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GOLD COMPLEXES AND ACTIVATION OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

DISSOCIATION OF CHANGES IN MEMBRANE POTENTIAL AND OXIDATIVE BURST

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Abstract—The effects of the gold compounds on the alteration of membrane potential of polymorphonuclear leukocytes (PMN) in response to various stimulants have been compared with their effects on the oxidative burst. The present studies have shown that gold complexes [auranofin (AF), aurothiomalate (Autm), aurocyanide ($Au(CN)_2^-$)] have contrasting effects on the membrane potential of 3,3'-dipentyloxacarbocyanine [di-O-C_5(3)] loaded PMN. $Au(CN)_2^-$ at concentrations which inhibit the oxidative burst of PMN did not affect the membrane depolarization after activation of PMN by phorbol myristate acetate (PMA) and *N*-formyl-methionyl-leucyl phenylalanine (FMLP); Autm slightly stimulated the oxidative burst but had no effect on the depolarization of PMN. In contrast, AF inhibited the depolarization of stimulated PMN to an extent depending upon the concentration of AF, the time of preincubation and the stimulus. The membrane depolarization of PMN caused by PMA, FMLP and concanavalin A (ConA) was inhibited by AF (5 μM) but the depolarization induced by calcium ionophore (A23187) was not affected. AF at the same conditions inhibits the oxidative burst of PMN induced by all these single stimuli including the calcium ionophore. Dissociation of membrane depolarization and superoxide generation caused by AF was also seen in PMN activated by two stimuli. AF (5 μM) had little initial inhibitory effect on the oxidative burst of PMN stimulated by combinations of PMA and ConA or PMA and FMLP whereas it almost totally blocked the depolarization caused by these combinations. Preincubation of cells with 5 μM AF for less than 5 min prior to the addition of PMA allowed membrane depolarization which was followed rapidly by repolarization. None of the gold complexes studied had any effect on the resting membrane potential of PMN.

Gold complexes such as auranofin (AF‖) and sodium aurothiomalate (Autm) are useful in the treatment of rheumatoid arthritis although their mechanism of action is unclear [1, 2]. The effects of AF and Autm on the functions of polymorphonuclear leukocytes (PMN), which are important cells in the inflammatory process, have been studied extensively *in vitro* in an attempt to explain the cellular and clinical effects of these drugs [3–7]. AF *in vitro* at pharmacological concentrations, inhibits many functions of PMN including the oxidative burst [8–10]. Autm has variable actions on the oxidative burst of PMN; it may be inactive or cause a slight stimulation or a moderate inhibition, depending on the source of the drug [11] and, possibly, the experimental conditions. Aurocyanide [$Au(CN)_2^-$] has been suggested to be formed *in vivo* from Autm and the cyanide generated from thiocyanate by activated PMN [12]. In serum-free medium, *in vitro*, $Au(CN)_2^-$ is a potent inhibitor of PMN functions [13] and may be the therapeutically active gold complex [14].

Although changes in membrane potential are often associated with activation of the PMN respiratory burst, there has been little investigation of the effect of gold complexes on the membrane potential of PMN [9]. In the present studies, we examined the effect of AF, Autm and $Au(CN)_2^-$ on the depolarization and the respiratory burst of PMN activated by addition of single or multiple stimuli. We have used the fluorescent carbocyanine probe [3,3-dipentyloxacarbocyanine; di-O-C_5(3)] to monitor the polarization of PMN.

When di-O-C_5(3) is added to the cell suspension, it enters the cell and equilibrates with the extracellular concentrations to a level depending on the membrane potential [15]. The fluorescence of this probe is enhanced intracellularly and hence its fluorescence can be used to monitor changes in the membrane potential of the PMN [16]. Stimulation of the normal human PMN by compounds such as *N*-formyl-methionyl-leucyl phenylalanine (FMLP), phorbol 12-myristate 13-acetate (PMA), concanavalin A (ConA) and the calcium ionophore (A23187) results in rapid depolarization. In the case of FMLP, this depolarization is followed by repolarization.

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‖ Abbreviations: AF, auranofin; Autm, sodium aurothiomalate; $Au(CN)^-_2$, aurocyanide; FMLP, *N*-formyl-methionyl-leucyl phenylalanine; PMA, phorbol 12-myristate 13-acetate; ConA, concanavalin A; A23187, calcium ionophore; di-O-C_5(3), 3,3'-dipentyloxacarbocyanine; PMN, polymorphonuclear leukocytes; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline without calcium and magnesium; PBSG, phosphate-buffered saline with calcium and magnesium containing 5.5 mM glucose.

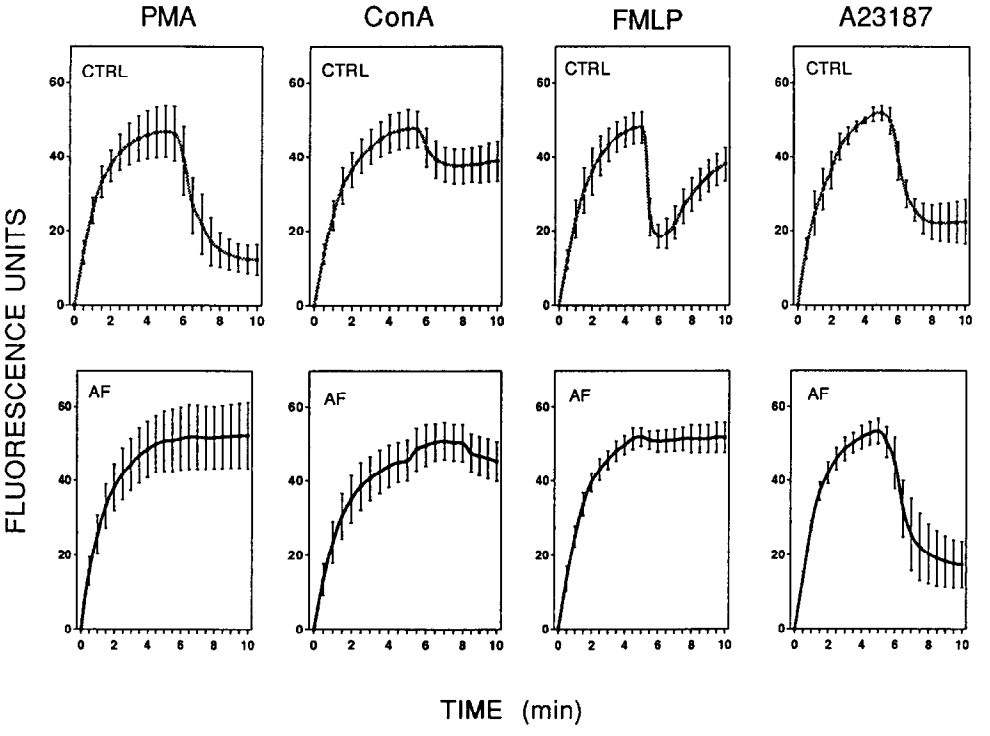

Fig. 1. The effect of various stimuli on the fluorescence of normal (upper panels) and AF-treated (lower panels) PMN loaded with di-O-C_5(3). Cells (2×10^5) and 5 μM AF (where appropriate) were added to buffer containing di-O-C_5(3) (50 nM) at time zero. Once the fluorescence stabilized (at 5 min) the stimulus was added. Data are presented as mean ± SD of 3–9 experiments.

Although the ionic basis of the membrane potential changes has not been fully elucidated, the depolarization induced by PMA, FMLP or ConA appears to be induced primarily due to an increase in sodium permeability and the resultant influx of sodium, while the depolarization in response to the calcium ionophore, A23187, is due to an influx of calcium to cytosol and has little sodium dependence [17]. The repolarization of the membrane potential in FMLP-activated PMN is due to potassium efflux from the cytosol [15] and is inhibited completely by inhibitors of the plasma membrane Na^+,K^+-ATPase [18] which is a primary contributor to the potassium distribution in the cells [19].

We reported recently the effect of gold complexes on several functions of PMN (glucose transport, hexose monophosphate shunt activity, superoxide generation and NADPH oxidase) [10, 11, 13] and have now extended these studies to an examination of the effect of these gold complexes on the membrane potential changes and oxidative burst of PMN after single and multiple stimulation in order to obtain more detailed information on the mechanisms by which gold complexes, particularly AF, affect the function of PMN.

MATERIALS AND METHODS

Materials. AF was obtained from Smith, Kline and French Laboratories (Sydney, Australia); Autm from May and Baker (Dagenham, U.K.) and from the Sigma Chemical Co. (St Louis, MO, U.S.A.); potassium aurocyanide from Enghelhard Ind. (Sydney, Australia); di-O-C_5(3) from Molecular Probe (Junction City, OR, U.S.A.); stimulants (PMA, FMLP, ConA and A23187) from Sigma; phosphate-buffered saline (PBS with and without calcium and magnesium) and heat inactivated foetal calf serum from Flow Laboratories (Sydney, Australia); Ficoll-Paque and Dextran T-500 from Pharmacia AB (Uppsala, Sweden). All other chemicals were of reagent grade. Stock solutions of PMA (1 mg/mL), calcium ionophore A23187 (5 mg/mL) and FMLP (1 mg/mL) were prepared in dimethyl sulphoxide (DMSO) and stored at −20°. AF (1 mg/mL) was prepared in DMSO then diluted in PBS to 0.1–100 μM and stored at 4°. Stock solutions of the fluorescence probe di-O-C_5(3) were made in DMSO and kept in the dark at 4°. Working solutions of probe were prepared by dilution (1:20) into PBS and were discarded after 5 hr of use. Solutions of Autm and $KAu(CN)_2$ were made up in PBS and stored at 4°. ConA solutions were prepared freshly in PBS before use and kept at room temperature.

Subjects. Studies were conducted on the PMN of healthy volunteers. The study was approved by the ethics committee of the Prince of Wales Hospital.

Isolation of PMN. PMN were isolated from 10 mL samples of heparinized blood from normal volunteers by standard Hypaque/Ficoll gradient techniques

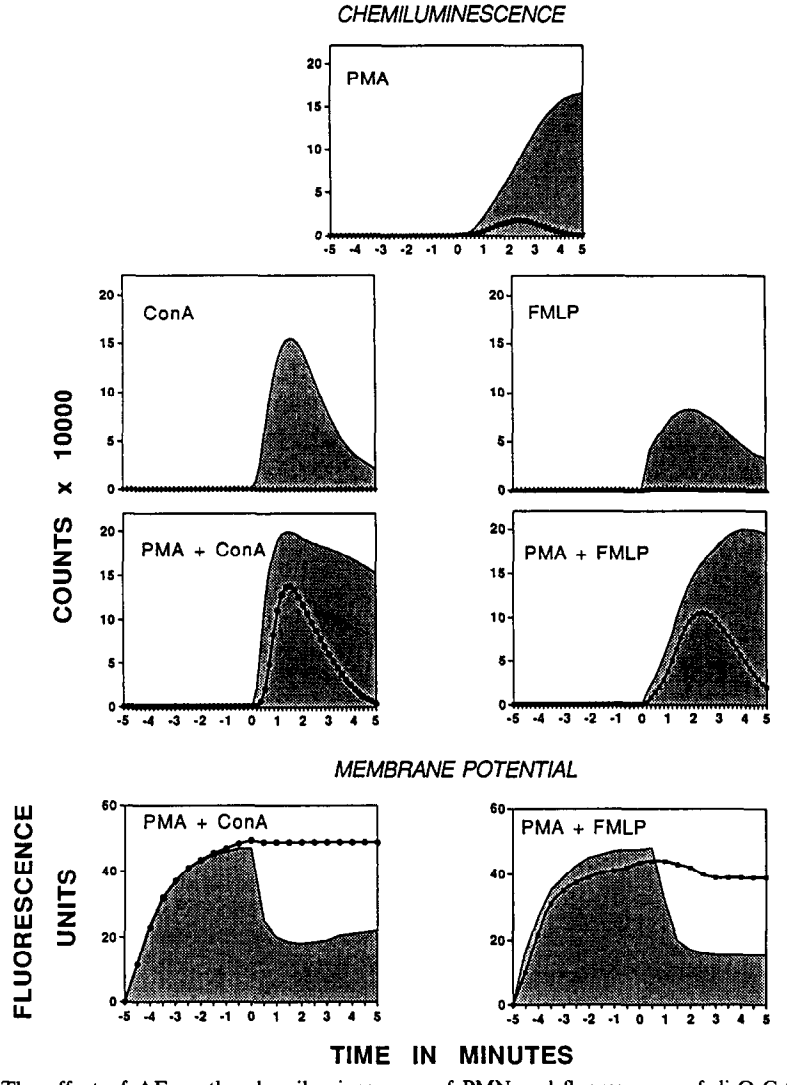

Fig. 2. The effect of AF on the chemiluminescence of PMN and fluorescence of di-O-C_5(3) loaded PMN activated by stimulants added singly and in combinations. Representative experiments are shown. Shaded areas indicate the response of cells in the absence of AF. In the chemiluminescence experiments cells were kept at 37° for 5 min in the absence (controls) or in the presence of 5 μM AF. Stimulants added at time zero were: PMA, ConA, FMLP, PMA + ConA and PMA + FMLP. Chemiluminescence was measured continuously for 10 min. In the fluorescence experiments, cells were exposed to di-O-C_5(3) and 5 μM AF (where appropriate) for 5 min at 37° and then stimulated by PMA, ConA and FMLP alone (see Fig. 1) or by combinations of stimuli: PMA + ConA and PMA + FMLP.

[20], followed by dextran sedimentation and hypotonic lysis of the remaining erythrocytes as described previously [13]. Cells were resuspended in PBS (with calcium and magnesium) containing 5.5 mmol/L glucose (PBSG) at a concentration of 2×10^6/mL, kept on ice and used within 5 hr. Cells stored for longer time exhibit age-related variability in depolarization to FMLP [15].

Determination of membrane potential changes. Changes in membrane potential were measured by using di-O-C_5(3) as described previously [21] with minor modifications. The fluorescence was measured with a luminescence spectrometer (Model LS 50, Perkin-Elmer), fitted with a magnetic stirrer and thermostatted cuvette holder (37°). The excitation and emisssion wavelengths were set at 460 and 512 nm, respectively. A volume of 2 mL PBSG was used in a 1 cm pathlength cuvette. Twenty microlitres of the working dye solution were added to this buffer, giving an initial dye concentration of 50 nM. A portion of dye was absorbed onto the cuvette and the stirrer, causing the fluorescence to decrease to a constant value within approximately 3 min. Once the fluorescence had stabilized, 200 μL of a suspension of PMN, prewarmed to 37°, was added to give a final concentration of 2×10^5 cells/mL. The temperature of the cuvette was maintained at 37°, and the cell suspension was maintained by a magnetic

stirrer (300 rpm). The uptake of dye by the PMN resulted in an increase in fluorescence. In control experiments (without the drug) the cells were incubated with the dye until the fluorescence level was approximately steady (after 5 min) at 37°. A stimulant was then added (50 μL), and the fluorescence was monitored continuously for approximately 10 min. In the experiments with gold complexes, the drugs were added to the cuvette either together with the cell suspensions or from 1 to 5 min prior to cell stimulation. The stimulants used were PMA (0.1 μg/mL), FMLP (5 μM), ConA (30 μg/mL) and A23187 (5 μM) or their combinations. The results are reported as relative fluorescence, which represents the ratio $F_2/F_1 \times 100$, where F_2 is the fluorescence measured after addition of the stimulant, and F_1 is the fluorescence of the PMN measured after equilibration with dye but before the addition of the stimulant.

To avoid interactions of gold complexes with plasma proteins, resulting in the reduction of their inhibitory effect on cells [9, 22], and because proteins also interfere with the fluorescence of the cyanine dye used to monitor the depolarization of the PMN [15], present experiments were conducted in a medium free of protein.

Chemiluminescence of PMN. Chemiluminescence was measured as described previously [10]. Briefly, PMN (2×10^5/mL) were preincubated with AF (0.1–5 μM), for 5 min at 37° before the addition of PMA (0.1 μg/mL), FMLP (5 μM), ConA (30 μg/mL) or their combinations. Concentrations of stimuli chosen were sufficient to elicit maximal chemiluminescence responses. The chemiluminescence was measured continuously at 37° in a Packard Picolite 6500 luminometer (modified to incorporate temperature control at 37°) for 5 min. The total chemiluminescence values were calculated.

PMN viability. This was assessed by the exclusion of trypan blue. Cell suspensions were mixed with an equal volume of 0.25% trypan blue in normal saline and the proportion of blue staining cells were determined.

Statistics. The significance of differences between treatments (among various sample means) was determined by one factor analysis of variance with repeated measures, while the Scheffé multiple comparison test, was used to examine differences between pairs of sample means whenever the ANOVA indicated that there was a significant difference. The Statview program (Brainpower, Inc., Calebases, CA, U.S.A.) was used.

RESULTS

The effect of stimuli on membrane potential of PMN

The addition of PMN to buffer containing di-O-C_5(3) resulted in a large increase in fluorescence, due to accumulation of di-O-C_5(3) in the cells. The final steady-state value of fluorescence reflects the resting membrane potential of PMN. The various stimulants decreased the fluorescence of the PMN suspension, consistent with depolarization of the cells, although the patterns of the fluorescence changes were different according to the stimulant used. In agreement with previous reports [21, 23], stimulation of di-O-C_5(3)-loaded PMN with PMA, A23187 and ConA all produced a rapid and sustained decrease in fluorescence while FMLP produced only a transient decrease in fluorescence followed rapidly by a rebound indicating repolarization of the cells (Fig. 1). The maximal decrease in fluorescence induced by PMA, A23187 and FMLP was much greater than that induced by ConA. The maximal changes in fluorescence (expressed as a percentage of the maximal fluorescence of the resting PMN), due to the stimulants were: PMA 75.8 ± 6.1 (N = 8), A23187 53.1 ± 8.3 (N = 6), FMLP 65.0 ± 4.7 (N = 7) and ConA 26.7 ± 6.3 (N = 8).

Effect of AF on membrane potential of PMN stimulated with PMA, FMLP, ConA and A23187

PMN were preincubated with 5 μM AF for 5 min at 37° prior to stimulation. As shown in Figs 1 and 2, AF did not affect the resting membrane potential of PMN but almost entirely inhibited the loss of fluorescence caused by the addition of PMA, ConA and FMLP. By contrast, 5 μM AF had no effect on the prompt depolarization response to A23187 (Fig. 1). Under the same conditions, AF inhibited the oxidative burst of the PMN after stimulation by all four compounds, including A23187, as shown by a marked inhibition of the lucigenin-dependent and luminol-dependent chemiluminescence [10].

Effect of AF on membrane potential changes and luminol-enhanced chemiluminescence of PMN stimulated by combinations of stimuli

While 5 μM AF markedly inhibited the luminol-dependent chemiluminescence after the single addition of PMA or ConA [10], it has a considerably lesser effect on chemiluminescence elicited by combinations of ConA and PMA. Following the simultaneous addition of these two stimuli, there was marked initial chemiluminescence, although there was later inhibition of the response. With the combination of stimulants in the absence of AF, the total chemiluminescence over 5 min was only slightly greater than the sum of that seen with PMA and ConA alone [21 ± 50% (N = 3)]. However, in the presence of 5 μM AF, the total chemiluminescence was very much greater than the corresponding sum produced by the individual stimulants [1310 ± 410% (N = 3)]. Similar results were seen when PMA and FMLP were used individually and in combination, i.e., the total chemiluminescence over 5 min was 16 ± 23% (N = 5) greater than the sum of that seen with PMA and FMLP alone in control cells and 209 ± 63% (N = 4) in AF-treated cells. Thus, the drug has a mild effect on the oxidative burst of PMN activated by the mixture of stimuli added simultaneously. Despite the substantial chemiluminescence seen with the combined stimulants, no significant depolarization of the PMN occurred in the presence of 5 μM AF (Fig. 2). Thus, it is apparent that in the presence of AF there is considerable dissociation between oxidative burst and membrane potential changes in PMN activated by a combination of certain stimuli.

Concentration- and time-dependent effects of AF on membrane potential of PMN stimulated with PMA

PMN were preincubated with varying con-

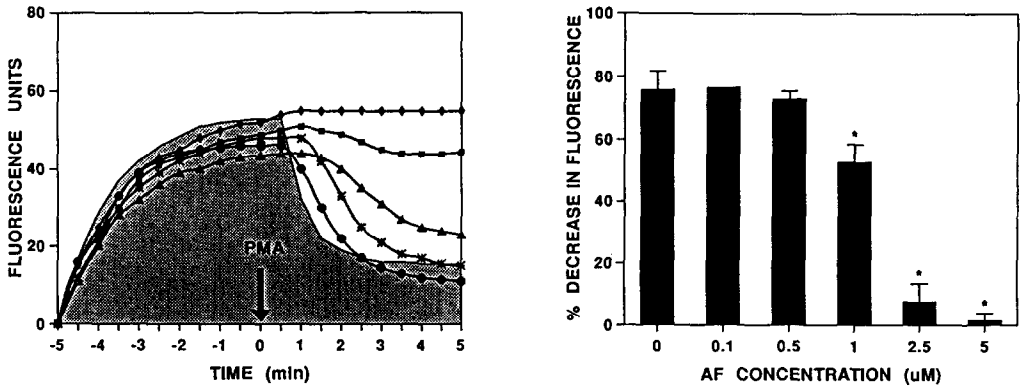

Fig. 3. The effect of different concentrations of AF on fluorescence of di-O-C_5(3)-loaded PMN. Experimental conditions are as described for Fig. 1. The left panel shows the data from representative experiment. Shaded areas indicate the response of cells in the absence of AF. The right panel shows the decrease in fluorescence of PMA-activated cells, expressed as a percentage of the maximal fluorescence of the resting cells. Data are presented as the mean ± SD of 3–8 experiments. Significant differences from control ($P < 0.5$) are shown by (*). Key: control (edge of the shaded area), 0.1 μM (●), 0.5 μM (*), 1 μM (▲), 2.5 μM (■), 5 μM (◆).

centrations of AF (0.1–5 μM) for 5 min at 37° prior to stimulation with PMA. At concentrations of 0.1 and 0.5 μM, AF had no significant effect on the membrane potential changes, whereas higher concentrations caused a dose-dependent inhibition of membrane depolarization (Fig. 3). By comparison, AF at a concentration of 0.5 μM markedly inhibited the luminol-dependent and lucigenin-dependent chemiluminescence after stimulation of the PMN by PMA [10].

A time-dependent effect of AF (5 μM) on membrane depolarization of PMN was demonstrated when AF was added to cells simultaneously with or from 1 to 5 min prior to PMA (Fig. 4). As noted previously, AF (5 μM) almost completely inhibited the depolarization when added 5 min before stimulation of cells by PMA. However, when PMA was added to cells preincubated with 5 μM AF for lesser periods, there was a smaller decrease in fluorescence, consistent with only partial depolarization, but the fluorescence recovered rapidly to some extent indicating repolarization (Fig. 4). Thus, PMA produced a biphasic response when the cells were incubated with AF (5 μM) for less than 5 min, i.e. depolarization which is followed by repolarization. This repolarization component is absent in control cells when PMA is used as the stimulant and was also absent in PMN preincubated with lower concentrations of AF for 5 min.

Effect of Autm and $Au(CN)_2^-$ on the membrane potential of PMN

Neither Autm (100 μM) nor $Au(CN)_2^-$ (50 μM) prevented the depolarization of PMN on addition of PMA (Fig. 5) or FMLP (results not presented). Neither gold complex had any effect on the resting membrane potential of the PMN.

DISCUSSION

The present experiments show that the three gold complexes studied, AF, Autm and $Au(CN)_2^-$, had different effects on the depolarization of PMN. AF was studied in most detail because of its much greater effects on both the oxidative burst and on depolarization.

There may be two separable effects of AF on the oxidative burst and depolarization of PMN elicited by PMA, FMLP and ConA. At low concentrations (up to about 0.5 μM), depolarization is not affected (Fig. 3) but there is a late inhibitory effect on the oxidative burst [13]. This latter effect may be mediated by inhibition of phosphofructokinase and the subsequent depletion of intracellular ATP [24]. At higher concentrations, AF inhibits the burst from its onset [13] and also inhibits the depolarization of PMN (Fig. 3). Recent work has implicated protein kinase C as a site of action of AF in the inhibition of the oxidative burst of PMN [4, 5] and since protein kinase C is involved in both the oxidative burst and changes in membrane potential [18, 21, 25, 26], it is reasonable to conclude that AF, at concentrations above 0.5 μM, may be inhibiting both the oxidative burst and depolarization, at least in part, by interacting with protein kinase C.

A sole interaction of AF with protein kinase C, however, does not explain all the effects of the higher concentrations of AF, particularly after stimulation by combinations of PMA with either ConA or FMLP. In the presence of AF, both combinations led to a substantial, although short lived, oxidative burst but without depolarization of the PMN. Thus, the use of AF revealed some dissociation of the oxidative burst from the depolarization of the cells under these conditions. A brisk oxidative burst has also been observed despite the presence of AF if it was added after ConA and before PMA when these two stimulants were added sequentially [10]. While AF is an inhibitor of protein kinase C, this enzyme may be less important in the transduction pathway with

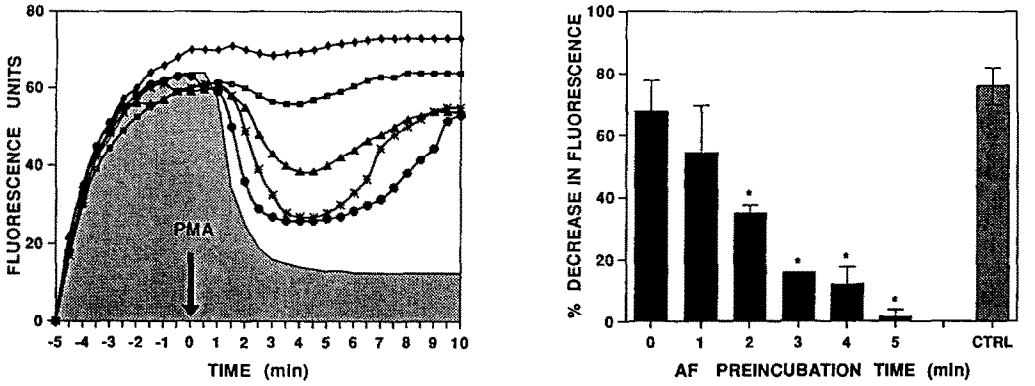

Fig. 4. The time-dependent effect of 5 μM AF on the fluorescence of di-O-C_5(3)-loaded PMN. Experimental conditions are as described in Fig. 1. The left panel shows the data from a representative experiment. Shaded areas indicate the response of cells in the absence of AF. The right panel shows the decrease in fluorescence of PMA-activated cells in the presence of AF. Data are calculated as a percentage of the maximal fluorescence of the resting cells and are presented as the mean ± SD of 2–8 experiments. Control response (CTRL) of PMN in the absence of AF is also shown. Significant differences from control ($P < 0.5$) are shown by (*). Key: control (edge of the shaded area), 0 min (●), 1 min (*), 2 min (▲), 4 min (■), 5 min (◆).

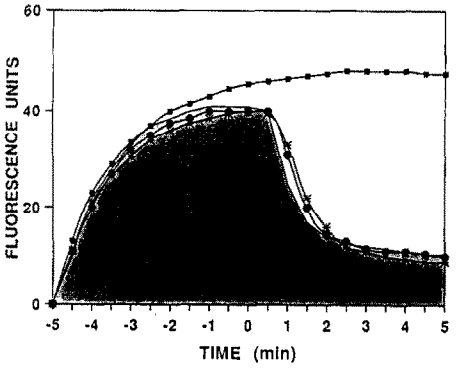

Fig. 5. The effects of 5 μM AF (■), 100 μM Autm (●) and 50 μM $Au(CN)_2^-$ (*) on the fluorescence of di-O-C_5(3)-loaded PMN stimulated with PMA. Shaded areas indicate the response of cells in the absence of gold complexes. Experimental conditions are as described for Fig. 1.

combined stimulants, either ConA or FMLP with PMA, than with these stimulants added singly. Furthermore, we suggest that the pathway of transduction of the oxidative burst with combined stimulants is also largely resistant to AF and is not involved in the depolarization of PMN. Two previous experimental results are consistent with these hypotheses. Firstly, the addition of FMLP has previously been shown to produce further stimulation of the oxidative burst of PMN even after maximal stimulation by PMA, a finding which Cooke and Hallett [27] suggested was due to the generation of an intracellular mediator separate from protein kinase C. Secondly, some inhibitors of protein kinase C do not alter the respiratory burst elicited by FMLP in PMN primed by low concentrations of PMA [28]. Although the present studies have not identified this AF more resistant pathway in PMN activated by simultaneous addition of two stimulants, the results indicate that the failure of single stimuli to activate PMN in the presence of high concentrations of AF cannot be attributed to a cytotoxic effect of AF but is due to inhibition of the activation signal.

There has been little previous evidence for the production of an oxidative burst without depolarization of PMN. However, an oxidative burst is produced without depolarization after activation of PMN by *Candida albicans* hyphae [29].

The time of preincubation had a marked effect on the actions of AF on the PMN. The decreasing effect and repolarization which was seen with PMA preincubated with 5 μM AF for less than 4 min prior to activation with PMA indicates a progressive interaction of AF with the PMN. Chemical studies of the binding of AF to a monocyte cell line clearly show time-dependent uptake of gold and loss of the ligands of AF [22] and the progressive pharmacological effects of AF demonstrate that similar processes may be occurring slowly in PMN.

The effect of AF on the oxidative burst and depolarization of PMN after stimulation by the calcium ionophore is markedly different to that seen with the other single stimulants. The calcium ionophore, A23187, induces a depolarization which is not inhibited by AF, indicating that AF had no effect on the transport of calcium into the cytosol by the ionophore. On the other hand, AF markedly inhibits the lucigenin-dependent chemiluminescence and luminol-dependent chemiluminescence after stimulation of the PMN by A23187 [10] showing that the depolarization can be produced without respiratory burst of PMN. However, while we have found that AF is a potent inhibitor of the oxidative

burst of PMN after the addition of A23187, this has not been a constant finding. Hafstrom *et al.* [9] reported that AF did not affect the production of superoxide by PMN after the stimulation by A23187 although no detailed results were reported. The reason for the contrasting results is not clear but we have recently found that AF inhibits the oxidative burst of PMN without influencing the increased cytosolic levels of calcium after the addition of another calcium ionophore, ionomycin. These results with ionomycin are consistent with our finding that the A23187-induced oxidative burst is inhibitable by AF [10]. Depolarization without an oxidative burst has been reported previously in a few situations, with high levels of potassium, with normal PMN [26] and in some cases with PMN from patients with chronic granulomatous disease. In the X-linked form of this disease, calcium ionophore but not FMLP induces depolarization of PMN [17, 30] while FMLP depolarizes PMN from patients with the autosomal recessive disease [31], without eliciting superoxide generation in cells from either type of patients.

At first sight, $Au(CN)_2^-$ also dissociated the oxidative burst from the depolarization of PMN. $Au(CN)_2^-$ (50 μM) has previously been shown to inhibit markedly both the superoxide production and chemiluminescence of PMN [13], but to have no effect on membrane potential changes induced by PMA or FMLP. However, $Au(CN)_2^-$ always had little effect on the initial development of the oxidative burst and the time at which inhibition occurred decreased with increase in $Au(CN)_2^-$ concentrations. Two hypotheses have been put forward to explain the late inhibition of the oxidative burst. Firstly, $Au(CN)_2^-$, possibly like low concentrations of AF, may not alter the transduction pathway involved in the initiation of the oxidative burst but rather may inhibit processes required for the maintenance of the burst [10]. A second suggestion is that $Au(CN)_2^-$ is not inhibitory by itself but is further metabolized to a inhibitory species.* The lack of effect of $Au(CN)_2^-$ on the depolarization of PMN is consistent with both hypotheses but does not distinguish between them.

The effect of Autm on the oxidative burst of PMN depends upon the source of the Autm [11]. Solutions were freshly prepared from solid Autm in the present work. Under the conditions of our experiments, we have previously found that 100 μM Autm slightly enhanced the luminol-dependent chemiluminescence [11, 13] but this effect was not mirrored by the membrane potential changes.

In the present studies, none of the gold complexes altered the resting fluorescence of the PMN indicating that they did not affect processes involved in the maintenance of a resting membrane potential. However, it was reported that exposure of PMN to AF (5.9 μM) caused slight depolarization of resting membrane [9] but the time of incubation was considerably longer (15 min) than in the present study (5 min).

Several suggestions can be made about the clinical relevance of the present data on the gold complexes. Our finding that in experiments *in vitro* PMN are less sensitive to AF after the addition of multiple stimulants, together with our previous finding that AF has a decreased effect on PMN which are already activated [10] indicates that, *in vivo*, AF may have restricted effects on the cells at the sites of inflammation, since, in synovial fluid of rheumatoid joints, PMN are continuously exposed to both priming and activating agents within rheumatoid joints [32]. The activation of PMN in synovial fluid is demonstrated by surface receptor expression [33] and the presence of myeloperoxidase in the synovial fluid [34]. These findings taken together indicate that potential effects of this drug *in vivo* may have been overestimated from studies *in vitro* in which AF was added to the cells prior to their activation by a single stimulus. It should also be noted that AF readily associates with serum proteins, decreasing the association of gold with cells [22] and thus reducing its inhibitory effect on PMN [9]. Likewise, but to a much higher degree, the inhibitory effect of $Au(CN)_2^-$ on the oxidative burst of PMN is decreased by serum, presumably by binding to serum proteins. However, AF and $Au(CN)_2^-$ have reduced although still significant activity in synovial fluid (unpublished observations).

Further work is necessary to identify other combinations of stimulus which are able to activate PMN in the presence of AF, elucidate the molecular bases of the activation pathways involved, and to determine how these phenomena are related to conditions *in vivo*.

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