Vasoactive Amines Directly Modify Endothelial Cells to Affect Polymorphonuclear Leukocyte Diapedesis In Vitro

By John Doukas, David Shepro, and Herbert B. Hechtman

Bovine aortic endothelial cells were cultured on the basement membrane surface of amnionic membrane and used as a substrate for polymorphonuclear leukocyte (PMN) diapedesis in vitro. Norepinephrine (NE), serotonin (5HT), or phallolidin treatment of the endothelial cells (ECs) reduces, whereas histamine or cytochalasin B increases, the number of PMNs migrating across the ECs and amnionic membrane. In contrast, amine treatment of PMNs or acellular amnionic membrane does not alter PMN diapedesis or chemotaxis. The NE and histamine effects are blocked by appropriate receptor antagonists, but the 5HT effect is not. All the agents' effects are also reversible.

The process of polymorphonuclear leukocyte (PMN) diapedesis, in which PMNs breach the endothelial cell (EC) barrier of the microvasculature and enter surrounding tissues, is of prime importance in the development of an inflammatory reaction. PMN diapedesis has been examined principally with regard to the earlier event of adherence to the endothelium,2,3 or with regard to PMN adhesion to foreign surfaces4 and chemokinesis.5 In the present study, however, we examine an active role for ECs in PMN diapedesis which, if demonstrated, would suggest that local metabolic regulation of ECs may help to maintain the microvascular barrier to PMNs.

We previously reported that vasoactive amines such as norepinephrine (NE), serotonin (5HT), and histamine modulate various EC functions such as permeability to macromolecules,6 the erythrocyte extravasation associated with severe thrombocytopenia,7 and surface area and stress fiber numbers. Stress fibers are microfilament bundles composed of actin, myosin, and other contractile proteins,8 which in ECs are believed to assist in maintaining normal barrier function to fluid and macromolecular permeability.9 An underlying hypothesis in our work is that exogenous metabolites such as NE and 5HT act as local regulators to maintain the normal barrier function of EC.

PMN diapedesis has been studied using pulmonary artery intimal explants15 as well as ECs cultured on glass16 and polycarbonate filters.17 These systems, however, have inherent limitations; for example, intimal explants retain a portion of the vessel wall, which can alter expected permeability changes18; cultures on glass coverslips do not possess a subendothelial connective tissue layer to be crossed by PMNs as occurs in vivo, and monolayers cultured on filters may show areas of attenuated cytoplasm overlying filter pores,19 an aberrant growth pattern not seen in ECs in vivo or cultured on substrates without pores. A more suitable substrate for use in PMN diapedesis studies is the acellular amnionic membrane, which provides normal EC monolayer growth over a continuous, native basement membrane and collagenous interstitial stroma.20 In this report, using ECs cultured on amnionic membrane mounted in modified chemotaxis chambers, we observed that the amines NE and 5HT significantly and reversibly decreased PMN diapedesis in vitro whereas histamine increased diapedesis through their direct effects on the EC.

Qualitatively similar effects on EC permeability to Evan’s blue-labeled albumin occur with all agents; however, PMN adhesion to ECs is not affected. Previously, we reported that NE and 5HT increase stress fiber numbers and decrease EC permeability to macromolecules in vitro, whereas histamine has the opposite effects, and that NE and 5HT eliminate the erythrocyte extravasation associated with thrombocytopenia in vivo. In this study, we propose that these vasoactive amines also alter PMN diapedesis in vitro through a direct effect on the EC, in part due to alterations in the EC cytoskeleton.

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MATERIALS AND METHODS

Tissue culture. Calf aortas were collected shortly after slaughter and transported on ice. After excess tissue was trimmed, the lumens were rinsed with phosphate-buffered saline (PBS) and exposed, and the intima was removed by gentle pressure with a scalpel. Individual cells were dispersed by treatment with 0.1% collagenase in Ca2+, Mg2+-free PBS at 37°C for 12 minutes. After centrifugation at 1,200 g for 4 minutes, the cell pellet was suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL), and plated at 1 to 2 aorta/25-cm2 flask. After 12 hours, debris was removed by rinsing the pellet with fresh medium. Cultures were refed every 3 days, and confluent monolayers were obtained in 5 to 7 days. ECs were confirmed by morphology, failure to overgrow as a monolayer, and positive staining for low-density lipoprotein (LDL) receptors using a fluorescent-labeled acetylated LDL (DiI-Ac-LDL).20

Amnionic membrane preparation. A modification of the method of Liotta and colleagues21 was used to prepare acellular amnionic membrane. Human placentas were obtained shortly after delivery and transported on ice. The amnion reflecta of the sac material was separated from the chorion, washed free of blood, and placed in two changes of 1 mmol/L of N-ethyl maleimide at 4°C over 60 minutes, with gentle agitation. The tissue was rinsed in 4°C distilled water, stretched loosely over plastic embroidery hoops, and the excess gelatinous stroma was removed by gentle rubbing. The hoops were submersed in 25°C 2% NH4OH for 30 minutes, with gentle agitation, after which the epithelium was removed with a rubber policeman. The amnionic membrane was then rinsed in distilled water and stored in 4°C distilled water for up to 12 hours prior to use.

PMN preparation. Heparinized human blood was obtained from healthy, drug-free donors and immediately used for isolating...
PMNs, using discontinuous density gradients of Ficoll-Hypaque.\textsuperscript{22} Contaminating erythrocytes were then removed by centrifugation (200 g for 3 minutes) through 3% Dextran 500 in Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-free Hank’s balanced salt solution (HBSS), and the PMNs were rinsed free of gradient fluid with HBSS (200 g for 5 min), and resuspended in buffer (DMEM containing 0.45% BSA, pH 7.4) at a final concentration of 6.25 x 10\textsuperscript{6} cells/mL. This procedure yielded >97% PMNs (Wright’s-Giemsa staining), with >98% viability as assessed by trypan blue exclusion.

**Permeability chamber assembly.** Amnionic membranes were arranged in modified chemotaxis chambers constructed in our laboratory from plastic test tube caps (Fig 1). Hoops of membrane, basement membrane side up, were placed on top of the open ends of lower caps, which had circular inner ridges extending 2 mm upward from the cap floors. Upper caps, which had outwardly flaring bases 1.5 mm thick, were pressed into the lower caps base first. In this way, upper and lower wells were formed (250-μL capacity each), separated by 50-mm\textsuperscript{2} circles of amnionic membrane held firmly in place between the bases of the upper caps and the ridges of the lower caps. The caps themselves fit tightly into each other, holding the assemblies firmly in place. The tops of the upper caps had been previously cut open to allow addition of cells and medium onto the basement membrane (formerly the epithelial surface) of the amnionic membrane; the lower wells were closed except for small holes previously drilled in their sides to allow emptying and refilling. After assembly, excess membrane was trimmed, the upper wells were filled with complete tissue culture medium (DMEM with FBS and antibiotics) and, if no leakage into the lower wells occurred due to a membrane tear, the lower wells were likewise filled by a syringe inserted through the side holes. The chambers were placed in 24-well tissue culture plates, the plates were filled with medium so as to cover the side holes of the chambers, and the plates were sterilized by exposure to a 60Co source for 3 to 5 minutes. The chambers were then stored at 4°C for 1 to 2 weeks until needed.

Bovine aortic endothelial cell (BAEC) monolayers were trypan-ized and seeded onto the basement membrane surface of amnionic membrane at a concentration of 1 x 10\textsuperscript{5} cells/chamber, at which time fresh complete medium was also added. This seeding density produced visual confluency within 12 hours; however, for maximum density and integrity, the monolayers were cultured for an additional 48 hours prior to use, with a medium change at 24 hours. For assessment of confluency, representative monolayers were stained with saturated auramine O (aqueous) for 1 minute and viewed by epifluorescence with a mercury light source. This staining procedure clearly delineates the cells in a confluent monolayer and any gaps that may be present; it was developed because the collagen fibrils of the amnionic stroma coupled with the plastic of the permeability chambers prevented effective transillumination.

Permeability chambers constructed in this manner routinely yielded comparable levels of PMN diapedesis. In addition, with Evan’s blue-labeled albumin used as a permeability tracer, the monolayers routinely demonstrated comparable barrier function to this macromolecule. The final passage number of the monolayers (passage 1, 2, or 3) did not affect these results. Transmission electron micrographs (not shown) reveal a continuous monolayer, an underlying basement membrane, and an interstitial stroma of collagen fibrils devoid of rents or tears, as has been previously described by Madri and Williams.\textsuperscript{19} In experiments in which membrane tears were purposely created, a greatly increased passage of PMNs into the lower well (~50% of all PMNs applied v the average of 8% for normal control conditions) of Evan’s blue-labeled albumin (EBA) occurred. Such results did not occur in experiments used for data analysis. Moreover, such permeability chambers (with membrane tears) did not respond significantly to the test agents.

**Diapedesis experiments.** BAEC monolayers cultured in permeability chambers were rinsed three times with buffer (DMEM containing 0.45% BSA, pH 7.4) at 37°C, the lower wells were rinsed and filled with 200 μL of buffer, and the side holes were stoppered with paraffin. Test agents dissolved in buffer were applied to the monolayers as 200-μL aliquots, and the permeability chambers were incubated at 37°C in a humidified 95% air/5% CO\textsubscript{2} atmosphere. After 30 minutes, the monolayers were rinsed three times with buffer and, if no further agents were to be applied, 1.25 x 10\textsuperscript{8} PMNs in a 200-μL aliquot of buffer (37°C) was applied to each monolayer and the chambers were again incubated in a 37°C tissue culture incubator. After 30 minutes, the monolayers were rinsed three times, the lower wells were sampled, and the number of PMNs in their 250-μL volume was determined with a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). The numbers of PMNs in the lower wells of experimental groups were then compared with values obtained for concurrently conducted controls (in which the monolayers were treated with buffer alone prior to addition of PMNs) and represented as percentages of control values.

In experiments to determine any direct amine influences on PMN diapedesis, PMNs suspended in DMEM buffer at 2 to 5 x 10\textsuperscript{8} PMNs/mL were treated with NE, 5HT, or histamine for 30 minutes at 25°C. The PMNs were then pelleted at 1.200 g for 4 minutes, resuspended in DMEM buffer without amines at 6.25 x 10\textsuperscript{6} cells/mL, and added to chambers containing monolayers that had not received any amine pretreatments. A diapedesis assay was then conducted as described above.

In addition, acellular amnionic membranes mounted in permeability chambers were directly treated with amines for 30 minutes, and a “diapedesis” assay was then conducted, except that 10\textsuperscript{-7} mol/L of N-formyl-methionyl-leucyl-phenylalanine (FMLP) was included in the lower wells as a chemoattractant.

**Permeability to EBA.** An aqueous solution of 5% BSA and 0.5% Evan’s blue dye was incubated at 37°C for 15 minutes. Protein was then precipitated repeatedly with 100% ethanol until no free dye was detectable in the washes. The resulting EBA was air dried and stored at 4°C. Gel electrophoresis under denaturing conditions revealed that EBA was a single blue band that comigrated with BSA standards.

BAEC monolayers cultured in permeability chambers were treated with pharmacological agents as previously described, and a 200-μL aliquot of 0.45% EBA in DMEM (37°C, filtered prior to use) was then applied to each monolayer. After a 30-minute incubation at 37°C, the upper wells were aspirated dry, the mono-

![Fig 1. Permeability chamber diagram.](image-url)
layers were rinsed three times, and the lower wells' volumes were sampled. These samples were then raised to 1.0 mL with distilled water and read for absorbance at 620 nm in a spectrophotometer. The lower the absorbance, the less EBA present, and therefore the less permeable the monolayer.

PMN adherence. PMN adherence to BAEC monolayers was determined using the method of Zimmerman and Hill.23 In brief, confluent monolayers cultured in 24-well plates were treated with pharmacological agents for 30 minutes at 37°C and then rinsed with buffer three times. A 500-μL aliquot containing 3.2 × 10⁶ PMNs was added to each well, and the plates were incubated at 37°C for 10 minutes. Supernatants were removed, the monolayers were gently rinsed with counting buffer, and the number of nonadherent PMNs was determined with a Coulter counter. Results are represented as the percentage of applied PMNs remaining adherent to the monolayers.

Statistical analysis. One-way analysis of variance and Duncan's multiple-range test were used to compare groups within experiments.24

Reagents. Reagents were obtained from the following sources: DMEM from Gibco, Grand Island, N.Y.; FBS from Hyclone Labs, Logan, UT; Dil-Ac-LDL from Biomedical Technologies, Cambridge, MA; Dextran T500 from Pharmacia Fine Chemicals, Piscataway, N.J.; BSA from Boehringer Mannheim, Indianapolis; phenytoin, diphenhydramine, and cimetidine (Tagamet) from Smith, Kline and French, Philadelphia; propranolol from Ayerst, New York; and ketanserin as a gift of Janssen Pharmaceutica, Beerse, Belgium; all other reagents were obtained from Sigma Chemical, St. Louis.

RESULTS

Under control conditions (30-minute monolayer pretreatment with buffer alone) 1.0 × 10⁶ PMNs (8.0% of the PMNs applied) migrate across a BAEC monolayer and the underlying amniotic membrane within 30 minutes (n = 78, SEM ± 3.494). (All percentages are given as mean values.) In the absence of this monolayer, 1.5 × 10⁶ PMNs (1.2% of the PMNs applied) migrate across the amniotic membrane (n = 10, SEM ± 1.073). These results agree with those of Russo and co-workers,25 who demonstrated that PMN diapedesis across intact amniotic membranes (ie, amnion separated from the chorion but otherwise untreated) is enhanced by a viable epithelial cell monolayer. These researchers also reported that the PMN transit occurs uniformly across the monolayer surface through intercellular junctions and closely mimics diapedesis as observed in vivo during inflammation.

If the length of time PMNs are incubated with BAEC monolayers is varied, a maximum level of PMN diapedesis is reached between 15 and 30 minutes and does not increase up to 60 minutes thereafter (n = 6 for each time point, data not shown). Cramer and colleagues26 found that PMN diapedesis across epithelial monolayers cultured on micropore filters was maximum within 15 minutes in the absence of a chemoattractant, whereas Meyerick and associates15 found that diapedesis across pulmonary artery explants was maximal by 60 minutes. Therefore, the present study agrees with these previous reports in that PMN diapedesis in in vitro systems reaches some maximum level, the timing of which probably depends on the nature of the substrate (eg, its thickness).

Either NE or 5HT treatment of a monolayer reduces the basal level of PMN diapedesis in a dose-responsive manner (Fig 2). For NE, the minimum significant effect occurs at 10⁻¹ mol/L (16% reduction over controls, SEM ± 3%), and the maximum effect occurs between 10⁻⁷ and 10⁻⁴ mol/L (58% reduction by 10⁻⁴ mol/L, SEM ± 6%). For 5HT, the minimum significant effect occurs at 10⁻⁷ mol/L (24% reduction over controls, SEM ± 4%), and the maximum effect occurs between 10⁻⁵ and 10⁻⁴ mol/L (39% reduction by 10⁻⁵ mol/L, SEM ± 2%). In contrast, histamine increases PMN diapedesis. The minimum significant effect occurs at 10⁻⁴ mol/L (27% increase over controls, SEM ± 3%), and the maximum effect occurs between 10⁻⁴ and 10⁻³ mol/L (97% increase by 10⁻⁴ mol/L, SEM ± 11%).

The addition of the chemoattractant peptide FMLP to the lower well of a permeability chamber significantly increases the number of PMNs migrating across the monolayer, from 1.1 × 10⁵ PMNs (8.8% of the PMNs applied) without FMLP to 1.9 × 10⁵ PMNs (15.2% of the PMNs applied) with FMLP (n = 6). FMLP, however, does not qualitatively alter the amine effects on the BAEC monolayers: NE and

<table>
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<tr>
<th>AMINE CONCENTRATION (Μ)</th>
<th>PMN IN LOWER WELL (% control)</th>
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<tr>
<td>10⁻⁶</td>
<td>50 ± 4</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>10⁻³</td>
<td>121 ± 3</td>
</tr>
</tbody>
</table>

Abbreviations: PMNs, polymorphonuclear leukocytes; BAEC, bovine aortic endothelial cells; NE, norepinephrine; 5HT, serotonin.

A PMN diapedesis experiment was conducted in which N-formylmethionyl-leucyl-phenylalanine (FMLP) (10⁻⁷ mol/L) was included in the lower wells of the permeability chambers. Data are expressed as percentages of control values ± SEM. All experimental groups differ significantly from controls (P < .01) and are n = 6, and all agents are used at 1 × 10⁻⁶ mol/L.
5HT again reduce diapedesis, whereas histamine increases it (Table 1).

If the length of time the monolayers are treated with amines is varied, NE and histamine produce maximal effects between 5 and 15 minutes (Fig 3). In contrast, the maximal effect of 5HT occurs between 15 and 30 minutes. For this reason, 30 minutes was used for treatment incubation times for all other experiments.

When PMNs are first treated with NE, 5HT, or histamine (10⁻⁶ mol/L) for 30 minutes at 25°C, and then used in diapedesis experiments with untreated BAEC monolayers, no changes in diapedesis are observed as compared with controls (n = 12, data not shown). Moreover, when acellular amionic membranes (without a BAEC monolayer) are directly treated with amines (10⁻⁶ mol/L), no changes in PMN chemotaxis across the membranes are observed as compared with controls (n = 6, data not shown).

When a BAEC monolayer is treated with the α-adrenergic antagonist phenoxybenzamine or the β-adrenergic antagonist propranolol and is then treated with both NE and the same receptor antagonist, the expected NE effect (a decrease in diapedesis) does not occur (Table 2). In contrast, treatment with the 5HT S₂ receptor antagonist ketanserin prior to ketanserin/5HT cotreatment does not significantly alter the expected 5HT effect of reduced diapedesis.

The histamine H₁ receptor antagonist diphenhydramine, when used as a pretreatment to diphenhydramine/histamine cotreatment, completely blocks the expected effect of increased diapedesis (Table 2). In contrast, cimetidine, a histamine H₂ receptor antagonist, does not block the histamine effect but slightly potentiates it.

Monolayers were also treated with two agents known to alter actin microfilaments directly, as a means of assessing any EC cytoskeletal influence on diapedesis. Phalloidin, which polymerizes G-actin to F-actin microfilaments and stabilizes these filaments, decreases diapedesis in a dose-responsive manner (Fig 4). The minimum significant effect occurs at 10⁻⁶ mol/L (21% reduction over controls, SEM ± 5%), and the maximum effect occurs between 10⁻⁴ and 10⁻³ mol/L (67% reduction over controls by 10⁻³ mol/L, SEM ± 2%). In contrast, cytochalasin B, which inhibits G-actin polymerization, increases diapedesis. The minimum effect occurs at 10⁻¹ mol/L (51% increase over controls, SEM ± 7%), and the maximum effect occurs between 10⁻³ and 10⁻² mol/L (128% increase over controls by 10⁻² mol/L, SEM ± 7%).

When monolayers are treated with either NE, 5HT, or

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**Table 2. PMN Diapedesis Following Treatment of BAEC Monolayers With Amines or Amine Receptor Antagonists/Amines**

<table>
<thead>
<tr>
<th>Monolayer Treatment</th>
<th>PMNs in Lower Well (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>NE</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>Phenoxybenzamine/NE</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>Propranolol/NE</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>5HT</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>Ketanserin/5HT</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>Histamine</td>
<td>140 ± 4</td>
</tr>
<tr>
<td>Diphenhydramine/histamine</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>Cimetidine/histamine</td>
<td>165 ± 3</td>
</tr>
</tbody>
</table>

Monolayers were pretreated for 30 minutes with a receptor antagonist followed by a 30-minute cotreatment with the same antagonist and the appropriately corresponding amine, or with buffer alone for 30 minutes followed by an amine for 30 minutes, or with buffer alone for 60 minutes (control). Data are expressed as percentages of control values ± SEM. NE, 5HT, ketanserin/5HT, histamine, and cimetidine/histamine differed significantly from controls (P < .01), and cimetidine/histamine differed significantly from histamine alone (P < .01). All agents were used at 1 × 10⁻⁷ mol/L, and all groups were n = 6.
histamine for 30 minutes and then with buffer alone for 30 minutes, no significant effects on PMN diapedesis are evident as compared with controls (ie, treatment with buffer alone for 60 minutes) (Fig 5). This demonstrates the reversibility of the amines' effects. Phalloidin's effect is also reversible within 30 minutes; the effect of cytochalasin B, however, is only partially reversed 30 minutes posttreatment (to 65% of the effect at 0 minutes posttreatment, SEM ± 6%).

Monolayer permeability to EBA is similarly affected by amine, receptor antagonist/amine, phalloidin, or cytochalasin B treatments (Table 3). NE reduces permeability, with the effect being completely blocked by propranolol and phenoxbenzamine; 5HT likewise reduces permeability, but its action is unaffected by ketanserin. Histamine increases permeability, and diphenhydramine completely blocks the effect. Finally, cytochalasin B increases permeability, whereas phalloidin decreases it.

PMN adhesion to BAEC monolayers is unaffected by previous monolayer treatment with NE, 5HT, histamine, cytochalasin B, or phalloidin (Table 4). The concentrations used significantly affect diapedesis, however.

**DISCUSSION**

These experiments demonstrate that specific vasoactive amines modulate the level of PMN diapedesis across a BAEC monolayer in vitro. The amines NE and 5HT significantly reduce diapedesis; histamine, conversely, increases diapedesis. These changes can be attributed to direct amine effects on the ECs, because amine treatment of EC produces the diapedesis changes whereas amine treatment of PMN does not affect their subsequent diapedesis across untreated monolayers. Also, amine treatment of amnionic membranes (without a BAEC monolayer) does not affect PMN chemotaxis across the membranes. This conclusion is supported in part by Clark and colleagues, who also reported no effect of histamine on PMN migration. Therefore, BAEC are capable of presenting a greater or lesser barrier to PMN diapedesis, depending on exposure to exogenous amines.

Unless otherwise stated, the permeability chambers contained buffer alone in their lower wells. Addition of a PMN chemoattractant (10−7 mol/L of FMLP) to the lower wells, however, does not qualitatively alter the amine effects on the BAEC monolayers. This further supports the relevance of the present work to PMN diapedesis during inflammatory states.

The amine-induced changes in diapedesis are reversible. A 30-minute monolayer incubation with buffer alone reverses any previous amine treatment, and PMN diapedesis returns to control values. Moreover, none of the amine treatments produced gross morphological changes as visible with light microscopy, and none of the amine or other treatments used decreased EC viability (as assessed by trypan blue and

**Table 3.** BAEC Monolayer Permeability to EBA Following Treatment With Various Agents

<table>
<thead>
<tr>
<th>Monolayer Treatment</th>
<th>Absorbance (% Control) ± SEM</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>NE</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Phenoxbenzamine/NE</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>Propranolol/NE</td>
<td>102 ± 2</td>
</tr>
<tr>
<td>5HT</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>Ketanserin/5HT</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>Histamine</td>
<td>121 ± 2</td>
</tr>
<tr>
<td>Diphenhydramine/histamine</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>123 ± 4</td>
</tr>
</tbody>
</table>

Abbreviation: EBA, Evans's blue-labeled albumin.

Monolayers were treated with amine receptor antagonists and amines as previously described (Table 2); phalloidin and cytochalasin B treatments were for 30 minutes. Data are expressed as percentages of control values for absorbance ± SEM. NE, 5HT, ketanserin/5HT, histamine, phalloidin, and cytochalasin B differed significantly from control (P < .01). All agents were used at 1 x 10−4 mol/L, except for phalloidin (1 x 10−5 mol/L); all groups were n = 6.

[Fig 5. Reversal of the amine, phalloidin, and cytochalasin B effects on polymorphonuclear leukocyte (PMN) diapedesis. His, histamine; Phal, phalloidin; and Cyto B, cytochalasin B. Solid bars, 30-minute treatment with agents; striped bars, 30-minute treatment with agent followed by a 30-minute treatment with buffer alone. Significance from controls is noted for P < .01 (**). In addition, cytochalasin B/buffer significantly differs from cytochalasin B alone (P < .01). All treatments are 1 x 10−6 mol/L, except for phalloidin (1 x 10−4 mol/L). Values are represented as mean ± SEM, and all are n = 6.]

**Table 4.** PMN Adhesion to BAEC Monolayers Following Treatment of Monolayers With Various Agents

<table>
<thead>
<tr>
<th>Monolayer Treatment</th>
<th>Adherent PMNs (% Applied) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>NE</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>5HT</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Histamine</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>12 ± 1</td>
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</table>

Data are expressed as the percentage of applied PMNs remaining adherent to the monolayers following a 10-minute incubation, ± SEM. Monolayers had been previously treated with amines or cytochalasin B (1 x 10−7 mol/L) or phalloidin (1 x 10−6 mol/L) for 30 minutes; all groups were n = 6.
fluorescein diacetate/ethidium bromide vital staining). Moreover, the amine concentrations used were comparable to levels ranging from normal conditions to those of thrombogenesis. These observations support our thesis that vasoactive amines alter EC physiology and that regulation of barrier function may occur in vivo, as we have previously suggested for monolayer permeability to macromolecules and the erythrocyte extravasation associated with severe thrombocytopenia.6,7 β-adrenergic receptors on BAEC has been demonstrated using a radiolabeled ligand.33 In the present study, the effects of NE on diapedesis and albumin permeability were blocked by both phenoxybenzamine and propranolol, suggesting that the amine may act through adrenergic receptors.

Histamine H1 receptors have also been localized on aortic EC using a radiolabeled ligand.34 In the present study, histamine-induced diapedesis and permeability increases were completely blocked by diphenhydramine, suggesting that they may be due to H1 receptor stimulation. Cimetidine's potentiation of histamine's effect on diapedesis may be in part to some H1 receptor agonistic activity or to a nonreceptor-mediated action.

The 5HT-induced diapedesis and permeability alterations appear to be nonreceptor mediated, in that the 5HT S2 receptor antagonist ketanserin does not block the amine's effects. This finding supports Makar's report of the lack of 5HT receptors on cultured BAEC,35 a conclusion also drawn in our own work using radiolabeled ligand localization and receptor binding and pulse-chase experiments. The longer time necessary for the appearance of a maximal 5HT effect on diapedesis, as compared with the time necessary for maximal NE and histamine effects, may reflect the nonreceptor-mediated action of 5HT versus the receptor-mediated action of the other two amines.

Histamine causes a transient dilation of EC junctions, leading to increased permeability to water and sucrose and albumin. In contrast, NE and 5HT decrease EC monolayer permeability to albumin and prevent the erythrocyte extravasation associated with thrombocytopenia in vivo, effects believed to reflect increased EC junctional integrity. Increased monolayer permeability as a result of decreased junctional integrity has been attributed to alterations in the EC cytoskeleton and Meza and colleagues39 related alterations in microfilament patterns to changes in epithelial cell monolayer electrical resistance, an indicator of junctional integrity. Direct manipulation of microfilaments with cytochalasin B also decreases monolayer junctional integrity and increases permeability to albumin, further establishing the connection among cytoskeletal arrangements, junctional integrity, and monolayer permeability.

Previously we showed that NE and 5HT increase, whereas histamine decreases, EC stress fiber numbers. We also showed that NE and 5HT act directly to increase G-actin to F-actin polymerization, as determined by G-actin inhibition of DNase I activity.38 We believe that the results reported here on amine-induced alterations in PMN diapedesis and monolayer permeability to albumin may be due to these actions: Amine-induced EC cytoskeletal alterations may alter monolayer junctional integrity as described above, leading to both diapedesis and permeability changes.

Because PMN diapedesis occurs intercellularly,22,38 as does the bulk of trypan blue-labeled albumin transport across BAEC monolayers,20 changes in monolayer junctional integrity would be expected to alter both PMN diapedesis and albumin permeability. The effects of phalloidin and cytochalasin B further support this conclusion: Stabilization of EC microfilaments by phalloidin decreases, whereas disruption of microfilaments by cytochalasin B increases, diapedesis and permeability. In addition, as with the amines, the effects of phalloidin and cytochalasin B are dose-responsive and reversible, which may further suggest a commonality of action between these agents and the amines. Wang and Gottlieb28 report a time course and effective concentration range for the disruption by cytochalasin B of the dense peripheral band of microfilaments in ECs similar to the time course in the present report for the agent's effects on diapedesis (ie, 10−4 mol/L is effective within 15 minutes of treatment, and the effect is reversible between 15 and 60 minutes following removal of the treatment).

Other mechanisms could contribute to the amine effects on EC barrier function. Because PMN adhesion to the endothelium occurs prior to diapedesis, alterations in PMN adhesion could increase or decrease diapedesis. Such a mechanism does not appear to be involved in the present work, however, as neither the amines, cytochalasin B, nor phalloidin affected PMN adhesion to BAEC monolayers at concentrations that affected diapedesis. Several reports have also suggested that ECs can elaborate factors capable of influencing PMN–EC interactions. Such factors would not be expected to alter both diapedesis and monolayer permeability to albumin, however, as evidenced in this study. We found that BAECs do release factor(s) that influence PMN diapedesis and that this release is influenced by NE and 5HT (J. Doukas, D. Shepro, H.B. Hechtman, unpublished observations). We are now examining the relationship between the release of these factors and the amine-induced cytoskeletal alterations.

In conclusion, we present experimental evidence that NE or 5HT treatment of a BAEC monolayer reduces subsequent PMN diapedesis across the monolayer whereas histamine increases subsequent diapedesis. These effects are suggested to reflect amine-induced alterations in the EC cytoskeleton.

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J Doukas, D Shepro and HB Hechtman