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Histamine selectively interrupts VE-cadherin adhesion independently of capacitive calcium entry

Michael C. Winter, Sandra S. Shasby, Dana R. Ries, and D. Michael Shasby

Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242

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Winter, Michael C., Sandra S. Shasby, Dana R. Ries, and D. Michael Shasby. Histamine selectively interrupts VE-cadherin adhesion independently of capacitive calcium entry. *Am J Physiol Lung Cell Mol Physiol* 287: L816–L823, 2004. First published June 25, 2004; 10.1152/ajplung.00056.2004.—Histamine is an important agent of innate immunity, transiently increasing the flux of immune-competent molecules from the vascular space to the tissues and then allowing rapid restoration of the integrity of the endothelial barrier. In previous work we found that histamine alters the endothelial barrier by disrupting cell-cell adhesion and identified VE-cadherin as an essential participant in this process. The previous work did not determine whether histamine directly interrupted VE-cadherin adhesion, whether the effects of histamine were selective for cadherin adhesion, or whether capacitive calcium flux across the cell membrane was necessary for the effects of histamine on cell-cell adhesion. In the current work we found that histamine directly interrupts adhesion of L cells expressing the type 1 histamine (H1) receptor and VE-cadherin to a VE-cadherin-Fc fusion protein. In contrast, integrin-mediated adhesion to fibronectin of the same L cells expressing the H1 receptor was not affected by histamine, demonstrating that the effects of histamine are selective for cadherin adhesion. Some of the effects of many edemagenic agonists on endothelium are dependent on the capacitive flux of calcium across the endothelial cell membrane. Blocking capacitive calcium flux with $LaCl_3$ did not prevent histamine from interrupting VE-cadherin adhesion of transfected L cells, nor did it prevent histamine from interrupting cell-cell adhesion of human umbilical vein endothelial cells. These data support the contentions that histamine directly and selectively interrupts cadherin adhesion and this effect on cadherin adhesion is independent of capacitive calcium flux.

endothelium; integrin; human histamine receptor H1; vascular endothelial cadherin

ACUTE INFLAMMATORY EDEMA is an important component of innate immunity that facilitates the transfer of immune-competent molecules from the vascular space to the tissues. Histamine and serotonin are well-acknowledged physiological agonists of acute inflammatory edema that disrupt cell-cell apposition in postcapillary venules (12, 13, 25). The disruptions in the integrity of the endothelial layer caused by these agonists are very small, <600 nm, with a half-life of ~2 min (4, 12, 13, 16, 25). Gaps also develop between adjacent endothelial cells after chelation of extracellular calcium, an intervention that interrupts sites of cell-cell and cell-matrix adhesion (21). Pretreatment of the endothelial cells with an inhibitor of myosin light chain kinase, with consequent reduction of the phosphorylation of myosin light chain and reduction of the basal tension within the cells, before interrupting cell adhesion, limits gap formation between adjacent cells (15, 22). Hence, the resting tension within the cells is sufficient for gap formation if sites of adhesion are interrupted.

Vascular endothelial (VE)-cadherin is an endothelial unique cadherin that mediates cell-cell adhesion of endothelial cells (17). Function-interrupting antibody to VE-cadherin increases permeability of endothelium in vivo, indicating that integrity of VE-cadherin adhesion is essential to the integrity of the endothelial barrier (9, 16). It was of note that although antibody to VE-cadherin increased in vivo endothelial permeability within 1 h of its administration, no light microscopic changes were evident in the endothelium even at 2 h, demonstrating that no large gaps had formed between the cells. However, similar to the effects of histamine and serotonin, transmission electron micrographs detected very small gaps in lung microvessels exposed to antibody to VE-cadherin (9).

ECV304 cells are a transformed bladder epithelial cell line that express the type 1 histamine receptor (H1) and P- and N-cadherin, but not VE-cadherin. When mock-transfected ECV304 cells were exposed to histamine, there was no change in the electrical resistance of the cell-cell barrier they created (24). However, when ECV304 cells transfected with VE-cadherin were exposed to histamine, the electrical resistance of the cell-cell barrier fell, identifying an important role for VE-cadherin in the response to histamine of a monolayer of polarized cells (24). ECV304 cells express claudin 2 and develop tight junctions (5). This tight junction is a necessary component of the electrical resistance decreased by histamine. The observation that histamine decreased the integrity of the cell-cell barrier created by VE-cadherin transfected ECV304 cells did not discriminate between effects of histamine on an interaction of VE-cadherin with the tight junction versus a direct effect of histamine on VE-cadherin adhesion.

Histamine and other agonists that increase endothelial permeability initiate signaling in endothelial cells that increases cell calcium (6, 7). Although some of the increase in cell calcium caused by these agonists reflects release of calcium from intracellular stores, capacitive flux of calcium across the cell membrane is also activated, and the capacitive calcium flux is necessary for some of the effects of these agonists on endothelial cells (23, 26).

In these investigations we used a model of cadherin adhesion that lacks tight junctions and asked whether histamine directly affects VE-cadherin adhesion as opposed to affecting an interaction between VE-cadherin and the tight junction. Our results indicate that histamine directly affects VE-cadherin adhesion. Using a similar model of integrin-based adhesion, we found

Address for reprint requests and other correspondence: D. M. Shasby, Dept. of Internal Medicine, Univ. of Iowa College of Medicine, 140E EMRB, Iowa City, IA 52242 (E-mail: michael-shasby@uiowa.edu).

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that histamine did not directly affect integrin adhesion. Hence, the effects of histamine are selective and do not affect all sites of adhesion. We also used the cadherin-based model to ask if the capacitive flux of calcium across the cell membrane initiated by histamine was important for the effect of histamine on VE-cadherin adhesion. The effects of histamine on VE-cadherin adhesion did not require capacitive calcium entry into the cells. Extending these observations to endothelium, we also found that the effects of histamine on endothelial cell-cell adhesion were not dependent on capacitive calcium entry into the endothelial cells.

MATERIALS AND METHODS

Materials. Fibronectin was from Collaborative Research (Bedford, MA). Tissue culture media and serum were from the Tissue Culture Core, University of Iowa. ECV304 cells and L cells were from ATCC (Rockville, MD). Antibody to VE-cadherin (mouse IgG, monoclonal, clone 55-7H1) was from PharMingen. Antibody to N-cadherin (mouse monoclonal) was a generous gift from Janne Balsamo (3). The pLKneo plasmid and the E-cadherin-human Fc fusion protein cDNA were generous gifts from W. James Nelson. Secondary antibody was sheep anti-mouse IgG conjugated with horseradish peroxidase from Amersham. Fura 2 was from Molecular Probes (Junction City, OR).

Cells. Primary cultures of human umbilical vein endothelial (HUVE) cells were grown as previously described (20). L cells were grown in DMEM with 10% FBS, penicillin (100 μ/ml), and streptomycin (100 μg/ml).

L cells transfected with VE-cadherin in the vector pLKneo were grown as above with the addition of G418 (1 mg/ml) for selection. L cells expressing VE-cadherin in pLKneo and the human histamine receptor (H1) in pcDNA 3.1 were grown as above with the addition of G418 (1.4 mg/ml) and zeocin (1 mg/ml) for selection. When zeocin was present penicillin and streptomycin were eliminated.

HEK-293 cells containing a cDNA for the VE-cadherin-human-Fc fusion protein were grown in DMEM with 10% FBS and hygromycin (200 mg/ml) as described by Chen and Nelson (8).

Plasmid preparation and transfection. A cDNA for the human histamine receptor was developed using primers based on the sequence published by Fukui et al. (10). RNA was made from ECV304 cells using RNA Stat (Tel-Test). ECV304 cDNA was made using the oligo(dt) primers in the Advantage RT for PCR kit (Clontech Laboratories). A PCR reaction was set up to amplify the H1 receptor using the cDNA from the above RT reaction and the oligonucleotides 5′-ATAACTGGCGGCTGCTCTTGCG-3′ and 5′-ATCCCCTCAGAGCCTCCCTTAG-3′ corresponding to the NH_2-terminal and COOH-terminal regions of the gene. The pFX platinum PCR kit (Invitrogen) was used. The product was run on a 1% agarose gel and then purified using β-agarose I (New England Biolabs). The purified PCR product was ligated into pCR-Blunt (Invitrogen). The directionality of human histamine receptor within pCR-Blunt was determined by restriction analysis. The human histamine receptor sequence was removed from the pCR-Blunt vector and ligated into pcDNA 3.1 (zeo) at the *Hin*dIII and *Xba*I sites. Sequence and directionality were verified by sequencing using the T7 promoter primer and the BGH reverse primer.

L cells were transfected with this plasmid using Lipofectamine Plus per the manufacturer's directions. Transfected cells were grown in DMEM with 10% FBS and selected with zeocin (1 mg/ml). Clones were isolated based on an increase in cell calcium in response to histamine.

James Nelson generously shared a cDNA for a chimeric protein whose NH_2 terminus is the extracellular domain of E-cadherin and whose COOH terminus is the human Fc domain (8). For the studies proposed in this paper we exchanged the E-cadherin extracellular domain with a VE-cadherin extracellular domain (including all but two terminal COOH amino acids of the fifth extracellular repeat). We created primers to amplify cDNA for the VE-cadherin extracellular domain from our own construct of VE-cadherin in pcDNA3 (24). The construct had an *Eco*RI restriction site in the 5′-region and we mutated the 3′-region to create a *Xho*I site. The 5′-primer was: 5′-GAACCCACTGCTTACTGGCTTAATCG-3′. The 3′-primer was: 5′-GTCTATTGCGGAGATCTCGAGGACCAGCTGGCC-3′. The PCR product was isolated and ligated into pCRBlunt using *Eco*RI and *Xho*I restriction sites. The cDNA was amplified and sequenced. It was then cut from pCR-Blunt using *Eco*RI and *Xho*I sites. The E-cadherin extracellular domain was removed from pUFc which contained the E-cadherin-Fc cDNA by cutting at *Eco*RI and *Xho*I sites (8). The E-cadherin was replaced with the VE-cadherin extracellular domain cDNA, also restricted at *Eco*RI and *Xho*I sites, 5′ and 3′, respectively. This created a VE-cadherin-Fc cDNA. After amplification and sequencing this was cut from pUFc at *Kpn*I and *Not*I sites and placed into pCEP4. After sequencing this product we noted that there was an extra start site 5′ to the desired start site. This had been incorporated during the pCR-Blunt shuttle, and it was in frame with the cadherin start site. A computer simulation of transcription indicated that the extra site might be the preferred start. Hence, we mutated this start (ATG) to an ATC using the Quick Change mutagenesis kit from Stratagene and the following primers: 5′-primer, 5′-CGTTGACGCAAATCGGCGGTAGGCGTGTACG-3′; 3′-primer, 5′-CGTACACGCCTACCGCCGATTTGCGTCAACG-3′. The entire mutated cDNA was again sequenced, and then HEK-293 cells were transfected with the sequence in pCEP4 as described (8). The protein was isolated on a protein A column and eluted with citric acid. After electrophoresis and transfer, the protein reacted with antibodies to VE-cadherin and to human Fc at the expected molecular mass for the chimeric protein (112 kDa).

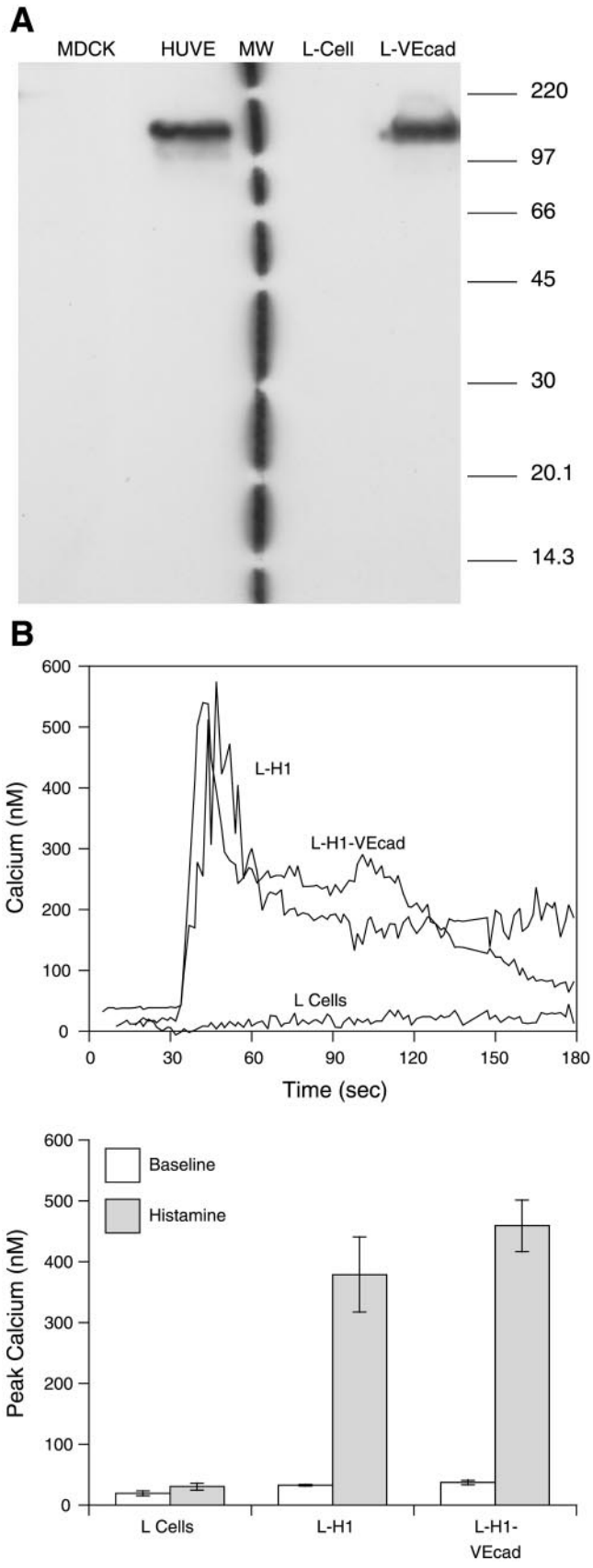
VE-cadherin, in the vector pLKneo, was used in ECV304 cells as previously described (24).

L cells expressing the histamine receptor in the vector pcDNA 3.1 zeo were transfected with the VE-cadherin-pLKneo plasmid using Lipofectamine Plus. Doubly transfected cells were selected with zeocin (1 mg/ml) and G418 (1.4 mg/ml). Clones were isolated based on expression of VE-cadherin.

Analysis of protein expression. Protein was solubilized from VE-cadherin and N-cadherin expressing L cells with SDS sample buffer, and equal masses of cell proteins were separated on 8% PAGE gels and transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine, 15% methanol, and 0.02% SDS buffer for 18 h at 4°C and 18 V. The membranes were blocked with 5% nonfat milk, 1% Tween 20 in 10 mM Tris, and 150 mM NaCl buffer. Blocked membranes were incubated with primary antibody in blocking buffer for 2 h at 27°C, washed 3× with blocking buffer, and then incubated with the secondary antibody (sheep anti-mouse IgG). Blots were examined with enhanced chemiluminescence (Amersham).

To detect surface expression of VE-cadherin, transfected and nontransfected cells were grown as indicated above. Cells expressing VE-cadherin in the dexamethasone-responsive vector pLKneo were cultured with the addition of dexamethasone (1 μM) for 18 h, cultured without dexamethasone for 24 h, washed in PBS, and released from the plates in 137 mM NaCl, 4.2 mM $NaHCO_3$, 5.4 mM KCl, 5.6 mM glucose, and 0.5 mM EDTA (Lifting solution). The cells were suspended in PBS, centrifuged at 150× gravity for 5 min, resuspended, and rotated in PBS containing primary antibody for 1 h at 27°C, washed 2× with PBS, and resuspended in PBS containing sheep anti-mouse IgG conjugated to FITC. Cells were rotated for 1 h at 27°C, resuspended in PBS with propidium iodide, and then analyzed for surface expression of VE-cadherin by fluorescence-activated cell sorting.

A reliable antibody for the human histamine receptor is not available. As a surrogate indicator of surface expression of the histamine receptor and of activation of signaling by ligation of the receptor, transfected L cells were loaded with fura 2 and examined for an



increase in cell calcium in response to histamine, as previously described (24).

Calcium measurements. Cells were grown on fibronectin-coated glass coverslips, loaded with fura 2-AM at 5 μg/ml for 30 min at 37°C, and rinsed three times with HBSCGA [(in mM) 135 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, and 0.05% bovine serum albumin, pH 7.4 with KOH]. EGTA (2 mM) was added in experiments with low (100 nM) free calcium (18). Fluorescence was measured at 480 and 460 nM using a Photon Technologies Imaging System. After background subtraction, ratios were compared with a standard curve to determine Ca^{2+} concentration.

Binding measurements. We adapted the adhesion assay used by Balsamo et al. (2) to measure cadherin-dependent adhesion to cadherin-Fc-coated microtiter plate surfaces. Protein G-coated 96-well plates were rinsed three times with 0.05% Tween 20 in PBS. One hundred microliters of the cadherin-Fc fusion protein (30 μg/ml) in PBS with 0.5 mM EGTA (PBS-EGTA) were added to the necessary wells and allowed to bind overnight at 5°C (PBS-EGTA alone is added to control wells). After two rinses with PBS containing 0.5 mM EGTA, HBSCGA was added to the wells and incubated at room temperature for 1 h. For experiments measuring integrin adhesion to fibronectin, 100 μl of either water, HBSGCA, or fibronectin (30 μg/ml in water) were added to tissue culture wells and incubated for 30 min at 37°C. After an additional rinse with HBSCGA, the wells were ready for the addition of cells.

Transfected L cells were seeded at 70% confluence in 60-mm tissue culture dishes. After 4 h the medium was replaced with medium containing 1 μM dexamethasone. Two days later we labeled the cells by replacing the medium with HBSGCA containing calcein AM (5 μg/ml) and incubated them for 30 min at 37°C. The cells were then rinsed with PBS, harvested (10-min incubation in 137 mM NaCl, 4.2 mM $NaHCO_3$, 5.4 mM KCl, 5.6 mM glucose, and 0.5 mM EDTA, pH 7.2), pelleted by mild centrifugation, and resuspended in HBSCGA at a final concentration of 100,000 cells/ml. For experiments using either the GRGDS or SDGRG peptides, peptide was added to the cell suspension at a final concentration of 10 μg/ml.

Two hundred microliters of the cell suspension were added to each well and allowed to bind for 45 min at 37°C. Each eight-well strip was then individually treated with histamine for the given time, removed from the cassette, and rinsed three times with HBSCGA to remove nonadherent cells. The fluorescence remaining in each well was then measured (EG&G Wallac Victor2, Gaithersburg, MD) and used as an indication of the relative number of adherent cells following background subtraction. Background binding was determined by pretreating the cadherin-Fc-coated wells with rabbit IgG to saturate the protein G, then soluble cadherin-Fc (150 μg/ml) to saturate the cadherin binding sites, and then measuring the number of cells that adhered to wells that had been pretreated in this manner. This pretreatment of the wells blocked 90 ± 2% of cell binding.

Cell impedance measurements. Cell-cell adhesion of HUVE cells was assayed by measuring the impedance of a cell-covered electrode and comparing it to the impedance of a cell-free electrode as described

Fig. 1. *A*: Western blot analysis shows expression of vascular endothelial (VE)-cadherin in transfected L cells (L-VEcad), but not in wild-type L cells (L Cell). Nontransfected L cells and Madin-Darby canine kidney (MDCK) cells are included as negative controls for VE-cadherin expression and human umbilical vein endothelial (HUVE) cells as a positive marker of VE-cadherin expression. MW, lane used for molecular weights. *B*: expression of the human histamine receptor H1 in L cells confers an increase in calcium when histamine is added. *Top*: addition of 10 μM histamine (30-s time point) to L cells does not alter intracellular calcium above baseline levels (measured just before addition of histamine). However, when expressing the H1 receptor either alone or in combination with VE-cadherin, histamine causes a significant increase in intracellular calcium. *Bottom*: peak calcium is determined as the maximum intracellular calcium concentration measured in the 2 min following histamine addition. VEcad, VE-cadherin.

(24). The total impedance was partitioned into its three components, the cell-cell resistance, the cell-matrix resistance, and the membrane capacitance as described. To do this we measured the impedance of the cell-covered electrode at five different frequencies (1, 2, 4, 8, and 16 kHz) every 2 s. For each of these time points, we used the downhill simplex method to determine the set of parameters (cell-cell resistance, cell matrix resistance, and membrane capacitance) that best fit the observed data (24). The software for data acquisition and analysis was written using LabVIEW (National Instruments, Austin, TX). Cells were plated on the electrode at 1.5×10^5 cells per cm^2.

Statistical analysis. Changes in cell-cell impedance and cell adhesion were compared by analysis of variance, and individual group comparisons were done using a Tukey honestly-significant-difference test for post hoc comparisons of means. Differences were considered significant at the $P < 0.05$ level.

RESULTS

Effects of histamine on cadherin adhesion. Our earlier experiments in which histamine decreased the cell-cell barrier created by ECV304 cells transfected with VE-cadherin, but not those transfected with lac-Z, identified an important role for VE-cadherin in the response of endothelial cell-cell barriers to histamine (24). However, ECV304 cells make tight junctions, and the electrical impedance signal we used to assess the cell-cell barrier could have been affected by a change in the integrity of VE-cadherin adhesion or by a change in the interaction of VE-cadherin with the tight junction. L cells do not express claudins and do not form tight junctions (11). L cells also do not express the H1 receptor as assessed by RT-PCR (D. M. Shasby, data not shown). To determine whether histamine directly affected VE-cadherin adhesion, we measured the effect of histamine on the adhesion of L cells transfected with the H1 receptor and VE-cadherin to microtiter plates coated with a VE-cadherin-Fc fusion protein. VE-cadherin expression in transfected L cells was detected by Western blotting (Fig. 1*A*). There are no effective antibodies to the H1 receptor. We confirmed its surface expression and ability to activate signaling in L cells transfected with a cDNA for the H1 receptor by examining the response of L cell calcium to histamine (Fig. 1*B*).

After confirming that the transfected cells expressed a functional H1 receptor and VE-cadherin, we examined the adhesion of L cells transfected with cadherin and the H1 receptor to a cadherin-Fc fusion protein bound to the surface of a protein A-coated microtiter plate. Pretreatment of the wells of the microtiter plate with soluble VE-cadherin-Fc blocked adhesion of VE-cadherin-expressing L cells, demonstrating that adhesion was dependent on homophilic cadherin binding (Fig. 2*A*). Similar treatment of the plates with soluble VE-cadherin-Fc did not affect adhesion of L cells not expressing VE-cadherin, demonstrating that other adhesion molecules, such as integrins, were not mediating adhesion of the cells to the VE-cadherin-Fc fusion protein (Fig. 2*A*).

Histamine interrupted the adhesion of L cells expressing the H1 receptor and VE-cadherin to the VE-cadherin-Fc fusion protein, but histamine did not affect adhesion to the VE-cadherin-Fc fusion protein of L cells expressing either the H1 receptor alone or VE-cadherin alone (Fig. 2*B*). Hence, histamine directly interrupts VE-cadherin adhesion, and the effects of histamine are not dependent on a link between VE-cadherin and the tight junction.

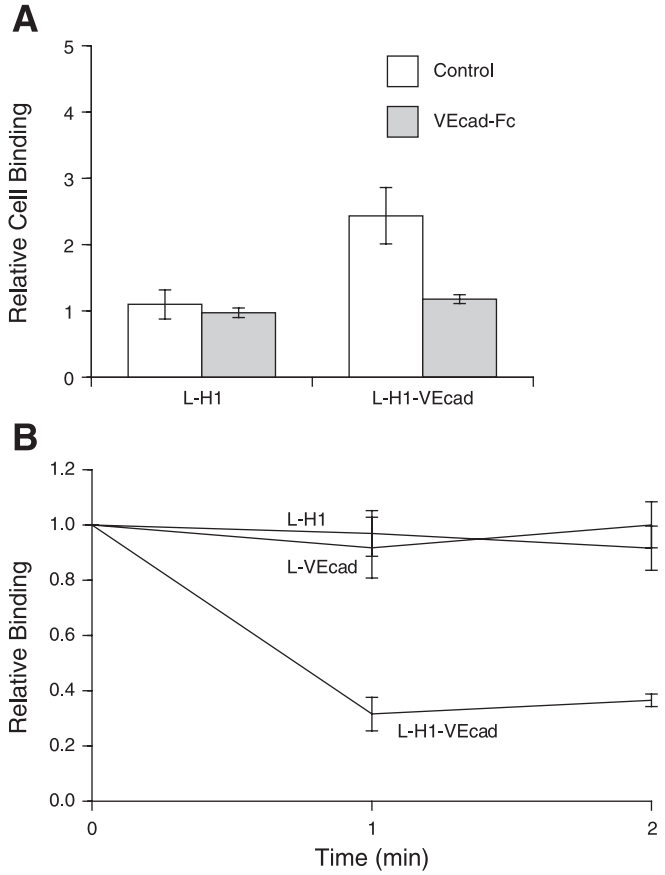


Fig. 2. *A*: binding of L cell transfectants to immobilized VEcad-Fc was measured as described and normalized to background binding in wells lacking VEcad-Fc. Under control conditions (open bars), L-H1 cells exhibited no increase in binding over background. However, there is a 2.5-fold increase in binding in cells expressing VEcad (L-H1-VEcad). Addition of excess soluble VEcad-Fc to competitively inhibit binding to the immobilized VEcad-Fc (solid bars) had no effect on L cells not expressing VE-cadherin (L-H1) but prevented binding of VE-cadherin expressing cells above background levels. *B*: histamine only decreases the binding of L cells expressing both H1 and VE-cadherin in combination. Binding of L cell transfectants was measured after 1 and 2 min of treatment with 10 μM histamine. The results are normalized to binding of cells treated with vehicle alone (zero time point). Histamine does not alter the binding of L cells expressing either H1 alone (L-H1, background binding) or VE-cadherin alone (L-VEcad). However, histamine does decrease binding of cells expressing both H1 and VE-cadherin (L-H1-VEcad) by >60%.

Selectivity of histamine's effects: integrin adhesion. The experiments just discussed demonstrate that histamine interrupts VE-cadherin adhesion. We asked whether these effects were specific to cadherin adhesion. L cells express several integrins, including $\alpha_5\beta_1$, that bind fibronectin (1). Four times as many L cells expressing the H1 receptor or the vector control (CAT) adhered to a fibronectin-coated plate compared with the same plate not coated with fibronectin (Fig. 3). Histamine did not affect the adhesion of L cells expressing the H1 receptor to fibronectin (Fig. 3). Integrin adhesion is dependent on calcium. In contrast to the lack of an effect of histamine, chelation of calcium with EGTA caused the cells to lose adhesion to the plates. Adhesion of the L cells to fibronectin was also interrupted by the peptide GRGDS, but not the peptide SDGRG, confirming the specificity of the integrin

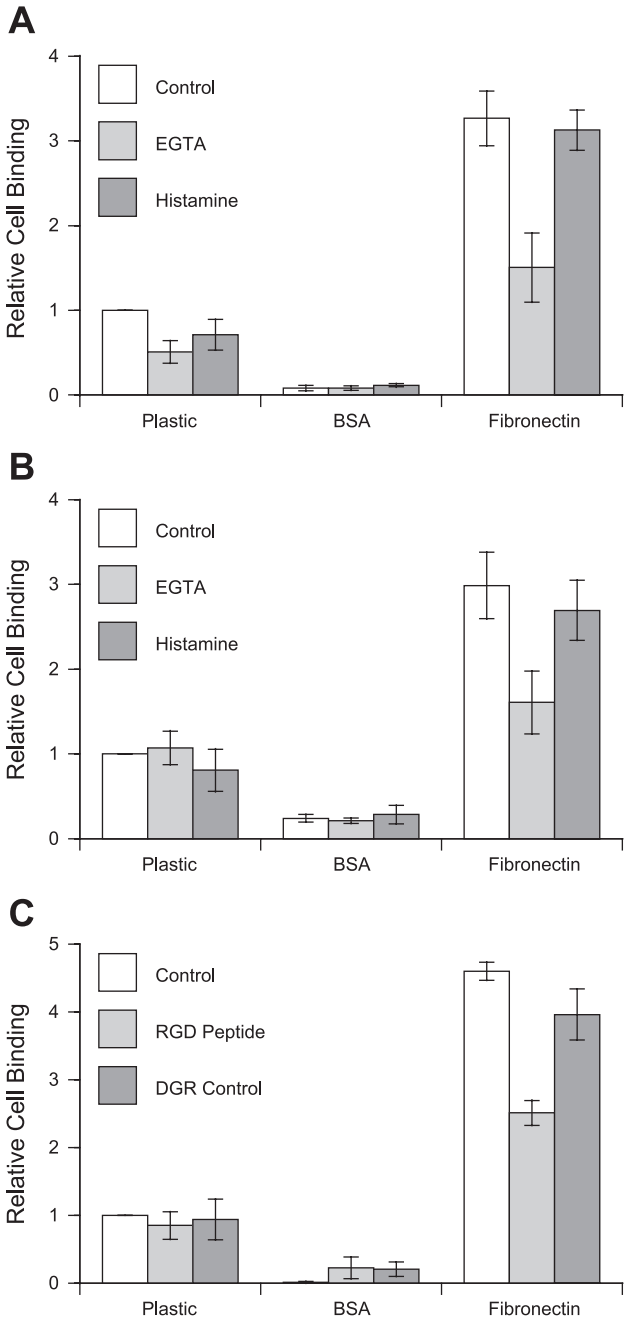


Fig. 3. Histamine does not alter the binding of L cells (*A*) or L cells expressing the H1 receptor (L-H1, *B*) to fibronectin-coated 96-well tissue culture plates. Binding of L-H1 cells to fibronectin can be inhibited by the GRGDS peptide (*C*) but not the reverse peptide (SDGRG). We measured cell binding to untreated tissue culture wells (Plastic), wells incubated with HBSCGA (BSA coated), and wells coated with fibronectin, with and without the addition of EGTA (5 mM for 5 min) or histamine (10 μM for 2 min). All values are normalized to the binding to plastic under control conditions (mock treated).

binding. Hence, the effects of histamine are selective and affect cadherin, but not integrin adhesion.

Role of capacitive calcium flux in effects of histamine on cadherin adhesion. Many agonists that alter the endothelial barrier increase cell calcium (6, 7). Some of the effects of these agonists require not only that signaling activate a release of calcium from intracellular stores, but also that there be a capacitive flux of calcium across the cell membrane (23, 26). We initially attempted to decrease extracellular calcium to between 100 and 400 nM to determine whether a flux of extracellular calcium across the cell membrane was important to the effects of histamine on adhesion of the VE-cadherin-expressing L cells to VE-cadherin. Although decreasing extracellular calcium was effective in blunting the increase in calcium (Fig. 4), these levels of extracellular calcium inhibited basal adhesion of cadherin-expressing L cells to the cadherin-Fc fusion protein to such an extent that it was not possible to get meaningful data.

As an alternative to low extracellular calcium concentrations, we used lanthanum chloride ($LaCl_3$) to block the capacitive calcium flux. $LaCl_3$ blocked the late phase of the increase in cell calcium caused by histamine. The effect of $LaCl_3$ on the calcium response of L cells and HUVE cells was similar to that of reduced extracellular calcium (Fig. 4*A*). Because $LaCl_3$ blocked the capacitive calcium influx, we asked whether $LaCl_3$ would alter the effects of histamine on the adhesion of VE-

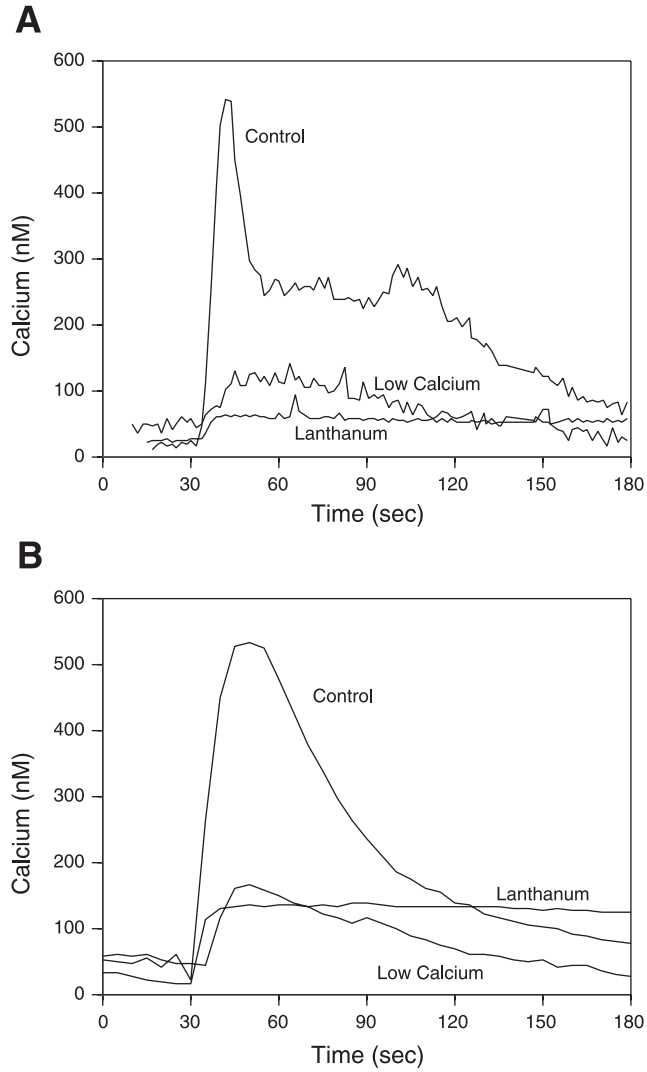


Fig. 4. Both lanthanum and low extracellular calcium (100 nM) prevent the histamine (10 μM)-induced capacitive calcium influx in both L-H1-VEcad (*A*) and HUVE (*B*) cells. Lanthanum (1 mM) was added just before the start of data acquisition. Histamine was added at 30 s.

cadherin expressing L cells to the VE-cadherin-Fc fusion protein. $LaCl_3$ did not prevent histamine from interrupting the adhesion of VE-cadherin expressing L cells to the VE-cadherin-Fc fusion protein (Fig. 5). Hence, the effects of histamine on VE-cadherin adhesion are independent of capacitive calcium flux across the cell membrane.

It was possible that L cells and endothelial cells would respond differently to $LaCl_3$, so we also examined the effects of $LaCl_3$ on the response of HUVE cells to histamine. $LaCl_3$ blocked the late phase of the increase in cell calcium in HUVE cells, the same as the effect of low extracellular calcium (Fig. 4*B*). Consistent with the lack of an effect of $LaCl_3$ on histamine's ability to interrupt VE-cadherin adhesion in L cells, $LaCl_3$ did not affect the change in cell-cell adhesion caused by histamine in HUVE cells (Fig. 6).

DISCUSSION

Inflammatory edema facilitates the transfer of immunologically active molecules and cells from the vascular space to the tissues. Although providing immunologically active molecules access to the tissues has potential benefit, excessive loss of molecules and water from the vascular space into the tissues can also impair organ physiology. Hence, it would be important for a system to evolve that would provide transient access to the tissues but rapidly restore normal barrier properties to preserve organ physiology. Histamine meets these requirements, as it rapidly decreases endothelial barrier properties but also allows the barrier to be restored within minutes (25). In this way histamine is representative of several molecules that rapidly and transiently increase endothelial permeability in postcapillary venules (4).

In an earlier report we found that histamine decreased endothelial adhesion predominantly at sites of cell-cell adhesion (16). ECV304 cells create tight junction structures, express claudin 2, and are a polarized monolayer (D. M. Shasby, unpublished data) (5). In a subsequent report using ECV304 cells transfected with and expressing VE-cadherin, we found that VE-cadherin played an essential role in the changes in cell-cell adhesion of the monolayer in response to histamine (24). Cadherins bind with proteins that also bind tight junction proteins, and the experiments with transfected ECV304 cells did not distinguish between an effect of histamine on a cadherin-tight junction interaction as opposed to a direct effect of histamine on cadherin adhesion.

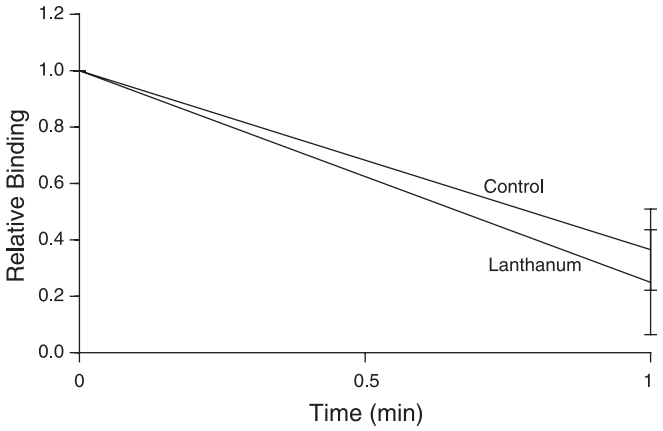


Fig. 5. The histamine (10 μM)-induced change in binding of L-H1-VEcad cells to immobilized VEcad-Fc was measured in the absence (Control) and presence of 1 mM lanthanum, and the results were normalized to mock-treated cells (zero time point). The presence of lanthanum does not significantly alter the change in binding caused by histamine.

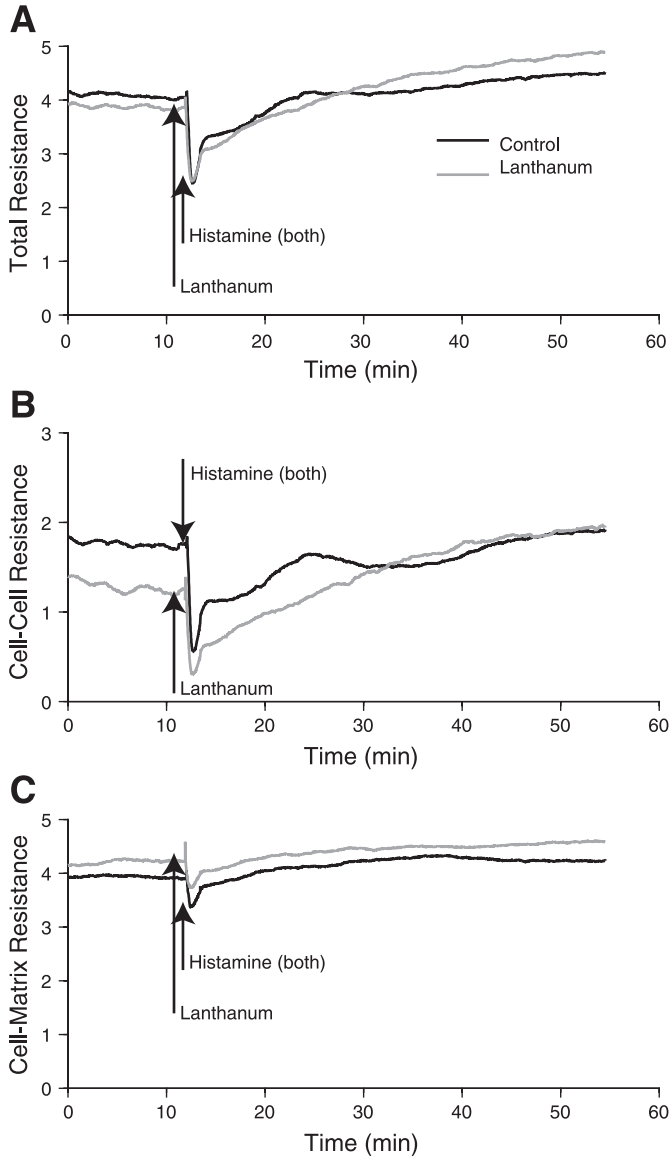


Fig. 6. Histamine-induced changes in HUVE cell total, cell-cell, and cell-matrix resistances were measured as described in MATERIALS AND METHODS. As previously shown, histamine (10 μM, added to all monolayers where indicated) causes a transient decrease in overall resistance (*A*) due to a decrease in cell-cell resistance (*B*). Cell-matrix resistance (*C*) is relatively unchanged. Blocking the capacitive calcium increase with lanthanum (1 mM, added where indicated to lanthanum monolayers only) does not inhibit this response. Resistances are measured in $\Omega \cdot cm^2$. The data presented are the electrical mean of 6 different control and 6 different lanthanum-treated monolayers from 2 separate experiments done on separate days.

In the experiments we report here, homotypic adhesion of L cells expressing the H1 receptor and VE-cadherin to a VE-cadherin-Fc fusion protein was interrupted by histamine. L cells do not express claudins or other cadherins (11). Adhesion of L cells expressing VE-cadherin to the respective cadherin-Fc fusion protein was specific, as it was blocked by soluble VE-cadherin-Fc fusion protein. Histamine altered ad-

hesion of VE-cadherin expressing L cells only if they expressed the H1 receptor, indicating histamine acted by specific receptor-dependent mechanisms. Hence, activation of the histamine receptor has a direct effect on VE-cadherin adhesion, which is not dependent on an interaction of VE-cadherin with the tight junction.

L cells express several integrins, including fibronectin-binding $\alpha_5\beta_1$ (1). The adhesion of L cells not expressing VE-cadherin to the plates coated with the VE-cadherin-Fc fusion protein was not affected by soluble VE-cadherin-Fc fusion protein. Hence, these integrins were not adhering to the VE-cadherin-Fc fusion protein. L cells and L cells expressing the H1 receptor adhered much more avidly to fibronectin-coated than to uncoated plates, a response consistent with the $\alpha_5\beta_1$-integrin binding the fibronectin. The peptide GRGDS, but not the peptide SDGRG, interrupted adhesion to fibronectin, confirming its specificity. Histamine did not affect the adhesion of L cells to fibronectin, indicating that the effects of histamine are selective for cadherin adhesion vs. integrin adhesion. Although these data indicate that histamine is selective for cadherin vs. integrin adhesion, they do not exclude the possibility that histamine affects other adhesion molecules we did not examine.

Histamine activates signaling that results in an increase in intracellular calcium in endothelial cells (6, 7). The increase in calcium is the result of release of calcium from intracellular stores and of a capacitive transmembrane flux of calcium. $LaCl_3$ blocks the transmembrane flux of calcium. In the L cells and HUVE cells used in these experiments, $LaCl_3$ blocked the late phase of the increase in cell calcium caused by histamine. Lanthanum also resulted in a decrease in the initial spike of calcium in response to histamine. This spike is usually associated with the release of intracellular calcium stores. Similar effects of lanthanum have been previously reported (19). We examined the effect of low extracellular (100 nM) calcium on the response to histamine. Low extracellular calcium caused a calcium response to histamine that was the same as that caused by lanthanum. Low extracellular calcium should not prevent the release of intracellular stores, especially when it has only been present for <2 min. The initial spike of calcium is clearly associated with the release of intracellular stores when single cells are studied. However, in our experiments, we examined the response of a population of cells, not all of which are activated at the same time. The experiments with low extracellular calcium suggest that the spike represents release of intracellular stores in some cells and early capacitive entry in others. Hence, although the data are clear that lanthanum prevented capacitive calcium entry, it is not certain whether there was or was not physiological release of intracellular stores. The data clearly demonstrate that histamine interrupts VE-cadherin adhesion independently of capacitive calcium entry. We cannot be certain whether this also occurs independently of the release of intracellular stores.

$LaCl_3$ also did not affect the histamine-initiated change in cell-cell resistance between HUVE cells, supporting the relevance of the L cell observations to the effects of histamine on endothelial permeability.

Tiruppathi et al. (23) found that the increase in endothelial permeability in response to thrombin was blunted in mice deficient in canonical transient receptor potential (TRPC) 4, the channel that conducts the capacitive calcium flux in endothelium. In these experiments, TRPC4-deficient preparations, or preparations pretreated with $LaCl_3$, exhibited a change in microvessel permeability that was about one-third that of wild type. In many reports, the effects of histamine on endothelial permeability are less than those of thrombin, the latter consistently producing a more persistent change in endothelial cell-cell adhesion (14, 16). Hence, our observations that capacitive calcium flux is not necessary for histamine to interrupt VE-cadherin adhesion in both the L cell model and in endothelium are consistent with Tiruppathi's observations in the TRPC4-deficient mice.

Our old reports demonstrate that the resting tension within endothelial cells is sufficient to cause significant cell retraction and gap formation when multiple sites of adhesion are interrupted by chelation of extracellular calcium (21, 22). The very limited gaps created by agonists like histamine and serotonin occur in cells with the same resting tension (15). The contrast with the large gaps created by chelation of extracellular calcium suggest that, unlike chelation of extracellular calcium, histamine selectively interrupts adhesion at one or a few sites and thereby limits the effects of the resting tension on cell retraction. This is supported by our observation that histamine affects cadherin, but not integrin adhesion.

The new data are also consistent with some of the differences between the response of endothelium to histamine and to thrombin. Histamine causes a very short-lived (<5 min) decrease in endothelial cell-cell adhesion and does not increase centripetally directed tension in endothelial cells (14–16). In contrast, thrombin causes a decrease in cell-cell adhesion that lasts at least 30 min and causes a large increase in centripetally directed tension that is dependent on capacitive calcium flux (14–16).

On the basis of this constellation of data we believe that histamine increases endothelial permeability by selectively interrupting VE-cadherin-based adhesion independently of capacitive calcium entry. This selective interruption of adhesion sites that occurs without increases in centripetal tension in the cells allows for the formation of very tiny gaps that close within a few minutes (4, 16). This makes histamine an effective agonist of innate immunity, facilitating the transport of immune-competent molecules from the vascular space to the tissues without producing so much edema that organ function is significantly impaired.

GRANTS

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