*Biochimica et Biophysica Acta* 861 (1986) 440–446
Elsevier

BBA 73314

# Effect of saturated and unsaturated fatty acids on the oxidative metabolism of human neutrophils. The role of calcium ion in the extracellular medium

Teruhide Yamaguchi, Mizuho Kaneda and Katsuko Kakinuma

*Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo-113 (Japan)*

(Received 8 April 1986)
(Revised manuscript received 28 July 1986)

Key words: Neutrophil; Oxidative metabolism; Fatty acid; Superoxide generation; $Ca^{2+}$

**The ability of fatty acids to stimulate the generation of superoxide anion ($O_2^-$) by human neutrophils was investigated with respect to their Krafft points. Saturated (myristic acid) and unsaturated (elaidic and oleic acid) induced a marked $O_2^-$ generation and release from human neutrophils at pH 7.4 in the absence of $Ca^{2+}$, while 0.3 mM $Ca^{2+}$ inhibited both myristic acid and elaidic acid-induced $O_2^-$ release. [$^{14}$C]Myristic acid association with neutrophils was reduced by addition of $Ca^{2+}$, whereas oleic acid association was not affected. When the pH of the reaction mixture was lowered to 6.4, 0.6 mM $Ca^{2+}$ did not inhibit the $O_2^-$ generation by human neutrophils. These results indicate that the inhibitory effect of $Ca^{2+}$ on the fatty acid-induced $O_2^-$ generation might be due to the ionic interaction between the carboxyl group of the fatty acid and $Ca^{2+}$. Furthermore, 11-methyltridecanoic acid, a branched isomer of myristic acid, which showed the low Krafft point even in the presence of $Ca^{2+}$, stimulated $O_2^-$ generation by human neutrophils not only in the absence but also in the presence of 0.6 mM $Ca^{2+}$. The effect of $Ca^{2+}$ on the fatty acid-induced $O_2^-$ generation by neutrophils was discussed with reference to its possible relationship to the Krafft point.**

## Introduction

Recently, fatty acids have been found to be able to modulate biological functions in certain tissues and cells, e.g., alterations of membrane-bound enzyme activity [1–3], lipogenesis in adipocytes [4], platelet aggregation [5], and surface receptor capping of lymphocytes [6–10]. In 1974, Kakinuma [11] showed that saturated and unsaturated fatty acids of suitable length caused an increase in cyanide-insensitive respiration of guinea pig neutrophils. This extra-respiration was accompanied by $O_2^-$ and $H_2O_2$ generation [12–14]. On the other hand, Karnovsky and his colleagues [15,16] reported that *cis*-unsaturated fatty acids, but not *trans*-unsaturated and saturated fatty acids, induced $O_2^-$ release from human neutrophils. This discrepancy is not due to a dissimilarity in either the $O_2^-$ generating enzyme or activation mechanisms known to exist in these cell types, in that Kakinuma et al. [17] demonstrated that myristic acid, a saturated fatty acid, was also capable of inducing $H_2O_2$ generation and release from human neutrophils.

In the present study, we have attempted to clarify the ability of fatty acids to stimulate $O_2^-$ release from neutrophils both in the presence and

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; myristic acid, tetradecanoic acid; stearic acid, octadecanoic acid; elaidic acid, 9-*trans* octadecenoic acid; oleic acid, 9-*cis* octadecenoic acid; linoelaidic acid, 9-*trans* 12-*trans* octadecadienoic acid; linoleic acid, 9-*cis* 12-*cis* octadecadienoic acid; anteiso-$C_{14}$, 11-methyltridecanoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

Correspondence address: Dr. T. Yamaguchi, Tokyo Metropolitan Institute of Medical Science, 3-8-22 Honkomagome, Bunkyo-ku, Tokyo-113, Japan.

absence of $Ca^{2+}$, since it is a well-known fact that $Ca^{2+}$ interacts strongly with anionic surfactant such as fatty acids. We showed and discussed the inhibitory effect of $Ca^{2+}$ on the fatty acid-stimulated $O_2^-$ release from human neutrophils.

## Materials and Methods

*Materials.* Fatty acids were purchased from Applied Science Laboratories Inc., Deerfield, IL. 11-Methyltridecanoic acid (anteiso-$C_{14}$), a branched saturated fatty acid, was obtained from Larodan Fine Chem. AB, Sweden. They were dissolved in dimethyl sulfoxide (DMSO), enveloped in a nitrogen stream, and stored at $-80°C$ until used. Assays after incubation with fatty acids dissolved in DMSO yielded essentially the same results as those obtained in preliminary experiments with the fatty acids dissolved in 50% ethanol and neutralized with NaOH. Cytochrome *c* (Type VI), Hepes and superoxide dismutase were obtained from Sigma Co., St. Louis, MO. [$^{14}$C]Myristic acid and [$^{14}$C]oleic acid were purchased from Amersham International, Amersham, U.K., through the Japan Isotope Association, Tokyo, and stored in the same manner as mentioned for unlabeled fatty acids. Atomlight, a liquid scintillation solution, was obtained from New England Nuclear, Boston, MA. Other chemical reagents were of analytical grade.

*Cell preparation.* Heparinized venous blood, obtained from healthy adult donors, was mixed with 2% dextran in saline and permitted to stand at room temperature. Neutrophils were prepared from the dextran-sedimented leukocytes as reported previously [18]. Finally, cells ($\approx$ 95% neutrophils) were suspended in 17 mM Hepes-buffered saline (pH 7.4) containing 5 mM KCl and 1.2 mM $MgCl_2$. Guinea pig perintoneal neutrophils and porcine blood neutrophils were collected as described previously [19,20] and suspended in Hepes-buffered saline.

*Measurement of $O_2^-$.* The rate of $O_2^-$ release from neutrophils was measured at 37°C by recording the reduction of cytochrome *c* [14] during continuous stirring of the cell suspension in a windmill type cell-mixer as described previously [17]. The basal assay mixture contained $2 \cdot 10^6$ cells, 50 $\mu$M cytochrome *c*, 5 mM glucose and 5 $\mu$g/ml catalase in 1.6 ml of Hepes-buffered saline. In some experiments, cells were suspended in 17 mM phosphate-buffered saline contained 5 mM KCl and 1.2 mM $MgCl_2$, pH 6.4, instead of Hepes-buffered saline.

*Association of fatty acid with neutrophils.* Neutrophils ($2 \cdot 10^6$ cells/1.6 ml) were preincubated in Hepes-buffered saline containing 5 mM glucose with or without $Ca^{2+}$ at 37°C for 5 min. Then, $^{14}$C-labeled fatty acids were added to the cell suspension and incubated for 2 min. Further incubation was not required, because in the preliminary experiments, the association of $^{14}$C-labeled fatty acids reached a plateau within 1 min. After the incubation, the cell suspension was loaded on a silicone oil (1.015 g/ml) layer in a siliconized tube and then centrifuged at $1500 \times g$ for 3 min to remove the unassociated fatty acids. The pellet was resuspended and dissolved in 0.1 M NaOH. An aliquot of the solubilized pellet was suspended in Atomlight scintillation fluid and the number of $^{14}$C counts determined.

*Krafft points determinations.* The Krafft point of each fatty acids was determined with or without equimolecular amounts of $Ca^{2+}$ photometrically, in principle according to the previous reports [21]. Assay mixture was prepared as follows; a 10% (w/v) solution of fatty acid in DMSO was diluted with 20-fold of Hepes-buffered saline, except for anteiso-$C_{14}$ (4% solution in DMSO was diluted with 20-fold of Hepes-buffered saline).

## Results

Fig. 1 shows the overall patterns of $O_2^-$ release from neutrophils induced by various amounts of fatty acids. Not only oleic acid (*cis*-unsaturated fatty acid) but also myristic acid (saturated fatty acid) and elaidic acid (*trans*-unsaturated fatty acid) induced a marked $O_2^-$ release from human neutrophils in the absence of $Ca^{2+}$; the maximum rate of $O_2^-$ generation was induced by about 25 $\mu$M oleic acid, 50 $\mu$M elaidic acid, or 70 $\mu$M myristic acid. The myristic and elaidic acid-induced $O_2^-$ generation by neutrophils was decreased in the presence of $Ca^{2+}$, the maximum rates of $O_2^-$ release being observed at higher concentrations of these fatty acids. On the other hand, the maximum rate of $O_2^-$ release induced by oleic acid

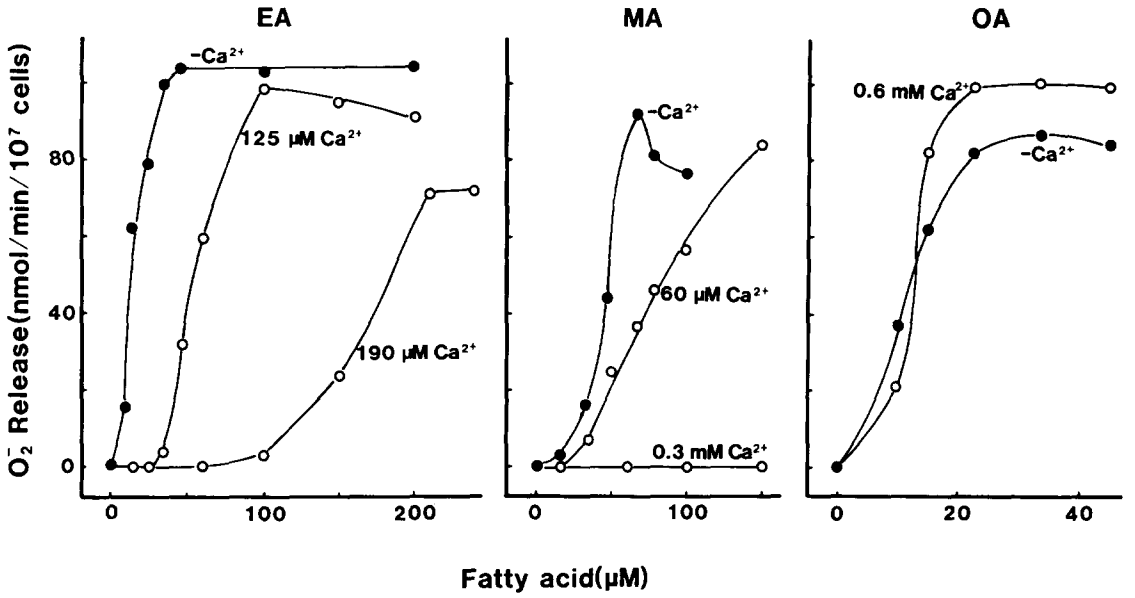


Fig. 1. Ability of various fatty acids to stimulate $O_2^-$ production by human neutrophils. The rate of $O_2^-$ production was measured in the standard assay mixture after stimulation with different concentrations of fatty acids as described under Methods. EA, MA and OA represent elaidic acid, myristic acid and oleic acid, respectively.

was slightly higher in the presence rather than absence of $Ca^{2+}$. Stearic acid, a $C_{18}$ saturated fatty acid failed to induce $O_2^-$ release under both conditions, with and without $Ca^{2+}$ (data not shown). The inhibitory effects of $Ca^{2+}$ on the $O_2^-$ generation by neutrophils induced by elaidic acid or myristic acid are compared in Fig. 2. The neutrophil $O_2^-$ generation induced by either elaidic acid (50 μM) or myristic acid (70 μM) was completely inhibited by 300 μM or 100 μM $Ca^{2+}$, respectively. The inhibitory effect of $Ca^{2+}$ on the $O_2^-$ generation induced by elaidic acid or myristic acid diminished following addition of 1 mM EGTA (data not shown). The $O_2^-$ generation by human neutrophils was also stimulated by linoelaidic acid(9,12-*trans*-octadecadienoic acid) or linoleic acid(9,12-*cis*-octadecadienoic acid) in the absence of $Ca^{2+}$, while the $O_2^-$ generation on stimulation by linoelaidic acid was completely inhibited by 0.6 mM $Ca^{2+}$ (data not shown).

It is well-known that fatty acids strongly interact with metal ions such as $Ca^{2+}$. To explore the inhibitory effect of $Ca^{2+}$ on the fatty acid-induced $O_2^-$ release from neutrophils, the abilities of fatty acids to associate with neutrophils were compared either in the presence or in the absence of $Ca^{2+}$, with the results summarized in Table I. The association of [$^{14}$C]myristic acid with neutrophils was inhibited about 43% by 60 μM $Ca^{2+}$ and about 58% 0.3 mM $Ca^{2+}$. However, the association of [$^{14}$C]oleic acid with neutrophils was rather

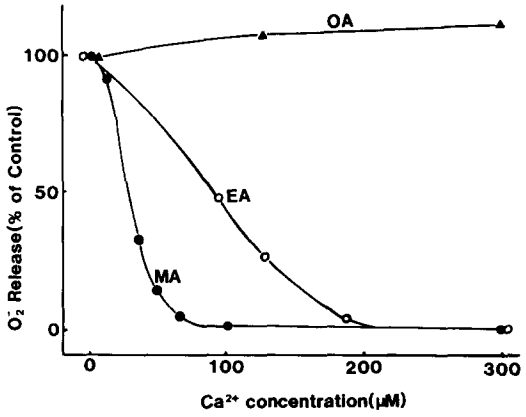


Fig. 2. Effect of $Ca^{2+}$ on the $O_2^-$ generation by human neutrophils stimulated with fatty acids. Human neutrophils were incubated with various concentrations of $Ca^{2+}$ in the standard assay mixture for 5 min prior to stimulation with myristic acid (MA, 70 μM), elaidic acid (EA, 50 μM) or oleic acid (OA, 25 μM).

TABLE I

ASSOCIATION OF MYRISTIC AND OLEIC ACIDS WITH HUMAN NEUTROPHILS

Association of fatty acids was measured as describd under Methods. Figures represent the means ± S.D. from six experiments.

| Condition | cpm | |
|---|---|---|
| | [$^{14}$C]myristic acid | [$^{14}$C]oleic acid |
| $-Ca^{2+}$ | 7520 ± 560 | 12560 ± 990 |
| 60 μM $Ca^{2+}$ | 4260 ± 150 | 13740 ± 670 |
| 0.3 mM $Ca^{2+}$ | 3190 ± 650 | 13860 ± 440 |

slightly enhanced by $Ca^{2+}$. There was more than 95% recovery of cell protein in all experimental conditions.

In aqueous solutions, the existing form of fatty acids varies with the pH, e.g. micelle, acid soap, or monomeric fatty acid form [22–24]. The ability of fatty acids to stimulate $O_2^-$ release from human neutrophils at pH 6.4 was compared in the presence and in the absence of $Ca^{2+}$. Fig. 3 shows the $O_2^-$ generation stimulated by various concentration of fatty acids at pH 6.4. It was noted that at pH 6.4, $Ca^{2+}$ did not inhibit the $O_2^-$ release from neutrophils induced by these fatty acids. The maximal level of $O_2^-$ release obtained at pH 6.4 was 29 to 48% below the level of pH 7.4 without $Ca^{2+}$, indicating that the NADPH oxidase activity itself or the activation process of the NADPH oxidase might be suboptimal at pH 6.4. Furthermore, the maximum $O_2^-$ release by neutrophils was found at a concentration of about 30 μM of any of these three fatty acids. At this pH, human neutrophils generated $O_2^-$ after an extended long lag time, compared to that at pH 7.4, except for the of case myristic acid in the absence of $Ca^{2+}$ (data not shown).

The Krafft point is, as is widely known, the temperature above which the solubility of a surfactant increases dramatically in aqueous systems, and is interpreted as representing the melting temperature of a hydrated solid surfactant

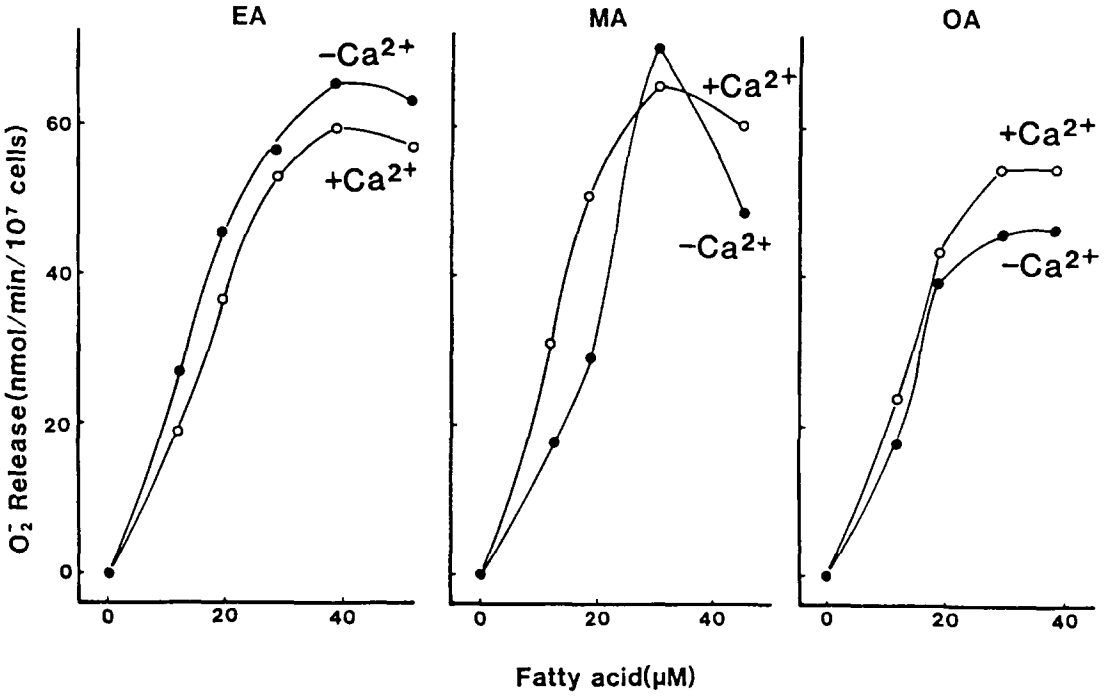


Fig. 3. Ability of various fatty acids to stimulate the $O_2^-$ generation by human neutrophils at pH 6.4. The reaction mixture contained 50 μM cytochrome *c*, 5 mM glucose, and 5 μg/ml catalase and $2 \cdot 10^6$ cells in 1.6 ml phosphate-buffered saline (pH 6.4) with or without 0.6 mM $Ca^{2+}$. The rate of $O_2^-$ generation was measured after stimulation with fatty acids at various concentrations described under Methods.

[25]. Furthermore, it is also well known that the Krafft point of anionic surfactant such as fatty acid is markedly enhanced by $Ca^{2+}$ but not $Mg^{2+}$ [25]. The melting points of branched saturated fatty acids are remarkably lower than those of non-branched fatty acids, thereby it was expected that the Krafft point of the former fatty acid even in the presence of $Ca^{2+}$ was lower than that of the latter one. We examined the stimulating ability of 11-methyltridecanoic acid (anteiso-$C_{14}$) which is an isomer of myristic acid. As shown in Fig. 4, anteiso-$C_{14}$ stimulated the $O_2^-$ release from neutrophils at lower concentration than myristic acid. It was noted, furthermore, that anteiso-$C_{14}$ caused $O_2^-$ generation not only in the absence but also in the presence of 0.6 mM $Ca^{2+}$, suggesting that the inhibitory effect of $Ca^{2+}$ is not ruled by saturated or unsaturated form of fatty acids. On the basis of present experiments, we classified fatty acid into three groups according to their abilities to stimulate the $O_2^-$ generation by human neutrophils. As shown in Table II, the Krafft points of all fatty acids were elevated by addition of $Ca^{2+}$. Group A-fatty acid, of which the Krafft point was very high even in the absence of $Ca^{2+}$, was unable to stimulate $O_2^-$ generation by human neutrophils in all condition tested. Group C-fatty acids, of which the Krafft points were very low in the presence of $Ca^{2+}$, were able to stimulate $O_2^-$ in all condition. Group B-fatty acids were able to stimulate $O_2^-$ generation by human neutrophils in the absence of $Ca^{2+}$, but were unable to stimulate in the presence of high concentration of $Ca^{2+}$.

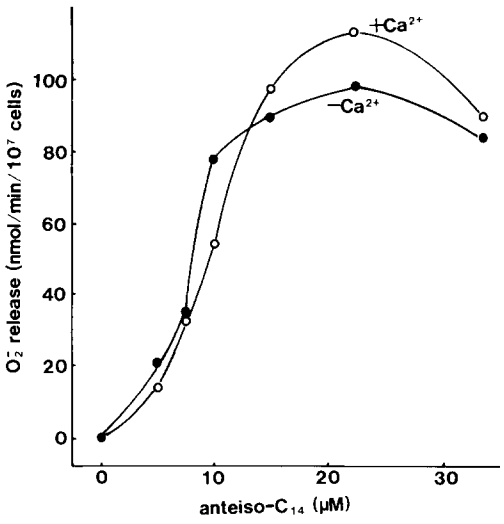


Fig. 4. Generation of $O_2^-$ by human neutrophils stimulated with 11-methyltridecanoic acid. The reaction mixture was the same as that described in Fig. 1. The rate of $O_2^-$ generation was measured after addition of various concentrations of anteiso-$C_{14}$ with or without 0.6 mM $Ca^{2+}$.

TABLE II

CLASSIFICATION OF FATTY ACIDS

| Fatty acid | Ability to stimulate the $O_2^-$ generation by human neutrophils | |
|---|---|---|
| | $-Ca^{2+}$ | $+Ca^{2+}$ |
| Group A | | |
| Stearic acid | − (62.5) * | − (82) |
| Group B | | |
| Myristic acid | + (42.5) | − (65) |
| Elaidic acid | + (40.5) | − (63) |
| Linoelaidic acid | + (22–25) | − (47) |
| Group C | | |
| Oleic acid | + (11) | + (31) |
| Linoleic aicd | + ( < 0) | + (9.0) |
| Anteiso-$C_{14}$ | + (14.3) | + (35) |

−, No stimulation of $O_2^-$ generation by human neutrophils.
+, Stimulate $O_2^-$ generation by human neutrophils.
* The Krafft point of each fatty acid was measured with or without equimolecular amounts of $Ca^{2+}$.

The $O_2^-$ generation by porcine neutrophils and guinea pig neutrophils, induced with elaidic acid or myristic acid, was inhibited by $Ca^{2+}$ as in the case of human neutrophils, and $Ca^{2+}$ did not inhibit the $O_2^-$ generation by neutrophils of these species, stimulated by oleic acid (data not shown).

## Discussion

Not only the *cis*-unsaturated fatty acid, but also the *trans*-unsaturated fatty acid and saturated fatty acid were able to stimulate $O_2^-$ generation and release by human neutrophils in the absence of $Ca^{2+}$. In the presence of 0.6 mM $Ca^{2+}$, however, neither elaidic acid nor myristic acid stimulated the $O_2^-$ generation by human neutrophils, whereas oleic acid proved to stimulate the $O_2^-$

generation. This inhibitory effect of $Ca^{2+}$ on the $O_2^-$ generation by neutrophils was diminished by addition of 1 mM EGTA to the reaction mixture. In the presence of low concentration of $Ca^{2+}$, a high concentration of elaidic acid or myrisitc acid was required to stimulate the maximum rate of $O_2^-$ generation by human neutrophils (Fig. 1). Furthermore, similar inhibitory effects of $Ca^{2+}$ on the fatty acid-induced $O_2^-$ generation were also observed in guinea pig and porcine neutrophils. These results suggest that this inhibitory effect of $Ca^{2+}$ is due to some interaction between fatty acid and $Ca^{2+}$. It is a finding of profound interest that not only *cis*-unsaturated fatty acids but also *trans*-unsaturated and saturated fatty acids are able to stimulate $O_2^-$ generation by neutrophils, in that it suggests that the fatty acid modulating site is not phosphatidylinositol specific phospholipase C, because this phospholipase is specifically activated by *cis*-unsaturated fatty acids [3].

The mechanism of the $Ca^{2+}$-dependent inhibition of $O_2^-$ generation by neutrophils stimulated by certain fatty acids remains unclear. Karnovsky's group [16] suggested that the melting point of fatty acids is closely correlated with their ability to stimulate $O_2^-$ generation by human neutrophils. This correlation seems to differ markedly with the presence or absence of $Ca^{2+}$ in the medium (Figs. 1 and 2). The present findings, together with a consideration of the previous reports [11,14], suggest that the ability of fatty acids to stimulate the $O_2^-$ generation by neutrophils is likely to be correlated with the Krafft point rather than with the melting point itself (Table II). It would appear probably that fatty acids are able to stimulate the $O_2^-$ generation by neutrophils only below a certain threshold of the Krafft point; this critical point might be between the Krafft point of myristic acid without $Ca^{2+}$ and that of linoelaidic acid with $Ca^{2+}$ (Table II). The elevation of the Krafft point of fatty acids implies a depression of their surface activities such as micelle-forming properties, thus in the presence of $Ca^{2+}$, elaidic acid or myristic acid might not cause enough perturbation of the plasma membrane in neutrophils. It would seen plausible to consider that the Krafft point of such fatty acids with very low melting points as oleic acid, linoleic acid and anteiso-$C_{14}$, might not be elevated up to the threshold even in the presence of 0.6 mM $Ca^{2+}$. Furthermore, $Ca^{2+}$ inhibited the association of [$^{14}$C]myristic acid with human neutrophils while that of [$^{14}$C]oleic acid with neutrophils was not inhibited by $Ca^{2+}$, indicating that $Ca^{2+}$ reduces the affinity of certain fatty acids for neutrophils, hence in support of the above postulation. Kakinuma and Minakami [14] reported that the increase in the length of the carbon chain of saturated fatty acids up to 14 carbons (myristic acid) was accompanied by a proportionate enhancement of the ability to stimulate the $O_2^-$ generation by guinea pig neutrophils. Increases in the length of the carbon chain of fatty acids parallels increases in their hydrophobicity [26]. Thus, the ability of fatty acid to stimulate the $O_2^-$ generation by neutrophils appears likely to be rules by both its hydrophobicity and the Krafft point in aqueous solution.

At pH 6.4, there was no appreciable inhibitory effect of $Ca^{2+}$ on the elaidic or myristic acid-induced $O_2^-$ generation by human neutrophils (Fig. 3). At pH 6.4, the solid phase of fatty acids exist as a free acid form (R-COOH), which is not affected by metal ions such as $Ca^{2+}$, at pH 7.4 the solid phase of fatty acids in aqueous solution is a 1 : 1 mixture of metal acid soap and free acid, which is affected by metal ions such as $Ca^{2+}$ [22–24]. Therefore, it would be reasonable to infer that, in the present experiment, all fatty acids tested might have been inserted into the plasma membrane of human neutrophils in a free acid form, at pH 6.4. Reversely, this result confirms that the inhibitory effect of $Ca^{2+}$ on the fatty acid-stimulated $O_2^-$ generation by human neutrophils is due to the ionic interaction between the carboxyl group of fatty acids and $Ca^{2+}$.

## Acknowledgments

This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan. We are very grateful to Dr. Tsukasa Chiba for critical review of the manuscript. We also thank Dr. Minoru Suzuki for his helpful suggestions. Thanks are also tendered to Dr. Kaoru Tsujii of the Tochigi Research Laboratories, Kao Co. Ltd., for providing us with his recent reports and helpful suggestions.

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