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Histamine reduces gap junctional communication of human tonsil high endothelial cells in culture

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Abstract

The regulation of gap junctional communication by histamine was studied in primary cultures of human tonsil high endothelial cells (HUTECs). We evaluated intercellular communication, levels, state of phosphorylation, and cellular distribution of gap junction protein subunits, mainly connexin (Cx)43. Histamine induced a time-dependent reduction in dye coupling (Lucifer yellow) associated with reduction in connexin43 localized at cell–cell appositions (immunofluorescence), without changes in levels and phosphorylation state of connexin43 (immunoblots). These effects were prevented with chlorpheniramine, an H_1 receptor blocker; indomethacin, a cyclooxygenase blocker; or GF109203X, a protein kinase C inhibitor. Treatment with phorbol myristate acetate, a protein kinase C activator, and 4bromo (4Br)-A23187, a calcium ionophore, mimicked the histamine-induced effects on dye coupling. 8Bromo-cAMP doubled the dye coupling extent and prevented the histamine-induced reduction in incidence of dye coupling. After 24-h histamine treatment, known to desensitize H_1 receptors, reapplication of histamine increased cell coupling in a way prevented by ranitidine, an H_2 receptor blocker. Thus, activation of H_1 and H_2 receptors, which increase intracellular levels of free Ca^{2+} and cAMP, respectively, may affect gap junctional communication in opposite ways. Stabilization of actin filaments with phalloidine diminished but did not totally prevent histamine-induced cell shape changes and reduction in dye coupling. Hence, the histamine-induced reduction in gap junctional communication between HUTEC is mediated by cytoskeleton-dependent and -independent mechanisms and might contribute to modulate endothelial function in lymphoid tissue.

Keywords: Connexin43; Dye coupling; Endothelium; Cytoskeleton; Gap junctions; Inflammation

Introduction

In humans, high endothelial venules (HEVs) are found in secondary lymphoid organs, such as lymph nodes, tonsils, adenoids in the pharynx, Peyer patches in the small intestine, appendix, and small aggregates of lymphoid tissue in the stomach and large intestine (Girard and Springer, 1995). An ultrastructural feature of HEVs is the presence of discontinuous 'spot-welded' junctions between endothelial cells (Anderson and Shaw, 1993). This property of HEVs is believed to facilitate the passage of lymphocytes between adjacent high endothelial cells and is likely to be one of the factors allowing massive lymphocyte emigration in HEVs.

Gap junction channels mediate one way of intercellular communication present in endothelial cells of various vascular territories, including aorta, umbilical vein, and the microvasculature (Haefliger et al., 2004). These channels communicate the cytoplasm of contacting cells allowing intercellular exchange of ions and small molecules,

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including second messengers (Sáez et al., 2003). Gap junctional communication between endothelial cells coordinates several physiological and pathophysiological processes such as endothelial cell migration, angiogenesis, endothelial growth and senescence, and coordination of vasomotor responses (Haefliger et al., 2004).

Each gap junction channel is formed by two hemichannels, and they correspond to dodecameric proteins constituted by protein subunits denominated connexins (Cxs) (Sáez et al., 2003). Cx43 is ubiquitously expressed in different mammalian cells, including endothelial cells of different territories of the circulatory system (Haefliger et al., 2004). Also, lymphoendothelial cells and vascular endothelial cells of human tonsil have been reported to present Cx43 immunoreactivity (Sáez et al., 2003). However, there are no reports of functional coupling between high endothelial cells and the possible modulation of this process by inflammatory agents.

Because Cx channels have a short half-life, intercellular communication can be altered by changes in the number of functional channels present at the plasma membrane. Moreover, most Cxs are phosphoproteins, and protein phosphorylation regulates gap junctional communication in many cellular systems (Sáez et al., 1998, 2003). A reduced gap junctional communication and/or expression of endothelial Cxs have been reported to occur in senescence and chronic diseases, including hypertension and diabetes (Haefliger et al., 2004). Accordingly, the extent of endothelial cells communication via gap junctions and expression of Cxs is affected by conditions of their microenvironment, such as laminar shear stress (DePaola et al., 1999) and by the action of several membrane receptor ligands (Haefliger et al., 2004). The latter includes growth factors and proinflammatory molecules, such as TNF-alpha, interleukin-1β, thromboxane A2, and bacterial lipopolysaccharide (Haefliger et al., 2004). Histamine has been long recognized as a potent inflammatory mediator causing increased permeability to macromolecules and favoring leukocyte adhesion (Majno and Palade, 1961; Ehringer et al., 1996). To our knowledge, the effect of histamine on gap junctions expressed by endothelial cells has not been reported. Under proinflammatory conditions, potential histamine sources are mast cells known to be present in human tonsil (Fureder et al., 1997).

Here, we report that cultured high endothelial cells from human tonsils express gap junction channels constituted at least of Cx43 and show histamine responses consistent with the presence of H_1 and H_2 histamine receptors. While gap junctional communication was reduced upon activation of H_1 receptors, it was increased upon activation of the signaling pathways of H_2 receptors or by histamine after desensitization of H_1 receptors. We propose that regulation of gap junctional communication by histamine might be involved in aspects of the inflammatory response. Preliminary results of this work have been previously communicated (Figueroa et al., 2001).

Materials and methods

Reagents and antibodies

RPMI 1640 medium, fetal bovine serum, penicillin, streptomycin, and trypsin-EDTA, were from GibcoBRL (Grand Island, NY, USA). Lucifer yellow-CH, phorbol 12-myristate 13-acetate (PMA), 8-bromo-cyclic adenosine monophosphate (8Br-cAMP), 4-bromo-A23187 (4Br-A23187), histamine, 1-octanol, ranitidine, monoclonal anti-alpha-tubulin antibody, anti-vimentin antibody, fluorescein isothiocyanate (FITC)-phalloidin, FITC-conjugated goat antirabbit IgG (Fab) antibody, FITC-conjugated anti-mouse IgG antibody, and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody were from Sigma (St. Louis, MO, USA). Rhodamine-labeled phalloidin was from Molecular Probes, Inc. (Eugene, OR, USA). $F(ab')_2$ fragments of anti-Cx43 antibodies were prepared as described previously by Brañes et al. (2002). $F(ab')_2$ fragments of anti Cx40 and Cx37 antibodies, previously characterized (Brañes et al., 2002), were also utilized.

Endothelial cells

Human tonsil endothelial cells (HUTECs) were obtained as previously described (Castro et al., 1996). These cells, have been characterized as of endothelial origin from high venules (named high endothelial cells), express intracellular Von Willebrand factor and LVAP-2, a surface adhesion molecule present in lymphoid organs, and are able to bind lymphocytes without need of prior stimulation (Castro et al., 1996; Reyes et al., 2002). HUTECs were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin, and streptomycin and maintained at 37°C in a 5% CO_2/95% air atmosphere at nearly 100% relative humidity. Cells between passages 5 and 10, 24 h after feeding, were used in all experiments.

Confluent cultures of HUTECs were poorly coupled, <20% incidence of dye coupling (Lucifer Yellow) to one or two neighboring cells (not shown). This condition was not modified after application of histamine (0.01–1 μM). In contrast, HUTEC cultures studied at 70–80% confluence (close to maximal growth) were well coupled. Therefore, we opted to study the effects of histamine under subconfluent conditions.

Dye coupling

The functional state of gap junctions between endothelial cells cultured on glass coverslips was tested by observing the transfer to neighboring cells of a fluorescent dye microinjected into one cell. The cultures were observed on an inverted microscope equipped with xenon arc lamp illumination and a Nikon B filter (excitation wavelength 450–490 nm; emission wavelength above 520 nm). The dye (5% w/v Lucifer yellow in 150 mM LiCl) was microinjected

through glass microelectrodes by brief overcompensation of the negative capacitance circuit in the amplifier until the impaled cell was brightly fluorescent. After 1 min of dye injection, cells were observed to determine whether dye transfer occurred. The number of coupled cells was scored by counting neighboring cells that accumulated the dye. The incidence of dye coupling was estimated by dividing the number of injected cells showing dye transfer to two or more neighboring cells by the total number of cells injected in each experiment. In coupled cells, the dye coupling index was calculated as the average number of cells to which the dye spread from the microinjected cell. In all experiments, dye coupling was tested by injecting a minimum of 10 cells.

Affinity and immunofluorescence detection of endothelial proteins

Endothelial cells cultured on No. 1 round glass coverslips were fixed and permeabilized in 70% ethanol in Ca^{2+}-free PBS for 20 min at −20°C. Cells were incubated in blocking solution (containing 5 mM EDTA, 1% fish gelatin, 1% BSA essentially immunoglobulin-free, and 1% goat serum or 20% rat serum) for 30 min at room temperature. Then, cells were incubated in diluted primary antibody [$F(ab')_2$ fragments of anti-Cx43 antibody, anti-Cx40, anti-Cx37, anti-alpha-tubulin, or anti-vimentin antibody] overnight at 4°C, rinsed in PBS (pH 7.4) for 1 h at room temperature, incubated with diluted FITC-conjugated goat anti-rabbit IgG secondary antibody or FITC-conjugated goat anti-mouse IgG for 1 h at room temperature, followed by another rinse period of 1 h. Microfilaments were stained with FITC-labeled phalloidin for 20 min followed by a rinse period of 30 min with PBS. After a brief wash in distilled water, coverslips were mounted using Gelvatol-Dabco (Sigma), observed under a Nikon labophot-2 microscope equipped with epifluorescent illumination and photographed. Specificity of the immunoreactivity was assessed by replacing the primary antibody or $F(ab')_2$ fragments of immunoglobulins by a nonimmune serum.

Immunoblotting

Endothelial cell cultures (60-mm dishes) were harvested by scraping with a rubber policeman in ice-cold 2 mM PMSF in PBS (pH 7.4), pelleted, and lysed in solubilization buffer by sonication. Solubilization buffer contained protease inhibitors (200 μg/ml soybean trypsin inhibitor, 1 mg/ml benzamidine, 1 mg/ml epsilon-aminocaproic acid, and 2 mM PMSF) and phosphatase inhibitors (20 mM $Na_4P_2O_7$ and 100 mM NaF). Alternatively, cells were suspended in a denaturing solution (containing 10 mM TCA and 10 mM DTT, pH 7.6), maintained on ice for 30 min, and centrifuged for 1 min at maximal speed in an Eppendorf 5415C centrifuge. The supernatant was discarded, and the pellet was washed with ethyl ether three times for 10 min each. Pelleted proteins were resuspended in a solution containing 8 M urea, 20 mM Tris pH 8.6, 23 mM glycine, 10 mM DTT, 3 mM PMSF, 10 μM/ml leupeptin, and 2 mg/ml soybean trypsin inhibitor. Proteins were measured in aliquots of cell lysates using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA), and Western blot analyses were performed as described previously (Martínez and Sáez, 1999). Briefly, 150 μg of protein was resolved in 8% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE). Gels were then blotted onto nitrocellulose and electrotransferred at 300 mA for 1.2 h. Equivalence of loading and transfer efficiency was confirmed by protein staining with Ponseau red (2% w/v in 30% trichloracetic acid). Gels showing lanes with equal amounts of proteins were then destained by washing several times with distilled water, incubated in 5% nonfat milk in Tris-buffered saline (TBS), pH 7.4 for 30 min at room temperature, and kept overnight at 4°C in anti-Cx43 antibody diluted in 5% nonfat milk in TBS. Blots were rinsed repeatedly in TBS and incubated for 1 h at room temperature in alkaline phosphatase-conjugated goat antirabbit IgG antibody diluted in 5% nonfat milk in TBS. After rinsing repeatedly in TBS, blots were incubated with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets, Sigma). Prestained low molecular weight markers and/or aliquots of rat heart homogenate were used to identify the electrophoretic mobility of Cx43 reactive bands. Resulting immunoblots signals were scanned to a 6360 Macintosh computer, and densitometric analysis was performed with the NIH-Image software.

Statistical analysis

Values are presented as mean ± SEM. Differences in dye coupling index were assessed by one- or two-way ANOVA, with appropriate post hoc tests of Neumann–Keuls in the case of multiple comparisons or Dunnett test for multiple comparisons against a single control, as needed. P values are indicated in the figures.

Results

Histamine reduces dye coupling between human tonsil endothelial cells

In resting conditions, HUTECs of 70–80% confluence were dye-coupled as evidenced by the intercellular transfer of Lucifer yellow microinjected into one cell to several neighboring cells (Fig. 1B). At this confluence condition, treatment for 1 h with 1 μM histamine caused a strong reduction in dye coupling (Fig. 1D). In 11 control series, the coupling index was 5.9 ± 0.24 (n = 98 cells), and the incidence of dye coupling was 90.8 ± 2.2% (n = 11 plates with 107 microinjected cells). One-hour treatment with histamine induced a reduction in coupling index and incidence (Fig. 2A), but no significant changes in dye

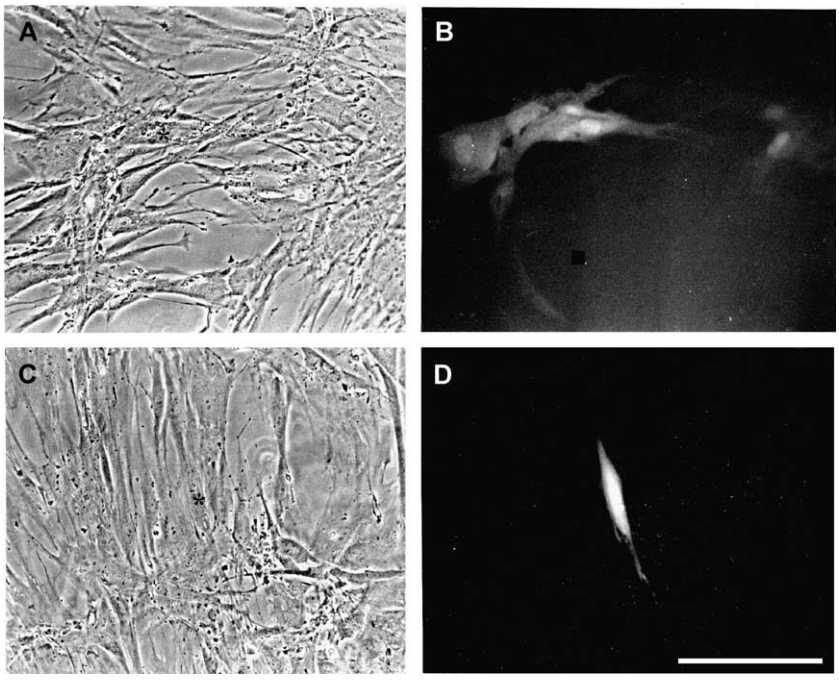

Fig. 1. Cultured HUTEC are dye-coupled, and gap junctional communication is reduced by histamine. Lucifer yellow was microinjected into one HUTEC (*) of a culture maintained under control conditions (A and B) or treated for 1 h with 1 μM histamine (C and D). B and D are fluorescent views of phase contrast showed in A and C. Bar: 100 μm.

coupling were observed between different histamine concentrations in the range of 10 nM to 1 μM (Fig. 2A), suggesting that maximal response might be attained at even lower concentrations. To explore the mechanisms involved in the uncoupling response, HUTECs were treated with 1 μM histamine in most experiments described below. Averaging all cultures treated with 1 μM histamine for 1 h, coupling index was 3.04 ± 0.18 (n = 47 cells), and the incidence of coupling was 46.4 ± 4.5% (n = 7 plates with 99 microinjected cells).

The histamine-induced reduction in dye coupling was time-dependent (Fig. 2B). After 30-min treatment, cell coupling was comparable to that of control cultures (Fig. 2B). Nevertheless, after 45-min treatment, the coupling index was drastically reduced, although the incidence of coupling was unaffected. Treatments for longer than 45 min, up to 3 h, reduced both the coupling index and incidence of dye coupling to about one half the control values (Fig. 2B). However, after 24-h incubation with 1 μM histamine, cells tended to be better coupled than control conditions (Fig. 2B).

H_1 receptors mediate cell uncoupling and H_2 receptors enhance cell coupling

We assessed the participation of H_1 receptors and their associated intracellular transduction pathways by using

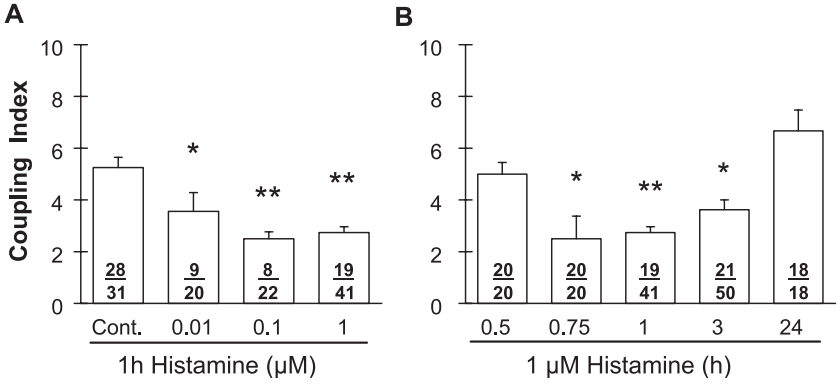

Fig. 2. Gap junctional communication between HUTECs is reduced by histamine in a time-dependent manner. Cell coupling was evaluated measuring dye transfer (LY). In panel A, HUTECs were treated for 1 h with culture medium (Cont.) or different concentrations of histamine. Panel B shows the coupling index of HUTECs treated for different periods of time (hours) with 1 μM histamine. Each bar corresponds to the mean ± SEM for coupling index. *$P < 0.05$, **$P < 0.01$ versus control, one-way ANOVA followed by Dunnett test. The number of coupled cells (numerator) and microinjected cells (denominator) within each column indicates the incidence of coupling.

chlorpheniramine, an H_1 receptor blocker, and GF109203X, a PKC inhibitor (Toullec et al., 1991). Neither 1 μM chlorpheniramine nor 2.5 μM GF109203X, applied for 1.5 h, affected the extent of dye coupling (Fig. 3A). However, both antagonists applied at the above mentioned concentrations 30 min before the addition of 1 μM histamine prevented the histamine-induced reduction in dye coupling (Fig. 3A). These results indicate that a PKC-dependent pathway triggered by H_1 receptors activation mediates the histamine-induced reduction in dye coupling between HUTECs. This assumption was further confirmed by the direct activation of PKC with PMA, a tumor promoter phorbol ester. Treatment for 1 h with 10–200 nM PMA caused a concentration-dependent reduction in cell coupling (Fig. 3B). Furthermore, the addition of 2 μM 4Br-A23187, a calcium ionophore, did not modify cell coupling within the period of time studied (1 h), but it enhanced the PMA-induced reduction in dye coupling (Fig. 3B), suggesting that a calcium-dependent PKC isoform is involved in the response.

To further characterize possible intracellular second messenger pathways that mediate the histamine-induced reduction in cell coupling, we explored the involvement of cyclooxygenases. Cells were treated with 10 or 50 μM indomethacin, a wide-range cyclooxygenase inhibitor, 30 min before the application of 1 μM histamine. Both indomethacin concentrations tested did not affect the intercellular dye transfer assessed 1.5 h later (Fig. 4); however, they prevented the reduction in dye coupling observed after 1-h treatment with 1 μM histamine (Fig. 4). These results indicate that a cyclooxygenase-dependent signal contributes to cell uncoupling induced by activation of H_1 receptors.

In contrast, activation of cAMP-dependent pathways, known to be activated by H_2 receptors, increased the extent of cell coupling in HUTECs. Treatment for 1.5 h with 1 mM 8Br-cAMP, a membrane-permeable analog of cAMP, caused an increment in the HUTEC coupling index, while maintaining a high incidence of dye coupling (Fig. 5A).

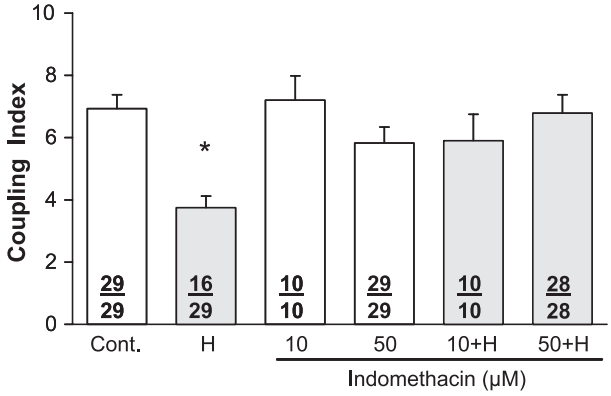

Fig. 4. Gap junctional communication between HUTECs is regulated by cyclooxygenase-dependent pathways. Inhibition of cyclooxygenases prevents the reduction in gap junctional communication induced by histamine in HUTECs. The dye coupling index of HUTECs was evaluated under control conditions (Cont.), after treatment with 1 μM histamine (H) for 1 h, 10 or 50 μM indomethacin for 1.5 h or 10 or 50 μM indomethacin for 1.5 h plus 1 μM H during the last 60 min. Each plotted value corresponds to the mean ± SEM. The number of coupled/injected cells is indicated in each column. (*$P < 0.01$ versus Cont., one-way ANOVA followed by Dunnett test).

Furthermore, cells treated with 1 mM 8Br-cAMP applied 30 min before the addition of 1 μM histamine showed similar dye coupling values as control cells, but significantly lower than cells treated only with 8Br-cAMP (Fig. 5A).

Since rapid desensitization of H_1 receptors has been reported (Hishinuma and Ogura, 2000), we explored whether H_2 receptor activation could be unmasked after prolonged (24 h) histamine application, perhaps contributing to dye coupling recovery or enhancement (Fig. 2B). Indeed, cells treated for 24 h with histamine followed by reapplication of 1 μM histamine for 1 h showed significant higher coupling index than control cells (Fig. 5B). In the presence of 10 μM ranitidine, an H_2 blocker, neither 24-h histamine, nor 24-h histamine followed by 1-h histamine reapplication caused an increase in cell coupling (Fig. 5B). Treatment with 10 μM ranitidine alone did not affect cell coupling (Fig. 5B).

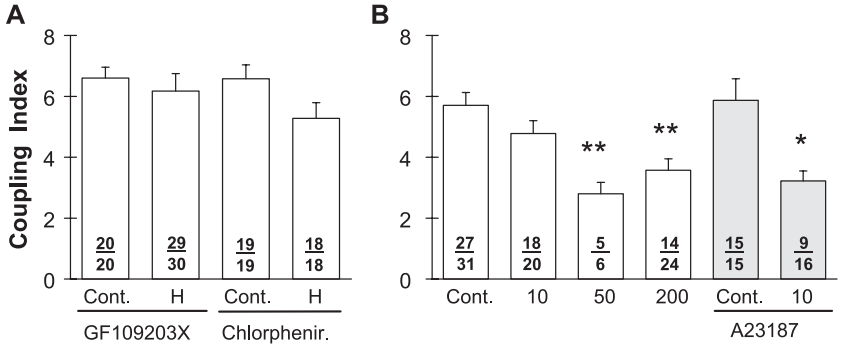

Fig. 3. The histamine-induced reduction in dye coupling is mediated by activation of H_1 receptors, and it is mimicked by activation of protein kinase C. Panel A depicts values obtained in HUTECs treated for 1.5 h with 1 μM chlorpheniramine (Chlorphenir.) or 2.5 μM GF109203X, with (H) or without (Cont.) the addition of 1 μM histamine during the last 60 min. Panel B shows values obtained in cells treated for 1 h with 10, 50, or 200 nM PMA, or with 2 μM 4Br-A23187 (A23187) with or without (Cont.) 10 nM PMA. The bars indicate mean ± SEM of coupling index. *$P < 0.05$, **$P < 0.01$ versus respective control, one-way ANOVA followed by Dunnett test. The incidence of coupling is indicated in each column by the number of coupled/microinjected cells.

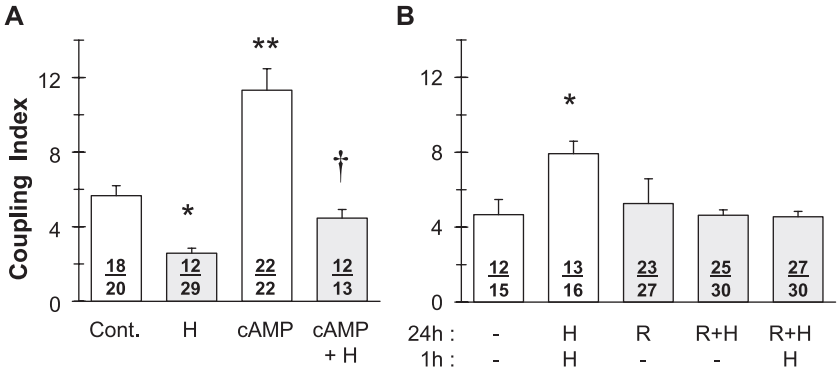

Fig. 5. Modulation of gap junctional communication by H_2 receptor pathways. Panel A. cAMP enhances cellular coupling and prevents the histamine-induced reduction in gap junctional communication between HUTECs. Dye coupling between HUTECs was evaluated under control conditions (Cont.), after treatment with 1 μM 8Br-cAMP (cAMP) for 1.5 h or 1 μM cAMP for 1.5 h plus 1 μM histamine (H) during the last 60 min. Each plotted value corresponds to the mean ± SEM of dye-coupling index. The incidence of coupling is indicated in each column by the number of coupled/injected cells. (*$P < 0.05$, **$P < 0.01$ versus Cont., $^{\dagger}P < 0.01$ versus cAMP, one-way ANOVA, Neumann–Keuls test). Two-way ANOVA indicated significant effects of histamine and cAMP ($P < 0.0001$) without significant interaction between both treatments. Panel B. Prolonged exposure to histamine reveals an H_2-mediated increase in cell coupling. Cells were incubated during 24 h with 1 μM histamine (H), 10 μM ranitidine (R), or both (R + H), followed by a 1-h period incubation with fresh medium with or without 1 μM histamine (H). Controls received just the vehicle (-) (*$P < 0.05$ versus control, one-way ANOVA followed by Dunnett test). Note that, in the presence of cAMP, histamine did not reduce the incidence of cell coupling.

Histamine induces Cx43 redistribution, but it does not significantly alter the levels of Cx43, nor affects Cx37 and Cx40

Since, in numerous cell types, PKC-mediated reduction in cellular coupling is associated to cellular redistribution and changes in phosphorylation state of Cx43 (Sáez et al., 2003), we studied whether these Cx43 features were affected by histamine. The reactivity of Cx43, a gap junction protein subunit, was detected by immunofluorescence and Western blot analyses. In fixed and permeabilized HUTECs under resting conditions, Cx43 showed a perinuclear distribution and was also detected in cell–cell membrane appositions (Fig. 6A). After 1-h application of 1 μM histamine, Cx43 was not detected at cell–cell interfaces and was localized in vesicle-like spots in addition to the perinuclear distribution (Fig. 6B). A similar Cx43 distribution was observed 1 h after the application of 1 μM 2-methyl histamine, a specific H_1 receptor agonist, or 200 nM PMA (Figs. 6E and F, respectively). Consistent with an H_1 receptor-mediated effect, Cx43 reactivity was clearly seen at cellular interfaces after coapplication of 1 μM histamine with 1 μM chlorpheniramine or 2.5 μM GF109203X (Figs. 6C and D, respectively). In contrast, treatment for 1 h with 1 mM 8Br-cAMP clearly increased the Cx43 labeling at cell–cell contacts (Fig. 6G), even in the presence of 1 μM histamine (Fig. 6H), in agreement with the enhanced cell coupling observed in the presence of 8Br-cAMP (Fig. 5A).

HUTECs were also positive for the gap junction subunits Cx37 and Cx40, as detected by indirect immunofluorescence (data not shown). However, both Cxs showed just a diffuse perinuclear and cytosolic distribution pattern. Furthermore, neither the intensity, nor the distribution of Cx37 or Cx40 labeling was modified after 1-h treatment with 1 μM histamine, suggesting that these proteins play a less significant role in cell coupling in this cell type, at least in the conditions of this study.

In total homogenates of HUTECs harvested under control conditions, three immunoreactive Cx43 bands were detected (Fig. 7). One band comigrated with the unphosphorylated (NP) form of Cx43 expressed in rat heart. The other two bands showed electrophoretic mobilities similar to that of the rat heart P2 and P3 phosphorylated forms. In HUTECs treated for 1 or 2 h with 1 μM histamine, the pattern and intensity of Cx43 bands were comparable to that of cells under control conditions (Fig. 7A, lanes 1 and 2 vs. lane 0). In contrast, after 1-h treatment with 200 nM PMA, both the pattern and intensity of Cx43 bands differed drastically from that of HUTECs under either control conditions or treated with histamine (Fig. 7A, lane PMA vs. lanes 0, 1, and 2, respectively). While the NP and P2 forms were less intense, the phosphorylated P1 and two hyperphosphorylated (HP) forms of Cx43 were resolved (Fig. 7A, lane PMA). In three independent experiments, the intensity and pattern of Cx43 reactive bands of HUTECs treated for 1.5 h with 1 mM 8Br-cAMP were slightly higher than that of HUTECs maintained under control conditions (Fig. 7B). According to a densitometric analysis, Cx43 immunoreactivity was 14.0 ± 5.5% higher in cAMP-treated cells than in controls, mainly due to a 15.4 ± 4.6% increase in the phosphorylated forms ($P = 0.06$, $n = 3$, paired t test).

Histamine induces changes in cell shape

It has been documented that histamine increases the permeability in endothelial cell monolayers by widening

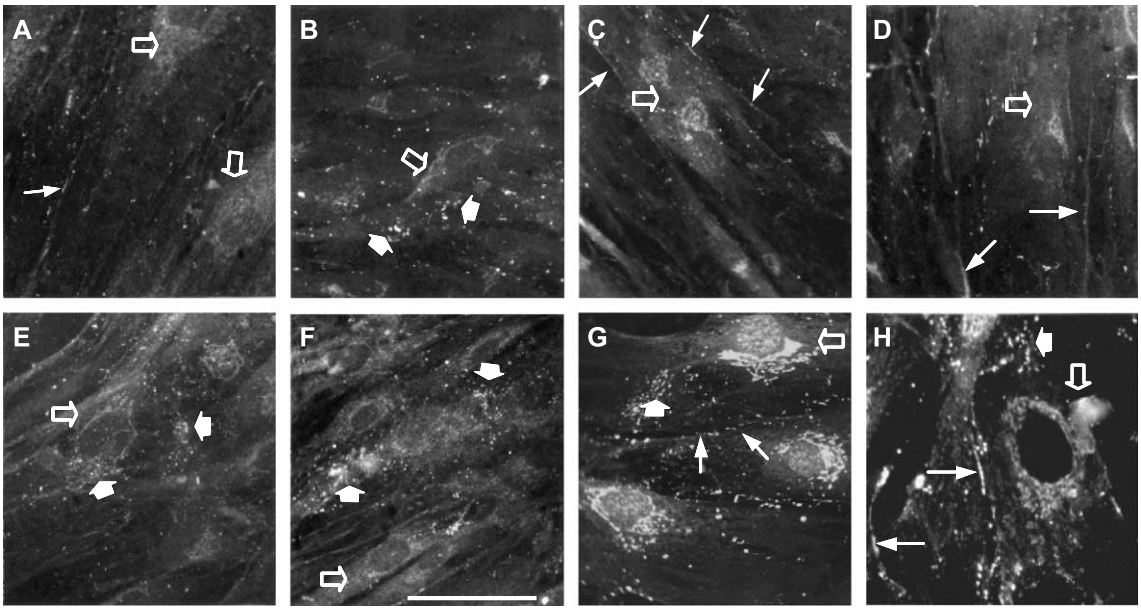

Fig. 6. Histamine induces cellular redistribution of Cx43, and its effect is not observed under conditions that prevent the reduction in gap junctional communication. The cellular distribution of Cx43 in HUTECs was determined by indirect immunofluorescence. Open arrows indicate diffuse perinuclear labeling; arrows denote labeling at cell–cell interfaces, and thick arrows show vesicle-like labeling. (A) Distribution of Cx43 in HUTECs under control conditions and (B) in cells treated for 1 h with 1 μM histamine. (C) and (D) show the distribution of Cx43 immunoreactivity in HUTECs pretreated with 1 μM chlorpheniramine or 2.5 μM GF109203X for 1.5 h and 1 μM histamine during the last 60 min, respectively. (E) and (F) are cells treated with 1 μM. 2-methyl histamine or 200 nM PMA for 1 h. (G) and (H) correspond to cells treated for 1.5 h with 1 mM 8Br-cAMP or 1 mM 8Br-cAMP for 1.5 h plus 1 μM histamine during the last 60 min, respectively. Bar: 130 μm for (A–D) and 65 μm for (G) and (H).

intercellular spaces, denominated "intercellular gaps," through a rearrangement of cytoskeletal stress fibers, which leads to cell retraction or contraction (Baldwin and Thurston, 1995; Mineau-Hanschke et al., 1990). Cell retraction could reduce the surface of intercellular contacts, and this process might imply a decrease in cell–cell adhesion leading to destabilization of gap junction channels and reduced intercellular communication. Therefore, we studied whether histamine affects the cell shape and/or the cytoskeleton in HUTECs. Phalloidin was used as an additional control to assess cytoskeletal changes. Phase contrast views of randomly chosen fields were photographed before and after 75-min incubation into a cell incubator (37°C, 95% air/5% CO_2 and 100% humidity) under control culture medium (n = 6) or with 0.1 μM phalloidin (n = 6); after 15-min, 1 μM histamine was added to three cultures in each condition. We choose 1-h histamine application because, at this time, we observed maximal reduction in gap junctional communication. Cells did not show noticeable shape changes in control conditions or with phalloidin treatment (Figs. 8A–D). But most cells showed drastic changes in shape after 1-h treatment with 1 μM histamine (Figs. 8E–F). In cells pretreated with phalloidin 15 min before treatment with 1 μM histamine, shape changes were much less pronounced than after treatment with histamine alone (Figs. 8G–H).

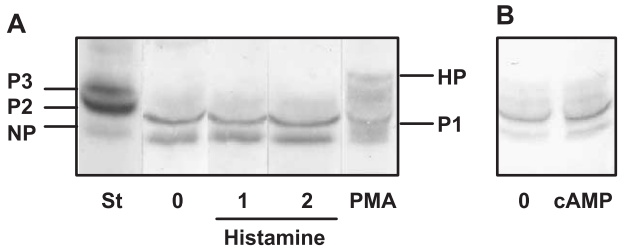

Fig. 7. Cx43 levels and phosphorylation state are not significantly affected by histamine and cAMP, but Cx43 phosphorylation state is enhanced by PMA in HUTEC. (A) Cultures of HUTECs were treated with 1 μM histamine for 0, 1, or 2 h, 200 nM PMA for 1 h, or (B) 1 mM 8Br-cAMP (cAMP) for 1.5 h. Cells were then harvested, homogenized, and analyzed by Western blot (samples of 150 μg of protein). A sample of rat heart homogenate (20 μg of protein) was used as standard (St). In A, the unphosphorylated form (NP), phosphorylated forms (P1, P2, and P3), and hyperphosphorylated forms (HP) of Cx43 are indicated.

Phalloidin abolishes the histamine-induced reduction in dye coupling

Due to the cell shape changes described above and because the formation of intercellular gap induced by inflammatory mediators is prevented by stabilization of actin filaments (Alexander et al., 1988; Goldblum et al., 1993a,b; Rasio et al., 1989), we used phalloidin to further assess the role of cytoskeletal and cell shape changes on the effects induced by histamine on dye coupling in HUTECs. Treatment for 1.5 h with 0.1 μM phalloidin did not affect either the coupling index or incidence of dye coupling in HUTECs (Fig. 9). However, in cells pretreated for 30 min with 0.1 μM phalloidin, 1-h treatment with 1 μM histamine

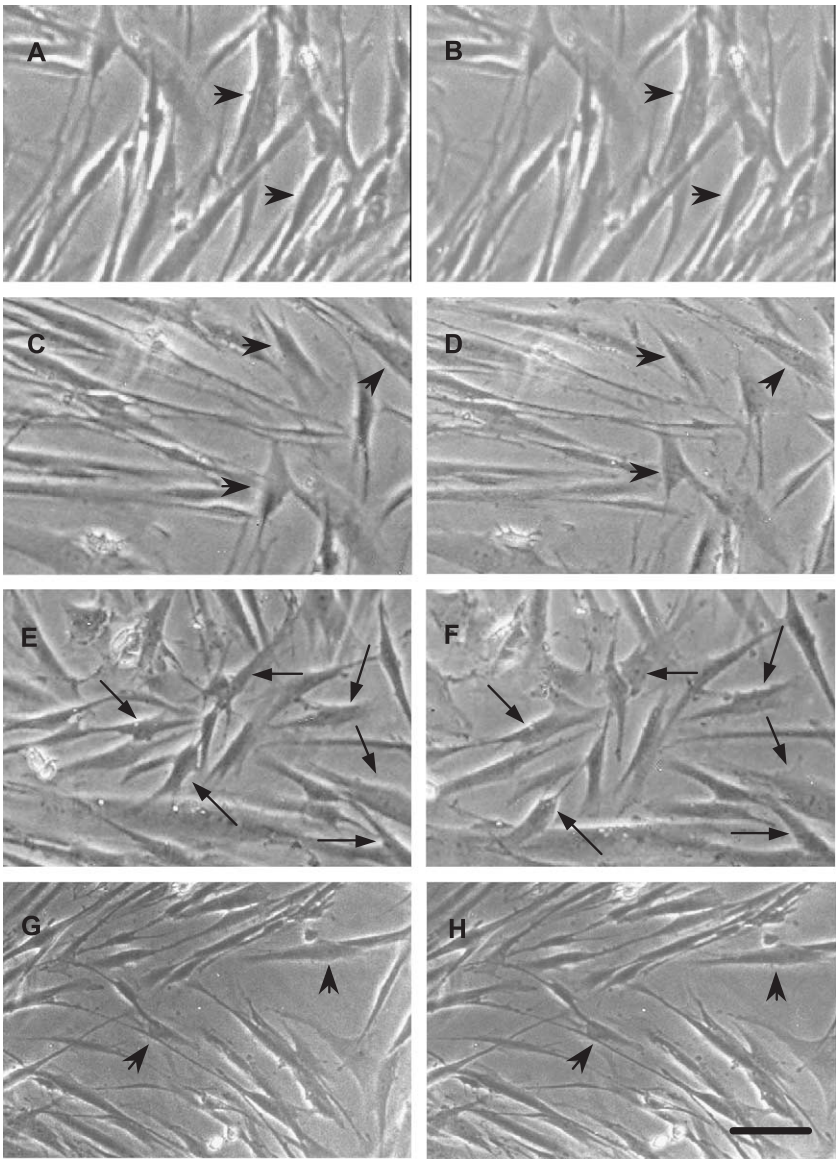

Fig. 8. Histamine induces cell shape changes, which are prevented by phalloidin. Phase-contrast photographs of given fields of HUTEC were taken 1 h apart. Cells did not show significant shape changes in control conditions (A–B) or with phalloidin treatment (C–D). Most cells showed major morphological changes after 1-h treatment with 1 μM histamine (E–F). In cells pretreated with phalloidin 15 min before treatment with 1 μM histamine, shape changes were much less evident than after treatment with histamine alone (G–H). Bar: 100 μm.

resulted in a coupling index and incidence of dye coupling similar to that of control cells (Fig. 9).

Discussion

The presence of Cx43 in cells of vascular endothelia, including HEVs of human tonsil, has been described previously (Krenács and Rosendaal, 1995), but conditions that regulate gap junctional communication in these cells were essentially unknown. Our results indicate that cultured HUTECs are responsive to histamine and express functional gap junction channels composed at least of Cx43. We also found that histamine acting on H_1 receptors triggers an intracellular pathway mediated by activation of PKC and cyclooxygenase, leading to a reduction in cell–cell communication via gap junctions. In contrast, a cAMP-dependent pathway, possibly triggered by the interaction of histamine with H_2 receptors, enhanced gap junctional communication. The inhibitory effect of histamine on gap junctional communication was also associated to changes in cell shape and was largely prevented by stabilization of F-actin microfilaments, suggesting the involvement of cytoskeleton-dependent mechanisms.

We found that HUTECs in primary culture are coupled to Lucifer yellow, a permeant dye for homotypic Cx43 gap junction channels (Elfgang et al., 1995). Accordingly, we found that HUTECs express Cx43 and also Cx37 and Cx40,

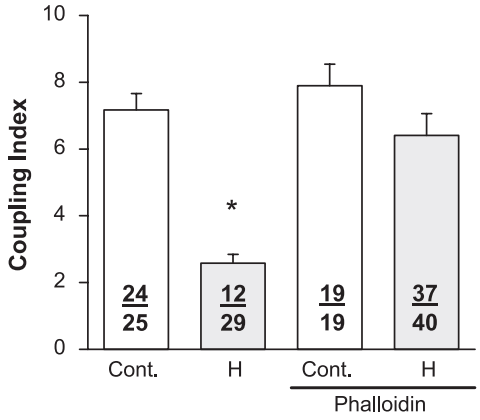

Fig. 9. Phalloidin prevents the reduction of dye coupling induced by histamine. Cell coupling was evaluated in HUTECs under control conditions (Cont.) or after treatment for 1 h with 1 μM histamine (H), both in absence (left) or presence of 0.1 μM phalloidin (right). Phalloidin was added 30 min before histamine. Each plotted value corresponds to the mean ± SEM (*$P < 0.001$ versus all other conditions, one-way ANOVA, Neumann–Keuls test). The incidence of coupling is indicated in each column by the number of coupled/injected cells.

as it has been reported for other types of endothelial cells (Haefliger et al., 2004). Cx40 is known to form heterotypic channels with Cx43, channels that are also permeable to Lucifer yellow (Valiunas et al., 2001).

Dye coupling was reduced by histamine at relatively low concentrations (0.1 μM), suggesting the involvement of plasma membrane receptors specific to histamine. Furthermore, the histamine-induced reduction in dye coupling was prevented by chlorpheniramine, indicating the participation of H_1 receptors. Interestingly, cells became refractory to histamine after 24-h treatment with the ligand. While histamine degradation was ruled out, the latter could be explained by several mechanisms, including desensitization and/or down-regulation of histamine receptors. In support of this notion, rapid desensitization of H_1 receptor through a Ca^{2+}/calmodulin-dependent kinase (Hishinuma and Ogura, 2000) and desensitization as well as a long-lasting down-regulation through a PKC-dependent mechanism have been described (Fujimoto et al., 1999; Pype et al., 1998). Down-regulation of H_1 receptors is inhibited by GF 109203X and by cycloheximide. Moreover, nuclear run-on assays have revealed a 50% reduction in the rate of histamine H_1-receptor gene transcription after roughly 24-h treatment with a tumor promoter phorbol ester, without changes in stability of the H_1 receptor mRNA (Pype et al., 1998). Thus, refractoriness to histamine effect on dye coupling could have resulted from a Ca^{2+}/calmodulin- and/or PKC-mediated desensitization of the histamine H_1-receptor and a transcriptional down-regulation of the histamine H_1-receptor gene expression.

A PKC-dependent pathway was also involved in the histamine-induced reduction in gap junctional communication because the PKC inhibitor, GF109203, prevented this effect. Moreover, the calcium ionophore, 4Br-A23187, enhanced the response induced by a low PMA concentration (10 nM), suggesting the involvement of a Ca^{2+}-dependent PKC isoform. In this regard, while activation of PKC with PMA induced a drastic modification of the Cx43 phosphorylation pattern in HUTECs, histamine did not affect it, suggesting that reduction of dye coupling in response to histamine was not due to direct phosphorylation of Cx43. In addition, the lack of effect of the calcium ionophore on both the coupling index and incidence of coupling of HUTECs suggests that increases in intracellular free Ca^{2+} concentration induced by histamine through activation of H_1 receptors (Grigorian et al., 1989) are required but not sufficient to explain the reduction in dye coupling observed in HUTECs treated with histamine.

Our observation of increased dye coupling in HUTECs treated with 8Br-cAMP agrees with previous reports showing increased intercellular communication in some cellular systems after activation of cAMP-dependent protein kinase (cAMP-dPK) (Sáez et al., 2003). This observation might also explain the increase in dye coupling found in cells treated for 24 h with histamine followed by 1-h histamine treatment, effect that was completely abolished by the coapplication of ranitidine, an H_2 blocker. As discussed before, H_1 receptors might have been desensitized and/or down-regulated, whereas the increase in dye coupling could have been due to an increase in endogenous cAMP mediated by activation of H_2 receptors. Nevertheless, in HUTECs, the increase in dye coupling induced by 8Br-cAMP was not associated to a relevant increase in levels and/or phosphorylation pattern of Cx43 detected by Western blot analysis, as it has been shown in other systems (Sáez et al., 2003). Our findings agree with the fact that Cx43 is a poor substrate for protein kinase A (Sáez et al., 2003). In HUTECs treated with 8Br-cAMP, the fluorescence labeling of cellular interfaces was increased, as it has been observed in other systems and attributed to an enhanced assembly of gap junction channels (Sáez et al., 2003). It is interesting to note that histamine was able to reduce the elevated coupling index of cells treated for 1 h with 8Br-cAMP back to control levels. This finding suggests that in control conditions, before desensitization, H_1 activation may effectively counteract H_2- or other agonist-induced pathways that elevate intracellular cAMP levels.

In HUTECs, the histamine-induced reduction in dye coupling was completely prevented by indomethacin, suggesting the involvement of arachidonic acid byproducts generated by a cyclooxygenase-dependent metabolism. In support to this notion, it is known that histamine induces the generation of arachidonic acid by activation of phospholipase A in endothelial cells (Millanvoye-Van Brussel et al., 1999). Moreover, as already mentioned, we found that histamine did not affect the pattern of Cx43 phosphorylation when assessed by Western blot analyses, but the dye uncoupling effect was associated to cellular redistribution of Cx43 from the plasma membrane to vesicle-like structures as previously shown for the arachidonic acid-induced dye uncoupling of cortical astrocytes (Martínez and Sáez, 1999).

The endothelium integrity is maintained by adhesive interactions at cell–cell and cell–matrix contacts via junctional proteins and focal adhesion complexes that are anchored to the cytoskeleton. Since gap junction formation and stabilization are favored by appropriate cell adhesion (Sáez et al., 2003), changes in cell adhesion proteins and their interaction with cytoskeleton might destabilize gap junction channels and induce their retrieval from the plasma membrane. In support to this notion, it is known that histamine-induced phosphorylation of adherents junction proteins modifies the interaction of VE-cadherin with the vimentin cytoskeleton (Andriopoulou et al., 1999; Shasby et al., 2002). Such general mechanism is consistent with the effect of different inflammatory mediators on various cell–cell junctions, including adherent junctions (Shasby et al., 2002), tight junctions (Gardner et al., 1996), and gap junctions, all of which require appropriate cell–cell adhesion. It is feasible that this mechanism may also operate within the specialized functions of high endothelial veins to facilitate passage of lymphocytes into the lymphoid tissue parenchyma.

It is known that phosphorylation cascades trigger biochemical and morphological changes of endothelial cells that ultimately lead to enhancing the paracellular pathway. In our studies, the histamine-induced reduction in cellular dye coupling occurred within the time course of the histamine-induced intercellular gaps (<1 h) observed in other types of cultured endothelial cells (Oliver, 1990). Moreover, our results show that the histamine effects on HUTECs were mediated by a PMA-activated PKC, and they were opposite to the effect of elevation of the intracellular cAMP concentration. Similarly, in some endothelial cells, activation of intracellular pathways mediated by cAMP-dependent protein kinase and PKC shows opposite actions on cellular junctions (Oliver, 1990) and on cytoskeleton organization (Meza et al., 1980; Sandoval et al., 2001), suggesting the involvement of the cytoskeletal changes downstream of PKC. In support of this, destabilization of gap junctions by disruption of actin microfilaments has been demonstrated (Theiss and Meller, 2002). Moreover, destabilization of microfilaments with cytochalasin B prevents formation of functional gap junctions between activated polymorphonuclear cells (Brañes et al., 2002) and inhibits recruitment and assembly of both Cx26 and Cx43 into gap junctions (Sáez et al., 2003). Likewise, in our HUTEC, the histamine-induced reduction in dye coupling was almost completely prevented by the actin filament stabilizer, phalloidin. Thus, the reduction in dye coupling induced by histamine could be in part due to cytoskeleton reorganization.

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