Calcium/Calmodulin Transduces Thrombin-stimulated Secretion: Studies in Intact and Minimally Permeabilized Human Umbilical Vein Endothelial Cells

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Abstract. Thrombin stimulates cultured endothelial cells (EC) to secrete stored von Willebrand factor (vWF), but the signal transduction pathways are poorly defined. Thrombin is known to elevate the concentration of intracellular calcium ($[Ca^{2+}]_i$) and to activate protein kinase C (PKC) in EC. Since both calcium ionophores and phorbol esters release vWF, both second messenger pathways have been postulated to participate in vWF secretion in response to naturally occurring agonists. We find that in intact human EC, vWF secretion stimulated by either thrombin or by a thrombin receptor activating peptide, TR(42–55), can be correlated with agonist-induced elevations of $[Ca^{2+}]_i$. Further evidence implicating calcium in the signal transduction pathway is suggested by the finding that MAPTAM, a cell-permeant calcium chelator, in combination with the extracellular calcium chelator EGTA, can inhibit thrombin-stimulated secretion. In contrast, the observation that staurosporine (a pharma-cological inhibitor of PKC) blocks phorbol ester- but not thrombin-stimulated secretion provides evidence against PKC-mediated signal transduction. To examine further the signal transduction pathway initiated by thrombin, we developed novel conditions for minimal permeabilization of EC with saponin (4–8 μg/ml for 5–15 min at 37°C) which allow the introduction of small extracellular molecules without the loss of large intracellular proteins and which retain thrombin-stimulated secretion. These minimally permeabilized cells secrete vWF in response to exogenous calcium, and EGTA blocks thrombin-induced secretion. Moreover, in these cells, thrombin-stimulated secretion is blocked by a calmodulin-binding inhibitory peptide but not by a PKC inhibitory peptide. Taken together, these findings demonstrate that thrombin-stimulated vWF secretion is transduced by a rise in $[Ca^{2+}]_i$ and provide the first evidence for the role of calmodulin in this process.

T HROMBIN, the ultimate serine protease activated by the coagulation cascade, is generated at sites of tissue injury and inflammation. In addition to its key role in converting plasma fibrinogen to fibrin, thrombin influences a diverse number of cellular activities. In human umbilical vein endothelial cells (EC)[1], thrombin rapidly induces both prostacyclin (Weksler et al., 1978) and platelet-activating factor (Prescott et al., 1984) synthesis, triggers cell shape changes (Laposata et al., 1983), and stimulates the release of von Willebrand factor (vWF) (Levine et al., 1982; Loesberg et al., 1983; deGroot et al., 1984) from Weibel-Palade bodies, an EC-specific secretory organelle (Weibel and Palade, 1964; Ewenstein et al., 1987). The vWF released from the Weibel-Palade body pool is composed chiefly of unusually large multimers (Sporn et al., 1986; Ewenstein et al., 1987) which are far more active in inducing platelet agglutination (Moake et al., 1986) and bind more avidly to extracellular matrix (Sporn et al., 1987) than smaller forms of vWF. Regulated secretion in EC also results in the translocation of a Weibel-Palade body membrane, protein, CD62 (alternatively named GMP-140 [McEver and Martin, 1984], PADGEM [Hsu-Lin et al., 1984], or P-selectin [Bevilacqua et al., 1991]), to the plasma membrane (McEver et al., 1989; Hattori et al., 1989a) where it is postulated to function as an adhesion molecule for neutrophils (Larsen et al., 1989; Geng et al., 1990; Hamburger and McEver, 1990). Thus,

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytosolic free calcium concentration; CaM, calmodulin; EC, endothelial cell; MAPTAM, 1,2-bis-5-methyl-amino-phenoxyethane-n,n,n'-tetraacetoxy-methyl acetate; ML-7, 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; MLCK, myosin light chain kinase; MLCK(796–817), A[796]RRKWQKTGHAVRAIGRLS-SMA[817]C; PKC, protein kinase C; TR(42–55), S[42]FLLRNPNDKYEPF[55]; vWF, von Willebrand factor; W-5, N-(6-aminohexyl)-1-naphthalenesulfon-amide; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

thrombin-stimulated exocytosis is likely to be an important component of endothelial regulation of both hemostasis and inflammation.

The intracellular events that transduce secretion in EC in response to thrombin have yet to be fully elucidated. Receptor-mediated processes in many cell types are thought to be initiated by the activation of a phosphatidylinositol-specific phospholipase C, thereby resulting in the generation of both inositol 1,4,5-trisphosphate and 1,2-diacylglycerol from phosphatidylinositol 4,5-bisphosphate (Berridge, 1984; Nishizuka, 1984a, b). Thrombin has been shown to activate this pathway in cultured human EC (Jaffe et al., 1987; Brock and Capasso, 1988). The thrombin-induced rise in inositol 1,4,5-trisphosphate precedes, and is believed to trigger, a subsequent rise in cytosolic-free calcium concentration ($[Ca^{2+}]_i$). The importance of calcium as a second messenger in endothelial secretion of vWF is suggested by several previous observations. First, calcium ionophore A23187, which elevates $[Ca^{2+}]_i$, also induces vWF release (Loesberg et al., 1983). Second, other agonists such as histamine (Hamilton and Sims, 1987), vascular permeability factor (Brock et al., 1991), and complement membrane attack complexes (Hattori et al., 1989b) which induce vWF release, also raise $[Ca^{2+}]_i$. Third, both thrombin- and histamine-stimulated vWF release is diminished in the presence of extracellular calcium chelators (deGroot et al., 1984; Hamilton and Sims, 1987). The mechanism by which calcium mediates secretion in response to thrombin is not known.

The 1,2-diacylglycerol generated by thrombin is thought, in the presence of elevated $[Ca^{2+}]_i$, to be an endogenous activator of protein kinase C (PKC). A possible role for PKC as a mediator of vWF secretion is suggested by the observation that the PKC activator, PMA, which does not cause elevation of $[Ca^{2+}]_i$ in human umbilical vein EC, is a potent secretogogue of vWF (deGroot et al., 1984; Ewenstein et al., 1987). In EC, thrombin has been shown to induce the translocation of PKC from cytosolic to membrane fractions (Lynch et al., 1990; Heller et al., 1991) and to stimulate the phosphorylation of a myristoylated, alanine-rich C kinase substrate (MARCKS) (Niedel and Blackshear, 1986; Jacobson et al., 1992). However, the role of PKC in thrombin-mediated secretion in EC has not been established.

Using both intact and saponin-permeabilized human umbilical vein EC, we present evidence here which demonstrates that thrombin-stimulated secretion of vWF is mediated primarily by calcium acting, at least in part, through calmodulin (CaM). In contrast, we find that PKC is not primarily involved in this process.

Materials and Methods

Cell Source and Culture

EC were isolated from three to five human umbilical veins by collagenase digestion as previously described (Gimbrone, 1976), pooled, and cultured in Medium 199 (M199) containing 20% heat-inactivated FCS supplemented with penicillin (125 U/ml), streptomycin (125 μg/ml), and 2 mM L-glutamine (all from Gibco Laboratories, Grand Island, NY). Cells were serially passaged under the conditions of Thornton et al. (1983), supplementing the medium with porcine heparin (100 μg/ml; Sigma Chemical Co., St. Louis, MO) and endothelial cell growth factor (50 μg/ml; Upstate Biotechnology, Lake Placid, NY). Tissue culture plasticware, including T75 flasks, 6- and 24-well plates (Corning Glass Works, Corning, NY), was coated with gelatin (Difco Laboratories, Detroit, MI) or fibronectin (New York Blood Cen-

ter, New York, NY) before plating. All cells used in these experiments were passaged two to four times from primary cultures.

Quantitation of vWF Secretion in Intact EC

Confluent cultures of EC were washed four times with M199 supplemented with 0.1% gelatin (M199/G) then incubated for 15 min with various concentrations of agonist in this buffer. Conditioned medium was then transferred to separate tubes, made 5 mM EDTA and 0.1 mM PMSF, and the quantity of vWF released into the conditioned medium was determined by an inhibition ELISA employing an avidin-biotin-peroxidase detection system as previously described (Ewenstein et al., 1987). In experiments involving pharmacologic inhibitors, EC were preincubated for 15–20 min at 37°C with these compounds in M199/G before stimulation with agonist. The quantity of vWF released per well of confluent EC varies among different cultures and passage levels; in order to allow comparisons among experiments, vWF secretion is normalized to maximal thrombin-stimulated vWF release (in response to a 3 or 10 U/ml thrombin) measured in the same experiment.

Cell Permeabilization

Confluent EC monolayers in 24-well tissue culture plates ($\sim$2 × 10^5 cells/well) were washed four times with cytosolic buffer (100 mM KCl, 20 mM NaCl, 30 mM Hepes, 1 Mm MgCl₂, 100 μM EGTA, and 0.1% gelatin, pH 7.0), then briefly (5–15 min) incubated at 37°C with the same buffer containing 4–8 μg/mL saponin as indicated. In some experiments, ATP (2.0 mM) was added to the permeabilization and stimulation buffers. At this concentration, ATP did not stimulate vWF release but potentiated secretion in response to thrombin. The precise concentration of saponin required for optimal permeabilization conditions varied with each stock solution and was adjusted for each new solution of saponin to a concentration which produced 5–10% release of total lactate dehydrogenase, as measured by the method of Wrobleski et al. (1955). After permeabilization, the saponin-containing solution was gently removed and replaced with cytosolic buffer containing the indicated concentrations of agonist. After an additional 15 min at 37°C, the medium was removed and vWF quantitated as described above. Calcium-EGTA buffers were prepared using the formulations of Fabiato and Fabiato (1979) and Miller and Smith (1984), and the free calcium concentrations in calcium-EGTA buffers ≥ 10 μM were verified using a calcium electrode (Orion Research, Inc., Boston, MA). In experiments involving pharmacologic inhibitors, inhibitory levels of EGTA, or peptide inhibitors, these compounds were present during saponin treatment and agonist stimulation.

Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ in suspensions of human umbilical vein EC (generally 1.2 × 10^5 cells/ml) were monitored using fura-2 essentially as described by Brock and Capasso (1988). Fluorescence measurements were made at 37°C in a SPEX Fluorolog II spectrofluorometer (model CM-1: SPEX Industries, Edison, NJ) equipped with a stirring apparatus and a thermostated cuvette holder. The sample cuvette was excited at 340 and 380 nm and emission was measured at 505 nm. Autofluorescence was subtracted from both the 340- and 380-nm tracings in each experiment before the calculation of the 340/380 ratio and conversion to calcium concentrations. The K_d of Ca^{2+} for fura-2 in the presence of 1 mM Mg^{2+} was assumed to be 224 nM (Grynkiewicz et al., 1985).

Materials

Staurosporine was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). W-7, W-5, and ML-7 were purchased from Seikagaku America, Inc. (St. Petersburg, FL). Fura-2/AM was purchased from Molecular Probes Inc. (Eugene, OR). MAPTAM was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Human α-thrombin (2,160 NIH clotting U/mg) was a gift of Dr. John W. Fenton II (New York Department of Health, Albany, NY). Bryostatin 1 was prepared by George Pettit (Arizona State University, Tempe, AZ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Three peptides were used in these studies. Thrombin receptor activating peptide, TR(42-55), representing the amino-terminal sequence generated by thrombin cleavage of the human DAMI cell thrombin receptor, S^{42}FLLRNPNDKYEPF^{55} (Vu et al., 1991), was synthesized by Immuno-Dynamics (La Jolla, CA). MLCK (796-817), a CaM-inhibiting peptide representing chicken smooth muscle myosin light chain kinase (MLCK) residues A^{796}RRKWQKTGHAVRAIGRLSSMA^{817}(C), was prepared and

characterized as previously described (van Berkum and Means, 1991). PKC(19-31), a PKC inhibitor derived from the pseudosubstrate inhibiting domain of the α-isoform of bovine brain protein kinase C, R[19]FARKGA-LRQKNV[31] (Parker et al., 1986; House and Kemp, 1987) was synthesized by the Protein Microsequencing Laboratory, Brigham and Women's Hospital. Its inhibitory activity in human umbilical vein EC was independently verified in PKC assays (Ritchie et al., 1991) of EC extracts.

Results

Comparison of vWF Secretion and Elevations of $[Ca^{2+}]_i$ Stimulated by Thrombin and Thrombin Receptor Activating Peptide

Thrombin has been previously shown to stimulate elevation of $[Ca^{2+}]_i$ as measured by calcium-sensitive fluors in human umbilical vein EC (Jaffe et al., 1987; Brock and Capasso, 1988). Using fura-2 fluorescence to measure $[Ca^{2+}]_i$, we compared thrombin-induced vWF secretion with peak elevations of $[Ca^{2+}]_i$. In agreement with our previous report (Zavoico et al., 1989), we found that thrombin stimulated vWF secretion in a dose-dependent fashion (half-maximum ≈0.1 U/ml). Thrombin-stimulated peak elevation of $[Ca^{2+}]_i$ required somewhat greater agonist concentrations (half-maximum ≈1.0 U/ml) (Fig. 1, A).

To explore further the relationship between thrombin-stimulated elevation of $[Ca^{2+}]_i$ and vWF secretion, we made use of a recently described thrombin receptor activating peptide, S[42]FLLRNPNDKYEPF[55] [TR(42-55)], representing the new amino terminus of the thrombin receptor created by cleavage of the peptide bond between R[41] and S[42] (Vu et al., 1991). TR(42-55) stimulates platelet aggregation, secretion (Vu et al., 1991), phospholipase C activation (Huang et al., 1991), and EC $[Ca^{2+}]_i$ elevation, prostacyclin production (Ngaiza and Jaffe, 1991; GBZ, our unpublished observations), and MARCKS phosphorylation (Jacobson et al., 1992). We found that TR(42-55) maximally stimulated vWF secretion comparable to that observed in response to proteolytic thrombin. However, in contrast to the actions of thrombin, the stimulation of peak $[Ca^{2+}]_i$ elevation in response to the thrombin receptor activating peptide exhibited concentration-dependence indistinguishable from that of vWF release (half-maximum ≈40 μM) (Fig. 1 B). The observed differences between TR(42-55) and thrombin suggest that thrombin may partly act to release vWF from cultured EC through a pathway which is independent of the described thrombin receptor.

Effect of PKC Inhibition on Thrombin-stimulated Secretion in Intact EC

The finding that half-maximal activity of thrombin stimulated vWF release was less than that observed for peak $[Ca^{2+}]_i$ elevation, raised the possibility that other signaling pathways may be involved in thrombin-mediated secretion in EC. In several other cell types, activation of PKC has been shown to increase the sensitivity to calcium of the secretory process to such an extent that secretion occurs at basal $[Ca^{2+}]_i$ (Rink and Knight, 1988). To investigate the possibility of a similar role for PKC in thrombin-stimulated vWF secretion, we utilized the pharmacologic PKC activator, PMA, and the PKC inhibitor, staurosporine (Tamoaki et al., 1986). PMA stimulated rapid vWF secretion in a dose-dependent fashion between 5 and 100 nM, a concentration


A

Thrombin (U/ml)

B
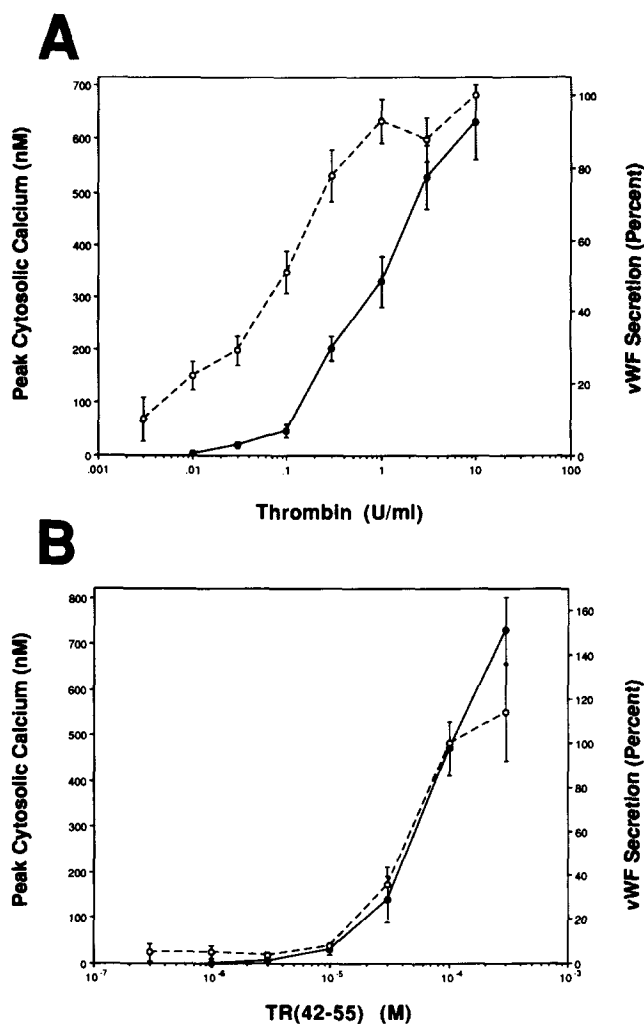
TR(42-55) (M)

Figure 1. Effect of thrombin and the thrombin receptor–activating peptide, TR(42–55) on vWF secretion (o) and $[Ca^{2+}]_i$ elevation (●). (A) Stimulation of vWF release and elevation of $[Ca^{2+}]_i$ in response to α-thrombin. Data for vWF secretion is expressed as a percentage of vWF released in response to 10 U/ml (means ±SEM; $n = 6$). Mean secretion in response to 10 U/ml thrombin was 85.5 ± 16.1 ng/well. $[Ca^{2+}]_i$ are expressed as mean (±SEM) increases from unstimulated (resting) levels ($n = 5$–9) in response to the indicated concentrations of thrombin. The mean resting $[Ca^{2+}]_i$ was 75 ± 7.0 nM. (B) Stimulation of vWF secretion and elevation of $[Ca^{2+}]_i$ in response to TR(42–55). Data for vWF secretion is expressed as the percentage of vWF released in response to 100 μM TR(42–55) (mean ± SEM; $n = 5$). Mean maximal secretion in response to 100 μM TR(42–55) was 97.7 ± 18.2 ng/well. $[Ca^{2+}]_i$ are expressed as mean (±SEM) increases from unstimulated (resting) levels ($n = 4$–8). Released vWF was determined by inhibition ELISA and $[Ca^{2+}]_i$ was determined by spectrofluorometry as described in Materials and Methods.

range previously shown to induce the phosphorylation of MARCKS, a PKC-specific substrate (Jacobson et al., 1992) and PKC translocation in EC (Ritchie et al., 1991). Staurosporine significantly blocked vWF secretion in response to PMA (Fig. 2 A). In contrast, staurosporine did not inhibit thrombin-induced vWF secretion, and in some experiments actually augmented thrombin-mediated responses (Fig. 2 B). Thus, staurosporine distinguishes thrombin-mediated from PMA-mediated secretion. Moreover, the

A / **B**
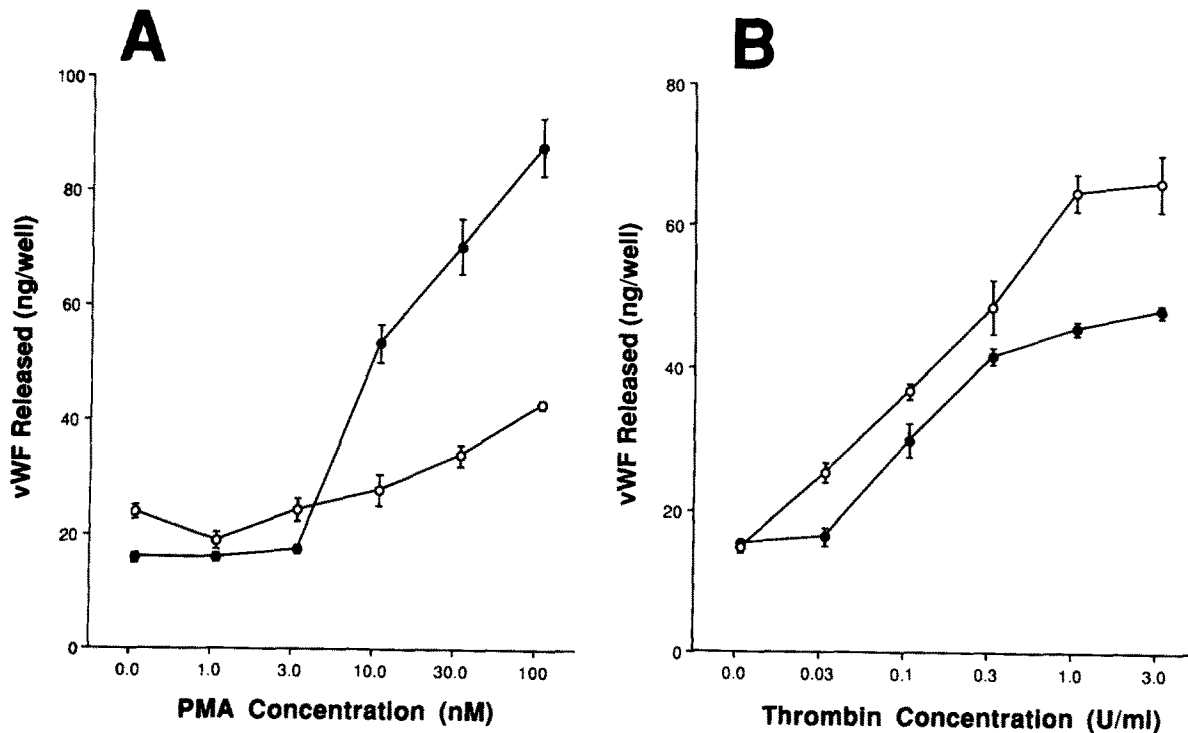
Figure 2. Effect of staurosporine on PMA- and thrombin-stimulated vWF secretion. EC were preincubated for 20 min in the presence (O) or absence (●) of staurosporine (30 nM) before the addition of the agonist. The vWF released in response to (*A*) PMA or (*B*) thrombin at the indicated concentrations was determined by inhibition ELISA (see Materials and Methods). Data points represent the means (±SEM) of quadruplicate wells from one of three similar experiments.

finding that staurosporine does not increase the thrombin concentration required for half-maximal secretion, suggests that thrombin-stimulated PKC activation is not responsible for the observed differences between dose responsiveness to vWF secretion and peak $[Ca^{2+}]_i$ elevation (Fig. 1 *A*). This contention is additionally supported by the finding that
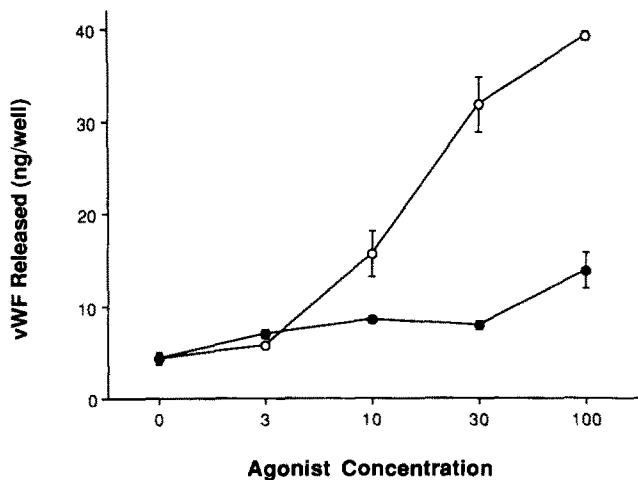

Figure 3. Comparison of bryostatin 1- and PMA-induced vWF secretion. EC were treated with 3–100 nM concentrations of either PMA (O) or bryostatin 1 (●) for 15 min and the quantity of vWF released into the conditioned media was determined by inhibition ELISA (see Materials and Methods). Data points represent the means (±SEM) of triplicate wells from one of three similar experiments.

threshold concentrations of PMA (5 nM) exert no net effect on thrombin-mediated vWF secretion in EC over a broad range of thrombin activities (0.03–3 U/ml) (data not shown).

The singular nature of phorbol ester-stimulated vWF secretion was further demonstrated in experiments with bryostatin 1, a macrocyclic lactone which is known to bind to and activate PKC (Kraft et al., 1986). When tested over the concentration range (3–100 nM) previously shown to induce maximal MARCKS phosphorylation in EC (Jacobson et al., 1992), bryostatin 1 was found to be an ineffective stimulator of vWF secretion (Fig. 3). Thus, PKC activation per se is not sufficient to stimulate significant endothelial vWF secretion. Collectively, these experiments suggest that PKC activation does not account for differences in secretion caused by thrombin and TR(42–55) and further suggest that PKC is not primarily involved in the signal transduction pathway utilized by thrombin.

Involvement of Calcium in Thrombin-stimulated Secretion in Intact EC

To determine if calcium is required for thrombin-stimulated secretion, we examined the effects of the cell-permeant calcium chelator, MAPTAM, and the cell-impermeant calcium chelator, EGTA. Previously, MAPTAM has been shown both to lower the resting level of $[Ca^{2+}]_i$ and to blunt the rise in $[Ca^{2+}]_i$ induced by thrombin in EC (Levin and Santell, 1991). In agreement with a previous report (deGroot et al., 1984), we found that EGTA inhibited thrombin-stimulated secretion by ~50%. Although 100 μM MAPTAM was ineffective when tested alone, it completely abrogated secretion in the presence of EGTA (Fig. 4). These
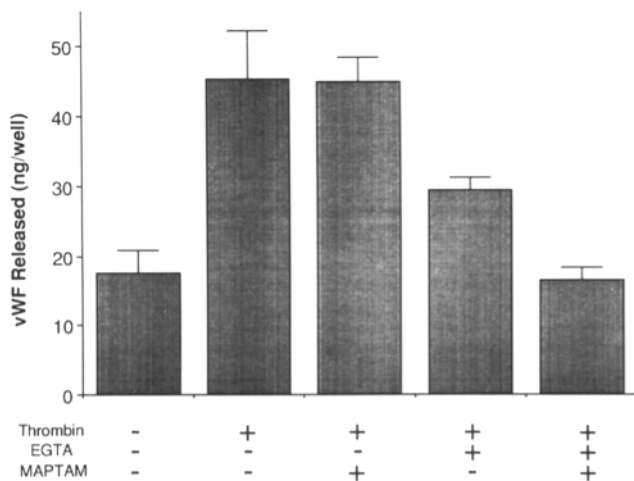
Figure 4. Effect of MAPTAM and EGTA on thrombin-stimulated vWF secretion. EC were preincubated with either 100 μM MAPTAM, 1 mM EGTA or both for 15 min, then stimulated with thrombin (1 U/ml) in the presence or absence of the indicated chelator(s) for an additional 15 min. Released vWF was measured by inhibition ELISA (see Materials and Methods). Data are expressed as means ($\pm$SEM) of triplicate wells from one of three similar experiments.

results suggest that calcium is necessary for secretion. However, these chelation experiments do not establish whether elevations in $[Ca^{2+}]_i$ are required.

Effect of a Calmodulin Antagonist (W-7) and a Myosin Light Chain Kinase Inhibitor (ML-7) on Thrombin- and PMA-stimulated Secretion

To investigate events in thrombin signal transduction distal to a stimulated rise in $[Ca^{2+}]_i$, we tested the effects of pharmacologic inhibitors of calcium-dependent processes. The calcium-binding protein, CaM, and the Ca^{2+}/CaM-activated enzyme, myosin light chain kinase (MLCK), have been implicated in secretion in a number of cell types (Nelson et al., 1987; Nakanishi et al., 1989; Kenigsberg and Trifaro, 1985; reviewed by Harper, 1988). The close correlation between the ability of thrombin to elevate $[Ca^{2+}]_i$ and stimulate vWF secretion led us to examine the possible role of CaM and MLCK in EC. Both the CaM antagonist W-7 (Hidaka et al., 1980), but not the less active analogue, W-5 (data not shown),

and the more specific MLCK inhibitor ML-7 (Hidaka and Tanaka, 1987), were found to inhibit thrombin-stimulated secretion in a dose-dependent manner (Table I). Unexpectedly, both W-7 (but not W-5) and ML-7 also inhibited PMA-induced secretion, a process which does not require elevated $[Ca^{2+}]_i$. These observations highlight the limited specificity of these commonly used pharmacologic inhibitors, and indicate a need for more specific reagents.

Development and Characterization of Minimally Permeabilized EC with Saponin

The most specific currently available inhibitors of intracellular signaling pathways are peptides that mimic protein–protein or protein–substrate binding sites. To use these highly specific, peptide-based reagents, we developed a minimal permeabilization protocol in which EC were rendered permeable to these molecules yet retained responsiveness to thrombin stimulation. Saponin is a cholesterol-dependent permeabilizing agent which produces stable pores in plasma membranes of eukaryotic cells (Akiyama et al., 1980; Brooks and Carmichael, 1983). Complete permeabilization of cells by saponin typically results in the loss of receptor-mediated responses. We therefore began our studies by establishing the minimal treatment with saponin that would produce permeabilization. The extent of saponin treatment was initially ascertained by measuring the release of the cytosolic enzyme lactate dehydrogenase during 15 min of treatment with varying concentrations of the detergent in a cytosolic buffer (Fig. 5 *A*). Saponin concentrations of 4–8 μg/ml typically produced 5–10% lactate dehydrogenase release and retain other large intracellular proteins as analyzed by SDS-PAGE of the cultured medium (data not shown). This degree of saponin exposure also appeared to permeabilize the cells to small extracellular molecules such as externally applied calcium leading to secretion (Fig. 5 *B*). Secretion in response to calcium was concentration dependent, beginning at ~1–2 μM and reaching a plateau near 10 μM (Fig. 5 *C*).

We next determined the effect of saponin incubation time at these low concentrations on agonist-induced vWF secretion. 5–10-min incubations with a low concentration of saponin (e.g., 6 μg/ml) preserved responsiveness to thrombin, and the magnitude of this response was found to be reproducibly augmented by the permeabilization procedure (Fig. 6).

Table I. Effect of a Calmodulin Antagonist (W-7) and a Myosin Light Chain Kinase Inhibitor (ML-7) on Thrombin-and PMA-stimulated vWF Secretion

| | | Inhibitor | | | |
| | | W-7 | | ML-7 | |
Agonist	None	10 μM	30 μM	10 μM	30 μM
Thrombin 1.0 U/ml	144 $\pm$ 9.8 (100)	84 $\pm$ 4.4 (58)	20.5 $\pm$ 1.7 (14)	96 $\pm$ 4.3 (67)	36 $\pm$8.5 (25)
PMA 100 nM	71 $\pm$ 4.2 (100)	46.5 $\pm$ 2.8 (65)	6.5 $\pm$ 2.2 (9)	40.5 $\pm$ 11.2 (57)	6.0 $\pm$ 3.2 (9)

EC were preincubated in the presence or absence of the indicated inhibitor for 15 min, then stimulated with either thrombin (1 U/ml) (expt. 1) or PMA (100 nM) (expt. 2) for an additional 15 min. The quantity of stimulated vWF release was derived by subtracting the quantity of vWF released in the absence of both agonist and inhibitor (basal secretion) from the measured vWF in each releasate. The data for each agonist are expressed as the mean ($\pm$SEM) of triplicate wells from one of three similar experiments. Numbers in parentheses represent residual agonist-induced vWF secretion (%) in the presence of the indicated inhibitor.
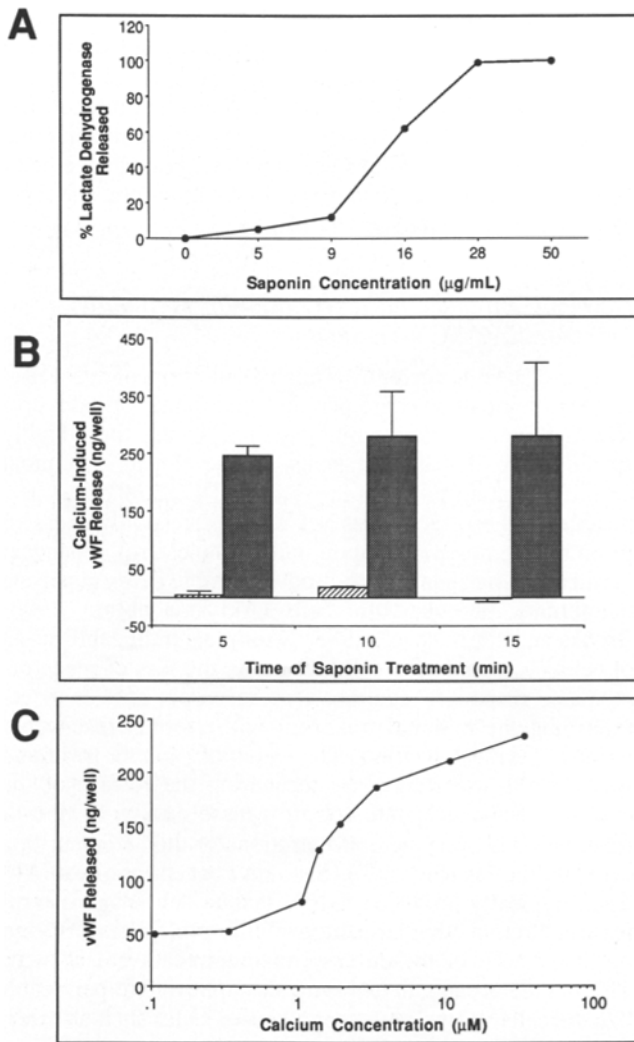
Figure 5. Development and characterization of EC minimally permeabilized with saponin. (A) EC were treated with saponin for 15 min at the indicated concentrations and released lactate dehydrogenase determined as described in Materials and Methods. The concentration dependence of saponin treatment varied slightly among four different experiments using different stock solutions; the results of a representative experiment are presented. (B) EC were treated with either 2.0 μg/ml (striped) or 4.0 μg/ml (shaded) saponin for the indicated time followed by a 15 min stimulation with pCa 5.5 calcium-EGTA buffer (3.2 μM free calcium). Stimulated vWF secretion was derived by substracting vWF released in the absence of added calcium (basal secretion) from the total vWF released during the incubation period. Results are expressed as the means (±SEM) of triplicate wells. (C) EC were treated with 6 μg/mL saponin for 10 min followed by stimulation with various calcium-EGTA buffers (see Materials and Methods). The results are the average of duplicate wells and are representative of ten similar experiments.

Longer exposure (20 min) to the same concentration of saponin, both increased unstimulated vWF release and abolished thrombin-mediated secretion although the response to calcium was retained (data not shown). In contrast to the initial enhancement of thrombin-stimulated secretion, PMA responsiveness was rapidly abolished under these same conditions lending additional support to the contention that
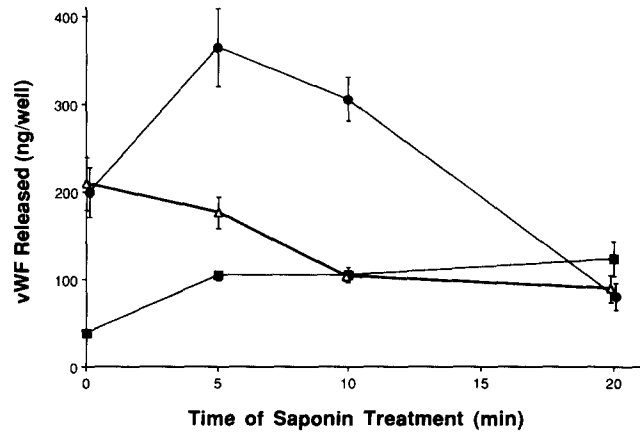
Figure 6. Time-dependent effect of saponin on agonist-induced vWF release. EC were treated with 6 μg/mL saponin for the indicated times and then stimulated for 15 min with either buffer alone (■), 100 nM PMA (Δ), or 1 U/mL thrombin (●). The quantity of vWF released during the stimulation period was determined by inhibition ELISA (see Materials and Methods). Data points represent the means (±SEM) of triplicate wells from one of six similar experiments.

thrombin- and PMA-stimulated vWF secretion are mediated by disparate signaling pathways.

Thrombin-induced secretion in the permeabilized cells occurred in the absence of extracellular calcium. Cells prelabeled with ^{45}Ca exhibited thrombin-induced calcium efflux (KAB; our unpublished observations) indicating the release of calcium from intracellular stores. To confirm that minimally-permeabilized EC utilize calcium in the signal transduction pathway of thrombin-induced vWF secretion, we performed experiments in permeabilized cells in the pres-
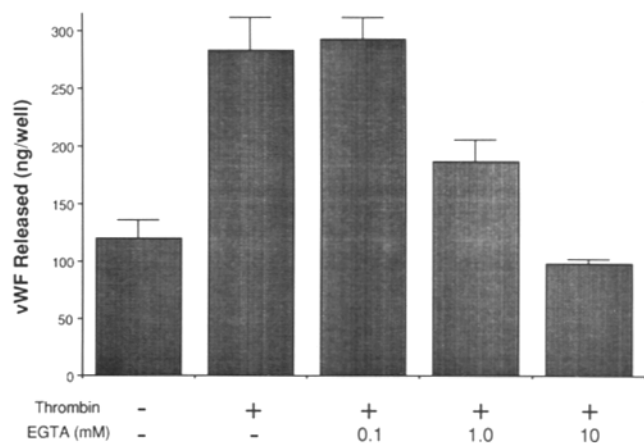
Figure 7. Effect of EGTA on thrombin-stimulated vWF release. EC were treated with 5 μg/mL saponin for 10 min in the presence of EGTA at the indicated concentrations and then stimulated with 0.3 U/mL thrombin for 15 min. Indicated EGTA concentrations represent the amount of chelator present in the permeabilization and stimulation buffers. The quantity of vWF released into the conditioned medium was determined by inhibition ELISA (see Materials and Methods). The data are represented as the means (±SEM) of triplicate wells from one of three similar experiments.

ence of increasing concentrations of EGTA. We found that EGTA, in a dose-dependent manner, inhibited thrombin-induced secretion (Fig. 7) implying a requirement for calcium in this response in permeabilized EC.

Effect of a Calmodulin Binding Peptide and a PKC Inhibitory Peptide on Thrombin-stimulated Secretion in Permeabilized EC

We utilized our minimally permeabilized EC system to examine the influence of two selective peptide inhibitors on thrombin-stimulated vWF secretion. MLCK(796–817), a peptide CaM antagonist (van Berkum and Means, 1991) patterned after the CaM binding region of chicken smooth muscle MLCK residues Ala^{796}-Ala^{817} (Lukas et al., 1986; Kemp et al., 1987), and PKC(19–31), an inhibitor of PKC derived from its endogenous pseudosubstrate domain (House and Kemp, 1987), were each introduced into EC by saponin permeabilization. We found that 100 μM MLCK(796–817), but not PKC(19–31), blocked thrombin-induced secretion by ~83% in permeabilized cells (Fig. 8). Access into the intracellular space was required for inhibition by MLCK(796–817) since this peptide had no effect on thrombin-stimulated secretion in intact cells. Secretion in response to externally applied calcium buffers (3.2 μM) was also blocked (66–82% inhibition; n = 2) by 100 μM MLCK (796–817) but not by PKC(19–31) (data not shown).

Discussion

Regulated secretion in eukaryotic cells requires the translocation of membrane-bound granules from the cytoplasm toward the plasma membrane and granule fusion with the plasma membrane. These events are presumed to be regulated by second messengers generated upon agonist stimulation (reviewed by Burgess and Kelly, 1987; Harper, 1988; Rink and Knight, 1988). Both calcium and PKC have been proposed as second messengers in stimulus-secretion coupling in EC (Loesberg et al., 1983; deGroot et al., 1984). The purpose of our study was to determine the relative importance of these two second messenger pathways in thrombin-stimulated secretion. Our data support a model in which thrombin-induced secretion proceeds primarily through a Ca^{2+}/CaM–dependent pathway. Evidence for this conclusion comes from experiments in both intact and saponin-permeabilized EC. In intact EC, we found a close correlation between the dose-response curves for vWF secretion and elevation of [Ca^{2+}]$_i$ and inhibition by the calcium chelators, MAPTAM and EGTA. In contrast, the finding that staurosporine inhibited PMA- but not thrombin-stimulated secretion suggests that different pathways are involved. In our minimally permeabilized cell model that retained thrombin-induced secretion, we observed an inhibitory effect of EGTA on thrombin-induced vWF release. Most convincingly, a specific CaM-binding peptide MLCK(796–817), but not a PKC inhibitory peptide, PKC(19–31), inhibited thrombin-stimulated secretion in these permeabilized cells.

Our studies confirm previous reports that the recently identified platelet thrombin receptor (Vu et al., 1991) is functional in human umbilical vein EC (Ngaiza and Jaffe, 1991). We (Zavoico, G. B., M. Gamberdella, A. J. Ritchie, and B. M. Ewenstein, manuscript in preparation) and others (Ngaiza and Jaffe, 1991) have shown that the kinetics of cal-
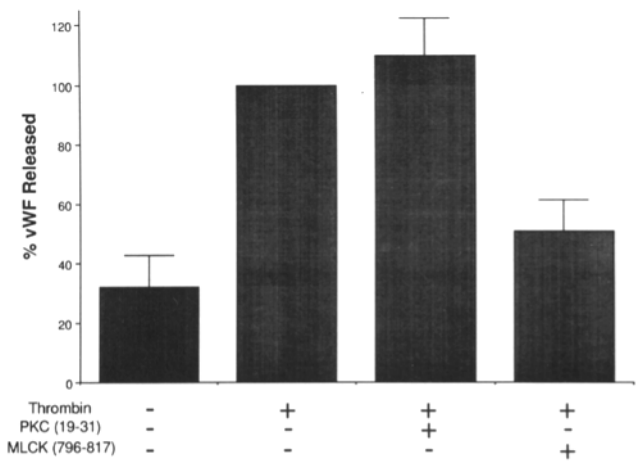
Figure 8. Effect of peptides MLCK(796–817) and PKC(19–31) on thrombin-stimulated vWF secretion in permeabilized cells. EC were treated for 15 min with a buffer containing 5 μg/ml saponin, 2 mM ATP, and the indicated peptide (100 μM), then stimulated for an additional 15 min with 1 U/ml thrombin in the same buffer (see Materials and Methods). The results (mean ± SEM; n = 3) are expressed as percentage of vWF secreted by thrombin-stimulated cells in the absence of peptide inhibitors.

cium responses following TR (42–55) and thrombin are similar. Yet a closer correlation between [Ca^{2+}]$_i$ and vWF secretion was observed in response to a specific peptide agonist, TR(42–55), than to proteolytically active thrombin. The reason for this disparity is not known but is unlikely to involve PKC because TR(42–55), like thrombin, stimulates PKC activation in this cell type (Jacobson et al., 1992). It is possible that the processing of EC for [Ca^{2+}]$_i$ measurements desensitized the thrombin receptor to stimulation by thrombin but not by TR(42–55). Alternatively, the possibility exists that thrombin activates additional signal transduction pathways, such as those involving cAMP or tyrosine kinases, which may not be stimulated by TR(42–55) and which may potentiate vWF secretion. These possibilities are under investigation.

Plasma membrane permeabilization has been used successfully to investigate stimulus-secretion coupling in many cell types (reviewed by Hersey and Perez, 1990) including EC (Suttorp et al., 1985; Martin and Michaelis, 1990; Van Geet et al., 1990). Saponin has been used typically at relatively high concentrations (25–100 μg/ml) (Smolen and Stoehr, 1985; Kimura et al., 1986), resulting in maximal permeabilization, with complete release of lactate dehydrogenase. Such preparations retain their ability to secrete in a regulated manner, but only to externally applied calcium-EGTA buffers and/or GTP analogues. In contrast, our cell preparations, generated by brief treatment with much lower saponin concentrations (4–8 μg/ml), retain their responsiveness to physiologic agonists such as thrombin and are susceptible to the introduction of small macromolecules. Our observations suggest that the pore size in such minimally permeabilized cells is smaller allowing retention of critical signaling components.

A signal transducing role for calcium in EC secretion was previously inferred from experiments using ionophores. Saponin permeabilization of the plasma membrane followed by the application of calcium-EGTA buffers confirmed these observations. It appears paradoxical that our saponin-treated

cells display intracellular calcium-mediated thrombin responses yet allow extracellular calcium to enter and directly cause secretion. We propose that the residual cell membrane in permeabilized cells creates a large "unstirred layer" which limits the rate of $[Ca^{2+}]_i$ equilibration. As a consequence, the cells can still produce transient and local elevations in $[Ca^{2+}]_i$, sufficient to activate calcium-dependent secretory components, before its dissipation by diffusion out of the cell. This residual membrane barrier may also explain the requirement for tenfold higher levels of calcium in the external medium to cause secretion than are generated in intact cells, as well as the requirement for high concentrations of peptide inhibitors. Similar calcium dose-response curves have been reported in many other permeabilized cell preparations (reviewed in Knight et al., 1989).

Our data do not support the primary involvement of PKC in thrombin-stimulated secretion in EC and is thus at variance with conclusions drawn from some other cell types. For example, platelet secretion has been shown to parallel the phosphorylation of p47, a PKC substrate in this cell type (Haslam and Davidson, 1984; Gerrard et al., 1989). Similarly, Ludowyke et al. (1990) have shown that histamine secretion from rat basophilic leukemia cells correlates with PKC-mediated phosphorylation of myosin heavy and light chains. In contrast to the conclusions of those studies and in agreement with the data presented in this report, H-7, a pharmacological inhibitor of PKC, was shown not to inhibit amylase secretion from pancreatic acini (Pandol and Schoeffield, 1986). Similarly, inhibition of PKC by either pharmacologic inhibitors such as staurosporine or K252a (Kase et al., 1987) or by peptide PKC(19–31) failed to block agonist-induced secretion in bovine adrenal chromaffin cells (Terbush and Holz, 1990; Isosaki et al., 1991). Taken together, these studies demonstrate that a primary role of PKC in secretion is not a universal requirement.

The mechanism by which PMA stimulates vWF secretion remains to be defined. PMA induces PKC translocation from cytoplasmic to membrane fractions in human EC over a range of PMA concentrations which parallel vWF secretion (Ritchie et al., 1991). Under identical conditions, in replicate wells, we have failed to observe translocation of PKC in response to thrombin at concentrations up to 10 U/ml (Ritchie, A. J., B. M. Ewenstein, and J. S. Pober, unpublished observations). Furthermore, as reported here, bryostatin 1 can activate PKC without inducing vWF secretion. Cumulatively, these observations provide support for the notion that PKC activation in response to PMA may be qualitatively different from that seen in response to naturally occurring agonists such as thrombin (Thomas et al., 1987).

The observation that PMA responsiveness is lost in our saponin-permeabilized EC preparation may be explained either by the loss of PKC activity or by specific inhibition of the phorbol ester by the detergent. Recent experiments have shown that PMA stimulates MARCKS phosphorylation in saponin-permeabilized EC and that PKC(19–31) inhibits this response (Birch, K. A., J. S. Pober, and B. M. Ewenstein, manuscript in preparation). Therefore, the failure of PKC(19–31) to block thrombin-induced vWF secretion further supports the argument that PKC activation is not an essential feature of this process.

A role for calmodulin in stimulus-secretion coupling in other cell types has been demonstrated using pharmaco-

logical inhibitors of calmodulin such as W-7 and trifluoperizine (Kimura et al., 1986; reviewed in Harper, 1988; Trifaro and Fournier, 1987). In addition, anti-CaM antibodies were shown to block cortical granule fusion in sea urchin oocytes (Steinhardt and Alderton, 1982) and acetylcholine- and K+-induced catecholamine secretion in chromaffin cells (Kenigsberg and Trifaro, 1985). In contrast to results obtained by Terbush and Holz (1990) in which they observed no effect of Ca^{2+}/CaM Kinase II(292–317), another CaM-binding peptide, on Ca^{2+}- or PMA-induced exocytosis from bovine adrenal chromaffin cells, we found that MLCK(796–817), blocked thrombin-stimulated secretion in minimally-permeabilized EC. We have since observed similar inhibition by other CaM-binding peptides (Birch, K. A., J. S. Pober, and B. M. Ewenstein, manuscript in preparation).

Ca^{2+}/CaM may regulate secretion at multiple sites along the secretory pathway. For example, it may be involved in activating contractile elements such as MLCK which may play a role in granule movement from the cytoplasm to the plasma membrane. The phosphorylation of myosin light chain by MLCK correlates with the exocytotic process in a number of other cell types (Inagaki et al., 1984; Saitoh et al., 1986) and more recently in EC retraction (Wysolmerski and Lagunoff, 1990), a process with which secretion may share certain common features. In addition, pharmacological inhibitors of MLCK, ML-7 and ML-9 (Hidaka and Tanaka, 1987), have been used to demonstrate the potential involvement of MLCK in catecholamine secretion from adrenal chromaffin cells (Nagatsu et al., 1987; Nakanishi et al., 1989). However, the inhibition by ML-7 and W-7 of both PMA- and thrombin-induced vWF secretion in EC suggests that either the signal transduction pathway utilized by PMA involves Ca^{2+}/CaM and possibly MLCK at some distal step, or more likely, that these agents block both calmodulin-dependent and independent kinases. The recent finding that W-7 inhibits phorbol ester-induced contraction in aortic smooth muscle cells by interfering with PKC translocation (Chuprun et al., 1991) lends support to the latter interpretation. Thus, these pharmacologic inhibitors are of limited utility in accessing the role of calmodulin and MLCK in signal transduction pathways, and such limited utility emphasizes the value of peptide-based, specific inhibitors.

Our observation that MLCK(796–817) blocks thrombin-stimulated secretion in permeabilized EC suggests that Ca^{2+}/CaM may act, at least in part, through the activation of MLCK or other Ca^{2+}/CaM-dependent protein kinases. Alternatively, Ca^{2+}/CaM may be involved in the dissolution of cortical actin networks which, under basal conditions, inhibit granule fusion with the plasma membrane. Previous studies have shown that CaM inhibits the interaction of fodrin, an actin-binding protein, with cortical actin filaments (Harris and Morrow, 1990) and antisera against brain-fodrin has been reported to partially inhibit Ca^{2+}-induced secretion from permeabilized adrenal chromaffin cells (Perrin et al., 1987). Fodrin, protein 4.1 and other actin binding proteins have been identified in EC (Leto et al., 1986; Pratt et al., 1984; J. S. Morrow, personal communication) and other secretory cells and may play a role in regulated secretion (reviewed by: Burgoyne, 1990; Trifaro, 1987; and Aunis and Bader, 1988).

In summary, the experiments reported here using both intact cells and a novel permeabilized preparation of human

umbilical vein EC indicate that Ca²⁺/CaM is a major second messenger system for transducing thrombin-stimulated vWF secretion. In contrast, PKC activity is not essential to this process although an adjuctive role cannot be ruled out. Finally, the thrombin-responsive, minimally-permeabilized preparation of EC described here should prove useful in future studies of stimulus-response coupling in this cell type.

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