0026-895X/03/6303-714–721$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 63:714–721, 2003

Vol. 63, No. 3
2182/1047486
Printed in U.S.A.

Tumor Necrosis Factor-α–Induced Activation of RhoA in Airway Smooth Muscle Cells: Role in the Ca^{2+} Sensitization of Myosin Light $Chain_{20}$ Phosphorylation

IRENE HUNTER, HANNAH J. COBBAN, PETER VANDENABEELE, DAVID J. MACEWAN, and GRAEME F. NIXON

Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom (I.H., H.J.C., D.J.M., G.F.N.); and Department of Molecular Biology, Flanders Interuniversity, Institute for Biotechnology, University of Gent, Ghent, Belgium (P.V.)

Received September 30, 2002; accepted December 6, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Tumor necrosis factor-α (TNF), an inflammatory cytokine, has a potentially important role in the pathogenesis of bronchial asthma and may contribute to airway hyper-responsiveness. Recent evidence has revealed that TNF can increase the Ca^{2+} sensitivity of agonist-stimulated myosin light $chain_{20}$ (MLC_{20}) phosphorylation and contractility in guinea pig airway smooth muscle (ASM). In the present study, the potential intracellular pathways responsible for this TNF-induced Ca^{2+} sensitization were investigated. In permeabilized cultured guinea pig ASM cells, recombinant human TNF stimulated an increase in Ca^{2+}-activated MLC_{20} phosphorylation under Ca^{2+} "clamp" conditions. This increased MLC_{20} phosphorylation was inhibited by preincubation with the Rho-kinase inhibitor Y27632. TNF also increased the proportion of GTP-bound RhoA, as measured using rhotekin Rho-binding domain, in a time course compatible with a role in the TNF-induced Ca^{2+} sensitization. In cultured human ASM cells, recombinant human TNF also activated RhoA with a similar time course. In addition, TNF stimulated phosphorylation of the regulatory subunit of the myosin phosphatase, which was inhibited by Y27632. Although human ASM cells expressed both receptor subtypes, TNF-R1 and TNF-R2, the activation of RhoA was predominantly via stimulation of the TNF-R1, although RhoA did not immunoprecipitate with the TNF-R1. In conclusion, the TNF-induced increase in the Ca^{2+} sensitivity of MLC_{20} phosphorylation is through stimulation of the TNF-R1 receptor and via a RhoA/Rho-kinase pathway leading to inhibition of the myosin light chain phosphatase. This intracellular mechanism may contribute to TNF-induced airway hyper-responsiveness.

The pathophysiology of bronchial asthma is regulated by the release of cytokines from inflammatory cells. There is increasing evidence that one of these cytokines, tumor necrosis factor-α (TNF), is directly linked to airway inflammation and the hyper-responsiveness observed in asthma (Broide et al., 1992; Amrani et al., 2000a). In vivo pretreatment of airways in several species, including human, to aerosolized TNF produced significant increases in bronchial airway resistance when challenged with endogenous agonists (Kips et al., 1992; Thomas et al., 1995). These observations, together with pharmacological evidence (Renzetti et al., 1996), suggest that TNF may be responsible for the bronchial smooth muscle hyper-responsiveness observed in asthma. However, the cellular mechanisms by which this TNF-induced hyperresponsiveness occurs is not clear. Longer incubations (18 h) with TNF in airway smooth muscle cells can produce increases in agonist-stimulated intracellular Ca^{2+} release (Amrani et al., 1996), possibly through increased expression of signaling proteins (Amrani et al., 1997; Hotta et al., 1999). However, in vivo effects are observed with TNF incubations of 1 h (Kips et al., 1992; Thomas et al., 1995). These effects could be caused by release of further inflammatory agents (Moore et al., 1991) and/or may be direct effects on bronchial smooth muscle cells.

It is generally accepted that the initiation of smooth muscle contractility is predominantly controlled by a Ca^{2+}-dependent increase in myosin light $chain_{20}$ (MLC_{20}) phosphorylation (Sellers, 1991). However, other pathways have now been described that may regulate the contractility of smooth muscle by regulating the phosphorylation level of MLC_{20} independently of a rise in intracellular Ca^{2+} (Himpens et al., 1990). These pathways are generally stimulated by contractile agonists that activate heterotrimeric G protein-coupled receptors, probably via $G_{12/13}$ stimulation of Rho guanine

This study was funded by The Wellcome Trust.

ABBREVIATIONS: TNF, tumor necrosis factor-α; MLC_{20}, myosin light $chain_{20}$; GEF, guanine exchange factor; TNF-R, tumor necrosis factor-α receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; RBD, Rho-binding domain; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; TRAF, tumor necrosis factor-α receptor-associating factor; Y27632, (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(L-aminoethyl)-cyclohexane carboxamide.

exchange factors (GEFs) (Hart et al., 1998; Kozasa et al., 1998). Activation of the monomeric G protein RhoA by seven-transmembrane G protein-coupled receptors may be of particular importance in many smooth muscle types (Somlyo and Somlyo, 2000). Activation of RhoA leads to subsequent activation of a recently isolated downstream target of Rho, p160 Rho-associated coiled-coil-containing protein kinase (Rho-kinase) (Leung et al., 1995; Matsui et al., 1996). Rho-kinase directly phosphorylates the regulatory subunit (MYPT-1) of the smooth muscle myosin light chain phosphatase (Feng et al., 1999), either directly or via an additional myosin phosphatase-associated kinase (Borman et al., 2002). This phosphorylation results in an inhibition of phosphatase activity leading to increased accumulation of phosphorylated MLC_{20} (Kimura et al., 1996) and subsequently an increased Ca^{2+} sensitivity to contraction. We have recently uncovered the first evidence of the cellular mechanism whereby TNF pretreatment of bronchial smooth muscle directly increases maintained agonist-induced responses (Parris et al., 1999). It is now clear that TNF, although not a Ca^{2+}-releasing agonist, potentiates the Ca^{2+}-activated contractile response via an increase in MLC_{20} phosphorylation. This TNF-induced increase in the Ca^{2+} sensitivity of MLC_{20} phosphorylation is the result of TNF receptor activation and intracellular signal transduction pathways that ultimately regulate smooth muscle contractility. TNF is known to activate a variety of signaling cascades in airway smooth muscle (Emala et al., 1997; Amrani et al., 2000b, 2001; McFarlane et al., 2001). Because there is no experimental evidence to suggest that TNF receptor subtypes (TNF-R1 and TNF-R2) can couple to heterotrimeric G proteins, the activation of these Ca^{2+}-sensitizing pathways is likely to occur via as yet undescribed signaling cascades, possibly involving either a potentiation of myosin light chain kinase activity or inhibition of the myosin light chain phosphatase activity.

In this study, we show that the TNF-induced Ca^{2+} sensitization of MLC_{20} phosphorylation in ASM cells from both guinea pig and human is regulated by activation of RhoA in a time course compatible with the TNF-induced airway hyper-responsiveness. This is predominantly via the TNF-R1 receptor. This Ca^{2+} sensitization is induced by an inhibition of the smooth muscle myosin light chain phosphatase activity, probably via Rho-kinase activation.

Materials and Methods

Reagents. Recombinant human TNF-α was purchased from R & D Systems (Abingdon, UK). Mutated forms ("muteins") of human TNF-α, which allow selective activation of either TNF-R1 (R32W, S86T/R1-TNF) or TNF-R2 (D143N, A145R/R2-TNF), have been described previously (Van Ostade et al., 1994). Monoclonal antibodies against RhoA (26C4) and TNF-R1 (H5) and a polyclonal antibody against TNF-R2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies against MYPT-1 and phospho-MYPT-1 (Thr696) were from Upstate Biotechnology (Lake Placid, NY). The Rho-kinase inhibitor Y27632 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Cell Isolation and Culture. Male Duncan Hartley guinea pigs were killed by cervical dislocation, and the trachea was removed and placed in Hanks' balance salt solution. After removal of fat and connective tissue, the smooth muscle was cut into small pieces and incubated in serum-free DMEM containing 1 mg/ml collagenase (type II), 0.2 mg/ml elastase (type IV), and 50 μg/ml soybean trypsin inhibitor at 37°C. The tissue was triturated every 30 min until complete dispersal had occurred (3–4 h); transferred into an 80-cm^2 tissue culture flask containing DMEM, 20% FBS, 2 mM L-glutamine, penicillin (10,000 units/ml), and streptomycin (10 mg/ml); and incubated at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, the medium was removed and cells were transferred to fresh DMEM, 10% FBS, 2 mM L-glutamine, penicillin (10,000 units/ml), and streptomycin (10 mg/ml). Cells were used for experimentation between passages 4 and 8.

Human bronchial smooth muscle cells, purchased from BioWhittaker UK Ltd. (Wokingham, UK), were grown in modified molecular cellular developmental biology 131 medium (Clonetics Corporation, San Diego, CA) containing 5% FBS, 0.5 μg/l epidermal growth factor, 5 mg/l insulin, 2 μg/l fibroblast growth factor, 50 mg/l gentamicin, and 50 mg/l amphotericin in a humidified 5% CO_2 atmosphere at 37°C. Routinely, cells were used between passages 4 and 8.

Cell Permeabilization and MLC_{20} Phosphorylation. Smooth muscle cells grown to 80% confluence in six-well tissue culture plates were transferred to 1 mM EGTA relaxing solution (G1) with pCa $<$ 8.0 (1 mM EGTA, 30 mM PIPES, 10 mM creatine phosphate, 7.3 mM Na_2ATP, and 85.8 mM potassium methane sulfonate) and permeabilized with 50 μg/ml *Staphylococcus aureus* α-toxin (Sigma-Aldrich, St. Louis, MO), for 2 h at RT. A23187 (5 μM) was added to release calcium from internal stores. After removal of α-toxin, cells were incubated in fresh G1 without or with TNF (200 ng/ml) for 1 h at RT, and then transferred to pCa 6.8 for 2 min. Details of the solutions have been described previously (Horiuti et al., 1986). The Ca^{2+} concentration of pCa buffers was regulated by the ratio of K_2EGTA and CaEGTA. The incubation in pCa 6.8 was chosen to activate myosin light chain kinase. Two minutes is the peak time course of MLC_{20} phosphorylation (data not shown). In some experiments, cells were preincubated with the rho-kinase inhibitor Y27632 (10 μM) for 30 min before the addition of TNF. Treatments were terminated by the addition of $HClO_4$ to a final concentration of 0.3 M, and cells were scraped from the dishes and centrifuged at 10,000*g* for 2 min. Cell pellets were washed with acetone to remove acid, air-dried, and extracted in 8 M urea, 20 mM Tris, 22 mM glycine, and 10 mM dithiothreitol for 1 h at RT. Cellular debris was removed by centrifugation at 10,000*g* for 2 min at room temperature and supernatants were analyzed by two-dimensional electrophoresis as described previously (Parris et al., 1999).

RhoA Activation Assay. RhoA activity was measured using a pull-down assay, based on the binding of active, GTP-bound RhoA to the Rho-binding domain of rhotekin (Ren et al., 1999). Smooth muscle cells, grown to 80% confluence in 10-cm^2 dishes, were transferred to serum-free medium for 48 h before treatment with TNF, TNF-R1 mutein, or TNF-R2 mutein (200 ng/ml) for the indicated times. Cells were washed twice with ice-cold Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and extracted with 50 mM Tris, pH 7.2, 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Cell extracts were incubated with 20 μg of rhotekin Rho-binding domain (RBD) coupled to glutathione-agarose (Upstate Biotechnology) for 45 min at 4°C. The beads were washed three times with 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Samples were analyzed by immunoblotting using a RhoA-specific monoclonal antibody. RhoA activity is determined as the amount of rhotekin-bound RhoA (GTP-RhoA) compared with the total amount of RhoA in cell lysates. Increases in RhoA activation for each experiment are expressed as fold increases from control (zero time point), normalized to "1".

Analysis of MYPT-1 Phosphorylation. Human airway smooth muscle cells, grown to 80% confluence in 10-cm^2 dishes, were deprived of serum for 48 h, before treatment with TNF (200 ng/ml) for the indicated times. Cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% Na-deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin; and cellular debris was removed by centrifugation at 15,000*g* for 15 min at 4°C, before analysis by immunoblotting.

Immunoprecipitation. Human airway smooth muscle cells, treated without or with TNF, as described above, were lysed with 50

mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin and clarified by centrifugation at 15,000*g* for 15 min at 4°C. Supernatants were incubated with either control IgG, anti-RhoA, or anti-TNF-R1 antibody. Immune complexes were collected by incubation with protein G-Sepharose beads, washed three times with lysis buffer, boiled in SDS sample buffer, and analyzed by immunoblotting. Control experiments demonstrated that, under these conditions, the anti-TNF-R1 antibody can coprecipitate proteins normally associated with TNF-R1.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting. Cell lysates were mixed with an equal volume of 2× SDS sample buffer and incubated at 100°C for 5 min. Lysates were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk powder in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 for 1 h at RT, blots were incubated with primary antibody for 1 h at RT or overnight at 4°C, washed, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at RT. Immunoreactive species were visualized using enhanced chemiluminescence and quantitated by scanning densitometry using a GS-690 imaging densitometer (Bio-Rad, Hercules, CA).

Results

TNF-Induced Increase in Ca^{2+}-Activated MLC_{20} Phosphorylation in Cultured Guinea Pig ASM Cells. We have previously demonstrated that TNF treatment of permeabilized guinea pig bronchial smooth muscle strips results in Ca^{2+} sensitization of the myofilaments and a corresponding increase in MLC_{20} phosphorylation (Parris et al., 1999). In attempting to dissect the underlying mechanism of this novel signaling pathway, we have examined the ability of TNF to stimulate MLC_{20} in permeabilized cultured guinea pig ASM. In initial studies, the effect of GTPγS, a nonhydrolysable analog of GTP, known to produce an increase in the Ca^{2+} sensitivity of MLC_{20} phosphorylation in permeabilized smooth muscle (Fu et al., 1998), was determined. After a 2-min incubation in pCa 6.8 with 100 μM GTPγS, the MLC_{20} phosphorylation was significantly increased compared with pCa 6.8 alone (Fig. 1). Because GTPγS is membrane-impermeant, these results demonstrate that the cells are effectively permeabilized and that the intracellular signaling pathways are intact.

To determine the ability of TNF to increase the Ca^{2+} sensitization of MLC_{20} phosphorylation, permeabilized cultured guinea pig ASM cells were incubated with 200 ng/ml recombinant human TNF for 45 min and exposed to pCa 6.8 for 2 min. TNF incubation produced a significant increase in Ca^{2+}-activated MLC_{20} phosphorylation compared with controls incubated in the absence of TNF (Fig. 1). Controls incubated with TNF but not stimulated with pCa 6.8 also showed no increase MLC_{20} phosphorylation (data not shown). This increase in MLC_{20} phosphorylation stimulated by TNF, is similar to that observed in TNF-treated guinea pig bronchial smooth muscle (Parris et al., 1999), indicating that cultured ASM cells represent a suitable model system in which to investigate the molecular mechanism of TNF-induced Ca^{2+}sensitization.

Involvement of the RhoA/Rho-Kinase Pathway in the TNF-Induced Ca^{2+} Sensitization of Cultured Guinea Pig ASM Cells. In smooth muscle, Ca^{2+} sensitization by a number of agonists has been shown to be dependent on the small G protein RhoA (Gong et al., 1997) and its downstream effector Rho-kinase. To examine the role of the RhoA/Rho-kinase pathway in TNF-induced MLC_{20} phosphorylation, permeabilized guinea pig ASM cells were treated with the Rho-kinase inhibitor Y27632, before incubation with 200 ng/ml TNF and subsequent stimulation with pCa 6.8 buffer as described above. The TNF-induced increase in the Ca^{2+}-activated MLC_{20} phosphorylation was significantly inhibited by preincubation with Y27632 (Fig. 2A). Y27632

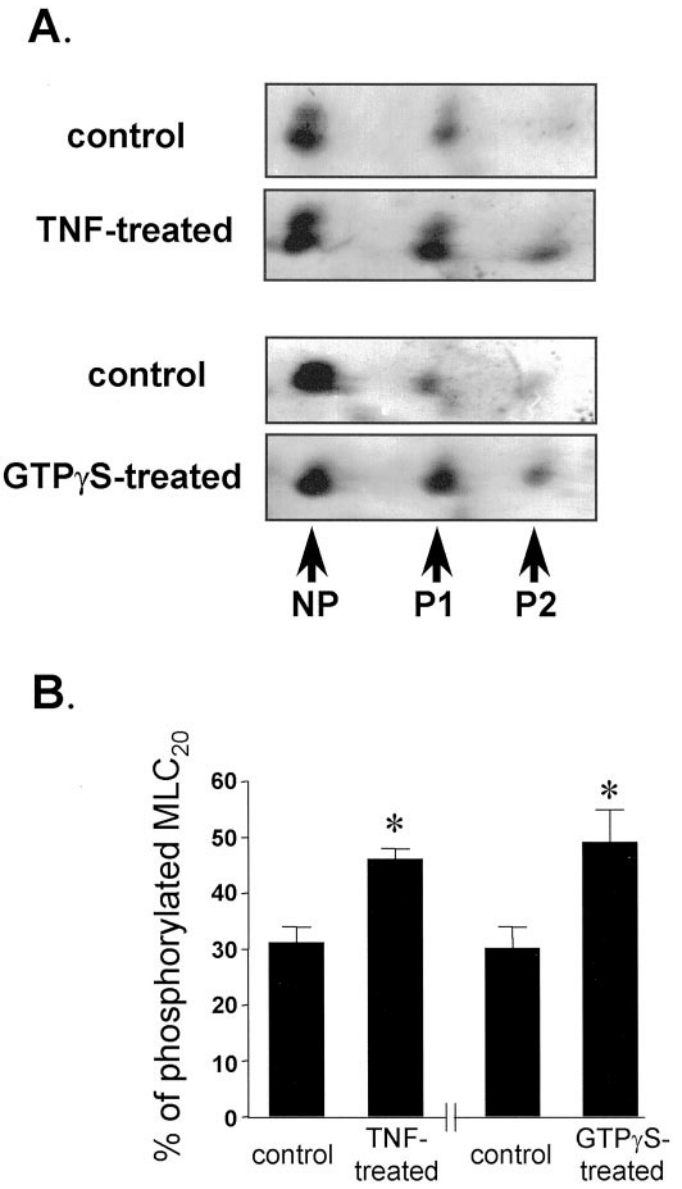

Fig. 1. Effect of TNF and GTPγS on Ca^{2+}-activated MLC_{20} phosphorylation in permeabilized guinea pig ASM cells. A, typical colloidal gold-stained membranes showing nonphosphorylated (NP), monophosphorylated (P1), and diphosphorylated (P2) MLC_{20} after a 2-min stimulation with pCa 6.8. Permeabilized cells were preincubated with 200 ng/ml TNF in G1 for 45 min and phosphorylation compared with controls (incubated in G1 alone for 45 min). Permeabilized cells were also incubated with 100 μM GTPγS for 2 min simultaneously with pCa 6.8. Controls were only incubated in pCa 6.8. As assessed by densitometric analyses, TNF preincubation increased the Ca^{2+}-activated MLC_{20} phosphorylation compared with control. GTPγS also increased the Ca^{2+}-activated MLC_{20} phosphorylation. B, graph showing mean MLC_{20} phosphorylation expressed as a percentage of the total MLC_{20}. Data are mean ± S.E.M. n = 8 for TNF-treated samples, and n = 5 for GTPγS-treated cells.

alone had no significant effect on Ca^{2+}-activated MLC_{20} phosphorylation in the absence of TNF.

To determine whether TNF can directly activate RhoA in cultured guinea pig ASM cells, a GST-rhotekin RBD pull-down assay was used (Ren et al., 1999). A detailed time course revealed that TNF caused a rapid and potent activation of RhoA in cultured guinea pig ASM cells (Fig. 2B). The level of GTP-RhoA was increased within 1 min, reaching peak activation of 3.5-fold within 15 min. The level of activated RhoA decreased sharply at 30 min, returning close to basal levels by 60 min. Taken together, these results support the notion that TNF-stimulated MLC_{20} phosphorylation is dependent on the RhoA/Rho-kinase pathway.

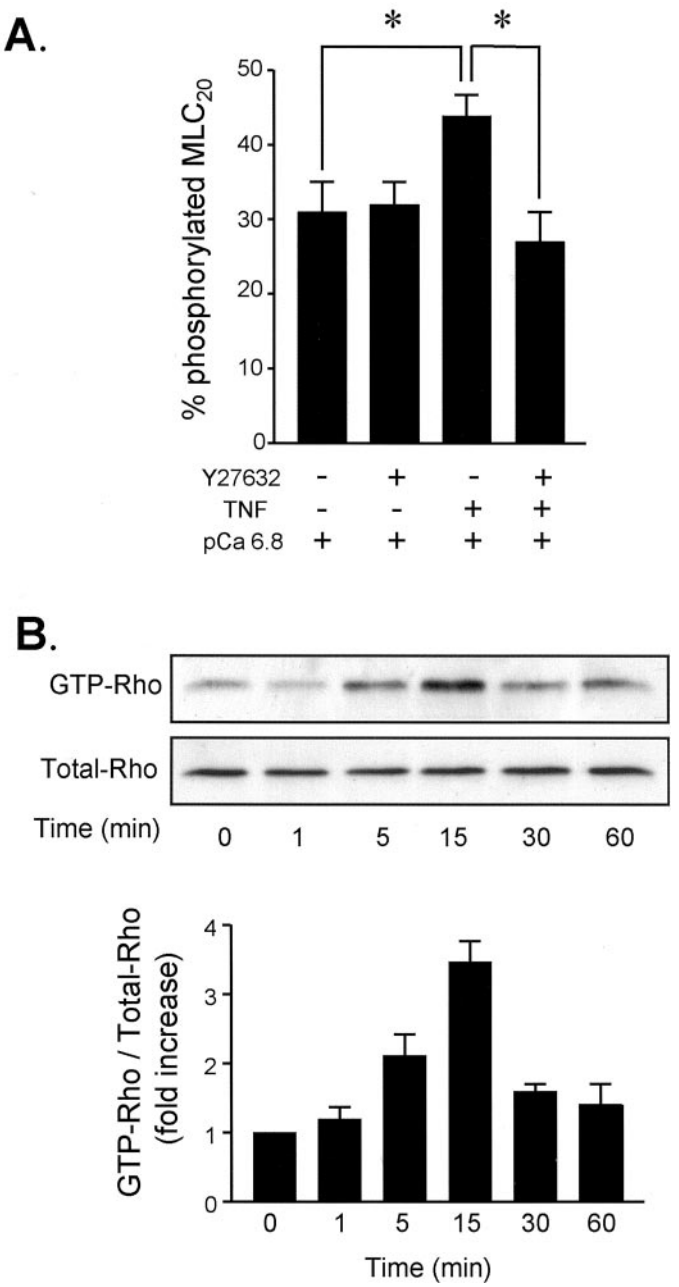

Fig. 2. Effect of Rho-kinase inhibition on Ca^{2+}-activated MLC_{20} phosphorylation and TNF-induced RhoA activation in guinea pig ASM cells. A, graph showing the inhibition of the TNF-induced increase in Ca^{2+}-activated MLC_{20} phosphorylation by Y27632. Permeabilized ASM cells were incubated with 10 μM Y27632 for 30 min followed by the addition of 200 ng/ml TNF for a further 45 min. Cells were then challenged with pCa 6.8 for 2 min. Mean MLC_{20} phosphorylation data are expressed as a percentage of the total MLC_{20}. $n = 5$ for each point. Asterisk denotes statistical significance. B, TNF-induced increase in GTP-bound RhoA from guinea pig ASM cells. Typical blots and graph of mean data showing GTP-bound RhoA ($n = 5$). The respective blot of total RhoA in each sample is also shown. Cells were stimulated at different time points with 200 ng/ml TNF and the rhotekin RBD binding measured. TNF caused an increase in GTP-bound (active) RhoA within 5 min, reaching a peak after 15 min of stimulation. This declined to baseline levels by approximately 30 min.

Involvement of the RhoA/Rho-Kinase Pathway in TNF-Induced Ca^{2+} Sensitization of Human Cultured ASM Cells. Because human TNF may have an altered affinity and/or efficacy when bound to guinea pig TNF receptors, the effects of human TNF on primary cultured human ASM cells were also investigated. In human cells, as in guinea pig ASM cells, TNF induced an increase in GTP-bound RhoA as assessed by the GST-rhotekin RBD pull-down assay (Fig. 3A). RhoA was activated more rapidly in human than guinea pig ASM cells, with a 2-fold increase in the level of GTP-RhoA detected within 1 min. Maximal RhoA activation (4-fold) was reached within 5 min, and thereafter activation declined, returning to basal levels by 60 min.

To determine potential involvement of TNF-induced RhoA activation of Ca^{2+} sensitization in human ASM cells, two-dimensional gel electrophoresis was carried out to determine the extent of MLC_{20} phosphorylation. In human ASM cells, a separation of MLC_{20} isoforms by two-dimensional gel electrophoresis revealed a complex pattern of spots at the pI and molecular weight of MLC_{20} (data not shown). These multiple spots were also recognized in immunoblots with a specific anti-MLC antibody. This suggests multiple isoforms and possibly additional phosphorylation states. TNF-induced Ca^{2+} sensitization of MLC_{20} phosphorylation in human ASM cells could therefore not be assessed further using this method.

As an alternative, we examined the effect of TNF on the phosphorylation of MYPT-1, the regulatory subunit of myosin phosphatase. Phosphorylation of MYPT-1 inhibits phosphatase activity and leads to an increase in MLC_{20} phosphorylation (Feng et al., 1999). Treatment with TNF resulted in a 2-fold increase in MYPT-1 phosphorylation within 1 min and reached a maximal 4.7-fold activation within 5 min (Fig. 3B). The level of phosphorylated MYPT-1 decreased slightly by 15 min but remained elevated even after 60 min. TNF-induced MYPT-1 phosphorylation was completely abrogated by pretreatment of cells with the Rho-kinase inhibitor Y27632 (Fig. 3C). These data demonstrate that in human ASM cells, TNF-induced activation of the RhoA/Rho-kinase pathway results in phosphorylation of MYPT-1.

TNF Receptor Subtypes Involved in the TNF-Induced Activation of RhoA. The effects of TNF are mediated by two receptors, TNF-R1 and TNF-R2 (MacEwan, 2002). To examine the expression of these receptors in cultured ASM cells, cell extracts were analyzed by immunoblotting with antibodies specific for the receptor subtypes. Hela cells, which have a previously defined expression of TNF-R1 and TNF-R2 (Grell et al., 1998), were used as a comparison. Both TNF-R1 and TNF-R2 were detected in human ASM cells and had apparent molecular weights identical to those in Hela cells (Fig. 4A). Human ASM cells contained approximately 3-fold less TNF-R1 and 1.5-fold more TNF-R2, compared with Hela cells. As previously published, Hela cells

have a TNF receptor subtype ratio of approximately 95% TNF-R1:5% TNF-R2 (Grell et al., 1998).

To determine the role of TNF receptor subtypes in the activation of the RhoA/Rho-kinase pathway, we have used muteins of TNF, which selectively bind to and activate either TNF-R1 or TNF-R2. Because these muteins are derived from the human TNF sequence and may therefore not be selective for guinea pig receptors, their effect was assessed on human ASM cells only. The TNF-R1 mutein stimulated an increase in GTP-RhoA in human ASM cells (Fig. 4B) with a time course identical to that observed for recombinant human TNF (Fig. 3A), although the magnitude of the increase was greater with the TNF-R1 mutein (6-fold increase compared with 4-fold increase for recombinant human TNF). The TNF-R2 mutein also stimulated an increase in GTP bound RhoA (Fig. 4C); however, this level was significantly lower than that stimulated by either the TNF-R1 mutein or recombinant TNF (peak increase 2-fold).

To determine whether TNF-R1 can interact with RhoA, extracts of human ASM cells, treated without or with TNF, were immunoprecipitated with anti-TNF-R1 receptor antibody. Subsequent immunoblotting failed to reveal coprecipitation of RhoA, although RhoA was effectively immunoprecipitated with the RhoA antibody (Fig. 5). We were also unable to detect any interaction between RhoA and the TNF-R1 adaptor proteins TNF receptor-associating factor

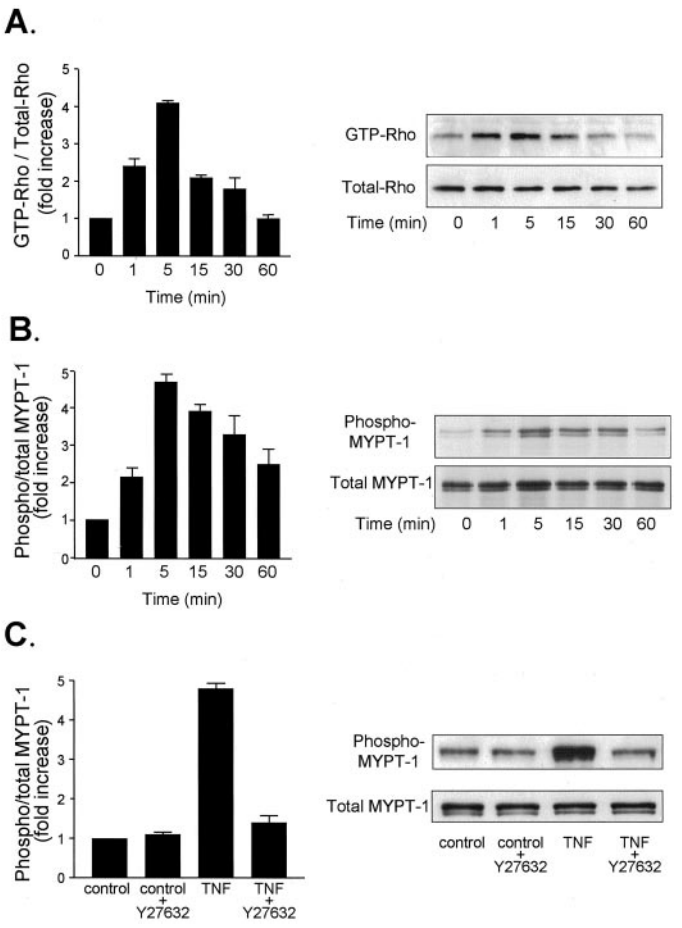

Fig. 3. TNF-induced RhoA activation and phosphorylation of MYPT-1 in human ASM cells. A, TNF-induced increase in GTP-bound RhoA from human ASM cells. Typical blots and graph of mean data showing GTP-bound RhoA ($n = 5$). The respective blot of total RhoA in each sample is also shown. Cells were stimulated at different time points with 200 ng/ml TNF and the rhotekin RBD binding measured. TNF caused an increase in GTP-bound (active) RhoA within 1 min, reaching a peak after 5 min of stimulation. This declined to baseline levels by approximately 60 min. B., typical blot and graph of mean data of TNF-induced phosphorylation of MYPT-1 in human ASM cells. Cells were stimulated at different time points with 200 ng/ml TNF and blotted with anti-phospho MYPT-1 antibody. Total MYPT-1 protein in each respective sample is also shown ($n = 3$). MYPT-1 phosphorylation peaked at 5 min and decreased slowly thereafter. Levels were still above baseline after 60 min of TNF incubation C, typical blot and graph of mean data showing inhibition of MYPT-1 phosphorylation by Y27632 in human ASM cells stimulated with 200 ng/ml TNF for 5 min. The rho-kinase inhibitor had no effect under control conditions (no TNF). However, a 30-min preincubation with 10 μM Y27632 almost completely abolished the TNF-induced phosphorylation of MYPT-1.

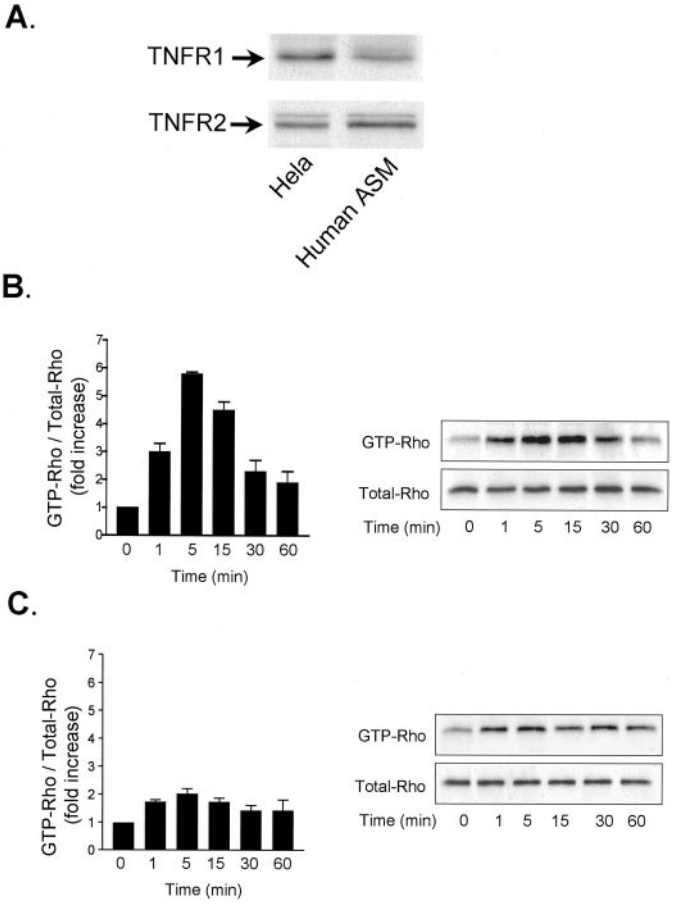

Fig. 4. TNF receptor subtypes involved in RhoA activation in ASM cells. A, expression of TNF receptor subtypes in human ASM cells. Typical blot of TNF-R1 and TNF-R2 expression in whole cell preparations with anti-human TNF receptor subtype-specific antibodies. Human ASM cells are compared with Hela cells. Human ASM cells express both TNF-R1 and TNF-R2. The immunoreactive bands corresponding to the TNR receptor subtypes have the same molecular weight as those observed in Hela cells. B, Rho activation in human ASM cells stimulated with TNF-R1 mutein. Typical blots and graph of mean data showing GTP-bound RhoA ($n = 4$). The respective blot of total RhoA in each sample is also shown. Cells were stimulated at different time points with 200 ng/ml TNF-R1 mutein and the rhotekin RBD binding measured. Selective activation of TNF-R1 produced an increase in GTP-bound RhoA in a time course and magnitude similar to recombinant human TNF: RhoA activation was increased at 1 min, reaching a maximum at 5 min. C, Rho activation in human ASM cells stimulated with 200 ng/ml TNF-R2 mutein. Typical blots and graph of mean data showing GTP-bound RhoA ($n = 6$). The respective blot of total RhoA in each sample is also shown. Cells were stimulated at different time points with TNF-R2 mutein and the rhotekin RBD binding measured. Selective activation of TNF-R2 produced only small increases in GTP-bound RhoA.

(TRAF)-2, receptor interacting protein, and TNF receptor-associated death domain binding protein (data not shown). In positive control experiments, RhoA was capable of coprecipitating with Rho-kinase.

Discussion

There is considerable in vivo evidence of an important role for TNF in airway hyper-responsiveness (Kips et al., 1992; Thomas et al., 1995; Renzetti et al., 1996). The present study describes a molecular mechanism that may be, at least in part, responsible for producing acute airway hyper-responsiveness (Thomas et al., 1995). TNF can increase the Ca^{2+} sensitivity of MLC_{20} phosphorylation in ASM cells, from both guinea pigs and humans, via a Rho-kinase-dependent pathway. In addition, TNF can activate RhoA in a time course compatible with a role in airway hyper-responsiveness and the TNF-induced potentiation of contractility previously observed in guinea pig bronchial smooth muscle (Parris et al., 1999). This activation is predominantly via TNF-R1.

Previous studies on the intracellular effects of TNF in ASM cells have concentrated predominantly on elucidating the mechanisms involved in longer term effects resulting in alterations of protein expression (e.g., heterotrimeric GTP-binding proteins) (Hotta et al., 2000) and intracellular adhesion molecule-1 (Amrani et al., 2000b, 2001). These TNF-induced effects are likely to be of importance in chronic inflammatory responses and possibly ASM cell proliferation; however, they do not address the potential mechanisms of acute airway hyper-responsiveness induced by TNF in a much shorter time course (Kips et al., 1992; Thomas et al., 1995). Our previous study suggested such a mechanism may occur via a TNF-induced increase in the Ca^{2+} sensitivity of ASM contraction (Parris et al., 1999), although TNF itself is not a contractile agonist and does not increase intracellular Ca^{2+} in ASM cells; i.e., TNF is acting only as a sensitizing agent. In permeabilized guinea pig bronchial smooth muscle strips, TNF incubation for 45 min significantly increased the Ca^{2+}-activated contractility via an increase in the Ca^{2+}-activated MLC_{20} phosphorylation. The increase in the MLC_{20} phosphorylation in guinea pig ASM cells in the present study was inhibited by preincubation with Y27632, a Rho-kinase inhibitor. Rho-kinase is known to play an important role in regulating contractility of airway (Iizuki et al., 1999, 2000; Ito et al., 2001) and other types of smooth muscle (Kureishi et al., 1997; Fu et al., 1998). To date, this regulation has been associated with contractile agonists activating seven-transmembrane receptors and heterotrimeric G proteins (Somlyo and Somlyo, 2000). This study now demonstrates a role for TNF, a noncontractile agonist, in regulating ASM contractility via activation of Rho-kinase. Further evidence for this mechanism is provided by TNF-induced activation of RhoA in both guinea pig and human ASM cells. The slower time of peak Rho activation in guinea pig ASM cells may represent a decreased relative efficacy and/or affinity of recombinant human TNF on guinea pig TNF receptors. However, this may also represent physiological differences in the TNF-induced Ca^{2+} sensitization in guinea pig versus humans. The fold levels of increase in GTP-bound RhoA observed here are similar in magnitude to those observed in other smooth muscles stimulated with contractile agonists such as U46619, norepinephrine, and endothelin-1 (Sakurada et al., 2001). Although our initial study first revealed the TNF-induced increase in Ca^{2+} sensitivity of MLC_{20} phosphorylation in guinea pig ASM, it is important to determine whether this effect occurs in human. TNF does produce an in vivo airway hyper-responsiveness in humans (Thomas et al., 1995). In cultured human ASM cells, it was not possible to determine TNF-induced MLC_{20} phosphorylation using two-dimensional gel electrophoresis until the apparently multiple isoforms of MLC_{20} have been further characterized. Phosphorylation of the MYPT-1 from the smooth muscle myosin light chain phosphatase, however, was assessed as an indicator of Ca^{2+} sensitization. TNF incubation increased phosphorylation of MYPT-1 in a time course compatible with TNF-induced Ca^{2+} sensitization; however, the phosphorylation levels remained elevated for a longer time period and were above control levels even after 1 h. This is in agreement with a recent study showing that dephosphorylation of MYPT-1 is relatively slow (Takizawa et al., 2002) and consistent with the prolonged Ca^{2+} sensitization observed after TNF incubation (Parris et al., 1999). Phosphorylation of MYPT-1 was blocked by the Rho-kinase inhibitor Y27632, suggesting that it is the result of Rho-kinase activation. This study therefore suggests that TNF induces activation of the RhoA/Rho-kinase pathway, leading to phosphorylation and inhibition of the smooth muscle myosin light chain phosphatase and a subsequent increase in the Ca^{2+} sensitivity of MLC_{20} phosphorylation.

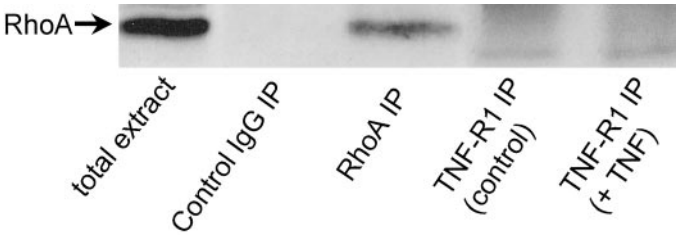

Fig. 5. TNF-R1 does not immunoprecipitate with RhoA in human ASM cells. Typical blot showing immunoprecipitation in ASM cell homogenates using anti-TNF-R1 antibody, anti-RhoA antibody, or control IgG. Total extract shows total RhoA present in the homogenate. As a positive control, anti-RhoA antibody was capable of immunoprecipitating RhoA. In nonstimulated cells, immunoprecipitation with the anti-TNF-R1 antibody did not pull down detactable levels of RhoA. This was also the case in ASM cells incubated with 200 ng/ml recombinant TNF for 5 min.

TNF effects are mediated by two receptor subtypes, TNF-R1 and TNF-R2 (MacEwan, 2002). Both TNF-R1 and TNF-R2 receptors are single transmembrane glycoproteins with 28% sequence homology and a marked delineation of function (Grell et al., 1994). As shown in this study by immunoblotting, both these receptor subtypes are present in cultured human ASM cells (Amrani et al., 2000b) and have been previously identified by us in guinea pig ASM cells (McFarlane et al., 2001). TNF receptor engagement activates a variety of signaling molecules. This study is the first to report activation of RhoA by TNF in ASM cells, predominantly via activation of the TNF-R1, with a smaller proportion via the TNF-R2. The intracellular mechanism by which TNF activates RhoA in these cells is not clear. TNF receptors activate downstream signaling pathways via interaction with a family of adaptor proteins (MacEwan, 2002). These proteins, known as TRAFs, bind to the cytoplasmic domain of TNF receptors (Wajant et al., 1999). To date, no direct interaction of TRAFs with either Rho or one of the several GEFs,

which mediate Rho activation, has been identified. TNF-induced RhoA activation has previously been implicated in cytoskeletal reorganization in serum-starved endothelial cells and fibroblasts over a relatively slow time course (approximately 30 min) (Wojciak-Stothard et al., 1998; Puls et al., 1999). This is possibly via a hierarchical cascade of structurally related monomeric G proteins (Puls et al., 1999). This study has directly measured the time course of TNF-induced RhoA activation and this reveals a rapid increase (within 1 min). This fast time course indicates that a relatively direct signaling pathway is probably involved. A direct interaction of RhoA has been previously observed with the neurotrophin receptor p75 (Yamashita et al., 1999), a related member of the TNF receptor family. We investigated whether this may also be the case for the TNF-R1 receptor. Under the conditions used in this study no interaction was observed between RhoA and TNF-R1, or the principal TNF-R1 adaptor proteins TRAF-2, receptor interacting protein and TNF receptor-associated death domain binding protein. A recent study in guinea pig ASM cells has suggested that the TNF-induced increase in MLC_{20} phosphorylation may be via formation of reactive oxygen species (Thabut et al., 2002), although the mechanism linking reactive oxygen species generation to increased MLC_{20} phosphorylation is not known. It is interesting to note that some effects of reactive oxygen species in the lung occur via Rho-mediated effects (Chiba et al., 2001). It is likely that the TNF receptor, predominantly TNF-R1 (or associated TRAFs), may activate one or more GEFs involved in Rho activation, although this remains to be established.

In conclusion, this study clearly demonstrates that TNF can activate RhoA in ASM cells. In addition, we have also shown that activation of the RhoA/Rho-kinase pathway contributes significantly to the TNF-induced Ca^{2+} sensitization of MLC_{20} phosphorylation. These effects are not species-specific and were observed in both guinea pig and human ASM cells. This TNF-activated intracellular mechanism may therefore contribute to the airway hyper-responsiveness observed in inflammatory diseases such as asthma.

References

Amrani Y, Ammit AJ, and Panettieri RA (2001) Tumor necrosis factor receptor (TNFR) 1, but not TNFR2, mediates tumor necrosis factor-α-induced interleukin-6 and RANTES in human airway smooth muscle cells: role of p38 and p42/44 mitogen-activated protein kinases. *Mol Pharmacol* **60:**646–655.

Amrani Y, Chen H, and Panettieri RA (2000a) Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? *Respir Res* **1:**49–53.

Amrani Y, Krymskaya V, Maki C, and Panettieri RA (1997) Mechanisms underlying TNF-α effects on agonist-mediated calcium homeostasis in human airway smooth muscle cells. *Am J Physiol* **273:**L1020–L1028.

Amrani Y, Lazaar AL, Hoffman R, Amin K, Ousmer S, and Panettieri RA (2000b) Activation of p55 tumor necrosis factor-α receptor-1 coupled to tumor necrosis factor receptor-associated factor 2 stimulates intercellular adhesion molecule-1 expression by modulating a thapsigargin-sensitive pathway in human tracheal smooth muscle cells. *Mol Pharmacol* **58:**237–245.

Amrani Y, Panettieri RA, Frossard N, and Bronner C (1996) Activation of the TNF-α-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth-muscle cells. *Am J Respir Cell Mol Biol* **15:**55–63.

Borman MA, MacDonald JA, Murayani A, Hartshorne DJ, and Haystead TAJ (2002) Smooth muscle myosin phosphatase-associated kinase induces Ca^{2+} sensitization via myosin phosphatase inhibition. *J Biol Chem* **277:**23441–23446.

Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federma EC, and Wasserman SI (1992) Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* **89:**958–967.

Chiba Y, Iishi Y, Kitamura S, and Sugiyama Y (2001) Activation of Rho is involved in the mechanism of hydrogen-peroxide-induced lung edema in isolated perfused rabbit lung. *Microvasc Res* **62:**164–171.

Emala CW, Kuhl J, Hungerford CL, and Hirshman CA (1997) TNF-α inhibits isoproterenol-stimulated adenylyl cyclase activity in cultured airway smooth muscle cells. *Am J Physiol* **272:**L644–L650.

Feng J, Ito M, Ichikawa K, Isaka N, Nishikawa M, Hartshorne DJ, and Nakano T (1999) Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J Biol Chem.* **274:**37385–37390.

Fu X, Gong MC, Jia T, Somlyo AV, and Somlyo AP (1998) The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPγS- and phorbol ester-induced Ca^{2+}-sensitization of smooth muscle. *FEBS Lett* **440:**183–187.

Gong MC, Fujihara H, Somlyo AV, and Somlyo AP (1997) Translocation of RhoA associated with Ca^{2+} sensitization of smooth muscle. *J Biol Chem* **272:**10704–10709.

Grell M, Wajant H, Zimmerman G, and Scheurich P (1998) The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. *Proc Natl Acad Sci USA* **95:**570–575.

Grell M, Zimmerman G, Hulser D, Pfizenmaier K, and Scheurich P (1994) TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. *J Immunol* **145:**1963–1972.

Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternwies PC, and Bollag G (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. *Science (Wash DC)* **280:**2112–2114.

Himpens B, Kitazawa T, and Somlyo AP (1990) Agonist dependent modulation of the Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle. *Pflueg Arch Eur J Physiol* **417:**21–28.

Horiuti K (1986) Some properties of the contractile system and sarcoplasmic reticulum of skinned slow fibres from *Xenopus* muscle. *J Physiol (Lond)* **373:**1–23.

Hotta K, Emala CW, and Hirshman CA (1999) TNF-α upregulates $G_i\alpha$ and $G_q\alpha$ protein expression and function in human airway smooth muscle cells. *Am J Physiol* **276:**L405–L411.

Hotta K, Hirshman CA, and Emala CE (2000) TNF-α increases transcription of $G\alpha_{i\text{-}2}$ in human airway smooth muscle cells. *Am J Physiol* **279:**L319–L325.

Iizuka K, Shimizu Y, Tsukagoshi H, Yoshii A, Harada T, Dobashi K, Murozono T, Nakazawa T, and Mori M (2000) Evaluation of Y27632, a rho-kinase inhibitor, as a bronchodilator in guinea pigs. *Eur J Pharmacol* **406:**273–279.

Iizuka K, Yoshii A, Samizo K, Tsukagoshi H, Ishizuki T, Dobashi K, Horie T, Nakazawa T, and Mori M (1999) A major role for the rho-associated coiled coil forming protein kinase in G-protein-mediated Ca^{2+} sensitization through inhibition of myosin phosphatase in rabbit trachea. *Br J Pharmacol* **128:**925–933.

Ito S, Kume H, Honjo H, Katoh H, Kodama I, Yamaki K, and Hayashi H (2001) Possible involvement of Rho kinase in Ca^{2+} sensitization and mobilization by MCh in tracheal smooth muscle. *Am J Physiol* **280:**L1218–L1224.

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, et al. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase. *Science (Wash DC)* **273:**245–247.

Kips JC, Tavernier J, and Pauwels RA (1992) Tumor necrosis factor causes bronchial hyperresponsiveness in rats. *Am Rev Respir Dis* **145:**332–336.

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, and Sternweis PC (1998) p115 RhoGEF, a GTPase activating protein for G α_{12} and $G\alpha_{13}$. *Science (Wash DC)* **280:**2109–2111.

Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, and Ito M (1997) Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem* **272:**12257–12260.

Leung T, Manser E, Tan L, and Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem* **270:**29051–29054.

MacEwan DJ (2002) TNF receptor subtype signaling: Differences and cellular consequences. *Cell Signal* **14:**477–492.

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, and Kaibuchi K (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. *EMBO (Eur Mol Biol Organ) J* **15:**2208–2216.

McFarlane SM, Jupp OJ, Cobban HJ, Hunter I, Anderson HM, Nixon GF, and MacEwan DJ (2001) Tumor necrosis factor receptor subtypes in airway smooth muscle stimulate stress-activated but not mitogen-activated protein kinases. *Biochem Pharmacol* **61:**749–759.

Moore KP, Sheron N, Ward P, Taylor GW, Alexander GIM, and Williams R (1991) Leukotriene and prostaglandin production after infusion of tumor-necrosis-factor in man. *Eicosanoids* **4:**115–118.

Parris JR, Cobban HJ, Littlejohn AF, MacEwan DJ, and Nixon GF (1999) Tumour necrosis factor-α activates a calcium sensitization pathway in guinea pig bronchial smooth muscle. *J Physiol (Lond)* **518:**561–569.

Puls A, Eliopoulis AG, Nobes CD, Bridges T, Young LS, and Hall A (1999) Activation of the small GTPase Cdc42 by the inflammatory cytokine TNF α and IL-1 and by the Epstein-Barr virus transforming protein LMP1. *J Cell Science (Wash DC)* **112:**2983–2992.

Ren XD, Kiosses WB, and Schwartz MA (1999) Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO (Eur Mol Biol Organ) J* **18:**578–585.

Renzetti LM, Paciorek PM, Tannu SA, Rinaldi NC, Tocker JE, Wasserman MA, and Gater PR (1996) Pharmacological evidence for tumor necrosis factor as a mediator of allergic inflammation in the airways. *J Pharmacol Exp Ther* **278:**847–853.

Sakurada S, Okamoto H, Takuwa N, Sugimoto N, and Takuwa Y (2001) Rho activation in excitatory agonist-stimulated vascular smooth muscle. *Am J Physiol* **281:**C571–C578.

Sellers JR (1991) Regulation of cytoplasmic and smooth muscle myosin. *Curr Biol* **3:**98–110.

Somlyo AP and Somlyo AV (2000) Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol (Lond)* **522:**177–185.

Takizawa N, Niiro N, and Ikebe M (2002) Dephosphorylation of the two regulatory components of myosin phosphatase, MBS and CPI17. *FEBS Lett* **515:**127–132.

Thabut G, El-Benna J, Samb A, Corda S, Megret J, Leseche G, Vicaut E, Aubier M, and Boczkowski J (2002) Tumor necrosis factor-α increases airway smooth muscle

oxidants production through a NADPH oxidase-like system to enhance myosin light chain phosphorylation and contractility. *J Biol Chem* **277:**22814–22821.
Thomas PS, Yates DH, and Barnes PJ (1995) Tumor-necrosis-factor-α increases airway responsiveness and sputum neutrophilia in normal human-subjects. *Am J Respir Crit Care Med* **152:**76–80.
Van Ostade X, Vandenabeele P, Tvernier J, and Fiers W (1994) Human tumor necrosis factor mutants with preferential binding to and activity on either the R55 or R75 receptor. *Eur J Biochem* **220:**771–779.
Wajant H, Grell M, and Scheurich P (1999) TNF receptor associated factors in cytokine signaling. *Cytokine Growth Factor Rev* **10:**15–26.
Wojciak-Stothard B, Entwhistle A, Carg R, and Ridley AJ (1998) Regulation of TNFα-induced reorganization of the actin cytoskeleton and cell-cell junctions by rho, rac and Cdc42 in human endothelial cells. *J Cell Physiol* **176:**150–165.
Yamashita T, Tucker KL, and Barde Y-A (1999) Neurontrophin binding to the p75 receptor modulates Rho activity and axonal growth. *Neuron* **24:**585–593.

Address correspondence to: Graeme F. Nixon, Ph.D., Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK. E-mail: g.f.nixon@abdn.ac.uk