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 $5 \times 10^{-6}$ 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 H$_1$ 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 $5 \times 10^{-6}$ 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 immunocytochemically indistinguishable from those of smooth muscle (3) and the preferential concentration of these in regions of interendothelial contact (30). Hel- tianu et al. (10) subsequently localized endothelial histamine receptors to the plasmalemma overlying 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-
bility that permeability changes result from cytoskeletal alterations governing cell–cell or cell–substrate interactions has received renewed attention. Indirect evidence in support of a calcium-mediated effect on the endothelial cell cytoskeleton includes the observations that endothelial cells undergo shape changes on exposure to the calcium ionophore A23187 or to histamine, and that changes in vascular permeability or cell shape on exposure to the latter agent are not seen when the experiments are conducted in the presence of cytochalasin B or in Ca\(^{2+}\)-free medium (16, 22, 27, 32). \(^{45}\)Ca\(^{2+}\) flux studies have demonstrated both influx and efflux of Ca\(^{2+}\) in endothelial cells exposed to inflammatory mediators (1, 2). Unfortunately, demonstration of isotope fluxes may be greatly influenced by a variety of technical considerations, and flux studies yield no direct information on the intracellular compartmentalization of the isotope in question.

We have recently used the fluorescent Ca\(^{2+}\) indicator, quin2, to examine endothelial cell responses to a variety of inflammatory mediators. In this report we describe a histamine-induced endothelial cell Ca\(^{2+}\) transient attributable to occupancy of specific H\(_1\) receptors, and demonstrate a decrease in endothelial cell F-actin content and enhanced diffusion of a macromolecular marker across endothelial monolayers exposed to H\(_1\) receptor activation and calcium ionophores.

### Materials and Methods

#### Materials

Assays were routinely conducted using Hanks’ balanced salt solution (HBSS; Whittaker M. A. Bioproducts, Walkersville, MD) containing 136.9 mM NaCl, 5.4 mM KCl, 0.34 mM NaH\(_2\)PO\(_4\), 1.3 mM Ca\(_{10}\), 0.8 mM MgSO\(_4\), 4.2 mM NaHCO\(_3\), and 5.6 mM glucose. In selected experiments modified balanced salt solutions were prepared using chemicals of reagent grade.

Experiments examining the effects of Ca\(^{2+}\) and Mg\(^{2+}\) were conducted in phosphate-, sulfate-, and bicarbonate-free HBSS prepared by substitution of MgCl\(_2\) for MgSO\(_4\) and buffered with 10 mM Hepes. In some experiments the concentration of potassium was varied from 5-120 mM at constant osmolarity 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, heparin, gelatin, BSA, Tris (Tris base), Triton X-100, oligomycin, toludine blue, quin2 aceoxymethyl ester (quin2/AM), quin2-free acid, DMSO, and verapamil (Sigma Chemical Co., St. Louis, MO); medium 199, FCS, l-glutamine (Gibco, Grand Island, NY); ionomycin (Calbiochem-Behring Corp., San Diego, CA); endothelial cell growth factor, human fibronectin (McIver Laboratories, Inc., Spring- field, VA); type I collagenase (Cooper Worthington, Freehold, NJ); EDTA (Fisher Scientific Co., Fairlawn, NJ); EGTA (Eastman Kodak Co., Rochester, NY); 24-well tissue culture plates (Costar, Cambridge, MA); 13-mm diameter 5-μm pore size polyvinylpyrrolidone-free polycarbonate filters (Nucleopore Corp., Pleasanton, CA); trypan blue (Allied Chemical Corp., Morris- town, NJ); \(\text{[Ca}^{2+}\text{]}_\text{o}\) (specific activity, 57 mCi/mmole) and \(\text{[H}^\text{3} \text{H]}_\text{o}\) (specific activity, 10.1 Ci/mmol; New England Nuclear, Boston, MA); Versilube F50 silicone fluid (General Electric, Waterford, NY); NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL); and 3a20 liquid scintillation counting fluid (Research Products International Corp., Mt. Prospect, IL).

**1. Abbreviation used in this paper:** NBD, nitrobenzoxadiazole.

#### Fluorescence Measurements

Fluorescence measurements were performed using a fluorescence spectrophotometer interfaced with an R-900A recorder (model LS5, 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 (Starstedt, Federal Republic of Germany) loaded with 2 ml of cell suspension, 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.
effective dissociation constant of 115 nM for Ca$^{2+}$ binding to quin2. In certain experiments, the Ca$^{2+}$ concentration of HBSS was rapidly decreased to <20 nM, as described by Sklar and Oades (31), by mild chelation with 5 mM EGTA, maintaining buffer pH at 7.4; calibrations were performed after reconstitution of the balanced salt solution by addition of sufficient CaCl$_2$ to yield final [Ca$^{2+}$] equivalent to that of HBSS. All stimuli, inhibitors, and calibrating reagents were added as 10-20-µl aliquots (0.5-1.0% of the cuvette sample volume); fluorescence values were not corrected for the resulting sample dilution. The Ca$^{2+}$-dependent fluorescence of quin2-loaded lysed cells in the modified balanced salt solutions used in selected experiments did not differ significantly from that obtained in HBSS. With the exception of cobalt and manganese, all compounds used as stimuli or inhibitors in fluorometric assays were shown not to alter the autofluorescence of unloaded cells or effect the Ca$^{2+}$-dependent fluorescence of quin2-free acid. Cellular quin2 contents were determined by comparing the fluorescence of lysed quin2-loaded cells with the fluorescence of known concentrations of quin2-free acid in the presence of lysed unloaded cells. Intracellular quin2 concentrations were calculated taking into account the known concentrations of quin2-free acid in the presence of lysed unloaded cells. Intracellular quin2 concentrations were calculated taking into account an absorption maximum at 590 nm. At Concentration, concentrations used in the diffusion experiments, compounds added to upper wells did not alter the spectral properties of the trypan blue-albumin complex.

**Preparation 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.

**F-actin Staining and Quantitation**

Endothelial cells grown on fibronectin-coated polycarbonate filters were exposed to 10$^{-3}$ 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 HBSS, 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 exposed 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.

**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.

**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 x 10$^5$ cells/ml, 5 x 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 ± 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 µl/10$^6$ cells, the

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**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 x 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.
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 lactic 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+]i 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 × 10^-5 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 × 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/10^6 cells (A40% × 740 pmol quin2/10^6 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 × 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 × 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.

Figure 2. Effect of extracellular Ca2+ on kinetics and magnitude of the histamine-elicited rise in endothelial cell [Ca2+]. (A) Representative tracing of quin2-loaded endothelial cells suspended in HBSS (Ca2+, 1.3 mM) without or with 5 mM EGTA (final pH 7.4, Ca2+ <20 nM) added 30 s before 10^-6 M histamine. Intracellular quin2 concentration in these cells was 0.37 mM; 740 pmol quin2/10^6 cells. (B) Data represent mean of triplicate cell suspensions in Ca2+-containing and Ca2+-free medium. * P < 0.05, Student's t test comparing geometric mean [Ca2+]i values with corresponding values for cells in Ca2+-containing medium.
Figure 3. Depletion of intracellular Ca\(^{2+}\) stores by histamine. Representative tracings of quin2-loaded endothelial cells suspended in HBSS and exposed to 5 × 10\(^{-6}\) M histamine followed at varying times by 5 mM EGTA (final pH 7.4) 15 s before addition of 10\(^{-6}\) M ionomycin. Release of residual intracellular Ca\(^{2+}\) stores is demonstrated by an increase in fluorescence at 2 min, but not at 4 min, after histamine.

Figure 4. Effect of Ca\(^{2+}\) channel blockers on histamine-elicited Ca\(^{2+}\) transients. Representative tracings of quin2-loaded endothelial cells suspended in modified HBSS (bicarbonate-, sulfate-, and phosphate-free) and exposed to 2 mM Co\(^{2+}\) or 2 mM Mn\(^{2+}\) 1 min before addition of 10\(^{-4}\) M histamine. The immediate step-off in fluorescence on addition of Co\(^{2+}\) or Mn\(^{2+}\) exceeds that seen with EGTA (Fig. 2) because Co\(^{2+}\) and Mn\(^{2+}\) quench both the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent fluorescence of extracellular (leaked) quin2 (II).

Figure 5. Effect of verapamil on histamine-stimulated Ca\(^{2+}\) transients. Representative tracing of quin2-loaded endothelial cells suspended in HBSS and exposed to histamine alone, 2 × 10\(^{-5}\) M verapamil 2 min before histamine, or to 2 × 10\(^{-5}\) M verapamil and increased extracellular Ca\(^{2+}\) before histamine. The inhibitory effects of verapamil were observed only at submaximal histamine concentrations, and were not overcome by raising extracellular Ca\(^{2+}\) to 10 mM.

(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\(^{-8}\) M), caused a rightward shift in the histamine dose-response curve (Fig. 6 A), and at higher concentrations (10\(^{-6}\) M pyrilamine, data not shown), completely blocked the rise in [Ca\(^{2+}\)], attributed to 10\(^{-4}\) 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.
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 [Ca\(^{2+}\)] of endothelial cells suspended in depolarizing buffers (22). When endothelial cells were suspended in 5 mM K\(^+\) or 120 mM K\(^+\) buffers, neither resting [Ca\(^{2+}\)] (97 ± 6 nM vs. 99 ± 2 nM, low vs. high K\(^+\), respectively, n = 3); peak stimulated [Ca\(^{2+}\)] (860 ± 31 nM vs. 898 ± 64 nM, low vs. high K\(^+\), respectively, n = 3); nor [Ca\(^{2+}\)] 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 monolayers in a concentration-dependent manner (Fig. 8), with a half-maximal effect (through the range of concentrations studied) at 5.6 x 10^{-6} M histamine, similar to that noted for the histamine-elicited rise in [Ca\(^{2+}\)]. To further probe the underlying mechanisms, parallel experiments were conducted with the addition of selective H\(_1\) and H\(_2\) agonists and antagonists, and the calcium ionophore, ionomycin (Fig. 9). Pyrilamine (10^{-7} M) blocked the effects of 10^{-5} M histamine (percent change in trypan–albumin diffusion, 7 ± 19, n = 2, histamine in the presence of pyrilamine vs. 172 ± 39, n = 6, histamine alone, P < 0.05). In contrast, the H\(_2\) antagonist cimetidine did not significantly
Alteration might underly the changes in endothelial monolayer permeability to albumin, we stained endothelial cells with NBD-phallacidin, a fluorescent marker of F-actin. Stained cells are readily extracted with methanol, yielding a quantitative 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, H₁ 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).

**Discussion**

In the present study we have employed the fluorescent Ca²⁺ indicator, quin2, to monitor changes in endothelial cytosolic free Ca²⁺ after histamine stimulation. Due to the relatively low quantum yield of quin2, measurements of [Ca²⁺]ᵢ 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 [Ca²⁺], in a range generally considered physiologic (5), and preservation of cellular mechanisms that effect a rapid decline in Ca²⁺ from peak-stimulated levels. In addition, in preliminary experiments, histamine increases [Ca²⁺]ᵢ in a concentration- and H₁-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 Ca²⁺ signals without apparent Ca²⁺ 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.

H₁ receptor occupancy elicited a concentration-dependent rise in endothelial cytosolic free calcium. Kinetic studies of histamine-stimulated cells revealed an initial rise in [Ca²⁺]ᵢ, 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 Ca²⁺ in the extracellular buffer. In the absence of extracellular Ca²⁺ 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 Ca²⁺ in these experiments in order to effectively lower extracellular Ca²⁺ below cytosolic concentrations yet not strip Ca²⁺ from intracellular sites. The stable fluorescence of quin2-loaded cells in the face of mild Ca²⁺ chelation is indirect evidence in support of the latter point.

In contrast, cells suspended in Ca²⁺-containing medium showed a short-lived decline from peak [Ca²⁺]ᵢ to a sustained level two- to threefold above resting; continued H₁ receptor occupancy was required for the latter. Intracellular Ca²⁺ stores were fully depleted within minutes of continuous H₁ receptor occupancy. The fact that [Ca²⁺]ᵢ remains stably elevated in the face of Ca²⁺ influx without repletion of intracellular pools indicates that histamine-induced Ca²⁺ efflux occurs concomitantly.

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Table I. Change in F-actin Content of Endothelial Cells Exposed to Histamine or Calcium Ionophore

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<thead>
<tr>
<th>Stimulus</th>
<th>Relative F-actin content</th>
<th>P*</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.05 (12)</td>
<td></td>
</tr>
<tr>
<td>10⁻³ M histamine</td>
<td>0.76 ± 0.07 (8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>10⁻⁵ M histamine</td>
<td>1.03 ± 0.05 (8)</td>
<td></td>
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<tr>
<td>+ 10⁻⁷ M pyrilamine</td>
<td>0.72 ± 0.06 (8)</td>
<td>&lt;0.01</td>
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<tr>
<td>10⁻⁷ M ionomycin</td>
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* Endothelial cells grown to confluency 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, stained with 80% acetone, and stained with NBD-phallacidin (1.65 × 10⁻⁶ M). Methanol-extractable fluorescence was measured by spectrophotometry 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.

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Rotrosen and Gallin Endothelial Ca²⁺ and Monolayer Permeability
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 extrusion from the cell via $\text{Ca}^{2+}$-ATPase pumps or Na$^+$/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 in tonic elevation 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, gelsolin (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 $^{45}\text{Ca}^{2+}$, 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 $[\text{Ca}^{2+}]$. Killacky 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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