 24 h of eosinophil co-incubation with IMR32 cells ($*P \leq 0.05$) (Figures 2A and 2B). Cellular protein was harvested and separated into cytoplasmic and membrane fractions. Western blot analysis using a polyclonal antibody to the human M_2 muscarinic receptor indicated that M_2 protein was expressed only in IMR32 cell membrane fractions (Figure 2C). Co-incubation of eosinophils with IMR32 cells for time periods between 1 and 24 h demonstrated that M_2 muscarinic receptor protein levels doubled after 24 h of co-incubation (Figures 2D and 2E).

Adhesion Is Essential but Not Sufficient for Eosinophil-Induced M_2 Protein Expression in Differentiated IMR32 Cells

In prior studies, we have shown that pretreatment of IMR32 cells with antibodies against both ICAM-1 and VCAM-1 completely inhibit eosinophil adhesion to nerves (6, 8). When eosinophils were co-incubated with IMR32 nerve cells in the presence of these antibodies, no increase in either M_2 mRNA or protein synthesis was observed (Figures 3A and 3B). We then investigated whether contact alone was sufficient to induce changes in M_2 receptor expression similar to those observed following treatment with whole eosinophils. Eosinophil cell membranes, which express eosinophil adhesion molecules but not eosinophil proteins nor RNA, were co-incubated with differentiated IMR32 cells for 1–24 h with no observed change in M_2 expression at 24 h (Figure 3A). These data suggest that adhesion is necessary but not sufficient to account for the eosinophil-induced changes in gene expression.

We have previously shown that eosinophil adhesion to nerves stimulates the release of eosinophil-derived factors. We then investigated if the changes in M_2 receptor expression were due to these released factors. Eosinophils synthesize, store, and release the neurotrophin NGF, which is known to influence cholinergic gene expression (16). We investigated the role of NGF on eosinophil induced changes in M_2 receptor expression by employing a specific NGF-neutralizing antibody that significantly reduced M_2 mRNA expression (Figure 3C). By contrast, a normal goat IgG control had no effect on eosinophil-induced upregulation of M2 receptor expression (Figure 3D).

Eosinophils contain four unique cationic proteins: eosinophil major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP). In the absence of specific neutralizing antibodies to these proteins, we studied the effect of MBP, EDN, and EPO, as well

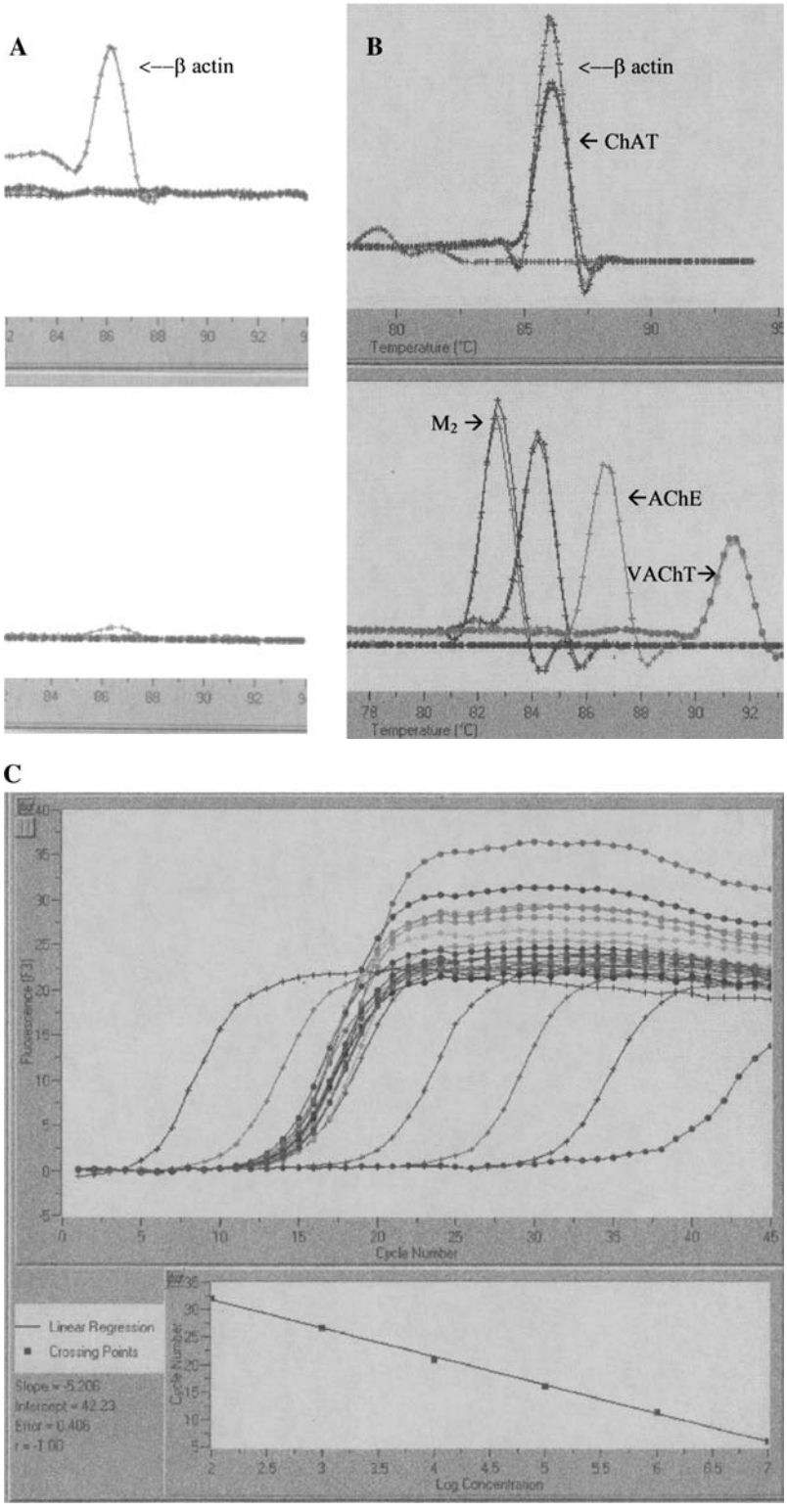

Figure 1. Human eosinophils do not express cholinergic genes. In *A*, eosinophil mRNA was extracted from 5×10^6 human eosinophils that had been isolated from a healthy donor, as described in MATERIALS AND METHODS. The RNA was then reverse-transcribed and subjected to real-time RT-PCR for M_2, ChAT, VAChT, and β-actin. In *B*, RNA was extracted from 5×10^6 IMR32 nerve cells, reverse-transcribed, and subjected to real-time PCR as described in MATERIALS AND METHODS. The PCR products for ChAT and β-actin (*upper panels*) and M_2, VAChT, and AChE (*lower panels*) were identifiable by their characteristic melting temperatures. The figures are representative of three independent experiments. *C* and *D* show representative amplification plots for the real-time PCR experiments on β-actin (*C*) and M_2 (*D*).

D

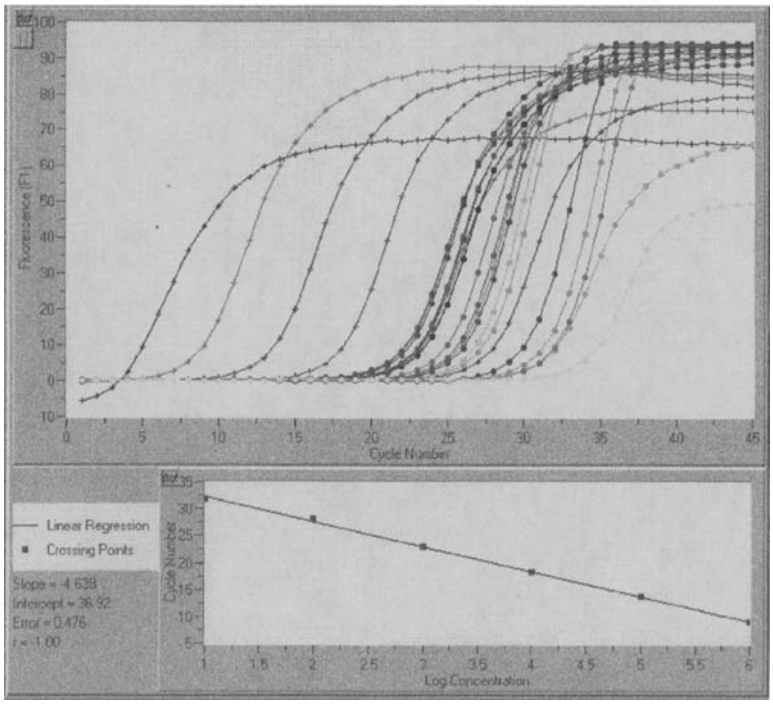

Figure 1. Continued

as all three proteins in combination, on M_2 gene expression in IMR32 cells. We demonstrated that, in combination (M/E/E), these proteins increased M_2 mRNA expression almost 2-fold (Figure 3E). The eosinophil protein concentrations chosen were similar to those released from eosinophils (1×10^6/ml) in contact with IMR32 cells (7).

Eosinophils Induce M_2 Receptor Expression via Transcription Start Site 3 in the Human M_2 Promoter in IMR32 Cells

The 5′ untranslated region of the M_2 muscarinic gene as expressed in IMR32 cells was identified using a combination of Rapid Amplification of 5′ cDNA Ends (5′ RACE) and reporter gene assays. Sequencing of successful 5′RACE clones confirmed five (A, B, C, E, and F) of the arrangements reported in human airway smooth muscle (HASM) cells (15). A new arrangement (G) was also identified (Figure 4A). All the 5′UTR arrangements identified as a result of 5′RACE experiments are shown in Figure 4B, together with their lengths. In total, five exons with alternative splicing patterns separated by introns ranging from 87 bp to > 145 kb were identified, in keeping with recent studies on both HASM and IMR32 cells. To identify the major regulatory region(s) of M_2 receptor expression in IMR32 cells and to also specifically identify the regions where eosinophils were exerting their effects, a series of promoter deletion constructs that spanned the three transcription start sites (TSS) were designed (Figure 4B). These were used in a series of transient transfection experiments on IMR32 cells that were exposed to eosinophils for 24 h; luciferase-based reporter assays were subsequently used to detect activity. The results obtained from IMR32 transfectants treated with 2×10^5 eosinophils for a 24-h period (Figure 4C) demonstrated construct C1 to have higher activity (6.34 ± 0.68 [+Eos]– versus 3.12 ± 3.30 [Control]–fold over empty vector [$n = 4$, $P < 0.05$]) than C2 (3.62 ± 1.65 [+Eos]– versus 1.45 ±1.41 [Control]–fold over empty vector [$n = 4$, $P = 0.05$]). Both of these regions contain sequence upstream of TSS3, suggesting that the major regulatory region for the muscarinic M_2 receptor expression in response to eosinophils in IMR32 cells lies immediately upstream of TSS3. With respect to the region upstream of TSS1, construct C5 showed a significant reduction in activity compared with controls (0.31± 0.13 [+Eos]– versus 0.53 ± 0.34 [Control]–fold over empty vector [$n = 4$, $P = 0.05$, ± SD]). As low promoter activity was obtained for all regions upstream of TSS1, sequences upstream of TSS1 in IMR32 cells may contain repressor elements.

Eosinophils Induce ChAT and VAChT mRNA and Protein Production in Differentiated IMR32 Cells

Eosinophils induced a 5-fold increase in ChAT gene expression relative to β-actin control after 24 h of co-incubation with IMR32 cells ($n = 5$, $^*P \leq 0.05$). Western blotting of harvested IMR32 cellular protein fractions (Figure 5A) demonstrated that ChAT protein was expressed only in the cytoplasmic fraction of IMR32 cells and not in either the membrane or nuclear fractions (Figure 5B). Eosinophil co-incubation with IMR32 cells induced a 2-fold increase in ChAT protein expression by 24 h (Figure 5C). Real-time RT-PCR demonstrated that eosinophils induced a 132 ± 12% increase in VAChT gene expression relative to β-actin control after 1 h of eosinophil co-incubation with IMR32 cells ($^*P = 0.05$). Western blotting of protein fractions from IMR32 cells demonstrated that VAChT was only expressed in the membrane fraction of IMR32 and not in the cytoplasmic fraction (Figure 5D). Eosinophils co-incubated with IMR32 cells induced a 2-fold increase in VAChT protein expression after 24 h of co-culture (Figures 5E and 5F).

Eosinophil Adhesion Reduces Acetylcholine Content and Increases Choline Content in IMR32 Cells

Eosinophil co-incubation with differentiated IMR32 cells over 24–48 h significantly decreased ACh content, with a concomitant significant increase in choline levels (Figures 6A and 6B).

Eosinophils induce AChE mRNA Production in Differentiated IMR32 Cells

Real-time RT-PCR demonstrated that eosinophils induced a modest change of ∼ 140% at 1 h and 12 h in AChE mRNA

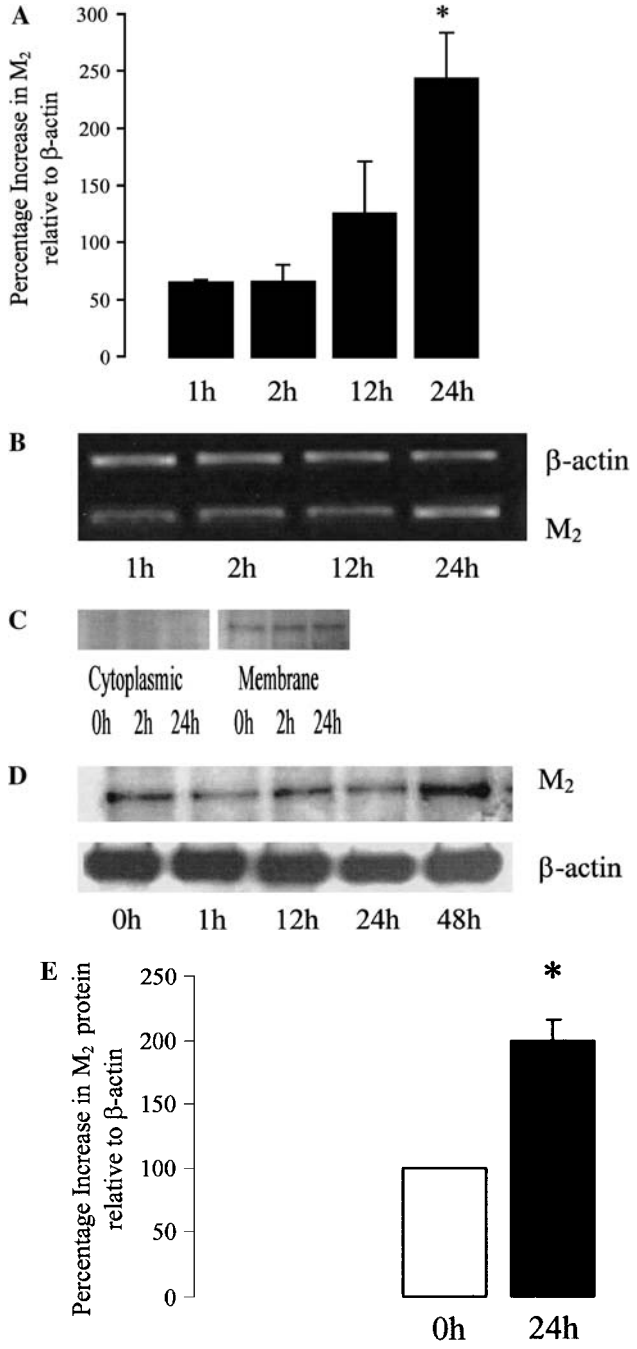

Figure 2. Eosinophils induce M_2 mRNA and protein production in differentiated IMR32 cells. Differentiated IMR32 cells were co-incubated with 2×10^5 human eosinophils for time periods of 1–24 h. The RNA was extracted, reverse-transcribed, and subjected to (*A*) quantitative real-time RT-PCR on LightCycler, where M_2 levels are normalized to β-actin (values are mean ± SD, $n = 3$, $^*P < 0.05$, relative to untreated control); or (*B*) semiquantitative PCR was performed and M_2 and β-actin PCR products were resolved on an agarose gel. In *C*, Western blot was performed on membrane and cytoplasmic protein fractions extracted from IMR32 cells that were exposed to eosinophils for the indicated time points, as described in MATERIALS AND METHODS. This experiment demonstrated that M_2 protein was detected only in membrane fractions. In *D*, Western blot analysis of IMR32 cell membrane protein fractions of IMR32 cells that were co-incubated with eosinophils for the indicated times is shown. Maximal M_2 protein expression was observed at 24 h. β-Actin was detected after stripping and re-probing of the blot shown in *D* (*upper panel*). In *E*, the eosinophil-induced change in M_2 protein from *D* is displayed graphically (values are mean ± SD, $n = 3$, $^*P < 0.05$).

relative to β-Actin control in IMR32 cells ($^*P \leq 0.05$, Figure 7A). However, functional studies revealed a significant decrease in the activity of AChE in IMR32 cells after 24 h of eosinophil co-incubation (Figure 7B).

Eosinophils Do Not Induce MnSOD mRNA Production in Differentiated IMR32 Cells

In prior studies it has been shown that contact between eosinophils and nerves led to the generation of oxygen free radicals in IMR32 nerve cells (8, 12). Furthermore, it has also been demonstrated that these free radicals were important intermediate signals involved in NF-κB activation in the nerve cells controlling nerve growth (9–11). Thus, we investigated whether the antioxidant gene, MnSOD, was also effected by eosinophil co-culture with nerves. RT-PCR demonstrated that in IMR32 cells eosinophils did not induce an increase in MnSOD mRNA at any of the time points studied (Figure 8).

DISCUSSION

The effects of eosinophil co-culture on cholinergic gene and protein expression in the cholinergic nerve cell line IMR32 were addressed in this study. Of particular interest was the muscarinic M_2 receptor, the cholinergic enzymes ChAT (instrumental in acetylcholine synthesis) and VaChT (responsible for ACh packaging), and the ACh hydrolysing enzyme AChE. The results obtained indicate that eosinophils alter the cholinergic phenotype of IMR32 cells. We observed that eosinophils increase M_2, ChAT, and VAChT mRNA and protein production after 24 h of co-culture. Furthermore, this led to increased ACh turnover and a significant decrease in AChE enzymatic activity. There was no alteration in gene expression of the antioxidant enzyme MnSOD at any of the time points studied, indicating that the observed changes in cholinergic genes were specific and not part of a generalized change in gene expression. Thus, eosinophils induce a muscarinic receptor that controls the release of ACh from nerves, induce the enzymes involved in the synthesis and storage of ACh, and cause a loss of function of the enzyme, which is responsible for the hydrolysis of acetylcholine. Together these data suggest that eosinophils promote and enhance the release of ACh from cholinergic nerve cell terminals.

It is known that the signaling molecules PI_3 kinase, PKA, and the MAP kinases are involved in regulating the expression of ChAT, VAChT, and the M_2 muscarinic receptor via a series of signaling pathways (17–19). *In vitro*, we have shown that eosinophils adhere to nerve cells via specific adhesion molecules and subsequently release factors that induce signaling pathways in nerve cells (9). Since eosinophils are known to promote airway remodeling in asthma, and since the expression of cholinergic genes is controlled by eosinophil-activated signaling pathways, we investigated whether eosinophils were responsible for the activation of these genes in cholinergic nerve cells.

In this study, M_2 receptor protein levels increased by at least 2.5-fold after 24 h of eosinophil co-culture. Experiments were undertaken to demonstrate the mechanisms underlying these changes. First, it was shown that eosinophils need to be adherent to nerves via the adhesion molecules ICAM-1 and VCAM-1 to induce M_2 receptor expression, as no alteration in gene expression was observed in the presence of adhesion inhibitors. However, adhesion alone was insufficient to induce M_2 expression, as a preparation of isolated eosinophil membranes did not induce these changes. Therefore, eosinophil adhesion led to the release of eosinophil factors that were responsible for changes in cholinergic phenotype, as we have previously described (8, 12). Functional studies revealed that this increased expression of M_2 was due to released NGF and eosinophil cationic proteins, as M_2

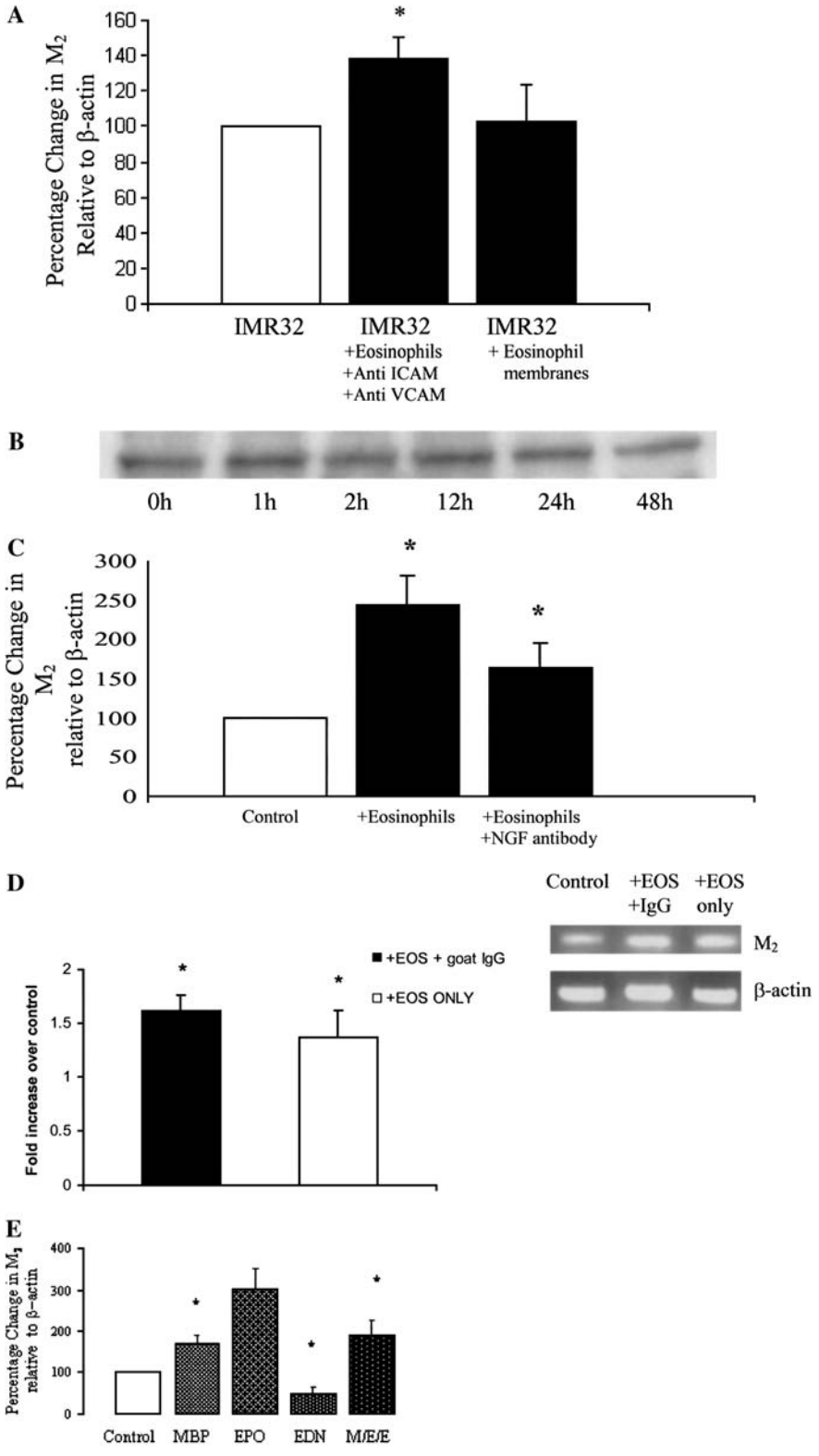

Figure 3. Eosinophil-induced M_2 gene and protein expression in differentiated IMR32 cells is dependent on eosinophil adhesion and released factors such as NGF and eosinophil proteins. In *A*, differentiated IMR32 cells were either pretreated with antibodies against VCAM-1 and ICAM-1 and then co-incubated with 2×10^5 isolated eosinophils for 24 h, or were co-incubated with purified membranes isolated from 2×10^5 human eosinophils for 24 h. RNA was extracted, reverse-transcribed, and subjected to quantitative real-time RT-PCR ($n = 3$, $^*P < 0.05$, relative to untreated control). Neither eosinophil membranes nor whole eosinophils in the presence of blocking antibodies induced M_2 mRNA expression in differentiated IMR32 cells. M_2 levels were normalized against the housekeeping gene β-actin. *B* shows Western blot analysis depicting M_2 protein levels in IMR32 cell membrane fractions that were pretreated with the blocking antibodies described above and then co-incubated with 2×10^5 human eosinophils for up to 48 h. Figure is representative of three independent experiments. (*C* and *D*) Differentiated IMR32 cells were either left untreated or were treated with (*C*) an NGF-neutralizing antibody (0.08 ng/ml) or (*D*) an equivalent amount of normal goat IgG, and subsequently co-incubated with 2×10^5 human eosinophils for 24 h. Cellular RNA was extracted, reverse-transcribed, and subjected to quantitative real-time RT-PCR ($n = 4$, $^*P = 0.05$, when compared with untreated control). In *E*, the effect of eosinophil proteins on M_2 receptor expression in IMR32 cells is shown. The eosinophil granule proteins MBP, EPO, and EDN were incubated with IMR32 cells both separately and in combination (M/E/E) for 24 h. Cellular RNA was extracted, reverse-transcribed, and subjected to quantitative real-time RT-PCR ($n = 3$, $^*P < 0.05$, when compared with untreated control).

gene expression was induced at 24 h by MBP (170%) and EPO (300%), suggesting these proteins play a part in the change induced by whole eosinophils (245%). Interestingly, treatment with EDN significantly decreased M_2 expression (50%). When cells were treated with a combination of all three proteins, M_2 expression was seen to increase significantly (190%), but not achieving levels as high as that of whole eosinophils.

From this study, it appears that M_2 expression is negatively regulated by factors as yet unidentified. It is possible that EDN could play an important role in this regulation. It is known to possess potent RNase activity which may explain in part the absence of the M_2 gene at the later time points. That is, it may specifically target a factor necessary for M_2 transcription or it may directly affect M_2 mRNA.

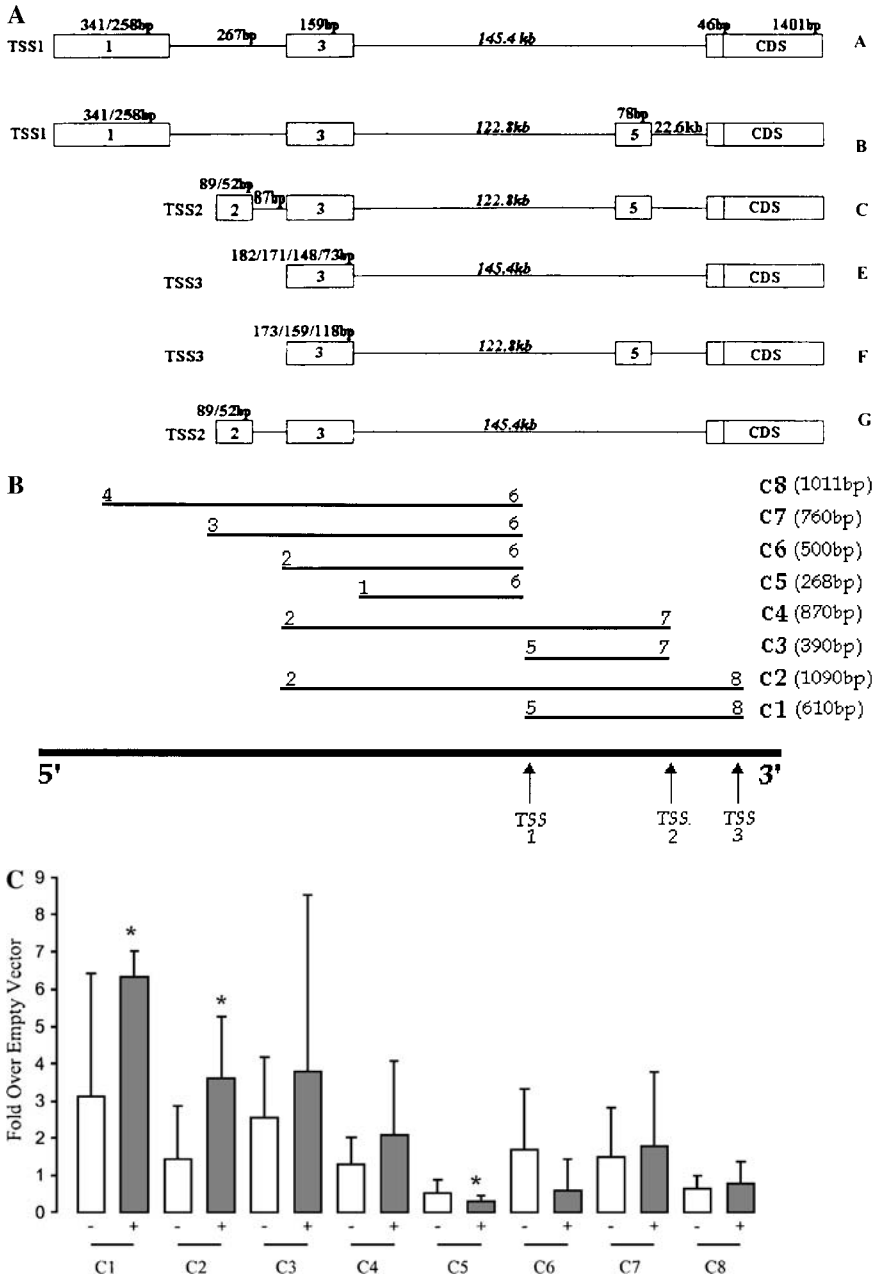

Figure 4. Eosinophil induced increase in promoter activity is due to activation of transcription factors in the TSS 3 region. (*A*) Identified arrangements, obtained by DNA sequencing analysis, in the human muscarinic M_2 receptor 5′UTR in IMR32 cells (CDS, coding sequences; *boxed region*, exonic sequence; *lined region*, intronic sequence). *B* shows the eight regions of the M_2 receptor 5′ UTR, which were amplified for pGL3E cloning. These different constructs cover the three main transcription start sites shown. Luciferase reporter activity for IMR32 transfectants (C) were cultured with (+) or without (−) eosinophils for 24 h. Data are expressed as fold values over empty vector baseline control ($n = 4$, ± SD, $^*P < 0.05$, when compared with nontransfected control).

To identify the promoter region(s) of IMR32 cells that exerts the most significant regulatory control over M_2 transcription in response to eosinophil co-culture, we initially identified the transcription starts sites in IMR32 cells and subsequently performed reporter gene studies. Analysis of the sequence data arising from 5′RACE experiments identified the presence of six different mRNA transcripts. The region also contains five exons of which Exon 2 and Exon 5 are alternatively spliced. The experiment confirmed the earlier published results (15, 20), and also identified one new transcript arrangement (G) (Figure 4D). We identified three regions of transcription initiation in the human muscarinic M_2 receptor gene 5′UTR, with each region containing a cluster of specific transcription start sites (TSSs) in close proximity to each other. The most 5′ TSS lies more than 146 kb upstream from the ATG start codon of the gene.

The M_2 coding sequence is preceded by a 46-bp exon that is expressed in all mRNA transcripts we obtained. Upstream of this, we have identified four additional exons, of which exons 5 and 2 are alternatively spliced. Our data suggest that the TSS1 region appears to be the most commonly used transcription start site (23 out of 46 clones), whereas TSS2 is the rarest (3 out of 46 clones).

Reporter gene expression analysis performed on IMR32 cells, transiently transfected with pGL3 Enhancer constructs, provided data that strongly suggest that the major regulatory region lies immediately upstream of TSS3. In addition, it would appear that repressor elements could operate upstream of TSS1, which is supported by the fact that construct 4 induces decreased expression of M_2 compared with construct 3. Also, all constructs containing regions upstream of TSS2 displayed low activity levels.

The human muscarinic M_2 receptor promoter, similar to all muscarinic receptor promoters identified, is TATA-less. Sp1, AP1, AP2, and GATA transcription factors have previously been cited as relevant for TATA-less promoters (21–26). In view of this it is interesting to note that the highest incidence of

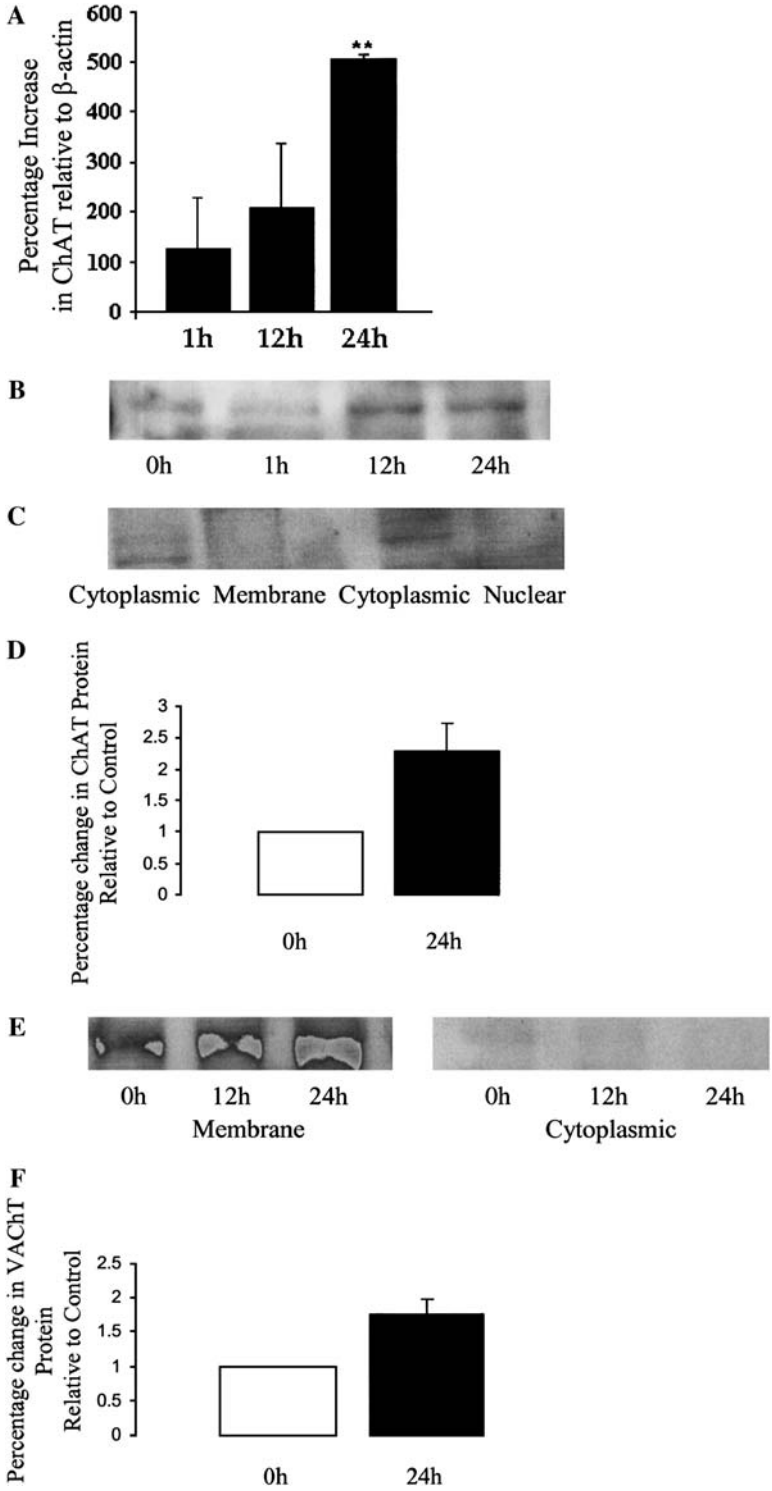

Figure 5. Eosinophils induce ChAT and VAChT production in differentiated IMR32 cells. Differentiated IMR32 cells were co-incubated with 2×10^5 human eosinophils for the indicated time points. In *A*, RNA was extracted, reverse-transcribed, and subjected to quantitative real-time RT-PCR and ChAT levels normalized against β-actin ($n = 3$, $^{**}P < 0.005$, when compared with untreated control). In *B*, Western blot analysis of IMR32 cell cytoplasmic, membrane, and nuclear fractions demonstrated that ChAT protein was expressed solely in cytoplasmic fractions. In *C*, co-incubation of IMR32 cells with eosinophils led to an increase in ChAT protein expression in cytoplasmic fractions, which was maximal at 24 h. In *D*, a graphical representation of Western blot analyses demonstrated that ChAT protein expression was significantly increased relative to control after 24 h of co-culture (Figures are representative of three independent experiments). In *E*, Western blot analysis membrane and cytoplasmic fractions from IMR32 cells demonstrated that VAChT protein was present only in membrane fractions and that eosinophil-induced increases in VAChT protein expression were maximal at 24 h. In *F*, co-incubation of IMR32 cells with eosinophils for 24 h led to a significant increase in VAChT protein expression relative to β-actin. Figures are representative of three independent experiments.

Sp1, GATA, and AP sites lies within the region of maximum transcriptional regulatory activity, immediately upstream of TSS3. We have previously shown that eosinophil MBP can induce AP transcription factor activation in IMR32 cells (9), but further studies will be required to establish which transcription factors are involved in eosinophil-induced activation of the M_2 receptor.

ACh is synthesized from choline and acetyl CoA under the enzymatic action of ChAT and is transported into the synaptic vesicle by the action of VAChT, where it is stored until required. Twenty-four hours of eosinophil co-culture with IMR32 nerve cells led to an increase in ChAT and VAChT protein levels. At the same time, choline levels are seen to increase by at least 3-fold. These facts suggest that the increased levels of intracellular choline are due to an increase in choline uptake in order for *de novo* ACh synthesis to occur. There is also a corresponding increase in ChAT enzyme levels, leading to increased amounts of ACh. However, VAChT protein levels are also increased, suggesting increased packaging of the newly synthesized ACh into vesicles. These facts suggest a rapid turnover of ACh such

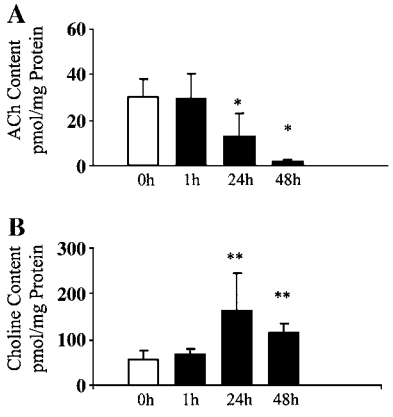

Figure 6. Eosinophil adhesion reduces intracellular acetylcholine content and concomitantly increases choline content in differentiated IMR32 cells. In *A*, ACh levels in differentiated IMR32 cells that have been treated with eosinophils for the indicated times were seen to decrease after 24 h of co-incubation. In *B*, choline levels in differentiated IMR32 cells that have been treated with eosinophils for the indicated times increased after 24 h of co-incubation. ACh and choline were quantified by HPLC, as described in MATERIALS AND METHODS ($n = 4$, $^*P = 0.05$, $^{**}P < 0.01$, when compared with untreated control).

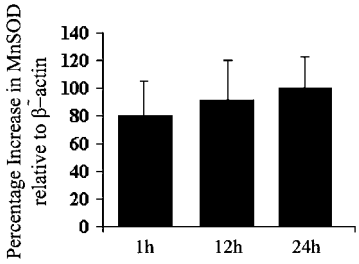

Figure 8. Eosinophils do not induce MnSOD mRNA production in differentiated IMR32 cells. Differentiated IMR32 cells were co-incubated with 2×10^5 human eosinophils for time periods of 1–24 h. RNA was extracted, reverse-transcribed, and subjected to quantitative real-time RT-PCR on LightCycler as described in MATERIALS AND METHODS. MnSOD levels were normalized against β-actin. Figure is representative of three independent experiments.

that intracellular ACh levels fall quickly and extracellular levels are high. Indeed, overexpression of VAChT in immature xenopus neurons increased the amount of neurotransmitters released by synaptic vesicles (27). Similar results were obtained for the related molecule VMAT2 (a monoamine transporter), which demonstrated that increasing its expression in mature synaptic vesicles actually increased transmitter release (28). This is supported by our prior experiments, which demonstrated an increase in the spontaneous release of ACh from nerves after exposure to eosinophils (6, 7). Specifically, we demonstrated that when eosinophils are added to IMR32 cells, they enhance acetylcholine release by 36% (7). We have also previously shown that eosinophils degranulate in response to adhesion to IMR32 cells (6, 8). MBP is a major eosinophil degranulation product and is a selective allosteric antagonist at the M_2 receptor (29–31), Thus release of MBP from eosinophils renders the M_2 receptor dysfunctional, resulting in the increased ACh release we refer to above. The effect of this protein is an important cause of M_2 receptor dysfunction and enhanced vagally mediated bronchoconstriction in asthma. It may be that the observed increase in M_2 expression in response to eosinophils and to MBP and EPO is an attempt to overcome this antagonistic effect.

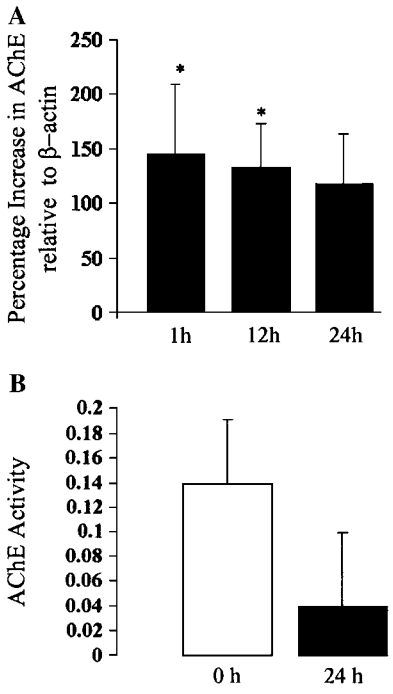

Figure 7. Eosinophils induce a minor increase in AChE mRNA production in IMR32 cells with an accompanying fall in its activity. In *A*, differentiated IMR32 cells were co-incubated with 2×10^5 human eosinophils for the indicated time points, and RNA was extracted, reverse-transcribed, and subjected to quantitative real-time RT-PCR on LightCycler as described in MATERIALS AND METHODS. AChE levels were normalized against β-actin ($n = 8$, $^*P < 0.05$, when compared with untreated control). In *B*, AChE activity was quantified as described in MATERIALS AND METHODS, with an observed decrease in AChE activity after 24 h of eosinophil co-incubation.

Levels of ACh esterase were seen to increase modestly between 1 and 12 h, but this was not maintained beyond 12 h. As an alternative to Western blotting for AChE protein, enzyme activity was measured, as results obtained would be of greater functional significance. After 24 h of eosinophil co-culture with nerve cells, there was a fall in AChE activity. This may be of some significance, as loss of AChE activity would be expected to lead to increased neurotransmission via ACh and augment the effect of the other changes seen in this system (i.e., increased cholinergic activity). There have been prior studies in a number of animal models of loss of function of acetylcholinesterase (27, 32–48). The observation of decreased acetylcholinesterase activity in the context of a modest increase in acetylcholinesterase protein expression may seem contradictory. Acetylcholinesterase exists in multiple molecular forms, which are differentially inactivated in different cell growth conditions (49). Therefore, depending on what molecular form is predominantly present, it could be inactivated in our cell system. However, another possible explanation is that in this manuscript we measured the expression of cell-associated acetylcholinesterase, not soluble cell-free acetylcholinesterase. In other cells, most acetylcholinesterase enzymatic activity ($\sim 80\%$) is detected in the cell growth medium as soluble acetylcholinesterase, both in humans and in other species (50, 51). Therefore, in this context it is not unexpected that increased acetylcholinesterase cell-associated protein expression does not correspond to increased activity.

Several control experiments were performed to verify the specificity of these results. First, we investigated whether the eosinophils caused nerve cell death. Previous studies and our own preliminary studies indicated that co-culture of eosinophils with IMR32 cells did not lead to significant apoptosis of either the nerves or the eosinophils when they were maintained in co-culture for as long as 96 h (12). Indeed, we have shown that eosinophils and their degranulation proteins protect IMR32 cells from apoptosis (52, 53). Second, to avoid potential contamination by gene products contained within eosinophils, mRNA extracted from eosinophils was subjected to RT-PCR. In all cases there was no detection of cholinergic genes or the M_2 muscarinic receptor. Therefore, the increase in M_2 receptor, ChAT, AChE, and VAChT mRNA expression observed were due to changes within IMR32 cells alone. Similar experiments were also performed on eosinophil total protein to verify the absence of the corresponding proteins. Western blotting analysis with specific antibodies also failed to detect any of the proteins of interest in eosinophil total protein. The choice of eosinophil concentration

used for these co-culture experiments was based on prior studies performed in the laboratory. Dose–response studies indicated that 2×10^5/ml of eosinophils was optimal for transcription factor activation in 5×10^5 IMR32 cells; therefore this number was used for gene expression studies. Also, to confirm that the changes in enzymes were of physiologic significance, we also measured the levels of ACh and enzymatic activity of the acetylcholinesterase activity. Finally, to assess the specificity of the changes induced by eosinophils, we measured changes in the antioxidant gene MnSOD. This was measured because adherence of eosinophils to IMR32 cells in culture induces free radical production in the IMR32 cells, as assessed by oxidation of dihydrorhodamine 123. However, no change in MnSOD mRNA levels was observed following eosinophil co-culture.

Since M_2 levels are increased, it would be expected that there would be a decrease in vagally mediated bronchoconstriction, as M_2 acts as an autoreceptor limiting the excessive release of ACh. However this does not seem to be the case in pathologic states such as asthma. Prior functional studies have shown that M_2 receptors are inhibited by eosinophil MBP (29–31). Thus, combining this data with the current observations, we suggest that the increased ACh released from eosinophil-stimulated nerves would be unable to bind M_2 receptors but still able to bind M_3 receptors, which are not inhibited by MBP, resulting in increased vagally mediated hyperreactivity (30).

The results of this study show that eosinophils have a profound effect on cholinergic gene expression in IMR32 cells, which tend to promote a cholinergic phenotype. These effects are exerted via eosinophil adhesion and factors released from eosinophil granules after adhesion, including the cationic proteins MBP and EPO and also NGF. Release of NGF and other neurotrophins may contribute significantly to nerve remodeling in asthma and other allergic diseases. Our results show that NGF released from eosinophils contributes significantly to the observed upregulation of M_2 receptor expression. Neurotrophins including NGF are survival factors for eosinophils (1); therefore, release of NGF would tend to perpetuate the effects of eosinophils on nerve cells, as well as exerting direct effects on the nerve cells. NGF has previously been implicated in the pathogenesis of allergic illness (reviewed Refs. 54 and 55). Its circulating levels are increased in allergy and it induces hyperresponsiveness of isolated human bronchus (56). Effects on bronchial hyperresponsiveness maybe due to NGF-induced increases in innervation. However, our results imply that in allergy, eosinophil-derived NGF may also directly affect nerve cholinergic phenotype, contributing in this way to ACh release, nerve hyperreactivity, and remodeling and thus to bronchial hyperresponsiveness.

To summarize, this work has demonstrated that increased expression of the M_2 muscarinic receptor in IMR32 cells results first from eosinophil adhesion to nerve cells and second from the products subsequently released by eosinophils; including the cationic proteins and NGF. The study characterized the 5′ UTR of the human muscarinic M_2 receptor gene and has defined sites of transcriptional regulation by eosinophils. In addition, the studies have shown an eosinophil-induced increase in the turnover and release of ACh and a reduction in acetylcholinesterase activity. Together these data suggest that eosinophils promote cholinergic nerve cell remodeling, a feature of many clinical manifestations (among them asthma).

Conflict of Interest Statement:: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Nassenstein C, Braun A, Erpenbeck VJ, Lommatzsch M, Schmidt S, Krug N, Luttman W, Renz H, Virchow JC. The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 are survival and activation factors for eosinophils in patients with allergic bronchial asthma. *J Exp Med* 2003;198:455–467.
2. Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000;105:651–663.
3. Rothenberg ME. Eosinophilia. *N Engl J Med* 1998;338:1592–1600.
4. Rothenberg ME, Mishra A, Brandt EB, Hogan SP. Gastrointestinal eosinophils in health and disease. *Adv Immunol* 2001;78:291–328.
5. Hogan SP, Foster PS, Rothenberg ME. Experimental analysis of eosinophil-associated gastrointestinal diseases. *Curr Opin Allergy Clin Immunol* 2002;2:239–248.
6. Sawatzky DA, Kingham PJ, Court E, Kumaravel B, Fryer AD, Jacoby DB, McLean WG, Costello RW. Eosinophil adhesion to cholinergic nerves via ICAM-1 and VCAM-1 and associated eosinophil degranulation. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L1279–L1288.
7. Sawatzky DA, Kingham PJ, Durcan N, McLean WG, Costello RW. Eosinophil-induced release of acetylcholine from differentiated cholinergic nerve cells. *Am J Physiol Lung Cell Mol Physiol* 2003;285: L1296–L1304.
8. Kingham PJ, McLean WG, Sawatzky DA, Walsh MT, Costello RW. Adhesion-dependent interactions between eosinophils and cholinergic nerves. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L1229–L1238.
9. Walsh MT, Curran DR, Kingham PJ, Morgan RK, Durcan N, Gleich GJ, McLean WG, Costello RW. Effect of eosinophil adhesion on intracellular signaling in cholinergic nerve cells. *Am J Respir Cell Mol Biol* 2004;30:333–341.
10. Gleich GJ, Adolphson CR. The eosinophilic leukocyte: structure and function. *Adv Immunol* 1986;39:177–253.
11. Curran DR, Morgan RK, Kingham PJ, Durcan N, McLean WG, Walsh MT, Costello RW. Mechanism of eosinophil induced signalling in cholinergic IMR32 cells. *Am J Physiol Lung Cell Mol Physiol* 2005; 288:L326–L332.
12. Kingham PJ, McLean WG, Walsh MT, Fryer AD, Gleich GJ, Costello RW. Effects of eosinophils on nerve cell morphology and development: the role of reactive oxygen species and p38 MAP kinase. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L915–L924.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
14. Potter LT. A radiometric microassay of acetylcholinesterase. *J Pharmacol Exp Ther* 1967;156:500–506.
15. Fenech AG, Billington CK, Swan C, Richards S, Hunter T, Ebejer MJ, Felice AE, Ellul-Micallef R, Hall IP. Novel polymorphisms influencing transcription of the human CHRM2 gene in airway smooth muscle. *Am J Respir Cell Mol Biol* 2004;30:678–686.
16. Solomon A, Aloe L, Pe'er J, Frucht-Pery J, Bonini S, Bonini S, Levi-Schaffer F. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. *J Allergy Clin Immunol* 1998;102:454–460.
17. Hausman RE, Ren Y, Ruiz JF, Shah BH. Insulin-mediated stimulation of ChAT and c-Jun in the developing retina neurons involves PI 3-kinase. *Biochem Soc Trans* 1998;26:S312.
18. Castell X, Cheviron N, Barnier JV, Diebler MF. Exploring the regulation of the expression of ChAT and VAChT genes in NG108–15 cells: implication of PKA and PI3K signaling pathways. *Neurochem Res* 2003;28:557–564.
19. Barnes PJ, Haddad EB, Rousell J. Regulation of muscarinic M2 receptors. *Life Sci* 1997;60:1015–1021.
20. Krejci A, Bruce AW, Dolezal V, Tucek S, Buckley NJ. Multiple promoters drive tissue-specific expression of the human M muscarinic acetylcholine receptor gene. *J Neurochem* 2004;91:88–98.
21. Klett CP, Bonner TI. Identification and characterization of the rat M1 muscarinic receptor promoter. *J Neurochem* 1999;72:900–909.
22. Wood IC, Roopra A, Harrington C, Buckley NJ. Structure of the m4 cholinergic muscarinic receptor gene and its promoter. *J Biol Chem* 1995;270:30933–30940.
23. Rosoff ML, Wei J, Nathanson NM. Isolation and characterization of the chicken m2 acetylcholine receptor promoter region: induction of gene transcription by leukemia inhibitory factor and ciliary neurotrophic factor. *Proc Natl Acad Sci USA* 1996;93:14889–14894.
24. Forsythe SM, Kogut PC, McConville JF, Fu Y, McCauley JA, Halayko AJ, Liu HW, Kao A, Fernandes DJ, Bellam S, *et al.* Structure and transcription of the human m3 muscarinic receptor gene. *Am J Respir Cell Mol Biol* 2002;26:298–305.
25. Zhou C, Fryer AD, Jacoby DB. Structure of the human M(2) muscarinic acetylcholine receptor gene and its promoter. *Gene* 2001;271:87–92.

26. Saffen D, Mieda M, Okamura M, Haga T. Control elements of muscarinic receptor gene expression. *Life Sci* 1999;64:479–486.
27. Song H, Ming G, Fon E, Bellocchio E, Edwards RH, Poo M. Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. *Neuron* 1997;18:815–826.
28. Pothos EN, Larsen KE, Krantz DE, Liu Y, Haycock JW, Setlik W, Gershon MD, Edwards RH, Sulzer D. Synaptic vesicle transporter expression regulates vesicle phenotype and quantal size. *J Neurosci* 2000;20:7297–7306.
29. Jacoby DB, Gleich GJ, Fryer AD. Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. *J Clin Invest* 1993;91:1314–1318.
30. Evans CM, Fryer AD, Jacoby DB, Gleich GJ, Costello RW. Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. *J Clin Invest* 1997;100:2254–2262.
31. Costello RW, Evans CM, Yost BL, Belmonte KE, Gleich GJ, Jacoby DB, Fryer AD. Antigen-induced hyperreactivity to histamine: role of the vagus nerves and eosinophils. *Am J Physiol* 1999;276:L709–L714.
32. Ayala LE, Ahmed T. Is there loss of protective muscarinic receptor mechanism in asthma? *Chest* 1989;96:1285–1291.
33. Bousquet J, Chanez P, Vignola AM, Lacoste JY, Michel FB. Eosinophil inflammation in asthma. *Am J Respir Crit Care Med* 1994;150:S33–S38.
34. Djukanovic R, Roche WR, Wilson JW, Beasley CR, Twentyman OP, Howarth RH, Holgate ST. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990;142:434–457.
35. Elbon CL, Jacoby DB, Fryer AD. Pretreatment with an antibody to interleukin-5 prevents loss of pulmonary M2 muscarinic receptor function in antigen-challenged guinea pigs. *Am J Respir Cell Mol Biol* 1995;12:320–328.
36. Frigas E, Loegering DA, Solley GO, Farrow GM, Gleich GJ. Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. *Mayo Clin Proc* 1981;56:345–353.
37. Fryer AD, el-Fakahany EE, Jacoby DB. Parainfluenza virus type 1 reduces the affinity of agonists for muscarinic receptors in guinea-pig lung and heart. *Eur J Pharmacol* 1990;181:51–58.
38. Fryer AD, Jacoby DB. Parainfluenza virus infection damages inhibitory M2 muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig. *Br J Pharmacol* 1991;102:267–271.
39. Fryer AD, Maclagan J. Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br J Pharmacol* 1984;83:973–978.
40. Fryer AD, Wills-Karp M. Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *J Appl Physiol* 1991;71:2255–2261.
41. Gambone LM, Elbon CL, Fryer AD. Ozone-induced loss of neuronal M2 muscarinic receptor function is prevented by cyclophosphamide. *J Appl Physiol* 1994;77:1492–1499.
42. Gies JP, Landry Y. Sialic acid is selectively involved in the interaction of agonists with M2 muscarinic acetylcholine receptors. *Biochem Biophys Res Commun* 1988;150:673–680.
43. Hu J, Wang SZ, Forray C, el-Fakahany EE. Complex allosteric modulation of cardiac muscarinic receptors by protamine: potential model for putative endogenous ligands. *Mol Pharmacol* 1992;42:311–321.
44. Minette PA, Barnes PJ. Prejunctional inhibitory muscarinic receptors on cholinergic nerves in human and guinea pig airways. *J Appl Physiol* 1988;64:2532–2537.
45. Okayama M, Shen T, Midorikawa J, Lin JT, Inoue H, Takishima T, Shirato K. Effect of pilocarpine on propranolol-induced bronchoconstriction in asthma. *Am J Respir Crit Care Med* 1994;149:76–80.
46. Scheid A, Caliguiri LA, Compans RW, Choppin PW. Isolation of paramyxovirus glycoproteins. Association of both hemagglutinating and neuraminidase activities with the larger SV5 glycoprotein. *Virology* 1972;50:640–652.
47. Schultheis AH, Bassett DJ, Fryer AD. Ozone-induced airway hyperresponsiveness and loss of neuronal M2 muscarinic receptor function. *J Appl Physiol* 1994;76:1088–1097.
48. Sorkness R, Clough JJ, Castleman WL, Lemanske RF Jr. Virus-induced airway obstruction and parasympathetic hyperresponsiveness in adult rats. *Am J Respir Crit Care Med* 1994;150:28–34.
49. Inestrosa NC, Perez CA, Simpfendorfer RW. Sensitivity of acetylcholinesterase molecular forms to inhibition by high MgCl2 concentration. *Biochim Biophys Acta* 1994;1208:286–293.
50. Velan B, Kronman C, Grosfield H, Leitner M, Gozes Y, Flashner Y, Sery T, Cohen S, Ben-Aziz R, Seidman S, *et al.* Recombinant human acetylcholinesterase is secreted from transiently transfected 293 cells as a soluble globular enzyme. *Cell Mol Neurobiol* 1991;11:143–156.
51. Talesa V, Romani R, Antognelli C, Giovannini E, Rosi G. Soluble and membrane-bound acetylcholinesterases in Mytilus galloprovincialis (Pelecypoda: Filibranchia) from the northern Adriatic Sea. *Chem Biol Interact* 2001;134:151–166.
52. Morgan RK, Kingham PJ, Walsh M-T, Curran DC, Durcan N, McLean WG, Costello RW. Eosinophil adhesion to cholinergic IMR-32 cells protects against induced neuronal apoptosis. *J Immunol* 2004;173:5963–5970.
53. Morgan RK, Costello RW, Durcan N, Kingham PJ, Gleich GJ, McLean WG, Walsh MT. Diverse effects of eosinophil cationic granule proteins on IMR-32 nerve cell signalling and survival. *Am J Respir Cell Mol Biol* 2005;33:169–177.
54. Bonini S, Lambiase A, Lapucci G, Properzi F, Bresciani M, Bracci Laudiero ML, Mancini MJ, Procoli A, Micera A, Sacerdoti G, *et al.* Nerve growth factor and asthma. *Allergy* 2002;57:13–15. [published erratum appears in *Allergy* 2003;58:366.
55. Frossard N, Freund V, Advenier C. Nerve growth factor and its receptors in asthma and inflammation. *Eur J Pharmacol* 2004;500:453–465.
56. Frossard N, Naline E, Olgart Hoglund C, Georges O, Advenier C. Nerve growth factor is released by IL-1beta and induces hyperresponsiveness of the human isolated bronchus. *Eur Respir J* 2005;26:15–20.