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ATP-induced endothelium-independent enhancement of lymphatic vasomotion in guinea-pig mesentery involves P_{2X} and P_{2Y} receptors

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1 The present study has investigated mechanisms underlying ATP-induced endothelium-independent enhancement of vasomotion in guinea-pig mesenteric lymphatic vessels.

2 Lymphatic vasomotion, vessel tone and smooth muscle $[Ca^{2+}]_i$ showed similar ATP concentration-response curves.

3 ATP, at 0.1 mM, caused a biphasic increase in tonic $[Ca^{2+}]_i$ and superimposed vasomotion-associated Ca^{2+} transients. All ATP-induced $[Ca^{2+}]_i$ changes were abolished by incubating the smooth muscle with suramin (0.1 mM).

4 α,β-MeATP (0.1 mM) and UTP (0.1 mM) caused similar changes in $[Ca^{2+}]_i$ but the responses to these agonists were smaller than to ATP.

5 The actions of α,β-MeATP (0.1 mM) were inhibited by suramin (0.1 mM) and PPADS (30 μM) but not by reactive blue 2 (30 μM).

6 In the presence of α,β-MeATP (0.1 mM), the increases in tonic $[Ca^{2+}]_i$ and vasomotion-associated Ca^{2+} transients induced by ATP (0.1 mM) were inhibited by U73122 (5 μM), CPA (20 μM) and heparin, whereas U73343 (5 μM) and pre-treatment with PTx (100 ng ml^{-1}) had no significant effects.

7 Depletion of the intracellular stores with CPA (20 μM) caused an increase in $[Ca^{2+}]_i$, which was not blocked by desensitization of P_{2X} receptors with α,β-MeATP.

8 The data indicate that ATP, at relatively high concentrations increases lymphatic smooth muscle $[Ca^{2+}]_i$ and vasomotion through activation of P_{2X1} and P_{2Y2} purinoceptors present on lymphatic smooth muscle. The increase in $[Ca^{2+}]_i$ is likely to result from Ca^{2+} release from inositol-1,4,5-trisphosphate-sensitive stores as well as Ca^{2+} influx through store-operated channels and P_{2X}-gated channels.

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Abbreviations: ACh, acetylcholine; α,β-MeATP, α,β-Methylene ATP; ATP, adenosine 5′-triphosphate; CPA, cyclopiazonic acid; IP_3, inositol-1,4,5-trisphosphate; PLC, Phospholipase C; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; SNP, sodium nitroprusside; SOCs, store-operated channels; UTP, uridine 5′-triphosphate

Introduction

Adenosine 5′-triphosphate (ATP) has been shown to be a sympathetic co-transmitter in lymphatic vessels where it subserves to modulate rhythmic contractile activity, termed vasomotion (Hollywood & Mchale, 1994). Our previous experiments (Gao *et al*., 1999) have indicated that extracellular ATP increases vasomotion in guinea-pig mesenteric lymphatic vessels through both endothelium-dependent and endothelium-independent pathways. The former action has been studied in detail and is mainly due to release of the endothelium-derived excitatory factor thromboxane A_2, whereas the mechanisms underlying the endothelium-independent action of ATP on vasomotion remain unknown.

ATP has been shown to cause or modulate vascular constriction through activation of P_2 purinoceptors, receptors that comprise both ligand-gated P_{2X} ion channel receptors and G protein-coupled P_{2Y} receptors. These receptors have been further classified at the molecular level (Ralevic & Burnstock, 1998) into various subtypes (P_{2X1-7} and $P_{2Y1,2,4,6}$). Activation of P_{2X} receptors leads to an increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) due to a direct Ca^{2+} influx through P_{2X} receptor-operated channels. α,β-MeATP is a specific agonist of P_{2X} receptors. P_{2X1} receptors have been shown to be the primary P_{2X} receptor subtypes expressed in vascular smooth muscle (Vulchanova *et al*., 1996). Binding studies have determined that P_{2X1} receptors have a high affinity for ATP and α,β-MeATP (Fredholm *et al*., 1994). Single channel analysis has shown that these receptors rapidly desensitize in the presence of α,β-MeATP (Surprenant *et al*., 1995; Werner *et al*., 1996). In contrast, at least three P_{2Y} receptor subtypes (P_{2Y2}, P_{2Y4} and P_{2Y6}) have been reported to be present on vascular smooth muscle where they are involved in modulation of vasoconstriction (Erlinge *et al*., 1998; Ralevic & Burnstock, 1998). P_{2Y1} receptors are also present in blood vessels but are mainly expressed in endothelial cells and contribute to ATP-induced vasodilation (Ralevic & Burnstock, 1998). P_{2Y} receptors have variable affinities for purine and pyrimidine

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nucleotides. P_{2Y1} receptors are ATP-specific receptors, whereas P_{2Y2} receptors have equal affinities for ATP and UTP (Lustig *et al*., 1993; Nicholas *et al*., 1996). P_{2Y4} receptors are UTP selective, while P_{2Y6} are UDP selective and are relatively insensitive to suramin (Boarder & Hourani, 1998; Hartley *et al*., 1998; Nicholas *et al*., 1996; Webb *et al*., 1996).

Lymphatic vasomotion has been indicated to arise through pacemaker Ca^{2+} release within the smooth muscle, causing depolarization and triggering Ca^{2+} action potentials (van Helden, 1993; van Helden, *et al*., 1996). The present study uses guinea-pig mesenteric lymphatic vessels to investigate the signal pathways underlying the endothelium-independent enhancement of vasomotion induced by ATP. Measurements of smooth muscle $[Ca^{2+}]_i$ suggest that extracellular ATP, at higher concentrations than required to activate the endothelium (Gao *et al*., 1999), enhances vasomotion of lymphatics by direct activation of P_{2X1} and P_{2Y2} purinoceptors on the smooth muscle. The resultant increases in $[Ca^{2+}]_i$ are due to Ca^{2+} influx through P_{2X1} ligand-gated channels and P_{2Y2}-receptor initiated Ca^{2+} release from IP_3-sensitive intracellular stores following activation of the G protein–PLC-IP_3 cascade. In addition, depletion of intracellular IP_3 receptor-operated stores activates a Ca^{2+} influx that is independent of P_{2X} ligand-gated channels.

Methods

Tissue preparation

Young guinea-pigs (3–10 days) of either sex were killed by an overdose of the inhalation anaesthetic, halothane (5–10% in air) followed by decapitation. Lymphatic vessels (diameter <300 μm) with associated arteries, veins and mesothelium from the ileal region of the intestine were dissected out and pinned flat onto the Sylgard-coated (Dow Corning) base of a small organ bath (volume 1.0 ml). The chamber was continuously superfused at a rate of 6 ml min^{-1} with a physiological saline solution containing (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1 and glucose 10. This solution was maintained at a pH of 7.2 by bubbling with a 95% : 5% O_2 : CO_2 gas mixture and heated to 34–36°C. Perfusion to remove the endothelium and/or to induce vasomotion was effected using a fine glass cannula loosely inserted into the distal end of the lymphatic vessel. The vessel was perfused with a low-calcium physiological saline solution ($CaCl_2$ 1.2 mM) pumped at a rate of approximately 2.5 μl min^{-1}. Tissues were routinely used within 1–4 h of isolation (except for pertussis toxin experiments) or stored from 2–6 h at 4°C in physiological saline until used.

Ratiometric measurement of $[Ca^{2+}]_i$ with Fura 2/AM

Experiments involving measurements of $[Ca^{2+}]_i$ changes in the smooth muscle were performed using the calcium sensing dye Fura 2. The smooth muscle of lymphatics was loaded by luminally perfusing endothelium denuded vessels at room temperature with the membrane permeant Fura 2/AM (2 μM) and pluronic acid (0.2%, w v^{-1}) for 30 min. Continued vessel perfusion for a further 10 min washed out the extraneous dye. The tissue was then left for an equilibration time of at least 20 min, allowing the intracellular esterases to cleave Fura 2/AM (Goldman *et al*., 1990). A metal ring (1 × 1 cm) bearing the Fura 2 loaded lymphatic vessels and associated arteries, veins and mesentery were placed into an organ bath (volume 0.5 ml) mounted onto an inverted microscope (Zeiss Axiovert 10). The tissue was superfused with physiological saline at a rate of 6 ml min^{-1} at 34–36°C and viewed through a ×40 oil immersion objective (NA 1.3). Movement artefacts were minimized by performing the experiments without luminal perfusion. The tissue was alternately illuminated with a xenon lamp at wavelengths of 340 and 380 nm for durations of 50 ms for each wavelength with an interval between wavelengths of 50 ms. This cycle was repeated at a frequency of 1–2 Hz. Emission light was passed through a dichroic mirror (490 nm) and a bandpass filter (510 nm) and collected by a photomultiplier with the output response captured by a computer.

Measurement of vasomotion

The constriction of lymphatic vessels was monitored by measurement of either vessel movement or the transient increase in $[Ca^{2+}]_i$ in the constricting smooth muscle. Vessel movement was monitored using a video camera attached to an inverted microscope (Nikon Diaphot, ×4 magnification) and analysed visually or videoscopically with vessel edges tracked at 25 Hz by a computer-based algorithm (Diamtrack, (Neild, 1989)). System limitations during Ca^{2+} photometry experiments prevented videoscopic edge-detection. However, measurement made at the isosbestic point of Fura 2 either directly at F_{360} (Furuya *et al*., 1994) or by calculating F_{360} from F_{340}/F_{380} data (Chiavaroli *et al*., 1994) allowed detection of vessel movement. We found that this movement correlated with the brief Ca^{2+} transients regularly recorded throughout our experiments. These F_{340}/F_{380} transients had a rapid rise phase of <0.5 s and duration at half amplitude between 0.5–1.0 s, values commensurate with independent measurements of vessel constriction (van Helden, 1993). Constriction was considered to have occurred when a Ca^{2+} transient of this wave shape presented with amplitudes >1.5 standard deviation of the baseline noise.

Confocal microscopy

Confocal imaging was performed using a BIORAD MRC600 or MRC 1000 laser scanning system attached to an inverted microscope (Zeiss Axiovert 10) with a ×40 oil immersion (NA 1.3) or a ×20 water immersion (NA 0.9) objective. Fluo3 or Oregon loaded tissues were excited at 488 nm using an argon ion laser. Emission fluorescence was collected through a 490 nm dichroic mirror and 515 nm bandpass filter. The AM form of the Ca^{2+} sensitive dye Oregon green (5 μM) was loaded into lymphatic smooth muscle by luminally perfusing endothelium-denuded vessels for 30 min.

Functional removal of endothelium

As described previously (Gao *et al*., 1999), The endothelium of lymphatics was removed *in vitro* by repeatedly passing brief streams of air through the lumen of the vessels at a rate of approximately 3 μl min^{-1}. Successful lysis was confirmed by application of acetylcholine (ACh, 100 μM) followed by

sodium nitroprusside (SNP, 100 μM). A negative response to ACh and a positive response to SNP were used as confirmation of successful removal of endothelium (von der Weid *et al*., 1996).

Pertussis toxin incubation

Tissues were superfused for 4 h at 35°C with a physiological saline solution containing pertussis toxin (PTx) (100 ng ml^{-1}) (Burch *et al*., 1986). Experiments to determine PTx sensitivities were performed subsequent to this incubation but in the absence of PTx in the bathing solution.

Heparin loading

Heparin (8 mg ml^{-1}) was loaded into lymphatic smooth muscle cells using a reversible permeabilization procedure (Kobayashi *et al*., 1988). Fluo3 (1 μM) salt was simultaneously loaded with heparin to confirm that the tissue had been reversibly permeabilized.

Experimental protocols

Experiments were routinely performed using a 15 min control perfusion period, a 5 min test period with agonists added to the perfusion media, followed by a 30 min washout period. Results were analysed by recording the vasomotion, measured as lymphangion constriction rates, for 4 min at the end of the initial control period, 1 min after application of the agonist and at the end of washout period. When a pharmacological inhibitor was used, the inhibitor was applied for a total of 20 min (15 min before and 5 min during application of agonist). In the case of reversible inhibitors, the antagonist was removed by a 30 min washout period before ATP was again used. Except where noted, analysis has been based on comparisons of the constriction rates of individual lymphangions with respect to the relevant control rates immediately preceding the test condition, both averaged over a 4 min period.

The Ca^{2+} photometry experiments followed the same experimental protocol. Responses were expressed as either a percentage of the control fluorescence ratio immediately preceding the test solution or as the number of Ca^{2+} transients per minute.

Chemicals

Chemicals used were acetylcholine (ACh), adenosine 5′-triphosphate (ATP), heparin, α,β-methylene ATP (α,β-MeATP), sodium nitroprusside (SNP), uridine 5′-triphosphate (UTP) from Sigma-Aldrich; suramin, cyclopiazonic acid (CPA), 1-(6-((17-beta-methoxyestra-1,3,5(10)trien-17-yl) amino)hexyl)-1H-pyrrole-2,5-dione (U73122), 1-[6-[[(17beta)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2, 5-pyrrolidinedione (U73343) from Biomol Pty Ltd; pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS); reactive blue 2 from Tocris Cookson Ltd; Pertussis toxin and ryanodine from RBI and Fura 2/AM, Fluo 3 and Oregon green/AM from molecular probes. Stock solutions of ACh, ATP, PPADS, reactive blue 2, SNP, suramin, UTP, α,β-MeATP (0.1 M) and Pertussis toxin (100 μg ml^{-1}) were made in distilled water. Stock solutions of CPA, ryanodine (20 mM), U73122, U73343 (5 mM), Fura 2/AM (2 mM) and Oregon green/AM (5 mM) were made in dimethylsulphoxide (DMSO). All stock solutions were stored at -20°C. The final concentration of DMSO used during experiments was always less than 0.1%, a concentration that was found to have no significant effects on lymphatic vasomotion or smooth muscle $[Ca^{2+}]_i$.

Statistical analysis

All constriction frequencies and fluorescence ratios (F_{340}/F_{380}) were normalized by using the following equation:

$$f_N = (f/f_i) \times 100\% \quad (1)$$

where f_N is the normalized value, f is the actual value and f_i is the initial value measured just before application of the test solution. The ATP-induced changes in relative tone (ΔT) were estimated during vasomotion by the following relationship:

$$\Delta T \propto [(D_i - D)/D_i] \times 100\%$$

Where D is the actual diastolic diameter and D_i is the initial diastolic diameter measured just before application of the test solution.

Dose-response curves were fit by a computer-based program (GraphPad prism 2.01) to the following equation:

$$Y = Ymax - \frac{Ymax - Ymin}{1 + \left(\frac{X}{EC_{50}}\right)^{H}} \quad (3)$$

where Y is the response at agonist concentration X, $Ymin$ and $Ymax$ are the minimum and maximum responses obtained, EC_{50} is the concentration giving half-maximal response and H is a Hill coefficient indicating the steepness of the curve.

Data are expressed as the mean$\pm$standard error of mean (s.e.mean). Experiments were performed using at least three tissue preparations. Individual n's are stated specifically. Statistical significance was determined using Student's t-test $P<0.05$ was considered significantly different and $P<0.01$ highly significantly different for all analyses.

Results

Effect of ATP on lymphatic vasomotion

ATP increased lymphatic vasomotion and tone in perfused lymphatic vessels, as indicated by a respective increase in the frequency of constrictions and decrease in resting diameter (Figure 1). Application of random concentrations of ATP ([ATP]) produced a concentration-dependent increase in both constriction frequency (Figure 2a solid curve) and relative tone, as estimated from vessel diameter ($(D_i - D)/D_i$; Figure 2b solid curve). Significant increases in both constriction frequency and tone were first observed at [ATP] of 10 nM ($110\pm3.4\%$ of control, $P<0.05$ and $2.2\pm1.5\%$ of control, $P<0.05$; $n=12$, respectively), with half maximal responses (i.e. EC_{50}) occurring at [ATP] of 17 and 19 μM, respectively (log EC_{50} -4.8 ± 0.3 and -4.9 ± 0.2). The maximum responses occurred near [ATP] of 3 mM with constriction frequency increasing to $207\pm12\%$ of control ($P<0.01$, $n=9$) and relative tone to $41\pm3.7\%$ of control ($P<0.01$, $n=9$).

Removal of the endothelium altered the concentration-response curves (Figure 2a,b, dashed curves), decreasing the maximum responses obtained. The minimum [ATP] required to produce significant increases in lymphatic vasomotion was now significantly higher than for control vessels (i.e. 10 μM *vs* 10 nM), though the respective EC_{50} values for vasomotion and tone of 14 and 19 μM (log EC_{50} -4.9 ± 0.1 and -4.7 ± 0.1) were not significantly different. There was a close link between relative tone and vasomotion in both control vessels (Figure 2c) and endothelium-denuded vessels (Figure 2d).

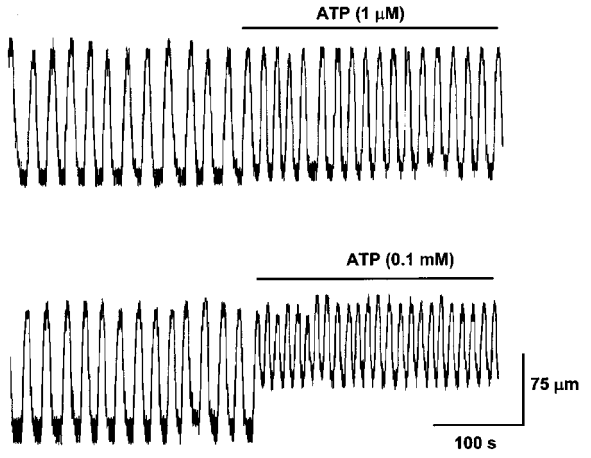

Figure 1 The effects of ATP on lymphatic vasomotion in a perfused vessel. Shown are sample traces of ATP-induced enhancement of lymphatic vasomotion and tone at concentrations of 1 μM (a) and 0.1 mM (b).

Effect of ATP on relative $[Ca^{2+}]_i$ in lymphatic smooth muscle

ATP was applied to non-perfused, endothelium-denuded vessels loaded with Fura-2 to monitor changes in relative $[Ca^{2+}]_i$. ATP (0.1 mM) caused a transient and significant increase in smooth muscle $[Ca^{2+}]_i$ achieving a peak of $154\pm6.4\%$ of control ($P<0.01$), which then gradually decreased to a plateau level. The plateau amplitude, measured 1–1.5 min after application of agonist was $141\pm5.2\%$ of control ($P<0.01$, $n=6$). ATP (0.1 mM) also initiated or enhanced oscillatory Ca^{2+} transients (frequency 0.7 ± 0.3 transients/min before and 9 ± 1.9 transients/min after addition of ATP, $n=6$; Figure 3a). Each transient corresponded to vessel constriction and hence was an indicator of vasomotion (von der Weid *et al*., 2001).

Applications of random [ATP] (0.1 μM–3 mM) caused concentration-dependent increases in smooth muscle $[Ca^{2+}]_i$ with significant responses first detected at an [ATP] of 10 μM (Figure 3b). Comparison of this curve to the corresponding ATP-induced increases in lymphatic constriction frequency and diameter, as obtained in endothelium-denuded vessels (Figure 2), shows close correspondences between both ATP-

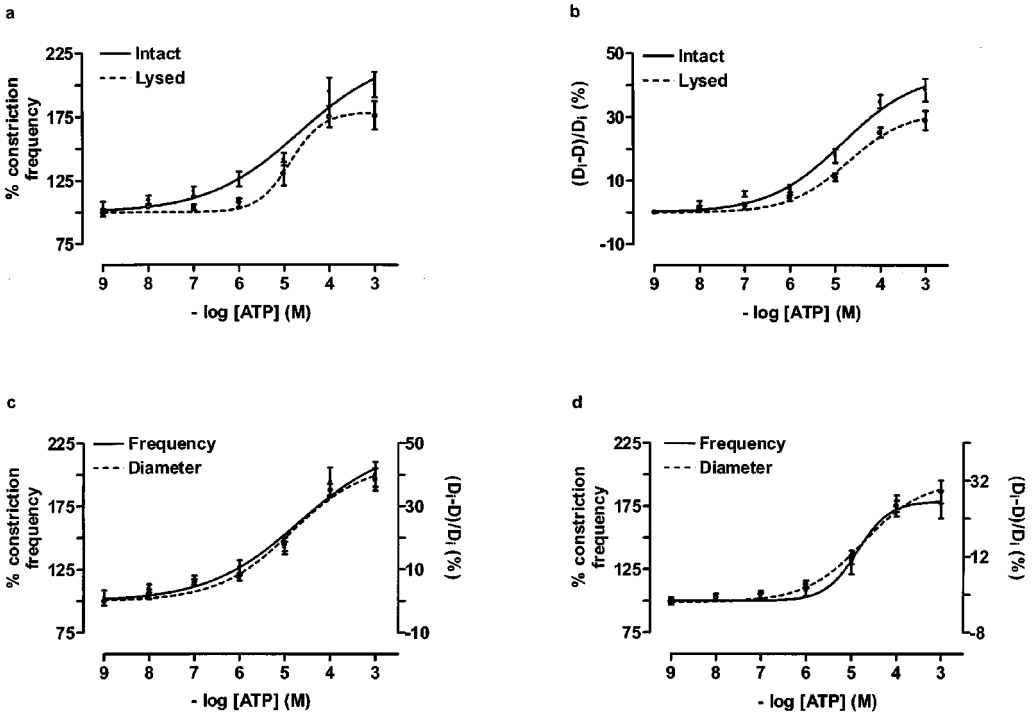

Figure 2 The effects of ATP on lymphatic vasomotion and tone in perfused vessels. (a) Concentration–response curves for ATP at concentrations in the range 1 nM–1 mM on lymphangion constriction frequency (i.e. vasomotion) in vessels with or without an endothelium. (b) The corresponding concentration–response plots of the relative change in vessel tone as reflected by the inverse diameter $[(D_i-D)/D_i]\times100\%$. Comparison of lymphatic constriction frequency (solid curve) and relative tone $[(D_i-D)/D_i]$ (dashed curve) in vessels with (c) and without (d) endothelium. Data were normalized with respect to the corresponding control lymphangion (chambers formed by two adjacent unidirectional valves (Mislin, 1983)) constriction rates or initial diameters (D_i) with $n=9-12$ lymphangions. Vertical lines denote s.e.mean.

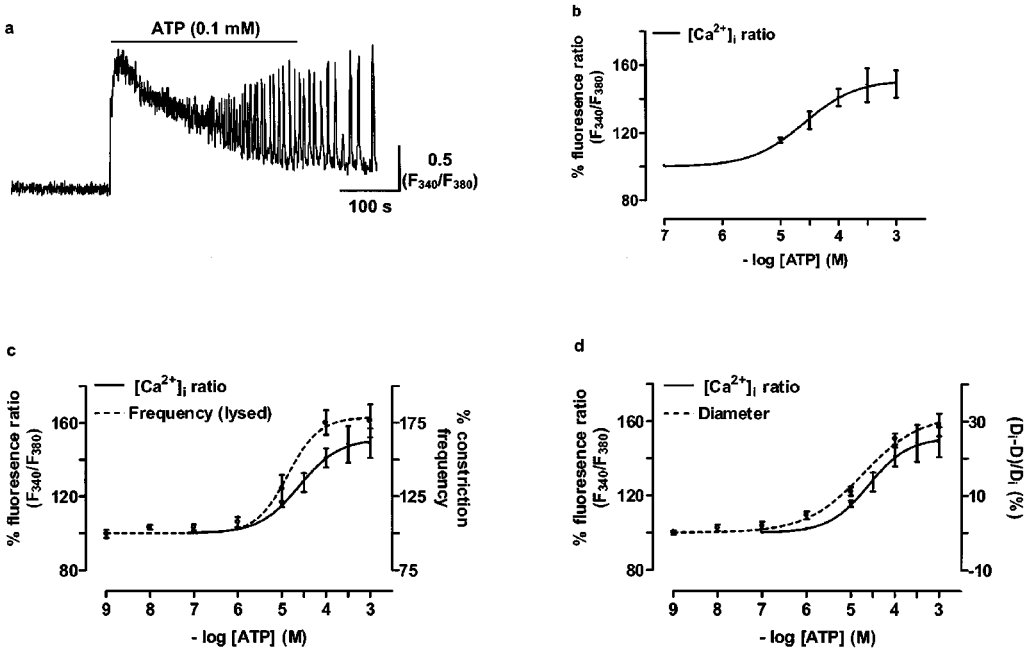

Figure 3 The effects of ATP on relative $[Ca^{2+}]_i$ in non-perfused lymphatic smooth muscle and the relationship to lymphatic vasomotion in perfused endothelium-denuded vessels. (a) Representative trace showing the effect of 0.1 mM ATP on relative $[Ca^{2+}]_i$. (b) The concentration–response curve for the ATP-induced response on smooth muscle $[Ca^{2+}]_i$. (c,d) Comparisons of relative $[Ca^{2+}]_i$ (solid curves) to lymphangion constriction frequency (c, dashed curve) and relative change in vessel tone (d, dashed curve) as reflected by the inverse diameter $(D_i-D)/D_i$. Data were normalized and expressed as a percentage with respect to the corresponding lymphangion constriction rate, initial diameter or fluorescence ratio of F_{340}/F_{380} just before application of ATP with $n=6-12$ lymphangions for all points. Vertical lines denote s.e.mean.

induced increases in $[Ca^{2+}]_i$ and vasomotion (Figure 3c) and relative tone (Figure 3d).

ATP receptor subtypes

Treatment of the tissue with 0.1 mM suramin, a non-specific P_2 purinoceptor antagonist (Dunn & Blakeley, 1988; Hoyle *et al*., 1990) prevented 0.1 mM ATP-induced increases in both tonic $[Ca^{2+}]_i$ ($141\pm8.3\%$ in control and $105\pm3.3\%$ in suramin, $P<0.01$, $n=6$) and the frequency of oscillatory Ca^{2+} transients (8.4 ± 1.4 transients/min in control and 0.8 ± 0.4 transients/min in suramin, $P<0.01$, $n=6$). Suramin itself had no significant effects on resting $[Ca^{2+}]_i$ or the frequency of the oscillatory Ca^{2+} transients ($103\pm4.8\%$ of control and 1.0 ± 0.4 transients/min, respectively; $n=6$, $P>0.05$). Smooth muscle $[Ca^{2+}]_i$ responses to ATP appear to involve contributions from both P_{2X} and P_{2Y} receptors, as indicated by applications of the P_{2X} receptor agonist α,β-MeATP (Burnstock & Warland, 1987; Dunn & Blakeley, 1988) and the P_{2Y} receptor agonist UTP (Nicholas *et al*., 1996; Webb *et al*., 1996). α,β-MeATP (0.1 mM) and UTP (0.1 mM) elicited comparable effects, significantly increasing tonic $[Ca^{2+}]_i$ to $122\pm1.0\%$ ($n=3$, $P<0.01$, Figure 4a,c) and $127\pm3.3\%$ of control ($n=3$, $P<0.01$, Figure 4b,c) and increasing the frequency of oscillatory Ca^{2+} transients to 4.5 ± 1.0 transients/min ($n=3$, $P<0.01$, Figure 4a,d) and 4.1 ± 1.3 transients/min ($n=3$, $P<0.01$, Figure 4b,d) respectively. The time course for the $[Ca^{2+}]_i$ response to α,β-MeATP (0.1 mM) to return to within 10% of baseline levels was 3.5 ± 0.8 min ($n=3$). In addition, α,β-MeATP increased vasomotion-associated Ca^{2+} transients as measured using a confocal laser scanning system (Methods). An EC_{50} of 1.9 μM ($n=3-5$) was obtained from concentration-response measurements (data not shown). The responses in smooth muscle $[Ca^{2+}]_i$ induced by 0.1 mM α,β-MeATP were abolished by 30 μM PPADS ($n=3$) but not by 30 μM reactive blue 2 ($n=3$). In the maintained presence of α,β-MeATP (0.1 mM), ATP (0.1 mM) still caused an increase in smooth muscle $[Ca^{2+}]_i$ and increased the frequency of oscillatory Ca^{2+} transients ($125\pm2.1\%$ of control and 4 ± 1.0 transients/min respectively; $n=4$, $P<0.01$, Figure 5a,c,d). Similar results were obtained when 0.1 mM UTP was applied in the maintained presence of 0.1 mM α,β-MeATP ($122\pm1.5\%$ of control and 4.8 ± 0.9 transients/min respectively; $n=4$). These data indicate that ATP-induced increases in smooth muscle $[Ca^{2+}]_i$ and associated vasomotion are mediated by both P_{2X} and P_{2Y} purinoceptors.

Effects of pertussis toxin

Measurements were made to determine whether PTx-sensitive G proteins were involved in the ATP-induced responses, as P_{2Y} receptors are coupled to G-proteins. Examination of P_{2Y} receptor-mediated responses, studied in the presence of 0.1 mM α,β-MeATP, demonstrated that the ATP-induced increase of smooth muscle $[Ca^{2+}]_i$ and the increase in the frequency of oscillatory Ca^{2+} transients were not abolished by pre-treatment with PTx (amplitude in tonic increase of $[Ca^{2+}]_i$ was $117\pm6.5\%$ of control and the frequency of Ca^{2+} oscillations was 5.0 ± 1.5 transients/min, $n=3$, Figure 5). These increases were not significantly different to the increases of $125\pm2\%$ of control and 4.7 ± 1.2 transients/

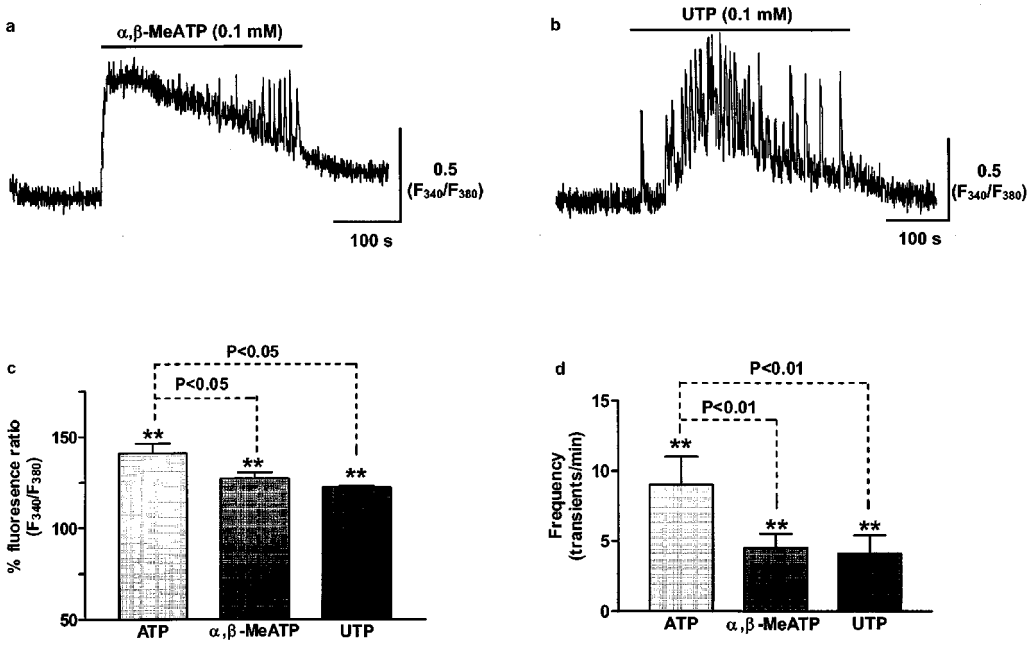

Figure 4 The effects of P_{2X} and P_{2Y} agonists on relative $[Ca^{2+}]_i$ in lymphatic smooth muscle. Sample traces representing the effects of α,β-MeATP (a) and UTP (b) on the F_{340}/F_{380} fluorescence ratio in lymphatic smooth muscle and summary data for these actions compared to the effects of ATP (c,d). Data were normalized with respect to the corresponding fluorescence ratios just before application of either agent. Each measurement is based on data from 3–6 vessels. Vertical lines denote s.e.mean. $^{**}P<0.01$.

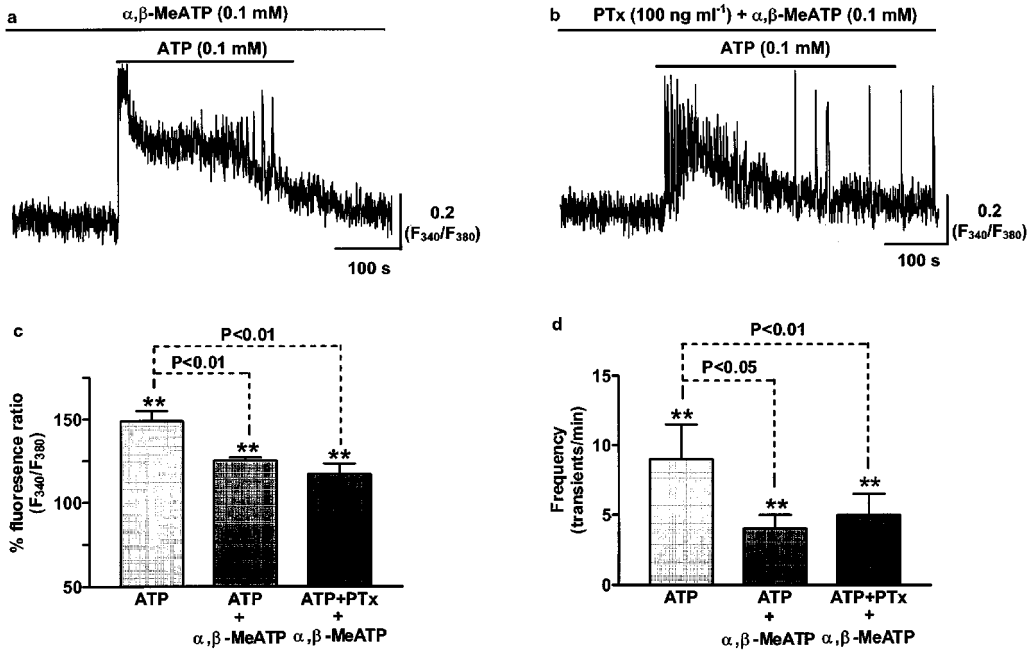

Figure 5 Effects of PTx agonists on relative $[Ca^{2+}]_i$ in lymphatic smooth muscle. Sample traces presenting the effects of ATP on smooth muscle fluorescence ratio (F_{340}/F_{380}) in the presence of α,β-MeATP (a) and α,β-MeATP with PTx (100 ng ml^{-1}, b) and summary data compared to the effects of ATP alone (c,d). Data were normalized with respect to the corresponding fluorescence ratio just before application of ATP or ATP plus other agents. Each measurement represents data from $n=3-6$ vessels. Vertical lines denote s.e.mean. $^{**}P<0.01$.

min measured in matched non-PTx treated tissues ($n=3$). The ability of ATP to cause a response in the presence of α,β-MeATP and PTx indicates that PTx-sensitive G proteins were not involved in the P_{2Y} receptor-mediated increases in smooth muscle $[Ca^{2+}]_i$ and vasomotion.

P_{2Y} receptor-mediated signalling

The signalling pathways underlying the P_{2Y} receptor mediated responses were first investigated by testing the effects of inhibiting the PLC-IP_3 pathway. Treatment of

lymphatic vessels with U73312 (5 μM, $n=3$), a blocker of PLC (Thompson *et al*., 1991), only partially inhibited the ATP-induced increases in smooth muscle $[Ca^{2+}]_i$ and frequency of oscillatory Ca^{2+} transients (Figure 6a,b). However, in the presence of a desensitizing concentration of α,β-MeATP (0.1 mM), 5 μM U73122 completely prevented the ATP-induced responses (Figure 6a,b). This was a PLC specific effect, as U73343 (5 μM), an inactive analogue of U73122 (Bleasdale *et al*., 1990; Thompson *et al*., 1991) had no significant effects on ATP-induced responses in the presence or absence of 0.1 mM α,β-MeATP (Figure 6a,b).

A role for IP_3-sensitive Ca^{2+} stores was further investigated using heparin, a specific antagonist of IP_3 receptors (Kobayashi *et al*., 1988). Heparin (8 mg ml^{-1}) was introduced into smooth muscle cells by a reversible permeabilization procedure (Kobayashi *et al*., 1988). In the presence of heparin, application of ATP (0.1 mM) caused only a sustained increase in $[Ca^{2+}]_i$ without inducing any oscillatory Ca^{2+} transients ($n=6$; Figure 7). In contrast, the biphasic increase in $[Ca^{2+}]_i$ and the frequency of Ca^{2+} transients induced by ATP ($n=6$, Figure 7a) were still evident in control tissues subjected to the same permeabilization treatment but without heparin. In addition, no evidence for an involvement of ryanodine receptors was found, as application of ryanodine (20 μM) had no significant effects on ATP-induced responses in $[Ca^{2+}]_i$ ($n=3$).

Basis for P_2 purinoceptor mediated mobilization of $[Ca^{2+}]_i$

Extracellular Ca^{2+} entry was found to be a primary contributor to the ATP-mediated increases in $[Ca^{2+}]_i$. The effects of omission of Ca^{2+} from the superfusate (Ca^{2+}-free solution with 1 mM EGTA) are shown in Figure 8. The absence of extracellular Ca^{2+} did not block the initial transient increases in $[Ca^{2+}]_i$ induced by application of 0.1 mM ATP, but markedly inhibited the maintained increases in $[Ca^{2+}]_i$ to $101\pm3\%$ of control ($n=6$), a mean value that was not significantly different from baseline levels before addition of ATP ($P>0.05$). These results suggest that the initial transient increase in $[Ca^{2+}]_i$ is predominantly due to release of Ca^{2+} from intracellular Ca^{2+} stores and the

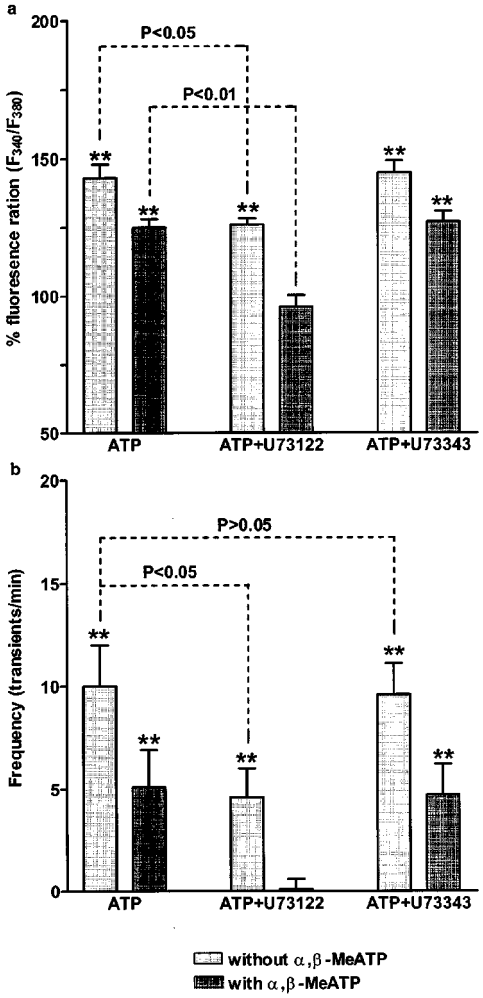

Figure 6 The effects of the PLC antagonist U73122 and its inactive analogue U73343 on ATP-induced responses of smooth muscle $[Ca^{2+}]_i$. Shown are the effects of U73122 (5 μM) and U73343 (5 μM) on the 0.1 mM ATP-induced changes in fluorescence ratio (F_{340}/F_{380}) and oscillatory Ca^{2+} transients (a,b) measured in the absence or presence of α,β-MeATP. All data have been normalized with respect to the control fluorescence ratio just before application of ATP or ATP plus antagonists. $n=3-6$ for all groups. Vertical lines denote s.e.mean. $^{**}P<0.01$.

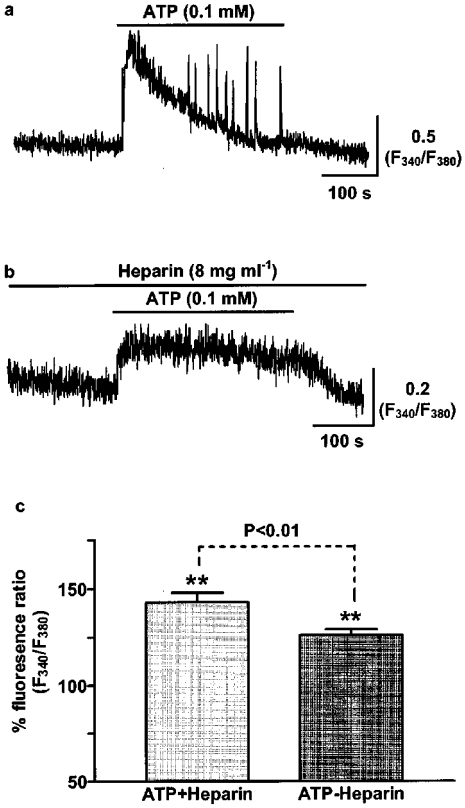

Figure 7 The effects of heparin on ATP-induced increases in relative $[Ca^{2+}]_i$ in lymphatic smooth muscle. Shown are representative traces of changes in the fluorescence ratio (F_{340}/F_{380}) in response to 0.1 mM ATP in lymphatic vessels subjected to permeabilization treatment of tissues without (a) or with (b) heparin in the external solution. (c) Group data for these responses. Data were normalized with respect to the corresponding control fluorescence ratio just before applications of ATP with $n=3-6$ for control and treatment groups. Vertical lines denote s.e.mean. $^{**}P<0.01$.

sustained component is due to influx of Ca^{2+} from the extracellular space.

The sources of Ca^{2+} underlying the ATP-induced $[Ca^{2+}]_i$ increases were also investigated by depleting intracellular Ca^{2+} stores using CPA, a blocker of SR Ca^{2+} ATPase (Darby *et al*., 1993). Incubation of the tissue in CPA (20 μM for 15 min) abolished both the initial transient phase of the ATP-induced increase in $[Ca^{2+}]_i$ and the oscillatory Ca^{2+} transients, consistent with the former response arising from Ca^{2+} release from intracellular stores (Figure 9a). In contrast, CPA significantly reduced, but did not abolish the sustained increase in $[Ca^{2+}]_i$ observed under control conditions (Figure 9a,c). However, in the presence of 0.1 mM α,β-MeATP (Figure 9b,c), CPA blocked the ATP-induced Ca^{2+} increase, indicating that when store function was interrupted, the residual sustained response was due to extracellular Ca^{2+} entry through P_{2X} receptor-gated channels. CPA itself caused a small but significant increase in resting $[Ca^{2+}]_i$ ($121 \pm 3.5\%$ of control, $n=3$, $P<0.01$, Figure 9d), possibly indicating store depletion-induced Ca^{2+} entry through SOCs. This action was not related to Ca^{2+} entry through P_{2X} receptor-gated channels since maintained application of α,β-MeATP (0.1 mM) had no significant effect on the CPA-induced increase in resting $[Ca^{2+}]_i$ (Figure 9d, $n=3$).

These data, together with the findings using U73122 and heparin (Figures 6 and 7), provide evidence that the initial increase in $[Ca^{2+}]_i$ is due to Ca^{2+} release from IP_3-sensitive Ca^{2+} stores and suggest that the maintained increase in $[Ca^{2+}]_i$ is the consequence of Ca^{2+} influx through both P_{2X}-gated channels and through an internal Ca^{2+} store associated pathway, possibly SOCs.

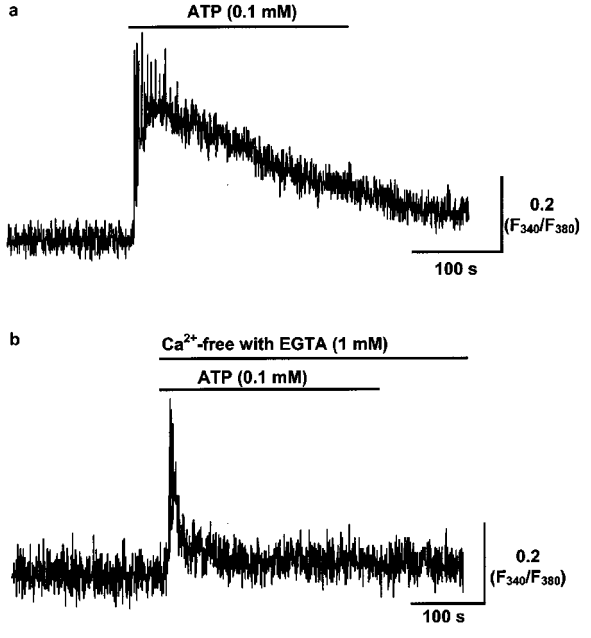

Figure 8 The effect of removal of extracellular Ca^{2+} on the ATP-induced increases in relative $[Ca^{2+}]_i$ in lymphatic smooth muscle. Pre-treatment of the tissue with Ca^{2+}-free solution containing 1 mM EGTA reduced the normal biphasic increase in relative $[Ca^{2+}]_i$ in response to 0.1 mM ATP (a) to a brief transient increase (b).

Discussion

This study investigated pathways by which ATP acts on lymphatic smooth muscle to increase $[Ca^{2+}]_i$ and enhance lymphatic vasomotion and tone. Unlike the previously reported endothelium-dependent effects of ATP (Gao *et al*., 1999), endothelium independent effects occur in response to higher concentrations of ATP (i.e. >1 μM). At these concentrations ATP binds to smooth muscle P_{2X} and P_{2Y} purinoceptors, leading to increases in smooth muscle $[Ca^{2+}]_i$ by Ca^{2+} influx through P_{2X}-gated channels and Ca^{2+} release from IP_3-sensitive stores.

Comparison of the ATP concentration–response curves for vasomotion, vessel tone and smooth muscle $[Ca^{2+}]_i$ in endothelium-denuded lymphatic vessels showed similar dependencies of these three parameters on [ATP]. This finding indicates that $[Ca^{2+}]_i$ is not only a key determinant of vessel tone but also of vasomotion, even though the curves do not perfectly overlie each other. This difference may in part relate to the vasomotion curve here being obtained in perfused vessels and the $[Ca^{2+}]_i$ curve from non-perfused vessels as the behaviour of smooth muscle in perfused vessels exhibits some differences to that observed in none perfused vessels. For example vasomotion in perfused vessels does not desensitise as rapidly as in non-perfused vessels (e.g. compare Figure 1 to Figure 3a). We have presented data on both perfused and non-perfused vessels, the former as this represents one extreme of *in vivo* conditions, when there is massive forward movement of lymph and the latter as it represents the other extreme when there is little movement of lymph. The differences in behaviour between perfused and non-perfused vessels are no doubt complex as the sensitivity of vasomotion to other key agents not dealt with in this paper (e.g. cyclopiazonic acid; Zhao & van Helden unpublished observations) is also different in perfused vessels. This alteration arises in part through perfusion-associated endothelial derived factors, but there seem to be perfusion-associated differences even in endothelium-denuded vessels.

P_{2X} receptors were found to be involved in the ATP action as the P_{2X} purinoceptor agonist α,β-MeATP (Fredholm *et al*., 1994) increased smooth muscle $[Ca^{2+}]_i$ as did ATP, the latter applied with the PLC/IP_3 cascade blocked. Lewis & Evans have found that in rat mesenteric artery smooth muscle the P_{2X1} receptor-activated current is very rapidly desensitized and has an EC_{50} of approximately 1–5 μM (Lewis & Evans, 2000b) (also for review see North & Surprenant, 2000). However P_{2X1} receptor activated constriction of arteries was demonstrated to follow a time course of about 2–5 min (Lewis *et al*., 1998; Gitterman & Evans, 2000; Lewis & Evans, 2000a), similar to the results recorded from our present experiments in mesenteric lymphatics and arteries (3.5 ± 0.5 min, $n=3$, unpublished observations). Further evidence indicating activation of P_{2X1} receptors by ATP in mesenteric lymphatics is provided by the finding that α,β-MeATP increased lymphatic vasomotion with a EC_{50} of 1.9 μM. This is similar to findings in vascular smooth muscle, where the responses have been shown to be P_{2X1}-mediated responses (Gitterman & Evans, 2000).

The sensitivity to α,β-MeATP and the effectiveness of PPADS and suramin but not reactive blue 2 in blocking this pathway further indicate the involvement of the P_{2X1} purinoceptor subtype in activation of lymphatic smooth

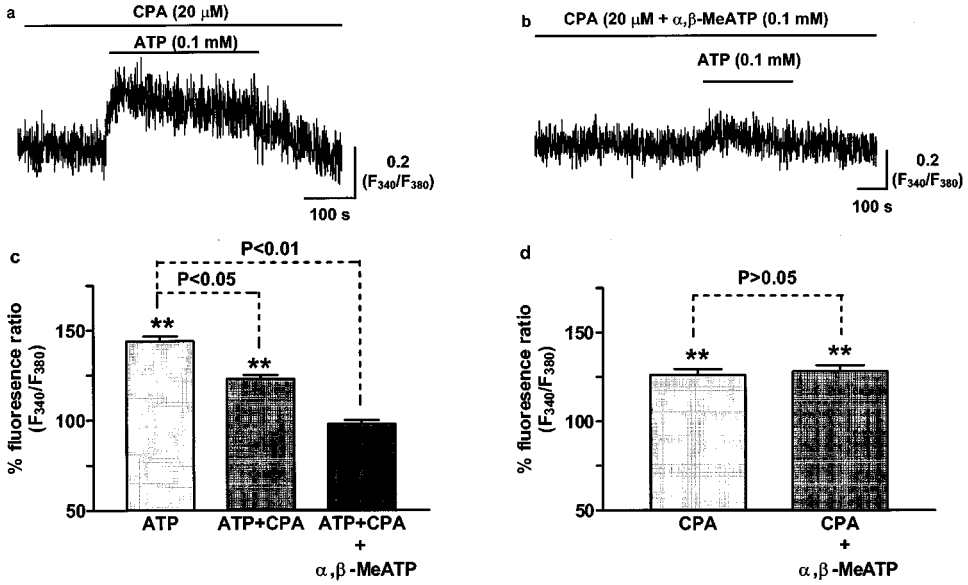

Figure 9 The effects of CPA on ATP-induced increases in relative $[Ca^{2+}]_i$ in lymphatic smooth muscle. Shown are representative traces of changes in the fluorescence ratio (F_{340}/F_{380}) induced by 0.1 mM ATP in the maintained presence of 20 μM CPA in the absence (a) and presence (b) of 0.1 mM α,β-MeATP. Summary data for these responses and for the effects of CPA on resting relative $[Ca^{2+}]_i$ with and without α,β-MeATP present are shown in (c) and (d) respectively. Data were normalized with respect to the corresponding control fluorescence ratio just before application of the various agents with $n=3-6$ for all groups. Vertical lines denote s.e.mean. $^{**}P<0.01$.

muscle by ATP. P_{2X1} receptors are known to be expressed at high levels in the smooth muscle cells of peripheral arteries (Evans & Surprenant, 1992; Vulchanova *et al*., 1996) and can be blocked by PPADS and suramin but not reactive blue 2 (Ralevic & Burnstock, 1998). In addition, all other receptor subtypes except for P_{2X3}, which are expressed in peripheral sensory ganglia, have been found to have low affinities to ATP and are insensitive to α,β-MeATP (Collo *et al*., 1996; Vulchanova *et al*., 1996). P_{2X4} and P_{2X5} have also been reported in smooth muscle (Gitterman & Evans, 2000; Lewis & Evans, 2000a). However they are unlikely to be involved in lymphatic vasomotion as they are relatively insensitive to α,β-MeATP and the P_{2X4} receptor isoform is suramin and PPADS-insensitive (Gitterman & Evans, 2000; Lewis & Evans, 2000a). A distinguishing feature of P_{2X3} receptors, which suggests they do not play a role in ATP activation of lymphatic smooth muscle, is that the receptors do not readily desensitize (Ralevic & Burnstock, 1998). Surprenant *et al*. (2000) have reported $P_{2X(1/5)}$ heterometric receptors in guinea-pig submucosal arteries. However it is unlikely that these receptors are involved in ATP-induced response, as these receptors exhibit a very high sensitivity to ATP (e.g. responses obtained at 3 nM) and have currents that do not desensitize. In contrast, our present experiments have shown that only high [ATP] (>1 μM) produced significant increases in lymphatic smooth muscle $[Ca^{2+}]_i$ which returned to baseline within 5 min.

The finding that ATP-induced increases in lymphatic smooth muscle $[Ca^{2+}]_i$ and vasomotion in vessels in which P_{2X} receptors had been desensitized by α,β-MeATP suggests a role for P_{2Y} receptors in these responses. In addition, responses similar to those induced by ATP were activated by UTP, an agonist of P_{2Y2}, P_{2Y4} and P_{2Y6} purinoceptors. P_{2Y2}, P_{2Y4} and P_{2Y6} purinoceptors have been reported to be present in vascular smooth muscle and there is functional evidence to suggest that they can modulate vasoconstriction (Erlinge *et al*., 1998; Ralevic & Burnstock, 1998). P_{2Y} receptors can be differentiated from each other by their pharmacological properties. UTP has been found to be an agonist of suramin-sensitive P_{2Y2} receptors in rat (Boarder & Hourani, 1998) and suramin-insensitive P_{2Y4} receptors. In contrast, P_{2Y6} receptors are UDP selective and insensitive to suramin (Webb *et al*., 1998). Therefore, on the basis of information presently available for this tissue, P_{2Y2} are the most likely receptor subtype involved in smooth muscle response to ATP. A possible involvement of P_{2Y1} receptors is unlikely as this receptor sub-type is sensitive to ATP but rather insensitive to UTP. In contrast, lymphatic P_{2Y} receptors were found to be equally sensitive to ATP and UTP.

Our previous experiments have shown that the P_{2Y} receptors in lymphatic endothelium exert their role through activation of a PTx-sensitive G protein (Gao *et al*., 1999). In contrast, our present results show that P_{2Y} receptors in lymphatic smooth muscle are PTx-insensitive, indicating the involvement of a different G protein.

The ATP-induced increases in mesenteric lymphatic smooth muscle $[Ca^{2+}]_i$ were found to be biphasic, exhibiting an initial transient followed by a sustained phase where $[Ca^{2+}]_i$ remained elevated. Such biphasic responses to ATP have been observed in a variety of tissues and have been reported to be due to a combination of internal Ca^{2+} release and Ca^{2+} influx. The initial transient of the ATP-induced increase in $[Ca^{2+}]_i$ is likely to be due to IP_3-modulated Ca^{2+} release from the SR as the response was blocked by the PLC inhibitor U73312, by the IP_3 receptor blocker heparin and by the store Ca^{2+} ATPase inhibitor CPA. Similar findings have been reported in vascular smooth muscle (el-Moatassim *et al*.,

1992). The sustained component of the ATP induced increase in $[Ca^{2+}]_i$ was found to be dependent on extracellular Ca^{2+} influx and resulted from P_{2X}-gated influx with a second pathway associated with functional Ca^{2+} stores. Evidence for this was provided by the finding that the sustained phase of ATP-induced increase in $[Ca^{2+}]_i$ was absent when external solutions were effectively free of Ca^{2+}. Part of this response arose through influx of Ca^{2+} through P_{2X}-gated channels as ATP caused a sustained increase in smooth muscle $[Ca^{2+}]_i$ after depletion of the intracellular stores with CPA, although the sustained increase was now significantly smaller. However, the combination of CPA-induced store depletion and desensitisation of P_{2X} receptors abolished the slow phase of the ATP response (Figure 9). Evidence for a sustained source of Ca^{2+} entry second to that resulting from P_{2X} receptors was also provided by the finding that ATP-induced increases in smooth muscle $[Ca^{2+}]_i$ in vessels in which P_{2X} receptors had been desensitized caused a transient and sustained response (Figure 5c). A likely candidate for this store-associated Ca^{2+} entry is SOCs.

The physiological concentration of ATP in human plasma is normally 0.1–1 μM (Forrester & Lind, 1969; Ryan *et al*., 1996). At these levels ATP would modulate lymphatic vasomotion by stimulating the release of both endothelium-derived relaxing and constrictive factors, though predominantly the latter (Gao *et al*., 1999). However, under some pathophysiological conditions (e.g inflammation and shock), the concentration of ATP may increase and reach much higher levels (e.g 0.1 mM) (Gordon, 1986). Such conditions may be particularly relevant to lymphatic function. In conclusion, our data indicates that ATP exerts its effects on smooth muscle by activating both P_{2X1} and P_{2Y2} purinoceptors. The resultant increases in smooth muscle $[Ca^{2+}]_i$, tone and vasomotion are likely to be due to P_{2Y2} receptor-mediated Ca^{2+} release from the SR and Ca^{2+} influx from the extracellular space by P_{2X1}-gated channels and SOCs.

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