Inhibition of E-selectin Gene Transcription through a cAMP-dependent Protein Kinase Pathway*

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Cytokines induce the expression of E-selectin, VCAM-1, and ICAM-1 on human umbilical vein endothelial cells (HUVECs). We show that expression of these surface proteins is differentially affected by cAMP. Increased cAMP levels decrease E-selectin and VCAM-1 but increase ICAM-1 expression. We demonstrate by mRNA half-life analysis and nuclear run-on assays that the cAMP repression of E-selectin occurs at the transcription level. This effect is abolished by protein kinase A inhibition, suggesting that repression is mediated by protein kinase A-driven phosphorylation. We found that a minimal E-selectin promoter sequence necessary to confer cytokine inducibility is also sufficient to mimic the cAMP effect in transfected HUVECs. Previously we characterized two regions (NF-xB and NF-ELAM1) of the minimal promoter that bind transcription factors necessary for E-selectin induction. Increased cAMP did not alter the binding of the complexes formed on either the NF-xB or NF-ELAM1 site. In contrast, in interleukin-1-treated HUVECs transactivity due to an NF-xB site is reduced by elevated cAMP. Increased cAMP in HUVECs appears to induce a protein kinase activity that reduces the cytokine signal for E-selectin and VCAM-1 expression. The reduction in signal may occur through an inhibitory phosphorylation of one or more of the factors responsible for regulating E-selectin expression.

Recent advances in understanding the inflammatory response have derived from the identification and characterization of the cellular adhesion molecules which determine cell-cell interactions. Indeed, the discovery that the endothelium expresses an array of adhesion molecules whose roles include attracting, binding, and allowing transmigration of leukocytes into sites of tissue inflammation has been a major contribution to the mechanisms of inflammation (see Refs. 1–4 for reviews). During the inflammatory response cytokine signals induce differential expression on the endothelium of the adhesion molecules ICAM-1 and VCAM-1, as well as the selectins P-selectin and E-selectin. While P-selectin is released from intracellular storage granules for a rapid and short response to cytokines (5), increased expression of E-selectin, ICAM-1, and VCAM-1 is transcriptionally regulated (6–9). In particular, cytokine induction of E-selectin expression is due to a very large increase in gene transcription rising from nearly undetectable levels prior to cytokine treatment (10).

Two principal transcription factors involved in E-selectin promoter up-regulation have been elucidated. They are NF-xB (10–12) and members of the transcription activating factor (ATF)1 family (13–15) which cooperate on the promoter to induce gene expression. In endothelial cells activation of NF-xB by cytokine induction appears to follow the established pathway of release from cytoplasmic sequestration to appear in nuclei where it binds to the E-selectin promoter (16, 17). Two NF-xB complexes have been identified that bind to two distinct sites on the E-selectin promoter, both containing subunits serologically related to p50 and p65 (18, 19). In contrast, DNA binding of ATFs to the E-selectin promoter element is constitutive (13). However, transcription ceases a few hours after cytokine induction, despite the continuing presence of NF-xB and ATFs, resulting in a transient expression of E-selectin (6, 10, 20).

Cytokines and bacterial lipopolysaccharide are physiological inducers of E-selectin. However, the phorbol ester PMA is also capable of inducing the expression of E-selectin as well as other adhesion molecules (21). While phorbol esters principally activate protein kinase C (22–24), the second messenger signal appears to induce a protein kinase activity that reduces the cytokine signal for E-selectin and VCAM-1 expression. The reduction in signal may occur through an inhibitory phosphorylation of one or more of the transcription factors necessary for gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by collagenase treatment. HUVECs were serially passaged (at 1:3 split ratio) and maintained using medium MCDB 131 supplemented with 2% fetal bovine serum, hydrocortisone (1 ng/ml), epidermal growth factor (10 ng/ml), bovine brain extract containing heparin, gentamycin, and amphotericin B (Clonetics Corp., San Diego, CA). Tissue culture dishes were pre-coated with human fibronectin (Boehringer Mannheim; 1 μg/cm²). Experiments were performed using HUVECs at passage 3–5.

Flow-cytometry Determination of E-selectin, VCAM-1, and ICAM-1—HUVECs were grown to confluence in 5-cm Petri dishes and then treated for 4 h in serum-free M199. For longer incubations a complete media change was performed.

1 The abbreviations used are: ATF, transcription activating factor; CAT, chloramphenicol acetyl transferase; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorter; H-88, N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide; H-89, N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide; HUVEC, human umbilical vein endothelial cell; IL, interleukin; NF-xB, nuclear factor xB; PKA, protein kinase A; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; CREB, cAMP response element binding factor.
CLONOTICS medium containing 2% fetal bovine serum was used. Cells were collected with the help of a rubber policeman in phosphate-buffered saline/EDTA (1 mM) pre-warmed at 37 °C. Cells were collected by spinning 5 min, 1,000 rpm at 4 °C, and re-suspended in phosphate-buffered saline + 1% bovine serum albumin + 0.1% NaN₃. Cells (4 × 10⁵ cells/ml) were then incubated for 30 min at 4 °C in the presence of the first monoclonal antibody, washed, and incubated for a further 15 min at 4 °C in the presence of a second antibody, anti-mouse IgG conjugated to fluorescein isothiocyanate. The cells were washed in PBS + 1% bovine serum albumin and submitted to FACS analysis. Data are expressed as mean fluorescence intensity or as percentage inhibition.

RESULTS

Effect of Forskolin on Intracellular cAMP Levels in Endothelial Cells—HUVECs were collected with trypsin, washed, re-suspended in serum-free culture medium, aliquotted at 2.5 × 10⁶ cells/tube, incubated at 37 °C in the presence of O₂, and treated with inducers. The incubation was stopped by addition of cold trichloroacetic acid (5% final), and the cells were lysed by freeze-thawing. After spinning at 13,000 rpm for 15 min at 4 °C, the supernatants were transferred to new tubes and extracted three times with 5 volumes of water-saturated ether. The aqueous phase was lyophilized, and the dried pellets were dissolved in 50 µl of Tris-EDTA (50 mM, pH 7.5) buffer. The cAMP concentration was measured using a radioimmunoassay kit (Amerham Corp.) as described by the manufacturer.

Preparation of RNA and Northern Blot Analysis—Total RNA was isolated from confluent HUVECs using the guanidine isothiocyanate/CsCl procedure (25). Total RNA (20 µg/lane) was fractionated on a 1% agarose gel containing 2.2 M formaldehyde, blotted onto nylon membrane filters (Gene Screen Plus, DuPont-NEN), and hybridized with a nick-translated E-selectin-1, ICAM-1, or human β-actin [32P]-labeled cDNA probes (26). Filters were washed twice for 30 min at 37 °C in 2 × SSC, 0.1% SDS, and for 15 min at 55 °C in 0.2 × SSC, 0.1% SDS, and then exposed for different times to Kodak XAR films at -80 °C. Results were visualized by autoradiography and quantified by densitometric scanning of the autoradiograms or quantified using an AMBIS, Inc. (San Diego, CA) radioactivity scanner.

Nuclear Run-on Transcription Assays—HUVECs were treated with human IL-1β (10 units/ml) in presence or absence of forskolin (10 µM), H-88 and H-89 (each at 5 µM), and nuclei were isolated after 1 h of treatment. Nuclear run-on assays were performed as described previously (20) starting with 3 × 10⁶ cells/treatment. For each assay, nuclei were re-suspended in 300 µl of transcription buffer containing 250 µCi of [α-32P]UTP (800 Ci/mmol) and incubated for 20 min at 30 °C. The radiolabeled RNA was purified using the guanidine isothiocyanate/CsCl procedure (27), and equal amounts of counts/min were used for hybridization to nitrocellulose filters that contained alkali-denatured target cDNA. The filters were blocked by 30% glycerol, 7.5% bovine serum albumin, 0.1% SDS, and then exposed for different times to Kodak XAR films at -80 °C. Results were visualized by autoradiography and quantified by densitometric scanning of the autoradiograms. Half-life studies were as described elsewhere (20).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—HUVECs were treated with recombinant human IL-1β (10 units/ml) or with forskolin (100 µM final). The protein kinase A inhibitors were added to the cells 30 min before the addition of IL-1β. Cells were collected after trypsin treatment and extensively washed, and nuclear extracts were prepared from single 10-cm diameter dishes as described previously (21). Extracts (about 2 µg of protein) were incubated for 25 min on ice in 25 µl of Pratt buffer (10 mM Tris-Cl, 7.5 mM EDTA, 10% glycerol, 0.1% Triton X-100, 5% glycerol, 80 mM NaCl, 3 mM MgCl₂, 0.5 µg/µl bovine serum albumin, 0.0025% bromphenol blue) supplemented with 3 µg of poly(dI-dC) (Pharmacia Biotech Inc.) and with an end-labeled, double-stranded β-globin oligonucleotide probe (18).

Plasmid Constructions—pUHG10-3-CAT was made by insertion of the CAT cDNA into pUHG-10-3. pUHG10-3 is identical to pHUH010-3 (31), except that its multiple cloning site has unique SacII, HindIII, and BamHI and inserted into pUC19. Plasmid pUHG15-1 is derived from pUHD15-1 (32) by replacement of the 3' p-globin tail with the E-selectin 5' intron plus the polyadenylation signal. An E-selectin promoter fragment was PCR-amplified from the -741-CAT construct described previously (10), using oligonucleotides TGAAATTCGAGTACCCACCTGTGAGAAC and TGCACTGAGAAGTCAGCATGGTGGATCC. The resulting fragment, corre-

RESULTS

Forskolin Increases cAMP Levels in HUVECs and Affects the Expression of E-selectin, VCAM-1, and ICAM-1—We wanted to determine the effects of activating the intracellular cAMP pathway on endothelial cell expression of adhesion proteins. To do this, we first tested whether forskolin, a direct activator of adenylyl cyclases, increases intracellular cAMP levels in endothelial cells. As Fig. 1 shows, forskolin increases, in a dose-dependent manner, the level of cAMP in endothelial cells. The values shown were measured 15 min after the addition of forskolin to the cultures. We had previously determined that for HUVECs this time point corresponds to the maximal increase in cAMP (data not shown). In contrast, cAMP levels were not
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**Table 1**

Effect of various agents on intracellular cAMP levels in endothelial cells

| Assay                        | cAMP (pmol/2.5 x 10⁴ cells) |
|------------------------------|-----------------------------|
| Untreated                    | 1.1 ± 0.5                   |
| IL-1                         | 1.8 ± 0.2                   |
| IL-1 + forskolin             | 43.6 ± 6.9                  |
| IL-1 + IBMX                  | 9.2 ± 0.2                   |
| Forskolin                    | 52.4 ± 1.1                  |
| IBMX                         | 8.7 ± 0.3                   |
| Forskolin + IBMX             | 67.6 ± 6.3                  |

We next tested the effect of increased cAMP levels produced by forskolin treatment or the induction of E-selectin. Forskolin alone was unable to induce any E-selectin protein expression as measured by flow cytometry (FACS; data not shown). When we measured the effect of forskolin on IL-1 induction of E-selectin, however, we found that forskolin inhibits its expression, in a dose-dependent manner (Figs. 1 and 2). We usually observed between 60 and 50% inhibition of the maximal expression of E-selectin using forskolin at 100 and 10 μM (Table I). These effects were measured at the time point (4 h after IL-1 induction) where maximal expression of E-selectin is seen (6, 10). Similar to E-selectin, expression of VCAM-1, another inducible cellular adhesion molecule, was also reduced by concomitant treatment with IL-1 and forskolin. Expression of VCAM-1 was suppressed to the same extent as E-selectin. In contrast, ICAM-1 expression was up-regulated on endothelial cells treated with forskolin alone (Fig. 2 and Table II). The forskolin-induced increase in ICAM-1 protein (reaching 50% higher levels) is particularly evident after 24 h of IL-1 treatment when ICAM-1 induction is maximal (Table II). However, forskolin alone is not sufficient to induce ICAM-1 expression over that found in untreated controls (data not shown).

The effect of forskolin treatment was different using IL-1, TNF, or PMA as the inducer of the adhesion proteins (Table II). In particular less suppression of E-selectin and VCAM-1 and less increased expression of ICAM-1 were observed with PMA plus forskolin (Table II). Thus PMA, a non-physiological inducer of protein kinase C, may use a different pathway to induce expression of the adhesion proteins than that used by cytokines.

To determine whether the observed effects of forskolin treatment were linked to the higher levels of cytosolic cAMP or to an indirect effect of forskolin, we tested cAMP analogues and other inducers of adenylate cyclase (forskolin analogues). 8-Bromo-cAMP and dibutyryl-cAMP are cAMP analogues that readily enter cells and are poorly hydrolyzed by phosphodiesterases. Following a 30-min preincubation and continuing treatment with either of these cAMP analogues, IL-1-induced expression of E-selectin was reduced (Table II). We next tested a series of forskolin analogues for their ability to decrease cytokine-induced E-selectin expression (Table III). All the forskolin analogues that induce adenyl cyclase also reduced IL-1 induction of E-selectin, although to differing degrees. The differences in inhibition observed may reflect the permeability of endothelial cells to the different analogues or some intrinsic characteristic of these compounds such as their degree of adenylate cyclase activation. The (1,9 dideoxy)-forskolin analogue, which does not activate adenylate cyclase, provided a negative control as it did not inhibit the cytokine-induced expression of E-selectin.

We also asked whether other means of increasing intracellular cAMP levels would have the same effects on the induction of E-selectin, VCAM-1, or ICAM-1 expression. Inhibition of cAMP hydrolysis by several different inhibitors of phosphodiesterases leads to increased intracellular cAMP. We used the phosphodiesterase inhibitors isobutylmethylxanthine, pentoxifylline, and rolipram to block cAMP hydrolysis in endothelial cells. These inhibitors increased the forskolin-mediated suppression of E-selectin and VCAM-1 and augmented the forskolin-mediated increased expression of ICAM-1 (Table IV). We conclude that increased intracellular levels of cAMP are responsible for altering the amplitude of the cytokine signal with regard to adhesion molecule induction on endothelial cells.

**Fig. 2.** Flow-cytometry analysis of E-selectin, VCAM-1, and ICAM-1 expression. HUVECs were left untreated (control) or incubated for 4 or 24 h with IL-1 (10 units/ml) in the absence or presence of forskolin (100 μM). At the end of the treatment cells were collected and subjected to FACS analysis.
E-selectin Gene Transcription Inhibition via a PKA Pathway

**TABLE II**

Effect of forskolin and CAMP analogues on the expression of E-selectin, VCAM-1, and ICAM-1 on endothelial cells induced by different stimuli

HUVECs were incubated for 4 or 24 h with IL-1β (10 units/ml), TNF-α (200 units/ml), or PMA (100 ng/ml). Forskolin was used at 100 or 10 μM and added concomitantly with the cytokines or PMA. The CAMP analogues were used at 100 μM and added 30 min before cytokine or PMA addition. Adhesion molecule expression was measured by FACS analysis. Results are expressed as percent inhibition (−) or percent increase (+) as compared to controls without forskolin or its analogues. The values shown are obtained from three experiments done in duplicate.

| Assay | E-selectin (4 h) | VCAM-1 (4 h) | ICAM-1 (4 h) |
|-------|------------------|--------------|--------------|
|       | IL-1 | TNF | PMA | IL-1 | TNF | PMA | IL-1 | TNF | PMA |
| µM | % | µM | % | µM | % | µM | % |
| Forskolin | 100 | -64.8 ± 4.8 | -49.1 ± 8.7 | -5.7 ± 2.3 | -63.9 ± 11.7 | -52.6 ± 1.4 | -42.5 ± 0.9 |
| 10 | -52.7 ± 10.4 | -32.8 ± 12.9 | -21.5 ± 1.3 | -40.6 ± 3.25 | -17.1 ± 10.3 | -29.2 ± 14.6 |
| cAMP analogues | 100 | -34.2 ± 9.2 | -19.8 ± 3.2 | -9.7 ± 2 |
| 8-Bromo-CAMP | 100 | -16.3 ± 10.1 | -5.7 ± 2.3 | -40.6 ± 3.25 | -17.1 ± 10.3 | -29.2 ± 14.6 |
| Forskolin | 100 | +11.5 ± 18.3 | +4.4 ± 15.9 | +4.4 ± 35.1 | +54.4 ± 14.2 | +14.1 ± 9.8 | +2.5 ± 3.3 | +29.8 ± 18.3 |
| 10 | +2.7 ± 10.8 | +8.4 ± 28.8 | +2.3 ± 6.7 |
| cAMP analogues | 100 | +22.7 ± 10.4 | +47.9 ± 3.2 |
| 8-Bromo-CAMP | 100 | -8.6 ± 3.5 | +47.9 ± 3.2 |

**TABLE III**

Effect of forskolin analogues on the expression of E-selectin and ICAM-1 induced by IL-1

HUVECs were incubated for 4 h with IL-1β (10 units/ml) plus forskolin analogues (all used at 100 µM final concentration). The values shown represent the percentage of increased or decreased expression as compared to IL-1 treatment alone; all are averages of three values.

| Forskolin analogues | E-selectin (4 h) | ICAM-1 (4 h) |
|---------------------|------------------|--------------|
| 1,9-Dideoxy-forskolin | 0 | 0 |
| 7-Deacetyl 6N-acetylglucosyl) forskolin | -27.9 ± 2.0 | +39.8 ± 0.1 |
| 7-β-Deacetyl 7β-(N-methylpipеразин)-butyryl forskolin | -52.9 ± 4.1 | +37.9 ± 6.9 |
| 7-c-Hexamcteclin-7 deacetyl forskolin | -55.7 ± 0.1 | +39.6 ± 6.0 |
| 6-Acetyl-7-deacetyl forskolin | -45.5 ± 3.1 | +45.2 ± 8.7 |
| Deacetyl forskolin | -36.9 ± 5.6 | +47.9 ± 3.9 |
| 7-Deacetyl-1,9-dideoxy-forskolin | 0 | +17.6 ± 2.5 |
| 7-Deacetyl-1-deoxy-forskolin | 0 | +21.5 ± 0.3 |
| 1-Deoxy-forskolin | 0 | +20.4 ± 3.3 |
| 6,β-Hydroxy-8,13-epoxy-labd-14-en-11-one | -12.2 ± 35.5 | -30.8 ± 37.8 |
| 9-α-Hydroxy-8,13-epoxy-labd-14-en-11-one | -22.7 ± 5.1 | -42.6 ± 4.3 |
| 7-β-Deacetyl 7β-(N-methylpipеразин)-butyryl, hydrochloride forskolin | -62.2 ± 1.0 | +3.6 ± 8.3 |
| 6,β-β-(Piperidino)propionate)-hydrochloride forskolin | -67.6 ± 0.8 | +5.4 ± 30.0 |

**TABLE IV**

Effect of phosphodiesterase inhibitors on the expression of E-selectin induced by IL-1

HUVECs were incubated for 4 h with IL-1β (10 units/ml) plus phosphodiesterase inhibitors. The values shown represent the percentage of increased or decreased expression as compared to IL-1 treatment alone; all are averages of three values.

| Assay | E-selectin (4 h) | ICAM-1 (4 h) |
|-------|------------------|--------------|
| µM | % | µM | % | µM | % |
| Forskolin | 100 | -64.8 ± 4.8 | +11.5 ± 18.3 | +54.4 ± 14.2 |
| Forskolin | 100 | -22.6 ± 7 | +21.6 ± 18.9 | +21.1 ± 12.8 |
| Phosphodiesterase inhibitors | 500 | -16.1 ± 0.8 | -2.9 ± 17.1 |
| Pentoxifylline | 500 | -55.1 ± 5.2 | +35.7 ± 10.8 |
| IBMX | 10 | -11.7 ± 5.7 | -8.6 ± 27.6 | -9.6 ± 2.3 |
| Forskolin + pentoxifylline | 100 | -63.4 ± 8.2 | +25.8 ± 1.1 |
| Forskolin + IBMX | 100 | -71.4 ± 0.2 | +10.5 ± 7.1 |
| Forskolin + rolipram | 10 | -59.5 ± 4.4 | +28.2 ± 9.8 | +23.9 ± 14.6 |

* IBMX, isobutylmethylxanthine.

We conclude that the reduction in E-selectin expression by forskolin treatment results in inhibition of the transcription rate of the E-selectin gene, we performed transcription run-on assays. Nuclei were purified from endothelial cells treated with IL-1 in the presence or absence of forskolin, and on-going E-selectin transcription was measured. The initial rate of E-selectin gene expression was reduced when cells were treated with IL-1 in the presence of forskolin (Fig. 5, lane 3). We conclude that the reduction in E-selectin expression caused by forskolin treatment results from a reduction in the rate of transcription of the gene.

Forskolin Inhibition of E-selectin Expression Involves Phosphorylation by PKA—Since in most cells a principal effect of saying the remaining message at different times following treatment of cells with actinomycin D, an inhibitor of further transcription. Although the absolute amounts of mRNA are lower in the forskolin treated samples, the rates of E-selectin and ICAM-1 mRNA decay appear similar with or without forskolin (Fig. 4, A and B). We conclude that the mechanism by which forskolin affects E-selectin and ICAM-1 expression does not involve significant alterations in mRNA stability.

To determine whether forskolin treatment results in inhibition of the transcription rate of the E-selectin gene, we performed transcription run-on assays. Nuclei were purified from endothelial cells treated with IL-1 in the presence or absence of forskolin, and on-going E-selectin transcription was measured. The initial rate of E-selectin gene expression was reduced when cells were treated with IL-1 in the presence of forskolin (Fig. 5, lane 3). We conclude that the reduction in E-selectin expression caused by forskolin treatment results from a reduction in the rate of transcription of the gene.

Forskolin Inhibition of E-selectin Expression Involves Phosphorylation by PKA—Since in most cells a principal effect of treated with both IL-1 and forskolin. We found that, with increasing doses of forskolin and a constant amount of IL-1 present, the E-selectin mRNA was reduced in a forskolin dose-dependent fashion (Fig. 3). We also measured the effect of forskolin on the induction of ICAM-1 mRNA. Steady-state levels of ICAM-1 mRNA increased in a dose-dependent manner in response to forskolin treatment (Fig. 3). The corresponding reduction or increase in mRNA closely parallels the changes in protein expression we observed by FACS analysis for these two proteins (see Fig. 1). From these results we conclude that forskolin acts principally at a step prior to translation.

The decreased E-selectin mRNA levels and increased ICAM-1 mRNA levels that we observed in forskolin treated cells, might be due either to variations in mRNA stability or to changed promoter activity. To assay the effect of increased cellular CAMP on E-selectin and ICAM-1 mRNA stability, we measured the mRNA half-life in the presence and absence of forskolin. The half-life of the message was determined by as—

Deacetyl forskolin
6-Acetyl-7-deacetyl forskolin
7-0-Hemisuccinyl-7 deacetyl forskolin
7-β-Deacetyl-7β-(N-acetylglycil)forskolin
7 Deacetyl 6(N-acetylglycil)forskolin
9-(r-Hydroxy-8,13-epoxy-labd-14-en-11-one
1,9-Dideoxy-forskolin
1-Deoxy-forskolin
1,9-Dideoxy-forskolin
7-p-Deacetyl-7-p-(y-N-methylpipera-zino)-butyryl forskolin
7 Deacetyl 6(N-acetylglycil)forskolin
7-Deacetyl-1,9-dideoxy-forskolin
7-Deacetyl-1-deoxy-forskolin
7-β-Deacetyl 7β-(N-methylpipera-zino)-butyryl, hydrochloride forskolin
6,β-β-(Piperidino)propionate)-hydrochloride forskolin
ruling cAMP levels is the activation of protein kinase A (PKA). We asked whether PKA mediates the inhibitory effect of cAMP on E-selectin expression. To answer this question, we used PKA-specific inhibitors in an attempt to reverse the forskolin effect. Two compounds, H-88 and H-89, have been described in the literature as highly specific inhibitors of PKA (34). When we pre-treated cells with these compounds, the inhibitory effect of forskolin on cytokine-induced E-selectin expression was significantly reduced. This reversal of the forskolin inhibition of E-selectin expression occurred at the level of the mRNA as shown by Northern blot analysis (Fig. 6). Moreover, we found that in the presence of H-88 and H-89 the inhibitory effect of forskolin on gene transcription is reversed (Fig. 5, lane 4). In contrast, inhibitors of protein kinase C (H7, bisindolylmaleimide, and calphostin) did not reverse the forskolin effect (data not shown). These results suggest that selective activation of PKA is involved in the modulation of E-selectin gene transcription.

To further explore the role of phosphorylation in forskolin's inhibitory effect, we examined dephosphorylation. We used okadaic acid, an inhibitor of protein phosphatase type 2A, to ascertain this enzyme's role in forskolin mediated effects. If forskolin activates PKA, then substrate phosphorylation might be important for the measured inhibition in E-selectin expression. Okadaic acid treatment prevented forskolin from inhibiting IL-1-induced E-selectin expression (Fig. 7). This effect of okadaic acid supports the role of phosphorylation in the regulation of transcription of E-selectin.

As a means to determine whether forskolin's effect on E-selectin transcription was direct, via a phosphorylation cascade or indirect, requiring newly synthesized factors, we measured its activity in the absence of protein synthesis. Previously, we have shown that cycloheximide is a potent inhibitor of protein synthesis in endothelial cells as well as a superinducer of E-selectin mRNA in the presence of IL-1 (20). Nonetheless, cycloheximide did not reverse the inhibition caused by forskolin (data not shown). These results indicate that protein synthesis is not required for the forskolin effect, further defining it as the result of direct phosphorylation(s).
Nuclear Factors That Mediate E-selectin Gene Activation—

Blot was probed with cDNA or vector DNA as indicated and exposed for 3 min (lane 3); were isolated from HUVECs untreated and H-88+H-89 (both at 10 μM) before the addition of IL-1β and forskolin for a further 1 h (lane 4). The blot was probed with cDNA or vector DNA as indicated and exposed for 3 h at ~80 °C. At the top is a graphic representation of the E-selectin hybridization.

Forskolin Does Not Alter the DNA Binding of the Principal Nuclear Factors That Mediate E-selectin Gene Activation—

Previously we have shown that a small fragment (approximately 200 bp) (10) of the E-selectin promoter sequence located 5′ of the transcription start site is sufficient to allow cytokine induction of gene transcription. Two of the four necessary transcription factors binding this promoter fragment have been identified as NF-κB and ATF (binding to the NF-ELAM1 sequence element) (10, 12). We decided to test whether their binding to the E-selectin promoter DNA is altered by forskolin treatment of the cells. Nuclear extracts from cells treated either with IL-1 plus forskolin or IL-1 alone were tested for the presence of factors which bind the κB and ATF sequence elements. Both factors binding these elements appear unaltered by forskolin in their ability to bind their respective elements as judged by gel mobility shift experiments (Fig. 8, A and B). Our results demonstrate that neither proteins' ability to bind DNA is lost nor is the nuclear localization of NF-κB inhibited by forskolin. We conclude that any alteration of these factors by forskolin treatment of the cells is more likely to affect their transactivating function than their ability to bind to promoter sequences.

The Cytokine-inducible Minimal E-selectin Promoter also Shows the Forskolin-induced Inhibition of Gene Expression—To determine whether the forskolin effect on E-selectin transcription could be mapped to a known region of the promoter, we tested different promoter fragments fused to a CAT reporter gene. Initially we were unable to reproduce the inhibition of expression seen with forskolin treatment with these constructs. To reproduce the forskolin effect we set up a two plasmid system in which different plasmid backbones are present than those used previously. We believe this to be important as the original parent plasmids have several CREB binding sites which may have been bound by CAMP-activated CREB thereby countering the negative effect of forskolin on the E-selectin promoter; a similar interference has been reported previously for standard, pUC-based CAP expression vectors (35, 36). Moreover, the two plasmids system appeared to amplify the reporter signal making a reduction in reporter expression easier to monitor. Thus the minimal cytokine-inducible E-selectin promoter was introduced in front of a gene encoding a constructed, hybrid transcription factor (31) (Fig. 9A). On a second plasmid a minimal promoter containing binding sites for the hybrid transcription factor was followed by the CAT reporter gene (Fig. 9A). When these two plasmids were co-transfected into HUVECs, the expected activation of the reporter gene by treatment of the cells with IL-1 was observed (Fig. 9B). Moreover, treatment of transfected cells with IL-1 plus forskolin resulted in a significant reduction in reporter activity (Fig. 9B, left panel). From these results we conclude that the minimal promoter necessary for cytokine induction also contains a site for forskolin-derived inhibition of gene activation. To demonstrate that cAMP modulation is not a general property of any promoter introduced into the cell, we also

2 P. Ghersa, J. Whelan, R. Pescini, J. F. DeLamarter, and R. Hooft van Huijsduijnen (1994) Gene (Amst.), in press.
tested a control construct in which the CMV promoter drives the tet-VP16 expression. This promoter is constitutively active and unaffected by cytokine treatment in the presence of forskolin (Fig. 9B, right panel). Thus the cAMP effect observed for the E-selectin promoter is therefore not a general one but specific to the elements present in the proximal E-selectin promoter.

As mentioned previously the E-selectin minimal promoter contains several elements that are necessary for cytokine inducibility of transcription. Two of these are recognized by the NF-κB and ATF transcription factors (13, 14). We have shown previously that the ATF binding site does not function as an independent enhancer (15). Rather ATPs act in concert with NF-κB to enhance transcription from the E-selectin promoter. In contrast, the NF-κB binding site is a cytokine-inducible enhancer on its own. Therefore, we decided to study the effect of forskolin treatment of endothelial cells on NF-κB transactivity. To do so we used the same two plasmid system as we had used with the E-selectin promoter fragment. However, the promoter fragment was replaced by a minimal promoter containing a TATA box preceded by two canonical NF-κB binding sites. When transfected into endothelial cells, this construct can indeed be induced by IL-1 to produce reporter gene activity (Fig. 9B, middle panel). Moreover, the effect of treating transfected cells with IL-1 plus forskolin is a significant reduction in the reporter gene activity (Fig. 9B). The generation of a similar reduction in transcription activity by forskolin treatment of cells carrying a reporter plasmid driven by the NF-κB enhancer suggests that transcription factor(s) binding there may be a target of the forskolin effect. As shown above the DNA binding activity of NF-κB isolated from cells treated with IL-1 plus forskolin is indistinguishable from that of cells treated with IL-1 alone. Taken together, the results we report here suggest that the ability of NF-κB to transactivate may be altered. Reduced transactivation of NF-κB may be caused by an inhibitory phosphorylation due to the PKA activity that is induced by increased cAMP levels. Alternatively, the induced phosphorylation may occur on a factor which interacts with NF-κB thus altering its transactivity.

**DISCUSSION**

In this study we have demonstrated a differential effect of forskolin on the cytokine induction of the expression of three cellular adhesion molecules on endothelial cells. The increase in intracellular cAMP levels generated by forskolin treatment inhibits the expression of E-selectin and VCAM-1, while increasing the expression of ICAM-1. For E-selectin we present strong evidence that the forskolin effect is at the level of gene transcription directly through PKA-mediated phosphorylation. Our results also demonstrate that transcription from a cytokine-responsive, minimal E-selectin promoter is reduced by forskolin treatment. This effect is likely the result of an altered phosphorylation of one or more of the required regulatory factors.

Recently another group has published on the effects of forskolin on E-selectin and VCAM-1 expression (37), but did not report changes in ICAM-1 expression or provide a mechanism for the changes they observed. We also found very modest effects of forskolin on ICAM-1 when we measured its expression at the time when E-selectin expression is maximal (4–6 h post induction). The major increase we observed in ICAM-1 expression occurs 24 h post induction. In addition we have investi-
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Fig. 9. The proximal E-selectin promoter is inhibited by forskolin. A, schematic representation of vectors containing the tetVP16 fusion cDNA, driven by the E-selectin promoter, by the minimal TK-promoter plus two NF-κB sites or by the CMV promoter. The tet-VP16 fusion protein, driven by these two promoters, binds and transactivates the tet-operator plus CAT reporter. B, CAT enzymatic activity of the operator/CAT fusions, directed by the (-383)-E-selectin promoter construct (ELA"tetVP16) (left), by the (NFκB) construct (middle), and by the CMV promoter (right), used as a negative control. Transiently transfected endothelial cells were treated as indicated in the figure (40 units/ml IL-1 β, 20 μM forskolin). For the CMV promoter, 1/100 of the cell extract was used to measure the CAT activity as compared to the two other promoters.

Gated the molecular mechanism operating in the forskolin-induced reduction of E-selectin expression. Our evidence that a PKA-induced phosphorylation event reduces E-selectin transcription and the activity of NF-κB in endothelial cells provides a possible mechanistic explanation for the reduction in E-selectin expression caused by forskolin treatment.

The existence of cAMP responsive elements in promoters and the transcription factors that bind them (CREB and ATF) is well documented (for reviews, see Refs. 38–40). In general CREB binds constitutively to the cAMP response element, transactivity being conferred by its PKA-dependent phosphorylation. ATFs have been divided into those responsive to cAMP and those not (41). We have shown recently that a CREB/ATF binding element exists in the E-selectin promoter and that this element (NF-ELAM1) binds non-cAMP responsive ATFs (15). In the same report we have demonstrated that NF-ELAM1 does not act as an independent enhancer. We show here no effect, positive or negative, of cAMP levels on the binding of ATFs to NF-ELAM1. Using our two-plasmid CAT-reporter system have also found that mutating the NF-ELAM-1 site in the E-selectin promoter does not abolish forskolin repression (data not shown). These results together strongly suggest that increased levels of cAMP do not exert their effect through the NF-ELAM1 site. We conclude that this element is unlikely to play a major role in the modification of E-selectin gene transcription caused by forskolin treatment of endothelial cells.

Some investigators have suggested that PKA is involved in IL-1 signaling (42). Our results using endothelial cells suggest that PKA activity acts rather as a "cross-talk" or overlay signal to the normal cytokine signal. Indeed, other investigations have suggested a protein kinase pathway for cytokine signaling different from either PKA or protein kinase C (43, 44). Moreover, the effect of increased cAMP levels is complex since the cytokine signal for E-selectin and VCAM-1 expression is reduced while it is increased for the expression of ICAM-1. The clear implication from our results is that increased cAMP induces PKA phosphorylation in treated endothelial cells. This in turn results in altered expression of adhesion molecules presumably by changing the phosphorylation state of one or more regulatory factors acting on the different adhesion molecule promoter sequences.

In support of the cross-talk hypothesis for the effects of forskolin that we observe are recent publications describing a cAMP-PKA pathway cross-talk to the ras pathway (45–49). These reports demonstrate that the activation of PKA by cAMP results in a phosphorylation of ras-1 that, in several examples, negates the positive signal given by ras-GTP catalyzed phosphorylation. Thus two pathways which have been studied independently have been shown to interact at the level of phosphorylation of a signaling intermediate. The modification of ras-1 signaling by PKA-directed phosphorylation is a model for the alteration of the cytokine signal which we have shown. Increased PKA-induced phosphorylation of one (or more) regulatory factors in the cytokine-signaling pathway may alter the signal as is the case for the ras pathway. Since a minimal promoter containing only NFκB enhancer elements is also repressed by forskolin (Fig. 9B), factor(s) binding this element are probably central to forskolin's repression. Bcl3 has recently been shown to inhibit nuclear NFκB (50), suggesting that NFκB is under control of events other than cytoplasmic sequestration. Alternatively, PKA-induced phosphorylation of the
transcription factor(s) themselves may directly change their gene-specific transactivation such that the end result is either increased or decreased transcription of individual genes.

Identifying the second messengers involved in cytokine signaling is important for determining the potential cross-talk intermediates in forskolin-induced alterations of the signal. Moreover, determining the differences in phosphorylation of the transcription factors following forskolin treatment will suggest if they are targets of PKA or PKC (or a PKA-activated kinase) modification as well. This information will clarify the step at which cross-talk occurs and whether the substrates provide targets for intervention in the induction of E-selectin and increased gene-specific transactivation such that the end result is either inhibition or induction.

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