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Role of cyclic AMP in promoting the thromboresistance of human endothelial cells by enhancing thrombomodulin and decreasing tissue factor activities

Gisèle Archipoff, [1]Alain Beretz, Katalin Bartha, Christine Brisson, Corinne de la Salle, Catherine Froget-Léon, Claudine Klein-Soyer & [2]Jean-Pierre Cazenave

Centre Régional de Transfusion Sanguine, INSERM U.311, 10 rue Spielmann, 67085 Strasbourg Cédex, France

1 The effects of forskolin, prostaglandin E_1 (PGE_1), dibutyryl cyclic AMP (db cyclic AMP), dibutyryl cyclic GMP (db cyclic GMP) and 3-isobutyl-l-methyl-xanthine (IBMX) were investigated on the expression of tissue factor and thrombomodulin activities on the surface of human saphenous vein endothelial cells (HSVEC) in culture.

2 Forskolin (10^{-6} to 10^{-4} M), PGE_1 (10^{-7} to 10^{-5} M) and db cyclic AMP (10^{-4} to 10^{-3} M) caused a concentration-dependent decrease of cytokine-induced tissue factor activity.

3 Similar concentrations of forskolin, PGE_1 and db cyclic AMP enhanced significantly constitutive thrombomodulin activity and reversed the decrease of this activity caused by interleukin-1 (IL-1).

4 IBMX (10^{-4} M) decreased tissue factor activity and enhanced the effect of forskolin on tissue factor and thrombomodulin activities.

5 Forskolin (10^{-4} M) decreased the IL-1-induced tissue factor mRNA and increased the thrombomodulin mRNA level. IL-1 did not change the thrombomodulin mRNA level after 2 h of incubation with HSVEC in culture.

6 Dibutyryl cyclic GMP (10^{-4} M to 10^{-3} M) did not influence tissue factor or thrombomodulin activity.

7 Our data suggest that elevation of intracellular cyclic AMP levels may participate in the regulation of tissue factor and thrombomodulin expression, thus contributing to promote or restore antithrombotic properties of the endothelium.

Keywords: Endothelial cells; thrombomodulin; tissue factor; cyclic AMP; forskolin; prostaglandin E_1; interleukin-1; thrombin; cyclic GMP

Introduction

One of the remarkable features of the coagulation system is its ability to respond rapidly to seal a wound site without the haemostatic plug extending to occlude the vessel lumen. This property is due to both surface properties and secreted products of endothelial cells. The endothelial cell surface participates in the activation or inactivation of coagulation factors and their cofactors, by binding them to receptors or to negatively charged membrane phospholipids. Endothelium synthesizes antithrombotic agents such as prostacyclin, endothelium-derived relaxing factor (EDRF), plasminogen activators, 13-hydroxyoctadecadienoic acid (13-HODE) and heparan sulphates (Fajardo, 1989). One of the anticoagulant mechanisms involves thrombomodulin, a membrane glycoprotein which is constitutively expressed by endothelium and is a specific receptor for thrombin. Thrombomodulin exhibits two complementary anticoagulant functions: one is to decrease the ability of thrombin to catalyze clot formation and the other is to convert thrombin into a potent protein C activator (Freyssinet & Cazenave, 1988; Esmon, 1989; Dittman & Majerus, 1990). Thrombin forms a 1:1 complex with thrombomodulin and this complex activates protein C into activated protein C. Activated protein C functions as an anticoagulant by inactivating activated factors V and VIII (FVa and VIIIa), two essential cofactors of two serine-proteases, respectively factors Xa and IXa. Activated protein C thus exerts a potent negative feedback control on the generation of thrombin.

Under normal conditions, endothelial cells present a non-thrombogenic surface, which is non-reactive to platelets or coagulation factors. But when injured, they rapidly become procoagulant and capable of initiating blood coagulation. In this activated state, they synthesize tissue factor (TF), a transmembrane glycoprotein which is essential in the initiation of the extrinsic coagulation pathway. Tissue factor is the receptor for factors VII and VIIa; the complex TF-FVII/VIIa catalyzes the conversion of factors X to Xa and IX to IXa (Nemerson, 1988). These activated factors contribute to the generation of thrombin at the surface of the cells Endothelium synthesizes tissue factor in some pathological situations or when exposed to stimuli such as cytokines, thrombin or endotoxin (Boeri *et al.*, 1989; Zuckerman & Surprenant 1989; Conway *et al.*, 1989; Almus *et al.*, 1989; Noguchi *et al.*, 1989; Crossman *et al.*, 1990; Archipoff *et al.*, 1991; Pettersen *et al.*, 1992). Some of these stimuli have also been described as reducing thrombomodulin activity *in vivo* or *in vitro*: endotoxin (Moore *et al.*, 1987), interleukin 1 (IL-1) (Nawroth *et al.*, 1986; Hirokawa & Aoki 1991), tumour necrosis factor (Gerlach *et al.*, 1989; Moore *et al.*, 1989; Scarpati & Sadler, 1989; Hirokawa & Aoki, 1991). The effect of thrombin is debated. Thrombin was found to cause the internalization of the thrombin:thrombomodulin complexes by human umbilical cord vein endothelial cells and A549 lung cancer cells (Maruyama & Majerus, 1985), but such endocytosis was not observed in human saphenous vein endothelial cells or in the EA.hy 926 cell line obtained by fusing human umbilical vein endothelial cells with the A549 lung cancer cells (Beretz *et al.*, 1989).

[1] Present address: Laboratoire de Pharmacologie Cellulaire et Moléculaire, URA CNRS 600, Université Louis Pasteur, Faculté de Pharmacie, BP 24, 67401 Illkirch-Graffenstaden, France.
[2] Author for correspondence.

Endothelial cells are thus able to regulate the expression of tissue factor and thrombomodulin, two glycoproteins with opposed functions. Under normal conditions, the constitutive expression of thrombomodulin and the absence of induced tissue factor on the intact endothelial cell surface contributes to the maintenance of an anticoagulant state of the endothelium. Abnormalities in the balance between pro and anticoagulant properties of the endothelium could contribute to the pathogenesis of human thrombotic diseases (Gimbrone, 1986). Pharmacological manipulation of these endothelial properties would therefore be useful in the prevention or treatment of such thrombotic disorders. Cyclic AMP is known to be an important intracellular second messenger involved in a wide variety of endothelial cell functions. Our aim was to study the role of cyclic AMP in the modulation of tissue factor and thrombomodulin activities. We show here that agents which elevate cyclic AMP enhance thrombomodulin activity in HSVEC and reverse the effects of cytokines on tissue factor and thrombomodulin activities.

Methods

Cell culture

Human endothelial cells (HSVEC) were collected from fragments of saphenous veins obtained during coronary-bypass surgery (Beretz *et al.*, 1989) and cultured as previously described (Klein-Soyer *et al.* 1986). The culture medium was M199/RPMI 1640 (1:1) containing 10 mM HEPES, 2 mM L-glutamine, antibiotics (100 units penicillin and 100 μg streptomycin ml^{-1}), fungizone (0.25 $\mu g\ ml^{-1}$) and 30% human serum. It was verified by a Limulus chromogenic assay (Coatest: Kabi Vitrum, Mölndal, Sweden) that all media and buffers had an endotoxin content which never exceeded 0.04 $ng\ ml^{-1}$. The cells were frozen at the second passage and used in experiments from the 3rd to the 10th passage. Preliminary experiments were carried out in order to verify that the number of passages did not alter cell responses. The cells were subcultured in 96-multiwell plates at an initial cell density of 10^4 cells cm^{-2} for concentration-effect and kinetics experiments and in 6-well plates for extraction of mRNA. All plates were precoated with purified human fibronectin (0.5 $mg\ ml^{-1}$).

Assays for thrombomodulin and tissue factor activities

For each experimental condition simultaneous measurements of tissue factor and thrombomodulin activities were performed on the same cell population.

Concentration-effect studies The culture medium was changed 24 h before each assay. For the 24 h incubation period, this medium contained either forskolin, PGE_1, dbcyclic AMP or dbcyclic GMP. Before the last 4 h of incubation, the cells were rinsed 3 times with RPMI/M199 (v/v) and then incubated with RPMI/M199 containing 10 $mg\ ml^{-1}$ HSA and either forskolin PGE_1, IBMX, dbcyclic AMP or dbcyclic GMP and Il-1, TNF or thrombin. In forskolin, PGE_1 and IBMX concentration-effect studies, IL-1 was used at 0.2 $u\ ml^{-1}$ to induce tissue factor activity and at 1 $u\ ml^{-1}$ to decrease thrombomodulin activity. In dbcyclic AMP and dbcyclic GMP concentration-effect studies, 1 $u\ ml^{-1}$ IL-1 (57 nM), 1 $ng\ ml^{-1}$ TNF (22 pM) and 1 $u\ ml^{-1}$ thrombin (8.8 nM) were used in both tissue factor and thrombomodulin assays. After 4 h of incubation at 37°C, the cells were rinsed 3 times with PBS. Tissue factor and thrombomodulin activities were then measured. For this purpose, the cell monolayers were covered with 200 μl of RPMI/M199 without phenol red, containing 3 $mg\ ml^{-1}$ HSA. For tissue factor assays, purified human factors VII (5 nM final concentration) and X (400 nM final concentration) were added to each well. After 20 min at 37°C, the supernatants were transferred into 96 multiwell plates for reading. The amidolytic activity of generated factor Xa was measured at 405 nm, using the specific chromogenic substrate S2765 (0.2 mM final concentration) in a kinetic microplate reader coupled to a microcomputer (Molecular Devices, Palo Alto, CA., U.S.A.). Thrombomodulin activity was evaluated in the same way by addition to the wells of thrombin (0.25 NIH $u\ ml^{-1}$, 2.2 nM final concentration) and protein C (65 nM final concentration). After 2 h of incubation at 37°C, the reaction was blocked by addition of hirudin (1.25 a.t.u. ml^{-1}). The amidolytic activity of generated activated protein C was measured as described above, using the substrate S2366 (0.1 mM final concentration). Each experimental condition was assayed in sextuplate wells. The concentration of APC or factor Xa formed was determined by reference to standard curves established with known amounts of factor Xa or APC. Cells from 3 wells per experimental condition were detached with trypsin-EDTA and then counted (Coulter Counter ZM, Coultertronics, Margency, France); the cell number was used to express thrombomodulin and tissue factor activities normalized per 10^4 cells. Cells from the remaining wells were fixed with 2% paraformaldehyde, 20 min at room temperature, and then stained with May-Grünwald Giemsa for subsequent control of the morphology of the cells in the monolayer.

Kinetic studies The cells were first incubated with RPMI/M199 containing 30% human serum and either forskolin (FK) (10^{-5} M), dbcyclic AMP (5×10^{-4} M), IBMX (10^{-4} M) or control buffer. Cells were incubated at 37°C for various times, all incubations being stopped at the same time. Before the last 4 h of incubation, the cell monolayers were washed 3 times with RPMI/M199 (v/v) and then incubated with RPMI/M199 containing 10 $mg\ ml^{-1}$ HSA and the same agents (FK, dbcyclic AMP, IBMX or buffer) plus IL-1 for 4 h at 37°C. IL-1 was used at 0.2 $u\ ml^{-1}$ to induce tissue factor activity and at 1 $u\ ml^{-1}$ to decrease thrombomodulin activity. Thrombomodulin and tissue factor activities were then measured as described above.

Cyclic AMP assay

HSVEC were cultured in 35 mm diameter culture dishes. At confluence the cells were washed 3 times in M199/RPMI medium (v/v), and then incubated at 37°C for 15 min with 1 ml of M199/RPMI containing 1% human albumin and 100 μM IBMX (final concentration), with or without forskolin or PGE_1. All following manipulations were done on ice. The cells were rinsed twice with ice cold PBS and 500 μl ice cold PBS plus 50 μl 6.6 N perchloric acid was added. After 10 min, the supernatant was removed and particulate material was pelleted by centrifugation. The supernatant was transferred to a 5 ml polystyrene tube and cyclic AMP was extracted as described by Khym (1975) with a mixture of trioctylamine and freon (33 ml/42 ml); 2 ml of this mixture were added to the supernatant and mixed thoroughly for 2 min. The upper aqueous phases was transferred to an Eppendorf tube, lyophilized and the dry extract dissolved in the buffer provided in the commercial radioimmunoassay kit (Amersham) used for cyclic AMP measurement. The cells were then fixed with 2% paraformaldehyde for 20 min at room temperature, stained with May-Grünewald Giemsa and counted in order to normalize the level of cyclic AMP per 10^6 cells.

Immunoelectron microscopy

HSVEC grown in 35 mm dishes were fixed in paraformaldehyde-glutaraldehyde mixture (2%–0.5%), followed by permeabilization with digitonin (10^{-6} M). The cells were incubated first with specific Fab-fragments of rabbit anti-TM antibodies (30 $\mu g\ ml^{-1}$). Fab-fragments of goat anti rabbit-IgG antibodies coupled to peroxidase (Biosys, Compiègne,

France) were used as a second antibody. Peroxidase activity was visualized according to Graham & Karnovsky (1966). Post fixation in osmium tetroxide and embedding in Epon were performed according to Tougard & Picart (1986). Ultrathin sections were counterstained with uranyl acetate and examined with a Siemens Elmiskop 10.

Extraction of RNA from endothelial cells

As for concentration-effect studies, after washing, cells were incubated with M199/RPMI containing 10 mg ml^{-1} HSA and either 10^{-4} M forskolin, 1 u ml^{-1} IL-1 or both or control buffer for 2 h at 37°C. RNA was extracted from about 4×10^5 cells from each well by a classical technique with guanidium isothiocyanate (Chirgwin *et al.*, 1979). Briefly, 1 ml of guanidium isothiocyanate (4 M) cellular lysate was overlaid onto a 0.5 ml caesium chloride (5.7 M) cushion and centrifuged 3 h at 55,000 r.p.m. in the TLS rotor of a Beckman TL 100 centrifuge. The pellet was resuspended in 200 µl of 0.3 M sodium acetate and precipitated with ethanol. After centrifugation, the pellet was dried and resuspended in water and the RNA concentration was determined by O.D. at 260 nm.

Reverse transcription and polymerase chain reaction method

For each assay condition, reverse transcription (RT) was performed on 100 ng of total cellular RNA. The mRNA was reverse transcribed directly in the polymerase chain reaction (PCR) buffer (50 mM KCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$, 0.001% gelatine, pH 8.3) with 3 pmol of the 3' PCR specific primer (respectively for thrombomodulin, tissue factor or glyceraldehyde phosphodeshydrogenase (GAPDH)), 1.25 mM of each dNTP and 20 units of MMLV reverse transcriptase in a final volume of 30 µl, at 37°C for 30 min. Then 10 pmol of each 3' and 5' primer was added and the reaction mixture was adjusted to a final volume of 50 µl with PCR buffer, pH 10. After an initial denaturation step of 1.5 min at 94°C, the tubes were put on ice and 1 unit of Taq polymerase was added. The mixture was then covered with about 40 µl of mineral oil to avoid evaporation and the tubes were transferred to a DNA thermal cycler for amplification by PCR (Perkin Elmer Cetus).

The primers used for thrombomodulin amplification were 5' primer: 5'CATGTGCGACCGGCTACCGGCTGGCGG 3', corresponding to nucleotides 1071 to 1100, and 3' primer: 5'AGGGGCTGGCACTGGTACTCGCAGTTGGC 3', corresponding to nucleotides 1262 to 1291, according to Wen *et al.* (1987). PCR conditions were 30 cycles, each consisting of 2 min denaturation at 94°C, 2 min hybridization at 51°C and 3 min elongation at 72°C.

The primers used for tissue factor amplification were 5' primer: 5' ACTACTGTTTCAGTGTTCAAGCAGTGATTC 3', corresponding to nucleotides 722 to 751, and 3' primer: 5' ATTCAGTGGGGAGTTCTCCTTCCAGCTCTG 3', corresponding to nucleotides 925 to 954, according to Scarpati *et al.* (1987). PCR conditions were 30 cycles of 1.5 min denaturation at 94°C, 2 min hybridization at 62°C and 3 min elongation at 72°C.

GAPDH mRNA was chosen as housekeeping gene (Maier *et al.*, 1990). The GAPDH mRNA level was thus used as a control to correct for errors in RNA concentration in the starting material an amplification of GAPDH being performed on the same aliquot of cellular RNA for each condition tested in thrombomodulin and tissue factor assay. The primers used were 5' primer: 5' CCACCCATGGCAAATTCCATGGCA 3' and 3' primer: 5' TCTAGACGGCAGGTCAGGTCCACC 3', according to Maier *et al.* (1990). PCR conditions were 30 cycles of 1.5 min denaturation at 94°C, 2 min hybridization at 62°C and 3 min elongation at 72°C.

An aliquot of each sample was loaded onto a 2% agarose gel. After migration the gel was stained with ethidium bromide and photographed with Polaroid 665 negative film. Quantification was performed by scanning the bands with a Biocom 2000 Image Analyzer (Les Ulis, France) and by comparison with the bands obtained for each condition with the GAPDH housekeeping mRNA on the same gel.

Reagents

Human protein C, α-thrombin (3000 NIH u mg^{-1}) and factors X and VII were purified from human plasma as previously described (Freyssinet *et al.*, 1988). Purified human fibronectin was prepared according to a published method (Ruoslahti *et al.*, 1982). Materials were obtained from the following sources: cell culture media (M199, RPMI 1640, HEPES, L-glutamine, penicillin, streptomycin, fungizone and trypsin-EDTA solution [trypsin (0.5 g l^{-1})/EDTA (0.2 g l^{-1})/NaCl (0.85 g l^{-1})]) were from Gibco, Paisley, UK; phosphate buffered saline (PBS) and endotoxin-free human serum albumin (HSA) were from the Centre Régional de Transfusion Sanguine, Strasbourg, France; hirudin was from Stago, Asnières, France; chromogenic substrates S2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride) and S2765 (N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride) were from Kabi Vitrum, Stockholm, Sweden; human recombinant tumour necrosis factor-α (TNF) (2.10^7 u mg^{-1}, Mr 45,000) and human recombinant interleukin-1-β (IL-1) (10^7 u mg^{-1}, Mr 17,500) were from Boehringer, Mannheim, Germany; forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), prostaglandin E_1. (PGE_1), N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dbcyclic AMP), N^2, 2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate (dbcyclicGMP), agarose type II_A and mineral oil were from Sigma, St. Louis, MO, U.S.A. Human serum was prepared from a pool of 100–150 healthy blood donor negative for hepatitis B virus or HIV. The serum was complement inactivated at 56°C for 30 min. Tris, sodium acetate, guanidium isothiocyanate, kalium chloride, magnesium chloride were obtained from Merck (Rathway, NY, U.S.A.). Caesium chloride was from BRL (Gaithersburg, MD, U.S.A.). SDS was obtained from Pierce Chemical Co (Paris, France). Triton X-100, DNA molecular weight markers VI, desoxyribonucleotides triphosphate and SP6-T7 transcription kit were from Boehringer (Mannheim, Germany). Taq polymerase was purchased from Cetus (Norwalk, CT, U.S.A.). Polaroid film type 665 were from Kodak Company (Rochester, NY, U.S.A.). The oligonucleotides were synthesized by the Laboratoire de Génétique Moléculaire des Eucaryotes (Strasbourg, France); a monoclonal antibody based ELISA-kit against human TM was a kind gift of Dr Amiral, Serbio, Paris, France.

Statistical analysis

The variations of tissue factor and thrombomodulin activities were compared by variance analysis followed by Duncan's multiple comparison test. *P* values less than 0.05 were considered to be statistically significant.

Results

Effects of forskolin

Forskolin (10^{-6} to 10^{-4} M) caused a statistically significant concentration-dependent decrease of tissue factor activity induced by IL-1 (Figure 1a). IL-1 (0.2 u ml^{-1}) induced tissue factor activity from 7.7 ± 1.6 (basal value) to 59 ± 12 pM generated factor Xa min^{-1} per 10^4 cells (mean ± s.e.mean, $n = 3$); 10^{-4} M forskolin caused 80 ± 5% inhibition of this activity after 4 h of incubation. Thrombomodulin activity, under basal conditions, was enhanced by 26 ± 8% after 4 h of incubation with 10^{-4} M forskolin (Figure 1b). One u ml^{-1} IL-1 caused a 15% decrease of basal thrombomodulin

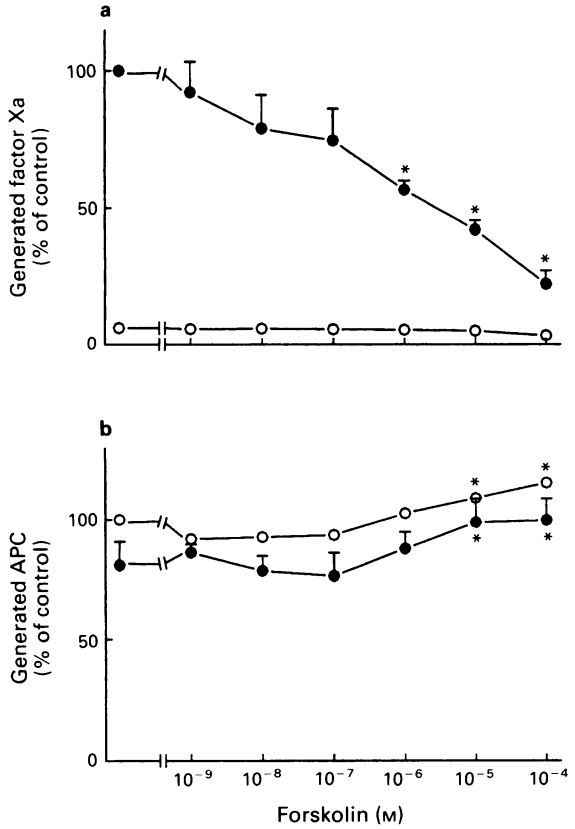

Figure 1 Concentration-effect relationship for forskolin on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC. Confluent monolayers of HSVEC were exposed to the concentrations of forskolin indicated for 4 h in the presence of either interleukin-1 (IL-1) (●) or control buffer (○) tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of three different experiments; each condition was performed in 6 wells. (a) Represents tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of HSVEC stimulated with 0.2 u ml^{-1} IL-1 in the absence of forskolin. (b) Represents thrombomodulin activities: results are expressed as % of control value of thrombomodulin activity at the surface of unstimulated HSVEC in the absence of forskolin. One u ml^{-1} IL-1 was used to decrease thrombomodulin activity. $^*P<0.05$ vs control (i.e. no forskolin) under the same experimental conditions.

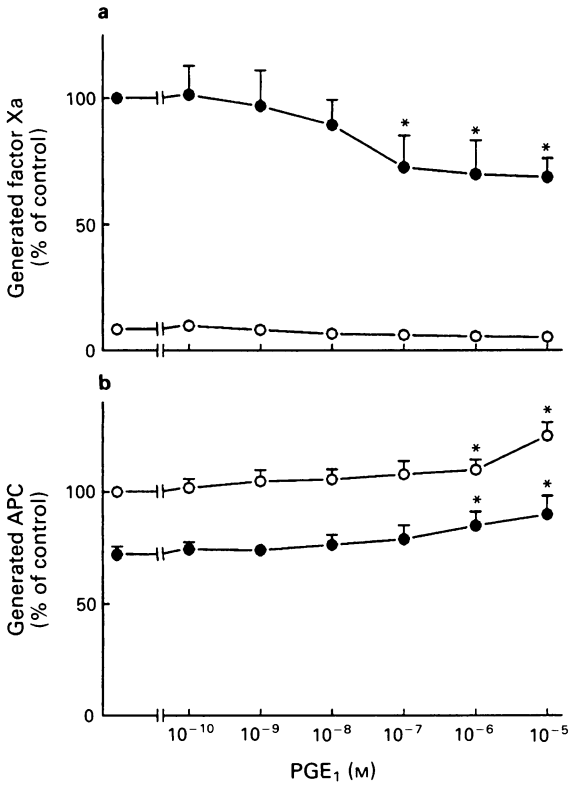

Figure 2 Concentration-effect relationship for prostaglandin E_1 (PGE_1) on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC. Confluent monolayers of HSVEC were exposed to the concentrations of PGE_1 indicated for 4 h in the presence of either interleukin-1 (IL-1) (●) or control buffer (○). Tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of four different experiments; each condition was performed in 6 wells. (a) Represents tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of HSVEC stimulated with 0.2 u ml^{-1} IL-1 in the absence of PGE_1. (b) Represents thrombomodulin activities: results are expressed as % of control value for thrombomodulin activity at the surface of unstimulated HSVEC in the absence of PGE_1. IL-1 (1 u ml^{-1}) was used to decrease thrombomodulin activity. $^*P<0.05$ vs control under the same experimental conditions.

activity from 22 ± 5 to 18.6 ± 7 pM generated APC min^{-1} per 10^4 cells (mean ± s.e.mean, $n = 3$). Forskolin caused an increase of thrombomodulin activity also in the IL-1 treated cells (Figure 1b). When forskolin at 10^{-4} and 10^{-5} M was added with IL-1, thrombomodulin activity recovered to the basal level of untreated cells.

Effects of prostaglandin E_1

PGE_1 caused a concentration-dependent decrease of induced tissue factor activity. The maximal decrease caused by 10^{-6} and 10^{-5} M PGE_1 reached, respectively, 30 ± 13% and 32 ± 7% after 4 h (Figure 2a). PGE_1 10^{-5} M caused a 25 ± 6% increase of thrombomodulin activity under basal conditions (Figure 2b). IL-1, 1 u mol^{-1} caused a 33 ± 3% decrease of basal thrombomodulin activity. Thrombomodulin activity was not significantly different from basal when PGE_1 10^{-5} M was added at the same time as IL-1.

Effects of 3-isobutyl-1-methylxanthine

IBMX caused a concentration-dependent decrease of induced tissue factor activity, the maximal decrease caused by 5 × 10^{-4} M IBMX reached 56 ± 3% (Figure 3a). IBMX caused a slight but not significant increase of basal thrombomodulin activity and of thrombomodulin activity decreased by IL-1 (Figure 3b).

Effects of dbcyclic AMP

dbCyclic AMP caused a concentration-dependent decrease of induced tissue factor activity and a simultaneous increase in thrombomodulin activity (Figure 4a and b).

dbCyclic AMP (10^{-3} M) decreased tissue factor activity induced by thrombin (to 45 ± 1%), by IL-1 (to 35 ± 12%) or by TNF (to 46 ± 13%) of controls. The same concentration of dbcyclic AMP increased basal thrombomodulin activity by 28 ± 3%. Thrombin alone did not alter constitutive thrombomodulin expression. dbCyclic AMP added with thrombin increased thrombomodulin activity more than dbcylic AMP alone, this increase reaching 45 ± 18% with thrombin plus 10^{-3} M dbcyclic AMP and 35 ± 12% with thrombin plus 5 × 10^{-4} M dbcyclic AMP. dbCyclic AMP, 10^{-3} M, only partially reversed the inhibition caused by IL-1 or TNF.

Concentration-effect studies of dbcyclicGMP were performed on the same cell population as those used to investigate the effect of dbcyclic AMP. In contrast to dbcyclic AMP, dbcyclicGMP (10^{-5} M to 10^{-3} M) did not modulate tissue factor or thrombomodulin activities (Figures 4c and d),

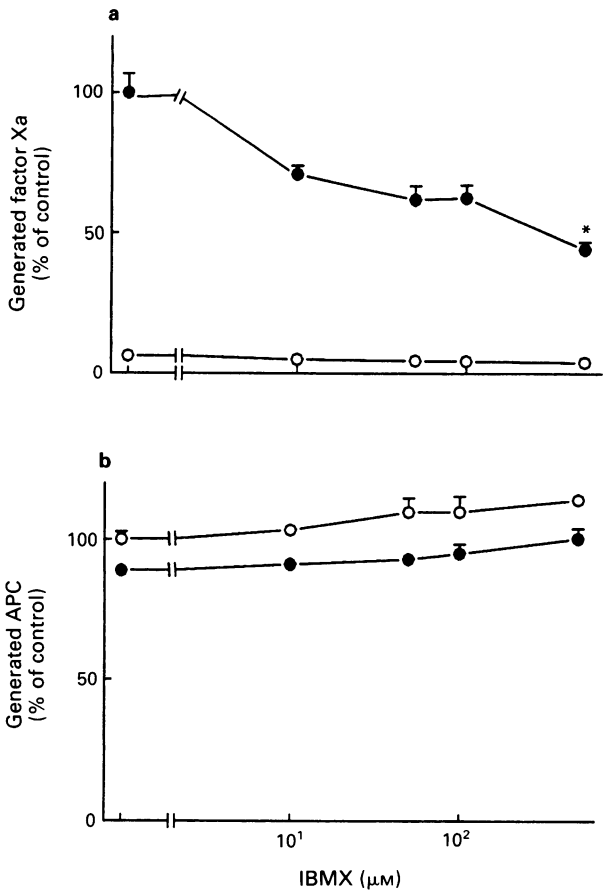

Figure 3 Concentration-effect relationship for 3-isobutyl-1-methyl xanthine (IBMX) on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC. Confluent monolayers of HSVEC were exposed to the concentrations of IBMX indicated for 4 h in the presence of either interleukin-1 (IL-1) (●) or control buffer (○). Tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of three different experiments; each condition was performed in 6 wells. (a) Represents tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of HSVEC stimulated with 0.2 u ml^{-1} IL-1 in the absence of forskolin. (b) Represents thrombomodulin activities: results are expressed as % of control value for thrombomodulin activity at the surface of unstimulated HSVEC in the absence of forskolin. IL-1 (1 u ml^{-1}) was used to decrease thrombomodulin activity. *$P<0.05$ vs control under the same experimental conditions.

the variations of these activities shown in the figures not being statistically significant.

Kinetic studies of the action of forskolin, IBMX and dbcyclic AMP

Forskolin (10^{-5} M), IBMX (10^{-4} M) or dbcyclic AMP (5×10^{-4} M) (final concentrations) were added to the cells for 4 to 48 h before measurement of tissue factor or thrombomodulin activities. At each time point, forskolin significantly decreased IL-1 induced tissue factor activity (from 69 ± 3% after 4 h to 64 ± 2% of control after 48 h of incubation) (Table 1A). The decrease caused by IBMX was maximal after 4 h (40 ± 18%) and was weaker and not statistically significant at the other incubation times. The effects of forskolin and IBMX added together were additive; the decrease of induced tissue factor activity in the presence of both agents reached 80% at each incubation time. Similar to forskolin, dbcyclic AMP inhibited induced tissue factor activity at each time point and by approximately the same extent. Forskolin and dbcyclic AMP increased thrombomodulin expression at each incubation time (Table 1b). The effect of forskolin was little different from 12 to 48 h (from 26 ± 7% increase at 12 h to 30 ± 9% at 48 h), but this increase was significant. IBMX alone did not influence basal thrombomodulin activity. However, addition of IBMX enhanced the increase of thrombomodulin expression induced by forskolin. At 4 h, thrombomodulin stimulated with forskolin was 108 ± 14% as compared to control; while in the presence of 10^{-4} M IBMX, this activity reached 129 ± 5%. At 24 h and 48 h, addition of IBMX also enhanced the increase induced by forskolin. This additive effect of forskolin was statistically significant after 4 and 48 h of incubation. Dibutyryl cyclic AMP exerted a similar effect to that of forskolin: dbcyclic AMP increased thrombomodulin activity with a maximal effect between 12 and 24 h. When thrombomodulin activity was decreased by IL-1, the presence of forskolin or dbcyclic AMP totally reversed this inhibition at 12 and 24 h. At 48 h only forskolin still exerted this effect; forskolin in combination with IBMX induced a 100% recovery of the basal activity after 4 or 12 h and a significant increase over basal values of thrombomodulin activity after 24 or 48 h, levels reaching respectively 121 ± 10% and 126 ± 16% of controls.

Thrombomodulin and tissue factor mRNA levels

The level of mRNA for tissue factor or thrombomodulin was assayed in endothelial cells using the reverse transcriptase-polymerase chain reaction method (RT-PCR). The amount of mRNA was normalized to that of the mRNA for GAPDH, a housekeeping gene which is not affected by the different agents used in the present study.

Resting endothelial cells showed no detectable expression of tissue factor mRNA. Incubation with 1 u ml^{-1} IL-1 for 2 h induced the appearance of tissue factor mRNA, while coincubation with 10^{-4} M forskolin inhibited by 1.5 fold this appearance of tissue factor mRNA (Figure 5). There was constitutive expression of thrombomodulin mRNA in endothelial cells and preincubation of the cells with 10^{-4} M forskolin induced a 2.5 fold increase in thrombomodulin mRNA levels. One u ml^{-1} IL-1 did not change the thrombomodulin mRNA level after 2 h of incubation; forskolin coincubated with IL-1 increased mRNA above the control level.

Ultrastructural localization of thrombomodulin

Immunoelectronmicroscopy was used to localize more precisely thrombomodulin in intact endothelial cells monolayers under basal conditions and after forskolin treatment. Under basal conditions, a thin labelling appeared on the plasma membrane (Figure 6). In forskolin-stimulated cells, this labelling was more dense and there was intense intracellular labelling, especially in the endoplasmic reticulum.

Effects of forskolin and PGE_1 on cyclic AMP levels

In an attempt to correlate changes in expression of thrombomodulin and tissue factor with actual rather than inferred changes in cyclic AMP levels, we carried out a small series of experiments in which intracellular cyclic AMP was measured following exposure of cells to either forskolin or PGE_1 (see Methods). As shown in Table 2, after a 15 min incubation, 10^{-4} M forskolin caused a 2.6 fold increase in total intracellular cyclic AMP levels, 10^{-5} M forskolin a 1.7 fold increase and 10^{-5} M PGE_1 a 1.4 fold increase compared with controls.

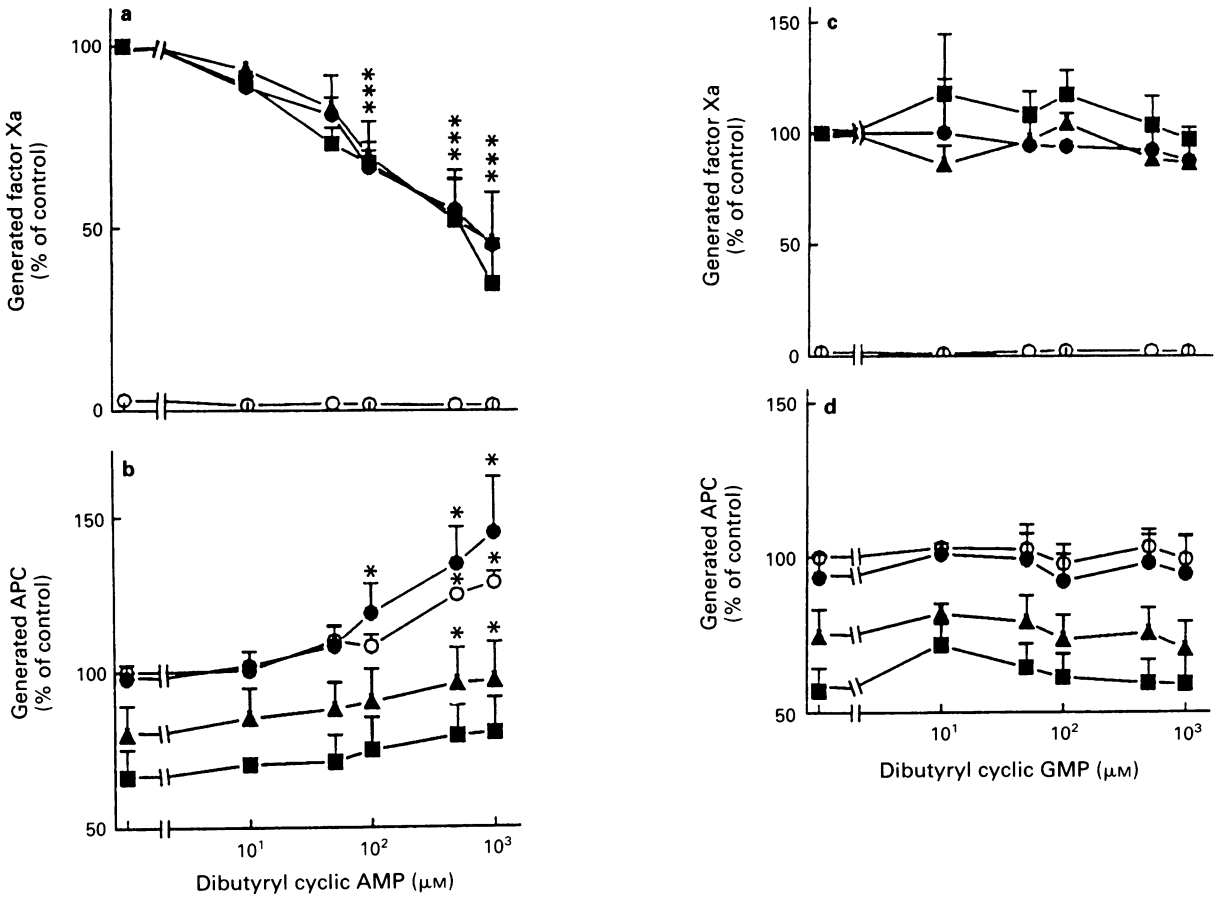

Figure 4 Concentration-effect relationship for dibutyryl cyclic AMP (dbcyclic AMP) and dbcyclic GMP on the expression of tissue factor and thrombomodulin activities at the surface of HSVEC, stimulated by either thrombin (1 u ml^{-1}), interleukin-1 (IL-1) (1 u ml^{-1}) or tumour necrosis factor (TNF) (1 ng ml^{-1}). Confluent monolayers of HSVEC were exposed to the concentrations indicated of dbcyclic AMP (a,b) or dbcyclic GMP (c,d) with either thrombin (●), IL-1 (■), TNF (▲) or control buffer (○) for 4 h. Tissue factor and thrombomodulin activities were measured as described in Methods. Points correspond to the mean + s.e.mean of five experiments; each condition was performed in triplicate. (a) and (c) represent tissue factor activities: results are expressed as % of control value for tissue factor activity at the surface of stimulated HSVEC. (b) and (d) represent thrombomodulin activities: results are expressed as % of control value for thrombomodulin activity at the surface of unstimulated HSVEC. *$P<0.05$ vs control under the same experimental conditions.

Table 1 Time course of the effect of forskolin (10^{-5} M), 3-isobutyl-1-methyl xanthine (IBMX) (10^{-4} M) and dbcyclic AMP (5×10^{-4} M) on tissue factor and thrombomodulin activities

A *Tissue factor activity* (% inhibition)

	4 h	12 h	24 h	48 h
Control	0	0	0	0
Forskolin	69 ± 3 *	54 ± 12*	63 ± 8 *	64 ± 2 *
IBMX	40 ± 18*	22 ± 5	15 ± 27	25 ± 12
Forskolin + IBMX	80 ± 2 *	68 ± 12*	78 ± 4 *	77 ± 4 *
dbcyclic AMP	30 ± 28*	45 ± 17*	55 ± 28*	47 ± 17*

Tissue factor activities were measured at the surface of HSVEC after stimulation during 4 h by 0.2 u ml^{-1} IL-1.
Results are the mean ± s.e.mean of three separate experiments.
*$P<0.05$ for significant difference from the control at the same time point.

B *Thrombomodulin activity* (% of control)

	IL-1 (u ml^{-1})	4 h	12 h	24 h	48 h
Control	0	100 ± 14	100 ± 5	100 ± 10	100 ± 12
	1	86 ± 14	89 ± 10	82 ± 8	82 ± 7
Forskolin	0	108 ± 16	126 ± 7 *	124 ± 9 *	130 ± 9 *
	1	91 ± 14	103 ± 9 *	102 ± 13*	112 ± 10*
IBMX	0	99 ± 14	99 ± 2	98 ± 4	106 ± 11
	1	81 ± 11	80 ± 8	78 ± 3	91 ± 5
Forskolin + IBMX	0	129 ± 5 *	126 ± 6 *	147 ± 19*	153 ± 12*
	1	105 ± 12*	103 ± 11*	121 ± 10*	126 ± 16*
dbcyclic AMP	0	112 ± 16	131 ± 5 *	128 ± 4 *	115 ± 9
	1	93 ± 5	106 ± 8	107 ± 8	94 ± 2

Thrombomodulin activities were measured at the surface of stimulated for 4 h with 1 u ml^{-1} IL-1 or unstimulated HSVEC.
Results are the mean ± s.e.mean of three separate experiments.
*$P<0.05$ for significant difference from the control at the same time point.

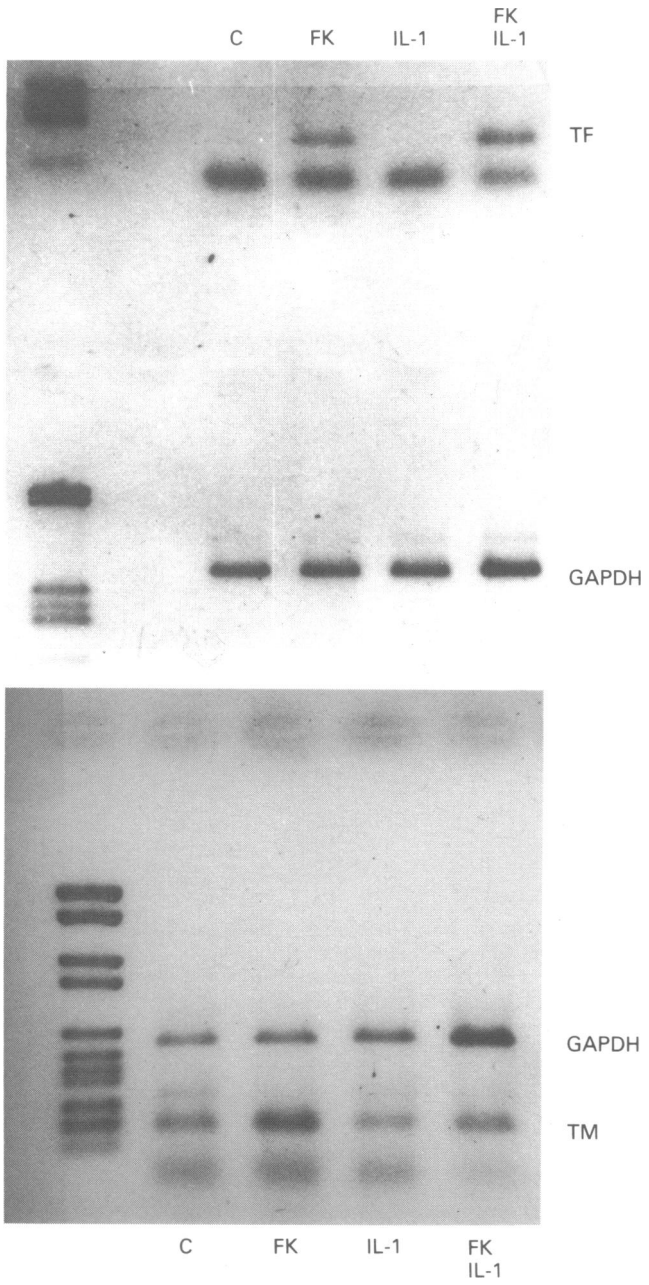

Figure 5 Effect of forskolin on thrombomodulin (TM) and tissue factor (TF) mRNA level in endothelial cells. Confluent monolayers of HSVEC were incubated for 2 h, at 37°C with interleukin-1 (IL-1) or without (C: control) 1 u ml^{-1} IL-1 and with or without 10^{-4} M forskolin (FK). Total cellular RNA was prepared and 100 ng was used for RT-PCR as described in Methods. GAPDH = glyceraldehyde phosphodehydrogenase.

Discussion

The aims of this study were to investigate if substances which are known to cause a rise in intracellular cyclic AMP levels could affect procoagulant and anticoagulant properties of endothelial cells. We found through the use of such agents, in this case forskolin and PGE_1, that it was possible to manipulate both tissue factor and thrombomodulin activities on HSVEC in a way that would be expected to preserve the antithrombotic potential of these cells. More specifically, we provide evidence that a rise in endothelial cell cyclic AMP levels caused an inhibition of tissue factor expression, enhanced thrombomodulin activity and reversed the decrease of thrombomodulin activity caused by cytokines.

Galdal *et al.* (1984, 1985) demonstrated an inhibition of the expression of tissue factor on human umbilical vein endothelial cells (HUVEC) by agents known to increase intracellular cyclic AMP levels. This effect has also been described for other cell types such as mouse trophoblasts (Dalaker & Prydz, 1986) or human monocytes (Lyberg, 1983). An increase of thrombomodulin activity in response to experimental manipulations has only recently been demonstrated. Foetomodulin, a surface protein marker of foetal development, was shown to be modulated by cyclic AMP (Imada *et al.*, 1987) and was later identified with thrombomodulin (Imada *et al.*, 1990). During the course of our study, other authors have described upregulation of thrombomodulin by cyclic AMP elevating agents in two human megakaryoblastic leukaemia cell lines (Ito *et al.*, 1990), in human umbilical vein endothelial cells (Ishii *et al.*, 1990; Hirokawa & Aoki, 1990; 1991; Maruyama *et al.*, 1991; Horie *et al.*, 1992), in mouse haemangioma cells (Maruyama *et al.*, 1991) and in F9 embryonal carcinoma cells (Weiler-Guettler *et al.*, 1992).

In contrast to the above mentioned studies, our experiments concerning the effects of cyclic AMP on tissue factor and thrombomodulin activities and mRNA levels were performed using the same cell population. This is of key importance for meaningful comparison of these results, since it has been shown that cell origin or cell culture conditions have major effects on the expression of tissue factor and thrombomodulin (Archipoff *et al.*, 1991).

IBMX reduced IL-1-induced tissue factor activity, while the coincubation of IBMX with forskolin showed an additive but not synergistic effect on the decrease of induced tissue factor activity. A synergistic effect would have been expected when combining a phosphodiesterase (PDE) inhibitor with an adenylate cyclase activator (Sutherland *et al.*, 1968). IBMX inhibits endothelial cell PDE (Lugnier & Schini, 1990), but also has other effects not mediated by cyclic AMP. Dasarathy & Fanburg (1988) have demonstrated that IBMX elevates angiotensin converting enzyme activity in endothelial cells by a calcium-calmodulin mediated process. The importance of calcium and calmodulin in the regulation of tissue factor expression in HUVEC has been stressed previously (Galdal *et al.*, 1984; 1985; Dalaker & Prydz, 1986; Callahan *et al.*, 1990; Bach & Rifkin, 1990). It is thus possible that IBMX does not regulate tissue factor expression on endothelial cells solely through inhibition of PDE, but also through other mechanisms that could involve interaction with calmodulin or other proteins.

Forskolin and PGE_1 decreased induced tissue factor activity and simultaneously increased thrombomodulin activity in a concentration-dependent manner. The down regulation of tissue factor and upregulation of thrombomodulin caused by forskolin (10^{-4} and 10^{-5} M) were statistically significant after 4 h of incubation. PGE_1 (10^{-5} M) was less effective. The difference in the effect of forskolin and PGE_1 may be due to the mechanisms through which these two drugs act on the cells; forskolin acts directly on the catalytic subunit of adenylate cyclase and desensitization to forskolin does not occur (Seamon & Daly, 1986), while PGE_1 may act through specific receptors on endothelial cells. We suggest that desensitization could explain the lower activity of PGE_1 compared to forskolin. A similar desensitization has been described in platelets (Ashby, 1989): activation of platelet adenylate cyclase by PGE_1 could be followed by a slow reversible transition of adenylate cyclase to an inactive form, through a distinct inhibitory receptor for PGE_1, leading to desensitization. Our results also showed no variation with time in the response to forskolin, while there was a time-dependent decrease in the effect of PGE_1 (data not shown). This adds support to the suggestion that the lower effect of PGE_1 is due to receptor desensitization more than to alteration of intracellular responses to cyclic AMP.

An increase of cyclic GMP *in vivo* in endothelial cells can be caused by EDRF/nitric oxide (NO) or by NO containing factors liberated by the cells themselves (Boulanger *et al.*,

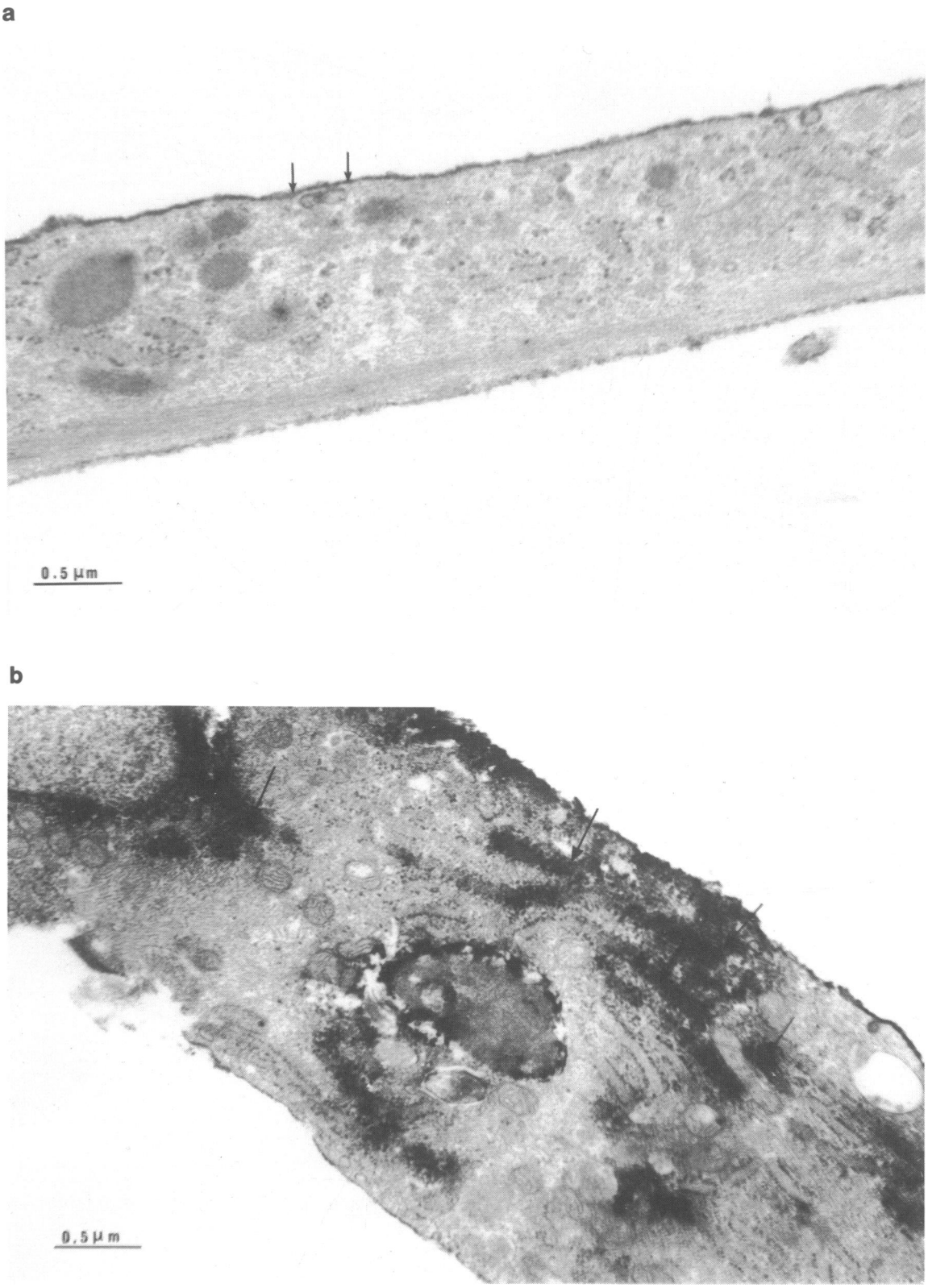

Figure 6 Immunoperoxidase staining of HSVEC under baseline and forskolin-stimulated conditions. HSVEC were permeabilized and incubated with antithrombomodulin antibodies. Unstimulated cells (a) show labelling on the apical plasma membrane, whereas forskolin (b)-stimulated cells contain dense staining in the reticulum in continuity with the plasma membrane.

1990). NO is known to induce vasodilatation of vessels and to have platelet antiaggregatory properties. Thus, it contributes to the antithrombotic properties of endothelium. In our experiments, the cyclic GMP at concentrations of up to 10^{-3} M had no significant effect on thrombomodulin and tissue factor activities (Figure 5c and d). Other compounds have been shown to increase NO and intracellular cyclic GMP in endothelial cells, for example, bradykinin, atriopeptin II or SIN-1 (Marin & Sanchez-Ferrer, 1990; Boulanger *et al.*, 1990; Needleman *et al.*, 1989; Gerzer *et al.*, 1988). However, although not reported here, we found that bradykinin (5×10^{-9}–5×10^{-7} M), atriopeptin II (5×10^{-8}–5×10^{-6} M)

Table 2 Effect of forskolin and prostaglandin E_1 (PGE_1) on cyclic AMP levels in HSVEC

	Cyclic AMP (pmol/10^6 cells)
Control	1.9 ± 0.16
Forskolin 10^{-4} M	5.0 ± 0.6*
Forskolin 10^{-5} M	3.2 ± 0.1
PGE_1 10^{-5} M	2.7 ± 0.2

HSVEC were incubated 15 min with forskolin, PGE_1 or control buffer and assayed for cyclic AMP by radioimmunoassay. Results are the mean ± s.e.mean of five separate experiments.
*$P < 0.05$ vs control.

or SIN-1 (10^{-8}–10^{-3} M) did not influence constitutive thrombomodulin or tissue factor induced by IL-1, thrombin or phorbol myristate acetate (Archipoff *et al.*, unpublished). Thus our results suggest that NO/EDRF does not play a major role in the regulation of tissue factor and thrombomodulin activities through stimulation of guanylate cyclase.

Sutherland *et al.* (1968) proposed four criteria to determine if cyclic AMP was involved in particular physiological responses: (i) drugs used should stimulate the formation of cyclic AMP, (ii) phosphodiesterase inhibitors should mimic or potentiate the action of the drug, (iii) cyclic AMP derivatives should cause the same effect as the drug, (iv) increased phosphorylation of protein(s) should be observed. In summary, our results confirm three of the four conditions proposed by Sutherland *et al.* (1968) to implicate cyclic AMP in a pharmacological regulation: (i) forskolin and PGE_1 caused an increase of intracellular cyclic AMP levels in HSVEC, (ii) IBMX enhanced the effects of forskolin and (iii) the addition of dbcyclic AMP, the permeant membrane analogue of cyclic AMP, caused the same effect on tissue factor and thrombomodulin activities as the stimulation of adenylate cyclase by forskolin and PGE_1. The increase of protein phosphorylation remains to be verified. We show here that the modulation of thrombomodulin or tissue factor activities is already observed at concentrations of PGE_1 and forskolin that have no significant effect on total cyclic AMP levels. A similar situation has often been described in other cell systems. For example, PDE inhibitors are potent inhibitors of platelet aggregation at concentrations that do not increase platelet cyclic AMP levels measured by a radioimmunoassay (Tsien *et al.*, 1982). Increases in cyclic AMP are usually observed at concentrations above those necessary for inhibiting platelet function. This could be caused by the existence of subcellular compartments for the cyclic nucleotide and/or lack of sensitivity of the assay (Ashida *et al.*, 1992). Also, correlation between cyclic AMP elevation and inotropic effects of PDE III has been equivocal. However it is widely accepted that these PDE inhibitors act through an increase in cyclic AMP, the discrepancy being explained in terms of compartmentation of cyclic AMP or cyclic AMP-dependent kinases (Nicholson *et al.*, 1991). Finally, only marginal increases of cyclic AMP were found at concentrations of forskolin that inhibit the permeability of endothelial cells monolayer (Langeler *et al.*, 1991); these authors suggest that this observation can be explained by local enhancements of cyclic AMP level by forskolin in endothelial cells. Our results add to the hypothesis that subcompartments for cyclic AMP exist in endothelial cells.

In conclusion, we have demonstrated that the enhancement of cell-surface thrombomodulin activity in HSVEC by agents which are known to increase intracellular cyclic AMP was associated with an increase in thrombomodulin mRNA. The localization of thrombomodulin by electron microscopy showed that newly synthesized protein was bound to the membrane structure. The discrepancy of the effect of forskolin on thrombomodulin activity (25% increase as compared to control) and on thrombomodulin mRNA level (250% increase as compared to control), could be explained by the fact that the newly synthesized thrombomodulin was stored in the reticulum and just partially exposed on the surface of the cell membrane. To confirm this hypothesis, we have performed preliminary experiments in order to evaluate thrombomodulin antigen levels in cell lysates using monoclonal antibody-based ELISA (Amiral *et al.*, 1991; Bartha *et al.*, 1993). Compared to the control conditions, addition of 10^{-4} M forskolin increased 1.6 times the thrombomodulin-antigen content of HSVEC lysates (from 266 ± 45 ng ml^{-1} thrombomodulin for control to 441 ± 18 ng ml^{-1} for forskolin, $n = 2$), while it did not influence the thrombomodulin-antigen level of conditioned medium (control = 10.8 ± 1.8 ng ml^{-1} thrombomodulin, forskolin = 9.9 ± 0.3 ng ml^{-1} thrombomodulin, $n = 2$). Although preliminary, these results are in accordance with the conclusions of the electron microscopy studies. Incubation of the cells with 1 u ml^{-1} IL-1 induced a decrease in thrombomodulin activity with no significant change in thrombomodulin mRNA levels (Figure 6). This suggests that IL-1 downregulates thrombomodulin activity in these conditions mainly through internalization and degradation, rather than through regulation of gene expression. However, it cannot be excluded that in other experimental settings, IL-1 can also influence thrombomodulin expression directly (Hirokawa & Aoki 1991). We also demonstrated that the inhibition of IL-1 induced tissue factor activity was associated with a decrease in tissue factor mRNA. This suggests that an increase in intracellular cyclic AMP: (i) enhances transcription of thrombomodulin mRNA and synthesis of the protein with subsequent increase of thrombomodulin on the cell surface: (ii) plays role in regulating transcription and/or degradation of tissue factor mRNA. Therefore, a rise in endothelial cyclic AMP levels has two complementary effects that could lead to decrease in the generation of thrombin at the endothelial cell surface during inflammatory or thrombotic episodes. Moreover, previous studies have shown that increased intracellular cyclic AMP levels in endothelial cells: (i) induce a reduction in plasminogen activator inhibitor-1 secretion coupled to a potentiation of tissue type plasminogen activator release (Santell & Levin, 1988), (ii) contribute to the regulation of permeability and maintenance of endothelial integrity (Yamada *et al.*, 1990) and provide protection against thrombin-induced permeability increases (Casnocha *et al.*, 1989) and (iii) increase 13-HODE synthesis, thus limiting endothelial cell reactivity towards platelets (Haas *et al.*, 1990). All these effects of cyclic AMP contribute to the antithrombotic properties of endothelial cells. Studies of the major PDE isoenzymes have shown that the characteristics of the enzymes isolated from endothelial cells differ from those of smooth muscle cells (Lugnier & Schini, 1990; Souness *et al.*, 1990; Kishi *et al.*, 1992). Since PDE types II and IV are the only isoenzymes present in endothelial cells, specific inhibition of these PDE forms could lead to an endothelium-specific increase in cyclic AMP. Although no data are yet available on the PDE isoforms of human endothelial cells, these data suggest that the use of such specific PDE inhibitors could ultimately lead to an enhancement of the antithrombotic capacity of the endothelium with only limited side effects.

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