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RESEARCH PAPER

Intermedin (adrenomedullin2) stabilizes the endothelial barrier and antagonizes thrombin-induced barrier failure in endothelial cell monolayers

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BACKGROUND AND PURPOSE

Intermedin is a member of the calcitonin gene-related-peptide (CGRP) family expressed in endothelial cells and acts via calcitonin receptor-like receptors (CLRs). Here we have analysed the receptors for intermedin and its effect on the endothelial barrier in monolayers of human umbilical vein endothelial cells (HUVECs).

EXPERIMENTAL APPROACH

We analysed the effect of intermedin on albumin permeability, contractile machinery, actin cytoskeleton and VE-cadherin in cultured HUVECs.

KEY RESULTS

Intermedin concentration-dependently reduced basal endothelial permeability to albumin and antagonized thrombin-induced hyperpermeability. Intermedin was less potent (EC_{50} 1.29 ± 0.12 nM) than adrenomedullin (EC_{50} 0.24 ± 0.07 nM) in reducing endothelial permeability. These intermedin effects were inhibited by AM_{22-52} and higher concentrations of $\alpha CGRP_{8-37}$, with pA_2 values of $\alpha CGRP_{8-37}$ of 6.4 for both intermedin and adrenomedullin. PCR data showed that HUVEC expressed only the CLR/RAMP2 receptor complex. Intermedin activated cAMP/PKA and cAMP/Epac signalling pathways. Intermedin's effect on permeability was blocked by inhibition of PKA but not of eNOS. Intermedin antagonized thrombin-induced contractile activation, RhoA activation and stress fibre formation. It also induced Rac1 activation, enhanced cell–cell adhesion and antagonized thrombin-induced loss of cell–cell adhesion. Treatment with a specific inhibitor of Rac1 prevented intermedin-mediated barrier stabilization.

CONCLUSION AND IMPLICATIONS

Intermedin stabilized endothelial barriers in HUVEC monolayers via CLR/RAMP2 receptors. These effects were mediated via cAMP-mediated inactivation of contractility and strengthening of cell–cell adhesion. These findings identify intermedin as a barrier stabilizing agent and suggest intermedin as a potential treatment for vascular leakage in inflammatory conditions.

Abbreviations

AJ, adherens junctions; CGRP, calcitonin gene related peptide; CLR, calcitonin receptor-like receptor; Epac, exchange protein directly activated by cAMP; HUVEC, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-Arginine methyl ester; L-NNA, N^G-nitro-L-Arginine; MLCs, myosin light chain; MLCK, myosin light chain kinase; MLCP myosin light chain phosphatase; RAMP, receptor activity modifying protein; Rock, Rho kinase

Introduction

Vascular endothelium forms a selective barrier and regulates trafficking of macromolecules and blood cells across the vessel wall (Mehta and Malik, 2006). The integrity of the endothelial barrier is highly dependent on actin–myosin-based contractile machinery and actin cytoskeleton-mediated endothelial adherens junctions consisting of vascular endothelial (VE)-cadherin linked to the actin cytoskeleton (Dejana *et al.*, 2008). Exposure of endothelial cells to inflammatory mediators like thrombin results in the activation of multiple signalling pathways, finally leading to endothelial barrier failure (Schnittler *et al.*, 1990; Rabiet *et al.*, 1996). The combined net effect of these phenomena is increased vascular permeability, which is the first step in the development of a variety of vascular anomalies including atherosclerosis and neointima formation (Mehta and Malik, 2006; Vandenbroucke *et al.*, 2008).

Accumulating evidence suggests a pivotal role of the calcitonin receptor-like receptor (CLR) signalling pathway in maintaining/stabilizing endothelial integrity (Hippenstiel *et al.*, 2002; Dackor *et al.*, 2006) and preventing oedema formation induced by a variety of inflammatory agents and sepsis (Hippenstiel *et al.*, 2002). These class B GPCRs are activated by members of the calcitonin gene-related peptide (CGRP) super-family and their activation results in a marked increase in cytosolic cAMP levels and subsequent activation of PKA (Hippenstiel *et al.*, 2002; Poyner *et al.*, 2002; Roh *et al.*, 2004). Intermedin (also called adrenomedullin 2) is a new member of the CGRP super-family which includes: calcitonin, amylin, CGRP and adrenomedullin, showing only 33% sequence homology to adrenomedullin (Roh *et al.*, 2004; Takei *et al.*, 2004b).

The CLR itself exhibits little affinity for CGRP, adrenomedullin or intermedin, but becomes ligand-selective when associated with one of the receptor activity-modifying proteins, RAMP1–3 (receptor nomenclature follows Alexander *et al.*, 2011). CLR, in association with RAMP1, forms CGRP receptors (Poyner *et al.*, 2002) which are sensitive to blockade by $\alpha CGRP_{8-37}$ (Hay *et al.*, 2005). CLR in combination with RAMP2 and 3 constitutes AM_1 and AM_2 receptors, respectively, which have low affinity towards $\alpha CGRP_{8-37}$ but are highly sensitive to block by the adrenomedullin fragment, AM_{22-52} (Hay *et al.*, 2003; 2004; Bell and McDermott, 2008). Previous studies show that adrenomedullin is an agonist for CLR/RAMP2 and RAMP3 (Fraser *et al.*, 1999; Takei *et al.*, 2004a), whereas intermedin signals mainly through CLR associated with RAMP1 or RAMP3, but also shows affinity towards RAMP2 (Roh *et al.*, 2004; Takei *et al.*, 2004a; Bell and McDermott, 2008). It has recently been shown that intermedin is cardioprotective in ischaemia-reperfusion injury (Yang *et al.*, 2005), and has vasodilatory (Burak *et al.*, 2006; Rademaker *et al.*, 2008) and hypotensive effects (Rademaker *et al.*, 2008). We have recently shown that intermedin mediated barrier-stabilizing effects in isolated mouse lungs and human pulmonary microvascular endothelial cells via CLR/RAMP complexes, which were mainly sensitive to $\alpha CGRP_{8-37}$ (Pfeil *et al.*, 2009). However, the signalling mechanisms underlying these actions have not yet been analysed.

In the present study, we analysed the effects of intermedin on the barrier function in a frequently used endothelial cell model, monolayers of human umbilical vein endothelial cells (HUVECs), and characterized the receptors which mediated this effect. Moreover, we analysed signalling mechanisms mediating these barrier-protective effects of intermedin. We showed that intermedin mediated its barrier protective effects mainly via CLR/RAMP2 (AM_1) receptors. Also intermedin activated cAMP signalling leading to inactivation of endothelial contractile machinery and enhanced cell–cell adhesion and thus protecting the endothelial barrier against inflammatory mediators.

Materials and methods

Cell culture

The study conforms to the principles outlined in the Declaration of Helsinki. HUVECs were isolated from umbilical cords derived from normal healthy, uncomplicated pregnancies obtained from the University Hospital Giessen, after approval from hospital ethics committee and cultured as described before (Aslam *et al.*, 2010). The experiments were performed with HUVECs at passage 1 or 2 (where mentioned).

Experimental protocols

The basal medium used in incubations was modified Tyrode's solution (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$ and 30 HEPES; pH 7.4, 37°C). Agents were added as indicated. Stock solutions of adrenomedullin, intermedin, myristoylated PKI, thrombin, $\alpha CGRP_{8-37}$, AM_{22-52} and Y27632 were prepared immediately before use with basal medium. Appropriate volumes of these solutions were added to the cells, yielding final solvent concentrations <0.1% (vol/vol). The same final concentrations of basal medium were included in all respective control experiments. Cells were incubated with serum-free Tyrode's solution 30 min prior to adding the drugs. In experiments where pharmacological inhibitors or peptide receptor antagonists (AM_{22-52} and $\alpha CGRP_{8-37}$) were used, the inhibitors or antagonists were added 30 min before adding intermedin or adrenomedullin. In the experiments where the PKA inhibitor peptide, PKI, was used, the cells were pre-incubated with PKI for 60 min followed by the addition of relevant stimulators as shown in the figure legends.

In a set of pilot experiments, concentration-response relationships were determined to find the optimal effective concentration. The following agents were applied in their optimal effective concentrations: intermedin (10 nM), adrenomedullin (10 nM), thrombin (0.2 $U \cdot mL^{-1}$), myristoylated PKI (20 μM), Y-27632 (10 μM).

Western blotting

Western blotting was performed as described previously (Aslam *et al.*, 2010). Briefly, cells were harvested in lysis buffer (composition: 2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol, 10 mM dithiothreitol (DTT), 5% β-mercaptoethanol) containing phosphatase and protease inhibitors. Equal amounts of lysates (30 μg) were subjected to SDS-PAGE (12.5% gel) and blotted. Membranes were incubated with rabbit phospho-specific anti-myosin light chain (MLC; Thr^{18}/Ser^{19}) overnight

at 4°C followed by HRP-labelled anti-rabbit IgG for 1 h at room temperature. Proteins were detected by using Bio-Rad luminescence imaging system (Discovery series; Hercules, CA, USA).

RhoA activity

Direct RhoA activation was measured by G-LISA assay (Cytoskeleton) according to the manufacturer's instructions. Briefly, the RhoA G-LISA kit used 96-well plates coated with the Rho-binding domain of the RhoA effector Rhotekin. Rho-GDP was removed during washing steps and Rho-GTP was detected with a RhoA-specific antibody and chemiluminescence. The level of RhoA activation was expressed relative to its basal level, in the absence of intermedin or thrombin.

Rac1 pulldown assay

The activation of Rac1 was assessed by pull-down assay in the cells stimulated with intermedin, thrombin or combination of both. HUVECs were washed with ice-cold PBS and lysed with 600 µL of lysis buffer on ice for 10 min. Lysate was centrifuged at 14 000× *g* for 1 min at 4°C. Cell lysates (500 µg) were incubated with 10 µg of GST-PAK beads (Cytoskeleton) at 4°C for 40 min. The beads were washed four times with wash buffer, heated to 95° for 5 min with 40 µL of Laemelli buffer and loaded on 12.5% SDS gel. Bound Rac1 protein was then detected by immunoblotting using polyclonal antibodies against Rac1 (Cytoskeleton). The total amount of Rac1 in cell lysates was used as a control for the cross-comparison of Rac1 activity (level of GTP-bound Rac1).

Macromolecule permeability measurement

The permeability of Trypan blue-labelled albumin across HUVEC monolayers was studied in a two-compartment system separated by a filter membrane as described previously (Pfeil *et al.*, 2009). Briefly, both compartments contained as basal medium modified Tyrode's solution (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$ and 30 HEPES; pH 7.4, 37°C) supplemented with 2% (vol/vol) normal calf serum. There was no hydrostatic pressure gradient between both compartments. The 'luminal' compartment containing the monolayer had a volume of 2.5 mL, and the 'abluminal' had 6.5 mL. The fluid in the 'abluminal' compartment was constantly stirred. Trypan blue-labelled albumin (60 µM) was added to the luminal compartment. The appearance of the labelled albumin in the abluminal compartment was continuously monitored by pumping the liquid through a spectrophotometer (Specord 10, Zeiss, Jena, Germany). Increase in the concentration of labelled albumin was detected with a time delay of less than 15 s.

cAMP assay

HUVECs were cultured in 6-well plates until confluence, washed once with modified Tyrode's buffer supplemented with the phosphodiesterase inhibitor, IBMX (0.1mM), to prevent the hydrolysis of cAMP and then incubated in the same solution for 30 min in the presence or absence of the other reagents. After the incubation, cAMP content of each well was determined by an acetylated version of colorimetric immunoassay method according to the manufacturer's instructions (Assay Designs, Loerrach, Germany) using 'Infinite® 200' fluorescent plate reader (Tecan, Austria). Forskolin (10 µM) was included as positive control on each plate. Three measurements per treatment group were performed on cells from an individual culture and averaged to yield one value. Experiments were performed on three individual cultures.

Immunocytochemistry and confocal microscopy

HUVECs were grown until confluence on glass cover slips. After treatment, cells were washed with PBS and fixed with 4% paraformaldehyde at 37°C for 20 min. Non-specific binding was blocked by incubating cells with blocking solution (5% BSA + 5% neonatal calf serum) for 1 h. Cells were incubated with the primary antibody (1:100) overnight at 4°C, washed three times with PBS and subsequently incubated with the secondary antibody (1:200) for 1 h at room temperature. For actin, cells were stained with phalloidine-TRITC (1:50) for 1 h at room temperature. The cover slips were embedded in fluorescent mounting medium (buffered glycerol pH 8.4) and put

Table 1

Primers used for RT-PCR

Gene	Species		Sequence	Product length	Accession number
β-actin	Human	Forward	TGGACTTCGAGCAAGAGATG	229 bp (975–746)	NM001101
		Reverse	TGTTGGCGTACAGGTCTTTG		
CL	Human	Forward	ATGGATGGCTCTGCTGGAA	147 bp (757–904)	NM005795
		Reverse	CTGTTGCTTGCTGGATGTC		
RAMP1	Human	Forward	CTCACCCAGTTCCAGGTAGA	156 bp (253–409)	NM005855
		Reverse	GAACCTGTCCACCTCTGCAT		
RAMP2	Human	Forward	GGGACGGTGAAGAACTATGA	166 bp (234–400)	NM005854
		Reverse	AAGCCCAGGTCAAACAACTC		
RAMP3	Human	Forward	AACTTCTCCCGTTGCTGCT	165 bp (90–255)	NM005856
		Reverse	ACACGATGAACTCGGACAG		

onto glass objective slides. Images were obtained using a Zeiss LSM 510 META confocal microscope.

RNA isolation and real-time RT-PCR

Total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) and genomic DNA was removed by treatment with DNase (Invitrogen, Karlsruhe, Germany). Total RNA (1 μg) was used in a 20 μL RT reaction to synthesize cDNA using Superscript RNase H-Reverse Transcriptase (200 U·μg^{-1} RNA; Invitrogen) and oligo dTs as primers. RT reactions were performed for 50 min at 42°C. Real-time quantitative PCR was performed using the I-cycler IQ detection system (Bio-Rad, Munich, Germany) in combination with the IQ SYBR Green Real-Time PCR Supermix (Bio-Rad). The thermal cycling programme consisted of initial denaturation in one cycle of 5 min at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. Primer sequences are provided in Table 1. The PCR products were separated by electophoresis on a 2% Tris-acetate-EDTA agarose gel.

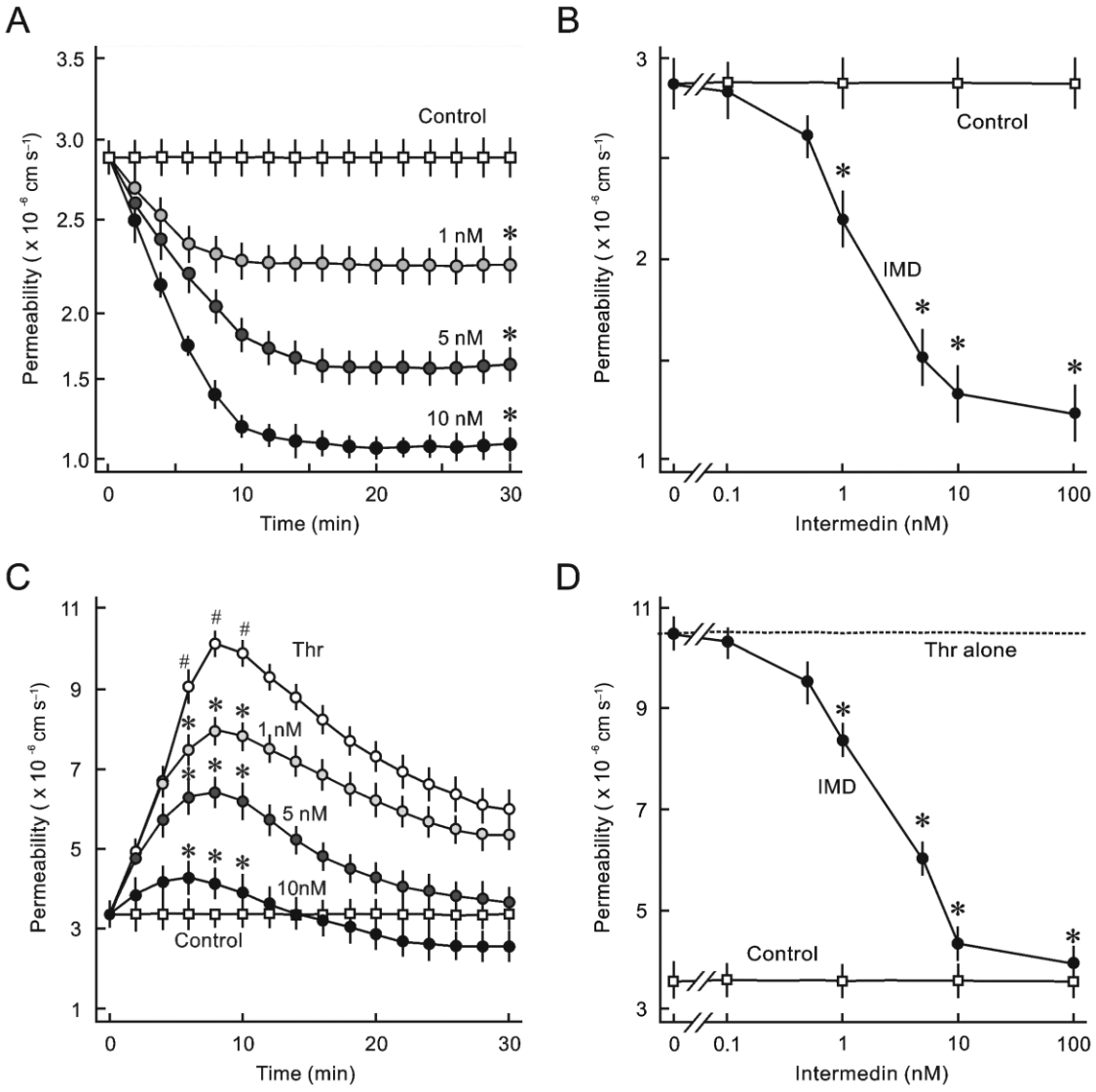

Figure 1

Intermedin (IMD) reduces permeability of HUVEC monolayers to macromolecules. (A) HUVEC monolayers were exposed to intermedin (1, 5 and 10 nM) or vehicle (control) as indicated. (B) Concentration-response curve for intermedin in experiments described in A on permeability after 10 min. HUVEC monolayers were exposed to intermedin as in A or vehicle (Control) as indicated. Mean ± SD of three experiments of independent cell preparations, *$P < 0.05$ significantly different from versus control. (C) Effect of intermedin on thrombin-induced hyperpermeability. HUVEC monolayers were exposed to (1, 5 and 10 nM) in the presence of thrombin (Thr; 0.2 IU·mL^{-1}) or vehicle (Control) as indicated. (D) Concentration-response curve of intermedin in experiments described in C on permeability after 10 min. HUVEC monolayers were exposed to intermedin as in C or vehicle (Control) as indicated. Mean ± SD of three experiments of independent cell preparations; $^{\#}P < 0.05$ vs. control, *$P < 0.05$ significantly different from thrombin alone.

Statistical analysis

Data are shown as means ± SD of *n* experiments using independent cell preparations. The comparison of means between groups was performed by one-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. *P* values of less than 0.05 were considered significant. For EC_{50} and pA_2 calculations, HUVECs were pre-incubated with antagonists for 30 min before adding the agonist. Permeability data after 10 min were calculated and normalized to control (taking control as 100%). A pA_2 value for each antagonist was determined from three different concentrations of the antagonist using Schild analysis using GraphPad prism software (version 5.0; GraphPad Inc., La Jolla, CA, USA) and are shown as means ± SEM.

Materials

HRP-conjugated anti-mouse IgG and rabbit IgG antibodies were from Amersham Biosciences (Heidelberg, Germany); human thrombin was from Behring (Marburg, Germany); human adrenomedullin (1–52) (cat. # H-2932), human intermedin-47 (cat. # H-6064), $\alpha CGRP_{(8-37)}$ and AM_{22-52}, were from Bachem AG (Zurich, Switzerland); anti VE-cadherin (clone TEA 1, mouse IgG) was from Beckman Coulter (Krefeld, Germany); benzonase, forskolin, myristoylated PKI and Y-27632 were from Calbiochem (Darmstadt, Germany); anti phospho-myosin light chain (MLC) and anti phospho-cAMP response element binding protein (CREB) were from Cell Signaling (Beverly, MA, USA); Rac1 activation assay kit and RhoA G-Lisa kit were from Cytoskeleton (Denver, CO, USA); Pierce® ECL solution was from Fischer Scientific (Niederlassung Nidderau, Germany); Alexa-Fluor labelled anti-mouse IgG and anti-rabbit IgG antibodies were from Invitrogen (Karlsruhe, Germany); endothelial cell basal medium plus supplement pack was from PromoCell (Heidelberg, Germany); Complete® protease inhibitor cocktail was from Roche (Mannheim, Germany); 3-isobutyl-1-methylxanthine (IBMX), Phalloidin-TRITC, L-NAME, L-NNA and anti-vinculin (clone hVIN-1, mouse IgG) were from Sigma (Steinheim, Germany); anti-phospho-myosin phosphatase targeting protein (MYPT)1 (Thr^{850}) was from Upstate (UK). Costar Transwell® polycarbonate membrane filters (24 mm round) were from VWR (Darmstadt, Germany); all other chemicals were of the best available quality, usually analytical grade.

Results

Effect of intermedin on endothelial barrier function

To analyse the effect of intermedin on endothelial barrier function, Trypan blue-labelled albumin flux across HUVEC monolayers was determined in the absence or presence of intermedin. Addition of intermedin to HUVEC monolayers reduced the permeability to albumin in a concentration-dependent manner (Figure 1A,B). Immediately upon addition of intermedin, macromolecular flux was reduced and the effect reached its maximum after 10 min with an EC_{50} of 1.29 ± 0.12 nM. To test whether intermedin could also antagonize hyperpermeability induced by inflammatory mediators, we investigated the effect of intermedin on thrombin-induced hyperpermeability. As shown in Figure 1C, thrombin treatment (0.2 IU·mL^{-1}) caused a rapid increase in macromolecule permeability which peaked in ~10 min after treatment. Co-treatment with intermedin antagonized the thrombin-induced hyperpermeability (Figure 1D).

Role of CGRP and adrenomedullin receptors in intermedin-mediated endothelial barrier protection

To analyse the role of CGRP and adrenomedullin receptors in this barrier-stabilizing effect of intermedin, two specific, truncated peptide, receptor antagonists, $\alpha CGRP_{8-37}$ and AM_{22-52}, were used. $\alpha CGRP_{8-37}$ in low concentrations is more specific for CGRP receptors, while AM_{22-52} antagonizes both AM_1 and AM_2 receptors showing higher affinity for AM_1 receptors. Pre-incubation (30 min) of endothelial cells with low concentrations $\alpha CGRP_{8-37}$ (0.1 μM) had no effect on intermedin-mediated reduction of basal permeability or

Figure 2

Role of CGRP and AM_1/AM_2 receptors in intermedin-mediated reduction in permeability. (A) Concentration-response curve of $\alpha CGRP_{8-37}$ effect on intermedin (IMD)-induced reduction in permeability. HUVEC monolayers were exposed to intermedin (10 nM), in the absence or presence of increasing concentrations of $\alpha CGRP_{8-37}$ or vehicle (Control) as indicated. $\alpha CGRP_{8-37}$ was added 30 min before addition of intermedin (mean ± SD of three experiments of independent cell preparations). *$P < 0.05$ significantly different from intermedin alone. (B) Concentration-response curve of $\alpha CGRP_{8-37}$ effect on intermedin-mediated reduction in thrombin (Thr)-induced hyperpermeability. HUVEC monolayers were exposed to thrombin (0.2 IU·mL^{-1}) plus intermedin (10 nM), in the absence or presence of increasing concentrations of $\alpha CGRP_{8-37}$ as indicated. $\alpha CGRP_{8-37}$ was added 30 min before addition of intermedin (mean ± SD of three experiments of independent cell preparations). *$P < 0.05$ significantly different from intermedin plus thrombin. (C) Concentration-response curve of AM_{22-52} effect on intermedin-induced reduction in permeability. HUVEC monolayers were exposed to intermedin (10 nM), in the absence or presence of increasing concentrations of AM_{22-52} or vehicle (Control) as indicated. AM_{22-52} was added 30 min before addition of intermedin (mean ± SD of three experiments of independent cell preparations). (D) Concentration-response curve of AM_{22-52} effect on intermedin-mediated reduction in thrombin-induced hyperpermeability. HUVEC monolayers were exposed to thrombin (0.2 IU·mL^{-1}) plus intermedin (10 nM), in the absence or presence of increasing concentrations of AM_{22-52} as indicated. AM_{22-52} was added 30 min before addition of intermedin (mean ± SD of three experiments of independent cell preparations). (E) Combined effect of $\alpha CGRP_{8-37}$ and AM_{22-52} on intermedin-induced reduction in permeability. HUVEC monolayers were treated with intermedin (10 nM) in the absence or presence of $\alpha CGRP_{8-37}$ (1 μM), AM_{22-52} (10 and 100 nM) or $\alpha CGRP_{8-37}$ (1 μM) plus AM_{22-52} (10 and 100 nM) as indicated. $\alpha CGRP_{8-37}$ and/or AM_{22-52} were added 30 min before addition of intermedin [mean ± SD of three experiments of independent cell preparations; *$P < 0.05$ significantly different from intermedin alone; $^{\#}P < 0.05$ significantly different from $\alpha CGRP_{8-37}$ plus AM_{22-52} (10 nM); n.s., not significantly different from AM_{22-52}(100 nM)]. (F) Expression of mRNA encoding RAMP1, 2, 3, CLR and β-actin in HUVEC (P0-P2) by RT-PCR. Human testis mRNA was used as positive control. MW; molecular weight marker.

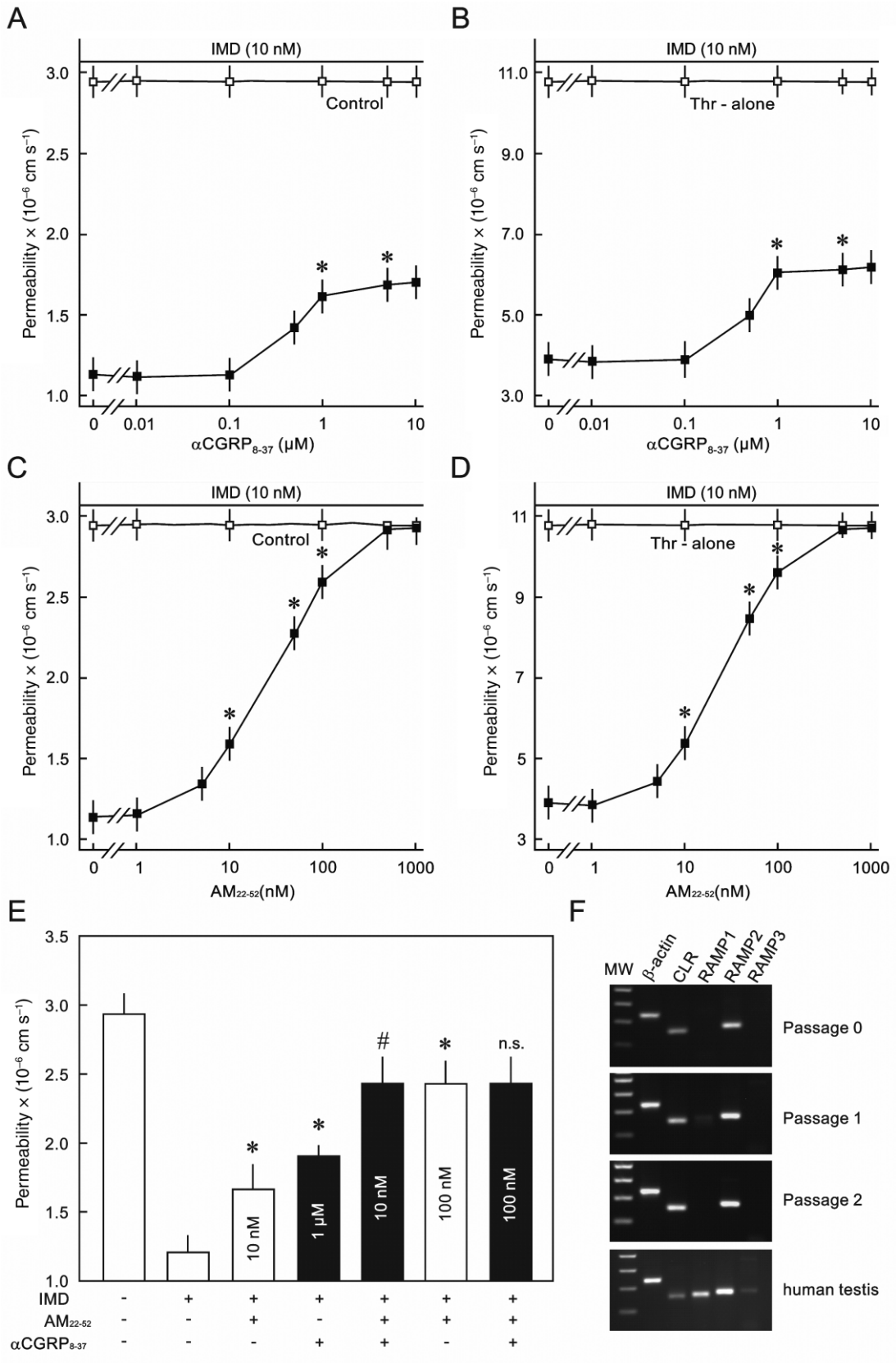
A
IMD (10 nM)
Control
Permeability × (10⁻⁶ cm s⁻¹)
αCGRP8-37 (μM)
B
IMD (10 nM)
Thr - alone
Permeability × (10⁻⁶ cm s⁻¹)
αCGRP8-37 (μM)
C
IMD (10 nM)
Control
Permeability × (10⁻⁶ cm s⁻¹)
AM22-52(nM)
D
IMD (10 nM)
Thr - alone
Permeability × (10⁻⁶ cm s⁻¹)
AM22-52(nM)
E
Permeability × (10⁻⁶ cm s⁻¹)
10 nM
1 μM
10 nM
100 nM
100 nM
n.s.
IMD
AM22-52
αCGRP8-37
F
MW
β-actin
CLR
RAMP1
RAMP2
RAMP3
Passage 0
Passage 1
Passage 2
human testis

Table 2

Relative expression of CLR and RAMP1/2/3 in HUVEC (passage 0–2)

C_T	β-actin	CLR	RAMP1	RAMP2	RAMP3
Passage 0	12.7	21.7	–	22.8	–
Passage 1	11.8	22.6	–	23.2	–
Passage 2	11.7	20.7	–	22.9	–
ΔC_T	CLR	RAMP1	RAMP2	RAMP3	
Passage 0	9.0	–	10.1	–	
Passage 1	10.8	–	11.4	–	
Passage 2	9.0	–	11.2	–	

Table 3

Summary of permeability data

Peptide	EC_{50}	RP	Antagonist (pA_2) $\alpha CGRP_{8-37}$	Antagonist (pA_2) hAM_{22-52}
IMD	1.29 ± 0.12	0.19	6.41 ± 0.07	7.36 ± 0.10
AM	0.24 ± 0.07	1.00	6.40 ± 0.09	6.60 ± 0.11

HUVECs were pre-incubated with antagonists for 30 min prior to adding the agonist, intermedin (IMD) or adrenomedullin (AM). Permeability data after 10 min were calculated and normalized to control (taking control as 100%). pA_2 values were determined from three different concentrations of the antagonist using GraphPad Prism software (version 5.0; Graphpad Inc., La Jolla, CA, USA). Data are means ± SEM from three independent experiments, each performed in triplicate. Experiments were performed with HUVECs, passage 1 and 2, with no significant difference in values.
RP, relative potency.

thrombin-induced hyperpermeability. However, at higher concentrations (1 μM), it could antagonize up to ~30% effect of intermedin (Figure 2A,B). On the other hand, pre-incubation of endothelial cells with AM_{22-52} antagonized intermedin in a concentration-dependent manner, producing complete inhibition of the effect with 0.5 μM (Figure 2C,D) with IC_{50} of ~30 nM (against 10 nM intermedin). Combining a low concentration (10 nM) but not higher concentrations (100 nM) of AM_{22-52} with higher concentrations of $\alpha CGRP_{8-37}$ (1 μM) had additive effects (Figure 2E). To further confirm the presence of CGRP and adrenomedullin receptors, we analysed the expression of CLRs and respective RAMPs by quantitative PCR. As shown in Figure 2F and Table 2, mRNA analysis showed expression of CLRs and RAMP2 in HUVECs (passage 0–2).

Comparison of th effects of adrenomedullin and intermedin on the endothelial barrier

In the next step, the effect of intermedin on endothelial permeability was compared with that of adrenomedullin. Like intermedin, adrenomedullin reduced albumin permeability in a concentration-dependent manner, with an EC_{50} of 0.24 ± 0.07 nM (Figure 3A). This effect was antagonized by AM_{22-52} in a concentration-dependent manner (Figure 3B), with IC_{50} of ~500 nM (for 10 nM AM). To compare the affinities of intermedin and adrenomedullin for AM_1 receptors, the concentration-response effects were determined for both intermedin and adrenomedullin in the presence of $\alpha CGRP_{8-37}$ and AM_{22-52}. In the presence of $\alpha CGRP_{8-37}$ (1 μM) and AM_{22-52} (1 μM), the EC_{50} of intermedin was shifted to ~5 and ~25 nM, respectively (Figure 3C), whereas that of adrenomedullin was shifted to ~1 and ~4 nM (Figure 3D) respectively. The pA_2 values of $\alpha CGRP_{8-37}$ were the same for intermedin and adrenomedullin but the pA_2 value of AM_{22-52} for intermedin was higher than that for adrenomedullin (Table 3).

Intermedin activates cAMP signalling in HUVECs

In the next step, we measured cAMP concentrations in HUVEC monolayers in the absence or presence of increasing concentrations of intermedin. As shown in Figure 4A, intermedin induced cAMP production in a concentration-dependent manner. To further confirm the activation of cAMP signalling pathways, the activation of two most important cAMP effectors, that is, PKA and the exchange protein directly activated by cAMP (Epac) (de Rooij *et al.*, 1998), were analysed. The activation of PKA was assessed by the phosphorylation state of CREB, the direct substrate of PKA, and the activation of Epac was analysed by the activation state of Rap1-GTP, a direct target of Epac (de Rooij *et al.*, 1998). Treatment of HUVEC monolayers for 10 min with increasing concentrations of intermedin induced CREB phosphorylation (Figure 4B) and ~2.2-fold Rap1 activation (Figure 4D) with 10 nM. To further analyse whether this cAMP/PKA activation played a role in intermedin-mediated barrier stabilization, the activity of PKA was blocked with PKI, a cell-permeable, specific, peptide inhibitor of PKA (Lum *et al.*, 1999; Aslam *et al.*, 2010). As shown in Figure 4C, PKI attenuated the barrier protective effects of intermedin by ~50%. Some recent studies reported that intermedin can stimulate the synthesis of NO in rat aortas and mediate the vasodilatory effects of intermedin. Therefore, in the next step we analysed the role of NO in intermedin-mediated barrier protection using two well-established inhibitors of NO synthase, N^G-nitro-L-Arginine methyl ester (L-NAME) and N^G-nitro-L-Arginine (L-NNA). As shown in Figure 4E, inhibition of endothelial NO synthase did not antagonize the intermedin-mediated reduction in macromolecule permeability.

Effect of intermedin on endothelial adherens junctions

One of the main regulators of the endothelial barrier function is the endothelial actin cytoskeleton-mediated adherens junctions consisting of VE-cadherin. Therefore, we analysed the intermedin effect on actin and VE-cadherin. As shown in Figure 5, the addition of intermedin to HUVEC resulted in loss of actin stress fibres and the appearance of actin and VE-cadherin at the cell periphery. By contrast, thrombin induced increased stress fibres formation, loss of peripheral

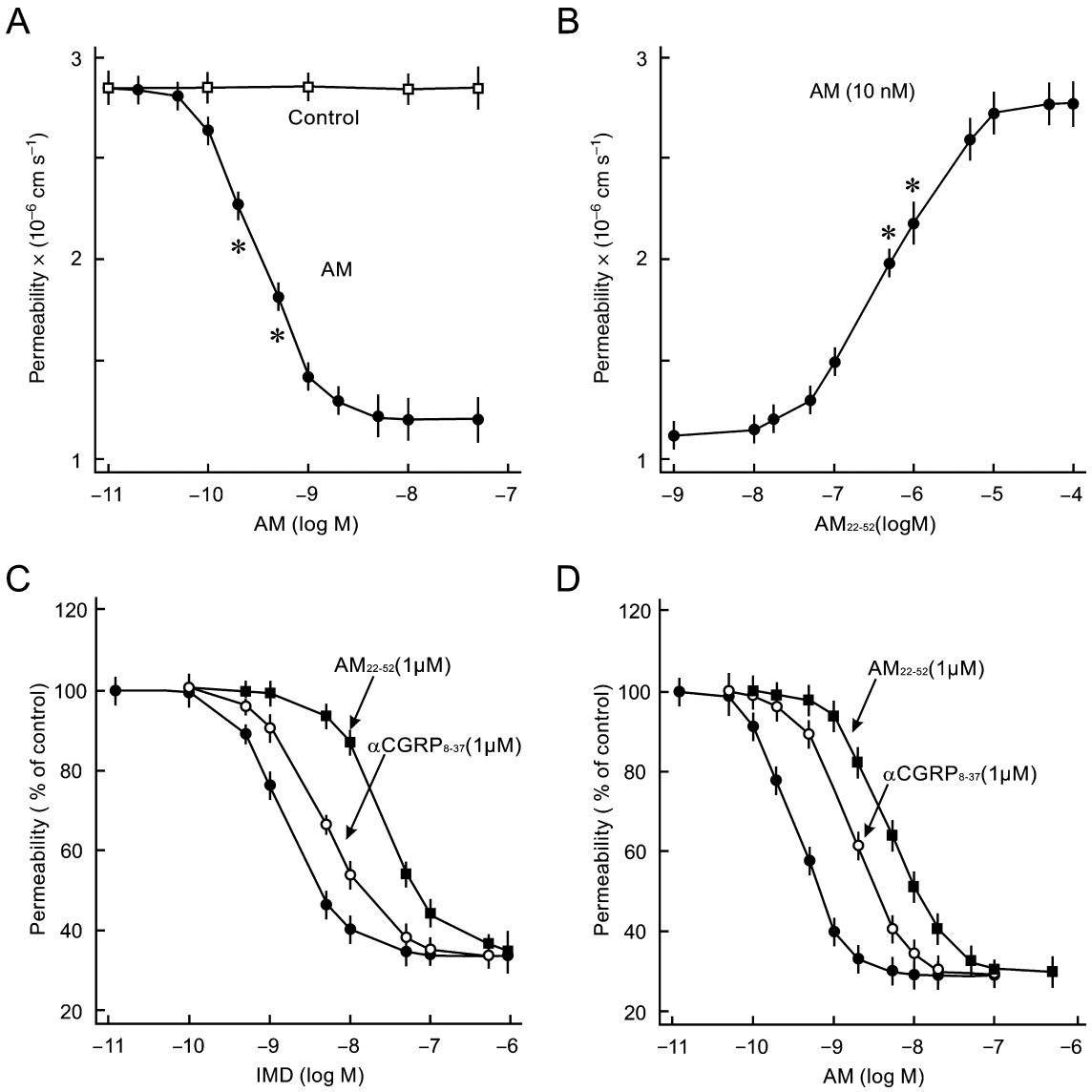

Figure 3

Effect of $\alpha CGRP_{8-37}$ and AM_{22-52} on intermedin (IMD) and adrenomedullin (AM)-mediated reduction in endothelial permeability. (A) Concentration-response curve of adrenomedullin on HUVEC permeability. HUVEC monolayers were exposed to increasing concentrations of adrenomedullin and the reduction in permeability after 10 min is shown. (B) Concentration-response curve of AM_{22-52} on AM-mediated reduction in permeability. HUVEC monolayers were exposed to adrenomedullin (10 nM), in the absence or presence of increasing concentrations of AM_{22-52} or vehicle (Control) as indicated. AM_{22-52} was added 30 min before addition of adrenomedullin (mean ± SD of five experiments of independent cell preparations; *$P < 0.05$ significantly different from adrenomedullin alone). (C) Permeability response of HUVEC to intermedin (increasing concentrations) in the absence or presence of $\alpha CGRP_{8-37}$ (1 μM) or AM_{22-52} (1 μM). (Mean ± SEM of three experiments of independent cell preparations). (D) Permeability response of HUVEC to adrenomedullin (increasing concentrations) in the absence or presence of $\alpha CGRP_{8-37}$ (1 μM) or AM_{22-52} (1 μM). (Mean ± SEM of three experiments of independent cell preparations).

actin and VE-cadherin and gap formation. These thrombin effects were completely abolished by co-incubation of HUVECs with intermedin.

Effect of intermedin on myosin light chains, myosin light chain phosphatase and RhoA

Endothelial actomyosin-based contractile machinery is another regulator of endothelial barrier function. As the phosphorylation state of the myosin light chain (MLC) controls the activation of endothelial contractile machinery, this was analysed using an antibody directed against phospho-Thr^{18}/Ser^{19} of MLC. Intermedin reduced basal as well as thrombin-induced MLC phosphorylation (Figure 6A). The dephosphorylation of MLC is initiated and triggered by the activation of MLC phosphatase (MLCP). The activity of MLCP is negatively regulated by the phosphorylation state of its regulatory subunit MYPT1 at Thr^{850}; and intermedin induced dephosphorylation of MYPT1 at Thr^{850}, in basal or thrombin-stimulated cultures (Figure 6A). MYPT1 is directly phosphorylated by Rho kinase (Rock), a

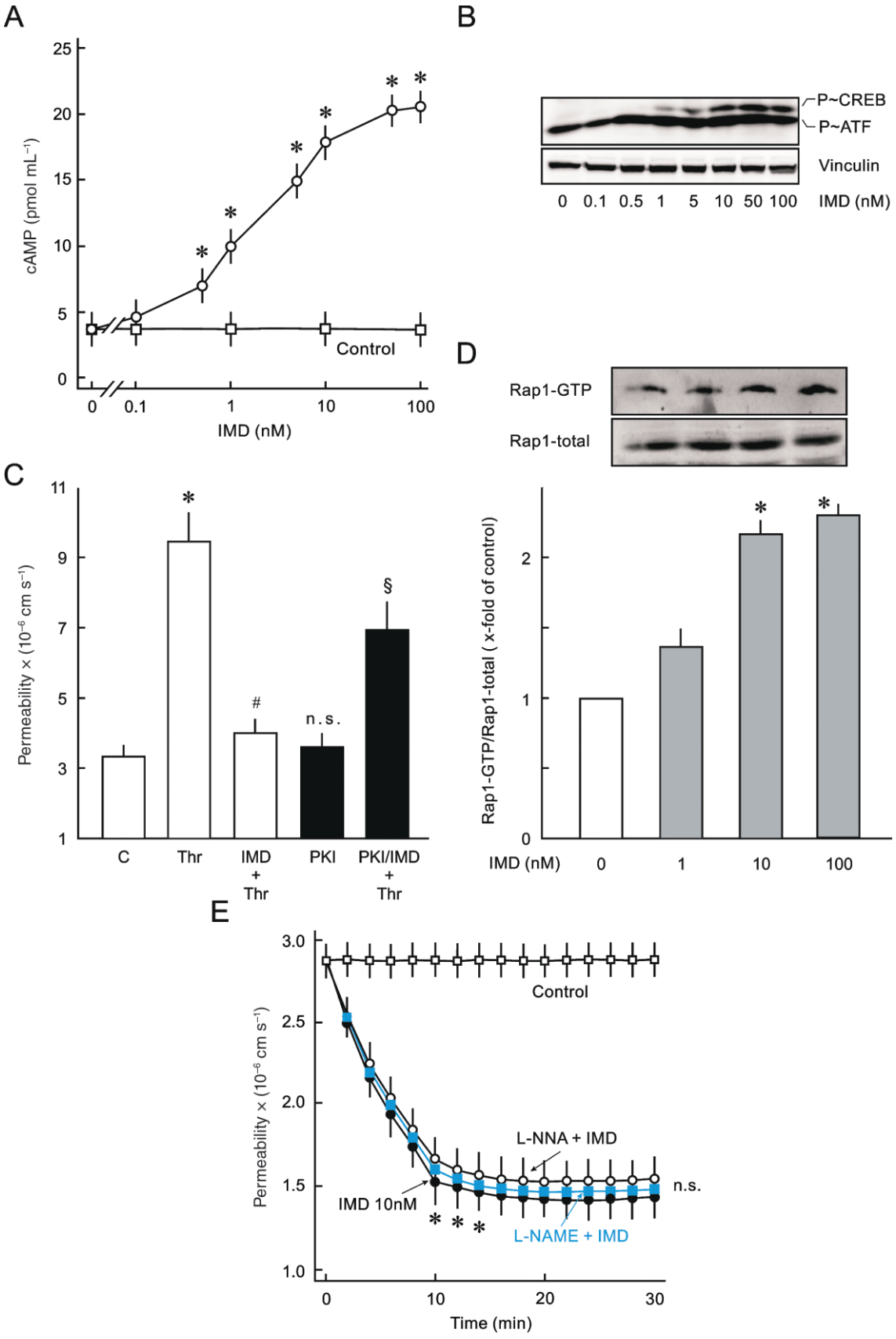
A
cAMP (pmol mL⁻¹)
25
20
15
10
5
0
Control
0
0.1
1
10
100
IMD (nM)
B
P~CREB
P~ATF
Vinculin
0 0.1 0.5 1 5 10 50 100 IMD (nM)
C
Permeability × (10⁻⁶ cm s⁻¹)
11
9
7
5
3
1
C
Thr
IMD + Thr
PKI
PKI/IMD + Thr
n.s.
D
Rap1-GTP
Rap1-total
Rap1-GTP/Rap1-total (x-fold of control)
2
1
0
IMD (nM) 0 1 10 100
E
Permeability × (10⁻⁶ cm s⁻¹)
3.0
2.5
2.0
1.5
1.0
Control
L-NNA + IMD
IMD 10nM
L-NAME + IMD
n.s.
0
10
20
30
Time (min)

Figure 4

Intermedin (IMD) activates the cAMP/PKA pathway. (A) Intermedin induces cAMP production in HUVEC. HUVECs were treated with increasing concentrations of intermedin for 10 min and the cAMP concentrations were measured by a colorimetric method. Data are means ± SD of three separate experiments with independent cell preparations. *P < 0.05 significantly different from control. (B) Effect of intermedin on CREB phosphorylation. Representative Western blots of CREB phosphorylation. HUVECs were exposed to increasing concentrations of intermedin for 10 min. The lower band represents phospho-ATF, another target of PKA, which is recognized by the antibody used. (C) Effect of PKA inhibition on intermedin-induced reduction in permeability. HUVEC monolayers were treated with intermedin (10 nM), thrombin (Thr; 0.2 IU·mL^{-1}), PKI (20 μM), intermedin plus thrombin, PKI plus intermedin plus thrombin or vehicle (Control), as indicated. Data are means ± SD of three separate experiments with independent cell preparations. *P < 0.05 significantly different from control; $^{\#}P$ < 0.05 significantly different from thrombin alone, §P < 0.05 significantly different from intermedin plus thrombin. (D) Effect of intermedin on Rap1 activation. HUVECs were exposed to increasing concentrations of intermedin for 10 min.The Western blots are representative of three separate experiments with independent cell preparations. *P < 0.05 significantly different from control. (E) Effect of eNOS inhibition on intermedin-induced reduction in permeability. HUVEC monolayers were treated with intermedin (10 nM) in the presence or absence of L-NAME (100 μM) and L-NNA (100 μM) or vehicle (Control), as indicated. Data are means ± SD of three separate experiments with independent cell preparations. *P < 0.05 significantly different from control; n.s., not significantly different from intermedin alone.

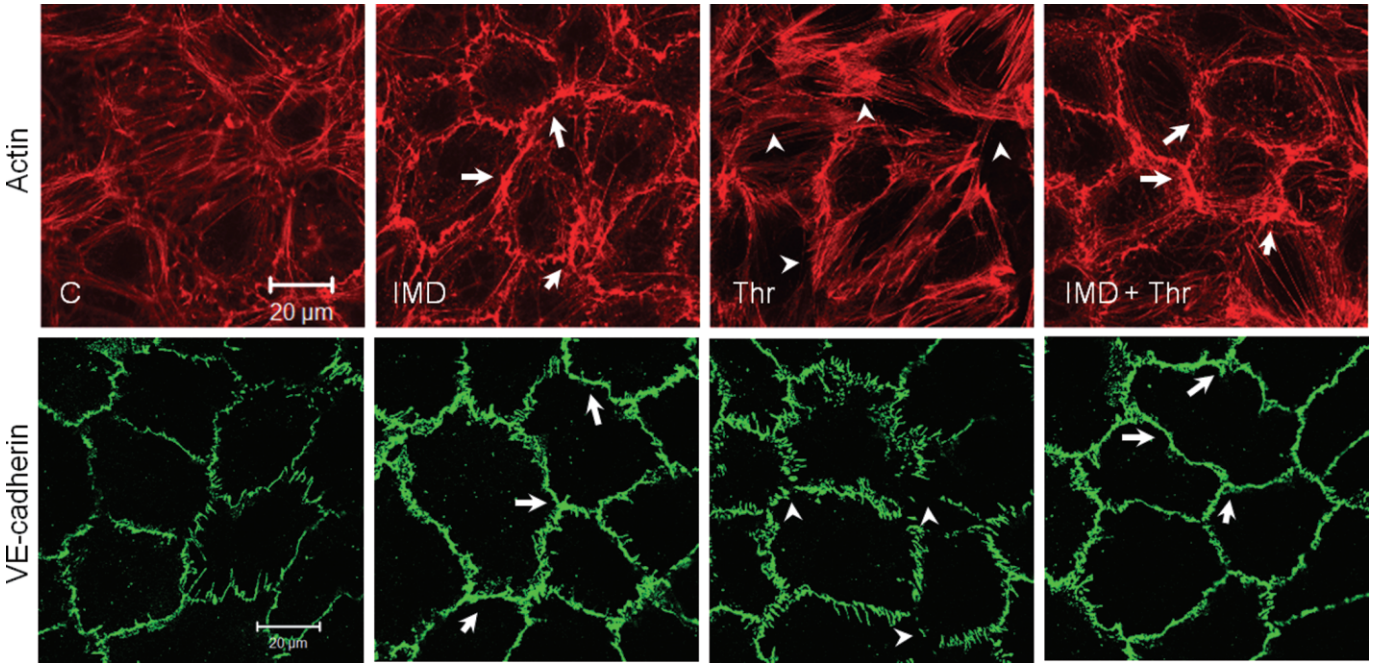

Figure 5

Effect of intermedin on VE-cadherin and actin cytoskeleton. HUVECs were treated with intermedin (10 nM), thrombin (Thr; 0.2 IU·mL^{-1}), intermedin plus Thr or vehicle (C, control) for 10 min and immunostained for VE-cadherin and F-actin (TRITC-labelled phalloidin). Arrows denote VE-cadherin localized at cell borders and arrowheads denote gaps between cells (scale bar 20 μm; representative immunostaining of five experiments of independent cell preparations).

downstream effector of RhoA and activation of RhoA was analysed by ELISA-based RhoA pulldown assay. Treatment of HUVECs with intermedin caused a 40% reduction in RhoA activity (Figure 6B). Thrombin induced a twofold activation of RhoA, which was completely blocked by pretreatment with intermedin.

Effect of intermedin on Rac1 activation

Rho GTPase Rac1 is a well-characterized regulator of cortical actin dynamics and thus cell–cell adhesion. Intermedin by itself caused a 2.5-fold increase in active Rac1 (Figure 6C) and thrombin caused a 60% reduction in active Rac1. Pre-treatment with intermedin abolished the effect of thrombin on Rac1 inactivation. The role of Rac1 activation in intermedin-mediated barrier stabilization was confirmed by using the Rac1 inhibitor, NSC23766. As shown in Figure 6D, pre-incubation of endothelial cells with NSC23766 abolished the effect of intermedin on the endothelial barrier.

Discussion

Intermedin is a new member of the CGRP super-family, showing 30% or lesser homology to other members of the family. It is known to act via the CLR/RAMP(1–3) receptor complex system but the role of CLRs and RAMPs in the

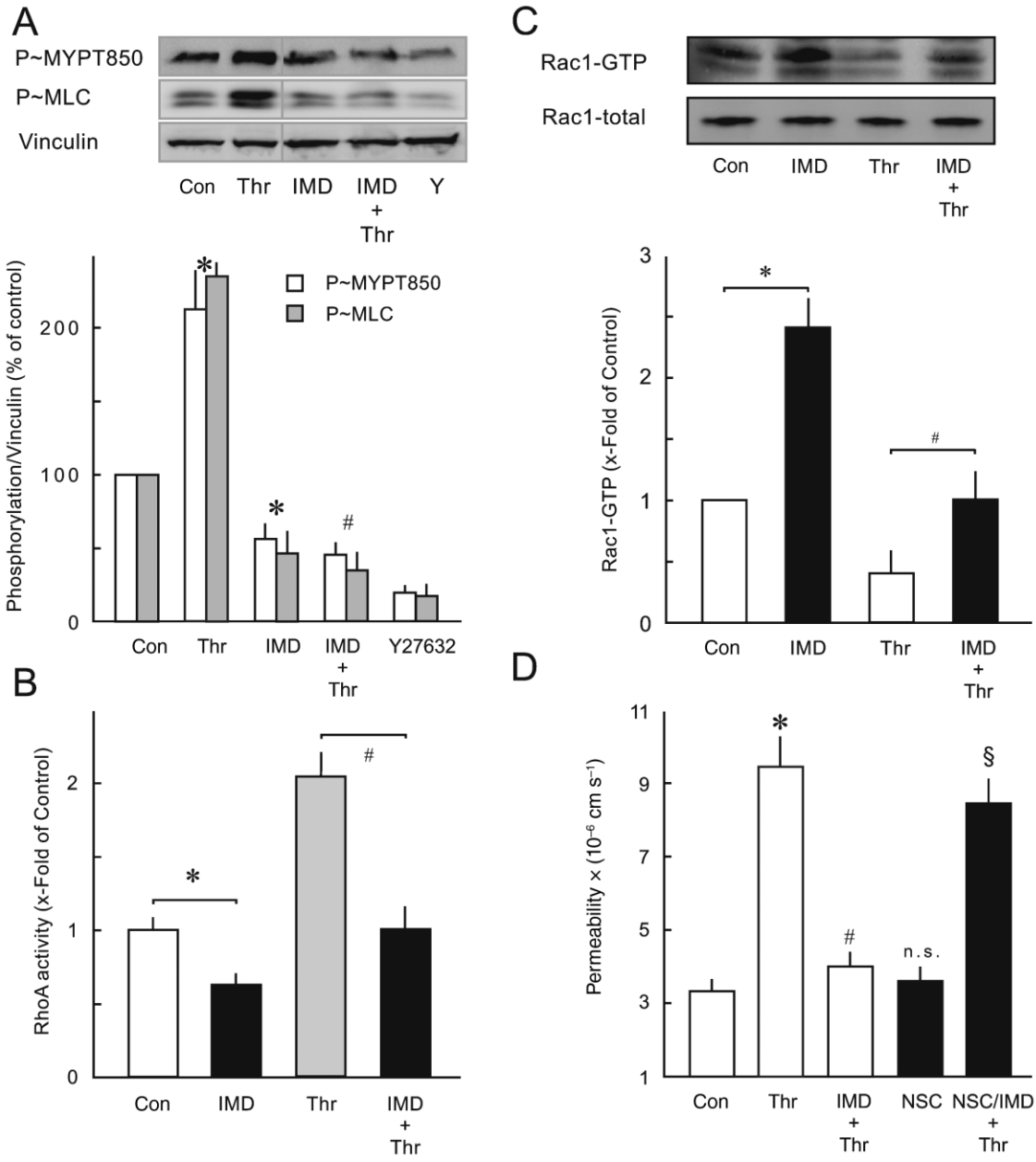

Figure 6

(A) Effect of intermedin on thrombin (Thr)-induced MLC and MYPT1 phosphorylation. Representative Western blots of MLC and MYPT1 phosphorylation. HUVECs were treated with intermedin (10 nM), thrombin (0.2 IU·mL^{-1}), intermedin plus thrombin, Y27632 (10 μM; a Rock inhibitor was used as positive control) or vehicle (Con, control) for 10 min. In HUVEC, phospho-MLC appears as a double band which indicates two isoforms of the protein and both are phosphorylated in response to thrombin. Y27632 in the concentration used is specific inhibitor of Rock (Ishizaki *et al.*, 2000). (B) Effect of intermedin on thrombin-induced RhoA activation. HUVECs were treated with intermedin (10 nM), thrombin (0.2 IU·mL^{-1}), intermedin plus thrombin or vehicle (Con, control) for 10 min and active RhoA was detected by an ELISA-based assay. The levels of total RhoA in the lysates were analysed by immunoblot and were used to normalize for loading. The active RhoA is given as x-fold of control. (mean ± SD of three experiments of independent cell preparations, $^{*\#}P < 0.05$). (C) Effect of intermedin on Rac1 activity. Representative Western blots of Rac1-GTP and Rac1 total. HUVECs were treated with intermedin (10 nM), thrombin (0.2 IU·mL^{-1}), intermedin plus thrombin or vehicle (Con, control) for 10 min and active Rac1 was detected by pulldown assay. The active Rac1 is given as x-fold of control. Mean ± SD of three experiments of independent cell preparations, $^{*\#}P < 0.05$. (D) Effect of the Rac1 inhibitor, NSC23766, on intermedin-mediated reduction of macromolecule permeability. HUVEC monolayers were treated with intermedin (10 nM), thrombin (0.2 IU·mL^{-1}), NSC23766 (10 μM), intermedin plus thrombin, NSC plus intermedin plus thrombin or vehicle (Con), as indicated. Data are means ± SD of three separate experiments with independent cell preparations. $^{*}P < 0.05$ significantly different from control; $^{\#}P < 0.05$ significantly different from. thrombin alone, §$P < 0.05$ significantly different from intermedin plus thrombin.

development and integrity of vasculature is still developing (Dackor *et al.*, 2006). Knockdown of CLR and RAMP2 is embryonically lethal and mice developed severe interstitial oedema. Thus, RAMP3 did not compensate for the absence of RAMP2 in the RAMP2$^{-/-}$ mice (Dackor *et al.*, 2006; Fritz-Six *et al.*, 2008). Although all the three RAMPs and CLRs are expressed differentially in all cells of vascular system, their optimal regulation is likely to be dependent on integrated release of different paracrine/autocrine ligands in a tissue-specific way. Although several studies have documented the barrier-protective effects of adrenomedullin (Hippenstiel *et al.*, 2002; Dunworth *et al.*, 2008), the role of intermedin in the regulation of endothelial barrier function is not well described. Recently, we have shown that expression of intermedin and its receptors was increased in hypoxic mouse lungs and intermedin infusion enhanced the endothelial barrier of isolated mouse lungs and pulmonary endothelial cells (Pfeil *et al.*, 2009). Similarly, circulating intermedin levels rise during gestation and decline at term in pregnant rats (Chauhan *et al.*, 2007) and infusion of an intermedin antagonist induced defects in placental vasculature and oedema (Chauhan *et al.*, 2006), indicating a critical role of intermedin and its receptors in the development of the vasculature and in the maintenance of the endothelial barrier.

Using well-established models of cultured human endothelial cells and *in vitro* measurement of endothelial permeability, here we show that intermedin is as effective as adrenomedullin, but less potent, in reducing endothelial permeability and antagonizing thrombin-induced hyperpermeability (Figures 1 and 3). Using the same functional readout, we characterized the receptors regulating this barrier-protective effect. This intermedin effect was insensitive to low concentrations of CGRP receptor antagonist $CGRP_{8–37}$, but sensitive to the AM_1 and AM_2 receptor antagonist $AM_{22–52}$, implying that CGRP receptors might not be involved in mediating these effects of intermedin. However, the permeability effect could be blocked, by up to 30%, with higher concentrations of $\alpha CGRP_{8–37}$ showing a non-selectivity of $\alpha CGRP_{8–37}$ at higher concentrations (Figure 2A–D). This is unlike our previous report where we showed that, in human pulmonary microvascular endothelial cells, the effect of intermedin was sensitive to lower concentrations of $\alpha CGRP_{8–37}$ (Pfeil *et al.*, 2009). In the present experiments, the greater sensitivity of intermedin to $AM_{22–52}$ suggested the involvement of AM_1 and/or AM_2 receptors which were further confirmed by real-time PCR. We used passage 1 cells in the present study and found that HUVEC (passage 0–2) only expressed high levels of CLR and RAMP2. This is in agreement with the results of Wunder *et al.* (2008) who found, in analysing several endothelial cell types, that HUVECs only expressed CLR/RAMP2 (although the data were not shown by the authors). Moreover, the pA_2 values of a receptor antagonist can also be predictive of receptor subtype. It is assumed that the majority of receptors with a pA_2 for $CGRP_{8–37}$ of more than 7 correspond to the CLR/RAMP1 type (Poyner *et al.*, 2002). The pA_2 values for $CGRP_{8–37}$ in HUVECs have not previously been reported but, for the human CLR/RAMP1 receptor, they range from 7.5 to 8.7 in different tissues and cell preparation (see Poyner *et al.*, 2002). Similarly, Owji *et al.* (2008), using rat embryonic spinal cord cells, which express both CGRP and AM_1 receptors (Takhshid *et al.*, 2006), reported pA_2 values of $CGRP_{8–37}$ of 7.24 and 7.91 for intermedin and adrenomedullin respectively. In the present study, we show, in HUVEC, the pA2 values for $CGRP_{8–37}$ for both intermedin and adrenomedullin were close to 6.4, which is far less than the presumptive value for CLR/RAMP1 receptors. This further strengthens the PCR data (Figure 2F) of the present study. Recently, Zheng *et al.* (2010) showed that CGRP promoted angiogenesis in HUVECs but because the authors used very high concentration of CGRP (0.1 μM), there is a real possibility that this effect most probably was mediated via AM_1 receptors as CGRP is known to activate these receptors at higher concentrations (Roh *et al.*, 2004; Bell and McDermott, 2008).

In contrast to $\alpha CGRP_{8–37}$, the pA_2 values for $AM_{22–52}$ against intermedin and adrenomedullin were distinctly different, 7.3 and 6.6 for intermedin and adrenomedullin, respectively, showing higher affinity for adrenomedullin over intermedin. This is in accordance to previously published reports (Roh *et al.*, 2004; Bell and McDermott, 2008) and the EC_{50} values calculated for these ligands in this study. The EC_{50} of intermedin is five times higher than adrenomedullin for HUVEC permeability, showing lower potency of intermedin over AM. This difference in potencies of intermedin and adrenomedullin is comparable, although relatively higher, to data from COS 7 cells expressing AM_1 receptors (Bailey and Hay, 2006) and rat embryonic spinal cord cells (Owji *et al.*, 2008).

Several recent studies demonstrate that intermedin can activate cAMP production in a variety of cells including endothelial cells (Yang *et al.*, 2005; Chen *et al.*, 2006). In HUVEC monolayers, intermedin induced cAMP production in a concentration-dependent manner (Figure 4A) and activated cAMP signalling pathways demonstrated by the activation of PKA and Epac/Rap1. Activation of PKA was demonstrated by CREB phosphorylation, which is a transcription factor directly phosphorylated by PKA and that of Epac was demonstrated by the levels of Rap1-GTP (Figure 4B,D). The role of PKA in the regulation of intermedin-mediated barrier stabilization was further demonstrated by experiments with PKI, a specific cell-permeable PKA inhibitor peptide (Figure 4C). The relative role of PKA and Epac in the cAMP-mediated regulation of endothelial barrier has recently been explained in detail (Aslam *et al.*, 2010; Birukova *et al.*, 2007; Netherton *et al.*, 2007). PKA regulated the endothelial barrier mainly via inactivation of contractile machinery by inhibiting RhoA/Rock signalling and, to some extent, by enhancing cell–cell adhesion (Aslam *et al.*, 2010), while Epac/Rap1 protects the endothelial barrier mainly via enhancing cell–cell adhesion, without affecting the contractile machinery (Birukova *et al.*, 2007; Aslam *et al.*, 2010).

Endothelial hyperpermeability is a typical response to inflammatory mediators like thrombin and the integrity of cellular junctions is essential for the maintenance of endothelial barrier function. Thrombin induces endothelial barrier failure via activation of the contractile machinery and disassembly of adherens junctions (Garcia *et al.*, 1986). Intermedin antagonized contractile activation (Figure 6A) and strengthened adherens junctions (Figure 5) in HUVEC monolayers, which explains the mechanistic aspects of this barrier stabilization. Endothelial contractile machinery is one of the main regulators of endothelial barrier function.

Activation of the contractile machinery is regulated by the phosphorylation state of the regulatory MLCs, which is precisely controlled by the balanced activities of MLCP and MLC kinase. The endothelial MLCP is a heterotrimeric enzyme composed of a catalytic subunit (PP1) and a regulatory myosin phosphatase targeting subunit (MYPT1). The activity of MLCP is controlled by Rock, which phosphorylates MYPT1 at its inhibitory sites (Kimura *et al.*, 1996). Our study showed that intermedin dephosphorylated both MYPT1 and MLC under basal conditions and after thrombin stimulation (Figure 6A,B). Furthermore, intermedin abolished RhoA activation, demonstrating that contractile inactivation is mediated via inhibition of RhoA/Rock signalling.

The stability of adherens junctions is highly dependent upon actin cytoskeleton-mediated organization of adherens junctions. Rho GTPases, RhoA and Rac1 have been well recognized as the main regulators of actin cytoskeleton dynamics and thus of the adherens junctions (Wojciak-Stothard and Ridley, 2002). Activation of RhoA induces the increased stress fibre formation and contraction via Rho kinase leading to increased gap formation, while the activation of Rac1 reorganizes actin cytoskeleton at the cell periphery and thus strengthens the adherens junctions (Wojciak-Stothard *et al.*, 2001). Our data showed that intermedin activated basal Rac1 and protected against thrombin-induced Rac1 inactivation. Rac1 has been shown to be activated by cAMP signalling via both PKA and Epac/Rap1 (Birukova *et al.*, 2007; Netherton *et al.*, 2007). As we show here that intermedin activated both PKA and Rap1, this suggests that activation of Rac1 most probably occurs via both signalling pathways. Although the precise mechanism of Rac1 activation is beyond the scope of this study, both PKA and Rap1 can activate Tiam1 and Vav2, the exchange factors of Rac1 (Birukova *et al.*, 2007). Pharmacological inhibition of these Rac1 exchange factors with the specific inhibitor, NSC23766, blocked the effects of intermedin, indicating a crucial role for of Rac1 in intermedin-mediated barrier stabilization (Figure 6C,D).

Some recent reports have shown that vasodilator effects of intermedin are partially mediated via activation of the NO/cGMP pathway (Burak *et al.*, 2006). We therefore assessed the effects of NO synthase inhibition with L-NAME and L-NNA on the barrier-protective effects of intermedin and were able to exclude the involvement of the NO/cGMP signalling pathway in mediating these responses to intermedin (Figure 4E), although a vasodilator response could involve NO/cGMP signalling (Kandilci *et al.*, 2008).

In conclusion, the present study shows that intermedin protects endothelial barrier of HUVEC monolayers via AM_1 receptors, inactivating endothelial contractile machinery and enhancing cell–cell adhesion. We show that HUVEC monolayers only express CLR/RAMP2 receptors and thus this model would be suitable to characterize CLR/RAMP2 receptors and their ligands, such as intermedin, adrenomedullin and CGRP, in the regulation of the endothelial barrier and angiogenesis, via genetic manipulations. Moreover, these findings identify a new function of intermedin and point to intermedin as a potential treatment for the reduction of vascular leakage in inflammatory conditions. Figure 7 summarizes the findings of the present study and presents possible mechanisms for intermedin-mediated barrier stabilization.

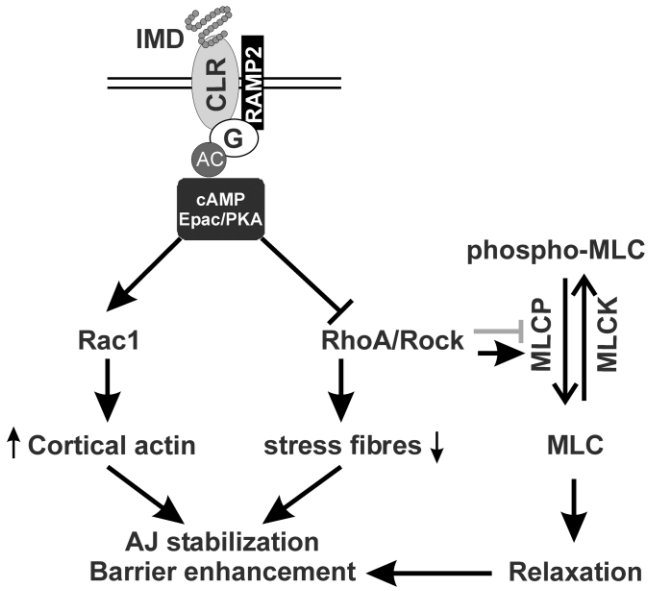

Figure 7

Summary of the results presented in this study. Intermedin (IMD), via CLR/RAMP2 receptors, activates cAMP signalling in HUVEC monolayers which on the one hand inactivates RhoA/Rock pathway leading to inactivation of contractile machinery. On the other hand, cAMP signalling activates Rac1, thus mediating adherens junction stabilization. The combined effect of this phenomenon is barrier stabilization. AJ, adherens junctions; MLC: myosin light chain; MLCK, MLC kinase; MLCP, MLC phosphatase.

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Conflicts of interest

None declared.

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