



HAL
open science

Reversible bleb formation in mast cells stimulated with antigen is Ca^{2+} -calmodulin –dependent and bleb size is regulated by ARF6

Yukki Yanase, Nicolas Carvou, Michael A. Frohman, Shamshad Cockcroft

► To cite this version:

Yukki Yanase, Nicolas Carvou, Michael A. Frohman, Shamshad Cockcroft. Reversible bleb formation in mast cells stimulated with antigen is Ca^{2+} -calmodulin –dependent and bleb size is regulated by ARF6. *Biochemical Journal*, 2009, 425 (1), pp.179-193. 10.1042/BJ20091122 . hal-00479236

HAL Id: hal-00479236

<https://hal.science/hal-00479236>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Reversible bleb formation in mast cells stimulated with antigen is Ca²⁺-calmodulin
-dependent and bleb size is regulated by ARF6**

Yuhki Yanase*, Nicolas Carvou*, Michael, A. Frohman[#], and Shamshad Cockcroft

Short Title: Antigen-stimulated blebs in mast cells

*Joint First Authors

Lipid Signalling Group, Dept. of Neuroscience, Physiology and Pharmacology,
University College London, University St. London WC1E 6JJ, UK

[#] Department of Pharmacology and Center for Developmental Genetics, Stony Brook
University, Stony Brook, NY 11794 USA

Correspondence to:

Shamshad Cockcroft, Dept. Neuroscience, Physiology and Pharmacology, University
College London UK; Phone: 0044-20-7679-6084; Email: s.cockcroft@ucl.ac.uk

Abbreviations used: PLD, Phospholipase D; PA, phosphatidic acid; DAG,
diacylglycerol; MLCK, myosin light chain kinase; PKC, protein kinase C; FIPI,
5-Fluoro-2-indolyl des-chloroahalopemide; PLC, phospholipase C; ROCK,
Rho-dependent kinase;

Synopsis

Mast cells stimulated with antigen undergo extensive changes in their cytoskeleton. We assess here the impact of actin-modifying drugs and report that in the presence of cytochalasin D, mast cells stop membrane ruffling but instead bleb. Bleb formation is reversible following washout of the cytochalasin D and occurs in an actin polymerization-dependent manner. Bleb formation is inhibited by expression of dominant-negative ezrin-T567D. Blebbing is also inhibited by blebbistatin, a myosin II inhibitor, implying myosin II activation in the process. We used a selection of inhibitors and observed that membrane blebbing is dependent mainly on calcium-calmodulin with only a small contribution from Rho kinase. The signalling pathways stimulated by antigen include phospholipase C and D. Bleb formation was dependent on activation of phospholipase C but not phospholipase D. Primary alcohols, previously used as a means to reduce phospholipase D-derived PA, were potent inhibitors of membrane blebbing, but a more selective inhibitor of phospholipase D, FIPI, was without effect. FIPI also did not inhibit membrane ruffling or degranulation of mast cells, indicating that inhibition by primary alcohols works through an unidentified mechanism rather than via diversion of phospholipase D activity as assumed. We also examined the requirement for ARF6 and observed its expression led to an increase in bleb size and a further increase observed with the dominant-active mutant, Q67L-ARF6. Since T27N-ARF6 had no effect on bleb size, we conclude that ARF6 needs to be active to regulate the size of the blebs.

Short Title: bleb formation in mast cells

Key words: membrane blebbing; calmodulin; phospholipase D; ARF6; phospholipase C;

Introduction

Mast cells are multi-functional effector cells of primary importance in innate and adaptive immune responses. They participate in allergic inflammation, host defense, immunological tolerance and autoimmune disease, atherosclerosis and cancer [1-5]. In addition to their ability to degranulate and synthesise mediators, mast cells are known to accumulate in tissues in both acute and chronic inflammation. Since the increase in mast cell numbers takes place within a matter of hours, it is believed that redistribution due to directed migration of mast cells within tissues is an important mechanism. Mast cell migration can be induced by a variety of ligands including chemokines, stem cell factor and antigen.

Mast cells cross tissue boundaries and this motility is driven by remodeling of the cytoskeleton and cell contacts with the extracellular matrix (integrin-driven). In our studies on mast cell signalling during membrane ruffling, a prelude to migration, we reported that ongoing activation of phospholipase D (PLD) was essential [6]. Moreover, we had proposed that local availability of phosphatidic acid (PA) and ARF6 were both required for regulating the synthesis of phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂) during membrane ruffling. Other studies have reached similar conclusions whereby PLD activity was required for PtdIns(4,5)P₂ synthesis for modulating the actin cytoskeleton in *Dictyostelium* [7]. Screening for genes involved in cell migration identified PLDB in *Dictyostelium* and migration speed was reduced in *pldb*-null cells [8]. The physical state of the actin (G-actin versus filamentous actin) also determines PLD activity. Both β -actin and α -actinin have been shown to associate with PLD2 and have been found to inhibit its activity in vitro [9,10]. Likewise PLD1 is also able to bind both monomeric actin and filamentous actin with monomeric actin inhibiting activity [11]. In order to understand the complex interplay between the cytoskeletal changes induced upon antigen stimulation of mast cells and the requirement for PLD activity, we have assessed the impact of latrunculin B and cytochalasin D, two actin-modifying drugs on several mast cell functions and on antigen-stimulated PLD activity. Cytochalasin D is a cell-permeable fungal toxin which binds to the barbed end of actin filaments inhibiting both the association and dissociation of subunits, leading to stabilization of the actin filaments. Under normal circumstances activation of mast cells with antigen leads to increased actin polymerization and this is inhibited by cytochalasin D pretreatment [12]. Latrunculin B is another compound from Red Sea sponges that also disrupts the actin cytoskeleton. It binds to monomeric actin, thus preventing actin polymerization. It too prevents antigen-stimulated actin polymerization

and thus is accompanied by increased degranulation [12].

In this study we report that although both cytochalasin D and latrunculin B treatment of RBL-2H3 mast cells lead to a robust increase in antigen-stimulated PLD activation, treatment with cytochalasin D, but not latrunculin B, results in the formation of membrane blebs. Blebs are spherical membrane protrusions that are formed when regions of the plasma membrane separate from the underlying cell cortex, which comprises of a layer of actin, myosin and associated proteins [13-15]. Normally, myosin motor proteins maintain the cortex under tension, but when the cortex is separated from the plasma membrane, cytoplasmic pressure leads to extrusion of the membrane that grow into spherical protrusions. Although blebbing occurs during apoptosis, blebs are also observed in healthy cells, i.e. during cytokinesis, cell spreading and in some cases of cell migration in three dimensional cultures and also *in vivo* [14,16]. Blebbing based motility has been observed in *Dictyostelium* [17], primordial germ cells from fish and amphibians and in amoebae [14]. Studies in mammalian cells are scant and are restricted to tumour cells. Blebs provide an alternative route to cell migration [16,18] and as such it is important to understand their dynamics and the molecular machinery that is required for the formation and retraction of a bleb. The main model system available for studying bleb formation in mammalian cells has been the human melanoma cell-lines, in particular the M2 cells which lacks the actin binding protein, ABP-280 (also known as filamin-A) [19,20]. Blebbing is observed spontaneously in these cells when allowed to re-attach i.e. during spreading. The cells initially show blebs and after 48-72 hrs the cells cease blebbing and develop membrane ruffles [19]. Loss of PIP5K1 γ , but not other PIP5K isoforms in megakaryotes show a blebbing phenotype similar to that seen with M2 cells [21]. An identical phenotype was also observed in megakaryotes lacking the PIP5K1 γ -binding partner, talin1 [21]. Reversible blebbing stimulated by an agonist has not been reported.

Migratory cells such as lymphocytes and neutrophils can switch to amoeboid migration when adhesion to the substrate is reduced as observed in neutrophils from patients where β 2 integrins are reduced [22] or in the Walker carcinosarcoma cells where sub-cell lines were identified that used an amoeboid migratory phenotype when the cells did not adhere [23]. Amoeboid movement is characterized by a rounded, blebbing morphology, independence from extracellular proteases, and a requirement for high levels of actomyosin contractility downstream of RhoA-ROCK signalling [16,18,24-26]. Here we analyse the signal transduction pathways involved in bleb formation in

antigen-stimulated RBL-2H3 mast cells and report that phospholipase C activation, and subsequently calcium-calmodulin are essential for bleb formation. Although butanol was found to inhibit membrane blebbing, a recently-synthesised small molecule inhibitor of PLD (FIPI) [27] was without effect suggesting that PLD activation is not required.

Experimental

Reagents

The chemicals used were obtained from the following sources: bovine serum albumin (BSA), dinitro-phenol-conjugated human serum albumin (DNP-HSA), anti-DNP IgE, calpain inhibitor I, quercetin, cytochalasin D and latrunculin B from Sigma-Aldrich; [³H]inositol was purchased from GE Healthcare, U73122, U73343, wortmannin, nocodazole, LY294002, PD98056 and calmidazolium chloride were purchased from Calbiochem (UK). SB-203580 (p38-MAPK inhibitor) and ATK (Arachidonyl Trifluoromethyl Ketone) was obtained from Cayman Chemical, Bisindolylmaleimide (GF 109203X) was obtained from Cell Signaling technology and Y27632 and blebbistatin were purchased from Tocris UK. A rabbit polyclonal antibody (Ab: 343) that stains tubulin was made in house.

Cell Culture

RBL-2H3 mast cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in DMEM supplemented with 12.5 % fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and 4 mM glutamine. One or 2 days before the experiments, the cells were harvested using trypsin and then cultured in culture plates with or without glass cover slip (thickness 0) in the presence of 5 ng /ml anti-DNP IgE.

Blebbing assay

RBL-2H3 cells primed with anti-DNP IgE were cultured on glass coverslips (thickness 0) and treated with 1µM cytochalasin D in the presence or absence of inhibitors for 15 min at 37°C and then stimulated with 5 ng/ml DNP-HSA for 20 min. After incubation at 37°C, the cells were washed twice with ice-cold PBS, and then fixed with 4 % paraformaldehyde. For measuring bleb retraction, the reaction medium containing cytochalasin D was removed 15 min after stimulation and replaced with medium with 50 nM latrunculin B. The cells were then washed with PBS and fixed at the indicated times.

Transfection with GFP-tagged proteins

To transfect cells with GFP-ezrin or ARF6-GFP, RBL-2H3 mast cells were resuspended in 100 μ l of AMAXA solution R to which was added 5 μ g of plasmid DNA. The cells were then electroporated in an AMAXA nucleofector (program T-20), resuspended in 2 ml of Growth medium and plated in 6-well plates containing glass coverslips. The cells were then incubated for 24 hrs in the presence of 5 ng/ml anti-DNP IgE.

Measurement of phospholipase C activity

For measurement of inositol phosphates, the cells were labeled for 2 days with [3 H] inositol (2 μ Ci/ml) in 6-well plates in the presence of 5 ng/ml anti-DNP IgE. On the day of the experiment, the cells were washed with HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 1 mg/ml glucose, 1 mg/ml BSA, PH 7.2) containing 10 mM LiCl (to inhibit the breakdown of inositol phosphates back to inositol). The cells were treated with inhibitors for 15 min at 37°C, and then stimulated with DNP-HSA for 15 min at 37°C in the presence of 10 mM LiCl. At the end of the incubation, the medium was replaced with 500 μ l of ice-cold methanol. The cells were then vigorously scraped and transferred to clean tubes. The tissue culture wells were rinsed with a further 500 μ l of methanol and the cellular extracts combined in a single tube. Chloroform (1 ml) was added to the methanol cell extracts followed by 1 ml of water. Following centrifugation, [3 H]inositol phosphates were recovered in the top hydrophilic phase and were analyzed on Dowex columns as described [28].

Measurement of phospholipase D activity

RBL-2H3 mast cells, seeded in 6-well plates at 2×10^5 per well in the presence of 5 ng/ml anti-DNP IgE the day before experiment were labeled with [3 H]myristic acid for 1 hr at 37°C in HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 1 mg/ml glucose, 1 mg/ml fatty acid free BSA, PH 7.2). After the labeling period, [3 H]myristic acid was removed and the cells were treated with inhibitors for 15 min, and then stimulated with 5 ng/ml DNP-HSA for 15 min in the presence of 1.0% ethanol. Reactions were terminated by adding 0.5 ml of ice-cold methanol to HCl at a 98:2 (v/v) ratio. Cells were then scraped and transferred to test tubes. The cells were rinsed with 500 μ l methanol and the volume was transferred to tubes. Two phases were generated by addition of 1 ml of chloroform and 1 ml of H₂O. The lower organic phase was removed and the chloroform evaporated. The lipids were resuspended in 20 μ l chloroform and spotted onto silica gel-60 TLC plates that were developed with chloroform: methanol: acetic acid : H₂O (75:45:3:0.4, v/v/v/v). The areas corresponding

to phosphatidylethanol and phosphatidylcholine were identified with authentic standards after iodine staining, scraped and counted for radioactivity.

Secretion assay

Degranulation in RBL-2H3 cells was measured by release of β -hexosaminidase. The day before the experiments, RBL-2H3 cells were seeded at 2×10^4 cells per well on a 96-well plate in the presence of 5 ng/ml anti-DNP IgE. Following 2 \times washes with HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml glucose, 1 mg/ml fatty acid free BSA, PH 7.2), cells were treated with inhibitors for 15 min at 37°C and then stimulated with 5 ng/ml DNP-HSA for 15 min. At the end of incubation, ice-cold buffer was added and then cells were centrifuged at $1000 \times g$ for 5 min. An aliquot of the supernatant (50 μ l) was analyzed for β -hexosaminidase as previously described [29].

Measurement of Akt phosphorylation

RBL-2H3 cells seeded in 6-well plates at 2×10^5 per well in the presence of 5 ng/ml anti-DNP IgE the day before experiment were pre-incubated for 10 min with or without cytochalasin D or latrunculin B and then stimulated with 5 ng/ml antigen for 10 min. The cells were washed twice in ice-cold PBS and lysed with ice-cold lysis buffer (20 mM Tris, pH 8.0 / 150 mM NaCl / 1.0% (v/v) NP-40 / 1 mM EDTA supplemented with mammalian cell and tissue extracts protease inhibitor cocktail (Sigma) (1:100 dilution) and phosphatase inhibitor cocktail 1 & 2 (1:100) (Sigma)). The lysed cells were scraped and diluted in reducing sample buffer. 50 μ g of cell lysates were run onto a 12% acrylamide SDS PAGE gels and treated for western blot. Total and phosphorylated AKT/PKB levels were monitored by immunostaining with anti-Akt and anti-phospho-Akt antibodies (Cell Signaling Technology).

Immunofluorescence Microscopy

RBL-2H3 mast cells cultured on glass cover slips (thickness 0) in 24 well-plates were pretreated with inhibitors and then stimulated with DNP-HSA. After incubation cells were washed with PBS twice and fixed with 4 % paraformaldehyde for 10 min at room temperature. Following two washes with PBS, the cells were permeabilised in 0.2 % TritonX-100, 0.2 M glycine in PBS for 10 min at room temperature. The cells were then blocked in PBS containing 0.5% BSA fraction V and 0.2 M glycine (blocking solution) for 30 min at room temperature and incubated with primary antibody diluted in the blocking solution for 1 hr at room temperature. The cells were then washed 4 times with

blocking solution and incubated with Alexa-Fluor® labeled secondary antibody (Invitrogen) for 30 min at room temperature. After 4 times washes with PBS, the coverslips were mounted onto a 10 μ l drop of mowiol and were left to set overnight at 4°C. For actin cytoskeleton and nuclear staining, the cells were treated with blocking solution containing tetramethylrhodamin B isothiocyanate (TRITC)-phalloidin and DAPI for 20 min. Three colour images were captured with an Olympus IX81 inverted microscope fitted with an X-Cite 120 light (Exfo), FITC, TRITC and DAPI HQ filter sets (Chroma) and a -20°C cooled Orca-ER camera (Hamamatsu). The microscope and camera were controlled with the images acquisition software Cell F 2.4 (Olympus).

Results

Induction of blebbing in antigen-stimulated RBL-2H3 cells pretreated with cytochalasin D

Quiescent mast cells are elongated when plated on plastic or glass and show a smooth actin cortex. Upon addition of antigen, membrane ruffles are observed within minutes in IgE-sensitized RBL-2H3 mast cells and this is accompanied by release of secretory granules. We have previously reported that during antigen-stimulated membrane ruffling, continual production of PA by PLD occurs. In addition, butan-1-ol but not butan-2-ol inhibited membrane ruffling in a reversible manner leading to the conclusion that PLD-derived PA was required for ongoing membrane ruffling. To examine whether the polymerization state of the actin cytoskeleton influenced PLD signalling, we examined two actin-disrupting drugs in antigen-activated RBL-2H3 mast cells on PLD activation and actin dynamics. IgE-sensitized RBL-2H3 mast cells were pretreated with cytochalasin D and latrunculin B for 15 min prior to stimulation with antigen. Both drugs dramatically enhanced antigen-stimulated PLD activation with cytochalasin D being more potent (Fig. 1A). Degranulation as monitored by β -hexosaminidase release was also enhanced two-fold compared to control cells (Fig. 1B). The addition of the drugs on their own did not have any significant effects on basal PLD activity or degranulation. This is in accordance with a previous report that showed that both drugs increased degranulation in RBL-2H3 mast cells [12]. We also examined the effect of cytochalasin D and latrunculin B on phospholipase C and Akt phosphorylation. Although both drugs enhanced phospholipase C activation, Akt phosphorylation was not significantly affected (Figs. 1C and D). In parallel studies, we observed that cytochalasin D or latrunculin B pre-treatment did not affect the typical elongated morphology of the cells prior to antigen activation (Fig. 1E). However, upon stimulation with antigen, ruffle formation was inhibited in both cytochalasin D- as well as in

latrunculin B-treated cells, as evidenced by staining with phalloidin for F-actin (Fig. 1E).

We also examined the cells for membrane ruffles by light microscopy and this revealed that whilst membrane ruffling was inhibited in cytochalasin D-treated cells, the cells had numerous blebs after antigen stimulation (Fig. 2A and see Movie 1 in supplementary material). In contrast, latrunculin B-treated cells did not show any blebs. (Latrunculin B was tested between 50nM – 2 μ M but was unable to induce membrane blebbing following antigen stimulation). Once formed, the blebs were quite mobile but did not retract (see Movie 1 in supplementary material). Most blebs were spherical with a straight base. The blebs were characterized with respect to the number of blebs per cell, the percentage of blebbing cells and the mean diameter of the blebs (Table 1). The blebs could be easily detached by vigorous shaking (data not shown).

To investigate the dynamics of bleb retraction, the cells were washed to remove cytochalasin D; the blebs started to retract spontaneously (Movie 2 in supplementary material and Fig. 2B). Retraction was complete 6 min after cytochalasin D removal. The cells were seen to ruffle after washout suggesting that removal of antigen was not as effective as removal of cytochalasin D (see Movie 2 in supplementary material). Retraction was dependent on actin polymerization as addition of latrunculin B in the wash buffer completely prevented bleb retraction (Fig. 2B). Furthermore several rounds of blebbing could be achieved by successive cycles of cytochalasin D addition and removal (data not shown), demonstrating that induced bleb formation in RBL-2H3 mast cells is a reversible process and not the result of the cells undergoing apoptosis. We also examined bleb initiation in non-adherent RBL-2H3 cells. RBL-2H3 mast cells were grown in cell suspension by coating the culture dishes with 2 % agarose to prevent cell adhesion [30]. In cytochalasin D-treated cells, blebs were readily observed upon antigen stimulation suggesting that bleb formation is anchorage-independent (Fig. 2C).

State of the actin cortex during bleb dynamics.

Blebs are defined as membrane protrusions which can be distinguished from other cellular protrusions such as lamellipodia or filopodia by the absence of polymerised actin filaments; cytosolic proteins are present in the blebs [13,15,31]. Antigen-stimulated blebs were examined by wide field fluorescence microscopy after fixation and staining with TRITC-phalloidin to visualize actin filaments and with an antibody to tubulin. The blebs stained with tubulin but no actin filaments were observed

in the blebs (Fig. 3A). Confocal analysis of the cells revealed that the blebs were devoid of actin filaments and that the actin cortex surrounding the cell body remained unaffected at the base of the bleb (Fig. 3B). Additionally, tubulin was present in the blebs although microtubules were not observed. Following cytochalasin D washout, actin filaments were observed to accumulate at the cortex of the retracting blebs (Fig. 3C).

Expression of Ezrin-T567D inhibits bleb formation.

For the membranes to bleb, local detachment of the membrane from the actin cortex has to occur [13,14]. The actin cortex makes many interactions with the overlying membrane and either loss of adhesive strength or gaps in the cytoskeleton (or both) enable the pressure to form blebs. To examine whether proteins that increase the attachment of the plasma membrane with the actin cytoskeleton will counteract bleb formation, we expressed an active form of ezrin. Ezrin belongs to the ERM family of proteins (ezrin-radixin-moesin) that can bind both the plasma membrane via their N-terminal ~300 residue FERM domain and the actin cytoskeleton via their C-terminal domain [32,33]. ERM proteins can exist in the dormant or in active conformations; in the dormant state, the F-actin binding site is masked. The activation of ERM proteins is mediated by both carboxy-terminal threonine phosphorylation and exposure to PI(4,5)P₂ [34]. Active ERM proteins could therefore maintain membrane attachment to the actin cortex and prevent blebbing. Over-expression of the phospho-mimetic, constitutively-active ezrin mutant (T567D) has been found to inhibit blebbing [20]. We therefore expressed GFP-tagged wild-type ezrin and ezrin-T567D in RBL-2H3 mast cells. Following transfection, many cells expressed the ezrin mutant, whilst the number of cells expressing the wild-type protein was always low. Wild-type ezrin localised mostly to the nucleus and the cytoplasm of resting cytochalasin D-treated cells, and upon antigen stimulation, moved to the plasma membrane (data not shown). In contrast, ezrin-T567D was concentrated at the plasma membrane prior to antigen stimulation (Fig. 4A).

Cells expressing the wild type protein still form blebs; wild-type ezrin was not observed at the rim of the blebs, although present in the bleb cytosol. Nonetheless, it was enriched at the plasma membrane at the base of the blebs (data not shown). In cells expressing T567D ezrin, no blebs were observed following antigen stimulation (Fig. 4A). Quantification showed that less than 10% of cells expressing ezrin-T567D produced blebs compared to 90% of control cells (Fig. 4B). Taken together, these results

demonstrate that ezrin resides in the cytosol of resting RBL-2H3 mast cells and upon cross-linking of the IgE receptor, it can translocate to the plasma membrane. Nonetheless, it does not prevent bleb formation. This may be explained by the rapid intrinsic turnover of T567 phosphorylation [35]. In contrast, over-expression of constitutively-active ezrin is sufficient to prevent antigen-stimulated bleb formation.

The activity of Myosin II is required for bleb formation but not membrane ruffling

Bleb nucleation is initiated by the rapid detachment of a local patch of membrane from the cortex and this requires intracellular pressure transients that are generated by myosin II contraction of the actin cortex; drugs that relax the cortex can inhibit blebbing [20]. In antigen-stimulated cells, inhibition of the ATPase activity of the myosin II motor protein with blebbistatin completely inhibited plasma membrane blebbing. In contrast, membrane ruffling was unaffected by blebbistatin (see Fig. 5A and B and Table 2).

Phospholipase C-dependent pathways are required for antigen-stimulated blebbing in RBL-2H3 cells

To examine which of the signalling pathways were required for bleb formation in RBL-2H3 mast cells we used a number of inhibitors that have been previously described to interrupt specific signalling pathways (Fig. 5A). The requirement for phospholipase C activity for bleb formation was tested using the inhibitor U-73122; it completely blocked bleb formation. As a control the inactive homologue U-73343 was used and it had no effect on blebbing. Wortmannin, a covalent inhibitor of PI3K, also inhibited blebbing and similarly, bleb formation was significantly reduced in cells treated with LY294002 (data not shown). The above inhibitors not only inhibited blebbing but inhibited membrane ruffling indicating that the upstream signalling events were identical for both (Fig. 5B and Table 2).

Since phospholipase C activation was needed, additional inhibitors were used to test the requirement for a rise in cytosol Ca^{2+} and for activation of protein kinase C by diacylglycerol (DAG). Treatment with the chelating agent EGTA, which prevents calcium entry, completely repressed blebbing, suggesting that calcium influx via plasma membrane calcium release activated channels is a requirement for bleb formation. Ca^{2+} can bind to calmodulin and an inhibitor of calmodulin, calmidazolium chloride was therefore tested; it was found to also inhibit bleb formation (Fig. 5A). Likewise, inhibition of protein kinase C by treatment with bisindolylamide (BIM) completely blocked membrane blebbing (Fig. 5A). However, phospholipase A_2 activation was not

required, as ATK was without effect on blebbing, and blocking MAP kinases p38 and ERK, which are late effectors downstream of PKC, had also no effect (Fig. 5A).

Two separate pathways can regulate myosin II activity – a Rho-Rho kinase pathway which is Ca^{2+} -independent and functions by phosphorylating myosin phosphatase to inhibit its activity, and a Ca^{2+} -dependent pathway that involves a Ca^{2+} -calmodulin complex that can activate myosin light chain kinase (MLCK) to phosphorylate the myosin light chain of myosin II. To examine the contribution of the Rho-Rho kinase pathway we used the ROCK inhibitor, Y-27632. This inhibitor only partially blocked membrane blebbing (Fig 5A). In Table 1 we compare the degree of inhibition by the calmodulin inhibitor and the ROCK inhibitor. We measured both the number of blebbing cells and also the number of blebs per cell. It is clear that the calmodulin pathway makes a substantial contribution compared to the Rho-ROCK pathway in the regulation of membrane blebbing. It should be noted that the ROCK inhibitor Y-27632, the myosin II inhibitor blebbistatin, and calcium sequestration by EGTA did not affect membrane ruffling, indicating that myosin II contractility is specifically required for blebbing (Fig. 5B and Table 2).

Finally, we checked whether calpain may be required for blebbing as they have been implicated in the control of cytoskeletal dynamics. However, Calpain Inhibitor I was unable to prevent antigen-stimulated membrane blebbing or membrane ruffling (Fig.5A and B).

Inhibitors of PA production via Phospholipase D activity block membrane blebbing

Phospholipase D-derived PA has been implicated to be an important signalling molecule that regulates the dynamic changes in the actin cytoskeleton that is obligatory for cell adhesion, spreading and migration [36]. We used butan-1-ol, a preferential substrate for PLD activity that inhibits PLD-mediated PA production, and looked at its effect on bleb formation. As shown in Fig. 6A and Table 2, bleb formation was inhibited in cells treated with butan-1-ol but not butan-2-ol, which is not a substrate for PLD. In our search for alternative inhibitors of PLD activity, we identified quercetin (Fig. 6B). At concentrations of quercetin ($10\mu\text{M}$) that inhibit PLD activation without effect on PLC activation (Fig. 6C) or membrane ruffling (Fig. 5B), quercetin was found to inhibit membrane blebbing (Fig. 5A). These data collectively suggest that PLD activation and therefore PA may be an important component required for bleb formation.

Use of a new PLD inhibitor, FIPI reveals that PLD activity is not required for many mast cell functions

To date, primary alcohols have been widely used to implicate the requirement for PLD activity for specific biological functions. Despite their limitations due to additional effects unrelated to PLD signaling, they have remained popular tools. 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) has been recently identified as an inhibitor of PLD activity [27,37]. It inhibits both PLD1 and D2 activity both in vitro and in vivo making it possible to reassess the requirement of PLD using more selective inhibitors.

We first characterized FIPI as an inhibitor of PLD activation in antigen-stimulated RBL-2H3 mast cells. FIPI inhibited antigen-stimulated PLD activity in a concentration-dependent manner with maximum inhibition achieved at 75 nM (Fig. 7A). At this concentration, 75 nM FIPI was found to inhibit both PMA- and cytochalasin D plus antigen-stimulated PLD activation (Fig. 7B). FIPI was introduced as a PLD inhibitor based on in vitro analysis, and its effects on phospholipase C activation when applied to intact cells have not been investigated. When tested at concentrations that maximally inhibited PLD activation, PLC signaling was not affected in RBL-2H3 mast cells stimulated with antigen (Fig. 7C). Based on this analysis, we were satisfied that FIPI is a potent inhibitor of antigen-stimulated PLD activity and that it is relatively specific as no significant inhibition of PLC was observed.

FIPI-treated cells were examined for membrane blebbing (in the presence of cytochalasin D), membrane ruffling and degranulation which are all responses that can be inhibited by primary alcohols but not secondary alcohols. To our surprise, 75nM FIPI did not prevent bleb formation (Fig. 7E), membrane ruffling (Figure 7F), or degranulation (Fig. 7D) in antigen-stimulated cells. In other experiments, we also used FIPI at 750 nM and even at this concentration, FIPI was without effect (e.g. Fig. 7D). Taken together, these results demonstrate that FIPI is a specific inhibitor of PLD activation in RBL-2H3 mast cells but that inhibition of PLD does not block membrane ruffling, blebbing or exocytosis.

ARF6 potentiates blebbing via PLD signaling.

To further explore the connection between PLD and membrane blebbing, we examined ARF6, a regulator of both PLD and of PIP5K activity that is also required for

membrane ruffle formation in HeLa cells [38]. We expressed four GFP-tagged ARF6 constructs, including wild type ARF6, inactive T27N-ARF6, constitutively-active Q67L-ARF6 and a double mutant, N48R/Q67L, which is constitutively-active but defective in PLD but not PIP5K activation [39-41]. Wild-type ARF6 was present at the rim of the blebs after antigen stimulation. Strikingly, the blebs were of a significantly bigger size in cells expressing wild type ARF6, compared to the neighbouring non-expressing cells (Fig. 8A). In contrast, inactive ARF6-T27N was enriched in the cytoplasm and did not localize to the bleb cortex upon antigen stimulation. Furthermore, cells expressing this construct were much thinner and more elongated than control cells and made blebs of normal sizes when stimulated (Fig. 8B). The constitutively-active Q67L-ARF6 localized to the plasma membrane and the cells had a wider body and shorter extensions than un-transfected neighbouring cells (Fig. 8C). More importantly, antigen stimulation resulted in the formation of giant blebs and ARF6-Q67L was enriched in the rim of the blebs.

Although PLD activity was not necessary for bleb formation, it was still possible that it could play a more subtle role in regulating the size of the bleb. To examine this possibility, we used the ARF6 double mutant (N48R/Q67L) which is unable to activate PLD. This mutant localized to the plasma membrane in resting cells and to the rim of the blebs upon stimulation. However, in cells expressing ARF6 N48R/Q67L, antigen stimulation resulted in the formation of blebs of a normal size, compared to control cells, suggesting that PLD activity could contribute to bleb size. In Fig. 8E, the bleb diameter was monitored. To quantitate the changes, we measured the bleb diameter of the transfected cells and compared them with their untransfected neighbors for each of the constructs. The data were collected from two separate experiments and the number of cells analysed for each construct and its untransfected neighbor is presented in the table and the data are plotted as a box plot that is a summary plot representing statistical values. The mean bleb diameter of all the non-transfected cells is $\sim 3.3\mu\text{M}$ and is seen to increase to 5.4 when wild type ARF6 is expressed and to 6.5 when the constitutively active form of ARF6-Q67L is expressed (Fig. 8E). The bleb diameter is considerably smaller when the ARF6 mutant that is unable to stimulate PLD activity was examined even when compared to wild type ARF6. These data support the suggestion that PLD activation may influence the bleb diameter.

Discussion

In this study we report that perturbation of the actin cytoskeleton by cytochalasin D treatment leads to a switch from ruffling to membrane blebbing in RBL-2H3 mast cell

stimulated with antigen. The blebs once formed remain stationary and are devoid of filamentous actin. Following washout of cytochalasin D, the blebs retract in a process dependent on actin polymerization; an actin cortex could be observed in the retracting blebs and addition of latrunculin B during bleb retraction was found to be inhibitory (Fig. 3C). For blebbing to occur, the local interaction between the cytoskeleton and the plasma membrane must be impaired. Nonetheless actin is observed at the base of the bleb (Fig. 3B), suggesting that the interactions between the actin cortex and the membrane have been disrupted. To test whether proteins that would strengthen the interaction between the plasma membrane and the actin cytoskeleton, we used an ezrin mutant, ezrin T567D. This phospho-mimetic form of ezrin is maintained in its open configuration and therefore can interact with both the plasma membrane and the actin cytoskeleton in a constitutive manner. The ezrin mutant, when expressed in RBL-2H3-mast cells, localises to the plasma membrane and inhibits bleb formation supporting the notion that maintaining the tight association between the plasma membrane and the actin cytoskeleton is inhibitory for bleb formation.

Since blebbing requires activation of the cells with antigen, we examined the signalling pathways that are required. We also monitored membrane ruffling in parallel to examine whether the signalling pathways leading to both these functional responses could be separated. Using a variety of inhibitors, we show that blebbing requires the activation of phospholipase C as well as the activity of PKC and a rise in cytosol calcium. Since chelation of extracellular calcium with EGTA, and the calmodulin inhibitor calmidazolium both inhibited bleb formation but not membrane ruffling, this suggested that calcium-calmodulin regulates a downstream event that is essential for bleb formation.

Bleb expansion is likely to be driven by hydrostatic pressure produced by cortical contraction involving myosin-II and this conclusion is supported by the studies with blebbistatin. Blebbistatin inhibits the activity of myosin II ATPase and was found to inhibit bleb formation. The activity of myosin II can be regulated by either calcium-calmodulin dependent phosphorylation of myosin light chain kinase or by the Rho-dependent kinase (ROCK). Since both EGTA and the calmodulin inhibitor blocked membrane blebbing we conclude that calcium-calmodulin is the main route to regulate myosin II-dependent contractility. To examine if Rho-dependent kinase made a contribution, we used Y27632. With this compound a partial inhibition was observed. Both the calmodulin inhibitor and the ROCK inhibitor led to a decrease in the number

of blebbing cells as well as in the number of blebs made per cell. Compared to the calmodulin inhibitor, the inhibition observed with the ROCK kinase inhibitor was modest. In most types of blebs reported so far bleb dynamics has involved signaling through the Rho-ROCK-myosin cascade in which the small GTPase Rho recruits and activates the Rho-associated kinase ROCK to phosphorylate myosin light chain thereby inducing myosin contraction and increase of local pressure [18,42,43]. In RBL-2H3 mast cells, it would appear that this pathway is less relevant, and that calcium-calmodulin regulation of myosin II plays a more dominant role. We cannot exclude the possibility that the kinase, MRCK (myotonic dystrophy-related Cdc42-binding kinase) that is regulated by Cdc42 could contribute to MLC2 phosphorylation and hence myosin II contractility in mast cells [24].

Requirement for Phospholipase D in membrane blebbing

Since our early studies on membrane ruffling and degranulation had clearly indicated that activation of PLD was required we explored the possibility that PLD activation was also essential for membrane blebbing. To examine this we used a primary alcohol, butanol and a secondary alcohol, butan-2-ol as a control. Butanol participates in transphosphatidylation leading to a decrease in PA production by PLD at the expense of the product, phosphatidylbutanol. PA is able to regulate the activity of PIP 5-kinase and hence increase the amount of PtdIns(4,5)P₂ locally [44]. Since PtdIns(4,5)P₂ is important in the regulation of the dynamics of the actin cytoskeleton, PLD could be an important regulator. Also in cytochalasin D-treated cells, the stimulation of PLD activity by antigen was enhanced. When antigen-stimulated cells were examined for bleb formation in the presence of butanol, blebbing was completely inhibited, but no inhibition was observed in the presence of the secondary alcohol.

To further evaluate the importance of PLD activity, we screened for inhibitors of PLD activity and identified that at low concentrations, quercetin was a potent inhibitor of antigen-stimulated PLD in mast cells. Quercetin did not inhibit phospholipase C activation and neither did it inhibit membrane ruffling. This further strengthened the suggestion that PLD activation was essential for membrane blebbing but also raised other issues. Since membrane ruffling was not affected by quercetin despite complete inhibition of PLD activity this implied that our previous studies which had relied on the use of alcohols may need to be re-evaluated [6].

Whilst this work was in progress, it was reported that 5-fluoro-2-indolyl

des-chlorohalopemide (FIPI) was identified as a potent inhibitor of both PLD1 and PLD2 both in vitro and in vivo [27]. PLD has been linked to regulated exocytosis in many cell types including neutrophils, mast cells and pancreatic β -cells, based on the observation that the agonist-stimulated exocytosis is inhibited by primary alcohols [45]. FIPI was tested for glucose-induced insulin secretion, and although FIPI inhibited PLD activation, no inhibition of glucose-stimulated insulin release was observed. From their study they concluded that PLD activity was not required for insulin release and that inhibition by butanol must occur through another mechanism.

We tested FIPI in RBL-2H3 mast cells and established that FIPI was a potent inhibitor of antigen-stimulated PLD activity. 75nM was sufficient for maximal inhibition. At this concentration, it also inhibited the enhanced activity observed in the presence of cytochalasin D and as well PMA-induced PLD activation. FIPI was without effect of phospholipase C activation. To our surprise, we have found that FIPI did not inhibit antigen-stimulated degranulation, membrane ruffling or membrane blebbing. From this data we conclude that previous studies which had relied on primary alcohols to demonstrate a role for PLD need to be re-evaluated. It is of interest that primary alcohols do inhibit exocytosis in many cell types and it would be of real interest to identify the targets of primary alcohols.

One of the reasons why PLD-derived PA was such an attractive candidate for modulating the actin cytoskeleton was the potential of PA to regulate PIP5K activity. It is now clear that PIP5K activity can be regulated independently of PA by the GTPase ARF6. Honda et al purified ARF6 as a co-factor acting with PA to stimulate PIP5K activity and ARF6 was identified as a requirement for membrane ruffle formation [38]. Subsequent work identified that PIP5K could be directly regulated by ARF proteins [46]. The ability of ARF proteins to regulate PIP5K activity is evolutionarily conserved – plasmodium PIP5K can be regulated by ARF proteins but not by PA [44,47]. It was therefore of interest to examine the importance of ARF6 in membrane blebbing. Expression of wild type ARF6 was found to affect the diameter of the bleb, and this was even more dramatic in cells where the constitutively-active ARF6-Q71L was used. To examine whether PLD activation in the bleb may have some subtle role, we used an ARF6 mutant that is unable to activate PLD whilst it remains competent to activate PIP5K. The bleb diameter was significantly reduced when this mutant ARF6 was expressed. We therefore conclude that although activation of PLD is not essential for bleb formation, it has an influence on the bleb diameter. We cannot exclude the

possibility that ARF6 has other as yet identified downstream effectors that this mutant is unable to activate. We would cautiously suggest that PA can influence the size of the membrane blebs. This would be very important particularly for cells that move by blebbing such as metastatic cells that can switch to the blebbing mode for migration when transferred to 3D-matrices [18]. Thus a recent study has described chemically-synthesized PLD inhibitors also based on halopemides and reported that PLD activity plays an essential role in invasiveness in highly metastatic breast cancer cell lines [48].

Tumour cell invasion through the extracellular matrix is accompanied by the formation of invadopodia, which are actin-rich protrusions at the adherent surface of cells at sites of extracellular degradation and ARF6 has been shown to be important in this process [49,50]. Interestingly, the effects of ARF6 are upstream to PLD activation. If bleb size determines migration speed then the increased invasiveness of cells expressing ARF6 using invadopodia might suggest that during blebbing-driven motility, the degree of ARF6 activation might determine the speed of movement.

Acknowledgements

We would like to thank Guillaume Charras (Institute of Nanotechnology, UCL) for suggestions, discussion and provision of the ezrin plasmids. Yuhki Yanase was supported by a grant from Uehara Memorial Foundation, Japan.

Figure legends

Figure 1. Treatment of RBL-2H3 mast cells with cytochalasin D or latrunculin B enhances antigen-stimulated PLD activation and degranulation whilst inhibiting membrane ruffling. IgE-sensitised RBL-2H3 mast cells were pretreated with cytochalasin D (1 μ M) or latrunculin B (500 nM) as indicated for 15 min and then stimulated with antigen (5 ng/ml) for 20 min. (A) Cells were prelabelled with [3 H]myristate for one hour prior to use for monitoring PLD activation. The production of phosphatidylethanol (PEt) was monitored in the presence of 1% ethanol. (B) Degranulation was assessed by measuring the release of β -hexosaminidase. (C) Phospholipase C activation was measured from cells prelabelled with [3 H]inositol for 48 hrs. Lithium chloride (10 mM) was present during stimulation to accumulate the inositol phosphates. (D) Cells were pretreated with cytochalasin D or latrunculin B for 15 min and then stimulated with antigen for 10 min. Cells were then lysed and analysed by western blot with anti-Akt and anti-phospho-Akt antibodies. Both the western blot and the ratio of P-Akt to total Akt is shown (E) The cells were fixed, and stained with TRITC-phalloidin to assess membrane ruffling.

Figure 2. Induction of membrane blebbing following stimulation with antigen in cytochalasin D-treated RBL-2H3 mast cells. (A) RBL-2H3 mast cells were pretreated with or without cytochalasin D (1 μ M) or latrunculin B (500 nM) for 15 min and then stimulated with antigen (5 ng/ml) for 20 min prior to fixation for analysis by differential interference contrast (DIC) microscopy. The percentage of cells that were observed to bleb was 86 ± 10 % (n=1290 cells) (See Table 1). The cells were counted from 10 random images in each of 3 independent experiments (Objective x40). No blebs were seen in control or latrunculin B-treated cells following stimulation with antigen. (B) Blebs retract upon washout of cytochalasin D and retraction is inhibited by latrunculin B. Left panel: Cells pre-treated with cytochalasin D for 15 min were subsequently stimulated with antigen for 20 min. Middle panel: 15 min after stimulation, cytochalasin D was removed and cells were fixed after 6 min. Right panel: 20 min after stimulation, cytochalasin D was removed and replaced with latrunculin B (50 nM). The cells were fixed after 6 min. (C) Blebs can be induced in cells in suspension. RBL-2H3 mast cells were cultured in tissue culture dishes coated with 2% agarose (5 mm thickness) to prevent cell adhesion. The cells were pretreated with cytochalasin D for 15 min and differential interference contrast images of live cells were taken 20 min after antigen stimulation (long distance objective $\times 40$).

Figure 3. Localisation of actin filaments and tubulin in blebbing cells. (A) Antigen-induced bleb formation in RBL-2H3 mast cells can be observed in fixed cells both by DIC and by staining with tubulin (green). No actin filaments are observed in the blebs. Images were acquired using wide field epifluorescence microscopy. (B) Cytochalasin D-treated RBL-2H3 mast cells were stimulated with antigen for 20 min, and then fixed and stained with anti-tubulin (green) and TRITC-phalloidin (red). Confocal images taken on a Zeiss LSM 510 Meta microscope. Pinhole 1.0, Objective X63. Actin microfilaments are absent from antigen-induced blebs. The inset shows that F-actin is absent from the cortex of the blebs but present at the plasma membrane at the point of origin of the bleb. Note that tubulin is present in the blebs but not microtubules. (C) F-actin is recruited to the bleb cortex during retraction. Cytochalasin D-treated cells were stimulated with antigen for 20 min, and the medium was replaced with medium without cytochalasin D. After 6 min incubation, cells were washed with PBS and then fixed and stained with anti-tubulin shown in green and TRITC-phalloidin (red).

Figure 4. Overexpression of constitutively-active ezrin prevents bleb formation. (A) RBL 2H3-mast cells expressing GFP-Ezrin-T567D were pretreated with cytochalasin D and then incubated in the presence or absence of antigen for 20 min and fixed for analysis by DIC and epifluorescence microscopy. Images were taken at x40 and x100 magnification. (B) Quantification of the number of blebbing cells for ezrin-T567D-expressing RBL-2H3 mast cells. n: number of cells counted compiled from 2 independent experiments.

Figure 5. Screening for inhibitors that can affect antigen-induced bleb formation and membrane ruffling. RBL-2H3 mast cells were treated with the indicated inhibitors in the presence (A) and absence of cytochalasin D (B) for 15 min prior to stimulation with antigen except for nocodazole. Cells were pre-incubated with nocodazole for 2 hrs. Drug concentrations are indicated in Table 2. The cells were fixed and the cytochalasin D-treated cells were examined for blebbing by DIC (A) and to examine membrane ruffling, the fixed cells were stained with TRITC-phalloidin (B).

Figure 6. (A) Inhibition of membrane blebbing by butanol. The cells were pretreated with cytochalasin D and then stimulated with antigen in the presence or absence of 0.5 % butan-1-ol or butan-2-ol. Objective x40. (B) Quercetin inhibits PLD but not phospholipase C activation in RBL-2H3 mast cells stimulated with antigen. The results

are from a representative experiment repeated on three occasions. Error bars indicate the S.D. (n=3).

Figure 7. FIPI inhibits antigen-stimulated PLD activation in RBL-2H3 mast cells but does not inhibit degranulation, membrane blebbing or membrane ruffling. RBL-2H3 mast cells were pre-incubated with FIPI for 30 min prior to stimulation with antigen. (A) Concentration dependence of FIPI for inhibiting antigen-stimulated PLD. (B) FIPI (75nM) inhibits antigen-, PMA- and antigen *plus* cytochalasin D-stimulated PLD activation. (C) FIPI (75 nM) does not inhibit phospholipase C activation (D) FIPI does not inhibit antigen-stimulated degranulation. (E) FIPI (75 nM) does not inhibit antigen-stimulated membrane blebbing or membrane ruffling.

Figure 8 Expression of ARF6 (wild-type and constitutively-active mutant regulates the size of the bleb in RBL-2H3 mast cells. RBL-2H3-cells expressing (A) GFP-ARF6-wild type (WT) or (B) T27N, (C) Q67L or (D) N48R/Q67L mutants were pretreated with cytochalasin D and then incubated in the presence or absent of antigen for 20 min. Epifluorescence images and DIC images show that, compared to non-expressing neighbouring cells, the blebs are more prominent in cells expressing ARF6-WT and ARF6-Q67L but not ARF6-T27N or the double mutant N48R/Q67L. (E) These images are representative and the data were quantitated by measuring the bleb diameter from the transfected population and compared with its neighboring non-transfected cells. The total number of cells counted in each condition is indicated in the Table. The data are plotted as a box plot which provides a summary plot representing statistical values. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers (error bars) above and below the box indicate the 90th and 10th percentiles. The outlying points are also included, represented by black circles. The mean values and the standard deviation is provided in the Table together with the Variance. The transfected population with wild type ARF6 and Q67L-ARF6 shows a high variance likely reflecting different levels of expression in the population.

No. of blebs per Cell	Percentage of Blebbing cells	Bleb Diameter (μM)	Number of cells counted	Treatment of cells
3.9 ± 1.0	86 ± 10	3.35 ± 1.0	1290	Control
2.5 ± 0.6	65 ± 14	ND	700	Y27632 (ROCK inhibitor)
1.4 ± 0.5	17 ± 6	ND	500	Calmidazolium chloride (calmodulin inhibitor)

Table 1

RBL-2H3 mast cells were treated with cytochalasin D for 15 min prior to stimulation with antigen. In addition cells were treated with the inhibitors ($20\mu\text{M}$) as indicated. The cells were fixed and subsequently examined by DIC.

Inhibitor	Target	Concentration used	Effect on Bleb formation	Effect on membrane ruffle formation
U73122	Phospholipase C	10 μ M	Inhibition	Inhibition
U73343	none	10 μ M	No effect	No effect
Bis indole-maleimide	Protein kinase C	5 μ M	Inhibition	Inhibition
Wortmannin	PI3K	1 μ M	Inhibition	Inhibition
ATK	PLA2	10 μ M	No effect	No effect
Quercetin	PLD but not PLC	10 μ M	Inhibition	No effect
Butan-1-ol	Inhibits PA production by PLD	0.5 %	Inhibition	Inhibition
Butan-2-ol	Control for butan-1-ol	0.5 %	Partial inhibition	Partial inhibition
SB203580	p38 (MAPK)	10 μ M	No effect	No Effect
PD98056	ERK (MAPK)	10 μ M	No effect	No Effect
Y27632	ROCK Kinase	20 μ M	Partial inhibition (25 %)	Slight inhibition
Blebistatin	Myosin-II	50 μ M	Inhibition	Slight inhibition
EGTA	Ca ²⁺ influx	0.5 mM	Inhibition	Slight Inhibition
Calpain inhibitor I	Calpain	100 μ M	No effect	No effect
Calmidazolium chloride	Calmodulin	20 μ M	Inhibition by 80 %	Slight inhibition

Table 2

Effects of several inhibitors on bleb formation and membrane ruffling. For the blebbing assay, RBL-2H3 mast cells were pre-incubated with reagents for 15 min (nocodazole for 2hr) in the presence of cytochalasin D and then stimulated with antigen for 20 min. For the membrane ruffling assay, RBL-2H3 mast cells were pre-incubated with reagents for 15 min (nocodazole for 2hr) and then stimulated with antigen for 10 min. The results have been compiled from three independent experiments.

Reference List

- 1 Kovanen, P. T. (2007) Mast cells: multipotent local effector cells in atherothrombosis. *Immunol.Rev.* **217**, 105-122
- 2 Brown, J. M., Wilson, T. M., and Metcalfe, D. D. (2008) The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clin.Exp.Allergy* **38**, 4-18
- 3 Sayed, B. A., Christy, A., Quirion, M. R., and Brown, M. A. (2008) The master switch: the role of mast cells in autoimmunity and tolerance. *Annu.Rev.Immunol.* **26**, 705-739
- 4 Galli, S. J., Tsai, M., and Piliponsky, A. M. (2008) The development of allergic inflammation. *Nature* **454**, 445-454
- 5 Guidolin, D., Nico, B., Crivellato, E., Marzullo, A., Vacca, A., and Ribatti, D. (2009) Tumoral mast cells exhibit a common spatial distribution. *Cancer Lett.* **273**, 80-85
- 6 O'Lunaigh, N., Pardo, R., Fensome, A., Allen-Baume, V., Jones, D., Holt, M. R., and Cockcroft, S. (2002) Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. *Mol.Biol.Cell* **13**, 3730-3746
- 7 Zouwail, S., Pettitt, T. R., Dove, S. K., Chibalina, M. V., Powner, D. J., Haynes, L., Wakelam, M. J., and Insall, R. H. (2005) Phospholipase D activity is essential for actin localization and actin-based motility in Dictyostelium. *Biochem.J.* **389**, 207-214
- 8 Nagasaki, A. and Uyeda, T. Q. (2008) Screening of genes involved in cell migration in Dictyostelium. *Exp.Cell Res.* **314**, 1136-1146
- 9 Park, J. B., Kim, J. H., Kim, Y., Ha, S. H., Kim, J. H., Yoo, J. S., Du, G., Frohman, M. A., Suh, P.-G., and Ryu, S.-H. (2000) Cardiac phospholipase D2 localizes to sarcolemmal membranes and is inhibited by α -actinin in an ADP-ribosylation factor-reversible manner. *J.Biol.Chem.* **275**, 21295-21301
- 10 Lee, S., Park, J. B., Kim, J. H., Kim, Y., Kim, J. H., Shin, K. J., Lee, J. S., Ha, S. H., Suh, P. G., and Ryu, S. H. (2001) Actin directly interacts with phospholipase D,

- inhibiting its activity. *J Biol Chem.* **276**, 28252-28260
- 11 Farquhar, M. J., Powner, D. J., Levine, B. A., Wright, M. H., Ladds, G., and Hodgkin, M. N. (2007) Interaction of PLD1b with actin in antigen-stimulated mast cells. *Cell Signal.* **19**, 349-358
 - 12 Frigeri, L. and Apgar, J. R. (1999) The role of actin microfilaments in the down-regulation of the degranulation response in RBL-2H3 mast cells. *J.Immunol.* **162**, 2243-2250
 - 13 Sheetz, M. P., Sable, J. E., and Dobereiner, H. G. (2006) Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. *Annu.Rev.Biophys Biomol.Struct.* **35**, 417-434
 - 14 Charras, G. and Paluch, E. (2008) Blebs lead the way: how to migrate without lamellipodia. *Nat.Rev.Mol Cell Biol* **9**, 730-736
 - 15 Charras, G. T., Coughlin, M., Mitchison, T. J., and Mahadevan, L. (2008) Life and times of a cellular bleb. *Biophys J.* **94**, 1836-1853
 - 16 Lammermann, T. and Sixt, M. (2009) Mechanical modes of 'amoeboid' cell migration. *Curr.Opin.Cell Biol*
 - 17 Langridge, P. D. and Kay, R. R. (2006) Blebbing of Dictyostelium cells in response to chemoattractant. *Exp.Cell Res.* **312**, 2009-2017
 - 18 Sahai, E. and Marshall, C. J. (2003) Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat.Cell Biol* **5**, 711-719
 - 19 Cunningham, C. C. (1995) Actin polymerization and intracellular solvent flow in cell surface blebbing. *J.Cell Biol* **129**, 1589-1599
 - 20 Charras, G. T., Hu, C. K., Coughlin, M., and Mitchison, T. J. (2006) Reassembly of contractile actin cortex in cell blebs. *J.Cell Biol* **175**, 477-490
 - 21 Wang, Y., Litvinov, R. I., Chen, X., Bach, T. L., Lian, L., Petrich, B. G., Monkley, S. J., Critchley, D. R., Sasaki, T., Birnbaum, M. J., Weisel, J. W., Hartwig, J., and Abrams, C. S. (2008) Loss of PIP5KIgamma, unlike other PIP5KI isoforms, impairs the integrity of the membrane cytoskeleton in murine megakaryocytes.

- J.Clin.Invest **118**, 812-819
- 22 Friedl, P. (2004) Prespecification and plasticity: shifting mechanisms of cell migration. *Curr.Opin.Cell Biol* **16**, 14-23
- 23 Sroka, J., von Gunten, M., Dunn, G. A., and Keller, H. U. (2002) Phenotype modulation in non-adherent and adherent sublines of Walker carcinosarcoma cells: the role of cell-substratum contacts and microtubules in controlling cell shape, locomotion and cytoskeletal structure. *Int.J.Biochem.Cell Biol* **34**, 882-899
- 24 Wilkinson, S., Paterson, H. F., and Marshall, C. J. (2005) Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nat.Cell Biol* **7**, 255-261
- 25 Wyckoff, J. B., Pinner, S. E., Gschmeissner, S., Condeelis, J. S., and Sahai, E. (2006) . *Curr.Biol* **16**, 1515-1523
- 26 Gadea, G., Sanz-Moreno, V., Self, A., Godi, A., and Marshall, C. J. (2008) DOCK10-mediated Cdc42 activation is necessary for amoeboid invasion of melanoma cells. *Curr.Biol* **18**, 1456-1465
- 27 Su, W., Yeku, O., Olepu, S., Genna, A., Park, J. S., Ren, H., Du, G., Gelb, M., Morris, A., and Frohman, M. A. (2009) FIPI, a Phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. *Mol Pharmacol.* **75**, 437-446
- 28 Cunningham, E., Thomas, G. M. H., Ball, A., Hiles, I., and Cockcroft, S. (1995) Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP₂. *Current Biol.* **5**, 775-783
- 29 Pandey, V., Mihara, S., Fensome-Green, A., Bolsover, S. R., and Cockcroft, S. (2004) Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosol Ca²⁺, degranulation, and membrane ruffling in the cultured RBL-2H3 mast cell-line. *J.Immunol.* **172**, 4048-4058
- 30 Friedman, A. and Beller, D. I. (1987) The effect of adherence on the in vitro induction of cytotoxic activity by macrophages. *Immunology* **61**, 469-474
- 31 Charras, G. T. (2008) A short history of blebbing. *J.Microsc.* **231**, 466-478

- 32 Bretscher, A., Edwards, K., and Fehon, R. G. (2002) ERM proteins and merlin: integrators at the cell cortex. *Nat.Rev.Mol Cell Biol* **3**, 586-599
- 33 Ivetic, A. and Ridley, A. J. (2004) Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology* **112**, 165-176
- 34 Fievet, B. T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D., and Arpin, M. (2004) Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. *J.Cell Biol.* **164**, 653-659
- 35 Zhu, L., Zhou, R., Mettler, S., Wu, T., Abbas, A., Delaney, J., and Forte, J. G. (2007) High turnover of ezrin T567 phosphorylation: conformation, activity, and cellular function. *Am.J.Physiol Cell Physiol* **293**, C874-C884
- 36 Rudge, S. A. and Wakelam, M. J. (2009) Inter-regulatory dynamics of Phospholipase D and the actin cytoskeleton. *Biochim Biophys Acta*
- 37 Monovich, L., Mugrage, B., Quadros, E., Toscano, K., Tommasi, R., LaVoi, S., Liu, E., Du, Z., LaSala, D., Boyar, W., and Steed, P. (2007) Optimization of halopemide for phospholipase D2 inhibition. *Bioorganic & Medicinal Chemistry Letters* **17**, 2310-2311
- 38 Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521-532
- 39 Jones, D. H., Bax, B., Fensome, A., and Cockcroft, S. (1999) ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatamer I. *Biochem.J.* **341**, 185-192
- 40 Skippen, A., Jones, D. H., Morgan, C. P., Li, M., and Cockcroft, S. (2002) Mechanism of ADP-ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J.Biol.Chem.* **277**, 5823-5831
- 41 Jovanovic, O. A., Brown, F. D., and Donaldson, J. G. (2006) An effector domain mutant of Arf6 implicates phospholipase D in endosomal membrane recycling. *Mol.Biol.Cell* **17**, 327-335

- 42 Morelli, A., Chiozzi, P., Chiesa, A., Ferrari, D., Sanz, J. M., Falzoni, S., Pinton, P., Rizzuto, R., Olson, M. F., and Di Virgilio, F. (2003) Extracellular ATP causes ROCK I-dependent bleb formation in P2X7-transfected HEK293 cells. *Mol Biol Cell* **14**, 2655-2664
- 43 Tournaviti, S., Hannemann, S., Terjung, S., Kitzing, T. M., Stegmayer, C., Ritzerfeld, J., Walther, P., Grosse, R., Nickel, W., and Fackler, O. T. (2007) SH4-domain-induced plasma membrane dynamization promotes bleb-associated cell motility. *J.Cell Sci.* **120**, 3820-3829
- 44 Cockcroft, S. (2009) Phosphatidic acid regulation of phosphatidylinositol 4-phosphate 5-kinases. *Biochim Biophys Acta* **1791**, 905-912
- 45 Cockcroft, S. (2001) Signalling roles of mammalian phospholipase D1 and D2. *Cell Mol.Life Sci.* **58**, 1674-1687
- 46 Jones, D. H., Morris, J. B., Morgan, C. P., Kondo, H., Irvine, R. F., and Cockcroft, S. (2000) Type I PIP 5-kinase directly interacts with ARF1 and is responsible for PI(4,5)P₂ synthesis in the Golgi compartment. *J.Biol.Chem.* **275**, 13962-13966
- 47 Leber, W., Skippen, A., Fivelman, Q. L., Bowyer, P. W., Cockcroft, S., and Baker, D. A. (2009) A unique phosphatidylinositol 4-phosphate 5-kinase is activated by ADP-ribosylation factor in *Plasmodium falciparum*. *Int.J.Parasitology* **39**, 645-653
- 48 Scott, S. A., Selvy, P. E., Buck, J. R., Cho, H. P., Criswell, T. L., Thomas, A. L., Armstrong, M. D., Arteaga, C. L., Lindsley, C. W., and Brown, H. A. (2009) Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. *Nat.Chem.Biol* **5**, 108-117
- 49 Tague, S. E., Muralidharan, V., and D'Souza-Schorey, C. (2004) ADP-ribosylation factor 6 regulates tumor cell invasion through the activation of the MEK/ERK signaling pathway. *Proc.Natl.Acad.Sci.U.S.A* **101**, 9671-9676
- 50 Muralidharan-Chari, V., Hoover, H., Clancy, J., Schweitzer, J., Suckow, M. A., Schroeder, V., Castellino, F. J., Schorey, J. S., and D'Souza-Schorey, C. (2009) ADP-ribosylation factor 6 regulates tumorigenic and invasive properties in vivo. *Cancer Res.* **69**, 2201-2209

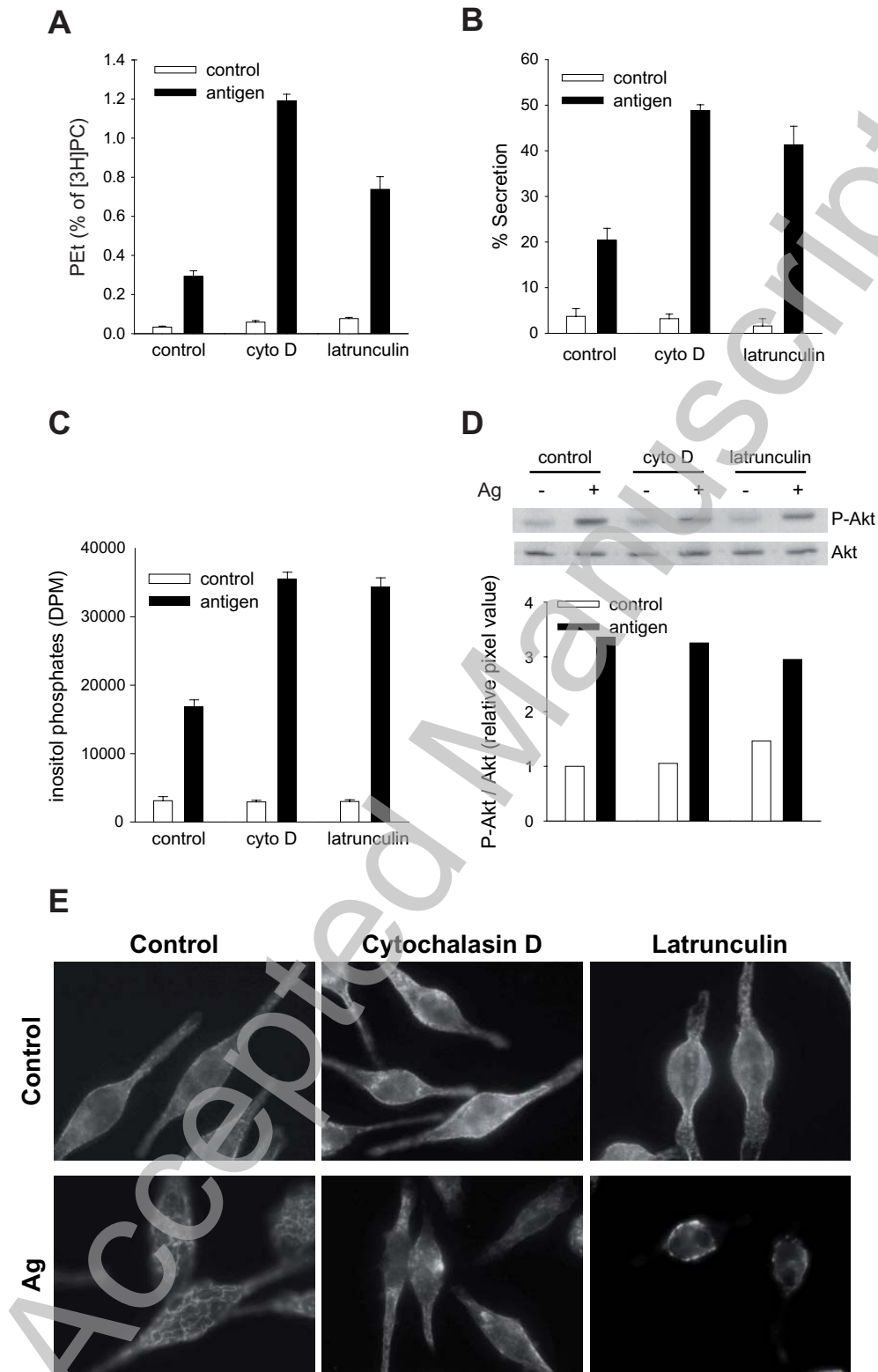


Figure 1

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122

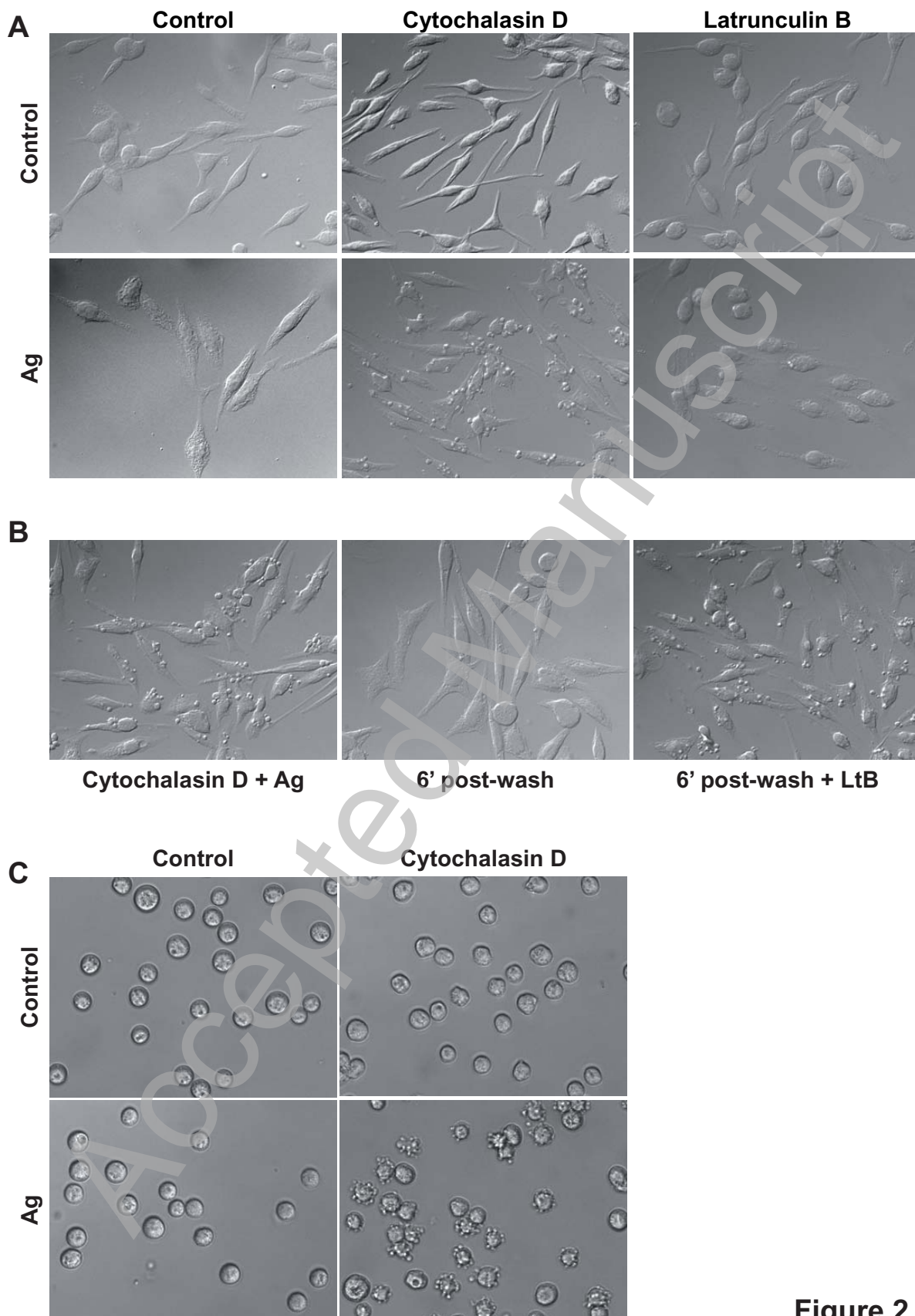


Figure 2

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122

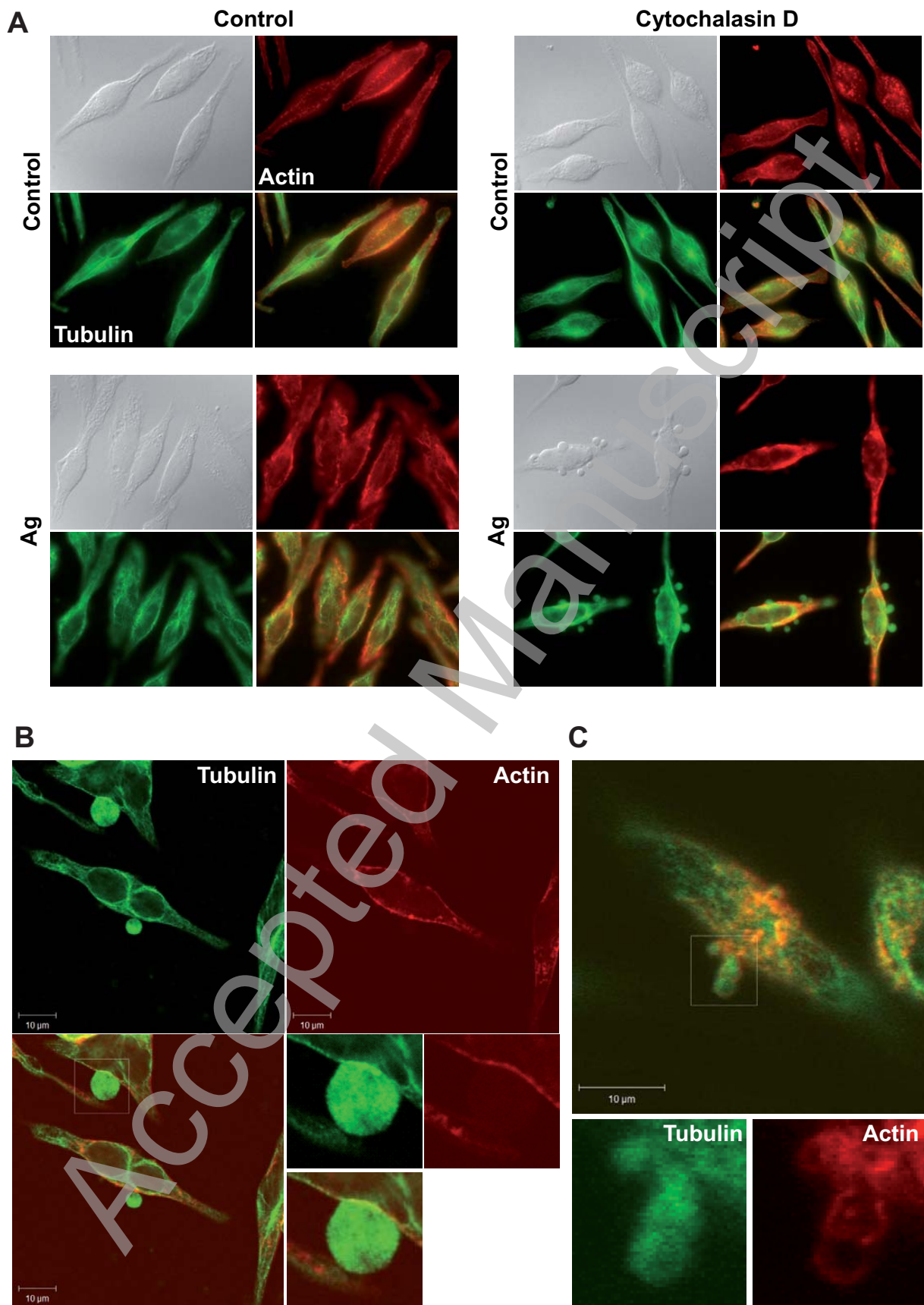


Figure 3

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122

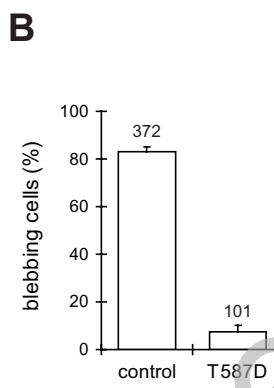
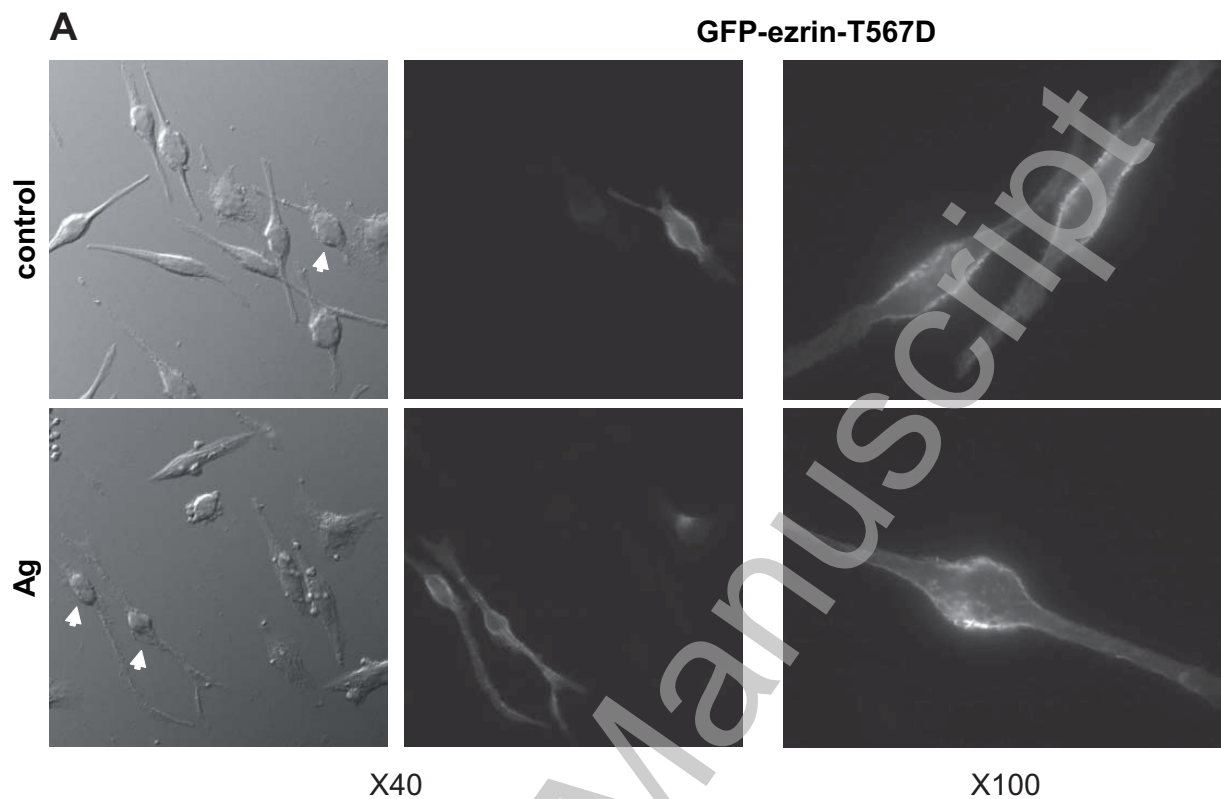


Figure 4

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122

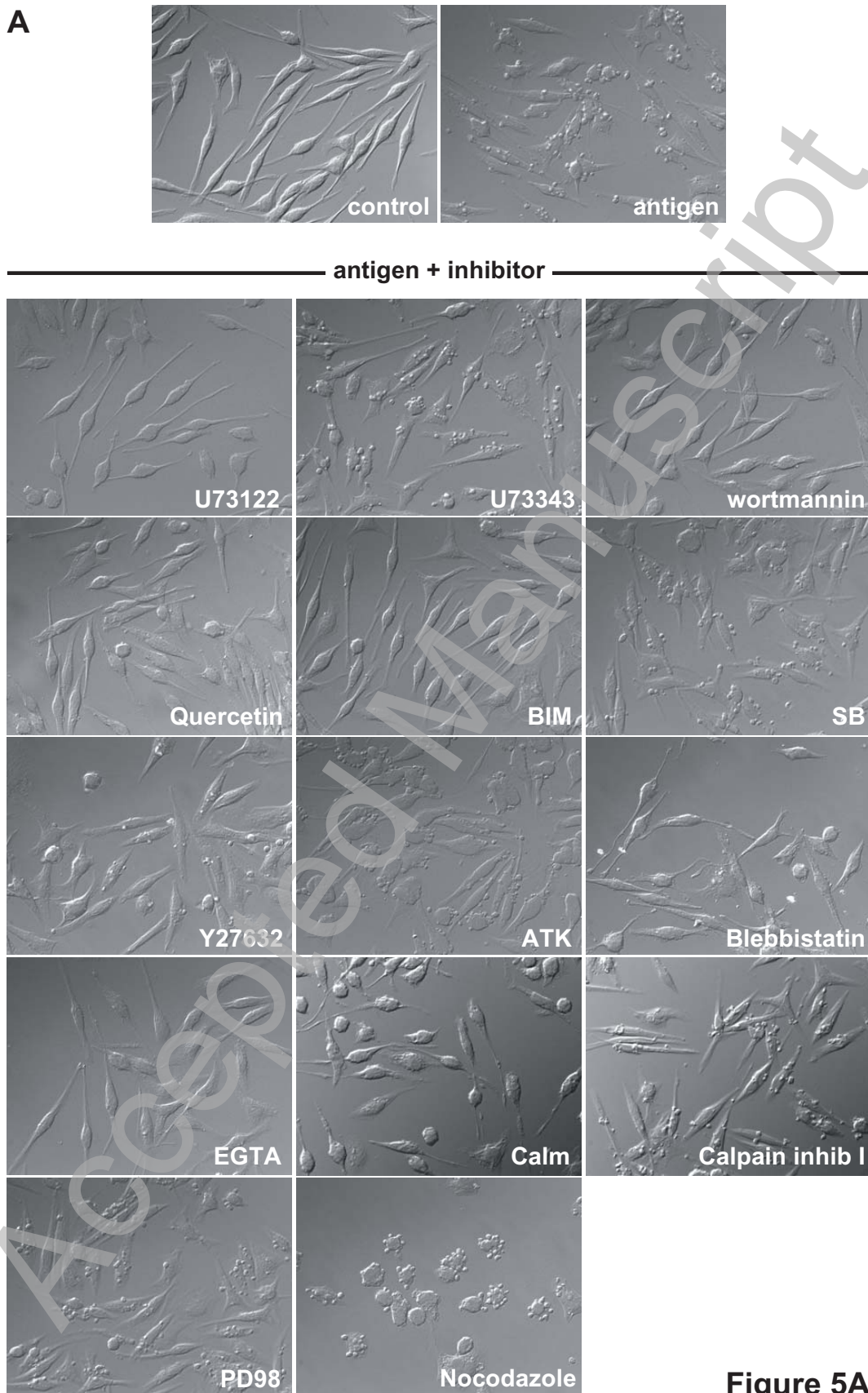


Figure 5A

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122

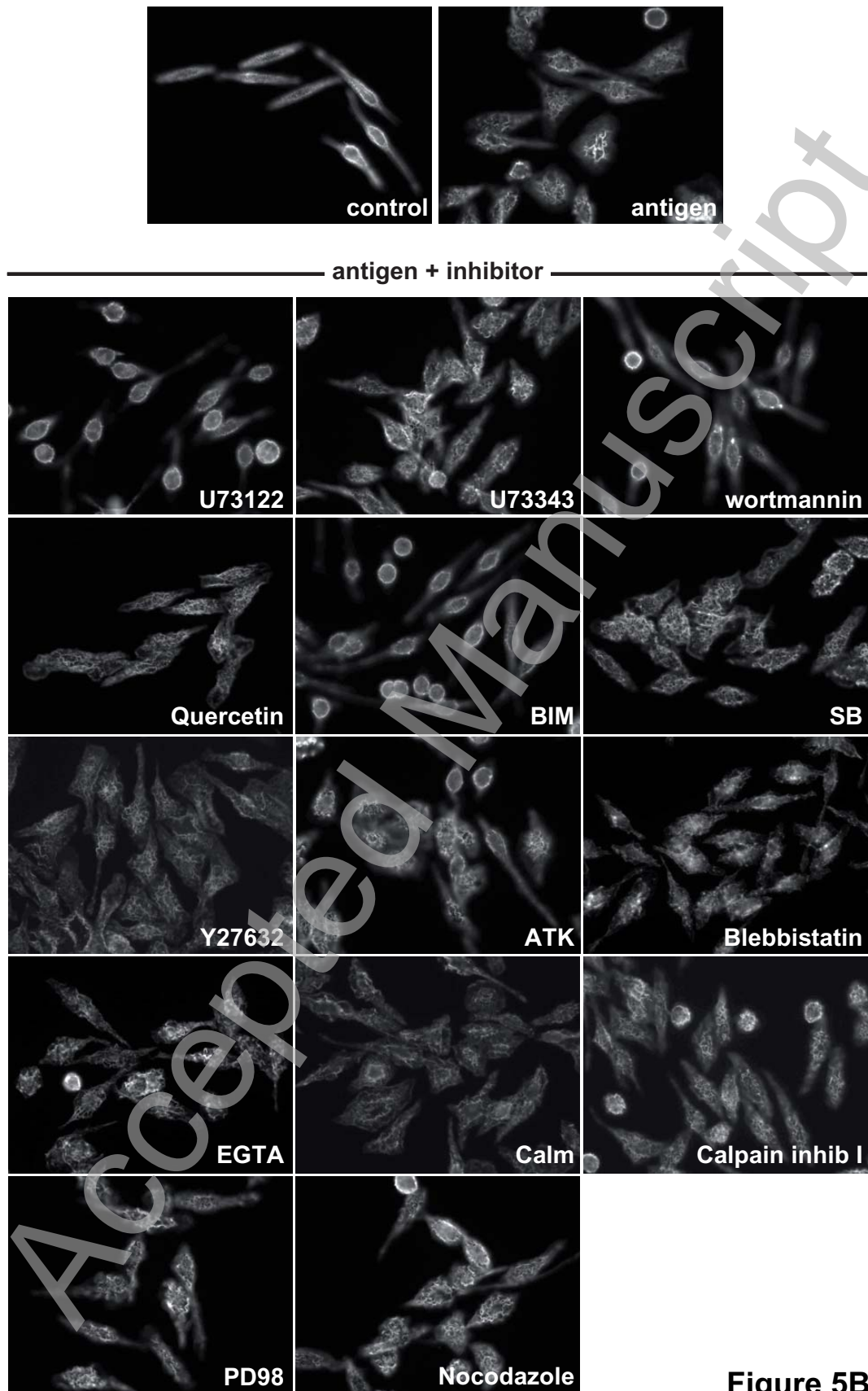


Figure 5B

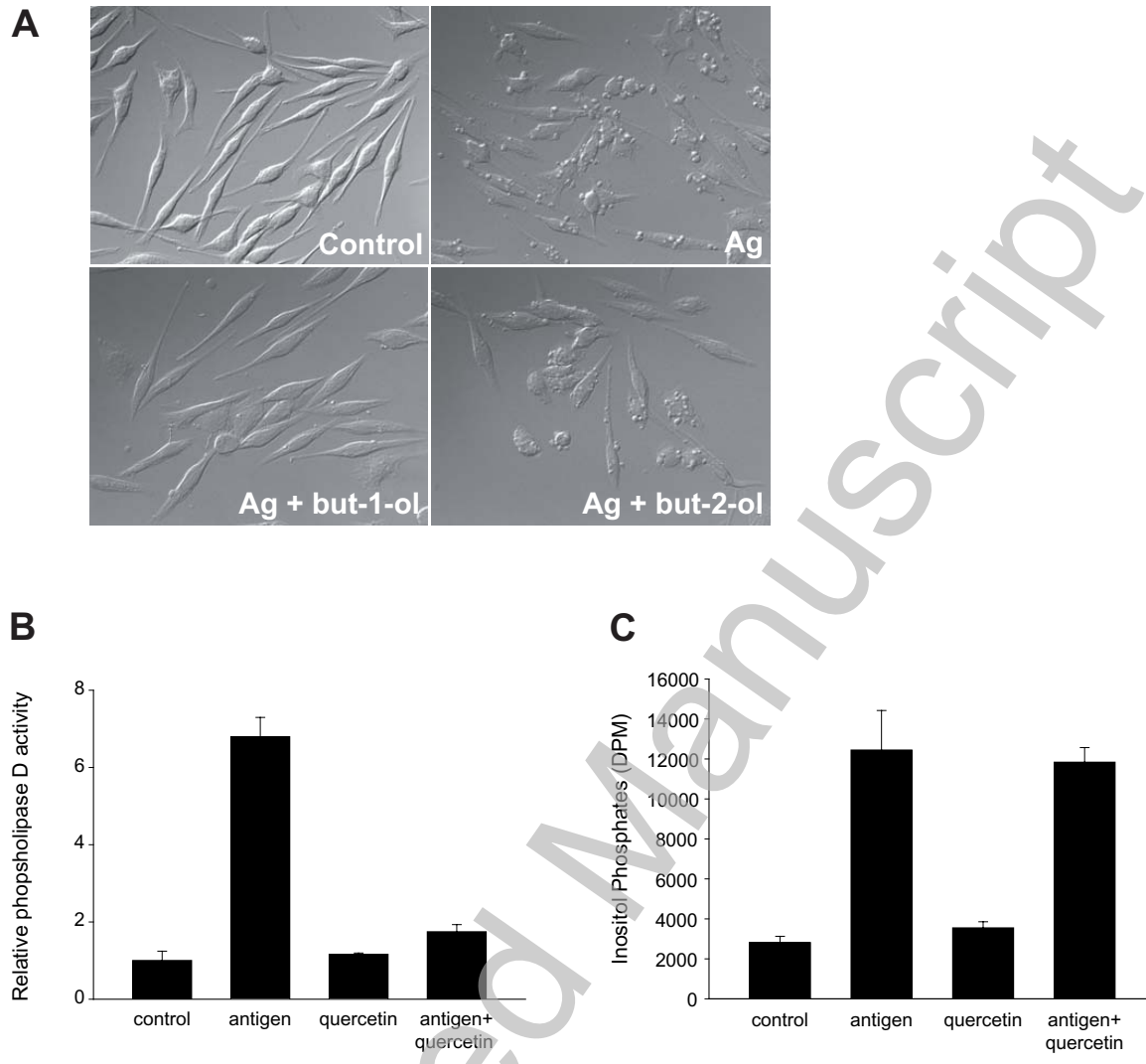


Figure 6

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122

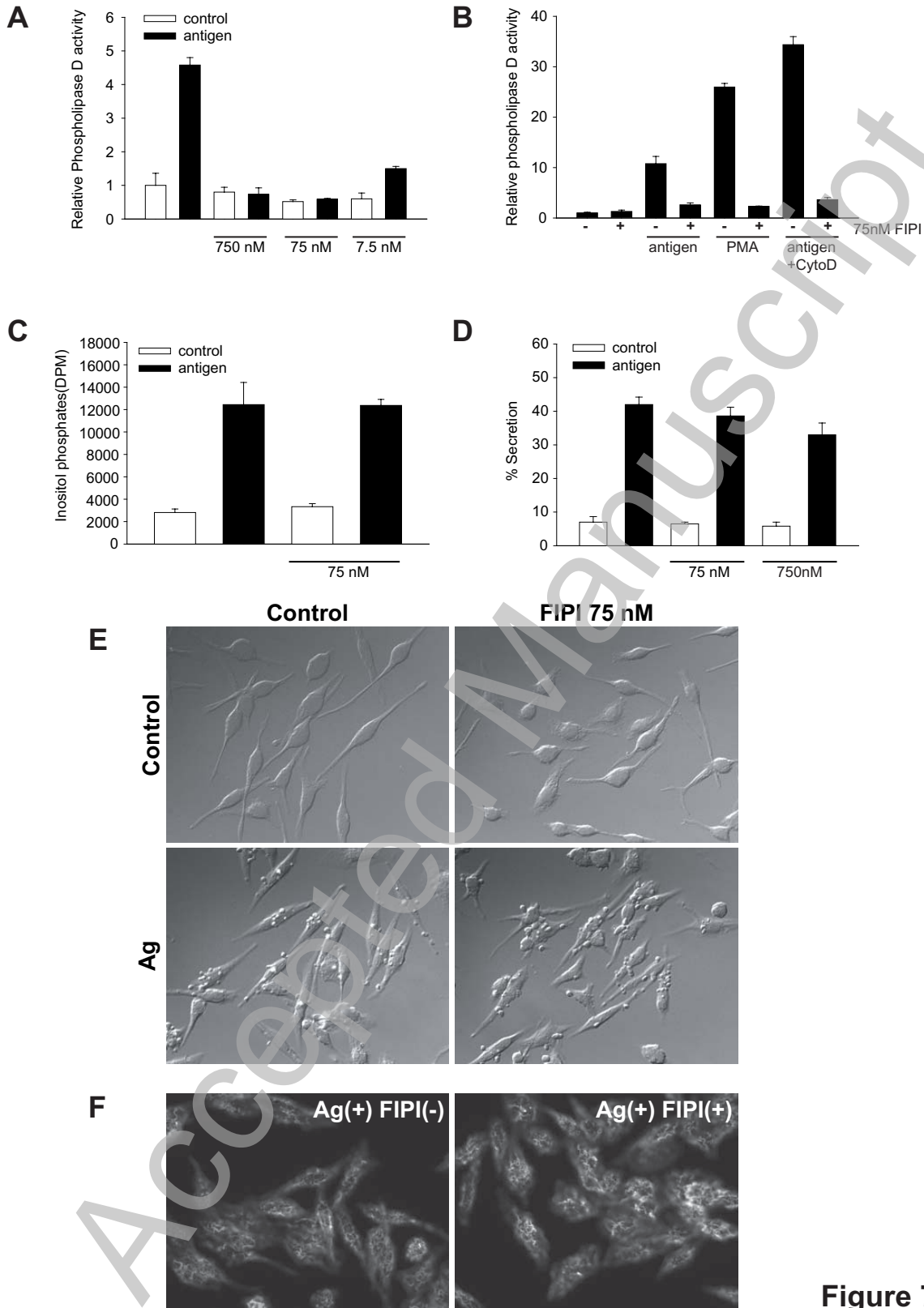
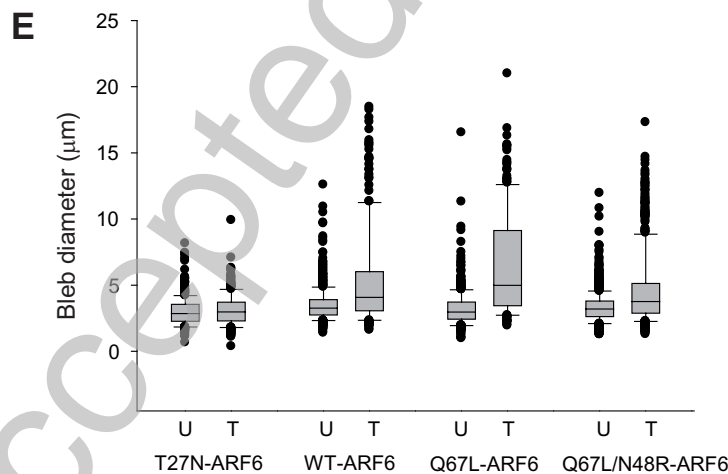
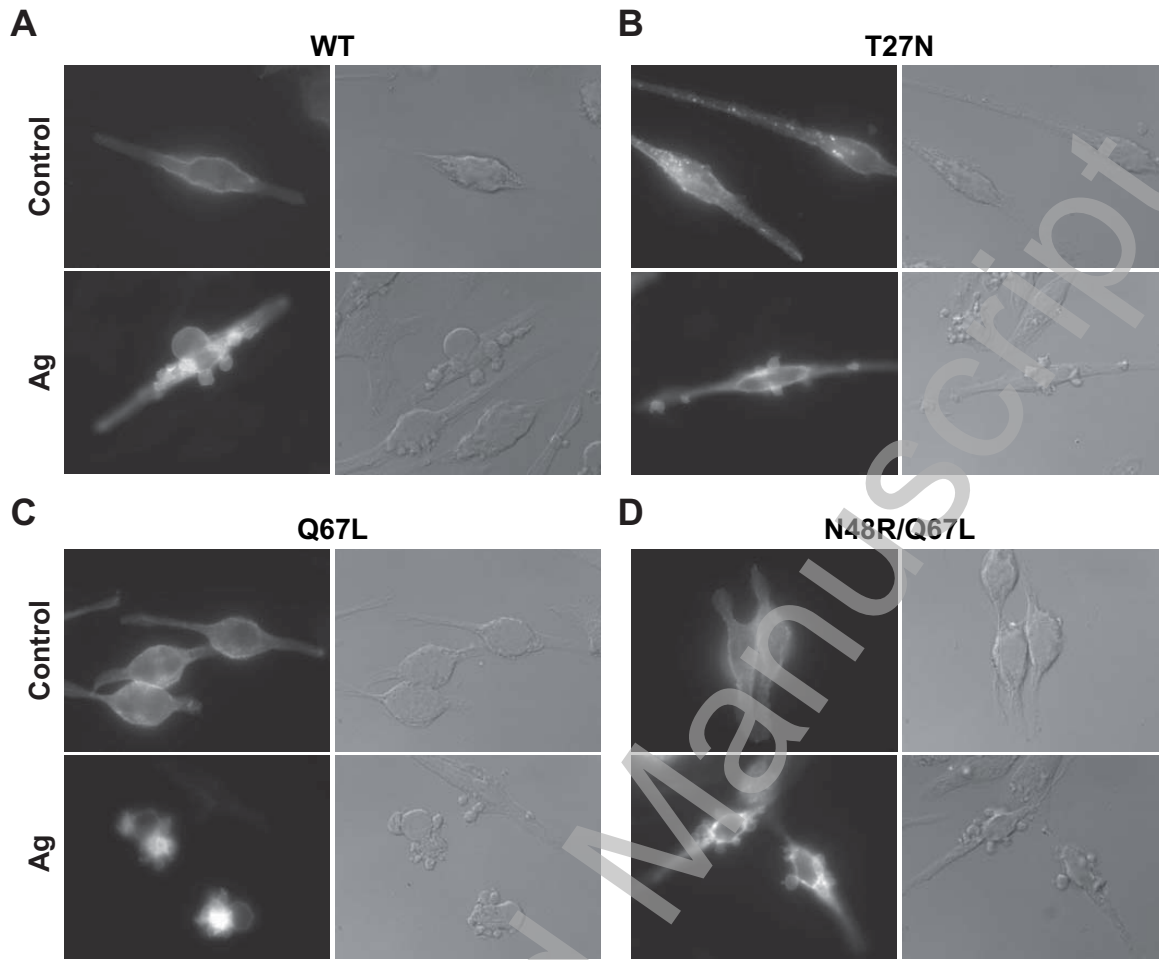


Figure 7

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20091122



	T27N-ARF6		WT-ARF6		Q67L-ARF6		Q67L/N48R-ARF6	
	U	T	U	T	U	T	U	T
Total cell count	649	446	664	249	524	235	946	546
Median	2.86	2.98	3.27	4.07	2.97	4.98	3.20	3.77
Mean	2.98	3.09	3.51	5.44	3.21	6.52	3.31	4.58
SD	0.98	1.12	1.24	3.77	1.31	3.87	1.11	2.70
Variance	0.96	1.26	1.55	14.19	1.72	14.96	1.22	7.30

Figure 8