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Activation of Human Vascular Endothelial Cells by Factor Xa: Effect of Specific Inhibitors

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ABSTRACT. Recently, human umbilical vein endothelial cells (HUVEC) have been shown to express functional high-affinity receptors for factor Xa, which may be of importance in the regulation of coagulation and homeostasis of the vascular wall. In this paper, we demonstrate that when added to cultured HUVEC, factor Xa was a potent mitogen, stimulating an increase in cell number at a 0.3 to 100 nM concentration. The same doses of factor Xa also increased intracellular free calcium levels and phosphoinositide turnover. When added to confluent HUVEC, factor Xa induced the expression of tissue factor and the release of tissue-type plasminogen activator and plasminogen activator inhibitor-1 without affecting urokinase expression. Indirect (antithrombin-pentasaccharide) and direct (DX9065) inhibitors of factor Xa affected all these activities of factor Xa in a dose-dependent manner. Taken together, these data show that the activities induced by factor Xa on HUVEC were dependent on its catalytic activity and could be inhibited by both direct and indirect factor Xa inhibitors. BIOCHEM PHARMACOL **57**;6:603–610, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. factor Xa, endothelial cells, calcium, proliferation, TPA, PAI-1, tissue factor

Among their numerous functions, vascular endothelial cells have been shown to provide an appropriate surface for the assembly of the prothrombinase complex which converts prothrombin into thrombin [1]. However, these cells have also been reported to synthesize both potent procoagulant and antithrombotic proteins [2], and the efficiency of endothelial cells as a procoagulant surface has not yet been clarified. In this context, considerable interest has been focused on the identification and characterization of binding sites for factor Xa on vascular endothelial cells. Factor Xa has been shown to bind to bovine aortic endothelial cells [3], alveolar macrophages [4], leukocytes [5–7], platelets [8], hepatoma cells, and bladder carcinoma J82 cells [9, 10]. It has been shown that factor Xa binds to endothelial cells both directly, via AT† [11], and through membrane-bound factor Va [3]. Moreover, Altieri and Edgington have recently reported that a membrane protein similar to the light chain of factor Va might be the membrane receptor of factor Xa on monocytes [7]. This protein, called EPR-1, behaved as a co-factor for factor Xa to catalyze prothrombin activation in the absence of added factor Va [12]. Recently, we and others demonstrated the existence of such a population of high-affinity functional factor Xa binding sites in human vascular endothelial cells and showed that exposure of HUVEC to factor Xa induced phosphoinositide turnover and an increase in $[Ca^{2+}]_i$ [13, 14]. Most importantly, through binding to this receptor, factor Xa was also a potent mitogen for endothelial cells [13]. Moreover, a recent paper by Papapetropoulos *et al.* [15] showed increased interleukin-6 release by HUVEC after treatment with factor Xa.

The aim of this work was to study the effects of factor Xa on other endothelial cell functions such as the release of proteases of the fibrinolytic system (tissue-type plasminogen activator or urokinase) and PAI-1 or tissue factor expression. We also determined the effect of two factor Xa inhibitors with regard to different factor Xa-induced cell responses. The inhibitors studied were SR34006, a newly developed pentasaccharide with high affinity for AT which has been found to elicit a high and specific AT-mediated anti-factor Xa activity *in vitro* [16], and DX9065, the first member of a newly developed series of synthetic, selective, and direct inhibitors of factor Xa [17].

MATERIALS AND METHODS

Materials

Factor Xa from human plasma was purchased from Enzyme Research Laboratories. Purified human AT was from Chromogenix. SR34006, a pentasaccharide which represents the minimal sequence on the heparin chains, interacting with

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† *Abbreviations:* HUVEC, human umbilical vein endothelial cells; AT, antithrombin III; EPR-1, effector protease receptor-1; TPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; FBS; fetal bovine serum; TNF-α, tumor necrosis factor-α; u-PA, urokinase; LPS, lipopolysaccharide; ECGS, endothelial cells growth supplement; OD, optical density; PSS, physiological salt solution; and $[Ca^{2+}]_i$, intra-cellular free Ca^{2+} concentration.

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AT and hirudin (rHV2-Lys 47 variant), was from Sanofi Recherche. DX9065 was from Daichi Pharmaceuticals Co. Ltd. RPMI 1640 medium and PBS were from GIBCO-BRL. Heparin, ECGS, endotoxin, and human fibronectin were from Sigma Chemical Co. FBS was from Boehringer Mannheim. HUVEC were from Clonetics. Fura-2 acetoxymethylester was from Molecular Probes. *myo*-[^{3}H]-inositol (100 Ci/mmol) and the Biotrak 6-keto-prostaglandin $F_{1\alpha}$ enzyme immunoassay system were from Amersham. TNF-α was from R & D Systems. Human α-thrombin (3000 NIH U/mg) was purchased from Centre Regional de Transfusion Sanguine (Strasbourg, France). Asserachrom TPA and PAI-1 kits were from Diagnostica Stago. Prothrombin complex concentrate was from Bio-Transfusion. Anti-tissue factor antibodies were prepared in goats with human tissue factor (Inovin–Baxter) as the antigen. The imubind u-PA Elisa kit was from American Diagnostica Inc. (Ortho Diagnostics Systems).

Cell Culture

HUVEC were routinely cultured in 75-cm^2 flasks coated with human fibronectin (5 μg/cm^2) in RPMI 1640 medium containing 10% FBS, 100 IU penicillin, 100 μg/mL streptomycin, 2 mM glutamine, heparin (100 μg/mL), and ECGS (30 μg/mL). For experiments, cells were detached by trypsin/EDTA (0.02%–0.05%) and seeded in fibronectin-coated 24-well plates. HUVEC were routinely used from the third to the sixth passage. For quantitation of TPA, u-PA, and PAI-1 released by cultured HUVEC, the cells were cultured under the same experimental conditions. After 24 hr, aliquots of medium (1 mL) were frozen at −70° in the presence of Tween 80 at a final concentration of 1/10,000. For proliferation assays, cells were plated sparsely (3×10^4 cells/well) in 24-well cluster plates in RPMI culture medium + 10% FBS. After 1 hr, the medium was removed and fresh RPMI medium supplemented with 0.5% FBS and factor Xa (100 nM) was added with or without increasing doses of DX9065 or AT/SR34006 (used at an equimolar ratio). After 1 day in culture, cells were detached from triplicate wells by trypsin treatment (0.05% trypsin–0.02% EDTA) and counted in a Coulter counter (Coultronics).

Quantitation of TPA, u-PA, and PAI-1

Quantitative determination of TPA and PAI-1 in the aliquots of medium was performed using Asserachrom TPA and PAI-1 ELISA kits from Stago. Urokinase release was determined with an Imubind u-PA Elisa kit.

$[Ca^{2+}]_i$ Measurements

HUVEC cultured in 75-cm^2 flasks were detached with a non-enzymatic cell dissociation solution (Sigma), scraped from the flasks, centrifuged, and resuspended in PSS (composition: NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 10 mM, glucose 5.6 mM, Hepes/NaOH 5 mM pH 7.4), containing fura-2 acetoxymethylester (1 μM) and incubated at 37° for 30 min as described [18]. The cell suspension was then diluted five times with PSS, and incubated for a further 60 min at 37°. After two washes with PSS to remove extracellular fura-2, cells were resuspended in PSS and kept in the dark at room temperature. Experiments were carried out under constant stirring in a PTI spectrofluorometer using around 75,000 cells in 3-mL fluorescence cuvettes at 37°. Data from several experiments were pooled and analyzed together by fitting the sigmodal equation to the data by non-linear regression, thus determining EC_{50} values and their standard errors using the Sigmaplot program (Jandel Scientific).

Measurement of Phosphoinositide Turnover

Confluent cell monolayers in 60-mm dishes were incubated for 72 hr in normal culture medium containing 5 μCi/mL of *myo*-[^{3}H]-inositol. The medium was then aspirated, and the cell monolayers were washed twice with PBS and incubated for 30 min with PSS containing 20 mM of LiCl. Cells were then stimulated in the same medium with 100 nM of factor Xa with or without increasing concentrations of DX9065 or AT/SR34006 for an additional 30 min at 37°. At the end of the incubation period, the buffer was aspirated and the cells were extracted with an ice-cold methanol/HCl 0.1 N (50/50) solution for 30 min. Extracts were then neutralized with 1 M Na_2CO_3, and [^{3}H]-inositol monophosphate separated as described by Berridge *et al.* [19], using columns containing 1 mL of AG1-X8 resin.

Determination of Tissue Factor Activity on HUVEC

Procoagulant activity was assayed according to Suprenant and Zuckerman [20]. Briefly, adherent cells (10^4 cells/well) were incubated for 5 hr at 37° in M-199 medium in the presence of the indicated concentrations of factor Xa, DX9065, and AT/SR34006. LPS (10 ng/mL) was used as a positive control. The medium was removed, wells were washed twice with 1 mL PBS, and 250 μL of M-199 containing prothrombin complex concentrate (a mixture of factors II, VII, IX, and X) (0.44 U/mL Factor VII) and 100 μg/mL of substrate S-2222 were added. The OD at 405 nm was measured as a function of time and increased in a linear manner for at least 20 minutes. The rate of change of the OD during these 20 minutes, expressed as OD/min and representative of the amount of tissue factor expressed by the cells, was plotted versus factor Xa concentrations and percent of inhibition was calculated.

RESULTS

Effect of DX9065 on Factor Xa-induced HUVEC Proliferation

We have previously shown that factor Xa stimulates the growth of HUVEC in a dose-dependent manner [13]. This

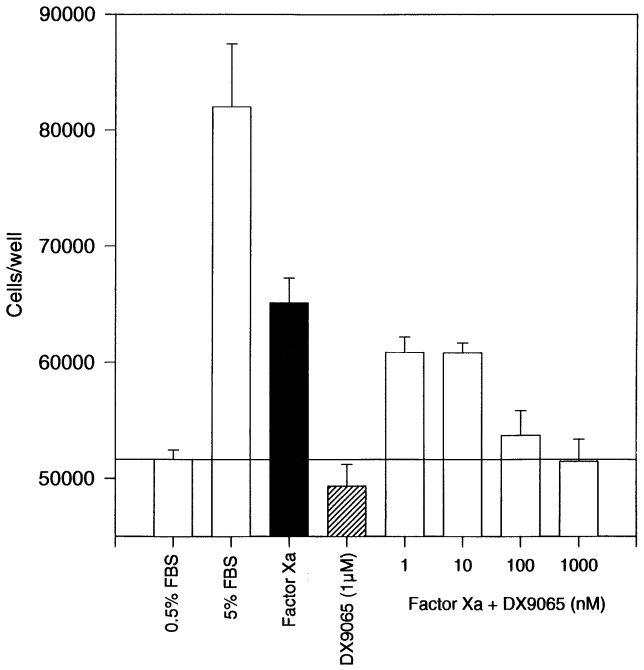

FIG. 1. Effect of DX9065 on factor Xa-induced HUVEC proliferation. HUVEC were seeded (40 × 10^3 cells/well) in culture medium containing 10% FBS and heparin/ECGS. After 1 hr, the culture medium was removed and replaced by fresh medium containing 0.5% FBS and factor Xa (100 nM) in the presence of the indicated doses of DX9065. Without factor Xa, DX9065 at 1 μM did not affect cell proliferation. After 1 day in culture, cells were trypsinized and counted. Data are reported as mean cell densities ± SEM (N = 6).

was confirmed in the present experiment where factor Xa (100 nM) induced a growth response representing 50% of the effect obtained with 5% FBS (Fig. 1). The concentration of factor Xa required to obtain optimal cell proliferation (Amax) was between 30 and 100 nM, with a half-maximal response (ED_{50}) of around 10 nM. Under these experimental conditions, DX9065 had no mitogenic effect of its own, but did inhibit in a dose-dependent manner, the proliferation of HUVEC induced by 100 nM of factor Xa (Fig. 1). The IC_{50} value was found to be 21 + 4 nM (N = 6).

Effect of DX9065 and AT/SR34006 on Factor Xa-induced TPA and PAI-1 Release

Because factor Xa was found to be a positive regulatory factor for HUVEC proliferation, we determined the effect of increasing doses of factor Xa on the release of TPA, u-PA, and PAI-1 by HUVEC. As expected from previous work [21], TPA release could be induced by either FBS (10% and 20%) or thrombin (12 nM), whereas TNF-α (200 U/mL) inhibited the release of TPA (Fig. 2). Factor Xa dose dependently stimulated the release of TPA in the cell supernatant. This effect was inhibited by DX9065 and AT/SR34006 (Fig. 2, inset). The IC_{50} values were found to be 20 + 2 and 5 + 1 nM for DX9065 and AT/SR34006, respectively.

PAI-1 release could be induced by either FBS, thrombin, or TNF [22]. As expected, TNF induced a strong release of PAI-1, in sharp contrast to its effects on TPA release. Factor Xa stimulated the release of PAI-1 into the cell supernatant in a dose-dependent manner (Fig. 3). The release of PAI-1 induced by factor Xa was dose dependently inhibited by DX9065 and AT/SR34006 (Fig. 3, inset). The maximum effect was comparable to the effect previously observed with thrombin. The IC_{50} values were found to be 28 + 4 and 23 + 5 nM for DX9065 and AT/SR34006, respectively. Under the same experimental conditions, factor Xa (100 nM) did not induce the release of u-PA into the supernatant (not shown).

Effect on Factor Xa-induced Increase in $[Ca^{2+}]_i$ Levels

In previous experiments, we showed that factor Xa induced a strong but transient increase in $[Ca^{2+}]_i$ in HUVEC monolayers [13] and Fig. 4A). The peak of the transient response was dose dependently increased by factor Xa, with a threshold of around 10 nM of factor Xa and a maximal effect reached at 1–3 μM. The EC_{50} value was 340 nM [13]. As shown in Fig. 4A, preincubation with DX9065A (1 μM) or the AT/SR34006 complex (1 μM) under these conditions nearly abolished the $[Ca^{2+}]_i$ response of factor Xa (1 μM). Actually, both DX9065 and AT/SR34006

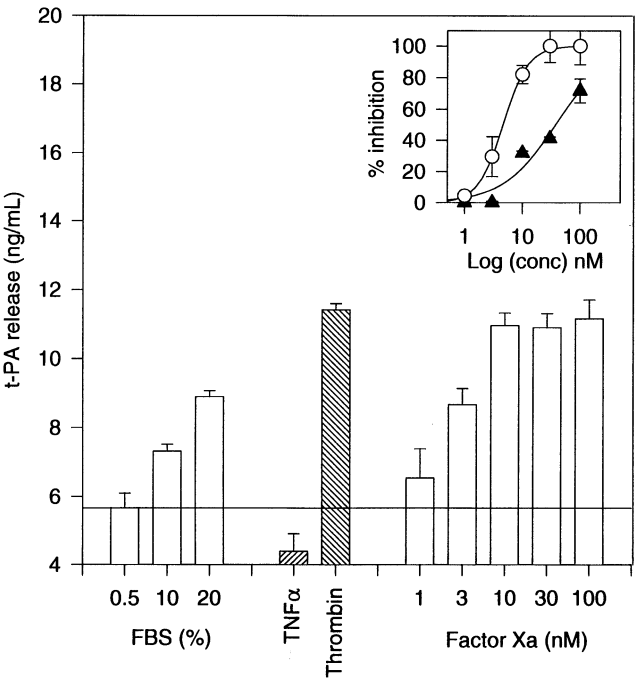

FIG. 2. Effect of factor Xa on TPA release in HUVEC. Non-confluent cells (2 × 10^5 cells/mL) were stimulated with FBS (10 or 20%), TNF-α (200 U/mL), thrombin (12 nM), or increasing concentrations of factor Xa in culture medium containing 0.5% FBS. After 24 hr, supernatants were collected and frozen at −70°. TPA was measured in the culture medium with the Asserachrom TPA kit. Data are reported as means + SEM (N = 6). Inset: Effect of DX9065 (▲) and AT/SR34006 (○) on TPA release. Results are expressed as percent inhibition of the response induced by 10 nM factor Xa. Without factor Xa, these compounds at 100 nM did not affect TPA release by HUVEC.

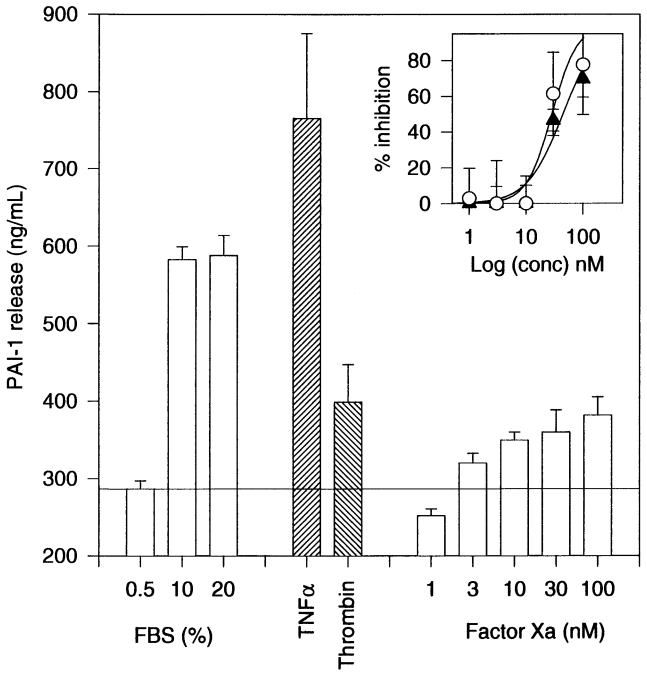

FIG. 3. Effect of factor Xa on PAI-1 release in HUVEC. Non-confluent cells (2 × 10^5 cells/mL) were stimulated with FBS (10 or 20%), TNF-α (200 U/mL), thrombin (12 nM), or increasing concentrations of factor Xa in culture medium containing 0.5% FBS. After 24 hr in culture, supernatants were collected and frozen at −70°. PAI-1 was measured in the culture medium with the Asserachrom PAI-1 kit. Data are reported as means + SEM (N = 6). Inset: Effect of DX9065 (▲) and AT/SR34006 (○) on PAI-1 release. Results are expressed as percent inhibition of the response induced by 10 nM factor Xa. Without factor Xa, these compounds at 100 nM did not affect PAI-1 release by HUVEC.

inhibited the peak increase in $[Ca^{2+}]_i$ levels induced by 1 μM of factor Xa in a dose-dependent manner. The IC_{50} values were found to be 230 + 31 and 690 + 24 nM for DX9065 and AT/SR34006, respectively (Fig. 4B).

Effect of DX9065 and AT/SR34006 on Factor Xa-induced Increase in Phosphoinositide Turnover

As previously shown, factor Xa increased phosphoinositide turnover in a concentration range slightly lower than the concentrations increasing $[Ca^{2+}]_i$ levels in HUVEC [13] and Fig. 5A). The concentration of factor Xa required to obtain an optimal effect was 100 nM (Fig. 5A). As shown in Fig. 5B, DX9065 and AT/SR34006 inhibited, in a dose-dependent manner, factor Xa-induced (100 nM) phosphoinositide turnover in HUVEC. The IC_{50} values were found to be 31 + 4 and 14 + 2 nM for DX9065 and AT/SR34006, respectively (Fig. 5B).

Effect of DX9065 and AT/SR34006 on Factor Xa-induced Tissue Factor Expression

After incubation with 5 ng/mL endotoxin or 100 nM factor Xa, HUVEC exhibited a significant tissue factor expression (Fig. 6). The expression of tissue factor at the surface of HUVEC was not affected by hirudin (50 nM), but its detection was totally blocked by the addition of an antitissue factor antibody. Results shown in Fig. 7 indicate that exposure of confluent HUVEC to increasing concentrations of factor Xa resulted in a dose-dependent increase in tissue factor expression on the cell surface. In this experiment, 100 nM of factor Xa exhibited a tissue factor expression corresponding to 93% of the effect of an optimal dose of endotoxin (10 ng/mL). As shown in the inset of Fig. 7, DX9065 and AT/SR34006 reduced, in a dose-dependent manner, factor Xa-induced tissue factor expression. Although neither of the inhibitors abolished the effect of factor Xa, half-maximal effects were obtained at micromolar concentrations (Fig. 6).

DISCUSSION

In agreement with Nicholson *et al.* [14], we recently reported that HUVEC express the EPR-1, a functional high-affinity receptor for factor Xa [13]. Moreover, we demonstrated a possible effect of factor Xa as a growth factor for human vascular endothelial cells.

In this work, we confirmed that factor Xa dose dependently induced the proliferation of HUVEC, phosphoinositide turnover, and the subsequent increase in intracellular free calcium. Moreover, in an attempt to find other activities of factor Xa on endothelial cells, we examined its effect on the release of fibrinolytic enzymes (TPA and u-PA) and of their inhibitor, PAI-1. Our results indicate for the first time that factor Xa induced the release of TPA and PAI-1 by HUVEC in a dose-dependent manner. These effects, which occurred at physiological concentrations of factor Xa (10 nM), seemed to be similar to the effect of thrombin [23, 24], another key component of the coagulation system. Such an effect could not be due to endogenous prothrombin cleavage because cell-associated factor Xa did not generate any thrombin under these experimental conditions [13]. Interestingly, factor Xa, even at high concentrations, did not induce the release of u-PA, thereby showing its specificity for TPA. The exact nature of such a selective effect remains to be determined. Since the release of fibrinolytic enzymes has been suggested to be important in the process of cell migration and proliferation [25], our results are in agreement with data obtained on other cell types [3, 6, 7], which clearly suggested the possibility of an active role of factor Xa in several physiopathological processes such as angiogenesis and atherogenesis, where abnormal cell proliferation and/or migration has long been acknowledged.

Most importantly, we demonstrated that stimulation of HUVEC by factor Xa induced the expression of tissue factor, thus showing for the first time that factor Xa could play a direct role in the regulation of the prothrombotic activity of endothelial cells. In this respect, it is known that prothrombin activation on HUVEC could not be solely mediated by released factor V/Va, as suggested earlier by the

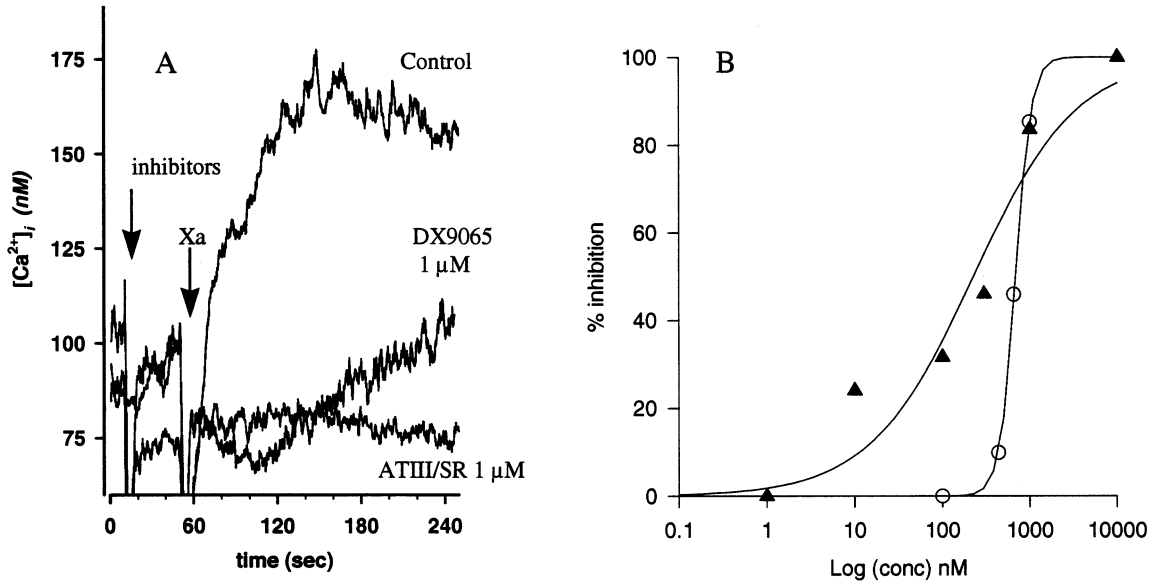

FIG. 4. Effect of DX9065 and AT/SR34006 on $[Ca^{2+}]_i$ levels induced by factor Xa in HUVEC. Increasing concentrations of DX9065 and AT/SR34006 were added to fura-2-loaded HUVEC and incubated for 1 min. Factor Xa (1 μM) was then added and $[Ca^{2+}]_i$ was measured as described under "Materials and Methods." (A) Traces of the calcium measurements after addition of factor Xa (1 μM) with or without DX9065 (1 μM) or AT/SR34006 (1 μM). (B) Dose–response curves obtained with DX9065 (▲) and AT/SR34006 (○). Data are from one experiment representative of 2 experiments performed on different batches of cells. Results are expressed as percent inhibition of the peak $[Ca^{2+}]_i$ response induced by 1 μM factor Xa. Without factor Xa, these compounds at 100 nM did not modify the $[Ca^{2+}]_i$ response.

absence of factor V/Va on normal unperturbed endothelium [26] and/or by the lack of factor Va transcription in these cells [27]. Moreover, we recently demonstrated that factor Xa bound to HUVEC was able to catalyze prothrombin activation, raising the interesting possibility of a major contribution of factor Xa in the thrombotic process that might occur at the endothelial cell surface [13].

Due to the importance of these factor Xa-induced events (proliferation, induction of tissue factor expression, and release of TPA and PAI-1), the effects of two selective factor Xa inhibitors, AT/SR34006 and DX9065, were examined. SR34006 is a newly developed pentasaccharide which shows high affinity for AT and behaves as a potent and selective catalyst of the anti-factor Xa activity of this serpin [16]. The high affinity of SR34006 for AT (K_d = 1.9 nM) allowed us to determine the concentration of SR34006 and AT in order to obtain 100% of AT/SR34006 complex. On the other hand, DX9065 is a synthetic compound which potently and selectively inhibits factor Xa directly at its catalytic site [17]. Since it has been suggested that factor Xa, like other serine proteases such as thrombin [28], might be affected differently by such compounds

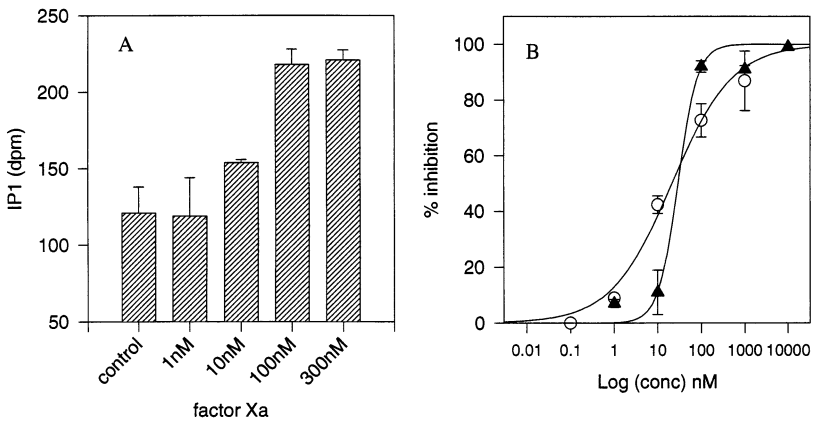

FIG. 5. Effect of DX9065 and AT/SR34006 on factor Xa-induced phosphoinositide turnover. Cell monolayers were incubated for 30 min with factor Xa with or without inhibitors. Inositol monophosphate accumulation was determined as described under "Materials and Methods." Data are reported as means ± SEM (N = 3). (A) Effect of increasing concentrations of factor Xa on phosphoinositide turnover. Results are expressed in dpm. (B) Effect of DX9065 (▲) and AT/SR34006 (○) on factor Xa (100 nM)-induced increase in phosphoinositide turnover. Results are expressed as percent inhibition of the response induced by 100 nM factor Xa. Without factor Xa, these compounds at 100 nM did not affect phosphoinositide turnover.

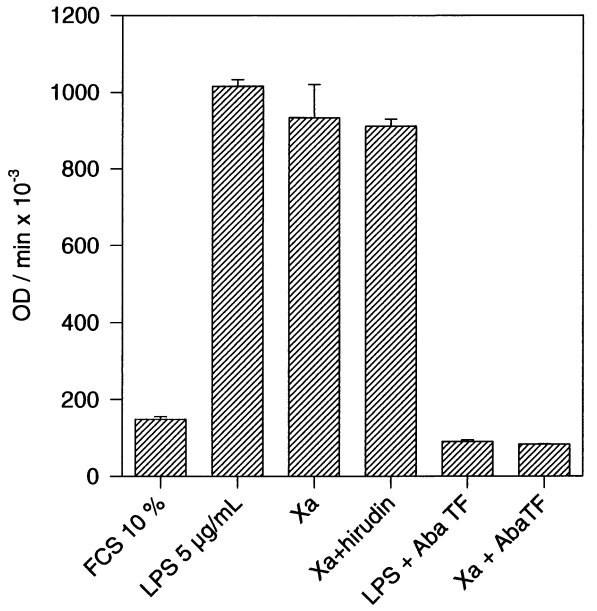

FIG. 6. Effect of factor Xa on tissue factor expression. Tissue factor expression was measured as described under "Materials and Methods" after incubation of adherent cells (10^4 cells/well) for 5 hr at 37° in M-199 in the presence of 10% FBS, endotoxin (5 μg/mL), factor Xa (100 nM), factor Xa (100 nM) + hirudin (50 nM), endotoxin (5 μg/mL) + antitissue factor antibody, and factor Xa (100 nM) + antitissue factor antibody. Results are expressed as OD/min × 10^{-3} due to the hydrolysis of the chromogenic substrate S-2222. Data are reported as means + SEM (N = 6).

depending on the accessibility of the active site of the enzyme, the effects of both inhibitors have been compared.

The data presented here indicate that the cellular responses induced by factor Xa were strongly affected by the addition of the two compounds, whatever their mode of inhibition. Indeed, both DX9065 and the AT/SR34006 complex dose dependently inhibited proliferation, the increase in $[Ca^{2+}]_i$, phosphoinositide turnover, the release of TPA and PAI-1, and the expression of tissue factor induced by factor Xa. Although DX9065 and AT/SR34006 inhibit factor Xa with a different mode of action, the IC_{50} values obtained were in the same range of concentrations, thereby showing that the active site of cell-bound factor Xa was accessible to both direct and AT-mediated inhibitors. However, the IC_{50} values of DX9065 and of the AT/SR34006 complex differed, depending on the cellular responses activated by factor Xa. Whereas cellular proliferation, TPA and PAI-1 release, and the increase in phosphoinositide turnover were blocked by low concentrations of inhibitors, much higher concentrations were needed to decrease the effects of factor Xa on $[Ca^{2+}]_i$ levels and tissue factor expression. Interestingly, the IC_{50} values of both inhibitors consistently varied in parallel across the whole range of cellular responses, suggesting that the differences in inhibitor potency essentially reflected the variable potency of factor Xa as an activator of these responses. Indeed, except for tissue factor expression, all responses activated by submicromolar concentrations of factor Xa were blocked by low concentrations of inhibitors. Furthermore, although it might at first sight seem astonishing that the $[Ca^{2+}]_i$ increase, which is a direct consequence of phosphoinositide turnover, required such high concentrations of factor Xa (1 μM) as well as of inhibitors, this may be explained by the fact that the cellular responses measured in our experiments required very different incubation times. $[Ca^{2+}]_i$ measurements took less than 1 min, whereas determination of phosphoinositide turnover was performed with incubation times at least 30 times longer. Evidently, as a consequence of the slow kinetics of receptor association [13], high concentrations of factor Xa are needed to induce significant receptor occupancy during the short incubation times necessarily used for $[Ca^{2+}]_i$ determinations. The induction of tissue factor expression by factor Xa was quite insensitive to inhibition by both antagonists. However, this is probably related to the fact that this response requires protein synthesis, which implicates long incubation times with somewhat unstable compounds.

Clearly, whatever the concentrations of factor Xa required to induce a given cellular response, the effects of factor Xa were always inhibited by both the competitive inhibitor DX9065 and the non-competitive inhibitor AT/SR34006. As already suggested in our previous work,

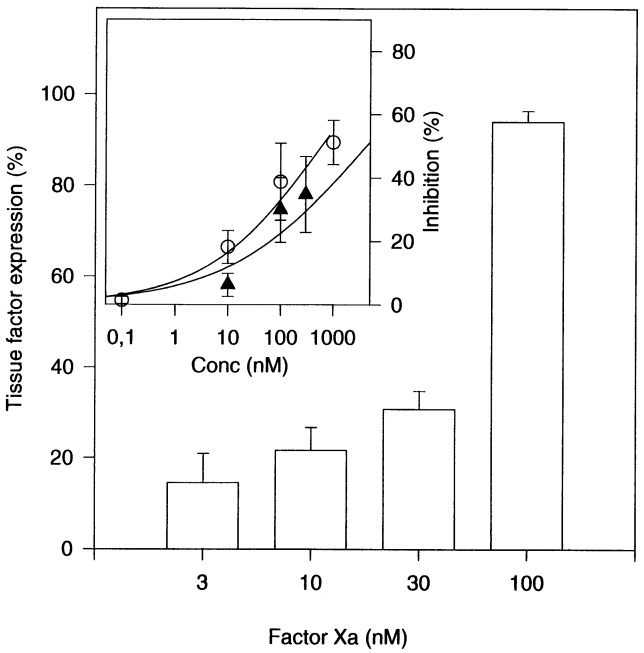

FIG. 7. Effect of DX9065 and AT/SR34006 on factor Xa-induced tissue factor expression. Adherent cells (10^4 cells/well) were incubated for 5 hr at 37° in M-199 in the presence of the indicated concentrations of factor Xa. LPS (5 μg/mL) was used as a positive control and taken as 100% expression of tissue factor. Tissue factor expression was measured as described under "Materials and Methods". Data are reported as mean % tissue factor expression + SEM (N = 6). Inset: Effect of DX9065 (▲) and AT/SR34006 (○) on factor Xa-induced tissue factor expression. Results are expressed as percent inhibition of the response induced by 100 nM factor Xa. Without factor Xa, these compounds at 100 nM did not affect tissue factor expression by HUVEC.

these results suggest that the protease activity of factor Xa is required for signal transduction and subsequent activation of vascular endothelial cells [13]. These responses observed in our studies were directly mediated by factor Xa but not via thrombin generation, as shown by the inability of the potent thrombin inhibitor hirudin to decrease factor Xa-induced responses in HUVEC [13]. These results are in agreement with those of Nicholson *et al*. [14], who showed that the mitogenic activity of factor Xa was highly dependent on its catalytic activity. However, since we have previously shown that these two inhibitors did not affect the binding of factor Xa to EPR-1 on HUVEC [13], it appears that postbinding signaling events (but not EPR1-Xa binding) require the active site of factor Xa to be functional. In this respect, factor Xa behaves like thrombin and trypsin, which require full catalytic activity to exhibit their various effects on cells [23, 24]; however, it differs from them in that both thrombin and trypsin receptors (protease-activated receptor-1 and protease-activated receptor-2, respectively) need to be specifically cleaved by these proteases to be activated. Indeed, because the amino-acid sequence of EPR-1 does not exhibit any consensus sequence for cleavage by serine proteases, factor Xa might activate HUVEC through another mechanism. In this respect, EPR-1 might be necessary to localize factor Xa in close proximity to the cellular membrane, where it may then activate another cleavable receptor such as protease-activated receptor-2, as suggested recently by Schaeffer *et al*. [29].

In conclusion, we have demonstrated here that, when added to cultured HUVEC, factor Xa exhibited a potent mitogenic activity, increased $[Ca^{2+}]_i$ levels and phosphoinositide turnover, and induced the expression of tissue factor and the release of TPA and PAI-1 without affecting urokinase expression. Using indirect and direct inhibitors, we have shown that these activities of factor Xa are dependent on its catalytic activity.

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