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Calcium Ionophore-Stimulated Chemiluminescence (CL) from Human Granulocytes: Evidence that A23187-Induced Chemiluminescence Originates from Arachidonic Acid Metabolism

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INTRODUCTION

In our original study, nonsteroidal antiinflammatory drugs (NSAID's) were shown to inhibit chemiluminescence (CL) from opsonized-zymosan-stimulated-human granulocytes (*17*). Our secondary study included the chemotactic peptide FMLP as the stimulant of granulocytes and similar conclusions regarding NSAID's were reached (*18*). In neither study was the source of chemiluminescence entirely clear nor, for that matter, has it ever been clear in the myriad publications concerning cellular CL. It was postulated that the CL produced might originate from one or two sources, either myeloperoxidase-chloride ion-hydrogen peroxide (oxidative burst) or arachidonate metabolism (*18*).

Arachidonate metabolism can be stimulated in human neutrophils by the addition of a calcium ionophore, A23187, in the presence of calcium (*3*). Such stimulation has been shown to greatly increase the production of slow reacting substances (leukotrienes) in an undegraded fashion (*9*). Stimulation of neutrophils with A23187 is different from zymosan or FMLP stimulation because it is relatively insensitive to azide (*19*).

It was the purpose of this investigation to investigate the origin of CL from nonreceptor, A23187-stimulated granulocytes and compare these findings with CL stimulation from FMLP, a known receptor stimulant. To accomplish this task we studied A23187 granulocyte CL with the potent calmodulin inhibitor R24571 (*15*). A potent inhibiton of CL with R24571 might show calcium-calmodulin dependency. We studied the effects of various nonsteroidal antiinflamatory drugs on ionophore CL with the idea that drugs that block either or both cyclooxygenase or lipoxy-

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genase pathways should help clarify the possible origin of CL. Because quinacrine is known to inhibit the activation of phospholipase A_2, an enzyme important in the release of arachidonate from membrane phospholipids, it should inhibit if A23187 produces free arachidonate involved in the production of CL. Since both lipoxygenase (*10*) and cyclooxygenase-peroxidase (*21*) have been shown to produce CL this would be a logical scenario.

Since many stimulants of CL from human granulocytes produce CL by activating the oxidative burst (*16*) after binding to a receptor, it would be important to investigate oxidative burst-related phenomena, e.g., oxygen consumption, production of superoxide, production of hydrogen peroxide, and membrane depolarization. In the following data we suggest that A23187 (a nonreceptor stimulant) produces CL without activating the oxidative burst. This could explain why the leukotrienes so produced are not degraded by oxidative burst metabolites (*9*).

MATERIALS AND METHODS

Isolation of cells. For the chemiluminescence assays, granulocytes were obtained from human blood and partially purified by dextran settling as described previously (*14*). Briefly, blood was drawn from human volunteers and collected into tubes containing anticoagulant (EDTA). Blood was mixed (3 ml blood:1 ml buffer) with Hepes-buffered medium (145 m*M* Nacl, 5 m*M* KCl, 10 m*M* Na-Hepes, and 5.5 m*M* glucose; pH 7.4) containing 6 mg of dextran/ml (MW = 100,000–200,000) and 10 mg of gelatin/ml. This suspension was drawn into syringes and stored tip up for 1 hr at 37°C go allow the red blood cells to settle. After settling, the supernatant solution was removed and centrifuged at 200*g* for 5 min. After centrifugation, the supernatant solution was removed and the cell pellet containing granulocytes was resuspended in Hepes-buffered medium containing 1 m*M* calcium chloride. The number of granulocytes in this preparation was determined under a light microscope using a hemocytometer.

For measurement of oxygen consumption, superoxide release, hydrogen peroxide release, and membrane potential, granulocytes were partially purified by dextran settling as described above and then further purified by centrifugal elutriation as described previously (*7*). Briefly, partially purified granulocytes were infused into an elutriator (Beckman Model J-21 centrifuge and Model JE-6 elutriation rotor, Beckman Instrument Co., Fullerton, Calif.) at a flow rate of 13 ml/min and a rotor speed of 2000 rpm. A total of 200 ml of Hepes buffer solution was infused at these conditions to remove contaminating red blood cells and lymphocytes. Granulocytes (approximately 95% pure) were then collected at a flow rate of 41 ml/min and a rotor speed of 2000 rpm. These cells were concentrated by centrifugation at 1000*g* for 5 min and the cell pellet re-

suspended in Hepes-buffered medium containing 1 m*M* calcium chloride. The number of granulocytes in this preparation was determined using an electronic cell counter (Coulter Model 2B, Coulter Instrument Co., Hialeah, Fla.).

Chemiluminescence. Chemiluminescence was measured using a liquid scintillation counter (Packard Model 2002) set in the out-of-coincidence mode as described previously (*14*). Briefly, granulocytes (1,000,000) were suspended in 5 ml of Hepes-buffered medium containing 1 m*M* calcium chloride and 0.01 μ*M* luminol and preincubated for 10 min at 37°C. At zero time 2 μ*M* A23187, a calcium ionophore, or 2 μ*M* FMLP, a chemotactic factor, was added to activate the cells and the chemiluminescence response was monitored with a liquid scintillation counter at various times. When the effects of inhibitors were studied, drugs were added during the preincubation period. The addition of stimulant, A23187 or FMLP, initiates the reaction.

Oxygen consumption. Oxygen consumption was measured with a Gilson K-IC oxygraph equipped with a Clark electrode (Gilson Medical Electronics, Middleton, Wisc.) as described previously (*4*). Granulocytes (6,500,000 cells) were suspended in 1.7 ml of Hepes-buffered medium containing 1 m*M* calcium chloride and transferred to a temperature-controlled chamber (37°C) equipped with a stirrer and resting oxygen consumption measured for 15 min. After this time, cells were activated with 2.0 μ*M* A23187 or 2.0 μ*M* FMLP and oxygen consumption measured for an additional time.

Superoxide anion release. Superoxide production was measured by monitoring cytochrome *c* reduction spectrophotometrically at 550 nm (*1*). Granulocytes (10,000,000 cells) were suspended in 2.5 ml of Hepes-buffered medium containing 1 m*M* calcium chloride and 0.12 m*M* cytochrome *c*. Resting superoxide release was monitored against a blank using an Aminco spectrophotometer Model DW-2A equipped with a stirrer and temperature controlled at 37°C (Silver Springs, Md.). Cells were activated with A23187 (2.0 μ*M*) or FMLP (2.0 μ*M*) and cytochrome *c* reduction monitored continuously with time.

Hydrogen peroxide release. Hydrogen peroxide release was measured by monitoring the fluorescence of scopoletin (*11*). Granulocytes (10,000,000 cells) were suspended in 2.5 ml of Hepes-buffered medium containing 1 m*M* calcium chloride, 3 μl of 2 m*M* scopoletin, and 10 μl of 10 mg/ml horseradish peroxidase (Sigma, 165 pur units/mg). Resting hydrogen peroxide release was monitored at 22°C with an excitation wavelength of 350 nm and an emission wavelength of 460 nm using a fluorometer equipped with a stirrer (Model MPE-3L, Perkin–Elmer Corp., Norwalk, Conn.). Cells were activated with A23187 (2.0 μ*M*(or FMLP (2.0 μ*M*) and fluorescence monitored continuously with time.

Membrane potential. Membrane potential was measured at 22°C using the fluorescent probe di-S-C_3 (*5*) as described previously (*10*). Granulocytes (23,000,000 cells) were suspended in 3 ml of Hepes-buffered medium containing 0.66 μg/ml di-S-C_3 (*5*). Fluorescence was measured at 22°C with an excitation wavelength of 622 nm and an emission wavelength of 665 nm using a fluorometer equipped with a stirrer (Model MPE-3L, Perkin–Elmer Corp.). After a resting level was attained, A23187 (2.0 μ*M*) or FMLP (2.0 μ*M*) was added to activate the cells and the fluorescence response was monitored continuously with time. In this system, an increase in fluorescence indicates membrane depolarization while hyperpolarization results in a decrease in fluorescence.

Chemicals. All chemicals were obtained from commercial sources and were highest grade obtainable. A23187 (calcium ionophore was purchased from Calbiochem, La Jolla, Calif., and FMLP (formylmethionyl leucyl phenylalanine), scopoletin, horseradish peroxidase, and cytochrome *c* were purchased from Sigma Chemical Company, St. Louis, Mo. Nonsteroidal antiinflammatory drugs including MK (+) and (−) 830 were kind gifts from Dr. T. Y. Shen, Vice-president of membrane and arthritis research of Merck Sharp & Dohme, Rahway, N.J. BW755C was a kind gift of Dr. John Vane of Burroughs-Welcome, Beckingham, Kent, England. Quinacrine was obtained from Winthrop-Sterling, New York, N.Y. R24571 was purchased from Janssen Pharmaceuticals, Beerse, Belgium.

Stock solutions. Stock solutions of the various nonsteroidal drugs, R24571, BW755C, and quinacrine were dissolved at 0.1 m*M* in 1 ml of dimethyl sulfoxide (DMSO) and brought up to 100 ml volume with physiological Hepes-buffered medium containing 1 m*M* calcium chloride (pH 7.4). If further dilution was necessary, more Hepes buffer was added. No effect from the DMSO was noted.

Data presentation. Due to the quantitative variation from person to person, data from single experiments are presented, and statistical analysis was not performed. However, these data are typical of at least 3–5 experiments for each assay.

RESULTS AND DISCUSSION

Recent studies have suggested that calmodulin is involved in the oxidative burst of granulocytes (*13, 16*). Data presented in Fig. 1 indicate that R24571, an inhibitor of calmodulin (*15*), can decrease the chemiluminescence induced by A23187 which Figs. 6–9 reveal to be unrelated to the oxidative burst. Although this inhibition of chemiluminescence is a consistent finding, the ED50 varies from 10–1.0 μ*M* depending on the donor. The reason for the variability between persons is unclear.

MK830 is an antiinflammatory drug. It is known that *in vivo* the (+) isomer is more potent than the (−) isomer (*18*). Data concerning the

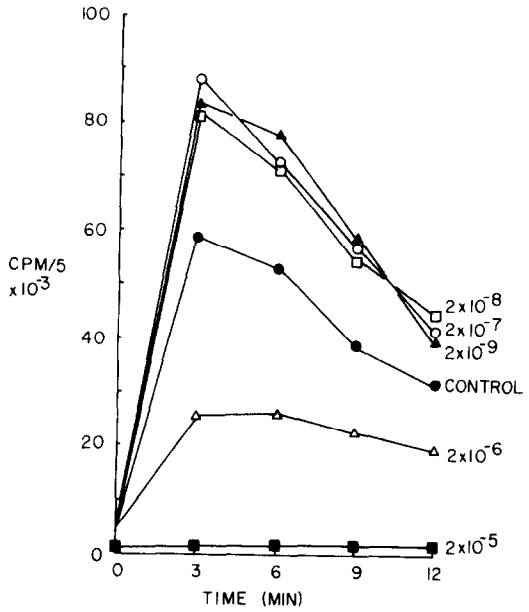

FIG. 1. Chemiluminescence of human granulocytes stimulated by A23187 (2 μM) in the presence and absence of R24571 (calmodulin inhibitor). The dose varies from 20 μM to 2.0 nM. Chemiluminescence blank at zero time was 5000 counts/0.2 min. Luminol concentration in all curves was 0.01 μM.

effects of these isomers on A23187-induced chemiluminescence by granulocytes is shown in Fig. 2. Note that the (+) isomer of MK830 is a more potent inhibitor of chemiluminescence than the (−) isomer. Therefore, there is a correlation between the actions of MK830 on chemiluminescence and inflammation. Apparently these actions of MK830 are mediated by inhibition of the cyclooxygenase pathway for arachidonic acid metabolism (*18*).

The inhibitory effects of various nonsteroidal drugs on A23187-induced chemiluminescence from human granulocytes are shown in Fig. 3. The sequence of potency is BW755C > sulindac sulfide > butazolidine > sulindac sulfone which is similar to indomethacin. It is of interest that inhibition of the cyclooxygenase pathway alone, as with indomethacin (*6*), is less effective than inhibition of both the lipoxygenase and cyclooxygenase pathways as with BW755C (*6*). It is also of interest that BW755C blocks almost all of the A23187-induced chemiluminescence, i.e., arachidonic acid metabolism accounts for almost all of the calcium ionophore-stimulated granulocyte response.

Quinacrine is an inhibitor of phospholipase A_2 (*2*). Therefore, it does not allow the production of arachidonate from phospholipids. If arachi-

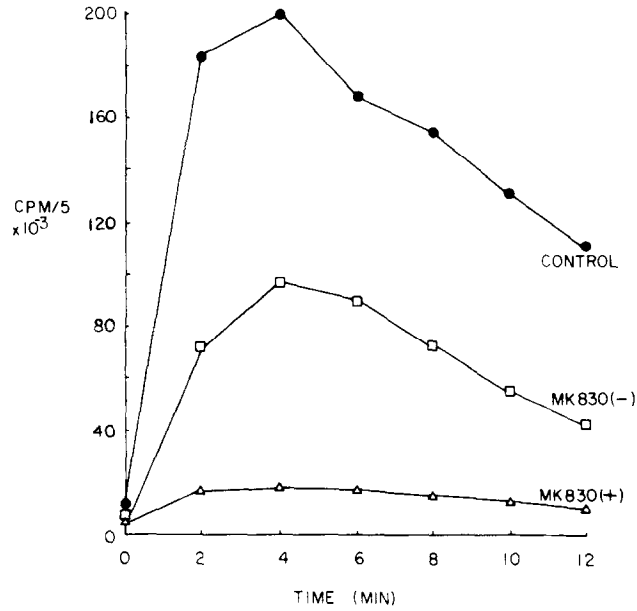

FIG. 2. Chemiluminescence of human granulocytes (1,000,000) stimulated by 23187 (2 μM) in the presence and absence of (+) and (−) MK830 (20 μM). Chemiluminescence blank at zero time was 12000 counts/0.2 min. Luminol concentration in all curves was 0.01 μM.

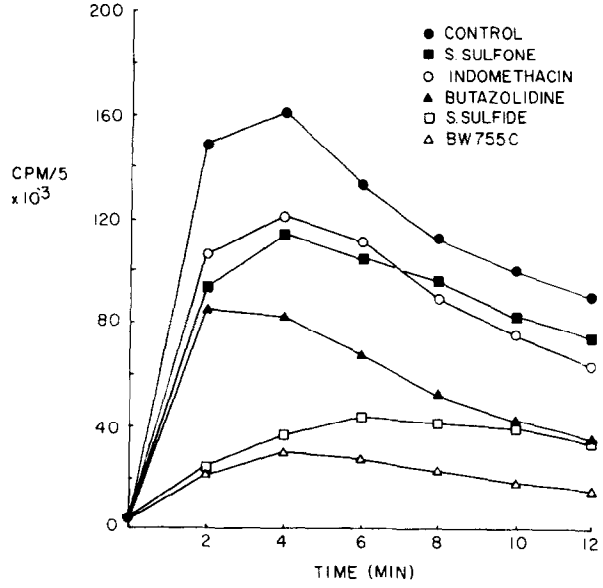

FIG. 3. Nonsteroidal drug effects (20 μM) on chemiluminescence of human granulocytes (1,000,000) stimulated by A23187 (2.0 μM). Chemiluminescence blank at zero time was 3000 counts/0.2 min. Luminol concentration for all curves was 0.01 μM.

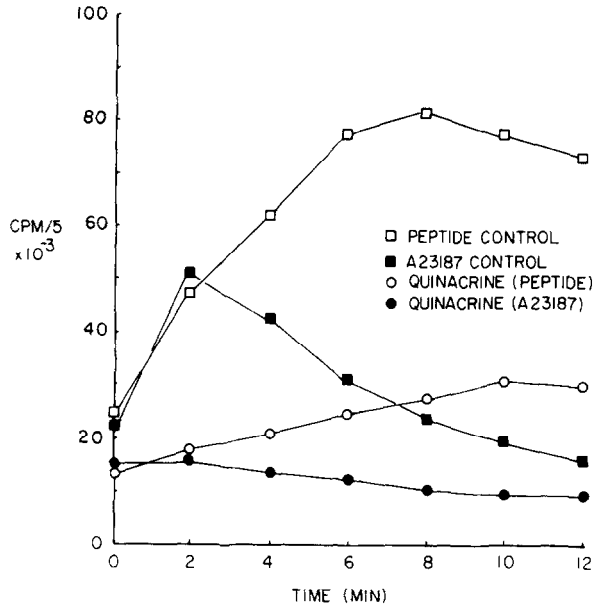

FIG. 4. Effect of quinacrine (0.1 *mM*) on chemiluminescence of human granulocytes stimulated by A23187 (2.0 μ*M*) or FMLP (2.0 μ*M*). Chemiluminescence blank at zero time was 22,000 counts/0.2 min for A23187 and 25,000 counts/0.2 min for FMLP. Luminol concentration for all curves was 0.01 μ*M*.

donic acid metabolism is the main source of A23187-induced chemiluminescence, then quinacrine should be a potent inhibitor. Figure 4 shows that quinacrine completely blocks A23187-stimulated chemiluminescence by human granulocytes. The ED50 for the inhibition is approximately 10 μ*M* quinacrine. In contrast, quinacrine only partially blocks chemiluminescence to FMLP. Therefore we suggest that while A23187-induced CL is almost totally arachidonate light; FMLP-stimulated CL has both an arachidonate and a nonarachidonate component. If this is the case, it may be possible to stimulate granulocytes successively with A23187 and then FMLP. These data are shown in Fig. 5. Granulocytes which respond to an initial addition of A23187 are still able to produce chemiluminescence in response to a second stimulant, i.e., FMLP. This secondary response to FMLP is smaller than the response to FMLP alone since only part of induced CL is nonarachidonate light. However, if FMLP is used as the primary stimulant, a secondary addition of A23187 is ineffective (data not shown). These data are consistent with the hypothesis that FMLP-induced light is due to both arachidonate metabolism and a second source while A23187-induced CL is essentially arachidonate light.

The next series of experiments were performed to determine if an oxidative burst could be the secondary source of FMLP-induced CL. Figure

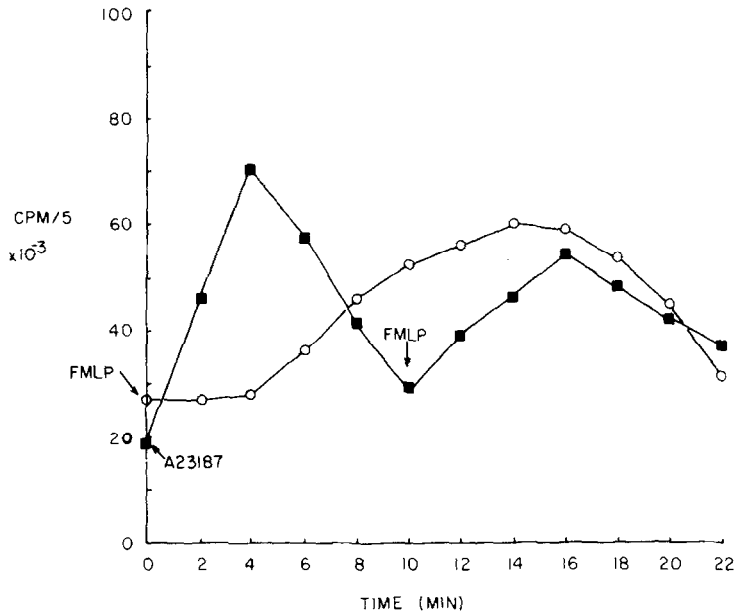

FIG. 5. Chemiluminescence of human granulocytes (1,000,000) stimulated by FMLP (2.0 μM) or A23187 (2.0 μM) and sequentially FMLP (2.0 μM). Chemiluminescence blank for A23187 at zero time was 19,000 and 28,000 counts/0.2 min. Luminol concentration for all curves was 0.01 μM.

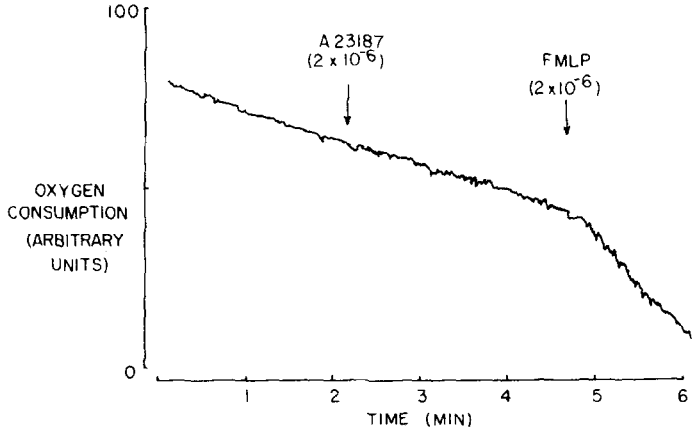

FIG. 6. The oxygen consumption of human granulocytes (6,500,000 cells/1.7 ml) vs time (min) measured: (1) in the absence of stimulant (initial slope, 0–2 min); (2) after A23187 (2.0 μM) was added (slope 2–4 min); and (3) after FMLP (2 μM) was added (slope 4.5–6 min).

6 shows that A23187 does not significantly enhance oxygen consumption by human granulocytes. However, subsequent addition of FMLP greatly enhances oxygen consumption. These data are consistent with measurement of superoxide release (Fig. 7). A23187 results in only a minor increase in superoxide release from human granulocytes. However, subsequent addition of FMLP greatly enhances the release of superoxide anion by human granulocytes. The effects of successive addition of A23187 and FMLP on hydrogen peroxide release by human granulocytes are shown in Fig. 8. As with oxygen consumption and superoxide release, A23187 results in little or no detectable hydrogen peroxide release. Therefore, A23187 does not initiate a major oxidative burst in human granulocytes while FMLP does. Thus, oxygen radical light may be the nonarachidonate component of FMLP-induced CL.

The differences in granulocyte stimulation by A23187 and FMLP are also evident by measuring the effects of these stimulants on membrane potential. These data are shown in Fig. 9. Initial stimulation of granulocytes by A23187 results in a small depolarization. This depolarization is the result of an increase in the membrane permeability to calcium (*8*).

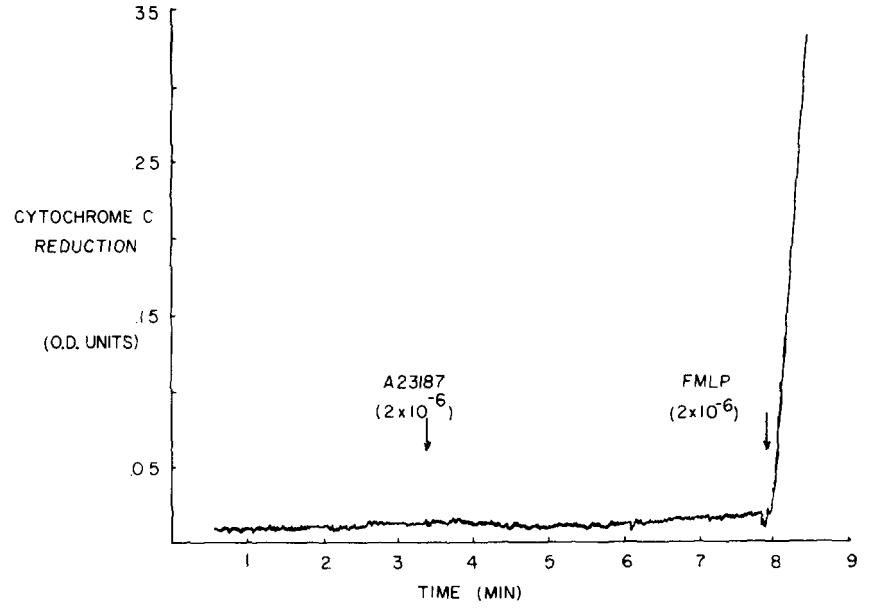

FIG. 7. Superoxide production by measurement of the reduction at 550 nm of cytochrome *c* (0.12 m*M*) in human granulocytes (10,000,000/2.5 ml) in absence and presence of stimulant. Cells were added and cytochrome *c* reduction measured from 1–3.25 min; A23187 (2.0 μM) added from 3.25–7.75 min; and FMLP (2.0 μM) added from 7.75–8.5 min.

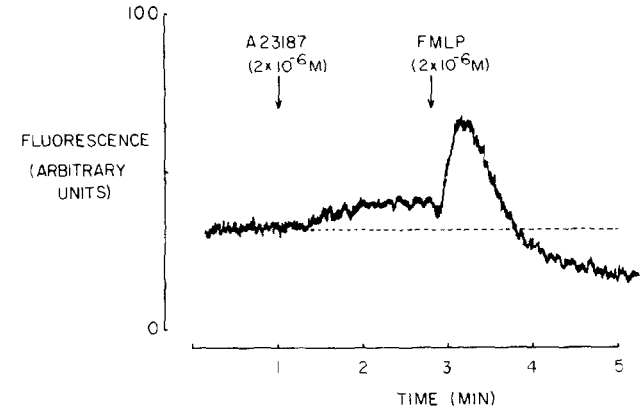

FIG. 8. Measurement of the generation of hydrogen peroxide in human granulocytes using the decrease of the fluorescence of scopoletin in the presence of horseradish peroxidase. The fluorescence of cells is measured (1) from 1–2 min for equilibration; (2) from 2–3.5 min when A23187 (2.0 μM) was added and; (3) from 3.75–4.25 when FMLP (2.0 μM) was added.

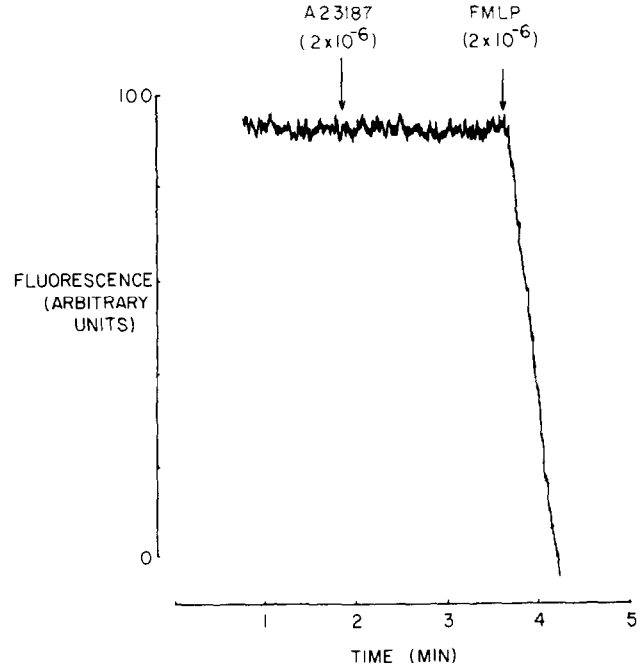

FIG. 9. Measurement of membrane depolarization of human granulocytes (23,000,000 cells/3 ml) vs time using the dye di-S-C_3(5) (0.66/ml). Initial reaction is with cells and dye 0–1 min; then after addition of A23187 (2.0 μM) from 1–3 min; and after addition of FMLP (2.0 μM) from 3–5 min.

Subsequent addition of FMLP results in additional depolarization which is sodium dependent (*8*). Therefore, the effects of A23187 and FMLP on membrane potential are separable and distinct.

In conclusion, we propose that A23187-induced CL in human granulocytes is mainly due to the metabolism of arachidonic acid. Our evidence for this conclusion is the following:

(1) BW755C, an inhibitor of both lipoxygenase and cyclooxygenase pathways, completely blocks A23187-induced CL,

(2) quinacrine, an inhibitor of arachidonate production, completely blocks A23187-induced CL,

(3) A23187 does not induce an oxidative burst, and

(4) subsequent addition of FMLP to A23187-stimulated cells results in an oxidative burst and additional membrane depolarization and CL.

Furthermore, it is suggested from the data that the FMLP-induced CL has an arachidonate and an oxygen radical component. This is further indicated because myeloperoxidase-deficient individuals respond to FMLP by producing CL and therefore indicating at least a second source of the production of light (*12*), i.e., arachidonate light.

SUMMARY

A23187 (calcium ionophore)-induced-granulocyte chemiluminescence is

(1) dependent on calmodulin (calcium binding protein) but independent of the oxidative burst,

(2) mainly due to arachidonate release from cellular phospholipids and subsequent metabolism,

(3) is not caused by the oxidative burst that accompanies various receptor stimulants.

Sequential addition of A23187 followed by FMLP produces two different depolarizations of the membrane and two sequential peaks of chemiluminescence. It may be suggested that the origin of A23187 CL is mainly arachidonate metabolism while FMLP CL has at least two origins; oxidative burst and arachidonate metabolism.

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