Biochemical Pharmacology, Vol. 38, No. 19, pp. 3383–3386, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

SHORT COMMUNICATIONS

Auranofin, gold thiomalate, and gold thioglucose inhibit protein kinase C

(*Received* 23 *August* 1988; *accepted* 24 *February* 1989)

The Au(I)-thio- compounds, including Auranofin (triethylphosphine gold thioglucose-tetraacetate), have been used for many years as antirheumatic therapeutic drugs in humans, yet their mode of action remains unclear. Oxygen radical production by neutrophils and macrophages is essential in immune defense by higher animals in order to kill invading bacteria. On the other hand, excessive production of these reactive metabolites generated through the respiratory burst in these cells has been linked to some types of chronic inflammation. Protein kinase C has been implicated in the regulation of the respiratory burst/reactive oxygen species generation in neutrophils [for review see Refs 2 and 3] and hence defects at the PKC level may be responsible for some types of chronic inflammation in which there is overproduction of reactive oxygen species. Activation of PKC by the PKC-specific phorbol diesters results in the stimulation of the respiratory burst in intact neutrophils which indicates that at least one of the pathways naturally bringing oxygen uptake to radical production involves PKC. It is possible that excess activation or the presence of constitutively active PKC *in vivo* may be responsible for excess reactive oxygen species generation and hence some types of chronic inflammation. This hypothesis suggests the possibility that some antirheumatic drugs may have a direct inhibitory interaction with PKC activity, thereby resulting in decreased reactive oxygen species generation and alleviation of some types of chronic inflammation. If this is the case, then it may be possible to utilize PKC as a target for anti-inflammatory drugs. In this paper we have examined the possible direct interaction with, and modulation of, the catalytic activity of PKC by the antirheumatic drugs, Auranofin, Au-*S*-Malate*, and Au-*S*-glucose.

Materials and methods

High purity phosphatidylserine (PS) was from Lipid Products (S. Nutfield, Surrey, U.K.), Au-*S*-glucose from Sigma Chemical Co. (St Louis, MO), and Auranofin and Au-*S*-malate were generous gifs from Smith Kline & French (Welwyn Garden City, U.K.) and BYK Gulden (Konstanz, F.R.G.).

Preparation of protein kinase C. (A) Bovine brain preparation. Extraction and 75% ammonium sulphate precipitation: protein kinase C was purified from bovine brain by a modification [4] of the procedure used by Walsh *et al.* [5]. All steps were carried out at 4°. Fresh bovine brain (500 g) was homogenized with a Waring blender (30 sec, twice) in 3 vol. 0.25 M sucrose, 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 2 mM β-mercaptoethanol (β-ME). The homogenate was centrifuged (30,000 *g*, 15 min) and the resultant supernatant was filtered through glass wool. The filtrate was then centrifuged (60,000 *g*, 105 min) and the resultant supernatant was filtered through glass wool and made 75% in ammonium sulphate (solid). After stirring in ammonium sulphate solution for 30 min the precipitated protein was collected by centrifugation (30,000 *g*, 45 min). The 75% ammonium sulphate pellet was resuspended and homogenized in 100–200 ml of 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM β-ME (Buffer A) and dialyzed extensively against the same buffer. The dialyzed protein solution was then clarified by centrifugation (30,000 *g*, 30 min) in preparation for phenyl Sepharose chromatography.

Phenyl Sepharose chromatography: the clarified supernatant from above was made 2 mM in $CaCl_2$ and $MgCl_2$ and loaded onto a 1.5 × 30 cm column of phenyl Sepharose in 20 mM Tris–HCl (pH 7.5), 0.1 mM $CaCl_2$, 2 mM β-ME (Buffer B). The column was then sequentially washed with 120 ml of Buffer B; 120 ml Buffer B, 1 M NaCl; and 120 ml 20 mM Tris–HCl (pH 7.5), 1 mM EGTA, 2 mM β-ME (Buffer C). The kinase active fractions were pooled and dialyzed overnight against Buffer C.

DE 52 chromatography: the dialyzed phenyl Sepharose pool was applied to a 1.5 × 20 cm column of DE 52 resin in Buffer C. The column was then washed with 120 ml of Buffer C and the kinase activity was eluted with a linear gradient created by mixing 200 ml of Buffer C with the same amount of Buffer C, containing 0.4 M NaCl. Protein kinase C active fractions were pooled and dialyzed against Buffer C and stored at 4°. Typical specific activities of 100–200 nmol P_i incorporated into histone/min/mg protein were obtained.

(B) Neutrophil preparation. Human neutrophils were isolated from freshly drawn whole blood by dextran sedimentation and lysis of contaminating red blood cells, according to the method of Markert *et al.* [6]. Cells were washed in extraction buffer (50 mM Tris–HCl, pH 7.5, 2 mM EGTA, 1 mM PMSF, 1 mg/ml soya bean trypsin inhibitor, 1 mM benzamidine and 50 mM β-ME) and resuspended in the same buffer to a final concentraion of approximately 4×10^7 cells/ml. The cells were disrupted by sonication in an ice bath for 25 sec and the sonicate was then centrifuged for 20 min at 20,000 *g* (4°). The supernatant (cytosolic fraction) was used to assay PKC activity.

Protein determination. Protein concentration was determined by the Coomassie Brilliant Blue (G) dye binding assay [7].

Kinase assay. Ca^{2+} and phospholipid dependent protein kinase activity for both bovine brain and neutrophil enzymes was determined by the procedure of Mahoney and Azzi [4]. The standard assay mixture (0.25 ml) contained 20 mM Tris–HCl (pH 7.5, 30°), 5 mM Mg acetate, 10 μM (^{32}P) ATP (100,000 cpm/nmol), 0.2 mg/ml histone III-S, ±0.5 mM $CaCl_2$ ± 0.04 mg/ml PS. When PS was used in the assay, a solution of it in $CHCl_3$ was dried under N_2, resuspended in 20 mM Tris–HCl (pH 7.5), and sonicated (30 sec, twice) under N_2 (4°). The sonicated PS solution was added to the balance of the assay mixture. The reaction was started by the addition of 20 μl of enzyme fraction and carried out at 30° for 8 min and then quenched with 0.35 ml cold 12% trichloroacetic acid, 2% sodium pyrophosphate and 100 μl 10 mg/ml bovine serum albumin. The precipitated protein solution was then quantitatively transferred to filters (Millipore HA, 0.45 μm). The filters were placed on holders connected with a vacuum device provided with multiple attachments and they were extensively washed with cold 6% trichloroacetic acid, 1% sodium pyro-

* Abbreviations: Au-*S*-malate, gold thiomalate; Au-*S*-glucose, gold thioglucose; PKC, protein kinase C; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonylfluoride; β-ME, β-mercaptoethanol; PS, phosphatidylserine.

phosphate. Subsequently they were counted in a liquid scintillation counter in the presence of 4 ml liquid scintillation cocktail.

Results and discussion

Auranofin in the 10–100 μM concentration range inhibits the production of superoxide radicals in neutrophils that have been stimulated by the PKC-specific phorbol diesters [8, 9].

These results have suggested a role for PKC in the chain of reactions leading to superoxide production and a potential role or co-role for PKC in the mechanism of Auranofin as an antirheumatic drug. The Ca^{2+}–PS-activated kinase activity of purified bovine brain PKC is similarly inhibited in a concentration-dependent manner by Auranofin, Au-*S*-glucose, and Au-*S*-malate in the 1 μM–1 mM concentration range (Fig. 1) and the control compounds HS-malate and malate show no inhibition of activity (Fig. 1) indicating that the (Au)I moiety is critical for the inhibition. When the kinase activity of the Ca^{2+}–PS-activated enzyme is assayed in the presence of β-ME it was found to be much more sensitive to inhibition by Au-*S*-glucose (IC_{50} = 2.7 μM) and Au-*S*-malate (IC_{50} = 3.8 μM) than Auranofin (IC_{50} = 360 μM) (Fig. 1). When phorbol ester, a PKC specific activator, was used to activate PKC, the enzyme was also inhibited in a similar concentration dependent manner and control compounds lacking Au(I) also showed no inhibition of activated kinase activity in this case (data not shown).

The results obtained with bovine brain PKC were reproduced also with the human neutrophil enzyme preparation. In Table 1 the inhibition by Auranofin, Au-*S*-glucose and stauroporine [10] (as control) are shown. The inhibition appeared to have qualitatively the same features observed with the brain enzyme. The data may suggest tentatively that the sensitivity of the neutrophil enzyme to Au-*S*-glucose is higher than for the bovine enzyme. This finding may be related to the suggestion that in neutrophils only one of the several isoforms of PKC is present [11, 12].

In order to further clarify the mechanism of inhibition of PKC by these compounds the effect of the presence or absence of β-ME and EGTA (200 μM and 100 μM respectively), which are normally present in our assay system, was examined with purified brain PKC (Fig. 2). In all cases

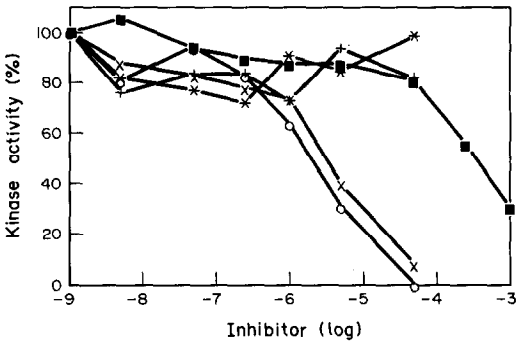

Fig. 1. Concentration dependent inhibition of protein kinase C activity by Au-*S*-glucose (○), Au-*S*-malate (×), Auranofin (■), and lack of inhibition by control compounds HS-malate (+), and malate (*). Assays were in the presence of 100 μM β-ME, 80 μM EGTA, 40 μg/ml PS, and 500 μM $CaCl_2$.

(Auranofin, Au-*S*-glucose, Au-*S*-malate) the presence or absence of 100 μM EGTA in the assay had no significant effect on the concentration dependent inhibition of kinase activity. In contrast to this, in the absence of β-ME, Auranofin (IC_{50} = 3.0–7.0 μM; Fig. 2A) and Au-*S*-malate (IC_{50} = 0.7–0.9 μM, Fig. 2B) were much more inhibitory than in the presence of β-ME (IC_{50} = 82–293 μM and 5–6 μM; Fig. 2A and B). Inhibition of PKC by Auranofin in the absence of β-ME occurred in the same concentration range as is the case with the inhibition of neutrophil superoxide production (IC_{50} = 23 μM) (data not shown). In contrast to the increased inhibitory potency of Auranofin and Au-*S*-malate in the absence of β-ME, Au-*S*-glucose showed the reverse effect, being more inhibitory in the presence of β-ME (IC_{50} = 3–4 μM) than in its absence (IC_{50} = 107–480 μM) (Fig. 2C). These data suggest that the mechanism of inhi-

Table 1. Inhibition of human neutrophil PKC by Au(I)-thio-compounds

Inhibitor	[Inhibitor]	Specific activity pmol/min/mg	Kinase activity (%)
None	—	286	100
Auranofin	200 μM	246	86
	500 μM	223	78
	1 mM	100	35
Au-*S*-glucose	2 μM	39	14
	5 μM	17	6
	10 μM	15	5
Staurosporine	2.1 nM	58	20
	4.2 nM	49	17
	8.4 nM	37	13

The standard assay mixture (0.25 ml) contained 20 mM Tris–HCl (pH 7.5, 30°), 5 mM Mg acetate, 10 μM (^{32}P) ATP (100,000 cpm/nmol), 0.2 mg/ml histone III-S, ±0.5 mM $CaCl_2$ ± 0.04 mg/ml phosphatidylserine and 4 mM β-ME. Specific activity in the absence of PS was 164.9 pmol/min/mg protein and in the absence of both Ca^{2+} and PS was 69.7 pmol/min/mg protein.

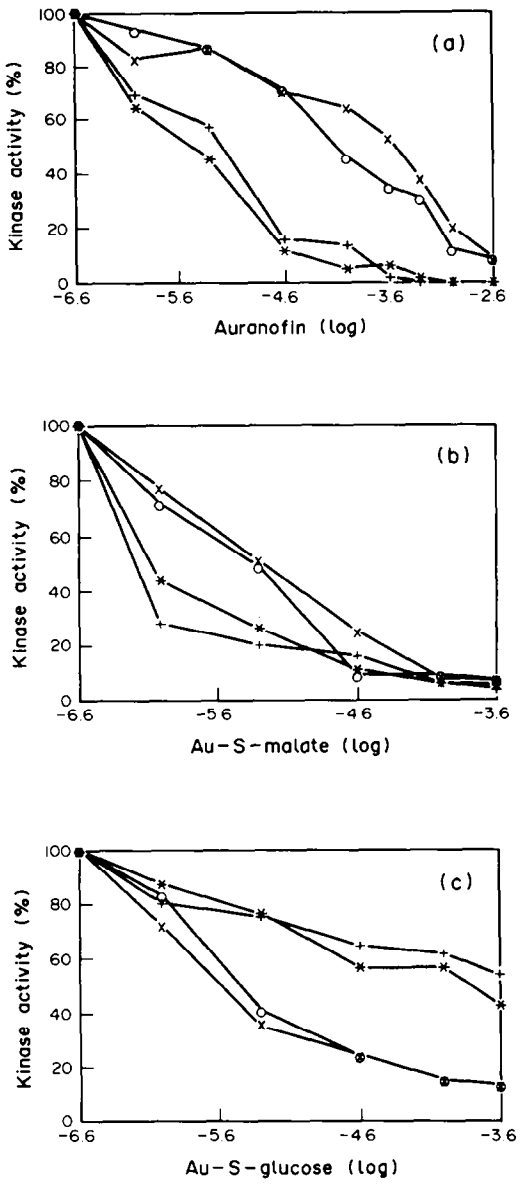

Fig. 2. Inhibition of protein kinase C activity by the Au(I)-thio- compounds and their dependence on β-ME and EGTA. PKC was dialyzed against 20 mM Tris–HCl, pH 7.5 ±2 mM β-ME, ±1 mM EGTA to give final assay concentrations of 200 μM β-ME and 100 μM EGTA (when present). The symbols × and ○ represent results obtained in presence of β-ME while + and * in its absence. The symbols × and + refer to results obtained in presence of 100 μM EGTA while ○ and * in its absence. (a) Auranofin, (b) Au-*S*-malate, (c) Au-*S*-glucose.

bition of PKC by Auranofin and Au-*S*-malate is different to that of Au-*S*-glucose. A reductive event or thiol exchange between the Au(I)-thio- of Au-*S*-glucose and β-ME may be required for inhibitory action whereas in the case of Auranofin and of Au-*S*-malate these events may prevent the inhibitory interaction of these compounds with the enzymatic system. Auranofin does not inhibit the NADPH oxidase of neutrophils [9], the key enzyme in the respiratory burst, which further supports the hypothesis of an activating role for PKC in the respiratory burst and an inhibitory effect of Auranofin at this level. The concentrations of gold derivatives required to inhibit the activity of both bovine brain and human neutrophil PKC and the respiratory burst of neutrophils are in the same order of magnitude as those found in the serum (30–40 μM) [13] and synovium (17–22 μM) [14] of patients undergoing treatment with these compounds. Hence the PKC inhibition reported here is apparently of physiological significance.

In a different system, i.e. intact platelets, Froscio *et al.* [15] have recently reported that Auranofin stimulates phorbol diester-specific protein phosphorylation (40 and 20 kDa M_r). Their results are in potential conflict to the results presented here. However, their results with intact platelets, although apparently in conflict with ours, may be potentially consistent with them if, for example, auranofin with intact platelets inhibited a protein phosphatase or activated another protein kinase thereby resulting in increased phosphorylation of the 40 and 20 kDa M_r proteins, despite inhibition of PKC. Auranofin and Au-*S*-glucose inhibition studies with the isolated PKC from neutrophils have confirmed the possible role of PKC in their inhibition of the respiratory burst.

In summary, Auranofin and Au-*S*-glucose, the anti-rheumatic therapeutic drugs, inhibited purified protein kinase C from bovine brain as well as from human neutrophils, which had been activated in the presence of Ca^{2+} and PS (IC_{50} = 360 and 2.7 μM, respectively) with or without phorbol diester. Au-*S*-malate was also used and found inhibitory with the bovine brain enzyme (I_{50} = 3.8 μM). The control compounds malate and HS-malate had no effect on PKC activity in the same concentration ranges (Fig. 1). Auranofin and Au-*S*-malate were more inhibitory in the absence of β-ME. In contrast, Au-*S*-glucose was more inhibitory in the presence of β-ME. These data suggest that the mechanism of inhibition is different for Auranofin and Au-*S*-malate as compared to Au-*S*-glucose and that a reductive–oxidative event may be involved in the inhibition. The interaction of Auranofin, Au-*S*-malate and Au-*S*-glucose with PKC and their inhibition of its catalytic activity may be responsible or co-responsible for the therapeutic antirheumatic action of these drugs.

Institut für Biochemie und Molekularbiologie
Universität Bern
CH-3012 Bern
Switzerland

CHARLES W. MAHONEY*
CARMEL E. HENSEY†
ANGELO AZZI‡

REFERENCES

1. Mahoney CW and Azzi A, The gold-I antirheumatic compounds inhibit protein kinase C. *Experientia* **44**: A82, 1988.
2. Rossi F, The O^{2-} forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. *Biochim Biophys Acta* **853**: 65–89, 1986.
3. Tauber AL, Protein kinase C and the activation of the human neutrophil NADPH-oxidase. *Blood* **69**: 711–720, 1987.
4. Mahoney CW and Azzi A, Vitamin E inhibits protein kinase C activity. *Biochem Biophys Res Commun* **154**: 694–697, 1988.

* Present address: National Institute of Child Health and Human Development, Bldg. 6, Room 126, National Institutes of Health, Bethesda, MD 20892, U.S.A.

† From the Department of Biochemistry, University College Dublin. These results were presented in part at the annual meeting of the Swiss Societies of Experimental Biology in Interlaken, Switzerland [1]. The support of the Schweizerische Krebsliga is gratefully acknowledged.

‡ To whom correspondence should be addressed.

5. Walsh MP, Valentine KA, Ngai PK, Carruthers AA and Hollenberg MD, Ca^{2+}-dependent hydrophobic-interaction chromatography. Isolation of a novel Ca^{2+}-binding protein and protein kinase C from bovine brain. *Biochem J* **224**: 117–127, 1984.
6. Markert M, Andrews PC and Babior BM, Measurement of O^{2-} production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. *Meth Enzymol* **105**: 358–365, 1984.
7. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
8. Hafstrom I, Seligmann BE, Friedman MM and Gallin JI, Auranofin affects early events in human polymorphonuclear neutrophil activation by receptor-mediated stimuli. *J Immunol* **132**: 2007–2014, 1984.
9. Parente JE, Wong K and Davis P, Effect of gold compounds on NADPH oxidase system of human neutrophils. *Inflammation* **10**: 303–310, 1986.
10. Tamaoki T, Takahashi I, Kato Y, Morimoto M and Tomita F, Staurosporin, a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase. *Biochem Biophys Res Commun* **135**: 397–402, 1986.
11. Sekiguchi K, Tsukuda M, Ogita K, Kikkawa U and Nishizuka Y, Three distinct forms of rat brain protein kinase C: differential response to unsaturated fatty acid. *Biochem Biophys Res Commun* **145**: 797–802, 1987.
12. Dianoux AC, Stasia MJ and Vignais PV, Purification and characterization of an isoform of protein kinase C from bovine neutrophils. *Biochemistry* **28**: 424–431, 1989.
13. Danpure CJ, Fyfe DA and Gumpel JM, Distribution of gold among plasma fractions in rheumatoid patients undergoing chrysotherapy compared with its distribution in plasma incubated with aurothiomalate *in vitro*. *Ann Rheum Dis* **38**: 364–370, 1979.
14. Gerber RC, Paulus HE, Bluestone R and Lederer M, Kinetics of aurothiomalate in serum and synovial fluid. Arthritis Rheum **15**: 625–629, 1972.
15. Froscio M, Solanki V, Murray AN and Hurst NP, Auranofin enhances phosphorylation of putative substrates of protein kinase C in human platelets. *Biochem Pharmacol* **37**: 366–368, 1988.

Biochemical Pharmacology, Vol. 38, No. 19, pp. 3386–3389, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

Effect of diabetes, starvation, ethanol and isoniazid on rat liver microsomal 12α-hydroxylase activity involved in bile acid biosynthesis

(*Received* 14 *September* 1988; *accepted* 11 *March* 1989)

12α-Hydroxylation is a step unique for cholic acid* biosynthesis and has been ascribed a role in the regulation of the ratio between cholic acid and chenodeoxycholic acid synthesized from cholesterol [1]. Experimental diabetes has been reported to increase the biosynthesis of cholic acid and decrease or not change the biosynthesis of chenodeoxycholic acid [2–4]. The change observed in bile acid biosynthesis may be due to effects of diabetes on the 12α-hydroxylating cytochrome P-450 [1, 5].

Recently, it has been shown that a species of cytochrome P-450 in rat liver microsomes, cytochrome P-450IIE1, is induced by starvation, diabetes, ethanol and isoniazid [6, 7]. No information is available concerning the possible activity of cytochrome P-450IIE1 towards C_{27}-steroids. In common with cytochrome P-450IIE1, the 12α-hydroxylating cytochrome P-450 is stimulated by starvation [1, 5]. It is not known whether other treatments inducing cytochrome P-450IIE1, e.g. experimental diabetes, have any effects on the 12α-hydroxylating system.

The present communication reports studies of the effect of diabetes, starvation, ethanol and isoniazid treatment on the 12α-hydroxylase system and the possible role of cytochrome P-450IIE1 in 12α-hydroxylation.

Materials and methods

The various ^{14}C- and ^{3}H-labeled substrates were obtained and prepared as described previously [8]. Streptozotocin and *N*-nitrosodimethylamine were from Sigma. Anti-P-450IIE1 IgG and preimmune IgG were generous gifts from Dr M. Ingelman-Sundberg, Stockholm. Other materials were obtained as described previously [9].

Sprague–Dawley male rats (150–200 g) were used. Liver microsomes were prepared from untreated, starved (72 hr), ethanol-treated (10% in drinking water for 3 weeks), isoniazid-treated (0.1% in drinking water for 10 days) and streptozotocin-treated (45 mg/kg body weight i.v. in tail vein) rats and from rats treated with a combination of ethanol and starvation as described previously [10]. An increase in the blood glucose concentration and in the serum cholesterol level, together with an increase in both the total amount as well as in the specific content of cytochrome P-450, confirmed the induction of diabetes in the streptozotocin-treated rats.

Microsomal cytochrome P-450 fractions were prepared from streptozotocin-treated and isoniazid-treated rats according to the procedures described by Ryan *et al.* [11]. The fraction eluted from the octylamine-Sepharose 4B column with the potassium phosphate buffer containing 0.08% Emulgen 911 and 0.33% sodium cholate [11], contained 12α-hydroxylase activity. The cytochrome P-450IIE1 fraction was eluted from the column and subsequently chromatographed on a hydroxylapatite column as described by Ryan *et al.* [11]. The partially purified cytochrome P-450IIE1 fraction from this step was not further purified but chromatographed on hydroxylapatite to remove detergents [10]. The resulting enzyme fraction was called cytochrome P-450IIE1 fraction.

The fractions that contained 12α-hydroxylase activity were purified on a hydroxylapatite column, equilibrated with 10 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 mM EDTA and 0.2% Emulgen 913. The column was washed with the same buffer con-

* The following trivial names and abbreviations used are: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid.