Coagulation Factors VII and X Induce Ca$^{2+}$ Oscillations in Madin-Darby Canine Kidney Cells Only When Proteolytically Active*

(Received for publication, June 27, 1996, and in revised form, August 8, 1996)

Eric Camerer‡§¶, John-Arne Røttingen‡§¶¶, Jens-Gustav Iversen, and Hans Prydz‡ ¶

From the ‡Biotechnology Centre of Oslo, N-0371 Oslo, Norway and the ¶Laboratory of Intracellular Signaling, Department of Physiology, Institute of Basic Medical Sciences, University of Oslo, N-0316 Oslo, Norway

We have recently reported that the activated serine protease and blood coagulation Factor VII (FVIIa) can induce Ca$^{2+}$ oscillations in Madin-Darby canine kidney cells. We now demonstrate a similar response by Madin-Darby canine kidney cells to the active coagulation Factor X (FXa), which is also a serine protease and a substrate of the tissue factor (TF)-FVIIa complex in the initiation of the coagulation cascade. The phosphatidyl inositol-specific phospholipase C inhibitor U73122 inhibited the signals elicited by both FVIIa and FXa. Lack of sensitivity to the tyrosine kinase inhibitors herbimycin A, genistein, and the tyrophostin AG18 and discordance between TF expression and FVIIa responsiveness argued against TF acting as a cytokine-like receptor, with tyrosine kinase-mediated activation by FVIIa. As demonstrated using the protease inhibitor benzamidine and by specific active site inhibition with 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone, both FVIIa and FXa lost their ability to elicit a calcium response when deprived of their proteolytic activity. Consistent with this, the native (zymogen) form of Factor X did not induce Ca$^{2+}$ transients. Homologous but not heterologous inhibition of FVIIa- and FXA-evoked Ca$^{2+}$ signals by 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone-inactivated FVIIa and FXa suggested that each factor had its own specific cell surface anchoring receptor. The two coagulation factors did not show homologous desensitization as seen for thrombin stimulation. Studies with hirudin excluded involvement of the established activation pathway through thrombin itself. Lack of desensitization of the response to FVIIa or FXa by thrombin ruled out any involvement of proteinase activated receptor-1 (PAR-1), the thrombin receptor. We speculate that FXa and FVIIa may work via a receptor (possibly common) analogous to PAR-1 or its functional homologue PAR-2. Although TF is essential for the FVIIa-induced signaling event, its role in the phosphatidyl inositol-specific phospholipase C-mediated Ca$^{2+}$ signal may be in anchoring FVIIa to the cell surface rather than in transmembrane signal mediation.

Tissue factor (TF) is an integral membrane protein that acts as an essential cofactor for the coagulation protease factor VIIa (FVIIa). The cloning of TF cDNA (1–4) revealed that it was a member of the cytokine receptor superfamily (5). Binding of FVIIa to TF activates the protease and also induces Ca$^{2+}$ oscillations in several different cell types (6). These oscillations were strictly dependent on TF (6). TF also plays a critical role in development as demonstrated by gene targeting experiments in mice (7).

It has been reported that coagulation Factor Xa (FXa) can also induce a Ca$^{2+}$ response in endothelial cells (8), and we report here that FXa induces Ca$^{2+}$ oscillations in MDCK cells in a similar manner to FVIIa. FX and FXa have a number of cell surface receptors including Factor Va, Mac-1 (CD11/CD18), and effector cell protease receptor 1 (9), and this activity may thus be independent of TF. Cross-linking of effector cell protease receptor 1 (but not FXa binding) has been shown to generate a Ca$^{2+}$ response (10).

The modulation of cellular function by thrombin is probably mediated by two receptors (11). One of them (the thrombin receptor, proteinase activated receptor-1 (PAR-1)) is G-protein linked and activates cells through a phosphatidic acid $P_{i}$, inositol (1,4,5)-trisphosphate pathway to give a strong Ca$^{2+}$ response (12).

In this report we use a digital imaging system to examine the response of fura-2-loaded MDCK cells to stimulation with these three coagulation proteases with the aim of further characterizing the pathway(s) leading to these changes in intracellular Ca$^{2+}$ levels. We have looked specifically at the convergence of activation pathways utilized by factors VIIa and Xa and compared these to Ca$^{2+}$ signals in response to thrombin stimulation.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, purified human Factor X (FX), hirudin, dimethyl sulfoxide, Russell’s viper venom (RVV), benzamidine, herbimycin A, genistein, ATP, and Hepes were obtained from Sigma; tyrophostin AG18 was kindly provided by Dr. Alexander Levitzki (Jerusalem, Israel); U73122 and U73343 was from Biomed (PA); bovine FX was kindly provided by Professor Johan Stenflo (Malma, Sweden); trypsin-EDTA and l-glutamine were from Flow (Irvine, Scotland) or Whittaker (Walkerville, MD); Dulbecco’s modified Eagle’s medium and fetal calf serum were from Life Technologies, Inc.; tissue culture plastic ware was from Nunc (Roskilde, Denmark); 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone (DEGRck) was from Calbiochem (San Diego, CA); recombinant human Factor VIIa and DEGRck-inactivated human FVIIa were from Novo-Nordisk ( Bagsvaerd, Denmark); substrate FXa-1 from Nycomed (Oslo, Norway); TF was isolated from human brain essentially as described by Hjort (13); purified RVV-activated human Factor VIIa, Factor Xa, FXa, Factor X, MDCK, Madin-Darby canine kidney; HSS, Hepes-buffered salt solution; PI-PLC, phosphatidylinositol-specific phospholipase C, [Ca$^{2+}$], cytosolic free Ca$^{2+}$ concentration; PAR, proteinase activated receptor; DEGRck, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone; RVV, Russell’s viper venom.

*This work was supported by the Research Council of Norway, the Norwegian Council for Cardiovascular Diseases, CLOTART project Contract BMH1-CT94-1202, and the Anders Jahre’s Foundation for the Norwegian Council for Cardiovascular Diseases.

**Recipient of a research studentship from the Research Council of Norway.

§ These authors have contributed equally to this publication.

¶ Research Fellow of the Norwegian Council for Cardiovascular Diseases.

¶¶ To whom correspondence should be addressed: Biotechnology Centre of Oslo, Gaustadalléen 21, N-0371 Oslo, Norway. Tel.: 47-22-95-87-55; Fax: 47-22-69-41-30; E-mail: hans.prydz@biotek.uio.no.
Factor X from Enzyme Research Laboratories (South Bend, IN); the fluorescent calcium indicator fura-2/acetoxymethyl ester and the surfactant Pluronic F-127 were from Molecular Probes (Eugene, OR); bacto-dextrose was from Difco Laboratories (Detroit, MI); Colorrapid was from Lucerna-Chem (Lucerne, Switzerland); mouse monoclonal antibodies against human FXa (5224) and human TF (4504) were from American Diagnostica (Greenwich, CT); mouse monoclonal (10G2) and rabbit polyclonal antibodies to human Factor VIIa were produced and purified in our laboratory; thrombin was kindly provided by Dr. J. W. Fenton II (New York State Department of Health, Albany, NY). The Hepes-buffered salt solution (HSS) consisted of (mM): NaCl, 136; KCl, 5; MgCl₂, 1.2; CaCl₂, 1.2; bacto-dextrose, 11; Hepes, 10; pH 7.35. All chemicals were of analytical quality.

**Cell Culture**—The constitutively TF-expressing MDCK type I distal tubule-derived epithelial cell line was obtained from Professor K. Prydz (Oslo, Norway) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and L-glutamine (380 µg/ml) in 30-mm tissue culture dishes. For experiments approximately 2 × 10⁵ cells/ml were seeded on glass coverslips 1 day prior to observation if not otherwise stated. Human fibroblasts isolated from a healthy individual were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and L-glutamine (380 µg/ml).

**Measurement of Cytosolic Ca²⁺ in Single Cells**—The measurement of cytosolic free Ca²⁺ in single cells was done as described previously (6). The cells were incubated for 60 min at 37 °C with a solution of 5 µM fura-2, 0.25% dimethyl sulfoxide, and 0.025% Pluronic F-127 in HSS. The cells were then washed once and incubated with 400 µl of HSS. Additions to the cell cultures were done by injection of 100 µl into the well. The Ca²⁺ imaging and registration software has been developed in our laboratory (14). Our equipment consisted of a Photon Technology International D-scan excitation device, a Nikon Diaphot-TMD inverted microscope, a Hamamatsu CCD video camera (C3077) and an intensifier head (C2400-8), a Sony u-matic SP video recorder, and a computer (DFI 386/33 MHz 12 Mb RAM) controlling a frame grabber and synchronizing the chopper speed with the video camera. The cytosolic Ca²⁺ concentration was calculated using the equation 

\[ [Ca^{2+}]_c = \frac{K_d}{R_{\min} - R_0} \]  

where \( R \) is the ratio of fluorescence signals and \( K_d \) is the dissociation constant. The experiments were carried out either at 35 or at 37 °C. Each set of data was internally consistent with respect to temperature.

**Quantitative Analysis and Definitions**—The fluorescence data sampled from the video tape were treated by a program (LICS) written in our laboratory. This program allows automated quantitative analysis of Ca²⁺ signals from case control or dose-response experiments based on preset criteria. The fluorescence signals and the calculated Ca²⁺ signals were smoothed by excluding higher frequencies than 0.33 and 0.20 Hz, respectively, by using a Hamming window low pass filter. The data are
presented as means and their standard error (S.E.). A Ca\(^{2+}\) signal was defined as a response if the difference between the maximal Ca\(^{2+}\) level after the application of agonist, and the mean before application was greater than 200 nm. The maximal increase in Ca\(^{2+}\) was calculated by computing the maximal Ca\(^{2+}\) after the application of agonist and then subtracting from this value the average Ca\(^{2+}\) level before application.

The integral of the part of the response after application of agonist, which was above the average Ca\(^{2+}\) level before application, was taken as the increase in cytosolic free Ca\(^{2+}\). This integral depended directly on the observation time, which was kept constant within but not between experiments. Spontaneous responders and cells with compromised permeability were excluded by not considering cells that during the first 35 s of observation had either a higher absolute Ca\(^{2+}\) level than 700 nm or a difference of more than 100 nm between the maximal and minimal Ca\(^{2+}\) level.

In the experiments on duration of the Ca\(^{2+}\) signal (see Fig. 4) and on desensitization (see Figs. 9 and 10), the cells were in a stimulated state at the start of the observation period, and the average Ca\(^{2+}\) level before this period could not be calculated. In these experiments the Ca\(^{2+}\) signal was defined as a response if the difference between the maximal and the minimal Ca\(^{2+}\) level in the observation period was greater than 300 nm. The integral of the Ca\(^{2+}\) response was calculated by computing the average Ca\(^{2+}\) level within the given observation period and then subtracting from this value the minimal Ca\(^{2+}\) level in the same span. Both integrals and response percentages are therefore comparable only within these experiments.

Nontoxic fura-2 was present in MDCK cells up to about 20% of total intracellular content, as demonstrated in digitonin permeabilization experiments (16). This will lead to an overestimation of the resting Ca\(^{2+}\) concentration by 200–300 nm. Because we were concerned about the Ca\(^{2+}\) signal, i.e., changes in the cytosolic Ca\(^{2+}\) concentration, this overestimation will not conceivably invalidate our findings.

Antibody Inhibition of FXa and FVIIa—FXa (500 nm) was incubated for 2 h at 37 °C with a rabbit polyclonal anti-FXa antibody produced and purified in our laboratory (final concentration, 2.5 mg/ml). As determined using a two-stage assay (16) with human brain extract as source of TF (0.25 unit/ml), limiting amounts of antibody-inhibited or normal FVIIa (10 nM), FX purified from human plasma (0.5 unit/ml, Sigma), and a chromogenic substrate for FXa (FXa-1, Nycodem), the antibody reduced the FVIIa coagulant activity by >90%. Similar results were obtained with a mouse monoclonal anti-FVIIa antibody (10G2). The antibody-inhibited FXa was used at a final concentration of 100 nm, with identically treated FXa or antibody alone as controls.

FXa (2.5 units/ml) was incubated for 1–2 h at 4 °C with a mouse monoclonal anti-FXa antibody (93 μg/ml, American Diagnostica 5224). The antibody reduced the activity of FXa by >70%, as determined using a one-stage chromogenic substrate assay for FXa (FXa-1, Nycodem). Cells were stimulated with FXa (0.5 unit/ml) and FXa (0.5 unit/ml) subjected to inhibition by antibody or antibody alone.

Duration of Response—Cells loaded for 1 h with fura-2 were rinsed briefly with HSS, fresh HSS with the appropriate agonist was added, and the cells were left at 37 °C for the time indicated (3, 30, or 60 min) before observation. No single well was observed more than once.

Internalization of TF in Transfected MDCK Cells—Iodinated FVIIa or an iodinated anti-TF monoclonal antibody (4504) was allowed to bind onto the surface of either untransfected MDCK cells or MDCK cells transfected with a construct coding for full-length human TF (MDCK hTF1–263) (17). After saturating the binding at 4 °C (1.5 h, 1 μg/ml FVIIa or 2.5 μg/ml anti-TF monoclonal antibody) and removal of unbound FVIIa or antibody, the cells were brought up to 37 °C and incubated further for up to 1 h. Cell surface-bound FVIIa or antibody was then removed with a pH 4.0 washing buffer, and what remained in the cell fraction was taken to be internalized. We corrected for the amount of radioactivity that had dissociated from the cells during the incubation period.

Inhibitors—Inhibitors (U73122, herbimycin, genistein, AG18, hirudin, and benzamide) and control (U73343 and HSS) at the given concentrations were added to the cells in HSS after the 1-h fura-2-loading period and 3–5 min before the start of the fluorescence observation, or, if indicated, added in a volume of 100 μl during observation. All the inhibitors were adjusted to the right osmolality prior to use.

RRV Activation of FX—RRV in cephalin was used to activate human and bovine FX. RRV (containing approximately 6 μg of FX-activating enzyme) was reconstituted in 3 ml of 0.15 M NaCl at 37 °C. One volume was mixed with nine volumes of a solution of 8.3 units/ml FX and incubated for 1 h at 37 °C. The one-stage chromogenic FXa substrate test showed essentially complete activation of FX using these conditions. At 30 s RRV-activated FX was added to a final concentration of 1.5 units/ml. Unactivated but otherwise identically treated FX or RRV were used as controls.

DEGRck Inactivation of FXa—FXa (5 units/ml) was incubated with DEGRck (5 nm) for 1 h at 37 °C in HSS. As control we used the same concentration of FXa without DEGRck. After the inactivation period the samples were passed through a filter with a 10-kDa cut-off and washed three times with 4 ml of HSS to remove excess DEGRck. The one-stage chromogenic FXa substrate test showed essentially complete activation of FXa, and control filtration of FXa alone excluded significant loss of DEGR-FXa during filtration. Cells were stimulated at 30 s with a final concentration of DEGR-FXa corresponding to 1 unit/ml of native FXa and

**Fig. 2. Specificity of FVIIa- and FXa-induced increase in cytosolic Ca\(^{2+}\).** Average of Ca\(^{2+}\) responses in MDCK cells treated with FVIIa (100 nm) (upper panel) or FXa (0.5 unit/ml) (lower panel). FVIIa and FXa were preincubated in the presence (dotted lines) or the absence (solid lines) of specifically neutralizing antibodies. The arrows indicate the addition of the preincubated agonists to the cells. For details see “Experimental Procedures.”

| Table II |
| --- |
| **Effect of cell density on [Ca\(^{2+}\)\]\(_{i}\), response to FVIIa and FXa in MDCK cells** |
| MDCK cells cultured for 2–5 days after seeding on glass coverslips were stimulated with FVIIa (200 nm) or FXa (1 unit/ml). The integral of the Ca\(^{2+}\) response was computed from the first 200 s after stimulation. The data are given as means ± S.E. |
| Treatment | Time after seeding | Responding cells | Maximal increase in Ca\(^{2+}\) | Integral of the Ca\(^{2+}\) response | Number of cells |
| --- | --- | --- | --- | --- | --- |
| FVIIa | 48 | 76 | 281 ± 31 | 15.5 ± 2.4 | 84 |
| FVIIa | 72 | 56 | 238 ± 22 | 11.9 ± 1.3 | 108 |
| FVIIa | 96 | 47 | 167 ± 16 | 7.5 ± 0.9 | 139 |
| FVIIa | 120 | 0 | 14 ± 2 | −0.7 ± 0.2 | 108 |
| FXa | 48 | 100 | 840 ± 66 | 33.2 ± 2.3 | 23 |
| FXa | 120 | 2 | 7 ± 4 | −4.9 ± 0.3 | 55 |
with identically treated FXa without DEGRck as control. DEGRck-inactivated human FVIIa was kindly provided by Novo-Nordisk.

**Desensitization Experiments**—If otherwise not stated, incubation for desensitization lasted 1 h prior to the start of observation. The agonist (in 100 μl of HSS) was added to the cells together with the 400 μl of HSS with fura-2 for loading. The cells were then rinsed three or four times with HSS, pH 7.4 (or HSS, pH 4.0, where stated), and observed either with HSS alone or with a repeated addition of agonist. The cells were stimulated at either 30 or 180 s after the start of observation.

**RESULTS**

**Effects of FVIIa and FXa on the \([Ca^{2+}]_{c}\) level of MDCK Cells**—We have recently reported (6) that human FVIIa induces dose-dependent synchronous calcium oscillations in MDCK cells. In further characterization of this signaling event, we included coagulation Factor Xa, which has been suggested to give a calcium response in endothelial cells (8). When MDCK cells were treated with increasing concentrations of FXa, dose-dependent \(Ca^{2+}\) responses were observed (Fig. 1 and Table I). The number of responding cells, the integral of the \(Ca^{2+}\) signal, the maximal amplitude of the response (Table I), and the frequency of the \(Ca^{2+}\) oscillations (not shown) all increased with increasing concentrations of FXa. As observed earlier for FVIIa (6), FXa also induced synchronous \(Ca^{2+}\) oscillations (not shown). The characteristics of the \(Ca^{2+}\) response to FXa (Fig. 1) were similar to those observed for FVIIa (6). \(Ca^{2+}\) spikes were observed even at 0.1 unit/ml of FXa, which is well below the normal plasma concentration of FX (1 unit/ml). At a concentration between 0.2 and 0.5 unit/ml FXa >90% of the cells showed \(Ca^{2+}\) transients. Above 0.5 unit/ml FXa, the cells would frequently not reach down to base-line levels before entering a new spike. Neither the FXa responses observed here nor the FVIIa evoked responses reported previously were due to contaminants in our preparations, as demonstrated by the substantial inhibition of the signals by antibodies neutralizing the respective factor activities (Fig. 2).

The FVIIa concentrations used (100–200 nM) are higher than the plasma concentration of Factor VII (10 nM). The \(Ca^{2+}\) effect is observed even at 2 nM but then only in a fraction (~40%) of the cells (6). To obtain the effect in all cells, a substantially higher concentration must be used. Binding of Factor VII to TF phospholipid membranes may increase the local concentration \textit{in vivo}. To mimic the effect of flowing blood providing new Factor VII to the local milieu, a higher concentration of FVIIa was necessary to obtain the response in all cells under the stationary conditions of our experiments.

Considering that both FVIIa and FXa can bind to TF and because both induce \(Ca^{2+}\) oscillations in MDCK cells, we...
looked to see if the two clotting factors had additive effects on the Ca\(^{2+}\) signal. When the cells were first treated with FVIIa and then after 5 min with FXa or vice versa, the frequency of the Ca\(^{2+}\) oscillations as well as the mean level of cytosolic free Ca\(^{2+}\) increased (not shown). Some of the base-line Ca\(^{2+}\) oscillations were also converted to a sustained Ca\(^{2+}\) response with sinusoidal oscillations when the second clotting factor was given.

**Cell Density**—Recent studies in our laboratory (17) have demonstrated that the major fraction of surface expressed TF (>90%) is localized on the basolateral surface of MDCK cells when these are grown under conditions allowing the formation of intercellular tight junctions. Many receptor-mediated signals are also down-regulated when cells are grown to confluence. We therefore examined whether cell density influenced the Ca\(^{2+}\) signals induced by FVIIa and FXa in MDCK cells. MDCK cells were seeded at low density and used for experiments 48, 72, 96, and 120 h after seeding. The cells were more than 80% confluent after 72 h, and the cell layers were completely confluent after 96 h as seen in the light microscope. After 120 h, when the cells were at maximal density, both FVIIa and FXa were unable to mount a Ca\(^{2+}\) response (Table II and Fig. 3). This was not because the cells had lost their potential to respond with a Ca\(^{2+}\) increase, because all cells responded to the addition of 10 \(\mu\)M ATP (Fig. 3). Consequently, the Ca\(^{2+}\) oscillations generated by FVIIa and FXa did not appear when the MDCK cells were at high density. At shorter times the Ca\(^{2+}\) signals were reduced (Table II).

**Duration of the Ca\(^{2+}\) Response**—Due to limitations in the capacity of our experimental setup and the problem of photobleaching and in order to avoid overexposure of the cells to incoming light, single cells were regularly not observed for more than 5–7.5 min. In order to investigate the duration of the Ca\(^{2+}\) response, we therefore observed the cells for 5 min at a series of time points after the addition of agonist. Each well was never observed more than once. We looked at the duration of Ca\(^{2+}\) signaling in cells stimulated with thrombin (1 unit/ml), FVIIa (200 nM), or FXa (1 unit/ml). In another cell line thrombin-induced Ca\(^{2+}\) transients lasted for less than 1 h (18). The observations were taken at 3, 30, and 60 min (Fig. 4). The synchronous Ca\(^{2+}\) oscillations observed in the presence of thrombin leveled off rather rapidly also in our system, whereas the signals generated by FVIIa and FXa were of a longer lasting nature (Fig. 4). The FVIIa-stimulated cells consistently showed strong, highly synchronous Ca\(^{2+}\) oscillations even 1 h after initiation. The amplitudes of the FVIIa-induced oscillations were reduced somewhat after 1 h. This may be a nonspecific effect related to fura-2 efflux and sequestration or desensitization. Also the FXa-stimulated cells appeared to oscillate with a certain synchronicity after 60 min, although not with the same consistency and amplitude as the FVIIa-induced cells.

**Internalization of Surface-bound FVIIa and TF in MDCK Cells**—The long duration of the FVIIa response suggested the lack of an efficient ligand-induced internalization mechanism for the FVIIa receptor, TF. To look at the turnover of FVIIa and TF on the cell surface within the time period studied, iodinated FVIIa or an iodinated anti-TF monoclonal antibody was al-

---

**Fig. 5.** An inhibitor of PI-specific phospholipase C inhibits FVIIa- and FXa-induced Ca\(^{2+}\) oscillations. Examples of single cell responses from cells treated with the phospholipase C inhibitor U73122 (5 \(\mu\)M, left column) or as control its close structural homologue U73343 (5 \(\mu\)M, right column) before the addition of either FVIIa (200 nM, upper panel) or FXa (0.5 unit/ml, lower panel). The arrows indicate additions.

**Fig. 6.** Effect of activation of FX by Russell's viper venom on induction of Ca\(^{2+}\) responses in MDCK cells. Average Ca\(^{2+}\) responses in MDCK cells treated with either human FX (1.5 units/ml, upper panel) or bovine FX (1.5 units/ml, lower panel) before (dotted lines) or after (solid lines) their activation by RVV or with RVV alone (dashed lines). The arrows indicate additions.
lowed to bind at 4 °C onto the surface of either untransfected MDCK cells or MDCK cells transfected with a construct coding for full-length human TF (MDCKhTF1–263) (17). A 37 °C internalization period followed. Within 1 h, only 20% of FVIIa and antibody were internalized (not shown). This is consistent with the lack of down-regulation of the response.

**Effects of FVIIa and FXa on Phosphatidyl Inositol-specific Phospholipase C**—To investigate whether the Ca²⁺ signals induced by FVIIa and FXa involved the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) and thereby an increase in inositol (1,4,5)-trisphosphate, we took advantage of the PI-PLC inhibitor U73122 (19). A close analogue (U73343) (19) with no observed effects on PI-PLC was used as control. Pretreatment (3–5 min) of the cells with 5 mM of the active inhibitor U73122 completely inhibited the appearance of Ca²⁺ transients after subsequent exposure to FVIIa or FXa, whereas the same concentration of the inactive analogue U73343 was without effect (Fig. 5). Application of the inhibitor to cells already stimulated with FVIIa or FXa and therefore oscillating, also blocked the signal in most cells (not shown).

**TF in FVIIa-induced Cellular Activation**—We have earlier presented clear evidence of a TF dependence for the FVIIa-induced Ca²⁺ signal (6). We then suggested, based on structural homology of the single transmembrane glycoprotein TF with the cytokine receptor family (5), that TF may interact with a cytosolic tyrosine kinase pathway. Two observations have made us question such a role for TF.

Firstly, inhibitors of tyrosine kinases did not inhibit the FVIIa-induced Ca²⁺ signal. 1-h incubation of MDCK cells with 10 or 100 μM genistein, 0.1 or 1.0 μg/ml herbimycin A, or 1 μM of the tyrophostin AG18 reduced neither the number of responding cells nor the increase in Ca²⁺ elicited by 100 or 200 nM FVIIa (not shown). This was in agreement with earlier findings where we could not detect any difference in the phosphorylation pattern between FVIIa-treated and untreated MDCK cells when examined by immunoblotting with anti-phosphotyrosine antibodies (6). How PI-PLC is activated remains an open question, although G-protein activation seems most likely.

In our earlier studies (6) there was a discordance between the number of cells expressing TF and those responding to FVIIa, especially evident in the case of J82 cells, where about 80% of the cells carry stable surface (20) TF, but only about 30% responded to FVIIa binding with Ca²⁺ signals. This suggested that TF and FVIIa alone may not be enough to induce the increase in [Ca²⁺]. Further evidence was obtained from studies of human fibroblasts, where no Ca²⁺ response to FVIIa was observed (2% responding cells, n = 42), although they clearly expressed TF on the surface (21) of almost all cells.

**Is the Proteolytic Activity of FVIIa and FXa Necessary for Inducing the Ca²⁺ Response?**—Both FVIIa and FXa are serine proteases, and their zymogens are prone to activation. Using a purified human FX preparation, we obtained variable and un-

---

**Table III**

Inhibition by benzamidine of FVIIa and FXa induced Ca²⁺ responses

| Benzamidine Treatment | Responding cells % | Maximal increase in Ca²⁺ | Integral of the Ca²⁺ response 10³ nM s | Number of cells n |
|-----------------------|---------------------|--------------------------|-----------------------------------------|------------------|
| HSS                   | FVIIa               | 93                       | 333 ± 12                                | 14.4 ± 1.1       | 72               |
| 0.3                   | FVIIa               | 63                       | 233 ± 11                                | 6.0 ± 1.1        | 72               |
| 1                     | FVIIa               | 69                       | 240 ± 12                                | 6.0 ± 0.8        | 72               |
| 10                    | FVIIa               | 6                        | 72 ± 7                                  | −1.6 ± 0.6       | 108              |
| 30                    | FVIIa               | 4                        | 59 ± 7                                  | −0.7 ± 0.8       | 72               |
| HSS                   | FXa                 | 69                       | 367 ± 27                                | 9.7 ± 0.6        | 72               |
| 10                    | FXa                 | 4                        | 38 ± 8                                  | −1.2 ± 0.3       | 72               |

**Fig. 7.** Effect of active site inhibition with DEGRx on FVIIa- and FXa-induced cytosolic Ca²⁺ responses. Examples of Ca²⁺ signals in single MDCK cells after treatment with DEGR-FVIIa (200 nM) or DEGR-FXa (0.5 unit/ml) followed by either FVIIa (200 nM) or FXa (0.5 unit/ml) as indicated by the arrows.
Ca\textsuperscript{2+} Signals Induced by Factors VIIa and Xa in MDCK Cells

reproducible Ca\textsuperscript{2+} responses. Substantial augmentation of the response was obtained by activation of the same FX preparation as well as a bovine FX preparation with RVV with essentially no Ca\textsuperscript{2+} response induced by the venom itself (Fig. 6).

We then tested whether inhibition of their serine protease activity would block the Ca\textsuperscript{2+} signal evoked by FVIIa and FXa. A general serine protease inhibitor, benzamidine, inhibited FVIIa-induced Ca\textsuperscript{2+} oscillations (Table III). Total inhibition was obtained at 10 mM, a concentration that also blocked the FXa-evoked Ca\textsuperscript{2+} response (Table III). DEGRck binds to and blocks the active sites of both FVIIa and FXa. Neither DEGR-FVIIa nor DEGR-FXa, both of which were inactive in a chromogenic substrate test, evoked Ca\textsuperscript{2+} signals in MDCK cells (Fig. 7). Both of these inactivated factors inhibited the effects of a subsequent addition of the homologous active factor (Fig. 8), suggesting the involvement of one or two saturable cell surface binding sites. Epinephrine and ATP still induced a Ca\textsuperscript{2+} response in these cells, indicating that there was no general inhibition of Ca\textsuperscript{2+} signaling (not shown). Little heterologous inhibition was observed for FXa addition after DEGR-FVIIa. DEGR FXa did not inhibit the action of FVIIa.

To see whether the long duration and the synchrony of the oscillations in cytosolic free Ca\textsuperscript{2+} generated by FVIIa was due to continuous signal generation by FVIIa or to the triggering of an intracellular cascade of calcium release and/or influx independent of the initial agonist, we added benzamidine to cells that had already been oscillating for 1 h due to the continuous presence of FVIIa. This led to an abrupt termination of the oscillations (Fig. 8). The same was seen for FXa-induced oscillations (not shown). This was not the case for cells stimulated with bradykinin (Fig. 8), another good inducer of synchronous Ca\textsuperscript{2+} oscillations in MDCK cells. A general effect of benzamidine on the ability of the cells to sustain Ca\textsuperscript{2+} oscillations was therefore unlikely.

Are the Effects of FVIIa and FXa Mediated through Throm-

![Fig. 8. Effect of benzamidine on synchronous Ca\textsuperscript{2+} oscillations induced by FVIIa or bradykinin in MDCK cells. Upper panel, average Ca\textsuperscript{2+} response of a single well of MDCK cells (n = 36) treated with FVIIa (200 nM) for 1 h prior to observation (to induce synchronous Ca\textsuperscript{2+} oscillations) and then treated with 10 mM benzamidine at 180 s as indicated by the arrow. Lower panel, average Ca\textsuperscript{2+} response of a single well of MDCK cells (n = 36) treated with bradykinin (10 nM) at 30 s (to induce synchronous Ca\textsuperscript{2+} oscillations) and then treated with 10 mM benzamidine at 180 s, as indicated by the arrows. Note the different ordinate scales.

![Fig. 9. Thrombin desensitization of Ca\textsuperscript{2+} responses to thrombin, FVIIa, and FXa. Average Ca\textsuperscript{2+} responses in MDCK cells pretreated for 1 h with thrombin (1 unit/ml, solid lines) or HSS (dotted lines), washed thrice with HSS pH 7.4, and restimulated at 30 s with thrombin (1 unit/ml, upper panel), FVIIa (200 nM, middle panel), or FXa (1 unit/ml, lower panel) as indicated by the arrows.]

| Pretreatment | Treatment | Responding cells | Maximal increase in Ca\textsuperscript{2+} | Integral of the Ca\textsuperscript{2+} response | Number of cells |
|--------------|-----------|------------------|---------------------------------------------|-----------------------------------------------|----------------|
| HSS          | thrombin  | 100              | 1455 ± 88                                   | 27.5 ± 2.8                                    | 36             |
| hirudin      | thrombin  | 28               | 179 ± 30                                    | −2.5 ± 2.8                                    | 36             |
| HSS          | FVIIa     | 91               | 349 ± 12                                    | 18.9 ± 0.9                                    | 93             |
| hirudin      | FVIIa     | 83               | 305 ± 11                                    | 17.0 ± 0.9                                    | 101            |
| HSS          | FXa       | 99               | 963 ± 36                                    | 63.3 ± 2.7                                    | 104            |
| hirudin      | FXa       | 90               | 718 ± 36                                    | 43.5 ± 2.7                                    | 100            |

Table IV

Effect of hirudin on Ca\textsuperscript{2+} responses induced by thrombin, FVIIa, and FXa

MDCK cells were preincubated 3–5 min with hirudin (5 units/ml) or HSS (control), before stimulation with thrombin (0.2 unit/ml), FVIIa (200 nM) or FXa (0.5 unit/ml). The integral of the Ca\textsuperscript{2+} response was computed from the first 230 s after stimulation. The data are given as means ± S.E.
bin or via Thrombin Receptors?—Thrombin is a powerful inducer of intracellular Ca\textsuperscript{2+} signals, and trace concentrations of prothrombin being converted to thrombin by FXa in our experiments should be excluded. Three lines of evidence indicate that this is indeed unlikely. First, the thrombin inhibitor hirudin (5 units/ml) inhibited almost completely the Ca\textsuperscript{2+} signals induced by thrombin (0.2 unit/ml), whereas only a very moderate reduction was observed in the response to FVIIa and FXa (Table IV). The number of responding cells decreased with 10% or less, the increase in Ca\textsuperscript{2+} levels decreased with 13–25%, and the total integral of the Ca\textsuperscript{2+} response decreased with 10–31%. This limited reduction may be accounted for by a small direct effect of hirudin on the response potential of the cells or on the activity/binding of FVIIa and FXa. Second, no thrombin-like activity was detectable in the cell culture medium even in prolonged incubations (not shown). Third, desensitization of the thrombin response (see Fig. 10) using 1 unit/ml thrombin for 1 h did not essentially impair the response to FVIIa or FXa. This also excluded direct activation of PAR-1 by FVIIa or FXa and made any contribution from a second thrombin receptor less likely (11).

Desensitization of FVIIa and FXa Responses—Homologous and heterologous desensitization by FVIIa and FXa were investigated after prior incubation with the respective agonist for 1 h. The cells were then washed three times with HSS, pH 7.4, or 4.0, as indicated, and fresh HSS without additions was added followed by observation of the cells for 180 s. The thrombin-induced (Fig. 9) and the FXa-induced (Fig. 10) cells remained silent with respect to Ca\textsuperscript{2+} oscillations during this period, whereas the FVIIa-induced cells showed synchronous oscillations even after the pH 7.4 washes (Fig. 10). The latter observation is consistent with earlier evidence from our laboratory that FVIIa is not effectively removed from the cell surface unless an acid wash (e.g. at pH 4.0) is performed. When washed at pH 4.0, the cells did not oscillate during the 180-s observation period (not shown). FVIIa-induced oscillating cells washed at pH 7.4 did not change their synchronous pattern upon restimulation with FVIIa, indicating that all available relevant binding sites were occupied. FXa was also unable to change the synchronous pattern of FVIIa-stimulated cells. Cells restimulated with FVIIa after a pH 4.0 wash responded erratically, most likely due to the low pH exposure. In many cells highly synchronous Ca\textsuperscript{2+} oscillations were re-established, showing that no homologous desensitization had occurred. Upon restimulation with FXa, FXa-induced and then silenced cells showed no homologous desensitization. The cells moved directly into highly synchronous oscillations. They were also stimulated by FVIIa, i.e. no cross-desensitization was observed. The slight reduction of the thrombin response after FVIIa and FXa incubation for 1 h was most likely due to the fact that the very strong normal response to thrombin requires cells that are unperturbed.

DISCUSSION

We demonstrated recently that binding of the coagulation factor VIIa to the surface of cells carrying TF elicited intracellular Ca\textsuperscript{2+} spikes (6). We demonstrate here that FXa has a similar effect on MDCK cells. For both factors their proteolytic activity was necessary for the effect to occur. The Ca\textsuperscript{2+} response depended on the concentration of agonist. Both responses were inhibited by neutralizing antibodies to the respective agonists. Possible involvement of thrombin in eliciting...
these responses has been excluded.

The induced oscillations gradually became synchronous for large numbers of cells. Similar synchrony induced by bradykinin was shown to depend on intercellular gap junctions. These synchronous signals go on for more than 1 h and can be stopped by washing off the agonist (for FXa) or by adding an inhibitor of serine proteases (benzamidine). In contrast, thrombin-induced signals decay with time and disappear completely after 30–45 min. A correspondingly low internalization of FVIIa and the absence of desensitization for FXa and FVIIa are consistent observations. The turnover may be slow because of lack of an essential component because it is likely to depend on the whole TF-FVIIa-FXa-tissue factor pathway inhibitor complex (22).

Increasing cell density led to decreasing signals, probably caused by formation of dense cell layers and tight junctions leading to down-regulation or basolateral sequestration of a factor essential for the signaling event. Studies of the TF distribution of the MDCK cell surface have revealed a predominant basolateral localization (17). Reduced Ca\textsuperscript{2+} signals would then result from reduced or abolished access of the agonist to the receptors on the basolateral surface. This is consistent with a role for TF in the generation of these signals. Several observations suggest that TF is not the sole cellular component involved in initiating the Ca\textsuperscript{2+} signal. Although constitutive TF producers, only a fraction of J82 cells responds to FVIIa binding with Ca\textsuperscript{2+} oscillations (6). Another example is human fibroblasts, which carry surface TF but do not respond to ligand binding with Ca\textsuperscript{2+} signals at all.

The obligatory proteolytic activity and the absence of tyrosine kinase activation suggest that a protease-activated receptor may be involved in generation of the Ca\textsuperscript{2+} signal. This is clearly not PAR-1. The role of the newly cloned orphan proteinase-activated receptor, PAR-2 (23), will be examined. Its distribution among different cell types (24–27) appears to be consistent with that of responsiveness to FVIIa. It is difficult, however, to reconcile these long lasting signals and their abrupt termination upon benzamidine addition with the hypothesis that a proteolytically activated receptor with a functional tethered ligand should be involved. There is no evidence of consumption or down-regulation of the putative receptor over a period of more than 1 h.

The intracellular pathway leading to the release of Ca\textsuperscript{2+} is not known. Our data suggest that PI-PLC is involved, although measurements of inositol (1,4,5)-trisphosphate levels have been inconclusive (not shown). There is no indication of an involvement of tyrosine kinases, neither from inhibitor studies nor from direct immunoblotting using anti-phosphotyrosine antibodies. G-protein-mediated activation remains an alternative.

Similarities in characteristics of and requirements for the FVIIa- and FXa-induced Ca\textsuperscript{2+} signals suggest that the pathway leading to these signals may converge early in the signaling cascade. The U73122 inhibitor studies show that this occurs before or on the activation of PI-PLC. If the activation of PI-PLC is G-protein-mediated, convergence may occur at the G-protein, the signaling receptor, or possibly a cell anchoring receptor.

Binding of DEGR-FVIIa to MDCK cells prevented signaling when subsequent binding of FVIIa was attempted. Similar observations were made for FXa. Binding of DEGR-FVIIa did not, however, prevent Ca\textsuperscript{2+} signaling induced by a subsequent addition of FXa. The simplest interpretation of these data is that FVIIa and FXa have separate and saturable binding sites, although not necessarily different receptors.

In conclusion, we suggest as a useful working hypothesis that the mode of action of FVIIa and FXa in eliciting the intracellular Ca\textsuperscript{2+} signal involves a receptor system, one anchoring receptor with a task of concentrating, activating (in the case of FVII), and presenting the protease to a second receptor, which after a proteolytic event triggers the pathway leading to PI-PLC activation and Ca\textsuperscript{2+} release.

Acknowledgments—We are grateful to Dr. K. Prydz for MDCK cells, Novo-Nordisk for recombinant human Factor VIIa and DEGR-FVIIa, Dr. J. Stenflo for bovine FX, Dr. A. Levitzki for tyrophostin AG18, and Dr. J. W. Fenton II for thrombin. Thanks also to Kristin Larsen for assistance in data processing. The imaging lab was established by Dr. J. S. Røtnes.

REFERENCES

1. Spicer, E. K., Horton, R., Bloom, L., Bach, R., Williams, K. R., Guha, A., Kraus, J., Lin, T. C., Nemerson, Y., and Koningsberg, W. H. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 5148–5152
2. Scarpati, E. M., Wen, D., Broze, G. J., Miletich, J. P., and Flandermeyer, R. R. (1987) Biochemistry 26, 5234–5238
3. Fisher, K., Gorman, C. M., Vehar, G. A., O’Brien, D. P., and Lawn, R. M. (1987) Thromb. Res. 48, 89–99
4. Morrissey, J. H., Fakhrui, H., and Edgington, T. S. (1987) Cell 50, 129–135
5. Bazan, J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 6934–6938
6. Røttingen, J.-A., Enden, T., Camerer, E., Iversen, J.-G., and Prydz, H. (1995) J. Biol. Chem. 270, 4650–4660
7. Carmeliet, P., Mackman, N., Wyns, S., Kleeckens, L., Edgington, T., and Collen, D. (1995) Thromb. Haemostasis 70, 1177 (Abstr. 1059)
8. Steinberg, S. F., Stern, D. M., Nawrot, P. P., and Bilezikian, J. P. (1985) Thromb. Haemostasis 54, 168 (Abstr. 994)
9. Esmon, C. T. (1995) Curr. Biol. 5, 743–746
10. Altieri, D. C., and Stamnes, S. J. (1994) Cell Calcium 15, 372–383
11. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V., Jr., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 3440–3450
12. Grand, R. J. A., Turnbull, A. S., and Graham, P. W. (1996) Biochem. J. 313, 353–368
13. Hjort, P. F. (1957) Scand. J. Clin. Lab. Invest. Suppl. 9
14. Røttingen, J.-A., Enden, T., Camerer, E., Iversen, J.-G., and Prydz, H. (1995) J. Biol. Chem. 270, 4650–4660
15. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
16. Roe, M. W., Lemasters, J. J., and Herman, B. (1990) Cell Calcium 11, 63–73
17. Camerer, E., Pringle, S., Skartlien, A. H., Wiiger, M., Prydz, K., Kolsto, A.-B., and Prydz, H. (1995) Blood 88, 1339–1349
18. Hein, L., Ishii, K., Coughlin, S. R., and Kobilka, B. K. (1994) J. Biol. Chem. 269, 27719–27726
19. Bleasdale, J. E., Thakur, N. R., Grebman, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J., and Bunting, S. (1990) J. Pharmacol. Exp. Ther. 255, 756–768
20. Drake, T. A., Ruf, W., Morrissey, J. H., and Edgington, T. S. (1989) J. Cell. Biol. 109, 39–395
21. Bach, R. (1988) J. Cell Biol. 107, 825 (abstr.)
22. Sevinsky, J. R., Rao, L. V. M., and Ruf, W. (1996) J. Cell. Biol. 133, 293–304
23. Nystedt, S., Emilsson, K., Wahlstedt, C., and Sundelin, J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9208–9212
24. Nystedt, S., Emilsson, K., Larsson, A.-K., Strombeck, B., and Sundelin, J. (1995) Eur. J. Biochem. 232, 84–89
25. Santulli, R. J., Derian, C. K., Darrow, A. L., Tomko, K. A., Eckardt, A. J., Seiberg, M., Scarborough, R. M., and Andrade-Gordon, P. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 9151–9155
26. Hwa, J. J., Ghibaudi, L., Williams, P., Chintala, M., Zhang, R., Chatterjee, M., and Sybertz, E. (1996) Circ. Res. 78, 581–588
27. Mirza, H., Yatsula, V., and Bahou, W. F. (1996) J. Clin. Invest. 97, 1705–1714