

## Inhibition of Superoxide Production and $Ca^{2+}$ Mobilization in Human Neutrophils by Halothane, Enflurane, and Isoflurane

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The inhibitory effects of three inhalation anesthetics, *i.e.*, halothane, enflurane, and isoflurane, on superoxide production and the intracellular mobilization of calcium in human neutrophils were studied. The superoxide production induced by N-formyl-methionyl-leucyl-phenylalanine (FMLP) was inhibited by the anesthetics, but the binding of FMLP<sup>3</sup>H to the cells and the superoxide-forming NADPH oxidase of the phagocytic vesicles were not inhibited. The inhibition of the cellular superoxide production was partially reversed by the addition of a calcium ionophore, A23187. The increase in intracellular free calcium monitored by a calcium-sensitive fluorescent probe, quin-2 and the release of calcium from hydrophobic environment monitored by chlortetracycline were inhibited dose dependently by the anesthetics. These observations suggest that decreased mobilization of intracellular  $Ca^{2+}$  is one of the mechanisms by which the anesthetics inhibited the superoxide production of human neutrophils stimulated by FMLP. (Key words: Anesthetics, volatile: enflurane; halothane; isoflurane. Blood: neutrophils. Ions: calcium. Infection: neutrophils.

NEUTROPHILS PLAY A CRUCIAL ROLE in host defense through their bactericidal<sup>1</sup> and tumoricidal<sup>2</sup> activities. The cellular response to microbial invasion consists of chemotaxis, ingestion, intracellular killing, digestion, and the release of lysosomal components.<sup>3</sup> The production of active oxygens is an essential event in bactericidal mechanisms<sup>4</sup> and the cells form active oxygens such as superoxide anions ( $O_2^{-1}$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $\cdot OH$ ) during phagocytosis or when they are stimulated by soluble agents such as N-formyl-methionyl-leucyl-phenylalanine (FMLP). The superoxide production is catalyzed by a superoxide-forming NADPH oxidase,<sup>5</sup> which is dormant in the resting cells. Calcium appears to act as a second messenger in the activation of the oxidase,<sup>6,7</sup> although the mechanism is still not clear.

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The intracellular mobilization of calcium can be ascribed to both influx through the plasma membrane and intracellular translocation from the storage sites.<sup>6,8</sup> Two new  $Ca^{2+}$ -sensitive fluorescent probes have become available: quin-2,<sup>9,10</sup> for monitoring intracellular free calcium ions, and chlortetracycline,<sup>11,12</sup> for monitoring the release of the ions from hydrophobic environment.

It has been reported previously that commonly used general anesthetics inhibit oxidative metabolism,<sup>13,14</sup> phagocytosis,<sup>15,16</sup> and microbicidal activity<sup>14,17</sup> of human neutrophils. The present report is concerned with the inhibition of superoxide production in human neutrophils by three volatile anesthetics, halothane, enflurane, and isoflurane, and partial recovery from the inhibition by a calcium ionophore A23187. The mobilization of intracellular calcium was inhibited by the anesthetics in a similar dose-dependent manner as the superoxide-releasing activity. The inhibition of calcium mobilization is suggested to be involved in the inhibition of oxidative metabolism by the anesthetics.

### Materials and Methods

#### MATERIALS

Halothane was obtained from Takeda Chemical Industries, Ltd., Japan, and enflurane and isoflurane were obtained from Dainabot Co., Ltd., Japan. Cytochrome *c* (type III), superoxide dismutase, N-formyl-methionyl-leucyl-phenylalanine, and chlortetracycline were obtained from Sigma Co., St. Louis, Missouri.  $Ca^{2+}$  ionophore A23187 was from Calbiochem-Behring, San Diego, California. Quin-2, quin-2 tetraacetoxymethylester (quin-2/AM) and ethyleneglycol-bis-( $\beta$ -aminoethyl-ether)-N,N'-tetraacetic acid (EGTA) were purchased from Dojin, Kumamoto, Japan. N-formyl-methionyl-leucyl[ring-2,6-<sup>3</sup>H]-phenylalanine was from New England Nuclear. Other reagents were of analytic grade.

#### CELL PREPARATION AND EXPOSURE TO HALOTHANE, ENFLURANE, AND ISOFLURANE

Human neutrophil suspensions were prepared from healthy adult donors by dextran sedimentation, hypotonic lysis, and the Conray-Ficoll method described by Böyum.<sup>18</sup> The cell analysis showed more than 98% as neutrophils and the trypan blue exclusion test showed that more than 98% of the cells were viable. The three anesthetics were

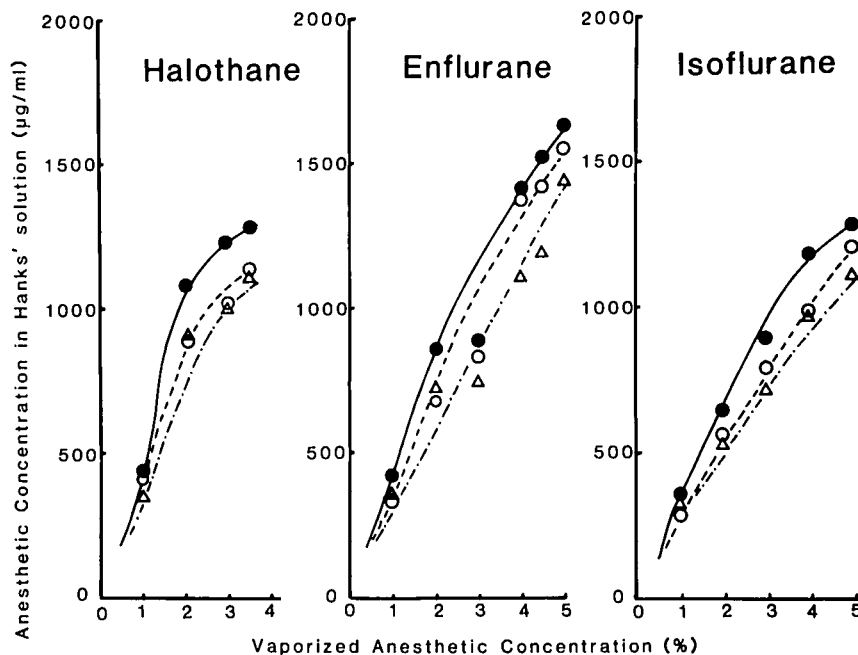


FIG. 1. Anesthetic concentrations in Hanks' salt solution. Closed circles (●), open circles (○), and open triangles (△) indicate the concentrations of each anesthetic after incubation at 37° C for 0, 10, and 20 min, respectively.

equilibrated in modified Hanks' salt solution (124 mM NaCl, 4 mM KCl, 0.64 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.66 mM K<sub>2</sub>HPO<sub>4</sub>, 15.2 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5 mM glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HEPES, pH 7.4) by bubbling each vaporized anesthetic with a carrier gas of air (4 l/min) at 4° C on a shaking plate. The concentration of each anesthetic (µg/ml) in the modified Hanks' salt solution in a cuvette sealed with parafilm was measured by gas chromatography, using a flame ionization detector, and the relationship to vaporized anesthetic concentration (%) is shown in figure 1. The values represent means of duplicate experiments. The decrease of the anesthetic concentrations in the salt solution did not exceed 22% after incubation for 20 min at 37° C. Neutrophils were preincubated in the salt solution, which was equilibrated with anesthetics at each concentration for 10 min at 37° C

before starting the reaction with the addition of FMLP in a cuvette sealed with parafilm.

#### ASSAY OF THE SUPEROXIDE PRODUCTION BY NEUTROPHILS

The activity of the superoxide release was measured by the reduction of superoxide dismutase-sensitive cytochrome *c* as previously described.<sup>19</sup> The cells (10<sup>6</sup> cells/ml) were incubated for 10 min at 37° C in a thermostatically controlled cuvette, which was placed in a Hitachi 557<sup>®</sup> dual-wavelength spectrophotometer and contained 1 ml of a control or an anesthetic-equilibrated modified Hanks' salt solution. The cuvette was sealed with parafilm during the incubation period in order to minimize loss of the anesthetic.

We studied whether anesthetic agents scavenge superoxide anions directly by using the xanthine-xanthine ox-

TABLE 1. Effects of Three Volatile Anesthetics on FMLP [<sup>3</sup>H]P Binding Activity to Human Neutrophils, the NADPH-Dependent Superoxide-forming Activity of Phagocytic Vesicles and Superoxide Anions Produced by Xanthine-Xanthine Oxidase System

	FMLP [ <sup>3</sup> H]P Binding Activity (cpm/10 <sup>6</sup> cells) × 10 <sup>2</sup>	NADPH-dependent Superoxide Forming Activity (nmol · min <sup>-1</sup> · mg of protein <sup>-1</sup> )	Superoxide Production by Xanthine-Xanthine Oxidase System (µmol/min)
Control	6.7 ± 0.4 (4)	44.5 ± 4.4 (4)	8.9 ± 1.3 (4)
Halothane (3.5%)	11.8 ± 0.8* (4)	34.6 ± 1.6 (4)	9.1 ± 1.5 (4)
Enflurane (5%)	15.7 ± 1.1* (4)	37.6 ± 3.3 (4)	9.2 ± 1.3 (4)
Isoflurane (5%)	14.1 ± 0.1* (4)	54.7 ± 5.4† (4)	8.8 ± 1.0 (4)

The number in parentheses represents the number of experiments, and the values represent mean ± SE.

\* Significantly different from control, *P* < 0.01.

† Significantly different from the activities in the presence of halothane and enflurane, *P* < 0.05.

TABLE 2. Recovery of Superoxide-releasing Activity by  $\text{Ca}^{2+}$  Ionophore A23187

	Control	Halothane (3.5%)	Enflurane (5%)	Isoflurane (5%)
FMLP ( $10^{-7}$ M)	$3.68 \pm 0.39$ (4)	$0.32 \pm 0.07^*$ (4)	$0.50 \pm 0.10^*$ (5)	$0.41 \pm 0.06^*$ (4)
A23187 ( $10^{-6}$ M)	$0.88 \pm 0.15$ (4)	$1.60 \pm 0.03^\dagger$ (5)	$0.01 \pm 0.01$ (5)	$0.04 \pm 0.04$ (4)
A23187 ( $10^{-6}$ M) + FMLP ( $10^{-7}$ M)	$6.47 \pm 0.59$ (4)	$4.26 \pm 0.75^*$ (4)	$1.73 \pm 0.13^*$ (5)	$2.23 \pm 0.41^*$ (4)

The number in parentheses represents the number of experiments, and the values represent mean  $\pm$  SE ( $\text{nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ ).

\* Significantly different from control,  $P < 0.01$ .

† Significantly different from control,  $P < 0.05$ .

idase system (table 1). The assay mixture consisted of the modified Hanks' salt solution, pH 7.4, equilibrated with the anesthetics, along with 0.25 mM xanthine and 50  $\mu\text{M}$  cytochrome *c*. The reaction was started by adding 50  $\mu\text{g}$  of xanthine oxidase after incubation for 10 min at 37° C.

In the experiments summarized in tables 2 and 3, neutrophils ( $1 \times 10^6$  cells) were preincubated in the modified Hanks' salt solution with (table 2) or without (table 3) 1 mM  $\text{Ca}^{2+}$  for 10 min at 37° C. The reaction was started by the addition of  $10^{-7}$  M FMLP,  $10^{-6}$  M A23187, or  $10^{-7}$  M FMLP 1 min after  $10^{-6}$  M A23187.

#### FMLP [ $^3\text{H}$ ]P BINDING ASSAY

Binding of FMLP to neutrophils was measured by the rapid filtration method as described by Rossi *et al.*<sup>20</sup> Neutrophils were suspended in a control-modified Hanks' salt solution or the salt solution bubbled with 3.5% halothane, 5% enflurane, or 5% isoflurane. A 200- $\mu\text{l}$  aliquot of neutrophil suspension ( $5 \times 10^6$  cells/ml) was incubated in an Eppendorf microtube for 10 min at 37° C and then FMLP [ $^3\text{H}$ ]P ( $5 \times 10^{-8}$  M, 48.3 Ci/mmol) was added. After further incubation for 10 min, the cell suspensions were rapidly filtered through Whatmann (Gf/B) glass fiber filters. The filters were washed twice with 5 ml of the ice-

cold modified Hanks' salt solution and dried and then placed in 5 ml scintillation liquid (Toluene including 4 g/l 2,5-diphenyloxazol). The samples were counted for [ $^3\text{H}$ ] in a liquid scintillation counter. Nonspecific binding was measured by saturating the binding sites with cold FMLP, and the value was subtracted from the value for total binding.

#### ASSAY OF THE NADPH-DEPENDENT SUPEROXIDE-FORMING ACTIVITY

Phagocytic vesicles of human neutrophils were prepared according to the method of Wakeyama *et al.*<sup>5</sup> Neutrophils were preincubated in polycarbonate centrifuge tubes ( $3 \times 10^9$  cells per tube) with 12 ml of phosphate Ringer's solution pH 7.4, and 2 mM-glucose at 37° C for 5 min. Then KCN was added to a final concentration of 1 mM and incubated with 2.9 ml of the oil droplets for 5 min. Oil droplets were prepared by modifying the method of Stossel *et al.*<sup>21</sup> Paraffin oil was replaced by 1,2,3,6-tetrahydrophthalic acid ester and serum albumin by immunoglobulin G. The tetrahydrophthalic acid ester (0.67 ml) was added to 2.2 ml of the phosphate Ringer's solution, pH 7.4, containing 5 mg pig immunoglobulin G/ml, and the mixture was sonicated for 90 s at 20 watts power with the standard probe tip of a Branson 200<sup>®</sup>

TABLE 3. Effects of A23187 Treatment without  $\text{Ca}^{2+}$  on Superoxide Release of Neutrophils Inhibited by Inhalation Anesthetics

	Control	Halothane (3.5%)	Enflurane (5%)	Isoflurane (5%)
FMLP ( $10^{-7}$ M)	$1.78 \pm 0.09$ (5)	$0.09 \pm 0.04^*$ (4)	$0.19 \pm 0.06^*$ (4)	$0.16 \pm 0.06^*$ (4)
A23187 ( $10^{-6}$ M)	<0.01	$0.08 \pm 0.02$ (4)	$0.06 \pm 0.02$ (4)	$0.04 \pm 0.02$ (4)
A23187 ( $10^{-6}$ M) + FMLP ( $10^{-7}$ M)	$2.83 \pm 0.48$ (3)	$0.12 \pm 0.02^*$ (4)	$1.30 \pm 0.24^*$ (4)	$1.38 \pm 0.38^*$ (4)

The number in parentheses represents the number of experiments and the values represent mean  $\pm$  SE ( $\text{nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ ).

\* Significantly different from control,  $P < 0.01$ .

sonifier set on pulsed power, 50% duty circle. The reaction was terminated by the addition of 15 ml ice-cold 0.9% NaCl. The cells were separated from the noningested oil droplets by centrifuging at 150 *g* for 10 min, and they were washed with 25 ml ice-cold 0.9% NaCl and then with 15 ml ice-cold distilled water. The washed cells were suspended in 8 ml ice-cold 0.34 M sucrose buffered with 5 mM HEPES, *pH* 7.5 (buffered 0.34 M sucrose) and homogenized by 10 strokes of a Teflon® pestle in a Potter-Elvehjem homogenizer with a tight clearance. The homogenate was centrifuged for 1 h at 76,500 *g* at 4° C with buffered 0.25 M sucrose solution layered on it. Vesicles filled with oil droplets floated to the top of the 0.25 M sucrose solution. The floating pellet was removed and was dispersed with a glass pestle in a Potter-Elvehjem homogenizer in 4 ml of the buffered 0.34 M sucrose.

The NADPH-dependent superoxide-forming activity was measured by the reduction of cytochrome *c* in the presence of 100 μM NADPH essentially as described by Kakinuma and Minakami.<sup>22</sup> In a typical experiment, phagocytic vesicles (4 mg protein/ml) were preincubated in 100 μl of the modified Hanks' salt solution equilibrated with each anesthetic agent for 10 min at 4° C. The reaction was started by the addition of the preincubated phagocytic vesicles into the cuvette, which contained 800 μl of a reaction mixture consisting of an anesthetic-equilibrated modified Hanks' salt solution and 50 μM cytochrome *c* and was placed in a thermostatically controlled cuvette holder of a Hitachi 556® dual wavelength spectrophotometer at 20° C. The cuvette was sealed with parafilm during the experiments. The absorbance change at 550 nm in reference to 540 nm ( $A_{550} - A_{540}$ ) was followed on a recorder. The superoxide-forming activity was calculated from the initial velocity of the reduction of cytochrome *c* by using a molar coefficient of  $19.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  and was corrected by subtracting the rate in the presence of superoxide dismutase.

#### LOADING OF QUIN-2

Neutrophils were loaded with quin-2 essentially as previously described.<sup>7,9</sup> Neutrophils were incubated with stirring in the modified Hanks' salt solution containing 15 μM quin-2/AM dissolved in dimethylsulfoxide for 10 min at 37° C and then diluted to 10 times with a warm salt solution. Complete hydrolysis of intracellular quin-2/AM to free quin-2 as monitored by the shift of emission peak at 430 nm (quin-2/AM) to 492 nm (quin-2) took about 40 min. The cells then were washed twice with the cold salt solution at 500 × *g* for 10 min at 4° C and suspended in the Ca<sup>2+</sup> free modified Hanks' salt solution ( $1 \sim 2 \times 10^8$  cells/ml). Quin-2 content in the loaded cells was 2 ~ 5 nmol per 10<sup>7</sup> cells, assuming that the cell volume is 0.35 μl/10<sup>6</sup> cells.<sup>23</sup> The superoxide-releasing activity of the cells was not affected by loading with quin-2.

#### MEASUREMENT OF QUIN-2 FLUORESCENCE CHANGES

Fluorescence measurements were performed using a Hitachi® fluorescence spectrophotometer 650-40 equipped with a thermostated cuvette holder.<sup>7</sup> The excitation and emission wavelengths were 339 nm and 492 nm, respectively, with 3 nm bandwidths. In a typical fluorescence experiment, quin-2 loaded neutrophils ( $1 \times 10^7$  cells/ml) were suspended in 1 ml of a modified Hanks' salt solution with or without 1 mM CaCl<sub>2</sub> and incubated for 10 min at 37° C in a cuvette, then stimulants were added and the changes of fluorescence were determined on a recorder. After completion of the fluorescence measurements, the cells were lysed with 1% of Triton X-100 and the fluorescence at high Ca<sup>2+</sup> (1.0 mM Ca<sup>2+</sup>;  $F_{\text{max}}$ ) and low Ca<sup>2+</sup> (2.0 mM EGTA + 50 mM TRIS;  $F_{\text{min}}$ ) was determined. From these values, the concentration of intracellular free Ca<sup>2+</sup>,  $[\text{Ca}^{2+}]_i$ , was calculated according to the following equation,<sup>9</sup>  $[\text{Ca}^{2+}]_i = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$ , where  $K_d$  is 115 nM and  $F$  is the fluorescence of the intracellular indicator before lysing the cells. The anesthetics did not affect the fluorescence *per se* of quin-2-Ca<sup>2+</sup> complex.

#### CHLORTETRACYCLINE LOADING AND FLUORESCENCE MEASUREMENT

Chlortetracycline (CTC) shows a high fluorescence, which is ascribed to binding of the probe to membrane-associated Ca<sup>2+</sup> and Mg<sup>2+</sup>. The Ca<sup>2+</sup> chelate gives stronger fluorescence than the Mg<sup>2+</sup> chelate, and the spectra of both chelates are different.<sup>11</sup> Therefore, CTC is used in the study of Ca<sup>2+</sup> mobilization in cellular and subcellular systems.

Neutrophils ( $1 \times 10^7$  cells/ml) were incubated with continuous stirring for 30 min at 30° C in the modified Hanks' salt solution containing 1 mM Ca<sup>2+</sup> and 100 μM chlortetracycline. After incubation, the cells were washed twice and resuspended in the salt solution. Then the CTC-loaded neutrophils ( $1 \times 10^7$  cells/ml) were incubated in an anesthetic-equilibrated or control salt solution for 10 min at 37° C in the presence of 1 mM Ca<sup>2+</sup>. The reaction was started by the addition of 10<sup>-7</sup> M FMLP. The changes of fluorescence were monitored by a Hitachi® fluorescence spectrophotometer 650-40 in a thermostatically controlled cuvette. The excitation and emission wavelength for Ca<sup>2+</sup> were 401 nm and 514 nm, respectively.<sup>12</sup> The activity of superoxide release of the cells was not affected by CTC treatment. There was no effect of the anesthetics on the fluorescence *per se* of CTC-Ca<sup>2+</sup> complex.

#### STATISTICAL ANALYSIS

Mean, standard error, and one-way analysis of variance were determined where appropriate for all experimental studies.

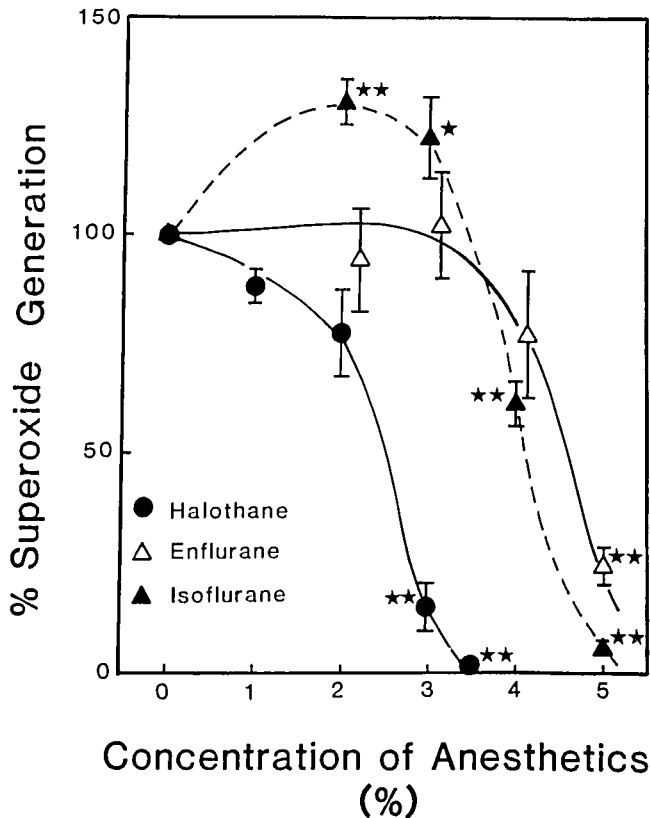


FIG. 2. Effects of halothane, enflurane, and isoflurane on the superoxide-releasing activity of human neutrophils. Values represent mean  $\pm$  SE ( $n = 4$ ). Single asterisk (\*) indicates significant difference from control ( $P < 0.05$ ). Double asterisks (\*\*) indicate significant difference from control ( $P < 0.01$ ).

## Results

### THE EFFECTS OF ANESTHETICS ON SUPEROXIDE RELEASE FROM HUMAN NEUTROPHILS STIMULATED BY N-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE

The effects of the three volatile anesthetics on superoxide production in human neutrophils are shown in figure 2. The anesthetics inhibited superoxide production dose dependently and halothane inhibited the production at lower concentrations than the two other anesthetics. An increase in superoxide release ( $P < 0.01$ ) was observed at low concentrations of isoflurane, while the two other anesthetics did not show any increases. These inhibitory effects on superoxide production were partially recovered when the anesthetics were removed by air bubbling for 10 min at 37° C. The superoxide-releasing activities of the cells treated by 2.5% halothane, 4.5% enflurane, and 4% isoflurane were recovered to  $65.2 \pm 13.8\%$ ,  $84.8 \pm 6.6\%$ , and  $81.6 \pm 6.4\%$  (mean  $\pm$  SE,  $n = 4$ ) of the activities of control cells, respectively.

The anesthetics did not inhibit the reduction of cyto-

chrome *c* in the xanthine-xanthine oxidase system, indicating that they do not scavenge superoxide radicals (table 1).

### BINDING OF FMLP[<sup>3</sup>H]P TO NEUTROPHILS AND NADPH-OXIDASE ACTIVITY IN PHAGOCYTIC VESICLES

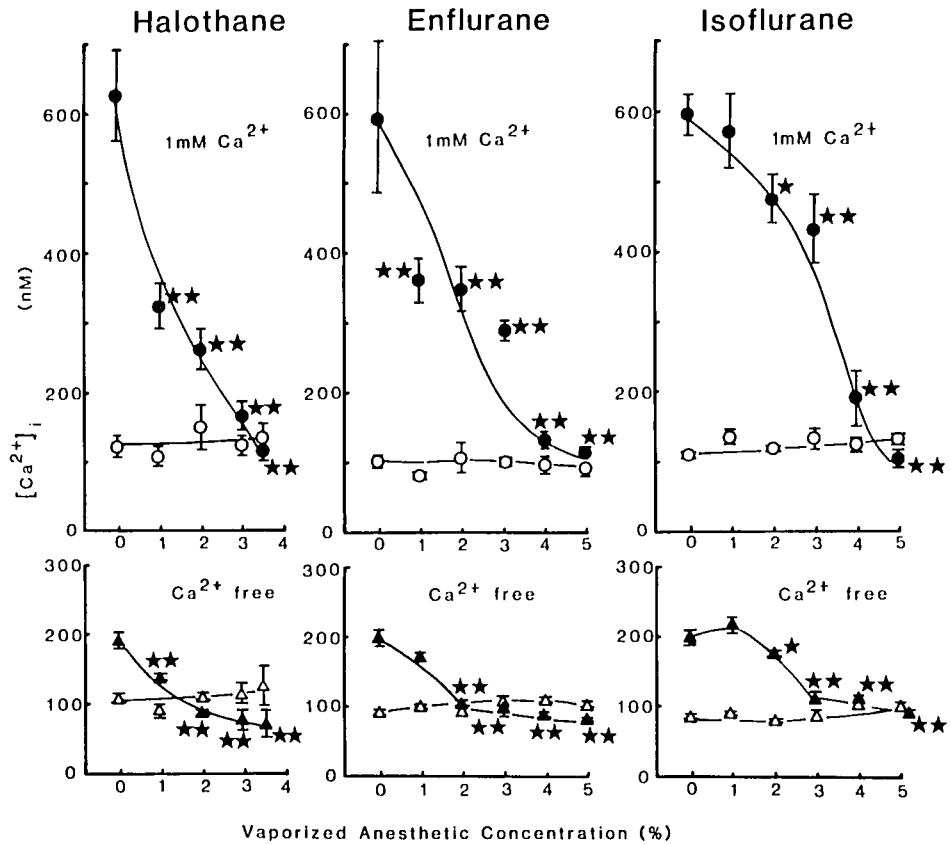
The inhibition of superoxide production by the anesthetics described above could not be ascribed either to the changes in the FMLP binding to neutrophils or to the direct effects on NADPH oxidase. The activities of neutrophils to bind FMLP[<sup>3</sup>H]P were not inhibited by the anesthetics but were accelerated ( $P < 0.01$ ) as shown in table 1. The effects of the anesthetics on the NADPH oxidase of the phagocytic vesicles prepared from the neutrophils ingesting oil droplets were not significantly different from the control by analyses of variance, although the increased activity in the presence of isoflurane was significantly higher than the activities in the presence of the two other anesthetics ( $P < 0.05$ ) (table 1).

### EFFECTS OF IONOPHORE A23187 ON FMLP-STIMULATED SUPEROXIDE PRODUCTION OF NEUTROPHILS TREATED WITH ANESTHETICS

Because  $\text{Ca}^{2+}$ -ionophore A23187 chelates divalent cations and transports them intracellularly,<sup>24</sup> we investigated whether the superoxide-producing activities inhibited by the anesthetics could be counteracted by treatment with the ionophore. The cells pretreated with the anesthetics in the presence of 1 mM  $\text{Ca}^{2+}$  were stimulated by FMLP, A23187, or the combination of both (table 2). A potentiating effect of the combination of FMLP and A23187 on superoxide production was observed: the production in the control cells stimulated by the combination of both reagents was about twice that when stimulated with FMLP alone and about sixfold that when stimulated with A23187 alone. The superoxide production induced by the combination was affected less by pretreatment with the anesthetics: more than half of the activity was observed in the halothane-treated cells and about one-third in the cells treated with enflurane or isoflurane, compared with the superoxide production induced by FMLP alone. The production induced by FMLP alone was decreased to about one-tenth by pretreatment with the anesthetics. The production of superoxide anions by the cells treated with halothane was twice that of the control cells when A23187 was used as an inducer, although the production of superoxide anions by the cells treated with enflurane and isoflurane was totally blocked.

We did similar experiments in the absence of added  $\text{Ca}^{2+}$  (table 3) in order to study the effects of  $\text{Ca}^{2+}$  release from intracellular storage sites. The ionophore A23187 is considered to be able to induce the superoxide release

FIG. 3. Effects of the volatile anesthetics on intracellular free  $\text{Ca}^{2+}$  of neutrophils monitored with quin-2. Open circle,  $[\text{Ca}^{2+}]_i$  at resting state in the presence of 1 mM  $\text{Ca}^{2+}$ ; closed circle,  $[\text{Ca}^{2+}]_i$  of the cells stimulated with  $10^{-7}$  M FMLP in the presence of 1 mM  $\text{Ca}^{2+}$ ; open triangle,  $[\text{Ca}^{2+}]_i$  at resting state in the absence of extracellular  $\text{Ca}^{2+}$ ; closed triangle,  $[\text{Ca}^{2+}]_i$  of the cells stimulated with  $10^{-7}$  M FMLP without extracellular  $\text{Ca}^{2+}$ . Values represent mean  $\pm$  SE ( $n = 4$ ). Single asterisks (\*) indicate significant difference from control ( $P < 0.05$ ). Double asterisks (\*\*) indicate significant difference from control ( $P < 0.01$ ).



without added  $\text{Ca}^{2+}$  by mobilizing intracellular  $\text{Ca}^{2+}$ .<sup>25</sup> The production by the control cells that were treated with enflurane and isoflurane and stimulated with A23187 or A23187 + FMLP was essentially similar in the presence (table 2) or absence (table 3) of calcium, whereas production by the halothane-treated cells decreased to very low levels (about 5%) when  $\text{Ca}^{2+}$  was omitted from the medium. A difference between halothane and the two other anesthetics, enflurane and isoflurane, was apparent: the cells treated with the latter two anesthetics partially recovered from the inhibition by A23187 in the presence or absence of  $\text{Ca}^{2+}$ , whereas the cells treated with halothane did not recover from the inhibition when  $\text{Ca}^{2+}$  was absent.

#### EFFECTS OF ANESTHETICS ON MOBILIZATION OF INTRACELLULAR CALCIUM

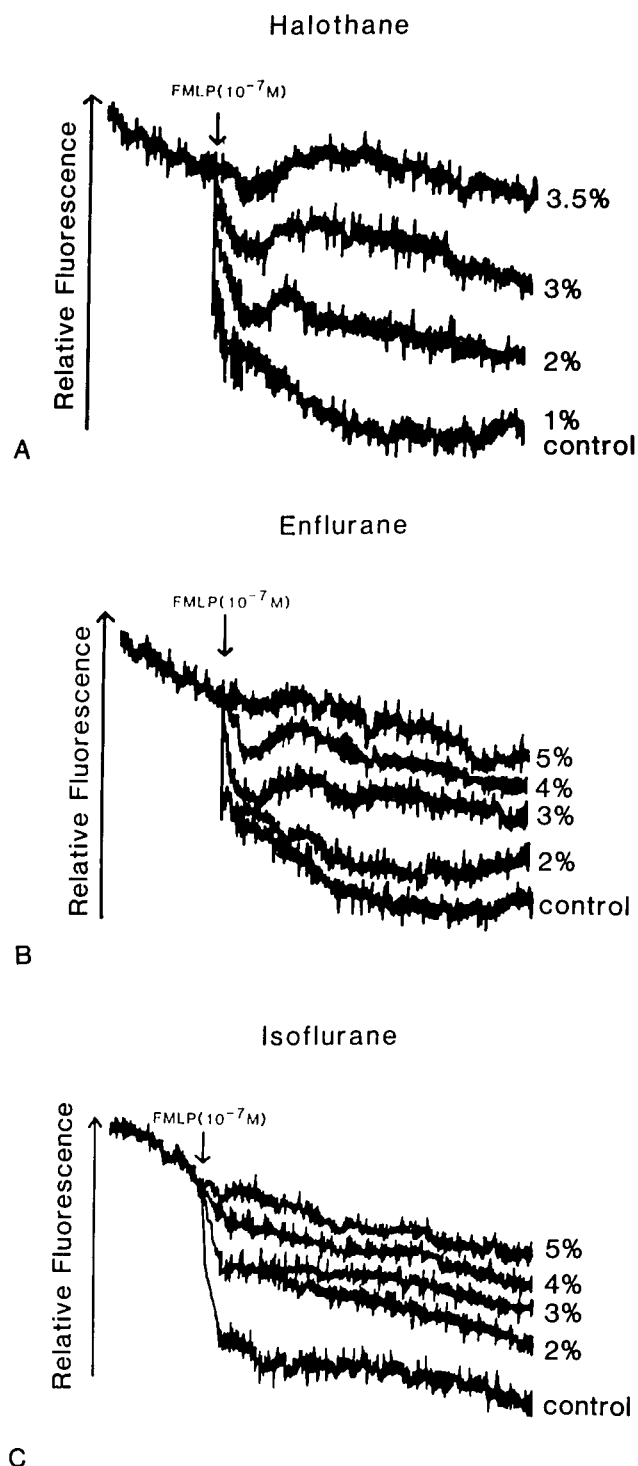
Intracellular free  $\text{Ca}^{2+}$ ,  $[\text{Ca}^{2+}]_i$ , monitored by quin-2 in the presence or absence of 1 mM  $\text{Ca}^{2+}$  is shown in figure 3. In the presence of 1 mM  $\text{Ca}^{2+}$ , the resting level of  $[\text{Ca}^{2+}]_i$  was about  $111 \pm 6$  nM (mean  $\pm$  SEM,  $n = 15$ ) and it increased to about  $609 \pm 27$  nM (mean  $\pm$  SEM,  $n = 15$ ) on stimulation with  $10^{-7}$  M FMLP, whereas in the absence of  $\text{Ca}^{2+}$  in the medium the former was about  $99 \pm 6$  nM (mean  $\pm$  SEM,  $n = 11$ ) and the latter was about

$195 \pm 9$  nM (mean  $\pm$  SEM,  $n = 11$ ). Halothane, enflurane, and isoflurane inhibited the increase of  $[\text{Ca}^{2+}]_i$  dose dependently both in the presence or absence of extracellular  $\text{Ca}^{2+}$ , indicating that the anesthetics inhibit both  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  release from the intracellular storage sites. A decrease of the level of  $[\text{Ca}^{2+}]_i$  to lower than that of the resting states on FMLP stimulation was observed when the cells were pretreated with higher concentrations of anesthetics, indicating that  $\text{Ca}^{2+}$  efflux becomes dominant due to the inhibition of  $\text{Ca}^{2+}$  influx by the anesthetics.

The release of  $\text{Ca}^{2+}$  from the intracellular storage sites of neutrophils during stimulation with FMLP was indicated by the fluorescence change of chlortetracycline (fig. 4). The decrease of chlortetracycline fluorescence on stimulation with FMLP due to the release of  $\text{Ca}^{2+}$  from the intracellular hydrophobic environment was reduced by an increase in the concentrations of anesthetics and disappeared when the cells were preincubated with 3.5% halothane, 5% enflurane, and 5% isoflurane. These results give further confirmation that mobilization of  $\text{Ca}^{2+}$  from the storage sites is also inhibited by the anesthetics.

#### Discussion

A decrease in neutrophil functions, such as chemotaxis, phagocytosis, and killing of microbes after anesthesia and

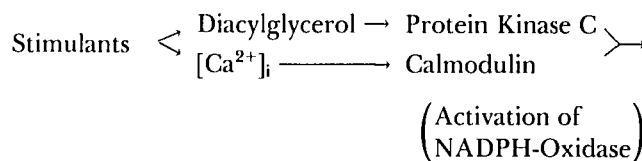


FIGS. 4A–C. Effects of the volatile anesthetics on the release of membrane-bound  $\text{Ca}^{2+}$ . Illustrated changes in chlortetracycline fluorescence of the cells on stimulation with  $10^{-7}$  M FMLP. Figures show typical patterns obtained from three experiments.

operation has been reported.<sup>15–17,26–28</sup> Recently, Welch has shown that commonly used general anesthetic agents such as halothane and enflurane exert potential adverse

effects on bacterial killing by neutrophils.<sup>14,17</sup> Microbicidal activity of neutrophils is suggested to be related to an increase in oxidative metabolism, the “respiratory burst.” Patients with chronic granulomatous disease (CGD), whose neutrophils lack the enzyme system for the generation of active oxygens, have recurrent infections.<sup>29</sup>

It has been reported by Welch and Zaccari<sup>13</sup> that *in vitro* exposure of neutrophils to 2 and 3% halothane resulted in 13 and 40% inhibition, respectively, of luminol-dependent chemiluminescence (LDCL) response in neutrophils stimulated with the opsonized zymosan or phorbol myristate acetate (PMA). He also observed a significant inhibition of the opsonized zymosan-stimulated LDCL response after exposure to 2% enflurane.<sup>14</sup> The mechanism of the impairment of oxidative metabolism by the anesthetics, however, has not been elucidated. We studied the effects of volatile anesthetics by determining superoxide generation because a probable first step in the respiratory burst is the formation of superoxide anions by the reduction of molecular oxygens in the NADPH-dependent oxidase system. The LDCL response represents not only superoxide generation but also several other reactions, which makes the analyses complicated.<sup>30</sup> Although the process of the activation of superoxide release in human neutrophils has not yet been clarified, a pathway currently considered responsible for the superoxide release by stimulation is as follows,



$\text{Ca}^{2+}$  is known to be essential in the induction of superoxide production in neutrophils stimulated by FMLP,<sup>7</sup> and both intracellular and extracellular  $\text{Ca}^{2+}$  are necessary to get full activity.<sup>7,8,31</sup> Inositol 1,4,5-trisphosphate, which is produced by the breakdown of phosphatidylinositol 4,5-bisphosphate is considered to regulate the release of calcium from the intracellular storage sites and increase the permeability of the plasma membrane to calcium.<sup>32</sup> The involvement of calmodulin and  $\text{Ca}^{2+}$ -sensitive, diacylglycerol-activating protein kinase C in the induction of superoxide release has been reported.<sup>33,34</sup> The present observations that the three volatile anesthetics caused the decrease of both  $\text{Ca}^{2+}$  influx and the release of  $\text{Ca}^{2+}$  from intracellular storage sites indicate that the inhibition of intracellular  $\text{Ca}^{2+}$  mobilization serves as one of the mechanisms for the anesthetics to inhibit the superoxide release. Other possibilities are as follows: 1) decrease in FMLP binding to the cells; 2) a direct inhibition of the NADPH oxidase; 3) effect on the protein kinase C<sup>33</sup>; 4) effect on calmodulin-dependent reaction, which seems to be involved in the activation of superoxide release<sup>34</sup>; and 5)

effect on fusion of granule membrane with plasma membrane, which is suggested to be an essential step in the activation of the neutrophil respiratory burst.<sup>35,36</sup> Our results may rule out the first two possibilities because the anesthetics did not inhibit either the binding of FML[<sup>3</sup>H]P to the neutrophils or the NADPH oxidase activity in the phagocytic vesicles but did not rule out the third, fourth, and fifth possibilities. Complete recovery of the superoxide-releasing activity could not be obtained (tables 2 and 3), probably because some additional mechanism other than Ca<sup>2+</sup> mobilization may be responsible for the inhibition. In addition, our results indicate that halothane seems to inhibit both Ca<sup>2+</sup> influx and Ca<sup>2+</sup> release from intracellular storage sites stronger than the other two anesthetics, enflurane and isoflurane: the recovery of superoxide production in the presence of extracellular Ca<sup>2+</sup> was much more pronounced with halothane (table 2), and no recovery was observed with halothane in the absence of extracellular Ca<sup>2+</sup> (table 3). The potency of the anesthetics as far as their effects on superoxide release and neutrophil Ca<sup>2+</sup> kinetics was approximately the same as their anesthetic potency.

General anesthetics are known to decrease movement of Ca<sup>2+</sup> across the vascular membranes and intracellularly.<sup>37</sup> Furthermore, Korenaga *et al.*<sup>38</sup> have reported that halothane has complex effects on the Ca<sup>2+</sup> economy: this agent releases Ca<sup>2+</sup> from the storage sites in the initial stage of the drug action and inactivates or reduces free Ca<sup>2+</sup> in the cytoplasm of the smooth muscle cells in the second stage. Malinconico *et al.*<sup>39</sup> have suggested that halothane affects myocardial beating intensity through its ability to disrupt fast calcium uptake. Thus, anesthetics appears to affect Ca<sup>2+</sup> metabolism in various cell types.

In conclusion, the parallel effects of the three volatile anesthetics on superoxide release in human neutrophils induced by FMLP and on the mobilization of intracellular calcium suggest that the inhibition of calcium mobilization is one of the mechanisms for the decrease in superoxide formation by neutrophils stimulated with FMLP.

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