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Research Article

$P2X_4$ and $P2X_6$ receptors associate with VE-cadherin in human endothelial cells

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Abstract. We investigated the expression of $P2X_4$ and $P2X_6$ receptors on human umbilical vein endothelial cells (HUVECs) and found that both P2X receptor subtypes on plasma membranes are largely restricted to areas of cell-cell contact. Co-labelling experiments at the confocal and electron microscopy levels revealed that $P2X_4$ and $P2X_6$ receptors are strongly co-localised with the cell adhesion molecule VE-cadherin. The $P2X_4$ and $P2X_6$ receptors on plasma membranes at cellular junctions are rapidly (within 5 min) internalised specifically after decreasing extracellular [Ca^{2+}]. Disruption of microfilaments, microtubules and integrin-mediated adhesion or stimulation of P2 receptors with ATP did not alter $P2X_4$ and $P2X_6$ receptor expression on HUVEC plasma membranes. Membraneous $P2X_4$ and $P2X_6$ receptors resisted extraction with Triton-X 100, whereas cytoplasmic P2X receptors were Triton-X 100 soluble. $P2X_4$ receptors, but not $P2X_6$ receptors, could be co-immunoprecipitated with VE-cadherin and vice versa. We conclude that $P2X_4$ and $P2X_6$ receptors are associated with VE-cadherin at HUVEC adherens junctions.

Key words. Cell-cell adhesion; VE-cadherin; purinergic signalling; P2X receptor; endothelial cell.

Extracellular purines and pyrimidines have a range of specific functions in intercellular signalling in the vasculature [1]. Purinergic signalling is involved in the control of vascular tone [2], activation of blood platelets [3], chemoattraction of immune cells [4] and paracellular permeability [5], and ATP acts as a growth factor for vascular smooth muscle [6]. ATP is actively secreted from endothelial cells in response to shear stress [7–9] and is released from smooth muscle cells [10], red blood cells [11], immune cells [12] and from blood platelets [13]. ATP is massively liberated during ischemia and from dying (necrotic) cells [14].

ATP stimulates two families of receptors: G-protein-coupled P2Y receptors and cation-selective channels called P2X receptors [15]. P2Y receptors (of which six mammalian subtypes exist) are well established in mediating NO production and, hence, inducing vasorelaxation [16]. Recent immunohistochemical studies have also shown the expression of P2X receptors (of which seven subtypes exist, $P2X_1$ to $P2X_7$) on endothelial cells [17–20]. Functional studies demonstrated $P2X_4$ receptor-mediated Ca^{2+} influx on human umbilical vein endothelial cells (HUVECs), and $P2X_4$ receptors were suggested to serve as transducers for the perception of shear stress on endothelial cells [21–23].

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P2X receptor subtypes consist of two transmembrane domains with a short intracellular amino terminus, a longer intracellular carboxy terminus and a large extracellular loop, which contains several putative glycosylation sites [24]. P2X receptors have a high affinity for ATP and work as intrinsic cation channels with permeability for Ca^{2+}, K^+ and Na^+ [24]. Subunits of P2X receptors probably assemble as trimers to form functional channels [25]. The P2X receptor subunits can assemble homologously (homomers) and heterologously (heteromers) [26, 27]. Only the $P2X_6$ receptor subtype does not form homomers, but assembles into functional channels after association with $P2X_2$ or with $P2X_4$ receptor subtypes [28, 29]. Antibodies which specifically detect recombinant and native human $P2X_4$ and $P2X_6$ receptors are held by our group [30].

Recent evidence from various cellular models indicates that P2X receptors are associated with the actin-based cytoskeleton and that stimulation of P2X receptors can initiate cytoskeletal remodelling and tyrosine phosphorylation of junctional proteins [31–33]. Furthermore, Loesch and Burnstock [18] demonstrated in rat brain that $P2X_2$ receptors are abundant in endothelial cell-cell junctions, in vivo. Therefore, we investigated the localisation of P2X receptors at endothelial intercellular junctions (i.e. the adherens junction molecule, VE-cadherin) and the cytoskeleton in more detail.

VE-cadherin is an endothelial cell-specific adhesion molecule, which is strictly located to intercellular junctions [34]. Extracellularly, cadherins engage in calcium-dependent, homophilic interactions, while intracellularly they are associated with the actin-based cytoskeleton through a number of adaptor proteins [35].

Cadherins are generally important in regulating tissue morphogenesis and homeostasis [35]. They are post-translationally modified in their adhesiveness under physiological and pathophysiological conditions [36]. Calcium influx has been shown to be a trigger for the modulation of cadherin-mediated adhesiveness [37–39]. Pey et al. [38] have suggested that localised calcium influx in close apposition to a cadherin-mediated junction and not a global intracellular calcium rise is the signal to modulate cell-cell adhesion. In the present study we show that $P2X_4$ and $P2X_6$ receptors in HUVECs are closely associated with VE-cadherin. These receptors can mediate a rise in intracellular calcium in close apposition to cadherin-mediated junctions. A role for P2X receptors in modulating cell-cell adhesion in HUVECs is discussed.

Materials and methods

Reagents

Reagents were purchased from the following sources: medium M199, RPMI 1640 and fetal calf serum from Gibco (UK), culture flasks from Falcon (Becton Dickinson, USA); collagenase II from Worthington (USA); Trizma, HEPES, HBSS, phosphate-buffered saline (PBS, with and without Ca^{2+} and Mg^{2+}), $MgCl_2$, $CdCl_2$, $MnCl_2$, $BaCl_2$, $La(NO_3)_3$, ATP (as lyophylised powder), colchicine, cytochalasine D, paraformaldehyde (PFA), Tween 20, Triton X-100 (TX-100), Igepal, sodium deoxycholate, ethyleneglycol-bis N,N, N′,N′, tetracetic acid (EGTA), ethylenediaminetetracetic acid (EDTA), methanol, glutaraldehyde, diaminobenzidine (DAB), sodium dodecylsulphate (SDS), glycine, aprotinin, sodium orthovanadate, PMSF and protein molecular weight markers from Sigma (UK); protein assay (Bio-Rad DC) and ready-made gels from BioRad (UK); chemiluminescence reagents (ECL and West-pico) and nitrocelluloses from Amersham (UK) and Pierce (UK); protein G Plus-Sepharose and non-immune goat IgG-Sepharose from Santa Cruz (USA); Immunocatcher kit from Autogen-Bioclear (UK); Melinex from (Marivac Ltd, Canada); silver enhancement kit from British Biocell (UK). RGD-sequence peptides and control peptides were custom-made by MWG-Biotech (Germany).

Antibodies

P2X receptor antibodies and their cognate peptides were obtained from Roche Bioscience (USA). Antibodies were raised in rabbit against a unique 15-amino-acid residue sequence at the carboxy termini of the rat $P2X_4$ and $P2X_6$ receptors. In previous immunohistochemical and Western blotting studies, these antibodies specifically detected native and recombinant human $P2X_4$ and $P2X_6$ receptors [30].

Polyclonal antibodies raised in goat for VE-cadherin and N-cadherin were purchased from Santa Cruz (USA); biotinylated rabbit antibody from donkey was purchased from Jackson (USA); streptavidin-Texas Red was purchased from Amersham (UK); FITC-labelled chicken anti-goat antibody was from ICN (USA). Secondary antibodies and reagents for Western blotting, chemiluminescence and for immunohistochemistry were streptavidin-horseradish-peroxidase (streptavidin-HRP), donkey anti-rabbit-HRP, donkey anti-goat-HRP from Amersham (UK) and 0.8-nm streptavidin-coupled gold particles from British Biocell (UK).

Cell culture

Endothelial cells were harvested from veins of umbilical cords after normal deliveries and obtained from the labour ward of the Royal Free Hospital in London. Cords were put in HEPES-buffered saline (HBS; 5.5 mM glucose, 137 mM NaCl, 4 mM KCl, 10 mM HEPES; pH 7.4) within 15 min of delivery and stored at 4 °C until use (up to 48 h).

To obtain HUVECs, the method described by Jaffe et al. [40] was used with minor modifications [7]. Cords were cannulated, ligated and flushed with HBS, filled with

HBS containing 1 mg/ml collagenase II and incubated at 37 °C. Detached endothelial cells from the umbilical vein were harvested and washed with Hanks' buffered saline. For immunofluorescent labelling, cells were plated on glass coverslips (with and without gelatine coating), and for electron microscopy, cells were plated on Melanex. For Western blotting experiments, cells were grown in 25-cm^2 (T25) or 75-cm^2 (T75) culture flasks. All HUVECs were seeded in M199 medium supplemented with 15% FCS, 0.1% penicillin, at a density of 100 µl cell suspension cm^{-2} and were incubated at 37 °C, 5% CO_2 and 95% humidity. After 24 h, HUVECs were washed with HBSS to remove residual blood and supplemented with fresh medium. Cultures reached confluency after 6–7 days. Cells were only used as confluent primary cultures.

Cells for immunofluorescence

HUVECs were quickly rinsed with PBS (containing calcium and magnesium) and fixed in 4% PFA in PBS, for 10 min at room temperature. Fixed cells were washed and permeabilised with PBS (containing 1% TX-100) and then washed with PBS. Cellular proteins were quenched for non-specific antibody binding with 10% normal horse serum (NHS) in PBS, primary antibodies were diluted in 10% NHS, secondary antibodies in 1% NHS and tertiary antibodies in PBS alone as described previously for the detection of antigens in vivo [20]. For double-labelling experiments, primary and secondary antibodies from different species were applied together. The cross-reactivity between co-applied antibodies was tested in single- and double-labelling experiments and was found to be absent. Photographs were taken with a Leica TCS SP or a Leica TCS 4D confocal microscope at the UCL Biomedical Imaging Unit.

Cells for electron microscopy

All experiments were performed at room temperature. Cells were rinsed as above and fixed in phosphate buffer (PB) containing 4% PFA and 0.2% glutaraldehyde for 10 min and then washed in PB. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide and 30% methanol in PB. Cells were then washed in PB, quenched with 10% NHS for 30 min and incubated with VE-cadherin antibody (diluted 1:200) in 10% NHS for 14 h. Bound antibody for VE-cadherin was detected with donkey anti-goat HRP-coupled antibody (incubation for 4 h at a dilution of 1:1500 in 1% NHS) using the DAB/horseradish peroxidase method as described previously [18]. After performing the DAB/horseradish peroxidase staining, antibodies for P2X receptors were applied (for 14 h in 10% NHS, at a concentration of 5 µg/ml) as described above and detected with biotinylated donkey anti-rabbit antibody (incubation for 4 h in 1% NHS at a dilution of 1:1500) and streptavidin-coupled immunogold (incubation for 4 h in PBS at a dilution of 1:400). The samples were washed with deionised-distilled water and the immunogold signal was enhanced using a silver enhancement kit. Cells were post-fixed with 1% osmium tetroxide (in 0.1 M cacodylate buffer, pH 7.4), dehydrated in ethanol and embedded in Araldite. Ultrathin 'en face' sections of HUVECs were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope.

Investigation of factors that may alter P2X receptor localisation to plasma membranes

Calcium dependence of $P2X_4$ and $P2X_6$ receptor localisation to HUVEC plasma membranes was investigated. Results were compared with controls incubated with PBS (+ 1 mM Ca^{2+} and 0.5 mM Mg^{2+}) for identical periods.

All cells were briefly rinsed with PBS and then challenged with: PBS (Ca^{2+} and Mg^{2+}-free) containing 1 µM EDTA for 5 min, 10 min and 20 min; PBS (Ca^{2+}-free, containing 0.5 mM Mg^{2+}) + 4 mM EGTA for 5 min, 10 min and 20 min; PBS (Ca^{2+} and Mg^{2+}-free) containing 1 mM Cd^{2+}, or 2 mM Mn^{2+} or La^{3+} [2 µM as $La(NO_3)_3$] for 10 min; PBS (Ca^{2+}-free, containing 0.5 mM Mg^{2+}) plus 1 mM Cd^{2+}, or 2 mM Mn^{2+} or La^{3+} [2 µM as $La(NO_3)_3$] for 10 min; Ringer solution (Ca^{2+} free, containing 0.5 mM Mg^{2+}) + 1.8 mM Ba^{2+} for 20 min; PBS (containing Ca^{2+} and Mg^{2+}) plus 1 mM ATP for 60 min; PBS (containing Ca^{2+} and Mg^{2+}) plus 10 µM colchicine (Sigma, USA) for 60 min; PBS (containing Ca^{2+} and Mg^{2+}) plus 1 µM cytochalasine D (Sigma, USA) for 60 min. Cells were then processed for immunohistochemistry as described.

Disruption of integrin-mediated adhesion

Integrin-dependency of $P2X_4$ and $P2X_6$ receptor staining on plasma membranes was investigated by incubating cells with RGD sequence-containing peptides and a control peptide. Peptide sequences used were: RGD-peptide: INRGDFSK, corresponding to a sequence in *Streptococcus* pyrogenic exotoxin, which was shown to bind to human integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ and to induce HUVEC detachment [41] and the integrin-disrupting sequence GRGD. Control peptides were GIESFNRK and GRED, respectively.

HUVECs (grown in four-well dishes) were incubated with the respective peptides as described previously [41]. Briefly, cells were washed with HBSS and incubated in complete, serum-free medium containing 10 µg/ml peptide for 24 h. Detached cells were washed off and cultures processed for immunohistochemistry as described.

Selective extractions with Triton X-100

Selectively extracted HUVECs for immunofluorescence were prepared as described [42], with minor modifications. Cells were grown on gelatine-coated glass coverslips and incubated with PBS containing 0.5% TX-100

for 10 min at 4 °C or with PBS alone and processed for immunofluorescence as described. Z-series of P2X receptor-stained HUVECs were taken by confocal microscopy and three-dimensional images were calculated using three-dimensional deconvolution.

Selectively extracted HUVECs for Western blotting studies were prepared with the method described by Hinck et al. [43]. Cells were grown to confluency in T25 culture flasks and harvested for the Triton-soluble fraction with 1% TX-100 in extraction buffer (EB: 50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 2 mM Ca^{2+},1% NP40, 10 µg/ml PMSF, 30 µl/ml aprotinin, 1 mM sodium orthovanadate) and for the Triton-insoluble fraction into 1% SDS in extraction buffer (EB). Western blots were then performed as described below. Expression of $P2X_4$ or $P2X_6$ receptors in the TX-100-soluble and -insoluble fractions was compared using identical cellular extracts for immunoblots of both P2X receptors.

Immunoprecipitation

Cells for immunoprecipitation were cultured to confluency in T75 culture flasks and cellular extracts were obtained by incubation with EB containing 1% TX-100 for 20 min on ice. Cells were scraped and the slurry was forced with a syringe through a 21-gauge needle. The slurry was cleared by centrifugation and the supernatant used for immunoprecipitation experiments. These extracts were precleared with protein G sepharose, and then measured for their protein content (Bio-Rad DC protein assay). Samples containing 250 µg of protein were incubated with 10 µg of the respective antibodies or, alternatively, the protein samples were used for control experiments. Negative controls included omission of the primary antibody where extracts were precipitated with non-immune goat IgG-sepharose or with protein G-sepharose. Immune complexes formed with primary antibody were precipitated using protein G-sepharose. All extracts were purified using the Immunocatcher kit according to the manufacturer's instructions. Proteins were released from the beads by boiling for 10 min in buffer containing 1% SDS and 26 mM dithiothreitol.

Western blotting

Cells were grown to confluency in T25 culture flasks, extracts were prepared as above except that incubation was performed with EB containing 1% SDS for 20 min on ice. The protein content of all cellular extracts was measured (Bio-Rad DC protein assay) and equal amounts of protein were loaded on Tris-HCl Ready Gels (7% and 10% gels). Samples were run under denaturing and reducing conditions (1% SDS and 26 mM dithiothreitol) in a Mini-Protean 2 Electrophoresis and Trans-Blotting Cell (Bio-Rad, USA) according to the manufacturer's instructions. Proteins were transferred onto Hybond ECL-nitrocellulose membranes, blocked with PBS containing 3% milk powder and 0.05% Tween 20 and incubated in fresh blocking solution with the primary antibodies (all at room temperature). For detection, a chemiluminescence method was performed using a peroxidase-linked donkey anti-rabbit IgG, peroxidase-linked streptavidin and the ECL substrate (for crude cellular extracts) or the West-pico substrate (for co-immunoprecipitation experiments). The signal was visualised by exposure to Hyperfilm ECL (Amersham).

Results

$P2X_4$ and $P2X_6$ receptors are highly co-localised with VE-cadherin

$P2X_4$ receptors are abundant on HUVEC cell membranes and in the perinuclear/cytosolic area. Membraneous expression of $P2X_4$ receptors was almost exclusively observed in areas of mutual cell contact and was virtually

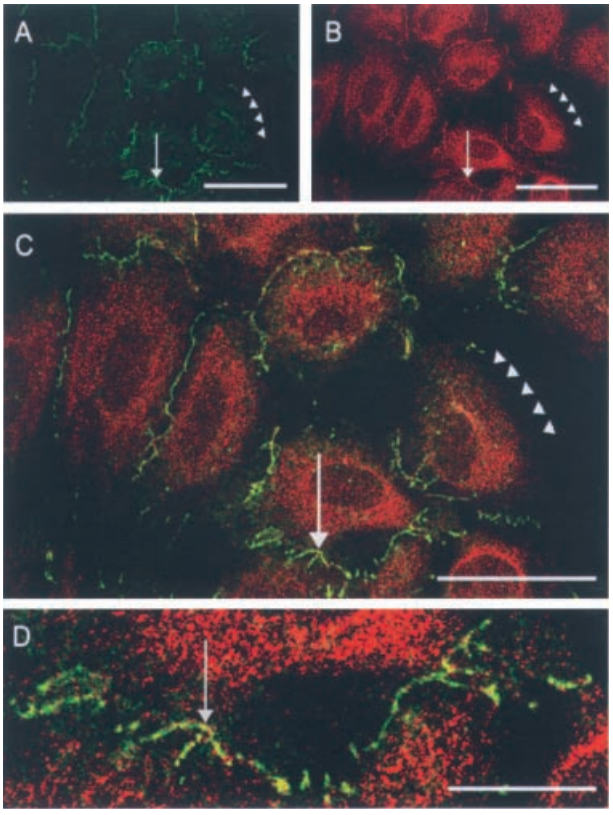

Figure 1. $P2X_4$ receptors on HUVECs are co-localised with VE-cadherin. $P2X_4$ receptors (Texas-Red) are co-localised with VE-cadherin (FITC); overlapping fluorescence appears yellow; note that immunolabelling for both molecules is largely restricted to cell-cell junctions and is absent from areas which are void of cell-cell contacts (arrowheads) (scale bars in *A*–*C*: 25 µm; *D*: 6 µm). (*A*) Single-channel visualisation of VE-cadherin (arrows point out corresponding areas in *A*, *B*, *C* and *D*). (*B*) Single-channel visualisation of $P2X_4$ receptors. (*C*) Merged immunofluorescence for VE-cadherin and $P2X_4$ receptors. (*D*) One cell-cell junction enlarged.

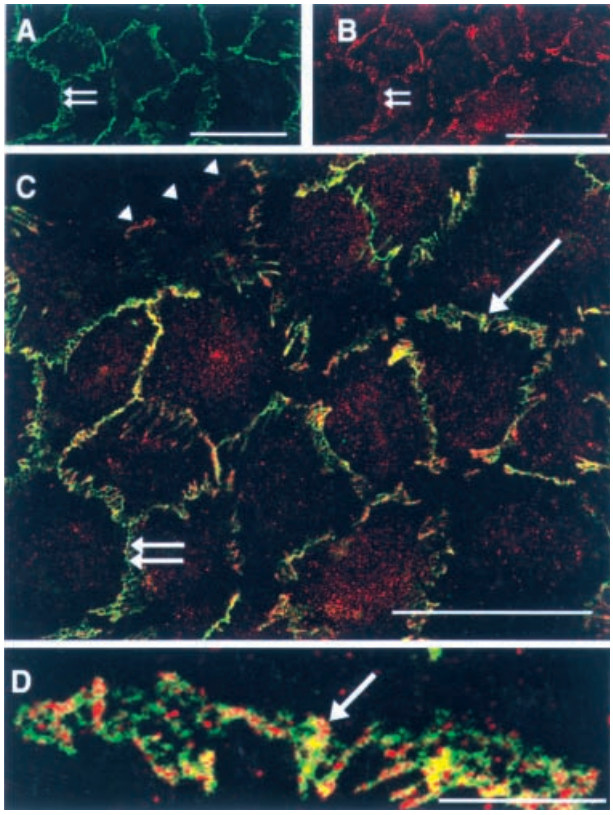

Figure 2. $P2X_6$ receptors on HUVECs are co-localised with VE-cadherin. $P2X_6$ receptors (Texas-Red) are co-localised with VE-cadherin (FITC); overlapping fluorescence appears yellow; note that immunolabelling for both molecules is largely restricted to cell-cell junctions and is absent from areas which are void of cell-cell contacts (arrowheads) (scale bars in *A*–*C*: 25 µm; *D*: 6 µm). (*A*) Single-channel visualisation of VE-cadherin (double arrows point out corresponding areas in *A*, *B* and *C*). (*B*) Single-channel visualisation of $P2X_6$ receptors. (*C*) Merged immunofluorescence for VE-cadherin and $P2X_6$ receptors. (*D*) One cell-cell junction (highlighted in *C* by an arrow) enlarged.

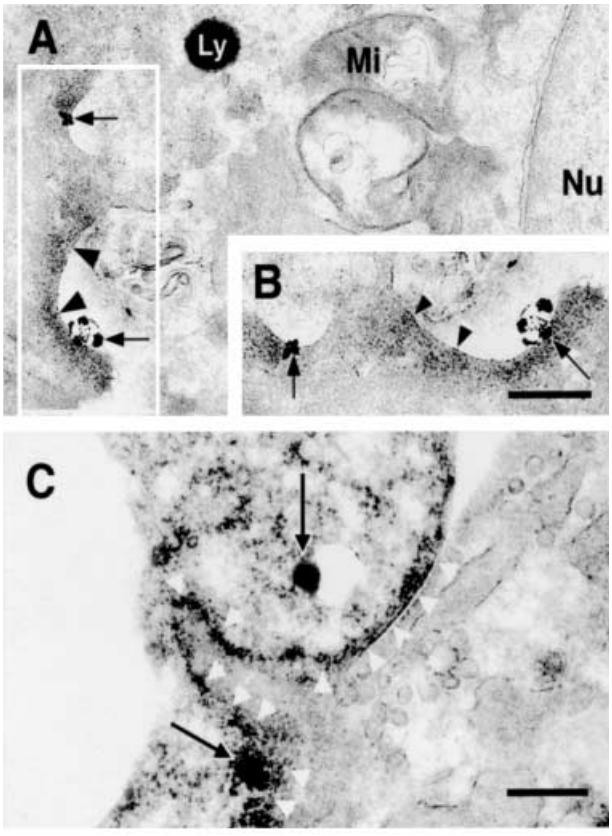

Figure 3. P2X receptors and VE-cadherin are co-localised at the ultrastructural level. The figures show HUVEC cell-cell contacts (peripheral regions of the cells) immunolabelled for VE-cadherin by the DAB/horseradish peroxidase method (arrowheads) and for P2X receptors with the silver-enhanced immunogold method (arrows); labelling for $P2X_4$ receptors is displayed in (*A*) and the framed part of (*A*) is magnified in (*B*); labelling for $P2X_6$ receptors is represented in (*C*); note that in labelled cells, co-localisation of immunoreactivity for $P2X_4$ receptors with VE-cadherin (*A, B*) and $P2X_6$ receptors with VE-cadherin (*C*) is seen in the vicinity of the plasmalemma (Mi, mitochondria; Nu, nucleus; ly, lysosome. Scale bars: 300 nm in (*A*) and 200 nm in *C*.

absent from cell surfaces not in contact with other cells. Confocal microscopy revealed the co-localisation of $P2X_4$ receptors with VE-cadherin in cell-cell junctions (fig. 1). Further magnification of an individual cell-cell junction showed that staining for VE-cadherin and $P2X_4$ receptors largely overlaps, but spots individually staining for either $P2X_4$ receptors or VE-cadherin are also present.

$P2X_6$ receptors are most abundantly expressed on HUVEC plasma membranes (fig. 2) and less intensely labelled in the perinuclear/cytosolic area. As described above for $P2X_4$ receptors, membraneous $P2X_6$ receptors were almost exclusively detected at cell-cell contacts and were closely co-localised with VE-cadherin (fig. 2).

Co-localisation studies of $P2X_4$ and $P2X_6$ receptors with VE-cadherin at the electron microscopic level demonstrated that both P2X receptors are inserted into the plasma membrane at cell-cell contacts and that both P2X receptors co-localised with VE-cadherin at the ultrastructural level (fig. 3).

Membraneous $P2X_4$ and $P2X_6$ receptors parallel the behaviour of cadherins, but not of integrins

Figure 4 shows rapid (10 min) internalisation of VE-cadherin and $P2X_4$ or $P2X_6$ receptors in low extracellular Ca^{2+} conditions. The initial co-labelling for $P2X_4$ receptors and VE-cadherin is separated, and both molecules are only marginally detected at cellular junctions. Co-localisation of $P2X_6$ receptors and VE-cadherin at cell-cell contacts is similarly dependent on extracellular Ca^{2+} as described for $P2X_4$ receptors.

Incubation of cells with RGD sequence-containing peptides led to massive cell detachment after 24 h (in contrast to the controls), but expression and localisation of $P2X_4$ receptors and VE-cadherin remained identical to controls (fig. 5). Localisation of $P2X_6$ receptors and VE-cadherin to junctions was again (as for $P2X_4$ receptors and VE-cad-

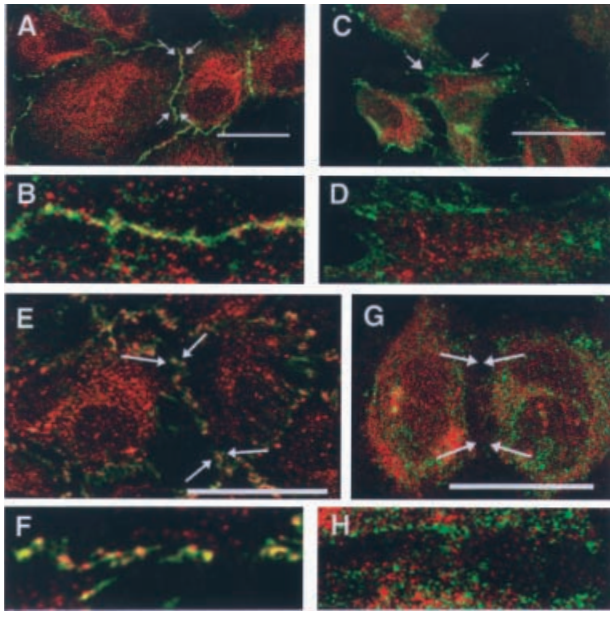

Figure 4. $P2X_4$ receptors and $P2X_6$ receptors on HUVECs are internalised from plasma membranes after disrupting cadherin-mediated cell adhesion. Cells were labelled for $P2X_4$ receptors or $P2X_6$ receptors (Texas-Red) and VE-cadherin (FITC); fluorescence overlap appears as yellow. (*A*) Control experiment incubating HUVECs for 10 min in PBS (containing 1.3 mM Ca^{2+}) showing co-localisation of VE-cadherin and $P2X_4$ receptors in cell-cell junctions (arrow); (*B*) magnified part of (*A*) corresponding to the highlighted cell-cell contact in (*A*); (*C*) HUVECs incubated in PBS containing 4 mM EGTA for 10 min show internalisation of both $P2X_4$ receptors and VE-cadherin; note that membrane location is gone; (*D*) magnified part of (*C*) corresponding to the disorganised cell-cell junction in (*C*); (*E*) control experiment incubating HUVECs for 10 min in PBS (containing 1.3 mM Ca^{2+}) showing co-localisation of VE-cadherin and $P2X_6$ receptors in cell-cell junctions (arrow); (*F*) magnified part of (*E*) corresponding to the highlighted cell-cell contact in (*A*); (*G*) HUVECs incubated in PBS containing 4 mM EGTA for 10 min show internalisation of both $P2X_6$ receptors and VE-cadherin; note that membrane localisation is gone (arrows); (*H*) magnified part of (*G*) corresponding to the disorganised cell-cell junction in (*G*) (scale bars: 30 µm).

herin) not affected by disruption of integrin-mediated adhesion.

Internalisation of $P2X_4$ and $P2X_6$ receptors is specific for low extracellular [Ca^{2+}]

Incubation of HUVECs with 1 mM ATP for 60 min did not have any effect on the membranous localisation of $P2X_4$ and $P2X_6$ receptors. In contrast, the whole receptor population was internalised to the perinuclear/cytosolic area after 5 min treatment with EGTA (shown for $P2X_6$ in fig. 6). The effect of P2X receptor internalisation was entirely calcium-dependent and could not be blocked by incubation with other di- or trivalent cations such as Mg^{2+}, Mn^{2+}, Cd^{2+}, Ba^{2+} or La^{3+} (not shown). Disruption of microtubules and microfilaments drastically changed the cellular morphology, but did not lead to $P2X_6$ receptor internalisation from the cytosolic membranes.

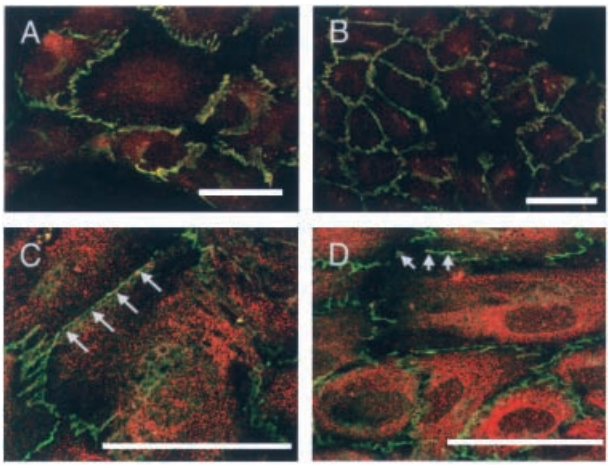

Figure 5. $P2X_4$ and $P2X_6$ receptors on HUVECs remain on plasma membranes after disruption of integrin-mediated cell adhesion. Cells were labelled for $P2X_4$ and $P2X_6$ receptors (Texas-Red) and VE-cadherin (FITC); fluorescence overlap appears as yellow. 24 h incubation with RGD-sequence-containing peptide. (*A*) Merged red and green immunofluorescence for $P2X_4$ receptors and VE-cadherin; many cells have detached from the dish after disrupting focal contacts, but staining for receptors and VE-cadherin remains confined to cell-cell junctions. (*B*) Immunofluorescence for $P2X_4$ receptors and VE-cadherin after 24-h incubation with a control peptide.
24-h incubation with RGD-sequence containing peptide. (*C*) Merged red and green fluorescence for $P2X_6$ receptors and VE-cadherin both labelling at adhesion junctions after many cells have detached from the culture-dish. (*D*) Immunofluorescence for $P2X_6$ receptors and VE-cadherin after 24-h incubation with a control peptide. (Scale bars: 30 µM).

P2X receptors on the plasma membrane are TX-100 insoluble

Immunofluorescence analysis of $P2X_4$ and $P2X_6$ receptor expression was performed on cells selectively extracted with TX-100 and on non-extracted control cells. Three-dimensional deconvolution of multiple optical sections of immunolabelled HUVECs, showed that both P2X receptor subtypes in the cytoplasm are extractable by TX-100, whereas $P2X_4$ and $P2X_6$ receptors on the plasma membrane resist extraction (fig. 7). However, extraction with TX-100 was also accompanied by massive cell loss. Therefore, selective extraction of $P2X_4$ and $P2X_6$ receptors was also analysed by Western blotting experiments (fig. 8).
Western blotting of crude HUVEC extracts (directly solubilised in 1% SDS) gave specific bands of approx. 64 and 120 kDa for $P2X_4$ receptors and resulted in specific bands of approx. 42 and 60 kDa for $P2X_6$ receptors. Staining of all bands specific for P2X receptors could be blocked by pre-absorption of antibodies with immunising peptides.
After selective extraction of HUVECs, the 64-kDa species of the $P2X_4$ receptor and the 60-kDa species of the $P2X_6$ receptor were detected in the TX-100-soluble fraction (fig. 8). The TX-100-insoluble fraction consisted of high-molecular-weight species of $P2X_4$ and $P2X_6$ receptors, both migrating at approx. 180 kDa.

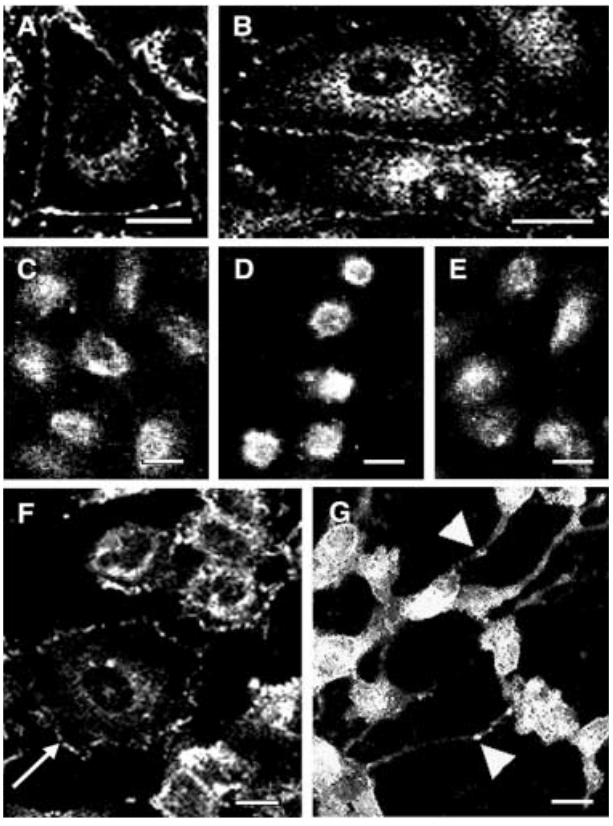

Figure 6. P2X receptor internalisation from the plasma membrane is rapid and entirely Ca^{2+}-dependent. HUVECs were incubated with buffer containing Ca^{2+} or with calcium-chelating agent, with buffer containing Ba^{2+} to replace Ca^{2+}, with buffer containing ATP or with buffer containing cytoskeleton-disrupting agents. Immunofluorescence for $P2X_6$ receptors was then detected by confocal microscopy (scale bars: 30 µm). (*A*) Control experiment showing HUVECs incubated in PBS (with normal Ca^{2+} [1 mM] and Mg^{2+} [0.5 mM]) for 1 h; note membranous localisation of $P2X_6$ receptors. (*B*) HUVECs incubated in PBS (+ normal Ca^{2+} and Mg^{2+}) plus 1 mM ATP for 1 h; note membranous localisation of $P2X_6$ receptors. (*C*) HUVECs treated for 5 min with PBS (Ca^{2+}-free, containing normal Mg^{2+}) plus 4 mM EGTA; note the large internalisation of $P2X_6$ receptors from the plasma membrane. (*D*) HUVECs treated for 10 min with PBS (Ca^{2+}-free, containing normal Mg^{2+}) plus 4 mM EGTA; note the complete internalisation of $P2X_6$ receptors from the plasma membrane. (*E*) HUVECs treated for 20 min with Ringer solution (Ca^{2+}-free, containing normal Mg^{2+}) plus 1.8 mM Ba^{2+}; note the large internalisation of $P2X_6$ receptors from the plasma membrane. (*F*) HUVECs treated for 60 min in PBS (containing Ca^{2+} and Mg^{2+}) plus 1 µM colchicine (which disrupts microtubules); note membraneous localisation of $P2X_6$ receptors (arrow). (*G*) HUVECs treated for 60 min in PBS (containing Ca^{2+} and Mg^{2+}) plus 1 µM cytochalasin D (disrupting microfilaments); some cellular processes with cell-cell junctions are still present (arrowheads).

$P2X_4$ receptors co-immunoprecipitate with VE-cadherin

Triton-soluble fractions of HUVEC crude extracts were immunoprecipitated with antibodies for VE-cadherin and N-cadherin and immunoblotted for $P2X_4$ and $P2X_6$ receptors. In the VE-cadherin-precipitated fraction, one

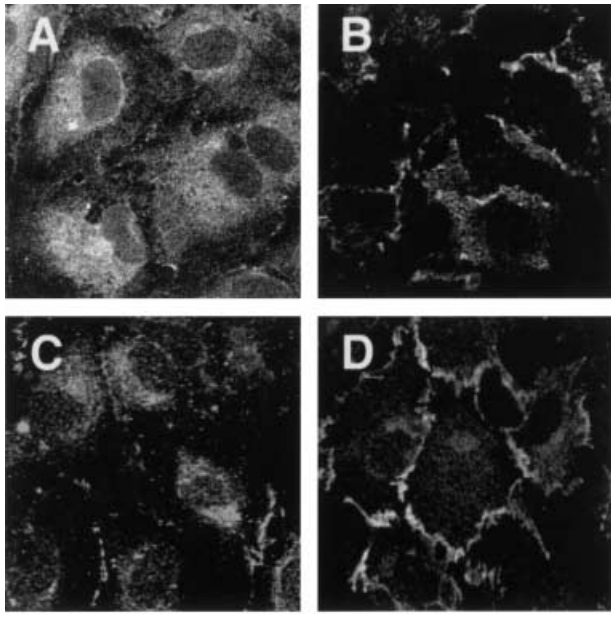

Figure 7. $P2X_4$ and $P2X_6$ receptors on the plasma membrane resist TX-100 extraction. HUVECs cultured on coverslips were either extracted with TX-100 (the TX-100-insoluble fraction remains visible) or washed with PBS (the TX-100-soluble and -insoluble fractions remain visible), cells were then fixed, multiple optical sections were taken and merged. (*A*) PBS-treated HUVECs stained for $P2X_4$. (*B*) TX-100-extracted HUVECs stained for $P2X_4$. (*C*) PBS-treated HUVECs stained for $P2X_6$. (*D*) TX-100 extracted HUVECs stained for $P2X_6$.

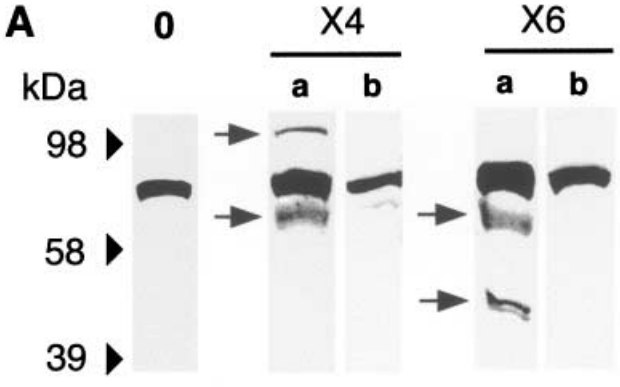

Figure 8. Western blotting of crude HUVECs extracts, selectively extracted HUVEC and of co-immunoprecipitated protein. (*A*) Western blotting of crude HUVEC extracts stained with antibody for P2X4 receptors (X4), antibody for $P2X_6$ receptors (X6) and after the omission of primary antibody (0); primary antibodies were used to detect native P2X receptors (a) or were pre-absorbed with their cognate antigen (10 µg antigenic peptide/1 µg antibody) (b); molecular weights are given in kDa. $P2X_4$ receptors migrated at approx. 64 and 120 kDa (arrows); $P2X_6$ receptors showed distinct bands at approx. 40 and 60 kDa (arrows); note that a single background band at about 70–80 kDa originating from the secondary antibody is visible; antibody binding to all polypeptide bands specific for $P2X_4$ and $P2X_6$ receptors could be pre-absorbed, whereas the background band remained visible.

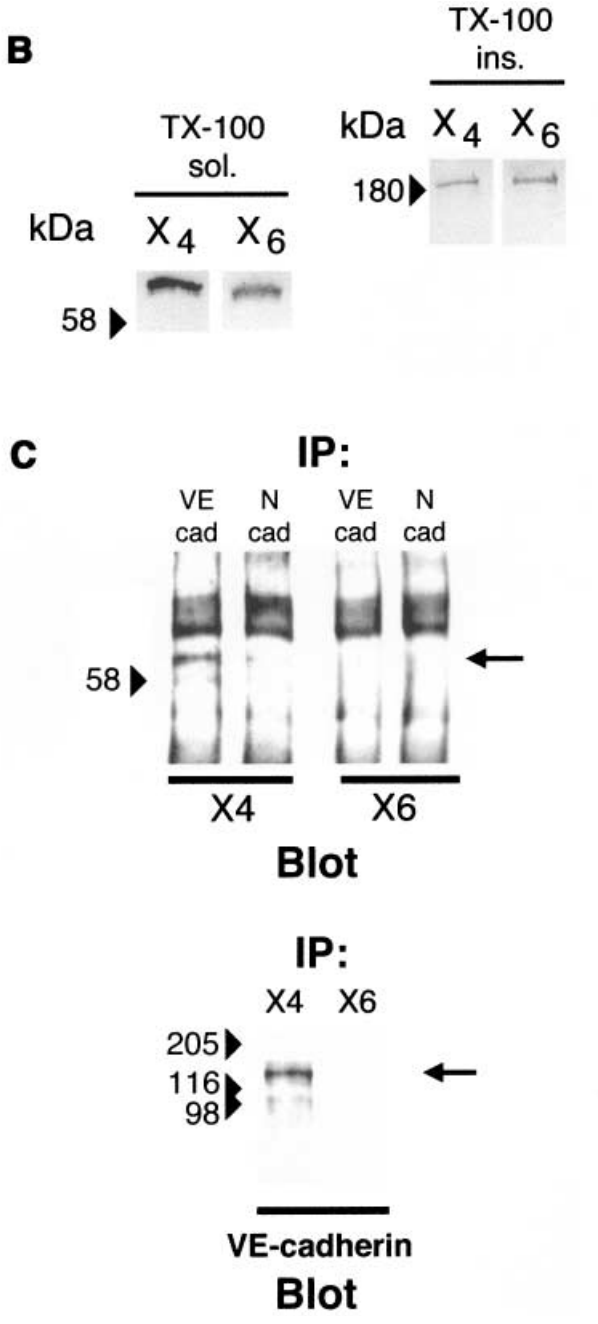

Figure 8 (continued) (*B*) Western blotting of HUVECs selectively extracted with TX-100. The TX-100-soluble fractions (TX-100 sol.) and the TX-100-insoluble fraction (TX-100 ins.) were stained for $P2X_4$ and $P2X_6$ receptors. $P2X_4$ and $P2X_6$ receptors in the TX-100-soluble fraction migrated at approx. 64 and 60 kDa, repectively, and both receptors were observed at approx. 180 kDa in the TX-100 insoluble-fraction. (*C*) Western blotting of immunoprecipitates from HUVECs. TX-100-soluble extracts from HUVECs were immunoprecipitated (IP) with antibody for VE-cadherin (VE cad) or with antibody for N-cadherin (N cad) and were then immunoblotted (Blot) with antibody for $P2X_4$ or $P2X_6$ receptors. In contrast, P2X receptor antibodies were used for immunoprecipitation and VE-cadherin antibody for immunoblotting. A single band of approx. 64 kDa for $P2X_4$ receptors was co-immunoprecipitated with VE cadherin (arrow) and a single band of approx. 130 kDa for VE-cadherin was co-immunoprecipitated with $P2X_4$ receptors (arrow). No other co-immunoprecipitates were detected.

specific band of approx. 64 kDa for $P2X_4$ receptors could be detected. Precipitates with N-cadherin antibody, which were immunoblotted for $P2X_4$ receptors, were immunonegative as were precipitates with N- and VE-cadherin antibody blotted for $P2X_6$ receptors. HUVECs were also immunoprecipitated with antibody for $P2X_4$ and $P2X_6$ receptors and immunoblotted for VE-cadherin. $P2X_4$ receptor precipitates showed immunoreactivity for one specific band of approx. 130 kDa for human VE-cadherin, whereas $P2X_6$ receptor precipitates were again immunonegative.

Discussion

$P2X_4$ and $P2X_6$ receptors are located to HUVEC cellular junctions where they colocalise with VE-cadherin

We have demonstrated in the present study, using confocal and electron microscopy, that $P2X_4$ and $P2X_6$ receptors are closely co-localised with VE-cadherin. Immunofluorescence for $P2X_4$ and $P2X_6$ receptors on plasma membranes was largely confined to areas of mutual cell contact. Both receptors show a similar expression pattern, similar dependency on extracellular calcium and similar resistance to TX-100 extraction of membranous $P2X_4$ and $P2X_6$ receptors. Thus our current working hypothesis is that both receptor subtypes form heterodimeric $P2X_{4/6}$ receptors. This view is consistent with a previous report [27], showing that $P2X_6$ receptors do not form homo-multimers and that the $P2X_6$ receptor subunit alone does not form functional ion channels. $P2X_4$ and $P2X_6$ receptor subunits together were shown to give rise to a functional $P2X_{4/6}$ heterodimeric channel [27]. Functional expression of $P2X_4$ receptors, but not of $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ receptors on HUVEC plasma membranes has been reported [22, 23], but expression of $P2X_6$ receptors was not investigated in that study. However, we cannot rule out that homo-oligomeric $P2X_4$ and $P2X_6$ receptors form on the plasma membrane and that only $P2X_4$ receptors assemble with VE-cadherin (independently of $P2X_6$ receptors). This form of homo-oligomeric assembly would be in accordance with our data from the immunoprecipitation experiments.

Localisation of $P2X_4$ and $P2X_6$ receptors to the plasma membrane is dependent on extracellular Ca^{2+}

$P2X_4$ and $P2X_6$ receptors on the plasma membrane were found to mimic the calcium dependency of membranous cadherin. The homotypic interactions of cadherin molecules in cellular junctions are dependent on the presence of extracellular calcium [44]. After chelation of calcium, cadherin-cadherin interactions become unstable and the molecules are rapidly (within 5 min) internalised from the plasma membrane to the perinuclear/cytosolic area [45].

$P2X_4$ and $P2X_6$ receptors mimic the behaviour of cadherins but not that of integrins

Integrin-mediated cell adhesion is also reported to be dependent on extracellular divalent cations [46, 47]. However, integrins are not as rapidly internalised after Ca^{2+} chelation as cadherins. However, possible coupling of $P2X_4$ and $P2X_6$ receptors to integrins was investigated by challenging endothelial cells with an RGD-sequence-containing peptide [41]. The disruption of integrin-mediated focal adhesion led to massive cell detachment, but neither VE-cadherin nor $P2X_4$ or $P2X_6$ receptor localisation to the plasma membrane were altered. Integrin-mediated focal adhesion is mainly stabilised by Mg^{2+} and Mn^{2+} [47], but both ions failed to replace the stabilising effects of Ca^{2+} on $P2X_4$ and $P2X_6$ receptor localisation to the plasma membrane. Disruption of microtubules and microfilaments has a greater effect on integrin-mediated cell adhesion than on cadherin-mediated cell adhesion [48]. Incubation of HUVECs with microfilament- or microtubule-disrupting agents strongly altered cell morphology, but did not lead to broad internalisation of $P2X_4$ or $P2X_6$ receptors. We conclude that $P2X_4$ and $P2X_6$ receptor localisation to HUVEC plasma membranes is restricted to areas of cadherin-mediated cell adhesion.

$P2X_4$ and $P2X_6$ receptors may not be present in all cadherin-mediated cell junctions

Epithelial tight junctions are controlled by cadherins and are reported to be dependent on the presence of extracellular cations [49]. In the frog urinary bladder epithelium, the function of tight junctions was dependent on the presence of extracellular calcium; however Ca^{2+} could be replaced by Cd^{2+}, Mn^{2+} and La^{3+} (but not by Ba^{2+} or Mg^{2+}) to prevent tight junction permeability. In the present study, none of these di- and trivalent cations could replace the stabilising action of Ca^{2+} on $P2X_4$ and $P2X_6$ receptor localisation to the plasma membrane. $P2X_4$ and $P2X_6$ receptors may therefore be absent from cadherin-mediated tight junctions (zonulae occludentes) and are potentially restricted to cadherin-mediated cell contacts at adherens junctions (zonulae adherentes).

Stimulation of $P2X_4$ and $P2X_6$ receptors on HUVEC cell junctions

Incubation of HUVECs with 1 mM ATP for 60 min did not affect cell morphology or result in $P2X_4$ and $P2X_6$ receptor internalisation. This indicates that $P2X_4$ and $P2X_6$ receptor internalisation after decreasing extracellular calcium is not a secondary event mediated by ATP secretion and receptor stimulation. $P2X_1$ receptor internalisation after stimulation with an ATP analogue has been previously reported [50].

Western blotting for detection of $P2X_4$ and $P2X_6$ receptors

Molecular weights for $P2X_4$ and $P2X_6$ receptors after Western blotting of HUVEC crude extracts were similar to previous findings with recombinant and native human $P2X_4$ and $P2X_6$ receptors. Molecular weights of approx. 60 kDa have repeatedly been described for both human (h) $P2X_4$ and $hP2X_6$ receptors [30, 51]. Higher-molecular-weight species of 120 and 160 kDa for native and recombinant hP2X receptors (run under denaturing and reducing conditions) have also been shown. These high-molecular-weight species were suggested to be multimerised receptors [30, 52]. Molecular weights for P2X receptors can differ, as these receptors are glycosylated on a number of N-glycosylation sites [53] and P2X receptor subunits assemble into multimers of probably three subunits [25].

The $hP2X_4$ receptor has a theoretical molecular weight of 43.5 kDa and contains seven putative N-glycosylation sites [54]. The band detected at 64 kDa is therefore concluded to be a glycosylated $P2X_4$ receptor and the band at 120 kDa is interpreted to be a dimer [30]. The $hP2X_6$ receptor has a theoretical molecular weight of 40 kDa and three putative N-glycosylation sites [55]. In the present study, the $hP2X_6$ receptor migrated at approx. 42 and 60 kDa; these bands are interpreted to represent the unglycosylated and the glycosylated $hP2X_6$ receptor, respectively. Recombinant $hP2X_6$ receptors previously migrated at 50 kDa [30] and native $hP2X_6$ receptors at 60 kDa [51].

$P2X_4$ and $P2X_6$ receptors on plasma membranes are associated with the underlying cytoskeleton

Selective extraction experiments of cellular proteins with TX-100 indicate whether cellular proteins are inserted into the plasma membrane and are associated with the actin-based cytoskeleton (TX-100-insoluble fraction) or if proteins do not make any contacts with actin filaments (TX-100-soluble fraction) [43]. Putative homomeric (non-functional) $P2X_4$ and $P2X_6$ receptors of 64 and 60 kDa, respectively, have been identified in the TX-100-soluble fraction. Putative trimeric $P2X_4$ and $P2X_6$ receptors of approx. 180 kDa have been detected in the TX-100-insoluble fraction, where functional/trimeric P2X receptors would be expected [25]. A Western blotting sample buffer containing 1% SDS may provide conditions mild enough for the conservation of these trimeric P2X receptors. Native expression of $P2X_{4/6}$ hetero-oligomers has previously been found to be abundant in mammals and $P2X_{4/6}$ hetero-oligomers have been shown to be functional after ectopic expression in *Xenopus* oocytes [29].

Selective extraction of $P2X_4$ and $P2X_6$ receptors on slides and subsequent analysis by confocal microscopy showed that cytoplasmic P2X receptors are extractable by TX-100 but that receptors on the plasma membrane are resistant to TX-100 extraction. We therefore conclude that

$P2X_4$ and $P2X_6$ receptors on the plasma membrane are associated with underlying microfilaments, as previously described for $P2X_1$ receptors in transfected cells [31]. Association of membranous protein with actin filaments is also an important feature for cadherin molecules [43].

$P2X_4$ receptors co-immunoprecipitate with VE-cadherin

Evidence for the association of P2X receptors with cadherin molecules has been obtained in co-immunoprecipitation experiments. $P2X_4$ and $P2X_6$ receptors have been investigated for association with VE-cadherin and N-cadherin. Both cadherins are expressed in human endothelial cells, but only VE-cadherin is expressed at cellular junctions; N-cadherin is diffusely expressed throughout the cytoplasm [56]. The $P2X_4$ receptor subtype co-immunoprecipitated only with VE-cadherin, but not with N-cadherin. This is in agreement with the localisation of membraneous $P2X_4$ receptors to cell-cell contacts. VE-cadherin could also be co-immunoprecipitated with antibodies for $P2X_4$ receptors. This is an important finding, as the molecular weight for P2X receptors has been found to vary depending on glycosylation of the receptor (see above). However, the molecular weight for VE-cadherin is constantly reported to be 130 kDa [57–59]. This 130-kDa band for VE-cadherin has also been found in our experiments after co-immunoprecipitation of VE-cadherin with $P2X_4$ receptor antibody.

$P2X_6$ receptors could not be co-immunoprecipitated with N- or VE-cadherin and VE-cadherin was not immunoprecipitatable with antibody for $P2X_6$ receptors. This seems to be in contrast to the dependency of $P2X_6$ receptors on $P2X_4$ receptors to form a functional channel, i.e. $P2X_6$ receptors should co-immunoprecipitate with $P2X_4$ receptors and should therefore also precipitate with VE-cadherin. However, the lack of immunoprecipitated $P2X_6$ receptor may be explained by the different intensity of the immunolabelling for the two P2X receptor subtypes in Western blots of the TX-100-soluble extracts. $P2X_4$ receptors are intensely labelled in the TX-100-soluble extracts, whereas labelling for $P2X_6$ receptors appears much weaker. The co-immunoprecipitation experiments were performed with TX-100-soluble extracts (as a stronger detergent would probably disrupt the interactions between the molecules). $P2X_4$ receptors associated with VE-cadherin are likely to be just above the detection limit of our immunoprecipitation procedure. Complexes formed by VE-cadherin and $P2X_6$ receptors may be just below the detection limit for co-immunoprecipitation, as $P2X_6$ receptors seem to give a generally weaker signal in the TX-100-soluble fraction.

Endothelial $P2X_4$ and $P2X_6$ receptors may be physically associated with VE-cadherin

The P2X receptor subtypes investigated in the present study have been shown to mimic the behaviour of cadherin molecules in many important aspects: $P2X_4$ and $P2X_6$ receptors are highly co-localised with VE-cadherin; $P2X_4$ and $P2X_6$ receptors are almost exclusively located to cell-cell junctions (as demonstrated for VE-cadherin) [60]. $P2X_4$ and $P2X_6$ receptor localisation to the plasma membrane is specifically dependent on extracellular Ca^{2+} (as described for VE-cadherin) [61]. $P2X_4$ and $P2X_6$ receptors are associated with microfilaments (as described for E-cadherin) [42]. The predominant P2X receptor subtype in the endoplasmic reticulum ($P2X_4$ receptor) assembles with VE-cadherin in the cytoplasmic cellular fraction (as shown for catenins and E-cadherin) [43]. We conclude that $P2X_4$ and $P2X_6$ receptors are linked to VE-cadherin either directly or through one or more adaptor proteins.

A potential role for purinergic signalling in modulating cell-cell adhesion

Yamamoto et al. [22] suggested that the $P2X_4$ receptor is a shear stress transducer in HUVECs. Shear stress is well known to modulate cadherin-mediated cellular junctions [58] and to cause ATP release [7]. Therefore, shear stress may be transduced to adherens junctions after ATP release, P2X receptor stimulation and possibly after tyrosine phosphorylation of cadherins. Phosphorylation of cadherins on tyrosine can modulate cell-cell adhesion [62, 63] and P2X receptors have previously been shown to induce tyrosine phosphorylation (probably via p60c-src [32]). A recent report [64] suggested that $P2X_4$ receptors can control neointimal proliferation after vascular injury. Lampugnani et al. [65] demonstrated that VE-cadherin is phosphorylated on tyrosine in proliferating endothelial cells after wounding experiments.

This effect on the appearance of cell-cell junctions may be mediated solely via purinergic signalling or (more likely) via a synergistic effect of purines with growth factors, which are present in the culture medium. Synergistic effects of purinergic signalling via P2X receptors and via growth factors have previously been described [66–68]. Growth factor receptors may be located in close apposition to $P2X_4$ and $P2X_6$ receptors in the endothelial cell-cell junction. The epidermal growth factor receptor has been demonstrated to be linked to the cadherin-catenin complex [69].

Recent studies show that Ca^{2+} signalling is an important event throughout the formation and remodelling of cadherin-mediated cell-cell junctions [37–39]. Purinergic signalling is likely to be a pathway for the mediation of a Ca^{2+} signal which can modulate cell-cell contacts for the following reasons; first, P2X receptors (which are known to mediate calcium signalling) were found in the present report to be located in close apposition to the cell-cell junctions; an important criterion previously suggested [38]. Second, P2X receptors can induce tyrosine phosphorylation of adhesion molecules [32]. Third, P2X receptors can mediate modulation of the actin-based cytoskeleton [33].

In summary, we have demonstrated for the first time that ligand-gated ion channels (the $P2X_4$ and $P2X_6$ receptors) are associated with VE-cadherin. We suggest a new physiological role for $P2X_4$ and $P2X_6$ receptors in modulating adhesion junctions in human endothelial cells.

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