J. Biochem. 2009;145(1)43–50
doi:10.1093/jb/mvn139

Inhibitory effect of *N*-palmitoylphosphatidylethanolamine on macrophage phagocytosis through inhibition of Rac1 and Cdc42

Akiko Shiratsuchi[1,2,*], Manami Ichiki[1], Yasuo Okamoto[2,3], Natsuo Ueda[3], Naotoshi Sugimoto[2], Yoh Takuwa[2] and Yoshinobu Nakanishi[1,2]

[1]Graduate School of Natural Science and Technology; [2]Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa, Japan; and [3]Department of Biochemistry, Kagawa University School of Medicine, Miki, Kagawa, Japan

Received May 29, 2008; accepted October 16, 2008; published online October 30, 2008

The production of *N*-acylethanolamine (NAE) is enhanced during inflammation. NAE is synthesized from phosphatidylethanolamine with *N*-acylphosphatidylethanolamine (NAPE) as a precursor. The amount of NAPE at the site of inflammation exceeds that of NAE. This evokes the possibility that NAPE possesses a biological function, as does NAE. We here examined if *N*-palmitoylphosphatidylethanolamine (NPPE), a precursor of *N*-palmitoylethanolamine, modulates the state of inflammation. We found that the level of the phagocytosis of latex beads, *Staphylococcus aureus*, *Escherichia coli*, or apoptotic cells by mouse peritoneal macrophages or J774A.1 macrophages was reduced in the presence of liposomes containing NPPE, while that of dextran remained unaffected. This action of NPPE seemed to be due to the inhibition of the activation of Rac1 and Cdc42 in macrophages. These results suggested that NAPE is bioactive lipid acting toward the termination of inflammation.

Key words: *N*-acylphosphatidylethanolamine, lipid mediator, macrophage, phagocytosis, Rho GTPase.

Abbreviations: NAE, *N*-acylethanolamine; NAPE, *N*-acylphosphatidylethanolamine; NPE, *N*-palmitoylethanolamine; NPPE, *N*-palmitoylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

INTORODUCTION

N-acylethanolamines (NAEs) are bioactive lipids synthesized from membrane phospholipids and possess various physiologic roles (*1–3*); *N*-arachidonoylethanolamine, also called anandamide, regulates neurotransmission in the brain (*4*, *5*); *N*-oleolylehtanolamine works as an anorexic substance (*6*); and *N*-palmitoylethanolamine (NPE) has an anti-inflammatory effect (*2*, *7*, *8*). In animal tissues, the production of NAE is enhanced upon the initiation of inflammation. Phosphatidylethanolamine (PE) first receives *N*-acylation, such as palmitoylation or arachidonoylation, giving rise to *N*-acylphosphatidylethanolamines (NAPEs). NAPEs are then hydrolysed to NAEs by the action of a phosphodiesterase known as NAPE-PLD (*9–15*). Anandamide, the best-characterized NAE, was originally found as an endogenous ligand for the cannabinoid receptors CB1 and CB2 (*16–18*) and afterwards it was shown that the vanilloid receptor TPRV1 also recognizes it (*19*). Other NAEs seem to serve as ligands for G protein-coupled receptors as well (*20*, *21*). In fact, GPR55 and GPR119 were recently found as novel cannabinoid receptors (*22*).

Macrophages sit on the front line of self-defense against pathogenic microorganisms (*23*). They phagocytose microbes, pathogen-infected cells, or apoptotic inflammatory cells, as part of the cellular innate immune response. This action of macrophages contributes to the removal of pathogens and at the same time phagocytosing macrophages produce various factors to regulate inflammation. It was recently reported that 2-arachidonoylglycerol, another endocannabinoid, stimulates the phagocytosis of zymosan by macrophages through the activation of dectin-1 (*24*) or integrin (*25*). The stimulation by 2-arachidonoylglycerol is mediated by CB2 and requires the activation of phosphatidylinositol 3-phosphate kinase (*24*, *25*). Anandamide also exerts inhibitory actions on macrophage phagocytosis in vivo (*26*), but the precise mechanisms of its action remain unclear. The fact that the concentration of NAPE is higher than that of its end product NPE at the site of inflammation (*16*) suggests roles for NAPE as an inflammation modulator (*21*, *27*, *28*). We here examined the effect of NPPE, a precursor of NAE having anti-inflammatory (*2*, *8*, *29*) and immunosuppressive activities (*30*), on the phagocytic action of macrophages.

MATERIALS AND METHODS

Materials—1,2-Dioleoyl-*sn*-glycerophosphoethanolamine and palmitic acid were purchased from Sigma-Aldrich (St Louis, MO). NPPE was synthesized with 1,2-dioleoyl-*sn*-glycerophosphoethanolamine and palmitic acid according to the method of Schmid *et al*. (*31*) and purified by thin-layer chromatography using a mixture of

*To whom correspondence should be addressed. Tel: +81-76-234-4424, Fax: +81-76-234-4480,
E-mail: shira@kenroku.kanazawa-u.ac.jp

chloroform, methanol, and 28% (v/v) ammonium hydroxide (40 : 10 : 1 in volume) as a solvent. The concentration of NPPE was determined by an assay for lipid phosphorus (*32*). 1,2-Dioleoyl-phosphatidic acid and *N*-palmitoylethanolamine were purchased from Sigma-Aldrich and Cayman Chemical (Ann Arbor, MI), respectively. Antibodies against Rac1 and Cdc42 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Texas Red-labelled dextran (molecular weight: 70,000, lysine-fixable) and Alexa546-labelled phalloidin were obtained from Molecular Probes (Eugene, OR). Cytochalasin B was obtained from Sigma-Aldrich. Latex beads were purchased from PolySciences (Warrington, PA). SR144528, a CB2 antagonist, was a gift from Sanofi-Synthelabo Recherche (Montpellier, France). Phosphatidylcholine (PC) (from chicken egg), phosphatidylserine (PS) (from porcine brain) and PE (from bovine liver) were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were prepared using a combination of PC and PE (PE liposomes), PC and NPPE (NPPE liposomes), or PC and PS (PS liposomes) at a molar ratio of 7 : 3, as described previously (*33*).

Cell Culture—Macrophages from the peritoneal cavity of thioglycolate-injected mice were prepared as described previously (*34–36*) and cultured in RPMI1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37°C with 5% (v/v) CO_2 in air. J774A.1 cells, a mouse macrophage cell line, were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C with 5% CO_2 in air. Thymocytes were prepared from ddY mice (female, 6–10 weeks old) and cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum in the presence of dexamethasone (10^{-5} M) for the induction of apoptosis, as described previously (*37*).

Assays for Phagocytosis, Endocytosis and Cell Adhesion—Phagocytosis reactions with peritoneal macrophages were carried out as described previously (*34–36*). *Staphylococcus aureus* (strain Smith) or *Escherichia coli* (strain W3110) at the logarithmic phase was labelled with fluorescein isothiocyanate (Molecular Probes) and used as the targets (*37*). The engulfment of latex beads or bacteria was examined by phase-contrast and fluorescence microscopy (×1,000 magnification) as described before (*37*). For the phagocytosis of apoptotic thymocytes, the sample after the reaction was fixed, stained with hematoxylin and examined under a light-field microscope (×400 magnification) (*37*). To examine the adhesion of apoptotic cells to macrophages, the cell mixtures were incubated on ice, fixed, stained with hematoxylin and examined by light-field microscopy. For the examination of the endocytosis of fluorescence-labelled dextran, macrophages were incubated with dextran (0.2 mg/ml), rinsed with phosphate-buffered saline and fixed and the level of fluorescence in macrophages was determined by fluorescence microscopy as described previously (*37*). J774A.1 macrophages were transfected with the plasmid vector pCMV containing cDNA coding for the dominant negative form of Rac1 (Rac1N17) or pME18S containing cDNA coding for the dominant negative Cdc42 (Cdc42N17) (*38*) together with the green fluorescent protein-expressing pCS2-venus (a gift from T. Miyawaki, RIKEN Brain Science Institute, Wako, Japan) using Fu GENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN). Those cells were incubated with latex beads (macrophages : targets = 1 : 11) and the number of latex beads engulfed by 100 or more green fluorescent protein-positive cells was determined by fluorescence-phase contrast microscopy. For the analysis of actin filaments, macrophages treated with phosphate-buffered saline containing 2% (w/v) paraformaldehyde, 0.7% (w/v) glutaraldehyde and 0.01% (v/v) Triton X-100 were incubated with fluorescence-labelled phalloidin and examined by fluorescence microscopy (*39*).

Determination of the Level of GTP-bound Rac1 and Cdc42—Macrophages after phagocytosis reactions were lysed with 50 mM Tris–HCl (pH 7.2) buffer containing 0.5 M NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 5 μg/ml each of leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride, and the lysates were centrifuged at $18{,}000 \times g$ at 4°C. The supernatants were incubated with glutathione-Sepharose 4B (Amersham-Pharmacia Biotech, Uppsala, Sweden) that had been conjugated with GST-p21 activated kinase at 4°C for 45 min. The Sepharose was washed with 50 mM Tris–HCl (pH 7.2) containing 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 5 μg/ml each of leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride, and incubated with SDS–PAGE buffer (*40*). Proteins released from the beads were subjected to a western blot analysis using monoclonal antibodies against either Rac1 or Cdc42, according to a standard protocol (*39*).

Statistical Analysis—Data are representative of at least three independent experiments that yielded similar results. Data from quantitative analyses are expressed as the mean ± SD ($n > 3$). Statistical analyses were performed using Student's *t*-test and *P* values of less than 0.05 were considered significant. The data significantly different from controls were marked with asterisks.

RESULTS

Inhibition of Macrophage Phagocytosis by NPPE-containing Liposomes—To examine the effect of NPPE on macrophage phagocytosis, we conducted phagocytosis reactions in the presence of liposomes consisting of NPPE and PC. Macrophages prepared from peritoneal fluids of thioglycolate-injected mice were subjected to an assay of phagocytosis with various targets, including latex beads, *S. aureus*, *E. coli* and apoptotic thymocytes. Both the ratio of macrophages that had incorporated latex beads and the number of latex beads engulfed by a given number of macrophages were reduced in the presence of NPPE-containing liposomes, while liposomes consisting of PE and PC did not affect the phagocytosis (Fig. 1A). NPPE in liposomes might be metabolized during incubation with macrophages, and the resulting metabolites could influence the phagocytosis. We thus tested the effect of 1,2-dioleoyl-phosphatidic acid and *N*-palmitoylethanolamine, major metabolites of NPPE after the cleavage by phospholipase D, on macrophage phagocytosis. However, neither substance seemed to influence

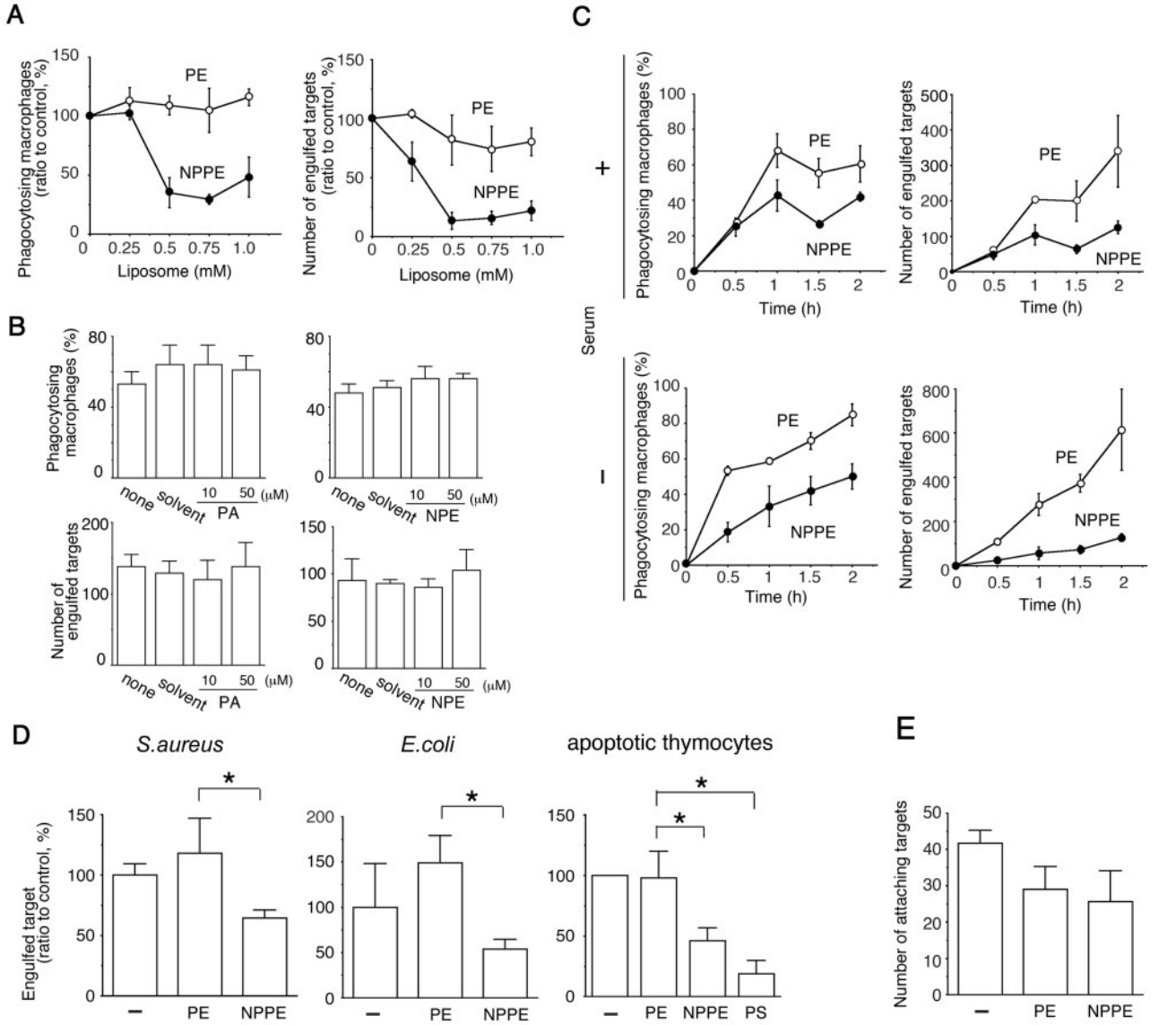

Fig. 1. **Inhibitory effect of NPPE on phagocytosis by mouse peritoneal macrophages**. (A) Dose response of NPPE-containing liposomes. Macrophages and latex beads were incubated for 1 h in the presence of increasing amounts of PC-based liposomes containing NPPE or PE, indicated as the concentration of total lipids, and the level of phagocytosis was determined and shown as the ratio of macrophages that have accomplished phagocytosis or the number of latex beads engulfed by 100 macrophages. (B) Effect of NPPE metabolites on phagocytosis. Phagocytosis reactions were conducted as in A in the presence of the indicated amounts of 1,2-dioleoyl-phosphatidic acid (PA) and *N*-palmitoylethanolamine (NPE), or solvent alone (methanol for PA and dimethylsulfoxide for NPE). (C) Time course of the phagocytosis of latex beads. Phagocytosis reactions were done with the indicated liposomes (0.75 mM) in the presence or absence of the serum [10% (v/v)]. (E) Effect of NPPE-containing liposomes on the phagocytosis of various targets. The indicated cells were used as targets in phagocytosis reactions for 1 h in the presence and absence of the indicated liposomes (0.75 mM). PS, phosphatidylserine-containing liposomes. (E) Effect of liposomes on the binding of apoptotic thymocytes to peritoneal macrophages. An assay for binding was carried out in the presence of the indicated liposomes (0.75 mM) and the number of thymocytes adhering to 100 macrophages is shown. All data are representative of two (A), two (B), one (C), two (D) and three (E) independent experiments that yielded similar results.

the phagocytosis (Fig. 1B), suggesting that NPPE not its metabolites is responsible for the inhibitory effect of NPPE-containing liposomes. The inhibition of phagocytosis by NPPE-liposomes was observed regardless of the presence of serum in the reactions, suggesting that serum components were not required for the action of NPPE-liposomes (Fig. 1C). The level of the phagocytosis of *S. aureus*, *E. coli* and apoptotic thymocytes was also decreased by the addition of NPPE-containing liposomes (Fig. 1D). Peritoneal macrophages phagocytose apoptotic thymocytes in a manner mediated by phosphatidylserine as a marker for phagocytosis (*37*). The level of this phagocytosis was reduced in the presence of phosphatidylserine-containing liposomes, as reported previously

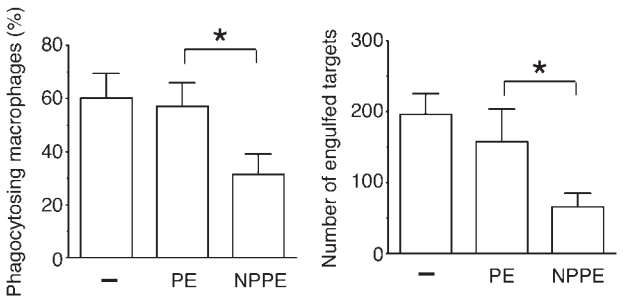

Fig. 2. **Inhibitory effect of NPPE-containing liposomes on phagocytosis by J774A.1 macrophages.** The level of the phagocytosis of latex beads by macrophages was determined in the presence and absence of the indicated liposomes (0.75 mM). Data are representative of three independent experiments that yielded similar results.

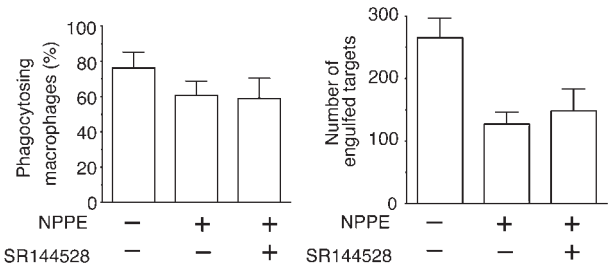

Fig. 3. **Effect of a cannabinoid receptor antagonist on the action of NPPE-containing liposomes.** The level of the phagocytosis of latex beads by mouse peritoneal macrophages was determined in the presence and absence of NPPE liposomes (0.75 mM) and the CB2 antagonist SR144528 (5 mM). As a negative control for SR144528, its solvent was present in the reactions (denoted with '–'). Data are representative of three independent experiments that yielded similar results.

(*37*) and the effect of NPPE-containing liposomes was smaller than that of liposomes containing phosphatidylserine (Fig. 1D). In contrast, the binding of apoptotic thymocytes to macrophages, which occurs prior to the engulfment, remained unaffected by NPPE-liposomes (Fig. 1E). The inhibitory effect of NPPE-liposomes was similarly observed when J774A.1 cells, a mouse macrophage cell line, were used as phagocytes (Fig. 2). To examine the possible involvement of known endocannabinoid receptors, phagocytosis reactions were conducted in the presence of an antagonist for CB2, which is highly expressed in macrophages (*41*). The inhibitory effect of NPPE-liposomes was unchanged in the presence of this antagonist, SR144528 (Fig. 3). These results indicated that NPPE-liposomes inhibit macrophage phagocytosis in a manner unrelated to the type of target and not mediated by CB2.

No Effect of NPPE on Endocytosis of Dextran by Macrophages—The phagocytosis reactions examined above were associated with the extension of pseudopods, filopodia and lamellipodia, as a result of the rearrangement of actin filaments in macrophages (*42*). We thus tested the effect of NPPE-liposomes on the endocytosis of dextran by macrophages, which event occurs without pseudopod extension (*39*). In the presence of NPPE-containing liposomes, the incorporation of fluorescent dextran into macrophages was not reduced, but rather slightly increased (Fig. 4). We next examined the effect of NPPE on the formation of actin filaments. When macrophages were stained with fluorescein-labelled phalloidin that selectively binds to F-actin, the pattern of the actin cytoskeleton was almost the same in macrophages treated and not treated with NPPE-liposomes (Fig. 5). On the other hand, cytochalasin B, an inhibitor of actin polymerization, retarded the formation of actin filaments in macrophages (Fig. 5). These results collectively suggested that NPPE-containing liposomes inhibit the pseudopod-mediated phagocytosis by macrophages.

Inhibition of the Activation of Rac1 and Cdc42 by NPPE—The rearrangement of actin cytoskeletons, which precedes the formation of pseudopods, is regulated by Rho family small G proteins including Rac1 and Cdc42

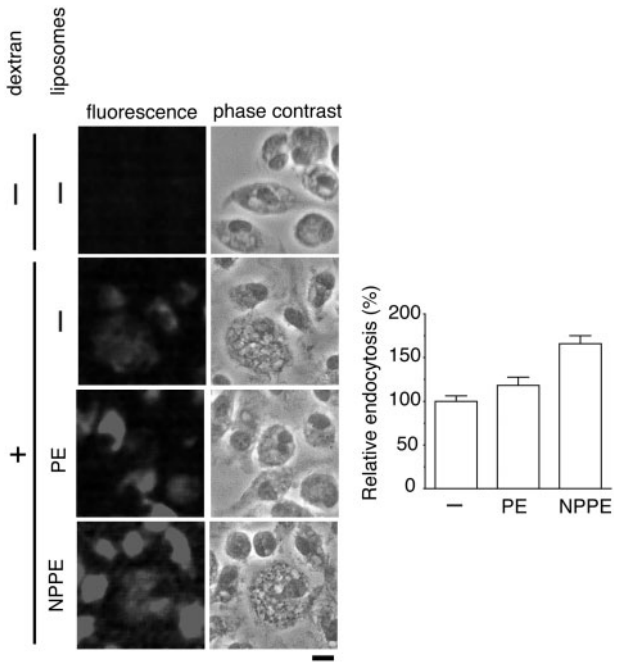

Fig. 4. **Effect of NPPE-containing liposomes on the endocytosis of dextran.** Mouse peritoneal macrophages were incubated with fluorescence-labelled dextran in the presence and absence of liposomes containing NPPE or PE (0.75 mM) and examined by fluorescence microscopy. (Left) Phase contrast and fluorescence views of the same microscopic fields are shown. Scale bar = 10 μm. (Right) The level of fluorescence in macrophages was determined and expressed relative (in percentage terms) to that in the reaction with no added liposomes. Data are representative of three independent experiments that yielded similar results.

(*42–46*). We thus tested the possibility that NPPE-liposomes inhibit the actions of these two small G proteins in macrophages. The involvement of Rac1 and Cdc42 in the phagocytosis of latex beads was first examined. The level of the active, i.e. GTP-bound, form of either Rac1 or Cdc42 transiently increased in peritoneal and J774A.1 macrophages with a peak at

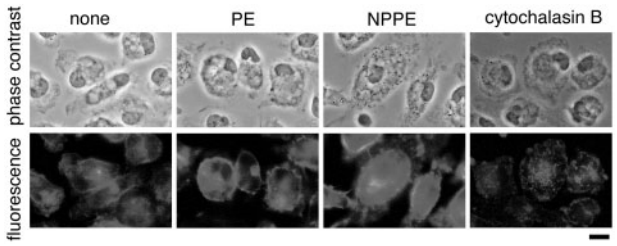

Fig. 5. **Effect of NPPE-containing liposomes on the organization of actin filaments in macrophages**. Mouse peritoneal macrophages were incubated with NPPE liposomes (0.75 mM), PE liposomes (0.75 mM), or cytochalasin B (50 mM) and actin filaments were visualized using fluorescence-labelled phalloidin. Phase contrast and fluorescence views of the same microscopic fields are shown. Scale bar = 10 μm.

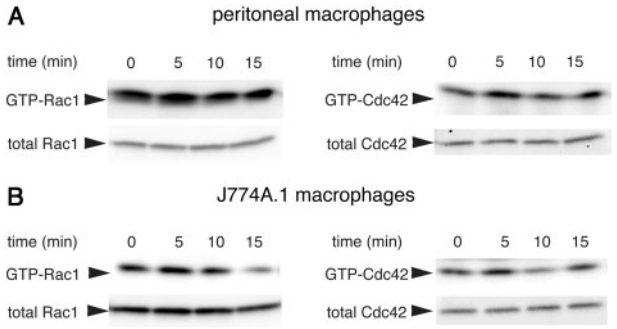

Fig. 6. **Transient increase of GTP-bound Rac1 and Cdc42 in phagocytosing macrophages**. Mouse peritoneal macrophages (A) and J774A.1 macrophages (B) were incubated with latex beads for the indicated periods, and their whole-cell lysates were subjected to an assay for GTP-bound Rac1 and Cdc42. The data for total (both GTP-bound and GDP-bound forms) proteins are also shown. Data are representative of three or more independent experiments that yielded similar results.

about 5 min of incubation with latex beads while total amount remained unchanged (Fig. 6). To know if Rac1 and Cdc42 are needed for the phagocytosis, J774A.1 macrophages after transfection with cDNA coding for the dominant negative form of Rac1 (Rac1N17) or Cdc42 (Cdc42N17) were used in phagocytosis reactions. We found that the level of the phagocytosis of latex beads was significantly lowered by the introduction of either DNA (Fig. 7), indicating that macrophages require the actions of both Rac1 and Cdc42 to phagocytose latex beads. We then examined the effect of NPPE on the activation of Rac1 and Cdc42 in macrophages during phagocytosis. The presence of NPPE-containing liposomes abolished an increase in the level of GTP-bound Rac1 and Cdc42 in either peritoneal or J774A.1 macrophages, whereas control PE-liposomes had no effect (Fig. 8). These results showed that NPPE-liposomes inhibit the activation of Rac1 and Cdc42, and suggested that this effect is a mechanism underlying the inhibition of pseudopod-mediated phagocytosis by the liposomes.

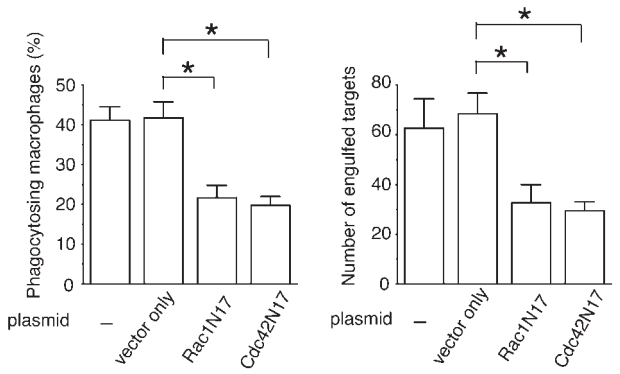

Fig. 7. **Requirement for Rac1 and Cdc42 in phagocytosis**. J774A.1 macrophages that had been transfected with DNA coding for the dominant-negative Rac1 (Rac1N17) or dominant-negative Cdc42 (Cdc42N17) were subjected to an assay for the phagocytosis of latex beads. Data are representative of three independent experiments that yielded similar results.

DISCUSSION

We found in this study that NPPE is involved in the regulation of inflammation by inhibiting the phagocytic activity of macrophages. This inhibition of phagocytosis seemingly owes to the action of NPPE to prevent the activation of the small G proteins Rac1 and Cdc42 in macrophages during phagocytosis. As a consequence, the level of the pseudopod-mediated phagocytosis by macrophages is reduced in the presence of NPPE-containing liposomes. NPE, an end product of NPPE, has been known to have an anti-inflammatory effect (*2*, *7*, *8*). Its production is stimulated upon the induction of inflammation (*16*), but NPE is rapidly hydrolysed giving rise to fatty acid and ethanolamine by the *N*-acylethanolamine-hydrolysing acid amidase and fatty acid amide hydrolase expressed in macrophages (*47*). As a result, the concentration of NPE at sites of inflammation is lower than that of its precursor NPPE. Our findings thus suggest that NPPE plays important roles in the regulation of inflammation as well as does NPE. The inhibitory effect of NPPE on macrophage phagocytosis most likely contributes to the termination of inflammation to protect tissues from being damaged by excess immune reactions.

The generation of NPPE starts with PE at the cytoplasmic side of the plasma membrane bilayer where both PE and the enzyme responsible, *N*-acyltransferase, are abundant (*9*–*15*). Presumably, NPE is liberated from the membrane and released into the extracellular space while more acylated NPPE remains as a component of the plasma membrane. There are two possible modes for the action of NPPE: one is that NPPE-containing membrane vesicles are released from cells and act on other cells, and the other is that NPPE functions only within the cells where it is synthesized. In order for NPPE to act outside, it needs to be first translocated from the inner to the outer leaflet of the plasma membrane, and then released from cells as membrane vesicles. In fact, the presence of NAPE in the outer leaflet of the plasma membrane has been suggested (*48*). It is widely appreciated that such vesicles called exosomes are produced and delivered from cells upon

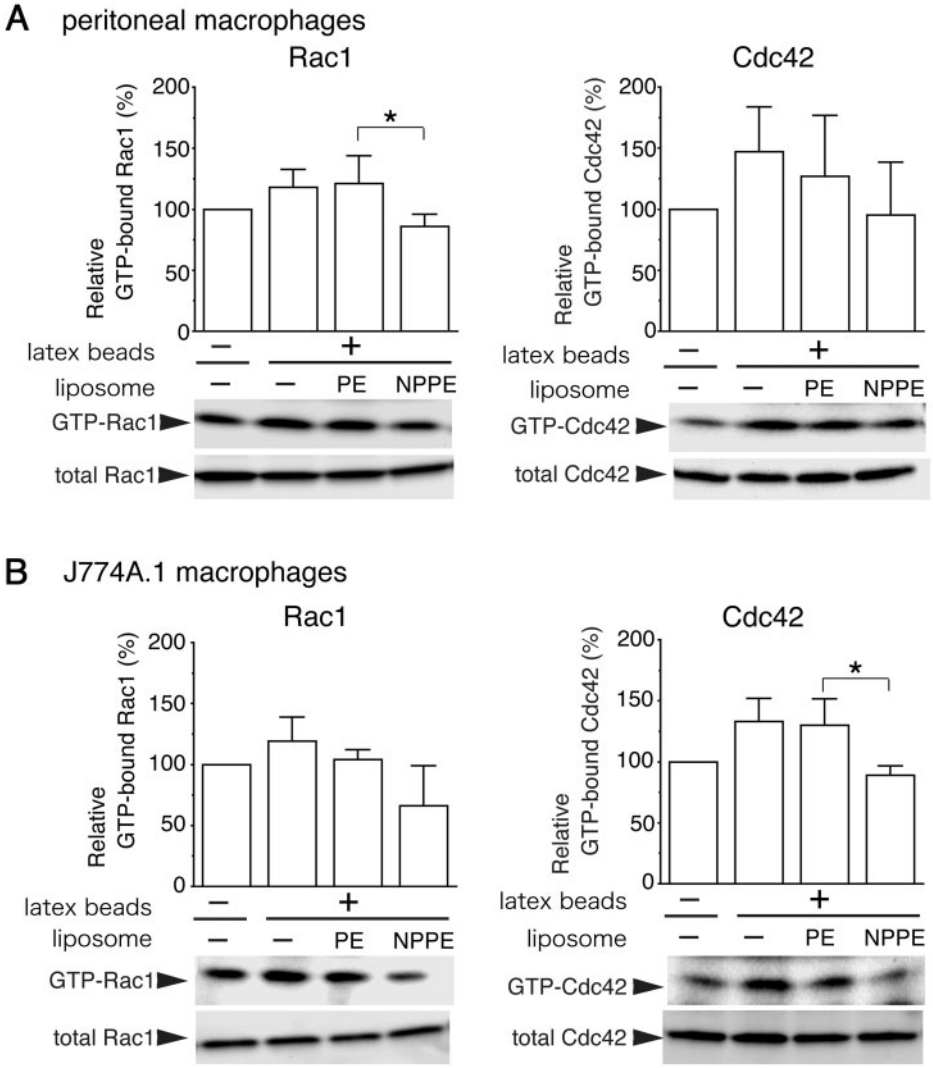

Fig. 8. **Inhibition of the formation of green fluorescent protein-bound Rac1 and Cdc42 in phagocytosing macrophages by NPPE liposomes**. Mouse peritoneal macrophages (A) and J774A.1 macrophages (B) were incubated with latex beads for 5 min in the presence and absence of the indicated liposomes (0.75 mM) and whole-cell lysates were analysed for the level of GTP-bound Rac1 and Cdc42. The intensity of the signals was determined and expressed relative (in percentage term) to that in the reaction with no added liposomes and beads. Data are representative of three independent experiments that yielded similar results.

activation (*49*, *50*). The exosomes produced in septic tissue contain milk fat globule epidermal growth factor VIII, a ligand for macrophage phagocytosis receptors, and alter the level of macrophage phagocytosis, leading to the attenuation of pro-inflammatory responses (*51*). The release of NPPE-containing exosomes could occur when macrophages are stimulated by microbial pathogens or inflammatory cytokines. In the second mode of action, NPPE acts intracellular as a signal mediator like phosphoinositides and therefore does not need to move to the cell surface. It is unclear whether or not NPPE requires a specific receptor to exert its function. We here showed that the cannabinoid receptor CB2 is not required for NPPE-liposomes to inhibit macrophage phagocytosis. The vanilloid receptor TRPV1 that also recognizes endocannabinoid seems to be not involved in the action of NPPE because the TRPV1 agonist resiniferatoxin did not alter the phagocytosis of latex beads by macrophages (data not shown). This suggests that the identification of a novel receptor(s), if it exists at all, is necessary. It is also possible that NPPE regulates macrophage phagocytosis without the aid of any receptors. As to how NPPE-containing liposomes repress the activation of Rac1 and Cdc42 remains to be elucidated. The conversion of small G proteins from the inactive to active GTP-bound form is mediated by guanine nucleotide-exchanging factors (*52–54*). NPPE-liposomes might somehow retard the actions of such factors specific to Rac1 and Cdc42. Many other lipid metabolites are produced in inflammatory cells and might act to regulate inflammation. In fact, 2-arachidonoylglycerol, known as an endocannabinoid, is synthesized from

phosphatidylinositol and stimulates macrophage phagocytosis in a manner mediated by CB2 (*24*, *25*). This means that a variety of lipid metabolites, which function to either stimulate or inhibit inflammation, exist at the site of inflammation. It is necessary for a better understanding of how inflammation is evoked and terminated, not only to clarify the actions of each lipid metabolite but also to determine the level of the metabolites at sites of inflammation.

ACKNOWLEDGEMENT

We thank T. Miyawaki for the gift of pCS-venus.

FUNDING

Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (16570112, 18570123, partial); Ministry of Education, Culture, Sports, Science and Technology-Japan (18057009 to A.S.).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Schmid, H.H.O. (2000) Pathways and mechanisms of *N*-acylethanolamine biosynthesis: can anandamide be generated selectively? *Chem. Phys. Lipids* **108**, 71–78
2. Lambert, D.M., Vandevoorde, S., Jonsson, K.O., and Fowler, C.J. (2002) The palmitoylethanolamide family: a new class of anti-inflammatory agents? *Curr. Med. Chem.* **9**, 663–674
3. Franklin, A., Parmentier-Batteur, S., Walter, L., Greenberg, D.A., and Stella, N. (2003) Palmitoylethanolamide increases after focal cerebral ischemia and cell motility. *J. Neurosci.* **23**, 7767–7775
4. Pacher, P., Batkai, S., and Kunos, G. (2006) The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* **58**, 389–462
5. Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**, 1946–1949
6. Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodriguez de Fonseca, F., Rosengarth, A., Luecke, H., Di Giacomo, B., Tarzia, G., and Piomelli, D. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature* **425**, 90–93
7. Facci, L., Toso, R.D., Romanello, S., Buriani, A., Skaper, S.D., and Leon, A. (1995) Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc. Natl Acad. Sci. USA* **92**, 3376–3380
8. Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A., and Piomelli, D. (2005) The nuclear receptor peroxysome proliferator-activated receptor-a mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol. Pharmacol.* **67**, 15–19
9. Okamoto, Y., Morishita, J., Wang, J., Schmid, P.C., Krebsbach, R.J., Schmid, H.H.O., and Ueda, N. (2005) Mammalian cells stably overexpressing *N*-acylphosphatidylethanolamine-hydrolysing phospholipase D exhibit significantly decreased levels of *N*-acylphosphatidylethanolamines. *Biochem. J.* **389**, 241–247
10. Ueda, N., Okamoto, Y., and Morishita, J. (2005) *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D: a novel enzyme of the beta-lactamase fold family releasing anandamide and other *N*-acylethanolamines. *Life Sci.* **77**, 1750–1758
11. Leung, D., Saghatelian, A., Simon, G.M., and Cravatt, B.F. (2006) Inactivation of *N*-acylphosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry* **45**, 4720–4726
12. Wang, J., Okamoto, Y., Morishita, J., Tsuboi, K., Miyatake, A., and Ueda, N. (2006) Functional analysis of the purified anandamide-generating phospholipase D as a member of the metallo-beta-lactamase family. *J. Biol. Chem.* **281**, 12325–12335
13. Liu, J., Wang, L., Harvey-White, J., Osei-Hyiaman, D., Razdan, R., Gong, Q., Chan, A.C., Zhou, Z., Huang, B.X., Kim, H.Y., and Kunos, G. (2006) A biosynthetic pathway for anandamide. *Proc. Natl Acad. Sci. USA* **103**, 13345–13350
14. Jin, X.-H., Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., and Ueda, N. (2007) Discovery and characterization of a Ca^{2+}-independent phosphatidylethanolamine *N*-acyltransferase generating the anandamide precursor and its congeners. *J. Biol. Chem.* **282**, 3614–3623
15. Wang, J., Okamoto, Y., Tsuboi, K., and Ueda, N. (2008) The stimulatory effect of phosphatidylethanolamine on *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD). *Neuropharmacology* **51**, 8–15
16. Hansen, H.H., Ikonomidou, C., Bittigau, P., Hansen, S.H., and Hansen, H.S. (2001) Accumulation of the anandamide precursor and other *N*-acylethanolamine phospholipids in infant rat models of *in vivo* necrotic and apoptotic neuronal death. *J. Neurochem.* **76**, 39–46
17. Sugiura, T., Kishimoto, S., Oka, S., and Gokoh, M. (2006) Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. *Prog. Lipid Res.* **45**, 405–446
18. Ligresti, A., Cascio, M.G., and Di Marzo, V. (2005) Endocannabinoid metabolic pathways and enzymes. *Curr. Drug Targets CNS Neurol. Disord.* **4**, 615–623
19. van der Stelt, M. and Di Marzo, V. (2004) Putative endogenous ligands of transient receptor potential vanilloid 1 channels. *Eur. J. Biochem.* **271**, 1827–1834
20. Schmid, H.H.O. and Berdyshev, E.V. (2002) Cannabinoid receptor-inactive *N*-acylethanolamines and other fatty acid amides: metabolism and function. *Prostaglandins Leukot. Essent. Fatty Acids* **66**, 363–376
21. Bradshaw, H.B. and Walker, J.M. (2005) The expanding field of cannabimimetic and related lipid mediators. *Br. J. Pharmacol.* **144**, 459–465
22. Brown, A.J. (2007) Novel cannabinoid receptors. *Br. J. Pharmacol.* **152**, 567–575
23. Aderem, A. and Underhill, D.M. (1999) Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* **17**, 593–623
24. Shiratsuchi, A., Watanabe, I., Yoshida, H., and Nakanishi, Y. (2008) Involvement of cannabinoid receptor CB2 in dectin-1-mediated macrophage phagocytosis. *Immunol. Cell Biol.* **86**, 179–184
25. Gokoh, M., Kishimoto, S., Oka, S., and Sugiura, T. (2007) 2-Arachidonoylglycerol enhances the phagocytosis of opsonized zymosan by HL-60 cells differentiated into macrophage-like cells. *Biol. Pharm. Bull.* **30**, 1199–1205
26. Sulcova, E., Mechoulam, R., and Fride, E. (1998) Biphasic effects of anandamide. *Pharm. Biochem. Behav.* **59**, 347–352
27. Schmid, H.H.O., Schmid, P.C., and Natarajan, V. (1990) *N*-acylated glycerophospholipids and their derivatives. *Prog. Lipid Res.* **29**, 1–43
28. Hansen, H.S., Moesgaard, B., Hansen, H.H., and Petersen, G. (2000) *N*-acylethanolamines and precursor

phospholipids-relation to cell injury. *Chem. Phys. Lipids* **108**, 135–150
29. Mazzari, S., Canella, R., Petrelli, L., Marcolongo, G., and Leon, A. (1996) *N*-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cells activation. *Eur. J. Pharmacol.* **300**, 227–236
30. Berdyshev, E.V., Boichot, E., Germain, N., Allain, N., Anger, J.-P., and Lagente, V. (1997) Influence of fatty acid ethanolamides and Δ9-tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells. *Eur. J. Pharmacol.* **330**, 231–240
31. Schmid, P.C., Reddy, P.V., Natarajan, V., and Schmid, H.H.O. (1983) Metabolism of *N*-acylethanolamine phospholipids by a mammalian phosphodiesterase of the phospholipase D type. *J. Biol. Chem.* **258**, 9302–9306
32. Bartlett, G.R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**, 466–468
33. Shiratsuchi, A., Umeda, M., Ohba, Y., and Nakanishi, Y. (1997) Recognition of phosphatidylserine on the surface of apoptotic spermatogenic cells and subsequent phagocytosis by Sertoli cells of the rat. *J. Biol. Chem.* **272**, 2354–2358
34. Shiratsuchi, A. and Nakanishi, Y. (1999) Phosphatidylserine-mediated phagocytosis of anticancer drug-treated cells by macrophages. *J. Biochem.* **126**, 1101–1106
35. Fujii, C., Shiratsuchi, A., Manaka, J., Yonehara, S., and Nakanishi, Y. (2001) Difference in the way of macrophage recognition of target cells depending on their apoptotic states. *Cell Death Differ.* **8**, 1113–1122
36. Watanabe, I., Ichiki, M., Shiratsuchi, A., and Nakanishi, Y. (2007) TLR2-mediated survival of *Staphylococcus aureus* in macrophages: a novel bacterial strategy against host innate immunity. *J. Immunol.* **178**, 4917–4925
37. Shiratsuchi, A., Watanabe, I., Takeuchi, O., Akira, S., and Nakanishi, Y. (2004) Inhibitory effect of Toll-like receptor 4 on fusion between phagosomes and endosomes/lysosomes in macrophages. *J. Immunol.* **172**, 2039–2047
38. Feig, L.A. and Cooper, G.M. (1998) Inhibition of NIH3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell Biol.* **8**, 3235–3243
39. Osada, Y., Shiratsuchi, A., and Nakanishi, Y. (2006) Involvement of mitogen-activated protein kinases in class B scavenger receptor type I-induced phagocytosis of apoptotic cells. *Exp. Cell Res.* **312**, 1820–1830
40. Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., and Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5, but not EDG1 or EDG3. *Mol. Cell Biol.* **20**, 9247–9261
41. Carlisle, S.J., Marciano-Cabral, F., Staab, A., Ludwick, C., and Cabral, G.A. (2002) Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *Int. Immunopharmacol.* **2**, 69–82
42. Small, J.V., Stradal, T., Vignal, E., and Rottner, K. (2002) The lamellipodium: where motility begins. *Trends Cell Biol.* **12**, 112–120
43. Ellis, S. and Mellor, H. (2000) Regulation of endocytic traffic by Rho family GTPases. *Trends Cell Biol.* **10**, 85–88
44. Qualmann, B. and Mellor, H. (2003) Regulation of endocytic traffic by Rho GTPases. *Biochem. J.* **371**, 233–241
45. Bokoch, G.M. (2005) Regulation of innate immunity by Rho GTPases. *Trends Cell Biol.* **15**, 163–171
46. Vega, F.M. and Ridley, A.J. (2007) Snapshot: rho family GTPases. *Cell* **129**, 1430
47. Sun, Y.X., Tsuboi, K., Zhao, L.Y., Okamoto, Y., Lambert, D.M., and Ueda, N. (2005) Involvement of *N*-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other *N*-acylethanolamines in macrophages. *Biochim. Biophys. Acta* **1736**, 211–220
48. Cadas, H., Schinelli, S., and Piomelli, D. (1996) Membrane localization of *N*-acylphosphatidylethanolamine in central neurons: studies with exogenous phospholipases. *J. Lipid Mediat. Cell Signal.* **14**, 63–70
49. Keller, S., Sanderson, M.P., Stoeck, A., and Altevogt, P. (2006) Exosomes: from biogenesis and secretion to biological function. *Immunol. Lett.* **1070**, 102–108
50. Li, X.-B., Zhang, Z.-R., Schluesener, H.J., and Xu, S.-Q. (2006) Role of exosomes in immune regulation. *J. Cell Mol. Med.* **10**, 364–375
51. Miksa, M., Wu, R, Dong, W., Das, P., Yang, D., and Wang, P. (2006) Dendritic cell-derived exosomes containing milk fat globule epidermal growth factor-factor VIII attenuate proinflammatory responses in sepsis. *Shock* **25**, 586–593
52. Côté, J.F. and Vuori, K. (2007) GEF what? Dock180 and related proteins help Rac to polarize cells in new ways. *Trends Cell Biol.* **17**, 383–393
53. Bos, J.L., Rehmann, H., and Wittinghofer, A. (2007) GEFs and GAPs: critical elements in the control of small G proteins. *Cell* **129**, 865–877
54. Cherfils, J. and Chardin, P. (1999) GEFs: structural basis for their activation of small GTP-binding proteins. *Trends Biochem. Sci.* **24**, 306–311