Published in final edited form as:

Exp Eye Res. 2013 August ; 113: 9–18. doi:10.1016/j.exer.2013.05.002.

PI 3-kinase independent role for AKT in F-actin regulation during outer segment phagocytosis by RPE cells

Ayelen Bulloj, Wei Duan, and Silvia C. Finnemann*

Department of Biological Sciences Center for Cancer, Genetic Diseases, and Gene Regulation, Fordham University, Bronx, NY10458, USA

Abstract

Daily phagocytosis of photoreceptor outer segment fragments (POS) by the retinal pigment epithelium (RPE) is essential for vision. RPE cells use an uptake machinery that is highly similar to the one macrophages use to phagocytose apoptotic cells. In both forms of phagocytosis, particle binding induces phagocyte signaling that is required for F-actin assembly and rearrangement beneath bound particles. Macrophage binding of apoptotic cells stimulates PI3 kinases (PI3K) and AKT kinases (AKT), which may be downstream of PI3K, and PI3K inhibition decreases engulfment. Here, we used specific inhibitory agents to investigate whether and how PI3K and AKT contribute to RPE phagocytosis. Either PI3K or AKT inhibition eliminated AKT activation by RPE cells in response to POS and increased the numbers of POS bound by RPE cells. Analyzing the quality of bound POS, we found a higher fraction of POS associated with F-actin phagocytic cups and myosin II in RPE receiving AKT inhibitor. In these cells, individual POS also recruited more F-actin and myosin II than POS in control cells. In contrast, PI3K inhibition did not alter frequency of phagocytic cups but individual cups contained less F-actin (but similar levels of myosin II) compared to control cups. Annexin AII, another phagocytic cup protein of RPE cells, associated with bound POS regardless of inhibitor treatment. POS engulfment proceeded normally if cells already carried surface-bound POS when receiving inhibitors. However, PI3K inhibition during POS binding blocked subsequent POS engulfment. In striking contrast, AKT inhibition had no effect on POS engulfment. Taken together, these results suggest distinct regulatory roles of PI3K and AKT during POS phagocytosis by RPE cells.

Keywords

signaling; actin cytoskeleton; photoreceptor outer segments; phagocytosis; retinal pigment epithelium

*Please address correspondence to: Silvia C. Finnemann PhD, Larkin Hall, 441 East Fordham Road, Bronx, NY10458, USA, Phone: 001-718-817-3630, Fax: 001-718-817-3514, finnemann@fordham.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors declare no conflict of interest.

1. Introduction

Recruitment of the F-actin cytoskeleton to particles tethered to specific surface receptors of phagocytic cells is a critical step preceding particle ingestion in all forms of phagocytosis. Complex signaling processes that may vary with different particles and/or phagocytes are required for appropriate F-actin rearrangement. The concerted activity of actin-associated proteins ultimately promotes particle engulfment. These include myosin motor proteins and the annexin family of Ca^{2+} - and phospholipid-binding proteins that regulate actin polymerization (Aderem and Underhill, 1999; Diakonova et al., 2002; Hayes et al., 2006; Stendahl et al., 1980; Swanson et al., 1999).

Phosphoinositide 3-kinases (PI3K) are activated in macrophages by phagocytic challenge following $\text{Fc}\gamma$ receptor engagement (Ninomiya et al., 1994) and associate with extending F-actin “phagocytic cup” structures (Kamen et al., 2007; Vieira et al., 2001). Studies of macrophages or fibroblasts including manipulations of PI3K either pharmacologically or genetically have further revealed that PI3K activity is required for engulfment by macrophages regardless whether the phagocytic particles are apoptotic cells (bound to integrins) or opsonized red blood cells (bound to $\text{Fc}\gamma$ receptors) (Leverrier et al., 2003). Inhibitors of PI3K prevent contraction of the phagocytic cup’s distal margin and fusion of membrane vesicles with cup membranes but permit initial F-actin assembly beneath bound particles (Araki et al., 1996; Cox et al., 1999). In addition to particle engulfment, PI3K also contribute to phagosome maturation in leukocytes (reviewed in (Stephens et al., 2002).

Serine-threonine kinases of the AKT family (from here on referred to as AKT) are key downstream effectors of PI3K. However, AKT also fulfills important signaling functions in cells that are independent of PI3K (Mahajan and Mahajan, 2012). Phagocytic challenge with apoptotic cells activates AKT and increases survival of macrophages in an AKT-dependent and growth factor-independent manner (Reddy et al., 2002). Whether AKT plays a role in the phagocytic uptake process itself has not yet been specifically studied.

In the retina, the daily clearance phagocytosis of shed photoreceptor outer segment fragments (POS) by the adjacent retinal pigment epithelium (RPE) is critical for photoreceptor function and, thus, vision. As RPE cells are post-mitotic in the mammalian eye they likely phagocytose more material over a lifetime than any other cell type. Even minor inefficiencies in POS clearance from the retina will gradually cause accumulation of undigested photoreceptor components in RPE cells, ultimately resulting in damage to photoreceptors and vision loss. RPE cells use a molecular mechanism for POS uptake that is very similar to the mechanism used by other cell types to take up apoptotic cells. Diurnal exposure of phosphatidylserine on rod outer segment tips allows binding of the phosphatidylserine binding protein MFG-E8 to tips (Nandrot et al., 2007; Ruggiero et al., 2012). Engagement of $\alpha\text{v}\beta 5$ integrin receptors of the RPE by MFG-E8-decorated rod tips initiates at least two distinct signaling pathways one of which activates RPE tyrosine kinases including focal adhesion kinase (FAK) and Mer tyrosine kinase (MerTK) (Feng et al., 2002; Finnemann, 2003; Nandrot et al., 2004) while the other one activates Rac1 promoting F-actin recruitment in phagocytic cups (Mao and Finnemann, 2012). Phosphorylated MerTK may interact with numerous cytoplasmic signaling and adaptor proteins that possess Src-

homology 2 domains including a regulatory subunit of PI3K (Shelby et al., 2013). Annexin AII and non-muscle myosin II have both been shown to localize to nascent phagosomes in RPE cells and both are required for internalization of RPE surface-bound POS (Law et al., 2009; Strick et al., 2009).

Here, we aimed to decipher contributions of PI3 and AKT kinases to POS phagocytosis by RPE cells. Dissecting effects of short-term inhibition of either kinase family using pharmacological agents during POS binding in synchronized POS uptake assays we found that, as seen in other forms of phagocytosis, PI3K inhibition weakens F-actin association with bound particles and decreases POS engulfment. In contrast, AKT inhibition enhances POS phagocytosis by specifically increasing POS binding and recruitment of F-actin and myosin II to bound POS. AKT inhibition does not alter the POS engulfment process in itself. These results imply an important role for AKT in regulating POS phagocytosis by RPE cells that is distinct and independent of PI3K function.

2. Materials and methods

Reagents were purchased from Sigma (St Louis, MO) or Invitrogen (Carlsbad, CA) unless otherwise stated.

2.1. Antibodies and pharmacological agents

Primary antibodies against the following proteins were used: total AKT, porin (both Cell Signaling, Danvers, MA), activated, phosphoserine-473-AKT (pAKT, Covance, Princeton, NJ), actin (Cytoskeleton, Denver, CO), myosin IIA (Sigma) and annexin AII (Genetex, Irvine, CA). Pharmacological agents were used at the following final concentration: PI3K inhibitor LY294002 (LY, 50 μ M) (Tocris Bioscience, Ellisville, MO), AKT inhibitors III (5 μ M), $\times$ (5 μ M), XI (100 nM), Integrin linked kinase inhibitor Cpd22 (ILK, 50 μ M (all EMD Millipore, Billerica, MA), mTORC2 inhibitor OSI-027 (mT, 25 μ M) (Selleckchem, Houston, TX). AKT inhibitors X and XI were stored frozen in ddH₂O as 1000-fold stock solutions. LY29002, AKT inhibitor III, Cpd22, and OSI-027 were stored frozen in DMSO as 1000-fold stock solutions. Control samples (CTL) received 0.1% DMSO solvent alone. No differences were observed in any experiment between samples that received 0.1% DMSO or 0.1% ddH₂O as control.

2.2. Cell culture

Rat RPE derived, immortalized RPE-J cells (ATCC, Manassas, VA) were routinely maintained at 32°C and 8% CO₂ in DMEM supplemented with 4% FBS (CELLect Gold, ICN, Irvine, CA) and were sub-cultured every 7 days. Before use for experiments, cells were grown in multi-well plates with or without glass coverslips to post-confluent, polarized monolayers for 6 days as described previously (Finnemann and Rodriguez-Boulan, 1999).

2.3. POS phagocytosis assays

POS were purified from porcine eyes obtained fresh from a local slaughterhouse according to established procedures (Finnemann et al., 1997). As appropriate, purified POS were covalently labeled with fluorescent dye by incubation with 0.1 mg/ml FITC isomer I for 1.5

hours on a rocker in the dark at room temperature. Cells were fed with 10 POS/cell in serum-free DMEM supplemented with 1.25 µg/ml recombinant mouse MFG-E8 (R&D Systems, Minneapolis, MN) either at 32°C for the duration of the experiment or, in pulse-chase experiments, for 1 hour at the restrictive temperature of 20°C that allows only POS binding (Finnemann and Rodriguez-Boulan, 1999). After POS incubation, cells were washed three times with phosphate buffered saline with 0.1 mM CaCl₂ (PBS-CM) before harvest or continued incubation at 32°C in DMEM with 5% delipidated FBS (Cocalico Biologicals, Reamstown, PA) to permit internalization of bound POS. For quantification of FITC-POS by fluorescence scanning, cells were fixed with ice-cold methanol. For exclusive detection of internalized particles, fluorescence of surface-bound FITC-POS was selectively quenched by incubation in 0.2% trypan blue in PBS-CM for 10 min before cell fixation. Total and internal FITC-POS were measured by fluorescence scanning using a Typhoon Trio Imager and quantified with ImageQuant™ TL 7.0 (both GE Healthcare, Waukeesha, WI) as described in detail previously (Finnemann and Rodriguez-Boulan, 1999).

2.4. Protein electrophoresis and immunoblotting

Samples obtained using a G-actin/ F-actin in vivo assay kit (Cytoskeleton) following the manufacturer's instructions or HNTG cell lysates were supplemented with reducing SDS sample buffer and boiled for 3 min before separation on SDS-polyacrylamide gels using standard protocols. For opsin detection, samples were not boiled. Proteins were transferred to nitrocellulose membranes, incubated with primary and appropriate horseradish peroxidase-conjugated secondary antibodies followed by ECL enhanced chemiluminescence detection (Perkin-Elmer, Waltham, MA). X-ray films were scanned and processed using Photoshop CS4 (Adobe, San Jose, CA). Bands were quantified by densitometry using ImageQuant.

2.5. Immunofluorescence microscopy

Polarized RPE-J cells on glass coverslips were treated as appropriate before fixation with 4% paraformaldehyde in PBS-CM for 20 minutes at room temperature. For F-actin and annexin AII staining, fixed cells were permeabilized with 0.5% Triton-X100 in PBS-CM for 15 minutes at room temperature before labeling with AlexaFluor594-conjugated phalloidin or annexin AII antibody and AlexaFluor594-conjugated secondary antibody. For myosin II staining, cells were incubated with pre-extraction buffer containing 50 mM MES, 5 mM MgCl₂, 3 mM EGTA, pH 6.4, 0.5% Triton X-100 for 30 seconds at room temperature before fixation as above and labeling with myosin II and AlexaFluor594-conjugated secondary antibody. Nuclei were counterstained with DAPI. Images were acquired on a Leica TSP5 laser scanning confocal microscopy system (Leica, Mannheim, Germany) using sequential scanning mode and compiled using Photoshop CS4. Quantification of F-actin or myosin II beneath bound POS was performed using Adobe Photoshop CS4 to quantify F-actin or myosin immunofluorescence signals in single x-y confocal scans beneath POS and normalizing for POS size.

2.6. Statistical analysis

All experiments were performed at least three times independently with triplicate samples. Samples were first analyzed using one-way ANOVA to determine significant variance

among groups. If significance was established, difference of selected treated samples to control sample was determined using Bonferroni's multiple comparison test. Student's *t*-test was used to determine the difference between two samples where appropriate. *P* values < 0.05 were considered a statistically significant difference.

3. Results

3.1. POS challenge activates AKT of RPE cells and its specific inhibition increases POS phagocytosis during prolonged POS challenge

To assess the role of AKT in RPE phagocytosis, we first tested if POS particle challenge induces AKT phosphorylation at its serine residue 473 (Ser473), which fully activates the enzymatic function of AKT kinases (Balendran et al., 1999; Persad et al., 2001). As experimental model, we chose the stable RPE-J cell line, which retains the POS binding and engulfment mechanism of primary RPE cells that uses the $\alpha v \beta 5$ integrin-FAK-MerTK/Rac1 pathway (Finnemann, 2003; Mao and Finnemann, 2012). Challenge with POS significantly increased AKT phosphorylation within 15 min as compared to control incubation with assay medium alone (Fig. 1). AKT phosphorylation declined if the duration of POS challenge was longer than 30 min suggesting that AKT activity might be relevant during early events of the phagocytosis process.

To study the effect of AKT inhibition during POS phagocytosis, we next tested AKT activation in the presence of pharmacological agents that act via different mechanisms to inhibit AKT: 1- LY294002 inhibits activity of PI3K and, indirectly, AKT activity (Vlahos et al., 1994); 2- AKT inhibitor III is a substrate competitive phosphatidylinositol analogue that inhibits PI3K and AKT activity (Hu et al., 2000); 3- AKT inhibitor X inhibits specifically AKT phosphorylation and kinase activity in a PH domain-independent manner (Thimmaiah et al., 2005); 4- AKT inhibitor XI is a copper complex that interacts with both the PH domain and the kinase domain specifically of AKT inhibiting its kinase activity (Hu et al., 2000). Figure 2A shows that each drug was effective at the concentration used (listed in *Materials and Methods*) in that it prevented AKT activation in response to POS. We next compared total (bound plus internalized) and internal POS content of RPE cells after continuous challenge for 7 hours with FITC-POS in the presence or absence of the different inhibitors. We chose this extended time period to allow both POS binding and engulfment to occur. As expected given its known effect on phagocytosis by other cell types, PI3K inhibitor significantly reduced the level of total POS and even to greater extent internal POS (Fig. 2B and C, bars LY). In contrast, the AKT specific inhibitors X and XI both significantly increased total and internal POS (Fig. 2B and C, bars X and XI). Moreover, the joint PI3K and AKT inhibitor III did not affect POS uptake. We previously established that RPE-J cells are slow phagocytes such that little degradation of engulfed POS occurs within 8 hours after initial POS challenge (Finnemann et al., 2002). However, PI3K inhibitor might promote earlier onset and faster degradation and thus loss of FITC-POS from the cells within the 7-hour period of our assays. To test this possibility, we repeated our uptake experiments for the much shorter time period of 3 hours. We found that the relative decrease compared to control in both total and internal POS caused by PI3K inhibitor during 3 hours was even greater than the decrease caused during 7 hours (Fig. 2, compare LY bars of 2D to

2B, and of 2E and 2C). Taken together, these results show that PI3K inhibition decreases POS internalization rather than accelerating degradation of engulfed POS. In contrast, AKT inhibition does not decrease POS internalization but increases total and internal POS acquired by cells fed for 7 hours.

3.2. AKT inhibition increases POS binding without effect on subsequent internalization

To get more precise insight into the contribution of AKT to POS phagocytosis, we next sought to separately analyze binding and internalization steps. We took advantage of the shared characteristic of primary and immortalized RPE cells in culture of normal POS binding capacity but inability of engulfment when the incubation temperature is lowered to 20°C (Mayerson and Hall, 1986; Finnemann and Rodriguez-Boulan, 1999). Like at physiological temperature, RPE cells bind POS via the MFG-E8- $\alpha v \beta 5$ integrin mechanism at 20°C (Mao and Finnemann, 2012). Raising the temperature to physiological levels rapidly and completely reverses the engulfment blockade and engages the RPE's known engulfment mechanisms via Rac1 and MerTK indicating that POS binding at 20°C is productive via physiologically relevant pathways (Mao and Finnemann, 2012). Here, we first corroborated that AKT is activated by POS at 20°C and that all inhibitors used are also effective under this experimental condition (Supp. Fig. 1). For the remainder of our study, we decided to focus on comparing specific inhibition of PI3K by LY294002 (but which also inhibits AKT activation by POS, see Fig. 2A, Supp. Fig. 1) to specific inhibition of AKT alone by AKT inhibitor XI. Quantifying POS binding by RPE cells during challenge with FITC-POS for 1 hour at 20°C we found that both PI3K and AKT inhibitor significantly increased POS binding (Fig. 3A). To determine whether the inhibitors specifically affected the internalization process, we first allowed POS pre-binding to the RPE cell surface for 1 hour at 20°C without inhibitors, washed off unbound POS, and then continued incubation with or without inhibitors for 5 hours at the permissive, normal growth temperature allowing engulfment of bound POS. Unexpectedly, cells internalized bound POS equally efficiently in the presence of solvent or when PI3K or AKT were inhibited (Fig. 3B). Next, we allowed POS binding at 20°C with or without inhibitors before removing unbound POS and continuing incubation at the permissive temperature without inhibitors. These experiments showed that PI3K inhibition during POS binding greatly reduced subsequent POS internalization (Fig. 3C). In contrast, AKT inhibition during POS binding did not significantly affect subsequent POS internalization (Fig. 3C).

Phosphorylation of AKT Ser473 does not occur by autophosphorylation but in other cell types requires activity of other cytosolic kinases such as Integrin linked kinase (ILK) (Nho et al., 2005) or mTORC2 (mT, the only kinase thus far shown to directly phosphorylate AKT Ser473) (Jacinto et al., 2006; Sarbassov et al., 2005). Here, we found that addition of ILK inhibitor during POS binding affected POS uptake like PI3K inhibitor, increasing POS binding and inhibiting internalization (Fig. 3D and E). In parallel experiments, mT inhibitor had no significant effect on POS binding or internalization (Fig. 3D and E). These intriguing results illustrate the complexity of phagocytic signaling pathways upstream of AKT that remain to be explored in depth in separate studies.

Taken together, these results show that inhibition of PI3K, ILK, or AKT alters POS phagocytosis during the binding phase. While inhibition of any of the three kinases during the binding phase increases POS binding itself, PI3K and ILK inhibition preclude while AKT inhibition does not affect internalization of bound particles. Notably, inhibiting either PI3K or AKT after POS binding to the RPE cell surface has no effect on POS engulfment.

3.3. Inhibition of AKT enhances F-actin recruitment to surface-bound POS

Subsequent to initial $\alpha\text{v}\beta 5$ -integrin dependent POS binding RPE cells assemble actin filaments beneath bound POS that mature to *bona fide* phagocytic cup structures, which eventually allow membrane fusion and thus engulfment (Chaitin and Hall, 1983; Mao and Finnemann, 2012). In macrophages, PI3K inhibition by LY294002 reduces phagocytosis by impeding phagocytic cup closure but not by blocking phagocytic cup formation (Araki et al., 1996). To optimize detection of phagocytic cups in our discontinuous phagocytosis assays we tested F-actin colocalization with bound POS in RPE cells after different times of POS challenge at 20°C. Because it showed a slightly (albeit not statistically significantly) higher percentage of bound POS with F-actin phagocytic cups we chose a 1-hour duration of POS challenge for all further experiments (Supp. Fig. 2). We now compared effects of PI3K and AKT inhibition on F-actin assembly beneath bound POS. Incubation of RPE cells with inhibitors without POS did not visibly alter F-actin organization or content of RPE cells (Fig. 4A). However, when co-incubating inhibitors and POS at 20°C and examining F-actin recruited to surface-bound POS, we observed that bound POS were associated with F-actin more frequently in cells treated with AKT inhibitor as compared to either control cells or cells treated with PI3K inhibitor (Fig. 4B). Differences in local F-actin beneath POS were not secondary to alterations in stress fibers, which were unaffected by the inhibitors (Fig. 4C). Quantification revealed that AKT inhibitor increased the fraction of F-actin-positive bound POS to 71% on average, from 38% and 41% in control and PI3K inhibited RPE cells, respectively (Fig. 5A). Furthermore, we observed brighter F-actin labeling beneath bound POS in cells treated with AKT inhibitor but fainter F-actin beneath bound POS in cells treated with PI3K inhibitor (Fig. 4B, compare insets). Quantification of F-actin intensity beneath POS revealed that on average, 45% more F-actin associated with bound POS in cells treated with AKT inhibitor than in control cells (Fig. 5B). In contrast, bound POS in cells treated with PI3K inhibitor were associated with 80% less F-actin than bound POS in control (Fig. 5B). In these experiments, inhibitor treatments did not cause changes in total actin protein content (Fig. 5C, upper panel), cellular F-actin/G-actin ratio (Fig. 5C, lower panel). These data suggest that AKT inhibition during the POS binding phase specifically facilitates F-actin assembly beneath particles increasing both the fraction of F-actin-associated bound POS and yielding more substantial phagocytic cups compared to solvent control.

F-actin recruitment to phagocytic cups is a dynamic process (May and Machesky, 2001). Accelerated recruitment of F-actin to bound POS or slower dissociation of F-actin from phagocytic cups or both could yield the higher fraction of bound POS with F-actin in cells treated with AKT inhibitor at the time point tested. To distinguish between these possibilities we challenged RPE cells with POS at 20°C for only 30 minutes with or without AKT inhibitor, washed away unbound POS and continued incubation at 20°C for another 30

minutes with solvent alone in control cells and with AKT inhibitor in cells that had bound POS with AKT inhibitor. After POS challenge for 30 minutes at 20°C the fraction bound POS associated with F-actin was the same whether or not the AKT inhibitor had been present (Fig. 5D, 0 min chase) suggesting that initial recruitment of F-actin occurs at a similar rate in both conditions. However, during the additional 30-minute incubation at 20°C, the fraction of bound POS associated with F-actin declined significantly in control cells (Fig. 5D, 30 min chase, white bar), illustrating the transient nature of F-actin association with bound particles (May and Machesky, 2001). In contrast, the frequency of F-actin associated bound POS not only did not decline but actually slightly increased in cells treated with AKT inhibitor (Fig. 5D, 30 min chase, black bar). These results suggest that AKT inhibition promotes recruitment of F-actin to bound POS at least in part by stabilizing F-actin beneath POS.

3.4. Neither PI3K nor AKT inhibition affect Annexin AII relocation to bound POS

The actin regulator annexin AII plays a role in F-actin phagocytic cup formation during RPE phagocytosis and localizes to nascent POS phagosomes (Law et al., 2009). Here, we tested whether annexin AII relocated to sites of surface-bound POS at the restricted temperature of 20°C and whether PI3K or AKT inhibition altered annexin AII localization. Annexin AII immunolabeling did not change if RPE cells were incubated with assay medium with solvent or either inhibitor (Fig. 6A). Furthermore, neither PI3K nor AKT inhibitor affected annexin AII redistribution to sites of surface-bound POS during a 1-hour challenge with POS at 20°C (Fig. 6B). Thus, differences in F-actin association with bound POS in inhibitor-treated cells are not due to lack of annexin AII mobilization. Furthermore, annexin AII relocates to sites beneath surface-bound POS at 20°C and even if F-actin phagocytic cup assembly is impaired by PI3K inhibition.

3.5. Inhibition of AKT increases myosin II localization beneath bound POS

The F-actin motor protein myosin II localizes to F-actin phagocytic cups in RPE cells (Strick et al., 2009). Like annexin AII, myosin II did not change localization in resting RPE cells incubated with PI3K or AKT inhibitor (Fig. 7A). Neither inhibitory agent changed total cellular myosin II protein levels of RPE cells (Supp. Fig. 3). After a 1-hour POS challenge at 20°C, we observed myosin redistribution to sites of bound POS in RPE cells regardless of inhibitor treatment (Fig. 7B). However, AKT inhibitor increased the frequency of bound POS that were associated with myosin II by 37% (Fig. 7C). In control cells, myosin II displayed faint staining beneath bound POS. In contrast, in cells challenged with POS in the presence of AKT inhibitor robust, bright myosin II rings formed around bound POS (Fig. 7B). On average, three times more myosin II associated with bound POS in cells treated with AKT inhibitor than in control cells (Fig. 7D). In contrast, PI3K inhibitor did not alter the percentage of bound POS associated with myosin II or myosin II staining intensity beneath each bound POS on average (Fig. 7, B – D). In agreement with earlier studies (Strick et al., 2009), differences in myosin protein levels beneath bound POS were not associated with detectable changes in phosphorylation and thus activation of myosin light chains indicating that myosin recruitment precedes and may occur independently of contractility and engulfment (data not shown).

Taken together, these results demonstrate that AKT inhibition during the POS binding phase alters myosin II recruitment just like it alters F-actin recruitment: a larger fraction of bound POS assembles myosin II and more myosin II accumulates beneath individual surface-bound POS particles.

4. Discussion

RPE cells are among the most active phagocytes known in nature. Their uptake machinery is regulated by cytoplasmic signaling mechanisms that ensure synchronized, swift and complete POS clearance in the retina. Understanding the pathways RPE cells employ to regulate their phagocytic activity may eventually allow development of targeted therapeutics that could counteract the deficiencies and delays in POS turnover known to contribute to human retinal diseases like retinitis pigmentosa and age-related macular degeneration (Gal et al., 2000; Sparrow et al., 2011).

We set out to determine the consequences of PI3K and AKT inhibition on RPE phagocytosis of POS. PI3K and AKT are ubiquitous families of kinases with multiple contributions to cell function. Earlier studies showed a requirement for PI3K signaling in several forms of macrophage phagocytosis but its relevance for RPE phagocytosis has remained largely unclear. Moreover, we did not find reports on a specific role of AKT activity in any phagocytic process. Here, we studied effects of short-term PI3K and AKT inhibition during either POS binding or POS engulfment on RPE phagocytosis.

As experimental approach we chose to use pharmacological agents to inhibit kinases in RPE cells to specifically and acutely prevent their activation during POS phagocytosis. Such approach has both strengths and weaknesses. First, we need to consider the possibility of off-target effects of pharmacological agents used. Fortunately, a very large number of reports have convincingly shown over the past decades that the PI3K inhibitor LY294002 is highly specific for PI3K. Here, we further showed that it inhibited its downstream effector AKT as expected. As AKT inhibitors have thus far been used less widely, we initially set out comparing three different agents with well-understood binding sites and mechanisms of action on AKT. All three inhibitors suppressed AKT activation as intended and ultimately affected POS binding in a similar fashion. It is thus highly likely that effects on phagocytosis we observed using these agents were due to AKT inhibition. Second, most cell types co-express several PI3K and AKT family members of each cell type. Leverrier and colleagues showed previously that the 110 β catalytic subunit of PI3K (but not 110 γ and 110 δ subunits) contribute to macrophage clearance phagocytosis (Leverrier et al., 2003). LY294002 used in our experiments inhibits PI3K with highest activity for class IA PI3K containing 110 α , 110 β , or 110 δ catalytic subunits. Testing efficacy of PI3K subunit-specific inhibitors on POS engulfment we found that inhibiting 110 β or 110 δ catalytic subunits during POS binding decreased POS internalization significantly and to similar extent as LY294002 (Supp. Fig. 4). 110 α catalytic subunit inhibitor reduced POS internalization only modestly and not significantly (Supp. Fig. 4). These results further support the important role of PI3K in POS internalization. They suggest that 110 α does not play a major role in this process. Further studies and genetic manipulations of catalytic subunits will be needed

to determine the precise contributions of $\alpha\text{v}\beta 5$ and $\alpha\text{v}\beta 3$ such as whether RPE cells may use either for engulfment or if activity of one requires the other.

A major advantage of the use of pharmacological agents is that they can be added and withdrawn from differentiated cells in culture to change cell activities only at relevant times and with immediate effect. PI3K and AKT pathways both together and independent of each other are important mediators of cell survival and proliferation. Our experiments that showed specific effects of inhibitor treatment during POS binding involved only 1-hour treatments. As a result, we did not observe adverse effects of inhibitor treatment, such as diminished survival, or cytoskeletal changes unrelated to the phagocytic process we focused on. Equally importantly, short-term drug use allowed us to interfere with kinase activities solely during either the POS binding or the engulfment phase.

The brief peak in AKT phosphorylation between 15 and 30 minutes after POS challenge suggests that AKT is activated primarily during POS recognition/binding. Even with continuous POS challenge, RPE cell phagocytosis, unlike macrophage phagocytosis, is a slow process in which binding precedes engulfment such that very little engulfment is detectable within the first 30 minutes of POS challenge (Finnemann et al., 1997; Finnemann and Rodriguez-Boulan, 1999). Lack of effect of either PI3K or AKT inhibitors on POS engulfment if present only during engulfment further support relevance of signaling activity mainly during POS binding.

Inhibition of either PI3K and indirectly AKT or of AKT alone during POS binding increases the numbers of bound POS by RPE cells. We speculate that PI3K inhibition increases POS binding via AKT. Earlier studies did not find a significant effect of LY294002 on apoptotic cell binding by macrophages in experiments, in which binding and engulfment phases of uptake overlapped (Leverrier et al., 2003). PI3K/AKT pathways may regulate specifically POS binding by RPE cells. As POS and apoptotic cells display the same 'eat me' signals and quantitatively compete for binding by RPE and by macrophages (Finnemann and Rodriguez-Boulan, 1999; Ruggiero et al., 2012), we propose that any discrepancy in signaling during particle binding would be due to differences between RPE and macrophage rather than due to particle differences. Indeed, differences are known between particles binding mechanisms of these two types of professional phagocytes. RPE cells solely use the integrin receptor $\alpha\text{v}\beta 5$ for particle binding (Finnemann et al., 1997). In contrast, macrophages may use $\alpha\text{v}\beta 3$ integrin and additional non-integrin tethering receptors unless $\alpha\text{v}\beta 5$ integrin is specifically activated (Bratton and Henson, 2011; Finnemann and Rodriguez-Boulan, 1999). Our experiments to date have not yet revealed the molecular mechanism through which AKT inhibition increases the POS binding activity of RPE cells. PI3K or AKT inhibitors do not visibly affect the levels or distribution at the apical plasma membrane of $\alpha\text{v}\beta 5$ integrin POS binding receptors (Supp. Fig. 5). It is however possible that AKT inhibition yields higher specific integrin binding activity for instance by affecting integrin-associated proteins as our live cell surface receptor labeling cannot discriminate integrin receptors by affinity state.

Having both promoted POS binding, PI3K and AKT inhibitors exert distinct effects on subsequent recruitment of F-actin beneath bound POS: POS bound by RPE cells while PI3K and AKT are inhibited show phagocytic cups as frequently as POS bound by control RPE

cells but with less F-actin per POS. POS bound while AKT alone is inhibited possess phagocytic cups at higher frequency and containing more F-actin and myosin II per cup. These results imply that PI3K activity promotes and AKT activity reduces phagocytic cup assembly. We propose that RPE cells may use a physiologically normal balance of PI3K and AKT activities induced upon POS recognition to assemble F-actin phagocytic cups of appropriate size and stability for subsequent POS engulfment. We previously showed that RPE cells possess molecular mechanisms to limit phagocytosis and AKT may contribute to them (Nandrot et al., 2012). Whether these pathways affect extent and duration of Rac1 and/or myosin II activity will be the subject of future experiments that are beyond the scope of the current study.

Our finding that PI3K inhibition blocks engulfment of surface-bound POS is in good agreement with earlier reports showing a requirement in engulfment of apoptotic cells and several other types of particles by macrophages (Allen et al., 2005; Araki et al., 1996; Cox et al., 1999; Khelef et al., 2001; Leverrier et al., 2003; Marshall et al., 2001). Moreover, Shelby and colleagues recently reported localization of PI3K regulatory subunit to POS phagosomes in RPE-J cells (Shelby et al., 2013). Our results are the first to show that PI3K inhibitor must be present during particle binding in order to block engulfment. Adding PI3K inhibitor after cells have tethered POS has no effect on engulfment. Given the multiple washes we perform to remove unbound POS at the end of the binding phase we feel that it is unlikely that residual inhibitor that is leftover after the washes affects engulfment. It is however possible that inhibitor that is present during the binding phase continues to alter cell signaling during engulfment in our discontinuous phagocytosis assays.

Our results imply that activities of PI3K in engulfment of bound POS are independent of AKT as inhibition of AKT has no effect on engulfment. We did not find precedent in other studies comparing effects of specific inhibitors of AKT to PI3K inhibition in any form of phagocytosis. Yet, PI3K activity controls proteins other than AKT that are important in phagocytic pathways. Regulator proteins of Rho and ARF family small GTPases may be directly affected by PI3K activity (Beemiller et al., 2006; Innocenti et al., 2003; Wertheimer, et al., 2012). These GTPases participate in phagocytosis mechanisms in general and Rac1 specifically is required for POS internalization by RPE cells (Mao and Finnemann, 2012). Furthermore, PI3K activity contributes to membrane delivery and extension around target particles and phagosome closure (Araki et al., 1996; Cox et al., 1999; Marshall et al., 2001). The specific targets that are modified by PI3K signaling to promote POS engulfment by RPE cells will require further studies. Yet, our results strongly suggest that their function in POS engulfment is independent of AKT activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Yingyu Mao for helpful discussions and advice on phagocytic cup staining and identification. This project was supported by NIH grant R01-EY013295.

References

- Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* 1999; 17:593–623. [PubMed: 10358769]
- Allen LA, Allgood JA, Han X, Wittine LM. Phosphoinositide 3-kinase regulates actin polymerization during delayed phagocytosis of *Helicobacter pylori*. *J. Leukoc. Biol.* 2005; 78:220–230. [PubMed: 15809290]
- Araki N, Johnson MT, Swanson JA. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol.* 1996; 135:1249–1260. [PubMed: 8947549]
- Balendran A, Casamayor A, Deak M, Paterson A, Gaffney P, Currie R, Downes CP, Alessi DR. PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr. Biol.* 1999; 9:393–404. [PubMed: 10226025]
- Beemiller P, Hoppe AD, Swanson JA. A Phosphatidylinositol-3-Kinase-dependent signal transition regulates ARF1 and ARF6 during Fcγ Receptor-Mediated phagocytosis. *PLoS Biol.* 2006; 4:e162. [PubMed: 16669702]
- Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol.* 2011; 32:350–357. [PubMed: 21782511]
- Chaitin MH, Hall MO. The distribution of actin in cultured normal and dystrophic rat pigment epithelial cells during the phagocytosis of rod outer segments. *Invest. Ophthalmol. Vis. Sci.* 1983; 24:821–831. [PubMed: 6345446]
- Cox D, Tseng C-C, Bjekic G, Greenberg S. A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *J. Biol. Chem.* 1999; 274:1240–1247. [PubMed: 9880492]
- Diakonova M, Bokoch G, Swanson JA. Dynamics of cytoskeletal proteins during Fcγ receptor-mediated phagocytosis in macrophages. *Mol. Biol. Cell.* 2002; 13:402–411. [PubMed: 11854399]
- Feng W, Yasumura D, Matthes MT, LaVail MM, Vollrath D. MerTK triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. *J. Biol. Chem.* 2002; 277:17016–17022. [PubMed: 11861639]
- Finnemann SC. Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. *EMBO J.* 2003; 22:4143–4154. [PubMed: 12912913]
- Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires αvβ5 integrin for binding but not for internalization. *Proc. Natl. Acad. Sci. U. S. A.* 1997; 94:12932–12937. [PubMed: 9371778]
- Finnemann SC, Leung LW, Rodriguez-Boulan E. The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99:3842–3847. [PubMed: 11904436]
- Finnemann SC, Rodriguez-Boulan E. Macrophage and retinal pigment epithelium phagocytosis: apoptotic cells and photoreceptors compete for αvβ3 and αvβ5 integrins, and protein kinase C regulates αvβ5 binding and cytoskeletal linkage. *J. Exp. Med.* 1999; 190:861–874. [PubMed: 10499924]
- Gal A, Li Y, Thompson DA, Weir J, Orth U, Jacobson SG, Apfelstedt-Sylla E, Vollrath D. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. *Nat. Genet.* 2000; 26:270–271. [PubMed: 11062461]
- Innocenti M, Frittoli E, Ponzanelli I, Falck JR, Brachmann SM, Di Fiore PP, Scita G. Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. *J. Cell Biol.* 2003; 160:17–23. [PubMed: 12515821]
- Hayes MJ, Shao D, Bailly M, Moss SE. Regulation of actin dynamics by annexin 2. *EMBO J.* 2006; 25:1816–1826. [PubMed: 16601677]
- Hu Y, Qiao L, Wang S, Rong S-b, Meuillet EJ, Berggren M, Gallegos A, Powis G, Kozikowski AP. 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J. Med. Chem.* 2000; 43:3045–3051. [PubMed: 10956212]

- Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates AKT phosphorylation and substrate specificity. *Cell*. 2006; 127:125–137. [PubMed: 16962653]
- Kamen LA, Levinsohn J, Swanson JA. Differential association of phosphatidylinositol 3-kinase, SHIP-1, and PTEN with forming phagosomes. *Mol. Biol. Cell*. 2007; 18:2463–2472. [PubMed: 17442886]
- Khelef N, Shuman HA, Maxfield FR. Phagocytosis of wild-type *Legionella pneumophila* occurs through a wortmannin-insensitive pathway. *Infect. Immun*. 2001; 69:5157–5161. [PubMed: 11447198]
- Law AL, Ling Q, Hajjar KA, Futter CE, Greenwood J, Adamson P, Wavre-Shapton ST, Moss SE, Hayes MJ. Annexin A2 regulates phagocytosis of photoreceptor outer segments in the mouse retina. *Mol. Biol. Cell*. 2009; 20:3896–3904. [PubMed: 19587120]
- Leverrier Y, Okkenhaug K, Sawyer C, Bilancio A, Vanhaesebroeck B, Ridley AJ. Class I phosphoinositide 3-kinase p110 β is required for apoptotic cell and Fc γ receptor-mediated phagocytosis by macrophages. *J. Biol. Chem*. 2003; 278:38437–38442. [PubMed: 12869549]
- Mahajan K, Mahajan NP. PI3K-independent AKT activation in cancers: a treasure trove for novel therapeutics. *J. Cell Physiol*. 2012; 227:3178–3184. [PubMed: 22307544]
- Mao Y, Finnemann SC. Essential diurnal Rac1 activation during retinal phagocytosis requires $\alpha\beta 5$ integrin but not tyrosine kinases focal adhesion kinase or Mer tyrosine kinase. *Mol. Biol. Cell*. 2012; 23:1104–1114. [PubMed: 22262456]
- Marshall JG, Booth JW, Stambolic V, Mak T, Balla T, Schreiber AD, Meyer T, Grinstein S. Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc γ receptor-mediated phagocytosis. *J. Cell Biol*. 2001; 153:1369–1380. [PubMed: 11425868]
- May RC, Machesky LM. Phagocytosis and the actin cytoskeleton. *J. Cell Sci*. 2001; 114:1061–1077. [PubMed: 11228151]
- Mayerson PL, Hall MO. Rat retinal pigment epithelial cells show specificity of phagocytosis in vitro. *J. Cell Biol*. 1986; 103:299–308. [PubMed: 3522605]
- Nandrot EF, Anand M, Almeida D, Atabai K, Sheppard D, Finnemann SC. Essential role for MFG-E8 as ligand for $\alpha\beta 5$ integrin in diurnal retinal phagocytosis. *Proc. Natl. Acad. Sci. U. S. A*. 2007; 104:12005–12010. [PubMed: 17620600]
- Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC. Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking $\alpha\beta 5$ integrin. *J. Exp. Med*. 2004; 200:1539–1545. [PubMed: 15596525]
- Nandrot EF, Silva KE, Scelfo C, Finnemann SC. Retinal pigment epithelial cells use a MerTK-dependent mechanism to limit the phagocytic particle binding activity of $\alpha\beta 5$ integrin. *Biol Cell*. 2012; 104:326–341. [PubMed: 22289110]
- Nho RS, Xia H, Kahm J, Kleidon J, Diebold D, Henke CA. Role of Integrin-linked kinase in regulating phosphorylation of AKT and fibroblast survival in type I collagen matrices through a $\beta 1$ integrin viability signaling pathway. *J. Biol. Chem*. 2005; 280:26630–26639. [PubMed: 15905178]
- Ninomiya N, Hazeki K, Fukui Y, Seya T, Okada T, Hazeki O, Ui M. Involvement of phosphatidylinositol 3-kinase in Fc γ receptor signaling. *J. Biol. Chem*. 1994; 269:22732–22737. [PubMed: 7521331]
- Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase. *J. Biol. Chem*. 2001; 276:27462–27469. [PubMed: 11313365]
- Reddy SM, Hsiao KH, Abernethy VE, Fan H, Longacre A, Lieberthal W, Rauch J, Koh JS, Levine JS. Phagocytosis of apoptotic cells by macrophages induces novel signaling events leading to cytokine-independent survival and inhibition of proliferation: activation of Akt and inhibition of extracellular signal-regulated kinases 1 and 2. *J. Immunol*. 2002; 169:702–713. [PubMed: 12097372]
- Ruggiero L, Connor MP, Chen J, Langen R, Finnemann SC. Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wild-type but not *Itgb5*^{-/-} or *Mfge8*^{-/-} mouse retina. *Proc. Natl. Acad. Sci. U. S. A*. 2012; 109:8145–8148. [PubMed: 22566632]

- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005; 307:1098–1101. [PubMed: 15718470]
- Shelby SJ, Colwill K, Dhe-Paganon S, Pawson T, Thompson DA. MERTK interactions with SH2-domain proteins in the retinal pigment epithelium. *PLoS One*. 2013; 8:e53964. [PubMed: 23390493]
- Sparrow JR, Gregory-Roberts E, Yamamoto K, Blonska A, Ghosh SK, Ueda K, Zhou J. The bisretinoids of the retinal pigment epithelium. *Prog. Retin. Eye Res.* 2011; 31:121–135. [PubMed: 22209824]
- Stendahl OI, Hartwig JH, Brotschi EA, Stossel TP. Distribution of actin-binding protein and myosin in macrophages during spreading and phagocytosis. *J. Cell Biol.* 1980; 84:215–224. [PubMed: 6991506]
- Stephens L, Ellson C, Hawkins P. Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr. Opin. Cell Biol.* 2002; 14:203–213. [PubMed: 11891120]
- Strick DJ, Feng W, Vollrath D. Mertk drives myosin II redistribution during retinal pigment epithelial phagocytosis. *Invest. Ophthalmol. Vis. Sci.* 2009; 50:2427–2435. [PubMed: 19117932]
- Swanson JA, Johnson MT, Beningo K, Post P, Mooseker M, Araki N. A contractile activity that closes phagosomes in macrophages. *J. Cell Sci.* 1999; 112:307–316. [PubMed: 9885284]
- Thimmaiah KN, Easton JB, Germain GS, Morton CL, Kamath S, Buolamwini JK, Houghton PJ. Identification of N10-substituted phenoxazines as potent and specific inhibitors of Akt signaling. *J. Biol. Chem.* 2005; 280:31924–31935. [PubMed: 16009706]
- Vieira OV, Botelho RJ, Rameh L, Brachmann SM, Matsuo T, Davidson HW, Schreiber A, Backer JM, Cantley LC, Grinstein S. Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. *J. Cell Biol.* 2001; 155:19–26. [PubMed: 11581283]
- Vlahos CJ, Matter WF, Hui KY, Brown RF. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 1994; 269:5241–5248. [PubMed: 8106507]
- Wertheimer E, Gutierrez-Uzquiza A, Rosemblyt C, Lopez-Haber C, Sosa MS, Kazanietz MG. Rac signaling in breast cancer: a tale of GEFs and GAPs. *Cell Signal.* 2012; 24:353–362. [PubMed: 21893191]

Highlights

- Photoreceptor outer segment (POS) challenge activates AKT of RPE cells.
- Inhibition of AKT or PI3K increases POS binding by RPE cells.
- PI3K inhibition during POS binding prevents F-actin recruitment to bound POS and their engulfment.
- AKT but not PI3K inhibition increases F-actin and myosin II recruitment to bound POS.

Figure 1. POS challenge activates AKT in RPE cells

A. Immunoblotting detection of activated pSer473-AKT (pAKT upper panel) and total AKT (lower panel) in RPE-J cells lysed following no treatment (/) or incubation with assay medium or POS in assay medium for different periods of time in hours as indicated. A representative blot membrane probed sequentially for pAKT and AKT of three independent experiments is shown. B. Results of densitometry quantification of activated pSer473-AKT. 15 and 30 minutes after POS challenge, AKT phosphorylation was significantly higher in cells fed with POS as compared to cells fed with medium. POS did not maintain

significantly increased AKT phosphorylation at later time points. Bars show relative levels of AKT phosphorylation at times indicated in the figure in cells fed with POS as compared to cells fed with medium for the same period of time (mean $\pm$ SD, n = 3). pAKT ratios were compared by ANOVA and found to significantly vary, with time points indicated by asterisks showing significant difference to the other time points.

Figure 2. Inhibition of either PI3K or AKT abolishes AKT activation by POS but only inhibition of PI3K decreases POS phagocytosis

A. Immunoblotting detection of activated pSer473-AKT (pAKT upper panel) and total AKT (lower panel) in RPE-J cells lysed following incubation for 0.5 hours with assay medium (med) or with POS in assay medium (POS) in the presence of 0.1% DMSO solvent as control (CTL), PI3K inhibitor (LY), or AKT inhibitors III, X, or XI as indicated. A representative blot membrane probed sequentially for pAKT and AKT of three independent experiments is shown. B – E. Quantification of total (bound plus internalized) POS (B, D) or internalized POS (C, E) acquired by RPE-J cells challenged continuously at 32°C with

FITC-POS in the presence of inhibitors or control solvent (as in A) for 7 hours (B, C) or for 3 hours (D, E). Bars show mean $\pm$ SD of three independent experiments with triplicate samples each. Results are shown relative to POS uptake by cells treated with solvent alone, which was set as 1. Asterisks indicate significant difference to control cells established by ANOVA.

Figure 3. Inhibition of PI3K, AKT, or ILK increases POS binding, and inhibition of PI3K or ILK but not AKT reduces POS internalization if inhibitors are present during POS binding
 A. Quantification of POS bound by RPE-J cells during 1-hour incubation with FITC-POS at the restricted temperature of 20°C in the presence of DMSO (CTL), PI3K inhibitor (LY), or AKT inhibitor XI (XI) as indicated. B. Quantification of POS internalized by RPE-J cells during 5-hour incubation at 32°C in medium with inhibitors as indicated subsequent to 1-hour POS binding at 20°C without inhibitors. C. Quantification of POS internalized by RPE-J cells during 5-hour incubation at the permissive temperature of 32°C in inhibitor-free medium subsequent to POS binding in the presence of inhibitors as indicated. D. Quantification of POS bound by RPE-J cells during 1-hour incubation with FITC-POS at the restricted temperature of 20°C in the presence of DMSO (CTL), ILK inhibitor (ILK), or mTORC2 inhibitor (mT) as indicated. E. Fluorescence scanning quantification of POS internalized by RPE-J cells during 5-hour incubation at the permissive temperature of 32°C in inhibitor-free medium subsequent to POS binding in the presence of inhibitors as indicated. Bars show mean $\pm$ SD of three independent experiments with triplicate samples each. Values are normalized to POS content of control cells in each experiment, which was set as 1. Asterisks indicate significant difference to control cells by ANOVA.

Figure 4. F-actin recruitment to bound POS is enhanced by AKT inhibition but decreased by PI3K inhibition

A. Maximal projections of x-y confocal image stacks showing F-actin (red) and cell nuclei (blue) of RPE-J cells fixed and stained immediately following incubation with DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI) for 1 hour at 20°C. Scale bar: 20 μm.

B. Large fields show single focal plane x-y images obtained from the apical cell surface showing bound FITC-POS (green), F-actin (red), and merged image of both as indicated of RPE-J cells challenged with FITC-POS for 1 hour at 20°C in the presence of DMSO (CTL, upper row), PI3K inhibitor (LY, center row) or AKT inhibitor XI (XI, lower row) followed

by immediate fixation and staining. In fields showing single stains, an x-y scan of the cell center of the same field was merged to show cell nuclei (blue) for the purpose of orientation. Arrows point out colocalization in merged images indicating apical F-actin recruited to bound POS to form phagocytic cups. Inset panels show higher magnification of one bound POS particle (top), associated F-actin (center), and merge of both (bottom). Representative fields are shown of a total of three independent experiments. Scale bars: large fields, 10 μm ; inset, 5 μm . C. Single focal plane x-y images showing F-actin stress fibers (red) of RPE-J cells fixed and stained immediately following incubation with POS for 1 hour at 20°C in the presence of DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI). Scale bar: 10 μm .

Figure 5. RPE cells generate more stable phagocytic cups if they bind POS in the presence of AKT inhibitor

A. Fraction of bound POS with associated F-actin in RPE-J cells incubated with FITC-POS for 1 hour at 20°C in the presence of DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI). B. Amount of F-actin associated with bound FITC-POS in RPE-J cells incubated with FITC-POS for 1 hour at 20°C in the presence of DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI). Levels of F-actin per POS are presented normalized for POS size and relative to levels in control cells, which were set as 1. C. Immunoblotting detection of actin, AKT, and porin, which served as loading control, in whole cell lysates obtained from

RPE-J cells after incubation for 1 hour at 20°C with FITC-POS in the presence of DMSO (CTL), PI3 kinase inhibitor (LY) or AKT inhibitor XI (XI) (upper panel). Immunoblotting detection of F-actin (F) and G-actin (G) isolated from RPE-J cells in the same conditions (lower panel). A representative blot membrane is shown of three independent experiments performed. D. Fraction of bound POS with associated F-actin in RPE-J cells incubated with FITC-POS for 30 minutes at 20°C in the presence of DMSO solvent (white bars) or AKT inhibitor XI (black bars) followed by immediate fixation (0 min chase) or wash and further incubation in medium with DMSO (white bar, 30 min chase) or AKT inhibitor XI (black bar, 30 min chase). A, B, D. All bars show mean $\pm$ SD of three independent experiments with duplicate samples each. 150 POS were evaluated in each sample. In A and B, asterisks indicate significant difference to control cells, $p < 0.01$ by ANOVA. In D, asterisks indicates significant difference in fraction of POS with F-actin after 30 minute 20°C chase compared to the same condition at 0 min chase, by Student's t -test, *, $p < 0.01$.

Figure 6. Inhibition of either AKT or PI3K has no effect on annexin AII recruitment to sites of bound POS

A. Maximal projections of x-y confocal image stacks showing annexin AII (red) and cell nuclei (blue) of RPE-J cells fixed and stained immediately following incubation with DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI) for 1 hour at 20°C. Scale bar: 10 μ m.

B. Single focal plane x-y images showing annexin AII (red) and bound FITC-POS at the apical surface of RPE-J cells following FITC-POS challenge for 1 hour at 20°C in the presence of DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI). Representative fields are shown from a total of three independent experiments. 30 POS were evaluated in each sample in each experiment. Scale bar: 5 μ m.

Figure 7. Inhibition of AKT but not of PI3K increases myosin II recruitment to sites of bound POS

A. Maximal projections of x-y confocal image stacks showing myosin II (red) and cell nuclei (blue) of RPE-J cells fixed and stained immediately following incubation with DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI) for 1 hour at 20°C. Scale bar: 10 µm. Representative fields of three independent experiments are shown. B. Single focal plane x-y images showing myosin II (red) and bound FITC-POS at the apical surface of RPE-J cells following FITC-POS challenge for 1 hour at 20°C in the presence of DMSO (CTL), PI3K inhibitor (LY) or AKT inhibitor XI (XI). Scale bar: 5 µm. C. Fraction of bound FITC-POS

with associated myosin II in experiments as in (B). D. Amount of myosin II associated with surface-bound FITC-POS in experiments as in (B). Levels of myosin II per POS are presented normalized for POS size and relative to levels in control cells, which were set as 1. B – D. Experiments were repeated three times independently. Representative images are shown. Bars show mean $\pm$ SD. 30 POS were evaluated in each sample in each experiment. Asterisks indicate significant difference to control cells by ANOVA, $p < 0.01$.