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Effect of Auranofin and Other Gold Complexes on the Activity of Phospholipase C

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SUMMARY

Auranofin (AF) is an orally active chrysotherapeutic agent used for the treatment of rheumatoid arthritis, a self-perpetuating inflammatory disease. Because of reports suggesting that AF and other gold complexes can, under certain circumstances, exacerbate rheumatoid inflammatory lesions in humans and adjuvant arthritic rats and that phospholipase C (PLC) and phospholipase A_2 activities are increased in rheumatoid patients, the effects of AF and a related gold complex on *in situ* mammalian and purified *Bacillus cereus* PLC were examined. Results of our studies show that 1) AF and triethylphosphine gold chloride (TEPG), an AF analog, stimulated PLC activity in the sonicate of RAW 264.7 macrophages; 2) AF and TEPG stimulated *B. cereus* PLC activity in a concentration-dependent manner, but the pattern of stimulation and concentrations of drugs required to stimulate the purified enzyme differ from those seen with the macrophage PLC; 3) metals (cobalt and zinc) and sulfhydryl reagents (*N*-ethylmaleimide, iodoacetic acid, and glutathione), tested at the same concentrations of AF that enhanced PLC activity, had no effect on the enzyme. These data suggest that stimulation of PLC may be a generic phenomenon since two divergent PLCs are affected by gold complexes. Additionally, these studies may provide one potential explanation for rheumatoid lesion flares seen in patients and animals on chrysotherapy.

Rheumatoid arthritis is a chronic autoimmune disease characterized by a destructive, self-perpetuating inflammatory response localized primarily in the synovium (1, 2). The unrestrained synovial inflammation is thought to be a consequence of abnormal macrophages, key effector and immunoregulatory cells involved in the initiation and maintenance of the disease (3–5). Eicosanoids are important mediators of the rheumatoid inflammatory response (6, 7). High concentrations of prostaglandin E_2, prostaglandin $F_{2\alpha}$, and thromboxane B_2 have been detected in the synovial fluid of rheumatoid patients (8–10). This may be a direct result of enhanced phospholipase A_2 (EC 3.1.1.4) and PLC (EC 3.4.1.5) activities observed in peripheral blood polymorphonuclear leukocytes and monocytes from patients with the disease (11, 12).

Auranofin, (AF) (Fig. 1), an orally absorbed chrysotherapeutic agent, possesses both antirheumatic and cytotoxic activities (13–15). Although much work has been done to elucidate the cellular interactions of AF, its precise mechanism of action with its putative target cell, the macrophage, is unknown (16–18). Numerous observations do, however, suggest that AF exerts its cytotoxic effect through interactions with membranes and membrane-associated enzymes. Mirabelli *et al.* (15) and Simon *et al.* (19) have reported that the *in vitro* treatment of cells with low concentrations of AF for short time periods caused extensive alterations in cell morphology including surface membrane pitting, cell rounding, blebbing, and membrane lysis. The blebbing phenomen has also been observed in lymphocytes of patients receiving AF (20). Others have reported that AF inhibits the incorporation of [^{3}H]thymidine, [^{3}H]deoxyglucose, and ^{14}C-amino acids through its actions at the cellular membrane level (21). Subcellular fractionation and electron microscopic analyses have also localized gold from AF and parenteral salts in various membrane fractions in a variety of cells (16, 22, 23).

Recent studies demonstrate that AF and gold sodium thiomalate, a parenterally administered chrysotherapeutic agent, can exacerbate the inflammation-mediated stage of adjuvant arthritis in rats (24).[1] Because of those reports and data indicating membrane localization of AF as well as increased PLC activity in rheumatoid patients, the effects of AF and related gold compounds on PLC were examined *in situ* with RAW 264.7 macrophages and *in vitro* using purified *Bacillus cereus* PLC, a membrane-associated enzyme (25).

[1] M. DiMartino, personal communication.

ABBREVIATIONS: PLC, phospholipase C; auranofin [(1-thio-β-D-glucopyranose 2,3,4,6-tetraacetato-*S*) (triethylphosphine)gold]; PC-2A, arachidonyl-1-[^{14}C]phosphatidylcholine, L-α-1-palmitoyl-2-arachidonyl; PI-2A, arachidonyl-1-[^{14}C]phosphatidylinositol, L-α-1-stearoyl-2-arachidonyl; PBS, phosphate-buffered saline; DAG, 1,2-diacylglyceride; TEPG, triethylphosphine gold chloride; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; GSH, glutathione.

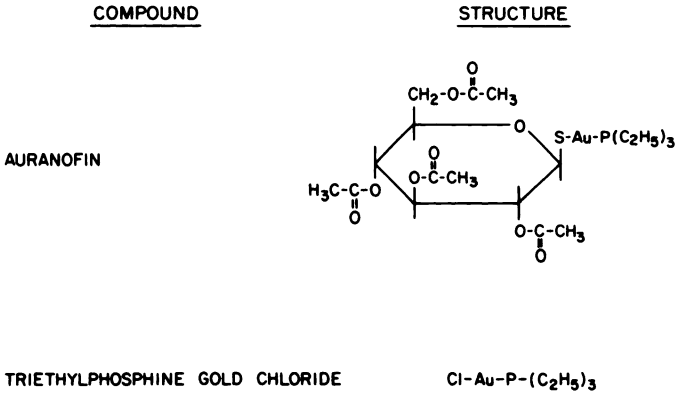

Fig. 1. Structures of auranofin (AF) and triethylphosphine gold chloride (TEPG).

Materials and Methods

PLC activity was quantitated using phospholipids containing ^{14}C-radiolabeled arachidonic acid at the R_2 position. PC-2A (52 mCi/mmol) and PI-2A (25 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Purified *B. cereus* PLC (molecular weight 20,000–25,000; phosphatidylcholine specific) was purchased from Boehringer Mannheim (Indianapolis, IN). Pentex reagent grade bovine albumin, fraction V, was obtained from ICN Immunobiological (Lisle, IL), and Dulbecco's low glucose minimal essential medium, PBS, and fetal calf serum were from Grand Island Biological Co. (Grand Island, NY). Silica gel thin layer chromatography plates were purchased from Analtech Corp. (Newark, DE), and Beckman HP/b scintillation fluid was from Beckman Diagnostic Products (Fullerton, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell culture techniques. RAW 264.7 cells (26), a murine macrophage-like cell line obtained from the American Type Culture Collection (ATCC TIB 71), were grown in monolayer in Dulbecco's low glucose minimal essential medium containing 10% fetal calf serum in a 5% CO_2 humidified incubator at 37°. The cells were determined to be mycoplasma free by the Hoescht 33258 procedure (27).

Cells were routinely split 1:3 into T-150 culture flasks 24 hr before harvesting for assay of *in situ* PLC activity. Macrophages were washed twice with PBS without calcium and magnesium (pH 7.0), scraped with a rubber policeman, combined, and placed in a 50-ml centrifuge tube. The cells were then centrifuged at 2000 rpm for 10 min at 4° in a Beckman tabletop centrifuge, the supernatant was removed, and the cells were resuspended in sufficient PBS to allow 200 μl of cell sonicate to be used per concentration of drug being tested. Cells were sonicated using six to nine bursts in a Branson Sonifer Cell Disruptor. Efficiency of lysis was determined by microscopic evaluation. The cell lysate was centrifuged at 2000 rpm for 10 min at 4° and the supernatant was removed. Two hundred μl of the cell-free supernatant and 50 μl of drug or PBS were added to a 1.5-ml Microfuge tube and incubated together for 30 min at 37°. The tube was removed from the incubator, placed on ice, and PLC activity was then measured.

Purified PLC preparation. Purified PLC was diluted 1/10,000 with PBS (without calcium and magnesium) containing 0.1% fatty acid-free bovine albumin (fraction V). Final enzyme dilutions were made with double distilled H_2O. The protein content of cell sonicates and purified PLC was determined by the method of Bradford (28) using a dye reagent supplied by Bio-Rad Laboratories with bovine albumin as a standard.

Preparation of radiolabeled substrate. The phospholipid substrates (PI-2A, PC-2A) were placed in Microfuge tubes and the solvent in which the lipids were dissolved was evaporated either by centrifugation for 30 min in a rotary evaporator or by evaporation under nitrogen. The phospholipid residue was dissolved in sodium deoxycholate (5 mg/ml) and sonicated for 60 min at room temperature in a Bransonic water bath sonicator. After sonication, the substrate was placed at 4° until use in the assay.

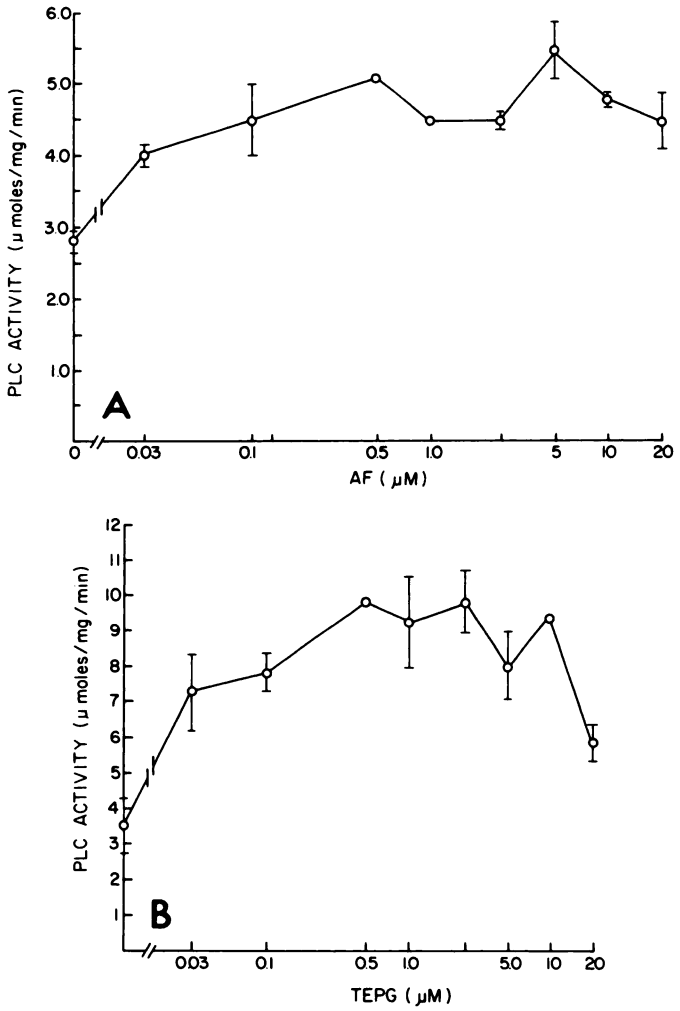

Fig. 2. Effect of increasing concentrations of AF (A) and TEPG (B) on PLC activity in sonicates of RAW 264.7 macrophages. Cell sonicates were preincubated with the drugs for 30 min at 37° and PLC activity was assayed for 60 min at 37°. Enzyme activity was determined as described in Materials and Methods. Values represent the means and standard deviations of triplicate samples from a representative experiment (one of four).

PLC assays. PLC activities were determined as described by Bomalaski *et al.* (11). PLC hydrolyzes the polar head group from membrane phospholipids to yield DAG. Analysis of PLC activity is based upon formation of radiolabeled DAG, which is separated from the parent compound by thin layer chromatography.

The reaction mixture for determining PLC activity in the pretreated cell sonicates contained 20 μl of sonicate, 10 μl of NaCl/$CaCl_2$ mixture (final concentration, 1 mM NaCl and 0.8 mM $CaCl_2$), 10 μl of radiolabeled PI-2A (10 μM), and 10 μl of Tris (pH 7.0). The reaction mixture for purified PLC contained 20 μl of purified enzyme (10 μM), 10 μl of NaCl/$CaCl_2$, 10 μl of radiolabeled PC-2A (10 μM). However, when AF and other compounds were tested, they were prepared in the Tris buffer and 10 μl of the drug/Tris were added to the reaction mixture. After incubation for 60 min (*in situ* PLC) and 30 min (purified PLC) at 37°, the reactions were terminated by addition of 50 μl of $CHCl_3$/methanol (1:2), $CHCl_3$, and 4 M KCl to each assay tube.

A 70-μl aliquot of the organic layer was spotted on silica gel thin layer chromatography plates. Cold phospholipid standards were also added to each lane to enhance visualization of the product. To separate the PLC reaction products, a mobile phase of petroleum ether/diethyl

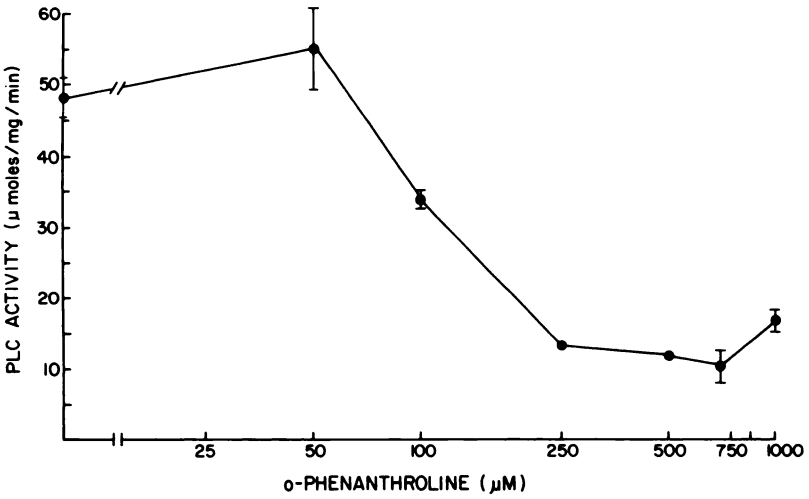

Fig. 3. Effect of *o*-phenanthroline on the activity of *B. cereus* PLC. The enzyme was preincubated for 10 min at 4° with increasing concentrations of *o*-phenanthroline. Activity was then measured as described in Materials and Methods. The data shown are from a representative experiment (one of four) performed in triplicate.

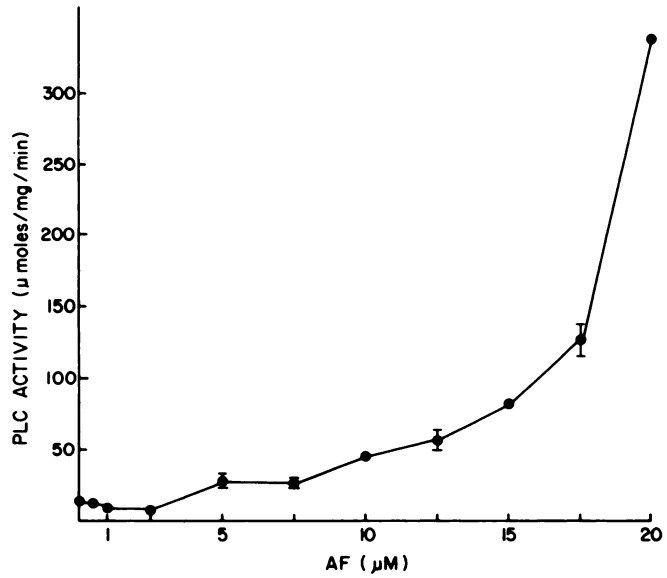

Fig. 4. Effect of AF on purified PLC activity. Procedures are as described previously in Materials and Methods. The data shown are means and standard deviations from a representative experiment (one of four) performed in triplicate.

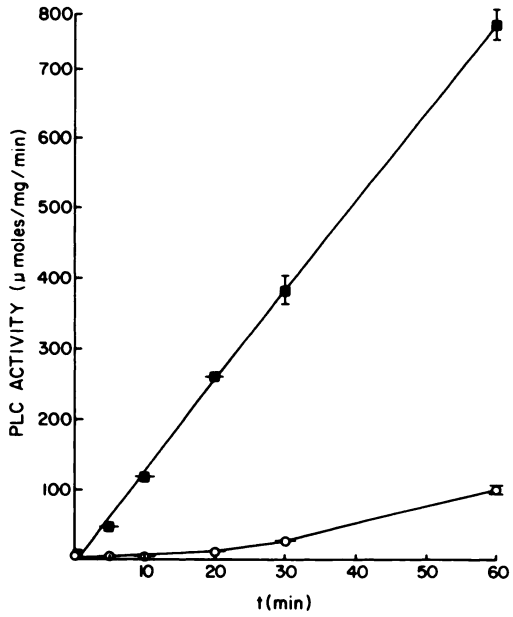

Fig. 5. Stimulation of purified PLC by AF as a function of time. Enzyme alone (10 pmol) (○) or enzyme plus 20μM AF (■) was incubated for varying times using conditions described previously in Materials and Methods. The data shown are means and standard deviations derived from two separate experiments performed in triplicate.

ether/acetic acid (70:30:1) was used. Plates were then developed in iodine vapor. After the iodine stains had disappeared, the spots were scraped from the plates into 20-ml scintillation vials. Five hundred μl of methanol and 10 ml of Beckman hp/B scintillation fluid were added to the vials and the mixture was counted in a Beckman scintillation counter.

Enzyme activity was expressed as μmol of DAG produced/mg of protein/min.

Results

Effect of AF and TEPG on PLC activity in RAW 264.7 macrophages. Before examining the effects of gold complexes on *in situ* PLC activity, assay conditions were standardized to study the production of radiolabeled DAG in RAW 264.7 macrophages. The preferred substrate for *in situ* PLC was determined to be PI-2A. The activity of phosphatidylinositol-specific PLC in the cell sonicates increased over time and with increasing protein concentration (data not shown). Final assay conditions are as described in Materials and Methods.

Fig. 2A shows the effect of preincubating cell sonicates with increasing concentrations of AF (0.03–20 μM). AF stimulated activity of the enzyme at all concentrations tested, with statistically significant stimulation occurring even at a 0.03 μM concentration of the drug. Like AF, TEPG (Fig. 1), the chloro analog of AF that also possesses antirheumatic activity (29) (Fig. 2B), stimulated PLC activity at all concentrations tested (0.03–20 μM).

Effect of AF and gold complexes on *B. cereus* PLC activity. To determine whether AF and analogs induce similar effects on different types of PLC and to better understand its mechanism of interaction with PLC, the effects of that drug and other gold complexes were studied on the well characterized, purified *B. cereus* PLC (25). As in the *in situ* PLC system,

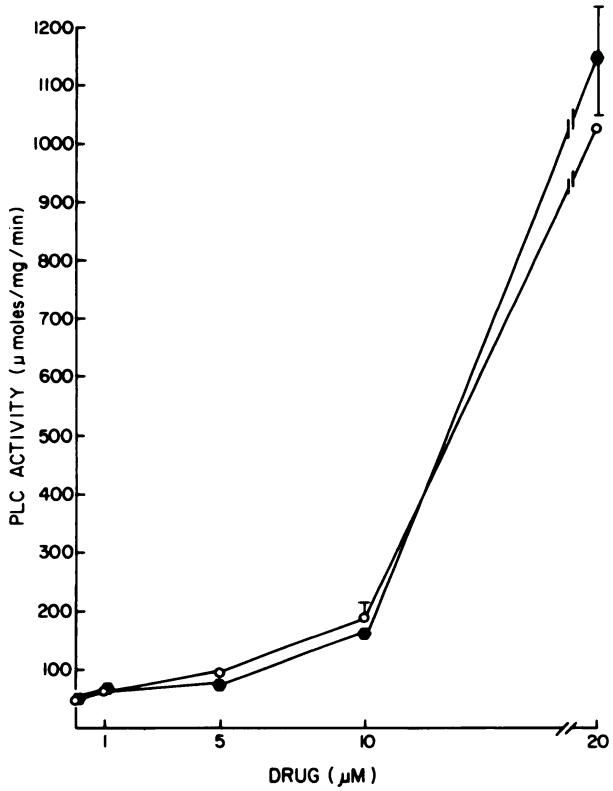

Fig. 6. Effect of increasing concentrations of AF and TEPG on purified PLC activity. The points are means and standard deviations derived from a representative experiment (one of four) performed in triplicate. Procedures are as described previously in Materials and Methods. ○, AF; ●, TEPG.

assay conditions were optimized for *B. cereus* enzyme activity. Unlike the *in situ* enzyme, PC-2A was the preferred substrate for purified PLC. The activity of the enzyme, as quantitated by the appearance of radiolabeled DAG, was linear up to 50 pmol of the enzyme and increased as a function of time over a 60-min incubation period (data not shown).

Incubation of purified PLC with *o*-phenanthroline, a zinc ion chelator, has been reported to inhibit enzyme activity (25). To ensure that the PLC used in these studies responded according to previous published reports, the enzyme was incubated with increasing concentrations of *o*-phenanthroline. As shown in Fig. 3, increasing concentrations of *o*-phenanthroline inhibited the *B. cereus* enzyme with 250–1000 μM *o*-phenanthroline causing a 3- to 4-fold decrease in PLC activity.

Incubation of the *B. cereus* enzyme with increasing concentrations of AF resulted in a concentration-dependent stimulation of PLC (Fig. 4), with a statistically significant increase in activity occurring between drug concentrations of 5 and 10 μM. In the experiment shown in Fig. 4, 20 μM AF resulted in 23-fold stimulation of activity. In other experiments, using different lots of purified PLC, even though absolute control and stimulated activities varied, AF consistently stimulated the enzyme producing 13- to 65-fold increases in activity. The kinetics of AF stimulation of PLC are shown in Fig. 5. The data demonstrate that 20 μM AF causes an approximately 40-fold increase in activity within 5 min of co-incubation of the enzyme and drug. At all time points (5–60 min), AF greatly stimulated PLC activity.

Fig. 6 compares the effects of AF and TEPG on purified PLC activity. The data demonstrate that these compounds stimulated the enzyme in a concentration-dependent manner, with a 24-fold increase in activity seen at the highest drug concentration tested (20 μM)

Effects of metals and sulfhydryl reagents on *B. cereus* PLC activities. To determine whether other metals could also stimulate activity of the enzyme, zinc and cobalt were incubated with purified PLC. Fig. 7 shows that incubation of those metals with PLC, at similar concentrations of AF and TEPG, did not stimulate activity. Significant stimulation of activity occurred only when the enzyme was incubated with 1 mM cobalt or zinc.

Because AF is a sulfhydryl-reactive compound (30, 31), other thiol reagents were tested to see if they would also stimulate PLC. As shown in Fig. 8, NEM, IAA, and GSH only stimulated PLC activity when 1 mM concentrations of these agents were incubated with the enzyme. Moreover, these compounds enhanced the activity of the enzyme only 3-fold, whereas AF induced as much as 65-fold stimulation. These data suggest

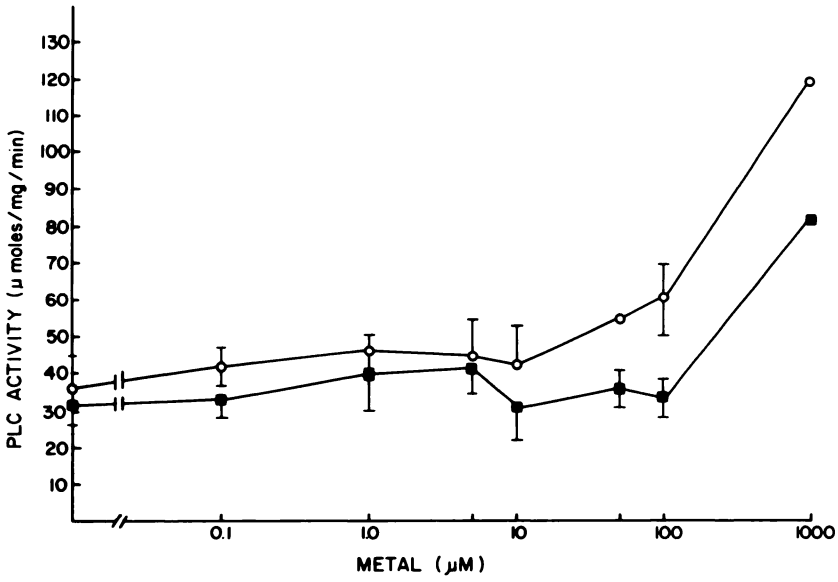

Fig. 7. Effect of cobalt and zinc on the activity of purified PLC. Procedures are as described in Materials and Methods. The points are means and standard deviations derived from a representative ■, Co^{2+}; ○, Zn^{2+}.

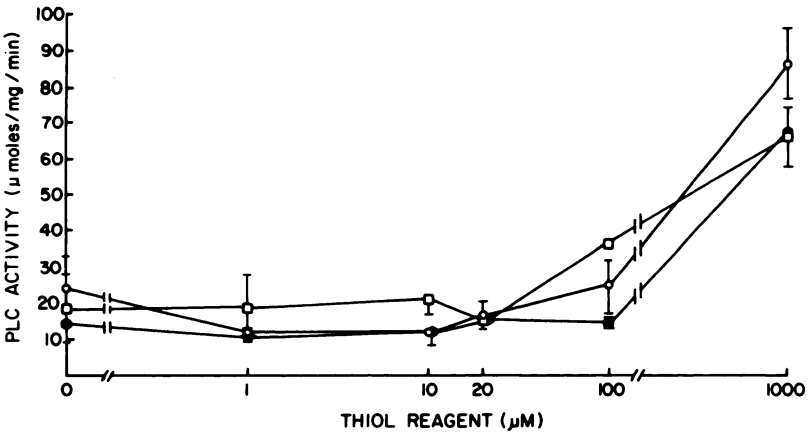

Fig. 8. Effect of thiol reagents on the activity of purified PLC. The points are means and standard deviations derived from a representative experiment performed in triplicate. Procedures are as described in Materials and Methods. ○, NEM; □, IAA; ●, GSH.

that AF's stimulatory effects are unique to the drug and are not solely due to nonspecific sulfhydryl interactions.

Discussion

AF has been shown to be highly cytotoxic against a variety of cells (14, 19, 20). Cell lysis occurs rapidly and is not explained by effects on DNA, RNA, and protein syntheses (15, 17). In addition, plasma membrane morphology has been reported to be altered in cells treated with AF for short periods of time (15, 20). These data suggest that the cytotoxic and perhaps the chrysotherapeutic effects of the drug may result from effects of membrane structures and/or the membrane activities of target cells. Because of the inflammatory nature of rheumatoid arthritis and data showing a significant association of gold and gold-triethylphosphine moieties of the AF molecule with the membranes of macrophages *in vitro* (16), we have examined the effects of AF on PLC, a membrane-associated enzyme that is involved in the inositol phosphate cycle, cellular Ca^{2+} fluxes, and release of arachidonic acid (32, 33).

The data in Fig. 2A indicate that AF stimulated PLC activity in the sonicate of RAW 264.7 macrophages. TEPG, the chloro analog of AF, also stimulated sonicate PLC activity (Fig. 2B), demonstrating that AF and TEPG display similar pharmacological behavior in this as well as other experimental systems (18).

To better understand the mechanism of PLC stimulation, AF and TEPG were tested in a less complicated system using the well characterized, purified *B. cereus* PLC. As seen with the cell sonicates, AF (Fig. 4) and TEPG (Fig. 6) stimulated *B. cereus* enzyme activity in a concentration-dependent manner. AF produced a statistically significant increase in activity at a 5 μM concentration of the compound and within 5 min of drug exposure (Fig. 5). These data show that AF interacted directly with the purified enzyme and that the pattern of stimulation and the concentration of AF required to stimulate the purified enzyme differ from those seen with the macrophage PLC. These differing sensitivities are not surprising since the PLCs are different enzymes with dissimilar characteristics, including molecular weight, source (mammalian, bacterial), and substrate specificities. Additionally, *in vivo* the activities of both phospholipase A_2 and PLC are regulated by lipomodulin (34, 35) or macrocortin (36, 37), antiphospholipase proteins which inhibit phospholipase activities. The greater sensitivity of PLC in the sonicates of macrophages to AF may result from effects of the drug on both the enzyme and/or its regulatory protein.

B. cereus PLC requires Zn^{2+} for normal enzyme function (25). To determine if metals, other than gold, could stimulate the enzyme, PLC activity was examined in the presence of zinc and cobalt, ions that reportedly activate the enzyme after removal of zinc by *o*-phenanthroline (25). Fig. 7 shows that Zn^{2+} and Co^{2+}, tested at the same concentrations of AF that enhanced PLC activity, had no effect on the enzyme, suggesting that gold complex stimulation of PLC is not simply the result of nonspecific metal-ion interactions. These experiments do not, however, exclude the possibility that gold derived from AF and TEPG replaces zinc atoms at the enzyme active site resulting in enhancement of activity.

AF interacts with and inhibits various enzymes by alkylation of available reactive sulfhydryl groups (30, 38). *B. cereus* PLC has been shown by amino acid analysis to contain one cysteine that is not at the active site of the enzyme (25, 39). One could suggest then, that AF and other gold compounds stimulate activity of PLC by coordinately binding to the cysteine in the molecule, allosterically modifying enzyme structure, and thereby enhancing activity. NEM, IAA, and GSH did not stimulate activity of the purified enzyme (Fig. 8). These data corroborate reports by Otnaess *et al.* (25) indicating that sulfhydryl reagents had no effect on *B. cereus* PLC. The data perhaps suggest that the gold-triethylphosphine or gold moieties derived from gold compounds represent better alkylators of PLC's cysteine sulfhydryl resulting from the unique non-ionic, lipophilic structure of AF and other gold alkylphosphines or to gold's strong attraction to reduced sulfhydryls.

Stimulation of both *in situ*, mammalian, and purified *B. cereus* PLC by AF and TEPG is significant for several reasons. First, the data show that PLC stimulation may be a generic phenomenon since two divergent PLCs, with differing physical characteristics and substrate specificities, are affected by the gold complexes. Second, in the past, AF has been shown to inhibit, not stimulate, enzyme activities (17, 30, 40). Third, significant stimulation *in situ* occurred at a drug concentration of 0.03 μM, a concentration of AF that is 20-fold lower than the IC_{50} value for cytotoxicity of the compound for these cells (unpublished data), and well within therapeutically attainable levels of gold in the rheumatoid joint and in blood. In addition, this is the lowest concentration of AF reported to have an effect on any *in vivo* and *in vitro* experimental parameter. Fourth, the perturbation of membrane structure, blebbing, is thought to be a consequence of the disruption of cellular thiol and Ca^{2+} levels (41). Since PLC is the key enzyme responsible for inositol

phosphate turnover and mobilization of intra- and extracellular Ca^{2+} (33), stimulation of the enzyme and subsequent disturbance of Ca^{2+} homeostasis may, in part, explain the events responsible for bleb formation. Fifth, and most importantly, the data may also provide one potential explanation for reports suggesting that AF and other gold compounds can, under certain conditions, exacerbate rheumatoid inflammatory lesions in humans and adjuvant arthritic rats (24)[2] since increased PLC activity and subsequent release of proinflammatory arachidonic acid metabolities would stimulate the inflammatory response in the rheumatoid synovium. The interaction of AF with PLC has important clinical ramifications and suggests that coadministration of non-steroidal anti-inflammatory drugs with AF is sound therapeutic practice, not only to reduce pain, but to prevent gold-induced inflammatory flares.

The mechanism by which AF and TEPG stimulate *in situ* and purified PLC activity is unclear. However, considering the crucial role that this enzyme plays directly in arachidonic acid and inositol phosphate metabolism and indirectly in cellular structure and function, the data reported here suggest that the interaction of AF with PLC has important implications for the pharmacology of that drug as well as other related gold complexes. Further studies with purified *B. cereus* and mammalian PLC will help elucidate AF's interaction with the enzyme and may provide further information regarding AF's chrysotherapeutic as well as cytotoxic effects on macrophages.

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