0026-895X/96/050781-07$3.00/0

MOLECULAR PHARMACOLOGY, 49:781–787 (1996).

Transforming Growth Factor-β1 Induces Transcriptional Down-regulation of m2 Muscarinic Receptor Gene Expression

EL-BDAOUI HADDAD, JONATHAN ROUSELL, JUDITH C. W. MAK, and PETER J. BARNES

Department of Thoracic Medicine, National Heart and Lung Institute, London SW3 6LY, United Kingdom

Received August 31, 1995; Accepted January 5, 1996

SUMMARY

In human embryonic lung fibroblasts, transforming growth factor-β1 (TGF-β1) induced a time-dependent down-regulation of M_2 muscarinic receptor binding sites as measured with the nonselective hydrophilic ligand [^{3}H]*N*-methylscopolamine (NMS). This down-regulation was slow, with 58% loss of all receptors after 24 hr of treatment. The affinity of [^{3}H]NMS for the remaining sites was unaltered by TGF-β1. The loss in [^{3}H]NMS binding was accompanied by reduced adenylyl cyclase activity and functional desensitization of M_2 muscarinic receptors. Northern blot analyses showed a 72% decrease in the steady state levels of m2 muscarinic receptor mRNA after 24-hr TGF-β1 treatment. Recovery of m2 muscarinic receptor mRNA after TGF-β1 treatment was slow, with a half-life of ~8 hr. There was no effect of TGF-β1 on the m2 muscarinic receptor mRNA half-life measured in the presence of actinomycin D, but the rate of m2 muscarinic receptor gene transcription measured with nuclear run-on assay was reduced by 50%, indicating reduced gene transcription. Cycloheximide (10 μg/ml) pretreatment abolished the TGF-β1 effect, indicating that *de novo* protein synthesis was required for receptor down-regulation. In summary, we have shown that TGF-β1 induced desensitization and down-regulation of M_2 muscarinic receptor protein and gene that was mediated through reduction in the rate of m2 receptor gene transcription.

The TGF-β family consists of a group of closely related proteins from mammals (TGF-β1, TGF-β2, and TGF-β3) and a large group of more distantly related members, including activins/inhibitins from a variety of species (1). TGF-βs occur as disulfide-linked proteins composed of 12.5-kDa homodimers that are synthesized and secreted by most cell types as latent high molecular weight complexes. They exert their action by binding to specific cell surface serine/threonine kinase receptors (2). The receptor or receptors for TGF-β1 are found on nearly all cell types (3, 4), but the nature of the biological response to TGF-β1 varies with the cell type. TGF-β1 has important physiological roles in the regulation of embryogenesis, tissue repair, inflammation, or cell adhesion, growth, and differentiation (5). TGF-β1 is stored in high concentrations in platelets and expressed in activated monocytes and macrophages at sites of wound healing or inflammation. TGF-β also is a potent chemotactic cytokine for fibroblasts, neutrophils, monocytes, lymphocytes, and alveolar macrophages. mRNA transcripts for TGF-β1, TGF-β2, and TGF-β3 isoforms are expressed in airway smooth muscle cells, fibroblasts, and epithelial cells, and high concentrations of TGF-β are found in the epithelial lining fluid in human lower airways (6).

The mechanisms by which TGF-β1 exerts its cellular effects are unclear, although one of the earliest effects of TGF-β1 involves regulation of gene expression. TGF-β1 stimulation of target cells results in increased expression of extracellular matrix-associated genes, such as collagen, fibronectin (7), and integrin (8, 9), as well as proto-oncogenes such as c-*sis*, c-*myc*, and c-*fos* (10, 11). TGF-β1 has also been reported to alter the gene expression of interleukin-1β (12), cyclooxygenases 1 and 2, and phospholipase A_2 (13).

Muscarinic acetylcholine receptors are encoded by five distinct and intronless genes (m1–m5). The expressed m1, m3, and m5 receptors are coupled to phosphoinositide hydrolysis, whereas the m2 and m4 receptors are coupled to the attenuation of adenylyl cyclase. Expression of muscarinic acetylcholine receptor genes in cell lines has made it possible to explore the pharmacological and molecular properties of the individual receptors (14, 15). However, much less is known about the factors that regulate the expression of muscarinic receptors, in part because the noncoding promoter and enhancer regions that directly control transcription of the individual muscarinic receptor genes have not been sequenced.

To gain a better understanding of the regulation of muscarinic receptor expression, particularly in inflammatory dis-

This work was supported by the Medical Research Council of the United Kingdom and by a European Union Fellowship (E.-B.H).

ABBREVIATIONS: TGF-β, transforming growth factor-β1; NMS, *N*-methylscopolamine; PGE_2, prostaglandin E_2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, standard sodium citrate; HEL, human embryonic lung; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

eases, we investigated the effect of TGF-β1 on the gene expression of M_2 muscarinic receptors in HEL 299 cells. Of the five muscarinic receptors, these cells express only the M_2 subtype (16). Moreover, HEL 299 cells have been shown to express high levels of TGF-β1 receptors that bind ^{125}I-TGF-β1 with very high affinity (4).

Experimental Procedures

Cell culture. HEL 299 cells were obtained from the American Type Culture Collection (code CCL 137; Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (GIBCO-BRL, Paisley, UK) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/liter amphotericin B in 95% air/5% CO_2 at 37°. All experiments were performed on cells at passage 9. The medium was replaced every 3–4 days, and on reaching confluence, cells were subcultured by detaching the monolayer with 0.05% trypsin/1 mM EDTA. Treatments were carried out so that cells could be harvested simultaneously at preconfluence.

Radioligand binding studies. All of the membrane preparation procedures were performed at 4°. Cells were washed twice with Hanks' balanced salt solution, harvested by cell scraping with ice-cold Tris buffer (25 mM, pH 7.4), and homogenized with an Ultra-Turax homogenizer (Kinematica, Littau, Switzerland). Membranes were pelleted by centrifugation at 40,000 × *g* for 20-min and resuspended in an appropriate volume of Tris buffer. The protein concentration was measured with the use of the Lowry assay (17). [^{3}H]NMS (80.4 Ci/mmol; New England Nuclear, Stevenage, UK) saturation curves were carried out as previously described (16). Binding data were analyzed with the computerized nonlinear regression program LIGAND as previously described (18).

cAMP measurements. After stimulation, the cells were washed, and the cAMP-phosphodiesterase inhibitor Org 20241 (30 μM) was added to fresh media for 30 min at 37°. From each treatment group, we measured both basal levels of cAMP and accumulation after forskolin exposure (100 μM) for 10 min in the presence and absence of carbachol (100 μM). Cells were harvested by the direct addition of 1 ml of boiling water to each well. Cells were then boiled for an additional 2 min before centrifugation at full speed in a microcentrifuge at 4° for 5 min. The supernatant was collected and stored at −20°. Aliquots (500 μl) of cell extracts were acetylated by the consecutive addition of triethylamine and acetic anhydride. cAMP content was measured with radioimmunoassay as described previously (19). Briefly, a 200-μl acetylated sample was added to 50 μl of adenosine-3′,5′-cyclic phosphoric acid-2-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (2000 Ci/mmol, Amersham, Amersham, UK) in 0.2% BSA (~2000 dpm) and 100 μl of anti-cAMP antibody in 0.2% BSA. Samples were incubated overnight at 4°, and free and antibody-bound cAMP were separated through charcoal precipitation in 100 mM phosphate buffer, pH 7.4. Protein assays were performed with a Bio-Rad protein assay (Hemel, Hempstead, UK) according to the manufacturer's instructions.

Adenylyl cyclase activity. Adenylyl cyclase activity was assayed in a 100-μl reaction mixture containing 25 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.5 mM ATP, 20 μM GTP, 5 mM phosphocreatine, 25 units/ml creatine phosphokinase, 0.5 mg/ml BSA, 5 mM SDS, 30 μM Org 20241, and the muscarinic receptor agonist carbachol (100 μM). Forskolin (100 μM) was added to the reaction mixture to amplify the muscarinic inhibition. The incubation was started by the addition of the membrane suspension (~5–10 μg protein) and was carried out at 30° for 10 min. The incubation was stopped by boiling, with subsequent cooling of the samples and centrifugation for 10 min at 12,000 × *g*. After storage at −20°, the amount of cAMP in the supernatant was determined with radioimmunoassay as described above.

Northern blot analysis. Cells were washed twice with Hanks' balanced salt solution, and total RNA was isolated according to the method of Chomczynski and Sacchi (20). Poly(A)$^+$ RNA was prepared with the PolyTract mRNA isolation kit (Promega, Southampton, UK) according to the manufacturer's instructions. Samples of mRNA were size fractionated on a 1% agarose/formaldehyde gel containing 20 mM morpholinosulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0, and blotted onto Hybond-N filters (Amersham) through capillary action with the use of 20× SSC (1× = 150 mM NaCl, 15 mM citric acid).

Cloned human m2 muscarinic receptor cDNA corresponding to the third intracellular loop and consisting of an *Eco*RI/*Pst*I fragment (550 base pair) was used as a probe. Prehybridizations and hybridizations were carried out at 42° with the probes labeled to ~1.5–2 × 10^6 cpm/ml in a buffer containing 50% formamide, 50 mM Tris·HCl, pH 7.5, 5× Denhardt's solution, 0.1% SDS, 5 mM EDTA, and 250 μg/ml denatured salmon sperm DNA. After hybridization, the blots were washed to a stringency of 0.1× SSC/0.1% SDS at 65° for 30 min before exposure to Kodak X-OMAT-S film. To account for differences in loading or transfer of the RNA, the blots were hybridized with a 1272-base pair *Pst1* fragment from rat GAPDH cDNA. The intensities of the signals were then quantified with the use of laser densitometry (Quantity One Software, PDI, New York, NY).

Nuclear run-on assay. For the measurement of gene transcription, nuclei were prepared according to the method of Greenberg and Ziff (21). Isolated nuclei were resuspended in 10 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 50% glycerol, 0.5 M sorbitol, 2.5% Ficoll, 0.008% permidine, and 1 mM dithiothreitol and stored at −70° until use. *In vitro* transcription was performed with nuclei (5 × 10^7) incubated for 30 min at 27° with 300 μCi [^{32}P]UTP, 0.625 mM ATP, 0.31 mM CTP, 0.31 mM GTP, 40 mM Tris·HCl, 150 mM NH_4Cl, 7.5 mM $MgCl_2$, and 120 units RNasin. DNA digestion was carried out with a 15-min incubation at 27° with RQ-1 DNase (75 units) and RNasin (40 units) before protein digestion for 3 hr at 37° with proteinase K (1 mg/ml) in buffer containing 10 mM Tris·HCl, pH 7.4, 15 mM EDTA, 3% SDS, and 3 mg/ml heparin. RNA extraction was then carried out with a phenol wash, a phenol/chloroform (1:1) wash, and a chloroform wash and then precipitated three times with 100% ethanol in the presence of 1.33 M ammonium acetate. The radiolabeled RNAs were dissolved in 100 μl of TE buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA) and added to 2 ml of hybridization solution [50% formamide, 5× SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris·HCl, pH 7.5, 5× Denhardt's solution, 50 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 0.02 μg poly(A)$^+$, and 0.02 μg of poly(G)$^+$ RNA]. After a 4-hr prehybridization in the above buffer, hybridization was carried out at 42° for 72 hr to 10 μg of the immobilized plasmid pGEM3Z as a control or to plasmids containing inserts of rat GAPDH and human m2 muscarinic receptor cDNAs. The filters were washed first in buffer A (300 mM NaCl, 10 mM Tris·HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, 1 μg/ml RNase A, and 10 units/ml RNase T1) at 37° for 30 min, washed second in buffer B (10 mM NaCl, 10 mM Tris·HCl, pH 7.4, 2 mM EDTA, and 0.4% SDS) to a stringency of 55° for 30 min, and autoradiographed.

Results

Effect of TGF-β1 on muscarinic receptor density and function. Saturation experiments performed with the hydrophilic nonselective muscarinic antagonist [^{3}H]NMS revealed a single class of binding site (B_{max}, 450 ± 35 fmol/mg protein) with a dissociation constant (K_D) of 0.21 ± 0.09 nM. Northern blot analyses on isolated mRNA revealed expression of the m2 muscarinic receptor mRNA transcript (6.1 kb) with no evidence of m1, m3, or m4 receptor mRNAs. Treatment of HEL 299 cells with TGF-β1 (2 ng/ml) induced a time-dependent decrease in the [^{3}H]NMS binding sites so that after a 24-hr incubation, 58% of the sites were down-regulated (Fig. 1). The affinity of [^{3}H]NMS for the remaining sites was unaltered by this treatment.

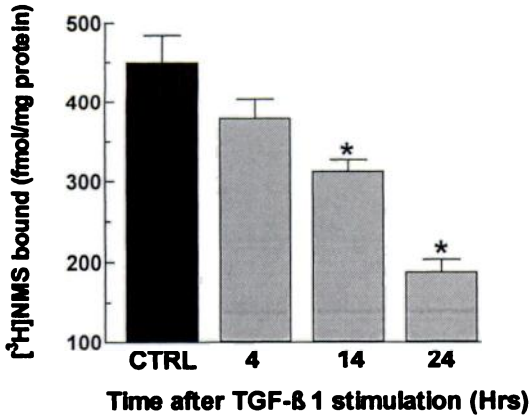

Fig. 1. Effect of TGF-β1 on the density of muscarinic receptors. Preconfluent cells were treated with vehicle (*CTRL*, control) or TGF-β1 (2 ng/ml) for the times indicated. The density and the affinity of muscarinic receptors were measured by saturation experiments with the hydrophilic nonselective muscarinic antagonist [^{3}H]NMS. Values are mean ± standard error of three to five separate experiments performed in duplicate. *, $p < 0.05$ compared with control.

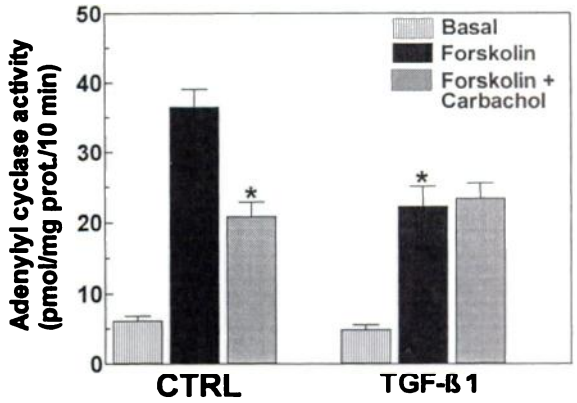

Fig. 3. Effect of TGF-β1 on forskolin-stimulated adenylyl cyclase activity. Modulation by TGF-β1 of adenylyl cyclase activity in membranes derived from control (*CTRL*) and TGF-β1-treated cells. Membranes from cells treated with or without TGF-β1 (2 ng/ml) for 24 hr were assayed under the conditions described in Experimental Procedures. Values are mean ± standard error of six determinations. *, $p < 0.01$ compared with forskolin in control cells.

Functional desensitization was assessed by measurement of cAMP accumulation after TGF-β1 stimulation for 24 hr. In untreated cells, forskolin induced a large increase in cAMP accumulation that was inhibited significantly by carbachol (Fig. 2). The inhibitory effect of carbachol on forskolin-induced cAMP accumulation was lost after a 24-hr TGF-β1 treatment. Higher concentrations of carbachol did not produce any inhibition of forskolin-induced cAMP accumulation in TGF-β1-treated cells. The basal level of cAMP accumulation was not affected by such treatment. The ability of the β_2-adrenergic receptor agonist procaterol (10 μM) to generate cAMP was also reduced after TGF-β1 treatment. This effect could be the result of β_2-adrenergic receptor decrease after TGF-β1 treatment (data not shown). Because cAMP accumulation experiments suggested that TGF-β1 altered the activity of the adenylyl cyclase, experiments were performed to directly measure the activity of the enzyme. The basal activity of adenylyl cyclase was not altered by 24-hr TGF-β1 stimulation. However, the forskolin-stimulated enzyme activity was decreased in membranes prepared from TGF-β1-treated cells (Fig. 3). The adenylyl cyclase activity data with forskolin are consistent with the decreased ability of forskolin to stimulate cAMP accumulation in intact cells (Figs. 2 and 3).

Because TGF-β1 alters the cAMP accumulation and adenylyl cyclase activity, it was difficult to speculate about functional cholinergic desensitization. In another set of experiments, we determined that, surprisingly, cAMP accumulation evoked by PGE_2 stimulation in intact cells was not altered by TGF-β1. Carbachol no longer inhibited PGE_2-induced cAMP accumulation in TGF-β1-treated cells (data not shown). Similar results were obtained at the level of adenylyl cyclase activity. These results suggest that functional desensitization of M_2 muscarinic receptors occurs after TGF-β1 stimulation of HEL 299 cells.

Effect of TGF-β1 on muscarinic m2 receptor gene expression. To address the question of whether the down-regulation of muscarinic M_2 receptors in HEL 299 cells is due to changes in the steady state levels of mRNA, the level of m2 receptor mRNA was measured with Northern blot analysis after TGF-β1 stimulation. TGF-β1 decreased the steady state levels of m2 muscarinic receptor mRNA by 68% and 72% after 12 and 24 hr of incubation, respectively (Fig. 4). These results suggest that the down-regulation of M_2 muscarinic receptors in HEL 299 cells by TGF-β1 involves a decrease in the receptor synthesis as a consequence of the decrease in its mRNA. Recovery of m2 muscarinic receptor mRNA was evaluated after 14 hr of TGF-β1 treatment. After TGF-β1 stim-

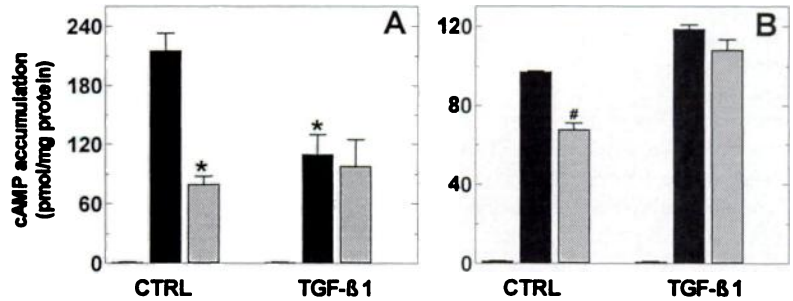

Fig. 2. Functional desensitization of muscarinic receptors by TGF-β1. Effect of TGF-β1 on forskolin-induced (A) and PGE_2-induced (B) cAMP accumulation in control (*CTRL*) and TGF-β1-treated cells. After stimulation with TGF-β1 (2 ng/ml) for 24 hr, cells were washed, and the phosphodiesterase inhibitor Org 20241 (30 μM) was added to fresh media for 30 min at 37°. cAMP was extracted and assayed with radioimmunoassay under basal conditions (*open bars*) or after forskolin (100 μM) or PGE_2 (1 μM) exposure for 10 min in the presence (*striped bars*) and absence (*solid bars*) of carbachol (100 μM). Values are mean ± standard error of six experiments performed in duplicate. *, $p < 0.005$ compared with forskolin stimulation in vehicle-treated cells. #, $p < 0.05$ compared with PGE_2 stimulation in vehicle-treated cells.

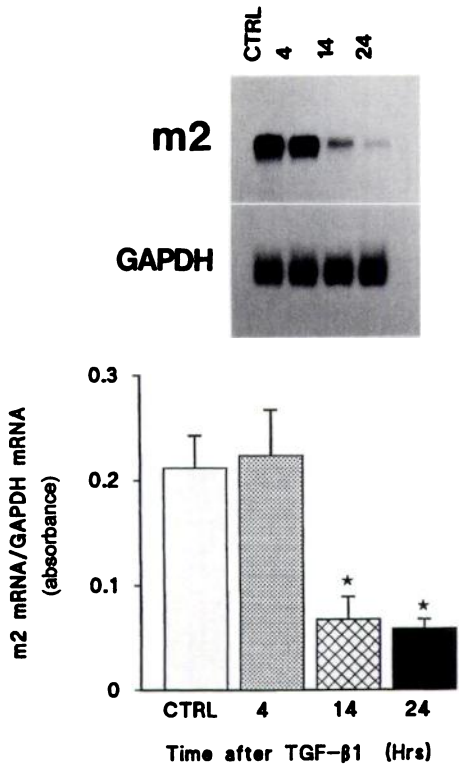

Fig. 4. Kinetics of TGF-β1-induced down-regulation of m2 receptor gene expression in HEL 299 cells. Preconfluent cells were treated with TGF-β1 for the indicated time period and harvested for Northern blot analyses. *CTRL*, control. GAPDH was used as an internal control. *Top*, representative Northern blot; *bottom*, densitometric measurements of the Northern blot data. Values are mean ± standard error of three to six independent experiments.

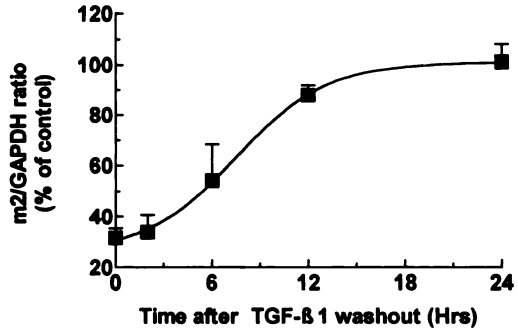

Fig. 5. Rate of m2 muscarinic receptor mRNA recovery after TGF-β1 removal. Cells were treated with TGF-β1 (2 ng/ml) for 14 hr. After this treatment, TGF-β1 was washed out, and recovery of m2 receptor mRNA was followed over a 24-hr period. Values are mean ± standard error of three or four determinations.

ulation, recovery of m2 receptor mRNA was followed over a 24-hr period. The results presented in Fig. 5 show that the kinetics of m2 receptor mRNA recovery were slow. The levels of m2 mRNA returned to control values after 12–24 hr of TGF-β1 washout, with an estimated half-life of 8 hr.

The mechanisms involved in m2 receptor down-regulation by TGF-β1 were also investigated. The protein synthesis inhibitor cycloheximide (10 μg/ml) suppressed the TGF-β1-induced down-regulation of muscarinic m2 receptor expression, whereas cycloheximide alone had no effect on the steady state levels of muscarinic m2 receptor mRNA (Fig. 6). This suggests that synthesis of at least one protein factor is required for TGF-β1 stimulation to alter m2 receptor mRNA levels. This was further substantiated with the use of a protocol in which the addition of cycloheximide was delayed until 4 hr after TGF-β1 stimulation. Steady state levels of m2 muscarinic receptor mRNA measured 10 hr after cycloheximide addition show that this translation inhibitor fully protected against TGF-β1-induced m2 receptor mRNA down-regulation (data not shown).

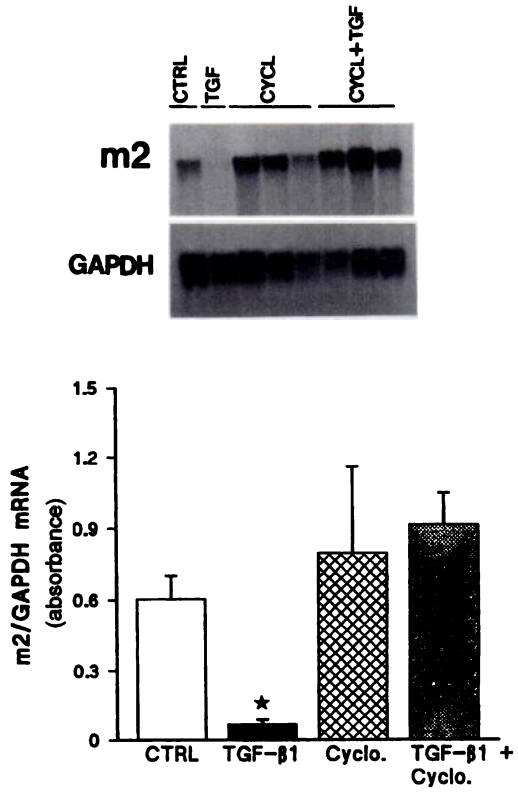

Fig. 6. Effect of cycloheximide on TGF-β1-induced down-regulation of m2 muscarinic receptor mRNAs. Cells were treated for 14 hr with vehicle (*CTRL*), TGF-β1 (*lane 2*), or cycloheximide (*CYCL*; *lanes 3–5*) or pretreated for 1 hr with cycloheximide (10 μg/ml) before incubation with TGF-β1 (*CYCL + TGF*; *lanes 6–8*). mRNA was then isolated and evaluated for m2 muscarinic receptor expression with Northern blot analyses. *Top*, representative Northern blot. *Bottom*, densitometric measurements of the Northern blot data. *Cyclo.*, cycloheximide. Values are mean ± standard error of five independent experiments. *, $p < 0.001$ compared with control.

The half-life of m2 mRNA was evaluated after TGF-β1 stimulation with the use of the RNA polymerase inhibitor actinomycin D. These experiments showed that TGF-β1 did not induce destabilization of the m2 message (Fig. 7). The half-life estimated from these experiments (3–4 hr) was not altered by TGF-β1 treatment. This result suggests that TGF-β1 induces down-regulation through a mechanism involving transcriptional rather than post-transcriptional alteration of the muscarinic m2 mRNA. This was confirmed with nuclear run-on assays. The rate of muscarinic m2 receptor gene transcription showed a 50% reduction after 18 hr of TGF-β1 treatment (Fig. 8).

Discussion

TGF-β1 affects the expression of multiple genes and may thereby regulate a broad spectrum of physiological re-

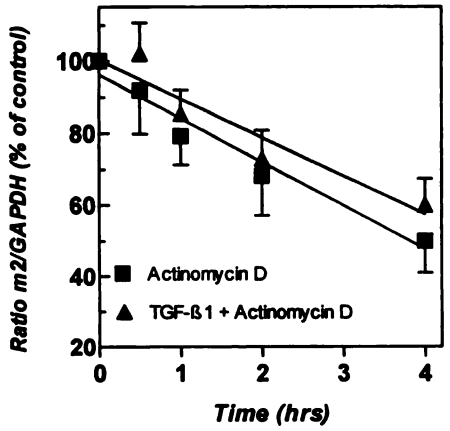

Fig. 7. Degradation rate of m2 muscarinic receptor mRNA. Cells were treated with vehicle or TGF-β1 for 14 hr. After this incubation period, cells were washed, and actinomycin D (5 μg/ml) was added for the times indicated. mRNAs were extracted and evaluated for m2 receptor mRNA expression with Northern blot. Values are mean ± standard error of three separate experiments.

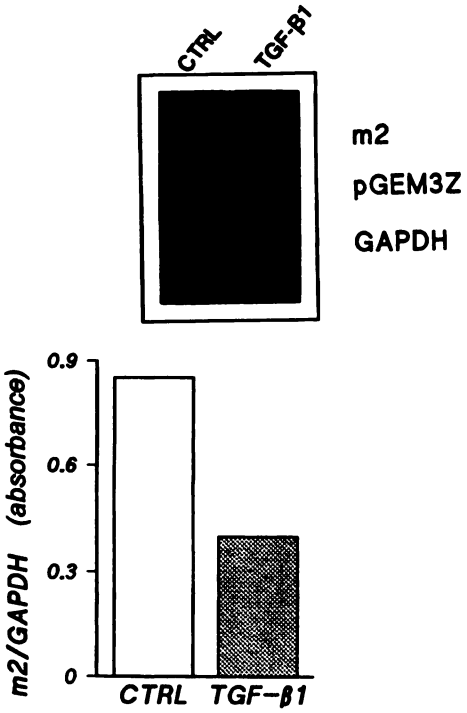

Fig. 8. Relative rate of nuclear transcription of the m2 gene after TGF-β1 treatment. Cells were treated for 18 hr with TGF-β1, and nuclei were collected for nuclear run-on assays. ^{32}P-labeled mRNA was transcribed *in vitro* from isolated cell nuclei, and 1.5 × 10^6 cpm of run-on products were hybridized to each blot as described in Experimental Procedures. The plasmids used were pGEM3Z without an insert (negative control) or containing m2 receptor and GAPDH cDNA inserts. Values are average of two separate experiments and represent the ratio of the absorbance of m2 and GAPDH in control (*CTRL*) and TGF-β1-treated cells.

sponses. In this study, we investigated its effect on the expression of M_2 muscarinic receptors in HEL 299 cells and provide evidence for the transcriptional down-regulation of muscarinic m2 receptor gene by TGF-β1.

HEL 299 cells constitutively express m2 receptors, with no evidence of other muscarinic receptor subtypes. In these cells, TGF-β1 induced a time-dependent decrease in muscarinic receptors. A marked reduction was seen with 58% loss of receptor number after a 24-hr incubation with TGF-β1. The loss of [^{3}H]NMS binding sites occurred slowly, which reflects a fall in the steady state levels of m2 receptor mRNA rather than internalization of the receptors through phosphorylation. The delay between protein loss and the fall in mRNA levels may be indicative of the rate of receptor turnover within the cell. Recovery of muscarinic receptor after TGF-β1 stimulation was investigated. The TGF-β1 effect was long lasting (half-life = 8 hr) as at least 12 hr was required for m2 receptor mRNA to return to basal levels after TGF-β1 washout. Previous results obtained in the same cell line have shown that the recovery of M_2 muscarinic receptor protein after receptor alkylation occurred mainly through the synthetic pathway, with an estimated receptor synthesis half-life of ~12 hr (22).

Functional desensitization of M_2 receptors after 24-hr TGF-β1 stimulation of the cells was evaluated with cAMP measurement. Basal concentrations of cAMP were not altered by TGF-β1 treatment. In control cells, forskolin-stimulated cAMP accumulation was significantly inhibited by carbachol, as expected. However, the inhibitory effect of carbachol was lost after exposure of the cells to TGF-β1, although the amount of cAMP generated by forskolin stimulation was also reduced after TGF-β1 stimulation. It is likely that the inhibitory effect of TGF-β1 on cAMP generation reflected a decrease in adenylyl cyclase activity. Adenylyl cyclase activity measured in membranes prepared from HEL 299 cells confirmed a reduced activity of the enzyme by TGF-β1. Our results differ with those obtained in human tracheal smooth muscle cells (23) but are in agreement with data obtained in cardiomyocytes, where forskolin-evoked cAMP accumulation and adenylyl cyclase activity were reduced by TGF-β1 (24).

Because the forskolin effect on cAMP accumulation and adenylyl cyclase activity was reduced by TGF-β1, it was not possible to draw a conclusion regarding functional responsiveness of M_2 muscarinic receptors after TGF-β1 treatment. Unlike forskolin, cAMP accumulation evoked by PGE_2 was not altered by TGF-β1. There was no significant difference in the amount of cAMP generated by the combination of carbachol and PGE_2 compared with PGE_2 alone in either control or TGF-β1-treated cells, confirming the functional desensitization of M_2 muscarinic receptors. The discrepancy in results obtained with TGF-β1 for forskolin and PGE_2 stimulation of intracellular cAMP and adenylyl cyclase activity is unclear but may reflect differences in the adenylyl cyclase isoforms recruited by the stimuli and their differential sensitivity to TGF-β1 (25, 26). Indeed, there is evidence of at least eight isoforms of adenylyl cyclase, which have been classified into three subfamilies based on sequence similarities. These isoforms differ in tissue distribution, subcellular location, and, most important, mode of regulation (25–27).

To gain insight into the mechanism by which TGF-β1 induced m2 receptor down-regulation, we investigated the effect of the potent protein synthesis (cycloheximide) and RNA polymerase (actinomycin D) inhibitors on receptor down-regulation. Cycloheximide abolished the reduction in the steady state levels of m2 mRNA induced by TGF-β1, whereas alone it had no effect on m2 receptor mRNA levels. This suggests that the synthesis of at least one protein is required for receptor down-regulation. This factor, or a sub-

sequently induced protein, might alter transcription of the m2 receptor gene directly or change the degradation rate of the m2 receptor mRNA. Recently, Lee *et al.* (28) identified a 261-base region in the 3′-untranslated region of the muscarinic m1 receptor gene that is essential in m1 mRNA destabilization. Actinomycin D was used to measure changes in the half-life of m2 receptor mRNA after TGF-β1 addition. These data indicate that the reduction in muscarinic m2 mRNAs after TGF-β1 treatment was not due to a change in the mRNA stability but rather reflects a decrease in the transcription rate of the gene. This was confirmed with nuclear RNA run-on assays. Fig. 6 shows that the basal expression of m2 receptor gene could be markedly reduced after TGF-β1 treatment. Thus, the reduction in m2 mRNA could be solely accounted for by transcriptional inhibition by this cytokine. This result agrees with the transcriptional regulation by TGF-β1 of other genes, including the matrix-degrading metalloprotease transin (29).

We have shown that *de novo* protein synthesis is required for receptor down-regulation. The nature of the protein(s) induced by TGF-β1 activation is not known. However, TGF-β1 is known to induce DNA binding activity of a number of proteins, including transcription factors such as activator protein-1. In the mouse hepatoma cell line BWTG3, TGF-β1 induced a rapid increase in Jun-B and Fos-B mRNAs (30). Furthermore, these authors concluded that this induction may participate in down-regulation of albumin synthesis. Similar results were obtained in mouse 3T3 fibroblasts (which are growth stimulated by TGF-β1) and in lung ML-CCL-64 epithelial cells (which are growth inhibited by TGF-β1), in which the c-Jun and Jun-B were induced by TGF-β1 (31). Electrophoretic mobility shift assays with nuclear extracts from HEL 299 cells treated with TGF-β1 showed a rapid increase in DNA-binding activity to the activator protein-1 site (data not shown). This suggests that TGF-β1 may alter the gene expression of the muscarinic m2 receptor subtype through increased expression of activator protein-1 binding proteins in a manner analogous to that described for the negative regulation of the transin/stromelysin gene expression by TGF-β1 (32). It was shown that TGF-β1 induces c-Fos and c-Jun proteins that bind the TGF-β1 inhibitory element (the consensus sequence GNNTTGGTGA is present in all genes repressed by TGF-β1) to induce transcriptional repression. However, direct interactions of these transcription factors with the m2 receptor gene promoter cannot be measured directly as no sequence data are available.

TGF-β1 signal transduction pathways are largely unknown. Cross-linking and immunoprecipitation studies have shown that the functional TGF-β1 receptor is a heteromeric complex (2). Because TGF-β1 receptors are transmembrane serine/threonine kinases, it is reasonable to suggest that stimulation of these receptors can lead to the activation of variety of protein kinases. The resulting phosphoproteins are likely to be associated with regulation of muscarinic m2 receptor gene through phosphorylation and then activation of transcription factors (33, 34). This is supported by evidence suggesting that type I and type II receptors for TGF-β fail to restore any response to TGF-β when a mutation in the ATP-binding site (which destroys the kinase activity) of either receptor was introduced (35).

In summary, we demonstrated that TGF-β1, a serine/threonine kinase receptor ligand, decreased the adenylyl cyclase activity and induced down-regulation of M_2 muscarinic receptor protein and mRNA through a reduction in the rate of m2 receptor gene transcription. Furthermore, we showed that protein synthesis is required for receptor down-regulation. We therefore suggest that TGF-β1, expressed and released by many cells within the airways, may modulate cholinergic function by reducing gene expression of M_2 receptors.

Acknowledgments

We thank Drs. I. M. Adcock and M. A. Giembycz for helpful discussion throughout the study. We are grateful to Dr. N. J. Buckley (National Institute for Medical Research, London, UK) for the gift of human muscarinic receptor cDNAs, to Hilary Lewis for her assistance with cell culture, and to Paul Seldon for printing the photographs.

References

1. Lyons, R. M., and H. L. Moses. Transforming growth factors and the regulation of cell proliferation. *Eur. J. Biochem.* **187**:467–473 (1990).
2. Derynck, R. TGF-β-receptor-mediated signalling. *Trends Biochem. Sci.* **19**:548–553, 1994.
3. Massagué, J., and B. Like. Cellular receptors for type β transforming growth factor. *J. Biol. Chem.* **260**:2636–2645 (1985).
4. Wakefield, L. M., D. M. Smith, T. Masui, C. C. Harris, and M. B. Sporn. Distribution and modulation of the cellular receptor for transforming growth factor-*beta*. *J. Cell Biol.* **105**:965–975 (1987).
5. Massagué, J. The transforming growth factor-β family. *Annu. Rev. Cell Biol.* **6**:597–641 (1990).
6. Kelley, J. *Cytokins in the Lung*. Marcel Dekker, New York, 101–137 (1993).
7. Ignotz, R. A., T. Endo, and J. Massagué. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-β. *J. Biol. Chem.* **262**:6443–6446 (1987).
8. Heino, J., R. A. Ignotz, M. E. Hemler, C. Crouse, and J. Massagué. Regulation of cell adhesion receptor by transforming growth factor-β. *J. Biol. Chem.* **264**:380–388 (1989).
9. Ignotz, R. A., J. Heino, and J. Massagué. Regulation of cell adhesion receptor by transforming growth factor-β. *J. Biol. Chem.* **264**:389–392 (1989).
10. Leof, E. B., J. A. Proper, A. S. Goustin, G. D. Shipley, P. E. DiCorleto, and H. L. Moses. Induction of c-*sis* mRNA and activity similar to platelet-derived growth factor by transforming growth factor-β: a proposed model for indirect mitogenesis involving autocrine activity. *Proc. Natl. Acad. Sci. USA* **83**:2453–2457 (1986).
11. Pietenpol, J., R. W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R. M. Lyons, M. R. Pittelkow, K. Münger, P. M. Howly, and H. L. Moses. TGF-β1 inhibition of c-*myc* transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61**:777–785 (1990).
12. Wahl, S. M., D. A. Hunt, L. M. Wakefield, N. McCartney-Francis, L. M. Wahl, A. B. Roberts, and M. B. Sporn. Transforming growth factor type β induces monocyte chemotaxis and growth factor production. *Proc. Natl. Acad. Sci. USA* **84**:5788–5792 (1987).
13. Jackson, B. A., R. H. Goldstein, R. Roy, M. Cozzani, L. Taylor, and P. Polgar. Effects of transforming growth factor-β and interleukin-1β on expression of cyclooxygenase 1 and 2 and phospholipase A_2 mRNA in lung fibroblasts and endothelial cells in culture. *Biochem. Biophys. Res. Commun.* **197**:1465–1474 (1993).
14. Hulme, E. C., N. J. M. Birdsall, and N. J. Buckley. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **30**:633–673 (1990).
15. Caulfield, M. P. Muscarinic receptors, characterization, coupling and function. *Pharmacol. Ther.* **58**:319–379 (1993).
16. Rousell, J., E.-B. Haddad, J. C. W. Mak, and P. J. Barnes. Transcriptional down-regulation of m2 muscarinic receptor gene expression in human embryonic lung (HEL 299) cells by protein kinase C. *J. Biol. Chem.* **270**:7213–7218 (1995).
17. Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol Chem.* **193**:265–275 (1951).
18. Haddad, E.-B., J. C. W. Mak, and P. J. Barnes. Characterization of [^{3}H]Ba 679 BR, a slowly dissociating muscarinic antagonist, in human lung: radioligand binding and autoradiographic mapping. *Mol. Pharmacol.* **45**: 899–907 (1994).
19. Haddad, E.-B., J. Rousell, J. C. W. Mak, and P. J. Barnes. Long-term carbachol treatment-induced down-regulation of muscarinic M_2 receptors but not m2 receptor mRNA in a human lung cell line. *Br. J. Pharmacol.* **116**:2027–2032 (1995).
20. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).

21. Greenberg, M. E., and E. B. Ziff. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (Lond.)* **311**:433–438 (1984).
22. Haddad, E.-B., J. Rousell, and P. J. Barnes. Muscarinic M_2 receptor synthesis: study of receptor turnover with propylbenzilylcholine mustard. *Eur. J. Pharmacol.* **290**:201–205 (1995).
23. Nogami, M., D. J. Romberger, S. I. Rennard, and M. L. Toews. TGF-β1 modulates β-adrenergic receptor number and function in cultured human tracheal smooth muscle cells. *Am. J. Physiol.* **266**:L187–L191 (1994).
24. Nair, B. G., Y. Yu, H. M. Rashed, H. Sun, and T. B. Patel. Transforming growth factor-β-1 modulates adenylyl cyclase signalling elements and epidermal growth factor signalling in cardiomyocytes. *J. Cell. Physiol.* **164**:232–239 (1995).
25. Iyengar, R. Molecular and functional diversity of mammalian Gs-stimulated adenylyl cyclases. *FASEB J.* **7**:768–775 (1993).
26. Taussig, R., and A. G. Gilman. Mammalian membrane-bound adenylate cyclases. *J. Biol. Chem.* **270**:1–4 (1995).
27. Cooper, D. M. F., N. Mons, and J. W. Karpen. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature (Lond.)* **374**: 421–424 (1995).
28. Lee, N. H., J. Earle-Hughes, and C. M. Fraser. Agonist-mediated destabilization of m1 muscarinic acetylcholine receptor mRNA: elements involved in mRNA stability are localized in the 3′-untranslated region. *J. Biol. Chem.* **269**:4291–4298 (1994).
29. Kerr, L. D., N. E. Olashaw, and L. M. Matrisian. Transforming growth factor-β1 and cAMP inhibit transcription of epidermal growth factor- and oncogene-induced transin RNA. *J. Biol. Chem.* **263**:16999–17005 (1988).
30. Beauchamp, R. D., H.-M. Sheng, J. Ishizuka, C. M. Townsend, and J. C. Thompson. Transforming growth factor (TGF)-β stimulates hepatic *jun*-B and *fos*-B proto-oncogenes and decreases albumin mRNA. *Ann. Surg.* **216**:300–308 (1992).
31. De Groot, R., and W. Kruijer. Transcriptional activation by TGF-β1 mediated by the dyad symmetry element (DSE) and the TPA responsive element (TRE). *Biochem. Biophys. Res. Commun.* **168**:1074–1081 (1990).
32. Kerr, L. D., D. B. Miller, and L. M. Matrisian. TGF-β1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence. *Cell* **61**:267–278 (1990).
33. Meek, D. W., and A. J. Street. Nuclear protein phosphorylation and growth control. *Biochem J.* **287**:1–15 (1992).
34. Hunter, T., and M. Karin. The regulation of transcription by phosphorylation. *Cell* **70**:375–387 (1992).
35. Massagué, J., L. Attisano, and J. L. Wrana. The TGF-β family and its composite receptors. *Trends Cell Biol.* **4**:172–178 (1994).

Send reprint requests to: Prof. Peter J. Barnes, Department of Thoracic Medicine, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY, UK. E-mail: p.j.barnes@ic.ac.uk