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## Effects of prostaglandin E<sub>2</sub> on the subcellular localization of Epac-1 and Rap1 proteins during Fcγ receptor mediated phagocytosis in alveolar macrophages

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### Abstract

Recent studies have demonstrated a central role for the exchange protein activated by cAMP (Epac) in the inhibition of Fcγ-receptor mediated phagocytosis and bacterial killing by prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in macrophages. However, the subcellular localization of Epac, and its primary target Rap1, has yet to be determined in primary macrophages. Therefore, we used immunofluorescent techniques and phagosome isolation to localize Epac-1 and Rap1 in alveolar macrophages. Epac-1 was predominantly expressed on punctate and tubular membranes throughout the cell body; on the plasma membrane; and co-localized with microtubule organizing centers (MTOCs). Rap1 was abundant on punctate membranes, less abundant on plasma membrane, and also found on MTOCs. Following PGE<sub>2</sub> treatment, Epac-1, but not Rap1, accumulated on the nuclear envelope and disappeared from MTOCs. By immunofluorescent microscopy, both Epac-1 and Rap1 were seen to associate with phagosomes containing IgG-opsonized beads, but this association appeared weak, as we failed to observe such interactions in phagosomes isolated from cells at various timepoints after bead ingestion. Strikingly, however, Epac-1, but not Rap1, appeared to accumulate on maturing phagosomes, but only after PGE<sub>2</sub> treatment (or treatment with a selective Epac-1 agonist). This association was confirmed in isolated phagosome preparations. The changes in Epac-1 localization were too slow to account for the inhibitory effects of PGE<sub>2</sub> on phagocytosis. However, the appearance of Epac-1 on late phagosomes following PGE<sub>2</sub> treatment might be important for suppressing H<sub>2</sub>O<sub>2</sub> production and inhibiting the killing of intraphagosomal pathogens. The absence of Rap1 on late phagosomes suggests that the effect of Epac-1 might not require Rap1.

### Introduction

The recognition and clearance of invading microorganisms is a critical function of phagocytic cells involved in innate immunity and occurs through both opsonin-dependent and -independent pathways. Phagocytes express a broad array of receptors that participate in particle recognition and internalization. Key opsonins include complement, fibronectin/vitronectin,

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and immunoglobulin (Ig), which bind corresponding complement, integrin, and Fc $\gamma$  receptors, respectively [1]. Interestingly, the molecular mechanisms facilitating opsonin-dependent phagocytosis are different for particular opsonin/receptor pairs [1]. For example, phagocytosis of IgG-opsonized pathogens, which occurs via the Fc $\gamma$  class of receptors (Fc $\gamma$ R), involves phagocyte membrane extension around the microbe and results in the production of pro-inflammatory mediators [1]. Complement receptor mediated pathogen ingestion, on the other hand, occurs without observable membrane extension (particles “sink” into the cell) and is not generally associated with a concomitant inflammatory mediator response [1].

Regardless of the opsonin/receptor pathway involved, phagocytosis is a highly regulated process subject to both positive and negative regulation. Our laboratory has focused on the regulation of Fc $\gamma$ R-mediated phagocytosis by lipid mediators derived from the cell membrane constituent arachidonic acid. Such metabolites, known as eicosanoids, are generated in abundance at sites of inflammation (*e.g.* the host-microbial interface) and influence key components of the innate immune system, namely, phagocytosis [2,3], intracellular microbial killing [4,5], and inflammatory mediator generation [6].

Prostaglandin (PG) E<sub>2</sub> is an immunomodulatory eicosanoid generated by the sequential oxygenation and isomerization of arachidonic acid by cyclooxygenase and PGE<sub>2</sub> synthase enzymes. The regulation of target cells by PGE<sub>2</sub> occurs via signaling through 4 distinct cell membrane-associated G-protein coupled E-prostanoid (EP) receptors, termed EP1, EP2, EP3, and EP4 [7]. EP1 receptor activation provokes G<sub>q</sub>-coupled increases in intracellular Ca<sup>2+</sup>, EP2 and EP4 receptors signal predominantly through G<sub>s</sub>, increasing cAMP, and the EP3 receptor most commonly reduces cAMP via G<sub>i</sub> coupling after PGE<sub>2</sub> ligation [7]. It is through its ability to stimulate the production of intracellular cAMP (via EP2 and/or EP4) that PGE<sub>2</sub> impairs Fc $\gamma$ R-mediated phagocytosis [2]. And though cAMP is a well known counterregulator of Fc $\gamma$ R phagocytosis [2,8-10], the precise mechanisms downstream of cAMP remain to be fully defined.

Classically, cAMP signaling involves the immediate activation of protein kinase A (PKA), which phosphorylates several downstream targets, including the cAMP response element binding protein. However, PKA-independent actions of cAMP have been recognized in various experimental systems and novel targets for cAMP signaling have been described. These include cyclic nucleotide gated channels involved in the transduction of olfactory and visual signals and the guanine exchange proteins directly activated by cAMP (Epac-1 and -2) [11,12]. Epac-1 is more generally expressed throughout cells and tissues than Epac-2 [12] and its expression has been described in diverse cell types, including cells of the innate immune system.

Recently, Aronoff *et al.* reported the PKA-independent, Epac-dependent suppression of Fc $\gamma$ R-mediated phagocytosis in rat alveolar macrophages (AMs) [4]. This effect involved Epac-1, as AMs did not express Epac-2 protein [4]. Subsequent publications have confirmed an important role for Epac-1 in both Fc $\gamma$ R-dependent and -independent phagocytic pathways [10,13], though the mechanisms involved remain undiscovered. Epac-1 activates the small GTPases Rap1 and Rap2 [11] and Rap1 was found to associate with late endocytic/phagocytic compartments of J774.A1 macrophage-like cells [14]. Rap2 associates with secretory pathways and has not been implicated in phagocytosis [14]. It is notable that neither Rap1 overexpression nor Rap1 inhibition significantly altered Fc $\gamma$ R-mediated phagocytosis in the J774.A1 cells [15]. Whether Epac-1-Rap1 signaling is involved in regulating Fc $\gamma$ R-mediated phagocytosis in primary macrophages remains unclear.

Given the involvement of Epac-1 in regulating Fc $\gamma$ R-mediated phagocytosis and bacterial killing in macrophages, and the ability of Epac-1 to activate Rap1, we hypothesized that these effector molecules would spatially and temporally associate with phagosomes during Fc $\gamma$ R-

mediated phagocytosis. We further speculated that such associations might be altered by treatment with PGE<sub>2</sub>. The following experiments were designed to test these hypotheses using two complementary approaches, immunofluorescence microscopy and dynamic phagosome isolation, to track Epac-1 and Rap1 before and during FcγR-mediated phagocytosis, in the presence or absence of PGE<sub>2</sub>.

## Materials and Methods

### Animals

Pathogen-free 125-150 gm female Wistar rats (Charles River Laboratories, Portage, MI) were utilized. Animals were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

### Reagents

RPMI-1640, and penicillin/streptomycin/amphotericin B solution were purchased from Gibco-Invitrogen (Carlsbad, CA). PGE<sub>2</sub> from Cayman Chemical (Ann Arbor, MI) was dissolved in N<sub>2</sub>-purged DMSO and stored under N<sub>2</sub> at -80 °C. The selective phosphodiesterase-resistant, cell permeable Epac-1 agonist 8-pCPT-2'-O-Me-cAMP was purchased from Biolog (Bremen, Germany). Dilutions were prepared immediately before use, with equivalent quantities of DMSO added to the appropriate controls. Rabbit polyclonal anti-Rap1, anti-Epac-1 and anti-α-tubulin antibodies were from Upstate (Charlottesville, VA).

### Cell isolation and culture

The rat alveolar macrophage cell line NR8383 was obtained from the American Type Culture Collection (Manassas, VA). Resident rat AMs were obtained via *ex vivo* lung lavage as previously described [16] and resuspended in RPMI to a final concentration of  $2 \times 10^6$  cells/ml. Cells were allowed to adhere to tissue-culture treated slides for 1 h (37°C, 5% CO<sub>2</sub>) followed by two washes with warm RPMI, resulting in > 99% of adherent cells identified as AMs by use of a modified Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGraw Park, IL) [16]. Cells were cultured overnight in RPMI containing 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B prior to use. The following day cells were washed 2x with warm medium to remove nonadherent cells.

### Microscopic phagocytosis assay

Rat AMs ( $10^5$ /well) were plated and cultured overnight in glass 4-well Falcon tissue culture slides (BD, Franklin Lakes, NJ) as described above. Cells were preincubated with compounds of interest at the concentrations and times indicated in figure legends prior to the addition of phagocytic targets. Superparamagnetic, polystyrene beads (2.8 μM diameter) covalently opsonized with affinity purified sheep anti-mouse IgG (Dynabeads M-280, Dynal Biotech, Oslo, Norway) were used to study FcγR-mediated phagocytosis [17]. Beads were suspended in sterile PBS containing 0.1% bovine serum albumin and added at a ratio of 4 beads per cell for the times indicated in figure legends. Slides were then rinsed with warm PBS to remove uningested beads and processed for immunofluorescent staining as described below.

### Immunofluorescent staining and fluorescence microscopy

Cells were cultured on chamber slides, fixed with 4% paraformaldehyde at room temperature for 30 min, permeabilized with 0.3% Triton X-100 in PBS, then blocked with 1% BSA in PBS containing non-immune goat serum. Rabbit anti-Epac-1 and rabbit anti-Rap1 (Abcam, Cambridge, MA) and rabbit anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were prepared in 1% BSA-PBS (1:200) and applied for 1 hour at 37 °C. Mounts

were washed three times with 1% BSA-PBS and rhodamine-conjugated goat anti-rabbit secondary (1:200) was added for 1 hour at 37 °C. After washing three times, preparations were mounted using Vectashield mounting media containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Fluorescence was visualized with a Nikon Labophot 2 microscope equipped for epifluorescence.

### Phagosome isolation and immunoblot analysis

Macrophages ( $3 \times 10^6$ /well) plated in a 6-well dish were washed twice with PBS, placed on ice for 10 min to synchronize phagocytosis, then incubated at 37°C for 10 min with 1  $\mu$ M PGE<sub>2</sub> prior to the addition of 10:1 IgG-conjugated, 3  $\mu$ m, paramagnetic beads (Dyna-Invitrogen Carlsbad, California). Cells were then incubated at 37°C and phagosomes recovered at intervals to obtain early, intermediate and late phagosomes. After [18,19], to isolate IgG-bead-containing phagosomes, macrophages were rinsed twice in PBS and scraped into 0.5 ml/dish ice-cold homogenization buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.2; protease inhibitors and 1% Triton X-100). AMs were lysed during 30 min incubation on ice and the beads/phagosomes were isolated from cellular debris using a magnet (Qiagen, Valencia, CA) and washed twice in 0.5 ml of homogenization buffer without Triton X-100. Phagosomal proteins were removed from the beads by sonication (2 min with cooling on ice) followed by boiling for 3 min. The beads were collected with the magnet, then counted with a hemacytometer, and the solubilized material from an equal number of phagosomes from each experimental condition was used as a source of phagosomal proteins for subsequent electrophoresis and immunoblot analysis. Plasma membrane contamination was determined by quantitating the integral plasma membrane protein CD45 (data not shown). For immunoblot analysis, equal volumes of proteins were mixed with loading buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM DTT, 10% glycerol, and 0.1% bromophenol blue), boiled, applied to 8% SDS-polyacrylamide gels and subjected to electrophoresis. The separated proteins were transferred to nitrocellulose membranes in Trans-blot SD-Semidry Transfer Cells (Bio-Rad; 15 min at 15 mV). After transfer, the membranes were incubated in TBST buffer (150 mM NaCl, 20 mM Tris, 0.01% Tween 20 (pH 7.4)) containing 5% fat-free dry milk. The blot was probed with primary antibodies (as described above, or with antibody against the late phagosome marker flotillin (BD Biosciences, Franklin Lakes, NJ)), washed three times with TBST, and incubated with 1/5000 dilutions of peroxidase-conjugated monoclonal anti-rabbit IgG for 1 h at room temperature. The immunocomplexed peroxidase-labeled Abs were visualized by an ECL chemiluminescence kit following the manufacturer's instruction (Amersham Biosciences). Densitometry was performed using ImageJ software.

## Results

To determine the localization of Epac-1 in AMs before and during Fc $\gamma$ R-mediated phagocytosis, rat AMs were adhered to glass chamber slides and incubated without or with IgG opsonized beads for 60 min. Immunofluorescent staining for Epac-1 in AMs without beads revealed strong membrane staining around the nucleus and throughout the cell body (Fig. 1A). Lighter staining for Epac-1 was also indicated at the plasma membrane at the margins of adherent, spread AMs. As Epac-1 has been reported to move during cell cycling [20], we considered the possibility that Epac-1 might move during phagocytosis. In AMs incubated with IgG opsonized beads, the fluorescent distribution of Epac-1 remained brightest on perinuclear membranes and on membranes throughout the cell body (Fig. 1B, C). Beads captured at the cell margin were outlined by Epac-1-positive plasma membrane (Fig 1B). IgG-opsonized beads that were not associated with cells did not fluoresce when excited with blue light (not shown), used to excite FITC-linked Epac-1 here.

Adherent AMs incubated without beads stained positively for Rap1 in a punctate pattern throughout the cell (Fig. 1D). Fluorescence at the plasma membrane was weak, as was staining at the nuclear envelope. Rap1 localization can be altered by phorbol esters [21], by growth factors [22] or during neutrophil degranulation [23], raising the possibility that Rap1 localization might change during Fc $\gamma$ R-mediated phagocytosis. In phagocytosing AMs, Rap1 remained distributed in a punctate fashion throughout the cell, with light staining at the plasma membrane and nuclear envelope (Fig. 1E). Closer inspection of phagosomes showed that antibody against Epac-1 positively stained the membranes of phagosomes surrounding captured beads (Fig. 2 A, C, D). Although other brightly fluorescing membranes appeared to be associated with the phagosomes (Fig. 2C), it was not clear whether these bright membranes had actively moved to the phagosome or if the phagosome moved from the cell surface toward these membranes. Rap1-positive membranes around phagocytosed particles were also observed (Fig. 2B, E), but this staining was consistently weaker than that for Epac-1. No significant change in the bright punctate pattern of Rap1 was evident following phagocytosis and punctate regions did not appear to significantly associate with phagosomes. For both Epac-1 and Rap1, then, plasma membrane-associated protein was observed to surround the captured target in the early phagosome.

The rat AM cell line NR8383 was utilized to further investigate the localization of Epac-1 and Rap1 in phagocytosing macrophages. As in primary AMs, Epac-1 was most abundant on membranes in the cell body of resting NR8383 cells and less abundant on plasma membrane (Fig. 3A). These membranes were more tubular than those observed in primary AMs. Opsonized beads, captured within 15 min by NR8383 cells, were outlined by positive Epac-1 staining (Fig. 3B). As seen in AMs at 60 min, brighter Epac-1 membranes could be found near the lighter Epac-1 staining that surrounded the beads (Fig. 3B, inset). With longer incubation times, internalized beads were surrounded by membranes that were strongly positive for Epac-1 (Fig. 3C). In NR8383 cells, Rap1 was largely diffuse with some punctate staining (Fig. 3D); no Rap1 was associated with internalized phagosomes. For comparison, the soluble protein GAPDH appeared soluble and diffusely distributed throughout the cells (Fig. 3D). Importantly, staining for GAPDH was evident around peripheral beads (arrow, Fig. 3D), indicating that soluble proteins will also be found to be associated with these beads.

As noted above, Epac-1 is known to be involved in the inhibition of Fc $\gamma$ R-mediated phagocytosis by cAMP-stimulating compounds such as PGE<sub>2</sub> [4,10]. Treatment of AMs with 1  $\mu$ M PGE<sub>2</sub> induced a time-dependent redistribution of Epac-1 to the nuclear envelope, with changes apparent within 30 min and more pronounced by 60 min (Fig. 4, left). Also, Epac-1 remained prominently associated with perinuclear membranes as macrophages spread in response to PGE<sub>2</sub>. In contrast, PGE<sub>2</sub> had no discernible effect on the distribution of Rap1 in AMs (Fig. 4, right). Thus, in addition to being distributed at different sites, with Epac-1 on tubular membranes and Rap1 on punctate membranes, only Epac-1 responds to PGE<sub>2</sub> by accumulating on the nuclear envelope and perinuclear membranes.

To determine the effect of PGE<sub>2</sub> pretreatment on Epac-1 and Rap1 localization during phagocytosis, AMs were incubated with IgG opsonized beads following the addition of PGE<sub>2</sub>. Again, Epac-1 was commonly found on the nuclear envelope of PGE<sub>2</sub>-pretreated AMs, even after phagocytosis (Fig. 5A, B). Peripheral phagosomes that formed on PGE<sub>2</sub>-pretreated AMs were clearly delimited by Epac-1-containing membrane, as was seen in AMs not receiving PGE<sub>2</sub> (Figs. 1, 2). Although the majority of phagocytosed beads appear to be restricted to the periphery of PGE<sub>2</sub>-pretreated AMs (Fig. 5), rather than moving deeper into the lysosomal field as seen in untreated AMs (Figs 1-3), this was an inconsistent finding. The distribution of Rap1 in PGE<sub>2</sub>-pretreated AMs paralleled that seen in AMs unexposed to PGE<sub>2</sub>, with abundant labeling of punctate membrane structures and weaker staining of plasma membrane, in the absence or presence of phagocytosis (Fig. 5). Thus, activation of Fc $\gamma$ R did not substantially

change the distribution of Epac-1 and Rap1 in PGE<sub>2</sub> treated AMs, as assessed by immunofluorescent staining.

To more clearly determine if PGE<sub>2</sub> altered the association of Epac-1 or Rap1 with intracellular phagosomes, we isolated phagosomes using magnetic, IgG-opsonized polystyrene microbeads at various times. Flotillin was used as a marker of phagosome maturation, as it has been identified on late phagosomes [24]. As shown by immunoblot, flotillin levels increased over time in phagosomes from untreated AMs (Fig. 6). Consistent with our previously published results [2], pretreatment of AMs with 1 μM PGE<sub>2</sub> for 5 min produced a significant decrease in the number of captured microbeads/phagosomes (data not shown). However, when equal numbers of untreated and PGE<sub>2</sub>-treated microbead/phagosomes were analyzed, flotillin increased similarly in both conditions, suggesting that PGE<sub>2</sub> treatment did not alter the rate of phagosome maturation. However, Epac-1 was not detected on phagosomes of untreated AMs, but was readily detected on late phagosomes of PGE<sub>2</sub> treated cells (Fig. 6). We observed a similar increase in Epac-1 association with late phagosomes when cells were treated with the selective Epac-1 agonist 8-pCPT-2'-O-Me-cAMP (1 mM) as we observed with PGE<sub>2</sub> (data not shown). We were unable to detect significant quantities of Rap1 using this technique, suggesting that the association between Rap1 and the phagosomal membrane is loose and/or transient.

Finally, Epac-1 has also been found to be associated with microtubules or centrosomes [20]. Dual staining of untreated AMs for these proteins and α-tubulin indicated co-localization of both Epac-1 and Rap1 at microtubule organizing centers (MTOC; Fig. 7). Pretreatment of AMs with PGE<sub>2</sub> (1 μM) for 60 min resulted in loss of Epac-1 staining at the MTOC, whereas Rap1 remained at the MTOC.

## Discussion

Although it is recognized that cAMP signaling, as is triggered by PGE<sub>2</sub> in macrophages, inhibits FcγR-mediated phagocytosis, the critical role of Epac has only recently been demonstrated. In this study, we sought to determine the subcellular localization of Epac and one of its major downstream targets, Rap1, before and after PGE<sub>2</sub> treatment. We find that both proteins are distributed on a variety of subcellular sites, but that only Epac-1 redistributes in response to PGE<sub>2</sub>.

### Epac-1 localization

Epac-1 is known to have a Dishevelled, Egl-10, Pleckstrin (DEP) domain, which is involved in membrane localization [25]. In fibroblast-like COS-7 cells, Epac-1 was found to be membrane-associated with a punctate distribution, with many of these co-localizing with a mitochondrial marker, as well as some localization to nuclear membranes [20]. There is also evidence that, in COS-7 cells, Epac-1 associates with the mitotic spindle and centrosomes during metaphase, that elevation of intracellular cAMP levels directs Epac-1 to nuclear membranes and that reduction of cAMP favors cytoskeleton association of Epac-1 [20].

To our knowledge, the spatial localization of endogenous Epac-1 has not previously been studied in phagocytic cells of the innate immune system. In resting AMs, Epac-1 is most abundantly distributed on punctate membranes throughout the cell body, including in the perinuclear region, with additional lower levels evident on the plasma membrane. A similar distribution for Epac-1 was observed in the NR8383 macrophage cell line, although there were additional tubular structures that stained strongly for Epac-1. These distributions were reminiscent of the tubular and spherical lysosomes that have been described in macrophages [26,27]. In addition to these sites, Epac-1 staining also overlapped with α-tubulin staining at the MTOC, but not with microtubules away from the MTOC.

Following cell treatment with PGE<sub>2</sub>, Epac-1 redistributed to the nuclear envelope and accumulated on late phagosomes. Both processes were relatively slow, requiring 30-60 min to produce changes that were observable by the methods used here. It is notable that Epac-1 and Rap1 appear to be associated with phagosomes from cells not exposed to PGE<sub>2</sub> when examined by immunofluorescence microscopy (Fig. 2), however we failed to observe this association when phagosomes were isolated from untreated cells and analyzed by Western blot (Fig. 6). We speculate that the association between Epac-1 and phagosomes seen by microscopy in non-PGE<sub>2</sub> treated cells is not a tight association, and the Epac-1 is therefore lost during the physical isolation of bead-containing phagosomes. Under the influence of PGE<sub>2</sub>, however, the Epac-1-phagosome association might become tighter and is not disrupted by phagosome isolation. This finding is further supported by our observing the soluble protein GAPDH in association with bead-containing phagosomes when observed by immunofluorescent staining (Fig. 3D) but not following phagosome isolation (not shown). The immunofluorescence data suggest that cytosolic proteins might non-specifically (and perhaps loosely) associate with phagosomes during the internalization of IgG-opsonized targets. This is in contrast to the finding that Epac-1 becomes specifically (and perhaps tightly) associated with phagosomes during Fc receptor mediated phagocytosis when cells are exposed to PGE<sub>2</sub> (or 8-pCPT-2'-O-Me-cAMP).

Although Epac-1 redistribution in response to PGE<sub>2</sub> may have begun earlier than 30 min, these changes may be too slow to account for the inhibitory effects of Epac-1 activation on phagocytosis. On the other hand, the striking recruitment of Epac-1 to maturing phagosomes following PGE<sub>2</sub> treatment places this signaling protein at a site that could be important for its known capacity to inhibit both H<sub>2</sub>O<sub>2</sub> production and bacterial killing [4]. Also, although Epac-1 was typically co-localized with MTOCs in untreated AMs, it did not co-localize with MTOCs following PGE<sub>2</sub> treatment. We find no evidence that Epac-1 associates with microtubules away from the MTOC in AMs. The loss of Epac-1 from MTOCs following PGE<sub>2</sub> treatment could possibly reduce the ability for Epac-1 to regulate microtubule function and this might play a role in altering phagosome internalization.

### Rap1 localization

Rap1 has been localized to a wide variety of membranes in different cell types. These include the golgi apparatus in rat-1 fibroblasts [28] and COS-7 cells [29], on secretory granules in parotid gland [30], on zymogen granules in pancreatic acinar cells [31], on specific granules in human neutrophils [21,23] on endosomes of fibroblasts and T cells [32] and COS-7 cells [29], in late endosomes in C2 (muscle) cells [33] as well as fibroblasts [14], on phagosomes in J774 macrophages [14] and in plasma membrane of fibroblasts and T cells [32]. Furthermore, Rap1 reportedly moves to the nucleus of cancer cells in response to growth factors [22] and to the plasma membrane of neutrophils following degranulation [21,23].

In this study, we localized Rap1 in primary AMs. Rap1 was, like Epac-1, most abundant on punctate membranes throughout the cell, with additional staining evident on the plasma membrane. Although positive staining for Rap1 on late phagosomes was apparent by immunofluorescent staining, Rap1 was not detected by immunoblot analysis of isolated, mature phagosomes. Rap1 was also found, by immunofluorescent staining, to co-localize with MTOCs. In our hands, Rap1 did not appear to move, either to the nucleus or to the plasma membrane, under any of the conditions examined here. Perhaps most surprisingly, we found little evidence that Rap1 accumulated on maturing phagosomes, as Epac-1 did. This raises the possibility that Epac-1 signals through pathways that are independent of Rap1. In addition, it is possible that the inhibition of killing of phagocytosed bacteria in AMs treated with PGE<sub>2</sub>, which is known to involve Epac-1 activation [4], does not require Rap1 activation. There is some precedent for Rap-independent Epac-1 action [34], though alternative targets for Epac-1 have not been convincingly defined.

In conclusion, we sought to determine the subcellular distribution of Epac-1 and its primary target, Rap1, in primary phagocytes, since activation of Epac-1 by PGE<sub>2</sub> significantly inhibits FcγR-mediated phagocytosis in these cells. We found that both enzymes were abundant on intracellular membranes that resembled the tubular and spherical lysosomes of macrophages, with lesser amounts also on plasma membrane and MTOCs. Treatment with PGE<sub>2</sub> led to a redistribution of Epac-1, but not Rap1, to the nuclear envelope and late phagosomes. The separation of effects on Epac-1 and Rap1 redistribution suggests that some effects of Epac-1, particularly in killing of phagocytosed bacteria, might be independent of Rap1. Earlier signaling events through Epac-1 that result in impaired phagocytosis remain to be elucidated.

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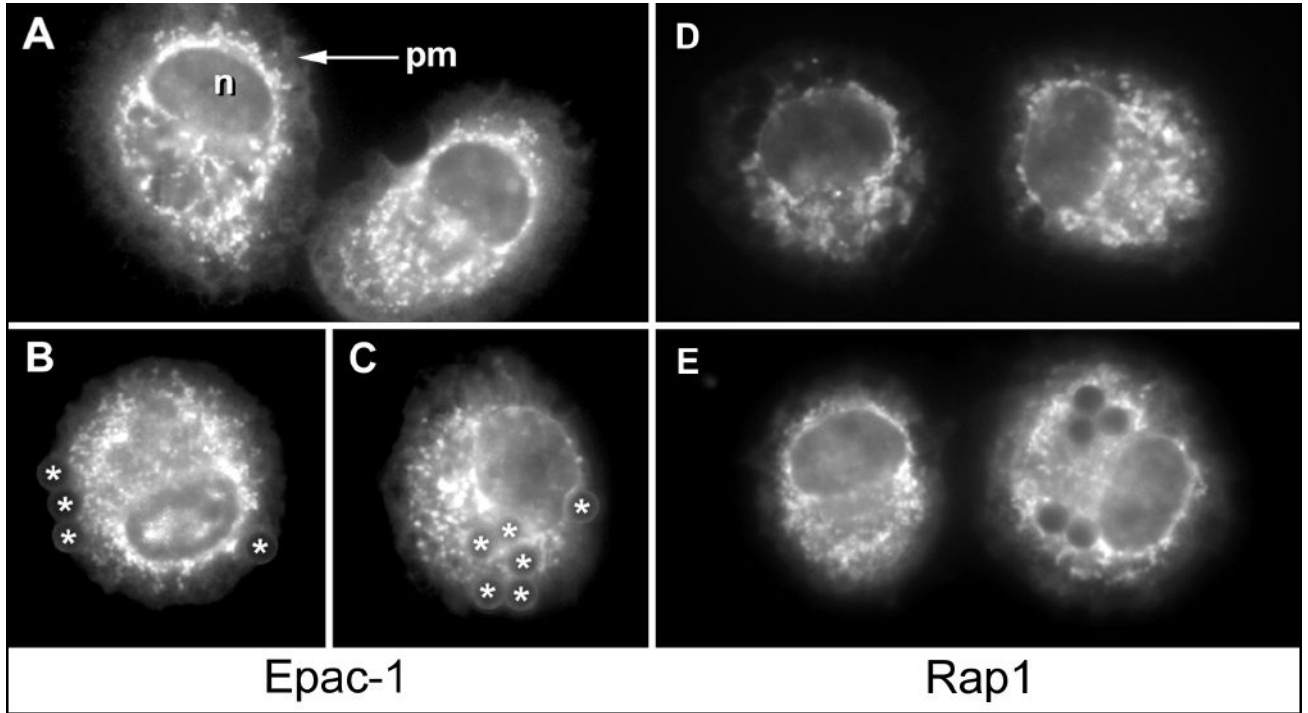
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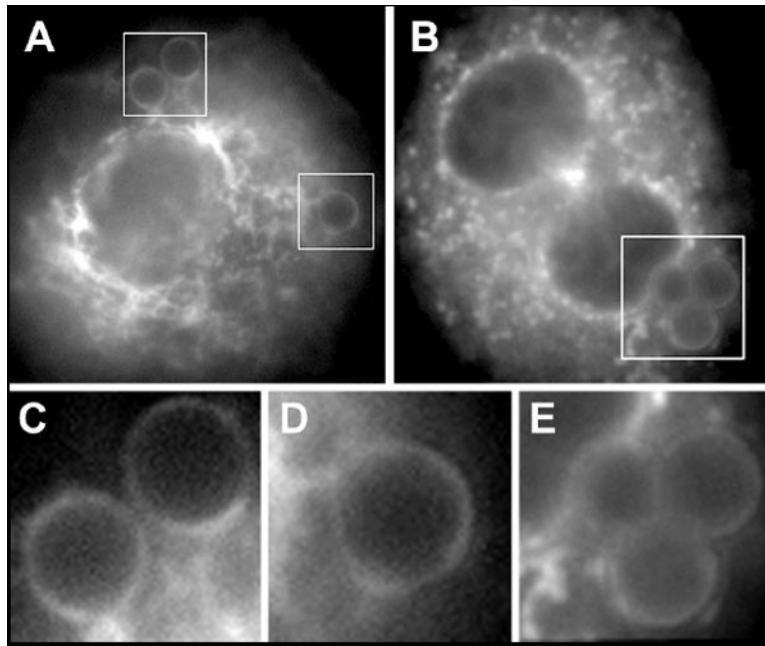
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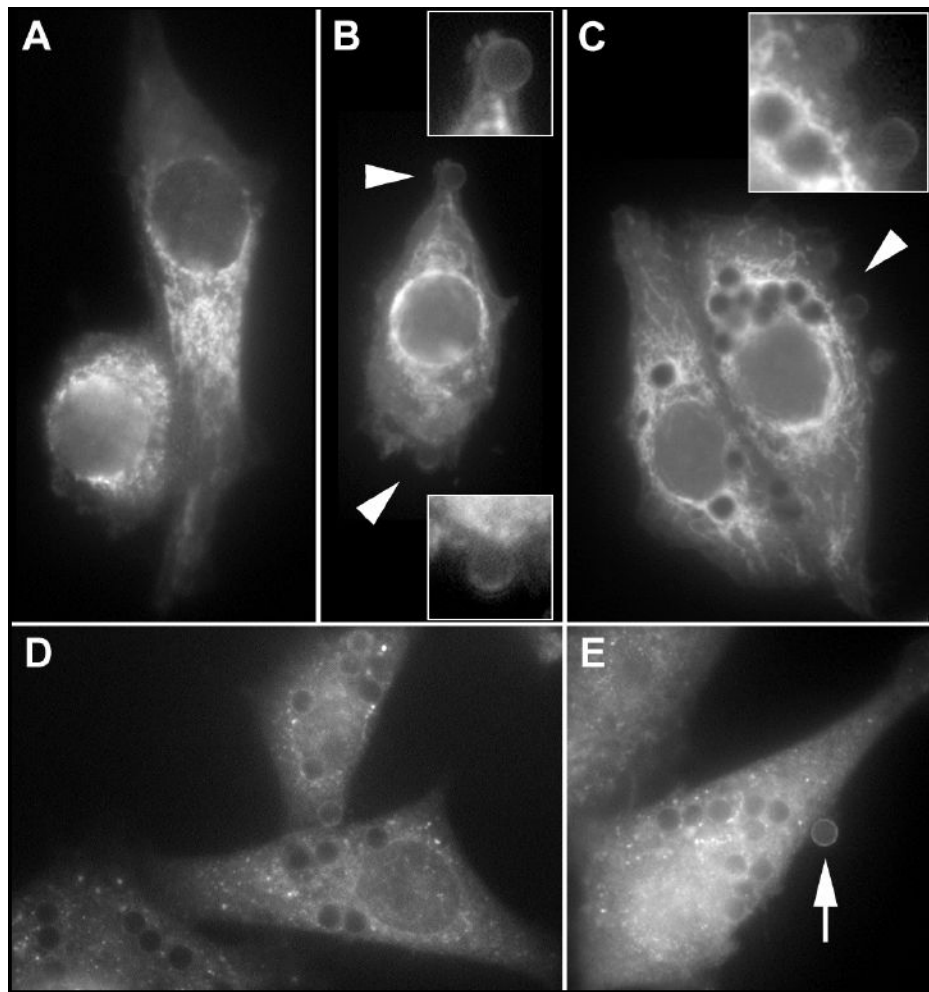


**Fig. 1. Subcellular localization of Epac-1 and Rap1 in AMs following phagocytosis of opsonized beads**

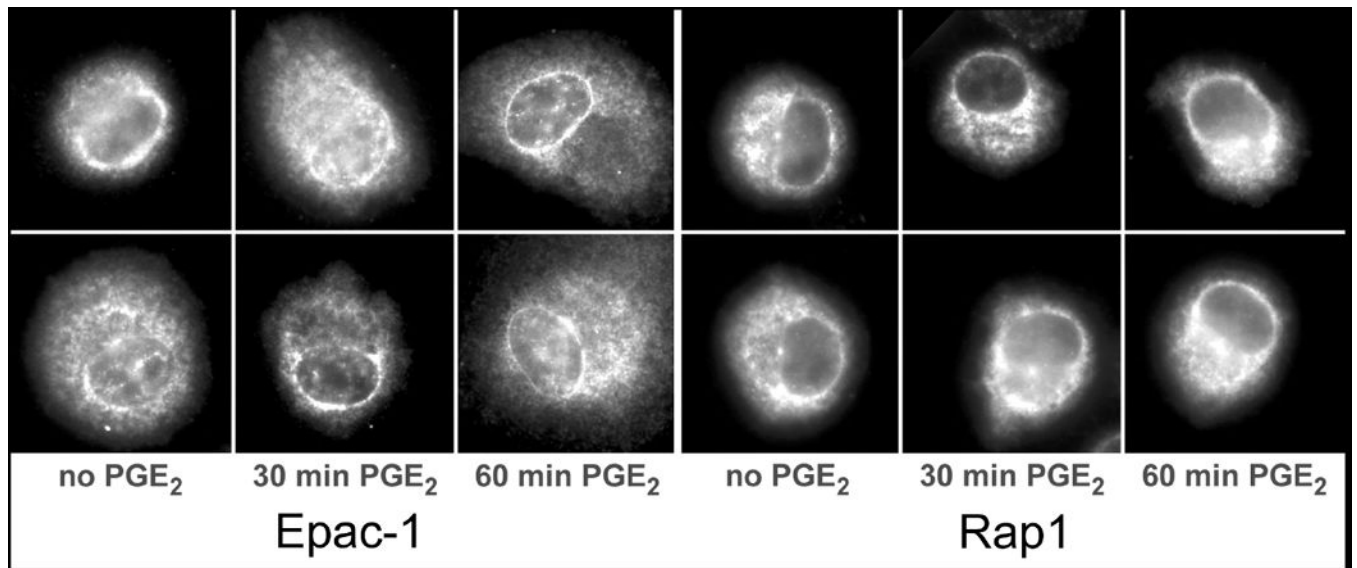
Rat AMs were incubated without (A,D) or with (B, C, E) opsonized beads for 60 min, then fixed and stained for Epac-1 (A-C) or Rap1 (D, E). Image E shows a cell that contains no beads (left) next to a cell that has engulfed five beads (right). n, nucleus; pm, plasma membrane; \*, phagocytosed bead. Results are from one experiment and are representative of three.



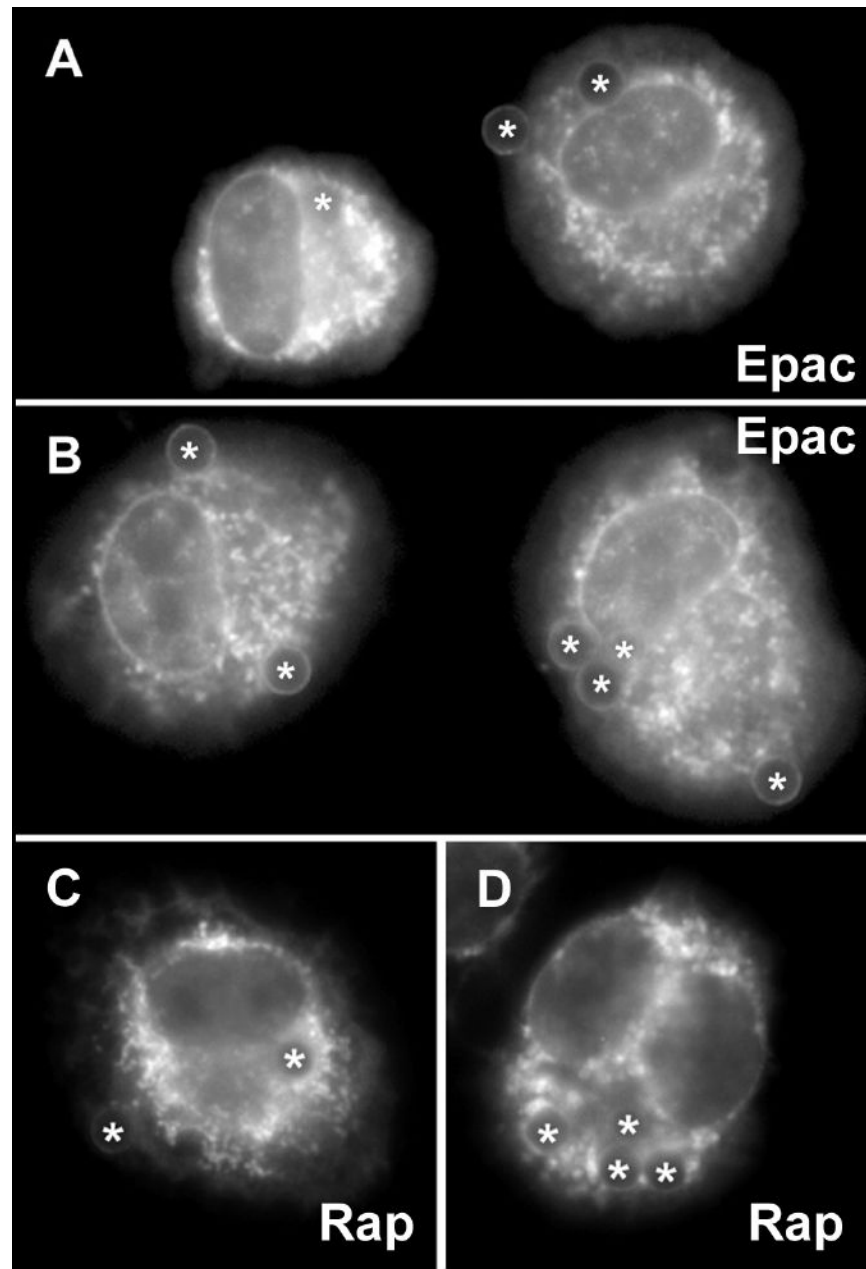
**Fig. 2. Detail of subcellular localization of Epac-1 or Rap1 near phagocytic cup**  
Following phagocytosis of opsonized beads for 60 min, AMs were fixed and stained for Epac-1 (A, C, D) or Rap1 (B, E). Images in C and D are details from A, and E is a detail of the indicated area in image B.



**Fig. 3. Subcellular localization of Epac-1, Rap1 and GAPDH in the AM cell line NR8383**  
Cells were incubated without beads (A) or with opsonized beads for 15 (B) or 60 (C-E) min. Insets in (B) and (C) are details of the regions indicated by arrowheads. Arrow in (E) indicates peripherally bound bead with positive staining for GAPDH.

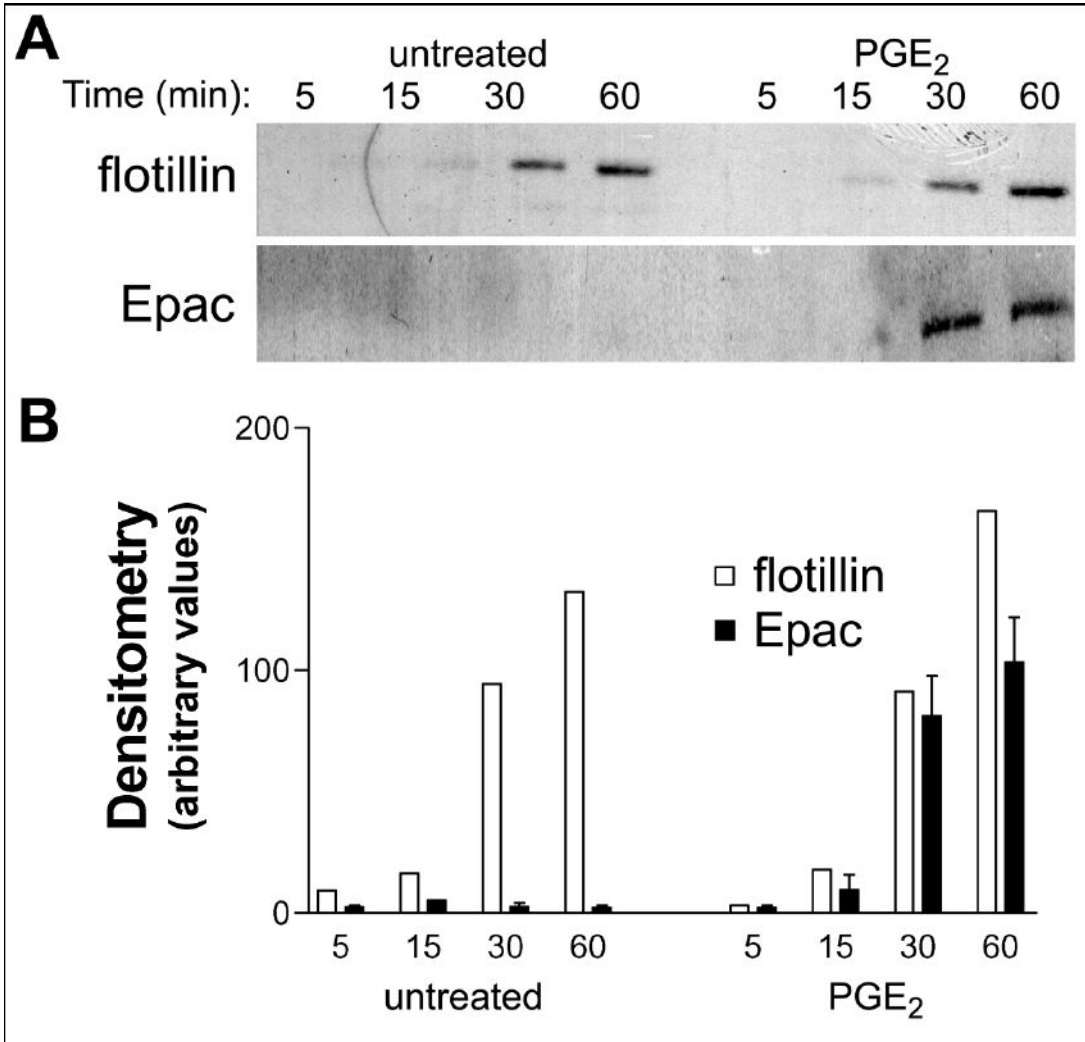


**Fig. 4. Subcellular localization of Epac-1 and Rap1 in AMs treated with PGE<sub>2</sub>**  
Cells were treated with 1  $\mu$ M PGE<sub>2</sub> for 0, 30 or 60 min, then fixed and stained for Epac-1 (left) or Rap1 (right) as described in Materials and Methods. Images are from one experiment and are representative of three independent experiments.



**Fig. 5. Localization of Epac-1 and Rap1 following phagocytosis of opsonized beads in AMs pretreated with PGE<sub>2</sub>**

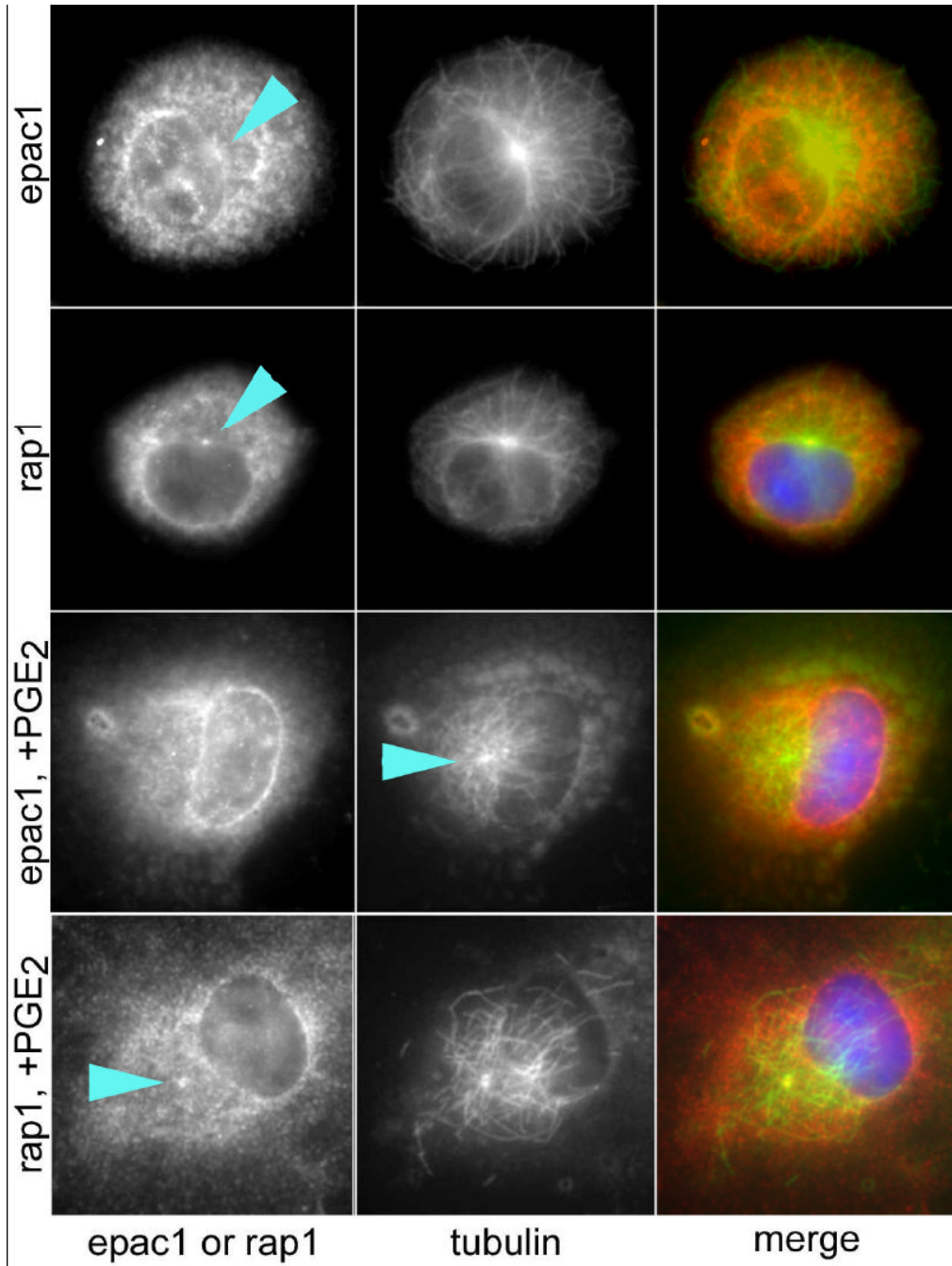
Cells were pretreated with 1  $\mu$ M PGE<sub>2</sub> for 15 min and then incubated with opsonized beads for 60 min. Two fields from independent experiments are presented for Epac-1 (A, B) and Rap1 (C, D). Asterisks (\*) indicate phagosomes containing beads.



**Fig. 6. Trafficking of Epac-1 to the phagosome following PGE<sub>2</sub> treatment**

Rat AMs were pretreated for 5 min with vehicle (DMSO) or PGE<sub>2</sub> (1 μM), followed by challenge with IgG-opsonized magnetic beads. Phagosomal membranes were harvested at the times noted and (A) assayed by immunoblot for the late phagosomal marker flotillin and for Epac-1. Densitometry (B) presents mean (+SE) data from independent experiments.





**Fig. 7. Trafficking of Epac-1 to the phagosome following PGE<sub>2</sub> treatment**

Rat AMs were pretreated for 5 min with vehicle (DMSO) or PGE<sub>2</sub> (1 μM), followed by challenge with IgG-opsonized magnetic beads. Phagosomal membranes were harvested at the times noted and (A) assayed by immunoblot for the late phagosomal marker flotillin and for Epac-1. Densitometry (B) presents mean (±SE) data from independent experiments.

**Co-localization of Epac-1 and Rap1 with MTOCs.** AMs were either untreated or treated for 60 min with 1 μM PGE<sub>2</sub> before fixing and staining for Epac-1 or Rap-1 and α-tubulin.

Individual stains (left, center) are provided in black and white for optimum presentation; merged images (right) use false coloring to show Epac-1 or Rap1 as red and α-tubulin as green. The positioning of the MTOC is indicated by an arrowhead. The Epac-1 + PGE<sub>2</sub> image is

identical to that shown in Fig. 5. Results are from one experiment and are representative of three independent experiments.