Ca²⁺ influx does more than provide releasable Ca²⁺ to maintain repetitive spiking in human umbilical vein endothelial cells

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We investigated why oscillations of intracellular Ca²⁺ concentrations ([Ca²⁺]_i) in endothelial cells challenged by submaximal histamine run down in Ca²⁺-free medium despite stores retaining most of their Ca²⁺. One explanation is that only a small subpopulation of the Ca²⁺ stores oscillate and are completely emptied of Ca²⁺. To investigate if influx refills an empty store subpopulation, we differentiated between cations entering the cell and those released from internal stores by using extracellular Sr²⁺ as a Ca²⁺ surrogate; we distinguished between [Sr²⁺]_i and [Ca²⁺]_i by using the larger effect of Sr²⁺ on fura 2 fluorescence at 360 nm (*F*₃₆₀). Ca²⁺ was still available for release when oscillations had run down since oscillations promptly reappeared on addition

of Sr²⁺_o and these were predominantly of Ca²⁺ (indicated by *F*₃₆₀ changes). Also, totally depleting Ca²⁺ stores inhibited Sr²⁺-induced oscillations, suggesting that Sr²⁺ entry leads to Ca²⁺ release. In contrast, Ba²⁺_o was unable to stimulate oscillations. Finally, oscillations generated by photolytic release of inositol trisphosphate (IP₃) analogues were similarly sensitive to extracellular Ca²⁺ and Sr²⁺. We conclude that stores (or a subpopulation) are not completely depleted of Ca²⁺ when oscillations run down in Ca²⁺-free medium. Bivalent cation entry therefore maintains sensitivity to IP₃, possibly by maintaining luminal bivalent cation levels.

INTRODUCTION

Human umbilical vein endothelial cells (HUVECs) and many other cell types respond to low concentrations of agonist with oscillations of intracellular free Ca²⁺ ([Ca²⁺]_i) [1–4] comprising repetitive Ca²⁺ spikes generated by brief periods of release from stores. There are several theories to explain the mechanism of repetitive spiking which of course may vary according to cell type. Some theories invoke the existence of two types of Ca²⁺ store [4]. However, it appears that HUVECs only possess an inositol trisphosphate (IP₃)-sensitive Ca²⁺ store (see below), so we will not consider 'two-pool' models any further. Another categorization is according to whether or not [IP₃]_i remains constant during the repetitive spiking cycle. Because we can stimulate oscillations in HUVECs with poorly metabolized IP₃ analogues (see the Results section), it is possible that agonist-induced oscillations are not accompanied by oscillations in [IP₃]_i. Consequently we assume constant [IP₃]_i, noting that an involvement of oscillating [IP₃]_i in generating the spikes would not affect our conclusions.

There is broad agreement that the initiation of a Ca²⁺ spike involves some form of Ca²⁺-induced Ca²⁺ release (CICR) although this need not be of the classical ryanodine receptor (RyR) type. The ability of IP₃ to bind to the IP₃ receptor–channel complex (IP₃R) and release Ca²⁺ is itself Ca²⁺ dependent, with release being potentiated by Ca²⁺ in the lower ranges of cytosolic Ca²⁺ [5–8], leading to positive feedback and CICR via the IP₃R.

More problematic is the mechanism by which the release of Ca²⁺ is terminated. In HUVECs the Ca²⁺ spikes are smaller and more abbreviated than the response to maximal stimulation, so that termination of release occurs before the stores are globally empty. Several explanations for the termination of release have

been proposed. Broadly speaking they can be divided into theories involving homogeneous or heterogeneous release [4,9,10]. Heterogeneous release explains the limited release of Ca²⁺ by postulating discrete stores of heterogeneous sensitivity to IP₃, with submaximal agonist stimulation discharging a subpopulation of the stores in an all-or-none fashion to form a spike. Homogeneous release explains spike formation in terms of a limited graded release (sometimes referred to as steady-state) that occurs from all stores (or perhaps from just one interconnected store); the termination of release can be explained by the inhibiting effect of high levels of cytosolic Ca²⁺ on IP₃-induced Ca²⁺ release [5–7,11,12] perhaps combined with a luminal effect on the sensitivity of the IP₃R to IP₃ [13,14]. These effects may account for the phenomenon of quantal Ca²⁺ release seen in permeabilized cells and vesicular preparations where submaximal [IP₃]_i discharges only a fraction of the stored Ca²⁺ even when the store Ca²⁺-ATPase [sarcoendoplasmic-reticulum Ca²⁺-ATPase, (SERCA)] is inhibited. However, a recent report has indicated that quantal release may not be related to IP₃ receptor regulation by cytosolic Ca²⁺ [15]. In the presence of an active SERCA, graded release could simply be due to a pump–leak balance, although in practice the imbalance between the very rapid rise in [Ca²⁺]_i due to discharge via the IP₃R compared with the relatively slow return of [Ca²⁺]_i to basal in Ca²⁺-free medium due to the combined activities of SERCA and the plasmalemmal Ca²⁺-ATPase (PMCA) suggests that graded release cannot be entirely due to pump–leak balance. However, there is some indication that quantal release may be an artifact of permeabilization [16] and, in the presence of SERCA pumping, partial release by submaximal [IP₃]_i could be influenced by SERCA activity [17].

We have investigated just one aspect of Ca²⁺ spiking, i.e. why spiking ceases in Ca²⁺-free medium, to clarify the mechanism of

Abbreviations used: HUVECs, human umbilical vein endothelial cells; Ba²⁺_o, extracellular Ba²⁺; [Ca²⁺]_i, intracellular Ca²⁺ concentration; Ca²⁺_o, extracellular Ca²⁺; [Sr²⁺]_i, intracellular Sr²⁺ concentration; Sr²⁺_o, extracellular Sr²⁺; *F*₃₄₀, *F*₃₆₀, *F*₃₈₀, fluorescences detected with 340, 360 or 380 nm excitation; *R*_{340/380}, ratio of *F*₃₄₀/*F*₃₈₀; HBSS, Hepes-buffered balanced salt solution; PMCA, plasma-membrane Ca²⁺-ATPase; SERCA, sarcoendoplasmic reticulum Ca²⁺-ATPase; SRCE, store-regulated cation entry; CICR, Ca²⁺-induced Ca²⁺ release; RyR, ryanodine receptor; IP₃, inositol 1,4,5-trisphosphate; IP₃R, the tetrameric IP₃ receptor channel complex; IP₃-5S, 1-*D*-myo-inositol 1,4-bisphosphate 5-phosphorothioate; GPIP₂, 1-(α -glycerophosphoryl)-*D*-myo-inositol 4,5-bisphosphate; CPA, cyclopiazonic acid; ISP, interspike period; PLC, phospholipase C.

spiking. One explanation for the eventual inhibition of spiking in Ca^{2+} -free medium is that there is no Ca^{2+} left in the stores, and, although this is the case in those cell types that pump most of the released Ca^{2+} out of the cell (e.g. pancreatic acinar cells [18]), this cannot explain the cessation of spiking in cell types such as HUVECs, where stores still contain substantial amounts of agonist-releasable Ca^{2+} after spiking has stopped [2]. However, it is possible that for a strictly heterogeneous mechanism, where the spikes are caused by a complete all-or-none discharge of a store subpopulation, spiking could cease when this subpopulation has lost all its Ca^{2+} . One approach that has been taken is to measure stored Ca^{2+} during oscillations [19,20], showing that a spike terminates before stores are completely depleted of Ca^{2+} . However, this approach does not resolve whether a spike terminates when a subpopulation of stores is completely depleted. In order to distinguish between the homogeneous and heterogeneous mechanisms, we have used Sr^{2+} as a Ca^{2+} surrogate to try to 'chase' Ca^{2+} from its stores.

Sr^{2+} behaves similarly to Ca^{2+} in many ways. It has been reported to potentiate [8,21,22] or inhibit [23] IP_3 -mediated Ca^{2+} release, potentiate cADP-ribose-induced Ca^{2+} release [24], serve as a Ca^{2+} -ATPase substrate [25–27], enter cells via a similar pathway to Ca^{2+} [28–31] and be taken up by and released from stores in response to either IP_3 or agonist [28,29,32–34]. Sr^{2+} appears to leak from stores in HeLa cells at the same rate as Ca^{2+} , and it has been argued that it will enter stores at the same rate as Ca^{2+} so that the final luminal concentration of Sr^{2+} -filled stores will be the same as that for Ca^{2+} -filled stores [35]. However, Sr^{2+} does not mimic Ca^{2+} in all respects, since some workers have found that refilling stores with Sr^{2+} does not completely inhibit store-regulated cation entry (SRCE) [33,34,36] nor does it display a biphasic concentration effect on IP_3 -mediated Ca^{2+} release, at least in well-buffered hepatocyte and cerebellar preparations [21,22].

We expected that, if a homogeneous mechanism is operating, then refilling stores with Sr^{2+} after cessation of spiking in Ca^{2+} -free medium would result in release of both Ca^{2+} and Sr^{2+} in the continued presence of agonist since incoming Sr^{2+} and residual stored Ca^{2+} would be located in the same stores. In contrast, if a heterogeneous mechanism were operating, then the Sr^{2+} would be filling stores devoid of Ca^{2+} , leading eventually to the release of Sr^{2+} alone.

We have exploited the different isoemissive wavelengths [29,30] of fura 2 for Ca^{2+} and Sr^{2+} to distinguish between these cations. We conclude that cessation of agonist-induced $[\text{Ca}^{2+}]_i$ spiking in Ca^{2+} -free medium is not due to the complete depletion of a subpopulation of stores and that cation influx does not simply serve to supply releasable cations but also maintains store sensitivity to agonist, possibly via a luminal effect. Our results also suggest that there are both homogeneous and heterogeneous elements in the spiking mechanism.

MATERIALS AND METHODS

Cell culture

Cells were isolated from human umbilical vein and cultured on glass coverslips at either passage 1 or 2 as previously described [2]. Cells were maintained at 37 °C in an atmosphere of 95% O_2 /5% CO_2 in medium 199 supplemented with 10% fetal calf serum, 10% newborn calf serum, 50 $\mu\text{g}/\text{ml}$ streptomycin, 50 IU/ml penicillin, 2 mM L-glutamine and 20 $\mu\text{g}/\text{ml}$ endothelial cell growth factor prepared from bovine brain. Experiments were performed on cells 2–6 days after plating at subconfluent density ($\sim 2 \times 10^4$ cells per coverslip).

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

For $[\text{Ca}^{2+}]_i$ determination, cells were loaded with 1 μM fura 2 acetoxymethyl ester for 30–45 min at room temperature as previously described [2,37]. After loading, cells were maintained in a Hepes-buffered balanced salt solution (HBSS) of the following composition (mM): 145 NaCl, 5 KCl, 1 MgSO_4 , 10 Hepes, 10 glucose, 1% (w/v) BSA, pH 7.4. Coverslips were then mounted on a heated stage of an inverted epifluorescence microscope and superfused by gravity feed at a flow rate of approx. 0.3 ml/min with HBSS containing a lower BSA concentration (0.1%). Complete exchange of bath volume was typically achieved in about 15 s. All experiments were conducted at 37 °C. For experiments with BaCl_2 (and parallel controls with SrCl_2), HBSS contained 1 mM MgCl_2 instead of MgSO_4 to avoid precipitation. Ca^{2+} -free media were prepared by omission of Ca^{2+} without addition of EGTA except where stated.

Single-cell measurements were carried out as before [2,37], fluorescence being measured at 340, 360 and 380 nm excitation (F_{340} , F_{360} and F_{380}) and detected from either single cells or patches of cells at wavelengths above 470 nm by a photomultiplier. Data were collected at 2 Hz. Experimental results are expressed as fluorescence ratio ($R_{340/380} = F_{340}/F_{380}$). However, calibration [38] gave basal $[\text{Ca}^{2+}]_i$ of about 60–70 nM and maximal histamine $[\text{Ca}^{2+}]_i$ responses of about 2 μM .

Imaging

In some experiments, fura-2-loaded cells were examined using imaging to analyse a large number of cells at a time. A ratio image was acquired approx. every 3 s using an LEP dual-filter wheel (Ludl, Hawthorne, NY, U.S.A.) equipped with 350 and 380 nm, 10 nm bandpass filters and a range of neutral density filters (Ealing ElectroOptics), and an extended Isis M intensified CCD camera (Photonic Sciences, Robertsbridge, E. Sussex, U.K.). Data were acquired using a Pixel Grabber board (Perceptics, Knoxville, TN, U.S.A.) hosted by a Macintosh Quadra 800 using Ionvision software (Improvision, Coventry, U.K.).

Microinjection and photolysis

Glass micropipettes (1 mm outside diameter) were pulled on a Mecnex (Geneva, Switzerland) BB-CH-PC puller to a tip diameter estimated to be approx. 0.2 μm using a bubble technique [39], backfilled and used to inject cells at room temperature in HBSS containing 1 mM Ca^{2+} . Acetoxymethyl ester loading of HUVECs resulted in an intracellular [fura 2] of 40–80 μM (as judged from hypotonic lysis experiments of cell populations assuming a cell volume of 1 pl). By injecting fura 2 free acid into loaded cells we estimated the injection volume to be 1–5% of the cell volume.

The analogues of IP_3 , 1-(α -glycerophosphoryl)-D-*myo*-inositol 4,5-bisphosphate (GPIP_2) (20 mM pipette concentration) or 1-D-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate (IP_3 -5S) (2 mM pipette concentration) were injected as their caged precursors (the $P^{(5)}$ -1-(2-nitrophenyl)ethyl esters of GPIP_2 [40], and the *S*-1-(2-nitrophenyl)ethyl ester of IP_3 -5S [41]), along with Calcium-Green-1 Dextran (M_r 10000; 1 mM pipette concentration) in injection buffer (27 mM K_2HPO_4 , 8 mM Na_2HPO_4 , 26 mM KH_2PO_4 , pH 7.3) [40]. The fluorescence signal was stable for 3000 s or more and was diffuse throughout the entire cell. After injection, cells were allowed to equilibrate for 5–10 min at 37 °C before the experimental run. Excitation was at 495 nm with emission measured above 520 nm, and slow, continuous photo-

lysis was achieved by constantly illuminating cells with UV light (360 and 380 nm) from the lamp used for the excitation at 495 nm. Prevention of continuous photolysis outside of the experimental run was achieved by placing a Schott GG420 filter in the exciting light path to block light below 400 nm. We adopted this approach because we could not titrate IP₃ analogue levels to give oscillations with repetitive *brief* flashes with a UV Xe arc lamp; indeed, we were surprised to observe only single transient spikes on photolytic release (not shown; $n \geq 5$ each for IP₃-5S and GPII₂). No sustained [Ca²⁺]_i response was stimulated by submaximal concentrations of analogues (see [40,41]).

Determination of the relative affinities of Calcium-Green-1 Dextran for Ca²⁺ and Sr²⁺

We compared the *in vitro* affinities of Calcium-Green-1 Dextran and fura 2 for Ca²⁺ and Sr²⁺. A bivalent-cation-free buffer was prepared (in mM): 145 KCl, 5 NaCl, 10 Mops, 10 EGTA, pH 7.10, at 37 °C. Bivalent-cation-containing buffer was prepared by adding 10 mM of either Ca²⁺ or Sr²⁺ together with sufficient KOH to maintain the pH at 7.10. Buffers with and without the bivalent cation were mixed in different proportions to give the appropriate free ion concentrations determined using Winmaxchelator (v1.2; Dr. C. Patton, Stanford, CA, U.S.A.) with stability constants from Martell and Smith [42]; we chose 8–12 concentrations extending one decade above and below the K_d for each dye. The dye was added at 1 μ M final concentration. The K_d for each ion was subsequently calculated by linear regression using a linear transformation of the Grynkiewicz equation [38]. The K_d values for fura 2 were calculated as 0.32 ± 0.03 μ M (Ca²⁺) and 10.2 ± 0.4 μ M (Sr²⁺), whereas for Calcium-Green-1 Dextran the values were 0.61 ± 0.02 μ M and 16.3 ± 0.2 μ M respectively (mean $\pm$ S.E.M., $n = 3$). These differed slightly from the published constants of 0.22 μ M (Ca²⁺) and 7.6 μ M (Sr²⁺) for fura 2 and 0.36 μ M (Ca²⁺) for Calcium-Green-1 Dextran [29,38,43]. Various factors could account for this, including impure EGTA, slight inaccuracies in pH and temperature, differences in Calcium-Green-1 Dextran batches, and the use of different ionic concentrations and stability constants. However, it was not our intention to accurately assess the individual K_d values but rather to assess the relative affinities for Ca²⁺ and Sr²⁺. We conclude that both dyes show a consistent approx. 30-fold difference in affinity for Ca²⁺ and Sr²⁺.

Isoemissive wavelengths

The fluorescence spectrum for fura 2 bound to Sr²⁺ differs slightly from fura 2 bound to Ca²⁺; whereas the isoemissive wavelength is close to 360 nm for Ca²⁺/fura 2 [38], that for Sr²⁺/fura 2, either *in situ* or *in vitro*, has been reported to be 364 nm [29,30]. Correspondingly, we observed larger changes in the F_{360} with increases in [Sr²⁺]_i than with [Ca²⁺]_i both *in vitro* and *in situ*. Figure 1 shows the *in vitro* relationship between percentage change in F_{360} and the free [Ca²⁺] or [Sr²⁺] that we observed while calibrating fura 2 affinities (see above). Note the much larger changes with Sr²⁺ than with Ca²⁺ even over an identical range of $R_{340/380}$. The maximum *in vitro* percentage change in F_{360} per unit ratio was approx. 6% for Ca²⁺ and approx. 32% for Sr²⁺. Assuming a nominal excitation of 360 nm and given that the $R_{\min}$ and $R_{\max}$ were similar for Sr²⁺ and Ca²⁺, these observations suggest that the isoemissive wavelength for Sr²⁺ binding to fura 2 lies further above 360 nm than does that for Ca²⁺ binding to fura 2. Similarly, when monitoring the fura 2 signals *in situ*, we saw larger changes in F_{360} for changes in [Sr²⁺]_i than for [Ca²⁺]_i (see Figures 4, 6 and 8). However, these changes differed from those *in vitro* in that when increases in $R_{340/380}$ were due to

Figure 1 Changes in F_{360} (percentage of value in Ca²⁺-free Sr²⁺-free buffer) seen after increases in free Sr²⁺ or Ca²⁺ concentration using an *in vitro* EGTA buffer system

Changes are plotted against $R_{340/380}$ on a logarithmic scale (top); scales at the bottom show corresponding calculated [Ca²⁺]_i and [Sr²⁺]_i. Horizontal error bars (usually smaller than symbol) refer to the $R_{340/380}$ measurement for a given [ion], whereas the vertical errors refer to the corresponding F_{360} values. Data represent the mean $\pm$ S.E.M. for three experiments.

changes in [Ca²⁺]_i, there were usually slight decreases in F_{360} . In contrast, when we knew that changes in $R_{340/380}$ were due to an increase in [Sr²⁺]_i (in experiments exploiting the selective discharge of Ca²⁺ compared with Sr²⁺ by ionomycin; see Figure 4) the F_{360} recording showed an appreciable increase, although smaller than expected from the *in vitro* calibration. The difference between *in vitro* and *in situ* changes probably reflects an *in situ* shift to a shorter wavelength in the fura 2 spectra for binding Sr²⁺ and Ca²⁺; such a shift will cause the slope of F_{360} against $R_{340/380}$ to become more negative. The variability of the slope of this relationship from cell to cell may reflect small shape changes which would give rise to small fluorescence changes due to either uneven distribution of the exciting light or a small part of the cell moving into or out of the fluorescence-collection area.

We quantified the F_{360} changes *in situ* as follows: since the analysis of F_{360} was complicated by a decline in F_{360} caused by leakage and photobleaching, we corrected for this decline by fitting a fifth-order polynomial to those regions of F_{360} corresponding to when $R_{340/380}$ was at its basal value. We then normalized the F_{360} trace by dividing each point by the corresponding point on the polynomial fit so that, apart from any excursions when $R_{340/380}$ was elevated, the trace maintained on average a constant value of 1.0. A good fit was not always obtained along the entire trace because of other factors affecting F_{360} (e.g. changes in focusing, air bubbles), and in longer experiments (> 1000 s) the fit was usually poorest at the end of the run, manifested as irregular excursions in the corrected trace. Regions of the F_{360} curves where poor fitting gives rise to large artefactual excursions are indicated by dashed lines. Accordingly, we did not quantify the data by comparing the ΔF_{360} with the $\Delta R_{340/380}$ but rather plotted the regression between the two traces which was more reliable (using more than 100 points instead of a few at the peaks). Even so, this method worked best with short-term changes in $R_{340/380}$ which minimized any contribution from extraneous influences on F_{360} . Thus the change

in F_{360} per unit change in $R_{340/380}$ was quantified as the slope of the regression of the normalized F_{360} values plotted against the corresponding $R_{340/380}$ values. These slopes were multiplied by 100 to give a percentage change in F_{360} per unit change in $R_{340/380}$. Typically, elevations in $[Ca^{2+}]_i$ gave a slope of -2% and elevations in $[Sr^{2+}]_i$ gave a slope of $+10\%$. No changes in autofluorescence were observed after maximal histamine stimulation in unloaded cells ($n \geq 4$).

Data analysis

Data were analysed using Student's t test which was two-tailed and unpaired except where stated. Data are presented as means $\pm$ S.E.M. In imaging experiments where up to 30 cells could be monitored in a single experiment, at least three experiments were run for any particular protocol.

Materials

Fura 2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.) or Calbiochem (Nottingham, Notts., U.K.). Calcium-Green-1 Dextran (M_r 10000), fura 2 (pentapotassium salt) and ryanodine were also from Molecular Probes. Cyclopiazonic acid (CPA), ionomycin (free acid) and caged GPIP₂ were from Calbiochem. Caffeine and histamine were from Sigma (Poole, Dorset, U.K.), $SrCl_2$ was from Fluka at puriss. grade and all other reagents were of analytical grade. Caged IP₃-5S was a gift from Dr. David Trentham (National Institute for Medical Research, Mill Hill, London, U.K.). Glass for micropipettes was obtained from Clark Electromedical Instruments (Reading, Berks., U.K.).

RESULTS

Cation influx maintains spiking frequency and amplitude

$[Ca^{2+}]_i$ spiking elicited by low histamine concentrations was well maintained in medium containing 1 mM Ca^{2+}_o (Figure 2A) but ran down in Ca^{2+} -free medium (Figure 2B). We measured the rate of run down by plotting the interspike periods (ISP) against the time at the end of the ISP and calculated the slope for each individual cell $[d(ISP)/dt]$; a positive value indicated run down. Cells were only included in the analysis if they were true baseline spikers and showed three or more spikes during 10 min of stimulation. In 1 mM Ca^{2+}_o , spiking was sustained $[d(ISP)/dt = -0.02 \pm 0.04, n = 70, P > 0.1]$, whereas in Ca^{2+} -free medium oscillations ran down $[d(ISP)/dt = 0.15 \pm 0.01, n = 99, P < 0.002]$ compared with control. This run down occurred despite the fact that stores still responded to a maximal concentration of histamine (10 μ M). Indeed, the size of the internal stores assessed with 10 μ M histamine was no different after spiking in Ca^{2+} -free compared with Ca^{2+} -containing medium ($\Delta R_{350/380}$: in 1 mM Ca^{2+}_o , $1.48 \pm 0.05, n = 76$ cells; in Ca^{2+} -free medium $1.49 \pm 0.07, n = 133$ cells, $P > 0.9$). This suggests that a low dose of agonist can only release a fraction of the intracellular stores, as has been previously observed in HUVECs [2]. A paradox therefore exists in HUVECs where spiking slows down or ceases despite the fact that stores appear full of Ca^{2+} . It is this paradox that we have set out to address. If $[Ca^{2+}]_o$ is restored to 1 mM, spiking returns [2], and, as shown below, Sr^{2+}_o can also restore spiking. Before we used the degree to which these spikes were spikes of Sr^{2+} rather than Ca^{2+} as a means of resolving homogeneous and heterogeneous mechanisms, we investigated the nature of store refilling with Sr^{2+} and its subsequent release.

Figure 2 Single-cell records of $[Ca^{2+}]_i$ spiking in the presence and absence of Ca^{2+}

(A) Maintained spiking in medium containing 1 mM Ca^{2+}_o , (B) spiking running down in Ca^{2+} -free medium. The size of the internal Ca^{2+} stores is indicated by the maximal histamine (his) response at the end of the trace. Records shown are representative of at least 50 cells.

Sr^{2+} refills and is released from internal stores

Sr^{2+} can enter HUVECs and lead to refilling of the intracellular stores (Figure 3). Ca^{2+} stores in patches of approx. 20 cells were emptied by exposure to 10 μ M histamine in Ca^{2+} -free medium and then, in the absence of agonist, were refilled by exposure to either Ca^{2+} or Sr^{2+} (both 5 mM). The $\Delta R_{340/380}$ response to the second histamine challenge was only $25.5 \pm 2.1\%$ ($n = 3$, Figure 3A) of the first response in control experiments when cells were not refilled with either Ca^{2+} or Sr^{2+} between challenges. After refilling with Ca^{2+} the second response increased to $89.7 \pm 5.2\%$ of the initial response ($n = 3, P > 0.1$; paired t test; Figure 3B). Refilling with Sr^{2+} also increased the second peak but by a lesser extent to $46.4 \pm 2.7\%$ ($n = 3, P < 0.01$; paired t test; $P < 0.01$ compared with control; Figure 3C). Given the approx. 30-fold lower affinity of fura 2 for Sr^{2+} than for Ca^{2+} ([28,29] and the Materials and methods section), the second response could reflect a substantial Sr^{2+} release that is poorly reported by the fura 2 [28]. In similar experiments, exposure to either cation at 1 mM gave more variable but still significant results and 10 mM Sr^{2+} gave no greater an effect than 5 mM Sr^{2+} (results not shown). The ability of Sr^{2+} to refill stores in the absence of agonist is consistent with it entering via an SRCE pathway.

To confirm that the increased peak truly represented release of sequestered Sr^{2+} rather than Ca^{2+} displaced from either the cytosol or stores, we exploited the ability of ionomycin to translocate Ca^{2+} more efficiently than Sr^{2+} [30,44,45]. All results are expressed as a percentage of the initial histamine response in experiments performed on either patches of cells ($n = 3$) or single cells ($n = 3-4$). Stores were refilled with Ca^{2+} using a similar protocol to that in Figure 3 except that, after repletion, 1 μ M ionomycin was applied for 5 min before histamine to selectively release Ca^{2+} . Ionomycin depleted stores of Ca^{2+} so that histamine had little further effect ($7.3 \pm 1.4\%$, $n = 6$; Figure 4B). In non-

Figure 3 Effect of Sr²⁺_o and Ca²⁺_o on refilling of intracellular stores in the absence of agonist

Internal stores were maximally depleted by challenging for approx. 2 min with 10 μ M histamine (his) in Ca²⁺-free medium and then refilled by applying 5 mM extracellular Ca²⁺ (B) or Sr²⁺ (C) for 200 s or not refilled (A). Records were obtained in patches of approx. 20 cells and are representative of at least three experiments.

refilled control cells, both the ionomycin and histamine responses were small ($15.7 \pm 2.3\%$ and $6.8 \pm 1.1\%$ respectively, $n = 6$; Figure 4A). However, after exposure to Sr²⁺, the ionomycin response was no different from that in control cells ($21.7 \pm 3.7\%$, $n = 7$, $P > 0.2$), whereas the 10 μ M histamine response was greatly enhanced ($29.2 \pm 3.8\%$, $n = 6$, $P < 0.003$; Figure 4C). In other similar experiments, Sr²⁺ could also be discharged by 0.3–0.5 μ M histamine after ionomycin ($\Delta R_{340/380}$: control, 0.08 ± 0.04 , $n = 5$; Sr²⁺-refilled, 0.57 ± 0.13 , $n = 7$, $P < 0.01$ compared with control; Ca²⁺-refilled, 0.16 ± 0.05 , $n = 6$, $P > 0.2$ compared with control).

We also monitored F_{360} , which showed changes consistent with Sr²⁺ being released by agonist but not by ionomycin (Figure 4). As indicated in the Materials and methods section we analysed the regression between the percentage change in F_{360} and $R_{340/380}$ for each stimulus. The only instance where there was a large positive slope was when the above interpretation indicated that Sr²⁺ was being released; there was only an increase in slope for the second compared with the first histamine response (Δ

slope response) after Sr²⁺ refilling (Δ slope response $N = 6$ or 7: control, $+1.9 \pm 1.8\%$; Ca²⁺-refilled, $-4.0 \pm 2.9\%$, $P > 0.05$ compared with control; Sr²⁺-refilled, $+10.9 \pm 2.3\%$, $P < 0.01$ compared with control; one-tailed t tests). Ionomycin did not elicit a significant increase in F_{360} versus $R_{340/380}$ slope under any circumstance; the large negative regression slope in control cells was due to poor curve fitting over the longer time that the ionomycin took to peak when stores were emptied. In the equivalent experiments with 0.3–0.5 μ M histamine after ionomycin, a similar pattern was seen for the F_{360} regression, i.e. the slope on release of Ca²⁺ was $-3.0 \pm 0.5\%$ compared with $+14.4 \pm 2.3\%$ on release of Sr²⁺ ($n = 6$, $P < 0.002$; paired one-tailed t test).

Taken together, these data suggest that (a) F_{360} can be used to assess Sr²⁺ against Ca²⁺ release, (b) ionomycin and histamine deplete the same store, (c) Sr²⁺ can refill stores and then be discharged by histamine but not by ionomycin, (d) Sr²⁺ can enter stores that are sensitive to submaximal doses of histamine and (e) Sr²⁺ does not displace a significant amount of Ca²⁺ from ionomycin-inaccessible sites to make it available for release since Sr²⁺ does not potentiate the response to ionomycin. In addition, in patches of cells, refilling stores with Sr²⁺ after discharge by histamine in Ca²⁺-free medium did not inhibit store-regulated Mn²⁺ entry as measured by a first-order rate constant for quench at 360 nm corrected for the basal rate [37]; Sr²⁺ refilling did not inhibit the quench ($4 \pm 16\%$, $n = 8$) whereas Ca²⁺ refilling resulted in $75 \pm 8\%$ inhibition ($n = 7$, $P < 0.003$; one-tailed t test), again inconsistent with the sequestered ion being Ca²⁺ that had been made available by being displaced by Sr²⁺.

Sr²⁺ enters via a capacitative entry pathway

The entry of Sr²⁺ in the absence of agonist (Figures 3 and 4) implies entry via an SRCE pathway as has been previously shown in HUVECs for Ca²⁺ [37]. To demonstrate SRCE for Sr²⁺ more directly, we applied Sr²⁺ to unstimulated cells and after depletion of stores by the SERCA inhibitor CPA (30 μ M) [46,47]. Exposure to Sr²⁺ had no effect on $R_{340/380}$ in unstimulated cells but raised $R_{340/380}$ (Figure 5) when Ca²⁺ stores had been discharged by CPA ($\Delta R_{340/380} = 0.13 \pm 0.05$, $n = 8$, $P < 0.05$; one-tailed t test) although by less than the rise in $R_{340/380}$ on exposure to Ca²⁺ after CPA (0.43 ± 0.18 , $n = 7$, $P < 0.05$ compared with Sr²⁺). In addition, emptying stores with ionomycin also appeared to facilitate Sr²⁺ entry even though ionomycin is a poor Sr²⁺ ionophore (see below). Taken together, these data argue in favour of an SRCE pathway(s) in HUVECs that is not selective for Ca²⁺, in agreement with a previous report [48].

Spiking can be restored by Sr²⁺

In experiments using the photometric system, spiking was induced by submaximal histamine concentrations which, in Ca²⁺-free solution, slowed down or ceased. Addition of 5 mM Sr²⁺ restored spiking frequency ($n = 32$) and removal of Sr²⁺ led to its run down again (Figures 6 and 7A). In similar experiments using imaging, increasing the concentrations of Sr²⁺_o (3–10 mM) during histamine stimulation caused a dose-dependent increase in spiking frequency (Figure 7B), although there was cell-to-cell variability in sensitivity: of the 144 cells analysed, 50 showed an increase in frequency for each increase in [Sr²⁺]_o, 38 showed an increase for [Sr²⁺]_o ≥ 5 mM and 44 showed an increase for [Sr²⁺]_o = 10 mM only. In other words, 67% of Sr²⁺-sensitive cells were affected by 5 mM Sr²⁺. Only 12 cells showed no obvious effects of Sr²⁺ at any concentration. Furthermore Sr²⁺-dependent spiking continued for as long as Sr²⁺ was present (up to 10 min; results not shown).

Figure 4 Sr^{2+} is sequestered by intracellular stores and is released by histamine but not by ionomycin

After depletion of stores with $10 \mu\text{M}$ histamine (his), stores were refilled with either 1 mM Ca^{2+} (B) or 5 mM Sr^{2+} (C) or not refilled (A). Ca^{2+} was selectively mobilized by $1 \mu\text{M}$ ionomycin (iono), and the remaining Sr^{2+} released by $10 \mu\text{M}$ histamine. (A) to (C) each show $R_{340/380}$ (top panel), corrected F_{360} (middle panel) and corrected F_{360} versus $R_{340/380}$ (bottom panel). The axes for the bottom panel, although extending over different limits and therefore differently labelled, are all plotted using a common scale so that the slopes are directly comparable. (D) Slopes (means $\pm$ S.E.M.) for percentage in change F_{360} versus $R_{340/380}$: $\square$, Control; $\square$, Ca^{2+} ; $\blacksquare$, Sr^{2+} . Graphs shown are representative of six or seven experiments.

We thought at first that these spikes were Ca^{2+} spikes, since they appeared to be similar in size, shape and duration to those seen in the absence of Sr^{2+} . However, examination of F_{360} showed variable contributions of Sr^{2+} and Ca^{2+} to the fluorescence signal. In all cells, the initial spike after addition of Sr^{2+} was predominantly Ca^{2+} since the F_{360} versus $R_{340/380}$ slope did not change significantly (mean slope before Sr^{2+} : $-2.6 \pm 0.5\%$; first spike with Sr^{2+} : $-2.1 \pm 1.2\%$, $n = 32$, $P > 0.6$; paired t test). As spiking continued in the presence of Sr^{2+} , the contribution of Sr^{2+}

increased markedly in some experiments (18 of 32 cells), confirmed by the gradual appearance of a positive F_{360} spike coincident with each $R_{340/380}$ spike. This phenomenon was variable and not as marked in 14 of the cells studied. Figures 6 and 7(A) show two extremes: in Figure 7(A) the cell responded to Sr^{2+} addition with continued Ca^{2+} spiking whereas in Figure 6 there is a gradual increase in the slope. The average slope for all cells was (for second, third and fourth spike): $+5.2 \pm 1.4\%$, $n = 32$; $+7.6 \pm 1.7\%$, $n = 23$; $+7.3 \pm 1.5\%$ ($n = 14$; all

Figure 5 Sr²⁺ and Ca²⁺ enter cells via an SRCE pathway

Single cells were exposed to 1 mM Ca²⁺ and 5 mM Sr²⁺ under basal conditions and after exposure to 30 μ M CPA in the absence of either ion. Reapplication of both cations evoked a significant change in fluorescence ratio. Trace typical of nine experiments.

Figure 6 Extracellular Sr²⁺ restores spiking with changes in F_{360} in some cells

Histamine (his)-stimulated [Ca²⁺]_i spiking was allowed to run down in Ca²⁺-free medium and then 5 mM Sr²⁺ was added. The top panel shows $R_{340/380}$, middle panel shows the corrected F_{360} and the bottom panel shows the change in F_{360} versus $R_{340/380}$ where the slope reflects the proportion of Sr²⁺ per spike. Horizontal bars labelled A–I indicate the period of time over which the lower regression plots were constructed: plots B–I use the same ratio scale as indicated for plot A but offset appropriately. The graph is representative of 18 experiments.

$P < 0.001$ compared with pre-Sr²⁺ spikes; paired t test. In the light of the results of Figure 4, these slopes are consistent with the later spikes containing substantial amounts of Sr²⁺. We could not discern a consistent relationship between the change in Sr²⁺ contribution and how the cell had responded before addition of Sr²⁺, i.e. it was independent of whether the oscillations had appeared to stop on removal of Ca²⁺.

Figure 7 Dose-dependent support of spiking by Sr²⁺, with little change in F_{360} in some cells

(A) Typical record of a cell not showing a marked increase in F_{360} spikes on Sr²⁺ addition. Histamine (his)-stimulated spiking runs down after Ca²⁺ withdrawal, but is restored by 5 mM Sr²⁺. (B) Cells were perfused in nominally Ca²⁺-free medium plus 100 μ M EGTA throughout the entire run. [Ca²⁺]_i spiking was stimulated by histamine, and then medium supplemented with 3–10 mM Sr²⁺ was added. Imaging was used for (B). Records are typical of 14 cells for (A) and 50 for (B).

Oscillations are not efficiently maintained by Ba²⁺

Sr²⁺ could be acting by competing with Ca²⁺ for binding sites thereby altering [Ca²⁺]_i by displacing bound Ca²⁺ as well as altering Ca²⁺ buffering; the degree of cytosolic Ca²⁺ buffering affects oscillations [49]. To test for this we repeated the oscillation experiments of Figures 6 and 7 using 2 or 5 mM Ba²⁺. Since Ba²⁺ is a poor Ca²⁺-ATPase substrate [28,29] and readily enters endothelial cells [28], its free ion concentration and therefore effects on Ca²⁺ displacement and buffering should be at least equal to, or even greater than, those of Sr²⁺. Although Ba²⁺ entry could be seen as a gradual rise in $R_{340/380}$, it did not affect oscillatory frequency. In control experiments, application of Sr²⁺ to cells spiking in response to 0.3 μ M histamine in Ca²⁺-free medium decreased the ISP by $75 \pm 6\%$ ($n = 7$). In contrast, application of Ba²⁺ did not decrease the ISP (2 mM Ba²⁺: $141 \pm 22\%$, $n = 3$; 5 mM Ba²⁺: $118 \pm 11\%$, $n = 13$), strength-

ening the argument against displacement and buffering. Ba^{2+} has been reported to enhance Ca^{2+} release from stores in oocytes [8] but the use of high Ba^{2+} (80 mM) in those experiments may have resulted in substantial displacement of Ca^{2+} from intracellular buffers, as argued by Marshall and Taylor [22], who saw no effect of Ba^{2+} on Ca^{2+} release in permeabilized cells. We conclude that substantially greater degrees of cation influx are required to 'indirectly' facilitate release by altering buffering or displacing Ca^{2+} than are observed in our experiments.

Spiking after Sr^{2+} addition to Ca^{2+} -depleted cells

Having established that Sr^{2+} could stimulate spiking in cells that still contained substantial amounts of stored Ca^{2+} , we then investigated whether Sr^{2+} could stimulate spiking in Ca^{2+} -depleted cells. In Ca^{2+} -free medium, stores were maximally depleted of Ca^{2+} with $10\ \mu\text{M}$ histamine for 2 min, and then exposed to $0.5\ \mu\text{M}$ histamine (Figure 8). Cells were then first exposed to Sr^{2+} or Ca^{2+} and then to the other cation. All ten cells exposed first to Sr^{2+} responded, with four cells showing repetitive baseline spiking (Figure 8A) and the other six showing a transient response. Of the seven cells exposed first to Ca^{2+} , all showed a sustained response in the presence of Ca^{2+} , which changed to spiking on removal of Ca^{2+} in four cells (Figure 8B).

Cells took substantially longer to respond to Sr^{2+} when stores were empty. Ca^{2+} -depleted cells responded to Sr^{2+} only after an extensive delay ($83 \pm 18\ \text{s}$, $n = 10$; Figure 8A), whereas the delay following the subsequent Ca^{2+} addition was only $17 \pm 2\ \text{s}$ ($n = 10$, $P < 0.005$ compared with Sr^{2+} ; paired t test). In contrast, Ca^{2+} -depleted cells responded (spiking or sustained) to a brief exposure to Ca^{2+} with a delay of $12 \pm 2\ \text{s}$ ($n = 7$; Figure 8B). If Sr^{2+} was then added to such cells, now with their stores substantially refilled with Ca^{2+} , a response occurred with a delay of $24 \pm 9\ \text{s}$ ($n = 7$, $P < 0.01$ compared with Sr^{2+} before Ca^{2+} , $P > 0.3$ compared with Ca^{2+}). These results indicate that stores need to contain significant amounts of Ca^{2+} in order to respond relatively promptly to Sr^{2+} .

Another difference between the responses to readdition of Sr^{2+} and Ca^{2+} was that the F_{360} versus $R_{340/380}$ slope when Ca^{2+} was added first ($-3.7 \pm 0.9\%$, $n = 7$) was significantly different from the slope during the first spike when Sr^{2+} was subsequently added in the same cell ($-0.4 \pm 0.8\%$, $P < 0.001$, paired t test), i.e. some Sr^{2+} was being released, but the negative slope suggested substantial amounts of Ca^{2+} were co-released. This was similar to Figures 5 and 6, as was the gradual build up of Sr^{2+} per spike; by the third spike, the F_{360} versus $R_{340/380}$ slope had increased to $+4.3 \pm 0.8\%$ in the four cells that spiked ($P < 0.01$; paired one-tailed t test).

On reversing the order of addition, application of Sr^{2+} to Ca^{2+} -depleted cells gave an initial $R_{340/380}$ spike accompanied by a more positive slope than the spike seen when Ca^{2+} was added first ($+1.2 \pm 1.2\%$, $n = 10$, $P < 0.01$ compared with Ca^{2+} first), i.e. some Sr^{2+} was released. However, this initial change in slope was not significantly different from that when Sr^{2+} was added after Ca^{2+} when stores were more full of Ca^{2+} ($P > 0.3$). Finally, exposure to Ca^{2+} after Sr^{2+} gave an F_{360} versus $R_{340/380}$ slope of $-0.5 \pm 1.7\%$ ($n = 9$).

There are two surprising features of these data. The first is the ability of Sr^{2+} to evoke a release from Ca^{2+} -depleted cells that appears to contain Ca^{2+} . The second is that the proportion of Ca^{2+} to Sr^{2+} in the signal generated by adding Sr^{2+} seems to be independent of whether the cells have been refilled with Ca^{2+} beforehand. The first feature probably reflects the release of the residual Ca^{2+} which is resequestered on removal of $10\ \mu\text{M}$ histamine (allowing a small reuptake into stores; note the small

Figure 8 Effect of store depletion on Sr^{2+} -stimulated spiking

Stimulating cells in Ca^{2+} -free medium for 2 min with $10\ \mu\text{M}$ histamine (his) depletes internal stores. A concentration of histamine normally producing spiking ($0.5\ \mu\text{M}$) was immediately perfused and then either Ca^{2+} (1 mM) or Sr^{2+} (5 mM) was added in the maintained presence of $0.5\ \mu\text{M}$ histamine where indicated. Lower traces show corresponding corrected F_{360} where dashed portions of the record indicate artifactual changes caused by poor polynomial fits used to correct the F_{360} trace (see the Materials and methods section). (A) Sr^{2+} added first; (B) Ca^{2+} added first. Each record is representative of at least seven experiments.

fall in $[\text{Ca}^{2+}]_i$ in Figures 3, 4 and 8) and can be re-released by histamine (Figure 3A). The second feature is tempered by standard errors equal to and double the mean values for the data being compared. In part these large standard errors arise from the difficulty in correlating changes in F_{360} and $R_{340/380}$ in the absence of the clearly defined short-term changes that occur during spiking. Indeed, comparing the slopes of F_{360} versus $R_{340/380}$ in those cells that spiked repetitively in response to Sr^{2+} , the slopes were $+3.7 \pm 0.7\%$ ($n = 4$) when Sr^{2+} was added first and $-1.9 \pm 0.8\%$ ($n = 4$) when Sr^{2+} was added second ($P < 0.002$). Thus in these cells, where we can more confidently interpret the F_{360} data, adding Sr^{2+} to cells with Ca^{2+} -filled stores produced fluorescence ratio changes that were indicative of a

Figure 9 Restoration of the second response to submaximal histamine concentration by refilling stores with Sr²⁺ or Ca²⁺

Internal stores were first partially depleted by a 5 min exposure to 0.3–0.5 μ M histamine (his) in Ca²⁺-free medium. Depletion of stores is indicated by a smaller response to a second challenge with 0.3–0.5 μ M histamine (A). Refilling stores by exposing cells to 1 mM Ca²⁺ (B) or 5 mM Sr²⁺ (C) between stimuli restores the second response. Traces are representative of at least 13 experiments.

higher Ca²⁺ content than those seen when Sr²⁺ was added to Ca²⁺-depleted cells.

Sr²⁺ can resensitize release by low doses of histamine by refilling internal stores

Up to this point, Sr²⁺ had only been applied during submaximal stimulation, and so we tested whether Sr²⁺ could resensitize partially depleted stores by refilling between stimuli. The protocol we adopted was to stimulate cells in Ca²⁺-free medium with 0.3–0.5 μ M histamine for 5 min to partially empty the stores. After a histamine-free period of 350 s, cells were rechallenged with the same concentration of agonist and the peak response measured as a percentage of the first. In cells with no refilling between stimuli (Figure 9A), the second challenge was significantly reduced ($49.9 \pm 7.8\%$, $n = 17$, $P < 0.0001$; paired t test), consistent with a loss of sensitivity and/or intraluminal Ca²⁺. As expected, refilling internal stores with Ca²⁺ resulted in

Figure 10 Sr²⁺ does not potentiate ionomycin-induced Ca²⁺ release

Stores were challenged in Ca²⁺-free medium by 0.4 μ M ionomycin (iono) resulting in partial discharge as indicated by the further response to 10 μ M histamine (his) (A) or 2.5 μ M ionomycin (B and C). Application of 5 mM Sr²⁺ for 5 min did not lead to any further 0.4 μ M ionomycin-induced Ca²⁺ release (C). However, the final 10 μ M histamine spike was larger in Sr²⁺-treated cells (C) than in controls (B). Traces show records typical of at least three experiments.

a second peak that was not significantly different from the first ($90.4 \pm 8.0\%$, $n = 13$, $P > 0.4$; Figure 9B), a result mimicked when Sr²⁺ was used instead to refill stores ($74.8 \pm 3.7\%$, $n = 14$, $P > 0.05$ compared with Ca²⁺; Figure 9C).

Sr²⁺-induced Ca²⁺ release requires elevated IP₃ levels

Given then that Sr²⁺ application results in enhanced liberation of Ca²⁺, we then examined the underlying mechanism by investigating whether Sr²⁺ could enhance release in non-agonist-stimulated cells. Cells were challenged with a submaximal dose of ionomycin which only partially depletes stores of Ca²⁺, as confirmed by the subsequent 10 μ M histamine response (Figure 10A). However, even a 5 min exposure to Sr²⁺ failed to enhance release of Ca²⁺, as judged by either the unchanged plateau ($P > 0.5$ compared with controls without Sr²⁺; $n = 7$; Figure 10C) or the same subsequent response to 2.5 μ M ionomycin

Figure 11 Effect of Ca^{2+}_o and Sr^{2+}_o on $[\text{Ca}^{2+}]_i$ spiking stimulated by photolytic release of caged IP_3 analogue

Cells were microinjected with the caged analogue together with Calcium-Green-1 Dextran, and test pulses of $10 \mu\text{M}$ histamine (indicated by thick bars below the trace) applied at the beginning and the end of the run in $1 \text{ mM } \text{Ca}^{2+}_o$ to determine maximal responses. Photolysis was initiated by applying light at $360\text{--}380 \text{ nm}$ ($h\nu$) which elicited $[\text{Ca}^{2+}]_i$ spikes. Removal of Ca^{2+}_o slowed or stopped the spiking which was restored by addition of $5 \text{ mM } \text{Sr}^{2+}_o$ or readdition of $1 \text{ mM } \text{Ca}^{2+}_o$. Trace shown using caged $\text{IP}_3\text{-5S}$ typical of four cells with $\text{IP}_3\text{-5S}$ and two with GPIP_2 .

($\Delta R_{340/380}$: control, 0.38 ± 0.05 ; post- Sr^{2+} , 0.34 ± 0.03 , $n = 3$, $P > 0.5$). To confirm that Sr^{2+} had indeed entered the cell and had been sequestered into stores, we again took advantage of the selective mobilization of Ca^{2+} by ionomycin (see Figure 4). Having released all the residual Ca^{2+} with $2.5 \mu\text{M}$ ionomycin (Figures 10B and 10C), there was only a substantial response to histamine in Sr^{2+} -treated cells ($\Delta R_{340/380}$: control, 0.26 ± 0.07 ; Sr^{2+} -treated cells, 0.64 ± 0.02 , $n = 3$, $P < 0.01$; Figure 10B compared with 10C), reflecting the discharge of sequestered Sr^{2+} . Hence, Sr^{2+} enters HUVECs via SRCE, after ionomycin-induced store depletion (compare with HUVEC cell line ECV304 [50]).

Although ionomycin clearly stimulates Sr^{2+} entry with no discernible effect on Ca^{2+} release, it could be argued that this protocol far from mimics the conditions prevailing during $[\text{Ca}^{2+}]_i$ spiking, either in terms of the nature of the stimulus or the monophasic $[\text{Ca}^{2+}]_i$ response itself. We therefore undertook experiments to address this point and at the same time identify the site(s) of action of Sr^{2+} . Up to this point we had assumed that IP_3 levels were more or less constant during our manipulations. However, it may have been that Sr^{2+} -induced enhancement of spiking was partly a consequence of increases in IP_3 levels since Sr^{2+} influx could either modulate production [via phospholipase C (PLC); see the Discussion] or metabolism of IP_3 . IP_3 is metabolized by either the 5-phosphatase or 3-kinase, with the latter higher-affinity enzyme probably predominating at the lower levels of IP_3 that would be expected to generate spiking [51]. Although Sr^{2+} could conceivably affect the 3-kinase by interfering with calmodulin or protein kinase regulation (Sr^{2+} did not support inositol 1,3,4,5-tetrakisphosphate production in adrenal glomerulosa cells [52]), it is less clear how Sr^{2+} could modify 5-phosphatase activity.

Nevertheless, to probe these possibilities, we photolytically released either GPIP_2 or $\text{IP}_3\text{-5S}$ from their microinjected caged precursors, allowing us to circumvent PLC activation and minimize changes in inositol phosphate metabolism. Although it was difficult to titrate precise amounts of analogues to give repetitive spiking (we often observed a sustained Ca^{2+} signal

using continuous illumination, $n \geq 5$), spiking of $[\text{Ca}^{2+}]_i$ was evoked in response to release of these analogues in six cells (two with GPIP_2 and four with $\text{IP}_3\text{-5S}$; Figure 11); these spikes were similar in shape and frequency to those evoked by histamine in Calcium-Green-1 Dextran-injected cells (not shown), suggesting a common mode of generation. As expected, removal of Ca^{2+}_o inhibited analogue-induced spiking in all six cells. The response to Sr^{2+}_o was more variable with three of the six cells responding with restoration of spiking (one with GPIP_2 and two with $\text{IP}_3\text{-5S}$). This is consistent with the percentage of cells sensitive to $5 \text{ mM } \text{Sr}^{2+}_o$ (Figure 7). Subsequent removal of Sr^{2+} inhibited any spiking and then a further application of Ca^{2+}_o restored spiking in all six cells, indicating that the IP_3 analogue was still active (Figure 11). As a control for injection damage and/or potential differences between Calcium-Green-1 Dextran and fura 2, the fluorescence response to histamine in control microinjected cells was similar in the presence of Sr^{2+}_o to that in the presence of Ca^{2+}_o ; this similarity was seen irrespective of whether the response in the presence of Ca^{2+}_o was of the peak-and-plateau type or oscillatory (not shown, $n \geq 3$).

Lack of evidence for functional RyRs in HUVECs

These results underscore the importance of IP_3 in the generation of oscillations in HUVECs. Nevertheless, we directly tested for the participation of RyRs in agonist-induced Ca^{2+} mobilization in HUVECs particularly in the light of the recent observation that Sr^{2+} sensitizes the RyR [24]. In contrast with others [53], we did not find an effect of the RyR activator, caffeine (10 mM), on $[\text{Ca}^{2+}]_i$ ($\Delta R_{340/380} = 0.03 \pm 0.02$, $n = 7$, $P > 0.1$). In addition, ryanodine itself ($0.05\text{--}10 \mu\text{M}$) did not lead to a substantial change in $[\text{Ca}^{2+}]_i$ in either the presence ($n = 7$, $P > 0.1$) or absence ($n = 3$, $P > 0.05$) of 10 mM caffeine, nor did $10\text{--}100 \mu\text{M}$ ryanodine concentrations affect $0.3 \mu\text{M}$ histamine responses over a period of $110\text{--}350 \text{ s}$ ($n = 8$ for $10 \mu\text{M}$; $n = 2$ for $100 \mu\text{M}$); our $2 \mu\text{M}$ ryanodine preparation gave a broad spike in some pancreatic β -cell preparations (K. C. Pedley, personal communication). The lack of an effect of micromolar ryanodine concentrations in HUVECs is supported by a previous report [54], although the authors noted some effects when Ca^{2+}_o was raised to 5 mM . However, we infer that with $1 \text{ mM } \text{Ca}^{2+}_o$ (and normal store loading) RyRs have little role in regulating Ca^{2+} in HUVECs.

DISCUSSION

The central question that we set out to answer is why HUVECs eventually stop spiking when challenged with submaximal histamine in Ca^{2+} -free solution. After spiking has stopped, a maximal dose of histamine still produces a substantial response, indicating that most of the IP_3 -releasable Ca^{2+} is still present (Figure 2). However, others have shown that submaximal agonist may only be capable of discharging a subpopulation of the IP_3 -sensitive Ca^{2+} stores [9,10], which is small enough to escape detection when stores are probed by maximal agonist; it may be the complete emptying of this subpopulation of the heterogeneous stores that leads to cessation of Ca^{2+} spiking in Ca^{2+} -free solution.

Stores still contain accessible Ca^{2+} when spiking runs down

We have found that spiking does not cease because of the complete depletion of subpopulation since, when Sr^{2+} restores

spiking, the initial spikes still predominantly consist of Ca²⁺. This was indicated by (a) the normally prompt response to Sr²⁺ in contrast with the prolonged delay when stores are depleted of Ca²⁺ and (b) the changes in F_{360} versus $R_{340/380}$ slope, which show that the initial phase of the Sr²⁺-induced spiking comprises [Ca²⁺]_i spikes.

Potentially these effects of Sr²⁺ could be due to Sr²⁺ displacing Ca²⁺. However, experiments with ionomycin do not reveal the presence of a substantial amount of displaced Ca²⁺ (Figure 4). Also, exposing cells to Ba²⁺ did not mimic Sr²⁺ even though Ba²⁺ can displace Ca²⁺ without affecting Ca²⁺ release *per se* [22]. This means that one or more Ca²⁺ spikes can be evoked by Sr²⁺ without refilling Ca²⁺ stores with Ca²⁺, strongly suggesting that Sr²⁺ is acting to resensitize the spiking mechanism. Thus the slowing down or cessation of spiking is not associated with the complete depletion of a subpopulation of stores, and the mechanism of spiking cannot be a simple heterogeneous mechanism with the all-or-none discharge of a subpopulation of stores.

A growing body of information has highlighted the role for [Ca²⁺]_i in positively modulating PLC activity [55]. Whether recruited from internal stores or from the extracellular medium, Ca²⁺ may enhance and sustain IP₃ accumulation during agonist stimulation [52,55,56]. In other words, repetitive spiking could run down simply as a result of a fall in [IP₃] [57]. However, the ability of Ca²⁺ and Sr²⁺ to promote spiking in the presence of poorly metabolizable IP₃ analogues indicates that they are acting either cytosolically or luminally at the level of the IP₃R and the Ca²⁺ stores, although we cannot rule out the possibility that, when stimulated by agonist, there may also be an additional effect of bivalent cation influx on PLC activity [58].

Influx may modulate sensitivity to IP₃ by maintaining luminal Ca²⁺ levels

Luminal Ca²⁺ has been reported to sensitize IP₃ receptors to IP₃ and to modulation by cytosolic Ca²⁺ [13,14,59], although this remains controversial [21,60], and it is this luminal effect that has been used to explain quantal release. Since Sr²⁺ enters the stores (Figures 3 and 4) and even spikes (Figures 6 and 8), it is feasible that Sr²⁺ restores spiking by entering the lumen and sensitizing IP₃ receptors to a given [IP₃]. Evidence for this comes from experiments where partially emptied stores were resensitized to a second application of histamine by refilling with Sr²⁺ between stimuli (Figure 9). Removal of Sr²⁺ 50 s before the second application leads to a rapid fall in cytosolic Sr²⁺ caused by the PMCA (note the rapid run down of spiking when Sr²⁺ is removed, e.g. Figure 7). Thus the sensitization by Sr²⁺ refilling is probably due to its presence luminally. Consistent with this is the delay between addition of Sr²⁺ and stimulation of Ca²⁺ release in Ca²⁺-depleted cells (Figure 8), probably reflecting the time taken to refill stores to a threshold level. Any potential detection of luminal cation levels by the IP₃ receptor would have to be via a different sensor from that employed by the SRCE mechanism, since Sr²⁺ filling only poorly inhibited Mn²⁺ entry. This inability is consistent with a study in A7r5 cells [36], although others have suggested a partial inhibition of Mn²⁺ entry by refilling platelet stores with Sr²⁺ [33,34].

Considering that Sr²⁺ entry results in Ca²⁺ release during agonist stimulation, the simplest explanation (assuming a simple luminal action) would be that stores had not been completely emptied of all their Ca²⁺ and that Sr²⁺ entry restores luminal cation levels, which in turn resensitizes stores to ambient IP₃ levels. Indeed, Berridge [61] has proposed that the frequency and latency of oscillations depends on the degree of filling of the stores. This interpretation rules out a simple heterogeneous

mechanism for graded release since refilling of discrete empty stores with Sr²⁺ would not promote release of Ca²⁺. However, it is possible that the release cannot be categorized simply as homogeneous or heterogeneous; the mechanism for graded release may be a hybrid with some stores being more sensitive to IP₃ than others but each sensitive store only releasing a fraction of its stored Ca²⁺. Consistent with this is the fact that stores appear little depleted when probed globally with 10 μM histamine (Figure 2) after spiking has run down in Ca²⁺-free medium, but depletion is more evident when stores are reprobed by the same low histamine concentration (Figure 9). The simplest explanation for this is that the submaximal histamine concentration is preferentially probing stores that are particularly IP₃-sensitive. Thus our data also suggest some store heterogeneity. The possibility that Ca²⁺ release may be partly homogeneous and partly heterogeneous has recently been proposed [14]. Indeed, the observation that Sr²⁺ itself can eventually contribute to a spike (e.g. Figure 6) presumably reflects those cells where a subpopulation of stores was more extensively depleted of Ca²⁺ but some Ca²⁺ was still trapped [14]; the variable Sr²⁺ contribution suggests that there may be cell-to-cell differences in the degree of Ca²⁺ emptying when oscillations run down.

Does Ca²⁺ influx affect IP₃ receptors cytosolically?

Poorly metabolized analogues of IP₃ gave rise to repetitive spiking, strongly suggesting that the feedback processes at the IP₃ receptor itself underlie spiking [5–8,12,13,22,59] as opposed to fluctuating [IP₃] [57,62]. Depending on its concentration, cytosolic Ca²⁺ can potentiate or inhibit IP₃-induced Ca²⁺ release [5–7,12,22]; Sr²⁺ can potentiate release but its ability to inhibit release is controversial (cf. [23] and [8,21,22]). Facilitation of internal release by cation entry has been suggested previously, be it during activation of IP₃ receptors [8,63,64] or RyRs [65]. There is ample precedent then for Sr²⁺ entry to sensitize the IP₃ receptor without needing to refill stores and, indeed in oocytes, Sr²⁺ was suggested to act in this way [8]. Despite the appeal of such a mechanism, there is little substantive evidence from our study to confirm such a role for Sr²⁺ during spiking. That is not to say it does not occur. We cannot investigate this issue in intact cells since blocking Sr²⁺ uptake via SERCA leads to large changes in the regulation of bivalent cation movement that would mask any subtle effects.

Using F_{360} to distinguish between Ca²⁺ and Sr²⁺ oscillations

Some of our interpretation relies on the use of F_{360} to resolve the contributions of Ca²⁺ and Sr²⁺ to changes in $R_{340/380}$. Although the *in vitro* calibration is quite clear, there is the possibility that fura 2 could behave differently *in situ*. Several lines of evidence suggest that the F_{360} is reliable. First, the conclusions drawn using F_{360} in the experiments in Figure 4 are paralleled by the $R_{340/380}$ changes seen in response to histamine and ionomycin. Secondly, during Sr²⁺-induced spiking, the F_{360} response indicates that the spikes are of Sr²⁺ when cells are depleted of Ca²⁺ (Figure 8A) and are, at least initially, of Ca²⁺ when the stores are nearly full of Ca²⁺ (Figures 6, 7A, and 8B). Thirdly, after run down of spiking in Ca²⁺-free solution, addition of Sr²⁺ in some cells causes a graded reversible transition from spikes with a small negative slope for F_{360} versus $R_{340/380}$ to spikes with a larger positive slope (Figure 6); in other words, changes in F_{360} are not an artifact associated with particular cells. Although it would be desirable to use the F_{360} versus $R_{340/380}$ slopes to derive values for [Sr²⁺]_i, we cannot do this because of the shift between the *in vitro* and *in situ* spectra.

It is perhaps surprising that cells can exhibit spikes (Figures 6, 8 and 11) that, in terms of the simple $R_{340/380}$ or F_{495} signals, look very similar to pure Ca^{2+} spikes despite the fact that they might contain substantial amounts of Sr^{2+} . Because the affinity of fura 2 (or Calcium-Green-1 Dextran) for Sr^{2+} is some 30-fold lower than for Ca^{2+} , these $R_{340/380}$ (or F_{495}) spikes would have to be $[\text{Sr}^{2+}]_i$ spikes that are 30-fold larger than the $[\text{Ca}^{2+}]_i$ spikes if they were generated purely by release of Sr^{2+} . However, because of the *in vitro* to *in situ* shift in spectrum we cannot quantify the relative amounts of Ca^{2+} and Sr^{2+} in these spikes, but, if the spikes are substantially of Sr^{2+} , several factors may contribute to the similarity. First, the IP_3R channel has a greater conductance for Sr^{2+} than for Ca^{2+} [21], favouring a larger release. Secondly, the SERCA has a lower affinity for Sr^{2+} than for Ca^{2+} (estimates range from a 10- to an 80-fold difference [26,27,66]), also favouring a larger net release of Sr^{2+} and at the same time allowing the SERCA to respond to a larger increase in $[\text{Sr}^{2+}]_i$ with kinetics equivalent to its response to smaller increases in $[\text{Ca}^{2+}]_i$. In addition the buffering of Sr^{2+} may also be less than for Ca^{2+} , in line with the consistently lower affinities for Sr^{2+} than for Ca^{2+} reported above for fura 2, Calcium-Green-1 Dextran, SERCA and IP_3 receptor [22], so that a relatively large increase in $[\text{Sr}^{2+}]_i$ does not necessarily imply a relatively large release of Sr^{2+} . The conductance differential of the IP_3R for Sr^{2+} and Ca^{2+} was in part due to the inability of Sr^{2+} to inhibit its own release in this system [21]. It is difficult to explain the spiking behaviour of these cells without invoking feedback inhibition and so it is likely that sufficient Ca^{2+} is co-released with Sr^{2+} to provide this.

Much of our interpretation assumes that there is only an agonist-sensitive Ca^{2+} store, based on the lack of response to ionomycin after exposure to histamine in Ca^{2+} -free medium. It is possible that the discharge of stores by histamine is only partly due directly to IP_3 -induced Ca^{2+} release and that the remainder of the discharge is via some other mechanism. One possibility is CICR via RyRs , as has been reported for several non-excitable cells [3,4]. However, neither caffeine nor ryanodine stimulated Ca^{2+} release in HUVECs and ryanodine did not modulate histamine-induced Ca^{2+} release.

Role of Ca^{2+} entry during repetitive spiking in non-excitable cells

Although in HUVECs Ca^{2+} influx appears to maintain oscillations via an effect on store sensitivity, the influence of Ca^{2+}_o on oscillations varies according to cell type. For some cells such as HeLa [67], AR42J [68] and avian nasal gland cells [69], the presence of Ca^{2+}_o is an absolute requirement, with Ca^{2+}_o removal immediately inhibiting oscillations; for these cells there may be a more direct involvement of Ca^{2+} influx in the initiation of a spike [67,69]. Other cell types such as pancreatic acinar cells [18] and salivary gland cells [61] show a less immediate dependence on Ca^{2+}_o . There are also substantial differences in the fraction of Ca^{2+} released to form a spike and the fate of the Ca^{2+} that is released. In the avian nasal gland cells the spikes appear to be due to the release of the entire IP_3 -sensitive Ca^{2+} stores [69], with most of the released Ca^{2+} being sequestered by the end of the spike, whereas in pancreatic acinar cells a large fraction of the stored Ca^{2+} , up to 70 %, is pumped out of the cell during a spike [18]. Clearly these very different features imply different roles for Ca^{2+} influx in different cell types. A sensitizing effect of Ca^{2+} influx on release has been suggested for other cell types [8,61,63,64,67].

Although the issues addressed here can be examined more directly using permeabilized cells or vesicles, we were concerned that permeabilization may alter the behaviour of Ca^{2+} stores [16]. Our approach has therefore been to see what we could ascertain

in the intact single cell and how this correlates with the literature on permeabilized cells. In summary, we suggest that spiking runs down in Ca^{2+} -free medium because of a loss of sensitivity to IP_3 caused by partial depletion of Ca^{2+} stores and not because of complete depletion of a subpopulation of the stores. This is consistent with observations in permeabilized cells and vesicular preparations showing a modulatory effect of luminal Ca^{2+} on IP_3 sensitivity. Moreover the sensitivity can be restored not only by refilling with Ca^{2+} but also by refilling with Sr^{2+} . Our data are therefore more consistent with models of spiking that explain the limited release of Ca^{2+} during a spike by inactivation of release while the store still contains releasable Ca^{2+} rather than all-or-none release from a subpopulation of stores.

We thank Dr. David Ogden (NIMR, Mill Hill, London, U.K.) for very generously loaning flash photolysis equipment and Dr. Kevin Pedley at this Institution for many helpful discussions. Cells were kindly provided by fellow members of the Vascular Biology Research Centre. We also gratefully acknowledge the staff of St. Mary's Hospital, London for the supply of umbilical cords. This work was funded by the Wellcome Trust and the British Heart Foundation.

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