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# Suppression of Inflammatory Responses by Surfactin,* a Selective Inhibitor of Platelet Cytosolic Phospholipase $A_2$

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**ABSTRACT.** Surfactin inhibits platelet and spleen cytosolic 100 kDa phospholipase $A_2$ ($PLA_2$). In contrast, this same compound enhances rat platelet group II $PLA_2$ activity by ~2-fold and slightly increases group I $PLA_2$ activity from porcine pancreas and *Naja naja* venom *in vitro*. Surfactin does not affect a $Ca^{2+}$-independent $PLA_2$ partially purified from bovine brain. Thus, this compound inhibits selectively the cytosolic form of $PLA_2$. Based on *in vitro* studies utilizing preincubation of surfactin with the enzyme, dialysis, and increased concentrations of substrates, the inhibitory effect of surfactin appears to be due to a direct interaction with the enzyme. Linear regression analysis of the linear portion of a concentration–response curve reveals an $IC_{50}$ of 8.5 μM. To further determine the inhibitory pattern, a Dixon plot was constructed to show that the inhibition by surfactin is competitive, but not uncompetitive, with an inhibition constant of $K_i$ = 4.7 μM in 50 mM Tris–HCl buffer, pH 8.0, at 37°. Surfactin blocked non-stimulated and calcium ionophore A23187-stimulated release of arachidonic acid from monkey kidney CV-1 cells, which contain a cytosolic 100 kDa $PLA_2$ as the major activity, as shown in an anionic exchange DEAE-5PW high performance liquid chromatography profile and western blotting analysis. Surfactin ameliorated inflammation induced by several chemicals. That is, it exhibited *in vivo* anti-inflammatory activity in several tested inflammatory reactions including 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema, carrageenan-induced rat paw edema, and acetic acid-induced mouse writhing. These results demonstrate that surfactin is a selective inhibitor for cytosolic $PLA_2$ and a putative anti-inflammatory agent through the inhibitory effect produced by direct interaction with cytosolic $PLA_2$, and that inhibition of cytosolic $PLA_2$ activity may suppress inflammatory responses. BIOCHEM PHARMACOL **55**;7: 975–985, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** surfactin; phospholipase $A_2$; inhibitor; arachidonic acid; inflammation

$PLA_2$s‖ are a family of enzymes that catalyze the hydrolysis of the fatty acid ester bond at the *sn*-2 position of membrane phospholipids. The AA that is released is then converted to inflammatory mediators, such as prostaglandins and leukotrienes, by the enzymes prostaglandin synthetase and lipoxygenase, respectively. The other product of $PLA_2$ activation, lysophospholipid, is the immediate precursor of platelet-activating factor, another potent inflammatory mediator [1, 2]. These mediators elicit inflammatory responses by inducing neutrophil and macrophage infiltration, cell proliferation, and vasomodulation [3, 4].

It has been known that mammalian cells contain several forms of $PLA_2$, which can be classified into secretory and cytosolic forms based on their biochemical properties, localization, and primary structures [5, 6]. Although it has been suggested that the secretory form of $PLA_2$ may be involved in inflammatory responses, much attention has been paid in recent years to the 100 kDa cytosolic $PLA_2$ since it is thought to be a major mediator of agonist-induced AA release implicated in signal transduction of many cell types [4, 6]. Whereas secretory forms of $PLA_2$ exhibit essentially no acyl-chain selectivity, cytosolic $PLA_2$ has a high selectivity for phospholipids with an *sn*-2 arachidonoyl chain. Furthermore, cytosolic $PLA_2$ is found in the cytosol and translocates to membranes in the presence of physiological calcium concentrations [7–10]. All of these results suggest that cytosolic $PLA_2$ plays a role in the signal-coupled release of AA and of the precursor of platelet-activating factor from the membrane phospholipid

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* Surfactin was formerly referred to as PI-003.

‖ *Abbreviations:* $PLA_2$, phospholipase $A_2$; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AA, arachidonic acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; and NSAID, non-steroidal anti-inflammatory drug(s).

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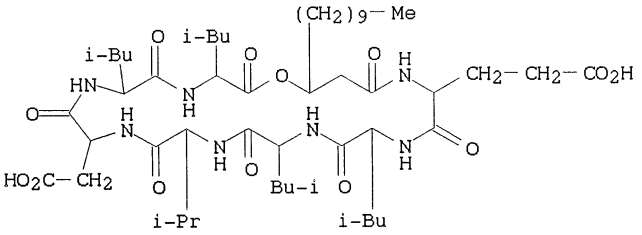

**FIG. 1. Structure of surfactin.**

pool for the production of the inflammatory mediators, thus making cytosolic $PLA_2$ an attractive potential target for anti-inflammatory therapeutics. Cytosolic $PLA_2$ has been purified from a number of mammalian cells including platelets [9, 11], kidney [12], and the human monocytic cell line U937 [10, 13].

Thus far, many attempts to screen an inhibitor against $PLA_2$ implicated in inflammatory reactions have been focused on a secretory form of group II $PLA_2$ as a target enzyme by evaluating its inhibitory ability in *in vitro* experiments using exogenous substrates or inflammatory animal models [14–16]. However, little is known about an inhibitor of cytosolic $PLA_2$ with anti-inflammatory activity. It has been shown that an analogue of AA, in which the COOH group is replaced by a trifluoromethyl ketone group ($COCF_3$), is an inhibitor of cytosolic $PLA_2$ [17].

During our investigations to screen an anti-inflammatory agent from soil microorganisms, we found that a component named PI-003 exhibits selective inhibition of cytosolic $PLA_2$ activity in *in vitro* assays, and subsequent analysis of the structure proved it to be surfactin, a surfactant lipopeptide (Fig. 1). This compound was originally isolated from *Bacillus subtilis* by Arima *et al.* [18] and consists of a heptapeptide and a lipid portion represented by a mixture of several β-hydroxy fatty acids with chain lengths of 13–15 carbon atoms. The main component is 3-hydroxy-13-methyltetradecanoic acid [19].

It is known that surfactin is one of the most powerful biosurfactants, possessing attractive therapeutical and biotechnological properties, such as antibiotic and antifungal activities [18, 20]. In addition, it has been suggested that surfactin may be used as an anti-coagulant for the prophylaxis of thrombosis, and generally for the prevention of diseases such as myocardial infarction and pulmonary embolism through its action of slowing down fibrin clot formation by an unknown mechanism [18].

Here, we report that surfactin inhibited cytosolic $PLA_2$ through its direct interaction with the enzyme using an *in vitro* assay that employs exogenous substrate and cytosolic $PLA_2$ enzyme purified from bovine platelets. The inhibitory effect was also observed by investigating the release of AA from calcium ionophore A23187-stimulated monkey kidney CV-1 cells. Furthermore, whereas surfactin ameliorated the inflammatory reactions induced by several chemicals, such as carrageenan-induced paw edema, acetic acid-induced writhing, and TPA-induced skin inflammation, it had a much lower effect on the AA-induced inflammatory reaction than on the TPA-induced response. These results demonstrate that surfactin is a potential anti-inflammatory agent through its selective inhibition on cytosolic $PLA_2$.

## MATERIALS AND METHODS

### *Animals and Materials*

Male rats (120–150 g) and male mice (25–30 g) were provided from Charles River Japan and were maintained under standardized conditions of light and temperature, with free access to animal chow and water. Surfactin was purified from *B. subtilis* as a compound termed PI-003, as described previously[22]. Rat platelet group II $PLA_2$ was partially purified as described previously [9]. $Ca^{2+}$-independent $PLA_2$ was partially purified from bovine brain according to the method of Ross *et al.* [23]. Purified porcine pancreatic and *Naja naja* snake venom $PLA_2$s were purchased from Sigma. Antiserum against pig spleen 100 kDa cytosolic $PLA_2$ was prepared as described previously [22]. TPA was purchased from Sigma. Prepared dialysis tubing (5961FA, 10,000–14,000 MW cutoff) was purchased from Life Technologies Inc. 1-Stearoyl-2-[1-$^{14}$C]arachidonoyl-*sn*-3-glycerophosphocholine (58.2 mCi/mmol; 2-[1-$^{14}$C]AA-GPC) and 1-acyl-2-[1-$^{14}$C]arachidonoyl-*sn*-3-glycerophosphoethanolamine (56.9 mCi/mmol; 2-[1-$^{14}$C]AA-GPE) were purchased from Amersham. [5,6,8,9,11,12,15-$^{3}$H]AA (230.50 Ci/mmol) was purchased from DuPont/NEN. 1-Stearoyl-2-arachidonoyl-*sn*-3-glycerophosphocholine (2-AA-GPC) was purchased from Sigma. The DEAE-5PW HPLC column was purchased from the Tosoh Co. All other chemicals were of the highest purity available from commercial sources.

### *Assay of $PLA_2$ Activity*

$PLA_2$ activity was assayed by measuring the hydrolysis of [1-$^{14}$C]AA from 2-[1-$^{14}$C]AA-GPC (substrate for cytosolic, pancreatic, and $Ca^{2+}$-independent $PLA_2$) or from 2-[1-$^{14}$C]AA-GPE (substrate for the secretory group II $PLA_2$). The assay was performed as described by Kim *et al.* [24]. It was initiated by the addition of purified bovine platelet cytosolic $PLA_2$ (approximately 10 ng of protein). The standard incubation system (100 μL) for the assay of $PLA_2$ activity contained 75 mM of Tris–HCl, pH 9.0, 5 mM of $CaCl_2$, and 0.45 nmol of radioactive phospholipids (approximately 55,000 cpm). For the Dixon plot, the concentration of 2-[1-$^{14}$C]AA-GPC as substrate of the standard assay was increased by adding 2-AA-GPC. The reaction was carried out at 37° for 30 min and stopped by adding 560 μL of modified Dole's reagent (*n*-heptane: isopropyl alcohol: 1 N of sulfuric acid; 400:390:10, by vol.). The [$^{3}$H]AA released was extracted as follows: Water (110 μL) was added, and the sample was vortex-mixed and centrifuged at 10,000 *g* for 2 min. Then 150 μL of the upper phase was transferred to a new tube, to which ~25 mg of silica gel and 800 μL of *n*-heptane were added. The samples were vortex-mixed and centrifuged again for 2 min, after which

800 μL of supernatant was counted for radioactivity in a liquid β-scintillation counter.

### *Dialysis Study for Binding of Surfactin to the Cytosolic $PLA_2$*

A mixture of 300 ng of the enzyme and 900 μg of bovine serum albumin was preincubated with 5 and 20 μM of surfactin at 37° for 30 min in 900 μL of reaction buffer (75 mM of Tris–HCl, pH 9.0, 5 mM of $CaCl_2$), respectively, and then 10 μg protein of the mixture was assayed for $PLA_2$ activity before dialysis. The remaining mixture was dialyzed against 1L of 50 mM of Tris–HCl (pH 7.5) buffer at 4° in dialysis tubing (MW cutoff 12,000–14,000) for 24 hr with two buffer changes. Protein (10 μg) of the dialyzed sample was assayed for $PLA_2$ activity after dialysis.

### *Inhibitory Effect of Surfactin on 100 kDa Cytosolic $PLA_2$ Detected in Monkey Kidney CV-1 Cells by DEAE-5PW HPLC and Western Blotting Analysis*

Monkey kidney CV-1 cells grown in six 10-cm culture dishes were washed twice with 5 mL of TBS (Tris-buffered saline; 25 mM of Tris–HCl, pH 8.0, 137 mM of NaCl, 2.7 mM of KCl) per dish and collected into 6.0 mL of homogenizing buffer (50 mM of Tris–HCl, pH 9.0, containing 1 mM of EDTA and 0.12 M of NaCl). The cells were disrupted with Tissue Tearor (Biospec Products, Inc., Model 985-370 Type 2) and centrifuged at 2000 *g* for 20 min at 4° to remove unlysed cells. The supernatant was ultracentrifuged at 100,000 *g* for 1 hr at 4°. The resulting supernatant was diluted with the same volume of buffer A (50 mM of Tris–HCl, pH 7.5, 1 mM of EDTA) and applied to a DEAE-5PW HPLC column (0.75 × 7.5 cm, Tosoh Co.) pre-equilibrated with buffer A. Bound fractions were eluted at a flow rate of 1 mL/min with a 20-mL linear gradient of 0 to 1.0 M of NaCl. Fractions (1.0 mL) were collected, and aliquots (30 μL) of each fraction were assayed for $PLA_2$ activity using 2-[1-$^{14}$C]AA-GPE as substrate for both the cytosolic and the secretory group II $PLA_2$. The bovine platelet 100,000 *g* supernatant obtained under the same procedures mentioned above was applied to the same column under the same conditions. To further examine the $PLA_2$ activity detected in CV-1 cells, western blot analysis was performed by using antiserum against pig spleen cytosolic $PLA_2$; each aliquot of the active $PLA_2$ pool partially purified from CV-1 cells and bovine platelets with the DEAE-5PW HPLC was concentrated into 20 μL and applied to a gel for SDS–PAGE. Immunoblotting was performed by using antiserum against pig spleen 100 kDa cytosolic $PLA_2$ [22].

### *Effect of Surfactin on AA Release from A23187-Stimulated Monkey Kidney CV-1 Cells*

The monkey kidney cell line CV-1 (ATCC CCL-70) was grown in 10-cm plastic dishes in a modified Eagle's basal medium supplemented with 10% fetal bovine serum. The cells were kept at 37° in a humidified atmosphere of 95% air and 5% $CO_2$. Confluent cultures were harvested by trypsinization and diluted with culture medium in plastic dishes with six 4-cm wells. The cells were incubated with the growth medium at 37° and used after 4–5 days, when they had reached 80–90% confluency. CV-1 cells grown in 4-cm dishes to near confluency were labeled by incubation with 0.3 μCi of [$^3$H]AA in 3 mL of growth medium for 20 hr. For measurements of the release of AA, the cells were washed twice free of unincorporated [$^3$H]AA with 5 mL of PBS, and 3 mL of fresh growth medium containing either ethanol (final concentration of 0.1% as vehicle) or various concentrations of surfactin was added 30 min before adding either ethanol (final concentration of 0.1% as vehicle) or 10 μM A23187. At the indicated time after stimulation, each aliquot (230 μL) of medium was removed and centrifuged for 5 min at 10,000 *g*. The released [$^3$H]AA was quantitated by counting radioactivity of the resulting supernatants (200 μL each) with a liquid β-scintillation counter. Following the last removal of medium, the residual medium was removed completely. The cells were disrupted by incubating at 37° for 6 hr with 1.0 mL of 0.5 N of NaOH solution, vortexed for 30 min, and centrifuged. After confirming complete disruption, each aliquot (100 μL) of the resulting supernatants was removed, and the total radioactivity of each dish was quantitated in a liquid β-scintillation counter.

### *TPA- and AA-Induced Edema in Mouse Ear*

This assay is a modification from Young *et al.* [25, 26]. Groups of ten mice (Swiss-derived, Charles River Laboratory, 25–30 g) were used. Compounds were prepared in acetone just prior to application. Ten microliters of TPA (1 μg/μL) or AA (100 μg/μL) dissolved in acetone was delivered to the right ear by micropipetting. Surfactin dissolved in the same volume of acetone was applied to the right ear just prior to challenge with TPA or AA. The vehicle-treated ear served as a control. The solutions were spread evenly over the whole surface of the ear. To measure the ear thickness, a dial caliper gauge with graduations of 0.01 mm was applied to the center of the ear. For TPA-induced edema, the thickness was measured at 1-hr intervals for up to 4 hr, and for AA-induced edema, the thickness was measured at 30-min intervals for up to 2 hr. The swelling evoked by inflammatory reaction was measured as the difference in thickness between the challenged and the unchallenged ear. Percent inhibition was calculated by using $(C-T)/C \cdot 100$ (%), where C and *T* indicate non-treated (vehicle) swelling and surfactin-treated swelling, respectively.

### *Carrageenan-Induced Edema in Rat Hind Paw*

Male Charles River Japan rats were given a single intraperitoneal dose (20 mg/kg) of surfactin suspended in 0.9% NaCl solution. Aspirin suspended in 0.9% NaCl solution was

administered orally 60 min before carrageenan injection. Paw edema was induced in the left paw by injecting 0.1 mL of carrageenan [1% (w/v) solution in 0.9% NaCl] into the plane area of the left hind paw. The volume of the injected paw was measured at 0, 0.5, 1, 2, 3, and 4 hr after carrageenan injection with a plethytometer. The percent edema of the hind paw evoked by the inflammatory reaction was measured from the difference in volume between the challenged and the unchallenged paw. Percent inhibition was calculated by using $(C-T)/C \cdot 100$ (%), where $C$ and $T$ indicate non-treated control (vehicle) percent edema and surfactin-treated percent edema, respectively.

### *Acetic Acid-Induced Writhing in Mice*

Groups of eight male mice (Charles River Japan, 25–30 g) were injected intraperitoneally with surfactin and indomethacin (0.5 mg/kg body weight each) suspended in 0.9% NaCl solution in a dose volume of 100 μL/30 g body weight; 0.5 hr later, each mouse was injected intraperitoneally with 0.25 mL of 0.6% acetic acid/30 g body weight. Mice were then placed in a cage, and the number of abdominal writhes of each mouse was recorded for 10 min starting 10 min after acetic acid treatment. The animals were killed immediately after measurements to minimize their sufferings. The average number of stretches of each group was compared with that of the control group, and the percent inhibition was calculated by the formula, $(C-T)/C \cdot 100$ (%), where C is the number of writhes of the non-treated control (vehicle) group and $T$ is that of the surfactin-treated group.

### *Statistical Analysis*

Computer-assisted statistical analysis using Pharm/PCS, MicroComputer Specialists, was performed to determine $IC_{50}$ and $ED_{50}$ values and the *t*-test.

## RESULTS

### *Effect of Surfactin on Activity of Various Forms of $PLA_2$*

Surfactin inhibited cytosolic 100 kDa $PLA_2$ purified from bovine platelets and pig spleen in a concentration-dependent manner. As shown in Fig. 2, cytosolic $PLA_2$ activity was inhibited completely at 50 μM. Linear regression analysis of the linear portion of the concentration–response curve revealed an $IC_{50}$ of 8.5 and 9.4 μM for bovine platelets and pig spleen, respectively. In contrast, while surfactin increased rat platelet secretory group II $PLA_2$ activity by ~2-fold, the compound did not affect the activities of pancreatic group I $PLA_2$ and a $Ca^{2+}$-independent form of $PLA_2$ from bovine brain, which was partially purified by sequential chromatographies of a heparin-affinity and Superose 12 gel filtration by a method described previously [23]. This result demonstrated that surfactin selectively inhibits cytosolic $PLA_2$.

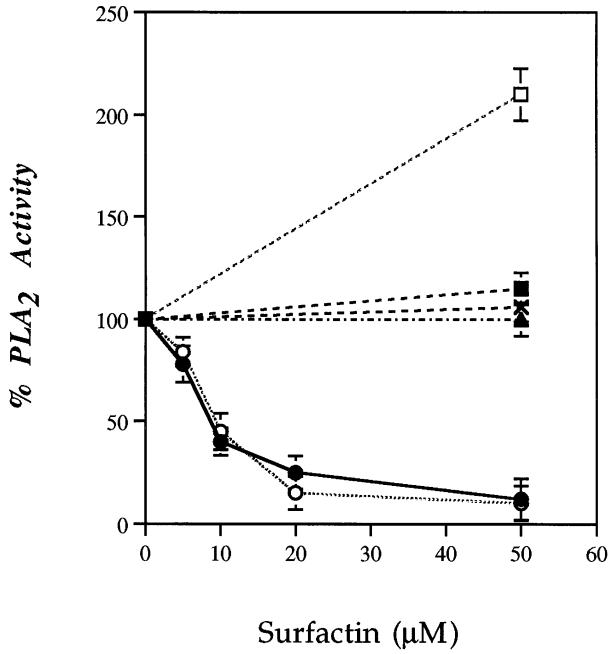


**FIG. 2. Concentration-dependent inhibition of various $PLA_2$s by surfactin. $PLA_2$ activity was assayed in the presence of increasing concentrations of surfactin as described under Materials and Methods. 2-[1-$^{14}$C]AA-GPC was used as substrate for the other $PLA_2$s except for using 2-[1-$^{14}$C]AA-GPE for rat secretory group II $PLA_2$. Surfactin was preincubated with various $PLA_2$ for 20 min at 37°. Each data point represents the mean ± SEM of three independent experiments. The surfactin-free activity of bovine platelet cytosolic $PLA_2$ (●), porcine spleen cytosolic $PLA_2$ (○), *Naja naja* venom $PLA_2$ (×), bovine pancreatic $PLA_2$ (■), rat platelet group II $PLA_2$ (□), and bovine brain $Ca^{2+}$-independent $PLA_2$ (▲) was 2980, 2710, 2010, 2540, 2750 and 2300 cpm, respectively, under the assay conditions.**

### *Evaluation for the Binding of Surfactin to Cytosolic 100 kDa $PLA_2$*

The inhibitory effect of surfactin on cytosolic $PLA_2$ activity was examined further in a time-dependent experiment. The enzyme was preincubated with 10, 20, and 50 μM of surfactin for the indicated periods, respectively, followed by addition of substrate. As shown in Fig. 3, the enzyme activity was inhibited in a time- and concentration-dependent manner; preincubation with 10, 20, and 50 μM of surfactin for 30 min at 37° abolished 78, 92, and 95% of control activity, respectively. Semilogarithmic plots of the residual activity versus incubation time were linear at all the concentrations tested, suggesting that the inhibitory effect during this period may occur by a pseudo-first-order process due to a direct effect on the enzyme. In addition, the initial steady-state rate was apparently preceded by a burst of inhibition as evidenced by the lack of intersection at 100% activity for any of the concentrations tested, suggesting a direct association of surfactin with the enzyme.

Since previous studies on the inhibition of phospholipases have shown an apparent inhibition due to nonspecific binding with the substrate vesicles, we examined

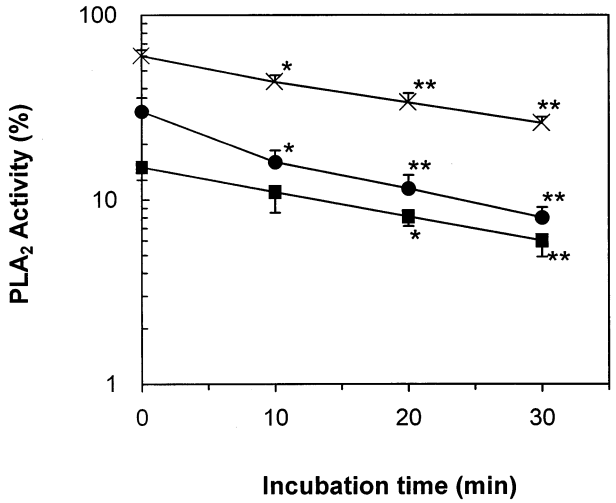


**FIG. 3. Time-dependent inhibition of bovine platelet cytosolic $PLA_2$ by surfactin. Purified bovine platelet cytosolic $PLA_2$ was preincubated with 10 μM (×), 20 μM (●), or 50 μM (■) of surfactin at 37° for the indicated times, and then the activity was measured using 4.5 μM of 2-[1-$^{14}$C]AA-GPC as substrate, as described under Materials and Methods. Each data point represents the mean ± SEM of three independent experiments. The surfactin-free $PLA_2$ activities at 0-, 10-, 20-, and 30-min preincubation times were 6250, 5630, 5250 and 4980 cpm, respectively, under the assay conditions. Significantly different from the control: ⋆$P < 0.05$ and ⋆⋆$P < 0.01$.**

whether surfactin inhibits substrate binding prior to enzyme association. As shown in Fig. 4, the inhibitory effect did not change even after increasing substrate concentrations by ~10-fold, suggesting that the effect is not due to a non-specific binding of surfactin to the substrate vesicles. Furthermore, to determine the inhibitory pattern on cytosolic $PLA_2$ by surfactin, Dixon plots

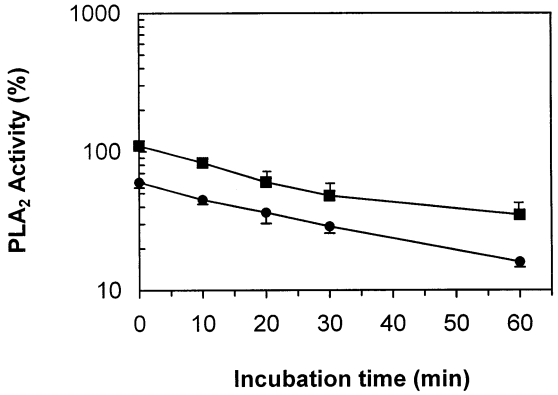


**FIG. 4. Effect of increased amounts of substrate on inhibition of bovine platelet cytosolic $PLA_2$ by surfactin. Surfactin (10 μM) was preincubated with purified $PLA_2$ for the indicated times at 37°, and the activity was measured after adding 3 μM of 2-[1-$^{14}$C]AA-GPC (●) or 30 μM of 2-[1-$^{14}$C]AA-GPC (■) as described under Materials and Methods. Each data point represents the mean ± SEM of three independent experiments. The surfactin-free $PLA_2$ activities at 0-, 10-, 20-, 30-, and 60-min preincubation times were 4130, 3850, 3610, 3510, and 2940 cpm, respectively, under the assay conditions.**

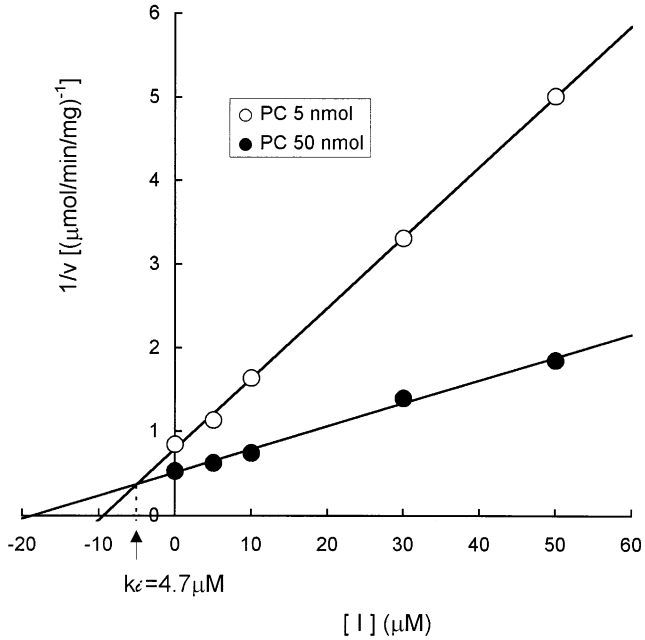


**FIG. 5. Determination of the inhibitory pattern on bovine platelet cytosolic $PLA_2$ by surfactin. The $PLA_2$ activity was assayed for 15 min at 37° in the presence of the indicated concentration of surfactin and 50 μM (○) or 500 μM (●) of 2-[1-$^{14}$C]AA-GPC as described under Materials and Methods. Shown are values from one experiment representative of three independent experiments producing similar results.**

were constructed from the hydrolysis rates of the substrate (2-[1-$^{14}$C]AA-GPC of 50 and 500 μM) by cytosolic $PLA_2$ at various surfactin concentrations. Figure 5 illustrates that the apparent $K_i$ value was 4.7 μM and that the inhibition was apparently competitive. Thus, these findings strongly support a direct binding of surfactin to the enzyme.

### *Dialysis Study for Binding of Surfactin to Cytosolic $PLA_2$*

To further confirm the direct association of surfactin with the enzyme, a mixture of surfactin and enzyme preincubated for 20 min at 37° was dialyzed against a 2000-fold volume of Tris–HCl buffer, pH 7.5, for 24 hr at 4°, and the enzyme activity in the dialysate was assayed by immediate addition of substrate. While no considerable effect of dialysis on the enzyme activity was observed (that is, surfactin-free dialysate retained 91% of activity prior to dialysis), the inhibitory effect of 20 μM of surfactin was observed both before and after dialyzing the mixtures (77.8 and 90.4%, respectively; Table 1). However, after dialyzing surfactin in the absence or in the presence of BSA using the same method, no inhibitory activity was observed when an aliquot of the dialysate was added to the assay system, as shown in Table 1. These results indicated a tight interaction of surfactin with the enzyme.

**TABLE 1. Irreversible inhibition of bovine platelet cytosolic $PLA_2$ by surfactin**

| Conditions | | | % Decrease of $PLA_2$ activity | |
|---|---|---|---|---|
| Surfactin (μM) | Cytosolic $PLA_2$ | BSA | Before dialysis | After dialysis |
| 0 | + | + | 0* | 2.0 |
| 5 | + | + | 66.3 | 77.1 |
| 20 | + | + | 77.8 | 90.4 |
| 20 | − | + | | 3.0† |
| 20 | − | − | | 2.0‡ |

After dialysis was performed as described in Materials and Methods, an aliquot of the dialysate was assayed for the $PLA_2$ activity.

*Cytosolic $PLA_2$ activity from 10 μg protein of the sample before dialysis was 3.2 pmol/min.

†‡When the enzyme was not added to the dialysis bag, $PLA_2$ activity was assayed with an aliquot of the dialysate following the addition of (†) the $PLA_2$ enzyme or (‡) the enzyme plus BSA.

### *Inhibitory Effect of Surfactin on 100 kDa Cytosolic $PLA_2$ Detected by DEAE-5PW HPLC and Western Blotting Analysis in Monkey Kidney CV-1 Cells*

To examine whether monkey kidney CV-1 cells contain $PLA_2$ activity similar to that of the bovine platelet cytosolic $PLA_2$, 100,000 *g* supernatants obtained from bovine platelets and monkey kidney CV-1 cells were applied to a DEAE-5PW anion exchange column. Both activities were found at the same fractions (Fig. 6). The activity of CV-1 cells eluted from the column exhibited dithiothreitol insensitivity, which is evidence that it is cytosolic $PLA_2$ (data not shown), and this activity could be inhibited 82% by preincubating for 20 min with 30 μM of surfactin. The inset of Fig. 6 shows a western blot of $PLA_2$ from the two enzyme sources using antiserum against pig spleen 100 kDa $PLA_2$. It shows that CV-1 cells contain a $PLA_2$ enzyme immunochemically related to the platelet 100 kDa $PLA_2$.

### *Inhibition of [$^3$H]AA Release from Monkey Kidney CV-1 Cells by Surfactin*

The inhibitory effect observed in an *in vitro* assay was examined by measuring the release of AA from the cultured cells. A23187, a $Ca^{2+}$-mobilizing agent, was added to the medium of the cultured monkey kidney CV-1 cells pretreated with surfactin. As shown in Fig. 7, the results indicate that the level of [$^3$H]AA in the cultured medium was increased in a time-dependent manner, and pretreatment of the cells with surfactin inhibited the release of [$^3$H]AA from A23187-stimulated cells as well as from non-stimulated cells at 30 min by 55 and 48%, respectively. Concentration-dependent inhibition was also observed in A23187-stimulated release of [$^3$H]AA with a saturation concentration at ~25 μM (Fig. 7B). Surfactin did not

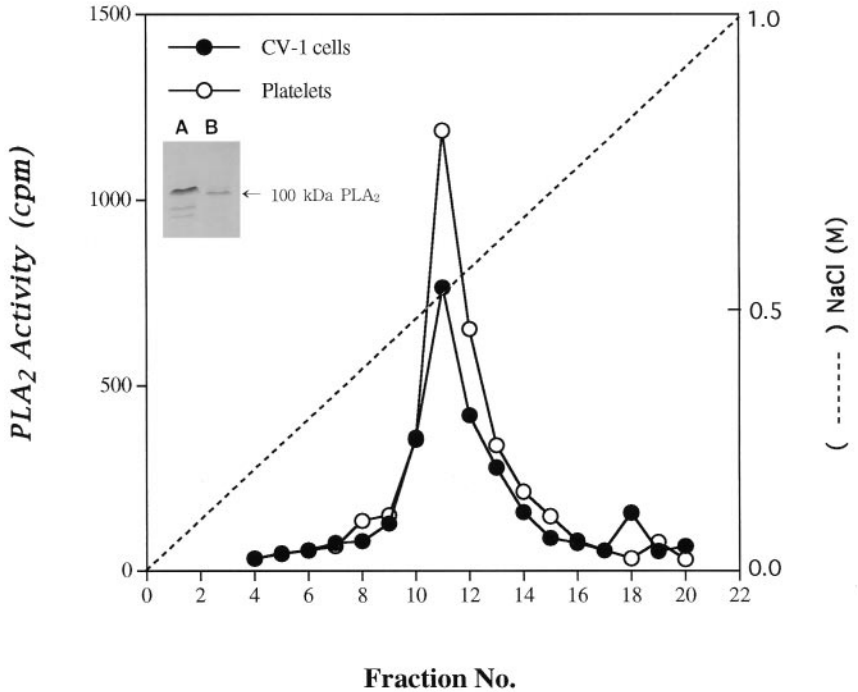


**FIG. 6. Detection of 100 kDa cytosolic $PLA_2$ in CV-1 cells by DEAE-5PW HPLC and western blot analysis. Supernatants (100,000 *g*) were prepared from bovine platelets (○) and monkey kidney CV-1 cells (●), as described in Materials and Methods, and directly loaded onto a DEAE-5PW column pre-equilibrated with 50 mM of Tris–HCl, pH 7.5, 1 mM EDTA. Proteins were eluted at a flow rate of 1.0 mL/min with a 20-mL linear gradient of 0 to 1.0 M of NaCl. Fractions (1.0 mL) were collected, and 30 μL of each fraction was assayed for $PLA_2$ activity using 2-[1-$^{14}$C]AA-GPE as substrate. The inset shows western blotting analysis; each active fraction of the $PLA_2$ from DEAE-5PW HPLC of bovine platelets (A) and monkey kidney CV-1 cells (B) was taken so that the total activity was 76 pmol/min and applied to SDS–PAGE after concentrating each sample to 20 μL with a Speed Vac dryer. Western blotting analysis was performed as described previously [22].**

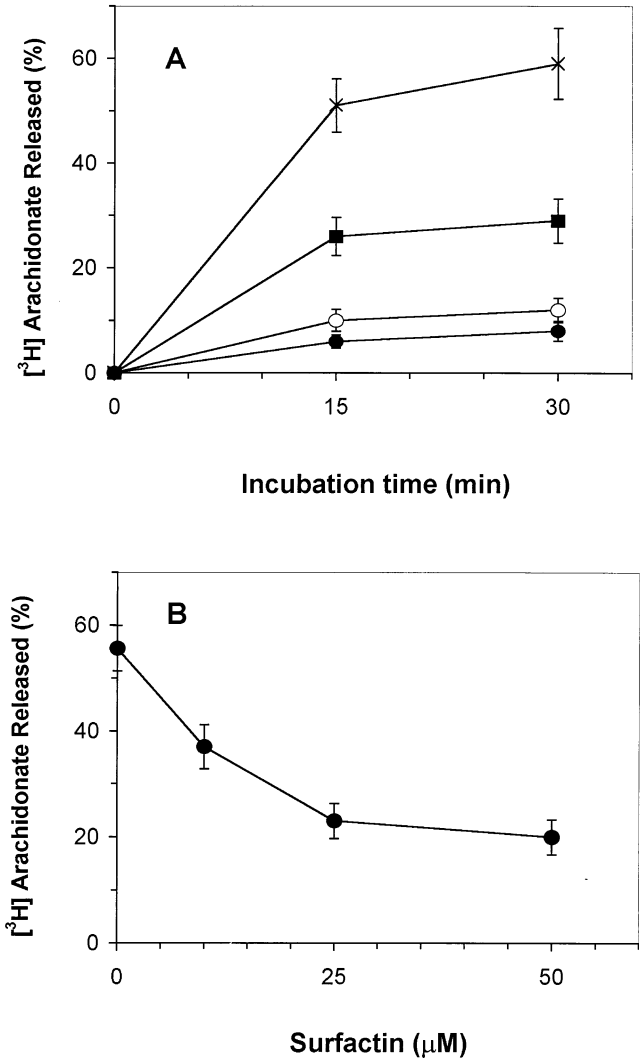


**FIG. 7. Inhibitory effect of surfactin on [$^3$H]AA release in monkey kidney CV-1 cells. Monkey kidney CV-1 cells were incubated with surfactin or vehicle and activated with 10 μM calcium ionophore A23187. The radioactivity of [$^3$H]AA released into the medium was measured as described under Materials and Methods. (A) A23187-stimulated (×), non-stimulated (vehicle) (○), surfactin-treated and A23187-stimulated (■), and surfactin-treated and non-stimulated (vehicle) (●). (B) CV-1 cells labeled with [$^3$H]AA were incubated for 20 min with the indicated concentrations of surfactin and then challenged for 15 min with 10 μM of A23187. Values represent the percentage of total radioactivity incorporated into the cells and are means ± SEM of three independent experiments in triplicate.**

affect the viability of the cells in the concentrations used (data not shown).

### *Suppression of Inflammatory Responses by Surfactin*

**Inhibition of mouse ear edema by surfactin.** Some *in vivo* experiments to support the inhibition of surfactin against cytosolic $PLA_2$ were performed. The TPA- or AA-induced mouse ear edema model was utilized as a model of skin inflammation [25, 26]. In this model, maximal edema occurred after 4 and 1.5 hr at a level of 140 and 129% swelling, respectively, when TPA and AA were applied. TPA-induced ear edema was inhibited dose-dependently by surfactin, and the $ED_{50}$ was calculated to be 230 μg/ear (Table 2). On the other hand, surfactin partially ameliorated AA-induced inflammation compared with inhibition of AA-induced edema by indomethacin. That is, surfactin inhibited AA-induced edema by 35% at 1000 μg/ear, which is a much higher concentration than TPA.

**TABLE 2. Inhibitory effect of surfactin on TPA- and AA-induced edema in mouse ear**

| Compound | TPA $ED_{50}$ *(μg/ear) | % Inhibition of AA-induced edema† |
|---|---|---|
| Surfactin | 230 ± 18 | 35 ± 4.1 |
| Indomethacin | 867 ± 51 | 40 ± 3.8 |

Mouse ear edema was measured 1 hr after application of TPA (10 μg/ear) and AA (1000 μg/ear), respectively. Surfactin or indomethacin was applied to the left ear of the mouse just prior to the application to the right ear of TPA or AA. The thickness of the treated ear was measured 1 hr after application of TPA or AA as described in Materials and Methods. Ear edema was calculated from the formula, $(R - L)/L \cdot 100(\%)$, where $R$ and $L$ were the thickness of the right and left ear, respectively. Each value represents the mean ± SEM.

*$ED_{50}$ values were calculated from the data obtained from three independent experiments using surfactin concentrations of 100, 200, and 400 μg/ear, and indomethacin concentrations of 500, 1000, and 2000 μg/ear, respectively.

†Percent inhibition was calculated from the data obtained from three independent experiments using surfactin and indomethacin of 1000 μg/ear. Percent inhibition was calculated by comparing individual mean values in the inhibitor-treated groups to the mean value of the control groups (% edema for TPA- and AA-treated group was 139.8 ± 9.7 and 128.9 ± 7.8%, respectively). Ten mice were tested for each group.

**Inhibition of carrageenan-induced rat paw edema by surfactin.** The anti-inflammatory action of surfactin was investigated further in a carrageenan-induced rat inflammation model. The paw hind cavity is known to be an inflammatory site in which accumulation of both exudate fluids and leukocytes can be observed as inflammatory responses to carrageenan [27]. The effects of surfactin on these responses were measured at 1-hr intervals up to 4 hr. As shown in Table 3, intraperitoneal administration of surfactin at 20 mg/kg markedly inhibited the carrageenan-induced paw edema by 45%, and, although low compared with the intraperitoneal route, oral administration dose-dependently inhibited the inflammatory reaction with a level comparable to that of the cyclooxygenase inhibitor aspirin.

**Analgesic effect of surfactin in acetic acid-induced writhing.** It has been known that intraperitoneal injection of acetic acid induces prostaglandin-mediated rapid onset of abdominal constriction [28]. Inhibition of the writhing response is a predictor of analgesic activity. A potent analgesic dose-dependent effect of inhibiting acetic acid-induced writhing was observed by intraperitoneal injection of surfactin (Table 4). Surfactin was more potent than the cyclooxygenase inhibitor indomethacin.

**TABLE 3. Inhibitory effect of surfactin on carrageenan-induced rat paw edema**

| | Dose (mg/kg) | Application route | % Edema | % Inhibition |
|---|---|---|---|---|
| Control | | i.p. | 63.9 ± 8.7 | 0 |
| | | p.o. | 64.4 ± 10.2 | 0 |
| Surfactin | 20 | i.p. | 35.1 ± 4.1 | 45.1 |
| | 40 | p.o. | 57.1 ± 5.6 | 11.3 |
| | 100 | p.o. | 41.3 ± 2.8 | 35.9 |
| Aspirin | 100 | p.o. | 46.9 ± 5.1 | 27.2 |

Surfactin and aspirin were administered intraperitoneally 30 sec prior to the injection of carrageenan. Oral administration was performed 1 hr prior to the injection of carrageenan. Hind paw edema was measured 4 hr after carrageenan application. The percent edema of the hind paw evoked by the inflammatory reaction was measured from the difference in volume between the challenged and the unchallenged paw. Each value represents the mean ± SEM ($N = 6$). Percent inhibition was calculated by the formula $(C - T)/C \cdot 100$ (%), where C and T indicate non-treated control (vehicle) percent mean edema and surfactin-treated percent mean edema, respectively.

## DISCUSSION

The present study reports that surfactin selectively inhibited cytosolic $PLA_2$ purified from bovine platelets through its direct interaction with the enzyme. Surfactin was identified from soil microorganisms as a component termed PI-003, which exhibits an inhibitory effect on the cytosolic $PLA_2$ activity *in vitro* assay to screen an anti-inflammatory agent. While the compound did not affect pancreatic group I $PLA_2$ from porcine pancreas and *Naja naja* venom and a $Ca^{2+}$-independent form of $PLA_2$ from bovine brain, it enhanced non-pancreatic group II $PLA_2$ activity from rat platelets by ~2-fold. This result suggests that the high selective inhibition of surfactin to the cytosolic form of $PLA_2$ may be due to differences in amino acid sequences and primary structures between the $PLA_2$s [23, 29], and not be dependent on the effect of the physical state of the substrates. Figure 2 shows the inhibitory effect of surfactin on various types of $PLA_2$. A $Ca^{2+}$-independent $PLA_2$ has been partially purified recently from human [23] and bovine (D. K. Kim *et al.*, unpublished data) brain and characterized. Neither the partially purified bovine brain $Ca^{2+}$-independent $PLA_2$ nor pancreatic group I $PLA_2$ is inhibited by surfactin. Thus, surfactin selectively inhibits 100 kDa cytosolic $PLA_2$. Interestingly, unlike the cytosolic $PLA_2$, rat platelet group II $PLA_2$ activity was enhanced by surfactin. It is unlikely that this inhibitory effect is due to the physical state of the substrate 2-[1-$^{14}$C]AA-GPE, since the compound also increased the 2-[1-$^{14}$C]AA-GPC-hydrolyzing activity of group II $PLA_2$ by ~2-fold even if lower than the 2-[1-$^{14}$C]AA-GPE-hydrolyzing activity (data not shown). Although a detailed kinetic study should be undertaken to elucidate the reason why surfactin increases the group II $PA_2$ activity, its contrary effects on the two $PLA_2$s will help distinguish their relative roles in cellular responses including platelet aggregation.

**TABLE 4. Inhibitory effect of surfactin on acetic acid-induced writhing in mouse**

| | Dose (mg/kg) | Number of writhes | % Inhibition |
|---|---|---|---|
| Control | | 23 ± 4 | 0 |
| Surfactin | 0.5 | 19 ± 3 | 17.4 |
| | 1.0 | 9 ± 3 | 60.9 |
| | 2.0 | 4 ± 2 | 82.6 |
| | 10.0 | 1 ± 0 | 95.7 |
| Indomethacin | 50.0 | 14 ± 2 | 39.1 |

Surfactin and indomethacin were administered intraperitoneally 30 sec prior to the application of acetic acid. The number of abdominal writhes of each mouse was counted for 10 min starting 10 min after the application of acetic acid. Each value represents the mean ± SEM ($N = 8$). Percent inhibition was calculated by the formula $(C - T)/C \cdot 100$ (%), where C and T indicate the mean of writhes of the non-treated control (vehicle) group and that of the surfactin-treated group, respectively.

It has been reported that a high molecular mass form of cytosolic $PLA_2$ is inhibited by a trifluoromethyl ketone analog of AA ($AACOCF_3$). $AACOCF_3$, which presumably binds directly to the active site of cytosolic $PLA_2$, was found to be a slow and tight-binding inhibitor [17, 30, 31]. As shown in Figs. 3 and 4, respectively, surfactin exhibited a time-dependent action on cytosolic $PLA_2$ activity, and the more extensive inhibitory activity was observed in the longer preincubation time of the enzyme and the inhibitor. This type of inhibition suggests that surfactin may be a direct-binding inhibitor to the enzyme. A similar type of suicide inhibition was shown in inhibition of BEL (bromoenol lactone) on myocardial $PLA_2$ [32] and chymotrypsin [22, 33]. It was known that the degree of inhibition observed for slow- and tight-binding inhibitors is independent of the amount of enzyme and duration of incubation [34]. In this context, since inhibition of $PLA_2$ by surfactin was time-dependent and also exhibited burst kinetics (Figs. 3 and 4), it is unlikely that inhibition of $PLA_2$ by surfactin is due to either slow binding or slow tight binding-reversible competitive inhibition. Furthermore, we determined the inhibitory pattern of surfactin on cytosolic $PLA_2$ from Dixon plots. Figure 5 illustrates that the apparent $K_i$ value was 4.7 μM, and the inhibition was apparently competitive. These results strongly indicate that the inhibition is due to a direct binding of surfactin to the enzyme, but not substrate depletion by surfactin.

Further study to support the inhibitory effect by the direct binding of surfactin to the enzyme was performed by utilizing dialysis experiments. Table 1 shows that no $PLA_2$ activity was detected in the dialysate when a mixture of the enzyme and surfactin was dialyzed, suggesting that surfactin tightly interacts with the enzyme. In an additional experiment, surfactin did not bind to bovine serum albumin when it was added to the purified enzyme preparations to avoid loss due to non-specific binding of an extremely low amount of the enzyme to the dialysis tubing.

As occasionally shown in an *in vitro* assay to screen an inhibitor against phospholipases, the apparant inhibitory effect may be caused by a non-specific interaction of the inhibitor with the exogenous phospholipid vesicle. To examine whether surfactin depletes the substrate available to the enzyme by binding to the phospholipid vesicle due to

the hydrophobic properties of the compound, the inhibitory effect was determined after increasing the substrate amounts by 10-fold. Figure 4 shows that the pattern of inhibition at the high concentration of substrate was similar to that at the 10-fold lower substrate concentration, suggesting that the apparent inhibition may not result from substrate depletion, but actually may be due to direct interaction between the enzyme and surfactin.

To summarize the above observations, surfactin inhibited cytosolic $PLA_2$ activity through a direct, possibly covalent, and irreversible binding to the enzyme. Thus, surfactin as a selective inhibitor for cytosolic $PLA_2$ can be expected to be a unique tool in the investigation to differentiate cytosolic $PLA_2$ from other secretory 14 kDa and $Ca^{2+}$-dependent forms of $PLA_2$ as well as in the elucidation of a cellular role of cytosolic $PLA_2$.

The effect of surfactin on the release of AA from A23187-stimulated monkey kidney CV-1 cells was determined to confirm its inhibitory effect on cytosolic $PLA_2$. First of all, we examined the presence of surfactin-inhibited 100 kDa cytosolic $PLA_2$ in the cells. Monkey kidney CV-1 cells contain a 100 kDa cytosolic form of $PLA_2$ similar to bovine platelet 100 kDa cytosolic $PLA_2$, as shown in an anionic exchange column DEAE-5PW HPLC profile and western blotting analysis (Fig. 6). The CV-1 cell $PLA_2$ eluted from the column was inhibited by preincubation for 10 min with 2 mM of dithiothreitol and 20 μM of surfactin by 87 and 82%, respectively, further supporting the similarity. There was no group II $PLA_2$ activity hydrolyzing 2-[1-$^{14}$C]AA-GPE in any fractions of the DEAE-5PW column (data not shown).

The release of AA was determined in the intact and cytosolic $PLA_2$-containing kidney CV-1 cells. As shown in Fig. 7, surfactin inhibited ~50% of the release of AA from both $Ca^{2+}$-mobilizing agent A23187-stimulated and non-stimulated CV-1 cells without any effect on the viability. The $IC_{50}$ for the release of AA from $Ca^{2+}$-mobilizing agent A23187-stimulated CV-1 cells was 10 μM, and the level of inhibition was not increased when tested at 50 μM. It was reported, however, that $AACOCF_3$, a selective inhibitor of cytosolic $PLA_2$, inhibits most of the release of AA from thrombin-stimulated human platelets [30, 31]. The limited inhibition in the CV-1 cells suggests the existence of a different form from cytosolic $PLA_2$. In fact, although group II 14 kDa $PLA_2$ activity was not detected in the cells, the membrane fraction of the cells contained a detectable $Ca^{2+}$-independent $PLA_2$ activity, which was not inhibited by surfactin (data not shown). Although the similarity between CV-1 and brain $Ca^{2+}$-independent $PLA_2$ remains to be determined, these results and those of Fig. 2 suggest that a $Ca^{2+}$-independent $PLA_2$ may be responsible for the surfactin-insensitive residual AA release from the CV-1 cells. Although the precise molecular mechanism by which surfactin inhibits $PLA_2$ action from participating in the release of AA from intact cells is not clear at present, these results suggest that surfactin is able to incorporate into the cytosol of the cells and interact with cytosolic $PLA_2$. The inhibitory effect of surfactin could be due to a direct interaction with the enzyme in the cytosol.

Since it is known that the release of AA by the action of $PLA_2$ might be a rate-limiting step in the biosynthesis of inflammatory mediators, such as prostaglandins, thromboxane $A_2$, leukotrienes, and platelet-activating factor [35], it is possible that cytosolic $PLA_2$ plays an important role in inflammatory responses. However, there has been much controversy about the relative contribution of the different types of $PLA_2$, 14 kDa secretory forms and 100 kDa cytosolic forms, to cellular AA release upon stimulus and the subsequent inflammatory response. Thus, these observations prompted us to examine anti-inflammatory activity of surfactin by utilizing several chemical-induced inflammatory animal models. In the present study, three animal models were utilized, i.e. TPA-induced mouse edema, carrageenan-induced rat edema, and acetic acid-induced mouse writhing. The TPA-induced edema model has been used to evaluate the anti-inflammatory activity of $PLA_2$ inhibitors. Prostaglandin $E_2$ accumulates concomitant with swelling in mouse ear administered TPA [36]. It has been reported that phorbol ester-activated protein kinase C stably enhances cytosolic $PLA_2$ activity through mitogen-activated protein kinase-mediated phosphorylation of the enzyme, which is known as a possible mechanism implicated in AA release by activation of cytosolic $PLA_2$ [37].

These observations are consistent with our results showing that surfactin ameliorates TPA-induced inflammation (Table 2). This anti-inflammatory effect of surfactin was also observed in AA-induced edema, although it was lower than that in TPA-induced edema. From this result, one possibility was suggested: AA added to the cells will be metabolized to eicosanoids, which are released to the extracellular medium. Then the released AA metabolites will interact with their receptors, which transduce a signal for the cytosolic $PLA_2$-mediated release of AA. The anti-inflammatory effect of surfactin was also observed in another animal model, carrageenan-induced rat paw edema. While intraperitoneal injection was more effective than oral administration, surfactin was more potent than aspirin, an anti-inflammatory drug. As the third animal model for testing anti-inflammatory effects, the acetic acid-induced abdominal constriction assay in mice has been commonly used to assess the analgesic effect of NSAID. Thus, the analgesic effect in this model could possibly be used to evaluate $PLA_2$ inhibition. Surfactin showed a potent dose-dependent analgesic effect when administered intraperitoneally (Table 4). Surfactin was more potent than indomethacin, a common NSAID, by ~10-fold.

These results demonstrated that surfactin showed anti-inflammatory activity through a possibly direct and selective action on cytosolic $PLA_2$, which could be responsible for the inflammatory reactions in all animal models utilized.

In conclusion, surfactin is a selective inhibitor of cytosolic $PLA_2$ distributed in a wide range of mammalian tissues. This compound inhibits the release of AA in calcium ionophore A23187-stimulated CV-1 cells through

the selective and direct inhibition of cytosolic $PLA_2$ in the cells. Finally, surfactin ameliorated the inflammatory reactions induced by several chemicals. These results demonstrate that surfactin is a putative anti-inflammatory agent and a useful tool for elucidating the role of other $PLA_2$s as well as cytosolic $PLA_2$ in cellular processes including inflammation and cell injury, and that inhibition of cytosolic $PLA_2$ activity may suppress inflammatory responses.

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## References


1. Van den Bosch H, Intracellular phospholipases A. *Biochim Biophys Acta* **604:** 191–246, 1980.
2. Hanahan DJ, Platelet activating factor: A biologically active phosphoglyceride. *Annu Rev Biochem* **55:** 483–509, 1986.
3. Vadas P and Pruzanski W, Role of secretory phospholipase $A_2$ in the pathobiology of disease. *Lab Invest* **55:** 391–404, 1986.
4. Bonventre JV, Phospholipase $A_2$ and signal transduction. *J Am Soc Nephrol* **3:** 128–150, 1992.
5. Kudo I, Murakami M, Hara S and Inoue K, Mammalian non-pancreatic phospholipases $A_2$. *Biochim Biophys Acta* **1170:** 217–231, 1993.
6. Dennis EA, Diversity of group types, regulation, and function of phospholipase $A_2$. *J Biol Chem* **269:** 13057–13060, 1994.
7. Kim DK, Kudo I and Inoue K, Detection in human platelets of phospholipase $A_2$ activity which preferentially hydrolyzes an arachidonoyl residue. *J Biochem (Tokyo)* **104:** 492–494, 1988.
8. Gronich JH, Bonventre JV and Nemenoff RA, Identification and characterization of a hormonally regulated form of phospholipase $A_2$ in rat renal mesangial cells. *J Biol Chem* **263:** 16645–16651, 1988.
9. Kim DK, Kudo I and Inoue K, Purification and characterization of rabbit platelet cytosolic phospholipase $A_2$. *Biochim Biophys Acta* **1083:** 80–88, 1991.
10. Kramer RM, Roberts EF, Manetta J and Putnam JE, The $Ca^{2+}$-sensitive cytosolic phospholipase $A_2$ is a 100-kDa protein in human monoblast U937 cells. *J Biol Chem* **266:** 5268–5272, 1991.
11. Kim DK, Suh PG and Ryu SH, Purification and some properties of a phospholipase $A_2$ from bovine platelets. *Biochem Biophys Res Commun* **174:** 189–196, 1991.
12. Gronich JH, Bonventre JV and Nemenoff RA, Purification of a high-molecular mass form of phospholipase $A_2$ from rat kidney activated at physiological calcium concentrations. *Biochem J* **271:** 37–43, 1990.
13. Clark JD, Milona N and Knopf JL, Purification of a 110-kDa cytosolic phospholipase $A_2$ from the human monocytic cell line U937. *Proc Natl Acad Sci USA* **87:** 7708–7712, 1990.
14. Miyake A, Yamamoto H, Kubota E, Hamaguchi K, Kuoda A, Honda K and Kawashima H, Suppression of inflammatory responses to 12-*O*-tetradecanoyl-phorbol-13-acetate and carrageenin by YM-26734, a selective inhibitor of extracellular group II phospholipase $A_2$. *Br J Pharmacol* **110:** 447–453, 1993.
15. Lindahl M and Tagesson C, Selective inhibition of group II phospholipase $A_2$ by quercetin. *Inflammation* **17:** 573–582, 1993.
16. Tanaka K, Matsutani S, Kanda A, Kato T and Yoshida T, Thielocin B3, a novel anti-inflammatory human group II phospholipase $A_2$-specific inhibitor from ascomycetes. *J Antibiotics (Tokyo)* **47:** 631–638, 1994.
17. Street IP, Lin HK, Laliberte F, Ghomashchi F, Wang Z, Perrier H, Tremblay NM, Huang Z, Weech PK and Gelb MG, Slow- and tight-binding inhibitors of the 85-kDa human phospholipase $A_2$. *Biochemistry* **32:** 5935–5940, 1993.
18. Arima K, Kakinuma A and Tamura G, Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochem Biophys Res Commun* **31:** 488–494, 1968.
19. Kakinuma A, Tamura G and Arima K, Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. *Experientia* **24:** 1120–1121, 1968.
20. Nakano MM and Zuber P, Molecular biology of antibiotic production in *Bacillus*. *Crit Rev Biotechnol* **10:** 223–240, 1990.
21. Lee DK, Jung JK, Kim KP, Yon CS, Suh JW, Kim CO, Hyun BC, Kim SH, Lim YH and Lee CH, PI-003, a novel cytosolic phospholipase $A_2$ inhibitor isolated from *Bacillus subtilis*. *Korean Biochem J* **26:** 625–631, 1993.
22. Kim DK and Bonventre JV, Purification of a 100 kDa phospholipase $A_2$ from spleen, lung and kidney: Antiserum raised to pig spleen phospholipase $A_2$ recognizes a similar form in bovine lung, kidney and platelets, and immunoprecipitates phospholipase $A_2$ activity. *Biochem J* **294:** 261–270, 1993.
23. Ross BM, Kim DK, Bonventre JV, and Kish SJ, Characterization of a novel phospholipase $A_2$ activity in human brain. *J Neurochem* **64:** 2213–2221, 1995.
24. Kim DK, Fukuda T, Thompson BT, Cockrill B, Hales C and Bonventre JV, Bronchoalveolar lavage fluid phospholipase $A_2$ activities are increased in human adult respiratory distress syndrome. *Am J Physiol* **269:** L109–L118, 1995.
25. Young JM, Wagner BM and Spires DA, Tachyphylaxis in 12-*O*-tetradecanoylphorbol acetate- and arachidonic acid-induced ear edema. *J Invest Dermatol* **80:** 48–52, 1983.
26. Young JM, Spires DA, Bedord CJ, Wagner B, Ballaron SJ and De Young LM, The mouse ear inflammatory response to topical arachidonic acid. *J Invest Dermatol* **82:** 367–371, 1984.
27. Murakami M, Kudo I, Nakamura Y, Yokoyama Y, Mori H and Inoue K, Exacerbation of rat adjuvant arthritis by intradermal injection of purified mammalian 14-kDa group II phospholipase $A_2$. *FEBS Lett* **268:** 113–116, 1990.
28. Deraedt R, Jouquey S, Delevallée F and Flahaut M, Release of prostaglandins E and F in an algogenic reaction and its inhibition. *Eur J Pharmacol* **61:** 17–24, 1980.
29. Clark JD, Lin L-L, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N and Knopf JL, A novel arachidonic acid-selective cytosolic $PLA_2$ contains a $Ca^{2+}$-dependent translocation domain with homology to PKC and GAP. *Cell* **65:** 1043–1051, 1991.
30. Riendeau D, Guay J, Weech PK, Laliberte F, Yergey J, Li C, Desmarais S, Perrier H, Liu S, Nicoll-Griffith D and Street IP, Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85-kDa phospholipase $A_2$, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-challenged platelets. *J Biol Chem* **269:** 15619–15624, 1994.
31. Bartoli F, Lin H-K, Ghomashchi F, Gelb MH, Jain MK and Apitz-Castro R, Tight binding inhibitors of 85-kDa phospholipase $A_2$ but not 14-kDa phospholipase $A_2$ inhibit release of free arachidonate in thrombin-stimulated human platelets. *J Biol Chem* **269:** 15625–15630, 1994.
32. Hazen SL, Zupan LA, Weiss RH, Getman DP and Gross RW, Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase $A_2$. Mechanism-based discrimination between calcium-dependent and -independent phospholipases $A_2$. *J Biol Chem* **266:** 7227–7232, 1991.
33. Baek D-J and Katzenellenbogen JA, Halo enol lactone inhibitors of chymotrypsin: Burst kinetics and enantioselectivity of

inactivation. *Biochem Biophys Res Commun* **178:** 1335–1342, 1991.
34. Stone SR and Morrison JF, Mechanism of inhibition of dihydrofolate reductases from bacterial and vertebrate sources by various classes of folate analogues. *Biochim Biophys Acta* **869:** 275–285, 1986.
35. Takamura H, Narita H, Park HJ, Tanaka K, Matsuura T and Kito M, Differential hydrolysis of phospholipid molecular species during activation of human platelets with thrombin and collagen. *J Biol Chem* **262:** 2262–2269, 1987.
36. Inoue H, Mori T, Shibata S and Koshihara Y, Modulation by glycyrrhetinic acid derivatives of TPA-induced mouse ear oedema. *Br J Pharmacol* **96:** 204–210, 1989.
37. Lin L-L, Wartmann M, Lin AY, Knopf JL, Seth A and Davis RJ, $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72:** 269–278, 1993.