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Effects of Gold Coordination Complexes on Neutrophil Function Are Mediated via Inhibition of Protein Kinase C

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SUMMARY

Previous studies have shown that the gold compounds auranofin (AUR) and gold sodium thiomalate (GST) inhibit responses of various cells and tissues. We found that superoxide anion generation induced in human neutrophils by the chemotactic tripeptide fmet-leu-phe (1 μM), fluoride (18 mM), or phorbol myristate acetate (PMA, 100 nM) was inhibited by pretreatment of cells with 5–100 μM AUR. The extent of inhibition was dependent on AUR concentration and duration of the preincubation. GST was much less potent, inasmuch as only weak effects were observed at 5 times higher concentrations. The ineffectiveness of GST was attributed to its slower rate of penetration into cells, compared with AUR. The finding that mobilization of internal Ca^{2+} stores was not blocked in AUR-treated cells suggests that phospholipase C-mediated hydrolysis of polyphosphoinositides to inositol 1,4,5-trisphosphate was not inhibited by the drug. Because PMA is known to mimic the action of diacylglycerol in activating protein kinase C (PKC), we investigated the possibility that gold compounds might be interfering with signal transduction at this level. Enzymatic assays indicated that both gold compounds reduced the level of PKC activity associated with the cytosol; however, translocation of PKC to the plasma membrane was not found. Immunoblot analyses carried out with polyclonal anti-PKC antisera revealed that the gold compounds did not cause degradation of PKC or increase translocation to the membrane. Further studies indicated that enhanced endogenous protein phosphorylation resulting from PMA stimulation was attenuated in cells co-treated with AUR. Finally, *in vitro* enzymatic assays showed that both AUR and GST inhibited partially purified PKC in a concentration-dependent manner. It is suggested that modulation of PKC represents a mechanism of action of gold coordination complexes at the cellular level.

AUR and GST are two chrysotherapeutic agents used in the treatment of rheumatoid arthritis. Clinical trials have shown AUR to be equivalent in efficacy to the more established GST by some clinical and laboratory parameters and less efficacious by other criteria (1). At present, the mechanism of action of gold compounds *in vivo* remains to be elucidated. Numerous *in vitro* studies have shown AUR and GST to differ in their modulation of cellular responses. For example, neutrophil responses such as phagocytosis, chemotaxis, adherence, degranulation, superoxide anion generation, and arachidonic acid metabolism are inhibited by AUR at micromolar concentrations (2–9). GST, in contrast, exerts only weak or insignificant inhibition at 5–10 times higher doses. GST is more potent in studies utilizing other cells inasmuch as both AUR and GST have been reported to inhibit lymphocyte, monocyte, and macrophage functions (10–13).

Because neutrophil responses to receptor-dependent stimuli are causally correlated with inositol phospholipid breakdown, PKC activation, and mobilization of internal Ca^{2+} stores (14, 15), the possibility that AUR may act by inhibiting key components in the signal transduction pathway was investigated. Experiments were carried out in which the effect of gold compounds on the kinetics of superoxide anion generation, the distribution of PKC activity, protein phosphorylation, and activity of partially purified platelet PKC was investigated. The present studies indicate that AUR blocked some cellular events after stimulation by direct inhibition of PKC.

Experimental Procedures

Materials. The gold compound, AUR, was a gift from Smith, Kline & French Laboratories (Philadelphia, PA). GST was a gift from Poul-

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ABBREVIATIONS: AUR, auranofin; GST, gold sodium thiomalate; PMA, phorbol myristate acetate; PKC, protein kinase C; fmet-leu-phe, *N*-formylmethionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β-aminoethyl-ether)-*N*,*N*,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; Me_2SO, dimethyl sulfoxide; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; HEPES, 4-2(-hydroxyethyl)-1-piperazineethanesulfonic acid; AM, acetoxymethyl ester.

enc (Montreal, Quebec, Canada). The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): ATP, Trizma base, histone type III-S, 1,2-diolein, phosphatidyl serine, fmet-leu-phe, ferricytochrome *c* type VI, PMSF, leupeptin, Triton X-100, dithiothreitol, and trichloroacetic acid. HBSS, HEPES, and fetal calf serum were purchased from GIBCO Laboratories (Grand Island, NY). Ficoll-Paque was obtained from Pharmacia Inc. (Dorvall, Quebec). The following reagents were purchased from Bio-Rad Laboratories (Richmond, CA): SDS, acrylamide, bis-acrylamide, TEMED, coomassie blue, R-250, molecular weight standards for gel electrophoresis, zeta-probe membrane, and glycine. Nitrocellulose membranes, 0.45 μm, were obtained from Mandel Scientific Co (Rockwood, Ontario, Canada). PMA and fura 2/AM were purchased from Calbiochem Brand Biochemicals (San Diego, CA). EGTA, sodium fluoride, and Whatman GF/F filters were supplied by Fisher Scientific Co. (Fairlawn, NY). Kodal XAR5 film was from Eastman Kodak (Rochester, NY). Carrier free ^{32}P (285 Ci/mg of P) was supplied by ICN Biomedicals Canada (Montreal, Quebec). [^{32}P]ATP (>5000 Ci/mmol) was obtained from Amersham Canada Ltd. (Oakville, Ontario). Sources for additional supplies and materials used in the isolation and assay of protein kinases and PKC immunoblotting were listed previously (16–18). All other chemicals were of reagent grade. Stock solutions of AUR, PMA, phosphatidyl serine, diolein, fura 2/AM, PMSF, and leupeptin were made up in Me_2SO. Fmet-leu-phe was dissolved in 95% ethanol. These reagents were diluted in appropriate aqueous buffers so that the total carrier solvent concentration did not exceed 0.5% (v/v).

Isolation of human neutrophils. Neutrophils were isolated from heparinized venous blood of normal healthy donors. Cell suspensions were prepared by centrifugation on Ficoll-Paque, followed by hypoosmotic lysis of erythrocytes (19). Subsequently, cells were washed with HBSS at pH 7.0 and resuspended in HBSS at the concentrations indicated below.

Assay for superoxide anion generation by neutrophils. Neutrophil superoxide anion generation was monitored continuously by its ability to reduce ferricytochrome *c* to ferrocytochrome *c*, the latter absorbing strongly at 550 nm (20). Cells were pretreated with AUR (0.1–100 μM) or GST (0.1–500 μM) for timed intervals up to 30 min at 37° before stimulation of the respiratory burst by fmet-leu-phe (1 μM), F^- (18 mM), or PMA (100 nM). Reaction mixtures consisted of 10^6 cells/ml in HBSS containing 10% fetal calf serum and 0.1 mM ferricytochrome *c*. Superoxide generation rates were determined by the division of the rate of the absorbance change (calculated as the slope of the linear portion of the tracing) by the molar extinction coefficient (21/cm/mM) for the absorbance difference between reduced and oxidized cytochrome *c*. Results are expressed as percentage of control rates by cells exposed to carrier solvent (ethanol) only.

Fura-2 fluorometric measurement of intracellular free Ca^{2+}. Changes in intracellular $[Ca^{2+}]_i$ were monitored by the fluorescent Ca^{2+} indicator fura-2 (21). Briefly, neutrophils (10^7/ml in HBSS) were loaded with 1 μM fura-2/AM (0.25% Me_2SO) for 30 min at 37°. Excess indicator was removed by two HBSS washes and the cells were resuspended in the same buffer at a density of 10^6/ml. Stock suspensions of loaded cells were kept at 20° and aliquots were prewarmed to 37° before assay. Cells were exposed to either fmet-leu-phe (0.2 μM) or F^- (18 mM) alone or in combination with AUR (20 μM). Fluorescence changes were monitored continuously using monochromator settings of 339 nm (excitation) and 505 nm (emission) on a Perkin Elmer model MKF-4 fluorescence spectrophotometer. Intracellular free $[Ca^{2+}]_i$, was calculated according to the method of Grynkiewicz *et al.* (21), based on principles and procedures laid down for quin2 studies (22, 23).

Partial purification of platelet PKC. PKC was partially purified from human platelets as follows. All steps were performed at 4°. Human platelets (100 units), obtained from the Red Cross Blood Bank, were lysed by freezing and thawing in 40 mM potassium phosphate, pH 7.0, 10 mM EDTA, 0.2 mM PMSF, 1 mM dithiothreitol, and centrifuged at 15,000 × *g* for 30 min. Solid ammonium sulfate was added to the supernatant slowly with stirring, to achieve a final saturation level of 58%, and allowed to stand for 20 min before centrifugation at 15,000 × *g* for 20 min. The supernatant was discarded and the pellet was redissolved in a minimum volume of 20 mM Tris·HCl, pH 7.5, 1 mM EGTA, 0.5 mM dithiothreitol, and dialyzed overnight against two changes (10 liters each) of the same buffer. The dialysis sample was loaded, at a flow rate of 50 ml/hr, on a column (2.5 × 40 cm) of diethylaminoethyl-Sephacel previously equilibrated with dialysis buffer. The column was washed with dialysis buffer until absorbance at 280 nm returned to baseline. Bound proteins were eluted with a linear gradient generated from 600 ml each of dialysis buffer and dialysis buffer containing 0.35 M NaCl. Selected fractions were assayed for Ca^{2+}- and phospholipid-dependent phosphorylation of type III-S histone. PKC eluted at 0.16 M NaCl and was well separated from its major substrate, P47. Enzyme activity was pooled separately and further purified by hydroxylapatite column chromatography (24). PKC-containing fractions were combined, glycerol and soybean trypsin inhibitor were added to final concentrations of 10% (v/v) and 0.2 mg/ml, respectively, and the fractions were stored in 1-ml aliquots in plastic Eppendorf vials at −80°. Enzymatic activity was stable for at least 2 years. Using platelet P47 as substrate, PKC exhibited the following activities (in nmol of P_i/min × ml): 4.38 (+Ca^{2+}, +phospholipid); 0.03 (+Ca^{2+},− phospholipid); 0.11 (−Ca^{2+}, +phospholipid); and 0.02 (−Ca^{2+}, −phospholipid). Using type III-S histone as substrate, PKC exhibited the following activities (in nmol of P_i/min × ml): 5.67 (+Ca^{2+}, +phospholipid); 0.98 (+Ca^{2+}, −phospholipid); 2.59 (−Ca^{2+}, +phospholipid); and 0.56 (−Ca^{2+}, −phospholipid). The PKC preparation contained no endogenous substrates.

Measurement of PKC activity. Neutrophil cytosolic and particulate fractions were prepared as described previously (25). These fractions were assayed for PKC activity by a modification of the method of Kishimoto *et al.* (26). The assay mixture contained 20 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.12 mg/ml type III-S histone, 20 μM [γ-^{32}P]ATP (50–100 cpm/pmol), 0.01% Triton X-100, ± 1.2 mM phosphatidyl serine, ± 15.4 μM diolein, ± AUR (10^{-8}–10^{-4} M), ± GST (10^{-8} – 10^{-4} M), and 50 μl of PKC fraction in a total volume of 250 μl. Histone phosphorylation was initiated by the addition of [γ-^{32}P]ATP and terminated 15 min later by simultaneous additions of 0.8% bovine serum albumin and 15% trichloroacetic acid. The precipitate was collected by filtration on 2.5-cm glass microfiber filters (Whatman GF/F) and subsequently assessed for radioactivity.

Partially purified PKC was assayed at 30° in 20 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 48.4 μM phosphatidylserine, 1.2 μM diolein, 0.2 mg/ml type III-S histone, 40 μl/ml PKC, 10 μM [γ-^{32}P] ATP (50–100 cpm/pmol), ± 0.01–50 μM AUR or GST. Reactions were started by the addition of [γ-^{32}P]ATP to a total reaction volume of 1.2 ml. Samples (0.2 ml) of reaction mixtures were withdrawn at selected times (1, 2, 3, 4, and 5 min) for quantification of protein-bound $^{32}PO_4$ (16) to enable calculation of the initial rate of histone phosphorylation. The maximum final concentration of ethanol in the reaction mixture was 0.95% (v/v); this concentration was without effect on PKC activity.

Immunological detection of PKC. Polyclonal antibodies raised in rabbits against purified porcine brain PKC were prepared according to the method of Girard *et al.* (17). Intact neutrophils at a concentration of 5 × 10^7 cells/ml were pelleted and homogenized by resuspension in hot SDS buffer (a solution containing 2% SDS, 10 mM Tris-HCl, pH 7.5, and 10 mM EGTA, 95°). The homogenates were heated for an additional 10 min at 95°. The cell homogenates were subjected to SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to Zeta-probe blotting membranes for subsequent treatment with antiserum, as previously reported (17). Neutrophil cytosolic and particulate fractions were subjected to the hot SDS treatment and similarly analyzed.

Endogenous protein phosphorylation. Neutrophils were adjusted to 10^7 cells/ml in a PO_4-free HEPES buffer (10 mM HEPES, pH 7.2, 140 mM NaCl, 5 mM KCl, 5.56 mM glucose, 0.33 mM $CaCl_2$). $^{32}PO_4$ was added to the cells as $H_3{}^{32}PO_4$ (0.5 mCi/ml) and the cells were

incubated for 1 hr at 37° in a shaking water bath. At the end of the incubation, the excess $^{32}PO_4$ was removed from the cells with three washes in PO_4-free HEPES buffer and resuspended in HBSS at a concentration of 10^7 cells/ml. Treatment with PMA (100 nM) in the presence and absence of AUR (20 μM) or GST (20 μM) was performed in HBSS at 37° for various times (2–30 min). Reactions were terminated by centrifugation and resuspension in 1 ml of 15% trichloroacetic acid. The proteins were allowed to precipitate for 30 min at 4°, then collected by centrifugation, and washed extensively with ice-cold HBSS. Each sample of phosphoprotein precipitate (approximately 50 μg of protein) was resuspended in 0.2 ml of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 95°) and heated. Electrophoresis was carried out by the technique of Laemmli (27) using 12% polyacrylamide gels. Gels were dried and autoradiographed using Kodak XAR5 film. In some cases, gels were blotted onto nitrocellulose membranes (0.45-μm pore size) for better resolution.

Protein quantitation. The method of protein estimation of Lowry *et al.* (28) was adopted to analyze various samples before PKC assay and electrophoretic analysis.

Results

Effect of gold compounds on neutrophil superoxide production. Expanding on previous studies (3–5), we investigated the effects of incubation time, concentration, and type of stimulus on the inhibition of the respiratory burst by gold compounds. We used the following three stimuli: fmet-leu-phe, a chemotactic tripeptide that acts through specific receptors on the plasma membrane (14); fluoride ions (in the form of AlF_4^-), which activate a guanine nucleotide-binding regulatory protein (G) coupled to phospholipase C (14, 29, 30); and PMA, which directly activates PKC (15). As the results demonstrate (Fig. 1), AUR exerted a biphasic effect on superoxide anion generation induced in neutrophils by each of the three stimuli. At lower AUR concentrations (0.1–1 μM), the rate of superoxide anion generation was slightly but significantly enhanced. Higher concentrations of AUR inhibited the reaction dose dependently. As can be seen, the extent of inhibition was also dependent on the exposure time of cells to AUR before the addition of stimulus.

On initial inspection, it appears that the F^--induced respiratory burst was more sensitive to AUR than were fmet-leu-phe- or PMA-mediated responses. However, it is pertinent to note that, with the concentration of stimuli used, the lag period for fmet-leu-phe- or PMA-induced responses is approximately 10–15 sec- and 30–60 sec, respectively, whereas that for F^- is 4–10 min (20). Therefore, the period of exposure of neutrophils to AUR was actually longer than was apparent in Fig. 1C. Compared with AUR, GST was much less potent in inhibiting superoxide anion release. GST at 100–200 μM exhibited only weak inhibition, a finding observed in previous reports on the comparative effects of gold compounds on neutrophil function (2–9).

Control studies showed that the inhibition mediated by AUR was not due to cytotoxicity of, or to scavenging of superoxide anions by, the drug. Cell cytotoxicity was assessed by the criteria of trypan blue uptake and release of lactic acid dehydrogenase; results showed that neutrophils remain viable up to 3 hr after exposure to the highest levels of gold compound applied in the present studies.

Effect of AUR on Ca^{2+} mobilization. The finding that the PMA-mediated response was inhibited by AUR implied that AUR was inhibiting PKC directly or at steps subsequent

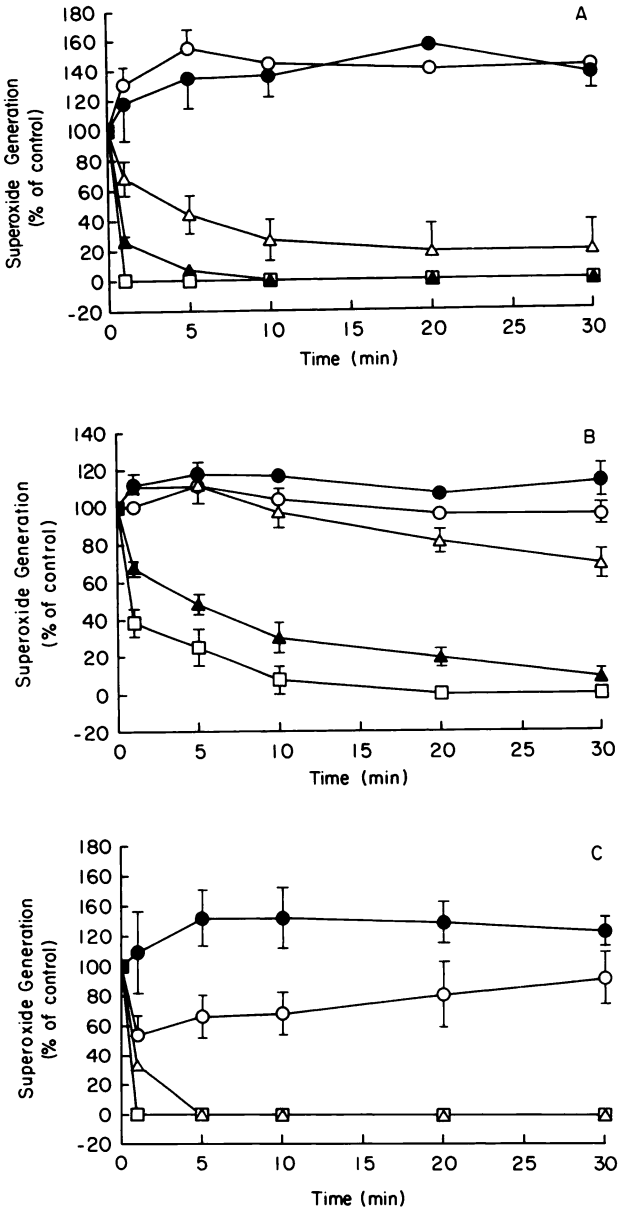

Fig. 1. AUR inhibition of the respiratory burst in human neutrophils. Neutrophils were preincubated in the presence of AUR for the indicated times. The stimulatory agent was added and the rate of superoxide anion generation was calculated and compared with control rates. A, stimulatory agent = fmet-leu-phe, 0.1 μM; B, stimulatory agent = PMA, 100 nM; C, stimulatory agent = NaF, 18 mM. AUR concentration (μM): 0.1 (○), 1 (●), 10 (△), 20 (▲), 100 (□). Mean control rates of superoxide generation were 12 ± 2, 10 ± 2, and 2.6 ± 0.7 nmol/min/10^6 cells, respectively, for fmet-leu-phe, PMA, and NaF (mean ± standard deviation, six separate experiments).

to its activation. However, it is possible that early signal transduction steps were also compromised. To test the latter possibility, the mobilization of intracellular Ca^{2+} stores was followed in cells loaded with the fluorescent Ca^{2+} chelator fura-2 (Fig. 2). Treatment of neutrophils with 20 μM AUR, which suppressed superoxide anion generation, did not affect Ca^{2+} mobilization caused by fmet-leu-phe or F^-. This suggests that AUR did not inhibit phospholipase C-mediated hydrolysis of polyphosphoinositides to inositol 1,4,5-trisphosphate. PMA was not included in this study because it is well documented that its action does not involve Ca^{2+} mobilization (14, 29–31).

Results illustrated in Fig. 2 also show that AUR alone induced a slow rise in intracellular Ca^{2+} concentrations, a result also noted by Zalewski *et al.* (32). The combination of AUR and fmet-leu-phe or F^- resulted in an enhanced fluorescence signal compared with either fmet-leu-phe or F^- alone.

Effect of gold compounds on the enzymatic and antigenic activity of PKC in subcellular fractions. The effect of gold compounds on the distribution of PKC activity was studied and the results are depicted in Figs. 3 and 4. Fig. 3 shows that cytosolic PKC activity, in cells treated with 20 μM AUR, decreased slowly with time compared with that observed for PMA. This correlated with the kinetics of inhibition of superoxide anion generation by AUR over the same period (Fig. 1). In agreement with previous studies (33, 34), PMA was found to cause rapid translocation of PKC to the particulate fraction, within minutes after addition to cells (Fig. 4).

To ascertain whether PKC translocation had occurred, PKC activity in particulate and cytosolic fractions isolated from cells treated with PMA, gold compounds, or a combination of both was compared. As Fig. 4 demonstrates, after a 10-min incubation with PMA, neutrophil cytosolic PKC was reduced to 20% of basal levels and particulate activity increased by more than 80%. After a 45-min incubation with AUR, a comparable reduction in cytosolic PKC had occurred but membrane-associated PKC was only slightly increased. In cells treated with a high concentration of GST (500 μM), a similar result was obtained. After 45 min, cytosolic PKC was reduced to approximately 60% of basal levels, whereas particulate PKC was not significantly altered. Under conditions in which PMA was added to cells after a 30-min preincubation with AUR or GST, only a small increase in particulate PKC was found.

These results could be interpreted in several ways. One is that gold compounds promote the translocation of PKC to the membrane, where the enzyme remains inactive or is inactivated by bound drug. Furthermore the possibility exists that PKC might be degraded as a result of its juxtaposition with proteases. A simpler interpretation is that gold compounds directly inhibit PKC *in situ* and that the interaction of either drug with PKC precludes PMA (or diacylglycerol) activation and translocation of PKC.

To distinguish between the models of AUR action, immunoblot analyses were carried out using polyclonal antisera directed against native (80-kDa) porcine brain PKC. Fig. 5 shows an autoradiogram of immunoreactive material present in whole cell and subcellular fractions of control (resting) neutrophils and cells treated with AUR. Cumulative results show that, in all cases, the major antigenic species was a 67-kDa fragment. Native (80-kDA) enzyme was not evident, although a faint 80-kDa band could be discerned in some experiments. Minor 50-kDa fragments can also be seen but their relative quantities varied between different experiments. All three immunoreactive species were detectable in brain homogenates (Fig. 5, *lane 1*), as reported previously (17).

Additional attempts to retard suspected proteolysis by the inclusion of diisopropyl fluorophosphate in resuspension medium failed to increase the yield of the 80-kDa native enzyme but did reduce the amount of immunoreactive 50-kDa fragments (data not shown). The pertinent finding here is that homogenates from cells treated with AUR for up to 45 min did not show increased fragmentation of PKC. Furthermore the relative distribution of PKC immunoreactive material between cytosolic and particulate fractions is similar in control and AUR-treated cells. Studies carried out with high concentrations of GST yielded the same results (data not shown). These findings support the conjecture that AUR did not catalyze PKC translocation or proteolysis of PKC.

Effect of gold compounds on protein phosphorylation. Inhibition of PKC activity in intact cells was assessed by comparing endogenous protein phosphorylation patterns in cells treated with combinations of PMA and gold compounds (Fig. 6). In agreement with previous reports (35–38), a range of proteins was phosphorylated in resting cells. PMA enhanced phosphorylation of over 10 protein bands and maximal levels were reached after 2-min exposure to the phorbol ester. Proteins of relative molecular mass (M_r) 40,000, 44,000, 47–50,000, 65,000, and 66–90,000 were most heavily labeled.

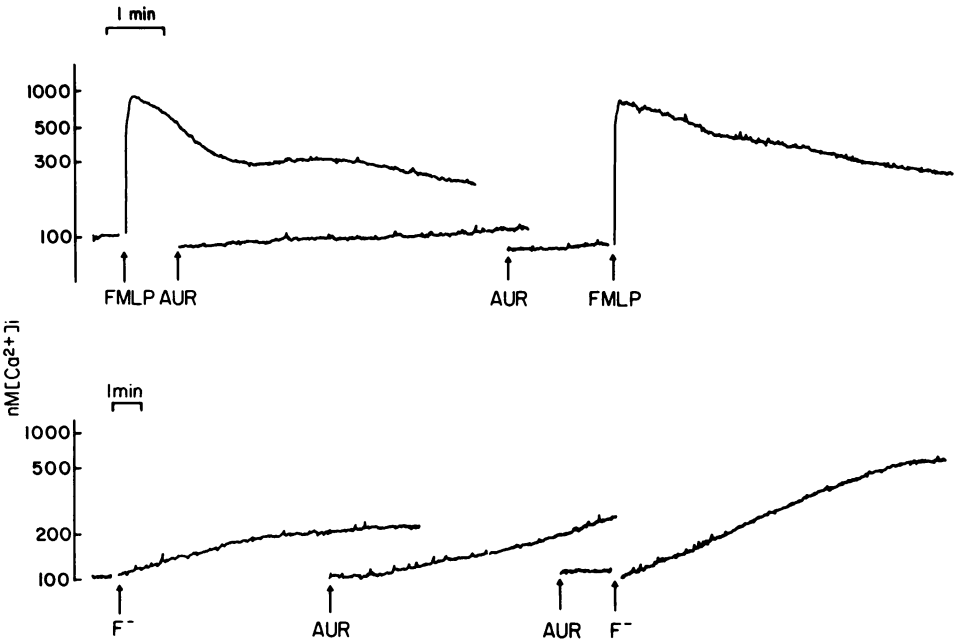

Fig. 2. Effect of AUR on calcium mobilization induced by fmet-leu-phe (*FMLP*) or F^-. Neutrophils loaded with the fluorescent calcium indicator fura-2 were exposed to 0.2μM fmet-leu-phe, 20 μM AUR, or 18 mM NaF at points indicated by *arrows*. Fluorescence changes were monitored continuously and intracellular $[Ca^{2+}]_i$ was calculated according to the formula $[Ca^{2+}]_i = 224\ \text{nM}\ (F - F_{min}/F_{max} - F)$ (21).

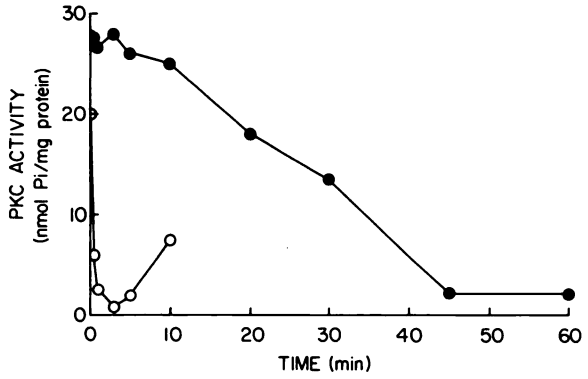

Fig. 3. Time course of the effect of PMA and AUR on neutrophil cytosolic PKC activity. Neutrophils were treated with PMA (○, 100 nM) or AUR (●, 20 μM), for the indicated periods of time before cellular disruption and ultracentrifugation. The 100,000 × *g* supernatant was assayed for calcium- and phospholipid-dependent kinase activity as described in Experimental Procedures. The results shown are representative for one of five experiments.

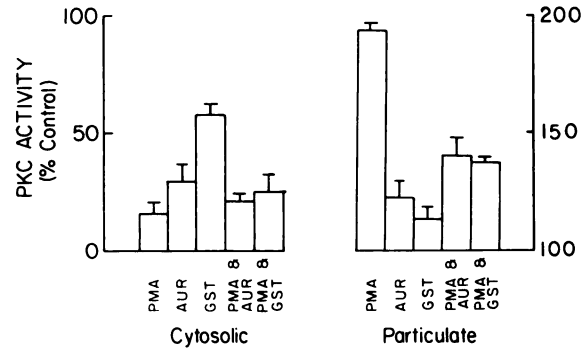

Fig. 4. PKC activity of neutrophil and subcellular fractions after prolonged exposure of cells to gold compounds. Cells were treated with 20 μM AUR or 0.5 mM GST for 40 min, or 10 min with 100 nM PMA. In combination studies, PMA was added 30 min after the gold compound and the results are the mean ± standard error of five determinations and are expressed as percentage of control activity found in the respective fractions of resting cells. Mean basal activity for cytosolic fractions was 20.3 nmol of ^{32}P incorporated/mg of protein and, for particulate fractions, 6.5 nmol of ^{32}P incorporated/mg of protein.

The addition of 20 μM AUR to labeled cell suspensions resulted in weaker enhancement of protein phosphorylation, compared with that observed with PMA (Fig. 6A). The exception was a protein band of M_r = 20–22,000, which was prominently phosphorylated after a 2-min incubation with AUR. The simultaneous addition of PMA and AUR to cells resulted in rapid enhancement of protein labeling, similar to that observed for PMA alone. With continued incubation, the extent of protein phosphorylation declined to levels close to that observed for control (carrier solvent alone). The rapidity of action of PMA likely accounts for the initial predominance of PKC-mediated effects. With prolonged incubation, AUR inhibition of PKC would assume precedence. Specifically, decreased labeling was noted for the 47–50-kDa protein, the phosphorylation of which is PKC dependent and necessary for activation of NADPH oxidase, the enzymatic system responsible for superoxide anion generation (35–38). GST (20 μM) also induced rapid phosphorylation of a 20–22-kDa protein (Fig. 6B). However, under conditions in which PMA and GST were added together to cells, reversal of labeling mediated by PMA was not found.

Effect of gold compounds on partially purified PKC. Direct evidence that AUR interfered with signal transduction at the level of PKC was obtained from *in vitro* assays of partially purified platelet PKC carried out in the presence of gold compounds. Results illustrated in Fig. 7 demonstrate that both AUR and GST inhibited PKC in a dose-dependent manner. The concentrations of AUR and GST that produced 50% inhibition were estimated to be 0.9 μM and 3.9 μM, respectively. Similar results (data not shown) were obtained using PKC purified from bovine brain (18). The gold compounds completely inhibited PKC activity at concentrations ≥10 μM (Fig. 7), indicating that they inhibit the basal activity levels observed in the absence of Ca^{2+} and/or phospholipid in addition to the Ca^{2+}- and phospholipid-stimulated activity. This implies a direct inhibitory effect of AUR and GST on the enzyme. In support of this conclusion, increasing the concentrations of phosphatidylserine and diolein up to their usual values had no effect on AUR inhibition of PKC. Furthermore, AUR was equally inhibitory when PKC activity was assayed by the mixed-micelle assay of Bell *et al.* (39), in which the concentrations of phosphatidylserine and diolein are considerably higher (3.71 mM and 0.93 mM, respectively, compared with 48.4 μM and 1.2 μM, respectively). On the other hand, inhibition by AUR could be reversed by increasing the concentration of platelet or brain PKC at a fixed AUR concentration under otherwise standard assay conditions (results not shown).[1]

The observed inhibitory effect of AUR appears to be specific for PKC because no significant inhibition of the catalytic subunit of cAMP-dependent protein kinase or Ca^{2+}- and calmodulin-dependent myosin light chain kinase was observed at concentrations of AUR as high as 10^{-4} M (results not shown).[1]

Discussion

In human neutrophils, fmet-leu-phe binding to its specific receptor is coupled via a guanine nucleotide-binding regulatory (G) protein to a phosphoinositide-specific phospholipase C, which generates the two intracellular second messengers inositol 1,4,5-trisphosphate and diacylglycerol (40). Inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+}- and diacylglycerol activates PKC. AUR inhibits superoxide anion generation by fmet-leu-phe, F^-, and phorbol esters. We have attempted to identify the site at which AUR exerts its action by examining the effects of AUR on 1) intracellular free [Ca^{2+}]; 2) translocation of PKC to the membrane; 3) degradation of PKC; 4) PKC-catalyzed phosphorylation of endogenous neutrophil proteins; and 5) the activity of PKC *in vitro*. The main conclusion from these studies is that the primary target of AUR is PKC itself, that is, the gold compound acts by inhibiting PKC. At the cellular level, AUR suppressed responses induced by PMA. PKC activity measurements and immunoblot analyses indicate that AUR inhibited PKC without promoting translocation or fragmentation of the enzyme. Furthermore, enhanced PKC activity and protein phosphorylation in PMA-stimulated neutrophils was reversed by the co-presence of AUR. Finally, *in vitro* assays provided direct evidence that AUR (and GST) inhibited PKC in a dose-dependent manner. It is pertinent to note that neutrophil responses elicited by the calcium ionophore A23187 are not affected by AUR treatment (4, 5). This is rationalized on the basis that the effects of A23187 are not mediated by PKC but rather by activation of other calcium-

[1] Manuscript in preparation.

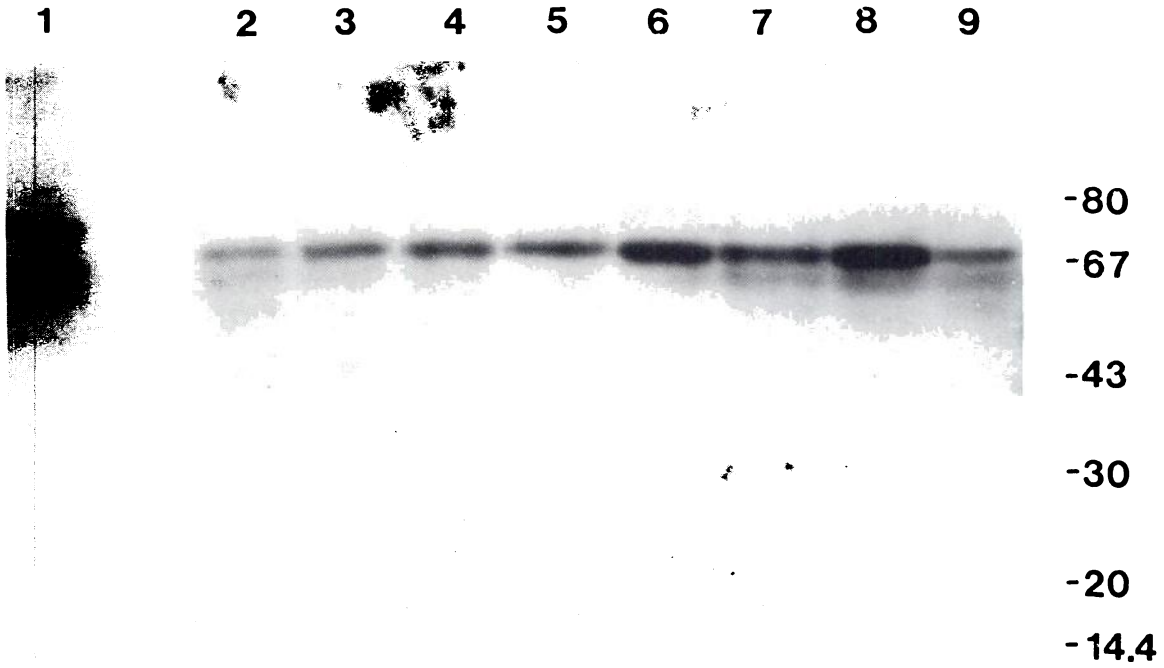

Fig. 5. Autoradiogram showing fractions in neutrophil homogenates immunoreactive with polyclonal antiserum directed against rat brain PKC. Intact neutrophils, resting or treated with AUR for 45 min at 37°, were pelleted and treated for 10 min with hot SDS as outlined in Experimental Procedures. Additional cell pellets were resuspended in Tris buffer (pH 7.4) containing 5 mM EGTA, 0.25 M sucrose, 10 mM 2-mercaptoethanol, 2 mM PMSF, and 1 mM leupeptin; cytosolic and particulate (membrane) fractions were obtained by sonication followed by centrifugation at 100,000 × *g*. Both the whole cell homogenate and subcellular fractions (200–250 μg of protein) were subjected to SDS polyacrylamide gel electrophoresis and blotted to a Zeta-probe membrane. The latter was incubated with PKC antiserum followed by ^{125}I-protein A and immunoreactive bands were visualized by autoradiography (17). Samples analyzed were as follows: pig brain PKC (*lane 1*); whole cell homogenate from resting cells (*lanes 2* and *3*), cells treated with 10 μM (*lane 4*) and 20 μM AUR (*lane 5*); cytosolic fractions from resting (*lane 6*) and AUR-treated cells (*lane 8*); and membrane fractions from resting (*lane 7*) and AUR-treated cells (*lane 9*). *Marks on the right* indicate positions of protein standards ($M_r \times 10^{-3}$).

dependent enzymatic processes brought about by elevated intracellular Ca^{2+} concentrations.

With respect to the immunoblot results (Fig. 5), both the 67- and the 50-kDa species likely derived from the 80-kDa native enzyme. The 50-kDa species probably corresponds to protein kinase M, a Ca^{2+}- and phospholipid-independent form of the enzyme generated by the action of Ca^{2+}-dependent proteases (26). In contrast to results obtained with neutrophils, HL60 and K562 leukemic cells contain the 50-kDa species instead of the 67-kDa species (17). The absence of significant amounts of 80-kDa enzyme was surprising, especially in the case of whole neutrophil homogenates, because the latter was treated with hot SDS, a procedure demonstrated previously to preserve the integrity of native PKC in other tissues (17). The fact that neutrophils are replete with lysosomal granules containing a large array of proteases may account for the present failure to block initial proteolysis.

In all likelihood, AUR also modulated other parts of the signal transduction pathway in the intact cell although to a lesser extent (e.g., the mobilization of intracellular Ca^{2+}). (Fig. 2). That internal Ca^{2+} stores were mobilized was evidenced by the finding that this event was not affected by the removal of extracellular Ca^{2+} by the presence of excess EGTA (results not shown). The possibility that AUR may interact with Ca^{2+}-gating mechanisms at the level of the endoplasmic reticulum is a subject of ongoing research. Alternatively, this phenomenon may be a consequence of PKC inhibition by AUR. For example, numerous studies suggest that PKC plays a role in active extrusion of Ca^{2+} immediately after its mobilization from intracellular stores and that the Ca^{2+}-transport ATPase is a substrate of PKC (15). If PKC inhibition is a factor, the spontaneous elevation of intracellular Ca^{2+} by AUR in neutrophils suggests that Ca^{2+} homeostasis is maintained in resting cells by a basal level of PKC activity.

The biphasic effect of AUR on cellular responses has been consistently reported with respect to neutrophils; a biphasic response was noted for lysosomal enzyme release, chemiluminescence, and superoxide anion generation (3–5). Low doses of AUR have been reported to enhance natural killer cell activity *in vitro*, whereas higher doses were inhibitory (41). The regulatory system potentiated by low (<1 μM) AUR remains to be elucidated.

The dependency of the inhibition of the respiratory burst on AUR preincubation time likely reflects the rate of entry of AUR into cells. In their studies on the distribution of labeled AUR in RAW 264.7 cells, Snyder *et al.* (42) found that the gold and triethylphosphine moieties of AUR appear in the cytosol within 1 min but that maximal levels are not attained until after 5–10 min. Although the kinetics of AUR entry into the neutrophils may differ, a parallelism exists in that maximal inhibition of superoxide anion generation occurred in cells preincubated with AUR for approximately 10 min (Fig. 1).

One question not answered by present results is why GST is a relatively ineffective inhibitor of neutrophils responses, although it is comparable to AUR as a PKC inhibitor. The answer may lie in the inability of GST to penetrate easily into the neutrophil cytosol. GST is a water-soluble compound whereas AUR is lipophilic; the latter is able to partition effectively into

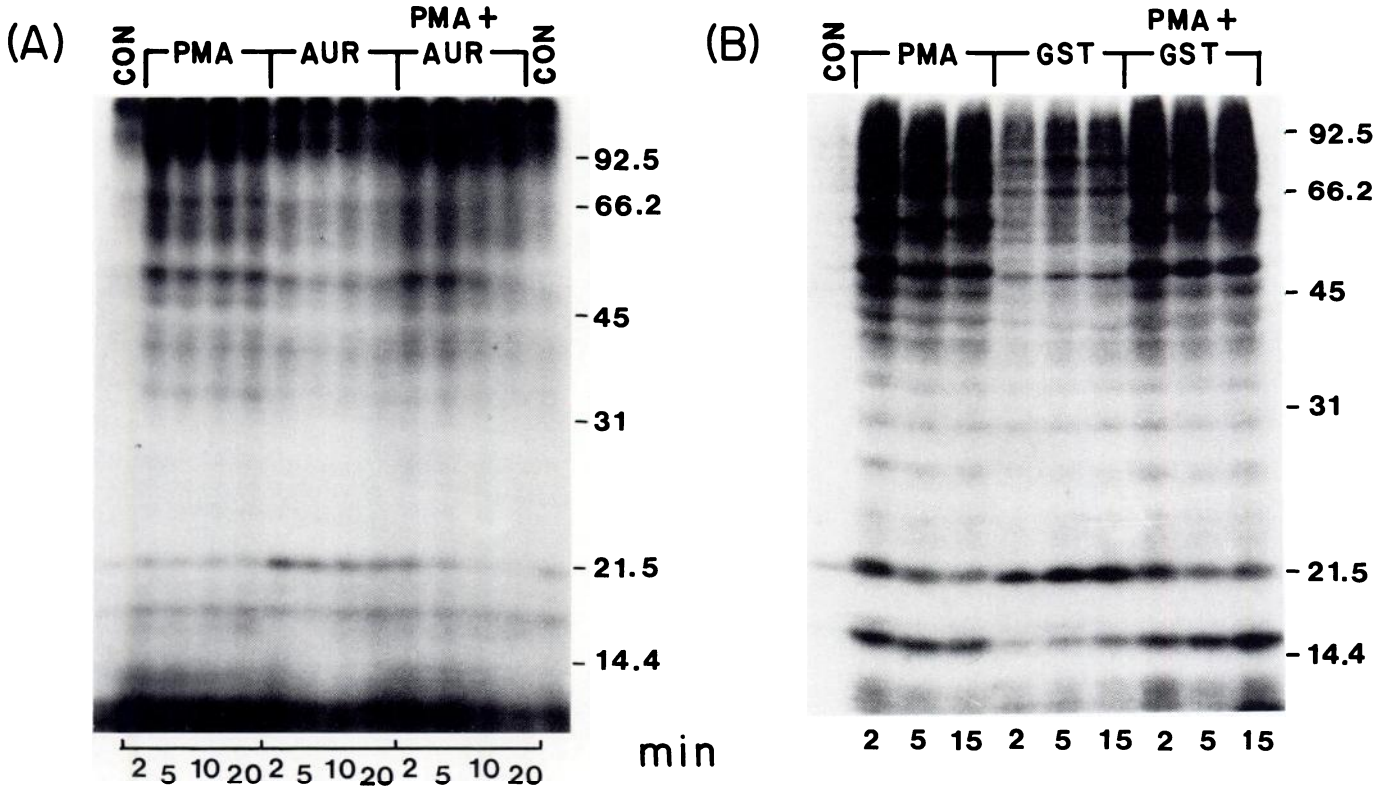

Fig. 6. Effect of gold compounds on endogenous protein phosphorylation in activated neutrophils. Neutrophils were prelabeled with $^{32}PO_4$ according to procedures outlined in Experimental Procedures. Cells were incubated at 37° with PMA (100 nM), AUR (20 μM), GST (20 μM), or combinations of PMA and gold compounds for durations shown on the *bottom* of autoradiograms. *Markers on the right* indicate positions of protein standards ($M_r \times 10^{-3}$). *A*, AUR experiments, 2–20-min incubation periods, *Con* = control cells exposed to solvent vehicles (0.1% ethanol and 0.1% Me_2SO) only. *B*, GST experiments, 2–15-min incubations, *Con* = control. Results shown are representative of six experiments, all demonstrating similar phosphorylation patterns.

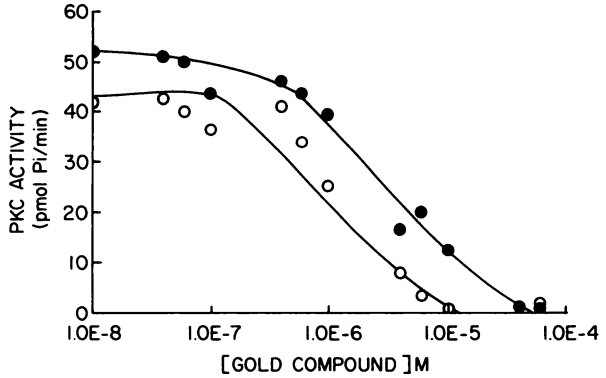

Fig. 7. Inhibition of semipurified platelet PKC by AUR and GST. Platelet protein kinase C was partially purified as described in Experimental Procedures. The initial rate of histone phosphorylation in the absence of gold compounds was 53.2 pmol of Pi/min. AUR (○) or GST (●) were added directly to the enzyme before assay. Data are representative of results obtained from three similar experiments.

lipid bilayers and from there to transfer to cytosolic targets. In fact, GST has been shown to form aggregates in solution and therefore may not be introduced to the cell in a monomeric form. Therefore, GST probably crosses the membrane barrier at a very slow rate, if at all, and time frames examined under the present protocol may be too brief to detect significant GST effects. Distribution studies of labeled GST along the lines carried out with AUR should bear this out.

In $^{32}PO_4$-loaded neutrophils, treatment with 20 μM AUR results in the phosphorylation of at least four proteins. The 20–22-kDa phosphoprotein is postulated to be the myosin light chain because it has been shown to be phosphorylated in the presence of micromolar Ca^{2+} by a calmodulin-dependent protein kinase (43–45). AUR, when added to fura-2/AM-loaded neutrophils, causes a slow rise in intracellular Ca^{2+}, peaking in the low micromolar range.[2] This rise in Ca^{2+} could be responsible for the effects observed. However, the time necessary for AUR-treated cells to reach micromolar levels of intracellular Ca^{2+} are in the order of 5–10 min (dependent upon concentration), whereas phosphorylation induced by AUR peaked at 2 min. Moreover, GST causes an increase in protein phosphorylation similar to that of AUR but does not elicit a rise in intracellular Ca^{2+}. The possibility that these compounds activate other kinases or inhibit cellular phosphatases does exist and requires further study. It should be noted that, when $^{32}PO_4$-loaded cells are co-stimulated with PMA and AUR, PMA-induced phosphorylation of proteins is reversed due to the proposed inhibition of PKC by AUR. Interestingly, the 20–22-kDa protein is also not phosphorylated under these conditions, suggesting that the kinase responsible for its phosphorylation may be under regulatory control by PKC or PMA. Examination of the phosphoprotein profiles under conditions in which AUR-pretreated $^{32}PO_4$-loaded neutrophils are stimulated with PMA

[2] Unpublished results.

at various times should provide useful information in this regard.

The mechanism of inhibition of PKC by AUR is under current investigation. Previous studies have shown that AUR interacts with proteins principally via ligand exchange with the sulfhydryl group of cysteine residues (46, 47). Because the tetraacetylthioglucose moiety of AUR is the better leaving group, complexes of PKC cysteine residues coordinated to gold-triethylphosphine alone is a distinct possibility. A possible target site on PKC may reside within the cysteine-rich domain in the amino terminal half of PKC (48).

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