Signal transduction events involved in TPA downregulation of SP-A gene expression

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Submitted 1 December 2003; accepted in final form 26 January 2004

Miakotina, Olga L., and Jeanne M. Snyder. Signal transduction events involved in TPA downregulation of SP-A gene expression. Am J Physiol Lung Cell Mol Physiol 286: L1210–L1219, 2004. First published January 29, 2004; 10.1152/ajplung.00416.2003.—Surfactant protein A (SP-A), the most abundant pulmonary surfactant protein, plays a role in innate host defense and blocks the inhibitory effects of serum proteins on surfactant surface tension-lowering properties. SP-A mRNA and protein are downregulated by phorbol esters (TPA) via inhibition of gene transcription. We evaluated the TPA signaling pathways involved in SP-A inhibition in a lung cell line, H441 cells. TPA caused sustained phosphorylation of p44/42 mitogen-activated protein kinase (MAPK), p38 MAPK, and c-Jun-NH2-terminal kinase. An inhibitor of conventional and novel isoforms of protein kinase C (PKC) and two inhibitors of p44/42 MAPK kinase partially or completely blocked the inhibitory effects of TPA on SP-A mRNA levels. In contrast, inhibitors of conventional PKC-α and -β, stress-activated protein kinases, protein phosphatases, protein kinase A, and the phosphatidylinositol 3-kinase 3-kinase pathway had no effect on the TPA-mediated inhibition of SP-A mRNA. TPA also stimulated the synthesis of c-Jun mRNA and protein in a time-dependent manner. Inhibitors of the p44/42 MAPK signaling pathway and PKC blocked the TPA-mediated phosphorylation of p44/42 MAPK and the increase in c-Jun mRNA. We conclude that TPA inhibits SP-A gene expression via novel isoforms of PKC, the p44/42 MAPK pathway, and the activator protein-1 complex.

12-O-tetradecanoylphorbol-13-acetate; surfactant protein A; signal transduction; protein kinase C; mitogen-activated protein kinases; H441 cells; activator protein 1

SURFACTANT IS A LIPOPROTEIN COMPLEX that forms a monolayer at the air-aqueous interface in the pulmonary alveolus and serves as a surface tension-reducing agent that facilitates the re-expansion of alveoli during inspiration (50). Surfactant consists primarily of phospholipids (~80%), cholesterol (~10%), and the surfactant-associated proteins (SP-A, -B, -C, and -D) (~10%), which contribute to surfactant biophysical properties and also function in innate host defense mechanisms in the lung (7, 48). The surfactant proteins are regulated during development and by cytokines, hormones, and growth factors (48). TPA is a structural homolog of diacylglycerol (DAG) that mimics its activation of protein kinase C (PKC). This kinase influences cell growth and differentiation and is part of the signal transduction pathways of many growth factors and cytokines involved in regulating gene expression in the lung, including SP-A. Treatment of rabbits with TPA in vivo causes lung pathology similar to that observed in acute respiratory distress syndrome (ARDS) (28). Phorbol ester (TPA) has been shown to inhibit SP-A and SP-B gene expression in lung epithelial cells at the level of gene transcription within a few hours (34, 49). TPA treatment decreases the nuclear content of thyroid transcription factor-1 and hepatocyte nuclear factor-3 transcription factors, and this in turn inhibits SP-B gene transcription in H441 cells (25). TPA has been shown to downregulate the SP-A promoter via an activator protein (AP)-1 response element in the first intron of the SP-A genes (18). There are no data available concerning the cellular signaling mechanisms by which phorbol esters inhibit SP-A in epithelial cells.

PKC is a family of lipid-regulated serine/threonine protein kinases that is divided into three classes: conventional or classical, novel, and atypical PKCs. Conventional PKC isoforms are activated by Ca2+ and diacylglycerol (DAG); novel isoforms are only dependent on DAG, whereas atypical PKC isoforms are not stimulated by either Ca2+ or DAG. Phorbol esters and DAG bind and activate conventional and novel PKC isoforms with high affinity. Phorbol esters also bind nonkinase DAG/phorbol ester receptors (37). Activation of PKC impairs the barrier function of epithelia and, in the lung, increases endothelial permeability and can lead to pulmonary edema and respiratory distress syndrome (RDS) (5, 43). Several studies have documented the involvement of certain PKC isoforms, i.e., PKC-μ and Ca2+-phosphatidylyserine (PS)-dependent PKC, in surfactant secretion in primary cultures of alveolar type II cells (14, 39).

Different isoforms of PKC act via different signal transduction pathways to regulate gene expression, cell survival, etc. (21). Activated PKC isoforms generally transduce their intracellular signal via either mitogen-activated protein kinases (MAPK) pathways, usually p44/42 MAPK and c-Jun NH2-terminal kinase (JNK), or via MAPK-independent pathways, for example the phosphatidylinositol 3-kinase (PI 3-kinase) pathway (11, 23, 36, 44, 46). Downstream targets of phorbol esters that are involved in gene regulation include transcription factors such as the AP-1 complex, cyclic AMP binding protein (CREB), nuclear factor (NF)-κB, or Sp1 (9, 23, 46, 52).

In the present study, we characterized the signaling pathways involved in TPA-mediated inhibition of SP-A mRNA levels in lung epithelial cells (H441 cells). SP-A is regulated by hormonal effectors such as insulin and retinoic acid in a similar manner in human fetal lung explants and the H441 cell line (10, 13, 29, 30). Thus H441 cells are a good in vitro model for studying the effects of regulatory agents on SP-A mRNA levels...

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in lung epithelial cells. In the present study, we used several specific inhibitors of signal transduction pathways. We confirmed the effects of these inhibitors by evaluating changes in the phosphorylation state and/or total amount of the various signal transduction protein kinases and their substrates. Our results indicate that TPA activates PKC isoforms, which in turn activate p44/42 MAPK and stimulate the synthesis of c-Jun mRNA and protein. We found that inhibitors of PKC and p44/42 MAPK blocked both c-Jun gene expression and the TPA-induced inhibition of SP-A mRNA levels. Thus TPA probably decreases SP-A mRNA levels by activating novel PKCs and p44/42 MAPK, followed by an increase in c-Jun levels that inhibits SP-A gene transcription via an interaction with AP-1 site(s) in the SP-A genes.

MATERIALS AND METHODS

Cell culture and reagents. Human lung epithelial cells, NCI-H441 cells, were maintained in monolayer culture in RPMI 1640 medium that contained 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2_. Cells were passed weekly, and medium was changed every 3 days.

For most experiments, subconfluent cells (~70% confluence) were incubated in serum-free medium for 24 h before the experiment, pretreated with either signal transduction inhibitors or vehicle for 1 h, and then incubated with TPA, 5–10 nM, in the presence or absence of the signal transduction inhibitors for an additional 24 h. To evaluate the phosphorylation state of the MAPK family protein kinases, we incubated serum-deprived cells in the presence or absence of TPA for 15 min, 30 min, 2 h, and 16 h.

PD-98059 and U-0126 were purchased from New England Biolabs (Beverly, MA). SB-203580, SP-600125, GF-109203X, myristoylated protein kinase C [20–28] (PKCI), okadaic acid, and H-89 were purchased from BioMol (Plymouth Meeting, PA). TPA, wortmannin, LY-294002, and rapamycin were obtained from Sigma Chemical (St. Louis, MO). Stock solutions of PD-98059, U-0126, SB-203580, SP-600125, GF-109203X, okadaic acid, wortmannin, LY-294002, rapamycin, TPA, and H-89 were prepared in DMSO as 10 mM, 1 mM, 5 mM, 20 mM, 5 mM, 1 mM, 100 μM, 50 mM, 50 μM, 1 mg/ml, and 40 mM stocks, respectively, and stored at –80°C in aliquots. These inhibitors have been shown previously to be relatively reliable and specific inhibitors of signal transduction protein kinases (8, 33, 47).

Antibodies directed against phosphorylated protein kinases, p44/42 MAPK, p38 MAPK, and SAPK/JNK, as well as against phospho-activating transcription factor (ATF)-2, phospho-c-Jun, and total c-Jun were purchased from Cell Signaling (Beverly, MA). Antibodies against total p44/42 MAPK (ERK1), p38 MAPK, and JNK1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoblot analysis. Immunoblotting was performed as described previously (31). In short, control and treated cells were rinsed twice with ice-cold phosphate-buffered saline and then lysed in a buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl_2, 50 mM NaF, 5 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C followed by centrifugation at 12,000 g for 10 min. The supernatant, which contained the protein lysate, was boiled with an equal volume of 2× electrophoresis sample buffer (250 mM Tris·HCl, pH 6.8, 2% 2-mercaptoethanol, 4% SDS, 10% glycerol, and 0.006% bromphenol blue) for 5 min, and then equal amounts of protein from each condition were separated on 10% SDS polyacrylamide gels and immunoblotted according to the instructions of the antibody supplier. Phosphospecific antibodies directed against p44/42 MAPK, p38 MAPK, and SAPK/JNK, ATF-2, c-Jun, and an antibody directed against c-Jun were used at dilutions of 1:1,000, whereas ERK 1, p38 MAPK, and JNK1 antibodies were diluted 1:1,000, 1:500, and 1:200, respectively. Primary antibodies were visualized with polyclonal anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibodies at a 1:2,000 dilution. Immunoblots were subsequently treated with chemiluminescence reagent (ECL Western blotting detection system; Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to X-ray film.

Northern blot analysis. Control and treated cells were harvested, and total RNA was isolated, separated on formaldehyde-containing agarose gel, and transferred to a nitrocellulose membrane according to previously described methods (31). Human SP-A cDNA, human c-Jun, or c-Fos cDNAs were labeled with [α-32P]dCTP and hybridized to membranes that contained the immobilized total RNA. Membranes were then washed, dried, and exposed to X-ray film. Radioactive bands were scanned and quantitated with Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA). Intensities of reactive bands were corrected to intensities of correspondent 18S rRNA bands (31). Each experiment was performed at least three times unless otherwise stated.

Quantification of results and statistical analysis. Results were quantitated relative to the densitometric data in the control condition, which was made equal to one. To estimate the statistical significance of the results, one-way analysis of variance (ANOVA) followed by Dunnett’s test or unpaired t-test was used (51).

RESULTS

To evaluate the viability of the H441 cells in the presence of the various signal transduction inhibitors, we treated cells with the highest concentration used of U-0126, SB-203580, SP-600125, GF-109203X, PKCI, PD-98059, wortmannin, LY-294002, rapamycin, DMSO, or TPA (10 nM) for 24 h. Other plates were preincubated with okadaic acid or H-89 for 1 h, then media were discarded, and the cells were exposed to fresh media for an additional 24 h. Examination of the cells under phase-contrast microscopy and trypan blue exclusion, as well as by measurement of the lactate dehydrogenase level in the media for some agents, revealed no difference in cell viability in the treated cells vs. controls.

TPA inhibits SP-A mRNA levels. To choose an optimal TPA concentration that would inhibit SP-A mRNA levels in the H441 cells, we applied 0.1–30 nM TPA to the cells for 24 h and then measured the amount of human SP-A mRNA present in the treated vs. control cells. Figure 1 shows that TPA caused a dose-dependent decrease in SP-A mRNA levels in H441 cells with maximum inhibition of ~90% by 30 nM TPA and a half-maximal effect (IC_{50}) at ~3–5 nM.

Inhibitors of PKC partially block the TPA inhibition of SP-A mRNA levels. Phorbol esters are well-characterized activators of PKC (21). To determine whether PKC activation is involved in the inhibition of SP-A mRNA levels by TPA, two well-characterized PKC inhibitors, i.e., PKCI and GF-109203X, were used. PKCI is a selective inhibitor of two conventional PKC isoforms, α and β, whereas GF-109203X blocks a wide spectrum of PKCs, including α-, βI-, βII-, δ-, and ε-isozymes, i.e., it inhibits both conventional and novel PKC isoforms. As shown in Fig. 2A, PKCI had no effect on the TPA-induced inhibition of SP-A mRNA levels. In contrast, GF-109203X partially blocked the TPA inhibition of SP-A in a dose-dependent manner at concentrations of 0.05–0.5 μM (Fig. 2B). Thus TPA probably inhibits SP-A mRNA levels via the activation of novel PKC isoforms but not via activation of conventional PKC-α or -β.
TPA H441 cells were pretreated with 0.1–30 nM TPA for 24 h. Cells were harvested, total RNA was isolated, and Northern blot analysis for human SP-A mRNA was performed. Reactive bands were detected by exposure to X-ray film and quantitated by densitometry. Data are normalized to values in untreated control cells and presented as the means ± SE. *Statistically significant difference from untreated controls (ANOVA, Dunnett’s test, P < 0.05, n = 3).

Fig. 2. The effects of protein kinase (PK) CI and GF-109203X (GF), both inhibitors of PKC, on SP-A mRNA levels in the presence of TPA. H441 cells were incubated in the presence of TPA for 15 min, 30 min, 2 h, and 16 h, and the protein lysates were used for immunoblotting with phoshospecific antibodies. Figure 3 demonstrates that TPA up-regulated the phosphorylation of all three MAPK family protein kinases in a time-dependent manner. TPA increased the phosphorylation of p44/42 MAPK as early as after 15 min, and this 1.5- to 4-fold increase in phosphorylation over control levels lasted for at least 16 h (Fig. 3A). TPA also increased the phosphorylation of p38 MAPK by a maximum of ~10-fold over controls at the 30-min time point (Fig. 3B). This increased level of phosphorylation also lasted for at least 16 h. TPA induced a 1.5- to 3-fold increase in the phosphorylation of SAPK/JNK with a temporal pattern that was similar to the increase in p44/42 MAPK phosphorylation (Fig. 3C). The effect was significant at 15 and 30 min. The addition of fresh media to the cells at the start of the experiment may have caused increased phosphorylation of p44/42 MAPK and SAPK/JNK during the initial 15-min incubation period. The level of phosphorylated enzyme in controls declined to baseline levels thereafter. This pattern has been observed in previous studies (2, 31). TPA had no effect on the total amount of p44/42 MAPK, p38 MAPK, or JNK1 proteins in the cells (data not shown).

**Inhibition of p44/42 MAPK blocks TPA-induced decrease in SP-A mRNA synthesis.** TPA signaling frequently involves the activation of the PKC-Ras-p44/42 MAPK pathway (12, 21, 40). The results of our initial experiments were suggestive that the classical MAPK pathway (p44/42 MAPK) is activated by TPA in H441 cells (Fig. 3A). To further test this hypothesis, we used U-0126, an inhibitor of MAPK kinase (MEK) 1/2, a dual protein kinase upstream from p44/42 MAPK. Cells were treated with U-0126 (0.01–3 μM) in the presence or absence of TPA (5 nM) for 24 h. Figure 4A shows that U-0126 dramati-
We have shown that TPA causes an inhibitory effect of TPA on SP-A mRNA levels. This effect is mediated by the PI 3-kinase pathway probably does not mediate the inhibitory effect of TPA on SP-A mRNA levels. JNK may be involved in the regulation of basal SP-A mRNA levels, however.

The PI 3-kinase pathway is not involved in SP-A inhibition by TPA. The PI 3-kinase signaling pathway may also be involved in TPA mechanisms of action (11). To examine whether this signaling pathway plays a role in TPA inhibition of SP-A mRNA levels, we evaluated the effects of three inhibitors of the PI 3-kinase pathway on the TPA-mediated decrease in SP-A mRNA levels. Wortmannin and LY-294002 are two structurally and mechanistically different inhibitors of PI 3-kinase with IC50 of ~5 nM and 1.4 μM, respectively (42). Rapamycin inhibits the mammalian target of rapamycin, a downstream PI 3-kinase effector that activates p70 S6 kinase (42). None of these PI 3-kinase pathway inhibitors modified the TPA-induced decrease in SP-A mRNA levels (Table 1). In addition, none of them had an effect on basal SP-A mRNA levels. We conclude that the PI 3-kinase pathway probably does not mediate the inhibitory effect of TPA on SP-A mRNA levels.

PKA or protein phosphatase does not affect TPA inhibition of SP-A. Previous studies have indicated that either protein phosphatases or PKA may modulate TPA signaling in cells (16, 20, 26, 40). To evaluate this possibility, we treated H441 cells with TPA plus or minus okadaic acid (1–200 nM), an inhibitor of protein phosphatase 2A, or H-89 (0.1–5 μM), a PKA inhibitor, and measured the levels of human SP-A mRNA. Neither okadaic acid nor H-89 had an effect on the TPA-mediated inhibition of SP-A mRNA levels (Fig. 6, A and B). In addition, neither inhibitor affected basal SP-A mRNA levels.

TPA stimulates the synthesis of c-Jun mRNA and protein. TPA can modify gene expression via AP-1, a transcription factor that consists of homo- or heterodimers of several proteins, i.e., c-Jun, JunB, JunD, c-Fos, or ATF-2 (22). Hoover...
and coworkers (18) have reported that TPA inhibits SP-A gene expression in H441 cells via an AP-1 response element located in the first intron of the human SP-A genes. We measured the levels of c-Jun and c-Fos mRNA in H441 cells at various times after TPA application and observed a significant increase in c-Jun mRNA levels after a 1-h incubation with TPA (Fig. 7A). In contrast, c-Fos mRNA was not detectable in the H441 cells in either the control or TPA-treated cells (data not shown). We also measured the total amount of c-Jun protein in TPA-treated and control cells at various time points. The maximal amount of c-Jun protein was detected after 2 h of incubation with TPA, whereas in control cells, c-Jun protein levels tended to decline over time (Fig. 7B). TPA inhibited SP-A mRNA levels during the 6- to 24-h time period (Fig. 7C). These results show that c-Jun mRNA and protein synthesis precede the inhibition of SP-A mRNA by TPA.

**Inhibition of phospho-p44/42 MAPK correlates with inhibition of c-Jun gene expression.** In further experiments, we evaluated the phosphorylation state of MAPK family protein kinases and their substrates in the presence of PKC and MAPK inhibitors and, in parallel experiments, examined the effect of these inhibitors on the TPA-mediated increase in c-Jun gene expression. TPA, when added alone, increased the phosphorylation of p44/42 MAPK, p38 MAPK, and JNK (Fig. 8A). TPA also increased the phosphorylation of ATF-2 and c-Jun, two proteins that can form AP-1 complexes and are targets of p38 MAPK and JNK (Fig. 8A). TPA increased the levels of c-Jun mRNA (Fig. 8B). Inhibition of p44/42 MAPK phosphorylation by U-0126 was accompanied by decreased JNK phosphorylation, as well as decreased phosphorylation of ATF-2 and c-Jun, both substrates of JNK. These effects correlated well with the complete inhibition of TPA-stimulated c-Jun gene expression by U-0126. An inhibitor of PKC, GF-109203X, partially blocked the TPA-induced increase in the phosphorylation of p44/42 MAPK and inhibited the TPA-mediated increase in c-Jun gene expression. Unexpectedly, GF-109203X completely blocked the TPA-induced phosphorylation of p38 MAPK. An α- and β-p38 MAPK inhibitor, SB-203580, did not affect the TPA-mediated increase in phosphorylated p38 MAPK or ATF-2 and partially inhibited the increased phosphorylation of c-Jun. An inhibitor of JNK, SP-600125, completely blocked the increased phosphorylation of JNK and its substrate, c-Jun. However, neither of the SAPK inhibitors, SB-203580 or SP-600125, had an effect on TPA-stimulated c-Jun gene expression (Fig. 8B). The total amounts of p44/42 MAPK, p38 MAPK, or JNK1 were not affected by any of the inhibitors (data not shown). Together these findings show that the TPA-stimulated c-Jun gene expression is probably mediated via the activation of PKC upstream of p44/42 MAPK activation. In contrast, activation of the two SAPKs, p38 MAPK and JNK, and subsequent phosphorylation of their substrates, ATF-2 and c-Jun, are probably not involved in the TPA-induced c-Jun gene expression.

**DISCUSSION**

PKC is a family of lipid-regulated serine/threonine protein kinases that is divided into three classes: conventional (α-, βI-, βII-, and γ-isozymes), novel (δ, ε, η, θ, and μ) and atypical (ζ and η). Conventional PKC isozymes are activated by Ca^{2+} and DAG, novel isozymes are only DAG dependent, whereas atypical isozymes are stimulated by neither Ca^{2+} nor DAG. The activity of PKC isozymes is regulated by phosphorylation, localization, and substrate specificity. Phorbol esters and DAGs bind to cysteine-rich domains with high affinity and activate conventional and novel PKC isozymes in the presence of PS. Phorbol esters are also known to bind to nonkinase, DAG/phorbol ester receptors (37, 38).

Rat type II alveolar epithelial cell lines, both adult and newborn, contain PKC-α, -βI, -βII, -δ, -η, -ζ, -θ, and -μ (15). PKC isozymes α, βI, δ, and ζ have been shown to be present in...
Table 1. Effects of inhibitors of the phosphatidylinositol 3-kinase signal transduction pathway on SP-A mRNA levels in the presence or absence of TPA in H441 cells

<table>
<thead>
<tr>
<th>Relative Amount of SP-A mRNA</th>
<th>Vehicle</th>
<th>LY-294002, 5 nM</th>
<th>Wortmannin, 200 nM</th>
<th>Rapamycin, 20 nM</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>0.99±0.11</td>
<td>0.99±0.11</td>
<td>0.97±0.13</td>
</tr>
<tr>
<td>TPA, 10 nM</td>
<td>0.35±0.02*</td>
<td>0.34±0.07*</td>
<td>0.31±0.06*</td>
<td>0.33±0.06*</td>
</tr>
<tr>
<td>TPA, 20 nM</td>
<td>0.35±0.02*</td>
<td>0.34±0.07*</td>
<td>0.31±0.06*</td>
<td>0.33±0.06*</td>
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Data are the result of 3 experiments, were normalized to controls, and are presented as means ± SE. H441 cells were pretreated with signal transduction inhibitors LY-294002 (5 nM), wortmannin (200 nM), rapamycin (20 nM), or vehicle for 1 h and subsequently exposed to TPA (10 nM) plus or minus the inhibitors for an additional 24 h. Cells were then harvested and subjected to Northern blot analysis for human surfactant protein (SP)-A mRNA. *Significantly different from the control condition (ANOVA and Dunnett’s test, P < 0.05).

H441 cells (2). In rat type II cells, the phorbol ester TPA activates PKC-α, -βI, -βII, -δ, and -θ and has been shown to target PKC-α to a membrane fraction (14). In fetal rabbit type II cells, TPA causes the translocation of PKC activity to a lamellar body fraction (39). TPA stimulates PKC activity associated with a membrane fraction rather than the cytosolic fraction of lung epithelial cell homogenates (2).

Activation of PKC-α by TPA increases the permeability of cellular tight junctions, which impairs the barrier function of epithelia and results in the movement of growth factors from luminal/apical fluids into lateral, intracellular spaces in epithelial cell lines and to interstitial fluid spaces in colon cancers (5). In acute lung injury, the activation of PKC, mainly PKC-α, can increase endothelial permeability, which can in turn lead to pulmonary edema and RDS. TPA, which is a potent activator of PKC, is used experimentally to simulate characteristics of acute lung injury, edema or RDS (43).

PKC isoforms are characterized by different signaling pathways that modulate gene expression and lead to terminal events such as neuronal differentiation, monocytic differentiation, and the induction of apoptosis (19, 27, 45). In two previous studies, it was shown that phorbol esters inhibit the gene expression of SP-A and -B in a dose- and time-dependent manner (34, 49). In H441 cells and human fetal lung explants, TPA profoundly decreases the SP-A transcription rate within 6–8 h of exposure; this effect lasts for 24 h (34, 49). However, there is no information available concerning the intracellular signaling pathways that are activated by TPA to inhibit surfactant protein gene expression.

To verify the involvement of certain PKC isoforms in TPA-mediated SP-A inhibition, we used two inhibitors of PKC. i.e., GF-109203X and PKC I. PKC I inhibits only conventional PKC-α and -β, whereas GF-109203X inhibits conventional and novel PKC isoforms, i.e., α, βI, βII, δ, and ε. Although PKC I failed to modify the TPA inhibition of SP-A, GF-109203X was able to partially restore SP-A mRNA levels decreased by exposure to TPA. We conclude that novel PKCs rather than the conventional α- and β-isoforms mediate the TPA inhibition of SP-A mRNA levels.

Several studies are suggestive that TPA activates protein kinases of the MAPK family (1, 2, 19, 41). Awasthi and King (2) have previously reported TPA-mediated activation of p44/42 MAPK and p38 MAPK in H441 cells. In the present study, we confirmed that the phosphorylation/activation of p44/42 MAPK, p38 MAPK, and JNK are increased by TPA in a time-dependent manner, reaching maximum after 15–30 min of application. To determine whether these protein kinases are involved in the TPA-mediated inhibition of SP-A mRNA levels, we used various inhibitors of the MAPKs. We found that neither an α- and β-p38 MAPK inhibitor, SB-203580, nor a JNK inhibitor, SP-600125, modified the TPA-mediated inhibition of SP-A mRNA levels. Because SB-203580 only inhibits the α- and β-isoforms of p38 MAPK, and SB-203580 did not inhibit the TPA-induced phosphorylation of ATF-2, it is possible that the H441 cells express the γ- or δ-forms of p38 MAPK. Further investigation will be necessary to evaluate whether the SB-203580-insensitive isoforms of p38 MAPK,
Fig. 6. The effects of a protein phosphatase inhibitor, okadaic acid (Ok), and a PKA inhibitor, H-89, on the TPA-mediated decrease in SP-A mRNA levels. H441 cells were preincubated with okadaic acid (1–200 nM) or H-89 (0.1–5 µM) for 1 h, then the media were changed, and the cells were incubated with TPA (10 nM) for an additional 24 h. Untreated cells (controls, no inhibitors or TPA) and treated cells were harvested and then RNA was isolated and used in Northern blots for human SP-A mRNA. Top: representative Northern blots and the ethidium bromide-stained rRNA of the corresponding samples. Bottom: densitometric data from several independent experiments presented as means ± SE (n = 2–5).

*Significant difference from untreated controls, which were made equal to 1 (ANOVA, Dunnett’s test, P < 0.05). A: Okadaic acid had no significant effect on either basal or TPA-inhibited SP-A mRNA levels at any concentration tested. B: H-89 had no effect on the TPA-induced decrease in SP-A mRNA levels. H-89 also had no significant effect on basal SP-A mRNA levels.

Fig. 7. TPA stimulates synthesis of c-Jun mRNA and protein in time-dependent manner. H441 cells were exposed to TPA (10 nM) for 5 min (A), 15 min, 1 h, 2 h, 6 h, and 24 h. The cells were harvested, and RNA was isolated and used in Northern blot analysis for human c-Jun mRNA (A) and SP-A mRNA (C). In another experiment, H441 cells were incubated with TPA (10 nM) for 15 min, 30 min, 2 h, and 16 h and harvested, and immunoblotting was performed for total c-Jun protein (B). Representative blots are shown on the left, and densitometric data from several independent experiments are depicted on the right. Data were normalized to values in untreated controls at the zero time point (A and C) or the 15-min time point (B). A: TPA increased c-Jun mRNA levels with maximum levels achieved after a 1-h incubation. *Significant difference from untreated controls at the zero time point (means ± SE, ANOVA, Dunnett’s test, P < 0.05, n = 4). B: TPA increased the amount of c-Jun protein in time-dependent manner. The maximal amount of c-Jun protein was detected after a 2-h incubation with TPA. In the untreated control cells, the amount of c-Jun protein tended to decrease over time. *Significant difference from respective untreated controls (unpaired t-test, P < 0.05, n = 3). C: TPA inhibited SP-A mRNA levels with maximal effect at the 24-h time point. Data from 2–3 experiments are expressed as means ± SE.
Fig. 8. Effect of signal transduction inhibitors on the phosphorylation of signal transduction protein kinases and their substrates and on the levels of c-Jun mRNA. H441 cells were pretreated with U-0126 (U0, 1 μM) and then exposed to TPA (10 nM) for an additional 30 min. Cells were harvested, and protein lysates used for immunoblotting using phosphospecific antibodies directed against p44/42 MAPK, p38 MAPK, JNK, activating transcription factor (ATF)-2, and c-Jun. In other experiments, cells pretreated with the indicated inhibitors or vehicle (controls) were incubated with TPA (10 nM) for an additional 30 min. Cell lysates were subjected to Western blot analysis and scanned by automatic laser densitometry.

**A**. Representative immunoblots from 2 experiments are shown for phospho-p44/42 MAPK, phospho-p38 MAPK, phospho-JNK, phospho-ATF-2, and phospho-c-Jun. The TPA-mediated increase in phospho-p44/42 MAPK was inhibited by U-0126 and GF-109203X; the increase in phospho-p38 MAPK was inhibited by GF-109203X but was not inhibited by SB-203580 or SP-600125. In contrast, when we used two inhibitors of the p44/42 MAPK pathway, i.e., PD-98059 and U-0126, which block MEK1 or MEK1/2, respectively, the TPA-mediated inhibition of SP-A mRNA levels was abolished. We conclude that p44/42 MAPK pathway plays a major role in transducing the TPA inhibitory signal to the SP-A gene.

**B**. Relative amount of c-Jun mRNA. Data are presented as means ± SE. *Significant difference from untreated controls, which were made equal to 1 (ANOVA, Dunnett’s test; \( P < 0.05, n = 3 \)). TPA-induced c-Jun gene expression was inhibited by U-0126 and GF-109203X but was not inhibited by SB-203580 or SP-600125.

Several other signal transduction pathways have been reported to be involved in TPA-mediated regulation of gene expression. These include the PI 3-kinase pathway, protein phosphatases, and the PKA pathway (3, 11, 16, 20, 26, 40). Our experimental results using multiple inhibitors of each of these signaling pathways rule out the possibility that any of them plays an important role in the TPA-mediated inhibition of SP-A mRNA levels.

In our study, TPA affected SP-A gene expression after prolonged exposure, for 24 h. TPA causes permanent activation of PKC and could potentially cause a depletion in PKC as a result of dephosphorylation and proteolysis (32). However, the low levels of TPA, 10 nM, used in our study are much less than the TPA levels required to deplete PKC in other studies, for instance, 200 nM in H441 cells (2), 1 μM in rat pituitary cells (6), or 2.5 μM in bovine luteal cells (4). In addition, the PKC isoforms that are most sensitive to depletion are the conventional isoforms, PKC-α and -β, which are probably not involved in inhibition of SP-A gene expression by TPA (24). Okadaic acid, which prevents dephosphorylation of PKC, failed to reverse the TPA inhibition of SP-A in the present study (16). Inhibitors of PKC usually cause the same effect as a depletion of PKC. In our study, both PKC inhibitors we used, GF-109203X and PKCl, added alone, did not affect SP-A mRNA levels. Finally, in the study of Awasthi and King (2), in H441 cells, PKC depletion resulted in fully inactivated p44/42 MAPK and partially inactivated p38 MAPK. In contrast, in our study, prolonged exposure of the H441 cells to low levels of TPA resulted in the activation of the three MAPKs to levels three- to fourfold above the controls. We conclude that PKC depletion is probably not a significant factor in the prolonged TPA-mediated inhibition of SP-A mRNA levels at low levels of the stimulus.
Downstream nuclear events in TPA signaling pathways leading to the regulation of gene expression include the activation of AP-1 complex, NF-κB, the CREB family of proteins, or Sp1 (9, 23, 46, 52). TPA causes organ-specific stimulation of AP-1 activity and phosphorylation of p44/42 and p38 MAPKs in transgenic mice that express an AP-1 luciferase reporter gene (53). Pryhuber and coworkers (35) showed that induction of NF-κB binding activity was not involved in the TPA-mediated inhibition of SP-A gene expression in H441 cells. Hoover and coworkers (18) have characterized a TPA-sensitive AP-1 binding element in the first intron of human SP-A genes and speculated that this response element binds an AP-1 complex consisting of a c-Jun homodimer. We found that TPA increases the expression of an early responding gene, c-Jun, with maximum effect at the 1-h time point followed by increase in the synthesis of c-Jun protein at the 2-h time point. c-Fos mRNA was not detectable in the cells. SP-A mRNA levels declined only after a 6-h exposure to TPA. In agreement with the hypothesis that TPA acts via c-Jun to decrease SP-A mRNA levels, we found that exposure of the H441 cells to U-0126, an MEK1/2 inhibitor, and GF-109203X, a PKC inhibitor, both of which block the inhibitory effects of TPA, decreased c-Jun mRNA levels. We conclude that TPA probably inhibits SP-A gene expression via a specific PKC isoform(s) that activates the p44/42 MAPK signaling pathway and increases c-Jun synthesis.

ACKNOWLEDGMENTS

The authors thank Jean Gardner for typing this manuscript.

GRANTS

The research was supported by National Institutes of Health Grants HL-50050 and DK-25295.

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