Dephosphorylation of the catenins p120 and p100 in endothelial cells in response to inflammatory stimuli

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Inflammatory mediators such as histamine and thrombin increase the tight-junction permeability of endothelial cells. Tight-junction permeability may be independently controlled, but is dependent on the adherens junction, where adhesion is achieved through homotypic interaction of cadherins, which in turn are associated with cytoplasmic proteins, the catenins. p120, also termed p120^{cas}/p120^{ctn}, and its splice variant, p100, are catenins. p120, originally discovered as a substrate of the tyrosine kinase Src, is also a target for a protein kinase C-stimulated pathway in epithelial cells, causing its serine/threonine dephosphorylation. The present study shows that pharmacological activation of protein kinase C stimulated a similar pathway in endothelial cells. Activation of receptors for agents such as histamine (H₁), thrombin and lysophosphatidic acid in the endothelial cells also caused serine/threonine dephosphorylation of p120 and p100,

suggesting physiological relevance. However, protein kinase C inhibitors, although blocking the effect of pharmacological activation of protein kinase C, did not block the effects due to receptor activation. Calcium mobilization and the myosin-light-chain-kinase pathway do not participate in p120/p100 signalling. In conclusion, endothelial cells possess protein kinase C-dependent and -independent pathways regulating p120/p100 serine/threonine phosphorylation. These data describe a new connection between inflammatory agents, receptor-stimulated signalling and pathways potentially influencing intercellular adhesion in endothelial cells.

Key words: adhesion, cadherin, histamine, protein kinase C, thrombin.

INTRODUCTION

Intercellular adhesion plays a crucial role in the development and maintenance of tissue architecture. Furthermore, regulation of adhesion is important in inflammation, tissue repair and remodelling. Aberrant control may contribute to pathologies such as cancer, where processes such as contact inhibition and cell motility are often misregulated. As such, there has been much interest in characterizing the proteins involved in intercellular adhesion and understanding the regulatory processes (for reviews, see [1–3]).

Cadherins are calcium-dependent adhesion molecules that play a key role in initiating and maintaining intercellular contact. The classic cadherins are single-pass transmembrane glycoproteins that interact homotypically with cadherins on neighbouring cells. Crucial for cadherin function is association via their cytoplasmic tail with a group of proteins termed catenins [4–6]. Cadherin binds directly to β - or γ -catenin [7,8], proteins related to the *Drosophila* segment polarity gene product Armadillo (see [9]), which then binds to α -catenin [8], a vinculin homologue, that links the complex to the actin-based cytoskeleton [10].

p120 was originally discovered as an Src substrate, tyrosine phosphorylation of which may be involved in cellular transformation [11,12]. It, too, is a member of the Armadillo family [9] and was subsequently realized to be associated with cadherins in both epithelial and endothelial cells [13–15]. A p120-related protein, p100, also exists [13,14], probably as a result of alternative splicing of the p120 gene [16]. Recently, a cadherin deletion

mutant that was designed to be defective in binding β -catenin could still cluster and support adhesion. This mutant was still capable of p120 binding, suggesting the importance of p120 in the regulation of cadherin function [17].

There is a fair amount of evidence that the cadherin–catenin complex is a target for signalling pathways (see [18–20]). As mentioned above, p120 is an Src substrate, and its tyrosine phosphorylation can also be stimulated by growth factors (see [20]). Tyrosine phosphorylation of β -catenin is also increased by *src* transformation but, although this correlates with disruption of cell–cell adhesion, its role in the regulation of intercellular adhesion is still questioned [21]. β -Catenin and p120/p100 tyrosine phosphorylation are also increased by treatment of epithelial cells with tyrosine phosphatase inhibitors, and again this correlates with disruption of intercellular adhesion [22]. p120 and p100 were also reported to be targets for a protein kinase C (PKC)-activated signalling pathway in epithelial cells [23]. In this case, PKC activation resulted in decreased serine/threonine phosphorylation of p120/p100. PKC activation must cause p120/p100 dephosphorylation by either p120/p100 kinase inhibition or activation of the corresponding phosphatase [23].

To explore the potential importance of the PKC–p120/p100 pathway, we investigated the regulation of p120/p100 phosphorylation in endothelial cells. The PKC–p120/p100 pathway is clearly present in these cells, as revealed by the use of phorbol 12,13-dibutyrate (PDB), a pharmacological activator of PKC. We then stimulated endothelial cells with agents that act via cell-surface receptors to trigger intracellular signalling. These cells possess receptors for agents such as histamine and thrombin,

Abbreviations used: BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; [Ca²⁺]_c, concentration of cytoplasmic free Ca²⁺; HUVEC, human umbilical-vein endothelial cells; LPA, lysophosphatidic acid; MLC, myosin light chain; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C; fura-2/AM, fura-2 acetoxymethyl ester; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; HAT, hypoxanthine/aminopterin/thymidine; BAEC, bovine aortic endothelial cells; MARCKS, myristoylated alanine-rich C-kinase substrate.

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known to be involved in the inflammatory response, and receptor activation seems to lead to PKC activation [24–27]. We found that p120/p100 phosphorylation in endothelial cells is clearly regulated by these inflammatory agents. However, they apparently act by a PKC-independent mechanism. p120/p100 serine/threonine phosphorylation is therefore regulated in endothelial cells by PKC-dependent and, yet-to-be-identified, -independent pathways.

EXPERIMENTAL

Chemicals and reagents

Bisindolylmaleimide I, fura-2 acetoxymethyl ester (fura-2/AM), the protein kinase inhibitor H-7, ionomycin, the kinase inhibitor Ro 31-8220, PDB, thapsigargin and thrombin were from Calbiochem–Novabiochem. ATP, histamine, and LPA (L- α -lysophosphatidic acid) were from Sigma Chemical Co. BAPTA [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrasodium salt] was from Molecular Probes. Cimetidine, pyrilamine and Ro 31-8425 were prepared by Tsukuba Research Laboratories, Eisai Co., Tsukuba, Japan.

Antibodies

Mouse monoclonal antibodies recognizing vacular endothelial cadherin (cadherin-5), p120 and β -catenin were from Transduction Laboratories. The PPMLC antibody was raised in rabbits against a peptide corresponding to myosin light chain (MLC) phosphorylated on Thr¹⁸ and Ser¹⁹. The peptide, RPQRA-pTpSNVFAMK, was coupled to keyhole-limpet haemocyanin (Pierce) using glutaraldehyde. Rabbits were immunized as described in [22], and specific antibodies in the immune serum were purified using peptide coupled to ethyl carbodi-imide hydrochloride–Sephacrose (Pharmacia). A similar strategy was used to raise antibodies to MLC using the peptide EFTRILK-HGAKDKDDK. Peptides were synthesized by ImmuneSystems (Paignton, Devon, U.K.). Horseradish-peroxidase-conjugated secondary antibodies were from Amersham, and fluorochrome-conjugated secondary antibodies and rabbit anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Cells and treatments

Endothelial cells were cultured in medium in the presence of 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C in humidified air with the indicated percentage of CO₂. The cells used were: EA.hy926 cells [28]; 10% CO₂/Dulbecco's modified Eagle's medium (DMEM)/10% (v/v) fetal-calf serum (FCS)/1 in 500 HAT [hypoxanthine (50 mM)/aminopterin (0.2 mM)/thymidine (8 mM)]; BAEC (bovine aortic endothelial cells, from Roy Bicknell, Institute of Molecular Medicine, Oxford, U.K.) grown on gelatin (5% CO₂/DMEM/10% FCS/30 μ g/ml endothelial-cell growth supplement/90 μ g/ml heparin); HUVEC (human umbilical-vein endothelial cells) were from Clonetics (distributed by TCS, Botolph Claydon, Buckingham, Bucks., U.K.), and more latterly BioWhittaker (Wokingham, Berks., U.K.), and cultured as instructed.

For experimental purposes, confluent monolayers of cells were equilibrated in fresh medium for at least 2 h before the start of the experiment. Compounds and agents that were soluble in DMSO were added to the culture medium of the cells in an equal volume of CO₂- and temperature-equilibrated medium containing twice the finally required concentration of compound. The final concentration of DMSO did not exceed 0.1% (v/v). Water-soluble agents were added at a 1:100 dilution directly to

the cultures. In all cases, appropriate vehicle controls were performed and found to have no effect.

Protein analysis

Whole-cell lysates were prepared by rapidly replacing the culture medium with hot sample buffer [29] supplemented with 5 mM EDTA (tetrasodium salt), followed by heating at 100 °C for 5 min. Proteins were resolved by gel electrophoresis using slab gels, 12 cm in length, and the buffer conditions described by Laemmli [29]. For p120/p100 analysis, 6%-(w/v)-acrylamide gels were used; for MLC, 12.5% gels were used. Gels were run overnight at 50–60 V. The gels were equilibrated in buffer containing 48 mM Tris, 39 mM glycine, 0.03% SDS (w/v) and 20% (v/v) methanol, and then transferred to nitrocellulose filters (Hybond ECL, Amersham). After Ponceau S staining, the filters were blocked in PBS containing 5% (w/v) non-fat dried milk, 1% (w/v) BSA and 0.05% (v/v) Tween-20. The filters were then incubated with primary antibody, rinsed, and immunoreactive bands were detected using appropriate horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL[®], Amersham).

Immunoprecipitations were performed at 4 °C. Cultures were rinsed with PBS and then lysed in either TX buffer [1% (v/v) Triton X-100/25 mM Hepes/2 mM EDTA/0.1 M NaCl/25 mM NaF/1 mM vanadate (pH 7.6) (adjusted with NaOH)/1 mM PMSF/10 μ g/ml soybean trypsin inhibitor/0.1 unit/ml α_2 -macroglobulin/10 μ g/ml leupeptin] or TDS buffer, which was identical with the TX buffer, except that it was supplemented with 0.5% (w/v) sodium deoxycholate and 0.2% (w/v) SDS. The cells were incubated with lysis buffer for 10–15 min and then scraped off. The lysates were collected and centrifuged at 14000 *g* for 20 min. The supernatant was precleared with Protein A–Sephacrose (Pharmacia) for 1–2 h and then incubated with primary antibody for 1 h followed by a further 1 h with Protein A–Sephacrose together with rabbit anti-mouse antibodies. After five washes in lysis buffer, immune complexes were dissociated by addition of Laemmli sample buffer followed by heating at 100 °C for 5 min. Protein analysis was by SDS/PAGE and immunoblotting as described above.

For phosphoaminoacid analysis, cultures of EA.hy926 cells were incubated overnight with 100 μ Ci/ml [³²P]P_i (Amersham) in phosphate-free DMEM medium containing 0.5% dialysed FCS and 1:500 HAT. After appropriate treatment, the cultures were rinsed in ice-cold PBS, lysed in TDS buffer and immunoprecipitated with the anti-p120 antibody. After separation by SDS/PAGE, protein was transferred to Immobilon P (Millipore), autoradiographed for [³²P]P_i-labelled protein and then immunoblotted to detect p120 and p100 protein. Densitometry of the autoradiogram and corresponding luminogram allowed determination of the ratio of incorporated label to protein. The area of filter containing p120 or p100 was then excised, and the protein was acid-hydrolysed to release phosphoamino acids which were then separated two-dimensionally (by high-voltage electrophoresis and chromatography) and detected by autoradiography [30].

Immunocytochemistry

Cells were fixed at room temperature for 15 min in 3% paraformaldehyde made up in PBS containing 0.5 mM CaCl₂ and 0.5 mM MgSO₄. Fixed cells were rinsed and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 min. After rinsing, the cells were incubated for 30 min in PBS containing 10% calf serum and 0.1 M lysine, pH 7.4. Incubation with primary antibody was in PBS containing 10% calf serum for 1 h.

After rinsing, the cells were then incubated for 30–60 min with a 1:100 dilution of FITC-conjugated anti-mouse IgG in PBS containing 10% calf serum. After rinsing, the filters were mounted with Citifluor and examined using a Nikon Microphot-FXA fluorescence microscope.

Cytoplasmic Ca^{2+}

The method to measure $[\text{Ca}^{2+}]_c$ (concentration of cytoplasmic free Ca^{2+}) in HUVECs was essentially a modification of that described in [31]. Loading of cells with fura-2 was achieved by incubation with $5 \mu\text{M}$ fura-2/AM for 30 min, followed by washing and then storage of the cells on ice. A Perkin–Elmer LS 50B fluorimeter with a cuvette holder maintained at 37°C was used to record fluorescence with excitation at 340 nm and emission at 510 nm. Fluorescence was converted into $[\text{Ca}^{2+}]_c$ according to published methods [32]. Saturation of intracellular fura-2 with Ca^{2+} to give maximum fluorescence (F_{max}) was achieved by application of $10 \mu\text{M}$ ionomycin, and minimum fluorescence (F_{min}) was subsequently determined by removal of Ca^{2+} from the fura-2 by chelation with BAPTA. Corrections were made for a fluorescence contribution from extracellular fura-2, and a K_d for Ca^{2+} of 220 nM was used in the calculations [32].

RESULTS

PKC activation in endothelial cells increases p120/p100 electrophoretic mobility

Confluent monolayers of a variety of cultured endothelial cell types [Figures 1A (HUVECs), 1B (EA.hy926), 1C and 1D (BAEC)] were incubated for 30 min with or without PDB, a pharmacological activator of PKC. The cultures were then extracted into sample buffer and analysed for p120/p100 by immunoblotting. As reported previously [14], endothelial cells express both p120 and p100 that migrate with a complex banding pattern on polyacrylamide gels. However, as in epithelial cells [23], after treatment of the cells with PDB, the mobility of p120 and p100 is altered to result in a faster and more tightly migrating protein (Figures 1A, 1B and 1C; Figure 1D is a longer exposure of Figure 1C that reveals p100 but overexposes the p120 signal). Therefore PDB treatment of endothelial cells triggers a mobility shift of p120 and p100. This effect could be inhibited by prior addition of the PKC inhibitor bisindolylmaleimide I, or rapidly reversed by subsequent addition of bisindolylmaleimide I (Figure 1E). This must reflect a dynamic interchange, not proteolysis, of p120 and p100 between faster- and slower-migrating forms of the protein.

Receptor activation in endothelial cells increases p120/p100 electrophoretic mobility

Endothelial cells express receptors for a variety of agents that are involved in the inflammatory response. These agents include the ligand histamine which through the H_1 receptor stimulates the phospholipase C-mediated generation of inositol lipids, leading to calcium mobilization, via $\text{Ins}(1,4,5)\text{P}_3$ [24,31,33], and PKC activation via diacylglycerol [26].

Incubation of HUVECs with $10 \mu\text{M}$ histamine resulted in a mobility shift of p120 and p100, seen within 30 s, the earliest time point examined, and sustained for up to 1 h (Figure 2A). The effect was stronger at earlier time points, with a trend back to the mobility of controls at later times (Figure 2A). The dose-dependence (Figure 2B) of histamine indicated very slight effects at $0.1 \mu\text{M}$ which were obvious at $1 \mu\text{M}$ and at higher concentrations. Furthermore, the H_1 antagonist pyrilamine blocked the

Figure 1 PKC activation stimulates a p120/p100 mobility shift in endothelial cells

Confluent monolayers of (A) HUVECs, (B) EA.hy926, (C and D) BAEC were incubated with (—) or without (+) 200 nM PDB for 30 min. The cells were extracted and p120 (○) and p100 (●) were detected by immunoblotting following SDS/PAGE. Two exposures are shown for the BAEC experiment, a short one (C) to highlight the p120 shift and a longer one (D) to reveal the p100 shift. HUVECs (E) were also incubated in the absence (Cont) or presence of 200 nM PDB for 15 min. In parallel, the cultures were pretreated with: $2.5 \mu\text{M}$ bisindolylmaleimide I for 15 min prior to the treatment with PDB (Bis I → PDB); 200 nM PDB for 15 min followed by co-incubation with either $2.5 \mu\text{M}$ bisindolylmaleimide (PDB → Bis I) or vehicle (PDB → Solv) for 15 min.

effect of histamine, whereas the H_2 antagonist cimetidine was ineffective (Figure 2C). The effect of histamine could also be reversed upon subsequent incubation with pyrilamine (result not shown). Other agents that can engage phosphoinositide signalling, including thrombin [24,26], LPA [34] and ATP, acting via purinergic receptors, also triggered the p120/p100 mobility shift (Figure 2D). Therefore, p120 and p100 are targets of what may be a common signalling pathway triggered by a variety of agents.

Dephosphorylation of p120/p100

Analysis of p120/p100 phosphorylation was performed using EA.hy926 cells. These cells respond to both PDB (Figure 1B), histamine (Figure 3A) and LPA (result not shown) with a p120/p100 mobility shift. Analysis of the $[\text{P}^{32}]$ phosphate content of these proteins after PDB, histamine or LPA treatment indicated that there was a loss of approx. 40% of signal when compared with control protein (Figure 3B). Therefore, as in the epithelial cells [23], these agents stimulated p120/p100 dephosphorylation.

Phosphoamino acid analysis revealed that, in unstimulated EA.hy926 cell, p120 (Figure 3C) and p100 (result not shown) were phosphorylated on serine and threonine residues with no detectable phosphotyrosine. Furthermore, the anti-phosphotyrosine antibodies PY20 and 4G10 could not immunoprecipitate p120 or p100 from SDS-solubilized cells (results not shown; see

Figure 2 Receptor activation stimulates a p120/p100 mobility shift in endothelial cells

(A) HUVECs were treated with $10 \mu\text{M}$ histamine for the times indicated. (B) HUVECs were treated for 15 min with the indicated concentrations of histamine. (C) HUVECs were preincubated for 10 min in the absence or presence of $1 \mu\text{M}$ pyrrolamine (Pyr, an H_1 antagonist), $1 \mu\text{M}$ cimetidine (Cim, an H_2 antagonist), and then incubated for a further 10 min in the absence or presence of $10 \mu\text{M}$ histamine (His). Control cells (Cont) received no treatment. (D) HUVECs were incubated in the absence (Cont) or presence of $10 \mu\text{M}$ histamine (His), $100 \mu\text{M}$ ATP, 10 units/ml thrombin (Thr) or $10 \mu\text{M}$ LPA for 10 minutes. In all cases, the cells were extracted into SDS sample buffer, proteins were separated by SDS/PAGE, and p120 (○)/p100 (●) mobility was then examined by immunoblotting following SDS/PAGE.

Figure 3 Serine/threonine dephosphorylation of p120/p100 in endothelial cells

(A) EA.hy926 cells were incubated in the absence (–) or presence (+) of $10 \mu\text{M}$ histamine for 15 min. The cells were extracted, and p120 (○) and p100 (●) were detected by immunoblotting following SDS/PAGE. (B) EA.hy926 were metabolically labelled with $[^{32}\text{P}]\text{P}_i$ and then received no further treatment (Cont), or were incubated with either 200 nM PDB, $10 \mu\text{M}$ histamine (His) or $10 \mu\text{M}$ LPA for 15 min. The cells were lysed, and the relative $[^{32}\text{P}]\text{phosphate}$ content of p120 and p100 was evaluated as described in the Experimental section. The data are means $\pm$ S.D. for two independent experiments, one done in duplicate, the other in triplicate. The $[^{32}\text{P}]\text{P}_i$ labelling by itself did not affect p120 or p100 mobility or the ability of the agents tested to cause a mobility shift. (C) In one experiment, phosphoamino acid analysis of p120 from the control cells (Cont) or the PDB-treated cells was then performed. In another (D), phosphoamino acids from p120 from control cells were compared with those from histamine (His) or LPA-treated cells. S, T and Y indicate respectively the migration of standards of phosphoserine, phosphothreonine and phosphotyrosine.

[22] for methodological details). In this case, for a positive control, cells were preincubated with pervanadate (see [22]), which resulted in tyrosine phosphorylation of p120 and p100, allowing its immunoprecipitation by PY20/4G10 (result not shown). Thus, in unstimulated endothelial cells, p120 and p100 appear to be phosphorylated exclusively on serine and threonine residues.

After treatment of EA.hy926 cells with PDB, histamine or LPA, phosphoserine and phosphothreonine were still the only phosphoamino acids detected in p120 (Figure 3C). Generally the phosphoserine and phosphothreonine levels were decreased, although it was difficult to see a clear difference between treated and control cells. However, since no phosphotyrosine was seen in p100/p120 from either control or treated cells by phosphoamino acid analysis (Figure 3C) or as inferred by the inability of PY20/4G10 to immunoprecipitate p100/p120 (result not shown), we conclude that the significant loss of phosphate from p100 and p120 seen in Figure 3(B) must be due to loss of serine/threonine phosphorylation.

It is perhaps not surprising that the phosphoamino acid signal did not change dramatically, as the phosphate-labelling data showed that p100/p120 was still substantially phosphorylated following treatment with PDB, histamine or LPA (Figures 3B and 3D). Also, the potential for slight variation in loading, differences in phosphoamino acid migration pattern, and overexposure of the film to ensure revelation of the weaker phosphothreonine all combine to make quantification from phosphoamino acid analysis difficult. Taken together, the loss in total phosphate content in p100/p120, along with the slight decrease in phosphoserine and phosphothreonine signal seen in phosphoamino acid analysis, and the complete absence of any phosphotyrosine in p100/p120 from control cells leads us to conclude that PDB, acting to directly activate PKC, and histamine, acting via the H_1 receptor, seem to produce similar loss of serine/threonine phosphorylation in p120 and p100. Preliminary studies based on Cleveland mapping indicate that the phosphorylation sites affected in response to both PDB and histamine may also be similar (results not shown).

PKC inhibitors do not completely block the effect of histamine

To test whether histamine acted on p120/p100 in HUVECs via PKC, the effects of PKC inhibitors were examined. Ro 31-8425 ([35]; Figure 4A) and bisindolylmaleimide I ([36]; Figures 1E and 4B) completely inhibited the ability of PDB to increase the electrophoretic mobility of p120/p100. In parallel, these same agents failed to suppress the effect of histamine (Figures 4A and 4B). In similar experiments, another PKC inhibitor, Ro 31-8220, also failed to inhibit the effect of histamine, but blocked that of PDB. These types of experiments were carried out several times with similar results. Therefore the effect of PDB and histamine can be distinguished pharmacologically, suggesting differences in pathways regulating p120/p100 phosphorylation.

To investigate the effects of histamine and the inhibitors on PKC activity, the phosphorylation of a PKC substrate, the myristoylated alanine-rich C-kinase substrate (MARCKS) protein, in the endothelial cells was determined (see [26,27]). Using the $[^{32}\text{P}]\text{P}_i$ labelling protocol described by Jacobson et al. [26], a 10 min incubation with 200 nM PDB or $10 \mu\text{M}$ histamine increased MARCKS phosphorylation by 1.78 ± 0.35 and 2.03 ± 0.29 -fold respectively (values are the means $\pm$ S.D., $n = 3$). Using Student's t test, both increases were significant at $P < 0.05$. Pretreatment with $2 \mu\text{M}$ bisindolylmaleimide for 15 min prior to 10 min treatment with vehicle actually suppressed MARCKS phosphorylation to $55 \pm 7\%$ (mean $\pm$ S.D., $n = 3$) of

Figure 4 Histamine-stimulated p120/p100 band shift is not blocked by PKC inhibitors

HUVECs were incubated in the absence (Cont) or presence of either 200 nM PDB or 10 μ M histamine (His) for 5 min. In some cases, the cells were also preincubated with 10 μ M Ro 31-8425 (A) or 2 μ M bisindolylmaleimide I (B) for 15 min prior to the treatments. The cells were extracted, and p120 (○) and p100 (●) were detected by immunoblotting following SDS/PAGE. Similar results were obtained using Ro 31-8220 as PKC inhibitor.

the control value. With PDB or histamine treatment, as described above, these agents were unable to stimulate MARCKS phosphorylation after pretreatment with bisindolylmaleimide (results not shown). Similar results (not shown) were obtained if the bisindolylmaleimide was replaced by 10 μ M Ro 31-8425. Stimulation of MARCKS phosphorylation is a reporter of PKC activation in cells. Therefore, the ability of histamine and PDB to stimulate MARCKS phosphorylation is consistent with PKC activation, and the inhibition by bisindolylmaleimide and Ro 31-8425 suggests inhibition of PKC activation in response to both of these agents. The inability of bisindolylmaleimide and Ro 31-8425 to block the histamine-induced increase in electrophoretic mobility of p120/p100 dissociates regulation of phosphorylation of these proteins from that of MARCKS.

Calcium mobilization and p120/p100 phosphorylation

Histamine, via H_1 receptors, is known to trigger an increase in $[Ca^{2+}]_i$ in endothelial cells (see Figure 5A). We investigated whether this Ca^{2+} signalling was involved in triggering p100/p120 dephosphorylation. Cellular calcium control was interfered with in several ways, and effects on p120/p100 were examined. In HUVECs, the histamine-triggered increase in $[Ca^{2+}]_i$ appears to initially involve inositol phosphate-mediated release from intracellular stores followed by Ca^{2+} influx from the extracellular medium [31]. Chelation of extracellular Ca^{2+} with BAPTA did not interfere with the initial increase in $[Ca^{2+}]_i$ in response to histamine, but attenuated the increase seen at later times (see Figure 5B). Extracellular BAPTA had no effect on the ability of histamine to produce the increase in electrophoretic mobility of p120/p100 (results not shown).

Thapsigargin, an inhibitor of the endoplasmic-reticulum Ca^{2+} -ATPase [37], can be used to manipulate $[Ca^{2+}]_i$. In endothelial cells, thapsigargin produces increases in $[Ca^{2+}]_i$ (Figure 5C). However, it was unable to trigger dephosphorylation of p120/

Figure 5 Calcium mobilization does not appear to regulate p120/p100 phosphorylation

In (A)–(C), HUVECs were loaded with fura-2 and fluorescence was measured as described in the Experimental section. In (A), Histamine (H) was added at the time indicated to a final concentration of 10 μ M, followed by ionomycin (I) also at 10 μ M. In B, BAPTA (B) was added (4 mM final) followed by histamine (H, 10 μ M), $CaCl_2$ (C, 4 mM) and ionomycin (I, 10 μ M). The immediate loss of fluorescence obtained upon addition of BAPTA and its regain after addition of $CaCl_2$ were due to the presence of a small amount of extracellular fura-2. In (C), thapsigargin (T, 1 μ M) was added, followed by histamine (H, 10 μ M) and ionomycin (I, 10 μ M). Resting values for $[Ca^{2+}]_i$ were $0.18 \pm 0.03 \mu$ M. In (A) the peak value after stimulation with histamine was 0.48 μ M, declining to 0.39 μ M in the plateau phase. In (B), a similar peak (0.53 μ M) was obtained, declining to 0.23 μ M just prior to addition of $CaCl_2$. In (C), thapsigargin treatment resulted in an increase in $[Ca^{2+}]_i$ to 0.59 μ M, a value similar to that obtained with histamine. Other experiments (not shown) yielded similar data. In (D), HUVECs were pretreated with the indicated concentrations of thapsigargin for 30 min, followed by incubation in the absence (C) or presence of either 10 μ M histamine (H) or 200 nM PDB (P) for 5 min. The cells were extracted and p120 (○) and p100 (●) were detected by immunoblotting following SDS/PAGE.

p100 (Figure 5D). Results of other experiments (not shown) indicated that, at earlier time points (0.5, 2, 5 and 15 min), 1 μ M thapsigargin failed to alter the electrophoretic mobility of p120/p100 when $[Ca^{2+}]_i$ was elevated to levels similar to those produced in response to histamine stimulation (see the legend to Figure 5). In cells pretreated with thapsigargin, the subsequent addition of histamine failed to further mobilize Ca^{2+} (Figure 5C), but still, like PDB, caused an increase in the electrophoretic mobility of p120/p100 (Figure 5D). These data indicate that Ca^{2+} is neither sufficient nor necessary for signalling leading to p120/p100 dephosphorylation.

Figure 6 PPMLC antibody

In order to study phosphorylation of MLC in endothelial cells, an antibody was raised against a peptide corresponding to Thr¹⁸/Ser¹⁹-phosphorylated MLC. (A) Shows an immunoblot of the migration of MLC protein from HUVECs as revealed by antiserum raised against EFTRILKHGAKDKDDK. The two bands (arrowheads) correspond to the 19 and 18 kDa isoforms of MLC in these cells. To detect Thr¹⁸/Ser¹⁹-phosphorylated MLC, antiserum was raised against RPQRAPTpSNVFAMK. (B) Shows an immunoblot of SDS extracts of HUVECs using the affinity-purified antiserum. Two bands corresponding to MLC were detected. Competition with RPQRAPTpSNVFAMK (pS19) failed to block antibody binding, whereas RPQRAPTpSNVFAMK (pT18/pS19) was completely effective. HUVECs were also incubated with 10 μM histamine for 2.5 min, 250 μM 8-(4-chlorophenylthio)cyclic AMP (CPT-cAMP), a membrane-permeant cyclic AMP analogue for 15 min and immunoreactivity with the anti-RPQRAPTpSNVFAMK serum is shown in (C).

p120/p100 and MLC phosphorylation

Histamine and thrombin are known to activate pathways leading to MLC phosphorylation in endothelial cells, which may be an important event in the regulation of cell contraction (see [38]). MLC phosphorylation appears to be controlled by $[Ca^{2+}]_i$, but it is also possible that Rho pathways play a regulatory role [39]. The relationship between p120/p100 dephosphorylation and pathways regulating MLC phosphorylation were investigated. This was achieved by using an antibody that recognizes Thr¹⁸- and Ser¹⁹-phosphorylated MLC (PPMLC), a form of the protein produced by stimulation of endothelial cells with histamine and thrombin (see [38]). A peptide, RPQRAPTpSNVFAMK, was used to immunize rabbits. Antibody was affinity-purified and shown to react with two bands of molecular mass 18 and 19 kDa (Figure 6B), corresponding to the MLC isoforms in endothelial cells (Figure 6A). Immunoreactivity was competed for by the immunizing peptide but not by Ser¹⁹-phosphorylated peptide (Figure 6B). The antibody therefore specifically recognizes MLC phosphorylated on both Thr¹⁸ and Ser¹⁹ residues. Incubation of HUVECs with 10 μM histamine produced an increase in Thr¹⁸-

Figure 7 MLC phosphorylation and p120/p100

(A) and (B) HUVECs were pretreated in the absence (Cont) or presence of 10 μM H-7 for 15 min and were either extracted for analysis or further treated with 10 μM histamine alone (His) or in the continued presence of H-7 (His + H-7) for 2 or 5 min. In (A), extracts were analysed for p120 (○) and p100 (●) by immunoblotting following SDS/PAGE, and in (B) for PPMLC (arrowheads).

and Ser¹⁹-phosphorylated MLC, and phosphorylation was suppressed by a cyclic AMP analogue (Figure 6C), further validating the use of the antibody as an experimental tool (see [40]).

Pathways regulating p120/p100 dephosphorylation and MLC phosphorylation were completely dissociable. Histamine stimulated a mobility shift in p120/p100 and an increase in PPMLC. The kinase inhibitor H-7 [41] inhibited the increase in PPMLC but had no effect on the ability of histamine to induce the p120/p100 mobility shift seen after 2 or 5 min incubation (cf. Figures 7A and 7B).

Cadherin association and cellular localization of p120/p100

In endothelial cells, as p120 and p100 are cadherin associated proteins [14], we investigated the possibility that altered phosphorylation resulted in regulation of association. VE-cadherin appears to be the major junctional cadherin in endothelial cells (see [19]). Endothelial cells were incubated with or without histamine, the cells were lysed in TX buffer (see the Experimental section) to preserve protein complexes and immunoprecipitated with anti-VE-cadherin antibody. Immunoblotting of the immunoprecipitates indicated altered p120/p100 mobility, but no change in the amount of protein associated with cadherin. Similarly, the amount of β-catenin associated with the VE-cadherin was not altered. We also examined p120/p100 localization by immunocytochemistry, and, again, found no evidence for altered association with intercellular junctions (results not shown).

DISCUSSION

The protein architecture of intercellular junctions is in the process of being elucidated. It is also becoming apparent that intercellular junctions are targets for signalling processes, suggesting that they are actively regulated (see [18–20]). We recently reported that, in epithelial cells, the cadherin-associated proteins p120 and p100

were targets for a PKC-activated pathway that lead to their dephosphorylation on serine and threonine residues [23]. Here we show that a similar pathway is present in endothelial cells. Moreover, inflammatory ligands that activate endogenous signalling pathways can also stimulate dephosphorylation of p120 and p100 on serine and threonine residues. However, these agents appear to act independently of PKC in that the PKC inhibitors tested so far, although blocking PDB effects, do not block the ability of the inflammatory agents to dephosphorylate p120/p100. This suggests that either histamine acts independently of PKC to induce p100/p120 dephosphorylation or that, given the diversity of the PKC family, [42], these particular PKC inhibitors were incapable of blocking the PKC isozyme activated by histamine. Whatever, PDB stimulated p120/p100 serine/threonine dephosphorylation can be pharmacologically distinguished from that triggered by histamine.

In looking for other pathways we found that Ca^{2+} mobilization in response to histamine could not account for effects on p120/p100. A variety of protocols were employed to either wilfully mobilize Ca^{2+} in cells or to interfere with Ca^{2+} mobilization in response to histamine, and were found to have no effect on p120/p100 phosphorylation. Likewise, histamine effects on pathways regulating MLC phosphorylation could not explain effects on phosphorylation of p120/p100. Thus, the kinase inhibitor H-7 inhibited phosphorylation of MLC in response to histamine but had no effect on the dephosphorylation of p120/p100. These data indicate that inflammatory agents trigger a signalling pathway that connects with proteins involved in cell-cell adhesion; however, the pathway itself seems to be novel, being inexplicable in terms of pathways known to be activated by these agents.

In terms of the function of phosphorylation of p120/p100 on serine/threonine residues, we explored the possibility that it regulated association of these proteins with the other members of the cadherin-catenin complex. By immunocytochemistry we could find no evidence for dissociation of p120/p100 from the complex at cell-cell contacts after treatment of the cells with agents known to alter serine/threonine phosphorylation of the protein. Furthermore, biochemical analysis of the cadherin-catenin complex after stimulation of the cells with the same agents indicated no effects on the stoichiometry of the complex. This parallels the situation in epithelial cells, where p100/p120 dephosphorylation also had no effect on association of these proteins with cadherin [23]. It is possible that other unknown components of the complex, if they exist, may have altered association after stimulation of the cells.

Physiologically, the role of the adherens junction in endothelial cells, as in epithelial cells (see [2]), is to initiate and maintain cell-cell contacts [43]. Tight-junction formation follows, and the endothelium acquires its barrier function, essential for the maintenance of tissue interstitial environment. Continued maintenance of tight-junction impermeability is dependent on the adherens junction (see [18,43]). Indeed, altered serine/threonine phosphorylation of p120/p100 was suggested to play a role in tight-junction permeability control in epithelial cells [23]. Given that agents such as histamine and thrombin are permeability-altering agents in endothelial cells, it is possible that regulation of p120/p100 serine/threonine phosphorylation is necessary in this regard. Yap et al. [17] have shown that the juxtamembrane p120/p100-binding region of cadherin may be involved in the regulation of clustering and adhesion of cadherins, and perhaps p120/p100 phosphorylation regulates this aspect of cadherin function. Another possibility is that inflammatory agents activate endothelial cells to recruit leucocytes to sites of inflammation. Transmigration, the process whereby these leucocytes cross the

endothelium, is a complex issue (see [44,45]). If leucocytes migrate through junctions, the role of p120/p100 phosphorylation may be to modulate cadherin function to facilitate this process. Furthermore, it has been proposed that neutrophil migration across endothelium involves activation of histamine-like signalling processes within the endothelial cells [44]. If so, we would predict that regulation of p120/p100 serine/threonine phosphorylation would also be involved in endothelial, and possibly epithelial, mechanisms involved in leukocyte transmigration. Further experiments will be needed to address these possibilities.

In summary, although p120 was initially reported as an Src substrate, a PKC activated pathway was then found in epithelial cells that caused serine/threonine dephosphorylation of p120 and the related protein p100. This pathway, as reported here, is also present in endothelial cells, suggesting some fundamental biological importance. p120/p100 serine/threonine dephosphorylation can also be stimulated by ligand-activated pathways, pathways that seem to be pharmacologically distinct from those activated by PDB. As these ligands are inflammatory agents, p120/p100 serine/threonine dephosphorylation may be an important regulator of endothelial function in the immune response. Many studies on catenin phosphorylation have employed rather extreme conditions, such as overexpression of tyrosine kinases or the use of broad-spectrum tyrosine phosphatase inhibitors. Importantly, the present study shows that p120/p100 phosphorylation can be regulated by physiologically relevant ligands acting through endogenous levels of signalling components, suggesting functional significance in the regulation of cell-cell adhesion.

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