Changes in Cytosolic Ca^{2+} Associated with von Willebrand Factor Release in Human Endothelial Cells Exposed to Histamine

Study of Microcarrier Cell Monolayers Using the Fluorescent Probe Indo-1

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Abstract

A method for measuring fluorescence in anchored monolayers of human endothelial cells is described and used to demonstrate changes in the cytosolic free-calcium concentration ($[Ca^{2+}]_c$) in these cells exposed to histamine and thrombin; some endothelial responses to both agonists (e.g., mitogenesis) have been suggested to be Ca^{2+}-mediated. Umbilical vein endothelial cells were cultured on microcarriers and loaded with the Ca^{2+} indicator, indo-1. Enzymatic cell detachment was avoided by monitoring the indo-1 fluorescence ratio (400/480 nm) of a stirred suspension of cell-covered microcarriers. Basal $[Ca^{2+}]_c$ was estimated to be 70–80 nM. Thrombin induced a transient dose-dependent increase in $[Ca^{2+}]_c$, which was active-site dependent. Histamine stimulated a dose-dependent increase in $[Ca^{2+}]_c$, which was reversed by removal of histamine and inhibited competitively by the H_1-receptor antagonist pyrilamine, but not by the H_2-receptor antagonist cimetidine. Furthermore, histamine induced a dose-dependent secretion of von Willebrand factor, which paralleled the rise in $[Ca^{2+}]_c$ and was similarly blocked by the H_1-receptor antagonist, and which may contribute to platelet deposition at sites of inflammation.

Introduction

Thrombin has a variety of effects on endothelium including stimulation of prostacyclin (PGI_2)[1] and platelet-activating factor (PAF) synthesis (1, 2), cell retraction (3), mitogenesis (4), and secretion of adenine nucleotides (5), von Willebrand factor (vWF) (6) and "endothelium-derived relaxant factor" (7). Because many effects of thrombin can be mimicked by calcium ionophore, a rise in cytosolic calcium has been suspected to be an important triggering event in the endothelium's response to this enzyme. Histamine has also been shown to elicit multiple responses from endothelial cells, including PGI_2 and PAF synthesis (8), disassembly of actin filaments (9), and cell retraction (10–12). Histamine-induced permeability changes and PGI_2 and PAF synthesis are inhibited by H_1-receptor antagonists, suggesting the existence of functional human endothelial H_1 receptors. On the other hand, both H_1- and H_2-specific agonists have been shown to bind to endothelium in the mouse (13). Nevertheless, intracellular events triggered by binding of histamine to its receptor, including possible coupling to cytosolic Ca^{2+} transients, have not been examined for endothelial histamine receptors.

Fluorescent calcium-sensitive intracellular probes have enabled receptor-mediated cytosolic calcium transients to be examined in a variety of cells (14–20). However, obtaining these measurements from anchorage-dependent cells has usually required that the cells be physically or enzymatically detached from their culture surface to form a suspension suitable for spectrofluorometry. For example, trypsinization has recently been used to form suspensions of rat smooth muscle cells (19) and renal tubular cells (20) for study using quin2 and fura-2. Such procedures alter the normal cell surface, abrogate cell polarity, and destroy normal intercellular junctions; results must therefore be interpreted cautiously with respect to conditions obtaining in anchorage-dependent cells in their characteristic attached state. Our interest in the role of cytosolic calcium in the endothelial responses to thrombin and histamine led us to develop a method using indo-1 for observing changes in intracellular calcium within human endothelial cells maintained as a confluent monolayer on microcarriers.

Methods

Materials. Histamine, cimetidine, pyrilamine, A23187, Medium 199 (with Earle's salts, L-glutamine, and 25 mM Hepes), endothelial cell growth supplement, porcine grade I heparin, and insulin-transferrin-sodium selenite media supplement were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was from Hazelton Dutchland Inc. (Denver, PA). Penicillin/streptomycin and trypsin/EDTA were from Gibco (Grand Island, NY). Type III collagenase was from Cappel Worthington Biochemicals (Malvern, PA). Human thrombin was provided by Dr. David Aronson, Bureau of Biologics, Federal Drug Administration, Bethesda, MD. Diisopropylphosphoryl (DIP)-thrombin was prepared by incubating thrombin (1 mg/ml in 0.1 M Tris, 0.15 M NaCl, pH 7.4) with 2 mM diisopropyl fluorophosphate (DFP) for 1 h at room temperature. Excess DFP was removed by dialysis. Glu-plasminogen was purified by affinity chromatography of fresh-frozen human plasma on lysine-agarose (21) and activated by urokinase (Sterling-Winthrop Pharmaceuticals, Rensselaer, NY). Indo-1 pentacetoxymethyl ester (indo-1 AM) and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) were from Calbiochem-Behring Corp. (La Jolla, CA). 2-deoxy-D-[1-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_c$, cytosolic free-calcium concentration; DFP, diisopropyl fluorophosphate; DIP-thrombin, diisopropylphosphoryl thrombin; [^{3}H]DOG, 2-deoxy-D-[1-^{3}H]glucose; PAF, platelet-activating factor; PGI_2, prostacyclin; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; vWF, von Willebrand factor.

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^{3}H]glucose ([^{3}H]DOG) was from Amersham Corp. (Arlington Heights, IL). Human albumin (Albuminar-25) was from Armour Pharmaceutical Co. (Kankakee, IL).

Cell culture. Human endothelial cells were obtained from umbilical veins by treatment with 0.1% collagenase. Cells from one cord were pelleted and resuspended in 5 ml of Medium 199 with 20% fetal bovine serum, 10 μg/ml endothelial cell growth supplement, 90 μg/ml porcine heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, bovine insulin 5 μg/ml, human transferrin 5 μg/ml, and sodium selenite 5 ng/ml. The cell suspension was added to 1.5 ml of a suspension of Cytodex 3 microcarriers (Pharmacia Fine Chemicals, Piscataway, NJ), prepared according to the manufacturer's instructions (1 g of dry beads/100 ml) and preincubated in culture medium. Cells were grown at 37°C in 5% CO_2 in 50-ml conical Falcon polypropylene tubes (Falcon Labware, Oxnard, CA). 2 ml of media was replaced each day. The cells were used after 4–6 d when confluent.

Cell loading with indo-1. Indo-1 AM was stored at − 20°C as a 1 mM stock solution in dimethyl sulfoxide. Approximately 2 ml of suspended cell-covered microcarriers were used for one set of experiments. Indo-1 AM was added to the medium at a final concentration of 5 μM, and the cells were returned to their incubator for 30–60 min. The microcarrier suspension was then poured into a 5-ml syringe fitted with 111 μm polyethylene Spectra/Mesh (Spectrum Medical Industries, Los Angeles, CA), which allowed the medium containing unincorporated indo-1 AM and any dead or unattached cells to drain through while retaining the cell-covered microcarriers. The microcarriers retained on the mesh were then washed rapidly with 25 ml of Hanks' balanced salt solution containing 25 mM Hepes (HBSS), suspended in 0.5 ml of HBSS containing 1% human albumin, and maintained at room temperature until used. The washed, indo-1–loaded cells were used within 1–2 h, during which time they remained viable, responsive, and attached to the microcarriers. Dye leakage during the course of these experiments never exceeded 8%. The intracellular indo-1 concentration after the above loading procedure was estimated to be 60–200 μM, based on an average cell density of 50,000/cm^2 and an estimated endothelial cell volume of 1.5 pl (22, 23).

Fluorescence measurements. Aliquots of indo-1–loaded cells attached to microcarriers were added to 2 ml of HBSS in a stirred, temperature-controlled (37°C) cuvette in the spectrofluorometer (SLM 8000, SLM-Aminco, Urbana, IL). The excitation wavelength was 355 nm with a 16-nm slit width. Crossed polarizers were used throughout to minimize the effects of light scattering. The indo-1 emission was monitored as the ratio of intensities recorded at 400/480 nm, using two double-grating emission monochromators (16-nm slit widths) placed in T format. Hamamatsu Corp. R928P photomultiplier tubes (Middlesex, NJ) in cooled housings were employed on both emission channels. Emission spectra were recorded with 4-nm slit widths and were corrected for the wavelength-dependent response of the photomultiplier tubes.

Roughly 5–10 μl of settled microcarriers (about 2,000 beads) containing 2–3 cm^2 surface area and 100,000–200,000 cells were used for each experiment. Therefore, one umbilical cord in 5 d yielded cells for about 20–30 experiments. The fluorescence data in the figures are each representative of experiments performed four or more times in different cell isolates.

Calcium calibration. The fluorescence recorded from the indo-1–loaded microcarrier-cell suspensions was found to be significantly affected by contributions from both the Raman peak and the intrinsic fluorescence of the endothelial cells (see Fig. 1). Thus, the equation developed by Grynkiewicz et al. (24) could not be used to calculate cytosolic calcium directly from the recorded ratio of 400/480-nm emission intensities. Accordingly, calibration of the 400/480-nm emission (obtained under the conditions of these experiments) to the cytosolic free-calcium concentration ($[Ca^{2+}]_c$) was performed empirically as follows: after dye loading, cell-covered microcarriers were washed and suspended in the cuvette by methods described above, except calcium-free HBSS containing 10 mM EGTA was substituted for HBSS. Incorporated indo-1 was released from the cells by addition of a lytic concentration of digitonin (final concentration, 0.1 mM), and the ratio of fluorescence at 400/480 nm was recorded before and after serial addition of 2-μl aliquots of 1 M $CaCl_2$. Free Ca^{2+} concentrations after each addition were calculated from the total added calcium by solving the metal–ligand equilibrium equations as described by Fabiato and Fabiato (25), using an apparent stability constant for Ca-EGTA (buffered to pH 7.3) of $10^{6.94}$ (26). Dye response obtained by these methods was found to be approximately linear for $[Ca^{2+}]_c$ ranging from 70 to 175 nM. Spectra obtained for aqueous solutions of indo-1 were unaffected by addition of digitonin (not shown).

vWF immunoassay. vWF antigen was quantitated using a modification of the enzyme-linked immunosorbent assay described by Fishman et al. (27). Microtiter plates were first coated with a goat anti-human factor VIII–related antigen (Atlantic Antibodies, Scarborough, ME), followed by incubation with test sample. The second antibody was an affinity-purified rabbit antibody to human vWF prepared in our laboratory and verified to be monospecific on Western blots of whole human plasma, human endothelial cell conditioned media, and endothelial cell detergent lysates. In control experiments, we confirmed that there is negligible binding of this antibody to bovine vWF (present in unconditioned culture medium). This rabbit antibody was followed by $F(ab')_2$ fragments of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chemical Co.). Finally, the alkaline phosphatase substrate used was *p*-nitrophenol phosphate 1 mg/ml in 0.1 M glycine, pH 10.4, with 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Dilutions of normal plasma pooled from five donors were used as the standards, and results are expressed as units per milliliter where 1 U is defined as the concentration of vWF in 1 ml of normal pooled plasma. The assay is linear in the range from 0.01 to 0.001 U. All samples were assayed in triplicate.

vWF release experiments. Cells were grown on 24-well tissue culture plates (2 cm^2 per well) and used at confluence (5–6 d). Monolayers were washed three times with 37°C HBSS, and 0.4 ml of medium or HBSS containing the appropriate stimulus and/or antagonist was added. The cells were then incubated at 37°C for 45 min, and the medium was removed, centrifuged for 4 min in a Beckman microcentrifuge to remove cell debri, and the supernatants stored at − 20°C until assayed. The monolayer was washed once with 1 ml of HBSS, and cell protein was assayed by the method of Lowry (28) using bovine albumin as a standard. Released vWF was expressed in units (as defined above) per milligram of total cell protein and compared with control wells in the same experiment. Results are reported as percent difference from control.

Cell cytotoxicity assay. Nonspecific cell toxicity was assessed by measuring release of [^{3}H]deoxy-D-glucose (29). Cells grown in 0.5-cm^2 wells were incubated for 18 h with 0.1 ml of medium containing 1 μCi of [^{3}H]DOG (15 Ci/mmol). Wells were then washed five times with 0.1 ml of HBSS-0.5% human albumin, and incubated for 45 min with histamine (10^{-3} to 10^{-8} M) or medium alone. After histamine treatment, the media was removed, and the wells were washed twice with 0.1 ml of HBSS with albumin. The cell monolayer was dissolved in 0.3 ml of 2% Triton X-100. Media and washes were pooled, and counted in 4 ml of Aquasol-2 in a Beckman LS98 beta counter (Beckman Instruments, Inc., Palo Alto, CA) with an efficiency of 36%; this count was designated released disintegrations per minute (R). Solubilized cells were similarly counted and designated cell-associated disintegrations per minute (C). Percent release was calculated as $[R/(R + C)] \times 100$. Mean release at each histamine concentration (six replicates) was compared with the mean of 12 control wells containing no histamine, and is reported as percent difference from control.

Data analysis. vWF and [^{3}H]DOG release data were analyzed using a standard one- and two-way analysis of variance (30). Differences in mean release from cells in different treatment groups (different histamine doses, histamine and/or histamine antagonists, and histamine in the presence or absence of EGTA) were compared by computing multiple t confidence intervals (reference 30, page 80). To achieve an overall confidence level of 95%, the t statistic, $t[1 - \alpha/2m, a(n - 1)]$, was used to compute each interval, where $\alpha = 0.05$, m = number of pairs of treatment groups being compared, a = total number of treatment groups, and n = number of replicates in each group.

Results

Fluorescence emission spectra of indo-1–loaded human endothelial cells

Fig. 1 shows the fluorescence emission spectra of indo-1–loaded endothelial cells in microcarrier suspension before and after cell lysis with digitonin. The intrinsic fluorescence of endothelial cells not containing fluorophore is shown for comparison and includes a contribution from the Raman peak (at 405 nm). Spectra shown were obtained from ~ 2,000 microcarriers covered with confluent endothelial cell monolayers, suspended in a total volume of 2.05 ml. The ratio of emission at the high-Ca^{2+} to low-Ca^{2+} peaks (400/480 nm) obtained from the indo-1–loaded cells suggested a basal cytosolic free-calcium concentration of 70–80 nm (see Methods).

Changes in $[Ca^{2+}]_c$ in response to thrombin

To maximize our ability to detect dynamic changes in $[Ca^{2+}]_c$, the ratio of the high- and low-calcium peaks of indo-1 (400/480 nm) was continuously monitored using two emission monochrometers in T format. In Fig. 2, changes in cytosolic calcium in response to selected concentrations of human thrombin are shown. The threshold dose at which a detectable change in fluorescence was reliably observed was 0.01 U/ml. Higher doses resulted in a higher peak $[Ca^{2+}]_c$, shorter time to peak, and a more prolonged response. The cells responded a second time to thrombin at the lower doses, but could not be restimulated after exposure to 1 U/ml thrombin (Fig. 2 *C*). Usually the $[Ca^{2+}]_c$ did not return completely to baseline during the subsequent 5–10 min of observation, although this ability to recover from thrombin varied from one cell preparation to another. When the experiment was performed in 10 mM EGTA (Fig. 2 *C*), the baseline ratio was lower and progressively declined (suggesting progressive leeching of calcium and/or loss of dye from the cells). Nevertheless, thrombin stimulated a marked rise in $[Ca^{2+}]_c$, even in the presence of subnanomolar extracellular Ca^{2+} (Fig. 2 *C*).

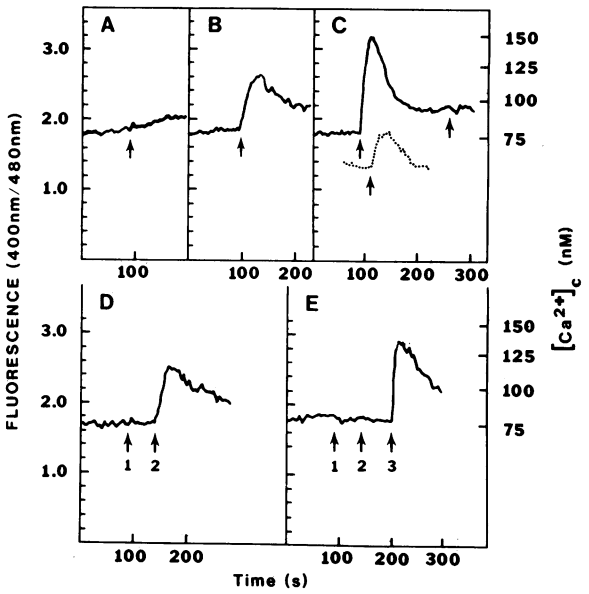

Figure 2. Changes in $[Ca^{2+}]_c$ in human endothelial cells in response to thrombin. Thrombin was added to the stirred microcarrier-cell suspension at times indicated by arrows in the following final concentrations: (*A*) 0.01 U/ml; (*B*) 0.1 U/ml; (*C*) 1 U/ml. For comparison, the response to a thrombin concentration of 1 U/ml by cells suspended in Ca^{2+}-free buffer containing 10 mM EGTA is also shown in *C* (*dotted line*). The absence of response to DIP-thrombin is demonstrated in *D*. DIP-thrombin, equivalent to 1 U/ml before inactivation, was added at arrow *1*. Active thrombin (0.1 U/ml final concentration) was added at arrow *2*. The effect of another active-site inhibitor of thrombin is shown in *E*. PPACK (final concentration 1.7×10^{-6} M) was added at arrow *1*, followed by thrombin (1 U/ml) at arrow *2* and histamine (10^{-5} M) at arrow *3*.

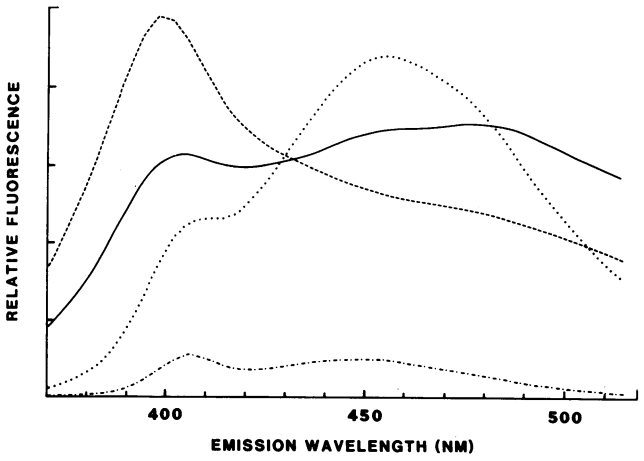

Figure 1. Fluorescence emission spectra of human endothelial cells attached to microcarriers after loading with indo-1 and subtraction of intrinsic cell fluorescence: (*solid line*) intact cells in 2 mM Ca^{2+} buffer, (*dashed line*) cells after lysis with 0.1 mM digitionin in 2 mM Ca^{2+}, (*dotted line*) cells lysed with digitonin in Ca^{2+}-free buffer containing 10 mM EGTA. Intrinsic fluorescence of the microcarrier-cell suspension before loading with indo-1 is shown for comparison (*combination dashed and dotted line*). All spectra are corrected for the wavelength-dependent response of the instrument, using correction factors supplied by the manufacturer.

Thrombin interacts with endothelial cells through several sites, including the active site, a heparin-like binding site, and thrombomodulin (1, 31, 32). Thrombin with its active site blocked by DFP was used to determine whether active enzyme was required for generation of the cytosolic calcium signal. DIP-thrombin did not stimulate a rise in $[Ca^{2+}]_c$ and did not inhibit the response to subsequent thrombin stimulation, even when added in a 10-fold molar excess (Fig. 2 *D*). Thus it appears that active thrombin is required for generation of the calcium transient in endothelial cells, and that the enzyme with its active site blocked probably does not function as a competitive antagonist. The participation of the active site was confirmed using another thrombin inhibitor, PPACK. PPACK added to the cuvette at 1.7×10^{-6} M completely inhibited subsequent activation of the cells by thrombin (1 U/ml) (Fig. 2 *E*). The addition of PPACK after thrombin did not blunt the response or increase the rate of return of $[Ca^{2+}]_c$ toward baseline (data not shown). The histamine response (described below) was not affected by PPACK (Fig. 2 *E*), indicating that PPACK does not alter cell activation by other agonists.

The effects of other serine proteases on endothelial cell calcium were also evaluated, for comparison with thrombin. Plasmin was found to have no immediate effect on endothelial cell $[Ca^{2+}]_c$ when evaluated at the molar concentrations which gave

peak thrombin response (10^{-8} M), but caused a slow rise in $[Ca^{2+}]_c$ at 10–20-fold higher concentrations (Fig. 3 *A*). We have observed cell detachment from culture flasks at these higher concentrations, probably due to digestion of cell adhesion proteins by plasmin. Nevertheless, these plasmin-treated cells remained responsive to thrombin (Fig. 3 *A*). Urokinase alone had no effect on intracellular calcium (at concentrations up to 100 Committee on Thrombolytic Agents [CTA] U/ml, data not shown). In contrast to plasmin, trypsin induced an immediate rise in $[Ca^{2+}]_c$ (Fig. 3 *B*). Also, in contrast to the largely reversible changes in $[Ca^{2+}]_c$ observed in response to thrombin, cells exposed to trypsin exhibited a sustained elevation of $[Ca^{2+}]_c$, suggesting that cells trypsinized from their adherent surface undergo pronounced changes in calcium homeostasis. In addition, a 5-min exposure to trypsin resulted in loss of 4%–8% of the fluorescent dye from the cells, which was insufficient to account for the observed ratio change but which suggests some cell membrane injury. It is of interest to note that the response illustrated in Fig. 3 *B* was obtained using trypsin at a final concentration 1/100th of that routinely employed to detach cell monolayers for subculturing.

Endothelial responses to histamine

Changes in $[Ca^{2+}]_c$. As shown in Fig. 4, histamine elicited a dose-dependent increase in cytosolic calcium in microcarrier-bound endothelial cells. The maximal rise in $[Ca^{2+}]_c$ consistently occurred at histamine concentrations of 10^{-5} to 10^{-4} M and responses to 10^{-3} M were essentially equivalent or slightly lower. The threshold dose of histamine that yielded a detectable change in indo-1 fluorescence was 2×10^{-8} M (Fig. 5 *C*). The peak increase in cytosolic calcium occurred within 15–30 s of histamine stimulation and was followed by partial recovery over 1–2 min and then a rather stable sustained phase of elevated $[Ca^{2+}]_c$ (Fig. 4 *D*). The calcium concentrations achieved during the rapid initial peak and during the sustained phase of the response were each dependent on the histamine dose (Fig. 4). The histamine concentrations at which calcium transients were observed would appear to be physiologically relevant: serum histamine concentrations in the range of 5–110 ng/ml (5×10^{-8} to 10^{-6} M) have been measured following drug-induced histamine release (by anthracyclines or anesthetic agents) (33). One can only speculate as to histamine concentrations transiently present at the site of mast cell degranulation, but they are likely to be orders of magnitude higher than histamine concentrations measured in serum samples.

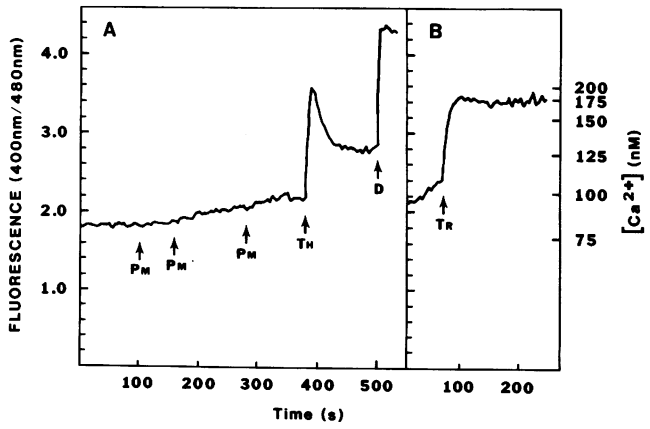

Figure 3. Effects of plasmin and trypsin on endothelial $[Ca^{2+}]_c$. (*A*) Plasmin was added to endothelial cells at times indicated (arrows *PM*) bringing the final concentration to 6, 36, and 66 μg/ml. Subsequently thrombin (2 U/ml, 1 μg/ml) was added (arrow *TH*), followed by digitonin 0.1 mM (arrow *D*). (*B*) Trypsin at a final concentration of 5 μg/ml was added at arrow *TR*.

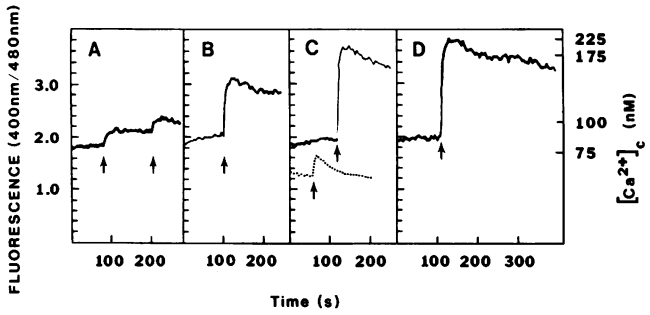

Figure 4. Dose-dependent effects of histamine on endothelial $[Ca^{2+}]_c$. Histamine was added at the arrows in the following concentrations: (*A*) 2×10^{-7} M; (*B*) 2×10^{-6} M; (*C*) 2×10^{-5} M; (*D*) 2×10^{-4} M. The response to histamine (2×10^{-5} M) in Ca^{2+}-free buffer containing 10 mM EGTA is shown in panel *C* (*dotted line*) for comparison with other responses observed in buffer containing 2 mM Ca^{2+}.

vWF release. Because endothelial vWF release has previously been induced by calcium ionophores, we wished to determine whether the histamine-stimulated rise in $[Ca^{2+}]_c$ is also accompanied by exocytosis of the vWF storage pool. As demonstrated in Fig. 5 *A*, histamine caused a dose-dependent release of vWF, with the maximal response (230% above control) observed at 10^{-5} M histamine, the same dose at which the peak elevations of cytosolic calcium were usually observed (Figs. 4 and 5 *C*). Higher doses of histamine (10^{-4} and 10^{-3} M) resulted in slightly less vWF secretion, although these responses varied from experiment to experiment and did not differ significantly ($P > 0.05$) from the response to 10^{-5} M. As noted above, the peak $[Ca^{2+}]_c$ was also usually recorded at 10^{-5} M histamine, and a decline at higher histamine concentrations was frequently, but not always, observed (compare Figs. 4 *D* and 5 *C*). At 10^{-8} M histamine (the threshold dose at which a change in indo-1 fluorescence was consistently detected), vWF release exceeded control levels by an average of 45%. Histamine-induced vWF release occurred both in medium containing fetal bovine serum and in serum-free medium. No cell detachment or morphologic changes were observed by phase-contrast microscopy even at the highest dose tested (not shown). vWF secretion in response to histamine was not affected by preloading the cells with indo-1 under the conditions used for measurement of $[Ca^{2+}]_c$: indo-1–loaded cells released 0.103±0.017 U/ng of cell protein; control cells 0.096±0.14 U/ng (mean±SD, $n = 6$). The release of vWF from histamine-treated endothelial cells was accompanied by a slight increase in the efflux of [^{3}H]DOG from the cells. Nevertheless, the efflux of this trapped aqueous marker was small in magnitude when compared with the concomitant release of vWF (cf. Fig. 5, *A* and *B*), suggesting that the observed histamine-induced release of vWF cannot be attributed simply to nonspecific membrane leakage.

The time course of histamine-induced vWF secretion is shown in Fig. 6 and is compared with secretion in response to the calcium ionophore A23187 (both agonists at 10^{-5} M). vWF release was detectable within 1 min of histamine stimulation (data now shown), and 25% of total release in these experiments occurred within the first 3 min (Fig. 6). Thus, secretion of vWF

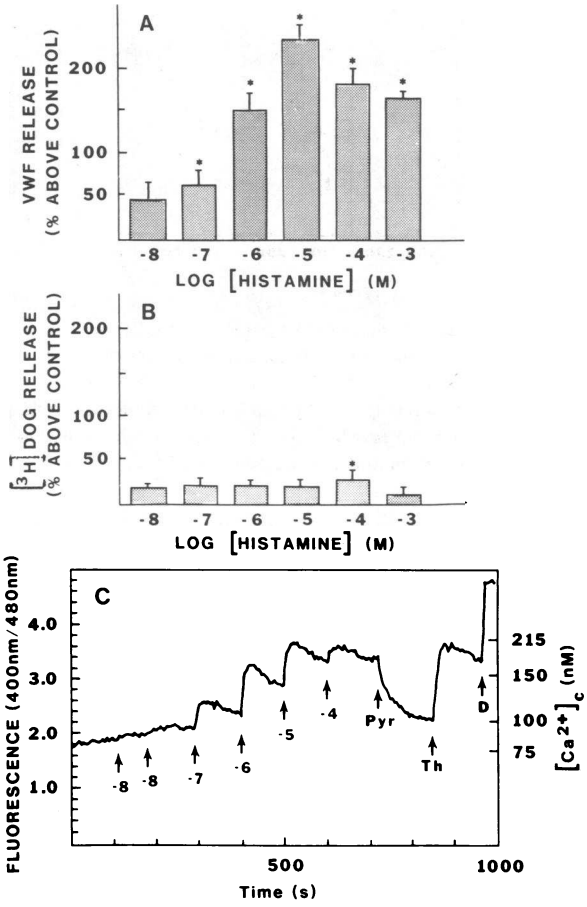

Figure 5. Dose-dependent histamine-induced vWF secretion. Release of (*A*) vWF and (*B*) [^{3}H]DOG from cultured endothelial cells is shown in response to increasing concentrations of histamine. Release was determined after a 45-min incubation. Results are means of six experiments. Error bars represent standard error of the mean. Significant differences from control cells are indicated by *asterisks* ($P \leq 0.05$). (*C*) Dose-dependent histamine-induced changes in $[Ca^{2+}]_c$ are shown for comparison. Arrows are labeled with the log [histamine] (10^{-8} to 10^{-4} M). Pyrilamine 10^{-6} M was added at arrow *Pyr*, thrombin 2 U/ml at arrow *Th*, and digitonin 0.1 mM at arrow *D*.

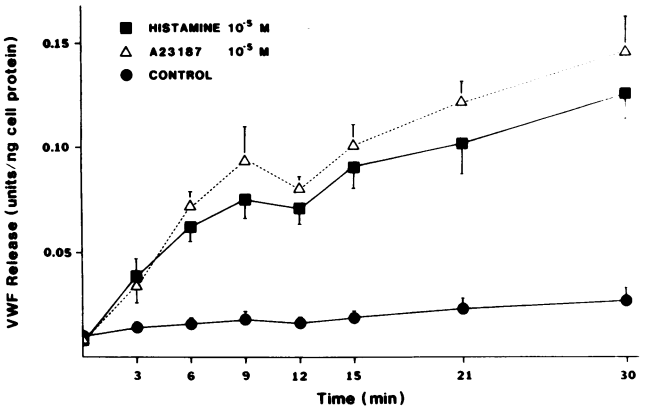

Figure 6. Time course of vWF secretion in response to histamine and calcium ionophore A23187. Cells were incubated with agonists (10^{-5} M) or control medium for the times indicated. Error bars represent standard error of the mean of data from four experiments.

follows within minutes of the rise in cytosolic Ca^{2+} induced by histamine (Fig. 4). Of interest, the magnitude and time course of secretion in response to histamine are similar to that induced by the calcium ionophore.

Contribution of sequestered intracellular Ca^{2+}. In order to determine the contribution of intracellular calcium stores to the observed rise in $[Ca^{2+}]_c$ in response to histamine, the microcarrier-bound cells were suspended in buffer containing 10 mM EGTA (to chelate external calcium) and then stimulated with histamine. As previously shown for thrombin (Fig. 2 *C* above), an elevation of $[Ca^{2+}]_c$ was observed upon exposure to histamine, even in the presence of 10 mM external EGTA (Fig. 4 *C*). Under these low-calcium conditions, the initial peak $[Ca^{2+}]_c$ is considerably depressed, and the sustained phase was absent, $[Ca^{2+}]_c$ returning within minutes to basal levels. These data suggest that the histamine-induced calcium transient is contributed at least in past by mobilization of calcium from intracellular stores.

Histamine-induced vWF release was also evaluated in medium containing 10 mM EGTA, to determine whether the secretion of this protein occurs when extracellular calcium is in the subnanomolar range. Histamine-stimulated secretion of vWF from cells in EGTA was reduced to approximately half that observed for cells suspended in 2 mM Ca^{2+} (Fig. 7). This diminished vWF release in the presence of EGTA, together with the lower peak and shorter duration of the increase in $[Ca^{2+}]_c$ measured by indo-1, suggests that the extent of secretory granule release may be a function of the peak and/or duration of the intracellular calcium signal. Alternatively, EGTA may also inhibit secretion by other mechanisms, for example by removing calcium from the cell membrane (thereby altering membrane protein function and interfering with exocytosis) or by gradually depleting intracellular calcium stores. Nevertheless, even in the presence of 10 mM EGTA, histamine increased vWF secretion 80% above control levels, suggesting that calcium released from intracellular stores may be sufficient to induce substantial vWF release or that another second messenger besides calcium may also be involved (34).

Effects of H_1- and H_2-receptor antagonists. Pyrilamine (H_1-receptor antagonist) and cimetidine (H_2-receptor antagonist) were used to determine the receptor type mediating the rise in

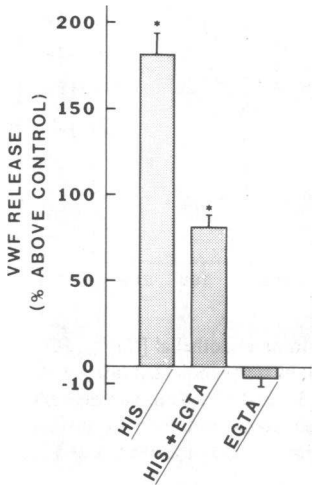

Figure 7. Effects of EGTA on histamine-induced vWF release. Cells were incubated without and with histamine (*HIS*) 10^{-5} M in Medium 199 plus 20% fetal bovine serum or the same medium containing 10 mM EGTA. Incubation was for 15 min. Results shown are means of six experiments; error bars indicate standard error of the mean, and treatments that differed from control ($P \leq 0.05$) are indicated by *asterisks*. The histamine-induced release in EGTA also differed significantly ($P \leq 0.05$) from histamine-induced release in the presence of Ca^{2+}. vWF release by controls in EGTA did not differ significantly from controls in the presence of Ca^{2+}.

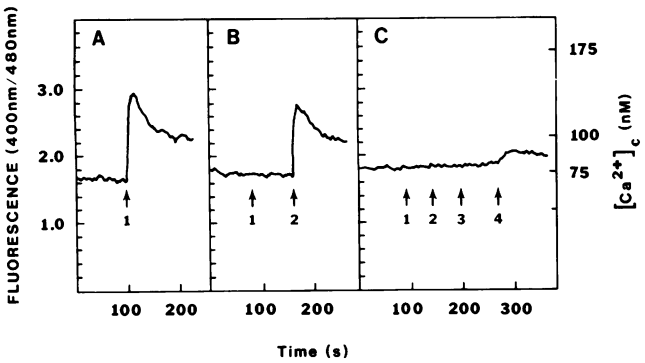

Figure 8. Effects of H_1- and H_2-receptor antagonists on histamine-induced endothelial Ca^{2+} transients. (*A*) Response to histamine 10^{-5} M (arrow *1*). (*B*) Cimetidine (2×10^{-4} M) was added (arrow *1*) followed by histamine 10^{-5} M (arrow *2*). (*C*) Pyrilamine 10^{-6} M was added (arrow *1*), followed by histamine 10^{-6} M (arrow *2*), 10^{-5} M (arrow *3*), and 10^{-4} M (arrow *4*).

cytosolic calcium and vWF release from histamine-stimulated endothelial cells. Pretreatment of cells with a 20-fold molar excess of cimetidine had a minimal effect on the magnitude or duration of the response to histamine, whereas pyrilamine completely prevented the histamine-induced rise in $[Ca^{2+}]_c$, even when used at $^1/_{10}$th of the histamine concentration (Fig. 8). These observations implicate the H_1 receptor in calcium messenger generation in these cells (consistent with the original definition of H_1 receptor) and suggest that pyrilamine has a much higher affinity for the endothelial H_1-receptor than histamine itself. The pyrilamine block is nevertheless reversible, and could be overcome by increasing the histamine concentration 100-fold (Fig. 8 *C*).

Histamine caused a dose-dependent elevation in cytosolic calcium which never returned to baseline during any 15-min experiment (cf. Fig. 4 *D*). This sustained elevation of $[Ca^{2+}]_c$ in response to histamine appears to require continued occupancy of the receptor by this agonist: addition of the H_1-receptor antagonist pyrilamine at any time after histamine activation of the cell promptly aborted the response and returned $[Ca^{2+}]_c$ essentially to basal levels (Figs. 5 *C* and 9). Furthermore, as shown by the data of Fig. 9 *C*, $[Ca^{2+}]_c$ could be repeatedly titrated between basal and stimulated levels by successive additions of histamine and pyrilamine, suggesting that the magnitude of the sustained phase of the calcium signal is determined by the proportion of receptors occupied by agonist vs. antagonist. The reversibility of the histamine-triggered rise in $[Ca^{2+}]_c$ was also demonstrated by removing histamine from the medium (Fig. 9 *A*), which returned $[Ca^{2+}]_c$ to baseline. This is in contrast to the thrombin-induced increase in cytosolic Ca^{2+}, which was not reversed by removal of thrombin from the medium (data not shown).

The effect of these specific H_1- and H_2-receptor antagonists on histamine-induced vWF secretion is shown in Fig. 10. Consistent with the histamine-induced changes in $[Ca^{2+}]_c$ observed in the presence of these drugs, histamine-stimulated vWF secretion was 85% inhibited by the H_1-receptor antagonist pyrilamine and was only 5% inhibited by cimetidine, when histamine and antagonist were used in equimolar concentrations. Neither antagonist significantly altered release of vWF from control cells not exposed to histamine.

Discussion

Advantages of microcarrier-bound cell monolayers in fluorescent calcium indicator studies. Alteration of cytosolic calcium concentration is one mechanism by which extracellular agonists can activate or inactivate selected cellular processes (for reviews, see references 35 and 36). Indirect evidence has suggested such a calcium second messenger might be important in a number of endothelial cell responses, including induction of PGI_2 and PAF synthesis, vWF secretion, and mitogenesis. For example, it has been previously shown that calcium ionophores will trigger—and EGTA abolish or diminish—some of these responses (34, 37) and that many agonists which cause endothelial cell proliferation (including thrombin and histamine) increase $^{45}Ca^{2+}$ flux across the plasma membrane of these cells (4). The fluorescent calcium indicators such as indo-1 (24) offer the opportunity to monitor $[Ca^{2+}]_c$ in cells which are relatively undisturbed except for the selected stimulus. Nevertheless, application of these probes to the study of anchorage-dependent cells such as endothelial cells has been limited, because of the need to obtain homogeneous cell suspensions for fluorescence measurements with readily available instrumentation.

Anchorage-dependent cells can be trypsinized from their attachment site to form suspensions for fluorescence studies (19, 20, 38). However, the changes in $[Ca^{2+}]_c$ we observed in cells exposed to this protease (Fig. 3) suggest that significant disruption of calcium homeostasis occurs during proteolytic detachment and thus limits extrapolation of observations made on such cells to the physiologic responses of the unperturbed endothelium. By contrast, use of microcarriers permits study of these cells

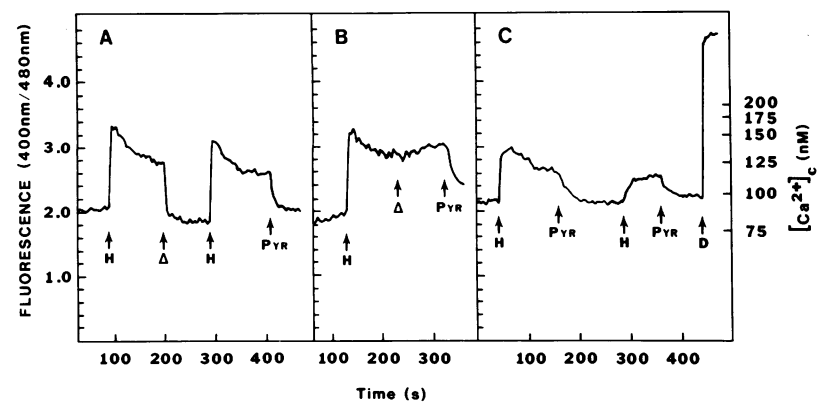

Figure 9. Reversibility of the histamine-induced changes in $[Ca^{2+}]_c$. (*A*) Histamine 10^{-5} M was added (arrow *H*). After 100 s (arrow Δ), fluorescence monitoring was interrupted, the microcarriers allowed to settle, the buffer exchanged for histamine-free buffer, and monitoring resumed (2.5 min later). Cells were then restimulated with the same dose of histamine (10^{-5} M, arrow *H*) followed by inhibition of the response by pyrilamine 10^{-6} M (arrow *PYR*). (*B*) Histamine 10^{-5} M was added (arrow *H*). At arrow Δ, the buffer was exchanged for new buffer, again containing 10^{-5} M histamine. Pyrilamine 10^{-6} M was added at arrow *PYR*. (*C*) Histamine and H_1-receptor antagonist were added sequentially at the arrows in the following concentrations: (*a*) histamine 10^{-6} M, (*b*) pyrilamine 10^{-6} M, (*c*) histamine 10^{-4} M, (*d*) pyrilamine 10^{-6} M. Cells were then lysed with digitonin (arrow *D*).

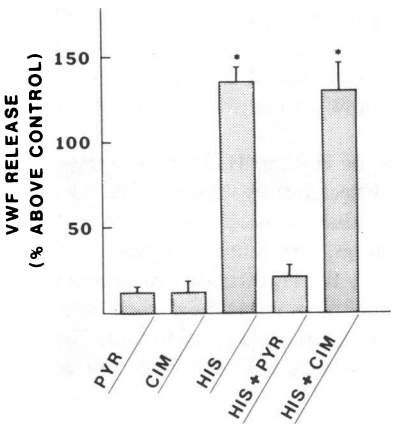

Figure 10. Effects of H_1- and H_2-receptor antagonists on histamine-induced vWF secretion. Cells were incubated with histamine (*HIS*), pyrilamine (*PYR*), and cimetidine (*CIM*) alone and in combinations for 45 min. The final concentrations of agonist and antagonist were 10^{-6} M. Results shown are means of four experiments, with error bars indicating standard error of the mean. Responses significantly different from control ($P \leq 0.05$) were designated by *asterisks*. Of note, the histamine plus cimetidine release did not differ significantly from that of histamine alone.

maintained in an adherent monolayer, undisturbed except for brief washing and gentle stirring during the experiment.

Measurement of cytosolic $[Ca^{2+}]_c$ in adherent cell monolayers has previously been reported for quin2-loaded fibroblasts grown on microcarriers (39) and bovine endothelial and smooth muscle cells grown on glass microscope slides (23, 40, 41). With the latter method, only a small, static field containing a single layer of cells is actually in the path of the exciting beam, resulting in a low signal-to-noise ratio, and increasing the artifacts of indicator photobleaching and cell phototoxicity. Furthermore, in our experience, human endothelial cells adhere poorly to glass surfaces and detach with minimal shear stress (e.g., rinsing, stirring). Although digital imaging systems for fluorescence microscopy offer an alternative approach to quantitative monitoring of fluorescence from cell monolayers (42), this instrumentation is not widely available, and permits sampling of only a small field of adherent cells.

The advantages of indo-1 and related tetracarboxylate stilbene fluorophores for the measurement of cell calcium have been discussed in detail by Grynkiewicz et al. (24). Our selection of indo-1 for use in endothelial cells was based on the following considerations: in preliminary experiments comparing indo-1 with quin2, the higher signal-to-noise ratio obtainable with indo-1 permitted detection of Ca^{2+} transients at the low histamine concentrations employed in these experiments (e.g., threshold for detection of a quin2 response was 10^{-6} M histamine, whereas responses were reliably observed with indo-1 at 10^{-8} M histamine, Fig. 5 *C*). Furthermore, indo-1 permitted continuous monitoring of emission at high and low Ca^{2+} wavelengths (in T format), enabling rapid changes in cell $[Ca^{2+}]_c$ to be resolved. The use of quin2 for these experiments was also precluded by its effects on cell secretion. At the concentrations of quin2 commonly required for loading cells (15–50 μM) (17, 23, 38), this indicator has been shown to inhibit platelet aggregation (17), perhaps by buffering $[Ca^{2+}]_c$ transients. In our experiments, quin2 at 5 μM inhibited histamine-stimulated vWF secretion by 24% (data not shown). By contrast, vWF release was unaffected by loading with indo-1 at the same concentration (see Results). This inhibition of endothelial cell secretion may be due to the higher affinity of quin2 for Ca^{2+} (resulting in buffering of Ca^{2+} transients) (17, 24), or possibly to greater cell uptake of quin2 relative to indo-1.

The fluorescence recorded from indo-1–loaded endothelial cells is composed of the signal from indo-1 distributed in various cell compartments and a small component of leaked dye, as well as a background of intrinsic cell fluorescence and scattered light. The relative contribution of the latter two components to the total signal depends on the concentration of intracellular indo-1 as well as the density of cells on the microcarriers, which cannot be rigorously controlled. It should be emphasized that calibration of the dye signal to $[Ca^{2+}]_c$ could only be empirically derived and is likely to represent at best a rough approximation of the absolute cytosolic concentration of this ion. Despite this inherent variability in cell density and dye uptake from experiment to experiment, the day-to-day reproducibility of calibrations of the dye signal to $[Ca^{2+}]_c$ suggests that the ratio of emission at 400/480 nm is essentially independent of these factors in the range of $[Ca^{2+}]_c$ experimentally observed (corresponding to between 70 and 300 nM). Furthermore, it is of interest to note that our estimate of resting $[Ca^{2+}]_c$ in human umbilical vein endothelial cells (70–80 nM) closely agrees with the 70 nM estimated by Luckhoff and Busse (23) using quin2-loaded calf endothelial cells attached to microscope slides. In contrast, resting $[Ca^{2+}]_c$ in bovine aortic endothelial cells after trypsinization has recently been reported to be 168±15 nM (38). Whether this difference in measured $[Ca^{2+}]_c$ is an effect of trypsin (Fig. 3 *B*) remains to be resolved.

Changes in $[Ca^{2+}]_c$ in response to thrombin. Endothelial cells respond to thrombin with synthesis of PGI_2 and PAF; release of adenine nucleotides, "endothelium-dependent relaxant factor," and vWF; increased protein synthesis (e.g., tissue plasminogen activator and the plasminogen activator inhibitor); cell retraction; and mitogenesis. Experiments using calcium ionophore and $^{45}Ca^{2+}$ have suggested that calcium plays a role in transduction of the thrombin stimulus in many of these responses. Although the high-affinity binding of thrombin to endothelial cells is not active-site dependent (1), most of the above physiologic responses to thrombin are not mediated through the high-affinity binding sites and do require active enzyme (1, 2, 6). In this paper, we demonstrate that thrombin triggers a dose-dependent increase in $[Ca^{2+}]_c$ (Fig. 2) and that the increase occurs at subnanomolar external calcium concentrations, suggesting that a portion of the thrombin-stimulated calcium transient is generated from intracellular stores. Moreover, the thrombin-induced rise in $[Ca^{2+}]_c$ is active-site dependent, consistent with the inability of DIP-thrombin to stimulate vWF release (6) or prostacyclin generation (1, 2). Brock et al. (43), using fura-2–loaded endothelial cell suspensions, recently reported a 300% increase in cytosolic calcium 15 s after stimulation with 1 U/ml of thrombin. Because their data is only available in preliminary form, we are unable to evaluate their method of suspending cells or compare the magnitude of observed responses with those we obtain from adherent cells.

Histamine-induced cytolsolic Ca^{2+} changes and vWF release. The most striking effect of histamine in vivo is an increase in vascular permeability, particularly in postcapillary venules. Disruption of interendothelial cell junctions by histamine has been repeatedly described (10, 11) and is felt to be the cellular basis for the permeability change. We now demonstrate that histamine induces characteristic changes in the $[Ca^{2+}]_c$ of this cell (Fig. 4): a rapid peak in $[Ca^{2+}]_c$ (15–30 s), partial recovery (1–2 min), and sustained moderate elevation in $[Ca^{2+}]_c$ (which persists for at least 15 min, not shown). The magnitude of each phase is dependent on histamine concentration, with maximal response

at 10^{-5} to 10^{-4} M. Calcium appearing in the cytosol during the early phase is derived, at least in part, from intracellular stores, as a substantial rise in $[Ca^{2+}]_c$ is detected in the presence of extracellular EGTA (Fig. 4 *C*). By contrast, the sustained phase of the response is completely eliminated by external EGTA, suggesting that extracellular Ca^{2+} may be required to maintain elevated $[Ca^{2+}]_c$. Alternatively, one can speculate that EGTA affects the response either by depleting intracellular stores or altering membrane function. The histamine-induced elevation in $[Ca^{2+}]_c$ is abruptly terminated when an H_1-receptor antagonist is added or when free histamine is removed by buffer exchange (Figs. 5 *C* and 9). These results suggest (*a*) that histamine binding to its endothelial H_1-receptor is freely reversible, and (*b*) that the sustained phase of the histamine response requires continued receptor occupancy. One might speculate that the rapid reversibility of histamine-stimulated elevation of $[Ca^{2+}]_c$ enables the endothelium to respond rapidly to changes in local histamine concentration, especially in that histamine-induced permeability changes have also been shown to reverse shortly after removal of histamine (10).

The histamine-induced rise in cytosolic Ca^{2+} in human endothelial cells was accompanied by secretion of vWF. Although our data suggest that calcium may be an important triggering event in secretion, studies of other cell types indicate that histamine binding to its H_1 receptor stimulates turnover of membrane phosphatidylinositol (44). Thus, other intracellular events (in particular activation of protein kinase C) are likely to play a role in histamine-induced secretion. Only three agents have previously been shown to induce exocytosis of stored vWF from cultured endothelial cells (thrombin, calcium ionophore, and the phorbol ester, 4-phorbol 12-myristate 13-acetate) (6, 34, 37) and only one of these (thrombin) is of potential physiologic significance in vivo. We have now demonstrated that histamine, an important modulator of endothelial function in vivo, induces vWF secretion from human endothelial cells in culture. Furthermore, histamine simultaneously triggers both endothelial cell retraction with exposure of subendothelium (predominantly in postcapillary venules), and release of the plasma protein (vWF) required for platelet adhesion to subendothelium at the high shear rates existing in the microcirculation (45, 46). The potential significance of this stimulated release of vWF to platelet deposition occurring at sites of inflammation remains to be explored.

Acknowledgments

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