Evidence that agonists stimulate bivalent-cation influx into human endothelial cells

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Human umbilical-vein endothelial cells stimulated with thrombin or histamine show an increase in $[Ca^{2+}]_i$ (cytoplasmic free calcium concn.) that is maintained well above the basal pre-stimulated value as long as agonist and a source of extracellular Ca^{2+} are present. These results provide circumstantial evidence that agonists stimulate influx of Ca^{2+} across the plasma membrane and into the cytoplasm. Here, we have used Mn^{2+} as the extracellular bivalent cation which can bind to the fluorescent Ca^{2+} indicator fura-2 to quench its fluorescence completely. Human umbilical-vein endothelial cells were loaded with fura-2 and, in the presence of extracellular Mn^{2+} , thrombin and histamine were shown to cause quenching of the intracellular dye. This result demonstrates conclusively that agonists can stimulate the influx of bivalent cations. Stimulated discharge of Ca^{2+} from intracellular stores and influx of Mn^{2+} were temporally resolved in the same cells to show that release of Ca^{2+} from intracellular stores clearly precedes influx. Influx of Mn^{2+} was also demonstrated when extracellular Mn^{2+} was added after agonist at a time when $[Ca^{2+}]_i$ had fallen back to the basal value, showing that influx is not dependent on elevated $[Ca^{2+}]_i$.

INTRODUCTION

When endothelial cells (from bovine or pig aortae, or human umbilical vein) loaded with the fluorescent Ca^{2+} -indicator dyes quin2, fura-2 or indo-1, are stimulated with histamine, bradykinin, thrombin, adenine nucleotides or PAF, an increase in $[Ca^{2+}]_i$ is observed [1–7]. In most of these studies attempts were made to determine the source of the Ca^{2+} required to sustain these elevations in $[Ca^{2+}]_i$ either by removing extracellular Ca^{2+} or by using organic channel blockers such as D600, diltiazem or dihydropyridines. However, the results are somewhat variable. As it is becoming increasingly clear that membrane-potential gated Ca^{2+} channels in various cells differ widely in their sensitivity to these organic Ca^{2+} -channel blockers, it is perhaps unwise to base assumptions of whether or not agonist-stimulated Ca^{2+} -entry mechanisms exist in endothelial cells on results obtained by using these tools. All studies with fluorescent cytoplasmic indicator dyes have shown that the initial rise in $[Ca^{2+}]_i$ after stimulation with agonists is, at least partially, independent of the extracellular Ca^{2+} concentration, and is therefore presumably due to the discharge of Ca^{2+} from an intracellular store. In most studies, the initial agonist-stimulated transient rise in $[Ca^{2+}]_i$ is followed by a maintained elevated value. This maintained elevation in $[Ca^{2+}]_i$ appears to be dependent on the presence of extracellular Ca^{2+} , and is therefore assumed to be the result of a stimulated Ca^{2+} -entry mechanism [3,7]. There are exceptions, however, where the maintained rise in $[Ca^{2+}]_i$ is very small or absent; these observations are particularly apparent with ATP and PAF, receptors for which typically desensitize relatively rapidly (see e.g., [2]).

Results obtained by using intracellular dyes to measure $[Ca^{2+}]_i$ are only indirect evidence for the existence of a

stimulated Ca^{2+} -entry mechanism, and there could be alternative explanations for the maintained rise in $[Ca^{2+}]_i$. For example, the extent of intracellular store discharge could be, in some way, dependent on extracellular Ca^{2+} . Observations that the organic Ca^{2+} -channel blockers are much less effective, i.e. are much less potent, at blocking endothelial responses [8–10] than depolarization-induced smooth-muscle contraction suggest that either there is no stimulated Ca^{2+} entry into endothelial cells or that Ca^{2+} entry is via some other form of channel distinct from these voltage-operated Ca^{2+} channels. Some direct evidence for stimulated Ca^{2+} entry has come from $^{45}Ca^{2+}$ -uptake studies [11,12], and we have provided electrophysiological evidence that cation channels open under shear stress [13]. Here, we have used the fluorescent Ca^{2+} -indicator dye fura-2 to provide an alternative way of demonstrating thrombin- or histamine-stimulated bivalent-cation entry into human umbilical-vein endothelial cells. Hallam & Rink [14] showed that agonists could stimulate the entry of Mn^{2+} across the plasma membrane of quin2-loaded human platelets to quench the intracellular dye fluorescence. Since Mn^{2+} is added extracellularly and there are no dischargeable intracellular stores of a quenching cation, it can be concluded that agonists stimulate Mn^{2+} entry. Recent evidence that human platelets and rat parotid acinar cells have different mechanisms for agonist-stimulated bivalent-cation entry have been based on this technique [15].

METHODS

Fura-2 AM was from Molecular Probes Inc. (Eugene, OR, U.S.A.), and ionomycin was from Calbiochem. Bovine thrombin and histamine were from Sigma.

Endothelial cells were obtained as described previously

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; PAF, platelet-activating factor (1-*O*-alkyl-2-acetylglucyl-3-phosphocholine).

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Fig. 1. Effect of thrombin and histamine on $[Ca^{2+}]_i$.

(a) The top panels show the effect of thrombin (1 unit/ml) or histamine (100 μ M) on endothelial-cell $[Ca^{2+}]_i$ in the presence of 1 mM extracellular Ca^{2+} (trace marked '1-Ca') or in the absence of extracellular Ca^{2+} with 1 mM-EGTA added 1–2 min before stimulation (trace marked '0-Ca'). The traces also show the effect of removal of extracellular Ca^{2+} by addition of 2 mM-EGTA after the stimulus; 2 mM-Tris base was added simultaneously to off-set any change in pH. (b) The lower panels show the effect of mepyramine (a selective histamine H_1 -receptor antagonist) and hirudin (an inhibitor of the active site of thrombin that prevents the binding of thrombin to its receptor) on the maintained elevated $[Ca^{2+}]_i$ produced by stimulation with either thrombin (1 unit/ml) or histamine (100 μ M) in the presence of 1 mM extracellular Ca^{2+} .

[7], which was essentially as follows. The lumen of human umbilical-cord veins was filled with Medium 199 (Flow Laboratories) containing 0.5 mg of collagenase/ml and incubated for 10 min at 37 °C. Endothelial cells were collected by centrifugation at 100 *g* for 5 min, resuspended in Medium 199 containing 10% (v/v) foetal-calf serum, 10% newborn-calf serum and 50 μ -units of penicillin and of streptomycin/ml and plated into 25 cm² flasks. When confluent (within 3–5 days) cells were detached by treatment with trypsin/EDTA in phosphate-buffered saline (Flow Laboratories) and re-seeded in complete medium on to 22 mm $\times$ 11 mm glass coverslips at a confluent density.

After 24 h the cells were loaded with fura-2 (see ref. [7]), and the coverslips containing confluent monolayers of cells were mounted across the diagonal of quartz cuvettes which were placed in the cuvette holder (thermostatically maintained at 37 °C) of a dual-excitation-wavelength Spex spectrofluorimeter. The cells were bathed and stimulated in a physiological saline consisting of 145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-Hepes, 10 mM-glucose, pH 7.4 at 37 °C; 1 mM-CaCl₂, 1 mM-EGTA, MnCl₂ or NiCl₂ were added to the cuvette as indicated and as specified in the Figures and their legends. In Fig. 1, $[Ca^{2+}]_i$ was calculated as previously described [7]. Cytoplasmic dye concentrations

were typically 50–100 μ M. All fluorescence records are typical of at least four others.

RESULTS

Fig. 1(a) shows the effect of thrombin (1 unit/ml) and histamine (100 μ M) on $[Ca^{2+}]_i$ in the presence or absence of extracellular Ca^{2+} (with 1 mM-CaCl₂ or 1 mM-EGTA added to the medium). Thrombin, as previously described [2,7], and histamine cause rapid elevations in human umbilical-vein endothelial-cell $[Ca^{2+}]_i$ from the resting value of around 100 nM to a peak of 2–3 μ M. The initial rise in $[Ca^{2+}]_i$ is only slightly smaller in the absence of extracellular Ca^{2+} , and is therefore presumed to be caused, at least in part, by the discharge of Ca^{2+} from an intracellular store. In the continuous presence of thrombin or histamine and absence of extracellular Ca^{2+} , $[Ca^{2+}]_i$ falls back to the resting value, but in the presence of extracellular Ca^{2+} $[Ca^{2+}]_i$ declines to a new maintained elevated value of 800–1000 nM. Fig. 1 also shows that the maintained elevated phase of the agonist-stimulated rise in $[Ca^{2+}]_i$ can be curtailed, and $[Ca^{2+}]_i$ rapidly returned to the pre-stimulated value, by removal of extracellular Ca^{2+} (addition of excess EGTA; 2 mM total), or by removal of the agonist (i.e. by addition of the thrombin ligand hirudin) or by blocking the effect of

Fig. 2. Effect of NiCl_2 on thrombin-stimulated changes in fura-2 fluorescence at 340 nm and 360 nm excitation

(a) Effect of thrombin (1 unit/ml) in the presence of 1 mM extracellular Ca^{2+} on fluorescence at 500 nm emission with 340 nm excitation in the top trace and 360 nm excitation in the lower trace. (b) In this experiment the endothelial cells were stimulated under the same conditions as in (a), except that 2 mM- Ni^{2+} was added where indicated. Ionomycin (1 μM) (from a 1 mM stock in dimethyl sulphoxide; final [dimethyl sulphoxide], 0.1 %) and MnCl_2 were added as indicated.

the agonist by using a specific antagonist (i.e. mepyramine specifically antagonizes the effect of histamine at the H_1 -receptor) (Fig. 1b).

Fig. 2 shows raw fluorescence traces (500 nm emission) at two excitation wavelengths (340 and 360 nm) from fura-2-loaded monolayers of confluent human umbilical-vein endothelial cells. Fig. 2(a) shows the effect of thrombin in the presence of 1 mM- Ca^{2+} . After the initial transient elevation in fluorescence with excitation at 340 nm (top trace), the signal remains elevated over basal. Addition of ionomycin, the bivalent-cation ionophore, further elevates $[\text{Ca}^{2+}]_i$, shown as a further increase in fluorescence at 340 nm. Subsequent addition of Mn^{2+} (1 mM) produces a rapid decline in fluorescence to well below the resting value; this is due to translocation

of Mn^{2+} across the plasma membrane by ionomycin, and quenching of the intracellular dye fluorescence by Mn^{2+} . This final value of quenched fluorescence is very close to the autofluorescence of the cells and the system (i.e. the fluorescence of the coverslips with monolayer of cells and the medium without any fura-2 present). Simultaneous measurement at 360 nm (lower trace) reveals no change in fluorescence on addition of thrombin or ionomycin; fura-2 fluorescence is insensitive to changes in $[\text{Ca}^{2+}]$ at this wavelength. This information is valuable, since it shows that there is no appreciable stimulated loss or leakage of dye (leaked dye would be diluted, which would decrease the fluorescence at 360 nm). Addition of Mn^{2+} in the continued presence of thrombin and ionomycin causes a rapid decrease in dye fluorescence as Mn^{2+} binds to fura-2 and quenches its fluorescence at all excitation wavelengths. In Fig. 2(b), a similar experiment is shown except that Ni^{2+} (2 mM) is added immediately before Ca^{2+} and thrombin. Under these conditions, the initial transient increase in $[\text{Ca}^{2+}]_i$ (fluorescence at 340 nm excitation) is not measurably affected, but the fluorescence trace (at 340 nm) then returns to the basal value, despite the continued presence of agonist and extracellular Ca^{2+} . No effect of Ni^{2+} is apparent on the fluorescence signal at 360 nm during stimulation. As Ni^{2+} normally quenches fura-2 fluorescence, this experiment suggests that Ni^{2+} cannot gain access to the cytoplasm of resting or stimulated endothelial cells. Addition of Mn^{2+} (3 mM) in the continued presence of ionomycin then quenches the dye fluorescence at both excitation wavelengths.

Fig. 3(a) shows the effect of thrombin (1 unit/ml) on fura-2 fluorescence at 340 and 360 nm excitation in nominally Ca^{2+} -free medium (i.e. in the absence of any extracellular added Ca^{2+} or chelator). Here $[\text{Ca}^{2+}]_i$ (fluorescence at 340 nm) transiently increases and then returns to the pre-stimulated value, presumably because the extracellular $[\text{Ca}^{2+}]$ is insufficient to support an influx. Fig. 3(b) shows the effect of addition of Mn^{2+} (100 μM) before thrombin. The fura-2 fluorescence at both 340 and 360 nm excitation begins to decline slowly after addition of extracellular Mn^{2+} , suggesting that Mn^{2+} is gradually leaking into the cells and quenching the fura-2 fluorescence. Addition of thrombin in the presence of Mn^{2+} (100 μM) results in a rapid increase in fluorescence at 340 nm (as Ca^{2+} is released from intracellular stores); however, the rise in fluorescence now appears smaller and shorter-lived than in the absence of Mn^{2+} , and then begins to decline rapidly to a final value well below the resting value. This thrombin-stimulated quench of fura-2 fluorescence is more clearly demonstrated at 360 nm, where the fluorescence is unaffected by concomitant changes in $[\text{Ca}^{2+}]_i$. Addition of ionomycin then has little further effect. This result demonstrates that thrombin can stimulate the quenching of fura-2 fluorescence in the presence of extracellular Mn^{2+} . Although this result is consistent with a stimulated uptake of Mn^{2+} across the plasma membrane, it is conceivable that stimulation of the cells may cause increased leakage of intracellular dye to the extracellular medium, which would have the same effect of quenching the dye fluorescence. However, (a) leakage of dye from cells in monolayer during stimulation would be diluted dramatically and would be very apparent as a loss of signal at 360 nm (Figs. 2 and 3a show that this is not the case), and (b) stimulation in the presence of extracellular

Fig. 3. Effect of extracellular Mn^{2+} on intracellular fura-2 fluorescence when cells are stimulated with thrombin

(a) Effect of thrombin (1 unit/ml) on the fluorescence of fura-2 with excitation wavelengths of 340 nm and 360 nm in the absence of any added $CaCl_2$ or chelator. (b) Effect of adding $MnCl_2$ (100 μM) to the medium before the addition of thrombin (1 unit/ml). Ionomycin (1 μM) was added where indicated. (c) A similar experiment but with the prior addition of 2 mM- $NiCl_2$. The concentrations of $MnCl_2$, thrombin and ionomycin were as stated above.

Ni^{2+} , which also quenches fura-2 fluorescence, has no effect on the fluorescence at 360 nm (Fig. 2b).

Fig. 3(c) shows the effect of Ni^{2+} at the same concentration that blocked the thrombin-stimulated maintained elevation in $[Ca^{2+}]_i$ (Fig. 2). Addition of Ni^{2+} (2 mM) in the presence of Mn^{2+} (100 μM) blocks the thrombin-stimulated quench in fluorescence (Fig. 3c). Under these conditions, the fluorescence is only quenched when ionomycin is subsequently added.

Fig. 4 shows stimulated Mn^{2+} entry experiments with

Fig. 4. Temporal resolution of thrombin- and histamine-stimulated quenching of intracellular fura-2 fluorescence by Mn^{2+}

Effect of thrombin (1 unit/ml; *a*) and histamine (100 μM ; *b*) on the fluorescence of fura-2 in the absence of added extracellular $CaCl_2$ or chelator but with 100 μM - $MnCl_2$ added where indicated. The continuous arrow between the traces in both panels marks the point of addition of agonist. In (*a*) the left-hand broken line shows the earliest detectable elevation in fura-2 fluorescence at 340 nm; the right-hand dashed line shows the earliest detectable decrease in fluorescence intensity at 360 nm excitation. The fluorescence intensities were measured at 100 ms time points.

thrombin and histamine as stimuli, but with an expanded time-base sampling fluorescence values every 100 ms. The top trace from each pair shows the $[Ca^{2+}]$ -sensitive 340 nm excitation signal, and the lower trace shows the 360 nm $[Ca^{2+}]$ -insensitive signal. Mn^{2+} (100 μM) was added extracellularly 1 min before the addition of agonist. When thrombin is added (Fig. 4*a*), the 340 nm trace increases 1–2 s after the addition of agonist and clearly precedes any decrease in the fluorescence signal detected at 360 nm excitation. This result shows that the elevation in $[Ca^{2+}]_i$ caused by the discharge of Ca^{2+} from the intracellular store precedes the stimulated quench of the dye caused by influx of Mn^{2+} through the bivalent-cation entry mechanism. It is clear that the onset of any response to thrombin occurs after a 1–2 s delay. With histamine (Fig. 4*b*), however, $[Ca^{2+}]_i$ rises to its peak value within the mixing time of about 0.5 s, and there is

Fig. 5. Effect of adding MnCl_2 after thrombin

(a) Fura-2 fluorescence with 340 nm and 360 nm excitation wavelengths where $100 \mu\text{M}$ - MnCl_2 was added after stimulation with thrombin (1 unit/ml). The experiment was performed without the addition of extracellular CaCl_2 or chelator. Ionomycin ($1 \mu\text{M}$) was added where indicated. (b) Results from a similar protocol but with the addition of 2 mM - NiCl_2 before thrombin.

no obvious delay before a stimulated Mn^{2+} influx can be detected. The resolution of the system is not adequate to determine whether histamine-stimulated release of Ca^{2+} from intracellular stores precedes influx. Although fura-2 is better than quin2 in rejecting Mn^{2+} , the $\text{Mn}^{2+}/\text{Ca}^{2+}$ preference is still 42-fold [16]. This makes it unlikely that the value of $[\text{Ca}^{2+}]_i$ will have significant effect on the onset or rate of Mn^{2+} quench. The delay in onset of the Mn^{2+} influx observed on stimulation with thrombin (Fig. 4a) cannot be attributable to the elevation in $[\text{Ca}^{2+}]_i$, because under similar conditions no delay is apparent, despite a similar elevation in $[\text{Ca}^{2+}]_i$, on stimulation with histamine (Fig. 4b).

Fig. 5(a) shows that Mn^{2+} influx can still be stimulated by thrombin when Mn^{2+} ($100 \mu\text{M}$) is added 2 min after the addition of thrombin; at this point $[\text{Ca}^{2+}]_i$ has returned to the basal value. This thrombin-stimulated Mn^{2+} entry is also blocked by Ni^{2+} (Fig. 5b), and the intracellular dye is only quenched on subsequent addition of ionomycin. The small response to ionomycin shows that the internal stores are substantially depleted after application of thrombin in Ca^{2+} -free medium.

DISCUSSION

The data presented here show that both thrombin and histamine cause a maintained elevated value of $[\text{Ca}^{2+}]_i$ in human umbilical-vein endothelial cells only in the presence of external Ca^{2+} . Rotrosen & Gallin [3] have previously shown a similar 'influx' component of the $[\text{Ca}^{2+}]_i$ response to histamine in quin2-loaded human umbilical-vein endothelial cells in suspension. However, using the same preparation, Jaffe and co-workers [5] failed to observe a maintained elevated $[\text{Ca}^{2+}]_i$ in response to thrombin. Our results support those of Rotrosen & Gallin [3], and dispell the notion that the two agonists are different in terms of their ability to elicit the maintained elevation in $[\text{Ca}^{2+}]_i$. A fundamental difference in the protocol of Jaffe and co-workers [5] was that their experiments were carried out at room temperature, whereas in Rotrosen & Gallin's study [3] and the present work all experiments were performed at 37°C . A maintained elevation in $[\text{Ca}^{2+}]_i$ is not necessarily a consequence of agonist-stimulated Ca^{2+} influx, as was discussed in the Introduction. However, the use of Mn^{2+} has allowed us to demonstrate more directly an agonist-stimulated bivalent-cation influx in response to both thrombin and histamine. Our data also clearly show that thrombin stimulation of the discharge of Ca^{2+} from intracellular stores precedes the influx of Mn^{2+} , and presumably therefore the influx of Ca^{2+} , across the plasma membrane. The response to histamine was generally faster, and we were not able to resolve any difference in the times of onset of the two responses. Since maximally effective concentrations of both histamine and thrombin were used, this difference may reflect faster binding of histamine to its receptor or more rapid signal transduction. We have also shown that thrombin can stimulate Mn^{2+} entry at a time when $[\text{Ca}^{2+}]_i$ has returned to the resting value. This result shows that bivalent-cation entry is not caused by the agonist-stimulated rise in $[\text{Ca}^{2+}]_i$.

The apparent lack of potency of the organic Ca^{2+} -channel blockers such as D600 [8] suggests that the mechanism for agonist-stimulated bivalent-cation entry in the endothelial cell may be through some form of entry mechanism distinct from the L-type voltage-operated channel. This is supported by observations made by us [1] and others [3,5] that the Ca^{2+} -channel agonist BayK 8644 or depolarization by high $[\text{K}^+]$ does not increase $[\text{Ca}^{2+}]_i$ in endothelial cells. The thrombin-stimulated rise in $[\text{Ca}^{2+}]_i$ was blocked by Ni^{2+} , which is consistent with the proposal that Ni^{2+} blocks Ca^{2+} entry. As expected, Ni^{2+} had no effect on discharge of Ca^{2+} from internal stores, showing that it does not interfere with binding of thrombin to its receptors or signal transduction. The same concentration of Ni^{2+} also blocked thrombin-stimulated Mn^{2+} influx. This result provides further evidence that Ca^{2+} and Mn^{2+} enter the cell by a similar mechanism.

Recently we have shown that agonists can stimulate Mn^{2+} influx into human platelets, but not parotid acinar cells [15]. This is of particular interest because, although there is evidence that the plasma membranes of both cells contain receptor-stimulated Ca^{2+} -entry mechanisms, the selectivity of these entry mechanisms appears to differ. Both cell types have also been analysed in terms of the kinetics of agonist-induced changes in $[\text{Ca}^{2+}]_i$ in the presence or absence of extracellular Ca^{2+} . Sage & Rink

[17] have shown that, in platelets, the rise in $[Ca^{2+}]_i$ stimulated by agonists in the presence of extracellular Ca^{2+} commences with less delay than that in its absence. This result suggests that agonist-evoked Ca^{2+} influx temporally precedes the discharge of Ca^{2+} from intracellular stores. However, in the parotid, studied by stopped-flow fluorimetry, there was no detectable difference in the delay period before stimulated elevations in $[Ca^{2+}]_i$ were observed [18]. That result is consistent with the model proposed by Putney [19], where stimulated influx occurs as a consequence of the partial or complete discharge of an intracellular store. Here we have shown that Mn^{2+} influx can be stimulated across the endothelial-cell plasma membrane, showing that the entry mechanism is similar, in this respect, to that in platelets. However, it appears (Fig. 4) that the thrombin-stimulated influx of Mn^{2+} occurs after discharge of Ca^{2+} from the intracellular store has commenced. This result is clearly different from stopped-flow studies with ADP-stimulated platelets, where Mn^{2+} entry precedes the discharge of Ca^{2+} from intracellular stores [20]. These results suggest that the Ca^{2+} -entry mechanism in endothelial cells is different again from that of both platelets and parotid acinar cells.

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