Histamine Type I Receptor Occupancy Increases Endothelial Cytosolic Calcium, Reduces F-Actin, and Promotes Albumin Diffusion Across Cultured Endothelial Monolayers

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Abstract. Considerable evidence suggests that Ca$^{2+}$ modulates endothelial cell metabolic and morphologic responses to mediators of inflammation. We have used the fluorescent Ca$^{2+}$ indicator, quin2, to monitor endothelial cell cytosolic free Ca$^{2+}$, [Ca$^{2+}$],, in cultured human umbilical vein endothelial cells. Histamine stimulated an increase in [Ca$^{2+}$] from a resting level of 111 ± 4 nM (mean ± SEM, n = 10) to micromolar levels; maximal and half-maximal responses were elicited by 10$^{-4}$ M and 5 × 10$^{-6}$ M histamine, respectively. The rise in [Ca$^{2+}$], occurred with no detectable latency, attained peak values 15-30 s after addition of stimulus, and decayed to a sustained elevation of [Ca$^{2+}$], two- to threefold resting. H$_{1}$ receptor specificity was demonstrated for the histamine-stimulated changes in [Ca$^{2+}$],. Experiments in Ca$^{2+}$-free medium and in the presence of pyrilamine or the Ca$^{2+}$ entry blockers Co$^{2+}$ or Mn$^{2+}$, indicated that Ca$^{2+}$ mobilization from intracellular pools accounts for the initial rise, whereas influx of extracellular Ca$^{2+}$ and continued HI receptor occupancy are required for sustained elevation of [Ca$^{2+}$]. Ionomycin-sensitive intracellular Ca$^{2+}$ stores were completely depleted by 4 min of exposure to 5 × 10$^{-6}$ M histamine. Verapamil or depolarization of endothelial cells in 120 mM K$^{+}$ did not alter resting or histamine-stimulated [Ca$^{2+}$],, suggesting that histamine-elicited changes are not mediated by Ca$^{2+}$ influx through voltage-gated channels. Endothelial cells grown on polycarbonate filters restricted the diffusion of a trypan blue-albumin complex; histamine (through an H$_{1}$-selective effect) promoted trypan blue-albumin diffusion with a concentration dependency similar to that for the histamine-elicited rise in [Ca$^{2+}$]. Exposure of endothelial cells to histamine (10$^{-5}$ M) or ionomycin (10$^{-7}$ M) was associated with a decline in endothelial F-actin (relative F-actin content, 0.76 ± 0.07 vs. 1.00 ± 0.05; histamine vs. control, $P < 0.05$; relative F-actin content, 0.72 ± 0.06 vs. 1.00 ± 0.05; ionomycin vs. control, $P < 0.01$). The data support a role for cytosolic calcium in the regulation of endothelial shape change and vessel wall permeability in response to histamine.

The vascular endothelial cell is uniquely situated to play an active role in the induction of the inflammatory response. The postcapillary venule (the primary site of neutrophil exudation and plasma protein leakage) displays only limited tight junctions (29, 30) and lacks a muscularis coat (38), affording the venular endothelial cell a central role in the barrier function of the vessel wall and allowing close approximation of the endothelial cell and subjacent mast cells, the predominant tissue source of vasoactive amines. Nearly a century ago, Metchnikoff suggested that endothelial cell motility and contractility directly influence the inflammatory response by modulating leukocyte emigration and plasma protein leakage (19). More recently, Majno and Palade (17) described the occurrence of interendothelial gaps after the local application of histamine, and argued, based on the ultrastructural alterations noted, that contraction of adjacent endothelial cells was responsible for interendothelial gap formation (18). They suggested that endothelial and smooth muscle cells share a similar contractile mechanism, a view supported by the finding of endothelial actin and myosin filaments immunohistochemically indistinguishable from those of smooth muscle (3) and the preferential concentration of these in regions of interendothelial contact (30). Heltianu et al. (10) subsequently localized endothelial histamine receptors to the plasmalemma overlaying this so-called perijunctional filament web. If endothelial cells employ a smooth muscle–like contractile apparatus, one would expect by analogy that occupancy of endothelial histamine receptors would lead to a rise in cytosolic free calcium, [Ca$^{2+}$],, which acts as the excitation-contraction coupler in smooth muscle (5). While recent reviews (9, 38) have doubted a functional role for active endothelial contractility, the possi-
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Materials and Methods

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were provided by John Paul (Smith, Kline, and French, Philadelphia, PA), 24-well tissue culture plates (Costar, Cambridge, MA); nitrobenzoxadiazole, Triton X-100, oligomycin, toluidine blue, quirt2 acetoxymethylester (NBD, nitrobenzoxadiazole).

Materials

Materials and Methods

Additions were routinely conducted using Hank's balanced salt solution (HBSS; Whittaker M. A. Bioproducts, Walkersville, MD) containing 136.9 mM NaCl, 5.4 mM KCl, 0.34 mM NaHPO4, 1.3 mM CaCl2, 0.8 mM MgSO4, 4.2 mM NaHCO3, and 5.6 mM glucose. In selected experiments modified balanced salt solutions were prepared using chemicals of reagent grade. Studies examining the effects of Ca2+ and Mg2+ were conducted in phosphate-, sulfate-, and bicarbonate-free HBSS prepared by substitution of MgCl2 for MgSO4 and buffered with 10 mM Hepes. In some experiments the concentration of potassium was varied from 5-120 mM at constant osmolality and fixed concentrations of sodium (10 mM) and chloride (120 mM) by reciprocally adjusting the concentration of KCl and choline chloride (26).

Obtained as follows were: histamine, peptidase, histamine, heparin, gelatin, BSA, Tris (Tris base), Triton X-100, oligomycin, toluidine blue, quirt2 acetoxymethylester (NBD, nitrobenzoxadiazole).

Confluent cultures were washed with Ca2+/Mg2+-free HBSS and incubated at 37°C for 10-15 min in the same buffer supplemented with 250 μM EDTA. Retracted cells were readily suspended by gentle agitation, diluted in an equal volume of HBSS, pelleted, washed, and resuspended in HBSS. Endothelial cells (5 × 106/million) were routinely loaded by incubation with quin2/AM (5 × 10-6 M) for 30 min at 37°C with gentle agitation (setting N° 1; multipurpose rotator; Scientific Industries, Inc., Bohemia, NY). In some experiments higher or lower quin2 loadings were achieved by altering the concentration of quin2/AM. Unloaded cells used in [Ca2+]i determinations were incubated in parallel in 0.1% DMSO without quin2/AM. After loading, cells were pelleted, washed, resuspended at 5 × 106/million in HBSS or modified balanced salt solutions, and stored on ice until used. Minimal quin2 leakage (<5 % after storage for up to 2 h) was evident in some batches of quin2-loaded cells (e.g., the abrupt step-off in quin2 fluorescence on addition of EGTA, Fig. 2). In preliminary experiments more extensive washing did not consistently eliminate extracellular quin2, suggesting that leakage of dye occurred during resuspension of cell pellets. Cells were routinely used within 2 h of quin2 loading; in occasional experiments cells stored for longer than 2 h were washed and resuspended before use.

Fluorescence Measurements

Fluorescence measurements were performed using a fluorescence spectrophotometer interfaced with an R-800A recorder (model L55; Perkin-Elmer Corp., Norwalk, CT) and equipped with a thermostatted, magnetically stirred cuvette holder. All assays were conducted at 37°C. Excitation and emission wavelengths were 339 ± 3 nm and 492 ± 10 nm, respectively, with the gain adjusted to provide a resting fluorescence of ~50% of full scale. Assays were conducted in disposable plastic cuvettes (Sarstedt, Federal Republic of Germany) loaded with 2 ml of cell suspension (final concentration 2.5 × 105/ml). The preparations were maintained as previously described (33). Maximum and minimum fluorescence values were recorded at ~10-3 M Ca2+ and ~10-7 M Ca2+, respectively, by lysing cells in 0.1% Triton X-100 followed by chelation of free Ca2+ on addition of 4 mM EGTA and sufficient Tris base to bring pH to >8.3. Observed values were corrected for the slightly enhanced fluorescence of Triton-lysed unloaded cells. Intermediate values of [Ca2+]: were calculated based on an

1. Abbreviation used in this paper: NBD, nitrobenzoxadiazole.
Figure 1. Kinetics and concentration dependency of histamine-elicited rise in endothelial cell $[Ca^{2+}]$. (A) Representative fluorescence tracing of quin2-loaded endothelial cells suspended in HBSS ($5 \times 10^5$ ml) and exposed to histamine ($10^{-7}$-$10^{-4}$ M). Fluorescence was measured at excitation and emission wavelengths of 339 ± 3 and 492 ± 10, respectively, and calibrated as described in the text. (B) Data represent mean ± SEM of four separate experiments, each performed in triplicate; abscissa, log scale. To control for variability between batches of endothelial cells loaded with quin2 on different days, ordinate data were normalized to a percent of the maximal increase in $[Ca^{2+}]$, which was consistently observed at $10^{-4}$ M histamine.

Figure 2. Preparati of Trypan Blue–Albumin Complex

Trypan blue (36 mg) and BSA (800 mg) were dissolved in 100 ml HBSS to yield a stable complex (trypan blue $>99.8\%$ protein bound as determined by TCA precipitation) with absorption maximum at 590 nm. At concentrations used in the diffusion experiments, compounds added to upper wells did not alter the spectral properties of the trypan blue–albumin complex.

Figure 3. F-actin Staining and Quantitation

Endothelial cells grown on fibronectin-coated polycarbonate filters were exposed to $10^{-5}$ M histamine with or without $10^{-7}$ M pyrilamine, or to $10^{-7}$ M ionomycin for 5 min at 37°C. The cells were washed with HBBS, fixed in 3.7% formaldehyde in PBS, extracted with 80% acetone, and stained with NBD-phallicidin (1.65 x 10^{-7} M, 20 min). To quantitate F-actin, stained monolayers were extracted with methanol (0.5 ml per filter, for 1 h at 37°C in the dark) and the extractable fluorescence was quantitated by fluorometry at excitation and emission settings of 465 and 535, respectively (12). F-actin content was expressed as the ratio of extractable fluorescence in stimulated compared with untreated cells.

Figure 4. Statistics

Standard error was used as an estimate of variance. Significance was determined by a two-tailed Student's t test or Dunnett's test where multiple experimental groups were compared with a control (4). Since $[Ca^{2+}]$ levels were displayed on a logarithmic scale, significance of the difference between widely divergent $[Ca^{2+}]$ values was determined by Student's t test of geometric means.

Figure 5. Results

Quin2 Loading

Endothelial uptake and hydrolysis of quin2/AM was readily monitored by a gradual shift in the emission spectrum of suspended cells from that of quin2/AM (peaking at 430 nm) to that of quin2 (peaking at 492 nm). Under the conditions routinely used ($5 \times 10^6$ cells/ml, $5 \times 10^{-6}$ M quin2/AM, 37°C), uptake and hydrolysis were rapid and complete within 30 min (data not shown). In four separate experiments, loading under these conditions yielded an incorporation of $0.81 \pm 0.07$ nmol quin2/10^6 endothelial cells (mean ± SEM of four batches of endothelial cells, loaded on separate days). Based on an endothelial urea volume of 2.0 $\mu$l/10^6 cells, the...
mean intracellular quin2 concentration in these cells was 0.40 ± 0.03 mM. Higher quin2 loadings, up to 4 mM, were readily achieved by increasing the initial concentration of quin2/AM. When quin2-loaded cells were stored on ice for 4 h, intracellular quin2 concentrations up to 4 mM were not toxic, as evidenced by trypan blue exclusion (94 vs. 92% viability, quin2-loaded vs. -unloaded cells, respectively) or lactate dehydrogenase release (4.4 vs. 4.6% of total cellular lactate dehydrogenase released per hour, quin2-loaded vs. -unloaded cells, respectively). Similarly, quin2-loaded cells re-attached to gelatin-coated tissue culture dishes (2-h plating efficiency, 70 vs. 74%, quin2-loaded vs. -unloaded cells, respectively) and proliferated to confluence.

**Histamine-dependent Increase In Endothelial [Ca2+].**

Endothelial cells suspended in HBSS had a resting [Ca2+] of 11 ± 4 nM (mean ± SEM, range 88–140 nM, 10 batches of endothelial cells loaded with quin2 on separate days). Histamine elicited a concentration-dependent rise in [Ca2+]i with maximal and half-maximal responses at 10⁻⁴ M and 5 x 10⁻⁶ M histamine, respectively (Fig. 1 A and B). The initial phase of the histamine response exhibited no detectable latency, peaked 15–30 s after the addition of a maximal stimulus, approached micromolar calcium levels, and gradually decayed. A second phase followed as a sustained elevation of cytosolic Ca²⁺ at two- to threefold above resting levels. At threshold histamine concentrations (10⁻⁷ M), a slower rise to maximum [Ca²⁺]i was noted. When extracellular Ca²⁺ was set at <20 nM by addition of EGTA immediately before stimulus, the initial response was unchanged. Under these conditions, histamine elicited a nearly identical rise in [Ca²⁺]i, which, however, declined within minutes and approached resting levels (Fig. 2, A and B). The initial rise in cytosolic Ca²⁺ could be accounted for largely, if not entirely, by mobilization of Ca²⁺ from intracellular pools, while sustained elevation of [Ca²⁺]i required influx from the extracellular space or inhibition of Ca²⁺ extrusion.

Intracellular calcium stores were rapidly depleted by histamine. For example, in Fig. 3, when extracellular Ca²⁺ was rapidly chelated with EGTA at varying times after histamine stimulation, and cells were immediately exposed to 10⁻⁶ M ionomycin, release of residual intracellular Ca²⁺ stores was demonstrated by a transient increase in fluorescence. At 2 min after 5 x 10⁻⁶ M histamine, ionomycin-sensitive Ca²⁺ stores were still demonstrable, whereas at 4 min or longer after histamine, cellular Ca²⁺ stores appeared fully depleted.

Since quin2 binds Ca²⁺ with 1:1 stoichiometry, the amount of Ca²⁺ released from intracellular stores can be estimated by the product of the percent change in quin2 saturation and intracellular quin2 concentration, as described by Lew et al. (15). For example, in Fig. 2 A, the amount of Ca²⁺ released is ~292 pmol/10⁶ cells (40% × 740 pmol quin2/10⁶ cells). As noted (15), only a lower limit of Ca²⁺ stores can be inferred from this approach, since the calculation assumes that endogenous Ca²⁺ buffering is negligible compared with that introduced by quin2.

**Inhibition of Histamine Response by Selective Ion Channel Blockers.**

Compounds characterized in other cellular systems as selective blockers of Ca²⁺ channels (25) were examined for their effects on the histamine-elicited rise in endothelial cytosolic free calcium. In the presence of the nonspecific calcium entry blockers cobalt and manganese (2 mM, Fig. 4), histamine elicited an early rise in [Ca²⁺]i, with kinetic parameters similar to controls. Both cobalt and manganese, however, prevented the sustained elevation of [Ca²⁺]i seen in their absence. Because cobalt and manganese quench both the calcium-dependent and calcium-independent fluorescence of quin2 (II), the immediate step-off in fluorescence on their addition exceeds that due to addition of EGTA alone; in addition, we cannot exclude the possibility that limited inward leakage of cobalt or manganese, as proposed in other cell types (II), contributes to the diminished fluorescent signal.

In the presence of 2 x 10⁻⁵ M verapamil (a plasma membrane Ca²⁺ entry blocker) the early and sustained responses to 10⁻⁴ M histamine were unchanged; apparent inhibition was, however, noted at 5 x 10⁻⁶ M histamine (Fig. 5). The latter effect was not seen at a 10-fold lower concentration of verapamil (not shown), could not be overcome by raising extracellular Ca²⁺ to 10 mM, and was obtained during the initial phase of histamine stimulation, when the rise in [Ca²⁺]i could be accounted for by release from intracellular pools.
(presumably a verapamil-resistant Ca$^{2+}$ flux) as opposed to influx of extracellular calcium. Viewed collectively, these observations suggest that verapamil acts at low histamine concentrations through a non-Ca$^{2+}$-specific mechanism (21).

**Specificity for $H_1$ vs. $H_2$ Receptor Subtype**

Compounds classified in other cellular systems as specific $H_1$ and $H_2$ receptor agonists and antagonists were used to characterize the endothelial receptor responsible for the histamine-stimulated rise in [Ca$^{2+}$]. The $H_1$ agonists 2-methylhistamine, 2-pyridylethylamine, and 2-(aminoethyl)thiazole each elicited an increase in endothelial [Ca$^{2+}$], with kinetics (not shown) and concentration dependency similar to that shown for histamine (Fig. 6, B-D). The $H_2$ antagonist, pyrilamine (10$^{-6}$ M), caused a rightward shift in the histamine dose-response curve (Fig. 6 A), and at higher concentrations (10$^{-5}$ M pyrilamine, data not shown), completely blocked the rise in [Ca$^{2+}$], attributed to 10$^{-6}$ M histamine. When addition of pyrilamine followed prior stimulation with histamine, the elevation of [Ca$^{2+}$], attributed to the latter agent was rapidly reversed (Fig. 7). In contrast, the $H_2$ agonist, dimaprit (data not shown), and the $H_2$ antagonist, cimetidine (Fig. 6 A), were without effect.
Figure 6. Effect of selective H₁ and H₂ agonists and antagonists on endothelial cell [Ca²⁺], or histamine-elicited rise in endothelial cell [Ca²⁺]. Data represent mean ± SEM of a single experiment performed in duplicate; abscissa, log scale. (A) Quin2-loaded endothelial cells suspended in HBSS were stimulated with histamine (10⁻⁷--5 × 10⁻⁴ M) alone (solid circle, solid line), 30 s after addition of 10⁻⁴ M cimetidine (solid circle, broken line), or 10⁻⁸ M pyrilamine (open circle, solid line). (B–D) Quin2-loaded endothelial cells suspended in HBSS were stimulated with the H₁ agonists 2-methylhistamine, 2-aminoethylthiazole, or 2-pyridylethylamine.

Figure 7. Sustained H₁ receptor occupancy is required for tonic elevation of endothelial cell [Ca²⁺], elicited by histamine. Representative tracing of quin2-loaded endothelial cells suspended in HBSS stimulated with 10⁻⁵ M histamine followed by 10⁻⁷ M pyrilamine.

Voltage Dependence of Histamine-activated Calcium Channels

Since voltage-gated calcium channels are involved in signal transduction in excitable cells (25) and electrophysiologic studies have demonstrated a histamine-induced endothelial depolarization (22), we examined resting and histamine-stimulated [Ca²⁺] of endothelial cells suspended in depolarizing buffers (22). When endothelial cells were suspended in 5 mM K⁺ or 120 mM K⁺ buffers, neither resting [Ca²⁺] (97 ± 6 nM vs. 99 ± 2 nM, low vs. high K⁺, respectively, n = 3); peak stimulated [Ca²⁺], (860 ± 31 nM vs. 898 ± 64 nM, low vs. high K⁺, respectively, n = 3); nor [Ca²⁺], 5-min poststimulation (291 ± 24 nM vs. 227 ± 26 nM, low vs. high K⁺, respectively, n = 3) differed significantly.

Histamine-induced Changes in Albumin Diffusion across Endothelial Monolayers

We prepared a trypan blue–albumin complex to facilitate measurement of the diffusion of a biologically relevant macromolecular species across endothelial monolayers. Spontaneous diffusion of the marker was minimal during the 30-min incubation routinely employed in these studies. In contrast, histamine promoted albumin diffusion across the monolayer was measured spectrophotometrically after a 30-min incubation. Data represent mean ± SEM of three separate experiments, each performed in triplicate, and are expressed as the histamine-stimulated change in trypan blue–albumin diffusion relative to simultaneous, unstimulated controls.
Table I. Change in F-actin Content of Endothelial Cells Exposed to Histamine or Calcium Ionophore

| Stimulus          | Relative F-actin content | P value |
|-------------------|--------------------------|---------|
| Control           | 1.00 ± 0.05 (12)         | -       |
| 10^{-5} M histamine | 0.76 ± 0.07 (8)         | <0.05   |
| 10^{-5} M histamine + 10^{-7} M pyrilamine | 1.03 ± 0.05 (8) | -       |
| 10^{-7} M ionomycin | 0.72 ± 0.06 (8)         | <0.01   |

* Endothelial cells grown to confluence on replicate fibronectin-coated polycarbonate filters were exposed to stimulus for 5 min at 37°C, fixed in 3.7% formaldehyde in Dulbecco’s PBS, extracted with 80% acetone, and stained with NBD-phallicidin (1.65 × 10^{-7} M). Methanol-extractable fluorescence was measured by spectrofluorometry at emission and excitation settings of 465 and 535, respectively (12).

+ Data are expressed as relative F-actin content normalized to a value of 1.00 for unstimulated cells, (number of replicate filters).

§ Significance of difference vs. control, two-tailed Dunnett’s test.

Discussion

In the present study we have employed the fluorescent Ca^{2+}-indicator, quin2, to monitor changes in endothelial cytosolic free Ca^{2+} after histamine stimulation. Due to the relatively low quantum yield of quin2, measurements of [Ca^{2+}], are more reliably calibrated using cell suspensions as opposed to adherent monolayers. In the case of the endothelial cell, this represents a clear-cut departure from the physiologic state. Nonetheless, calcium homeostasis in suspended cells appears intact, as evidenced by resting and stimulated [Ca^{2+}], in a range generally considered physiologic (5), and preservation of cellular mechanisms that effect a rapid decline in Ca^{2+} from peak-stimulated levels. In addition, in preliminary experiments, histamine increases [Ca^{2+}]; in a concentration- and H_{1}-dependent fashion in adherent endothelial cells grown on fibronectin-coated spectrofluorometer cuvettes (Rotrosen and Gallin, unpublished data). Therefore, we believe that correlation of quin2 studies performed on cell suspensions with functional studies of cells in monolayer (as done here) is both valid and informative.

Quin2 loading was not toxic to cultured umbilical vein endothelial cells as evidenced by trypan blue exclusion, lactic dehydrogenase release, reattachment of quin2-loaded cells to tissue culture dishes, and subsequent proliferation. Intracellular quin2 concentrations were easily titrated by varying loading conditions. Quin2 loadings sufficient to generate measurable Ca^{2+} signals without apparent Ca^{2+} buffering were readily achieved. While we sought to avoid high quin2 loading, intentional buffering of cytosolic free calcium may be of use in other studies examining the role of calcium in endothelial stimulus-response coupling.

H_{1} receptor occupancy elicited a concentration-dependent rise in endothelial cytosolic free calcium. Kinetic studies of histamine-stimulated cells revealed an initial rise in [Ca^{2+}], with no detectable latency, attaining peak values within 10–15 s after addition of the stimulus. The initial response was not significantly effected by the presence of Ca^{2+} in the extracellular buffer. In the absence of extracellular Ca^{2+} there followed a gradual decay of the quin2 signal to near resting levels. Of note, we used relatively mild chelation (5 mM EGTA, final pH 7.4) of Ca^{2+} in these experiments in order to effectively lower extracellular Ca^{2+} below cytosolic concentrations yet not strip Ca^{2+} from intracellular sites. The stable fluorescence of quin2-loaded cells in the face of mild Ca^{2+} chelation is indirect evidence in support of the latter point.

In contrast, cells suspended in Ca^{2+}-containing medium showed a short-lived decline from peak [Ca^{2+}]; to a sustained level two- to threefold above resting; continued H_{1} receptor occupancy was required for the latter. Intracellular Ca^{2+} stores were fully depleted within minutes of continuous H_{1} receptor occupancy. The fact that [Ca^{2+}]; remains stably elevated in the face of Ca^{2+} influx without rep influx of intracellular pools indicates that histamine-induced Ca^{2+} efflux occurs concomitantly.

 alter the response to histamine, and albumin diffusion was not significantly effected by the H_{2} agonist dimaprit. In the presence of albumin (which binds ionomycin) 10^{-7} M ionomycin increased endothelial cell [Ca^{2+}], more than twofold and promoted albumin diffusion (percent change in trypan--albumin diffusion, 206 ± 46, n = 2, P < 0.05, 10^{-7} M ionomycin vs. control).

F-Actin Content of Histamine-stimulated Endothelial Cells

To determine whether a histamine-induced cytoskeletal alteration might underly the changes in endothelial monolayer permeability to albumin, we stained endothelial cells with NBD-phallicidin, a fluorescent marker of F-actin. Stained cells are readily extracted with methanol, yielding a qualitative index of actin polymerization (12). The F-actin content of endothelial cells exposed to 10^{-5} M histamine for 5-min was significantly decreased (relative F-actin content, 0.76 ± 0.07, n = 8, vs. 1.00 ± 0.05, n = 12, histamine-exposed vs. -unexposed cells, respectively, P < 0.05). Of note, H_{1} receptor occupancy was required for the histamine-elicted changes in F-actin content, and changes of a similar magnitude were seen when endothelial cells were exposed to the calcium ionophore, ionomycin (Table I).

Figure 9. Effect of histamine, H_{1} and H_{2} agonists and antagonists, or ionomycin on trypan blue–albumin diffusion across endothelial cell monolayers. Endothelial cell monolayers were exposed for 30 min at 37°C to 10^{-5} M histamine ± 10^{-7} M pyrilamine or 10^{-4} M cimetidine, 10^{-4} M dimaprit, or 10^{-7} M ionomycin, as indicated. Trypan blue–albumin diffusion was measured as in Fig. 8. Data represent mean ± SEM of six experiments (control vs. histamine-stimulated) or two experiments (control vs. dimaprit, ionomycin, or histamine plus pyrilamine or cimetidine) each performed in triplicate. * P < 0.05 vs. control, two-tailed Dunnett’s multiple comparison test. In parallel experiments, exposure of endothelial cell monolayers to pyrilamine or cimetidine alone did not significantly alter trypan blue–albumin diffusion.

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The exact mechanisms underlying the initial decline in \([\text{Ca}^{2+}]\) from peak levels cannot be discerned from data presented here, but may include buffering by intracellular calcium binding proteins, and sequestration within the cell or efflux from the cell via \(\text{Ca}^{2+}\)-ATPase pumps or \(\text{Na}^{+}/\text{Ca}^{2+}\) exchange.

The calcium channel blockers cobalt and manganese each inhibited the sustained rise in \([\text{Ca}^{2+}]\), attributed to histamine. As noted above, although the presence of cobalt or manganese clearly precludes quantitative determination of \([\text{Ca}^{2+}]\), the qualitative assessment of their effects (i.e., that \(\text{Ca}^{2+}\) influx is required for sustained elevation of \([\text{Ca}^{2+}]\), after histamine) is consistent with experiments conducted in the absence of extracellular calcium (Fig. 2).

In prior electrophysiologic studies (23), endothelial cells were depolarized by histamine, an effect attributed to passage of the inward calcium current. In those studies, histamine-stimulated cells remained depolarized so long as the agonist and extracellular \(\text{Ca}^{2+}\) were present. Our results are in accord with that notion of \([\text{Ca}^{2+}]\) required extracellular calcium and continued histamine receptor occupancy. It is noteworthy that depolarization by pharmacologic agents or high K\(^+\) buffers promoted lateral diffusion of integral membrane proteins, thus favoring dissociation of tight junctions in freshly isolated epithelial cells (37). To our knowledge, analogous studies have not been conducted with endothelial cells.

Since Majno and Palade (17) initially proposed a role for active endothelial contraction in histamine-induced alterations in vascular permeability, the underlying mechanisms have been the subject of numerous studies. Ultrastructural and functional studies have shown that changes in endothelial cell shape, formation of interendothelial gaps, and altered vascular permeability follow the local application of histamine (10, 16, 17, 18, 29, 30) or stimuli of mast cell degranulation (32). The endothelial cytoskeleton is comprised, in part, of actin and myosin filaments immunohistochemically indistinguishable from contractile elements found in smooth muscle (3). Simionescu et al. (30) localized these filaments (the perijunctional filament web) to regions of interendothelial contact, and showed preferential distribution of histamine receptors to the overlying plasmalemma (10).

The early mobilization of calcium from intracellular pools followed by influx of extracellular calcium are remarkably similar in kinetics and molar histamine dependency to events after histamine stimulation of smooth muscle, in which \(\text{Ca}^{2+}\) is thought to be the excitation-contraction coupler (20). In smooth muscle a rise in cytosolic \(\text{Ca}^{2+}\) leads to an increase in \(\text{Ca}^{2+}\) bound to calmodulin. The \(\text{Ca}^{2+}\)-calmodulin complex activates myosin light chain kinase and the resulting phosphorylation of myosin light chains permits myosin-actin cross bridge cycling, or contraction (5). Alternatively, cytosolic \(\text{Ca}^{2+}\) might indirectly modulate endothelial cytoskeletal architecture by altering the activity of the F-actin fragmenting protein, gelosin (36). Several lines of evidence support a role for \(\text{Ca}^{2+}\) in the regulation of cytoskeletal structure or as an excitation-contraction coupler after histamine stimulation. D’Amore and Shepro (2) showed that histamine stimulated an early rise in endothelial cell-associated \(4^{\text{Ca}}^+\), though histamine effects on \(\text{Ca}^{2+}\) efflux, total cellular \(\text{Ca}^{2+}\), and cytosolic \(\text{Ca}^{2+}\) were not examined. In other studies the changes in endothelial permeability and cell shape attributed to histamine were mimicked by calcium ionophores, and were not observed if the experiments were conducted in \(\text{Ca}^{2+}\)-free medium or in the presence of calcium channel blockers (16, 27, 32).

Based on the results of our quin2 experiments, we designed functional studies to examine the role of \(\text{Ca}^{2+}\) in modulation of endothelial monolayer permeability. Since ionophores and calcium agonists, including histamine, promote subtle and inconsistently observed changes in endothelial cell shape (8, 14), we used a model of albumin diffusion across endothelial monolayers grown on polycarbonate filters as an indirect means to monitor alterations in endothelial cell shape, cell–cell, or cell–substratum interactions. In this model, histamine enhanced albumin diffusion in a concentration-dependent fashion. Concentrations of histamine required to augment monolayer permeability were of the same order of magnitude as those shown to elevate endothelial \([\text{Ca}^{2+}]\). Killackey et al. (14) have recently demonstrated histamine-induced interendothelial gap formation and dye diffusion between endothelial cells grown on microcarrier beads. In that study, histamine elicited minimal alterations in morphology, despite relative increases in dye diffusion of a similar magnitude, and at similar histamine concentrations to those we noted. Another study presented in abstract demonstrated less subtle calcium-dependent alterations in bovine pulmonary endothelial cell architecture but required considerably higher concentrations of extracellular calcium to consistently observe the effect (35). Using a model similar to ours, Shasby et al. (27) demonstrated a calcium-dependent enhancement of albumin diffusion across monolayers of pulmonary endothelial cells exposed to reversible oxidative stress. In that study, calcium-dependent changes in stress fiber staining and architecture were thought to underlie the changes noted in cell shape and monolayer permeability. Using a digitized image analysis system, Shepro and Hechtman (28) have documented a decrease in F-actin in endothelial cells exposed to agents known to increase vascular permeability. In the present study we have shown a decrease in endothelial F-actin in cells exposed to histamine or ionomycin. It is not known whether the histamine-induced changes in endothelial cell shape noted in vivo (or the changes in albumin diffusion in the present study) truly reflect alterations in intercellular junctions, active endothelial cell contractility, or passive retraction consequent to an altered cell–substratum attachment. Nonetheless, while induction of the inflammatory response depends upon complex interactions involving circulating cells, endothelial cells, soluble mediators, and nonendothelial cells of the vessel wall, the data presented here support a central role for cytosolic \(\text{Ca}^{2+}\) in the histamine-elicited endothelial changes that most likely contribute to altered vascular permeability.

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