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Unstimulated platelets evoke calcium responses in human umbilical vein endothelial cells

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Abstract

Interactions between human platelets and human umbilical vein endothelial cells (HUVEC) were studied by monitoring changes in cytosolic $[Ca^{2+}]_i$ in both cell types. Confluent monolayers of Fura-2-loaded HUVEC, grown on gelatin-coated coverslips, responded to repeated addition of a suspension of unstimulated platelets by transient increases in cytosolic $[Ca^{2+}]_i$. These platelet-evoked Ca^{2+} responses were not caused by products secreted from the platelets and were insensitive to inhibitors of platelet activation and/or platelet aggregation. The platelet-evoked rises in $[Ca^{2+}]_i$ in endothelial cells, similarly as the thrombin-evoked rises, were blocked by preincubation of HUVEC with the phospholipase C inhibitor U73122 or the Ca^{2+} influx blocker Ni^{2+}. In contrast, treatment with the protein tyrosine kinase inhibitor genistein was without effect. Video imaging experiments, in which the fluorescence signal was analysed from the individual cells of an endothelial monolayer, showed that only 2–20% of the cells, scattered over the monolayer, responded to the addition of platelets by a transient increase in $[Ca^{2+}]_i$, whereas most of the cells responded to thrombin. This leads to the conclusion that unstimulated platelets can activate HUVEC putatively by mechanical interaction with individual endothelial cells in the monolayer.

Keywords: Platelet; Umbilical vein; Calcium ion; Endothelial cell; Fura-2; (Human)

1. Introduction

Endothelial cells lining the blood vessel play a crucial role in maintaining blood fluidity and in permitting haemostasis after the injury of a vessel wall. In controlling these processes, the endothelial cells interact extensively with circulating blood platelets. For instance, in case of endothelial damage platelets rapidly adhere to the subendothelial matrix and aggregate there with other platelets to form a thrombus. Undamaged, healthy endothelium, on the other hand, produces large amounts of nitric oxide (NO) and prostacyclin (PGI_2), which autacoids prevent the adhesion and aggregation of platelets by raising the intraplatelet levels of cGMP and cAMP, respectively [1–3]. It has been demonstrated by several authors that this endothelial PGI_2 and NO production can be stimulated by the presence of vasoactive agents like thrombin, ATP and bradykinin [4–6], but also by an increased fluid shear stress at the endothelial surface [7–9]. The stimulated autocoid release is considered to be mediated by a phospholipase C-dependent rise in cytosolic $[Ca^{2+}]_i$, regardless of whether the stimulus is provided by receptor agonists [4,5,10], high shear stresses [9,11,12] or mechanical forces [13,14]. A number of other interactions between these two cell types emerge from the literature, including an activating effect of ADP and ATP [15], once secreted from activated platelets of the endothelial P_{2Y}-purinoceptors, resulting in an elevation in $[Ca^{2+}]_i$ and production of PGI_2 [4,8]. In addition, some authors have proposed that platelets may directly adhere to cultured monolayers of endothelial cells, although the physiological importance of this binding is still unclear [16,17].

Here, we report evidence for yet another type of interaction between platelets and endothelial cells. We detected in confluent monolayers of human umbilical vein endothelial cells (HUVEC) multiple, transient increases in $[Ca^{2+}]_i$ that were caused by the repeated addition of a suspension of unstimulated platelets. The experimental data suggested

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that the platelet-evoked Ca^{2+} signals involved endothelial phospholipase C activity, but were independent of platelet activation or adhesion. We assume that the endothelial cells were activated by mechanical interaction with colliding platelets. This intercellular contact may contribute to the factors responsible for the continuous production of autacoids by healthy endothelial cells.

2. Materials and methods

2.1. Cell culturing

HUVEC were isolated from human umbilical cords and cultured as described before [18]. Glass coverslips were cut to sizes (14 × 24 mm), treated with 1% (w/v) glutardialdehyde and coated with 1% (w/v) gelatin at 37°C for 30 min. The gelatin was cross-linked with 0.5% (w/v) glutardialdehyde in de-ionized water for 30 min. Cultured HUVEC (usually passage 1) were seeded on these gelatin-coated coverslips at $1 \cdot 10^5$ cells per coverslip, and the cells were grown until confluency was reached. Confluent monolayers contained approx. $2.5 \cdot 10^5$ cobblestone-shaped cells per coverslip.

2.2. Loading of HUVEC with Fura-2

Confluently grown cells on coverslip were incubated with 2 μM Fura-2 pentaacetoxymethyl ester, 1.8 mM $CaCl_2$ and 1% (w/v) bovine serum albumin in buffer pH 7.45 under CO_2-containing, humidified air at 37°C for 45 min. Buffer pH 7.45 consisted of 150 mM NaCl, 5 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, 5 mM glucose, 4 mM L-glutamin and 0.25% (w/v) bovine serum albumin. After Fura-2 loading, the HUVEC were re-incubated with culture medium at 37°C for another 15 min to complete the hydrolysis of acetoxymethyl ester groups. The cells were then washed twice with buffer pH 7.45 that was supplemented with 1.8 mM $CaCl_2$, and were kept strictly at 37°C until use (within 1 h). In specific experiments, HUVEC on coverslip were treated with genistein (ICN, Cleveland, OH, USA) during the loading with Fura-2 or, alternatively, were treated with U73122 (Biomol, Plymouth Meeting, PA, USA) for 2 min before start of the activation.

2.3. Isolation of platelets and loading with Fura-2

Human, unloaded and Fura-2-loaded platelets were obtained as described before [19,20]. The platelets were treated with aspirin [20]. After a wash step platelets were resuspended ($1 \cdot 10^9$ cells/ml) in buffer pH 7.45, supplemented with apyrase (0.1 U ADPase and 0.2 U ATPase/ml). Platelet suspensions were kept at room temperature and used within 3 h; the cells were warmed to 37°C immediately before use.

2.4. Measurements of $[Ca^{2+}]_i$

Fura-2 fluorescence was recorded at 37°C either from HUVEC on coverslip or from platelets in suspension, using the spectrofluorimetric equipment described before [21]. A coverslip with HUVEC was diagonally placed in a 1-cm cuvette in such a way that the cells were directly exposed to the excitation light (without interference of the glass) and the reflecting light was minimal. Pre-warmed buffer pH 7.45 (2.5 ml), supplemented with 1.8 mM $CaCl_2$, was immediately added to the cuvette. The coverslip and cuvette were immobilised in the light beam by a black, home-made coverslip holder. Fluorescence intensities were measured and corrected for the light reflected by the coverslip. Since the reflection was sensitive to small changes in the position of the coverslip, it was always verified that the calculated rises in $[Ca^{2+}]_i$ were caused by an increased fluorescence at 340 nm and decreased fluorescence at 380 nm excitation. For calibration of fluorescence values to $[Ca^{2+}]_i$, the cells were lysed with 0.1% (w/v) Triton X-100 [21].

Calcium measurements to individual, Fura-2-loaded, endothelial cells (HUVEC) on coverslip were carried out with a Quanticell fluorometric system (Applied Imaging, Sunderland, UK), following earlier described procedures [20]. The excitation light was passed alternately through filters of 340 and 380 nm and the fluorescent light above 505 nm was observed by a charge-coupled device camera, working at standard video rate and connected to an inverted microscope. Fluorescence images were digitised and background-subtracted, ratios were calculated, and the resulting ratio images (obtained every 1–3 s) were stored to a 650 MByte read/write optical disk. Geometric regions matching individual cells were defined and analysed for changes in fluorescence ratio. Separate observation of the images taken at 340 or 380 nm excitation showed small cell variation in fura-2 fluorescence intensity, but did not reveal fluorescence hot spots. It was checked that increases in 340 nm/380 nm fluorescence ratio were always due to an increase in 340 nm fluorescence and a decrease in 380 nm fluorescence. In this type of experiment, calibration of ratio values to $[Ca^{2+}]_i$ was omitted, because of the unequal amount of fluorescence per cell. Therefore, these single cell data are represented as changes in ratio values, which are indicative for changes in $[Ca^{2+}]_i$.

2.5. Activation studies

Platelet suspensions and agonist solutions were added to a coverslip-containing cuvette by the following procedure: (1) gentle removal of the buffer medium out of the cuvette with a plastic pipette, (2) mixing the buffer with a concentrated stock of agonist or suspended platelets (kept at 37°C), and (3) gentle addition of the mixture back to the coverslip-containing cuvette. Removal and re-addition occurred by placing the pipette at the cell-free side ('back

side') of the coverslip. Microscopic inspection showed that this procedure did not influence the morphology of the HUVEC. Where indicated, the platelets were pretreated with relevant activation or adhesion inhibitors: platelet-rich plasma was incubated with dimethyl-BAPTA, as described before [19]; washed platelets were preincubated with neuraminidase from *C. perfringens* (1 U/ml, Sigma, St. Louis, MO, USA) at 37°C for 60 min [22] and then washed again. These neuraminidase-treated platelets were reduced in collagen- or thrombin-evoked aggregation tendency by 70% or more. Ilomedine (Schering, Berlin, Germany) and RGDS (Bachem, Bubendorf, Switzerland) were added to the platelet suspension [21] at 5 min before start of the measurement.

3. Results

3.1. Addition of platelets evokes a transient Ca^{2+} response in HUVEC

HUVEC were grown to confluency on glass coverslips coated with gelatin, and the cells were loaded with Fura-2. When ATP (20 μM), a suboptimal dose of thrombin (5 nM) or ionomycin (see below) was added to such a confluent monolayer, this resulted in an immediate, transient increase in $[Ca^{2+}]_i$ (Fig. 1A). Similar transient Ca^{2+} responses have been observed by others [4,5,10,23]. The endothelial cells on coverslip did not respond to the addition of thromboxane A_2 analogue U46619 (10 μM) (data not shown). However, they responded by a significant rise in $[Ca^{2+}]_i$ of 37 ± 7 nM (mean ± S.E., $n = 10$), when a suspension of aspirin-treated platelets ($5 \cdot 10^7$/ml, final concentration) was added. The level of this Ca^{2+} signal increased dose-dependently with the number of platelets added (not shown). Removal and re-addition of the platelets resulted in a second Ca^{2+} response in the endothelial cells (Fig. 1B). Addition of thrombin after stimulating the cells with platelets caused a rapid increase in $[Ca^{2+}]_i$ of 158 ± 33 nM ($n = 10$), demonstrating that the HUVEC were still highly responsive to this receptor agonist (Fig. 1C). In contrast, addition of platelet-free buffer solution evoked in the HUVEC an increase in $[Ca^{2+}]_i$ of only 5 ± 2 nM (Fig. 1D), while the concerning cells responded normally to thrombin by a $[Ca^{2+}]_i$ rise of 114 ± 5 nM ($n = 5$). Typically, non-confluent HUVEC monolayers failed to respond to the addition of platelets.

3.2. The platelet-evoked Ca^{2+} signal in HUVEC is not caused by platelet activation

We searched for the signalling factors responsible for the platelet-evoked $[Ca^{2+}]_i$ transients in endothelial cells. The addition of Fura-2-loaded platelets to a gelatin-coated, cell-free (not shown) or HUVEC-containing (Fig. 2) coverslip did not evoke a significant Ca^{2+} response in the platelets, whereas thrombin caused the expected, high Ca^{2+} signal. Thus, the platelets were unlikely to be activated by the addition procedure. It was also unlikely that the platelet-evoked Ca^{2+} signal in HUVEC was mediated by ADP and/or ATP that were secreted from the platelets, since the platelets were suspended in a medium containing

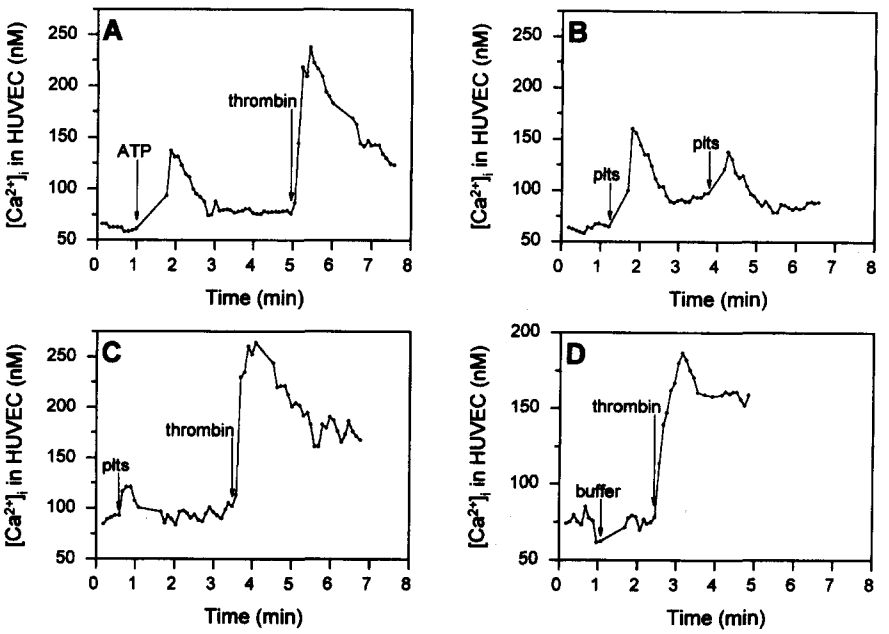

Fig. 1. Calcium responses in monolayers of endothelial cells. Coverslips with monolayers of confluently grown, Fura-2-loaded HUVEC were placed in a thermostated cuvette and changes in endothelial $[Ca^{2+}]_i$ were recorded. Representative Ca^{2+} responses ($n = 4$–9) are shown of the addition of: (A) 20 μM ATP and 5 nM thrombin; (B) a suspension of platelets added twice to the same monolayer (plts, finally $1.5 \cdot 10^8$/3 ml); (C) a platelet suspension followed by 5 nM thrombin; (D) 100 μl buffer and 5 nM thrombin.

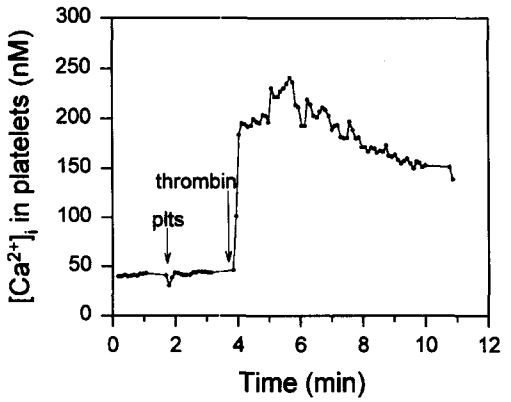

Fig. 2. Calcium responses of platelets in the presence of a monolayer of endothelial cells. A coverslip with confluently grown HUVEC was placed in a cuvette containing Fura-2-loaded platelets ($1.5 \cdot 10^8$/3 ml), and intraplatelet $[Ca^{2+}]_i$ was measured. The platelets were removed and placed back (plts), and thrombin (5 nM) was added, as indicated.

sufficient amounts of apyrase to degrade these nucleotides immediately. This was confirmed by the observation that addition to the HUVEC of cell-free supernatant from a centrifuged platelet suspension gave a rise in endothelial $[Ca^{2+}]_i$ of only 5 ± 2 nM ($n = 3$) (Fig. 3A). In addition, the Ca^{2+} response was probably not caused by the presence of traces of (pro)thrombin in the platelet suspension, since it was not influenced with the thrombin scavenger huridin (data not shown). Thus, the platelet-evoked Ca^{2+} response in HUVEC seemed to be independent of platelet activation and platelet secretion products.

So far, these experiments did not exclude the possibility that a small subfraction of the platelets might be activated by direct contact with the endothelial monolayer, which may lead to a local release of secretion products (e.g., ADP and ATP) and a Ca^{2+} response in the HUVEC. To investigate this, we used platelets that were pretreated in various ways. Platelet activation was suppressed by loading the platelets with the intracellular Ca^{2+}-chelator dimethyl-BAPTA [19] or by treating the cells with the PGI_2 analogue ilomedine [21]. Glycoprotein-mediated adhesion events were blocked by preincubation of the platelets with neuraminidase, which strips sialic acid residues from the surface membrane glycoproteins [22], or by addition of the tetrapeptide RGDS, which suppresses fibrinogen- and von Willebrand factor-mediated adhesion [24]. However, as shown in Fig. 3A, none of these treatments could significantly reduce the platelet-evoked rises in $[Ca^{2+}]_i$ in the HUVEC.

3.3. The Ca^{2+} signal in HUVEC is phospholipase C-mediated and is partially dependent on extracellular Ca^{2+}

Preincubation of the Fura-2-loaded HUVEC with U73122, a known phospholipase C inhibitor [21], completely blocked the $[Ca^{2+}]_i$ rises caused by the addition of platelets (Fig. 3B) or thrombin (data not shown), suggesting phospholipase C activation was required for the generation of both Ca^{2+} signals. In contrast, preincubation of the HUVEC with the protein tyrosine kinase inhibitor genistein was without effect on the platelet-evoked Ca^{2+} signal (Fig. 3B).

As shown in Fig. 4A, repeated administration of platelets to a single monolayer gave progressively smaller Ca^{2+} signals with each addition, although the HUVEC response to ionomycin (a Ca^{2+}-ionophore) was still high. When $NiCl_2$ was added to the incubation mixture to block the influx of extracellular Ca^{2+} [23], the Ca^{2+} signal evoked by the first addition of platelets was only slightly reduced

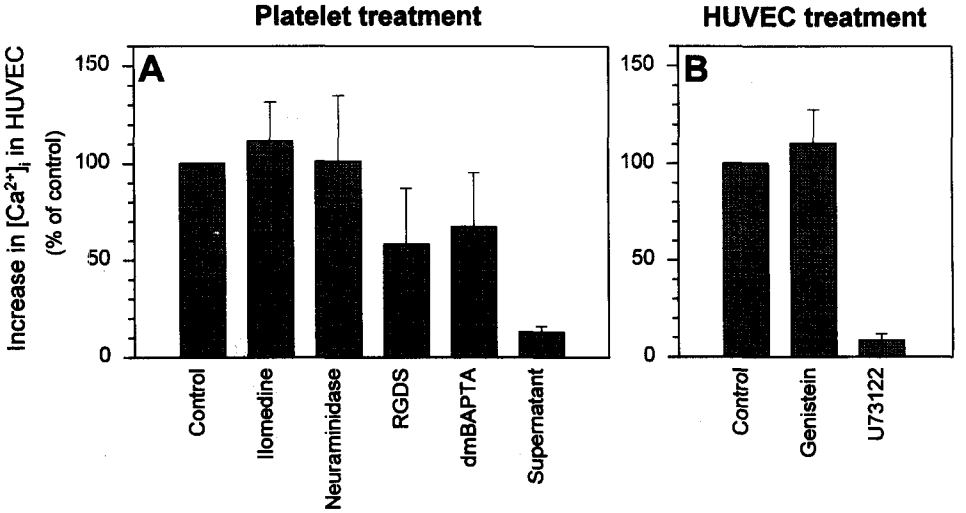

Fig. 3. Effects of inhibitors on platelet-evoked calcium responses in endothelial cells. Coverslips with confluently grown, Fura-2-loaded HUVEC were placed in a cuvette and maximal rises in endothelial $[Ca^{2+}]_i$ were calculated, evoked by the addition of platelets ($1.5 \cdot 10^8$/3 ml). (A) Platelets remained untreated (control), or were treated with ilomedine (5 μM), neuraminidase (1 U/ml), RGDS (20 μM) or dimethyl-BAPTA (10 μM), as described in Section 2. In addition, the platelet suspension was centrifuged and platelet-free supernatant was added to the HUVEC. (B) HUVEC were untreated (control) or were treated with genistein (100 μM) or U73122 (10 μM). Data are expressed as percentages of control incubations (mean $\pm$ S.E., $n = 3$–6).

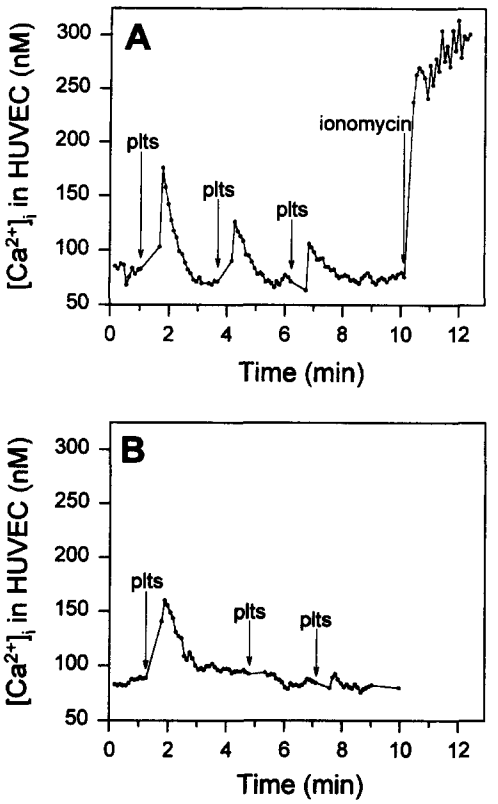

Fig. 4. Calcium responses in monolayers of endothelial cells. Coverslips with confluently grown, Fura-2-loaded HUVEC were placed in a cuvette and changes in endothelial $[Ca^{2+}]_i$ were recorded. Representative Ca^{2+} responses are shown of the addition of a platelet suspension added three times to the same monolayer (plts, finally $1.5 \cdot 10^8$/3 ml), followed by 1 μM ionomycin as indicated. Incubations contained 1.8 mM $CaCl_2$ and were in the absence (A) or presence (B) of 5 mM $NiCl_2$.

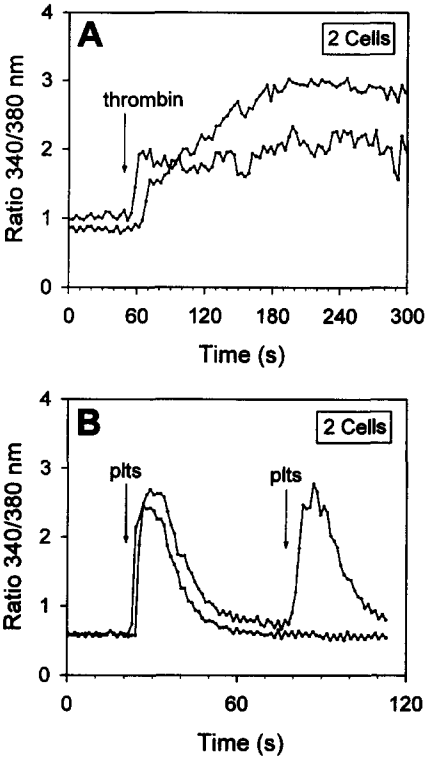

Fig. 5. Calcium responses of individual cells in a confluently grown monolayer of Fura-2-loaded endothelium. Changes in 340 nm/380 nm fluorescence ratio are given, indicative for changes in $[Ca^{2+}]_i$. (A) Representative traces of 2 cells from the monolayer responding to thrombin (5 nM). (B) Traces from 1 cell responding twice and 1 cell responding once to the addition of a platelet suspension (plts, $5 \cdot 10^7$/ml).

(suggesting it was largely due to the release of Ca^{2+} from intracellular stores), whereas the signals evoked by the second and third addition were blocked completely (Fig. 4B). It is known from the literature that in endothelial cells the phospholipase C-mediated rises in $[Ca^{2+}]_i$ are accompanied by generation of PGI_2 and NO [5,9,11,12]. Indeed, preliminary experiments where we measured the formation of the PGI_2-derivative 6-keto prostaglandin $F_{1\alpha}$ by HPLC suggested that gentle shaking of a HUVEC monolayer with platelets resulted in increased PGI_2 formation (W.

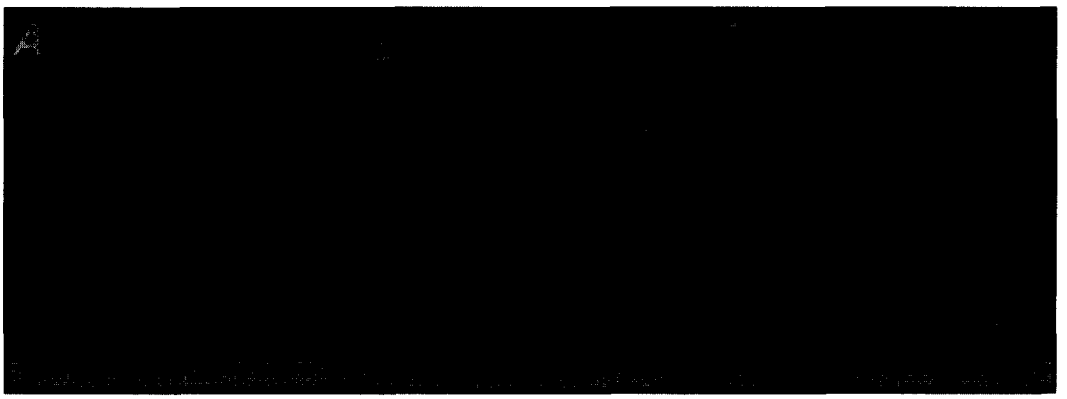

Fig. 6. Identification of individual cells from a monolayer of fura-2-loaded HUVEC responding to the addition of platelets. Changes in 340 nm/380 nm fluorescence were monitored every 3 s by video imaging, as described for Fig. 5. Video frames were taken from a confluent part of the HUVEC monolayer with almost homogenous fluorescence intensity all over the microscope field. However, the images were processed in such a way that only pixels with a higher 340/380 nm ratio than 2 × the basal ratio were coloured. Thus, white areas represent cells with a high fluorescence ratio, which are elevated in $[Ca^{2+}]_i$. Consecutive images are given from an endothelial cell monolayer immediately before adding platelets (A), and after 6 s after a first (B) and 6 s after a second (C) addition of platelets. Frame width is 0.35 × 0.35 mm.

Engels, P. Lemmens and J.W.M. Heemskerk, unpublished results).

To reveal the spatial characteristics of these Ca^{2+} responses in Fura-2-loaded HUVEC, we performed a number of fluorescence video imaging experiments. Analysis of the fluorescent signals from individual cells of a confluent monolayer showed that more than 85% of the cells responded to thrombin by a rapid increase in $[Ca^{2+}]_i$ (Fig. 5A). The gentle addition of unstimulated platelets evoked a Ca^{2+} signal of relatively short duration in only 2–20% of the observed endothelial cells, some of which also responded to a second addition of platelets (Fig. 5B). Usually, the responding cells seemed to be scattered over the endothelial monolayer (Fig. 6).

4. Discussion

In the present study, we describe that HUVEC in a confluent monolayer are activated by the addition of a suspension of unstimulated platelets. Multiple additions evoked multiple Ca^{2+} signals in the endothelial cells, although the strength of the Ca^{2+} response tended to decrease with each addition (Fig. 1B and 4A). Analysis of the Ca^{2+} signals from individual cells showed that only a subset of the cells from a monolayer responded to platelets by a $[Ca^{2+}]_i$ transient (Fig. 6). Platelet-free buffer was unable to evoke a significant Ca^{2+} response in the endothelial cells (Fig. 1D), indicating that the observed rises in $[Ca^{2+}]_i$ were not a consequence of the addition procedure per se. The platelet-evoked response was inhibitable by U73122 (Fig. 3B), suggesting it was phospholipase C-mediated, and it was partially blocked by Ni^{2+} (Fig. 4), which points to the contribution of the influx of extracellular Ca^{2+} to the signal. The Ca^{2+} signal in HUVEC was most probably not caused by products secreted from the platelets, since the platelet-free supernatant from a centrifuged platelet suspension was completely inactive (Fig. 3A). Moreover, the signal was unlikely to be due to the activation of platelets during the addition procedure, since (i) all experiments were carried out with aspirin-treated platelets that remained discoid and low in $[Ca^{2+}]_i$ (Fig. 2), and (ii) the HUVEC response was not significantly influenced by compounds (apyrase, ilomedine) or procedures (loading with dimethyl-BAPTA) which potently inhibit the platelet activation process (Fig. 3A).

A number of mechanisms may account for this platelet-evoked effect on endothelial cells. Firstly, it is conceivable that HUVEC respond to the products secreted from those platelets that collide with the cell monolayer. This would imply that endothelial cells have an activating effect on platelets, which contrasts with the general idea that the predominant influence of the endothelium and the endothelial-derived autacoids is suppression of platelet adhesion, activation and secretion processes [1–3]. In addition, it is not easy to see why only confluent monolayers of HUVEC would show such a Ca^{2+} response.

Secondly, platelets might directly interact with HUVEC via their glycoproteins, e.g., the fibrinogen or von Willebrand receptors, and then stimulate the endothelium. However, when we tried in our experiments to reduce the glycoprotein-mediated interactions by using platelets that were treated with neuraminidase or RGDS, this hardly influenced the rises in $[Ca^{2+}]_i$ in HUVEC (Fig. 3A). Moreover, microscopic observations showed that platelets were greatly reluctant to adhere to the HUVEC, as long as the endothelial cells were not retracted.

Thirdly, it is possible that platelets stimulate the HUVEC via their mechanical sensing elements. Stretch-activated ion channels, putatively connected to mechanosensors, have been detected in vascular endothelium as early as in 1987 [25]. Since that time, it has been observed that mechanical strain can increase $[Ca^{2+}]_i$ in endothelial cells from various sources [13,14]. Although the biochemical nature of the putative mechanosensors is still unknown, there is now sufficient evidence that mechanical forces can stimulate HUVEC and other endothelial cells to produce PGI_2 via a pathway involving the activation of phospholipase C and a Ca^{2+} influx-dependent increase in $[Ca^{2+}]_i$ [7,9,11–14,26]. Platelet addition apparently evokes an activation pathway in HUVEC with the same characteristics. Therefore, it is tempting to suggest that mechanical contact with colliding platelets stimulates these mechano-sensing and signal-transducing elements in the endothelial monolayer. This suggestion is further supported by the observation that only a few individual cells from a confluent monolayer became activated with platelets and not the HUVEC monolayer as a whole. It should be stressed that the experiments were performed under essentially static conditions and thus HUVEC were exposed to only a minimal fluid shear stress which, by itself, was unable to activate the endothelial cells. Especially HUVEC grown to confluency may be subjected to this type of stimulation, since there is evidence that in confluent monolayers shear stress resistance is effectively conveyed by cell to cell and cell to matrix interactions to the whole sheet of the endothelium [27]. We speculate that this type of mechanical stimulation of endothelium is a factor contributing to autacoid release under physiological conditions, in particular at those sites in the vaculature where the shear stress is low.

In conclusion, we have demonstrated that the list of interactions between endothelium and platelets [28] can be extended with a new element: unstimulated platelets appear to activate a phospholipase C-mediated, Ca^{2+}-entry-dependent signal transduction pathway in endothelial cells putatively by mechanical interaction with these cells.

Acknowledgements

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