

Pulmonary surfactant inhibits monocyte bactericidal functions by altering activation of protein kinase A and C

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SUMMARY

Pulmonary surfactant, the main function of which is to reduce surface tension in the alveoli, is also known to affect the functions of monocytes. Two protein kinases play a role in the regulation of the bactericidal functions of phagocytes, i.e. cAMP-dependent protein kinase A (PKA), which is involved in inhibition, and Ca^{2+} /phospholipid-dependent PKC, which is involved in stimulation of these functions. In the present study we investigated whether altered activation of PKA and/or PKC plays a role in the surfactant-induced inhibition of both the intracellular killing of *Staphylococcus aureus* and the production of reactive oxygen intermediates (ROI) by monocytes. The significance of increased activation of PKA was demonstrated by the following findings. Firstly, surfactant induced a sustained increase in the intracellular cAMP concentration in monocytes. Secondly, dibutyryl-cAMP (db-cAMP), a membrane-permeable cAMP analogue, mimicked the inhibitory effects of surfactant on both the killing capacity and the production of ROI by monocytes. Thirdly, an inhibitor of PKA partially restored the impaired bactericidal functions of monocytes incubated with surfactant. The involvement of decreased activation of PKC in the impaired bactericidal functions of monocytes incubated with surfactant was evident from two findings. Firstly, surfactant attenuated the phorbol myristate acetate (PMA)-mediated translocation of PKC. Secondly, surfactant inhibited the production of O_2^- by monocytes upon stimulation with PMA. Therefore, the mechanism involved in the surfactant-induced inhibition of the bactericidal functions of monocytes comprises both activation of an inhibitory pathway, which includes cAMP and PKA, and inactivation of a stimulatory pathway, in which PKC is involved.

INTRODUCTION

Alveoli are lined with surfactant which consists of about 90% lipids and 5–10% surfactant-specific proteins.¹ The major function of this material is to reduce the surface tension in the alveoli, thereby preventing alveolar collapse and oedema.¹ Alveolar macrophages reside in this surfactant-rich micro-environment and contain surfactant.^{2,3} Under steady-state

conditions, monocytes leave the circulation and enter the alveoli where they differentiate into alveolar macrophages.^{4,5} These macrophages are impaired in their bactericidal functions compared to monocytes.^{6,7} This can be explained by the intracellular presence of surfactant, since monocytes incubated *in vitro* with surfactant display impaired intracellular killing of bacteria due to a reduced production of reactive oxygen intermediates (ROI).⁷

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Abbreviations: BAL, broncho-alveolar lavage; db-cAMP, N⁶, 2'-O-dibutyryl-adenosine 3':5'-cyclic monophosphate; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; FURA-2/AM, 1-[2-(5-carboxy-oxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy)-ethane-N,N,N'-tetraacetic acid, penta-acetoxymethyl ester; H89, N-[2-((3-(4-bromophenyl)-2-propenyl)-amino)-ethyl]-5-isoquinolinesulphonamide, di-hydrochloride; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROI, reactive oxygen intermediates.

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At least two protein kinases, i.e. cAMP-dependent protein kinase A (PKA) and Ca^{2+} /phospholipid-dependent PKC, are thought to play a role in the regulation of the production of ROI by monocytes and granulocytes. Agents that increase intracellular cAMP concentrations in monocytes and granulocytes, such as prostaglandin E₂, histamine and adenosine, can inhibit the production of ROI by these cells.^{8–11} Activation of PKC by phorbol esters or membrane-permeable diacylglycerol-analogues results in enhanced production of ROI by phagocytes,^{11–16} whereas inhibition of PKC impairs the respiratory burst.^{11,12,15,16} Evidence exists that PKA and PKC are also involved in regulation of the intracellular killing of bacteria by monocytes and granulocytes, i.e. both the activation of PKA^{17–19} and inactivation of PKC^{12,20} result in impaired intracellular killing of bacteria by these cells.

The aim of the present study was to investigate whether changes in the activation of PKA and/or PKC account for the impaired intracellular killing of *Staphylococcus aureus* and the reduced production of ROI by monocytes incubated with surfactant.

MATERIALS AND METHODS

Surfactant

Human surfactant was isolated from broncho-alveolar lavage (BAL) fluid derived from lung segments with no underlying pathological condition, collected after informed consent from patients who underwent BAL for diagnostic reasons. After BAL fluid was centrifuged at 750 *g* for 20 min to remove cells and debris, the supernatant was centrifuged at 21 000 *g* for 2 hr. The resulting pellet was resuspended in a small volume of saline and stored at -20° . The concentration of surfactant, expressed in mM organic phosphate, was determined according to the method of Bartlett.²¹ The surfactant preparations contained about 4 ng lipopolysaccharide (LPS)/mM phospholipid, as quantified by the Limulus assay.

Monocytes

Buffy coats from blood of healthy donors were diluted in phosphate-buffered saline (PBS) and subjected to differential centrifugation at 400 *g* for 20 min on a Ficoll-Amidotriazoate gradient ($\rho = 1.077$ g/ml; Pharmacia, Uppsala, Sweden).²² The monocyte-lymphocyte interphase was washed four times with PBS containing 0.5 U heparin/ml. This cell preparation consisted of about $28 \pm 8\%$ monocytes, $71 \pm 7\%$ lymphocytes and $2 \pm 1\%$ granulocytes. For intracellular cAMP, Ca^{2+} and PKC measurements, the monocytes were purified by elutriation centrifugation using a JE-6 Elutriation Rotor (Beckman Instruments Inc., Palo Alto, CA). The monocyte-rich fractions were pooled and washed with PBS; the final preparation consisted of $89 \pm 6\%$ monocytes, the remaining cells being lymphocytes. Viability of the monocyte preparations exceeded 95%, as determined by trypan blue exclusion.

Incubation of monocytes with surfactant

Monocytes at a concentration of 1×10^7 /ml Hanks' balanced salt solution containing 0.1% gelatin (gel-HBSS), were incubated with various concentrations of surfactant, ranging from 1 to 4 mM, for 30 min under slow rotation (4 r.p.m.) at 37° . Excess surfactant was removed by three washes with gel-HBSS and centrifugation at 250 *g*. Viability of the monocytes incubated with surfactant exceeded 90%, as measured by trypan blue exclusion. Control monocytes were incubated under identical conditions with saline, the diluent of surfactant.

Incubation of monocytes with db-cAMP or H89

To mimic an increase in the intracellular cAMP concentration, monocytes at a concentration of 1×10^7 /ml gel-HBSS were incubated with 0.5–1.0 mM dibutyryl(db)-cAMP (Sigma Chemical Co., St Louis, MO) for 15 min under slow rotation (4 r.p.m.) at 37° . To inhibit PKA, 1×10^7 monocytes/ml gel-HBSS was incubated with 30 μM H89 (Calbiochem, San Diego, CA), a selective inhibitor of PKA,²³ for 10 min under slow rotation (4 r.p.m.) at 37° . Viability of the monocytes incubated with db-cAMP or H89 exceeded 90%, as measured by trypan blue exclusion.

Production of O_2^-

O_2^- -production by resting monocytes and upon stimulation with 2.5 ng phorbol myristate acetate (PMA)/ml (Sigma) or 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma) was determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C.²⁴ For optimal stimulation with FMLP, cells were incubated with 10 μg cytochalasin E/ml (Sigma) for 5 min at 37° prior to the addition of FMLP. The results are expressed as nmol O_2^- /(10^6 cells \times 60 min).

Opsonization of *S. aureus*

Staphylococcus aureus (type 42D) were cultured overnight in Nutrient broth no. 2 (Oxoid Ltd, Basingstoke, UK) at 37° . Bacteria were harvested by centrifugation at 1500 *g*, washed twice with PBS, and resuspended in gel-HBSS to a concentration of 1×10^7 bacteria/ml. Bacteria were opsonized with 10% human serum for 30 min at 37° under rotation (4 r.p.m.), excess serum removed by centrifugation, and the bacteria resuspended in gel-HBSS to a concentration of about 1×10^7 /ml.

Intracellular killing of *S. aureus*

The assay for intracellular killing was performed as described elsewhere.²⁵ Equal volumes of pre-opsonized bacteria (1×10^7 /ml) and monocytes (1×10^7 /ml) were mixed and incubated for 3 min at 37° under slow rotation. The phagocytosis was terminated by shaking the tube in crushed ice. The non-ingested bacteria were removed by differential centrifugation and washing. Then monocytes were reincubated with 10% human serum at 37° under rotation (4 r.p.m.). At the start of the assay and at 60 min, a sample of the cell suspension was transferred to ice-cold water with 0.01% bovine serum albumin (BSA) and the monocytes were disrupted by vigorous shaking on a vortex mixer for 1 min. The numbers of viable intracellular bacteria in the samples were determined microbiologically. The serum-stimulated intracellular killing of *S. aureus* by monocytes was expressed as the percentage decrease in the number of viable intracellular bacteria according to the formula:

$$\text{intracellular killing} = \left(1 - \frac{N_{s,t=60}/N_{s,t=0}}{N_{c,t=60}/N_{c,t=0}} \right) \times 100\%$$

in which $N_{s,t=60}$ and $N_{s,t=0}$ are the respective numbers of viable cell-associated bacteria at 60 and 0 min in the presence of serum, and $N_{c,t=60}$ and $N_{c,t=0}$ are the respective numbers of viable cell-associated bacteria in the absence of a stimulus for killing at 60 and 0 min.

Measurement of the intracellular cAMP concentration in monocytes

Fifty microlitres of 1×10^8 monocytes/ml PBS was incubated with surfactant for various intervals at 37° ; the reaction was stopped by addition of 450 μl ice-cold 1-propanol. After 20 min at 4° the propanol samples were sonified for 5 min and centrifuged at 7000 *g* for 1 min. After lyophilization of the supernatants, the residues were dissolved in 100 μl distilled water and the cAMP concentration of these samples was determined with a competition binding assay.²⁶ Briefly, 20 μl of the cell sample was incubated with 20 μl of 0.1 M sodium acetate (pH 4), 20 μl of bovine adrenocortical preparation (about 10 mg protein/ml) and 20 μl of 5',8-[^3H]cAMP (1500 c.p.m., specific

activity 40–60 Ci/mmol; Amersham, Amersham, UK) for 30 min at 4°. Bound [³H]cAMP was separated from free [³H]cAMP by centrifugation at 7000 *g* for 1 min; the pellet was resuspended in 100 µl of 0.1 M Tris–HCl buffer (pH 9) and mixed with 1.0 ml Ultimagold (Packard Chemical Operations, Groningen, the Netherlands) for liquid scintillation counting. The intracellular cAMP concentration was calculated from the cAMP value extrapolated from a standard curve made with serial dilutions of cAMP and the mean volume of monocytes.²⁷

Assessment of the intracellular free Ca²⁺ concentration

Monocytes at a concentration of 1 × 10⁷ cells/ml were resuspended in a buffer, pH 7.4, containing 138 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1.1 mM CaCl₂, 1 mM NaH₂PO₄, 5.5 mM D-glucose, 0.1 mM EGTA, 20 mM HEPES and 0.1% BSA, called Ca²⁺-medium. Then the cells were loaded with 1 µM FURA-2/AM (Sigma) for 30 min at 37° in the dark, washed once and resuspended in Ca²⁺-medium. Next, 2 ml of a suspension of 5 × 10⁶ FURA-2-loaded cells/ml were transferred to a cuvette and the fluorescence was measured at 340, 360 and 380 nm excitation and at 500 nm emission on a Perkin-Elmer 3000 spectrofluorometer (Perkin-Elmer, Ueberlingen, Germany) equipped with a magnetic stirrer. The intracellular free Ca²⁺ concentration ([Ca²⁺]_i) was calculated from the ratio of the fluorescence at 340 nm and 380 nm, as previously described.²⁸ Calibration of the FURA-2 fluorescence was performed by lysing the cells with 0.1% Triton X-100 (Sigma) in the presence of 1 mM extracellular Ca²⁺ followed by the addition of EGTA to a final concentration of 10 mM.

Fractionation of monocytes for PKC measurements

Monocytes 0.5–1 × 10⁷ were suspended in cold lysis buffer (20 mM Tris–HCl, pH 7.4, 330 mM sucrose, 2 mM EDTA, 0.5 mM EGTA, 5 mM β-mercaptoethanol and 0.5 mM PMSF) and disrupted by sonication on ice using a Sonifier 250 (Branson Ultrasonics, Danbury, CT; 2 min at 20% duty cycle at 15 kHz). The sonicates were then centrifuged at 10 000 *g* for 5 min to remove nuclei and intact cells. The supernatant was centrifuged at 100 000 *g* for 1 hr at 4° to obtain a cytosolic (supernatant) and a membranous (pellet) fraction. PKC activity in the membranous fraction was extracted with lysis buffer containing 1% Nonidet P-40 (Sigma). PKC in the cytosolic and membranous fractions was partially purified using DEAE Cellulose (DE52; Whatman BioSystems Ltd, Maidstone, UK) columns pre-equilibrated with lysis buffer

without sucrose, further referred to as buffer. Unbound proteins were removed by washing the columns with buffer and fractions containing PKC were eluted with 500 µl buffer containing 200 mM NaCl. Samples were stored at 4° for at least 18 hr prior to PKC measurements.

PKC measurement

Ca²⁺- and phospholipid-dependent protein kinase activity was measured as described elsewhere²⁹ with minor modifications. In short, 20 µl of cytosolic or membranous fraction was added to a 40 µl assay solution containing final concentrations of 200 µg histone type IIIS/ml (Sigma), 95 µg phosphatidylserine/ml (Sigma), 383 µg phosphatidylcholine/ml (Sigma), 3.2 µg 1,2-dioleoin/ml (Sigma), 2 mM CaCl₂, 15 mM MgCl₂, 10 µM [γ-³²P]-ATP (specific activity 1 Ci/mmol; Amersham) and 10 µg leupeptin/ml in 30 mM Tris–HCl (pH 7.5). The reaction was carried out for 20 min at 30°. Fifty microlitres of this suspension was spotted onto Whatman P81 filters (Whatman); unbound [γ-³²P]ATP was removed by washing the filters four times in PBS; the filters were then put in vials containing 2 ml Ultimagold for liquid scintillation counting. PKC activity was expressed as picomoles P_i incorporated into histone per 1 × 10⁷ cell equivalents during 20 min. Incorporation of P_i into histone was negligible in the absence of essential cofactors, achieved by omission of phospholipids and dioleoin and addition of 10 mM EDTA and 10 mM EGTA, indicating that the protein kinase activity depended on phospholipids and Ca²⁺.

Statistics

The significance of differences between control cells and cells incubated with surfactant, db-cAMP, or H89 was determined with the Mann–Whitney *U*-test. Results are means and SD.

RESULTS

Effect of surfactant on the intracellular killing of *S. aureus* and the production of O₂⁻ by monocytes

Incubation of monocytes with surfactant significantly (*P* < 0.05) inhibited the intracellular killing of *S. aureus* by these cells at concentrations as low as 1 mM surfactant; however, the inhibition was greater after incubation of monocytes with 4 mM surfactant (Table 1). On the basis of these data, all further experiments were performed with 4 mM

Table 1. Effect of surfactant on the intracellular killing of *S. aureus* and the production of O₂⁻ by monocytes*

Incubation	Intracellular killing	<i>n</i>	O ₂ ⁻ production upon stimulation with FMLP	<i>n</i>	O ₂ ⁻ production upon stimulation with PMA	<i>n</i>
Saline	60 ± 20	5	15.0 ± 4.3	5	15.6 ± 7.0	6
1 mM surfactant	40 ± 23†	5	ND‡		ND‡	
4 mM surfactant	17 ± 19†	5	9.8 ± 2.1†	5	5.9 ± 3.4†	6

* Monocytes were incubated with surfactant for 30 min at 37°. Excess surfactant was removed by washing. Intracellular killing is expressed as the percentage decrease in the number of viable intracellular bacteria at 60 min, and O₂⁻ production as nmol O₂⁻/(1 × 10⁶ monocytes × 60 min).

† *P* < 0.05 for monocytes incubated with surfactant and saline.

‡ ND, not done.

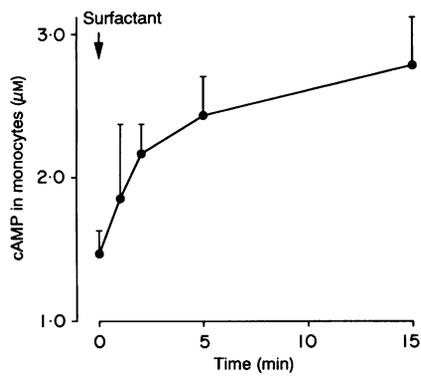


Figure 1. Effect of surfactant on the intracellular cAMP concentration in human monocytes. Monocytes were incubated with 4 mM surfactant for various intervals at 37°. The reaction was stopped by the addition of propanol, and the cAMP concentration of these samples was measured by a competition-binding assay. Experiments were performed in triplicate. Results are one representative out of five experiments.

surfactant unless otherwise indicated. Incubation of monocytes with surfactant inhibited the FMLP-stimulated production of O_2^- by these cells by $33 \pm 14\%$. The PMA-stimulated production of O_2^- by monocytes was more severely inhibited by surfactant, i.e. the inhibition amounted to $61 \pm 17\%$ (Table 1).

Effect of surfactant on the intracellular cAMP concentration of monocytes

The intracellular cAMP concentration in resting monocytes amounted to $1.3 \pm 0.4 \mu\text{M}$ ($n = 5$), and surfactant induced a time-dependent increase in the cAMP concentration (Fig. 1). Incubation of monocytes with 4 ng LPS/ml, which is equivalent to the concentration of LPS found in 4 mM surfactant, did not induce an increase in the intracellular cAMP concentration; the value amounted to 107% relative to control cells after 15 min ($n = 2$). This indicates that the increase in cAMP concentration was not due to LPS contamination of surfactant.

Effect of db-cAMP on the intracellular killing of *S. aureus* and the production of O_2^- by monocytes

If an increase in the intracellular cAMP concentration is involved in the impaired bactericidal functions of monocytes incubated with surfactant, db-cAMP could mimic the effects of

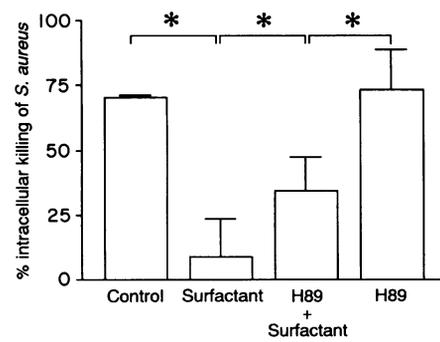


Figure 2. Effect of H89 on the surfactant-impaired intracellular killing of *S. aureus* by monocytes. Monocytes were preincubated with $30 \mu\text{M}$ H89 for 10 min at 37° and then 4 mM surfactant was added for an additional incubation period of 30 min. Excess surfactant was removed by washing; H89 was present during the killing assay. In order to determine intracellular killing, monocytes and pre-opsonized *S. aureus* were incubated at a ratio of 1:1; after 3 min of phagocytosis, extracellular bacteria were removed by washing and killing of the ingested bacteria was initiated by the addition of 10% human serum. * $P < 0.05$; $n = 3$.

surfactant. Incubation of monocytes with db-cAMP inhibited the intracellular killing of *S. aureus* by about 55% and the FMLP-stimulated production of O_2^- by 30% (Table 2). The PMA-stimulated production of O_2^- was not affected by incubation of monocytes with db-cAMP (Table 2).

Effect of H89 on the intracellular killing of *S. aureus* and the production of O_2^- by monocytes

To find out to what extent the increased intracellular cAMP concentration in monocytes incubated with surfactant accounts for the impaired bactericidal functions of these cells, monocytes were incubated with H89, a selective inhibitor of PKA,²³ prior to the addition of surfactant. The results revealed that H89 restored the intracellular killing of *S. aureus* by surfactant-incubated monocytes to about 50% of that by control cells either incubated with H89 or with saline (Fig. 2). H89 did not affect the intracellular killing of *S. aureus* by control monocytes. The impaired production of O_2^- by monocytes incubated with surfactant and then stimulated with FMLP was almost completely restored by H89 (Fig. 3a). However, H89

Table 2. Effect of db-cAMP on the intracellular killing of *S. aureus* and the production of O_2^- by monocytes*

Incubation	Intracellular killing	<i>n</i>	O_2^- production upon stimulation with FMLP	<i>n</i>	O_2^- production upon stimulation with PMA	<i>n</i>
Saline	63 ± 13	4	9.4 ± 1.3	4	17.5 ± 7.9	5
db-cAMP	$29 \pm 13^\dagger$	4	$6.5 \pm 0.1^\dagger$	4	16.9 ± 7.7	5

* Monocytes were incubated with 1 mM db-cAMP for 15 min at 37° prior to the killing assay and with 0.5 mM db-cAMP prior to the O_2^- production assay. Excess db-cAMP was removed by washing. Intracellular killing is expressed as the percentage decrease in the number of viable intracellular bacteria at 60 min, and O_2^- production as $\text{nmol } O_2^- / (1 \times 10^6 \text{ monocytes} \times 60 \text{ min})$.

† $P < 0.05$ for monocytes incubated with db-cAMP and saline.

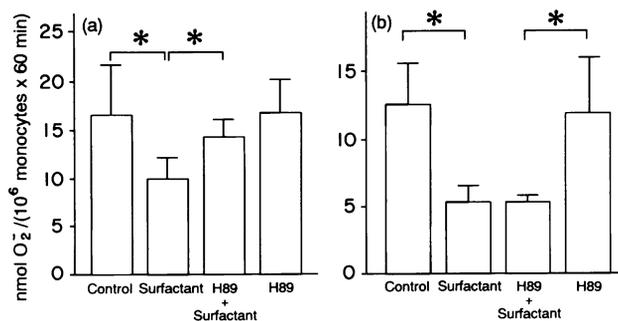


Figure 3. Effect of H89 on the surfactant-impaired production of O₂⁻ by monocytes stimulated with FMLP or PMA. Monocytes were preincubated with 30 μM H89 for 10 min at 37° and then 4 mM surfactant was added for an additional incubation period of 30 min. Excess surfactant was removed by washing; H89 was present during stimulation of monocytes with 10⁻⁷ M FMLP (a) or 2.5 ng PMA/ml (b). **P* < 0.05; *n* = 4.

did not affect the reduced production of O₂⁻ by monocytes incubated with surfactant when these cells were stimulated with PMA (Fig. 3b). H89 did not affect the production of O₂⁻ by control monocytes stimulated with FMLP or PMA (Fig. 3).

Effect of surfactant on the intracellular free Ca²⁺ concentrations in monocytes

Since the FMLP-induced production of O₂⁻ by granulocytes can be suppressed by inhibiting the accompanying rise in the [Ca²⁺]_i in these cells,³⁰ we investigated whether surfactant affects the [Ca²⁺]_i in monocytes. The [Ca²⁺]_i in resting monocytes amounted to 102 ± 15 nM (*n* = 5) and this value did not change (*P* > 0.05) after addition of 1 mM surfactant. In monocytes incubated with 1 mM surfactant and then stimulated with 10⁻⁷ M FMLP, a transient increase in the [Ca²⁺]_i was found, of 315 ± 64 nM (*n* = 4), which was not significantly (*P* > 0.05) different from the value found for control cells, i.e. 379 ± 70 nM (*n* = 5). Therefore, it is not likely that the reduced FMLP-induced production of O₂⁻ by monocytes incubated with surfactant is due to an impaired rise in [Ca²⁺]_i.

Effect of surfactant on the distribution of PKC in monocytes

Since PMA primarily acts through activation of PKC,³¹ inhibition of the PMA-stimulated production of O₂⁻ by monocytes incubated with surfactant indicated that activation of PKC was impaired in these cells. To investigate the effects of surfactant on activation of PKC, the subcellular distribution of this enzyme was determined. In control monocytes, about 80% of PKC activity was present in the cytosol (Fig. 4). Incubation of monocytes with PMA resulted in a significantly reduced activity of PKC in the cytosolic fraction and a two- to threefold increase of PKC activity in the membranous fraction (Fig. 4). In monocytes incubated with surfactant, PKC activity was slightly reduced in the cytosolic and increased in the membranous fraction (Fig. 4). The increase in PKC activity in the membranous fraction was much lower in monocytes that had been incubated with surfactant prior to stimulation with PMA, indicating that surfactant attenuates the PMA-induced translocation of PKC (Fig. 4). Surfactant added to the

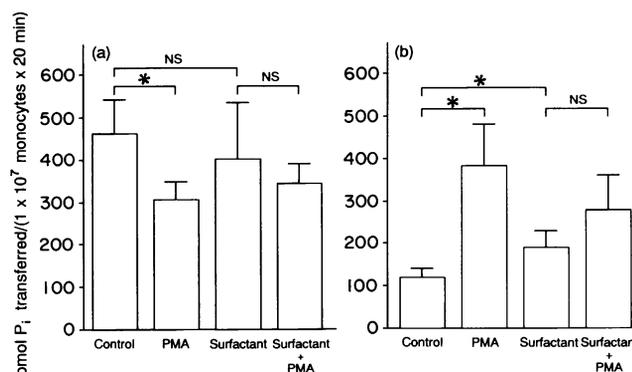


Figure 4. Effect of surfactant on distribution of PKC in monocytes. Monocytes were incubated with 4 mM surfactant for 30 min; excess surfactant was removed by washing, and the cells were incubated with 10 ng PMA/ml for 5 min. After two washes to remove PMA, cells were lysed by sonication and then separated into a cytosolic and membranous fraction by ultracentrifugation. PKC activity was measured in triplicate in the cytosolic (a) and membranous (b) fractions as Ca²⁺/phospholipid-dependent histone III phosphorylating activity. **P* < 0.05; NS not significant; *n* = 4.

membranous or cytosolic fraction of control monocytes did not affect the PKC activity in these fractions (data not shown), indicating that surfactant, the main components of which are phospholipids, has no cofactor-activity for PKC.

DISCUSSION

The main conclusion of the present study is that both activation of PKA and inhibition of PKC are involved in the impaired intracellular killing of *S. aureus* by monocytes incubated with surfactant. The impaired production of O₂⁻ by monocytes containing surfactant stimulated with PMA is attributable to inhibition of PKC activity, whereas the impaired O₂⁻ production by these cells upon stimulation with FMLP is mediated by enhanced activity of PKA.

The conclusion that PKA plays a role in the impaired bactericidal functions of monocytes incubated with surfactant is based on the following lines of evidence. Firstly, surfactant induced sustained elevated levels of intracellular cAMP in monocytes. Secondly, db-cAMP, a membrane-permeable analogue of cAMP, mimicked the inhibitory effect of surfactant on the intracellular killing of *S. aureus* and the FMLP-induced O₂⁻ production by monocytes. Thirdly, inhibition of PKA partially restored the reduced intracellular killing of *S. aureus* and the FMLP-induced production of O₂⁻ by monocytes containing surfactant. The mechanism for the inhibitory effect of PKA on the respiratory burst of monocytes is not yet known. It could be that activation of PKA desensitizes FMLP receptors, as has been described for β₂-adrenergic receptors.³² The observation that surfactant did not affect the FMLP-induced rise in [Ca²⁺]_i in monocytes does not support this possibility and indicates that the PKA-mediated inhibition of the production of ROI by monocytes most probably does not occur at the FMLP receptor level. Another explanation may be that PKA inhibits the respiratory burst at the NADPH oxidase level. In this respect, it is relevant that PKA phosphorylates rap1A, a low molecular weight G protein

associated with the cytochrome b558;³³ however, it is not known whether phosphorylation of this protein mediates the reduced production of ROI by phagocytes.

The involvement of PKC in the impaired bactericidal activities of surfactant-incubated monocytes follows from the attenuated PMA-induced translocation of PKC in these cells and their impaired production of O₂⁻ upon stimulation with PMA. The identity of the PKC isozyme(s) affected by surfactant is not yet known. Stimulation of granulocytes with PMA induces translocation of the major isozyme β -PKC from the cytosolic to the membranous fraction, resulting in the phosphorylation of a number of cytosolic proteins, including a 47000 MW protein.³⁴ This protein may be similar to p47^{phox}, one of the cytosolic components of the NADPH oxidase involved in O₂⁻ generation.³⁵ The observation that H89 almost completely restored the impaired FMLP-induced production of O₂⁻ by monocytes incubated with surfactant indicates that inhibition of PKC played a limited role in this process. The discrepancy in the involvement of inhibition of PKC in the impaired FMLP- and PMA-induced respiratory burst by monocytes incubated with surfactant indicates that the FMLP-induced production of O₂⁻ is less sensitive to PKC inhibition than that induced by PMA. Consistent with this finding is the fact that a higher concentration of a relatively selective PKC inhibitor is required to impair the production of O₂⁻ by granulocytes upon stimulation with FMLP compared to PMA.¹¹

Activation of PKC is essential for the serum-stimulated intracellular killing of *S. aureus* by monocytes, since this process can be inhibited by inhibitors of PKC²⁰ or with agents that activate and subsequently deplete PKC, such as PMA and membrane-permeable diacylglycerol analogues.¹² On the basis of these arguments, the surfactant-induced inactivation of PKC in monocytes is most probably involved in the inhibited intracellular killing of *S. aureus* by these cells. Since this study was performed to elucidate the role of surfactant in the decreased microbicidal functions of alveolar macrophages, it is relevant to report that preliminary experiments have shown that about 40% of PKC activity in human alveolar macrophages is membrane-associated (data not shown). Consistent with this finding is the fact that a higher proportion of PKC activity is membrane-associated in rat alveolar macrophages than in peritoneal macrophages.^{36,37} Together, these results indicate that the impaired bactericidal functions of alveolar macrophages may be due to translocation of PKC induced by exposure of these cells to surfactant *in vivo*.

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