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SPECIAL REPORT

Leukaemia inhibitory factor (LIF) upregulates excitatory non-adrenergic non-cholinergic and maintains cholinergic neural function in tracheal explants

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The effect of leukaemia inhibitory factor (LIF) in modulating cholinergic and sensory nerve function was examined using guinea-pig tracheal explants. Specific LIF receptors (LIFR) were immunolocalized to both cholinergic and sensory nerves. Release of SP in culture was not influenced by LIF. Similarly, maximum contraction to carbachol (C_{max}) was not influenced by LIF. After 3 h, maximum (E_{max}) eNANC-induced contraction in controls was $32 \pm 2.5\%$ of C_{max}. In LIF-treated preparations, E_{max} was enhanced to $50 \pm 4.5\%$ C_{max} ($P < 0.05$). Cholinergic nerve-induced contractions after 3 h incubation with LIF were similar to control. After 24 h, control E_{max} was $25 \pm 4.5\%$ C_{max} (58% smaller than E_{max} at 3 h). In contrast, in LIF-treated preparations, E_{max} was $37 \pm 2.5\%$ C_{max}, (24% smaller than at 3 h, $P < 0.05$). This did not appear to be due to the effect of LIF on muscarinic M_2 receptor expression or function. Thus LIF appears to differentially influence the function of airway nerves and thus may provide an important link between the immune and neural systems.

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Abbreviations: ChAT, choline acetyltransferase; EFS, electrical field stimulation; EIA, enzyme immunoassay; eNANC, excitatory non adrenergic non-cholinergic; FITC, fluorescein isothiocyanate; IR-SP, immunoreactive substance P; LIF, leukaemia inhibitory factor; LIFR, leukaemia inhibitory factor receptor; L-NAME, L-nitro-arginine-methyl-ester; M_2R, muscarinic M_2 receptor, PBS, phosphate buffered saline; RT–PCR, reverse transcription-polymerase chain reaction; SP, substance P; TRITC, tetramethylrhodamine isothiocyanate

Introduction Leukaemia inhibitory factor (LIF) is a member of the IL-6 cytokine family, is a secreted glycoprotein, existing in both soluble and bound forms (Gearing, 1993) and exerts a wide array of biological activities (Kishimoto *et al*., 1995). LIF is released from a variety of cell types *in vitro*, including airway smooth muscle cells (Knight *et al*., 1999), eosinophils (Zheng *et al*., 1999), T-cells and monocyte-macrophages (Takashima & Klagsbrun, 1996).

There appears to be a complex bi-directional interaction between the inflammatory process and neural responses and it is likely that substances derived from the inflamed tissue are responsible for inducing the trophic changes seen in neurotransmitter phenotype. LIF has been shown to increase the expression of tachykinin receptor mRNA as well as synthesis and release of tachykinins while co-ordinately inhibiting the expression of muscarinic M_2 receptors (M_2R; Ludlam *et al*., 1994; 1995) in neural tissue. Furthermore, LIF has been shown to maintain the expression of the eyzyme choline acetyltransferase (ChAT) in axotomized nerves (Cheema *et al*., 1998). There are several mechanisms by which altered neural function contributes to airway inflammation. However, whether LIF influences nerve functions and induces neural plasticity of peripheral nerves within the airway is unknown.

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We hypothesized that LIF receptors (LIFR) would be present on cholinergic and sensory nerves and that *via* these receptors, LIF would upregulate the contractile effects of excitatory sensory nerves and cholinergic nerves.

Methods *Tracheal explants* Guinea-pigs weighing between 300–800 g were euthanased with an overdose of sodium pentrobarbitone (Nembutal; 100 mg kg^{-1}, i.p.) and tracheal tissue removed. Tracheal rings were removed and explants established as previously described (Knight *et al*., 1997).

Briefly, tracheae were placed in L-15 medium supplemented with 100 units ml^{-1} of penicillin and 100 μg ml^{-1} streptomycin and all connective tissue and visible blood vessels were removed. One ring from each trachea was placed in organ baths for immediate use. Randomly chosen rings were allocated into pairs for explant culture. Within each pair, one ring served as the time control, while the remaining ring was incubated in the presence of 0.5 or 5 ng ml^{-1} LIF for a pre determined period of time. Tracheal rings were placed individually in 35 mm plastic tissue culture dishes containing 1.5 ml of CRML-1066 medium supplemented with 2 μg insulin, 200 units penicillin, 200 μg streptomycin, 2 mM glutamine and 10% (v v^{-1}) FBS, in the presence or absence of LIF. Dilutions of LIF were made in a 0.1% (w v^{-1}) BSA/CRML-1066 solution. In some studies, explants were preincubated with a neutralizing antibody to the LIFR (α-LIFR), for 30 min prior to the addition of LIF. Tissue culture dishes were placed in a controlled atmosphere chamber which was flushed with a mixture of 45% O_2, 50% N_2 and 5% CO_2 at a flow rate of 4 l min^{-1} for 15 min. The chamber was then

placed in a 37°C incubator on a rocking platform set at 10 cycles min^{-1} so that the tracheal lumen was intermittently exposed to media and gas mixture. The chamber was flushed with fresh gas mixture every 16 h.

Functional studies Tracheal rings were suspended under 0.5 g tension in KBS, containing indomethacin (3 μM) and propranolol (1 μM) and aerated with 5% CO_2 in O_2 at 37°C. Changes in isometric tension were measured *via* an FT03 force-displacement transducer (Grass Instruments) linked to a preamplifier and a computer-based data acquisition system. Preparations were exposed to carbachol (10 μM) to establish tissue viability and obtain an estimate of the maximal contractile capacity of the tissue (C_{max}). Repeated washing over the ensuing 30 min re-established baseline tone. At this time, L-nitro-arginine-methyl-ester (L-NAME; 100 nM) was added to all organ baths. Electrical field stimulation (EFS) was delivered by a Grass S44 stimulator connected to a Stimu Splitter and stimulus isolation unit (SIU5, Grass Instruments) with an automated timing device. Stimuli were delivered *via* two parallel platinum electrodes.

Cholinergic nerve stimulation Stimulation parameters for cholinergic nerve-mediated contractions were 13 V, 0.5 ms pulse width, 10 s train, 1, 2, 4, 8, 16, 32 and 64 Hz at 3 min intervals.

Excitatory non-adrenergic non-cholinergic (eNANC) nerve activation In these experiments, atropine (1 μM) and the peptidase inhibitors α-chymotrypsin (2 units ml^{-1}) and phosphoramidon (10 μM) were present in the organ bath. Stimulation parameters were 13 V, 1 ms pulse width, 15 s train at 1, 2, 4, 8, and 16 Hz at 3 min intervals.

Substance P (SP) release Tracheal explants were placed in culture dishes as described above and exposed to LIF or media alone for 3 or 24 h. At each of these times, an aliquot of culture media was taken and stored at −70°C for subsequent measurement of SP by enzyme immunoassay (EIA).

Confocal microscopy The distribution of LIFR was compared to that for ChAT a marker for cholinergic nerves and SP, a marker for eNANC nerves. Whole mounts were fixed in 2% (w v^{-1}) paraformaldehyde for 30 min at room temperature, washed several times in phosphate buffered saline (PBS; pH 7.6), before being stored in PBS at 4°C until required. Tissues were incubated with primary antibody combinations of LIFR and ChAT or LIFR and SP for 24 h at room temperature in a humidified chamber. All antibodies were used at a dilution of 1 : 200. Preparations were washed extensively over a 16 h period before being incubated with the fluorochrome-labelled secondary antibody for a further 2 h. Following another extended wash (16 h), preparations were mounted in 90% (v v^{-1}) glycerol containing *p*-phenylethylenediamine to reduce fading. Each specimen was individually slide mounted and coverslips were raised to prevent specimen compression. Specimens treated as above, but without the primary antibody served as negative controls.

Fluorescent images of LIFR as well as ChAT and SP-containing nerves in double stained whole mounts were obtained using a confocal laser scanning microscope (BioRad MRC 2400) using COMOS software. Whole mounts were optically sectioned at 1 μm to follow the paths of the nerves. The fluorescein isothiocyanate (FITC; ChAT or SP)-associated fluorescence image of the nerves was then merged with the tetramethyrhodamine isothiocyanate (TRITC; LIFR)-associated fluoresence image to determine whether LIFR were present on cholinergic and/or excitatory sensory nerves. Image processing was performed using Confocal Assistant software.

Reverse transcription-polymerase chain reaction (RT–PCR) Total RNA was obtained from explants using RNAsol-B™. RNA concentration was determined spectrophotometrically while integrity was judged by inspection of 28S and 18S ribosomal bands after electrophoresis on a 1% agarose-5% formaldehyde denaturing gel. Samples of RNA (10 μg each), together with random hexamers were subjected to first strand cDNA synthesis as described (Fryer *et al*., 1996). A volume of this cDNA equating to 1 μg of starting RNA was then amplified by PCR. In all cases, PCR was optimized such that measurements of cDNA were taken during the exponential phase of amplification. Gene-specific, intron-spanning primers were used to amplify specific sequences of guinea-pig M_2R (Fryer *et al*., 1996). Following PCR amplification, aliquots were electrophoresed on a 2% (w v^{-1}) agarose gel and visualized by staining with ethidium bromide.

Cytokines and reagents Recombinant human LIF was obtained from Pharmingen (San Diego, CA, U.S.A.). The EIA kit for SP was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Goat anti-human LIFR antibody was obtained from R&D systems. Rabbit anti-human SP and Rabbit anti-human ChAT antibodies were purchased from Chemicon (Temecula, CA, U.S.A.).

Statistical analysis Differences in contractile responses to EFS were calculated and subjected to ANOVA and Dunnett's test performed to correct for multiple comparisons (GraphPad Prism, San Diego, CA, U.S.A.).

Results *Localization of LIFR to eNANC and cholinergic nerves* Specific staining for LIFR was found on cholinergic ganglia (Figure 1a–c), cholinergic nerves and excitatory non-cholinergic nerves (Figure 1d–f).

Effect of LIF and OSM on SP release in culture Levels of IR-SP released from tracheal explants exposed to LIF (0.5 and 5 ng ml^{-1}) were not statistically different to IR-SP release from control preparations (data not shown).

Effect of LIF on tracheal smooth muscle responses to carbachol Incubation for up to 24 h culture in medium alone did not significantly affect the mean maximum contractile response of tracheal rings to a bolus dose of carbachol (10 μM). The mean maximum contractile responses of preparations exposed to LIF at concentrations of 0.5 or 5 ng ml^{-1} were not significantly different to control values.

Effect of LIF on eNANC-induced contractions of guinea-pig tracheal explants EFS on tracheal preparations pretreated with atropine to prevent cholinergic influence produced frequency-dependent monophasic contractions with a slow decay phase. The effect of exposure to LIF on eNANC-induced smooth muscle contractions is shown in Figure 2a. The magnitude of these contractile responses reached $32 \pm 2.5\%$ of the response to 10 μM carbachol. Following 3 h exposure to LIF (0.5 ng ml^{-1}) the maximum contractile response was significantly enhanced to $50 \pm 4.5\%$ ($P < 0.05$). Increasing the concentration of LIF to 5 ng ml^{-1} did not

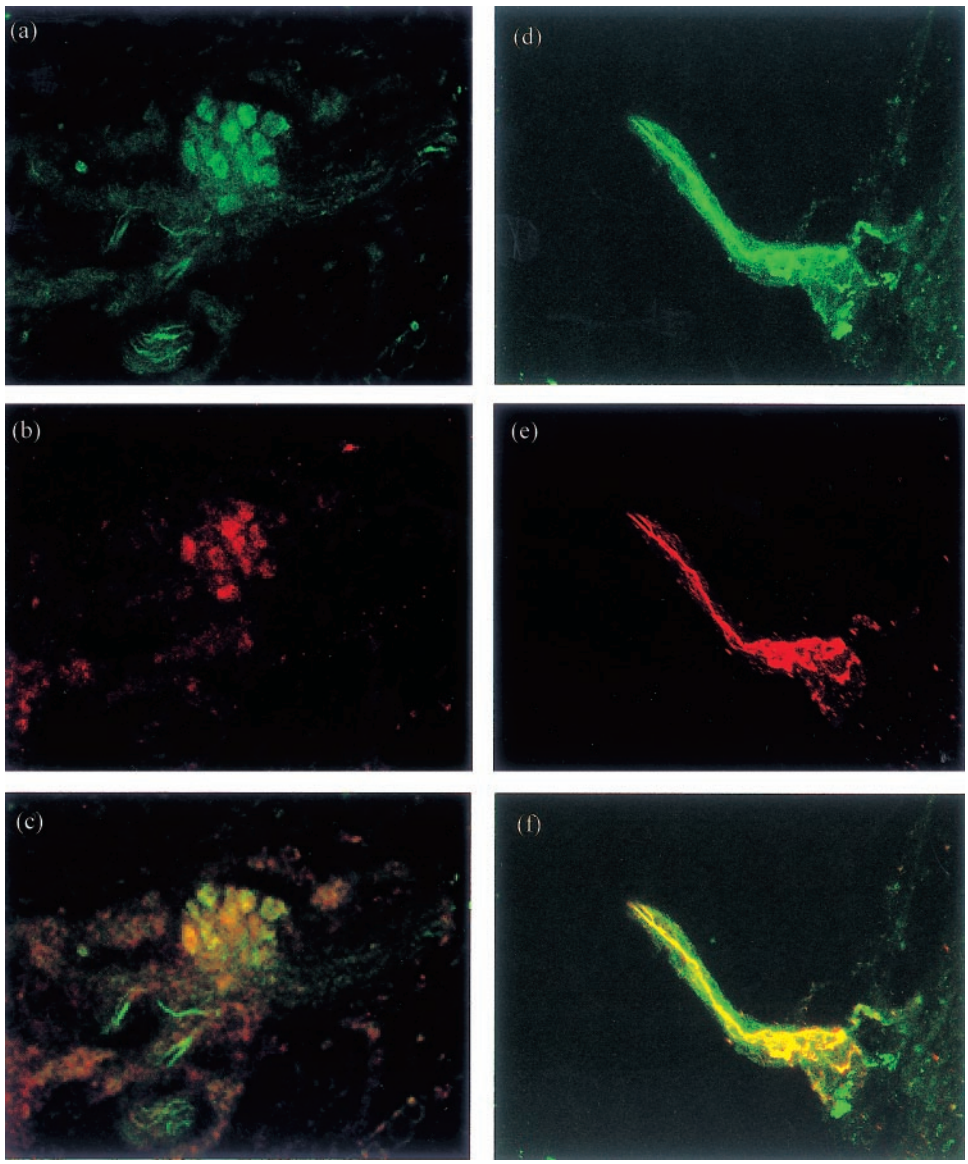

Figure 1 Localization of LIFR to neural tissue in whole mount preparations of guinea-pig trachea. Figures are presented as a montage of confocal images of preparations exposed to an antibody against ChAT (a) or SP (d) conjugated to FITC-(green), and LIFR (b,e) conjugated to TRITC-(red) and the merged image (c,f). Areas of orange/yellow colouring demonstrate co-localization of LIFR.

result in a significantly greater effect than that served at 0.5 ng ml^{-1}. The amplifying effects of LIF were completely inhibited by a LIF receptor neutralizing antibody (Figure 2a). After 24 h in culture, contractile responses to eNANC-stimulation were not observed in either control or cytokine-treated tissues.

Contractile responses to carbachol were not significantly different compared to the 3 h time point, suggesting that tissue responsiveness *per se* was not adversely effected by time in culture.

Effect of LIF on cholinergic nerve-induced contractions After 3 h incubation, cholinergic nerve stimulation produced a monophasic contractile response that reached a maximum of $59 \pm 4\%$ of the response to carbachol (Figure 2b). Incubation of explants with LIF for 3 h did not influence tracheal responses to cholinergic nerve stimulation. After 24 h incubation, contractile responses of control preparations were significantly lower than the corresponding responses after 3 h incubation, declining to $25 \pm 4.5\%$ of the response to carbachol. This represented a decline of 58% in responsiveness compared to control tissues at 3 h. In contrast, in preparations exposed to LIF (0.5 ng ml^{-1}) for 24 h, contractions in response to cholinergic nerve stimulation were maintained and represented $37 \pm 2.5\%$ of the response to carbachol. This represented a decline of only 24% from the corresponding value after 3 h incubation ($P < 0.05$). This effect was completely abolished by pre-incubation with an α-LIF-R antibody (Figure 2c).

Effect of LIF on M_2R function Addition of the M_2R agonist pilocarpine in concentrations ranging from 0.1 – 100 μM, resulted in highly variable contractions in the absence of other stimuli. In the majority of cases, the magnitude of these contractions was greater than 30% of the carbachol-induced contraction and thus masked other events. In other experiments ($n = 3$), the effects of LIF on M_2R function was investigated in the presence of the specific antagonist gallamine (30 μM). In these experiments, incubation with gallamine resulted in a weak enhancement

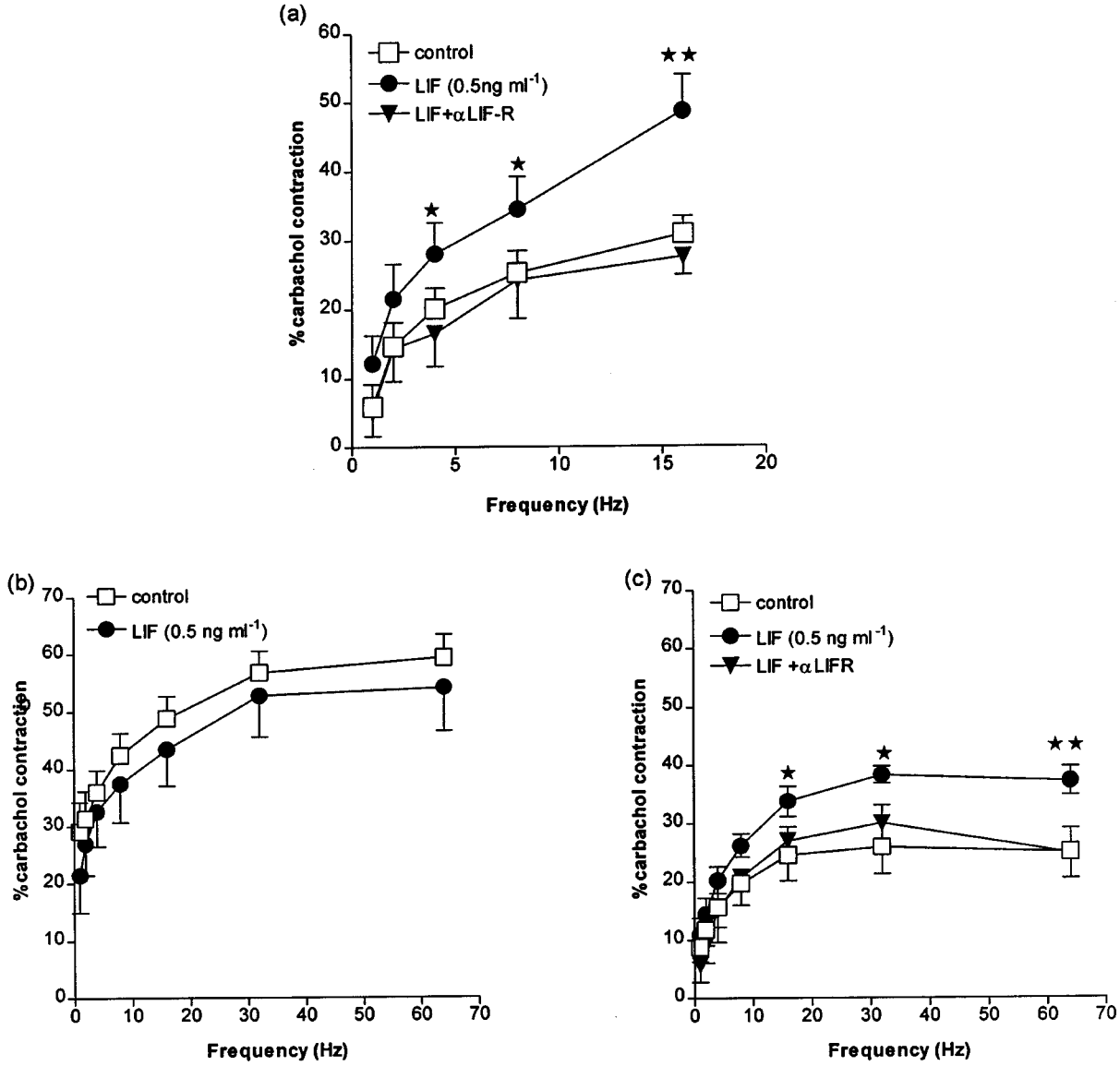

Figure 2 Effects of LIF on eNANC and cholinergic nerve-induced contractions of guinea-pig trachea. (a) Exposure to LIF (0.5 ng ml^{-1}) for 3 h significantly enhanced eNANC-induced contraction compared to control responses. The effect was completely inhibited by pre-treatment with α-LIFR. $^{**}P<0.01$, $^{*}P<0.05$ compared to control. (b) Incubation with LIF (0.5 ng ml^{-1}) for 3 h did not influence contractile responses to cholinergic nerve stimulation above control. (c) Incubation with LIF (0.5 ng ml^{-1}) for 24 h appeared to maintain cholinergic nerve mediated contraction. The effect of LIF was completely inhibited by pre-treatment with a specific LIFR antibody. $^{**}P<0.01$, $^{*}P<0.05$ compared to control responses ($n=6$ for all experiments).

of the EFS-induced contraction. This effect was not significantly influenced by 24 h exposure to LIF (data not shown).

Effect of LIF on M_2R gene expression RNA taken from tracheal explants incubated with LIF for 3 or 24 h was subjected to RT–PCR. However, the intensity of bands corresponding to mRNA from the LIF-treated preparations was not different to the control, suggesting that LIF does not down-regulate M_2R expression (data not shown).

Discussion We have previously shown that LIF augments contractile responses of guinea-pig trachea to capsaicin as well as exogenously applied tachykinins (Knight *et al*., 1997), suggesting that this cytokine may influence the activity of sensory nerves as well as the end-organ response to sensory neuropeptides. The present study confirms and extends this hypothesis by demonstrating for the first time that specific receptors for LIF are present on both sensory and cholinergic nerves and that LIF upregulates eNANC and appears to preserve cholinergic nerve activity over time. This effect appears to be pre-junctional since contractile responses to the cholinergic agonist carbachol were not effected.

Using confocal microscopy, we localized specific receptors for LIF on both SP-positive nerves as well as ChAT-positive nerves and ganglia in whole mounts of guinea-pig trachea. These findings extend our previous work in which the mRNA for LIFR was shown to be widespread in lung tissue, being expressed in both resident structural cells as well as infiltrating inflammatory cells (Knight *et al*., 1999).

Incubation of tracheal explants with LIF for as little as 3 h resulted in a marked augmentation in the contractile response to excitatory non-cholinergic nerve stimulation. This finding is in agreement with a previous study that demonstrated that exposure to LIF for 3–6 h augments the contractile responses to capsaicin (Knight *et al*., 1997). Together these data suggest

that LIF may act to enhance the release of sensory neuropeptides. This effect does not appear to be related to incubating tracheal explants with LIF *per se*, since there were no observable increases in IR-SP in explant media in preparations exposed to LIF compared to time controls. However, after 24 h in culture, contractile responses to sensory nerve stimulation were abolished, both in control and LIF-treated preparations, suggesting neuronal degeneration. Indeed, studies using retrograde tracing and immunohistochemistry demonstrate that synthesis of tachykinins occurs in cell bodies within the jugular ganglia or dorsal root ganglia (Kummer *et al*., 1992) and thus would not present in these whole mount preparations.

In contrast with the acute effects seen on sensory nerve function, contractile responses to cholinergic nerve stimulation were not significantly altered by exposure to LIF. However, after 24 h in culture, LIF appeared to preserve cholinergic nerve function and/or activity. Although cholinergic nerve-mediated responses were observed in control tissues the amplitude was significantly reduced compared to responses at 3 h. However, in preparations incubated with LIF for 24 h, the contractile responses to cholinergic nerve stimulation were maintained at 60% of values at 3 h, suggesting that LIF maintain or increase cholinergic nerve activity. The mechanisms involved in this process are unknown, although effects on M_2R expression (Ludlam *et al*., 1994) and ChAT levels (Cheema *et al*., 1998) have been suggested as targets for the activity of LIF. The reports of LIF down regulating M_2R mRNA are intriguing and provide a plausible mechanism by which LIF may maintain cholinergic nerve activity. Incubation of rat sympathetic cervical ganglia with LIF down-regulates the expression of muscarinic M_2R mRNA, while coordinately increasing the expression of substance P and the NK-1 receptor (Ludlam *et al*., 1994). In peripheral cholinergic nerves, stimulation of M_2R mediates a feedback inhibitory response to inhibit the release of more neurotransmitter. Thus, down-regulation of these receptors would then lead to an uninhibited release of ACh. In asthmatic airways, eosinophils are often found in close proximity to neural tissue and it is thought that eosinophil-derived granular proteins accounted for the down-regulation of M_2R (Evans *et al*., 1997; Fryer *et al*., 1997). More recently, eosinophils have been demonstrated to synthesize and release LIF (Zheng *et al*., 1999), suggesting that LIF released from adjacent eosinophils or from neural tissue itself may also contribute to this effect. However, in the current study, LIF did not appear to influence either the function or gene expression of M_2R in guinea-pig tracheal explants after 3 or 24 h exposure.

In cholinergic nerves of the medial septal nucleus, LIF maintains levels of the enzyme ChAT (Cheema *et al*., 1998) following axotomy, suggesting that in these types of nerves at least, LIF influences several intracellular mechanisms to maintain cholinergic function. Although we did not examine the effects of LIF on the activity of ChAT in airway nerves in this investigation, it is an intriguing hypothesis and currently being investigated.

In conclusion, this study has demonstrated, for the first time, that LIFR are present on excitatory non-cholinergic nerves as well as cholinergic nerves and ganglia in the airways. In agreement with our previous studies, incubation of trachea explants with LIF did not influence carbachol-induced contraction. In contrast, short-term incubation (3 h) of tracheal explants with LIF significantly increased the contractile response to sensory nerve stimulation, without affecting cholinergic nerve-mediated contraction. However, long term (24 h) incubation with LIF appeared to preserve cholinergic nerve activity. This effect did not appear to involve down-regulation of M_2R. These results suggest that LIF has important neuromodulatory roles in the airways and thus may be an important effector molecule in the airways response to inflammation.

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