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Inhibitory effects on protein kinase C activity by lipophosphoglycan fragments and glycosylphosphatidylinositol antigens of the protozoan parasite *Leishmania*

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Fragments of the lipophosphoglycan of *Leishmania donovani* were generated by phospholipase C digestion and mild acid hydrolysis. The fragments were purified and examined for inhibitory activity on protein kinase C isolated from rat brains. On a molar basis, the 1-*O*-alkylglycerol portion of LPG exhibited the most inhibitory activity, whereas the carbohydrate domain was not as effective. In addition, several glycolipid antigens from *L. major*, which contain short carbohydrate chains attached to phosphatidylinositol, were also efficient inhibitors of the enzyme. These results are consistent with the hypothesis that protein kinase C may be a key target for the parasites to overcome within host macrophages.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the causative agents of human leishmaniasis. A striking feature of these parasites is their ability to survive in hydrolytic environments throughout their life cycle, such as within the phagolysosomal system of mammalian macrophages. Macrophage cells undergo an oxidative burst during phagocytosis, leading to the production of several species of toxic oxygen radicals. Stimulation of the oxidative burst is believed to be mediated by protein kinase C (PKC) [1–3]. To avoid destruction by the cytocidal elements of the oxidative burst in activated macrophages, it has been postulated that the lipophosphoglycan (LPG) of the parasites functions as an inhibitor of PKC [4]. The net result is an impairment of the PKC-initiated oxidative-burst mechanism, enabling the parasites to survive.

LPG is the major cell-surface glycoconjugate of leishmanial parasites [5]. In *L. donovani* it is a polymer of repeating phosphorylated disaccharide units of [$PO_4 \rightarrow 6Gal\beta 1,4Man\alpha 1$] linked via a phosphosaccharide core to a novel lyso-1-*O*-alkylphosphatidylinositol lipid anchor [6,7]. The antigenically distinct *L. major* LPG appears to have multiple phosphorylated tri- and tetrasaccharide units containing galactose, mannose, glucose and arabinose [8]. In addition to LPG, three leishmanial glycosylphosphatidylinositol antigens (GPI-A, -B and -C) that are able to bind antibodies in immune sera from patients with cutaneous leishmaniasis have been reported [9]. These glycolipids contain an alkylacylphosphatidylinositol anchor linked to the glycan through an unacetylated hexosamine (G. Rosen and M. V. Londner, unpublished work).

In a previous study [10], intact LPG was found to be a potent inhibitor of isolated PKC. The glycoconjugate was a competitive inhibitor with respect to diolein (dioeoylglycerol) and a non-competitive inhibitor with respect to phosphatidylserine. Here we show that the lipid domain of LPG is primarily responsible for suppression of PKC activity, although the glycan region also caused appreciable inhibition. Smaller leishmanial GPI lipids were also found to be capable inhibitors.

MATERIALS AND METHODS

Materials

All materials were obtained from Sigma Chemical Co., except as follows: phosphatidylserine (pig brain) from Serdary Research Laboratories, London, Ont., Canada; GFA filters and 3MM paper from Whatman; [γ-^{32}P]ATP (7000 Ci/mmol) from ICN Radiochemicals; and trichloroacetic acid from Fisher. Phosphatidylinositol-specific phospholipase C (PI-PLC) (from *Staphylococcus aureus*) was generously given by Dr. Martin Low (Columbia University, New York, NY, U.S.A.

Fragmentation of LPG

LPG was extracted and purified from *L. donovani* as described previously [6]. Fragmentation of LPG was achieved as illustrated in Scheme 1, and quantification of the fragments was obtained after phosphate analysis [11]. Mild acid hydrolysis (0.02 M-HCl, 5 min, 100 °C) cleaves LPG at the mannose 1-phosphate site in the repeating units, generating phosphorylated disaccharides and phosphosaccharidyl-PI as products. The two products were separated by passage of the hydrolysate through a column of phenyl-coupled Sepharose equilibrated in 0.5 M-NaCl/0.1 M-acetic acid. The phosphorylated disaccharides did not bind to the hydrophobic support and were eluted in the breakthrough eluate. The phenyl-Sepharose was washed with water, and the phospho-

Abbreviations used: LPG, lipophosphoglycan; PG, phosphoglycan; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol(PI)-specific phospholipase C; PKC, protein kinase C.

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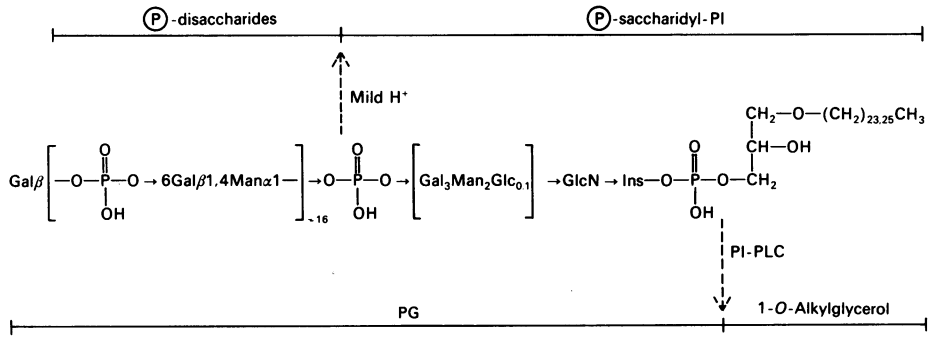

Scheme 1. Structure of LPG and its fragmentation by mild acid and by PI-PLC

Abbreviations: Ins, inositol; Ⓟ, phosphate.

saccharidyl-PI was eluted with the addition of water/ethanol/diethyl ether/pyridine/NH_3 ($d = 0.88$) (15000:15000:5000:1000:17, by vol.).

Treatment of LPG with PI-PLC [6] liberated the 1-*O*-alkylglycerol from the entire carbohydrate portion termed 'phosphoglycan' (PG). The products were separated by partitioning with the addition of 2 ml each of chloroform and water.

Preparation of GPIs from *L. major*

GPI antigens A, B and C from *L. major* were extracted and separated by t.l.c. as previously described [9]. Each glycolipid was purified by gel-filtration chromatography on a column (1 cm × 10 cm) of LH-20 equilibrated in methanol.

PKC assay

PKC from rat brain was partially purified by chromatography on DEAE-cellulose and phenyl-Sepharose as previously described [12]. The PKC was stored at −70 °C in the presence of 2 mM-EGTA/1 mM-dithiothreitol/2 mM-EDTA/20 mM-Tris/HCl, pH 7.4. The kinase was used within 2 weeks of isolation. PKC was assayed in a total volume of 100 μl, containing 5 μl of the PKC preparation, 40 μg of histone (type III-S, lysine-rich), 10 μM-ATP (containing 0.3 μCi of [γ-^{32}P]ATP), 5.0 mM-$MgCl_2$ and either (*a*) 1.0 mM-$CaCl_2$, 36 μM-phosphatidylserine and 0.15 μM-diacylglycerol or (*b*) 5 μM-$CaCl_2$, 18 μM-phosphatidylserine and 0.5 μM-diacylglycerol. The variability in Ca^{2+} concentration in the assay depended on the concentration of EDTA/EGTA used during the purification of the enzyme. It was found that the stability of PKC varied from preparation to preparation and, consequently, adjusting the amounts of the chelators was necessary to retard loss of enzymic activity. Lipids in chloroform/methanol (2:1, v/v) were dried under N_2 and resuspended by vortex-mixing and sonication with a Tekmar Sonic Disruptor in 25 mM-Tris/HCl, pH 7.5, containing 0.03% Triton X-100. The final concentration of Triton X-100 was not more than 0.003% in the PKC assay. The assay was initiated by addition of $MgCl_2$/ATP to the other reagents. The reaction continued for 4 min at 30 °C. At that time, 10 μl of reaction solution was removed and spotted on to the bottom of a rectangular piece of 3MM filter paper which was previously spotted with 20% (w/v) trichloroacetic acid. The filter paper was then developed in 5% trichloroacetic acid containing 0.2 M-KCl to remove [^{32}P]ATP that had not reacted. The spot containing ^{32}P-labelled histone was cut out and measured for ^{32}P emission in the presence of 0.2 ml of 1% SDS and 3.6 ml of scintillation fluid. Alternatively 1 ml of 10% trichloroacetic acid was added to the reaction mix to stop the reaction, and the total reaction mixture filtered over GFA filters. The filters were washed twice with 10 ml of 10% trichloroacetic acid and once with 10 ml of diethyl ether. The filter papers were measured for ^{32}P emission in the presence of 3.6 ml of scintillation fluid. In either case, ^{32}P emission was detected by using a Prias II liquid-scintillation spectrophotometer. Non-specific enzymic activity was measured in the absence of lipid and subtracted from total activity. ATPase activity in the purified PKC preparation was negligible as demonstrated by t.l.c. of the [γ-^{32}P]ATP substrate after a 20 min incubation with PKC (results not shown).

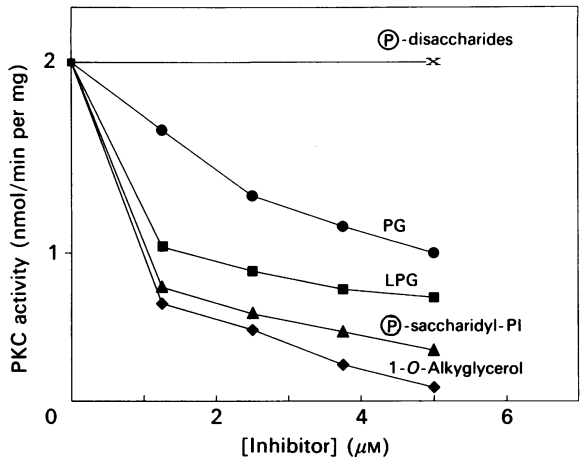

Fig. 1. Inhibition of PKC activity by LPG and LPG fragments

PKC was assayed in the presence of 1.0 mM-$CaCl_2$, 5.0 mM-$MgCl_2$, 36 μM-phosphatidylserine, and 0.15 μM-diacylglycerol with the designated concentrations of LPG and LPG fragments as described in the Materials and methods section. Abbreviation: Ⓟ, phosphate.

RESULTS

Inhibitory effects of LPG fragments

LPG was fragmented as described in Scheme 1. The fragments were purified and PKC was assayed in the presence of these fragments (Fig. 1). As reported pre-

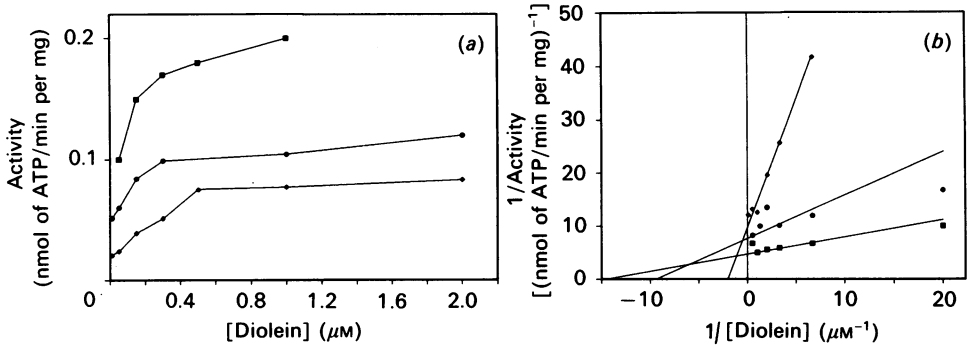

Fig. 2. Effect of the 1-*O*-alkylglycerol from LPG on PKC activity as a function of diolein concentration

(*a*) The enzyme was assayed in the presence of 1.0 μM-$CaCl_2$, 5.0 mM-$MgCl_2$, 18 μM-phosphatidylserine, 0.5 μM-diacylglycerol and 0 μM (■), 0.1 μM (●), or 1 μM (◆) -1-*O*-alkylglycerol as described in the Materials and methods section. (*b*) Double-reciprocal plot of the data in (*a*). Values plotted are the mean values from three experiments.

Table 1. Effect of LPG fragment analogues on PKC activity

The analogues were added at a final concentration of 5 μM to a PKC assay mixture containing 5 μM-$CaCl_2$, 18 μM-phosphatidylserine and 0.5 μM-diolein. Other reagents and protocols were as described in the Materials and methods section. Values are shown with the S.D. for three to six determinations. Values indicated by the asterisk designate those values that were significantly different from the control value as determined by Student's *t* test. The values in parentheses are the percentage activities relative to the control value.

Analogue	PKC activity (nmol of ATP/min per mg of histone)
None	0.219±0.018 (100%)
LPG	0.045±0.002* (20.0%)
1-*O*-Alkylglycerol (from LPG)	0.041±0.002* (18.7%)
1-*O*-Octadecyl-*rac*-glycerol	0.119±0.019* (54.2%)
1-*O*-Hexadecyl-*rac*-glycerol	0.123±0.019* (56.2%)
1-*O*-Dodecyl-*rac*-glycerol	0.206±0.013* (94.1%)
1-Monopalmitoyl-*rac*-glycerol	0.181±0.026 (82.6%)
1,2-Di-*O*-hexadecyl-*rac*-glycerol	0.219±0.019 (100%)
Diacyl-PI	0.219±0.0189 (100%)
Lyso-1-acyl-PI	0.166±0.014* (75.8%)
Galactose 6-phosphate	0.221±0.031 (100.9%)
Galβ1,4Man	0.204±0.012 (93.2%)
PO_4→6Galβ1,4Man	0.219±0.022 (100%)

viously [10], intact LPG is a potent inhibitor of the enzyme, even at a relatively high concentration of phosphatidylserine (36 μM). The 1-*O*-alkylglycerol fragment of LPG [6], which consists of either a saturated, unbranched C_{24} (78%) or C_{26} (22%) hydrocarbon, had a greater inhibitory effect on PKC than had intact LPG under these assay conditions (Fig. 1). The phosphosaccharidyl-PI fragment also had a greater inhibitory effect than intact LPG. Although the entire carbohydrate portion of LPG (phosphoglycan) also suppressed the activity of PKC, it was not nearly as effective as LPG or its lipid-containing fragments. Addition of the individual phosphorylated disaccharide fragments isolated from LPG had no effect on PKC activity.

To pursue the inhibitory nature of LPG's lipid fragment, the effect of the 1-*O*-alkylglycerol moiety was measured as a function of diolein concentration. As shown in Fig. 2(*a*), increasing concentrations of diolein, with constant levels of phosphatidylserine and other reagents, resulted in an increase in PKC activity. The stimulation by diolein was counteracted by increasing concentrations of the 1-*O*-alkylglycerol obtained from LPG. A double-reciprocal plot of the data (Fig. 2*b*) revealed that the apparent K_m for diolein significantly increased [K_m values for diolein were calculated as 0.050±0.005 μM ($n = 6$), 0.055±0.006 μM ($n = 5$) and 1.000±0.108 μM ($n = 6$) in the presence of 0 μM-, 0.1 μM- or 1 μM-1-*O*-alkylglycerol respectively]. The $V_{max.}$ decreased as a function of 1-*O*-alkylglycerol concentration [the $V_{max.}$ values for PKC were calculated as 0.200±0.022 ($n = 6$), 0.114±0.012 ($n = 5$) and 0.101±0.011 nmol of ATP/min per mg ($n = 6$) in the presence of 0 μM-, 0.1 μM- or 1 μM-1-*O*-alkylglycerol respectively].

A series of compounds structurally related to the fragments from LPG were examined for their effect on PKC activity. As shown in Table 1, the monoalkylglycerols exhibited significant inhibitory activity under the assay conditions used, whereas the dialkylglycerol showed no effect. Comparing 1-*O*-hexadecyl-

Table 2. Effect of GPIs from *L. major* on PKC activity

The GPIs were added at a final concentration of 10 μM to a PKC assay mixture containing 5 μM-$CaCl_2$, 18 μM-phosphatidylserine and 0.5 μM-diolein. Other reagents and protocols are as described in the Materials and methods section. Values are shown with the standard deviation for three determinations. All values obtained for the GPIs were significantly different from the control value as determined by Student's *t* test. Value in parentheses are the percentage activities relative to the control value.

GPI	PKC activity (nmol of ATP/min per mg of histone)
None	0.219±0.018 (100%)
GPI-A	0.099±0.044 (45.2%)
GPI-B	0.110±0.002 (50.2%)
GPI-C	0.116±0.002 (52.9%)

glycerol with 1-monopalmitoylglycerol, the results suggested that an aklyl group at the C-1 position of glycerol is important to inhibitory activity. Although not directly proportional in general, the greater the alkyl chain length, the greater the degree of inhibition. The effect of several relatively simple carbohydrates related to LPG are also listed in Table 1. No appreciable inhibitory activity was associated with any of the carbohydrates tested.

Inhibitory effects of leishmanial GPI lipids

Three isolated glycosylphosphatidylinositol antigens from *L. major* [9] were examined for their effect on PKC. All three GPIs at a concentration of 10 μM showed significant inhibitory activity toward the enzyme, as shown in Table 2.

DISCUSSION

Since LPG is the major surface glycoconjugate of leishmanial parasites, it is very possible that LPG plays a critical role in the survival of the parasites within host cells. One important survival mechanism would be the potential abrogation or mediation of the oxidative burst of the macrophage cells in which the parasites reside. It has been shown that intracellular *Leishmania* parasites significantly decrease the oxidative burst of mouse peritoneal macrophage cells when infected macrophages are stimulated by phorbol myristic acid or zymosan [13]. Also, Eilam and co-workers [14] have reported that human erythrocytes coated with 'excreted factor' (i.e. LPG) are protected from destruction within macrophages, possibly due to suppression of oxidative-burst induction. We have been investigating the molecular basis for how LPG might accomplish this suppression.

Induction of the oxidative burst in macrophages is believed to be mediated by PKC [1–3]. Recently, the *L. donovani* LPG was demonstrated to be a potent inhibitor of purified PKC [10]. The results from that report indicate that the 1-*O*-alkylglycerol portion of LPG is primarily responsible for the inhibitory activity. This was not unexpected, since ether-for-acyl substitutions have been reported to result in a potent decrease in PKC-activating ability [15,16]. The type of inhibition displayed by the 1-*O*-alkylglycerol appears to be complex with respect to diolein concentration, since both the apparent K_m and $V_{max.}$ were affected. A similar type of inhibition has been previously reported to occur in the presence of alkylacylglycerols [17].

A potentially important observation concerning LPG that actually accounted for its original name of 'excreted factor' [18] is its release from the surface of the parasite and its appearance in the culture medium. One of the released forms of LPG is a hydrophilic form [5,18]. Although the identity of the cleavage reaction has not yet been demonstrated, the resulting hydrophobic fragment may actually be the biological inhibitor of PKC within infected macrophages.

In another potential mechanism of inhibition, it is conceivable that LPG (or PG), due to the presence of many charged phosphate groups, could chelate significant amounts of free Ca^{2+}, or other important bivalent cations, which are present in small concentrations intracellularly. Indeed, when assayed in the presence of micromolar levels of Ca^{2+} (Table 1), the difference in inhibitory activity between LPG and 1-*O*-alkylglycerol became almost negligible, perhaps due in part to chelation of the calcium.

To determine whether other leishmanial surface molecules are potentially PKC inhibitors, three GPI antigens were also examined. They were isolated from *L. major* and shown to be effective antigens in sera from patients with cutaneous leishmaniasis [9]. These glycolipids, which contain an alkylacylphosphatidylinositol anchor, showed a half maximal inhibition of protein kinase C at 10 μM. Synthetic alkyl-linked diacylglycerols were reported to inhibit PKC activation by diacylglycerol in HL-60 cells at a similar concentration [17]. It is not yet known whether they are biosynthetic precursors to LPG or have distinct biological roles. In the PKC assay, three of the GPIs were shown to have inhibitory activity toward the enzyme.

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