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The Effect of Anionic Amphiphiles on the Recruitment of Rac in Neutrophils

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Anionic amphiphiles have been shown to influence the NADPH oxidase system. Although one target of the amphiphile action is p47^{phox}, the cell-free activation of the enzyme in the absence of p47^{phox} is also influenced. In the present study, we examined the actions of sodium dodecyl sulfate (SDS) on the NADPH oxidase system *in vivo*. Treatment of guinea pig neutrophils with the amphiphile caused the translocation of Rac to a membrane fraction and its conversion to the GTP-bound form. Because SDS had little effect on p47^{phox}, it increased the superoxide production only when p47^{phox} was otherwise activated. Inhibitors of phosphoinositide 3-kinases had no effect on the SDS-induced translocation of Rac to the membrane. However, the inhibitors prevented the conversion of Rac to its GTP-bound form, indicating that these two processes can be controlled separately. In a cell-free system, SDS induced the binding of p47^{phox} and Rac to the membrane preparation. The SDS concentration inducing the Rac binding was lower than that inducing the p47^{phox} binding. Thus we observed that Rac is more sensitive to SDS than p47^{phox} both *in vivo* and *in vitro*. The results suggest a role of natural amphiphiles such as unsaturated fatty acids in regulation of Rac activation.

Key words: anionic amphiphiles, NADPH oxidase, neutrophil, Rac, sodium dodecyl sulfate.

Phagocytic cells including neutrophils produce oxygen radicals, which are essential for host-defense against invading bacteria, fungi and parasites. NADPH oxidase is a multi-component enzyme responsible for production of the superoxide anion, a precursor of more reactive oxygen species. The catalytic core of the enzyme is cytochrome b_{558}, an integral membrane protein consisting of gp91^{phox} and p22^{phox} subunits. The enzyme is inactive in the absence of at least three cytosol proteins, p47^{phox}, p67^{phox} and Rac. Upon cell activation, these regulatory proteins move to cell membrane to make up the active complex of NADPH oxidase with cytochrome b_{558} (for review Ref. *1*).

It has been shown that p67^{phox} directly interacts with gp91^{phox} and regulates its catalytic activity (*2–4*). *In vitro* studies indicate that this interaction is enhanced by addition of the activated form of p47^{phox} (*4*). Because neutrophils from individuals with genetic deficiencies of p47^{phox} lack the ability to generate the superoxide anion (*5–8*), this adaptor protein is considered to play a key regulatory role under physiological conditions. Recruitment of p47^{phox} is considered to be under the control of protein kinases, as suggested by the effect of inhibitors on both the phosphorylation of p47^{phox} and the superoxide generation (*9–11*). In a cell-free system consisting of purified components of NADPH oxidase, on the other hand, anionic amphiphiles such as sodium dodecyl sulfate (SDS) and arachidonic acid (AA) are known to facilitate the formation of an active complex of the enzyme by causing a conformational change of p47^{phox} (*12–17*). Although the phosphorylation of p47^{phox} causes similar changes, the effect is reported to be much weaker than that of the amphiphiles (*18*, *19*). Because the sensitivity of p47^{phox} to AA increases after the phosphorylation, it has been suggested that the phosphorylation and AA regulate p47^{phox} in a cooperative manner under physiological conditions in cells (*20*).

In human neutrophils, expression of a dominant negative form of Rac2 impairs the generation of the superoxide anion (*21*, *22*). The analysis of Rac2 (–/–) mice provides further evidence for the essential role of this GTP-binding protein (*23–26*). In a cell-free system, the activation of NADPH oxidase occurs even in the absence of p47^{phox} when Rac is supplemented with high concentrations of p67^{phox} (*17*, *27–29*). Thus, Rac is another essential regulator of the NADPH oxidase system. Activators of the superoxide production have been shown to induce the conversion of Rac to its GTP-bound form (*25*, *30–32*). Prevention of the Rac activation by use of inhibitors of phosphoinositide 3-kinase (PI 3-kinase) causes a parallel loss of fMLP-stimulated production of the superoxide anion (*25*, *30*, *31*). The need for the GTP-bound Rac in the activation of the NADPH oxidase system has been also shown in cell-free reconstitution systems (*33–35*). However, it has been reported that Rac in its GDP-bound form can activate the NADPH oxidase under some conditions (*29*, *36*, *37*). Rac is known to associate with RhoGDI in cytosol, and this complex has been shown to dissociate in the presence of anionic amphiphiles (*38*, *39*). In an *in vitro* study, on the other hand, Rac–RhoGDI complex has

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been reported to activate the NADPH oxidase without the dissociation (*37*).

In the present study, we examined the actions of SDS on the NADPH oxidase system. Treatment of guinea pig neutrophils with the amphiphile caused the translocation of Rac to a membrane fraction and its conversion to the GTP-bound form. SDS increased the superoxide production of the neutrophils only when $p47^{phox}$ was otherwise activated by calyculin A. These results indicate that SDS influences the NADPH oxidase system by affecting the Rac recruitment to the system and has little effect on $p47^{phox}$. Our results also indicate that the Rac recruitment occurs in two steps: SDS causes the translocation of Rac in a PI 3-kinase-independent process, while the conversion of Rac to its GTP-bound form is dependent on the PI 3-kinase activity in the cells.

EXPERIMENTAL PROCEDURES

Materials—Neutrophils were obtained from the peritoneal cavities of female guinea pigs of the Hartley strain as reported previously (*40*). The content of neutrophils in the preparation was more than 90%. Rabbit anti-$p47^{phox}$ antibody was prepared as described previously (*41*). The cDNA encoding the Rac-binding domain (RBD) of human PAK2 (amino acids 66–147) in pGEX-4T–based expression vector was a gift from Dr. Hideki Sumimoto (Kyushu University, Japan). Glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* and adsorbed onto gluthatione-Sepharose 4B beads (Amersham Pharmacia) in accordance with the manufacturer's instructions. The beads were stored at –30°C in phosphate-buffered saline containing 50% glycerol. Other materials were from the commercial sources: ferricytochrome *c* (horse heart type III), superoxide dismutase (SOD) and type III-S histone from Sigma; calyculin A, SDS (electrophoresis grade) and okadaic acid from Wako Pure Chemical; arachidonic acid from Funakoshi Pharmaceutical; LY294002 from Cayman Chemical; dipalmitoyl (di C_{16}) PtdIns (*3*,*4*,*5*) P_3 from Matreya; anti-Rac and anti-$p67^{phox}$ antibodies from Transduction Laboratories.

O_2^- Production of Neutrophils—O_2^- production by neutrophils was measured on the basis of SOD-inhibitable reduction of ferricytochrome *c*, as reported previously (*42*). Neutrophils (10^6 cells / ml) suspended in Hanks'-PIPES buffer (137 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 1.28 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.43 mM Na_2HPO_4, 5.5 mM glucose and 8 mM PIPES, pH 7.3) were preincubated at 37°C for 5 min with or without 0.1 μM calyculin A. LY294002 or wortmannin was included during this period when the effects of the inhibitors were examined. After the addition of 0.1 mM ferricytochrome *c* and SDS, the mixture was further incubated at 37°C for 10 min, chilled to stop the reaction, and quickly centrifuged at 1,000 × *g* for 10 min. The supernatant was analyzed for the reduced cytochrome *c* by measuring the increase in absorbance at 550 nm. In time-course experiments, the reaction mixture in a cuvette was thermostatically controlled, and the absorbance at 550 nm was monitored continuously in reference to the SOD-containing cuvette using a Hitachi U3210 spectrophotometer. When the effect of di-C_{16} PtdIns (3,4,5) P_3 was examined, the lipid was added as a mixture with 10 μM histone (*43*) 3 min before the addition of SDS.

Phosphorylation of $p47^{phox}$—Neutrophils were labeled with carrier-free $^{32}P_i$, as described previously (*44*). The ^{32}P-labeled neutrophils (10^7 cells/ml) suspended in phosphate-free Hanks'-PIPES buffer were incubated with or without 0.1 μM calyculin A at 37°C for 5 min. After the addition of 50 μM SDS, the mixture was incubated at 37°C for 10 min, then rapidly centrifuged at 3,000 × *g* for 10 s. The cells were lysed in an ice-cold lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 200 μM phenylmethansulfonyl fluoride (PMSF), 2 μM pepstatin, 2 μM leupeptin, 20 μM (4-amidinophenyl)-methanesulfonyl fluoride (pA-PMSF), 0.1 μM staurosporine, and 0.1 μM calyculin A. After centrifugation at 10,000 × *g* for 10 min, the supernatant was incubated with a rabbit anti-$p47^{phox}$ antibody for 1.5 h at 4°C, then mixed with Protein G-Sepharose. The immunoprecipitate was washed three times with the lysis buffer and boiled for 5 min in SDS sample buffer. The solubilized proteins were separated by SDS-PAGE and detected by autoradiography.

Preparation of Membrane Fraction from Activated Neutrophils—Neutrophils were suspended in Hanks'-PIPES buffer and incubated with or without 0.1 μM calyculin A at 37°C for 5 min. After the addition of 50 μM SDS or 2.5 μM AA, the mixture was further incubated for the indicated periods before the reaction was stopped by chilling. The mixture was quickly centrifuged (120 g for 5 min), and the precipitate was suspended in a relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 10 mM PIPES, pH 7.3) containing 0.2 mM PMSF, 2 μM pepstatin, 2 μM leupeptin and 20 μM pA-PMSF. The cells were disrupted by sonication (5 s × 5 bursts) at 0°C using an Olympus Bioruptor. The homogenate was centrifuged (900 × *g* for 10 min) to remove unbroken cells and nuclei, and the supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet was suspended at the concentration equivalent to 10^8 cells/ml in the same buffer.

In Vivo Translocation of Cytosolic Factors to the Membrane—The crude membrane fraction, prepared as described above, was mixed with 3× SDS-PAGE sample buffer and boiled for 5 min. Each sample was subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). After blocking, the membrane was incubated with a rabbit polyclonal antibody against $p47^{phox}$, a monoclonal antibody against Rac, or a monoclonal antibody against $p67^{phox}$. The membrane was washed and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG. The second antibodies were located using the enhanced chemiluminescence detection system (PerkinElmer).

NADPH Oxidase Activity of the Membrane Fraction—NADPH oxidase activity of the membrane fraction was measured on the basis of SOD-inhibitable reduction of ferricytochrome *c* at 25°C. The membrane fraction (50 μg of protein) was incubated with 0.1 mM ferricytochrome *c* and 0.2 mM NADPH in the relaxation buffer. Absorbance change at 550 nm in reference to the SOD-supplemented mixture was monitored.

Rac Activation Assay—Neutrophils suspended in Hanks'-PIPES buffer were stimulated as described above.

At the appropriate time, the reaction was stopped by mixing with a half volume of a buffer consisting of 75 mM Tris (pH 7.6), 225 mM NaCl, 15 mM $MgCl_2$, 2.7 mM EGTA, 3% Nonidet P-40, 15% glycerol, 0.6 mM PMSF, 6 μM pepstatin, 6 μM leupeptin and 60 μM pA-PMSF. The lysate was centrifuged at 10,000 × *g* for 1 min, and the supernatant was incubated with GST-PAK2-RBD–bound beads for 10 min. The beads were washed twice with the lysis buffer and boiled in SDS sample buffer for 5 min. The bound Rac were quantified by Western blotting with an anti-Rac monoclonal antibody.

In Vitro Translocation of the Cytosolic Factors to the Membrane Fraction—The cytosol and the plasma membrane fractions were prepared from resting neutrophils as described previously (*45*). A mixture of the cytosol and membrane fractions (100 μg/ml and 30 μg/ml of protein, respectively) in the relaxation buffer was incubated at 25°C in the presence or absence of SDS. After 3 min, the reaction mixture was carefully layered on the same volume of the relaxation buffer containing 15% (w/v) sucrose and centrifuged at 100,000 × *g* for 2 h. The membrane fraction at the bottom of the centrifugation tube was subjected to Western blotting with anti-$p47^{phox}$ or anti-Rac antibodies.

RESULTS

We have reported that calyculin A, a potent inhibitor of protein phosphatases, induced a marked phosphorylation of $p47^{phox}$ in guinea pig neutrophils (Ref. *46*, see Fig. 3A). The level of phosphorylation was higher than that attained by 0.1 μM PMA, a potent activator of superoxide anion production of the cells (*46*). Nevertheless, treatment of the neutrophils with calyculin A did not induce the superoxide anion production (Fig. 1A), indicating that the phosphorylation of $p47^{phox}$ is not enough to activate NADPH oxidase in the intact cells. Interestingly, stimulation of the calyculin A–treated cells with SDS triggered a marked superoxide production, even though SDS alone showed no effect on control cells (Fig. 1A). A similar effect of SDS was observed when okadaic acid was used as an inhibitor of protein phosphatases (data not shown).

In the experiment shown in Fig. 1B, the neutrophils were disrupted after treatment with calyculin A and/or SDS, and the NADPH oxidase activity of membrane fractions therefrom was measured. The activity *in vitro* was little affected by *in vivo* treatment with SDS or calyculin A alone, but it was increased markedly by their combination. The result indicates that SDS and calyculin A possess a synergistic effect on the formation of the active complex of NADPH oxidase in the membranes. Figure 1C shows the dose-dependent effect of SDS. In control cells, SDS alone had only a marginal effect even at 100 μM. However, it induced a marked production of superoxide anion at a concentration as low as 10 μM in the calyculin A–treated neutrophils.

Arachidonic acid at its higher concentration (5 μM) caused a marked superoxide production by itself (Fig. 2A), in agreement with previous studies (*47–50*). AA at its lower concentration (2.5 μM) showed a faint effect by itself but caused marked superoxide production in the presence of calyculin A. The synergism was observable when oleic acid, linolenic acid, or palmitoleic acid was used as an amphiphile (data not shown). A saturated fatty acid, stearic acid, caused no effect on the superoxide

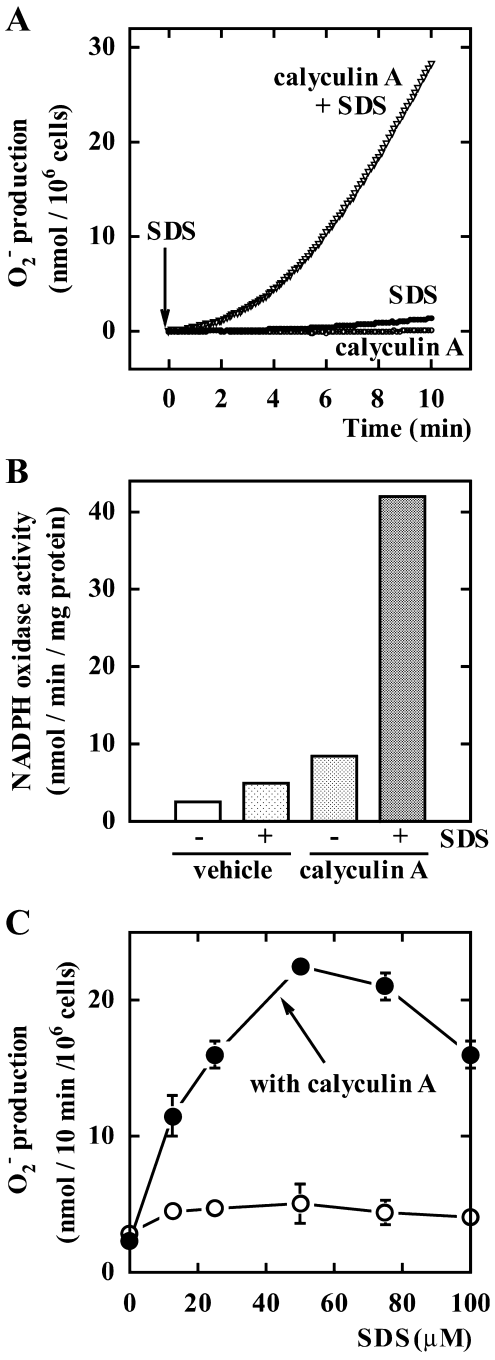

Fig. 1. **Effect of SDS on the NADPH oxidase activity of calyculin A–treated neutrophils.** Neutrophils were incubated with 0.1 μM calyculin A or DMSO at 37°C for 5 min. A, Production of O_2^- was monitored with or without the addition of 50 μM SDS. B, The cells were further incubated with or without 50 μM SDS for 10 min. The cells were then disrupted and the membrane preparations therefrom were analyzed for NADPH oxidase activity. C, The cells were further incubated for 10 min with or without the addition of various concentrations of SDS. Production of O_2^- during the incubation was measured.

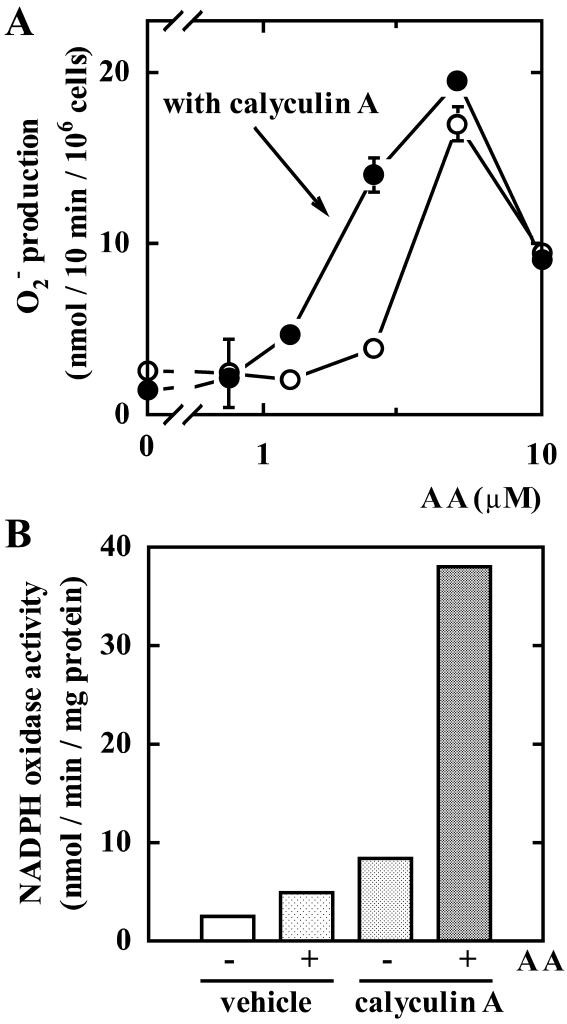

Fig. 2. **Effect of AA on the NADPH oxidase activity of calyculin A–treated neutrophils.** Neutrophils were incubated with 0.1 μM calyculin A or DMSO at 37°C for 5 min. A, The cells were further incubated for 10 min with or without the addition of various concentrations of AA. Production of O_2^- during the incubation was measured. B, The cells were further incubated with or without 2.5 μM AA for 10 min. The cells were then disrupted and the membrane preparations therefrom were analyzed for NADPH oxidase activity.

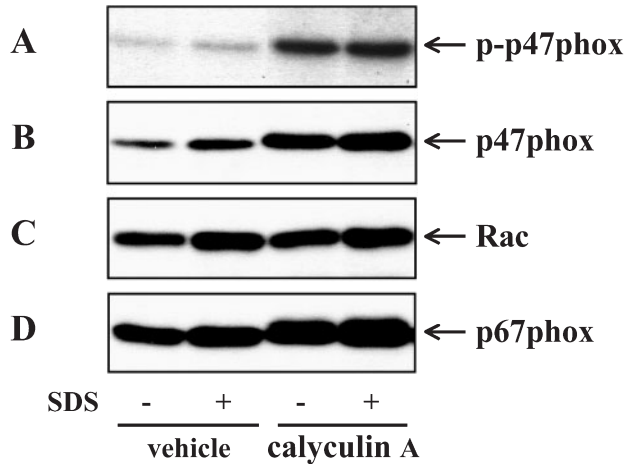

Fig. 3. **Effects of SDS and calyculin A on the translocation of each component of NADPH oxidase.** A, ^{32}P-labeled neutrophils were treated with 0.1 μM calyculin A or DMSO at 37°C for 5 min, and then for 10 min with or without the addition of 50 μM SDS. The lysate of the cells was mixed with the anti-mouse $p47^{phox}$ antibody. The proteins in the immune complex were separated by SDS-PAGE and analyzed for the radioactivity. B, C, D, Neutrophils were incubated with 0.1 μM calyculin A or DMSO at 37°C for 5 min, and then for 10 min with or without the addition of 50 μM SDS. The cells were disrupted to prepare the membrane fraction. The peptides in the fraction were subjected to SDS-PAGE, transferred to PVDF membranes, and analyzed with antibodies against $p47^{phox}$ (B), Rac (C), or $p67^{phox}$ (D).

anion production even in the presence of calyculin A (data not shown). Treatment of the neutrophils with 2.5 μM AA caused a slight increase in the NADPH oxidase activity of the membrane from the cells (Fig. 2B). The activity increased markedly when the cells were treated with both AA and calyculin A. Thus the synergism with calyculin A was observed for many anionic amphiphiles. However, we utilized SDS for further examination because the interaction with calyculin A was prominently observed over a wide range of its concentration.

An inhibitor of tyrosine kinase has been shown to inhibit receptor-mediated recruitment of Rac to the NADPH oxidase system, while it has no effect on $p47^{phox}$ (*51*). The result indicates that two essential regulators, $p47^{phox}$ and Rac, are controlled separately in cells. Thus, we examined the effect of calyculin A and SDS on these regulators. Calyculin A induced a marked phosphorylation of $p47^{phox}$ (Fig. 3A), which was accompanied by its translocation to the membrane fraction (Fig. 3B). SDS showed only minor effects on the phosphorylation (Fig. 3A) and the translocation (Fig. 3B) of $p47^{phox}$ regardless of whether the cells were treated with calyculin A or not. Thus the synergistic action of SDS and calyculin A on the superoxide production was not due to their combined effect on $p47^{phox}$. SDS increased the amount of Rac in the membrane fraction (Fig. 3C). The increase was slight but was observed repeatedly (1.3- to 3.3-fold in five separate experiments). In contrast, calyculin A did not increase the translocation (Fig. 3C). These results indicate that the actions of SDS and calyculin A are complementary in recruiting two essential regulators of the NADPH oxidase. The translocation of $p67^{phox}$ to the membrane fractions was increased by the treatment of cells with either calyculin A or SDS (Fig. 3D). This result may reflect the fact that $p67^{phox}$ possesses the affinity to both $p47^{phox}$ and Rac *in vitro* (*12*, *52–58*).

Activators of superoxide production have been shown to induce the conversion of Rac to its GTP-bound form (*25*, *30–32*). Because this change has been reported to occur by both PI 3-kinase–dependent and independent mechanisms (*30*, *31*), we next examined the possible involvement of the lipid kinase in the SDS-induced Rac recruitment. As shown in Fig. 4A, the SDS-induced translocation of Rac was accompanied by its conversion to the GTP-bound form. Calyculin A decreased the basal amount of the GTP-bound Rac. This inhibitory effect of calyculin A may be one reason why it decreased the superoxide production under some conditions (*41*, *51*, *59*). In spite of this basal inhibition, SDS-induced activation

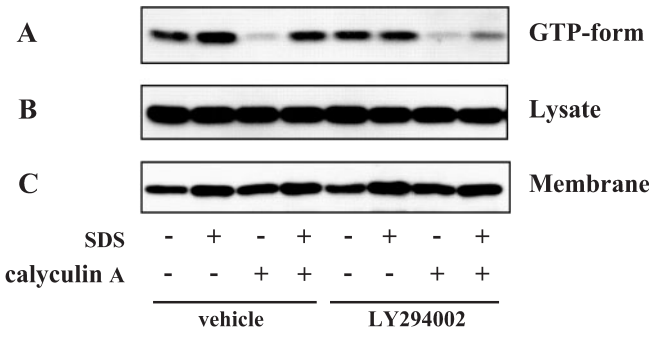

Fig. 4. **Inhibitory effect of LY294002 on the SDS-induced activation of Rac.** Neutrophils were treated in the presence or absence of 0.1 μM calyculin A with or without 100 μM LY294002 at 37°C for 5 min. The cells were further incubated for 10 min with or without the addition of 50 μM SDS. A, The cell lysate was incubated with PAK2-RBD–bound beads for 10 min. Proteins bound to the beads were solubilized, subjected to SDS-PAGE, transferred to PVDF membrane, and analyzed with the antibody against Rac. B, The cell lysate of was subjected to Western blotting analysis with anti-Rac. C, The membrane fraction from the cells was subjected to the Western blotting analysis with anti-Rac.

of Rac was still prominent in the calyculin A–treated cells. This effect on Rac, one of the essential factors of NADPH oxidase, may be the reason why SDS caused a marked production of superoxide anion when $p47^{phox}$, another essential factor of the oxidase, had been activated by calyculin A. LY 294002, an inhibitor of PI 3-kinase, blocked the SDS-induced activation of Rac both in the calyculin A–treated and untreated cells (Fig. 4A). The inhibitor showed no effect on the SDS-induced translocation of Rac to the membrane fraction (Fig. 4C). Similar results were obtained when wortmannin was used as an inhibitor of PI 3-kinase (data not shown).

The above results suggest that the first step of the SDS-induced Rac activation (*i.e.*, translocation to the plasma membrane) occurs by a PI 3-kinase–independent mechanism. However, the inhibition of PI 3-kinase effectively blocked the SDS and calyculin A–induced superoxide production (Fig. 5A). The effect was accompanied by impaired formation of the active complex of NADPH oxidase in membranes (data not shown). In the experiment shown in Fig. 5B, the neutrophils were pretreated with wortmannin and calyculin A, then the effects of SDS and PtdIns 3,4,5-P_3, a product of PI 3-kinase, were examined. Although PtdIns 3,4,5-P_3 by itself caused no superoxide production by the wortmannin-treated cells, it caused a marked activation when Rac had been recruited to the membrane by the use of SDS. The results confirm the role of the lipid products of PI 3-kinase in the activation of the membrane-anchored Rac.

We next examined the action of SDS on the translocation of Rac and $p47^{phox}$ in a cell-free system. The membrane and the cytosol fractions from the guinea pig neutrophils were mixed and incubated in the presence or absence of SDS, and the amount of cytosolic factors associating with the membrane fraction was estimated. Significant translocation of Rac occurred at 25 μM SDS (Fig. 6A), whereas only minor translocation of $p47^{phox}$ was evident at 50 μM SDS (Fig. 6B). Thus, Rac is found to be

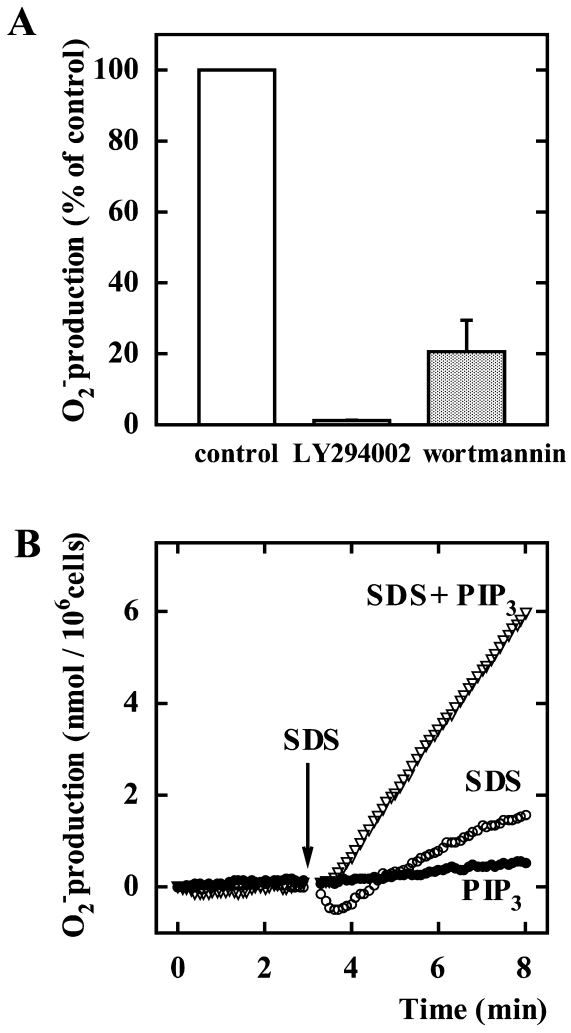

Fig. 5. **Effect of PtdIns 3,4,5-P_3 on the superoxide production of wortmannin-treated neutrophils.** A, Neutrophils were incubated with 0.1 μM calyculin A in the presence of 100 μM LY294002, 1 μM wortmannin or DMSO (control) at 37°C for 5 min. After the addition of 50 μM SDS, production of O_2^- during a 10-min incubation was measured. B, Neutrophils were incubated with 0.1 μM calyculin A and 0.5 μM wortmannin at 37°C for 7 min. At time 0 in the figure, 5 μM PtdIns (3,4,5) P_3 or empty carrier (10 μM histone) was added. The cells were then incubated for 3 min before the addition of 50 μM SDS or H_2O (control).

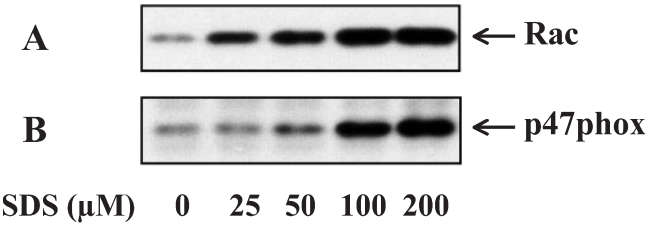

Fig. 6. **Effect of SDS on the in vitro translocation of Rac and $p47^{phox}$ to the plasma membrane.** A, B, The membrane and the cytosol fractions from neutrophils were mixed and incubated at 25°C for 3 min with various concentrations of SDS. The membrane fraction was collected and analyzed by anti-Rac (A) or anti-$p47^{phox}$ (B).

more sensitive than p47^{phox} to the SDS-induced translocation both *in vivo* (Fig. 3) and *in vitro*.

DISCUSSION

Anionic amphiphiles including unsaturated fatty acids, phosphatidic acid and SDS have been shown to influence the NADPH oxidase system. Analysis of the enzyme activation in cell-free systems indicates that one target of the amphiphile action is p47^{phox} (*12–14*, *16*, *17*). In a masking-unmasking model of p47^{phox} activation (*12*), the amphiphiles cause a conformational change of p47^{phox} by disrupting the intramolecular interaction between the C-terminal region and the SH3 domain of the protein. In the present study, we examined the action of SDS on the superoxide anion production of neutrophils, and obtained results suggesting that its *in vivo* action is mainly through its effect on the Rac activation.

We observed that SDS stimulated the superoxide production only when the cells had been treated with calyculin A, a protein phosphatase inhibitor (Fig. 1). Because p47^{phox} is a critical regulatory factor of *in vivo* activation of NADPH oxidase (*5*, *6*, *8*, *60*), one possible explanation for this phenomenon is that SDS acts on p47^{phox} in intact cells. In agreement with this hypothesis, an *in vitro* study showed that the conformational change of p47^{phox} is induced in a synergistic manner with an amphiphile (AA) and the phosphorylation of the protein (*20*). However, we were able to detect only a minimal effect of SDS on the translocation of p47^{phox} both in the calyculin A–treated and untreated cells (Fig. 3, A and B). Furthermore, the concentrations of SDS used in the present study were lower than was sufficient to trigger the superoxide generation in a cell-free system; 50 μM SDS fully activated the superoxide generation in the calyculin A–treated neutrophils (Fig. 1C), while its *in vitro* effect on p47^{phox} was minimal (Fig. 6B). Thus the effect of SDS on a factor other than p47^{phox} was considered to be the basis of its *in vivo* action.

We observed that treatment of cells with calyculin A caused the phosphorylation of p47^{phox} and its translocation to the cell membrane (Fig. 3, A, and B). Interestingly, the calyculin A-induced translocation of p47^{phox} was not accompanied by the translocation of Rac (Fig. 3C) and caused only a marginal generation of superoxide anion (Fig. 1A). Because SDS induced the translocation of Rac without affecting p47^{phox} or the superoxide generation, it is suggested that calyculin A and SDS synergistically stimulate the superoxide production in intact cells by recruiting separately the two regulator proteins, neither of which alone is enough to stimulate the superoxide generation. In a cell-free experiment, 25 μM SDS caused the Rac binding to the membrane fraction with minimum effect on the p47^{phox} binding (Fig. 6A). Thus SDS possesses both *in vivo* and *in vitro* ability to recruit Rac to the membrane fraction, where the active complex of the oxidase is formed. Rac in a cytosol fraction is known to make a complex with RhoGDI. A cell-free experiment has shown that the complex dissociates at 86 μM SDS (*39*). Thus, one possible explanation for the SDS action is that it causes the Rac binding to the membrane by facilitating the dissociation. Alternatively, the lower concentration of SDS used in the present study may facilitate the binding of Rac-RhoGDI to cell membrane, where Rac is freed and activated.

Inhibition of PI 3-kinase has been shown to inhibit receptor-mediated conversion of Rac to its GTP-bound form and activation of superoxide anion production (*30*, *31*). It has been also shown that the product of PI 3-kinase activates Rac by enhancing the activity of GEF (*61*). In the present study, we observed that the inhibitors of PI 3-kinase prevent the SDS-induced formation of the GTP-bound Rac (Fig. 3C). An intriguing observation is that the inhibitors did not affect the SDS-induced translocation to the membrane fraction (Fig. 3C). This result indicates that the Rac recruitment to the oxidase system can be understood as a two-step process. SDS triggers the translocation of the GDP-bound Rac in a PI 3-kinase–independent manner, and the conversion of the membrane-anchored Rac to the GTP-bound form depends on the PI 3-kinase activity. In this process, the dissociation of the Rac-RhoGDI complex should occur before the conversion, because the efficient activation of Rac1 by the DH-PH domain of Tiam1, a Rac-specific GEF, requires the dissociation of the complex and the membrane translocation of Rac (*62*).

We showed that recruitment of Rac and p47^{phox} to the NADPH oxidase complex can be manipulated separately. We used SDS as a tool to examine the mechanism of Rac activation because this amphiphile has, in clear contrast to AA, little effect on p47^{phox} *in vivo*. This may be because AA, but not SDS, has the ability to activate PKC, which phosphorylates p47^{phox}. However, it is intriguing to note that the synergism between amphiphiles and protein phosphatase inhibitors could be observed when lower concentrations of unsaturated fatty acids (Fig. 2) or phosphatidic acid (data not shown) were used. In fact, we have observed that 0.25 μM AA activates Rac in guinea pig neutrophils (data not shown). Thus, it is attractive to speculate that these natural amphiphiles play some role in regulation of Rac under physiological conditions, although further examination is necessary to confirm this possibility.

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