Ionomycin enhances Ca^{2+} influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane

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In fura-2-loaded ECV304 cells ionomycin elicited a saturable biphasic change in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), where the initial phase represented mobilization of intracellular stores and the sustained component represented Ca^{2+} influx. To examine whether ionomycin could stimulate influx via a store-dependent mechanism, Mn^{2+} entry was monitored by the quenching of fura-2 fluorescence: influx was enhanced even after ionomycin wash-out, provided that internal stores were not refilled with Ca^{2+} . Moreover, the maximal rate of histamine-stimulated Mn^{2+} entry was unaffected by ionomycin, suggesting a common route of entry. The Ca^{2+} -entry blocker SK&F 96365 inhibited both the ionomycin-induced Mn^{2+} entry and the sustained $[\text{Ca}^{2+}]_i$ response to the ionophore (leaving the initial

peak $[\text{Ca}^{2+}]_i$ response unaffected). In other experiments, although addition of ionomycin further increased the plateau phase induced by 100 μM histamine, the increase was completely abolished by pretreatment with the store Ca^{2+} -ATPase inhibitor cyclopiazonic acid (CPA). Furthermore, in store-depleted cells, re-addition of 1 mM extracellular Ca^{2+} (in the presence of CPA plus histamine) led to a rapid rise in $[\text{Ca}^{2+}]_i$, dependent on Ca^{2+} influx, with kinetics that were not enhanced by ionomycin. These data suggest that ionomycin acts primarily at the level of the internal Ca^{2+} stores, so that, at the concentrations used here ($\leq 1 \mu\text{M}$), it increases Ca^{2+} (and Mn^{2+}) influx via activation of endogenous entry pathways and not by plasmalemmal translocation.

INTRODUCTION

Determining the key position that Ca^{2+} holds in controlling many cellular pathways has been greatly aided by the availability of Ca^{2+} ionophores such as A23187 and ionomycin. Given the earlier studies in cells and artificial membranes (Pressman, 1976; Pressman and Fahim, 1982), it has generally been assumed that these antibiotics directly facilitate the transport of Ca^{2+} across the plasma membrane, circumventing natural entry pathways. However, more recently, some reports have suggested this assumption may be incorrect: in bovine adrenal chromaffin cells (Stauderman and Pruss, 1989) and fibroblasts (Byron et al., 1992), ionomycin mobilized intracellular Ca^{2+} stores without stimulating a Ca^{2+} influx. Similarly, studies in pituitary cell lines suggested that ionomycin primarily discharged the agonist-sensitive Ca^{2+} pools, whereas the smaller Ca^{2+} -influx component was assumed to be due to ionophore action at the plasma membrane (Albert and Tashjian, 1984, 1986). Furthermore, ionomycin-induced cation entry in human umbilical-vein endothelial cells (HUVEC) may share a common route of entry with histamine-induced entry, since both responses were inhibited by thrombin (Neylon and Irvine, 1991). On the other hand, others have shown that inhibitors of Ca^{2+} entry such as SK&F 96365 (Davies et al., 1992; Graier et al., 1992) or phorbol ester (McCarthy et al., 1989) had no effect on ionophore responses, while decreasing agonist-driven effects, which is more consistent with the ionophores directly translocating bivalent cations across the plasma membrane.

We have undertaken a more rigorous examination of ionomycin stimulation of Ca^{2+} influx in a HUVEC-derived cell line, ECV304. This is a spontaneously transformed cell line that

resembles endothelial cells in several aspects, including antigenic markers, polyamine uptake and endothelial-specific enzymes (Takahashi et al., 1990). In this paper, we show that apparent ionomycin-mediated translocation of cations across the plasma membrane is by a store-regulated Ca^{2+} -influx mechanism (Putney, 1992; Jacob, 1990; Schilling et al., 1992) and not the expected ionophore action at the plasma membrane.

MATERIALS AND METHODS

Materials

Fura-2/AM and fura-2 (penta-potassium salt) were from Molecular Probes (Eugene, OR, U.S.A.). Hepes (free acid), cyclopiazonic acid (CPA) and ionomycin were from Calbiochem. Histamine phosphate, digitonin, pyrilamine and BSA (fraction V) were from Sigma (Poole, Dorset, U.K.). EGTA was obtained from Fluka at puriss. grade. SK&F 96365 (1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride) was given by Dr. Paul England, SmithKline Beecham, Welwyn, Herts., U.K. All other reagents were from Sigma, Fisons and BDH at the equivalent of AnalaR grade.

Cell culture

The HUVEC line ECV304 was obtained from Dr. Kimiko Takahashi (Department of Biochemistry, National Defense Medical College, Saitama, Japan) and used between passages 90 and 120. Cells were cultured in Medium 199 supplemented with 10% foetal-calf serum, 2 mM L-glutamine, 50 i.u./ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin, and passaged by using trypsin/

Abbreviations used: BSS, balanced salt solution; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; Ca^{2+}_o , extracellular Ca^{2+} ; CPA, cyclopiazonic acid; HUVEC, human umbilical-vein endothelial cells; SRCE, store-regulated cation entry.

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EDTA. Cells were seeded on glass coverslips and used 2–4 days after plating.

Determination of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

Fura-2 was loaded by incubating cells in 80% Dulbecco's Modified Eagle's Medium/20% foetal-calf serum with $2\text{ }\mu\text{M}$ fura-2/AM at room temperature for 60 min, after which the cells were maintained at room temperature in balanced salt solution (BSS) supplemented with 1% (w/v) BSA before use (< 3 h after loading). The BSS composition was (mM): NaCl 145, KCl 5, MgSO_4 1, CaCl_2 1, Hepes 10, glucose 10, BSA 0.1% (w/v), pH 7.4. Measurement of $[\text{Ca}^{2+}]_i$ was performed essentially as described previously (Jacob, 1990), by using epifluorescence microscopy, or a similar system connected to a Cairn cuvette holder for population studies. The rate of data acquisition was generally 2 Hz, except in those stated instances where a 5 Hz collection rate was employed. The entry of Mn^{2+} was inferred from the quenching of fluorescence at 360 nm, the isoemissive wavelength for fura-2 with respect to Ca^{2+} (Meritt et al., 1989). At the end of each run, autofluorescence was estimated by addition of $0.5\text{--}1\text{ }\mu\text{M}$ ionomycin and 2 mM Mn^{2+} (or 8 mM when 2 mM EGTA was present), which quenches fura-2 (Merritt et al., 1989) and calibrated as described by Grynkiewicz et al. (1985).

Data were usually converted into $[\text{Ca}^{2+}]_i$, except for the data reported in Figures 1, 2 and 4, where calibrations were not made. Where cited, the peak changes in $[\text{Ca}^{2+}]_i$ or 350/380 nm ratio elicited by addition of histamine, ionomycin or CPA refers to the maximum change as defined by the peak height minus the value immediately before the addition. Both ratio and $[\text{Ca}^{2+}]_i$ increases in the absence of extracellular Ca^{2+} (Ca^{2+}_o) will reflect the amount of Ca^{2+} released from internal stores, although neither is likely to be linearly related to the amount released.

To test for fura-2 compartmentation, perfusion of cells with $\sim 5\text{ }\mu\text{M}$ digitonin in Ca^{2+} -containing BSS discretely permeabilized the plasma membrane (indicated by a substantial increase in $[\text{Ca}^{2+}]_i$), leaving fura-2 trapped in the cell. Under these conditions, $89.5 \pm 1.3\%$ of the dye could be quenched by 2 mM Mn^{2+} within 100 s ($n = 4$). Increasing the digitonin 3-fold to $\sim 15\text{ }\mu\text{M}$ released $92.6 \pm 1.2\%$ of cell-bound fluorescence ($n = 8$) within 100 s. Thus, at least 90% of fura-2 appeared to be cytosolic.

Data analysis

Student's *t* test was used and data were paired where appropriate. Analysis was two-tailed, except where stated. Data are presented as means $\pm$ S.E.M. within one batch of cells, except where indicated, and results obtained in one preparation were confirmed in at least one other preparation.

RESULTS

Effects of ionomycin and CPA on $[\text{Ca}^{2+}]_i$

Ionomycin and the endoplasmic-reticulum Ca^{2+} -ATPase inhibitor CPA both mobilized Ca^{2+} (Figure 1), with the response to ionomycin being comparable with the response to histamine (see below, Figure 3). In nominally Ca^{2+} -free medium the Ca^{2+} responses were transient, falling back to baseline, indicating mobilization of intracellular stores. The ability of CPA to mobilize internal stores, albeit slowly, suggests that there is a continuous leak from the stores which is only observed when uptake into the stores is inhibited. When Ca^{2+}_o was present, both

Figure 1 Effect of Ca^{2+}_o on the $[\text{Ca}^{2+}]_i$ response to ionomycin and CPA

Cells were exposed to either (a) $0.5\text{ }\mu\text{M}$ ionomycin or (b) $20\text{ }\mu\text{M}$ CPA either in BSS containing 1 mM CaCl_2 or in nominally Ca^{2+} -free buffer. Traces typical of 3 or more experiments in single cells (a) or populations of cells (b) are shown.

Figure 2 Effect of store depletion on histamine- and ionomycin-stimulated $[\text{Ca}^{2+}]_i$ responses

(a) Internal Ca^{2+} stores of single cells were fully discharged by a short exposure to $100\text{ }\mu\text{M}$ histamine in nominally Ca^{2+} -free solution, and then challenged with $0.5\text{ }\mu\text{M}$ ionomycin. (b) Ionomycin ($0.5\text{ }\mu\text{M}$) was used to deplete stores, and then the cells were stimulated with $100\text{ }\mu\text{M}$ histamine. The Figure represents records typical of 5 or more experiments performed with cells from at least 2 cultures.

the CPA and ionomycin responses became more sustained, indicating a Ca^{2+} influx (Demaurex et al., 1992; Schilling et al., 1992).

The nature of the intracellular store(s) that these two agents were mobilizing was examined by completely discharging the agonist-sensitive store with $100\text{ }\mu\text{M}$ histamine in Ca^{2+} -free solution (Figure 2a: histamine followed by ionomycin). This had the effect of substantially decreasing the subsequent ionomycin- and CPA-evoked changes in 350/380 nm fluorescence ratio, expressed as a percentage of the initial histamine peak response [ionomycin $13.4 \pm 3.6\%$ ($n = 5$); CPA $17.0 \pm 3.5\%$ ($n = 4$); $P < 0.0002$ and $P < 0.02$ respectively (paired, one-tailed)]. Similarly, when the order of addition was reversed (Figure 2b: ionomycin followed by histamine), depletion of stores by ionomycin or CPA inhibited the histamine peak, expressed as a percentage of the initial peak response [ionomycin depletion $26.8 \pm 3.9\%$ ($n = 7$); CPA depletion $11.4 \pm 2.8\%$ ($n = 4$); $P < 0.0001$ and $P < 0.005$ respectively]. These observations suggest that all three agents mobilize the same intracellular Ca^{2+} pools. The ability of ionomycin and CPA to elevate $[\text{Ca}^{2+}]_i$ slightly, even after histamine-induced store depletion, does not necessarily suggest that there is more than one store: re-

Figure 3 Concentration–response relationship for ionomycin

(a) Single cells were stimulated with 100 μM histamine for 60 s in Ca^{2+} -containing solution as a reference stimulus (histamine was then removed), and then exposed to the indicated concentrations of ionomycin for 1–2 min after recovery for ≥ 120 s. Data are expressed as the peak height of the ionomycin response as a percentage of the peak height of the histamine reference response. Data are means $\pm$ S.E.M. of 5–9 experiments using up to 5 different cell preparations. (b) Cumulative additions of 1 μM ionomycin (iono) to cell populations in Ca^{2+} -containing solution. A portion of 1 mM CaCl_2 was added at end of the run. A trace typical of 3 experiments is shown.

Figure 4 Stimulation of Mn^{2+} quenching of fura-2 by histamine, ionomycin and CPA

In Ca^{2+} -free solution, 100 μM Mn^{2+} was used to assess basal cation influx rate: 100 μM histamine (a), 0.5 μM ionomycin (b, c) or 20 μM CPA (d) was added to discharge internal stores for the times indicated. Then 100 μM Mn^{2+} was again added, and the second challenge was initiated once a linear rate of quench had been established. Data collection rate was 5 Hz. 'Ratio' refers to fluorescence ratio 350/380 nm. F_{360} is the fluorescence at 360 nm (arbitrary units). Rates of stimulated cation entry were examined by a linear regression analysis of the quench of the F_{360} traces before and after the second stimulus. The ratio traces (a) and (c) are shortened because of the noisy signal at high quench. Traces are shown of runs with single cells typical of at least 5 others (a, b, c), or 1 other (d) on different batches of cells.

application of histamine itself after histamine depletion also produces a small rise in $[\text{Ca}^{2+}]_i$ comparable with that seen with ionomycin [percentage of first histamine response $11.9 \pm 6.1\%$ ($n = 6$); $P < 0.0001$].

The concentration–response relationship to ionomycin was established in single cells by using the response to 100 μM histamine as a reference (see Figure 3 legend). Figure 3 shows saturation of the peak ionomycin response at 0.5–1 μM

($\text{EC}_{50} \sim 0.3 \mu\text{M}$) (Figure 3a), where the large errors indicate the inherent variability in the responses in individual cells. Saturation of the plateau phase of the response is also achieved by addition of 1 μM ionomycin, since there is no further effect of repeated additions of ionomycin beyond 1 μM in populations of cells (Figure 3b).

For further experiments it was first necessary to verify that ionomycin was reversible, and so histamine was applied before

and 240 s after a brief exposure to 0.5 μM ionophore. Since $[\text{Ca}^{2+}]_i$ returned to basal after removal of ionomycin and the ratio of the two histamine responses was found to be insignificantly different from the appropriate controls without ionomycin (control $94 \pm 8\%$; +ionomycin $82 \pm 4\%$; $n = 4$, $P > 0.2$), a 240 s wash-out in BSA-containing buffer was deemed sufficient to remove ionomycin.

Stimulation of Mn^{2+} entry after store depletion

Given that both CPA and ionomycin could discharge the agonist-sensitive pool, we next examined whether they could, in turn, trigger cation influx in a manner analogous to histamine (Jacob, 1990). The unidirectional entry of Mn^{2+} , monitored by the quenching of fura-2, has frequently been used as an index for stimulated cation entry (Merritt et al., 1989; Jacob, 1990; Montero et al., 1990; Neylon and Irvine, 1991; Davies et al., 1992). Figure 4 indicates the ability of histamine, ionomycin and CPA to stimulate Mn^{2+} influx by depletion of internal stores in Ca^{2+} -free solution. As has been observed previously (Jacob, 1990), histamine activated Mn^{2+} influx even after the removal of agonist. Moreover, once a rate of quench was established, the addition of ionomycin did not alter this rate of Mn^{2+} entry (pre-ionomycin 0.0149 ± 0.0023 V/s; post-ionomycin 0.0151 ± 0.0020 V/s; paired two-tailed, $P > 0.9$, $n = 10$) (Figure 4a). Similarly, when ionomycin was used to deplete stores, and then removed, a stimulation of Mn^{2+} entry was again seen, which was not significantly enhanced upon addition of histamine (pre-histamine 0.0152 ± 0.0030 V/s; post-histamine 0.0273 ± 0.0051 V/s; $P > 0.05$, $n = 8$) (Figure 4b), although in these experiments the responses were somewhat more variable, with 3/8 cells exhibiting an enhancement of the quench rate with histamine. Similarly, no significant effect was observed upon ionomycin-stimulated Mn^{2+} entry if ionomycin itself was re-applied instead of histamine (pre-ionomycin re-application 0.0127 ± 0.0019 V/s; post-ionomycin re-application 0.0123 ± 0.0012 V/s; $P > 0.7$, $n = 6$) (Figure 4c). To strengthen further the idea that store depletion activated Mn^{2+} entry, refilling the intracellular pools after ionomycin removal by a 2 min exposure to 1 mM Ca^{2+} , substantially decreased the stimulated rate of quench [$85 \pm 2\%$ inhibition; $n = 13$, $P < 0.0001$ (paired, two-tailed)]. In other experiments, CPA also activated Mn^{2+} entry. However, the effect of histamine on this stimulated Mn^{2+} -entry rate was somewhat variable. In some cells histamine had little effect on the rate of quench ($n = 2$) (Figure 4d), whereas in two other cells agonist gave a more substantial change in $[\text{Ca}^{2+}]_i$, which was accompanied by a faster rate of Mn^{2+} entry (results not shown).

Block by SK&F 96365 of ionomycin-induced Ca^{2+} and Mn^{2+} entry

Although a Mn^{2+} -entry pathway was activated by ionomycin, the data did not address whether the sustained phase of the ionomycin-induced $[\text{Ca}^{2+}]_i$ response was mediated by this pathway: although ionomycin may not translocate significant amounts of Mn^{2+} across the plasma membrane, it may still be capable of translocating significant amounts of Ca^{2+} . Given that ionomycin can stimulate store-regulated cation entry (SRCE), this begs the question, to what extent is the sustained $[\text{Ca}^{2+}]_i$ response to ionomycin mediated by physical translocation across the plasma membrane, as opposed to a store-regulated influx?

SRCE can be blocked with the compound SK&F 96365 (Merritt et al., 1990; Davies et al., 1992; Schilling et al., 1992; Graier et al., 1992), and thus, if ionomycin-induced cation influx

Figure 5 Effect of SK&F 96365 on $[\text{Ca}^{2+}]_i$ responses to histamine, ionomycin and CPA

In cell populations, 75 μM SK&F 96365 was added either 60 s before or 6 min after stimulus with (a) 100 μM histamine, (b) 1 μM ionomycin or (c) 20 μM CPA in Ca^{2+} -containing solution. Experiments shown are typical of 2–4 others; traces are from experiments with same batch of cells for comparison.

is mediated by such a mechanism, SK&F 96365 should inhibit the response. Indeed, when fura-2 quenching in single or small groups of cells was measured, 75 μM SK&F 96365 inhibited the entry of 100 μM Mn^{2+} stimulated by either 100 μM histamine [$108 \pm 12\%$; $P < 0.005$ (paired two-tailed), $n = 6$] or by 1 μM ionomycin ($84 \pm 6\%$; $P \leq 0.001$, $n = 6$).

With respect to Ca^{2+} itself, a 60 s preincubation with 75 μM SK&F 96365 completely blocked the sustained phase of the responses to histamine, ionomycin and CPA such that $[\text{Ca}^{2+}]_i$ fell back to near basal levels within 3 min (Figure 5). The initial mobilization was unaffected in each case (Table 1). Furthermore, the addition of SK&F 96365 6 min after a plateau phase had been established by ionomycin or CPA also inhibited the sustained responses. However, when a plateau was established by an exposure to histamine, the response remained relatively insensitive to SK&F 96365. As a whole, the data suggest that

Table 1 Effect of 75 μM SK&F 96365 on peak and plateau responses to 100 μM histamine, 1 μM ionomycin and 20 μM CPA

Responses are expressed as $[\text{Ca}^{2+}]_i$ (μM). Pre-SK&F refers to the final plateau value after a 60 s preincubation with SK&F 96365, and Post-SK&F refers to the new plateau when the blocker was added 6 min after histamine, ionomycin or CPA. Unpaired two-tailed test with respect to appropriate control: * $P < 0.01$; ** $P < 0.001$. ND: because the CPA response is monophasic, the peak $[\text{Ca}^{2+}]_i$ is not determinable.

	Peak		Plateau		
	Control	+ SK&F	Control	Pre-SK&F	Post-SK&F
Histamine	0.83 ± 0.07 (5)	0.97 ± 0.14 (4)	0.26 ± 0.02 (5)	0.11 ± 0.01 (4)*	0.21 ± 0.03 (5)
Ionomycin	1.29 ± 0.22 (4)	1.04 ± 0.28 (5)	0.49 ± 0.02 (4)	0.17 ± 0.02 (5)**	0.21 ± 0.02 (4)**
CPA	ND	0.42 ± 0.01 (3)	0.66 ± 0.04 (3)	0.19 ± 0.01 (3)*	0.21 ± 0.00 (3)*

Figure 6 Effect of CPA on ionomycin and histamine additivity

(a) In buffer containing 1 mM CaCl_2 , cell populations were stimulated with 100 μM histamine for 120 s, after which 0.3 μM and 1 μM ionomycin were added. Ca^{2+}_o was then increased to 3 mM by two additions of 1 mM CaCl_2 . (b) After addition of 20 μM CPA, histamine and ionomycin were added as for (a). (c) as (b), except that the order of addition of histamine and 0.3 μM ionomycin was reversed. Traces are representative of 5 experiments with 3 different batches of cells.

ionomycin acts via an SK&F 96365-sensitive mechanism, and therefore via SRCE.

Prevention of store refilling with CPA

The action of ionomycin seemed to depend substantially on its ability to mobilize intracellular stores, and thereby activate channels in the plasma membrane. Thus it was assumed that ionomycin would have no further effect on the Ca^{2+} plateau established with a maximal concentration of histamine which would presumably fully discharge the store. However, this was not the case, since application of increasing concentrations of ionomycin during the sustained phase resulted in concomitant elevations in $[\text{Ca}^{2+}]_i$ (Figure 6a) [0.3 μM ionomycin 0.18 ± 0.07 μM ($P = 0.05$); 1 μM ionomycin 0.39 ± 0.11 μM ($P < 0.05$); $n = 5$]. At first sight, this might be taken as evidence in support of ionomycin translocating Ca^{2+} across the plasma membrane, but it is open to further interpretation. Given the continual action of store Ca^{2+} -ATPases, it may be that a combination of histamine and ionomycin empties stores more fully than does agonist alone, and therefore the above additivity protocol was repeated after preincubation with the Ca^{2+} -ATPase inhibitor CPA, to ensure complete discharge of the internal pools. In Figure 6(b), the expected elevation of $[\text{Ca}^{2+}]_i$ due to the inhibition of uptake into the stores was observed, followed by a sharp rise in $[\text{Ca}^{2+}]_i$ seen on addition of histamine (1.15 ± 0.21 μM ; $P < 0.01$, $n = 6$). However, the rise in $[\text{Ca}^{2+}]_i$ caused by histamine was transient and soon returned to levels seen with CPA alone. Further addition of 0.3 or 1 μM ionomycin did not lead to any further increase in $[\text{Ca}^{2+}]_i$ [0.3 μM ionomycin 0.10 ± 0.06 μM ($P > 0.1$); 1 μM ionomycin 0.01 ± 0.02 ($P > 0.7$); $n = 6$], nor was there any response to histamine or 1 μM ionomycin after a similar initial transient elicited by 0.3 μM ionomycin (Figure 6c) [0.3 μM ionomycin 0.78 ± 0.21 ($P < 0.05$); histamine 0.15 ± 0.15 ($P > 0.3$); 1 μM ionomycin 0.03 ± 0.01 μM ($P = 0.05$); $n = 5$]. Nevertheless, the elevation in $[\text{Ca}^{2+}]_i$ caused by raising the extracellular Ca^{2+} concentration to 3 mM [1.20 ± 0.21 μM ($P < 0.01$) (Figure 6b); 1.46 ± 0.30 μM ($P < 0.05$) (Figure 6c); $n = 5-6$] suggested that this was not an artefact of saturation of the system. This further supports a predominantly store-dependent effect of ionomycin.

Ionomycin does not affect rate of Ca^{2+} entry

Another way to assess whether ionomycin is translocating Ca^{2+} across the plasma membrane is to compare the kinetics of influx in the presence and absence of the ionophore. After complete depletion of intracellular stores, the rate of store-regulated entry will be maximal, and therefore any potentiation by ionomycin of such an influx rate could not be due to an effect on the degree of

Figure 7 Effect of ionomycin on the rate of rise in $[Ca^{2+}]_i$ in store-depleted cells

Histamine (100 μM) and CPA (40 μM) were simultaneously added to populations of cells in nominally Ca^{2+} -free medium to deplete intracellular stores. Ionomycin (1 μM) or vehicle [0.1% (v/v) dimethyl sulphoxide] was added 100 s before 1 mM $CaCl_2$; 2 mM EGTA was added 100 s later. In those experiments examining the effect of agonist on rates of rise, histamine was antagonized with 10 μM pyrilamine, an H_1 -antagonist, 300 s before repletion of Ca^{2+}_o . To examine the effect of ATPase inhibition on rates of rise, CPA was omitted and replaced by dimethyl sulphoxide (0.1%) (see Table 2). Traces are typical of 8–9 experiments performed on the same batch of cells.

Table 2 Effect of ionomycin, pyrilamine and $[Ca^{2+}]_o$ on the rate of rise and fall of $[Ca^{2+}]_i$ in store-depleted cells

The protocol was as described for Figure 7. ND, not determined. * $P \leq 0.02$ when compared with control. All other values were not significantly different from control ($P > 0.4$: unpaired, two-tailed).

	Rate of rise ($\mu M/s$)	Rate of fall ($\mu M/s$)
1 mM Ca^{2+}_o (Control)	0.087 ± 0.011 (8)	0.135 ± 0.028 (8)
1 mM Ca^{2+}_o + ionomycin	0.100 ± 0.012 (9)	0.139 ± 0.023 (9)
1 mM Ca^{2+}_o + pyrilamine	0.087 ± 0.009 (3)	0.157 ± 0.040 (3)
3 mM Ca^{2+}_o	0.116 ± 0.015 (3)	ND
10 mM Ca^{2+}_o	0.270 ± 0.024 (3)*	ND

store filling. Thus the agonist-sensitive pool was discharged with a combination of histamine and CPA in Ca^{2+} -free solution, and then 1 mM Ca^{2+} was added in the presence or absence of ionomycin (Figure 7). CPA was included to prevent rapid sequestration of the Ca^{2+} into the internal stores that might otherwise (a) inhibit the store-dependent influx rate as the pool partially fills and (b) render the rises in $[Ca^{2+}]_i$ a composite of the relative rates of influx into the cell and the release of Ca^{2+} which had been rapidly sequestered into the stores. Indeed, the rate of rise of $[Ca^{2+}]_i$ was slower when CPA was absent (histamine $0.009 \pm 0.001 \mu M/s$; histamine + CPA $0.033 \pm 0.005 \mu M/s$; $P < 0.02$, $n = 3-4$).

When the effect of ionomycin was examined, the rise in $[Ca^{2+}]_i$ with a combination of histamine and CPA was not significantly increased by the inclusion of ionomycin (Table 2). Given that these protocols do not strictly measure unidirectional uptake across the plasma membrane, it was necessary to verify that ionomycin did not stimulate extrusion of Ca^{2+} from the cell (which would otherwise dampen increases in the rate of rise in $[Ca^{2+}]_i$). This was accounted for by addition of excess EGTA

100 s after Ca^{2+} was included and monitoring the rate of fall in $[Ca^{2+}]_i$. No difference was observed between the rates of removal in either the presence or the absence of ionomycin. This confirms that there was no significant change in influx rate with ionomycin.

Using a similar protocol, experiments were also designed to test whether the presence of histamine could stimulate Ca^{2+} influx additional to that caused by discharge of the internal stores, complementary to the Mn^{2+} -quench experiments of Figures 4(b) and 4(d). Accordingly, pyrilamine (an H_1 -antagonist) was used at 10 μM , which completely antagonizes histamine responses in these cells (results not shown). Pylamine was thus added 5 min before re-exposure to 1 mM Ca^{2+}_o and the increase in $[Ca^{2+}]_i$ compared with that in cells where histamine action was not removed. No effect of agonist on the rate of rise or fall could be detected. To confirm that increases in influx rate could be seen by using this protocol, stores were depleted as described above with a combination of histamine and CPA, and 3 or 10 mM $CaCl_2$ was applied. Although no significant increase in the rate of rise was observed with 3 mM Ca^{2+}_o , an approx. 3-fold increment in rate of rise was seen with 10 mM Ca^{2+}_o , confirming the previously reported non-linear relationship between rate of influx and $[Ca^{2+}]_o$ in endothelial cells (Schilling et al., 1989).

DISCUSSION

The purpose of this study was to define the mechanism underlying ionomycin-stimulated cation influx in human endothelial cells. A wealth of evidence has accumulated to suggest that agonist-stimulated Ca^{2+} -influx in a variety of cell types, including vascular endothelial cells (and HUVEC in particular), is a consequence of store depletion itself and not merely the activation of receptors (Putney, 1992; Jacob, 1990; Schilling et al., 1992). Therefore, our working hypothesis was that ionomycin-stimulated Ca^{2+} influx might be due to SRCE.

One major Ca^{2+} pool in ECV304 cells

In the absence of extracellular Ca^{2+} , histamine, ionomycin and CPA clearly discharge internal stores which appear to be common to all three agents. In this regard, the ECV304 cell line resembles bovine aortic endothelial cells (Schilling et al., 1989), whereas PC12 cells (Fasolato et al., 1991) and rat parotid acinar cells (Merritt and Rink, 1987) appear to possess more than one intracellular store distinguishable by their accessibility to pharmacological agents. Although others have shown that Ca^{2+} -ATPase inhibitors can distinguish subsets of intracellular Ca^{2+} pools (Robinson et al., 1992; Røtnes and Iversen, 1992; Islam and Berggren, 1993), which may reflect the inhibitor-sensitivity of some Ca^{2+} -ATPase isoforms (Papp et al., 1992), we cannot infer that there is only one homogeneous pool, since the ability of CPA to distinguish ATPase populations is not well characterized.

Store-regulated Mn^{2+} influx

Consistent with the capacitance model for the regulation of Ca^{2+} entry (Putney, 1992), we have shown that depletion of the stores by histamine, ionomycin and CPA stimulates Mn^{2+} influx, as has been demonstrated in other cell types (Jacob, 1990; Schilling et al., 1992), and by a mechanism which is shared by all three agents: in additivity experiments, the rate of ionomycin-stimulated Mn^{2+} entry was unaffected by agonist, suggesting that no additional entry pathways are recruited by histamine over and above those invoked by store discharge (at least, that are detectable by this protocol). Complementary to this, the rate of

rise in $[Ca^{2+}]_i$ (equivalent to Ca^{2+} influx) was unaffected by the presence of histamine, which suggests that there is no major Ca^{2+} -specific entry pathway coupled tightly to receptors that is not detected by Mn^{2+} quenching (Hoth and Penner, 1992) (unless it is rapidly desensitising; Fonteriz et al., 1992).

Moreover, Mn^{2+} quenching of fura-2 also gave some insight into how ionomycin stimulates Mn^{2+} uptake: it could be envisaged that either ionomycin acts as a ' Mn^{2+} ionophore' (a direct translocation across the plasma membrane), or that it stimulates influx by SRCE. Therefore it is noteworthy that the maximal rate of quench established after complete discharge of the stores with histamine was not increased further by ionomycin, suggesting that ionomycin did not provide an additional route of entry across the plasma membrane. In addition to this, Mn^{2+} entry was still seen after ionomycin wash-out, indicating that ionomycin did not need to be present (at least at concentrations that elevate $[Ca^{2+}]_i$) for influx to occur. In further support of a SRCE mechanism, either refilling of internal stores with Ca^{2+} or treatment with SK&F 96365 could almost completely abolish ionomycin-stimulated Mn^{2+} entry. Ionomycin not being a true Mn^{2+} ionophore is consistent with previous data where thrombin inhibited both histamine- and ionomycin-stimulated Mn^{2+} influx, suggesting a common mechanism of Mn^{2+} entry (Neylon and Irvine, 1991). It is also consistent with ionophores activating similar inward cation conductances to agonists in whole-cell recordings in HUVEC (Bregotovski et al., 1988).

Ionomycin-induced Ca^{2+} entry is not mediated by ionophore action at the plasma membrane

Given that the above demonstrates ionomycin-stimulated cation (Mn^{2+}) entry, two mechanisms of ionomycin-induced Ca^{2+} entry may therefore be possible: the predicted direct facilitation across the plasma membrane and/or via SRCE. However, several lines of evidence suggest that ionomycin acts primarily on internal membranes and not at the plasma membrane. Firstly, if Ca^{2+} influx was due to direct translocation across the plasma membrane, we would not expect that the ionomycin concentration-response relationship would saturate for influx at such a low concentration of ionomycin. In other cell types, saturation of the ionomycin-stimulated Ca^{2+} response (Albert and Tashjian, 1984) or cell swelling (Artalejo and García-Sancho, 1988) has been previously observed. This suggests that another factor(s) is limiting, but the response has not been saturated, since ratio changes are well below the R_{max} . Also, saturation is not due to marked activation of extrusion processes, since CPA can further increase the Ca^{2+} plateau induced by 1 μM ionomycin (results not shown), and elevating Ca^{2+}_o to 3 mM increased steady-state $[Ca^{2+}]_i$ in the presence of micromolar levels of ionomycin (Figure 6).

Secondly, channel antagonists can be used to discriminate between store-regulated Ca^{2+} entry and ionophore action at the plasma membrane. Although transition-metal ions are commonly employed as non-specific blockers of Ca^{2+} entry (Rink, 1990), their potential for competing with Ca^{2+} for the co-ordination sites in the ionomycin molecule (Stiles et al., 1991) rendered them unsuitable agents. Instead, the store-regulated Ca^{2+} -entry blocker SK&F 96365 was used (Merritt et al., 1990; Davies et al., 1992; Schilling et al., 1992; Graier et al., 1992) and, consistent with its blockade of influx, was found to inhibit the plateau, but not the initial mobilization, of intracellular stores by histamine, CPA or ionomycin. Curiously, with histamine, inhibition was only significant after a preincubation with the blocker. However, there was little difference between the uninhibited histamine

plateau value and the inhibited plateau of ionomycin or CPA, suggesting that there may be a small influx component common to all three agents that remains resistant to these concentrations of SK&F 96365. Nevertheless, our data remain consistent with SK&F 96365 blocking an ionomycin-induced Ca^{2+} influx that is mediated by cellular pathways, since both CPA and histamine responses can be antagonized. This is consistent with earlier work, demonstrating the effects of SK&F 96365 on CPA-stimulated Ca^{2+} entry in endothelial cells (Schilling et al., 1992) or FRTL cells (Törnquist, 1993) and of other SRCE blockers on the store-dependent effects of ionomycin, thapsigargin and CPA (Alonso et al., 1991; Sargeant et al., 1992; Vostal and Fratantoni, 1993).

Thirdly, in additivity experiments, the ionomycin enhancement of the histamine response was abolished by treatment with CPA (Figure 6). Although it might be argued that the CPA-raised baseline before histamine stimulation may have activated extrusion mechanisms that obscured elevations in $[Ca^{2+}]_i$, this is unlikely, since (i) an increase in $[Ca^{2+}]_i$ could still be evoked upon raising Ca^{2+}_o to 2–3 mM (Figure 6b and 6c), (ii) histamine and 0.3 μM ionomycin could transiently raise $[Ca^{2+}]_i$ in the presence of CPA (Figures 6b and 6c), and (iii) raising the baseline with histamine to a similar degree as for CPA (Figure 6a) did not in itself attenuate the subsequent response to 1 μM ionomycin. It ought to be emphasized that the state of the agonist-sensitive store during stimulation in the continued presence of Ca^{2+}_o is unknown in human endothelium, but, by analogy with the work in other non-neuronal cells (Kwan et al., 1991; Muallem et al., 1988), we suspect that the continuous refilling action of the Ca^{2+} -ATPase during agonist stimulation does not allow the pool to discharge completely. Thus the elevation of the histamine-induced plateau by ionomycin seemed dependent on the residual Ca^{2+} left in the stores: had the sustained phase of the ionomycin response been substantially attributable to direct plasmalemmal translocation, then a further increase in $[Ca^{2+}]_i$ would have been predicted, irrespective of the state of the internal store.

If ionomycin translocated Ca^{2+} directly across the plasma membrane in addition to any endogenous entry pathway, then an increased rate of Ca^{2+} influx would have been expected, but this was clearly not the case (Figure 7). Since the elevation of $[Ca^{2+}]_i$ by 1 μM ionomycin is a relatively fast process (0.035–0.096 $\mu M/s$) and comparable with the absolute rates of rise seen by using this protocol (0.025–0.127 $\mu M/s$), it would seem reasonable to suggest that ionomycin-induced potentiation of the influx rate should be detectable by using the rate-of-rise protocol. Indeed, from these values one might have predicted a doubling of the influx rate by ionomycin, which would have been detectable by this protocol (a 3-fold increase in rate of rise was seen with 10 mM Ca^{2+}_o). These experiments at least suggest that the major component of Ca^{2+} entry with ionomycin is via a store-dependent entry mechanism. Moreover, the data with ionomycin and CPA indicate that receptor activation (and the production of inositol phosphates) is not mandatory for the stimulation of influx processes in ECV304 cells [unlike some other cells (Irvine, 1992; Putney, 1992)].

Very recently, Mason and Grinstein (1993) have investigated the action of ionomycin on lymphocyte Ca^{2+} entry by using SK&F 96365 and by varying the membrane potential. They concluded that at higher concentrations (1 μM) ionomycin stimulated Ca^{2+} influx mainly by ionophoretic action at the plasma membrane, whereas at low concentrations (100 nM) this was less dominant, revealing an SRCE component. In contrast, we have established that ionomycin can be used to activate Ca^{2+} entry maximally via SRCE without translocating Ca^{2+} directly across the plasma membrane. This may reflect a difference in

experimental approach or a real difference between lymphocytes and endothelial cells.

Route of cation entry

As recently pointed out by Cabello and Schilling (1993), the route of cation entry into the cytoplasm of vascular endothelial cells had not been entirely resolved. In agreement with their conclusion, our data also suggest that cations enter the cytoplasm directly rather than passing into the store via a restricted channel (Merritt and Rink, 1987). Firstly, histamine did not potentiate the Mn^{2+} quench evoked by either ionomycin or CPA. As argued by Putney for Ca^{2+} (Takemura et al., 1989), if Mn^{2+} enters the cytoplasm from the store via a leak pathway, then activation of the $InsP_3$ receptor upon addition of histamine would enhance the rate of quench by providing an additional route from the store to the cytoplasm, since the $InsP_3$ -receptor channel conducts Mn^{2+} ions (Glennon et al., 1992; Joseph and Williamson, 1986). The lack of effect of histamine therefore implies that Mn^{2+} enters the cytosol directly.

Secondly, although in store-depleted cells re-addition of Ca^{2+} normally can refill the intracellular Ca^{2+} pools with little change in basal $[Ca^{2+}]_i$ [results not shown, but see Jacob (1990) for HUVEC], when the Ca^{2+} -ATPase is inhibited, application of Ca^{2+} results in a rapid rise in $[Ca^{2+}]_i$, even when histamine is antagonised by pyrilamine (Figure 7). This can be explained in two ways: either (i) Ca^{2+} enters the cytoplasm directly via a restricted space or (ii) there is a direct entry into the internal pool, but there must also be a considerable and rapid leak pathway from the store to the cytoplasm (unlikely to be the activated $InsP_3$ receptor, since histamine stimulation of phospholipase C was removed). However, the elevation of $[Ca^{2+}]_i$ with CPA appears to be due to a slow leak from stores, and not from an inherently slow model of action (A. J. Morgan and R. Jacob, unpublished work). Such a slow leak (maximum initial rate $0.0095 \pm 0.0009 \mu M/s$; $n = 15$) could not account for the rate of rise in $[Ca^{2+}]_i$ (~ 10 -fold greater) produced on replenishing Ca^{2+} . The results are more consistent with Ca^{2+} directly entering the cytoplasm into a restricted domain below the plasma membrane (Putney, 1986, 1990; Cabello and Schilling, 1993), where it is rapidly sequestered into the intracellular stores by a Ca^{2+} -ATPase (Lückhoff and Busse, 1990).

We have demonstrated that ionomycin predominantly stimulates cation entry indirectly by activation of SRCE, not by a direct translocation across the plasma membrane. The data also further support the hypothesis that cations do not first enter endothelial cells via the internal stores, but by a direct route into the cytoplasm (Putney, 1986, 1990). Although our evidence strongly suggests a selectivity of ionomycin for endomembranes [as has previously been only hinted at (Stauderman and Pruss, 1989; Albert and Tashjian, 1986; Neylon and Irvine, 1991)], there are reports of ionophore being unaffected by conditions which can nevertheless blunt the agonist-stimulated Ca^{2+} entry (McCarthy et al., 1989; Davies et al., 1992; Graier et al., 1992). We cannot fully explain such discrepancies in the light of our data, but suggest that there may be differences in experimental protocols (e.g. relative concentration of ionophores or Mn^{2+}) or in agonist-specific pathways in these systems. Why ionomycin should selectively act on internal membranes is unclear, but ionic or pH differences may induce variations in the affinity of ionomycin for Ca^{2+} (Fasolato and Pozzan, 1989). The ability of ionomycin to mobilize selectively intracellular Ca^{2+} by an altern-

ative mechanism to $InsP_3$ elevation or Ca^{2+} -ATPase inhibition could provide a useful tool for investigating Ca^{2+} regulation.

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