Membrane capacitance changes induced by thrombin and calcium in single endothelial cells cultured from human umbilical vein

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1. Vesicular secretion from single human umbilical vein endothelial cells (HUVECs) was monitored by changes in membrane capacitance (C_m). Secretion was evoked by dialysis with strongly buffered intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), flash photolysis of Ca^{2+}-loaded DM-nitrophen or caged InsP_3, or by thrombin. $[Ca^{2+}]_i$ was monitored spectrofluorimetrically with furaptra. The results show that a large, slowly rising component of vesicular secretion requires prolonged exposure to high $[Ca^{2+}]_i$.

2. C_m increased during intracellular perfusion with $[Ca^{2+}]$ buffered in the range 1·0–20 μM. Changes in C_m comprised an initial slowly rising small component of 0·1–0·5 pF followed by a faster rising larger component of up to ~7 pF, seen when $[Ca^{2+}]_i > 2$ μM and which was maximal at 10–20 μM Ca^{2+}.

3. Thrombin evoked rapid initial elevations of $[Ca^{2+}]_i$ to a peak of 7·1 ± 1·5 μM (mean ± S.E.M., $n = 5$) that declined within ~20–30 s with thrombin present either to resting levels or to a maintained elevated level of 2·0 ± 0·7 μM (mean ± S.E.M., range 1·0–3·6 μM, $n = 3$). Transient $[Ca^{2+}]_i$ rises were associated with small, slowly rising increases in C_m of 0·1–0·2 pF, that recovered to pre-application levels over 2–3 min. Maintained elevations of $[Ca^{2+}]_i$ caused larger, faster-rising sustained increases in C_m to 1·14 ± 0·12 pF (mean ± S.E.M., $n = 3$). Separate specific enzyme-linked immunosorbent assay (ELISA) showed that 1·0 U ml^{-1} thrombin produced secretion of von Willebrand factor in HUVEC cultures.

4. Short-lived $[Ca^{2+}]_i$ elevations with a peak of 3–25 μM and a duration of approximately 20 s generated by flash photolysis of caged InsP_3 or DM-nitrophen produced either no net change in C_m, or small slow increases of ~0·1–0·6 pF at up to 5 fF s^{-1} that recovered to pre-flash levels over 2–3 min.

5. Maintained elevations of $[Ca^{2+}]_i$ in the range 1–28 μM produced by flash photolysis of DM-nitrophen caused large increases in C_m, up to ~4 pF, corresponding to ~25–30 % of the initial cell C_m. The maximum rate of change of C_m was up to 50 fF s^{-1} at steady $[Ca^{2+}]$ up to 20 μM; C_m recovered towards pre-flash levels only when $[Ca^{2+}]$ had declined.

Vascular endothelial cells regulate blood flow and haemostasis by releasing several locally acting mediators in response to mechanical, biochemical or neural signals. Some mediators are made and released 'on demand' by the endothelium, including prostacyclin, nitric oxide and platelet-activating factor. Others are stored in preformed storage granules and secreted by exocytosis. These are von Willebrand factor (vWf), tissue plasminogen activator (t-PA), tissue factor pathway inhibitor (TFPI), endothelin-1 (ET) and Protein S. These mediators function to maintain blood as a fluid and control blood flow under normal conditions, but also prevent leaks by promoting the formation of platelet plugs at sites of vascular injury (reviewed by Mann, 1997).

With the exception of ET, a rise in endothelial intracellular free calcium ion concentration ($[Ca^{2+}]_i$) produced by Ca^{2+}-mobilizing hormones is thought to play a central role in the regulated release of each of these mediators (Stern *et al.* 1986; Hamilton & Simms, 1987; Carter & Pearson, 1992; Birch *et al.* 1992; Kooistra *et al.* 1994; Frearson *et al.* 1995; Lupu *et al.* 1995; van den Eijnden-Schrauwen *et al.* 1997; Emeis *et al.* 1997). Limitations in the sensitivity of

detection assays prevent direct simultaneous measurements of secretion and $[Ca^{2+}]_i$ at the single cell level. However, morphological and biochemical evidence suggests that regulated secretion of vWf, t-PA, TFPI and Protein S from endothelial cells involves the fusion with and subsequent recycling of secretory vesicles with the surface membrane (Stern *et al.* 1986; Wagner, 1990; Emeis *et al.* 1997). It is likely that this would result in changes of cell C_m which could be detected electrophysiologically and provide a means by which regulated secretion from storage granules could be followed in single endothelial cells. vWf, t-PA and TFPI are stored in their own separate storage granules within endothelial cells (Emeis *et al.* 1997). The best characterized of these storage granules is the Weibel-Palade (WP) body that contains vWf (Wagner, 1990). WP bodies are tubular structures (1–4 μm in length, 0·1–0·2 μm in diameter) that show characteristic longitudinal striations, thought to be condensed polymers of vWf. The WP body membrane also contains the leucocyte adhesion molecule P-selectin, which appears on the cell surface as a result of exocytosis during regulated secretion of vWf (Wagner, 1990). The t-PA storage granules are much smaller, ~0·1 μm in diameter, and are similar in size to those described for ET and Protein S (Harrison *et al.* 1995; Stern *et al.* 1986).

In the experiments reported here changes in human endothelial cell C_m were measured during whole-cell recording as a single cell index of secretion (Neher & Marty, 1982). The $[Ca^{2+}]$ dependence, extent and time course of C_m changes were investigated in response to (1) intracellular dialysis with buffered Ca^{2+} solutions in the range known to trigger vWf secretion (Scrutton & Pearson, 1989; Birch *et al.* 1992), (2) responses to the vasoactive hormone thrombin, (3) controlled elevations of $[Ca^{2+}]_i$ produced by intracellular flash photolysis of caged InsP_3 or caged Ca^{2+} (DM-nitrophen) to mimic the initial agonist-evoked Ca^{2+} spike, and (4) photolysis of caged Ca^{2+} to produce a maintained Ca^{2+} rise. These results show that during the initial InsP_3-evoked release of Ca^{2+} from internal stores the C_m change is small, suggesting that initially there is little vesicular secretion. In contrast, a large C_m change, indicating extensive vesicular secretion, is seen when $[Ca^{2+}]_i$ is elevated for longer, a situation that may arise during strong activation, e.g. by thrombin or by cell damage.

A preliminary account of this work has been published (Smith *et al.* 1997; Carter *et al.* 1997).

METHODS

Tissue culture

Human umbilical vein endothelial cells (HUVECs) were isolated and grown as previously described (Hallam *et al.* 1988). Primary isolates of HUVECs in medium M199 supplemented with 10% fetal calf serum and 10% newborn calf serum were seeded onto 35 mm diameter glass or quartz coverslips and incubated at 37 °C in an atmosphere of 95% air–5%CO_2. Cells were used 24–72 h after isolation. Experiments were carried out in a Hepes-buffered physiological saline solution containing (mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 10 glucose, 10 Hepes (pH 7·4) at room temperature (25–28 °C).

Assay of vWf secretion

HUVEC cultures were grown to confluence in 24-well tissue culture trays. Cells were washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM; pH 7·4) containing 20 mM Hepes prior to addition of DMEM (control) or DMEM containing thrombin 1·0 U ml^{-1}. The supernatant fraction was removed 10 min later and the release of vWf measured by enzyme-linked immunosorbent assay (ELISA) as previously described (Wheeler-Jones *et al.* 1996*b*). The mouse monoclonal anti-vWf coating antibody (CLBRAg35) was a gift from Dr A. J. Van Mourik (Central Blood Laboratory, Amsterdam, The Netherlands).

Fluorescence measurements and flash photolysis

Changes in cytosolic free $[Ca^{2+}]$ were measured with furaptra as previously described (Konishi *et al.* 1991; Ogden *et al.* 1995). Furaptra free acid (500 μM) was introduced into the cell together with caged compounds (DM-nitrophen or caged InsP_3) by diffusion from a patch pipette during whole-cell recording. Diffusional equilibration between pipette solution and cell was determined from the fluorescence record. Microspectrofluorimetry was performed with a Nikon TMD microscope with a ×40, 1·3 NA objective lens. Excitation (400–440 nm) was from a 100 W quartz halogen lamp, and light emitted from a single cell viewed via long-pass filters at > 470 nm. Emitted light was detected by a photomultiplier operated in photon counting mode. Photon counts were converted to an analog signal by an integrating amplifier with correction for missed counts (Cairn Research, Faversham, Kent, UK), stored on FM tape and digitized at 100 Hz (CED1401, Cambridge Electronic Design, Cambridge, UK) and then stored on an AT bus computer. The method for converting the fluorescence change to free $[Ca^{2+}]$ has been described in detail previously (Ogden *et al.* 1995; see Konishi *et al.* 1991). Briefly, furaptra fluorescence with 400–440 nm excitation is quenched at high $[Ca^{2+}]$, and therefore the intrinsic fluorescence recorded before whole-cell recording is the fluorescence at saturating free $[Ca^{2+}]$, $F_{Ca,max}$. The free $[Ca^{2+}]_i$ in HUVECs has been estimated as ~0·1 μM (Hallam *et al.* 1988). The Ca^{2+} dissociation constant, K, for furaptra was assumed to be the *in vitro* value of 48 μM (Ogden *et al.* 1995; see also Konishi *et al.* 1991); therefore, the fluorescence of furaptra under resting conditions can be taken as an estimate of $F_{Ca,min}$. The free $[Ca^{2+}]$, Ca_f, was calculated from the fluorescence, F, by means of the relation:

$$Ca_f = K(F_{Ca,min} - F)/(F - F_{Ca,max}). \quad (1)$$

Photolysis of DM-nitrophen or caged InsP_3, the P-5 1-(2-nitrophenyl)ethyl ester of InsP_3 (kindly provided by Dr D. R. Trentham, NIMR, Mill Hill, London), was by a 1 ms pulse of near-UV light (280–360 nm with a Schott UG11 filter) from a short arc xenon flashlamp (Cairn Research) focused to produce an image of the arc 3–4 mm across at the cell as described previously (Ogden *et al.* 1990). The output of the lamp was fully adjustable up to 270 J, a range producing 0–30% photolysis of caged InsP_3. Photolysis of caged InsP_3 in the experimental microsope was calibrated by HPLC analysis of 5 μl droplets of 1 mM caged ATP irradiated at the experimental lamp intensities. Caged ATP and caged InsP_3 have similar quantum yields and extinctions in the near-UV and therefore the same conversion (Walker *et al.* 1989; Ogden *et al.* 1990). Photolysis of the caged compounds by fluorescence excitation was minimized by shuttering the excitation when not recording.

Electrophysiological recording

The whole-cell patch-clamp technique (Hamill *et al.* 1981) was used for electrical recording and to introduce Ca^{2+} buffers, or furaptra and caged compounds into the cytosol of single HUVECs. Normal internal solution contained (mM): 153 potassium gluconate, 8 Hepes, 3 $MgSO_4$, 3 Na_2ATP (pH 7·2). The cells were voltage clamped at a holding potential of −50 mV and current was recorded with a patch-clamp amplifier (Axon Instruments Axopatch 200A). Recordings were made with pipettes of resistance 1·5–6 MΩ, and access conductances in whole-cell recording were between 90 and 278 nS.

C_m measurement

The two-phase lock-in amplifier was constructed to the circuit described by Lindau & Neher (1988), and the whole-cell C_m calculated from the output as described by Lindau & Neher (1988). The zero phase angle of the lock-in amplifier was initially adjusted with patch-clamp capacitance compensation switched off and an open circuit headstage until an addition of fast capacitance compensation resulted in changes only in the imaginary output. The setting was then verified as described by Lindau & Neher (1988). Pipette capacitance was cancelled in cell-attached recording prior to membrane rupture. A sinusoidal voltage command of frequency 800 Hz and amplitude 2 mV peak to peak was applied. Membrane current and signals from the lock-in amplifier, corresponding to the real and imaginary components of whole-cell admittance, were low-pass filtered (30 Hz, 3 dB) and digitized at 100 Hz. Real time calculation of the passive cell parameters from equations given by Lindau & Neher (1988) used the programme CAP3 written by Dr John Dempster (University of Strathclyde, UK). The mean membrane potential, −40 mV (see Results), was taken as the reversal potential for membrane current when calculating the conductance. Traces of the cell capacitance (C_m), access conductance (G_a) and the combined leak and membrane conductance (G_m) were calculated on-line.

DiBrBAPTA-buffered Ca^{2+} solutions and DM-nitrophen solutions

5′,5′-Dibromo-BAPTA (DiBrBAPTA) stock was prepared in internal solution (10·78 mM; determined from absorbance at 263 nm (molar extinction coefficient ϵ_{263} for free anion $= 2 \times 10^4$ M^{-1} cm^{-1}; data from Molecular Probes). Solutions containing 5 mM DiBrBAPTA and Ca^{2+} were prepared to yield free $[Ca^{2+}]$ in the range 0·1–20 μM. The required free $[Ca^{2+}]$ was calculated from:

$$Ca_t = (B_t Ca_f K_a)/(1 + (K_a Ca_f), \quad (2)$$

where Ca_t is total $[Ca^{2+}]$, B_t is total [DiBrBAPTA], K_a is 625 000 μM^{-1} (Tsien, 1980) and Ca_f is free $[Ca^{2+}]$.

A stock of 147 mM DM-nitrophen (provided by Drs D. R. Trentham & G. Reid, NIMR, Mill Hill, London) was used to make Ca^{2+}-loaded DM-nitrophen solutions. Internal solutions were prepared containing 4 mM DM-nitrophen, 3 mM Mg^{2+}, 3 mM ATP, 500 μM furaptra and either 4 mM or 0 mM Ca^{2+}. The two solutions were mixed to vary the DM-nitrophen : Ca_t ratio. The equilibrium free $[Ca^{2+}]$ and $[Mg^{2+}]$ for a given DM-nitrophen : Ca_t ratio were calculated using the Ca^{2+} and Mg^{2+} dissociation constants for DM-nitrophen (Ellis-Davies *et al.* 1996), ATP (Sillén & Martell, 1971) and furaptra (Konishi *et al.* 1991; Ogden *et al.* 1995). For 4 mM DM-nitrophen : 2·4 mM Ca^{2+} these were $Ca_f = 0·12$ μM and $Mg_f = 12·2$ μM, respectively (equilibrium Mg^{2+}–ATP concentration 1·5 mM). Generally, the first pulse of UV light (at full output, 270 J) produced a transient elevation of free $[Ca^{2+}]$ that rose to a peak within the resolution of the fluorescence detection system (< 1 ms) and decayed with a half-time of ~10 s back to resting levels. A second pulse generally produced an initial transient followed by a decline to a maintained elevated free $[Ca^{2+}]_i$, the magnitude of which could be varied by varying either the initial DM-nitrophen : Ca^{2+} ratio or (more usually) the energy of the second light pulse.

Data are expressed as means ± S.E.M.

RESULTS

Electrical properties of HUVECs; membrane potential, membrane conductance and C_m

Cells selected for recording were not connected by gap junctions to neighbouring cells, based both on a large physical separation and no transfer to neighbours of fluorescent indicator from the perfused cell, thus avoiding the electrical complications arising from cell–cell coupling (Carter & Ogden, 1994; Carter *et al.* 1996). The resting electrical parameters determined with whole-cell recording were as follows. The mean resting membrane potential was −40 ± 3 mV (range −19 to −65 mV, $n = 20$). The input conductance including leak in the pipette–membrane seal at the holding potential of −50 mV was 1·16 ± 0·14 nS ($n = 50$, range 0·04–4·62 nS). To optimize voltage clamp, small cells were visually identified and used for the C_m measurements. The initial C_m recorded had a mean of 23·8 ± 1·6 pF ($n = 50$, range 7·9–48·1 pF). In the same cells the access conductance was 144 ± 7 nS ($n = 50$, range 90–278 nS).

Ca^{2+}-dependent exocytosis in HUVECs

Figure 1*A* shows the membrane currents, access conductances and C_m after establishing whole-cell recording in two HUVECs. With the pipette solution buffered to free $[Ca^{2+}]$ of 0·1 μM (left-hand panel), there was little change in C_m from the initial level of 16·0 pF. C_m increased slightly over 100 s from 16·0 to 16·2 pF and then declined to 15·1 pF over 500 s. In contrast, the cell shown in the right-hand panel, with 10 μM free $[Ca^{2+}]$ in the pipette, showed a continuous, mainly monotonic, increase of C_m over 500 s from the initial level of 16·5 to 23·1 pF. The time courses of C_m changes with buffered free $[Ca^{2+}]_i$ in the range 0·1–20 μM applied by pipette perfusion are shown for six cells in Fig. 1*B*. The C_m change is normalized to the initial value after establishing whole-cell recording. At free $[Ca^{2+}]_i$ levels of 0·1 and 1 μM there was little or no change in C_m; at 2 μM $[Ca^{2+}]_i$ there was a monotonic increase in C_m, and at 5, 10 and 20 μM $[Ca^{2+}]_i$ a more complex time course with initial slow change followed by a faster change for 20–50 s and finally a slower rise to reach a steady level after approximately 200 s. The increase in amplitude of the C_m (in pF) measured at 400–600 s close to or at a steady state, is plotted against free $[Ca^{2+}]_i$ for 29 cells in Fig. 1*C* ($n = 3$–9 cells at each $[Ca^{2+}]_i$). No increase in C_m is seen at free $[Ca^{2+}]_i$ of less than 1–2 μM, and at high free $[Ca^{2+}]_i$ C_m increases to a maximum averaging 4 pF above the initial level. The

maximal increase in C_m at high $[Ca^{2+}]_i$ represents an increase of ~25–35% over the initial membrane area.

Changes in $[Ca^{2+}]_i$ and C_m evoked by thrombin

Thrombin is a strong stimulant of vWf secretion from endothelium during coagulation. At 1·0 U ml^{-1}, thrombin has been shown to produce strong or maximal secretion of stored mediators (e.g. Hamilton & Simms, 1987) and was used here to evoke capacitance changes associated with vesicular secretion. In cultures of HUVECs thrombin (1·0 U ml^{-1}) stimulated release of vWf as shown by specific ELISA. Basal vWf release was 0·41 ± 0·13 mU ml^{-1} and in thrombin-stimulated cultures it was 0·93 ± 0·07 mU ml^{-1} ($n = 4$ wells). In single HUVECs, this concentration of thrombin has been reported to produce an initial rise in $[Ca^{2+}]_i$ followed by a maintained elevated phase (e.g. Birch *et al.* 1994).

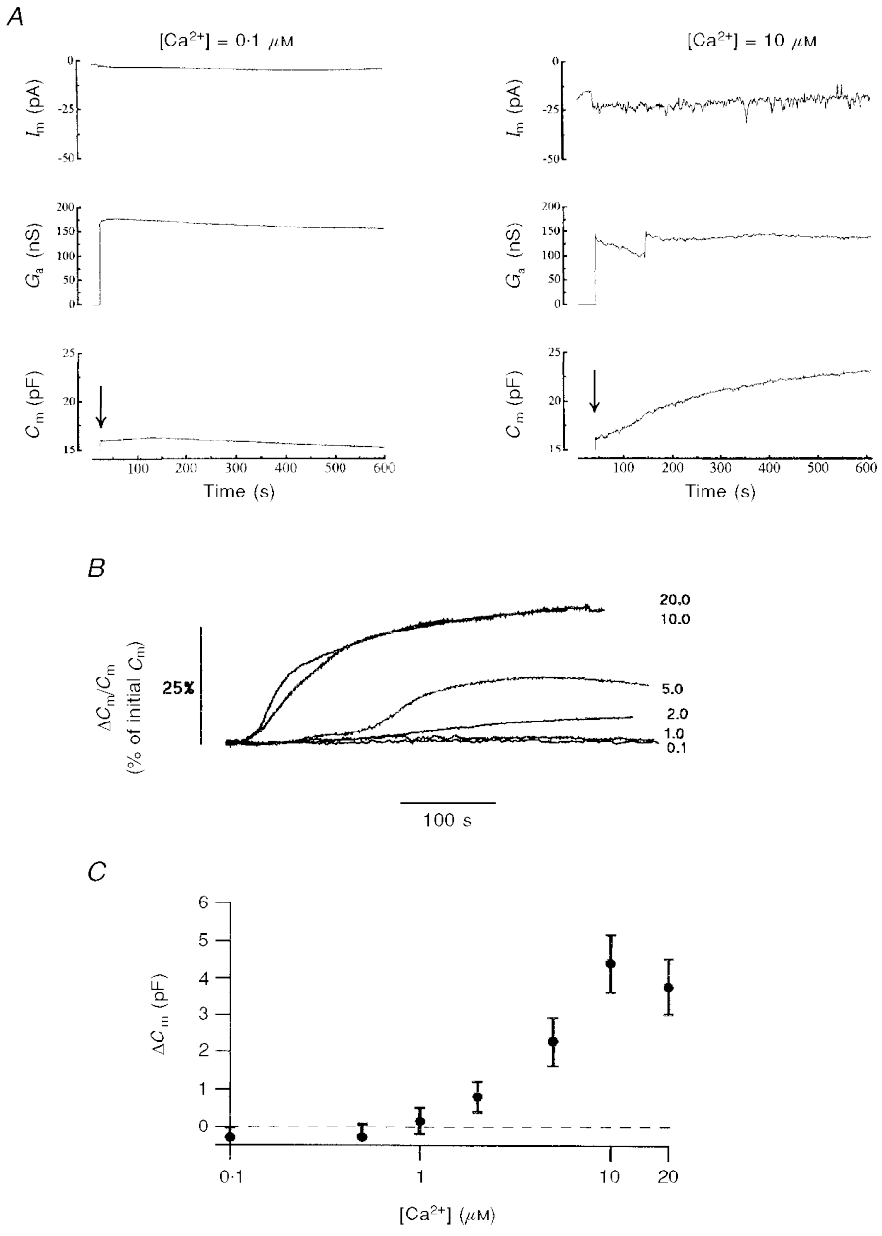

Figure 1. Ca^{2+}-dependent exocytosis in HUVECs

A shows continuous recordings of membrane current, I_m (upper traces), access conductance, G_a (middle traces), and membrane capacitance, C_m (lower traces), in two separate HUVECs following establishment of whole-cell recording (arrow). The intracellular solution contained 0·1 μM (left panel) or 10 μM (right panel) free $[Ca^{2+}]$. *B* shows the time course of C_m changes in six HUVECs at different free $[Ca^{2+}]$ ranging from 0·1 to 20 μM. The C_m is normalized to the initial C_m following establishment of whole-cell recording. *C* shows the maximal change in C_m plotted against the free $[Ca^{2+}]$ of the dialysing pipette solution (mean ± S.E.M., $n = 3$–9 cells at each $[Ca^{2+}]$).

Thrombin was applied to single HUVECs with a puffer pipette placed approximately two cell diameters from the patch-clamped cell. Application of external solution alone produced no change in $[Ca^{2+}]_i$ or C_m. Application of thrombin caused a large initial rise in intracellular free $[Ca^{2+}]$ to $7{\cdot}1 \pm 1{\cdot}5$ μM (range 2·4–11·4 μM, $n = 5$) (Fig. 2). Following the initial rise, $[Ca^{2+}]_i$ declined in three of five cells tested to a sustained level of $2{\cdot}0 \pm 0{\cdot}8$ μM ($n = 3$, range 1·0–3·6 μM) (Fig. 2*A*). In the remaining two cells, $[Ca^{2+}]_i$ returned to pre-stimulus levels despite the continued presence of thrombin (e.g. Fig. 2*B*). In the two cells with no prolonged elevation of $[Ca^{2+}]_i$, the C_m comprised either no change (one cell), or a small slow increase of ~0·1–0·2 pF following the initial $[Ca^{2+}]_i$ rise which then declined back to pre-stimulus levels over 3–4 min (e.g. Fig. 2*B*). In contrast, the three cells with a sustained elevated $[Ca^{2+}]_i$ had large increases in C_m ($1{\cdot}14 \pm 0{\cdot}12$ pF, $n = 3$) with a more complex time course (Fig. 2*A*). After removal of thrombin, $[Ca^{2+}]_i$ declined to resting levels and C_m decreased more slowly to pre-stimulus levels.

Flash photolysis of caged InsP_3 or DM-nitrophen

Thrombin-evoked changes in $[Ca^{2+}]_i$ comprise two components, identified by their sensitivity to removal of external Ca^{2+}: an initial spike insensitive to external Ca^{2+} followed by a Ca^{2+}-sensitive maintained elevation of $[Ca^{2+}]_i$ (Hamilton & Simms, 1987; Hallam *et al.* 1988). To determine how the size and duration of the change in C_m evoked by thrombin were related to the amplitude and time course of the $[Ca^{2+}]_i$ elevation, C_m was monitored during a $[Ca^{2+}]_i$ spike and during maintained intracellular $[Ca^{2+}]_i$. Both were produced by flash photolysis of caged InsP_3 or DM-nitrophen and were made to simulate the $[Ca^{2+}]$ increase during the two components of the thrombin-evoked response. Control experiments with no cage present or caged phosphate (150 μM) showed that brief UV irradiation with the flash lamp pulse or release of the photolysis byproducts had no effect on $[Ca^{2+}]_i$ or C_m.

Transient elevation of $[Ca^{2+}]_i$ evoked by flash photolysis of caged InsP_3 or DM-nitrophen

The small, slowly rising increase in C_m seen with transient elevations of $[Ca^{2+}]_i$ during the early phase of the response produced by thrombin suggests that vesicular secretion may be small during short-lived high $[Ca^{2+}]_i$ but greater when $[Ca^{2+}]_i$ is maintained. To test this hypothesis, C_m was monitored during elevation of $[Ca^{2+}]_i$ by flash photolysis of either caged InsP_3 or DM-nitrophen under conditions when both compounds produce a short-lived spike of $[Ca^{2+}]_i$ in the range of peak $[Ca^{2+}]_i$ produced by thrombin (or other agonists) in endothelium of large vessels (~1–30 μM; Carter & Ogden, 1994; Carter *et al.* 1996). Only the change in C_m resulting from the first flash in each cell is shown here because evidence given below suggests that on second and subsequent flashes the increase in C_m is affected by the initial elevation of $[Ca^{2+}]_i$. Figure 3*A* shows the change in C_m evoked by transient changes in $[Ca^{2+}]_i$ resulting from flash photolysis of caged InsP_3 (left-hand records; InsP_3 concentrations indicated) or DM-nitrophen (right-hand records; 4 mM DM-nitrophen–2·4 mM Ca^{2+}). The photolytically induced Ca^{2+} spikes had amplitudes and half-durations (approximately 10–20 s) similar to the initial Ca^{2+} spike evoked hormonally (cf. Fig. 2; Carter & Ogden, 1994; Carter *et al.* 1996). Small brief increases in $[Ca^{2+}]_i$ (5–10 μM; upper records) produced by low intensity light pulses evoked little change, < 0·1 pF, in C_m. At higher light intensities, larger increases in $[Ca^{2+}]_i$ (10–30 μM), whether due to photolysis of caged InsP_3 (left-hand records) or Ca^{2+}-loaded DM-nitrophen (right-hand records), produced small, slow increases of C_m, up to 0·6 pF, with rates of up to ~4 fF s^{-1},

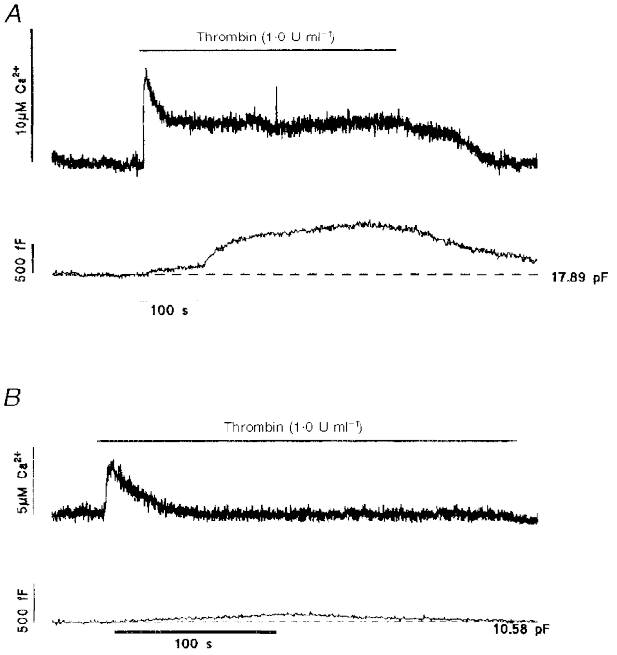

Figure 2. Effect of thrombin on $[Ca^{2+}]_i$ and C_m in single HUVEC

A and *B*, thrombin-evoked changes of $[Ca^{2+}]_i$ (upper records) and C_m (lower records) in two HUVECs. The C_m prior to application of thrombin is indicated by the dashed line. Thrombin (1·0 U ml^{-1}) was applied during the period indicated by the bar. The cells were voltage clamped at −50 mV and the external medium contained 1·8 mM Ca^{2+} in both cases.

that recovered to pre-flash levels over 2–3 min. The results are summarized in Fig. 3*B* where the peak increase in C_m and the rate of increase of C_m, measured as the maximum slope, are plotted against peak $[Ca^{2+}]_i$. There was a significant correlation between the amplitude or rate of change of C_m with peak $[Ca^{2+}]_i$ (see figure legend). The results show only small slowly increasing C_m at peak $[Ca^{2+}]$ of 10–30 μM, suggesting that the pool of vesicles available for very rapid secretion is small.

C_m changes resulting from large prolonged elevations of $[Ca^{2+}]_i$

Large C_m changes were seen during prolonged Ca^{2+} elevation by thrombin and at high buffered $[Ca^{2+}]_i$ (Figs 1*A* and 2*A*).

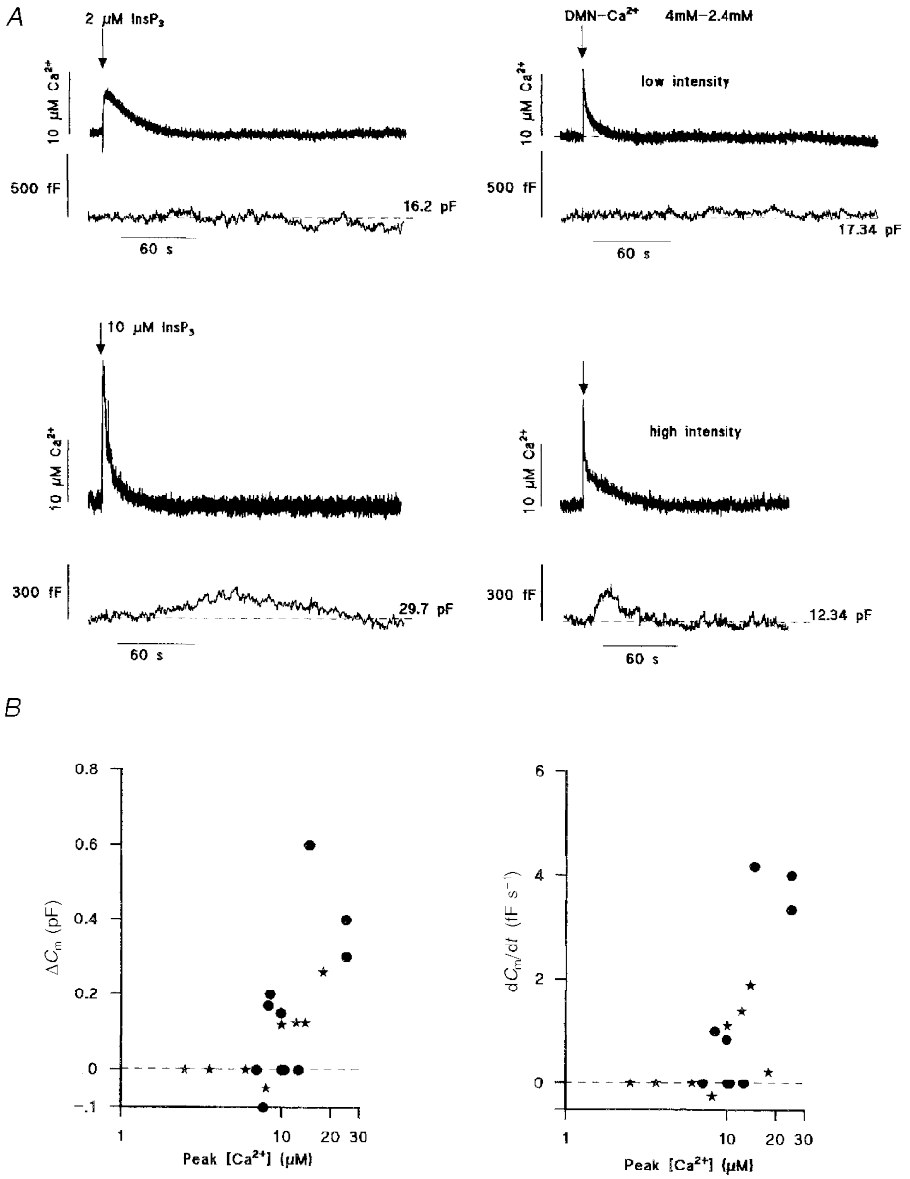

Figure 3. C_m changes evoked by transient elevations of $[Ca^{2+}]_i$ produced by photolysis of caged InsP_3 or DM-nitrophen

A shows changes in $[Ca^{2+}]_i$ (upper records of pairs) and C_m (lower records of pairs) in four separate HUVECs following intracellular photolytic release (at times indicated by arrows) of InsP_3 (left) or Ca^{2+} from DM-nitrophen (DMN; right). Cells were pre-equilibrated with internal solutions containing either 50 μM caged InsP_3 or 4 mM DM-nitrophen–2·4 mM Ca^{2+} and 500 μM furaptra. The extent of photolysis was controlled by varying the light intensity of the UV pulse to give smaller (upper records) or larger (lower records) increases in $[Ca^{2+}]_i$. The C_m prior to photolysis is indicated by the dashed line. *B* summarizes the extent (left panel) and rate of change (right panel) of C_m *versus* peak $[Ca^{2+}]_i$ produced by photolysis of caged InsP_3 (★) or DM-nitrophen (●) in individual HUVECs. Regression analysis of the data in *B* gave correlation coefficients (r) of 0·7 ($P < 0·001$; left panel) and 0·49 ($P < 0·03$; right panel).

This suggests that high $[Ca^{2+}]_i$ must be maintained for long periods to evoke large vesicular secretion. High levels of $[Ca^{2+}]_i$ in the range seen here during thrombin activation are known to drive vWF secretion in permeabilized endothelial cells (e.g. Scrutton & Pearson, 1989). To test this idea, prolonged elevations of $[Ca^{2+}]$ were established rapidly by high-intensity photolysis of DM-nitrophen. In some experiments a prolonged elevation of $[Ca^{2+}]_i$ was generated with the first flash, in other experiments by a second high intensity flash ~5 min after an initial low intensity flash. The data are distinguished as open circles (first flash) and filled circles (second flash) in Fig. 4*B*. Illustrative traces of $[Ca^{2+}]_i$ and C_m are shown in Fig. 4*A* for three cells with differing steady free $[Ca^{2+}]$ levels produced by photolysis. Data from 16 cells are summarized in Fig. 4*B*. Comparison of the rate of increase of C_m for similar steady-state $[Ca^{2+}]_i$ levels (2–5 μM) produced by a first or a second flash showed a larger change on the second flash. This difference indicates that there may be a priming effect of the first flash. Figure 4*B* also shows that when $[Ca^{2+}]_i$ was maintained at levels greater than ~3–4 μM the maximum rate of increase of C_m (lower graph) and the steady amplitude (upper graph) both increased to levels much greater than those seen with transient elevations of $[Ca^{2+}]_i$ (cf. Fig. 3). This result is similar to that for perfusion of buffered Ca^{2+} (Fig. 1) but in this case it is clear that the rise in C_m was not slowed by equilibration of $[Ca^{2+}]_i$ by perfusion into the cytosol. The changes in C_m seen with prolonged high $[Ca^{2+}]_i$ stimulation are very large, up to 4 pF in Fig. 4*B*, equivalent to ~30% of the initial C_m, and similar to those changes seen in the cell perfusion experiments of Fig. 1*C* (of up to 7 pF). Furthermore, the rates of increase in C_m evoked by high sustained $[Ca^{2+}]$ are large compared with those seen in response to transient elevations of $[Ca^{2+}]_i$, (up to 50 fF s^{-1} in Fig. 4*B* compared with 4 fF s^{-1} in Fig. 3*B*).

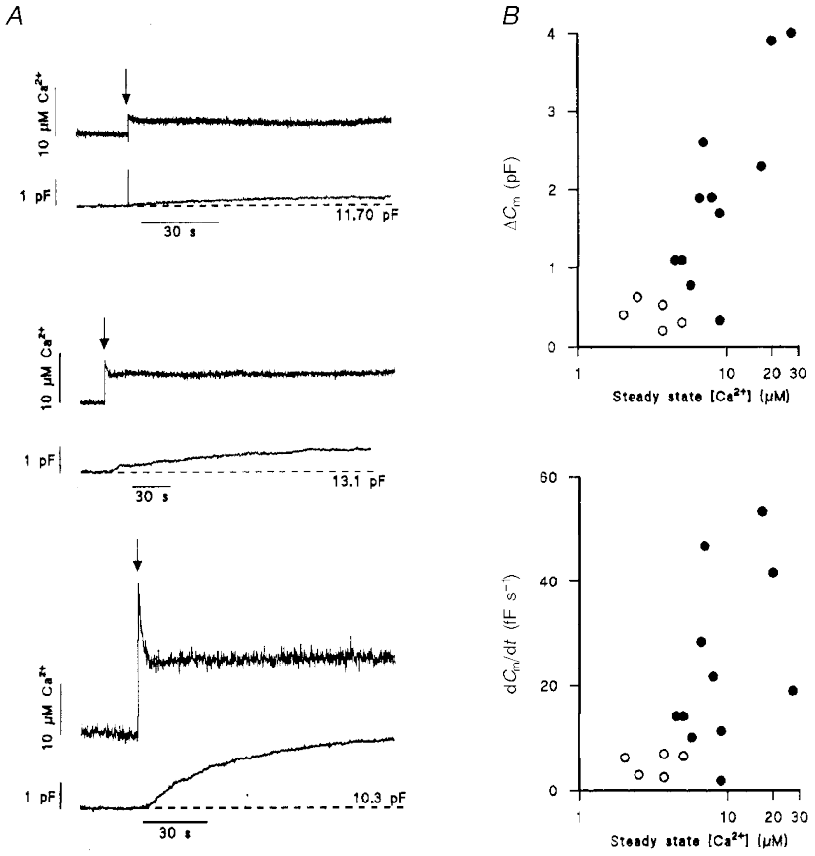

Figure 4. Capacitance changes in response to maintained elevations of $[Ca^{2+}]_i$ produced by photolysis (arrows) of DM-nitrophen

A shows the changes in capacitance (lower record of each pair) produced by different steady free $[Ca^{2+}]$ levels (upper traces of each pair) in three different cells. The C_m prior to photolysis is indicated by the dashed line. *B* summarizes the extent (upper panel) and rate of change (lower panel) of capacitance *versus* steady free $[Ca^{2+}]$ levels produced by photolysis of DM-nitrophen in individual HUVECs. ○, prolonged Ca^{2+} elevation produced by a first flash; ●, prolonged Ca^{2+} elevation produced by a second flash. Regression analysis of the data in *B* gave correlation coefficients (r) of 0·86 ($P < 0{\cdot}001$; upper panel) and 0·53 ($P < 0{\cdot}001$, lower panel).

DISCUSSION

Vesicular secretion of vasoactive mediators was monitored in single endothelial cells by detecting changes in membrane capacitance and was used to study the mechanisms underlying regulated secretion. In many cell types an increase in $[Ca^{2+}]_i$ provides a trigger for secretion (reviewed in Burgoyne & Morgan, 1993). In this study, the $[Ca^{2+}]_i$ of single isolated endothelial cells was manipulated by intracellular perfusion with buffered $[Ca^{2+}]_i$, by hormonal stimulation with thrombin, or by flash photolysis of caged $InsP_3$ or Ca^{2+}-loaded DM-nitrophen.

Intracellular perfusion of endothelial cells with buffered $[Ca^{2+}]_i$ in the range corresponding to resting $[Ca^{2+}]_i$ (100 nM) evoked little or no change of C_m over many minutes of recording, indicating that exocytosis and endocytosis under resting conditions were occurring at approximately equal rates. At high $[Ca^{2+}]_i$, corresponding to the range that triggers vWF secretion (0·5–20 μM $[Ca^{2+}]$; Scrutton & Pearson, 1989; Birch *et al.* 1992; Frearson *et al.* 1995), large net increases in C_m of up to 30% of resting C_m were seen. The time course of the changes in C_m were complex at $[Ca^{2+}]_i > 2$ μM (e.g. Fig. 1*B*), and difficult to interpret. First, during intracellular perfusion the free $[Ca^{2+}]_i$ equilibrates slowly over a period of up to 90 s, during which time secretory granules experience a continually changing $[Ca^{2+}]_i$. Second, endothelial cells contain different secretory granules for t-PA, TFPI, Protein S and vWF, each of which may have different sensitivities to Ca^{2+}. Biochemical studies in cell cultures have shown that the time course of procoagulant vWf secretion differs from that of the anticoagulants TFPI, t-PA and Protein S. Secretion of TFPI, t-PA and Protein S occurs early (peaking in 30–60 s) but declines to low levels within 2–3 min (Stern *et al.* 1986; Kooistra *et al.* 1994; Lupu *et al.* 1995) and might be expected to produce an early transient increase in C_m. Indeed, TFPI storage granules have been identified in clusters at plasma membrane caveolae, suggesting that they may be available for early release (Lupu *et al.* 1995). On the other hand, the large vWF storage granules are generally sequestered further from the plasma membrane (Weibel & Palade, 1964), which may account in part for their much slower time course of secretion, detected ~1–3 min after stimulation (Hamilton & Simms, 1987; Hattori *et al.* 1989; Frearson *et al.* 1995). Unlike the secretion of anti-coagulants, vWF secretion can be maintained for long periods in the continued presence of a stimulus, and might be expected to produce a delayed, larger and more maintained change in C_m, similar to those described here for free $[Ca^{2+}]_i$ buffered to > 2 μM. Similar data have been obtained in neutrophils, which like endothelial cells contain several distinct granule populations that can undergo regulated secretion during specific phases of neutrophil activation (Nuesse *et al.* 1998). In that study, it was argued that the early smaller component and the delayed larger component of the change in C_m seen at higher $[Ca^{2+}]_i$ reflects release of distinct granule populations with different Ca^{2+} affinities. Further work is needed to determine if the time course of changes in C_m seen here reflects secretion from different pools of granules.

Thrombin is generated at high levels at wound sites during blood coagulation and is a strong secretogogue for vWF, t-PA and TFPI from endothelial cells (Hamilton & Simms 1987; Lupu *et al.* 1995; van den Eijnden-Schrauwen *et al.* 1997). The predominant $[Ca^{2+}]_i$ response in single intact HUVECs at high thrombin concentrations (1·0 U ml^{-1}) consists of a biphasic elevation, an initial release of Ca^{2+} from internal stores followed by a maintained elevated component due to Ca^{2+} influx (Birch *et al.* 1994). In single patch-clamped cells, thrombin at 1·0 U ml^{-1} produced a biphasic elevation of $[Ca^{2+}]_i$, and a large maintained C_m increase (Fig. 2*A*). In two cells there was only a transient increase in $[Ca^{2+}]_i$ and in these cells the change in C_m was slow and small (Fig. 2*B*). Similar results were obtained when $[Ca^{2+}]_i$ was increased in a controlled manner either as a maintained elevation or as a transient spike, by flash photolysis of caged $InsP_3$ or DM-nitrophen. The amplitude of the $[Ca^{2+}]_i$ spike produced by photolysis (~1–30 μM) was in the physiological range since similar large-amplitude Ca^{2+} transients are evoked by thrombin or other agonists in endothelial cells in culture and *in situ* (Carter & Ogden, 1994; Carter *et al.* 1996). The similar rates and extent of changes in C_m following transient elevations of $[Ca^{2+}]_i$ evoked by photolysis of caged $InsP_3$ (where Ca^{2+} release occurs over 50–100 ms) or DM-nitrophen (where Ca^{2+} is released in less than 1 ms; Kaplan & Ellis-Davies, 1988) suggest that secretion may not be triggered by the rate of rise of $[Ca^{2+}]_i$, as has been suggested for presynaptic transmitter release (Hsu *et al.* 1996).

Studies based on kinetic analysis of changes in C_m during controlled changes in $[Ca^{2+}]_i$ by flash photolysis in chromaffin cells and melanotrophs have led to the idea that secretory granules proceed through a number of stages prior to exocytosis (see Henkel & Almers, 1996). Following a rapid increase in $[Ca^{2+}]_i$, the most advanced (docked) granules are secreted rapidly, while those at preceding stages are secreted more slowly. The small slow (up to 5 fF s^{-1}) increases in C_m seen here following a transient elevation of $[Ca^{2+}]_i$ suggest that in endothelial cells the pool of vesicles available for rapid secretion is small. Similar conclusions have been reached for other cells in which little secretion is seen during an initial agonist-evoked Ca^{2+} spike (Tse *et al.* 1993, 1997; Kim *et al.* 1997). The large component of secretion requires a more prolonged exposure to high $[Ca^{2+}]_i$, the slow time course of the changes in C_m indicating a slow recruitment of secretory vesicles into a releasable pool. The results presented in Fig. 4*B* suggest that exposure to high $[Ca^{2+}]_i$ several minutes prior to a second prolonged Ca^{2+} rise may have a priming effect on secretion which persists during the relatively long interval between first and second flashes (~5 min). Similar observations have been reported in neurohypophysial nerve terminals and in other systems (see Seward *et al.* 1995). This priming effect may underlie the delay before the high secretion rate seen during

dialysis with solutions buffered to high $[Ca^{2+}]_i$ (Fig. 1). In summary, to produce a high level of secretion, such as that seen with thrombin stimulation (Fig. 2*A*) it may be necessary to have a slow step that can be primed and a prolonged high elevation of $[Ca^{2+}]_i$.

There is considerable evidence that secretion of vWf, t-PA, TFPI and Protein S is triggered by an increase in $[Ca^{2+}]_i$ (Stern *et al.* 1986; Hamilton & Simms, 1987; Scrutton & Pearson, 1989; Birch *et al.* 1992, 1994; Kooistra *et al.* 1994; Frearson *et al.* 1995; Emeis *et al.* 1997; van den Eijnden-Schrauwen *et al.* 1997) and that this may be mediated via calmodulin (Birch *et al.* 1992; van den Eijnden-Schrauwen *et al.* 1997). In mast cells, the recruitment of secretory vesicles is thought to be due to a slow Ca^{2+}-dependent activation of protein kinase C (PKC), the most important factor being the amplitude rather than the pattern of the Ca^{2+} rise in this process (Kim *et al.* 1997). However, there is little evidence that other signalling pathways, including the PKC pathway, play an important role in triggering secretion in endothelial cells (Carew *et al.* 1992; Kooistra *et al.* 1994; Wheeler-Jones *et al.* 1996*a*,*b*; van den Eijnden-Schrauwen *et al.* 1997). Exocytosis in all cells requires several well-defined proteins (Hanson *et al.* 1997). A number of these proteins, required for vesicle trafficking and fusion in neurons and exocrine cells, are now known to be present in endothelial cells (e.g. VAMP-2, cellubrevin, NSF, SNAP, annexins and trimeric and monomeric GTPases; Schnitzer *et al.* 1995). Although some have been implicated in membrane retrieval and transcytosis, their role in regulated vesicular secretion has not yet been determined.

The maximum rate of change of C_m, during secretion in HUVECs, of 50 fF s^{-1} is much less than that seen in pituitary melanotrophs (5 pF s^{-1}; Thomas *et al.* 1993), adrenal chromaffin cells (0·5–1 pF s^{-1}; Neher & Zucker, 1993) or gonadotrophs (50–300 fF s^{-1}; Tse *et al.* 1993). However, vWf release is sustained at a high rate for minutes rather than the milliseconds to seconds seen in neuroendocrine cells. Following a large increase in C_m evoked by thrombin the recovery by endocytosis of C_m to the initial level is slower than the recovery of $[Ca^{2+}]_i$ and occurs at 1–5 fF s^{-1} (see Fig. 2*A*), a rate similar to the compensatory rate of endocytosis seen in chromaffin cells (Smith & Neher, 1997).

Local hormones (e.g. ATP, ADP, kinins or thrombin), generated at high concentrations close to or at a wound site, are likely to produce a prolonged rise in endothelial $[Ca^{2+}]_i$ as seen here. A maintained elevated $[Ca^{2+}]_i$ under these conditions may be important for strong secretion, particularly of vWF, by endothelial cells at wound sites. Further away agonist concentrations will be lower because of dilution and constitutive metabolic degradation by endothelial cells (see Mann, 1997). At low agonist concentrations endothelial cells generally respond with repetitive Ca^{2+} spikes (Jacob *et al.* 1988; Carter *et al.* 1991; Carter & Ogden 1994), and this might modify the pattern or extent of secretion. Recent studies suggest that Ca^{2+} spiking plays a central role in controlling cell processes such as mitochondrial metabolism (Pralong *et al.* 1994; Hajnoczky *et al.* 1996) and gene expression (Dolmetsch *et al.* 1998; Li *et al.* 1998). In some cells vesicular secretion is closely coupled to intracellular Ca^{2+} spikes or repetitive Ca^{2+} influx (Tse *et al.* 1993; Seward *et al.* 1995) while in others the amplitude rather than the specific pattern of Ca^{2+} elevation appears to be important (Kim *et al.* 1997). Indirect evidence suggests that Ca^{2+} spiking in endothelial cells may provide a mechanism for controlling secretion of the procoagulant molecule vWF. Secretion of vWF can be maintained for longer periods in the presence of an agonist and differs from t-PA and TFPI secretion which is temporally coupled to the early phase of agonist-evoked Ca^{2+} responses. Low agonist concentrations, which produce Ca^{2+} spiking in single endothelial cells, produce less vWF secretion than high agonist concentrations, which cause prolonged $[Ca^{2+}]_i$ elevations (Birch *et al.* 1994). It seems likely that the priming effect seen in two-pulse experiments described above may have a role in potentiating vWF secretion during spiking. Current work is aimed at addressing the role of Ca^{2+} spiking in the control of secretion in single endothelial cells in culture.

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