Agonists for endothelial P₂ purinoceptors trigger a signalling pathway producing Ca²⁺ responses in lymphocytes adherent to endothelial cells

James S. WILEY,* Joan R. CHEN, Gary P. JAMIESON and Peter J. THURLOW

Department of Haematology, Austin Campus, Austin and Repatriation Medical Centre, Heidelberg, Victoria 3084, Australia

Recirculation of lymphocytes through the body involves their frequent adhesion to endothelial cells but little is known of the signalling pathways between these two cell types. Lymphocytes from patients with chronic lymphocytic leukaemia were loaded with the Ca²⁺-sensitive indicator, fura 2, and allowed to adhere to either glass or monolayers of human umbilical-vein endothelial cells. Addition of ATP or UTP (1–10 μ M) to the superfusate produced a transient rise in cytosolic Ca²⁺ concentration in the lymphocytes adherent to endothelium (24 of 35 cells). In contrast, ATP or UTP (1–10 μ M) had no effect on the cytosolic Ca²⁺ of lymphocytes attached to glass. As the only lymphocyte receptor for ATP (P_{2z} class) requires higher ATP concentrations

(> 50 μ M) for Ca²⁺ influx and is unresponsive to UTP, the involvement of a lymphocyte P_{2z} purinoceptor is unlikely. Various agonists including ATP, UTP, 2-methylthioATP, ADP and histamine all stimulated increases in endothelial cytosolic Ca²⁺ but only ATP and UTP (both agonists for endothelial P_{2u} purinoceptors) triggered Ca²⁺ transients in adherent lymphocytes. Removal of extracellular Ca²⁺ did not abolish the ATP-induced rise in cytosolic Ca²⁺ concentration in lymphocytes adherent to endothelial cells. These findings show that stimulation of endothelial P_{2u} purinoceptors triggers an endothelial–lymphocyte signalling pathway which releases internal Ca²⁺ in adherent lymphocytes.

INTRODUCTION

The role of extracellular ATP as a mediator of intercellular signalling is now established in a range of cells and tissues [1–3]. In the autonomic and central nervous systems, synaptic transmission can be mediated by ATP via ligand-gated ion channels which, although non-selective, have a high permeability to Ca²⁺ ions [4]. Other cell types may also communicate by purinergic mechanisms. For example, cell-to-cell spread of Ca²⁺ signals through a monolayer of mast cells is mediated by ATP release from one cell causing a Ca²⁺ transient in the neighbouring cell which leads to further release of ATP [5]. Vascular endothelial cells also release significant concentrations of ATP into plasma [6] which raises the possibility that purinergic mechanisms may be involved in endothelial signalling to adherent leucocytes. In one study of human polymorph neutrophils adherent to human umbilical vein endothelial cells (HUVECs), it was shown that Ca²⁺-mediated signalling was necessary for transmigration to proceed [7]. Similar studies with human lymphocytes are not available and it is not known if Ca²⁺ transients or other second-messenger pathways are involved in communication between endothelial cells and adherent lymphocytes.

Endothelial P₂ purinoceptors are of at least two types classified as P_{2y} and P_{2u} on the basis of their specificity for ATP and other nucleotide agonists [8–11]. Although ATP activates both P_{2y} and P_{2u}, these two purinoceptors can be distinguished by the specificity of P_{2y} for 2-methylthioATP (2-MeSATP) and of P_{2u} for UTP respectively. Activation of either P_{2y} or P_{2u} purinoceptors initiates the phosphoinositide signalling cascade with Ins(1,4,5)P₃ production and subsequent release of Ca²⁺ from intracellular stores. Lymphocytes do not possess either P_{2y} or P_{2u} purinoceptors but do express a purinoceptor of a separate subclass termed P_{2z} which functions as an ATP-receptor-operated ion channel. This purinoceptor is characterized by specificity for the fully ionized ATP⁴⁻ and relatively low affinity

for this nucleotide agonist with an EC₅₀ of about 100 μ M total ATP [12–18]. In this study of cell–cell signalling, human lymphocytes loaded with fura 2 were attached to monolayers of HUVECs. Lymphocytes were taken from patients with chronic lymphocytic leukaemia (CLL), as this cell population represents a clonal expansion of mature B-lymphocytes with well-characterized adhesive properties and purinoceptors [12–16]. Intracellular Ca²⁺ concentration in individual lymphocytes was measured by single-cell fluorimetry after addition of agonists known to produce Ca²⁺ transients in endothelial cells. The data show that low concentrations of ATP or UTP (1–10 μ M) that are unable to activate lymphocyte P_{2z} purinoceptors directly can nevertheless activate endothelial P₂ purinoceptors and trigger a signalling pathway causing release of internal Ca²⁺ in lymphocytes adherent to endothelial cells.

MATERIALS AND METHODS

Materials

Ficoll-Paque (density 1.077) was obtained from Pharmacia (Uppsala, Sweden). ATP, ADP, UTP, histamine, BSA, Hepes, endothelial-cell growth supplement, collagenase, 2% gelatin solution, poly-L-lysine, glutamine, bovine calf serum, trypsin and Medium 199 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-MeSATP was from Research Biochemicals (Natick, MA, U.S.A.). Heparin, penicillin, streptomycin and Fungizone were from Gibco-BRL (Gaithersburg, MD, U.S.A.). Fura 2 acetoxymethyl ester (fura 2/AM), fluo 3 acetoxymethyl ester (fluo 3/AM) and Fura-Red acetoxymethyl ester (Fura-Red/AM) were from Molecular Probes (Eugene, OR, U.S.A.). CS-1 peptide [19] was kindly provided by Dr. Ann Ager, National Institute for Medical Research, Mill Hill, Middx., U.K. Monoclonal antibody to very late antigen 4 (VLA-4) (CD49d) was from Immunotech (Marseilles, France).

Abbreviations used: fura 2/AM, fura 2 acetoxymethyl ester; fluo 3/AM, fluo 3 acetoxymethyl ester; Fura-Red/AM, Fura-Red acetoxymethyl ester; HUVECs, human umbilical vein endothelial cells; CLL, chronic lymphocytic leukaemia; 2-MeSATP, 2-methylthioATP; oxidized ATP, 2',3'-dialdehyde ATP; VLA-4, very late antigen-4 (CD49d/CD29).

* To whom correspondence should be addressed.

Cell preparation and culture

Heparinized venous blood from patients with B-cell CLL was diluted with 2 vol. of Hepes-buffered saline (145 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 10 mM Hepes, pH 7.4, 5 mM D-glucose, 1 g/l BSA), and mononuclear cells were separated by density-gradient centrifugation over Ficoll-Paque. Cells were washed twice and resuspended to 10^7 /ml in Hepes-buffered saline. Cytochrome preparations and immunophenotyping showed that more than 99% of the mononuclear cells were small mature B-lymphocytes ($\text{CD}20^+$, $\text{CD}5^+$). HUVECs were isolated from human umbilical-cord vein as described [20] and grown in culture flasks precoated with gelatin using M199 medium containing 20% iron-supplemented bovine calf serum, 50 $\mu\text{g}/\text{ml}$ endothelial-cell growth supplement, 5 units/ml heparin, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 2.5 $\mu\text{g}/\text{ml}$ Fungizone. Endothelial cells from between the second and sixth passage were seeded on to poly-L-lysine-coated glass coverslips (3.2 cm diameter; 100 μm thick) at approx. 30% confluent density and cultured under 10% CO_2 /air for between 3 and 5 days to form a confluent monolayer.

Measurement of cytosolic Ca^{2+} responses by fluorimetry

Lymphocytes (10^7 cells/ml) were loaded with 2 μM fura 2/AM for 20 min at 37 °C. Coverslips with endothelial-cell monolayers were rinsed and placed in 2 ml of RPMI 1640 medium containing 2 g/l BSA. Lymphocytes (10^7 cells per coverslip) were added and gently centrifuged on to the endothelial-cell monolayer at 100 g for 10 min followed by a further incubation of 2 h at 37 °C. Non-adherent lymphocytes were gently removed by rinsing and the coverslip was transferred to a heated chamber in which the coverslip formed the base of the chamber (Biophysica Technologies, Baltimore, MD, U.S.A.). Hepes-buffered saline at 37 °C was then continuously superfused over the lymphocyte-endothelial conjugates. After a period of equilibration, ATP, ADP, UTP, 2-MeSATP or histamine was added as indicated to the superfusion of Hepes-buffered saline for a period 2–3 min. In some experiments endothelial-cell monolayers growing on coverslips were loaded with 2 μM fura 2/AM for 30 min at 37 °C. The effect of these agonists on the cytoplasmic free Ca^{2+} in single endothelial cells was then tested. In some experiments in which a Hepes-buffered Ca^{2+} -free medium was used, 0.3 mM Mg^{2+} was added to the Ca^{2+} -free medium. In control experiments, fura 2-loaded lymphocytes were attached to glass coverslips without the endothelial-cell monolayer by incubation for 30 min at 37 °C. Fura 2 fluorescence was measured from a circular area (20 μm in diameter) which encompassed a single lymphocyte attached to an endothelial cell, a single lymphocyte attached to glass or a single endothelial cell using a MPM200 photometry attachment to a Zeiss epifluorescent microscope. Measurements of fluorescence emission at 510 nm were collected every 5 s from two excitation wavelengths (340 nm and 380 nm). The ratio of fluorescence intensities at these two wavelengths was monitored as an estimate of cytoplasmic free Ca^{2+} concentration [21]. The Figures show typical results from a minimum of three experiments.

Measurement of cytosolic Ca^{2+} responses by flow cytometry

Lymphocytes (10^7 cells/ml) were loaded with 2 μM fluo 3/AM and 2 μM Fura-Red/AM for 20 min at 37 °C, and then attached to monolayers of endothelial cells as described above. Non-adherent lymphocytes were removed by thorough washing with Hepes-buffered saline containing 1 mM Ca^{2+} . The adherent lymphocytes and the endothelial cells were harvested by either

brief exposure to trypsin/EDTA or exposure to 5 mM EGTA for 20 min at 37 °C. Cells were analysed on an Elite ESP flow cytometer (Coulter) with excitation light at 488 nm. Forward-angle and side-scatter gates were used to ensure that only fluorescence emissions from lymphocytes were analysed. Fluorescence emissions were monitored at 530 nm (fluo 3) and 675 nm (Fura-Red). The ratio of fluorescence intensities at these two wavelengths was monitored as an estimate of cytoplasmic free Ca^{2+} concentration [22].

Fixation of endothelial-cell monolayers

In some assays the monolayers of HUVECs were fixed before the addition of lymphocytes with a solution containing 2% paraformaldehyde, 0.075% L-lysine monohydrochloride and 2.1 mg/ml sodium metaperiodate for 5 min at room temperature. This fixative is known to preserve the adhesive properties of HUVECs for the binding of human T-lymphocytes [23].

RESULTS

Nucleotide-induced Ca^{2+} transients in lymphocytes adherent to endothelium

Basal cytosolic Ca^{2+} concentration of individual fura 2-loaded lymphocytes adherent to endothelium was stable for up to

Figure 1 Effect of (a) ATP and (b) UTP on intracellular Ca^{2+} levels of single lymphocytes attached to endothelial cell monolayers

Fura 2-loaded lymphocytes obtained from one of eight different lymphocyte donors with CLL were centrifuged gently on to confluent monolayers of endothelial cells on glass coverslips. After 2 h incubation at 37 °C, non-adherent cells were washed away. The coverslips were then superfused with Hepes-buffered medium in a microfluorimeter and measurements of fluorescence emission at two excitation wavelengths (340 and 380 nm) were collected. The ratio of fluorescence emissions (340 nm/380 nm) is a measure of intracellular Ca^{2+} . ATP (a) or UTP (b) was added at the indicated concentrations for periods of 2–3 min.

Figure 2 Effect of ATP and UTP on intracellular Ca²⁺ levels in single lymphocytes attached to glass

Fura 2-loaded lymphocytes from patients with CLL were allowed to settle on to glass coverslips for 20 min at 37 °C before microfluorimetry as described above. ATP and UTP were added at the indicated concentrations for periods of 2–3 min.

20 min during superfusion with Ca²⁺-containing medium at 37 °C. Addition of low concentrations of ATP or UTP (1 or 10 μM) to the superfusate caused a rapid increase in cytosolic free Ca²⁺ in 24 of 35 lymphocytes adherent to endothelium (Figures 1a and 1b). The duration of the Ca²⁺ transient varied from 1 to 3.5 min and in many instances cytosolic Ca²⁺ levels returned to prestimulation values despite the continued presence of ATP or UTP. After 3–5 min 'washout' with agonist-free medium,

another Ca²⁺ transient could be induced by reapplication of either ATP or UTP (Figures 1a and 1b).

Lymphocytes adherent to glass are insensitive to low ATP concentrations

Addition of 500 μM ATP to lymphocytes adherent to glass increased lymphocyte cytosolic Ca²⁺ concentrations by opening a P_{2z} purinergic ion channel which has been well characterized in this cell type [12–18]. Lower concentrations of ATP (1–10 μM) and any concentration of UTP (1–1000 μM) failed to increase cytosolic Ca²⁺ in glass-attached lymphocytes (Figure 2). Furthermore adhesion of fura 2-loaded lymphocytes to glass coverslips coated with gelatin or fibronectin failed to confer any sensitivity to 10 μM ATP or UTP.

Lack of involvement of lymphocyte P_{2z} purinoceptor

Although the lymphocyte P_{2z} purinoceptor requires high concentrations of ATP (over 50 μM) to open its associated ion channel, the adhesion of lymphocytes to endothelial cells may increase the affinity of this receptor for ATP. Lymphocytes were incubated for 60 min with and without 300 μM 2',3'-dialdehyde ATP (oxidized ATP), an irreversible inhibitor of the P_{2z} purinoceptor of lymphocytes [16,24], before loading with fura 2 and adhesion to endothelial cells. The pretreatment with oxidized ATP failed to inhibit the Ca²⁺ transients induced by 10 μM ATP in lymphocytes adherent to endothelium (Figure 3). Parallel experiments confirmed the effectiveness of the oxidized ATP, as it blocked ATP-induced Ca²⁺ influx in lymphocytes attached to glass (results not shown). Thus the lymphocyte P_{2z} purinoceptor is not involved in the ATP- or UTP-induced signalling pathway between endothelium and adherent lymphocytes.

Figure 3 Effect of oxidized ATP pretreatment of lymphocytes on ATP-induced Ca²⁺ transients in lymphocytes adherent to endothelial cells

Lymphocytes were preincubated with 300 μM oxidized ATP at 37 °C for 60 min, washed, loaded with fura 2 and centrifuged on to confluent monolayers of endothelial cells. Changes in intracellular Ca²⁺ levels were measured in single adherent lymphocytes. Similar responses to a 2 min superfusion of 10 μM ATP were observed with both untreated (a) and oxidized-ATP-treated (b) adherent lymphocytes.

Figure 4 Comparison of the effects of ATP and histamine on intracellular Ca^{2+} in single endothelial cells attached to glass (a) and in single lymphocytes adherent to endothelial cell monolayers (b)

In (a) endothelial cells were loaded with fura 2 and changes in intracellular Ca^{2+} levels in single endothelial cells were measured by microfluorimetry. In (b) lymphocytes were loaded with fura 2 and gently centrifuged on to confluent endothelial cell monolayers. ATP (10 μM) and histamine (100 μM) were added as indicated.

Involvement of endothelial P_2 purinoceptors

As the lymphocyte $\text{P}_{2\text{Z}}$ purinoceptor is not involved in the Ca^{2+} transients triggered by ATP or UTP, the possible involvement of endothelial P_2 purinoceptors was studied. Addition of ATP (1–10 μM) and histamine (10–100 μM) both gave major Ca^{2+} transients in fura 2-loaded endothelial monolayers (Figure 4a) in six of six experiments. In parallel experiments carried out on the same day, fura 2-loaded lymphocytes adherent to endothelial cells consistently gave Ca^{2+} responses to ATP but not to histamine (Figure 4b).

Endothelial $\text{P}_{2\text{U}}$ but not $\text{P}_{2\text{Y}}$ purinoceptors are involved

At least two different purinoceptors are present on endothelial cells, $\text{P}_{2\text{U}}$ for which both ATP and UTP are agonists and $\text{P}_{2\text{Y}}$ for which 2-MeSATP and ADP as well as ATP are agonists [10,11]. Figure 5(a) shows that ATP, ADP and UTP (all at 10 μM) triggered Ca^{2+} transients in endothelial cells. Whereas ATP and UTP elicited a Ca^{2+} transient in lymphocytes adherent to endothelial cells, ADP failed to give this response (Figure 5b). A similar result was obtained with 2-MeSATP, addition of this analogue producing a Ca^{2+} transient in endothelial cells but not in lymphocytes adherent to endothelial cells (Figures 5c and 5d).

Extracellular Ca^{2+} is not required for endothelial–lymphocyte signalling

All the previous data were obtained in media containing 1.0 mM Ca^{2+} . The effect of removal of extracellular Ca^{2+} on ATP-induced endothelial–lymphocyte signalling was studied. Figure 6 shows that superfusion with Ca^{2+} -free media did not abolish the Ca^{2+} transient induced by 10 μM ATP in lymphocytes adherent to endothelial cells. Figure 6 also shows that the presence of 0.3 mM Mg^{2+} which was added to the superfusate in this experiment had no effect on ATP-induced Ca^{2+} transients in lymphocytes adherent to endothelial cells.

Lymphocytes detached from endothelial cells are insensitive to low ATP concentrations

Lymphocytes (loaded with fluo 3 and Fura Red) were attached to monolayers of endothelial cells for 2 h at 37 °C. Non-adherent lymphocytes were then removed by thorough washing and discarded. The adherent lymphocytes together with the endothelial cells were harvested. These two cell types were then separated and the cytosolic Ca^{2+} of the detached lymphocytes was measured by flow cytometry. Addition of 10 μM ATP or 10 μM UTP had no effect on the cytosolic Ca^{2+} of these detached lymphocytes. In other experiments, lymphocytes (loaded with fluo 3 and Fura-Red) were incubated for 2 h at 37 °C with either anti-VLA-4 monoclonal antibody or 500 μM CS-1 peptide, which represents a major adhesion domain on fibronectin [19]. Flow-cytometric analysis showed that these cells did not have increased sensitivity to the purinergic agonists, ATP or UTP.

Fixation of endothelial cells abolishes signalling

Monolayers of endothelial cells were treated with the fixative, periodate/lysine/paraformaldehyde, which preserves the adhesive properties of these cells [23]. Fura 2-loaded lymphocytes were then allowed to adhere to the fixed endothelial cells for 2 h at 37 °C. Addition of ATP or UTP (10 μM) did not cause Ca^{2+} transients in lymphocytes adherent to fixed endothelial cells. Thus the ATP/UTP-induced endothelial–lymphocyte signalling described above is dependent on the viability of the endothelial cells.

DISCUSSION

This study shows that very low concentrations of extracellular ATP or UTP (1–10 μM) can trigger an endothelial–lymphocyte signalling pathway leading to the release of Ca^{2+} from internal stores of lymphocytes adherent to endothelial cells. Considerable evidence suggests that this Ca^{2+} transient in adherent lymphocytes is triggered first by the action of ATP or UTP on endothelial cells. Thus Figure 5 shows that ATP or UTP at 10 μM produces Ca^{2+} transients in endothelial cells and Figure 2 shows that the same concentration of nucleotides has no effect on the cytosolic Ca^{2+} concentration of lymphocytes attached to glass. Previous studies have shown that both human and murine lymphocytes possess ATP-operated ion channels (of $\text{P}_{2\text{Z}}$ purinoceptor subclass) which require extracellular ATP in the concentration range 50–500 μM (EC_{50} approx. 100 μM) for opening and cannot be opened at all by extracellular UTP [12–18]. However, our findings exclude any possibility that the lymphocyte $\text{P}_{2\text{Z}}$ purinoceptor is involved in the observed ATP-induced endothelial–lymphocyte signalling. Figure 3 shows that pretreatment of lymphocytes with oxidized ATP, an irreversible inhibitor of the lymphocyte $\text{P}_{2\text{Z}}$ purinoceptor [16,24], had no effect on ATP-induced Ca^{2+} transients in lymphocytes adherent to endothelial cells. In addition, Figure 6 shows that these Ca^{2+} transients induced by

Figure 5 Effect of nucleotide agonists specific for P_{2Y} and P_{2U} purinoceptors on cytosolic Ca²⁺ in endothelial cells (a and c) and in lymphocytes adherent to endothelial cells (b and d)

In (a) and (c), endothelial cells were loaded with fura 2. In (b) and (d) lymphocytes were loaded with fura 2 and allowed to attach to endothelial cell monolayers. The effect of 10 μ M ATP, UTP, ADP and 2-MeSATP on the cytosolic Ca²⁺ levels of single endothelial cells (a and c) or of single lymphocytes attached to endothelial cells (b and d) was measured.

Figure 6 Effect of removal of extracellular Ca²⁺ on ATP-induced Ca²⁺ responses of lymphocytes adherent to endothelial cells

Fura 2-loaded lymphocytes attached to endothelial cell monolayers were superfused first with Hepes-buffered medium containing 1 mM CaCl₂, then with Hepes-buffered medium containing 0.3 mM MgCl₂ in place of CaCl₂, and finally with Hepes-buffered medium containing 1 mM CaCl₂. Addition of 10 μ M ATP (as indicated) gave Ca²⁺ transients in the presence and absence of extracellular Ca²⁺. The addition of 0.3 mM MgCl₂ during the 'Ca²⁺-free' superfusion was to prevent contraction of the endothelial cell monolayer in the absence of Ca²⁺.

10 μ M ATP in adherent lymphocytes did not require the presence of extracellular Ca²⁺, a result that is inconsistent with a direct effect of ATP on a lymphocyte P_{2Z} purinergic ion channel.

A number of nucleotide agonists are known to produce Ca²⁺ transients in endothelial cells and were tested for their ability to give Ca²⁺ transients in adherent lymphocytes. ATP, UTP, 2-MeSATP and ADP all produce Ca²⁺ transients in endothelial cells from a variety of sources [25,26] including HUVECs [27,28]. At least two subclasses of P₂ purinoceptors are involved in these responses, a conclusion based on the differential production of prostacyclin or Ins(1,4,5)P₃ in response to the above four agonists [10,11]. This study confirmed that all four nucleotide agonists gave Ca²⁺ transients in HUVECs and that homologous desensitization [27,28] could be avoided by allowing at least a 4 min 'wash-out' interval between successive additions of the same or different agonists. However, of the four nucleotide agonists above, only ATP and UTP were able to elicit a Ca²⁺ transient in lymphocytes adherent to endothelial cells (Figures 1, 4 and 5). This observation together with the ineffectiveness of 2-MeSATP and ADP suggests that stimulation of endothelial P_{2U} purinoceptors generates the signal that induces a Ca²⁺ transient in adherent lymphocytes.

The exact mechanism by which endothelial cells signal to adherent lymphocytes is still unclear although active metabolism of endothelial cells was necessary, as fixation of endothelial cells abolished signalling. Both ATP and UTP are known to stimulate the release of low-molecular-mass mediators such as prostacyclin and NO which are involved in short-range signalling between endothelial cells and either blood platelets or smooth muscle [29,30]. Although neither prostacyclin nor NO causes Ca²⁺ transients in fura 2-loaded human lymphocytes (J. Chen and G. Jamieson, unpublished work), many other low-molecular-mass

Figure 7 Possible signalling pathways by which agonists of endothelial cell P_{2U} purinoceptors give rise to the transient increase in intracellular Ca^{2+} concentration observed in adherent lymphocytes

Dashed lines indicate known ligand–receptor interactions that appear not to be involved in producing the lymphocyte Ca^{2+} transient. H_1 is the endothelial histamine receptor. Solid lines indicate potential signalling pathways, one of which may involve diffusion of an endothelial second messenger which causes direct stimulation of the lymphocyte. Another signalling pathway may involve interaction of a second messenger with adhesion molecules which indirectly causes a Ca^{2+} transient in the adherent lymphocyte.

mediators may be released on stimulation of endothelial cells. However, attempts to capture released products during UTP stimulation by superfusing endothelial cells with media containing albumin concentrations (20–40 mg/ml both standard and fatty acid-free albumin) were unsuccessful, as subsequent addition of superfusate to fura 2-loaded lymphocytes had no effect on their cytosolic Ca^{2+} . Our data also show that signalling from endothelial cells to adherent lymphocytes was triggered only by ATP and UTP and not by histamine. Histamine gave major and reproducible Ca^{2+} responses in single cells of an endothelial cell monolayer as has been previously reported [31–33]. However, concentrations of histamine as high as 100 μ M failed to elicit Ca^{2+} responses in lymphocytes adherent to endothelial cells (Figure 4). Thus a Ca^{2+} transient in endothelial cells may be necessary but not sufficient for the signalling process. Indeed it is possible that second messengers other than endothelial-cell Ca^{2+} transients are involved in generating the Ca^{2+} response in the adherent lymphocyte (Figure 7). Direct cytoplasmic contact between endothelial cells and adherent lymphocytes can be excluded as a possible explanation, as Ca^{2+} transients produced by histamine, 2-MeSATP or ADP in endothelial cells are not transmitted to adherent lymphocytes.

Another mechanism of endothelial–lymphocyte signalling may be via adhesive-molecule interactions. Lymphocytes in the body undergo continuous recirculation between the blood and tissues [34] and the first step in this process is the adhesion of lymphocytes to vascular endothelial cells. This adhesion involves an array of interactions of which the most important is between the lymphocyte integrin VLA-4 and its respective ligand vascular cell adhesion molecule 1 on endothelium [35,36]. One potential mechanism of endothelial–lymphocyte signalling may involve the adhesive molecules themselves stimulating the phosphoinositide pathway and Ca^{2+} release on binding to their ligands. It is interesting that the P_{2U} purinoceptor itself has in its first

extracellular loop an RGD sequence [37] which may adhere to a counter-receptor on the lymphocyte surface. However, neither an anti-VLA-4 antibody nor engagement of the adhesive domain of VLA-4 with a complementary CS-1 peptide could transduce a Ca^{2+} signal in the B-lymphocytes in this study. Although the second messenger involved in endothelial–lymphocyte signalling has not been identified, our data suggest that stimulation of the endothelial cell P_{2U} purinoceptor is a primary trigger for this cell–cell communication.

We thank Ms. Brenda Huckstepp for typing the manuscript. This work was supported by a grant from the National Health and Medical Research Council of Australia.

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