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Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26

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Abstract

Calcium signals can be communicated between cells by the diffusion of a second messenger through gap junction channels or by the release of an extracellular purinergic messenger. We investigated the contribution of these two pathways in endothelial cell lines by photoliberating $InsP_3$ or calcium from intracellular caged precursors, and recording either the resulting intercellular calcium wave or else the released ATP with a luciferin/luciferase assay. Photoliberating $InsP_3$ in a single cell within a confluent culture triggered an intercellular calcium wave, which was inhibited by the gap junction blocker α-glycyrrhetinic acid (α-GA), the connexin mimetic peptide gap 26, the purinergic inhibitors suramin, PPADS and apyrase and by purinergic receptor desensitisation. $InsP_3$-triggered calcium waves were able to cross 20 μm wide cell-free zones. Photoliberating $InsP_3$ triggered ATP release that was blocked by buffering intracellular calcium with BAPTA and by applying gap 26. Gap 26, however, did not inhibit the gap junctional coupling between the cells as measured by fluorescence recovery after photobleaching. Photoliberating calcium did not trigger intercellular calcium waves or ATP release. We conclude that $InsP_3$-triggered ATP release through connexin hemichannels contributes to the intercellular propagation of calcium signals.

Keywords: ATP release; Inositol trisphosphate; Calcium signalling; Flash photolysis; Gap junctions; Hemichannels

1. Introduction

Intercellular calcium waves, i.e., cell-to-cell propagating changes of intracellular free calcium, are an ubiquitous form of cell–cell communication observed in cell cultures, slices and retina [1–3]. Intercellular calcium waves can be communicated by an intracellular pathway that involves the passage of a calcium mobilising second messenger like inositol-1,4,5-trisphosphate ($InsP_3$) through gap junctions [4–6], or an extracellular pathway that involves the release of a purinergic messenger like ATP acting on P_{2Y} receptors in a paracrine way [7–12]. The involvement of an extracellular purinergic messenger-dependent pathway has been investigated more directly using bioluminescent ATP measurements, demonstrating ATP release in response to mechanical stimulation [3,10], electrical stimulation [8], the application of UTP [9] or by lowering extracellular calcium [13]. We have previously demonstrated that increasing the $InsP_3$ concentration in a single cell, by photolytic cleavage of its caged form, is a potent stimulus to initiate intercellular calcium waves that, in astrocytes, appear to be mainly carried by the gap junctional pathway [5,14]. The aim of the present work was to determine whether an intracellular elevation of $InsP_3$ or calcium is also able to incite the extracellular/purinergic pathway and thus trigger the release of ATP.

Purinergic messengers like ATP or ADP are long known to be released by vesicular release from dense core vesicles in platelets upon activation [15], a process mediated by $InsP_3$ and calcium [16]. ATP is equally well known to be co-released from neurons together with other neurotransmitters like noradrenaline [17,18]. ATP release from endothelial cells in response to shear stress or hypotonic stress has recently been suggested to be vesicular in nature [19,20]. However, hypotonic stress-induced ATP release from endothelial and/or epithelial cells has also been reported to be mediated by other release pathways [21] including anion channels [22,23], the cystic fibrosis transmembrane conductance regulator (CFTR) [24,25], the multidrug-resistance

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P-glycoprotein [26] or, most recently, by means of so called connexin hemichannels, i.e., channels composed of six connexin subunits that do not connect to their counterparts in adjacent cells [9,11,13,27,28].

In the present work we investigated the ability of $InsP_3$ and calcium to trigger purinergic messenger release in several cell lines of endothelial origin using photoliberation of these two messengers. Our experiments on calcium wave propagation and direct measurements of released ATP demonstrate that $InsP_3$ and the subsequent calcium increase are able to trigger the release of ATP that is largely inhibited by the connexin mimetic peptide gap 26. Connexin mimetic peptides interact with the extracellular loops of the connexin subunit and some of them, like gap 27, have been demonstrated to be effective blockers of gap junction channels and thus gap junctional coupling [29]. Interestingly, our results show that gap 26 does not influence gap junctional dye coupling but has a drastic inhibitory effect on ATP release, suggesting a role for connexin hemichannels in the release process.

2. Materials and methods

2.1. Cell cultures

In this study we used two rat brain endothelial cell lines: RBE4 [30] (kind gift of Dr. F. Roux, Neurotech, Evry, France) and SV-ARBEC (kind gift of Dr. D. Stanimirovic, Institute for Biological Sciences, NRC-CNRC, Ottawa, Canada), and the cell line ECV304 (European Collection of Animal Cell Cultures, Salisbury, UK). ECV304 cells, originally identified as endothelial cells from human umbilical vein [31], have now been shown to be epithelial bladder cancer cells [32]. ECV304 cells are interesting in that they have a purinergic receptor pharmacology that is similar to primary brain capillary endothelial cells [33–38] and they develop tight junctions and a low permeability barrier in co-culture with glial cells making them useful in blood–brain barrier models [39,40]. Clone 9 cells (European Collection of Animal Cell Cultures, Salisbury, UK) contain connexin 43 as the major connexin [41] and were used as a positive control for the immunoblottings. Culture media (all from Gibco, Merelbeke, Belgium) were alpha-MEM/Ham's F10 (1:1) with 10% foetal bovine serum (FBS), 1 μg/ml bFGF (Boehringer Mannheim, Brussels, Belgium) and 30 mg/ml geneticin (Gibco, Merelbeke, Belgium) for RBE4, Medium-199 with 10% FBS, 100 mg/l peptone (Sigma, Bornem, Belgium), BME amino acids (Sigma, Bornem, Belgium) and BME vitamins (Gibco, Merelbeke, Belgium) for SV-ARBEC and Medium-199 with 2 mM glutamine and 10% FBS for ECV304, and DMEM/Ham's F12 (1:1) with 2 mM glutamine and 10% FBS for clone 9. Cells were grown (except for immunoblotting) on glass bottom microwells (MatTek Corporation, Ashwood, MA, USA) coated with collagen type I (Boehringer Mannheim, Brussels, Belgium) and used for experiments upon confluency.

2.2. Agents

Fluo-3 acetoxymethyl ester (fluo-3 AM), D-*myo*-inositol-1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester trisodium salt (NPE-caged $InsP_3$), *o*-nitrophenyl EGTA acetoxymethyl ester (NP-EGTA-AM), BAPTA acetoxymethyl ester (BAPTA-AM), DMNB-caged fluorescein dextran (3000 MW), tetramethylrhodamine dextran (10,000 MW) and 5-carboxyfluorescein diacetate (5-CFDA) were obtained from Molecular Probes (Leiden, The Netherlands). Probenecid, apyrase grade III, 18-α-glycyrrhetinic acid (α-GA), pyridoxal phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS) and adenosine 5′ trisphosphate disodium salt (ATP) were from Sigma (Bornem, Belgium). Suramin hexasodium salt was from RBI (Natick, MA). The peptides gap 26 (VCYDKSFPISHVR) and des 5 (LEGHGDPLHLEEC) [42] were synthesised by the Biochemistry Department of Ghent University as a lyophilised compound at 95% purity.

2.3. Western blotting

Proteins were extracted from cells by treatment with LDS buffer (Invitrogen, Groningen, The Netherlands) containing 50 μg/ml proteinase inhibitor cocktail (Sigma, Bornem, Belgium) and sonicated for two 10 s pulses. Total protein was determined with the BioRad (Nazareth, Belgium) DC protein assay. Proteins were separated on 10% Bis Tris gels and transferred to nitrocellulose membranes. Blots were probed with polyclonal rabbit anti-rat connexin 43 antibody (Sigma, Bornem, Belgium) (1:10,000) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, Bornem, Belgium) (1:5000) and detection by the ECL chemiluminescent detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.4. Calcium imaging

Cytoplasmic free calcium was measured using the calcium-sensitive dye fluo-3 in combination with epifluorescence video microscopy and digital imaging. Cell cultures were loaded with fluo-3 for 1 h at room temperature in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS–HEPES, pH 7.4) containing 10 μM fluo 3-AM (Molecular Probes, Leiden, The Netherlands) and 1 mM probenecid. Cultures were then washed with HBSS–HEPES and left at room temperature for 30 min for de-esterification. HBSS–HEPES was the bathing solution for all calcium imaging experiments Cells were viewed with an inverted epifluorescence microscope (Nikon Eclipse TE300, Analis, Ghent, Belgium) using a 40× oil immersion lens (CFI Plan Fluor, Nikon). Fluo-3 fluorescence images were obtained by excitation at 485 nm, reflection of a dichroic mirror with cut-off at 510 nm, and emission bandpass filtering at 535 nm (485DF22, 505DRLPXR and 535DF35 filters, respectively from Omega Optical, Brattleboro, VT). Images were captured using an intensified CCD (Extended Isis

camera, Photonic Science, East Sussex, UK) and stored on an S-VHS videorecorder (Panasonic, Avicom, De Pinte, Belgium) or directly to a PC equipped with an image acquisition and processing board (DT3155, Data Translation, Marlboro, MA). Each experiment was concluded by recording background images in a culture dish containing only medium and no cells; background images were subtracted from the fluo-3 fluorescence image sequences.

2.5. Caged compound loading, electroporation and UV spot illumination

The cells were loaded with caged $InsP_3$ by electroporation. Cultures were briefly rinsed with electroporation buffer (300 mM sorbitol, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1 mM $MgCl_2$ and 2 mM HEPES, pH 7.20) and thereafter a small volume (5 μl) of caged $InsP_3$ (200 μM) in electroporation buffer was added. This solution contained in addition dextran-rhodamine (100 μM) to visualise the electroporation zone. Electroporation was done with a parallel wire electrode (Pt-Ir, wire separation 0.8 mm, length 10 mm) positioned close to the cell surface (200 μm) and to which an oscillating high-frequency electric field was applied (20 repeats of ten 50 kHz bursts of 10 ms duration at 400 V/cm field strength). The electroporation solution was then removed and the culture was washed three times with HBSS–HEPES. The zone of directly electroporated cells was, as estimated from the zone of dextran-rhodamine loaded cells, approximately 1 mm wide and 10 mm long. Caged $InsP_3$ (635 MW) can permeate through gap junctions and the distance to which significant caged $InsP_3$ spread occurred was determined by applying UV spot illumination (see below) at various distances away from the border of the dextran-rhodamine loaded zone and measuring the size of the calcium wave triggered in this way. The calcium wave size sharply decreased with the distance from the border of the dextran-rhodamine loaded zone and no waves could be triggered at approximately 150 μm away from the border. Caged $InsP_3$ thus spreads out from the electroporated zone, making the total zone of caged $InsP_3$ loading to be in the order of 1.3 mm × 10 mm.

Caged calcium loading was done by ester loading the cells with 5 μM NP-EGTA-AM for 10 min in 1 ml HBSS–HEPES containing probenecid (1 mM), followed by 30 min de-esterification (all at room temperature).

UV spot illumination for photorelease in calcium imaging experiments was performed with an Hg-arc lamp coupled to the microscope epifluorescence input through a dichroic mirror with cut-off at 400 nm (400DCLP02, Omega Optical, Brattleboro, VT) and focused to the field diaphragm plane by means of two plano-convex fused silica lenses (SPX046 and SPX049, Newport, Leuven, Belgium). The UV beam was bandpass filtered at 330 nm (330WB80, Omega Optical, Brattleboro, VT) and the exposure time (0.2 s) was controlled by a mechanical iris diaphragm shutter (Wollensak, Rochester, NY). The UV spot had a half-energy diameter of 10 μm, as determined by flashing a thin layer of DMNB-caged fluorescein dextran at 3 mM mixed (1:1) with Dako Glycergel (Dako Corporation, Carpinteria, CA). Regular inspection of the UV spot was done by illuminating a thin layer of Lucifer Yellow solution (as illustrated in Fig. 1H). To determine whether the UV spot only covered a single cell, we overloaded the cultures with NP-EGTA (10 μM NP-EGTA-AM for 60 min), resulting in heavy calcium buffering in all the cells loaded because of the high calcium affinity of NP-EGTA in its caged non-photolysed form. UV exposure liberates calcium only in the illuminated cell, while non-illuminated cells cannot display any propagated calcium signal because of effective buffering by non-photolysed NP-EGTA. Applying this kind of protocol showed that the photolytic UV light was only directed to a single target cell. Electroporation loading and flash photolysis are described in further detail in Leybaert and Sanderson [43].

2.6. Cell-free zones and mechanical cell stimulation

Cell-free lanes were brought about by linearly scratching the cell cultures over approximately 10 mm with a thin syringe needle 4 h before the actual experiment. The same procedure was used to elicit ATP release, as a positive control condition, in the experiments where ATP was measured (see below). Mechanical stimulation of a single cell was used in the experiments where the side effect of loading with NP-EGTA on cytoplasmic calcium buffering was tested. This was done by gently touching the cell with a glass micropipette (2 μm tip size) mounted on a micromanipulated piezoelectric device and driven to produce a short (100 ms) tip displacement over approximately 5 μm.

2.7. Fluorescence recovery after photobleaching (FRAP)

Gap junctional coupling was investigated in ECV304 cells using the FRAP technique. Confluent monolayer cultures were loaded with the gap junction permeable fluorescent tracer 5-CFDA (25 μM) for 30 min at room temperature and kept 10 min in HBSS–HEPES without 5-CFDA. Cultures were then transferred to the microscope stage and fluorescence within a single cell was photobleached by spot exposure to 500 nm light (500DF50, Omega Optical, Brattleboro, VT) over a period of 10 s. The set-up for spot illumination was the same as the one used for flash photolysis, except for the bandpass filter (500 instead of 330 nm) and a 50/50 beamsplitter (XF121, Omega Optical, Brattleboro, VT) replacing the dichroic. Recovery of fluorescence through dye influx from neighbouring cells was quantified at 9 min after the start of photobleaching. A correction was made for spontaneous bleaching that occurred because of continuous exposure to the 485 nm excitation light. This was done by recording the fluorescence profile in cells not exposed to the 500 nm spot bleach and correcting the recovery signal in proportion to the spontaneous fluorescence decay.

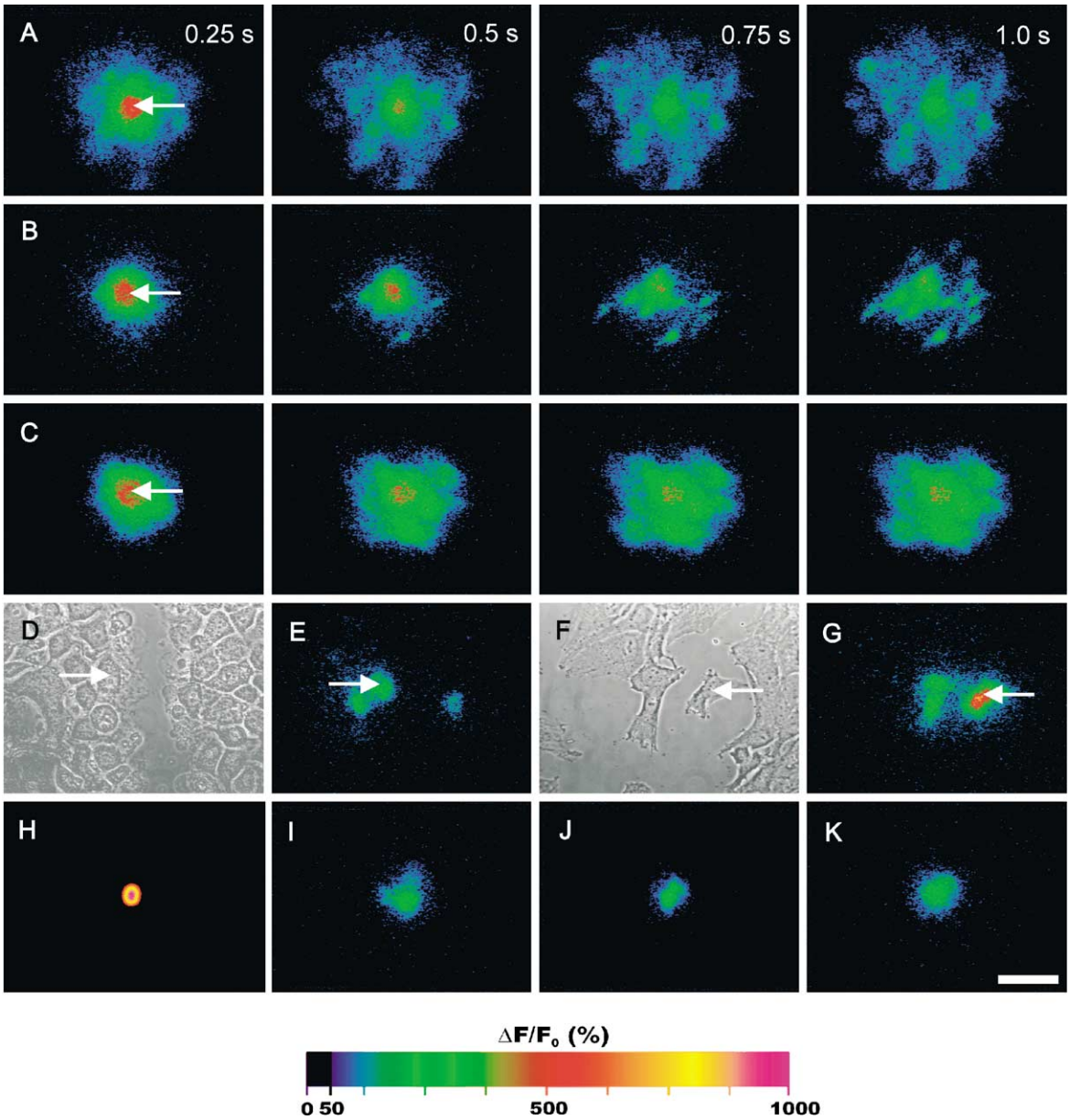

Fig. 1. $InsP_3$-triggered cell-to-cell propagating calcium signals. (A–C) Time sequences of intercellular calcium waves triggered by photoliberation of $InsP_3$ in confluent cultures of ECV304 (A), RBE4 (B) and SV-ARBEC (C). The images depict relative fluo-3 fluorescence changes ($\Delta F/F_0$) colour coded as indicated in the colour bar. The numbers in the right corner of each panel indicate the time after the start of photostimulation. The arrows indicate the position of the UV photostimulation spot. (D–E and F–G) $InsP_3$-triggered calcium signals propagate across cell-free zones. Photoliberation of $InsP_3$ in a single cell (arrow) at the left side of the cell-free lane induced calcium changes in a cell at the opposite side of the lane (ECV304). Photoliberation of $InsP_3$ in a single isolated cell (arrow) in a subconfluent culture triggered calcium changes in a neighbouring cell (SV-ARBEC). D and F are phase contrast images. (H) Image of the photostimulation spot (half-energy diameter approximately 10 μm). (I–K) Photolytic release of caged calcium (NP-EGTA) triggered a calcium increase in the stimulated cell but did not induce calcium changes in surrounding cells (ECV304 in I, RBE4 in J and SV-ARBEC in K). The calibration bar in panel K measures 50 μm and applies to all panels.

2.8. Extracellular ATP measurements

ATP release in response to photoliberation of $InsP_3$ or calcium was determined in ECV304 cultures using an ATP bioluminescent luciferin/luciferase assay kit (product no. FL-AA, Sigma, Bornem, Belgium). The photolytic UV light in these experiments was applied as a *field* illumination (not spot illumination) exposing multiple cells. To that purpose, light from an Hg-arc lamp with quartz collector lens was directed through a shutter, a 330 nm bandpass filter and a mirror (10D510AL.2, Newport, Leuven, Belgium) to form an image of the arc at the cell culture (covering an area of approximately 6 mm × 8.0 mm). The total energy dose per unit of surface area applied in UV field illumination experiments was adjusted so that it was equal to the energy dose per unit of surface in the spot illumination experiments. This

was done by adjusting the UV exposure time so that the fluorescence increase brought about by photoreleasing fluorescein from thin layers of caged fluorescein dextran (prepared as described above) was the same in both spot and field illumination set-ups. The UV exposure time for field illumination was 2 s determined in this way. The photolytic efficiency with this exposure time was in the order of the quantum efficiency of caged $InsP_3$ (0.65), indicating that all of the caged probe present in the cultures was effectively photocleaved upon a single field illumination exposure.

Immediately after UV exposure, 100 μl of 200 μl supernatant was collected from the cell cultures and transferred to 100 μl ATP assay mix solution (used at 5-fold dilution). Light emission from the 200 μl mix was then measured with a custom build luminometer consisting of a photomultiplier tube (9924B, Thorn-Emi Electron Tubes, Middlesex, UK) mounted in a light-tight housing with a sample holder. Luminescence was quantified as photon counts within a time window of 100 ms and average photon count values were calculated from 100 repeated measurements on the same specimen (10 s measurement time per sample). Mechanical movement of the cell cultures during manipulation was strictly minimised as small shocks to the cultures or solution changes produced by themselves measurable ATP release [26]. Calibration curves with ATP were performed in a concentration range of 50–1000 nM. The pharmacological agents used in the ATP measurement experiments were tested for side effects on the luciferin/luciferase assay, but none of them had any significant effect on the ATP-calibration curves.

To make the experimental conditions of the different cell culture groups comparable, all cultures received UV field illumination, which was applied just before collection of the supernatant. Baseline (non-triggered, spontaneous) ATP release was slightly but not significantly affected by exposure to the photolytic UV light (100% without illumination versus 88 ± 15% with UV illumination; $n = 7$; $P = 0.23$). A group of cell cultures received a linear scratch (applied as described above), which served as a positive control condition because scratching the cultures liberates ATP from destroyed cells. The scratch was applied immediately after UV illumination and just before collection of supernatant. NP-EGTA loading and caged $InsP_3$ loading was done as described above (ester loading and electroporation loading respectively). Cell loading with the calcium buffer BAPTA was done before electroporation loading with caged $InsP_3$, by ester loading with 25 μM BAPTA-AM for 45 min in HBSS–HEPES containing probenecid (1 mM), followed by 30 min de-esterification (all at room temperature). Alpha-GA and gap 26 were applied after electroporation, for the time and concentrations indicated in Section 3, and were removed just before photolytic illumination.

It should be considered that in the ATP release experiments some photoliberated $InsP_3$ will diffuse, through gap junctions, to cells beyond the borders of the caged $InsP_3$ loaded zone, thereby extending the zone of cells potentially contributing to ATP release and introducing a gap junction-dependent component to the $InsP_3$-triggered ATP release. The extent of $InsP_3$ spread over the culture via gap junctions is expected to be comparable to the extent of caged $InsP_3$ spread (both have similar molecular weights, 437 and 635, respectively), which was determined to be in the order of 150 μm as described above. Such spread will occur at both lateral sides of the caged $InsP_3$ loaded zone (measuring 1.3 mm × 10 mm). The gap junction-dependent component in the $InsP_3$-triggered ATP release signal will derive from this diffusion zone and its relative contribution to the $InsP_3$-triggered ATP release signal was calculated to be in the order of 20–25%. This means that the inhibitory effect of the gap junction blocker α-GA on $InsP_3$-triggered ATP release per se will be overestimated by a similar percentage.

2.9. Data analysis and statistics

Calcium changes are expressed as relative fluo-3 fluorescence changes, i.e., $\Delta F/F_0 = [F - F_0]/F_0$ with F_0, the fluorescence before stimulation and F, the time-dependent fluorescence signal after stimulation.

The magnitude of $\Delta F/F_0$ changes triggered by photorelease of $InsP_3$ or calcium in the photostimulated cell was determined 2.5 s after the end of the illumination artefact. The extent of cell-to-cell propagation of calcium changes was determined from the size of the intercellular calcium wave at its maximal state of extension and was quantified by determining the surface area where $\Delta F/F_0$ was above a threshold of 50%. Average $\Delta F/F_0$ changes for the whole calcium wave were calculated as the average of all above threshold pixel values.

The data are expressed as mean ± S.E.M. with n denoting the number of experiments. Statistical significance was tested using a t-test for unpaired observations with $P < 0.05$. Multiple groups were compared using variance analysis and the Student–Newman–Keuls test.

3. Results

3.1. Photoreleasing $InsP_3$ triggers intercellular calcium waves

Photoreleasing $InsP_3$ in a single endothelial cell triggered a transient increase of cytoplasmic free calcium propagating to neighbouring cells as an intercellular calcium wave (Fig. 1A–C). Application of the photolytic UV light to cultures not loaded with caged $InsP_3$ did not trigger any calcium change. At their maximal extent of propagation, the induced calcium waves had an above threshold ($\Delta F/F_0 >$ 50%) surface area of 4836 ± 409 μm^2 ($n = 50$) for RBE4, 6982 ± 375 μm^2 ($n = 55$) for SV-ARBEC and 7595 ± 341 μm^2 ($n = 29$) for ECV304 (RBE4 wave size significantly smaller than SV-ARBEC and ECV304 wave sizes [$P < 0.0001$]; SV-ARBEC not different from ECV304). There were no significant differences between the cell lines

in the magnitude of the average calcium increase taken over all cells contributing to the calcium wave ($\Delta F/F_0$ averaged over all cells at maximal wave extension amounted to 111.0±2.0% [$n = 134$]; pooled data for all three cell lines).

$InsP_3$-triggered intercellular calcium waves were able to cross cell-free zones (19.0 ± 2.6 μm wide [$n = 10$]) (Fig. 1D–G). This indicates that the wave propagation mechanism also contains an extracellular, non-gap junction dependent component, as has been demonstrated for mechanically triggered calcium waves [8–10,12,22]. We investigated the mechanisms of $InsP_3$-triggered intercellular calcium waves using pharmacological agents.

3.2. Alpha-GA and gap 26 reduce the size of $InsP_3$-triggered intercellular calcium waves

The contribution of the intracellular/gap junctional pathway to calcium signal propagation was studied with the gap junction blocker α-GA and the connexin mimetic peptide gap 26, a peptide that mimics a 13 amino acid sequence on the first extracellular loop of connexin 43 [42]. Connexin 43 immunoreactivity was present in the three cell lines used as evidenced by Western blotting (Fig. 2). Alpha-GA (50 μM, 30 min) reduced the wave size in the three cell lines (Fig. 3A) but the effect was the most pronounced in RBE4 cells (approximately 60% reduction). The effect of gap 26 (0.25 mg/ml, 30 min) more or less paralleled the effects of α-GA except in ECV304 where its inhibitory influence was stronger (approximately 60% reduction) as compared to α-GA. The control peptide des 5, which mimics a 13 amino acid sequence on the intracellular and thus inaccessible loop of connexin 32 [42], had no effects on the size of intercellular calcium waves (not shown).

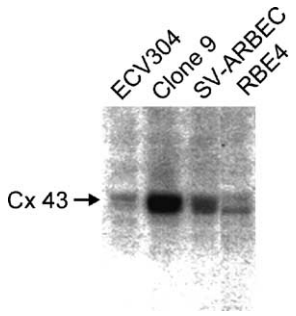

Fig. 2. Immunoblotting of connexin 43 in the different cell lines used. The second lane (clone 9; liver epithelial cells) represents the positive control condition. Connexin 43 expression could be detected in all the cell lines used, but at lower levels as compared to clone 9.

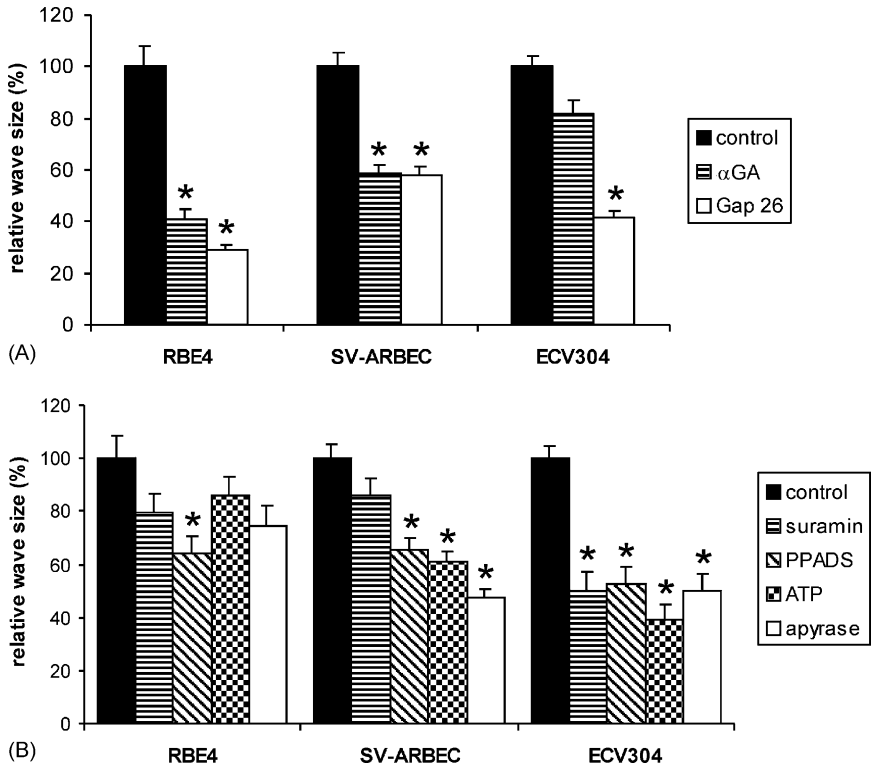

Fig. 3. Effects of gap junction blockers and purinergic inhibitors on intercellular calcium wave propagation. (A) Effects of the gap junction blocker α-glycyrrhetinic acid (α-GA; 50 μM, 30 min) and the connexin mimetic peptide gap 26 (0.25 mg/ml, 30 min) on the calcium wave size (surface area) expressed relative to the control condition. (B) Effects on the calcium wave size of the purinergic receptor antagonists suramin (100 μM, 10 min) and PPADS (10 μM, 10 min), of receptor desensitisation with ATP (10 μM, 10 min) and of ATP/ADP degradation by the enzyme apyrase (40 IU/ml, 10 min). *Significantly below control ($P < 0.01$).

Table 1
Effects of gap junction blockers and purinergic inhibitors on gap junctional communication studied with FRAP

Experimental condition	Fluorescence recovery (%), mean ± S.E.M. (*n*)
Control	20 ± 5 (11)
α-GA	7 ± 2[a] (10)
Gap 26	21 ± 3 (11)
Suramin	17 ± 7 (11)
PPADS	18 ± 3 (8)
Apyrase	26 ± 6 (8)

Alpha-GA significantly reduced fluorescence recovery, while gap 26 had no effect. Suramin, PPADS and apyrase did not significantly affect fluorescence recovery, indicating that these substances acting on the purinergic pathway have no side effects on the gap junctional pathway.
[a] Significantly below control ($P < 0.05$).

Alpha-GA and gap 26 were tested for their effects on gap junctional coupling using the fluorescence recovery after photobleaching (FRAP) technique on 5-CFDA labelled ECV304 cells [44]. Alpha-GA significantly reduced the gap junctional coupling between the cells, while gap 26 had no effects (Table 1). Prolonging the incubation period with gap 26 to 2 h did neither inhibit the gap junctional coupling. The control peptide des 5 had no effects on coupling as expected (not shown). In order to rule out the possibility that α-GA or gap 26 inhibited the extracellular/purinergic pathway, we checked whether these agents influenced calcium transients triggered by ATP superfusion (10 μM ATP, applied 1 min). The two compounds had no significant effects on the peak amplitude of ATP-triggered calcium transients in ECV304 cells ($\Delta F/F_0 = 138.4 \pm 8.4\%$ in control, $120.0 \pm 13.4\%$ with α-GA and $137.8 \pm 12.2\%$ with gap 26; $n = 6$ for each), indicating that these agents do not interfere with the cascade downstream from ATP release. The influence of these agents on the primary event in the extracellular/purinergic pathway, namely the release of ATP by the cells, was also studied and the results of these experiments are shown below.

3.3. Purinergic inhibitors also reduce the size of $InsP_3$-triggered intercellular calcium waves

The contribution of the extracellular/purinergic pathway was investigated with the purinergic receptor antagonists suramin (P_{2Y} and P_{2X} blocker) and PPADS (non-specific P_{2Y} blocker), the purine degrading enzyme apyrase and receptor desensitisation with ATP. Suramin (100 μM, 10 min), PPADS (10 μM, 10 min), apyrase (40 IU/ml, 10 min) and ATP (10 μM, 10 min) all decreased the extent of wave propagation in the three cell lines but the potency of this effect varied between the cell lines used (Fig. 3B). The effect was most pronounced in ECV304 cells (approximately 50% reduction) and the least pronounced in RBE4 (approximately 25% reduction). Exposing ECV304 cells to the combination of α-GA, PPADS and apyrase (concentrations and exposure time as mentioned above) significantly reduced the calcium wave size to $34.9 \pm 4.3\%$ of the 100% control size ($n = 29$; $P = 0.0001$; the remaining response comprised approximately 5 ± 1 cells, meaning that only the cells immediately adjacent to the stimulated cell contributed).

To rule out the possibility that some of the purinergic inhibitors acted directly on the intracellular calcium signalling cascade downstream from the purinergic receptor, we investigated whether these substances influenced calcium transients triggered by photorelease of $InsP_3$. None of the agents used had a significant effect on the peak amplitude of $InsP_3$-triggered calcium transients, indicating that these agents do not interfere with the cascade downstream from $InsP_3$-triggered store release (data not shown). We also determined whether the purinergic inhibitors influenced gap junctional coupling: suramin, PPADS and apyrase did not significantly affect the FRAP signal (Table 1), ruling out side effects of these purinergic inhibitors on gap junction channels.

3.4. Photoreleasing calcium does not trigger intercellular calcium waves

Photoreleasing calcium ions in a single cell, using the caged calcium compound NP-EGTA (5 μM AM ester, 10 min loading time), triggered an increase of intracellular free calcium in the photostimulated cell but did not trigger any calcium signal in neighbouring cells (Fig. 1I–K). The magnitude of the induced calcium increase in these experiments was not different from the calcium increase triggered by photoreleasing $InsP_3$ ($\Delta F/F_0 = 196.8 \pm 7.2\%$ [$n = 71$] for photorelease of calcium versus $184.8 \pm 7.6\%$ [$n = 84$] for photoliberation of $InsP_3$; pooled data from the three cell lines). Subsequent mechanical stimulation (with a micropipette) of the photostimulated cell always triggered an intercellular calcium wave, excluding the possibility that NP-EGTA in its caged, non-UV exposed and hence high-calcium-affinity form, substantially buffered cytoplasmic calcium. A separate series of experiments confirmed that the size of calcium waves triggered by mechanical cell stimulation was not significantly influenced by the short protocol of NP-EGTA loading used in these experiments (control size $19{,}920 \pm 824\ \mu m^2$ [$n = 25$] versus $18{,}524 \pm 967\ \mu m^2$ [$n = 19$] following NP-EGTA loading; pooled data from the three cell lines). Calcium waves triggered by photorelease of $InsP_3$ were neither affected by NP-EGTA loading ($n = 11$).

3.5. Photoreleasing $InsP_3$ triggers ATP release that is blocked by calcium buffering, α-GA and gap 26

The role of $InsP_3$ and calcium in triggering the release of a purinergic messenger was further investigated in experiments directly measuring the release of ATP in the extracellular medium in response to photolytic liberation of these two messengers using a bioluminescent ATP assay. The experiments were performed on ECV304 cells because calcium waves in these cells were the most affected by

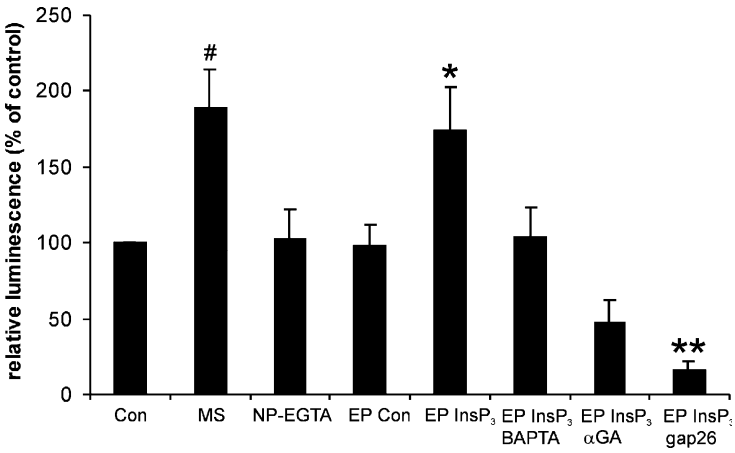

Fig. 4. $InsP_3$ triggers ATP release in luciferin/luciferase bioassay experiments. Control cell cultures (Con) produced baseline (spontaneous) ATP release. Mechanically stimulating the cultures (MS) by applying a linear scratch with a needle to release intracellular ATP, produced additional ATP release that was significantly above the baseline level. Cultures loaded with caged calcium (NP-EGTA) or cultures loaded by electroporation with vehicle (EP Con) showed ATP release upon photolytic stimulation that was not different from the baseline ATP release. By contrast, cultures loaded by electroporation with caged $InsP_3$ produced significantly increased ATP release following photolysis (EP $InsP_3$). The net component of $InsP_3$-triggered ATP release can be estimated from the difference between EP $InsP_3$ and EP Con. The $InsP_3$-triggered ATP release was abolished (ATP signal back to baseline level) by loading the cells with the calcium buffer BAPTA before photolysis (EP $InsP_3$ BAPTA). Interestingly, the $InsP_3$-triggered ATP release was also reduced by the connexin mimetic peptide gap 26 (EP $InsP_3$ gap26) and the gap junction blocker α-GA (EP $InsP_3$ α-GA). The ATP signal in the presence of gap 26 or α-GA dropped below the baseline ATP level, and a separate series of experiments demonstrated that gap 26 significantly reduced the baseline component of ATP release (see text). #Significantly above Con ($P < 0.05$), *significantly above EP Con ($P < 0.05$), **significantly below EP $InsP_3$ ($P < 0.01$).

purinergic inhibition (see Fig. 3B). Scratching the cultures with a needle (positive control condition) produced ATP release that was significantly above the background level recorded in control cultures (Fig. 4). Photolytically increasing calcium did not produce any significant ATP release in comparison to control. Increasing the amount of photolytically liberated calcium by increasing the NP-EGTA concentration and loading time (10 μM concentration and 60 min loading time as compared to 5 μM and 10 min loading time in the standard protocol) was neither effective in triggering any ATP release (data not shown). By contrast, photoliberating $InsP_3$ produced a large ATP signal, significantly above control. Preventing the $InsP_3$-triggered calcium increase by ester loading the cells with the calcium chelator BAPTA reduced the $InsP_3$-triggered ATP release back to the control level. Incubation of the cells with α-GA or gap 26 (50 μM and 0.25 mg/ml, respectively, 30 min) completely abolished the $InsP_3$-triggered ATP response and reduced the ATP release to below the control level, indicating that the basal ATP release is also affected. In a separate series of experiments, α-GA and gap 26 reduced the basal non-stimulated ATP release to 55.7 ± 22.9% ($n = 5$) and 39.8 ± 6.8% ($n = 4$) of the control level, respectively ($P < 0.001$ for the gap 26 effect). Alpha-GA and gap 26 had no influence on the bioluminescent ATP assay per se (see Section 2).

4. Discussion

The present work was undertaken to investigate the pathways of calcium signal communication in response to elevations in cytoplasmic $InsP_3$ or calcium, and to determine whether such stimulation is able to trigger purinergic messenger release. The main finding of these experiments is that photoliberating $InsP_3$ triggers intercellular calcium waves that, in addition to the known involvement of the intracellular/gap junctional pathway, also invoke the extracellular/purinergic pathway. Increasing $InsP_3$ was demonstrated to directly trigger the release of ATP, and this stimulated release was dependent on the downstream calcium increase and was largely suppressed by the gap junction blocker α-GA and the connexin mimetic peptide gap 26.

Intercellular calcium waves were triggered by spot release of $InsP_3$ or calcium ions using flash photolysis, a technique we have used in previous work [5,14,43]. $InsP_3$-triggered intercellular calcium waves differed in size between the different cell lines, with ECV304 displaying the largest and RBE4 the smallest extent of wave propagation. The contribution of the two signalling pathways that mediate wave propagation was also different: the intracellular/gap junctional pathway was, judged from the effects of the gap junction blocker α-GA, the most pronounced in RBE4 while the extracellular/purinergic pathway appeared to be the most pronounced in ECV304 (Fig. 3). Exposure of ECV304 cells to a cocktail consisting of purinergic blockers (PPADS and apyrase) and α-GA demonstrated an additive effect compatible with two separately acting signalling pathways: α-GA alone removed 18% of the calcium wave size, the purinergic inhibitors removed another 50% (giving a summed inhibitory effect of 68%) and the cocktail accordingly reduced the calcium wave size over approximately 65%. Because the contribution of the extracellular purinergic pathway was so clear

in ECV304 cells, we used these cells to investigate whether ATP release could be demonstrated more directly by using the luciferin/luciferase bioluminescent ATP assay. Our experiments clearly demonstrate that photoliberation of $InsP_3$ is sufficient to trigger ATP release that, estimated from the ATP assay calibration curves, attained a magnitude in the order of 200 nM in the 200 μl solution bathing the cells. Local concentrations close to the plasma membrane will be expected to be much higher immediately following release and before diffusion distributes the liberated ATP over the extracellular solution [3].

The most notable results in this study derive from the effect of the connexin mimetic peptide gap 26. Connexin mimetic peptides are short synthetic peptides that mimic a sequence on one of the extracellular loops of the connexin subunit [29]. The most frequently used peptide is gap 27 which mimics a highly conserved SRPTEK sequence present in the second extracellular loop and which has been demonstrated to inhibit gap junctional coupling [45,46]. Gap 26 is a tridecapeptide (13 amino acids) composed of residue numbers 63–75 of the first extracellular loop of connexin 43, containing the SHVR amino acid motif [47]. We used gap 26 in our experiments because this peptide has been reported to be an effective inhibitor of mechanically triggered intercellular calcium waves in airway epithelial cells [42]. Gap 26 did indeed significantly inhibit our $InsP_3$-triggered intercellular calcium waves, but it did not have any effect on dye coupling through junctional channels as evidenced by the FRAP experiments, despite the fact that connexin 43 was present in the cell lines used. Long incubations (2 h) with the peptide were neither effective in blocking gap junction channels. Connexin mimetic peptides are hypothesised to prevent the docking of two hemichannels on adjacent cells, thereby preventing the formation of a functional gap junction channel between the two cells [48]. Presumably, the connexin turnover time in our conditions is so slow that still longer incubations are needed to reveal the channel blocking effect. The most remarkable finding in our experiments was, however, that the peptide produced a complete inhibition of $InsP_3$-stimulated ATP release and also strongly inhibited the basal non-triggered ATP release. These findings thus indicate that the inhibitory effect of gap 26 on the $InsP_3$-triggered calcium waves is not the result of decreased gap junctional coupling, but of a selective inhibition of the ATP release pathway. Effects of gap 26 downstream the paracrine ATP signalling cascade, i.e. at the level of the purinergic receptors or on intracellular signal transduction were excluded in appropriate control experiments.

Photoreleasing calcium from NP-EGTA in a single cell, did not trigger any propagating calcium changes in nearby cells, although the calcium concentration in these experiments was increased to the same level as obtained following photoliberation of $InsP_3$. This is in line with previous observations made in astrocytes [5,49], where it was concluded that calcium by itself is (at least in those cells) not the messenger that communicates calcium signals via the intracellular/gap junctional pathway because of slower cytoplasmic diffusion as compared to $InsP_3$ [50]. The present work indicates that photolytically increasing calcium is neither sufficient to invoke the extracellular/purinergic pathway of calcium signal propagation. This is confirmed by the bioluminescence experiments that showed no ATP response upon photoliberation of calcium. A similar conclusion has been reported by Wang et al. [51] based on direct imaging of extracellular ATP concentration and mechanically stimulated calcium waves, and more recently by Arcuino et al. [13]. Calcium is however necessary as our experiments demonstrate that $InsP_3$-triggered ATP release was largely inhibited by buffering intracellular calcium with BAPTA (Fig. 4). Most probably, the $InsP_3$-triggered calcium release from the stores occurs focally and in close association to the calcium-sensitive release machinery [52,53], while photoreleasing calcium from its caged precursor brings about a global calcium elevation in the cytoplasm that is insufficient to reach concentrations high enough at the release site [54]. Consistent with this picture are the reports that calcium entry from the extracellular space, which occurs in close proximity to the target release site, is an effective stimulus of ATP release in epithelial and endothelial cells [55,56].

A consequence of the finding that increasing $InsP_3$ triggers the release of ATP is that this can contribute to regenerative calcium wave propagation. In general, regenerative calcium wave propagation can occur by three distinct mechanisms: first, calcium ions diffusing through gap junctions might trigger calcium-induced calcium release [57], thereby regenerating a calcium signal in adjacent cells [58–60]. As argued in the previous paragraph, this kind of regeneration does not occur under our experimental conditions and in the endothelial cells used. One should, however, take care in the interpretation of the experiments with NP-EGTA: we excluded any buffering effect of this compound in its caged form on macroscopic calcium signals but we cannot exclude more subtle influences on microdomain calcium signals. Second, a cytoplasmic calcium increase might activate PLC [61,62], thereby regenerating $InsP_3$ that diffuses to adjacent cells via gap junctions [63]. Third, $InsP_3$ and its subsequent calcium increase might release a paracrine messenger that acts on metabotropic receptors of adjacent cells, thereby activating PLC and regenerating $InsP_3$ [8]. Our present results indicate that this last pathway contributes to regenerative calcium wave propagation in the cell lines used.

Our experiments indicate that gap 26, at the concentration and incubation time used, is an interesting tool to dissect the pathways of intercellular calcium signal communication, as it selectively suppresses the ATP release pathway without affecting the gap junction channel conduit. This effect is likely to be mediated by specific interactions of the peptide with the first extracellular loop of connexin 43, as the control peptide des 5 had no effects. ATP release thus appears to be connexin-related, but the exact nature of this relation is not clear at present. Connexin hemichannels have been implicated in the release of nucleotides by the cell, and

several authors have recently given experimental evidence for the involvement of such release pathway that is activated by lowering extracellular calcium [9,13,27,28,64,65]. Our present work suggests that an increase of intracellular $InsP_3$ and calcium might transduce to connexin-related ATP release, but this notion is difficult to combine with the well documented evidence that an increased cytoplasmic calcium concentration, with or without the help of downstream calcium-dependent protein kinases, results in inhibition of gap junction channels or hemichannels [66–68]. However, one aspect that should be taken into account is that channel closure only occurs after prolonged (minutes) exposure to the increased calcium condition while short lasting calcium transients (several tens of seconds), e.g., as occurs in the course of an intercellulary propagating calcium wave, are ineffective in blocking the channel [69]. A point noteworthy to mention is that the first extracellular loop on the connexin subunit, which contains the gap 26 mimetic motif, is part of a sequence involved in voltage gating [70]. Interaction of gap 26 with this loop, which on connexin hemichannels is not occupied by an apposed hemichannel and hence freely accessible, can thus be hypothesized to alter the gating properties of connexin hemichannels. Further work will be needed in order to characterise the nature of $InsP_3$-triggered and gap 26-inhibitable ATP release and its possible relation to other candidate ATP release pathways such as vesicular, CFTR-related, P-glycoprotein related or anion channel mediated release.

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