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Expression of tissue factor procoagulant activity: Regulation by cytosolic calcium

(blood coagulation/membrane protein/phospholipid asymmetry/A23187/calmodulin)

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ABSTRACT **Intact bovine fibroblasts, pericytes, and kidney cells manifested significantly less tissue factor procoagulant activity than their disrupted counterparts. Addition of calcium ionophore A23187 rapidly and reversibly enhanced the cell-surface expression of tissue factor in intact cells up to the level achieved by disruption. Inhibitors of calmodulin blocked the ionophore-dependent enhancement of procoagulant activity. Similar kinetic parameters were obtained for factor X hydrolysis by tissue factor–factor VIIa on unperturbed pericytes and phosphatidylcholine vesicles. Increase in V_{max} and decrease in apparent K_m for this reaction were seen after either disruption or ionophore stimulation of the pericytes. Addition of phosphatidylserine to the reconstituted phospholipid vesicles also increased the V_{max} and decreased the apparent K_m for factor X hydrolysis. These data agree with the hypothesis that the expression of tissue factor procoagulant activity on cell surfaces is modulated by calcium-mediated changes in the asymmetric distribution of phosphatidylserine in plasma membrane.**

Tissue factor (TF) is a membrane-anchored cell-surface protein that initiates coagulation when blood contacts a damaged tissue (1, 2). The expression of TF procoagulant activity is essential in the hemostatic response to vascular injury. TF may also trigger the clotting associated with heart attacks, strokes, cancer, and other thrombohemorrhagic diseases. Thus, the description of how TF activity is regulated *in vivo* is basic to understanding the biology and pathology of coagulation.

TF-initiated coagulation begins with plasma factor VII (FVII) or factor VIIa (FVIIa) binding to TF (3, 4). The TF–FVII/FVIIa complex catalyzes peptide bond cleavages in two other plasma proteins, factor IX (FIX) and factor X (FX), both zymogens of serine proteases, which are converted to active enzymes (FIXa and FXa) by limited proteolysis (5–7).

The interactions between these clotting factors have been studied extensively *in vitro* (3–12). Nevertheless, several fundamental questions regarding the initiation of coagulation *in vivo* remain unresolved. For example, how the expression of TF activity on cell surfaces is modulated so that clotting occurs only when and where it is needed is unclear.

One model for the biological regulation of TF-initiated coagulation is that the endothelium forms a TF-deficient barrier between FVII in the plasma and underlying cells that contain TF. Thus, coagulation may be triggered by damage to the endothelium, causing the association of plasma FVII and TF on cell surfaces outside the blood. Anatomical localization, however, may not be the only mechanism controlling TF-initiated coagulation. TF in unperturbed cells appears cryptic (13). In this study we have examined the relationship between number of TF molecules in a cell and expressed procoagulant activity to understand the mechanism of TF encryption and assess its importance as a physiological modulator of coagulation.

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MATERIALS AND METHODS

TF was purified from bovine brain and reconstituted into phospholipid vesicles as described (3, 14, 15). The isolation of FVII, FIX, and FX from bovine plasma, and the conversion of FVII to FVIIa were accomplished by established protocols (11, 16–18). Triton X-100, octyl β-D-glucopyranoside, dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone (DEGR), and calcium ionophore A23187 were obtained from Calbiochem. Scintillation mixture/formula 963 and NaB^3H_4 (≈70 Ci/mmol; 1 Ci = 37 GBq) were acquired from New England Nuclear. Bovine phosphatidylserine (PtdSer) and egg phosphatidylcholine (PtdCho) were from Supelco. Horseradish peroxidase conjugated to goat anti-rabbit IgG was from Bio-Rad. Polyvinyl microtiter plates (no. 2595) used in the TF ELISA were purchased from Costar. Trifluoperazine and bovine serum albumin (BSA) were from Sigma. Penfluridol was a gift of Janssen Pharmaceutical. All other chemicals were of reagent grade or better and were obtained from standard sources.

Functional Assays for TF. The two-stage coagulation assay for TF was done essentially as described (14). The isotonic buffer used in these studies was 137 mM NaCl/5.36 mM KCl/5.5 mM glucose/10 mM Hepes, pH 7.4 (HBS). Proteins were diluted in HBS/0.1% BSA (HBSA). The assay was calibrated with TF reconstituted into phospholipid vesicles composed of 30% PtdSer and 70% PtdCho. The procoagulant activity expressed by cultured bovine cells was identified as TF by the following criteria: No activity was manifested by the disrupted cells when FVIIa was omitted from the two-stage clotting assay, and the activity was completely inhibited by polyclonal rabbit antibodies against pure bovine TF (14).

The radiometric assays measuring the cleavage of ^3H-labeled factor FIXa or ^3H-labeled FX were done as described (4–6). Calcium ionophore A23187 in ethanol and trifluoperazine in H_2O were diluted 1:1000 from concentrated stock solutions. In experiments using ^3H-labeled FX as substrate, the feedback activation of FX by FXa was prevented by adding DEGR to a final concentration of 1 μM from a 1.0 mM stock in 10^{-3} M HCl. We determined that, under the described conditions, 1 μM DEGR inhibited the hydrolysis of FX by FXa but had no effect on the rate of FIX cleavage by TF–FVIIa (data not shown). Thus, with DEGR the observed activation of FX was solely from cleavage by TF–FVIIa. This

Abbreviations: TF, tissue factor; FVII, factor VII; FVIIa, activated FVII; FIX, factor IX; FIXa, activated FIX; FX, factor X; FXa, activated FX; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; BSA, bovine serum albumin; HBS, Hepes-buffered saline; HBSA, HBS/0.1% BSA; TBS, Tris-buffered saline; DEGR, dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone.

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result agrees with previous experiments in which a similar chloromethyl ketone inhibited the cleavage of FX by FXa without affecting the activation of FIX by TF–FVIIa (4).

Cell Culture. Bovine cells (embryonic skin fibroblasts, aortic endothelial cells, pericytes, smooth muscle cells, and Madin-Darby bovine kidney cells) were grown and passaged as described (19, 20). The cells were grown to confluence in 35-mm dishes. Twenty-four hours before an experiment, fresh medium was added to each dish. Cell density was determined by using a hemocytometer after trypsinizing duplicate cultures. Cells were disrupted by freezing at −80°C and thawing at 37°C three times. Variability of cell number and TF content (antigen and activity) per dish of the fibroblasts or pericytes was routinely <20%. Therefore, in the kinetics experiments we did not normalize the rate of FIX or FX hydrolysis to cell number.

TF ELISA. The murine monoclonal antibody that binds and inhibits bovine TF (TF1-1F7) has been described (15). Microtiter plates were prepared by adding 250 μg of the antibody in 0.1 M NaCl/0.05 M Tris, pH 7.5 (TBS) to each well. The monoclonal antibody was adsorbed to the plate overnight, and the plates were then blocked with 1% BSA/TBS for 48 hr. Cells were scraped into TBS/0.1% Triton X-100/1% BSA and diluted in the same buffer. The cell samples and the assay standard, pure bovine TF (0–200 pg/ml), were added to the plates for 20 hr. After rinsing the cells with TBS/0.1% Triton X-100 and then with TBS, polyclonal antibodies directed against bovine TF (3.5 μg of IgG/ml in TBS/1% BSA) were added for 5 hr. After rinsing the cells with TBS, goat anti-rabbit IgG–horseradish peroxidase conjugate, 1/2000, in TBS/1% BSA was added for 2 hr. After rinsing the cells with TBS, the color reaction was done as described (21).

RESULTS

Correlation Between TF Antigen and TF Activity Levels in Cultured Bovine Cells. Previous studies have shown considerable variations in the level of TF activity manifested by different cultured cells (13, 22). To investigate the physical basis of these observations, we examined five bovine cells. The cells were assayed for TF antigen and TF activity levels. Our aim was to determine whether the TF concentration in disrupted cell membranes directly correlated with TF activity.

Results of this experiment are summarized in Table 1. Pericytes, fibroblasts, and bovine kidney cells all contained 50,000–100,000 molecules of TF per cell as judged by both assays. Smooth muscle cells contained only 700 copies of TF per cell as measured by the clotting assay. The antigen concentration in these cells was below the limit of sensitivity of the ELISA. TF was not detected in the endothelial cells by either assay. This result is consistent with previous studies of unstimulated endothelial cells from other sources (22–24). The specific TF activity (TF activity/TF antigen) of membrane fragments derived from cells producing high levels of TF was indistinguishable from that of pure TF reconstituted into vesicles composed of 30% PtdSer and 70% PtdCho.

Table 1. TF content of five bovine cells as determined by immunoassay and functional assay

Cell type	Molecules of TF per cell, no. ± SD	
	Antigen	Activity
Pericyte	71,000 ± 11,000	62,000 ± 5,400
Kidney	88,000 ± 14,000	100,000 ± 4,000
Fibroblast	60,000 ± 5,900	60,000 ± 4,600
Smooth muscle	<1,000	700 ± 29
Endothelial	<400	<200

These data suggest that variations in procoagulant activity may be attributed solely to the TF content of the disrupted cell membranes.

Encryption of TF Activity in Bovine Fibroblasts. From the results described above, TF is evidently available to initiate coagulation once a cell is disrupted. However, other work (13) has shown that TF activity in unperturbed cells is not fully expressed. With bovine fibroblasts in HBSA/5 mM $CaCl_2$/140 nM FIX/3.4 nM FVIIa, the rate of FIX hydrolysis was 0.024 nM·min^{-1} for intact fibroblasts and 0.40 nM·min^{-1} for an identical plate containing fibroblasts disrupted by freezing and thawing. Thus, in accord with previous work, the disrupted cells manifested significantly greater TF activity than unperturbed cells.

Calcium Ionophore A23187 Stimulated Expression of TF Activity by Intact Cells. To understand how TF activity is masked in intact cells, we searched for a stimulus that would enhance the expression of TF activity within minutes, thus eliminating protein synthesis as a contributor to the change in procoagulant activity. We examined various ionophores (A23187, valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone, nigericin, and gramicidin), Bt_2-cAMP, colchicine, and cytochalasin B for effects on TF activity. Of these agents, the calcium ionophore A23187 was the only compound that had an effect. Addition of A23187 to fibroblast cultures enhanced the hydrolysis of FIX in a dose-dependent fashion (Fig. 1). At ionophore concentrations ≥10 μM the TF activity of the fibroblasts approached the value achieved by freeze–thaw disruption. With disrupted fibroblasts, the rate of FIX cleavage was 0.19 nM·min^{-1} for both the control and ionophore-treated samples. Similar results were obtained when bovine pericytes or kidney cells were substituted for the fibroblasts and when FX was the substrate (see below). Therefore, the phenomenon was not restricted to a particular cell or substrate.

The rate of change in TF expression after ionophore stimulation is illustrated in Fig. 2. There was an immediate 32-fold increase in the rate of substrate hydrolysis (0.032 vs. 1.03 nM·min^{-1}) after exposure to the ionophore. This abrupt increase shows that TF activity can change in a time scale inconsistent with dependence on new protein synthesis.

Calmodulin and TF Encryption. Under the conditions used in these experiments, calcium ionophore A23187 produces an influx of calcium ions into the cytosol (25). Because calmodulin is a principal intermediary in calcium-regulated intracellular events (26), we evaluated the effect of a calmodulin inhibitor, trifluoperazine. This drug is taken up by cells, binds tightly to calmodulin, and effectively blocks its

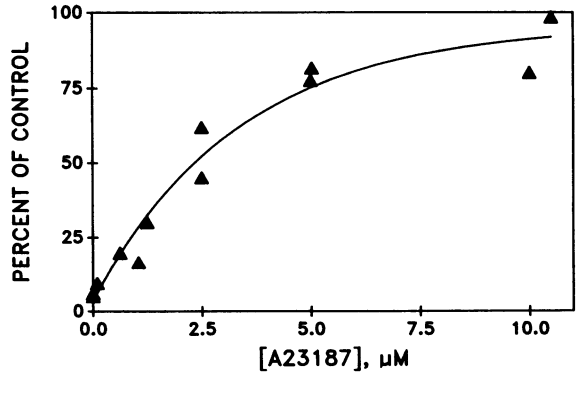

FIG. 1. Calcium-ionophore-A23187 stimulation of cell-surface TF activity. Bovine fibroblasts in HBSA/5 mM $CaCl_2$ were pretreated for 15 min with the indicated concentrations of ionophore. Reactions were initiated by adding 150 nM FIX/3.4 nM FVIIa. Results are presented as percent of the rate of FIX hydrolysis seen in a reaction containing disrupted fibroblasts.

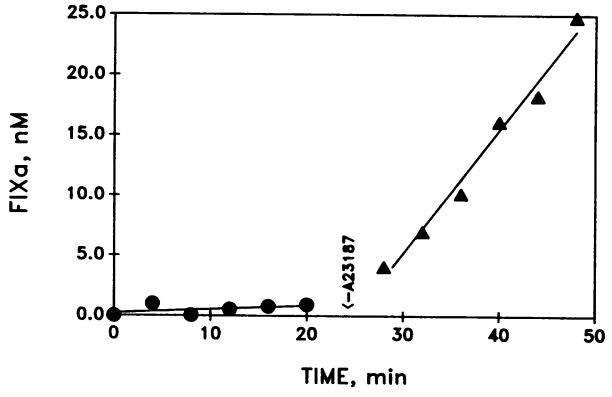

FIG. 2. Hydrolysis of FIX before and after stimulating bovine fibroblasts with calcium ionophore A23187. Intact fibroblasts were assayed in HBSA/5 mM $CaCl_2$/600 nM FIX/3.4 nM FVIIa. Unstimulated velocity (●) was measured for 20 min, and then 20 μM A23187 was added at 24 min. Stimulated velocity (▲) was measured from 28 to 48 min.

action (27). With disrupted fibroblasts as the TF source in a reaction containing 2 μM FIX and 3.8 nM FVIIa, the rate of FIX hydrolysis was 1.24 nM·min^{-1} for the untreated control and 1.34 nM·min^{-1} for the reaction containing 40 μM trifluoperazine. Therefore, trifluoperazine did not directly inhibit TF. Intact fibroblasts were then treated with trifluoperazine, and the rate of FIX hydrolysis before and after exposure to A23187 was determined. The results of this experiment are presented in Fig. 3. By itself, trifluoperazine had essentially no effect. However, at concentrations of trifluoperazine ≥20 μM, the control and stimulated velocities were essentially indistinguishable.

The specificity of trifluoperazine is not unique (27). Thus, another inhibitor of calmodulin, penfluoridol (27), was used in a similar experiment. This compound was chosen because it is structurally distinct from phenothiazines such as trifluoperazine. The ionophore-induced increase in TF activity was also blocked by this second inhibitor (data not shown). The ability of these agents to inhibit the ionophore effect strongly suggests that calmodulin is an intermediary in the process whereby increasing cytosolic calcium enhances the cell-surface expression of TF activity.

Reversal of the Ionophore-Stimulated Increase in TF Expression. All subsequent experiments were done with bovine pericytes as the TF source and FX as the substrate. The higher cell density of the pericyte cultures increased the TF content per dish, and FX is more rapidly hydrolyzed by TF–FVIIa than FIX (5, 6). The faster reactions resulting from these changes made the experiments described below technically feasible.

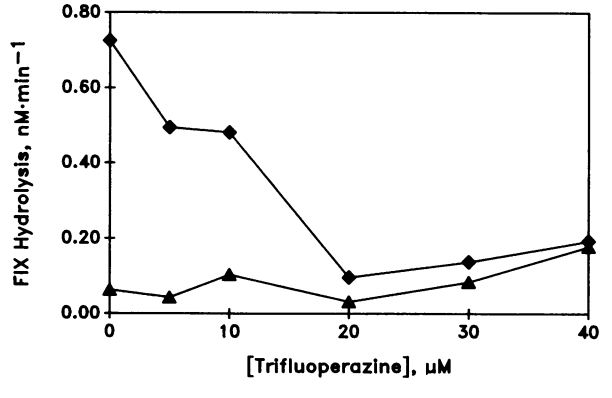

FIG. 3. Inhibition of calcium-ionophore-A23187 stimulation with trifluoperazine. Thirty minutes before assay the cells were treated with trifluoperazine at the indicated concentrations. Reactions were initiated by adding 600 nM FIX/3.4 nM FVIIa. The unstimulated velocities (▲) were determined by the standard protocol from 0 to 35 min. At 35 min, A23187 was added to a final concentration of 10 μM. The ionophore-stimulated velocities (◆) were then measured from 40 to 70 min.

The reversibility of the ionophore-induced stimulation of TF expression was investigated by using pericytes that had been pretreated with A23187 (20 μM) for 20 min. The medium was then aspirated, the monolayers were washed with HBS, and Dulbecco's modified Eagle's medium/10% fetal calf serum was added. The cell monolayers were subsequently assayed as described. As illustrated in Fig. 4, the rate of FX hydrolysis by the stimulated cells decreased as a function of time after A23187 withdrawal. One hour after removing the ionophore, the rate of FX hydrolysis had declined to a basal level that remained unchanged for 4 hr. This decay of activity was well described by a single exponential equation (see legend for Fig. 4). The observed and calculated values for the reaction velocity at zero time were 2.05 nM·min^{-1} and 2.02 ± 0.13 nM·min^{-1}, respectively. After removal of the stimulus, the reaction velocity decreased to a final value of 0.34 ± 0.08 nM·min^{-1}. The activity decayed at a rate of 0.04 min^{-1}.

Under the conditions described above, untreated pericytes activated FX at a rate of 0.41 ± 0.08 nM·min^{-1} (±SD, n = 4), and cells that were stimulated with ionophore just before assay hydrolyzed FX at a rate 2.07 ± 0.33 nM·min^{-1} (±SD, n = 4). These values are not significantly different from the basal and stimulated velocities shown in Fig. 4. In addition, the pericytes responded to a second round of ionophore stimulation. After the manipulations described above, the cells were again exposed to A23187 at 0, 1, 3, and 5 hr and assayed. These cells yielded an average velocity of 1.80 ± 0.37 nM·min^{-1} (±SD, n = 4).

PtdSer and TF Encryption. To explore further the phenomenon of encrypted TF, we performed a kinetic analysis of FX hydrolysis comparing pericytes (intact, ionophore-stimulated, and disrupted) and pure TF reconstituted into phospholipid vesicles containing various amounts of PtdSer. Michaelis–Menten kinetics predicts that transition from unperturbed to stimulated cells solely from increase in number of TF–FVIIa complexes will increase V_{max} for FX hydrolysis but will not change the apparent K_m. Thus, when the apparent K_m changes, another mechanism seems to be operating. In this three-component reaction the simplifying assumptions of

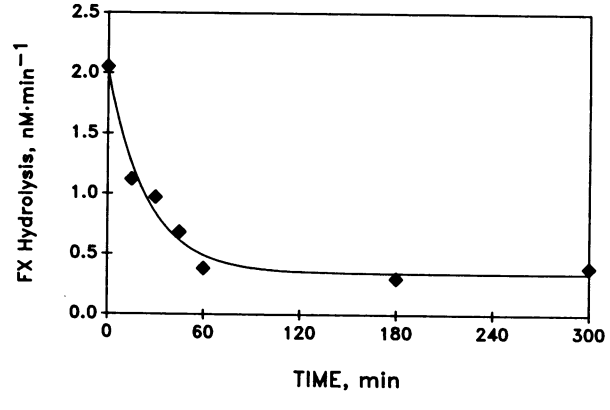

FIG. 4. Decay of the ionophore-stimulated expression of cell-surface TF activity. Pericyte cultures were exposed for 20 min to 20 μM A23187 in HBSA/5 mM $CaCl_2$, rinsed twice with HBS, and returned to the growth medium for the indicated times. Assays were done in HBSA/5 mM $CaCl_2$/1 μM DEGR/200 nM FX/10 nM FVIIa. The following equation was fitted to the data: $Y = ae^{-kt} + b$, where Y = reaction velocity, t = time after removal of A23187, a = the Y intercept, k = first-order rate constant, b = the value of Y as $t \rightarrow \infty$. A nonlinear least-squares algorithm was used in calculating these parameters.

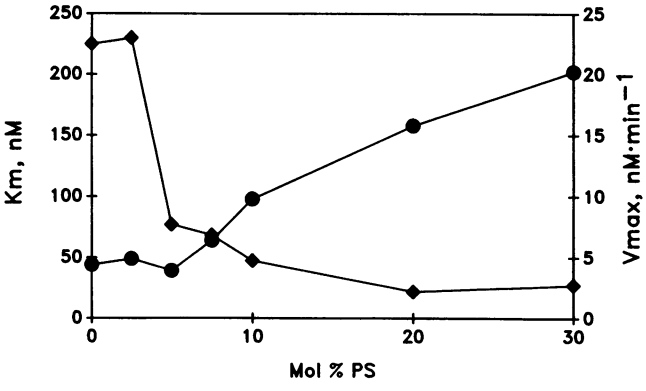

FIG. 5. Changes in apparent K_m and V_{max} for FX hydrolysis by TF–FVIIa as a function of increased vesicle charge. The TF reconstititutions and the calculation of apparent K_m (◆) and V_{max} (●) were described (3, 4). The assays were performed at 37°C in TBS/0.1% BSA/5 mM $CaCl_2$/2 μM DEGR/0.05 nM TF/1 nM FVIIa. Concentrations of substrate used in these assays were well distributed above and below the calculated apparent K_m. PS, PtdSer.

Michaelis–Menten kinetics clearly may not apply (4). However, under our conditions of enzyme excess the three-component system reduces to a simple Michaelis–Menten form.

The kinetic parameters for FX hydrolysis were determined with untreated, ionophore-stimulated, and disrupted pericytes as the TF source. Unperturbed cells were pretreated with solvent for 10 min before adding FX, 1 μM DEGR, and 10 nM FVIIa. These reactions yielded an apparent K_m of 294 ± 62.6 nM and a V_{max} of 1.98 ± 0.10 nM·min^{-1}. Stimulating the cells with 20 μM A23187 for 10 min before initiating the reactions lowered apparent K_m to 99.7 ± 14.9 nM and increased V_{max} to 4.46 ± 0.22 nM·min^{-1}. K_m (apparent) for disrupted cells was 30.3 ± 0.42 nM, and V_{max} was 4.68 ± 0.17 nM·min^{-1}. Because the increase in TF activity after cell disruption or ionophore stimulation was produced by an alteration in both kinetic parameters, the enhanced activity evidently cannot be simply explained by an increase in the number of catalytic complexes.

For more than two decades PtdSer has been known to accelerate TF-dependent reactions (28). Therefore, we considered the possibility that changes in the phospholipid composition of the plasma membrane could alter the cell-surface expression of TF activity. To measure the effect of PtdSer on TF activity, pure bovine TF was reconstituted into vesicles ranging from 100% PtdCho to 30% PtdSer/70% PhtCho (3). The apparent K_m and V_{max} for hydrolysis of FX are plotted against the PtdSer content of the vesicles (Fig. 5).

Comparable results have been obtained using FIX as the substrate (data not shown). As PtdSer increased, the apparent K_m decreased and a 4-fold rise in V_{max} occurred. Changes in the apparent K_m and V_{max} for FX hydrolysis seen after cell stimulation and the addition of PtdSer to reconstituted vesicles were similar in both direction and magnitude. This correlation suggests that the effect of the ionophore on cell-surface expression of TF activity may result from an increase in the PtdSer content on the plasma-membrane outer leaflet.

DISCUSSION

Although TF is generally recognized as the principal biological initiator of coagulation (1, 2), the mechanisms that regulate TF-initiated coagulation *in vivo* remain obscure. TF activity may be controlled, in part, by the sequestering of TF on cell surfaces outside the vasculature. The data on the TF content of cultured cells presented here and elsewhere are consistent with this hypothesis (22–24, 29–31). Also, studies of human tissues have found TF antigen and TF mRNA only in abluminal cells (32–34). Furthermore, recent evidence that coagulation on deendothelialized umbilical artery segments is initiated by TF (35) is consistent with the thesis that sequestration of TF on subendothelial cells inhibits coagulation in an undamaged blood vessel.

From the experiments just described, it is evident that anatomical localization may play a significant role in regulating TF-initiated coagulation. There is, however, additional evidence that indicates that other factors may contribute to the control of coagulation. When lymph plasma is used for blood plasma in standard clinical coagulation assays, clotting is seen (36). This fact raises the possibility that some extravascular cells are continuously exposed to FVII and the other components of the coagulation cascade. Why then is clotting not observed in undamaged tissues?

That intact cells in culture express substantially less TF activity than their disrupted counterparts (13) suggests the regulated expression of TF activity on cell surfaces may contribute to the control of TF-initiated coagulation *in vivo*. Previous studies have not elucidated the mechanism of this encryption of TF activity. However, binding studies with specific antibodies directed against TF (37) and FVII/FVIIa (12, 38, 39) have shown that TF is principally on the cell surface of unperturbed cells and that it can bind both FVII and FVIIa. Therefore, TF encryption appears not to be from the failure of the formation of TF–FVIIa complexes on the surface of intact cells. The changes reported here in the kinetic parameters for FX hydrolysis after disruption or ionophore stimulation of cells also indicate that the increase in TF activity after perturbation of cells cannot be solely from an increase in the number of TF–FVIIa complexes.

In our study of the mechanism of TF encryption, we have shown that calcium ionophore A23187, under conditions that produce an influx of calcium into the cytosol, enhances cell-surface expression of TF activity up to the level obtained with disruption. The rapid stimulation of cell-surface TF activity by calcium ionophore A23187, illustrated in Fig. 2, suggests that the stimulation is not from protein synthesis. Additionally, the stimulation is reversible (Fig. 4), inconsistent with proteolysis as the mechanism of activation. The evidence that ionophore stimulation of TF activity is reversible further suggests that this effect is not simply an artifact of cell trauma. Blocking the ionophore stimulation with calmodulin inhibitors, trifluoperazine and penfluoridol, suggests that calmodulin is an essential link between cytosolic calcium and TF encryption. Agents that increase cytosolic calcium in TF-containing cells may be the biological counterparts of calcium ionophore. Hormones, growth factors, proteases, and histamine, all of which are known to increase cytosolic calcium, are a few of the possible candidates (40–44).

Previous studies of plasma membrane structure have shown that PtdCho is the predominate phospholipid in the outer leaflet of the plasma membrane, and acidic lipids such as PtdSer are sequestered on the cytoplasmic face of the bilayer (45, 46). The stimulation of platelets with calcium ionophore A23187 or thrombin plus collagen as well as treatment with sulfhydryl-reactive compounds results in the appearance of PtdSer on the outer leaflet of the plasma membrane (47, 48). The restoration of PtdSer asymmetry on the surface of the oxidized platelets, after dithiothreitol addition, occurs at a rate similar to the decay of ionophore-stimulated TF activity described in this report. Recently, tumorigenic undifferentiated murine erythroleukemic cells have been shown to express substantial amounts of PtdSer on the cell surface, and PtdSer asymmetry is reestablished in these cells after differentiation to a nontumorigenic state (49). Thus, nucleated cells appear to have the ability to modulate

the distribution of PtdSer in the plasma membrane. These observations in conjunction with the evidence that PtdSer is a potent accelerator of TF-initiated coagulation *in vitro* (28) and that A23187 stimulates the expression of TF activity by cultured cells led us to consider the possibility that TF activity is modulated on cell surfaces by PtdSer.

The hypothesis was tested in an experiment comparing the kinetics of FX activation by TF-FVIIa on the surface of reconstituted vesicles and cells. The kinetic effects of varying the PtdSer content of reconstituted vesicles are summarized in Fig. 5. With increased PtdSer, there was a decrease in apparent K_m and a rise in V_{max} for the TF–FVIIa-catalyzed cleavage of FX. The apparent K_m for substrate hydrolysis by unperturbed cells was similar to that obtained with TF reconstituted into PtdCho vesicles. Therefore, before stimulation, cell-surface TF may be in an environment principally composed of neutral phospholipids. The decrease in apparent K_m and increase in V_{max} produced by cell disruption and ionophore stimulation were similar in both direction and magnitude to the changes induced by adding PtdSer to TF-containing vesicles. This correlation suggests that the PtdSer content of the outer leaflet of the plasma membrane modulates TF expression.

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