Influx of bivalent cations can be independent of receptor stimulation in human endothelial cells

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Stimulation of human umbilical-vein endothelial cells by agonists such as histamine or thrombin promotes an influx of Ca^{2+} , causing an increase in cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) that is dependent on the continued presence of both agonist and extracellular Ca^{2+} . This influx can also be clearly detected by using Mn^{2+} as a marker for Ca^{2+} entry, since Mn^{2+} quenches fura-2 fluorescence. The internal stores can be discharged in nominally Ca^{2+} -free solution by stimulation for a brief period by $100\ \mu\text{M}$ -histamine, with the stimulation being terminated by addition of $20\ \mu\text{M}$ of the H_1 antagonist mepyramine. After this (i.e. in the continuous presence of antagonist) a stimulated bivalent-cation influx can still be detected, as evidenced by the following observations: (a) addition of Mn^{2+} produces a stimulated quench, (b) addition of Ca^{2+} produces a transient rise in $[\text{Ca}^{2+}]_i$, (c) addition of 1 unit of thrombin/ml produces a much attenuated response unless the cells are exposed for a short period to 1 mM extracellular Ca^{2+} . These results imply that stimulated bivalent-cation influx may be a direct consequence of the discharge of the internal Ca^{2+} stores rather than a direct consequence of the presence of agonist.

INTRODUCTION

Evidence that inflammatory mediators such as histamine, thrombin, bradykinin, platelet-activating factor (PAF) and ATP can stimulate the influx of bivalent cations into the cytoplasm of endothelial cells comes from observations made by three techniques. First, $^{45}\text{Ca}^{2+}$ uptake can be stimulated by thrombin, bradykinin or histamine [1,2]. Second, currents carried by bivalent cations across cell-attached patches of bovine pulmonary-artery endothelial cells are stimulated by bradykinin or thrombin [3]. Third, measurement with fluorescent cytoplasmic Ca^{2+} indicators such as fura-2 show a prolonged elevation of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in the presence, but not in the absence, of extracellular Ca^{2+} , suggesting a stimulated influx of Ca^{2+} across the plasma membrane [4–8]. This last piece of evidence is circumstantial, but recently [8] we have shown that the fluorescent dye fura-2 can be used in conjunction with Mn^{2+} to demonstrate an agonist-stimulated bivalent-cation influx, a technique first used in quin2-loaded human platelets [9]. In human umbilical-vein endothelial cells, cytoplasmic fura-2 fluorescence was quenched after stimulation by histamine or thrombin in the presence of extracellular Mn^{2+} (a bivalent cation which quenches fura-2 fluorescence), suggesting that influx of Mn^{2+} had occurred by a mechanism analogous to that which normally translocates Ca^{2+} ions [8].

Although it is clear from these data that stimulated influx of bivalent cations can occur, the mechanism underlying the influx is somewhat obscure. If one sets aside voltage-operated channels or $\text{Na}^+/\text{Ca}^{2+}$ exchange, for which there is no evidence in agonist-stimulated responses in endothelial cells, there are several ways in which bivalent-cation influx can be evoked.

(a) Influx of Ca^{2+} may occur through a plasma-membrane channel that is itself part of, or is in close vicinity to, the receptor. The best evidence for such a mechanism, which is often referred to as a receptor-operated Ca^{2+} channel, is the ATP-stimulated Ca^{2+} influx in rabbit ear-artery smooth-muscle cells [10]. Here, the crucial experimental manipulation was to show that exposure of excised patches of plasma membrane to ATP opened a Ca^{2+} channel, suggesting that the receptor and channel are in close proximity and can still interact under conditions where it is unlikely that soluble second messengers could play a role.

(b) A second possibility is that agonist–receptor complexes cause the generation of a soluble second messenger, which in turn acts to open the channel. Irvine and colleagues [11] have suggested that inositol 1,3,4,5-tetrakisphosphate acting in-conjunction with inositol 1,4,5-trisphosphate could fulfil such a function.

(c) Both of the above mechanisms assume that bivalent-cation influx can be stimulated independently of, although probably concomitantly with, the discharge of Ca^{2+} from intracellular stores. This is not an unreasonable assumption if one considers that influx of Ca^{2+} into the human platelet appears to precede the discharge of Ca^{2+} from intracellular stores [12,13]. However, Putney [14] has described a model of Ca^{2+} influx in parotid acinar cells, further substantiated by work with dyes [15], where influx is dependent on the state of the intracellular Ca^{2+} store and is only evoked to re-fill this store. In this case it would be expected that influx would follow the discharge of Ca^{2+} from intracellular stores. There is some evidence to suggest that this is the case in endothelial cells, because after thrombin stimulation there is a time lag of 2–3 s between the initial release of Ca^{2+} from internal stores and the stimulated bivalent-cation

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

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entry monitored by the stimulated quench of fura-2 fluorescence by Mn^{2+} .

In this paper we have manipulated the extracellular Ca^{2+} and Mn^{2+} concentrations to determine whether stimulated bivalent-cation influx requires the continuous presence of agonist (i.e. histamine), or whether depletion of the intracellular stores is a sufficient stimulus alone.

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 [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 M199 containing 10% (v/v) foetal-calf serum, 10% newborn-calf serum and 50 μ -units of penicillin/ml and 50 μ g 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) 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 glass 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 and 10 mM-glucose, pH 7.4 at 37 °C. CaCl_2 , EGTA or MnCl_2 was added to the cuvette as indicated and as specified in the Figures and their legends. In Figs. 1 and 4 $[\text{Ca}^{2+}]_i$ was calculated as previously described [7,8]. 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 elevation of $[\text{Ca}^{2+}]_i$ in response to a maximally effective dose of histamine (100 μ M) in the presence of 1 mM extracellular Ca^{2+} . $[\text{Ca}^{2+}]_i$ increased to a peak of nearly 3 μ M and then declined to a maintained value of around 1 μ M in the continuous presence of histamine and extracellular Ca^{2+} . Removing extracellular Ca^{2+} by adding EGTA caused a rapid decline back to the initial basal value. Stimulation of cells with the same dose of histamine in the absence of extracellular Ca^{2+} (Fig. 1b) caused a transient increase in $[\text{Ca}^{2+}]_i$, owing to discharge of Ca^{2+} from intracellular stores, which then returned towards the basal pre-stimulated value. The maintained elevated $[\text{Ca}^{2+}]_i$, normally observed in the presence of extracellular Ca^{2+} , could be reconstituted simply by re-adjusting the extracellular Ca^{2+} concentrations back to near-physiological, i.e. 1 mM. Fig. 1(c) shows the effect of adding 20 μ M-mepyramine to antagonize completely the H_1 -receptor after histamine had completely discharged the intracellular pool of Ca^{2+} and $[\text{Ca}^{2+}]_i$ had returned to near the basal value. (See Figs. 3b and 4b for confirmation that 20 μ M-mepyramine

Fig. 1. Histamine-evoked $[\text{Ca}^{2+}]_i$ changes: role of extracellular Ca^{2+}

(a) Effect of stimulation by a maximal dose of histamine (100 μ M) in the presence of 1 mM extracellular Ca^{2+} . The plateau is abolished on removal of extracellular Ca^{2+} by addition of 2 mM-EGTA with 2 mM-Tris base to offset any change in pH. $[\text{Ca}^{2+}]_i$ is calculated from the ratio of the fluorescence intensities measured at 500 nm emission with 340 and 380 nm excitation. (b) Effect of stimulation by 100 μ M-histamine in the absence of extracellular Ca^{2+} (with 1 mM-EGTA). There is no plateau after the peak, but the plateau can be restored by adding back 2 mM- Ca^{2+} . (c) Effect of adding back extracellular Ca^{2+} (2 mM) after a brief period of stimulation by 100 μ M-histamine in the absence of extracellular Ca^{2+} (with 1 mM-EGTA). The stimulation was terminated before the addition of Ca^{2+} by addition of 20 μ M of the H_1 -receptor antagonist mepyramine.

Fig. 2. Histamine stimulates Mn²⁺ influx

(a) Raw fluorescence data, measured at 340 nm and 360 nm, showing little quench in the presence of 100 μ M extracellular Mn²⁺ until the cells are stimulated by 100 μ M-histamine. The experiment was performed in nominally Ca²⁺-free medium with no added EGTA. Ionomycin, 1 μ M (from a 1 mM stock in dimethyl sulphoxide; final [dimethyl sulphoxide] 0.1%), was added as indicated. (b) Raw fluorescence data showing that stimulated entry of Mn²⁺ persists after Ca²⁺ has been discharged from the internal stores.

Fig. 3. Effect of mepyramine on histamine-stimulated Mn²⁺ influx

(a) Raw fluorescence data showing that a stimulated influx of Mn²⁺ (100 μ M added) after 100 μ M-histamine in nominally Ca²⁺-free solution persists after the action of histamine has been blocked by 20 μ M-mepyramine. (b) Raw fluorescence data obtained in nominally Ca²⁺-free medium showing that 20 μ M-mepyramine completely antagonizes the ability of 100 μ M-histamine to promote Mn²⁺ influx. Subsequent addition of 1 unit of thrombin/ml stimulates a release of Ca²⁺ from internal stores and promotes Mn²⁺ entry.

completely antagonizes 100 μ M-histamine.) Mepyramine caused a small but immediate decrease in [Ca²⁺]_i. This suggests that most of the Ca²⁺ normally stored in the intracellular store has been pumped out of the cell, but that, for example, a small amount is left recycling between the store and the cytoplasm, or the plasma-membrane Ca²⁺-ATPases are less active in the continued presence of histamine. On adding back Ca²⁺ to the medium, [Ca²⁺]_i rose immediately, reached a peak and then declined back towards the resting value. Because this transient was evoked in the presence of mepyramine, it suggests a Ca²⁺-influx mechanism which is not dependent on continuous stimulation of the receptor by agonist.

Confirmation that influx of bivalent cations is occurring through the plasma membrane under these conditions can be obtained by detecting the stimulated entry of the extracellular quenching ion, Mn²⁺, as shown in Fig. 2. Fig. 2(a) shows raw fluorescence intensity traces at 340 nm and 360 nm excitation. Addition of 100 μ M-Mn²⁺ to the cuvette in the absence of extracellular Ca²⁺ caused a slow decrease in fluorescence intensity at both wave-

lengths, owing to a slow influx of Mn²⁺, which quenches the cytoplasmic dye. Addition of 100 μ M-histamine caused a rapid increase in fluorescence intensity at 340 nm, showing that [Ca²⁺]_i has increased. Because there is insufficient extracellular Ca²⁺ to support the influx of Ca²⁺, this [Ca²⁺]_i transient must be caused by the discharge of Ca²⁺ from intracellular stores. Simultaneously (within the time resolution of this technique), the fluorescence intensity at 360 nm rapidly decreased, suggesting that histamine stimulated a Mn²⁺ influx. The 340 nm trace is also quenched. Fig. 2(b) shows the effect of adding Mn²⁺ after addition of histamine. As expected, addition of Mn²⁺ in the continuous presence of histamine caused a rapid quench of the intracellular fluorescence, indicating that the stimulated bivalent-cation entry mechanism remains active. Fig. 2(b) is also a control for Fig. 2(a), showing that histamine does not stimulate a quench in the absence of extracellular Mn²⁺.

Fig. 3(a) shows the effect of adding Mn²⁺ in an experiment analogous to that shown in Fig. 1(c). Mepyramine (20 μ M) was added to block completely the

Fig. 4. Effect of re-filling internal Ca^{2+} stores

(a) Stimulation by $100\ \mu\text{M}$ -histamine in Ca^{2+} -free medium (1 mM-EGTA) almost completely discharges the agonist-releasable internal Ca^{2+} store, since subsequent addition of 1 unit of thrombin/ml causes virtually no response. (b) The control experiment for (a), showing that in the absence of any prior discharge, 1 unit of thrombin/ml produces a normal response. The ability of $20\ \mu\text{M}$ -mepyramine to antagonize $100\ \mu\text{M}$ -histamine is also demonstrated. (c) In Ca^{2+} -free medium a brief stimulation by $100\ \mu\text{M}$ -histamine (curtailed by $20\ \mu\text{M}$ -mepyramine) is sufficient to deplete the internal store, since subsequent stimulation by 1 unit of thrombin/ml produces virtually no response. (d) The same protocol as in (c), but with a period of raised extracellular Ca^{2+} produced by first adding 2 mM- Ca^{2+} to the medium (already containing 1 mM-EGTA) and then 2 mM-EGTA. Associated with this period is a $[\text{Ca}^{2+}]_i$ transient. The subsequent addition of 1 unit of thrombin/ml now produces a normal response, indicating that the internal stores had been refilled.

effects of histamine before adding Mn^{2+} . Clearly, stimulated Mn^{2+} influx could be monitored under conditions where the H_1 -receptor had been functionally blocked. These experiments suggest that the endothelial cell has some form of 'memory' that remains after removal of the stimulation to keep the influx mechanism activated. The transient nature of the stimulated influx on adding back Ca^{2+} under these conditions (cf. Fig. 1b) suggests that the influx may somehow be related to the state of the intracellular store. The ability of $20\ \mu\text{M}$ -mepyramine to block completely the action of histamine is shown in Fig. 3(b), where its application before that of $100\ \mu\text{M}$ -histamine blocked both the initial store release of Ca^{2+} and the stimulated Mn^{2+} entry. Subsequent addition

of 1 unit of thrombin/ml confirmed that both these mechanisms could be stimulated in this preparation.

In Fig. 4, we tested (a) whether the intracellular store was emptied by treatment with $100\ \mu\text{M}$ -histamine and (b) whether the transient elevation of $[\text{Ca}^{2+}]_i$ on re-addition of extracellular Ca^{2+} in the absence of receptor stimulation was sufficient to re-fill the intracellular store completely. Fig. 4(a) shows that, after stimulation by $100\ \mu\text{M}$ -histamine for 7 min in the absence of extracellular Ca^{2+} , 1 unit of thrombin/ml caused only a small transient elevation in $[\text{Ca}^{2+}]_i$ compared with the control (Fig. 4b), suggesting that the intracellular stores that are normally mobilized by thrombin had been discharged by the prior stimulation with histamine. Fig. 4(c) shows that stimulation with histamine for only 2 min, during which time the transient elevation of $[\text{Ca}^{2+}]_i$ falls back to basal, is sufficient to deplete the intracellular store substantially. Stimulation with histamine was curtailed by addition of $20\ \mu\text{M}$ -mepyramine, which was able to block the effects of histamine completely (see Fig. 4b for control). Fig. 4(d) shows the effect of the same depletion protocol as in Fig. 4(c), but with the inclusion of a 3 min period in between the stimulations by histamine and by thrombin where the cells were exposed to 1 mM extracellular Ca^{2+} . As shown in Figs. 1(c) and 3(a), addition of Ca^{2+} to cells where the internal store was previously depleted appears to cause an elevation in $[\text{Ca}^{2+}]_i$ that is dependent on an influx of bivalent cations into the cell in the absence of agonist. This transient elevation of $[\text{Ca}^{2+}]_i$, and therefore presumably transient influx of Ca^{2+} , appears to be sufficient to refill the intracellular store completely, because stimulation with thrombin now caused a large transient elevation of $[\text{Ca}^{2+}]_i$ (in the absence of extracellular Ca^{2+}) similar to that observed in controls (Fig. 4b).

DISCUSSION

The data presented in this paper provide clear evidence for the first time of a bivalent-cation flux into human endothelial cells under conditions where there is no stimulation of cell-surface receptors. The idea that Ca^{2+} influx is only stimulated as a consequence of partial or complete emptying of intracellular stores is not a novel one. Perhaps the best and most widely cited example of this sort of influx mechanism is Putney's [14] model of Ca^{2+} -influx in parotid cells, a model further substantiated and refined by fura-2 measurements [15]. Parotid cells can be shown to re-fill their intracellular Ca^{2+} stores in the absence of an extracellular stimulus after exposure to carbachol followed by the antagonist atropine, analogous to the histamine and mepyramine treatment used here with endothelial cells [15]. However, there is a significant difference between endothelial and parotid cells. Here, on adding back Ca^{2+} after depleting the intracellular stores, there is a significant increase in $[\text{Ca}^{2+}]_i$, suggesting that the re-filling of the intracellular stores might occur by sequestration of Ca^{2+} from the cytoplasm. However, in parotid cells only a very small increase in $[\text{Ca}^{2+}]_i$ is observed on adding back Ca^{2+} under these conditions [15]. These observations in parotid have led to the proposal that influx of Ca^{2+} occurs either directly into the intracellular store or into a restricted space bounded by the plasma membrane and intracellular membrane-bounded organelles that constitute the intracellular Ca^{2+} stores [14,15]; Ca^{2+} is pumped into the store from this

restricted space after influx across the plasma membrane, so that the influx of Ca^{2+} has little effect on $[\text{Ca}^{2+}]_i$.

A second important difference between endothelial cells and parotid cells is that stimulated influx of Mn^{2+} is not observed in parotid cells [16]; stimulation of rat parotid cells with either carbachol or substance P in the presence of extracellular Mn^{2+} failed to cause any significant quenching of intracellular fura-2. In this respect the influx of bivalent cations appears to be more reminiscent of that stimulated by thrombin, PAF and ADP in human platelets [9]. However, there are also clear differences between the controlling mechanisms for stimulated bivalent-cation influx into human platelets and endothelial cells. Sage *et al.* [13] have recently shown, by using stopped-flow fluorimetry, that the influx of Mn^{2+} into platelets clearly precedes the discharge of Ca^{2+} from intracellular stores on stimulation with ADP. This result is clearly the converse of what is observed on stimulating endothelial cells with thrombin, where influx of Mn^{2+} lags the discharge of Ca^{2+} from intracellular stores [8]. In platelets, then, it appears that, although Mn^{2+} influx can be stimulated, the influx is unlikely to be triggered by a partially depleted intracellular store, but is more closely linked to stimulation of receptor.

Modelling the mechanism underlying the influx of bivalent cations into human endothelial cells is complex. The results in Fig. 3(a) show that flux of bivalent cations into endothelial cells can occur in the absence of receptor stimulation, and those in Fig. 4(d) suggest that this influx is curtailed as soon as the intracellular store is re-filled. So influx is controlled not by the stimulation of cell-surface receptors but by the state of fullness (or depletion) of intracellular stores of Ca^{2+} .

Merritt & Rink [15] suggested in their modified Putney model of influx into parotid, where Ca^{2+} fluxes directly into the intracellular store, that Ca^{2+} concentrations inside the store feed back to switch off influx when the store has re-filled. The difficulty with applying this explanation to endothelial cells is that influx of Ca^{2+} (and of Mn^{2+}) appears to be via the cytoplasm, and one envisages certain problems with the necessary communication between the intracellular store and the plasma-membrane influx site.

One possible resolution of this conceptual problem is that influx of bivalent cations in endothelial cells might occur directly into the intracellular store, in close analogy with parotid, but that there is some overshoot of ions into the cytoplasm. Consistent with this are the results of

single-cell measurements of $[\text{Ca}^{2+}]_i$ on human endothelial cells [17], showing that, although many cells display an elevated $[\text{Ca}^{2+}]_i$ during re-filling of the intracellular stores on re-addition of extracellular Ca^{2+} , several cells displayed virtually no rise in $[\text{Ca}^{2+}]_i$. Clearly, more complete understanding of the mechanisms underlying bivalent-cation influx will come from single-cell observations.

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